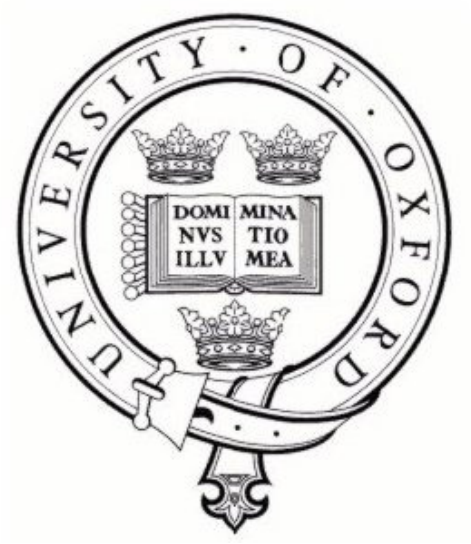
Amino acid utilisation and apoplast colonisation by *Pseudomonas syringae*.



Rachel Jones

St John's College

Thesis submitted for the degree of Doctor of Philosophy at the University of Oxford

Trinity Term

2009

Abstract

Rachel Jones
St John's College
Amino acid utilisation and apoplast colonisation by *Pseudomonas syringae*.
Trinity 2009

The aim of this study was to increase understanding of amino acid utilisation and amino acid permease (aa_permease) genes in the plant pathogen *Pseudomonas syringae*, which inhabits the nutrient-limited environment of the plant surface and intracellular apoplast. aa_permease genes are significantly reduced in *P. syringae* genomes compared to non plant-pathogenic *Pseudomonas*. Accordingly, this work demonstrates that *P. syringae* can utilise a restricted number of amino acids for growth compared to non plant-pathogenic *Pseudomonas*. The remaining aa_permease genes in *Pseudomonas syringae* pv. tomato are annotated as transporting GABA, ethanolamine, proline and aromatic amino acids. Sequence analyses, phylogenetic analyses, chemotaxis assays and gene expression analyses supported the functional annotations of the GABA, ethanolamine and aromatic aa_permeases, but showed that expression of the proline permease was specifically induced by histidine, which suggests that this encodes a histidine transporter. Expression of the GABA and histidine permeases was also induced in the presence of tomato apoplast, indicating that these amino acids may be assimilated during apoplast colonisation. *P. syringae* pv. tomato exhibited chemotaxis towards tomato apoplast and several constituent amino acids, which may be important for invasion of the apoplast from the plant surface. Uptake of several amino acids including GABA and histidine appeared to be negatively affected in alkaline media. The aa_permeases use electrochemical potential energy to transport substrates and this mechanism may be affected by pH. The plant apoplast becomes increasingly alkaline during infection and this may select against the retention of particular transporters in *P. syringae*. As GABA is an abundant amino acid in the apoplast and can be used by *P. syringae* strains for growth, the growth of *P. syringae* pv. tabaci was monitored in transgenic tobacco plants with increased GABA. Although growth was similar in all plants, significantly fewer symptoms and more callose deposition was observed upon infection.

Contents.

1. Chapter 1. Introduction.

1.1 Overview.

1.2 *Pseudomonas* species.

1.3 Plant pathogenic *Pseudomonas syringae.*

1.4 Infection of plants by *Pseudomonas syringae.*

1.5 Physiological adaptations to a plant-associated lifestyle.

1.6 Plant physiology during infection.

1.7 The plant apoplast and *Pseudomonas syringae*.

1.8 *Pseudomonas syringae*: adaptations to the apoplast?

1.9 Amino acid permease (aa_permease) genes.

1.10 The GABA permease has been extensively characterised in non-*Pseudomonas* species.

1.11 The role of GABA in plants.

1.12 Conclusions.

Chapter 2. General materials and methods.

2.1 Introduction.

2.2 Bacteria, growth conditions and media.

- 2.2.1 Bacterial strains and abbreviations.
- 2.2.2 Bacterial growth media.
- 2.2.3 Antibiotic concentrations.

2.4 Manipulation of DNA.

- 2.4.1 Plasmids.
- 2.4.2 Isolation of genomic DNA.
- 2.4.3 Ethanol precipitation of DNA.
- 2.4.4 Agarose gel electrophoresis.
- 2.4.5 DNA quantification.
- 2.4.6 Primers.
- 2.4.7 Polymerase chain reaction (PCR).

2.5 Cloning techniques.

- 2.5.1 Purification of PCR products.
- 2.5.2 Ligation of PCR products into plasmids.
- 2.5.3 Restriction enzyme digests of DNA.
- 2.5.4 Dephosphorylation of DNA.

2.5.5 Phenol/chloroform purification of DNA.

2.5.6 DNA Sequencing.

2.5.7 Dialysis of DNA.

2.5.8 Production of electrocompetent *Pseudomonas.*

2.5.9 Production of electrocompetent *Escherichia coli.*

2.5.10 Transformation of electrocompetent *Escherichia coli* and *Pseudomonas.*

2.6 Bioinformatics.

2.6.1 Statistical analysis and result presentation.

2.6.2 Manipulation and analysis of DNA sequences.

2.7 Plant growth conditions and apoplast extraction techniques.

2.7.1 Extraction of apoplast fluid.

2.7.2 Estimation of apoplast dilution.

2.7.3 Plant growth conditions.

2.8 Imaging.

2.8.1 Digital photography.

2.8.2 Microscopy.

2.8.3 Photon camera imaging.

2.9 Spectrophotometric measurements.

Chapter 3: Bioinformatics and phylogenetic analysis of amino acid permease genes in *Pseudomonas.*

3.1 Overview.

3.2 Introduction.

3.3 Objectives.

3.4 Materials and methods.

3.4.1 Domain analysis.

3.4.2 Analysis of orthologs and horizontal evolution (horizontal gene transfer).

3.4.3 Phylogenetic analysis.

3.4.4 Identification of predicted transmembrane domains.

3.4.5 Identification of predicted sigma^{54} (RpoN̄)-binding sites.

3.4.6 Genomic context.

3.4.7 Advanced operon prediction.

3.4.8 Statistical analysis.

3.4.9 Abbreviations.

3.5 Results.

3.5.1 Sequence analysis of aa_permease domains.

3.5.2 *Pseduomonas syringae* is underrepresented in several transporter domains of the APC superfamily, including the amino acid permease domain.

3.5.3 The aa_permeases in *Pseudomonas syringae* are a subset of those found in other *Pseudomonas*.

3.5.4 Experimental evidence for the function of *Pseudomonas* aa_permease genes is rare.

3.5.5 The aa_permease genes localise to the cytoplasmic membrane.

3.5.6 Identification of transmembrane domains.

3.5.7 Several aa_permease genes are potentially regulated by RpoN

3.5.8 The *Pseudomonas stutzeri* genome contains five aa_permease genes.

3.5.9 Synteny of the *Pseudomonas syringae* GABA permease is well conserved between *Pseudomonas syringae* pathovars but not across all *Pseudomonas* species.

3.5.10 The ProY permease falls within the histidine utilisation (*hut*) operon.

3.5.11 The synteny of the ethanolamine permease region is conserved between *Pseudomonas syringae* pathovars but not across all *Pseudomonas* species.

3.5.12 Synteny of the aromatic amino acid permease region is conserved between *P. syringae* pathovars but not across all *Pseudomonas* species.

3.5.13 The *proY* aa_permease gene has been functionally demonstrated to be a histidine transporter in *Pseudomonas fluorescens* SBW25.

3.5.14 The ProY aa_permease gene has orthologs in all *Pseudomonas* except *Pseudomonas fluorescens* Pf0-1.

3.5.15 The GABA aa_permease genes appear to be evolutionarily related in *Pseudomonas*, with one exception.

3.5.16 One GABA permease present in *Pseudomonas putida* species appears to have been acquired from *Burkholderia* species.

3.5.17 All aromatic aa_permeases in *Pseudomonas* are evolutionarily related.

3.5.18 Ethanolamine permeases group within two clades.

3.5.19 *Pseudomonas fluorescens* Pf-5 is the only *Pseudomonas* to have more than one proline permease, and this does not group with the others.

3.5.20 The histidine (*hut*) operon, containing the ProY aa_permease, has undergone several rearrangements in *Pseudomonas*.

3.5.21 Horizontal evolution of aa_permease genes appears to be rare.

3.5.22 The aa_permease genes encode a diverse family of transporters

3.5.23 The aa_permease genes in *Xylella fastidiosa* appear to be a subset of those found in *Xanthomonas* species.

3.5.24 APC superfamily domains are reduced in *Xylella fastidiosa* and *Xanthomonas* species.

3.6 Discussion.

Chapter 4: Amino acid utilisation by *Pseudomonas syringae*.

4.1 Overview.

4.2 Introduction.

4.3 Objectives.

4.4 Materials and methods.

4.4.1 Bacterial strains.

4.4.2 Growth *in vitro.*

4.4.3 Phenoarrays.

4.4.4 Extraction of tomato apoplast fluid and estimation of apoplast dilution.

4.5 Results.

4.5.1 *Pseudomonas syringae* can utilise fewer amino acids for growth than *Pseudomonas aeruginosa* or *Pseudomonas fluorescens*.

4.5.2 pH affects the growth of *Pseudomonas syringae* more than *Pseudomonas fluorescens* or *Pseudomonas aeruginosa*.

4.5.3 The pH of unbuffered LB media varied over time and appeared to be correlated with growth of *Pseudomonas.*

4.5.4 Some amino acids significantly increased the growth of *Pseudomonas syringae* pv. tomato, but this was not correlated with their abundance in tomato apoplast.

4.5.5 *Pseudomonas syringae* pv. tomato can only derive a growth benefit from certain amino acids in acidic conditions.

4.5.6 Development of synthetic apoplast and synthetic guttation fluid as a growth medium for *Pseudomonas syringae.*

4.5.7 Final composition of synthetic apoplast media.

4.5.8 Growth of *Pseudomonas syringae* pv. tomato in synthetic apoplast media.

4.5.9 Incubation of *Pseudomonas syringae* pv. tomato in synthetic tomato apoplast results in a similar nutrient utilisation profile to incubation in real tomato apoplast.

4.5.10 Growth of *Pseudomonas syringae* in synthetic guttation fluid.

4.5.11 Comparison of *Pseudomonas syringae* growth in synthetic apoplast media.

4.5.12 Glutamine, serine and proline contribute significantly towards increased growth of *Pseudomonas syringae* pv. tomato in synthetic *Arabidopsis thaliana* apoplast.

4.5.13 GABA, aspartic acid and glutamic acid contribute significantly towards the increased growth of *Pseudomonas syringae* pv. tomato in synthetic tomato apoplast.
4.5.14 Growth in synthetic media at different pH values.
4.5.15 Metabolism of compounds as carbon sources is increased at acidic pH when bacteria are pre-incubated in synthetic tomato apoplast.
4.5.16 Metabolism of compounds as carbon sources is increased at alkaline pH when bacteria are pre-incubated in LB.
4.5.17 Metabolism of amino acids as carbon sources by *Pseudomonas syringae* is affected by the media used for pre-incubation and the pH of that media.
4.6 Discussion.

Chapter 5: Chemotaxis by *Pseudomonas syringae* pv. tomato.

5.1 Overview.
5.2 Introduction.
5.3 Objectives.
5.4 Materials and methods.
5.4.1 Bacterial strains and abbreviations.
5.4.2 Growth in vitro.
5.4.3 Chemotaxis assay.
5.5 Results.
5.5.1 Literature survey of previous research on chemotaxis to amino acids by *Pseudomonas* species.
5.5.2 Chemotaxis by *Pseudomonas syringae* pv. tomato to amino acids.
5.5.3 Analysis of amino acid chemotaxis by *Pseudomonas syringae* pv. tomato.
5.5.4 Chemotaxis by *Pseudomonas syringae* pv. tomato towards synthetic media and extracted tomato apoplast.
5.5.5 Bioinformatic comparisons of chemotaxis-related proteins in Pseudomonas.
5.6 Discussion.

Chapter 6: Expression analysis of aa_permease genes in *Pseudomonas syringae* pv. tomato and *rpoN* mutant characterisation.

6.1 Overview.
6.2 Introduction.
6.3 Objectives.
6.4 Materials and methods.
6.4.1 Bacterial strains and abbreviations.
6.4.2 Growth - *in vitro.*

6.4.3 Growth – *in planta.*
6.4.4 Motility assay.
6.4.5 P.s.t-*rpoN* mutant complementation.
6.4.6 RT-PCR.
6.4.7 Primers used in this study.
6.5 Results.
6.5.1 Expression of aa_permease genes in wild type *Pseudomonas syringae* pv. tomato, in synthetic tomato apoplast and LB media.
6.5.2 Development of a simple method to analyse gene expression in planta.
6.5.3 Expression of aa_permease genes in the presence of selected amino acids, in extracted tomato apoplast and 'in planta'.
6.5.4 Comparison of aa_permease gene expression in synthetic tomato apoplast and extracted tomato apoplast.
6.5.5 P.s.t- rpoN can be complemented by expression of the *Pseudomonas fluorescens* SBW25 rpoN gene.
6.5.6 Expression of aa_permease genes in the P.s.t-rpoN mutant and the P.s.t-rpoN mutant complemented with pJJP6.
6.5.7 Expression of *Pseudomonas syringae* pv. tomato aa_permease genes in high and low nitrogen media.
6.5.8 Analysis of genes in the histidine (hut) operon in P.s.t.
6.6 Discussion.
Chapter 7: Infection of glutamate decarboxylase-overexpressing tobacco plants with *Pseudomonas syringae* pv. tabaci.
7.1 Overview.
7.2 Introduction.
7.2.1 The metabolism of GABA in plants.
7.2.2 Possible roles for GABA and the GABA shunt in plants.
7.2.3 Previous studies using transgenic GAD plants.
7.3 Objectives.
7.4 Materials and methods.
7.4.1 Plant material.
7.4.2 Bacterial strains and abbreviations.
7.4.3 Extraction of apoplast fluid and estimation of apoplast dilution.
7.4.4 Infection assays.
7.4.5 Cell biology assays.

7.4.6 Measuring salicylic acid (SA) production by tobacco plants.

7.4.7 Identification of amino acids by HPLC.

7.4.8 Reverse transcriptase (RT-PCR) of plant gene expression.

7.5 Results.

7.5.1 *Pseudomonas syringae* pv. tabaci causes fewer symptoms in transgenic GAD tobacco than the wild type or plant-vector control plants, without differences in bacterial growth.

7.5.2 GAD plants produce more callose than WT plants in response to P.s.t.-hrcC.

7.5.3 GAD plants do not differ in H_2O_2 production in response to *Pseudomonas syringae* pv. tabaci and *Pseudomonas syringae* pv. tomato.

7.5.4 GAD plants may produce an earlier superoxide response than wild type plants.

7.5.5 Trypan blue staining.

7.5.6 Transgenic plants do not produce significantly more salicylic acid (SA) in response to pathogen inoculation.

7.5.7 PR1α gene expression is similar in all plants in response to *Pseudomonas syringae* pv. tabaci and *Pseudomonas syring*ae pv. tomato inoculation.

7.5.8 The apoplast of tobacco and tomato leaves contain similar amino acids.

7.5.9 GABA is the most abundant amino acid in the both the tobacco and tomato leaf apoplast.

7.5.10 GABA is more abundant in the apoplast of $MgCl_2$-treated GAD and GADΔC plants than in wild type plants.

7.5.11 The GABA content of the GAD and GADΔC apoplast significantly decreases when treated with *Pseudomonas syringae* pv. tabaci.

7.6 Discussion.

Chapter 8: General discussion of results.

8.1 Discussion.

Appendix: Mutant construction.

A.1 Overview.

A.2 Introduction.

A.3 Objectives

A.4 Materials and Methods.

A.4.1 Bacterial strains, plasmids and abbreviations.

A.4.2 Antibiotics and supplements.

A.4.2 Bacterial growth conditions.

A.4.3 Primers used in this study.

A.4.4 Cloning to create constructs for aa_permease gene deletion knockout.

A.4.5 Plasmid conjugation and allelic exchange.

A.5 Results.

A.5.1 Creation of deletion constructs in pUIC3.

A.5.1 Allelic exchange of deletion constructs.

A.6 Discussion.

References.

List of abbreviations used in this thesis.

aa_permease	-amino acid permease
ABA	-abscinic acid
APC	-amino acid/polyamine/organocation
Avr protein	-avirulence protein
C	-control
COR	-coronatine
Che	-chemotaxis protein
EPS	-extracellular polysaccharides
ET	-ethylene
GABA	-gamma-aminobutyric acid
GABA-T	-gamma-aminobutryic acid transaminase
GAD	-glutamate decarboxylase
GAD∆C	-GAD enzyme lacking Ca^{2+}/calmodulin binding domain
GC-MS	-gas chromatography-mass spectrophotometry
GF	-guttation fluid
GS	-glutamine synthetase
HR	-hypersensitive response
HIM	-Hrp-inducing minimal media
HMM	-hidden Markov model
Hut	-histidine utilisation
JA	-jasmonic acid (jasmonate)
MP	-maximum parsimony
MCP	-methyl-accepting chemotaxis protein
NJ	-neighbour joining
OD	-optical density
PNP	-para-nitrophenol
PCD	-programmed cell death
Pfam	-online protein families database maintained by NCBI
PR	-pathogenesis related
QS	-quorum sensing
R gene	-resistance gene
RBBH	-reciprocal best BLAST hit
RCR	-relative chemotactic response
SA	-salicylic acid
SAA	synthetic *A. thaliana* apoplast
STA	-synthetic tomato apoplast
T3SS	-type three secretion system
TA	-tomato apoplast
Tn	-transposon
WT	-wild type

1. Introduction.

1.1 Overview.

Pseudomonas syringae (*P. syringae*) is a biotrophic plant pathogen capable of epiphytic and endophytic growth on a taxonomically broad range of flowering plants. *P. syringae* is capable of epiphytic growth on the surface of both host and non-host plants, and endophytic growth in the intercellular fluid (apoplast) of host plants.

The ability of some *P. syringae* pathovars to infect the model plant *Arabidopsis thaliana*, (*A. thaliana*) combined with the availability of genome sequence data and the relative amenability of these bacteria to molecular genetic techniques, has resulted in *P. syringae* becoming a model organism for the study of plant-pathogen interactions. To date, the majority of research into *P. syringae*-plant interactions has focused on the mechanisms by which these bacteria are able to interact with and modulate plant cells, while themselves remaining outside of the plant cell wall in the apoplast. In particular, attention has concentrated on the ways in which pathogen-derived or secreted molecules, such as pathogen-associated molecular patterns (PAMPs) and effectors, influence pathogen-host specificity between *P. syringae* strains and plants (Mudgett, 2005; Grant et al., 2006; Truman et al., 2006; Cunnac et al., 2009). However, we still know relatively little about the apoplast environment encountered by *P. syringae*. In particular, it is still not clear which nutrient sources are available to *P. syringae* in the apoplast, how these may change during pathogenesis, or the ways in which *P. syringae* may be adapted to utilise the available nutrient sources.

In this chapter, the well-studied mechanisms by which *P. syringae* infects host plants and by which plants defend themselves against infection are briefly reviewed before looking at the less studied area of how these bacteria may be ecologically adapted to life in the plant environment and for assimilation of plant-derived nutrients. In particular, as the plant apoplast is the site of bacterial growth for *P. syringae in planta*, the nutrient composition of this environment is reviewed.

1.2 *Pseudomonas* species.

Pseudomonas species are ubiquitous bacteria in nature. They possess a range of metabolic abilities allowing them to utilise a range of organic compounds and they occupy a wide range of environmental niches. The genus *Pseudomonas* includes opportunistic pathogens, plant pathogens, plant growth-promoting and saprophytic species (Holloway and Morgan, 1986) and

has been shown to constitute a monophyletic group on the basis of *gyrB* and *rpoD* sequencing (Yamamoto et al., 2000). The interrelationships of several major *Pseudomonas* species discussed in this study are shown in Figure 1.1 below.

Figure removed for copyright reasons

Figure 1.1
The relationship of major *Pseudomonas* species, based on 1,705 conserved genes. Adapted from Silby et al., 2009. Numbers on nodes represent percentages of individual trees containing that relationship. The scale bar corresponds to the number of substitutions per site. *P. fluorescens* strains SBW25 (SBW25), Pf0-1 (Pf01) and Pf-5 (Pf5); *P. aeruginosa* strains PAO1 (P_aer_PAO1), PA14 (P_aer_PA14) and PA7 (P_aer_PA7); *P. syringae* pv. *syringae* B728a (P_syr_syr), pv. *tomato* DC3000 (P_syr_tom) and pv. *phaseolicola* 1448A (P_syr_pha); *P. putida* strains GB1 (P_put_GB1), F1 (P_put_F1), W619 (P_put_W619) and KT2240 (P_put_KT24); and *P. stutzeri* strain A1501 (P_stut).

Complete or draft genome sequences are now available for at least 19 *Pseudomonas* strains, including plant pathogens, opportunistic pathogens and non-pathogenic plant-associated strains (Table 1.1). More recently, high-throughput sequencing technologies have begun to reduce the costs associated with genome sequencing, with the result that several more draft genome sequences have been published for *Pseudomonas,* including *P. syringae* pv. tomato T1 (Almeida et al., 2009), and *P. syringae* pv. oryzae (Reinhardt et al., 2009). In addition, multiple online resources are available for searching and comparing multiple *Pseudomonas* sequence data and annotations, including the *Pseudomonas* Genome Database (Winsor et al., 2005; Winsor et al., 2009), xBASE (http://xbase.bham.ac.uk/), and the National Centre for Biotechnology Information (NCBI) database (Sayers et al., 2009).

Pseudomonas species **(abbreviation)**	**Description**	**Reference or source**
P. syringae pv. tomato DC3000 (P.s.t)	Plant pathogen	(Buell et al., 2003)
P. syringae pv. phaseolicola 1448A (P.s.ph)	Plant pathogen	(Joardar et al., 2005)
P. syringae pv. syringae B728a (P.s.s)	Plant pathogen	(Feil et al., 2005)
P. syringae pv. tomato T1 (T1)	Plant pathogen	(Almeida et al., 2009) (draft)
P. syringae pv. oryzae (P.s.oryzae)	Plant pathogen	(Reinhardt et al., 2009) (draft)
P. aeruginosa PA14 (PA14)	Opportunistic pathogen	http://pga.mgh.harvard.edu/Parabiosys/projects/
P. aeruginosa PAO1 (PAO1)	Opportunistic pathogen	(Stover et al., 2000)
P. aeruginosa PA7 (PA7)	Opportunistic pathogen	http://msc.jcvi.org/pseudomonas_aeruginosa/index.shtml
P. aeruginosa LESB58 (LESB58)	Opportunistic pathogen	(Winstanley et al., 2009)
P. stutzeri A1501 (A1501)	Plant commensal	(Yan et al., 2008)
P. entomophila L48 (L48)	Entomopathogen	(Vodovar et al., 2006)
P. fluorescens Pf-5 (Pf-5)	Plant commensal	(Paulsen et al., 2005)
P. fluorescens SBW25 (P.fl.SBW25)	Plant commensal	(Silby et al., 2009)
P. fluorescens Pf0-1 (Pf0-1)	Plant commensal	(Silby et al., 2009)
P. putida KT2440 (KT2449)	Soil saprophyte	(Nelson et al., 2002)
P. putida F1 (F1)	Soil saprophyte	http://genome.jgi-psf.org/finished_microbes/psepu/psepu.home
P. putida GB1 (GB1)	Soil saprophyte	http://genome.jgipsf.org/finished_microbes/psepg/psepg.home.html
P. putida W619 (W619)	Soil saprophyte	http://genome.jgipsf.org/finished_microbes/psepw/psepw.home.html
P. mendocina ymp (P.men)	Soil saprophyte	http://genome.jgipsf.org/finished_microbes/pseme/pseme.home.html

Table 1.1
Sequenced *Pseudomonas* species, including published draft and complete genomes, and complete genomes not yet published, available from the Genomes Online database (Kyrpides, 1999)

The natural diversity of *Pseudomonas* species, combined with the tools available to mine the genome sequence data, makes these bacteria an ideal model for comparative studies. In particular, such resources have been of particular use to researchers seeking to identify the genetic basis for pathogenesis by the plant pathogenic species *P. syringae*.

1.3 Plant pathogenic *Pseudomonas syringae.*

P. syringae is a biotrophic plant pathogen capable of epiphytic and endophytic growth on a range of flowering plants. The species is divided into approximately 50 pathogenic varieties [pathovars (p.v.)], many of which demonstrate a high degree of host specificity and cause distinct disease symptoms (Hirano and Upper, 1990; Alfano and Collmer, 1996). The availability of multiple *P. syringae* genomes including the strains tomato DC3000 (P.s.t), phaseolicola 1448A (P.s.ph), and syringae B728a (P.s.s), has provided researchers with the opportunity to identify many factors contributing to plant pathogenicity and virulence. In particular, the ability of P.s.t to infect the model plant *A. thaliana* has made the *P. syringae* / *A. thaliana* interaction the archetype for plant-pathogen interactions. This pathogen/host system provides the possibility to directly compare interactions resulting in basal resistance, susceptibility, and gene-specific resistance by using different genotypes of *P. syringae,* or *P. syringae* mutants, on the same host. Furthermore, by comparing sequenced *P. syringae* genomes with those of non plant-pathogenic *Pseudomonas*, researchers have been able to identify genomic differences, and make hypotheses about the way these might contribute to the plant-pathogenic lifestyle (Preston, 2004; Studholme et al., 2005).

P. syringae is capable of epiphytic growth on both host and non-host plants (Figure 1.2). Bacteria may arrive on the surface of a plant by a variety of mechanisms including rain, insect vectors or by growth on the surface of seeds. Following a period of epiphytic growth, *P. syringae* may gain entry into a plant through wound sites or natural openings (Hirano and Upper, 2000). However, successful endophytic growth is only achieved within host plants (Truman et al., 2006). Inside a host plant, *P. syringae* lives within the intercellular apoplast. From here, bacteria manipulate aspects of plant metabolism, toxins and hormones, to overcome plant defences and obtain nutrients, by injecting protein ‘effectors’ into the cells through a specialised needle-like structure called the type three secretion system (T3SS) (Alfano and Collmer, 2004; Cui et al., 2005).

The ability of *P. syringae* to cause disease on crop plants has lead to extensive, and almost exclusive, research on this bacterium in an agricultural setting as both an epiphyte and endophyte of cultivated plants (Hirano and Upper, 2000). However, a recent study found strains of *P. syringae* in rain, snow, lakes and wild plants, which were genetically almost identical to the sequenced *P. syringae* pathovars P.s.t, P.s.s, and P.s.ph, revealing that this species is also widespread in a range of non-agricultural niches and has been detected in forests, clouds and snow (Morris et al., 2007; Morris et al., 2008).

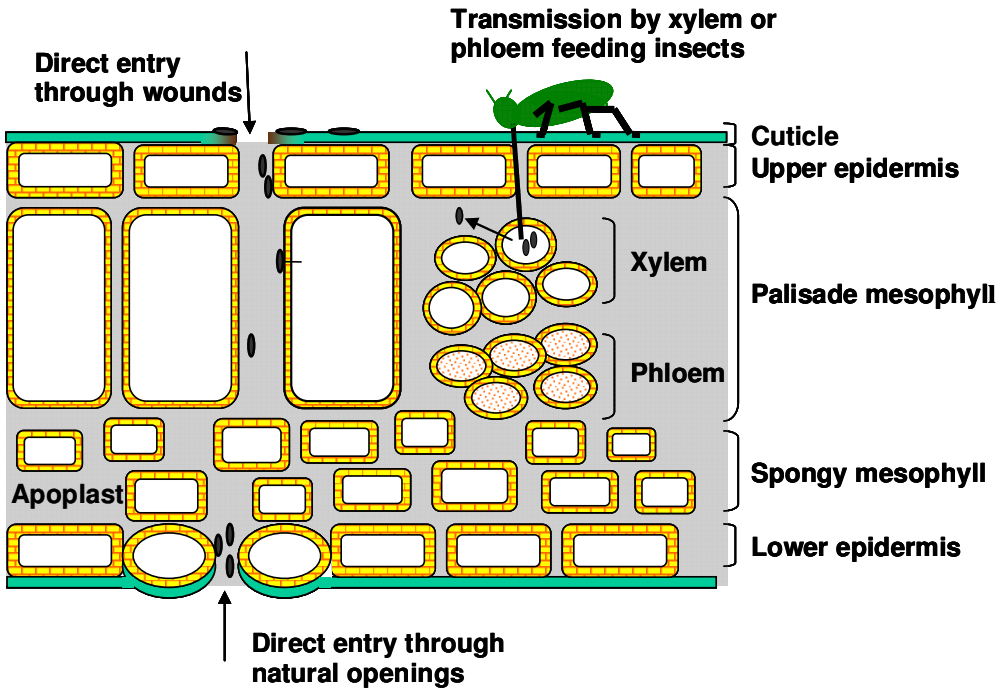


Figure 1.2
The infection cycle of *P. syringae* on and within plants. Bacteria can arrive on the surface of a leaf through a number of ways, including rain, water splash or vectors. *P. syringae* is capable of epiphytic colonisation on the surface of host and non host plants. *P. syringae* can enter a leaf through natural openings such as stomata, or through would sites, entry can also be facilitated by vectors such as xylem-feeding insects. Inside a host plant *P. syringae* colonises the intercellular apoplast. *P. syringae* is a biotrophic pathogen which needs living plants cells for nutrients, initially proliferation is asymptomatic however, eventually extensive necrosis, and frequently chlorosis, are visible on the surface infected tissue. Figure by Rachel Jones, published in: Adaptation to the plant apoplast by plant pathogenic bacteria (Rico et al., 2008).

In addition, a new host for P.s.s was recently discovered: Stavrinides and coworkers (2009), reported that P.s.s (but not P.s.t) can infect the pea aphid *Acyrthosiphon pisum*, which feeds on a range of crop plants (Stavrinides et al., 2009). These findings suggest that the number and variety of environments potentially encountered by *P. syringae* may be far larger than previously thought, and emphasise the potential complexity of the selection pressures which shape the genome of this bacterium.

1.3.1 Plant defences and *P. syringae.*

In order to successfully infect host plants, *P. syringae* must suppress the basal plant defences, which are activated against all microbes, while evading or suppressing pathogen-specific plant defences (Heil and Bostock, 2002; Kim et al., 2008; Vlot et al., 2008). In response to pathogen attack, plants have evolved a range of strategies to defend themselves against infection, these defences are summarised below (Table 1.2).

Resistance	Specificity	Location	Mechanism
Constitutive basal/non-host resistance	Most microorganisms	Throughout plant	Synthesis of antimicrobial chemicals and physical barriers to infection.
Induced basal/non-host resistance	Most microorganisms	Extracellular	Recognition of PAMPs (pathogen-associated molecular patterns)
Host-specific	Non-host bacteria with corresponding effector proteins	Intracellular, (possibly also extracellular)	Direct or indirect recognition of effectors by R genes.
Induced resistance I (SAR)	Host-specific pathogens or cell damage by necrosis	Can be transmitted through whole plant	Up regulation of defence gene expression by signaling molecules. Requires SA signal transduction pathway.
Induced resistance II (ISR)	Detection of PAMPs,	Can be transmitted through whole plant	Priming of defence gene expression. Requires JA / ET signal transduction pathways.

Table 1.2
Constitutive and induced plant defences which may be employed against *P. syringae*. These defences are reviewed in the text.

Basal defences.

Basal, or non-host defences, are expressed by most plants towards the majority of potential pathogens. They are stimulated from outside of plant cells by 'general elicitors' of pathogen or plant origin. Pathogen-derived elicitors are also known as pathogen-associated molecular patterns (PAMPs) (Li et al., 2005). PAMPs are defined as invariant epitopes within molecules that are fundamental to the pathogen's fitness, found widely distributed among different microbes and absent in, but recognised by the host (Reviewed in (Schwessinger and Zipfel, 2008)). *Pseudomonas* flagellin is a well-characterised PAMP. Flagellin elicitor activity has been shown to reside in a conserved N-terminal domain of 22 amino acids known as flg22 (Gomez-Gomez, 2004; Zipfel and Felix, 2005). It has been demonstrated that the *A. thaliana* resistance gene NONHOST1 (NHO1) is transcriptionally activated by flagellin and limits the ability of the non-host *P. syringae* pv. tomato to infect *A. thaliana* (Li et al., 2005). Not all PAMPs are extracellular molecules; the bacterial translation elongation factor Tu (EF-Tu) is an example of an intracellular PAMP, but the N-acetylated N-terminal 18 amino acids of EF-Tu (elf18) are recognised by the *Brassicacae* (Zipfel and Felix, 2005)

Plant-derived elicitors are 'self' molecules that are displaced, degraded or inappropriately processed, potentially by pathogens. These are recognised by plant 'danger' receptors, which

then elicit the immune response. Plants have been shown to recognise and respond to pectate oligomers released through pathogen degradation of the cell wall (Dumville and Fry, 2000). Although *P. syringae* does not produce pectate lyase in particular, these bacteria do produce lipases and proteases which could have similar consequences by damaging membranes or releasing peptides (Kaffarnik et al., 2009). Basal defences are rapidly deployed and can be detected as changes in plant signal transduction and gene expression after only 2 and 15 minutes exposure to bacterial elicitors, respectively (de Torres et al., 2003; Gomez-Gomez, 2004). Resistance is highly localised and is manifested as structural, chemical and transcriptional changes. Cell wall alterations and the formation of callose-rich papilla are thought to restrict water and nutrient supply to the bacteria (Truman et al., 2006), while the production of ROS is an important stress signal. Increased defence gene expression has also been widely reported (reviewed in (Dangl and Jones, 2001).

Host-specific and induced defences.

Host-specific defence occurs when injected bacterial 'effector' proteins are detected inside a resistant cell by a plant 'surveillance system' of resistance (R) proteins and in this context effector proteins are generally referred to as avirulence or Avr proteins. Plant R proteins are believed to guard components of the plant defence machinery, known as virulence targets, which are targeted by Avr proteins (Alfano and Collmer, 2004; Jones and Dangl, 2006). Recognition of Avr proteins is gene-for-gene dependent, occurring only if the plant possesses a corresponding R gene (Grant et al., 2006; Cunnac et al., 2009), and many of these Avr/R-gene combinations have now been characterised (Dangl and Jones, 2001). In the simplest known example, the R protein Pto directly recognises the *P. syringae* effector AvrPto and initiates a signalling transduction cascade through another R protein Prf that leads to disease resistance (Jones and Dangl, 2006). For the majority of known examples it appears that recognition of effectors occurs by an indirect mechanism which has been termed the 'guard hypothesis' (Dangl and Jones, 2001). This hypothesis proposes that R proteins monitor the homeostasis of their virulence target and activate plant defences when they detect an Avr protein-induced change. For example, in *A. thaliana* the R proteins RPS2 and RPM1 trigger plant defences in response to the disappearance of their virulence target RIN4, caused by the P.s.t effector AvrRpt2 (Ellis and Dodds, 2003). More recently the alternative, (although not mutually exclusive) 'decoy hypothesis' has been proposed which argues that some virulence targets act as decoys to detect pathogen effectors via R proteins (Van der Hoorn and Kamoun, 2008) although this remains to be experimentally demonstrated (Hogenhout et al., 2009).

Recognition of a bacterial Avr protein by the corresponding plant R protein initiates a wave of plant defences including the hypersensitive response (HR), a rapid and localised programmed cell death (PCD) of plant cells in the region around the bacteria (He, 1996). The primary plant cells undergoing PCD release reative oxygen species (ROS) and nitric oxide, thereby stimulating additional responses in surrounding cells (Bretz et al., 2003). The ultimate effect of the HR is to limit further growth of the bacteria within the plant. The precise mechanism by which this limitation occurs remains unclear, but is most likely as a result of increased water stress (Wright and Beattie, 2004; Freeman and Beattie, In press), accumulation of phytoalexins and other toxic compounds, such as cyanide (a co-product of ethylene synthesis) in the apoplast (Tsuji et al., 1992; Hammerschmidt, 1999; Adie et al., 2007), and / or trapping bacteria in the outer plant cell wall (Agrios, 2005)

Induced defences are also stimulated by cell damage, regardless of the cause and whether alternative defences are activated. Generally this defence is systemic and increases the level of plant resistance against future pathogen attack in any part of the plant. Systemic acquired resistance (SAR) confers long-lasting protection against a broad spectrum of microorganisms (Vlot et al., 2008). SAR requires the signal molecule salicylic acid (SA) and is associated with accumulation of pathogenesis-related proteins, which are thought to contribute to defence. Resistance is long-lasting, sometimes for the lifetime of the plant, and effective against a broad spectrum of pathogens including viruses, bacteria (including *P. syringae*), fungi, and oomycetes.

Understanding the array of defence pathways is complicated by the cross talk which occurs naturally between them. Research into the plant hormones ethylene (ET), jasmonic acid (JA) and salicylic acid (SA), which are important signals for inducing defence responses through hormone signalling pathways, has indicated that cross talk between these pathways allows the plant to tailor the defence response towards a particular threat. It is generally accepted that the SA pathway is primarily linked to biotrophic pathogen resistance while the JA and ET pathways mediate resistance to necrotrophic pathogens. This indicates that the activation of a particular defence pathway depends on the particular pathogen and its lifestyle. In addition, the SA and JA/ET pathways are mutually antagonistic (Figure 1.3). For example, in *A. thaliana*, ET-sensitivity mutants show increased SA-dependent defences, while transgenic plants affected in SA accumulation or signalling show increased expression of ET-dependent PR genes (Adie et al., 2007; Lopez et al., 2008). However, cooperative interactions have also been reported between SA and ET, including the *hrl1* (*hypersensitive response-like lesions1*) mutant in *A. thaliana*, which shows constitutive expression of SA and ET/JA defence genes, increased

accumulation of both SA and ET, and increased resistance to *P. syringae* (Devadas and Raina, 2002).

Figure removed for copyright reasons

Figure 1.3
Plant defence responses involving jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and salicylic acid (SA). Synthesis of the hormones JA, ET, ABA or SA can be induced by biotic stress including pathogen attack, herbivory and wounding. In general ET and JA are considered to cooperate, (for example through ERF1 above), in the induction of defences against necrotrophic pathogens while repressing defences against biotrophic pathogens and wounding. In contrast SA induces defences against biotrophic pathogens (for example via WRKY70 above) while repressing defences against necrotrophic pathogens. Defence in response to wounding is dependent on JA, which can regulate genes through intermediates such as the transcription factor AtMYC2. Adapted from (Adie et al., 2007)

The natural cross talk that occurs in plants during fine-tuning of defence responses can also be exploited by pathogens as a virulence strategy (Lopez et al., 2008; Spoel and Dong, 2008). For example, using coronatine-deficient *P. syringae* mutants and plants impaired in JA signalling, it has been shown that P.s.t exploits the reciprocal inhibition of the SA and JA pathways by using the JA-mimic coronatine (COR) to suppress SA-mediated defences, thereby inducing systemic susceptibility to future bacterial infection (Zhao et al., 2003; Cui et al., 2005). It is also interesting to note that production of ET has been reported by the fungal pathogen *Botrytis cinerea* and the bacterial pathogens *Ralstonia solanacearum* and certain pathovars of *P. syringae* (Weingart et al., 1999) representing another potential mechanism for pathogens to interfere with host-defence signalling.

As well as modulation by pathogens, plant defence responses have also been shown to vary according to the age, nutrition and environmental conditions of the plant and the number of cell damage sources occurring at once, such as damage by herbivores or pathogens, and environmental damage such as frost (Bolwell et al., 2002; Heil and Bostock, 2002; Alfano and Collmer, 2004; Spoel and Dong, 2008).

Several plant species have been reported to show increased pathogen resistance as they mature. A number of genes with possible defence functions are known to be expressed late in plant development including some pathogenesis-related (PR) and PR-like genes (Hanfrey et al., 1996), and this correlates with increased resistance to some bacterial and fungal pathogens. However, an age-related increase in resistance to P.s.t was seen in *A. thaliana npr1-1* plants which were deficient in PR-gene expression (Cao et al., 1994) but not in *NahG* plants which were defective in both PR-gene expression and SA production (Kus et al., 2002). *NahG* plants also failed to accumulate extracellular antimicrobials in response to P.s.t (Kus et al., 2002). These results therefore highlight the importance of a functional SA pathway in defence responses.

1.4 Infection of plants by *Pseudomonas syringae.*

Plant-pathogenic bacteria such as *P. syringae* have evolved an arsenal of mechanisms in order to overcome the defences of host plants (summarised in Figure 1.4). These include the type three secretion system (T3SS), used to deliver effector proteins directly into plant cells, along with secreted proteins such as phytotoxins which are reviewed in more detail below.

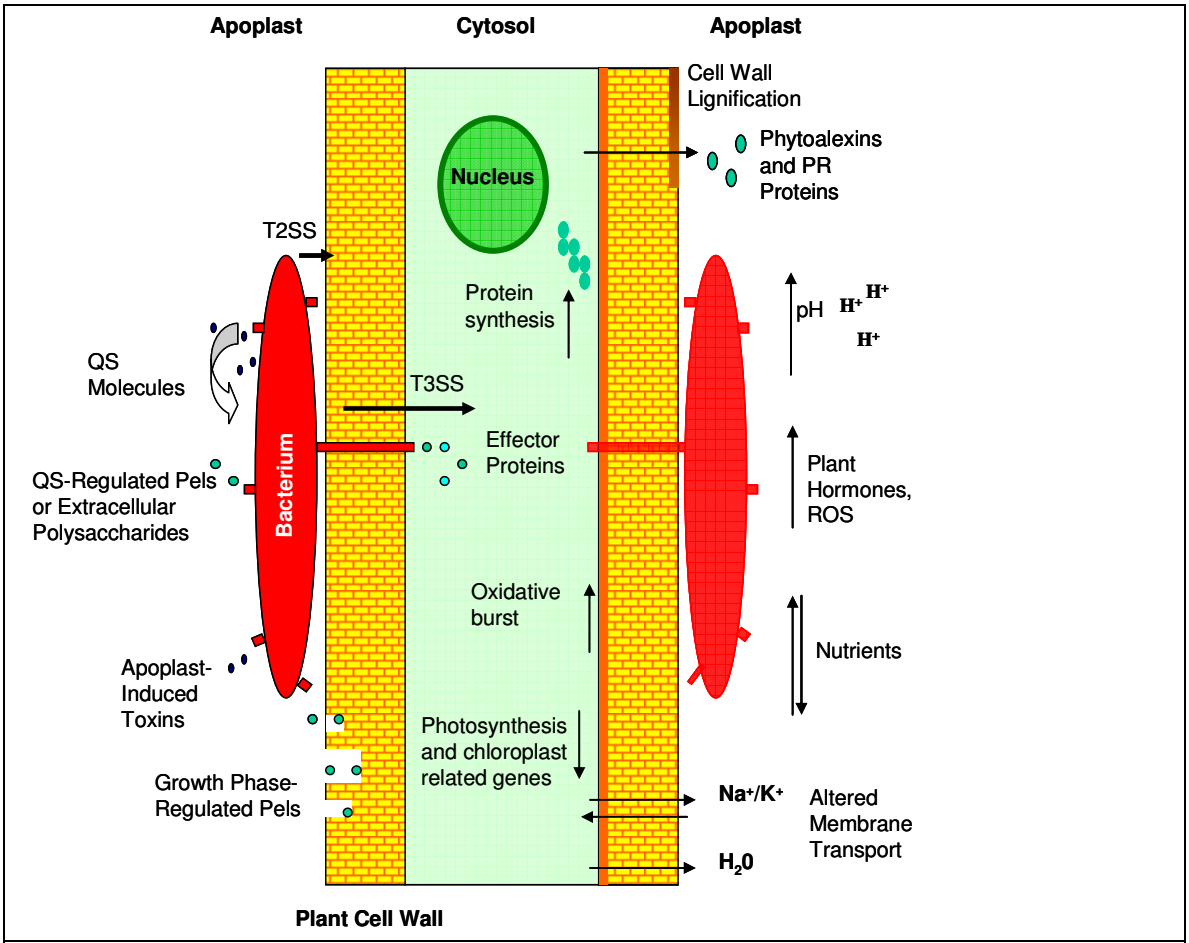


Figure 1.4
Summary of bacterial-plant interactions in the apoplast. *P. syringae* can interact with host cells through the T3SS which is used to inject effector proteins into the host cell cytoplasm. These proteins have a range of effects in host cells including suppressing plant defences such as PR proteins. *P. syringae* can also secrete proteins directly into the apoplast. The net result of this modulation is that components of the apoplast environment, such as the nutrient composition or pH, are altered during infection. QS=quorum sensing, ROS=reactive oxygen species, PR= pathogenesis-related. Figure by Rachel Jones, published in: Adaptation to the plant apoplast by plant pathogenic bacteria (Rico et al., 2008)

1.4.1 The type three secretion system (T3SS).

The injection of effector proteins into plant cells is a vital step in pathogenesis for many phytopathogens (Alfano and Collmer, 2004). These effectors are secreted through the type T3SS, encoded by conserved *hrp* (hypersensitive response and pathogenicity) genes. Environmental conditions within the apoplast, particularly the low pH and low nitrogen content, induce *hrp* gene transcription leading to T3SS construction and, subsequently, effector secretion. Around 60 Hrp-regulated proteins and effectors have been identified in *P. syringae* that can travel the T3SS into host cells (Ramos et al., 2007). The T3SS is a widely used virulence strategy and *hrp* genes are thought to be present in the majority of *P. syringae*

pathovars, although naturally occurring *P. syringae* isolates have been found to lack both the T3SS and effectors (Mohr et al., 2008). The T3SS has also been reported in many other phytopathogens, and has been particularly well characterised in species of *Xanthomonas, Ralstonia* and plant-pathogenic enterobacteria (reviewed by Mudgett (2005)). T3SS secreted effectors are often unique to particular pathogens (Cunnac et al., 2009). T3SS genes have also been found in non-pathogenic *Pseudomonads* and symbiotic bacteria, such as nitrogen-fixing *Rhizobia*, where they may mediate interactions with other microorganisms, mediate symbiotic interactions with plant cells, or reflect an underlying predisposition to pathogenicity (Preston, 2004).

1.4.2 T3SS- effectors.

Effectors target a diverse range of plant cell components and processes including signalling molecules, parts of the cytoskeleton and photosynthesis, with the overall aim of subverting host defences, signalling and metabolism to the benefit of the bacterium situated outside the cell (Grant et al., 2006; Thilmony et al., 2006; Cunnac et al., 2009). Mutants which are unable to produce structural components of the TTSS, such as the major subunit protein HrpA, are non-pathogenic (Hueck, 1998), and no longer secrete effectors (Wei et al., 2000). Mutants unable to express one or more effectors have typically subtle phenotypes, thought to be due to effector redundancy, usually only seen as reductions in pathogen growth in host tissues (Deng et al., 1998). However, the importance of individual effector proteins in controlling the outcome of pathogenesis can be seen when specific effectors from non-host pathogens are transformed into other pathovars of *P. syringae* or non pathogenic *P. fluorescens*; when these bacteria are inoculated into the host plant the HR replaces pathogenesis as the outcome of infection (Huang et al., 1988; Badel et al., 2002). Although effectors have been the subject of studies for many years it is only recently that details about the precise action of these effector proteins inside host cells have begun to be elucidated. For example, the *P. syringae* effector HopM1 is known to target the *A. thaliana* defence protein AtMIN7 (underlined in Figure 1.4). AtMIN7 normally functions as part of the vesicle trafficking pathway that builds up cell-wall defences in response to a pathogen (DebRoy et al., 2004). Correspondingly, *P. syringae* mutants that were unable to produce HopM1 triggered enhanced callose deposition during infection of wild type *A. thaliana* (Nomura et al., 2006). Using a series of *A. thaliana* AtMIN7 and *P. syringae* HopM1 mutants, Nomura and coworkers (2006) were able to demonstrate that HopM1 functions as a 'targeting' protein that facilitates the destruction of AtMIN7 by marking it for destruction by the host ubiquitination/proteosome system (Nomura et al., 2006).

The interactions between *P. syringae* effectors and their host virulence targets are complex. In several cases, it appears that multiple effectors from within the same *P. syringae* strain may all target a single protein. For example, the *A. thaliana* virulence target RIN4 is targeted by the three unrelated P.s.t effectors; AvrRpt2, AvrB and AvrRpt2 (Figure 1.5), and is guarded by at least two host R proteins (RPM1 and RPS2) (Ellis and Dodds, 2003; Kim et al., 2005). However, despite extensive research in this area, the molecular basis by which effectors attempt to modulate virulence targets, and by which R proteins detect each effector, remains largely unclear (Hogenhout et al., 2009).

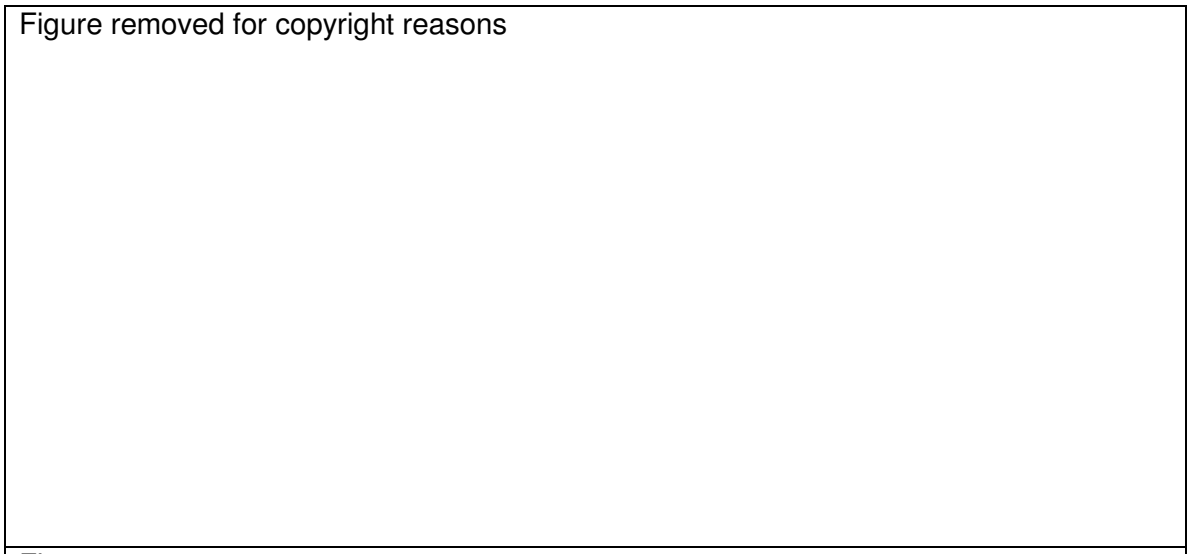


Figure 1.5
Effectors of plant pathogenic bacteria (red) and their targets within plant cells (yellow). Adapted from Speth and coworkers (2007). The *P. syringae* effector HopU1 is an ADP-ribosyltransferase which is thought to interfere with RNA binding (Fu et al., 2007). HopI1 has been shown to target the chloroplast and is thought to interfere with plant photosynthesis (Jelenska et al., 2007). HopM1 targets AtMIN7, located in the plant secretion pathway, to the proteome (Nomura et al., 2006). *P. syringae* effectors AvrPto, AvrPtoB, and HopAI1all interfere with signal transduction initiated by the flagellin receptor FLS2. AvrRpt2, AvrB, and AvrRpm1 target RIN4, a negative regulator of plant defence (Ellis and Dodds, 2003; Kim et al., 2005). In addition several *Xanthomonas* effectors, XopD, PthXo1 and AvrBs3, have been shown to localize to the plant cell nucleus where they stimulate transcription of specific host genes.

The pathogenicity of *P. syringae* is determined by the evolution of novel effectors that evade or suppress detection by existing R proteins. Conversely, plant resistance requires the innovation of complementary R proteins for these effectors (Figure 1.6). This has led to an evolutionary 'arms race' between pathogens and disease resistant plants. Evidence of this was demonstrated experimentally by Pitman and coworkers (2005). The AvrPphB protein of P.s.ph triggers defence in beans that carry the R3 resistance protein. When passaged through

resistant plants P.s.ph rapidly lost the *avrPphB* gene and after the 5th passage 98 % of colonies were capable of causing disease (Pitman et al., 2005).

Figure removed for copyright reasons

Figure 1.6
Disease resistance and susceptibility. Plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, diamonds) to trigger PAMP-triggered immunity (PTI). Successful pathogens overcome PTI by delivering effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal; resulting in effector-triggered susceptibility (ETS). However, plants are constantly evolving to detect effectors, and when detected by the plant an effector (indicated by a circle) activates effector-triggered immunity (ETI) in the plant, (an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR)). The detection of an effector results in selection for pathogen isolates which have lost that effector, (and perhaps gained new effectors through horizontal gene transfer— these can help pathogens to suppress ETI. Selection then favours new plant R-genes that can recognize one of the newly acquired effectors, resulting again in ETI (Jones and Dangl, 2006)

1.4.3 Phytotoxins.

In addition to a functional T3SS, phytotoxins are known to contribute to the virulence of most strains of *P. syringae* within plants (Gross, 1991). The most intensively-studied *P. syringae* phytotoxins are coronatine (COR), syringomycin, syringotoxin, tabtoxin, and phaseolotoxin. Some of these phytotoxins, like COR, are associated with numerous *P. syringae* pathovars while the distribution of others, like tabtoxin, is more restricted (Bender et al., 1999).

Phytotoxins exert a range of effects on plants, many of which are still being discovered. Probably the most widely studied *P. syringae* phytotoxin is COR, a molecular mimic of the plant hormone jasmonic acid (JA). JA is most commonly induced during wounding or herbivore attack but does not appear to contribute to plant resistance against *P. syringae*. The initiation of JA-signalling in host plants appears to indirectly benefit *P. syringae*, by suppressing salicylic acid (SA)-mediated defences, such as pathogenesis-related (PR) gene expression, which contribute to plant resistance against bacteria (Brooks et al., 2005). COR is also known to affect other host pathways *in planta*, including polyamine biosynthesis genes, which are upregulated in *A. thaliana* infected with P.s.t but not with a P.s.t-COR mutant (Uppalapati et al., 2008).

Syringomycin and syringotoxin are produced by most strains of P.s.s, (Morgan and Chatterjee, 1988) and form pores in plasma membranes leading to electrolyte leakage, while tabtoxin (P.s.tabaci) and phaseolotoxin (P.s.ph) are strongly antimicrobial, and function by inhibiting glutamine synthetase and ornithine carbamoyltransferase, respectively (Bender et al., 1999).

Although the contribution of phytotoxins to bacterial virulence has been mainly studied in an endophytic context, the actions of some phytotoxins may also be important for survival on the leaf surface. For example, syringomycin is synthesised by non-pathogenic, as well as pathogenic, strains of P.s.s at levels high enough to form pores in plant cell membranes (Hutchison et al., 1995). As non-pathogenic strains of P.s.s are restricted to the epiphytic niche, syringomycin synthesis may trigger the release of plant metabolites from epidermal cells onto the leaf surface. Additionally, syringomycin may act as a surfactant for *P. syringae*; the leaf cuticle is hydrophobic and it is thought that increasing the wettability of this environment allows the solubilisation and diffusion of substrates, making them more readily available to epiphytic bacteria. Surfactants may also increase the movement of *P. syringae* across the leaf surface (Hutchison et al., 1995; Lindow and Brandl, 2003).

Recently a novel function for the phytotoxin COR was reported in the reopening of stomata by P.s.t following PAMP-induced closure (Melotto et al., 2006). Melotto and coworkers infected light-adapted *A. thaliana*, in which 70-80 % of leaf stomata were open, with P.s.t, a P.s.t COR-deficient mutant, or the human pathogenic *E. coli* O157:H7 which is also unable to produce COR. When inoculated with either P.s.t or *E. coli* (or bacterial PAMPs), extensive stomatal closure was observed within one hour. However, for P.s.t inoculations this closure was transient and stomata re-opened within three hours, while for *E. coli* stomatal closure persisted for up to 8 hours post inoculation. This led to the hypothesis that stomatal closure is a non specific defence response to both plant and human pathogenic bacteria, but that plant-pathogenic P.s.t has evolved a way to induce reopening of stomata. The role of COR in stomatal reopening was seen when light-adapted *A. thaliana* was inoculated with the COR-deficient mutant, as in this interaction stomata failed to reopen again within three hours. Further experiments demonstrated that an avirulent P.s.t-*hrp* mutant, which was unable to produce the T3SS but could still produce COR, retained the ability to induce stomatal reopening within three hours. These results demonstrate that COR is at least one of the virulence factors involved in suppressing stomatal closure in P.s.t, and that this ability is not reliant on a functional T3SS (Melotto et al., 2006). This finding is particularly interesting due to the fact that COR production is widespread among *P. syringae* pathovars (Bender et al., 1999). Unexpectedly, one of the two gene clusters

required for coronatine synthesis in *P. syringae* was also detected in the soft-rot bacterium *Pectobacterium atrosepticum* (formerly *Erwinia carotovora* subsp. *atroseptica*) and was subsequently shown to be important for virulence, possibly through the production of an alternative phytotoxin (Bell et al., 2004; Liu et al., 2008).

It was also recently demonstrated that the vascular pathogen *Xanthomonas campestris* (*X. campestris*) possesses a similar capacity to re-open stomata (Gudesblat et al., 2009). An *X. campestris*-secreted extract inoculated at the same time as the P.s.t COR-deficient mutant on to *A. thaliana*, resulted in enhanced stomatal re-opening after three hours and subsequently enhanced disease (Gudesblat et al., 2009). The identity of the active molecule or molecules present in the *X. campestris* extract remains unknown. However, as *X. campestris* does not produce COR, or possess orthologs of the *P.syringae* coronatine-synthesis genes, these results indicate that the ability to overcome defence-induced stomatal closure may be common to many bacterial phytopathogens, through many different mechanisms.

1.5 Physiological adaptations to a plant-associated lifestyle.

The plant phyllosphere (aerial parts) provides an important niche for microbial inhabitation (Lindow and Brandl, 2003). *P. syringae* occupies two ecological niches within the phyllosphere, the epiphytic foliar surfaces and endophytic apoplast (Preston, 2004). The leaf surface has been extensively studied in terms of the number, composition and distribution of species present (Beattie and Lindow, 1999; Hirano and Upper, 2000). *P. syringae* can grow epiphytically on both host and non-host plants, and the propensity for epiphytic populations to provide inoculum for subsequent infection of the plant under appropriate conditions has stimulated research in this area (Hirano and Upper, 2000).

1.5.1 Life on the surface – epiphytic growth.

There are many ways in which *P. syringae* may arrive on the leaf surface (Hirano and Upper, 2000). For example, bacteria may be present on the surface of seeds; once the seedlings emerge *P. syringae* is able to colonise leaves as soon as they appear (Hirano et al., 1997). *P. syringae* may also arrive through aerial dispersion, either by long distance mechanisms of evaporation and dissemination throughout clouds (Morris et al., 2008), or locally from neighboring plants (Lindemann et al., 1984). Alternatively, bacteria may be transported between plants by vectors such as insects (Beattie and Lindow, 1995; Hirano and Upper, 2000).

Environmental conditions on the surface of the leaf fluctuate massively, particularly on a microbial scale. Exposed to the atmosphere, epiphytes experience rapid changes in microclimate such as temperature, UV radiation, relative humidity and moisture (Marco et al., 2005; Gunasekera and Sundin, 2006). In addition, the uneven topography of the leaf, comprising a variety of surface structures such as trichomes and veins, along with surface waxes, means that exposure to these environmental conditions is not experienced evenly by all bacteria inhabiting the surface (Hirano and Upper, 2000).

Nutritional availability on the leaf surface also appears to be extremely heterogeneous. Leveau and coworkers (2001) monitored sugar availability on the surface of bean plants using a *Pantoea agglomerans* GFP bioreporter. Although for the first hour after inoculation, nearly all bioreporter cells appeared to be actively engaged in fructose consumption, only 1% of bacteria were in areas of the leaf surface where fructose was abundant enough to support consumption for more than 24 hours (Leveau and Lindow, 2001). Furthermore, even when nutrients are present on the surface of leaves, studies have hypothesised that much of this may be in locations that are inaccessible to the majority of bacteria (Mercier and Lindow, 2000). Some authors have indirectly measured amino acid abundance on the leaf surface; for example, the histidine utilisation genes of *P. fluorescens* SBW25 were active on the plant surface. However, the amount of histidine present in the plant environment was estimated to be low, 0.6 µg histidine ml^{-1} (3 µM), and the histidine utilisation genes were not essential for plant colonisation by P.fl SBW25 (Zhang et al., 2006).

However, some researchers believe that the actual conditions experienced by epiphytic bacteria may be quite different from those estimated from large-scale measurements, since the scale of the bacterial microhabitat is smaller than that of even the smallest physical probes (Lindow and Brandl, 2003). Several results support this argument. For example, Beattie and coworkers (2002) inoculated the surface of leaves with *Pantoea agglomerans* containing a GFP reporter, which responded to water availability. Results showed that the majority of *P. agglomerans* cells on dry leaves did not experience the water stress that might be expected, suggesting that the surface of the leaf may be buffered from the atmosphere away from the leaf (Beattie and Axtell, 2002). In addition, the most common sites of bacterial colonisation on the leaf are in the epidermal cell wall junctions, along veins, at the base of trichomes, and around natural openings in the leaf such as hydathodes and stomata, areas which may be relatively protected or nutritionally abundant (Figure 1.7), also reviewed by Whipps (2008).

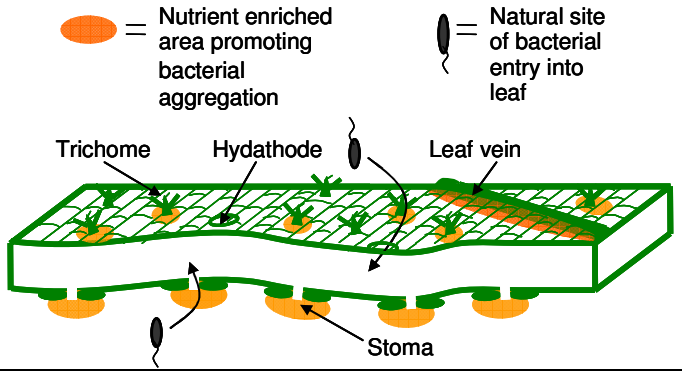


Figure 1.7
Potential sites of increased nutrient availability on the leaf surface. Nutrients are thought to accumulate around leaf-surface structures such as trichomes or leaf veins. In addition nutrients may 'leak' onto the surface from natural openings such as hydathodes or stomata. Figure by Rachel Jones, published in; Adaptation to the plant apoplast by plant pathogenic bacteria (Rico et al., 2008).

Although the plant cuticle presents a considerable barrier to the diffusion of large molecules such as sugars from within the plant to the leaf surface, nutrients can seep out of the interior of a plant through natural openings including hydathodes and stomata. Therefore the areas around these natural openings may constitute replenishable nutrient patches for those bacteria that land nearby or are capable of moving towards them (Lindow and Brandl, 2003; Melotto et al., 2008). This may have the additional benefit to phytopathogenic bacteria, like *P. syringae*, of providing a chemotactic signal towards a natural or wound site for entry into the leaf apoplast (Schulze-Lefert and Robatzek, 2006; Melotto et al., 2008).

Non-pathogenic, epiphytic *Pseudomonas* species also appear to possess mechanisms to gain apoplastic nutrients without crossing the leaf epidermis. When *P. fluorescens* Bk3, marked with a lux reporter, was inoculated onto the surface of extracted, intact, plant cuticle membranes, the bacteria were able to access nutrient solutions placed in contact with the apoplastic side of the cuticle, as measured by an increase in lux activity (Singh et al., 2004). In addition, more than 46 bacterial extracellular proteins were detected to have crossed the cuticle into the nutrient solution, including predicted amino acid binding proteins, enzymes and a porin-like protein, which are thought to contribute to nutrient leakage onto the cuticle surface (Singh et al., 2004).

A habitat-inducible promoter-trapping strategy revealed that a diverse range of genes are specifically activated in P.s.s during epiphytic colonisation of bean leaves. These genes were associated with diverse cellular functions including virulence, transcriptional regulation, transport and nutrient acquisition (Marco et al., 2005). In particular, various mechanisms of

stress tolerance or avoidance are employed by *P. syringae* during epiphytic colonisation (Figure 1.8). These include increased cell aggregation (Monier and Lindow, 2003; Monier and Lindow, 2005), inducible exopolysaccharides (EPS) (Keith and Bender, 1999), surfactants (Hutchison et al., 1995), UV-protective pigments (Jacobs and Sundin, 2001), and auxin production (Glickmann et al., 1998), many of which contribute to epiphytic fitness and may be regulated by quorum sensing, a system whereby the release and detection of signaling molecules allows bacteria to regulate gene expression in a density-dependent manner (Quinones et al., 2005; Dulla and Lindow, 2008).

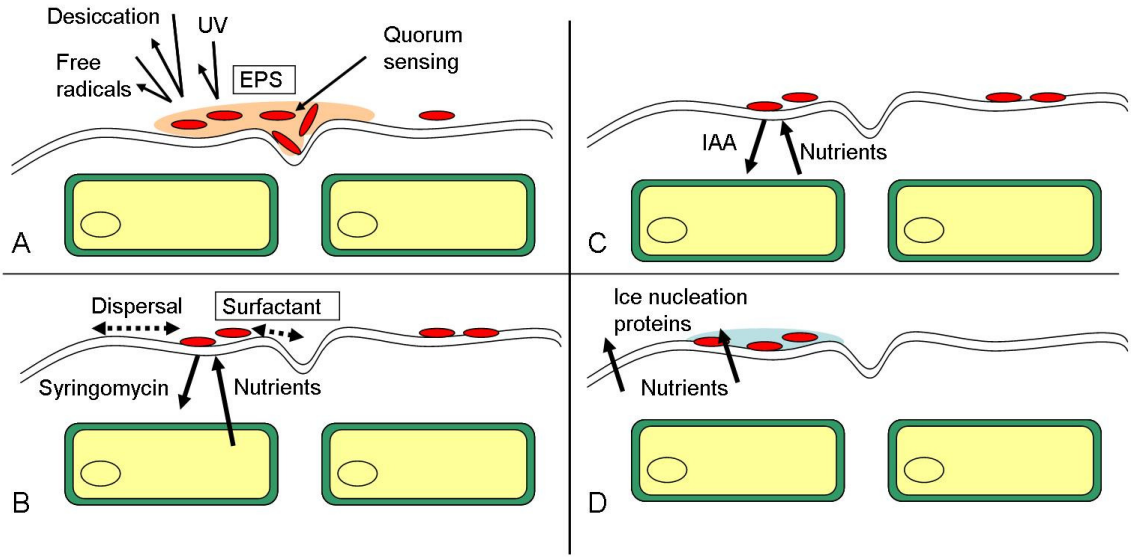


Figure 1.8
P. syringae may employ numerous mechanisms of tolerance, avoidance or habitat alteration in the epiphytic niche. For example: protection from environmental stresses via production of extracellular polysaccharides (EPS) in bacterial aggregates (A). The P.s.s-produced toxin syringomycin may stimulate the release of nutrients from plant cells by forming pores in their cell membrane, and potentially acts as a surfactant thereby aiding dispersal of bacteria across the cuticle (B). *P. syringae* may secrete auxin, causing the release of saccharides from the plant cell wall (C). Ice nucleation proteins promote the formation of ice thereby damaging plant epithelia and making nutrients available to bacteria on the surface (D). Figure by Rachel Jones, adapted from (Lindow and Brandl, 2003).

Epiphytic populations of *P. syringae* provide inoculum for outbreaks of disease (Hirano et al., 1999) and a large epiphytic population precedes disease outbreaks (Rouse et al., 1985). Therefore, following a successful period of proliferation as an epiphyte, and with the right environmental conditions, *P. syringae* may gain entry to the leaf apoplast and initiate a period of endophytic growth.

1.5.2 Life within a leaf – apoplast colonisation.

Arguably the most effective form of abiotic stress avoidance is localisation within a leaf. Many of the damaging environmental conditions faced by epiphytes do not apply or are reduced in the apoplast, and conditions are therefore comparatively stable. Unlike colonisation of the epiphytic niche, the array of innate and induced plant defences prevents most bacteria from proliferating within the plant interior (Sigee, 1993; Wilson et al., 1999). For example, studies have shown that although many saprophytic and human-pathogenic bacteria are capable of colonising the plant surface, they are unable to proliferate within the plant apoplast, even when manually infiltrated (Beattie and Lindow, 1995; Wilson et al., 1999; Brandl, 2006). Similarly, the majority of naturally plant-associated bacteria are strictly epiphytic, completing their life cycle on the plant surface without colonising, or without being able to colonise, the leaf apoplast (Beattie and Lindow, 1995; Beattie and Lindow, 1999).

The T3SS and related virulence determinants discussed above allow virulent strains of *P. syringae* to overcome or suppress the host plant defences and proliferate in the host plant apoplast. However, in order to colonise the apoplast, *P. syringae* must first cross the surface cuticle and epidermis. This may be achieved though wound sites and/or natural openings. Wound sites can be caused by feeding insects, mechanical or frost damage (natural or through ice-nucleation activities), or by natural leaf abscission. Natural openings include hydathodes (water-exuding pores at the edges of leaves), trichomes (leaf hairs formed by specialised epidermal cells) and stomata (openings in the leaf surface that control gas exchange and transpiration between the plant interior and the environment) (Hirano and Upper, 1990; Hirano and Upper, 2000). In addition, and in contrast to many plant-pathogenic bacteria, *P. syringae* pv. mori has been shown to infect the leaves of mulberry plants through cystoliths, inorganic crystals on the leaf surface, rather than trichomes or stomata (Gupta et al., 1995).

Stomata are the most common natural openings on the mature leaf surface and are therefore thought to represent one of the most important routes of bacterial entry. This hypothesis was supported by the discovery of a role for the P.s.t toxin coronatine (COR) in re-opening stomata following PAMP-triggered closure (Melotto et al., 2006). Trichomes also represent an important route, particularly into seedlings, which have few stomata (Schneider and Grogan, 1977), and it has been observed that *A. thaliana* mutants without trichomes are rarely infected by P.s.t in seedling infection assays (Preston and McWatters, unpublished).

Furthermore, modulation of host metabolism by T3SS-secreted effector proteins and phytotoxins may result in the release of nutrients into the apoplast from plant cells, thereby creating an ideal apoplast environment for *P. syringae*. We know very little about the physiological adaptations that allow *P. syringae* to successfully survive conditions within the endophytic niche. However, *P. syringae* must be able to tolerate the environmental conditions initially present in the apoplast including constitutive and induced antimicrobial compounds, and factors such as low pH, high osmolarity, and restricted nutrient types and/or abundance (Alfano and Collmer, 1996).

Recently, Rico and Preston (2008), used a series of BIOLOG assays to demonstrate that *P. syringae* is adapted to use amino acids that are abundant in the tomato leaf apoplast (Rico and Preston, 2008). Firstly, a comparative assay was carried out to compare the ability of *P. syringae*, *P. fluorescens, P. putida* and *P. aeruginosa* to metabolise compounds present in BIOLOG plates as carbon or nitrogen sources. Results showed that *P. syringae* used a restricted range of nutrient sources compared to other *Pseudomonas,* particularly amino acids. Secondly, in a modified BIOLOG assay nutrient assimilation pathways were 'pre-induced' in *P. syringae* by growing bacteria in extracted tomato apoplast, or the synthetic media Hrp-inducing minimal medium (nutrient poor) or LB (nutrient rich), and tetracycline was then added to prevent further protein synthesis. Bacteria were subsequently assayed for their ability to metabolise a range of carbon or nitrogen sources (Rico and Preston, 2008). Results demonstrated that *P. syringae* was able to utilise different amino acids depending on whether the pre-incubation step was carried out in apoplast extract or synthetic media, and also that the amino acids which *P. syringae* could assimilate following apoplast incubation correlated with those which are known to be abundant in the tomato apoplast (Rico and Preston, 2008). Therefore it was hypothesised that the nutrient assimilation pathways present in *P. syringae* have evolved to reflect the nutrients available in the plant apoplast, and many of these assimilation pathways are specifically activated in response to components of the apoplast, rather than other synthetic media (Rico and Preston, 2008).

Various studies have indicated the ability of *P. syringae* to modify the apoplast environment. Wright and coworkers (2004) used a water stress-responsive transcriptional fusion to show that P.s.t can modify the water potential of the apoplast during pathogenesis. The water potential sensed by wild type P.s.t, or a P.s.t mutant containing an avirulence gene, was monitored during the resulting compatible (pathogenesis) and incompatible (HR) interactions with *A. thaliana*. During an incompatible interaction, the apoplastic water potential decreased rapidly,

and the avirulent P.s.t encountered low water potentials at values which were shown to dramatically hinder growth *in vitro*. In contrast, during pathogenesis, the water potentials experienced by wild type P.s.t were significantly higher, at values similar to those which supported optimal growth of this bacterium *in vitro* (Wright and Beattie, 2004).

In addition, there is growing evidence that one of the effects of T3SS effector-modulation of plant signalling and metabolism is to promote pathogen nutrition. For example, a major target for effectors secreted by *P. syringae* is the abscisic acid (ABA) signalling pathway, and biosynthesis of ABA increases dramatically in *A. thaliana* plants infected with P.s.t (de Torres-Zabala et al., 2007). ABA is normally associated with responses to abiotic stress and so T3SS effector-mediated increases in ABA may exploit endogenous stress pathways to compensate for an enhanced metabolic load associated with pathogen nutrition in the apoplast (de Torres-Zabala et al., 2007). Similarly, the *P. syringae* effector AvrRpt2 targets the auxin pathway (Chen et al., 2007). Increased auxin levels are known to stimulate various physiological effects including cell wall loosening and changes in membrane permeability (Rose et al., 2004), which may result in the leakage of water and/or nutrients into the apoplast (Chen et al., 2007). Most recently, *P. syringae* effector proteins have been shown to alter the protein composition of the apoplast during pathogenesis of *A. thaliana* (Kaffarnik et al., 2009). The extracellular accumulation of 45 proteins was monitored during the compatible and incompatible infection of *A. thaliana* with various strains of *Pseudomonas.* In particular, when plants were infected with virulent P.s.t (a compatible interaction resulting in disease), researchers observed the extracellular accumulation of a particular subset of host proteins, all lacking signal peptides, indicating that P.s.t may be manipulating host secretion to promote pathogenesis (Kaffarnik et al., 2009). However, the precise way in which the altered apoplast proteome may benefit the colonising bacteria was not established.

Apoplast alkalinization by *P. syringae* has also been hypothesised to modify the nutritional environment of the apoplast. Raising the apoplastic pH has been shown to decrease the activity of symporters directing sucrose into the phloem, and therefore increase the apoplastic sucrose levels (Atkinson and Baker, 1987).

1.6 Plant physiology during infection.

We know most about the wide range of metabolic and physiological changes occurring in plants during infection, rather than the colonising bacteria. Affymetrix chip analysis revealed 2800

A. thaliana genes with a diverse range of functions that were regulated in response to P.s.t infection (Thilmony et al., 2006); however at 12 hours post inoculation (hpi) P.s.t had suppressed at least 20% of those plant genes induced at 2 hpi (Truman et al., 2006). In particular the genes suppressed by P.s.t encoded leucine rich repeat receptor proteins and protein phosphatases leading to the suggestion that P.s.t may avoid detection through diminution of extracellular receptors and attenuation of plant kinase signalling pathways (Truman et al., 2006).

The nutritional status of the infected plant has a key role in disease progression. Lesion size during infection changes depending on plant nutrition, and reduced availability of nitrogen has been shown to increase plant susceptibility to diseases (Dietrich et al., 2004). The over application of ammonium-based fertilisers has also been shown to enhance disease susceptibility to both fungal (Long et al., 2000) and bacterial pathogens. Growing fungal and bacterial phytopathogens in nitrogen-limited *in vitro* cultures induces the same pathogenicity genes as those expressed during infection (Solomon and Oliver, 2001). Therefore it has been proposed that entry into the nitrogen limited environment of the plant apoplast is a cue for the expression of genes involved in the early stages of infection. The phytopathogenic fungus species *Colletotrichum* stimulates the accumulation of ammonia in the apoplast of host cells, which enhances virulence by raising the pH to the fungus-secreted pectate lyase optimum, and possibly by altering nutrient availability (Prusky et al., 2001). This raises an interesting question as to whether altering nitrogen availability in the apoplast could be an important trait for successful bacterial pathogens. Use of nitrogen fertilizers has long been linked to increased severity of disease for crop plants and high nitrogen levels *in planta* are known to increase susceptibility of tomato and *A. thaliana* to P.s.t. infection (Rico, Steinbrenner and Preston, unpublished). Use of nitrogen fertilizers has been shown to increase the available nitrogen in the apoplast (see Section 1.7.2 below), suggesting that bacteria may be benefiting from increased nutrients in the apoplast as a result of fertilizer application, possibly resulting in increased proliferation.

Infection by phytopathogenic bacteria alters nitrogen metabolism within plant cells in a manner similar to that seen during natural senescence, particularly in the latter stages of infection. P.s.t infection of tomato induces a novel cytosolic GS1 (glutamine synthetase 1) isoform, highly similar to GS1 which accumulates during natural senescence in tobacco (Pérez-García et al., 1998). GS1 functions in the main pathway for ammonia assimilation in plants, resulting in glutamine and glutamate production, and is thought to generate compounds for nitrogen

transport (Pérez-García et al., 1998). In more recent studies, asparagine synthase (AS) has also been shown to be upregulated, suggesting the existence of a GS1/AS pathway, thus representing a metabolic route for transferring ammonium released from protein catabolism into asparagine, an amino acid that may have a major role in nitrogen mobilisation from diseased tissues (Olea et al., 2004). Moving amino acids out of diseased tissues would involve secreting these compounds into the apoplast where they could be 'hijacked' by proliferating bacteria. Therefore *P. syringae* may be deliberately activating particular senescence pathways within the plant cell, which result in the liberation of nutrients into the apoplast.

1.7 The plant apoplast and *P. syringae*.

Many gaps still exist in our understanding of the plant apoplast, not just as a habitat for pathogens, but also as a storage compartment and dynamic site for reactions and signalling (reviewed in (Sattelmacher, 2001)). Many cases still exist where basic information for apoplast conditions, such as ionic concentrations, are still relatively unknown, or in some cases, have produced widely differing experimental results depending on the technique used. For example, estimates of apoplastic Ca^{2+} range from between 10–30 µM (fluorochrome method) (Mühling et al., 1997), to between 300–800 µM (infiltration/centrifugation method) (Mühling and Sattelmacher , 1995) using different techniques. However, although much still remains to be elucidated, it is fairly well established that the apoplast environment changes throughout infection as a consequence of the metabolic changes occurring within the surrounding plant cells. The pH of the apoplast increases and nutrients differ in both composition and abundance. Other compounds such as ascorbic oxidase, which alters the ascorbic acid-dependent redox state of the apoplast, also change during infection (Pignocchi et al., 2006), although in all of these cases it remains unclear to what degree these changes are the result of deliberate bacterial manipulation or plant defence responses.

1.7.1 The apoplast during infection.

Variation in apoplast pH.

Within the apoplast, pH is an important parameter as it optimises both membrane transport and enzyme activities (Felle et al., 2004). Recording pH measurements from the apoplast has generally relied on the cheap and relatively simple method of extracting the apoplast fluid and measuring the extracted pH *in vitro*. More recently accurate pH measurements have been made *in vivo* by inserting microelectrodes through stomata of intact plants (Felle and Hanstein, 2007). However, results from these experiments have generally confirmed the accuracy of previous pH measurements using apoplast extracts.

The pH of the apoplast is acidic, due to the abundance of organic acids, such as citric or malic acid, and due to proton pumping from nearby cells (Grignon and Sentenac, 1991). The acidity of the apoplast also varies diurnally during natural growth, reflecting changes in membrane permeability, fluctuating between around pH 4.5 during the day and pH 6.5 at night (Grignon and Sentenac, 1991). An increase in apoplastic pH is also known to occur during infection with many endophytic pathogens. When pH was measured in barley leaves infected with the brown rust fungus (*Puccinia hordeii*), the pH of apoplast from infected leaves was ~7·3, compared to ~6·6 in controls (Tetlow and Farrar, 1993).

The naturally acidic apoplast environment has been proposed as one of the reasons why only a small number of bacterial genera have evolved to become plant pathogens, compared to animal pathogens (Llama-Palacios et al., 2003). Interestingly, plants are more resistant to bacteria in the light, when the apoplast is more acidic, than in the dark when the apoplast is more alkaline (Roberts and Paul, 2006). Exactly how light might modulate disease resistance remains unclear. However, research has shown that *A. thaliana* phytochrome mutants are hyper-susceptible to infection with avirulent P.s.t-*avrRpt2* mutants, suggesting that light might be important in the regulation of R-genes (Genoud et al., 2002). In addition, at least one *P. syringae* effector, HopI1, has been shown to target host photosynthesis via chloroplasts (Jelenska et al., 2007). Consequently, alkalinization of the apoplast during infection, caused by photosynthesis suppression by effectors like HopI1, could mimic susceptible night time conditions, thus enhancing the potential for bacterial proliferation. In addition to being a result of photosynthesis suppression, apoplast alkalinization could also be due to bacterial modulation of membrane permeability by phytotoxins, resulting in a K^+ efflux/H^+ influx (Alfano and Collmer, 1996).

Apoplast pH is also an important signalling regulator during the defence responses associated with an incompatible plant-pathogen interaction. Apoplast alkalinization is a result of many defence related signalling changes, including the proton-coupled uptake of salicylic acid (SA). The extent to which SA accumulates in cells depends on the pH of the apoplast (a linear decrease in SA uptake occurs between pH 3.5 and 8.5), and the magnitude of the proton gradient that drives the import mechanism. Extracellular alkalinization induced by the bacterial elicitor harpin during an incompatible plant/pathogen interaction, inhibits SA uptake, promoting SA export. Apoplastic SA can then diffuse into uninfected cells with a lower apoplastic pH and accumulate inside these uninfected cells to activate defence responses (Clarke et al., 2005).

Tolerance of the apoplast environment.

The mechanisms used by *P. syringae* to tolerate pH fluctuations in the apoplast environment are still unknown. However, a vast body of research has been carried out into pH homeostasis in enteric bacteria. One mechanism used by many bacteria to tolerate extreme pH values is the use of decarboxylase and deaminase enzymes to regulate pH. Cells respond to acid stress by consuming acids and excreting volatile CO_2 with alkaline products, whereas under alkali stress cells produce acids while excreting volatile NH_3. The use of amino acid deaminase and decarboxylase enzymes to regulate pH has been extensively studied in enteric bacteria such as *E. coli* (Merrell and Camilli, 2002; Stancik et al., 2002; Saito and Kobayashi, 2003). In acidic environments amino acid decarboxylase genes are expressed at higher levels. Decarboxylation involves splitting of the carboxyl (-COOH) group of an amino acid as CO_2 to produce an amine. The activity of decarboxylases both consumes protons and produces alkaline amines, the net effect of which is to increase (alkalinise) the intracellular pH. Conversely, in alkaline environments the expression of deaminase genes increases. Amino acid deaminases decrease (acidify) the surrounding pH, by removing the amino group of an amino acid and converting it to ammonia, while coordinately channeling the carbon skeleton of the amino acid into acids, which can both be exported from the cell (Gut et al., 2006). In addition many enteric bacteria possess ion pumps such as Na^+/H^+ antiporters which are regulated by pH. The two Nha ion pumps in *E. coli* have been well characterised and are essential for Na^+ tolerance and the capacity to grow at alkaline pH (Hunte et al., 2005).

In *Pseudomonas* the arginine decarboxylase pathway, which includes the *arcDABC* operon, has been characterised in *P. aeruginosa* PAO1. This operon is regulated by pH and oxygen availability and as such may be important for adaptation to acid environments (Gamper et al., 1991; Bourdineaud et al., 1993). However, of the sequenced *P. syringae* pathovars, only P.s.s appears to possess orthologous genes to the *P. aeruginosa arcDABC operon* (Psyr_2683 to Psyr_2686).

In several enteric bacteria, including *Salmonella typhimurium, Neisseria meningitidis* and *Yersinia pestis*, the PhoP-PhoQ two-component system is important for acid tolerance (Bearson et al., 1998; Nachin and Barras, 2000). Furthermore, this system is known to be a key factor in pathogenicity in the plant-pathogenic bacteria *Dickeya dadantii* EC16 (Llama-Palacios et al., 2003) and *Xanthomonas oryzae* pv. oryzae (*X. oryzae*) (Lee et al., 2008). For example, *D. dadantii* EC16 mutants that contained insertions in *phoQ* showed decreased survival at acid pH and in plant tissues; showed decreased virulence *in planta*; and showed reduced ability to

alkalinise plant tissues after infection (Llama-Palacios et al., 2003). In *X. oryzae*, *PhoP* was found to be an important component of virulence because strains lacking *phoP* produced smaller lesions *in planta* and reduced acid tolerance *in vitro.* In addition, both *phoP* and *phoQ* mutants showed reduced tolerance to antimicrobial peptides (Lee et al., 2008). *X. oryzae* has been shown to experience a change in acidic conditions during infection of host rice plants and therefore the PhoPQ system is hypothesised to play an important role in the survival and proliferation of this bacterium during pathogenesis (Lee et al., 2008). Interestingly a further role for PhoP in *X. oryzae* has been proposed as a regulator of the TTSS. This is through regulation of *hrpG* gene expression, as HrpG controls the expression of *hrp* genes. It has been hypothesised that in *X. oryzae, hrpG* may be regulated in response to calcium levels in the rice apoplast. However, as the calcium concentration of the rice apoplast has not yet been established, this remains a topic for further research (Lee et al., 2008).

1.7.2 The apoplast environment during infection – nutrient composition.

The metabolite composition of the uninfected apoplast varies between plant species (Lohaus et al., 2001), and according to factors such as plant age, sampling time (Lohaus et al., 2001) and nutritional status, including iron deficiency (Lopez-Millan et al., 2000) or fertilizer application (Tejera et al., 2006). For example, the total apoplast amino acid content in sugarcane was six to nine times lower in non-nitrogen fertilized plants than in fertilized ones (Tejera et al., 2006). These variables can add considerable complications when trying to identify pathogen-induced changes in metabolite composition.

The apoplast appears to be nitrogen limited.

Many authors have reported that low nitrogen in the apoplast is a limiting factor for pathogens within plants (Hirano and Upper, 1990). For example, many of the genes transcribed in the tomato pathogen *Fusarium oxysporum* (*F. oxysporum*) during plant colonisation, were also transcribed during growth in nitrogen starvation conditions *in vitro*. Several of these genes were identified and shown to function in organic nitrogen acquisition, including a general amino acid permease and a peptide transporter (Divon et al., 2008). Similarly, evidence suggests that *Cladosporium fulvum* (*C. fulvum*) may experience nitrogen-limitation during growth in tomato. The *C. fulvum* avirulence gene *avr9* is not expressed under optimal growth conditions *in vitro*, but is highly expressed when the fungus grows inside the tomato leaf. This gene was specifically induced in low nitrogen media *in vitro* and was found to be regulated by the nitrogen-response factor NRF1, which suggests that *C. fulvum* lacks nitrogen *in planta*, and that nitrogen status is used to regulate *avr9* (Perez-Garcia et al., 2001).

Many bacteria also appear to use nitrogen-limitation as a cue to activate nitrogen-scavenging transporters, to facilitate the uptake of nitrogen-containing compounds that may be present in the surrounding environment (Leigh and Dodsworth, 2007). Differential uptake of amino acids by bacteria in conditions of carbon or nitrogen starvation has long been observed, for example; in 1969 Kay and coworkers reported that during carbon or nitrogen deprivation *P. aeruginosa* transported more glutamate, alanine, and glycine, while the transport rates for the aliphatic amino acids leucine and valine decreased (Kay and Gronlund, 1969). This activity was proposed to provide *P. aeruginosa* with a survival advantage during starvation, as increased transporter activity would maximise amino acid scavenging of metabolically preferred amino acids. In *E. coli*, nitrogen limitation has been shown to control the expression of about 100 nitrogen-regulated (Ntr) genes, which have many roles in assimilating ammonia and scavenging nitrogen (Reitzer, 2003). Many Ntr genes encode transport systems and therefore nitrogen scavenging has been proposed as a major function of the Ntr response (Reitzer, 2003). The Ntr response is regulated by glutamine, which is used as a measure of nitrogen sufficiency. When glutamine is low, this acts as a signal of nitrogen limitation to activate the Ntr response. Two regulators, NtrC and NtrB, are known to be important in regulating this response. NtrC is a response regulator which, along with an environmental sensor NtrB, forms a two-component regulatory system capable of regulating the transcriptional activities of various nitrogen assimilation-associated genes. Under conditions of nitrogen limitation, the environmental sensor NtrB interacts with the regulatory domain of NtrC, which consequently becomes activated and can activate NtrC-regulated genes (Xiao et al., 1994; Snoeijers et al., 2000). Expression of Ntr genes often also requires the involvement of the alternate sigma factor RpoN. For example, in *E. coli*, about half of the products of RpoN-dependent genes are known to be involved in nitrogen metabolism (Reitzer and Schneider, 2001).

Low nitrogen conditions in the apoplast may also provide an important regulatory cue for important pathogenicity genes in *P. syringae*, such as the *hrp* genes (Alfano and Collmer, 2004). In general, *hrp* genes are more highly expressed in minimal medium, such as M9, compared to nutrient rich media like KB or LB (Snoeijers et al., 2000). For example, in P.s.ph, the expression of the *hrp* genes *hrpA, hrpB, hrpC, hrpD, hrpL, hrpR* and *hrpS* was up to 700 times higher in M9, compared to their expression in KB (Rahme et al., 1992). Minimal medium has long been cited as providing the closest synthetic equivalent to apoplast conditions (Yucel et al., 1989). Using reporter fusions, it was subsequently demonstrated that *in planta*, the maximum expression levels of these seven *hrp* genes was equivalent or higher than in M9, confirming that for these genes the expression levels in M9, rather than rich media, more

closely reflect those *in planta* (Rahme et al., 1992). Regulation of *hrp* genes has been studied extensively in P.s.s. As such, in P.s.s the products of the *hrpR* and *hrpS* genes have been shown to be related to the NtrC class of nitrogen regulators discussed above, (in P.s.s. these genes lack the N-terminal regulatory domain, although they have both the RpoN-activating and DNA-binding domains). The NtrC/NtrB complex therefore controls the expression of numerous *hrp* and *avr* genes in response to nitrogen availability, activating these genes in response to nitrogen limitation in the apoplast (Xiao et al., 1994). NtrC is also thought to be the master regulator of nitrogen-responsive genes in *P. putida* (Hervas et al., 2008). Transcriptome analysis of *P. putida* during nitrogen limitation revealed 58 operons which showed increased expression under nitrogen-limited conditions, and also showed increased expression in wild-type *P. putida* compared to a *P. putida* NtrC mutant. These NtrC-activated operons included several operons involved in amino acid transport, including nonspecific amino acids (PP1297 to PP1300), branched-chain amino acids (PP1138 to PP1141, PP4841 to PP4845, and PP4863 to PP4867), and transporters for individual amino acids such as a proline ABC transporter (PP0296) (Hervas et al., 2008).

Apoplast extraction techniques are a useful method to study the nutritional composition of the apoplast.

In order to study the nitrogen and other nutrients available to pathogens in the apoplast, studies have predominantly used apoplast extraction techniques, followed by techniques such as HPLC and NMR, to measure the metabolite content in the extracted apoplast. A viable method for extracting apoplast from plants was first demonstrated by Klement (1965). Later, this method was verified by demonstrating that extracted apoplast was not contaminated by components of the cytoplasm. These experiments used the cytosolic enzyme glucose-6-phosphate dehydrogenase as a marker for cytoplasmic contamination of apoplast, and levels of this enzyme in extracted apoplast fluid were found to be negligable (Rathmell and Sequeira, 1974). Variations and improvements on this original method are still employed today (Solomon and Oliver, 2001; Rico and Preston, 2008).

However, compared to whole-tissue metabolite studies, there have been relatively few studies investigating the metabolite composition of the apoplast, and of those, few have measured a significant number of metabolites. Therefore, information on the metabolite composition of the apoplast must be largely pieced together from separate studies, and furthermore, as the composition of extracted apoplast varies widely between plant species, (Lohaus et al., 2001) results can only be combined from studies working with the same species. Therefore, there

remains a need for more data on the metabolite content of apoplast extracted from different plant species.

The healthy apoplast.

Probably the most abundant apoplast metabolite information is available for the tomato apoplast (Joosten et al., 1990; Valle et al., 1998; Solomon and Oliver, 2001; Rico and Preston, 2008). The amino acid composition of the healthy tomato apoplast was recently quantified and GABA, aspartate, glutamine and glutamate were found to be the predominant amino acids (Rico and Preston, 2008). This finding was in agreement with previous reports that glutamine and glutamate were the most abundant free amino acids in exudate from cut tomato leaves (Valle et al., 1998) (GABA abundance was not measured in this study), and that GABA was used as a major nitrogen source by the biotrophic fungus *C. fulvum* in the tomato apoplast (Solomon and Oliver, 2002). However, as can be seen from Table 1.3 below, there is variation in the absolute values reported for each amino acid in the tomato apoplast. Studies have also reported sucrose, glucose and fructose as the most abundant tomato apoplast sugars (Joosten et al., 1990), while malic acid is reportedly the most abundant organic acid (White, 1981). Malic along with citric acid, were also the most abundant organic acid sugar beet (Lopez-Millan et al., 2000) and kiwi plant apoplast (Clarke et al., 1986). Interestingly, a recent Tn5 mutant screen revealed that P.s.t with mutations in the malate:quinone oxidoreductase gene (mqo), which encodes an enzyme of the tricarboxylic acid cycle, displayed reduced virulence on the host plants *A. thaliana* and tomato (Mellgren et al., 2009). In addition to growing more slowly than wild type P.s.t *in planta*, P.s.t-mqo mutants also grew more slowly than wild type *in vitro* when dicarboxylates, such as malate, citrate and succinate, were the only carbon source. Based on these results it was suggested that dicarboxylates may be important for the growth of P.s.t in the apoplast (Mellgren et al., 2009). However, subsequent experiments have demonstrated that the P.s.t-mqo mutant did not exhibit a significant growth defect during growth in extracted apoplast (Rico, Steinbrenner and Preston, unpublished), suggesting that the reasons for the inhibited growth *in planta* of this mutant may be more complex than previously speculated.

Metabolite information is also available for the amino acid content in leaf apoplast from sugar beet, (Lopez-Millan et al., 2000), potato, (Heineke et al., 1992), sugarcane, (Tejera et al., 2006) barley, (Lohaus et al., 1995) spinach (Lohaus et al., 1995) and *A. thaliana* (Pilot et al., 2004) (summarised in Table 1.3 below).

	values in µM/ml										nmol/mg
Apoplast source	Tomato	Tomato	Barley	Spinach	*A. thaliana*	sugarcane (variety) (NCo310)	sugarcane (variety) (RD 75-11)	sugarcane (variety) (PR 60-170)	Bean	Sugar beet	Potato
Reference	1	2	3		*4*	5			6	7	8
Amino acids											
Asp	350	145.4±46	290	420	93.7±27	10.52±0.51	6.02±0.32	6.70±0.72	0.37±0.12	262±20	617
Asn	150	9.5±2	40	90	0	–	–	–	4.28±1.31	–	60
Glu	700	137.4±45	780	430	201±47	3.24±0.21	3.62±0.21	3.29±0.12	0.60±013	320±3	2006
Gln	650	34.0±9	240	630	53±14	–	–	–	0.58±0.13	450±180	317
Ser	600	51.2±12	630	500	60.5±18	38.77±1.21	23.93±0.88	46.39±1.31	0.74±0.22	121±42	504
Thr	310	22.0±9	170	130	9.3±9	5.39±0.21	5.60±0.19	4.74±0.18	0.16±0.03	44±15	–
Gly	250	28.8±10	100	130	7.7±13	1.88±0.11	2.02±0.1	4.15±0.13	0.16±0.04	56±8	191
Ala	620	67.3±14	550	350	62.8±54	16.27±0.42	16.51±0.38	11.77±0.44	1.54±0.39	–	146
Cys	0	0	–	–	0	2.61±0.15	2.15±0.09	2.31±0.08	0	–	–
Val	200	24.7±0.1	80	240	0	4.76±0.26	4.27±0.24	3.82±0.13	0.11±0.02	60±55	51
Met	0	24.8±9	0	0	–	2.12±0.09	1.99±0.0.08	2.17±0.11	0.03±0.01	–	–
Ile	50	19.4±23	50	100	2.4±5	2.32±0.12	3.03±0.1	1.87±0.08	0.04±0.01	16±16	25
Leu	0	31.8±6	50	90	0	2.80±0.12	3.10±0.14	2.67±0.11	0.05±0.02	<1	12
Tyr	0	16.0±3	30	50	0	6.43±0.21	6.07±0.14	6.64±0.20	0.03±0.01	–	13
Phe	100	25.2±3	–	–	0	2.73±0.13	2.87±0.14	2.84±0.16	0.04±0.01	–	–
GABA	800	575.5±83	–	–	0	–	–	–	0.13±0.03	56±11	–
His	0	6.9±2	–		28.6±6	2.53±0.09	2.63±0.11	–	0.08±0.03	<1	12
Lys	0	22.5±2	70	80	0	1.72±0.13	1.36±0.0.1	1.36±0.14	0.03±0.01	31±0.5	19
Arg	0	–	–	–	40.3±24	–	–	3.48±0.12	0.02±0.01	–	–
Pro	40	12.8±2	–	–	38.2±20	18.08±0.72	16.97±0.64	14.05±0.61	–	<1	28
Trp	–	–	–	–	–	–	–	–	0.02±0.01	–	–

Table 1.3
Review of apoplast amino acid abundance data to date. All data is from the leaf apoplast of untreated wild type plants. The six most abundant amino acids are highlighted. A dash (-) means the amino acid was not measured in this study. References are: 1: (Solomon and Oliver, 2001), 2: (Rico and Preston, 2008), 3: (Lohaus et al., 1995), 4: (Pilot et al., 2004), 5: (Tejera et al., 2006), 6: (Struck et al., 2004), 7: (Lopez-Millan et al., 2000), 8: (Heineke et al., 1992).

The infected apoplast.

Most of the information we have regarding the nutrient composition of infected apoplast comes from studies done with fungal pathogens. For example, the total apoplast amino acid abundance in bean leaves infected with the hemibiotrophic fungus *Colletotrichum lindemuthianum* (C. *lindemuthianum*) was five-fold higher after 12 days compared to control leaves, with glutamine and glutamate as the most abundant individual amino acids (Tavernier et al., 2007). Glutamine in particular represented 33 % of the total apoplast amino acids in infected plants compared to only 1 % in controls. Interestingly, this increase was found to correlate with the accumulation of glutamine synthetase (*GS1α*) mRNA, an enzyme that catalyzes the formation of glutamine from glutamate and ammonia, although it was not established whether this accumulation was the result of deliberate host cell modulation by *C. lindemuthianum* to liberate glutamine into the apoplast (Tavernier et al., 2007).

Of these fungal studies, probably the most useful for inferring the potential changes in apoplastic nutrients during *P. syringae* infection are those using the biotrophic fungal pathogen *C. fulvum*. *C. fulvum* colonises and proliferates in the apoplast of tomato plants, a host of *P. syringae* pv. tomato, and is hypothesised to obtain nutrients from the apoplast environment in a similar manner to *P. syringae* (Solomon and Oliver, 2001).

Studies following the sugar composition of tomato apoplast inoculated with virulent races of *C. fulvum* have found that during infection, the amount of sucrose decreased, while glucose, fructose and mannitol increased (Joosten et al., 1990). In contrast, when tomato apoplast was infected by avirulent races of *C. fulvum*, the apoplast composition closely mirrored that of water-inoculated plants, suggesting that in non-host plants, defence responses prevent the fungus from modulating plant cells to release sugars into the apoplast (Joosten et al., 1990). Experiments have also been able to demonstrate that the concentration of amino acids varies during the course of a compatible infection with several being massively upregulated, particularly the non protein amino acid GABA, which increased from 0.8 mM to 2.5 mM between 7 and 14 days post inoculation, respectively (Solomon and Oliver, 2001).

The function of GABA in plants is unconfirmed but may have roles in intercellular signalling, nitrogen storage or osmoprotectancy (Shelp et al., 1999). GABA is also known to be utilised by *C. fulvum* as a major source of nitrogen in the tomato apoplast (Solomon and Oliver, 2002). Therefore, the up-regulation of GABA synthesis during compatible *C. fulvum* infection has been suggested by some authors to demonstrate the ability of

C. fulvum to modulate the physiology of the tomato apoplast for its own benefit, or to take advantage of a naturally occurring response (Solomon and Oliver, 2002).

However, fungal studies can only be used as an indirect inference of the nutrient changes which occur during *P. syringae* pathogenesis. Many hemibiotrophic and necrotrophic fungal pathogens possess cell-wall degrading enzymes which allow then to liberate nutrients from host cells into the apoplast in a way which is unavailable to *P. syringae*. Evidence of this can be seen from research following changes in the nutrient composition of the wheat apoplast during infection by the necrotrophic fungus *Mycosphaerella graminicola*. Keon and coworkers (2007) found that the wheat apoplast failed to show any significant changes in the amino acids measured prior to the initial breakdown of plant cell membranes at day nine. After this time there was a massive increase in apoplastic amino acids, peaking at the onset of advanced host programmed cell death (PCD) at day 13 (Keon et al., 2007). Furthermore, although the exact role of *P. syringae* virulence factors in releasing nutrients into the apoplast is still unknown, it is unlikely to be identical to the way in which fungal pathogens modulate host cells (which is also still being elucidated). Therefore, there remains a need for information about the specific changes induced in the apoplast during *P. syringae* infection.

1.8 *P. syringae*: adaptations to the apoplast?

The genes induced in *P. syringae* during infection of host plants are well studied and include those involved in producing the T3SS, and secreted effector proteins and phytotoxins. *In vivo* expression technology (IVET) revealed that over 500 P.s.t promoters are expressed *in planta* (Boch et al., 2002). Analysis of these promoters identified corresponding genes with either predicted or known roles in virulence, and also metabolic genes, presumably important for adaptation to growth in plant tissue, including genes involved in amino acid uptake and metabolism (Boch et al., 2002). However, although it is known that the collective effect of these expressed genes is to enhance bacterial growth in the apoplast, the contribution of individual genes has not been well established. For example, although T3SS-secreted effectors have been well studied, and have been shown to target a diverse range of plant cell components, their role in contributing to the release of nutrients into the apoplast remains largely speculative (Cunnac et al., 2009). Furthermore, we still lack answers to basic questions regarding the nutrition of *P. syringae* in the apoplast, for example; in what form does *P. syringae* obtain sulphur? We know from extracted healthy apoplast that concentrations of the sulphur-containing amino acids cysteine and methionine are relatively low, (Table 1.3, above) and are utilised poorly by *P. syringae* as sole carbon and nitrogen sources (Rico

and Preston, 2008). However, further research is needed to investigate whether these amino acids increase during infection, and whether they can benefit the growth of *P. syringae*, even though they can't be used as carbon or nitrogen sources for growth; i.e. by directly incorporating these amino acids into proteins or by breaking them down for other uses.

Furthermore, most probably influenced by the lack of available data on the nutrient composition of the plant apoplast, few studies appear to have investigated the ways in which the transport systems of *P. syringae* may have adapted to take up available nutrients from the apoplast. Bacteria take up nutrients from their environment via a range of membrane-bound transporters, powered by different passive or active mechanisms such as ATP-utilisation or proton gradients, and with different specificities for the transported compound (Hosie and Poole, 2001). The efficiency of a particular type of transporter can depend on the external environment, for example; H^+-coupled symporters or antiporters, which utilise the movement of a proton down its concentration gradient to bring a desired compound into the cell, are less efficient in acidic environments when the external H^+ concentration is high (Konings, 2006). The type of bacterial transporters can also depend on the nutrients available in the bacterial environment; transporters for scarce nutrients or for ones which can be easily synthesised by the bacteria themselves are costly in terms of the energy expended to make them.

The availability of genome sequences for multiple *Pseudomonas* species, including multiple *P. syringae* pathovars have provided researchers with the opportunity to make bioinformatic comparisons of these genomes, and relate any differences in gene distribution, to the lifestyle of a particular species (Lindeberg et al., 2008). Proteins are made up of constituent domains which can be classified into many thousands of families. Rearrangement of these domains may provide proteins with novel functions; therefore the identification of proteins with domain architectures that are exclusive to particular bacterial lifestyle may identify proteins that are specifically important for that lifestyle. The Pfam database is a widely used tool for investigating bacterial protein domains (Bateman et al., 2004) and includes the protein domains present in all sequenced *Pseudomonas*. In order to identify candidate proteins with an involvement in the plant-pathogenic lifestyle the Pfam database was mined for over and under-represented protein domain architectures in *P. syringae* compared to non-plant pathogenic *Pseudomonas* (Studholme et al., 2005).

One area in which genomes differed markedly was nutrient utilisation. For example, the sugar_tr and BPD_transp_2 domains, found in sugar transporters and ATP-dependent high-affinity branched-chain amino acid transporters, respectively, were identified as being present in significantly higher numbers in *P. syringae* than in other *Pseudomonas*. Domains were also identified as being underrepresented in *P. syringae* compared to non-plant pathogenic *Pseudomonas*. Particularly striking was the difference in the number of aa_permease (amino acid permease) domains, of which there were significantly fewer in *P. syringae* genomes (Table 1.4). The reduction in aa_permease domains is interesting given the large number of *Pseudomonas* domains that have expanded in size relative to other bacteria (Studholme et al., 2005). This level of gene reduction is usually seen in highly specialised obligate plant pathogens such as *Xylella fastidiosa* (*X. fastidiosa*), which possesses three aa_permeases (Table 1.5). *X. fastidiosa* is an obligate plant pathogen, confined to the xylem vessels of host plants and transported by xylem-feeding insects.

Pseudomonas species **(abbreviation)**	**aa_permease**
P. syringae pv. tomato DC3000 (P.s.t)	4
P. syringae pv. phaseolicola 1448A (P.s.ph)	5
P. syringae pv. syringae B728a (P.s.s)	6
P. aeruginosa PA14 (PA14)	21
P. aeruginosa PAO1 (PAO1)	22
P. aeruginosa PA7 (PA7)	17
P. stutzeri A1501 (A1501)	5
P. entomophila L48 (L48)	19
P. fluorescens Pf-5 (Pf-5)	21
P. fluorescens Pf0-1 (Pf0-1)	14
P. putida KT2440 (KT2440)	21
P. putida F1 (F1)	21
P. putida GB1 (GB1)	29
P. putida W619	18

Table 1.4
Numbers of amino acid permease (aa_permease) genes in sequenced *Pseudomonas* species, from the PFAM database 23.0 (July 2008).

Bacteria	aa_permease
Xylella fastidiosa Temecula 1	3
Xylella fastidiosa Ann-1	3
Xylella fastidiosa Dixon	3
Xanthomonas campestris pv. campestris 8004	11
Xanthomonas oryzae pv. oryzae MAFF 311018	10
Xanthomonas campestris pv. vesicatoria 85-10	11
Xanthomonas axonopodis pv. citri 306	12

Table 1.5
Numbers of amino acid permease (aa_permease) genes in sequenced *Xanthomonas* and *Xylella* species, from the PFAM database 23.0 (July 2008).

The usual explanation offered for genome reduction in obligate pathogens such as *X. fastidiosa*, is that the ability of these specialised pathogens to parasitise host genomes has rendered numerous genes expendable (Moran, 2002). For example, *X. fastidiosa* lacks biosynthetic enzymes for serine and methionine which it is thought to obtain directly from the xylem sap (Lemos et al., 2003). This makes *X. fastidiosa* particularly difficult to culture in the laboratory, and synthetic media must be designed to contain essential nutrients which *X. fastidiosa* cannot make itself, and would usually obtain directly from the host plant (Lemos et al., 2003; Almeida et al., 2004). In contrast to obligate pathogens, generalist species need to adapt to a wide range of environmental conditions and may undergo frequent horizontal gene transfer, often resulting in genome expansion (Stepkowski and Legocki, 2001).

P. syringae has long been considered to be a relatively specialist species due to the host specificity demonstrated by individual pathovars. In addition, the nutritional specialisation showed by *P. syringe* has traditionally been used for classification of *Pseudomonas* species, which can involve phenotypic tests based on their differential abilities to use different nutrients as sole sources of energy (Thornley, 1960; Lelliott et al., 1966; Misaghi and Grogan, 1969; Palleroni and Moore, 2004). Most recently, analysis of the presence and distribution of metabolic pathways in sequenced *Pseudomonas* using information extracted from the KEGG database (http://www.genome.jp/kegg/), has demonstrated that the *P. syringae* genome shows significantly more deletion events relative to other *Pseudomonas* (Mithani et al., manuscript submitted), which is consistent with the authors' hypothesis that *P. syringae* is adapted for growth in the nutritionally restricted plant apoplast. Therefore an interesting question is whether the apparent reduction of aa_permease genes in *P. syringae* compared to other *Pseudomonas* species represents specialisation to the nutrients available in the apoplast environment

1.9 Amino acid permease (aa_permease) genes.

The aa_permease proteins have been classified within the amino acid/polyamine/organocation (APC) superfamily of transporters. This is one of the largest superfamilies of transporters containing five large families, each with many subfamilies (Saier, 2000). APC transporters are electrochemical potential-driven porters, meaning they utilise a carrier-mediated process to facilitate either antiport (two or more substrates are transported in opposite directions), and / or symport (two or more substrates transported together in the same direction) not coupled to a direct form of energy other than chemiosmotic energy (Saier, 2000). This superfamily is one of the largest yet identified, and all of its functionally characterised members transport amino acids and/or polyamines or organocations (Chang et al., 2004). However, the substrate specificities of APC superfamily transporters vary, with some having exceptionally broad specificity, and others being restricted to just one or a few related compounds (Jack et al., 2000).

Within the APC superfamily, the aa_permease proteins have been classified within the APC family, and the AAT (amino acid transporter) subfamily. The majority of APC family transporters comprise twelve hydrophobic transmembrane sections interspersed by hydrophilic sections (Jack et al., 2000). This predicted topology has been experimentally verified for several bacterial transporters including the *E. coli* GABA permease GabP (Hu and King, 1998). Within the AAT subfamily, transporters are classified on the basis of the substrate or range of substrates transported, as well as the polarity of transport (in or out) (Saier, 2000). Several aa_permease transporters have been experimentally characterised and the transported substrate(s) established (Table 1.6). However, due to the large size of the APC superfamily, the number of characterised aa_permease transporters, one of many subfamilies belonging to one of five families, is comparatively low.

APC family	Example permease types	Reference
Amino acid transporter **subfamily** (AAT) (all contain the aa_permease domain)	Phenylalanine:H^+ symporter, PheP of *E. coli*	(Pi et al., 1991)
	Aromatic amino acid:H^+ antiporter, AroP of *E. coli*	(Cosgriff and Pittard, 1997)
	GABA:H^+ symporter, GabP of *E. coli*	(Niegemann et al., 1993)
	Lysine:H^+ symporter, LysP of *E. coli*	(Steffes et al., 1992)
	β-alanine / GABA:H^+ symporter of *Bacillus subtilis*	(Brechtel and King, 1998).
	Proline permease, ProY, of *Salmonella typhimurium*	(Liao et al., 1997)
	D-Serine / D-alanine / glycine:H^+	(Schneider et al., 2004)

	symporter, CycA of *E. coli*	
	S-Methylmethionine permease, MmuP, of *E. coli*	(Gal et al., 2002)
	L-Arginine permease, RocE, of *Bacillus subtilis*	(Gardan et al., 1995)
	Putrescine importer, PuuP, of *E. coli*	(Kurihara et al., 2009)

Table 1.6
Amino acid permease transporters are classified within the AAT family by the Transport Classification Database (http://www.tcdb.org/). Within this family permeases are separated according to the substrate(s) transported and the direction of transport. Some transporters which have been classified into the AAT family by this database have been characterised and the transported substrate(s) identified. Representative examples from the database are show above and the transported substrate is underlined.

However, several aa_permease genes in bacterial species other than *Pseudomonas* have been characterised and shown to contribute to survival. For example, the virulence of a *Staphylococcus aureus* (*S. aureus*) ProP (proline aa_permease) mutant was attenuated in animals (Schwan et al., 1998), while the phenylalanine aa_permease PheP, was found to contribute to survival of *S. aureus in vivo* and during starvation *in vitro* (Horsburgh et al., 2004). In the former example, although *S. aureus* is not a phenylalanine auxotroph, Schwan and coworkers (1998) hypothesised that in the oxygen-limited environment of an abscess, biosynthesis of phenylalanine would not be enough to supply cellular requirements. This would create a requirement for additional phenylalanine during infection, which in turn would provide a selection pressure to maintain phenylalanine transporters in *S. aureus*.

Within *Pseudomonas* few aa_permeases have been experimentally characterised. The arginine/ornithine antiporter, ArcD has been studied in *P. aeruginosa* PAO1, where it is found as part of the *arcDABC* operon of the arginine decarboxylase pathway. The ArcD permease has been shown to mediate a stoichieometric and energy-independent exchange between ornithine and arginine, with ornithine being transported out of the bacterial cytosol and arginine imported (Itoh and Nakada, 2004). The *arcDABC* pathway is known to contribute to the survival of PAO1 in acidic conditions by neutralizing acidity via the production of ammonia (Itoh and Nakada, 2004) and is upregulated during pathogenesis of the lungs in patients with cystic fibrosis (Son et al., 2007). This operon was found to be regulated by pH and oxygen availability (Gamper et al., 1991; Bourdineaud et al., 1993). Of the sequenced *P. syringae* pathovars, only P.s.s. appears to possess orthologous genes to the *P. aeruginosa arcDABC* operon (Psyr_2683 to Psyr_2686) (Joardar et al., 2005).

In P.fl SBW25, HutT, located within the histidine (*hut*) operon has been experimentally characterised and demonstrated to transport histidine (Zhang and Rainey, 2007). The histidine utilisation genes of P. fl. SBW25, including *hutT*, are active on plant surfaces suggesting that histidine is available in the plant environment. However, the competitive fitness of a *hutT* deletion mutant was not significantly affected during colonisation of sugar beet plants, suggesting that this gene was not essential for plant colonisation (Zhang et al., 2006).

In general, it appears that aa_permease genes in *Pseudomonas* may not produce a detectable phenotype when mutated. Johnson and coworkers (2008) used Biolog phenotype microarrays to identify phenotypes of *P. aeruginosa* PAK Mini-Tn*5*-Tc^r gene knockout mutants. Seventy-eight mutants defective in predicted sugar and amino acid membrane transporter genes were screened, including 19 aa_permease gene mutants (Table 1.7). Of the aa_permease mutants only one, PA0220 (annotated as an ethanolamine transporter), showed a phenotype when disrupted. PA0220 was investigated further using RT-PCR to measure gene expression in the presence of the putative transported substrate. Expression of PA0220 was strongly induced by histamine. In addition this gene appeared to be co-transcribed in an operon alongside two genes that potentially form a novel histamine utilisation pathway (Johnson et al., 2008). Particularly interesting is the report that PA5097 did not show a phenotype in Biolog assays. This gene, annotated as a proline aa_permease, is located in the middle of the *hut* operon. Therefore, disruption of PA5097 might be expected to produce a loss of histidine utilisation-phenotype, even if this substrate is not transported by PA5097, due to the Tn5 transposon knocking out the function of the entire operon. This may be due to the presence of a promoter within the transposon which allows the transcription of downstream genes when the transposon is oriented in the same direction as the gene. However, this cannot be confirmed as the mutants used by Johnson and coworkers were bought ready-made and therefore specific details of the transposon's internal construction were not provided.

Gene	Predicted substrate(s)	Biolog phenotype
PA0129	GABA	No phenotype
PA0220	Ethanolamine	Can't use histamine as a sole nitrogen source
PA0322		No phenotype
PA0789		No phenotype
PA1147		No phenotype
PA1194	Arginine/Ornithine	No phenotype
PA1485		No phenotype
PA1819		No phenotype
PA2041		No phenotype
PA2079		No phenotype
PA3000	Aromatic	No phenotype
PA3597		No phenotype
PA4023	Ethanolamine	No phenotype
PA4072		No phenotype
PA4628	Lysine	No phenotype
PA4804		No phenotype
PA5097	Proline	No phenotype
PA5170	Arginine/Ornithine	No phenotype
PA5510		No phenotype

Table 1.7
Phenotypes of *P. aeruginosa* PAK Mini-Tn*5*-Tc^r gene knockout mutants in aa_permease genes, from Johnson and coworkers (2008). Phenotypes were assessed in Biolog carbon and nitrogen microarray plates and confirmed by assessing growth in minimal media supplemented with the test compound as a sole carbon or nitrogen source. Of the aa_permease mutants only PA0220 showed a detectable phenotype. This mutant lost the ability to use histamine as a sole nitrogen source. Interestingly PA5097 did not show a phenotype, this gene is located in the centre of the *hut* operon.

1.10 The GABA permease has been extensively characterised in non-*Pseudomonas* species.

Probably the most extensively studied aa_permease transporter is GabP. In particular, GabP has been extensively studied in *E. coli* and *B. subtilis* (Niegemann et al., 1993; Brechtel and King, 1998). Mutations in *gabP* in *E. coli* and *B. subtilis* result in a loss of ability to utilise GABA as a carbon or nitrogen source (Metzer et al., 1979; Niegemann et al., 1993; Brechtel and King, 1998). In both species GabP appears be a symporter, transporting GABA into the cell in exchange for a proton; however, in *B. subtilis* GABA is transported into the cell at the same time as β-alanine (Brechtel and King, 1998). Interestingly, although overexpression of *gabP* in *E. coli* JM103 increased the ability of cells to take up GABA, strong overexpression of *gabP* using an inducible *tac* promoter resulted in severe growth inhibition, which may have been due to excess GABA accumulation in cells (Niegemann et al., 1993).

In *E. coli,* the *gabP* gene belongs to the *gab* gene cluster, *gabCDPT*, which specifies the synthesis of the enzymes of the GABA degradation pathway (Schneider et al., 2002). In this bacterium the GABA-catabolism genes, *gabT* and *gabD,* and the GABA-permease, *gabP*, have been shown to be induced by nitrogen limitation *in vitro* (Zimmer et al., 2000). Within *Pseudomonas* the organisation of the *gabCDPT* cluster is different to that of *E. coli* or *B. subtilis,* with only *gabD* (NADP-dependent succinic semialdehyde dehydrogenase) and *gabT* (GABA transaminase) appearing together in the genome (although both *gabC* and *gabP* genes are present elsewhere) (Ahn et al., 2007). In PAO1, a *gabD* (PA0265) mutant was unable to grow on GABA, and showed slower growth on putrescine. The growth of a *gabT* (PA0266) mutant was significantly slower on GABA than the wild type, but was not abolished. This appears to be due to redundancy by a second *gabT* gene in PAO1. When a PAO1 *gabT-gabT* double mutant was constructed, it was unable to grow on GABA as the sole source of carbon and nitrogen (Chou et al., 2008).

GABA-catabolism appears to be important for *Pseudomonas* in the environment. In *P. putida* KT2440, the GabT protein, which catalyses the conversion of GABA into succinic semialdehyde, was induced during growth in the rhizosphere (Espinosa-Urgel et al., 2002), while in *P. putida* 06909, *gabD* induction has been reported during the colonisation of *Phytopthora infestans* mycelia (Ahn et al., 2007). In addition, GABA was found to be the predominant amino acid in the healthy tomato apoplast (Rico and Preston, 2008), and this abundance increased during *C. fulvum* infection (Solomon and Oliver, 2002). The sequenced *P. syringae* pathovars all possess one permease predicted to transport GABA and multiple copies of both a GABA transaminase (although P.s.ph PSPPH3457 contains an authentic frameshift mutation), and succinate-semialdehyde dehydrogenase (P.s.ph PSPPH5038 contains an authentic frameshift mutation) (Joardar et al., 2005). However, only P.s.ph appears to possess a putative glutamate decarboxylase gene *gadB*. Therefore, P.s.t and P.s.s appear unable to synthesise GABA, and the ability of P.s.ph to synthesise GABA is speculative (Alan Collmer, personal communication). *B. subtilis* has also been reported to lack any glutamate decarboxylase genes (Brechtel and King, 1998) but possesses the GABA-catabolism genes *gabT* and *gabD*. As intracellular accumulation of GABA appears to be toxic to many microorganisms, it has been hypothesised that the GABA-catabolism genes in *B. subtilis* may function to maintain the intracellular GABA pool at a low level (Belitsky and Sonenshein, 2002).

Furthermore, modulation of GABA metabolism by pathogens has been hypothesised to further benefit virulence, as the GABA shunt may serve as part of a cellular scavenger system for oxidative stress signals (Solomon and Oliver, 2002). The role of ROS in plant defence signalling is well established; therefore successful modulation of GABA metabolism may also subvert plant defence signals in addition to providing nutrients (Solomon and Oliver, 2002).

1.11 The role of GABA in plants.

The potential role played by GABA in plants has received increased attention in recent years. GABA has previously been linked with plant senescence, with studies showing increased GABA concentration (Diaz et al., 2005) and expression of the GABA-catabolism enzyme, GABA-transaminase (Gab-T) in senescent leaves (Ansari et al., 2004). GABA has also been shown to regulate 14-3-3 gene expression in *A. thaliana* (Lancien and Roberts, 2006). These 14-3-3 proteins are ubiquitous regulatory proteins known to be important in the regulation of carbon and nitrogen metabolism in plants (Comparot et al., 2003). Furthermore, 14-3-3 genes are known to be differentially regulated during a range of environmental, metabolic and nutritional stress responses (Roberts et al., 2002; Roberts, 2003) including defence responses to fungal and bacterial pathogen infection (Roberts and Bowles, 1999; Chen et al., 2006). Overexpression of 14-3-3 genes has been shown to result in improved stress tolerance. For example, overexpression of the *A. thaliana gf14* gene results in improved tolerance to water stress, and a 'stay-green' phenotype (Yan et al., 2004). Fifteen 14-3-3 genes, named *Gf1* to *Gf15*, have so far been identified in *A. thaliana*. Lancien and co-workers (2006) investigated the effect of GABA on nine of these genes, *Gf1* to *Gf9,* in *A. thaliana* seedlings. In the presence of calcium (which itself does not affect 14-3-3 expression), GABA, but no other tested nitrogen source, caused a significant reduction in the transcription of *Gf3* to *Gf9,* and a corresponding decrease in the levels of Gf3 to Gf9 protein (Lancien and Roberts, 2006). The authors of this work therefore speculated that GABA-regulated gene expression may function as a component of the plant stress response, with 14-3-3 proteins as one important target. Further to these results, global microarray analysis was used to compare gene expression in *A. thaliana* seedlings grown under normal conditions, or in the presence of 10 μM GABA (Roberts, 2007). Analysis identified 71 genes that were downregulated by 2.5-fold or more in the presence of GABA, and 36 genes that were upregulated by 2.5-fold or more in the presence of GABA. Interestingly 26 out of the 36 GABA-upregulated genes correlated with genes upregulated during senescence in tissue cultured cells (Roberts, 2007).

1.12 Conclusions.

Plant-pathogenic *P. syringae* is capable of colonising the aerial surfaces of host and non-host plants and the intracellular apoplast of host plants. Although many species of bacteria are able to survive on the plant surface (in either natural or experimental conditions), the ability to proliferate within the apoplast is comparatively rare. One reason for this is the array of constitutive and induced defences employed by plants to defend themselves from pathogen attack, which must be suppressed or overcome for a plant-pathogen such as *P. syringae* to survive and proliferate. The mechanisms by which *P. syringae* suppresses and evades plant defences, including secreted hormones, toxins and effector proteins, have been well studied, although the function of many effector proteins in particular is still being elucidated. In contrast, relatively little attention has been paid to other ways in which *P. syringae* may have adapted to the apoplast environment, particularly with respect to nutritional adaptations.

In contrast to the heterogeneous environments encountered by many non plant-pathogenic *Pseudomonas* species, the apoplast appears to be a relatively constant, nutrient limited, low nitrogen environment. Researchers have long been aware that *P. syringae* could be identified from other *Pseudomonas* species by the reduced ability of this species to utilise certain nutrient sources, particularly amino acids, and the reduced tolerance of this species for conditions such as extreme pH. However, it is only recently that researchers have begun to identify the amino acids present in the plant apoplast and to compare these with the amino acids metabolised by *P. syringae*, and far more work in this area is required to profile all available nutrient sources. It is also only relatively recently that genome sequencing has advanced to the point where multiple *Pseudomonas* genomes have become available, facilitating comparisons between the genes present in plant-pathogenic and non plant-pathogenic species. In one study, the aa_permease genes were highlighted as being significantly underrepresented in *P. syringae* compared to non plant-pathogenic *Pseudomonas*, thereby providing a potential genetic basis for the observed differences in amino acid assimilation between these species. This finding also raises interesting questions as to the nature of the evolutionary selection pressures acting on these genes in *P. syringae* to produce the presently observed difference. Various hypotheses can be proposed to explain this difference in aa_permease gene number between *P. syringae* and non plant-pathogenic *Pseudomonas* species. These can be categorised as positive, neutral, negative or 'evolutionary' selection hypotheses (Figure 1.9).

Briefly, positive selection hypotheses are based on the assumption that there is a fitness advantage associated with the remaining aa_permease genes in *P. syringae* which has led to these genes in particular being retained in the genome while others have been lost. For example, the amino acids transported by the aa_permease genes in *P. syringae* may reflect those that are abundant in the plant environment and the nutritional benefit of uptake may outweigh the metabolic cost of uptake, or may have a beneficial non-nutritional role such as osmoprotection. Alternatively, uptake of these particular amino acids may indirectly benefit *P. syringae* by interfering with aspects of plant signalling or homeostasis.

Negative selection hypotheses assume that there is a cost to particular aa_permease genes in the apoplast environment, and that this led to selection for bacterial strains that had lost those aa_permease genes. Particular 'costs' to bacteria could include: the co-transport of toxic compounds alongside an imported amino acid; the cost of amino acid uptake being greater than the metabolic benefit to the bacteria; or that the assimilation pathway for an amino acid is no longer present in the bacteria and so the amino acid cannot be metabolised once taken up.

Positive selection hypotheses:
There is a fitness advantage to the remaining aa_permease genes.

- The number of aa_permease genes may reflect the amino acids that are available in the niches colonised by *P. syringae*. The benefit of their assimilation exceeds the cost of uptake.
- It may be advantageous for *P. syringae* to take up amino acids that play a role in plant signalling, such as GABA, a positive nutritional benefit will have reinforced this.
- Certain amino acids may have additional non-nutritional roles in the maintenance of bacterial homeostasis, for example proline is a known osmoprotectant.

Neutral selection hypotheses:
There is no fitness advantage or disadvantage to genes in a particular environment; these genes are generally inactivated through the accumulation of mutations.

- Not all amino acids are available in the niches colonised by *P. syringae* and so there is no selection to maintain transporters for these amino acids.

Negative selection hypotheses:
There is a fitness cost associated with certain aa_permease genes.

- The cost of uptake for some amino acids exceeds the metabolic benefit
- Particular amino acids co-transport toxic compounds, compromise bacterial signalling or cellular homeostasis. Alternatively certain transporters exhibit broad specificity which can result in uptake of toxic substances.
- Loss of function mutations in assimilation pathways mean that exogenous metabolites cannot be metabolised, or can only be partially metabolised and the metabolite, or degradation intermediates may be toxic or inhibit metabolism. An effective way to remove this inhibition is to mutate nutrient uptake pathways.

Evolutionary hypotheses:
- The current number of genes reflects those present in founding or surviving individuals following an evolutionary bottleneck, such as that which might have occurred as a result of some individuals acquiring the ability to infect plants.

Figure 1.9
Hypotheses proposed to explain the distribution of aa_permease domains between *P. syringae* and generalist *Pseudomonas*

Neutral selection hypotheses assume that there is neither a fitness advantage nor disadvantage to aa_permease genes in the plant environment, but that as the apoplast is thought to be a nutrient limited environment, mutations have gradually accumulated in aa_permease transporters for which a particular amino acid is not present, eventually leading to the loss of these transporters from the *P. syringae* genome. Finally, evolutionary hypotheses do not invoke selection arguments, but are based on the idea that the present number of aa_permease genes in *P. syringae* reflect those present in the first individuals capable of infecting plants.

The aa_permease transporters have not been extensively studied in bacteria and very few have been experimentally characterised in *Pseudomonas.* Therefore, the overall aim of this thesis is to gain an understanding of the aa_permease genes in *P. syringae*. In particular, the model strain P.s.t will be studied as this strain can infect the model plant *A. thaliana* as well as its host plant tomato, and has previously proven genetically amenable to mutation. Bioinformatic analyses will be carried out to investigate location and putatively transported substrates of the aa_permease genes in *P. syringae* and non plant-pathogenic *Pseudomonas* genomes. In particular, the evolutionary history of these genes can be modelled using phylogenetic methods in order to investigate the frequency of events such as horizontal evolution. In order to gain a better understanding of the role these genes might play during apoplast colonisation, the amino acids assimilated by P.s.t for growth will be investigated and the role of conditions such as pH on amino acid assimilation will be studied. In addition, the ability of P.s.t to chemotax towards amino acids and extracted plant apoplast will be investigated in order to investigate the role of these compounds in providing a signal for P.s.t to enter the plant apoplast from the leaf surface, and to investigate a connection between energy taxis and amino acid uptake and utilisation. In order to facilitate apoplast studies, a 'synthetic' apoplast will be created to mimic the *in planta* amino acid abundance. Finally, the aa_permease genes will be directly investigated through mutation and expression studies in order to identify the transported amino acids and the fitness consequences of the loss of these transporters.

Chapter 2. General materials and methods.

2.1 Introduction.

This chapter describes the general materials and methods which are employed in two or more chapters of this thesis. Materials and methods that are specific to a single chapter will be described at the beginning of the appropriate chapter.

2.2 Bacteria, growth conditions and media.

Pseudomonas and *Escherichia coli* (*E. coli*) strains were maintained as stocks in 20 % glycerol, at -80°C. Bacteria were routinely streaked to single colonies from the -80°C stocks onto Luria-Bertani (LB) agar plates (Sambrook et al., 1989), and incubated at 28°C (*Pseudomonas*) or 37°C (*E.coli*). Liquid cultures were taken from plates that were a maximum of three days old (*P. syringae* requires at least two days incubation to produce sizable colonies). Unless otherwise stated, 5ml liquid cultures were maintained in sterile 50ml universal tubes, inoculated from a single colony with a sterile pipette tip. Liquid cultures were shaken overnight at 200 rpm.

2.2.1 Bacterial strains and abbreviations.

The bacterial strains and abbreviations used for experimental work in this study are described below in Table 2.1. Abbreviations for bacterial strains used in bioinformatic studies or general discussions are described in Table 2.2

Bacterial strain	**Abbreviation**	**Reference or source**
P. syringae pv. tomato DC3000	P.s.t	(Buell et al., 2003)
P. syringae pv. tomato DC3000- hrcC	P.s.t-hrcC	(Yuan and He, 1996)
P. syringae pv. tabaci ATCC 11528	P.s.tabaci	American Type Culture Collection
P. aeruginosa PAO1	PAO1	(Stover et al., 2000)
P. fluorescens Pf-5	Pf-5	(Paulsen et al., 2005)
P. fluorescens SBW25	SBW25	(Bailey et al., 1995)
Acinetobacter sp. ADPWH_*lux*	ADPWH_*lux*	(Huang et al., 2005)
Agrobacterium tumefaciens C58 derivative *A.tumefaciens* GV3101	AtGV3101	(Koncz and Schell, 1986)
E. coli DH5α λ-pir	E. coli DH5α	(Mahan et al., 1993)
E. coli DH5α-pRK2013	pRK2013	(Friedman et al., 1982)
P. syringae pv. tomato DC3000 *rpoN* [Km^r]	P.s.t-*rpoN*	Arun Chatterjee, personal gift
P. syringae pv. tomato DC3000 *rpoN* complemented with pJJP6 [Km^r / Gm^r]	P.s.t-*rpoN* (pJJP6)	This study
E. coli DH5α (pJJP6) [Gm^r]	*E. coli* (pJJP6)	(Jones et al., 2007)

Table 2.1
Bacterial strains used in this study.

Bacteria	Abbreviation
*P. syringa*e pv. tomato	P.s.t / PSPTO
P. syringae pv. phaseolicola	P.s.ph / PSPPH
P.syringae pv. syringae	P.s.s / PSS
P. fluorescens Pf-5	Pf-5 / PFL
P. fluorescens Pf0-1	Pf0-1 / Pfl
P. fluorescens SBW25	SBW25
P. putida F1	F1 / PPF1
P. putida KT2440	KT2440 / PP
P. aeruginosa PAO1	PAO1
P. aeruginosa PA14	PA14
P. entomophila L48	L48 / PSEEN
P. mendocina ymp	P.men

Table 2.2
Abbreviations for bacterial strains used in bioinformatic analyses and general discussions in this study

2.2.2 Bacterial growth media.

All chemicals were obtained from Sigma-Aldrich Ltd (Poole, UK) or BHD Laboratory Supplies (Poole, UK) unless otherwise stated, Details of the composition of each growth medium are provided below. Where appropriate the pH of a growth medium was adjusted by addition of dilute HCL or NaOH as appropriate. Media was then filter sterilised using a sterile 0.2 µm Whatman syringe filter (Whatman Plc, UK) attached to a sterile 20 ml syringe (Fisher, UK). The pH of the media was measured using a Jenway 3510 pH meter (Bibby Scientific Ltd T/As Jenway, UK).

SOC media

SOC media was made up according to the protocol described in Sambrook, (1989). One liter of SOC media comprised: 900 ml of distilled H_2O, 20 g Bacto Tryptone, 5 g Bacto Yeast Extract, 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$, 20 ml of 1 M glucose. These ingredients were adjusted to one liter with distilled H_2O and sterilised by autoclaving.

M9 minimal media

M9 minimal media was made up according to the protocol described in Sambrook, (1989). One hundred millilitres of M9 minimal media consisted of the following ingredients: 10 ml of 10 x salts (68 g Na_2HPO_4, 30 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl in a final volume of 1000 ml), 200 µl of 1 M $MgSO_4$, 10 µl of 1 M $CaCl_2$, 5 ml 1 M glucose, 85 ml of dH_2O. In addition, for solid media 1.5 g agar was added. M9 minimal media was

made by autoclaving all ingredients separately and adding them together once cooled. M9 minimal media lacking a nitrogen source was made by omitting NH_4Cl from the 10 x minimal salts, and M9 minimal media lacking a carbon source was made by omitting glucose.

LB media

LB media consists of 10 g NaCl, 10 g tryptone and 5 g yeast extract in 1000 ml final volume. For LB agar 11 g of agar were added. The media was autoclaved before use.

Synthetic apoplast and guttation fluid.

Synthetic apoplast (intracellular fluid) and guttation fluid (drops of xylem sap on the tips or edges of leaves) media was based on M9 minimal media with ammonia, with the following modifications. Five millilitres of 1 M glucose were replaced with a combination of glucose, sucrose, fructose, malate and citrate all at 1 M concentration in the ratio 1:1:1:0.5:0.5 respectively (1.25 ml of glucose, sucrose and fructose and 0.625 ml malate and citrate in 1000 ml). After the M9 media with the sugar combination had been assembled, media was divided in to 10 ml aliquots and sterile stock solutions of 40 mM amino acids were added in the following amounts to make the different synthetic media:

- **Synthetic tomato apoplast**

All amounts in µl: GABA 125; aspartic acid 39; glutamic acid 37.5; alanine 17; serine 12.5; glutamine 12.5; leucine 12.5; glycine 7.5; phenylalanine 6.3; methionine 6.3; valine 6.3; lysine 6.3; threonine 5; isoleucine 5; proline 2.5; asparagine 2.5; histidine 1.3.

- **Synthetic *Arabidopsis thaliana* apoplast**

All amounts in µl: proline 57; glutamine 56.5; serine 43; glutamic acid 32.5; alanine 34.5; aspartic acid 25.5; glycine 23; threonine 8; histidine 4.2.

- **Synthetic *Arabidopsis thaliana* guttation fluid**

All amounts in µl: glutamine 28; histidine 16; aspartic acid 10.5.

Completed media was filter sterilised and stored at 4°C until use (no more than 1 week). The order to which components were added was found to affect their solubility. Components were added to sterile distilled water in the order illustrated in Figure 2.1 Synthetic media was allowed to reach room temperature before bacteria were added.

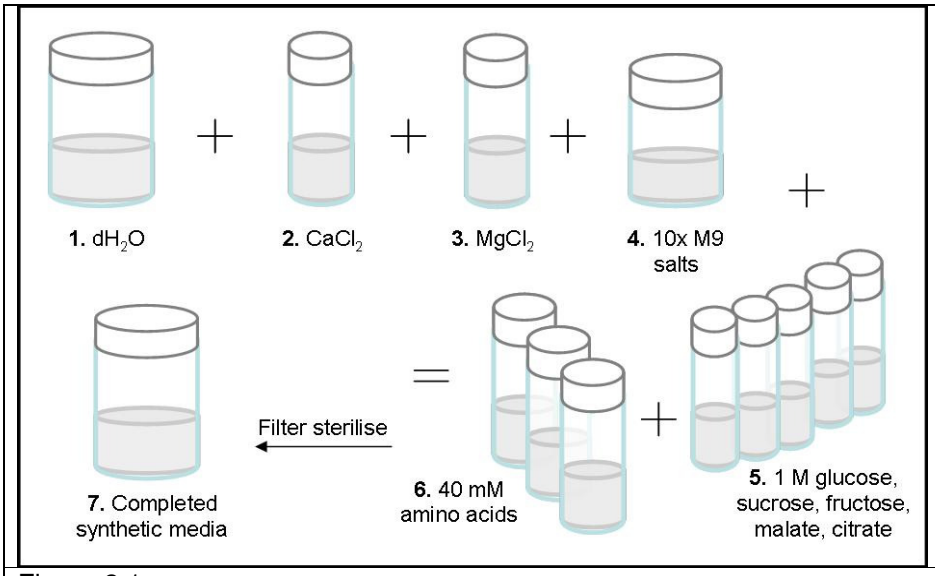


Figure 2.1
Synthetic apoplast and guttation fluid media was based on M9 minimal media, modified with a combination of sugars and amino acids. Components were added as illustrated.

2.2.3 Antibiotic concentrations.

Antibiotics were prepared in 1000 x stock solutions as described in Table 2.3 below and added in the ratio of 1 µl per 1 ml of growth media.

Antibiotic	Abbreviation	Stock concentration
Kanamycin	Km	50 mg / ml (in dH_20)
Gentamicin	Gm	10 mg / ml (in dH_20)
Tetracycline	Tet	12.5 mg / ml (in 70 % EtOH)
5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside	Xgal	40 mg / ml (in dimethylformamide)
Nitrofurantoin	NF	50 mg / ml (in dimethylsulfoxide)
Chloramphenicol	Chl	25 mg / ml (in EtOH)

Table 2.3
Antibiotics used in this study

2.4 Manipulation of DNA.

2.4.1 Plasmids.

The plasmids used in this study are described in Table 2.4 below.

Plasmid	Markers	Reference or source
pUIC3	Tet / LacZ	(Rainey, 1999)
pCR®2.1	Km / LacZ	Invitrogen, UK

Table 2.4
Plasmids used in this study

2.4.2 Isolation of genomic DNA.

Genomic DNA was isolated from *Pseudomonas* using the DNeasy Extraction Kit (Qiagen, UK). For each preparation, 500 µl of a 5 ml overnight culture was used and cells were lysed using Proteinase K (Quiagen, UK), according to the DNeasy Extraction Kit instructions for Gram-negative bacteria.

2.4.3 Ethanol precipitation of DNA.

DNA was precipitated for concentration by addition of 0.1 volumes of 3 M sodium acetate (pH 5.3) and 2 volumes of ice cold 100 % ethanol. Samples were placed on ice and centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed twice with ice cold 70 % ethanol. Tubes were air dried for 15 minutes to remove residual ethanol after which DNA was resuspended in DNA Hydration Solution (Qiagen, UK).

2.4.4 Agarose gel electrophoresis.

Agarose gels were prepared by melting high gel strength agarose to a concentration of 0.8 % (w/v) in 1 x Tris-Acetate-EDTA (TAE) buffer. When examining RT-PCR products the gel concentration was increased to 2 %. Molten gels were allowed to cool to below 50°C before being supplemented with ethidium bromide to a final concentration of 0.2 mg/ml and poured into an appropriate casting tray to set. Gels were then transferred to an electrophoresis tank and immersed in 1 x TAE. Prior to loading one-fifth volume of 5x loading buffer (25 % glycerol, 0.21 % brophenol blue, 0.21 % xylene cyanol) was added to each sample. A standard DNA marker ladder (0.5 µg of 1 kbp or 100 bp as appropriate; New England Biolabs, UK) was added to an empty well to allow a comparison of band sizes. A standard potential difference of 100 V was applied to the gel for one hour. If increased resolution of bands was required then the voltage was lowered to 60 V for 90 minutes. Gels were visualised using a BioRad UV transilluminator (wavelength 245 nm) (BioRad Laboratories, UK) and photographed digitally using BioRad Quantity One software.

2.4.5 DNA quantification.

A Nano-Drop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA) was used for precise quantification of DNA following the manufacturer's instructions.

2.4.6 Primers.

Primers were synthesised by MWG Biotech (Germany) and were used at a final concentration of 10 pmol / reaction. Primers were designed using sequence data available from the *Pseudomonas* genome database and the Primer3 (v 0.4.0) software (Rozen and Skaletsky, 2000).

2.4.7 Polymerase chain reaction (PCR) and Real Time PCR (RT-PCR).

PCR was performed using Taq DNA polymerase (Invitrogen, UK). One tenth dilutions of genomic DNA or plasmid preparations were used as templates. For colony PCR a single colony was picked directly from an agar plate into 50 µl dH2O and boiled for 10 minutes on a heat block. The heat treated bacterial suspension was subsequently used as a DNA template.

The reaction mixture used was as follows:

16.3 µl dH_2O; 2.5 µl 10 x buffer; 0.5 µl dNTP Mix (10 mM each); 0.75 µl MgCl2 (50 mM)
0.2 µl Taq DNA polymerase (1 unit); 1.25 µl 1% W1 solution; 1.25 µl Primer 1 (10 µM)
1.25 µl Primer 2 (10 µM); 1 µl template

Control PCR reactions were included for new primer pairs in which only one primer was used at a time, thereby allowing the specificity of the PCR to be gauged. PCR reactions were run on a Peltier Thermo Cycler (MJ Research, USA) using the following programme:

- 94°C for 3 minutes
- 94°C for 30 seconds
- X°C for 30 seconds
- 72°C for 1 minute / kbp of expected product
- Go to step 2, 29 times
- 72°C for 5 minutes
- 4°C forever

Thermocycler conditions were adjusted according to primer melting temperatures, with X, the annealing temperature, 5°C below the Tm of the primer set. The gradient function of the PCR machine was used to establish the optimum primer annealing temperature where necessary. For RT-PCR reactions the cycle extension step at 72°C was set to 30 seconds. For TA cloning the time at step 6 was increased to 10 minutes as recommended in the TA Cloning Kit (Invitrogen, UK).

2.5 Cloning techniques.

2.5.1 Purification of PCR products.

PCR products were purified by excision of bands from gels using a razor blade, samples were exposed to UV light for the minimum time possible to decrease the risk of DNA damage. Extracted bands were purified using the QIAquick PCR purification Kit (Qiagen, UK) according to the manufacturer's instructions.

2.5.2 Ligation of PCR products into plasmids.

All PCR products were initially cloned into the pCR2.1 vector available as part of the TA Cloning Kit (Invitrogen, UK). Control reactions were performed as described in the manfacturer's instructions. PCR products generated for TA cloning were produced using the polymerase chain reaction described above with a final extension step of 10 minutes. DNA ligations were performed in a 10 µl final volume. For each reaction 1 µl of T4 DNA ligase and 1 µl 10x T4 DNA ligase buffer (both New England Biosciences, UK) were added. Insert and vector were added in the ratio 3:1. Samples were ligated overnight at 14ºC in a Peltier Thermo Cycler (MJ Research, USA). Ligated samples were dialysed as described below to remove salt and transformed into competent cells by electroporation. Aliquots were plated out onto LB agar supplemented with Km and X-gal. Positive transformants were screened by the loss of their ability to break down β-galactosidase (blue-white screening) according to the manufacturer's instructions. Plasmids from positive transformants were extracted and sent for sequencing. In some cases the insert was subsequently removed from pCR2.1, using restriction enzyme digests and ligated into the pUIC3 vector as described.

2.5.3 Restriction enzyme digests of DNA.

DNA molecules were digested with restriction endonucleases according to the manufacturer's recommendations for conditions of digestion. Enzymes were used at a concentration of 1 unit / 25µl reaction volume. Digests were carried out for 1-3 hours. Digested DNA was purified using a QIAquick^{R} Nucleotide Removal Kit (Qiagen, UK) according to the manufacturer's instructions before use in cloning.

2.5.4 Dephosphorylation of DNA.

DNA fragments were dephosphorylated by treatment with Calf Intestinal Phosphatase (CIP,New England Biolabs, UK) to remove terminal 5' phosphate groups and prevent self

ligation. Generally 20 µl of DNA was treated with 1 unit of CIP at 37ºC for 1 hour, before being purified using a QIAquick^{R} purification kit (Qiagen, UK)

2.5.5 Phenol/chloroform purification of DNA.

Restriction endonucleases and CIP were inactivated and removed using phenol/chloroform extraction. All work apart from the centrifugation steps was carried out in a fume hood (Fisher, UK). Samples were mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then centrifuged at 16000 rpm for 1 minute. The phenol layer was discarded and the procedure was repeated. To remove residual phenol the aqueous layer was mixed with an equal volume of chloroform and centrifuged for a further 1 minute. The chloroform layer was then removed and the procedure was repeated. Finally the aqueous layer (containing DNA) was removed to a fresh tube.

2.5.6 DNA Sequencing.

Sequencing reactions were carried out using a Peltier Thermo Cycler (MJ Research, USA) in 10 µl reactions containing the following:

1.5 µl of 5x sequencing buffer

0.3 µl of primer

1 µl of big dye

2 µl of purified plasmid

5.2 µl of H_2O.

The conditions for the sequencing reaction were 96ºC for 10 seconds, 50ºC for 20 seconds, 60ºC for 4 minutes, 25 cycles. Following the sequencing reaction samples were precipitated by the addition of 1 µl of 125 mM EDTA, 1 µl of 3M NaOAc and 25 µl of 100 % ethanol. Samples were gently mixed by pipetting and incubated at room temperature for 15 minutes before centrifugation for 30 minutes at 7,000 rpm. The supernatant was removed immediately and the pellet washed with 70 % ethanol. Samples were centrifuged for 1 minute at 13,000 rpm, the supernatant removed and the samples allowed to air dry. Nucleotide sequence was determined using a 3100 16 capillary big-dye sequencer (Applied Biosystems, USA) in the Department of Zoology, Oxford.

2.5.7 Dialysis of DNA.

Salt-free DNA was obtained using 0.025 µm Millipore (Millipore, UK) 'V' series dialysis membranes and the 'drop dialysis' protocol obtained from the Millipore website:

www.millipore.com: A Petri dish was filled with sterile water. Using stainless steel forceps a membrane filter disc was placed on the surface of the water, shiny side up. The sample (5-100 µl) was placed on the center of the membrane with a micropipet. The petri dish was sealed to eliminate loss of sample to evaporation during the procedure. Samples were dialysed for 30 minutes and the desalted sample was then recovered with a micropipet

2.5.8 Production of electrocompetent *Pseudomonas.*

Electrocompetent *Pseudomonas* were generated according to the protocol described by Choi and coworkers (2006). All chemicals were obtained from Sigma-Aldrich, UK. Briefly 1.5 ml of an overnight bacterial culture were transferred to a chilled microfuge tube. Cultures were centrifuged and washed twice in 1 ml of ice-cold, sterile 10 % Glyceol, 1mM HEPES solution. Cultures were centrifuged again and the bacterial pellet was re-suspended in 100 mM Glycerol / HEPES and stored on ice. Two microlitres of salt-free DNA (dialysis performed as described above) were added to the re-suspended bacterial pellet. The contents of the microfuge tube were then transferred to an ice-cold Gene Pulser 1 mm electroporation cuvette (BioRad, UK) and electroporated with a BioRad Gene Pulser electroporation system at 1.75 V, 200 Ohm. Following electroporation, cells were immediately mixed with 1 ml SOC medium and transfered to a sterile microfuge tube. Cells were incubated at 28°C for 2 hours with shaking (200 rpm). Four 100 µl aliquots of cells were plated out at 10^{-1} dilution onto LB medium supplemented with half-strength CFC (Oxoid Limited, UK), a combination of antibiotics which preferentially select for the growth of *Pseudomonas* species, and the appropriate selective antibiotics. The remaining cells were centrifuged for 1 minute at 13,000 rpm, resuspended in 100 µl SOC medium and plated out onto the selective medium.

2.5.9 Production of electrocompetent *Escherichia coli.*

Batches of electrocompetent *E.coli* were prepared in 100 µl aliquots according to the following protocol: A single *E.coli* colony was inoculated into 50 ml of no-salt LB media (10 g tryptone, 5 g yeast extract per 1000 ml), and grown overnight at 37°C with shaking (200 rpm). On day two, 25 ml of the overnight culture were added to 1000 ml of no-salt LB, pre-warmed to 37°C. This culture was incubated at 37°C with shaking (200 rpm) until the optical density (OD_{600}) reached 0.6 - 0.75. Immediately, the culture was chilled on ice for 15 min to prevent further growth. The culture was divided into chilled, sterile 50 ml tubes and centrifuged at 5000 g for 10 minutes at 4°C in a Beckman J2-MC centrifuge. The supernatant was removed and the bacterial pellet was resuspended in a total volume of 200 ml ice-cold 10 % glycerol. The centrifugation step was then repeated for

another 5 minutes. This process was repeated three more times, resuspending the pellet in decreasing volumes of 50 ml, 100 ml and finally 2 ml of ice-cold 10 % glycerol. Finally, 100 µl aliquots were transferred into frozen 1.5 ml microfuge tubes and stored at -80°C until use.

2.5.10 Transformation of electrocompetent *Escherichia coli* and *Pseudomonas.*

Aliquots of electrocompetent cells stored at -80°C were thawed on ice before use. This step did not apply to electrocompetent *Pseudomonas* which were freshly made. One microliter of plasmid prep or dialysed ligation was added to the electrocompetent cells and gently mixed with a sterile pipette tip. The cells were transferred to a sterile, pre-chilled 0.2 cm electroporation cuvette (BioRad, UK). Cuvettes were kept on ice until electroporation at 2.5 V and 200 Ohms using a Gene Pulser electroporation system (BioRad, UK). Immediately after electroporation 800 µl of SOC medium was added to the cuvette and the cells were transferred to a sterile 1.5 ml microfuge tube. Cells were incubated for one hour at 37°C for *E.coli*, or 2 hours at 28°C for *Pseudomonas*, with shaking (200rpm), after which aliquots were plated out onto appropriate selective media.

2.6 Bioinformatics.

2.6.1 Statistical analysis and result presentation.

Basic statistics (mean, standard deviation etc) and graphs were performed in Microsoft Excel. Advanced statistics were performed in Microsoft Excel (Student's t-tests) and Minitab (Minitab Inc., State College, PA, USA) (analysis of variance [ANOVA] and calculation of confidence intervals).

2.6.2 Manipulation and analysis of DNA sequences.

- **Validation of positive transformants from vector ligations.**

Following vector ligations, positive transformants were selected by blue/white colony screening, followed by sequencing. The resulting sequence file was opened in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html, Hall 1999), and manually examined for sequence quality (percentage of unknown 'N' bases in the sequence). The sequence was extracted as a FASTA file and the closest match for the sequence determined by BLASTn search (Altschul et al 1990) of the National Centre for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The returned sequence was manually checked for point mutations when compared to the database sequence.

- **General genome visualisation methods.**

The Artemis genome viewer and annotation tool (Rutherford et al., 2000) (http://www.sanger.ac.uk/Software/Artemis/) was used for genome visualisation. Examination of gene orthologs among the *Pseudomonas* and of gene synteny were performed using the utilities available from www.pseudomonas.com (Winsor et al., 2009) and the WebACT tool (Abbott et al., 2005) (http://www.webact.org/WebACT/home).

2.7 Plant growth conditions and apoplast extraction techniques.

2.7.1 Extraction of apoplast fluid.

Apoplastic fluid was extracted from uninfected tomato plants using vacuum infiltration as described in Rico and Preston (2008). Apoplastic fluid was extracted from tobacco plants using this method with slight modifications (Figure 2.2). Briefly, each tobacco leaf was detached from the plant, cut into four equal pieces, and placed in ~200 ml of distilled water in a 1litre conical flask with a side arm, attached to a Welch Vacuum pump (Gardner Denver, Il, USA). Cycles of pressure (60-90psi) and vacuum were applied to until the leaf was completely infiltrated. Infiltrated leaf sections were then blotted dry, rolled and introduced into 5 ml tips. The tip was placed inside a 50 ml conical tube (Corning, NY, USA) containing a 1.5 ml microfuge tube. Apoplast samples were then collected by centrifugation of the conical tubes at 2000 rpm for 5 min at 4°C. The apoplast present in the microfuge tube was centrifuged again for 10 min at 3000 rpm at 4°C. The supernatant was decanted into a clean 1.5 ml microfuge tube and freeze dried overnight using an ALPHA 2-4LD freeze drier (Christ, Osterode am Harz, Germany).

Figure removed for copyright reasons

Figure 2.2: Flow diagram illustrating the complete procedure for obtaining a concentrated apoplast extract as used in this study. (A) Apoplast infiltration, (B) Apoplast extraction, and (C) Apoplast extract concentration protocols are based on protocols used previously by Rico & Preston, (2008), and Solomon & Oliver, (2001). Figure adapted from Stuart King (unpublished)

2.7.2 Estimation of apoplast dilution.

During the apoplast extraction method, the apoplast metabolites become diluted with the infiltrated water. In order to measure the amount of metabolites in the healthy apoplast before the apoplast extraction process, the diluted apoplast therefore needs to be freeze dried and resuspended at the original concentration. In order to do this, the dilution of the apoplast fluid by the infiltrated water must first be calculated. The estimation of apoplast dilution was carried out as described in Rico and Preston (2008). Briefly, pre-weighed leaves were infiltrated with a high viscosity silicone fluid (AP100, polydimethymethyphenylsiloxane, density 1.07 g/cm^3; Wacker, Munich, Germany). The air volume was calculated as the increase in the weight of the leaves after the infiltration, corrected with the density of the silicone oil (V_{air}cm^3 of air g FW^{-1}).

The apoplastic water volume was calculated by infiltrating pre-weighed leaves with indigo carmine (50 mM indigo5,5'disulfonic acid, disodium salt) dissolved in 50mM phosphate buffer at pH6.2. Infiltrated leaves were immediately blotted dry and reweighed, the difference in weight before and after infiltration was used for calculation of the infiltration volume **(V_i)**, assuming a leaf density of 1 gcm^{-1}. The dye was then extracted by centrifugation as described above, and the dilution of the extracted dye **(D_{dye})** was determined spectrophotometrically at 610nm by comparing the absorbance of the extracted dye with known concentrations of indigo carmine. The apoplastic water volume was calculated using the following equation:

$$V_{apo} = D_{dye} \times V_i / (1 / D_{dye})$$

The equation for calculating the dilution factor of apoplast extracts is:

$$F_{dil} = (V_{apo} + V_{air}) / V_{apo}$$

The apoplast dilution factor was calculated separately for different plant species. Freeze dried apoplast was then resuspended in MilliQ water using the formula:

$$V_{orig} = V_{ex} / F_{dil}$$

Where V_{orig} is the 'full strength' apoplast, V_{ex} is the amount of apoplast extracted from the infiltrated tobacco leaves and F_{dil} is average the dilution factor of the apoplast extraction process. From: Rico and Preston 2008.

2.7.3 Plant growth conditions.

Seeds were grown on a 3:1 mixture of peat based compost (Goundrey's, UK) and vermiculite (Sinclair, UK). Seeds were soaked overnight in a solution of 0.5 mg / ml^{-1} gibberelic acid (Sigma Aldrich, UK) made up in water, and germinated in a plant growth chamber set at a constant temperature of 21°C and a photoperiod of 16 hours (16 hours light, 8 hours dark). After 21 days seedlings were transplanted into individual pots

containing soil/vermiculite mixture and moved to a transgenic greenhouse set at 21°C and a 16 hours photoperiod. Tobacco plants were used in experiments at twelve weeks old. Tomato plants were used in experiments at eight weeks old for apoplast extraction experiments and *in planta* bacterial growth assays. Two replicate plants were used for each experiment, and each experiment was independently performed at least twice as indicated.

2.8 Imaging.

2.8.1 Digital photography.

Images were acquired using a Fujifilm FinePix S7000 digital camera (Fujifilm, UK). All images were taken by the author or John Baker (Plant Sciences Department, Oxford).

2.8.2 Microscopy.

An Olympus BX50 microscope was used for fluorescence and light microscopy (Olympus, USA). Images were taken using an Optronics camera system and analysed using MagnaFire software (Optronics, USA).

2.8.3 Photon camera imaging.

Luminescence was imaged using a photon-counting camera (Photek Ltd., East Sussed, UK). Luminescence counts were recorded every second for 200 seconds. The total points and pixel intensity for each inoculated area was obtained using Photek IFS32 image processing and data acquisition software (Photek Ltd.)

2.9 Spectrophotometric measurements.

The optical density of bacterial solutions was routinely measured using a Sanyo SP BIO spectrophotometer (Gallenkamp, UK). The sample was contained in a 1 cm cuvette and measured at a wavelength of 590 nm. When a sample produced an optical density reading greater than 0.3, then the sample was diluted 1 in 10 or 1 in 100 and measured again. The optical density of bacteria in 96-well plates was measured using a Tecan Infinite M200 (Tecan, UK) at a wavelength of 590 nm. When plates were measured following incubation at 28°C, the Tecan reader was also set to 28°C to reduce risk of condensation.

Chapter 3: Bioinformatics and phylogenetic analysis of amino acid permease genes in *Pseudomonas*.

3.1 Overview.

The availability of genome sequence data offers new opportunities to gain deeper insight into niche specificity and plant colonisation by *Pseudomonas syringae* (*P. syringae*), and to identify candidate genes which may contribute to *Pseudomonas*~plant interactions. It has previously been demonstrated that the amino acid permease domain (aa_permease), found in aa_permease genes, is under-represented in *P. syringae* (Studholme et al., 2005). Chapter 1 proposed several hypotheses to explain this distribution, including the suggestion that most aa_permease genes are of little or no importance during plant colonisation compared to other ecological niches, resulting in gene loss in plant-pathogenic bacteria. Conversely, the aa_permease genes may be important for colonisation of heterogeneous environments by non plant-pathogenic *Pseudomonas* leading to aa_permease genes being retained in these species or even increased in number by duplication or gene acquisition.

In order to learn more about the aa_permease genes in *Pseudomonas,* and *P. syringae* in particular, bioinformatic analyses were carried out. This chapter reviews the distribution of aa_permease genes across the sequenced *Pseudomonas*. Bioinformatics software was used to identify the number and context of these genes in *Pseudomonas* species and annotate features such as RpoN-binding sites, which may contribute to their regulation or function. The prevalence of horizontal gene transfer of aa_permeases was investigated and found to be relatively rare. Phylogenetic tree-building software was used to place the number and diversity of aa_permeases into an evolutionary context.

3.2 Introduction.

Pseudomonads are ubiquitous bacteria in the environment, able to metabolise a wide range of compounds and forming an important part of the carbon cycle (Alfano and Collmer, 1996). In recent years the number of sequenced *Pseudomonas* genomes has expanded considerably to include three plant-pathogenic *P. syringae* pathovars, (with two more draft sequences published in 2009) and a range of non plant-pathogenic *Pseudomonas* species including saprophytes, biotrophs and opportunistic pathogens (see Chapter 1, Table 1.1). The availability of genome sequences for *Pseudomonas* species from a diverse range of habitats makes this genus an excellent tool for bioinformatic studies, in particular, genome sequences have facilitated comparative analyses between sequenced *P. syringae* strains (Feil et al., 2005; Joardar et al., 2005; Lindberg et al., 2008) and between *P. syringae* and non-plant pathogenic *Pseudomonas* such as *P. aeruginosa*, *P. putida* or *P. fluorescens* (Studholme and Dixon, 2003; Studholme et al., 2005). In particular Studholme and coworkers (2005) identified the amino acid permease (aa_permease) genes as being significantly underrepresented in plant pathogenic *P. syringae* compared to non plant-pathogenic *Pseudomonas*.

P. syringae occupies two plant-associated niches; the foliar surface and the intercellular apoplast. Comprehensive data describing the nutrient composition and abundance in either niche is relatively scarce, although both are thought to be nutrient limited (see Chapter 1). Nutrients available on the foliar surface are thought to arise from leaching of sugars and amino acids from epidermal plant tissues, particularly around the bases of trichomes and around hydathodes where *P. syringae* is found to aggregate (Beattie and Lindow, 1999). Studies describing the nutrient content of the plant apoplast in particular are relatively rare, and even when data can be found, researchers have rarely measured more than a few metabolites in a single study. The apoplast amino acid content of several plant species, including tobacco, bean and tomato, has been described in the literature (Table 1.3 Chapter 1). Of these studies, the majority of data describes the compounds present within the tomato apoplast (Joosten et al., 1990; Valle et al., 1998; Solomon and Oliver, 2001; Rico and Preston, 2008). As such, the most abundant nutrients in the tomato apoplast were found to be hexose sugars like sucrose, fructose and glucose, the polyol inositol, and dicarboxylic acids such as malic and citric acid, while of the amino acids the apoplast contained predominantly GABA, aspartic acid, glutamic acid and alanine.

Rico and Preston (2008) compared the amino acids detected in the healthy tomato apoplast, with those amino acids which could be metabolised by three non plant-

pathogenic *Pseudomonas* species (*P. fluorescens*, *P. putida* and *P. aeruginosa*) or three pathovars of plant-pathogenic *P. syringae*. Results indicated that although *P. syringae* pathovars could metabolise fewer amino acids than the generalist species, those amino acids which were metabolised by *P. syringae* corresponded to ones which were abundant in the tomato apoplast. These results have been suggested to provide further evidence that *P. syringae* is adapted to utilise nutrients which are available in the plant apoplast (Rico and Preston, 2008).

P. syringae has long been viewed as a specialist species and prior to DNA sequencing *P. syringae* was commonly identified from other *Pseudomonas* species using phenotypic tests based on their differential abilities to use different nutrients as sole sources of energy (Thornley, 1960; Lelliott et al., 1966; Palleroni and Moore, 2004). As more *Pseudomonas* genomes are published and annotated, researchers have been able to compare *P. syringae* and generalist (non plant-pathogenic) *Pseudomonas* genomes to investigate the genomic basis for this specialisation.

In particular, Studholme and coworkers (2005) identified a significant difference in protein domains found in transporters associated with nutrient assimilation when comparing the genomes of *P. syringae* with more generalist *Pseudomonas* species. Proteins are generally composed of one or more functional regions known as domains. Different combinations of domains give rise to the diverse range of proteins found in nature. The identification of domains that occur within proteins can therefore provide insights into their function. Pfam is a collection of protein domain families along with relevant functional annotations, literature references and database links. The *Pseudomonas* genomes listed in Chapter 1, Table 1.4, have all been annotated in Pfam.

P. syringae acquires amino acids from its environment via membrane bound transporters belonging to several protein domain families including ABC (ATP-Binding Cassette) transporters, branched chain amino acid transporters and permeases. Several of these transporters have been shown to be powered by 'active' mechanisms such as ATP utilisation or proton gradients (Hosie and Poole, 2001) although the exact mechanism of many transport proteins has yet to be elucidated. The Pfam database (http://www.sanger.ac.uk/Software/Pfam/) (Bateman et al., 2004) is a useful tool with which to identify and catalog similar transport proteins.

In their 2005 study, Studholme and coworkers computationally searched the Pfam database to look for over and underrepresented protein domain architectures in

P. syringae compared to other *Pseudomonas*, or in all *Pseudomonas* compared to other bacteria (Studholme et al., 2005). The rational behind this experiment was that expansion of a domain family may correspond to an increase in functional properties, such as an improvement in regulatory or nutritional flexibility or an enhanced stress response, while underrepresented domains are likely to represent loss of function (Studholme et al., 2005).

One significant area in which *P. syringae* and non plant-pathogenic *Pseudomonas* genomes differed was nutrient utilisation. For example, the sugar transport domains sugar_tr and BPD_transp_2 were both overrepresented in *P. syringae* compared to other *Pseudomonas* species. Particularly striking however was the reduction in the number of aa_permease (amino acid permease) domains in *P. syringae* compared to non plant-pathogenic *Pseudomonas* (Table 3.1). This reduction appears to have occurred alongside an expansion of aa_permease domains in non plant-pathogenic *Pseudomonas* compared to other bacteria (Studholme et al., 2005). The reduction in *P. syringae* aa_permease domains is particularly interesting due to the large number of domains that have increased in number in all *Pseudomonas* species (i.e. including *P. syringae*) in comparison to all sequenced bacteria in the Pfam database (Studholme et al., 2005).

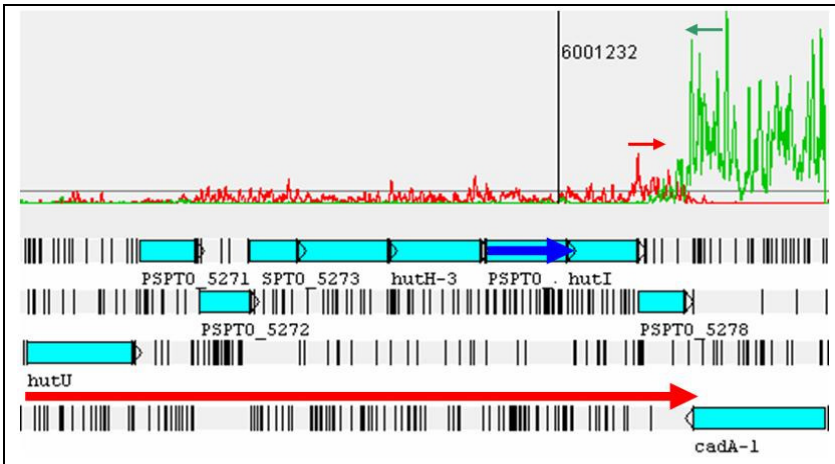
Gene reduction to the extent where closely related bacteria are left with statistically different numbers of genes can be seen in highly specialised bacteria such as *Xylella fastidiosa*, which contains significantly fewer aa_permease than species from the closely related *Xanthomonas* genus of plant pathogens (Vauterin et al., 1995; Moreira et al., 2005) (Chapter 1, Table 1.5).

The explanation offered for genome reduction in obligate species such as *Xylella*, is that the ability of these specialised pathogens to parasitise host organisms has rendered numerous genes redundant, costly or deleterious, and therefore expendable. In contrast, generalist species need to adapt to a wide range of environmental conditions and may undergo gene duplication and horizontal gene transfer resulting in genome expansion (Stepkowski and Legocki, 2001). With this in mind a number of hypotheses were proposed in Chapter 1 to explain the distribution of aa_permease domains within *Pseudomonas* (Chapter 1, Figure 1.10).

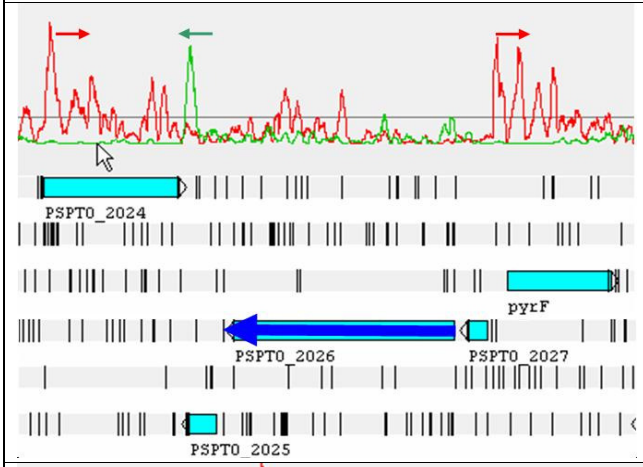
Within the *Pseudomonas*, few aa_permease transporters have been extensively characterised; ArcD in *P. aeruginosa* PAO1 is an arginine/ornithine antiport protein which mediates an energy-independent, stoichieometric exchange between ornithine and

arginine, with ornithine being transported out of the bacterial cytosol and arginine imported (Itoh and Nakada, 2004). HutT in *P. fluorescens* SBW25 is a histidine-inducible transporter located within the histidine (hut) operon of *P. fluorescens* SBW25 (Zhang and Rainey, 2007). In addition, a screen of transposon mutants in *P. aeruginosa* PAK revealed that a mutant carrying the transposon in PA0220 (annotated as an ethanolamine transporter), was unable to metabolise histamine as a sole nitrogen source (Johnson et al., 2008). The aa_permease transporters have been classified by the Transport Classification Database (http://www.tcdb.org/index.php) into the Amino acid-Polyamine-organo Cation (APC) superfamily on the basis of sequence and functional similarity to characterised transporters (Saier, 2000). Transporters of the APC superfamily are electrochemical potential-driven porters, meaning they utilise a carrier-mediated process to facilitate either antiport and/or symport using chemiosmotic energy (Saier, 2000).

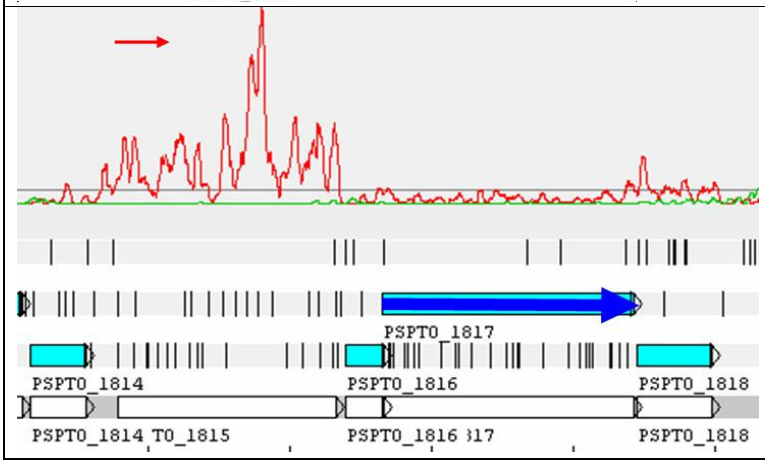
Recently additional information has come to light regarding the aa_permease genes in P.s.t, in the form of a more advanced technique of operon prediction used to address the question of which genes are co-transcribed in particular conditions. Briefly, this technique involves Solexa sequencing of RT-PCR transcripts generated from mRNA expressed in Mannitol Glutamate media, as described by Bronstein and coworkers (2008). The amount of transcription occurring in each region under these conditions is indicated by the height of the sequence trace. RT-PCR transcript traces were generated by Magdalen Lindeberg for PSPTO_2026, PSPTO_5276 and PSPTO_5356 as these have adjacent genes in the same orientation which might therefore form an operon. Figure 3.1 shows the sequencing traces for the genes surrounding the P.s.t. proline (A), ethanolamine (B) and aromatic (C) aa_permease genes during growth in mannitol glutamate medium (a low nutrient minimal media). Traces were supplied by Magdalen Lindeberg (personal communication) methods are described in detail by Bronstein and coworkers (2008). The aa_permease genes were not expressed in the mannitol glutamate media used. However, we can see that the genes of the *hut* operon, with the exception of the final gene PSPTO_5278, were all equally inactive in this media. PSPTO_5278 did show slightly more expression than the other genes, although the level of transcription was still very low compared to the expression of PSPTO_5279 (CadA-1), shown in green, and did not necessarily represent independent transcription of this gene from the rest of the *hut* genes. It will be interesting to see if other researchers repeat this technique in the future using other media.



A. PSPTO_5276
proline
aa_permease



B. PSPTO_2026
ethanolamine
aa_permease



C. PSPTO_1817
aromatic
aa_permease

Figure 3.1
Operon prediction based on Solexa sequencing of RT-PCR transcripts expressed in mannitol glutamate medium (a minimal medium). The red trace shows the (+) strand and the green trace shows the (-) strand, the height of the trace indicates the strength of gene expression. Red and green arrows indicate which genes are predicted to be expressed under these conditions. Genes of the P.s.t. *hut* operon are indicated by the red arrow in A. The aa_permease genes (blue arrows in A, B C) are not highly expressed under low iron conditions.

One way to learn more about the aa_permease transporters in *Pseudomonas* and the possible substrate(s) they transport is through bioinformatic and phylogenetic analyses. These analyses can aid our understanding of aa_permeases in several ways. Bioinformatics programmes can locate signal peptides and transmembrane domains to support the identity of these proteins as transporters. In addition, comparative searches looking at the genomic context of each gene may reveal clues to their function and the degree to which the synteny of surrounding genes has been conserved during evolution. In relation to the hypotheses discussed in Chapter 1, seeking to explain the distribution of aa_permeases in *Pseudomonas*, the question which can be immediately addressed using bioinformatics is whether the observed gene distribution is the result of gene loss in *P. syringae*, or gene duplication in other *Pseudomonas*. Orthology analysis can identify whether the aa_permeases found in *P. syringae* are present in other *Pseudomonas*, and comparison of sequences to databases can indicate whether genes have been acquired from other bacterial species through horizontal evolution. Phylogenetic tree building can be used to support these findings and relate them to an evolutionary framework. Where experimental evidence is available for the substrate(s) transported by a particular aa_permease, then phylogenetically related sequences could also be hypothesised to perform the same function.

3.3 Objectives.

- Collect protein sequence data for all *Pseudomonas* genes containing an aa_permease domain.
- Search for signal peptides and transmembrane domains to identify the cellular localisation of these proteins.
- Compare the genomic context of aa_permeases and synteny of the surrounding genes in *Pseudomonas*
- Use bioinformatic techniques to identify gene duplication and horizontal evolution within the aa_permease genes.
- Construct phylogenetic trees showing the evolutionary relationship of the aa_permease genes in *P. syringae*, and how these genes are related to those found in other *Pseudomonas*.

3.4 Materials and methods.

3.4.1 Domain analysis.

The Pfam (protein families) database is a collection of protein sequences which have been annotated with their constituent protein domains and separated into families. Pfam lists 153 aa_permease domains from the *Pseudomonas* species listed in Figure 3.1. As this information only corresponds to the protein sequence of the aa_permease domain, not the whole gene, sequence accession numbers were obtained and used to locate the protein sequences of the corresponding aa_permease gene. The complete gene sequences were used in sequence alignment.

The information in the Pfam database includes the length of each aa_permease domain in amino acids. The majority of aa_permease domains span the length of the corresponding aa_permease gene; however some domains only span fragments of the full gene sequence (Figure 3.2). The allocation of domains in the Pfam database has been done using automated computational techniques and therefore some errors may exist. Domains identified by Pfam as being shorter than 200 amino acids in length were reviewed as described in Figure 3.2 to establish whether they should be included in further analysis.

>PSPTO_5276
MTSQDGLKRGLSAR**HIRFMALGSAIGTGLFYGSASAIQQAGPAVLLAYLIGGAAVYMVMRA**
LGEMAVHDPVSGSFSHYATRYMGPLAGFVLGWTYAFEMIIVCLADVTAFGIYMGFWFPEVP
RWIWVLGIVFLIGALNLCNVKVFGETEFWLSILKVSAIVAMIVAGFGIMIFGIGSSTSGTEIGIS
NLWAHGGFMPNGVTGLIASFAVVMFAFGGIEIIGITAGEAKDPQRSLPQAINAVPLRILLFYV
LTLFVLMCIYPWPQIGTQGSPFVQIFDNLGIASAATILNIVVISAAVSAINSDIFGAGRMMYGL
ARDGQAPASFARLSRQGVPWMTVLVMGVTLLGGVLLNYLIPKDVFLLIASLATFATVWVW
LMILLTQVAMRRSMSREEVTQLKFAVPFWPYGPAAAIAFMLFIFGVLGYFPDNRAALIVGAI
WIALLLIAYGLWVKPKALNKTH

>Psyr_2683
MSTPPGKLRLGALVALVVGSMIGGGIFSLPQNMAASAEVGAVLVGWAITAIGMLTLAFVFQTL
ANRKPDLDGGVYVYAKAGFGDYMGFSSAWGYWISAWLGNVGYFVLLFSTLGYFFPVFGEG
NTVAAVIGASVLLWGVHFLVLRGIQEAAFINLVTTVAKVVPLILFVLIAVFAFKLDIFTTDIWGLE
KPDMGSVMNQVRHMMLVTVWVFIGIEGASIFSARASKRSDVGKATVIGFITVLLLLVLVNVLS
LGVMTQPELAKLQNPS**MAAVLEHIVGPWGAALISVGLIISLLGALLSWVLLCAEIMFAAAKD**
HTMPEFLSRENANHVPANALWLTNAMVQVFLVITLFSSSTYLSLIYLATSMILIPYLWSAAYA
LRLAIGGEGYENARRERRKDLFIAAVALLYAIWLIYAGGVKYLLLSALLYAPGVILFAKAKLEAK
KPVFTHLENMMFVAVLIGALIAAYGLYDGFLTL

Figure 3.2
The majority of Pfam-identified aa_permease domains span the length of the corresponding gene. This has been illustrated above using PSPTO_5276 as a representative example. The aa_permease domain of PSPTO_5276 is highlighted in blue on top of the corresponding amino acid sequence for the full gene. However, the aa_permease domain in Psyr_2683 is only 100 amino acids long (highlighted in blue); this corresponds to a fragment of the Psyr_2683 gene.

3.4.2 Analysis of orthologs and horizontal evolution (horizontal gene transfer).

Many aa_permease genes with an annotated transport function (e.g. GABA) are present in more than one species of *Pseudomonas*. To identify whether these genes were orthologous, the BLASTP program (http://www.ncbi.nlm.nih.gov/) was used to perform a reciprocal best blast hits (RBBH) method. Amino acid sequences corresponding to the aa_permease transporters were downloaded from the Pfam database. These sequences were entered, one at a time, into the BLASTP programme in FASTA format, where each sequence was compared against protein sequences from all organisms in the NCBI database using the default settings. The most similar sequence (as measured by a high percentage identity and an E-value (confidence measure) of close to 0.00) was put back into BLASTP. When the most similar sequence resulting from this search was the original input sequence, then the sequences are RBBH, and the corresponding genes were identified as orthologs. This method has been criticised on the basis that the closest BLAST neighbour is too commonly reported as an ortholog without checking that a similar phylogenetic relationship exists between the two species in question (Koski and Golding, 2001). However, these arguments do not apply to the *Pseudomonas*, whose close phylogenetic relationship is well established (Yamamoto et al., 2000) and therefore confidence that RBBH in other *Pseudomonas* are phylogenetically closely related is high.

RBBH results were compared to ortholog predictions in the *Pseudomonas* Genome Database (http://v2.pseudomonas.com/), where some *Pseudomonas* genomes have been analysed using the high-throughput Ortholuge programme (http://www.pathogenomics.ca/ortholuge/). The ortholog predictions made by a manual RBBH approach were supported by the Ortholuge predictions in the *Pseudomonas* Genome Database.

When a gene has been acquired from non-*Pseudomonas* bacteria through horizontal gene transfer, it is expected that the sequence of this gene will more closely resemble the gene in the original bacterial species than any *Pseudomonas* genes. To investigate possible horizontal evolution of aa_permease genes, the protein sequence of each gene was compared with all of the proteins in the NCBI database using the BLASTP programme. The bacterial species of the most similar matches to the input sequence were recorded. When one of the top matching sequences was present in a species other than *Pseudomonas* then the gene in the other species was investigated to see if it could be identified as the ortholog of the *Pseudomonas* sequence. This was investigated further by phylogenetic analysis; if the *Pseudomonas* gene was found to consistently

group with genes from non-*Pseudomonas* bacteria then horizontal transfer of the gene is thought to have occurred.

3.4.3 Phylogenetic analysis.

Protein sequences were aligned using ClustalX (http://www.clustal.org/) with the gap penalties adjusted as necessary to obtain improved alignments. Protein sequences were used primarily because the level of sequence diversification between genes made it hard to align DNA sequences. Outgroups were selected according to the out-group rooting method described in Harrison and Langdale 2006. Manual adjustments to the alignments were performed in BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html), truncated sequences and gapped areas present in more than 80% of sequences were removed from the alignment. The alignment file was exported to PAUP*4.0 (http://paup.csit.fsu.edu/) for tree building. Phylogenetic trees were constructed using the maximum parsimony method (MP) with neighbour joining (NJ) methods used for a comparison of accuracy. Trees were bootstrapped with 500 replicates to give an estimate of alignment reliability. Output trees were presented using TreeView: (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

3.4.4 Identification of signal peptides and localisation of proteins within the cell.

The amino acid permeases are predicted to be transporters; one way of supporting this is to demonstrate the presence of signal peptides. Two applications, SignalP and PSORTB were used to predict the presence of signal peptides and the subcellular localisation of proteins. Each application bases its prediction on a different algorithm. The SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson et al., 2007) uses a neural network and hidden Markov model algorithm to predict the presence and location of signal peptide cleavage sites in amino acid sequences, and predict the cellular localisation of the subsequent protein. The PSORTBV2.04 server (http://www.psort.org/psortb/) uses a hidden Markov model and support vector machine algorithm to predict cellular localisation based on amino acid sequences. Sequences were entered in FASTA format.

3.4.5 Identification of predicted transmembrane domains.

Further support of the aa_permease role as transporters can be gained by demonstrating the presence of transmembrane domains, which are only present in membrane bound proteins. The number of transmembrane domains in aa_permease sequences was predicted using the TMHMM2.0 server (Sonnhammer et al., 1998). Amino acid sequences were entered in FASTA format. The output format is a probability plot

showing the posterior probabilities of inside / outside / TM helix giving an idea of the certainty of each segment in the prediction. At the top of the plot (between 1 and 1.2) the best transmembrane domain prediction is shown. An example output is shown below (Figure 3.3). These results were also confirmed using the HMMTOP programme (Tusnády and Simon, 1998), which uses Hidden Markov Model-based predictions in a similar manner to TMHMM2.0, and PredictProtein (Rost et al., 2004) which predicts transmembrane domains based on the alternative method of artificial neural networks.

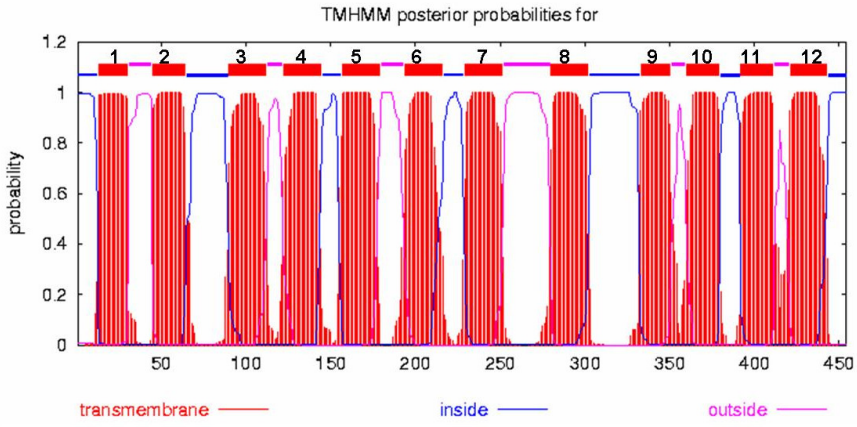


Figure 3.3
An example output from the TMHMM2.0 server.
This sequence is predicted to have 12 transmembrane domains. The confidence in each transmembrane domain is indicated on the y axis.

3.4.6 Identification of predicted sigma^{54} (RpoÑ)binding sites.

The PromScan program scans DNA sequences for characteristic sequence motifs which represent potential sigma^{54} (RpoÑ)binding sites Predicted RpoN binding sites from three *Pseudomonas* genomes; PAO1, KT2440 and P.s.t were collected from the PromScan database (http://www.promscan.uklinux.net/RpoN/data.html). Data for P.s.ph, P.s.s, Pf0-1, Pf-5 and PA14 genomes was provided by David Studholme (personal communication).

3.4.7 Genomic context.

Knowledge of the function and organization of the genes in the surrounding area can be used to indicate the function of a gene of interest. In addition, if the synteny of the surrounding genes is conserved across several species this can suggest co-regulation of this group of genes. The genomic context and surrounding gene synteny of the aa_permease genes was examined using an online version of the Artemis Comparison Tool (WebACT) (http://www.webact.org/WebACT/home), which facilitates the alignment of genomes and indicates the percentage identity shared by genes in a particular region.

3.4.8 Statistical analysis.

Statistics were carried out using Minitab and Excel programs. For large datasets a general linear model was carried out in Minitab. Smaller datasets were analysed using Student's t-tests in Excel. In both cases significance at the 95% confidence interval was judged by a p value <0.05.

3.4.9 Abbreviations.

During discussions in the text *Pseudomonas* species are frequently referred to by abbreviations as shown below. In Tables or Figures *Pseudomonas* species may also be annotated by their gene names (Table 3.1).

***Pseudomonas* species**	**Abbreviation**	**Gene name**
P. syringae pv. tomato DC3000	P.s.t	PSPTO
P. syringae pv. phaseolicola 1448A	P.s.ph	PSPPH
P. syringae pv. syringae B728a	P.s.s	Psyr
P. aeruginosa PA14	PA14	PA14
P. aeruginosa PAO1	PAO1	PA
P. entomophila L48	L48	PSEEN
P. fluorescens Pf-5	Pf-5	PFL
P. fluorescens Pf0-1	Pf0-1	Pfl
P. putida KT2440	KT2440	PP
P. putida F1	F1	Pput

Table 3.1
Pseudomonas species used in this chapter and their abbreviations

3.5 Results.

3.5.1 Sequence analysis of aa_permease domains.

The first stage in bioinformatic analysis was to gather the amino acid sequences for the aa_permease-domain containing genes. Initially only genes containing an aa_permease domain longer than 200 amino acids in length were collected for further analysis. However, the technique of automatically excluding short aa_permease domains was shown to be flawed after it was observed that several highly similar aa_permease genes were annotated in Pfam as having aa_permease domains of dramatically different lengths. For example, the KT2440 genes PP1002 and PP1003 are very similar; they align well with each other in BLAST2 and ClustalX analysis along their entire length and the BLAST2 alignment has a good confidence value of E=6.0E-172. But while PP1003 has been annotated with an aa_permease domain which spans the full length of the gene, PP1002 has only been annotated as having a short aa_permease domain of less than 100 amino acids (Figure 3.4).

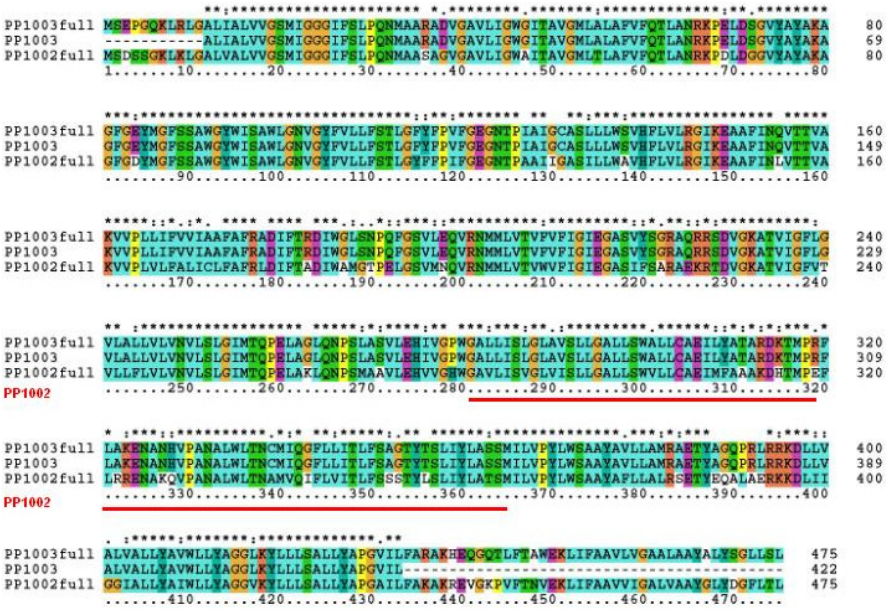


Figure 3.4
Pfam identifies the PP1002 gene (PP1002full) as containing a aa_permease domain less than 200 amino acids long (red line). However the highly similar PP1003 gene (PP1003full) has been identified as containing an aa_permease domain (PP1003) which spans almost the full length of the gene. As PP1003 aligns closely with PP1002 in ClustalX then PP1002 is treated as having the same aa_permease domain annotation as PP1003, and the full gene sequence of PP1002 is used in sequence alignments.

Domains are identified in Pfam by comparing each sequence to Hidden Markov Models (HMM) and multiple sequence alignments. Therefore it is likely that the similarity of some regions in these genes causes them to fall just on the cut-off values for these programmes. This hypothesis is supported by other researchers who have reported a high stringency of the Pfam cut-off values (Studholme et al., 2005). However, when the amino acid sequence was similar enough to align closely with the other aa_permease genes in *Pseudomonas*, these sequences were retained in the dataset (similarity was judged by an alignment in ClustalX between the sequence of interest and sequences annotated with a full aa_permease domain. Sequences containing a domain of less than 200 amino acids, which could not be aligned with any sequences containing the full aa_permease domain, were excluded from the analysis. A full list of sequences retained or lost in this way is provided in Appendix Figure A1.

The Pfam database identified some genes as containing 'split' aa_permease domains, for example PA14_72710 has an aa_permease domain stretching from amino acid residues 52-249, then again from residues 305-386, the total length of the region

containing these split domains was always greater than 200 amino acids and so the corresponding genes were included in further analysis. The presence of split domains supports the hypothesis that sections of some protein sequences may miss the cut off for domain identification because Pfam uses automated computational techniques to locate domains, highlights the need to manually investigate sequences before discarding them from analysis. A full list of 'split domain' sequences is provided in Appendix Figure A1.

3.5.2 *P. syringae* is underrepresented in several transporter domains of the APC superfamily, including the amino acid permease domain.

The Pfam database has classified the aa_permease domain within the APC superfamily, which contains a total of 14 domain families (Table 3.2). These are electrochemical potential-driven porters which catalyse either antiport, and / or symport of a substrate (Saier, 2000). Of the 14 Pfam domain families found within the APC superfamily, 10 are found within *Pseudomonas* species (Table 3.2).

As these transporters are all classified by their ability to utilise electrochemical potential energy, and the aa_permease transporters have been shown to be underrepresented in *P. syringae*, it was interesting to investigate whether other transporter families which utilise the same mechanism were also underrepresented.

A general linear model was performed on the total number of APC-superfamily domains (the sum of all domains within the 10 domain families) in *P. syringae* compared to all other *Pseudomonas*. There was a significant difference between the total number of domains, indicated by a p-value <0.05. Upon further investigation, it was found that *P.syringae* was significantly underrepresented in five of these APC-superfamily domains; Transp_cyt_pur; BenE; Xan_ur_permease; SSF; and aa_permease, as indicated by a p-value <0.05 in Student's t-tests. In particular, the aa_permease domain shows the biggest disparity in number between *P. syringae* and other *Pseudomonas* species. The statistical differences between domain numbers remained significant when differences in genome size were taken into account.

As *P. syringae* appears to be underrepresented in several transport domains which utilise the same mechanism (electrochemical-potential energy), compared to non plant-pathogenic *Pseudomonas* species, this may indicate a selective disadvantage of this transport mechanism for transport during colonisation of leaf surfaces or in the plant apoplast.

APC superfamily		*P.Putida* KT2440	*P.putida* F1	*P.fluorescens* Pf-5	*P.fluorescens* Pf-01	*P.aeruginosa* PA01	*P.syringae pv tomato* DC3000	*P.syringae pv phaseolicola* 1448a	*P.syringae pv syringae* B728a		
Pfam domain	**Pfam annotation**	**Number of genes**								**is *P.syringae* significantly over or underrepresented?**	**p-value**
Transp_cyt_pur	Substrate:H^+ symporter for cytosine/purines, uracil, thiamine, allantoin	**7**	**7**	**8**	**9**	**6**	**2**	**2**	**3**	***P.syringae* under**	**0.000**
Sulfate_transp	Sulfate transporter family	**4**	**5**	**6**	**3**	**4**	**4**	**4**	**4**	not significant	0.578
Na_Ala_symp	Na^+:alanine symporter family	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	not significant	0.482
Branch_AA_trans	Branched-chain amino acid:Na^+/H^+ symporter	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	not significant	0.482
aa_trans	Transmembrane amino acid transporter	**1**	0	0	0	0	0	0	0	not significant	0.482
BenE	Benzoate:H^+ symporter	**3**	**3**	**3**	**3**	**1**	**1**	**1**	**1**	***P.syringae* under**	**0.024**
Trp_Tyr_perm	Tryptophan / tyrosine permease family	0	**1**	**1**	**1**	**3**	**1**	**1**	**1**	not significant	0.770
Xan_ur_permease	Xanthine / uracil permease family	**6**	**6**	**8**	**8**	**7**	**3**	**3**	**3**	***P.syringae* under**	**0.000**
SSF	Na^+:solute symporter family	**7**	**6**	**7**	**5**	**6**	**3**	**4**	**3**	***P.syringae* under**	**0.002**
aa_permease	Amino acid permease	**21**	**21**	**21**	**14**	**21**	**4**	**5**	**6**	***P.syringae* under**	**0.000**
Spore_permease	Spore germination protein	0	0	0	0	0	0	0	0	not present in any *Pseudomonas*	
HCO3_cotransp	HCO3- transporter family	0	0	0	0	0	0	0	0		
DUF1468	Protein of unknown function (DUF1468)	0	0	0	0	0	0	0	0		
CbiQ	Cobalt transport protein	0	0	0	0	0	0	0	0		
	Total	**51**	**51**	**56**	**45**	**53**	**20**	**22**	**23**	***P.syringae* under**	**0.000**

Table 3.2
P. syringae is underrepresented in several families within the APC superfamily, all encoding various transport proteins, but the difference in the aa_permease domain numbers (highlighted) is the most striking. Values for each Pfam domain in *P. syringae* was compared to the other *Pseudomonas* indicated above in Student's 2-tailed t-tests. H_0=no difference in domains between *P. syringae* strains and other *Pseudomonas*. Tests had 6 degrees of freedom. P-values were as indicated; a value of $p \leq 0.05$ was taken as significant.

3.5.3 The aa_permeases in *P. syringae* are a subset of those found in other *Pseudomonas*.

The putative substrates transported by the aa_permease genes in sequenced *P. syringae* pathovars include GABA, proline, ethanolamine, aromatic amino acids and arginine/ornithine. Significantly more aa_permease genes are present in non-plant pathogenic *Pseudomonas* species. However, it is not known whether any of these aa_permease genes are orthologs of those present in the sequenced *P. syringae* pathovars. Furthermore, many of the aa_permease genes in non-plant pathogenic *Pseudomonas* have not been annotated with a putative transported substrate at all. Orthology analysis was therefore used to identify which, if any, aa_permease genes in non plant-pathogenic *Pseudomonas* species were reciprocal best blast hit (RBBH) orthologs of the aa_permease genes in *P. syringae*. Secondly, in order to annotate as many aa_permease genes with a putative transported substrate, where a transporter had been annotated with a putative substrate in one *Pseudomonas* species, this annotation was extended to all RBBH orthologs of that transporter.

There are RBBH orthologs of all of the *P. syringae* aa_permease genes in almost all the investigated *Pseudomonas* species. The most parsimonious explanation for such a distribution may be that these genes were present in the common ancestor of all *Pseudomonas* species. Table 3.3 illustrates the distribution of aa_permease genes across the three sequenced *P. syringae* strains and seven non plant-pathogenic sequenced *Pseudomonas* species. Each column depicts the aa_permeases in a particular *Pseudomonas*, while rows represent the RBBH orthologs of these genes in other *Pseudomonas*. Where information was available these RBBH were verified against the Ortholuge results in the *Pseudomonas* Genome Database. Interestingly, KT2440, F1, Pf0-1 and L48 all appear to possess multiple GABA permeases.

The genes listed in Table 3.4 have been grouped according to their putative transported substrate as annotated in the Pfam database. Of the 153 aa_permease domains, found in the 10 published *Pseudomonas* genomes, 85 of the corresponding genes are not annotated with a specific transport function. These are commonly annotated as ‘amino acid permease protein’ or ‘amino acid permease associated-region’. Of those genes which have been assigned a putative function, the most commonly predicted substrate was GABA, followed by aromatic amino acids.

Transported amino acid (putative) Pfam annotation	*Pseudomonas*	*P.s.tomato* DC3000	*P.s.phaseolicola* 1448A	*P.s.syringae* B728a	*P.fluorescens* Pf-5	*P.fluorescens* Pf-O1	*P.putida* KT2440	*P.putida* F1	*P.aeruginosa* PA01	*P.aeruginosa* PA14	*P.entomophila* L48	Ortholog annotation
	aa_permease domains	4	5	6	21	14	21	21	22	22	19	
	Gene number:	PSPTO	PSPPH	Psyr	PFL	Pfl	PP	Pput	PA	PA14	PSEEN	
	GABA	5356	4937	4909	0344	0313	0284	0302	0129	01580	5205	A1
							4106	1758			3473	A2
							4407	1447				A3
							2911	2780				A4
							2543	3171			2395	A5
						2293	4756	4627			0737	A6
	Ethanolamine	2026	1795	1835	4298	4034	0544	0583	4023	11790	0633	B1
					3522	4990			0220	02690	2919	B2
	Aromatic	1817	3536	3580	1605	1493	4495	1417	0866	53050	3899	C1
							0927	0967			1067	C2
					0756							C3
									3000	25270		C4
	Unknown		2512	2379	6168	2793	1229	1258	2041	38130	4074	U1.1
							2586	3130	0322	04210		U1.2
	Proline	5276	4866	4834	0408		5031	4905	5097	67310	5095	D1
					6228							D2
	Arginine / **Ornithine**			2683	4634	4385	1002	1039	5170	68300	4427	E1
					4633	4384	1003	1040			4426	E2
	Unknown				2710		3727	2036	4981	65850		U2
	D-ser/-ala/-gly				3642		4840	4718			4885	F
	Unknown				3813		0660	0694				U3
	Unknown				3216				4072	11210	3103	U4
	Unknown				4897	4556	2802		3597	17740		U5
	Unknown				2243	2055			5510	72710		U6
	Unknown				4906	4561	1059	1100	0789	54040	1179	U7
	Unknown				0763				2079	37630		U8
	Unknown				2914	1043			1147	49570		U9
	Unknown				3212				1485	45240	2882	U10
	Unknown								4804	63480		U11
	Aspartate*				1414	2309	1259	1286			4049	U12
	Unknown						5297	5206			5442	U13
	Lysine								4628	61250		G
	Unknown								1194	48850		U14
	Unknown							2445	1819	41010		U15
	Unknown					2343						U16
	Unknown										3580	U17

Table 3.3
Orthology analysis of aa_permease genes in *Pseudomonas*. Orthology analysis was used to investigate the distribution of aa_permease genes across *Pseudomonas*; the aa_permease genes present in *P. syringae* have orthologs in almost all other *Pseudomonas*. The putative transported substrate was obtained from the Pfam database, 'unknown' indicates that the transporter was not annotated with a putative substrate. D-ser/-ala/-gly=D-serine/D-alanine/D-glycine. Aspartate* annotation based on characterisation in *Bacillus subtilis* (Lorca et al., 2003)

Hypothetical transport role, as annotated in Pfam	Number
Aromatic amino acids	14
GABA	23
Arginine / ornithine (antiporter)	13
Putrescine / ornithine	1
Proline	5
Lysine	1
Ethanolamine	8
D-serine/D-alanine/D-glycine	3
None stated	**85**
Total	153

Table 3.4
Pfam annotation of 153 aa_permease domains from the 10 sequenced *Pseudomonas* genomes. Eighty-five of 153 aa_permease domains are not annotated with a hypothetical transport function.

3.5.4 Experimental evidence for the function of *Pseudomonas* aa_permease genes is rare.

Several aa_permease genes in *Pseudomonas* have been annotated with a putative transported substrate. This is due to their similarity (measured by BLAST analysis) to transporters in other bacterial species, such as *E. coli*, for which the function has been demonstrated experimentally. A literature review was carried out to investigate the similarity of the *Pseudomonas* transporters to those which have been experimentally characterised in other bacterial species.

Table 3.5 lists the amino acid permease genes present in *Pseudomonas*, and the corresponding experimental evidence available for their function obtained by searching the literature. The *P.aeruginosa* aa_permease PA5170 (ArcD), was shown to be an arginine/ornithine permease with proton motive force-driven arginine import and ornithine export activity (Verhoogt et al., 1992; Bourdineaud et al., 1993). In *P. fluorescens* SBW25 *hutT* was been experimentally characterised as a histidine-inducible transporter (Zhang and Rainey, 2007). BLASTP analysis demonstrated that HutT shares high homology with the *P. fluorescens* Pf-5 ProY aa_permease PFL_0408, suggesting that this gene may be presently misannotated in the majority of *Pseudomonas* species. All other aa_permeases in *Pseudomonas* have been annotated on the basis of their similarity to permeases in other species, predominantly *E. coli and S. cerevisiae*. (Table 3.5) Many aa_permease genes in *Pseudomonas*, such as the ethanolamine permease in *P. syringae*, do not share a high homology with the characterised gene and therefore may not transport the same substrate.

Pseudomonas	*P.s.tomato* DC3000	% *identity*	*P.s.phaseolicola* 1448a	% *identity*	*P.s.syringae* B728a	% *identity*	*P.fluorescens* Pf-5	% *identity*	*P.fluorescens* Pf-01	% *identity*	*P.putida* KT2440	% *identity*	*P.putida* F1	% *identity*	*P.aeruginosa* PA01	% *identity*	*P.aeruginosa* PA-14	% *identity*	*P.entomophila* L48	% *identity*	**Annotated protein and the organism in which this function has been demonstrated. From the Transport Classification Database (TCDB)**	**Protein accession number**
Gene number:	PSPTO	%	PSPPH	%	Psyr	%	PFL	%	Pfl	%	PP	%	Pput	%	PA	%	PA14	%	PSEEN	%		
GABA	5356	67	4937	67	4909	66	0344	68	0313	66	0284	69	0302	69	0129	67	01580	67	5205	68		
											4106	60	1758	60					3473	62		
											4407		1447									
											2911	71	2780	71								
											2543	55	3171	55					2395	54		
									2293	60	4756	59	4627	59					0737	59	GABA:H+ symporter GabP: *E.coli*	P25527
Ethanolamine	2026	37	1795	37	1835	37	4298	38	4034	39	0544	63	0583	63	4023	63	11790	63	0633	64	Ethanolamine import permease: *Rhodococcus erythropolis*	Q53148
							3522		4990						0220		02690		2919			
Aromatic	1817	66	3536	65	3580	65	1605	67	1493	66	4495	57	1417	66	0866	68	53050	67	3899	66		
											0927	69	0967	69					1067	69		
							0756	68													Aromatic amino acid:H+ symporter, AroP: *E. coli*	P15993
															3000	69	25270	69				
Unknown			2512		2379		6168		2793		1229	62	1258	62	2041	78	38130	78	4074	62	Putrescine importer, PuuP: *E. coli*	P76037
											2586		3130		0322		04210					
Proline	5276	82	4866	82	4834	82	0408	81			5031	98	4905	98	5097	76	67310	76	5095	96	Histidine permease, Hut-T: *Pseudomonas fluorescens* SBW25	O31199
							6228	68														
Arginine / Ornithine					2683	82	4634	85	4385	85	1002	85	1039	84	5170	100	68300	100	4427	84	Arginine/ ornithine transporter, ArcD: *Pseudomonas aeruginosa*	P18275
							4633	76	4384	75	1003	74	1040	74					4426	76		
Unknown							2710	54			3727	54	2036	54	4981	54	65850	54			L-arginine permease, RocE: *Bacillus subtilis*	P39137

Pseudomonas	*P.s.tomato* DC3000	% *identity*	*P.s.phaseolicola* 1448a	% *identity*	*P.s.syringae* B728a	% *identity*	*P.fluorescens* Pf-5	% *identity*	*P.fluorescens* Pf-O1	% *identity*	*P.putida* KT2440	% *identity*	*P.putida* F1	% *identity*	*P.aeruginosa* PA01	% *identity*	*P.aeruginosa* PA-14	% *identity*	*P.entomophila* L48	% *identity*	**Annotated protein and the organism in which this function has been demonstrated. From the Transport Classification Database (TCDB)**	**Protein accession number**
Gene number:	PSPTO		PSPPH		Psyr		PFL		Pfl		PP		Pput		PA		PA14		PSEEN			
D-ser/-ala/-gly							3642	**57**			4840	**75**	4718	**75**					4885	**77**	D-serine/-D-alanine/D-glycine transporter: *E. coli*	P0AAE0
Unknown							3813	**58**			0660	**58**	0694	**58**							S-methylmethionine permease, MmuP: E.coli	Q47689
Unknown							3216								4072		11210		3103			
Unknown							4897	**45**	4556	**44**	2802	**43**			3597	**44**	17740	**43**			Putrescine importer, PuuP: *E.coli*	P76037
Unknown							2243		2055						5510		72710					
Unknown							4906	**41**	4561	**41**	1059	**41**	1100	**41**	0789	**42**	54040	**42**	1179	**41**	Asparagine permease, AnsP: *Salmonella typhimurium*	P40812
Unknown							0763								2079		37630					
Unknown							2914		1043						1147		49570					
Unknown							3212								1485		45240		2882			
Unknown															4804		63480					
Unknown							1414		2309		1259		1286						4049			
Unknown											5297		5206						5442			
Lysine															4628	**71**	61250	**71**			Lysine: H^+ symporter, LysP: *E. coli*	P25737
Unknown															1194		48850					
Unknown													2445	**54**	1819		41010				Putrescine-ornithine antiporter PotE: *E. coli*	P76037
Unknown									2343													
Unknown																			3580			

Table 3.5
Several aa_permease genes have been identified and functionally demonstrated in other organisms, although few of these have been demonstrated in *Pseudomonas*. The percentage identity from BLASTP analysis (NCBI), of the *Pseudomonas* aa_permease to the experimentally demonstrated transporter is indicated to the right of each gene. Genes whose function remains speculative are coloured in light grey.

3.5.5 The aa_permease genes localise to the cytoplasmic membrane.

Several aa_permease transporters in *Pseudomonas* have a low sequence identity, measured by BLASTP analysis, with characterised aa_permease transporters. Therefore, in order to confirm the identity of the aa_permease proteins as transporters, the amino acid sequences of the aa_permease genes from a representative selection of sequenced *Pseudomonas* genomes, P.s.t, Ps.s., P.s.ph, KT2440, Pf-5, PAO1 and L48, were analysed using the SignalP and PSORTB web servers. All aa_permease proteins were predicted to contain a signal peptide, which is likely to target the protein to the cytoplasmic membrane.

3.5.6 Identification of transmembrane domains.

The aa_permease transporters have been classified within the APC superfamily. Most superfamily members contain 12 transmembrane domains (Saier, 2000). The aa_permease genes from the sequenced *Pseudomonas* species were manually entered into the TMHMM2.0 and HMMTOP programs to identify the number of transmembrane domains in each aa_permease sequence. All contained transmembrane domains and the majority of sequences were predicted to contain 12 transmembrane domains (Table 3.6).

3.5.7 Several aa_permease genes are potentially regulated by RpoN.

During adaptation and growth, bacteria use several mechanisms to differentially regulate gene transcription. One of the most sophisticated regulatory mechanisms is enhancer-dependent transcription, whereby a special form of RNA-polymerase containing the sigma^{54} (RpoN) subunit, encoded by the *rpoN* gene, interacts with an enhancer-binding protein upstream of the RNA-polymerase binding site. RpoN is known to be required for expression of many genes that are activated in response to low nitrogen availability and other nutritional and environmental conditions, and is required for virulence in *Pseudomonas* including P. *syringae* pv maculicola M4 (Hendrickson et al., 2000). Potential RpoN binding sites can be identified by characteristic features such as consensus DNA sequences (Studholme and Dixon, 2003). Three *Pseudomonas* genomes were available on the PromScan website(http://www.promscan.uklinux.net/), where the genes had been automatically scanned for potential RpoN-binding sites. RpoN scans of a further seven *Pseudomonas* genomes using this software were obtained from David Studholme (Sainsbury Laboratory). PromScan identified several aa_permease genes as potential candidates for RpoN regulation, including the ethanolamine permease (PSPTO_2026) in P.s.t (Table 3.6).

Transported amino acid (putative) Pfam annotation	*Pseudomonas*	*P.s.tomato* DC3000	*P.s.phaseolicola* 1448A	*P.s.syringae* B728a	*P.fluorescens* Pf-5	*P.fluorescens* Pf-O1	*P.putida* KT2440	*P.putida* F1	*P.aeruginosa* PA01	*P.aeruginosa* PA14	*P.entomophila* L48
	aa_permease domains	4	5	6	21	14	21	21	22	22	19
	Gene number:	PSPTO	PSPPH	Psyr	PFL	Pfl	PP	Pput	PA	PA14	PSEEN
	GABA	5356	4937	4909	0344	0313	**0284**	0302	0129	01580	**5205**
							4106	1758			3473
							4407	1447			
							2911	2780			
							2543	3171			2395
						2293	4756	4627			0737
	Ethanolamine	**2026**	**1795**	**1835**	**4298**	**4034**	**0544**	**0583**	**4023**	**11790**	**0633**
					3522	4990			0220	02690	2919
	Aromatic	1817	3536	3580	1605	1493	4495	1417	0866	53050	3899
							0927	0967			1067
					0756						
									3000	25270	
	Unknown		2512	2379	6168	2793	1229	1258	2041	38130	4074
							2586	3130	0322	04210	
	Proline	5276	4866	4834	0408		5031	4905	5097	67310	5095
					6228						
	Arginine / Ornithine			2683	4634	4385	1002	1039	**5170**	**68300**	4427
					4633	4384	1003	1040			4426
	Unknown				2710		**3727**	2036	4981	65850	
	D-ser/-ala/-gly				3642		4840	4718			4885
	Unknown				3813		0660	0694			
	Unknown				3216				4072	11210	**3103**
	Unknown				4897	**4556**	2802		3597	17740	
	Unknown				2243	2055			5510	72710	
	Unknown				**4906**	4561	1059	1100	0789	54040	1179
	Unknown				0763				2079	37630	
	Unknown				2914	1043			1147	49570	
	Unknown				3212				1485	45240	2882
	Unknown								4804	63480	
	Unknown				1414	2309	1259	1286			4049
	Unknown						5297	5206			5442
	Lysine								4628	61250	
	Unknown								1194	48850	
	Unknown							2445	1819	41010	
	Unknown					2343					
	Unknown										3580

Table 3.6
Transmembrane domain and RpoN binding site predictions in aa_permease genes. Transmembrane domain (TMD) predictions are colour coded as follows: Cream=12 TMD, green= 13 TMD, blue=14 TMD. Genes containing a predicted RpoN binding site, indicated by analysis with PromScan, are shown in red.

3.5.8 The *Pseudomonas stutzeri* genome contains five aa_permease genes.

In 2008 the *P. stutzeri* A1501 genome was published (Yan et al., 2008). Subsequent Pfam annotations of the domains present in *P. stutzeri* A1501 indicated the presence of five aa_permease genes in this bacterium. As the number of aa_permease genes in *P. stutzeri* A1501 was more similar to the number found in *P. syringae*, than to non plant-pathogenic *Pseudomona*s species, the *P. stutzeri* A1501 aa_permease sequences were investigated further using the analyses described above.

Table 3.7 details the five aa_permease genes in *P. stutzeri* A1501. Four genes; ethanolamine, aromatic and two arginine/ornithine genes, share a high percentage of sequence identity with similarly-annotated genes in other *Pseudomonas s*pecies. Therefore these aa_permease genes represent a sub set of those found in other *Pseudomonas*. However, one gene is only found in *P. mendocina* ymp*,* with the next closest BLASTP hit found in a non-*Pseudomonas* species. This gene is not annotated with a predicted substrate and may have been acquired by horizontal evolution.

The number of APC superfamily domains in *P. stutzeri* A1501, were compared with the number in *P. syringae* and the representative non plant-pathogenic *Pseudomonas* analysed previously in Table 3.2 above. The number of APC superfamily domains in *P. stutzeri* closely resembled the numbers present in *P. syringae*. An analysis of variance (ANOVA, carried out in the Minitab program) comparing the means of the total *P. stutzeri* and *P. syringae* APC domains, and the total domains present in the other representative *Pseudomonas*, indicated that the number of domains in *P. stutzeri* and *P. syringae* was significantly different to that of other *Pseudomonas* species. The different domain types were then investigated individually using Student's 2-tailed t-tests comparing the number of each domain type in *P. stutzeri* and *P. syringae*, with the number in other *Pseudomonas* species. *P. stutzeri* and *P. syringae* have significantly reduced numbers of Transp_cyt_pur, BenE, Xan_ur_permease and aa_permease domains compared to other *Pseudomonas* species. Only one APC domain, SSF (found in sodium/solute symporters), is reduced in *P. syringae* but not in *P. stutzeri*. These results indicate that APC domain-containing transporters in *P. stutzeri* have experienced a similar reduction in number to *P. syringae* (Table 3.8).

P. stutzeri A1501 (formerly *Alcaligenes faecalis* A1501) was originally isolated from the roots of rice plants in China (You et al., 2005) but can also colonise wheat plants (Yan et al., 2008). *P. stutzeri* was found proliferate both on the rice root surface, and in the intercellular space between cells of the root cortex, where it has been shown to fix

atmospheric nitrogen (You et al., 2005; Yan et al., 2008). The reduction of aa_permease genes in this bacterium may therefore reflect another example of adaptation to a lifestyle intimately associated with plants

Amino acid permease	**Pfam annotation**	**TM**	**Closest BLASTP hit**	**Notes**
PST_3023	Amino acid permease	12	67% similar to *P. mendocina* Pmen_3544 amino acid permease associated region	Not present in any other *Pseudomonas* species. 63% similar to Bpet4853 amino acid permease *Bordetella petrii* DSM 12804
PST_3680	Ethanolamine permease	12	84% similar to PP_0544 ethanolamine permease	Orthologs in *Pseudomonas* spp.
PST_2613	Aromatic amino acid permease	12	81% similar to PA3000 aromatic amino acid permease	Orthologs in *Pseudomonas* spp.
PST_0677	Arginine/ ornithine antiporter (Arc D-1)	13	78% similar to PP_1002 arginine/ornithine permease	Orthologs in *Pseudomonas* spp.
PST_3977	Arginine/ ornithine antiporter (Arc D-2)	13	78% similar to PP_1003 arginine/ornithine permease	Orthologs in *Pseudomonas* spp.

Table 3.7
Analysis of the five aa_permease genes in *P. stutzeri* A1501. BLASTP analysis showed the ethanolamine and aromatic aa_permease gene is present in almost all *Pseudomonas* genomes. An ortholog of least one arginine/ornithine antiporter was found in all *Pseudomonas* genomes except P.s.t and P.s.ph. An ortholog of PST_3023 is only present in *P. mendocina*. TM= transmembrane domains.

APC superfamily		*P.Putida* KT2440	*P.putida* F1	*P.fluorescens* Pf-5	*P.fluorescens* Pf-01	*P.aeruginosa* PA01	*P.syringae pv tomato* DC3000	*P.syringae pv phaseolicola* 1448a	*P.syringae pv syringae* B728a	*P.stutzeri* A1501	
Pfam domain	**Pfam annotation**	**Number of genes**									Are *P.stutzeri* and *P.syringae* combined significantly over or underrepresented? (p-value)
Transp_cyt_pur	Substrate:H^+ symporter for cytosine/purines, uracil, thiamine, allantoin	**7**	**7**	**8**	**9**	**6**	**2**	**2**	**3**	**1**	**Under (p=0.000)**
Sulfate_transp	Sulfate transporter family	**4**	**5**	**6**	**3**	**4**	**4**	**4**	**4**	**5**	not significant (p=0.815)
Na_Ala_symp	Na^+:alanine symporter family	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**2**	not significant (p=0.774)
Branch_AA_trans	Branched-chain amino acid:Na^+/H^+ symporter	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	not significant (p=0.407)
Aa_trans	Transmembrane amino acid transporter	**1**	0	0	0	0	0	0	0	0	not significant (p=0.407)
BenE	Benzoate:H^+ symporter	**3**	**3**	**3**	**3**	**1**	**1**	**1**	**1**	**2**	**Under (p=0.031)**
Trp_Tyr_perm	Tryptophan / tyrosine permease family	0	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**0**	not significant (p=0.476)
Xan_ur_permease	Xanthine / uracil permease family	**6**	**6**	**8**	**8**	**7**	**3**	**3**	**3**	**3**	**Under (p=0.000)**
SSF	Na^+:solute symporter family	**7**	**6**	**7**	**5**	**6**	**3**	**4**	**3**	**6**	**Under (p=0.022)**
aa_permease	Amino acid permease	**21**	**21**	**21**	**14**	**21**	**4**	**5**	**6**	**5**	**Under (p=0.000)**
	Total	**51**	**51**	**56**	**45**	**53**	**20**	**22**	**23**	**25**	**Under (p=0.000)**

Table 3.8
APC superfamily domains in *P. stutzeri* A1501 compared to those in *P. syringae* and other *Pseudomonas.* The total number of APC domains in *P. stutzeri* A1501 (25) is very similar to those in the sequenced *P. syringae* strains (20, 22 and 23). The combined number of Transp_cyt_pur, BenE, Xan_ur, Tryp_Tyr and aa_permease domains in *P. stutzeri* and *P. syringae* are underrepresented compared to other *Pseudomonas.* Values for each Pfam domain in *P. syringae* and *P. stutzeri* strains was compared to the other *Pseudomonas* indicated above in Student's 2-tailed t-tests. Tests had 7 degrees of freedom. H_0=no difference in domains between *P. syringae* / *P. stutzeri* and other *Pseudomonas.*
P-values were as indicated above and a value of p≤0.05 was taken as significant.

3.5.9 Synteny of the *P. syringae* GABA permease is well conserved between *P.syringae* pathovars but not across all *Pseudomonas* species.

All the aa_permease genes in *P. syringae* have been shown to have orthologs in other *Pseudomonas*. However, it is not known whether the genomic context of the aa_permeases is the same in each *Pseudomonas* and if so whether the synteny of the surrounding genes has been preserved. When the synteny of genes is preserved this can indicate a relationship between these genes such as co-transcription or co-regulation, while rearrangements of neighbouring genes between related species suggests that there is not strong selection to retain the order of these genes. The genomic context of each of the *P. syringae* aa_permeases in comparison to other *Pseudomonas*, was visualised using WebACT. This program allows genomic regions to be compared between multiple *Pseudomonas* species. In addition to revealing whether the synteny of genes in the region surrounding each aa_permease is preserved across all genomes, the putative or known function of the genes surrounding each aa_permease was noted, to reveal whether each aa_permease is found in a region of genes performing a similar function.

Figure 3.5 is a WebACT screenshot of the region surrounding the GABA permease in *P.syringae*. There has been an insertion of an effector protein and its chaperone, (which have no orthologs in other *Pseudomonas*) in P.s.t. Figure 3.6 is a schematic of the WebACT output for this region across all sequenced *Pseudomonas*. The GABA permease is frequently found alongside an ABC transporter of unknown function, however, this synteny is not conserved across all *Pseudomonas* species, particularly *P. aeruginosa*.

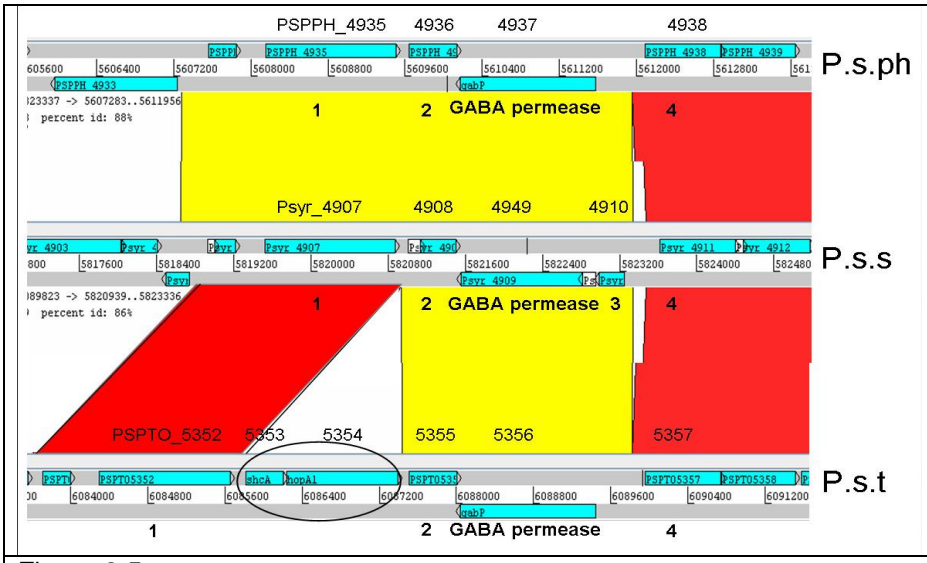


Figure 3.5
The GABA permease *gabP* is not in an operon. Synteny appears to be conserved in the region surrounding *gabP* in P.s.ph and P.s.s, but there has been an insertion of genes encoding the effector *hopA1* and chaperone *shcA* (PSPTO5353, PSPTO5354, circled) in P.s.t. This effector does not closely resemble any other *Pseudomonas* genes using BLASTX analysis. The genes surrounding *gabP* are annotated as follows: 1=methyl-accepting chemotaxis transducer/sensory box protein, 2= hypothetical protein; 3= hypothetical protein, 4= ABC transporter.

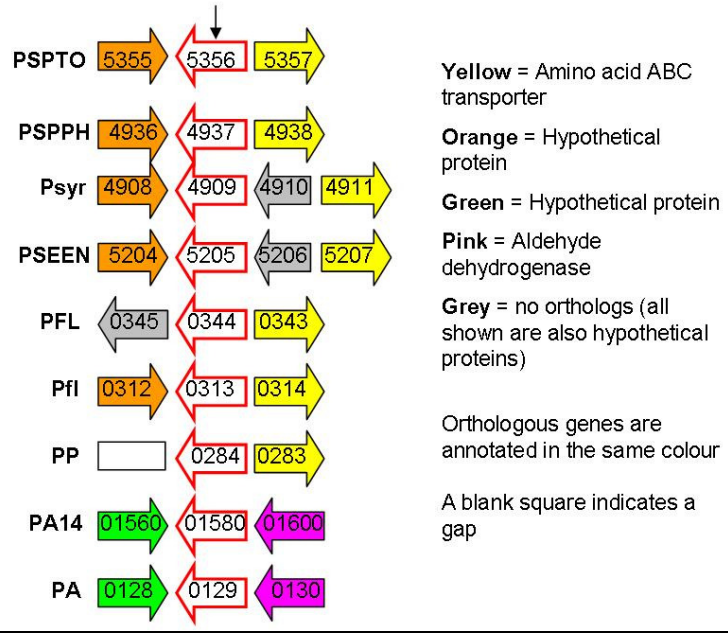


Figure 3.6
Schematic of the genes immediately surrounding the GABA permease (arrow) in all sequenced *Pseudomonas*. The 'gap' indicated by a blank square was investigated using BLASTX analysis and does not appear to be a pseudogene. Genes are represented by arrows, forwards facing arrow indicates gene is on the (+) strand, backwards arrow indicates gene is on the (-) strand. Genes annotated in the same colour are RBBH orthologs.

3.5.10 The ProY permease falls within the histidine utilisation (*hut*) operon.

The synteny of the genes surrounding the ProY permease in the sequenced *P. syringae* pathovars, and in other *Pseudomonas* species was investigated. The genes surrounding the ProY permease in sequenced *P. syringae* pathovars are connected to histidine transport and utilisation. Figure 3.7 shows a schematic of the WebACT output for the all sequenced *Pseudomonas*. The synteny of genes in this region is conserved. The surrounding histidine genes have been previously reported to constitute an operon in P. fl. SBW25 (Zhang and Rainey, 2007). The proximity of these genes to one another in *P. syringae* suggests that they may also form an operon in this species, a hypothesis which can be investigated later on. As seen in Figure 3.7, although the same genes are present in almost all *Pseudomonas* genomes, there appears to have been rearrangements to the order of the genes, and in *P. putida*, several genes appear to have been lost. This indicates that although the surrounding genes have been annotated with a similar function, the order of these genes within the operon may be flexible. The majority of *Pseudomonas* species possess two copies of the *hutH* gene, encoding a histidine ammonia-lyase (Figure 3.8). In P.fl SBW25, *hutH-1* was incapable of complementing a *hutH-1/H-2* double mutant, even when expressed from a constitutive promoter, suggesting that the protein is not a functional histidine ammonia lyase (Zhang and Rainey, 2007). However, copies of *hutH-1* share a high sequence similarity between *Pseudomonas* species and all lack the exact same three amino acid residues (Ser–Gly–Asp) that span the active site (Zhang and Rainey, 2007). Zhang and Rainey (2007) proposed a number of evolutionary scenarios to explain the distribution of this gene, including an ancient duplication or gene-acquisition event (which is supported by the fact that HutH1 and HutH2 share only 37% sequence identity), or alternatively, suggested that this gene may have an as yet undetermined functional role in the metabolism of histidine, for example, as a protein that binds (but does not metabolise) histidine and is involved in controlling the upper limit of *hut* activity.

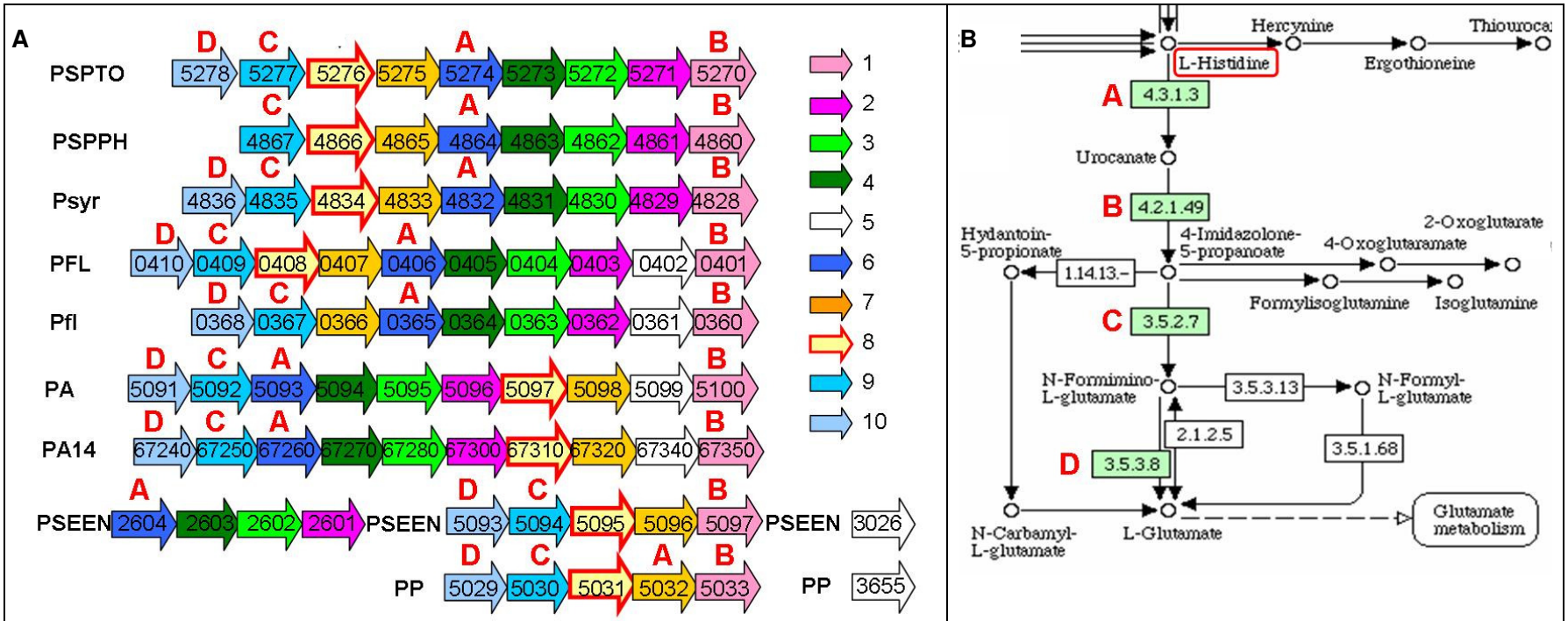


Figure 3.7

A: Synteny of the *hut* operon is not strictly conserved across all *Pseudomonas,* although it is only in *P. putida* that there are not orthologs of most *hut* genes elsewhere in the genome. 1= *hutU* (urocanate hydratase), 2=histidine binding protein, 3= histidine ABC transporter, 4= histidine ABC transporter, 5= cytosine/purine/uracil/thiamine/allantoin permease 6= *hutH-2* (his ammonia-lyase) 7= *hutH-3*, (his ammonia-lyase) 8= *proY*, (aa_permease) 9 = *hutI*,(imidazolonepropionase) 10=*hutG* (formimidoylglutamase, this gene is a pseudogene in P.s.ph). The Pfam annotation for the gene represented by a white arrow is Transp_cyt_pur: a permease for cytosine/purines, uracil, thiamine and allantoin. There are no orthologs of this gene in *P. syringae*. The Transp_cyt_pur domain is one of the ABC-transport domain families that are under-represented in *P. syringae*. Right: Histidine assimilation pathway from KEGG. Enzymes A-D are superimposed above their relevant positions in the *hut* operon (B) P.s.ph is missing enzyme D from this pathway (*hutG*). All other *Pseudomonas* have retained all enzymes in this pathway, despite the rearrangements of the *hut* operon.

3.5.11 The synteny of the ethanolamine permease region is conserved between *P.syringae* pathovars but not across all *Pseudomonas* species.

The synteny of the genes surrounding the ethanolamine permease in the sequenced *P. syringae* pathovars, and in other *Pseudomonas* species was investigated. The aim was to identify whether this gene was found in a region of genes annotated with a similar function, and whether there appeared to be a selective pressure for the aa_permease to fall alongside the same genes. The ethanolamine permease in *P. syringae* or in the non plant-pathogenic *Pseudomonas* did not fall within a region of genes targeted towards a particular function.

Figure 3.8 is a schematic of the WebACT output for this region in all *Pseudomonas.* In several *Pseudomonas* species, including *P. putida* and *P. aeruginosa* the ethanolamine permease is located next to a gene annotated as an ethanolamine ammonia-lyase, which catalyses the conversion of ethanolamine into acetaldehyde + NH_3. Predicted orthologs of this gene are present in *P. syringae*, but not close to the ethanolamine permease.

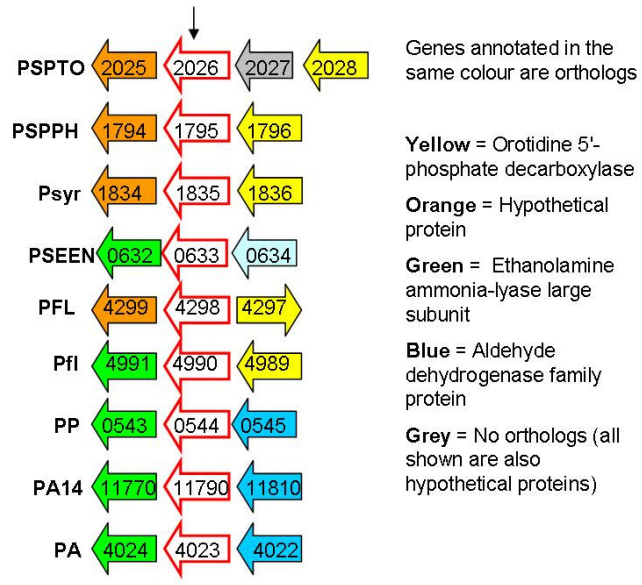


Figure 3.8
Schematic of the WebACT output for the region surrounding the ethanolamine permease gene in all *Pseudomonas.* Genes are represented by arrows, forwards facing arrow indicates gene is on the (+) strand; backwards arrow indicates gene is on the (-) strand. Genes annotated in the same colour are RBBH orthologs. Ethanolamine ammonia-lyase catalyses the conversion of ethanolamine into acetaldehyde + NH_3. Predicted orthologs of this gene are present in *P. syringae*, but not close to the ethanolamine permease. For example in P.s.t the ethanolamine ammonia-lyase ortholog is PSPTO_0727. Aldehyde dehydrogenases are a family of enzymes that catalyse the oxidation of aldehydes.

3.5.12 Synteny of the aromatic amino acid permease region is conserved between *P. syringae* pathovars but not across all *Pseudomonas* species.

The synteny of the genes surrounding the aromatic aa_permease in the sequenced *P. syringae* pathovars, and in other *Pseudomonas* species was investigated. As before the aim was to identify whether this gene was found in a region of genes annotated with a similar function, and whether there appeared to be a selective pressure for the aa_permease to fall alongside the same genes. Synteny of the aromatic amino acid permease region is conserved in the sequenced *P. syringae* pathovars investigated.

Figure 3.9 is a schematic of the WebACT output for this region in all sequenced *Pseudomonas*. Although synteny was conserved between *P. syringae* pathovars, the surrounding genes did not appear to share similar functions. Synteny of the region surrounding the aromatic aa_permease was not found to be conserved across multiple *Pseudomonas* species.

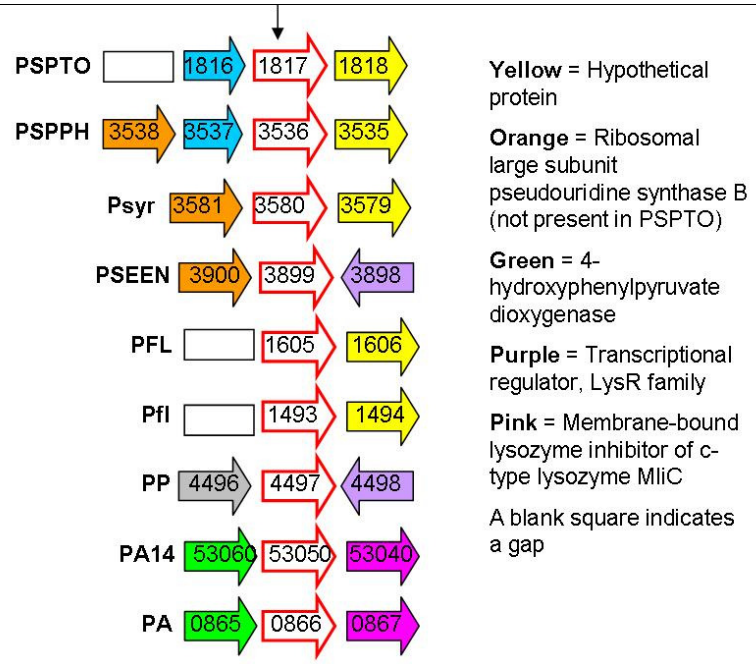


Figure 3.9
Schematic of the WebACT output for the region surrounding the aromatic amino acid permease gene in all *Pseudomonas.*
Genes are represented by arrows, forwards facing arrow indicates gene is on the (+) strand; backwards arrow indicates gene is on the (-) strand. Genes annotated in the same colour are RBBH orthologs. A small hypothetical protein (approximately 200 residues long) has been annotated next to the aromatic aa_permease gene in both P.s.t and P.s.ph (blue arrow). This protein is not present in P.s.s and does not share high sequence similarity with any annotated genes in *P. syringae*.

3.5.13 The *proY* aa_permease gene has been functionally demonstrated to be a histidine transporter in *P.fluorescens* SBW25.

The *P. fluorescens* SBW25 genome has been sequenced, and was recently published (Silby et al., 2009). The unannotated genome sequence has been made available for BLAST searching on the Sanger website (www.sanger.ac.uk). BLASTP analysis of the P.fl SBW25 genome, using the ProY sequence from Pf-5, indicated the presence of a protein in P.fl SBW25 which was 79% similar (percentage identity) to the Pf-5 ProY. Although the P.fl SBW25 *hutT* was shown to share 93% and 80% identity to the experimentally characterised *proY* from *Salmonella* and *E. coli* respectively (Rainey, 1999), this protein was later experimentally characterised as a histidine transporter in P.fl. SBW25 (Zhang et al., 2006). Zhang and coworkers (2006) reported that *hutT* was induced in the rhizosphere, and mutation of *hutT* demonstrated this to be a histidine transporter found in the P.fl SBW25 *hut* operon, as the non-polar deletion mutant was unable to assimilate histidine (Zhang et al., 2006). Therefore the ProY permease may be misannotated in *Pseudomonas* species. In order to confirm that the *hutT* gene identified by Zhang and coworkers was an ortholog of *proY*, reciprocal best blast hit orthology analysis was used as before. The *hutT* gene in P.fl.SBW25 was found to be an ortholog of the *proY* gene in other sequenced *Pseudomonas*. In addition, the hut operon of P. fl. SBW25 was compared to that of related *P. fluorescens* Pf-5 (PFL) and Pf-01 (Pfl) (Figure 3.10). The synteny of genes in this operon is well conserved between P.fl. SBW25 and Pf-5. This, in addition to the 79% sequence identity between *hutT* and the corresponding *proY* gene in Pf-5, indicates that *proY* may have been misannotated in *Pseudomonas*, and may therefore transport histidine.

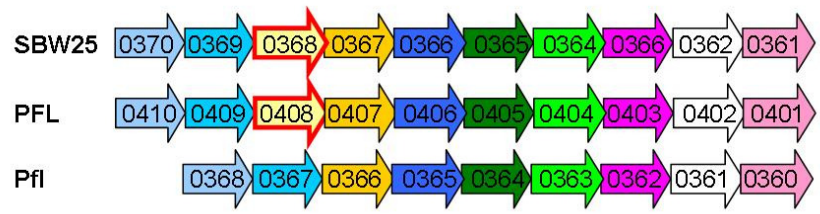


Figure 3.10
The *P. fluorescens* SBW25 *hutT* gene (ORF number 0368, outlined in red) has been experimentally demonstrated to be a histidine transporter. The organisation of the *hut* operon in P.fl SBW25 is shown alongside the *hut* operon from *P. fluorescens* Pf-5 and *P. fluorescens* Pf0-1. The P.fl. SBW25 *hutT* gene shares 79% BLAST similarity to PFL_0408

3.5.14 The ProY aa_permease gene has orthologs in all *Pseudomonas* except *P. fluorescens* Pf0-1.

The aa_permease genes in *P. syringae* appear to have orthologs in almost all other *Pseudomonas* with one exception. BLAST analysis failed to reveal an ortholog of the proline aa_permease in *P. fluorescens* Pf0-1. This was supported by alignment of the *hut* operon region of P.fl SBW25, Pf-5 and Pf0-1 (Figure 3.10, above).

Alignment of the Pf0-1 genome with the closely related *P. fluorescens* Pf-5 revealed that although synteny was conserved in the genes of the *hut* operon surrounding *proY*, the *proY* gene itself is missing from Pf0-1 (Figure 3.11). There was no evidence that the *proY* gene remained in the Pf0-1 genome as a pseudogene as there are no similar BLASTX hits to the Pf-5 *proY* gene in Pf0-1.

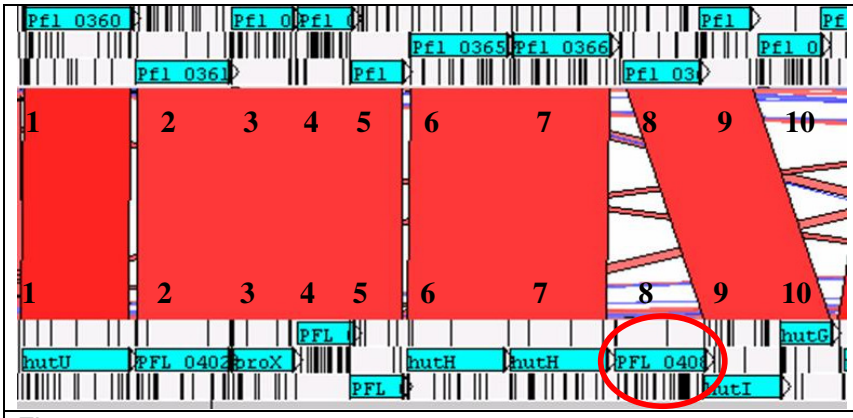


Figure 3.11
Alignment of the *hut* operon in Pf0-1 (top) and Pf-5 (bottom) in WebACT.
The alignment shows the genes of the Pf-5 *hut* operon (1-10), and the corresponding genes in the Pf0-1 *hut* operon. The *proY* gene (8) is missing from Pf0-1.

3.5.15 The GABA aa_permease genes appear to be evolutionarily related in *Pseudomonas*, with one exception.

All *Pseudomonas* species contain aa_permease genes (based on domain analysis and homology to aa_permease genes in other species) and putatively transported substrates have been identified. However, very few aa_permease transporters in *Pseudomonas* have been experimentally characterised. The aim of these experiments was to investigate the evolutionary relationship between these genes in *Pseudomonas*. In particular, to identify evolutionary processes which may have been acting on these genes such as horizontal evolution or gene duplication. These experiments focussed on the aa_permease genes found in P.s.t, predicted to transport GABA, proline, aromatic amino acids and ethanolamine.

All *Pseudomonas* genomes examined possessed at least one ortholog of a GABA permease. In addition, sequenced *P. putida* and *P. entomophila* genomes contained three and five additional GABA permeases respectively. In order to study these additional GABA permease genes in *P. putida* and *P. entomophila*, and in particular identify any genes which may have been acquired by horizontal evolution, a phylogenetic tree was constructed. Two alternate phylogenetic trees, constructed by maximum parsimony and neighbour joining methods as described in the methods above, showed that one of the GabP sequences, only present in *P. putida* species, consistently groups away from the other GABA permeases. Figures 3.12 and 3.13 show all GabP sequences from the sequenced *Pseudomonas* rooted using an aa_permease from the bacterium *Streptomyces pristinaespirali*s. This outgroup was chosen according to the methods described in Harrison and Langdale (2006). Briefly, the aa_permease gene of interest was first compared to all sequenced bacterial species in the NCBI database using BLASTP. Then several sequences of interest were chosen which shared less similarity to the aa_permease gene of interest than any other *Pseudomonas* aa_permease gene. These sequences were then aligned with the *Pseudomonas* aa_permease genes and a single outgroup sequence was chosen (Harrison and Langdale, 2006). Figures 3.12 and 3.13 represent two alternative trees for the GabP sequence constructed by different methods. Both trees were constructed in PAUP*.

In both trees the main clade contains all GabP sequences except for one GabP sequence only found in KT2440 and F1 (highlighted with an asterix in Figures 3.12 and 3.13). Therefore we can hypothesise that the majority of GABA permease sequences from the sequenced *Pseudomonas* are evolutionarily related, as they fall within the main clade. This suggests that where *Pseudomonas* genomes contain multiple *gabP* genes, these have been brought about through gene duplication rather than acquisition of genes from other species (i.e. horizontal evolution). However, the outlying GabP sequences from the KT2440 and F1 genomes do not appear to be evolutionarily related to the other *Pseudomonas* sequences, and therefore we can hypothesise that these genes were acquired by the ancestor of KT2440 and F1, by horizontal evolution. This hypothesis can now be investigated further.

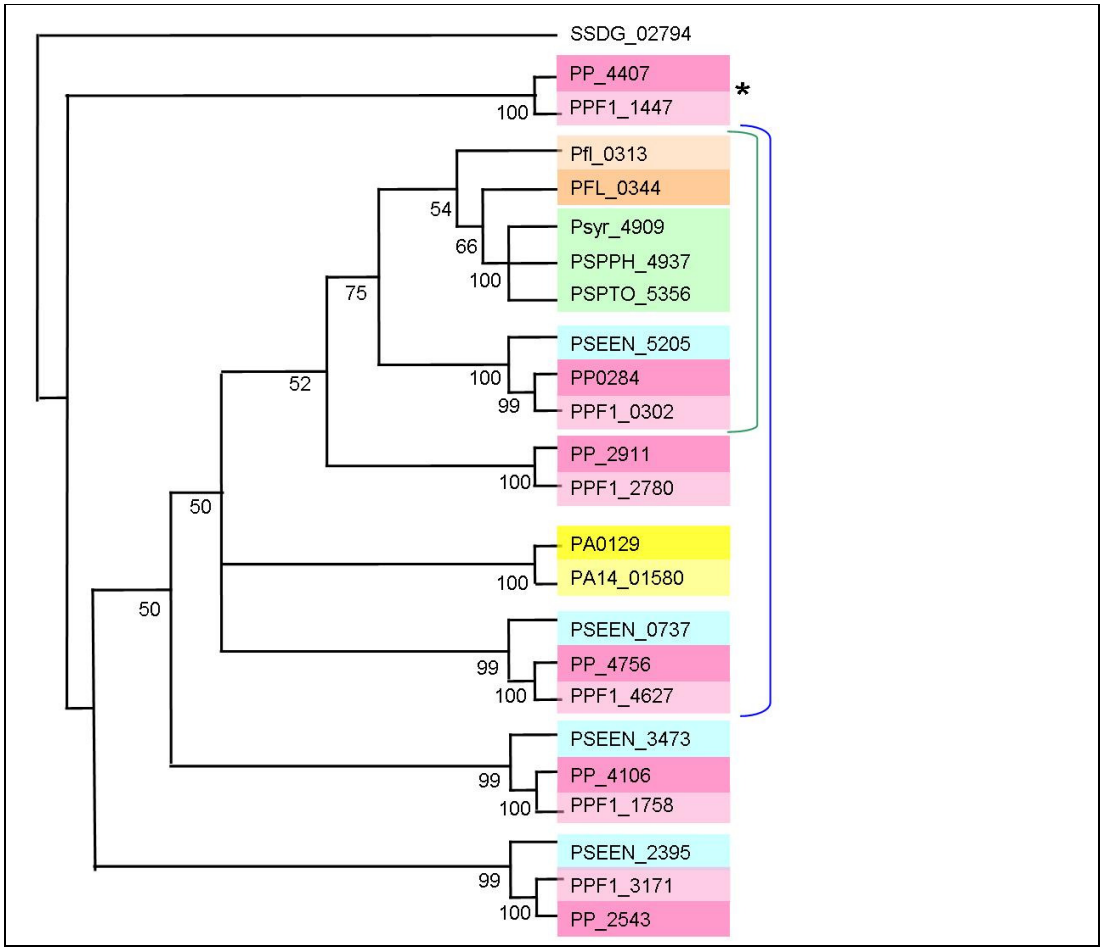


Figure 3.12
Maximum parsimony tree of GABA aa_permeases from *Pseudomonas* constructed using PAUP*: *P. syringae* pv. tomato DC3000 (PSPTO) *P.s.*pv. syringae B728a (Psyr), *P.s.*pv. phaseolicola 1448A (PSPPH), *P. aeruginosa* PAO1 (PA), *P. aeruginosa* PA14 (PA14), *P. entomophila* L48 (PSEEN), *P. fluorescens* Pf-01 (Pfl), *P. fluorescens* PF-5 (PFL), *P. putida* KT2440 (PP) and *P. putida* F1 (Pput). The sequences from PSPTO, PSPPH, Psyr, PSEEN, PP, PPF1, Pfl and PFL form a clade (green bracket); however the sequences from PA01 and PA14 fall outside this clade along with a second sequence from PP, Pput and PSEEN. To illustrate the multiplicity of GABA permeases in some *Pseudomonas*, the genes from each species have been coloured: *P. syringae* genes are green, *P. putida* genes are pink, *P. aeruginosa* genes are yellow, *P. fluorescens* are orange and *P. entomophila* are blue. Rooted with *Mycobacterium tuberculosis* H37Rv, aa_permease. Bootstrapped with 500 replicates *One GABA permease present in *P. putida* does not group with the other sequences.

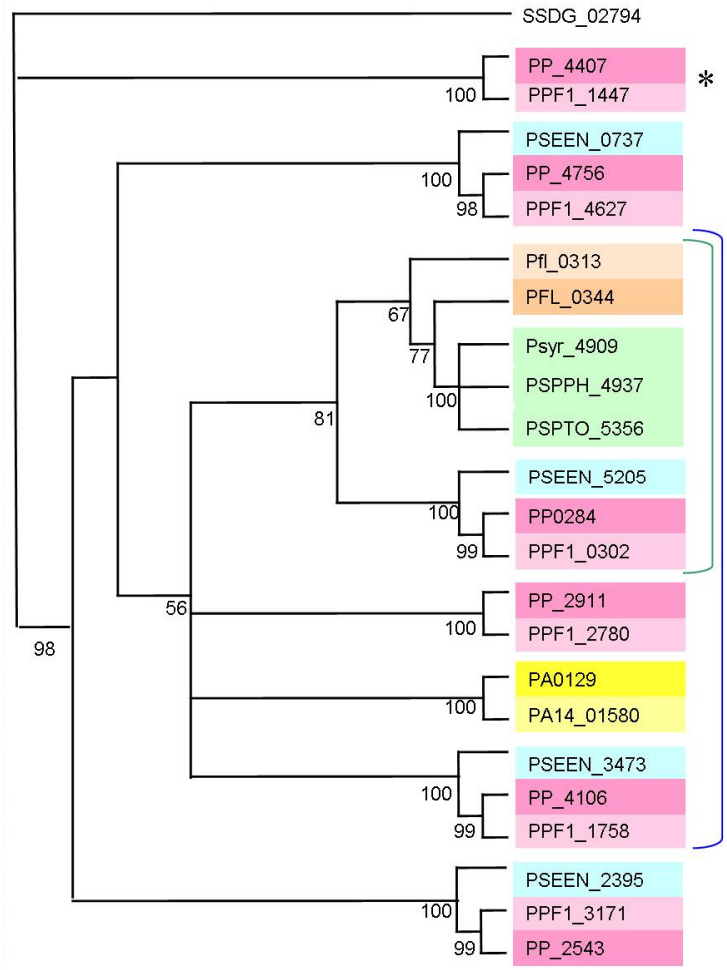


Figure 3.13
Neighbour joining (NJ) tree of GABA aa_permeases from all sequenced *Pseudomonas* constructed using PAUP*: *P. syringae* pv. tomato DC3000 (PSPTO) *P. syringae* pv. *syringae* B728a (Psyr), *P. syringae* pv. *phaseolicola* 1448A (PSPPH), *P. aeruginosa* PAO1 (PA), *P. aeruginosa* PA14 (PA14), *P. entomophila* L48 (PSEEN), *P. fluorescens* Pf-01 (Pfl), *P. fluorescens* PF-5 (PFL), *P. putida* KT2440 (PP) and *P. putida* F1 (Pput). The NJ method produces the same groupings as maximum parsimony (green and blue brackets). Rooted with *Streptomyces pristinaespiralis* ATCC 25486, annotated as a GABA permease, bootstrapped with 500 replicates. Colour coding as before.
*One GABA permease present in *P. putida* does not group with the other sequences.

3.5.16 One GABA permease present in *P. putida* species appears to have been acquired from *Burkholderia* species.

In order to test the hypothesis that one GabP sequence, only found in *P. putida* KT2440 and F1 species, was acquired by horizontal evolution from a species other than *Pseudomonas*, BLASTP analysis was used to compare the GabP sequence against all amino acid sequences in the NCBI database. The closest matches, as measured by percentage sequence identity, were sequences from *Burkholderia* species. These sequences were also annotated as GABA permeases. The top three *Burkholderia* BLASTP hits were included in

an alignment with GABA permeases from KT2440 and F1. Sequences from *P. entomophila* L48 were also included, because this species is known to share many similar genes with *P. putida* and also has multiple copies of the GABA permease.

The outlying GabP sequence from *P. putida* KT2440 (PP) and *P. putida* F1 (Pput) grouped with those from *Burkholderia* with good bootstrap support (Figure 3.14). In contrast, the remaining GabP sequences in KT2440, F1 and L48 (PSEEN) all group separately and form a well-supported clade.

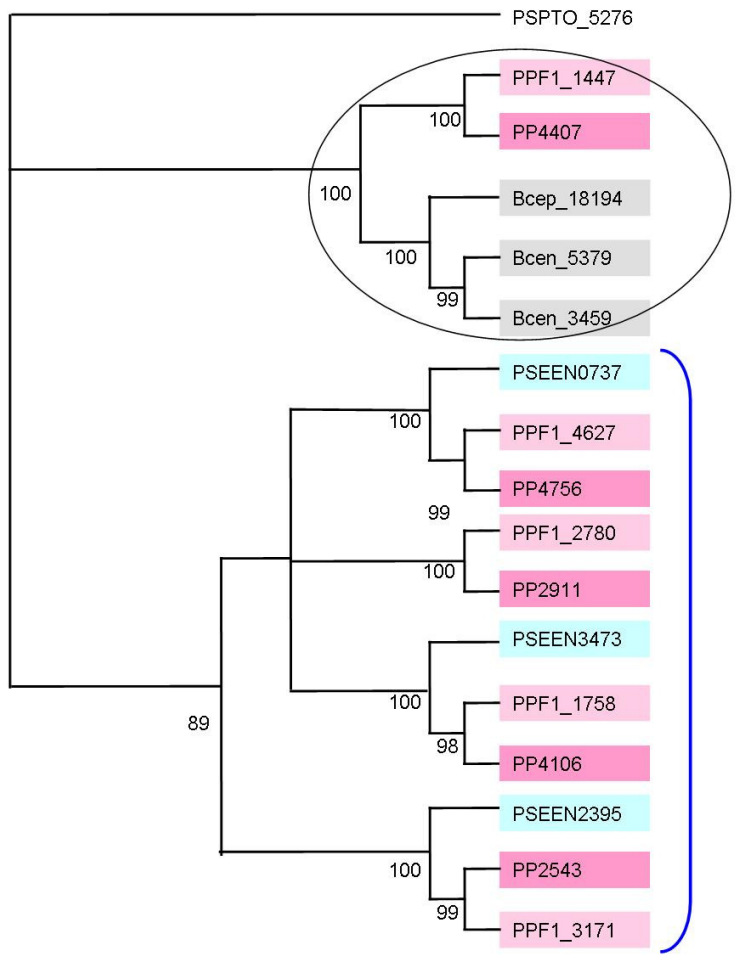


Figure 3.14
Maximum parsimony tree of the multiple GABA aa_permeases from *P. putida* KT2440, (PP) *P. putida* F1 (Pput), *P. entomophila* L48 (PSEEN), along with sequences from *Burkholderia* species, constructed using PAUP*. One of the GABA permeases found in *P. putida* is more similar to GABA permeases in *Burkholderia* species. The other sequences form a distinct clade (blue bracket). Rooted using P.s.t. proline amino acid permease. Bootstrapped with 500 replicates support. Colour coding as before. Bcep18194= *Burkholderia* sp. 383 Bcen5379 = *Burkholderia cenocepacia* MC0-3 Bcen3459 = *Burkholderia cenocepacia* AU 1054

3.5.17 All aromatic aa_permeases in *Pseudomonas* are evolutionarily related.

All *Pseudomonas* genomes examined possessed at least one aromatic aa_permease. These sequences appeared to be closely related and formed a clade (Figure 3.15). In addition, KT2440, F1, PAO1, PA14 and Pf-5 genomes contained a second aromatic aa_permease. In general, these sequences grouped alongside the sequences of the first aromatic aa_permease within an extended clade. Although the sequences from KT2440 and F1 do not group within this extended clade, they do not appear to have been acquired by horizontal evolution as measured by the high bootstrap value where these two clades separate. In addition the closest BLASTP hits for the KT2440 and F1 sequences (PP0927 and Pput_0967, respectively) was the aromatic aa_permease gene *P. entomophila* (PSEEN3899).

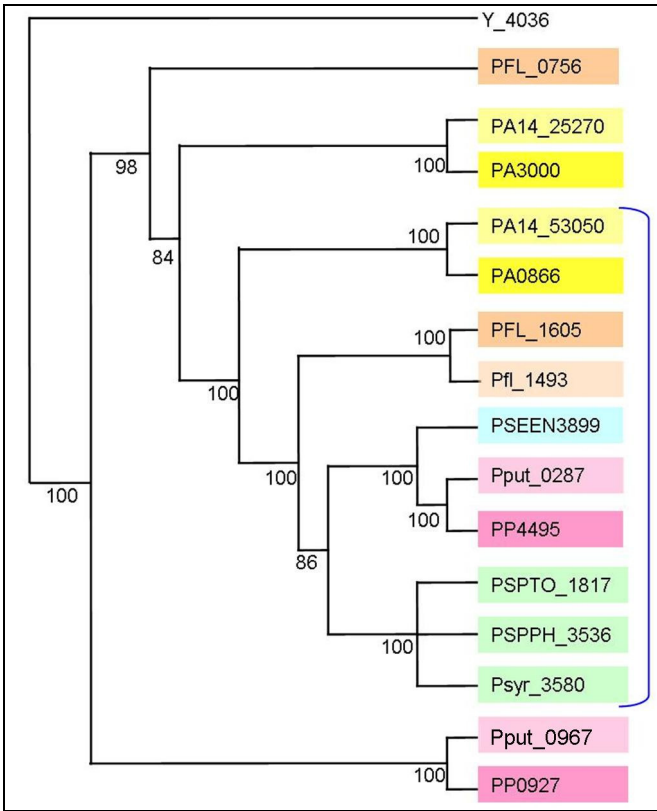


Figure 3.15
Maximum parsimony tree of aromatic aa_permeases from all sequenced *Pseudomonas*: *P. s.* pv. tomato DC3000 (PSPTO) *P. s.* pv. syringae B728a (Psyr), *P. s.* pv. phaseolicola (PSPPH), *P. aeruginosa* PAO1 (PA), *P. aeruginosa* PA14 (PA14), *P. entomophila* L48 (PSEEN), *P. fluorescens* Pf0-1 (Pfl), *P. fluorescens* PF-5 (PFL), *P. putida* KT2440 (PP) and *P. putida* F1 (Pput) with 500 bootstrap replicate support. Every sequenced *Pseudomonas* possesses at least one aromatic aa_permease and these form a group (indicated). In order to clearly illustrate the presence of multiple aromatic permease in some *Pseudomonas*, the genes from each species have been coloured: *P. syringae* genes are green, *P. putida* genes are pink, *P. aeruginosa* genes are yellow, *P. fluorescens* are orange and *P. entomophila* are blue. The second copy of each aa_permease falls outside this main grouping. Rooted using *Yersinia pestis* KIM y_4036, annotated as aa_permease.

3.5.18 Ethanolamine permeases group within two clades.

All *Pseudomonas* genomes examined possessed at least one ethanolamine permease. In addition, PAO1, PA14, Pf-5, Pf0-1 and L48 genomes contained a second copy of the ethanolamine permease. In order to investigate whether the second ethanolamine aa_permease gene was the result of gene duplication of the first ethanolamine gene, a phylogenetic tree was generated using all sequences annotated as transporting ethanolamine.

Figure 3.16 shows the maximum parsimony tree generated from all *Pseudomonas* ethanolamine aa_permease sequences. These sequences separated into a main clade, containing one copy of an ethanolamine permease from all sequenced *Pseudomonas*, and a second clade containing the second ethanolamine permease. Although there was good bootstrap support for the grouping of sequences within each clade, there was not good resolution for whether these two clades are evolutionarily related. There does not appear to be any evidence that either permease was obtained by horizontal evolution, as all sequences have their closest BLASTP hits in other *Pseudomonas*. However, there does not appear to be support from this tree that the observed distribution of ethanolamine permease genes arose from an ancestral duplication of a single ethanolamine permease.

In *P. aeruginosa* PAK, the aa_permease PA0220 transposon mutant lost the ability to utilise histamine as a nitrogen source, suggesting that this gene encoded a histamine transporter (Johnson et al., 2005). *P. aeruginosa* genomes contain two genes annotated as transporting ethanolamine. PA0220 falls within a clade alongside permeases from *P. fluorescens* and *P. entomophila*, but this clade does not include the *P. syringae* ethanolamine permease. If the presence of two ethanolamine permease genes in *P. aeruginosa*, *P. fluorescens* and *P. entomophila* is the result of a gene duplication event, then there may have been substrate diversification between the duplicated copies with one copy transporting histamine and another ethanolamine.

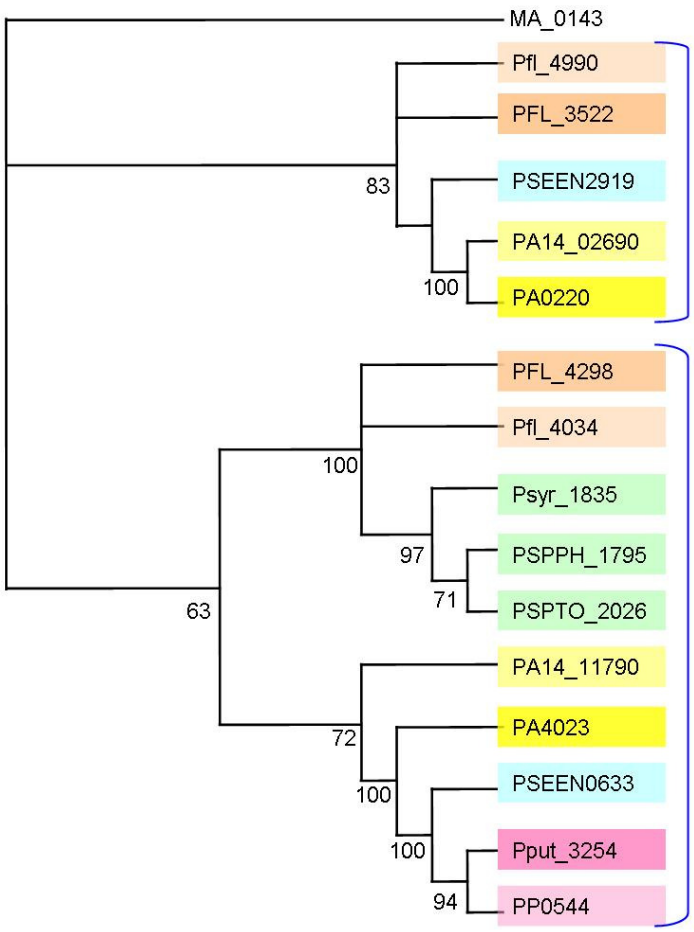


Figure 3.16
Maximum parsimony tree of ethanolamine aa_permeases from all sequenced *Pseudomonas, P. syringae* pv. tomato DC3000 (PSPTO) *P. syringae* pv. syringae B728a (Psyr), *P. syringae* pv. phaseolicola 1448A (PSPPH), *P. aeruginosa* PAO1 (PA), *P. aeruginosa* PA14 (PA14), *P. entomophila* L48 (PSEEN), *P. fluorescens* Pf0-1 (Pfl), *P. fluorescens* PF-5 (PFL), *P. putida* KT2440 (PP) and *P. putida* F1 (Pput) with 500 bootstrap replicate support. Every sequenced *Pseudomonas* possesses at least one ethanolamine aa_permease and these form a clade (bottom blue bracket). Several *Pseudomonas* possess a second gene annotated as an ethanolamine permease. The second copy of each ethanolamine aa_permease appears to be unrelated and forms a distinct clade. The genes from each species have been coloured: *P. syringae* genes are green, *P. putida* genes are pink, *P. aeruginosa* genes are yellow, *P. fluorescens* are orange and *P. entomophila* are blue. Rooted using *Methanosarcina acetivrans* C2A aa_permease.

3.5.19 *P.fluorescens* Pf-5 is the only *Pseudomonas* to have more than one proline permease, and this does not group with the others.

Only one sequenced *Pseudomonas* genome contained more than one proline aa_permease gene. Figure 3.17 shows a maximum parsimony tree of the proline aa_permease genes in all sequences *Pseudomonas* species investigated. The majority of sequences form a single well-supported clade. However, one of the Pf-5 sequences, PFL_6228 did not fall within this clade. Further analysis revealed that there were no Ortholuge-predicted orthologs of

PFL_6228, in the *Pseudomonas* Genome Database. The most likely hypotheses explaining PFL_6228 are that this gene was acquired by horizontal evolution, or that PFL_6228 is the result of a lineage-specific duplication in Pf-5. BLASTP analysis indicated that although PFL_6228 was not orthologous to any other *Pseudomonas* sequence, *Pseudomonas* genes are the closest hits in a BLASTP search of all organisms in the NCBI database. In addition this gene was over 70% similar to proline permeases from P.s.t, KT2440 and F1. Therefore this gene is unlikely to have been acquired by horizontal evolution from another species. Therefore PFL_6228 may have been acquired from another *Pseudomonas* species or an as-yet uncharacterised bacterium, or alternatively, may be the result of a lineage-specific duplication is Pf-5.

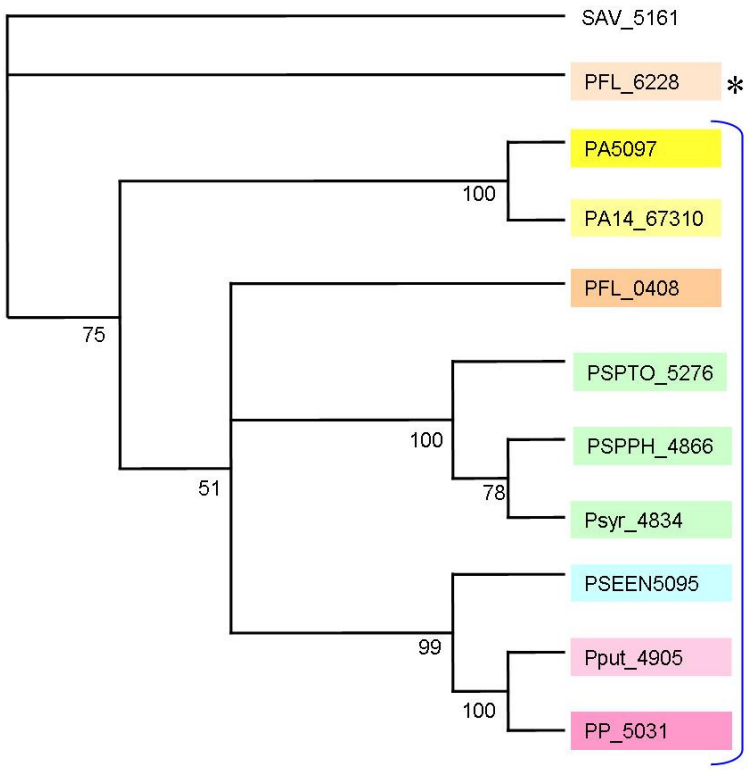


Figure 3.17
Maximum parsimony tree of proline aa_permeases from all sequenced *Pseudomonas*: *P. syringae* pv. tomato DC3000 (PSPTO) *P. syringae* pv. syringae B728a (Psyr), *P.* syringae pv. phaseolicola 1448A (PSPPH), *P. aeruginosa* PAO1 (PA), *P. aeruginosa* PA14 (PA14), *P. entomophila* L48 (PSEEN), *P. fluorescens* Pf0-1 (Pfl), *P. fluorescens* PF-5 (PFL*), P. putida* KT2440 (PP) and *P. putida* F1 (Pput) with 500 bootstrap replicates support. Every sequenced Pseudomonas possesses at least one ethanolamine aa_permease and these form a group (indicated). The second proline permease, present only in *P. fluorescens* Pf-5 appears to be unrelated to this group. Rooted using *Streptomyces avermitilis* MA-4680, proline permease

3.5.20 The histidine (*hut*) operon, containing the ProY aa_permease, has undergone several rearrangements in *Pseudomonas.*

The ProY aa_permease appeared to be relatively well conserved within *Pseudomonas*, with all sequenced strains except Pf0-1 possessing one gene, which appears to be orthologous. The *proY* gene was consistently found within an operon which had been experimentally demonstrated to be involved in the transport and assimilation of histidine (but not urocanate or proline) (Zhang and Rainey, 2007). However, the number and arrangement of genes in this operon appeared to have altered during *Pseudomonas* evolution, and the *hut* operon now differs between *Pseudomonas* species.

In order to track the rearrangement of genes within this operon, and the loss of genes including *proY*, a phylogenetic tree was built based on the *hutU* sequence, annotated as a urocanate hydratase (Figure 3.18). This sequence was chosen for the phylogenetic tree as it was consistently found within the *hut* operon and was conserved in sequence identity between all sequenced *Pseudomonas*. The current order of genes in the *hut* operon is shown next to each *Pseudomonas* and the most parsimonious rearrangement and gene loss events that would have resulted in this order are indicated in the branches. Gene loss is indicated by a cross. A gene is annotated as being lost from the genome is no orthologous genes can be found anywhere in the genome (i.e not just that is has been lost from within the *hut* operon). Where orthologous genes are present in the genome but not within the hut operon, these are indicated by separating them from the other genes by a vertical line.

Results indicate that rearrangements in the *hut* operon have occurred at least twice during *Pseudomonas* evolution, (at points indicated below). At the same time several gene loss events have occurred, mainly in *P. putida* KT2440 and F1. Therefore it appears that two rearrangements of genes in the *hut* operon can explain the presently observed arrangement and loss of *hut* genes appears to be relatively rare in *Pseudomonas*.

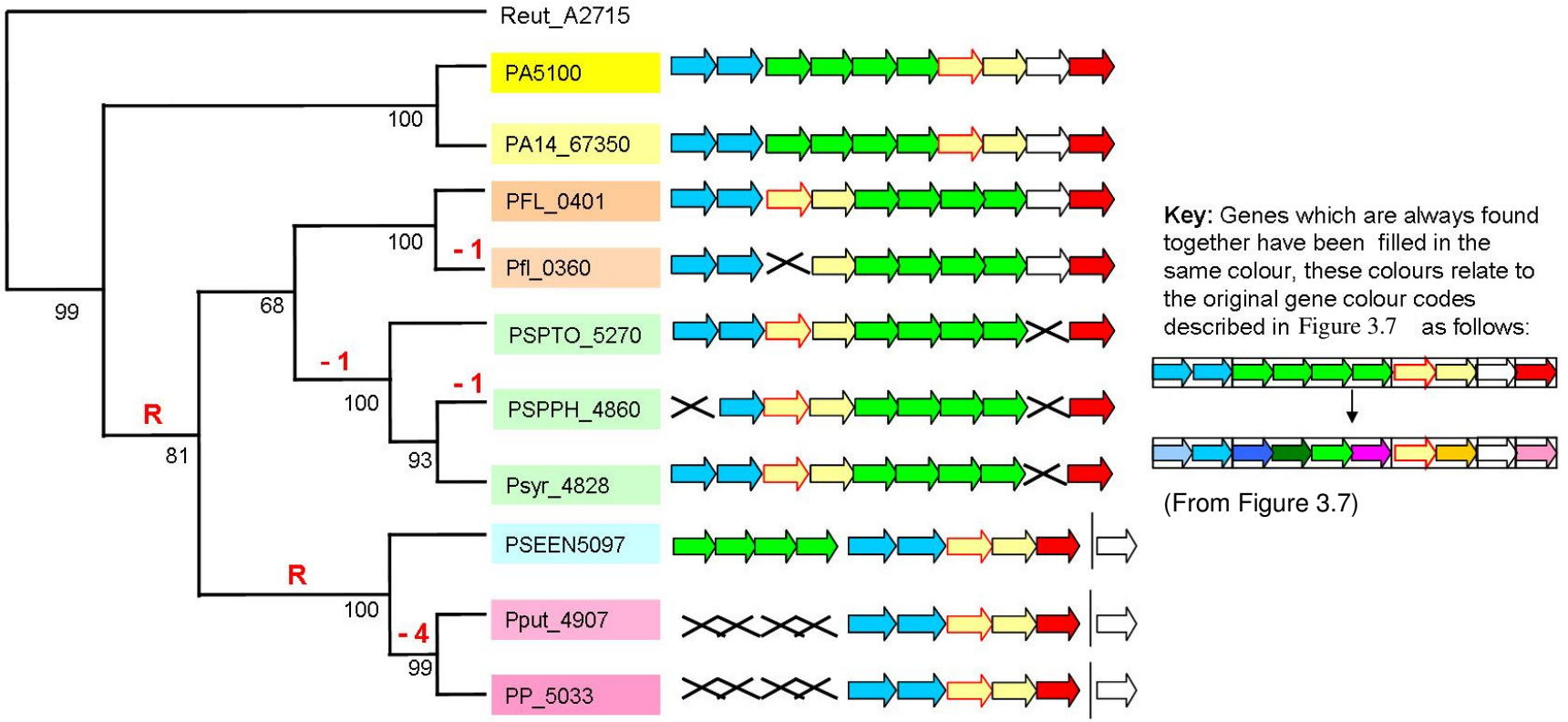


Figure 3.18
Rearrangements and gene loss in the *hut* operon of pseudomonads plotted onto a phylogenetic tree of the *hutU* gene (red arrow), which is consistently found in the *hut* operon and whose evolution is used as representative for the operon as a whole. **R** indicates a rearrangement of the genes in the *hut* operon while numbers indicate gene loss. Genes are represented as arrows. A cross indicates a missing gene. A vertical line indicates that the genes are separate in the genome. The tree was generated in PAUP* and supported with 500 bootstrap replicates. The outgroup used for rooting the tree is *Ralstonia eutropha* JMP134, a urocanate hydratase.

3.5.21 Horizontal evolution of aa_permease genes appears to be rare.

Horizontal gene transfer can have a major impact on phylogenetic inference (Philippe and Douady, 2003). Therefore the identification and removal of horizontally acquired sequences can be essential for producing a high quality phylogenetic alignment. To detect horizontal gene transfer in the aa_permease sequences found in sequenced *Pseudomonas* other than *P. syringae* (i.e not GABA, aromatic, proline or ethanolamine permease sequences) the method of abnormal sequence similarity was used as described in Phillippe and Douady (2003), detailed in the Materials and Methods above. Briefly, reciprocal BLASTP searches were carried out using each aa_permease sequence to find any sequences which were more similar to a gene from a non *Pseudomonas* species.

There are two sequences which are present in one sequenced *Pseudomonas*, found in Pf0-1 and L48 genomes respectively, where the closest BLASTP hits are found in non-*Pseudomonas* species. The most parsimonious explanation for this result is horizontal gene transfer from other species. This conclusion is supported by the generalist lifestyles of both Pf0-1 and L48, which would bring them into contact with a range of bacteria. Furthermore, as shown in Table 3.9, the closest sequences were found in *Rhodococcus* sp. RHA1, a generalist gram-positive bacterium found in a broad range of habitats including soil, water and eukaryotic cells (McLeod et al., 2006) and the gram-negative soil bacterium *Myxococcus xanthus* DK 1622 (Vos and Velicer, 2006), which appear to share similar habitats those encountered by both Pf0-1 and L48 (Vodovar et al., 2006; Silby et al., 2009).

In addition several sequences are found in multiple sequenced *Pseudomonas* genomes, but all from the same species. All additional BLASTP hits are found in species other than *Pseudomonas*. In this case the argument for gene acquisition by horizontal evolution is slightly less clear cut; however, the most parsimonious explanation may still be that the gene was acquired by horizontal evolution. For example, the *P. putida* KT2440 gene PP_4407 (annotated as an amino acid transporter) has an ortholog only in *P. putida* F1 (Pput_1447). The genome sequences of KT2440 and F1 are closely related, with many genes sharing high sequence identity (Nelson et al., 2002). This indicates relatively recent divergence between KT2440 and F1 from a common ancestor. The most parsimonious explanation may be that the common ancestor of KT2440 and F1 acquired this gene by horizontal evolution and it was passed on after KT2440 and F1 diverged.

Pseudomonas	**Gene**	**Closest BLAST hit**	**Gene**	**% ID**	**Closest non-*Pseudomonas* BLAST ortholog**	**Class**	**Habitat**	**Gene**	**% ID**	**Comment**
P.fluorescens Pf-01	Pfl_2343	*Myxococcus xanthus* DK 1622	MXAN_1558	42		4	A			Not present in other *Pseudomonas*
P.entomophila L48	PSEEN3580	*Rhodococcus sp.* RHA1	RHA1_ro03033	58		5	A			Not present in other *Pseudomonas*
P.putida KT2440	PP4407	*P.putida* F1	Pput_1447	98	*Burkholderia* sp. 383	2	A	Bcep18194_B0786	78	Only present in *P.putida*
P.aeruginosa PAO1	PA4804	*P.aeruginosa* PA14	PA14_63480	100	*Parvibaculum lavamentivorans* DS-1	3	A	Plav_1143	44	Only present in *P.aeruginosa*
P.aeruginosa PAO1	PA1819	*P.aeruginosa* PA14	PA14_41010	100	*Chromobacterium violaceum* ATCC 12472	2	A	CV_3432	51	Only present in *P.aeruginosa*
P.aeruginosa PAO1	PA4628	*P.aeruginosa* PA14	PA14_61250	100	*Burkholderia multivorans* ATCC 17616	2	A	Bmul_5011	73	Next 16 BLAST hits are to *Burkholderia* spp.
P.aeruginosa PAO1	PA1194	*P.aeruginosa* PA14	PA14_48850	99	*Photobacterium profundum* 3TCK	1	B	na	65	Only present in *P.aeruginosa*
P.aeruginosa PAO1	PA1916	*P.aeruginosa* PA14	PA14_39750	99	*Enterobacter sakazaki* ATCC BAA-894	1	C	ESA_04280	51	Only present in *P.aeruginosa*
P.entomophila L48	PSEEN5442	*P.putida* F1 and *P.putida* KT2440	Pput_5206 and PP5297	93	*Arthrobacter aurescens* TC1	5	A	AAur_0973	59	Only present in *P.entomophila* and *P.putida*
P.fluorescens Pf-5	PFL_3813	*P.putida* F1 and *P.putida* KT2440	Pput_0694 and PP0660	79	*Bacillus pumilus* SAFR-032	6	A	BPUM_0223	61	Only present in *P.flourescens* and *P.putida*

Table 3.9

Section 1 (yellow) indicates genes found only in a single strain of sequenced *Pseudomonas.* Section 2 (green) indicates genes that are restricted to only 1, or 2 closely related *Pseudomonas* species. PP4407 is a GABA permease previously demonstrated to group in the same clade as GABA permeases from *Burkholderia* species. Class 1= γ-Proteobacteria, 2= β-Proteobacteria 3= □-Proteobacteria, 4= Δ/E Proteobacteria 5= Actinobacteria, 6=Bacilli. Habitat A= free living, commonly found in soil, B= Marine, C= opportunistic pathogen (source: NCBI)

3.5.22 The aa_permease genes encode a diverse family of transporters

In order to investigate the evolutionary relationship of the aa_permease sequences to each other, a phylogenetic tree was constructed using all aa_permease sequences from all representative *Pseudomonas* species. The aa_permease sequences from P.s.t, P.s.s and P.s.ph were used, along with all PAO1, KT2440, Pf-5 permeases, to generate a maximum parsimony tree (Figure 3.19). This tree indicates good support for the groupings of each type of aa_permease, but resolution of the relationship between permease types is not clear.

In general, the tree supports the ortholog groupings shown in Table 3.3 above, and these are marked alongside the tree below. Furthermore, the functional annotations of these genes appear to be broadly correct, with transporters annotated with a similar transported solute function grouping together on the tree (indicated by colour). It appears that the aa_permease genes encode a diverse family of transporters, and the aa_permease genes found in *P. syringae* represent a subset of those found in other sequenced *Pseudomonas* species.

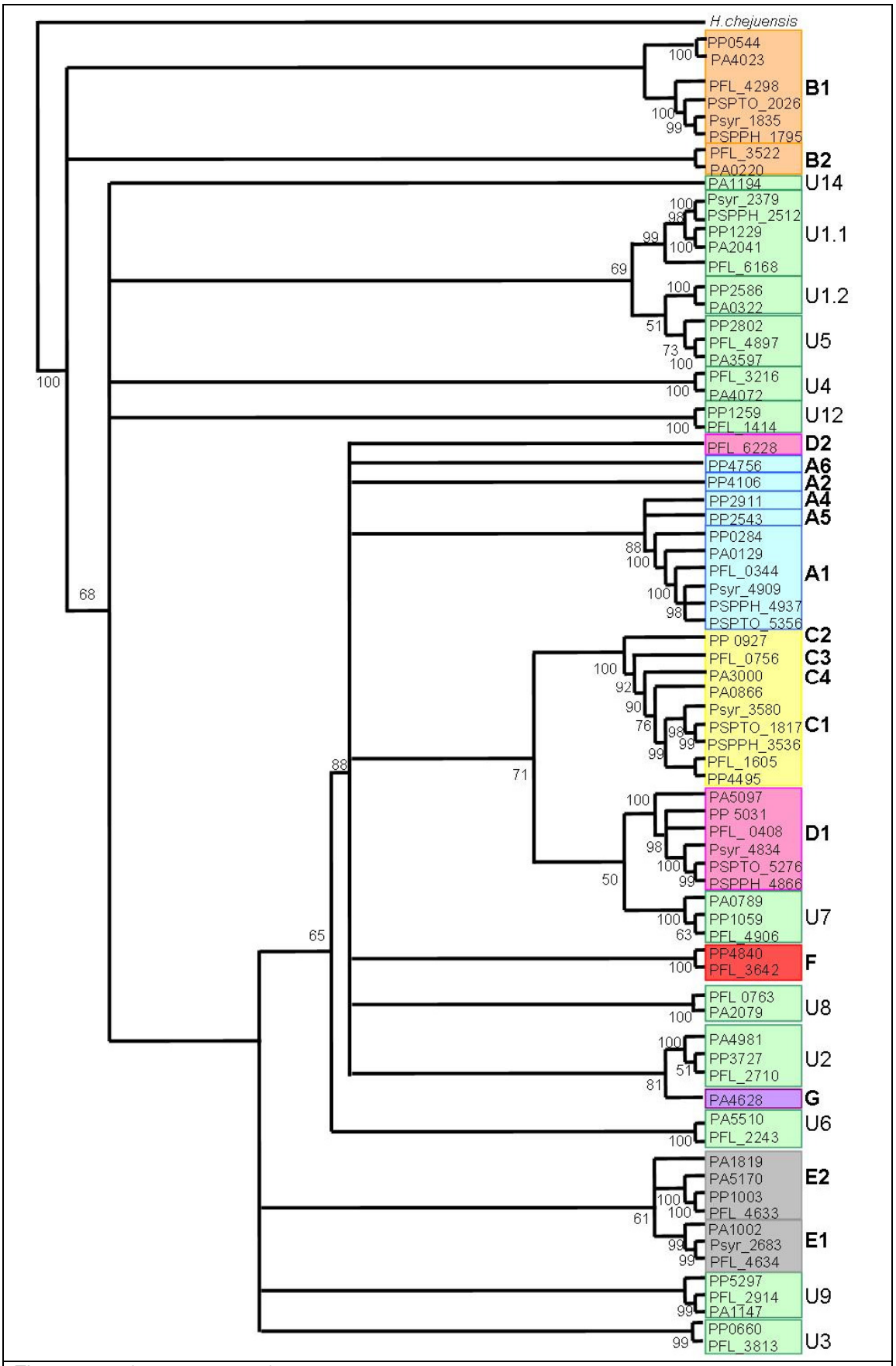


Figure 3.19 (see next page)

Figure 3.19Maximum parsimony tree of aa_permease protein sequences from all three sequenced *P. syringae* strains along with representatives from the other sequenced *Pseudomonas*: *P. putida* KT2440 (PP)*, P. aeruginosa* PAO1 (PA)*, P. fluorescens* Pf-5 (PFL). Colours and letters indicate the annotated substrates for each aa_permease: B1-2 orange= ethanolamine, A1-6 blue= GABA, C1-4 yellow= aromatic, D1-2 pink= proline, U1-15 green= unknown, F red= D-serine/D-alanine/D-glycine, G purple= lysine, E1-2 grey= arginine/ornithine. Bootstrapped with 500 replicates. Outgroup *Hahella chejuensis* KCTC 2396 (*H. chejuensis*) HCH_06322: aa_transporter.

3.5.23 The aa_permease genes in *Xylella fastidiosa* appear to be a subset of those found in *Xanthomonas* species.

Xanthomonas and *Xylella fastidiosa* are closely related species, and, like *P. syringae* colonise the nutrient-limited plant interior (Vauterin et al., 1995). However, while *Xanthomonas* species can colonise the plant surface and apoplast, *X. fastidiosa* species have become particularly specialised and are now limited to the xylem of host plants. In doing so, the genome of *X. fastidiosa* has reduced in size, as genes which are not needed in the xylem environment have gradually been lost. The distribution of aa_permease genes between *X. fastidiosa* and *Xanthomonas* appears to mirror that seen between *P. syringae* and the non plant-pathogenic *Pseudomonas* species, and *X. fastidiosa* contains significantly fewer aa_permease genes than *Xanthomonas* species, even when the difference in genome size is taken into account.

Therefore it was interesting to compare the aa_permease genes in *Xanthomonas* species compared to *X. fastidiosa* in particular to investigate the substrate(s) transported by aa_permease genes in these species, and also to investigate whether the aa_permease genes in *X. fastidiosa* species are a subset of those found in *Xanthomonas* species. Sequences from *Xanthomonas* and *X. fastidiosa* species were downloaded from the Pfam database and used to construct a maximum parsimony tree (Figure 3.20). Results indicate that the aa_permease genes present in *X. fastidiosa*, are a subset of those present in *Xanthomonas* species. Many transporters do not have annotated substrates, however those which have been annotated with a predicted transport function include GABA and ethanolamine and seen in *P. syringae* and D-serine/D-alanine/D-glycine as seen in other sequenced *Pseudomonas*. One sequence, present in *Xanthomonas axonopodis* pv. citri may have been acquired by horizontal evolution as this sequence is closely related to an aa_permease in *Solibacter usitatus*.

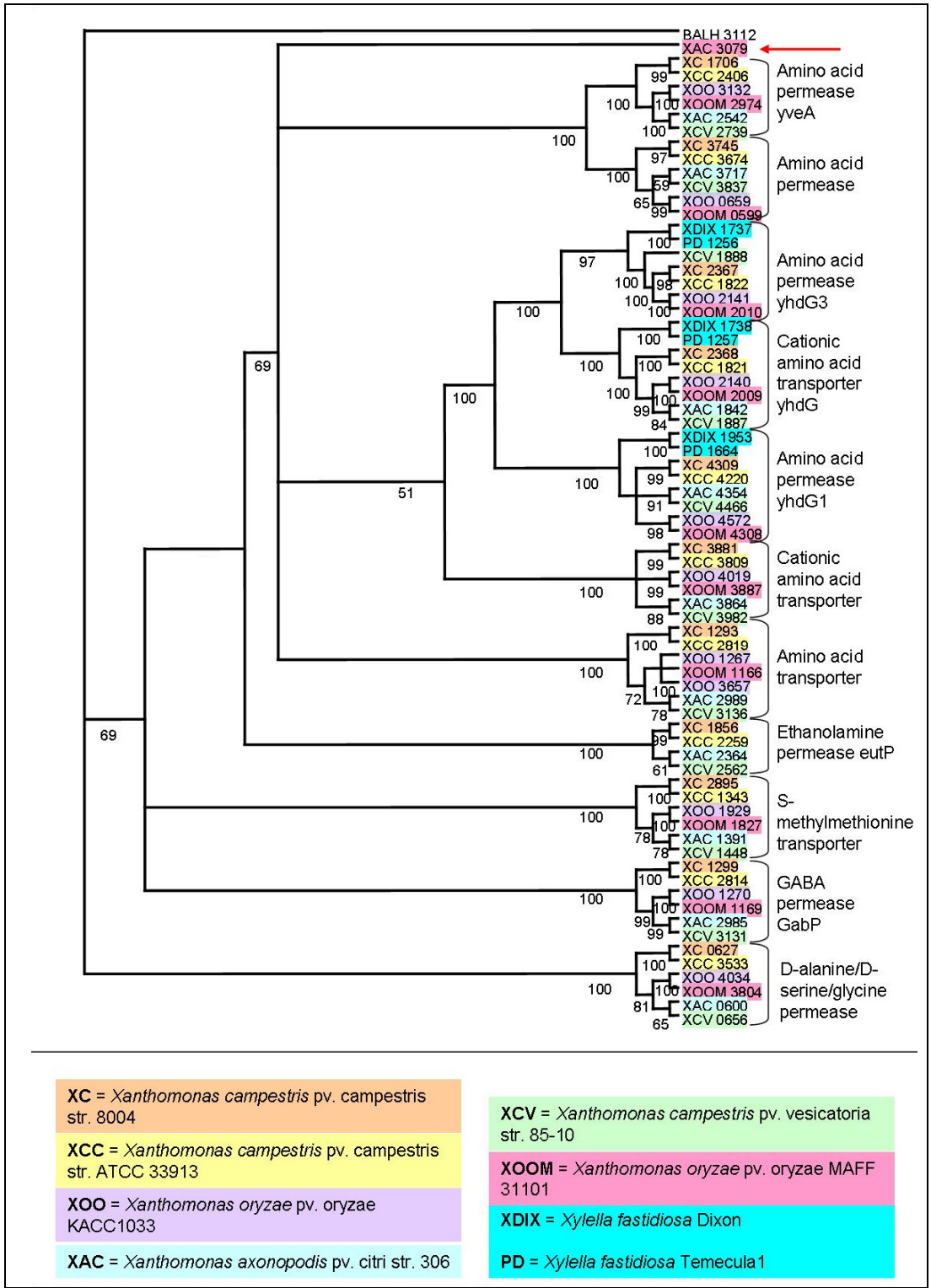


Figure 3.20
Maximum parsimony tree of aa_permease sequences in *Xanthomonas* and *X. fastidiosa* coded as in the key above. As in the *Pseudomonas*, the relationship between aa_permease sequences annotated with a similar transport function is better resolved than between sequences with different transport functions. One sequence, XAC_3079 (red arrow) is 50% similar to *Solibacter usitatus* (Acid_1927 aa_permease associated region) and may have been acquired by horizontal evolution.

3.5.24 APC superfamily domains are reduced in *Xylella fastidiosa* and *Xanthomonas* species.

P. syringae is underrepresented in several APC superfamily domains in addition to the aa_permease domain (Table 3.2). One hypothesis is that a selective advantage is conferred on bacteria in the plant environment that lose transporters containing these domains, possibly because APC transporters do not function as well within plants as they do in other environments. One way of investigating this hypothesis is to compare the number of APC domains in other plant-pathogenic bacteria.

The number of APC-domains in *Xanthomonas* and *X. fastidiosa* species was extracted from the Pfam database (Table 3.10). These numbers were compared using the statistics package Minitab as described in the Materials and Methods. The total number of APC domains was significantly reduced in *X. fastidiosa* compared to *Xanthomonas*. In addition, the total number of APC domains was also significantly reduced in *Xanthomonas* compared to the non plant-pathogenic *Pseudomonas*. Both plant-pathogenic *P. syringae* and *Xanthomonas* appear to have lost APC-domain containing transporters compared to non plant pathogens.

Interestingly, although the total number of APC domains is similar in *P. syringae* and *Xanthomonas*, the distribution of APC domains was different in these species. Unlike *P. syringae*, *Xanthomonas* species do not contain any Transp_cyt_pur, Na_Ala_symp or Tryp_try_perm domains. Also, *Xanthomonas* species have significantly more aa_permease domains than *P. syringae* ($p=0.002$), although still significantly less than non plant-pathogenic *Pseudomonas* species ($p=0.000$). The only APC domains in *X. fastidiosa* are the aa_permease and SSF domains. The numbers of both types of domain are reduced in *X. fastidiosa* compared to *Xanthomonas* species.

APC superfamily	***P. putida* KT2440**	***P. putida* F1**	***P. fluorescens* Pf-5**	***P. fluorescens* Pf0-1**	***P. aeruginosa* PAO1**	***P. syringae* pv. phaseolicola 1448A**	***P. syringae* pv. Syringae B728a**	***P. syringae* pv. tomato DC3000**	***Xanthomonas campestris pv.* Vesicatoria 85-10**	***Xanthomonas axonopodis pv.* citri KACC1033**	***Xanthomonas oryzae pv.* oryzae MAFF**	***Xanthomonas campestris pv.* campestris 8004**	***Xylella fastidiosa* Dixon**	***Xylella fastidiosa* Temecula-1**	***Xylella fastidiosa* Ann-1**	
Pfam domain	**Number of domains**															Is *X. fastidiosa* over or underrepresented compared to *Xanthomonas* (p-value)
Transp_cyt_pur	**7**	**7**	**8**	**9**	**6**	**2**	**3**	**2**	0	0	0	0	0	0	0	not present in *Xanthomonas* or *Xylella*
Sulfate_transp	**4**	**5**	**6**	**3**	**4**	**4**	**4**	**4**	**4**	**3**	**3**	**3**	0	0	0	under (p=0.000)
Na_Ala_symp	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	0	0	0	**1**	0	0	0	not significant
Branch_AA_trans	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	0	0	0	0	0	0	0	not present in *Xanthomonas* or *Xylella*
AA_trans	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	not present in *Xanthomonas* or *Xylella*
BenE	**3**	**3**	**3**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	0	0	0	under (p=0.000)
Trp_Try_permease	0	**1**	**1**	**1**	**3**	**1**	**1**	**1**	0	0	0	0	0	0	0	not present in *Xanthomonas* or *Xylella*
Xan_ur_permease	**6**	**6**	**8**	**8**	**7**	**3**	**3**	**3**	**1**	**1**	0	**1**	0	0	0	under (p=0.036)
SSF	**7**	**6**	**7**	**5**	**6**	**4**	**3**	**3**	**3**	**3**	**3**	**3**	**1**	**2**	**1**	under (p=0.045)
AA_permease	**21**	**21**	**21**	**14**	**21**	**5**	**6**	**4**	**11**	**12**	**10**	**11**	**3**	**3**	**3**	under (p=0.000)
Spore permease	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*not present in Xanthomonas* or *Xylella*
HCO3_cotransp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
DUF1468	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
CbiQ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Total	**51**	**51**	**56**	**45**	**53**	**22**	**23**	**20**	**20**	**20**	**18**	**20**	**4**	**5**	**4**	under (p=0.000)

Table 3.10APC-superfamily domains in *Xanthomonas* and *X. fastidiosa*, alongside the number in *P. syringae* and non plant-pathogenic *Pseudomonas* (for interest. These were not included in the statistics). *Xanthomonas* and *X. fastidiosa* genomes contain fewer types of APC domain than *Pseudomonas*. In particular APC domains are almost entirely absent in *X. fastidiosa.* However, *Xanthomonas* genomes contain more aa_permease domains than *P. syringae.* For statistical analysis, the number of Pfam domains in *Xanthomonas* and *X. fastidiosa* strains was compared in Student's 2-tailed t-tests. Tests had 6 degrees of freedom. H_0=no difference in domains between *Xanthomonas* and *X. fastidiosa.* P-values were as indicated above and a value of $p \leq 0.05$ was taken as significant.

3.6 Discussion.

The aa_permease (amino acid permease) domain, found within aa_permease transporters, is significantly underrepresented in *P. syringae* genomes compared to other sequenced *Pseudomonas* species (Studholme et al., 2005). Domain loss or gain can be associated with adaptation by bacteria to a particular environment, such as a plant-associated niche (Studholme et al., 2005). The aim of the work described in this chapter was to investigate the distribution and diversity of aa_permease genes in *P. syringae* and the other sequenced *Pseudomonas,* and interpret the findings with reference to the niche inhabited by *P. syringae* and the evolutionary processes which may have occurred to produce the current distribution.

The location of the aa_permease proteins was confirmed using bioinformatic programs to identify signal peptides targeting these proteins to the cytoplasmic membrane, and the presence of transmembrane domains which cross the cytoplasmic membrane. Several *Pseudomonas* permeases were predicted to be regulated by the global response regulator RpoN. In particular, the putative ethanolamine permease, present in all sequenced *P. syringae* genomes, was predicted to be RpoN-regulated. RpoN regulates a variety of genes involved in nitrogen assimilation and adaptation to complex natural environments, which suggests that the main function of these permeases may be in nitrogen assimilation. Consistent with this a *P.fluorescens* SBW25-*rpoN* mutant was impaired in the ability to assimilate a wide range of amino acids as carbon or nitrogen sources including ethanolamine and GABA (Jones et al., 2007). The importance of RpoN to nutrient uptake has been demonstrated in a range of *Pseudomonas* species including *P.aeruginosa* PAO1 (Hendrickson et al., 2001), *P. putida* KT2440 (Kohler et al., 1989), and *P. syringae* pv. maculicola (Hendrickson et al., 2000). However the creation of a *P. syringae* pv. tomato RpoN mutant has yet to be reported.

The aa_permease proteins are classified by the Transporter Classification Database (whose classifications are used by Pfam) as one of 14 domain families belonging to the APC superfamily of transport proteins (Saier, 2000). APC superfamily transporters are electrochemical potential-driven porters, which utilise a carrier-mediated process to facilitate either antiport, and/or symport, not coupled to a direct form of energy (e.g. ATP) other than chemiosmotic energy (Saier, 2000).

Ten APC superfamily domains are present in the genomes of sequenced *Pseudomonas* species (Table 3.2). Statistical analysis revealed that *P. syringae* is underrepresented in five of these domain families, including the aa_permease domains. The underrepresented domains are found in transporters for a diverse range of substrates including uracil, xanthine

and benzoate along with non-specific amino acid transporters. The common factor among transporters containing these domains appears to be their mechanism of action (i.e. the use of electrochemical-potential energy to drive substrate transport), rather than the particular substrate transported. These results indicate that there is a significant reduction in the number of APC electrochemical potential-driven transporters in general, which appears to have occurred regardless of the substrate transported.

The most striking disparity in domain number between *P. syringae* and other *Pseudomonas* is found between the numbers of aa_permease domains. This may be because the aa_permease domain is simultaneously underrepresented in *P. syringae* compared to non plant-pathogenic *Pseudomonas* and overrepresented in non plant-pathogenic *Pseudomonas* compared to other bacteria (Studholme et al., 2005). One hypothesis for this disparity is that the non plant-pathogenic *Pseudomonas* species have expanded their aa_permease genes by gene duplication and/or horizontal gene transfer from other bacterial species encountered in their environment. Horizontal gene transfer has frequently been assumed to be a dominant force in the shaping of prokaryotic genomes although its actual contribution remains unclear (Philippe and Douady, 2003).

In order to address this hypothesis and to indicate how common this process might be in *Pseudomonas*, the aa_permease sequences were analysed for evidence of horizontal evolution, as measured by abnormal sequence similarity. Results highlighted several sequences, which have reciprocal best blast hit (RBBH) orthologs in non-*Pseudomonas* species, predominantly in generalist soil-dwelling bacteria. The most parsimonious explanation for this result is that these genes were not present in the common ancestor of *Pseudomonas,* and they have been acquired at some point since the divergence of different *Pseudomonas* species by horizontal gene transfer from a non-*Pseudomonas* species. For the majority of sequences the closest BLASTP match in the non-*Pseudomonas* species had a relatively low sequence similarity to the *Pseudomonas* gene, as measured by percentage identity. This is probably because the majority of soil bacteria still remain uncharacterised (Fierer et al., 2007) and therefore it is possible that as more sequences become available a closer match will be found. A likely exception to this is a GABA permease found in *P. putida* species, identified as an ortholog of a GABA permease found in *Burkholderia* species with 73 % BLASTP identity. *Burkholderia* species are ubiquitous in the environment and many have now been sequenced and added to the NCBI genomes database. The *P. putida* sequence matches a GABA permease in three sequenced *Burkholderia* genomes with over 70% identity. This strongly implicates *Burkholderia* species as the original source for this

gene. However, as more bacteria are sequenced it is possible that a permease with even higher identity may be identified.

The majority of *Pseudomonas* aa_permease genes which have orthologs in non-*Pseudomonas* species are restricted to the genomes of a single *Pseudomonas* species. One exception is a gene which is found in both *P. putida* (Pput_5206, PP5297) and *P. entomophila* (PSEEN_5442) species. However as *P. putida* and *P. entomophila* have been shown to be closely related, with over 70% of genes being orthologs and more than 96% in synteny (Vodovar et al., 2006), the most likely explanation here may be horizontal acquisition of this gene in the last common ancestor of *P.putida* and *P.entomophila*.

None of the aa_permeases present in *P. syringae* appear to have been acquired by horizontal gene transfer. Ortholog and phylogenetic analysis shows that these genes constitute a sub-set of the aa_permeases possessed by other *Pseudomonas,* many of which have unknown transport functions or low similarity to characterised transporters in other bacterial species such as *E. coli* (Table 3.5).

In P.s.t., the aa_permease genes are annotated as transporting GABA, ethanolamine, proline and aromatic amino acids based on their homology to aa_permease transporters in other bacteria. The ProY permease was annotated as such in *Pseudomonas* due to its homology to permeases in *Salmonella* and *E. coli* which have been experimentally demonstrated to transport proline. However this gene was subsequently shown to transport histidine, rather than proline, in *P. fluorescens* SBW25 (annotated as *hutT*) (Zhang and Rainey, 2007). The similarity between *hutT* in P.fl.SBW25 and the orthologous gene (*proY*) in other *Pseudomonas* species is much greater than the similarity between *proY* in *Pseudomonas* and *proY* in *E. coli* or *Salmonella*. This finding suggests that BLAST homology of sequences does not necessarily imply shared function of the protein, when the genes being compared are from two different species, and it seems likely that the *proY*-like gene in P.s.t. is actually involved in histidine transport.

Between the closely related *P. syringae* pathovars the synteny of genes surrounding the aa_permeases is generally preserved, however this synteny is not preserved across all *Pseudomonas* indicating that aa_permease genes do not need to be transcribed alongside the genes that surround them in the *P. syringae* genome. For the aromatic and ethanolamine genes in P.s.t (PSPTO_1817 and PSPTO_2026) this conclusion is supported by sequenced RT-PCR transcripts which show that in mannitol-glutamate minimal medium the genes either side of each permease are transcribed independently of the permease itself

(Figure 3.1). The ProY permease falls between histidine-utilisation and transport genes which have been shown to be transcribed in an operon in P.fl.SBW25 (Zhang and Rainey, 2007). Although almost all *Pseudomonas* species share the same *hut* operon genes, the organisation of this operon appears to be quite flexible and has undergone at least two rearrangements between *Pseudomonas* species (Figure 3.7). However in *P. syringae* the sequenced RT-PCR transcripts in Figure 3.1 seem to support the annotation of this region as still being an operon, as all genes behaved similarly, except for the last gene of the operon (PSPTO_5278), which may be transcribed independently. This could be confirmed experimentally by sequencing RT-PCR transcripts from P.s.t grown in a media known to activate *hut* gene expression.

The *hut* operon appears to vary greatly in length between species, ranging from ten genes in *P. aeruginosa* PAO1 and PA14 and *P. fluorescens* Pf-5 to five in *P. putida*. In order to model the process of rearrangement and gene loss in this operon a phylogenetic tree was created using the *hutU* sequence, as this gene is found in the *hut* operon of all species. On to this tree the most parsimonious rearrangement and gene loss events were superimposed (Figure 3.7). As the *P. aeruginosa* sequences formed an outgroup to the other *Pseudomonas*, and this species contains all ten *hut* operon genes, the organisation of genes in the *P. aeruginosa* operon was taken as ancestral. From here it only required two rearrangements to produce the current operon organisation. Gene loss appeared to be highly species specific and had occurred mainly in *P. putida*. Extensive rearrangements have occurred in the *hut* operon in both the *P. putida* KT2440 and F1 genomes, along with the loss of four genes present in the *hut* operon of PAO1. However, as KT2440 can still use histidine as a sole source of carbon and nitrogen for growth (Rico and Preston, 2008), it can be hypothesised that *P. putida* still contains the minimum number of genes for a functional *hut* operon. This is supported by the fact that the KT2440 *hut* operon contains genes for enzymes which have previously been identified as necessary for histidine degradation in *P. fl* SBW25 (*hutG, hutI, hutH and hutU*) (Zhang and Rainey, 2007), in addition to a probable histidine transporter (*proY*). Therefore, the genes missing from the KT2440 *hut* operon, which include two histidine ABC transporters and a histidine binding protein, may represent genes which are not essential for histidine utilisation by *P. putida*. In addition *P. putida* lacks a second copy of the *hutH* gene. All other sequenced *Pseudomonas* species studied in this chapter contained two copies of the *hutH* gene (Figure 3.7), indicating an ancestral duplication event. In *P. fl* SBW25 one copy of *hutH* has been shown to be non-functional (Zhang and Rainey, 2007), it can be hypothesised that during rearrangement of the *hut* operon, *P. putida* lost this non functional copy and retained instead a single *hutH* gene within the reduced *hut* operon.

Interestingly, despite these rearrangements the *proY* gene is retained within the *hut* operon in all *Pseudomonas* except *P. fluorescens* Pf0-1, where it has been lost. Unlike in P.s.ph where the first gene in the *hut* operon (PSPPH_4865) can be seen to contain a premature stop codon making it a non-functional pseudogene, there is no evidence for the past existence of the *proY* gene in Pf0-1. The most parsimonious explanation, given that this gene is present in all other *Pseudomonas*, is gene loss in Pf0-1, possibly facilitated by the rearrangements occurring in the *hut* operon. The lack of evidence remaining in the Pf0-1 genome for the existence of a *proY* ortholog implies this event may have happened a long time ago.

Phylogenetic trees for each of the aa_permease genes in P.s.t were constructed including orthologous sequences from all other sequenced *Pseudomonas*. In addition, if multiple aa_permease sequences from other *Pseudomonas* shared a high homology to a P.s.t sequence, despite not being orthologous, then these were also included. For example, there are multiple copies of GabP in *P. entomophila* L48, all of which share a high homology to the GabP in P.s.t so these sequences were all included in the GabP sequence alignment.

The GABA tree was constructed using two different methods: neighbour joining (NJ) and maximum parsimony (MP). Both methods have their advantages and disadvantages. The MP method is more computationally demanding, while NJ has been criticised for being too simple a model and so prone to more errors (Tateno et al., 1994). Although both tree-building methods produced the same groupings for the GabP sequences it was decided to continue with the more rigorous MP method for the remaining trees and to use NJ as a quick and informal 'double check' (Harrison and Langdale, 2006). For all trees the groupings proposed by the NJ method were highly similar to those proposed by MP.

For the GABA trees shown in Figures 3.12 and 3.13, the orthologous sequences identified by reciprocal BLAST analysis grouped together on the tree. In general, the non-orthologous GABA sequences also grouped closely with these sequences. However, one GABA permease from *P. putida* consistently grouped away from all other GabP sequences, indicating that this particular sequence was not evolutionarily related to the others, and may have been acquired by horizontal evolution. In order to test this hypothesis a phylogenetic tree was generated using the *P. putida* GabP sequence and sequences from the closest BLASTP hits in the NCBI database, which were from *Burkholderia* species. The GabP sequence from *P. putida* grouped within a clade alongside the *Burkholderia* sequences (Figure 3.14), and separate from other *P. putida* GabP sequences. One reason for the

additional *gabP* genes in *P. putida* compared to the most other *Pseudomonas* species appears to be that at least one *gabP* gene has been acquired through horizontal evolution.

However, it is clear that not all the additional *gabP* genes can be explained in this way. There is no evidence of horizontal evolution for the remaining GabP sequences in *P. putida* or for the many GabP sequences in *P. entomophila* L48. As *P. putida* and *P. entomophila* are closely related (Vodovar et al., 2006), the most likely explanation for the presence of additional *gabP* genes in both these species is ancestral duplication. One reason for this could be that the ancestral strain encountered high levels of GABA in their environment (or compounds structurally related to GABA, such as gamma-hydroxybutyrate), which might be transported by the additional permeases. Although we have no way of knowing which environments this ancestral strain lived in, we can look for data on the presence of GABA in the environments regularly encountered by *P. putida* and *P. entomophila*. These bacteria are generalists and will therefore encounter a range of environments along with other microorganisms. In *P. putida* KT2440, an IVET strategy identified the *gabP* gene PP2543 as being highly induced in the presence of maize seedlings. This gene was also significantly induced in the rhizosphere (Ramos-Gonzalez et al., 2005). In addition, in KT2440, the GabT protein, which catalyses the conversion of GABA into succinic semialdehyde, was induced during growth in the rhizosphere (Espinosa-Urgel et al., 2002), while in *P. putida* 06909, *gabD* induction has been reported during the colonisation of *Phytopthora infestans* mycelia (Ahn et al., 2007). The GabD enzyme converts succinic semialdehyde, the product of GABA-catabolism, into succinate which can enter the TCA cycle and it has been suggested that either GABA or the products of GABA catabolism are present on mycelial surfaces (Ahn et al., 2007). Therefore it appears that many sources of GABA, or related products, may be present in the environments inhabited by *P. putida* and *P. entomophila*. This could have provided a selective advantage to bacteria with multiple GABA transporters and led to the numbers presently observed.

The phylogenetic tree based on the aromatic amino acid sequences (Figure 3.15) shows that sequences designated as orthologs by RBBH analysis group together in a well supported clade, with the second copy of this permease, present in only some *Pseudomonas* genomes, falling outside of this clade. The phylogenetic tree generated by ethanolamine permease sequences (Figure 3.16) also follows this pattern, with the orthologous genes grouping together, and the second copies grouping together but elsewhere. These results indicate that one copy of the aromatic or ethanolamine gene is most likely the 'original' with the second copy arising as an ancestral duplication (i.e. a paralog). In addition the duplicated and the original gene had diverged enough in the

ancestral species so that when the *Pseudomonas* diverged into the present species, the original or duplicated genes grouped more closely with the respective copies in other *Pseudomonas*, than with each other. The tree of proline aa_permease sequences (Figure 3.17) shows the orthologous genes grouping together in a well supported clade, with a second copy of this permease, found only in Pf-5 falling outside. This sequence does not appear to have been acquired by horizontal evolution, and the most parsimonious explanation in this case is gene duplication within the Pf-5 lineage.

Within the *Pseudomonas* there is little experimental evidence for the substrate transported by any of the permeases. In addition, the ProY permease, (the ortholog of which has been experimentally demonstrated in P.fl SBW25 as a histidine transporter), appears to have been misannotated and may in fact transport histidine. So we cannot be sure that 'ethanolamine' permease genes will actually transport ethanolamine in *Pseudomonas*. This caveat extends to second copies of genes; just because a permease sequence has a close BLASTP similarity to a (putatively) annotated ethanolamine permease, or is the reciprocal best blast hit of an annotated ethanolamine permeasem does not necessarily make its function an ethanolamine transporter (for example, in *P. aeruginosa* PAK PA0220 may function as a histamine, rather than an ethanolamine transporter). For these reasons, bioinformatic analysis regarding the predicted substrate transported by a transporter must be treated with caution.

A tree containing all aa_permeases from *P. syringae* and representative non plant-pathogenic *Pseudomonas* demonstrated that aa_permease sequences are diverse, but that in general sequences annotated with the same functional annotation group together (Figure 3.20). Where an ancestral duplication was hypothesised to have occurred, such as in the aromatic and ethanolamine permeases, both copies grouped together, indicating that the sequences are more similar to each other than to other aa_pemeases. The main exception is the second copy of the ProY gene in *P. fluorescens* Pf-5. This sequence groups away from all other ProY genes and does not fall within a defined clade. This indicates that the second ProY has diverged in structure, and possibly in function, from the original, and consequently this gene may have been missannotated.

The reduction in aa_permease genes in *P. syringae* compared to non plant-pathogenic *Pseudomonas* appears to mirror the reduction seen in species of the obligate plant pathogen *X. fastidiosa*, compared to the closely related, but non obligate plant pathogen *Xanthomonas*. This was interesting as it suggested the reduction of aa_permease genes in *P. syringae* may be mirroring the gene reduction undergone by highly specialised obligate

pathogens. The aa_permease genes present in *X. fastidiosa* genomes were significantly reduced compared to *Xanthomonas* genomes (Table 3.10). In addition, the aa_permease genes present in *Xanthomonas* were significantly reduced compared to non plant-pathogenic *Pseudomonas* species. Therefore it appeared that the reduction in aa_permease genes may be common to plant-pathogenic species. However, for *Xanthomonas* we do not have a non plant-pathogenic species with which to compare the number of aa_permease genes. This hypothesis could be further developed by comparing the number of aa_permease genes in plant-pathogenic and non plant-pathogenic bacterial species within the same genera, although there are very few genera which inhabit both plant and non-plant associated environments, and are as well sequenced and studied as *Pseudomonas* species.

Phylogenetic analysis indicated that the aa_permease genes in *X. fastidiosa*, were a subset of those present in *Xanthomonas* species (Figure 3.20). The majority of *Xanthomonas* permeases are not annotated with putatively transported substrates. However those which have been annotated include GABA and ethanolamine permeases, (which are also present in *P. syringae*) and D-serine/D-alanine/D-glycine (which is found in other sequenced *Pseudomonas* such as *P. putida*). As aa_permease transporters in both *P. syringae* and *Xanthomonas* have been annotated with GABA or ethanolamine transport functions, uptake of these compounds may be important in the plant environment. *Xanthomonas* aa_permease transporters are also annotated as transporting substrates which have not been annotated for any *Pseudomonas* permease, such as S-methylmethionine. These sequences are not similar to any *Pseudomonas* aa_permease sequence so *Pseudomonas* aa_permeases may not transport S-methylmethionine. One permease, present in *Xanthomonas axonopodis* pv. citri may have been acquired by horizontal evolution as this sequence is closely related to an aa_permease in the soil dwelling bacterium *Solibacter usitatus*. Horizontal evolution of aa_permease genes in *Xanthomonas*, as in *Pseudomonas* species, appears to be relatively rare.

A comparison of APC domains in Table 3.2 indicated that the reduction in aa_permease genes in *P. syringae* compared to non plant-pathogenic *Pseudomonas* may contribute towards a more general reduction in APC-superfamily transporters. APC transporters function as symporters or antiporters and use chemiosmotic potential energy to power the transport rather than ATP. Therefore a reduction in these transporters in general could indicate that chemiosmotic potential energy does not work as well in the plant environment as it does in niches inhabited by non plant-pathogenic species. Table 3.10 demonstrated that in general APC family transporters were also significantly reduced in *X. fastidiosa* genomes, and that *X. fastidiosa* genomes only contain SSF (Na^+/solute symporter family)

and aa_permease domain-containing transporters. In addition, *Xanthomonas* genomes contained fewer types of APC domain than *P. syringae*, although significantly more aa_permease domains in particular. This distribution appears to represent a general reduction in the number of APC-domain containing transporters in plant-pathogenic bacteria.

In conclusion, there is little experimental information available for the aa_permease transporters, particularly with reference to the *Pseudomonas* where only the *arcD,* PA0220 and *hutT* genes have been experimentally investigated. This chapter has shown that the amino acid permeases to constitute a relatively diverse family of transporters, whose transported substrates are generally not known. There appears to be flexibility as to the genomic context of these transporters within *Pseudomonas*, as measured by the rearrangements of the genes either side, which may indicate these genes are generally transcribed independently rather than alongside other genes. In addition, there appears to be a more general reduction in the number of APC-domain containing transporters in plant-pathogenic bacteria. This may be due to negative selection for transporters which function using chemiosmotic potential energy in the plant environment. Therefore it may be interesting to influence the effect of pH on the aa_permeases, as this is known to fluctuate diurnally within the plant environment and may effect the functioning of these transporters. However, for some aa_permeases the benefit to the bacteria of taking up the transported substrate may outweigh any negative effects. For these transporters, there may be positive selection operating to maintain them in the genome of plant-pathogenic bacteria, thereby explaining why aa_permease transporters remain even in obligate plant pathogens like *X. fastidiosa*.

In P.s.t there are four remaining aa_permease genes, encoding transporters currently predicted to transport GABA, aromatic amino acids, ethanolamine and proline, (although the latter possibly transports histidine based on characterisation of the orthologous gene in P. fl. SBW25). These amino acids have all been detected in the tomato apoplast (Chapter 1, Table 1.3), suggesting that these are available for P.s.t during colonisation of tomato. However, many other amino acids were also detected in the tomato apoplast for which P.s.t does not have permeases. For example, lysine has been detected in the healthy tomato apoplast and was more abundant than either histidine or proline (Rico and Preston., 2008). *P. aeruginosa* PAO1 and PA14 genomes both contain genes encoding a putative lysine aa_permease but orthologous genes are not present in *P. syringae* genomes. Therefore, the reason why transporters remain for these amino acids in particular remains unclear. One way to investigate why aa_permease transporters for particular amino acids have been retained in *P. syringae*, and P.s.t in particular, is to investigate the response of P.s.t amino

acids which might be transported by the permeases. In particular, it is logical to test the ability of P.s.t to utilise different amino acids as a source of carbon or nitrogen for metabolism or growth. It can be envisaged that there may be negative selection for *P. syringae* to maintain transporters for amino acids which are present in the apoplast, but cannot be utilised, while conversely there may be positive selection operating on transporters for amino acids which provide good carbon or nitrogen sources. In addition, it will also be interesting to test the ability of P.s.t to chemotax towards different amino acids, as the ability to move towards compounds which can then be taken up and utilised for energy (energy taxis) may represent another survival strategy for *P. syringae* in the plant environment.

Appendix.

	Gene	Pfam length of domain	
A	Psyr_2683	269-368	
	PA5170	283-354	
	PP1002	282-366	
	PSEEN4427	227-383	
	PA14_68300	283-354	
	Pfl_4384	230-366	
	Pput1039	282-366	
	PSEEN4426	200-366	
	PFL_4633	230-360	
B	PA14_72710	52-249	305-386
	PFL_2243	14-249	305-387
	Pfl_2055	46-256	305-387
	PA5510	52-249	305-386
	PSEEN3580	45-140	193-230
	PA1147	75-130	202-386
	PA14_49570	75-130	202-386
C	PA1485	19-96	
	PFL_3212	19-96	
	Pput_2445	202-329	
	PA14_45240	19-96	
	PSEEN2282	19-96	

Figure A1
List of genes containing aa_permease domains identified by Pfam as being less than 200 amino acids in length. Category A contains domains which aligned well to sequences containing an aa_permease domain which ran the full length of the sequence, and were left in. Category B contains 'split domains' which were identified more than once by Pfam, these were also left in. Category C contains domains which were removed from further analysis.

Chapter 4: Amino acid utilisation by *Pseudomonas* syringae.

4.1 Overview.

The plant-pathogenic bacterium *Pseudomonas. syringae* (*P. syringae*) has long been considered a nutritionally specialist species compared to non plant-pathogenic *Pseudomonas*, which typically inhabit more heterogeneous environments. A survey of amino acid utilisation by *Pseudomonas* species revealed that *P. syringae* can use a restricted number of amino acids for growth and metabolism compared to those used by other *Pseudomonas* species. However, the amino acids utilised by *P. syringae* pv. tomato (P.s.t) for growth correlated with those which were abundant in the apoplast of the host plant tomato. In addition, the growth of three *P. syringae* strains was inhibited in acidic and alkaline conditions outside the pH range typically found in the apoplast which were not found to inhibit the growth of representative non plant-pathogenic *Pseudomonas* spp. Alkaline pH also influenced the ability of *P. syringae* to assimilate amino acids, possibly by affecting the ionic form of amino acids or by directly inhibiting the activity of amino acid transporter. As aa_permease transporters function using chemiosmotic energy, rather than ATP, the efficacy of these transporters may be affected more by fluctuating pH than other types.

Apoplast extraction is a labour intensive process requiring large amounts of plant material. Therefore synthetic apoplast and guttation fluid media were created as a cheap and simple alternative, using data describing the amino acid content of tomato and *A. thaliana* leaf apoplast, and of *A. thaliana* guttation fluid (exudate from hydathodes). These media comprised a common base of M9 minimal media with added plant-derived sugars, organic acids, and amino acids. As the composition and pH of synthetic apoplast media could be varied, experiments were then carried out to investigate the contribution of different amino acids to the growth of *P. syringae*, and the importance pH for the assimilation of metabolites. Both tomato and *A. thaliana* synthetic apoplast media supported increased growth of *P. syringae* compared to M9 media, although growth was lower compared to extracted tomato apoplast. Incubating *P. syringae* in synthetic tomato apoplast prior to nutrient utilisation assays, resulted in a similar profile compared to extracted tomato apoplast, indicating that these media activated similar nutrient utilisation pathways. The compounds utilised by *P. syringae* as a carbon source were affected by the pH of the synthetic tomato apoplast media.

4.2 Introduction.

The genus *Pseudomonas* represents one of the most diverse groups of bacteria on the planet and inhabits a broad range of environmental niches (Spiers et al., 2000). The plant-pathogenic species *P. syringae* has long been considered to be a relatively specialist *Pseudomonas* and the distinctive performance of *P. syringae* in phenotypic and biochemical tests has traditionally formed the basis for classification of this species (Thornley, 1960; Lelliott et al., 1966; Misaghi and Grogan, 1969). In addition the nutritional specialisation of *P. syringae* has traditionally been used for classification of *Pseudomonas* species which can involve phenotypic tests based on their differential abilities to utilise carbon sources as sole sources of energy (Palleroni, 1984). More recently, bioinformatic analysis of *Pseudomonas* genomes has provided researchers with a genetic basis for hypotheses regarding differences in nutrient utilisation between plant-pathogenic and non plant-pathogenic *Pseudomonas* species. In particular, comparison of protein domains in plant-pathogenic *P. syringae* compared to non plant-pathogenic *Pseudomonas* species, revealed differences in the number of domains found in proteins with a role in nutrient utilisation (Studholme et al., 2005). For example, the sugar_tr and BPD_transp_2 domains, found in sugar transporters and ATP-dependent high-affinity branched-chain amino acid transporters, respectively, were identified as being present in significantly higher numbers in *P. syringae* than in other *Pseudomonas*. In contrast, the aa_permease domain, found in amino acid permease transporters, was significantly underrepresented in *P. syringae* compared to other *Pseudomonas* species (Studholme et al., 2005).

The plant apoplast environment colonised by *P. syringae* is thought to contain a limited number of plant-derived nutrients present in a low abundance (Alfano and Collmer, 1996; Lindow and Brandl, 2003). In contrast, non plant-pathogenic *Pseudomonas* species are thought to encounter a range of nutritionally heterogeneous niches. Therefore it has been hypothesised that the nutrient specialisation of *P. syringae* results from the restricted range of nutrients available in the apoplast environment (Rico and Preston, 2008). Studies have begun to address the question of which nutrients are potentially available for *P. syringae* to utilise in this environment, particularly amino acids. Rico and Preston (2008) compared the ability of *P. syringae* and non plant-pathogenic *Pseudomonas* species, to metabolise amino acids as sole sources of carbon or nitrogen for growth, in Biolog plate-based assays. The results of these assays have been reproduced in Tables 4.1 and 4.2 below.

	P. syringae pathovars				*P. fluorescens*			*P. putida*		*P. aeruginosa*		*P. entomophila*	
	tomato	syringae	phaseolicola	tabaci									
Amino Acid	**DC3000**	**B728a**	**1448A**	**11528**	**Pf-5**	**PfO-1**	**SBW25**	**KT2440**	**F1**	**PA14**	**PAO1**	**L48**	**Key**
Alanine	w	w	w	w	+	+	+	+	+	+	w	+	☆
Arginine	+	+	+	+	+	+	+	+	+	+	+	+	
Asparagine	+	+	+	+	+	+	+	+	+	+	+	+	
Aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+	
Glutamine	+	+	+	+	+	+	+	+	+	+	+	+	
Glycine	–	w	–	+	+	w	+	+	+	+	+	+	—
Histidine	w	+	+	+	+	+	w	+	+	+	+	+	
Isoleucine	–	–	w	+	+	+	+	+	+	+	+	+	—
Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	
Leucine	w	–	w	w	+	w	+	+	+	+	w	+	☆
Cysteine	–	–	w	–	+	–	+	–	+	+	w	+	☆
Lysine	w	–	–	w	+	w	w	+	+	+	w	+	☆
Methionine	w	–	–	w	w	w	+	+	+	w	–	+	☆
Phenylalanine	w	–	w	w	+	+	+	+	+	+	+	+	☆☆
Proline	+	+	w	+	+	+	+	+	+	+	+	+	
Serine	+	+	w	+	+	+	+	+	+	+	+	+	
Threonine	–	w	w	+	+	w	+	+	+	+	+	+	—
Tryptophan	–	–	w	w	+	–	+	–	+	+	–	–	☆
Valine	–	–	–	+	+	+	+	+	+	+	w	+	—
GABA	+	+	+	+	+	+	+	+	+	+	+	+	

Table 4.1

Amino acid metabolism as a nitrogen source in Biolog GN2 plates after 24 hours by 12 *Pseudomonas* strains. Results are scored as follows : + = positive result (OD_{620} > 0.2), w = weak result (OD_{620} 0.2-0.05), ~ = negative result (OD_{620} < 0.05). Data reproduced from Rico and Preston 2008. Differences are highlighted according to the following scale (Key) **= Negative or weak in all *P. syringae* and positive in all other *Pseudomonas*; *= Negative or weak result in all *P. syringae* strains and positive in at least 50 % of other *Pseudomonas*; — = negative or weak in all except one *P. syringae* strains and positive in at least 50 % of other *Pseudomonas*.

	P. syringae pathovars				*P. fluorescens*			*P. putida*		*P. aeruginosa*		*P. entomophila*	
	tomato	syringae	phaseolicola	tabaci									
Amino Acid	**DC3000**	**B728a**	**1448A**	**11528**	**Pf-5**	**PfO-1**	**SBW25**	**KT2440**	**F1**	**PA14**	**PAO1**	**L48**	**Key**
Alanine	+	+	w	+	w	+	+	+	+	w	w	+	
Arginine	w	w	w	+	+	+	+	+	+	+	+	+	—
Asparagine	+	+	+	+	+	+	+	+	+	+	+	+	
Aspartic acid	+	+	+	+	+	+	+	+	+	w	+	+	
Glutamine	+	+	+	+	+	+	+	+	+	+	+	+	
Glycine	–	w	–	–	–	+	–	–	w	–	w	+	
Histidine	w	+	+	+	+	+	+	+	+	+	+	+	
Isoleucine	–	–	w	w	–	+	+	+	+	–	w	+	☆
Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	
Leucine	w	w	–	w	+	+	w	+	w	–	w	+	☆
Lysine	–	w	–	–	–	w	w	+	+	–	w	+	
Methionine	–	w	–	–	–	w	w	–		–	–	–	
Phenylalanine	w	w	–	w	–	w	w	–	w	–	–	w	
Proline	+	+	w	+	+	+	+	+	+	+	+	+	
Serine	+	+	w	+	+	+	+	+	+	–	w	+	
Threonine	–	w	w	–	–	w	w	–	–	–	w	w	
Tryptophan	–	–	w	–	+	–	w	–	–	–	w	–	
Valine	–	w	w	–	+	+	+	+	+	–	w	+	☆
GABA	+	+	+	+	+	+	+	+	+	+	+	+	

Table 4.2

Amino acid metabolism as a carbon source in Biolog GN2 plates after 24 hours by 12 *Pseudomonas* strains. Results are scored as follows: + = positive result (OD_{620} > 0.2), w = weak result (OD_{620} 0.2-0.05), ~ = negative result (OD_{620} < 0.05). Data reproduced from Rico and Preston 2008. Differences are highlighted according to the following scale (Key) *= Negative or weak result in all *P. syringae* strains and positive in at least 50 % of other *Pseudomonas*; — = negative or weak in all except one *P. syringae* strains and positive in at least 50 % of other *Pseudomonas*.

Rico and Preston (2008) compared the metabolism of 19 amino acids by four *P. syringae* strains to their metabolism by *P. fluorescens*, *P. aeruginosa*, *P. putida* and *P. entomophila* species. In contrast to non plant-pathogenic *Pseudomonas* species, *P. syringae* was only capable of metabolising a restricted number of amino acids as sole sources of carbon or nitrogen, indicating that assimilation pathways for these amino acids as carbon or nitrogen sources were not present. Five amino acids; namely asparagine, aspartic acid, glutamine, glutamic acid and GABA, were strongly metabolised by all tested *P. syringae* pathovars as carbon or nitrogen sources (Table 4.1 and Table 4.2). Interestingly, these amino acids are also the most abundant in the tomato leaf apoplast (Rico and Preston, 2008) and have been detected in the apoplast of other plant species (Chapter 1, Table 1.3). Although Biolog plate-based assays can be used to detect metabolism of a compound as a carbon or nitrogen source, this does not necessarily mean that these compounds could be used by bacteria as a carbon or nitrogen source for growth. This is because the Biolog assay does not require bacteria to grow using the test compound, but rather measures metabolism of that compound. Therefore, one area of further study is to investigate whether *P. syringae* is also only capable of using a restricted number of amino acids for growth compared to non plant-pathogenic *Pseudomonas*, and whether there is a correlation between the amino acids that *P. syringae* can use for growth and those which are abundant in the apoplast.

Rico and Preston (2008) also demonstrated that different nutrient assimilation pathways are activated when *P. syringae* is incubated in extracted tomato apoplast compared to synthetic LB (rich) or *hrp*-inducing (minimal) media (HIM) (Rico and Preston, 2008). This study highlighted the choice of growth media as an important consideration for nutrient utilisation studies. HIM is widely believed to mimic the apoplast environment due to being both nutrient poor and acidic (pH 5.5). In particular, HIM has provided a useful tool for *in vitro* expression studies of *hrp* regulons as this media induces the type three secretion system (T3SS) (Lindgren, 1997). However, there has been no attempt to further mimic the nutrient composition of the apoplast environment by supplementing a synthetic, nutrient poor media such as HIM with appropriate apoplastic amino acids, organic acids or sugars. In addition, the growth of *P. syringae* in HIM is poor compared to growth in extracted apoplast, indicating that HIM lacks nutrients that are available to the bacteria *in planta* (Appendix Figure A1).

P. syringae has been shown to grow faster, reach a higher overall density and activate different nutritional pathways when cultured in extracted apoplast compared to synthetic media such as HIM (Appendix Figure A1 and Rico and Preston,. 2008). This suggests

that to closely model *in planta* nutrient assimilation and associated gene expression extracted apoplast should be used. However, the time taken to extract apoplast from plants is by no means trivial and a large number of plants are required to extract even a few millilitres of apoplast. Therefore a 'synthetic' apoplast would be a quick, inexpensive and useful proxy in which to study the growth of *P. syringae*. In addition, a synthetic apoplast could be used to explore the relationship between amino acid abundance and growth by artificially modifying the abundance of various components.

Several synthetic growth media comprising a common salt, sugar and vitamin composition supplemented with different amino acids have previously been designed to study the *in vitro* growth of the xylem-limited phytopathogen *Xylella fastidiosa* (Lemos et al., 2003). Here, genetic analysis was used to identify amino acids for which complete degradation pathways occur in the *X. fastidiosa* genome, highlighting these as suitable components for use in a synthetic media. The most effective media for growth of *X. fastidiosa* contained two of the most abundant amino acids in xylem sap; namely asparagine and glutamine, for which complete degradation pathways are present in *X. fastidiosa* (Lemos et al., 2003).

In addition to the tomato apoplast, amino acid abundance data is available for the *A. thaliana* leaf apoplast (Guillaume Pilot, personal communication) and guttation fluid, (Pilot et al., 2004). Both tomato and *A. thaliana* are hosts of *P. syringae* pv. tomato DC3000 (P.s.t) and P.s.t is able to utilitse extracted tomato apoplast as a complete medium for growth (Rico and Preston, 2008). Therefore, these data could be used in the design of a new synthetic growth media for *P. syringae*, designed to replicate the nutrients encountered during infection of, and growth within plants.

The apoplast is also known to provide a relatively constant acidic environment, commonly between pH 5 and pH 6.5 in uninfected plants, although this is known to become progressively more alkaline during infection (Grignon and Sentenac, 1991; Tetlow and Farrar, 1993). Previous work has indicated that *P. syringae* shows a decreased ability to grow in extremely acidic or basic conditions not found in plants, compared to non-plant-pathogenic *Pseudomonas*, and also that the pH of the growth media may affect the ability of *P. syringae* to utilise certain compounds *in vitro* (Stuart King, unpublished data). Therefore, specialisation for growth and nutrient assimilation in a restricted range of acidic or alkaline environments may represent another way in which *P.syringae* has become adapted to the plant apoplast. The effect of pH on *P. syringae* growth can be investigated in more detail; in addition it will be interesting to assess the

effect of pH on nutrient assimilation as bioinformatic studies in Chapter 3 suggested that APC transporters, which function using electrochemical potential energy to drive transport (and therefore may be effected by pH), are significantly reduced in *P. syringae* compared to non plant-pathogenic *Pseudomonas.*

4.3 Objectives.

- Profile the ability of *P. syringae* pv. tomato DC3000 (P.s.t) to utilise amino acids as sole carbon or nitrogen sources for growth, and compare this information to the Biolog data described by Rico and Preston (2008) on the metabolism of these amino acids; to the data describing the abundant amino acids in the tomato apoplast; and to the predicted function of aa_permeases in *P. syringae*.
- Create synthetic tomato apoplast, synthetic *A. thaliana* apoplast and synthetic *A.thaliana* guttation fluid, based on the available information regarding the amino acids, organic acids and sugars present in these locations.
- Profile the ability of P.s.t to grow in synthetic guttation fluid and synthetic *A.thaliana* and tomato apoplast, compared to growth in extracted tomato apoplast.

4.4 Materials and methods.

4.4.1 Bacterial strains.

Bacterial strains were used as described in Chapter 2.

4.4.2 Growth *in vitro*.

All strains were routinely cultured on LB agar medium at 28°C as described in Chapter 2.2. Overnight cultures were washed twice in the media of interest and resuspended at a starting density of $1x10^5$ cfu ml^{-1} unless otherwise stated.

- **pH**

To profile the growth of bacteria at a range of pH values, dilute sodium hydroxide or hydrochloric acid were added to the media to achieve the desired pH value. For synthetic apoplast or M9 minimal media, dilute sodium hydroxide or hydrochloric acid were added to the fully assembled media, which was then filter sterilised using a sterile 0.2 µm Whatman syringe filter (Whatman Plc, UK) attached to a sterile 20 ml syringe (Fisher, UK). The pH of the media was measured using a Jenway 3510 pH meter (Bibby Scientific Ltd T/As Jenway, UK).

4.4.3 Phenoarrays.

- **Custom carbon and nitrogen phenoarrays**

To assess the ability of P.s.t, Pf-5 and PAO1 to utilise amino acids as carbon, nitrogen or carbon and nitrogen sources for growth, strains were grown in M9 minimal media with an amino acid replacing glucose (the carbon source), ammonia (the nitrogen source) or both glucose and ammonia. Amino acid solutions were made up at 40mM (double the final concentration) in distilled H_2O (dH_2O) and adjusted to pH7 using dilute sodium hydroxide or hydrochloric acid as appropriate. The pH was measured using a Jenway 3510 pH meter (Bibby Scientific Ltd T/As Jenway, UK). Amino acid solutions were filter sterilised using a 0.2µm Whatman syringe filter (Whatman Plc, UK) attached to a sterile 20 ml syringe (Fisher, UK). Bacterial strains were grown overnight in LB at 28°C, washed twice and resuspended at $2x10^5$ cfu ml^{-1} in double strength M9 media lacking the appropriate carbon, nitrogen or carbon and nitrogen source. Seventy five microlitre aliquots of bacterial suspension were inoculated into each well of a 96 well plate using a multichannel pipettor. Seventy five microlitre aliquots of amino acid solution were then inoculated into the same plate resulting in final concentrations of 20 mM amino acid, $1x10^5$ cfu ml^{-1} bacteria and 1xM9 media. Plates were incubated for 24 hours at 28°C with shaking and the OD_{590} of each plate was then read using a microplate reader (MRXSpec; Dynatech Laboratory). A control well of sterile H_2O in place of the amino acid was included in each plate and the value of this well was subtracted from the data prior to analysis.

- **Biolog assays – carbon source utilisation after growth in synthetic tomato apoplast or LB.**

Biolog GN2 plates were used to assess the ability of P.s.t to metabolise a range of compounds as carbon sources. GN2 plates comprise 95 wells containing different compounds and a control well. If bacteria are able to metabolise the compound, a redox dye (tetrazolium violet) is reduced, changing from clear to purple (Figure 4.1). GN2 plates were inoculated according to the manufacturer's instructions (BIOLOG, Hayward, CA, USA) with slight modifications. Bacteria were grown overnight in STA at 28°C with shaking, washed twice in inoculating fluid (IF) (BIOLOG) and subsequently resuspended in IF to a final OD_{620} of 0.28. At this stage tetracycline was added at a final concentration of 12.5 µg / $µl^{-1}$ to prevent further protein synthesis, according to the method described in Rico and Preston (2008). One hundred and fifty microlitre aliquots of bacteria suspended in IF were inoculated into each well using a multichannel pipettor. Plates were sealed with Parafilm (Fisher, UK) to prevent evaporation and incubated statically at 28°C for 24 hours. The OD_{620} of each plate was then read using a microplate reader (MRXSpec; Dynatech Laboratory). When analysing data the value of the control well, which

contained no carbon or nitrogen source and was used as a measure of residual metabolism, was subtracted from each result.

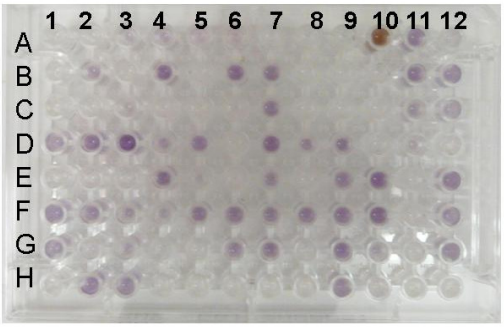


Figure 4.1
Example output of a Biolog GN2 plate after incubation with P.s.t. Purple colour indicates positive metabolism of the carbon source. Well A1 is a control.

- **Biolog assays – carbon source utilisation after growth in synthetic tomato apoplast at pH 6 compared to pH 7**

Biolog GN2 plates were also used to assess the ability of P.s.t to use a range of compounds as carbon sources following a pre treatment in synthetic tomato apoplast (STA) at either pH 6 or pH 7. Bacteria were grown overnight at 28°C in STA (~pH 6.5) with shaking. Subsequently, 1.5 ml aliquots of bacteria were washed twice with STA at pH 6 or pH 7 before being resuspended in 2ml of STA at pH 6 or pH 7. Bacteria were then incubated in STA with shaking for 2 hours at 28°C. Bacteria were then washed twice in IF and subsequently resuspended in IF to a final OD_{620} of 0.28. Bacteria were then added to GN2 plates as before. For inhibition treatments tetracycline was added to IF at a final concentration of 12.5µg/ μl^{-1} following the 2 hour pre-treatment period and prior to inoculation into GN2 plates.

4.4.4 Extraction of tomato apoplast fluid and estimation of apoplast dilution.

Apoplastic fluid was extracted from uninfected tomato plants using the vacuum infiltration method as described in Chapter 2, Section 2.7.1. The average apoplast dilution for tomato leaves was calculated from five independent experiments as 2.5±0.40. Therefore freeze-dried apoplast was resuspended in dH_2O using the formula:

$$V_{orig} = V_{ex}/2.5$$

Where V_{orig} is the 'full strength' apoplast and V_{ex} is the amount of apoplast extracted from the infiltrated tomato leaves.

4.5 Results.

4.5.1 *P. syringae* can utilise fewer amino acids for growth than *P. aeruginosa* or *P. fluorescens*.

P. syringae has long since been regarded as a relatively specialist species based on phenotypic tests which include the ability to utilise various nutrients (Palleroni, 1984). Rico and Preston (2008) previously used Biolog GN2 plates to profile the ability of 12 *Pseudomonas* to metabolise amino acids as carbon or nitrogen sources. Positive results from Biolog GN2 plates are indicative of amino acid metabolism but not necessarily of the ability of *Pseudomonas* to use that amino acid as a sole carbon or nitrogen source for growth. Therefore, the growth of three representative *Pseudomonas* species; *P. syringae* pv. tomato DC3000 (P.s.t), *P. fluorescens* Pf5 (Pf5) and *P. aeruginosa* PAO1 (PAO1) was determined for the19 amino acids used in the assays of Rico and Preston (2008). Ethanolamine was also included as this is the putatively transported substrate of the P.s.t aa_permease: PSPTO_2026.

Growth assays compared the ability of P.s.t, Pf-5 and PAO1 to utilise amino acids as a sole carbon, nitrogen, or combined carbon and nitrogen source for growth in M9 minimal media. Complete M9 minimal media contained glucose and ammonia as a carbon and nitrogen source, respectively. For carbon source utilisation assays, M9 media was supplemented with 20 mM amino acid instead of glucose; for nitrogen source utilisation assays an amino acid was used in place of ammonia; and for carbon and nitrogen source utilisation assays the amino acid replaced both glucose and ammonia.

P.s.t can use fewer types of amino acids for growth than Pf-5 and PAO1 (Table 4.4). P.s.t is capable of utilising 10 of the tested amino acids as a sole carbon source, 9 as a sole nitrogen source and 6 as a combined carbon and nitrogen source. In comparison PAO1 is capable of using 16 of the tested amino acids as a sole carbon source, 17 as a sole nitrogen source and 10 as a combined carbon and nitrogen source while Pf-5 can use 16, 19 and 16 amino acids, respectively. Comparing these results (Table 4.4) with Tables 4.1 and 4.2 indicated that P.s.t can metabolise more amino acids than it can use for growth. This suggested that the ability to metabolise an amino acid as a sole carbon or nitrogen source, did not necessarily correlate with the ability to use this amino acid for growth. One particular example for P.s.t is histidine, which is metabolised as a sole carbon or sole nitrogen source but does not support growth as a sole carbon, nitrogen or combined carbon and nitrogen source.

	P. syringae pv. tomato DC3000			*P. fluorescens* Pf-5			*P. aeruginosa* PAO1		
Amino Acid	**C**	**N**	**C / N**	**C**	**N**	**C / N**	**C**	**N**	**C / N**
Alanine	+	w	–	+	+	w	+	+	+
Arginine	w	w	–	–	+	w	+	+	+
Asparagine	+	+	+	w	+	+	+	+	+
Aspartic acid	+	+	+	+	+	+	+	+	+
Glutamine	+	+	+	+	+	+	+	+	+
Glycine	–	–	–	w	+	–	w	w	–
Histidine	–	–	–	+	+	+	+	+	+
Isoleucine	–	w	–	+	+	+	w	w	–
Glutamic acid	+	+	+	+	+	+	+	+	+
Leucine	–	–	–	w	w	–	w	–	–
Cysteine	–	–	–	w	–	w	–	w	–
Lysine	–	–	–	–	w	w	w	–	–
Methionine	w	–	–	w	w	w	–	–	–
Phenylalanine	–	w	–	+	w	w	–	w	–
Proline	+	w	–	+	w	+	+	+	+
Serine	+	+	+	w	+	+	w	w	w
Threonine	–	–	–	–	w	–	–	w	–
Tryptophan	–	–	–	w	+	w	w	w	–
Valine	–	–	–	w	w	–	w	w	–
GABA	+	+	+	+	+	+	+	+	+

Table 4.4

Growth (measured by optical density OD_{590} after 24 hours) by P.s.t, Pf-5 and PAO1 using 20 mM amino acids as a carbon, nitrogen or carbon and nitrogen source in M9 minimal media with the amino acid replacing either glucose, ammonia or both glucose and ammonia. Results are scores as follows: + = good growth (OD_{590}>0.8); w = weak growth (OD_{590}0.1-0.6); – = no growth (OD_{590}<0.1).

4.5.2 pH affects the growth of *P. syringae* more than *P. fluorescens* or *P. aeruginosa*.

The ability to survive in a range of acidic and alkaline conditions has been reported in generalist *Pseudomonads* that inhabit heterogeneous environments, such as *P. aeruginosa*. In contrast the leaf apoplast colonised by *P. syringae* is a relatively constant environment with an acidic pH commonly ranging between 5 and 6.5 (Grignon and Sentenac, 1991), although this is known to become more alkaline when infected with fungal or bacterial phytopathogens (Tetlow and Farrar, 1993; Nachin and Barras, 2000)

In order to investigate the ability of *P. syringae* species to grow in acidic and alkaline conditions compared with non-plant-pathogenic *Pseudomonas,* bacteria were grown in LB media at pH 4.5, pH 5, pH 5.5, pH 7, pH 8, pH 9 or pH 10. 'Good' growth was measured as an OD_{590} after 24 hours of more than 80 % of the highest OD_{590} reached at any pH. Growth of the *P. syringae* pathovars: *P. syringae* pv. tomato DC3000 (P.s.t); *P. syringae* pv. phaseolicola 1448A (P.s.ph) and *P. syringae* pv. syringae B728a (P.s.s), was compared to the growth of the generalist *Pseudomonas* species *P. fluorescens* SBW25 (P. fl SBW25) and *P. aeruginosa* PAO1 (PAO1).

The growth of all three *P. syringae* pathovars was inhibited in media below pH 5 and above pH 8 (Figure 4.2). In comparison P. fl. SBW25 and PAO1 were capable of growth at all acidic pH values tested and alkaline conditions up to pH 10 (Figure 4.3).

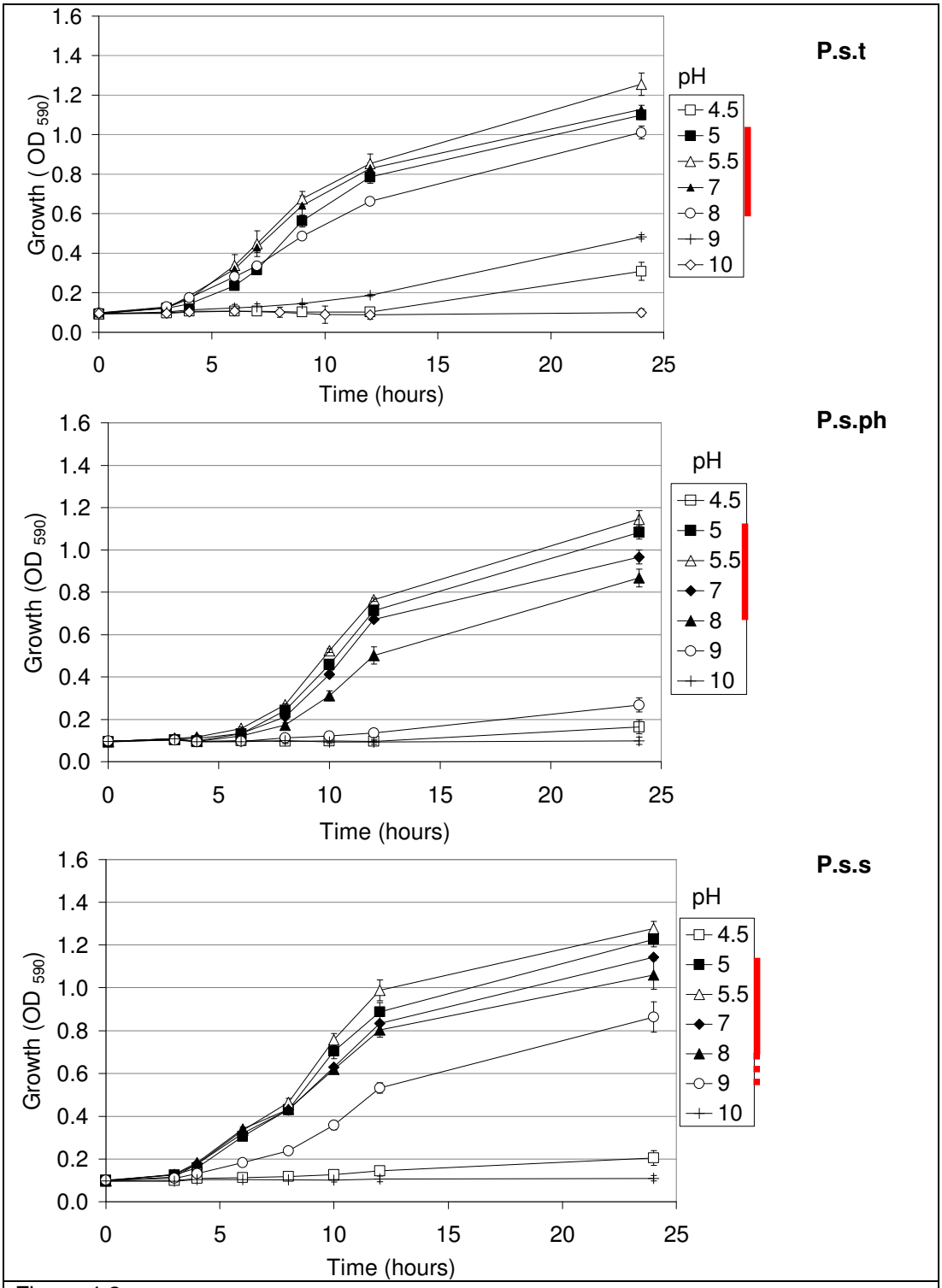


Figure 4.2
Growth *of P. syringae* pathovars P.s.t, P.s.ph and P.s.s in LB media at a range of pH values between pH 4.5 and pH 10. The range of pH values at which each strain grows well (measured as a final optical density ≥ 80% of the highest OD_{590} reached at any pH) are shown by a red line. pH values which result in a final OD_{590} that is 50-80% of the maximum, is shown by a dashed red line. Error bars represent standard deviations from three replicates. Experiments were repeated twice with similar results.

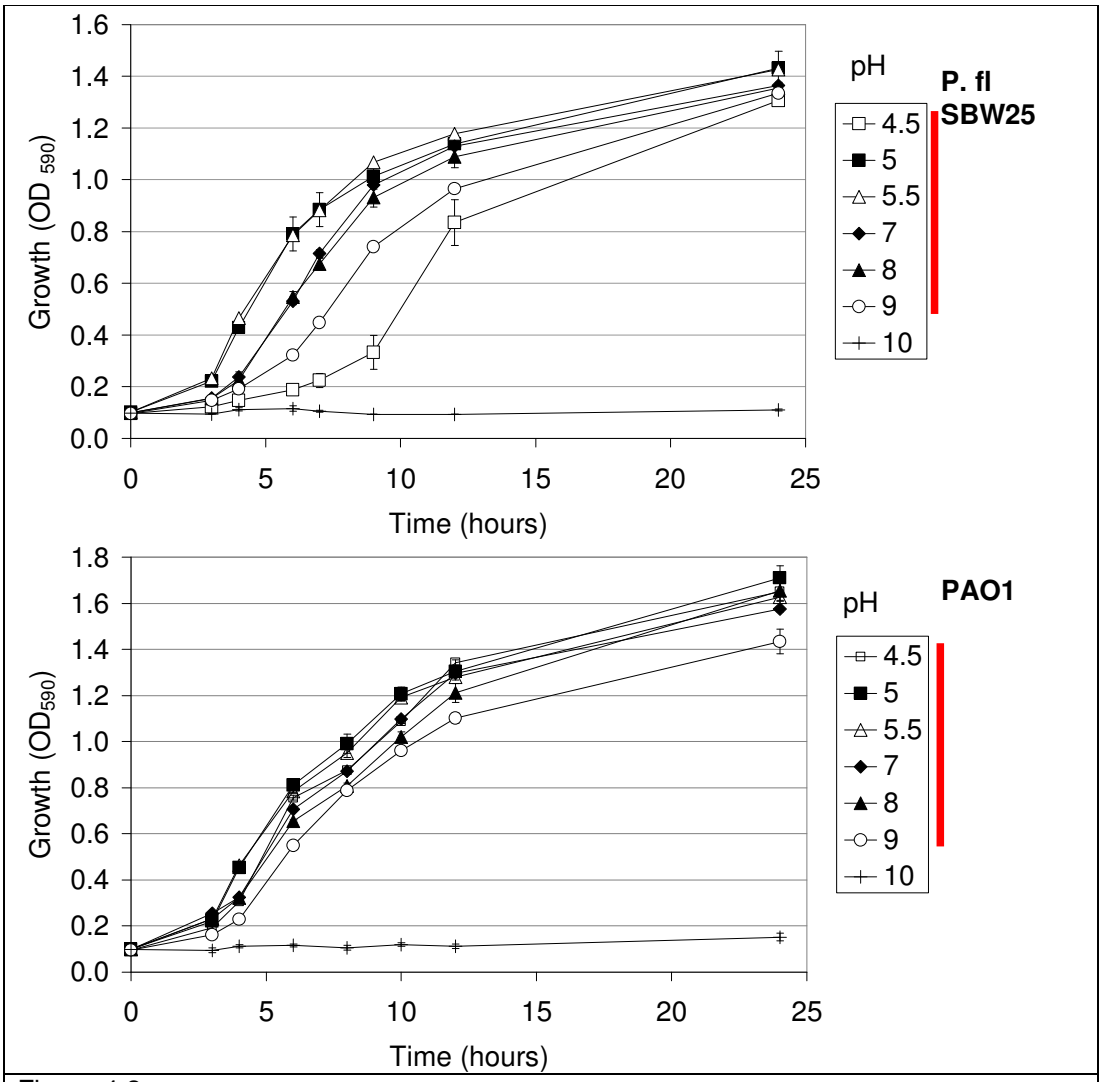


Figure 4.3
Growth of P.fl SBW25 and PAO1 in LB from pH 4.5 to pH 10. The range of pH values at which each strain grows well (measured as a final optical density ≥ 80% of the highest OD_{590} reached at any pH) are shown by a red line. Error bars represent standard deviations from three replicates. Experiments were repeated twice with similar results.

4.5.3 The pH of unbuffered LB media varied over time and appeared to be correlated with growth of *Pseudomonas.*

The plant apoplast is known to become progressively more alkaline during *P. syringae* pathogenesis (Alfano and Collmer, 1996), and alkalinisation of growth media has been widely reported during *in vitro* growth of *Pseudomonas* species including *P. syringae* (Bultreys and Gheysen, 2000) *P. putida* (Romano and Kolter, 2005), and *P. aeruginosa* (Yates et al., 2002).

In order to investigate whether alkalinisation of growth media is a consequence of bacterial growth, such as the production of waste products, the pH of unbuffered LB media was monitored over 24 hours during the growth of three *P. syringae* pathovars: P.s.t; P.s.ph; P.s.s, and two representative non-plant pathogens, P. fl SBW25 and PAO1.

The growth of all tested *Pseudomonas* in unbuffered LB caused an initial acidification of the growth media, followed by an increase in alkalinity over the next 24 hours (Figure 4.4). The growth of PAO1 resulted in the largest increase in alkalinity, however this species also reached the highest optical density. When the differences in growth were taken into account, no *Pseudomonas* species significantly alkalinised the growth media more than any other over the course of the experiment (Figure 4.5). Therefore, both plant-pathogenic and non plant-pathogenic *Pseudomonas* alkalinise the culture media during growth. Interestingly, during the earliest time point (0-2 hours) all *Pseudomonas* species acidify the growth media, with P.s.t acidifying the growth media significantly more than any other species (Figure 4.5). This may indicate that during early growth uptake of compounds in the media by *Pseudomonas* exceeds excreted compounds, temporarily acidifying the media.

Based on this result, we can speculate that during growth in the plant apoplast *P. syringae* may be contributing to the overall alkalinisation of the apoplast which is observed. But alkalinisation of the growth media does not appear to be an effect which is specific to plant-pathogenic *Pseudomonas*.

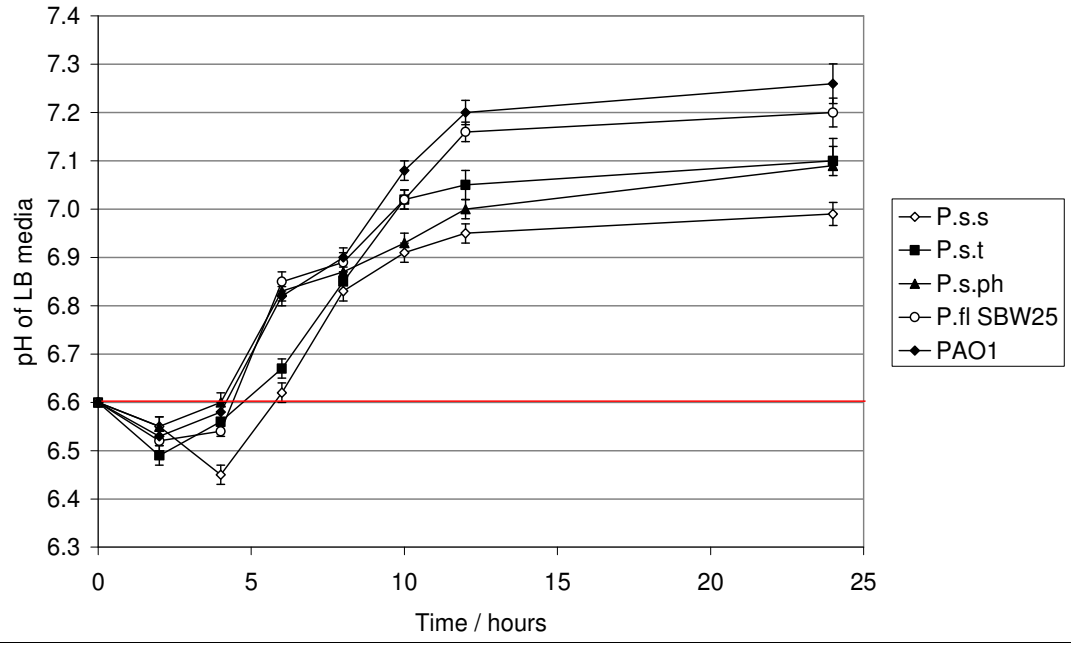


Figure 4.4
Pseudomonas growing in unbuffered LB media (starting pH 6.6 – indicated by red line). The media becomes briefly more acidic before it increases in alkalinity over time. Error bars are standard deviations from three replicate cultures. Experiments were repeated twice with similar results.

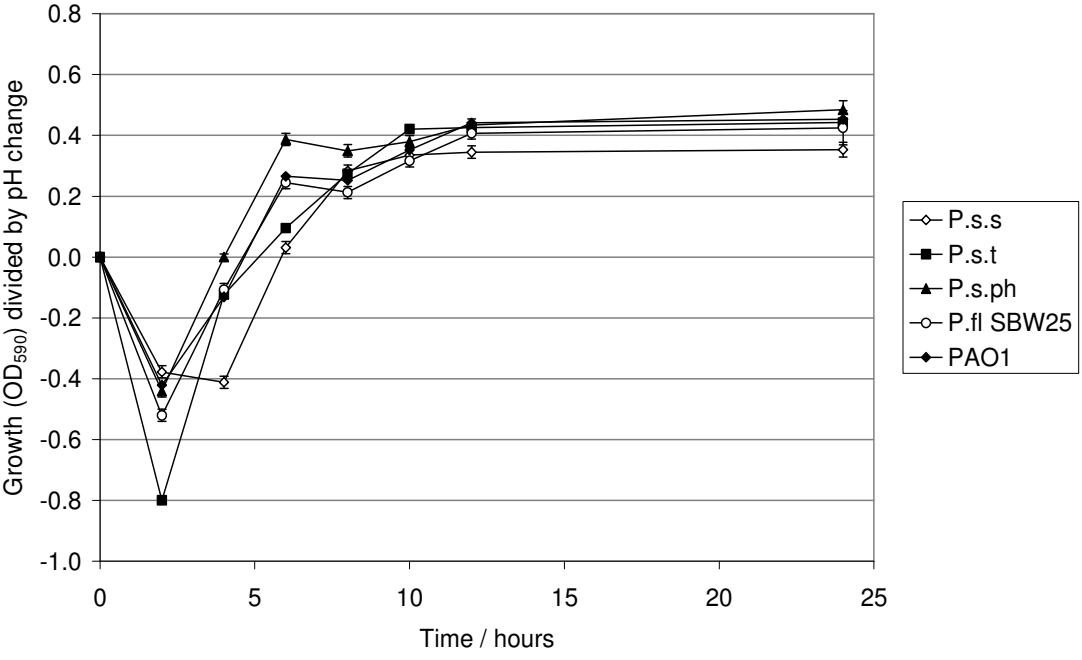


Figure 4.5
PAO1 makes the media the most alkaline, however it also grows more. Dividing growth (OD_{590}) by the change in pH at each time point reveals that PAO1 does not alter the overall pH significantly more or less than *P. syringae*. Error bars are standard deviations from three replicate cultures. Experiments were repeated twice with similar results.

4.5.4 Some amino acids significantly increased the growth of *Pseudomonas syringae* pv. tomato, but this was not correlated with their abundance in tomato apoplast.

P. syringae pv. tomato (P.s.t) was unable to use certain amino acids to support growth (Table 4.4). For example, P.s.t did not grow when histidine and threonine were provided as sole nitrogen sources, while leucine, histidine and phenylalanine could not be utilised for growth as sole nitrogen sources. However positive metabolism results for these amino acids in Biolog assays (Table 4.1 and 4.2) indicated that P.s.t may be able to benefit from the metabolism of some of these amino acids in an environment supplemented with alternative sources of carbon and nitrogen.

The addition of 0.5 mM of 11 amino acids; glutamic acid, serine, glutamine, aspartic acid, GABA, asparagine, proline, alanine, arginine, leucine or histidine to M9 minimal media (where glucose and ammonia provided the carbon and nitrogen sources, respectively) significantly increased the growth of P.s.t compared to the growth in M9 without any amino acid supplement (Figure 4.6 A). In particular, this result demonstrated that although histidine and leucine were not utilised as carbon or nitrogen sources for growth, these amino acids could increase the growth of P.s.t when alternative carbon and nitrogen sources were available. In addition to growth benefits to P.s.t from metabolising these amino acids, this may also be because P.s.t could be using histidine and leucine directly as amino acid precursors for protein synthesis.

The addition of 0.5 mM glycine, phenylalanine, valine, isoleucine, cysteine, tryptophan, threonine, lysine or methionine to M9 media did not significantly increase the growth of P.s.t compared to M9 without an amino acid supplement (Figure 4.6 A).

Rico and Preston (2008) reported that P.s.t predominantly metabolised amino acids which are abundant in the host tomato apoplast. These results demonstrate that many of the abundant tomato apoplast amino acids also significantly increase the growth of P.s.t (Figure 4.6 A). However, the apoplastic amino acids differ in abundance, while these experiments used a fixed value of 0.5mM. Therefore, the growth increase and abundance of each amino acid were tested for correlation, in order to investigate whether P.s.t gains the largest growth increase from amino acids which are most abundant in the tomato apoplast (Figure 4.6 B). There is not a strong correlation between the growth increase afforded by each amino acid and its abundance in the tomato apoplast.

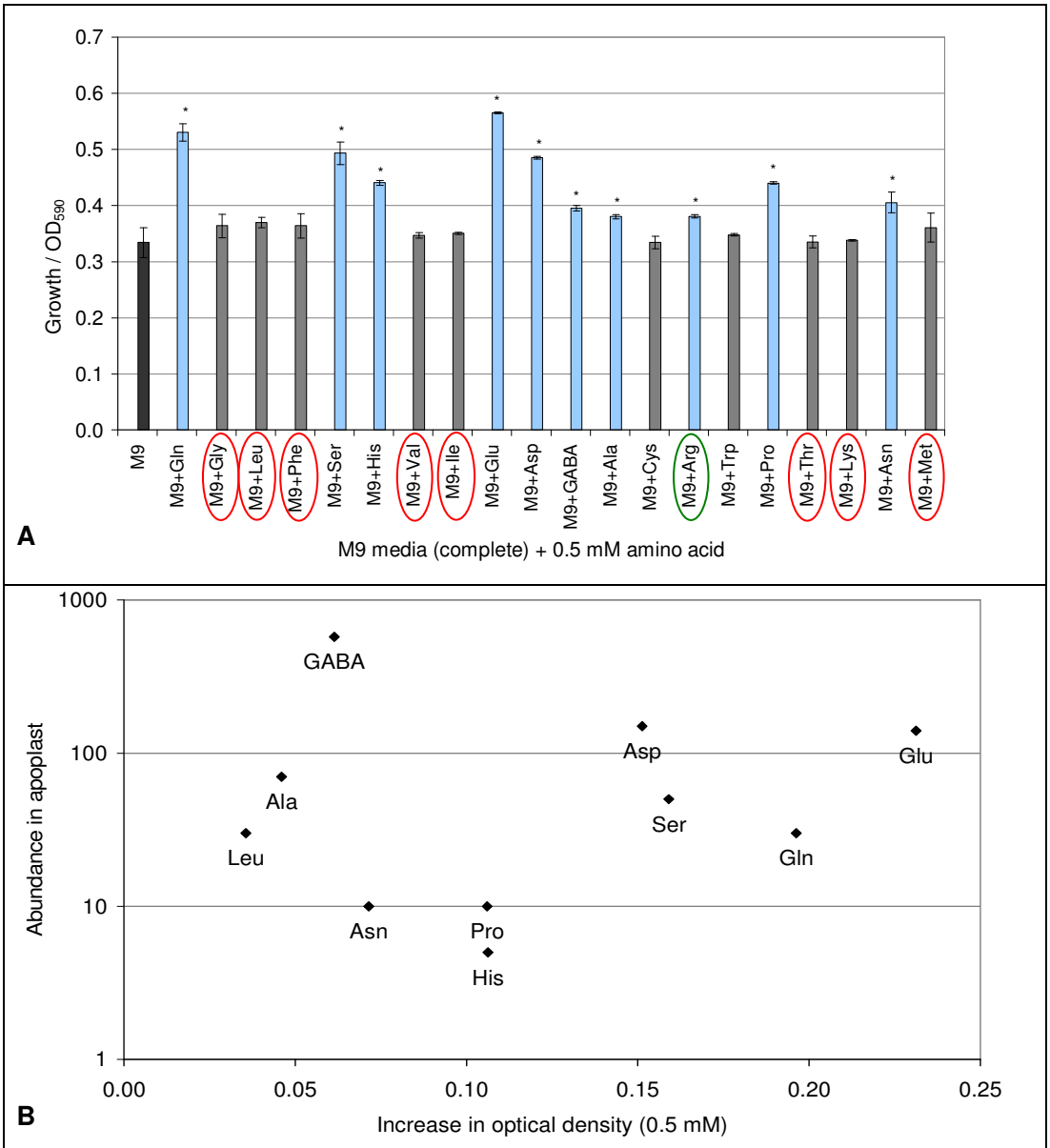


Figure 4.6

A. Growth of P.s.t at 24 hours in complete M9 minimal media (with NH_4 and glucose) supplemented with 0.5 mM amino acid. Error bars indicate the standard deviation of three replicate experiments. Amino acids that significantly increased growth are highlighted in blue *=Growth is significantly increased compared to M9 $p \leq 0.05$. Student's 2-tailed t-tests, H_0= no difference between M9 and M9+amino acid. Ten degrees of freedom. Amino acids which are present in the tomato apoplast, but do not significantly increase growth of P.s.t at 0.5 mM are circled in red. Amino acids which significantly increase the growth of P.s.t, but are not present in the apoplast are circled in green

B. Growth increase provided by amino acids compared with the abundance of each amino acid in the tomato apoplast. Abundance is shown on a log scale to clarify data points. Growth increase is measured as the increase in optical density (OD_{590}) when bacteria are grown in complete M9 media supplemented with 0.5 mM of amino acid, compared to complete M9 media not supplemented with an amino acid.

4.5.5 *Pseudomonas syringae* pv. tomato can only derive a growth benefit from certain amino acids in acidic conditions.

The plant apoplast encountered is an acidic environment which becomes more alkaline as pathogenesis progresses. Within the plant, regulation of apoplastic pH is important due to the physiological effects of altered pH on many important processes, including the efficiency of proton-driven membrane transporters (Grignon and Sentenac, 1991). The aa_permease transporters belong to the APC superfamily. These transporters are driven by a variety of ATP-independent mechanisms including proton-driven symport or antiport, which may be affected by changes in external pH.

P. syringae obtained a growth 'boost' when cultured in complete M9 minimal medium supplemented with some amino acids (Figure 4.6 A). In addition *P. syringae* grew similarly well over 24 hours in mildly acidic (pH5.5) and alkaline (pH 8) conditions (Figure 4.2) which are known to occur in the plant apoplast during pathogenesis (Grignon and Sentenac, 1991). As amino acid uptake occurs via membrane bound transporters that may be affected by pH changes, it was interesting to compare whether the growth boost provided by the addition of amino acids to M9 medium, was affected in acidic (pH 5.5) and alkaline (pH 8) media.

Complete M9 media was supplemented with 20 mM amino acids as before. Ten millimolar HEPES was added to each M9-amino acid solution and adjusted to pH 5.5 or pH 8. Growth was compared after 24 h (Table4.5). The growth increase to P.s.t of several amino acids was reduced at pH 8, with the biggest disparity between the growth boost at pH 5.5 and pH 8 seen for several of the most abundant apoplastic amino acids. The amino acids which were affected by pH included the acidic amino acids glutamic acid and aspartic acid. In contrast, the growth boost provided by arginine (not found in the tomato apoplast) or asparagine was not affected by pH. *P. syringae* contains many amino acid transporters, driven by many different processes, not all of which are expected to be influenced by external pH. However, these results suggest that the majority of transporters for glutamic acid and aspartic acid are driven by mechanisms which are affected by pH, while transporters for alanine and arginine may include at least some which are not influenced by external pH. In addition, the pH of the media may also be affecting the ionic form of the carboxyl group of particular amino acids (between COO- [alkaline] and COOH [acid]) which may affect their transport.

Amino Acid	Abundance in apoplast	P.s.t pH 5.5	P.s.t. pH 8.0	
Alanine	***	3	1	*
Arginine		3	3	
Asparagine	*	3	3	
Aspartic acid	***	4	1	*
Glutamine	**	4	3	
Glycine	**	2	1	
Histidine	*	3	2	
Isoleucine	**	1	1	
Glutamic acid	***	4	2	*
Leucine	**	2	1	
Cysteine		1	1	
Lysine	**	1	1	
Methionine	**	1	1	
Phenylalanine	**	1	1	
Proline	*	3	1	*
Serine	***	4	2	*
Threonine	**	1	1	
Tryptophan		1	1	
Valine	**	1	1	
GABA	***	3	2	

Table 4.5
Growth of P.s.t. in complete M9 minimal media (with both ammonium salts and glucose), supplemented with 20 mM of amino acids at pH 5.5 or pH 8. Numbers indicate growth in M9 supplemented with amino acid (measured by optical density OD_{590}) after 24 hours, in relation to growth in complete M9 at the same pH, without any amino acid supplement. 1=OD_{590} not significantly different to M9, 2= OD_{590} increased by up to 0.1, 3= OD_{590} increased by 0.1-0.2, 4= OD_{590} increased by ≥ 0.2. Results are representative of three independent replicates with similar results. Amino acids marked with an asterix in the final column show the biggest disparity in the growth boost they provide at pH 5.5 and pH 8.

4.5.6 Development of synthetic apoplast and synthetic guttation fluid as a growth medium for *Pseudomonas syringae*.

The plant apoplast is an acidic, nitrogen-limited environment containing plant-derived sugars, organic acids and amino acids (Alfano and Collmer, 1996). A recent paper demonstrated that different nutrient assimilation pathways were activated when *P.syringae* was incubated in tomato apoplast compared to LB or *hrp*-inducing minimal media (HIM) (Rico and Preston, 2008). This result suggested that LB and HIM do not accurately mimic nutrient conditions in the apoplast, and in order to study nutrient assimilation mechanisms extracted apoplast should be used. However, as the apoplast extraction process is both time and labour intensive, a 'synthetic' apoplast containing the major sugars, organic and amino acids would be a useful proxy. Other plant-associated environments could also be modeled in this way. *P. syringae* enters the plant apoplast through wounds or natural openings such as hydathodes. Hydathodes exude a liquid called guttation fluid through pores in the epidermis or margin of leaves; by simulating guttation fluid it may be possible to ascertain whether *P. syringae* exhibits chemotaxis to any of the predominant amino acids and hypothesise what role this behavior might play in infection through hydathode openings.

Three synthetic media were created: synthetic tomato and *A. thaliana* apoplast and *A. thaliana* guttation fluid. These media were all based on a modified version of M9 minimal media, which provided carbon and nitrogen sources in the form of plant-derived sugars and organic acids, and ammonia, respectively. The intention was for the nutrient content of this media to resemble plant apoplast or guttation fluid by the addition of appropriate amino acids. This was carried out as follows:

- **Amino acids**

The amino acid composition of tomato apoplast was published in Rico and Preston (2008). These values differ from those published by Solomon and Oliver for the tomato apoplast reproduced in Table 1.3, Chapter 1, which may be due to differences such as sampling methods or sampling time. However, the values reported in Rico and Preston will be used to create the synthetic apoplast in this study as the raw data is available and the sampling methods the same as used elsewhere in this study. The amino acid composition of *A. thaliana* apoplast was published by Pilot and coworkers (2004). Data describing the abundant amino acids present in *A. thaliana* guttation fluid were kindly provided by Guillaume Pilot, Stanford University (Table 4.6). Concentrations of each amino acid were rounded up to the nearest whole number and this amount was added to the synthetic media (Table 4.7).

A	Tomato apoplast		**B**	*A. thaliana* apoplast		**C**	*A. thaliana* guttation fluid
Amino acid	**Average concentration (µM)**	**±SD**	**Amino acid**	**Average concentration (µM)**	**±SD**	**Amino acid**	**Average concentration (µM)**
GABA	575.47	82.88	Proline	227.76	109.71	Glutamine	2.84
Aspartic acid	145.36	45.50	Glutamine	226.35	93.94	Histidine	1.58
Glutamic acid	137.37	45.02	Serine	172.07	73.33	Aspartate	1.06
Alanine	67.30	13.86	Alanine	137.76	12.84	Threonine	nd
Serine	51.23	12.28	Glutamate	129.54	57.83	Glutamate	nd
Glutamine	33.95	9.44	Aspartate	102.28	44.95	Alanine	nd
Leucine	31.78	5.68	Glycine	91.97	17.23	Serine	nd
Glycine	28.81	9.67	Threonine	30.97	43.80	Proline	nd
Phenylalanine	25.19	2.60	Histidine	16.81	3.77	Glycine	nd
Methionine	24.88	8.56	GABA	na		Cytosine	nd
Valine	24.73	0.09	Asparagine	nd		GABA	nd
Lysine	22.49	2.10	Cytosine	nd		Leucine	nd
Threonine	21.98	8.88	Tyrosine	nd		Phenylalanine	nd
Isoleucine	19.37	4.24	Methionine	nd		Methionine	nd
Tyrosine	16.00	2.58	Valine	na		Valine	nd
Proline	12.77	1.73	Phenylalanine	nd		Isoleucine	nd
Asparagine	9.49	2.74	Leucine	nd		Asparagine	nd
Histidine	6.83	2.01	Ornithine	na		Tyrosine	nd
Arginine	nd		Lysine	na		Lysine	nd
Tryptophan	nd		Arginine	nd		Tryptophan	na
Cysteine	nd		Tryptophan	na		Cytosine	na

A. Data provided by Arantza Rico, Oxford, UK **B** and **C**. Data provided by Guillaume Pilot, Stanford University, USA

Table 4.6

Amino acids, in order of abundance detected by HPLC analysis in healthy, wild type tomato apoplast (Rico and Preston 2008)(**A**); *A. thaliana* apoplast (Pilot et al 2004)(**B**) and *A.thaliana* guttation fluid (Guillaume Pilot, Stanford University)(**C**). na= not analysed, nd=not detected. SD= standard deviations, calculated from three independent replicates. For C data was already provided as an average and no information was provided on the SD.

A	Synthetic tomato apoplast
Amino acid	**Concentration (µM)**
GABA	575
Aspartic acid	150
Glutamic acid	140
Alanine	70
Serine	50
Glutamine	30
Glycine	30
Leucine	30
Methionine	25
Valine	25
Phenylalanine	25
Isoleucine	20
Threonine	20
Lysine	20
Tyrosine	15
Asparagine	10
Proline	10
Histidine	5

B	Synthetic *A. thaliana* apoplast
Amino acid	**Concentration (µM)**
Proline	228
Glutamine	227
Serine	170
Alanine	138
Glutamic acid	130
Aspartatic acid	100
Glycine	92
Threonine	30
Histidine	17

C	Synthetic *A. thaliana* guttation fluid
Amino acid	**Concentration (µM)**
Glutamine	3
Histidine	1.5
Aspartate	1

Table 4.7
Amounts of amino acid added to synthetic media. **A**= amounts added to synthetic tomato apoplast. **B**= amounts added to synthetic *A. thaliana* apoplast. **C**= amounts added to synthetic *A. thaliana* guttation fluid.

- **Sugars and organic acids.**

A literature survey was used to identify the major sugars and organic acids present in plant leaves (Table 4.8). A valuable source of information were metabolic studies where transgenic plants were the subject of interest but information on wild type plants was provided for comparison. Data were collected from studies looking at apoplast, whole leaves and leaf exudates. Unlike the comprehensive data available for the amino acids in the apoplast, there was relatively limited information available in published studies detailing the organic acid or sugar content of the plant apoplast. Therefore data from several studies was combined.

Glucose, sucrose and fructose sugars and the organic acid salts D,L-malate and citrate were identified as abundant in tomato plants. These sugars and organic acids have also been identified in the apoplast of sugar beet (Lopez-Millan et al., 2000), and sucrose, glucose and fructose are the predominant sugars in barley leaf apoplast (Tetlow and Farrar, 1993).

The growth of P.s.t in M9 medium with ammonia as a nitrogen source and each of the sugars glucose, sucrose or fructose or the organic acids D,L-malate and citrate as a carbon source was investigated to see which supported most growth (Figure 4.7). Results showed that the organic acid salt citrate, and the sugars glucose and fructose supported the highest growth of P.s.t. after 24 hours. Next, the growth of P.s.t in M9 medium with ammonia as a nitrogen source and a combination of the above sugars and organic acids was investigated (Figure 4.8). Results showed that the carbon source combination of glucose, sucrose, fructose, malate and citrate in the ratio 1:1:1:0.5:0.5 supported the maximum growth of P.s.t over 24 hours (Figure 4.8). This carbon source combination and ratio also supported the highest growth of P.s.t when amino acids were added to the medium in tomato apoplast concentrations (Figure 4.9).

	Abundant sugars and organic acids				
Reference	**1**	**2**	**3**	**4**	**5**
Glucose	*	*	*	*	*
Fructose	*	*	*	*	*
Sucrose	*	*	*	*	*
Malate	*	*		*	
Citrate	*	*		*	
Maltose		*			*
Fucose		*			
Maleic					
Xylose					*
Location within tomato plant	L	L	A	L,A	E

Table 4.8
Literature survey of sugars identified in tomato plants. Studies which have specifically looked at the sugar composition of the tomato leaf apoplast are highlighted in yellow. L= leaf, A= apoplast, E= root exudate.
References: 1= (Schauer et al., 2005), 2 = (Roessner-Tunali et al., 2003), 3 = (Joosten et al., 1990), 4 = (Liao et al., 1988), 5 = (Lugtenberg et al., 1999).

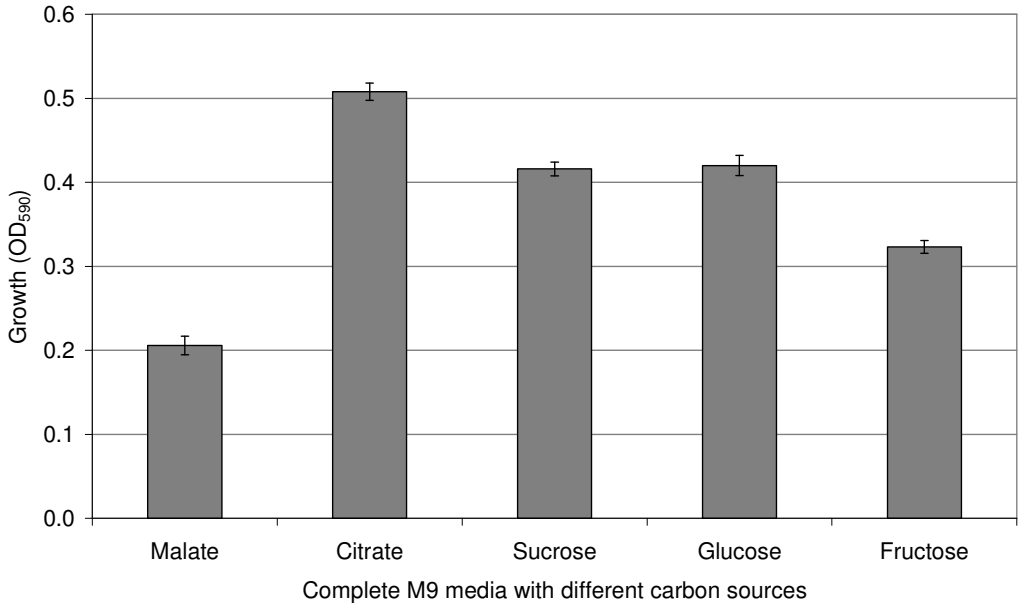


Figure 4.7
Growth of P.s.t after 24 hours in complete M9 minimal media with ammonia and a selection of different carbon sources. Complete M9 medium contains a final carbon source (frequently glucose) concentration of 0.4 % w / v (Sambrook et al., 1989). Therefore all alternative carbon sources were added to complete M9 media at a final concentration of 0.4 % w / v. Error bars indicate standard deviations of three replicates and are representative of at least two independent experiments with similar results.

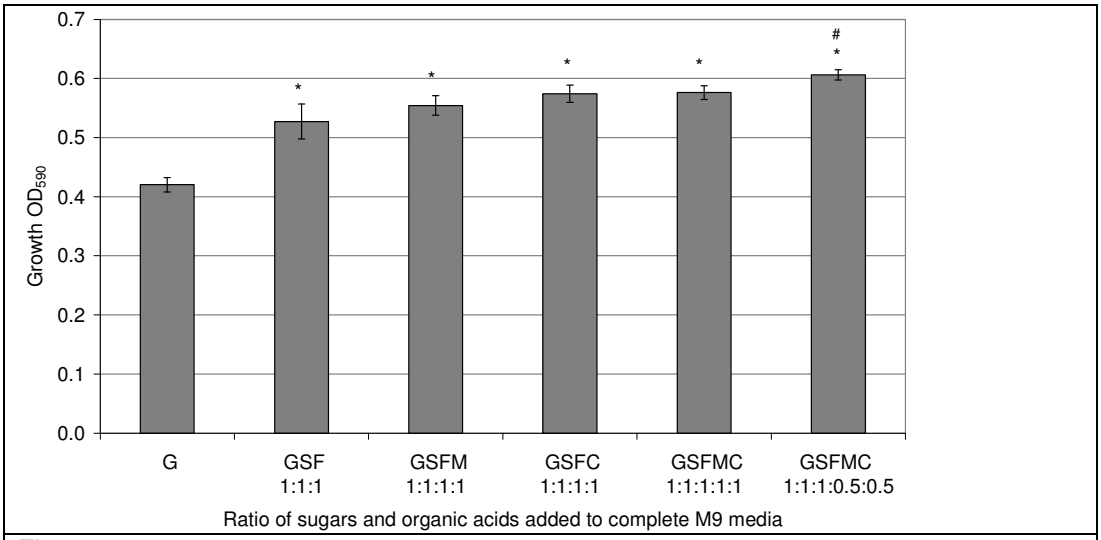


Figure 4.8
Growth of P.s.t in complete M9 minimal media with a combination of sugar and organic acid carbon sources in different ratios as indicated. G= glucose, S= sucrose, F= fructose, M= malic acid, C= citric acid. All carbon sources were added at a total of 0.4 % w / v. Error bars indicate standard deviations of three replicates and are representative of at least two independent experiments with similar results. * = Carbon source combination results in significantly higher growth than complete M9 with glucose (G) #= Carbon source combination results in significantly higher growth than any other combination ($p \leq 0.05$).

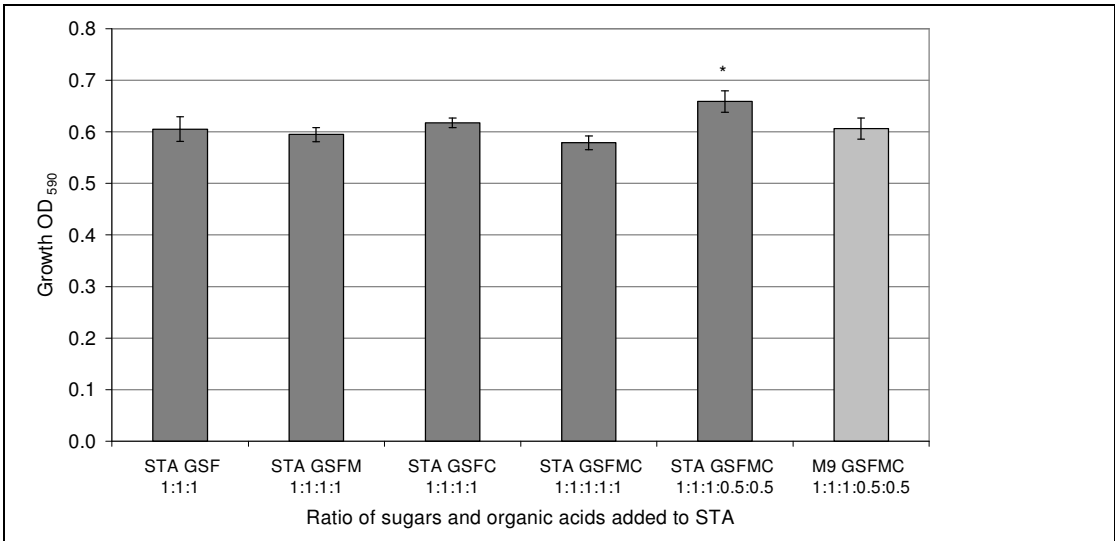


Figure 4.9
Growth of P.s.t in synthetic tomato apoplast made using different carbon source combinations as indicated. All carbon sources were added at a total of 0.4 % w / v. Complete M9 minimal media was supplemented with the amino acids present in tomato apoplast (Table 4.6), with a combination of sugars and organic acids providing the carbon source. Error bars indicate standard deviations of three replicates and are representative of at least two independent experiments with similar results. * = Carbon source and amino acid combination results in significantly increased growth compared to other combinations ($p \leq 0.05$). Growth of P.s.t in M9 GSFMC (which has the same composition as STA GSFMC but without any amino acids) is provided as a comparison.

4.5.7 Final composition of synthetic apoplast media.

The final composition of synthetic apoplast media contained amino acids, organic acids and hexose sugars in a minimal media base (Table 4.9). Amino acids were made up in 40 mM stock solutions at pH 7. When amino acids were added to the M9 minimal media base the pH was approximately 6.5.

The same 'base' of M9 minimal media and a combination of five hexose sugars and organic acids (M9-GSFMC) was used for synthetic tomato and *A. thaliana* apoplast, and for synthetic *A.thaliana* guttation fluid media. These media differed in their amino acid content.

	Synthetic media			
	M9-GSFMC	**Apoplast**		**Guttation Fluid**
Based on		**Tomato**	***A. thaliana***	***A. thaliana***
Amino acid	**Amount (ml)**	**Amount (ml)**	**Amount (ml)**	**Amount (ml)**
GABA		1.2500		
Aspartic acid		0.3740	0.2560	0.1060
Glutamic acid		0.3740	0.0324	
Alanine		0.1740	0.0344	
Serine		0.1250	0.0430	
Glutamine		0.0750	0.0566	0.2800
Leucine		0.0750		
Glycine		0.0750	0.0230	
Phenylalanine		0.0625		
Methionine		0.0625		
Valine		0.0625		
Lysine		0.0625		
Threonine		0.0500	0.0760	
Isoleucine		0.0500		
Proline		0.0250	0.0569	
Asparagine		0.0250		
Histidine		0.0125	0.0042	0.1580
Sugars (total 0.4 %)				
Glucose	0.500	0.500	0.500	0.500
Sucrose	0.500	0.500	0.500	0.500
Fructose	0.500	0.500	0.500	0.500
Malate	0.250	0.250	0.250	0.250
Citrate	0.250	0.250	0.250	0.250
10 x Minimal salts	10.000	10.000	10.000	10.000
1M $MgSO_4$	0.200	0.200	0.200	0.200
1M $CaCl_2$	0.010	0.010	0.010	0.010

Table 4.9
The amount of each constituent chemical used to make 100 ml of synthetic media and M9-GSFMC on which each synthetic media is based.

4.5.8 Growth of *Pseudomonas syringae* pv. tomato in synthetic apoplast media.

Tomato apoplast has been shown to provide a complete growth medium for P.s.t (Rico and Preston, 2008). In order to investigate the ability of the newly created synthetic tomato and *A. thaliana* apoplast to provide a complete growth medium for P.s.t, the growth of P.s.t over 24 hours in synthetic apoplast was compared to the growth in LB, modified M9 media M9-GSFMC (on which the synthetic apoplast media is based) and extracted tomato apoplast (Figure 4.10).

The growth of P.s.t was significantly increased in synthetic apoplast media compared to modified M9 (Figure 4.10). This indicates that the added amino acids provide a growth benefit to P.s.t. However, the growth of P.s.t in extracted tomato apoplast is significantly better than in either synthetic apoplast media. This may be due to as yet-unidentified components of the apoplast which provide a growth benefit to P.s.t. Preliminary apoplast analysis has indicated compounds such as trigonelline and inositol which may provide sources of nutrients for *P. syringae* in the apoplast (Arantza Rico and Gail Preston, personal communication). Arantza Rico and Gail Preston have also detected 'phases' of nutrient assimilation by P.s.t in apoplast, with some compounds, such as inositol being utilised only once preferred carbon sources have been used up. The presence of these later-used sources of carbon could also explain the difference in growth between APO and synthetic apoplast.

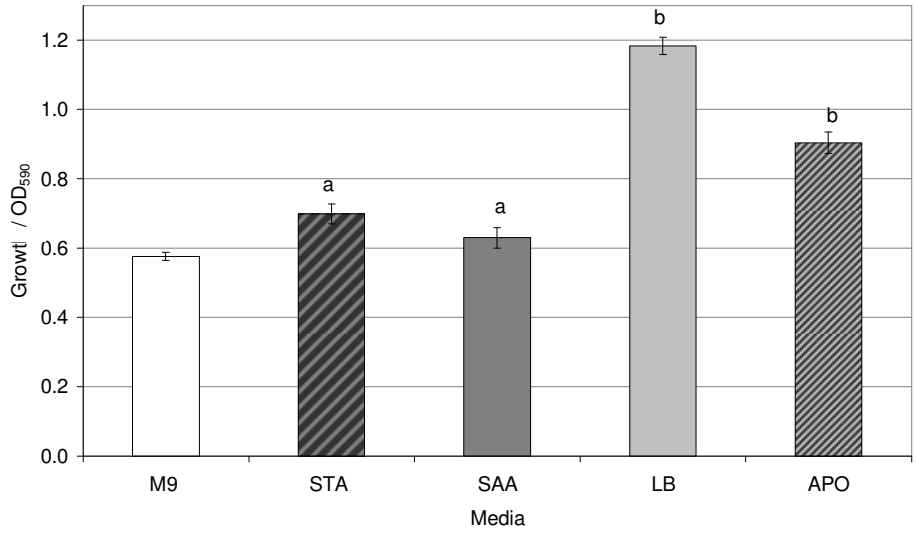


Figure 4.10
The growth of P.s.t in modified M9, M9-GSFMC (M9), synthetic tomato apoplast (STA), synthetic *A. thaliana* apoplast (SAA), LB and extracted tomato apoplast (APO) at 24 hours. a=growth is significantly different to M9 ($p<0.05$), b=growth is significantly different to synthetic apoplast media ($p<0.05$). Student's 2-tailed t-test. Ho=no difference between growth in M9 and synthetic media. Error bars indicate standard deviations from three replicates. Experiment was repeated twice with similar results.

Subsequently, the growth of P.s.t in synthetic tomato apoplast (STA), tomato apoplast (APO), M9-GSFMC (M9) and LB was studied in detail in a 24-hour time course experiment (Figure 4.11 A). The growth of P.s.t in STA was similar in STA and SA between 0 and 4 hours. However the growth of P.s.t in STA was less than in TA after this time point and bacteria reached a lower optical density after 24 hours. The growth rate of P.s.t in each media was calculated between 0 to 6 hours, 6 to 10 hours and 10 to 24 hours (Figure 4.11 B). Interestingly the growth rate of P.s.t in STA slowed after 6 hours to levels comparable with growth in M9 (the media on which STA is based). This suggested that the nutrients in STA are used up in the first six hours of growth. In contrast the growth rate of TA remained high between 6 and 10 hours thereby supporting the hypothesis that compounds are present in tomato apoplast which can be used as carbon sources during later growth by P.s.t.

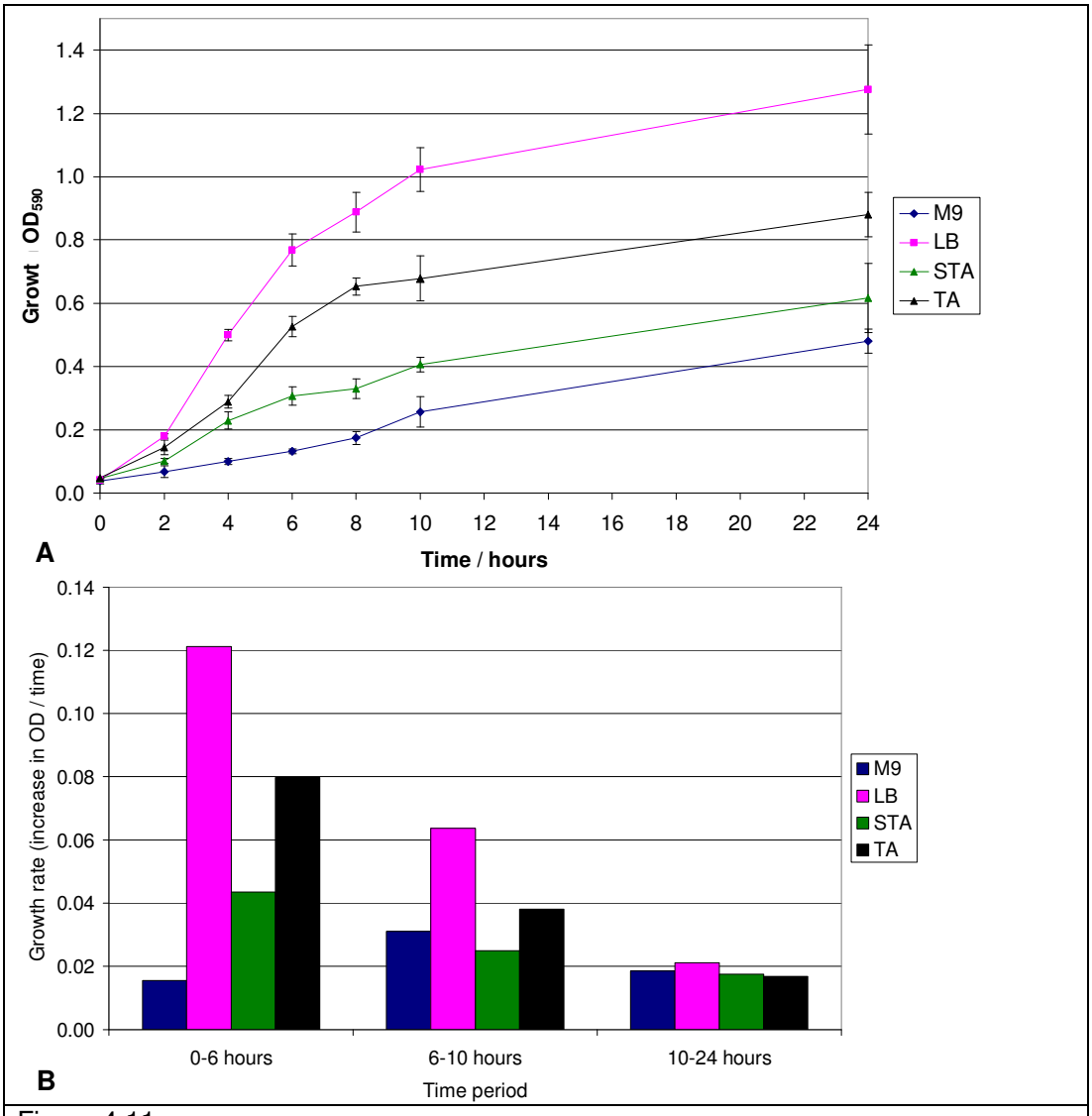


Figure 4.11

(Figure 4.11)
A. Twenty four hour time course of P.s.t growth in M9-GSFMC (M9), LB, synthetic tomato apoplast (STA) and extracted tomato apoplast (APO). Error bars indicate standard deviations from three replicates. This experiment was independently repeated twice with similar results. **B**. Growth rate of P.s.t in M9, LB, STA and APO at different time points during the experiment in part A. Calculated as the increase in optical density of the culture between the time points indicated. The growth rate in TA is higher than in STA between 0-6 and 6-10 hours indicating that there are more nutrients available in TA.

4.5.9 Incubation of *Pseudomonas syringae* pv. tomato in synthetic tomato apoplast results in a similar nutrient utilisation profile to incubation in real tomato apoplast.

Rico and Preston (2008) demonstrated that incubation of *P. syringae* pv. tomato (P.s.t) in extracted tomato apoplast (APO) prior to inoculation in Biolog GN2 plates limited the nutrients which could be metabolised as carbon sources. STA was designed to mimic APO. One way to test how well STA mimics APO was to repeat the experiments of Rico and Preston (2008) using STA, to assess the effectiveness of STA as an inducer of nutrient utilisation pathways in P.s.t.

P.s.t was incubated in either APO or STA prior to inoculation into Biolog GN2 plates and results were read after 24 hours. Results indicated that pre-incubation of P.s.t in STA results in a similar Biolog nutrient utilisation profile as pre-incubation in APO (Table 4.10). This suggested that STA is inducing similar nutrient utilisation pathways in P.s.t as APO. In particular, fructose, citric acid and malonic acid were utilised by P.s.t following incubation in STA and APO. Assimilation of these compounds was found to be 'induced' by pre-incubation in tomato apoplast, rather than being constitutive (Rico and Preston, 2008). Inositol was not utilised by P.s.t after pre-incubation in APO or STA, however this compound is relatively abundant in the tomato apoplast (Gail Preston, personal communication), and can be utilised by P. *syringae* when tetracycline is not added to apoplast (Rico and Preston, 2008). This result is consistent with a hypothesis that inositol is one of the later constituents of the apoplast to be utilised by P.s.t.

Unlike pre-incubation in APO, pre-incubation in STA did not result in metabolism of galactose, formic acid, uridine or bromosuccinic acid. However, this may be due to the presence of as-yet unidentified compounds in APO which have not been added to STA. Interestingly there are also two compounds which are metabolised by P.s.t after

incubation in STA but not APO: α-keto valeric acid and L-pyroglutamic acid possibly suggesting an effect on nutrient utilisation pathways of the M9 minimal media.

Well	Carbon source	Synthetic apoplast	Tomato apoplast*		Well	Carbon source	Synthetic apoplast	Tomato apoplast*	
A1	Water				E1	p-Hydroxy Phenylacetic Acid			
A2	α-Cyclodextrin				E2	Itaconic Acid			
A3	Dextrin			C	E3	α-Keto ButyricAcid			
A4	Glycogen				E4	α-Keto Glutaric Acid			C
A5	Tween 40				E5	á-Keto Valeric Acid			N
A6	Tween 80				E6	D,L-Lactic Acid			
A7	N-Acetyl-D-Galactosamine				E7	Malonic Acid			I
A8	N-Acetyl-D-Glucosamine				E8	PropionicAcid			
A9	Adonitol				E9	Quinic Acid			
A10	L-Arabinose				E10	D-Saccharic Acid			
A11	D-Arabitol				E11	Sebacic Acid			
A12	D-Cellobiose				E12	Succinic Acid			C
B1	i-Erythritol				F1	Bromosuccinic Acid			C
B2	D-Fructose			I	F2	Succinamic Acid			
B3	L-Fucose				F3	Glucuronamide			I
B4	D-Galactose			I	F4	L-Alaninamide			
B5	Gentiobiose				F5	D-Alanine			
B6	α-D-Glucose			C	F6	L-Alanine			C
B7	Myo-Inositol				F7	L-Alanyl-glycine			
B8	α-D-Lactose				F8	L-Asparagine			C
B9	Lactulose				F9	L-Aspartic Acid			C
B10	Maltose				F10	L-Glutamic Acid			C
B11	D-Mannitol				F11	Glycyl-L-Aspartic Acid			
B12	D-Mannose			C	F12	Glycyl-L-Glutamic Acid			
C1	D-Melibiose				G1	L-Histidine			
C2	α-Methyl-D-Glucoside				G2	Hydroxy-L-Proline			
C3	D-Psicose				G3	L-Leucine			
C4	D-Raffinose				G4	L-Ornithine			
C5	L-Rhamnose				G5	L-Phenylalanine			
C6	D-Sorbitol				G6	L-Proline			
C7	Sucrose				G7	L-Pyroglutamic Acid			N
C8	D-Trehalose			I	G8	D-Serine			
C9	Turanose				G9	L-Serine			C
C10	Xylitol				G10	L-Threonine			
C11	Pyruvic Acid Methyl Ester				G11	D,L-Carnitine			
C12	Succinic Acid M-M-Ester				G12	γ-Amino ButyricAcid			C
D1	Acetic acid				H1	Urocanic Acid			
D2	Cis-Aconitic acid				H2	Inosine			
D3	Citric acid			I	H3	Uridine			I
D4	Formic acid			I	H4	Thymidine			
D5	D-Galactonic acid				H5	Phenyethyl-amine			
D6	D-Galacturonic acid				H6	Putrescine			
D7	D-Gluconic acid				H7	2-Aminoethanol			
D8	D-Glucosaminic acid				H8	2,3-Butanediol			
D9	D-Glucuronic acid			I	H9	Glycerol			
D10	α-HydroxybutyricAcid				H10	D,L-α-Glycerol Phosphate			
D11	β-HydroxybutyricAcid				H11	α-D-Glucose-1-Phosphate			
D12	γ-HydroxybutyricAcid				H12	D-Glucose-6-Phosphate			

OD_{620} 0.05-0.20	<0.05

Table 4.10

Comparison of carbon source metabolism in GN2 Biolog™ plates, by P.s.t pre-grown in synthetic tomato apoplast or real tomato apoplast, with the addition of tetracycline. Purple colour equals a positive result and metabolism of the C-source. (*=Data provided by Arantza Rico, Oxford UK) Succinic Acid M-M-Ester = Succinic Acid Mono-Methyl-Ester. C, I or N in the final column indicate whether this compound was found to be utilised constitutively, induced (following incubation in apoplast extract) or was not utilised in the experiments reported in Rico and Preston (2008). This experiment was repeated twice with similar results.

4.5.10 Growth of *Pseudomonas syringae* in synthetic guttation fluid.

Synthetic *A. thaliana* guttation fluid (GF) was created using the amino acid data of Pilot and coworkers (2004). In order to gauge the ability of the amino acids present in GF to enhance the growth of P.s.t, the growth of P.s.t over 24 hours in GF was compared to the growth of P.s.t in the modified M9 minimal media (M9-GSFMC) on which it is based. For comparison, the growth of the non plant-pathogen P.fl SBW25 was also assessed.

The growth of P.s.t or P.flSBW25 in GF was not significantly different to M9-GSFMC (Figure 4.12). This may be because GF was only reported to contain relatively low concentrations of three amino acids; glutamine, histidine and aspartate. However, as hydathodes are known to provide a natural site of entry for *P. syringae* during infection (Lindow and Brandl, 2003); this media could be used to assess chemotaxis of *P. syringae* towards these sites. Future experiments could establish realistic estimates of ammonia and nitrate in guttation fluid and adjust the media accordingly.

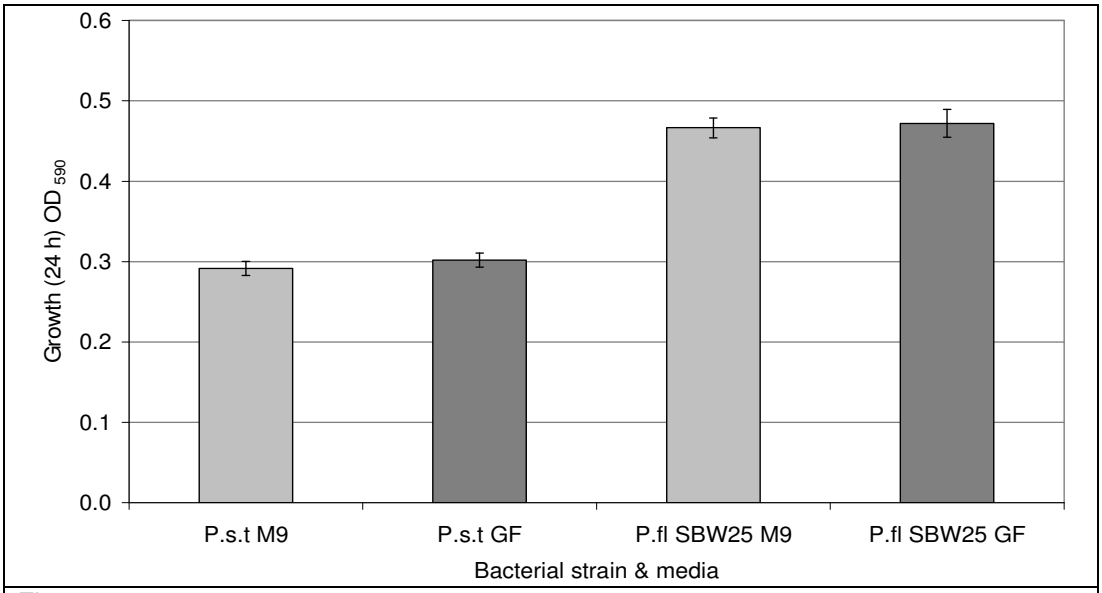


Figure 4.12
The growth of P.s.t and P.fl SBW25 was not significantly different in guttation fluid (GF) from M9-GSFMC (M9). Error bars are standard deviations from three replicates. This experiment was repeated twice with similar results.

4.5.11 Comparison of *Pseudomonas syringae* growth in synthetic apoplast media.

P. syringae pathovars infect different host plants; P.s.t is a pathogen of tomato plants, while P.s.ph, P.s.s and P.s.m are host specific for bean plants and crucifers respectively, and are unable to infect tomato plants. In addition P.s.m and P.s.t can infect the model plant *A. thaliana*. The apoplast of different plant species, including tomato, barley and *A. thaliana*, appeared to differ in nutritional composition, particularly the abundance of amino acids (Chapter 1, Table 1.4). Therefore it was interesting to compare the ability of *P. syringae* strains with different host plants, to grow in synthetic apoplast based on tomato or *A. thaliana* (Figure 4.13). P.fl SBW25 was included as a representative non plant-pathogenic *Pseudomonas*. In addition the ability of these bacteria to grow in M9 and synthetic guttation fluid (GF) was compared.

None of the strains tested grew differently in GF compared to M9. This supported the previous conclusion that with only three added amino acids, GF does not contain enough added nutrients to support significantly increased growth compared to M9. P. fl SBW25 grew well in all synthetic media tested, but this growth was not significantly different between the different media. This may be because *P. fluorescens* strains are better adapted to use ammonium as a sole or main nitrogen source, Pf-5 has six loci annotated as putative glutamine synthetases (the main route for ammonium assimilation), compared to three in P.s.t and four in P.s.ph.

The growth of P.s.t, P.s.m, P.s.ph and P.s.s was significantly increased in STA compared to M9 minimal media. The growth of P.s.t, P.s.m and P.s.s was increased in SAA compared to M9. The growth of P.s.ph was not significantly different in SAA compared to M9 (Figure 4.13), however P.s.ph has been shown to metabolise the fewest amino acids as carbon or nitrogen sources (Table 4.2 and 4.3), and therefore may represent the most nutritionally restricted *P. syringae* strain tested.

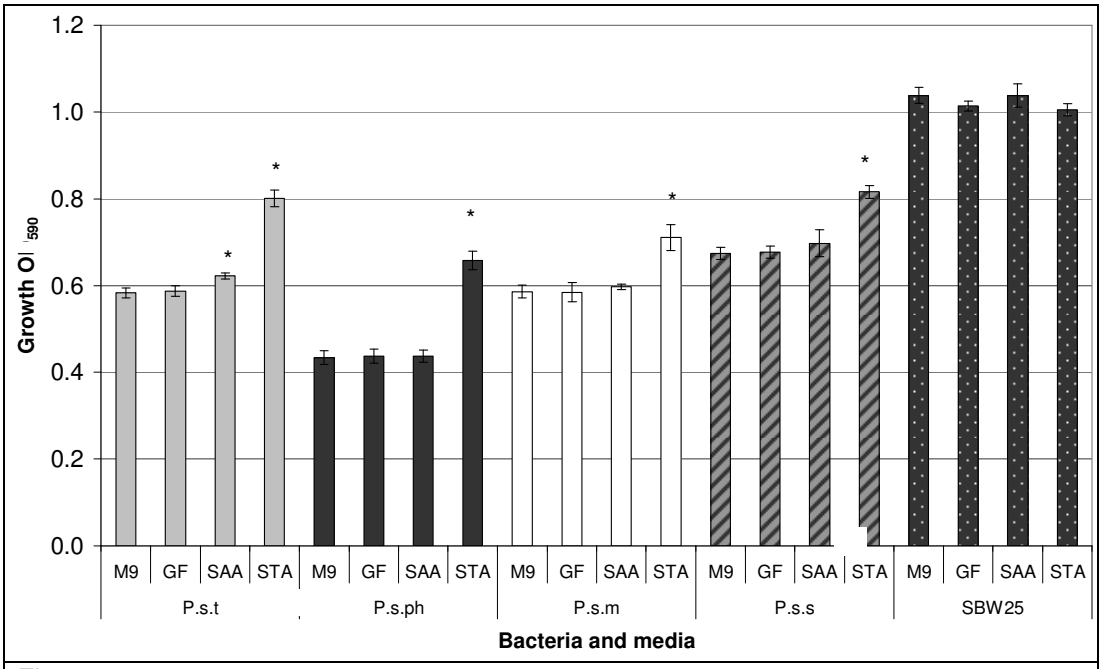


Figure 4.13
Growth of P.s.t, P.s.ph, P.s.s, P.s.m and P.fl SBW25 after 24 hours in synthetic media. M9= M9-GSFMC, GF= synthetic *A. thaliana* guttation fluid, STA= synthetic tomato apoplast, SAA= synthetic *A. thaliana* apoplast. *= Growth was significantly different to M9 $p \leq 0.05$. Student's 2-tailed t tests, 4 degrees of freedom, H_0= no difference between growth in M9 and in synthetic apoplast. Error bars are standard deviations of three replicates. This experiment was repeated twice with similar results.

4.5.12 Glutamine, serine and proline contribute significantly towards increased growth of *Pseudomonas syringae* pv. tomato in synthetic *Arabidopsis thaliana* apoplast.

Synthetic *A. thaliana* apoplast (SAA) was shown to support significantly higher growth of P.s.t, than the modified M9-GSFMC media on which it is based. Furthermore, synthetic tomato apoplast (STA) has been shown to support increased growth of P.s.t, relative to SAA. The only difference between these two media is the amino acids added. To investigate the contribution of each amino acid in SAA to the growth of P.s.t, each of the amino acids present in SAA were added to M9-GSFMC media at the same concentration as in SAA (Figure 4.14).

Results showed that three amino acids, proline, glutamine and serine, all significantly increased the growth of P.s.t compared to M9-GSFMC media after 24 hours. The growth of P.s.t in complete SAA was significantly higher after 24 hours than in any M9-GSFMC + single amino acid combination. However, a combination of serine, proline and glutamine added to M9-GSFMC media resulted in growth after 24 hours which was not significantly different to the growth of P.s.t in complete SAA (Figure 4.14). These

three amino acids are the most abundant in *A. thaliana* apoplast. Therefore, the majority of the growth increase seen in SAA compared to M9, appears to come from the addition of the three most abundant amino acids.

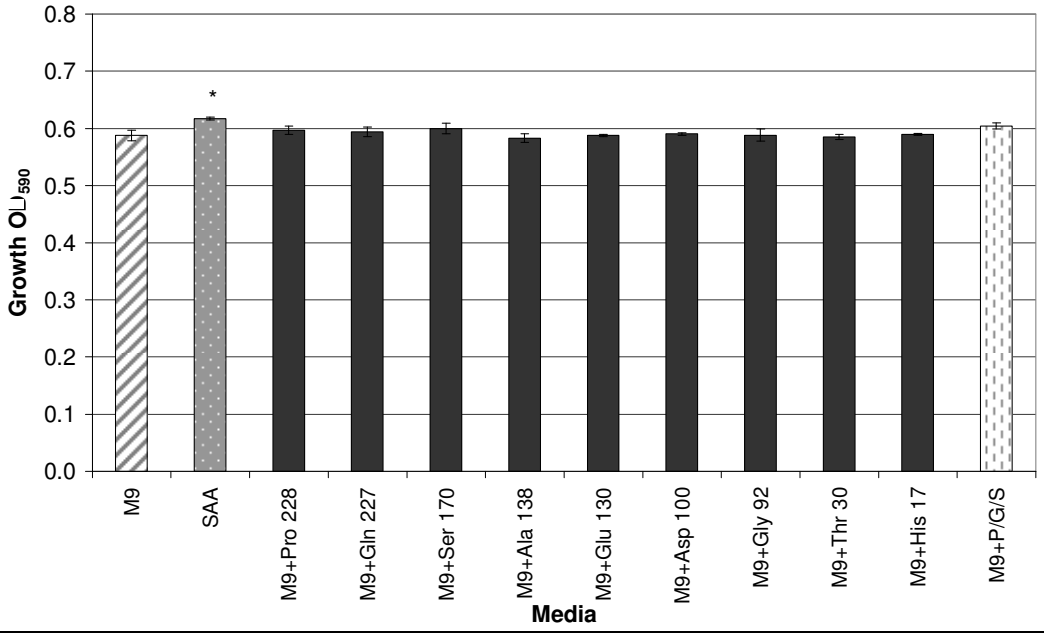


Figure 4.14
Growth at 24 hours of P.s.t in M9-GSFMC (M9), synthetic *A. thaliana* apoplast (SAA), and M9-GSFMC plus an amino acid at SAA concentration (M9+amino acid). Numbers indicate the concentrations of amino acids in □M. M9+P/G/S= M9 + proline (228μM), serine (170μM), glutamine (227μM). * = Growth was significantly different to M9 p≤0.0.5. Error bars are standard deviations of three replicates. This experiment was repeated on three independent occasions with similar results. Significance was measured using Student's 2 tailed t-tests on data with 4 degrees of freedom. H_0= no difference in growth in M9 and synthetic media. The p-value of SAA compared to M9 was 0.006.

4.5.13 GABA, aspartic acid and glutamic acid contribute significantly towards the growth of *Pseudomonas syringae* pv. tomato in synthetic tomato apoplast.

Synthetic tomato apoplast (STA) supports significantly higher growth of P.s.t than the modified M9-GSFMC media on which it is based. This media also supports increased growth of P.s.t compared to SAA. To investigate the contribution of individual amino acids to the growth of P.s.t, each of the amino acids present in STA was individually added to M9-GSFMC media at the same concentration as in STA (Figure 4.15).

The addition of GABA, aspartic acid and glutamic acid added together, all significantly increased the growth of P.s.t compared to M9-GSFMC media after 24 hours. These amino acids are the most abundant in the tomato apoplast. The growth of P.s.t in complete STA was significantly higher after 24 hours than in any M9 + amino acid combination.

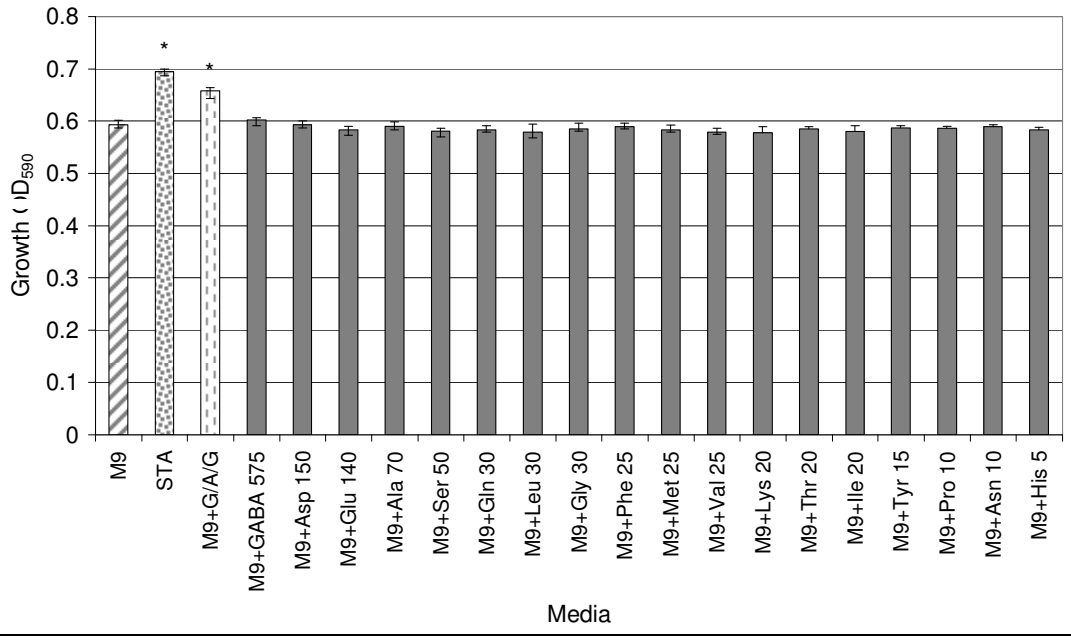


Figure 4.15
Growth of P.s.t in M9 with a combination of plant sugars (GSFMC 1:1:1:0.5:0.5), synthetic tomato apoplast (STA) and M9 with combination sugars plus an amino acid at STA concentration. Numbers indicate the concentrations of amino acids in µM. M9+G/A/G= M9+GABA (575µM), aspartic acid (150µM) and glutamic acid (140µM). Significance was measured using Student's 2-tailed t-tests with 10 degrees of freedom. H_0= no difference in growth. * =Growth was significantly different to M9 $p \leq 0.05$. Growth of STA was significantly different to M9 (p=0.000), Growth of M9+G/A/G was significantly different to M9 (p=0.000). Error bars are standard deviations of three replicates. This experiment was repeated twice with similar results.

4.5.14 Growth in synthetic media at different pH values.

As pH appeared to be important for amino acid uptake in *P.syringae* (Table 4.5), the pH of synthetic tomato apoplast (STA) and synthetic *A. thaliana* apoplast (SAA) was adjusted to a range of pH values likely to be encountered by *P. syringae* during growth *in planta* (pH 5 to pH 7.5), and the growth of P.s.t in these synthetic media was compared to that in M9-GSFMC at the same pH (Figure 4.16). At pH values between 5.5 and 6.5 the growth of P.s.t in STA and SAA was significantly higher than in M9 media. However at values outside of this range, the growth of P.s.t was not significantly different to that in M9 media. This suggests that at pH 5, and at pH values above 6.5 P.s.t cannot efficiently take up the amino acids present in STA and SAA.

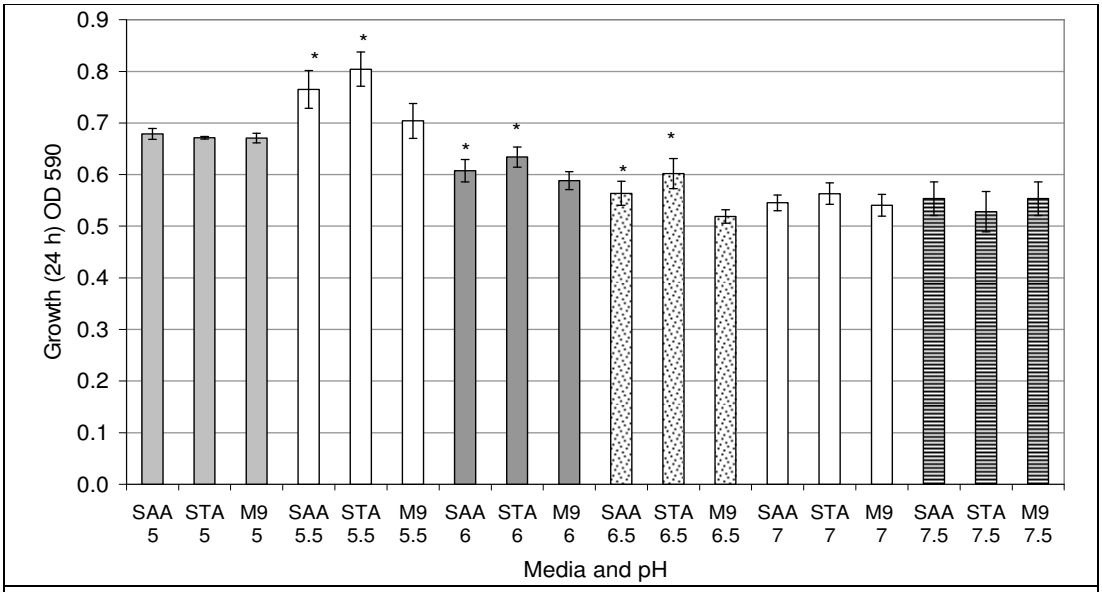


Figure 4.16
The growth of P.s.t in synthetic *A. thaliana* apoplast (SAA), synthetic tomato apoplast (STA) and M9-GSFMC minimal media (M9), at a range of pH values as indicated.
*=Growth is significantly different to that in M9, p≤0.05 in Student's 2-tailed t-tests comparing M9 to SAA or STA at each pH. Twenty two degrees of freedom. H_0=no difference between growth in M9 and synthetic media. After Error bars represent the standard deviations of three replicate cultures. The experiment was repeated twice with similar results. 48 hours the growth of P.s.t in STA at pH 5 was significantly higher than M9.

As the pH of the apoplast changes during infection (Grignon and Sentenac, 1991), and alters during the growth of *P.syringae* in LB media (Figure 4.4 and 4.5), the pH of STA, SAA and M9 was tested at 24 and 48 hours to investigate whether a pH change was occurring during this experiment which could be affecting the growth of *P.syringae* (Figure 4.17). The pH of each media did not vary significantly during growth. This is in agreement with previous results (Figure 4.9) which showed that although there was a

pH change resulting from the growth of P.s.t in LB, this was by less than one pH unit. Furthermore, he final optical density reached by P.s.t in any of these synthetic medias was lower than in LB (tested previously), which would explain why we see less of a pH change following growth in STA and SAA compared to LB.

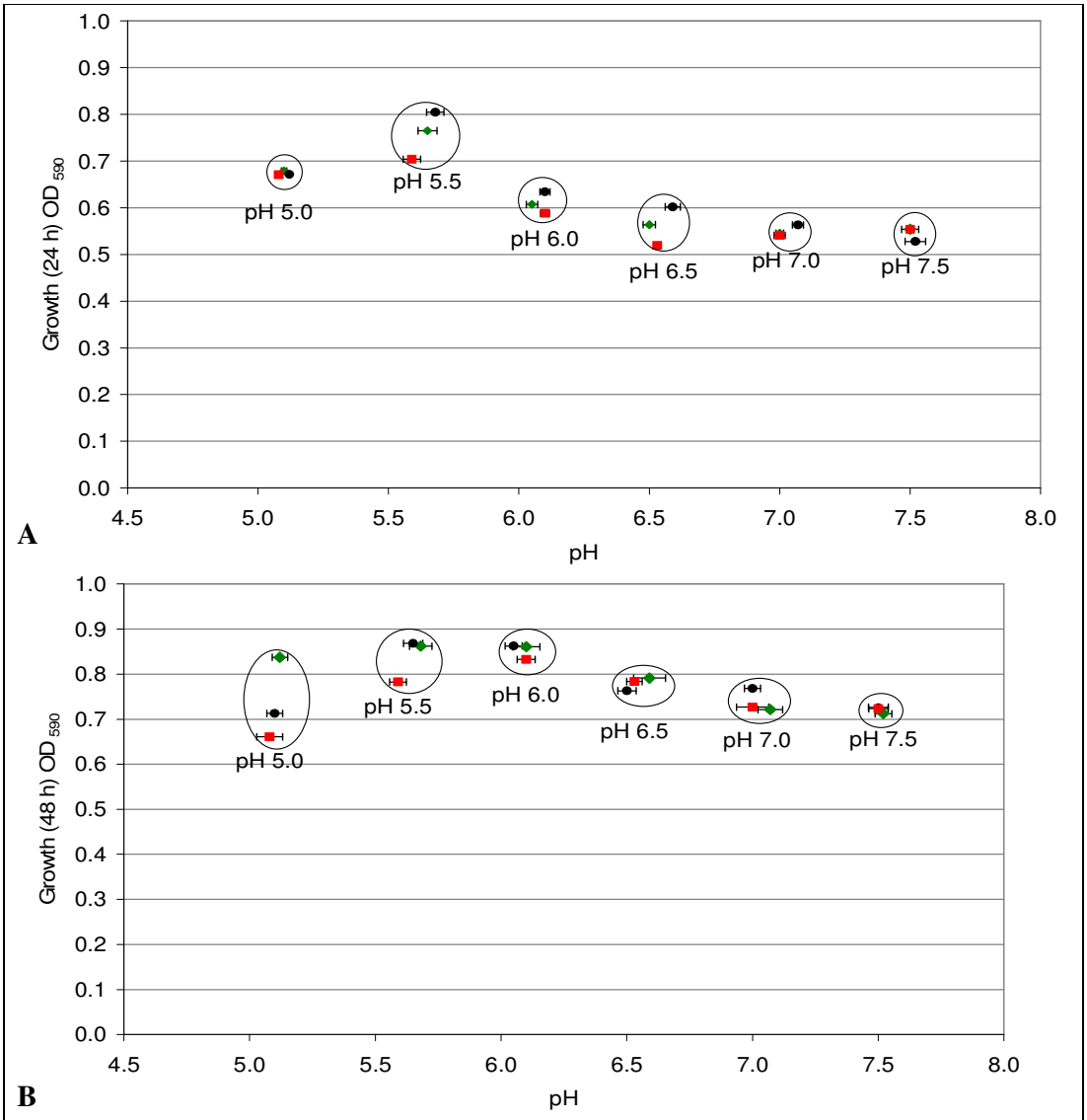


Figure 4.17
pH of synthetic media during the growth of P.s.t over 24 (A) and 48 (B) hours. The pH of SAA (black), M9-GSFMC (red) and STA (green) was adjusted to different staring pH values between pH 5 and pH 7.5 (but not buffered). P.s.t was then grown in these media for 24 and 28 hours and the final pH of the media was measured. Although the pH of the media did vary, at both 24 (A) and 48 (B) hours, (as shown by the error bars), this was not significant enough to overlap with the next pH value. Error bars indicate standard deviations of three replicates. Experiments were repeated independently twice with similar results.

4.5.15 Metabolism of compounds as carbon sources is increased at acidic pH when bacteria are pre-incubated in synthetic tomato apoplast.

The growth of P.s.t was significantly increased in synthetic tomato apoplast (STA) compared to M9 media at pH5.5, pH 6 and pH 6.5, but not at pH 5, pH7 or pH7.5 (Figure 4.16). The pH of the surrounding media may be important for nutrient uptake, particularly when transporters are driven by the movement of protons or other small molecules, rather than ATP.

In order to further investigate the effect of pH on nutrient utilisation by P.s.t a Biolog GN2 plate metabolism assay was used. Bacterial suspensions were pre-incubated in STA at two different pH values: pH6 (at which the growth of P.s.t was significantly increased compared to M9) or pH7 (where there was no significant growth increase). Tetracycline was added prior to the inoculation of P.s.t into GN2 Biolog plates to prevent new protein synthesis according to the method described in Rico and Preston 2008.

The metabolism of many compounds was significantly increased when P.s.t was pre-incubated at pH6 compared to pH 7 (Table 4.11). Pre-treatment affects the expression of bacterial genes. This suggests that at pH 7 the expression of nutrient assimilation transporters may be compromised. Alternatively the pH of the media could be exerting an effect on the compounds to be transported. Therefore at pH 7 compounds may have an altered ionic form which means they do not enter cells as efficiently and induce nutrient assimilation pathways. These results are in agreement with those shown in Table 4.5, which demonstrate that at ph 5.5 P.s.t can gain a growth boost from the addition of certain amino acids, but for many this effect is lost at alkaline pH (pH 8).

Well	Carbon source	p-value		pH 6	pH 7	Well	Carbon source	p-value		pH 6	pH 7
A1	Water					E1	p-HydroxyPhenylacetic Acid				
A2	α-Cyclodextrin					E2	Itaconic Acid				
A3	Dextrin	0.175				E3	α-Keto ButyricAcid				
A4	Glycogen	0.129				E4	α-Keto Glutaric Acid	0.174			
A5	Tween 40					E5	α-Keto Valeric Acid	0.246			
A6	Tween 80					E6	D,L-Lactic Acid				
A7	N-Acetyl-D-Galactosamine					E7	Malonic Acid	0.139			
A8	N-Acetyl-D-Glucosamine					E8	PropionicAcid				
A9	Adonitol					E9	Quinic Acid				
A10	L-Arabinose					E10	D-Saccharic Acid				
A11	D-Arabitol					E11	Sebacic Acid				
A12	D-Cellobiose					E12	Succinic Acid	0.002	7		
B1	i-Erythritol					F1	Bromosuccinic Acid				
B2	D-Fructose	0.012	6			F2	Succinamic Acid				
B3	L-Fucose					F3	Glucuronamide				
B4	D-Galactose					F4	L-Alaninamide				
B5	Gentiobiose					F5	D-Alanine				
B6	α-D-Glucose	0.039	6			F6	L-Alanine	0.048	6		
B7	m-Inositol					F7	L-Alanyl-glycine				
B8	α-D-Lactose					F8	L-Asparagine	0.153			
B9	Lactulose					F9	L-Aspartic Acid	0.008	6		
B10	Maltose					F10	L-Glutamic Acid	0.009	6		
B11	D-Mannitol					F11	Glycyl-L-Aspartic Acid				
B12	D-Mannose	0.029	6			F12	Glycyl-L-Glutamic Acid				
C1	D-Melibiose					G1	L-Histidine				
C2	α-Methyl-D-Glucoside					G2	Hydroxy-L-Proline				
C3	D-Psicose					G3	L-Leucine				
C4	D-Raffinose					G4	L-Ornithine				
C5	L-Rhamnose					G5	L-Phenylalanine				
C6	D-Sorbitol					G6	L-Proline				
C7	Sucrose					G7	L-Pyroglutamic Acid	0.183			
C8	D-Trehalose					G8	D-Serine				
C9	Turanose					G9	L-Serine	0.183			
C10	Xylitol					G10	L-Threonine				
C11	Pyruvic Acid Methyl Ester					G11	D,L-Carnitine				
C12	Succinic Acid M-M-Ester					G12	γ-Amino ButyricAcid	0.023	6		
D1	Acetic acid					H1	Urocanic Acid				
D2	Cis-Aconitic acid					H2	Inosine				
D3	Citric acid	0.089				H3	Uridine				
D4	Formic acid					H4	Thymidine				
D5	D-Galactonic acid					H5	Phenyethyl-amine				
D6	D-Galacturonic acid					H6	Putrescine				
D7	D-Gluconic acid					H7	2-Aminoethanol				
D8	D-Glucosaminic acid					H8	2,3-Butanediol				
D9	D-Glucuronic acid					H9	Glycerol				
D10	α-HydroxybutyricAcid					H10	D,L-α-Glycerol Phosphate				
D11	β-HydroxybutyricAcid					H11	α-D-Glucose-1-Phosphate				
D12	γ-HydroxybutyricAcid					H12	D-Glucose-6-Phosphate				

OD_{620} 0.05-0.20	<0.05

Table 4.11

Metabolism of compounds by P.s.t pre-incubated in synthetic tomato apoplast (STA) pH 6 or STA pH 7. Tetracycline was added to bacteria prior to inoculation into Biolog GN2 plates to prevent further protein synthesis. Student's 2-tailed t-tests were carried out on the results comparing the optical density at pH6 with that at pH 7 (three independent replicates for each). p-values are indicated alongside. Tests had 4 degrees of freedom. Significant p-values ($p \leq 0.05$) are highlighted in pink and the pH at which the metabolism was significantly higher is indicated alongside. Results were scored at 24 hours, minus water control. Succinic Acid M-M-Ester = Succinic Acid Mono-Methyl-Ester

4.5.16 Metabolism of compounds as carbon sources is increased at alkaline pH when bacteria are pre-incubated in LB.

To investigate whether the metabolism of compounds was also similar at pH 6 and pH 7 in LB media the same Biolog GN2 plate metabolism assay was used as before. Bacterial suspensions were pre-incubated in LB at pH 6 or pH7 and tetracycline was added prior to the inoculation of P.s.t into GN2 Biolog plates to prevent new protein synthesis.

Interestingly the metabolism of many compounds was found to be significantly increased when P.s.t was pre-incubated at pH7 compared to pH 6 (Table 4.12). This result is the opposite of the result seen when P.s.t was pre-incubated in STA. The reasons for this remain unknown, but it is speculated that the high sodium chloride (NaCl) content of LB may influence the action of Na^+-linked transporters in *P. syringae*, including ABC family members (which function using ATP and therefore whose function should not be directly affected by pH) producing this unexpected result. This hypothesis could be tested in future using LB with lower sodium chloride content, such as the no salt LB used for electroporation.

Well	Carbon source	p-value		pH 6	pH 7	Well	Carbon source	p-value		pH 6	pH 7
A1	Water					E1	p-HydroxyPhenylacetic Acid				
A2	α-Cyclodextrin					E2	Itaconic Acid				
A3	Dextrin	0.164				E3	α-Keto ButyricAcid				
A4	Glycogen					E4	α-Keto Glutaric Acid	0.048	7		
A5	Tween 40					E5	α-Keto Valeric Acid				
A6	Tween 80					E6	D,L-Lactic Acid				
A7	N-Acetyl-D-Galactosamine					E7	Malonic Acid				
A8	N-Acetyl-D-Glucosamine					E8	PropionicAcid				
A9	Adonitol					E9	Quinic Acid				
A10	L-Arabinose					E10	D-Saccharic Acid	0.044	7		
A11	D-Arabitol					E11	Sebacic Acid				
A12	D-Cellobiose					E12	Succinic Acid	0.124			
B1	i-Erythritol					F1	Bromosuccinic Acid	0.037	6		
B2	D-Fructose					F2	Succinamic Acid				
B3	L-Fucose					F3	Glucuronamide	0.006	7		
B4	D-Galactose					F4	L-Alaninamide	0.014	7		
B5	Gentiobiose					F5	D-Alanine	0.019	7		
B6	α-D-Glucose	0.087				F6	L-Alanine	0.131			
B7	m-Inositol					F7	L-Alanyl-glycine	0.164			
B8	α-D-Lactose					F8	L-Asparagine	0.018	7		
B9	Lactulose					F9	L-Aspartic Acid	0.164			
B10	Maltose					F10	L-Glutamic Acid	0.042	7		
B11	D-Mannitol					F11	Glycyl-L-Aspartic Acid				
B12	D-Mannose	0.016	7			F12	Glycyl-L-Glutamic Acid	0.004	7		
C1	D-Melibiose					G1	L-Histidine				
C2	α-Methyl-D-Glucoside					G2	Hydroxy-L-Proline				
C3	D-Psicose					G3	L-Leucine	0.074			
C4	D-Raffinose					G4	L-Ornithine	0.007	7		
C5	L-Rhamnose					G5	L-Phenylalanine				
C6	D-Sorbitol					G6	L-Proline				
C7	Sucrose					G7	L-Pyroglutamic Acid				
C8	D-Trehalose	0.013	7			G8	D-Serine				
C9	Turanose					G9	L-Serine	0.084			
C10	Xylitol					G10	L-Threonine	0.178			
C11	Pyruvic Acid Methyl Ester					G11	D,L-Carnitine				
C12	Succinic Acid M-M-Ester					G12	γ-Amino ButyricAcid	0.174			
D1	Acetic acid					H1	Urocanic Acid				
D2	Cis-Aconitic acid					H2	Inosine				
D3	Citric acid					H3	Uridine				
D4	Formic acid					H4	Thymidine				
D5	D-Galactonic acid					H5	Phenyethyl-amine				
D6	D-Galacturonic acid					H6	Putrescine				
D7	D-Gluconic acid					H7	2-Aminoethanol				
D8	D-Glucosaminic acid					H8	2,3-Butanediol				
D9	D-Glucuronic acid					H9	Glycerol				
D10	α-HydroxybutyricAcid					H10	D,L-α-Glycerol Phosphate				
D11	β-HydroxybutyricAcid					H11	α-D-Glucose-1-Phosphate				
D12	γ-HydroxybutyricAcid					H12	D-Glucose-6-Phosphate				

OD_{620} 0.05-0.20	<0.05

Table 4.12

Metabolism of compounds by P.s.t pre-incubated in LB pH 6 or LB pH 7. Tetracycline was added to bacteria prior to inoculation into Biolog GN2 plates to prevent further protein synthesis. Student's 2-tailed t-tests were carried out on the results comparing the optical density at pH6 with that at pH 7 (three independent replicates for each). p-values are indicated alongside. Tests had 4 degrees of freedom. Significant p-values ($p \leq 0.05$) are highlighted in pink and the pH at which the metabolism was significantly higher is indicated alongside. Succinic Acid M-M-Ester = Succinic Acid Mono-Methyl-Ester

4.5.17 Metabolism of amino acids as carbon sources by *Pseudomonas syringae* is affected by the media used for pre-incubation and the pH of that media.

The previous two experiments demonstrated that the composition and pH of the media used for pre-incubation are important for determining the ability of *P.syringae* to utilise certain compounds as carbon sources.

Purchased Biolog GN2 plates only contain a sub-set of amino acids. Therefore, to study the effect of media composition and pH on amino acid utilisation in particular, custom Biolog plate assays were used. P.s.t was pre-incubated in synthetic tomato apoplast (STA) or LB media at pH 6 (which is most similar to the uninfected apoplast colonised by P.s.t) or pH 7 (which resembles the apoplast after a period of bacterial proliferation), prior to inoculation into custom Biolog plates. The metabolism of amino acids was recorded after 24 hours.

In the first assay no tetracycline was added to the bacteria thereby allowing the synthesis of new proteins after inoculation. Results showed no difference in amino acid metabolism whether bacteria were pre-incubated in LB or STA, or whether the media was at pH 6 or pH 7 (Table 4.13).

In a second assay tetracycline was added to the bacteria prior to inoculation preventing further protein synthesis, according to the method of Rico and Preston (2008). The metabolised compounds should therefore reflect the nutrient utilisation pathways which are active in the bacteria after incubation in each treatment. Results show that the metabolism of glutamic acid, glutamine, aspartic acid, ethanolamine, asparagine, alanine, serine and GABA was increased after pre-incubation in STA (at either pH 6 or pH 7) compared to LB. In addition the metabolism of aspartic acid, asparagine, alanine and serine was specifically increased in STA at pH 6 compared to LB at pH 6 or STA at pH 7 (Table 4.14).

These results further demonstrate that the pH of the culture media affects the ability of P.s.t to assimilate amino acids. In addition, these results show that pre incubation in STA activated nutrient assimilation pathways for certain amino acids, including abundant apoplastic amino acids such as aspartic acid, asparagine and serine, more strongly than in LB.

	Media			
Carbon source	**STA pH 6**	**STA pH 7**	**LB pH 6**	**LB pH 7**
Proline				
Leucine				
Histidine				
Glutamic Acid				
Glutamine				
Aspartic Acid				
Ethanolamine				
Asparagine				
Urocanate				
Alanine				
GABA				
Serine				
Glycine				

OD_{620} > 0.03	0.015 - 0.03	< 0.015

Table 4.13
Custom Biolog plate using amino acids as carbon sources. P.s.t was pre-incubated in synthetic tomato apoplast (STA) or LB at pH 6 or pH 7 as indicated. No tetracycline was added prior to inoculation into custom Biolog plates. Results are averages of three independent experiments. Metabolism of amino acids is not significantly different across all treatments.
STA= synthetic tomato apoplast. Result scored at 24 hours, minus water control.

	Media			
Carbon source	**STA pH 6**	**STA pH 7**	**LB pH 6**	**LB pH 7**
Proline				
Leucine				
Histidine				
Glutamic Acid	#	#		
Glutamine	*			
Aspartic Acid	*			
Ethanolamine				
Asparagine	*			
Urocanate				
Alanine	*			
GABA	#	#		
Serine	*			
Glycine				

OD_{620} > 0.03	0.015 - 0.03	< 0.015

Table 4.14
Custom Biolog plate using amino acids as carbon sources. P.s.t was pre-incubated in synthetic tomato apoplast (STA) or LB at pH 6 or pH 7 as indicated. Tetracycline was added prior to inoculation into custom Biolog plates to prevent further protein synthesis. Results are averages of three independent experiments. Metabolism of amino acids is not significantly different across all treatments. Results were scored at 24 hours, minus water control. * = Treatment produced significantly increased metabolism $p \leq 0.05$, Student's 2-tailed t-tests. Four degrees of freedom. # = No significant difference between pH treatments using the same media for pre-incubation, but there is a significant difference between the two media treatments. The symbol is placed in the media and pH combination which resulted in increased metabolism.

4.6 Discussion.

Evidence of the nutritional specialisation of *P. syringae* pathovars can be seen found in many taxonomic studies, particularly from the late 1960s when the species and pathovar designation of *Pseudomonads* were established on the basis of their performance in nutrient utilisation and specific phenotypic tests rather than DNA sequence data (Thornley, 1960; Lelliott et al., 1966; Misaghi and Grogan, 1969). These studies of plant-pathogenic and non plant-pathogenic *Pseudomonas*, demonstrated that *P. syringae* shows a reduced ability to assimilate nutrients, particularly amino acids, compared to other *Pseudomonas* species. More recently these results have been supported by bioinformatic analysis of sequenced *Pseudomonas* genomes, which have shown reductions and diversifications in different protein families in plant-pathogenic compared to non plant-pathogenic *Pseudomonas* species. One particular example is the reduction in the amino acid permease domain proteins in *P. syringae* compared to other *Pseudomonas* species (Studholme et al., 2005), which has been hypothesised to provide a genomic basis for the restricted ability of *P. syringae* to assimilate amino acids compared to non plant pathogenic *Pseudomonas* species (Rico and Preston, 2008).

One explanation which has recently been suggested in order to explain the observed genomic and experimental data is that *P. syringae* has become specialised to use a limited number of nutrients present in the plant apoplast (Rico and Preston, 2008). This hypothesis has long been considered by researchers, but for the first time studies have specifically begun to provide experimental data describing the nutrient composition of the leaf environment, including the apoplast, guttation fluid droplets and the leaf surface, all of which indicate that the plant niche is nutrient limited compared to other *Pseudomonas*-inhabited niches such as the rhizosphere (Andrews and Harris, 2000; Solomon and Oliver, 2001; Pilot et al., 2004; Rico and Preston, 2008).

The aim of this chapter was to support and build on previous findings that *P. syringae* is nutritionally limited, particularly with respect to amino acid utilisation. Furthermore, this chapter aimed to use data describing the nutrient composition of the leaf environment to create new synthetic media which can be used to mimic guttation fluid and apoplast. Finally, as the pH of the plant apoplast is known to alter both during infection and diurnally (Grignon and Sentenac, 1991), and pH is predicted to influence the efficacy of certain types of transporters, the effect of pH on nutrient utilisation was investigated.

Results indicated that P.s.t can use fewer amino acids for growth compared to the representative non plant-pathogenic species, PAO1 and Pf-5 (Table 4.4). These results are complimentary to previous work which describes that *P. syringae* pathovars can metabolise fewer amino acids as carbon or nitrogen sources than other *Pseudomonas* species (Rico and Preston, 2008). In addition, the growth of *P. syringae iṇvitro* was pH limited compared to non plant-pathogenic *Pseudomonas*. The growth of three sequenced *P. syringae* pathovars was inhibited in acidic media (pH 4.5) or alkaline media (pH 9). In contrast, the non plant-pathogenic species PAO1 and P. fl. SBW25 were capable of similar growth at pH 4.5 and pH 9 as they were in the intermediate conditions of pH 5 to pH 8. These results support a hypothesis that non plant-pathogenic *Pseudomonas,* which generally inhabit a broad range of environmental niches, may encounter a correspondingly heterogeneous range of acidic and alkaline conditions. In contrast, the apoplast niche colonised by *P. syringae* is reported to be a relatively constant, mildly acidic environment (Grignon and Sentenac, 1991), thereby potentially reducing the selection pressure on *P. syringae* to possess extreme pH tolerance systems.

The growth media used in pH tolerance experiments was unbuffered. Therefore an additional experiment was carried out to investigate whether the observed results were due to non plant-pathogenic *Pseudomonas* modifying the pH of the media over time, either deliberately or as a side effect, which would give the appearance of an ability to grow in a wider range of conditions than *P. syringae.* The pH of unbuffered LB media (pH 6.6) was monitored for 24 hours during the growth of three *P. syringae* pathovars (Ps.t., P.s.ph and P.s.s), and the non plant-pathogenic species PAO1 and P. fl SBW25. All *Pseudomonas* species were observed to alkalinise the growth media over time, although this change was by less than 1 pH unit in all cases. The largest change in pH (from 6.6 to 7.3) was observed during the growth of PAO1. However, this species also reached the highest optical density after 24 hours growth. When bacterial growth (measured by the increase in optical density) was taken into account, the pH change in the growth media was found to be similar for all *Pseudomonas* species, indicating that no one species was altering the pH of the media significantly more or less than any other. Previous reports exist of *P. aeruginosa* (Yates et al., 2002) and *P. fluorescens* (Christen and Marshall, 1984) alkalinising the pH of LB over time. In both cases the pH increase is stated as being due to the bacteria utilising nutrients present in the media, such as the decarboxylation of amino acids over time to produce amines (Christen and Marshall, 1984).

The tomato apoplast has been shown to contain 17 amino acids, plus the non-protein amino acid GABA (Rico and Preston, 2008). Within the nutrient-limited environment of the tomato apoplast amino acids may provide an important source of carbon or nitrogen for growth or metabolism. The ability of P.s.t to derive a growth benefit from the present of any of the amino acids present in the tomato apoplast was investigated. Each amino acid was added at 0.5 mM concentrations to M9 minimal media and the growth increase, if any, was recorded in comparison to M9 without any amino acid added.

Nine amino acids present in the tomato apoplast significantly increased the growth of P.s.t (Figure 4.6A). Of these, glutamic acid, serine, glutamine, aspartic acid and GABA could also be used as the sole source of carbon, nitrogen or carbon and nitrogen for growth by P.s.t (Table 4.4) and were also metabolised as a carbon or nitrogen source in Biolog metabolism assays (Tables 4.1 and 4.2). These amino acids were among the most abundant in the tomato apoplast (Rico and Preston, 2008) and uptake of these amino acids may therefore benefit the growth of P.s.t *in planta*. Other amino acids which increased the growth of P.s.t included amino acids such as proline, alanine, and arginine which could be metabolised by P.s.t. but could not be used as both a carbon and nitrogen source for growth. These results indicated that even those amino acids which cannot be used by *P. syringae* to support growth, may provide some fitness benefit *P. syringae* in the apoplast, (in this case benefit was measured as an increase in growth), when the amino acid can be metabolised by *P. syringae*, possibly through being directly incorporated into proteins after uptake.

There was not a strong correlation between the abundance of an amino acid in the apoplast and the size of the growth increase of P.s.t when this amino acid was added to M9 (Figure 4.6B). However, it was interesting to note that seven out of the nine amino acids which significantly increased the growth of P.s.t, were the most abundant amino acids in the tomato apoplast. P.s.t has previously been demonstrated to possess nutrient utilisation pathways for amino acids that are abundant in the tomato apoplast, as measured by the metabolism of those amino acids (Rico and Preston, 2008). Therefore we can hypothesise that *P. syringae* may derive a growth benefit from the presence of these amino acids in the apoplast, even though some of these amino acids cannot be used directly as a source of energy for growth. In addition, eight of these amino acids could be used as substrates which are ultimately converted to ammonia or enter the TCA cycle, in 2 of fewer steps making them metabolically efficient sources of energy (Table 4.15, below). Therefore, *P. syringae* appears to possess nutrient

utilisation pathways for amino acids which are abundant in the tomato apoplast, and which can be converted from the raw amino acid, to the beneficial product (energy, via the TCA cycle, or ammonia) in the smallest number of steps.

		Minimum number of reactions from the amino acid to:	
	Amino acid	Ammonia (NH_3)	TCA Cycle
Amino acids which increase growth	GABA	3	2
	Asp	1	1
	Glu	2	1
	Gln	1	2
	His	1	5
	Leu	3	**0**
	Asn	1	1
	Pro	4	3
	Ser	1	2
	Ala	4	2
Amino acids which do not increase growth	Gly	3	3
	Phe	3	5
	Val	**0**	**0**
	Ile	**0**	**0**
	Cys	**0**	**0**
	Trp	2	3
	Thr	4	4
	Lys	**0**	**0**
	Met	**0**	**0**

Table 4.15
Table showing amino acids and the minimum number of metabolic reactions (from the Kegg database (Kanehisa et al., 2006)) between the amino acid and either the TCA cycle or NH_3. A pathway is said to exist between the amino acid and either the TCA cycle or NH_3 if there is a connected sequence of distinct reactions between these two metabolites such that the product of one reaction acts as a substrate in the next reaction. Amino acids are divided into those which significantly increased the growth of P.s.t when added to complete M9 minimal media (top), and those which did not significantly increase growth (bottom). Amino acids which are predicted to be assimilated in 2 or fewer steps have been coloured yellow. In general amino acids which significantly increased the growth of P.s.t can be assimilated into either the TCA cycle or converted to ammonia in 2 or fewer steps. Data from (Mithani et al., Manuscript in preparation)

In order to mimic the changing apoplastic pH during infection, the pH of M9 minimal media was adjusted to pH 5.5 or pH 8. The ability of *P. syringae* to gain a growth benefit (measured as a growth increase), from the presence of amino acids at each of these pH values was then investigated. As the pH of the culture media was increased from pH 5.5 to pH 8, the ability of P.s.t to benefit from the addition of several amino acids decreased. In particular, the ability of P.s.t to gain a growth benefit from the addition of aspartic or glutamic acid dropped markedly (Table 4.5). This effect was not seen for all amino acids tested, for example P.s.t gained the same increase in growth when arginine or asparagine were added to M9 media at pH 5.5 or at pH 8.

The *P. syringae* genome contains many amino acid transporters, which function by different mechanisms such as ATP hydrolysis, chemiosmotic energy, or passive processes such as diffusion (Saier, 2000). Furthermore, the same amino acid may be transported by transporters powered by different mechanisms, only some of which may be influenced by external pH. These results suggest that for some amino acids all transporters capable of transporting the amino acid may be powered by pH-sensitive mechanisms. Alternatively, these results could indicate a pH effect on the ionic form of certain amino acids, which might reduce their likelihood of being transported into the cell.

In contrast, for amino acids such as arginine, there was minimal or no pH effect on growth. Examples of transporters which might function independently of pH include the ABC (ATP-binding component) transporters, which utilise ATP-hydrolysis to transport substrates (Saier, 2000). Transporters which might be influenced by external pH include those of the APC superfamily, which function by co-transporting small molecules such as protons or sodium across a gradient (Saier, 2000). The APC superfamily includes the amino acid permeases, so it can be hypothesized that a reduced efficacy of aa_permease transporters in certain conditions, such as the increasingly alkaline environment of the apoplast during infection, may result in a selection pressure for the loss of such transporters. Interestingly, the utilisation of both histidine and GABA appeared to be slightly inhibited at pH 8, leading to the hypothesis that the histidine and GABA aa_permeases, (along with any other transporters for these amino acids) may be inhibited in alkaline conditions, such as those observed in the apoplast during the latter stages of infection.

Using a combination of plant species-specific amino acid data, a literature survey of abundant plant-derived sugars, and M9 minimal media, three synthetic growth media were developed. These were based on the amino acids present in the tomato apoplast as reported by Rico and Preston (2008) and *A. thaliana* apoplast and guttation fluid as reported by Pilot and coworkers (2004). As Table 1.3 in Chapter 1 shows, there can be considerable variation in measurements of amino acid abundance between studies. Therefore it is important to remember that the synthetic media created in this study reflects the amino acid abundance measured in these particular study conditions.

The tomato apoplast has been previously reported to provide a complete growth medium for *P. syringae* (Rico and Preston, 2008). Furthermore P.s.t achieved better growth, (measured by a higher optical density), in extracted apoplast compared to

minimal media such as HIM or M9 (Figure A1, Appendix). Therefore the growth of P.s.t in the newly created synthetic media was investigated and compared to the growth in modified M9 minimal media on which the synthetic media was based.

A. thaliana guttation fluid was reported to contain only three amino acids, histidine, glutamine and aspartic acid (Pilot et al., 2004). The addition of these amino acids to M9 minimal media, customised with a combination of plant-derived sugars to create a synthetic *A. thaliana* guttation fluid (GF), did not result in significantly increased growth of P.s.t. In contrast, the growth of P.s.t was significantly increased compared to M9 in both synthetic *A. thaliana* (SAA) and synthetic tomato (STA) apoplast media. The amino acids which contributed most significantly towards the growth of P.s.t in each of the synthetic media, were those which were most abundant (abundant amino acids in the tomato apoplast can all be used by P.s.t for growth). In SAA these amino acids were glutamine, serine and proline while in STA they were GABA, aspartic acid and glutamic acid. All of these amino acids have been shown to provide a growth increase to P.s.t when added as a supplement to complete M9 minimal media, and all can support the growth of P.s.t as a sole carbon or nitrogen source.

In order to compare STA as a proxy for extracted tomato apoplast, the growth of P.s.t in both these media was compared. The growth of P.s.t was significantly less in the STA than in extracted apoplast. This was probably due to the presence of additional compounds the tomato apoplast which were not added to the synthetic medium. However, the nutrient utilisation profile of P.s.t pre-incubated in STA and extracted tomato apoplast was very similar (Table 4.10) indicating that the presence of amino acids in synthetic apoplast are important for activating the nutrient utilisation pathways which are seen following incubation in extracted tomato apoplast (Rico and Preston, 2008). Therefore, STA may represent a cheap and easily made mimic for extracted apoplast in studies investigating nutrient utilisation pathways which may be activated *in planta*. Furthermore as more data becomes available describing the

The pH of STA, M9 and SAA was increased between pH 5 and pH 7.5 and the growth of P.s.t at each pH was monitored. At pH 5.5, pH 6 and pH 6.5 the growth of P.s.t in STA and SAA was significantly higher than in M9 media. However at values outside of this range, the growth of P.s.t was not significantly different to that in M9 media. This indicated that in conditions of pH 5 or pH 7, the added amino acids in STA and SAA did not provide any growth benefit to P.s.t. This result is in keeping with previous findings

that the growth of P.s.t was increased in the presence of certain amino acids at pH 5.5, but not pH 8. Therefore, a relatively constant pH may be important for *P. syringae* to efficiently take up amino acids *in planta*.

Using the synthetic tomato apoplast (STA) in a Biolog assay format it was then possible to investigate whether the nutrient utilisation profile of P.s.t varied when bacteria were pre-incubated in STA at pH 6 compared to pH 7. These conditions were chosen to mimic the apoplast pH immediately following colonised by P.s.t or after alkalinisation as a result of bacterial proliferation, respectively. Results indicated that the metabolism of many compounds was significantly higher at pH 6 including several plant-derived sugars and the amino acids alanine, aspartic acid, glutamic acid and GABA. Again, this was in keeping with previous data which indicated that amino acid transporters may be inhibited in more alkaline environments. Conversely, pre-incubating P.s.t in LB media at pH 6 pr pH 7 resulted in an increase in metabolism of compounds at pH 7. However, LB is a complex media and the effect on nutrient assimilation pathways of any component of LB is not known. Subsequent experiments analysing the metabolism of amino acids after pre-incubation in LB compared to STA, at pH 6 or pH 7, revealed that most amino acids were metabolised more after incubation in STA than LB, and at pH 6 than pH 7 (Table 4.14). Therefore, in general metabolism of compounds as carbon sources by P.s.t is increased following incubation in STA and at pH 6, conditions most similar to those of the plant apoplast.

The amino acid composition of the tomato apoplast is known to alter during infection, with several amino acids becoming less abundant (Arantza Rico, personal communication). Therefore, when observing the present distribution of aa_permease transporters, which function using chemiosmotic energy and may be affected by increasing pH of the apoplast, it is possible to hypothesise that a decrease in efficacy of transporters at increased pH may coincide with the increasing scarcity of particular amino acids which might make the cost of their uptake proportionately higher than the benefit of amino acid assimilation. This would therefore provide a positive selection pressure on bacteria which had lost these transporters.

In conclusion, the results presented in this chapter demonstrate that P.s.t can utilise a restricted number of amino acids for growth. However, these amino acids are present in the tomato apoplast. Furthermore, amino acids which cannot be used for growth, but

can be metabolised by P.s.t, may still provide a growth benefit to bacteria in the apoplast. In addition, this chapter has demonstrated the importance of pH for amino acid assimilation, possibly due to the effect of external pH on the mechanism of particular transporters. pH, along with media composition, has also been shown to play a role in the activation of nutrient assimilation pathways in P.s.t, and therefore the pH of the apoplast may represent another important cue for the induction nutrient uptake or assimilation mechanisms. Finally, three synthetic media were developed. In particular, the synthetic apoplast mimics STA and SAA were shown to provide a complete growth medium for P.s.t, and support increased growth compared to minimal media. In addition, pre-incubation of P.s.t in STA produced a similar carbon utilisation profile as extracted apoplast. Therefore this media may be a useful apoplast mimic for future studies.

Appendix.

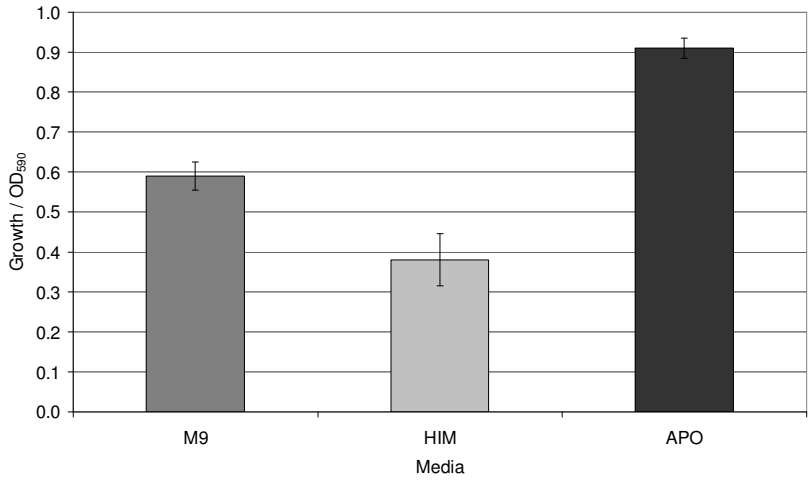


Figure A1.
Comparison of the growth of P.s.t at 24 hours, in M9, hrp-inducing minimal media (HIM) and extracted tomato apoplast (APO). The growth of P.s.t in HIM is significantly less than M9 minimal media. The growth of P.s.t in APO is significantly higher than in M9 or in HIM.

Carbon source	Same media but different pH		Same pH in different media	
	STA pH 6 vs STA pH 7	LB pH 6 vs LB pH 7	STA pH 6 vs LB pH 6	STA pH 7 vs LB pH 7
Proline	0.073	0.057	0.246	0.250
Leucine	0.207	0.188	0.235	0.078
Histidine	0.162	**7 0.021**	0.334	0.124
Glutamate	0.101	0.214	**STA 0.009**	**STA 0.008**
Glutamine	0.189	**6 0.026**	0.158	**STA 0.007**
Aspartate	**6 0.012**	**7 0.047**	**STA 0.000**	0.161
Ethanolamine	0.103	0.476	0.096	0.476
Asparagine	**6 0.001**	0.055	**STA 0.010**	**LB 0.001**
Urocanate	0.279	0.145	0.303	**STA 0.004**
Alanine	**6 0.037**	0.087	**STA 0.050**	0.359
GABA	0.474	0.391	0.094	**STA 0.012**
Serine	**6 0.050**	0.413	0.389	**STA 0.037**
Glycine	0.309	0.429	0.186	0.079

Carbon source	Media			
	STA pH 6	STA pH 7	LB pH 6	LB pH 7
Proline				
Leucine				
Histidine				
Glutamic Acid				
Glutamine				
Aspartic Acid				
Ethanolamine				
Asparagine				
Urocanate				
Alanine				
GABA				
Serine				
Glycine				

OD_{620} > 0.03	0.015 - 0.03	< 0.015

Table A1

A. p-values for custom BIOLOG plate with tetracycline. p-values were generated using Student's T-tests, comparing the OD_{620} values for metabolism by P.s.t. in the same media at different pH values (eg STA pH 6 vs STA pH 7 compares the metabolism in synthetic tomato apoplast at pH 6 and pH 7) and in different media at the same pH (eg. LB pH 6 vs LB pH 7). A significant result is indicated by a number (eg 6 indicates metabolism was higher at pH 6), or the name of the media (STA or LB). B. The OD_{620} values are presented as a reference.

Chapter 5: Chemotaxis by *Pseudomonas syringae* pv. tomato.

5.1 Overview.

Chemotaxis is the process by which motile bacteria sense changes in certain chemicals present in their environment and respond to these changes by moving to more favourable conditions. Plant-pathogenic *P. syringae* is nutritionally specialised compared to non plant-pathogenic *Pseudomonas* species, particularly with respect to amino acid metabolism (Rico and Preston, 2008). In addition, *P. syringae* was previously shown to be capable of using amino acids which are abundant in the plant apoplast as sources of carbon and nitrogen for growth (Chapter 3). In this chapter, the ability of *P. syringae* to chemotax towards apoplastic amino acids was tested. Furthermore, bioinformatics tools were used to investigate whether differences exist between *P. syringae* and non plant-pathogenic *Pseudomonas*, in protein domains with chemotaxis related functions.

P. syringae pv. tomato (P.s.t) was attracted to solutions of GABA, aspartate, glutamate, alanine, serine and isoleucine at the concentrations present in the tomato apoplast. In addition, P.s.t was attracted to synthetic tomato apoplast and synthetic *A. thaliana* apoplast, which contain organic acids, plant sugars and different combinations of amino acids. However, P.s.t was attracted more strongly to extracted tomato apoplast than any synthetic apoplast media, indicating that additional chemoattractants for P.s.t are present *in planta*.

Bioinformatic comparisons of *Pseudomonas* genomes indicated that *P. syringae* was overrepresented in several chemotaxis-related protein domains compared to non plant-pathogenic *Pseudomonas*. Consequently two operons with potential chemotaxis-related functions were identified in the *P. syringae* genome. These operons are present in various plant associated bacteria but are notably absent in the opportunistic human pathogen *P. aeruginosa*. Therefore genes within these operons may have functions relating to a plant-associated lifestyle.

5.2 Introduction.

Chemotaxis is the ability of motile bacteria to detect and respond directionally to specific chemicals in the environment (Wadhams and Armitage, 2004). As defined for *Escherichia coli*, chemotaxis refers to a response that is independent of uptake or metabolism of the chemoattractant (Adler, 1973). However, bacteria may also exhibit a metabolism-dependent form of chemotaxis known as energy taxis to migrate towards compounds which can be utilised as sources of energy (Wadhams and Armitage, 2004). The mechanism by which chemotaxis occurs in bacteria has been well studied and is now one of the best understood signalling pathways in biology. For a bacterial cell to respond chemotactically to its environment, it must be able to detect and respond to specific chemicals and changes in their concentration. Motile bacteria bias their movement towards an attractant gradient and away from a repellent. However, bacteria are too small to detect a spatial concentration gradient along their length. Therefore bacterial swimming consists of smooth 'runs' interrupted by 'tumbles' (Figure 5.1).

Figure removed for copyright reasons

Figure 5.1
Bacterial chemotaxis (from Webre *et al.*, (2003)).
Motile bacteria such as *E. coli* migrate towards attractants and away from repellents using a combination of runs and tumbles (A). Cells tend to exhibit longer periods of smooth swimming (runs) when migrating towards attractants; in contrast when swimming away from attractants they tend to tumble and change direction (B).

In the presence of a chemoattractant, bacteria bias their swimming pattern so that smooth runs are favoured and the tumbling frequency is decreased. In *E. coli*, the mechanics of directional swimming have been well-studied. Smooth swimming is the result of flagella all rotating in a counter-clockwise direction and coming together as a rotating bundle. Conversely, when heading towards a repellent the flagellar motors switch to a clockwise direction which forces the flagellar bundle apart and results in a tumble. Tumble frequency is the mechanism controlled in bacterial chemotaxis (Webre et al., 2003; Wadhams and Armitage, 2004).

The concentration of chemoattractant or repellent molecules is detected by methyl accepting chemotaxis proteins (MCPs). Detection of the chemoattractant or repellent may be via direct binding of the ligand to the MCP, or through periplasmic binding proteins, which act as an intermediate between the external environment and MCPs embedded in the inner membrane of the periplasm (Dwyer and Hellinga, 2004). Once a ligand activates an MCP it stimulates a signal transduction cascade resulting in a change of rotation of the flagellar motor, a switch between a 'run' and a 'tumble', and consequently a change of direction for the bacterium. Six cytoplasmic chemotaxis (Che) proteins, CheW, CheY, CheZ, CheR, and CheB are involved in the pathway between MCP and flagellum (Figure 5.2).

Briefly, CheA is the first protein to receive the chemotaxis signal inside the cytoplasm. CheA interacts with the MCPs, autophosphorylates, and then transfers the phosphate group to CheY and CheB. Phosphorylated CheY then interacts with the flagellar switch to increase the tumble frequency. CheB is involved in the adaptation response and phosphorylated CheB competes with a constitutive methyltransferase, CheR, to control the adaptation of the MCP to the attractant. CheZ interacts directly with CheY to remove the phosphate group and counteract the tumble signal. Although CheW is not directly involved in the signal cascade, this protein is essential for chemotaxis as cells lacking CheW can only rotate counterclockwise and therefore cannot tumble (Webre et al., 2003; Dwyer and Hellinga, 2004; Wadhams and Armitage, 2004).



Figure 5.2
Chemotaxis proteins in bacteria, adapted from Wadhams, *et al.,* (2004).
A dimeric methyl-accepting chemotaxis protein (MCP) is shown interacting with a primary receptor for chemotaxis a periplasmic binding protein. Methyl-accepting chemotaxis proteins (MCP's) are chemoreceptors which adopt a conformational change upon an attractant (or repellent) binding which initiates an intracellular signalling cascade resulting in either clockwise rotation of the flagella (and bacterial 'tumbling' leading to a change in direction) or counterclockwise rotation of the flagella (leading to continued swimming in the same direction). Chemotaxis proteins CheW and CheA interact with a highly conserved signalling domain of the MCP in the cytoplasm. A change in attractant concentration induces autophosphorylation of CheA, which phosphorylates the response regulator CheY. Phosphorylated CheY then binds to the flagellar motor to bring about a change in direction. The dephosphorylation of phosphorylated CheY is accelerated by CheZ. Phosphorylated CheA also phosphorylates another response regulator the methylesterase CheB. Phosphorylated CheB competes with a constitutive methyltransferase, CheR, to control the adaptation of the MCP to the attractant.

Chemotaxis by plant-pathogenic bacteria may be important at various stages of the infection cycle. *P. syringae* occupies two environmental niches; the epiphytic foliar surface and the endophytic intercellular apoplast. Bacteria initially colonise the leaf surface prior entering the apoplast. The epiphytic environment has been demonstrated to be nutritionally heterogeneous (Leveau and Lindow, 2001; Beattie and Axtell, 2002) with the majority of nutrients found in particular locations on the leaf surface such as the base of trichomes, or around natural openings in the leaf such as hydathodes and stomata (Beattie and Axtell, 2002). Therefore, chemotaxis by *P. syringae* towards these locations may represent an important epiphytic survival strategy (Haefele and Lindow, 1987). Secondly, in order to gain entry to, and proliferate within the leaf apoplast, bacteria must first cross the leaf epidermis

through sites of natural entry such as hydathodes or stomata, or wound sites. In particular, stomata represent a major route of entry for *P. syringae* into the leaf apoplast. Although it is not yet known precisely how plant-pathogenic bacteria locate these openings, *P.syringae* has recently been shown to preferentially move towards open stomata (Melotto et al., 2006) leading to speculation that this could be driven by chemotaxis towards nutrients from the leaf interior (Schulze-Lefert and Robatzek, 2006).

The ability of plant-pathogenic or plant-associated species to chemotax towards plant-derived compounds has been demonstrated for numerous bacterial species. For example plant-pathogenic *Xanthomonas campestris* exhibited positive chemotaxis towards guttation fluid exuded from hydathodes (Hugouvieux et al., 1998), while the non-pathogenic rice epiphyte *Bacillus pumilus* was strongly attracted to rice exudates in the rhizosphere (Bacilio-Jiménez et al., 2003). The ability to chemotax was found to be essential for virulence of plant-pathogenic *Ralstonia solanacearum* on tomato (Yao and Allen, 2006; Tans-Kersten et al., 2001).

Studies focusing on chemotaxis by plant-pathogenic *Pseudomonas* species have demonstrated that *P. syringae* pv. glycinea exhibited positive chemotaxis towards apoplast extracted from the host plant soybean (Hattermann and Ries, 1989). It has also been noted that the guttation fluid secreted by hydathodes contains trace amounts of substances that may serve as chemical attractants as well as nutrient sources, and that chemotaxis by *Pseudomonas lachrymans* was directly correlated with the concentrations of amino acids and carbohydrates in these droplets (Chet et al., 1973). More recently it was also reported that the plant hormone ethylene serves as a chemoattractant for several *Pseudomonas* including *P. syringae* and also the plant-associated species *P. fluorescens* and *P. putida* (Kim et al., 2007). Finally, a non-motile mutant of *P. syringae* pv. phaseolicola 1448A was unable to chemotax towards plant exudates and showed a decreased ability to cause disease in bean leaves compared to the wild-type, even when bacteria were manually inoculated into leaves (Panopoulos and Schroth, 1974). Similarly, the loss of flagella reduced the virulence of *P. syringae* pv. tabaci in tobacco plants (Ichinose et al,. 2006). Taking all these studies together it appears reasonable to propose that chemotaxis plays an important role in colonisation and infection by plant-associated and plant-pathogenic bacteria.

Chemotaxis towards plant-derived compounds has also been demonstrated for plant-associated (non plant-pathogenic) *Pseudomonas* species. For example, *P. fluorescens* WCS365, used as a biocontrol strain, was chemotactically attracted to the exudate of tomato

seeds and roots (Lugtenberg et al., 1999), along with individual organic acids, particularly malic and citric acids, and some amino acids present in tomato root exudate (de Weert et al., 2002). Chemotaxis was not detected by *P. fluorescens* WCS365 towards any of the major sugars present in tomato root exudate (de Weert et al., 2002). This result agreed with the previous finding that mutants of *P. fluorescens* WCS365, defective in sucrose, glucose, and fructose catabolism, colonised the roots of tomato plants as effectively as the wild-type (Lugtenberg et al., 1999) indicating that these components of the root exudate are not essential for nutrition. In contrast, a *P. fluorescens* WCS365 mutant with reduced malic acid utilisation was impaired in tomato root colonisation (Lugtenberg et al., 2001).

Plant associations with plant growth-promoting rhizobacteria such as *P. fluorescens*, provide protection from soil-borne plant pathogens by mechanisms such as competitive exclusion, and also through colonising the root systems, can trigger induced systemic resistance (ISR) in aerial parts of the plant (Heil and Bostock, 2002). Recently, it has been demonstrated that plants are capable of exploiting bacterial chemotaxis in order to selectively recruit plant growth-promoting rhizobacteria. Rudrappa and coworkers (2008) reported that *A. thaliana* plants infected with plant-pathogenic P.s.t secreted L-malic acid from the roots. L-Malic acid specifically attracted and enhanced root binding and biofilm formation on infected seedlings by the plant growth-promoting rhizobacterium *Bacillus subtilis* strain FB17. A malic acid transporter mutant of *B. subtilis* no longer exhibited positive chemotaxis towards infected roots (Rudrappa et al., 2008). Therefore root-secreted L-malic acid was proposed as a potential example of a plant-derived compound which is secreted in order to stimulate chemotaxis of beneficial bacteria towards plant roots (Rudrappa et al., 2008).

Some authors have suggested that plant pathogenic bacteria may be able to distinguish resistant and susceptible cultivars and chemotax towards the susceptible cultivar (or may be repelled more by the resistant cultivar). For example, experiments with *P. lachrymans* demonstrated that this species is attracted more to exudates from susceptible rather than resistant soybean cultivars (Chet et al., 1973). In addion, *Ralstonia solanacearum*, is more strongly attracted to exudates of the host plant tomato, than the non-host plant rice (Yao and Allen, 2006), and *Xanthomonas oryzae* is attracted to hydathode exudates from susceptible rice cultivars more strongly than resistant cultivars (Feng and Kuo, 1975). However, two studies have indicated that *P.syringae* pathovars tomato and glycinea are unable to distinguish between resistant and susceptible exudates of the host plants tomato and soybean respectively (Cuppels, 1988; Hattermann and Ries, 1989).

Chemotaxis has also been reported by bacterial strains towards other microorganisms in their environment. In particular, *P. fluorescens* LAM has been shown to be chemotactically

attracted to the fungus *Macrophomina phaseolina* in soil (Singh et al., 2002). *P. fluorescens* WCS365 was chemotactically attracted towards hyphae of *Fusarium oxysporum,* and in particular fusaric acid, a secondary metabolite secreted by *Fusarium* strains was an excellent chemoattractant (de Weert et al., 2004).

Bacterial chemotaxis towards certain compounds may be correlated with the ability of individual species to utilise those compounds as sources of energy for growth. For example, chemotaxis of *Pseudomonas* species B4 towards polychlorobiphenyls and chlorobenzoates was correlated with the ability of B4 to use these as carbon and energy sources (Gordillo et al., 2007), while *P. putida* G7 was shown to be attracted to the aromatic hydrocarbon biphenyl, a compound that does not serve as a growth substrate for this strain (Grimm and Harwood, 1997). In addition, *P. fluorescens* WBC-3 is attracted to multiple aromatic compounds, including methyl parathion or para-nitrophenol (PNP) which could be used as a sole source of carbon or nitrogen for growth. Interestingly, the disruption of PNP catabolic genes had no effect on chemotaxis towards these compounds suggesting that the chemotactic response was metabolism-independent (Zhang et al., 2008).

The amino acid content of the uninfected tomato apoplast has recently been established (Rico and Preston, 2008). In particular, it has been reported that several of the most abundant amino acids in the tomato apoplast could be metabolised and used for growth by *P. syringae* pv. tomato DC3000 (P.s.t), a host pathogen of tomato plants. However, compared to non plant-pathogenic *Pseudomonas*, *P. syringae* was nutritionally restricted and used fewer amino acids for metabolism or growth (see Chapter 4). This nutritional specialisation by *P. syringae* is hypothesised to result from inhabiting the nutritionally restricted plant apoplast (Rico and Preston, 2008). Inhabiting a nutritionally restricted niche such as the apoplast may also influence chemotaxis, as there may be fewer compounds available to act as chemoeffectors and bind to MCP proteins (classical chemotaxis), or be utilised as a source of energy (energy taxis). However, components of the apoplast may provide important cues for bacteria gaining entry into the endophytic niche. Therefore it is interesting to compare the chemotactic response of P.s.t to various amino acids present in the plant apoplast, to synthetic apoplast and guttation fluid media, and to extracted tomato apoplast. In addition, as bioinformatic studies have revealed a potential genetic basis for the observed nutritional specialisation of *P. syringae*, a bioinformatic comparison of chemotaxis proteins in *P. syringae* and non plant-pathogenic *Pseudomonas* genomes will be carried out. A previous bioinformatic survey of signal transduction proteins in 167 genomes from a diverse range of bacteria and archaea suggested that *P. syringae* genomes contained an unusually high number of MCP proteins (Galperin, 2005). Therefore further bioinformatic

analysis of chemotaxis-related proteins in *P. syringae* and non plant-pathogenic *Pseudomonas* genomes may reveal significant underlying differences for future studies.

5.3 Objectives.

- Profile the ability of P.s.t to exhibit positive chemotaxis towards amino acids, synthetic tomato and synthetic *A. thaliana* apoplast, synthetic *A. thaliana* guttation fluid and extracted tomato apoplast.
- Use bioinformatic techniques to compare the genomes of sequenced *Pseudomonas* species and highlight any differences in the number and nature of chemotaxis-related proteins.

5.4 Materials and methods.

5.4.1 Bacterial strains and abbreviations.

Bacterial strains and abbreviations were used as shown in Chapter 2.

5.4.2 Growth *in vitro.*

All strains were routinely cultured on LB agar medium at 28°C as described in Chapter 2.2. Overnight cultures were washed twice in the media of interest and resuspended at a starting density of $1x10^6$ cfu ml^{-1} unless otherwise stated.

5.4.3 Chemotaxis assay.

A chemotaxis assay originally developed for *E. coli* by Adler (1973) and optimised for *P. syringae* by Cuppels (1988) was used with modifications (Figure 5.1). Briefly, for each replicate assay $5x10^6$ cfu ml^{-1} bacteria were suspended in chemotaxis buffer PME (10 mM potassium phosphate (pH 7.2) 1 mM $MgCl_2$, and 0.1 mM EDTA). Twenty five mililitres of bacteria-PME solution was placed in a sterile 90 mm petri dish (Scientific Laboratory Supplies, UK) in a Horizontal HA448BO laminar airflow cabinet (Thermo Fisher Scientific, MA, USA). Two hundred microlitres of test compound made up in PME buffer was taken up into a sterile 1ml syringe (Fisher, UK) and submerged in the bacterial-PME solution using sterile tweezers. After 60 minutes incubation at room temperature the contents of the syringe were expelled into a sterile 1.5ml microfuge tube within the laminar airflow cabinet. Samples were then serially diluted and 100µl aliquots were spread onto LB agar plates supplemented with half-strength CFC (Oxoid Limited, UK), a combination of antibiotics which preferentially select for the growth of pseudomonads. The number of bacteria per syringe was determined after 48 hours static incubation at 28°C Results were expressed as the mean of five to ten

replicate assays (duplicate plate counts for each assay). Control syringes, which contained PME alone, were included with each assay.

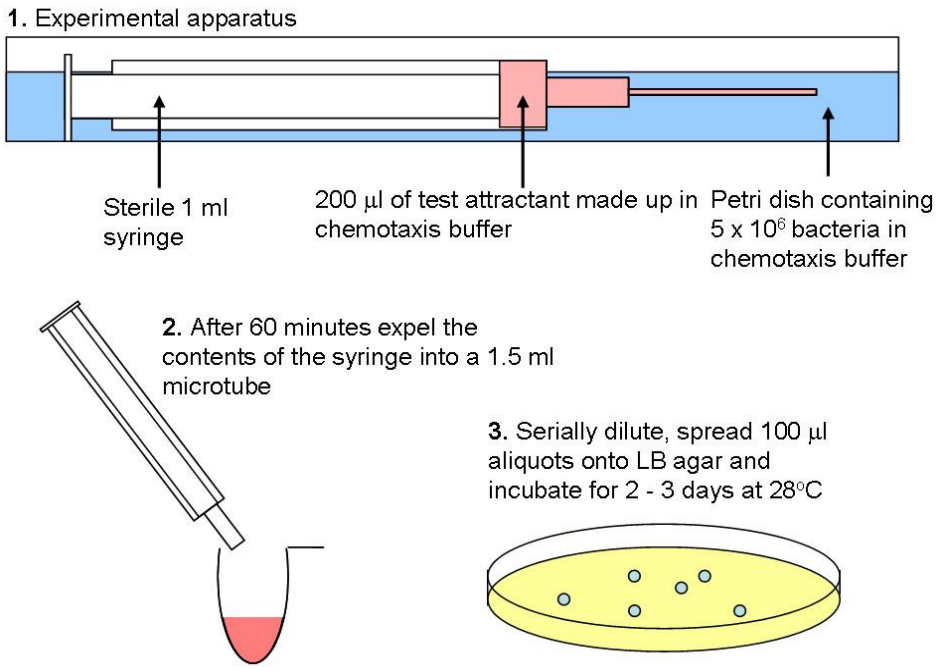


Figure 5.1
Chemotaxis assay used in this chapter. 1. Test attractant is taken up into a sterile syringe and submerged in a bacterial solution. 2. After 60 minutes the contents of the syringe are expelled. 3. Serial dilutions are plated out onto agar.

5.5 Results

5.5.1 Literature survey of previous research on chemotaxis to amino acids by *Pseudomonas* species.

Chemotaxis towards amino acids has been demonstrated in several species of *Pseudomonas*. In order to gain an overview of the number and concentration of amino acids which have stimulated a positive chemotactic response, a literature survey was carried out (Table 5.3). This survey was not intended to be exhaustive, and has focused on papers which test multiple amino acids, and also those which have tested species from across the *Pseudomonas* genus.

Results indicate that *P. aeruginosa* and *P. fluorescens* species are capable of chemotaxis to a wide range of amino acids. However, the chemotactic response of *P. syringae* species to amino acids has not been comprehensively assessed.

Attractant	(+) = chemotaxis					nt = not tested in this study			
***Pseudomonas* species**	PAO1	PAO1	PAO1	Pfl WCS365	Pfl LAM1 / 2	Pfl E-20	Pfl 1013	P.s.t	P.s.g
Reference	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Alanine	+	nt	nt	+	+	nt	+	nt	nt
Arginine	+	+	+	+	+	+	–	nt	nt
Asparagine	+	nt	nt	+	nt	nt	nt	+	+
Aspartate	+	nt	nt	+	+	nt	–	+	nt
Cysteine	+	nt	nt	+	nt	nt	–	nt	nt
Glutamine	+	nt	nt	+	nt	nt	nt	+	nt
Glutamate	+	nt	nt	+	+	+	+	+	nt
Glycine	+	+	nt	+	nt	nt	+	nt	nt
Histidine	+	nt	nt	+	+	nt	+	nt	nt
Isoleucine	+	nt	nt	+	nt	nt	+	nt	nt
Leucine	+	+	nt	+	+	+	+	nt	nt
Lysine	+	nt	nt	+	+	nt	+	nt	nt
Methionine	+	+	nt	+	nt	nt	nt	nt	nt
Phenylalanine	+	nt	nt	+	nt	nt	+	nt	nt
Proline	+	nt	nt	+	nt	nt	+	+	nt
Serine	+	+	nt	+	nt	+	+	+	nt
Threonine	+	nt	nt	+	nt	nt	–	nt	nt
Tryptophan	+	nt	nt	+	nt	nt	nt	nt	nt
Tyrosine	+	nt	nt	+	nt	nt	nt	nt	nt
Valine	+	+	nt	+	nt	nt	nt	nt	nt
GABA	nt	nt	nt	+	nt	nt	nt	+	nt

Table 5.3
Summary of data extracted from previous studies describing the chemotactic response of various *Pseudomonas* species to amino acids. KEY: PAO1= *P. aeruginosa* PAO1 Pfl = *P. fluorescens* species, P.s.t= *P. syringae* pv. tomato, P.s.g= *P. syringae* pv. glycinea. nt= amino acid was not tested in this study. Reference key: 1= (Taguchi et al., 1997), 2= (Kelly-Wintenberg and Montie, 1994), 3= (Alvarez-Ortega and Harwood, 2007), 4= de Weert et al 2002, 5= (Singh et al., 2002), 6= (Lynch, 1980), 7= (Sood, 2006), 8= (Cuppels, 1988), 9= (Hattermann and Ries, 1989).

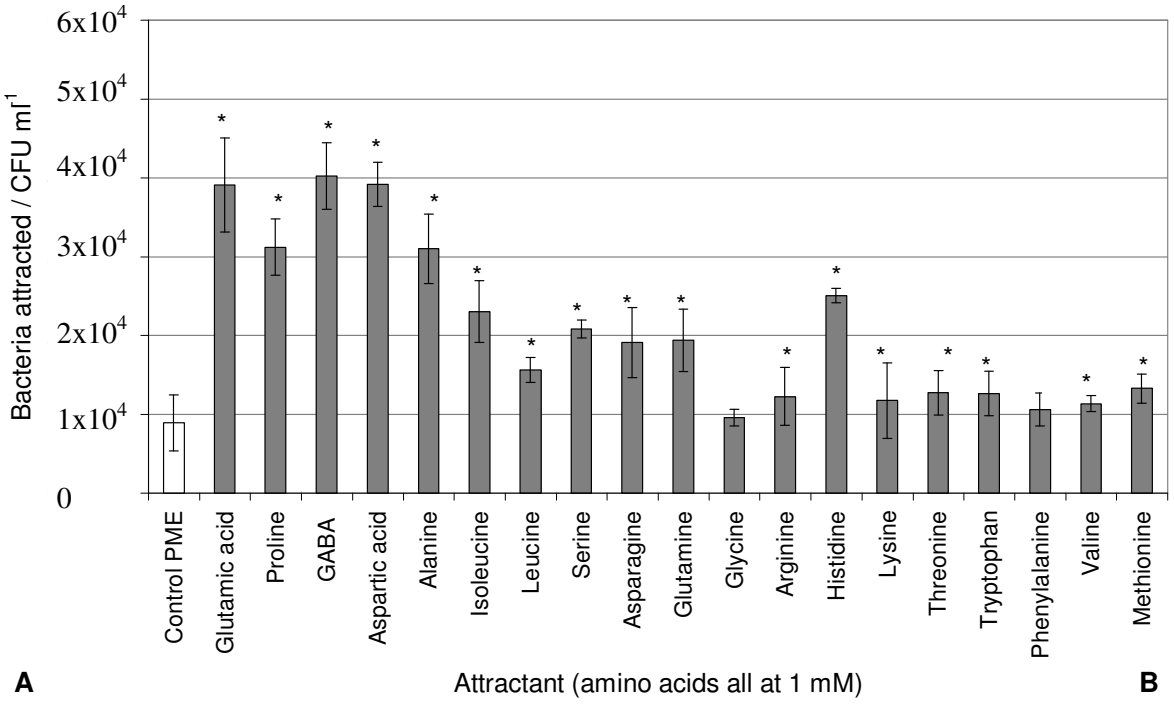
5.5.2 Chemotaxis by *P. syringae* pv. tomato to amino acids.

Bacteria sense and swim up gradients of chemicals to accumulate near the source of an attractant. Chemotaxis assays can exploit this fundamental characteristic of the chemotactic response. In a capillary assay, a microcapillary tube containing a solution of attractant is placed into a suspension of motile bacteria. Chemotactic cells respond to the concentration gradient that is formed as the attractant diffuses from the microcapillary tube by swimming up the gradient and into the tube. The tube is then removed and the number of cells it contains counted (Adler, 1973)

A modified capillary assay was used to assess the response of P.s.t towards a variety of amino acids, and particularly those amino acids that are abundant in tomato leaf apoplast. This assay used a syringe needle, attached to a syringe, as a microcapillary thereby allowing a defined amount of test attractant to be taken up prior to the assay. After 60 minutes the test attractant (along with any attractant-responsive cells) was easily dispensed into microfuge tubes for serial dilution. Diluted suspensions of test attractant and bacteria were plated out onto semi-selective agar plates and counted after 48 hours (see Figure 5.1).

Twenty amino acid solutions were made up in PME buffer. Each amino acid was tested at 1 mM to get an overview of the relative strength of chemotaxis towards each amino acid. Amino acids were also tested at the concentration found in the tomato apoplast, to assess chemotaxis towards these amino acids at biologically relevant concentrations. Chemotaxis towards each amino acid was compared to chemotaxis towards a control of sterile PME buffer and expressed as the relative increase in chemotaxis compared to this control (Relative Chemotactic Response - RCR).

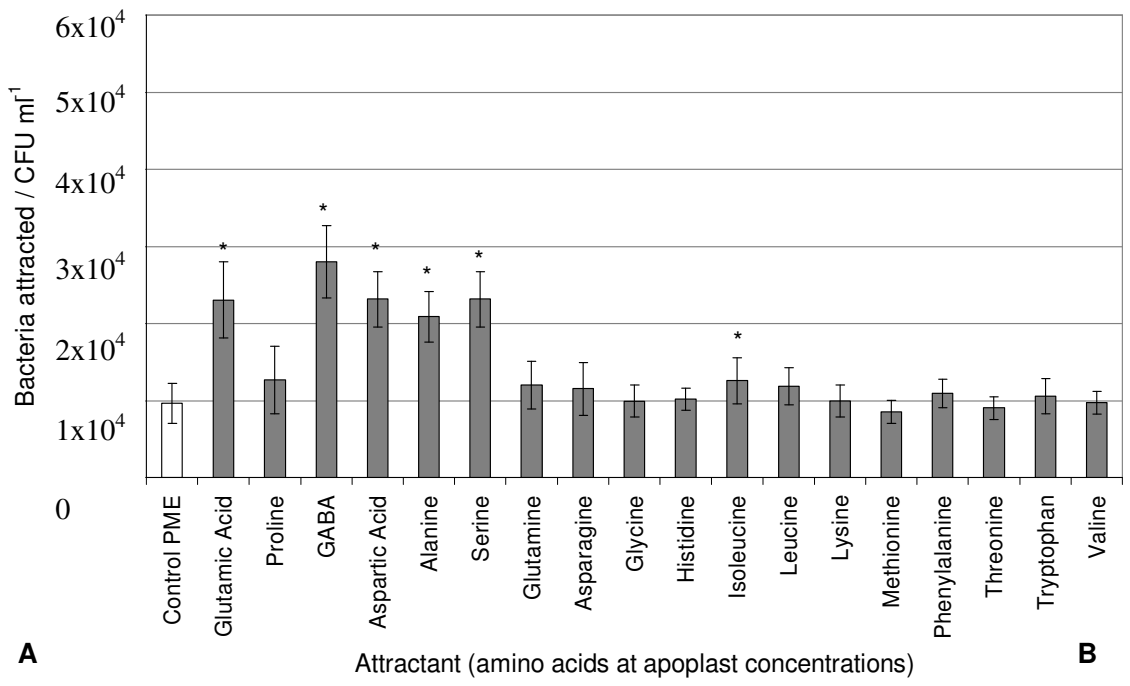
At 1 mM, all tested amino acids except glycine provoked a significant chemotactic response by P.s.t compared to the control (Figure 5.3). When amino acid solutions were made up at the concentrations present in the tomato apoplast, P.s.t exhibited significant chemotaxis towards glutamic acid, GABA, aspartic acid, alanine, serine and isoleucine (Figure 5.4 A and B). Glutamic acid, GABA, aspartic acid, alanine and serine represent the five most abundant amino acids in the tomato apoplast. Therefore the 'apoplastic concentration' solutions of these amino acids were the highest. In contrast, isoleucine is not abundant in the tomato apoplast. However, chemotaxis towards isoleucine by P.s.t was only just significant at the 95% confidence interval, as measured by a p-value of 0.049 in Student's T-tests.



Amino Acid	RCR	p-value
Glutamic Acid	4.59	0.000
Proline	3.61	0.000
GABA	4.67	0.000
Aspartic Acid	4.52	0.000
Alanine	3.57	0.000
Isoleucine	2.67	0.000
Leucine	1.84	0.000
Serine	2.40	0.000
Asparagine	2.21	0.004
Glutamine	2.28	0.000
Glycine	1.07	0.453
Arginine	1.40	0.002
Histidine	2.90	0.000
Lysine	1.38	0.039
Threonine	1.38	0.004
Tryptophan	1.50	0.000
Phenylalanine	1.19	0.053
Valine	1.27	0.020
Methionine	1.54	0.000

Figure 5.3

A. Chemotaxis by P.s.t towards amino acids at 1 mM. *=significant chemotaxis ($p \leq 0.05$) Student's 2-tailed t-tests comparing chemotaxis to PME with chemotaxis towards each amino acid. Error bars indicate standard deviations from at least five independent replicates. Tests have between 11 and 16 degrees of freedom depending on replicates. H_0=no increase in chemotaxis towards amino acids. **B.** Individual p-values are listed in Table B. Significant p-values are highlighted in pink. The relative chemotactic response (RCR) for each amino acid is the average increase in chemotaxis towards the amino acid, divided by chemotaxis towards the control of PME buffer. An RCR of 1 indicates no difference in the strength of chemotaxis towards the control and the amino acid.



Amino acid	Concentration (μM)	RCR	p-value
GABA	575	2.91	0.000
Aspartic Acid	145	2.49	0.000
Glutamic Acid	137	2.80	0.000
Alanine	67	2.22	0.000
Serine	51	2.14	0.003
Glutamine	34	1.25	0.078
Leucine	32	1.24	0.063
Glycine	29	1.03	0.145
Methionine	25	0.88	0.422
Phenylalanine	25	1.17	0.210
Valine	25	0.98	0.068
Lysine	22	1.04	0.523
Threonine	22	0.94	0.132
Isoleucine	19	1.73	0.049
Tryptophan	16	1.07	0.194
Proline	13	1.31	0.057
Asparagine	9	1.20	0.053
Histidine	7	1.06	0.432

Figure 5.4

A. Chemotaxis by P.s.t towards amino acids at the concentration they are found in the tomato apoplast (concentrations listed in decreasing order in Table B). *=significant chemotaxis ($p \leq 0.05$). Student's 2-tailed t-tests comparing chemotaxis to PME with chemotaxis towards each amino acid. Error bars indicate standard deviations from at least five independent replicates. Tests have between 11 and 16 degrees of freedom depending on replicates. H_0=no difference in chemotaxis towards amino acids compared to PME. **B.** Individual p-values are listed in Table B. Significant p-values are highlighted in pink. The relative chemotactic response (RCR) for each amino acid is the average increase in chemotaxis towards the amino acid, divided by chemotaxis towards the control of PME buffer. An RCR close to one indicates no difference in the strength of chemotaxis towards the control and the amino acid.

5.5.3 Analysis of amino acid chemotaxis by *Pseudomonas syringae* pv. tomato.

In Chapter 4, it was demonstrated that P.s.t was able to derive a growth benefit from the addition of certain amino acids, when they were added as a supplement to minimal media where alternative sources of carbon and nitrogen were provided. These amino acids included some which could be metabolised by P.s.t, in Biolog assays but were not used as a sole source of carbon or nitrogen for growth (e.g. histidine). These results suggested that P.s.t may derive a growth benefit from the presence of an amino acid which it can metabolise, even though it cannot utilise this amino acid directly for growth. There was not a strong correlation between the growth increase resulting from the addition of an amino acid to minimal media, and the abundance of that amino acid in the apoplast (Chapter 4, Section 4.5.4). However, we can now re-examine this data in combination with the chemotaxis data of Section 5.5.2 above, to test the hypothesis that P.s.t is more strongly attracted to those amino acids which it can derive a growth benefit from.

In Figure 5.5A, chemotaxis by P.s.t towards 1 mM of an amino acid was compared to the growth increase that the amino acid provided when added to complete M9 minimal media. There does not appear to be a strong correlation between chemotaxis and growth increase. In particular, chemotaxis towards GABA is similar to that of aspartic or glutamic acid, yet the latter two amino acids provide a much bigger increase in growth. This may be because P.s.t is unable to synthesise GABA (Alan Collmer, personal communication). However, there is relatively little chemotaxis to the majority of amino acids that are poor nutrient sources, which suggests that P.s.t may lack chemoreceptors for these amino acids and cannot show energy taxis towards them. In Figure 5.5B, chemotaxis towards 1mM of amino acid was compared to the abundance (in µM) of that amino acid in the tomato apoplast. The three most abundant amino acids (GABA, aspartic acid and glutamic acid) are also the three which P.s.t shows the strongest chemotaxis. However there are also examples of strong chemotaxis towards amino acids which are only weakly abundant in the apoplast such as histidine or asparagine. In Figure 5.6A, chemotaxis by P.s.t towards amino acids at the concentration found in the tomato apoplast, was compared to the growth increase that the amino acid provided when added to complete M9 minimal media. In Figure 5.6B, chemotaxis towards amino acids at the concentration found in the tomato apoplast, was compared to the abundance of that amino acid in the tomato apoplast. P.s.t was capable of significant chemotaxis towards GABA, aspartic acid, glutamic acid, alanine, serine and isoleucine. Of these, all except isoleucine are the most abundant amino acids in the tomato apoplast.

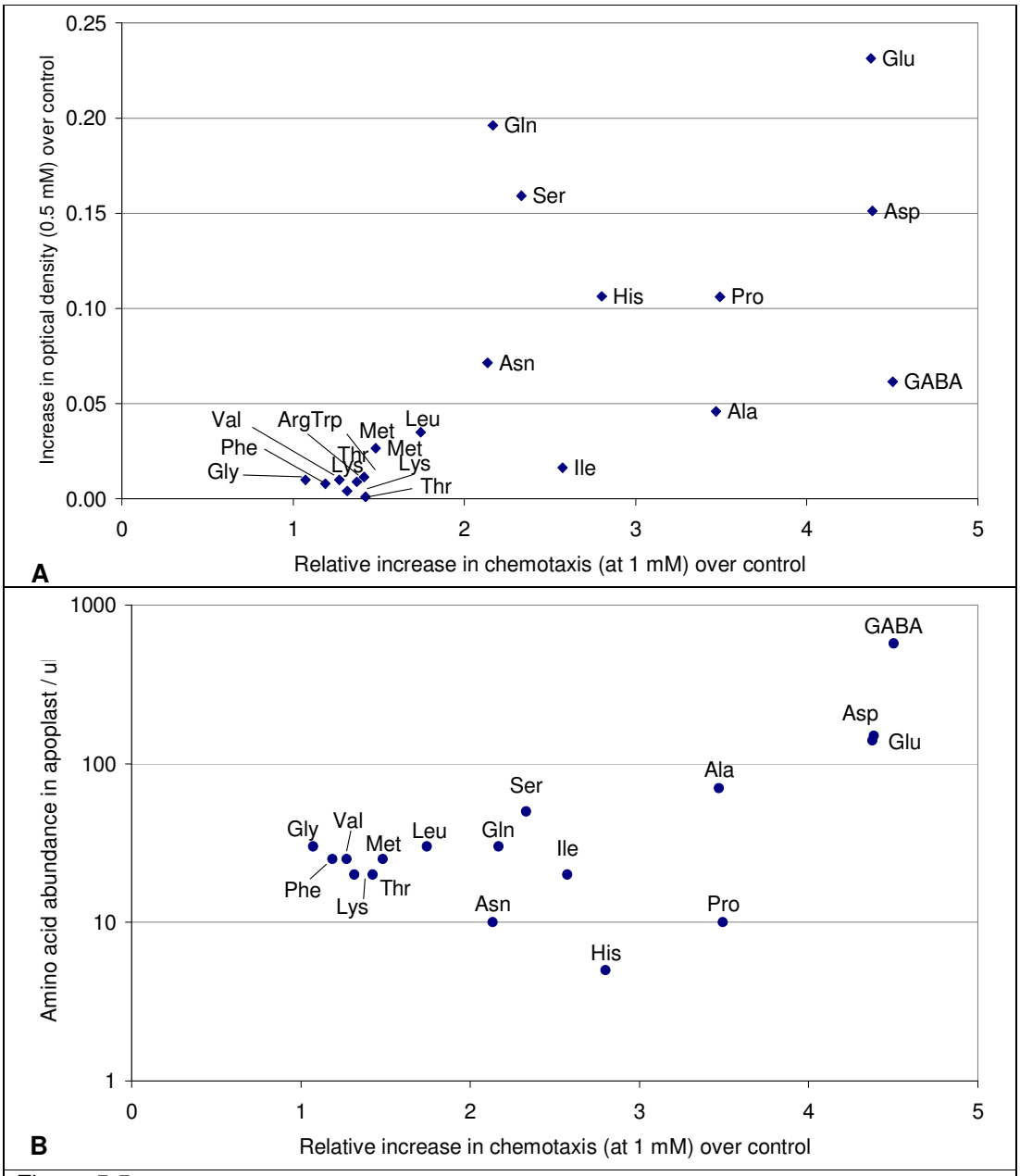


Figure 5.5

A. Comparison of the increase in optical density (growth boost) caused by the addition of an amino acid to complete M9 medium and the ability of P.s.t to chemotax towards these amino acids. Relative increase in chemotaxis is measured as the average chemotaxis towards 1 mM amino acid (made up in PME chemotaxis buffer), compared to the average chemotaxis towards a PME control. Growth boost is measured as the increase in optical density when bacteria are grown in complete M9 media supplemented with 0.5 mM amino acid, compared to complete M9 not supplemented with an amino acid. **B.** Graph comparing the abundance of amino acids in the tomato apoplast (in µM) plotted on a log scale, and the ability of P.s.t to chemotax towards these amino acids at 1 mM.

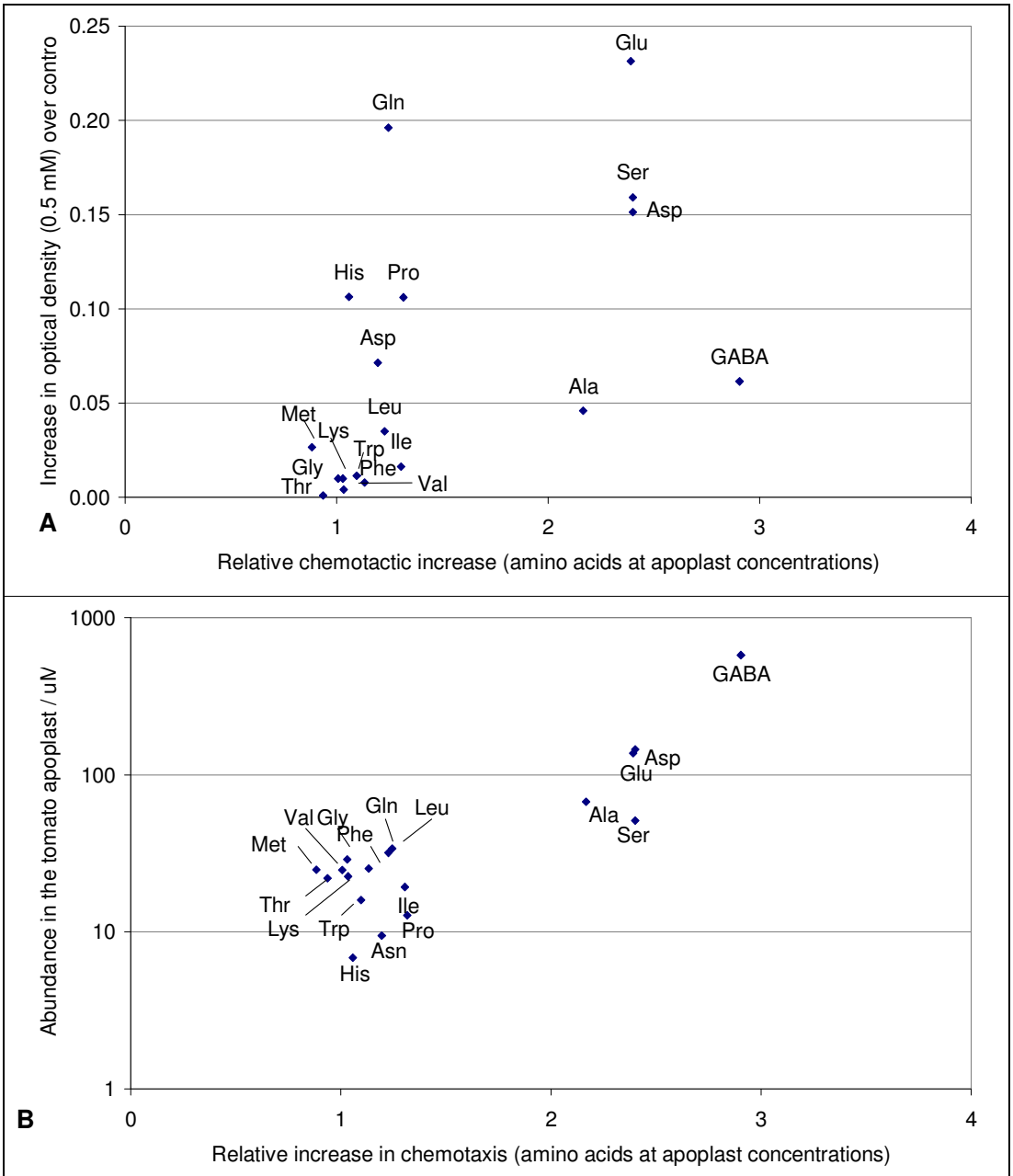


Figure 5.6

A. Graph comparing the increase in optical density (growth boost) following the addition of 0.5 mM amino acid, and the relative increase in chemotaxis towards that amino acid at apoplast concentrations. **B.** Graph comparing the abundance of each amino in the tomato apoplast (in µM, on a log scale) and chemotaxis of P.s.t towards that amino acid at the concentration found in the tomato apoplast.

5.5.4 Chemotaxis by *Pseudomonas syringae* pv. tomato towards synthetic media and extracted tomato apoplast.

Synthetic tomato and *A.thaliana* apoplast, and synthetic *A.thaliana* guttation fluid media have been created (Chapter 4, Section 4.5.7). These media contain a common 'base' of hexose sugars and organic acids, and different types and concentrations of amino acid. It was also demonstrated that *P.syringae* pv. tomato (P.s.t) was capable of chemotaxis towards certain amino acids at apoplast concentrations (Figure 5.4). It was therefore interesting to investigate whether P.s.t. was capable of significant chemotaxis towards these synthetic media.

Chemotaxis of P.s.t was tested towards the synthetic apoplast and guttation fluid media: synthetic *A.thaliana* guttation fluid (GF), synthetic *A.thaliana* apoplast (SAA), synthetic tomato apoplast (STA). In addition chemotaxis was tested towards the common 'base' M9-GSFMC minimal media (made using the carbon source combination glucose, sucrose, fructose, malate and citrate) on which all synthetic media is based, and LB media (Figure 5.7).

P.s.t did not show significant chemotaxis towards GF media or M9-GSFMC. M9 minimal media is nutrient poor and therefore may be expected to contain low concentrations of any chemical which might act as a chemoattractant. Furthermore, GF contains the same basic composition as M9-GSFMC with the addition of only three amino acids (glutamine, histidine and aspartate) added in low concentrations (3 µM, 1.5 µM and 1 µM respectively). Chemotaxis by P.s.t towards any of these amino acids at the concentration they are found in GF has not been tested. However, chemotaxis by P.s.t towards amino acids at tomato apoplast concentrations has been tested previously (Figure 5.4) and P.s.t did not chemotax towards glutamine or histidine at the concentrations found in tomato apoplast (34 µM and 7 µM, respectively). Therefore we would not expect P.s.t to demonstrate positive chemotaxis towards these amino acids at the (even lower) concentrations present in GF. P.s.t did show significant chemotaxis towards aspartate at the concentration present in the tomato apoplast, however this concentration was 145 µM, around 145 times more concentrated than the amount found in GF.

P.s.t showed significant chemotaxis towards STA and SAA. Both STA and SAA are based on nutrient poor M9-GSFMC, however these media are supplemented with at least nine additional amino acids. As P.s.t had previously shown significant chemotaxis towards six of the constituent amino acids in STA when tested individually (Figure 5.4), it was not surprising that P.s.t was significantly attract towards STA media. Chemotaxis by

P.s.t to SAA was slightly (although not significantly) less than to STA. SAA contains fewer amino acids and in particular lacks GABA. P.s.t also showed significant chemotaxis towards LB. The composition of LB was defined and found to be rich in amino acids, many above 1 mM concentrations (Sezonov et al., 2007), therefore it is possibly not surprising that P.s.t was strongly attracted towards this medium.

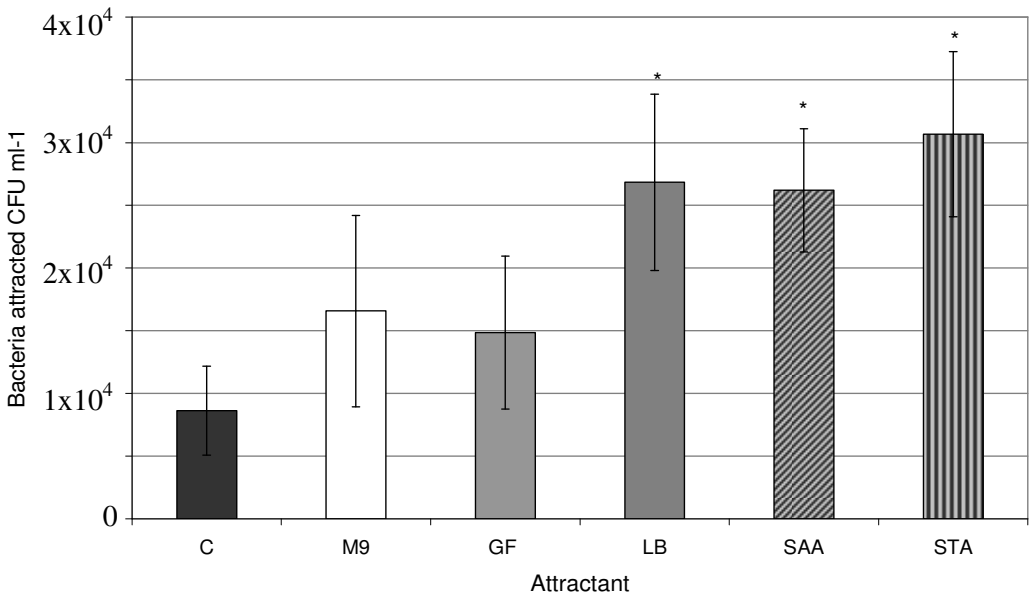


Figure 5.7
Chemotaxis by P.s.t to different synthetic media. Chemotaxis towards M9-GSFMC (M9), synthetic guttation fluid (GF), LB, synthetic *A. thaliana* apoplast (SAA) and synthetic tomato apoplast (STA). (C), PME buffer control. *= Chemotaxis is significantly different to the control $p \leq 0.05$, Student's 2-tailed t-test. Error bars represent the standard deviations of at least five replicates. This experiment was independently repeated twice with similar results.

To further investigate the strength of chemotaxis by P.s.t towards synthetic tomato apoplast (STA) compared to *A.thaliana* apoplast (SAA), these media were serially diluted and the chemotactic response of P.s.t towards the dilutions was tested. In addition the chemotactic response of P.s.t to extracted tomato apoplast was tested.

Results showed that P.s.t can significantly chemotax towards a 1/10 dilution of SAA ($p=0.003$), but not a 1/100 dilution ($p=0.132$) (Figure 5.8). P.s.t can significantly chemotax towards a 1/10 dilution of STA ($p=0.000$), however chemotaxis towards a 1/100 dilution of STA is only significant at the 90% confidence interval ($p=0.065$) (Figure 5.9 A). These results indicate that P.s.t can chemotax towards STA media at lower dilutions than SAA, probably because STA contains more amino acids than SAA. In contrast to the synthetic media, P.s.t can still significantly chemotax towards extracted

tomato apoplast at the 1/100 dilution (p=0.000) (Figure 5.9 B). This suggests that there are chemoattractants present in tomato apoplast, other than the amino acids, sugars and organic acids which have been added to STA, and that P.s.t can sense and respond to the present of these chemoattractants in very low concentrations.

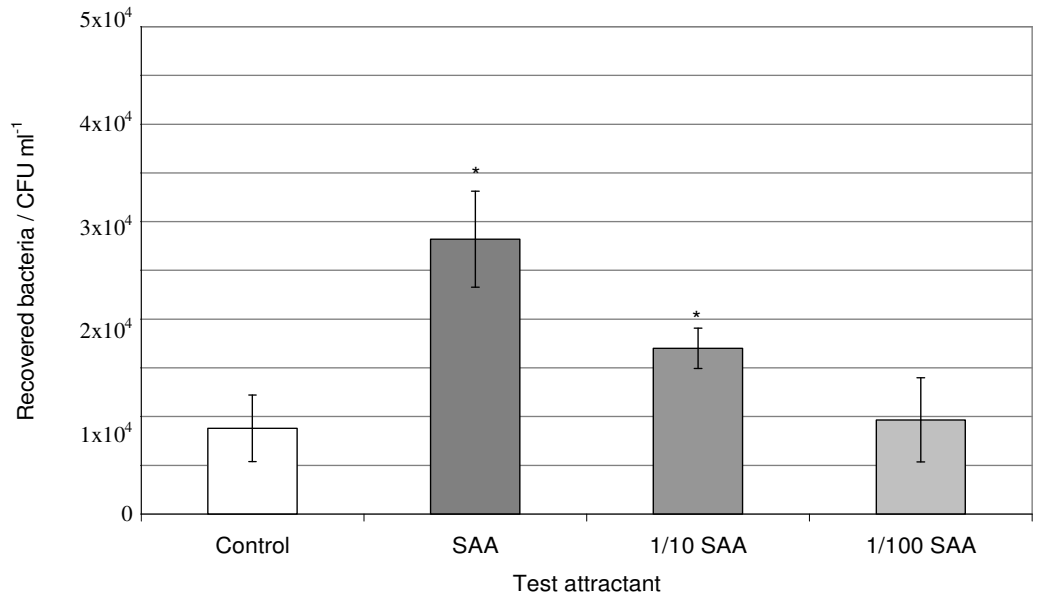


Figure 5.8
Chemotaxis by P.s.t. to a dilution series of synthetic *A. thaliana* apoplast media (SAA) and a control of PME chemotaxis buffer. *=Chemotaxis is significantly different to the control p≤0.05. Student's one tailed t-test. H_0= chemotaxis towards APO is greater than towards the control. Error bars represent standard deviations from at least five replicates. Experiments were independently replicated twice with similar results.

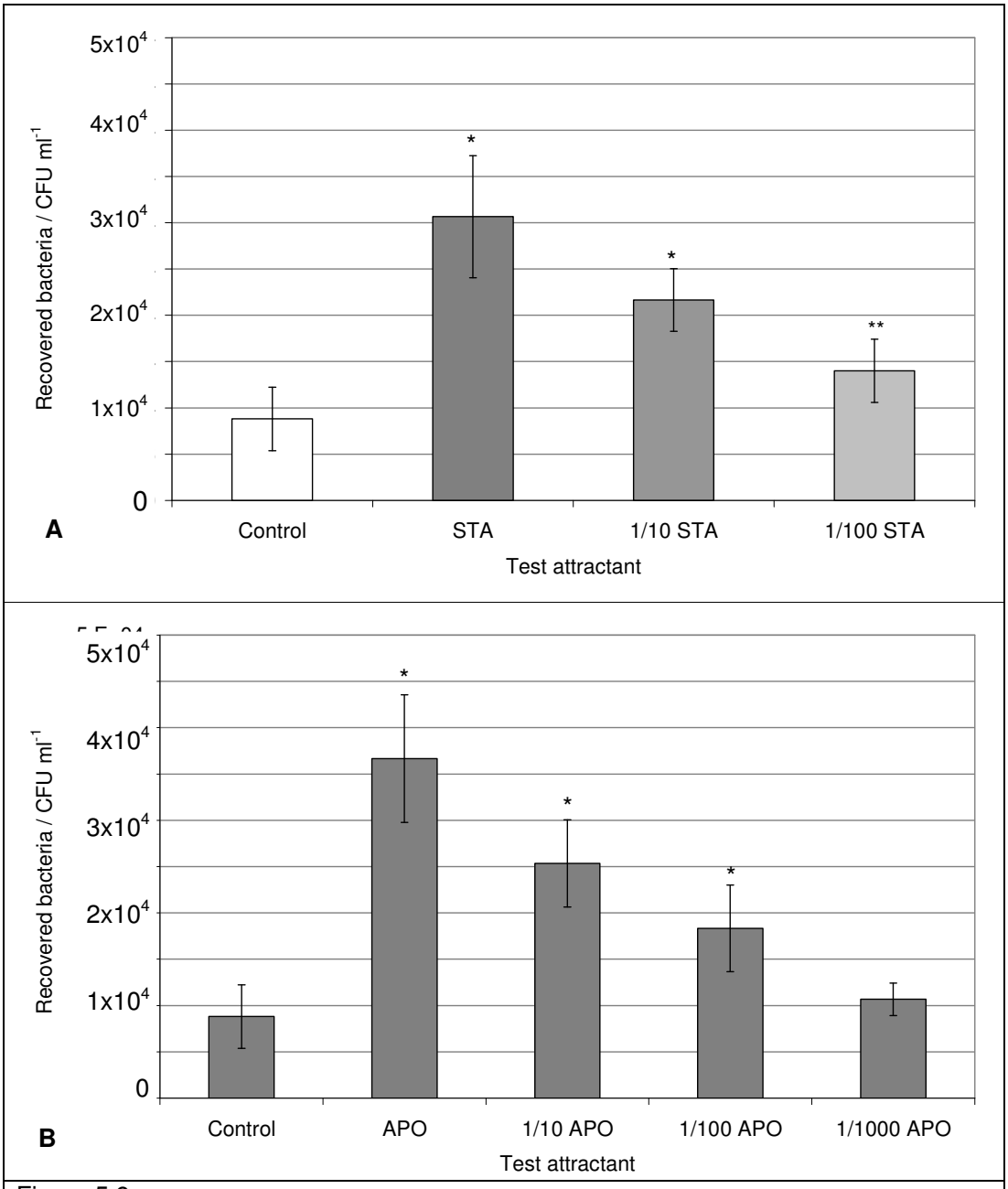


Figure 5.9

A. Chemotaxis by P.s.t. to a dilution series of synthetic tomato *a*poplast (STA) and a control of PME chemotaxis buffer. **B**. Chemotaxis by P.s.t. to a dilution series of tomato apoplast (APO) and a control of PME chemotaxis buffer. *=Chemotaxis is significantly different to the control $p \leq 0.05$. Student's one tailed t-test. H_0= chemotaxis towards APO is greater than towards the control. Error bars represent standard deviations from at least five replicates. Experiments were independently replicated twice with similar results.

5.5.5 Bioinformatic comparisons of chemotaxis-related proteins in *Pseudomonas* species.

The availability of sequence data for many *Pseudomonas* species has facilitated genome comparisons between plant-pathogenic and non-plant-pathogenic *Pseudomonas*. Numerous genes have been found to be unique to *P. syringae*, such as 98 pathogenicity-associated genes, which are thought to play roles specific to plant-bacterial interactions (Buell et al., 2003). These comparisons have also provided a genetic basis for phenotypic differences, such as differences in nutrient assimilation abilities, between plant-pathogenic and non plant-pathogenic *Pseudomonas*. For example, *P. syringae,* which occupies the nutrient-restricted apoplast environment (Alfano and Collmer, 1996; Lindow and Brandl, 2003), can metabolise fewer amino acids as carbon or nitrogen sources compared to non plant-pathogenic *Pseudomonas* (Rico and Preston, 2008). Consistent with this finding, genome comparisons have revealed that the *P. syringae* genome contains significantly fewer amino acid transporter (aa_permease) genes compared to other *Pseudomonas* species (Studholme et al., 2005).

- **Chemotaxis-related protein domains in *P. syringae* and non plant-pathogenic *Pseudomonas*.**

As chemotaxis is closely connected to energy taxis, the nutrient restricted plant environment may also have influenced the chemotaxis proteins present in the *P. syringae* genome. In order to investigate whether the plant-pathogenic lifestyle of *P. syringae* has resulted in chemotaxis specialisation, the number of chemotaxis-related protein domains in the *P. syringae* genome were compared to the numbers present in the genomes of non plant-pathogenic *Pseudomonas*. As before, data were extracted from the Pfam database. There were 15 protein domains annotated with a chemotaxis-related function in the Pfam database. The numbers of these domains in 11 sequenced *Pseudomonas,* including three *P.syringae* pathovars, were compared (Table 5.5). *P.syringae* genomes were not significantly underrepresented in any chemotaxis-related domain compared to non plant-pathogenic *Pseudomonas*. However *P. syringae* appeared to be overrepresented in four chemotaxis-related domains; CheW, Methyl-accepting Chemotaxis Protein (MCP), chase 3 and peripla_bp_1, compared to non plant-pathogenic *Pseudomonas* (Table 5.5). Therefore, contrary to the nutritional specialisation of *P. syringae* compared to other *Pseudomonas*, *P. syringae* genomes appeared to have increased in proteins associated with chemotaxis. Such an increase can be hypothesised to be associated with an increased ability for *P. syringae* to detect and respond to plant-derived compounds.

	cheW	cheZ	MCP	cheR	cheD	chase3	peripla_BP_1	filL	flgM	cheB	response_reg	HAMP
P.men	11	1	34	4	0	2	4	2	1	3	80	44
L48	12	1	32	4	0	1	5	2	1	3	86	49
PA14	12	1	26	4	1	0	5	2	1	4	90	46
PAO1	13	1	32	4	1	0	6	2	1	4	104	56
F1	12	1	27	3	0	0	6	2	1	3	103	50
KT2440	12	1	27	3	0	1	6	2	1	3	95	50
PfO-1	12	1	27	3	0	0	9	2	1	3	108	63
Pf-5	12	1	42	3	0	3	9	2	1	3	120	66
P.s.t	**15**	1	**49**	4	1	**2**	**14**	2	1	4	93	59
P.s.s	**15**	1	**51**	4	1	**3**	**14**	2	1	4	93	62
P.s.ph	**15**	1	**50**	4	1	**2**	**14**	2	1	4	91	57
p-value	**0.000**	–	**0.000**	0.243	0.124	**0.033**	**0.000**	–	–	0.267	0.233	0.11

Table 5.5

A survey of the number of chemotaxis-related domains in 11 *Pseudomonas* species, collected from the Pfam database. Student's 2 tailed t-tests were used to compare the number of domains in the three sequenced *P. syringae* pathovars (yellow) compared to the non plant-pathogenic *Pseudomonas*. H_0= no difference in chemotaxis related domains between *P. syringae* and other *Pseudomonas*. Significant p-values are highlighted in pink ($p \leq 0.05$). The numbers of four chemotaxis-related domains, MCP, peripla_BP_1, chase3 and chew, are significantly different in the *P. syringae* genome.

- **Similarity of *Pseudomonas syringae* chemotaxis protein organisation to the *Pseudomonas aeruginosa* PAO1 'chemotaxis clusters'.**

As *P. syringae* genomes were overrepresented in some chemotaxis-related domains, chemotaxis proteins in *P. syringae* were investigated in further detail. First the *P. syringae* genomes were investigated for the presence of 'chemotaxis clusters'; operons of genes involved in chemotaxis and twitching motility which have been well-studied in *P. aeruginosa* PAO1. Secondly, the genes within these clusters were studied to investigate whether they included the chemotaxis-related domains listed in Table 5.5. In particular, it was interesting to investigate whether the chemotaxis clusters had expanded in size in *P. syringae* genomes, and therefore included the overrepresented chemotaxis-related domains of Table 5.5, or whether the genes containing these domains were located elsewhere in the genome. Finally, where the chemotaxis-related domains of Table 5.5 were located in genes which did not fall within a 'chemotaxis cluster', the location of these genes in the *P. syringae* genome was investigated in more detail.

Within *Pseudomonas* the genomic basis for chemotaxis has been best studied in *P. aeruginosa* PAO1. PAO1 has been shown to have homologues of *E. coli* chemotaxis-related genes organised into five chemotaxis clusters (Ferrandez et al., 2002) (Figure 5.10). No detailed reports have been published on the presence of equivalent chemotaxis clusters in *P. syringae* or other *Pseudomonas*. Therefore the reciprocal best blast hit (RBBH) method described in Chapter 3 Section 3.4.2, was used to identify orthologous genes of those in the five PAO1 chemotaxis clusters in other *Pseudomonas* species. In addition the percentage identity was calculated for each amino acid sequence between *P. syringae* and the orthologous gene in other *Pseudomonas* species.

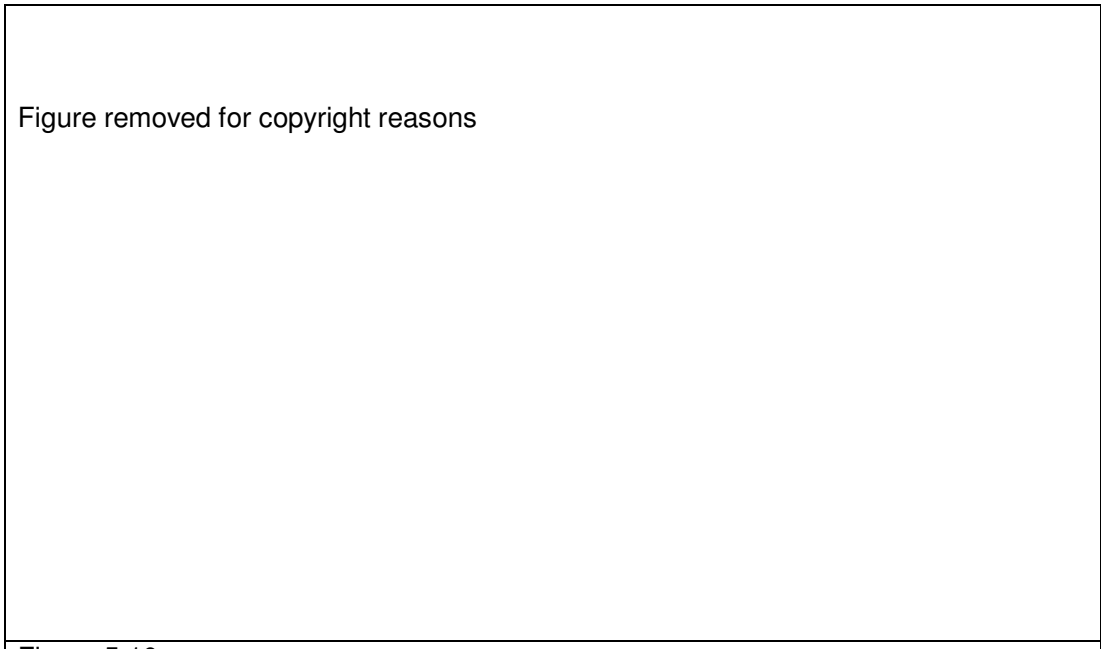


Figure 5.10
Five chemotaxis clusters have been identified in *P.aeruginosa* PAO1. Genes in cluster I, II and V have been shown to be essential for chemotaxis and cluster IV has been shown to be involved in twitching motility (reviewed in Ferrandez et al., (2002)). Cluster III has been studied in *P.fluorescens* (Rainey and Travisano, 1998; Spiers et al., 2002) and *P.aeruginosa* (Guvener and Harwood, 2007) and is predicted to encode a signal transduction system homologous to a bacterial chemotaxis system responsible for the wrinkly spreader morphology. Image reproduced from Ferrandez et al., (2002).

Orthologs of genes found in four PAO1 chemotaxis clusters (I, III, IV and V) were found in all the *P. syringae* genomes investigated, in addition, the presence and synteny of these genes was generally well conserved in all the *Pseudomonas* species investigated (Figure 5.11, Figure 5.12, Figure 5.13 and Figure 5.14, respectively).

Chemotaxis cluster II was present in *P. syringae*, *P. aeruginosa* and *P. mendocina* species but missing from *P. fluorescens, P. entomophila* and *P. putida* genomes (Figure 5.12). The genes within cluster II in P.s.t, did not appear to be orthologs of those found in PAO1. Each of the cluster II genes *P. syringae* shared a low percentage identity with the same genes in *P. aeruginosa* and *P. mendocina* species. The most similar genes to those present in P.s.t, according to BLASTP analysis of all sequenced bacteria, are found in *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966, a ubiquitous water-borne bacterium (Seshadri et al., 2006).

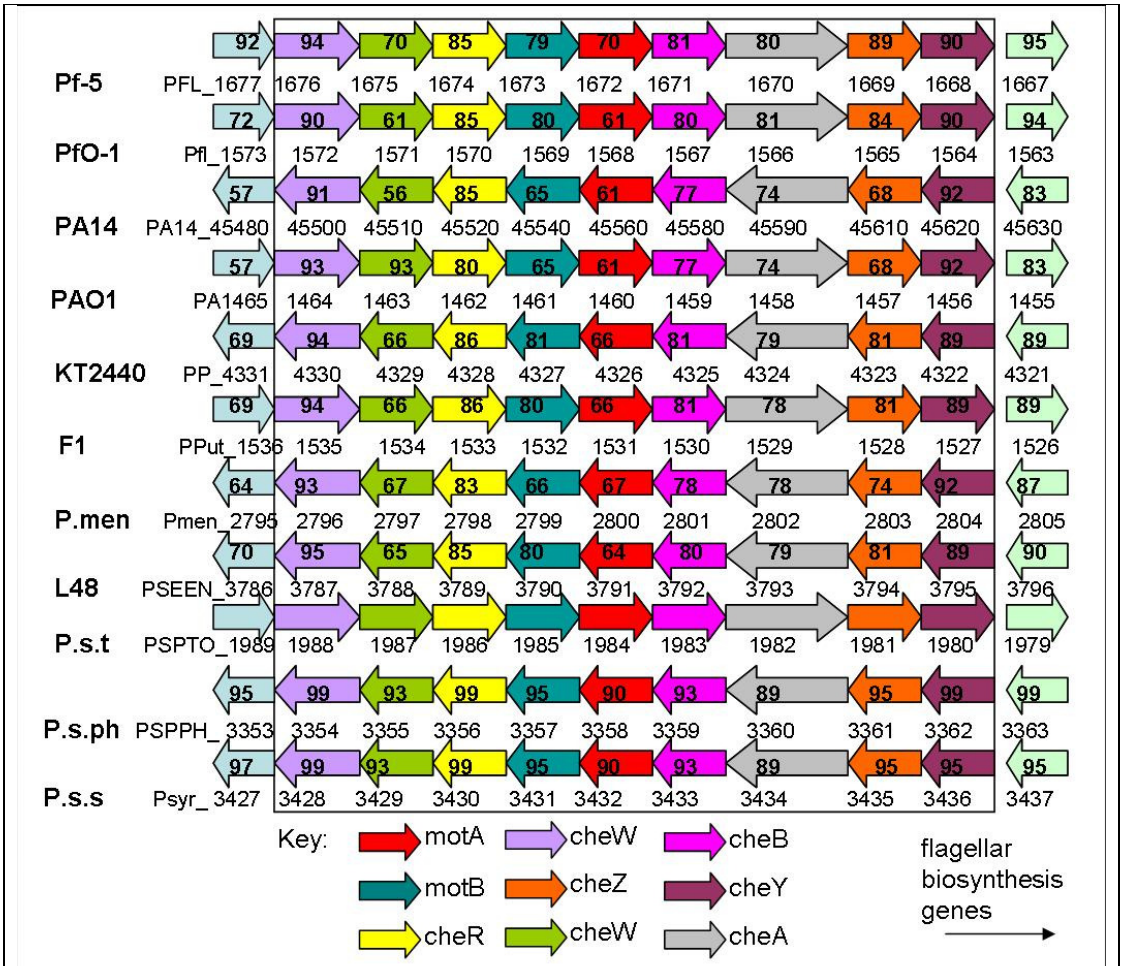


Figure 5.11

Chemotaxis cluster I orthologs in *Pseudomonas*. Chemotaxis cluster I is found next to flagellar biosynthesis genes as indicated and has previously been reported to be essential for chemotaxis (Masduki et al., 1995). Numbers in bold indicate the percentage identity of each gene to the orthologous gene in P.s.t. Genes filled with the same colour are orthologs. The box encloses the genes that make up chemotaxis cluster I as described in Ferrandez et al 2002. The genes found either side of this cluster are provided for information as to the preservation of synteny outside this region.

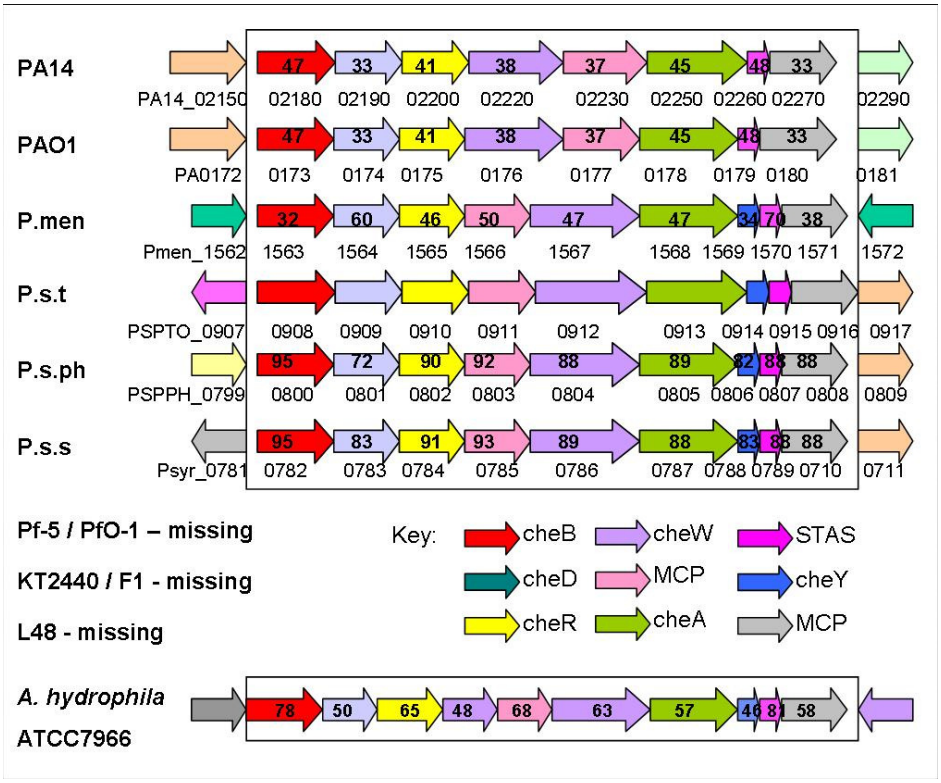


Figure 5.12
Chemotaxis cluster II orthologs in *Pseudomonas*. Chemotaxis cluster II appears to be missing in *P.fluorescens, P.entomophila* and *P.putida*. The genes within this cluster in *P.syringae* are not closely related to those in *P.aeruginosa*. The most similar genes according to BLASTP analysis are found in *Aeromonas hydrophila subsp. hydrophila* ATCC 7966, a ubiquitous water-borne bacterium (Seshadri et al., 2006). Numbers in bold indicate percentage identity between the gene in P.s.t and orthologous genes in other bacterial species. Genes have been represented by arrows. Arrows filled in the same colour are orthologs. The key refers to the Pfam protein domain annotation for each gene.

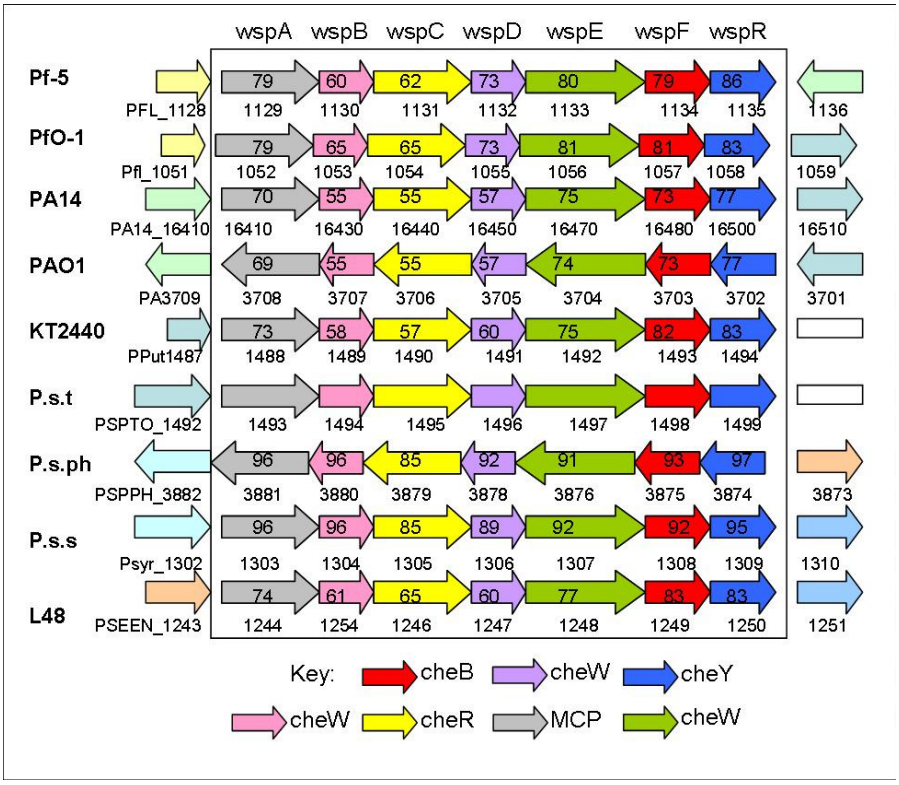


Figure 5.13
Chemotaxis cluster III orthologs in *Pseudomonas*. Chemotaxis cluster III contains genes that are predicted to encode a signal transduction system responsible for the wrinkly spreader phenotype in P.fluorescens SBW25 (Rainey and Travisano, 1998; Spiers et al., 2002). This system has previously been reported as conserved in all sequenced Pseudomonas species (Guvener and Harwood, 2007). Genes have been represented by arrows. Arrows filled in the same colour are orthologs. Numbers within each arrow indicate the percentage identity between the gene in P.s.t and orthologous genes in other bacterial species. The key refers to the Pfam protein domain annotation for each gene.

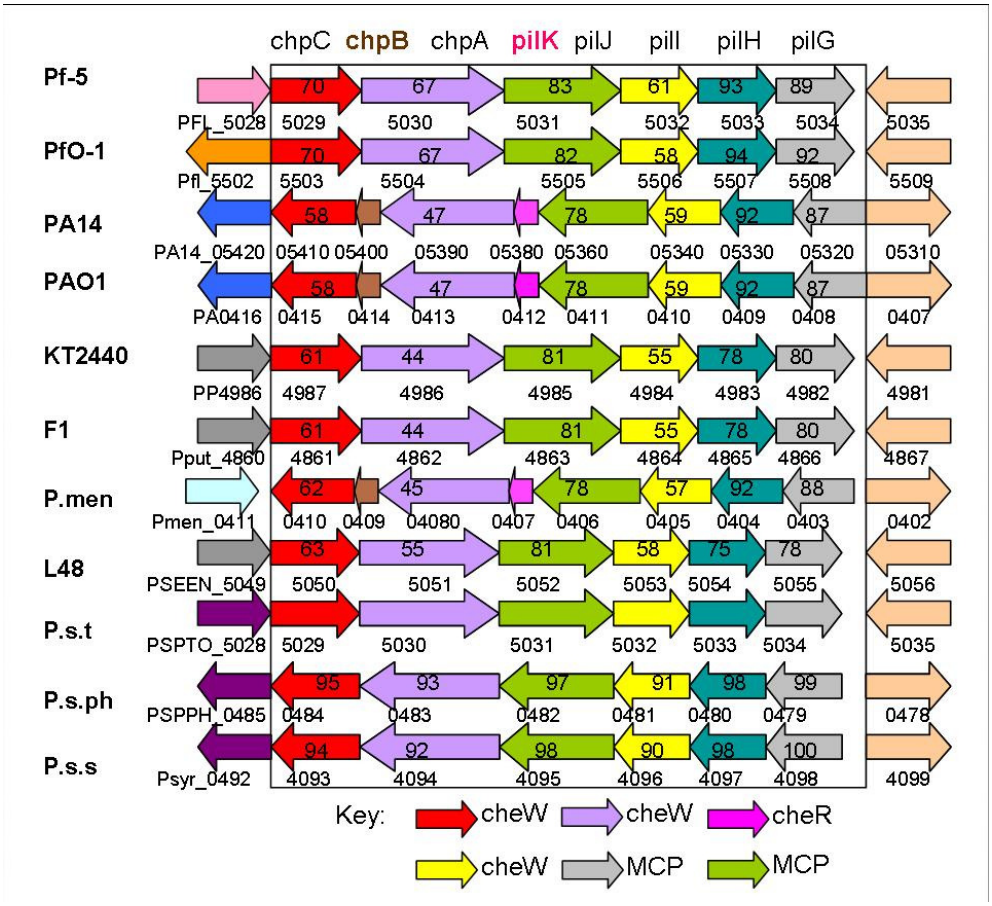


Figure 5.14
Chemotaxis cluster IV orthologs in *Pseudomonas.* Chemotaxis cluster IV has previously been shown to be involved in twitching motility in *P.aeruginosa* (Darzins, 1994; Kearns et al., 2001). This cluster has orthologs in all sequenced *Pseudomonas* species. Genes have been represented by arrows. Arrows filled in the same colour are orthologs. Numbers within each arrow indicate the percentage identity between the gene in P.s.t and orthologous genes in other bacterial species. The key refers to the Pfam protein domain annotation for each gene.

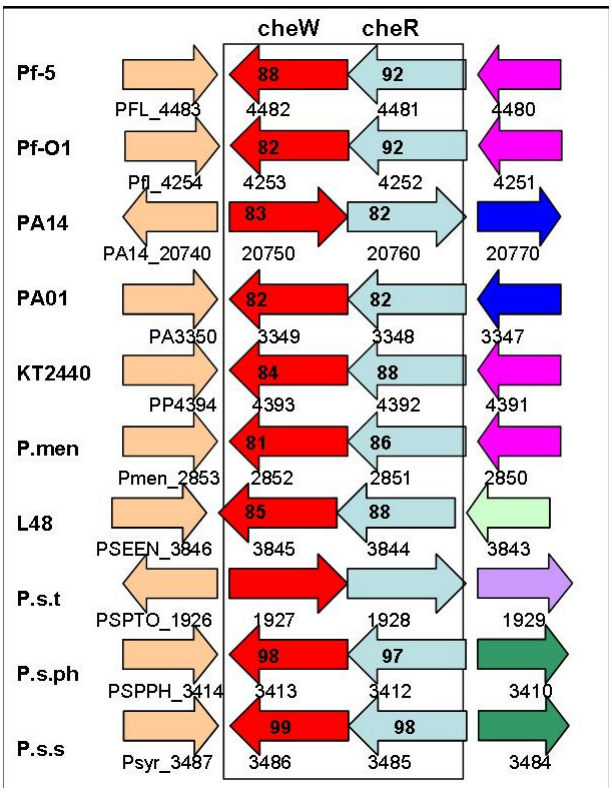


Figure 5.15
Chemotaxis cluster V orthologs in *Pseudomonas.* Chemotaxis cluster V contains genes essential for chemotaxis (Masduki et al., 1995). This cluster is present in all sequenced *Pseudomonas* species (Ferrandez et al., 2002). Genes have been represented by arrows. Arrows filled in the same colour are orthologs. Numbers within each arrow indicate the percentage identity between the gene in P.s.t and orthologous genes in other bacterial species. The key refers to the Pfam protein domain annotation for each gene.

There were no duplications of chemotaxis-related genes within the chemotaxis clusters in the *P. syringae* genome which could account for the observed differences in the number of these proteins between the *P. syringe* and non plant-pathogenic *Pseudomonas*. However, of the chemotaxis related domains listed in Table 5.5, relatively few were found in genes located within chemotaxis clusters. For example, three out of the four cheR-domain and cheB domain-containing proteins in *P. syringae* were found in chemotaxis clusters II, III and V, while the one cheD-domain containing protein was present in cluster II. However, only four out of the 49 *P syringae* MCP-domain containing proteins were found within chemotaxis clusters.**Therefore the genomic location of each of the overrepresented chemotaxis-domain containing proteins (MCP, cheW, chase 3 and peripla_bp_1) not already known to be present within a chemotaxis cluster, was investigated in the P.s.t genome.** Investigation of the position of the MCP domain, cheW domain and peripla_bp_1 domain-containing proteins in the P.s.t genome revealed that these occur throughout the

genome, rather than clustered together. These proteins are not found in operons (measured as occurring within 100 bp of another gene on the same strand), other than when they occur in chemotaxis clusters I-V, and do not appear to be concentrated next to genes with a particular function.

However, tracking the genomic location of the chase 3 domain-containing proteins revealed two potential new chemotaxis-related operons found in *P. syringae* but not present in *P. aeruginosa* (Figure 5.16). Operon A encodes four proteins, including a response regulator domain-containing protein and the only cheR and cheB domain-containing proteins not found in the chemotaxis clusters described in PAO1. Orthologous genes of those found in operon A are present in all *Pseudomonas* species except *P. aeruginosa*. Operon B also encodes four proteins, including two response regulator domain-containing proteins and a protein containing the chemotaxis-related domain cheA. Orthologs of genes found in operon B are present in *P. fluorescens* Pf-5 and *P. mendocina* ymp.

Two hypotheses can be proposed to explain the presence of operon A in all *Pseudomonas* species except *P. aeruginosa*. Firstly, the operon may have acquired from another bacterial species by horizontal evolution. Alternatively this operon may have been present in the common ancestor of all *Pseudomonas* and has been subsequently lost in *P. aeruginosa*. Referring to the *Pseudomonas* family tree in Figure 1.1, Chapter 1, it can be seen that the *P. aeruginosa* lineage diverged from the other *Pseudomonas* early in their evolutionary history suggesting that gene loss of this operon may be the most parsimonious explanation for the current distribution. Interestingly, as this operon is present in all plant-associated and plant-pathogenic species it may possibly function in responding to plant-derived compounds. The evolutionary history of operon B is harder to predict as it is found in only three *Pseudomonas* species, meaning that a greater number of *Pseudomonas* species would have had to undergo gene loss in order to produce the current distribution. However, none of the genes in this operon share a high percentage identity with genes in non-*Pseudomonas* species which suggests they were not acquired by horizontal gene transfer. Therefore the loss of this operon may have occurred in other *Pseudomonas* species. Operon B is found in plant-pathogenic and plant-associated species, however it is also notably absent from the plant-associated species *P. putida*. Therefore the association between genes within operon B and the plant-associated lifestyle is harder to predict.

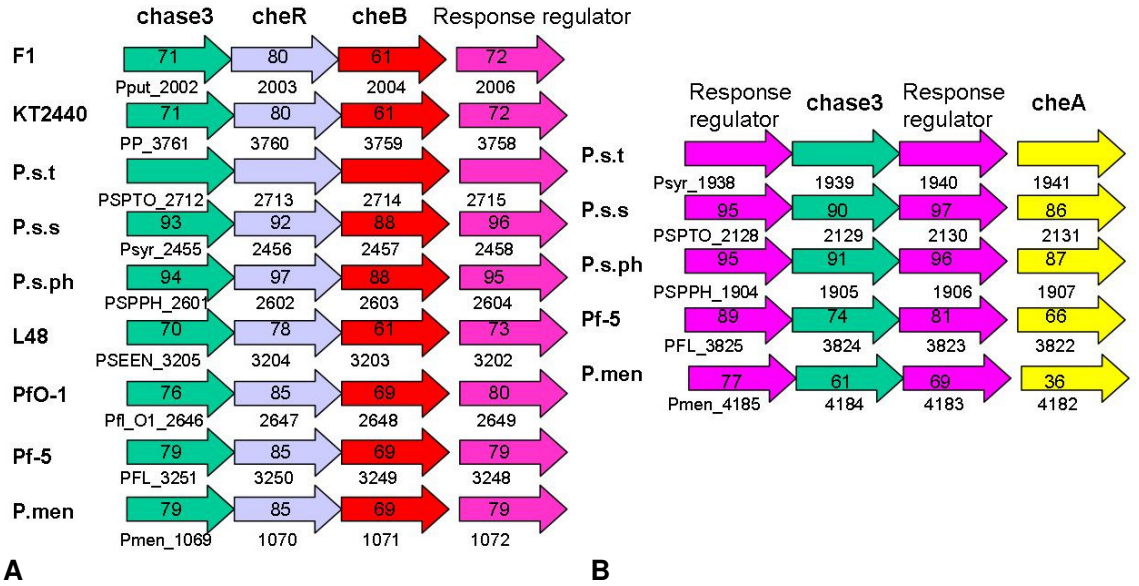


Figure 5.16
Additional chemotaxis clusters in some *Pseudomonas* species. *P.syringae* is overrepresented in chase 3 domain containing proteins. Two of these are found alongside proteins containing known chemotaxis-related domains (bold). These genes are all transcribed in the same direction, and may constitute an operon as the distance between them is less than 100 bp. Operon A is found in all sequenced *Pseudomonas* except *P.aeruginosa*. Operon B appears to be restricted to a few *Pseudomonas* species and in particular is not found in *P. aeruginosa* or *P. putida*. Genes have been represented by arrows. Arrows filled in the same colour are orthologs.

5.6 Discussion.

Bacterial chemotaxis is the detection and directional movement in response to specific chemicals in the environment. As defined for *E. coli*, chemotaxis does not require the metabolism of the chemoattractant (or repellent) as an energy source, the binding of a chemoattractant molecule to a chemotaxis receptor is sufficient to initiate the chemotaxis response. However, chemotaxis may also be metabolism-dependent whereby bacteria sense a change in metabolism (energy) rather than the chemoattractant as such. These forms of chemotaxis may interact to produce the overall chemotactic response observed.

P. syringae inhabits the epiphytic leaf surface, prior to gaining entry into the endophytic niche of the leaf apoplast through wounds or natural openings. On the leaf surface, nutrients are thought to be heterogeneously dispersed and concentrated in certain areas such as the bases of trichomes or around open stomata (Leveau and Lindow, 2001; Beattie and Axtell, 2002). Natural openings and wound sites are also thought to 'leak' nutrients onto the leaf surface. Chemotaxis towards plant-derived compounds is therefore hypothesised to play a role in both the epiphytic and endophytic stages of the *P. syringae* life cycle. As an epiphyte, chemotaxis towards patchily dispersed nutrients may provide a survival advantage, while

chemotaxis towards apoplast-derived compounds may provide a signal directing bacteria towards a route of entry into the endophytic niche.

Chemotaxis towards root and seed exudates and extracted apoplast has been demonstrated for several pathovars of *P. syringae* (Panopoulos and Schroth, 1974; Cuppels, 1988; Hattermann and Ries, 1989). As the amino acid content of the tomato apoplast has recently been elucidated, one aim of this chapter was to investigate the chemotactic response of *P. syringae* pv. tomato (P.s.t) towards the amino acids present in tomato apoplast. In addition, the chemotactic response of P.s.t was tested in response to the synthetic apoplast and synthetic guttation fluid media made in Chapter 4, and this was compared to the response to extracted tomato apoplast. Finally, the genomic basis for the chemotaxis response was compared in *P. syringae* and the non plant-pathogenic *Pseudomonas* species, in order to address the hypothesis that the plant-pathogenic lifestyle within the nutrient limited apoplast may have reduced the number of chemotaxis proteins in the *P. syringae* genome.

In order to profile the response of P.s.t to amino acids present in the tomato apoplast, a capillary assay was used. Unlike the commonly used swarm plate assay, the capillary assay does not depend on metabolism to generate a concentration gradient. Instead, diffusion of the amino acid from the mouth of the syringe needle (acting as a microcapillary) sets up the concentration gradient. Bacteria respond to a chemoattractant by swimming up the gradient and into the tube. After a 60 minute incubation period, the number of bacteria in the needle was determined by plate counts.

The concentration of amino acids in the tomato apoplast varies widely (Rico and Preston, 2008). Therefore chemotaxis by P.s.t towards each constituent amino acid at a concentration of 1 mM was tested. At 1 mM concentrations P.s.t exhibited the strongest chemotaxis towards GABA, aspartic acid and glutamic acid, while chemotaxis towards proline, alanine and histidine was also comparatively good (Figure 5.3). In general there was not a strong correlation between the chemotaxis by P.s.t towards 1 mM concentrations of amino acids, and the abundance of each amino acid in the tomato apoplast (Figure 5.5A), or between chemotaxis by P.s.t towards 1 mM concentrations of amino acids and the growth increase observed after adding 0.5 mM of each amino acid as a supplement to complete M9 minimal media (containing glucose as a carbon source and ammonia as a nitrogen source) (Figure 5.5B). However, it was noted that there was generally poor chemotaxis towards amino acids which are poorly used as carbon or nitrogen sources, suggesting that P.s.t may lack chemoreceptors for these amino acids. Conversely there was generally good

chemotaxis towards amino acids which provided a good source of carbon or nitrogen for metabolism or growth. This suggests that energy taxis may play a role in the movement of P.s.t towards amino acids, with increased movement towards amino acids which can be used as carbon or nitrogen sources.

In general, there was also not a good correlation between chemotaxis by P.s.t towards amino acids at the concentration present in the tomato apoplast, and the growth increase provided by these amino acids (Figure 5.6A), or the abundance of these amino acids in the tomato apoplast (Figure 5.6B). However, the five most abundant amino acids in the tomato apoplast, GABA, aspartic acid, glutamic acid, serine and alanine provoked the largest RCR from P.s.t at apoplast concentrations, and these amino acids in particular showed a fairly clear correlation between apoplast abundance and chemotaxis (Figure 5.6B). This may be due to the fact that the concentrations tested for these amino acids (the apoplast concentrations) were the largest, and these amino acids can be used for growth and metabolism, indicating that P.s.t is demonstrating energy taxis towards these amino acids. The exception to this trend was isoleucine, which P.s.t was significantly attracted toward despite not being able to utilise this amino acid for growth or metabolism. In addition, P.s.t was not capable of metabolising isoleucine as a carbon or nitrogen source or as a source of carbon or nitrogen for growth (Chapter 4 Tables 4.1 - 4.3). Therefore the chemotaxis towards isoleucine by P.s.t is particularly intriguing. A bioinformatic search of the P.s.t genome revealed the presence of transporters for isoleucine uptake (PSPTO_4110, PSPTO_4111); therefore this may represent a particularly desirable amino acid in the apoplast environment. However the reasons why *P.syringae* may have retained both the ability to chemotax towards this amino acid and transporters for its uptake when there is no obvious energy benefit in terms of growth or metabolism are unknown. However, these results are similar to those observed in *P. fluorescens* WBC-3, which was attracted to para-nitrophenol (PNP) even when PNP-catabolic genes had been mutated, suggesting that chemotaxis towards this compound was metabolism-independent (Zhang et al., 2008).

At 1 mM concentrations all tested amino acids except glycine and phenylalanine provoked a significant chemotactic response by P.s.t. However, at 1 mM concentrations the response of P.s.t to serine and isoleucine was lower than to amino acids such as proline or histidine, which did not provoke a significant chemotactic response when tested at apoplast concentrations. This result suggests that some amino acids may be inhibitory at high concentrations, for example isoleucine may inhibit leucine and valine synthesis. The benefit of testing amino acids at both 1 mM and at apoplast concentrations was to demonstrate that P.s.t is capable of movement towards a range of amino acids at high concentrations, but for

some amino acids, such as proline or histidine the concentrations present in the apoplast are too low to provoke a detectable chemotactic response. However, it can be envisaged that significant chemotaxis by epiphytic bacteria to one component of the mixture of compounds present in apoplast may provoke a chemotactic response which could result in the bacteria moving towards the opening and eventually entering the apoplast. It is also important to remember that the amino acids were tested at the concentrations found in healthy tomato apoplast. The concentration of amino acids in the diseased tomato apoplast is presently under investigation (Arantza Rico, personal communication). However, Solomon and coworkers (2001) previously reported large increases in several apoplastic amino acids over the course of tomato infection with the biotrophic fungus *C. fulvum*. Therefore the chemotactic environment experienced by *P. syringae* may alter during the course of infection.

The chemotactic response of P.s.t towards synthetic guttation fluid (GF) synthetic tomato apoplast (STA) and *A. thaliana* apoplast (SAA) was tested. P.s.t did not show significant chemotaxis towards GF media. This result was not unexpected given that P.s.t did not chemotaxis towards the modified M9 minimal media on which GF was based, and GF contains the same basic composition as M9 with the addition of only glutamine, histidine and aspartate added in low concentrations. In contrast, P.s.t showed significant chemotaxis towards both STA and SAA. P.s.t had previously shown significant chemotaxis towards six of the constituent amino acids in STA when tested individually (Figure 5.4). Therefore it was not surprising that P.s.t was significantly attracted towards STA media. Chemotaxis by P.s.t to SAA was slightly (although not significantly) less than to STA. SAA contains fewer amino acids and in particular lacks GABA, which is a strong chemoattractant for P.s.t. When the strength of chemotaxis by P.s.t towards STA and SAA was tested further, using a dilution series, P.s.t was able to significantly chemotax towards STA at a lower concentration than SAA. This was thought to be because STA contains more amino acids than SAA. Tomato apoplast contains many compounds which have not been replicated in STA, and which may serve as chemoattractants for P.s.t either individually or as a cumulative effect. Significant chemotaxis by P.s.t towards diluted tomato apoplast is particularly interesting as this suggests that in the leaf environment P.s.t may be able to move towards apoplast which has been diluted, for example as nutrients diffuse away from wounds or natural openings in the plant surface.

P. syringae has been shown to be nutritionally restricted in comparison to non plant-pathogenic *Pseudomonas*, particularly for amino acid utilisation (Rico and Preston, 2008). A bioinformatic comparison of *P. syringae* and non plant-pathogenic *Pseudomonas* genomes

revealed a potential genetic basis for this difference, as *P. syringae* was underrepresented in the amino acid permease family of amino acid transporters (Studholme et al., 2005). The nutrient-limited environment of the plant apoplast may also have influenced the numbers of other genes in the *P. syringae* genome, such as chemotaxis-related proteins. Chemotaxis may be metabolism independent, or metabolism dependent (energy taxis). Chemotaxis requires the binding of a chemoattractant (or repellent) to an MCP protein. A connection between adaptation to the apoplast environment and chemotactic specialisation can be proposed. Encountering a restricted range of nutrient sources could restrict the potential number of chemoattractants which could bind to MCP proteins and provide a selective pressure for the loss of the cognate MCPs which detect compounds not present in the apoplast.

The Pfam database was searched for chemotaxis-related protein domains. Data was extracted for the three sequenced *P. syringae* pathovars, along with eight non plant-pathogenic *Pseudomonas* species. Results showed that the three sequenced *P. syringae* pathovars were overrepresented in several chemotaxis-related protein domains compared to non plant-pathogenic *Pseudomonas*. These are the MCP, cheW, Peripla_BP_1 and chase 3 domains. In particular, the significant increase in MCP and Peripla_BP_1-domains might indicate that *P.syringae* may be able to detect an increased number of compounds compared to non plant-pathogenic *Pseudomonas.* These domains may allow P.s.t to detect compounds present in tomato apoplast which were not present in STA, which would support the observation that P.s.t was able to chemotax towards extracted tomato apoplast at lower concentrations that STA.

The genomic location of these genes in *P. syringae* was studied in more detail. In particular the *P. syringae* genome was compared to *P. aeruginosa* PAO1, where chemotaxis proteins have been studied in the most detail within *Pseudomonas*. The *P. aeruginosa* PAO1 genome contains five chemotaxis clusters each containing chemotaxis (*che*) genes. In particular, the genes present in chemotaxis clusters one and five have been previously shown to essential for chemotaxis (Masduki et al., 1995). *P. syringae* genomes contained orthologs of all the genes present in the chemotaxis clusters identified in PAO1.

In general, the overrepresented proteins found in the *P. syringae* genome were dispersed throughout the *P. syringae* genome. However, several chemotaxis-related proteins, including some che proteins, were found together in two clusters within the *P. syringae* genome (Figure 5.16). These clusters were present in plant-associated bacteria such as *P. putida* and *P. fluorescens*, but were not present in the *P. aeruginosa* genome. One cluster in

particular (Figure 5.16 B) was restricted to *P. syringae*, *P. fluorescens* Pf-5 and *P. mendocina* genomes. The genes within both clusters are close enough together (less than 100 bp) that they may form an operon, and therefore may constitute two new chemotaxis clusters.

The cognate chemoeffectors for the majority of MCP's have not been identified. *P. aeruginosa* encodes 26 MCP genes, however, so far, chemoeffectors for just six of these MCPs have been reported; three MCP's has been identified as detecting malate, ethylene and inorganic phosphate, and another three as detecting 18, seven and two naturally occurring amino acids each (Taguchi et al., 1997; Alvarez-Ortega and Harwood, 2007).

Other overrepresented domains in *P. syringae* include the CheW protein. CheW is closely associated with the MCP complex *in vitro* (Figure 5.2) and has been previously shown to be essential for chemotaxis (Webre et al., 2003). Very little is known about Chase 3-domain proteins except that they are transmembrane receptors found in signal transduction pathways in bacteria. However nothing is yet known about the environmental factors that are recognised by chase 3 domains (Zhulin et al., 2003)

In conclusion, this chapter demonstrated the ability P.s.t to chemotax towards a range of amino acids. In particular, the five most abundant amino acids in the tomato apoplast were excellent chemattractants for P.s.t at the concentrations present in the tomato apoplast. Furthermore, bioinformatic comparisons between the genome of *P. syringae* and non plant-pathogenic *Pseudomonas* species indicated that *P. syringae* may be overrepresented in several chemotaxis-related domains. This may represent an increase in the number of chemoeffectors which *P. syringae* can bind to cognate MCP proteins and therefore an increase in the chemotactic ability of this bacterium. The ability to chemotax towards compounds, such as certain amino acids, which are abundant in the plant environment, may provide an advantage to epiphytic *P. syringae*, as this may lead the bacteria towards openings in the leaf surface and access to the apoplast. In addition, the ability to detect and move towards amino acids which can be utilised as carbon or energy sources may increase the ability of *P. syringae* to colonise and grow in the leaf environment. P.s.t demonstrated positive chemotaxis towards amino acids which are putatively transported by the aa_permease genes, including GABA and isoleucine (an aromatic amino acid). Moving towards areas of increased amino acid concentration would increase the efficiency of amino acid uptake. Therefore at this point it is logical to try and establish exactly which amino acids are transported by the aa_permease genes in P.s.t.

Chapter 6: Expression analysis and mutant characterisation.

6.1 Overview.

The genome of sequenced plant-pathogenic *Pseudomonas syringae* (*P. syringae*) contains significantly fewer amino acid permease (aa_permease) domains, found in aa_permease genes, than non plant-pathogenic *Pseudomonas* species. Domain loss is thought to represent a loss of function; for *P. syringae* in particular this is possibly due to being associated with a function that is of little or no importance in the plant environment. Conversely, genes which are retained in the *P. syringae* genome may have some beneficial function (Studholme et al 2005). The four aa_permease genes found in *P. syringae* pv. tomato DC3000 (P.s.t) have been provisionally annotated in the Pfam database as transporting proline (PSPTO_5276), GABA (PSPTO_5356), aromatic amino acids (PSPTO_1817) and ethanolamine (PSPTO_2026) but this has yet to be experimentally demonstrated.

This chapter uses expression analysis by RT-PCR to indicate conditions under which the P.s.t aa_permease genes are expressed or upregulated. Expression of a gene indicates that the gene is functional and can provide insights into its function. The expression of each aa_permease gene was established in exponential growth phase in a range of media, at different pH values and in the presence of certain amino acids including those annotated as solutes for these transporters. The expression of each aa_permease gene in these conditions was compared and discussed. This chapter also documents the complementation and characterisation of a P.s.t–*rpoN* mutant. The alternative sigma factor RpoN is known to be involved in regulating the expression of many genes in response to nutritional and environmental conditions. The P.s.t aa_permease PSPTO_2026 contains a predicted RpoN-binding site but a P.s.t-*rpoN* mutant has yet to be published. A P.s.t-*rpoN* mutant was obtained as a gift, characterised, and complemented with the P.fl SBW25 *rpoN* gene. Expression of the P.s.t aa_permease genes was compared in P.s.t and P.s.t–*rpoN*.

Finally, an RT-PCR-based strategy was used to investigate the expression of genes in P.s.t putatively annotated as belonging to the *hut* operon. Primers successfully amplified regions spanning adjacent *hut* genes demonstrating the presence of cDNA, and therefore RNA, corresponding to these regions, indicating that these genes were transcribed together during growth *in vitro*.

6.2 Introduction.

Plant-pathogenic *P. syringae* is capable of colonising the phyllosphere of host and non-host plants and the intracellular apoplast of host plant species. In order to colonise and proliferate in the plant environment, *P. syringae* must be able to acquire and assimilate nutrients present in these environments. However, many studies have suggested that the plant environment is relatively nutrient poor.

On the leaf surface nutritional availability appears to be extremely heterogeneous. For example, when sugar availability on the surface of bean plants was monitored using an *Erwinia herbicola* GFP bioreporter, for the first hour after inoculation nearly all bioreporter cells appeared to be actively engaged in fructose consumption, but only 1% of bacteria were in areas of the leaf surface where fructose was abundant enough to support consumption for more than 24 hours (Leveau and Lindow, 2001). Furthermore, even when nutrients are present on the surface of leaves, studies have hypothesised that due to the uneven topography of the leaf surface at the microbial scale much of this may be in locations that are inaccessible to the majority of bacteria (Mercier and Lindow, 2000).

The plant apoplast environment is thought to contain a relatively limited number of nutrients in a low abundance (Alfano and Collmer, 1996; Lindow and Brandl, 2003). Many studies have provided evidence in support of the view that nitrogen is limited within the apoplast environment. For example, many of the genes transcribed in the fungal tomato pathogen *Fusarium oxysporum* during plant colonisation were also transcribed during growth in nitrogen starvation conditions *in vitro* (Divon et al., 2008). Nitrogen limitation appears to be an important regulatory cue for the expression of certain virulence and pathogenicity-associated genes in *P. syringae*, such as the *hrp* genes which have been shown to be more highly expressed in minimal medium, *hrp*-inducing minimal medium (HIM), compared to nutrient rich media like KB or LB (Snoeijers et al., 2000). For example, in P.s.ph, the expression of the *hrp* genes *hrpA, hrpB, hrpC, hrpD, hrpL, hrpR* and *hrpS* was up to 700 times higher in HIM, compared to their expression in KB (Rahme et al., 1992).

One regulatory protein which is important for controlling gene expression in response to nitrogen availability is the alternate sigma factor RpoN. RpoN has been shown to control the expression of a variety of genes in bacteria in response to nutritional and environmental conditions, particularly in response to nitrogen. For example, In *E. coli*, about half of the products of RpoN-dependent genes are known to be involved in nitrogen metabolism (Reitzer and Schneider, 2001). In *P. syringae*, *rpoN* also controls *hrp* gene expression and influences virulence *in planta*. *P. syringae* pv. maculicola *rpoN* mutants were nonmotile,

displayed a reduced ability to utilise nutrient sources, and were unable to produce the phytotoxin coronatine, cause disease or HR, or induce host defense mRNAs (Hendrickson et al., 2000b). Similar phenotypes have been reported for *rpoN* mutants of other *Pseudomonas* species, including *P. putida* (Kohler et al., 1989) and *P. aeruginosa* (Totten et al., 1990; Hendrickson et al., 2001).

P. syringae has long been considered to be a relatively specialist *Pseudomonas*, particularly with respect to nutrient assimilation. Recently, comparative analyses of nutrient assimilation by *P. syringae, P. fluorescens, P. putida* and *P. aeruginosa*, demonstrated that *P. syringae* used a restricted range of nutrient sources compared to other *Pseudomonas,* particularly amino acids. However, those amino acids which were used by *P. syringae* correlated with those which were abundant in the tomato apoplast (Rico and Preston, 2008). Furthermore, when *P. syringae* was pre-incubated in extracted apoplast prior to inoculation into a Biolog plate assay to assess carbon source utilisation, Rico and Preston (2008) demonstrated that nutrient assimilation pathways were activated which corresponded to the nutrients present in extracted apoplast. Therefore it has been hypothesised that nutritional specialisation of *P. syringae* has evolved to reflect the nutrients available in the plant apoplast (Rico and Preston, 2008). The availability of many sequenced *Pseudomonas* genomes, including several *P. syringae* strains, has allowed researchers to investigate the genetic basis for the observed nutritional specialisation in *P. syringae* through comparative genome analyses. A bioinformatic comparison of the number of protein domains in *P. syringae* compared to non plant-pathogenic *Pseudomonas* species, revealed differences in the number of domains found in proteins with a role in nutrient utilisation (Studholme et al., 2005). In particular, the aa_permease domain, found in amino acid permease transporters, was significantly underrepresented in *P. syringae* compared to other *Pseudomonas* species (Studholme et al., 2005). The genome of P.s.t contains four aa_permease transporters, predicted to transport ethanolamine (PSPTO_2026), GABA, (PSPTO_5356), proline (PSPTO_5276) and aromatic amino acids (PSPTO_1817), based on homology to characterised proteins in other bacterial species.

Genes involved in the transport of metabolism of specific substrates are likely to be induced in the presence of that substrate; therefore it is logical to test the expression of the aa_permease genes in the presence of their putatively transported substrates. In P.fl.SBW25, the PSPTO_5276 ortholog, *hutT* was experimentally demonstrated to be a histidine-inducible gene required for histidine utilisation *in vitro* (Zhang et al., 2006). This gene was not induced in the presence of proline. PSPTO_5276 is more closely related to P.fl.SBW25 *hutT*, than to the experimentally characterised transporters in other bacterial

species such as *E. coli*, on which the annotation of PSPTO_5276 as a proline transporter was based. Therefore PSPTO_5276 may also transport histidine rather than proline. In order to test this hypothesis the expression of PSPTO_5276 will be monitored in the presence of histidine and proline. The expression of PSPTO_2026, PSPTO_1817 and PSPTO_5356 will also be monitored in the presence of their putatively transported substrates ethanolamine, aromatic amino acids and GABA, respectively.

A limited number of studies have reported the expression of *Pseudomonas* aa_permease genes in the plant environment. For example, Zhang and coworkers (2006) reported that in P.flSBW25, *hutT* was expressed in the phyllosphere, rhizosphere and root exudates, although the level of expression was low (measured as the amount of β-galactosidase activity). Mark and coworkers (2005) reported that in PAO1, the aa_permease genes PA1485 and PA4804 (which have no annotated substrates, see Chapter 3) were upregulated in the presence of root extracts from sugar beet (the expression of PA4804 was specifically upregulated in the presence of a single tested cultivar, namely Roberta).

If the aa_permease genes are involved in plant colonisation we would expect to see them expressed in the plant environment and upregulated in apoplast extracts. Therefore it will be interesting to compare the expression of each P.s.t aa_permease gene following incubation of bacteria in tomato apoplast and in synthetic tomato apoplast, and to relate the observed expression levels to the abundance of amino acids known to be present. As previous authors have indicated that pH may influence the efficacy of transporters powered using chemiosmotic energy (Saier, 2000) such as the aa_permeases, and as previous work in this thesis has demonstrated that the ability of P.s.t to gain a growth benefit from the presence of certain amino acids is influenced by pH, the expression of each aa_permease gene at different pH values will also be investigated.

Finally, genes involved in nitrogen assimilation and uptake are frequently regulated by RpoN. At least one aa_permease in P.s.t was predicted to contain a binding site for RpoN, and therefore may be regulated by this protein (PSPTO_2026; Chapter 3, Section 3.5.7). Furthermore, the P.fl.SBW25 *hutT* gene was found to be regulated by RpoN (Zhang and Rainey, 2007) and a P.fl.SBW25-*rpoN* mutant displayed a loss of histidine-utilisation phenotype (Jones et al., 2007). The ortholog of *hutT* in the P.s.t genome is PSPTO_5276; therefore this gene may also transport histidine and be regulated by RpoN. Interestingly the P.fl.SBW25-*rpoN* mutant also lost the ability to utilise ethanolamine and GABA, suggesting these genes may be regulated by RpoN. Therefore, it is logical to test whether the aa_permease genes are expressed in an RpoN-dependent manner. The role of RpoN in

controlling the expression of aa_permease genes in P.s.t will be investigated; P.s.t-rpoN mutant will be complemented and characterised, and the expression of each permease compared in wild type P.s.t and P.s.t-*rpoN*.

6.3 Objectives

- Analyse the expression of the four aa_permease genes in P.s.t *in vitro* in a range of media, and in the presence of putatively transported amino acids.
- Develop a simple method for analysing the expression of aa_permease genes *in planta***.**
- Compare the expression of aa_permease genes *in planta* and in apoplast extracted from tomato plants.
- Investigate the ability of synthetic tomato apoplast to induce gene expression compared to extracted apoplast.
- Complement the P.s.t *rpoN* mutant (P.s.t-*rpoN*) mutant with the *rpoN* gene from P.fl SBW25.
- Characterise the performance of the P.s.t-*rpoN* mutant and complemented mutant (P.s.t-*rpoN* (pJJP6)) *in vitro* and *in planta.*
- Investigate the expression of aa_permease genes in P.s.t-*rpoN* and P.s.t-*rpoN* (pJJP6), particularly those which are predicted to have RpoN binding sites.

6.4 Materials and methods.

6.4.1 Bacterial strains and abbreviations.

Bacterial strains and abbreviations were used as in Chapter 2.

6.4.2 Growth - *in vitro.*

For RT-PCR, bacteria were grown overnight in LB media at 28°C with shaking at 200 rpm. Cells were washed twice with the appropriate test media, and resuspended in the test media at an OD_{590} of 0.2. Test media comprised LB, M9 minimal media, tomato apoplast (extracted as described in Chapter 2) or synthetic tomato apoplast (STA) media (made as described in Chapter 4). Where appropriate the pH of the media was adjusted using sodium hydroxide or hydrochloric acid as necessary. Bacteria were then returned to the incubator for two hours before RNA was extracted according to the protocol described below.

6.4.3 Growth – *in planta.*

For *in planta* assays, cells were grown overnight in LB media at 28°C with shaking at 200 rpm. Cells were washed twice with 10 mM $MgCl_2$ and resuspended in 10 mM $MgCl_2$ at a concentration of $1x10^5$ colony forming units (cfu) ml^{-1}. Bacterial suspensions were inoculated into the underside of fully expanded leaves using a 1 ml syringe without needle (Fisher Scientific, UK). Symptoms in tomato plants were photographed at three days post inoculation. Symptoms in tobacco plants were photographed at 24 hours post inoculation.

6.4.4 Motility assay.

Motility assays were carried out as previously described (Jones et al., 2007). Briefly, overnight cultures were adjusted to an OD_{590} of 0.5 and stabbed into the centre of 1/10 LB, 0.3% agar plates. Plates were incubated at room temperature and measured and photographed at 48 hours and 72 hours post inoculation.

6.4.5 P.s.t-*rpoN* mutant complementation.

An RpoN mutant of P.s.t, (P.s.t-*rpoN*) was obtained as a gift from Arun Chatterjee (University of Missouri, USA). P.s.t-*rpoN* was subsequently complemented by transforming electrocompetent cells with the pJJP6 plasmid containing the *rpoN* gene of *P. fluorescens* SBW25 cloned into pBroadgate (Jones et al., 2007). In addition, a pJJP6 control was created by transforming electrocompetent wild type P.s.t with the pBroadgate-D plasmid, which contains a small insert encoding gentamycin resistance in the cloning site in place of the *ccdB* gene to avoid lethality.

Electrocompetent cells were created according to the protocol described in Chapter 2, Section 2.5.10. The plasmid pJJP6, containing the *rpoN* gene from *P. fluorescens* SBW25 (P. fl. SBW25) (Jones et al., 2007), was extracted from an overnight culture of *E. coli* DH5α λ pir using a QIAprep Spin Miniprep Kit (Qiagen, UK). The pJJP6 plasmid was dialysed for one hour to remove salt using 0.025 µm Millipore (Millipore, UK) 'V' series dialysis membranes and the 'drop dialysis' protocol described in Chapter 2, Section 2.5.7 One microlitre of dialysed plasmid prep was added to the electrocompetent cells and gently mixed with a sterile pipette tip. The cells were transferred to a sterile, pre-chilled 0.2 cm electroporation cuvette (BioRad, UK). Cuvettes were kept on ice until electroporation at 2.5 V and 200 Ohms using a Gene Pulser electroporation system (BioRad, UK). Immediately after electroporation 800 µl of SOC medium was added to the cuvette and the cells were transferred to a sterile 1.5 ml microfuge tube. Cells were incubated for 2 hours at 28°C with shaking (200 rpm), after which aliquots were plated out onto appropriate selective media.

6.4.6 RT-PCR.

- **Extraction of bacterial RNA and cDNA synthesis.**

RNA was extracted from lysed bacteria using the RNeasy Protect Bacteria Mini Kit (Qiagen, UK). In order to extract RNA from bacterial cultures, single bacterial colonies were inoculated into 5 ml of LB media and incubated overnight at 28°C with shaking (200 rpm). On day two, bacterial cultures were washed twice in the media of interest and resuspended in that media at an optical density OD_{590} of 0.2. Cultures were returned to a shaking incubator at 28°C for two hours. To harvest the cells, cultures were removed from the incubator and 400 µl was immediately resuspended in 800 µl of RNAProtect Bacteria Reagent (Qiagen, UK). Cells were incubated for 5 minutes at room temperature, centrifuged for 20 minutes at 5000 g and the supernatant decanted. TE buffer was prepared containing 15 mg ml^{-1} lysozyme (Sigma-Aldrich, UK) and 100 µl proteinase K solution (Quiagen, UK). Pellets were resuspended in 110 µl of this mixture and incubated for 15 minutes at room temperature, mixed by vortexing every 2 minutes. Then, 350 µl of buffer RLT (from the RNeasy Protect Bacteria Mini Kit) containing 10 µl ml^{-1} β-mecaptoethanol was added. The sample was mixed by vortexing and 250 µl 100 % ethanol was added.

RNA was then purified from this bacterial lysate using the RNeasy Protect Bacteria Mini Kit according to the manufacturer's instructions, with the following modifications. RNA was eluted in 30 µl of nuclease-free water; the eluate was then passed through the elution column for a second time to remove residually bound RNA. Then a 16 µl aliquot of each RNA sample was treated with 2 units of RQ1 RNase-free DNase 1 (Promega, UK), which was then inactivated according to the manufacturer's instructions.

To assess the quantity and quality of the RNA yield, 2 µl of DNase-treated RNA was quantified using a NanoDrop ND-100 UV-Vis Spectrophotometer according to the manufacturer's instructions. RNA quality with respect to DNA contamination and residual ethanol was assessed using the ratio of absorbance at 260/A280 and 260/A230. Ratios above 2.0 were judged to be of good quality. RNA samples were normalised to the lowest sample concentration using nuclease-free water. Negative controls were produced by treating one aliquot of each sample with 0.5 µl of nuclease-free water containing a 1 in 40 dilution of RNase A (Quiagen, UK). A second sample was treated with 0.5 µl of nuclease-free water without RNase A. Both aliquots were incubated at 37°C for 30 minutes. Normalised RNA samples were used as a template for cDNA synthesis using the Improm-II reverse transcription system and random primers (Promega, UK) according to the manufacturer's instructions. Each growth condition was assayed in four replicates and

results are presented as the average of these replicates. After synthesis, an aliquot of cDNA was diluted 1 in 10 with nuclease-free water. Samples were then stored at -20°C.

- **Agarose gel electrophoresis and quantification of RT-PCR products.**

For examining RT-PCR products agarose gels were prepared by melting high gel strength agarose to a concentration of 2 % (w/v) in 1 x Tris-Acetate-EDTA (TAE) buffer. Molten gels were allowed to cool to below 50°C before being supplemented with ethidium bromide to a final concentration of 0.2 mg ml^{-1} and poured into an appropriate casting tray to set. Gels were then transferred to an electrophoresis tank and immersed in 1 x TAE. Prior to loading one-fifth volume of 5x loading buffer (25 % glycerol, 0.21 % brophenol blue, 0.21 % xylene cyanol) was added to each sample. A standard 100 bp DNA marker ladder (0.5 µg; New England Biolabs, UK) was added to an empty well to allow a comparison of band sizes. A standard potential difference of 60 V was applied to the gel for 90 minutes.

Gels were visualised using a BioRad UV transilluminator (wavelength 245 nm) (BioRad Laboratories, UK) and photographed digitally using BioRad Quantity One software. All RT-PCR gels were photographed immediately after agarose gel eletrophoresis, and within 10 minutes of being exposed to UV illumination to minimise the loss of band intensity. Quantification of RT-PCR products was carried out using the densitometry option within the BioRad Quantity One software. Briefly, bands were first analysed for pixel saturation and camera settings for aperture and time of exposure were adjusted as appropriate. If necessary the PCR program was repeated with a fewer cycles to produce less intense bands. The total pixel density was calculated by the Quantity One software for each band and the average background pixel density was subtracted. Results were presented as the density of each RT-PCR product divided by the density of the housekeeping gene. Results represent the values obtained from three independent experiments.

- **Maintenance of a RNA-free area.**

Following harvesting of RNA from bacteria, all steps were carried out in an area designated for RNA work. Prior to use this area was thoroughly wiped with RNase-Zap (Ambion Inc, USA) and RNase-free filter tips were used throughout. PCR and 1.5 ml microfuge tubes were treated overnight in a solution of 0.1 % (w/v) diethylpyrocarbonate (DEPC) in distilled water. These were then rinsed twice with autoclaved DEPC-treated water and then autoclaved at 121°C for 30 minutes to remove residual DEPC. Nuclease-free water (Qiagen, UK) was used where appropriate for all RNA extraction steps.

6.4.7 Primers used in this study.

Primers were manufactured by MWG (Germany) and were used at a final concentration of 10 pmol / reaction. RT-PCR primers were designed to amplify fragments between 100 and 200 bp. Housekeeping primers were designed by Jake Jones, Oxford UK, for the housekeeping gene *groEL* (a molecular chaperone), to allow assessment of normalisation between samples. All primers were checked against genomic DNA for specific amplification of the gene in question. Sequences of all primers are provided below. The expression of *groEL* was found to be similar and constant in P.s.t during growth in LB and M9 minimal media.

Primer name	**Sequence (5'-3')**
PSPTO_1817_RTL	TGGAAATGCTCGGTTTTACC
PSPTO_1817_RTR	TCAGCGACAACAGCACTACC
PSPTO_5296_RTL	ACCTGGAACTGATGGACAGC
PSPTO_5296_RTR	CAGCCAGGCATTATTGAGGT
PSPTO_5356_RTL	GGCCACCAATTCAGTGATCT
PSPTO_5356_RTR	AGCACGGTCTGATACGAACC
PSPTO_2026_RTL	TTTGAACTGGTGGTGTGCAT
PSPTO_2026_RTR	AGGAAAAACCAGATGGCAAA
GroEL_RT1_R	CATGCTTTCCGGCTTGTTG
GroEL_RT1_F	TGTAGAAGGCATGCAGTTCGA

Table 6. 2
RT-PCR primers used in this study.

Primer name	**Sequence (5'-3')**
PSPTO_5270L	CTGTGACGGTACGGACGAG
PSPTO_5271R	CGGATACCAAGGTGGTCAAC
PSPTO_5271L	AGCTGGCGAAGATTTTCCTT
PSPTO_5272R	GTCGCCATACTGAGTCACCA
PSPTO_5272L	GTCATTCTGGCGGTCGTC
PSPTO_5273R	GTCTGCGCCAGTACCTGAT
PSPTO_5273L	GTGTTCATCACCCACGACCT
PSPTO_5274R	CACCGAAGACGACCTGTTCT
PSPTO_5274L	TGCTCAAAGACGAAACATCG
PSPTO_5275R	CTGTCGTCGATCTGCTGGT
PSPTO_5275L	CAGTCAGCTGTTGTCCTCCA
PSPTO_5276R	AGCCGTAGAACAACCCTGTG
PSPTO_5276L	TTGCCTTCATGCTGTTCATC
PSPTO_5277R	GCTTGCGATGTGACAGTGTT
PSPTO_5277L	ATTTTGTCGCCTGGAACATC
PSPTO_5278R	ACCGGTGTCAGCTTCAGG

Table 6.3
Primers used in this study.

6.5 Results.

6.5.1 Expression of aa_permease genes in wild type *Pseudomonas syringae* pv. tomato in synthetic tomato apoplast and LB media.

Bacteria continually monitor their extracellular environment and intracellular physiological status. Based on this information they can appropriately modulate their gene expression in response to changes in their environment (Camilli and Bassler, 2006). Reverse transcriptase PCR (RT-PCR) is commonly used to monitor bacterial gene expression, and can be used to indicate conditions under which genes are expressed or upregulated. In order to investigate whether the aa_permease genes in P.s.t (PSPTO_5276, PSPTO_5356, PSPTO_2026 and PSPTO_1817) were upregulated in nitrogen limited conditions, the expression of these genes was monitored in 'rich' LB media compared to synthetic tomato apoplast (STA). In addition, Chapter 4 Section 4.5.17 previously indicated that pH may affect the metabolism of some compounds. Therefore, expression was monitored in P.s.t grown in LB and STA at pH 6 and pH 7, to investigate whether the differences in metabolism were correlated with differences in expression of transporters importing compounds into the cell.

In this chapter, gene expression was monitored using a semi quantitative RT-PCR method in which RNA expression was monitored indirectly by measuring the amount of cDNA in each sample compared to that of the housekeeping gene *groEL*. A computer program was used to measure cDNA in samples which had been run out on an agarose gel by the intensity of the ethidium bromide staining (densitometry). This technique is relatively simple and cheap, but has several limitations which need to be remembered when analysing results. Mainly, the resolution of this technique is low compared to alternative techniques such as real-time reverse-transcription PCR (qRT-PCR), this is because the intensity of ethidium bromide staining decreases over time and therefore slight differences in results could be due to differences in the amount of time that samples were run out on agarose gels. This was controlled as much as possible in this chapter by running all samples on agarose gels for the same length of time. In addition, it was possible that highly expressed genes could saturate the densitometry program. Therefore fewer PCR cycles were used in certain cases in order to produce a weaker band.

In general, results indicated that the expression of all the aa_permease genes was low in exponentially growing bacteria in LB and STA relative to the expression of the housekeeping gene *groEL*. The expression of PSPTO_5276 and PSPTO_5356 was significantly higher in synthetic tomato apoplast (STA) than in LB media. In contrast, the expression of

PSPTO_1817 and PSPTO_2026 was not significantly different in LB compared to STA and expression was not significantly affected by the pH of the media. These results are discussed in detail below.

- **Expression of PSPTO_5276 in LB and STA at different pH values.**

The expression of PSPTO_5276 was significantly increased when cells were grown in slightly acidic (pH 6) media compared to media at pH 7, but expression was not affected by the composition of the culture media (i.e there were no significant differences between the expression of PSPTO_5276 in STA compared to LB at the same pH)(Figure 6.1). LB has been reported to contain small amounts of histidine and proline (Sezenov et al., 2006) which may be sufficient to induce expression of this gene. The increase in expression of PSPTO_5276 at pH 6 is concurrent with results presented in Chapter 4, Section 4.5.5, which demonstrated that P.s.t could gain a larger growth benefit (measured as an increase in optical density), from the addition of certain amino acids (including histidine and proline) to M9 media at acidic pH 5.5 compared to alkaline pH 8.

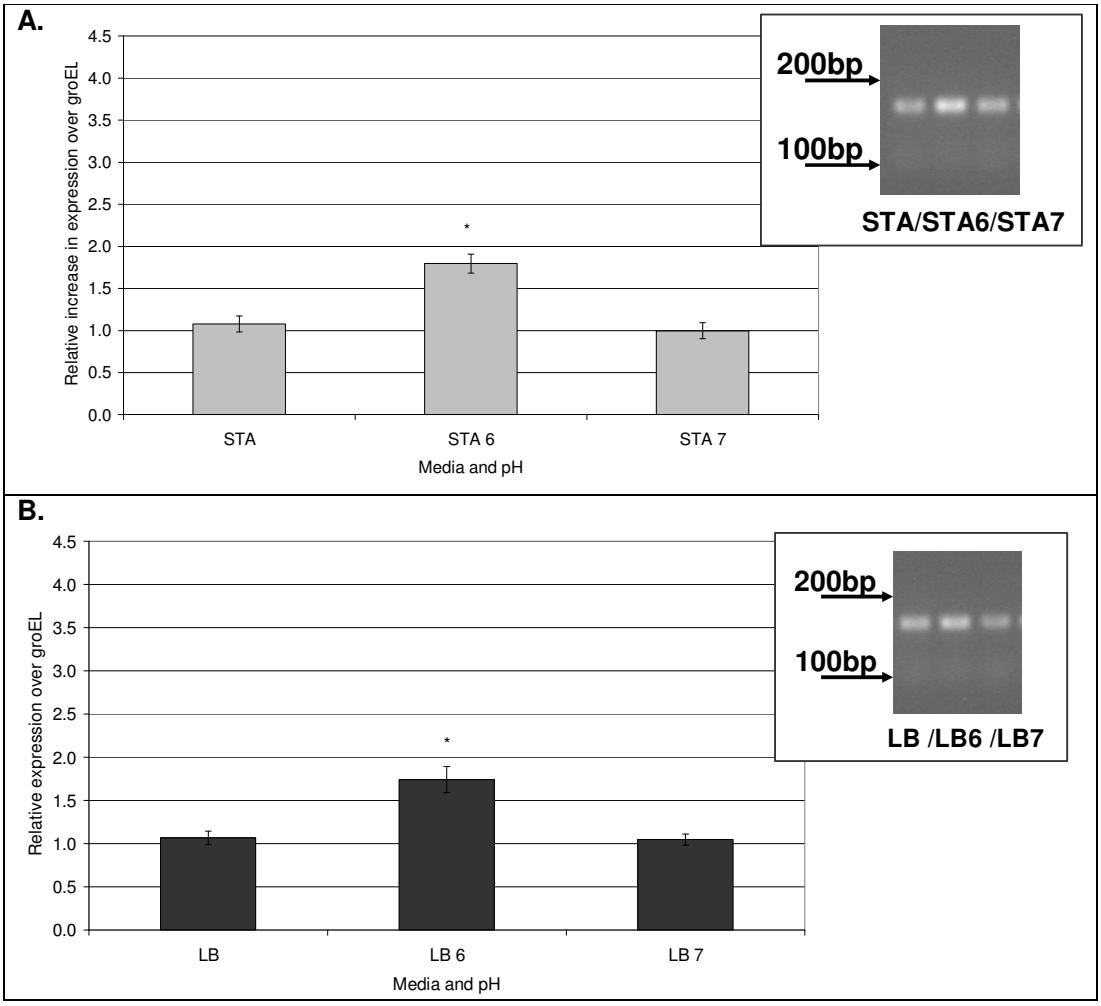


Figure 6.1
The expression of PSPTO_5276 in **A.** synthetic tomato apoplast (STA) and **B.** LB media at pH 6, 6.6 and 7. STA and LB are at pH 6.6. Error bars represent standard deviations of RNA extracted from three cultures. The experiment was independently repeated twice. Significant ($p \leq 0.05$) results in Student's 2-tailed t-tests are marked with an asterix. H_0= no difference in expression at different pH. **A.** * = Expression of PSPTO_5276 was significantly increased in STA pH 6 compared to STA pH 6.6 and STA pH 7. **B.** *= Expression of PSPTO_5356 was significantly increased in LB pH 6 compared to LB pH 6.6. Expression of PSPTO_5356 in LB pH 6 and LB pH 7 was not significantly different. There were no significant differences between the expression of PSPTO_5276 in STA compared to LB at the same pH.

- **Expression of PSPTO_5356 in LB and STA at different pH values.**

In general, the expression of PSPTO_5356 in STA was significantly higher than in LB media. PSPTO_5356 has been provisionally annotated as a GABA transporter. GABA was detected in the tomato apoplast and was therefore added to STA media; however although LB media contains the GABA precursor, glutamic acid, GABA was not been detected (Sezenov et al., 2006). Therefore the presence of GABA in STA may explain the increased expression of PSPTO_5356 in this media compared to LB.

Results also indicate a significant effect of pH on gene expression. Increased expression of PSPTO_5356 was observed in cells incubated in both STA at pH 6 compared to pH 7 (Figure 6.3). It is worth noting here that results from Chapter 4, (Section 4.5.15) demonstrated that metabolism of GABA by P.s.t was significantly increased ($p=0.023$) following pre-incubation of cells in STA at pH 6 compared to STA pH 7. Therefore, these results indicate that pre-incubation of P.s.t in STA at pH 6, may result in increased metabolism of GABA due to the increased expression of at least one GABA transporter at acidic pH values. The expression of PSPTO_5356 was also significantly increased in LB at pH 6 compared to the expression at pH 7. This result agrees with the above hypothesis that PSPTO_5356 expression is increased in slightly acidic conditions.

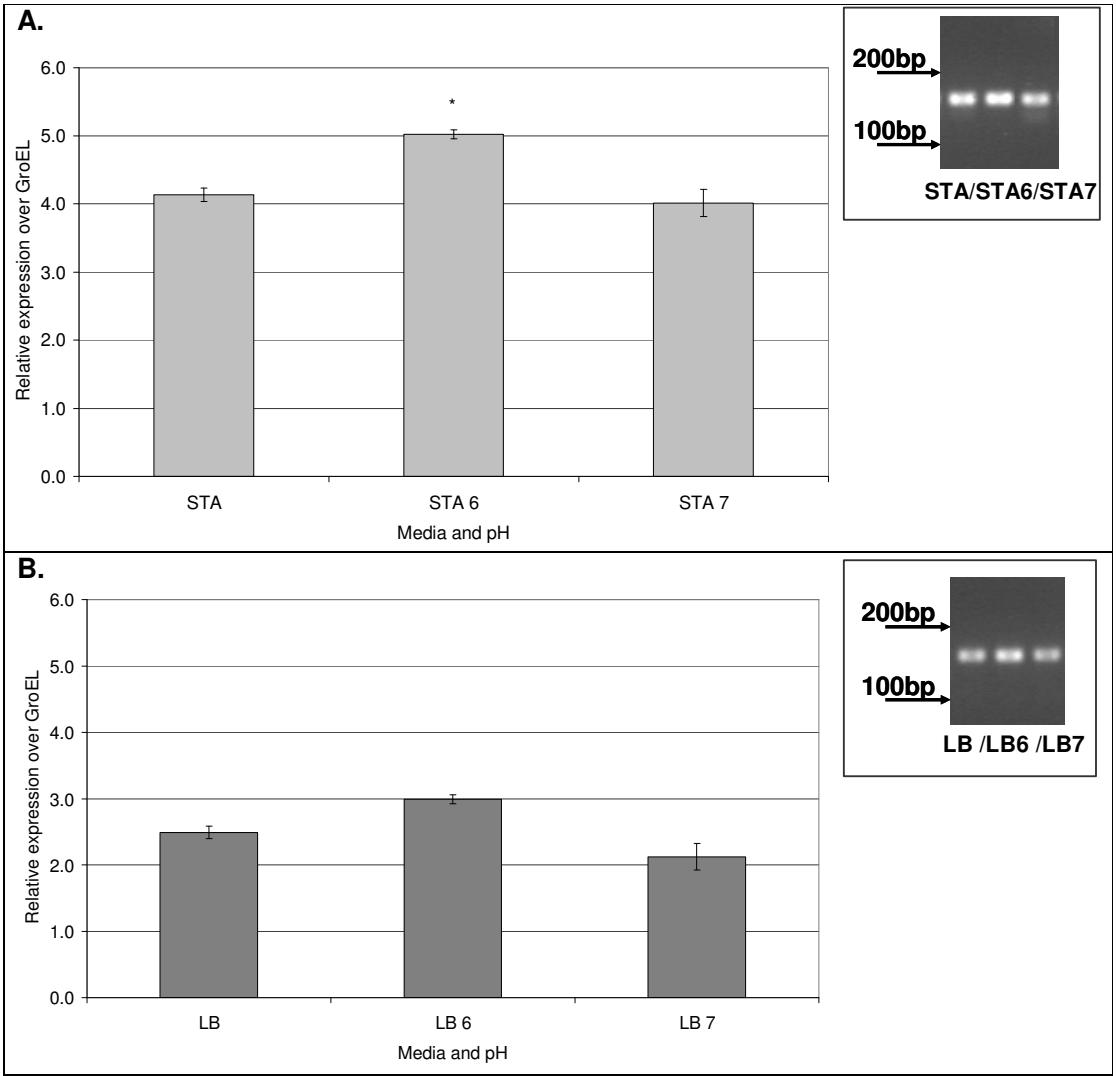


Figure 6.2

The expression of PSPTO_5356 in **A.** synthetic tomato apoplast (STA) and **B.** LB media at pH 6, 6.6 and 7 (STA and LB are at pH 6.6). RNA was extracted from P.s.t in exponential growth phase at 28°C. Error bars represent standard deviations of three cultures. The experiment was independently repeated twice. PCR transcripts of PSPTO_5356 cDNA are shown inset. Significant ($p \leq 0.05$) results in Student's 2-tailed t-tests are marked with an asterix. H_0= no difference in expression at different pH.

A. * = Expression of PSPTO_5356 was significantly increased in STA pH 6 compared to STA at pH 7 (p=0.000) and STA pH 6.6 (p=0.001).

B. *= Expression of PSPTO_5356 was not significantly different.

Differences between expression in STA and LB: The expression of PSPTO_5356 was significantly higher in STA compared to LB, at all pH values tested. Expression of PSPTO_5356 in STA pH 6 was significantly higher compared to LB pH 6 (p=0.00). Expression in STA was significantly increased compared to LB (p.0.01). Expression of PSPTO_5356 in STA at pH 7 was significantly increased compared to LB media at pH 7 (p=0.001)

- **Expression of PSPTO_1817 in LB and STA at different pH values.**

In general, the expression of PSPTO_1817 was low in both LB and STA media. As bacteria were extracted during exponential phase growth, this result could indicate that PSPTO_1817 is expressed at a different time point to the one sampled. Alternatively, this result may indicate the absence of an inducing compound for PSPTO_1817 expression in STA and LB. Therefore it will be interesting to investigate the expression of PSPTO_1817 in extracted apoplast, as a significant increase in these conditions would indicate that expression of PSPTO_1817 was induced by a compound(s) not presently added to STA.

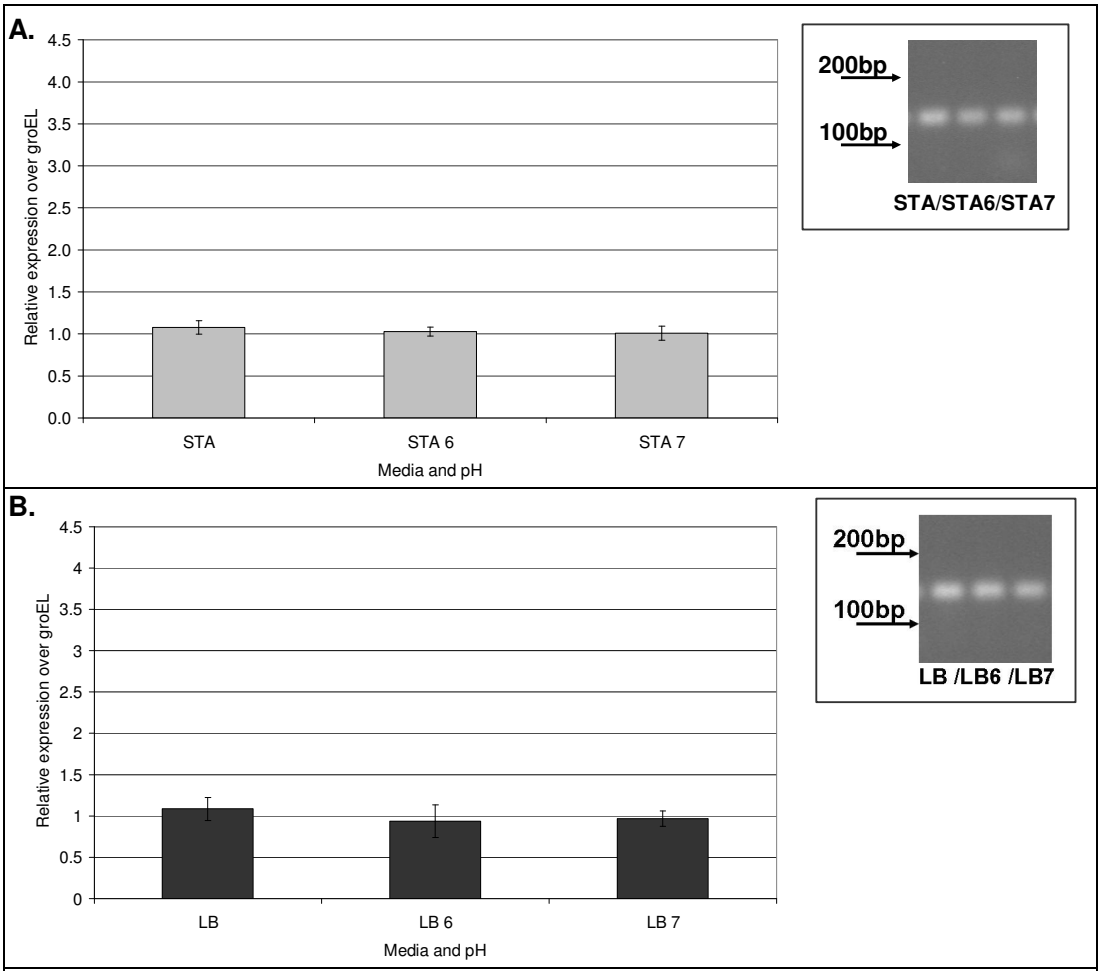


Figure 6.3
The expression of PSPTO_1817 in **A.** synthetic tomato apoplast (STA) and **B.** LB media at pH 6, 6.6 and 7. STA and LB are at pH 6.6. PCR transcripts of PSPTO_1817 cDNA are shown inset. RNA was extracted from P.s.t in exponential growth at 28°C. Error bars represent standard deviations from three replicate cultures. The experiment was independently repeated twice with similar results. **A.** The expression of PSPTO_1817 was not significantly different in STA at any pH tested. **B.** The expression of PSPTO_1817 was not significantly different in LB at any pH tested. $p \leq 0.05$, Student's 2-tailed t-tests, H_0= no difference in expression between media.

- **Expression of PSPTO_2026 in LB and STA at different pH values.**

The expression of PSPTO_2026 was low in both LB and STA media (Figure 6.4). As before this result may indicate that PSPTO_2026 is not expressed during exponential growth. Alternatively, this result may indicate the absence of an inducing compound in STA and LB. Ethanolamine was not analysed by Rico and Preston (2008) in the tomato apoplast, and therefore was not added to STA media. Therefore, it will be interesting to investigate the expression of PSPTO_2026 in extracted apoplast, which may contain ethanolamine or other inducing compounds.

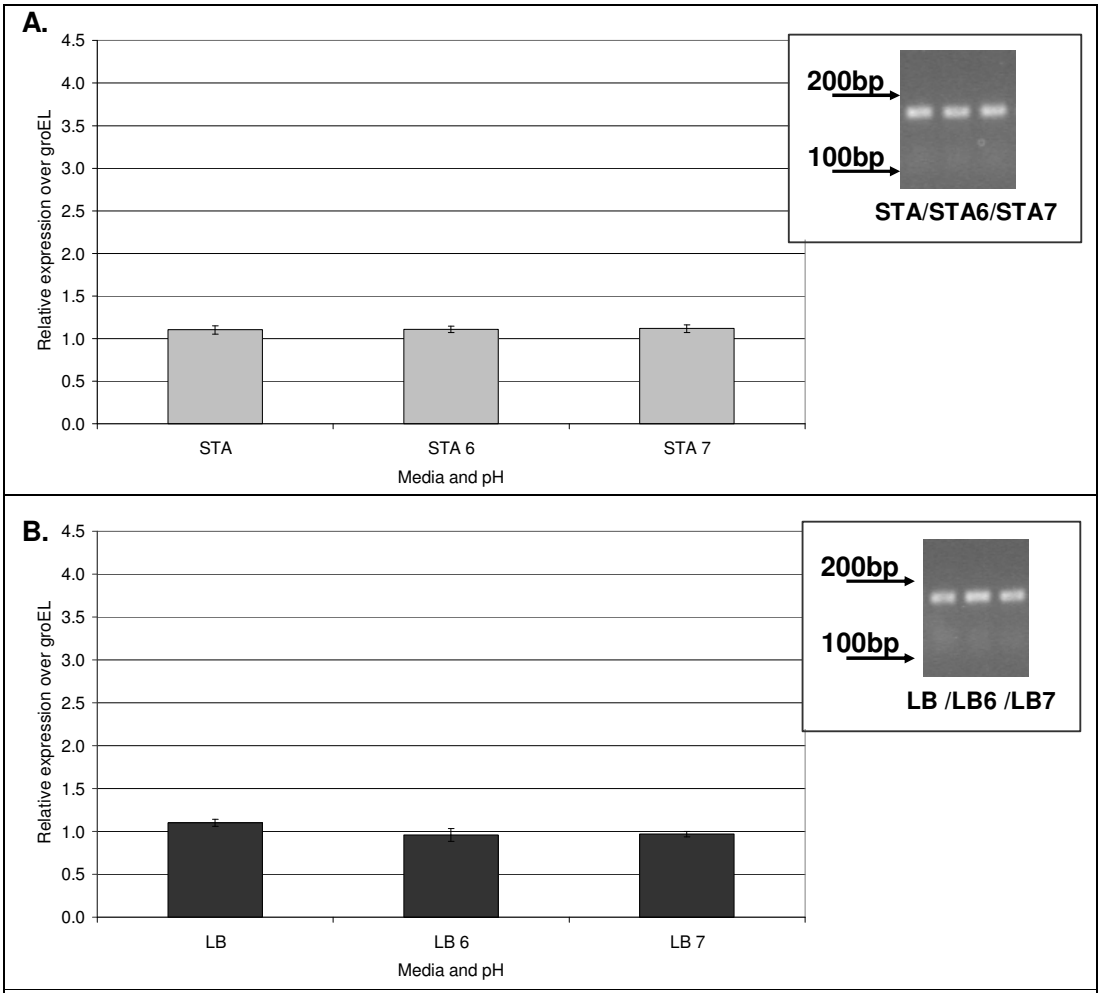


Figure 6.4
The expression of PSPTO_2026 in **A.** synthetic tomato apoplast (STA) and **B.** LB media at pH 6, pH 6.6 and pH 7. STA and LB are at pH 6.6. RNA was extracted from P.s.t in exponential growth phase at 28°C. Error bars represent standard deviations of three replicate cultures. PCR transcripts of PSPTO_2026 cDNA are shown inset. **A.** The expression of PSPTO_2026 was not significantly different in any of the conditions tested. **B.** The expression of PSPTO_2026 was not significantly different in any of the conditions tested. $p \leq 0.05$, Student's 2-tailed t-tests, H0= no difference in expression between media.

6.5.2 Development of a simple method to analyse gene expression *in planta.*

The expression of several aa_permease genes in synthetic media is low. This may be due to the absence of an inducing compound for these genes in the conditions tested. It would therefore be desirable to measure the expression of these genes *in planta* by measuring RNA abundance. Although it is possible to extract RNA from both plant tissues and bacterial cultures independently, there are few methods published to extract RNA from bacterial cells *in planta*. Published methods which have reported successful separation of bacterial and plant RNA are time consuming and expensive (Schenk et al., 2008). Possibly due to these reasons, research on P.s.t has so far focused on the use of transcriptional fusions to measure *in planta* gene expression (Keith et al., 2003; Weingart et al., 2004).

An apoplast extraction technique which facilitates the removal of plant apoplast with minimal cytoplasmic contamination has recently been published (Rico and Preston, 2008). Using this, an alternative method to monitor gene expression *in planta* will be investigated and developed. It is envisaged that this method will be based on infiltrating bacteria into the plant apoplast, and then centrifuging cells out of leaves along with the apoplast fluid into an RNA-stabilising buffer. Although such a method could be criticised for extracting RNA from bacteria that had been centrifuged out of a leaf rather than being stabilised and extracted *in situ*, it would offer the advantage that the stabilised RNA could be processed quickly and relatively cheaply using a commercially available RNA extraction kit. As the apoplast extraction process has been shown to involve negligible contamination of the apoplast with cytoplasmic components of plant cells, this method would also avoid contamination of the sample with plant RNA.

In order to investigate the feasibility of such a method several preliminary experiments were carried out: The aim of these preliminary experiments was to assess the optimal cell density of P.s.t to infiltrate into the apoplast, so that following centrifugation enough cells would be recovered to extract a usable quantity of RNA. P.s.t was inoculated into tomato leaves at densities of $2.5x10^9$, $2.5x10^8$ and $2.5x10^7$ cfu ml^{-1}. Approximately 1 ml of bacterial suspension was used to inoculate each fully expanded tomato leaf. These densities were chosen based on the RNeasy Protect Bacteria Mini Kit (Qiagen, UK), which recommends using $2.5x10^8$ of bacteria per column. Although lower than the recommended density, leaves were also inoculated with $2.5x10^7$ cfu ml^{-1} of bacteria in the hope that RNA might be recovered from inoculation densities closer to those used in standard infection assays.
Bacteria were extracted by centrifugation 30 minutes after inoculation. A 30-minute time interval was chosen because after this length of time the leaves were still damp, and apoplast could be extracted without the need for infiltration with water, which may have

resulted in further changes in bacterial gene expression. The average volume of apoplast extracted from one leaf was 404 µl ± 110 µl (from twelve replicate leaves).

The numbers of inoculated and recovered bacteria were compared (Figure 6.5). Results indicate that the number of recovered bacteria per leaf is generally one to two orders of magnitude lower than the number inoculated. This data was then used to calculate the number of leaves that would need to be inoculated in order to recover sufficient bacteria for RNA extraction (Table 6.2). From these results it can be seen that the pooled apoplast extracted from 2.5 leaves inoculated with $2.5x10^9$ bacteria should recover enough cells for RNA extraction.

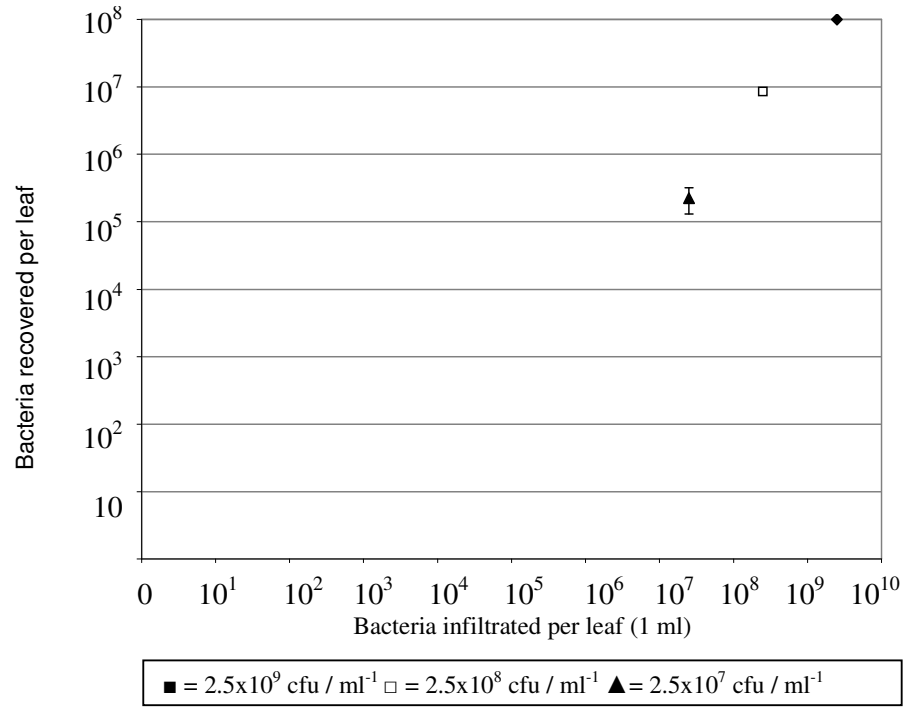


Figure 6.5
Bacteria were infiltrated into fully expanded leaves at $2.5x10^9$, $2.5x10^8$ and $2.5x10^7$ cfu ml^{-1}. After 30 minutes the leaves were detached and bacteria were extracted by centrifugation. Samples were serially diluted and plated out onto LB agar supplemented with the *Pseudomonas*-selective antibiotic CFC. Colonies from at least six plates were counted after two days growth at 28°C. Error bars represent standard deviations from two independent experiments. Both axes use a log scale.

Bacteria inoculated		Bacteria recovered		
OD_{590}	cfu ml^{-1}	Bacteria recovered per leaf	Volume of apoplast recovered per leaf (ul)	Leaves needed to recover $2.5x10^8$ bacteria
4.5	$2.5x10^9$	$1x10^8$	400	2.5
0.45	$2.5x10^8$	$8.5x10^6$	400	29
0.045	$2.5x10^7$	$2.3x10^5$	400	1087

Table 6.2
Amount of leaf tissue needed in order to extract $2.5x10^8$ bacteria for RT-PCR. Bacteria were infiltrated into fully expanded leaves at $2.5x10^9$, $2.5x10^8$ and $2.5x10^7$ cfu ml^{-1}. After 30 minutes the leaves were detached and bacteria were extracted by centrifugation. Samples were serially diluted and plated out onto LB agar supplemented with the *Pseudomonas*-selective antibiotic CFC. Based on the number of bacteria recovered from each leaf sample, the number of leaves which need to be inoculated can be calculated. Leaf calculations are for fully-expanded tomato leaves weighing approximately 1 g per leaf.

These results were then used to develop the following assay for recovering RNA from bacteria infiltrated into the leaves of tomato plants:

A fully expanded tomato leaf was infiltrated with a 1 ml suspension of $2.5x10^9$ bacteria. This suspension was distributed evenly throughout the leaf using a 1 ml syringe without a needle pressed against the underside of the leaf. This process was repeated for two replicate leaves of a similar size and age. The plants were incubated for 30 minutes. Cells were then extracted from 2.5 leaves using the apoplast recovery method from Rico and Preston (2008) with modifications. Briefly, leaves were placed within 5 ml pipette tips, the end of which was inserted into a 50 ml centrifuge tube. Bacterial expression needed to be stabilised in order to obtain high quality RNA; therefore RNAprotect Bacterial Reagent (supplied with the RNeasy Protect Bacteria Mini Kit, Qiagen, UK) was added to the 50 ml microfuge tube, so that RNA was stabilised as soon as the bacteria left the leaf. According to the manufacturer's instructions, two volumes of RNAprotect Bacterial Reagent must be added for every volume of bacterial culture. The average volume of apoplast extracted from each leaf was 400 µl; therefore at least 800 µl of RNAprotect Bacteria Reagent was needed to stabilise the bacterial RNA in this volume. The RNAprotect Bacteria Reagent was placed in the bottom of the 50 ml centrifuge tube and apoplast was centrifuged directly into this. Following the stabilisation of bacterial RNA, the RNeasy Protect Bacteria Mini Kit protocol was followed without modifications.

6.5.3 Expression of aa_permease genes in the presence of selected amino acids, in extracted tomato apoplast and '*in planta'*.

The solute(s) transported by four aa_permease genes in P.s.t have been provisionally annotated in the Pfam protein database as: GABA (PSPTO_5356), proline (PSPTO_5276), aromatic amino acids (PSPTO_1817) and ethanolamine (PSPTO_2026). However this has yet to be experimentally demonstrated in any *P. syringae* strain. In addition, these annotations, based on the homology of *P. syringae* aa_permease genes to aa_permeases which have been experimentally characterised in other bacterial species, may be incorrect. For example, in P.fl.SBW25 an ortholog of PSPTO_5276, SBW25_0368 (*hutT*) has been experimentally demonstrated by Zhang and coworkers (2006) to transport histidine, not proline. Zhang and coworkers (2006) used a reporter gene fusion to monitor *hutT* induction by different amino acids, and an in-frame *hutT* deletion mutant, to demonstrate that *hutT* was strongly induced by histidine (but not significantly induced by proline) and that *hutT* activity was required for histidine utilisation in P.fl.SBW25.

The expression of each aa_permease was monitored in the presence of 1 mM concentrations of the potentially transported amino acids: GABA (for PSPTO_5356), histidine and proline (for PSPTO_5276), ethanolamine, (for PSPTO_2026), and the aromatic amino acids phenylalanine and tryptophan (for PSPTO_1817).

In addition, gene expression was analysed in extracted tomato apoplast (annotated as '*in vitro'* expression) and in apoplast extracted from tomato plants according to the method described in Section 6.5.2 above (annotated as '*in planta'* expression). For the latter, three fully-expanded tomato leaves were inoculated with a suspension of 2.5×10^9 cfu ml^{-1} bacteria in 10 mM $MgCl_2$. Apoplast was extracted after 30 minutes by centrifugation into a 50 ml centrifuge tube containing RNA Protect Bacterial Reagent as described above.

- **Expression of PSPTO_5356 in extracted apoplast, '*in planta*' and in the presence of GABA.**

The expression of PSPTO_5356 was monitored following incubation in extracted apoplast; in the presence of the predicted transported substrate GABA; and in bacteria which had been incubated for 30 minutes in the leaves of healthy tomato plants. The expression of PSPTO_5356, annotated as a GABA permease, was significantly increased in the presence of both 1 mM and 0.5 mM GABA compared to the expression of this gene in M9.

Expression of PSPTO_5356 was also significantly increased *in vitro* (when P.s.t was incubated in extracted apoplast) and *in planta* (when P.s.t was infiltrated into plants, incubated for 30 minutes, centrifuged out and RNA immediately extracted).

Uninfected tomato apoplast has been reported to contain 0.5 mM GABA (Rico and Preston, 2008). Concurrent with this result, it can be seen from Figure 6.6, that the expression of PSPTO_5356 was not significantly different in M9 supplemented with 0.5 mM GABA compared to the expression *in planta* or in extracted apoplast (*in vitro*). Interestingly, increasing the concentration of GABA to 1 mM did not significantly increase the expression of PSPTO_5356 compared to the expression in the presence 0.5 mM GABA, indicating a maximum expression for this gene might exist.

In order to check that the observed increase in expression of PSPTO_5356 in the presence of 1 mM or 0.5 mM GABA was not a general response to increased nitrogen in M9 media with GABA compared to M9 minimal media without GABA, the expression of PSPTO_5356 was investigated in the presence of 1 mM histidine (Figure 6.6 C). The expression of PSPTO_5356 was not significantly different in M9 plus histidine compared to M9 without any amino acid.

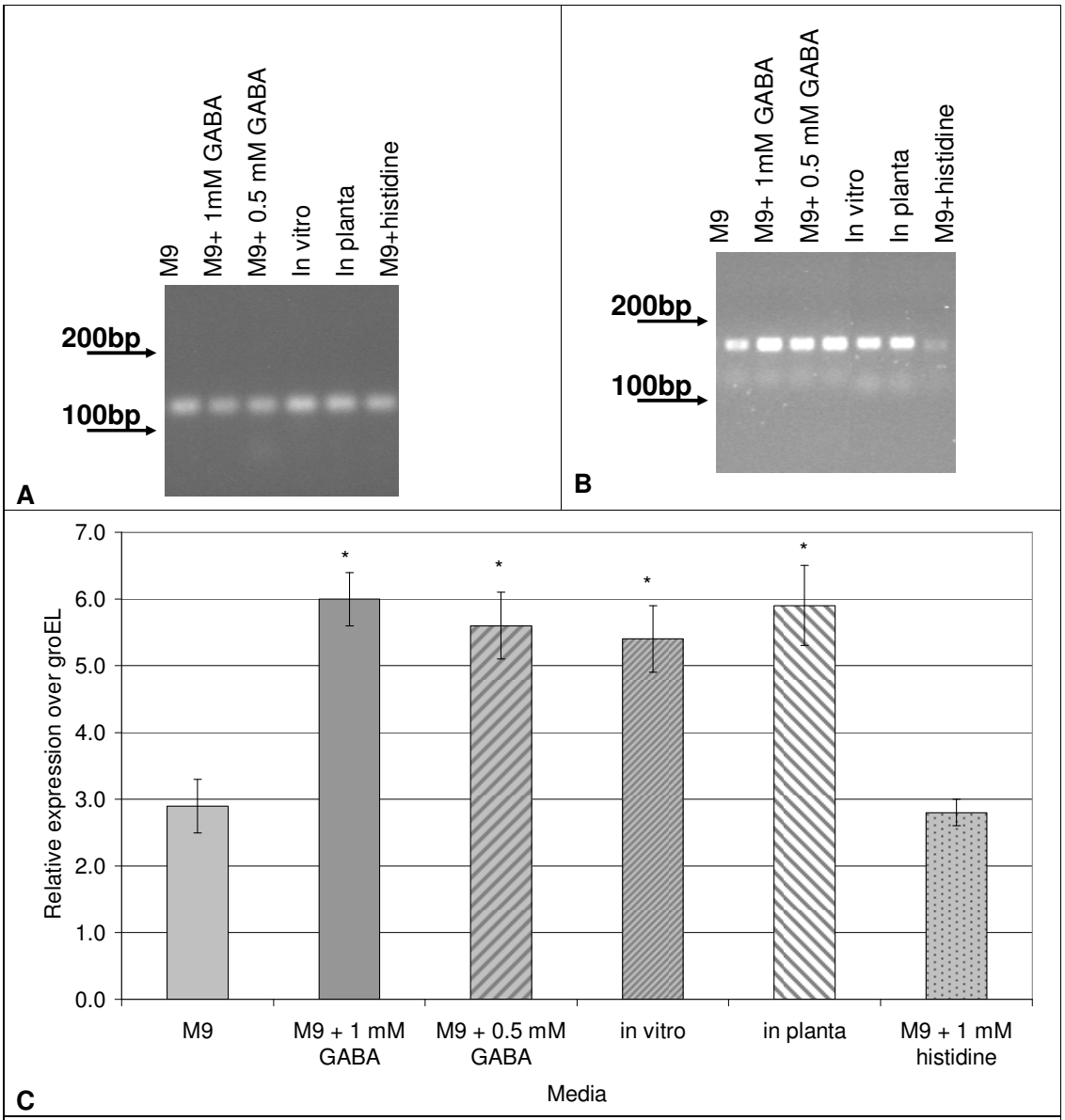


Figure 6.6
Expression of PSPTO_5356 (*gabP*) in a range of media.
A. Expression of GroEL in complete M9 media, M9+1 mM GABA, M9+ 0.5 mM GABA, in vitro (extracted apoplast), in planta and in and 1 mM histidine (control).
B. Expression of PSPTO_5356 complete M9 media, M9+1 mM GABA, M9+ 0.5 mM GABA, in vitro (extracted apoplast), in planta and in and 1 mM histidine (control).
C. Expression of PSPTO_5356 in M9 minimal media, M9 + 1 mM GABA, M9 + 0.5 mM GABA, extracted tomato apoplast (*in vitro*), in tomato plants (*in planta*) and in M9 + 1 mM histidine (control), relative to the expression of the housekeeping gene *groEL*. Error bars indicate the standard deviations of three replicates. Experiments were independently repeated twice. Significance was calculated using Student's t-tests *= expression was significantly different to the expression in M9 minimal media ($p \leq 0.05$). Expression of PSPTO_5356 was not significantly different in the presence of 1 mM or 0.5 mM GABA, in vitro or in planta. The expression of PSPTO_5356 was not significantly different in M9 compared to M9 plus 1mM histidine.

- **Expression of PSPTO_5276 in extracted apoplast, '*in planta*' and in the presence of histidine and proline.**

PSPTO_5276 has been provisionally annotated as transporting proline, based on homology to characterised proline transporters in other bacterial species. However an ortholog of this gene in P. fl. SBW25 was demonstrated to transport histidine (Zhang et al., 2006). Proline and histidine have both been detected in the tomato apoplast and therefore both may conceivably be transported during colonisation of the apoplast. In order to establish whether proline or histidine was likely to be transported by PSPTO_5276, the expression of this gene was monitored in the presence of 1 mM histidine or 1 mM proline. In addition, the expression was monitored in the presence of 1 mM ethanolamine. PSPTO_5276 is not predicted to transport ethanolamine and therefore this amino acid is included as a control. The expression of PSPTO_5276 was also monitored in extracted apoplast (*in vitro*) and following incubation in the leaves of healthy tomato plants (*in planta*).

The expression of PSPTO_5276 was significantly increased in the presence of 1 mM histidine but not 1 mM proline (Figure 6.7). This result was in agreement with the results of Zhang and coworkers (2006) who reported that expression of the orthologous gene in P.fl SBW25 was significantly increased in M9 supplemented with 15.5 µg ml^{-1} histidine, but not other tested amino acids (Zhang et al., 2006). The expression of PSPTO_5276 was also not significantly increased in the presence of 1 mM ethanolamine compared to the expression in M9. This supports the hypothesis that observed induction of this gene in the presence of histidine is due to the presence of that amino acid, and not due to the added nitrogen provided by the addition of the amino acid. Expression of PSPTO_5276 was significantly increased in cells which had been incubated in healthy tomato plants (*in planta*) and in cells which had been incubated in extracted tomato apoplast (*in vitro*), compared to the expression in M9. However, the expression of PSPTO_5276 both *in planta* and *in vitro* was lower than in the presence of 1 mM histidine. Uninfected tomato apoplast has been shown to contain an average of 6.83 µM histidine (Rico and Preston, 2008). Therefore this may suggest that the expression of PSPTO_5276 is concentration dependent and is increased following exposure to increased concentrations of histidine.

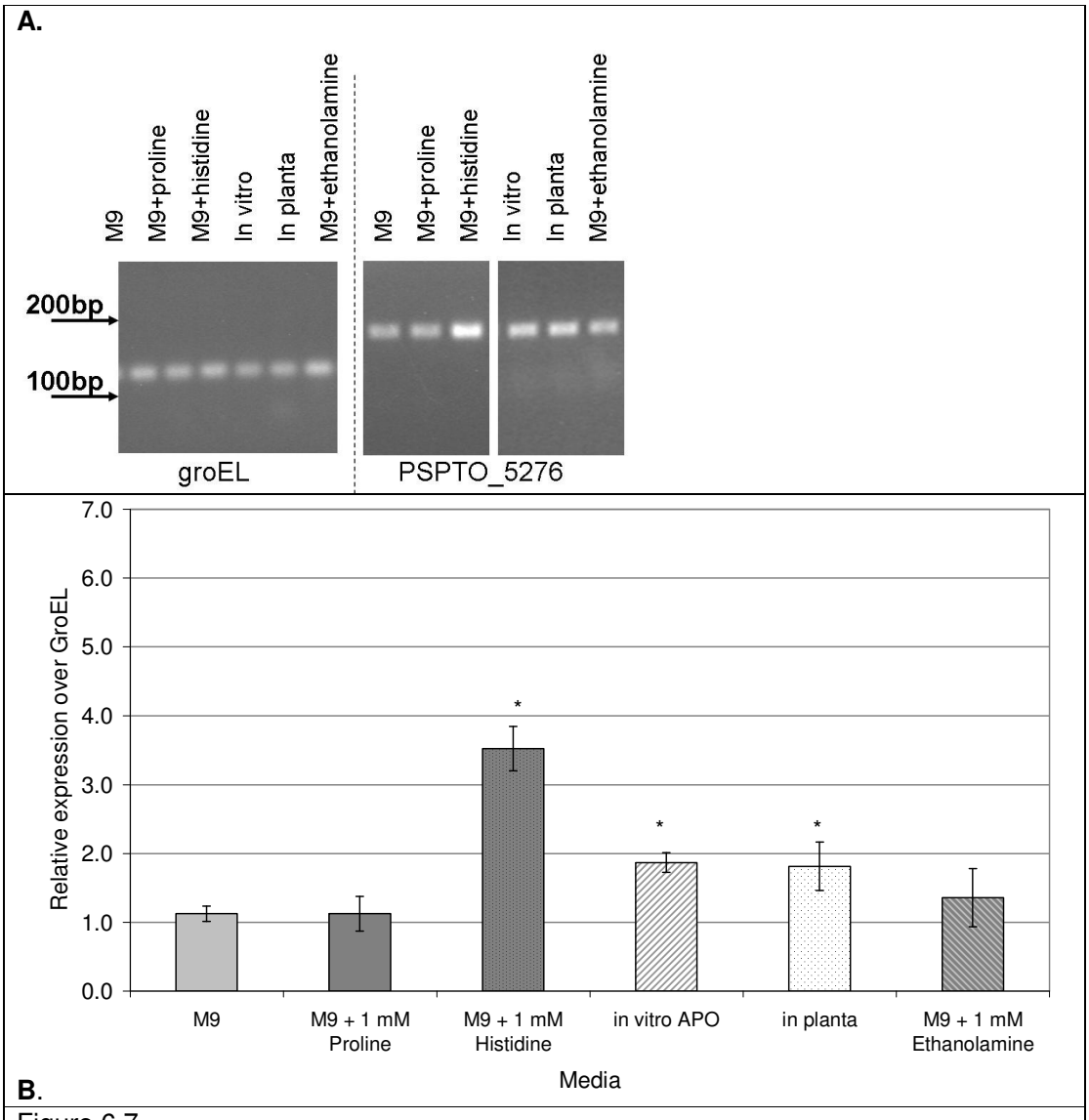
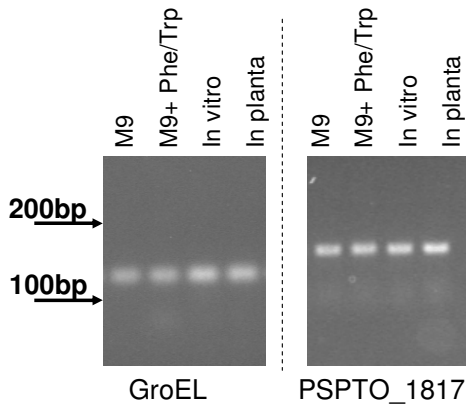


Figure 6.7
Expression of PSPTO_5276 in various media.
A. RT-PCR transcripts of PSPTO_5276 expression. B. Graph showing the expression of PSPTO_5276 (*proY*) in complete M9, M9 supplemented with 1 mM proline, M9 supplemented with 1 mM histidine, in extracted tomato apoplast (APO) and *in planta* (using the method described in 6.5.2). Expression in M9 supplemented with 1 mM ethanolamine is provided as a control. Error bars indicate the standard deviations of three replicates. Experiments were independently repeated twice. Significance was calculated using Student's t-tests *= expression is significantly higher than in M9 minimal media ($p \leq 0.05$). The expression of PSPTO_5276 was not significantly different in vitro compared to in planta.

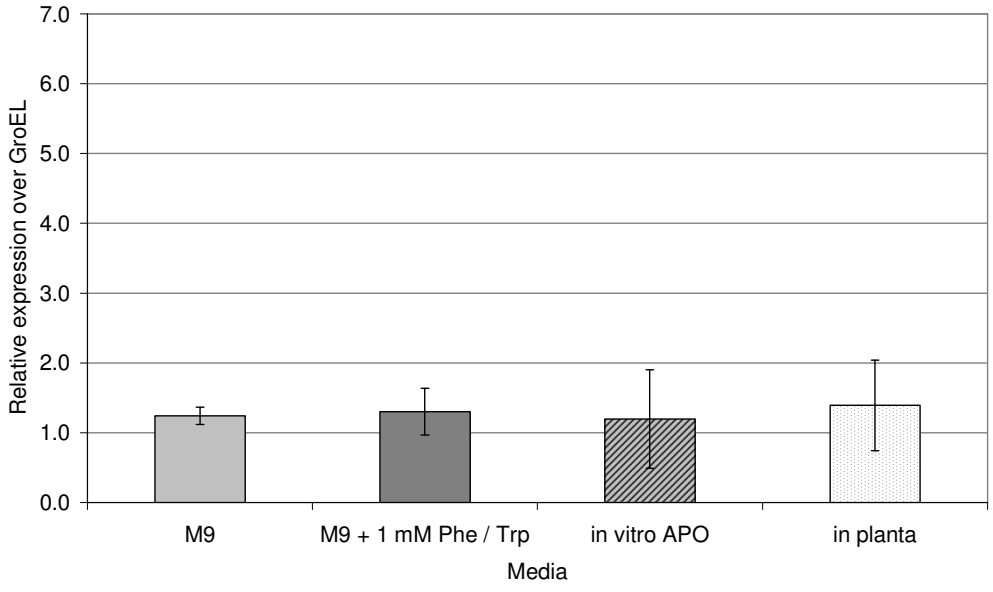
- **Expression of PSPTO_1817 in extracted apoplast, '*in planta*' and in the presence of aromatic amino acids.**

The expression of PSPTO_1817, which has been annotated as an aromatic amino acid permease, was monitored in the presence of a combination of the aromatic amino acids tryptophan and phenylalanine. The aromatic amino acid tyrosine was not tested due to its insolubility in M9 minimal media. The expression of PSPTO_1817 was also investigated in the presence of extracted apoplast (*in vitro*) or following incubation of bacteria in healthy tomato leaves (*in planta*). The expression of PSPTO_1817 was not significantly increased in M9 minimal media supplemented with 1 mM tryptophan and 1 mM phenyalanine; therefore PSPTO_1817 may not transport these aromatic compounds. However the aromatic amino acid tyrosine was not tested. Tyrosine and phenylalanine have been shown to be present in the tomato apoplast using HPLC analysis whereas tryptophan was not detected (Rico and Preston, 2008). However, more recent GC-MS analysis has demonstrated that tyrosine is present in the healthy tomato apoplast (Gail Preston, personal communication).

The expression of PSPTO_1817 was not significantly elevated either *in vitro* or *in planta* apoplast (Figure 6.8), suggesting that the concentration of aromatics in healthy plants was not enough to induce expression. However, Solomon and Oliver (2001) reported an increase in apoplastic tyrosine and phenylalanine concentrations following infection with the biotrophic fungus *Cladosporium fulvum*, which inhabits the tomato apoplast. In particular, concentrations of tyrosine increased from almost undetectable levels to ~0.2 mM concentrations. Provisional NMR analysis of infected tomato apoplast suggests that tyrosine concentrations are also significantly increased following infection with P.s.t (Gail Preston, personal communication). Therefore, several days post infection, P.s.t may be exposed to much higher concentrations of tyrosine than when infiltrated into healthy plants.



A.



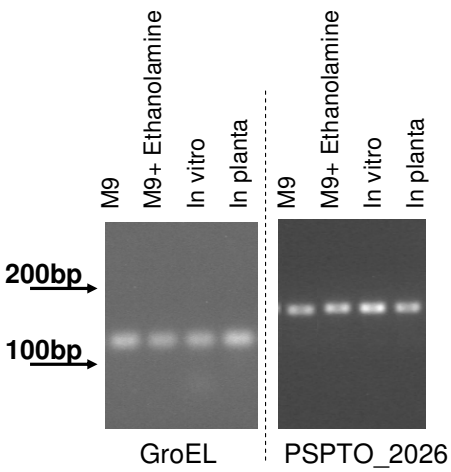
B.

Figure 6.8
Expression of PSPTO_1817 (annotated as transporting aromatic amino acids).
A. RT-PCR transcripts of PSPTO_1817. B. Graph of the expression of PSPTO_1817 in complete M9, M9 supplemented with 1 mM phenylalanine and 1 mM tryptophan (aromatic amino acids), in extracted tomato apoplast (*in vitro*) and *in planta* (using the method described in 6.5.2). Error bars indicate the standard deviations of three replicates. Experiments were independently repeated twice. Significance was calculated using Student's 2-tailed t-tests. The expression was not significantly different than in M9 minimal media in any condition tested ($p \leq 0.05$).

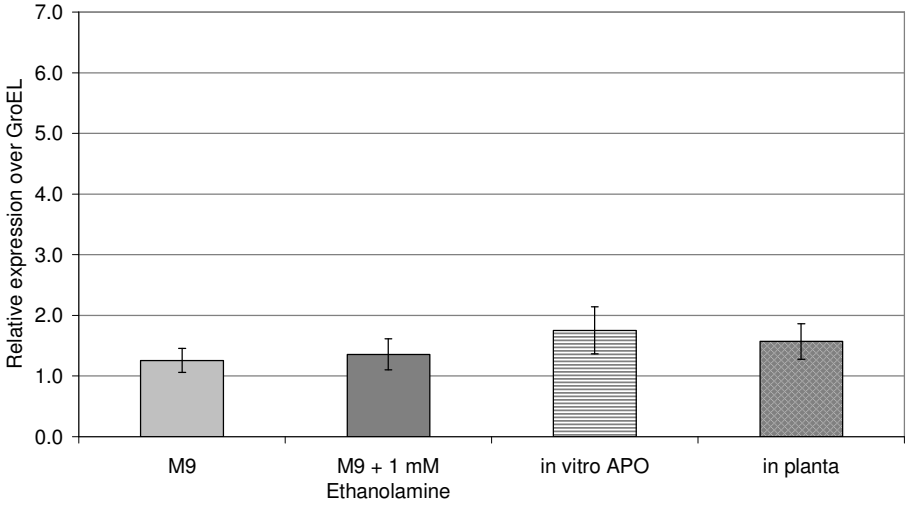
- **Expression of PSPTO_2026 in extracted apoplast, *in planta*, and in the presence of ethanolamine.**

The expression of PSPTO_2026, annotated as an ethanolamine transporter, was investigated in the presence of ethanolamine, in extracted apoplast (*in vitro*) and in cells incubated in healthy tomato leaves (*in planta*). The expression of PSPTO_2026 was not significantly increased in the presence of 1 mM ethanolamine compared to M9 without any amino acid (Figure 6.9). The concentration of ethanolamine in the plant apoplast has not been reported (see Table 1.4, Introduction), although recent GC-MS analysis indicates that ethanolamine is present in the healthy tomato apoplast (Gail Preston, personal communication). The expression of PSPTO_2026 was not significantly increased in bacteria that had been incubated *in planta* (Figure 6.9). Therefore we can hypothesise that the substrate transported by PSPTO_2026 may be induced in the apoplast during later colonisation by P.s.t. The substrate(s) transported by this permease remain unknown.

Interestingly, *P. aeruginosa* PAO1 contains two aa_permease genes annotated as transporting ethanolamine (see Chapter 3). The expression of one of these, PA0220, was increased in the presence of histamine rather than ethanolamine (Johnson et al., 2008). Therefore an interesting future experiment would be to investigate the expression of PSPTO_2026 in the presence of histamine, and also to analyse extracted apoplast for the presence of histamine.



A.



B.

Figure 6.9

A. RT-PCR transcripts of PSPTO_2026 expression. **B**. Graph of the expression of PSPTO_2026 in complete M9, M9 supplemented with 1 mM ethanolamine, in extracted tomato apoplast (*in vitro*) and *in planta* (using the method described in 6.5.2). Error bars indicate the standard deviations of three replicates. Experiments were independently repeated twice.

- **Comparison of gene expression in extracted apoplast (*in vitro*) and in tomato plants (*in planta*).**

Previously a novel method for monitoring gene expression in planta was developed. In order to investigate this method as an effective way of monitoring gene expression *in planta* in the early stages of infection, the expression of the P.s.t aa_pemease genes in extracted apoplast and following incubation in tomato leaves was compared. The data presented below are the same as those shown in Figures 6.6-6.9, but the expression of each permease gene has been shown in a single graph (Figure 6.10). The expression of each aa_permease gene was not significantly different in extracted apoplast compared to the expression following incubation in healthy tomato plants, suggesting that the centrifugation process for extracting the bacteria from the leaves did not significantly alter gene expression in P.s.t. There was also a slight increase in the expression of PSPTO_5356 (*gabP*) following incubation in tomato leaves. As the levels of various amino acids have been shown to alter during infection, it is tempting to hypothesise that this increase may be due to an alteration in the levels of GABA *in planta*. However, this would be contrary to the previous result that expression of PSPTO_5356 was not significantly different in 0.5 mM GABA compared to 1 mM GABA, and the bacteria were probably inoculated into the leaves for too short a time period to result in a significant change in apoplast amino acid composition.

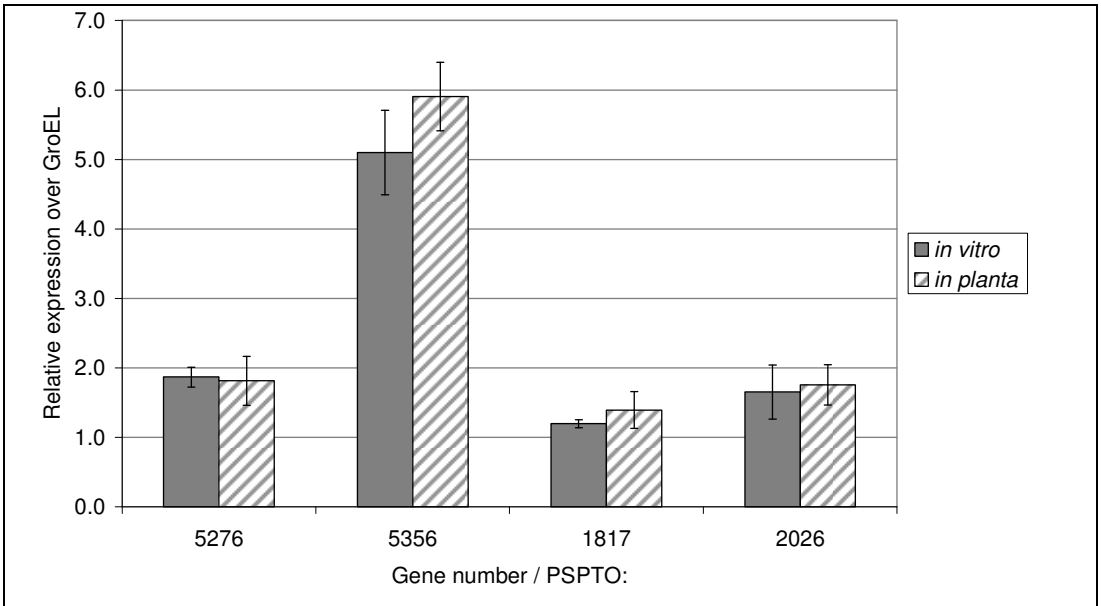


Figure 6.10
Expression of P.s.t aa_permease genes in extracted apoplast (*in vitro*) and following incubation in healthy tomato plants (*in planta*). The expression of each aa_permease gene was not significantly different *in vitro* to *in planta* expression. Therefore, the expression of these genes *in vitro* is a good reflection of their expression *in planta* after 30 minutes ($p \leq 0.05$).

6.5.4 Comparison of aa_permease gene expression in synthetic tomato apoplast and extracted tomato apoplast.

In the previous Section 6.5.3, the expression of the four P.s.t aa_permease genes in extracted apoplast (*in vitro*) and following incubation in healthy tomato plants (*in planta*) was reported (Figures 6.11). Generally it was observed that the expression of each aa_permease gene *in vitro* closely mirrored the expression *in planta*. This result suggests that extracted healthy apoplast can be used as a proxy to monitor gene expression *in planta*, at least for the early stages following bacterial inoculation. In Chapter 4 a synthetic tomato apoplast was developed with the intention of creating a cheap and quick alternative to extracting apoplast from leaves. In order to test the similarity of this media to extracted tomato apoplast, the expression of each permease gene in the synthetic tomato media (STA) and in extracted apoplast (TA) was compared (Figure 6.10)

For the genes PSPTO_2026 and PSPTO_5276 the expression in extracted tomato apoplast was significantly higher than in STA. This indicates that for these genes STA is not sufficient to mimic the inducing conditions *in planta*. This may include factors such as pH, which are known to alter both diurnally and during infection in plants, and are also thought to affect the uptake of amino acids by transporters such as the aa_permeases which function using chemiosmotic energy rather than ATP hydrolysis. The expression of PSPTO_5256 is not significantly different in tomato apoplast compared to STA. This gene has previously been shown to be induced in the presence of M9 + 0.5 mM GABA, which is the amount of GABA reported to be present in the healthy tomato apoplast and was therefore the amount added to STA. This result therefore further confirms the induction of PSPTO_5356 by GABA. The expression of PSPTO_1817 was not significantly increased in STA compared to tomato apoplast.

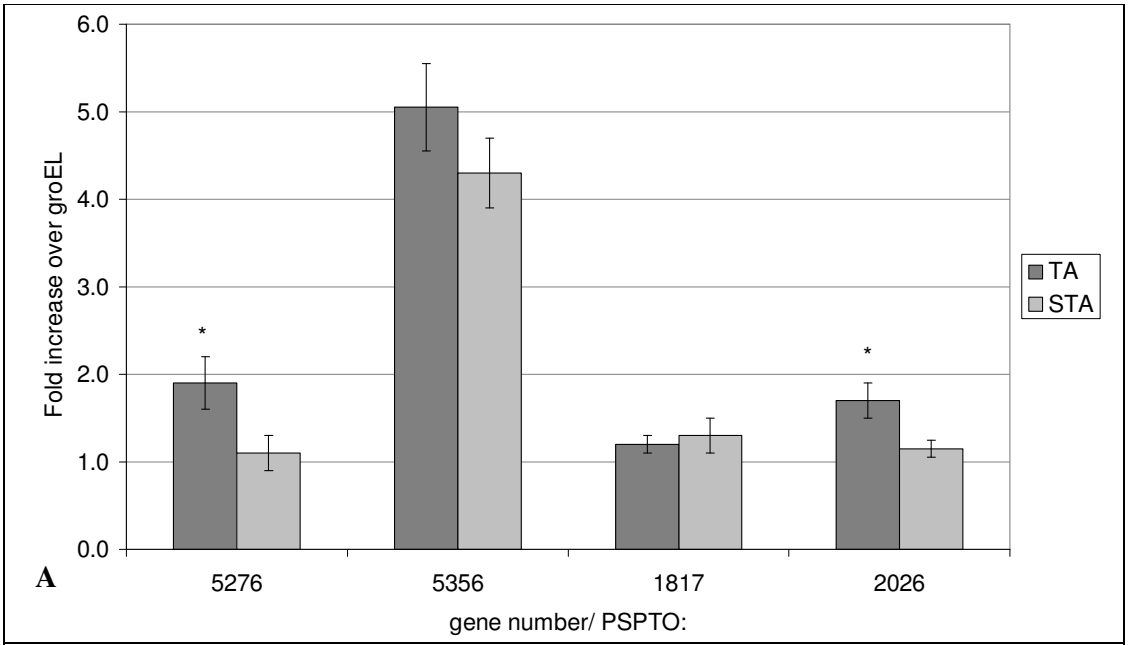


Figure 6.11
Comparison of P.s.t aa_permease gene expression in extracted healthy tomato apoplast (TA) and in synthetic tomato apoplast (STA)
A. Expression of the four P.s.t aa_permease genes and the housekeeping gene *groEL* in TA and STA as indicated.*= Expression is significantly different to the expression (of the same gene) in STA ($p \leq 0.05$).

6.5.5 P.s.t- rpoN can be complemented by expression of the P.fl SBW25 *rpoN* gene.

The global response regulator RpoN is known to be involved with the regulation of many *Pseudomonas* genes (Kohler et al., 1989; Totten et al., 1990; Hendrickson et al., 2001; Hendrickson et al., 2001) including some aa_permease genes (see Chapter 3 Table 3.9). In particular the P.s.t aa_permease PSPTO_2026 contains a predicted RpoN binding site. Although *rpoN* mutants have been reported for many *Pseudomonas* species, including *P. putida* (Kohler et al., 1989), *P. aeruginosa* (Totten et al., 1990; Hendrickson et al., 2001) and *P. syringae* pv. maculicola M4 (Hendrickson et al., 2000b), a characterized P.s.t *rpoN* mutant has yet to be published. However, a P.s.t-*rpoN* mutant has been generated and was obtained as a gift from Arun Chatterjee (University of Missouri, USA). This mutant was successfully complemented with pJJP6, which contains the P.fl SBW25 *rpoN* gene cloned into the plasmid pBroadgate (Jones et al., 2007). P.fl SBW25 *rpoN* shares 93 % amino acid sequence identity over the full length P.s.t. *rpoN* gene, according to BLASTP analysis.

Four different assays were used to confirm successful complementation of the P.s.t-*rpoN* mutant (P.s.t-*rpoN* –pJJP6): A motility assay, a growth assay, a virulence assay and a carbon source utilisation assay were selected, as used by Jones and coworkers (2007), to

confirm the complementation of a P.fl.SBW25 *rpoN* mutant with the P.fl.SBW25 *rpoN* gene (plasmid pJJP6) (Jones et al., 2007).

- **Motility assay**

All *Pseudomonas rpoN* mutants generated so far, including *P. fluorescens*, *P. aeruginosa, P. putida* and *P. maculicola* species, have been non-motile (Kohler et al., 1989; Totten et al., 1990; Hendrickson et al., 2000b; Hendrickson et al., 2001; Jones et al., 2007). Therefore a motility assay was used to investigate the motility of the P.s.t-*rpoN* mutant.

In agreement with results from other *Pseudomonas* species, the P.s.t-*rpoN* mutant was non motile (Figure 6.12 A). Subsequently the motility assay was used to confirm that P.s.t-*rpoN*–pJJP6 was motile (Figure 6.12 D), and the motility of this strain was similar to that of wild type P.s.t.

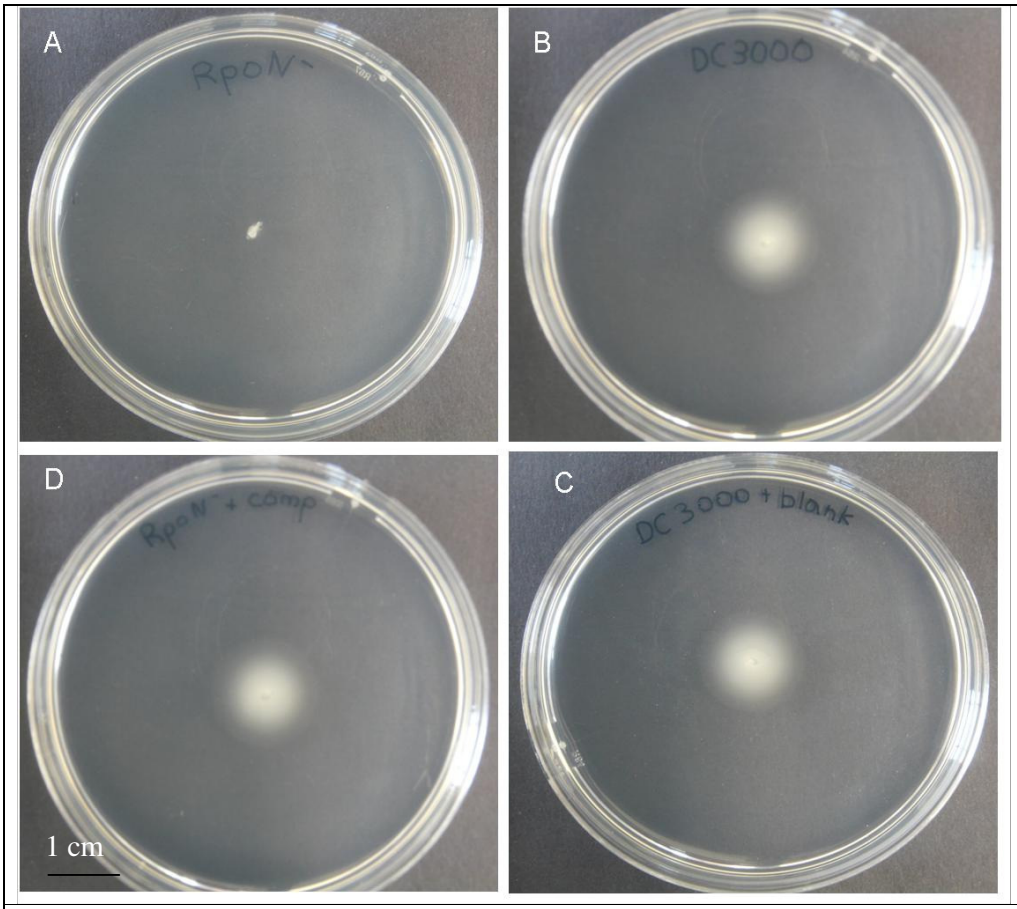


Figure 6.12
Motility assay. Swarm plate assay comparing motility in P.s.t-*rpoN* (A), wild type P.s.t (B), the wild type P.s.t containing the blank control plasmid (C) and P.s.t-*rpoN* pJJP6, complemented with Pfl-SBW25 *rpoN* (D). Motility was lost in P.s.t-*rpoN*, but could be restored by complementation with the *rpoN* gene from P.fl SBW25, expressed on the vector pBroadgate. The pBroadgate-D plasmid expressed in wild type P.s.t demonstrated that the presence of the pBroadgate plasmid alone did not enhance the motility of P.s.t.

- **Virulence assay**

The virulence of plant-pathogenic *P. maculicola* has been shown to be RpoN-dependent. A *P. maculicola-rpoN* mutant did not multiply or elicit disease symptoms when infiltrated into *A. thaliana*, and did not elicit a hypersensitive response (HR) when infiltrated into tobacco (Hendrickson et al., 2000b). A virulence assay was used to investigate the ability of P.s.t-rpoN to elicit disease symptoms in the host tomato plant, and to elicit the HR in tobacco (Figure 6.13). P.s.t-*rpoN* failed to cause disease symptoms in tomato or elicit the HR in tobacco. These abilities were restored when P.s.t-*rpoN* was complemented with pJJP6. Therefore in P.s.t, as in *P. maculicola*, RpoN is required for virulence *in planta*.

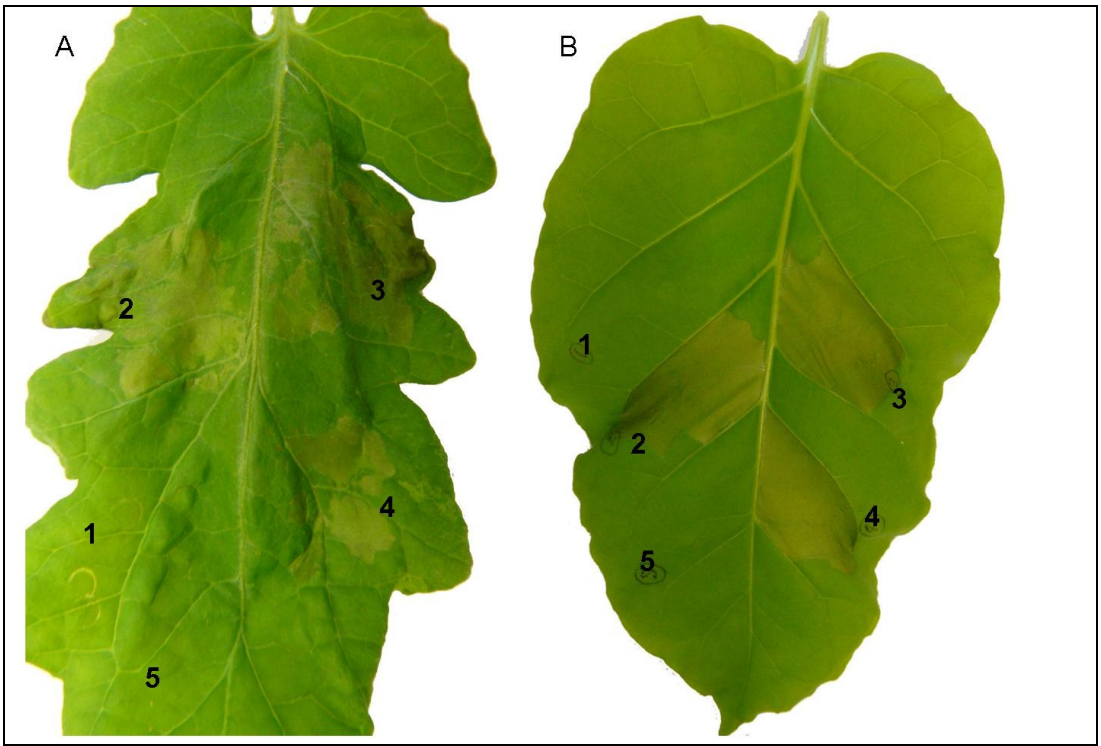


Figure 6.13
Virulence assay. Photograph showing symptoms in tomato and tobacco plants. P.s.t-*rpoN* (1) had lost the ability to cause disease symptoms in tomato (**A**) and the hypersensitive response in tobacco (**B**). However the complemented P.s.t-*rpoN* pJJP6 (2) caused similar symptoms to wild type P.s.t (3) and a P.s.t-control containing the blank pBroadgate plasmid (4) in both tomato and tobacco. 10 mM $MgCl_2$ was included as an additional control. In tomato plants bacteria were inoculated at $1x10^5$ cfu ml^{-1} and images were taken at 3 days. In tobacco plants bacteria were inoculated at $1x10^7$ cfu ml^{-1} and images were taken after 24 hours.

- **Growth assay**

RpoN mutants in other *Pseudomonas* species have been shown to exhibit severe growth defects compared to the wild type strain (Kohler et al., 1989; Totten et al., 1990; Hendrickson et al., 2000b; Hendrickson et al., 2001; Jones et al., 2007). A 24-hour growth assay was carried out to investigate the growth of the P.s.t-*rpoN* mutant, and to compare this growth to that of wild type P.s.t and the complemented P.s.t-*rpoN* mutant.

Results showed that the growth of P.s.t-rpoN was significantly less than wild type P.s.t in LB media. However, complementing this mutant with pJJP6 restored the growth of P.s.t-*rpoN* to levels comparable with wild type P.s.t (Figure 6.14).

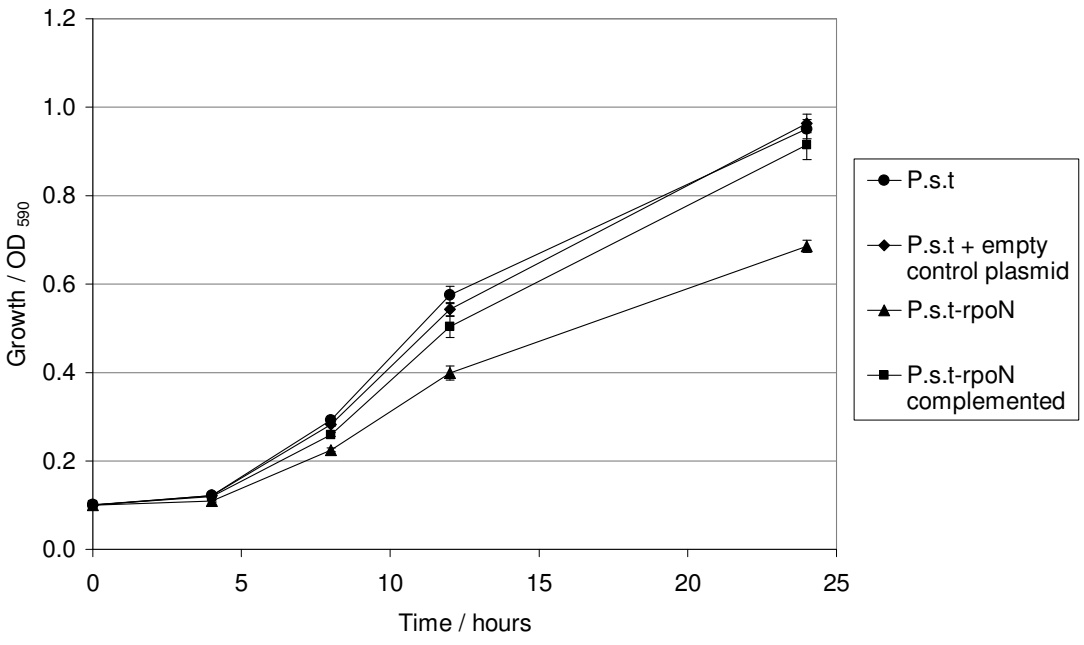


Figure 6.14
Growth assay. Time course of growth in LB media of wild type P.s.t, P.s.t-*rpoN*, P.s.t-*rpoN* pJJP6 complemented with the pBroadgate vector and wild type P.s.t transformed with the empty pBroadgate vector. Error bars are standard deviations from three replicates.

- **Carbon source utilisation assay**

As RpoN mutants in other *Pseudomonas* species have been shown to lose the ability to utilise certain carbon or nitrogen sources compared to the wild type strain (Kohler et al., 1989; Totten et al., 1990; Hendrickson et al., 2000b; Hendrickson et al., 2001; Jones et al., 2007), a carbon source utilisation assay was used to investigate the ability of P.s.t-*rpoN* to metabolise the compounds present in a Biolog GN2 plate as carbon sources.

P.s.t-*rpoN* had lost the ability to metabolise a large number of compounds as carbon sources compared to wild type P.s.t (Figure 6.15). In order to investigate the extent to which pJJP6 restored the ability of P.s.t-*rpoN* to metabolise these compounds, the ability of wild type P.s.t, P.s.t-*rpoN* and P.s.t-*rpoN* pJJP6 to metabolise the compounds present in the Biolog GN2 plate was compared. P.s.t-*rpoN* pJJP6 could metabolise significantly more compounds than P.s.t-rpoN. However the ability of P.s.t-*rpoN* pJJP6 to metabolise certain compounds, such as sucrose and GABA, was not fully restored to the extent seen in wild type P.s.t (Table 6.3). This may be due to the differences in sequence identity between the P.fl.SBW25 *rpoN* gene used for complementation and the *rpoN* gene in P.s.t, and/or due to the complexity of certain metabolic pathways meaning they are not operating correctly when regulated by *rpoN* expressed on a plasmid, rather than the native gene. Alternatively this could be due to multiple copies of the pBroadgate plasmid in complemented cells leading to differential expression of the *rpoN* gene compared to in the wild type cell, or as a result of the complemented *rpoN* gene being expressed under the pBroadgate rather than the native promoter (Jones et al., 2007).

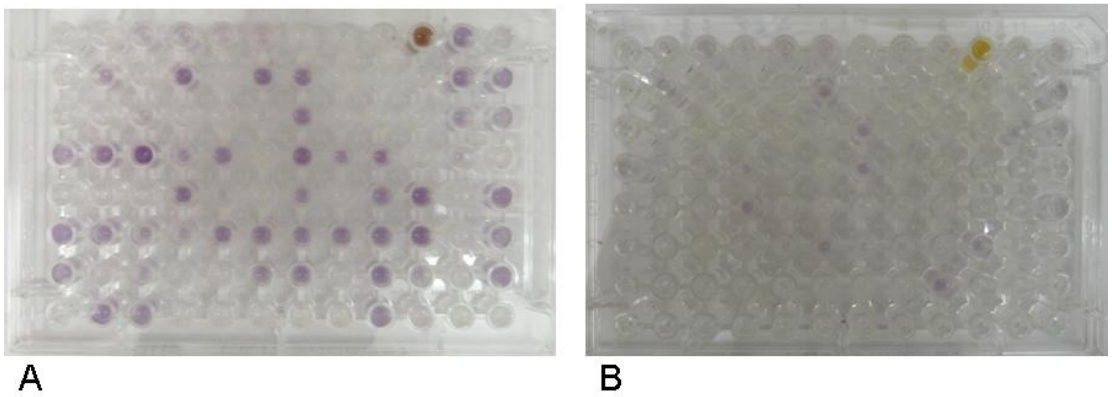


Figure 6.15
Carbon source utilisation assay part 1.
Example output from Biolog GN2 plates containing **(A)** wild type P.s.t and **(B)** P.s.t-*rpoN* after 24 hours incubation. Each well contains a different carbon source. Metabolism of the carbon source produces an purple substrate, which can be quantified by spectrophotometry. Wild type P.s.t can metabolise a wider range of carbon sources compared to P.s.t-*rpoN*

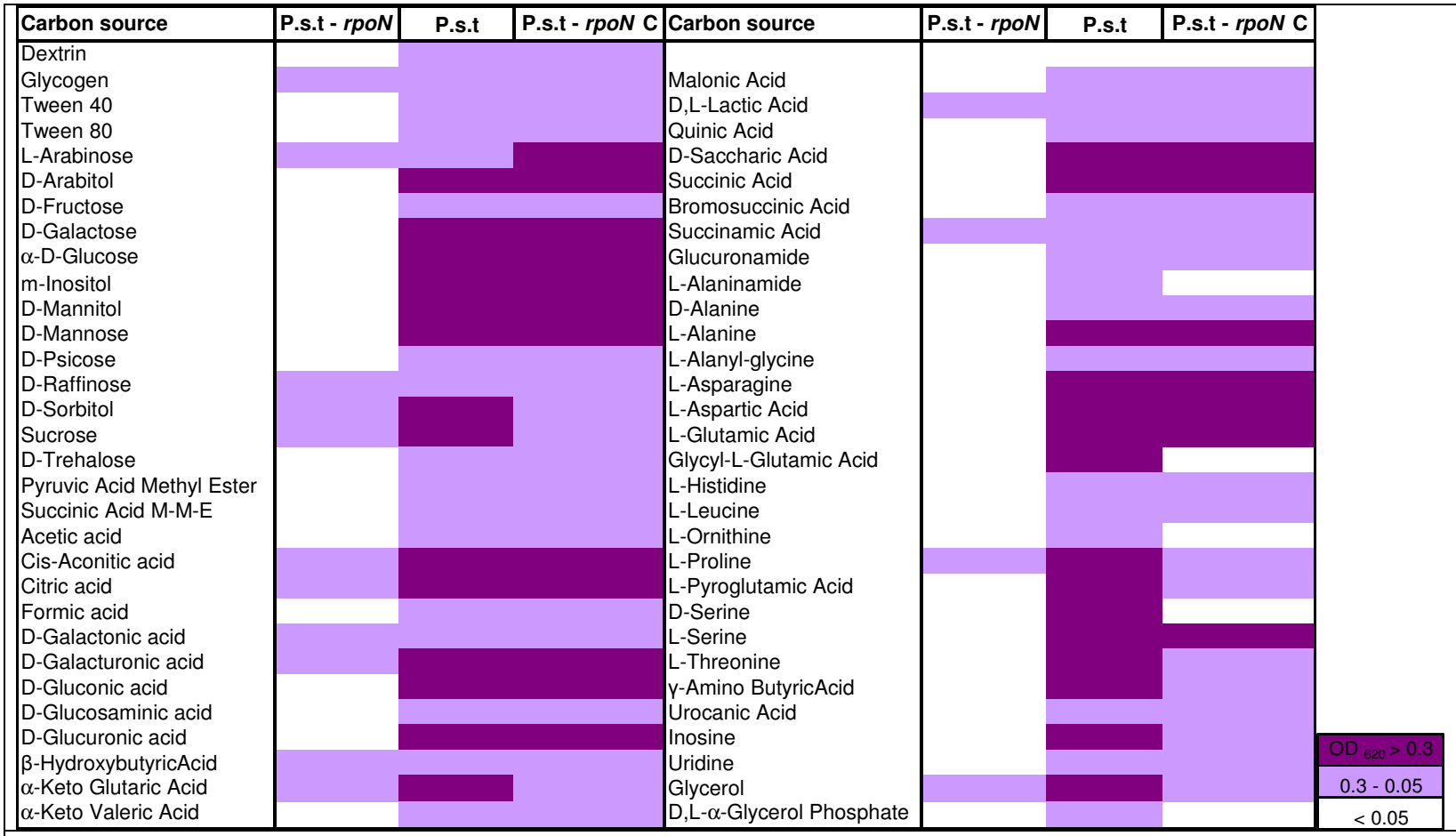


Table 6.3
Carbon source utilisation assay. GN2 Biolog plate carbon source utilisation for P.s.t-*rpoN*, wild type P.s.t and P.s.t-*rpoN* pJJP6, complemented with the Pfl SBW25 *rpoN* gene (P.s.t-*rpoN* C). Dark purple OD_{620} > 0.3 =strong utilisation of carbon source, light purple OD_{620} 0.3-0.05 = weak utilisation of carbon source, white OD_{620}<0.05 = no utilisation. Results are representative of two independent experiments with similar results.

6.5.6 Expression of aa_permease genes in the *Pseudomonas syringae* pv. tomato-*rpoN* mutant and the mutant complemented with pJJP6.

The P.s.t-*rpoN* mutant demonstrated a severely reduced ability to metabolise a range of compounds as carbon sources, including amino acids (Section 6.5.5, above). In particular P.s.t-*rpoN* could not metabolise GABA or histidine. According to bioinformatic prediction software (Chapter 3, Section 3.5.7) at least one aa_permease, (PSPTO_2026) is predicted to contain an RpoN-binding site, and therefore the expression of this gene may be regulated by RpoN. One way in which the loss of RpoN could affect the ability of P.s.t to metabolise amino acids as carbon sources, is if the transporters for these amino acids were not expressed in P.s.t-*rpoN*. Therefore, to investigate the role of RpoN in regulating aa_permease transporters in P.s.t, the expression of aa_permease genes was compared in wild type P.s.t and P.s.t-*rpoN*. In addition, the expression of the aa_permease genes was investigated in the complemented P.s.t-*rpoN* (Figure 6.16). Gene expression was compared between bacteria in exponential-phase growth in STA media, as the expression of several aa_permease genes in STA media was shown to be increased compared to other synthetic media such as LB.

The expression of PSPTO_2026 was significantly reduced in P.s.t-*rpoN*. This result is in agreement with the bioinformatic prediction that PSPTO_2026 contained an RpoN-binding site, and that this gene may therefore be regulated by RpoN. Interestingly, the expression of PSPTO_5356 was also significantly reduced in P.s.t-RpoN indicating that this gene may also be RpoN-dependent. This result was supported by the findings that the P.s.t-*rpoN* mutant had lost the ability to utilise GABA as a carbon source (Table 6.3), and a P. fl.SBW25-*rpoN* had lost the ability to utilise GABA as a carbon or nitrogen source (Jones et al., 2007). Furthermore, a putative RpoN-binding site was subsequently identified upstream of PSPTO_5356 (TGGCGTAATTCTTGCG), which contains the minimum GG-10 base pairs-GC format of the RpoN-binding site (Gail Preston, personal communication). The expression of PSPTO_1817 and PSPTO_5276 was not significantly different in any bacterial strain.

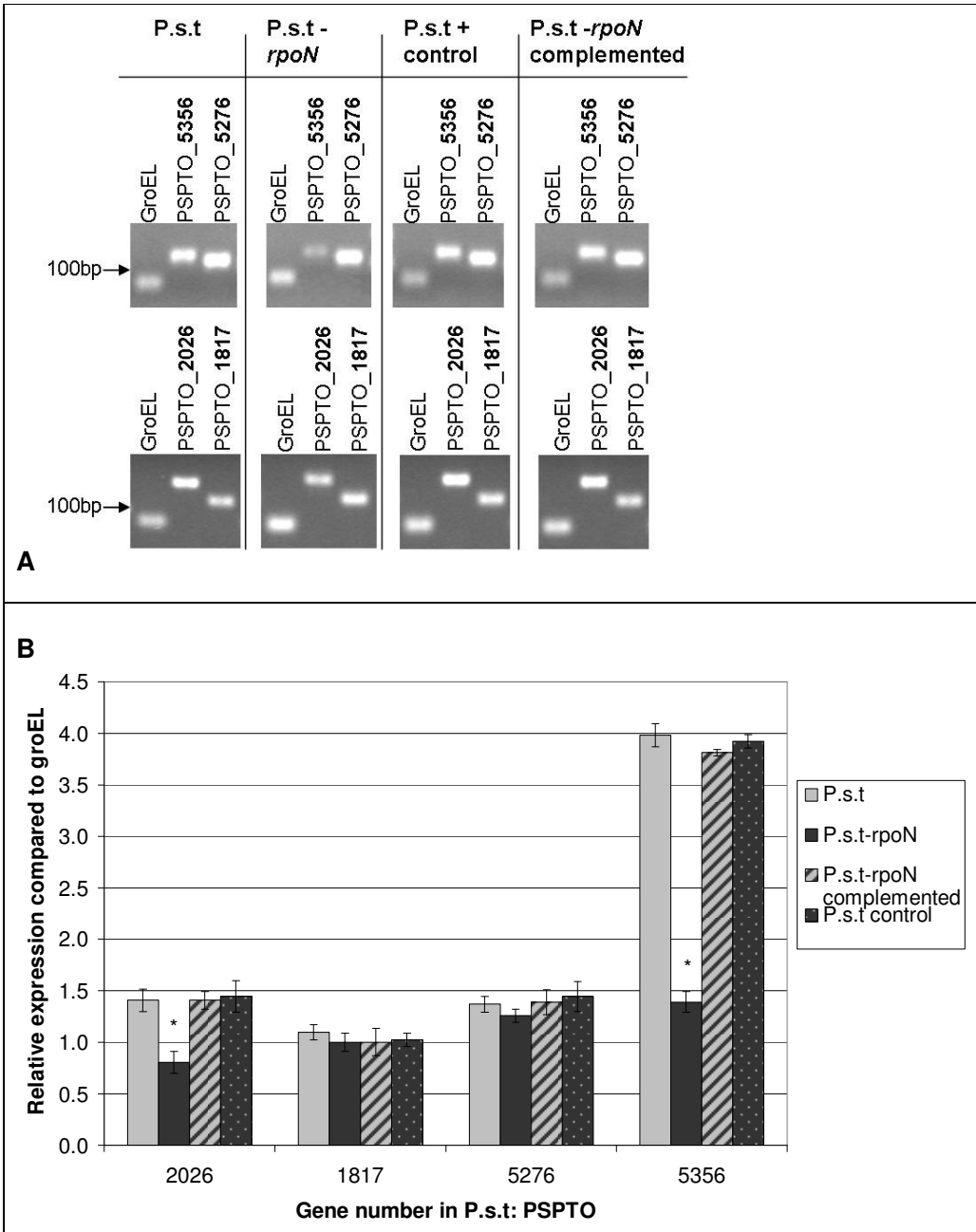


Figure 6.16
Expression of aa_permease genes in wild type P.s.t; P.s.t-*rpoN*; P.s.t-*rpoN* complemented with pJJP6 and P.s.t containing the empty pBroadgate plasmid.
A. RNA was extracted from bacteria in exponential phase growth in synthetic tomato apoplast (STA) media.
B. Gene expression presented as the relative increase relative to the housekeeping gene *groEL*. Error bars represent standard deviations of three replicate experiments.
*= expression was significantly different to wild type P.s.t ($p \leq 0.05$).

6.5.7 Expression of P.s.t aa_permease genes in high and low nitrogen media.

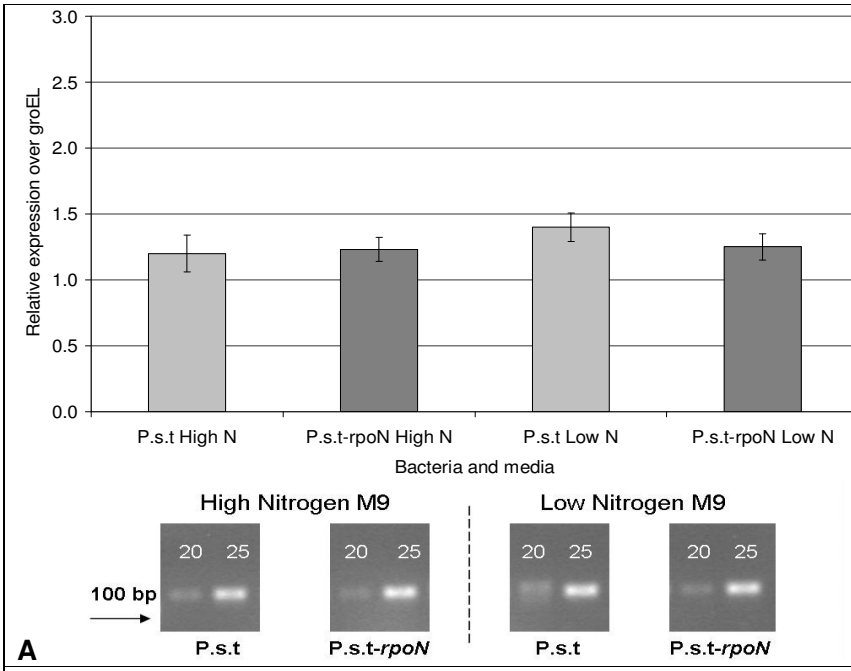
Previously, it was shown that the expression of PSPTO_2026 and PSPTO_5356 was significantly reduced in a P.s.t-*rpoN* mutant compared to wild type P.s.t. PSPTO_2026 had previously been predicted to contain an RpoN binding site, indicating that this gene was one of the many genes regulated by RpoN. In addition, the ability of P.s.t-*rpoN*, to metabolise amino acids as carbon sources was found to be severely limited compared to wild type P.s.t. RpoN-regulation of amino acid transporters, such as the aa_permeases, may therefore represent one hypothesis to explain the reduced ability of P.s.t-*rpoN* to metabolise amino acids as carbon sources.

RpoN was originally described as a factor involved in the expression of nitrogen-regulated genes, (although since then multiple and diverse physiological functions have been found to depend on this factor). Therefore, to further investigate the role of RpoN in regulating the expression of aa_permease genes in P.s.t, the expression of these genes in P.s.t-*rpoN* and wild type P.s.t was compared in high and low nitrogen M9 minimal media. For high nitrogen M9 media the nitrogen concentration was increased to 0.2 % NH_4Cl, while for low nitrogen M9 media, the nitrogen concentration was reduced to 0.025 % NH_4Cl.

As observed previously, the expression of PSPTO_1817 was not significantly different in wild type P.s.t compared to P.s.t-*rpoN*, and there was no significant increase in the expression of PSPTO_1817 in wild type P.s.t compared to P.s.t-*rpoN* when bacteria were grown in low nitrogen media (Figure 6.17 A). The expression of PSPTO_5276 was significantly increased in wild type P.s.t compared to P.s.t-*rpoN* when bacteria were grown in low nitrogen media ($p=0.009$), suggesting that expression of PSPTO_5276 may be regulated by RpoN, and this regulation has been lost in P.s.t-*rpoN* (Figure 6.17 B). Furthermore, the expression of PSPTO_5276 was significantly increased in wild type P.s.t grown in low nitrogen conditions compared to wild type P.s.t grown in high nitrogen conditions, suggesting that in wild type bacteria expression of this gene is increased in conditions of nitrogen limitation.

PSPTO_5356 was not predicted by the bioinformatics program PromScan to contain a putative RpoN-binding site. However, the expression of this gene was significantly increased in wild type P.s.t compared to P.s.t-*rpoN* in low nitrogen conditions, suggesting that this gene is increased during nitrogen limitation, and that the ability to respond to nitrogen limitation has been lost in the P.s.t-*rpoN* mutant. Similarly, the expression of PSPTO_2026, which was predicted by the bioinformatic program PromScan to contain an RpoN binding site, was also significantly increased in wild type P.s.t compared to P.s.t-*rpoN* when bacteria were grown in low nitrogen media ($p=0.000$), again suggesting that expression of this gene

RpoN-regulated (Figure 6.19). The expression of PSPTO_2026 was significantly increased in wild type P.s.t grown in low nitrogen conditions compared to wild type P.s.t grown in high nitrogen conditions, suggesting that in wild type bacteria expression of this gene is increased under nitrogen limitation. However, interestingly the expression of this gene was also significantly higher in wild type P.s.t in high nitrogen conditions compared to P.s.t-*rpoN*. This suggests that there may be some increase in gene expression in high nitrogen conditions, although far less of an increase than is observed in low nitrogen conditions, and this may also be connected to RpoN as this increase is not observed in the P.s.t-r*poN* mutant.



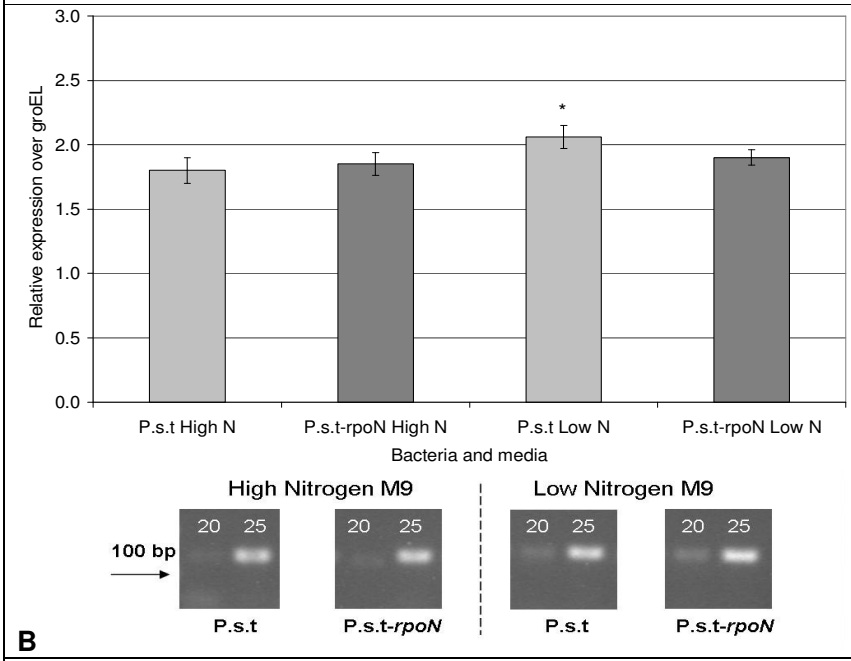
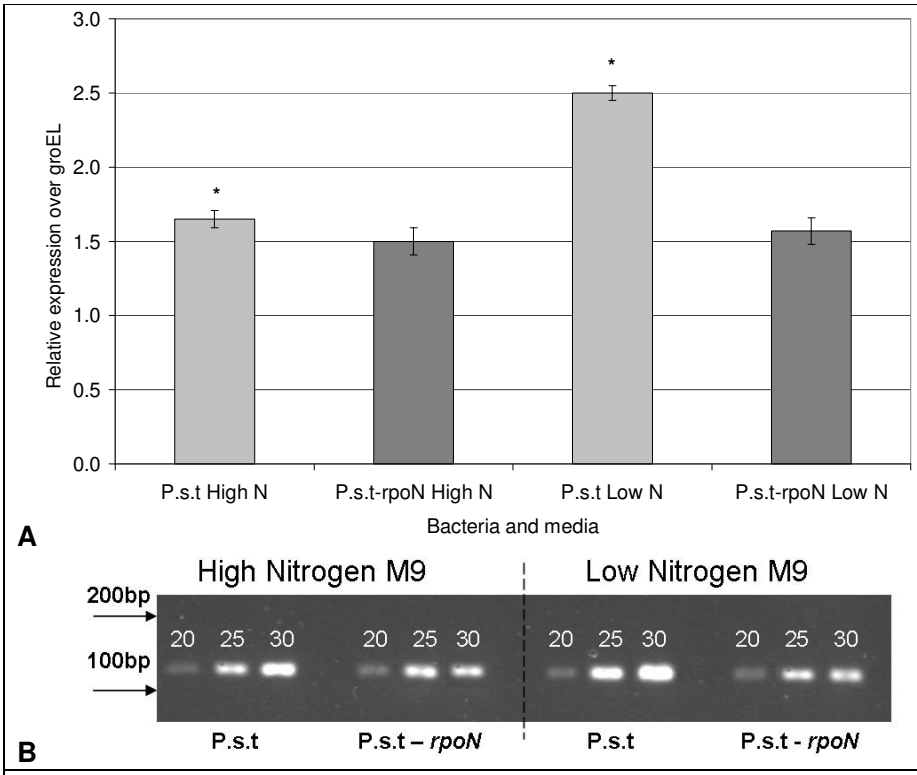


Figure 6.17
Expression of PSPTO_1817 (**A**) and PSPTO_5276 (**B**) in wild type P.s.t P.s.t-*rpoN*, in high and low nitrogen M9 media. **A**. Expression of PSPTO_1817 was not significantly different in low nitrogen M9 media compared to high nitrogen M9 media or in wild type P.s.t compared to P.s.t-*rpoN* in the same media ($p \leq 0.05$). **B**. Expression of PSPTO_5276 was significantly different in wild type P.s.t compared to P.s.t-*rpoN* when bacteria were cultured in low nitrogen M9 media ($p=0.009$). The expression of PSPTO_5276 was also significantly increased in wild type P.s.t grown in low nitrogen media compared to high nitrogen media ($p=0.001$). Results were based on densitometry analysis using Quantity One software of RT-PCR products with 25 PCR cycles. Error bars represent the average of three samples. Experiments were repeated twice with similar results



A

B

Figure 6.18

Expression of the PSPTO_5356 aa_permease in wild type P.s.t and the P.s.t-*rpoN* mutant, in high and low nitrogen M9 media. **A.** The expression of PSPTO_5356 in wild type P.s.t was significantly increased in low nitrogen M9 media compared to the P.s.t-*rpoN* mutant (p=0.000). Expression was also significantly increased in wild type P.s.t in high nitrogen media (p=0.041). *=Expression was significantly different compared to P.s.t-*rpoN* in the same media, in Student's t-tests (p≤0.05). Error bars represent the average of three samples. Experiments were repeated twice with similar results. The expression of PSPTO_5356 in wild type P.s.t was also significantly higher in bacteria grown in low nitrogen media than in high nitrogen media. Results represent the average of three independent experiments. **B**. Analysis was based on 25 PCR cycles to prevent pixel saturation of the Quantity One analysis program.

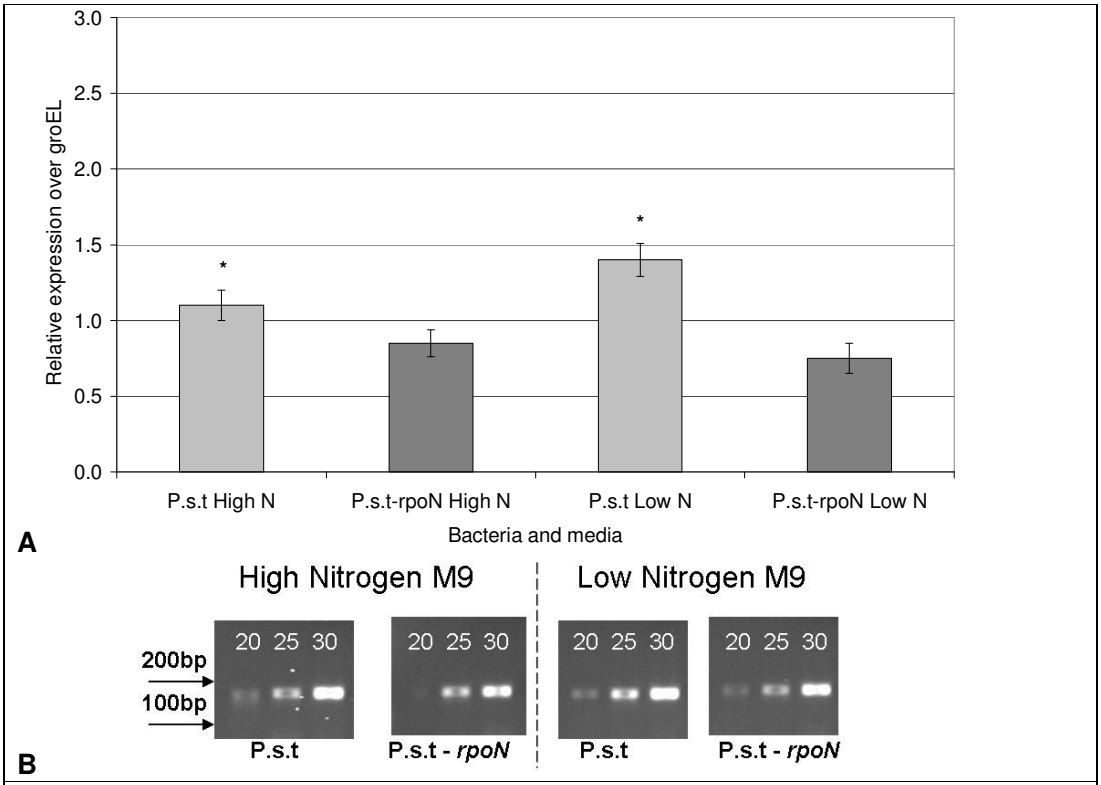


Figure 6.19
Expression of the PSPTO_2026 aa_permease in wild type P.s.t and the P.s.t-*rpoN* mutant, in high and low nitrogen M9 media. **A**. * = expression was significantly different compared to P.s.t-*rpoN* in the same media in Student's t-tests ($p \leq 0.05$). Expression of PSPTO_2026 in wild type P.s.t was significantly increased in low nitrogen M9 media compared to the P.s.t-*rpoN* mutant in low nitrogen media ($p=0.000$). Expression was also significantly higherr in wild type P.s.t than the P.s.t-*rpoN* mutant in high nitrogen ($p=0.021$). The expression of PSPTO_2026 was also significantly higher in wild type P.s.t cultured in low nitrogen than in high nitrogen media ($p=0.000$) **B**. Results were calculated from densitometry analysis of RT-PCR products with 25 PCR cycles to avoid pixel saturation of the Quantity One software. Error bars represent the average of three samples. Experiments were repeated twice with similar results

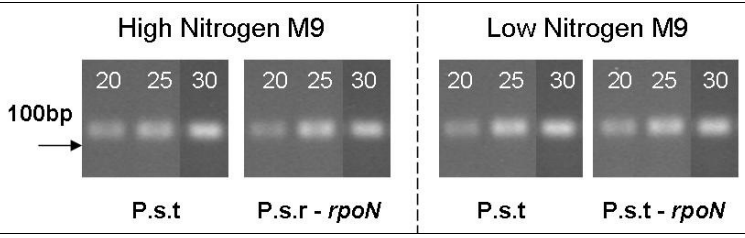


Figure 6.20
groEL expression in high and low nitrogen conditions.
Expression of this gene was not affected by culturing P.s.t in high or low nitrogen media. Numbers refer to the PCR cycles used to demonstrate that the observed constant expression was not the result of saturation. As such, the expression of *groEL* can be seen to be constant following 20, 25 and 30 PCR cycles.

6.5.8 Analysis of genes in the histidine (*hut*) operon in P.s.t.

The histidine (*hut*) operon has been characterised in several *Pseudomonas* species including *P. fluorescens* and *P. putida* (Hu and Phillips, 1988; Zhang and Rainey, 2007) and contains genes essential for histidine utilisation. However, in Chapter 3, (Section 3.5.10) it was demonstrated that the orthologous genes of this operon vary in number and synteny in different *Pseudomonas* species. It is possible that such rearrangements could have resulted in a loss of operon function, particularly for the 'classic' operon organisation with a single promoter controlling the expression of multiple downstream genes. In order to confirm that the histidine utilisation genes were transcribed together in an operon in *P. syringae*, primers were designed which amplified a region corresponding to the end of a gene in the operon, and the start of the adjacent gene (Figure 6.21). These primers were used to amplify cDNA obtained from RNA extracted from P.s.t cultured in M9 supplemented with 1 mM histidine, conditions where one putative gene of the hut operon PSPTO_5276 was previously shown to be expressed. The amplification of a fragment indicated that RNA spanning the adjacent genes was produced, and therefore indicated that these genes were expressed together in an operon in P.s.t (Figure 6.21).

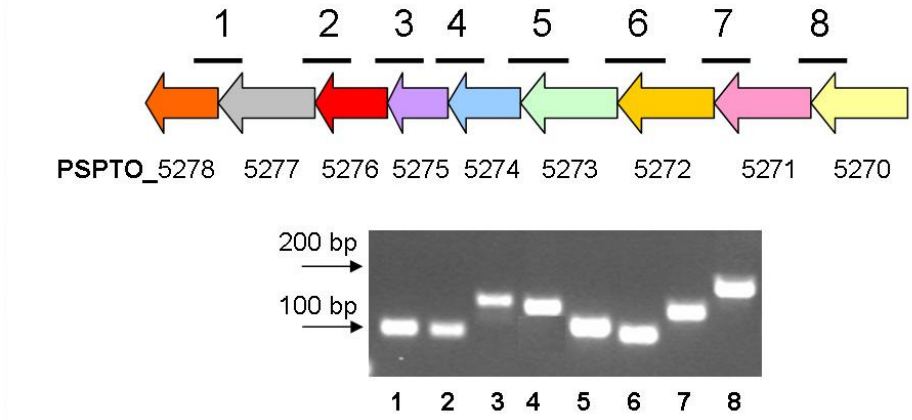


Figure 6.21
RT-PCR transcripts of the *hut* operon genes in P.s.t
The expression of genes PSPTO_5270 to PSPTO_5278 as an operon was investigated using primers designed to amplify overlapping regions between adjacent genes. The primers were used to amplify cDNA from RNA extracted from P.s.t grown in M9 + 1 mM histidine. The amplification of a fragment indicated that RNA spanning the region between genes was produced in P.s.t cultured in 1 mM histidine, and therefore can be used to predict that these genes were expressed together.
Primer key: 8=PSPTO_5270 L / R, 7=PSPTO_5271 L / R 6=PSPTO_5272 L / R
5=PSPTO_5273 L / R, 4=PSPTO_5274 L / R, 3=PSPTO_5275 L / R
2=PSPTO_5276 L / R, 1=PSPTO_5277 L / R

6.6 Discussion.

Bacteria continually monitor their extracellular environment and intracellular physiological status and can modulate their gene expression in response to changes in their environment (Camilli and Bassler, 2006). Reverse transcriptase PCR (RT-PCR) is commonly used to

monitor bacterial gene expression, and can be used to indicate conditions under which genes are expressed or upregulated. In this chapter the expression of the aa_permease genes was investigated in P.s.t in a range of media and in the presence of certain amino acids thought to be substrates transported by the permeases (Table 6.4). Also in this chapter, the role of the global response regulator RpoN in regulating the expression of the aa_permease genes was investigated using a P.s.t-*rpoN* mutant. This mutant was successfully complemented using the *rpoN* gene from P.fl.SBW25 and expression of each permease gene was compared in wild type P.s.t and P.s.t-*rpoN*.

	Expression conditions tested														
Gene PSPTO:	STA	STA pH6	STA pH7	LB	LB pH6	LB pH7	M9	*in vitro'*	*in planta'*	M9 + 1mM GABA	0.5 mM GABA	M9 + 1mM his	M9 + 1mM pro	M9 + 1mM eth	M9 +1mM phe/trp
5356	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓			
2026	✓	✓	✓	✓	✓	✓	✓	✓	✓					✓	
1817	✓	✓	✓	✓	✓	✓	✓	✓	✓						✓
5276	✓	✓	✓	✓	✓	✓	✓	✓	✓			✓	✓	✓	

Table 6.4
Summary of expression conditions tested in this chapter. STA= synthetic tomato apoplast, *in vitro*= extracted apoplast, *in planta*= method developed in Section 6.5.2, his=histidine, pro=proline, eth=ethanolamine, phe/trp= phenylalanine/tryptophan.

The expression of each permease in the conditions tested is summarised below before being discussed in more detail.

- **PSPTO_5356**

Annotated as a putative GABA permease; the expression of this gene was increased in the presence of both 0.5 mM and 1 mM GABA. Expression was also increased in extracted apoplast, and when bacteria were inoculated into tomato plants. Gene expression was increased during growth at pH 6 compared to pH 7. Gene expression was significantly reduced in P.s.t-*rpoN* compared to wild type P.s.t and gene expression was significantly increased during growth in low nitrogen, compared to high nitrogen minimal medium.

- **PSPTO_2026**

Annotated as a putative ethanolamine permease; however, the expression of this gene was not significantly increased during growth in the presence of 1 mM ethanolamine. Gene expression was significantly decreased in P.s.t-*rpoN* compared to P.s.t, and expression was significantly increased during growth in low nitrogen, compared to high nitrogen minimal medium

- **PSPTO_1817**

Annotated as a putative aromatic amino acid permease. Gene expression was not significantly increased in the presence of 1 mM phenylalanine or 1 mM tryptophan; however

expression in the presence of tyrosine was not tested. Gene expression was not significantly different in P.s.t-*rpoN* compared to P.s.t or in low nitrogen compared to high nitrogen minimal medium.

- **PSPTO_5276**

Annotated as a putative proline permease; however the expression of this gene was not significantly increased in the presence of 1 mM proline. Gene expression was significantly increased in the presence of 1 mM histidine. Expression was also increased in extracted healthy tomato apoplast. Expression was increased during growth at pH 6 compared to pH 7. Expression was decreased in P.s.t-*rpoN* compared to P.s.t, and expression was significantly increased during growth in low nitrogen, compared to high nitrogen minimal medium.

Gene expression in STA and LB, at pH 6 and pH 7.

Initially the expression of the aa_permease genes PSPTO_5276, PSPTO_5356, PSPTO_1817 and PSPTO_2026, annotated as transporting proline, GABA, aromatic amino acids and ethanolamine, respectively, was investigated in LB and synthetic tomato apoplast (STA) media. Furthermore, as work in Chapter 4 had indicated that pH might influence the ability of P.s.t to assimilate amino acids, possibly through affecting the efficacy of amino acid transporters, the expression of each permease gene was compared at pH 6 and pH 7. Results indicated that in general the expression of PSPTO_5276 and PSPTO_5356 was higher in STA than in LB. In particular, the expression of these genes was significantly higher when cultured in slightly acidic STA (pH 6) compared to STA at pH 7. Furthermore, the expression of PSPTO_5356 was significantly higher in STA at all tested pH values compared to the expression at the respective pH values in LB. Taking these results together it appears that the expression of both PSPTO_5356 and PSPTO_5276 is influenced by pH, with expression being increased in slightly acidic conditions (pH 6). Furthermore, the expression of PSPTO_5356 is also influenced by the composition of the culture media, as expression was significantly higher in STA compared to LB, at all pH values. This suggested that the expression of PSPTO_5356 was induced by a component of STA media. In particular, STA contains GABA, the predicted substrate transported by this permease, and therefore it was tempting to speculate that GABA in STA was inducing the expression of this gene. Furthermore the finding that PSPTO_5356 expression was increased in STA at pH 6 supported the finding reported in Chapter 4, that metabolism of GABA by P.s.t was significantly increased following pre-incubation of cells in STA at pH 6 compared to STA at pH 7 (Chapter 4, Section 4.5.15). Taking the results of PSPTO_5356 expression analysis with those of Chapter 4, Section 4.5.15 together, we can hypothesise that pre-incubation of P.s.t in STA at pH 6 may result in increased metabolism of GABA because of the increased

expression of at least one GABA transporter (PSPTO_5356). The expression of PSPTO_2026 and PSPTO_1817 was not significantly different in STA compared to LB, and expression was not significantly different at any pH tested. This result suggested that the increase in expression at pH 6, observed for PSPTO_5356 and PSPTO_5276, does not apply to the permease genes in general.

Development of an *in planta* gene expression protocol.

It was decided to develop a method to monitor the expression of the aa_permease genes *in planta*. This method was based on infiltrating bacteria into the plant apoplast, and then centrifuging cells out of leaves along with the apoplast fluid into an RNA-stabilising buffer. The benefit of this method was its simplicity compared to other published methods for analysing bacterial gene expression *in planta* (Keith et al., 2003; Weingart et al., 2004; Schenk et al., 2008). Bacteria were inoculated at a very high density compared to standard infection assays ($2.5x10^9$ cfu ml^{-1}), and therefore the gene expression recorded as a result of this method may not reflect the exact conditions during natural infection. However, when the expression of the permease genes was monitored *in planta* (using the above method) compared to *in vitro* (extracted apoplast), the expression of each permease gene was not significantly different in each condition tested. As the *in vitro* apoplast was extracted from healthy tomato plants, this indicates that the amino acid content of the apoplast after 30 minutes of incubation with P.s.t was similar to that of a healthy plant. It may be expected that 30 minutes incubation *in planta* was not enough time for even a high density of bacteria to significantly alter the concentration of available nutrients in the apoplast, particularly if we assume that there will be a lag following inoculation before genes to transport and assimilate particular nutrients are expressed. Therefore, the inducing compounds for these permeases may have been present in similar amounts to that found in extracted apoplast.

Expression of aa_permease genes in the presence of putatively-transported amino acids.

The expression of each permease gene was also tested in the presence of the annotated substrate. Furthermore, where expression was shown to increase in the presence of this amino acid, the expression was investigated in the presence of a second amino acid to confirm that the observed increase was not just a response to increased nitrogen in the media.

The expression of PSPTO_5276 was significantly increased in the presence of 1 mM histidine but not 1 mM proline (Figure 6.7). Therefore this gene appears to be a histidine transporter. This result is concurrent with the finding that expression of the orthologous gene

in P.fl SBW25 was significantly increased in M9 supplemented with 15.5 µg ml^{-1} histidine, but not other tested amino acids (Zhang et al., 2006). The expression of PSPTO_5276 was not significantly increased in the presence of 1 mM ethanolamine compared to the expression in M9, supporting a hypothesis that the observed increase in expression was due to the presence of histidine rather than the added nitrogen provided by the added amino acid (compared to M9 without any amino acids added). The expression of PSPTO_5276 was significantly increased *in planta* and *in vitro*, compared to the expression in M9. However, this expression was significantly lower than in the presence of 1 mM histidine. As uninfected tomato apoplast contains an average of 6.83 µM histidine (Rico and Preston, 2008), far lower than the 1 mM concentration tested here, this may suggest that the expression of PSPTO_5276 is concentration dependent. The concentration of amino acids in the tomato apoplast is known to vary following inoculation with P.s.t (Arantza Rico, personal communication). Therefore it is tempting to speculate that a concentration-dependent increase in expression would allow increased expression of this gene in conditions where the concentration of the transported amino acid were increased and therefore more readily available to be transported. Furthermore, when Zhang and coworkers (2006) monitored the activity of the PSPTO_5276-ortholog *hutT* in P.fl.SBW25 on sugar beet seedlings, the levels of *hutT* expression in the phyllosphere, rhizosphere and in root exudates was found to be low, indicating that in the natural environment bacteria are exposed to widespread but low levels of histidine (Zhang et al., 2006). This result is concurrent with the present finding that expression in planta and in extracted apoplast is relatively low. Zhang and coworkers (2006) also reported that the expression of *hutT* was increased when P.fl.SBW25 were grown in M9 minimal media supplemented with 15.5 µg ml^{-1} histidine. Therefore further experiments investigating the expression of PSPTO_5276 in the presence of decreasing amounts of histidine may indicate the minimum amount needed to induce expression, which can then be compared to the amount of histidine present in the apoplast at different stages of infection.

The expression of PSPTO_5356 was significantly increased in the presence of 0.5 mM and 1 mM GABA, although not 1 mM histidine. Therefore this gene appears to be a GABA transporter. Interestingly the expression of PSPTO_5356 was not significantly increased in the presence of 1 mM GABA compared to 0.5 mM GABA, indicating that there is a maximum expression of PSPTO_5356. This may be due to negative consequences caused by an excess of intracellular GABA. Such an effect has been shown in *A. thaliana*, where succinic semialdehyde dehydrogenase (SSADH) mutants, (which can no longer convert GABA to succinic semialdehyde), show severe morphological defects, an effect attributed to the accumulation of intracellular GABA (Ludewig et al., 2008). Furthermore, GABA has been

demonstrated to partially inhibit the growth of P.s.t in mannitol-glutamate (MG) minimal media. In experiments involving growing P.s.t in MG media in the presence of 5 mM GABA, the growth of P.s.t was partially reduced in the presence of 5 mM GABA, suggesting an inhibitory effect of high concentrations of this amino acid on bacteria growth (Allan Collmer, personal communication). The expression of PSPTO_5356 was similar in the presence of GABA to the expression observed *in planta* and *in vitro*. As the tomato apoplast has been shown to contain approximately 0.5 mM GABA (Rico and Preston, 2008) this result supports a hypothesis that PSPTO_5356 is induced by the presence of GABA during colonisation of the plant apoplast.

The expression of PSPTO_1817 and PSPTO_2026 was not significantly increased following incubation in extracted tomato apoplast (*in vitro*) or *in planta*. This may be because PSPTO_1817 and PSPTO_2026 are expressed at a different time point to the one investigated. For example, these genes might be expressed on the leaf surface during epiphytic growth, or at a later time point following colonisation of the apoplast; therefore the uninfected apoplast (*in vitro*), or the apoplast following 30 minutes incubation with P.s.t (*in planta*), may not contain the inducing compound, or may not contain a high enough concentration of the inducing compound, for these genes to be expressed. Further experiments detailing the nutrient content of the apoplast at various time points during P.s.t colonisation may help to elucidate the time these genes are expressed and the substrate transported.

Comparison of gene expression in STA and extracted apoplast.

The synthetic tomato apoplast (STA) media was developed as a cheap and simple alternative to extracting tomato apoplast. Previous results in Chapter 4 indicated that similar nutrient assimilation pathways were induced following incubation of P.s.t in STA compared to extracted tomato apoplast. This result suggested that STA was a good substitute for extracted tomato apoplast in nutrient utilisation assays. In order to further test the properties of STA as a substitute for extracted tomato apoplast, the expression of each aa_permease gene was compared. The expression of PSPTO_5356 and PSPTO_1817 was not significantly different in STA and tomato apoplast, indicating that these genes are expressed to similar levels in synthetic and real tomato apoplast. However, the expression of PSPTO_5276 was significantly increased in extracted tomato apoplast compared to the synthetic alternative. The expression of PSPTO_5276 was increased in the presence of histidine, but not proline, and therefore appears to be a histidine transporter. The concentration of histidine in the healthy tomato apoplast was reported to average 6.83 µM

(Rico and Preston, 2008) and therefore this was the concentration added to STA. However, there may be other components of the apoplast which affect the expression of this gene, such as total nitrogen content or pH. For example, in Section 6.5.1 of this Chapter, it was shown that the expression of PSPTO_5356 was sensitive to the pH of the culture medium, with expression increasing in LB and STA at pH 6 compared to pH 7. An effect of pH on amino acid transporters was also hypothesised in Chapter 4, Section 4.5.5 following the observation that P.s.t can only derive a growth benefit (measured as an increase in optical density) from the presence of certain amino acids, including histidine, in acidic (pH 5.5) rather than alkaline (pH 8) conditions. Therefore, these additional components of the apoplast, which have not been replicated in STA, may result in the increased expression of PSPTO_5276. This therefore represents an avenue for further research concerning STA.

Motility, virulence, nutrient assimilation and growth of P.s.t-*rpoN*.

The global response regulator RpoN is known to be involved with the regulation of many *Pseudomonas* genes including those required for assimilation of a wide variety of nutrient sources such as inorganic nitrogen, amino acids, sugar alcohols and dicarboxylic acids (Kohler et al., 1989; Jones et al., 2007). Furthermore, RpoN is predicted to regulate the expression of several *Pseudomonas* aa_permease genes (see Chapter 3, Section 3.5.7), based on the presence of putative RpoN-binding sites. In particular, the P.s.t permease PSPTO_2026 contains a predicted RpoN binding site. Although *rpoN* mutants have been reported for many *Pseudomonas* species, including *P. putida* (Kohler et al., 1989), *P. aeruginosa* (Totten et al., 1990; Hendrickson et al., 2001) and *P. syringae* pv. maculicola M4 (Hendrickson et al., 2000b), a characterized P.s.t *rpoN* mutant has yet to be published. A P.s.t-*rpoN* mutant was obtained and successfully complemented with the *rpoN* gene from P.fl SBW25, cloned into the pBroadgate plasmid (Jones et al., 2007).

Initially, a motility assay, growth assay, virulence assay and carbon source utilisation assay were used to confirm successful complementation of the P.s.t-*rpoN* mutant with the P.fl.SBW25 *rpoN* gene. Previous reports had indicated that *rpoN* was conserved between bacterial species, allowing successful complementation of an *rpoN* mutant with a non-native *rpoN* gene. For example, an *rpoN* gene from *P. syringae* pv. maculicola ES4326 was used to successfully complement a *P. aeruginosa rpoN* mutant, restoring virulence, motility and the ability to assimilate a range of nutrients (Hendrickson et al., 2000a). P.fl SBW25 *rpoN* shares 93 % amino acid sequence identity over the full length P.s.t. *rpoN* gene, according to BLASTP analysis. Accordingly, the P.fl SBW25 *rpoN* was able to restore motility, virulence *in planta*, growth *in vitro,* and the ability to assimilate many compounds as carbon sources.

The effect of RpoN on aa_permease gene expression.

The expression of the permease genes was compared in wild type P.s.t and P.s.t-*rpoN*. Initially, the expression was compared following incubation in STA (Figure 6.16). The expression of PSPTO_2026, which contained a putative RpoN binding site, was reduced in P.s.t-*rpoN* compared to wild type P.s.t. Interestingly the expression of PSPTO_5356 was also significantly recued in P.s.t-*rpoN* compared to wild type P.s.t suggesting that this gene is also RpoN regulated. Bioinformatic analysis in Chapter 3, Section 3.5.7, did not identify the presence of a putative RpoN binding site in PSPTO_5356. However, sites were identified in GABA permease genes in other *Pseudomonas* including *P. putida* and *P. entomophila*, and a putative RpoN-binding site was subsequently identified upstream of PSPTO_5356 (Gail Preston, personal communication).

When the expression of the permease genes in wild type P.s.t and P.s.t-*rpoN* was compared in high and low nitrogen media, several interesting results were observed. In general, the expression of each permease in wild type P.s.t was highest in low nitrogen media, suggesting that these genes may be upregulated in response to low nitrogen conditions. This result has been documented previously for several genes, such as the *hrp* genes of P.s.s (Ramos et al., 2007) which are thought to use the low nitrogen conditions of the apoplast as a cue to signal their transcription. This result is also concurrent with the hypothesis that some permease genes may be regulated by RpoN, which is known to regulate a variety of genes in response to nitrogen. For PSPTO_2026, which was previously predicted to contain an RpoN-binding site, and PSPTO_5356, gene expression was significantly increased in wild type P.s.t compared to P.s.t-*rpoN* in low nitrogen conditions. This result suggested that P.s.t-*rpoN* had lost the ability to increase gene expression in response to low nitrogen suggesting a requirement for RpoN. Interestingly, there was not a significant difference in PAPTO_5276 expression in wild type P.s.t compared to P.s.t-*rpoN* when cells were cultured in STA (Figure 6.16). However, there was a significant increase in PSPTO_5276 expression in low nitrogen compared to high nitrogen minimal medium (Figure 6.16). One hypothesis is that P.s.t experiences STA as a ‘high’ nitrogen medium (there was no difference in expression in P.s.t and P.s.t-*rpoN* in high nitrogen minimal media). As STA was designed to mimic the low nitrogen conditions of the apoplast this result remains largely unexplained. However, the ammonium and nitrate composition of the apoplast remains unestablished and therefore the amount of nitrogen in STA may still be higher than that of the apoplast. Therefore future experiments could establish the amount of ammonia and nitrate in the apoplast and refine STA accordingly.

Finally, using RT-PCR transcripts the histidine utilisation genes in P.s.t were shown to be transcribed as an operon. This technique used primers which were designed to span adjacent genes in the putative P.s.t *hut* operon. These primers were used to amplify cDNA obtained from extracted RNA. Therefore cDNA would only have been amplified for the regions spanning adjacent genes if an RNA transcript was produced for this region, i.e if the genes were transcribed together, such as in an operon. The expression of PSPTO_5270 to PSPTO_5278 appears to occur together as an operon in P.s.t. This result is concurrent with the predictions obtained from Magdalen Lindberg in Chapter 3, which used Solexa sequencing of RT-PCR transcripts to predict the presence of an operon.

In conclusion, the present chapter has investigated the expression of the four aa_permease genes in P.s.t in a range of conditions. The expression of PSPTO_5276 was shown to be increased by the presence of histidine while PSPTO_5356 expression was increased by GABA, indicating a role for these permeases in transporting these amino acids. A new method for monitoring gene expression *in planta* was developed and results indicate that the permease genes are expressed similarly after 30 minutes *in planta* compared to in apoplast extracted from healthy (uninfected) plants, indicating that following a 30 minute incubation with P.s.t, the apoplast contains similar amounts of amino acids or other permease-inducing compounds to the healthy apoplast. The expression of aa_permease genes in synthetic tomato apoplast and extracted tomato apoplast was significantly different for PSPTO_5276 and PSPTO_2026, with expression lower in STA, suggesting that additional components of the apoplast, not replicated in STA, may contribute to the expression of these genes. The expression of permease genes in P.s.t-rpoN suggested that at least two (PSPTO_2026 and PSPTO_5356) may be regulated by RpoN, and that permease genes in general appear to be increased in low nitrogen conditions. This result is concurrent with the widely held view of the apoplast as a low nitrogen environment and suggests that these genes may be induced upon entry to the apoplast. Finally, using RT-PCR transcripts the histidine utilisation genes in P.s.t were shown to be transcribed in an operon, indicating that following a 30 minute incubation with P.s.t, the apoplast contains similar amounts of amino acids or other permease-inducing compounds to the healthy apoplast. The expression of aa_permease genes in synthetic tomato apoplast and extracted tomato apoplast was significantly different for PSPTO_5276 and PSPTO_2026, with expression lower in STA, suggesting that additional components of the apoplast, not replicated in STA, may contribute to the expression of these genes. The expression of permease genes in P.s.t-rpoN suggested that at least two (PSPTO_2026 and PSPTO_5356) may be regulated by RpoN, and that permease genes in general appear to be increased in low nitrogen conditions. This result is concurrent with the widely held view of the apoplast as a low nitrogen environment and

suggests that these genes may be induced upon entry to the apoplast. Finally, using RT-PCR transcripts the histidine utilisation genes in P.s.t were shown to be transcribed in an operon.

Chapter 7: Infection of glutamate decarboxylase-overexpressing tobacco plants with *P. syringae* pv. tabaci.

7.1 Overview.

In higher plants GABA is synthesised rapidly and in great quantities in response to a variety of biotic and abiotic stresses, including pathogen attack. This can occur on a scale of a 20-40 fold increase in GABA concentration within minutes of the stimulus and is achieved by positive regulation of the enzyme glutamate decarboxylase (GAD) by calcium/calmodulin (Ca^{2+}/CAM). Transgenic tobacco plants containing an additional copy of GAD have previously been shown to constitutively produce more GABA and display enhanced disease resistance when challenged with a variety of pathogens, including the Northern root-knot nematode, phytophagous insects and the bacterial pathogen *Agrobacterium tumefaciens.* The resistance of these plants to *P. syringae* has not been previously investigated. GABA can be utilised by *P. syringae* strains as a carbon and nitrogen source for both metabolism and growth and therefore may provide a nutrient source for *P. syringae* growing in the plant apoplast. However, the increase in GABA production following pathogen attack suggests this amino acid may play a role in plant defence against pathogens. This chapter describes experiments investigating the resistance of GAD plants to the bacterial tobacco pathogen *Pseudomonas syringae* p.v. tabaci (P.s.tabaci), and the growth of this bacterium in planta and in extracted apoplast.

The *in planta* growth of P.s.tabaci was found to be similar in wild type (WT) and GAD plants. However, whereas infected WT leaves showed extensive cell death five days post-inoculation, GAD plants were consistently less necrotic, and remained so after seven days. HPLC analysis of extracted apoplast demonstrated that GABA was more abundant in GAD lines than in the WT or plant vector control plants, contributing an average of 47% of the total measured amino acids, compared to 28% and 36% respectively. A range of cell biology and infection assays were used to investigate further the behaviour of P.s.tabaci *in planta,* and develop a hypothesis by which elevated GABA levels may contribute to reduced physical symptoms during infection.

7.2 Introduction.

7.2.1 The metabolism of GABA in plants.

GABA is a non-protein amino acid present in a large range of organisms including bacteria, plants, yeast and animals (Chevrot et al., 2006). Historically, research into the mechanism and function of GABA has focused on animals, where it is recognised as the most important inhibitory neurotransmitter in the central nervous system. More recently, interest has shifted to the role of GABA in plants, after it was discovered that the typically low levels of GABA in plant tissues rapidly increase in response to many diverse stimuli including hypoxia, low temperatures, pathogen attack and drought (Bown and Shelp, 1997; Kinnersley and Turano, 2000).

In both plants and animals GABA is mainly synthesised by the decarboxylation of glutamate by the cytosolic enzyme glutamate decarboxylase (GAD), in an essentially irreversible reaction (Figure 7.1). Genomic DNA analysis indicates there are at least four tobacco GADs, existing in pairs of highly identical genes (Yevtushenko et al., 2003).

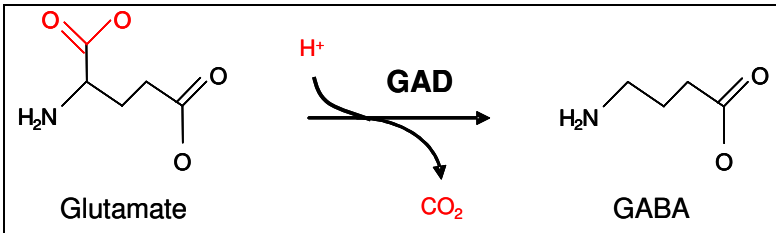


Figure 7.1
The primary route of GABA synthesis in higher plants.

GABA is then metabolised by two mitochondrial enzymes: GABÃtransaminase (GABÃT) and succinic semialdehyde dehydrogenase (SSADH). GABÃT exists in two isoforms that can use either pyruvate / glyoxylate, or Õoxoglutarate as an acceptor, converting GABA nitrogen to alanine or glutamate respectively, both of which result in the production of succinate which can enter the TCA cycle (Clark et al., 2009). Together these three reactions make up a conserved metabolic pathway known as the GABA shunt, so called because it bypasses two successive steps in the TCA cycle (Bown and Shelp, 1997). Figure 7.2 shows the enzymes and metabolites of the GABA-shunt in eukaryotes in relation to the TCA cycle and related reactions.

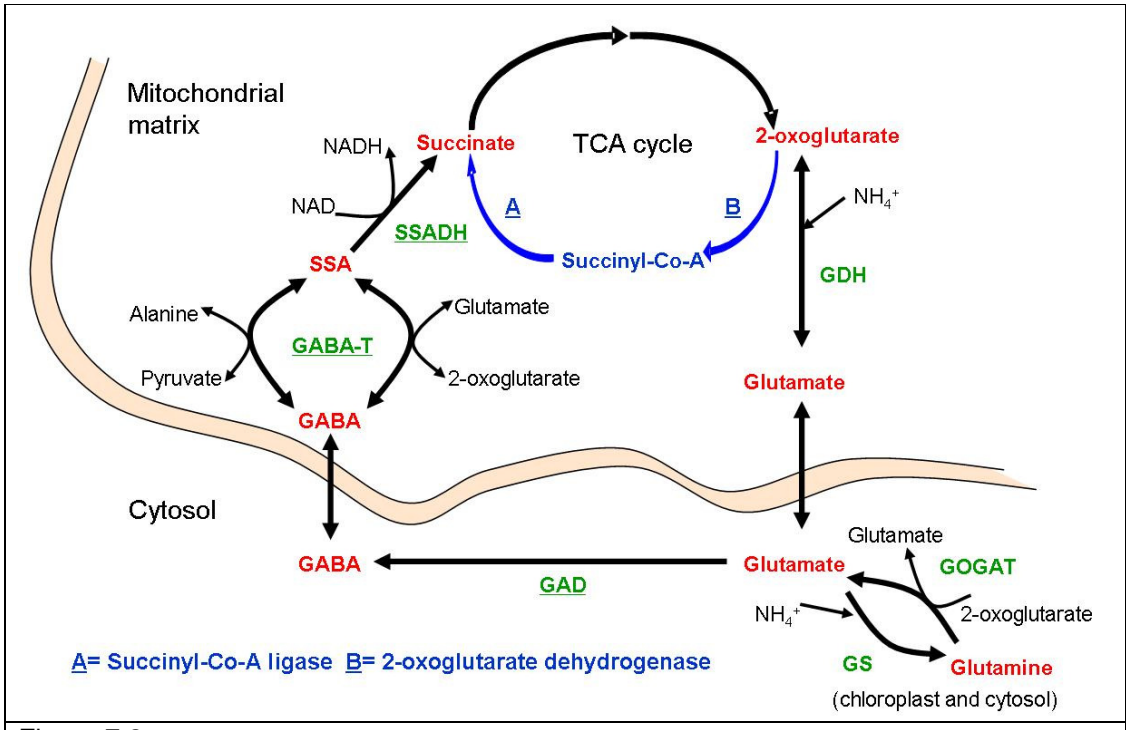


Figure 7.2
Schematic diagram of the GABA shunt and related enzymes. Enzymes are shown in green typeface. The three enzymes of the GABA-shunt are underlined. **GAD**; glutamate decarboxylase EC4.1.1.15, **GABA-T**; GABAtransaminase EC2.6.1.19, **SSADH**; succinic semialdehyde dehydrogenase EC1.2.1.16, **GDH**; glutamate dehydrogenase EC1.4.1.3, **GS**; glutamine synthase EC 6.3.1.2, **GOGAT**; glutamate synthase EC 1.4.7.1 / 1.4.1.14. TCA cycle, tricarboxylic acid cycle; SSA, succinic semialdehyde. Arrows within the TCA cycle indicate the overall direction of carbon flux.

The speed with which GABA accumulates following an appropriate stimulus led to the hypothesis that GAD enzymes are upregulated in response to the stimulus, rather than an increase in *de novo* synthesis of GAD (Bown and Shelp, 1997). *A.thaliana* GAD knockouts have dramatically reduced GABA levels and are unable to accumulate GABA in response to stress (Bouche and Fromm, 2004). In vitro, GAD has an enzyme optimum of pH~5.8, well below normal physiological pH. As GAD is a cytosolic enzyme it has been proposed that cytosolic acidosis, which occurs in response to stresses causing metabolic or mechanical disruptions such as insect feeding or anoxia, may stimulate GAD to synthesise stress-induced GABA. *In vivo* experiments have now shown that artificially increasing the cytosolic H^+ levels, (decreasing the cytosolic pH), precedes GABA accumulation, thereby demonstrating a mechanism by which the stresses that cause cytosolic acidification can result in GABA accumulation (Shelp et al., 1999). However not all stimuli which result in an increase of GABA are accompanied by a decrease in cytosolic pH. Cloning and characterising the first GAD gene, from a petunia, showed this enzyme to be regulated by a Ca^{2+}/CAM binding domain (Ca^{2+}/CAMBD), which is autoinhibited in the absence of Ca^{2+}/CAM (Baum et al 1993). All GAD enzymes from dicotyledonous plants, including

tobacco, have been shown to carry a Ca^{2+}/CAMBD, which is lacking in bacterial and animal GADs (Akama and Takaiwa, 2007). Many stress factors, such as touch or cold-shock, cause a transient increase in cytosolic Ca^{2+} levels, thus a model has subsequently been proposed whereby rapid GABA synthesis is mostly the result of CAM activation directly associated with the cytosolic Ca^{2+} fluxes, which occur as a result of biotic and abiotic stress (Figure 7.3)

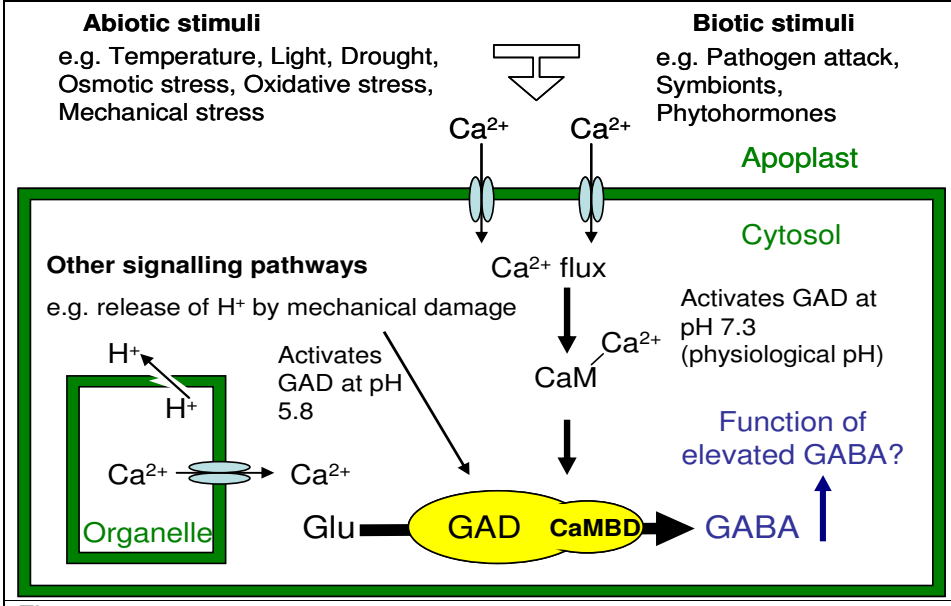


Figure 7.3
Model of stimulus-response signalling mediated by Ca^{2+}/CaM and H^+. The majority of environmental stresses evoke a transient change in cytosolic Ca^{2+} levels, which are transduced by Ca^{2+}/CaM interacting with a Ca^{2+}/CaM-BD within the GAD enzyme. This relieves the autoinhibition of GAD resulting in increased decarboxylation of glutamate and corresponding increases in GABA levels. During particular stresses, such as mechanical damage or anoxia, GAD is activated in the absence of Ca^{2+}/CaM, by an increase in cytosolic H^+.

7.2.2 Possible roles for GABA and the GABA shunt in plants.

The finding that GABA accumulates rapidly in stressed plant tissue, leads to the question of what function this compound may play in mitigating such stresses. Many roles have been suggested and these are summarised below:

- **Nitrogen storage**

The conversion of glutamate to GABA is increased under conditions that inhibit glutamine synthesis, reduce protein synthesis or enhance protein degradation, leading to the hypothesis that GABA is a temporary nitrogen store (Shelp et al., 1999). It is also suggested that when 2oxoglutarate is the amino donor for GABAT (Figure 7.2), the resulting glutamate can be used to make more GABA, therefore 'cycling' the amino group between these two metabolites and ridding plants of excess carbon (Bown and Shelp, 1997). Approximately 50

% of glutamate and GABA is found within the vacuole and accumulated GABA is believed to be sequestered within organelles (Shelp et al., 1999).

- **GABA as an osmolyte.**

Plant cell dehydration as a result of stresses such as drought or freezing can cause the concentration of cellular constituents to rise to levels that cause membrane damage or cell death. It has been suggested that an accumulation of GABA in the cytosol would balance the decrease in water potential that occurs during cellular dehydration (Shelp et al 1999). Evidence has also been provided of GABA protecting biological membranes from inactivation during freezing (Heber et al., 1971).

- **Regulation of cytosolic pH.**

In addition to stimulation by protons (H^+), the decarboxylation of glutamate by GAD also consumes them (Figure 7.1). When asparagus cells were continually exposed to weak acids a cytosolic pH decrease was associated with increased GAD activity which decreased as the pH recovered. Stress-induced GABA accumulation is therefore believed to play a role in maintaining cytosolic pH in a normal physiological range by acting as a biochemical pH stat (Janzen et al., 2001).

- **Tricarboxylic acid (TCA) cycle bypass.**

In addition to entering the TCA cycle as 2-oxoglutarate, the GABA shunt allows glutamate to enter the TCA cycle further along as succinate (Figure 7.2). The metabolism of 2-oxoglutarate to succinate in the TCA cycle is tightly regulated, and involves two enzymes (A and B in Figure 7.2) which are sensitive to stress conditions such as oxygen deprivation. Therefore this additional route may be important in conditions such as hypoxia, where respiration and the NAD/NADH ratio are reduced, because such conditions inhibit certain enzymes of the TCA cycle (A and B in Figure 7.2) and limit the production of succinate (Bown and Shelp, 1997).

- **Protection against oxidative stress.**

The GABA shunt is activated, via Ca^{2+} regulation of GAD, under stress conditions which cause enhanced production of reactive oxygen intermediates (ROIs) (Shelp et al., 1999). Compromising the function of the GABA shunt in yeast (Coleman et al., 2001) and *A. thaliana* (Bouche et al., 2003; Bouche and Fromm, 2004), caused ROI accumulation and increased sensitivity to environmental stresses. As these same conditions inhibit certain enzymes of the TCA cycle (above) the GABA shunt may serve to limit the accumulation of ROIs by ensuring the continued generation of NAD(P)H to maintain reducing conditions when the TCA cycle is compromised. However, more recent findings have thrown doubt on this hypothesis. Ludewig and coworkers (2008) demonstrated that the severe oxidative stress and growth retardation phenotype of *A. thaliana* ssadh mutants was not caused by a lack of succinate and NADH supplied to the TCA cycle. Instead it was suggested that the

accumulation of succinic semialdehyde (SSA), gamma-hydroxybutyrate (GHB), or both, (produced downstream of the GABA-T transamination step), is toxic to the plants resulting in the observed high ROI levels and impaired development of ssadh mutants (Ludewig et al., 2008).

- **Signalling molecule.**

Kinnersley and Turano (2000) define three characteristics of signalling molecules: they alter metabolism and/or growth alter gene expression and/or activate enzymes and have receptor molecules. GABA has been shown to alter gene expression in *A.thaliana* (Lancien and Roberts, 2006), activates enzymes in sunflower (Kathiresan et al., 1997) and the existence of GABÃlike receptors currently being investigated (reviewed in Kinnserley and Turano,. 2000). It has also been discovered that GABA indirectly promotes ethylene biosynthesis in sunflower by promoting the de novo protein synthesis of the enzymes responsible for ethylene production (Kathiresan et al., 1997). If GABA-induced ethylene production can be confirmed in other plants, it would connect the stress-induced changes in intracellular Ca^{2+} to functional stress responses.

- **Plant defence.**

GABA increases in response to insect feeding, and also has been shown to increase in *A.thaliana* whole leaf tissue 10 hours post inoculation with P.s.t. (Murray Grant, personal communication). The role of GABA in plant defence against pathogens and feeding invertebrates has been studied using tobacco plants which overexpress the GAD enzyme, resulting in constitutively elevated GABA levels. As these are the same transgenic GAD plants used in this chapter the results of these experiments will be described in detail below.

7.2.3 Previous studies using transgenic GAD plants.

- **The effect of GABA on phytophagous invertebrates.**

In animals, GABA functions as an inhibitory neurotransmitter acting at GABÃgated Cl^- channels. Phytophagous activity by invertebrates such as insects destroys vacuolar compartmentalisation, causing cytosolic acidification, and consequently H^+stimulation of GAD enzymes (Shelp et al., 1999). When ingested the resulting high levels of GABA are thought to have a damaging affect on the neuromuscular activity of invertebrates as the GABA receptors of the nervous system are exposed to the haemolymph, and are not protected by a blood-brain barrier. This point was experimentally demonstrated by feeding the phytophagous larvae of the oblique-banded leaf roller (*Choristoneurarosaceana*) an artificial diet containing levels of GABA equivalent to the amounts found in GAD-activated leaves. The result was reduced growth rates, developmental rates and survival rates of the larvae (Ramputh and Bown, 1996).

These results led to the hypothesis that GABA accumulation may deter feeding by invertebrates. In their 2003 paper, Mc Lean and coworkers described the creation of phenotypically normal, homozygous transgenic lines of tobacco (*Nicotianatabacum*L.cv.Delgold) overexpressing a single copy of a full-length GAD, or a truncated GADΔC lacking the Ca^{2+}/CaM domain. The levels of GABA in transgenic plants were statistically higher in GAD and GADΔC plants than the WT, in twoweekold whole seedlings, and the shoots and roots of twelve-week-old plants (McLean et al., 2003), and were comparable to the amounts which inhibited larval growth and development in an artificial diet: 1.6–2.6 μM g^{-1} of fresh weight (FW) (Ramputh and Bown, 1996), particularly in the roots where GABA reached ~ 3.5 μM g^{-1} FW.

Transgenic plants were tested for resistance against the northern root-knot nematode by assessing the number of egg masses present on the root surface after nine to twelve weeks' growth in nematode-infected soil. Transgenic plants supported significantly fewer egg masses than WT plants, suggesting that female nematodes were reduced in number or fecundity. Unfortunately GABA levels in infected plants were not determined due to the complicating factors of nematode presence and root damage during sampling (McLean et al 2003).

Following these results, two of the GAD lines generated by McLean and coworkers, GAD2 and GAD5 (two independently transformed lines), were used to determine the effect of enhanced GABA on feeding by phytophagous insect larvae of the tobacco budworm *Heliothis virescens* (MacGregor et al., 2003). Although *in planta* GABA concentrations were not determined, GAD2 and GAD5 were found to have twice the GAD enzyme activity of WT plants. After 4 hours of feeding on a choice of GAD or WT leaves, phytophagous larvae had consumed 6-12 times more leaf tissue from WT than GAD plants (MacGregor et al., 2003).

The conclusion of these two studies was that GABA accumulation in response to pest activity increases plant resistance, either directly by affecting invertebrate growth and development, or through the GABA-mediated activation of local or systemic plant defences.

- **The effect of GABA on plant-pathogenic bacteria.**

The damaging neuromuscular effects of GABA cannot be expected to apply to prokaryotic pathogens. Therefore GAD3, GAD7, GADΔC1 and GADΔC2 generated by McLean and coworkers were used to investigate the effect of enhanced GABA on the virulence of the bacterium *Agrobacterium tumefaciens* (Chevrot et al., 2006).

A.tumefaciens is a soilborne pathogen which infects tobacco plants at sites of wounding, causing crown gall tumours. Chevrot and coworkers reported that crown gall tumours were significantly reduced in GADΔC1 and GADΔC2 lines compared to GAD3, GAD7 and WT plants. The mechanism of GABA action in this instance was proposed to be due to GABAstimulation of the *A.tumefaciens* AttKLM operon, which contains a lactonase (*Att*M) capable of degrading quorum-sensing (QS) N-acylhomoserine lactones (AHLs). *A.tumefaciens*, in common with many bacteria, uses the release and subsequent detection of QS signals to monitor population density *in planta.* When QS molecules reach a threshold concentration they stimulate a range of adaptive responses, such as Ti plasmid conjugation, which enhance the virulence of *A.tumefaciens in planta* and therefore the severity of symptoms. High levels of GABA, such as those found in wounded tissues, stimulate the degradation of QS molecules by *Att*M and prevent them reaching this threshold concentration. In WT plants this system is thought to be advantageous to *A.tumefaciens* by breaking down QS molecules at stages of the infection process when they might be detected by plant defence systems, or by improving the *in planta* growth of *A.tumefaciens* by degrading QS molecules from competing bacteria (Chevrot et al., 2006). In WT plants as pathogenesis progresses the abundance of GABA in developing tumours decreases and QS molecules can accumulate. It is this step that is thought to be inhibited by transgenic GAD plants.

An isogenic mutant of *A.tumefaciens*, lacking the *Att*M lactonase grew equally well in all transgenic GAD plants (Chevrot et al., 2006), indicating that the inhibitory effects may be specific this particular gene. This raises the question of how plant pathogens which do not use GABA-regulated accumulation of QS molecules as part of their infection process, such as *P. syringae*, would respond to transgenic tobacco plants with elevated GABA.

The aim of this chapter is to investigate whether GAD plants show enhanced resistance in response to the plant pathogenic bacterium *P.syringae*. *P. syringae* grows epiphytically on the surface of leaves prior to dissemination into the leaf apoplast, mainly through natural openings such as stomata or hydathodes. AHLs have been demonstrated to be important in regulating traits involved in epiphytic fitness and virulence in *P.syringae* pv. syringae B728a (Quinones et al., 2005). However, BLAST analysis indicates that no *Pseudomonas* species possess a gene of significant similarity to *A.tumefaciens Att*M and therefore GABA may not affect QSsignalling *in planta*, although little is known about this area. In contrast to *A.tumefaciens*, which mainly assimilates tumour-synthesised opines, *P. syringae* is thought to utilise nutrients available in the plant apoplast, and of these, GABA has been shown to provide an excellent carbon and nitrogen source for both growth and metabolism (Chapter

4). In addition, in P.s.t the expression of PSPTO_5356, encoding the GABA permease transporter GabP was highly expressed in extracted tomato apoplast (Chapter 6). Therefore it will be particularly interesting to investigate the growth of *P. syringae in planta* and in extracted apoplast of transgenic GAD plants which have increased GABA.

7.3 Objectives.

- Compare the growth of P.s.tabaci in GAD, GADΔC, wild type (WT) and plant-vector control (control or C) plants *in planta* and in extracted apoplast.
- Use a range of cell biology assays to investigate plant defence responses to infection by pathogenic and non-pathogenic bacteria.
- Using HPLC analyse the amino acid content of WT tobacco plants and compare the abundance of major amino acids to those found in the tomato (*Solanum lycospersicum L. cv. Rio Grande*) apoplast.
- Analyse the amino acid content of the GAD, GADΔC, WT and control plant apoplast after infection with P.s.tabaci, or a control treatment of $MgCl_2$.

7.4 Materials and methods.

7.4.1 Plant material.

Transgenic tobacco (*Nicotiana tabacum* L cv. Delgold) plants were constructed as previously detailed (McLean et al., 2003). Seeds for GAD, GADΔC and WT plants (cv. Delgold), were supplied by Barry Shelp (University of Guelph). The four homozygous glutamate decarboxylase (GAD)-overexpressing plants all contain one additional copy of the GAD enzyme; **GAD1** and **GAD2** (called GAD3 and GAD7 in McLean et al 2003), express a full length tobacco GAD enzyme. **GADΔC1** and **GADΔC2** express a truncated enzyme that lacks the autoinhibitory $Ca2^{+}$/CAMBD (called GADΔC1 and GADΔC2 in McLean et al 2003). A vector control plant was also obtained which contained the pMDM8 plasmid without a GAD or GADΔC enzyme (McLean et al., 2003).

Seeds were grown on a 3:1 mixture of peat based compost (Goundrey's, UK) and vermiculite (Sinclair, UK). Seeds were soaked overnight in a solution of 0.5 mg / ml^{-1} gibberelic acid (Sigma Aldrich, UK) made up in water, and germinated in a plant growth chamber set at a constant temperature of 21°C and a photoperiod of 16 hours (16 hours light, 8 hours dark). After 21 days seedlings were transplanted into individual pots containing soil/vermiculite mixture and moved to a transgenic greenhouse set at 21°C and a 16 hours photoperiod. Plants were used in experiments at twelve weeks old. Two replicate plants

were used for each experiment, and each experiment was independently performed at least twice as indicated.

7.4.2 Bacterial strains.

Bacterial strains and abbreviations were used as described in Chapter 2. All strains were routinely cultured in LB agar medium at 28°C as described in Chapter 2. Overnight bacterial cultures were washed twice in 10 mM $MgCl_2$ then resuspended in 10mM $MgCl_2$ at the appropriate density prior to inoculation unless otherwise stated. Antibiotics were added where appropriate.

7.4.3 Extraction of apoplast fluid and estimation of apoplast dilution.

Apoplastic fluid was extracted from uninfected tobacco plants using the vacuum infiltration method modified from Rico and Preston (2008) as described in Chapter 2.7.1. The average apoplast dilution for tobacco leaves was calculated from five independent experiments as 1.4±0.05. Therefore freeze-dried apoplast was resuspended in MilliQ water using the formula:

$$V_{orig} = V_{ex}/1.4$$

Where V_{orig} is the 'full strength' apoplast and V_{ex} is the amount of apoplast extracted from the infiltrated tobacco leaves.

7.4.4 Infection assays.

Growth *in planta.*

A leaf disc method was used to monitor the *in planta* growth of P.s.tabaci (Figure 7.4). *P.s.*tabaci was infiltrated into leaves four and five, (counted from the base of the plant) at a concentration of $1x10^5$ colony forming units (cfu)ml^{-1} using a blunt 1ml syringe. Immediately after infiltration three leaf discs were taken, per leaf, using a 1cm diameter cork borer (Fisher Scientific, UK) and separately homogenised in 1ml 10mM $MgCl_2$ using a plastic microcentrifuge pestle (Fisher Scientific, UK). Samples were then serially diluted and 100µl aliquots were spread onto LB agar plates supplemented with half-strength CFC (Oxoid Limited, UK), a combination of antibiotics which preferentially select for the growth of *Pseudomonads*. This procedure was repeated on day three, five and seven. Plates were incubated at 28°C for two days and those dilutions which provided 50-100 colonies per plate were counted. Data from each of the three leaf discs was averaged per time point. The selectivity of LB-CFC plates was tested by taking leaf discs from an uninfected leaf and processing them as described in the leaf disc protocol, no colonies were detected after two days growth at 28°C.

Growth *in vitro* – apoplast extracts.

Apoplast was extracted and pooled from leaves three, four, five and six for each tobacco plant; this was considered one biological replicate. Two biological replicates were used per experiment. P.s.tabaci was resuspended in 1 ml of extracted apoplast at $1x10^7$colony forming units (cfu)ml^{-1} ($OD_{=}0.02ml^{-1}$) and 150 µl aliquots were pippetted into six wells of a Corning 96 well plate (Sigma Aldrich, UK). The wells surrounding the apoplast were filled with 150µl aliquots of water, to prevent evaporation of the apoplast. Plates were incubated overnight at 28ºC, with shaking, in a Dynatech multiwell plate reader. Optical density was recorded every hour as a measure of growth.

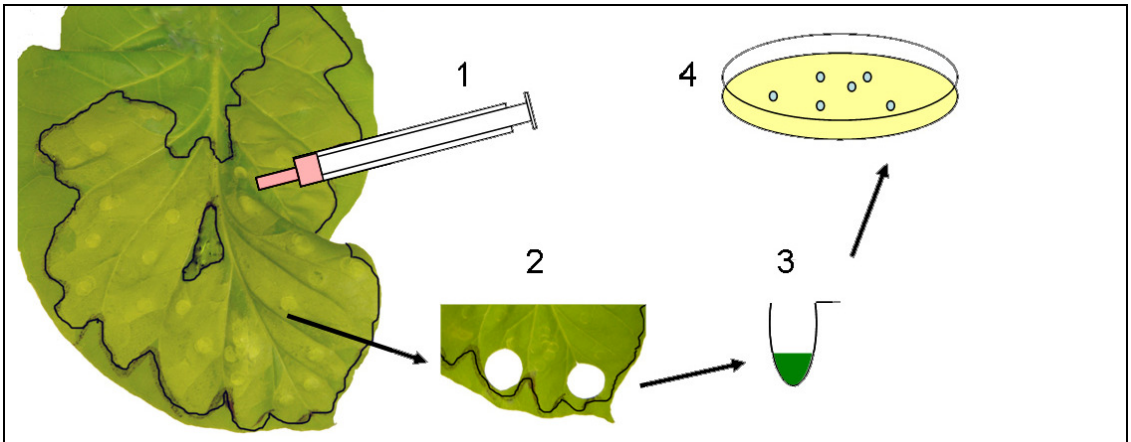


Figure 7.4
The leaf disc protocol used to monitor bacterial growth *in planta*. Leaves were infiltrated with a bacterial suspension in 10mM $MgCl_2$ (1). Leaf discs were taken from three points across each leaf (2) and homogenised in 10mM $MgCl_2$ (3). 100µl aliquots were spread onto LBCFC agar plates and incubated at 28ºC for two days.

7.4.5 Cell biology assays.

In all cell biology assays, leaves were inoculated with P.s.tabaci ($1x10^5cfuml^{-1}$), P.s.t.($1x10^5cfuml^{-1}$), P.s.t-hrcC ($1x10^7cfuml^{-1}$), or 10mM $MgCl_2$ (negative control). Six leaf discs were taken from leaves four and five of each tobacco plant (Figure 7.5). Two plants of each transgenic line or wild type cultivar were used in each experiment. Each experiment was independently replicated twice. Between three and five fields of view were randomly photographed per leaf disc at 10x magnification, using an Optronics camera system with MagnaFire software (Optronics, USA) attached to an Olympus BX50 microscope (Olympus, USA).

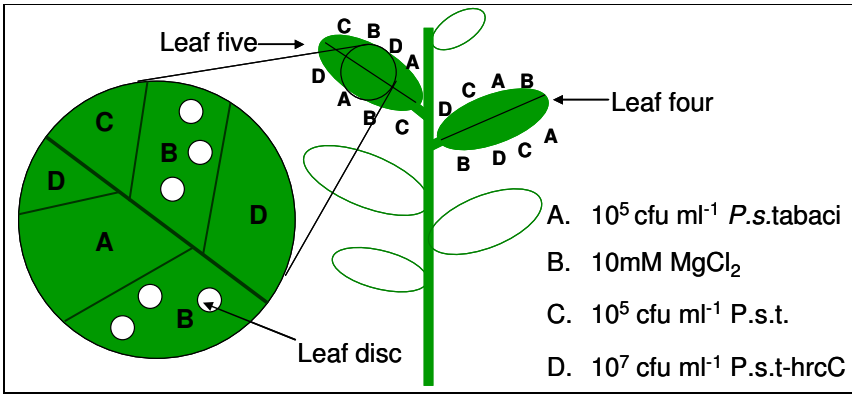


Figure 7.5
Inoculation of bacteria for cell biology assays. Bacteria were resuspended in 10mM $MgCl_2$ and inoculated in two positions per leaf, using the natural sections formed by the secondary veins. At appropriate time points leaf discs were removed.
cfu = colony forming units

- **Detection of basal defences: Callose staining.**

Leaf discs were removed at six and 24 hours post-inoculation and cleared in methanol before being stained overnight with 0.05% aniline blue in 50mM potassium phosphate buffer (pH8). Leaves were then examined for callose staining by UV microscopy using a broad-UV filter (U-MWU, Olympus, USA). All chemicals Sigma-Aldrich, UK.

- **Detection of reactive oxygen species: Hydrogen peroxide (H_2O_2) detection.**

Leaf discs were removed at one, two and six hours post-inoculation and placed in 1mgml^{-1} 3,3'diaminobenzidine tetrahydrochloride (DAB) pH3.8 for 8 hours. Leaf discs were then cleared in 99.5% ethanol, rinsed in distilled water, and examined for DAB staining by light microscopy. All chemicals Sigma-Aldrich, UK.

- **Detection of reactive oxygen species: Superoxide (O_2^-) detection.**

Leaf discs were removed at one and three hours post-inoculation and placed in 0.1 % (w/v) nitroblue tetrazolium (NBT), 10 mM sodium azide, 50 mM potassium phosphate (pH 6.4) (Schraudner et al 1998) overnight. Leaf discs were then cleared in 99.5% ethanol, rinsed in distilled water, and examined for NBT staining by light microscopy. All chemicals Sigma-Aldrich, UK.

- **Detection of cell death: Trypan blue staining.**

Leaf discs were boiled in lactophenol-trypan blue solution (10 mL of lactic acid, 10 mL of glycerol, 10 mL of liquid phenol, and 10 mL of distilled H_2O) containing 10 mg of trypan blue for 1 min (all chemicals Sigma-Aldrich, UK). Disks were cleared of chlorophyll by boiling in menthol for 2 min and stored in water. For analysis disks were mounted on microscope slides and examined for trypan blue staining by light microscopy

7.4.6 Measuring salicylic acid (SA) production by tobacco plants.

Salicylic acid (SA) production by the different tobacco lines was examined in response to two treatments. Leaves were either 'primed' before inoculation with *A.tumefaciens* GV3101 (AtGV3101), a strain containing a Ti plasmid lacking a T-DNA region and therefore unable to transfer T-DNA, or treated with 10mM $MgCl_2$ as a blank. The purpose of the AtGV3101 pre-treatment was twofold, to support previous observations that *A.tumefaciens* suppresses SA production in response to *P.syringae*, and to investigate any differences in basal defence responses between transgenic lines and WT plants in their SA response. An overnight culture of AtGV3101 was resuspended in 10mM $MgCl_2$ with 0.5mM acetosyringone ($MgCl_2$AS) and infiltrated into leaves at approximately $1x10^7$ cfuml^{-1}; 10mM $MgCl_2$AS was used as a negative control. Two leaves (four and five) were inoculated per tobacco line. Plants were incubated in the laboratory at 21°C for 72 hours. Overnight cultures of P.s.tabaci were resuspended in 10mM $MgCl_2$ and infiltrated at $1x10^5$ cfuml^{-1} for *P.s.*tabaci and P.s.t. and $1x10^7$ cfuml^{-1} for P.s.t-hrcC. Plants were incubated for a further 24 hours before the biosensor ADPWH_*lux* was infiltrated to measure SA.

The lux reporter *Acinetobacter* ADPWH_*lux* was used to measure SA in planta as described by Huang and coworkers (2006) with slight modifications. Briefly, an overnight culture was diluted in 100ml of LB broth and incubated with shaking at 37°C for approximately 2hr to reach OD_{600} ~ 0.4, at which point ADPWH_*lux* was infiltrated directly into leaves. Plants were maintained at 21°C for one hour prior to imaging. SA-induced luminescence was imaged using a photon-counting camera (Photek Ltd., East Sussed, UK). Luminescence counts were recorded every second for 200 seconds. The total points and pixel intensity for each inoculated area was obtained using Photek IFS32 image processing and data acquisition software (Photek Ltd.) and normalised luminescence values were obtained by dividing the total counts by number of pixels in the inoculated area. SA ladders were included *in vitro* as a control for exposure length to allow the comparison of bioluminescence between separate images, and *in planta* to provide a calibration between bioluminescence and SA concentration (Figure 7.6 and Appendix Figure A3).

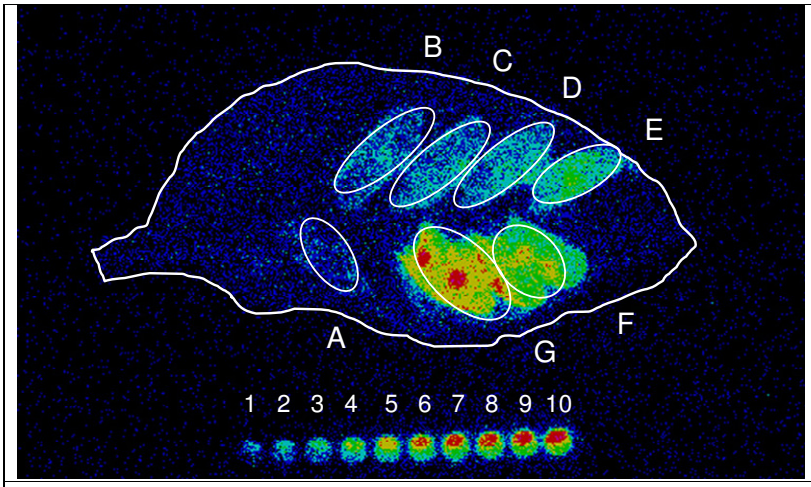


Figure 7.6
In planta and *in vitro* SA ladders.
The *In planta* leaf ladder containing the following amounts of SA in µM: A=0, B=2.5, C=50, D=100, E=200, F=300, G=400. *In planta* ladders were infiltrated in each tobacco line and the averaged bioluminescence intensities were plotted against SA concentration. The *in vitro* SA ladder contains the following amounts of SA µM: 1=0, 2=0.02, 3=0.05, 4=0.1, 5=0.2, 6=0.3, 7=0.4, 8=0.5, 9=1, 10=2.

7.4.7 Identification of amino acids by HPLC.

Apoplast extracts were subjected to high performance liquid chromatography (HPLC) to detect protein amino acids and GABA. Apoplast samples were obtained from two leaves of two replicate plants, for each tobacco line. Each leaf was infiltrated with a 10mM $MgCl_2$ control in one side of the leaf mid-vein, and $1x10^5 cfuml^{-1}$ P.s.tabaci on the other side of the leaf vein. This allows data describing the amino acid abundance in P.s.tabaci and $MgCl_2$treated plants to be directly compared. After 24 hours the leaves were cut in half along the mid vein and the apoplast from each treatment was extracted separately. Apoplast from leaves with the same treatment, on the same plant were pooled to form one biological replicate (Figure 7.7). Samples were derivitised according to the following method provided by Dr Laurent Miguet (Oxford University) (all chemicals Sigma-Aldrich, UK). Ten microlitres of apoplast was mixed with 10□l 0.1 N hydrochloric acid (HCl), 5□l 0.52 M N-isobutyryl-D-cysteine (IBDC), 5□l 0.32M o-phthaldialdehyde (OPA) and 55□l 0.4 N borate buffer, pH 10.4. This mixture was incubated for 2 minutes at room temperature. 80□l was injected into a HPLC reverse phase C-18 column. The HPLC program used was: 0 to 80% buffer B for 80min, 80to 100% buffer B for 10min, 100 to 0% buffer B for 10min. The flow rate was 1 $mlmin^{-1}$. The relative percentages of buffer B were achieved by altering the flow of this buffer with respect to buffer A. Buffer A consisted of 23mM sodium acetate trihydrate and 10% HPLC grade methanol. Buffer B consisted of 60% HPLC grade methanol, 23mM sodium acetate trihydrate. The fluorescence detector was set at 230nm excitation and 445nm emission. An internal standard of 10 mM GABA was run on the column to establish the position of the GABA peak in the HPLC spectrum profile. The positions of all other amino

acids in the HPLC profile were provided by Arantza Rico (Oxford, UK). Amino acid abundance was automatically calculated from the peak area by the HPLC analysis programme ANAL2 provided by Dr Laurent Miguet (Oxford University).

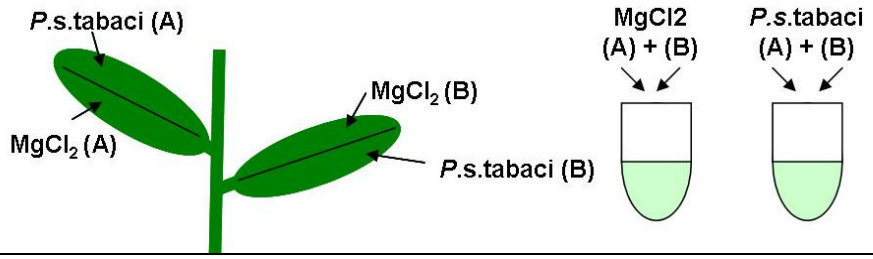


Figure 7.7
Two leaves per plant were inoculated. Half of each leaf received $MgCl_2$-treatment and half P.s.tabaci. After 24 hours the apoplast was extracted separately and the samples from the same treatment were pooled.

7.4.8 Reverse transcriptase (RT-PCR) of plant gene expression.

The expression of pathogenesis-related protein-1a (PR1a) was compared between tobacco lines. P.s.tabaci (10^5cfu ml^{-1}) was inoculated into leaves four and five of two replicate plants, per tobacco line as previously described. Plants were incubated for 24 hours at 21°C on the bench. 100mg of leaf tissue was cut from each leaf, snap frozen in liquid nitrogen and stored at -80°C until use (no longer than 1 month later). Leaf samples were homogenised using a plastic microcentrifuge pestle while still frozen, and the two samples from each leaf were pooled. Total RNA was isolated using RNeasy(R) Plant Mini kit (Quiagen GmbH, Hilden, Germany). RNA was treated with DNase and quantified on a nanodrop ND-1000 (Thermo Fisher Scientific, UK). Random primed first-strand cDNA was generated using the ImpProm-II™ Reverse Transcription System (Promega Corp., Madison, WI, USA). The primers used for detecting the relative transcripts of PR1α and the housekeeping gene EF1 were; PR1αF (5'GCGCAAAATTATGCTTCCCA3'), PR1 R(5'CCGTCATGAAATCGCCACTT3'),EF1αF(5'TCTGTTGAGATGCACCACGAAG3'), EF1αR(5'ACAAACCCACGCTTGAGATCC3').

The thermal cycling conditions used were:

- 95°C for 2 minutes, 1 cycle
- 95°C for 1 min, 60°C for 1 min, 72°C for 1.30 mins, 29 cycles
- 72°C for 5 mins, 1 cycle
- 4°C to end

7.5 Results.

7.5.1 P.s.tabaci causes fewer symptoms in transgenic GAD tobacco than the wild type or plant-vector control plants, without differences in bacterial growth.

Transgenic plants which overexpress the GABA-synthesis enzyme glutamate decarboxylase show fewer symptoms upon infection with the bacterial phytopathogen *A. tumefaciens*. However this phenotype was subsequently found to be due to the effect of GABA accumulation on the regulation of the *A. tumefaciens Att*KLM operon, which regulates certain virulence factors (McLean et al., 2003). The objective of this experiment was to describe the infection of transgenic GAD plants by the bacterium *Pseudomonas syringae* pv tabaci ATCC 11528 (P.s.tabaci), which does not contain homologous genes to those of the *Att*KLM operon, by following bacterial growth *in planta* and in extracted apoplast, and scoring the severity of symptoms on infected leaves.

Assays were carried out on four homozygous transgenic tobacco lines that had enhanced capacity to accumulate GABA (McLean et al., 2003). In accordance with previous reports by McLean and coworkers all transgenic and control plants were indistinguishable in size to the wild type (WT) (Figure 7.8).

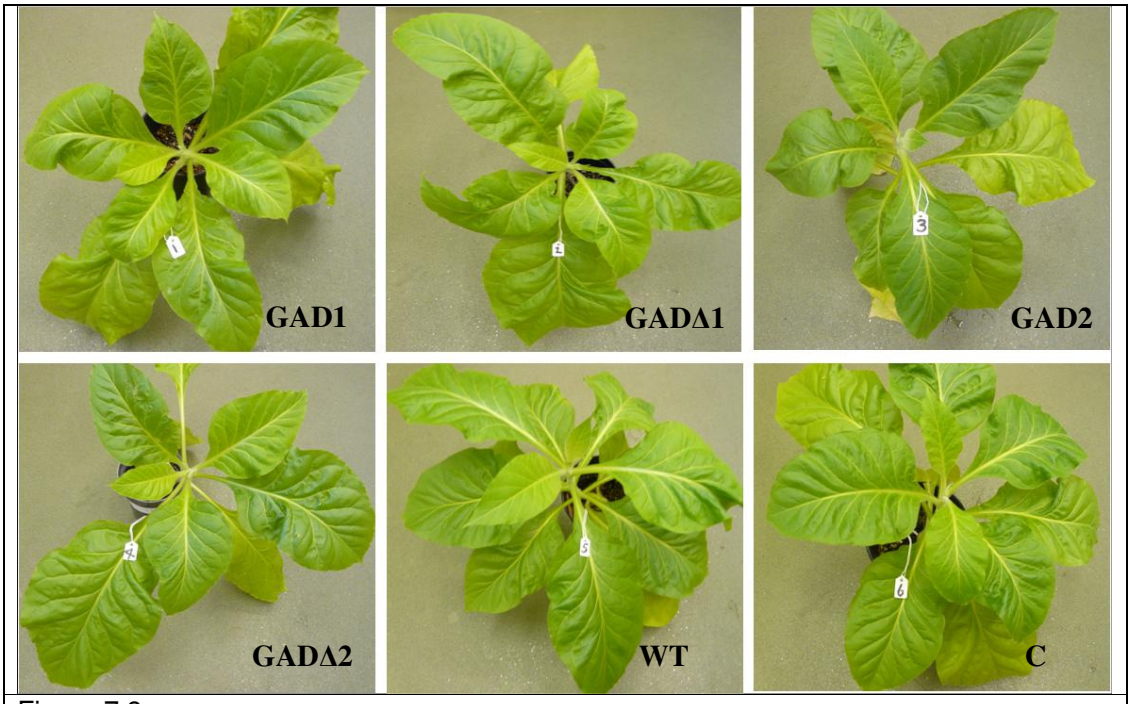


Figure 7.8
Phenotype of GAD and GADΔC plants.
GAD and GADΔC plants are healthy, and indistinguishable in size from the WT and plant-vector control lines.

In order to check that transgenic lines were not compromised in their ability to undergo the hypersensitive response (HR) as part of their defence response against bacterial pathogens, plants were inoculated with the non-host pathogen *P. syringae* pv. tomato DC3000 (P.s.t). After 24 hours all plants had produced a visible HR (Figure 7.9).

No differences in the growth of P.s.tabaci were observed *in planta* at two or five days post inoculation (Figure 7.10), or during 24 hours growth in apoplast extracts from uninfected plants (Figure 7.11). However, transgenic plants, particularly the GADΔC lines, displayed fewer symptoms (necrosis and chlorosis) when infected with P.s.tabaci compared to WT and control plants (Figure 7.12). Photographs in Figure 7.12 were taken at seven days post inoculation when leaves were detached, however differences were apparent in the severity of symptoms between transgenic and WT plants at five days post inoculation.

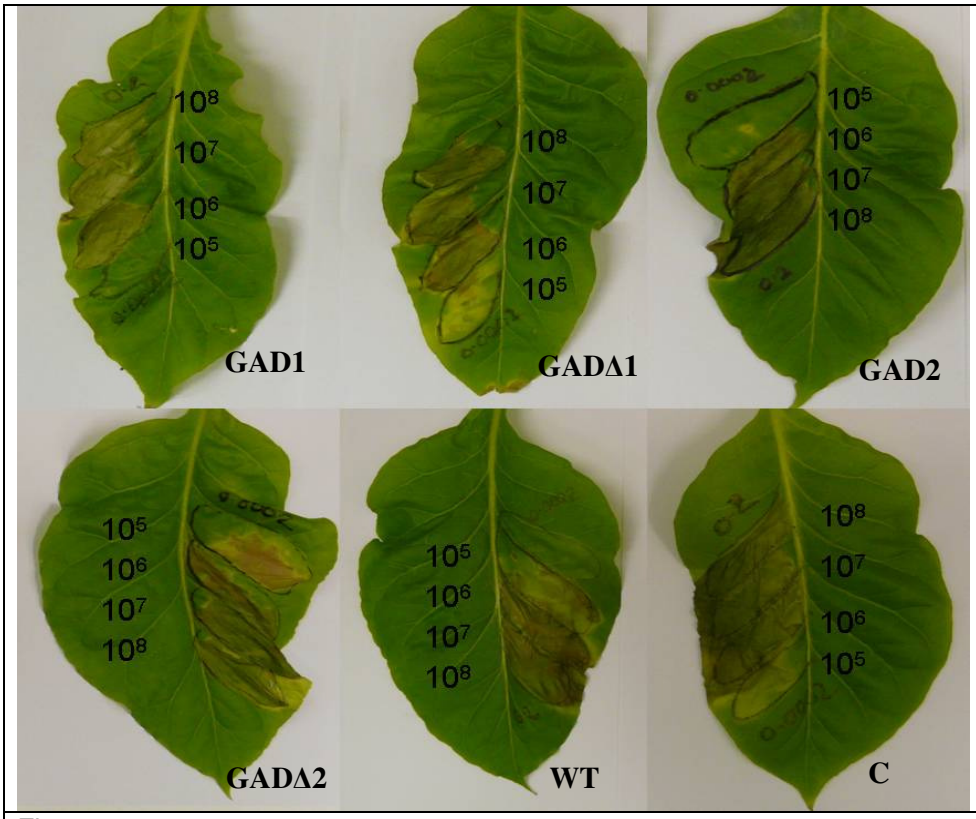


Figure 7.9
Hypersensitive response (HR) competence of tobacco lines. Plants were inoculated with a dilution series of P.s.t at $1x10^8$, $1x10^7$, $1x10^6$ and $1x10^5$ colony forming units (cfu)ml^{-1}. Two independent experiments were carried out with similar results. C= plant-vector control, WT= wild type.

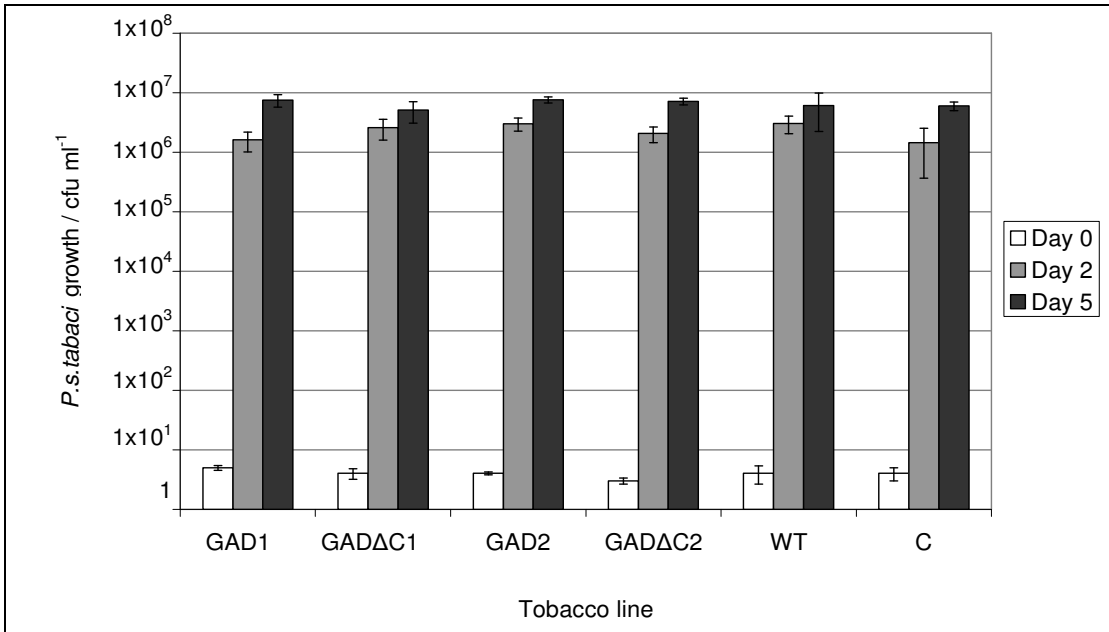


Figure 7.10
Growth of P.s.tabaci *in planta* in the transgenic tobacco lines, wild type and control. Plants were inoculated with $1x10^5$ colony forming units (cfu) ml^{-1} and growth was measured by a leaf disc assay using two replicate plants per transgenic line. Error bars represent the standard deviations of the means. Two independent experiments were carried out with similar results. Differences are not statistically significant at the 95% confidence interval ($p \leq 0.05$) as measured by Student's t-tests.

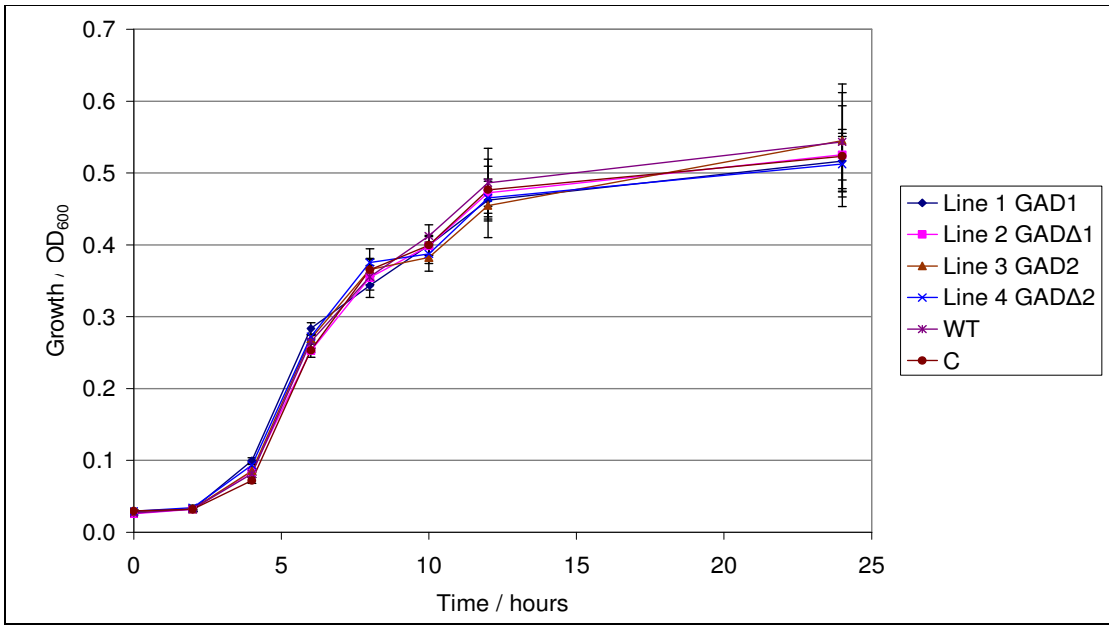


Figure 7.11
Growth of P.s.tabaci in apoplast extracted from uninfected tobacco plants. Error bars represent the standard deviations of the means. Differences at 8, 12 and 24 hours are not statistically significant at the 95% confidence interval ($p \leq 0.05$) as measured by Student's t-tests.


GAD1
GAD2
GADΔC1
GADΔC2

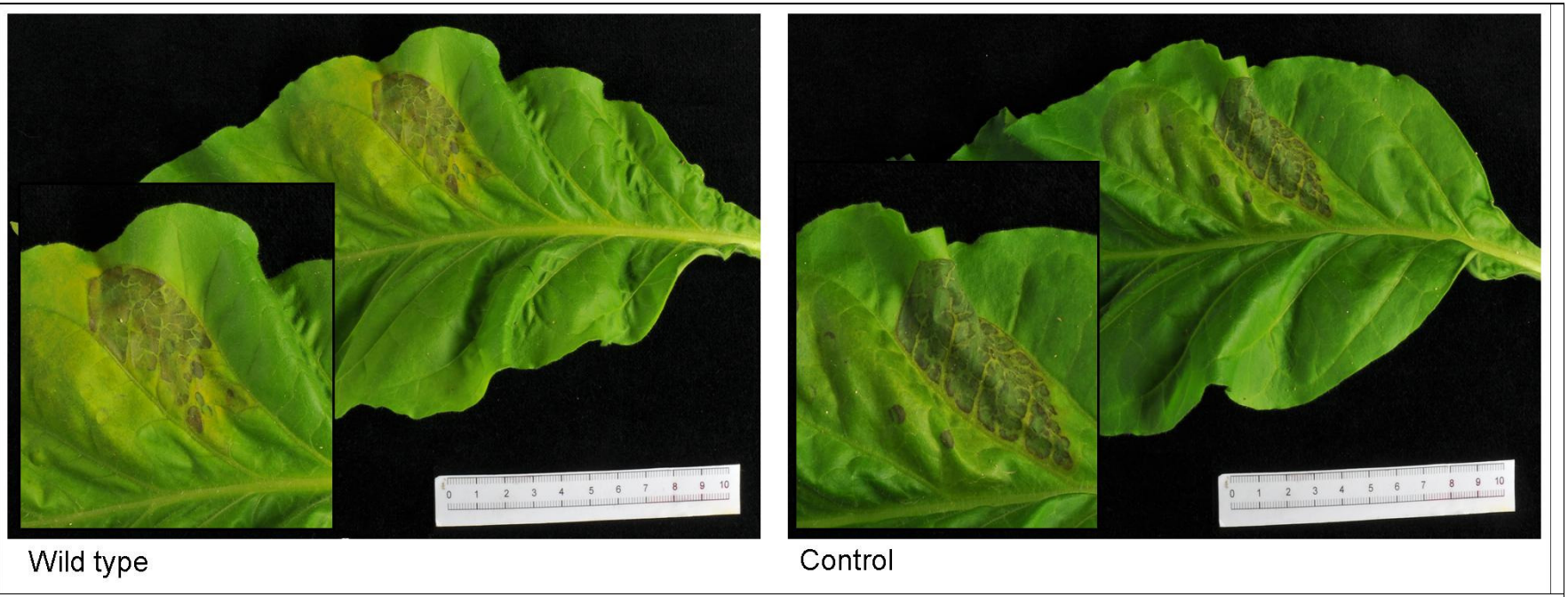


Figure 7.12
Symptoms caused by P.s.tabaci, inoculated at $1x10^5$ colony forming units (cfu)ml^{-1} after 5 days growth *in planta*. A representative leaf is shown, with a closer view of the symptoms, in each picture. Two leaves were inoculated per plant and three independent experiments were carried out with similar results. The same trend was observed after 7 days WT= wild type, C= plant-vector control. For further images see Appendix Figure A7.

A

Score	Disease assessment key
0	No chlorosis or necrosis after 7 days
1	Evidence of chlorosis in the infected area < 10 % necrosis
2	Evidence of chlorosis. 10 - 40 % of infected area is necrotic
3	Evidence of chlorosis. 40 - 90 % of infected area is necrotic
4	Evidence of chlorosis. > 90 % of infected area is necrotic

B

Transgenic line	Average score	p-value (from t-tests comparing each line to the WT)
GADΔC1	1.25	0.002
GAD1	2.25	0.016
GADΔC2	2	0.049
GAD2	2.5	0.034
C	3.5	0.195
WT	3.75	na

Table 7.2
A. A disease assessment key was used to score each replicate leaf for disease severity seven days after inoculation with P.s.tabaci. **B**. These values were then used in Student's t-tests comparing the symptoms of transgenic plants to those of the wild type (WT). There was no significant difference between symptoms in WT and vector control (C) lines. All transgenic GAD lines display significantly reduced symptoms to the WT.

7.5.2 GAD plants produce more callose than WT plants in response to P.s.t.-hrcC.

The deposition of callose is an important component of basal resistance, and an importance determinant for the outcome of a plant–pathogen interaction (Flors et al., 2005). Callose deposition is suppressed by P.s.tabaci effectors during infection of WT plants (Oh and Collmer, 2005). As transgenic GAD plants display reduced symptoms upon infection with P.s.tabaci, it was decided to investigate the strength of the callose response in these lines in comparison to the WT, to four different treatments. *P.*s.tabaci is a virulent tobacco pathogen triggering a compatible reaction which results in disease. P.s.t is avirulent, triggering an incompatible reaction on tobacco characterised by the development of a typical hypersensitive response (HR). P.s.̃thrcC is a *hrp* mutant unable to produce the type three secretion system (T3SS), it triggers an incompatible reaction but no HR. $MgCl_2$ was used as a control treatment. To check the efficacy of callose staining, leaf discs were examined by eye for areas which had been damaged by the blunt syringe during inoculation. These areas were found to contain stained callose deposits (Appendix Figure A2).

When inoculated with P.s.̃thrcC, GADΔC1 and GADΔC2 lines produced significantly more callose in comparison to WT plants. The WT and plant-vector control lines did not differ significantly in their response to any of the treatments (p=0.112). GAD and WT plants showed no differences in their callose response to inoculation with P.s.tabaci, or the $MgCl_2$ control treatments. When inoculated with P.s.t, GAD1, GADΔC1 and GADΔC2

lines produced significantly more callose in comparison to WT plants. This significant result was observed only in one experiment but a similar, non-significant trend was observed in a second independent experiment (Figure 7.13, 7.14).

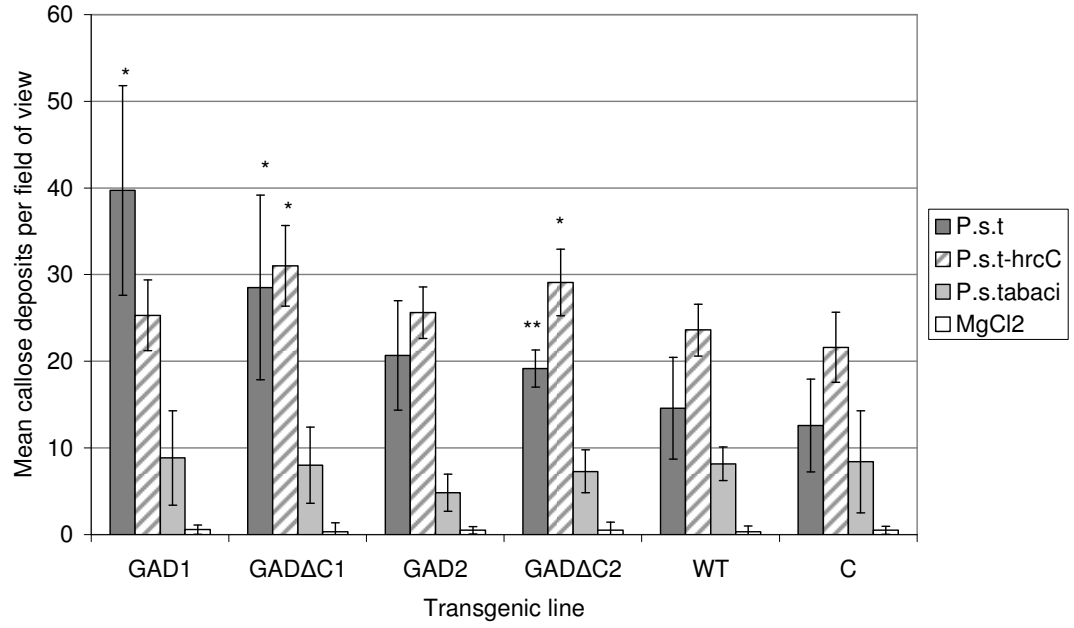


Figure 7.13
Callose response from all transgenic lines 24 hours after inoculation with P.s.t, P.s.tabaci, P.s.t-hrcC or $MgCl_2$. GADΔC1 and GADΔC2 plants produced significantly more callose in response to P.s.t-hrcC. GAD1, GADΔC1 and GADΔC2 produced significantly more callose in response to the P.s.t. Error bars represent the standard deviations of the means. Data from 36 fields of view was analysed per treatment and the average is presented above. Two independent experiments were carried out. * = result was significantly different from the equivalent treatment in wild type plants ($p \leq 0.05$). **=$p \leq 0.1$ Wild type and plant vector control plants did not differ significantly in their response to any treatment.

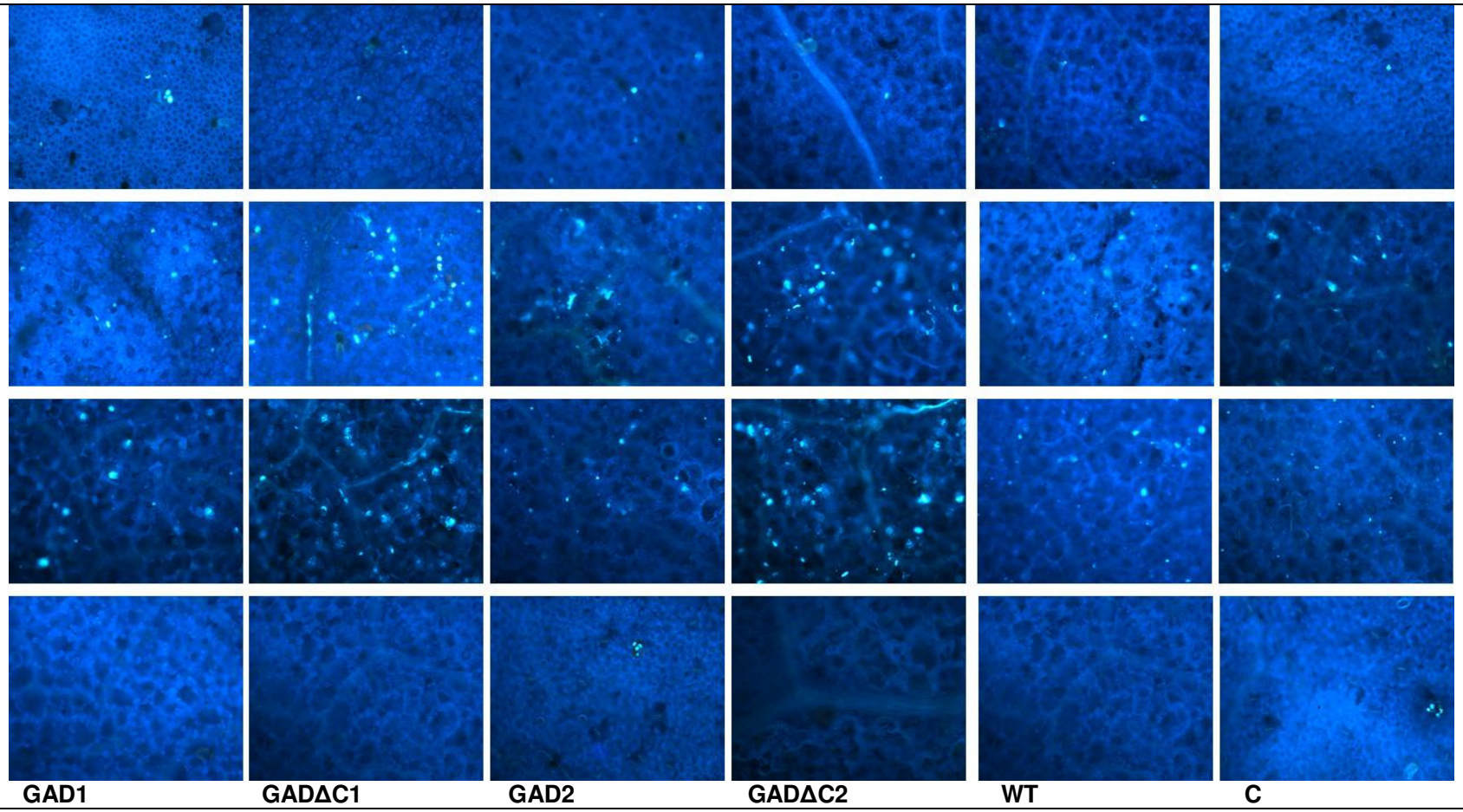


Figure 7.14
Callose deposits in GAD, WT, and plant-vector control (C) plants at 24 hours in response to infiltration with (1) $1x10^5$ cfu / ml^{-1} P.s.tabaci, (2) $1x10^5$ cfu / ml^{-1} P.s.t, (3) $1x10^7$ cfu / ml^{-1} P.s.t-hrcC, (4)10 mM $MgCl_2$

7.5.3 GAD plants do not differ in H_2O_2 production in response to P.s.tabaci and P.s.t.

Plant defence responses are accompanied by the accumulation of active oxygen species including hydrogen peroxide (H_2O_2). H_2O_2 production can be monitored histochemically with diaminobenzidine (DAB) which forms a brown precipitate after oxidation by H_2O_2.

There was not a significant difference in the amount of H_2O_2 produced one, two or six hours post inoculation with the virulent pathogen P.s.tabaci, the hypersensitive response (HR)-inducing avirulent P.s.t, or the HR-negative mutant P.s.t-hrcC (Figure 7.15).

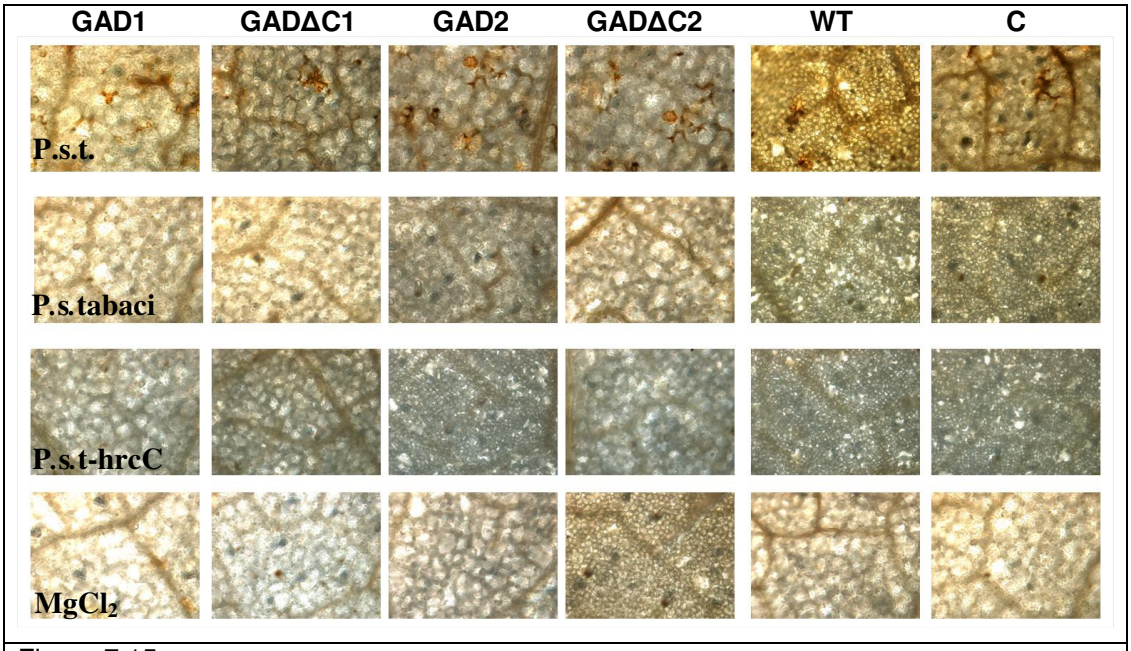


Figure 7.15
Representative pictures of DAB staining one hour after inoculation with $1x10^5$ cfu / ml^{-1} P.s.t, $1x10^5$ cfu / ml^{-1} P.s.tabaci, $1x10^7$ cfu / ml^{-1} P.s.t-hrcC or 10 mM $MgCl_2$. Pictures are representative of 20 fields of view and is representative of two independent experiments

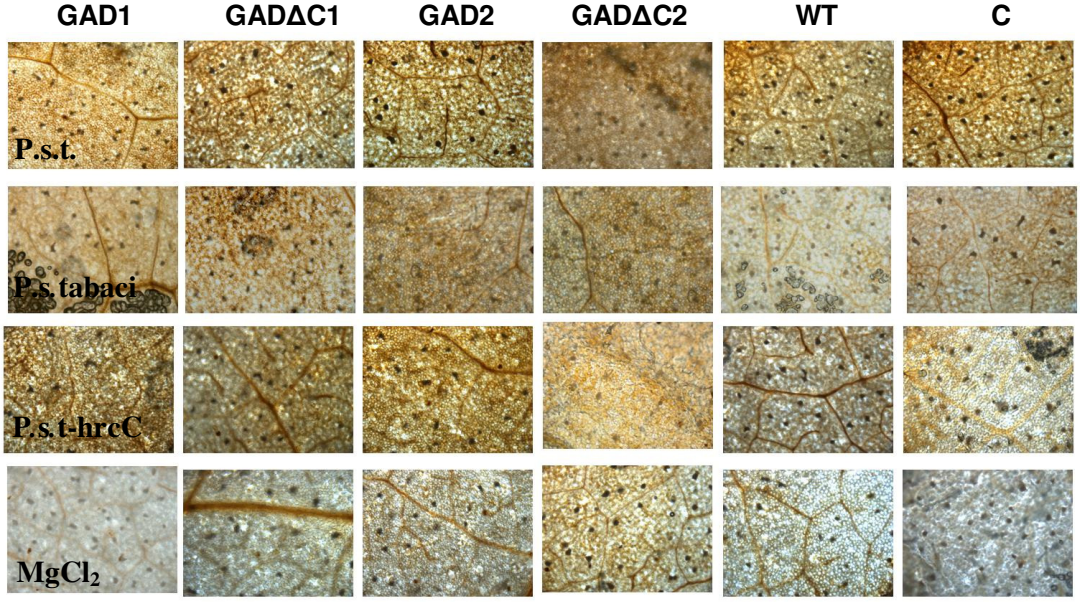


Figure 7.16
Representative pictures of DAB staining two hours after inoculation with $1x10^5$ cfu / ml^{-1} P.s.t, $1x10^5$ cfu / ml^{-1} P.s.tabaci, $1x10^7$ cfu / ml^{-1} P.s.t-hrcC or 10 mM $MgCl_2$. Pictures are representative of 20 fields of view and is representative of two independent experiments

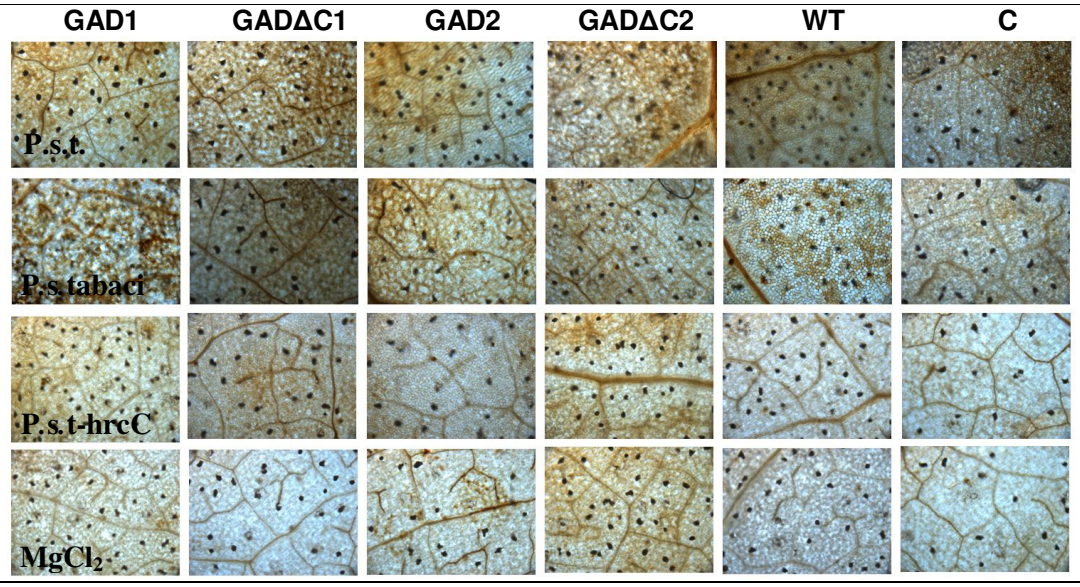


Figure 7.17
Representative pictures of DAB staining six hours after inoculation with $1x10^5$ cfu / ml^{-1} P.s.t, $1x10^5$ cfu / ml^{-1} P.s.tabaci, $1x10^7$ cfu / ml^{-1} P.s.t-hrcC or 10 mM $MgCl_2$. Pictures are representative of 20 fields of view and is representative of two independent experiments

7.5.4 GAD plants may produce an earlier superoxide response than wild type plants.

In addition to H_2O_2, a second type or reactive oxygen species can be detected histochemically. Superoxide (O_2^-) production can be monitored using nitroblue tetrazolium (NBT) which forms a blue precipitate when reduced to formazan (Fryer et al., 2002).

Previous work has indicated that O_2^- production peaks within two hours following the application of treatment stress (Fryer et al 2002; Helen Fones, Oxford University, unpublished data). Therefore leaf discs were removed at one and three hours post inoculation with P.s.t, P.s.tabaci, P.s.t-hrcC or $MgCl_2$. Results from this experiment were inconclusive, as one replicate experiment did not indicate any difference between transgenic lines. However, results of a second replicate indicate GAD plants may be able to mount a superoxide response to P.s.tabaci earlier than wild type plants. Further experiments may be able to confirm this observation.

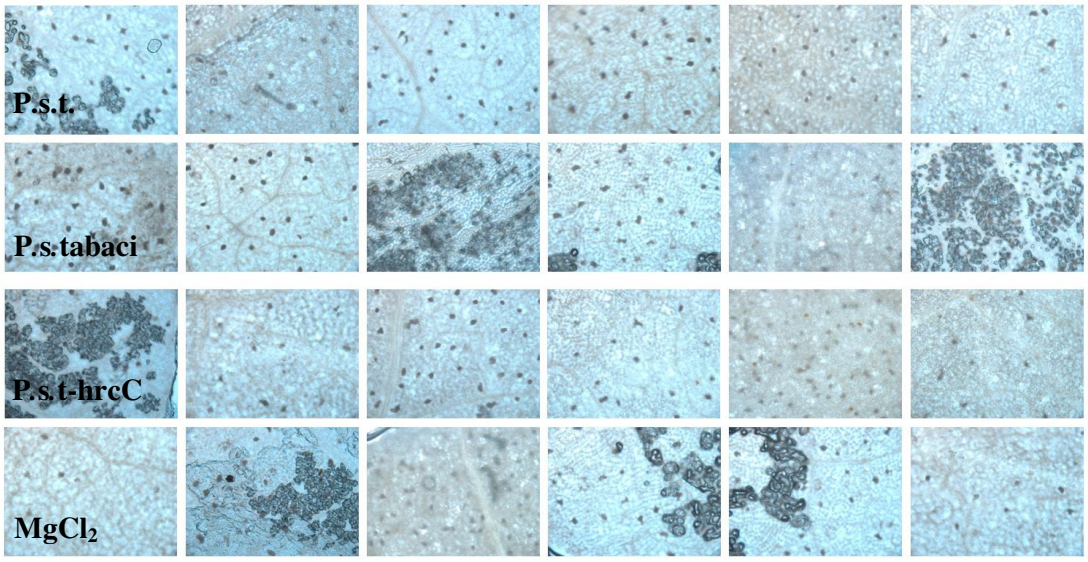


Figure 7.18
Representative pictures of NBT staining one hour after inoculation with $1x10^5$ cfu / ml^{-1} P.s.t, $1x10^5$ cfu / ml^{-1} P.s.tabaci, $1x10^7$ cfu / ml^{-1} P.s.t-hrcC or 10 mM $MgCl_2$. Pictures are representative of 36 fields of view and is representative of two independent experiments. Leaf discs from GAD and GADΔC lines appeared to show darker staining one hour after inoculation with P.s.tabaci. However, this was only observed in one out of two replicate experiments.

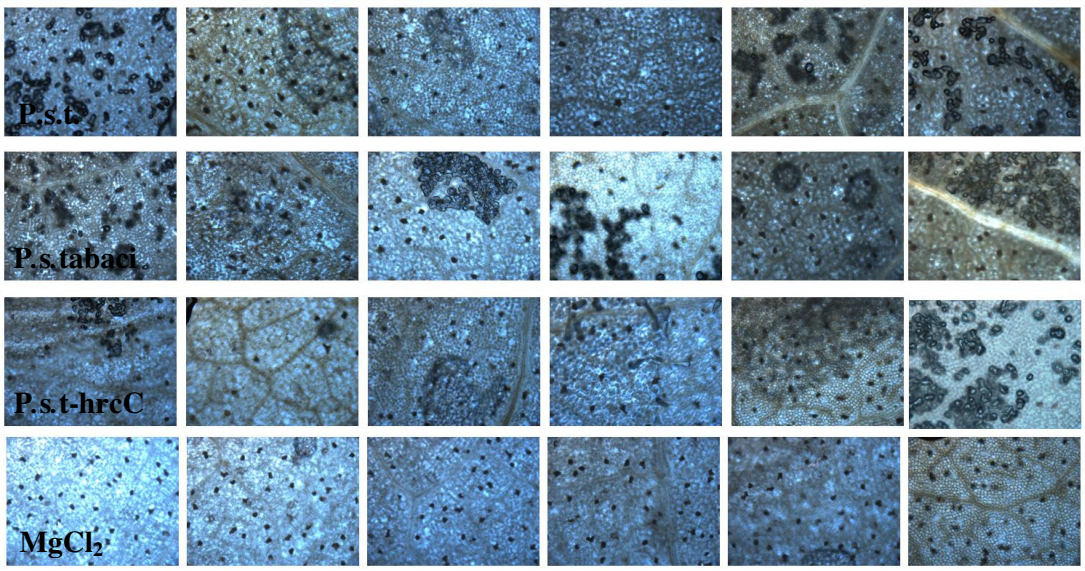
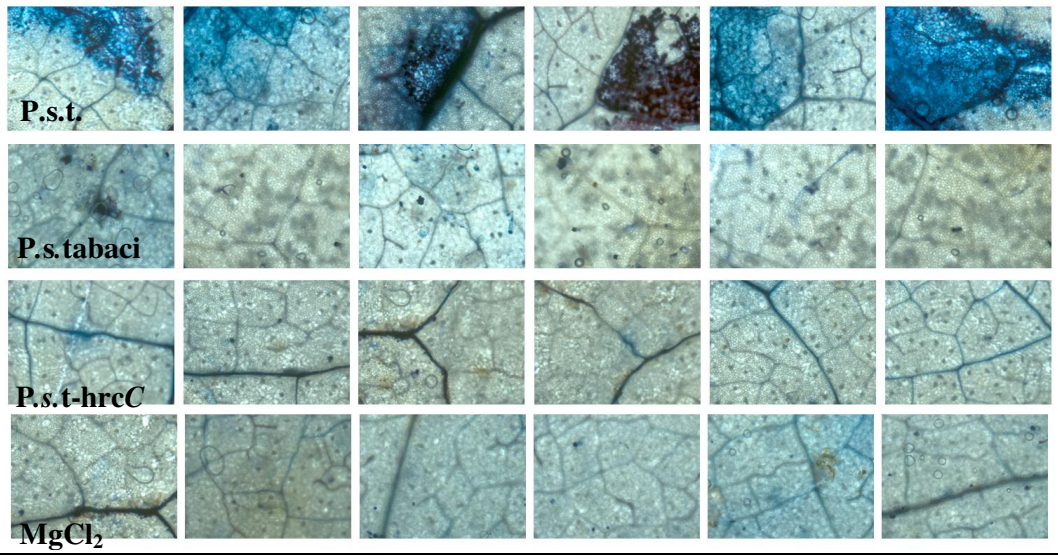


Figure 7.19
Representative pictures of NBT staining three hours after inoculation with $1x10^5$ cfu / ml^{-1} P.s.t, $1x10^5$ cfu / ml^{-1} P.s.tabaci, $1x10^7$ cfu / ml^{-1} P.s.t-hrcC or 10 mM $MgCl_2$. Pictures are representative of 36 fields of view and is representative of two independent experiments

7.5.5 Trypan blue staining for reactive oxygen species.

The generation of ROS such as H_2O_2 and O_2^- is expected to be closely related to the occurrence of cell death (Mehdy, 1994). For this reason, histochemical staining methods for the detection of cell death were performed. Trypan blue staining revealed extensive cell death in all tobacco lines 24 hours post-inoculation with P.s.t. Little cell death was detected after inoculation with P.s.tabaci, P.s.t-hrcC or $MgCl_2$. The differences in the area of leaf stained by trypan blue were not statistically significant (Figure 7.20). However, there is an indication that P.s.t inoculation in transgenic lines may result is less cell death compared to WT, which would be in keeping with the observation that some transgenic lines produce more callose in response to P.s.t or P.s.t-hrcC.



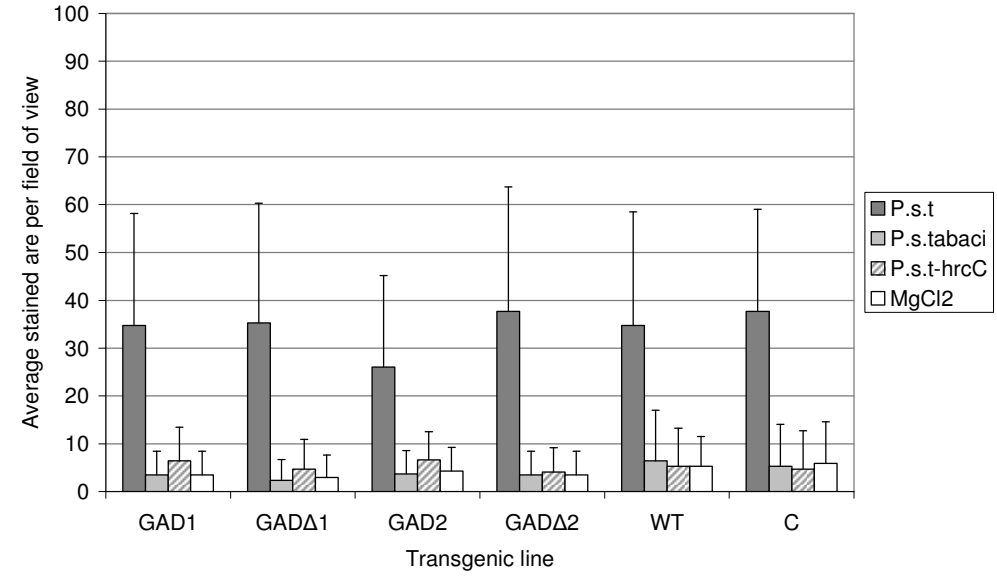


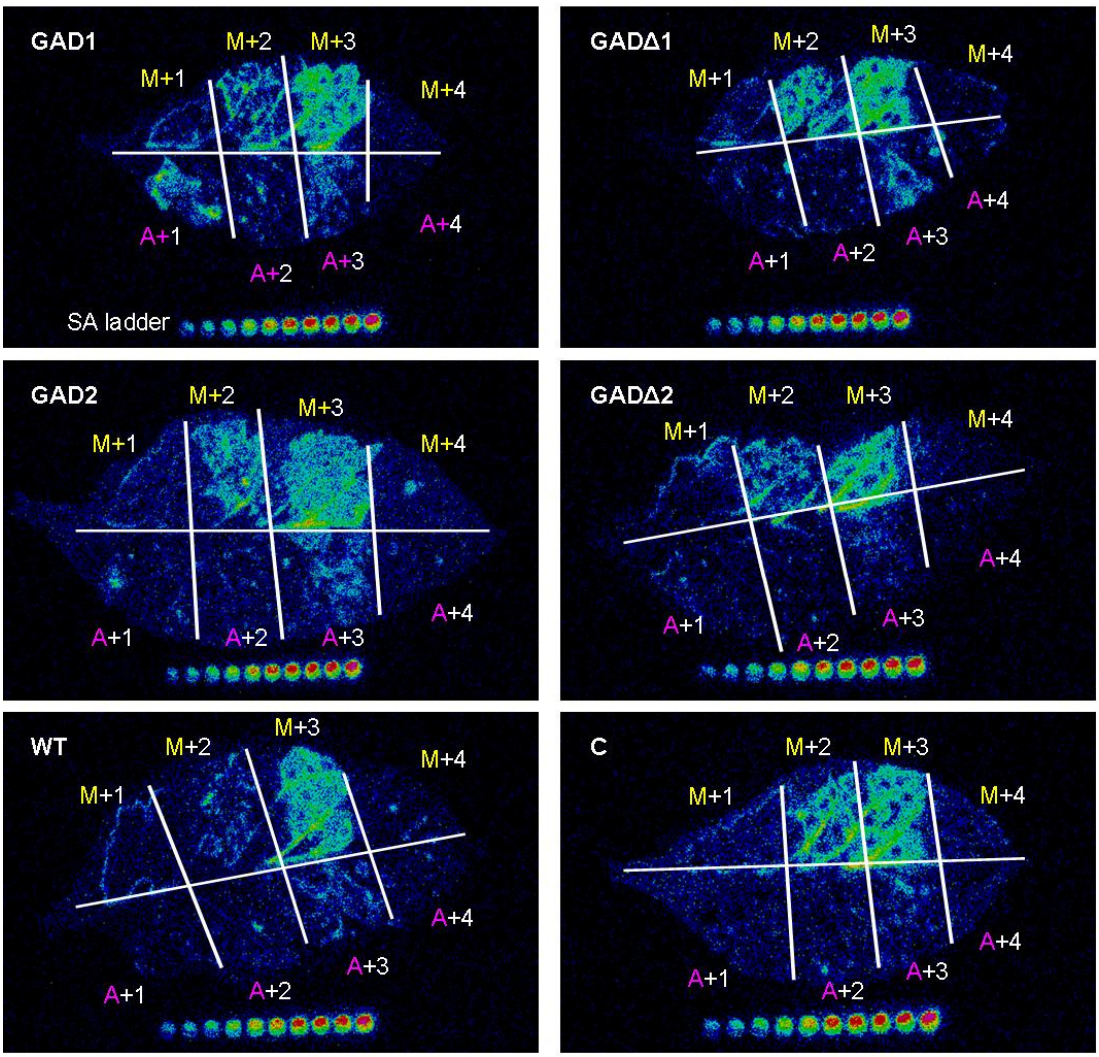
Figure 7.20
Representative pictures of trypan blue staining three hours after inoculation with $1x10^5$ cfu / ml^{-1} P.s.t, $1x10^5$ cfu / ml^{-1} P.s.tabaci, $1x10^7$ cfu / ml^{-1} P.s.t-hrcC or 10 mM $MgCl_2$. Data represents the average of 36 fields of view from one experiment.

7.5.6 Transgenic plants do not produce significantly more salicylic acid in response to pathogen inoculation.

Salicylic acid (SA) is known to accumulate in plants in response to pathogen attack (10 to 50-fold over background levels), and is essential to the establishment of both local and systemic-acquired resistance (Cameron et al., 1999). As SA and callose deposition pathways are known to be inversely regulated (Nishimura et al., 2003), and previous results had suggested that GADΔC lines were capable of producing increased callose response compared to wild type plants (Figure 7.14), the strength of the SA response in transgenic and WT plants was compared. This was investigated using the *A. tumefaciens*

mutant AtGV3101, which is non-pathogenic but capable of eliciting a basal defence response. AtGV3101 'primes' basal defences in tobacco and has been shown to reduce the strength of the SA response to subsequent pathogen inoculation, compared to a pre-treatment of $MgCl_2$ (Rico and Preston, personal communication).

Figure 7.21 shows representative images from each of the four transgenic tobacco lines. When plants were pre-treated with AtGV3101, the SA response by all tobacco lines was statistically lower than when pre-treated with $MgCl_2$. This result supported the previous findings by Rico and Preston, and indicated that the ability of AtGV3101 to 'prime' basal defences was not altered in the transgenic lines. The amount of SA produced in response to each treatment did not statistically differ between tobacco lines (Appendix Figure A4). This result was interesting as previous data had indicated that P.s.t, particularly a *hrcC* mutant, provoked an increased callose response in GADΔC lines compared to the wild type and therefore we might expect to see reduced SA in these lines following inoculation with these strains. Therefore the present result suggests that priming plants with *A. tumefaciens* may affect the ability of the transgenic lines to produce a callose response to P.s.t-hrcC. This could be investigated in further experiments.



M = $MgCl_2$ pre-treatment, **A** = *A.tumefaciens* pre-treatment,

plus treatments: **1** = P.s.t-hrcC, **2** = *P.s.tabaci*, **3** = P.s.t, **4** = MgCl2

Figure 7.21 (continued overleaf)
SA production by transgenic plants, wild type (WT) and plant-vector control (C).
Initially leaves were inoculated with either *A.tumefaciens* AtGV3101 or $MgCl_2$, on day two,
Figure 7.21 continued
plants were inoculated with P.s.t, P.s.t-hrcC, P.s.tabaci or $MgCl_2$. On day three the SA biosensor ADPWH_*lux* was infiltrated into leaves and images were taken after one hour using a photon-counting camera (see Materials and methods). Images are representative of three leaves per plant, from two replicate plants, per tobacco line.

7.5.7 PR1α gene expression is similar in all plants in response to P.s.tabaci and P.s.t inoculation.

Increases in the expression of genes encoding the pathogenesis-related (PR) proteins coincide with the activation of disease resistance in pathogen-inoculated plants (Flors et al., 2005). Transgenic GAD and GADΔC and WT tobacco plants show differences in the severity of symptoms when infected with P.s.tabaci. In order to investigate disease resistance in these plants, the expression of the PR gene PR1α was measured in each line.

RTPCR analysis of RNA extracted from whole leaf samples 24 hours after inoculation with P.s.tabaci showed no significant difference in the amount of PR1α gene expression. RNA extracted from whole leaf samples 24 hours after inoculation with P.s.t.-hrcC showed that PR1α gene expression was not constitutively higher in transgenic plants (Figure 7.22). These results are consistent with previous observations that there was no difference in the SA response to these bacteria in transgenic lines.

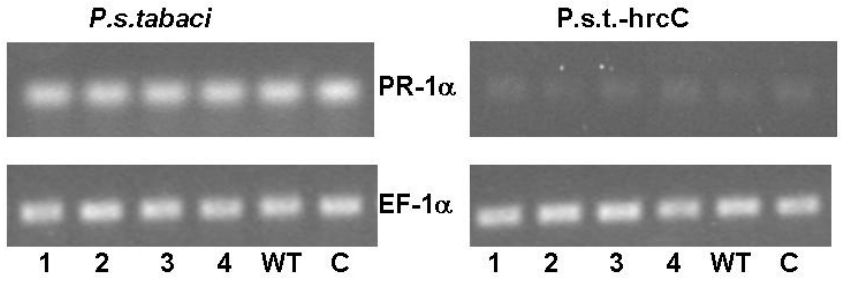


Figure 7.22
PR1α expression in tobacco plants 24 hr after inoculation with 10^5 cfuml^{-1} P.s.tabaci (left) or 10^7 cfuml^{-1} P.s.t-hrcC (right).
1=GAD1, 2=GADΔ1, 3=GAD2, 4=GADΔ2, WT = wild type, C = plant-vector control. The housekeeping gene elongation factor (EF-1□) was provided as a control. Images are representative from two independent replicates with similar results

7.5.8 The apoplast of tobacco and tomato leaves contain similar amino acids.

The amino acid content of the tobacco apoplast has not been previously reported. HPLC analysis was carried out on apoplast extracted from healthy, uninfected wild type plants and compared to the amino acid content of the tomato apoplast, previously reported by Rico and Preston (2008).

The abundant amino acids detected in the wild type (WT) tobacco apoplast by HPLC analysis were GABA, serine, valine, glutamine, glutamate, asparagine, aspartate and alanine + arginine. In this system alanine and arginine elute together from the HPLC column giving rise to a single 'ala + arg' peak. Small amounts of phenylalanine, isoleucine, tyrosine and leucine, glycine and threonine were also detected in all samples. These amino acids did not appear to vary between $MgCl_2$ and P.s.tabaci-treated plants. As the peaks were small the HPLC analysis programme failed to automatically detect them, therefore the decision was made to exclude these amino acids from the analysis. Results were presented as the percentage that each amino acid contributes to the 'total measured amino acids' i.e. the eight amino acids consistently detected in HPLC samples (for the purpose of analysis alanine and arginine are considered a together as only a single value of abundance is available).

Table 7.3 lists the amino acids detected in WT tobacco leaf apoplast samples, in order of abundance, alongside those which are present in the apoplast of tomato leaves. In the tomato apoplast, the nine most abundant amino acids constitute 87% of the total amino acids detected (data in Appendix Figure A1). All of these amino acids were found in the tobacco apoplast. Similarly the most abundant amino acids in the tobacco apoplast; GABA, aspartate, glutamate, serine and glutamine, were also abundant in tomato leaf apoplast.

Samples of apoplast from each tobacco line have been sent to the MeT-RO facility in Rothampstead for NMR analysis. This will allow quantification of the amino acids present in apoplast from each transgenic line, and also should identify all major compounds present in apoplast from tobacco plants.

	Amino acid	Tomato apoplast	Tobacco apoplast		Amino acid	Tomato apoplast	Tobacco apoplast
1	GABA	+	+	12	Thr	+	+
2	Asp	+	+	13	Ile	+	+
3	Glu	+	+	14	Lys	+	na
4	Ala	+	+	15	Tyr	+	+
5	Ser	+	+	16	Pro	+	na
6	Gln	+	+	17	Asn	+	+
7	Leu	+	+	18	His	+	na
8	Gly	+	+	19	Arg *	nd	+
9	Phe	+	+	20	Cys	na	na
10	Met	+	na	21	Tryp	na	na
11	Val	+	+				

Table 7.3
A comparison of the amino acids detected in WT tobacco and WT tomato (tomato data reproduced from Rico and Preston 2008). The amino acids are listed in their order of abundance in the tomato leaf apoplast. Data for the tobacco apoplast is from two independent experiments which produced similar results. += detected by HPLC, nd=not detected, na=not analysed. *Arginine was detected independently from alanine in the tomato apoplast.

7.5.9 GABA is the most abundant amino acid in the both the tobacco and tomato leaf apoplast.

GABA was found to be the most abundant amino acid in both the uninfected wild type (WT) tobacco, and WT tomato apoplast. Raw HPLC data describing the amino acids present in the tomato apoplast (provided by Arantza Rico, Oxford) was re-analysed in the same way as the tobacco data, by calculating the percentage that each amino acid contributes to the total of the measured amino acids. There was proportionately more GABA in the WT tomato apoplast than WT tobacco (Table 7.4).

Aspartate and glutamate made up similar proportions of the measured amino acids contributing 13.6% and 12.9% in tomato apoplast, and 14.5%, and 10.3% in tobacco apoplast respectively. Glutamine, valine and asparagine were the least abundant of the measured amino acids in both species. The major difference between the tomato and tobacco apoplast was in the relative proportion of serine, which was three times higher in the tobacco leaf apoplast. There was also a difference in the relative proportion of alanine in tomato compared to the alanine + arginine peak in tobacco, however these particular peaks were not measured in the same way, due to being analysed on a more sensitive HPLC machine in the case of the tomato apoplast data. Therefore, it was difficult to compare data directly between the two species. It is worth mentioning however, that even if arginine is not present in the tobacco apoplast, the difference between the samples

would still remain, because the measured peak in the tobacco apoplast would consist only of alanine.

WT tomato leaf apoplast	% of measured amino acids	WT tobacco leaf apoplast	% of measured amino acids
GABA	55.3 ± 2.35	GABA	36.4 ± 2.36
Asp	13.6 ± 2.10	Ser	15.9 ± 0.06
Glu	12.9 ± 2.23	Asp	14.5 ± 1.65
Ala*	6.4 ± 0.63	Ala+Arg	12.9 ± 1.39
Ser	4.9 ± 1.20	Glu	10.3 ± 2.36
Gln	3.3 ± 1.10	Gln	4.3 ± 0.23
Val	2.4 ± 0.49	Val	3.1 ± 1.04
Asn	0.9 ± 0.14	Asn	2.26 ± 0.07

Table 7.4
A comparison between the relative abundance of the eight amino acids detected in uninfected tobacco leaf apoplast and corresponding data for the uninfected tomato leaf apoplast (± standard deviations). Data for the amino acid abundance in the tomato leaf apoplast was provided by Arantza Rico (Oxford University). Each of the eight amino acids is presented as a percentage of those eight amino acids. Error bars are the standard deviations of three independent replicates for the tomato leaf apoplast, and two independent replicates for the tobacco leaf apoplast. *HPLC analysis of the tomato leaf apoplast was carried out in the Department of Biochemistry, Oxford University, here it was possible to separate the alanine and arginine peaks and ascertain that only alanine was present.

Interestingly GABA does not appear to be the most abundant amino acid in all plant species. The major amino acids in healthy *A. thaliana* apoplast were found to be proline, glutamine, serine, alanine and aspartate, while GABA was not detected (Guillaume Pilot, Personal communication). In addition, GABA was the 9th most abundant amino acid in the apoplast of *Vivia faba* (Struck et al., 2004), and the 6th most abundant in the apoplast of sugar beet (Lopez-Millan et al., 2000) (see also, Chapter 1, Table 1.3).

7.5.10 GABA is more abundant in the apoplast of GAD and GADΔC plants than in wild type plants.

Whole leaf tissue analysis of transgenic GAD and GADΔC lines previously demonstrated significantly increased levels of GABA in root and shoot tissue in these lines compared to wild type plants (McLean et al., 2003). However, as the site of GABA synthesis is the cytosol, it is not known whether an increase in cytosolic GABA abundance is accompanied by a concurrent increase in the apoplast environment inhabited by P.s.tabaci. Therefore, HPLC analysis was carried out on apoplast extracted from healthy, uninfected plants from GAD and GADΔC lines, wild type and plant vector control plants.

Analysis of HPLC data from $MgCl_2$ treated tobacco apoplast revealed that the abundance of glutamine, asparagine and serine was significantly lower in the apoplast of transgenic lines compared to the WT. In contrast, the abundance of GABA was significantly higher in all transgenic lines, particularly in GAD1 and GADΔ1 where it accounted for over 50% of the measured amino acids (Figure 7.23). These two lines also had significantly lower glutamine and glutamate than WT plants, which lead to the suggestion that more of these amino acids are being converted to GABA by the overexpressed GAD, and this was providing the higher GABA levels observed in these lines.

The plant-vector control line had significantly different proportions of asparagine, aspartate and glutamate compared to the WT. However the abundance of GABA in particular was not significantly different compared to the WT (Figure 7.23).

7.5.11 The GABA content of the GAD and GADΔC apoplast significantly decreases when treated with P.s.tabaci.

The abundance of GABA in the apoplast of GAD and wild type (WT) plants following infection by P.s.tabaci was investigated and compared to the observed abundance following the $MgCl_2$ control treatment (Section 7.5.10, above) In Figure 7.25 it can be seen that the abundance GABA in the WT apoplast was similar in plants treated with $MgCl_2$ and P.s.tabaci. This result was also observed in healthy tomato apoplast before and after infection with *P. syringae* pv. tomato DC3000 (Arantza Rico, personal communication).

However, the GABA abundance in the apoplast of transgenic lines was significantly lower after treatment with P.s.tabaci, compared to treatment with $MgCl_2$ (Figure 7.25). One hypothesis is that this result may be due to the action of the phytotoxin, tabtoxin, produced by P.s.tabaci. Tabtoxin inhibits glutamine synthetase, reducing the synthesis of

glutamine, a precursor of GABA (Chapter 1, Section 1.4.3). As transgenic lines synthesise more GABA, they require increased GABA precursors and therefore the effect of tabtoxin may be increased in these lines compared to WT plants.

When the amino acid abundance was compared between $MgCl_2$-treated and P.s.tabaci-treated plants, for each tobacco line there were several amino acids in addition to GABA which differed significantly between the two treatments. It was also possible to identify general trends for the abundance of each amino acid in the two treatments, even though these differences may not be statistically significant (Figure 7.24). The abundance of serine was higher in GAD plants treated with P.s.tabaci, compared to $MgCl_2$. This trend was present in all GAD lines and was high enough to be significant in GAD2 and GADΔ1. In contrast, the WT apoplast contained a significantly lower abundance of serine in plants treated with P.s.tabaci. Similarly, all GAD lines showed a trend for lower GABA in plants treated with P.s.tabaci, compared to $MgCl_2$, this trend was significant in GAD2 and GADΔ1 plants and again contrasts with the abundance in WT plants where there was no significant alteration in GABA abundance.

Figure 7.25 and Table 7.5 summarise the changes in amino acid abundance when plants are treated with P.s.tabaci or $MgCl_2$. In general WT plants have a lower abundance of the measured amino acids after infection with P.s.tabaci, although the abundance of GABA increases slightly. In contrast, transgenic plants have a higher abundance of many amino acids in P.s.tabaci-treated apoplast and a lower abundance of GABA.

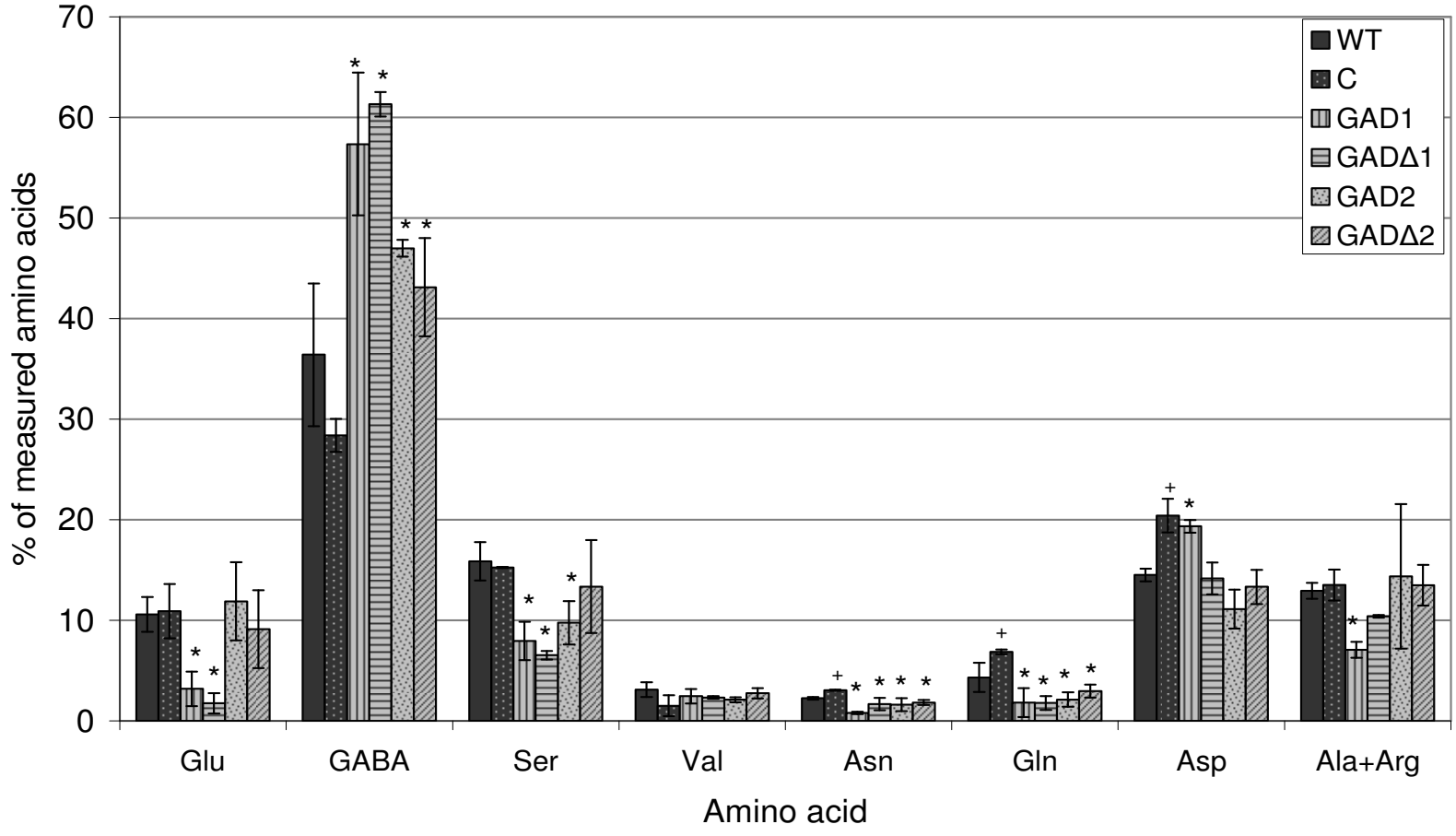


Figure 7.23

The abundance of eight amino acids in $MgCl_2$–treated tobacco plants. Apoplast samples were taken 24 hours after $MgCl_2$–treatment. Student's t-tests were applied to the data, comparing each amino acid within each transgenic line with the abundance of that amino acid in the WT.

* = GAD line is significantly different to the WT ($p \leq 0.05$) += Plant-vector control line is significantly different to the WT ($p \leq 0.05$). Data is averaged from two independent experiments. Error bars represent standard deviations.

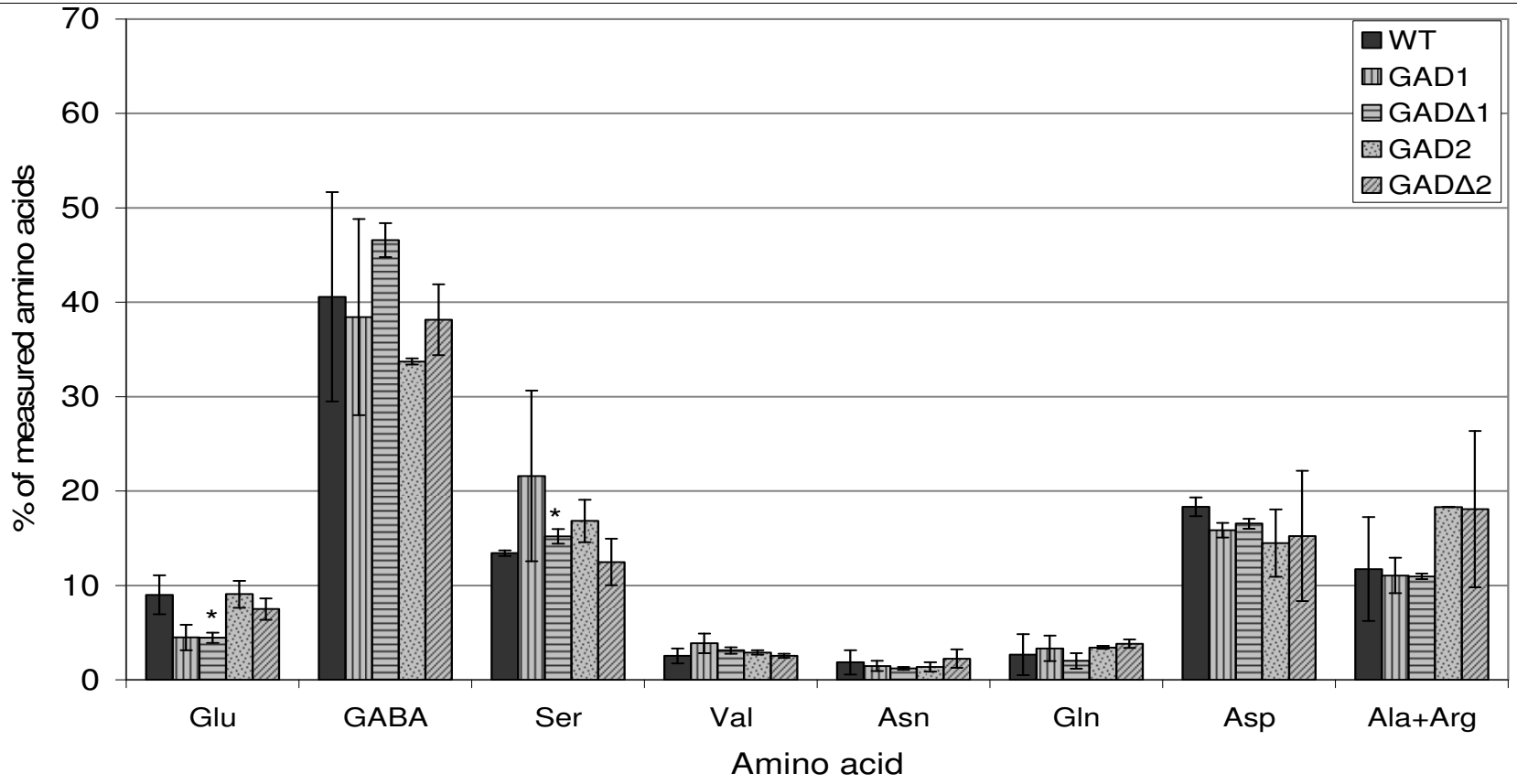


Figure 7.24
The abundance of eight amino acids in P.s.tabaci-treated tobacco plants. Apoplast samples were taken 24 hours after P.s.tabaci treatment. Student's t-tests were applied to the data, comparing each amino acid within each GAD line with the abundance of that amino acid in the WT. *=GAD line was significantly different to the WT ($p \leq 0.05$). Data was averaged from two independent experiments. Error bars represent standard deviations.

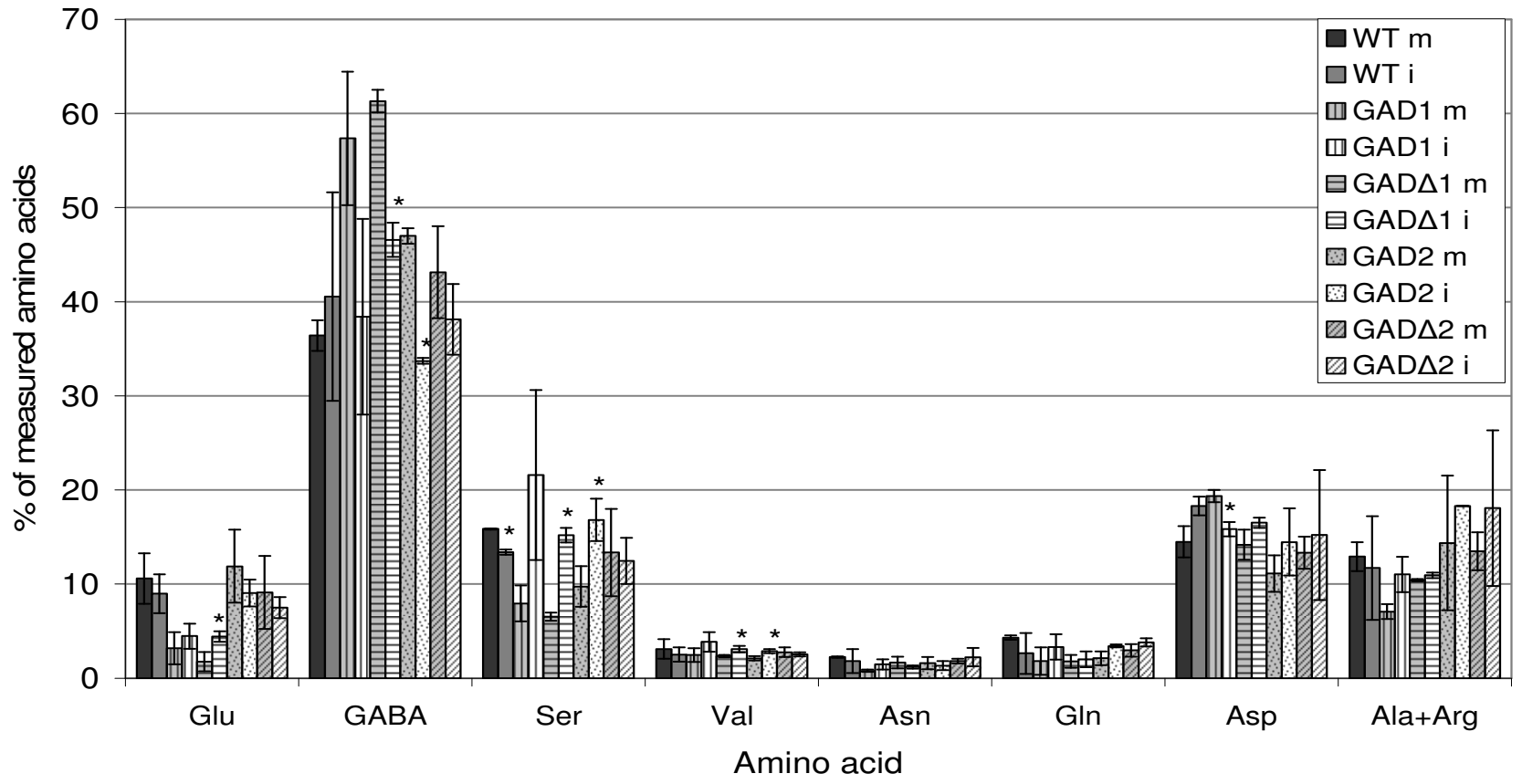


Figure 7.25
A comparison showing the abundance of each measured amino acid in $MgCl_2$-treated, and P.s.tabaci-treated plants. i= apoplast from P.s.tabaci-treated (infected) plants, m= apoplast from $MgCl_2$-treated plants. *=Abundance was significantly different between $MgCl_2$ and P.s.tabaci treatments ($p \leq 0.05$). Data was averaged from two independent experiments. Error bars represent standard deviations.

Measured amino acid	WT		GAD1		GADΔ1		GAD2		GADΔ2		General trend?
	p-values										
Glu	0.302	m	0.245	i	0.041	i	0.421	m	0.314	m	
GABA	0.327	i	0.118	m	0.005	m	0.001	m	0.185	m	yes
Ser	0.004	m	0.086	i	0.003	i	0.043	i	0.416	i	yes
Val	0.295	m	0.129	i	0.048	i	0.042	i	0.314	i	yes
Asn	0.342	m	0.113	i	0.207	m	0.335	m	0.314	m	
Gln	0.196	m	0.197	i	0.400	i	0.067	i	0.136	i	yes
Asp	0.054	i	0.019	m	0.092	i	0.182	i	0.371	i	
Ala+Arg	0.396	m	0.055	i	0.071	i	0.260	i	0.263	i	yes

Table 7.5
Differences in amino acid abundance between $MgCl_2$-treated and P.s.tabaci-treated plants. p-values of Student's t-tests detailing which amino acids differed significantly in their abundance at 24 hours in $MgCl_2$-treated plants, compared to P.s.tabaci-treated plants (significant values where $p \leq 0.05$ are coloured yellow). The treatment with the highest amino acid abundance is indicated as follows: i=amino acid was higher in *P.s.*tabaci-treated (infected) plants, m=amino acid was higher in $MgCl_2$-treated plants. General trends for an amino acid to be higher in $MgCl_2$ or P.s.tabaci treatments are highlighted in pink and blue respectively.

7.6 Discussion.

This chapter describes the response of transgenic plants overexpressing either a full length GAD enzyme or a truncated GAD (GAD and GADΔC plants respectively) to infection by the tobacco pathogen *P. syringae* pv. tabaci 11528 (P.s.tabaci). In addition it describes the abundance of eight amino acids: GABA, glutamate, glutamine, asparagine, aspartate, serine, valine and alanine/arginine, in the apoplast of uninfected wild type (WT), GAD and GADΔC plants, and the biological and metabolic response of these plants to P.s.tabaci infection compared to a $MgCl_2$ control treatment. Interestingly, although GAD and GADΔC plants did not significantly inhibit the growth of this pathogen *in planta* or *in vitro*, they displayed fewer physical symptoms than WT plants, possibly as a consequence of the increased GABA abundance observed in the apoplast.

From now on, the term GAD plants will be used to refer to both GAD and GADΔC lines unless otherwise stated

Three previous studies have reported reduced symptoms on pathogen-infected GAD plants, however two of these focused on invertebrate pathogens where GABA is a known antagonist of receptors at neuromuscular junctions and there have been previous reports of developmental or growth defects as a result of GABA consumption (MacGregor et al., 2003; McLean et al., 2003). Conversely, fungal (*Cladosporum fulvum*) and bacterial (*Pseudomonas syringae*) pathogens are known to utilise GABA as a major nitrogen source during infection (Solomon and Oliver, 2002; Rico and Preston, 2008). A third

study found GADΔC plants attenuated the virulence of the bacterium *A.tumefaciens*. This phenotype was subsequently found to be due to negative regulation by GABA, of an A.*tumefaciens*-specific lactonase (*Att*M) which regulates virulence of this bacterium *in planta*. A homolog of this gene was not found in the P.s.tabaci genome and an isogenic mutant of *A.tumefaciens*, lacking the *Att*M lactonase, grew equally well all GAD plants (Chevrot et al., 2006), suggesting that the effect of GABA on P.s.tabaci might be different to that observed for *A.tumefaciens*.

GAD plants were not affected in their SA production to virulent and avirulent pathogens, nor were they affected in SA-related PR1α gene expression in response to P.s.tabaci. This latter result was supported by the finding that although the closely related isomer of GABA, βaminobutyric acid (BABA) stimulates PR1α gene expression, GABA itself was found to have no effect on the expression of this gene (Siegrist et al., 2000). These results indicated that the reduced symptoms of GAD plants might not be the result of an enhanced defence response to the proliferating pathogen, a hypothesis supported by the uninhibited growth of P.s.tabaci *in planta*, and the finding that P.s.tabaci was able to suppress the callose response in transgenic lines.

One particularly interesting result was the enhanced production of callose by GADΔC plants in response to the P.s.t and P.s.t *hrcC*. GADΔC plants do not contain the Ca^{2+}/CaM-BD on the overexpressed GAD, which leads to constitutive GABA expression even in the absence of Ca^{2+}. Differential response to pathogens by GAD and GADΔC plants was observed during *A.tumefaciens* infection, when only GADΔC plants displayed significantly reduced symptoms (Chevrot et al., 2006). However, recent results suggest an alternative explanation for the enhanced production of callose in GADΔC plants. In P.s.t, the addition of 5 mM GABA to M9 minimal medium downregulated *hrp* gene expression; particularly in a ΔgabT2/T3/T1 mutant which accumulated intracellular GABA (Park et al., manuscript submitted). GABA was subsequently proposed to act as a direct metabolite repressor of the Hrp regulon in *P. syringae* (Park et al., manuscript submitted). This finding may be relevant to the present observations, because *hrp* mutants in *P. syringae* and *X. campestris* have been shown to provoke an enhanced callose response *in planta* (Hauck et al., 2003 and references therein). Therefore in GADΔC plants, increased GABA in the plant apoplast may suppress P.s.t *hrp* gene expression, leading to a reduced ability to suppress callose deposition. However, increased callose deposition was not observed in GAD plants following inoculation with P.s.tabaci suggesting that *hrp* expression may not be affected in this strain. Future experiments could investigate *hrp* expression in P.s.t and P.s.tabaci in GAD plants.

HPLC analysis of $MgCl_2$-treated (uninfected) apoplast showed that all four GAD lines had increased GABA in comparison to the WT and plant-vector control. Data describing the GABA content of the plant-vector control line has not previously been published but the results of this chapter show that the apoplast abundance is statistically similar to that of WT plants. Preliminary NMR analysis of apoplast samples subsequently confirmed that the GABA abundance was significantly high in GADΔC1 plants (Figure A8). These plants in particular displayed fewer symptoms following infection by P.s.tabaci (Figures 7.12 and A7). Although serine, asparagine and glutamine were found to vary significantly between WT and plant-vector control plants, the abundance of these amino acids was always more similar to that of WT than GAD lines. This result may suggest an effect of the empty plant-vector on amino acid abundance, possibly to be expected from introducing a foreign vector into the plant cell. Alternatively, this may represent 'noise' and natural variation in the amino acid content of apoplast samples, which tend to be quite variable. Quantitative NMR analysis of apoplast samples should help to clarify this.

Despite the significant differences in GABA concentration between tobacco lines, there was not a significant difference in the growth of P.s.tabaci in extracted apoplast. This result was also observed between growth of P.s.t in apoplast from healthy and diseased tomato plants, where the amino acid content is known to differ considerably (Arantza Rico, personal communication). One explanation for this result might be that nutrients in WT apoplast extracts were present at levels in excess of what the pathogen can utilise. Therefore, even though apoplast from GAD plants contained increased GABA compared to WT plants, it might be present in excess of what *P. syringae* can metabolise or use for growth. Future experiments could look into diluting extracted apoplast from GAD and WT lines and seeing if there is a point at which the enhanced GABA in GAD lines provides an additional growth benefit compared to the WT apoplast.

An interesting observation was that apoplastic GABA abundance did not differ between uninfected GAD and uninfected GADΔC plants. GADΔC should be able to synthesise GABA in the absence of Ca^{2+}/CaM stimulation so we might expect the abundance of GABA in GADΔC apoplast to be constitutively higher than in the GAD apoplast. There are several hypotheses which may explain these results. Firstly, when looking at GABA abundance in the GAD and GADΔC apoplast, we must consider that GAD is a cytosolic enzyme and a difference in GABA abundance within the cytosol of these plants may not necessarily translate into a difference in the GABA found in the apoplast. This may be due to increased sequestration of GABA into organelles, or increased flux through the

GABA shunt. For example, Scott-Taggart and coworkers (1999) demonstrated that when cytosolic glutamate was elevated, there was an increase in carbon flux through the GABÃshunt, without any significant change in GABA abundance.

Secondly, the plants were grown outside in greenhouses, rather than in growth rooms or tissue culture conditions (as in the original study), and here they will have undoubtedly encountered a variety of small stresses, such as exposure to invertebrates or fluctuations in temperature, which may have stimulated GAD over the course of their development. We know that GAD is stimulated to produce GABA during the growth of WT plants in greenhouse conditions, as it can be detected in the uninfected WT apoplast. This stimulus would also stimulate the second copy of this enzyme in GAD plants. It is therefore possible that the amount of GABA produced by two stimulated enzymes in GAD plants was not statistically different to the amount synthesised by the native and constitutively expressed GAD in GADΔC plants during these growth conditions (Figure 7.26). It is also possible that the syringe-infiltration method of infecting plants, used to infiltrate P.s.tabaci into tobacco plants, might have stimulated GAD activity. The syringe-infiltration method was used in this study because it is a reliable method to infiltrate a known density of bacteria, without the associated safety hazards and humidity requirements needed for spray inoculation (plants were too large for dip inoculating). However, when infiltrating bacteria, pressure on the leaves from the syringe may stimulate GAD activity leading to increased GABA in the apoplast before the bacteria had acclimatised to the apoplast conditions. Therefore, although the results seen in this chapter are valid for the commonly used infiltration method, we may observe different results if the bacteria are allowed to gain entry to the plant under more natural conditions, where GAD stimulation may not occur until several hours post infection.

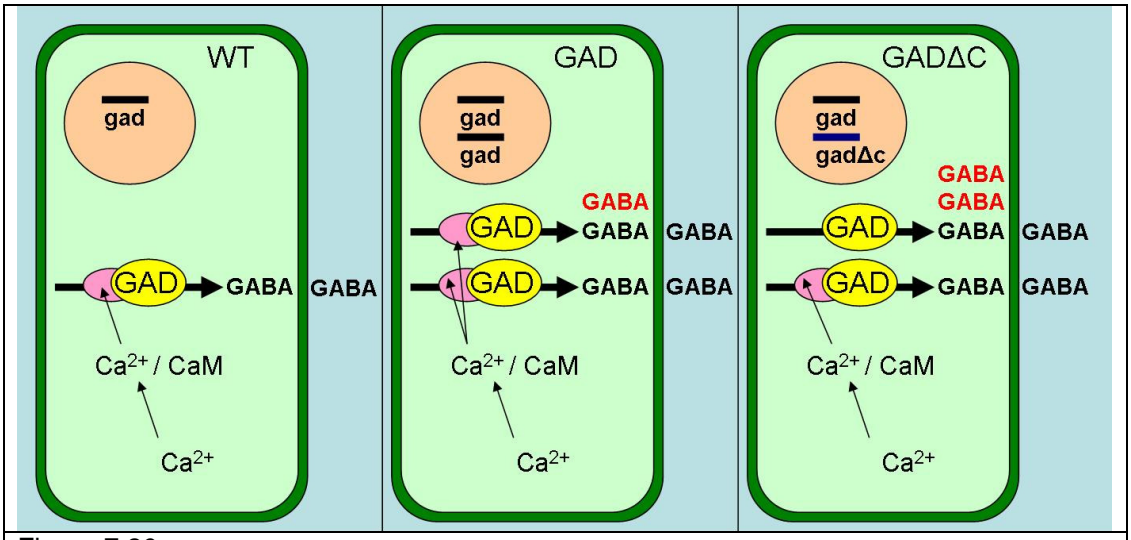


Figure 7.26
Explanations why GAD and GADΔC plants may have a similar apoplastic GABA content. Differences in the cytosolic GABA content may not necessarily translate into significant differences in apoplastic GABA, this may be due to processes such as increased sequestration of GABA in organelles of GADΔC plants (red font). Alternatively, when all plants are exposed to Ca^{2+} stimulation, the GABA synthesised by the native and additional enzymes in GAD plants may be similar the that produced by the native and constitutively expressed enzymes in GADΔC plants. Apoplast = blue; cytosol= green.

Interestingly, the original data (reproduced in Figure 7.27) show that the abundance of GABA in the shoots of GADΔC plants was low, whereas the abundance of GABA in the roots was approximately 10 times higher than in the roots of GAD plants. As the original paper used whole tissue samples for analysis, this may be due to the effect of pooling the cytosol, apoplast and vacuolar compartments, all of which may accumulate GABA to different extents, diluting any significant differences between GAD and GADΔC lines. This explanation would also support the idea that GABA is often sequestered within organelles, rather than the apoplast.

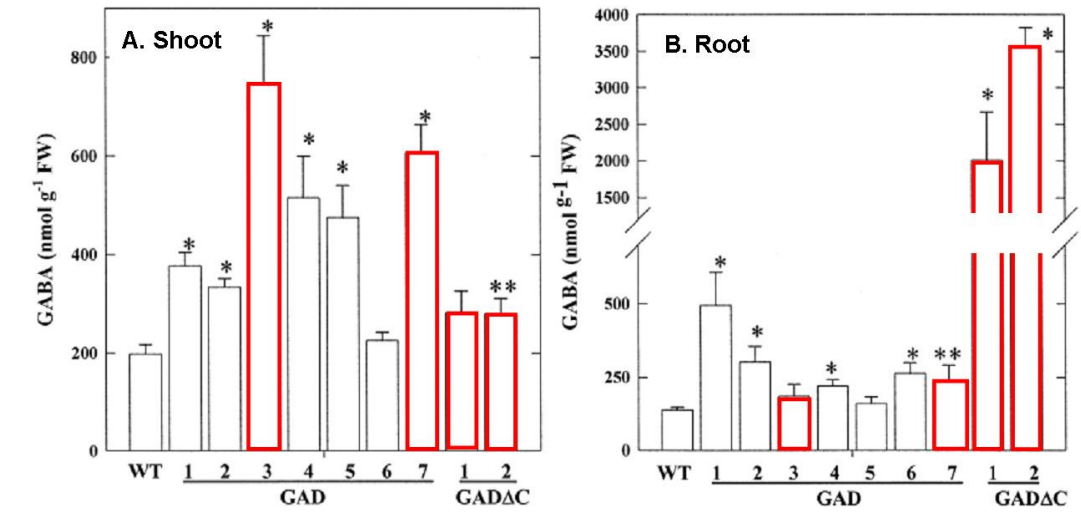


Figure 7.27
Reproduced from McLean and coworkers (2003). GAD lines used in this chapter have been highlighted in red, GAD3 and GAD7 are called GAD1 and GAD2 in this chapter. HPLC analysis of GABA in twelve-week-old shoots (A) and roots (B). Prior to amino acid determination samples were subjected to a 'freeze-thaw' cycle designed to enhance the GABA response. * = p≤0.05, ** = p≤0.1. Error bars are standard error.

Enhanced GABA accumulation in plants containing a truncated GAD has been shown in rice (Akama and Takaiwa, 2007) and tobacco (Baum et al., 1996) plants, where the enzyme is controlled by the cauliflower mosaic virus (CaMV) 35S promoter. However both of these studies also report abnormal plant morphology which is believed to be due to extreme overexpression by the promoter causing a damaging level of GABA synthesis, whereas the plants used in this chapter are phenotypically normal (Figure 7.8).

GABA abundance in the WT apoplast did not significantly differ between P.s.tabaci and $MgCl_2$ treatments. This result has also been observed in tomato plants infected with P.s.t (Arantza Rico, personal communication) and may indicate that *P. syringae* is consuming any additional GABA synthesised by the plant, or suppressing GABA synthesis. In comparison, the abundance of GABA in the infected GAD apoplast was significantly lower than the uninfected GAD apoplast. GABA abundance was reduced in the uninfected GAD apoplast to the extent that after P.s.tabaci treatment, GABA abundance in the GAD and WT apoplast was no longer significantly different.

In summary, GABA abundance in the apoplast was observed to vary in response to *P.s.*tabaci in the following ways: In the WT apoplast, which initially had low GABA abundance, levels did not vary, and plants supported pathogen proliferation and displayed disease symptoms. However, in the infected GAD apoplast there was a

significant decrease in GABA abundance, and although bacteria proliferated normally within the plant, the physical symptoms usually associated with increased bacterial load were reduced. This latter result may be connected to the suppression of *hrp* gene expression in P.s.tabaci by GABA, thereby reducing or preventing the expression of effector proteins. Such an effect was observed in P.s.t, whereby the addition of GABA to M9 minimal medium reduced the expression of both the *hrp* gene *hrpL* and the *avrPto* effector gene (Park et al., manuscript submitted).

Appendix.

	Amino acid		**Sample 1**	**Sample 2**	**Sample 3**	**Average**	**SD**
1	Aspartic acid	Asp	178.34	93.45	164.30	145.36	45.50
2	Glutamic acid	Glu	177.20	88.52	146.37	137.37	45.02
3	Asparagine	Asn	12.43	7.01	9.04	9.49	2.74
4	Serine	Ser	42.03	46.48	65.18	51.23	12.28
5	Glutamine	Gln	42.79	35.06	24.01	33.95	9.44
6	Histidine	His	7.40	4.60	8.50	6.83	2.01
7	Glycine	Gly	19.22	28.65	38.56	28.81	9.67
8	Threonine	Thr	12.68	22.88	30.37	21.98	8.88
9	Alanine	Ala	68.21	53.02	80.69	67.30	13.86
10	GABA	Gaba	623.73	479.76	622.90	575.47	82.88
11	Tyrosine	Tyr	14.42	14.60	18.97	16.00	2.58
12	Methionine	Met	23.65	17.00	33.99	24.88	8.56
13	Valine	Val	24.63	24.74	24.80	24.73	0.09
14	Phenylalanine	Phe	22.85	24.74	27.98	25.19	2.60
15	Isoleucine	Ile	17.33	16.54	24.24	19.37	4.24
16	Leucine	Leu	33.85	25.36	36.14	31.78	5.68
17	Lysine	Lys	23.34	20.10	24.03	22.49	2.10
18	Proline	Pro	11.12	14.57	12.62	12.77	1.73

	Sample 1	**Sample 2**	**Sample 3**	**Average**
Total amino acids (µM)	1355.22	1017.09	1392.70	1255.00
Total amino acids 1-9 (µM)	1208.22	875.05	1206.14	1096.47
% of total amino acids made up of amino acids 1-9	**89.15**	**86.03**	**86.60**	**87.37**

Figure A1
Raw data of three independent replicates of tomato leaf apoplast, provided by Arantza Rico, Oxford. The percentage of the total amino acids contributed by the 9 most abundant has been calculated for each sample and averaged.

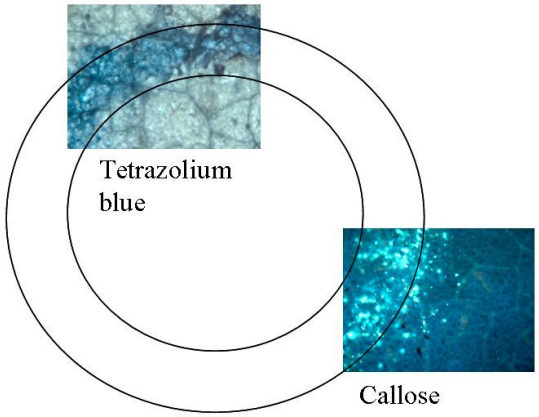


Figure A2
The efficacy of callose and tetrazolium blue staining was checked by examining leaf discs for areas where the leaf has been marked by the syringe during inoculation of bacteria. In these damaged areas callose deposits can clearly be seen. These areas could then be avoided when counting callose deposits. The outline of the tip of a 1 cm syringe is superimposed on the images.

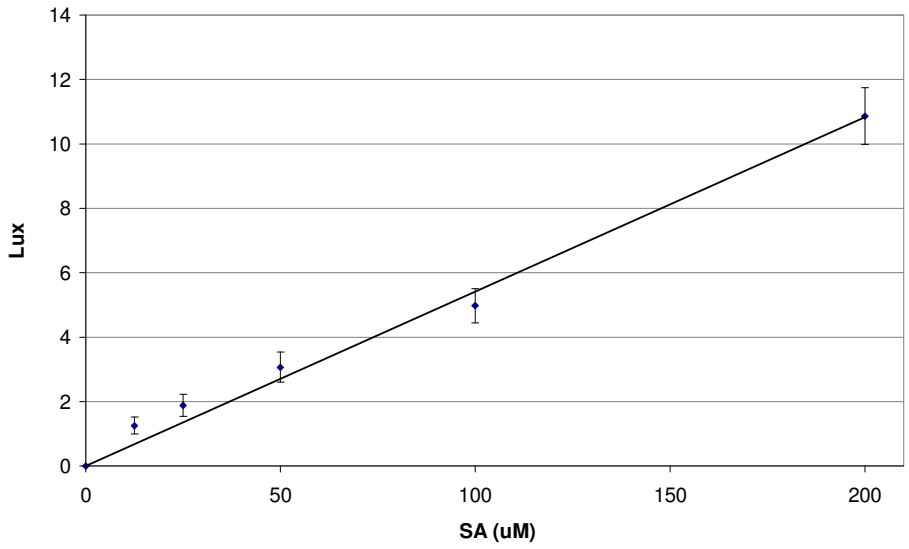


Figure A3
Conversion from Lux to SA, y=0.0542x. The R_2 for the trendline is 0.9877

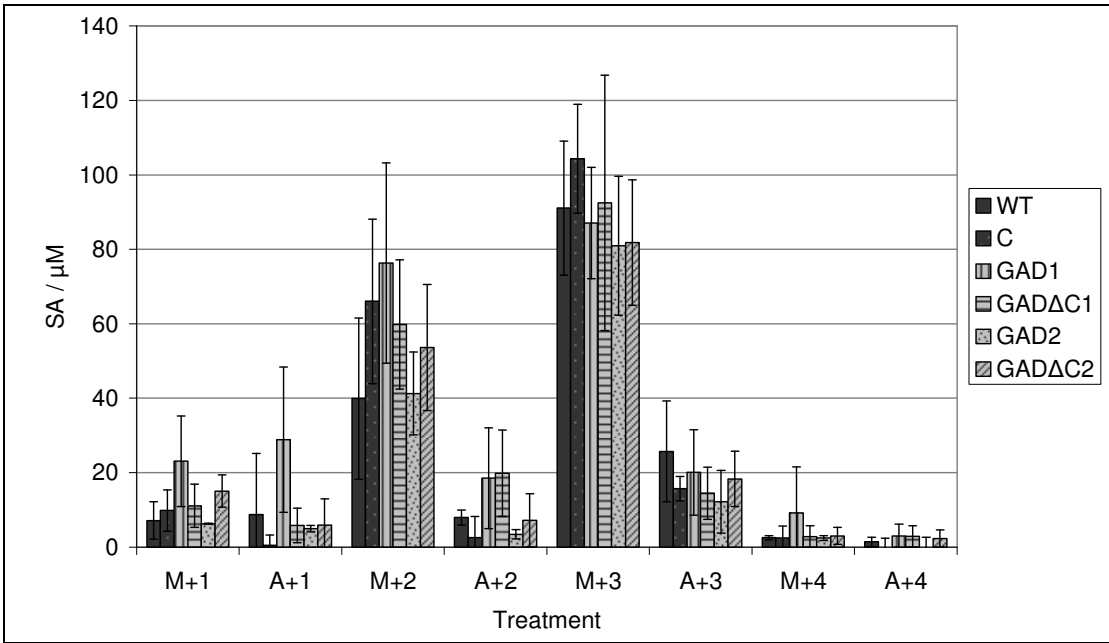


Figure A4
Graph showing average SA production by all tobacco lines. Initially leaves were inoculated with either AtGV3101 or $MgCl_2$, on day two leaves were inoculated with P.s.t, P.s.t-hrcC, P.s.tabaci or $MgCl_2$. On day three the SA biosensor ADPWH_*lux* was infiltrated into leaves and images were taken after one hour using a photon camera.
M=$MgCl_2$ pre-treatment, A=AtGV3101 pre-treatment, followed by treatments 1 – 4:
1=P.s.t-hrcC, 2=P.s.tabaci, 3=P.s.t. 4=$MgCl_2$. Graphs are the average of two independent experiments. Error bars show the standard deviation of two replicate leaves.

Treatment	p-value
$MgCl_2$ - *P.s.tabaci*	0.137
A.tumefaciens - *P.s.tabaci*	0.470
$MgCl_2$ - P.s.t.-hrcC	0.099
A.tumefaciens - P.s.t.-hrcC	0.408
$MgCl_2$ - P.s.t.	0.254
A.tumefaciens - P.s.t.	0.243
$MgCl_2$ - $MgCl_2$	0.716
A.tumefaciens - $MgCl_2$	0.455

Table A1
A General Linear Model applied to the data. The SA response by all tobacco lines is compared, for all treatments. In all cases $p>0.05$. Therefore there is no significant relationship at the 95% confidence interval. Three replicates were used for each treatment, per tobacco line.

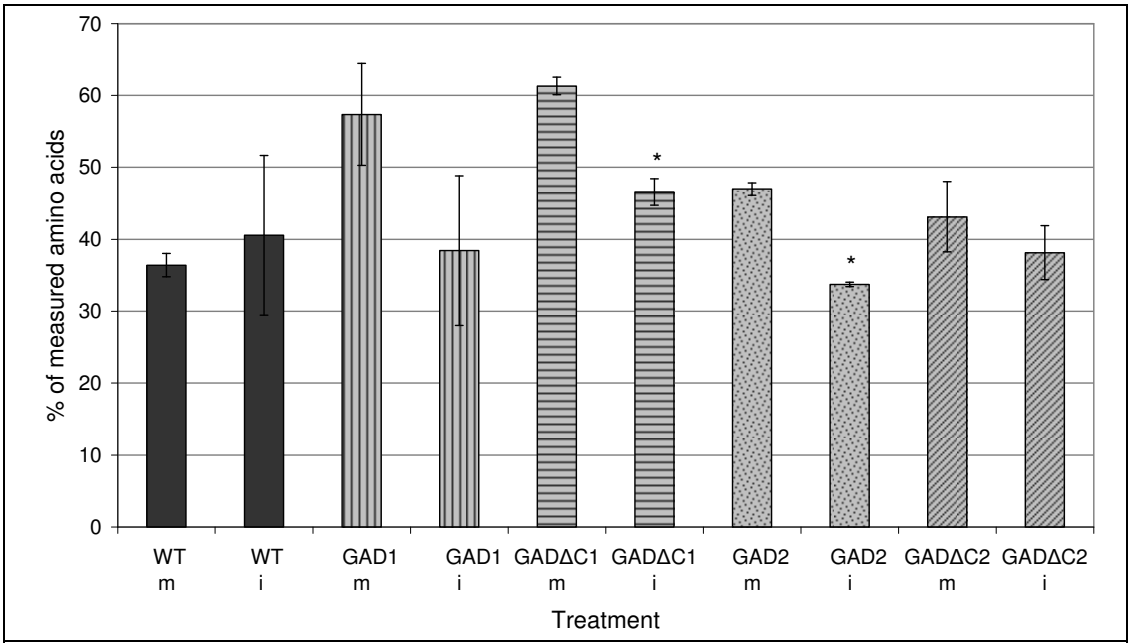


Figure A5
The abundance of GABA in relation to the other measured amino acids decreases during infection in GAD lines. i=apoplast from plants infected for 24 hours with P.s.tabaci, m=apoplast from plants treated with $MgCl_2$ control.

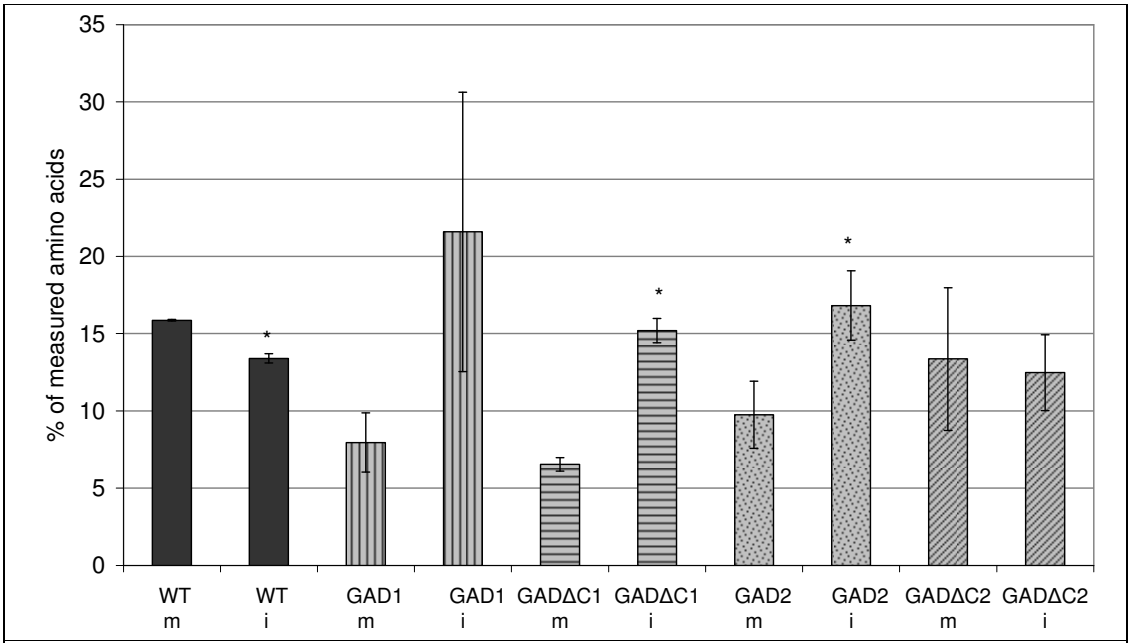


Figure A6
The abundance of serine in relation to the other measured amino acids increases during infection in GAD lines. i=apoplast from plants infected for 24 hours with P.s.tabaci, m=apoplast from plants treated with $MgCl_2$ control.



Figure A7

Symptoms caused by *P.s.*tabaci, inoculated at $1x10^5$ colony forming units (cfu)ml^{-1} after 7 days growth *in planta*. A representative leaf is shown on the top line, with a closer view of the symptoms underneath each picture. Two leaves were inoculated per plant and three independent experiments were carried out with similar results. In this experiment the same leaves were used to measure growth of *P.s.*tabaci *in planta* by leaf disc assay so that a direct comparison can be made between bacterial proliferation and symptoms. WT= wild type, C= plant-vector control.

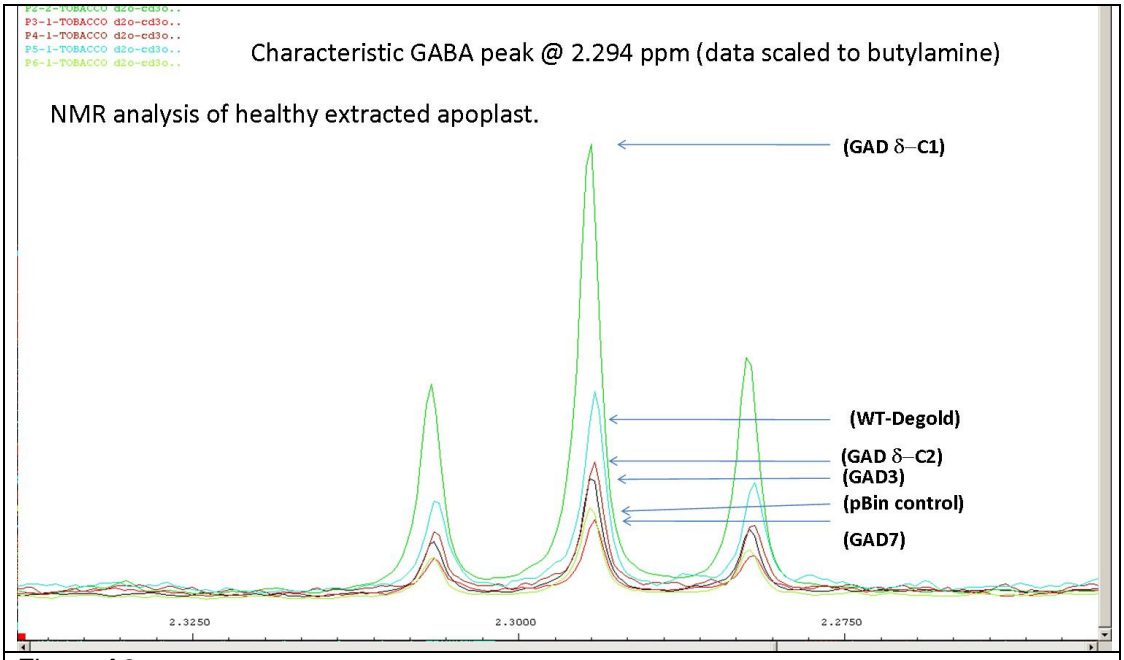


Figure A8.
NMR analysis of apoplast samples extracted from healthy tobacco leaves. The region including a characteristic GABA peak is shown. The concentration of GABA was found to be increased in the apoplast of GADΔC1 plants. However, the concentration of GABA was not significantly increased in any other transgenic line. Butylamine was infiltrated into the apoplast along with distilled water and the concentration of this compound was subsequently used to standardize samples. However, the peak for butylamine (not shown) coincides with another GABA peak making interpretation of results difficult. Analysis was carried out by MeT-RO (Metabolomics at Rothamsted)

Appendix: Mutant construction.

A.1 Overview.

There are significantly fewer aa_permease genes in *P. syringae* compared to non-plant pathogenic *Pseudomonas* species. In particular, the genome of P.s.t contains four aa_permease genes: PSPTO_5276, PSPTO_5356, PSPTO_2026 and PSPTO_1817. Previous work in this thesis identified that the expression of PSPTO_5356 and PSPTO_5276, was upregulated in the presence of the amino acids GABA and histidine, respectively, suggesting that these genes encode transporters for these amino acids. These amino acids are known to be present in the tomato apoplast and therefore uptake of GABA and histidine may be transported by P.s.t during apoplast colonisation. However the solute transported by the remaining aa_permease genes remains unknown. Furthermore, it is not known whether uptake of any amino acid transported by the aa_permease genes is essential for growth or virulence of P.s.t in the apoplast. One strategy to establish the amino acid(s) transported by a particular aa_permease gene is to create deletion knockouts and assay the mutated bacteria for a reduced ability to utilise amino acids for metabolism or growth. Furthermore, the virulence and competitive ability of mutant and wild type bacteria can be compared to assess the importance of amino acid transport by aa_permeases *in vitro* or *in planta*.

In this chapter, constructs for creating unmarked deletion mutants in P.s.t were created in the suicide reporter plasmid pUIC3. Allelic exchange was then performed and single-crossover colonies of P.s.t and pUIC3 were generated. However, despite repeated attempts deletion mutants were unable to be recovered.

A.2 Introduction.

Within the *Pseudomonas* very few aa_permease genes have been experimentally characterised. The *P. aeruginosa* PAO1 aa_permease ArcD (PA5170) has been characterised as an arginine/ornithine antiporter (Verhoogt et al., 1992; Bourdineaud et al., 1993), while a *P. aeruginosa* PAK mutant containing the Mini-Tn*5*-Tc^r transposon in the aa_permease gene PA0220 was unable to use histamine as a sole nitrogen source. In addition, in *P. fluorescens* SBW25 (P.fl.SBW25) the aa_permease *hutT* was characterised as a histidine transporter (Zhang et al., 2006).

The characterisation of *hutT* in P.flSBW25 was particularly interesting as the orthologous gene in other *Pseudomonas* species, including PSPTO_5276 in P.s.t, had been

annotated as transporting proline. One element of gene characterisation involves knocking out the gene of interest and assaying the resultant mutant strain for a phenotype. Therefore, in order to investigate the substrate transported by the aa_permease genes it was desirable to create knock out mutants of the aa_permease genes.

Many mutation strategies exist. One widely used strategy involves inserting a physical barrier, such as a transposon, into the gene of interest in order disrupt the transcription of the native protein. Transposons can be provided with a marker, such as the *lux* gene which can produce a detectable signal when transcription of the mutated gene is initiated. However, there are several disadvantages to an insertion-mutation based strategy. Firstly, the creation of multiple knockout mutant strains is limited by each transposon (located with in a different genes) requiring a different selective marker, and where appropriate a different detectable signal for gene expression. Furthermore, insertion-mutation strategies can also result in the unwanted disruption of genes downstream to the gene of interest. This effect might be particularly significant for the P.s.t aa_permease PSPTO_5276, which was previously demonstrated to fall within the histidine (*hut*) operon (Chapter 6), as the creation of a PSPTO_5276 insertion mutant might prevent transcription of downstream histidine utilisation genes.

A second mutation strategy therefore is based on the complete excision of the gene of interest to produce an unmarked deletion mutant. Such a strategy is specific to the gene of interest and therefore will not disrupt downstream genes, and as mutants do not carry selective markers such as antibiotics, there is no limit to the number of genes which can be knocked out in the same strain using this method.

Unmarked deletion mutants will be generated in P.s.t for each of the aa_permease genes (PSPTO_5356, PSPTO_2026, PSPTO_1817 and PSPTO_5276) using a two-step allelic exchange strategy (Gal et al., 2003; Zhang et al., 2006) using the suicide reporter plasmid pUIC3 (Rainey, 1999). This strategy has previously been successfully used by different research groups to generate clean knockouts in P.fl.SBW25 (Spiers et al., 2002; Zhang et al., 2006; Jones et al., 2007).

Briefly, the region either side of the gene of interest is amplified by PCR and checked by sequencing. These fragments are subsequently joined together in the suicide plasmid pUIC3, in the same orientation as in the genome. Homologous recombination occurs between one of the fragments in pUIC3 and the corresponding region of the genome.

This event can be detected using the tetracycline and lacZ genes present on pUIC3 which provide once-recombined cells with tetracycline resistance and a blue coloration when plated on the substrate X-gal. These marked cells are then encouraged to undergo a second recombination event, between the second pUIC3 fragment and the corresponding region of the genome. While excising the pUIC3 plasmid some cells will revert back to wild type while some will also excise the gene of interest producing an unmarked deletion mutant.

A.3 Objectives.

- Generate unmarked deletion mutants for the aa_permease genes present in P.s.t; PSPTO_1817, PSPTO_5356, PSPTO_5276, PSPTO_2026.
- Characterise the ability of these mutants to grow on amino acids as a sole carbon, nitrogen or combined carbon and nitrogen source *in vitro*
- Characterise the ability of these mutants to grow and cause disease *in planta*.

A.4 Materials and Methods.

A.4.1 Bacterial strains, plasmids and abbreviations.

In this chapter the following bacterial strains and abbreviations were used.

Bacterial strain	**Abbreviation**	**Reference**
P. syringae pv. tomato DC3000	P.s.t	(Buell et al., 2003)
E. *coli* DH5α λ-pir	E. coli DH5α	(Mahan et al., 1993)
E. *coli* DH5α-pRK2013	pRK2013	(Friedman et al*.,* 1982)

Table A.1
Bacterial strains and abbreviations used in this study.

In this chapter the following plasmids were used.

Plasmid	**Markers**	**Notes**	**Reference**
pUIC3	Tet/LacZ	Blue white selection	(Rainey, 1999)
pCR2.1	Kan/Amp	Tc^r, Tra^-, Mob^+, R6K replicon	Invitrogen, UK
pCR2.1-2026L	Kan/Amp	Vector containing region flanking PSPTO_2026	This study
pCR2.1-2026R	Kan/Amp	Vector containing region flanking PSPTO_2026	This study
pCR2.1-1817L	Kan/Amp	Vector containing region flanking PSPTO_1817	This study
pCR2.1-1817R	Kan/Amp	Vector containing region flanking PSPTO_1817	This study
pCR2.1-5356L	Kan/Amp	Vector containing region flanking PSPTO_5356	This study
pCR2.1-5356R	Kan/Amp	Vector containing region flanking PSPTO_5356	This study

pCR2.1-5276L	Kan/Amp	Vector containing region flanking PSPTO_5276	This study
pCR2.1-5276R	Kan/Amp	Vector containing region flanking PSPTO_5276	This study
pUIC3-2026	Tet/LacZ	Vector containing both regions flanking PSPTO_2026	This study
pUIC3-1817	Tet/LacZ	Vector containing both regions flanking PSPTO_1817	This study
pUIC3-5356	Tet/LacZ	Vector containing both regions flanking PSPTO_5356	This study
pUIC3-5276	Tet/LacZ	Vector containing both regions flanking PSPTO_5276	This study
pRK2013	Kan	'Helper' strain for triparental mating	(Figurski and Helinski, 1979)

Table A.2
Plasmids used in this study. Construction of plasmids created in this study is detailed in the relevant methods section.

A.4.2 Antibiotics and supplements.

Antibiotics and supplements were added at appropriate concentrations as detailed in Chapter 2. Tetracycline was used for selection of the plasmid pUIC3. Kanamycin was used for selection of the plasmid pCR2.1. Antibiotics were added to sterilised LB or KB media which had been cooled to 50°C.

A.4.2 Bacterial growth conditions.

P.s.t was routinely cultured on LB agar medium at 28°C as described in Chapter2. *E. coli* was cultured on LB agar medium at 37°C. Overnight bacterial cultures were grown at the appropriate temperature in a shaking incubator with shaking at 200rpm.

The recipe of nematode growth medium (NGM), used by Peter Burlinson (University of Toronto, Canada) in the allelic exchange protocol is as follows:

3 g NaCl

2.5 g Difco bacto peptone

1 ml cholesterol (5 mg ml^{-1} in 100 % ethanol)

975 ml dH_2O

(17 g Agar)

Autoclaved, followed by aseptic addition of:

1 ml 1 M Cacl2

1 ml 1 M MgSO4

25 ml 1 M potassium phosphate pH 6.0 (3.3 ml 1M K_2HPO_4, 21.7 ml KH_2PO_4)

A.4.3 Primers used in this study.

Primers were manufactured by MWG (Germany) and were used at a final concentration of 10 pmol / reaction. P.s.t-specific primers were designed using the P.s.t genome sequence obtained from NCBI (AE016853) and downloaded into Artemis 10 genome visualisation software (Sanger, USA) and Primer3 primer design software (Rozen and Skaletsky, 2000).

Primer name	Sequence (5'–3')	Use
M13 forward	GTAAAACGACGGCCAG	Insert sequencing PCR2.1
M13 reverse	CAGGAAACAGCTATGAC	Insert sequencing PCR2.1
IVETbla	CAGGGTTATTGTCTCATGAGG	Insert sequencing pUIC3
IVETblarev	CTTTCATCGGTTGTCGGATC	Insert sequencing pUIC3
PSPTO1817_Rightfor	TGCTGGTGATTTCCAACAAT	Cloning right gene region
PSPTO1817_Rightback	GGTTATGTCCACAGCGACCT	Cloning right gene region
PSPTO1817_Leftfor	CCGGGCTATCAAGTATTTCG	Cloning left gene region
PSPTO1817_Leftback	TGATCATGAAGGCGATGAAG	Cloning left gene region
PSPTO5276_Rightfor	ACGCTCTGGAAACACTGTCA	Cloning right gene region
PSPTO5276_Rightback	TCAGCACATGGCTGTAATCC	Cloning right gene region
PSPTO5276_Leftfor	TGCTCAAAGACGAAACATCG	Cloning left gene region
PSPTO5276_Leftback	GCGTTTCAAACCATCTTGTG	Cloning left gene region
PSPTO2026_Rightfor	GAAACATCCTGTTGCAGAGC	Cloning right gene region
PSPTO2026_Rightback	AGGTCGATACCAGCCTTGC	Cloning right gene region
PSPTO2026_Leftfor	GTGCGAAACGTTGTTTGAGA	Cloning left gene region
PSPTO2026_Leftback	GGCGCTTACACTTGAACCAT	Cloning left gene region
PSPTO5356_Rightfor	GGCAATCGACAGCATCGTA	Cloning right gene region
PSPTO5356_Rightback	GATTTGCCAGTCAGGCTTTC	Cloning right gene region
PSPTO5356_Leftfor	CATTCAGTCACGCTTCTCCA	Cloning left gene region
PSPTO5356_Leftback	CTCTGCGTCAGCACGATCT	Cloning left gene region

Table A.3
Primers used in this study.
Primers were designed to amplify the regions either side of the gene of interest. These are referred to as the left and right regions. Left and right refer to the left or right hand side of the gene when viewing the gene oriented in a left to right direction (i.e the end of the gene at the left and the start of the gene at the right)

A.4.4 Cloning to create constructs for aa_permease gene deletion knockout .

A three-step cloning procedure was used in order to generate constructs for aa_permease deletion knockouts in P.s.t.

1. **Cloning of flanking regions in the vector pCR2.1.**

DNA flanking either side of each aa_permease gene was amplified using the primers detailed above. The primers PSPTO_XXXXRightfor and PSPTO_XXXXRightback were used to amplify the downstream region of the PSPTO_XXXX gene (where XXXX is 2026,

1817, 5276 or 5356) and PSPTO_XXXXLeftfor and PSPTO_XXXXLeftback were used to amplify the upstream region.

The flanking DNA regions were cloned into pCR2.1 (Invitrogen, UK) according to the manufacturer's instructions. Transformants were selected on LB / kanamycin / X-Gal plates at 37°C. X-Gal blue/white selection was used to identify pCR2.1 clones containing an insert. Transformants were sequenced using M13 forward and reverse primers to confirm presence of the insert. This yielded the plasmids pCR2.1-2026L, pCR2.1-2026R, pCR2.1-1817L, pCR2.1-1817R, pCR2.1-5356L, pCR2.1-5356R, pCR2.1-5276L and pCR2.1-5276R in Table 8.2. All plasmids contained 1800 bp inserts corresponding to a single region either side of each gene of interest, flanked by *Xba*I and *Spe*I sites in the pCR2.1 vector.

2. Joining of flanking regions in the vector PCR2.1

Next, the pCR2.1 vector which contained the left flanking region of DNA (for example pCR2.1-2026L) was cut with *Spe*I according to the manufacturer's instructions to linearise the construct. A double digest using *Xba*I and *Spe*I was performed on the pCR2.1 vector which contained the right flanking region of DNA (for example pCR2.1-2026R) yielding two bands. The 3.6 kbp band corresponding to the right aa_permease flank and the linearised construct corresponding to the left aa_permease flank and vector were excised from a gel using minimal UV exposure. The linearised construct was dephosphorylated with Calf Intestinal Phosphatase (CIP) (New England Biolabs, USA) as described in Chapter 2. Both fragments were phenol-chloroform extracted and ligations were set up to join them together. Ligations were transformed by electroporation into *E. coli* DH5α and positive transformants were selected on LB / kanamycin plates. Correct clones containing both fragments were identified by PCR using the primers M13 forward and M13 reverse and sequenced to identify clones in which the inserted right flank was correctly oriented with respect to the *Xba*I / *Spe*I sites as shown in Figure 8.1.

3. Transferring flanking regions into the vector PUIC3

To clone the joined fragments into a vector suitable for transformation into *Pseudomonas*, the combined left and right flanks in pCR2.1 were digested together out of pCR2.1 using *Xba*I / *Spe*I (Figure A.1 part 4) and gel extracted. Vector pUIC3 was cut with *Spe*I, gel extracted, dephosphorylated and phenol-chloroform extracted and ligations were set up with the insert extracted from pCR2.1. Clones containing the insert were identified using the primers IVETbla and IVETblarev and sequenced.

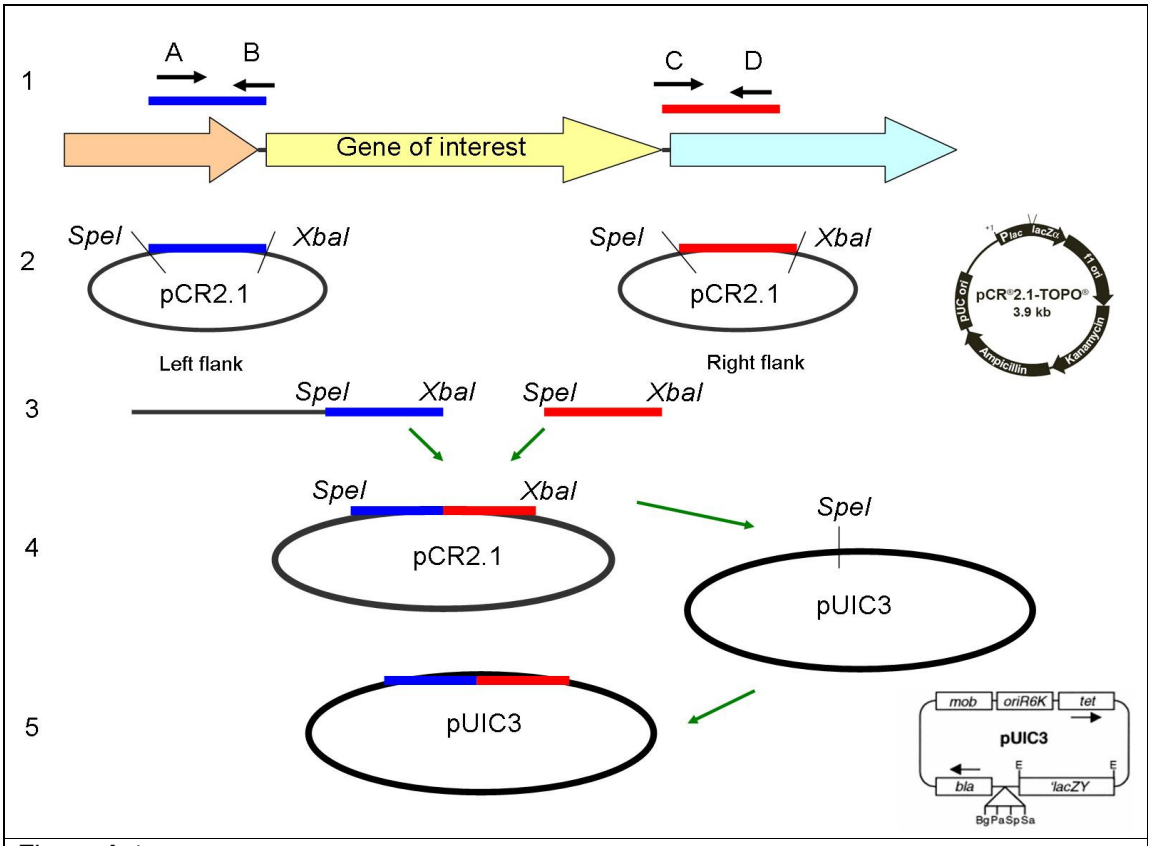


Figure A.1
Cloning scheme for the creation of a deletion knockout construct in the vector pUIC3. **1.** DNA either side of the gene of interest is amplified using the downstream primers PSPTO_XXXXLeftfor (A) and PSPTO_XXXXLeftback (B), and upstream primers PSPTO_XXXXRightfor (C) and PSPTO_XXXXRightback (D) Where XXXX indicates the number of the gene of interest. **2.** The upstream and downstream fragments are cloned into the vector pCR2.1. **3.** The downstream (left) fragment is cut with *Xba*I and the upstream (right) fragment is cut with *Xba*I / *Spe*I. **4.** The fragments are ligated together with the loss of the *Xba*I / *Spe*I sites located at the join. **5.** Vector pUIC3 is cut with *Spe*I, vector pCR2.1 is cut with *Xba*I / *Spe*I and the joined fragment is ligated into pUIC3. Images of pUIC3 reproduced from Rainey (1999) and pCR2.1 reproduced from Invitrogen.com

A.4.5 Plasmid conjugation and allelic exchange.

1. Generation of single crossover mutants.

Triparental plate mating was used to conjugate pUIC3 into P.s.t for the generation of single crossover (one homologous recombination event) mutants. The donor, recipient and helper (*E. coli* DH5α harboring pRK2013) bacterial strains were grown overnight in LB supplemented with the appropriate antibiotic. Cells were prepared for conjugation by centrifuging 3 ml of each culture at 8000 g for 4 minutes. Cells were resuspended in 1.5 ml of LB and recentrifuged twice. Next P.s.t cells were heat-shocked at 42°C for 15 minutes (*E. coli* were incubated at 37°C during this time) and conjugation mixes were set up containing 700 µl P.s.t, 300 µl donor *E. coli* and 100 µl helper *E. coli* strain.

Conjugation mixes were centrifuged at 8000g for 2 minutes and the pellet was resuspended in 50 µl of LB, which was pipetted onto the surface of an LB plate and allowed to dry. Plates were incubated overnight at 28°C and the conjugation mix then resuspended in 2 ml LB using a glass spreader. Fifty microlites of conjugation mix was spread onto selective tetracycline and X-gal-containing media for *Pseudomonas* containing the conjugated plasmid. Chloramphenicol (25µg/ml^{-1} of LB) was added as a counterselection against *E. coli*. Plates were incubated for 48 hours and single colonies were restreaked onto new plates or picked into growth media supplemented with the appropriate antibiotics. Cells which had undergone homologous recombination with pUIC3 were tetracycline resistant and blue on X-gal plates. As a further check that the cultures were not contaminated by *E. coli* strains, these cells were tested for the ability to produce fluorescent siderophores on KB plates and the inability to grow at 37°C. Positive transformants were subjected to the following protocol to produce cells which had undergone a second recombination event and lost the plasmid pUIC3, either producing reversions to the wild type P.s.t or mutants lacking the gene of interest.

2. Enrichment of double crossover mutants

The protocol used by Zhang and Rainey (2007) was used to try and obtain double crossover mutants of P.s.t. This protocol exploits the use of the *lacZ* marker gene and tetracycline resistance provided by the pUIC3 suicide vector that must integrate into the P.s.t genome by homologous recombination (Figure A.2).

To enrich for colonies that had undergone a second recombination step, a single transconjugant (single crossover of pUIC3 into the P.s.t genome), identified by tetracycline resistance and blue colouration on X-Gal plates was inoculated into 200 ml of LB broth with no antibiotic and incubated at 28°C in a shaking incubator at 200rpm. At this stage P.s.t will have no selection pressure to retain the pUIC3 plasmid and this plasmid should be lost in some clones. Furthermore, during the excision of pUIC3 clones will either undergo a reversion to the wild type P.s.t strain, or (ideally) excise pUIC3 through a second homologous recombination event which results in the excision of the gene of interest.

On day two, 500 µl of this broth was transferred to 25 ml of LB pre warmed to 28°C and shaken at 28°C for 30 minutes. At this point bacteria have been provided with fresh nutrients and should be actively growing. Tetracycline was then added to a final concentration of 25 µg ml^{-1} and incubated for another 2 hours. During this step clones that retained the integrated pUIC3 vector should continue to grow whilst those that have lost pUIC3, and therefore have lost the tetracycline resistance cassette are unable to multiply. D-cycloserine was then added to a final concentration of 800 µg ml^{-1} and the incubation continued for another three hours. D-cycloserine inhibits bacterial cell wall biosynthesis and acts as a bacteriocidal antibiotic towards actively growing cells. D-cycloserine therefore acts on the tetracycline resistant cells but not on those which have undergone a second recombination event, losing the pUIC3 vector to become tetracycline sensitive because these will not be growing in the presence of tetracycline.

Cells were then washed once with sterile distilled water and serial dilutions were plated onto LB / X-gal plates. Positive transformants were white as these must have undergone a second recombination event to lose *lacZ* contained on the pUIC3 plasmid. Positive transformants were restreaked onto LB / X-gal plates to confirm colony colour. Mutants which had lost the aa_permease gene of interest could then be confirmed by PCR.

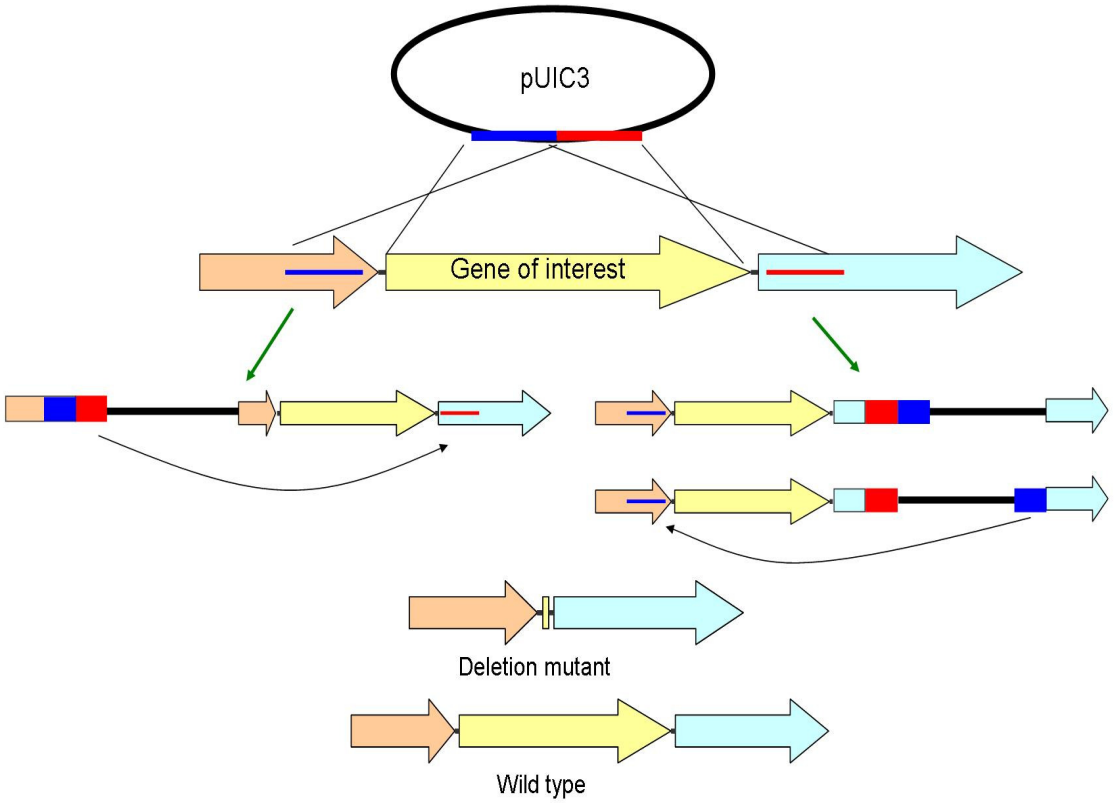


Figure A.2
Mutation strategy. The pUIC3 plasmid containing the flanking regions either side of the gene of interest (blue and red) was conjugated into P.s.t by triparental mating to produce single crossover mutants containing the pUIC3 plasmid. Positive transformants were incubated in LB overnight without tetracycline to allow P.s.t to excise the pUIC3 plasmid from the genome. This could either be achieved through reverting to the wild type P.s.t, or through a second homologous recombination step between pUIC3 and subsequent excision of the gene of interest from the genome. Cells were then treated to a tetracycline D-cycloserine protocol to enrich for cells which had lost the pUIC3 plasmid. Cells which were white on X-gal plates had lost the pUIC3 plasmid. These cells were either wild type reversions or mutants. White cells were analysed by PCR for the presence or absence of the gene of interest.

A.5 Results.

A.5.1 Creation of deletion constructs in pUIC3.

In order to try and create unmarked deletion knockouts of the P.s.t aa_permease genes PSPTO_2026, PSPTO_5276, PSPTO_5356 and PSPTO_1817 an allelic exchange protocol was used (Spiers et al., 2002; Zhang et al., 2006; Jones et al., 2007). Primers were designed to amplify ~1800bp regions either side of each aa_permease gene in the P.s.t genome. The amplified fragments were successfully generated, cloned into the

plasmid PCR2.1, and checked by sequencing using the primers M13 forward and M13 reverse (Table A.3). The left and right fragments either side of each gene of interest were subsequently joined together in the plasmid pCR2.1 (in the same orientation as in the genome). This double fragment was excised by virtue of *Xba1 / Spe1* cloning sites and introduced into the *Spe1* site of plasmid pUIC3 to produce a deletion construct. This deletion construct was checked by sequencing using the primers IVETbla and IVERblarev.

A.5.1 Allelic exchange of deletion constructs.

Deletion constructs were successfully recombined into P.s.t through triparental mating. Positive transformants were identified by their resistance to tetracycline and blue colouration on X-gal, which confirmed the presence of the pUIC3 plasmid. The identity of transformants as P.s.t, rather than *E. coli*, was checked by monitoring the ability of cells to produce siderophores on KB agar plates and to grow at 28°C, but not 37°C.

Positive transformants were subjected to the enrichment protocol detailed above. Following this protocol approximately 1% of recovered cells were white. This percentage was similar to that reported previously (Jones et al., 2007). However, PCR of white cells revealed that all white colonies checked still contained the aa_permease gene.

At this point various modifications of the enrichment protocol were tried. Following each modification the enrichment protocol was retried. No significant increase in the number of while colonies was observed and PCR amplifications revealed that all white colonies had retained the gene of interest.

:

- P.s.t grows more slowly in LB than *P. fluorescens* for which this protocol was previously used. Therefore, culturing P.s.t overnight might not provide sufficient time for these bacteria to excise the pUIC3 plasmid. Therefore the incubation time during which P.s.t could excise this plasmid was increased. Cells incubated in LB without tetracycline for 36, 48 and 72 hours (subculturing 500 µl of cells into fresh LB every 24 hours). No increase in the number of white colonies was observed and all white cells tested by PCR had retained the aa_permease gene.

- The recombination efficiency of the pUIC3 plasmid into the P.s.t genome was quite low using triparental mating. As an alternative the deletion constructs were transformed into P.s.t by electroporation. This method was less efficient than triparental mating at producing transformed cells.
- If the concentration of tetracycline added to the bacteria was too high, then the growth of both P.s.t and P.s.t containing pUIC3 plasmid (P.s.t-pUIC3) would be inhibited. Therefore tetracycline resistance of P.s.t and P.s.t-pUIC3 was investigated using a tetracycline sensitivity assay. This revealed that wild type P.s.t was sensitive to tetracycline added to liquid LB at concentrations of 25 μg ml^{-1}, 12.5 μg ml^{-1} and 6.25 μg ml^{-1}. Although P.s.t-pUIC3 was resistant to 25 μg ml^{-1} tetracycline on LB agar, the resistance of P.s.t-pUIC3 in LB broth decreased to approximately half this value. Therefore during enrichment assays 12.5 μg ml^{-1} tetracycline was used. No increase in the number of white cells was observed, and all white cells tested contained the aa_permease gene.
- D-cycloserine is a β-lactam antibiotic which interferes with bacterial cell wall synthesis. In order for this antibiotic to be effective bacteria must be incubated for a sufficient time that synthesis of bacterial cell walls is negatively affected. Therefore the period of D-cycloserine incubation was increased to six hours to maximise the time for this antibiotic to take effect. No increase in the number of white cells was observed, and all white cells tested contained the aa_permease gene.
- The rate of excision of the pUIC3 plasmid appeared to be low, as very few white colonies were observed following the enrichment steps. Therefore, the experiment was scaled up to maximise the number of white cells which were assayed for the loss of the gene of interest. This strategy increased the number of white colonies recovered; however all colonies retained the aa_permease gene.

The deletion constructs were sent to Peter Burlinson (University of Toronto, Canada) for further work in generating the P.s.t mutants. A putative PSPTO_1817 (aromatic aa_permease) mutant was subsequently generated by Peter Burlinson using the following modified protocol:

pUIC3 deletion constructs were conjugated into P.s.t by triparental mating as above; however the mixture of *E. coli* donor/helper and wild type P.s.t was incubated on nematode growth medium (NGM) overnight as this appears to suppress *Pseudomonas* antibiotic production. Following incubation mating mixtures were plated onto M9 medium

containing X-gal and 5 µg ml $^{-1}$ tetracycline. A few blue colonies were recovered which appeared to contain pUIC3. Single crossover mutants were incubated for 48 hours in KB medium, subculturing twice. Three hundred microlitres was transferred to 30 ml of KB medium for enrichment. After 2 hours 30 minutes, tetracycline was added to a final concentration of 10 µg ml $^{-1}$. After a further 2 hours 30 minutes, D-cycloserine was added to a final concentration of 400 µg ml $^{-1}$. Bacteria were incubated for a further 3 hours before being washed in dH_2O to remove D-cycloserine. Bacteria were plated onto KB-agar plates. Very few white colonies were observed following enrichment. However, one white colony appeared to have lost the aromatic aa_permease gene when checked by PCR.

A.6 Discussion.

Although the allelic exchange strategy has previously been successfully used to produce unmarked deletion mutants *P. fluorescens* SBW25, (Spiers et al., 2002; Zhang et al., 2006; Jones et al., 2007), this method was not very successful when applied to P.s.t. In this chapter, pUIC3 deletion constructs were successfully generated containing the regions either side of the aa_permease genes in P.s.t. These constructs were successfully integrated in the P.s.t genome by homologous recombination to produce blue, tetracycline resistant cells. The identities of these cells were repeatedly verified as being *Pseudomonas*. However, despite repeated efforts the number of cells which subsequently lost the pUIC3 plasmid remained extremely low. Furthermore, the majority of cells which lost the pUIC3 plasmid did so by reverting to wild type P.s.t, rather than excising the aa_permease gene.

The number of P.s.t cells which retained the pUIC3 plasmid was high. This was surprising as the plasmid is large and would be expected to confer an added metabolic cost the bacteria carrying it. However, the aa_permease genes are not strongly expressed in LB (Chapter 6) which may influence the recombination efficiency of these genes. An interesting future experiment would be to grow P.s.t in a medium where the aa_permease genes are highly expressed, such as the synthetic tomato apoplast medium developed in Chapter 4. However, this might also increase the selection pressure on P.s.t to retain the aa_permease gene in order to assimilate amino acids in the growth medium. Therefore, the best approach might be to identify a rich medium where these genes are strongly expressed, but where P.s.t would not suffer a selective

disadvantage by losing these genes. For PSPTO_5356, one explanation for the failure of the pUIC3 plasmid to undergo a second homologous recombination event might be due to the identity of one of the flanking genes. In P.s.t, but not closely related P.s.s or P.s.ph, the PSPTO_5356 gene is flanked on one side by genes encoding the effector *hopA1* and its chaperone *shcA* (Chapter 3, Section 3.5.9). It can be hypothesised that this region may be particularly resistant to recombination, possibly due to the presence of insertion sequences which might affect the secondary structure.

From the results of these experiments it appears that the rate of recombination in *P. syringae* may be lower than for *P. fluorescens*, where this strategy has previously been successful. Low numbers of single-crossover mutants were recovered following integration of the pUIC3 plasmid by triparental mating, suggesting a low frequency of recombination between pUIC3 and P.s.t. While low numbers of white colonies were recovered following enrichment for P.s.t which had lost pUIC3, suggesting that once the pUIC3 plasmid had recombined into the genome, the rate of excision was low.

Future strategies for generating deletion knockout mutants in *P. syringae*, may wish to consider using an alternative plasmid to pUIC3 containing the counterselectable marker *sacB* (encoding levansucrase) which confers sucrose sensitivity, in addition to an antibiotic. Levansucrase activity is lethal in the presence of sucrose for most gram-negative bacteria and therefore sucrose can be used to screen much larger numbers of bacteria than the blue-white selection of pUIC3. The three *gabT* genes were recently successfully knocked out of P.s.t using a similar allelic exchange strategy to the one described in this chapter (Allan Collmer, personal communication). However, in place of pUIC3 the mobile suicide vector pK18*mobsacB* (Schafer et al., 1994) containing the *sacB* and kanamycin genes was used. Alternatives for future work also include heterologous expression of the aa_permease genes in bacteria which are unable to assimilate certain amino acids, such as GABA. For example, Johnson and coworkers (2005) have created transposon mutants in *P. aeruginosa* PAK which include several aa_permease genes. In particular PA0220 was unable to utilise histamine as a nitrogen source. One interesting approach would be to investigate whether the orthologous aa_permease gene from P.s.t (PA2026) could complement this phenotype. In conclusion, this chapter details the construction of deletion knockout constructs for the P.s.t aa_permease genes. Knockout mutants were not generated during the

experimental work of the present thesis, possibly due to the low recombination activity of P.s.t. However the deletion knockout strategy is presently being continued by Pete Burlinson.

8. General discussion

Many bacteria are capable of proliferating in the plant phyllosphere under natural or experimental conditions. However, unlike many bacteria and uniquely among *Pseudomonas* species, plant-pathogenic *P. syringae* can also proliferate within the nutrient-poor intracellular apoplast of host plants. *P. syringae* has long been considered a nutritionally specialist species compared to non plant-pathogenic *Pseudomonas* and the ability of *P. syringae* to utilise a restricted number of nutrient sources formed part of the traditional (pre-sequencing) methods for classifying *Pseudomonas,* which were based on phenotypic characteristics. However, the genetic and evolutionary basis behind the nutritional specialisation of this species is only recently being studied.

The work in this thesis follows on from a number of experiments which have hypothesised that the ability of *P. syringae* to utilise a restricted number of nutrient sources may be linked to the apoplast-colonising lifestyle of this bacterium. In particular, a bioinformatic study by Studholme and coworkers (2005) provided a genetic basis for the observation that *P. syringae* could utilise fewer amino acids than non plant-pathogenic *Pseudomonas*, as genomes of sequenced *P. syringae* pathovars contained significantly fewer aa_permease protein domains, found in aa_permease transporters. Subsequently, Rico and Preston (2008) demonstrated that although *P. syringae* could metabolise a restricted number of amino acids as carbon or nitrogen sources compared to non plant-pathogenic *Pseudomonas*, the utilised amino acids correlated with those which were abundant in the tomato apoplast, suggesting that *P. syringae* is adapted to use the available nutrient sources in this niche.

Within the nutrient-limited apoplast the ability to assimilate amino acids may be an important trait for the survival and proliferation of plant-pathogenic species. Therefore, there may be positive selection operating on transporters for amino acids which can be utilised by plant pathogens for growth or metabolism. For example, GABA was shown to be utilised as a major source of nitrogen by the biotrophic fungal pathogen

Cladosporium fulvum during colonisation of the tomato apoplast (Solomon and Oliver., 2002). Work in this thesis complemented the findings of Rico and Preston above, by demonstrating that P.s.t could also only utilise a restricted number of amino acids as carbon or nitrogen sources for growth compared to non plant-pathogenic *Pseudomonas*. The amino acids utilised by P.s.t also correlated well with those which were abundant in the plant apoplast, particularly GABA, serine, glutamine, glutamate asparagine and aspartate, all of which could be used as a combined carbon and nitrogen source for growth.

The observed difference in aa_permease transporters between *P. syringae* and non plant-pathogenic *Pseudomonas*, along with the potential importance of amino acid uptake for colonisation of the plant apoplast, highlighted these transporters as an interesting area for further research. As such, the broad aims of this thesis were; to investigate the number, type and distribution of aa_permease genes in the genomes of sequenced *P. syringae* and non plant-pathogenic *Pseudomonas* species; to identify the amino acid(s) transported by the four aa_permease transporters in P.s.t by mutagenesis and expression analysis; to create a synthetic apoplast media to model amino acid abundance in the apoplast environment; and to investigate the effect of increased amounts of the amino acid GABA on pathogenesis of tobacco plants by *P. syringae* pv. tabaci 1448A.

Bioinformatic analysis revealed that orthologous aa_permease genes to those present in *P. syringae* genomes were found in the genomes of non plant-pathogenic *Pseudomonas*, including *P. aeruginosa*, *P. putida*, *P. entomophila* and *P. fluorescens* species. From an evolutionary standpoint, the most parsimonious explanation for this distribution was that these genes were present in the common ancestor of all *Pseudomonas* species. However, from this point there are two different scenarios which would provide the presently observed distribution. Firstly, that the common ancestor contained a number of aa_permease genes similar to that found in *P. syringae*, and additional genes have been acquired by non plant-pathogenic *Pseudomonas* species by gene duplication or horizontal evolution. Conversely, the common ancestor may have a number of aa_permease genes similar to that found in non plant-pathogenic *Pseudomonas*, and additional genes have been lost by *P. syringae*. The results from this work favor the latter explanation.

In particular, there was little evidence of the two major mechanisms by which bacteria can expand the numbers of particular genes; horizontal evolution and gene duplication. In Chapter 3, horizontal evolution of aa_permease genes in *Pseudomonas* species was found to be relatively rare and the few genes acquired from other bacteria were readily identifiable by their increased sequence similarity to genes in non-*Pseudomonas* species. In addition, phylogenetic trees constructed using aa_permease sequences from representative *Pseudomonas* did not suggest extensive gene duplication within this family. For example, in addition to a first gene annotated as transporting ethanolamine which was present in all *Pseudomonas* species investigated, there was a second gene also annotated as an ethanolamine permease in the genome of *P. fluorescens*, *P. aeruginosa* and *P. entomophila* species. However, a phylogenetic tree generated using all aa_permease sequences annotated as transporting ethanolamine did not suggest a common ancestry for these two genes, as the copies grouped within separate clades. This result was particularly interesting as in *P. aeruginosa* PAK, a transposon mutant in the 'ethanolamine' permease PA0220 could no longer assimilate histamine, suggesting that this gene has been misannotated. However, most recent GC-MS analysis of the tomato apoplast has shown that it does contain a metabolite predicted to be ethanolamine (Rico and Preston, personal communication), and P.s.t has been shown to metabolise ethanolamine as a nitrogen source (Baker, personal communication). Therefore it will be interesting in the future to see whether the ethanolamine permease gene in *P. syringae* also transports histamine (particularly as this work showed that the expression of this gene was not induced by ethanolamine), or whether these transporters have diverged in substrate specificity.

One particular exception where gene duplication may have occurred was in the GABA aa_permeases, which were present in multiple copies in *P. putida* and *P. entomophila* genomes. These genes shared a high amino acid similarity to one another, and grouped within a single extended clade in a phylogenetic tree of GABA permease sequences, the exception being one GABA aa_permease gene in *P. putida* which may have been obtained by horizontal evolution. In addition, the multiple GABA permeases in *P. putida* and *P. entomophila* genomes were identified as orthologous, suggesting a shared evolutionary history for these sequences. As these species have only recently diverged, based on the high sequence similarity of many genes (Vodovar et al., 2006), we can

hypothesise that a GABA permease duplication occurred in the ancestor of these two species following their divergence from the other *Pseudomonas,* but prior to their differentiation into the separate *P. putida* and *P. entomophila* species observed today.

Transported amino acid (putative) Pfam annotation and annotation based on this thesis (bold)	*Pseudomonas*	*P.s.tomato* DC3000	*P.s.phaseolicola* 1448A	*P.s.syringae* B728a	*P.fluorescens* Pf-5	*P.fluorescens* Pf-O1	*P.putida* KT2440	*P.putida* F1	*P.aeruginosa* PA01	*P.aeruginosa* PA14	*P.entomophila* L48
	aa_permease domains	4	5	6	21	14	21	21	22	22	19
	Gene number:	PSPTO	PSPPH	Psyr	PFL	Pfl	PP	Pput	PA	PA14	PSEEN
	GABA	5356	4937	4909	0344	0313	0284	0302	0129	01580	5205
							4106	1758			3473
							2911	2780			
							2543	3171			2395
						2293	4756	4627			0737
	Unknown (histamine?)	2026	1795	1835	4298	4034	0544	0583	4023	11790	0633
					3522	4990			0220	02690	2919
		1817	3536	3580	1605	1493	4495	1417	0866	53050	3899
							0927	0967			1067
	Unknown (tyrosine?)				0756						
									3000	25270	
	Unknown		2512	2379	6168	2793	1229	1258	2041	38130	4074
							2586	3130	0322	04210	
	Histidine	5276	4866	4834	0408		5031	4905	5097	67310	5095
					6228						
	Arginine / Ornithine			2683	4634	4385	1002	1039	5170	68300	4427
					4633	4384	1003	1040			4426
	Unknown				2710		3727	2036	4981	65850	
	D-ser/-ala/-gly				3642		4840	4718			4885
	Unknown				3813		0660	0694			
	Unknown				3216				4072	11210	3103
	Unknown				4897	4556	2802		3597	17740	
	Unknown				2243	2055			5510	72710	
	Unknown				4906	4561	1059	1100	0789	54040	1179
	Unknown				0763				2079	37630	
	Unknown				2914	1043			1147	49570	
	Unknown				3212				1485	45240	2882
	Unknown				1414	2309	1259	1286			4049
	Unknown								1194	48850	
	Unknown							2445	1819	41010	
	GABA						4407	1447			
	Unknown	Genes which appear to have been acquired by horizontal gene transfer from non-*Pseudomonas* spp							4804	63480	
	Unknown						5297	5206			5442
	Lysine								4628	61250	
	Unknown										3580
	Unknown					2343					

Table 1
Updated orthology analysis table of aa_permease genes in *Pseudomonas*. Genes are annotated with the putatively transported substrate, as reported in the NCBI database, or, where appropriate the putative substrate based on the findings of this thesis (bold). Genes which appeared to have been acquired by lateral gene transfer have been moved to the bottom of the table.

Therefore, the most parsimonious explanation for the distribution of aa_permease genes in *P. syringae* and non plant-pathogenic *Pseudomonas* appears to be that there has been a reduction of these genes in *P. syringae*, along with a few occurrences of gene acquisition or duplication in certain non-plant pathogenic *Pseudomonas* species.

In order to address the question of why aa_permease genes would be lost from the genome of *P. syringae* and conversely why they would be retained in the genomes of non plant-pathogenic *Pseudomonas*, the four aa_permease genes in the genome of P.s.t were studied in more detail. The findings of this thesis regarding the aa_permease genes are summarised in the form of questions in Table 1 below before being discussed in more detail below.

	Gene	PSPTO_5356	PSPTO_2026	PSPTO_1817	PSPTO_5276
	Annotated substrate	**GABA**	**Ethanolamine**	**Aromatic aa's**	**Proline**
	Predicted substrate based on the results of this thesis	**GABA**	**Unknown**	**Unknown**	**Histidine**
Gene expression	Was gene expression increased in the presence of any amino acids?	GABA	No	No	Histidine
	Was gene expression increased in extracted tomato apoplast?	Yes	No	No	Yes
	Was gene expression increased *in planta*?	Yes	No	No	Yes
	Was there an effect of pH on gene expression?	Increased at pH6	No	No	Increased at pH 6
	Was expression different in P.s.t and P.s.t-*rpoN*?	Decreased in P.s.t-rpoN	Decreased in P.s.t-rpoN	No	No
	Was expression different in high and low nitrogen media?	Increased in low nitrogen	Increased in low nitrogen	No	No
Chemotaxis	Did P.s.t exhibit significant chemotaxis towards the putative substrate at 1mM?	Yes	Not tested	Yes to tryptophan. No to phenylalanine (tyrosine not tested)	Yes
	Did P.s.t exhibit significant chemotaxis towards the putative substrate at apoplast concentrations?	Yes	Not tested	No	No
Amino acid utilisation	Can the putative substrate be used by P.s.t as a source of carbon, nitrogen or carbon and nitrogen for growth?	Yes to all	Weakly metabolised as an N and C source	Weakly phenylalanine (tyrosine not tested)	No to all
	Does adding the putative substrate to M9 media significantly increase P.s.t growth?	Yes	Not tested	No (tyrosine not tested)	Yes

Table 1
Summary of what has been discovered about the P.s.t aa_permeases and candidate substrates

The aa_permease genes P.s.t are annotated as transporting GABA, ethanolamine, proline and aromatic amino acids. These functional annotations can now be revisited in light of the findings in this work and related studies.

- **PSPTO_5356 – GABA permease (GabP).**

All *Pseudomonas* genomes investigated contained at least one GABA permease, and phylogenetic analysis indicated that the majority of these sequences grouped together in a single clade, indicating a common ancestry for the GABA permeases in *Pseudomonas*. In addition, *P. aeruginosa, P. entomophila* and *P. putida* genomes contained multiple GABA permease genes, indicating that that the ability to utilise GABA may be important for both plant pathogenic and non plant-pathogenic species.

As the expression of a transporter is likely to be increased in the presence of the transported substrate, RT-PCR was carried out to investigate the expression of the aa_permease genes in the presence of putatively transported amino acids. In P.s.t, expression of the GABA permease was significantly increased in the presence of GABA, suggesting that the annotation of this gene as encoding a GABA transporter was correct. The ability to utilise GABA might be particularly important for P.s.t in the plant apoplast as this amino acid was the most abundant in the host plant tomato apoplast (Rico and Preston., 2008). P.s.t was also capable of metabolising GABA as a carbon or nitrogen source (Rico and Preston., 2008), and the present study demonstrated that GABA could also be utilised by P.s.t as a sole carbon, nitrogen or carbon and nitrogen source for growth. In addition, expression of the GABA permease was increased in the presence of apoplast extracted from tomato plants. These results suggest that GABA may provide a major nutrient source for P.s.t in the tomato apoplast in a similar manner to that reported for *C. fulvum* by Solomon and coworkers (2002). In keeping with these results, the present work also identified a role for the nitrogen regulator RpoN in regulating the GABA permease. Although automated bioinformatic analysis did not detect a putative binding site for RpoN in Chapter 3, expression analysis of the GABA permease in Chapter 6 indicated that expression of this gene was significantly reduced in the P.s.t-*rpoN* mutant compared to wild type P.s.t and may therefore be regulated by RpoN. A putative RpoN-binding site was subsequently identified upstream of PSPTO_5356. Therefore RpoN may play a role in regulating the expression of this gene in response to nitrogen availability in the plant environment.

P.s.t was also able to chemotax towards GABA at tomato apoplast concentrations, indicating that P.s.t may demonstrate energy taxis towards this amino acid. This may

be particularly important in the phyllosphere where the distribution of nutrient sources heterogeneous and may be concentrated in certain structures such as around trichomes (Mercier and Lindow, 2000; Leveau and Lindow, 2001). P.s.t was also able to chemotax significantly towards extracted tomato apoplast, even at dilute concentrations. Therefore chemotaxis towards apoplast and abundant amino acids such as GABA may represent one mechanism whereby P.s.t is able to move towards openings in the leaf surface such as wounds or open stomata.

Very recently a P.s.t GABA permease mutant was generated by Duck Hwan Park (Cornell University, USA). Preliminary data for this mutant suggested that the P.s.t-*gabP* mutant had lost the ability to derive a growth 'boost' from the addition of GABA to minimal medium (as shown in Chapter 4 for wild type P.s.t). However, the growth of P.s.t-*gabP* in *A. thaliana* was not significantly different to that of wild type P.s.t (Duck Hwan Park, personal communication). In combination, these results indicate that although GABA is capable of providing a nitrogen and carbon source for P.s.t, the ability to utilise GABA in the plant apoplast is not essential for growth of this bacterium *in planta*. However, as GABA levels were reported to be low in *A. thaliana*, it may be interesting to repeat this experiment with tomato plants.

There was also preliminary evidence to suggest that P.s.t-*gabP* produced a slightly stronger HR when inoculated in tobacco, and increased symptoms when inoculated into tomato (Duck Hwan Park, personal communication). These results are particularly interesting in light of the findings presented in Chapter 7; that tobacco plants with increased GABA produced fewer symptoms upon inoculation with the host pathogen P.s.tabaci. The role of GABA in plants has been the subject of many lines of investigation but remains largely speculative. One role proposed for GABA is as a component of plant defence. Results in Chapter 7 suggest that transgenic plants containing increased GABA may have an increased ability to respond to pathogen infection.

- **PSPTO_2026 – ethanolamine permease.**

The substrate(s) transported by this permease remain speculative. Expression of PSPTO_2026 was not significantly increased in the presence of ethanolamine, suggesting that the substrate annotation for this transporter may be incorrect. One possible explanation for this may be that this gene is a histamine transporter, based on a transposon mutant in *P. aeruginosa* PAK, PA0220 (Johnson et al., 2005). Like PSPTO_2026, PA0220 has also been annotated as an ethanolamine transporter

(Johnson et al., 2005). The expression of PSPTO_2026 was not increased in the presence of tomato apoplast extracted from (uninfected) tomato plants. Low levels of ethanolamine have recently been detected in the uninfected tomato apoplast (Rico and Preston, personal communication). However, the apoplastic amino acid abundance is known to alter during pathogen infection (Solomon and Oliver., 2001; Rico and Preston, personal communication); therefore PSPTO_2026 may be expressed later in colonisation in response to altered conditions in the tomato apoplast. Bioinformatic and expression analysis both indicated that gene was be regulated by RpoN. These findings support a hypothesis that this gene is regulated in response to changes in nitrogen availability, such as those which occur in the apoplast during infection.

- **PSPTO_1817 – aromatic amino acid permease.**

The expression of the aromatic permease was not increased in the presence of the aromatic amino acids tryptophan or phenylalanine. However, expression in the presence of the aromatic amino acid tyrosine was not tested and therefore this amino acid remains a candidate substrate. Tyrosine was not measured in the tomato apoplast by Rico and Preston (2008); however NMR analysis has subsequently identified this amino acid in infected and uninfected apoplast (Gail Preston, personal communication). Tyrosine was also reported to increase in the tomato apoplast during by *C. fulvum*; in this interaction tyrosine was observed to increase from almost-undetectable levels to approximately 0.2 mM concentrations (Solomon and Oliver., 2001). Therefore tyrosine is an obvious candidate for further work to try and identify the transported solute for PSPTO_1817.

- **PSPTO_5276 – proline permease (ProY).**

This gene was annotated as a proline permease in P.s.t. However, expression analysis subsequently demonstrated that PSPTO_5276 was upregulated in the presence of histidine but not proline, suggesting that this gene encodes a histidine transporter. This conclusion was supported by the genomic location of this gene between histidine utilisation and transport genes (Chapter 3), and by the characterisation of the orthologous gene in P.fl.SBW25, *hutT*, which was experimentally demonstrated to transport histidine (Zhang and Rainey, 2007). The histidine (*hut*) gene operon has not been studied in P.s.t in particular; however, results in Chapter 6 demonstrate that the genes of the P.s.t *hut* operon are co-transcribed, supporting previous bioinformatic predictions in Chapter 3 that these genes form an operon in P.s.t.

Histidine was not found to be abundant in the tomato apoplast (Rico and Preston, 2008) and P.s.t was unable to chemotax towards this amino acid at tomato apoplast concentrations in Chapter 5. However, histidine can be metabolised by P.s.t as a carbon and nitrogen source (Rico and Preston., 2008), and the addition of histidine to M9 minimal media significantly increased the growth of P.s.t in Chapter 4. Therefore the uptake of histidine may be advantageous to P.s.t in the plant environment. Characterisation of the *hutT* mutant in P.fl.SBW25 demonstrated that this gene was upregulated on the plant surface (where histidine abundance was also found to be low), but that *hutT* was not required for colonisation of sugar beet (Zhang et al., 2006). Complementary to this, the present study demonstrated that PSPTO_5276 expression was induced in the presence to extracted tomato apoplast, where histidine is also low. Together these results suggest that histidine is available in both the epiphytic and endophytic plant niches, and that histidine is taken up by *Pseudomonas* species in both environments.

- **The aa_permease transporters in general.**

The aa_permease transporters belong to the APC transporter superfamily, which harness the movement of small charged ions (such as H^+ or Na^+) across a membrane to transport an amino acid substrate. Bioinformatic analysis of protein domains found in APC transporters, Bioinformatic analysis indicated that transporters containing APC-domains were significantly underrepresented in *P. syringae* genomes compared to non plant-pathogenic *Pseudomonas*. This trend was also observed in the plant-pathogenic species *Xanthomonas* and *Xylella fastidiosa*. Different APC transporters transport a range of substrates but all utilise the same transport mechanism. Therefore, we can speculate that there may be a selective disadvantage to this type of transporter in the plant environment.

In keeping with this hypothesis, the results of this thesis indicated an effect of pH on nutrient utilisation by P.s.t. In particular the utilisation of GABA and histidine, which are known to be transported by permeases, was slightly inhibited in alkaline media. This may indicate a pH effect on the ionic form of certain amino acids, which might inhibit their uptake, or an effect on particular amino acid transporters themselves. As APC superfamily transporters utilise electrochemical potential energy, rather than, for example, ATP hydrolysis, these may be particularly sensitive to environmental pH. Therefore the fluctuating pH of the plant apoplast, and the increasingly alkaline conditions during pathogenesis, may negatively affect the functioning of these transporters more than those which function using alternate mechanisms. This study

also demonstrated that the growth of *P. syringae* was limited to a narrower range of acid and alkaline pH values compared the growth of *P. aeruginosa* and *P. fluorescens*. Therefore one explanation for the poor growth of *P. syringae* at alkaline pH may result from a reduced ability to assimilate nutrients. One interesting point is that the original study by Solomon and coworkers (2005), which identified the aa_permease genes as being overrepresented in *P. syringae*, also identified overrepresented protein domains found in ATP-driven transporters. These included the BPD_transp_1 and BPD_transp_2 protein domains which are found in some amino acid transporters.

Finally, the work in this thesis allows us to speculate about the evolutionary fate of some of the aa_permease transporters in *Pseudomonas*. In general it appears as though the current distribution of aa_permease genes in *P. syringae* compared to non plant pathogenic *Pseudomonas* is the result of gene loss in *P. syringae*, rather than extensive duplication in non plant pathogenic *Pseudomonas* species. The selective pressure behind this loss is still unknown, as is whether the process is ongoing and the remaining four permease genes will be lost in the future, but the results of this thesis suggest that for the GABA and histidine permeases at least, there may be a benefit to *P. syringae* in retaining these genes.

In summary, the work in this thesis has helped to further the understanding of how P.s.t assimilates nutrients in the plant apoplast. In particular, the interaction between P.s.t and GABA is an interesting topic for future research. GABA has long been recognised as an interesting molecule within plants, and this is also seen in Chapter 7, which shows that plants overexpressing GABA show fewer symptoms upon infection with *P. syringae*. However, this thesis also shows that GABA may be an important molecule for plant pathogenic bacteria such as *P. syringae*, who may be exploiting this molecule for their own ends within the plant. The successful creation of a P.s.t aa_permease mutant, lacking some or all of its aa_permease transporters, will aid this future work.

Abbott J.C., Aanensen D.M., Rutherford K., Butcher S., and Spratt B.G. (2005). WebACT--an online companion for the Artemis Comparison Tool. Bioinformatics **21,** 3665-3666.

Abramovitch R.B. and Martin G.B. (2004). Strategies used by bacterial pathogens to suppress plant defenses. Curr. Opin. Plant Biol. **7,** 356-364.

Adie B., Chico J.M., Rubio-Somoza I., and Solano R. (2007). Modulation of plant defenses by ethylene. J. Plant. Growth. Regul **26,** 160-177.

Adler J. (1973). A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli* . J. Gen. Microbiol. **74,** 77-91.

Agrios G.N. (2005). Plant pathology, 5th ed. (: Academic Press).

Ahn S.J., Yang C.H., and Cooksey D.A. (2007). *Pseudomonas putida* 06909 genes expressed during colonization on mycelial surfaces and phenotypic characterization of mutants. J. Appl. Microbiol. **103,** 120-132.

Akama K. and Takaiwa F. (2007). C-terminal extension of rice glutamate decarboxylase (OsGAD2) functions as an autoinhibitory domain and overexpression of a truncated mutant results in the accumulation of extremely high levels of GABA in plant cells. J. Exp. Bot. **58,** 2699-2707.

Alfano J.R. and Collmer A. (1996). Bacterial pathogens in plants: Life up against the wall. Plant Cell **8,** 1683-1698.

Alfano J.R. and Collmer A. (2004). Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu. Rev. Phytopathol. **42,** 385-414.

Almeida N.F., Yan S., Lindeberg M., Studholme D.J., Schneider D.J., Condon B., Liu H., Viana C.J., Warren A., Evans C., Kemen E., Maclean D., Angot A., Martin G.B., Jones J.D., Collmer A., Setubal J.C., and Vinatzer B.A. (2009). A draft genome sequence of *Pseudomonas syringae* pv. tomato T1 reveals a type III effector repertoire significantly divergent from that of *Pseudomonas syringae* pv. tomato DC3000. Mol. Plant Microbe Interact. **22,** 52-62.

Almeida R.P., Mann R., and Purcell A.H. (2004). *Xylella fastidiosa* cultivation on a minimal solid defined medium. Curr. Microbiol. **48,** 368-372.

Alvarez-Ortega C. and Harwood C. (2007). Identification of a malate chemoreceptor in *Pseudomonas aeruginosa* by screening for chemotaxis defects in an energy taxis-deficient mutant. **73,**(23): 7793–7795

Andreote F.D., de Araujo W.L., de Azevedo J.L., van Elsas J.D., da Rocha U.N., and van Overbeek L.S. (2009). Endophytic colonization of potato (*Solanum tuberosum L.*) by a novel competent bacterial endophyte, *Pseudomonas putida* strain P9, and the effect on associated bacterial communities. Appl. Environ. Microbiol. Published online ahead of print.

Andrews J.H. and Harris R.F. (2000). The Ecology and Biogeography of Microorganisms on Plant Surfaces. Annu. Rev. Phytopathol. **38,** 145-180.

Ansari M.I., Ruey-Hua L., and Shu-Chen G.C. (2004). A novel senescence-associated gene encoding γ-aminobutyric acid (GABA):pyruvate transaminase is upregulated during rice leaf senescence. Physiologia Plantarum **123,** 1-8.

Arnold D.L., Jackson R.W., Waterfield N.R., and Mansfield J.W. (2007). Evolution of microbial virulence: the benefits of stress. Trends Genet. **23,** 293-300..

Atkinson M.M. and Baker C.J. (1987). Association of host plasma-membrane K+/H+ exchange with multiplication of *Pseudomonas syringae* pv. syringae in *Phaseolus vulgaris*. Phytopathol. **77,** 1273-1279.

Atkinson M.M. and Baker C.J. (1987). Alteration of plasmalemma sucrose transport in *Phaseolus vulgaris* by *Pseudomonas syringae* pv. syringae and its association with H+/K+ exchange. Phytopathol. **77,** 1573-1578.

Bacilio-Jiménez M., Aguilar-Flores S., Ventura-Zapata E., Pérez-Campos E., Bouquelet S., and Zenteno E. (2003). Chemical characterization of root exudates from rice and their effects on the chemotactic response of endophytic bacteria. **249,** 271-277.

Badel J.L., Charkowski A.O., Deng W.L., and Collmer A. (2002). A gene in the *Pseudomonas syringae* pv. tomato Hrp pathogenicity island conserved effector locus, *hopPtoA1*, contributes to efficient formation of bacterial colonies in planta and is duplicated elsewhere in the genome. Mol. Plant Microbe Interact. **15,** 1014-1024.

Bailey M.J., Lilley A.K., Thompson I.P., Rainey P.B., and Ellis R.J. (1995). Site directed chromosomal marking of a fluorescent pseudomonad isolated from the phytosphere of sugar beet; stability and potential for marker gene transfer. Mol. Ecol. **4,** 755-763.

Bateman A., Coin L., Durbin R., Finn R.D., Hollich V., Griffiths-Jones S., Khanna A., Marshall M., Moxon S., Sonnhammer E.L., Studholme D.J., Yeats C., and Eddy S.R. (2004). The Pfam protein families database. Nucleic Acids Res. **32,** 138-141.

Baum G., Lev-Yadun S., Fridmann Y., Arazi T., Katsnelson H., Zik M., and Fromm H. (1996). Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. EMBO J. **15,** 2988-2996.

Bearson B.L., Wilson L., and Foster J.W. (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. J. Bacteriol. **180,** 2409-2417.

Beattie G.A. and Lindow S.E. (1995). The secret life of foliar bacterial pathogens on leaves. Annu. Rev. Phytopathol. **33,** 145-172.

Beattie G.A. and Lindow S.E. (1999). Bacterial colonization of leaves: a spectrum of strategies. Phytopathol. **89,** 353-359.

Beattie G.A. and Axtell C.A. (2002). The use of *proU-gfp* transcriptional fusion to quantify water stress on the leaf surface. In Biology of Plant-Microbe Interactions. Vol. 3, S. A. Leong, C. Allen and E. W. Triplett, eds pp. 235-240.

Behrendt U., Ulrich A., Schumann P., Meyer J.M., and Sproer C. (2007). *Pseudomonas lurida* sp. nov., a fluorescent species associated with the phyllosphere of grasses. Int. J. Syst. Evol. Microbiol. **57,** 979-985.

Belitsky B.R. and Sonenshein A.L. (2002). GabR, a member of a novel protein family, regulates the utilization of gamma-aminobutyrate in *Bacillus subtilis* . Mol. Microbiol. **45,** 569-583.

Bell K.S., Sebaihia M., Pritchard L., Holden M.T., Hyman L.J., Holeva M.C., Thomson N.R., Bentley S.D., Churcher L.J., Mungall K., Atkin R., Bason N., Brooks K., Chillingworth T., Clark K., Doggett J., Fraser A., Hance Z., Hauser H., Jagels K., Moule S., Norbertczak H., Ormond D., Price C., Quail M.A., Sanders M., Walker D., Whitehead S., Salmond G.P., Birch P.R., Parkhill J., and Toth I.K. (2004). Genome sequence of the enterobacterial

phytopathogen *Erwinia carotovora* subsp. atroseptica and characterization of virulence factors. Proc. Natl. Acad. Sci. U. S. A. **101,** 11105-11110.

Bender C.L., Alarcon-Chaidez F., and Gross D.C. (1999). *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol. Rev. **63,** 266-292.

Beuve N., Rispal N., Laine P., Cliouet J.B., Ourry A., and Le Deunff E. (2004).Putative role of γ-aminobutyric acid (GABA) as a long-distance signal in up-regulation of nitrate uptake in *Brassica napus L.*. Plant. Cell. Environ. **27,** 1035-1046.

Block A., Schmelz E., O'Donnell P.J., Jones J.B., and Klee H.J. (2005). Systemic acquired tolerance to virulent bacterial pathogens in tomato. Plant Physiol. **138,** 1481-1490.

Boch J., Joardar V., Gao L., Robertson T.L., Lim M., and Kunkel B.N. (2002). Identification of *Pseudomonas syringae* pv. tomato genes induced during infection of *Arabidopsis thaliana* . Mol. Microbiol. **44,** 73-88.

Bolton M.D. and Thomma B.P.H.J. (2008). The complexity of nitrogen metabolism and nitrogen regulated gene expression in plant pathogenic fungi. Phys. Mol. Plant. Path. **72,** 104-110.

Bolwell G.P., Butt V.S., Davies D.R., and Zimmerlin A. (1995). The origin of the oxidative burst in plants. Free Radic. Res. **23,** 517-532.

Bolwell G.P., Blee K.A., Butt V.S., Davies D.R., Gardner S.L., Gerrish C., Minibayeva F., Rowntree E.G., and Wojtaszek P. (1999). Recent advances in understanding the origin of the apoplastic oxidative burst in plant cells. Free Radic. Res. **31,** 137-45.

Bolwell G.P., Bindschedler L.V., Blee K.A., Butt V.S., Davies D.R., Gardner S.L., Gerrish C., and Minibayeva F. (2002). The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. J. Exp. Bot. **53,** 1367-1376.

Bouche N., Lacombe B., and Fromm H. (2003). GABA signaling: a conserved and ubiquitous mechanism. Trends Cell Biol. **13,** 607-610.

Bouche N. and Fromm H. (2004). GABA in plants: just a metabolite? Trends Plant Sci. **9,** 110-115.

Bourdineaud J.P., Heierli D., Gamper M., Verhoogt H.J., Driessen A.J., Konings W.N., Lazdunski C., and Haas D. (1993). Characterization of the arcD arginine:ornithine exchanger of *Pseudomonas aeruginosa*. Localization in the cytoplasmic membrane and a topological model. J. Biol. Chem. **268,** 5417-5424.

Bown A.W. and Shelp B.J. (1997). The metabolism and functions of *γ*-aminobutyric acid. Plant Physiol **115,** 1-5.

Brandl M.T. (2006). Fitness of human enteric pathogens on plants and implications for food safety. Annu. Rev. Phytopathol. **44,** 367-392.

Brechtel C.E., Hu L., and King S.C. (1996). Substrate specificity of the *Escherichia coli* 4-aminobutyrate carrier encoded by *gabP*. Uptake and counterflow of structurally diverse molecules. J. Biol. Chem. **271,** 783-788.

Brechtel C.E. and King S.C. (1998). 4-Aminobutyrate (GABA) transporters from the amine-polyamine-choline superfamily: substrate specificity and ligand recognition profile of the 4-aminobutyrate permease from *Bacillus subtilis* . Biochem. J. **333** (Pt 3), 565-571.

Bretz J.R., Mock N.M., Charity J.C., Zeyad S., Baker C.J., and Hutcheson S.W. (2003). A translocated protein tyrosine phosphatase of *Pseudomonas syringae* pv. tomato DC3000 modulates plant defence response to infection. Mol. Microbiol. **49,** 389-400.

Brooks D.M., Bender C., and Kunkel B.N. (2005). The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid dependent defences in *Arabidopsis thaliana*. Mol. Plant. Pathol. **6,** 629-639.

Brown D.G. and Allen C. (2004). *Ralstonia solanacearum* genes induced during growth in tomato: an inside view of bacterial wilt. Mol. Microbiol. **53,** 1641-1660.

Brugiere N., Dubois F., Limami A.M., Lelandais M., Roux Y., Sangwan R.S., and Hirel B. (1999). Glutamine synthetase in the phloem plays a major role in controlling proline production. Plant Cell **11,** 1995-2012.

Buell C.R., Joardar V., Lindeberg M., Selengut J., Paulsen I.T., Gwinn M.L., Dodson R.J., Deboy R.T., Durkin A.S., Kolonay J.F., Madupu R., Daugherty S., Brinkac L., Beanan M.J., Haft D.H., Nelson W.C., Davidsen T., Zafar N., Zhou L., Liu J., Yuan Q., Khouri H., Fedorova N., Tran B., Russell D., Berry K., Utterback T., Van Aken S.E., Feldblyum T.V., D'Ascenzo M., Deng W.L., Ramos A.R., Alfano J.R., Cartinhour S., Chatterjee A.K., Delaney T.P., Lazarowitz S.G., Martin G.B., Schneider D.J., Tang X., Bender C.L., White O., Fraser C.M., and Collmer A. (2003). The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. Proc. Natl. Acad. Sci. U. S. A. **100,** 10181-10186.

Bultreys A. and Gheysen I. (2000). Production and comparison of peptide siderophores from strains of distantly related pathovars of *Pseudomonas syringae* and *Pseudomonas viridiflava* LMG 2352. Appl. Environ. Microbiol. **66,** 325-331.

Cameron R.K., Paiva N.L., Lamb C.J., and Dixon R.A. (1999). Accumulation of salicylic acid and *PR-1* gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv tomato in *Arabidopsis*. Phys. Mol. Plant. Path. **55,** 121-130.

Camilli A. and Bassler B.L. (2006). Bacterial small-molecule signaling pathways. Science **311,** 1113-1116.

Cao H., Bowling S.A., Gordon A.S., and Dong X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583-1592.

Carapito R., Hatsch D., Vorwerk S., Petkovski E., Jeltsch J.M., and Phalip V. (2008). Gene expression in *Fusarium graminearum* grown on plant cell wall. Fungal Genet. Biol. **45,** 738-748.

Chang A.B., Lin R., Keith Studley W., Tran C.V., and Saier M.H.,Jr (2004). Phylogeny as a guide to structure and function of membrane transport proteins. Mol. Membr. Biol. **21,** 171-181.

Chen F., Li Q., Sun L., and He Z. (2006). The rice 14-3-3 gene family and its involvement in responses to biotic and abiotic stress. DNA Res. **13,** 53-63.

Chen Z., Agnew J.L., Cohen J.D., He P., Shan L., Sheen J., and Kunkel B.N. (2007). *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. Proc. Natl. Acad. Sci. U. S. A. **104,** 20131-20136.

Chet I., Zilberstein Y., and Henis Y. (1973). Chemotaxis of *Pseudomonas lachrymans* to plant extracts and to water droplets collected from the leaf surfaces of resistant and susceptible plants. **3,** 473-479.

Chevrot R., Rosen R., Haudecoeur E., Cirou A., Shelp B.J., Ron E., and Faure D. (2006). GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens* . Proc. Natl. Acad. Sci. U. S. A. **103,** 7460-7464.

Chisholm S.T., Dahlbeck D., Krishnamurthy N., Day B., Sjolander K., and Staskawicz B.J. (2005). Molecular characterization of proteolytic cleavage sites of the *Pseudomonas syringae* effector AvrRpt2. Proc. Natl. Acad. Sci. U. S. A. **102,** 2087-2092.

Choi K.H., Kumar A., and Schweizer H.P. (2006). A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64,** 391-397.

Chou H.T., Kwon D.H., Hegazy M., and Lu C.D. (2008). Transcriptome analysis of agmatine and putrescine catabolism in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **190,** 1966-1975.

Christen G.L. and Marshall R.T. (1984). Selected properties of lipase and protease of *Pseudomonas fluorescens* 27 produced in four media. J Dairy Sci **67,** 1680-1687.

Clark S.M., Di Leo R., Dhanoa P.K., Van Cauwenberghe O.R., Mullen R.T., and Shelp B.J. (2009). Biochemical characterization, mitochondrial localization, expression, and potential functions for an *Arabidopsis* gamma-aminobutyrate transaminase that utilizes both pyruvate and glyoxylate. J. Exp. Bot. **60,** 1743-1757.

Clarke A., Mur L.A., Darby R.M., and Kenton P. (2005). Harpin modulates the accumulation of salicylic acid by *Arabidopsis* cells via apoplastic alkalization. J. Exp. Bot. **56,** 3129-3136.

Clarke C.J., Holland P.T., and Smith G.S. (1986). Chemical composition of bleeding xylem sap from kiwifruit vines. Ann. Bot. (Lond) **58,** 353-362.

Coleman S.T., Fang T.K., Rovinsky S.A., Turano F.J., and Moye-Rowley W.S. (2001). Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae* . J. Biol. Chem. **276,** 244-250.

Comparot S., Lingiah G., and Martin T. (2003). Function and specificity of 14-3-3 proteins in the regulation of carbohydrate and nitrogen metabolism. J. Exp. Bot. **54,** 595-604.

Cosgriff A.J. and Pittard A.J. (1997). A topological model for the general aromatic amino acid permease, AroP, of *Escherichia coli* . J. Bacteriol. **179,** 3317-3323.

Cowley T. and Walters D.R. (2002). Polyamine metabolism in barley reacting hypersensitively to the powdery mildew fungus *Blumeria graminis* f. sp. hordei. P. Cell & Environ. **25,** 461-468.

Cui J., Bahrami A.K., Pringle E.G., Hernandez-Guzman G., Bender C.L., Pierce N.E., and Ausubel F.M. (2005). *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. Proc. Natl. Acad. Sci. U. S. A. **102,** 1791-1796.

Cunnac S., Lindeberg M., and Collmer A. (2009). *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. Curr. Opin. Microbiol. **12,** 53-60.

Cuppels D. (1988). Chemotaxis by *Pseudomonas syringae pv*. tomato. **54** (3):629–632

da Silva A.C.R., Ferro J.A., Reinach F.C., Farah C.S., Furlan L.R., Quaggio R.B., Monteiro-Vitorello C.B., Van Sluys M.A., Almeida N.F., Alves L.M.C., do Amaral A.M., Bertolini M.C., Camargo L.E.A., Camarotte G., Cannavan F., Cardozo J., Chambergo F., Clapina L.P., Cicarelli R.M.B., Coutinho L.L., Cursino-Santos J.R., El-Dorry H., Faria J.B., Ferreira A.J.S., Ferreira R.C.C., Ferro M.I.T., Formighieri E.F., Franco M.C., Greggio C.C., Gruber A., Katsuyama A.M., Kishi L.T., Leite R.P., Lemos E.G.M., Meidanis J., Menck C.F.M.,

Miyaki C.Y., Moon D.H., Moreira L.M., Novo M.T.M., Okura V.K., Oliveira M.C., Oliveira V.R., Pereira H.A., Rossi A., Sena J.A.D., Silva C., de Souza R.F., Spinola L.A.F., Takita M.A., Tamura R.E., Teixeira E.C., Tezza R.I.D., dos Santos M.T., Truffi D., Tsai S.M., White F.F., Setubal J.C., and Kitajima J.P. (2002). Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. **417,** 459-463.

Dangl J.L., Dietrich R.A., and Richberg M.H. (1996). Death don't have no mercy: Cell death programs in plant-microbe interactions. Plant Cell **8,** 1793-1807.

Dangl J.L. and Jones J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature **411,** 826-833.

Darzins A. (1994). Characterization of a P*seudomonas aeruginosa* gene cluster involved in pilus biosynthesis and twitching motility: sequence similarity to the chemotaxis proteins of enterics and the gliding bacterium *Myxococcus xanthus*. Mol. Microbiol. **11,** 137-153.

de Torres M., Sanchez P., Fernandez-Delmond I., and Grant M. (2003). Expression profiling of the host response to bacterial infection: the transition from basal to induced defence responses in RPM1-mediated resistance. Plant J. **33,** 665-676.

de Torres-Zabala M., Truman W., Bennett M.H., Lafforgue G., Mansfield J.W., Rodriguez Egea P., Bogre L., and Grant M. (2007). *Pseudomonas syringae* pv. tomato hijacks the *Arabidopsis* abscisic acid signalling pathway to cause disease. EMBO J. **26,** 1434-1443.

de Weert S., Vermeiren H., Mulders I.H., Kuiper I., Hendrickx N., Bloemberg G.V., Vanderleyden J., De Mot R., and Lugtenberg B.J. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens* . **15,** 1173-1180.

de Weert S., Kuiper I., Lagendijk E.L., Lamers G.E., and Lugtenberg B.J. (2004). Role of chemotaxis toward fusaric acid in colonization of hyphae of *Fusarium oxysporum* f. sp. radicis-lycopersici by P*seudomonas fluorescens* WCS365. Mol. Plant Microbe Interact. **17,** 1185-1191.

DebRoy S., Thilmony R., Kwack Y.B., Nomura K., and He S.Y. (2004). A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. Proc. Natl. Acad. Sci. U. S. A. **101,** 9927-9932.

Deng W.L., Preston G., Collmer A., Chang C.J., and Huang H.C. (1998). Characterization of the hrpC and hrpRS operons of *Pseudomonas syringae* pathovars syringae, tomato, and glycinea and analysis of the ability of hrpF, hrpG, hrcC, hrpT, and hrpV mutants to elicit the hypersensitive response and disease in plants. J. Bacteriol. **180,** 4523-4531.

Devadas S.K. and Raina R. (2002). Preexisting systemic acquired resistance suppresses hypersensitive response-associated cell death in *Arabidopsis hrl1* mutant. Plant Physiol. **128,** 1234-1244.

Diaz C., Purdy S., Christ A., Morot-Gaudry J.F., Wingler A., and Masclaux-Daubresse C. (2005). Characterization of markers to determine the extent and variability of leaf senescence in *Arabidopsis*. A metabolic profiling approach. Plant Physiol. **138,** 898-908.

Dietrich R., Ploss K., and Heil M. (2004). Constitutive and induced resistance to pathogens in *Arabidopsis thaliana* depends on nitrogen supply. Plant, Cell, Environ. **27,** 896-906.

Divon H.H., Ziv C., Davydov O., Yarden O., and Fluhr R. (2006). The global nitrogen regulator, FNR1, regulates fungal nutrition-genes and fitness during *Fusarium oxysporum* pathogenesis. Mol. Plant. Pathol **7,** 485-497.

Divon H.H., Rothan-Denoyes B., Davydov O., DiPietro A., and Fluhr R. (2008). Nitrogen-responsive genes are differentially regulated in planta during *Fusarium oxyspsorum f. sp. lycopersici* infection. Mol. Plant. Pathol **6,** 459-470.

Dos Santos V.A., Heim S., Moore E.R., Stratz M., and Timmis K.N. (2004). Insights into the genomic basis of niche specificity of *Pseudomonas putida* KT2440. Environ. Microbiol. **6,** 1264-1286.

Dulla G. and Lindow S.E. (2008). Quorum size of *Pseudomonas syringae* is small and dictated by water availability on the leaf surface. Proc. Natl. Acad. Sci. U. S. A. **105,** 3082-3087.

Dumville J.C. and Fry S.C. (2000). Uronic acid-containing oligosaccharins: Their biosynthesis, degradation and signaling roles in non-diseased plant tissues. Plant Physiol. Biochem **38,** 125-140.

Dwyer M.A. and Hellinga H.W. (2004). Periplasmic binding proteins: a versatile superfamily for protein engineering. Curr. Opin. Struct. Biol. **14,** 495-504.

Ellis J. and Dodds P. (2003). Plant pathology: monitoring a pathogen-targeted host protein. Curr. Biol. **13,** 400-402.

Emanuelsson O., Brunak S., von Heijne G., and Nielsen H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. Nat. Protoc. **2,** 953-971.

Espinosa-Urgel M., Kolter R., and Ramos J.L. (2002). Root colonization by *Pseudomonas putida*: love at first sight. Microbiology **148,** 341-343.

Fabro G., Kovacs I., Pavet V., Szabados L., and Alvarez M.E. (2004). Proline accumulation and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions in *Arabidopsis* . Mol. Plant Microbe Interact. **17,** 343-350.

Feil H., Feil W.S., Chain P., Larimer F., DiBartolo G., Copeland A., Lykidis A., Trong S., Nolan M., Goltsman E., Thiel J., Malfatti S., Loper J.E., Lapidus A., Detter J.C., Land M., Richardson P.M., Kyrpides N.C., Ivanova N., and Lindow S.E. (2005). Comparison of the complete genome sequences of *Pseudomonas syringae* pv. syringae B728a and pv. tomato DC3000. Proc. Natl. Acad. Sci. U. S. A. **102,** 11064-11069.

Felle H.H. and Hanstein S. (2007). Probing apoplastic ion relations in *Vicia faba* as influenced by nutrition and gas exchange In The Apoplast of Higher Plants: Compartment of Storage, Transport and Reactions, B. Sattelmacher and W. J. Horst, eds pp. 295-306.

Felle H.H., Herrmann A., Hanstein S., Huckelhoven R., and Kogel K.H. (2004). Apoplastic pH signaling in barley leaves attacked by the powdery mildew fungus *Blumeria graminis* f. sp. hordei. Mol. Plant Microbe Interact. **17,** 118-123.

Feng T.Y. and Kuo T.T. (1975). Bacterial leaf blight of rice plant. VI. Chemotactic responses of *Xanthomonas oryzae* to water pores on the leaf of rice plants. Bot. Bull. Acad. Sin **16,** 126-136.

Ferrandez A., Hawkins A.C., Summerfield D.T., and Harwood C.S. (2002). Cluster II che genes from *Pseudomonas aeruginosa* are required for an optimal chemotactic response. J. Bacteriol. **184,** 4374-4383.

Fierer N., Bradford M.A., and Jackson R.B. (2007). Toward an ecological classification of soil bacteria. Ecology **88,** 1354-1364.

Figurski D.H. and Helinski D.R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. U. S. A. **76,** 1648-1652.

Fitch W.M. (2000). Homology a personal view on some of the problems. Trends Genet. **16,** 227-231.

Flors V., Ton J., Jakab G., and Mauch-Mani B. (2005). Abscinic acid and callose: Team players in the defence against pathogens? J Phytopathol. **153,** 377-383.

Fox K.A., Ramesh A., Stearns J.E., Bourgogne A., Reyes-Jara A., Winkler W.C., and Garsin D.A. (2009). Multiple posttranscriptional regulatory mechanisms partner to control ethanolamine utilization in *Enterococcus faecalis* . Proc. Natl. Acad. Sci. U. S. A. **106,** 4435-4440.

Freeman B.C. and Beattie G.A. (2009). Bacterial growth restriction during host resistance to *Pseudomonas syringae* is associated with leaf water loss and localized cessation of vascular activity in *Arabidopsis thaliana*. M. P. M. I. **22,** (7) 857–867.

Fryer M.J., Oxborough K., Mullineaux P.M., and Baker N.R. (2002). Imaging of photo-oxidative stress responses in leaves. J. Exp. Bot. **53,** 1249-1254.

Fu Z.Q., Guo M., Jeong B.R., Tian F., Elthon T.E., Cerny R.L., Staiger D., and Alfano J.R. (2007). A type III effector ADP-ribosylates RNA-binding proteins and quells plant immunity. Nature **447,** 284-288.

Gal J., Szvetnik A., Schnell R., and Kalman M. (2002). The *metD* D-methionine transporter locus of *Escherichia coli* is an ABC transporter gene cluster. J. Bacteriol. **184,** 4930-4932.

Gal M., Preston G.M., Massey R.C., Spiers A.J., and Rainey P.B. (2003). Genes encoding a cellulosic polymer contribute toward the ecological success of *Pseudomonas fluorescens* SBW25 on plant surfaces. Mol. Ecol. **12,** 3109-3121.

Galperin M.Y. (2005). A census of membrane-bound and intracellular signal transduction proteins in bacteria: bacterial IQ, extroverts and introverts. BMC Microbiol. **5, (**35).

Gamper M., Zimmermann A., and Haas D. (1991). Anaerobic regulation of transcription initiation in the arcDABC operon of *Pseudomonas aeruginosa*. J. Bacteriol. **173,** 4742-4750.

Gardan R., Rapoport G., and Debarbouille M. (1995). Expression of the *rocDEF* operon involved in arginine catabolism in *Bacillus subtilis* . J. Mol. Biol. **249,** 843-856.

Genoud T., Buchala A.J., Chua N.H., and Metraux J.P. (2002). Phytochrome signalling modulates the SA-perceptive pathway in *Arabidopsis* . Plant J. **31,** 87-95.

Girousse C., Bournoville R., and Bonnemain J.L. (1996). Water Deficit-Induced Changes in Concentrations in Proline and Some Other Amino Acids in the Phloem Sap of Alfalfa. Plant Physiol. **111,** 109-113.

Gits J.J. and Grenson M. (1967). Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. 3. Evidence for a specific methionine-transporting system. Biochim. Biophys. Acta **135,** 507-516.

Glickmann E., Gardan L., Jacquet S., Hussain S., Elasri M., Petit A., and Dessaux Y. (1998). Auxin production is a common feature of most pathovars of *Pseudomonas syringae* . Mol. Plant Microbe Interact. **11,** 156-162.

Goel A.K., Lundberg D., Torres M.A., Matthews R., Akimoto-Tomiyama C., Farmer L., Dangl J.L., and Grant S.R. (2008). The *Pseudomonas syringae* type III effector HopAM1 enhances virulence on water-stressed plants. Mol. Plant Microbe Interact. **21,** 361-370.

Gohre V. and Robatzek S. (2008). Breaking the barriers: microbial effector molecules subvert plant immunity. Annu. Rev. Phytopathol. **46,** 189-215.

Gomez-Gomez L. (2004). Plant perception systems for pathogen recognition and defence. Mol. Immunol. **41,** 1055-1062.

Goodman R.N. (1972). Electrolyte leakage and membrane damage in relation to bacterial population, pH, and ammonia production in tobacco leaf tissue inoculated *Pseudomonas pisi*. Phytopathol. **62,** 1327-1331.

Gordillo F., Chavez F.P., and Jerez C.A. (2007). Motility and chemotaxis of *Pseudomonas* sp. B4 towards polychlorobiphenyls and chlorobenzoates. FEMS Microbiol. Ecol. **60,** 322-328.

Grant S.R., Fisher E.J., Chang J.H., Mole B.M., and Dangl J.L. (2006). Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. Annu. Rev. Microbiol. **60,** 425-449.

Grenson M. (1966). Multiplicity of the amino acid permeases in Saccharomyces cerevisiae. II. Evidence for a specific lysine-transporting system. Biochim. Biophys. Acta **127,** 339-346.

Grignon C. and Sentenac H. (1991). pH and ionic conditions in the apoplast. . Annu. Rev. Plant Phys. **42,** 103-128.

Grimm A.C. and Harwood C.S. (1997). Chemotaxis of *Pseudomonas spp*. to the polyaromatic hydrocarbon naphthalene. Appl. Environ. Microbiol. **63,** 4111-4115.

Griswold A., Chen Y.Y., Snyder J.A., and Burne R.A. (2004). Characterization of the arginine deiminase operon of *Streptococcus rattus* FA-1. Appl. Environ. Microbiol. **70,** 1321-1327.

Gross D.C. (1991). Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. Annu. Rev. Phytopathol. **29,** 247-278.

Gudesblat G.E., Torres P.S., and Vojnov A.A. (2009). *Xanthomonas campestris* overcomes *Arabidopsis* stomatal innate immunity through a DSF cell-to-cell signal-regulated virulence factor. Plant Physiol. **149,** 1017-1027.

Gunasekera T.S. and Sundin G.W. (2006). Role of nucleotide excision repair and photoreactivation in the solar UVB radiation survival of *Pseudomonas syringae* pv. syringae B728a. J. Appl. Microbiol. **100,** 1073-1083.

Gupta V.P., Tewari S.K., and Datta R.K. (1995). Surface ultrastructural studies on ingress and establishment of *Pseudomonas syringae* pv. mori on mulberry leaves. J. Phytopathol. **143,** 415-418.

Gut H., Pennacchietti E., John R.A., Bossa F., Capitani G., De Biase D., and Grutter M.G. (2006). *Escherichia coli* acid resistance: pH-sensing, activation by chloride and autoinhibition in GadB. EMBO J. **25,** 2643-2651.

Guvener Z.T. and Harwood C.S. (2007). Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. Mol. Microbiol. **66,** 1459-1473.

Haefele D.M. and Lindow S.E. (1987). Flagellar motility confers epiphytic fitness advantages upon *Pseudomonas syringae* . **53,** .

Hammerschmidt R. (1999). PHYTOALEXINS: What Have We Learned After 60 Years? Annu. Rev. Phytopathol. **37,** 285-306.

Hammes U.Z., Nielsen E., Honaas L.A., Taylor C.G., and Schachtman D.P. (2006). AtCAT6, a sink-tissue-localized transporter for essential amino acids in *Arabidopsis* . Plant J. **48,** 414-426.

Hanfrey C., Fife M., and Buchanan-Wollaston V. (1996). Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins. Plant Mol. Biol. **30,** 597-609.

Hanlon D.W., Marquez-Magana L.M., Carpenter P.B., Chamberlin M.J., and Ordal G.W. (1992). Sequence and characterization of *Bacillus subtilis* CheW. J. Biol. Chem. **267,** 12055-12060.

Hare P.D. and Cress W.A. (1997). Metabolic implications of stress-indiced proline accumulation in plants. Plant Growth Regulation **21,** 72-102.

Harrison C.J. and Langdale J.A. (2006). A step by step guide to phylogeny reconstruction. Plant J. **45,** 561-572, doi/10.1111/j.1365-313X.2005.02611.x.

Harwood C.S., Rivelli M., and Ornston L.N. (1984). Aromatic acids are chemoattractants for *Pseudomonas putida* . **160,**(2): 622–628.

Harwood C.S., Parales R.E., and Dispensa M. (1990). Chemotaxis of *Pseudomonas putida* toward chlorinated benzoates. Appl. Environ. Microbiol. **56,** 1501-1503.

Hasne M.P. and Ullman B. (2005). Identification and characterization of a polyamine permease from the protozoan parasite *Leishmania major*.. J. Biol. Chem. **280,** 15188-15194.

Hattermann D.R. and Ries S.M. (1989). Motility of *Pseudomonas syringae pv.* glycinea and its role in infection.Am. Phytopathol.**79**(33), 284-289.

Hauck P., Thilmony R and He S.E. (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defenses in susceptible *Arabidopsis thaliana* plants. PNAS. **100** (14) 8577-8582.

He J., Baldini R.L., Deziel E., Saucier M., Zhang Q., Liberati N.T., Lee D., Urbach J., Goodman H.M., and Rahme L.G. (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc. Natl. Acad. Sci. U. S. A. **101,** 2530-2535.

He S.Y. (1996). Elicitation of Plant Hypersensitive Response by Bacteria. Plant Physiol. **112,** 865-869.

Heber U., Tyankova L., and Santarius K.A. (1971). Stabilization and inactivation of biological membranes during freezing in the presence of amino acids. Biochim. Biophys. Acta **241,** 578-592.

Heil M. and Bostock R.M. (2002). Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. Ann. Bot. (Lond) **89,** 503-512.

Heineke D., Sonnewald U., Bussis D., Gunter G., Leidreiter K., Wilke I., Raschke K., Willmitzer L., and Heldt H.W. (1992). Apoplastic expression of yeast-derived invertase in potato: Effects on photosynthesis, leaf solute composition, water relations, and tuber composition. Plant Physiol. **100,** 301-308.

Hendrickson E.L., Guevera P., and Ausubel F.M. (2000). The alternative sigma factor RpoN is required for hrp activity in *Pseudomonas syringae* pv. maculicola and acts at the level of hrpL transcription. J. Bacteriol. **182,** 3508-3516.

Hendrickson E.L., Guevera P., Penaloza-Vazquez A., Shao J., Bender C., and Ausubel F.M. (2000). Virulence of the phytopathogen *Pseudomonas syringae* pv. maculicola is rpoN dependent. J. Bacteriol. **182,** 3498-3507.

Hendrickson E.L., Plotnikova J., Mahajan-Miklos S., Rahme L.G., and Ausubel F.M. (2001). Differential roles of the *Pseudomonas aeruginosa* PA14 rpoN gene in pathogenicity in plants, nematodes, insects, and mice. J. Bacteriol. **183,** 7126-7134.

Hervas A.B., Canosa I., and Santero E. (2008). Transcriptome analysis of *Pseudomonas putida* in response to nitrogen availability. J. Bacteriol. **190,** 416-420.

Hildebrand D.C., Palleroni N.J., Hendson M., Toth J., and Johnson J.L. (1994). *Pseudomonas flavescens* sp. nov., isolated from walnut blight cankers. Int. J. Syst. Bacteriol. **44,** 410-415.

Hirano S.S. and Upper C.D. (1990). Population biology and epidemiology of *Pseudomonas syringae* . **28,** 155-170.

Hirano S.S., Ostertag E.M., Savage S.A., Baker L.S., Willis D.K., and Upper C.D. (1997). Contribution of the Regulatory Gene lemA to Field Fitness of *Pseudomonas syringae* pv. syringae. Appl. Environ. Microbiol. **63,** 4304-4312.

Hirano S.S., Charkowski A.O., Collmer A., Willis D.K., and Upper C.D. (1999). Role of the Hrp type III protein secretion system in growth of *Pseudomonas syringae* pv. syringae B728a on host plants in the field. Proc. Natl. Acad. Sci. U. S. A. **96,** 9851-9856.

Hirano S.S. and Upper C.D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*-a pathogen, ice nucleus, and epiphyte. Microbiol. Mol. Biol. Rev. **64,** 624-653.

Hoffland E., Jeger M., and Van Beusichem M.L. (2000). Effect of nitrogen supply rate on disease resistance in tomato depends on the pathogen. Plant and Soil **218,** 239-247.

Hoffmann N. and Rehm B.H. (2005). Nitrogen-dependent regulation of medium-chain length polyhydroxyalkanoate biosynthesis genes in pseudomonads. Biotechnol. Lett. **27,** 279-282.

Hogenhout S.A., Van der Hoorn R.A., Terauchi R., and Kamoun S. (2009). Emerging concepts in effector biology of plant-associated organisms. Mol. Plant Microbe Interact. **22,** 115-122.

Holloway B.W. and Morgan A.F. (1986). Genome organization in *Pseudomonas*. Annu. Rev. Microbiol. **40,** 79-105.

Horsburgh M.J., Wiltshire M.D., Crossley H., Ingham E., and Foster S.J. (2004). PheP, a putative amino acid permease of *Staphylococcus aureus*, contributes to survival in vivo and during starvation. Infect. Immun. **72,** 3073-3076.

Hosie A.H. and Poole P.S. (2001). Bacterial ABC transporters of amino acids. Res. Microbiol. **152,** 259-270.

Hu L. and Phillips A.T. (1988). Organization and multiple regulation of histidine utilization genes in *Pseudomonas putida* . J. Bacteriol. **170,** 4272-4279.

Hu L.A. and King S.C. (1998). Membrane topology of the *Escherichia coli* gamma-aminobutyrate transporter: implications on the topography and mechanism of prokaryotic and eukaryotic transporters from the APC superfamily. Biochem. J. **336 (Pt 1),** 69-76.

Hu W.S., Lin Y.H., and Shih C.C. (2007). A proteomic approach to study *Salmonella enterica* serovar Typhimurium putative transporter YjeH associated with ceftriaxone resistance. Biochem. Biophys. Res. Commun. **361,** 694-699.

Huang H.C., Schuurink R., Denny T.P., Atkinson M.M., Baker C.J., Yucel I., Hutcheson S.W., and Collmer A. (1988). Molecular cloning of a *Pseudomonas syringae* pv. syringae gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. J. Bacteriol. **170,** 4748-4756.

Huang W.E., Wang H., Zheng H., Huang L., Singer A.C., Thompson I., and Whiteley A.S. (2005). Chromosomally located gene fusions constructed in *Acinetobacter* sp. ADP1 for the detection of salicylate. Environ. Microbiol. **7,** 1339-1348.

Huber D.M. and Thompson I.A. (2007). Nitrogen and plant disease In Mineral Nutrition and Plant Disease, L. E. Datnoff, W. H. Elmer and D. M. Huber, eds. The American Phytopathological Society.

Hueck C.J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62,** 379-433.

Hugouvieux V., Barber C.E., and Daniels M.J. (1998). Entry of *Xanthomonas campestris* pv. campestris into hydathodes of *Arabidopsis thaliana* leaves: a system for studying early infection events in bacterial pathogenesis. Mol. Plant Microbe Interact. **11,** 537-543.

Hutchings H., Stahmann K.P., Roels S., Espeso E.A., Timberlake W.E., Arst H.N.,Jr, and Tilburn J. (1999). The multiply-regulated *gabA* gene encoding the GABA permease of *Aspergillus nidulans*: a score of exons. Mol. Microbiol. **32,** 557-568.

Hutchison M.L., Tester M.A., and Gross D.C. (1995). Role of biosurfactant and ion channel-forming activities of syringomycin in transmembrane ion flux: a model for the mechanism of action in the plant-pathogen interaction. Mol. Plant Microbe Interact. **8,** 610-620.

Ichinose Y., Shimizu R., Ikeda Y., Taguchi F., Marutani M., Mukaihara T., Inagaki Y., Toyoda K., and Shiraishi T. (2003). Need for flagella for complete virulence of *Pseudomonas syringae* pv. tabaci: genetic analysis with flagella-defective mutants *fliC* and *fliD* in host tobacco plants. J. Gen. Plant. Pathol. **69,** 244-249.

Ishiga Y., Uppalapati S.R., Ishiga T., Elavarthi S., Martin B., and Bender C.L. (2009). The phytotoxin coronatine induces light-dependent reactive oxygen species in tomato seedlings. New Phytol. **181,** 147-160.

Itoh Y. and Nakada Y. (2004). Arginine and polyamine metabolism In *Pseudomonas*: Biosynthesis of Macromolecules and Molecular Metabolism, J. L. Ramos, ed (Birkhäuser) pp. 243-273.

Jack D.L., Paulsen I.T., and Saier M.H. (2000). The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. Microbiology **146 (Pt 8),** 1797-1814.

Jackson R.W. and Giddens S.R. (2006). Development and application of in vivo expression technology (IVET) for analysing microbial gene expression in complex environments. Infect. Disord. Drug Targets **6,** 207-240.

Jacobs J.L. and Sundin G.W. (2001). Effect of solar UV-B radiation on a phyllosphere bacterial community. Appl. Environ. Microbiol. **67,** 5488-5496.

Jakob K., Kniskern J.M., and Bergelson J. (2007). The role of pectate lyase and the jasmonic acid defense response in *Pseudomonas viridiflava* virulence. Mol. Plant Microbe Interact. **20,** 146-158.

Janzen D.J., Allen L.J., MacGregor K.B., and Bown A.W. (2001). Cytosolic acidification and gamma-aminobutyric acid synthesis during the oxidative burst in isolated *Asparagus sprengeri* mesophyll cells. Can. J. Bot. **79,** 438-443.

Jauniaux J.C. and Grenson M. (1990). GAP1, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. Eur. J. Biochem. **190,** 39-44.

Jelenska J., Yao N., Vinatzer B.A., Wright C.M., Brodsky J.L., and Greenberg J.T. (2007). A J-domain virulence effector of *Pseudomonas syringae* remodels host chloroplasts and suppresses defenses. Curr. Biol. **17,** 499-508.

Jennings M.P., Anderson J.K., and Beacham I.R. (1995). Cloning and molecular analysis of the *Salmonella enterica ansP* gene, encoding an L-asparagine permease. Microbiology **141 (Pt 1),** 141-146.

Joardar V., Lindeberg M., Jackson R.W., Selengut J., Dodson R., Brinkac L.M., Daugherty S.C., Deboy R., Durkin A.S., Giglio M.G., Madupu R., Nelson W.C., Rosovitz M.J., Sullivan S., Crabtree J., Creasy T., Davidsen T., Haft D.H., Zafar N., Zhou L., Halpin R., Holley T., Khouri H., Feldblyum T., White O., Fraser C.M., Chatterjee A.K., Cartinhour S., Schneider D.J., Mansfield J., Collmer A., and Buell C.R. (2005). Whole-genome sequence analysis of *Pseudomonas syringae* pv. phaseolicola 1448A reveals divergence among pathovars in genes involved in virulence and transposition. J. Bacteriol. **187,** 6488-6498.

Johnson D.A., Tetu S.G., Phillippy K., Chen J., Ren Q., and Paulsen I.T. (2008). High-throughput phenotypic characterization of *Pseudomonas aeruginosa* membrane transport genes. PLoS Genet. **4,** 1-11.

Jones J.D.G. and Dangl J.L. (2006). The plant immune system. Nature **444,** 323-329.

Jones J., Studholme D.J., Knight C.G., and Preston G.M. (2007). Integrated bioinformatic and phenotypic analysis of RpoN-dependent traits in the plant growth-promoting bacterium *Pseudomonas fluorescens* SBW25. Environ. Microbiol. **9,** 3046-3064.

Joosten M.H.A.J., Hendrick L.J.M., and De Wit P.J.D. (1990). Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent of avirulent races of *Cladosporium fulvum* (sy. Fulvia fulva). Euro. J. Plant. Pathol. **96,** 929-1873.

Kaffarnik F.A., Jones A.M., Rathjen J.P., and Peck S.C. (2009). Effector proteins of the bacterial pathogen *Pseudomonas syringae* alter the extracellular proteome of the host plant, *Arabidopsis thaliana* . Mol. Cell. Proteomics **8,** 145-156.

Kanehisa M., Goto S., Hattori M., Aoki-Kinoshita K.F., Itoh M., Kawashima S., Katayama T., Araki M., and Hirakawa M. (2006). From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. **34,** 354-357.

Kannan K., Janiyani K.L., Shivaji S., and Ray M.K. (1998). Histidine utilisation operon (hut) is upregulated at low temperature in the antarctic psychrotrophic bacterium Pseudomonas syringae. FEMS Microbiol. Lett. **161,** 7-14.

Kathiresan A., Tung P., Chinnappa C.C., and Reid D.M. (1997). gamma-aminobutyric acid stimulates ethylene biosynthesis in sunflower. Plant Physiol. **115,** 129-135.

Kato J., Nakamura T., Kuroda A., and Ohtake H. (1999). Cloning and characterization of chemotaxis genes in *Pseudomonas aeruginosa* . Biosci. Biotechnol. Biochem. **63,** 155-161.

Kay W.W. and Gronlund A.F. (1969). Influence of carbon or nitrogen starvation on amino acid transport in *Pseudomonas aeruginosa* . J. Bacteriol. **100,** 276-282.

Kearns D.B., Robinson J., and Shimkets L.J. (2001). *Pseudomonas aeruginosa* exhibits directed twitching motility up phosphatidylethanolamine gradients. J. Bacteriol. **183,** 763-767.

Keith L.M. and Bender C.L. (1999). AlgT (sigma22) controls alginate production and tolerance to environmental stress in *Pseudomonas syringae*. J. Bacteriol. **181,** 7176-7184.

Keith R.C., Keith L.M., Hernandez-Guzman G., Uppalapati S.R., and Bender C.L. (2003). Alginate gene expression by *Pseudomonas syringae* pv. tomato DC3000 in host and non-host plants. Microbiology **149,** 1127-1138.

Kelly-Wintenberg K. and Montie T.C. (1994). Chemotaxis to oligopeptides by *Pseudomonas aeruginosa* . Appl. Environ. Microbiol. **60,** 363-367.

Keon J., Antoniw J., Carzaniga R., Deller S., Ward J.L., Baker J.M., Beale M.H., Hammond-Kosack K., and Rudd J.J. (2007). Transcriptional adaptation of *Mycosphaerella graminicola* to programmed cell death (PCD) of its susceptible wheat host. Mol. Plant Microbe Interact. **20,** 178-193.

Kim B.J., Park J.H., Park T.H., Bronstein P.A., Schneider D.J., Cartinhour S.W., and Shuler M.L. (2009). Iron concentration limits growth rate and the expression of virulence factors in hrp-inducing minimal medium with *Pseudomonas syringae* . Appl. Environ. Microbiol. **25**, 2720-2726

Kim H.E., Shitashiro M., Kuroda A., Takiguchi N., and Kato J. (2007). Ethylene chemotaxis in *Pseudomonas aeruginosa* and other *Pseudomonas* species.

Kim M.G., da Cunha L., McFall A.J., Belkhadir Y., DebRoy S., Dangl J.L., and Mackey D. (2005). Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. Cell **121,** 749-759, doi/10.1016/j.cell.2005.03.025.

Kim M.G., Kim S.Y., Kim W.Y., Mackey D., and Lee S.Y. (2008). Responses of *Arabidopsis thaliana* to challenge by *Pseudomonas syringae* . Mol. Cells **25,** 323-331.

Kinnersley A.M. and Turano F.J. (2000). Gamma aminobutyric acid (GABA) and plant responses to stress. CRC Crit Rev Plant Sci **19,** 479-509.

Klement Z. (1965). Method of obtaining fluid from the intercellular spaces of foliage and the fluid's merit as a substrate for bacterial phytopathogens. Phytopathol. **55,** 1033-1034.

Kobayashi Y., Fling M., and Fox S.W. (1948). Antipodal specificity in the inhibition of growth of *Escherichia coli* by amino acids. J. Biol. Chem. **174,** 391-398.

Kohler T., Harayama S., Ramos J.L., and Timmis K.N. (1989). Involvement of *Pseudomonas putida* RpoN sigma factor in regulation of various metabolic functions. J. Bacteriol. **171,** 4326-4333.

Koncz C. and Schell J. (1986). The promoter of the T_L-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet **204,** 383-396.

Konings W.N. (2006). Microbial transport: adaptations to natural environments. Antonie Van Leeuwenhoek **90,** 325-342,

Koski L.B. and Golding G.B. (2001). The closest BLAST hit is often not the nearest neighbour. J. Mol. Evol. **52,** 540-542.

Kunkel B.N. and Brooks D.M. (2002). Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. **5,** 325-331.

Kurihara S., Oda S., Kato K., Kim H.G., Koyanagi T., Kumagai H., and Suzuki H. (2005). A novel putrescine utilization pathway involves gamma-glutamylated intermediates of *Escherichia coli* K-12. J. Biol. Chem. **280,** 4602-4608.

Kurihara S., Tsuboi Y., Oda S., Kim H.G., Kumagai H., and Suzuki H. (2009). The putrescine Importer *PuuP* of *Escherichia coli* K-12. J. Bacteriol. **191,** 2776-2782.

Kus J.V., Zaton K., Sarkar R., and Cameron R.K. (2002). Age-related resistance in Arabidopsis is a developmentally regulated defense response to *Pseudomonas syringae* . Plant Cell **14,** 479-490.

Lalucat J., Bennasar A., Bosch R., Garcia-Valdes E., and Palleroni N.J. (2006). Biology of *Pseudomonas stutzeri* . Microbiol. Mol. Biol. Rev. **70,** 510-547.

Lancien M. and Roberts M.R. (2006). Regulation of *Arabidopsis thaliana* 14-3-3 gene expression by gamma-aminobutyric acid. Plant. Cell. Environ. **29,** 1430-1436.

Lange R. and Hengge-Aronis R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. **5,** 49-59.

Lee S.W., Jeong K.S., Han S.W., Lee S.E., Phee B.K., Hahn T.R., and Ronald P. (2008). The *Xanthomonas oryzae* pv. oryzae PhoPQ two-component system is required for AvrXA21 activity, hrpG expression, and virulence. J. Bacteriol. **190,** 2183-2197.

Leigh J.A. and Dodsworth J.A. (2007). Nitrogen regulation in bacteria and archaea. Annu. Rev. Microbiol. **61,** 349-377.

Lelliott R.A., Billing E., and Hayward A.C. (1966). A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. **29,** 470-489.

Lemos E.G., Alves L.M., and Campanharo J.C. (2003). Genomics-based design of defined growth media for the plant pathogen *Xylella fastidiosa* . FEMS Microbiol. Lett. **219,** 39-45.

Leveau J.H. and Lindow S.E. (2001). Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. Proc. Natl. Acad. Sci. U. S. A. **98,** 3446-3453.

Li W. and Lu C.D. (2007). Regulation of carbon and nitrogen utilization by CbrAB and NtrBC two-component systems in *Pseudomonas aeruginosa* . J. Bacteriol. **189,** 5413-5420.

Li X., Lin H., Zhang W., Zou Y., Zhang J., Tang X., and Zhou J.M. (2005). Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. Proc. Natl. Acad. Sci. U. S. A. **102,** 12990-12995.

Liao C.H., Hung H.Y., and Chatterjee A.K. (1988). An extracellular pectate lyase is the pathogenicity factor of the soft-rotting bacterium *Pseudomonas viridiflava*. MPMI **1,** 199-206.

Liao M.K., Gort S., and Maloy S. (1997). A cryptic proline permease in *Salmonella typhimurium* . Microbiology **143** (Pt 9), 2903-2911.

Lin N.C., Abramovitch R.B., Kim Y.J., and Martin G.B. (2006). Diverse AvrPtoB homologs from several *Pseudomonas syringae* pathovars elicit Pto-dependent resistance and have similar virulence activities. Appl. Environ. Microbiol. **72,** 702-712, doi/10.1128/AEM.72.1.702-712.2006.

Lindeberg M., Schneider D.J., Cartinhour S., and Collmer A. (2008). Genomic analysis of *Pseudomonas syringae* pathovars: Identification of virulence genes and associated regulatory elements using pattern-based searches and genome comparison. In: *Pseudomonas syringae* pathovars and related pathogens – Identification, epidemiology and genomics, B. Fatmi, A. Collmer, N. S. Iacobellis, J. W. Mansfield, J. Murillo, N. W. Schaad and M. Ullrich, eds (Springer) pp. 207-220.

Lindemann J., Arny D.C., and Upper C.D. (1984). Epiphytic populations of *Pseudomonas syringae* pv. syringae on snap bean and nonhost plants and the incidence of bacterial brown spot disease in relation to cropping patterns. Phytopathol. **74,** 1329-1333.

Lindgren P.B. (1997). The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathol. **35,** 129-152.

Lindow S.E. and Brandl M.T. (2003). Microbiology of the phyllosphere. Appl. Environ. Microbiol. **69,** 1875-1883.

Liu H., Coulthurst S.J., Pritchard L., Hedley P.E., Ravensdale M., Humphris S., Burr T., Takle G., Brurberg M.B., Birch P.R., Salmond G.P., and Toth I.K. (2008). Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum* . PLoS Pathog. **4,** 1-11.

Llama-Palacios A., Lopez-Solanilla E., Poza-Carrion C., Garcia-Olmedo F., and Rodriguez-Palenzuela P. (2003). The *Erwinia chrysanthemi* phoP-phoQ operon plays an important role in growth at low pH, virulence and bacterial survival in plant tissue. Mol. Microbiol. **49,** 347-357.

Llama-Palacios A., Lopez-Solanilla E., and Rodriguez-Palenzuela P. (2005). Role of the PhoP-PhoQ system in the virulence of *Erwinia chrysanthemi* strain 3937: involvement in sensitivity to plant antimicrobial peptides, survival at acid pH, and regulation of pectolytic enzymes. J. Bacteriol. **187,** 2157-2162.

Locher K.P. (2009). Review. Structure and mechanism of ATP-binding cassette transporters. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **364,** 239-245.

Lohaus G., Winter H., Riens B., and Heldt H.W. (1995). Further studies of the phloem loading process in leaves of barley and spinach. The comparison of metabolite concentrations in the apoplastic compartment with those in the cytosolic compartment and in the sieve tubes. Plant Physiol. **108,** 270-275.

Lohaus G., Pennewiss K., Sattelmacher B., Hussmann M., and Hermann Muehling K. (2001). Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. Physiol. Plant. **111,** 457-465.

Long D.H., Lee F.N., and TeBeest D.O. (2000). Effect of nitrogen fertilization on disease progress of rice blast on susceptible and resistant cultivars. Plant Dis. **83,** 403-409.

Loper J.E., Kobayashi D.Y., and Paulsen I.T. (2007). The genomic sequence of *Pseudomonas fluorescens* Pf-5: Insights into biological control. Phytopathol. **97,** 233-238.

Lopez M.A., Bannenberg G., and Castresana C. (2008). Controlling hormone signaling is a plant and pathogen challenge for growth and survival. Curr. Opin. Plant Biol. **11,** 420-427.

Lopez-Millan A.F., Morales F., Abadia A., and Abadia J. (2000). Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport. Plant Physiol. **124,** 873-884.

Lorca G., Winnen B., and Saier M.H.,Jr (2003). Identification of the L-aspartate transporter in *Bacillus subtilis* . J. Bacteriol. **185,** 3218-3222.

Lorenzo O., Piqueras R., Sanchez-Serrano J.J., and Solano R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15,** 165-178.

Ludewig F., Huser A., Fromm H., Beauclair L., and Bouche N. (2008). Mutants of GABA transaminase (POP2) suppress the severe phenotype of succinic semialdehyde dehydrogenase (ssadh) mutants in *Arabidopsis* . PLoS ONE **3,** 1-11.

Lugtenberg B.J., Kravchenko L.V., and Simons M. (1999). Tomato seed and root exudate sugars: composition, utilization by *Pseudomonas* biocontrol strains and role in rhizosphere colonization. Environ. Microbiol. **1,** 439-446.

Lugtenberg B.J., Dekkers L., and Bloemberg G.V. (2001). Molecular determinants of rhizosphere colonization by *Pseudomonas* . Annu. Rev. Phytopathol. **39,** 461-490.

Lynch W.H. (1980). Effect of temperature on *Pseudomonas fluorescens* chemotaxis. J. Bacteriol. **143,** 338-342.

MacGregor K.B., Shelp B.J., Peiris S., and Bown A.W. (2003). Overexpression of glutamate decarboxylase in transgenic tobacco plants deters feeding by phytophagous insect larvae. J. Chem. Ecol. **29,** 2177-2182.

Mahan M.J., Slauch J.M., and Mekalanos J.J. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259,** 686-688.

Marco M.L., Legac J., and Lindow S.E. (2003). Conditional survival as a selection strategy to identify plant-inducible genes of *Pseudomonas syringae* . Appl. Environ. Microbiol. **69,** 5793-5801.

Marco M.L., Legac J., and Lindow S.E. (2005). *Pseudomonas syringae* genes induced during colonization of leaf surfaces. Environ. Microbiol. **7,** 1379-1391.

Marina M., Maiale S.J., Rossi F.R., Romero M.F., Rivas E.I., Garriz A., Ruiz O.A., and Pieckenstain F.L. (2008). Apoplastic polyamine oxidation plays different roles in local responses of tobacco to infection by the necrotrophic fungus *Sclerotinia sclerotiorum* and the biotrophic bacterium *Pseudomonas viridiflava* . Plant Physiol. **147,** 2164-2178.

Masduki A., Nakamura J., Ohga T., Umezaki R., Kato J., and Ohtake H. (1995). Isolation and characterization of chemotaxis mutants and genes of *Pseudomonas aeruginosa* . J. Bacteriol. **177,** 948-952.

McLean M.D., Yevtushenko D.P., Deschene D.V.C.,O.R., Makhmoudova A., Potter J.W., Bown A.W., and Shelp B.J. (2003). Overexpression of glutamate decarboxylase in transgenic tobacco plants confers resistance to the northern root-knot nematode. Mol Breed **11,** 277-285.

McLeod M.P., Warren R.L., Hsiao W.W., Araki N., Myhre M., Fernandes C., Miyazawa D., Wong W., Lillquist A.L., Wang D., Dosanjh M., Hara H., Petrescu A., Morin R.D., Yang G., Stott J.M., Schein J.E., Shin H., Smailus D., Siddiqui A.S., Marra M.A., Jones S.J., Holt R., Brinkman F.S., Miyauchi K., Fukuda M., Davies J.E., Mohn W.W., and Eltis L.D. (2006). The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. Proc. Natl. Acad. Sci. U. S. A. **103,** 15582-15587.

Mehdy M.C. (1994). Active oxygen species in plant defense against pathogens. Plant Physiol. **105,** 467-472.

Mellgren E.M., Kloek A.P., and Kunkel B.N. (2009). Mqo, a tricarboxylic acid cycle enzyme, is required for virulence of *Pseudomonas syringae* pv. tomato strain DC3000 on *Arabidopsis thaliana* . J. Bacteriol. **191,** 3132-3141.

Melotto M., Underwood W., Koczan J., Nomura K., and He S.Y. (2006). Plant stomata function in innate immunity against bacterial invasion. **126,** (5) 969-980.

Melotto M., Underwood W., and He S.Y. (2008). Role of stomata in plant innate immunity and foliar bacterial diseases. Annu. Rev. Phytopathol. **46,** 101-122.

Mercier J. and Lindow S.E. (2000). Role of leaf surface sugars in colonization of plants by bacterial epiphytes. Appl. Environ. Microbiol. **66,** 369-374.

Merrell D.S. and Camilli A. (2002). Acid tolerance of gastrointestinal pathogens. Curr. Opin. Microbiol. **5,** 51-55.

Metzer E., Levitz R., and Halpern Y.S. (1979). Isolation and properties of *Escherichia coli* K-12 mutants impaired in the utilization of gamma-aminobutyrate. J. Bacteriol. **137,** 1111-1118.

Meyer A., Eskandari S., Grallath S., and Rentsch D. (2006). AtGAT1, a high affinity transporter for gamma-aminobutyric acid in *Arabidopsis thaliana* . J. Biol. Chem. **281,** 7197-7204.

Misaghi I. and Grogan R.G. (1969). Nutritional and biochemical comparisons of plant-pathogenic and saprophytic fluorescent pseudomonads. Phytopathology **59,** 1436-1450.

Mithani A., Hein J., and Preston G.M. (Manuscript in preparation). Comparative analysis of metabolic networks provides insight into the evolution of pathogenic and non-pathogenic lifestyles in *Pseudomonas*..

Mohr T.J., Liu H., Yan S., Morris C.E., Castillo J.A., Jelenska J., and Vinatzer B.A. (2008). Naturally occurring nonpathogenic isolates of the plant pathogen *Pseudomonas syringae* lack a type III secretion system and effector gene orthologues. J. Bacteriol. **190,** 2858-2870.

Monds R.D., Newell P.D., Schwartzman J.A., and O'Toole G.A. (2006). Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0-1. Appl. Environ. Microbiol. **72,** 1910-1924.

Monier J.M. and Lindow S.E. (2003). Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces. Proc. Natl. Acad. Sci. U. S. A. **100,** 15977-15982.

Monier J.M. and Lindow S.E. (2005). Aggregates of resident bacteria facilitate survival of immigrant bacteria on leaf surfaces. Microb. Ecol. **49,** 343-352, doi/10.1007/s00248-004-0007-9.

Moran N.A. (2002). Microbial minimalism: genome reduction in bacterial pathogens. Cell **108,** 583-586.

Moreira L.M., De Souza R.F., Digiampietri L.A., Da Silva A.C., and Setubal J.C. (2005). Comparative analyses of *Xanthomonas* and *Xylella* complete genomes. OMICS **9,** 43-76.

Morgan M.K. and Chatterjee A.K. (1988). Genetic organization and regulation of proteins associated with production of syringotoxin by Pseudomonas syringae pv. syringae. J. Bacteriol. **170,** 5689-5697.

Morris C.E., Kinkel L.L., Xiao K., Prior P., and Sands D.C. (2007). Surprising niche for the plant pathogen *Pseudomonas syringae*. Infect. Genet. Evol. **7,** 84-92.

Morris C.E., Sands D.C., Vinatzer B.A., Glaux C., Guilbaud C., Buffiere A., Yan S., Dominguez H., and Thompson B.M. (2008). The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. ISME J. **2,** 321-334, doi/10.1038/ismej.2007.113.

Mota L.J. and Cornelis G.R. (2005). The bacterial injection kit: type III secretion systems. Ann. Med. **37,** 234-249.

Mudgett M.B. (2005). New insights to the function of phytopathogenic bacterial type III effectors in plants. Annu. Rev. Plant. Biol. **56,** 509-531.

Mühling K.H. and Sattelmacher B. (1995). Apoplastic ion concentration of intact leaves of field bean (*Vicia faba*) as influenced by ammonium and nitrate nutrition. J. Plant. Phys. **147,** 81-86.

Mühling K.H., Wimmer M., and Goldbach H. (1997). Apoplastic and membrane-associated Ca^{2+} in leaves and roots as affected by boron deficiency. Physiologia Plantarum **102,** 179-184.

Nabais C., Hagemeyer J., and Freitas H. (2005). Nitrogen transport in the xylem sap of *Quercus ilex*: the role of ornithine. J. Plant Physiol. **162,** 603-606.

Nachin L. and Barras F. (2000). External pH: an environmental signal that helps to rationalize pel gene duplication in *Erwinia chrysanthemi* . Mol. Plant Microbe Interact. **13,** 882-886.

Navarro L., Bari R., Seilaniantz A., Nemri A., and Jones J.D.G. (2008). Roles of plant hormones in plant resistance and susceptibility to pathogens In Genomics of Disease, Anonymous (: Springer) pp. 1-10.

Nelson K.E., Weinel C., Paulsen I.T., Dodson R.J., Hilbert H., Martins dos Santos V.A., Fouts D.E., Gill S.R., Pop M., Holmes M., Brinkac L., Beanan M., DeBoy R.T., Daugherty S., Kolonay J., Madupu R., Nelson W., White O., Peterson J., Khouri H., Hance I., Chris Lee P., Holtzapple E., Scanlan D., Tran K., Moazzez A., Utterback T., Rizzo M., Lee K., Kosack D., Moestl D., Wedler H., Lauber J., Stjepandic D., Hoheisel J., Straetz M., Heim S., Kiewitz C., Eisen J.A., Timmis K.N., Dusterhoft A., Tummler B., and Fraser C.M. (2002). Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. **4,** 799-808.

Niegemann E., Schulz A., and Bartsch K. (1993). Molecular organization of the *Escherichia coli* gab cluster: nucleotide sequence of the structural genes *gabD* and *gabP* and expression of the GABA permease gene. Arch. Microbiol. **160,** 454-460.

Nishimura M.T., Stein M., Hou B.H., Vogel J.P., Edwards H., and Somerville S.C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science **301,** 969-972.

Nomura K., Debroy S., Lee Y.H., Pumplin N., Jones J., and He S.Y. (2006). A bacterial virulence protein suppresses host innate immunity to cause plant disease. Science **313,** 220-223.

Oh H.S. and Collmer A. (2005). Basal resistance against bacteria in *Nicotiana benthamiana* leaves is accompanied by reduced vascular staining and suppressed by multiple *Pseudomonas syringae* type III secretion system effector proteins. Plant J. **44,** 348-359.

Olea F., Perez-Garcia A., Canton F.R., Rivera M.E., Canas R., Avila C., Cazorla F.M., Canovas F.M., and de Vicente A. (2004). Up-regulation and localization of asparagine synthetase in tomato leaves infected by the bacterial pathogen *Pseudomonas syringae* . Plant Cell Physiol. **45,** 770-780.

Pallen M.J. and Wren B.W. (2007). Bacterial pathogenomics. Nature **449,** 835-842.

Palleroni N.J. (1984). Pseudomonadaceae. In: Bergey's Manual of Systematic Bacteriology, B. Tansill, ed (: Williams & Wilkins) pp. 141-199.

Palleroni N.J. and Moore E.R.B. (2004). Taxonomy of Pseudomonads: Experimental approaches In *Pseudomonas*: Volume 1: Genomics, Life Style and Molecular Architecture, J. L. Ramos, ed (: Birkhäuser) pp. 3-47.

Panopoulos N.J. and Schroth M.N. (1974). Role of flagellar motility in the invasion of bean leaves by *Pseudomonas phaseolicola*. **76**, 177-188

Parales R.E., Ditty J.L., and Harwood C.S. (2000). Toluene-degrading bacteria are chemotactic towards the environmental pollutants benzene, toluene, and trichloroethylene. Appl. Environ. Microbiol. **66,** 4098-4104.

Parales R.E. and Harwood C.S. (2002). Bacterial chemotaxis to pollutants and plant-derived aromatic molecules. Curr. Opin. Microbiol. **5,** 266-273.

Park D.H., Mirabella R., Bronstein., P. Preston., Lim. C., Collmer A., Schuurink R., Mutations in amma-aminobutaric acid (GABA) transaminase genes in plants or Pseudomonas syringae reduce bacterial virulence. Manuscript submitted to The Plant Journal.

Park S.W., Kaimoyo E., Kumar D., Mosher S., and Klessig D.F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science **318,** 113-116.

Pate J.S. (1976). Nutrients and metabolites of fluids recovered from xylem and phloem; significance in relation to long distance transport in plants In , Anonymous () pp. 235-281.

Paulsen I.T., Sliwinski M.K., Nelissen B., Goffeau A., and Saier M.H.,Jr (1998). Unified inventory of established and putative transporters encoded within the complete genome of *Saccharomyces cerevisiae*. FEBS Lett. **430,** 116-125.

Paulsen I.T., Press C.M., Ravel J., Kobayashi D.Y., Myers G.S., Mavrodi D.V., DeBoy R.T., Seshadri R., Ren Q., Madupu R., Dodson R.J., Durkin A.S., Brinkac L.M., Daugherty S.C., Sullivan S.A., Rosovitz M.J., Gwinn M.L., Zhou L., Schneider D.J., Cartinhour S.W., Nelson W.C., Weidman J., Watkins K., Tran K., Khouri H., Pierson E.A., Pierson L.S.,3rd, Thomashow L.S., and Loper J.E. (2005). Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. Nat. Biotechnol. **23,** 873-878.

Perez-Garcia A., Snoeijers S.S., Joosten M.H., Goosen T., and De Wit P.J. (2001). Expression of the Avirulence gene Avr9 of the fungal tomato pathogen *Cladosporium fulvum* is regulated by the global nitrogen response factor NRF1. Mol. Plant Microbe Interact. **14,** 316-325.

Pérez-García A., Pereira S., Pissarra J., García Gutiérrez A., Cazorla F.M., Salema R., de Vicente A., and Cánovas F.M. (1998). Cytosolic localization in tomato mesophyll cells of a novel glutamine synthetase induced in response to bacterial infection or phosphinothricin treatment. Planta **206,** 426-434.

Philippe H. and Douady C.J. (2003). Horizontal gene transfer and phylogenetics. Curr. Opin. Microbiol. **6,** 498-505.

Pi J., Wookey P.J., and Pittard A.J. (1991). Cloning and sequencing of the *pheP* gene, which encodes the phenylalanine-specific transport system of *Escherichia coli* . J. Bacteriol. **173,** 3622-3629.

Pieterse C.M.J., Van Der Ent S., Van Pelt J.A., and van Loon L.C. (2007). The role of ethylene in rhizobacteria-induced systemic resistance (ISR) In Advances in Plant Ethylene Research, A. Ramina, C. Chang, J. Giovanni, H. Klee, P. Perata and J. Woltering, eds (: Springer) pp. 325-331.

Pignocchi C., Kiddle G., Hernandez I., Foster S.J., Asensi A., Taybi T., Barnes J., and Foyer C.H. (2006). Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defense processes in tobacco. Plant Physiol. **141,** 423-435.

Pilot G., Stransky H., Bushey D.F., Pratelli R., Ludewig U., Wingate V.P., and Frommer W.B. (2004). Overexpression of GLUTAMINE DUMPER1 leads to hypersecretion of glutamine from Hydathodes of *Arabidopsis* leaves. Plant Cell **16,** 1827-1840.

Pitman A.R., Jackson R.W., Mansfield J.W., Kaitell V., Thwaites R., and Arnold D.L. (2005). Exposure to host resistance mechanisms drives evolution of bacterial virulence in plants. Curr. Biol. **15,** 2230-2235.

Preston G.M. (2004). Plant perceptions of plant growth-promoting *Pseudomonas* . Philos. Trans. R. Soc. Lond. B. Biol. Sci. **359,** 907-918.

Prusky D., McEvoy J.L., Leverentz B., and Conway W.S. (2001). Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. Mol. Plant Microbe Interact. **14,** 1105-1113.

Quinones B., Dulla G., and Lindow S.E. (2005). Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae* . Mol. Plant Microbe Interact. **18,** 682-693.

Rahme L.G., Mindrinos M.N., and Panopoulos N.J. (1992). Plant and environmental sensory signals control the expression of hrp genes in *Pseudomonas syringae* pv. phaseolicola. J. Bacteriol. **174,** 3499-3507.

Rainey P.B. and Travisano M. (1998). Adaptive radiation in a heterogeneous environment. Nature **394,** 69-72.

Rainey P.B. (1999). Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. Environ. Microbiol. **1,** 243-257.

Rainey P.B. (1999). Evolutionary genetics: The economics of mutation. Curr. Biol. **9,** 371-373.

Ramos A.R., Morello J.E., Ravindran S., Deng W.L., Huang H.C., and Collmer A. (2007). Identification of *Pseudomonas syringae* pv. syringae 61 type III secretion system Hrp proteins that can travel the type III pathway and contribute to the translocation of effector proteins into plant cells. J. Bacteriol. **189,**(15): 5773–5778.

Ramos-Gonzalez M.I., Campos M.J., and Ramos J.L. (2005). Analysis of *Pseudomonas putida* KT2440 gene expression in the maize rhizosphere: In vitro expression technology capture and identification of root-activated promoters. **187**, (12) 4033-4041.

Ramputh A.I. and Bown A.W. (1996). Rapid gamma-aminobutyric acid synthesis and the inhibition of the growth and development of oblique-banded leaf-roller larvae. Plant Physiol. **111,** 1349-1352.

Rathmell W.G. and Sequeira L. (1974). Soluble peroxidase in fluid from the intercellular spaces of tobacco leaves. Plant Physiol. **53,** 317-318.

Regenberg B., During-Olsen L., Kielland-Brandt M.C., and Holmberg S. (1999). Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae* . Curr. Genet. **36,** 317-328.

Reig N., del Rio C., Casagrande F., Ratera M., Gelpi J.L., Torrents D., Henderson P.J., Xie H., Baldwin S.A., Zorzano A., Fotiadis D., and Palacin M. (2007). Functional and structural characterization of the first prokaryotic member of the L-amino acid transporter (LAT) family: a model for APC transporters. J. Biol. Chem. **282,** 13270-13281.

Reinhardt J.A., Baltrus D.A., Nishimura M.T., Jeck W.R., Jones C.D., and Dangl J.L. (2009). De novo assembly using low-coverage short read sequence data from the rice pathogen *Pseudomonas syringae* pv. oryzae. Genome Res. **19,** 294-305.

Reitzer L. and Schneider B.L. (2001). Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli* . Microbiol. Mol. Biol. Rev. **65,** 422-44.

Reitzer L. (2003). Nitrogen assimilation and global regulation in *Escherichia coli* . Annu. Rev. Microbiol. **57,** 155-176.

Ren Q. and Paulsen I.T. (2007). Large-scale comparative genomic analyses of cytoplasmic membrane transport systems in prokaryotes. J. Mol. Microbiol. Biotechnol. **12,** 165-179.

Rico A. and Preston G.M. (2008). *Pseudomonas syringae* pv. tomato DC3000 uses constitutive and apoplast-induced nutrient assimilation pathways to catabolize nutrients that are abundant in the tomato apoplast. Mol. Plant Microbe Interact. **21,** 269-282.

Rico A., Jones R., and Preston G.M. (2008). Adaptation to the plant apoplast by plant pathogenic bacteria. In, Plant Pathogenic Bacteria: Genomics and Molecular Biology, R. W. Jackson, ed (Norwich, UK.: Horizon Scientific Press).

Roberts M.R. (2007). Does GABA act as a signal in plants? Hints from molecular studies. Plant Signaling & Behaviour **2,** 408-409.

Roberts M.R. and Bowles D.J. (1999). Fusicoccin, 14-3-3 proteins, and defense responses in tomato plants. Plant Physiol. **119,** 1243-1250.

Roberts M.R., Salinas J., and Collinge D.B. (2002). 14-3-3 proteins and the response to abiotic and biotic stress. Plant Mol. Biol. **50,** 1031-1039.

Roberts M.R. (2003). 14-3-3 proteins find new partners in plant cell signalling. Trends Plant Sci. **8,** 218-223.

Roberts M.R. and Paul N.D. (2006). Seduced by the dark side: integrating molecular and ecological perspectives on the influence of light on plant defence against pests and pathogens. New Phytol. **170,** 677-699.

Robert-Seilaniantz A., Navarro L., Bari R., and Jones J.D. (2007). Pathological hormone imbalances. Curr. Opin. Plant Biol. **10,** 372-379.

Roessner-Tunali U., Hegemann B., Lytovchenko A., Carrari F., Bruedigam C., Granot D., and Fernie A.R. (2003). Metabolic profiling of transgenic tomato plants overexpressing hexokinase reveals that the influence of hexose phosphorylation diminishes during fruit development. **133,** 84-91.

Romano J.D. and Kolter R. (2005). *Pseudomonas-Saccharomyces* interactions: influence of fungal metabolism on bacterial physiology and survival. J. Bacteriol. **187,** 940-948.

Rose J.K., Saladie M., and Catala C. (2004). The plot thickens: New perspectives of primary cell wall modification. Curr. Opin. Plant Biol. **7,** 296-301.

Rost B., Yachdav G., and Liu J. (2004). The PredictProtein server. Nucleic Acids Res. **32,** 321-326.

Rouse D.I., Nordheim E.V., Hirano S.S., and Upper C.D. (1985). A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant-pathogens. Phytopathol. **75,** 505-509.

Rozen S. and Skaletsky H.J. (2000). Primer 3 on the WWW for general users and for biologist programmers. **132**, 65-386.

Rudrappa T., Czymmek K.J., Pare P.W., and Bais H.P. (2008). Root-secreted malic acid recruits beneficial soil bacteria. Plant Physiol. **148,** 1547-1556.

Rutherford K., Parkhill J., Crook J., Horsnell T., Rice P., Rajandream M.A., and Barrell B. (2000). Artemis: sequence visualization and annotation. Bioinformatics **16,** 944-945.

Saier M.H.,Jr (2000). A functional-phylogenetic classification system for transmembrane solute transporters. Microbiol. Mol. Biol. Rev. **64,** 354-411.

Saito H. and Kobayashi H. (2003). Bacterial responses to alkaline stress. Sci. Prog. **86,** 271-282.

Sambrook J., Maniatis T., and Fritsch E.F. (1989). Molecular cloning: a laboratory manual.(:Cold Spring Harbor Laboratory Press).

Sanders J.W., Leenhouts K., Burghoorn J., Brands J.R., Venema G., and Kok J. (1998). A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation. Mol. Microbiol. **27,** 299-310.

Sattelmacher B. (2001). The apoplast and its significance for plant mineral nutrition. New Phytol. **149,** 167-192.

Sayers E.W., Barrett T., Benson D.A., Bryant S.H., Canese K., Chetvernin V., Church D.M., DiCuccio M., Edgar R., Federhen S., Feolo M., Geer L.Y., Helmberg W., Kapustin Y., Landsman D., Lipman D.J., Madden T.L., Maglott D.R., Miller V., Mizrachi I., Ostell J., Pruitt K.D., Schuler G.D., Sequeira E., Sherry S.T., Shumway M., Sirotkin K., Souvorov A., Starchenko G., Tatusova T.A., Wagner L., Yaschenko E., and Ye J. (2009). Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. **37,** 5-15.

Schäfer, A., Tauch, A., Jäger, W., Kalinovski, J., Thierbach, G., and Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamican*. *Gene* **145:** 69-73.

Schauer N., Zamir D., and Fernie A.R. (2005). Metabolic profiling of leaves and fruit of wild species tomato: a survey of the *Solanum lycopersicum* complex. J. Exp. Bot. **56,** 297-307.

Schenk A., Weingart H., and Ullrich M.S. (2008). Extraction of high-quality bacterial RNA from infected leaf tissue for bacterial in planta gene expression analysis by multiplexed fluorescent Northern hybridization. Mol. Plant. Pathol. **9,** 227-235,.

Schneider B.L., Ruback S., Kiupakis A.K., Kasbarian H., Pybus C., and Reitzer L. (2002). The *Escherichia coli* gabDTPC operon: specific gamma-aminobutyrate catabolism and nonspecific induction. J. Bacteriol. **184,** 6976-6986.

Schneider F., Kramer R., and Burkovski A. (2004). Identification and characterization of the main beta-alanine uptake system in *Escherichia coli* . Appl. Microbiol. Biotechnol. **65,** 576-582.

Schneider R.W. and Grogan R.G. (1977). Tomato leaf trichomes, a habitat for resident populations of *Pseudomonas* tomato. Phytopathol. **67,** 898-902.

Schulze-Lefert P. and Robatzek S. (2006). Plant pathogens trick guard cells into opening the gate. **126,** (5) 831-834

Schwan W.R., Coulter S.N., Ng E.Y., Langhorne M.H., Ritchie H.D., Brody L.L., Westbrock-Wadman S., Bayer A.S., Folger K.R., and Stover C.K. (1998). Identification and characterization of the PutP proline permease that contributes to in vivo survival of *Staphylococcus aureus* in animal models. Infect. Immun. **66,** 567-572.

Schwessinger B. and Zipfel C. (2008). News from the frontline: recent insights into PAMP-triggered immunity in plants. Curr. Opin. Plant Biol. **11,** 389-395,

Seshadri R., Joseph S.W., Chopra A.K., Sha J., Shaw J., Graf J., Haft D., Wu M., Ren Q., Rosovitz M.J., Madupu R., Tallon L., Kim M., Jin S., Vuong H., Stine O.C., Ali A., Horneman A.J., and Heidelberg J.F. (2006). Genome sequence of *Aeromonas hydrophila* ATCC 7966T: jack of all trades. J. Bacteriol. 188, 8272-8282.

Sezonov G., Joseleau-Petit D., D'Ari R. (2007). *Escherichia coli* physiology in Luria-Bertani broth. J. Bacteriol. **189** (23), 8746-8749.

Shelp B.J., Bown A.W., and McLean M.D. (1999). Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. **4,** 446-452.

Shitashiro M., Kato J., Fukumura T., Kuroda A., Ikeda T., Takiguchi N., and Ohtake H. (2003). Evaluation of bacterial aerotaxis for its potential use in detecting the toxicity of chemicals to microorganisms. J. Biotechnol. **101,** 11-18.

Siegrist J., Orober M., and Buchenauer H. (2000). β-Aminobutyric acid-mediated enhancement of resistance in tobacco to tobacco mosaic virus depends on the accumulation of salicylic acid. Phys. Mol. Plant. Path. **56,** 95-106.

Sigee D.C. (1993). Bacterial plant pathology: cell and molecular aspects (:Cambridge University Press) pp. 325.

Silby M.W. and Levy S.B. (2004). Use of in vivo expression technology to identify genes important in growth and survival of *Pseudomonas fluorescens* Pf0-1 in soil: discovery of expressed sequences with novel genetic organization. J. Bacteriol. **186,** 7411-7419.

Silby M.W., Cerdeno-Tarraga A.M., Vernikos G.S., Giddens S.R., Jackson R.W., Preston G.M., Zhang X.X., Moon C.D., Gehrig S.M., Godfrey S.A., Knight C.G., Malone J.G., Robinson Z., Spiers A.J., Harris S., Challis G.L., Yaxley A.M., Harris D., Seeger K., Murphy L., Rutter S., Squares R., Quail M.A., Saunders E., Mavromatis K., Brettin T.S., Bentley S.D., Hothersall J., Stephens E., Thomas C.M., Parkhill J., Levy S.B., Rainey P.B., and Thomson N.R. (2009). Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. Genome Biol. **10,** R10-51

Simons M., Permentier H.P., de Weger L.A., Wijffelman C.A., and Lugtenberg B.J. (1997). Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. MPMI **10,** 102-106.

Singh P., Piotrowski M., Kloppstech K., and Gau A.E. (2004). Investigations on epiphytic living *Pseudomonas* species from *Malus domestica* with an antagonistic effect to *Venturia inaequalis* on isolated plant cuticle membranes. Environ. Microbiol. **6,** 1149-1158.

Singh T. and Arora D.K. (2001). Motility and chemotactic response of *Pseudomonas fluorescens* toward chemoattractants present in the exudate of *Macrophomina phaseolina* . Microbiol. Res. **156,** 343-351.

Singh T., Srivastava A.K., and Arora D.K. (2002). Horizontal and vertical movement of *Pseudomonas fluorescens* toward exudate of *Macrophomina phaseolina* in soil: influence of motility and soil properties. Microbiol. Res. **157,** 139-148.

Snoeijers S.S., Pérez-García A., and Joosten M.H.A.J. (2000). The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. Euro. J. Plant Path. **106,** 493-506.

Soksawatmaekhin W., Kuraishi A., Sakata K., Kashiwagi K., and Igarashi K. (2004). Excretion and uptake of cadaverine by CadB and its physiological functions in *Escherichia coli* . Mol. Microbiol. **51,** 1401-1412.

Solomon P.S. and Oliver R.P. (2001). The nitrogen content of the tomato leaf apoplast increases during infection by *Cladosporium fulvum*. Planta **213,** 241-249.

Solomon P.S. and Oliver R.P. (2002). Evidence that gamma-aminobutyric acid is a major nitrogen source during *Cladosporium fulvum* infection of tomato. Planta **214,** 414-420.

Son M.S., Matthews W.J.,Jr, Kang Y., Nguyen D.T., and Hoang T.T. (2007). In vivo evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. Infect. Immun. **75,** 5313-5324.

Sonnhammer E.L., von Heijne G., and Krogh A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. Proc. Int. Conf. Intell. Syst. Mol. Biol. **6,** 175-182.

Sood S.G. (2006). Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. **45,** 219-227.

Speth E.B., Lee Y.N., and He S.Y. (2007). Pathogen virulence factors as molecular probes of basic plant cellular functions. Curr. Opin. Plant Biol. **10,** 580-586.

Spiers A.J., Buckling A., and Rainey P.B. (2000). The causes of *Pseudomonas* diversity. **146,** 2345-2350.

Spiers A.J., Kahn S.G., Bohannon J., Travisano M., and Rainey P.B. (2002). Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. Genetics **161,** 33-46.

Spoel S.H. and Dong X. (2008). Making sense of hormone crosstalk during plant immune responses. Cell. Host Microbe **3,** 348-351.

Stancik L.M., Stancik D.M., Schmidt B., Barnhart D.M., Yoncheva Y.N., and Slonczewski J.L. (2002). pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli* . J. Bacteriol. **184,** 4246-4258.

Stavrinides J., McCann H.C., and Guttman D.S. (2008). Host-pathogen interplay and the evolution of bacterial effectors. Cell. Microbiol. **10,** 285-292.

Stavrinides J., McCloskey J.K., and Ochman H. (2009). Pea aphid as both host and vector for the phytopathogenic bacterium *Pseudomonas syringae* . Appl. Environ. Microbiol. **75,** 2230-2235.

Steffes C., Ellis J., Wu J., and Rosen B.P. (1992). The lysP gene encodes the lysine-specific permease. J. Bacteriol. **174,** 3242-3249.

Stepkowski T. and Legocki A.B. (2001). Reduction of bacterial genome size and expansion resulting from obligate intracellular lifestyle and adaptation to soil habitat. Acta Biochim. Pol. **48,** 367-381.

Stojiljkovic I., Baumler A.J., and Heffron F. (1995). Ethanolamine utilization in *Salmonella typhimurium*: nucleotide sequence, protein expression, and mutational analysis of the c*chA cchB eutE eutJ eutG eutH* gene cluster. J. Bacteriol. **177,** 1357-1366.

Storz G. and Hengge-Aronis R. (2000). Bacterial stress responses. ASM Press. Washington.

Stover C.K., Pham X.Q., Erwin A.L., Mizoguchi S.D., Warrener P., Hickey M.J., Brinkman F.S., Hufnagle W.O., Kowalik D.J., Lagrou M., Garber R.L., Goltry L., Tolentino E., Westbrock-Wadman S., Yuan Y., Brody L.L., Coulter S.N., Folger K.R., Kas A., Larbig K., Lim R., Smith K., Spencer D., Wong G.K., Wu Z., Paulsen I.T., Reizer J., Saier M.H., Hancock R.E., Lory S., and Olson M.V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406,** 959-964.

Struck C.S., Mueller E., Martin H., and Lohaus G. (2004). The *Uromyces fabae* UfAAT3 gene encodes a general amino acid permease that prefers uptake of *in planta* scarce amino acids. Mol. Plant. Pathol. **5,**(33) 183-189,

Studholme D.J. and Dixon R. (2003). Domain architectures of sigma 54-dependent transcriptional activators. J. Bacteriol. **185,** 1757-1767.

Studholme D.J., Downie J.A., and Preston G.M. (2005). Protein domains and architectural innovation in plant-associated Proteobacteria. BMC Genomics **6,** (17) 1-15.

Taguchi K., Fukutomi H., Kuroda A., Kato J., and Ohtake H. (1997). Genetic identification of chemotactic transducers for amino acids in *Pseudomonas aeruginosa.* Microbiol. **143,** 3223-3229.

Tans-Kersten J., Huang H., and Allen C. (2001). *Ralstonia solanacearum* needs motility for invasive virulence on tomato. **183,** 3597-3605.

Tateno Y., Takezaki N., and Nei M. (1994). Relative efficiencies of the maximum-likelihood, neighbor-joining, and maximum-parsimony methods when substitution rate varies with site. Mol. Biol. Evol. **11,** 261-277.

Tavernier V., Cadiou S., Pageau K., Lauge R., Reisdorf-Cren M., Langin T., and Masclaux-Daubresse C. (2007). The plant nitrogen mobilization promoted by *Colletotrichum lindemuthianum* in *Phaseolus* leaves depends on fungus pathogenicity. J. Exp. Bot. **58,** 3351-3360.

Tejera N., Ortega E., Rodes R., and Lluch C. (2006). Nitrogen compounds in the apoplastic sap of sugarcane stem: some implications in the association with endophytes. J. Plant Physiol. **163,** 80-85.

Tetlow I.J. and Farrar J.F. (1993). Apoplastic sugar concentration and pH in barley leaves infected with brown rust. J Exp Bot **44,** 929-936.

Thilmony R., Underwood W., and He S.Y. (2006). Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. tomato DC3000 and the human pathogen *Escherichia coli* O157:H7. Plant J. **46,** 34-53.

Thornley M.J. (1960). The differentiation of *Pseudomonas* from other gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. **23,** 37-52.

Tian Q., Uhlir N.J., and Reed J.W. (2002). *Arabidopsis* SHY2/IAA3 inhibits auxin-regulated gene expression. Plant Cell **14,** 301-319.

Totten P.A., Lara J.C., and Lory S. (1990). The rpoN gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. **172,** 389-396.

Truman W., de Zabala M.T., and Grant M. (2006). Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. Plant J. **46,** 14-33.

Tsuji J., Jackson E.P., Gage D.A., Hammerschmidt R., and Somerville S.C. (1992). Phytoalexin Accumulation in *Arabidopsis thaliana* during the Hypersensitive Reaction to *Pseudomonas syringae* pv syringae. Plant Physiol. **98,** 1304-1309.

Tusnády G.E. and Simon I. (1998). Principles governing amino acid composition of integral membrane proteins: Applications to topology prediction. J. Mol. Biol. **283,** 489-506.

Underwood W., Melotto M., and He S.Y. (2007). Role of plant stomata in bacterial invasion. Cell. Microbiol. **9,** 1621-1629.

Uppalapati S.R., Ishiga Y., Wangdi T., Urbanczyk-Wochniak E., Ishiga T., Mysore K.S., and Bender C.L. (2008). Pathogenicity of *Pseudomonas syringae* pv. tomato on tomato seedlings: phenotypic and gene expression analyses of the virulence function of coronatine. Mol. Plant Microbe Interact. **21,** 383-395.

Valle E.M., Boggio S.B., and Heldt H.W. (1998). Free amino acid composition of phloem sap and growing fruit of *Lycopersicon esculentum* . Plant & Cell Physiology **39,** 458-461.

Van der Hoorn R.A. and Kamoun S. (2008). From Guard to Decoy: a new model for perception of plant pathogen effectors. Plant Cell **20,** 2009-2017.

van Loon L.C., Bakker P.A., and Pieterse C.M. (1998). Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. **36,** 453-483, doi/10.1146/annurev.phyto.36.1.453.

van Loon L.C., Geraats B.P., and Linthorst H.J. (2006). Ethylene as a modulator of disease resistance in plants. Trends Plant Sci. **11,** 184-191.

Vardar G., Barbieri P., and Wood T.K. (2005). Chemotaxis of *Pseudomonas stutzeri* OX1 and *Burkholderia cepacia* G4 toward chlorinated ethenes. Appl. Microbiol. Biotechnol. **66,** 696-701.

Vauterin L., Hoste B., Kersters K., and Swings J. (1995). Reclassification of *Xanthomonas*. Int. J. Syst. Evol. Microbiol. **45,** 472-489.

Verhoogt H.J., Smit H., Abee T., Gamper M., Driessen A.J., Haas D., and Konings W.N. (1992). arcD, the first gene of the arc operon for anaerobic arginine catabolism in Pseudomonas aeruginosa, encodes an arginine-ornithine exchanger. J. Bacteriol. **174,** 1568-1573.

Vilchez S., Molina L., Ramos C., and Ramos J.L. (2000). Proline catabolism by *Pseudomonas putida*: cloning, characterization, and expression of the put genes in the presence of root exudates. J. Bacteriol. **182,** 91-99.

Vlot A.C., Klessig D.F., and Park S.W. (2008). Systemic acquired resistance: the elusive signal(s). Curr. Opin. Plant Biol. **11,** 436-442.

Vodovar N., Vallenet D., Cruveiller S., Rouy Z., Barbe V., Acosta C., Cattolico L., Jubin C., Lajus A., Segurens B., Vacherie B., Wincker P., Weissenbach J., Lemaitre B., Medigue C., and Boccard F. (2006). Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila* . Nat. Biotechnol. **24,** 673-679.

Von Bodman S.B., Bauer W.D., and Coplin D.L. (2003). Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. **41,** 455-482.

Von Rad U., Mueller M.J., and Durner J. (2005). Evaluation of natural and synthetic stimulants of plant immunity by microarray technology. New Phytol. **165,** 191-202.

Vos M. and Velicer G.J. (2006). Genetic population structure of the soil bacterium *Myxococcus xanthus* at the centimeter scale. Appl. Environ. Microbiol. **72,** 3615-3625.

Wadhams G.H. and Armitage J.P. (2004). Making sense of it all: bacterial chemotaxis. Nat. Rev. Mol. Cell Biol. **5,** 1024-1037.

Walters D.R. (2003). Resistance to plant pathogens: possible roles for free polyamines and polyamine catabolism. New Phytol. **159,** 109-115.

Wang E.L.H. and Bergeson G.B. (1974). Biochemical changes in root exudate and xylem sap of tomato plants infected with *Meloidogyne incognita*. 194-201.

Webre D.J., Wolanin P.M., and Stock J.B. (2003). Bacterial chemotaxis. Curr. Biol. **13,** R47-9.

Wehrmann A., Morakkabati S., Kramer R., Sahm H., and Eggeling L. (1995). Functional analysis of sequences adjacent to *dapE* of *Corynebacterium glutamicum* reveals the presence of *aroP*, which encodes the aromatic amino acid transporter. J. Bacteriol. **177,** 5991-5993.

Wei W., Plovanich-Jones A., Deng W.L., Jin Q.L., Collmer A., Huang H.C., and He S.Y. (2000). The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. tomato. Proc. Natl. Acad. Sci. U. S. A. **97,** 2247-2252.

Weingart H., Volksch B., and Ullrich M.S. (1999). Comparison of ethylene production by *Pseudomonas syringae* and *Ralstonia solanacearum* . Phytopathology **89,** 360-365.

Weingart H., Stubner S., Schenk A., and Ullrich M.S. (2004). Impact of temperature on in planta expression of genes involved in synthesis of the *Pseudomonas syringae* phytotoxin coronatine. Mol. Plant Microbe Interact. **17,** 1095-1102.

Whipps J.M., Hand P., Pink D., and Bending G.D. (2008). Phyllosphere microbiology with special reference to diversity and plant genotype. J. Appl. Microbiol. **105,** 1744-1755.

White M.C. (1981). Metal complexation in xylem fluid : I. Chemical composition of tomato and soybean stem exudate. Plant Physiol. **67,** 292-300.

Wilson M., Hirano S.S., and Lindow S.E. (1999). Location and survival of leaf-associated bacteria in relation to pathogenicity and potential for growth within the leaf. Appl. Environ. Microbiol. **65,** 1435-1443.

Winsor G.L., Lo R., Sui S.J., Ung K.S., Huang S., Cheng D., Ching W.K., Hancock R.E., and Brinkman F.S. (2005). *Pseudomonas aeruginosa* Genome Database and PseudoCAP: facilitating community-based, continually updated, genome annotation. Nucleic Acids Res. **33,** 338-343

Winsor G.L., Van Rossum T., Lo R., Khaira B., Whiteside M.D., Hancock R.E., and Brinkman F.S. (2009). Pseudomonas Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes. Nucleic Acids Res. **37,** D483-488,

Winstanley C., Langille M.G., Fothergill J.L., Kukavica-Ibrulj I., Paradis-Bleau C., Sanschagrin F., Thomson N.R., Winsor G.L., Quail M.A., Lennard N., Bignell A., Clarke L., Seeger K., Saunders D., Harris D., Parkhill J., Hancock R.E., Brinkman F.S., and Levesque R.C. (2009). Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa* . Genome Res. **19,** 12-23

Wright C.A. and Beattie G.A. (2004). *Pseudomonas syringae* pv. tomato cells encounter inhibitory levels of water stress during the hypersensitive response of *Arabidopsis thaliana* . Proc. Natl. Acad. Sci. U. S. A. **101,** 3269-3274.

Xiao Y., Heu S., Yi J., Lu Y., and Hutcheson S.W. (1994). Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. syringae Pss61 *hrp* and *hrmA* genes. J. Bacteriol. **176,** 1025-1036.

Xu Y., Chang P., Liu D., Narasimhan M.L., Raghothama K.G., Hasegawa P.M., and Bressan R.A. (1994). Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell **6,** 1077-1085.

Yamakawa H., Kamada H., Satoh M., and Ohashi Y. (1998). Spermine is a salicylate-independent endogenous inducer for both tobacco acidic pathogenesis-related proteins and resistance against tobacco mosaic virus infection. Plant Physiol. **118,** 1213-1222.

Yamamoto S., Kasai H., Arnold D.L., Jackson R.W., Vivian A., and Harayama S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology **146** (Pt 10), 2385-2394.

Yan J., He C., Wang J., Mao Z., Holaday S.A., Allen R.D., and Zhang H. (2004). Overexpression of the *Arabidopsis* 14-3-3 protein GF14 lambda in cotton leads to a "stay-

green" phenotype and improves stress tolerance under moderate drought conditions. Plant Cell Physiol. **45,** 1007-1014.

Yan S., Liu H., Mohr T.J., Jenrette J., Chiodini R., Zaccardelli M., Setubal J.C., and Vinatzer B.A. (2008). Role of recombination in the evolution of the model plant pathogen *Pseudomonas syringae* pv. tomato DC3000, a very atypical tomato strain. Appl. Environ. Microbiol. **74,** 3171-3181.

Yan Y., Yang J., Dou Y., Chen M., Ping S., Peng J., Lu W., Zhang W., Yao Z., Li H., Liu W., He S., Geng L., Zhang X., Yang F., Yu H., Zhan Y., Li D., Lin Z., Wang Y., Elmerich C., Lin M., and Jin Q. (2008). Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501. Proc. Natl. Acad. Sci. U. S. A. **105,** 7564-7569.

Yang C.H., Gavilanes-Ruiz M., Okinaka Y., Vedel R., Berthuy I., Boccara M., Chen J.W., Perna N.T., and Keen N.T. (2002). hrp genes of *Erwinia chrysanthemi* 3937 are important virulence factors. Mol. Plant Microbe Interact. **15,** 472-480.

Yang S., Perna N.T., Cooksey D.A., Okinaka Y., Lindow S.E., Ibekwe A.M., Keen N.T., and Yang C.H. (2004). Genome-wide identification of plant-upregulated genes of *Erwinia chrysanthemi* 3937 using a GFP-based IVET leaf array. Mol. Plant Microbe Interact. **17,** 999-1008.

Yao J. and Allen C. (2006). Chemotaxis is required for virulence and competitive fitness of the bacterial wilt pathogen *Ralstonia solanacearum.* . **188,** .

Yates E.A., Philipp B., Buckley C., Atkinson S., Chhabra S.R., Sockett R.E., Goldner M., Dessaux Y., Camara M., Smith H., and Williams P. (2002). N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa* . Infect. Immun. **70,** 5635-5646.

Yevtushenko D.P., McLean M.D., Peiris S., Van Cauwenberghe O.R., and Shelp B.J. (2003). Calcium/calmodulin activation of two divergent glutamate decarboxylases from tobacco. J. Exp. Bot. **54,** 2001-2002.

You C.B., Song W., Wang H.X., Li J.P., Lin M., and Hai W.L. (2005). Association of *Alcaligenes faecalis* with wetland rice. Plant & Soil **137,** 81-85.

Young J.M. and Triggs C.M. (1994). Evaluation of determinative tests for pathovars of *Pseudomonas syringae* van Hall 1902. **77,** 195-207.

Yu Q., Tang C., and Kuo J. (2000). A critical review on methods to measure apoplastic pH in plants. **219,** 29-40.

Yuan J. and He S.Y. (1996). The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. J. Bacteriol. **178,** 6399-6402.

Yucel I., Xiao Y.X., and Hutcheson S.W. (1989). Influence of *Pseudomonas syringae* culture conditions on initiation of the hypersensitive response of culture tobacco cells. Appl. Environ. Microbiol. **55,** 1724-1729.

Zhang J., Xin Y., Liu H., Wang S., and Zhou N. (2008). Metabolism-independent chemotaxis of *Pseudomonas sp*. strain WBC-3 toward aromatic compounds. J. Environ. Sci. (China) **20,** 1238-1242.

Zhang X.X., George A., Bailey M.J., and Rainey P.B. (2006). The histidine utilization (hut)genes of *Pseudomonas fluorescens* SBW25 are active on plant surfaces, but are not required for competitive colonization of sugar beet seedlings. **152,** 1867-1875

Zhang X.X. and Rainey P.B. (2007). Genetic analysis of the histidine utilization (hut) genes in *Pseudomonas fluorescens* SBW25. Genetics **176,** 2165-2176.

Zhao Y., Thilmony R., Bender C.L., Schaller A., He S.Y., and Howe G.A. (2003). Virulence systems of *Pseudomonas syringae* pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. Plant J. **36,** 485-499.

Zhulin I.B., Nikolskaya A.N., and Galperin M.Y. (2003). Common extracellular sensory domains in transmembrane receptors for diverse signal transduction pathways in bacteria and archaea. J. Bacteriol. **185,** 285-294.

Zimmer D.P., Soupene E., Lee H.L., Wendisch V.F., Khodursky A.B., Peter B.J., Bender R.A., and Kustu S. (2000). Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. Proc. Natl. Acad. Sci. U. S. A. **97,** 14674-14679.

Zipfel C. and Felix G. (2005). Plants and animals: a different taste for microbes? Curr. Opin. Plant Biol. **8,** 353-360.