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Colicin translocation through the *E. coli* cell envelope

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Declaration

I hereby confirm that the work presented in this thesis, submitted for the degree of Doctor of Philosophy, is my own original work. Work that is not my own is duly referenced. This thesis has not been previously submitted at this or any other university.

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Abstract

Gram-negative bacteria have evolved mechanisms to kill other microbial species to compete for space and resources. One mechanism exploited by *Escherichia coli* is the production of bacteriocins called colicins, which target closely-related sensitive *E. coli* strains. Colicins bind with high affinity to outer membrane proteins on the surface of *E. coli*, and following contact with proteins in the periplasm and inner membrane, translocate a cytotoxic domain to kill the cell. Killing displays first-order kinetics which has been suggested to mean a single molecule is sufficient to kill a cell, although this has yet to be demonstrated.

The mechanism by which a folded colicin can cross two membranes to kill cells is still unknown, due to the lack of tools available. The aim of this research was to develop fluorescence-based microscopy tools to visualise colicin entry into *E. coli* and probe the mechanism of translocation *in vivo*.

ColE9 was labelled with organic fluorophores within its cytotoxic domain and retained its catalytic activity *in vitro*, however its *in vivo* activity was reduced. Microscopy was optimised using a ColE9 active site mutant and the AlexaFluor-647 dye, to generate a fluorescent probe capable of translocating into but not killing cells.

Translocation of ColE9*^{AF647} across the outer membrane (OM) was dependent on the PMF, in particular the electrical potential. Translocation of single-molecules of ColE9*^{AF647} to the cytoplasm was visualised for the first time *in vivo*; ~4-5 molecules were observed per cell, in 30-minutes at 37 °C. Two populations of ColE9*^{AF647} molecules were present; a mobile fraction with a diffusion coefficient similar to cytoplasmic GFP (~4-10 $\mu\text{m}^2/\text{s}$) and an immobile fraction, the origins of which are unknown. Finally, the rate-limiting step for ColE9 translocation across the OM was shown to be passage through OmpF or OmpC, which could be modulated by the fluorophore used. The present work demonstrates that Tol-dependent colicins translocate across the OM in an energy-dependent manner and must be completely unfolded in order to translocate through OmpF or OmpC.

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Abbreviations

ΔpH	Proton concentration gradient
$\Delta\psi$	Electrical potential
ADP	Adenosine diphosphate
AF	AlexaFluor (AF488, AF568, AF647)
Amp	Ampicillin
Å	Angstrom
APS	Ammonium persulfate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AU	Absorbance unit
β -ME	β -Mercaptoethanol
BRP	Bacteriocin release protein
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CD	Circular dichroism
CDI	Contact dependent inhibition
C-domain	Cytotoxic domain
CFU	Colony forming units
Chl	Chloramphenicol
ColB	Colicin B
ColE9	Colicin E9
ColE9*	ColE9 H551A
CPS	Counts per second, counts/s
CTD	C-terminal domain
CV	Column volume
Da	Dalton
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNP	2,4-dinitrophenol
dNTPs	deoxynucleotide triphosphates
dsDNA	double-stranded DNA
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
eGFP	Enhanced green fluorescent protein
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EMCCD	Electron multiplying CCD camera
ESI-MS	Electrospray ionisation mass spectrometry
ϵ	Extinction coefficient
FepA	Ferric enterobactin
FMN(H ₂)	Flavin mononucleotide
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
H ⁺	Hydrogen/proton
H-N-H	Histidine Asparagine Histidine motif
IM	Inner membrane
Im (9)	Immunity (9)
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ITC	Isothermal titration calorimetry

IUTD	Intrinsically Unstructured Translocation Domain
k	Rate constant
K ⁺	Potassium
Kan	Kanamycin
KDa	kilodalton
LB	Lysogeny Broth
LPS	Lipopolysaccharide
LUVs	Large unilamellar vesicles
MALDI-TOF MS	Matrix-assisted laser desorption ionisation-time of flight mass spectrometry
MDR	Multidrug resistant
MSD	Mean square displacement
MWCO	Molecular weight cut off
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleoid excision repair
NMR	Nuclear magnetic resonance
OBS	OmpF binding site
OD ₆₀₀	Optical density at 600 nm
OM	Outer membrane
OMP(s)	Outer membrane protein(s)
Pal	Peptidoglycan-associated lipoprotein
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PFA	Paraformaldehyde
PG	Peptidoglycan
Pi	Phosphorylate
PL	Phospholipid
PMF	Proton motive force
PMSF	Phenylmethylsulfonyl fluoride
PSF	Point-spread function
R-domain	Receptor domain
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNase	Ribonuclease
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
SEM	Standard error of mean
SEC	Size exclusion chromatography
SIM	Structured illumination microscopy
SMT	Single-molecule tracking
ssDNA	Single-stranded DNA
ssRNA	single-stranded RNA
t _{1/2}	Half-life (time)
TBE buffer	Tris-borate EDTA
TBE	TolB binding epitope
T-domain	Translocation domain
TEA	Triethanolamine
TEMED	N, N, N', N'-tetramethyl ethylenediamine
Tet	Tetracycline
TIRF(M)	Total internal reflection fluorescence (microscopy)
TM	transmembrane
tRNA	Transfer RNA

tRNase	Transfer ribonuclease
T6SS	Type 6 secretion system
UV	Ultraviolet
WT	Wild-type
w/v	Weight per volume

1 Chapter 1 - General Introduction

1.1. Antibiotic resistance and the search for alternative therapies

Penicillin was the first antibiotic to be used and was discovered in 1928, it targets cell wall synthesis in bacteria by blocking the cross-linking of peptidoglycan units (Kohanski, Dwyer, and Collins 2010). Its discovery proved crucial in the fight against bacterial infections and revolutionised modern medicine. Since then, several new classes of antibiotics have been discovered that target a wide range of bacterial species. Several existing classes were identified as small molecule natural products. Over time, their scaffolds have been synthetically tailored to generate successive generations for each antibiotic. However, this cannot go on indefinitely and highlights the need for new antibiotic core scaffolds (Walsh and Wencewicz 2014).

Antibiotics tend to target cell wall biosynthesis (peptidoglycan synthesis), protein synthesis, inner membrane integrity, DNA replication, RNA transcription and metabolic pathways (Kapoor, Saigal, and Elongavan 2017) (Walsh and Wencewicz 2014). Resistance mechanisms include preventing the accumulation of the antibiotic, either through decreasing membrane permeability by decreasing the number of porins on the surface, or increasing efflux of the antibiotic through multi-drug efflux pumps in the inner membrane. Other resistance mechanisms include enzymes for the inactivation of antibiotics through hydrolysis and modification of the antibiotic target site to prevent binding (Kapoor, Saigal, and Elongavan 2017). These resistance mechanisms can either be acquired through chromosomal mutations or the acquisition of mobile genetic elements between species by horizontal genetic transfer, resulting in the spread of antibiotic resistance.

In the past 30 years, only a few new classes of antibiotics have been introduced. Alongside the rapidly declining identification of new antibiotics has been the alarming increase in antibiotic resistance. In some cases, there are multi-drug resistant (MDR) bacteria resistant against all known classes of antibiotic. In the US and Europe, there is an estimated 50,000 people

that die annually from infections caused by common strains of resistant bacteria, with a predicted 10 million deaths by 2050 (O'Neil 2014). Gram-negative bacterial antibiotic resistance is of particular concern due to their highly impermeable outer membrane (OM) which prevents the entry of multiple classes of antibiotics.

Gram-negative bacterial pathogens that pose a serious threat include *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, in all of these species multi-drug resistance occurs (Boucher et al. 2009). In the EU, 58.6 % of *E. coli* isolates reported to the ECDC were resistant to at least one of aminopenicillins, fluoroquinolones, third-generation cephalosporins, aminoglycosides and carbapenems ((ECDC) 2017). There is therefore a critical need for new antibiotics targeting these bacteria. Bacteriocins offer a potential alternative therapeutic strategy to current antibiotics. This will be addressed in Section 1.2.3.

1.2. Bacterial competition systems

Gram-negative bacteria exist in a broad range of ecological niches. They have developed the ability to thrive in their environments by evolving and adapting to their surroundings, through the acquisition of genes from other organisms by horizontal genetic transfer (Wiedenbeck and Cohan 2011). This leads to the sharing of genes for virulence and antibiotic resistance between bacteria, thereby allowing other ecological niches to be inhabited or improve survival in its current niche.

Several different related and unrelated species of bacteria can inhabit the same ecological niche but usually will have to compete for space and resources. Bacteria have therefore developed mechanisms that either confer a competitive advantage passively, known as exploitative competition, or impair or kill other microbial species to survive, known as interference competition (Hibbing et al 2010).

An example of a competitive advantage to bacteria for survival includes the acquisition of nutrients. Bacteria that have the ability to uptake certain scarce nutrients, or do so at a faster

rate, will deplete the availability to other competitors, giving a higher chance of survival and reproduction. An example of this is the uptake of iron, either through the use of siderophores or the use of transporters for iron uptake on the cell surface (Wandersman and Delepelaire 2004). Bacteria that are adapted to produce surface adhesins and receptors have a higher chance of colonising a more challenging ecological niche (Hibbing et al 2010). Some bacteria like *Lactobacillus*, produce specific exterior glycoproteins forming an S-layer, to act as a protective layer that excludes enzymes and antimicrobials to protect themselves from dispersal (Sleytr et al. 2014).

Alternatively, bacteria can compete through the production of antimicrobials that are either bacteriostatic or bacteriolytic to stimulate dispersal of colonised bacteria, for example *Streptomyces coelicolor* and its production of antibiotics (Hibbing et al. 2010). Antimicrobials can either be released into the surrounding environment to diffuse and bind their target neighbours, through the use of Type 1 and Type 2 secretion systems, or mediated through physical cell-cell adhesion using Type 3, Type 4, Type 5 or Type 6 secretion systems (Green and Mecsas 2016).

1.2.1. Type 6 secretion system (T6SS)

The type 6 secretion system (T6SS) in bacteria is found in around 25 % of Gram-negative bacteria (Pukatzki et al. 2007). It is a trans-envelope syringe-like structure composed of a membrane-associated baseplate, a sheath and tube topped with a spike. The T6SS has a lot of structural and mechanistic homology to contractile nanomachines like the T4 phage tail (Pukatzki et al. 2007). On contraction of the sheath, the tube and spike are forced out of the cell to penetrate the membrane of its target cell, where the effectors are injected directly into the cytoplasm in a contact-dependent manner. It can target both eukaryotic and more commonly prokaryotic cells. The effector activities include peptidoglycan hydrolases, phospholipases and nucleases. Several of these effectors also have genes for immunity against them (Russell, Peterson, and Mougous 2014).

1.2.2. Contact-dependent inhibition (CDI)

Another form of bacterial competition is contact-dependent inhibition (CDI) systems. In *E. coli*, CDI involves CdiA hemagglutinin-repeat protein and CdiB β -barrel protein two-partner secretion proteins, which are a subfamily of the T6SS (Aoki et al. 2005). CdiA effector extends as a β -helical structure through CdiB on the inhibitor cell, to bind its receptor on the surface of a neighbouring cell. Receptors in *E. coli* include BamA, OmpC, OmpF and Tsx (Ruhe et al. 2017). CDI requires the direct cell-cell contact of CdiA with its target cell, which then leads to the cleavage of the C-terminal toxin and its translocation into the cell. Translocation occurs by a poorly understood mechanism, however the T-domain is predicted to interact with a series of specific inner membrane (IM) proteins (Willett et al. 2015) and translocation requires the proton motive force (PMF) for entry across the IM and does not use the Tol or Ton system (Ruhe et al. 2014). There are several CDI toxins, which encode nucleases, pore-formers, adenosine deaminases, ADP-ribosyl cyclases and metalloproteases. A third CDI gene is CdiI, which encodes an immunity protein to protect cells from the various CdiA toxins (Ruhe, Low, and Hayes 2013).

1.2.3. Bacteriocins

Bacteriocins are peptide or protein antibiotics that are produced by bacteria in response to competition within their ecological niche. They are present in both Gram-positive and Gram-negative bacteria and Archaea. Bacteriocins have been found in almost every bacterial species and each species can contain many different bacteriocins. They are one of the most diverse and naturally abundant classes of antimicrobials (Sharp et al. 2017). Bacteriocins vary in their size, cell target and mode of action.

The naming of protein bacteriocins, the focus here, often reflects the species that produced them and the bacteria they target; for example, pesticins target *Yersinia pestis*, diffocins target *Clostridium difficile*, pyocins target *Pseudomonas pyogenes*, cloacins target *Enterobacter cloacae*, klebicins target *Klebsiella pneumoniae* and colicins target *Escherichia coli*

(Kim, Tarr, and Penfold 2014). Bacteriocin gene clusters can be encoded exclusively on plasmid replicons like *E. coli*, on the chromosome like *P. aeruginosa* and in some cases on both, like *S. marcesens* (Riley and Wertz 2002).

Bacteriocins in Gram-positive bacteria are very diverse and tend to be small peptides. Their production and release from the cell are not necessarily lethal. Whereas in Gram-negative bacteria, bacteriocin release involves the lysis of the producer cell, in Gram-positive bacteria bacteriocins can use either the sec-dependent export pathway or cells have evolved specific transport systems for release. Generally, their expression in Gram-positive bacteria is controlled by quorum-sensing, regulated by the culture-density with stationary-phase inducing their expression (Riley and Wertz 2002). Gram-negative bacteriocins are either small peptides called microcins or large protein bacteriocins. Microcins are small peptides <10 kDa that are mainly hydrophobic, have high stability to heat, pH and proteases. Microcins are both plasmid and chromosomally-encoded as gene clusters for the precursor microcin, immunity factors and secretion proteins (Duquesne et al. 2007).

Colicins are the most well-studied bacteriocin in Gram-negative bacteria. They are large 40-70 kDa proteins, that belong to the Enterobacteriaceae family and Gammaproteobacteria class and are highly abundant. Several of the colicins identified are thought to have evolved through the swapping of domains and/or the positive selection of mutations in both the immunity proteins and colicins. A two-step process of colicin evolution has been proposed, in which a point mutation occurs within the immunity gene, broadening its immunity function. The producer cell is now immune to its ancestral colicin and to other similar colicins, increasing its fitness and chance of survival. This is followed by a second mutation within the colicin gene, which produces a novel colicin no longer recognised by its ancestral immunity, thereby generating new colicin-immunity proteins (Riley and Wertz 2002).

Colicins are highly specific proteins that are produced by one *E. coli* strain and target closely-related sensitive *E. coli* strains via specific cell surface receptors (Hibbing et al. 2010). Colicins are thought to play a role in pathogenesis, through the displacing of bacterial flora and enhancing colonisation and therefore infection. For example, strains of *Shigella sonnei*, that causes dysentery, were isolated in Vietnam and found to have acquired E5-type colicin within its first evolutionary bottleneck, leading to the killing of *E. coli* within the gut and its establishment in a host population (Holt et al. 2013).

The use of broad-spectrum antibiotics kills both pathogenic and commensal bacteria, leading to an increase in the occurrence of nosocomial infections and an intense selection pressure for the evolution of antibiotic resistance, thereby reducing the shelf-life of the drug. Because of their high abundance, high specificity and diverse range of modes of cytotoxicity, bacteriocins show huge potential as narrow-spectrum antibiotics against specific pathogens, which would reduce dysbiosis and the intensity of the selection pressure and therefore rate of developing resistance.

In addition to the potential of bacteriocins as antibiotics against human infections, they are also of commercial interest for farming and agriculture practices. Bacteriocins are being investigated for their use in preventing food contamination as an alternative to thermal inactivation. Colicins have been shown to be expressed within edible plants and this leads to the death of Enterohemorrhagic *Escherichia coli* (EHEC), improving food safety (Schulz et al. 2015). In addition, nisin is a Gram-positive bacteriocin approved by the FDA that is used in preventing food spoilage in milk based products (Riley and Wertz 2002). Colicins are also being tested for their use in animal feed as an alternative to antibiotics. Colicin E1 has been shown to kill enterotoxigenic *E. coli* in weaning pigs (Cutler et al. 2007), colicinogenic *E. coli* strains that produce Colicin E7 added to cattle feed have been shown to reduce the level of *E. coli* 0157:H7

within cattle (Schamberger et al. 2004). Bacteriocins are also being tested for their use in agriculture against phytopathogens that cause plant disease (Grinter, Milner, and Walker 2012).

1.3. The cell envelope of Gram-negative bacteria

The cell envelope of Gram-negative bacteria is composed of the outer membrane (OM), the periplasmic compartment containing a peptidoglycan (PG) layer and the inner membrane (IM), all surrounding the cytoplasmic compartment (Figure 1. 1).

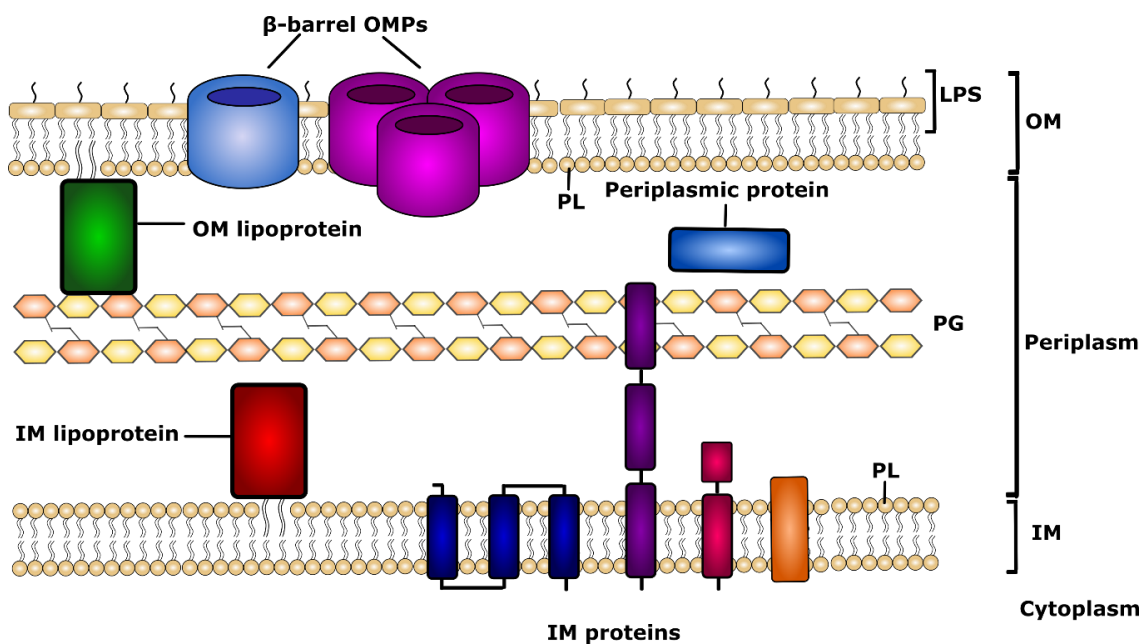


Figure 1. 1 - The Gram-negative bacterial cell envelope.

The cell envelope of Gram-negative bacteria is composed of an OM, periplasmic compartment, containing a peptidoglycan (PG) layer, an inner membrane (IM), and a cytoplasmic compartment. Cell envelope components included lipopolysaccharide (LPS), phospholipids (PL), IM and OM lipoproteins and periplasmic proteins (Adapted from (Ruiz, Kahne, and Silhavy 2009)).

1.3.1. Outer membrane (OM)

The outer membrane of bacteria acts as a permeability barrier to cells protecting them from the entry of harmful substances like antimicrobials, whilst allowing the passive and active diffusion of nutrients. The OM of Gram-negative bacteria is ~7 nm thick and is composed of an asymmetric bilayer that contains phospholipids and lipoproteins on the inner leaflet of the membrane and lipopolysaccharides (LPS) on the outer leaflet of the membrane (Figure 1. 1) (Ruiz, Kahne, and Silhavy 2009). LPS is essential for cell survival and is used to protect bacteria from the entry of

antibiotics and other toxic substances. LPS is composed of lipid A, which acts as a hydrophobic anchor for the LPS in the membrane, a core oligosaccharide made up of an outer and inner core and an O-antigen, which is highly variable and elicits an immune response (Figure 1. 2) (Li et al. 2015). Lipid A and core polysaccharide carry a large number of negative charges. Lab *E. coli* K-12 strains do not produce an O-antigen and are referred to as rough LPS. The amount of LPS in the OM has been estimated to be $\sim 10^6$ molecules (Raetz and Whitfield 2002) and is balanced with the number of phospholipids (PLs). A shift in the LPS:PL ratio is lethal to the cell. The composition can vary between different Gram-negative species and can be modulated as a result of changing environments (Langklotz, Schakermann, and Narberhaus 2011).

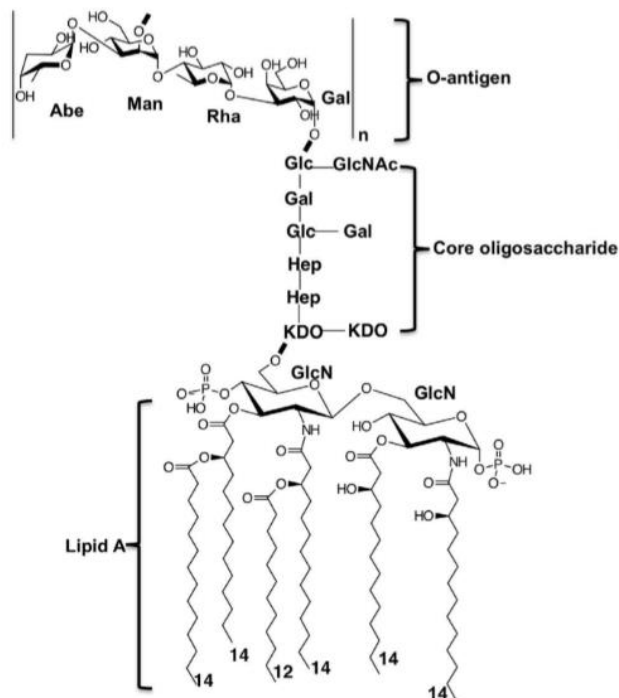


Figure 1. 2 - Structure of lipopolysaccharide (LPS).

LPS is composed of an O-antigen, a core oligosaccharide made up of an outer and inner core and lipid A (Li et al. 2015).

Common OM phospholipids in the inner leaflet include ~ 80 % phosphatidylethanolamine (PE), ~ 15 % phosphatidylglycerol (PG) and ~ 5 % cardiolipin (CL) (Raetz 1978). Their hydrophobic fatty acid acyl chains are protected by their polar head groups. Lipoproteins like murein lipoprotein (Lpp) play a role in maintaining the integrity of the OM, through their interaction with PG in the periplasm (Delcour 2009). Recently, it has been shown

that a combination of LPS, OMPs and the OM contributes to the mechanical rigidity, strength and prevention of cell lysis (Rojas et al. 2018).

Proteins within the OM are involved in regulating the transport of substances across the membrane, these include integral transmembrane proteins, also referred to as outer membrane proteins (OMPs). Most OMPs form β -barrels and function as either porins or ligand-gated transporters. Porins act as non-specific aqueous diffusion channels, with a size exclusion limit for diffusion of ~ 600 Da and are involved in the passive diffusion of ions, amino acids, and small sugars. Porins can exist as monomers or trimers. For example, OmpF, OmpC and PhoE are trimeric porins containing 16-antiparallel β -strands that form β -barrel general diffusion porins (Cowan et al. 1992). They contain a central hydrophilic pore, which is partially occluded by an extracellular loop. The lumen contains a series of charged residues that allow for cation or anion selectivity. They are very abundant in the OM at $\sim 10^5$ copies per cell (Li et al. 2014).

Ligand-gated transporters act as specialised receptors for the active transport of specific nutrients. They are larger β -barrel proteins with 20-24 β -strands and contain an N-terminal globular plug domain that occludes the barrel of the pore. Examples include BtuB for the uptake of vitamin B₁₂ and FepA, FhuA and Cir for the uptake of iron siderophores (Nikaido 2003). They are present at much lower levels with ~ 2500 copies of BtuB, ~ 70 copies of FepA, ~ 1200 copies of FhuA and ~ 100 copies of LamB (Li et al. 2014). Ligands for these receptors bind with high affinity and specificity and their entry is dependent on interactions with the Ton system for their PMF-dependent uptake into cells (Nikaido 2003). There are also several enzymatic β -barrel proteins that function as proteases like OmpT, and phospholipases like PldA (Silhavy, Kahne, and Walker 2010).

1.3.2. Periplasm

The periplasm is a highly viscous oxidising aqueous compartment located between the OM and IM (Figure 1. 1). The size of the periplasmic space can vary and is controlled by periplasmic

lipoproteins and osmotic pressure, but is on average ~15 nm thick (Bayer 1991) (Leduc et al. 1989). The periplasm has many different functions including the folding, quality control and transport of proteins from the IM to the OM. The periplasm aids cells in their ability to withstand turgor pressure by providing structural systems like PG. It also sequesters enzymes that are toxic to the cytoplasm, including proteases, nucleases, phosphatases and phospholipases. The periplasm contains periplasmic binding proteins and chaperones for the transport of small molecules, assembly platforms for the secretion of β -barrel proteins and LPS for insertion in the OM, cell division machinery and trans-periplasmic secretion systems. It also contains environmental sensing modules to sense and respond to changes like toxic compounds or damage to the cell envelope (Miller and Salama 2018).

1.3.3. Peptidoglycan (PG)

Peptidoglycan is present within the periplasm (Figure 1. 1) and is composed of glycan strands made of repeating units of alternating N-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurMac), which are cross-linked by pentapeptide side chains. The thickness of the PG is ~ 6 nm (Vollmer, Blanot, and de Pedro 2008) (Vollmer 2008). The length and composition of the glycan strands and the degree of crosslinking varies between bacterial strains and under different growth conditions. The PG is attached to the OM through the covalent attachment of the major OM lipoprotein Lpp and through non-covalent attachment of Lpp, OmpA and porins in the OM (Lazzaroni et al. 1999). PG plays a role in determining the shape of bacteria due to its rigidity and helps prevent lysis by withstanding turgor. It also acts as a scaffold for various envelope components (Vollmer, Blanot, and de Pedro 2008). The PG acts as a molecular sieve that limits the diffusion of proteins within the periplasm to <50 kDa (Demchick and Koch 1996).

1.3.4. Inner membrane (IM)

The IM is a symmetrical phospholipid bilayer that surrounds the cytoplasm (Figure 1. 1), containing the same composition of phospholipids as the inner leaflet of the OM (Raetz 1978).

It acts as a selectively permeable membrane for the passive diffusion of small and lipid-soluble molecules (Ruiz et al. 2009) or active transport powered by ATP, PMF or ion transport (Pugsley 1993). Functions of the IM include peptidoglycan synthesis during cellular growth and division, lipid biosynthesis, protein transport and secretion, signal transduction and energy production through the electron transport chain. The proteins that carry out these diverse functions are anchored within the membrane by one or several transmembrane α -helices (Silhavy, Kahne, and Walker 2010).

1.3.5. Cytoplasm

The cytoplasm is the central compartment of bacteria protected by the IM (Figure 1. 1). The cytoplasm is highly viscous, but less so than the periplasm as reflected by the diffusion coefficients of proteins. It is the location of DNA replication, transcription and translation. There is high molecular crowding in the cytoplasm, contributed to by the presence of the nucleoid which acts as a molecular sieve for diffusion (Mika and Poolman 2011).

1.4. Energy-transducing systems in Gram-negative bacteria

1.4.1. Proton motive force (PMF)

The electron transport chain is a series of membrane-bound respiratory enzyme complexes in the IM, including iron-sulphur clusters, quinones and cytochromes (Anraku 1988). Under aerobic conditions during glycolysis and the Krebs cycle, glucose is oxidised to produce nicotinamide adenine dinucleotide (NADH) and succinate. These act as substrates and initial electron donors for the respiratory enzymes whose oxidation leads to the generation of electrons. These electrons are then transferred between the enzyme complexes via redox reactions acting as electron donors and acceptors, which reach the terminal electron acceptor, molecular oxygen, to generate water (Jormakka, Byrne, and Iwata 2003).

The transfer of electrons between the respiratory enzymes releases free energy which is used to transfer a proton (H^+) across the IM from the cytoplasm into the periplasm against its

membrane potential, effectively acting as a proton pump. Protons are positively charged and unable to diffuse freely through membranes, so they require a pore or protein to assist their movement across the proton-impermeable IM (Cook et al. 2014).

This transfer of protons across the IM generates two trans-membrane gradients; a proton concentration gradient (ΔpH), where there is a high concentration of protons in the periplasm and low concentration in the cytoplasm. The second gradient is an electrical potential ($\Delta\Psi$), due to a charge separation where there is a high concentration of positively charged ions (usually protons) in the periplasm and fewer positively charged ions in the cytoplasm. These two gradients (ΔpH and $\Delta\Psi$) compose the electrochemical proton motive force (PMF, Δp) (Cook et al. 2014).

Bacteria use this proton gradient to generate energy for the cell. Protons in the periplasm are passed by passive diffusion down their electrochemical gradient by chemiosmosis across the IM into the cytoplasm using ATP synthase. ATP synthase uses the kinetic energy of this proton transfer to phosphorylate (P_i) adenosine diphosphate (ADP), generating adenosine triphosphate (ATP) which is the energy source of the cell for biosynthesis, cell growth and cell division (Deckers-Hebestreit and Altendorf 1996). The protons then get transferred back into the periplasm by the electron transport chain to generate a continuous cycle of protons and generation of ATP. The PMF is also used by bacteria for solute transport, flagella rotation, protein secretion and membrane-related processes (Henkel et al. 2014).

Bacteria do not contain an energy source at the OM due to the lack of ATP-hydrolysing proteins and inability to generate a proton gradient across this membrane. Therefore, bacteria have developed periplasmic transmembrane complexes to access and harness the energy produced by the PMF in the IM to drive processes at the cell surface. Two such systems are Ton and Tol.

1.4.2. Ton system

The Ton system is composed of TonB, ExbB and ExbD within the IM. ExbB and ExbD are structurally and functionally homologous to TolQ and TolR of the Tol system, respectively (Figure 1. 3). The role of the Ton system in Gram-negative bacteria is the active transport of iron siderophores, vitamin B₁₂, metals and carbohydrates (Schauer, Rodionov, and de Reuse 2008). TonB extends into the periplasm where it contacts TonB-dependent transporters (TBDTs) in the OM. ExbB and ExbD are thought to act as a scaffold and aid in harnessing the PMF (Jana, Manning, and Postle 2011), which is propagated between the IM and receptors at the OM through a conformational change of TonB, for the uptake of nutrients (Bradbeer 1993) (Noinaj et al. 2010). The Ton system is also used by group B colicins for translocation across the OM, addressed later in Section 1.5 (Lazdunski et al. 1998).

1.4.3. Tol system

The Tol system is composed of five proteins; TolQ, TolR, TolA, peptidoglycan-associated lipoprotein (Pal) and TolB (Figure 1. 3). TolQ and TolR are embedded within the IM, TolA has a domain anchored within the IM and extends into the periplasm. Pal resides within the periplasm through its anchor at the periplasmic side of the OM and is in complex with TolB (Lazzaroni et al. 1999). TolQRA proteins interact and form a complex within the IM. The role of the Tol system in Gram-negative bacteria is the stabilisation and maintenance of OM integrity (Lloubes et al. 2001) and maintenance of the structure during cell division (Gray et al. 2015). Mutations of these proteins leads to hypersensitivity towards detergents and antibiotics, OM blebbing, the release of periplasmic contents and defects in the synthesis of smooth LPS containing O-antigens (Lazzaroni et al. 1999). The Tol system has been shown to function similarly to the Ton system in the access and harnessing of the PMF and the propagation to the OM through a conformational change of TolA (Germon et al. 2001).

The Tol system is parasitized by filamentous phages for OM translocation and for release of bacteriophage DNA into the cytoplasm during infection (Click and Webster 1998). The Tol

system is also used by group A colicins for translocation across the OM, addressed later in Section 1.5 (Lazdunski et al. 1998).

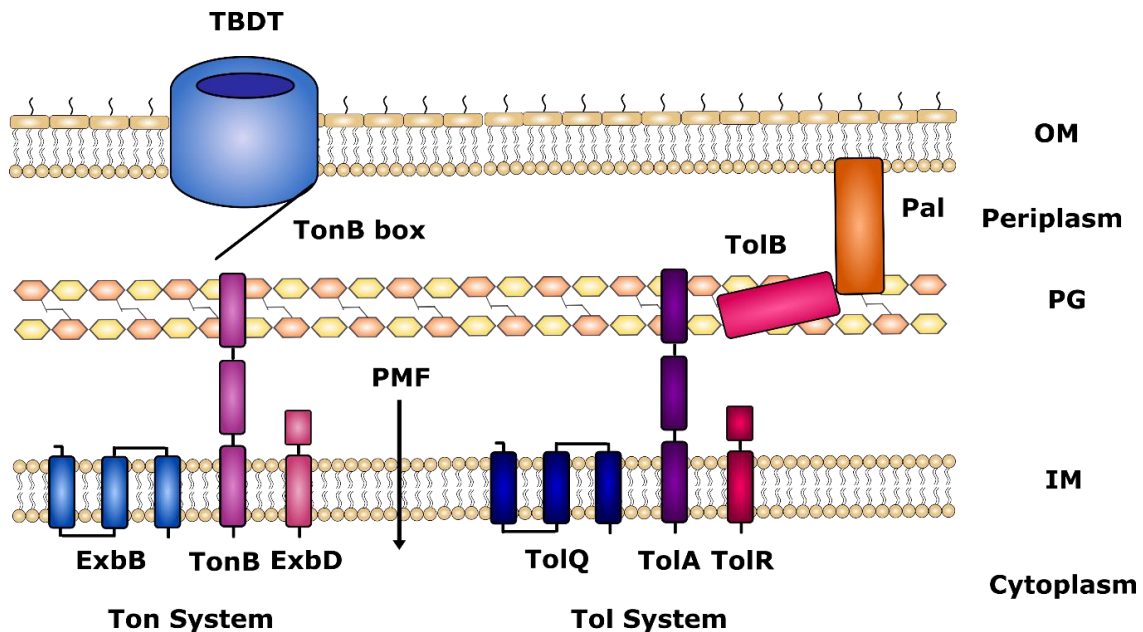


Figure 1. 3 - The Ton and Tol systems.

The Ton system is composed of TonB, ExbB and ExbD within the IM. TonB extends into the periplasm where it contacts TonB-dependent transporters (TBDTs) via the TonB box in the OM. The Tol system is composed of TolQ, TolR, TolA, peptidoglycan-associated lipoprotein (Pal) and TolB.

1.4.4. Nutrient uptake

The entry of small solutes and nutrients across the OM into cells occurs via passive diffusion through porins or active transport through OMPs, which is the case for essential nutrients that exist in low external concentrations, such as iron.

Vitamin B₁₂, also known as cobalamin, is a nutrient taken up by *E. coli* and used as a cofactor in several enzyme systems, in the biosynthesis of methionine (Ahmed 1973), the use of ethanolamine and the synthesis of the modified tRNA base queine (Kadner 1990). Vitamin B₁₂ binds to BtuB as its receptor on the OM with ~0.3 nM affinity in the presence of calcium ions (Reynolds, Mottur, and Bradbeer 1980) (White et al. 1973) (Bradbeer et al. 1986) (Figure 1. 4). BtuB is a member of the TonB dependent OM transporters (TBDTs), along with Cir, FepA and FhuA (Kadner 1990). It is a 22-stranded β -barrel that contains an N-terminal globular plug

domain that occludes the barrel and prevents the free diffusion of nutrients across the membrane. This N-terminal globular domain contains a highly conserved pentapeptide sequence called the TonB box (Pressler et al. 1988), which was identified through a mutation in BtuB that allowed the binding but not the TonB-dependent transport of vitamin B₁₂ (Heller and Kadner 1985). This TonB box is found in all TonB-dependent OM receptors.

When vitamin B₁₂ binds to BtuB in the extracellular loops, the TonB box becomes disordered and undergoes large structural changes moving away from the barrel interior (Fanucci et al. 2003). TonB undergoes a PMF-dependent conformational change that extends into the periplasm (Larsen, Letain, and Postle 2003) and allows the formation of a non-covalent complex between the TonB box of the barrel plug and TonB in the IM (Shultis et al. 2006). This contact allows the transduction of the PMF from the IM to the OM TonB-dependent receptors and the energy-dependent transport of vitamin B₁₂ into the cell (Bassford et al. 1976). The PMF-dependence for vitamin B₁₂ uptake was shown through PMF inhibition, either using pore-forming colicins ColK or ColE1 (Di Masi et al. 1973), through the deletion or mutation of TonB (Bassford et al. 1976) and through chemical PMF inhibition using cyanide, arsenite or 2,4-DNP (Di Girolamo and Bradbeer 1971). The exact mechanism of force transduction remains unresolved however.

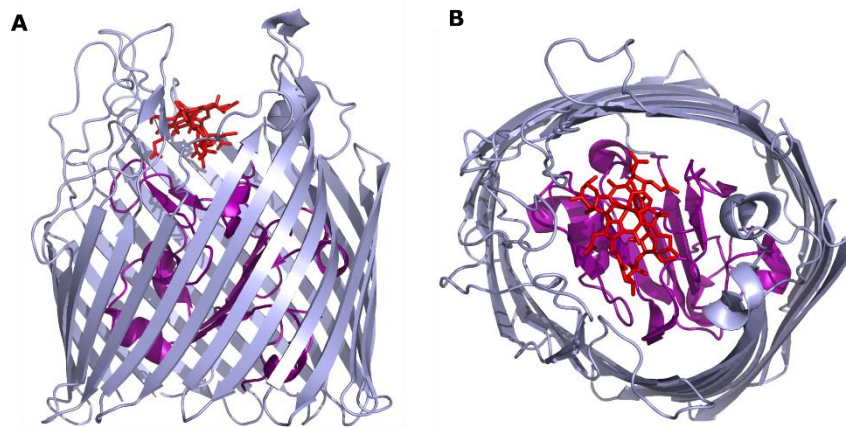


Figure 1. 4 - Structure of BtuB and vitamin B₁₂.

The structure of BtuB (light blue) with its N-terminal globular plug domain (purple) containing the TonB box for opening and entry of vitamin B₁₂ (red). A) side view of vitamin B₁₂ bound to BtuB, B) top view of vitamin B₁₂ bound to BtuB (PDB; 1nqh).

1.5. Colicins

1.5.1. Genetic organisation of colicin operons

Gene clusters for colicins are exclusively encoded on colicinogenic plasmids called pCol. There are two types of pCol plasmids; Type I pCol plasmids are small (6-10 kb) multicopy plasmids and present in ~20 copies/cell. These plasmids mainly encode group A colicins and are non-transmissible in the absence of a plasmid for their conjugation. Type II pCol plasmids are much larger (~40 kb) and present as a single copy. These plasmids mainly encode group B colicins. These plasmids can also encode multiple colicin operons for expression in cells and are transmissible (Hardy et al. 1973) (Lehrbach and Broda 1984).

The colicin operon contains one to three genes depending on the group it belongs to and its cytotoxic activity. All colicin operons contain the *cxa* gene, standing for colicin x activity, which codes for the structure of the colicin. This gene is under the control of the SOS promoter (P_{SOS}) (Lloubes, Baty, and Lazdunski 1986). The second gene in the operon is *cxi* gene, standing for colicin x immunity, which codes for a specific immunity protein downstream of the structural

gene. The third gene is the *cxl* gene, standing for colicin x lysis protein, which is involved in the release of the colicin from the producing cell (Figure 1. 5) (Riley 1993).

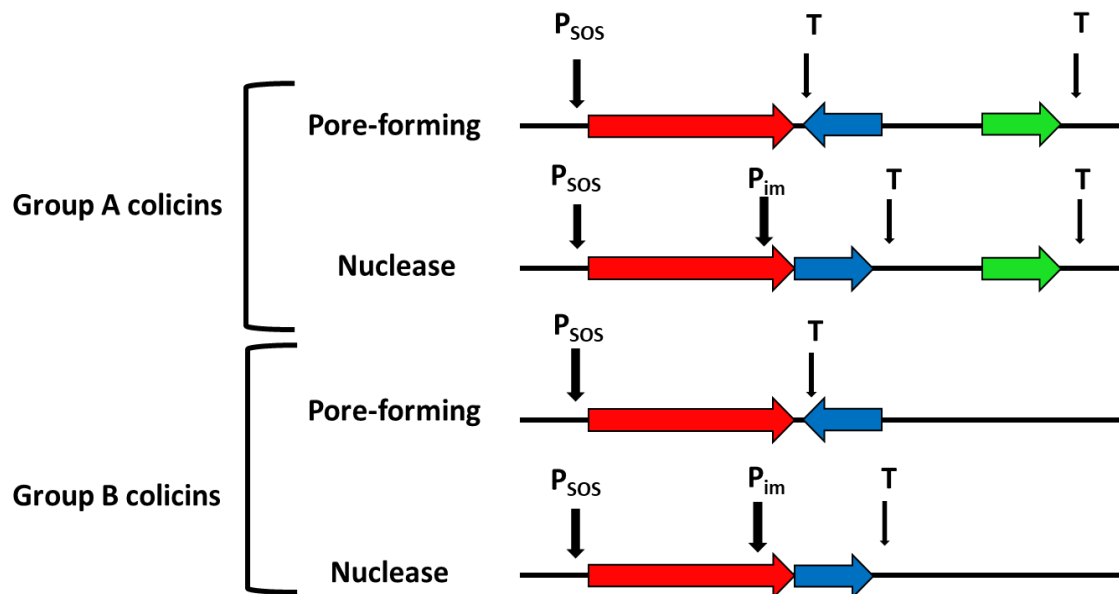


Figure 1. 5 - Genetic organisation of colicin operons.

The different colicin operon contains up to three genes. The *cx_a* gene (red), standing for colicin x activity, codes for the structure of the colicin. This gene is under the control of the SOS promoter (P_{SOS}). The *cx_i* gene (blue), standing for colicin x immunity, codes for a specific immunity protein downstream of the structural gene. It is under the control of both P_{SOS} and immunity promoter (P_{im}). The *cx_l* gene (green), standing for colicin x lysis protein, is involved in the release of the colicin from the producing cell. T stands for transcription terminator sites (Adapted from (Cascales et al. 2007)).

Only nuclease colicins of group A and B contain the *cx_i* gene for immunity on their operon. This gene is under the control of two promoters; the SOS promoter (P_{SOS}) of the entire operon and its own immunity promoter (P_{im}) to ensure an excess of immunity protein is present to inhibit the cytotoxicity of the structural colicin gene (Lloubes, Baty, and Lazdunski 1986). Group A and B pore-forming colicins do not contain an immunity gene within their colicin operon, but it is present and transcribed from the opposite DNA strand in the intergenic space between the *cx_a* and *cx_l* genes under constitutive regulation (Riley 1993).

The *cx_l* lysis gene is present mainly in group A pore-forming and nuclease colicins, occasionally in the operon of group B colicins. This gene does not contain its own promoter and due to the favoured transcription of the major mRNA transcript, which contains a termination

site before the lysis gene, the lysis gene is present at low concentrations compared to the colicin and immunity. Its expression is dependent on transcription of the minor mRNA transcript which has its terminator after the lysis gene (Lloubes, Baty, and Lazdunski 1988) (Cascales et al. 2007).

1.5.2. Regulation of colicin transcription

Colicins are mainly under the control of the bacterial SOS response, a global response to DNA damage, with 60-75 % of colicin operons containing dual overlapping SOS boxes for binding two LexA dimers (Mavridou et al. 2018) (Gillor, Vriezen, and Riley 2008). During normal cellular growth, LexA repressor binds to a 20 bp SOS box promoter sequence of the SOS regulon and negatively regulates expression of approximately 50 SOS genes. There are several inducers of DNA damage and thereby the SOS response. These include exogenous triggers like UV irradiation, oxidative compounds, mitomycin C and antibiotics (Zgur-Bertok 2013). DNA damage leads to an accumulation of single-stranded DNA (ssDNA) regions at the replication fork where DNA polymerase activity is halted. RecA protease protomers assemble to form filaments at the sites of ssDNA on the chromosome, these interact with LexA dimer and induce its autocleavage and its dissociation from the SOS operon, thereby relieving its repression and allowing RNA polymerase to bind and initiate transcription of the SOS genes (Janion 2008) (Little and Mount 1982).

LexA repressor has different affinities for different SOS boxes. Operons with weak affinity for LexA are the first genes to be expressed in response to DNA damage and bacteria can therefore sequentially and temporally activate different responses and mechanisms of repair (Zgur-Bertok 2013). The first SOS genes induced are the *uvr* genes which encode proteins involved in nucleoid excision repair (NER) to try to repair damaged DNA. Other genes encode error-prone repair DNA polymerases, *suIA* which binds FtsZ and prevents cell division, proteins that promote biofilm formation and proteins that decrease the PMF and ATP levels to induce cell dormancy (Zgur-Bertok 2013). The affinity of LexA to colicin SOS boxes has been predicted

to be weak, allowing their expression even in the absence of environmental cue and their rapid expression in response to DNA damage (Mavridou et al. 2018).

The expression of some colicins is under the control of stress regulators like CRP, IscR and Fur (Mavridou et al. 2018). Certain stress conditions can induce colicin expression, like thymine starvation, nutrient deprivation, high temperature, the stringent response, catabolite repression, stationary phase of growth, anaerobiosis and *ompR* mutation (Cascales et al. 2007) (Ghazaryan et al. 2014).

In a population of *E. coli* there is always a subpopulation that express colicin in the absence of an environmental cue or threat. This phenotypic heterogeneity ranges from 0.5-3 % of producers in planktonic populations and 7-9 % of producers in sessile populations. It is proposed that stochastic mutation of DNA triggers the SOS response and thereby colicin expression in a small subpopulation (Bayramoglu et al. 2017). Colicin expression is a costly trait, with cells showing slower growth and elongation. This subpopulation is maintained through autoinduction of neighbouring clonemate cells by the release and uptake of the colicin. The advantage of this subpopulation is the generation of colicins for a pre-emptive attack, the low level of colicin production is sufficient to kill a sensitive competitor (Mavridou et al. 2018). Cells have been shown to undergo huge coordinated attacks on competitor strains through the sensing of competitor toxins through SOS regulated DNA damage and the autoinduction of neighbouring cells of their colony to propagate the release of large amounts of colicin (Mavridou et al. 2018).

1.5.3. Colicin release

After induction of colicin expression, group A colicins are released from the cell via their lysis protein also known as bacteriocin release protein (BRP). This gene is co-expressed on the minor mRNA of the colicin operon and therefore present at low concentrations. This ensures that there is a high level of colicin expression before its release into the surrounding environment and

concomitant death of the producer cell. Lysis proteins are small lipoproteins (~4.5 kDa) with a high degree of sequence similarity (Cascales et al. 2007). They are synthesised as precursors in the cytoplasm with signal sequences that direct them to the IM, where they translocate across by Sec-dependent and independent routes. All lysis proteins contain an N-terminal cysteine which becomes acylated for its insertion and anchoring in the periplasmic leaflet of either the IM or OM, with most released from the IM and located in the OM by recognition and shuttling by the Lol system (Cavard et al. 1987) (Gennity, Kim, and Inouye 1992) (Silhavy, Kahne, and Walker 2010).

The sequence of events that leads to cell lysis are not well understood. Release of the lysis protein leads to quasilysis, which is a sharp decline in the optical density following a delay from lysis induction, without observing lysed bacterial cells. In addition, modifications and destabilisation of the IM and OM are observed possibly through formation of pores, followed by the activation of OmpLA, outer membrane phospholipase A. OmpLA is proposed to form lysophospholipids, which act as detergents and permeabilise the IM and OM, increasing cell permeability and the release of colicin from the cytoplasm (Pugsley and Schwartz 1984; van der Wal, Luirink, and Oudega 1995).

Group B colicins do not encode lysis proteins. There is evidence to suggest that group B colicins use phage-encoded lysis proteins for their release. Prophages comprise ~20 % of bacterial genomes and lysogenic phages can be transcribed under the SOS response (van Raay and Kerr 2016) (Nedialkova et al. 2016).

1.5.4. Colicin domain organisation

Most colicins are composed of three main architectural domains; an N-terminal translocation domain (T), a central receptor binding domain (R) and a C-terminal cytotoxic domain (C) (Appendix I Figure 1). The N-terminus is usually unstructured and contains an intrinsically unstructured translocation domain (IUTD) (Figure 1. 6). Although colicins vary in their size,

structure and cytotoxicity, they contain a combination of the three domains and use similar routes for their translocation into cells.

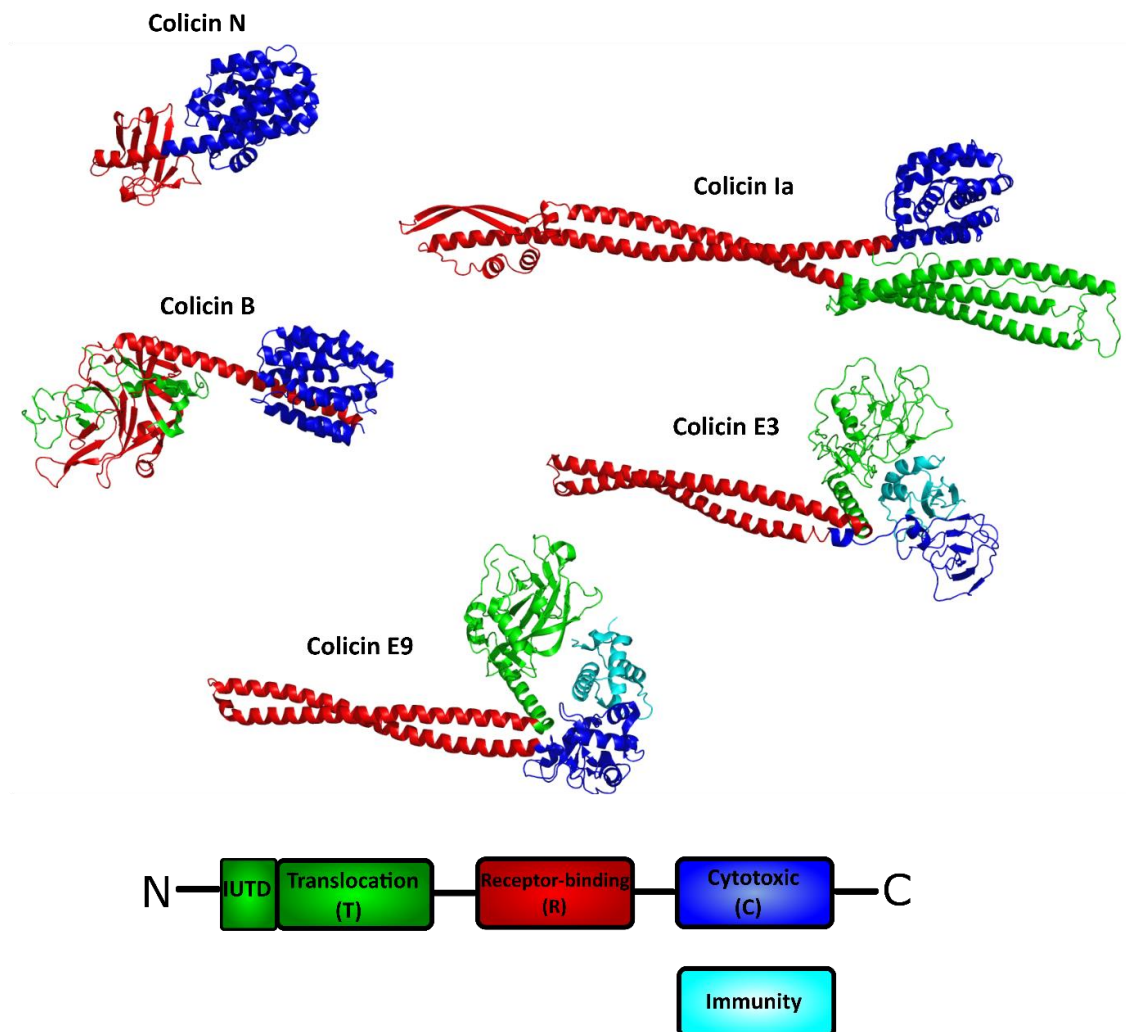


Figure 1. 6 - Colicin domain organisation and crystal structures.

Colicins are composed of an N-terminal Translocation domain (T) (green) with an IUTD, a central receptor binding domain (R) (red) and a C-terminal cytotoxic domain (C) (blue). Some colicins also have immunity proteins (Im) (Cyan). The structure of Colicin N (PDB; 1a87), Colicin B (PDB; 1rh1), Colicin Ia (PDB; 1cii), and Colicin E3 (PDB; 1jch) are shown and demonstrate the wide range of shapes, sizes and organisations that exist.

Receptor binding domain (R-domain)

The receptor binding domain of colicins binds with high affinity to specific OMP receptors and localise the colicin to the cell surface. These receptors are usually monomeric 22-stranded β -barrel proteins that are used to transport certain vitamins and nutrients into cells, including the vitamin B₁₂ receptor BtuB, the iron receptor Cir, ferric enterobactin receptor FepA and

ferrichrome-iron receptor FhuA. Porins like OmpF can also act as specific receptors for colicins (Cascales et al. 2007). A list of the specific receptors used by colicins is present in Table 1. 1.

BtuB was originally found to be the receptor for certain colicins (Colicin A and group E colicins ColE1-E9) through the protection of cells against colicin activity by binding of vitamin B₁₂ and also the lack of sensitivity of cells in the absence of BtuB in plate killing assays (Di Masi et al. 1973) (Kadner and Liggins 1973). This indicated that the two substrates shared a common site on BtuB (Di Masi et al. 1973). In addition, it was found that *btuB* was mapped to the same chromosome position that had previously been found to be the locus of colicin E receptors (Kadner and Liggins 1973) and the overexpression of BtuB also led to an increase in colicin sensitivity (Cavard 1994).

The structure of the central receptor binding domain is quite diverse amongst colicins (Figure 1. 6). The differences in the length and shape of the R-domain structures, reflects the different binding specificities for receptors and the use of a second OMP as a translocator. For colicins E2, E3, E9 (also referred to as ColE2, E3, E9) and Ia, the R-domains form an α -helical elongated coiled-coil structure between ~100-160 Å long, that forms an antiparallel alacoil or hairpin (Kurusu et al. 2003) (Klein et al. 2016) (Wiener et al. 1997).

Based on the crystal structures of the R-domains of ColE2 and ColE3 bound to BtuB and the structural similarity of ColE3 with ColE9, the R-domains bind their receptors at an acute ~45° angle to the membrane, with their T-domains and C-domains at the other end of the colicin, protruding from the cell surface (Figure 1. 7).

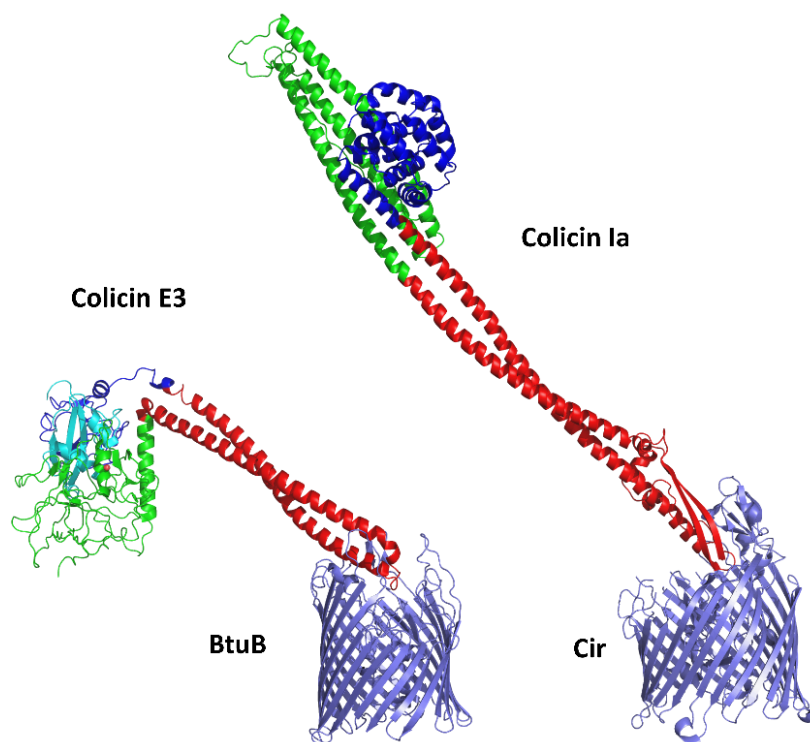


Figure 1. 7 - Structure of Colicin E3 and Colicin Ia bound to their primary receptors BtuB and Cir, respectively.

The R-domains of Colicin E3 and Colicin Ia are α -helical elongated structure with a coiled-coil hairpin (red), which bind to their receptors at an acute $\sim 45^\circ$ angle to the cell surface. This is proposed to aid the T-domain (green) at the distal end of the colicin in the recruitment and translocation using their second OMP as a translocator. BtuB (PDB; 1ujw), Cir (PDB; 2hdi), Colicin Ia (PDB; 1cii), Colicin E3 (PDB; 1jch).

The binding of the R-domain overlaps the binding site of the ligand on the receptor (Penfold et al. 2000), in addition binding is accompanied by conformational changes and ordering of extracellular regions (Kurisu et al. 2003). Translocation of these colicins does not occur using the receptor protein, demonstrated by the lack of displacement of the N-terminal globular plug domain occluding the lumen of their barrel on binding the R-domain, usually observed for ligand binding and entry. The binding of the colicin at an acute angle is proposed to aid the T-domain at the distal end of the colicin in the recruitment of and translocation through the second OMP translocator.

The structure of colicin B (ColB) is composed of a single 74 Å long α -helical R-domain, with its T-domain and C-domain at opposite ends of the R-domain (Figure 1. 6). Both the

T-domain and R-domain are involved in binding to FepA receptor, thereby using a different mechanism for binding and entry than colicins with elongated coiled-coil structures (Hilsenbeck et al. 2004). Evidence for translocation through its receptor FepA is lacking and there are conflicting reports for the displacement of the N-terminal globular plug domain of FepA on binding ColB. Therefore, it is unclear if ColB translocates through FepA lumen (Smallwood et al. 2009) (Devanathan and Postle 2007).

The structure of colicin N (ColN) R-domain is composed of a 6-stranded β -sheet around a 63 Å long α -helix that extends into the cytotoxic pore-forming domain at the C-terminus. The T-domain is unstructured at the N-terminus (Vetter et al. 1998). Binding of ColN to its receptor OmpF has been shown to occur at the external face of the OmpF barrel (Baboolal et al. 2008). Evidence for translocation through its receptor is conflicting as OmpF exists as a trimer which does not contain a globular plug-domain and is partially occluded by an extracellular loop (Loop L3).

Table 1. 1 - Colicin receptors, translocators and modes of cytotoxicity.

Colicins are grouped into group A and group B by their use of the Tol and Ton systems for entry, respectively. Listed in the table is their known surface receptor and various translocators as well as their mode of cytotoxicity.

Colicin	OM receptor	Translocation proteins	Cytotoxic activity
Group A (use of Tol system)			
ColE1	BtuB	TolC, TolAQ	Pore
ColE2, E7, E8, E9	BtuB	OmpF, TolQRAB	DNase
ColE3, E4, E6	BtuB	OmpF, TolQRAB	rRNase
ColE5	BtuB	OmpF, TolQRAB	tRNase
ColA	BtuB	OmpF, TolQRAB	Pore
ColK	Tsx	OmpF, TolQRAB	Pore
ColN	OmpF	OmpF, TolQRA	Pore
ColS4	OmpW	OmpF, TolQRAB	Pore
Group B (use of Ton system)			
ColIa, ColIb	Cir	Cir, TonB, ExbBD	Pore
ColB	FepA	TonB, ExbBD	Pore
ColD	FepA	TonB, ExbBD	tRNase
ColM	FhuA	TonB, ExbBD	PG synthesis inhibition
Col5, Col10	Tsx	TolC, TonB, ExbBD	Pore

Translocation domain (T-domain)

Once the colicin is bound to the cell surface, the translocation domain (T-domain) is used to locate and recruit a second specific outer membrane protein as its translocator. The N-terminal ~80 residues are part of the intrinsically unstructured translocation domain (IUTD) and contain several glycine residues making it highly flexible. The IUTD threads through the lumen of its translocator protein to deliver a linear epitope to the periplasm, which is used to recruit a periplasmic or IM translocator belonging to the Ton and Tol protein systems (Kleanthous 2010). Threading and binding of the IUTD through the lumen of its translocator has been shown for several colicins using channel conductance measurements, crystal structures and fluorescence

microscopy (Housden et al. 2005) (Zakharov et al. 2004) (Yamashita et al. 2008) (Housden et al. 2018).

Colicins have been classified into group A or B depending on the system they parasitize within the periplasm. Group A colicins parasitize the Tol system and Group B colicins parasitize the Ton system, both of which are coupled to the PMF across the IM (Figure 1. 8) (Cascales et al. 2007) (Kleanthous 2010).

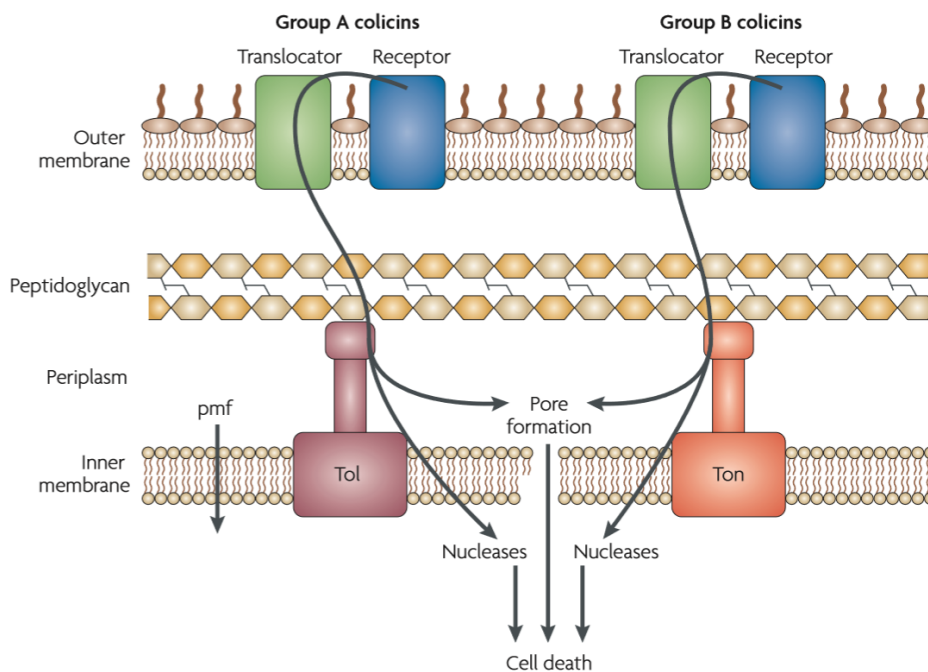


Figure 1. 8 - Use of the Tol or Ton systems for cell entry by group A and group B colicins, respectively.

Colicins are classified into group A or B depending on the system they parasitize within the periplasm. Group A colicins parasitize the Tol system and Group B colicins parasitize the Ton system, both of which are coupled to the PMF across the IM. Reproduced from (Kleanthous 2010).

Within the N-terminus of the IUTD is a series of conserved residues that confer binding specificity to either the Tol or Ton system. For most group A colicins, Tol-dependent entry requires a pentapeptide sequence DGSGW called the TolB box, part of a 16-residue TolB binding epitope (TBE) that is highly conserved in the T-domain of enzymatic E colicins (Loftus et al. 2006). Some group A colicins make direct contact via their T-domain with TolA and TolB, like ColA,

whereas ColE1 and ColN make contact with only TolA (Cascales et al. 2007). For Ton-dependent entry, this is called the TonB box (Cascales et al. 2007). Following the interaction with the Tol/Ton system, the PMF is transduced to the colicin at the cell surface, triggering the release of the immunity protein for nuclease colicins and the subsequent translocation of the colicin across the OM (Vankemmelbeke et al. 2009). A list of the specific translocator proteins and Tol/Ton specificity is shown in Table 1. 1.

Cytotoxic domain (C-domain)

The cytotoxic domains (C-domain) of colicins kill cells by different modes of action. The cytotoxicity is either enzymatic leading to the inhibition of peptidoglycan biosynthesis in the periplasm, rRNA, tRNA or DNA hydrolysis in the cytoplasm, or alternatively it can act as a pore-forming ionophore that depolarises the IM (Kleanthous 2010) (Table 1. 1).

Pore-formation

Pore-forming domains insert in the IM, forming a voltage-gated channel that leads to the diffusion of ions and dissipation of the PMF, which ultimately leads to cell death. Structures of the cytotoxic domains of several pore-forming colicins, including ColA, ColIa, ColE1, ColN and ColB, show them to be composed of 10 tightly packed α -helices. There is a hydrophobic segment that forms a helical hairpin which is surrounded by the remaining 8 mostly amphipathic helices (Cascales et al. 2007). How these pore-forming domains insert and form channels in the IM is not well understood. The proposed model is that the two hydrophobic helices insert in the IM and interact with the hydrophobic core of the bilayer (Song, Cohen, and Cramer 1991). The remaining 8-helices seem to retain their secondary structure but have flexibility in their arrangement on the surface of the IM, forming a closed channel (Parker et al. 1989). Application of positive voltage to the closed channel, is thought to cause several of the amphipathic helices on the surface to lose their helical structure and insert into the membrane and either span the membrane or traverse to the cytoplasmic side of the IM. This generates an open channel that is

estimated to be ~7-10 Å wide, allowing the diffusion of ions across the IM and dissipation of the PMF (Krasilnikov et al. 1998; Cascales et al. 2007).

Peptidoglycan biosynthesis inhibition

Colicin M (ColM) inhibits the synthesis of peptidoglycan (PG) in the periplasm, leading to cell lysis. It does so by enzymatic degradation of undecaprenylphosphate-linked peptidoglycan precursors (El Ghachi et al. 2006), preventing the regeneration of the lipid carrier from bactoprenolpyrophosphate to bactoprenolphosphate for the transfer of peptidoglycan precursors from the cytoplasm to the periplasm for PG layer insertion (Harkness and Braun 1989a). In addition, this lipid carrier is also involved in the transfer of LPS O-antigen precursors, thereby inhibiting LPS O-antigen biosynthesis (Harkness and Braun 1989b).

tRNases

tRNase domains cleave single phosphodiester bonds in anticodon loops of specific tRNAs. Different tRNases have different specificities based on their different active sites and different substrate recognition methods. For example, ColE5 cleaves tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn} and tRNA^{Asp} (Ogawa et al. 2006), ColD cleaves 3-4 of the tRNA^{Arg} (Tomita et al. 2000). This affects the incorporation of amino acids during protein synthesis, arresting protein synthesis and ultimately leading to cell death.

rRNases

The mechanism of cleavage of rRNA was first identified using colicin E3 (ColE3), the first rRNase to be isolated and characterised. The rRNase domain binds to the 30S subunit of bacterial ribosomes and cleaves a single phosphodiester bond between residue A1493 and G1494 in helix 44 of the 16S rRNA at the A-site of the ribosome (Bowman et al. 1971) (Senior and Holland 1971) (Ng et al. 2010). This cleavage affects the rate of initiation of protein synthesis by reducing the rate and stability of the codon-recognition complex of tRNAs in the A-site, leading to a higher rejection rate. This low occupancy of the A-site predominantly affects the elongation step of

translation during protein synthesis. This leads to the generation of short incomplete peptides, which ultimately leads to cell death (Lancaster et al. 2008).

1.5.5. The cytotoxic DNase domain of E group colicins

The cytotoxic domains of group E colicins, ColE2, ColE7, ColE8 and ColE9, share 65 % sequence identity (Appendix I Figure 1). The active sites of these enzymes are composed of two antiparallel β -strands, an α -helix containing a metal ion (Kleanthous et al. 1999) and is structurally related to the $\beta\beta\alpha$ -Me motif in metal-dependent endonucleases (Kuhlmann et al. 1999). The active site is a 32-amino acid sequence that contains a H-N-H motif. This is structurally and mechanistically homologous to the highly conserved H-N-H family of homing endonucleases, DNA repair enzymes and the apoptotic caspase activated DNase (CAD) and is common across all three kingdoms of life (Mate and Kleanthous 2004). This active site H-N-H sequence is not just confined to E group colicins, but also other bacteriocins like pyocins and klebicins (Figure 1. 9). The residues of the H-N-H motif of ColE9 correspond to H551, N566 and H575, numbered based on the full-length colicin sequence.

Protein	Sequence				
	550	551			
	548		566	575	579
Colicin E9	YEL <u>H</u> <u>H</u> DKPISQGGEVYDMD <u>N</u> IRVTTPKR <u>H</u> ID <u>I</u> H				
Colicin E2	FELH <u>H</u> DKPISQDGGVYDMN <u>N</u> IRVTTPKR <u>H</u> ID <u>I</u> H				
Colicin E7	FELH <u>H</u> EKPISQNGGVYDMD <u>N</u> ISVVTPKR <u>H</u> ID <u>I</u> H				
Colicin E8	FELH <u>H</u> DKPISQDGGVYDMD <u>N</u> LRITTPKR <u>H</u> ID <u>I</u> H				
Klebicin B	VELH <u>H</u> KVEISKGGDVYNVD <u>N</u> LNALTPKR <u>H</u> IE <u>I</u> H				
Pyocin AP41	FEIH <u>H</u> VVPLESGGALYNID <u>N</u> LVIVTPKR <u>H</u> SE <u>I</u> H				
Pyocin S2	IEIH <u>H</u> KVRIADGGGVYNMGN <u>N</u> LVAVTPKR <u>H</u> IE <u>I</u> H				
Pyocin S1	IEIH <u>H</u> KVRVADGGGVYNMGN <u>N</u> LVAVTPKR <u>H</u> IE <u>I</u> H				
T4 DNase	TETH <u>H</u> IIPRCMGG TDDKT <u>N</u> LVLLTPEE <u>H</u> FTA <u>H</u>				
McrA	LEVH <u>H</u> VIPLSSGG ADTTD <u>N</u> CVALCPN <u>H</u> REL <u>H</u>				
PsbA Orf	MEKH <u>H</u> IIPRHAGG PDEK <u>N</u> LISLTPED <u>H</u> IEA <u>H</u>				
RTase pO157	WQVH <u>H</u> IIRVDGG SNCL <u>N</u> LIMLHPM <u>H</u> TLV <u>H</u>				
Usp	FQIH <u>H</u> VVAIEHGGGVYDID <u>N</u> FRIVTPRL <u>H</u> DE <u>I</u> H				

Figure 1. 9 - Sequence alignment of the active site of ColE9 and endonucleases containing the H-N-H motif.

The conserved residues of the active site of ColE9 are underlined, residues of the H-N-H motif are in bold; H551, N566 and H575. Numbering of residues refers to their position in the full-length colicin, in the DNase H551 corresponds to H103, N566 corresponds to N118 and H575 corresponds to H127. Figure adapted from (Walker et al. 2002).

The current understanding of the structure, function and mechanism of action of the DNase domain in DNA binding and hydrolysis, has been largely investigated using ColE9 and ColE7. There are several important residues that play a role in the binding and hydrolysis of DNA, the locations of these in ColE9 are shown in Figure 1. 10. The binding of DNA to ColE9 and ColE7 H-N-H is metal independent, however enzymatic activity is dependent on the presence of a divalent metal ion within its active site (Pommer et al. 1999) (Ku et al. 2002).

The role of residues D499 and R502 is formation of a network of hydrogen bonds between the DNA and DNase, which was identified from the structure of ColE9 bound to dsDNA in the presence of Mg^{2+} (Mate and Kleanthous 2004). Residue R453 was shown to play a role in stabilising the pentacoordinate transition state of phosphodiester hydrolysis. Its mutation to alanine led to a loss of biological activity by plate killing assay and inactivity in the presence of Mg^{2+} by plasmid nicking assay (Walker et al. 2002). Residues R544, E548 and H575 were

identified as active-site residues by random and site-directed mutagenesis (Garinot-Schneider et al. 1996). Their mutation to alanine led to loss of biological activity (Walker et al. 2002) and lack of DNA cleavage *in vitro* by plasmid nicking or Kunitz assays (Walker et al. 2002) (Garinot-Schneider et al. 1996). Residues E548A and H575A were shown to decrease binding of Zn^{2+} >1000-fold (Walker et al. 2002) and H575 was shown to coordinate Ni^{2+} and Zn^{2+} transition metals and Mg^{2+} for the biological activity of ColE9 (Kleanthous et al. 1999).

Residue H550 was shown through alanine mutagenesis, to be essential for biological activity and cleavage by Kunitz or nicking assays. It was shown to be important for the binding of Ni^{2+} and Zn^{2+} transition metals and Mg^{2+} , which was confirmed by protease protection assay (Walker et al. 2002). Mutation of residue H551 of the H-N-H motif to alanine still allowed DNA and metal binding but lost its biological activity by plate killing, nicking and Kunitz assays. It was shown to act as a general base in the activation of a water molecule for hydrolysis of a phosphodiester bond (Walker et al. 2002).

Residue N566 was shown to have a structural role which stabilises the β -strands through formation of bridging hydrogen bonds to backbone atoms (Kleanthous et al. 1999). Mutation of residue N566 of the H-N-H motif to alanine had its biological activity reduced to <10 % (Walker et al. 2002). Mutation of residue H579 to alanine showed loss of biological activity to <2 % by plate killing assay and no cleavage by nicking assay. H579A was unable to bind Zn^{2+} transition metal (Walker et al. 2002). In ColE7, position H579 corresponds to a residue for the coordination of Zn^{2+} (Ko et al. 1999).

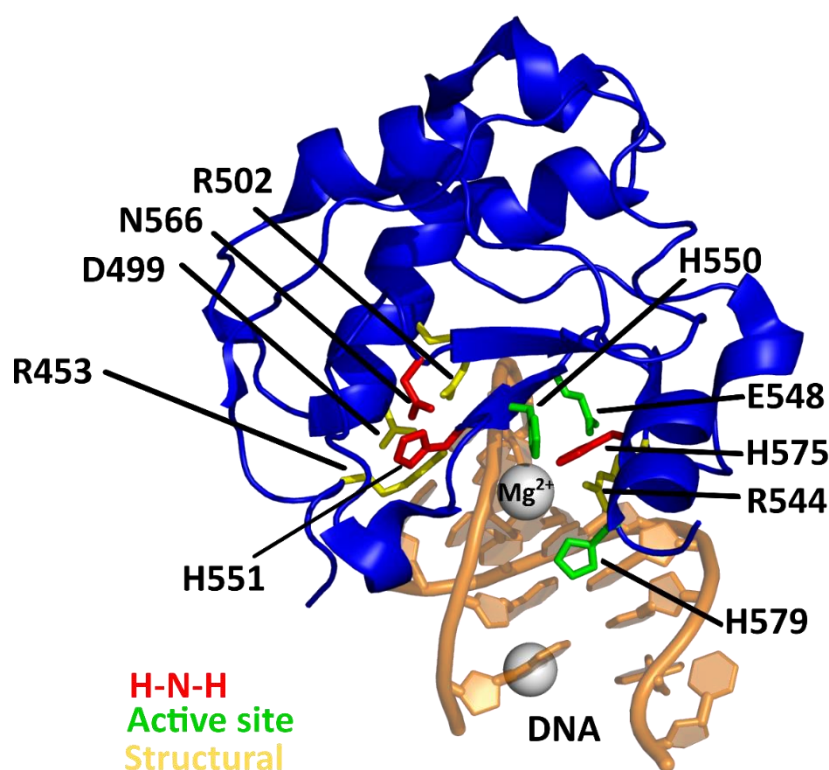


Figure 1. 10 - ColE9 DNase domain bound to dsDNA.

ColE9 DNase domain in complex with dsDNA, residues highlighted play a role in hydrolysis of DNA. Residues in red are the conserved H-N-H motif within the active site, residues in green represent other important/conserved residues within the 34-amino acid active site, residues in yellow are not within the active site sequence but contribute to the bending and stabilisation of dsDNA for its hydrolysis (PDB; 1v14).

Differential cleavage of substrates by DNase

ColE9 acts as a non-specific endonuclease that is able to hydrolyse different substrates by different mechanisms using different metal ions (Pommer et al. 2001). ColE9 cleaves single-stranded RNA (ssRNA) in the absence of divalent cations. ColE9 binds single-stranded DNA (ssDNA) with a stoichiometry of 1:1, an affinity of $<2.3 \mu M$ and in a sequence-independent manner with an optimum length >10 bp. Cleavage of ssDNA is dependent on the presence of transition metals Ni^{2+} or Co^{2+} in the active site (Pommer et al. 2001), which bind to ColE9 with an affinity of $0.7 \mu M$ and $1.8 \mu M$, respectively (Pommer et al. 1999). ColE9 cannot cleave mononucleotide or dinucleotide substrates. ColE9 also cleaves double-stranded DNA (dsDNA) with a base specificity ($\sim 56\%$) after thymine (T), in a Mg^{2+} -dependent manner, which could

reflect the cleavage of A/T-rich bacterial genome of *E. coli*. Optimal activity is in the presence of Mg^{2+} or Ca^{2+} at pH 8-9 by Kunitz assay (Pommer et al. 2001).

Residues E548, H550, H551 and H575 are essential for Mg^{2+} and Ni^{2+} dependent activity. However, additional residues R453, R544 and H579 are required for Mg^{2+} dependent activity. Positively charged residues on both sides of the active site cleft are required for Mg^{2+} dependent activity and cleavage of dsDNA, whereas only one side of the active site cleft is required for Ni^{2+} dependent activity and cleavage of ssDNA (Walker et al. 2002). Thereby explaining the use of different metal ions for different substrate specificities. ColE9 can bind Zn^{2+} with an affinity of 1 nM (Pommer et al. 1999), however, the DNase is biologically inactive against dsDNA (Pommer et al. 1998). This is due to the coordination of Zn^{2+} by residues H550, H575, H579 and an oxygen atom from the phosphate scissile bond of DNA. The additional coordinating histidine cannot coordinate a water molecule for DNA hydrolysis (Mate and Kleanthous 2004).

For ColE7, the requirement of a divalent cation for the cleavage of dsDNA, a minimum DNA sequence > 8 bp for cleavage and RNase activity was also observed (Hsia et al. 2004). Metal ions are coordinated by residues H544, H569, H573 and a water molecule or phosphate ion from the DNA in a tetrahedral geometry. These are the equivalent residues for the coordination of Zn^{2+} in ColE9. ColE7 can coordinate Zn^{2+} , Mn^{2+} , Ni^{2+} and Mg^{2+} , with Zn^{2+} and Ni^{2+} having the largest effect on stabilising ColE7 and Zn^{2+} proposed to be of physiological relevance (Doudeva et al. 2006). In addition, ColE7 retained catalytic activity in the presence of all metals, with an order of $Ni^{2+} > Mn^{2+} \sim Mg^{2+} > Zn^{2+}$ (Doudeva et al. 2006). Thereby, nuclease colicins coordinate different metal ions and activate endonuclease activity differently.

Mechanism of DNA binding and cleavage

The mechanism of dsDNA binding and distortion and the role of active site residues was determined largely from the crystal structure of ColE9 DNase in complex with dsDNA and Mg^{2+} (Mate and Kleanthous 2004) and ColE7 DNase in complex with dsDNA and Zn^{2+} (Hsia et al. 2004) (Doudeva et al. 2006). The active site of ColE9 DNase forms a rigid V-shape with a highly positive charge and binds to DNA using electrostatically driven interactions (Walker et al. 2002). On binding dsDNA, there is no major conformational change in the DNase active site. For ColE9, Mg^{2+} is coordinated by H550 and H575 of the H-N-H motif and the scissile phosphate ion of the DNA backbone (Mate and Kleanthous 2004) (Figure 1. 11). Residue R544 and E548 form a salt-bridge that inserts into the minor groove of dsDNA and distorts it by widening the groove from 5.9-~9 Å. This conformation is stabilised by a network of hydrogen bonds between the DNA and residues of the DNase. The deformation positions the scissile phosphodiester of the DNA backbone close to the metal ion in the DNase active site (Mate and Kleanthous 2004). The equivalent residues of ColE7 behave similarly (Hsia et al. 2004).

The proposed mechanism of cleavage involves the metal ion (Mg^{2+} for ColE9, Zn^{2+} for ColE7) polarising the scissile phosphate, stabilising the phosphoanion transition state and activating a water molecule. A histidine residue (H551 for ColE9, H545 for ColE7) acts as a general base to activate the hydrolytic water molecule and generate a nucleophile to hydrolyse the phosphodiester bond. The water molecule protonates the leaving group (Mate and Kleanthous 2004). The DNase generates a series of nicks in the dsDNA, which by themselves would be unlikely to kill the cell, as various DNA repair machineries would fix these nicks. It is therefore likely that repeated nicking of the DNA would lead to a dsDNA break, which would be lethal (Cascales et al. 2007).

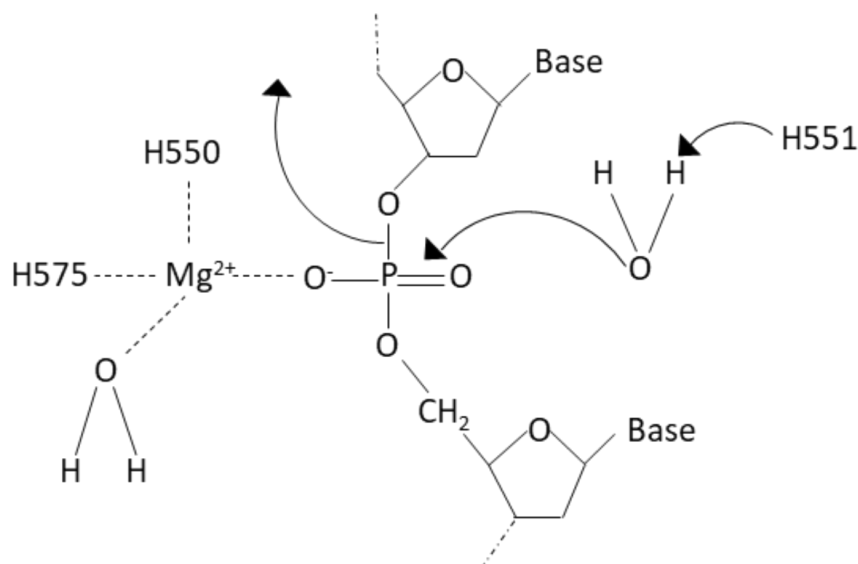


Figure 1. 11 - Proposed mechanism of DNA hydrolysis by ColE9.

The Mg^{2+} ion polarises the scissile phosphate, stabilising the phosphoanion transition state and activating a water molecule, in which residue H551 acts as a general base to activate the hydrolytic water molecule and generate a nucleophile to hydrolyse the phosphodiester bond (Adapted from (Pommer et al. 2001)).

1.5.6. Immunity proteins

To inhibit the cytotoxic activity of nuclease colicins, the C-domain binds with high affinity to an immunity protein (Im) which is co-expressed with the colicin, to prevent suicide of the producer cell. The Im protein associates rapidly with the cytotoxic domain. Endonuclease immunity proteins are ~9.5 kDa antiparallel 4-helix bundles (Osborne et al. 1996). The endonuclease group E colicins, ColE2, ColE7, ColE8 and ColE9, have cognate Im proteins, Im2, Im7, Im8 and Im9. These immunity proteins share ~50 % sequence identity (Kleanthous et al. 1999).

The binding of Im to DNase is through a dual-recognition mechanism. Helix III of the Im protein acts as a binding site anchor and is responsible for the affinity to the DNase domain and stability of the complex. It has a series of conserved residues that form a binding energy hotspot, which is composed primarily of hydrogen bonds and hydrophobic interactions, to anchor Im to DNase. Within this hotspot is a conserved tyrosine on helix III, which forms a hydrophobic contact with a bulky variable amino acid of the DNase. This interaction directs and aids in the presentation of amino acids of the DNase towards specificity residues in helix II of Im. These Im

Helix II specificity residues influence binding and define DNase specificity, through neutral, positive or negative contributions to binding free energy for the complex. A series of water molecules are also involved in the interaction and contribute to the number of intramolecular hydrogen bonds at the DNase-Im interface, thereby contributing to the interface shape complementarity (Kuhlmann et al. 2000) (Meenan et al. 2010).

In the case of ColE9 DNase, the Im9 binding site is a cleft composed of a short helix, a β -strand and two loops, which are adjacent to the active site (Kleanthous et al. 1999) (Figure 1.12), this region is strongly positively charged (Kuhlmann et al. 2000). For Im9, the E9 DNase binding site are helix II and III, which has a net negative charge and which straddle the cleft of the DNase. Therefore, they associate by an electrostatic steering mechanism in a diffusion-controlled association (Kuhlmann et al. 2000). This is consistent with their rapid bimolecular association rate constant of $\sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$, that is reduced by increasing salt concentration. The equilibrium dissociation constant K_d for ColE9-Im9 is $9.3 \times 10^{-17} \text{ M}$, at 25 °C pH 7 (Wallis, Moore, et al. 1995). When in complex, the buried surface area is $\sim 1575 \text{ \AA}^2$. Im binding to an exosite on the DNase, causes steric and electrostatic hinderance by its net negative charge to DNA binding in the active site (Kleanthous et al. 1999) (Kleanthous and Walker 2001).

These group E colicins have also been shown to bind non-cognate immunity proteins that are capable of inhibiting their cytotoxicity, but with lower affinity. This is because the Im Helix III can still act as the anchor for the affinity of Im to DNase, even in the absence of Helix II specificity residues for that DNase which would contribute to the stability. In addition, other Im proteins may insert charged or polar specificity residues from its helix II, which would further destabilise the affinity of the interaction. For example, ColE9 DNase binds Im2 with a K_d of 10^{-7} M and binds Im8 with a K_d of 10^{-6} M , with a range of 6-10 orders of magnitude weaker non-cognate binding than binding Im9 (Wallis, Leung, et al. 1995). The structure of cognate DNase-Im are not identical, for example the superposition of ColE7 DNase-Im7 with ColE9 DNase-Im9, showed that

the Im proteins are displaced due to a 19° rotation by conserved helix III tyrosines. The reason for this is the presentation of different regions of Im helix II to the DNase for their specificity (Kuhlmann et al. 2000). However, the structures of non-cognate complexes can be almost identical to their cognate complex, as is the case for ColE9 DNase-Im2 (Meenan et al. 2010). In this case, the reason for the near identical structure but >7-orders of magnitude weaker affinity, is due to the affinity of the complex tolerating unfavourable specificity contacts at the interface, which result in a weaker non-cognate complex which experiences frustration (Meenan et al. 2010).

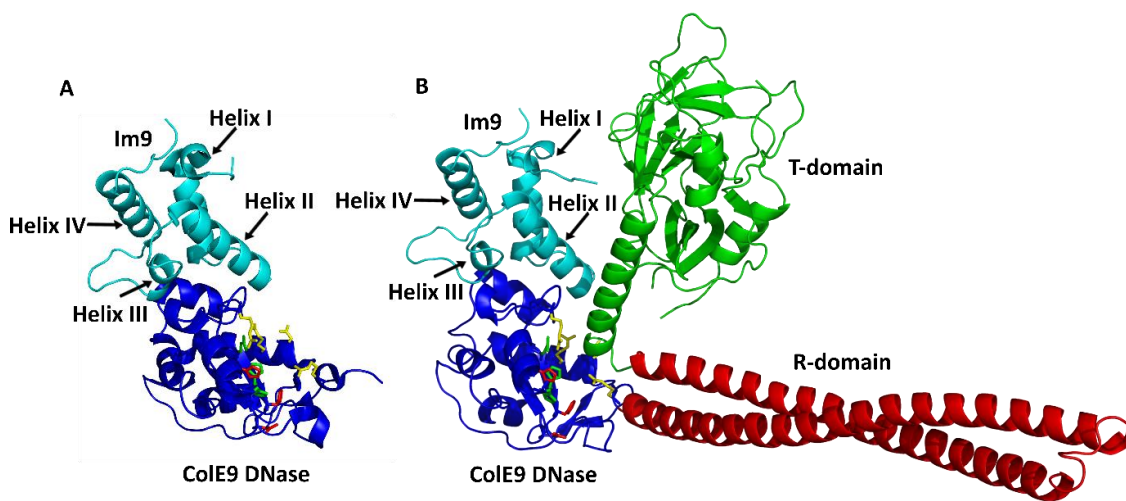


Figure 1. 12 - Structure of ColE9 in complex with Im9.

ColE9 DNase (blue) highlights all residues involved in the active site for DNA binding (red; H-N-H motif, green; active site residues, yellow; structural). Im9 binds to an exosite of ColE9 DNase away from the active site. A) ColE9 DNase-Im9 (PDB; 1emv) B) full-length ColE9-Im9 (PDB; 5ew5).

The immunity proteins of rRNases also inhibit the cytotoxicity of rRNases, by binding to an exosite opposite the active site by steric and electrostatic occlusion. They bind with high affinity, for example colicin E3 rRNase-Im3 has a K_d of 10^{-12} M (Walker et al. 2003). However, the immunity proteins of DNases and rRNases have no sequence or structural similarity (Cascales et al. 2007).

This mode of inhibition is not the case for all nuclease colicins. For certain tRNases it was found that their immunity proteins bind directly at the active site. This is the case for ColD-ImD and ColE5-Im5, where the immunity proteins are negatively charged and mimic the substrate RNA binding in the positively charged active site with minimal conformational changes (Cascales et al. 2007).

The mechanism of protection of cells against pore-forming colicins is poorly understood. Pore-forming colicins do not contain an immunity gene within their colicin operon, but it is present and transcribed from the opposite DNA strand (Riley 1993). The immunity proteins have transmembrane helices and are localised in the IM and are proposed to inactivate the pore-forming colicin there. They are thought to either prevent channel opening or block it to inhibit cytotoxicity (Cascales et al. 2007).

1.6. Proposed mechanism of ColE9 translocation

ColE9 is a 61.5 kDa enzymatic nuclease colicin and binds to BtuB as its receptor on the cell surface (Figure 1. 15) with an affinity of 2.4 nM in the presence of Ca^{2+} ions as shown by ITC (Housden et al. 2005). Recruitment of OmpF porin was shown through affinity purification of colicin bound protein complexes isolated from the OM using a colicin that was unable to translocate into the cell and identified through SDS-PAGE and MALDI-TOF MS. The N-terminal 83-residue IUTD of the T-domain of ColE9 recruits and passes through the OM into the periplasm, through the lumen of OmpF (or OmpC); this was demonstrated by deletion analysis and affinity purification (Housden et al. 2005). The first 61-residues become inaccessible and occluded within OmpF as shown by the proteolytic protection of engineered enterokinase cleavage sites (Zhang et al. 2008).

The IUTD OmpF-binding site 2 (OBS2, residue 54-63) binds within the lumen of the barrel with an affinity of 24 μM . The extreme N-terminus of the IUTD threads back into the lumen of another OmpF monomer within the trimer from the periplasmic side in the reverse orientation

and the OBS1 (residue 2-18) binds with an affinity of 2 μ M, as shown by ITC with isolated peptides (Housden et al. 2013). This allows the presentation of the TolB binding epitope (TBE, residue 32-47), found between OBS1 and OBS2, in a fixed orientation to the periplasm for binding with TolB (Housden et al. 2010) (Housden et al. 2005). The orientation and direction-specific binding of OBS1 from the periplasmic face and OBS2 binding the extracellular face of OmpF was recently demonstrated by fluorescence microscopy, ITC and channel conductance measurements (Housden et al. 2018) (Figure 1. 13).

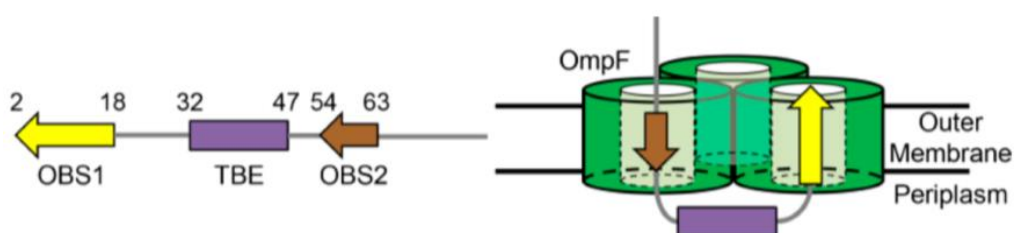


Figure 1. 13 - The N-terminal IUTD of ColE9 containing OBS1, OBS2 and the TBE threading through OmpF.

The IUTD of ColE9 contains OmpF-binding site 2 (OBS2, residue 54-63) which threads through and binds within the lumen of an OmpF barrel monomer. Arrows show orientation of binding. The OBS1 (residue 2-18) at the N-terminus of the IUTD threads back into the lumen of another OmpF monomer within the trimer in the opposite orientation (Housden et al. 2010). This allows presentation of the TolB binding epitope (TBE, residue 32-47), found between OBS1 and OBS2, in a fixed orientation to the periplasm for binding with TolB (Housden et al. 2018).

TolB is composed of an N-terminal α/β domain and a C-terminal 6-bladed β -propeller domain (Bonsor et al. 2009). TolB has been shown to bind Pal with an affinity of 47 nM by ITC (Loftus et al. 2006). The TBE of ColE9 is unstructured and contains hydrophobic clusters, as shown by NMR (Hands et al. 2005). It was shown to bind TolB within the narrow canyon channel of the β -propeller domain in a distorted hairpin conformation (Loftus et al. 2006). This binding site on TolB was shown to overlap that of Pal through mutation of TolB residues, which affected the binding of both ColE9 and Pal. ColE9 is able to competitively recruit TolB from Pal in the presence of divalent cations that bind within the TolB β -propeller channel, which changes the electrostatics and increases the binding of negatively charged ColE9 >10-fold (Loftus et al. 2006).

ColE9 binds TolB via its pentapeptide TolB box (residue 35-39, DGSGW), within its 16-residue TolB binding epitope (TBE) (residue 32-47), that is highly conserved in the T-domain of enzymatic E colicins. ColE9 binds TolB with a K_d of $\sim 1 \mu\text{M}$ as shown by ITC and SPR (Loftus et al. 2006) (Papadakos et al. 2012). When ColE9 binds TolB, it promotes disorder at the N-terminus of TolB as shown by NMR (Bonsor et al. 2009). TolB makes contact with the C-terminal periplasmic globular domain of TolA using 12 residues at its N-terminus, called the TolA box. TolB binds TolA weakly ($\sim 40 \mu\text{M}$ by ITC), this interaction is increased ~ 3 -fold to $13 \mu\text{M}$ in the presence of ColE9 (Bonsor et al. 2009). TolA undergoes a PMF-dependent conformational change through its interaction with TolQ and TolR in the IM, extending into the periplasm. This aids in the presentation of TolA for interaction with TolB (Germon et al. 2001).

Flexibility and unfolding of the R-domain of ColE9 is essential for cell entry and therefore its biological cytotoxicity. This was shown through the introduction of a disulphide within the R- domain of ColE9 (ColE9^{S-S}), which could still bind BtuB and TolB and retain its DNase activity *in vitro* but was prevented from translocating, thereby showing that a conformational change is essential for translocation (Penfold et al. 2004). It was shown through ITC that this receptor unfolding was not induced by binding to BtuB, but after assembly of the translocon (Housden et al. 2005). As this would require an input of energy, it is proposed to occur after interaction with the Tol system in the IM and thereby transduction of the PMF. In addition, it was shown that receptor binding alone was not sufficient for Im9 release to occur, demonstrated by the presence of the immunity after affinity purification of the translocon using ColE9^{S-S} and by the lack of enthalpic change by ITC of BtuB binding ColE9 +/- Im9 (Housden et al. 2005).

The dissociation of Im9 from ColE9 requires proteins of the Tol system and through them access to the PMF. This was demonstrated by the development of a fluorescence-based assay through the labelling of Im9 with AF⁵⁹⁴ and determining the conditions which led to its release into the surrounding media and subsequent cytotoxicity of ColE9. Vankemmelbeke et al.

demonstrated that physical interactions were required between the N-terminal 83 residues of ColE9 T-domain, including the TBE for TolB binding and the TolQRA proteins, as demonstrated through their deletion or mutation. The authors showed through chemical inhibition of the PMF using CCCP, a dose-dependent increase in Im9 release inhibition with increasing CCCP (Vankemmelbeke et al. 2009). These protein-protein interactions are necessary to harvest the PMF and its energy from the IM and transduce it to ColE9 at the OM for Im9 dissociation and the concomitant translocation of ColE9. Im9 release occurs rapidly as demonstrated by the use of ColE9^{S-S}-Im9^{AF594} and the rapid detection of fluorescence on treatment with DTT to reduce the R-domain disulphide (Zhang et al. 2008).

Im9 is bound with fM affinity to ColE9 (Wallis, Moore, et al. 1995), which equates to a dissociation half-life of ~4.1 days. Atomic force microscopy (AFM) along with the introduction of disulphides in the DNase domain, have shown that application of low force <20 pN to the ColE9 DNase-Im9 complex, distal to the interface, is capable of increasing the rate of dissociation ~10⁶ fold to 12 ms (Figure 1. 14). The first 20 residues of the DNase (residue 449-468) acts as a transducer of the force which remodels an allosteric activator between residues 21-30 (residue 469-478), termed a trigger switch, which lies close to the DNase-Im9 interface, which becomes kinetically unstable and triggers Im9 release. This triggering force could be provided by conformational changes induced by the PMF via the Tol system, for example the unfolding of the R-domain that gets transmitted to the DNase domain (Farrance et al. 2013).

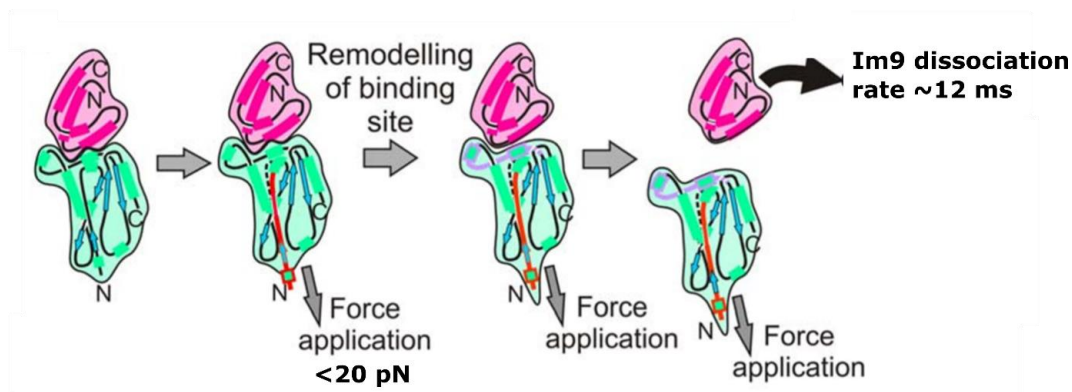


Figure 1. 14 - The application of low force triggers the dissociation of Im9 from ColE9.

The application of low force <20 pN to the E9 DNase-Im9 complex, distal to the interface, remodels an allosteric activator trigger switch, becomes kinetically unstable and increases the rate of dissociation $\sim 10^6$ fold to 12 ms demonstrated by AFM. Adapted from (Farrance et al. 2013).

Unfolding of the R-domain and release of Im9 is followed by ColE9 translocation across the OM. Once inside the periplasm, ColE9 DNase domain with a net positive charge of +7, is electrostatically attracted towards the negatively charged IM. This was shown through both the mutation of the DNase domain to decrease its net positive charge and depletion of negatively charged anionic phospholipids, decreasing the net negative charge of the IM, which both led to a reduction in the rate of cell killing (Walker et al. 2007).

ColE9 DNase domain interacts through electrostatic interactions with negatively charged phospholipids in the IM, as shown by a shift in the intrinsic tryptophan fluorescence towards exposure of the residues on protein-lipid complex formation (Mosbahi et al. 2004). This leads to the destabilisation of the DNase domain and loss of its secondary and tertiary structure, as shown by CD, chemical and thermal denaturation and an increase in the proteolytic susceptibility. Once destabilised, ColE9 DNase domain then forms a short-lived non-voltage-gated channel in the IM. This was shown through the interaction of ColE9 DNase with the membrane of planar lipid bilayers, where random fluctuating channels formed which were open for milliseconds. Introduction of a disulphide in a highly mobile region of the DNase had little effect on activity, but abolished the channel forming activity and thereby its cytotoxicity, as did Im9 binding (Mosbahi et al. 2002).

ColE9 DNase domain binds the IM with $\sim 1 \mu\text{M}$ affinity, demonstrated by the use of a membrane surface potential sensor on large unilamellar vesicles (LUVs) containing synthetic and *E. coli* lipids (Vankemmelbeke et al. 2012). The DNase domain was shown to bind via a cooperative mechanism and insert only to the interfacial region of bilayer, shown through tryptophan fluorescence quenching analysis using brominated lipids. In addition, dye leakage assays showed that membrane permeation was minimal, suggesting more of an effect on lipid packing and arrangement than pore formation (Vankemmelbeke et al. 2012).

Once bound to the IM, ColE9 DNase is proteolytically cleaved from the rest of the colicin by FtsH, allowing retro-translocation and release into the cytoplasm. The putative processing site was identified as residues 424-446 within the C-terminus of the R-domain through *in vitro* cleavage experiments using ColE3, which is essentially identical in the T-domain and R-domain to ColE9 (de Zamaroczy and Buckingham 2002). FtsH is an essential ATP-dependent IM AAA+ (ATPases associated with diverse cellular activities) ATPase/protease that is responsible for the post-translational ATP-dependent unfolding and proteolysis of regulatory proteins and key biosynthetic pathway enzymes from the IM, to control the levels and quality within the cell for survival (Langklotz, Baumann, and Narberhaus 2012). FtsH is anchored to the IM and composed of a β -barrel that forms a pore in the IM and an ATPase and protease domain that is on the cytoplasmic side of the IM (Tomoyasu et al. 1993). Its cleavage is sequence independent and can degrade from the N- or C-terminus or within internal sites (Ito and Akiyama 2005) (Akiyama 2009). The role of FtsH in the retro-translocation of ColE9 DNase into the cytoplasm was shown through a series of FtsH deletion and mutation strains and its lack of cytotoxicity and therefore entry into the cytoplasm (Walker et al. 2007). It seems likely that when the DNase is destabilised and inserted within the IM, it is recognised by FtsH, which dislocates it from the membrane for its proteolytic cleavage from the rest of the colicin and release into the cytoplasm.

To avoid complete proteolytic degradation, the DNase would have to spontaneously rapidly refold in the cytoplasm, where FtsH is unable to degraded tightly folded substrates (Herman et al. 2003). The DNase domain then diffuses within the cytoplasm to bind and nick its dsDNA target, leading to the accumulation of double-stranded breaks and eventually cell death.

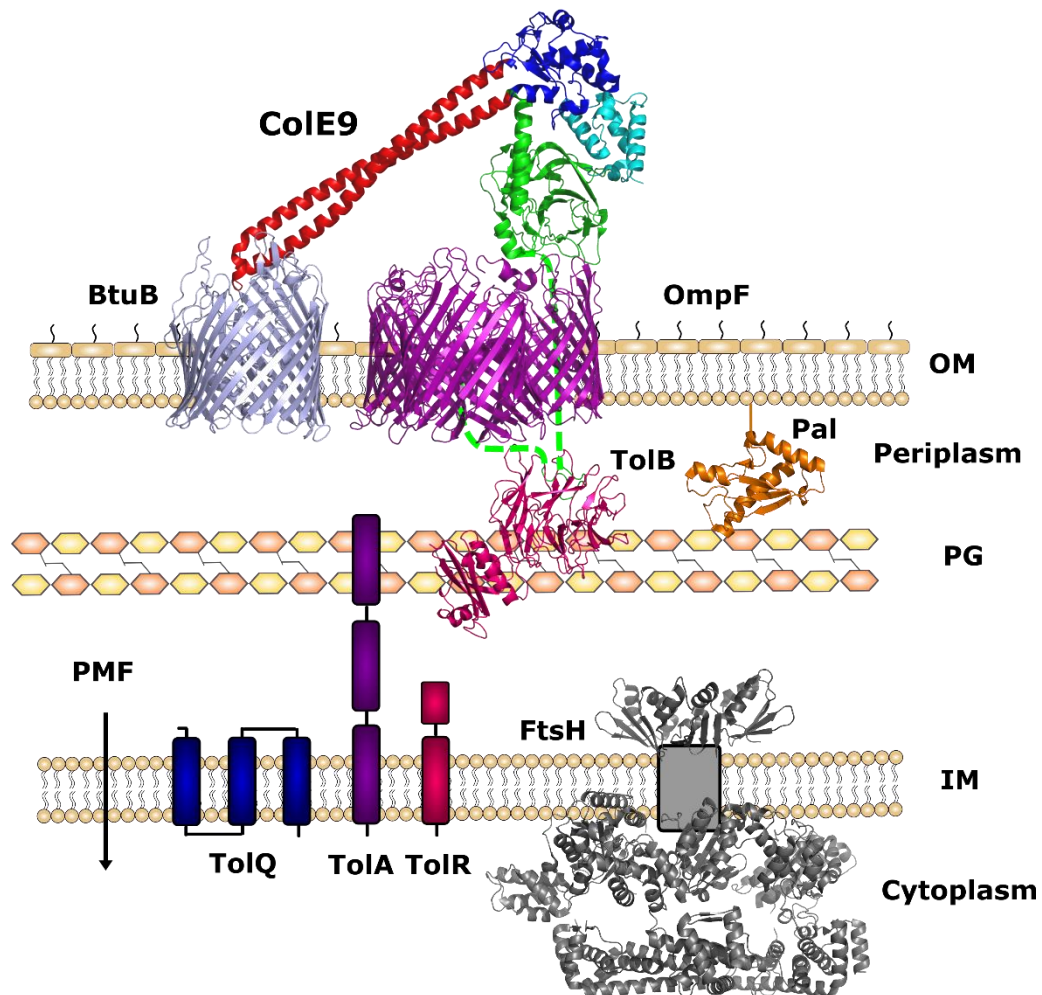


Figure 1. 15 - Proposed model for the translocation of ColE9 into *E. coli* cells.

ColE9 binds to BtuB as its receptor on the cell surface, it uses its N-terminal IUTD (green dotted line) to thread through the lumen of OmpF as its translocator and deliver its TolB box epitope to the periplasm, to recruit TolB. TolB makes contact with TolA via its N-terminal TolA box, which allows transduction of the PMF via its interaction with TolQR in the IM. This contact causes the dissociation of its immunity protein Im9 (cyan), unfolding of the colicin within its R-domain (red) and translocation of ColE9 across the OM by an unknown mechanism. Once inside the periplasm, the DNase domain (blue) is electrostatically attracted to the IM, where it unfolds and inserts into the membrane. The DNase domain gets proteolytically cleaved from the rest of the colicin by FtsH during translocation across the IM into the cytoplasm, where it re-folds and cleaves its dsDNA target to kill cells. (PDB; ColB 1rh1, FepA 1fep, BtuB-ColE3 R-domain 1ujw, ColE9 5ew5, OmpF 3o0e, Pal 2w8b, TolB 4jml, FtsH ATPase and protease 2cea, periplasmic loops 4q0f).

1.7. Use of fluorescently-labelled colicins to study OMP island organisation

As colicins bind to specific receptors on the cell surface of *E. coli*, a technique was developed to study their localisation in the OM (Figure 1. 16) (Rassam et al. 2015). ColE9 and Colla, which bind to BtuB and Cir, respectively, were engineered with a disulphide within their R-domains to prevent translocation. They were then fluorescently labelled within their cytotoxic domains with AlexaFluor dyes.

Using these labels, it was found that the OMPs were clustered and co-localised with each other and the BAM OMP biogenesis machinery in the OM (Figure 1. 16 A) (Rassam et al. 2015). During cell growth, the labelled OMPs migrated to the cell poles. A subsequent labelling of the OMPs after a period of time showed that newly generated OMPs were formed and inserted at the midcell, pushing older OMPs to the poles in a gradient of OMP biogenesis (Figure 1. 16 B and C) (Rassam et al. 2015). By following several cell divisions starting with a single cell, the old OMPs remained sequestered at the poles of the single cell and new OMPs inserted at the midcell. This led to daughter cells containing newly generated OMPs (Rassam et al. 2015) and thereby a proposal for a mechanism of OMP turnover by binary partitioning (Figure 1. 16 D) (Kleanthous, Rassam, and Baumann 2015).

The co-localised OMPs formed islands/puncta by promiscuous protein-protein interactions (Kleanthous, Rassam, and Baumann 2015), with a mean of ~7 copies of BtuB and Cir per island, and many other OMPs. BtuB and Cir had a restricted diffusion diameter of ~0.5 μm , roughly equivalent to the size of the islands, with diffusion coefficients of 0.013 and 0.019 $\mu\text{m}^2/\text{s}$, respectively, using single-molecule tracking (SMT)-TIRF microscopy (Rassam et al. 2015).

This technique of fluorescently-labelling ColE9 to study OMP organisation, forms the basis of this thesis, to develop a fluorescence-based assay to monitor ColE9 translocation into the cell.

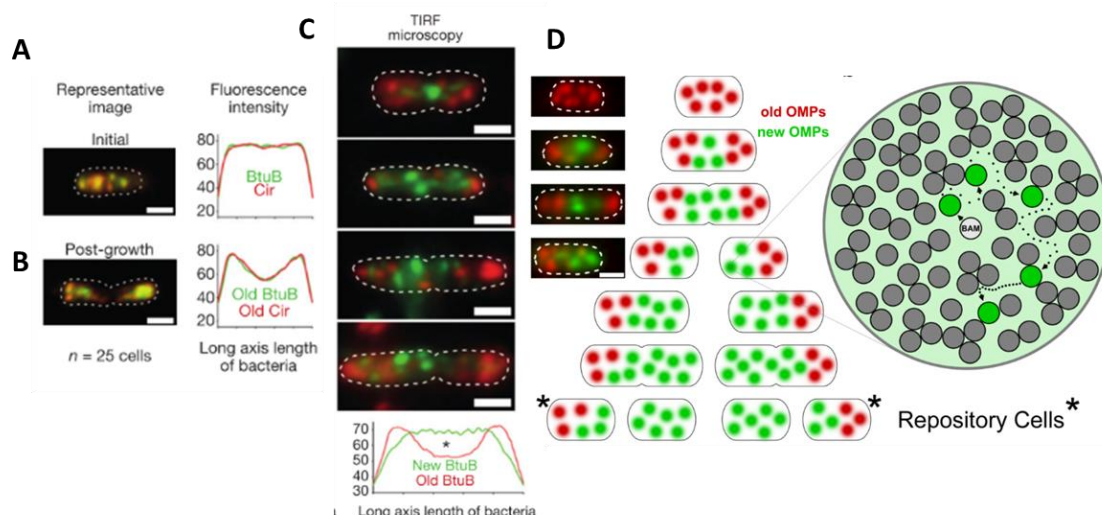


Figure 1. 16 - Fluorescent labelling of ColE9 as a method to study OMPs on the surface of *E. coli*.

A method was developed to study OMPs on the surface of *E. coli*, by fluorescently-labelling ColE9 and Colla in the cytotoxic domain with AlexaFluor dyes and engineering a disulphide in the R-domain to prevent translocation (Rassam et al. 2015). ColE9 and Colla bind specifically to BtuB and Cir, respectively. (A) The fluorescently-labelled OMPs BtuB and Cir were clustered and co-localised. (B and C) Over time the OMPs migrated to the cell poles (red) and a subsequent cell labelling using colicin labelled with another colour (green) showed that newly generated OMPs were formed predominantly at midcell. (D) By following several cell divisions starting with a single cell, the old OMPs remained sequestered at the poles of the single cell and new OMPs inserted at the midcell. This led to daughter cells containing newly generated OMPs and thereby a proposal for a mechanism of OMP turnover by binary partitioning. This led to the formation of daughter cells containing only new OMPs and the proposal for a mechanism of OMP turnover by binary partitioning. Reproduced from (Rassam et al. 2015).

1.8. Aims of this study

Colicins are the best studied bacteriocins, in particular ColE9. There are a wide range of techniques that have been used and developed to better understand their structure, function and mechanism of action of colicin binding, translocation and cytotoxicity. However, there are still a lot of outstanding questions to be addressed on the mechanism of translocation of ColE9.

The principal aim of this research was to develop fluorescence-based microscopy tools to follow the translocation of ColE9 to the cytoplasm of single, live *E. coli* cells. Many of the proteins involved in the translocation of ColE9 into the cell, including BtuB, OmpF, TolQRAB and FtsH, have been identified and their interactions and role in the cytotoxicity of ColE9 has been well studied using a range of *in vitro* and *in vivo* techniques (Cascales et al. 2007) (Kleanthous 2010) (Papadakos, Wojdyla, and Kleanthous 2012).

These new microscopy-based tools were used to elucidate the requirements for binding and translocation of ColE9 into the cytoplasm by direct visualisation, including the involvement of specific surface receptors, PMF-dependence and the requirement for periplasmic and IM proteins. In addition, fluorescence microscopy was used to characterise the diffusive behaviour of ColE9*^{AF647} after translocation to the *E. coli* cytoplasm using Total internal reflection fluorescence (TIRF) and widefield microscopy, so as to better understand the fate of these molecules and the kinetics of this process. Finally, fluorescence microscopy was used to show that ColE9 translocates through the lumen of the porins OmpF or OmpC in order to cross the OM.

2 Chapter 2 - Materials and Methods

2.1 Plasmids, bacterial strains and media

All *E. coli* strains and plasmids used in this study are listed in Table 2. 1. Strains were routinely grown at 37°C in either Lysogeny broth (LB) or M9-minimal media, or plated on LB-agar or M9-agar. LB contained 10 g tryptone, 5 g yeast extract and 10 g NaCl per litre of media at pH 7.2 (Miller 1972). Supplemented M9-minimal media contained 47.78 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.56 mM NaCl, 2 mM MgSO₄, 0.1 mM CaCl₂, 18.7 mM NH₄Cl, 0.4 % (w/v) D-glucose, 0.05% (w/v) casamino acids and 0.0002 % (w/v) thiamine. Selective media were supplemented with 100 µg/ml ampicillin (Amp), 20 µg/ml chloramphenicol (Chl), 50 µg/ml kanamycin (Kan) or 10 µg/ml tetracycline (Tet) where indicated.

Table 2. 1 - Plasmids and *E. coli* strains used in this study.

Strain	Genotype and description	Antibiotic	References
NEB 5α	F ⁻ φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK ⁻ , mK ⁺) phoA supE44 λ ⁻ thi-1 gyrA96 relA1 Competent <i>E. coli</i> cells used for transformation with plasmids, made by ligation or whole plasmid mutagenesis, and the preparation of plasmid DNA for sequencing.	-	Invitrogen
BL21 (DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3) Competent cells used for induced protein expression from recombinant plasmids under the regulation of T7 promoter. Protein expression controlled by isopropyl-β-D-thiogalactopyranoside (IPTG).	-	Novagen
JM83	rpsL ara Δ(lac-proAB) φ80dlacZΔM15 Wild-type <i>E. coli</i> strain, used for colicin toxicity assays and microscopy. Competent JM83 used for transformation with Im9-GFP carrying plasmid for microscopy.	-	(Yanisch-Perron, Vieira, and Messing 1985)
MG1655	F ⁻ λ ⁻ <i>ilvG⁻ rfb-50 rph-1</i> <i>E. coli</i> strain used for colicin toxicity assays and microscopy.	-	(Blattner et al. 1997)
RK5016	Δ <i>lac</i> (U169) <i>araD139 rpsL gyrA thi non metE argH recA ΔbtuB</i> BtuB knockout used to show ColE9 receptor specificity by toxicity assays and microscopy.	Kan	(Heller, Mann, and Kadner 1985)
DPD1718	<i>lac Cam^r lacZ::recA φluxCDABE</i>	Chl	

	Used to study ColE9 toxicity <i>in vivo</i> by bioluminescence.		(Davidov et al. 2000)	
JW2203	F-, $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta ompC768::kan$, <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, <i>hsdR514</i>	Kan	Keio (Baba et al. 2006)	
	OmpC porin knockout used to show ColE9 translocation by toxicity assays, CFU counts and microscopy.			
JW0912	F-, $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta ompF746::kan$, <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, <i>hsdR514</i>	Kan	Keio (Baba et al. 2006)	
	OmpF porin knockout used to show ColE9 translocation by toxicity assays, CFU counts and microscopy.			
BZB1107	ompR $\Delta lamB$ ompF::Tn5	Kan	(Jeanteur et al. 1994)	
	OmpF, OmpC, LamB porin knockout used to show ColE9 translocator specificity by toxicity assays and microscopy.			
JW5086	F-, $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, $\Delta fepA721::kan$, λ^- , <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, <i>hsdR514</i>	Kan	Keio (Baba et al. 2006)	
	FepA knockout used to show ColB receptor specificity by toxicity assays and microscopy.			
HDL11	<i>pgsA::kan</i> ϕ (<i>lacOP-pgsA⁺</i>)1 <i>lacZ'</i> <i>lacY::Tn9 lpp2 zdg::Tn10</i>	Kan, Tet, Chl	(Kusters, Dowhan, and de Kruijff 1991)	
	<i>E. coli</i> strain depleted in anionic phospholipids, containing a single copy of the IPTG-inducible <i>pgsA</i> gene encoding the major anionic phospholipid phosphatidylglycerol, to control the inner membrane charge. Used to show ColE9 toxicity.			
AR3289	W3110 <i>sfhC21 zad220::Tn10</i>	-	(Tatsuta et al. 1998)	
	<i>E. coli</i> strain with an <i>sfhC21</i> mutant background encoding FabZ enzyme, which is involved in fatty acid biosynthesis. Used as parent strain to show FtsH involvement in ColE9 toxicity by toxicity assays and microscopy.			
AR3291	W3110 $\Delta ftsH3$ <i>sfhC21 zad220::Tn10</i>	Kan	(Ogura et al. 1999)	
	AR3291 is an FtsH chromosomal knockout with the <i>sfhC21</i> mutant background encoding FabZ, used to suppress FtsH deletion lethality by upregulating FabZ activity to restore balance to LPS:phospholipid ratio. Used to show FtsH involvement in ColE9 toxicity by toxicity assays and microscopy.			
Plasmid name	Plasmid description	Properties		References
pET21a		T7 promoter (IPTG inducible) Amp ^R		Novagen
pBAD24		Arabinose inducible, Amp ^R		ATCC

pUC19		Amp ^R	NEB
pTrc99A		Amp ^R	(Amann, Ochs, and Abel 1988)
pCS4	pET21a-ColE9-Im9	Expression construct for IPTG-inducible ColE9-Im9 wild-type inserted into the NdeI/XhoI site of pET21a, containing a C-terminal hexahistidine tag, Amp ^R	(Garinot-Schneider et al. 1997)
pMLF07 (pCS4NK3)	pET21a-ColE9-K469C-Im9	Expression construct for IPTG-inducible ColE9-Im9, with DNase domain K469C mutation for fluorescent labelling, containing a C-terminal hexahistidine tag, Amp ^R	This work and Nadine Kirkpatrick
pCS4 H551A	pET21a-ColE9-H551A-Im9	Expression construct for IPTG-inducible ColE9-Im9, with DNase domain H551A mutation to inactivate cytotoxicity, containing a C-terminal hexahistidine tag, Amp ^R	(Walker et al. 2002)
pMLF08	pET21a-ColE9-H551A-K469C-Im9	Expression construct for IPTG-inducible ColE9-Im9, with DNase domain H551A mutation to inactivate cytotoxicity and K469C mutation for fluorescent labelling, containing a C-terminal hexahistidine tag, Amp ^R	This work
pBH29NK3	pET21a-ColE9-Y324C-L447C-K469C-Im9	Expression construct for IPTG-inducible ColE9-Im9, with DNase domain K469C mutation for fluorescent labelling, and R-domain Y324C, L447C internal disulphide to block cell entry, containing a C-terminal hexahistidine tag, Amp ^R	(Rassam et al. 2015)
pREN94	pQE2-Im9-eGFP	Expression construct for IPTG-inducible Im9-eGFP, containing an N-terminal hexahistidine tag-Im9-factor Xa linker-eGFP, Amp ^R	Unpublished data, Renata Kaminska
pMLF09	pBAD24-Im9-eGFP	Expression construct for arabinose-inducible Im9-eGFP, containing an N-terminal hexahistidine tag-Im9-factor Xa linker-eGFP, Amp ^R	This work
pREN113	pET21a-ColE9-K298C-Im9	Expression construct for IPTG-inducible ColE9-Im9, with T-domain K298C mutation for fluorescent labelling, containing a C-terminal hexahistidine tag, Amp ^R	Unpublished data, Renata Kaminska
pREN115	pET21a-ColE9-S420C-Im9	Expression construct for IPTG-inducible ColE9-Im9, with R-domain S420C mutation for fluorescent	Unpublished data, Renata Kaminska

		labelling, containing a C- terminal hexahistidine tag, Amp ^R	
pREN116	pET21a-ColE9-S451C-Im9	Expression construct for IPTG-inducible ColE9-Im9, with R-domain S451C mutation for fluorescent labelling, containing a C- terminal hexahistidine tag, Amp ^R	Unpublished data, Renata Kaminska
pREN118	pET21a-ColE9-W39A-K469C-Im9	Expression construct for IPTG-inducible ColE9-Im9, with DNase domain K469C mutation for fluorescent labelling and TolB box W39A mutation to prevent TolB binding, containing a C-terminal hexahistidine tag, Amp ^R	Unpublished data, Renata Kaminska
pIFH108 WT		pET29b derivative carrying the T7-promoter, expression construct for IPTG-inducible WT FtsH plasmid, Amp ^R	(Karata et al. 1999)
pIFH108 E255Q		pET29b derivative carrying the T7-promoter, expression construct for IPTG-inducible FtsH ATPase mutant plasmid, Amp ^R	(Karata et al. 2001)
pIFH108 F228R		pET29b derivative carrying the T7-promoter, expression construct for IPTG-inducible FtsH pore entrance mutant plasmid, Amp ^R	(Yamada-Inagawa et al. 2003)
pIFH108 H421Y		pET29b derivative carrying the T7-promoter, expression construct for IPTG-inducible FtsH active site protease mutant plasmid, Amp ^R	(Karata et al. 1999)
pPW13	pACYCDuet-1-ColB-TR-Cys	Expression construct for IPTG-inducible ColB TR domain construct, with R domain K342C mutation for fluorescent labelling, containing a C-terminal hexahistidine tag, Chl ^R	Unpublished data, Paul White
pEE01	pACYCduet-1-ColB	Expression construct for IPTG-inducible ColB wild-type, containing a C-terminal hexahistidine tag, Chl ^R	Unpublished data, Emma Elliston
pRJ353	pET21d-ColE9-DNase-Im9	Expression construct for IPTG-inducible ColE9 DNase-Im9, containing a C- terminal hexahistidine tag, Amp ^R	(Garinot-Schneider et al. 1996)
pBC1	pET21d-ColE9-DNase-K469C-Im9	Expression construct for IPTG-inducible ColE9 DNase-Im9, with DNase domain K469C mutation for fluorescent labelling, containing a C-terminal hexahistidine tag, Amp ^R	Unpublished data, Boonya-porn Chin-thammit

2.2 Molecular biology techniques

2.2.1 Purification of plasmid DNA

Plasmids were purified from NEB 5 α cultures using Qiagen QIAprep spin Miniprep or Plasmid Plus Midiprep kit following the manufacturer's protocol. DNA concentrations were determined by absorbance at 260 nm using the Beer-Lambert Law.

2.2.2 Polymerase chain reaction (PCR)

Whole-plasmid site-directed mutagenesis and the amplification of genes and gene fragments of interest was performed by polymerase chain reaction (PCR). The composition of standard PCR reactions was assembled as described in Table 2. 2 and run according to the program in Table 2. 3. A negative control was conducted where no polymerase was added. Mutagenesis primer sets were designed and purchased from Eurofins MWG Operon or Sigma-Aldrich (Table 2. 4). Their design was aimed to be between 18-30 nucleotides, contain the mismatch in the centre of the sequence and to display a melting temperature greater than 60°C, calculated using the formula $T_m = 4(G + C) + 2(A + T) ^\circ C$. The GC content was designed to have 40-68 % GC content with the 3' end ending with C/G. PCR was performed with an Eppendorf Mastercycler Personal Thermal Cycler in a total volume of 50 μ l.

Table 2. 2 - Composition of a typical PCR reaction for whole plasmid site-directed mutagenesis.

Reagent	Volume (μ l)
5 x HF NEB Phusion reaction buffer (1x)	10
100 ng dsDNA template	1.2
125 ng oligonucleotide forward primer	2
125 ng oligonucleotide reverse primer	2
10 mM dNTP mix	1
ddH ₂ O	32.8
HF Phusion DNA polymerase	1

Table 2. 3 - PCR programme thermocycling conditions for whole plasmid site-directed mutagenesis.

Step	Cycles	Temperature (°C)	Time
Initial denaturation	1 cycle	98°C	30 seconds
Denaturation	30 cycles	98°C	30 seconds
Annealing		55°C (primer Tm-5°C)	30 seconds
Extension		72°C	~3.5 minutes (30s/kb plasmid)
Final extension	1 cycle	72°C	10 minutes
	Hold	4°C	Hold

Table 2. 4 - List of PCR primers used in this study.

Primer	Mutagenesis primer sequence
K469C F	5'-ACCAGTTGGTGATTGTTGGCTGGATGATGC-3'
K469C R	5'-GCATCATCCAGCCAACAATCACCAACTGGT-3'
H551A F	5'-GTCTATGAACTTCATGCTGACAAGCCAATTAGTCAAGGTGGTGAGG-3'
H551A R	5'-CCTCACCACCTTGACTAATTGGCTTGTCAGCATGAAGTTCATAGAC-3'
EcoRI-6His-Im9 F	5'-CCGGAATTCTACCATCACCATCACCATCAC-3'
GFP-HindIII R	5'-CCCAAGCTTAGGATCCGCGACCCATTTGC-3'
K298C F	5'-AGTCCTGACCAGGTATGTCAACGTCAGG-3'
K298C R	5'-CCTGACGTTGACATACCTGGTCAGGACT-3'
S420C F	5'-GCTGCAAAAGAGAAGTGTGATGCTGATGC-3'
S420C R	5'-GCATCAGCATCACACTTCTCTTTGCAGC-3'
S451C F	5'-TTAGCCATGGAGTGTAACGGAATAAGCCAGGGAAGG-3'
S451C R	5'-CCTTCCCTGGCTTATTCCGTTTACACTCCATGGCTAA-3'

Products were run on a 1.5 % (w/v) agarose gel to check for amplification of the plasmid relative to the negative control. To digest the plasmid template DNA after the PCR reaction, the samples were incubated with 2 µl NEB *DpnI* restriction enzyme at 37°C for 2 hours. 10 µl *DpnI* treated PCR product were used to transform 100 µl competent NEB 5α cells, plated on LB-agar and incubated overnight at 37 °C.

Mutations were confirmed by DNA sequencing (Source Bioscience Sequencing). T7F and T7R primers were used for sequencing pET-based plasmids and pBADF and pBADR primers for sequencing pBAD based plasmids. E9_seq primer was used for ColE9 pET-based plasmids for

complete read-through of the sequence shown in Table 2. 5. The amino acid sequences of the proteins that are encoded by these plasmids are detailed in Appendix I.

Table 2. 5 - Sequencing primers used in this study.

Sequencing primers		
	Forward	Reverse
T7	5'-TAATACGACTCACTATAGGG-3'	5'-GCTAGTTATTGCTCAGCGG-3'
pBAD	5'-ATGCCATAGCATTTTATCC-3'	5'-GATTTAATCTGTATCAGG-3'
E9_Seq	5'-GACAGGCTAAAGCTGTTCAGG-3'	-

2.2.3 DNA agarose gel electrophoresis

Electrophoresis grade agarose (Invitrogen) was dissolved in 0.5x TrisBorate-EDTA-buffer (TBE) (4.45 mM Tris-borate pH 8.3, 0.1 mM EDTA) to make a 1.5 % (w/v) gel. 5 µl of SYBR Safe (10000x concentrate Invitrogen) were added per 50 ml of gel. A 0.1-10 kb ladder (NEB, Quick-Load 2-Log) was used as a molecular weight marker size standard. DNA was mixed 1:5 with 6x DNA loading buffer (30 % (w/v) glycerol, 6 mM EDTA, 0.4 % (w/v) bromophenol blue), loaded onto the gel and run in 0.5x TBE buffer at 80 V for 1 hour until resolution had been achieved. DNA was visualised with a UV transilluminator.

2.2.4 Restriction enzyme digests

Restriction digests of plasmids and PCR products were performed using enzymes and buffers purchased from New England Biosciences (NEB). Typically, reactions were performed containing components in Table 2. 6, for 2 hours or overnight at 37 °C. Subsequently, digested DNA was purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol.

Table 2. 6 - Composition of a typical restriction digest reaction using NEB restriction enzymes.

Reagent	Volume (µl)
NEB buffer 2.1	10
5 µg PCR product or plasmid	5
Restriction enzyme	5
ddH ₂ O to final volume 50 µl	30

2.2.5 DNA gel extraction

Linearized vectors and digested PCR products were purified by DNA agarose gel electrophoresis (Section 2.2.3) followed by extraction using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

2.2.6 DNA ligation

Ligation of the PCR product insert and linearized vector was performed in a ratio of 3x excess insert to vector, containing components from the Quick Ligation kit (NEB) in Table 2. 7, for 30 minutes at room temperature. Negative controls were conducted where no ligase or no insert were added. Ligation mix was transformed into competent NEB 5 α cells, Section 2.2.8.

Table 2. 7 - Composition of a typical ligation reaction using the Quick Ligation kit (NEB).

Reagent	Volume (μ l)
10x Quick ligation buffer	1
100 ng vector DNA	4
300 ng insert DNA	4
Quick T4 DNA ligase	1
ddH ₂ O to final volume 30 μ l	20

2.2.7 Preparation of competent cells

E. coli BL21 (DE3), NEB 5 α and JM83 cells, were made competent by growing cells in a 50 ml culture to an OD₆₀₀ of 0.3-0.6 in LB medium, before harvesting 35 ml by centrifugation (10 minutes at 5000 xg, 4 °C). The cells were re-suspended in 20 ml of ice-cold competent cell solution 1 (20 mM Tris-HCl pH 7.5, 50 mM CaCl₂) and incubated at 4 °C for 1 hour. Cells were pelleted by centrifugation (10 minutes at 5000 xg, 4 °C), and re-suspended in 2 ml ice-cold competent cell solution 2 (20 mM Tris-HCl pH 7.5, 50 mM CaCl₂, 20 % v/v glycerol). Aliquots of 200 μ l were flash frozen in liquid nitrogen and stored at – 80 °C.

2.2.8 Transformation of competent cells

Transformation competent cells were thawed on ice, mixed with 100 ng of plasmid DNA per 100 μ l of competent cells and incubated on ice for 30 minutes. Cells were subjected to a heat

shock at 42 °C for 1 minute, then ice for 5 minutes, followed by the addition of 1 ml LB medium and incubated for 1 hour at 37°C with shaking, before plating 100 µl onto LB-agar and incubating at 37 °C for ~16 hours.

2.3 Biochemical methods

2.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gels were prepared using a stock of 30 % (w/v) acrylamide and 0.8 % bis-acrylamide (Protogel, National Diagnostics) which was diluted into 375 mM Tris-HCl pH 8.8 and 0.1 % (w/v) sodium dodecyl sulphate (SDS) to give final acrylamide concentrations of 12 % and 16 %, where specified as required for the running gel. Ammonium persulfate (APS) and N, N, N', N'-tetramethyl ethylenediamine (TEMED) were added to a final concentration of 0.08 % w/v and 0.13 % v/v, respectively. Stacking gels were prepared similarly but contained 5 % acrylamide and 250 mM Tris-HCl pH 6.8.

Equal volumes of protein samples and 2x sample loading buffer (100 mM Tris-HCl pH 6.8, 4 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 20 % (v/v) glycerol, 200 mM β -ME) were mixed. Samples were boiled for 5 minutes prior to gel loading in 8 µl aliquots. Some samples were resuspended in the absence of β -ME and without boiling to reduce unfolding. Unstained protein molecular weight marker with a range of 14.4 to 116 kDa (Fermentas #SM0431) was run alongside samples to enable approximate molecular weight determination of protein samples. Gels were run in 25 mM Tris-HCl, 192 mM glycine, 0.1 % (w/v) SDS at a constant current of 30 mA per gel for 30 minutes.

Proteins were visualised by staining the gel with 0.2 % (w/v) Coomassie brilliant blue R250 (Pierce) in 10 % v/v acetic acid and 50 % v/v ethanol for 30 minutes and de-stained in 10 % v/v acetic acid and 10 % v/v ethanol.

2.3.2 Colicin expression and purification

pET21a-ColE9-Im9 (pMLF07 or pMLF08) plasmids were transformed into competent *E. coli* BL21 (DE3) cells (Section 2.2.8). A single colony was selected to inoculate a 50 ml overnight culture. Typically, 6 flasks containing 800 ml of LB media were inoculated with a 1:100 dilution of the 50 ml overnight culture and supplemented with 100 µg/ml Amp. The culture was grown to OD₆₀₀ ~0.6 with shaking at 120 rpm at 37 °C, before inducing expression with 1 mM IPTG (Melford) for 3 hours at 37 °C. The cells were harvested by centrifugation at 6250 xg for 20 minutes at 4 °C. The cell pellet was re-suspended in 35 ml lysis buffer (20 mM potassium phosphate pH 7, 500 mM NaCl, 1 mM PMSF, 10 mg lysozyme). The suspension was sonicated on ice using a sonicator (Misonix S-4000 ultrasonic liquid processor fitted with 3/8 inch stud probe) for 2 minutes (3 seconds pulse on, 7 seconds pulse off) at 60 % amplitude. The cell lysate was centrifuged for 30 minutes at 15,000 xg at 4 °C to pellet cellular debris and collect the supernatant, which contained His-Im9-ColE9 complex. The lysate was filtered (0.45 µm), before loading via a 50 ml superloop onto a 5 ml HisTrap FF HP nickel column (GE Healthcare) equilibrated with binding buffer (20 mM potassium phosphate pH 7, 500 mM NaCl, 5 mM imidazole), at a rate of 1 ml/min.

Protein binding and elution was monitored by UV absorbance at 280 nm. The column was washed in ~25 column volumes (CV) of wash buffer (20 mM potassium phosphate pH 7, 500 mM NaCl, 5 mM imidazole) to remove unbound and non-specifically bound protein until the UV signal had returned to A280 nm ~0. ColE9 was dissociated and eluted from His-Im9 through unfolding using a step gradient of 6 M guanidine hydrochloride at 1 ml/min for 3 CVs (20 mM potassium phosphate pH 7, 500 mM NaCl, 6 M guanidine pH 7). Eluted fractions were collected and run on 12 % SDS-PAGE gels to locate protein and dialysed overnight at 4 °C in dialysis buffer (20 mM potassium phosphate pH 7, 500 mM NaCl, 10 mM DTT) using Spectra/Por membrane with a molecular weight cut off (MWCO) of 12-14 kDa, to remove guanidine and allow protein refolding whilst preventing dimer formation.

ColE9 was concentrated (using Vivaspin 20 30 kDa MWCO) to ~5 ml before further purification by size-exclusion chromatography, using a S200 HL 26/600 column (GE Healthcare) equilibrated with size- exclusion buffer (20 mM potassium phosphate pH 7, 500 mM NaCl 10 mM DTT). DTT reducing agent is present in the buffer to prevent the formation of dimers through disulphide bond formation of the free cysteine residues on each ColE9. 5 ml of ColE9 was loaded and eluted isocratically at 1 ml/min, protein elution was monitored by absorbance at 280 nm. Eluted fractions were selected and run on SDS-PAGE gels to locate protein, before concentrating and freezing at -20 °C.

2.3.3 Im9-eGFP expression and purification

pQE2-Im9-eGFP plasmid (pREN94) was transformed into competent *E. coli* BL21 (DE3) cells and was expressed using the same method as ColE9 (Section 2.3.2) using 1 mM IPTG. Im9-eGFP with an N-terminal Hexahistidine tag was loaded onto a 5 ml HisTrap FF HP nickel column (GE Healthcare) equilibrated with binding buffer (20 mM potassium phosphate pH 7, 500 mM NaCl, 5 mM imidazole), at a rate of 1 ml/min. Protein loading was monitored by UV absorbance at 280 nm and 488 nm on an AKTA Purifier. The column was washed in ~25 CVs of buffer to remove unbound and non-specifically bound protein (20 mM potassium phosphate pH 7, 500 mM NaCl, 2 mM imidazole). Im9-eGFP was eluted from the column using a 2-50 % (20-500 mM) imidazole gradient at 1 ml/min over 20 CVs (20 mM potassium phosphate pH 7, 500 mM NaCl, 1 M imidazole). Eluted fractions were selected and run on 12 % SDS-PAGE gels to locate protein visualised by both UV and Coomassie, which were collected and dialysed overnight at 4 °C in dialysis buffer (20 mM potassium phosphate pH 7, 500 mM NaCl) using Spectra/Por membrane with a molecular weight cut off (MWCO) of 12-14 kDa, to remove imidazole.

Im9-eGFP was concentrated (using Vivaspin 20 10 kDa MWCO) to ~5 ml before further purification by size-exclusion chromatography, using an S75 HL 26/600 column (GE Healthcare) equilibrated with 20 mM potassium phosphate pH 7, 500 mM NaCl. 5 ml of Im9-eGFP was loaded and eluted using an isocratic gradient at 1 ml/min. Protein elution was monitored by absorbance

at 280 nm and at 488 nm. Eluted fractions were selected and run on SDS-PAGE gels to locate protein, before concentrating and freezing at -20°C.

2.3.4 Protein concentration measurements

Protein concentrations were determined using the Beer-Lambert law ($A = \epsilon \cdot c \cdot l$, where A = absorbance, ϵ = molar extinction coefficient ($M^{-1} \text{ cm}^{-1}$), c = concentration (M) and l = pathlength (1 cm)) at 280 nm, where absorbance values were measured using a biophotometer (Eppendorf), in combination with amino acid sequence based theoretical extinction coefficients (Appendix I) using <http://protcalc.sourceforge.net/> presented in Table 2. 8.

The theoretical molar extinction coefficients were calculated according to the formula:

$$\epsilon_{\text{protein}} = N_{\text{Tyr}} \cdot \epsilon_{\text{Tyr}} + N_{\text{Trp}} \cdot \epsilon_{\text{Trp}} + N_{\text{Cys}} \cdot \epsilon_{\text{Cys}}$$

Where N_{Tyr} , N_{Trp} , N_{Cys} is the number of tyrosine, tryptophan or cysteine residues in the protein sequence, and ϵ_{Tyr} , ϵ_{Trp} , ϵ_{Cys} are the molar extinction coefficients for tyrosine, tryptophan and cysteine (1280, 5690, and 120 $M^{-1} \text{ cm}^{-1}$ respectively, as determined by (Gill and von Hippel 1989)).

Table 2. 8 - Theoretical extinction coefficients and molecular weights for proteins used in this study.

Protein	ϵ Theoretical extinction coefficients ($M^{-1} \text{ cm}^{-1}$)	Molecular weight (kDa)
ColE9	46,075	61.6
ColE9 Y324C L447C K469C	46,350	61.5
ColE9 K469C	47,510	61.5
ColE9 H551A	47,510	61.5
ColE9 H551A K469C	47,510	61.5
Im9	9,970	9.8
Im9-eGFP	30,580	39.7
ColE9 K298C	47,510	61.5
ColE9 S420C	47,510	61.5
ColE9 S451C	47,510	61.5
ColE9 W39A	41,820	61.4
ColE9 W39A K469C	41,820	61.4
ColB	62,160	54.9
ColB-TR-Cys	39,970	36.7
ColE9 DNase	15,220	15.1
ColE9 DNase K469C	15,220	15.1

2.3.5 Colicin cysteine maleimide-fluorophore labelling

Purified colicins with mutated cysteines for labelling were desalted on a 5 ml HiTrap column (GE Healthcare) equilibrated with binding buffer (20 mM potassium phosphate pH 7, 500 mM NaCl) to remove DTT, as the free thiols of DTT react with maleimide groups of the dyes, preventing fluorescent labelling of ColE9. The protein was diluted to 900 µl of 50 µM and labelled in a 5:1 molar excess of fluorophore:protein with AlexaFluor 488 C₅-Maleimide (Invitrogen) (AF⁴⁸⁸), AlexaFluor 568 C₅-Maleimide (Invitrogen) (AF⁵⁶⁸), or AlexaFluor 647 C₂-Maleimide (Invitrogen) (AF⁶⁴⁷), re-suspended in DMSO as a 10 mM stock.

Labelling was performed for 1-2 hours at room temperature with rotation on a rotary wheel in the dark. The reaction was quenched using 2 mM DTT for 15 minutes, before dialysing overnight at 4°C in dialysis buffer (20 mM potassium phosphate pH 7, 500 mM NaCl) using Spectra/Por membrane with a molecular weight cut off (MWCO) of 6-8 kDa, to remove excess dye. Labelled protein was separated from further excess dye by size-exclusion chromatography using an S200 HL 10/300 column (GE Healthcare) equilibrated with 20 mM potassium phosphate pH 7, 500 mM NaCl. ~1 ml of labelled colicin was loaded and eluted isocratically at 1 ml/min. Protein elution was monitored by absorbance at 280 nm and fluorescence by absorbance at 494 nm, 575 nm, and 651 nm for AF⁴⁸⁸, AF⁵⁶⁸ and AF⁶⁴⁷, respectively. Eluted fractions were selected and run on 12 % SDS-PAGE gel, using loading buffer to visualise labelled protein and excess dye by UV and protein by Coomassie blue staining. All labelling and subsequent purification steps were performed in the dark.

2.3.6 Measurement of protein labelling efficiency

The protein concentration and labelling efficiency was determined by either a spectrophotometer (ND-1000 Nanodrop) or UV-VIS (JASCO), using the protein and fluorophore molar extinction coefficients and absorbances at 280 nm and 494 nm, 575 nm and 651 nm for AF⁴⁸⁸, AF⁵⁶⁸ and AF⁶⁴⁷, respectively, as described below (Table 2. 9).

Protein concentration is calculated first:

$$\text{Protein concentration (M)} = \frac{A_{280} - (A_{\text{dye}} \times \text{correction factor})}{\text{Protein molar extinction coefficient } (\epsilon) \text{ (M}^{-1} \text{ cm}^{-1})}$$

Then the degree of labelling is calculated:

$$\text{Moles dye per mole of protein} = \frac{A_{\text{dye}}}{\text{Dye molar extinction coefficient } (\epsilon) \text{ (M}^{-1} \text{ cm}^{-1}) \times \text{protein concentration (M)}}$$

Table 2. 9 - Extinction coefficients, molecular weights and correction factors for fluorophores used in this study.

Extinction coefficients and molecular weights (Invitrogen 2010a), correction factors (invitrogen 2012).

Fluorophore	ϵ Extinction coefficients (M ⁻¹ cm ⁻¹)	Molecular weight (kDa)	Correction factor
AF ⁴⁸⁸ C ₅ -maleimide	72,000 at 494 nm	0.721	0.11
AF ⁵⁶⁸ C ₅ -maleimide	92,000 at 575 nm	0.881	0.46
AF ⁶⁴⁷ C ₂ -maleimide	265,000 at 651 nm	1.25	0.03

2.3.7 Analytical Size Exclusion Chromatography

To show complex formation of Im9-eGFP and ColE9 DNase^{AF568}, proteins were buffer exchanged on a 5 ml HiTrap column (GE Healthcare), 5 μ M Im9-eGFP, 5 μ M ColE9 DNase^{AF568} and 5 μ M Im9-eGFP in the presence of 5 μ M ColE9 DNase^{AF568} were prepared in 20 mM potassium phosphate, pH 7, 100 mM NaCl. Each single protein or complex was loaded onto an S75 10/300 GL Increase column (GE Healthcare) equilibrated with 20 mM potassium phosphate pH 7, 100 mM NaCl. Proteins were eluted isocratically at 1 ml/min by size-exclusion chromatography. Protein elution was monitored by absorbance at 280 nm and fluorescence by absorbance at 488 nm for eGFP and 575 nm for ColE9 DNase^{AF568}, respectively.

2.4 Biophysical methods

2.4.1 Electrospray-Ionisation Mass spectrometry

The masses of the fluorescently labelled proteins used during this study were obtained using electrospray ionisation mass spectrometry (ESI-MS), by Dr David Staunton (Molecular Biophysics Suite, University of Oxford). Samples were diluted to ~80 µg/ml in 500 µl containing 20 mM potassium phosphate pH 7, 500 mM NaCl. Samples were desalted on a Merck Chromolith C18 2 x 5 mm guard column and run on a Waters LCT Premier XE using 0.1 % formic acid and acetonitrile solvents. ESI-MS of the samples showed the main species to be 1 colicin to 1 dye (Appendix I Figure 2). The predicted and observed molecular weights are shown in Appendix I Table 1.

2.4.2 Fluorimetry

To determine if Im9-eGFP and ColE9 DNase^{AF568} form a FRET pair when in a complex, their spectral overlap was checked by fluorimetry. Each individual protein and the complex was prepared in 20 mM potassium phosphate pH 7, 100 mM NaCl, at a final concentration of 5 µM in 3 ml. Each sample was then loaded into a 3 ml quartz cuvette with a 10 mm path length and placed in a FluoroMax-4 spectrophotometer (HORIBA Scientific). Im9-eGFP emission scans were performed at a wavelength of 500-700 nm using an excitation of 488 nm. For ColE9 DNase^{AF568} emission scans were performed at a wavelength of 585-700 nm using an excitation of 575 nm. This was repeated to look at spectral overlap exciting at 488 nm and scanning emission 498-700 nm. The complex emission scans were performed at a wavelength of 500-700 nm using an excitation of 488 nm. The CPS (counts per second) were below 1 million and used slit widths of 2 nm for both excitation and emission monochromators. Scan speeds were 1 nm/s and integration time of 0.1 s.

2.5 Microscopy

2.5.1 Preparation of microscopy slides

A 1.5 x 1.6 cm Gene Frame matrix (Thermo Scientific) was adhered onto a 1.2-1.5 mm thick microscope slide (Thermo Fisher). A 1 % agarose gel was made by adding agarose (Invitrogen) to supplemented M9 minimal media. 190 µl of this agarose was added within the Gene Frame matrix and a 22 x 22 mm glass coverslip (Menzel Gläser #1) was applied to the top of the Gene Frame to flatten the agarose pad and remove excess gel.

2.5.2 Cell growth and labelling of cells with ColE9 (AF⁴⁸⁸, AF⁵⁶⁸, AF⁶⁴⁷)

E. coli cells, transformed with various plasmids described in the results, were inoculated from an overnight LB culture into 10 ml of supplemented M9 minimal media (Section 2.1) with the appropriate antibiotics and grown at 37 °C until they reached exponential phase OD₆₀₀ 0.4. 1600 µl of cells were pelleted by centrifugation at 7000 xg for 3 mins at 17 °C, the supernatant was removed and the pellet re-suspended in 200 µl M9 with 1.5 µM fluorescently-labelled colicin and 20 µM Hoechst 33342 (Thermofisher) for 30 minutes at room temperature with rotary inversion in a black tube. Subsequent steps were performed in the dark. Cells were pelleted by centrifugation at 7000 xg for 3 minutes at 17 °C, the supernatant removed and the pellet gently re-suspended in 400 µl supplemented M9 minimal media to wash the cells and remove excess dye, followed by centrifugation, removal of the supernatant and repeating the wash stage three times. Cells were gently re-suspended in a small volume ~10-100 µl of M9 before loading onto a 1 % agarose pad on a microscope slide (Section 2.5.1).

2.5.3 Fixing cells with Paraformaldehyde (PFA)

After growth in M9 to exponential phase OD₆₀₀ 0.4 (Section 2.5.2), cells were re-suspended in 400 µl 4 % PFA (Thermofisher) for 15 minutes at 4 °C with rotation to fix cells. Cells were pelleted by centrifugation at 7000 xg for 3 minutes at 17 °C, the supernatant removed and pellet re-suspended in 200 µl M9 and labelled with colicin as above (Section 2.5.2).

2.5.4 Treating cells with Carbonyl cyanide m-chlorophenyl hydrazine (CCCP)

After growth in M9 to exponential phase OD₆₀₀ 0.4 (Section 2.5.2), cells were re-suspended in 400 µl 100 µM CCCP (Sigma) for 5 minutes at room temperature. Cells were pelleted by centrifugation at 7000 xg for 3 minutes at 17 °C, the supernatant removed and pellet re-suspended in 200 µl M9 with 100 µM CCCP and labelled with 1.5 µM fluorescently-labelled colicin and 20 µM Hoechst as above. All wash stages in M9 were in the presence of 100 µM CCCP and the 1 % agarose pad was made with M9 with 100 µM CCCP.

2.5.5 Treating cells with nigericin and valinomycin

After growth in M9 to exponential phase OD₆₀₀ 0.4 (Section 2.5.2), cells were permeabilised using 1 mM EDTA pH 8 for 5 minutes, before treating with 5 µM nigericin (Sigma) or 5 µM valinomycin (Sigma) in the presence of 150 mM KCl for 5 minutes at room temperature. Cells were pelleted by centrifugation at 7000 xg for 3 minutes at 17 °C, the supernatant removed and pellet re-suspended in 200 µl M9 with nigericin or valinomycin and labelled with 1.5 µM fluorescently-labelled colicin and 20 µM Hoechst as described. All wash stages in M9 were in the presence of nigericin or valinomycin and the 1 % agarose pad was made with M9 with nigericin or valinomycin.

2.5.6 Treating cells with trypsin

After labelling cells for 30 minutes with fluorescently-labelled colicin and Hoechst, cells were washed three times to remove excess dye (Section 2.5.2). Cells were gently re-suspended in 200 µl M9 media with 50 µg/ml of fresh trypsin (Sigma) from its stock in 20 mM CaCl₂, 1 mM HCl pH 3, in a 2:1 colicin:trypsin ratio. Cells were incubated with trypsin for 1 hour at room temperature with rotary inversion, before washing three times in M9 as described above.

2.5.7 Spheroplast generation

Cells were grown to an OD₆₀₀ of 0.4, labelled with fluorescently-labelled colicin and Hoechst (Section 2.5.2), before treating with trypsin (Section 2.5.6). Cells were re-suspended in 400 µl ice-cold hypertonic 0.5 M sucrose in M9 pH 8, lysozyme (Sigma) was added at 150 µg/ml and incubated on ice for 5 minutes. Cells were then treated with 400 µl 10 mM EDTA pH 8 for 30 minutes at room temperature. Cells were pelleted by centrifugation at 3000 xg for 15 minutes, the supernatant was removed and the pellet re-suspended in a small volume ~10 µl of M9 media.

2.5.8 Total internal reflection fluorescence (TIRF) microscopy

Cells were grown and treated as in Section 2.5.2 and 2.5.6. and were visualised at room temperature using a 100x oil-immersion objective (NA 1.40, UPLSAPO, Olympus) on a custom-built TIRF microscope developed by the Kapanidis research group (Oxford). Brightfield transmission illumination imaging used an Olympus IX2 condenser and LED light source. A multi-laser engine was used (iChrome, Toptica) with 405 nm, 488 nm, 561 nm and 640 nm lasers as the excitation light sources. Laser excitation was controlled using Toptica iChrome software and image acquisition was visualised and controlled using Andor Solis software. The 405 nm laser line was used at ~10-15 % laser intensity, the 488 nm laser line was used at ~20-40 % laser intensity, the 561 nm laser line was used at ~30-40 % laser intensity and the 640 nm laser was used at ~30-40 % intensity. The illumination angle was optimised empirically between experiments. Fluorescence was recorded at room-temperature at a rate of 20 ms/frame under continuous excitation for 500 frames on an electron multiplying CCD (EMCCD) camera (iXon 897 Ultra, Andor, 512x512 pixels), with an image pixel size of 96 nm. The schematics of the optical configuration and details of the custom-built microscope can be seen in (Wegel et al. 2016).

2.5.9 TIRF tracking and data analysis

Fluorescently-labelled colicins were analysed and tracked from TIRF frames using a custom software called PaTrack (Dosset et al. 2016) and implemented in visual C++. The centre of each

fluorescence peak was determined with sub-pixel resolution by fitting to a two-dimensional elliptical Gaussian function. The software parameters for tracking were as follows; video rate of 0.02 s, resolution of 0.1 $\mu\text{m}/\text{px}$ (the size of one pixel), Dim Part. of 0.7 μm (the approximate size of the particle tracked), Max part. disp. of 0.7 μm (the maximum distance that any of the tracked particles are likely to cover between frames from the centre of the PSF), death of 3 frames (how many frames during which a tracked particle can be lost before tracking abortion), Short trajectories of 41 frames (the minimum number of consecutive frames that validates a trajectory) and an Eccentricity max. of 1.7 (A particle is considered as valid if its eccentricity is smaller or equal to this value. The 2D trajectories of the single molecules were tracked frame-by-frame. The analysis function of PaTrack plots the Mean Square Displacement (MSD) of the diffusing tracked molecules and the apparent diffusion coefficient was calculated from a linear fit to the MSD plot between the first and fourth time points using the equation $\text{MSD}(t) = 4Dt$. An algorithm based on a back-propagation neural network allows the automatic detection of Brownian, confined and direction motion types within the trajectories.

2.5.10 TIRF single-molecule photobleaching analysis

The single-molecule photobleaching data were generated from the frames collected using TIRFM. Using ImageJ, the molecule of interest was circled in the first frame of the video and the function Plot Z-axis Profile was used to generate a list of the pixel intensity for each frame, which was plotted in excel. This was performed for >30 molecules from >10 separate TIRF videos recorded at 20 ms/frame for ~500 frames.

2.5.11 3D Structured Illumination microscopy (3D-SIM)

Cells were grown in M9 minimal media to OD_{600} 0.4, before re-suspending in 400 μl 4 % PFA for 15 minutes at 4 °C with rotation to fix cells (Section 2.5.3). Cells were pelleted by centrifugation at 7000 $\times g$ for 3 minutes at 17 °C, the supernatant removed and pellet re-suspended in 200 μl M9 and labelled with colicin as above (Section 2.5.2). 3D-SIM was performed with CoIEG^{S-5 AF488}.

Cells were visualised at room temperature using a 60x oil-immersion objective (Oil UPlanSApo) (NA 1.42) on an OMX V3 BLAZE (GE) optical microscope with Deltavision OMX software. The 488 nm (FITC filter) laser line was used at 10-31.5 % laser intensities and an exposure of 30 ms. Cell sample thickness was set to 3 μm , with 25 optical Z-sections and therefore an optical section spacing of 0.125 μm . Five illumination phases are recorded at three angles of 60° (180° total) with a total of 15 recorded images per Z-section. This is repeated for the entire cell thickness at each optical section through the Z-axis to generate 3D image stacks. Fluorescence was detected on a sCMOS camera.

The cell image stacks were reconstructed using DeltaVision OMX SoftWoRx 6.1.1 software with a Wiener filter of 0.002 using wavelength specific experimentally determined OTF functions (Gustafsson et al. 2008). This leads to a halving of the pixel size and a doubling of the X and Y direction pixel number (i.e. four times as many pixels), but no change in Z-steps in the image stacks. This generates 3D image stacks at 488 nm excitation with a lateral (x-y) resolution of ~ 120 nm and an axial (z) resolution of ~ 340 nm.

2.5.12 Widefield microscopy and image analysis

Cells were grown and treated as in Section 2.5.2 and Section 2.5.6. and were visualised at room temperature using a 60x oil-immersion objective (Oil UPlanSApo) (NA 1.42) on an OMX V3 BLAZE Optical microscope with Deltavision OMX software. The 405 nm (DAPI filter), 488 nm (FITC filter), 593 nm (615/24 filter) or 642 nm (Cy5 filter) laser lines were used. Lasers were used at 10-31.5 % laser intensities and an exposure time of 30 ms. Single images were taken or videos were recorded at a frame rate of ~ 30 ms/frame for 100-500 frames. The resolution of this microscope based on the Rayleigh Criterion using AF⁶⁴⁷ dye is ~ 288 nm.

All widefield data in the fluorescence channels (405 nm, 488 nm, 593 nm and 642 nm) were smoothed using the ImageJ function and had the brightness and contrast altered to enhance visualisation and to normalise the data between experiments. Brightness and contrast was set at a minimum of ~ 100 units/pixels to remove background.

The distribution of fluorescence intensity across the width of the bacterial cells was determined in ImageJ. A line of 2.5 μm was drawn across the width of the cell and the thickness of the line was made to be the length of the cell to avoid bias. The ImageJ function using analyse plot profile, provided the distance across the width of the cell against pixel intensity, which was plotted in excel. This was performed for 15 cells for each condition and the average was plotted with the standard error of mean (SEM) as the error bars.

The number of single colicin molecules per cell was determined from the widefield data by smoothing the data and setting the brightness and contrast of the images in each fluorescence channel to a minimum pixel intensity of ~ 100 units/pixels, as described above to normalise the data. The fluorescence dots present above ~ 100 units/pixels were counted as a single molecule. The number of dots were counted visually, if molecules were in close proximity and their airy discs overlapped, only those at the resolution limit or above were resolved and recorded as two molecules. Anything overlapping more than this was recorded as a single molecule. The resolution limit of this microscope based on the Rayleigh Criterion using AF⁶⁴⁷ dye is ~ 288 nm.

An additional method to compare and validate the number of molecules between different cell conditions was through pixel intensity. Using ImageJ, cell outlines were drawn around, then using the functions -analyse and -measure, allowed the calculation of the mean of each cell intensity, which was plotted as a box and whisker plot. This was performed on 50 cells per condition.

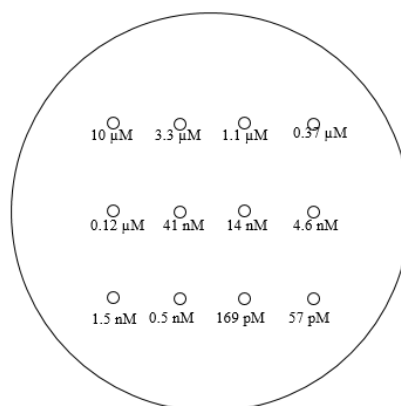
2.6 Colicin cytotoxicity and activity assays

2.6.1 Plate killing assay

The plate contained a 20 ml bottom layer consisting of 1.5 % (w/v) agar in LB, containing the appropriate antibiotics. *E. coli* cells, containing plasmids as indicated in results, were inoculated into 10 ml LB and grown at 37 °C until OD₆₀₀ ~ 0.4 from an overnight culture. 5 ml of 0.75 % agar

in LB was added to 200 μ l of cells, mixed by inversion and poured on top of the LB layer of the plate and allowed to cool and dry.

Three-fold serial dilutions of colicin were made, spanning a concentration range of 10 μ M to 57 pM. Once the soft agar was dried, 2.5 μ l of each of the 12 serial dilutions was spotted onto the plate, allowed to dry and incubated at 37 °C for 16 hours overnight. Colicin induced cell killing was indicated as zones of clearance in the bacterial lawn.



2.6.2 Liquid culture killing assay

E. coli cells, containing plasmids as indicated in results, were inoculated into 10 ml LB and grown at 37 °C until OD₆₀₀ ~0.4, containing the appropriate antibiotics. 80 nM colicin was added and the growth of each culture was monitored by OD₆₀₀ at 0, 5, 10, 15, 20, 25, 30, 40, 60, 80, 100, 120, 180 minutes and every hour up to 520 minutes incubation with colicin.

2.6.3 Plasmid nicking assay

pUC19 DNA was used at 1 μ g/ml in 50 mM triethanolamine (TEA) (Fisher Scientific) pH 7.5 with 20 mM MgCl₂. The reaction was performed at room temperature and a 5 μ l sample was taken in the absence of colicin. 200 nM of ColE9 DNase was added and 5 μ l samples were taken at time-points of 0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 minutes, the reactions were quenched using 37.5 mM EDTA pH 8. 2 μ l of DNA loading dye were added and samples were run on a 1.2 % agarose gel in 0.5x TBE for 1 hour at 80 V.

2.6.4 Bioluminescence assay

All media and plasticware used was pre-warmed to 37 °C to minimise stress on the cells and thereby induction of the SOS response. *E. coli* DPD1718 cells were inoculated from an overnight culture into 10 ml LB and grown at 37 °C until OD₆₀₀ ~0.4, before diluting 1:2 in LB in 100 μ l into a black Nunc microwell 96 well optical bottom polystyrene plate. Cells were diluted so that they

were present in exponential phase of growth but at a low density to follow the effect of ColE9 on growth for longer. ColE9 was used at 0, 0.5, 1, 2, 20 and 200 nM. The plate was loaded into a plate incubator at 37 °C with gentle shaking using a Freedom Evo 150 robot, before being loaded into a Tecan Infinite M200 Pro plate reader. The plate was monitored every 10 minutes for a total of 4 hours for cell density at OD₆₀₀ and induction of bioluminescence at OD₄₉₀. SOS stress controls were performed using 0.6 µg/ml mitomycin C (Sigma), exposure to UV for 2 and 5 minutes in a hood using four G15T8 GERMICIDAL 18 15W T8 UVC bulbs, 2 mM H₂O₂ (Sigma), and exposure to 40°C for 10 minutes.

2.6.5 Inner membrane charge induction killing assay

E. coli HDL11 cells were inoculated from an overnight culture into 10 ml M9 minimal media and grown at 37 °C until OD₆₀₀ ~0.4 in the presence of 0, 20, 30, 40 and 50 µM IPTG. 80 nM colicin was added and the OD₆₀₀ was measured every 30 minutes for 330 minutes.

2.7 Kinetics of cell killing

E. coli JM83 cells were grown in LB to OD₆₀₀ ~0.4 (~10⁸ cells) at 37 °C, 80 nM of colicin was added to a 25 ml culture and incubated at 37 °C. After 0, 5, 10, 15, 20, 25, 30, 40, 60, 80, 100, 120, 180, 240, 300 and 360 minutes incubation with colicin, a 900 µl sample of culture was taken and added to 100 µl of trypsin at 1 mg/ml in a stock buffer of 20 mM CaCl₂, 1 mM HCl pH 3. Samples were further incubated with the trypsin at 37 °C for 30 minutes. 100 µl of each time point were diluted in LB between 10⁰ and 10⁶-fold, before plating 90 µl of a range of dilutions on LB-agar plates with the appropriate antibiotics, in triplicate, and incubated for 16 hours at 37 °C. Colonies were counted and averaged from the triplicate plates. The number of CFUs were calculated using the equation below:

$$\text{CFU} = \frac{\text{number of colonies (from average of triplicate)} \times \text{dilution factor (from } 10^8 \text{ starting culture)}}{\text{Volume plated}}$$

As it was a first-order reaction, the Ln of each CFU value was plotted against time (mins). The data were fitted to the equation of a straight line to obtain the rate constant (k) of the reaction.

From the equation of the straight line $y = kx + c$, where k, the gradient, is the rate constant (k).

The half-life of the reaction in cell killing kinetics are quoted as $t_{1/2}$ values, where $t_{1/2} = \ln 2/k$

For JW2203 and JW0912 strains, cells were grown to OD₆₀₀ 0.4, they were treated with 20 µg/ml chloramphenicol to inhibit cell division, before treating with 80 nM colicin.

3 Chapter 3 - Development of a fluorescence-based assay to dissect the entry mechanism of ColE9 translocation

3.1 Introduction

Of the bacteriocins, colicins are currently the most studied and thereby best understood. There has been a wide range of techniques used and developed that have proved invaluable in contributing to our understanding of their structure, function and mechanism of action (Section 1. 5 and 1. 6). The *in vivo* activity of colicins has been studied in killing assays. Through deletion or mutation of the colicin and *E. coli* strains used, these methods have provided information on features that are important or essential for the binding, translocation and cytotoxicity, the rate at which it occurs and its cellular location. Many of the individual protein-protein interactions have been dissected *in vitro* through various biophysical techniques. These methods have provided detailed information on molecular interactions, their stoichiometry and affinity.

Past *in vivo* methods that have been used to study colicin translocation include indirect approaches, like the detection of K⁺ efflux using a K⁺-selective electrode on addition of the pore-former ColA (Bourdineaud et al. 1990). The kinetics of ColA translocation and the factors that affected this, including colicin concentration, pH, temperature, membrane potential and conformational changes through urea denaturation and disulphide bond engineering, were followed by the release of K⁺ into the surrounding media (Bourdineaud et al. 1990) (Benedetti et al. 1992) (Duche et al. 1994).

Regions of colicin still accessible at the cell surface after receptor binding, were identified through the introduction of enterokinase cleavage sites in ColE9 (Zhang et al. 2008). In addition, the use of trypsin against ColA bound to cells, showed that domains of ColA remained at the surface after translocation which was necessary for cytotoxicity and to maintain the pores, measured by potassium efflux (Benedetti et al. 1992).

Another method used was an eGFP-Im2 fusion complex with ColE2 and detection of Im2 release by western blot analysis. Conditions for Im2 release were studied, including the requirement of a functional translocon and Tol system (Duche et al. 2006). Using a similar approach, conditions for the release of Im9 from a disulphide-locked ColE9 (ColE9^{S-S}), were studied using Im9-AF⁵⁹⁴ and detection of relative fluorescence units (RFU) on its release into the surrounding supernatant. ColE9 binding to BtuB was not sufficient for Im9 release and required an unfolding of the colicin within the R-domain (Zhang et al. 2008), a complete network of ColE9 interactions with the Tol system and use of the PMF (Vankemmelbeke et al. 2009).

An SOS-inducible lux reporter strain has been used as a method to probe the mechanism and rate of DNA damage by the emission of bioluminescence on addition of ColE9 and its translocation to the cytoplasm (Vankemmelbeke et al. 2005). In addition, the use of radioactively-labelled DNA in *E. coli* cells has been used to follow the rate of entry and DNA degradation by ColE2 (Nose and Mizuno 1968).

These methods are indirect and are unable to follow colicin translocation in real-time at the single-cell single-molecule level *in vivo*. To answer some of the many outstanding questions left to address and to further enhance our understanding of this system, it requires the development of a new technique.

Fluorescent proteins like GFP are currently widely used in fluorescent microscopy. They are generally bright, non-toxic, environment insensitive and have little non-specific binding, depending on the fluorescent protein. This makes them very useful tools for time-course microscopy and tracking. However, they have low photostability, they need to be fused to the protein of interest through genetic engineering and usually attached at the N-termini or C-termini of the protein. They can often affect or inhibit the structure or function of the protein. In addition, some can have long maturation times and they are also quite large at ~3 nm wide and ~27 kDa (Jensen 2012) (Crivat and Taraska 2012) (Sustarsic and Kapanidis 2015). They are

usually used to study intracellular processes (e.g. cell division), or cell surface processes, however they would be unable to translocate across the cell membrane. This is because in their mature form, their molecular mass and dimensions are essentially equivalent to the OM porins that they would need to pass through to cross the OM and they would be unable to cross the IM (White et al. 2017).

Synthetic fluorescent dyes are relatively bright, photostable, insensitive to a range of pH values 4-10, have well differentiated excitation and emission spectra and are commercially available in a wide range of colours, sizes and methods of conjugation. In addition, they can be easily conjugated anywhere on the protein of interest through genetic engineering and are much smaller than fluorescent proteins (Invitrogen 2010b) (Berlier et al. 2003). The solute exclusion size of OMPs like OmpF have been shown to be ~600-700 Da (Nikaido and Vaara 1985). Given the problems of fluorescent proteins to look at colicin import, synthetic dyes offered a potential route forward. However, the molecular masses of the most commonly used dyes (AlexaFluor) are 720-1250 Da and above the cut-off for porins. Hence it was unclear if translocation of the labelled colicin would occur.

Previously, research was performed using ColE3 fluorescently-labelled at position K549C within the cytotoxic rRNase domain with Org⁴⁸⁸, which is in a similar location to position K469C in ColE9 (Rassam et al. 2015). The purpose of this construct was to develop a FRET pair with a fluorescent label in the T-domain to look at colicin unfolding on receptor binding but was not used for following translocation into the cell (Zakharov et al. 2008). Recently, research has also been performed using fluorescently-labelled pyocins. Pyocin S2 R-domain was fluorescently-labelled with AF⁴⁸⁸ (pyoS2^{NTD}-AF488). The purpose of this construct was to study its binding and the requirements for TonB-dependent import into *Pseudomonas aeruginosa* cells (White et al. 2017).

Another example of a bacteriocin being fluorescently-labelled is Plantaricin EF (PlnEF), a two-peptide bacteriocin that targets Gram-positive *Lactobacillus plantarum*. PlnEF was labelled with FITC dye for the purpose of studying its mode of action. Its rapid accumulation in cells observed by microscopy, followed by the uptake of the usually membrane impermeable dye propidium iodide (PI), was used to visualise the rapid damage to the cell membrane caused by PlnEF, thereby determining its cytotoxic activity against cells (Zhang et al. 2016).

Fluorescently-labelled ColE9 has previously been used to probe the organisation of the outer membrane with small synthetic dyes (Rassam et al. 2015), as described in Chapter 1 Section 1. 7. In the present work, similarly labelled colicin constructs were used to develop a fluorescence-based microscopy technique for probing the mechanism of translocation through the cell envelope. If successful, this could allow direct visualisation of single-colicin molecules at the single-cell level, using live *E. coli* cells.

The aim of this chapter was to develop such a fluorescence-based microscopy assay, to monitor the translocation of ColE9 into the cytoplasm of live *E. coli* cells. The chapter concerns the development of a fluorescently-labelled ColE9 for import assays and in particular *in vivo* and *in vitro* characterisation of binding and cytotoxicity.

3.2 Results

3.2.1 Expression, purification and fluorescent labelling of ColE9 using AlexaFluor-maleimide dyes AF⁴⁸⁸, AF⁵⁶⁸ and AF⁶⁴⁷

Identification of the optimal location for fluorescent labelling on ColE9 DNase

The DNase domain of ColE9 has previously been extensively mutated to produce the single cysteine mutations at positions S451C, K469C, S478C, S528C and S556C suitable for the attachment of fluorescent dyes (Lewin 2002) (White et al. 2007) (Farrance et al. 2013) (Rassam et al. 2015). In addition to this, an undergraduate student supervised during the course of this work (Boonyaporn Chinthammit), mutated residue S510C (Figure 3. 1). The locations of these residues were chosen due to their surface exposure for ease of fluorescent labelling and their lack of involvement in ColE9 DNA binding and catalysis or the binding of Im9. Boonyaporn Chinthammit purified and fluorescently labelled these ColE9 constructs as isolated DNases with AlexaFluor647 dye (AF⁶⁴⁷) using the same method as full-length ColE9 (Section 2.3.5). These constructs were assayed for activity and results are described in the undergraduate project report of Boonyaporn Chinthammit. Labelling efficiencies for all the constructs was ~80-100 %, except for ColE9 DNase S451C^{AF647}, which had a labelling efficiency of 22 % and was therefore discarded. *In vitro* plasmid nicking assays were performed using these fluorescently-labelled constructs and compared to wild-type ColE9 DNase (Section 2.6.3). ColE9 DNase S556C^{AF647} was the slowest at nicking with very little linear DNA generated within 100 minutes. This was followed by ColE9 DNase S510C^{AF647} and ColE9 DNase S478C^{AF647}, which generated linear DNA but did not reach completed digestion within 100 minutes. ColE9 DNase S528C^{AF647} completely digested the linear DNA by ~20-25 minutes. The most efficient however was ColE9 DNase K469C^{AF647}, which completely digested linear DNA in ~15 minutes, which was comparable to wild-type (Boonyaporn Chinthammit, data not shown). *In vivo* bioluminescence assays as a reporter of DNA damage, was also performed using full length ColE9 S528C and ColE9 K469C

labelled with AF⁶⁴⁷ (Section 2.6.4). Data indicated that both labelled constructs were poor at translocating and damaging DNA and there was very little difference between the two constructs *in vivo* (Boonyaporn Chinthammit, data not shown). Plate killing assays also confirmed that ColE9 K469C^{AF647} was more efficient at killing than ColE9 S528C^{AF647} (Boonyaporn Chinthammit, data not shown). These preliminary data suggested K469C was the best location for fluorescent labelling of ColE9, with minimal perturbation to its DNA binding and catalysis functions.

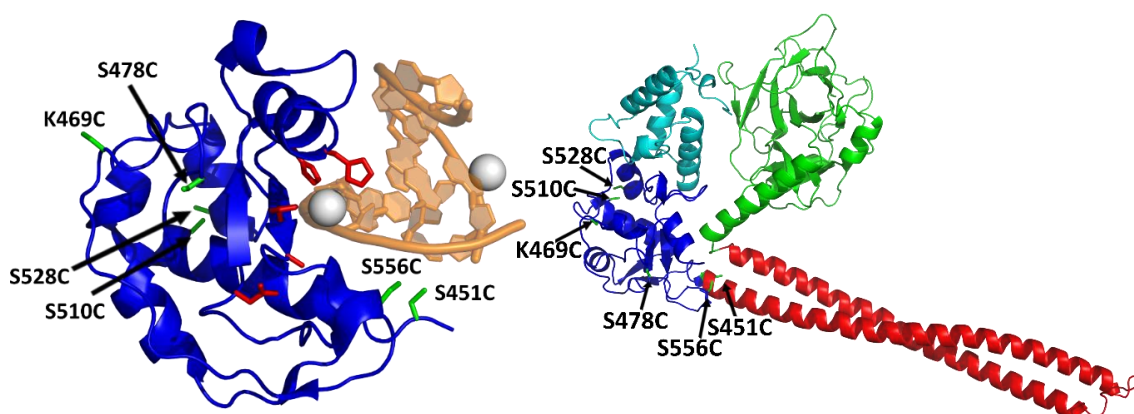


Figure 3. 1 - Location of potential cysteines tested for labelling with AlexaFluor 647.

ColE9 DNase domain (blue) bound to DNA (orange) with the locations of the catalytic HNH motif (red), Mg²⁺ ion (grey sphere) and the locations of all cysteine mutants tested (green sticks). For the full-length ColE9, the T-domain (green), R-domain (red) C-domain (blue) and Im9 (cyan) are shown (PDB; ColE9 DNase- 1V14, full-length ColE9-5ew5).

Based on the results described above, ColE9 K469C within the cytotoxic DNase domain, was selected for labelling with synthetic fluorescent dyes (Figure 3. 2). This is the only cysteine present in ColE9, to ensure labelling is selective.

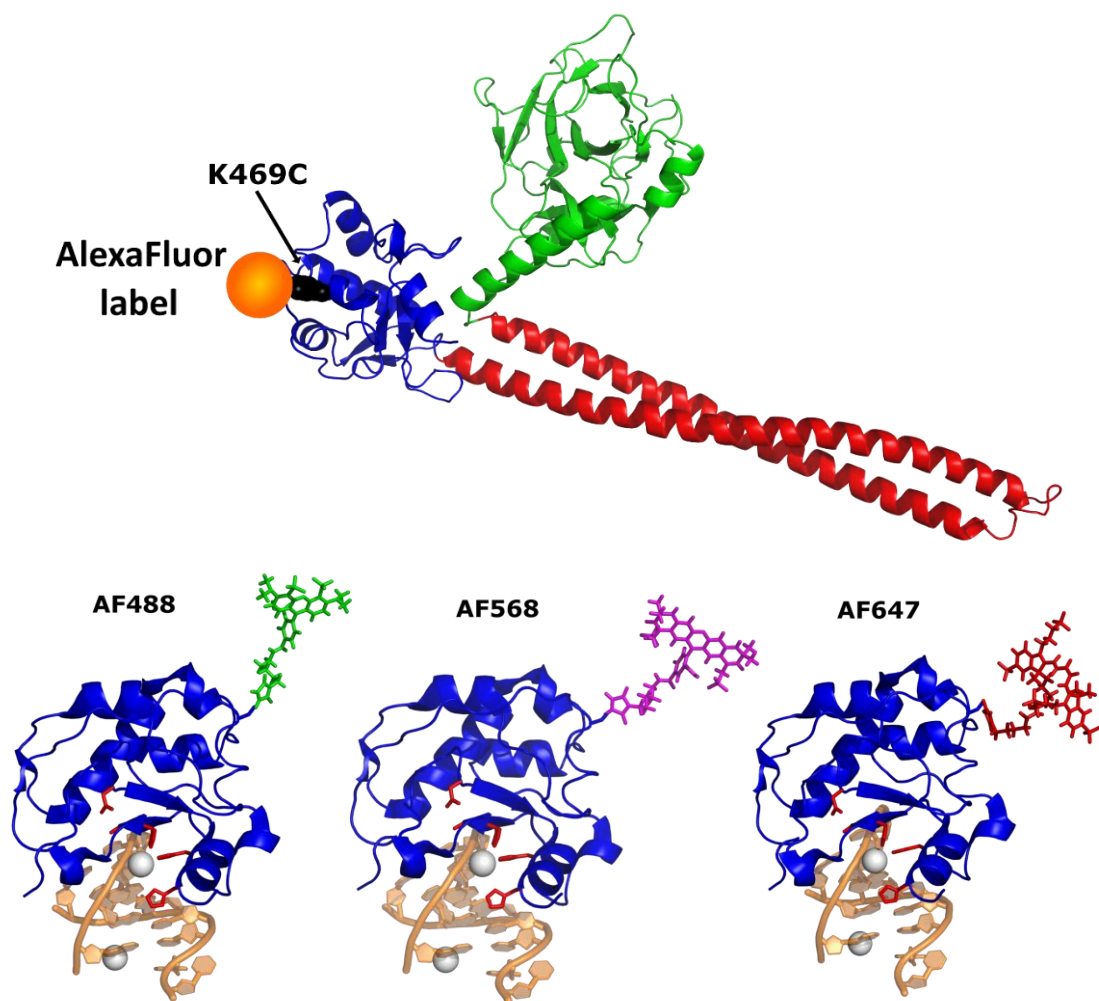


Figure 3. 2 - ColE9 mutated at K469C within the cytotoxic DNase domain for labelling with synthetic fluorescent dyes.

(Top) ColE9 was mutated within the cytotoxic DNase domain (blue) from lysine (K) K469 to a cysteine (C) (black sphere) for labelling with the AlexaFluor dyes (orange sphere) via maleimide conjugation. The T-domain (green) and R-domain (red) are shown. (Bottom) The DNase domain bound to DNA, with the 3D structure of each fluorophore used, AF488, AF568 and AF647, for size comparison.

Expression and purification of ColE9

ColE9-Im9 complex with a C-terminal hexahistidine tag on Im9, was expressed from an IPTG-inducible pET-21a(+) plasmid in *E. coli* BL21 (DE3) cells (Section 2.3.2). The complex was loaded onto a nickel affinity chromatography column and ColE9 was eluted from Im9 by a guanidine denaturation step elution (Figure 3. 3 A and B).

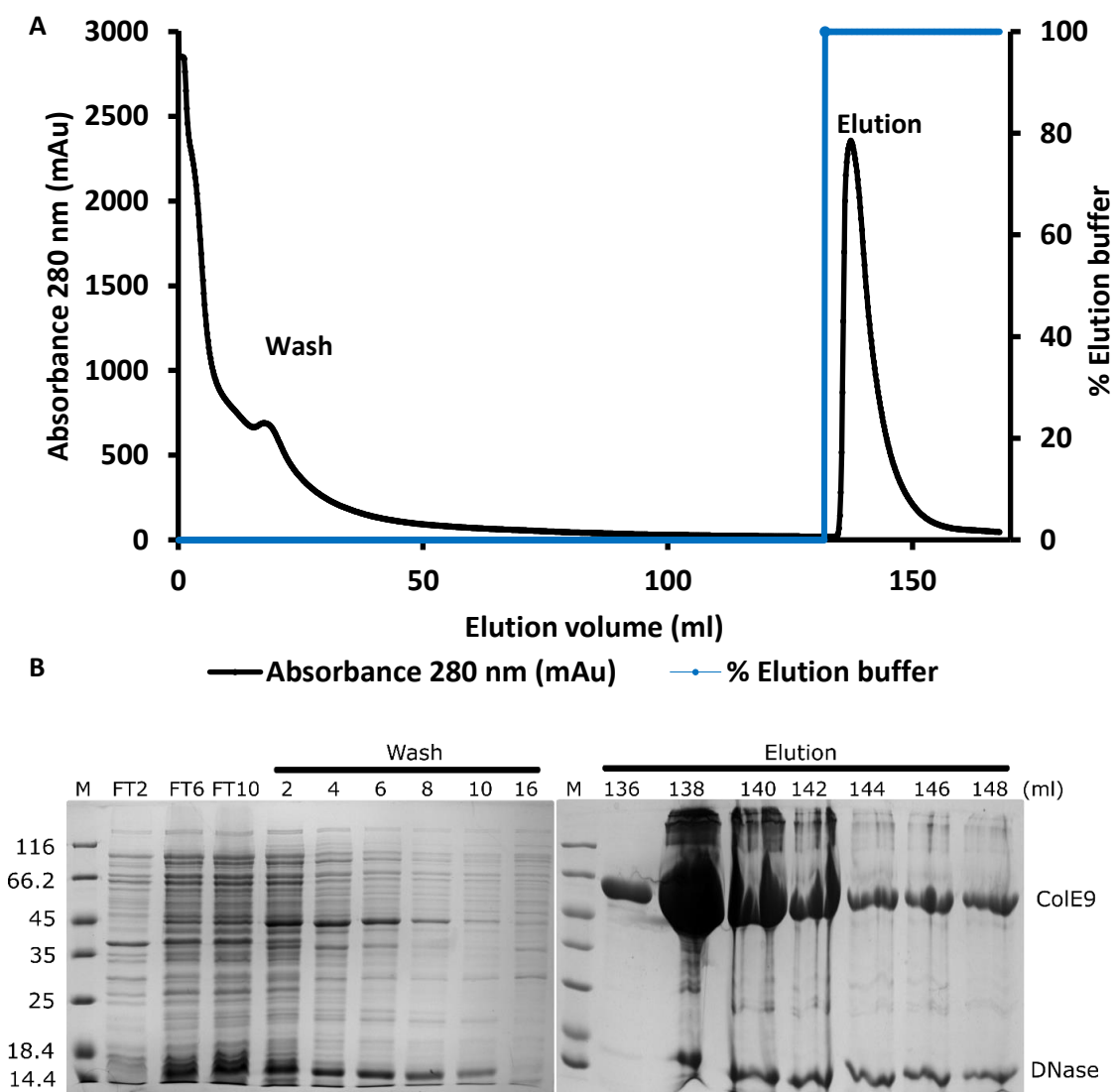


Figure 3. 3 - ColE9 HisTrap FF HP nickel affinity chromatography purification.

(A) Nickel affinity purification trace for ColE9 (Section 2.3.2). (B) Fractions of the flow-through (FT), wash and elution of ColE9 were run on 12 % SDS-PAGE to visualise ColE9 and demonstrate its purity (Section 2.3.1). Fractions 136-148 ml from the elution were pooled and concentrated. M stands for Marker.

ColE9 was refolded by dialysis and further purified by size-exclusion chromatography (Figure 3. 4 A and B).

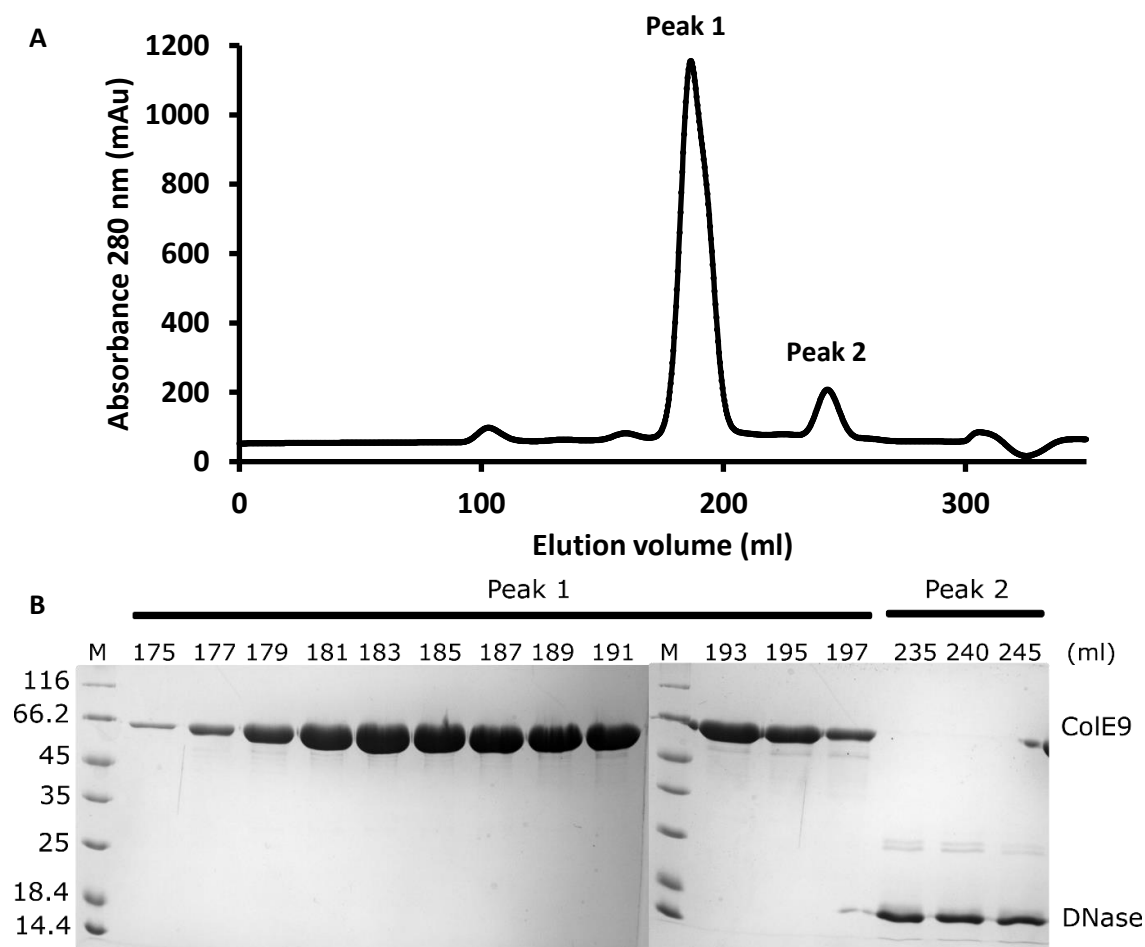


Figure 3. 4 - ColE9 size-exclusion chromatography purification.

(A) Size-exclusion purification trace for ColE9 (Section 2.3.2). (B) Fractions of the elution were run on 12 % SDS-PAGE to visualise ColE9 and demonstrate its purity (Section 2.3.1). Peak 1 represents ColE9, Peak 2 represents DNase. Fractions 175-197 ml from the elution were pooled and concentrated. M stands for Marker.

ColE9 K469C was labelled with AlexaFluor dyes AF⁴⁸⁸, AF⁵⁶⁸ or AF⁶⁴⁷ via maleimide conjugation chemistry (Section 2.3.5), the structures of the dyes used can be seen in Figure 3. 5 A-C, respectively. ColE9 was labelled with three different dyes with varying properties and wavelength emissions, to identify the optimal dye with which to develop a fluorescence-based assay. The maleimide group of the dye reacts with the deprotonated sulfhydryl group of the surface-exposed cysteine residue between pH 6.5 and 7.5, to form an irreversible thioether bond through alkylation (Figure 3. 5 D).

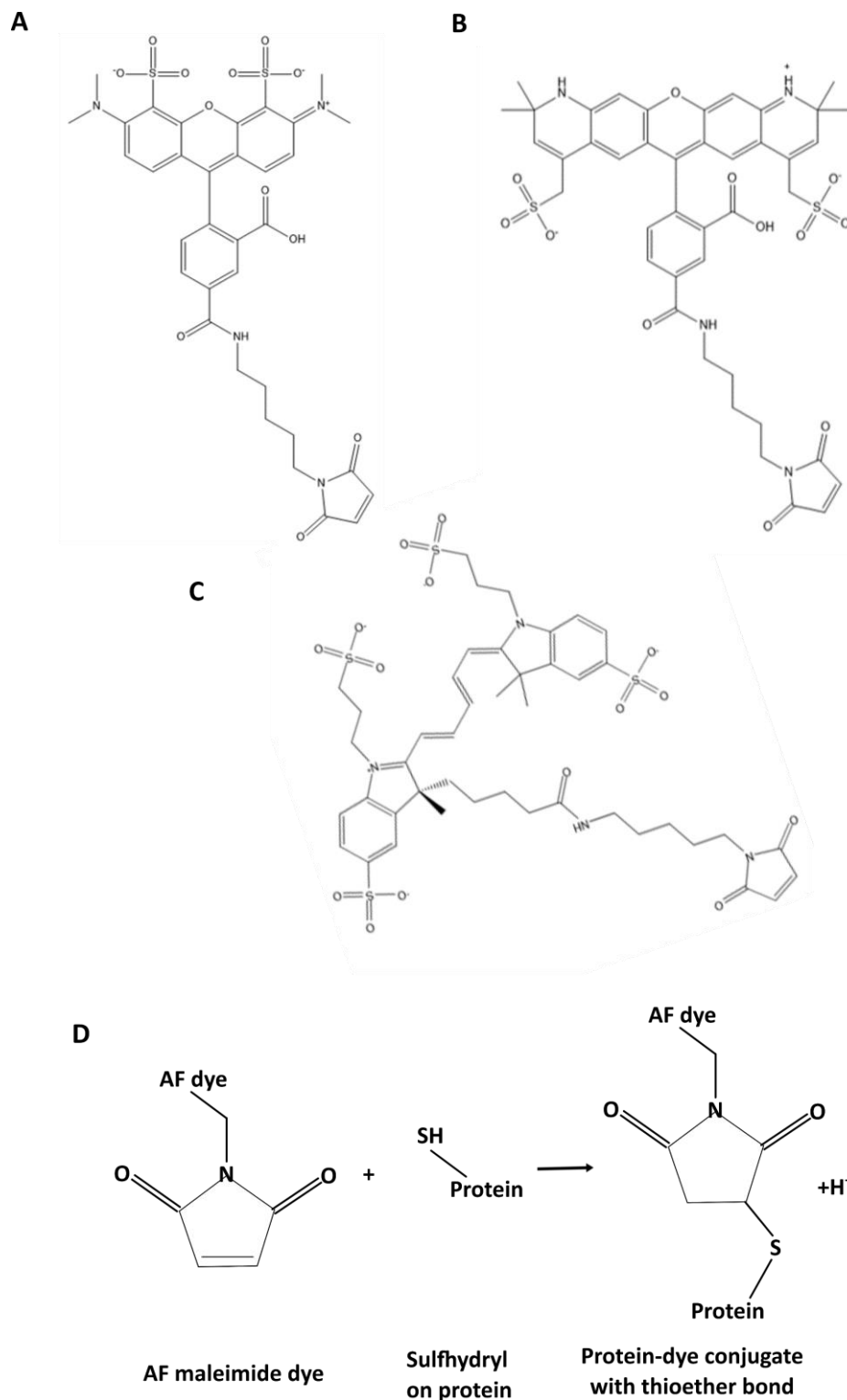


Figure 3. 5 - The Structure of AlexaFluor (AF) maleimide dyes AF⁴⁸⁸, AF⁵⁶⁸ and AF⁶⁴⁷ and the Maleimide conjugation chemistry.

AF⁴⁸⁸ C₅-maleimide (A), AF⁵⁶⁸ C₅-maleimide (B), AF⁶⁴⁷ C₂-maleimide (C). The maleimide reaction chemistry (D) involves the maleimide group of the AF dyes conjugating to the sulfhydryl (-SH) group of the cysteine in ColE9 via alkylation, to form an irreversible thioether bond.

An example of the purification of AF⁶⁴⁷ fluorescently-labelled ColE9 K469C is shown in Figure 3. 6 A. Size-exclusion was used to remove excess dye (peak 2) from the labelled colicin (peak 1) (Section 2.3.5). Figure 3. 6 B shows selected fractions from the purification trace run on 12 % SDS-PAGE gel to visualise labelled protein (peak 1) and excess dye (peak 2) by UV and protein by Coomassie blue staining (Section 2.3.1). Fluorescently-labelled proteins are called ColE9^{AF488}, ColE9^{AF568} or ColE9^{AF647}.

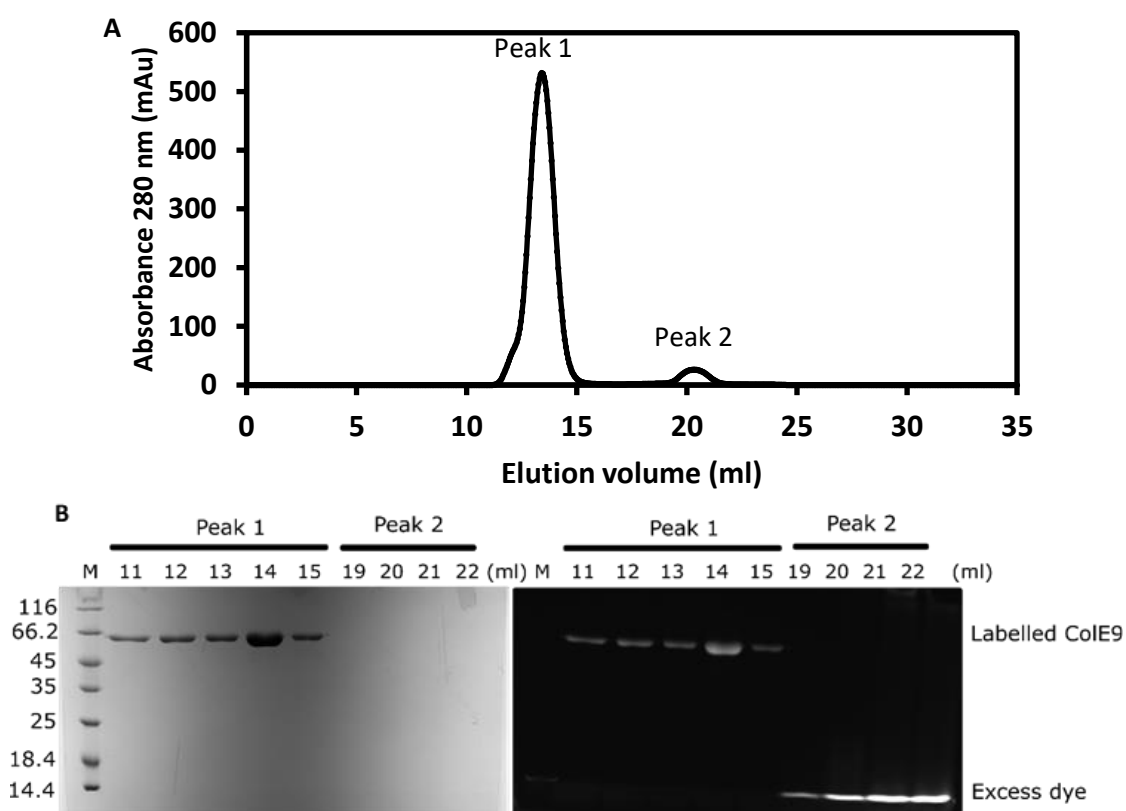


Figure 3. 6 - Purification of AlexaFluor-647 labelled ColE9 K469C.

(A) fluorescent labelling size-exclusion purification trace for ColE9 K469C AF⁶⁴⁷ (Section 2.3.5). (B) Fractions of peak 1 and peak 2 were run on a 12 % SDS-PAGE gel and visualised by UV (right) and Coomassie (left) to identify labelled protein from excess dye (Section 2.3.1). An example elution profile, UV and Coomassie gel of ColE9 is shown.

Protein concentrations and fluorescent labelling efficiencies were determined spectrophotometrically by UV-VIS (Section 2.3.6). An example spectrophotometry profile of ColE9^{AF647} can be seen in Appendix I Figure 3. Labelling efficiencies were typically >90 %. Labelled ColE9 samples were verified using ESI-MS (Appendix 1 Figure 2) (Section 2.4.1). The expected

and observed molecular weights (Appendix I Table 1) are consistent with ColE9 being labelled with one fluorescent dye.

3.2.2 Fluorescently labelled ColE9 retains *in vivo* cytotoxicity

Based on the work of Boonyaporn Chinthammit in Section 3.2.1, position K469C was identified as the optimal location for fluorescent-labelling of ColE9 with AF⁶⁴⁷ dye. Compared to the other positions tested, K469C had the least impact with AF⁶⁴⁷, *in vitro* and *in vivo*. However, the reason for the reduced *in vivo* activity compared to wild-type ColE9 was not investigated. In addition, the effect and optimization of other fluorescent-dyes at position K469C was not explored.

The first question to address using the fluorescently-labelled ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} constructs, was if they have retained their cytotoxicity against *E. coli* cells. This was investigated by plate killing assays using *E. coli* JM83 cells (Section 2.6.1), with wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} (Figure 3. 7). The clear circular zones on the bacterial lawn represent cell growth inhibition and therefore death by the colicin. The plates therefore showed that all of the colicin constructs, wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647}, were capable of causing cell death as seen by the presence of circular clear zones. The plate for ColE9^{AF568} had the contrast enhanced to observe circular clear zones and is shown in Figure 3. 7, as the plate taken at the same contrast and enhanced contrast (inset).

This result suggested that fluorescently-labelled ColE9 is still capable of translocating into live *E. coli* cells to cause cell death, however, cytotoxicity is reduced. *E. coli* JM83 cells were sensitive to ColE9 down to ~57 pM, ColE9^{AF488} down to ~0.5 nM (~8.8x reduction), ColE9^{AF568} down to ~0.12 µM (~2100x reduction) and ColE9^{AF647} down to ~4.6 nM (~81x reduction).

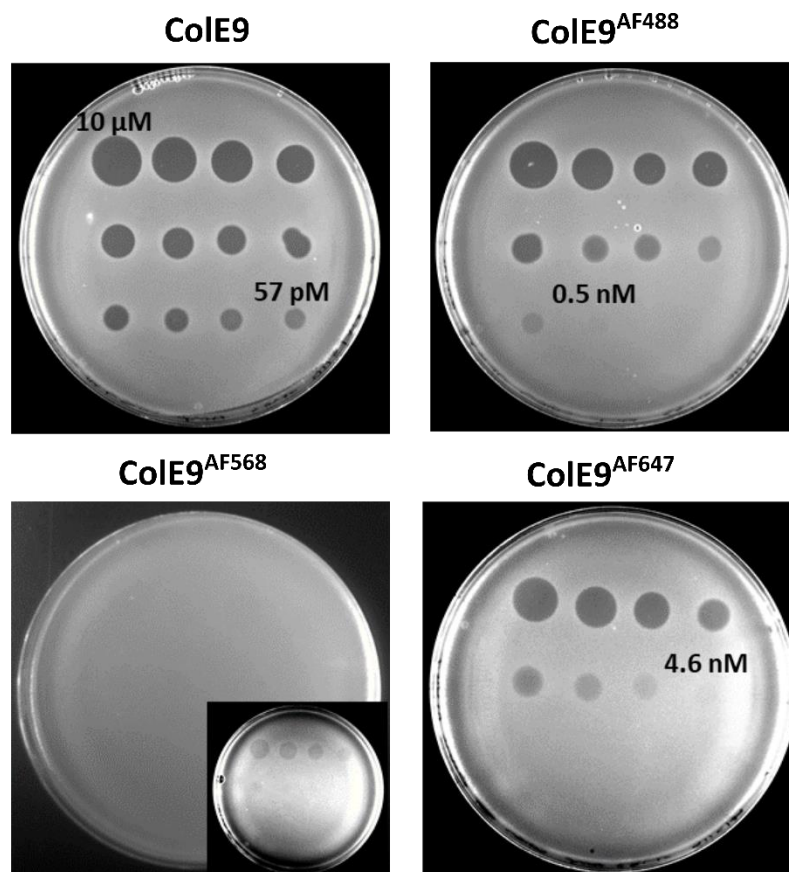


Figure 3. 7 - *In vivo* plate killing assay comparison of wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647}.

E. coli JM83 cells were used to perform plate killing assays, using wild-type ColE9 or fluorescently-labelled ColE9 from 10 μ M in 3-fold serial dilutions to 57 pM (Section 2.6.1). Colicin induced cell killing is indicated as zones of clearance in the bacterial lawn. JM83 cells were sensitive to ColE9 down to $\sim$ 57 pM (top left), ColE9^{AF488} down to $\sim$ 0.5 nM (top right), ColE9^{AF568} down to $\sim$ 0.12 μ M (bottom left) and ColE9^{AF647} down to $\sim$ 4.6 nM (bottom right), showing fluorescent-labelling of ColE9 has an impact on cytotoxicity *in vivo*.

It was unclear from this result if the presence of the AlexaFluor fluorescent dyes and the different properties of those dyes (Figure 3. 5 and Table 3. 1), were affecting either the cytotoxic nuclease activity of ColE9, or the ability of ColE9 to translocate into *E. coli* across the OM, reducing the level of killing.

Table 3. 1 - Properties of synthetic fluorescent AlexaFluor dyes.

The molecular weight, charge, theoretical extinction coefficient, quantum yield and fluorescence lifetimes are published by the manufacturer ThermoFisher (Invitrogen 2010a), the dimensions (width, height and depth) are approximate calculations from the structure imported into PyMOL.

	AF488	AF568	AF647
X-axis (width) nm	1.32	1.57	1.63
Y-axis (height) nm	1.58	1.23	1.9
Z-axis (depth) nm	1.86	1.69	1.15
mW (Da)	720.66	880.92	1250
Charge	-2	-2	-3
ϵ Theoretical extinction coefficients ($M^{-1} cm^{-1}$)	72,000 (at 494 nm)	92,000 (at 575 nm)	265,000 (at 651 nm)
Quantum yield (Φ)	0.92	0.69	0.33
Fluorescence lifetime (τ ns)	4.1	3.6	1

3.2.3 Fluorescently labelled ColE9 retains enzymatic nuclease activity *in vitro*

The synthetic fluorescent dyes used in these experiments clearly had an effect on the *in vivo* activity of ColE9, by reducing the cytotoxicity. To investigate if the fluorescent dyes had an effect on the *in vitro* activity of ColE9, a plasmid nicking assay was performed (Section 2.6.3).

The DNase domain of ColE9 was purified using the same method as full-length ColE9 and fluorescently-labelled to generate ColE9 DNase^{AF488}, DNase^{AF568} and DNase^{AF647} (Section 2.3.2 and 2.3.5). A plasmid nicking assay was performed using wild-type ColE9 DNase and the fluorescently-labelled ColE9 DNase constructs (Figure 3. 8). The DNA was present in three forms; a tightly-supercoiled form (S), open-circular form (C) and a linear form (L). These represent the progressive nicks to the DNA by the nuclease and their different migration properties (Pommer et al. 1998).

A comparison of these gels by wild-type ColE9 DNase, DNase^{AF488}, DNase^{AF568} and DNase^{AF647} (Figure 3. 8), showed that the rate of DNA nicking and hydrolysis by the fluorescently-labelled

DNase domains was the same as that of wild-type DNase. The circular form of DNA was completely hydrolysed by 10 minutes and the linear form was completely degraded into smaller fragments by 20 minutes for all of the DNase domains. By ~90 minutes, essentially all of the DNA is degraded into small fragments which run as a smear. This result is in agreement with previous experimental data using nicking assays performed with ColE9 (Pommer et al. 1998) (Pommer et al. 2001).

This result therefore showed that the presence of a fluorescent label on ColE9 and the different properties of those labels did not have an effect on the *in vitro* nuclease activity of ColE9.

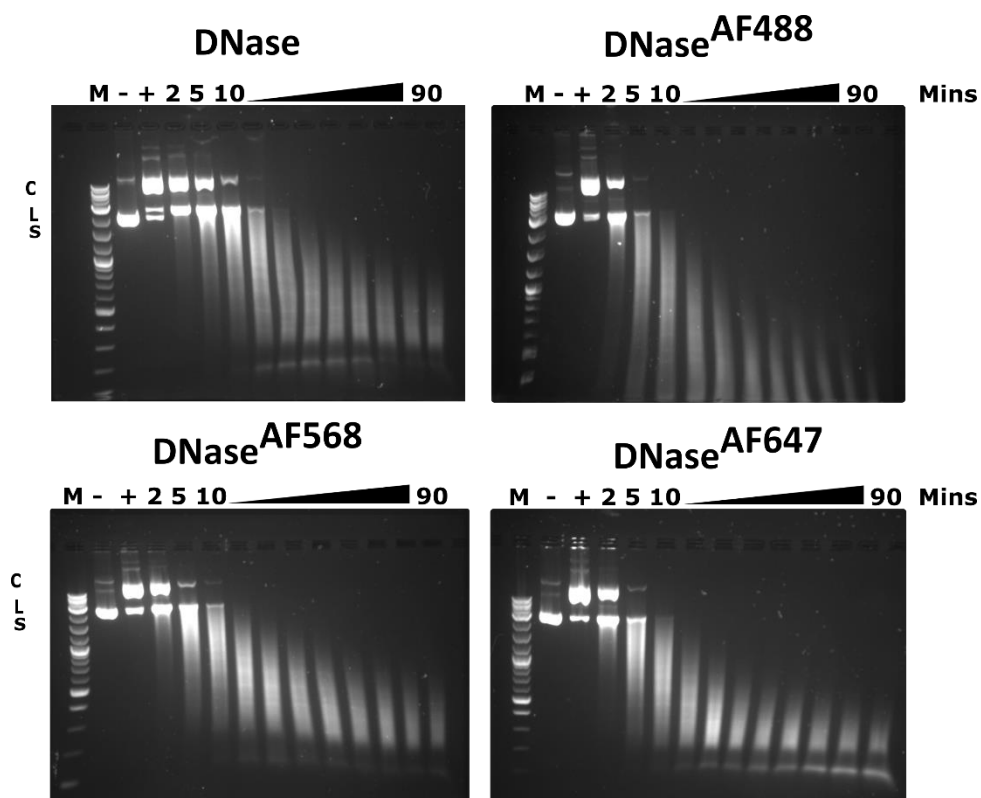


Figure 3. 8 - Plasmid nicking assay of pUC19 DNA by ColE9 DNase domain, DNase^{AF488}, DNase^{AF568} and DNase^{AF647}.

Plasmid nicking assay was performed using wild-type ColE9 DNase domain, DNase^{AF488}, DNase^{AF568} and DNase^{AF647} (Section 2.6.3). Samples of each reaction were taken between 0-90 minutes and separated by agarose gel. DNA was present as Supercoiled (S), circular (C) and linear (L) forms. (M) stands for Marker, (-) contains only DNA, (+) contains DNA mixed with the DNase domain at time point 0. Remaining lanes contain DNA incubated with the DNase domains for 2-90 minutes. DNA cleaved into small fragments run as a smear. The rate of DNA hydrolysis was the same for wild-type ColE9 DNase and DNase^{AF488}, DNase^{AF568} and DNase^{AF647}.

3.2.4 Fluorescently-labelled ColE9 cleaves DNA *in vivo*

To investigate the ability of fluorescently-labelled ColE9 to translocate to the cytoplasm and cleave DNA, bioluminescence was used. *E. coli* DPD1718 is a strain previously generated to assay genotoxicity, to use as a rapid, sensitive *in vivo* lux-based reporter of DNA damage (Davidov et al. 2000). It contains a fusion of the LexA regulated *recA* promoter region of the SOS operon of *E. coli*, fused to the *Photorhabdus luminescens luxCDABE* reporter system, integrated into the *lacZ* locus of the *E. coli* strain DPD1692 (Davidov et al. 2000). The strain has been used as a method to probe the mechanism and rate of DNA damage on addition of ColE9 previously (Vankemmelbeke et al. 2005).

When ColE9 translocates across the OM of the cell into the cytoplasm, it leads to DNA hydrolysis and the generation of single-stranded DNA (ssDNA) which induces the SOS response (Figure 3. 9) (Cascales et al. 2007). The accumulation of ssDNA leads to the activation of RecA, a specific protease (Vollmer et al. 1997). RecA protomers assemble to form filaments at the sites of ssDNA on the chromosome, these interact with LexA dimer which acts as a repressor of the SOS operon usually bound to a 20 bp SOS box, inhibiting the binding of RNA polymerase for the transcription of the SOS genes. RecA induces the autocleavage of LexA dimer and its dissociation from the SOS operon, thereby relieving its repression and allowing RNA polymerase to bind and initiate transcription of the SOS genes (Janion 2008).

Through the fusion of the *luxCDABE* genes to the SOS operon, they too get transcribed. LuxA and LuxB encode the heterodimer luciferase subunits A and B. LuxC, LuxD, and LuxE encode the fatty acid reductase complex enzymes; a reductase, transferase, and synthetase, respectively. These are responsible for the generation of the fatty aldehyde substrate (RCHO) for luciferase. Luciferase catalyses the reduction of oxygen and oxidation of reduced flavin mononucleotide (FMNH₂) and the fatty aldehyde substrate to produce flavin mononucleotide (FMN), water and fatty acid with the emission of bioluminescence blue-green light at ~490 nm. The fatty acid can

be recycled by LuxCDE to generate more substrate for continuous light emission (Meighen 1991).

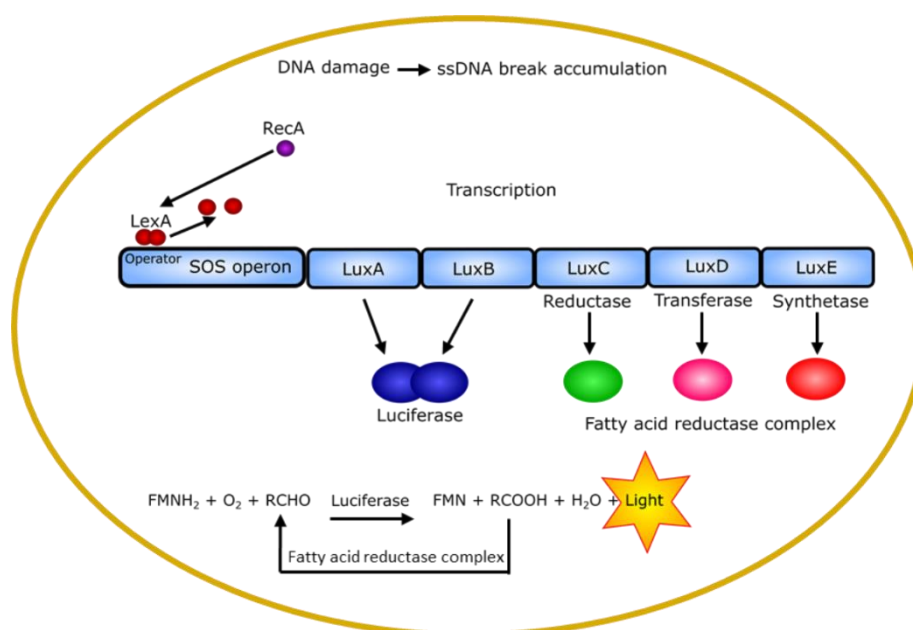


Figure 3. 9 - Mechanism for the emission of bioluminescence from DNA damage by ColE9.

DPD1718 strain contains a fusion of LexA regulated *recA* promoter region of the SOS operon of *E. coli*, fused to the *Photorhabdus luminescens luxCDABE* reporter system, integrated into the *lacZ* locus (Davidov et al. 2000). When ColE9 translocates into the cytoplasm and nicks DNA generating ssDNA, it induces the SOS response. This leads to activation of RecA, autocleavage of LexA and its dissociation from the SOS operon, allowing RNA polymerase to bind and initiate transcription of the SOS genes. The *luxCDABE* genes also get transcribed. Luciferase catalyses the reaction that generates emission of bioluminescence blue-green light at ~490 nm.

Bioluminescence was performed with *E. coli* DPD1718 cells, using full-length wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} constructs, measuring both the cell density at OD₆₀₀ and the emission of bioluminescence at OD₄₉₀ (Figure 3. 10) (Section 2.6.4). The results showed that in the absence of ColE9, there was no emission of bioluminescence, hence there was no induction of the SOS response. Further controls were performed using a range of DNA damage inducing agents, including mitomycin C, UV exposure, H₂O₂ and exposure to 40 °C (Appendix I Figure 5). Mitomycin C was the most efficient at damaging DNA and emitting bioluminescence, whereas the other agents had little to no effect on DNA.

For all of the constructs, as the concentration of ColE9 increased, the rate of cell growth and the OD₆₀₀ decreased. This can be seen for wild-type ColE9 in Figure 3. 10 A, where at 20 nM and 200 nM, cell growth was completely inhibited by ~100 minutes. At ≤ 2 nM, there was an insufficient number of ColE9 molecules to kill each cell before division or DNA repair occurred, whereas at ≥ 20 nM, cell death was rapid. When comparing the fluorescently-labelled constructs to wild-type ColE9 at 20 nM (Figure 3. 10 C), it was clear that wild-type ColE9 was the most rapid and efficient at inhibiting cell growth, whereas the labelled constructs were much less efficient. The effect of ColE9^{AF488} on inhibiting the rate of cell growth started to emerge from the control (absence of ColE9) after ~90 minutes. Whereas the effect of ColE9^{AF568} and ColE9^{AF647} on reducing cell growth is not seen within the 4-hour experiment. This result is in agreement with the plate killing assay (Figure 3. 7).

The bioluminescence data showed that all the constructs emitted bioluminescence after 20-30 minutes as a result of DNA damage (Figure 3. 10 B and D). For wild-type ColE9 at concentrations ≤ 2 nM, bioluminescence emission increased until it reached its peak at ~110-120 minutes and then started to plateau or to decline (Figure 3. 10 B), at ≥ 20 nM, bioluminescence emission peaked at ~90 minutes, before declining rapidly. When comparing the fluorescently-labelled constructs to wild-type ColE9 at 20 nM (Figure 3. 10 D), it was again clear that wild-type ColE9 was the most rapid and efficient at causing DNA damage and bioluminescence emission. ColE9^{AF488} bioluminescence increased over time, slower than wild-type ColE9. ColE9^{AF568} and ColE9^{AF647} had much lower levels of bioluminescence emission that increased slowly over the experiment. This showed that although capable of causing DNA damage by the emission of any bioluminescence, they are much less efficient at doing so within the same period of time than wild-type ColE9 or ColE9^{AF488}, with ColE9^{AF568} being the poorest construct. The range of ColE9 concentrations used are shown in Appendix I Figure 4.

This data therefore shows that the fluorescently-labelled constructs are still capable of translocation and cell death, in agreement with the plate killing assay (Figure 3. 7). However, the fluorescent label, and the different properties of those dyes affects the ability of ColE9 to translocate into cells, reducing the level of killing. ColE9^{AF488} had the least impact on translocation, whereas ColE9^{AF568} and ColE9^{AF647} had their translocation severely impacted, especially ColE9^{AF568}.

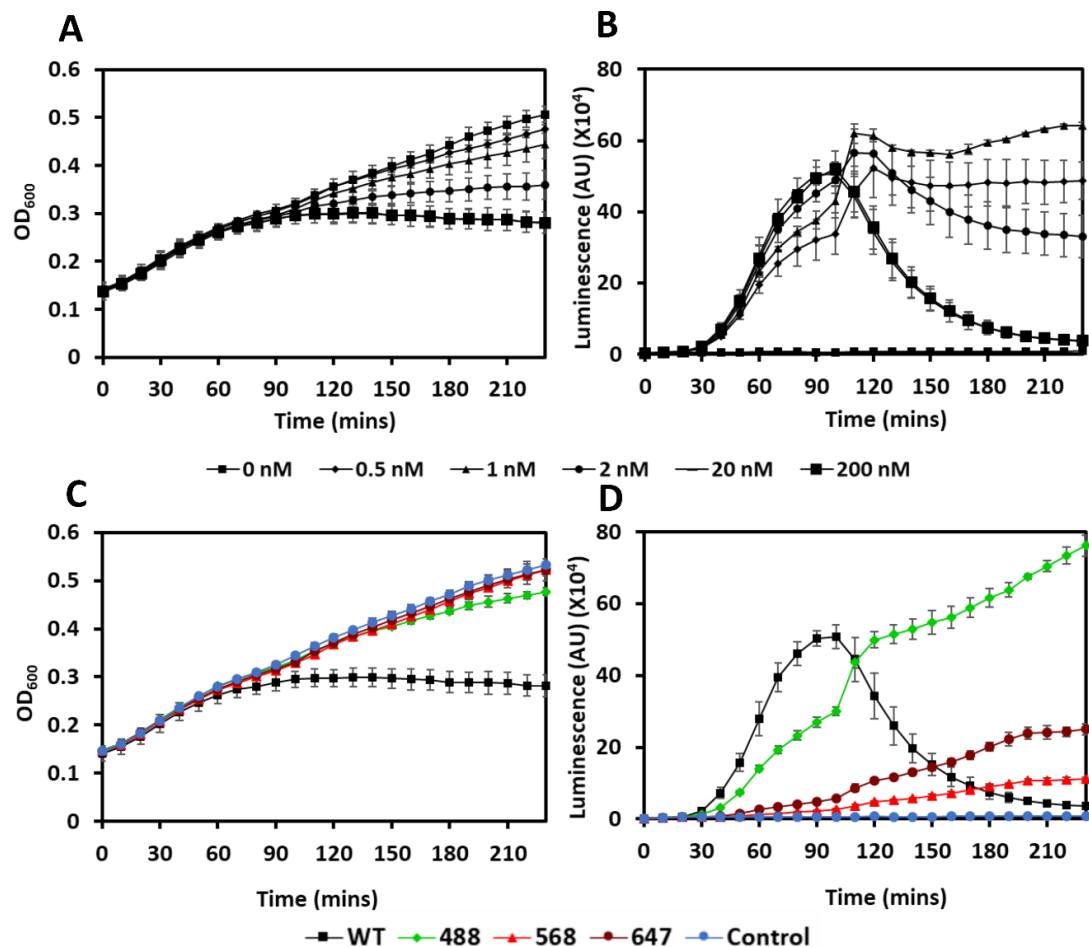


Figure 3. 10 - Effect of ColE9 constructs on bioluminescence emission and cell density.

E. coli DPD1718 cells were used to measure the effect of cell growth and DNA damage through the emission of bioluminescence, using wild-type ColE9 (WT) (black ■), ColE9^{AF488} (green ◆), ColE9^{AF568} (red ▲) and ColE9^{AF647} (maroon ●) and a control (●) (Section 2.6.4). Constructs were used at concentrations of 0 (■), 0.5 (◆), 1 (▲), 2 (●), 20 (-) and 200 nM (■). On addition of ColE9, the cell density at OD₆₀₀ and the emission of bioluminescence at OD₄₉₀ was reported. Top two graphs show the effect of wild-type ColE9 at the range of concentrations used on both OD₆₀₀ (A) and bioluminescence (B), the bottom two graphs show a comparison of all the constructs at 20 nM on OD₆₀₀ (C) and bioluminescence (D).

3.2.5 BtuB is required for outer membrane labelling by ColE9

The bioluminescence and plasmid nicking data (Section 3.2.3 and 3.2.4) clearly indicated that the fluorescently-labelled colicins were cleaving DNA. Therefore, we wanted to inhibit this in order to follow colicin import by fluorescence microscopy.

When H551 within the catalytic H-N-H motif of the DNase domain (Pommer et al. 2001) is mutated to alanine, the enzymatic activity is inhibited, as demonstrated by plate killing, kunitz and plasmid killing assays. The ability to bind its metal cofactor and DNA is retained however (Walker et al. 2002). Fluorescent labelling of K469C in the context of the H551A mutation was performed to allow monitoring of colicin uptake in the absence of toxicity. Wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} labelled constructs were mutated at position H551A (Figure 3. 11), to inhibit DNA hydrolysis and therefore the cytotoxicity of the constructs for use in microscopy. Constructs with the H551A mutation are labelled as ColE9*, ColE9*^{AF488}, ColE9*^{AF568} and ColE9*^{AF647}.

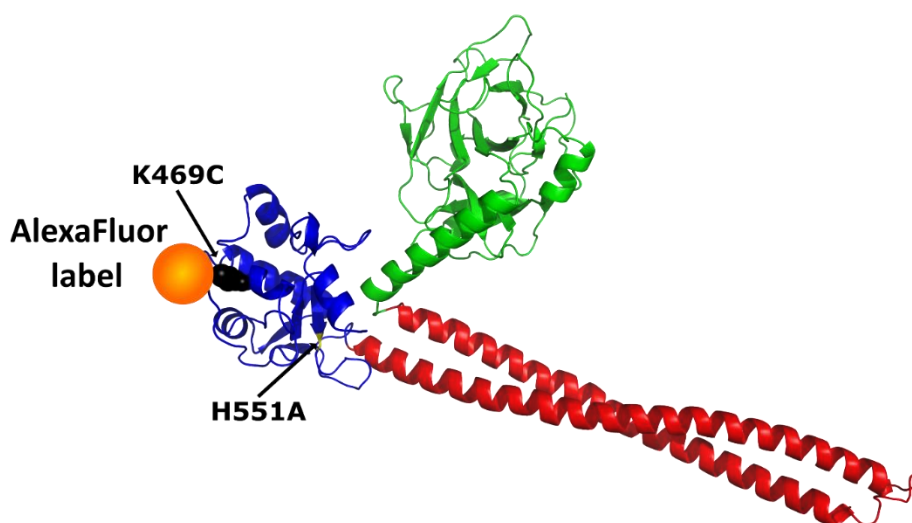


Figure 3. 11 - ColE9 mutated at K469C within the cytotoxic DNase domain for labelling with synthetic fluorescent dyes and position H551A to inactivate DNase activity.

ColE9 was mutated within the cytotoxic DNase domain (blue) with K469C for labelling with the AlexaFluor dyes (orange sphere) and H551A (yellow) to inactivate DNA hydrolysis and therefore cytotoxicity. The T- domain (green) and R-domain (red) are shown.

Inactivation of H551A in ColE9*, ColE9*^{AF488}, ColE9*^{AF568} and ColE9*^{AF647} constructs was confirmed through plate killing assays using *E. coli* JM83 cells (Section 2.6.1) (Figure 3. 12 and Appendix I Figure 6). The ColE9* plates had no circular clear zones on the bacterial lawns compared to wild-type ColE9, showing that cytotoxicity had been abolished.

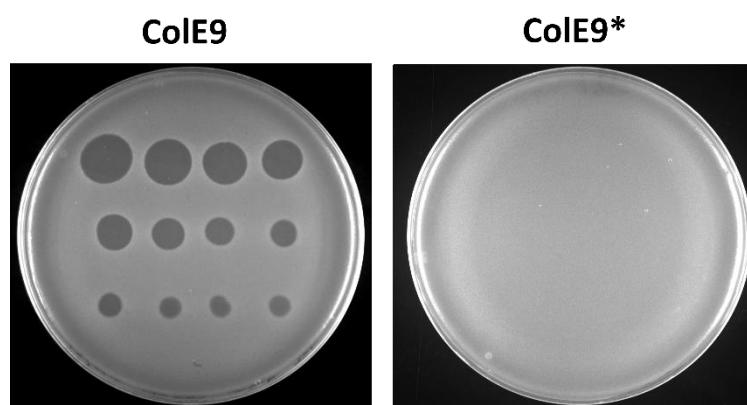


Figure 3. 12 - *In vivo* plate killing assay comparison of wild-type ColE9 and ColE9*.

E. coli JM83 cells were used to perform plate killing assays, using wild-type ColE9 and ColE9*, or fluorescently-labelled ColE9* (Appendix I Figure 6), from 10 μ M in 3-fold serial dilutions to 57 pM (Section 2.6.1). ColE9 H551A (*) mutation abolished colicin activity.

The use of BtuB as a receptor for enzymatic E colicins has been known since the 70's (Di Masi et al. 1973). Recent methods have shown that binding of ColE9 to BtuB is subnanomolar by ITC (Housden et al. 2005), and more recent techniques have shown binding through the labelling of cells with fluorescent ColE9 by microscopy (Rassam et al. 2015). I repeated this using ColE9*^{AF488}, ColE9*^{AF568} and ColE9*^{AF647} to confirm that these new constructs could be visualised binding to BtuB *in vivo* and imaged using the different fluorescent labels. *E. coli* JM83 cells were grown in M9 media and labelled with Hoechst stain for DNA and fluorescently-labelled colicin (Section 2.5.2). The reason for using M9 minimal media for microscopy experiments is that it produces little to no autofluorescence as background compared to other rich medias like LB, allowing a higher signal-to-noise ratio from the fluorescent dyes and therefore better imaging. Cells were visualised by widefield fluorescence microscopy (Section 2.5.12).

Figure 3. 13 showed the outer surface of the cell by widefield in grey, the Hoechst DNA stain in blue and the fluorescence of the different fluorophores. Overlays of the DNA and fluorescent

colicin channels are also shown. Figure 3. 13 showed that all of the fluorescently-labelled colicins, ColE9*AF488, ColE9*AF568 and ColE9*AF647 bound to the surface of *E. coli* cells *in vivo* and displayed the characteristic OMP islands or puncta, which are clusters of OMPs as seen by the brighter spots on the OM that have been described previously (Rassam et al. 2015).

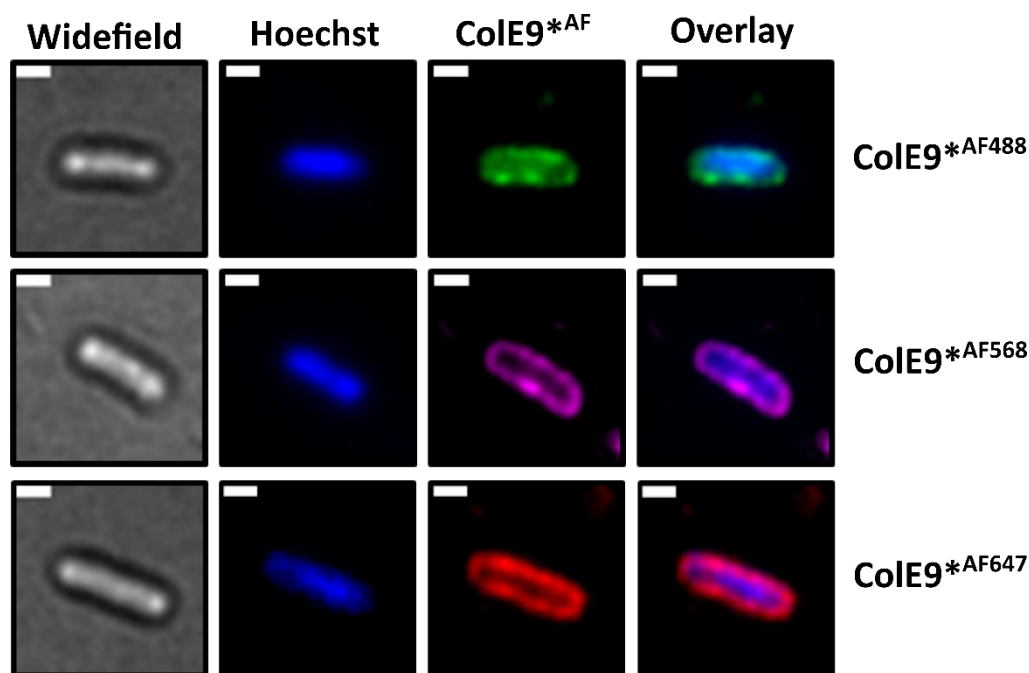


Figure 3. 13 - *E. coli* JM83 cells outer membrane labelled with ColE9*AF488, ColE9*AF568 and ColE9*AF647.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF488, ColE9*AF568 and ColE9*AF647 and 20 μ M Hoechst and imaged using widefield fluorescence microscopy (Section 2.5 and 2.5.12). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF488 (green), ColE9*AF568 (pink) and ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ColE9*AF488, ColE9*AF568 and ColE9*AF647 bound to the surface of *E. coli* cells *in vivo* and displayed the characteristic OMP islands. Scale bar is 1 μ m.

ColE9*AF488 had the least impact on translocation into cells than ColE9*AF568 and ColE9*AF647, as shown by plate killing assay (Figure 3. 7), OD₆₀₀ and bioluminescence (Figure 3. 10). However, due to the visualisation of autofluorescence from cells, visible < 500 nm, ColE9*AF488 was not the best suited to use in the development of a fluorescence-based microscopy assay to follow colicin translocation into live cells. This is because both emissions are detected using the same

microscopy channel, interfering with the detection and visualisation of fluorescence from CoIE9*AF488.

To get a better understanding of the size, shape, number and distribution of these BtuB receptor islands on the surface of *E. coli*, super-resolution structured illumination microscopy (SIM) was performed using the OMX V3 BLAZE microscope with the AF⁴⁸⁸ dye (Section 2.5.11). Cells were fixed with PFA to prevent cell growth and the translocation of the labelled colicin, before labelling cells using the construct CoIE9^{S-S AF488} (Section 2.5.2 and 2.5.3), a construct that contains a disulphide bond in the R-domain to prevent entry into the cell. This construct was generated and used previously (Rassam et al. 2015).

CoIE9^{S-S AF488} was used for this super-resolution 3D SIM imaging, because of the three fluorescent labels used in these experiments, AF⁴⁸⁸ is the smallest, the brightest, has the highest quantum yield and fluorescence lifetime (Table 3. 1) of the dyes. This means that it was the best suited to undergo repeated imaging using SIM without photobleaching, thereby allowing complete collection of the fluorescence data of the cell for reconstruction. In addition, cell fixation and the colicins disulphide (S-S) bond between the R-domain coils prevented the translocation of the colicin and ensured its localisation to the outer membrane. As it is the shortest wavelength of the dyes, it is also capable of giving the highest resolution. Using Super-resolution SIM imaging and the reconstruction software it was possible to visualise the fluorescence of the AF⁴⁸⁸ dye in these intense OMP islands without the autofluorescence (Figure 3. 14).

Figure 3. 14 showed that the OMP islands were fairly similar in their size, ranging ~120-200 nm diameter and are evenly distributed before cell growth and division. Previously, the size of OMP islands have been published to be ~0.5 µm, using fluorescently-labelled CoIE9 and ColIa bound to BtuB and Cir, respectively, by widefield and TIRF microscopy (Rassam et al. 2015). In addition, assembly precincts of BAM complexes have been published to be 100-300 nm, using fluorescently-labelled antibodies bound to BamA and BamC by dSTORM microscopy

(Gunasinghe et al. 2018). Therefore, in close agreement with the range of small islands of OMPs on the cell surface. The 3D image reconstruction in super-resolution gives a much clearer picture of the size, shape, number and distribution of these OMP islands than what has been seen in 2D by widefield microscopy (Figure 3. 13).

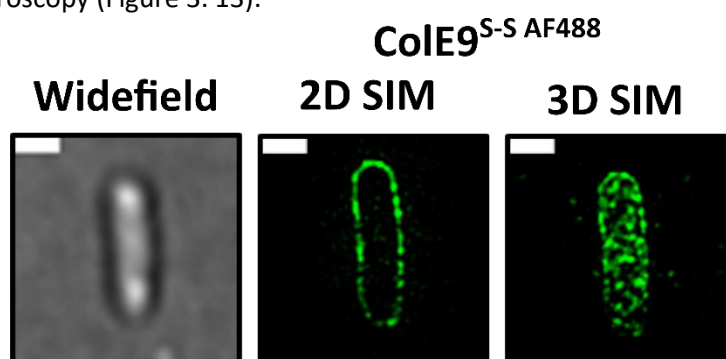


Figure 3. 14 - Super-resolution 2D and 3D SIM imaging of ColE9^{S-S AF488} bound to the surface of JM83 *E. coli* cells.

E. coli JM83 cells were fixed by PFA, labelled with ColE9^{S-S AF488} and imaged using Super-resolution SIM on the OMX V3 BLAZE microscope (Section 2.5). The cell is seen in DIC (grey) and the fluorescence of ColE9^{S-S AF488} (green). ColE9^{S-S AF488} bound to the surface of *E. coli* cells *in vivo* and displayed the characteristic OMP islands, which ranged in size between 120-200 nm with an even distribution. Scale bar is 1 μ m.

Both ColE9^{*AF568} and ColE9^{*AF647} produced good signal-to-noise ratios due to their far-red fluorescence properties. They emit light >500 nm, and so can be detected without the collection of autofluorescence from the cells. However, due to the large effect the AF⁵⁶⁸ dye had on dramatically reducing the rate of translocation of ColE9 into cells by plate killing and bioluminescence assays (Section 3.2.3 and 3.2.4), ColE9^{*AF568} was not the best-suited fluorescent label to use in the development of the translocation assay. For this reason, the subsequent data on the development of the assay was performed using ColE9^{*AF647}.

As proof-of principle of this technique and the specificity of binding, ColE9^{*AF647} was incubated with RK5016, a BtuB knockout strain of *E. coli* and visualised by widefield microscopy (Section 2.5). Figure 3. 15 shows that using JM83 cells that have BtuB, fluorescence was observed, but RK5016 cells are not fluorescent, confirming the construct's specificity.

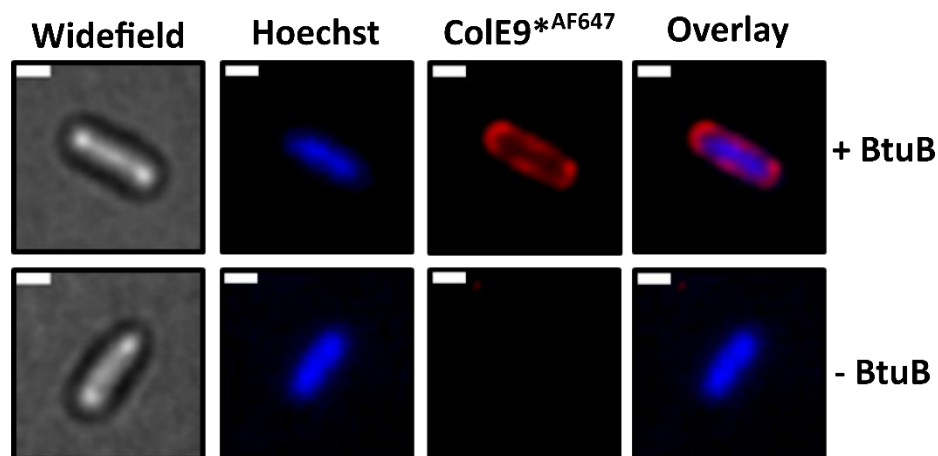


Figure 3. 15 - ColE9*AF647 binding to BtuB as its receptor on the cell surface of *E. coli* by widefield microscopy.

E. coli JM83 cells (+ BtuB) and *E. coli* RK5016 cells (- BtuB) were labelled with ColE9*AF647 and 20 μ M Hoechst and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. Widefield microscopy showed fluorescence of ColE9*AF647 binding cells in the presence of BtuB, but not in the deletion mutant. Scale bar is 1 μ m.

Microscopy was repeated using *E. coli* JM83 cells that were fixed using PFA followed by the addition of ColE9*AF647 (Section 2.5). Figure 3. 16 shows that ColE9*AF647 still bound and labelled the OM of *E. coli* even when fixed, producing the characteristic islands or puncta.

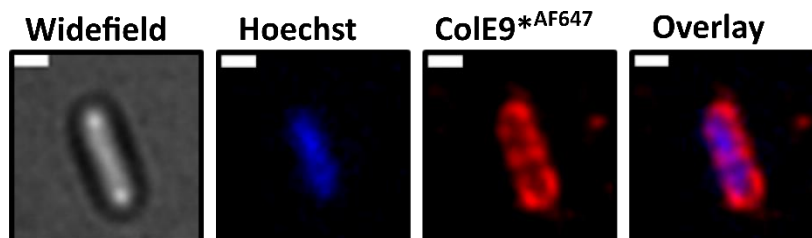


Figure 3. 16 - *E. coli* JM83 cells fixed with PFA and labelled with ColE9*AF647.

E. coli JM83 cells were fixed by PFA and labelled with ColE9*AF647 and 20 μ M Hoechst and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. Widefield microscopy showed fluorescence of ColE9*AF647 binding cells when fixed. Scale bar is 1 μ m.

3.2.6 Proteolysis of cell surface-exposed colicin allows visualisation of translocated ColE9*AF647

Microscopy data shows that ColE9*AF647 produced a good signal-to-noise ratio, making clear the binding of the colicin and the specificity for BtuB. Plate killing and bioluminescence assays have shown that the AF647 fluorescently-labelled construct is capable of translocating across the OM into the cell. Further to this, it has been shown by plate killing assay that introduction of the mutation H551A inactivates the colicin, preventing cell death and enabling the study of translocation. Due to the large number of BtuB receptors on the cell surface, as a consequence, a large number of fluorescently-labelled colicins can bind. Microscopy has shown that these form islands or puncta with high intensity fluorescence at the OM. This obscures visualisation of translocated ColE9 molecules. Therefore, proteolysis was used to remove surface-bound colicin molecules.

Using the same method as described above, *E. coli* JM83 cells in M9 minimal media were incubated with 1.5 μM (~92 $\mu\text{g/ml}$) ColE9*AF647 and 20 μM Hoechst for 30 minutes to allow enough time for colicins to translocate into cells, before washing thoroughly to remove excess dye (Section 2.5.2). Cells were then gently re-suspended in M9 minimal media and incubated with 50 $\mu\text{g/ml}$ of fresh trypsin, chymotrypsin, proteinase K or different combinations of those enzymes, in a 2:1 colicin:trypsin ratio. Cells were incubated for 1 hour at room temperature to allow sufficient proteolytic cleavage, before washing cells to remove cleaved, surface-exposed colicins (Section 2.5.6). Cells were then visualised using widefield microscopy (Figure 3. 17) (Section 2.5.12).

A relatively high amount of protease was used in these experiments to ensure that all of the surface-exposed colicins were degraded quickly. The use of trypsin in the rapid proteolytic cleavage of colicins from the cell surface has been used extensively in the past (Walker et al. 2007) (Braun et al. 1980).

Chymotrypsin treatment was insufficient for cleaving ColE9*AF647 from the cell surface, as seen in Figure 3. 17 where there is still patchy OM labelling visible and consequently some background fluorescence. Proteinase K was sufficient at cleaving ColE9*AF647 from the cell surface with loss of all OM fluorescence, however it led to cell damage and lysis as observed in the widefield and also by the loss or non-uniform appearance of the blue Hoechst stain (also present in the surrounding background of the cells). Trypsin was optimal for cleaving ColE9*AF647 from the cell surface as judged by the loss of all OM fluorescence and background whilst keeping the cells intact. Trypsin was therefore chosen as the protease for degrading surface bound colicins during translocation studies.

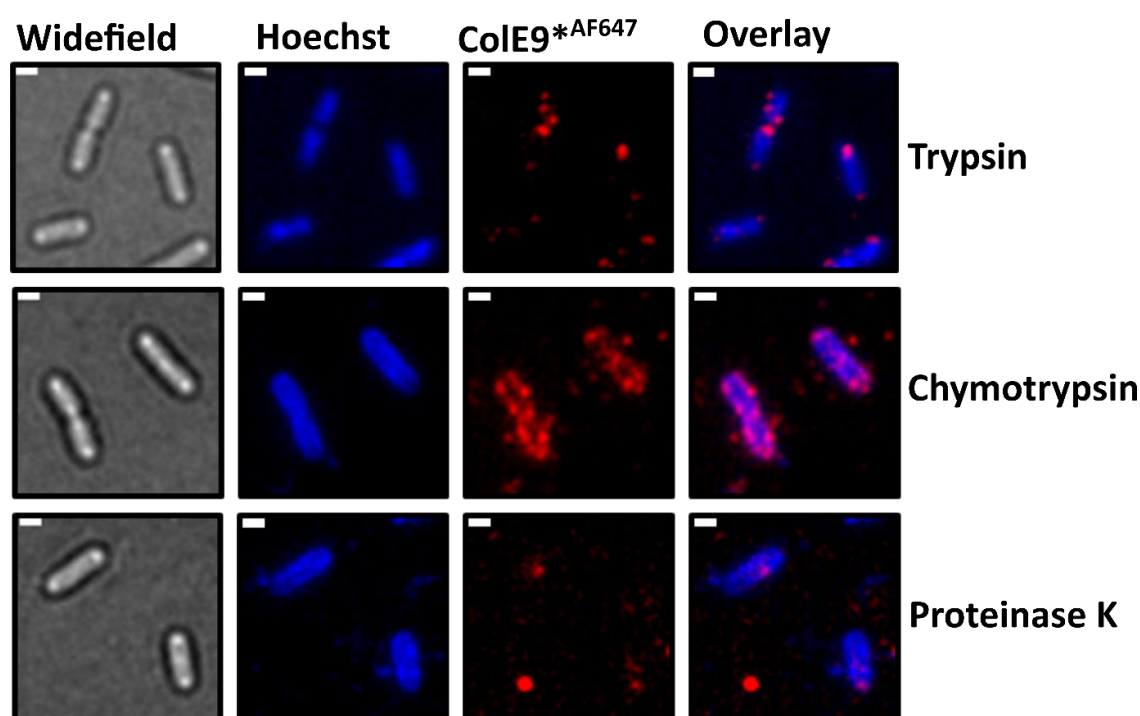


Figure 3. 17 - *E. coli* JM83 cells labelled with ColE9*AF647 followed by treatment with trypsin, chymotrypsin and proteinase K proteases.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF647 and 20 μ M Hoechst, followed by 50 μ g/ml protease treatment in a 2:1 colicin:trypsin ratio and thoroughly washed before imaging using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. Chymotrypsin treatment was insufficient at cleaving ColE9*AF647 from the cell surface, Proteinase K was sufficient at cleaving ColE9*AF647 but led to cell damage and lysis. Trypsin was sufficient at cleaving ColE9*AF647 from the cell surface whilst keeping the cells intact. Scale bar is 1 μ m.

To test if *E. coli* cells were under any stress by the presence of high levels of trypsin, *E. coli* JM83 cells were grown in LB to an OD₆₀₀ ~0.6 and supplemented with 50 µg/ml and 250 µg/ml of trypsin at 37 °C. The OD₆₀₀ was measured every 10 minutes (Figure 3.18). It was clear that the OD₆₀₀ and therefore rate of cell growth was not affected by the presence of trypsin relative to a control.

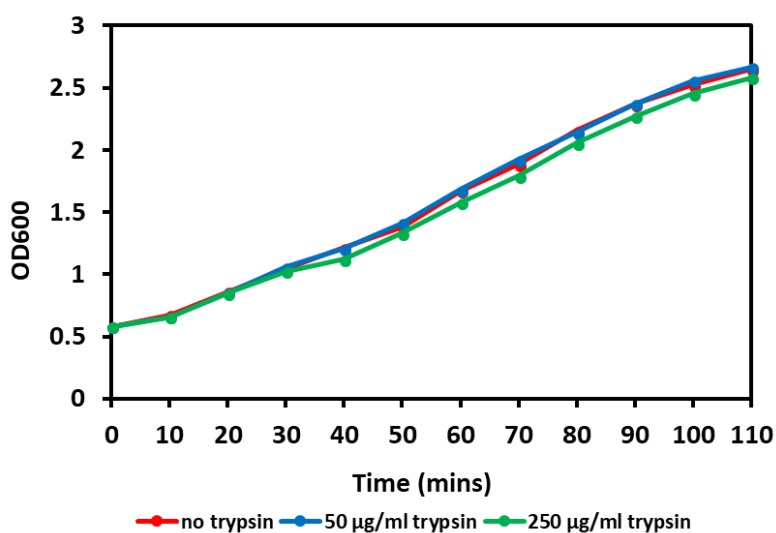


Figure 3. 18 - Effect of trypsin protease on cell growth.

E. coli JM83 cells were grown in LB to an OD₆₀₀ ~0.6 and then supplemented with 50 µg/ml and 250 µg/ml of trypsin at 37 °C. The OD₆₀₀ was measured every 10 minutes. The OD₆₀₀ and therefore rate of cell growth was not affected in the absence or presence of 50 µg/ml and 250 µg/ml of trypsin.

Data in Figures 3. 17 and 3. 19 show clearly the presence of internalised ColE9*AF⁶⁴⁷ fluorophores within *E. coli* JM83 cells following trypsin treatment. These fluorescent dots have radii of ~250- 750 nm wide, with an average of 450 nm. The resolution limit for these experiments by widefield microscopy using the AF⁶⁴⁷ dye is ~288 nm, based on the Rayleigh Criterion. The nature of these molecules will be further addressed in Chapters 4 and 5.

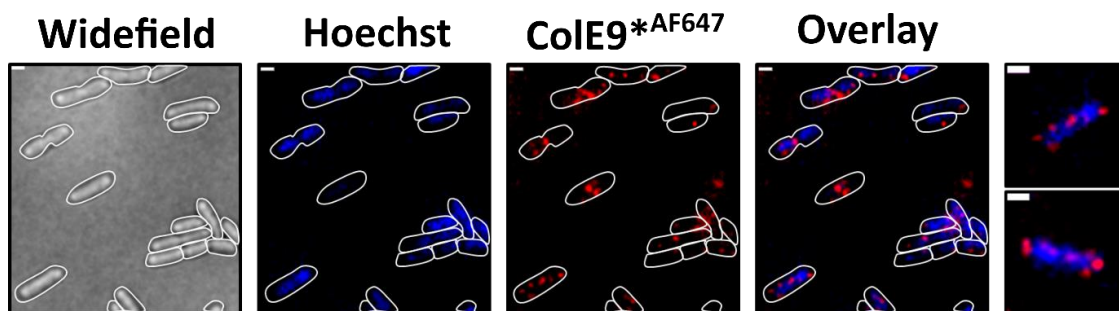


Figure 3. 19 - Protocol for observing ColE9 translocation in live *E. coli* cells.

E. coli JM83 cells were labelled with 1.5 ColE9*AF647 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown and two enlarged images of cells. Scale bar is 1 μ m.

This methodology was repeated using ColE9*AF488 and ColE9*AF568, to determine if the nature of the fluorophore affected internalisation. *E. coli* JM83 cells were labelled with ColE9*AF488 and ColE9*AF568 and treated with trypsin (Section 2.5) (Figure 3. 20). Here again, internalised fluorophores were observed although these were less easily visualised for ColE9*AF488 due to cell autofluorescence. Nevertheless, these results show that different fluorophores can be translocated into *E. coli* JM83 cells.

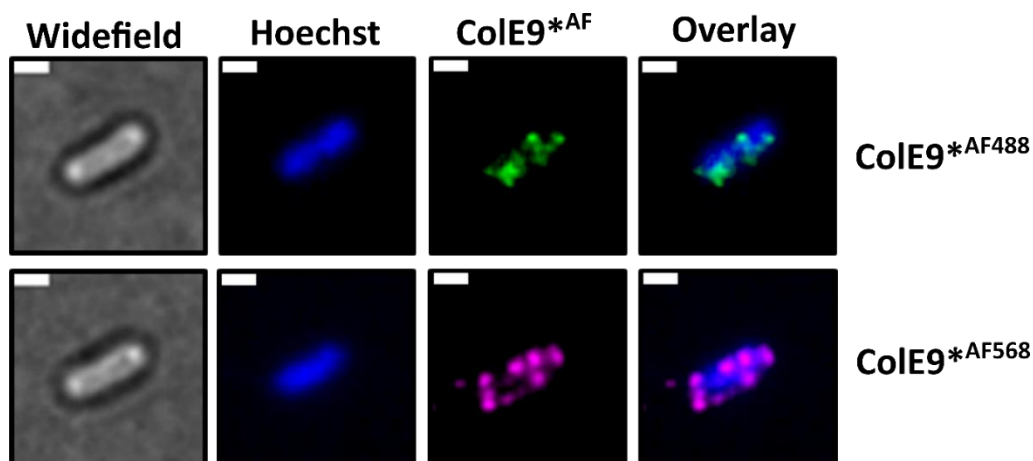


Figure 3. 20 - *E. coli* JM83 cells incubated with ColE9*AF488 and ColE9*AF568 followed by trypsin treatment.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF488 and ColE9*AF568 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF488 (green) and ColE9*AF568 (pink). Overlays of the DNA and fluorescent colicin are also shown. Scale bar is 1 μ m.

3.3 Discussion

The results in this chapter demonstrated that the presence of a fluorescent label on ColE9 and the use of different fluorophores with different properties, had varying effects on reducing *in vivo* cytotoxicity, which has been reported previously (Zakharov et al. 2008). ColE3 was fluorescently-labelled at position K549C within the cytotoxic rRNase domain with Org488 and at positions A85C/A86C within the T-domain with AF568. Plate killing assays showed that the presence of the C-domain label reduced killing by ~8x, the T-domain label reduced killing by 64- 128x using Org488 and AF488 labels, respectively. ColE3 labelled at K549C with Org488 and at A85C/A86C with AF568 reduced killing >250x. They regarded these last two constructs with reduced killing as inactive and concluded that their translocation through OmpF must be hindered by the attachment of the fluorescent dyes and did not pursue following cell entry (Zakharov et al. 2008).

The present studies are consistent with this earlier work and indicate that fluorescently-labelling ColE9 can dramatically reduce cytotoxicity and that different dyes can influence this differently. The nicking assay showed that the fluorescent labels on ColE9 have no effect on colicin activity, whereas the bioluminescence assay showed that the fluorescence labels have an effect on the rate of entry into the cell to cause DNA damage. Therefore, a fewer number of labelled colicins are likely translocating into cells. In addition, it suggests that there are differences in the rate of cell entry between the fluorescently-labelled colicins, with ColE9^{AF488} having the least impaired translocation of the labelled colicins, followed by ColE9^{AF647} and then ColE9^{AF568}. This is most likely due to their difference in size, charge and dimensions (Table 3. 2).

The use of the bioluminescence strain as a method to probe the mechanism and rate of DNA damage on addition of ColE9 was developed previously (Vankemmelbeke et al. 2005). This work demonstrated the ability to observe DNA damage from the emission of bioluminescence in as little as 15 minutes on addition of ColE9 and showed the effect of increasing colicin

concentration on the rate of bioluminescence emission. The result obtained in this chapter using this method for the concentrations used and rates of bioluminescence emission using this strain, are in close agreement with those previously published (Vankemmelbeke et al. 2005).

Further evidence in support of fluorophores affecting bacteriocin translocation was shown using a fluorescently-labelled pyocin. pyoS2^{NTD}-AF488 was used to show FpvAI receptor specificity in *Pseudomonas aeruginosa*, for the binding and translocation to the periplasm through confocal microscopy (White et al. 2017). Further to this, the authors showed that fusion of GFP to pyoS2 (pyoS2^{NTD}-GFP), prevented its translocation through the lack of recovery after FRAP, as a result of the large fluorescent protein blocking entry.

In addition, the fluorescent-labelling of ColE3 was shown to have no effect on binding to BtuB through competition assays using wild-type ColE3 (Zakharov et al. 2008). Although no affinity measurements have been performed using ColE9*^{AF647} binding BtuB in this study, microscopy has shown the labelled construct to bind tightly to the OM of JM83 cells and survive repeated centrifugation and washing steps without its dissociation.

3.4 Conclusions

Data collected by Boonyaporn Chinthammit identified position K469C as the optimal location for fluorescent labelling of ColE9. This chapter showed that when ColE9 is fluorescently-labelled, the different fluorescent dyes used do not affect the nuclease activity but do have an effect on the ability of ColE9 to translocate. All of the fluorescently-labelled constructs were capable of translocating into the cell and causing DNA damage. The cytotoxicity of ColE9^{AF488} was only slightly reduced compared to wild-type ColE9, the cytotoxicity of ColE9^{AF568} was nearly completely inhibited. The cytotoxicity of ColE9^{AF647} was also dramatically reduced, but not to the extent of ColE9^{AF568}. These fluorescently-labelled colicins were chosen to pursue as probes for following cell entry, because compared to using fluorescent proteins, fluorescently-labelled antibodies or quantum dots, the AF dyes are the smallest available fluorescent-probe that could

theoretically enter the cell and be used to follow translocation. The dyes do impact cell entry, as demonstrated by plate killing and bioluminescence assays for ColE9 and previous data for ColE3 (Zakharov et al. 2008). However, although their rate of entry is affected, they do still translocate and can therefore still be pursued as probes to follow translocation.

In the development of the fluorescence-based microscopy method to follow colicin translocation, ColE9*^{AF488}, ColE9*^{AF568} and ColE9*^{AF647} constructs all bound to the OM. Microscopy using ColE9*^{AF488} was complicated by the issue of autofluorescence, whereas ColE9*^{AF647} produced good signal-to-noise and so was chosen to use for further analysis. ColE9*^{AF647} binds specifically to BtuB on the OM. This will allow us to confirm the involvement of other proposed proteins in the translocation of ColE9, described in Chapters 4 and 6. The technique to observe the translocation of ColE9*^{AF647} was optimised using trypsin. One interesting observation from the widefield microscopy was the presence of fluorescent populations of ColE9*^{AF647} molecules inside the cell. This suggests that it is possible to see translocated ColE9*^{AF647} molecules inside live *E. coli* cells even though the fluorophore may slow the rate of import. This will be further addressed in Chapter 4 and 5.

4 Chapter 4 - Following the translocation of ColE9 to the cytoplasm

4.1 Introduction

The cytotoxic mode of action of ColE9 is a DNase and consequently kills by degrading DNA in the cytoplasm, therefore at least the cytotoxic domain must reach the cytoplasm. ColE9 translocation to the cytoplasm has been studied indirectly through the degradation of DNA and the emission of bioluminescence (Vankemmelbeke et al. 2005). Similarly, the degradation of DNA by ColE2 in the cytoplasm, has been studied through the inhibition of synthesis of C¹⁴-uracil radioactively-labelled DNA after ColE2 addition (Nomura 1963). In addition, the DNase domains of ColE2 and ColE7 have been identified in the cytoplasm after translocation, through western blotting (Mora and de Zamaroczy 2014).

The entry of nutrients and vitamins like vitamin B₁₂ across the OM, requires interaction with the Ton system (Bassford et al. 1976) and access to the PMF (Di Girolamo and Bradbeer 1971) (Di Masi et al. 1973). The entry of colicins into bacteria requires either the Tol or Ton systems. The use of TolB and other members of the Tol system as essential components for ColE9 entry has been shown previously (Hands et al. 2005; Loftus et al. 2006) (Bonsor et al. 2009) (Carr et al. 2000). It was assumed that colicins that parasitize the energy-transducing Tol or Ton systems would necessarily need the PMF for entry, however, scant evidence exists in the case of Tol-dependent colicin entry.

The requirement for a complete network of protein interactions in the Ton/Tol system and the dependence on the presence of the PMF for entry has been studied using other bacteriocins. The requirement of the PMF for the translocation of Ton-dependent colicins ColB, Colla and ColD and the Tol-dependent colicin ColE5, was shown through inhibition of the PMF using CCCP (Nairn et al. 2017) (Ruhe et al. 2014). The requirement for protein interactions between the colicin and the Ton system for entry has been observed for ColB through mutation of its TonB box (Mende and Braun 1990), for all group B Ton-dependent colicins through

mutation of TonB (Bassford et al. 1976) and for ColE5 and ColD through deletion of TolA and TonB, respectively (Ruhe et al. 2014). The necessity of the PMF and protein interactions with the Ton system for the translocation of other colicin-like bacteriocins was demonstrated using pyocins against *Pseudomonas aeruginosa* cells (White et al. 2017). Pyocin S2-AF488 remained on the cell surface but was unable to translocate into the cell after inhibition of the PMF using CCCP or deletion of the TonB box, demonstrated by the lack of FRAP by microscopy, ITC and cross-linking.

The first direct evidence for use of the PMF for ColE9 translocation, was shown through the fluorescent-labelling of Im9 and determining conditions for its release through measuring relative fluorescence units (RFU) (Vankemmelbeke et al. 2009). Im9 release required multiple components (TBE, TolB, TolA, TolQR). In addition, the authors showed a dose-dependent decrease in Im9 release using CCCP ionophore and that Im9 dissociation appeared to be dependent mainly on the electrical potential ($\Delta\Psi$) of the PMF, through the use of valinomycin (Vankemmelbeke et al. 2009). The PMF is transduced via the Tol system to the cell surface, for the dissociation of Im9 and presumably translocation of the colicin.

FtsH is an ATP-dependent IM AAA+ ATPase/protease that functions to detect and remove misfolded IM proteins and degrade them. FtsH plays an essential role in the translocation of ColE9 DNase domain across the IM into the cytoplasm (Walker et al. 2007). Walker et al. showed that certain mutations in FtsH that rendered the cell inactive against its cytoplasmic substrate σ^{32} , also rendered the cell tolerant to nuclease colicins. The authors showed sensitivity to pore-forming colicins, Colla and Collb, in the absence of FtsH and tolerance to nuclease colicins like ColE3, D, and E9, regardless of their use of the Tol or Ton system for entry of their specific nuclease (Walker et al. 2007). Since this finding, the use of FtsH for the proteolytic cleavage of other nuclease colicins for their entry into the cytoplasm has been found,

like ColE2 and ColE7 (Mora and de Zamaroczy 2014). In addition, ColE3 and ColD were shown to require both FtsH and the IM signal protease LepB (Chauleau et al. 2011).

The aims of this chapter were to use the fluorescence-based microscopy method developed in Chapter 3, to demonstrate the translocation of ColE9 DNase^{AF647} to the cytoplasm of *E. coli* cells. Next, to use this method to investigate the PMF-dependence for the translocation process, dissect its energy requirements, and additionally, look at the impact of TolB and FtsH on the translocation of fluorescently-labelled ColE9 into the cell. Finally, this chapter aimed to investigate the effect of fluorescently-labelling the T-domain and R-domain of ColE9.

4.2 Results

4.2.1 ColE9*AF647 translocates to the cytoplasm of *E. coli*

In the previous chapter (Chapter 3), a method was developed to observe ColE9*AF647 translocation into cells, where the fluorophore was attached to the DNase domain. The next question to address was where these molecules were located inside the cell. This was investigated by dual-labelling spheroplasting experiments using ColE9*AF647 and ColB*AF488.

Colicin B (ColB) is a pore-forming colicin that uses Ferric enterobactin receptor (FepA) as its receptor on the cell surface of *E. coli*. ColB uses the Ton system to translocate to the periplasm, where its cytotoxic pore-forming domain inserts into the IM to depolarise and kill the cell (Hilsenbeck et al. 2004). A ColB-TR construct was engineered (pPW13), containing the translocation and receptor binding domains (TR) shown in green and red, respectively (Paul White, unpublished result) (Figure 4. 1). This construct is therefore inactive due to the lack of a cytotoxic domain. A mutation was introduced at position D342C at the end of the receptor binding domain to provide a surface-exposed cysteine for labelling with organic fluorophores. In the present work, ColB-TR was labelled with both AF⁴⁸⁸ and AF⁶⁴⁷ using the same method as for ColE9 (Section 2.3.5), to produce ColB*AF⁴⁸⁸ and ColB*AF⁶⁴⁷, respectively.

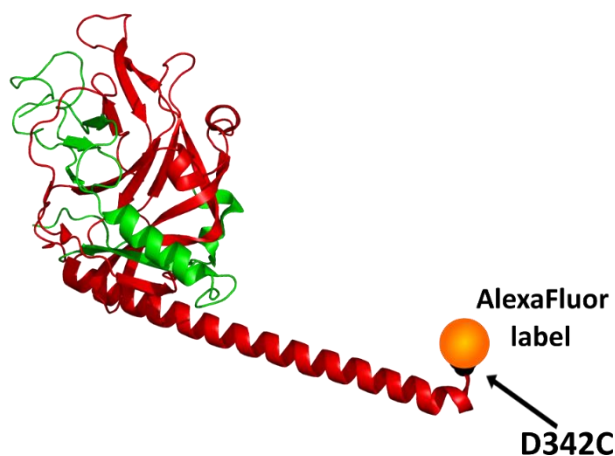


Figure 4. 1 - ColB-TR construct with a mutation at position D342C within the receptor binding domain for labelling with synthetic fluorescent dyes.

ColB is a pore-forming colicin that uses FepA as its cell surface receptor. Figure shows a ColB construct containing only the T-domain and R-domain (ColB-TR) and a mutation at the end of the R-domain (red) at position D342C for labelling with fluorescent dyes (orange sphere) (right image). (PDB; 1rh1-ColB, 1fep - FepA).

First, it was confirmed that ColB*^{AF647} bound to FepA expressing *E. coli* cells. Full-length ColB was active against *E. coli* JM83 cells that have FepA (+FepA) but not the *E. coli* Keio FepA knockout strain JW5086 (-FepA) (Figure 4. 2 A) (Section 2.6.1). Widefield microscopy was performed incubating ColB*^{AF647} with both JM83 cells and JW5086 cells (Figure 4. 2 B) as described in (Section 2.5). ColB*^{AF647} was used for these experiments and clearly binds to FepA expressing cells, but not cells where the FepA gene was deleted.

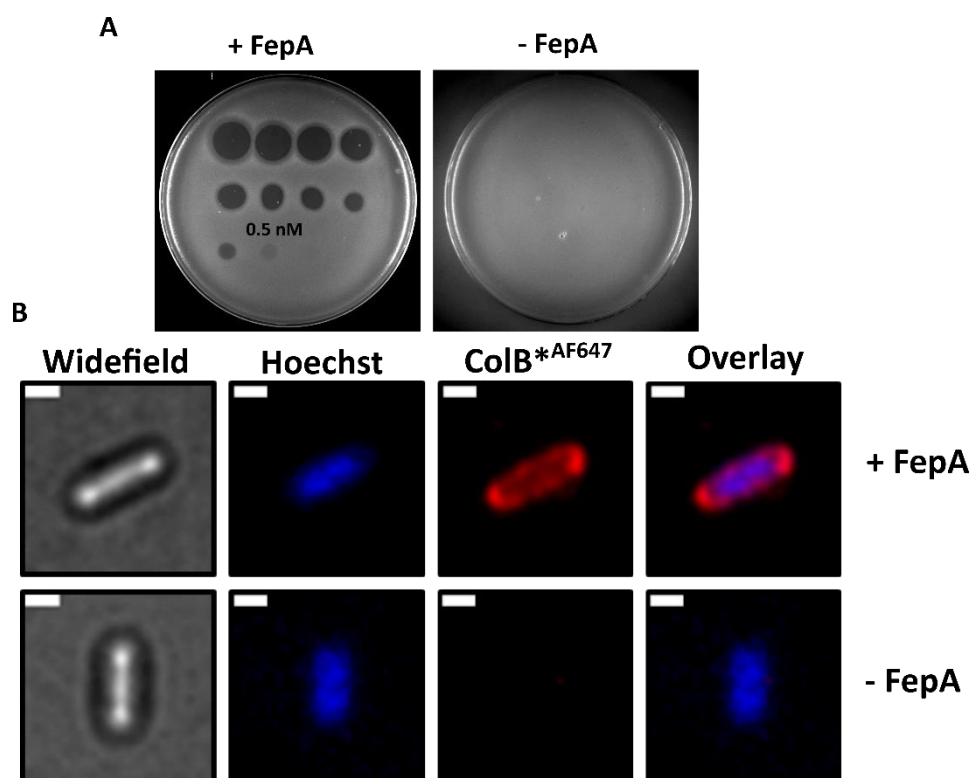


Figure 4. 2 - Plate killing assay and widefield microscopy to show FepA receptor specificity for ColB.

E. coli JM83 cells (+FepA) and *E. coli* JW5086 (- FepA) were used to perform a plate killing assay using full-length ColB (Section 2.6.1). (A) ColB induced cell killing in the presence of FepA (+ FepA) down to ~0.5 nM and no killing in the absence of FepA (- FepA). (B) JM83 cells (+FepA) and JW5086 (- FepA) were labelled with 1.5 μ M ColB*^{AF647} and 20 μ M Hoechst and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColB*^{AF647} (red). Overlays of the DNA and fluorescent colicin are also shown. ColB*^{AF647} bound to the surface of *E. coli* cells in the presence of FepA, but not in its absence. Scale bar is 1 μ m.

Next, *E. coli* JM83 cells were labelled either with ColE9*^{AF647} or ColB*^{AF488}, followed by trypsin treatment (Section 2.5) and their fluorescence distributions compared by widefield microscopy (Figure 4. 3).

For both ColE9*AF647 and ColB*AF488 labelled cells, there was remaining internal fluorescence observed after trypsin treatment. For ColB*AF488 treated cells, autofluorescence was evident throughout the cell, but the most intense fluorescence was confined to the perimeter of the cell (Figure 4. 3). In addition, videos of these ColB*AF488 treated cells collected by widefield microscopy, showed the photobleaching of the autofluorescence at the centre of the cell and the fluorescence at the perimeter moving as dots in a track-like motion (Appendix I Figure 7). This was also observed using ColB*AF647 (data not shown). This behaviour is consistent with the location of ColB*AF488 in the periplasm of the cell, which was its expected destination as a pore-forming colicin. For ColE9*AF647 treated cells, the same result was observed as previously seen at the end of Chapter 3, there was internal fluorescence throughout the cell that appeared as dots (Figure 4. 3).

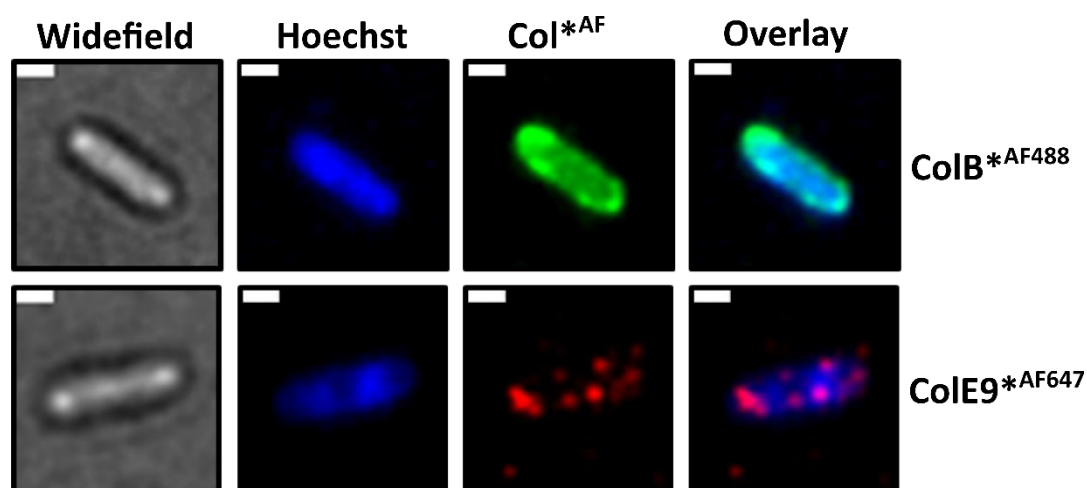


Figure 4. 3 - *E. coli* JM83 cells incubated with either ColB*AF488 or ColE9*AF647 followed by trypsin treatment.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF647 or ColB*AF488 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColB*AF488 (green) and ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. For both ColE9*AF647 and ColB*AF488 labelled cells, there was remaining internal fluorescence after trypsin treatment. Scale bar is 1 μ m.

E. coli JM83 cells were then incubated with both ColE9*AF647 and ColB*AF488 together, so that ColE9*AF647 could translocate to the cytoplasm and ColB*AF488 to the periplasm. Cells were thoroughly washed and treated with trypsin as described above (Figure 4. 4). ~84 % of cells imaged contained both ColE9*AF647 and ColB*AF488 and ~16 % cells appeared to be labelled with only ColB*AF488.

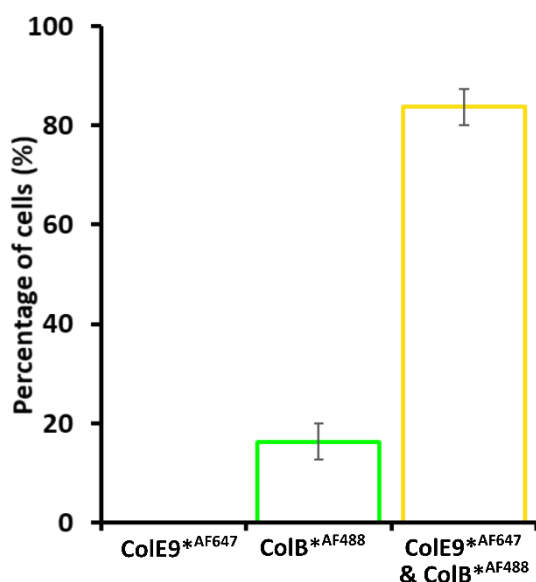
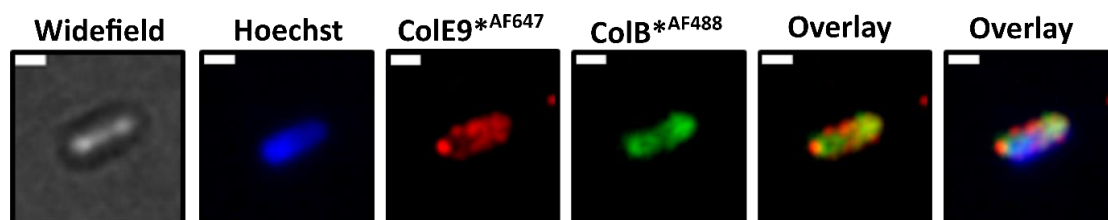


Figure 4. 4 - *E. coli* JM83 cells incubated with ColB*AF488 and ColE9*AF647 followed by trypsin treatment.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF647 and ColB*AF488 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColB*AF488 (green) and ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ~84 % of cells imaged contained both ColE9*AF647 and ColB*AF488 and ~16 % that appeared labelled with only ColB*AF488. Error bars represent % SEM, n = 62 cells. Scale bar is 1 μ m.

Having shown that JM83 cells could be simultaneously labelled with both ColE9*AF647 and ColB*AF488, the next step was to determine their relative locations by spheroplasting experiments.

Spheroplasts are cells in which the OM has been perforated and partly removed and the peptidoglycan (PG) in the periplasm hydrolysed, which releases membrane tension causing cells to become spherical. The protocol for the generation of spheroplasts uses a hypertonic sucrose to help stabilise the damaged cells and prevent lysis occurring, EDTA for chelating divalent

cations at the OM and lysozyme to hydrolyse the PG layer in the periplasm. Spheroplasting leads to the rapid removal of ~30-50 % LPS from the OM, increasing cell permeability to lysozyme leading to further hydrolysis. This exposes the periplasm and all of its contents including proteins within it (Leive 1965). The IM has been shown previously to remain intact during spheroplast formation and appear unaffected by the sucrose, lysozyme or EDTA (Birdsell and Cota-Robles 1967). This means the IM and cytoplasm and all of the contents within it will remain in the spheroplasted cells. The generation of spheroplasts is therefore a useful method to determine the location or compartment that proteins reside within (Vazquez-Laslop et al. 2001) (Mansell et al. 2010). The presence of OMPs within the remaining OM of spheroplasted cells has been observed, for example the major OMP PorA in strains of *C. jejuni* (Hobb et al. 2009) and CrcA in *E. coli* (Tefsen et al. 2005).

Cells fluorescently-labelled with both ColE9*^{AF647} and ColB*^{AF488} were spheroplasted and visualised by widefield microscopy (Section 2.5.7). Cells labelled with ColE9*^{AF647} and ColB*^{AF488} showed near complete loss of ColB*^{AF488} but retention of ColE9*^{AF647} (Figure 4. 5); ~78 % of cells imaged contained only ColE9*^{AF647}, ~20.4 % contained both ColE9*^{AF647} and ColB*^{AF488} and ~1.4 % contained only ColB*^{AF488}. This therefore indicated that ColB*^{AF488} was present within the periplasm, which had been perforated and exposed to the environment was lost. In contrast, ColE9*^{AF647} was within the cytoplasm of the cells and hence retained. The 20 % of spheroplasted cells that still contained both ColE9*^{AF647} and ColB*^{AF488} may not have had as much LPS removed and therefore not as permeable for complete loss of ColB*^{AF488} from the periplasm. The ~1.4 % of spheroplasted cells that contained only ColB*^{AF488} may not have been labelled with ColE9*^{AF647} to begin with, in agreement with the ~16 % of non-spheroplasted cells labelled with only ColB*^{AF488} in Figure 4. 4.

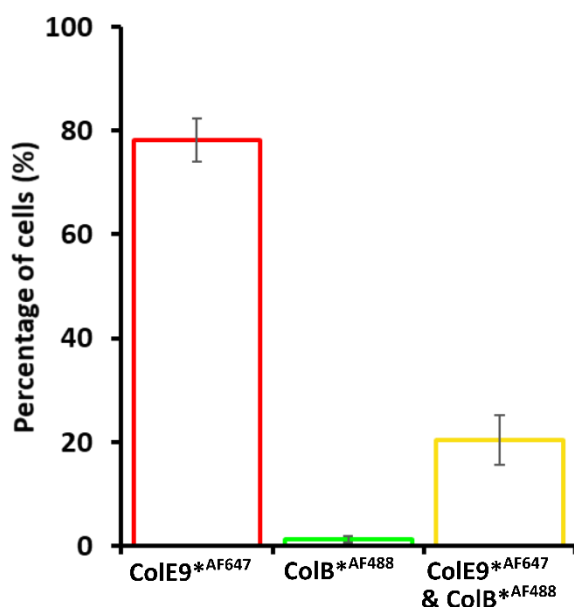
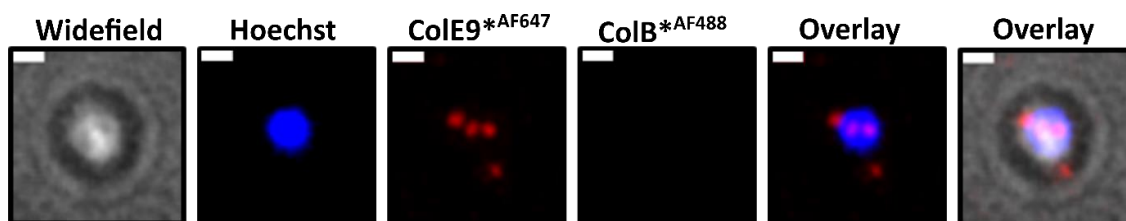


Figure 4. 5 - *E. coli* JM83 cells incubated with ColB*AF488 and ColE9*AF647, followed by trypsin treatment and spheroplasting.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF647 and ColB*AF488 and 20 μ M Hoechst before treating with trypsin and spheroplasting and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColB*AF488 (green) and ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ~78.2 % of cells imaged contained only ColE9*AF647, 20.4 % contained both ColE9*AF647 and ColB*AF488 and 1.4 % contained only ColB*AF488. Error bars represent % SEM, n = 57 cells. Scale bar is 1 μ m.

These data suggest that the ColE9*AF647 molecules observed after trypsin treatment inside the cell, were located primarily in the cytoplasm. Although this result is indirect, in combination with the previous plate killing and bioluminescence assays in Chapter 3, which showed ColE9*AF647 translocating into the cytoplasm to bind and hydrolyse DNA and cause cell death, it indicated that the molecules observed are ColE9 DNase*AF647. From this spheroplasting data we can also show that ColB*AF488 translocates to the periplasm, where it would target the IM as a pore-forming colicin.

4.2.2 The translocation of ColE9*^{AF647} across the outer membrane is dependent on the proton motive force

To further investigate the role of the PMF in colicin translocation, the microscopy method developed in Chapter 3 with ColE9*^{AF647} was used. There are many chemicals capable of inhibiting the PMF in bacteria. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) is an uncoupling agent that acts as an anionic protonophore to inhibit the PMF (Figure 4. 6). CCCP is negatively charged but becomes neutralised by protonation (CCCPH). This neutral molecule is driven across the IM to the cytoplasm by the ΔpH where the proton dissociates and CCCP is then driven to the periplasm by the $\Delta\Psi$ to bind more protons (Hards and Cook 2018). CCCP therefore alters both the proton concentration gradient and electrical potential by moving the protons down their electrochemical gradient, thereby inhibiting the PMF. By bypassing ATP synthase in the movement of protons to the cytoplasm, formation of ATP is also inhibited (Orlov et al. 1994).

There are also chemicals that are capable of inhibiting the individual gradients of the PMF. Valinomycin is a macrocyclic dodecadepsipeptide ionophore that is highly selective for potassium (K^+) ions (Figure 4. 6). Valinomycin binds K^+ ions in the periplasm before diffusing across the IM, releasing K^+ and returning to the periplasm in a cyclic action. By moving K^+ ions down their electrochemical gradient, it disrupts the charge distribution by having more K^+ in the cytoplasm and more H^+ in the periplasm, thereby negating the charge distribution and inhibiting the electrical potential of the PMF ($\Delta\Psi$), whilst maintaining the proton concentration gradient (ΔpH) (Strahl and Hamoen 2010) (Orlov et al. 1994).

Nigericin is a polyether ionophore K^+/H^+ antiporter. It binds H^+ ions in the periplasm and transports them across the IM where it releases them in the cytoplasm, and binds K^+ ions in the cytoplasm where it transports them across the IM, where it releases them in the periplasm (Figure 4. 6). By moving H^+ ions down their electrochemical gradient it disrupts the proton concentration gradient (ΔpH), but by replacing the lost positive charge of the H^+ with K^+ by

electroneutral exchange in the periplasm it maintains the electrical potential of the PMF ($\Delta\Psi$) (Strahl and Hamoen 2010).

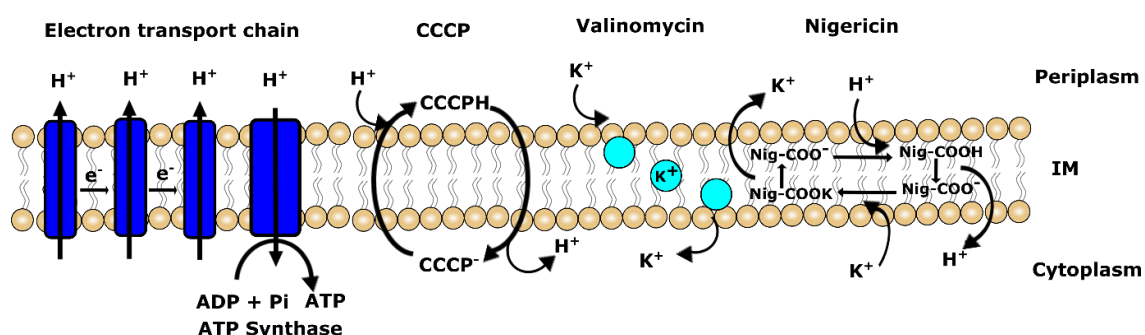


Figure 4. 6 - The generation of the proton motive force and its inhibition by protonophores.

The electron transport chain is a series of enzymes in the IM which transfer electrons (e^-) between them, releasing free energy to transfer a proton (H^+) across the IM from the cytoplasm to the periplasm. This generates two trans-membrane gradients; a proton concentration gradient (ΔpH), and an electrical potential ($\Delta\Psi$), which compose the electrochemical proton motive force (PMF, Δp). CCCP is a negatively charged protonophore that binds positively charged protons (H^+) in the periplasm and is driven across the IM to the cytoplasm by the ΔpH , where the proton dissociates. CCCP is then driven to the periplasm by the $\Delta\Psi$ to bind more protons (Hards and Cook 2018). This alters both the (ΔpH) and ($\Delta\Psi$), thereby inhibiting the PMF and ATP synthesis. Valinomycin is an ionophore that binds K^+ in the periplasm, diffusing across the IM, releasing K^+ and returning to the periplasm in a cyclic action. It disrupts the charge distribution and inhibits the electrical potential ($\Delta\Psi$) only. Nigericin is an ionophore K^+/H^+ electroneutral antiporter, it binds H^+ in the periplasm and transports them to the cytoplasm and binds K^+ ions in the cytoplasm and transports them to the periplasm. It disrupts the proton concentration gradient (ΔpH) only. Adapted from (Hards and Cook 2018).

To investigate the energy requirements of ColE9 translocation, *E. coli* JM83 cells were treated with CCCP to inhibit the PMF and labelled with ColE9*^{AF647} (Section 2.5). These CCCP treated cells were further treated with trypsin to see if translocation of ColE9*^{AF647} occurred and visualised by widefield microscopy (Figure 4. 7) (Section 2.5). In the presence of CCCP, ColE9*^{AF647} still bound and labelled the OM producing islands or puncta. When cells were further treated with trypsin, all ColE9*^{AF647} fluorescence was lost. When labelling and trypsin treatment was repeated in the absence of CCCP, ColE9*^{AF647} was observed and present as fluorescent dots inside the cell. This indicated that the translocation of ColE9*^{AF647} across the OM was dependent on the presence of the PMF.

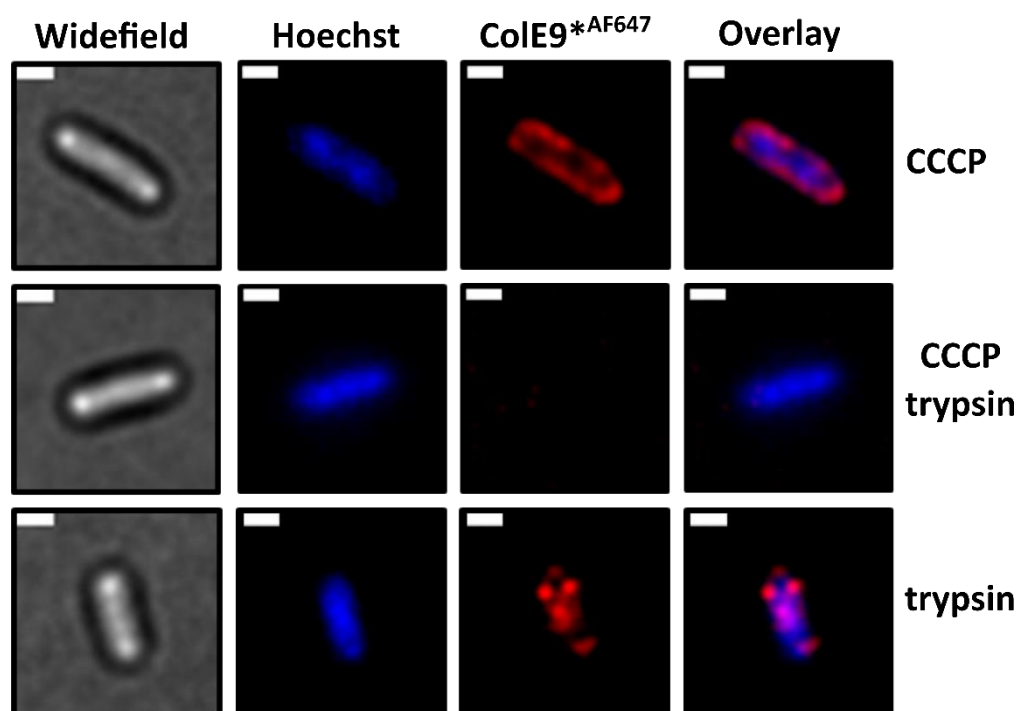


Figure 4. 7 - *E. coli* JM83 cells treated with CCCP and labelled with ColE9*AF647 followed by trypsin treatment.

E. coli JM83 cells were treated with CCCP, labelled with 1.5 μ M ColE9*AF647 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ColE9*AF647 still bound to the OM in the presence of CCCP, but fluorescence was lost after trypsin treatment. Labelling and trypsin treatment in the absence of CCCP, showed ColE9*AF647 fluorescence. Scale bar is 1 μ m.

The distribution of fluorescence intensity across the width of cells for OM labelling and trypsin treatment was determined (Section 2.5.12) (Figure 4. 8). CCCP treated cells showed fluorescence intensity for ColE9*AF647 in the OM in the cross-section of the cell, whereas the trypsin treated cells showed fluorescence intensity in the central region of the cell. CCCP and trypsin treated cells showed no fluorescence intensity across the cell.

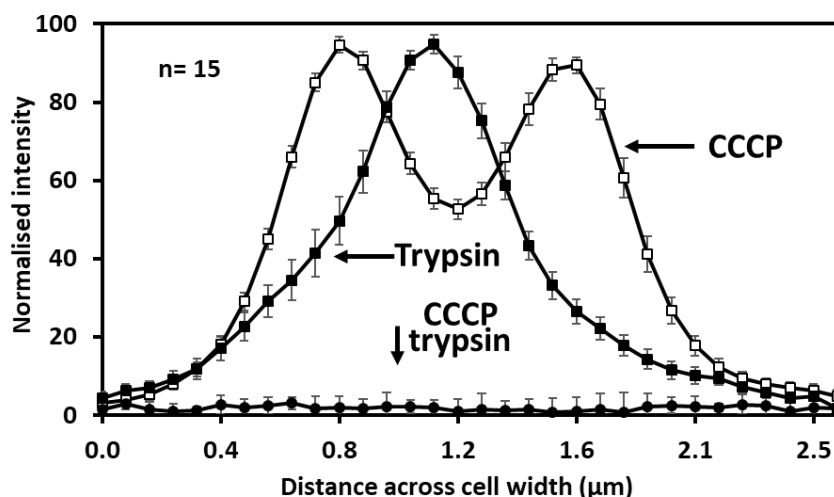


Figure 4. 8 - The distribution of CoIE9*AF647 fluorescence intensity across the width of bacterial cells when treated with CCCP or trypsin.

The fluorescence intensity of each cell condition was measured in ImageJ, $n = 15$ cells per condition (Section 2.5.12). CCCP treated cells showed fluorescence intensity of CoIE9*AF647 in the OM, the trypsin treated cells showed fluorescence intensity in the central region of the cell. CCCP and trypsin treated cells showed no fluorescence intensity across the cell. Mann-Whitney U-test performed between the CCCP and the trypsin data sets between cell distances of 0.8-1.6 μm , gave a value of $U = 0.04$, showing that there is a significant difference between these two data sets. Error bars represent % SEM.

To exclude the possibility that the observed effect of PMF dependent translocation was caused by the AF⁶⁴⁷ dye, this experiment was repeated using CoIE9*AF488 and CoIE9*AF568 using the same method. Figure 4. 9 shows that when *E. coli* JM83 cells are in the presence of CCCP, both CoIE9*AF488 and CoIE9*AF568 bind to the OM and form islands or puncta. When cells are further treated with trypsin, all fluorescence for CoIE9*AF488 and CoIE9*AF568 is lost. These results demonstrate the translocation of CoIE9 across the OM is dependent on the presence of the PMF irrespective of the fluorophore used.

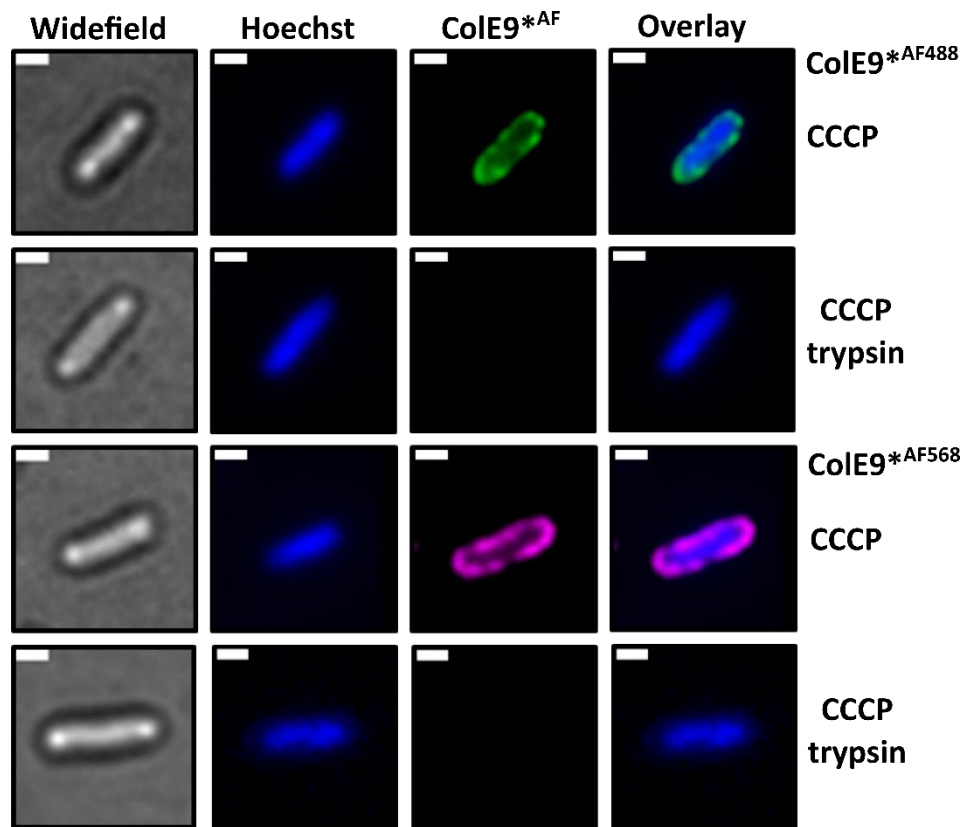


Figure 4. 9 - *E. coli* JM83 cells treated with CCCP and labelled with ColE9*AF488 and ColE9*AF568 followed by trypsin treatment.

E. coli JM83 cells were treated with CCCP, labelled with 1.5 μ M ColE9*AF488 or ColE9*AF568 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF488 (green) or ColE9*AF568 (pink). Overlays of the DNA and fluorescent colicin are also shown. ColE9*AF488 and ColE9*AF568 still bound to the OM in the presence of CCCP, but fluorescence was lost after trypsin treatment. Scale bar is 1 μ m.

To exclude the possibility that the observed effects of CCCP were non-specific, rather than PMF inhibition, the experiment was repeated using ColB, the pore-forming colicin that dissipates the PMF. *E. coli* JM83 cells were incubated with 2.5 μ M full-length ColB for 30 minutes, before labelling with ColE9*AF647 and further treated with trypsin to determine if translocation of ColE9*AF647 occurred (Figure 4. 10) (Section 2.5).

In the presence of ColB, ColE9*AF647 still bound and labelled the OM producing islands or puncta. When cells were further treated with trypsin however, all fluorescence for ColE9*AF647 was lost.

These data together show that translocation of ColE9*AF647 across the OM is dependent on the PMF.

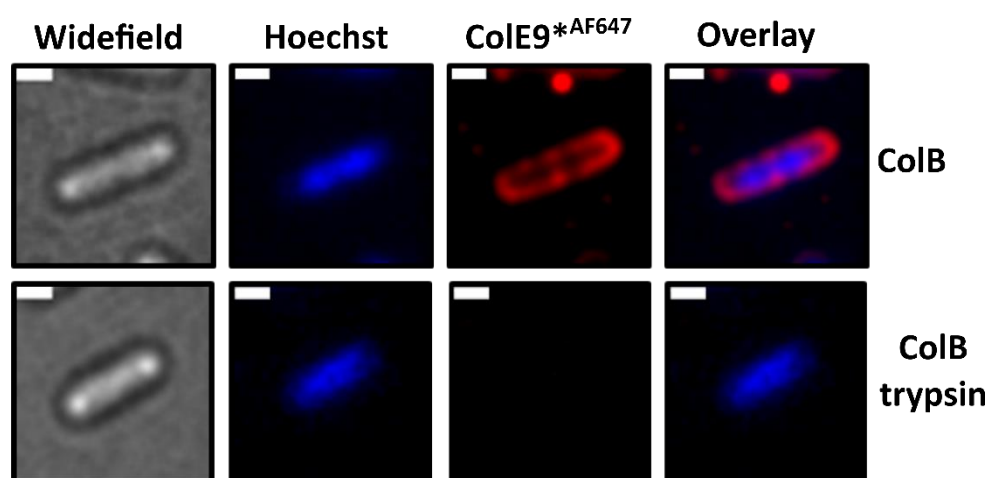


Figure 4. 10 - *E. coli* JM83 cells first treated with ColB and then labelled with ColE9*AF647 followed by trypsin treatment.

E. coli JM83 cells were treated with 2.5 μ M full-length ColB, labelled with 1.5 μ M ColE9*AF647 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ColE9*AF647 still bound to the OM in the presence of ColB, but fluorescence was lost after trypsin treatment. Scale bar is 1 μ m.

This method was repeated using CCCP to inhibit the PMF of *E. coli* JM83 cells, before incubating with ColB*AF647 to see if the translocation of ColB pore-forming colicin is also PMF-dependent. In the presence of CCCP, ColB*AF647 bound the OM and formed islands or puncta (Figure 4. 11). When cells were further treated with trypsin, all fluorescence for ColB*AF647 was lost. When labelling and trypsin treatment was repeated in the absence of CCCP, ColB*AF647 was observed inside the cell and videos showed movement in a track-like motion in the periplasm (data not shown). These data demonstrate conclusively that the translocation of ColB across the OM was also dependent on the PMF, which is in agreement with previously published data (Nairn et al. 2017), and therefore not confined to ColE9*AF647 or DNase colicins.

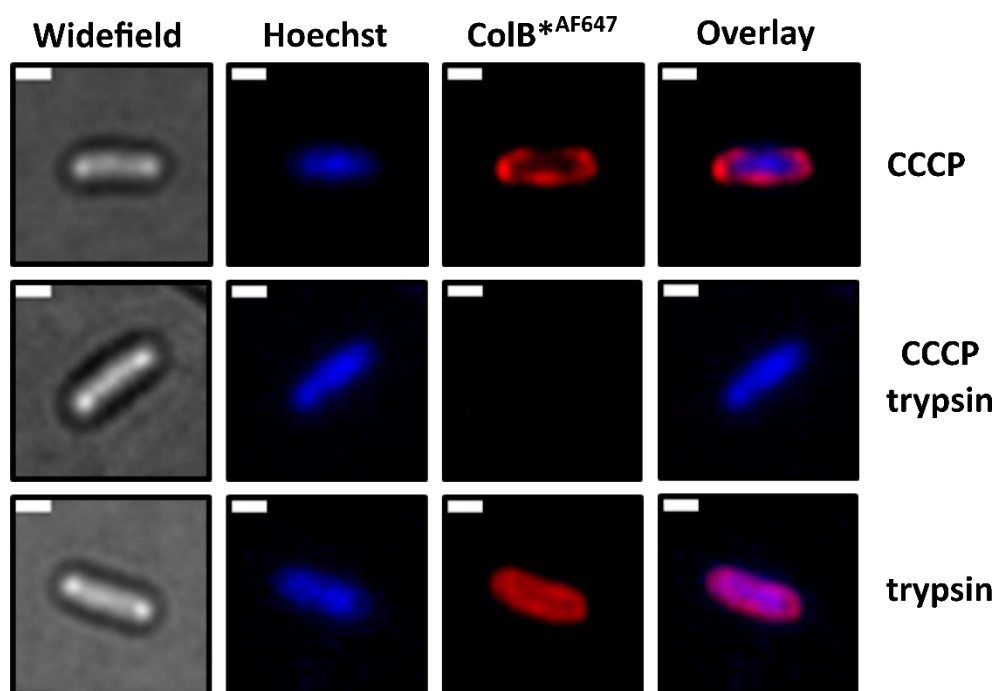


Figure 4. 11 - *E. coli* JM83 cells treated with CCCP and labelled with ColB*AF647 followed by trypsin treatment.

E. coli JM83 cells were treated with CCCP, labelled with 1.5 μ M ColB*AF647 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColB*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ColB*AF647 still bound to the OM in the presence of CCCP, but fluorescence was lost after trypsin treatment. Labelling and trypsin treatment in the absence of CCCP, showed ColB*AF647 fluorescence. Scale bar is 1 μ m.

ColE9 translocation is dependent on the electrical potential ($\Delta\Psi$) for translocation into *E. coli* at neutral pH

To further dissect the energy requirements of ColE9 translocation and identify if one of the two components of the PMF was dominant, experiments were performed with nigericin and valinomycin. *E. coli* JM83 cells were treated with either valinomycin or nigericin, which inhibits the electrical potential ($\Delta\Psi$) and the proton concentration gradient (Δ pH) of the PMF, respectively. Cells were labelled with ColE9*AF647 and further treated with trypsin to see if translocation of ColE9*AF647 occurred (Figure 4. 12) (Section 2.5).

In the presence of nigericin and valinomycin, ColE9*AF647 still bound and labelled the OM producing islands or puncta (Figure 4. 12). When cells were further treated with trypsin, all fluorescence for ColE9*AF647 with valinomycin treatment was lost, whereas internal ColE9*AF647

dots with nigericin treatment remained. This suggested that ColE9*AF647 could translocate when the proton concentration gradient (ΔpH) was inhibited but the electrical potential ($\Delta \Psi$) remained and was therefore using the electrical potential component of the PMF for cell entry.

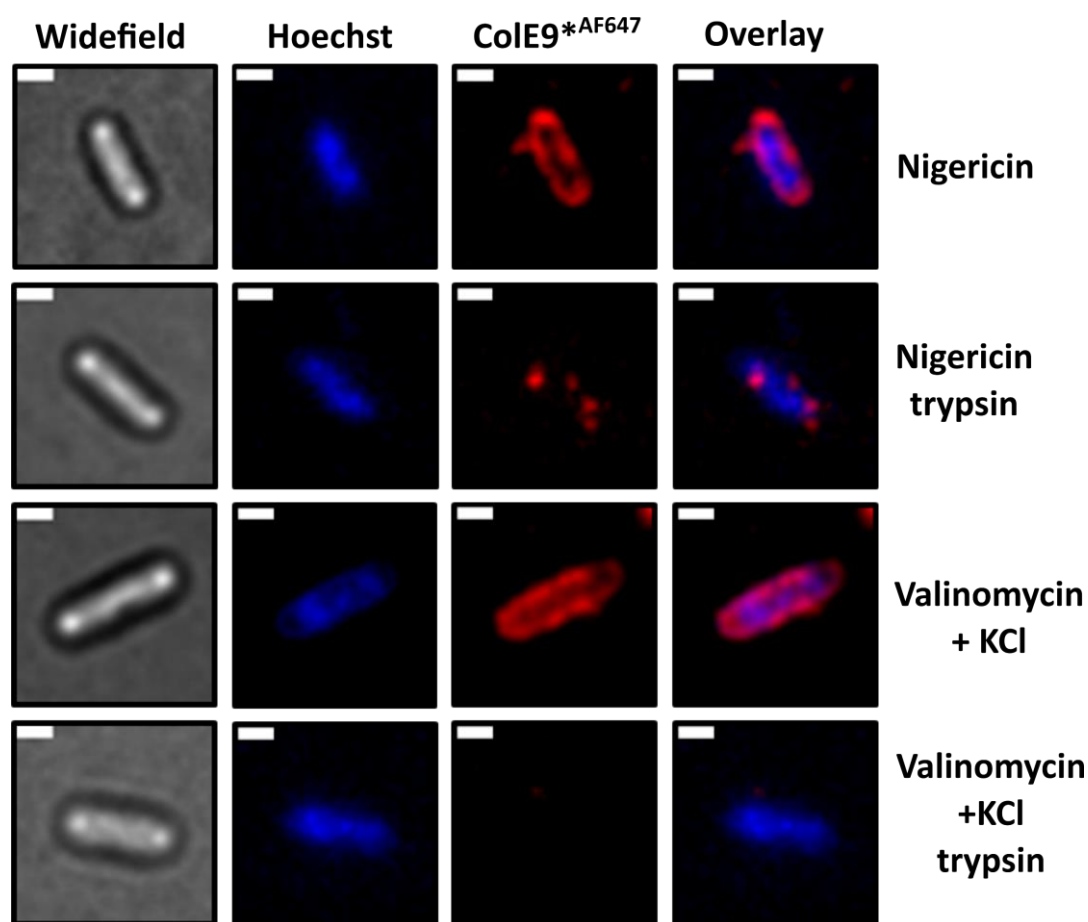


Figure 4. 12 - *E. coli* JM83 cells treated with nigericin or valinomycin and labelled with ColE9*AF647 followed by trypsin treatment.

E. coli JM83 cells were treated with nigericin or valinomycin, labelled with 1.5 μM ColE9*AF647 and 20 μM Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ColE9*AF647 still bound to the OM in the presence of nigericin and valinomycin. Fluorescence was lost after trypsin treatment with valinomycin, but remained with nigericin. Scale bar is 1 μm .

4.2.3 Investigating the impact of other proteins on the translocation of fluorescently-labelled ColE9

The impact of TolB on ColE9^{AF647} translocation

To assess the impact of TolB on ColE9^{AF647} translocation by microscopy, a mutation at position W39A within the TolB box of ColE9 (W39A) was used that abolishes the ColE9-TolB interaction (Hands et al. 2005; Loftus et al. 2006). A plate killing assay was performed using *E. coli* JM83 cells with ColE9 W39A^{AF647} (Section 2.6.1). Figure 4. 13 showed that there were no circular clear zones on the bacterial lawn and showed that in the absence of the interaction of ColE9 with TolB, no translocation and therefore no killing occurred.

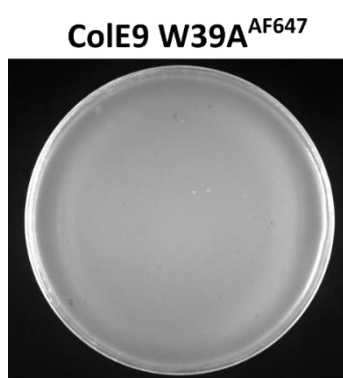


Figure 4. 13 - *In vivo* plate killing assay using ColE9 W39A^{AF647} on *E. coli* JM83 cells.

E. coli JM83 cells were grown and spotted with purified ColE9 W39A^{AF647} (Section 2.6.1). No zones of killing are evident.

Widefield microscopy was performed to identify if translocation of ColE9 W39A^{AF647} could nevertheless be visualised in *E. coli* JM83 cells. JM83 cells were labelled with ColE9 W39A^{AF647}, treated with trypsin and imaged using widefield microscopy (Figure 4. 14) (Section 2.5). ColE9 W39A^{AF647} still bound and labelled the OM of JM83 cells producing islands or puncta. When cells were further treated with trypsin all fluorescence was lost. Hence, OM translocation of ColE9 requires contact with TolB, as has been shown by earlier studies.

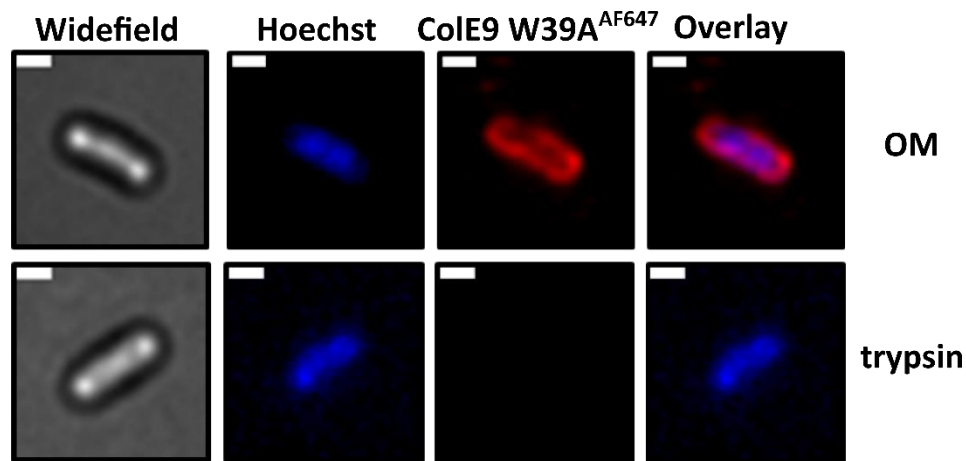


Figure 4. 14 - *E. coli* JM83 cells labelled with ColE9 W39A^{AF647} and treated with trypsin.

E. coli JM83 cells were labelled with 1.5 μ M ColE9 W39A^{AF647} and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9 W39A^{AF647} (red). Overlays of the DNA and fluorescent colicin are also shown. ColE9 W39A^{AF647} still bound to the OM, but fluorescence was lost after trypsin treatment. Scale bar is 1 μ m.

The impact of FtsH on ColE9^{AF647} translocation

FtsH is an essential ATP-dependent IM AAA+ ATPase/protease (Tomoyasu et al. 1993), that plays a key role in maintaining the balanced ratio of synthesised lipopolysaccharide (LPS) and phospholipids (PL) on the cell surface (Ogura et al. 1999; Langklotz, Baumann, and Narberhaus 2012). A shift in the LPS:PL ratio is lethal to the cell, therefore deletion of FtsH is lethal (Langklotz, Schakermann, and Narberhaus 2011).

FtsH also plays an essential role in the translocation of ColE9 DNase domain across the IM into the cytoplasm for cytotoxicity to occur (Walker et al. 2007). This was shown using a series of FtsH deletion and mutation strains that have been well characterised, that allow cell survival in the absence of FtsH through a suppressor mutation. *E. coli* strain AR3291 contains a null mutant FtsH (Δ *ftsH*). To prevent the lethal effect of FtsH deletion in this strain, a suppressor mutation *sfhC21* (Suppressor of FtsH) was found to upregulate FabZ, which assists in re-balancing the LPS:PL ratio (Ogura et al. 1999). Strain AR3289 contains a functional chromosomal FtsH and the suppressor mutation FabZ *sfhC21* (Ogura et al. 1999). AR3289 (*sfhC21*) was shown to proteolytically cleave σ^{32} , whereas AR3291 (*sfhC21* Δ *ftsH*) showed ~15-20-fold higher levels

of σ^{32} in comparison, due to the lack of cleavage (Tatsuta et al. 1998) and showed a colicin tolerance phenotype (Ogura et al. 1999). The PL levels in these two strains were not significantly affected, LPS levels were similar with 7.8 $\mu\text{g}/\text{mg}$ and 10.3 $\mu\text{g}/\text{mg}$ protein for AR3289 and AR3291, respectively (Ogura et al. 1999). Plasmid pIFH108 was generated containing the *ftsH* gene to complement its deletion in strain AR3291 (*sfhC21 Δ ftsH*) (Karata et al. 1999). Uninduced leaky expression from the IPTG-inducible plasmid was sufficient to provide levels of FtsH comparable to chromosomal FtsH (Karata et al. 1999). Further FtsH mutant plasmids were constructed to elucidate the function of regions of FtsH.

To further investigate the use and importance of FtsH for the translocation of ColE9 DNase domain across the IM, fluorescence microscopy was performed with ColE9*^{AF647}. Several FtsH mutant plasmids were used; pIFH108 F228R (Yamada-Inagawa et al. 2003) the normal function of which is as a bulky uncharged residue within the conserved pore 1 motif (Wang et al. 2001) (Figure 4. 15), in a loop involved in controlling the passage of substrates through the central pore of FtsH to the proteolytic chamber for cleavage. It is proposed to be important for the regulation of ATP hydrolysis, substrate binding and possibly unfolding (Yamada-Inagawa et al. 2003). Mutation F228 to arginine affects the ability of the loop to undergo conformational changes on ATP hydrolysis and therefore assistance with substrate translocation. F228R has a reduction in ATPase activity and inhibition of proteolytic activity against soluble substrates (Yamada-Inagawa et al. 2003). This mutation is referred to as the pore mutant.

Plasmid pIFH108 E255Q (Karata et al. 2001). E255 is a residue in the Walker B motif (Figure 4. 15), involved in the coordination of Mg^{2+} and a catalytic water molecule for the proteolytic cleavage of substrates (Karata et al. 2001). Mutation of E255 to glutamine prevents ATP hydrolysis, which prevents the conformational change of the central pore loops and translocation of substrates. In addition, protease activity is ATP-dependent, so it inhibits the

proteolytic cleavage of soluble substrates (Karata et al. 2001). This mutation is referred to as the ATPase mutant.

Plasmid pIFH108 H421Y (Karata et al. 1999). H421 is within the zinc-binding motif of the protease active site (Figure 4. 15) and coordinates the zinc ion for activity (Langklotz, Schakermann, and Narberhaus 2011). Mutation of H421 to tyrosine results in loss of proteolytic cleavage of soluble substrates *in vitro* and *in vivo* and a 75 % reduction in ATPase activity (Karata et al. 1999). This mutation is referred to as the protease mutant.

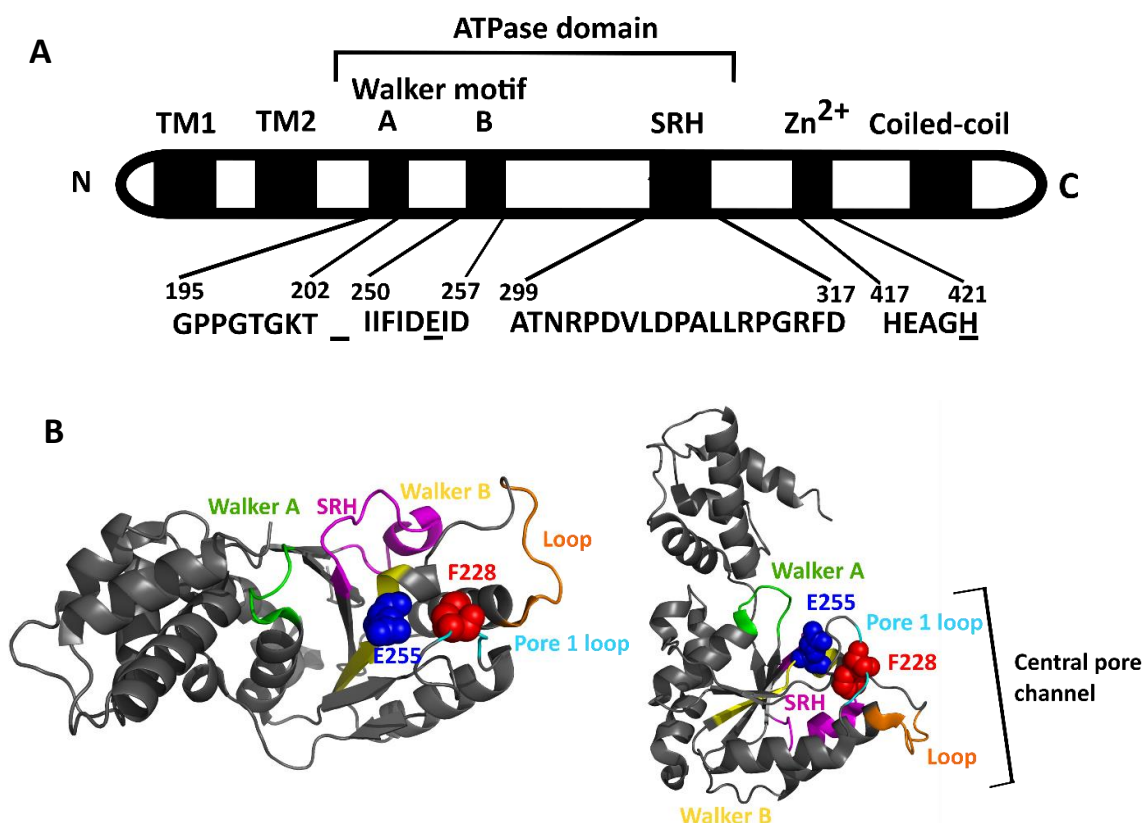


Figure 4. 15 - Structure and features of the FtsH AAA⁺ ATPase domain monomer in *E. coli*.

(A) The features of *E. coli* FtsH (Figure adapted from (Karata et al. 2001). (B) The structure of the FtsH ATPase domain monomer from residue 144-399 (Krzywda et al. 2002), PDB; 1LV7). FtsH is composed of two N-terminal transmembrane regions (TM1 and TM2), followed by the Walker A motif (residues G195-T202, green), Walker B motif (residues I250-D257, yellow), Second region of homology (SRH) (residues A299-D317, pink) of the AAA module, the pore 1 loop (residues 227-230, cyan), the loop (residues 266-273, orange), and a C-terminal Zinc-binding motif (Zn^{2+}) (residues H417-H421). Mutated residues used in this research are underlined in (A) at positions F228, E255 and H421. In (B) residue F228 is shown as a red sphere and E255 is shown as a blue sphere. The location of the central pore channel residues/loops is indicated.

To demonstrate the involvement of FtsH in ColE9 transport, plate killing assays were performed using the various *E. coli* wild-type, deletion, complementation (with wild-type FtsH, +WT) and mutant strains and plasmids of FtsH (Figure 4. 16). All strains were grown in both LB media and supplemented M9 minimal media. One observation was that their rate of growth was markedly different (Appendix I Table 2). Strain AR3289 containing the *sfhC21* suppressor mutation and chromosomal FtsH, grew at rates in both LB and M9 that was comparable to *E. coli* JM83 cells. Strain AR3291 Δ (*sfhC21* Δ *ftsH*) that contained no FtsH but the suppressor mutation, displayed markedly reduced growth, ~2.5-fold reduction in both LB and M9. Strain AR3291 Δ complemented with plasmid encoded wild-type FtsH (+WT), had a reduced growth ~1.5-fold in both LB and M9. All of the plasmid-encoded mutant strains, AR3291 +F228R/E255Q/H421Y had reduced growth rates, ~2.5-fold reduction in both LB and M9, like the AR3291 Δ strain. This is consistent with previous findings, where AR3291 strain reportedly grew ~2-fold slower than wild-type or AR3289 (Ogura et al. 1999). This showed that FtsH is important and plays a role in cell growth. FtsH deletion, mutation or FtsH plasmid expression levels severely impacted the rate of cell growth.

Plate killing assays were performed in LB with all strains and plasmids and spotted with wild-type ColE9 or ColE9^{AF647} (Figure 4. 16) (Section 2.6.1). AR3289 (*sfhC21*) showed very good killing by both wild-type ColE9 and ColE9^{AF647} to 57 pM, which is in contrast to JM83 cells where ColE9^{AF647} only killed down to 4.6 nM. This improved killing in the presence of both chromosomal FtsH and the hyperactive FabZ may be the result of a shift in the LPS:PL ratio towards more PLs, or an effect on the OMPs as an indirect result of the suppressor mutation. Alternatively, it could reflect slight differences between *E. coli* strains and their susceptibility.

AR3291 Δ (*sfhC21* Δ *ftsH*) showed no killing by wild-type ColE9 or ColE9^{AF647} in the absence of FtsH. AR3291 + WT showed killing by wild-type ColE9 down to 57 pM and by ColE9^{AF647} down to 4.6 nM, demonstrating that the plasmid-encoded wild-type FtsH can be expressed at levels to

complement its deletion from the strain. This also demonstrated that FtsH is essential for the translocation and killing by ColE9. AR3291 + F228R (pore), + E255Q (ATPase) or + H421Y (protease) mutant FtsH plasmids showed no killing by wild-type ColE9 or ColE9^{AF647}. The result of cell killing by wild-type ColE9 and ColE9^{AF647} using the AR3291 strains with the wild-type and mutant FtsH plasmids, was the same using leaky expression or with IPTG induction of the plasmid (data not shown).

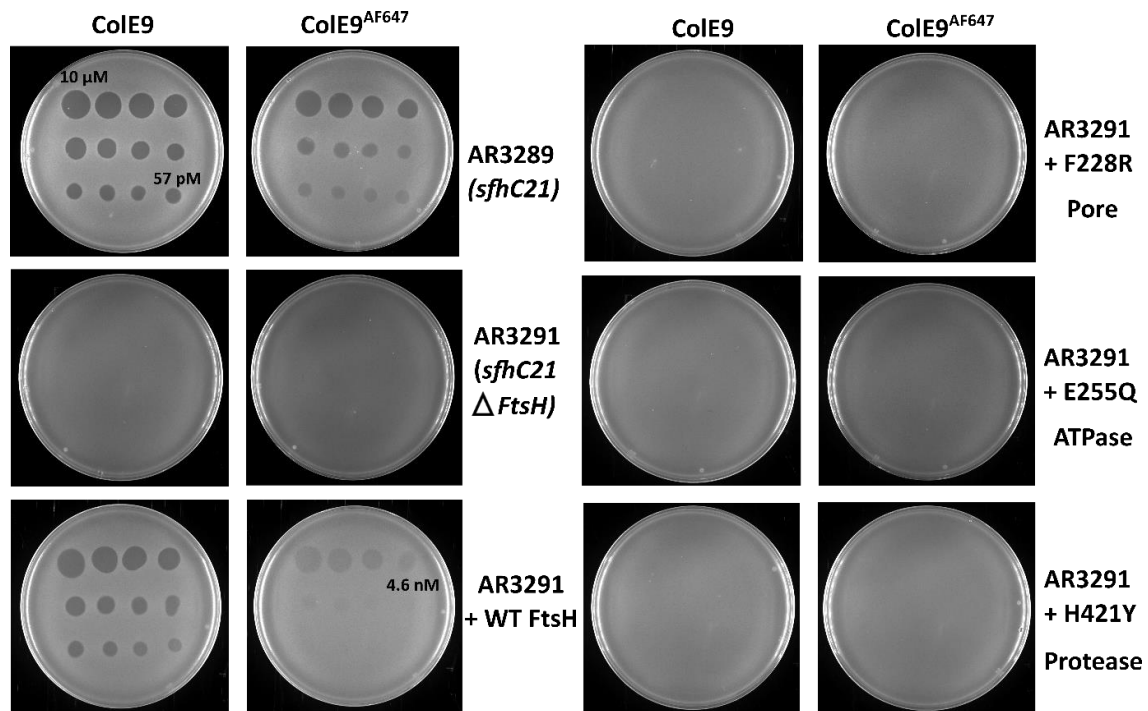


Figure 4. 16 - *In vivo* plate killing assay comparison of wild-type ColE9 and ColE9^{AF647} with *E. coli* FtsH strains in LB.

E. coli FtsH strains were spotted with wild-type ColE9 or ColE9^{AF647} from 10 μ M in 3-fold serial dilutions to 57 pM (Section 2.6.1). Colicin induced cell killing is indicated as zones of clearance in the bacterial lawn. AR3289 (*sfhC21*) showed very good killing by both wild-type ColE9 and ColE9^{AF647} to 57 pM. AR3291 (*sfhC21* Δ *FtsH*) showed no killing by wild-type ColE9 or ColE9^{AF647} in the absence of FtsH. AR3291 + WT FtsH (plasmid encoded wild-type FtsH) showed killing by wild-type ColE9 down to 57 pM and by ColE9^{AF647} down to 4.6 nM. AR3291 + F228R, + E255Q or + H421Y mutant FtsH plasmids showed no killing by wild-type ColE9 or ColE9^{AF647}.

Plate killing assays were repeated in M9 minimal media to confirm the effect seen was the same, as microscopy will be performed in M9 using ColE9*^{AF647} (Appendix I Figure 8). The only difference observed using M9 minimal media in plate killing assays, was the killing range. In LB with wild-type ColE9 this is usually 57 pM, in M9 it is ~4.6 nM. The reason for this is unknown

and may be down to the difference in salt affecting the ratio of OMPs for ColE9 translocation or the reduced rate of cell growth.

The lack of killing of the AR3291 Δ strain and the mutant FtsH plasmid strains (+F228R, +E255Q and +H421Y) by wild-type ColE9 and ColE9^{AF647} was expected as it had been published previously using ColE9 (Walker et al. 2007). It suggests that inhibition of substrate entry into the pore (F228R), lack of ATPase activity (E255Q), or the lack of proteolytic activity (H421Y), would all prevent the entry of ColE9 as a substrate into FtsH for the proteolytic cleavage of the cytotoxic domain, to pass across the IM into the cytoplasm and lead to cell death. Similar results were obtained in liquid culture experiments in M9 minimal media (Appendix I Figure 9).

The next question was whether these colicin insensitive cells still showed evidence of OM translocation by ColE9^{AF647}. However, imaging experiments with the FtsH deletion strain AR3291 and complementation with the FtsH mutants proved impossible due to aberrant cell growth, cell phenotypes, the small dimensions of the cells and the poor fluorescent labelling by ColE9^{AF647}. See Appendix II for details.

Widefield microscopy was performed to investigate the binding and translocation of ColE9^{AF647} using the various *E. coli* FtsH strains. Cells were incubated with ColE9^{AF647} before treating with trypsin (Section 2.5). The wild-type FtsH strains, AR3289 (chromosomal FtsH) and AR3291 + WT (plasmid FtsH), showed labelling of the OM and islands or puncta as seen previously in JM83 cells (Figure 4. 17). When cells were further treated with trypsin, internal fluorescence was observed, the same result as seen previously. This showed that in the presence of the suppressor mutation and the plasmid-encoded FtsH complement, ColE9^{AF647} can still bind and translocate into cells.

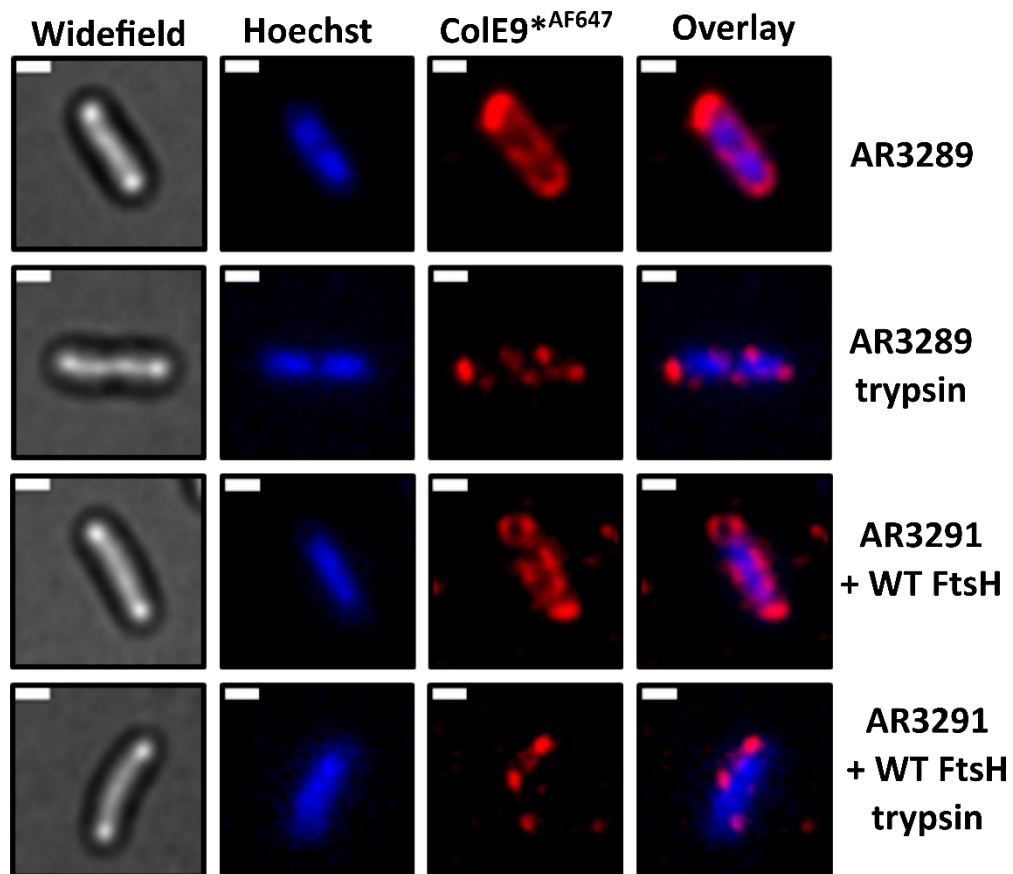


Figure 4. 17 - *E. coli* AR3289 (*sfhC21*) and AR3291 (*sfhC21 ΔftsH*) + WT FtsH cells treated with ColE9*AF647 followed by trypsin treatment.

E. coli AR3289 (*sfhC21*) and AR3291 (*sfhC21 ΔftsH*) complemented with wild-type FtsH plasmid (+WT) cells were labelled with 1.5 μ M ColE9*AF647 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ColE9*AF647 bound to the OM and there was remaining internal fluorescence after trypsin treatment. Scale bar is 1 μ m.

4.2.4 Investigating the effect of fluorescent labelling of ColE9 T-domain and R-domain on ColE9 translocation

Previous studies suggest that the T-domains and R-domains of ColE2 (Duche 2007) and ColA (Benedetti et al. 1992) remain bound to the cell surface after translocation of the C-domain, which in some cases is required for cytotoxicity. In the case of ColE9, evidence has shown that the C-domain translocates into the cytoplasm (Section 4.2.1), however the location of the T- domain and R-domain after translocation are unknown. They could remain on the cell surface,

or translocate with the C-domain into the periplasm. In addition, it is unknown how much of each domain enters the cell during translocation.

To investigate their location, ColE9 was mutated to introduce cysteines within the T-domain and R-domain for fluorescent labelling, to investigate the effect this had on cell entry of ColE9 into *E. coli*. ColE9 had a cysteine introduced at position K298C within the T-domain, position S420C within the R-domain, which is before the FtsH proteolytic cleavage site, and at position S451C in the R-domain, after the FtsH proteolytic cleavage site (de Zamaroczy and Buckingham 2002). The proteolytic cleavage site is the region of the colicin that is cleaved and which translocates to the cytoplasm to cause cell death (Figure 4. 18). These constructs were labelled with AF⁶⁴⁷ dye (Section 2.3.5) and called ColE9 K298C^{AF647}, ColE9 S420C^{AF647} and ColE9 S451C^{AF647}.

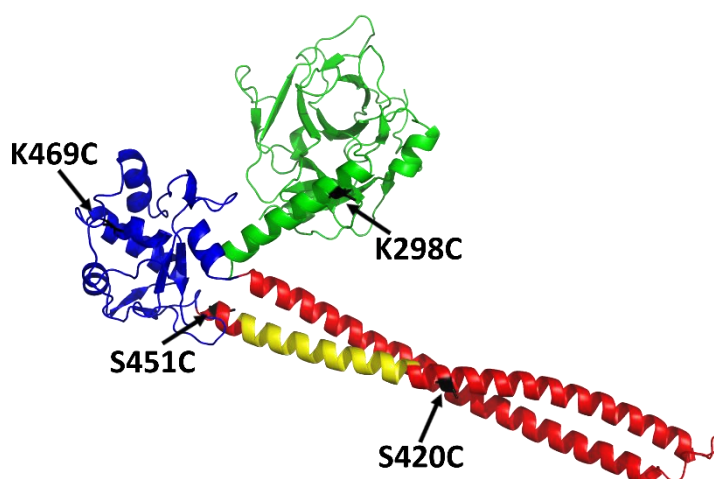


Figure 4. 18 - Alternative labelling sites in ColE9 with AF⁶⁴⁷ dye.

ColE9 was mutated at position K298C within the T-domain (green), position S420C within the R-domain (red) before the proteolytic cleavage region (yellow) and at position S451C in the R-domain after the proteolytic cleavage site. Position K469C within the cytotoxic DNase domain (blue) is shown for comparison.

Plate killing assays were performed using either *E. coli* JM83 cells or *E. coli* JW2203 OmpF⁺ expressing cells, with unlabelled or fluorescently-labelled constructs ColE9 K298C, ColE9 S420C and ColE9 S451C (Section 2.6.1). JW2203 OmpF⁺ cells were used, as ColE9 has a preference for OmpF (Housden et al. 2010), allowing the visualisation of killing if cytotoxicity is poor. Figure

4. 19 showed that using OmpF⁺ cells, ColE9 and the fluorescently-labelled constructs all killed efficiently down to ~57 pM.

Using JM83 cells, all of the unlabelled constructs killed, but the range of cytotoxicity was reduced compared to wild-type ColE9. ColE9 K298C killed down to ~4.6 nM, ColE9 S420C killed down to ~57 pM with faint circles and therefore less efficiently than wild-type ColE9 and ColE9 S451C killed down to ~1.5 nM. When fluorescently-labelled, all of the constructs killed JM83 cells, with little difference with the attached fluorescent label for ColE9 K298C^{AF647} and ColE9 S420C^{AF647} constructs, however ColE9 S451C^{AF647} killed very poorly with a very faint clearance zone at 10 µM. This suggests that introduction of cysteines at position K298C, S420C and S451C within the T-domain and R-domain, reduced the cytotoxicity but that all constructs were still capable of translocating and causing cell death. This result also showed that the addition of the AF⁶⁴⁷ dye had a large effect on reducing the cytotoxicity of ColE9 S451C with >6600x reduction. The location of the dye clearly has an impact on cell entry, as ColE9 S451C^{AF647} had dramatically reduced cytotoxicity in JM83 cells, but had near wild-type level killing using OmpF⁺ cells, as did ColE9 K298C^{AF647} and ColE9 S420C^{AF647}.

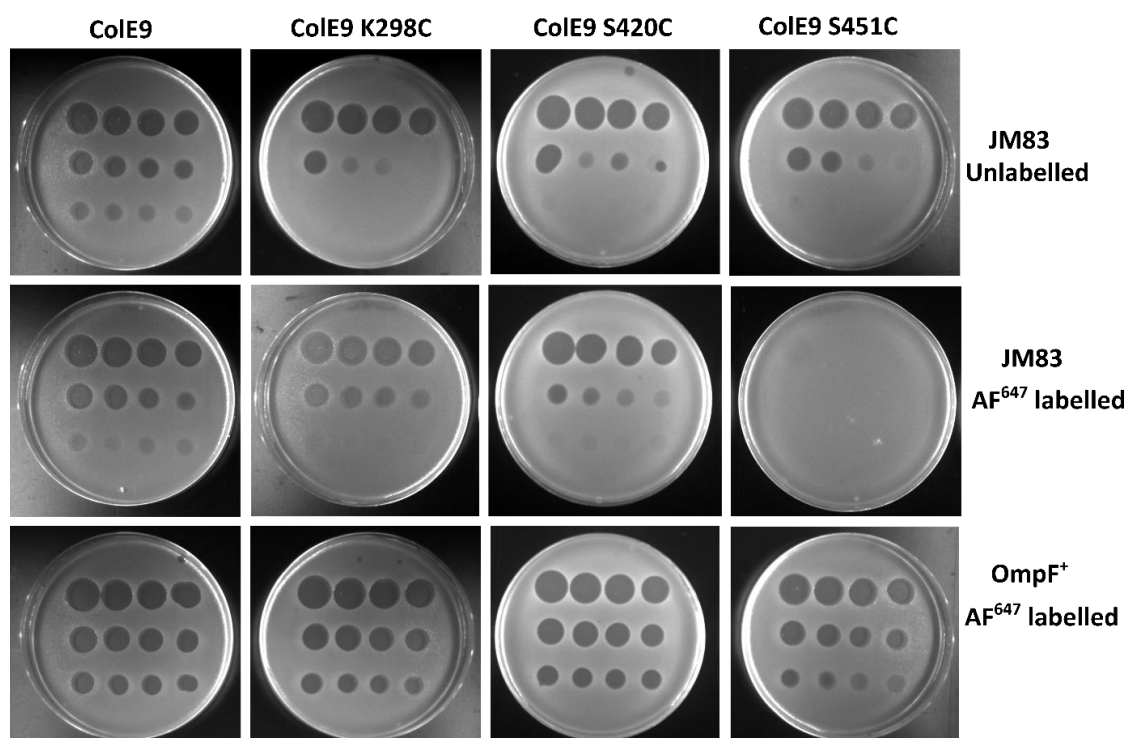


Figure 4. 19 - *In vivo* plate killing assay comparison of wild-type ColE9, unlabelled and fluorescently-labelled ColE9 K298C, ColE9 S420C and ColE9 S451C.

E. coli JM83 cells and *E. coli* JW2203 OmpF⁺ cells were spotted with wild-type ColE9, unlabelled or AF⁶⁴⁷ labelled ColE9 constructs from 10 μ M in 3-fold serial dilutions to 57 pM (Section 2.6.1). Colicin induced cell killing is indicated as zones of clearance in the bacterial lawn. All of the unlabelled constructs killed JM83 cells, with wild-type ColE9 killing down to 57 pM, ColE9 K298C to $\sim$ 4.6 nM, ColE9 S420C to $\sim$ 57 pM and ColE9 S451C to $\sim$ 1.5 nM. All of the fluorescently-labelled constructs killed JM83 cells, with little difference with the attached fluorescent label for ColE9 K298C^{AF647} and ColE9 S420C^{AF647} constructs, however ColE9 S451C^{AF647} killed very poorly with a very faint clearance zone at 10 μ M. Using OmpF⁺ cells, all constructs killed efficiently down to 57 pM.

Widefield microscopy was performed on *E. coli* JM83 cells using ColE9 K298C^{AF647}, ColE9 S420C^{AF647} and ColE9 S451C^{AF647} and further treated with trypsin (Section 2.5) (Figure 4. 20). All constructs labelled the surface of JM83 cells and fluorescence remained for all the constructs after trypsin treatment, suggesting translocation of the T-domain and R-domain into the cell. Typical OM labelling and translocated molecules were observed with ColE9 K298C^{AF647} and ColE9 S451C^{AF647}. However, ColE9 S420C^{AF647} gave a poor signal-to-noise ratio with very high background and anomalous OM labelling and internal fluorescence after trypsin treatment. This could suggest that the construct had a higher tendency to adhere to the cells and the background. This is preliminary data and requires further investigation, including identifying the location of the fluorescence of each construct through spheroplasting experiments. The results suggest translocation of the entire molecule across the OM, which would discount previous models for the translocation of other colicins, where the colicin remains bound to the OM while killing. However, this would need further investigation through fluorescent-labelling of other positions of the T-domain and R-domain.

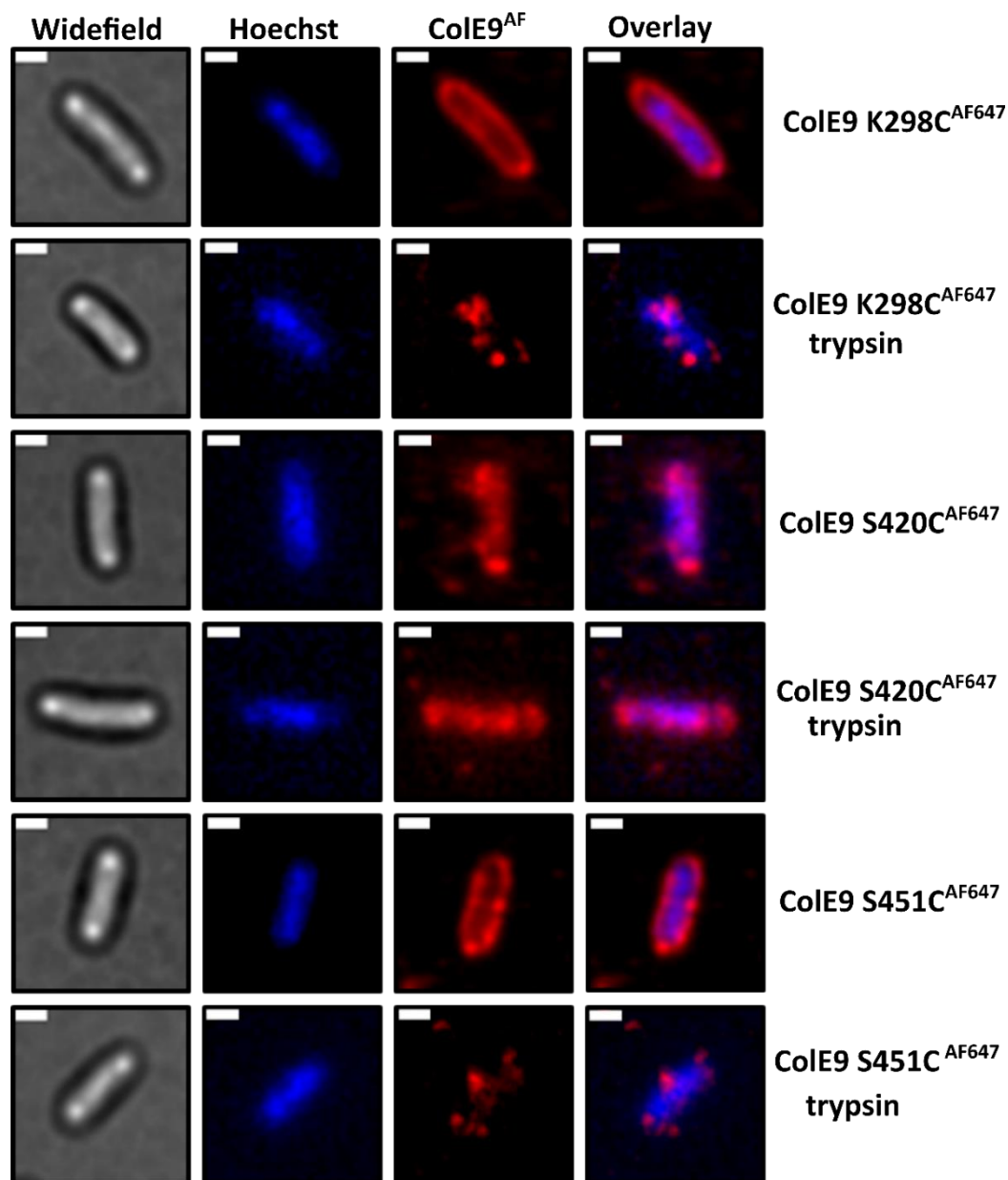


Figure 4. 20 - *E. coli* JM83 cells labelled with ColE9 K298C^{AF647}, ColE9 S420C^{AF647} and ColE9 S451C^{AF647} followed by trypsin treatment.

E. coli JM83 cells were labelled with 1.5 μ M ColE9 K298C^{AF647}, ColE9 S420C^{AF647} or ColE9 S451C^{AF647} and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of AF⁶⁴⁷ (red). Overlays of the DNA and fluorescent colicin are also shown. All constructs bound to the OM and there was remaining internal fluorescence after trypsin treatment, suggesting translocation of the T-domain and R-domain into the cell. ColE9 S420C^{AF647} gave a poor signal-to-noise ratio and anomalous OM labelling and internal fluorescence after trypsin treatment. Scale bar is 1 μ m.

4.3 Discussion

Questions that this chapter aimed to address, were to use the fluorescence-based microscopy assay to demonstrate where the fluorescently-labelled ColE9 molecules were located inside cells. Next, to investigate the PMF-dependence for the translocation process of ColE9 and dissect its energy requirements. In addition, to investigate the impact of FtsH on translocation of fluorescently-labelled ColE9 into the cell. Finally, to investigate the effect of fluorescently-labelling the T-domain and R-domain of ColE9 on its translocation.

ColE9*^{AF647} translocates to the cytoplasm of *E. coli*

The first question, was to identify the location of fluorescently-labelled ColE9 inside the cell. The lethal function of ColE9 is due to its nuclease activity, therefore the cytotoxic domain must reach the cytoplasm. The spheroplasting results of this chapter demonstrate that AF⁶⁴⁷ labelled molecules conjugated to the DNase do indeed reach the cytoplasm of the cell. Additional evidence to support this contention will be presented in Chapter 5, through tracking of individual molecules and through its complex with Im9-eGFP which leads to protection of the cells.

The translocation of ColE9 across the OM is dependent on the proton motive force

The next question, was to investigate the energy-dependence of ColE9 translocation. This chapter demonstrated that the translocation of ColE9*^{AF647} across the OM was dependent on the PMF, shown both by its inhibition using CCCP and the pore-forming colicin ColB. In addition, the electrical potential ($\Delta\Psi$) appears to be the dominant transmembrane gradient used for entry, based on the selective inhibition by valinomycin. The importance of TolB in the translocon and its role in the transduction of the PMF for ColE9 translocation, was observed through its mutation by microscopy. In addition, the data showed that ColB translocation in the periplasm was also PMF-dependent.

The requirement for ColE9 translocation to have a complete network of protein-protein interactions involving TolQRAB has been shown previously. Recently, this was shown along with

the PMF-dependence of these interactions by fluorescence microscopy (Rassam et al. 2018). TolA-GFP freely diffuses in the IM (demonstrated by FRAP), however on addition of fluorescently-labelled ColE9^{S-5}, TolA-GFP becomes restricted and co-localised with ColE9 (loss of FRAP). Therefore, intermembrane crosstalk between ColE9 and the Tol system is needed for entry. Moreover, inhibition of the PMF by CCCP prevented the interaction of ColE9 with TolA-GFP (Rassam et al. 2018). These findings by Rassam et al. are therefore in agreement with the work of Vankemmelbeke et al., who showed the requirement of the complete network of interactions of the Tol system and the PMF for Im9 release and presumably translocation of ColE9 (Vankemmelbeke et al. 2009). In addition to this, the findings of this work showed that translocation of ColE9 across the OM is dependent on the PMF, in particular the electrical potential ($\Delta\Psi$).

Therefore, these findings for the use of the PMF for cell entry is not confined to just ColE9. It agrees with the findings for OM translocation of the Ton-dependent bacteriocins; ColB, Colla, ColD and Pyocin S2, the Tol-dependent bacteriocin ColE5 and the IM translocation step for CDIs, as described in Introduction Section 4.1 (Nairn et al. 2017) (Ruhe et al. 2014) (Mende and Braun 1990), (Bassford et al. 1976) (White et al. 2017). This shows that use of PMF for cell entry is independent on their use of the Tol or Ton system and their modes of cytotoxicity in cells and may be a common mechanism for entry of other protein toxins.

The translocation of ColE9 is dependent on the electrical potential ($\Delta\Psi$) at neutral pH

To further dissect the energy requirements of ColE9 translocation and identify if one of the two components of the PMF was dominant, experiments were performed with nigericin and valinomycin. The results in this chapter demonstrate that ColE9^{*AF647} can translocate when the proton concentration gradient (ΔpH) is inhibited by nigericin and the electrical potential ($\Delta\Psi$) is present. ColE9 therefore uses the electrical potential of the PMF for cell entry.

Although both the proton concentration gradient (ΔpH) and the electrical potential ($\Delta\Psi$) gradient are present simultaneously, the dominance and relative amount of energy distributed between the two gradients can differ. When cells are grown at neutral pH the PMF is predominantly the electrical potential ($\Delta\Psi$) (Borisov et al. 2011) (Padan, Zilberstein, and Rottenberg 1976). When the surrounding environment becomes more acidic, there are more protons available to shift the balance predominantly to the proton concentration gradient (ΔpH). As one gradient increases, the other decreases to maintain a constant PMF energy level (Trchounian, Blbulyan, and Trchounian 2013) (Bakker and Mangerich 1981). It would therefore not be surprising that experiments carried out at pH 7-7.5 using the PMF, proteins including ColE9 would predominantly use the electrical potential for entry. ColE9 entry may shift to use of the proton concentration gradient at more acidic pH and this could be further investigated to see the environmental dependence of energy-driven entry into cells.

In addition to showing the requirement of the PMF for Im9 dissociation, Vankemmelbeke et al. used nigericin and valinomycin at pH 7.6, to show Im9 dissociation appeared to be dependent mainly on the electrical potential ($\Delta\Psi$) of the PMF (Vankemmelbeke et al. 2009). This finding is therefore in agreement with the finding of this thesis that the electrical potential is used not only for Im9 dissociation, but also for ColE9 translocation.

The dominant use of the electrical potential of the PMF for proteins has been reported. For example, Strahl et al. studied the effect of the PMF on the localisation of cell division related proteins in *Bacillus subtilis* using GFP/YFP-tagged MinD, FtsA and MreB. They showed using CCCP, nisin, pore-forming Colicin N, nigericin and valinomycin at pH 7.5, that the electrical potential was directly involved in protein localisation. The authors suggested that the electrical potential may play a role in stimulating an interaction between the amphipathic helix of the proteins and the lipid bilayer or through a change in membrane fluidity (Strahl and Hamoen 2010).

The use of the electrical potential for protein transport has also been seen in other protein systems. In the case of mitochondria, protein import across the inner mitochondrial membrane has been shown to use both ATP hydrolysis and the PMF. In some cases this has been shown to use the electrical potential (Voos et al. 1999). ATP is used to power the import motor matrix heat shock protein 70 (mtHsp70) in association with the translocase of the IM (TIM). The electrical component is suggested to exert an electrophoretic effect on positively charged polypeptides to promote their initial insertion in the IM membrane and their unfolding (Huang, Ratliff, and Matouschek 2002). This has been shown for the import of mitochondrial precursor proteins like ADP/ATP carrier using CCCP, nigericin and valinomycin (Schleyer, Schmidt, and Neupert 1982) (Martin, Mahlke, and Pfanner 1991) (Pfanner and Neupert 1985).

For chloroplasts, it has been reported that only ATP is required for the import of precursor proteins into the stroma across the envelope membranes. This was shown for precursors to ferredoxin and ribulose-1,5-bisphosphate carboxylase/oxygenase (Theg et al. 1989). Proteins that are transported into the thylakoid lumen have been shown to use several pathways including the Sec and SRP-like pathway which uses ATP and ΔpH , spontaneous insertion or a Tat ΔpH (Twin-arginine translocation) pathway that uses only the proton concentration gradient for entry. Therefore, energy requirements for thylakoid transport appears to be protein specific. Use of only the proton gradient (ΔpH) for thylakoid translocation was shown for the 23 kDa and 17 kDa proteins of the photosynthetic oxygen-evolving complex using nigericin and valinomycin (Mould and Robinson 1991) (Cline, Ettinger, and Theg 1992). This may reflect the difference in the orientation of the PMF, where in bacteria and mitochondria protons are pumped out of the matrix/cytoplasm, whereas in the thylakoid membrane of chloroplasts, protons are pumped into the thylakoid lumen. It could also reflect the balance and contribution of the two PMF components, in which the electrical potential is said to be small under steady-state conditions (Mould and Robinson 1991) (Mant et al. 1995).

Therefore, across the different protein translocation systems in bacteria, mitochondria and chloroplasts, there are examples of proteins being dependent on ATP hydrolysis, the proton gradient (ΔpH), the electrical potential ($\Delta\Psi$) or both as the PMF.

The impact of FtsH on ColE9^{AF647} translocation

Another question this chapter aimed to address was to investigate the impact of FtsH on translocation of ColE9^{AF647}. FtsH plays an essential role in the retro-translocation of ColE9 DNase domain across the IM into the cytoplasm for cytotoxicity to occur (Walker et al. 2007). To further investigate the use and importance of FtsH for ColE9 retro-translocation, plate killing assays and microscopy was performed with ColE9^{AF647}. Strain AR3289 (*sfhC21*) and AR3291 (*sfhC21 ΔftsH*) were used, in combination with the plasmid-encoded wild-type FtsH and mutant plasmids pIFH108 F228R (pore), E255Q (ATPase) and H421Y (protease).

Plate killing assays showed efficient killing of strains AR3289 and AR3291 + WT FtsH (plasmid-encoded WT FtsH) by wild-type ColE9 and ColE9^{AF647}. AR3291 Δ and the complemented plasmid-encoded mutant FtsH plasmids showed no killing, in agreement with the findings of walker et al. that a functional FtsH is required for ColE9 translocation (Walker et al. 2007). Widefield microscopy was performed using AR3289 and AR3291 + WT FtsH and showed OM labelling and translocation of ColE9^{AF647}. However, FtsH deletion strain AR3291 and FtsH mutant plasmids, all showed aberrant cell growth, cell phenotypes, small cell dimensions and poor fluorescent labelling by ColE9^{AF647}, making microscopy impossible. There are several possible biological explanations for why these cell phenotypes could have occurred in these strains, which is further addressed in Appendix II.

The effect of fluorescent labelling of ColE9 T-domain and R-domain on ColE9 translocation

The main focus of this chapter was to identify the location of the fluorescently-labelled DNase domain within the cell, which was found to be located in the cytoplasm. To contribute to the understanding of the mechanism of ColE9 translocation, I aimed to investigate the location of the T-domain and R-domain of ColE9 after translocation.

The location of the T-domain and R-domain after translocation of other colicins has been investigated previously. The T-domain and R-domain of ColE2 were shown to remain bound to the surface via OmpF and BtuB, respectively, even after translocation of the C-domain into cells (Duche 2007). This was shown through competition experiments using cells pre-incubated with ColE2, followed by addition of ColA and ColE1, that use the same receptors and Tol system for entry. No pore-forming cytotoxicity by ColA or ColE1 was observed and therefore no entry. Further to this, incubation of cells with active or inactive ColE2 for long periods to allow for complete translocation, still prevented group A colicins from cell entry. Trypsin protection experiments showed the R-domain remained bound to BtuB at the surface and the T-domain remained bound to the Tol system, even after translocation of its cytotoxic domain.

In addition to this, domains of ColA remained at the surface after translocation which was necessary for cytotoxicity (Benedetti et al. 1992). Through the method of detection of potassium efflux after pore-formation by ColA, the authors showed that on addition of trypsin, efflux arrested, suggesting ColA was still in contact with the outside of the cell which was necessary for cytotoxicity. Trypsin treatment of ColA *in vitro* cleaves into 15-18 kDa fragments. When ColA bound to cells was incubated with trypsin, a 38 kDa fragment was obtained from the cell envelope and a 15-18 kDa fragment from the supernatant, showing that part of ColA is protected within the cell envelope when bound and part is exposed to the surface which gets cleaved.

Both of these examples suggested that the T-domain and R-domain translocates into the cell and that parts of the domains remain bound to the surface even after colicin translocation. However, it has not been determined how much of each domain translocates and how much remains surface-bound.

To investigate the effect on cell entry of ColE9 into *E. coli* cells, cysteines were introduced within the T-domain and R-domain for fluorescent labelling. The results in this chapter demonstrated that fluorescently-labelling the T-domain and R-domain did not restrict ColE9 entry, as shown by plate killing assays. Constructs were incubated with cells, followed by trypsin treatment and imaged by widefield microscopy. Fluorescence could be visualised after trypsin treatment. This finding suggests that the whole colicin (including the T-domain and R- domain) translocates into the cell.

When ColE9 binds to BtuB on the cell surface, the distal region of the T-domain, the R- domain and the C-domain are localised at the cell surface. This was shown through the introduction of enterokinase (EK) cleavage sites in ColE9^{S-S} and testing exposure *in vitro* and *in vivo* (Zhang et al. 2008). The authors identified that the first 61-residues of the T-domain were protected from cleavage, due to its translocation through OmpF. The extreme distal region of the T-domain at residue 83 showed partial protection from EK cleavage and was therefore not occluded by OmpF. Whereas the ordered T-domain, R-domain and C-domain showed no protection from EK cleavage (Zhang et al. 2008). Showing that when ColE9 first binds, the R- domain, C-domain and ordered T-domain are surface-exposed. In addition, Housden et al. showed that residues 1-83 of the IUTD were important for the recruitment of OmpF through deletion analysis and complex formation (Housden et al. 2005). Also that the IUTD contained two OmpF binding sequences (OBS1 and OBS2) that bound within OmpF by ITC (Housden et al. 2013) and that the two OBS sequences occluded the lumen of OmpF in differing binding

orientations, through channel conductance measurements and fluorescence microscopy (Housden et al. 2018) (Figure 1. 15).

The logical model for colicin translocation, is that the entire unfolded colicin gets pulled through OmpF from the N-terminal IUTD, T-domain, R-domain through to the C-terminal cytotoxic domain, via its interaction with the energised Tol/Ton system. It is hypothesised that following colicin binding, the long coiled-coil R-domains, in the case of ColIa ~160 Å (Wiener et al. 1997) and for ColE3 and ColE9 ~100 Å (Soelaiman et al. 2001) (Klein et al. 2016), unfold, translocate into the cell for the purpose of spanning the periplasmic space (>150 Å) to deliver the C-domain to the IM. This model would support the translocation of the T-domain and R-domain into the cell and therefore explain the presence of the fluorescent labels after trypsin treatment. However, the microscopy results are not conclusive of translocation of the entire colicin and could still show that part of the T-domain and R-domain could remain bound to the cell surface, but cannot be visualised as only three residues are fluorescently-labelled. This could be further investigated by the introduction of fluorescent labels elsewhere in the T-domain and R-domain and probing their fluorescence after trypsin treatment.

One finding from the microscopy data collected for ColE9*^{AF647} translocation in Chapters 3 and 4, was that from the initial high OM labelling by ColE9*^{AF647}, only a few molecules of ColE9*^{AF647} translocate inside the cytoplasm after trypsin treatment, which is further addressed in Chapters 5 and 6. It is unclear if this small fraction that translocates remains bound to the cell surface via its T-domain and R-domain. One way in which this could be investigated is through the saturation of the OM with unlabelled ColE9*, followed by addition of ColE9*^{AF647}, in which fluorescence would only be observed if the fraction of unlabelled ColE9* that translocates had entirely dissociated from the OM.

Due to the similarity between ColE9 and ColE2 (Appendix I Figure 1), it could be assumed that only a small fraction of ColE2 would translocate into the cell. If this were the case, it could

suggest that the majority of receptors would be occupied by un-translocated ColE2, which would block further binding and cytotoxicity by ColA/ColE1 (Duche 2007). Therefore, it is unclear if the work of Duche et al. shows that translocated ColE2 dissociates entirely or remains surface-bound.

It could also be possible that the fluorescently-labelled T-domain and R-domain have remained on the surface after trypsin treatment. It is unclear how much of the colicin gets cleaved off the surface after translocation and fragments of the T-domain and R-domain could remain bound to BtuB or OmpF. This could be investigated by saturating the OM of cells with unlabelled ColE9*, treating with trypsin, followed by addition of ColE9*^{AF647} and seeing if fluorescence is observed. In addition, spheroplasting experiments could be used to identify if the T-domain and R-domain have translocated into the periplasm. It is unlikely that the fluorescence of the T-domain and R-domain could remain on the outside of the cell after trypsin treatment, due to the high level of trypsin used and presence of multiple trypsin cleavage sites on ColE9.

To summarise, the observation of remaining fluorescence after trypsin treatment using fluorescently-labelled T-domain and R-domain of ColE9, suggests that the whole colicin translocates into the cell. However, more controls would be required for it to be conclusive. In addition, this finding using ColE9 may not be representative of all colicins, including ColE2 and ColA. Colicins may differ in their entry mechanisms, which may result from their differing modes of cytotoxicity.

4.4 Conclusions

The work presented in this chapter showed the translocation of ColE9*^{AF647} into the cytoplasm *in vivo* and ColB to the periplasm using the fluorescence microscopy method. Investigation into the energy-dependence *in vivo*, showed that translocation of ColE9*^{AF647} was dependent on the PMF and suggested that the electrical potential ($\Delta\Psi$) was the dominant transmembrane gradient used. The effect of PMF-dependence for cell entry was found to be irrespective of the fluorophore used to study import and of the choice of protonophore or ionophore used to inhibit the PMF. ColB translocation into the periplasm was also PMF-dependent.

The requirement of TolB to have a complete network of protein-protein interactions for the translocation of ColE9 was confirmed using the *in vivo* fluorescence microscopy method, thereby providing proof-of principle evidence that this technique can be used to elucidate the mechanism of cell entry and the proteins involved. The impact of FtsH in the translocation of ColE9 was observed through repetition of plate killing assays, previously reported. Research into the involvement of FtsH and its different features using fluorescence microscopy was hampered however by distorted cell morphologies. Finally, work in this chapter provided *in vivo* evidence to suggest that the T-domain and R-domain of ColE9 translocates across the OM into the cell to allow the DNase domain to access the cytoplasm to cause cell death.

5 Chapter 5 - Analysing ColE9*^{AF647} diffusive behaviour when translocated in to live *E. coli* cells

5.1 Introduction

The ability to observe fluorescently-labelled colicins in live cells *in vivo* for the first time, can provide invaluable mechanistic information on numbers of colicins, their location, transport kinetics and diffusive behaviour and the properties of the colicin and cell that can influence these. This can be used to identify interactions of the protein with the membrane, with DNA or with other proteins at the single-molecule and single-cell level and identify processes or mechanisms that have not been observed before using ensemble techniques.

There are several techniques that are used to determine the diffusive mobility of proteins in bacteria. Some of the major fluorescence microscopy-based methods and their advantages and limitations have been discussed, see (Mika and Poolman 2011). The most commonly used method to determine the mobility of molecules inside cells is through FRAP. This technique is easy to use when high concentrations of proteins are present in the cell that can withstand bleaching and recovery. It lacks the single-molecule sensitivity and can only provide information on all of the molecules together, rather than the motion and behaviour of each single molecule. Single-molecule tracking (SMT) is a very sensitive technique that allows determination of diffusion coefficients of individual molecules within a population and can provide information on the motion type, localisation and behaviour of molecules. The only experimental data collected for mobility using ColE9, was for the indirect determination of the diffusion coefficient of BtuB bound to ColE9^{S-S^{TMR}}, which gave a value of 0.013 $\mu\text{m}^2/\text{s}$, typical for proteins confined to the OM of bacterial cells (Rassam et al. 2015).

Both widefield microscopy and total internal reflection fluorescence microscopy (TIRFM) techniques, have their advantages and disadvantages for fluorescent imaging. Using widefield microscopy, it is possible to visualise individual live bacterial cells, observe labelling of the DNA

by Hoechst staining and visualise clearly the OM labelling of cells by a range of fluorescent labels. One advantage of using this technique is the ability to move in the axial (Z) dimension throughout the cell and choose the focal point, whether it be at the cell surface or directly in the centre of the cell, which in this case allowed imaging of ColE9 in the cytoplasm. It is also possible to collect multiple frames in short videos of the fluorescence of the cell to show the translocated ColE9 inside the cell. However, its disadvantages are its resolution, with the XY resolution at 180- 250 nm and the Z resolution at 500-700 nm due to its illumination pattern, and diffraction-limited spatial resolution due to the wave nature of light (Schermelleh, Heintzmann, and Leonhardt 2010). The light waves emitted from a fluorescent point object on the specimen plane, converge and interfere at the image plane to produce a diffraction pattern consisting of a bright point surrounded by rings known as airy discs in the XY dimension and a point-spread function (PSF) in the Z dimension. This prevents the ability to resolve structures that are approximately half the wavelength of light, leading to the object to appear blurry (Rottenfusser et al. 2017). The other disadvantage is that due to the lasers focussing directly onto the specimen focal plane, it leads to rapid photobleaching of the AlexaFluor synthetic dyes used, preventing multiple frames to be collected as videos.

TIRFM uses a laser that is illuminated at an oblique angle at the glass-medium interface between two media of differing refractive indices, that reaches a critical incident angle resulting in the reflection of the light. This generates an evanescent wave of illumination confined to a thin region at the coverslip ~100-200 nm deep. This selectively excites fluorophores in the ~100- 200 nm restricted region, whilst the exponential decay of the evanescent wave and its energy means fluorophores further than this region are not excited. Thereby reducing the fluorescent signal from the out of focus region of the cell and improving the signal-to-noise ratio of the fluorescent specimen plane. This improves the axial resolution of the technique to below the diffraction limit, with an XY resolution of 180-250 nm and a Z resolution of 100-400 nm. The other advantage of this method is that due to the generation of an evanescent wave and

reflection of the incident beam, lower laser powers can be used and less photobleaching occurs, allowing longer imaging and multiple frames of videos to be collected. The disadvantage to this method is that due to the restricted excitation ~100-200 nm depth, this technique is used to image the cell surface and plasma membrane at the coverslip interface and restricts visualisation of the centre of the cell (Schermelleh, Heintzmann, and Leonhardt 2010). Therefore, a combination of microscopy techniques was used in the present study to investigate the translocation of ColE9 into the cytoplasm of *E. coli*.

The aim of this chapter was to characterise the diffusive behaviour of ColE9*^{AF647} after translocation in live *E. coli* cells using TIRF and widefield microscopy, so as to better understand the fate of these molecules and the kinetics of this process.

5.2 Results

5.2.1 Two populations of ColE9*AF647 molecules can be observed inside *E. coli* cells

Previously in Chapters 3 and 4, experiments were performed using *E. coli* JM83 cells incubated with ColE9*AF647 followed by trypsin treatment and imaged using widefield fluorescence microscopy (Section 2.5). A fluorescent population of ColE9*AF647 molecules were observed, as dots inside the cell. These dots had radii of ~250-750 nm wide, with an average of 450 nm. Chapter 4 demonstrated that these molecules appeared to be inside the cytoplasm.

This experiment was repeated and cells imaged using TIRFM. The fluorescent population of ColE9*AF647 molecules were observed inside cells (Figure 5. 1). This demonstrated that ColE9 translocation could be visualised by other fluorescent microscopy techniques. From the TIRFM data, these dots had radii of ~250-870 nm wide, with an average of 500 nm. This range of molecule size was comparable to that visualised by widefield microscopy (Figure 3. 19).

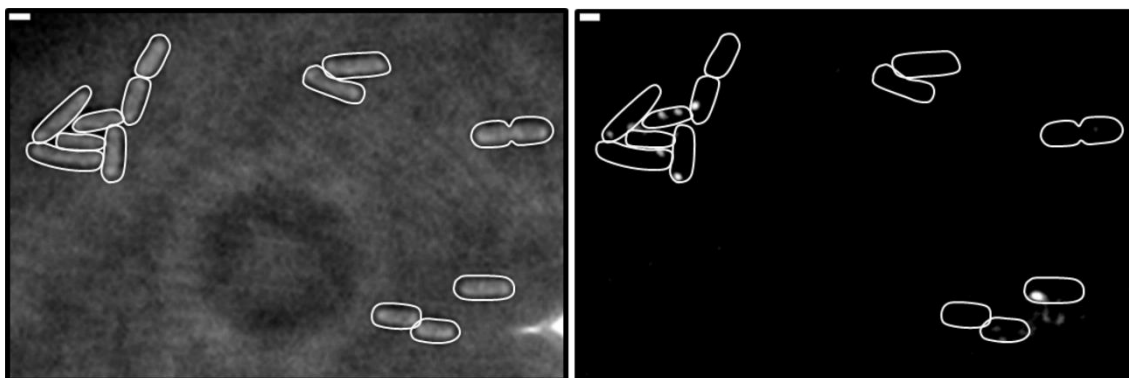


Figure 5. 1 - TIRFM of *E. coli* JM83 cells incubated with ColE9*AF647 followed by trypsin treatment.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF647 before treating with trypsin and imaged using TIRF fluorescence microscopy (Section 2.5). The cell is seen in Brightfield LED light (grey) and the fluorescence of ColE9*AF647 (white). There was remaining internal fluorescence in the cell present as dots. Scale bar is 1 μ m.

One interesting observation from both the widefield and TIRF microscopy, was that the ColE9*AF647 molecules inside cells were present as two populations; a mobile and an immobile population (Figure 5. 2 A and B). This distinction is best seen in TIRFM images. The cells have been outlined and the arrow and white circle represents the initial position of the ColE9*AF647

fluorophore (white dot) from frame 1 at 0 seconds (s). The subsequent images showed the fluorophore position (white dot) relative to its initial position (white circle) every 0.5 seconds (s). The mobile ColE9*AF647 population diffused rapidly within the cell boundary between frames (Figure 5. 2 A). The immobile population of ColE9*AF647 was confined within the cell boundary between frames (Figure 5. 2 B).

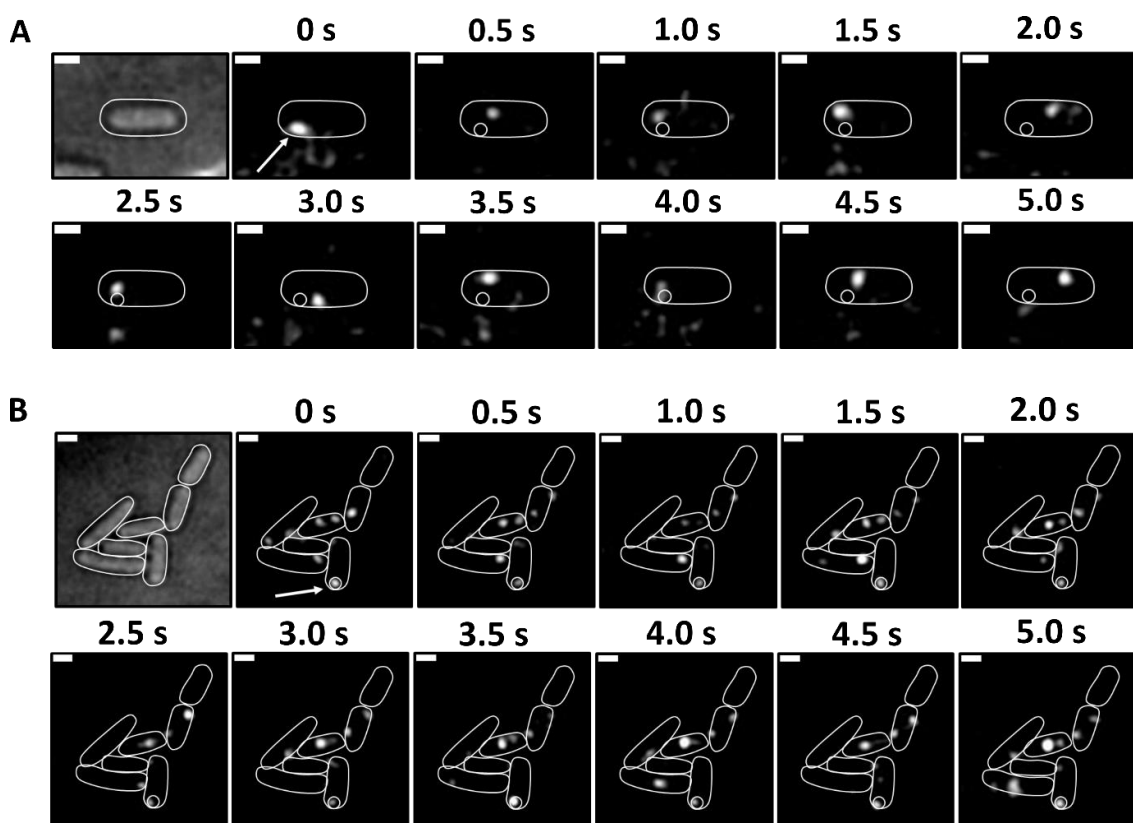


Figure 5. 2 - TIRF microscopy time-course of *E. coli* JM83 cells incubated with ColE9*AF647 and treated with trypsin showing mobile and immobile fluorophores.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF647 before treating with trypsin and imaged using TIRF fluorescence microscopy (Section 2.5). The cell is seen in Brightfield LED light (grey) and the fluorescence of ColE9*AF647 (white). ColE9*AF647 molecules were visualised as both mobile (A) and immobile (B) populations. Images were collected every 20 ms, with every 25 frames or 0.5 seconds shown above. The initial position of the ColE9*AF647 molecule (white dot) has been demonstrated by a white arrow and white circle from frame 1 at 0 seconds (s), the subsequent images show the molecules position (white dot) relative to its initial position (white circle) every 0.5 seconds (s). Scale bar is 1 μ m.

Average number of ColE9*AF647 molecules per cell

Widefield microscopy was used to visualise the centre of the cytoplasm, where it was expected that the majority of the ColE9*AF647 molecules would be present to image, as opposed to the TIRFM, which predominately images at the basal membrane where fewer molecules were likely to be present. The average number of ColE9*AF647 molecules present in each cell was determined using the widefield microscopy data, to get a better idea of the number of translocated ColE9*AF647 molecules. The average number of ColE9*AF647 molecules per JM83 cell was between 0 and 10 ColE9*AF647 molecules after 30 minutes of incubation with 1.5 μ M ColE9*AF647, based on >100 cells across 3 experiments (Section 2.5.12) (Figure 5. 3). Approximately 4 % of cells had no ColE9*AF647 molecules visible and 1 % of cells contained 10 molecules, the mode was 4 ColE9*AF647 molecules per cell (mean of 4.4 molecules/cell). This range in number of ColE9*AF647 molecules could represent the different stages of cell growth and division, a difference in the number of receptor or translocator proteins between cells, or the location or diffusion of ColE9*AF647 in the cytoplasm not visualised at the centre of the cytoplasm. This therefore does not represent an absolute number of ColE9*AF647 molecules present in the entire cytoplasm of each cell, but provides an idea of what can be visualised under certain conditions. In addition to this measurement, the mean intensity of 50 cells for this condition was plotted in Figure 5. 5 to provide an alternative measurement method and for comparison to other cell conditions.

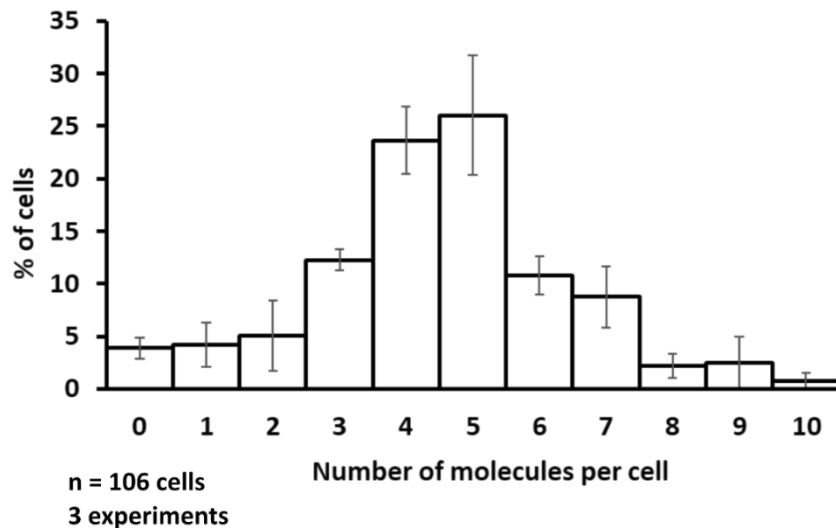


Figure 5. 3 - Number of ColE9*AF647 molecules per cell in 30 minutes of incubation with 1.5 μ M ColE9*AF647 in *E. coli* JM83 cells.

E. coli JM83 cells were labelled with 1.5 μ M ColE9*AF647 for 30 minutes, before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The number of ColE9*AF647 molecules per cell was counted (Section 2.5.12). This is based on a total of 106 cells from 3 experiments containing 466 molecules and plotted as an average of these as a percentage of the total. The error bars represent the Standard error of mean (SEM) of these averages.

Although populations of mobile and immobile ColE9 molecules could be observed from the widefield microscopy data, the number or percentage of each population could not be accurately determined from these data due to the high number of molecules, the poor resolution and rapid photobleaching. The vast majority of molecules appeared to be immobile for the length of the videos taken by widefield microscopy.

Widefield microscopy was repeated using another common laboratory *E. coli* strain MG1655, for comparison to the JM83 data. A plate killing assay demonstrated the killing of MG1655 by ColE9^{AF647}, producing the same result as JM83 (Figure 5. 4 A). Widefield microscopy showed OM labelling of MG1655 cells by ColE9*AF647 and the dependence of the PMF for translocation, by CCCP and trypsin (Section 2.5) (Figure 5. 4 B). Based on the widefield microscopy, the average number of ColE9*AF647 molecules per MG1655 cell was between 0 and 10 ColE9*AF647 molecules in 30 minutes of incubation with 1.5 μ M ColE9*AF647, based on >60 cells across 2 experiments. Approximately 4 % of cells had no ColE9*AF647 molecules visible and 3 % of cells contained

10 molecules, but the mode (and mean) was 5 ColE9*AF647 molecules per cell (Figure 5. 4 C). Again, this does not represent an absolute number of ColE9*AF647 molecules present in each cell. The vast majority of molecules appeared to be immobile for the length of the videos taken by widefield microscopy, similar to JM83 cells.

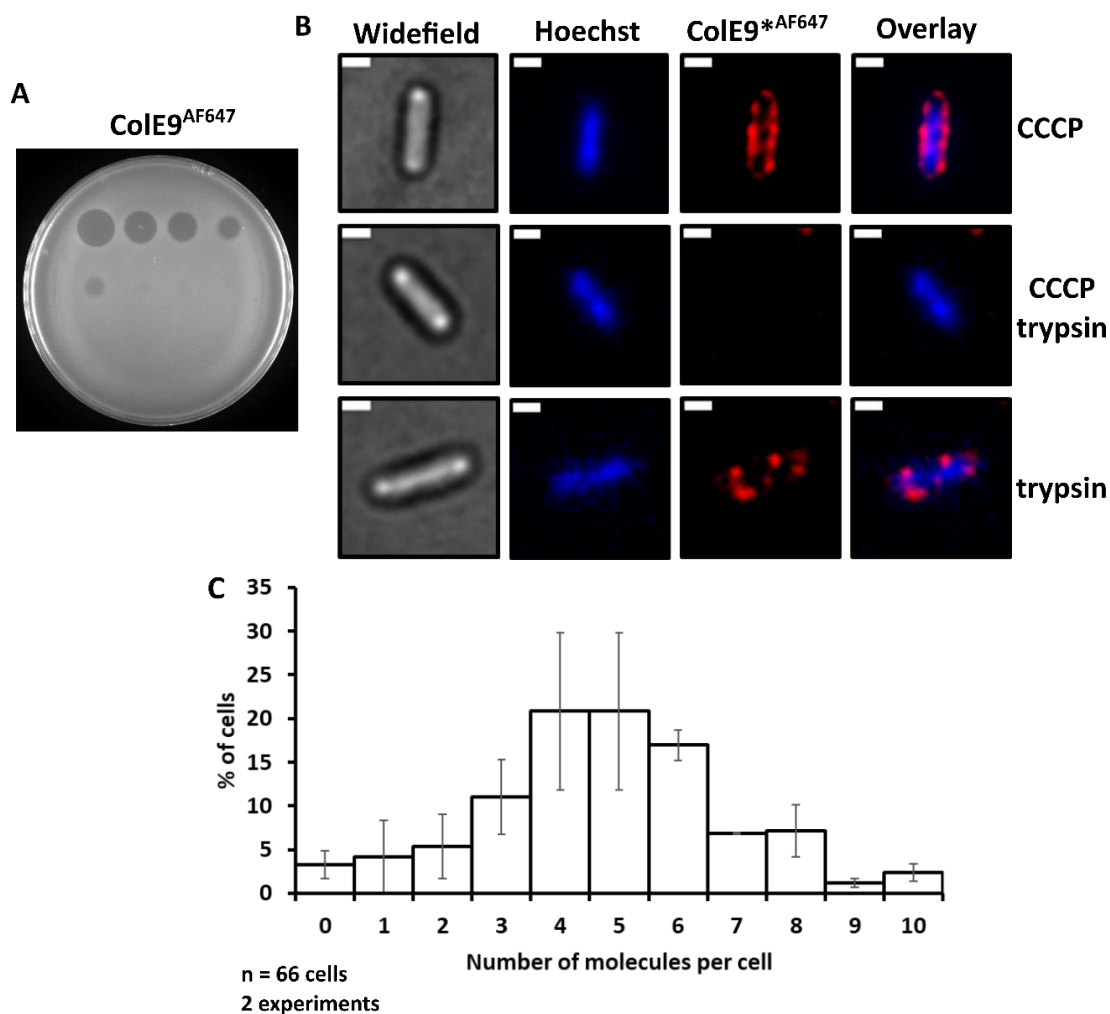


Figure 5. 4 - *E. coli* MG1655 cells treated with CCCP, ColE9*AF647 before trypsin treatment and the number of ColE9*AF647 molecules per cell in 30 minutes of incubation.

(A) *E. coli* MG1655 cells were sensitive to ColE9^{AF647} by plate killing assay (Section 2.6.1). (B) *E. coli* MG1655 cells were treated with CCCP, labelled with 1.5 μ M ColE9*AF647 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ColE9*AF647 still bound to the OM in the presence of CCCP, but fluorescence was lost after trypsin treatment. Labelling and trypsin treatment in the absence of CCCP, showed ColE9*AF647 fluorescence. Scale bar is 1 μ m. (C) The number of ColE9*AF647 molecules per cell was counted (Section 2.5.12). This is based on a total of 66 cells from 2 experiments containing 330 molecules and plotted as an average of these as a percentage of the total. The error bars represent the Standard error of mean (SEM) of these averages.

The mean intensity of 50 cells for this condition was plotted in Figure 5. 5 to provide an alternative measurement method and for comparison to other cell conditions. Figure 5. 5, shows a very similar result to what was observed using JM83 cells using both the method of counting and measurement of the mean pixel intensity, therefore a similar number of molecules translocate.

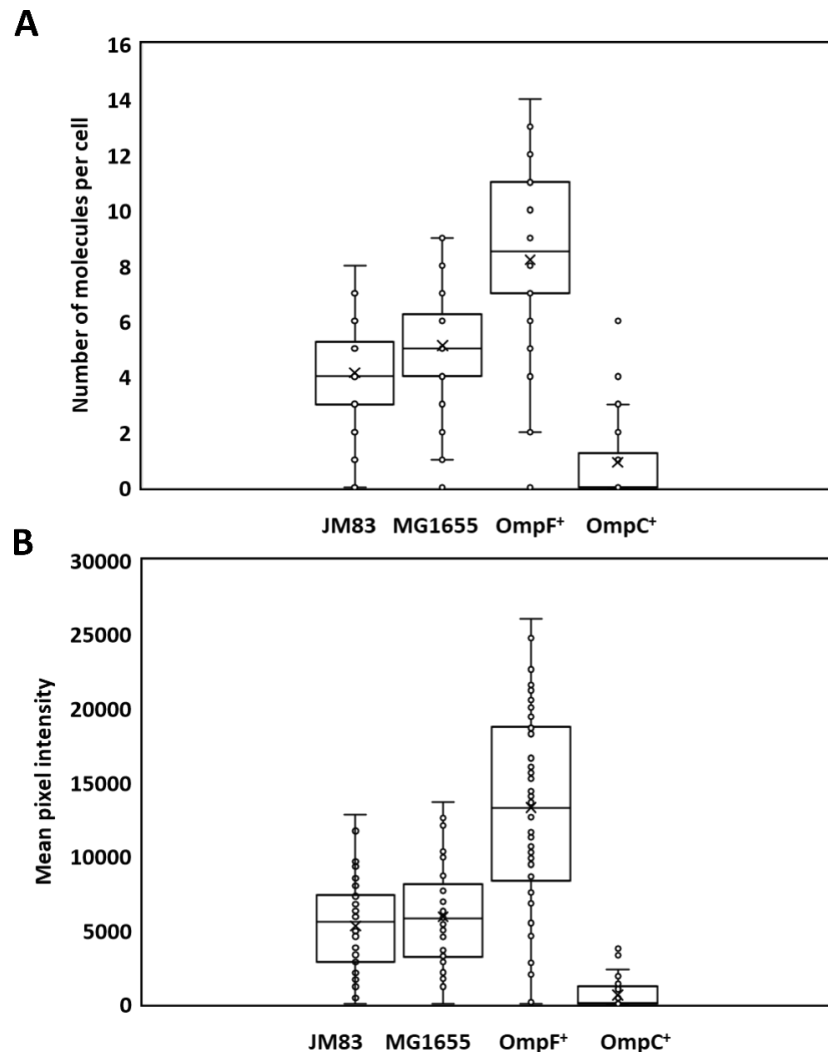


Figure 5. 5 - Box and whisker plot comparison of the number of ColE9*AF647 molecules per cell in *E. coli* JM83, MG1655, JW2203 OmpF⁺ and JW0912 OmpC⁺ cells in 30 minutes of incubation.

Widefield microscopy data was collected for *E. coli* JM83, MG1655, JW2203 (OmpF⁺) and JW0912 (OmpC⁺) cells, which were labelled with 1.5 μ M ColE9*AF647 for 30 minutes at 37 $^{\circ}$ C, before treating with trypsin (Section 2.5). (A) The number of ColE9*AF647 molecules per cell was counted (Section 2.5.12) for 50 cells for each cell condition. (B) The mean pixel intensity of ColE9*AF647 per cell was measured (Section 2.5.12) for 50 cells for each cell condition. Each circle represents a data point for the 50 cells per condition, the whiskers represent the minimum and maximum value plotted, the box shows the 1st and 3rd quartile with the mean shown as a line. Comparisons of the number of molecules counted versus the mean intensity of the cells are comparable for each condition and show that JM83 and MG1655 cells translocate a similar number of ColE9*AF647 molecules, JW2203 (OmpF⁺) cells overlap, but contain a slightly higher number of ColE9*AF647 molecules, whereas JW0912 (OmpC⁺) cells contain much less ColE9*AF647 molecules per cell.

5.2.2 Single fluorescently-labelled ColE9*AF647 molecules can be visualised *in vivo*

Using the TIRFM data, the intensity of the ColE9*AF647 molecules, both mobile and immobile, were plotted against each frame (Figure 5. 6). The average intensity of these molecules was ~290 AU and when bleached was ~230 AU, which is the intensity of the background. These molecules went from a bright fluorescing state to a dark photobleached state in a single-step and therefore showed that these ColE9*AF647 molecules were single-molecules. The tracking of mobile molecules was only possible in the bright state. Background fluorescent molecules surface adhered to the agarose pad not associated with a cell, also showed single-molecule photobleaching.

It is reasonable to assume, from the initial fluorescence intensity of the immobile molecules versus the mobile population, that they are also single-molecules. The mobile molecules had slightly greater variation in their average bright state intensity, most likely due to their movement around the focal plane of the TIRF microscope.

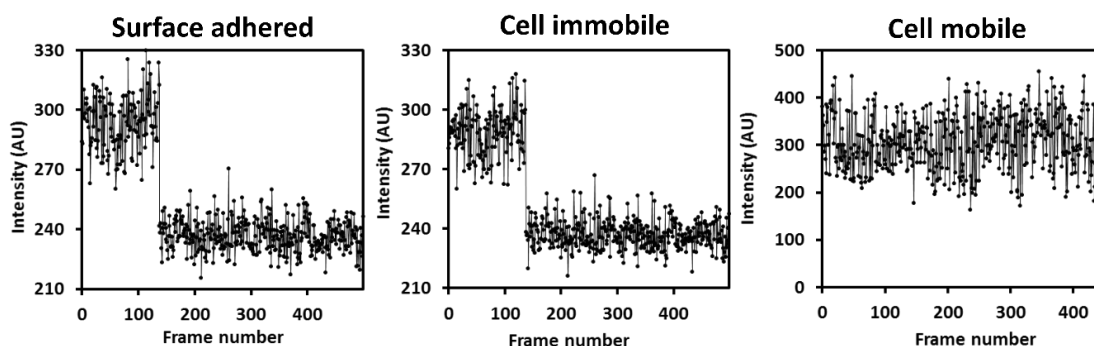


Figure 5. 6 - Intensity profiles of ColE9*AF647 molecules by TIRFM showed ColE9*AF647 are single-molecules inside live *E. coli* JM83 cells by their single-step photobleaching.

The intensity of ColE9*AF647 molecules, both mobile and immobile, were plotted against each frame collected by TIRFM (Section 2.5.10). The average intensity of the molecules when fluorescing was ~290 AU and when bleached was ~230 AU. Immobile molecules went from a bright state to a dark photobleached state in a single-step and therefore showed that these ColE9*AF647 molecules were single-molecules. This was seen for both the immobile ColE9*AF647 populations and background fluorescent molecule surface adhered to the agarose pad not associated with a cell.

Due to the large number of molecules observed by widefield microscopy, their poor resolution and their rapid movement and photobleaching, single-step photobleaching using this data was

not possible. The similar size of the molecules seen in both TIRFM and widefield microscopy suggests that single-molecules are also visible by widefield microscopy. This means using TIRFM, it is possible to image single colicin molecules within live *E. coli* cells for the first time.

5.2.3 Single, translocated, fluorescently-labelled ColE9*AF647 molecules can be tracked in the cytoplasm of *E. coli*

The mobile ColE9*AF647 population of single-molecules in live *E. coli* cells were tracked using the TIRFM data. Each frame was taken at a 20 ms interval for ~500 frames (10 seconds). PaTrack is an automatic particle tracking software that can be used to track and analyse the diffusion of molecules in two-dimensions (2D) (Dosset et al. 2016). PaTrack was used to track these mobile ColE9*AF647 single-molecules in 2D. Figure 5. 7 shows a selection of 2D ColE9*AF647 tracks and an overlay of the track with its Brightfield LED image of the cell. It demonstrated the Brownian diffusion of ColE9*AF647 inside the cell. In some cells, it showed the occasional period of 2D confinement for a few frames, before returning to Brownian diffusion.

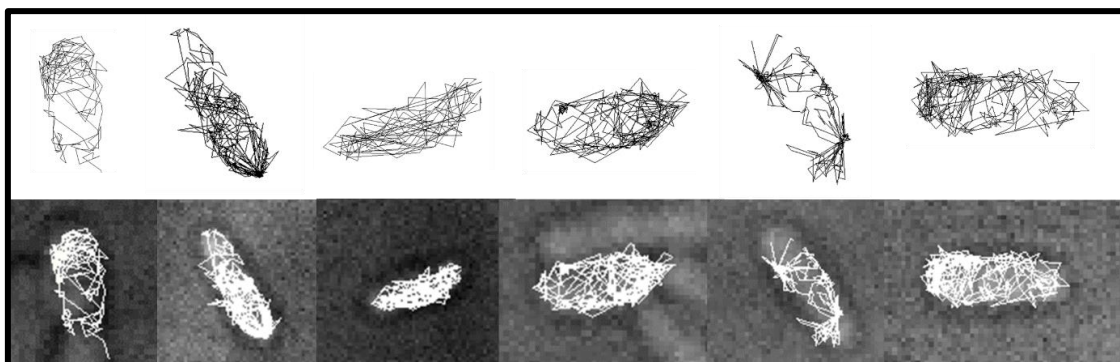


Figure 5. 7 - 2D tracks of ColE9*AF647 molecules collected by TIRFM in live *E. coli* JM83 cells and analysed by PaTrack.

PaTrack was used to track ColE9*AF647 from the TIRFM data in 2D frame-by-frame. The data are a selection of 2D ColE9*AF647 tracks and an overlay of the track with its Brightfield LED image of the cell. ColE9*AF647 showed brownian diffusion across the cell and in some instances, there was evidence of occasional confinement.

PaTrack analysis function used the tracks to plot the mean square displacement (MSD) of the diffusing molecules as a function of time and calculate the apparent diffusion coefficient. Figure 5.8 A shows a representative MSD plot for the track shown inset in red. Figure 5.8 B shows the range of apparent diffusion coefficients calculated from the TIRFM tracks. The diffusion coefficients ranged from 1.7 $\mu\text{m}^2/\text{s}$ to 17.1 $\mu\text{m}^2/\text{s}$ with an average of 6.2 $\mu\text{m}^2/\text{s}$ (Figure 5.8 B). This represents the apparent diffusion coefficient, which would otherwise take into consideration the viscosity of the cytoplasm using references inside the cell to serve as diffusion standards.

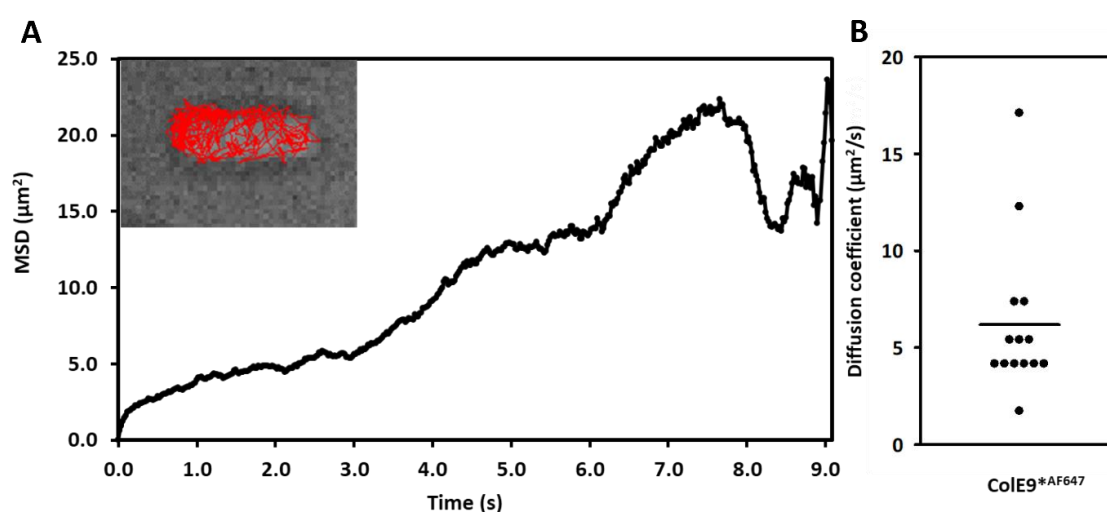


Figure 5.8 - Mean Square Displacement plot and diffusion coefficients of translocated ColE9*AF647 obtained from TIRFM data in *E. coli* JM83 cells.

PaTrack analysis function used the tracks to plot the Mean Square Displacement (MSD) of the diffusing molecules as a function of time. Each point represents 20 ms intervals. (A) A representative MSD plot for the track shown inset in red. (B) The range of apparent diffusion coefficients calculated from the TIRFM tracks with the average shown by the line.

Investigating what the two populations of colicin molecules represent

As described in Section 5.2.1, both widefield and TIRF microscopy demonstrated that ColE9*AF647 single-molecules within cells were present as two populations; mobile and immobile. It was unclear what these different ColE9*AF647 populations represented. One possibility was that the

DNase domain was bound to its target DNA in the cytoplasm and was therefore immobilised for the length of frames imaged by widefield microscopy.

To investigate this possibility, an Im9-eGFP construct was designed in both an IPTG-inducible plasmid (pREN94) and arabinose-inducible plasmid (pMLF09). ColE9 DNase binds to its Immunity protein Im9 forming a tight complex with femtomolar (fM) affinity. Expressing Im9-eGFP in the cytoplasm of *E. coli* should form a complex with ColE9 DNase and prevent it from binding to its target DNA.

To demonstrate the formation of the Im9-eGFP-ColE9-DNase complex and its inability to bind DNA and cause cell death, a plate killing assay was performed. *E. coli* JM83 cells transformed with Im9-eGFP (pREN94), were grown in the absence of IPTG for leaky Im9-eGFP expression and in the presence of 1 mM IPTG. Wild-type ColE9 and ColE9^{AF647} constructs were spotted onto the plates (Figure 5. 9) (Section 2.6.1), which had no clear zones on the bacterial lawns, showing that ColE9 was not capable of causing cell death and therefore cytotoxicity had been inhibited by Im9 complex formation. The result was the same using leaky expression of Im9-eGFP and with IPTG induction (data not shown).

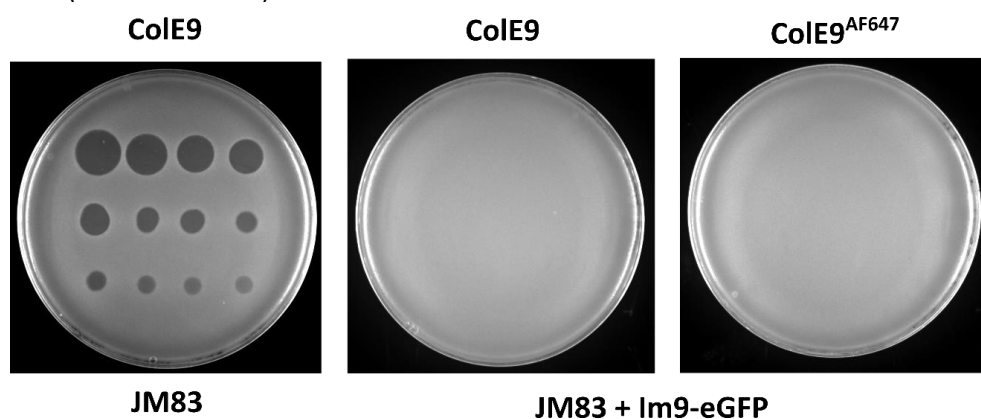


Figure 5. 9 - *In vivo* plate killing assay comparison of wild-type ColE9 and ColE9^{AF647} on *E. coli* JM83 cells expressing Im9-eGFP.

E. coli JM83 cells transformed with Im9-eGFP (no IPTG) were used to perform plate killing assays in LB, using wild-type ColE9 or ColE9^{AF647} from 10 μ M in 3-fold serial dilutions to 57 pM (Section 2.6.1). Colicin induced cell killing is indicated as zones of clearance in the bacterial lawn. (Left) JM83 cells in the absence of Im9-eGFP showed cells were sensitive to wild-type ColE9 down to ~57 pM. (Right) JM83 Im9-eGFP cells showed no zones of killing were evident by wild-type ColE9 or ColE9^{AF647}, therefore cytotoxicity had been inhibited by Im9.

To demonstrate complex formation between Im9-eGFP and Cole9 DNase^{AF568} *in vitro*, Im9-eGFP with an N-terminal hexahistidine tag was expressed from the IPTG-inducible plasmid pREN94 in *E. coli* BL21 (DE3) cells (Section 2.3.3). Im9-eGFP was loaded onto a nickel affinity chromatography column and eluted by an imidazole gradient (Figure 5. 10 A and B).

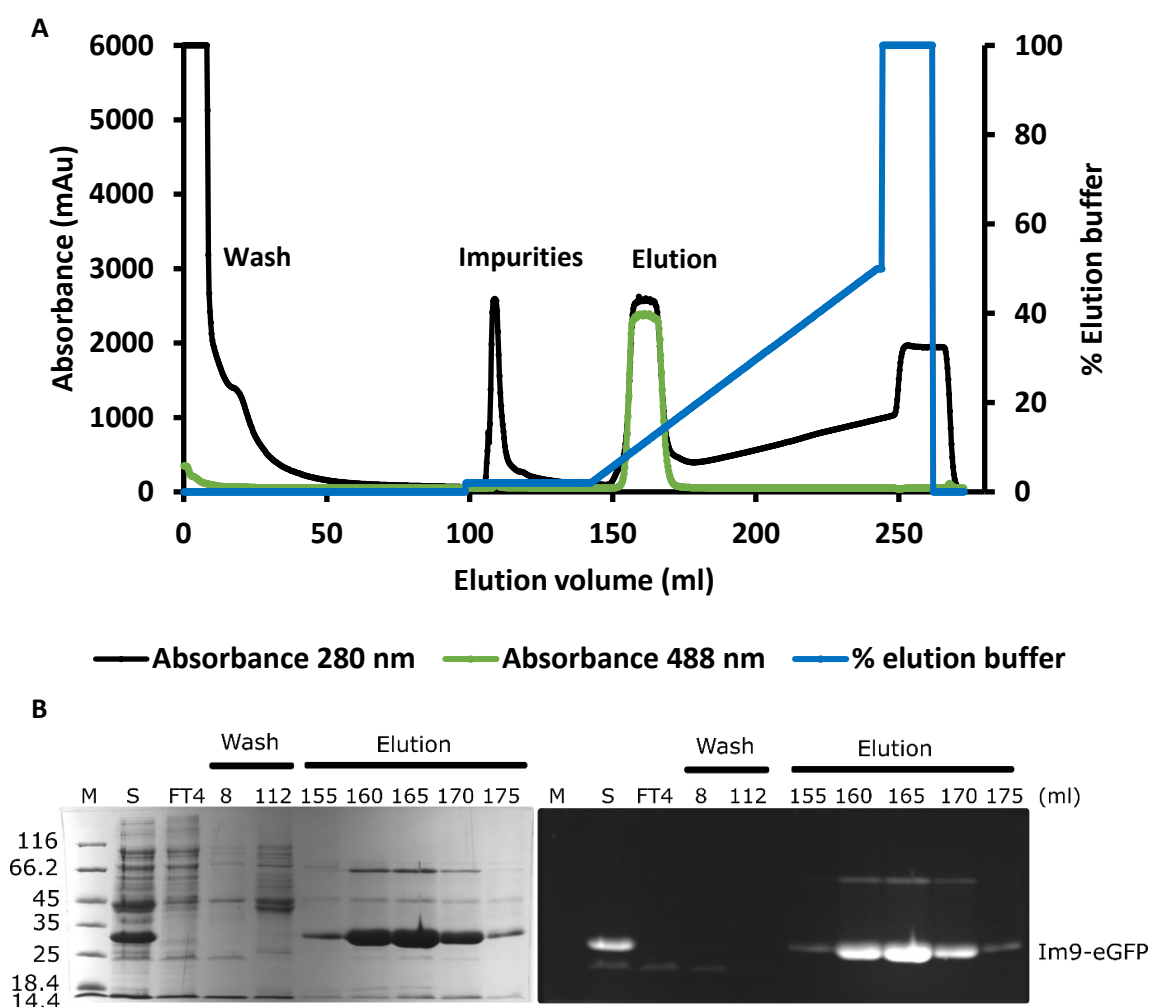


Figure 5. 10 - Im9-eGFP HisTrap FF HP nickel affinity chromatography purification.

(A) Nickel affinity purification trace for Im9-eGFP, using a 2-50 % (20-500 mM) imidazole gradient (Section 2.3.3). (B) Fractions of the supernatant (S), flow-through (FT), wash and elution of Im9-eGFP were run on 12 % SDS-PAGE to visualise Im9-eGFP and demonstrate its purity and visualised by UV (right) and Coomassie (left). Fractions 155-175 ml from the elution were pooled and concentrated. M stands for Marker.

Im9-eGFP was dialysed and further purified by size-exclusion chromatography (Figure 5. 11 A and B).

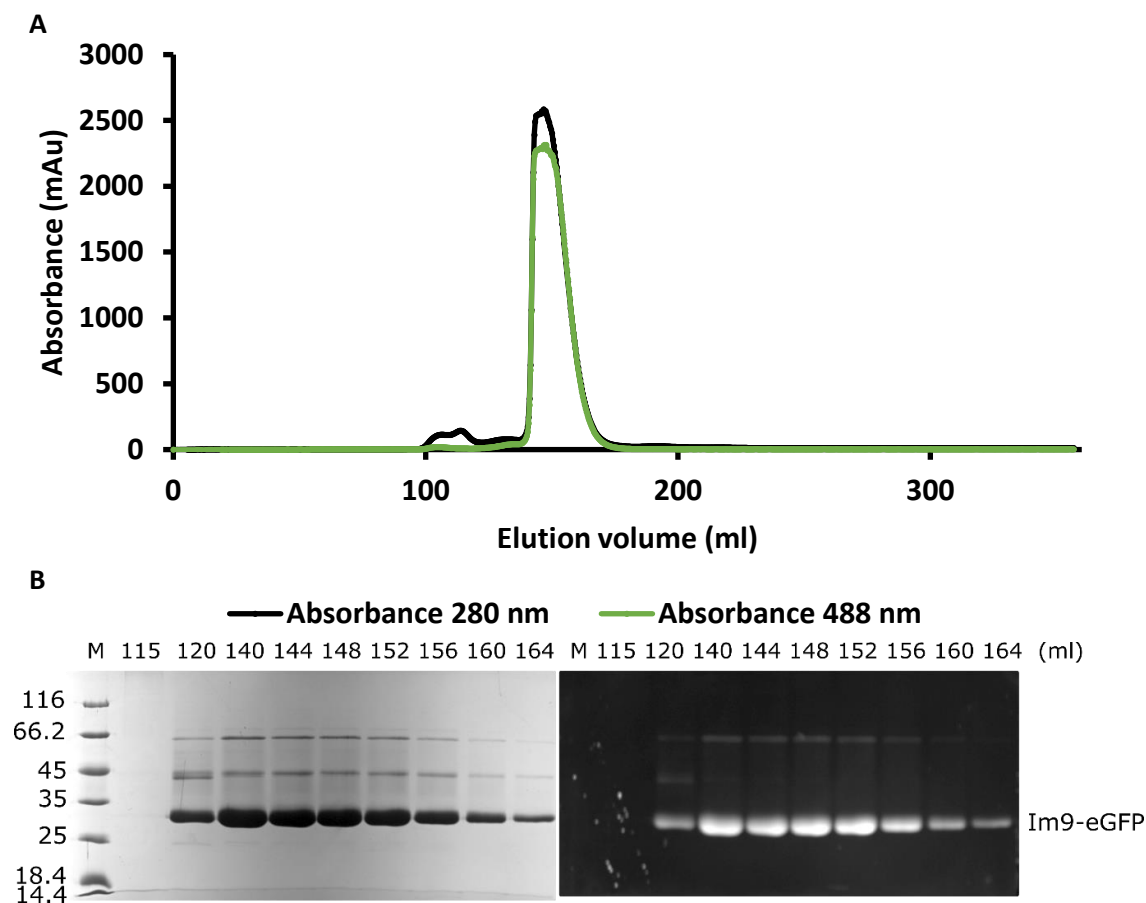


Figure 5. 11 - Im9-eGFP size-exclusion chromatography purification.

(A) Size-exclusion purification trace for Im9-eGFP (Section 2.3.3). (B) Fractions of the elution were run on 12 % SDS-PAGE to visualise Im9-eGFP and demonstrate its purity by Coomassie (left) and UV (right). Fractions 140-164 ml from the elution were pooled and concentrated. M stands for Marker.

ColE9 DNase^{AF568} was also expressed and purified (Section 2.3.2 and 2.3.5), previously used in the plasmid nicking assay in Chapter 3. ColE9 DNase^{AF568} was used (instead of ColE9 DNase^{AF647}) in order to eventually perform single-molecule FRET (SM-FRET) experiments *in vivo*, since eGFP and AF⁵⁶⁸ form a FRET pair (Invitrogen 2010c).

Im9-eGFP, ColE9 DNase^{AF568} and a complex of both were prepared and eluted on an analytical size-exclusion column (Section 2.3.7). Protein elution was monitored by absorbance at 280 nm and fluorescence by absorbance at 488 nm for eGFP and 575 nm for ColE9 DNase^{AF568}, respectively. Figure 5. 12 A shows the absorbance of the single proteins and complex at 280 nm,

Figure 5. 12 B shows the absorbance of the single proteins and complex at the wavelength of their respective fluorescent dyes. Figure 5. 12 A and B shows the peak of elution of Im9-eGFP (MW 39.7 kDa) eluting at ~10.5 ml and Cole9 DNase^{AF568} (MW 15.1 kDa) eluting at ~13.5 ml. The complex (MW 54.8 kDa) showed a shift in the elution peak and its elution at ~9.5 ml. Im9-eGFP therefore formed a complex with Cole9 DNase^{AF568} *in vitro*.

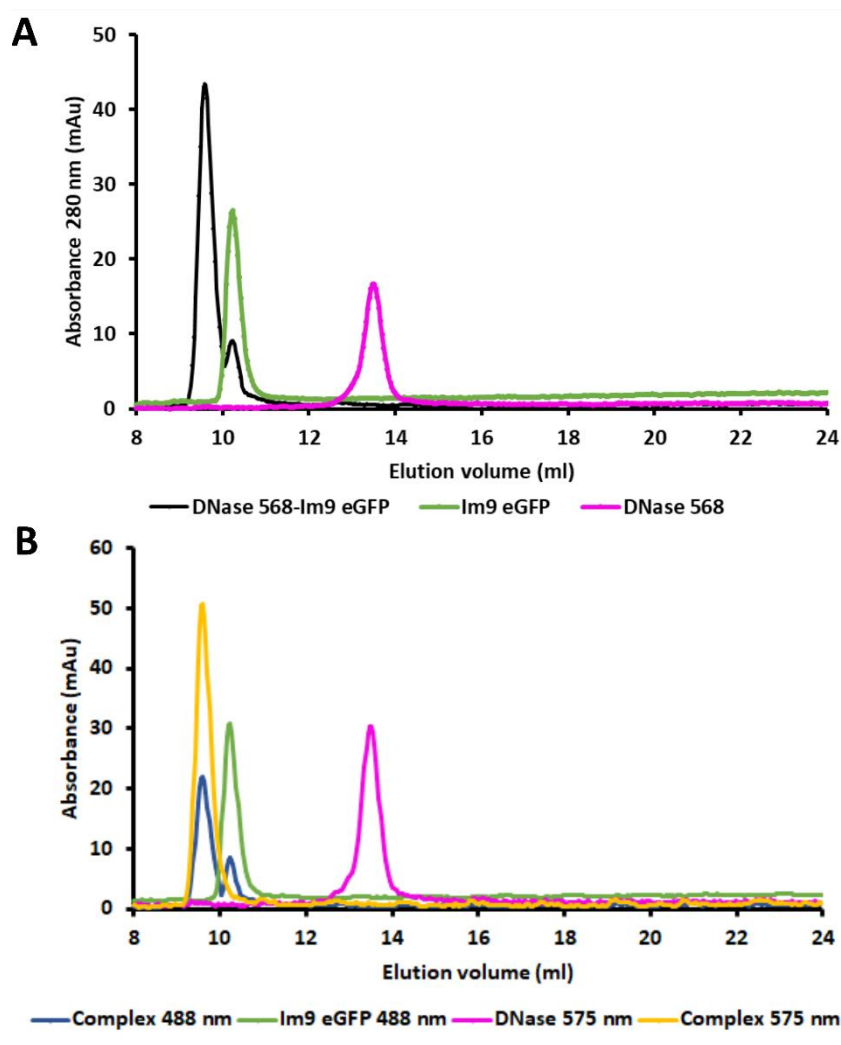


Figure 5. 12 - Im9-eGFP-Cole9 DNase^{AF568} complex formation followed by analytical size-exclusion chromatography.

Im9-eGFP, Cole9 DNase^{AF568} and a complex of both were prepared and eluted on an analytical size-exclusion column (Section 2.3.7). (A) Protein elution was monitored by absorbance at 280 nm, with Im9-eGFP (green), Cole9 DNase^{AF568} (pink) and the complex (black). (B) Protein elution was monitored by absorbance at the excitation wavelength of the fluorophore, with Im9-eGFP at 488 nm in green, Cole9 DNase^{AF568} at 575 nm in pink and the complex at 488 nm in blue and at 575 nm in yellow, respectively.

Due to the much higher level of Im9-eGFP expressed from the IPTG-inducible pREN94, even from leaky expression, the arabinose-inducible plasmid pMLF09 was chosen for microscopy, since expression is more tightly regulated.

The expression level of Im9-eGFP in the cytoplasm was optimised for microscopy by test expressions using plate killing assays. *E. coli* JM83 cells transformed with arabinose-inducible Im9-eGFP (pMLF09), were grown in M9 minimal media using arabinose concentrations at 0 %, 0.0002 %, 0.002 %, 0.02 % and 0.2 % for 1 to 3 hours, which is the length of time JM83 cells take to grow to OD₆₀₀ 0.4 in M9 minimal media. Plate killing assays were performed against wild-type ColE9 (Section 2.6.1). Figure 5. 13 A showed that in the absence of arabinose induction, wild-type ColE9 killed cells to 4.6 nM on M9 minimal media agar plates. With 0.0002 % arabinose for 1 to 3 hours induction, ColE9 killed down to ~14 nM and 41 nM, respectively. For 0.002 % and 0.02 % arabinose for 1 to 3 hours induction, the concentration at which ColE9 killed cells slowly declined progressively (data not shown). Figure 5. 13 B showed at the highest concentration of arabinose at 0.2 %, killing by ColE9 could faintly be seen at ~10 µM after 1 hour, but after 2 and 3 hours killing by ColE9 was completely inhibited. This shows the level of induction of Im9-eGFP needed to form a complex with different concentrations of ColE9 in M9 minimal media.

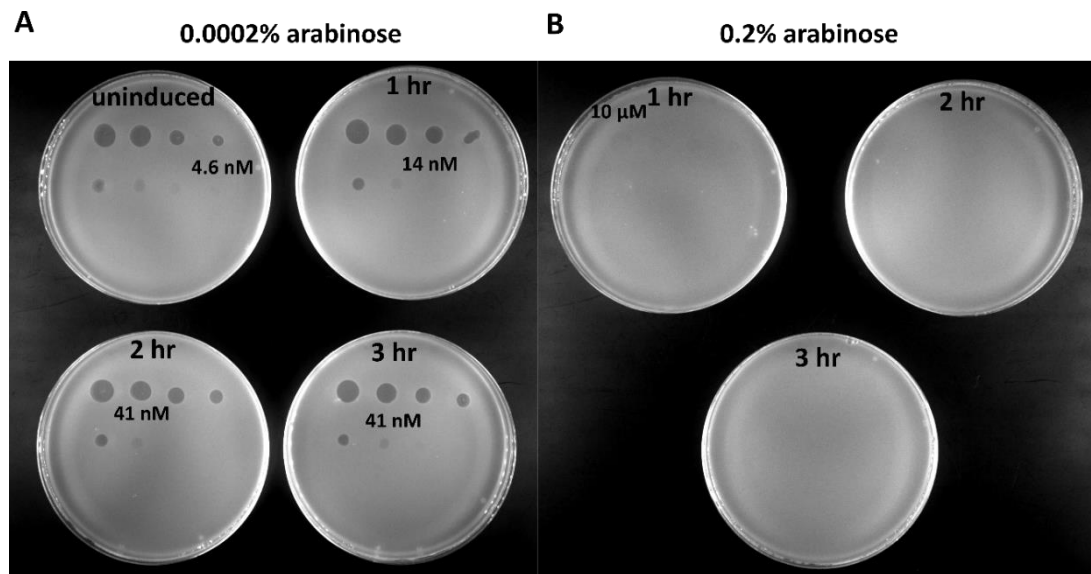


Figure 5. 13 - *In vivo* plate killing assay in M9 minimal media of wild-type ColE9 against *E. coli* JM83 cells expressing Im9-eGFP at a range of arabinose concentrations.

E. coli JM83 cells transformed with Im9-eGFP and grown in supplemented M9 minimal media in the absence (uninduced) and presence of 0.0002 % and 0.2 % arabinose for 1 to 3 hours. Cells were used to perform plate killing assays, using wild-type ColE9 from 10 μM in 3-fold serial dilutions to 57 pM (Section 2.6.1). Colicin induced cell killing is indicated as zones of clearance in the bacterial lawn. (A) uninduced expression of cells showed killing by wild-type ColE9 down to 4.6 nM. With 0.0002 % arabinose for 1 to 3 hours induction, wild-type ColE9 killed down to ~14 nM and 41 nM, respectively. (B) At 0.2 %, killing by wild-type ColE9 could faintly be seen at ~10 μM after 1 hour, but after 2 and 3 hours killing by wild-type ColE9 was completely inhibited by Im9-eGFP.

For microscopy, *E. coli* JM83 cells transformed with the arabinose-inducible Im9-eGFP (pMLF09) were grown with and without arabinose, using the same conditions as tested in the plate killing assays. In the absence of arabinose there was low levels of expression of Im9-eGFP. Although the overall intensity of fluorescence was much lower compared to arabinose induced cells, it was not possible to observe single Im9-eGFP molecules diffusing in the cytoplasm (data not shown). So as single-molecules were not possible, Im9-eGFP was over-expressed using 0.2 % arabinose to ensure high levels of Im9-eGFP to form rapid complexes with ColE9*AF568 and act as the donor for FRET.

The following microscopy was only initial preliminary data over a small data set and requires much further optimisation and repetition. *E. coli* JM83 cells transformed with Im9-eGFP were grown in the presence of 0.2 % arabinose and incubated with ColE9*AF568 before treating with trypsin (Section 2.5). Cells expressing Im9-eGFP showed uniform fluorescence; the level was too high to observe single Im9-eGFP molecules (Figure 5. 14 A).

JM83 cells with induced Im9-eGFP and labelled with ColE9*AF568 before and after trypsin treatment (Figure 5. 14 B), showed that ColE9*AF568 can bind to the OM and translocate in the presence of cytoplasmic Im9-eGFP, as expected. ColE9*AF568 molecules were observed inside cells and it appeared from the small data set collected that both mobile and immobile populations were still present. This suggests that the immobile ColE9 population might not be molecules bound to DNA. As this was only preliminary data over a small data set, further work needs to be done to confirm this. In addition, complex formation needs to be confirmed *in vivo* through SM-FRET. Eventually this experimental setup could be used to track translocated ColE9*AF568 DNases in complex with Im9-eGFP.

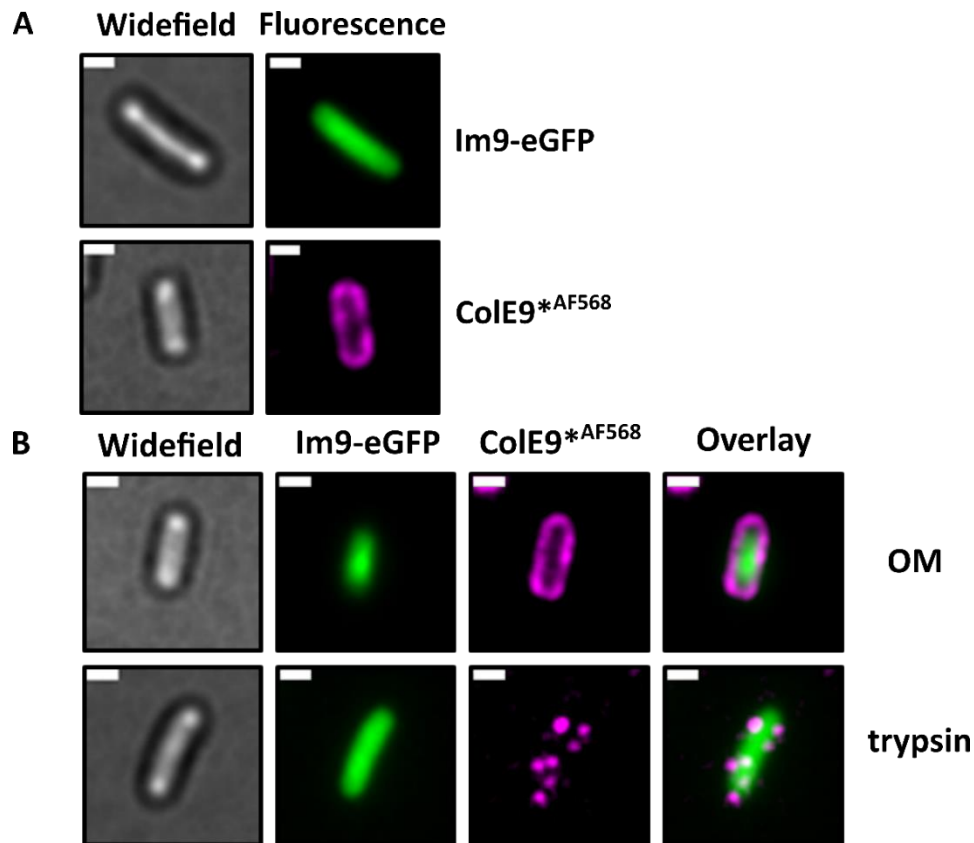


Figure 5. 14 - *E. coli* JM83 cells transformed with Im9-eGFP labelled with ColE9*AF568 followed by trypsin treatment.

E. coli JM83 cells transformed with Im9-eGFP grown in supplemented M9 minimal media in the presence of 0.2 % arabinose. Cells were labelled with 1.5 μ M ColE9*AF568 before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey) and the fluorescence of ColE9*AF568 (pink). Overlays of the Im9-eGFP and fluorescent colicin are also shown. For both Im9-eGFP and ColE9*AF568 labelled cells, there was remaining internal fluorescence after trypsin treatment. Scale bar is 1 μ m.

Although analytical size-exclusion data had shown Im9-eGFP and ColE9 DNase^{AF568} could form a complex *in vitro*, FRET was not observable between the fluorescent dye and fluorescent protein by widefield microscopy *in vivo*. It was previously indicated that of the fluorescent dyes used in this research, AF⁵⁶⁸ was the only dye that had spectral overlap with eGFP, although this was still poor as show by fluorimetry (Appendix I Figure 10). The development of a SM-FRET method to observe this was beyond the scope of this project.

5.3 Discussion

Observing single ColE9*AF647 molecules inside *E. coli* cells

This chapter demonstrated the use of the fluorescence microscopy method developed in Chapter 3, to observe single-colicin molecules diffusing at the single-cell level in live *E. coli* cells, for the first time. The ability to observe and count only a few ColE9*AF647 molecules per cell was surprising. It was expected that due to the thousands of receptors and translocators on the cell surface for colicin translocation, and the huge excess of colicin added to cells, that hundreds of colicin molecules would translocate under these experimental conditions. Yet, for ColE2 indeed it has been reported that a minimum of 2000-3000 colicins bound per cell and ~100 ColE2 molecules were capable of causing cell death (Nomura 1964) (Maeda and Nomura 1966) (Shannon and Hedges 1967). Observing the translocation of a large number of colicins was the case in chapter 3, for ColB*AF488 and ColB*AF647 entry into the periplasm. Although the periplasm is a smaller compartment, such a large number of colicins had translocated into the periplasm that it was not possible to observe single-molecules unless severe photobleaching occurred.

It is therefore surprising that as few as 1-10 ColE9*AF647 molecules, in 30 minutes of incubation, are observed per cell in the present study. This could either be a function of the different colicins, their different cytotoxicity's or a function of their different cellular compartments. It could suggest that due to the further processing of ColE9 and its retro-translocation across the IM before entry into the cytoplasm, that fewer colicin molecules translocate into the cell than those destined for the periplasm. Additionally, the presence of the fluorophore which restricts entry into the cell could be reducing the number that translocate into the cytoplasm.

It is important to note that the number of molecules counted per cell represents an underestimation. Rapidly diffusing molecules moving away from the focal plane may not have been counted or may have appeared at the same localisation as another molecule, showing one dot. The axial thickness of the image taken of the focal plane and out of focus light in the Cy5

channel using the AF⁶⁴⁷ dye is $\sim 0.9 \mu\text{m}$, which represents roughly the entire cytoplasm (Spitzer and Poolman 2013). The ability to detect these molecules within the out-of-focus light may be affected. In addition, the technique used to count the number of dots seen per cell is used to give an idea as to how many dots can be resolved and would therefore underestimate the actual number.

Killing by colicins is single-hit and from this it is proposed that a single molecule could be sufficient to kill a cell, although this has yet to be formerly demonstrated (Nomura 1963). Perhaps it is logical to observe only a few molecules that have translocated simultaneously to ensure rapid cell death. Alternatively, these experiments were performed using a cytotoxically inactive colicin construct, that is still capable of binding DNA. Perhaps as a single-colicin does not lead to cell death, several subsequent colicins were translocated to try to aid in cell death. It is also possible that the number of colicins observed per cell could be a result of the high concentration added experimentally as opposed to what might occur in nature.

The technique used in this thesis to show that single ColE9*AF⁶⁴⁷ molecules were present in each fluorescent spot using the TIRFM data, is commonly used for this purpose to provide an accurate determination of the number or stoichiometry of single-molecules. For example, using stepwise photobleaching to count the number of fluorophores per fluorescent spot has been used to demonstrate that the number of copies of GFP-MotB stator proteins in the flagellar motor (Leake et al. 2006), the number of TatA-YFP subunits per complex in the Tat system (Leake et al. 2008) and number of α -Hemolysin-AF647 subunits within β -barrel forming toxin complexes within the membrane (Das et al. 2007). Alternatively, techniques like fluorescence correlation spectroscopy (FCS) and ratiometric comparison of fluorescence intensity to known standards is also used (Verdaasdonk, Lawrimore, and Bloom 2014).

Two populations of ColE9*AF647 can be observed inside the cell

The results in this chapter provided the first observation that translocated ColE9 molecules exist as two populations, a mobile and immobile population. It is unclear what these two ColE9*AF647 populations represent, although one of these is almost certainly cytoplasmic. One possibility for the immobile population, is that the DNase domain is bound to its target DNA in the cytoplasm and therefore immobilised for the length of frames imaged by widefield microscopy. It may also be that due to the construct used and its inability to nick the dsDNA with the H551A mutation, the DNase domain remains bound to DNA. Another possibility is that due to the size of the AlexaFluor dyes on the DNase domain, ColE9*AF647 may be stuck in OMPs, or in the IM or FtsH. Unfortunately, studies using FtsH strains in Chapter 4 were unsuccessful due to their narrow, elongated phenotype impairing visualisation. The use of OMPs will be further addressed in Chapter 6.

The presence of two fluorescently-labelled populations of molecules has been observed for other bacterial proteins within the cytoplasm. Stracy et al. studied the localisation and dynamics of fluorescently-labelled RNA polymerase (RNAP) and DNA in live *E. coli* cells, using PALM-TIRF, SMT and 3D-SIM. The authors showed an average of ~2700 copies of RNAP/cell, of which 48 % were immobile and 52 % mobile. The immobile population were RNAP molecules bound to DNA for transcription, with a high proportion of clusters located at the nucleoid periphery at the ribosomes, abolished by rifampicin treatment. The mobile population were diffusing through the nucleoid in search of promoters to initiate transcription, making transient non-specific interactions, demonstrated through the blocking of transcription using rifampicin, which shifted the amount of mobile RNAP to 84 % (Stracy et al. 2015). In addition, the authors found a difference to these observations when performed in rich versus minimal media, due to the rate of cell growth and organisation of the nucleoid. The immobile population was 63 % in rich media, versus 48 % in minimal media and the size and number of RNAP molecules in each

cluster differed, with ~50 molecules/cluster in minimal media versus ~500 molecules/cluster in rich media (Stracy et al. 2015).

Additionally, research on the intracellular diffusion and subcellular localisation of electroporated Cy5-labelled tRNA in the bacterial cytoplasm using SMT, showed that it had a fast and slow mode of diffusion (Plochowietz et al. 2017). The fast mode represented >70 % of tRNA freely diffusing and was enriched at the nucleoid. The slow mode represented ribosome-bound tRNA located at the periphery of the cell for protein synthesis, which was reduced when treated with chloramphenicol (Plochowietz et al. 2017).

The possibility that colicin diffusion could follow a similar mode of action, that mobile colicins are freely diffusing in the cytoplasm ready to bind non-specifically and nick DNA and the immobile colicins are already bound, is unlikely. The preliminary Im9-eGFP data still shows immobile molecules even when excess Im9 is present, suggesting that immobile molecules are not bound to DNA.

One thing to note is the length of the videos taken in TIRF is up to 10 seconds long. It is possible that the immobile colicins are temporarily restricted in the cytoplasm by molecular crowding, this could be investigated using longer time-courses and active colicins. The finding of Stracy et al., that the proportion of mobile and immobile molecules changed in different media due to the rate of cell growth and nucleoid organisation, could also be a factor for colicin organisation and could be further investigated in the future.

Tracking of single fluorescently-labelled ColE9*^{AF647} molecules *in vivo*

In this chapter, I developed a method to fluorescently-label ColE9 and track single translocated colicin molecules diffusing within the cytoplasm of live *E. coli* using TIRFM. Tracking of these single-colicin molecules within the cytoplasm gave an average apparent diffusion coefficient of $6.2 \mu\text{m}^2/\text{s}$. This value is similar to that for GFP inside *E. coli* by FRAP. GFP-TorA (in $\Delta\text{tatABCDE}$ cells) in the cytoplasm had an average diffusion coefficient of $9 \pm 2.1 \mu\text{m}^2/\text{s}$. GFP-TorA exported

into the periplasm, gave an average diffusion coefficient of $2.6 \pm 1.2 \mu\text{m}^2/\text{s}$. GFP-TatA in the IM had an average diffusion coefficient of $0.13 \pm 0.03 \mu\text{m}^2/\text{s}$ (Mullineaux et al. 2006). Elowitz et al. used photobleaching and photoactivation of a red-emitting state of GFP using FRAP to show GFP had an average diffusion coefficient of $7.7 \pm 2.5 \mu\text{m}^2/\text{s}$ in the cytoplasm (Elowitz et al. 1999). In addition, factors that affected the diffusion coefficient was protein expression levels ($3.6 \pm 0.7 \mu\text{m}^2/\text{s}$), Hexa-His tags ($4.6 \pm 2 \mu\text{m}^2/\text{s}$) and size dependence of the GFP-labelled protein (GFP-Maltose binding protein, MW 72 kDa, $2.5 \pm 0.6 \mu\text{m}^2/\text{s}$) (Elowitz et al. 1999). Nenninger et al. further investigated size dependence for GFP diffusion in the cytoplasm using FRAP, using TorA-GFP multimers, showing that as you increased the molecular weight from 57 to 138 kDa the diffusion coefficient decreased from 8.3 ± 4.2 to $2.8 \pm 1.5 \mu\text{m}^2/\text{s}$, most likely due to a molecular sieving effect (Nenninger, Mastroianni, and Mullineaux 2010).

The value obtained for the diffusion coefficient using ColE9*^{AF647} is therefore in agreement with the published value of cytoplasmic proteins like GFP. In addition, it supports the findings of Chapters 3 and 4, that ColE9*^{AF647} is inside the cytoplasm. In the case of this research, with up to 10 observed single-molecules in cells, there is little chance of overcrowding having an effect on the diffusion of ColE9. In addition, by using a small synthetic dye with a molecular weight of 1.25 kDa covalently attached to the DNase domain with a molecular weight of ~15 kDa, there is little chance of the dye contributing to an effect on the diffusion of ColE9. As the synthetic dye is not indigenous to the cell, it would be unlikely to have any specific interactions and therefore the diffusive behaviour would be a result of the ColE9 DNase domain alone.

The current approach to enable the study of protein expression, localisation, copy number, and diffusion profiles is the fusion of fluorescent proteins like GFP, which can be expressed with the protein of interest *in vivo*. However, fluorescent proteins are a lot less bright, photostable and are much larger in comparison to synthetic organic dyes. The complication of using organic dyes is that the protein of interest needs to be labelled *in vitro* and then internalised into cells. A method has recently been developed by Crawford et al. for the

internalisation of DNA and proteins fluorescently-labelled with organic dyes via electroporation (Crawford et al. 2013). This allowed for an increased localisation precision, slower photobleaching of molecules, FRET and *in vivo* tracking of their protein of interest. Crawford et al. tracked a 45 bp dsDNA molecule, fluorescently-labelled with -Cy3B/AF647/ATTO647N to provide mean diffusion coefficients ranging from 0.61-0.92 $\mu\text{m}^2/\text{s}$ in the cytoplasm (Crawford et al. 2013). This electroporation method was further employed by Di Paolo et al. for the internalisation and tracking of the chemotactic signalling response regulator CheY, fluorescently labelled with Atto647. Tracking of the freely-diffusing species of Atto647-CheY in the cytoplasm gave a diffusion coefficient of $1.95 \pm 0.05 \mu\text{m}^2/\text{s}$ (Di Paolo et al. 2016). In addition, tRNA-Cy5 was electroporated and tracked with a diffusion coefficient of 8 $\mu\text{m}^2/\text{s}$ in the cytoplasm (Plochowietz et al. 2017).

The advantage of the method developed in this thesis, is the internalisation of the AlexaFluor dyes via specific transport through Cole9 translocation. In addition, cell integrity is not compromised and does not require cell recovery before imaging. Further to this, the number of molecules observed inside the cell, is not varied on the concentration of external agent added and likely reflects the number of naturally translocated molecules.

From the videos collected in widefield and TIRF microscopy and from the tracks of the single-molecules, it was clear to see that the diffusion of the mobile colicins was predominately Brownian. From the tracks of some of the molecules, it appeared as if there were occasional periods of confinement before returning to Brownian diffusion, or that the molecule sometimes briefly returned to the same or similar location of the cell, as demonstrated by the highly concentrated points of the tracks. The reason for this is unclear, it may be a result of interactions with cell components like proteins or DNA, or it may be due to the complex macromolecular crowding of the cytoplasm restricting its path of diffusion. Alternatively, it may not represent confinement but the diffusion of the molecule in 3D in the axial dimension not detected in the

2D tracking. The range in diffusion coefficients could reflect the difference in ColE9*^{AF647} tracks where there was possible confinement of the molecule or 3D axial diffusion.

5.4 Conclusions

The work presented in this chapter showed that the translocation of ColE9*^{AF647} can be observed using both widefield and TIRF microscopy. The molecules observed were shown to be single-molecules by their ability to photobleach in a single-step by TIRFM. There was between 0 and 10 molecules per cell, with a mode of 4/5 which was observed in JM83 and MG1655 lab *E. coli* strains, respectively, as counted from widefield microscopy data. In addition, two populations of ColE9*^{AF647} molecules were observed; a mobile and immobile population, with the vast majority immobile. Mobile single-molecules were tracked *in vivo*, which displayed Brownian diffusion with an average apparent diffusion coefficient of 6.2 $\mu\text{m}^2/\text{s}$ within the cytoplasm. This chapter showed that the microscopy method developed in Chapter 3 allowed the visualisation of single fluorescently-labelled colicin molecules diffusing in live *E. coli* cells for the first time.

6 Chapter 6 - ColE9 translocates through the general porins OmpF or OmpC to cross the outer membrane

6.1 Introduction

Alternate routes have been proposed as to how colicins translocate across the OM, there are three suggested routes. First translocation across the OM at the lipid-protein interface of porins (Baboolal et al. 2008); second, translocation through the channel of its receptor (Fourel et al. 1993); third, binding a receptor and translocation through the lumen of a porin (Housden et al. 2010). This chapter aims to address the route of entry of ColE9 across the OM once the colicin has bound its receptor.

Colicin N (ColN) is a unique group A pore-forming colicin, that uses OmpF as both a receptor and a translocator for cell entry. There is evidence to suggest that ColN translocates through the lumen of OmpF (Fourel et al. 1993) (Jeanteur et al. 1994) (Zakharov et al. 2004), but there is also evidence that ColN traverses down the side of the porin at the interface with LPS (Baboolal et al. 2008) (Bainbridge et al. 1998) (Clifton et al. 2012). There is evidence in support of entry of ColE3, ColE9 and ColE1 colicins, or part of it, through the lumen of OmpF and TolC, respectively (Zakharov et al. 2004) (Zakharov et al. 2006), further addressed in the discussion. There are therefore conflicting ideas and a lack of direct evidence for the route of translocation across the OM for colicins.

The similarities and differences between OmpF and OmpC

ColE9 can enter *E. coli* cells through OmpF, OmpC or PhoE, with OmpF ~1 order of magnitude more efficient than OmpC, which is in turn ~1 order of magnitude more efficient than PhoE (Housden et al. 2010) (Mock and Pugsley 1982). This chapter investigates how this impacts the translocation of fluorescently-labelled ColE9, using OmpF and OmpC. PhoE was not investigated in these studies.

OmpF and OmpC are non-specific, general porins that allow passive diffusion of many different hydrophilic molecules into *E. coli*, including nutrients. OmpF has been shown to be advantageous to the cell for glucose uptake, whereas OmpC has been shown to be advantageous for using histidine nitrogen source and reducing the entry of β -lactam antibiotics (Basle et al. 2006).

OmpF and OmpC are composed of 16-stranded anti-parallel β -sheets that form barrel proteins with a central pore, the β -strands are connected by extracellular loops on the surface and short turns in the periplasmic side (Figure 6. 1). The porins share 60 % sequence identity, with 74 % identity of the residues lining their aqueous pore. The extracellular loops of both porins are closely packed, and act as a screen for substrates in both their size and charge (Cowan et al. 1992). Both porins have an extracellular loop L3 that extends into and constricts the central pore which significantly contributes to the exclusion size of solutes (Basle et al. 2006). Acidic negatively charged residues of the loop interact with basic positively charged residues on the barrel wall (Figure 6. 1). There are equivalent charged residues in the constriction site of both OmpF and OmpC (Yamashita et al. 2008) (Ziervogel and Roux 2013) (Lou et al. 2011). The loop also contributes to the stability and shape of the pore (Cowan et al. 1992). The solute exclusion size of OmpF and OmpC has been shown to be ~600-700 Da (Nikaido and Vaara 1985) (Nikaido and Rosenberg 1983).

The dimensions of the central pore of OmpF are elliptical and between 11-19 Å at the extracellular face of the pore, 11x7 Å at the constriction site caused by loop L3 and 22x15 Å at the periplasmic end. This constriction site is ~ 9 Å long and at the centre of the barrel (Cowan et al. 1992). OmpF pore diameter is reported as ~11.6-12 Å and OmpC is ~10.8-11 Å (Hancock 1987), which is not a large difference, but enough to contribute to reducing or inhibiting diffusion of nutrients (Figure 6. 1).

Another important difference between OmpF and OmpC, is that whilst having very similar central pores and periplasmic outlets, the lining of the pore residues, charge distribution and loop conformations at the extracellular entrance of OmpC are structurally different (Basle et al. 2006). The channel of OmpC contains a larger number of ionizable residues that affect the electrostatic profile of the pore (Figure 6. 1). The pore of OmpC is more negatively charged explaining why OmpC is more cation-selective than OmpF. These residues may affect the interaction of the solutes within the pore or the organisation of the pore itself. Replacement of these residues in OmpC or the presence of higher salt concentrations, lead to similar permeability properties to those of OmpF (Basle et al. 2006) (Kojima and Nikaido 2014). The cation selectivity for OmpF and OmpC is 4.5 and 5.8, based on the anion/cation ratio. The single channel conductance in 1M NaCl is 0.8 nS and 0.5 nS and the voltage-gating threshold potential is 90 mV and 160 mV, for OmpF and OmpC, respectively (Prilipov et al. 1998) (Buehler et al. 1991).

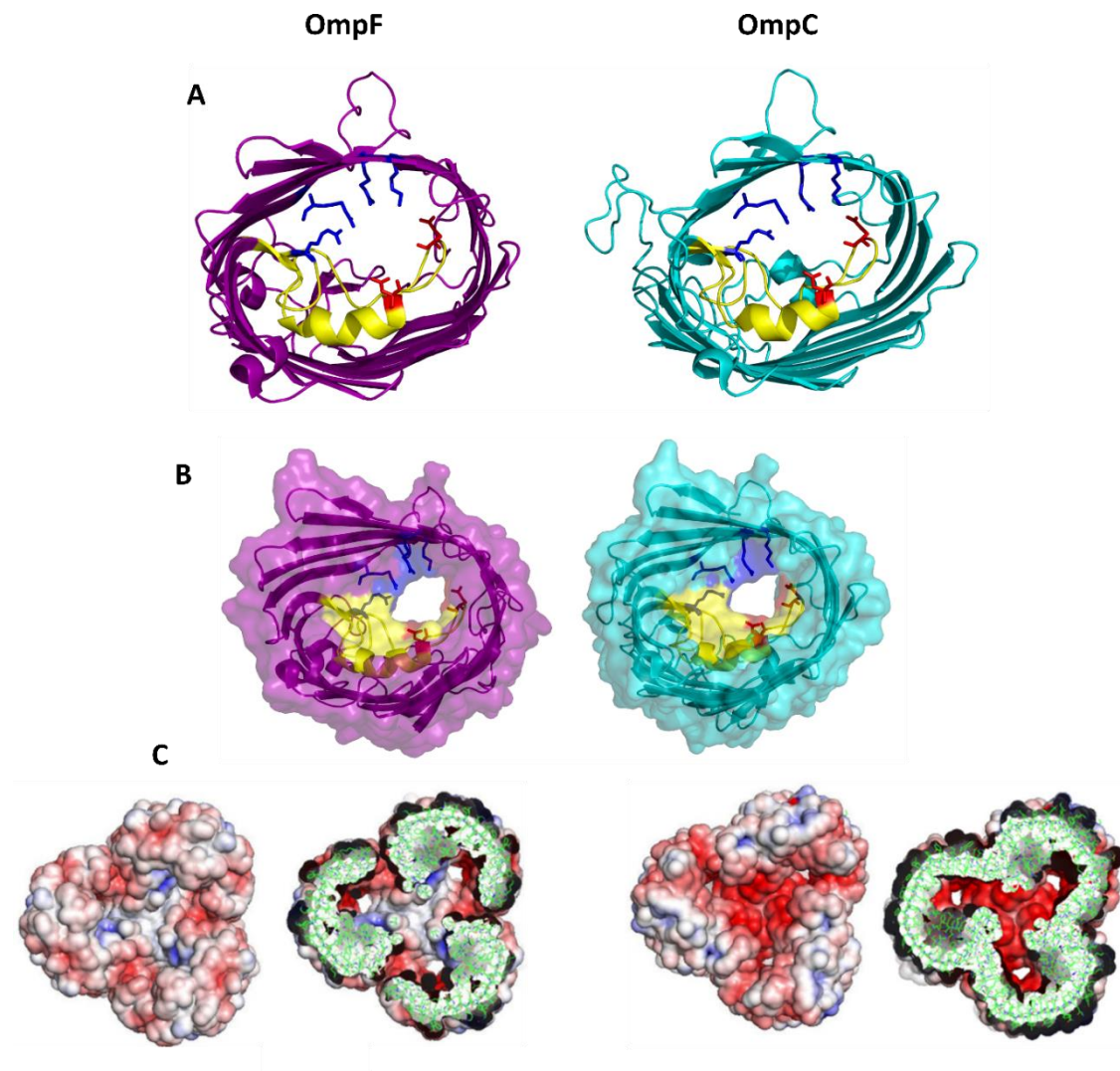


Figure 6. 1 - Comparison of the similarities and differences between OmpF and OmpC porins.

OmpF is shown in purple on the left and OmpC in cyan on the right. A) Comparison of the structure and residues involved in the occlusion site of the barrel caused by loop L3. Loop L3 (yellow), acidic residues on the loop (red) and basic residues on the barrel wall (blue). B) Comparison of the size of the lumen of OmpF and OmpC as a result of the occlusion site. The constriction site of OmpF is $\sim 11.6\text{-}12\text{ \AA}$ and OmpC is $\sim 10.8\text{-}11\text{ \AA}$ (Hancock 1987). C) Comparison of the electrostatic properties of the lumen of OmpF and OmpC (Kojima and Nikaido 2014). All views are from the extracellular side. PDB; 3o0e for OmpF, 2j1n for OmpC.

OmpF and OmpC are differentially expressed and tightly regulated in response to different stimuli. Osmolarity is monitored by the EnvZ/OmpR two-component regulatory system. Under low osmolarity, OmpF transcription is enhanced and OmpC transcription is repressed (Forst et al. 1988). Acidic pH leads to the repression of OmpF and increased expression

of OmpC through the EnvZ/OmpR two-component system (Heyde and Portalier 1987). Nutrient limitation like glucose starvation leads to an increase in the expression of OmpF, whereas nitrogen starvation leads to an increase in the expression of OmpC (Liu and Ferenci 1998). Temperature also plays a role, where high temperatures increase the transcription of *micF* antisense RNA which inhibits the translation of *ompF* mRNA (Delihais 1995). Therefore, small changes in experimental conditions can shift the amount of both porins.

The kinetics of cell entry of colicins

Colicin cytotoxicity occurs in two stages, the first is the adsorption of the colicin to the cell surface, in which cell survival and colony formation is rescuable by trypsin treatment, the second is the translocation across the OM, in which cells are no longer protected from being killed (Plate and Luria 1972) (Nomura 1964; Maeda and Nomura 1966).

From previous data and those shown in this chapter from CFU experiments, on addition of colicin to cells, cell survival decreases exponentially with time. When plotted logarithmically against time, the curve appears as a straight line to fit to the first-order integrated rate law to determine the rate constant and $t_{1/2}$. The kinetics of cell killing by colicins is first-order and the rate of killing is directly proportional to the concentration of colicin until an excess colicin is used and a plateau is reached (Reeves 1965).

Colicin action has been previously shown to have single-hit rather than multi-hit kinetics. Meaning that, although hundreds of colicin molecules bind to cell surface receptors, they each act non-cooperatively to translocate and kill the cell. Theoretically, a single unit of colicin can cause, with a certain probability, the death of a sensitive cell (Nomura 1963).

Previous experiments with ColE2 led to the estimation that there was a minimum of ~20-30 units of colicin bound per cell (Maeda and Nomura 1966). This was calculated based on the initial concentration of colicin added to cells and the amount removed from solution, which was analysed through spot test activity. In addition, radioactively-labelled (H^3) ColE2 was added

to cells and increased linearly up to a minimum of 20 units. The amount of protein that corresponds to a 'killing unit' was calculated from the experimental kinetic curves of ColE2 and estimated to be 100 molecules of ColE2 per unit (40-300/per unit depending on the experimental conditions used). Therefore, based on this there was an estimated 2000-3000 ColE2 molecules adsorbed to the surface cell (Maeda and Nomura 1966) (Shannon and Hedges 1967). The adsorption of a single killing unit (~100 ColE2 molecules) was capable of causing an observable biochemical effect on its target slowly, which occurred faster with more colicin (Nomura 1964). A similar result was observed with other colicins, for example ColD was shown to follow single-hit kinetics with an average of 100 ColD molecules required to kill a cell, as calculated from survival ratios (Timmis and Hedges 1972).

The aims of this chapter were to confirm and characterise the translocation of ColE9 through the lumen of both OmpF and OmpC as their route of entry across the OM. Next, to determine the kinetics of entry of ColE9 and the fluorescently-labelled ColE9 constructs across the OM using either OmpF or OmpC and investigate the role of the IM on the rate of entry of the fluorescently-labelled ColE9 constructs.

6.2 Results

6.2.1 Fluorescence labelling impacts ColE9 mediated killing of OmpF/OmpC expressing *E. coli* cells

To demonstrate the use of OMPs as the route of entry of ColE9 across the OM, plate killing assays were performed using wild-type ColE9 and fluorescently-labelled ColE9 using *E. coli* strain BZB1107, an OmpF, OmpC and LamB porin knockout (OmpF⁻ OmpC⁻), *E. coli* strain JW0912, OmpF knockout (contains OmpC, referred to as OmpC⁺), and *E. coli* strain JW2203, OmpC knockout strain (contains OmpF, referred to as OmpF⁺) (Section 2.6.1). Cells were treated with wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} constructs (Figure 6. 2).

Using OmpF⁻ OmpC⁻ cells, wild-type ColE9 cannot kill the cells. Wild-type ColE9 killed both OmpF⁺ and OmpC⁺ cells very efficiently and can therefore use either major porin for translocation into *E. coli*, as shown previously (Housden et al. 2010). For the fluorescently-labelled ColE9 constructs, OmpF⁺ cells showed dramatically higher killing than JM83 cells for ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647}, nearly as effective as wild-type ColE9. OmpC⁺ cells showed dramatically reduced killing for ColE9^{AF488}, inhibited killing by ColE9^{AF568} and very poor killing by ColE9^{AF647}. Again, showing the preference for OmpF over OmpC for translocation by the fluorescently-labelled colicins.

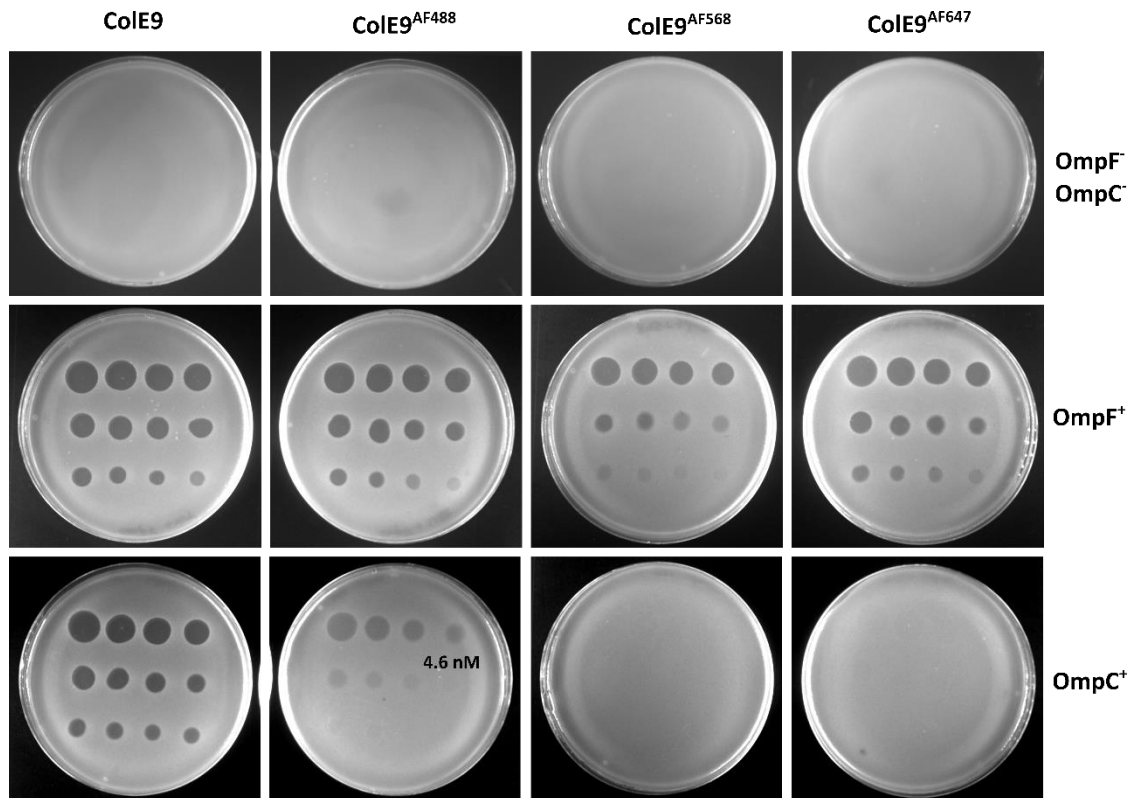


Figure 6. 2 - *In vivo* plate killing assay comparison of wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} on *E. coli* BZB1107 OmpF⁻ OmpC⁻, JW2203 OmpF⁺ and JW0912 OmpC⁺ cells.

E. coli BZB1107, JW2203 and JW0912 cells were used to perform plate killing assays, using wild-type ColE9 or fluorescently-labelled ColE9 from 10 μ M in 3-fold serial dilutions to 57 pM (Section 2.6.1). Colicin induced cell killing is indicated as zones of clearance in the bacterial lawn. None of the constructs killed cells in the absence of major OMP porins (OmpF⁻ OmpC⁻). Wild-type ColE9 killed both OmpF⁺ and OmpC⁺ cells very efficiently, the fluorescently-labelled ColE9 constructs showed dramatically improved killing with the OmpF⁺ cells and the OmpC⁺ cells showed dramatically reduced killing for ColE9^{AF488} and inhibited killing by ColE9^{AF568} and ColE9^{AF647}.

Killing experiments were also performed in liquid cultures (Section 2.6.2), using the colicin constructs against OmpF⁺ and OmpC⁺ expressing *E. coli* cells (Figure 6. 3). The time-courses showed that both OmpF⁺ and OmpC⁺ cultures grew at similar rates in the absence of ColE9. For OmpF⁺ cells, there was a 40-minute delay from addition of wild-type ColE9 to observing a reduction in the OD₆₀₀, in OmpC⁺ cells, there was an 80-minute delay. This showed that although wild-type ColE9 can use either OmpF or OmpC to translocate into cells, OmpF is preferred, in agreement with (Housden et al. 2010).

Using OmpF⁺ cells with the fluorescently-labelled constructs, ColE9^{AF488} was the fastest at killing, followed by ColE9^{AF647} and then ColE9^{AF568} as the slowest. This was the same observation with JM83 cells by both liquid culture killing assay and bioluminescence emission (Figure 3. 10). In OmpC⁺ cells, all of the fluorescently-labelled constructs showed very poor or no killing. ColE9^{AF488} showed a ~3-hour delay from addition of the colicin to observing a reduction in the OD₆₀₀. ColE9^{AF568} cannot be seen on the graph as it overlaps the control (no colicin). This result shows that killing is slower, so growth wins over killing in liquid culture. It indicates that the labelled colicins can pass more easily through OmpF, but with an observable difference between the dyes, whereas they get restricted or blocked in OmpC.

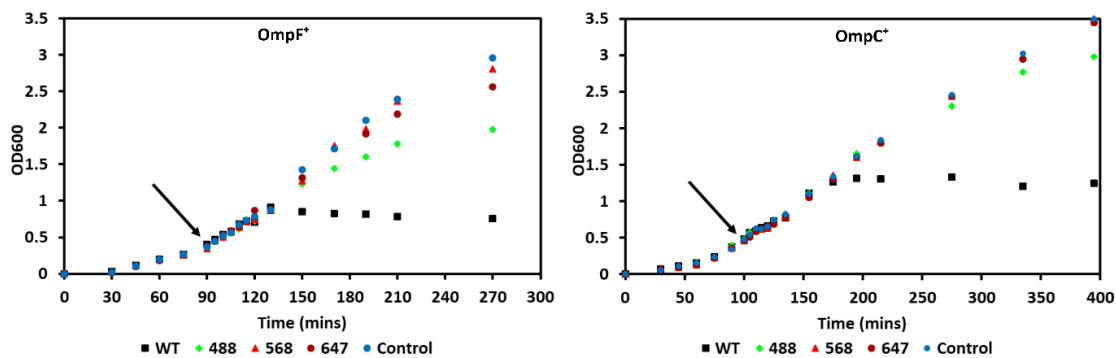


Figure 6. 3 - *In vivo* liquid culture killing assay comparison of wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} on *E. coli* JW2203 OmpF⁺ and JW0912 OmpC⁺ cells.

E. coli JW2203 and JW0912 cells were used to compare cell killing using OmpF and OmpC, using wild-type ColE9 (■), ColE9^{AF488} (◆), ColE9^{AF568} (▲) and ColE9^{AF647} (●) and a control (●) as indicated by the black arrow. There was a longer delay in cell killing using OmpC by wild-type ColE9. Wild-type ColE9 can use both OmpF or OmpC to enter cells, but enters quicker using OmpF. In both strains, using the fluorescently-labelled constructs, ColE9^{AF488} was the fastest at killing, followed by ColE9^{AF647} and then ColE9^{AF568} as the slowest (not seen on graph of OmpC⁺ cells dye to overlap with control).

Widefield microscopy was performed to identify if translocation of ColE9^{AF647} could be visualised in both OmpF⁺ cells and OmpC⁺ cells. *E. coli* JW2203 OmpF⁺ cells and *E. coli* JW0912 OmpC⁺ cells were labelled with ColE9^{AF647} before treating with trypsin (Section 2.5). ColE9^{AF647} still bound and labelled the OM of both OmpF⁺ cells and OmpC⁺ cells producing islands or puncta (Figure 6. 4). For OmpF⁺ cells, when cells were further treated with trypsin, there was remaining

internal fluorescence observed. For OmpC⁺ cells, there was little or no internal fluorescence remaining.

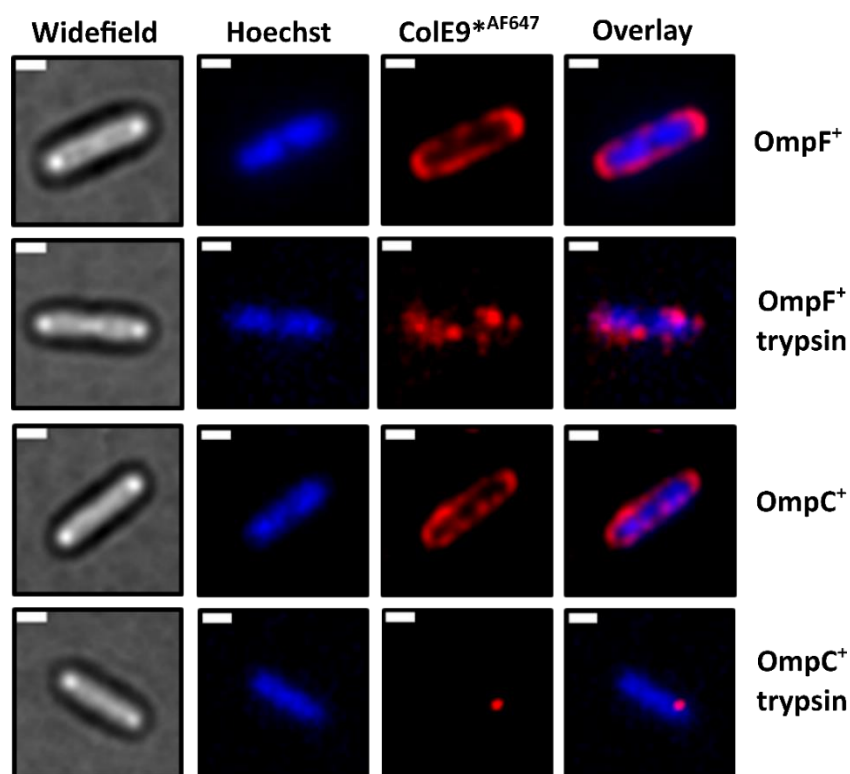


Figure 6. 4 - *E. coli* JW2203 OmpF⁺ and JW0912 OmpC⁺ cells labelled with ColE9*AF647 followed by trypsin treatment.

E. coli JW2203 (OmpF⁺) and JW0912 (OmpC⁺) cells were labelled with 1.5 μ M ColE9*AF647 and 20 μ M Hoechst before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the DNA and fluorescent colicin are also shown. ColE9*AF647 bound to the OM of both strains. When further treated with trypsin, OmpF⁺ cells showed remaining internal fluorescence, for OmpC⁺ cells, there was little to no internal fluorescence remaining. Scale bar is 1 μ m.

The average number of ColE9*AF647 molecules present in each cell was determined using widefield microscopy (Section 2.5.12). For OmpF⁺ cells there was between 0 and 15 ColE9*AF647 molecules in 30 minutes of incubation with 1.5 μ M ColE9*AF647, based on 92 cells across 2 experiments (Figure 6. 5 A). Approximately 3 % of cells had no ColE9*AF647 molecules visible and 1 % of cells contained 14/15 molecules, the mode was 8 ColE9*AF647 molecules per cell (mean of 7.9 molecules/cell). This does not represent an absolute number of ColE9*AF647 molecules present in the entire cytoplasm of each cell. The vast majority of molecules appeared to be

immobile for the length of the videos taken by widefield microscopy, similar to *E. coli* JM83 cells. The mean intensity of 50 cells for this condition was plotted in Figure 5. 5 to provide an alternative measurement method and for comparison to other cell conditions (Section 2.5.12). It showed that the overall number of molecules per cell using OmpF⁺, was higher than using JM83 or MG1655 cells.

For OmpC⁺ cells there was between 0 and 8 ColE9*AF647 molecules in 30 minutes of incubation with 1.5 µM ColE9*AF647, based on 91 cells across 2 experiments (Figure 6. 5 B). Approximately 55 % of cells had no ColE9*AF647 molecules visible and 1 % of cells contained 8 molecules, but the mode was 0 ColE9*AF647 molecules per cell (mean of 1.6 molecules/cell). In the cells that contained ColE9*AF647 molecules, the vast majority appeared to be immobile for the length of the videos taken, with the occasional mobile molecule, but these were less frequent due to the absence or low number of colicins per cell. The mean intensity of 50 cells for this condition was plotted in Figure 5. 5. It showed that the overall number of molecules per cell using OmpC⁺, was much lower than using *E. coli* JM83, MG1655 or OmpF⁺ cells.

The microscopy was therefore in agreement with the plate and liquid culture killing assays, demonstrating that ColE9*AF647, and the other fluorescently labelled colicins, can translocate across the OM through OmpF, however OmpC restricts their cell entry.

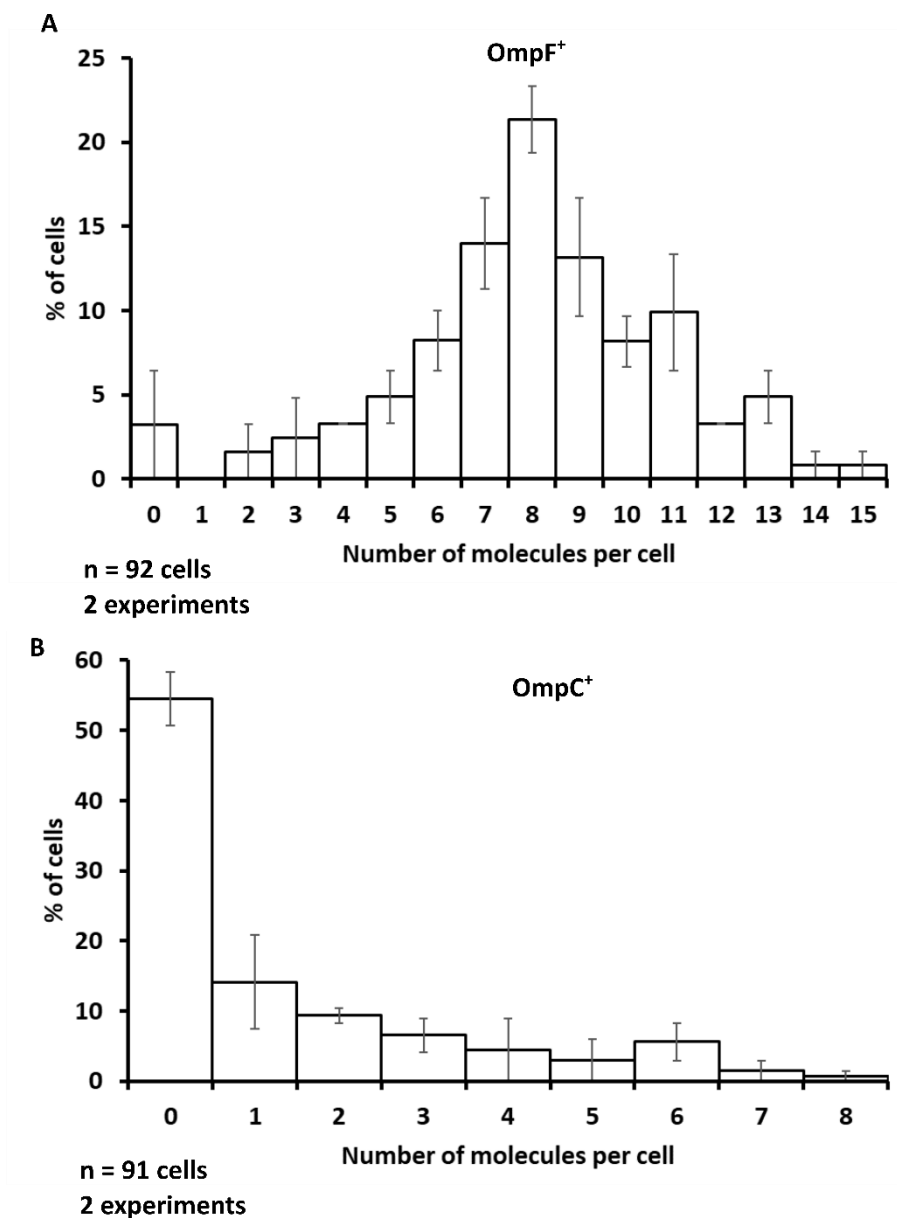


Figure 6. 5 - Number of ColE9*AF647 molecules per cell in *E. coli* JW2203 OmpF⁺ and JW0912 OmpC⁺ cells, in 30 minutes of incubation with 1.5 μ M ColE9*AF647.

E. coli JW2203 and JW0912 cells were labelled with 1.5 μ M ColE9*AF647 for 30 minutes, before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The number of ColE9*AF647 molecules per cell was counted (Section 2.5.12). (A) For OmpF⁺ cells, this is based on a total of 92 cells from 2 experiments containing 725 molecules. (B) For OmpC⁺ cells, this is based on a total of 91 cells from 2 experiments containing 139 molecules. This was plotted as an average of these as a percentage of the total. The error bars represent the Standard error of mean (SEM) of these averages.

6.2.2 Kinetics of entry of ColE9 across the outer membrane

To further investigate the rate of translocation of ColE9 and the fluorescently-labelled constructs across the OM, colony forming unit (CFU) experiments were performed using *E. coli* JM83 (OmpF⁺ OmpC⁺), *E. coli* JW0912 (OmpC⁺) and *E. coli* JW2203 (OmpF⁺) cells. *E. coli* JM83 cells were grown in LB to OD₆₀₀ ~0.4 (~10⁸ cells), 80 nM colicin added and samples taken over a series of time-points and inhibited using trypsin to cleave surface-exposed colicin and prevent further translocation (Section 2.7). Each time point was diluted in LB between 10⁰-10⁶ -fold and plated on LB-agar plates. Colonies were counted and averaged from triplicate plates. As it is a first-order reaction, the Ln of each CFU value was plotted against time (mins) and fitted to the equation of a straight line to obtain the rate constant (k) of the reaction.

Previously, it was shown that *E. coli* cells were under limited stress by the presence of high levels of trypsin with 50 µg/ml and 250 µg/ml of trypsin at 37 °C (Figure 3. 18). This was repeated using 1 mg/ml of trypsin used for the CFU experiment to ensure there was no effect on cell growth and viability. It was clear that the OD₆₀₀ and therefore rate of cell growth was not affected by the high levels of trypsin. Therefore, any affect observed from the CFU experiment was a result of the colicin and not the trypsin (data not shown).

The result of the CFU experiments on *E. coli* JM83 (OmpF⁺ OmpC⁺), JW2203 (OmpF⁺) and JW0912 (OmpC⁺) cells with wild-type ColE9 and fluorescently-labelled constructs can be seen in Figure 6. 6 and Figure 6. 7. The fit of the data to a straight line for all the constructs gave R² values of >0.92, indicating a very good fit. From this, the rate constant (k) and t_{1/2} values, or time it takes to kill half of the cells, were determined for all the constructs and are summarised in Table 6. 1.

For *E. coli* JM83 cells, wild-type ColE9 translocated across the OM and killed cells rapidly (Figure 6. 6), this was indicated by the sharp decline in the CFUs over time. The t_{1/2} was ~8.5 ± 0.04 minutes. For ColE9^{AF488}, the t_{1/2} was ~24.1 ± 1 minutes, nearly 3x slower than wild-

type ColE9. For ColE9^{AF647}, the $t_{1/2}$ was $\sim 49.5 \pm 0$ minutes, nearly 6x slower than wild-type ColE9. The slow rate of entry for ColE9^{AF647} demonstrated that for this construct there were two competing rates; a rate of cell growth and rate of cell death. Due to the slow rate of entry and cell death, cells had time to divide, thereby gradually increasing the OD₆₀₀, reflected by the slight incline in the Ln CFU over time. The rate of entry was not determined for ColE9^{AF568} on JM83 cells for this reason, as the previous data showed this construct to be the poorest at translocating.

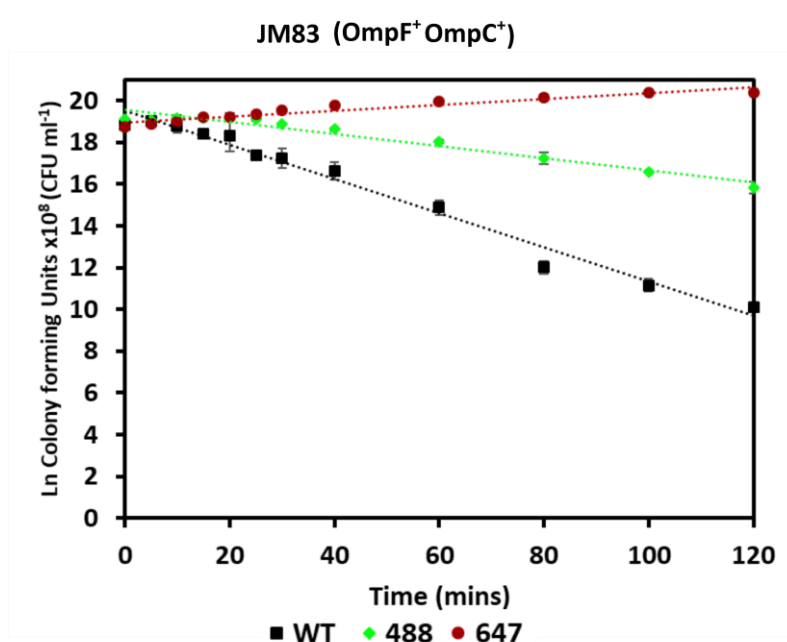


Figure 6. 6 - CFU rates of translocation across the OM of *E. coli* JM83 (OmpF⁺ OmpC⁺) cells using wild-type ColE9, ColE9^{AF488} and ColE9^{AF647}.

E. coli JM83 cells (OmpF⁺ and OmpC⁺), were used to measure the rate of colicin entry into cells, using wild-type ColE9 (■), ColE9^{AF488} (◆) and ColE9^{AF647} (●) (Section 2.7). The data was fitted to the equation of a straight line to obtain the $t_{1/2}$; the $t_{1/2}$ for wild-type ColE9 was $\sim 8.5 \pm 0.04$ minutes, $\sim 24.1 \pm 1$ minutes for ColE9^{AF488} and $\sim 49.5 \pm 0$ minutes for ColE9^{AF647}.

Table 6. 1 - Rates of translocation of ColE9 across the OM using OmpF⁺ and OmpC⁺ in *E. coli* JW2203 and JW0912 cells.

	JW2203 OmpF⁺	JW0912 OmpC⁺
	t_{1/2} (mins)	t_{1/2} (mins)
ColE9 WT	2.5 ± 0.3	7.5 ± 0.3
ColE9^{AF488}	4.1 ± 0.7	-
ColE9^{AF568}	78.8 ± 25	-
ColE9^{AF647}	29.6 ± 2.6	-

Because of the presence of both the cell growth and death rate, the method for measuring CFU was altered for OmpF⁺ and OmpC⁺ strains. *E. coli* JW2203 cells (OmpF⁺) and JW0912 cells (OmpC⁺) cells were grown to OD₆₀₀ 0.4, treated with 20 µg/ml chloramphenicol to inhibit cell growth, then treated with 80 nM of colicin (Section 2.7). This allowed the study of just the rate of cell death. Liquid growth assays confirmed that the addition of 20 µg/ml chloramphenicol inhibited cell growth immediately and no delay occurred (data not shown).

For *E. coli* JW2203 cells (OmpF⁺), wild-type ColE9 translocated across the OM and killed cells very rapidly with a t_{1/2} of ~2.5 minutes (Figure 6. 7). For ColE9^{AF488}, the t_{1/2} was ~4.1 minutes, only ~1.5x slower than wild-type ColE9 in these cells. For ColE9^{AF568}, the t_{1/2} was ~78.8 minutes. This was >30x slower than wild-type ColE9 in these cells and showed that regardless of the OMP, the AF⁵⁶⁸ label severely impacted the rate of entry of ColE9 across the OM. For ColE9^{AF647}, the t_{1/2} was ~29.6 minutes, ~12x slower than wild-type ColE9 in these cells. CFU experiments using chloramphenicol with *E. coli* JW0912 cells (OmpC⁺) was performed with only wild-type ColE9, which translocated across the OM and killed cells rapidly with a t_{1/2} of ~7.5 minutes (Figure 6. 7). This represents a <3x slower killing using OmpC⁺ cells than OmpF⁺. CFU experiments were not performed using these cells with the fluorescently-labelled constructs, as they would be as slow or slower than the result observed in JM83 cells.

Together, these experiments showed that wild-type ColE9 used large OM porins for its entry across the OM and that translocation across the OM by wild-type ColE9 and the fluorescently-labelled constructs, was much faster using cells with only OmpF, than cells with only OmpC or a mixture of OmpF and OmpC.

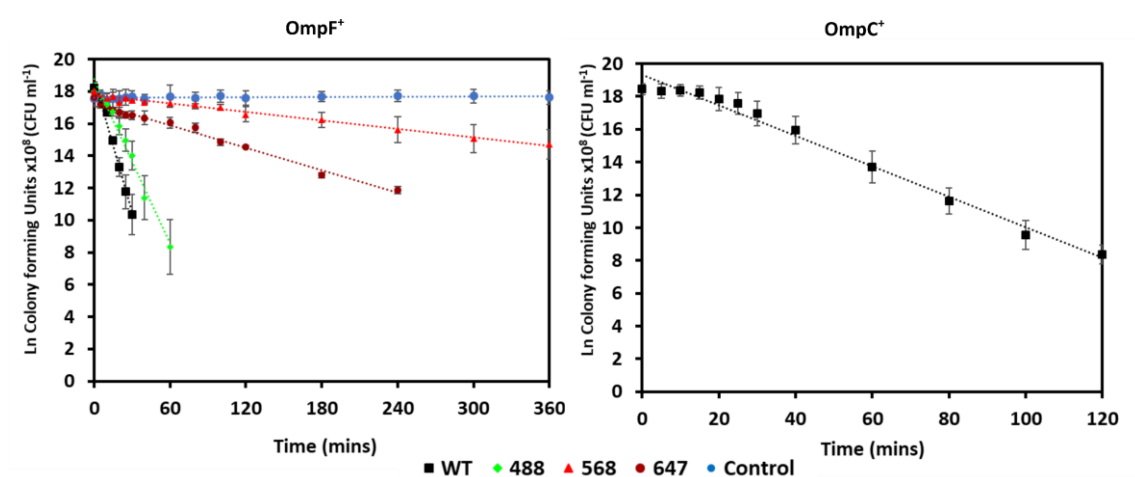


Figure 6. 7 - CFU rates of translocation across the OM of *E. coli* JW2203 OmpF⁺ and JW0912 OmpC⁺ cells using wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647}.

E. coli JW2203 OmpF⁺ and JW0912 OmpC⁺ cells were used to measure the rate of colicin entry into cells that had been treated with chloramphenicol to inhibit cell division. Wild-type ColE9 (■), ColE9^{AF488} (◆), ColE9^{AF568} (▲), ColE9^{AF647} (●) and a control (●) were used (Section 2.7). The data was fitted to the equation of a straight line to obtain the $t_{1/2}$ (Table 6. 1).

6.2.3 Influence of the inner membrane charge on cell killing by fluorescently-labelled colicins

E. coli strain HDL11 is depleted of negatively charged anionic phospholipids and has a single copy of an IPTG-inducible *pgsA* gene (Kusters et al. 1991). The product of this essential gene is phosphatidylglycerol-phosphate synthase (PGP-synthase), and is responsible for producing the major IM phospholipid phosphatidylglycerol (PG). In the absence of IPTG and *pgsA* induction, there is still some anionic phospholipids; ~2 % PG or ~1 % cardiolipin (CL) and ~6 % phosphatidic acid (PA), therefore a total of ~ 10 % anionic phospholipid content (Kusters et al. 1991). Increasing the IPTG concentration between 0-50 μ M gradually increases the amount of anionic phospholipids to ~15 % PG and ~20 % total anionic phospholipid content, which is similar to wild-type *E. coli* K-12 strains (Kusters et al. 1991).

It was previously shown that the net positive charge of the ColE9 DNase domain is attracted to the negative charge of the IM through electrostatically-driven protein-lipid interactions, before retro-translocation to the cytoplasm (Walker et al. 2007). Using strain HDL11, it was demonstrated through liquid culture killing assays, that as the level of anionic phospholipids and thereby the negative charge of the IM was increased, the rate of cell death by ColE9 increased. In addition, they showed that altering the DNase domain to both increase and decrease the overall net positive charge, increased and decreased the kinetics of cell killing, respectively.

AF⁴⁸⁸, AF⁵⁶⁸ and AF⁶⁴⁷ dyes have charges of -2, -2 and -3, respectively. To investigate if the different negative charges of the ColE9 AlexaFluor dyes had an impact on the rate of translocation across the negatively charged IM, HDL11 strain was used. *E. coli* HDL11 cells were grown in M9 minimal media in the presence of 0-50 μ M IPTG, before addition of wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} (Section 2.6.5).

For wild-type ColE9 and ColE9^{AF488}, in the absence of IPTG there was a marked decrease in OD₆₀₀, which reflected the small amount (~10 %) of remaining anionic phospholipids allowing colicin translocation and death (Figure 6. 8 A). As the concentration of IPTG increased from 0 to 50 μ M, there was a correlative decrease in the OD₆₀₀ (Appendix I Figure 11 A and B), with 50 μ M IPTG having the largest effect on colicin induced cell death (Figure 6. 8 B). This showed that the rate of cell entry across the IM and therefore cell death was increased with the increase in IM negative charge for electrostatic attraction of ColE9 DNase domain. For ColE9^{AF568} and ColE9^{AF647}, there was no real change in the OD₆₀₀ in the absence of colicin or 0-40 μ M IPTG (Figure 6.8 A, Appendix I Figure 11 C and D). There was only a slight reduction of OD₆₀₀ when 50 μ M IPTG was used (Figure 6. 8 B).

Altering the amount of negative anionic phospholipids did not increase or decrease killing by the different labelled colicins. This result would suggest that rate of cell translocation and killing by

the fluorescently-labelled colicins, was not affected by the charge of the fluorescent dyes at the IM or did not have an obvious contribution.

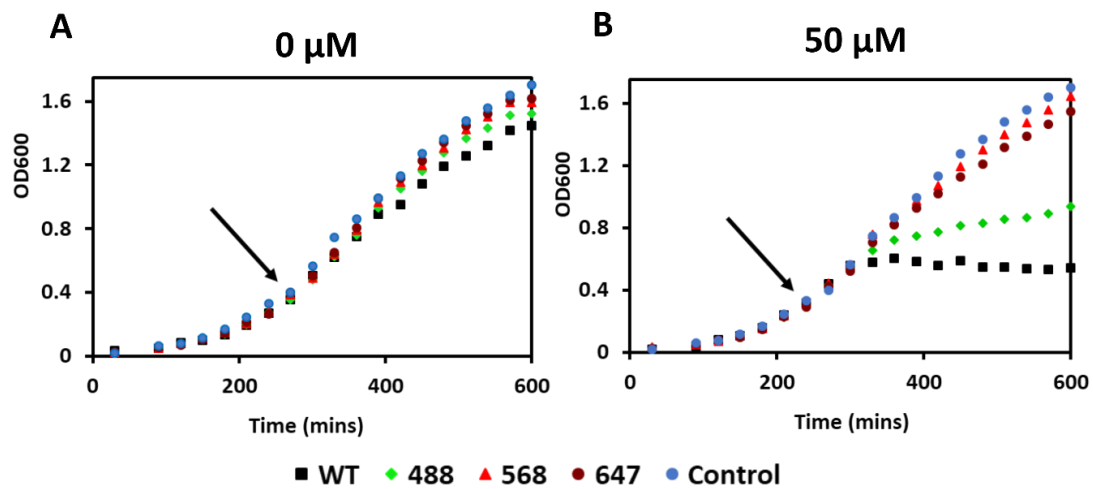


Figure 6. 8 - Effect of IM charge on the rate of translocation of wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647}.

E. coli HDL11 cells were used to look at the effect of IM charge on translocation, using wild-type ColE9 (■), ColE9^{AF488} (◆), ColE9^{AF568} (▲) and ColE9^{AF647} (●) and a control with no added colicin (●) (Section 2.6.5). (A) For wild-type ColE9 and ColE9^{AF488}, with 0 μM IPTG there was a marked decrease in OD₆₀₀, for ColE9^{AF568} and ColE9^{AF647}, there was no real change. (B) 50 μM IPTG, had a large effect on increasing killing by wild-type ColE9 and ColE9^{AF488}, for ColE9^{AF568} and ColE9^{AF647} there was only a slight reduction of OD₆₀₀. This suggests that rate of cell translocation and killing by the fluorescently-labelled colicins was not affected by the charge of the fluorescent dyes at the IM.

6.3 Discussion

The aims of this chapter were to confirm and characterise the translocation of ColE9 through the lumen of both OmpF and OmpC as their route of entry across the OM, determine the kinetics of entry and investigate the role of the IM on the rate of entry of the fluorescently-labelled ColE9 constructs.

It has previously been shown that ColE9 N-terminal IUTD is required for the recruitment of OmpF (Housden et al. 2005) (Housden et al. 2013), before threading through and binding via OBS1 and OBS2 sequences in the IUTD to the lumen of OmpF monomers (Figure 1. 13). The structure for ColE9 OBS1 peptide within the lumen of OmpF barrel has been determined (Housden et al. 2010). LPS was shown to have no effect on OBS1-OmpF binding, indicating its location within the lumen of OmpF away from LPS association (Housden et al. 2010). The occlusion of OmpF lumen by ColE9 IUTD OBS1 and OBS2 peptides, and their differing binding orientations, was recently shown through channel conductance measurements and fluorescent labelling of the OBS sequences and visualisation by microscopy (Housden et al. 2018).

Similar observations were found for ColE3, essentially identical in the T-domain and R- domain to ColE9 (Appendix I Figure 1). The N-terminus of the T-domain of ColE3 occluded and induced closing of all 3 channels of OmpF only when added from the trans-side with cis-negative potentials, using channel conductance measurements in planar lipid bilayers (Zakharov et al. 2004). An increase in the salt concentration prevented the occlusion of OmpF by ColE3, indicating that interactions with OmpF were primarily electrostatic. Similarly, the N-terminus of the T-domain of ColE1 showed occlusion of TolC (Zakharov et al. 2004). Residue 1-83 of ColE3 IUTD has been co-crystallised within OmpF, identified by its electron density (Yamashita et al. 2008). In addition to this, following release of immunity protein from ColE3, the C-domain undergoes a large unfolding and decrease in stability, shown by near-UV and far-UV circular dichroism (CD) and intrinsic tryptophan fluorescence. The isolated C-domain in the absence of

Im3 was capable of occluding and closing the channels of OmpF, from the trans-side, with a cis-negative potential (Zakharov et al. 2006). The recent method developed by Lonescu for determining the orientation of OmpF in planar lipid bilayers, suggests that the channel conductance experiments described were performed with the extracellular loops on the trans-side, which was shown as the side in which ColE3 T-domain and C-domain, ColN T-domain and ColE9 T-domain occluded OmpF (Lonescu et al. 2017). Taken together, this suggests that the pore of OmpF could support both the insertion of the N-terminal T-domain or C-domain if in a mostly unfolded state.

Following formation of the translocon at the cell surface, the colicin has to somehow translocate the cytotoxic domain across the OM. Although threading of the T-domain through OmpF does not necessarily provide evidence of translocation through the lumen, the data for the C-domain of ColE3 occluding OmpF and the results in Chapter 4 that suggested that the T-domain and R-domain of ColE9 translocate across the OM, in addition to the C-domain, it is possible that the entire colicin could be unfolded and passed through OmpF for entry into the cell.

The work in this chapter showed that the translocation of ColE9 across the OM and its subsequent cytotoxicity, is dependent on the presence of OM porins and occurred much faster using OmpF than OmpC. This is in agreement with the work of (Mock and Pugsley 1982), who showed that porins are required for the cytotoxicity of enzymatic group A colicins and sensitivity was dependent on the type of porin. The preference for use of OmpF over OmpC for rate of entry of ColE9 has been seen previously (Housden et al. 2010).

Using fluorescently-labelled ColE9 constructs, a difference in the translocation of ColE9 using OmpF and OmpC was observed through plate killing assays, microscopy and CFU experiments. The plate killing assays suggested that the fluorescently-labelled ColE9 constructs were able to pass through OmpF porin with less hinderance to enter and kill the cell than with

OmpC porin only. ColE9^{AF488} with the smallest of the fluorophores could still translocate into cells but rate of entry was reduced, whereas ColE9^{AF568} and ColE9^{AF647} with the larger fluorophores could either no longer translocate or were severely hindered, suggesting that they were getting stuck in the lumen of OmpC during translocation. This would explain why in *E. coli* JM83 cells, containing both OmpF and OmpC, the cytotoxicity is reduced with only a proportion of the colicins passing into the cell to kill and the other proportion remaining at the OM.

This was further demonstrated in this chapter by the determination of the kinetics of entry. Using CFU experiments, the results demonstrated that using *E. coli* JM83 cells (OmpF⁺ and OmpC⁺), wild-type ColE9 was fastest at killing, followed by ColE9^{AF488} and ColE9^{AF647}. Using JW2203 (OmpF⁺), again wild-type ColE9 was the fastest at killing, followed by ColE9^{AF488}, ColE9^{AF647} and ColE9^{AF568}. In comparison, using JW0912 cells (OmpC⁺), wild-type ColE9 killed slower than using OmpF⁺ cells. Therefore, wild-type ColE9 translocates into cells faster using OmpF, which also improves the rate of entry using fluorescent-labelled colicins, which are still reduced in comparison to wild-type ColE9. The value for the $t_{1/2}$ of wild-type ColE9 determined here was in agreement with the kinetics previously determined, where 3 different *E. coli* strains showed that ColE9 had a $t_{1/2}$ of 13-18 minutes (Walker et al. 2007). The preference for OmpF for improved kinetics of entry is thereby in agreement with the findings of (Housden et al. 2010).

The microscopy data using OmpF⁺ or OmpC⁺ expressing cells with ColE9*^{AF647}, showed that after trypsin treatment, there is remaining internal fluorescence with a higher number of molecules using OmpF⁺ cells, whereas using OmpC⁺, >55 % of cells had no fluorescence and the remaining cells had very few molecules (mean of 1.6 molecules/cell). It suggests that using OmpC⁺ cells, ColE9*^{AF647} is exposed to trypsin on the cell surface for its cleavage and loss from the cell, whereas using OmpF⁺ cells, ColE9*^{AF647} remains protected. This could suggest that the ^{AF647} fluorophore can pass with hinderance through OmpF, but cannot translocate, or does so very poorly and therefore on very rare occasions through OmpC. If this were the case, this could

suggest that the mobile population are colicin molecules that have translocated fully into the cell and the immobile colicin populations have translocated but the fluorophores are stuck in OmpF (rarely OmpC). This could also explain why such a small number of molecules of ColE9*AF647 are seen in JM83 cells, as the number of molecules that translocate would be entirely dependent on the number of OmpF molecules expressed in cells, which is further hindered by the dye.

Based on the differences between OmpF and OmpC, the size of the central pore is likely to have an effect, but the contribution may only be minor. It is probably a combination of the size of the pore with the cation-selectivity and difference in charged residues in the OmpF and OmpC central pores that affect entry of ColE9, which could be further affected by the different properties of the fluorescent dyes, including size, charge and dimensions.

It is a possibility that instead of passing through the central pore, the cytotoxic domain is passing down the OmpF-LPS interface, as is suggested for ColN, and that the presence of the different fluorescent dyes retards their entry across the lipid membrane. However, this is unlikely, as the external β -barrels of OmpF and OmpC are similar, so a similar result should have been observed using the OmpC-LPS interface.

This could therefore provide additional evidence to suggest that ColE9 passes through the central pore of the OMP, as the use of fluorescent dyes attached to the colicins restricts or completely inhibits their translocation across the OM by blocking the pore.

In the case of ColN, there is conflicting evidence for the translocation route across the OM. There is evidence that ColN passes down the OmpF-LPS interface and other evidence to suggest it passes through the OmpF lumen. A ColN-RC (receptor and cytotoxic domain construct) has been shown to bind to the external face of the OmpF barrel at the interface between two monomers and cause LPS displacement (Baboolal et al. 2008) (Clifton et al. 2012). The OmpF barrel is proposed to create a gap in the LPS to aid in exposure of the base of LPS for ColN

R- domain to bind (Johnson et al. 2014). Baboolal et al. propose that following this, the C-domain unfolds and translocates across the membrane at the OmpF-LPS interface. OmpF-OmpC hybrids were used to show that ColN binding and translocation was dependent on residues on the OmpF barrel outer wall which correspond to proposed LPS-binding sites (Fourel et al. 1990) and that genes for LPS biosynthesis are required for ColN cytotoxicity (Sharma et al. 2009). However, there is also evidence that the R-domain of ColN occludes OmpF, which could indicate a more central binding of ColN to OmpF at the entrance of the barrel, like other colicins and their respective receptors (Stora, Lakey, and Vogel 1999).

As a route for translocation, there is evidence to suggest that ColN does not pass through the lumen of OmpF. Mutations introduced into loop L3 in the centre of the lumen of OmpF to fix its conformation and prevent conformational changes or its displacement, still showed sensitivity to ColN (Bainbridge et al. 1998). It was suggested that due to the lack of space in the lumen and the extent of unfolding of ColN required for entry, that ColN does not translocate through the lumen of OmpF. However, there is also evidence to support ColN entry through the lumen OmpF. Mutation at position G119D on loop L3 within the barrel of OmpF, which had no effect on barrel external surface but dramatically altered the cross-sectional area of the lumen, led to ColN resistance (Fourel et al. 1993). A mutation within the centre of OmpF would not reduce ColN translocation if it occurred at the OmpF-LPS interface and could suggest that ColN, or some part of it binds within or traverses through the porin of OmpF to cross the OM (Jeanteur et al. 1994). In addition, planar lipid bilayer experiments using OmpF showed that the T-domain of ColN occluded and induced closing of all 3 channels of OmpF, suggesting that at least part of ColN passes through OmpF (Zakharov et al. 2004). In agreement with this is the identification of an OBS1 sequence in the unstructured T-domain of ColN, which was essential for OmpF binding *in vitro* and became ordered upon complex formation. Suggesting that similar to what has been shown for ColE9, the T-domain of ColN threads through and binds in OmpF, presumably for binding to the Tol system for translocation (Johnson et al. 2013). For ColN, there

is therefore a lot of conflicting evidence for binding and the route of entry using OmpF across the cell. In addition, there is a lack of evidence for how the C-domain translocates across the membrane.

From our current understanding of the range of different colicins, they have similarities and differences in their modes of entry, including the receptors and translocators, use of Tol/Ton system and their different target locations and modes of cytotoxicity. It is therefore possible that whilst the use of OMPs as translocators is essential, they have different modes of translocation using the OMPs, or alternatively OMPs may only be essential as translocators for Tol-dependent colicins. This could also be a function of their structure and size, ColN is small ~40 kDa (Vetter et al. 1998) with a short R-domain with reduced flexibility, which may prevent the ability to search for and translocate through the lumen of OmpF. Another example of this is ColB, a 55 kDa dumbbell shaped colicin that uses FepA as a receptor. There is conflicting evidence for the displacement of the N-terminal globular plug domain from FepA and therefore the potential for ColB to translocate through the pore using a single channel for binding and translocation (Smallwood et al. 2009) (Devanathan and Postle 2007).

In addition to determining the kinetics of entry of ColE9 ($t_{1/2}$ of 13-18 minutes), Walker et al. also determined kinetics for ColE7 and ColE8, using both trypsin protection cell survival assays and the rate of DNA damage using a lux reporter. Using three lab strains and the two methods, ColE7 and ColE8 gave a $t_{1/2}$ of 1-7 minutes and 10-15 minutes, respectively. The difference in rate was attributed to the difference in the overall net positive charge of the DNase domain, with ColE9, ColE7 and ColE8 having charges of +7, +13 and +9, respectively. Altering the overall charge of ColE9 DNase by both increasing and decreasing the net positive charge, led to enhanced and reducing killing, respectively (Walker et al. 2007).

The role of the charge of the IM phospholipids for ColE9 was demonstrated using strain HDL11 (Walker et al. 2007). In the absence of IPTG and therefore reduced anionic phospholipids,

ColE9 gave a $t_{1/2}$ of 60 minutes, a > 3-fold reduction. Taken together, this showed the relationship and importance between the overall net positive charge of the DNase domain and negative charge of the IM for the electrostatically-driven interaction and entry into the cytoplasm to kill the cell (Walker et al. 2007). Based on the finding that charge contributed to kinetics of entry, the difference between the different negative charges of the AlexaFluor dyes on ColE9 kinetics was investigated. Altering the amount of negative anionic phospholipids did not increase or decrease killing by the different labelled colicins, thereby suggesting that rate of cell translocation and killing by the fluorescently-labelled colicins was not affected by the charge of the fluorescent dyes at the IM or did not have an obvious contribution.

6.4 Conclusions

The work presented in this chapter showed that ColE9 can use either OmpF or OmpC to translocate across the OM, but that the rate of translocation and thereby killing by ColE9 was faster in cells with OmpF. Plate killing assays using fluorescently-labelled ColE9 suggested constructs were able to pass through OmpF with less hinderance than OmpC, where ColE9^{AF488} could still translocate but was reduced, whereas ColE9^{AF568} and ColE9^{AF647} were essentially completely inhibited. This was also reflected in the kinetics of OM entry by CFU experiments, where wild-type ColE9 and fluorescently-labelled ColE9 translocated and killed cells faster in cells containing only OmpF than in cells containing OmpF and OmpC. Widefield microscopy data using OmpF⁺ or OmpC⁺ expressing cells with ColE9*^{AF647}, showed that after trypsin treatment, there is remaining internal fluorescence with a higher number of molecules using OmpF⁺ cells, whereas using OmpC⁺, >55 % of cells had no fluorescence and the remaining cells had very few molecules, suggesting lack of or very poor translocation of ColE9*^{AF647} through OmpC. The results of this chapter provided additional evidence that ColE9 translocates through the central pore of OmpF and OmpC, which was demonstrated through the use of the fluorescently-labelled ColE9 constructs, with their different size AF dyes, reducing or inhibiting the rate of entry of ColE9 across the OM. The charges of the fluorescent dyes did not seem to have an obvious impact on the rate of translocation of ColE9.

7 Chapter 7- General Discussion

7.1 General discussion

ColE9 is the best studied bacteriocin and our current understanding has been elucidated by a wide range of *in vitro* and *in vivo* techniques to better understand its structure, function and mechanism of action. However, a limitation of the current methods available, is the lack of approaches to directly visualise colicin translocation at the single-molecule level in live *E. coli* cells. Therefore, the principal aim of this research was to develop fluorescence-based microscopy tools, through which ColE9 transport to the cytoplasm of live *E. coli* cells could be followed.

The energy requirements for ColE9 translocation

One of the findings that this thesis has further dissected and expanded on is the energy requirements for ColE9 translocation. As previously discussed, Vankemmelbeke et al. showed that the PMF, in particular the electrical potential, was required for the dissociation of Im9 from ColE9 at the cell surface (Vankemmelbeke et al. 2009). The work of Rassam et al. showed that the PMF was necessary for the formation of the translocon between the Tol system and ColE9 (Rassam et al. 2018). The work of this thesis has now shown that the PMF is also required for the transport of ColE9 across the OM.

This suggests that the energy-dependence and the role of the PMF has several successive steps in the translocation of ColE9 across the OM. Initially, the PMF is necessary for the conformational change of TolA for its extension into the periplasm (Germon et al. 2001) and its presentation for interaction with TolB to form a translocon with ColE9 (Rassam et al. 2018). These interactions allow the transduction of the PMF to ColE9 at the cell surface by an unknown mechanism.

Additional conformational changes have been reported which are conceivable to be a result of transduction of the PMF. The R-domain of ColE9 is flexible and requires unfolding

(Penfold et al. 2004), which was only induced on formation of the translocon with the Tol system (Housden et al. 2005). Following this, the PMF has been shown to be required for Im9 dissociation (Vankemmelbeke et al. 2009). Low levels of force have been shown to be transduced by the first 20 residues of the DNase domain to undergo conformational changes and remodel a trigger switch, for the rapid dissociation of Im9 from ColE9 (Farrance et al. 2013). It is conceivable that this force is provided by the unfolding of the R-domain on contact with the PMF through the T-domain. The findings of this thesis showed that following immunity release, the PMF is still required for colicin entry, as experiments were performed in the absence of Im9 and PMF and translocation was inhibited. This suggests that the PMF is either necessary to maintain the translocon for entry, or the PMF plays a role in the unfolding of the colicin and its DNase domain for entry into cell, as the DNase domain does not spontaneously unfold in the absence of Im9 when purified *in vitro*.

How much of the colicin translocates into the cell? And how does it cross the OM?

Following Im9 dissociation, the colicin has to translocate across the OM. The narrow lumen of OmpF and OmpC is able to accommodate unstructured polypeptides, as demonstrated by the IUTD of ColE9 (Housden et al. 2010) (Zhang et al. 2008). In combination with the evidence of R- domain unfolding at the cell surface on translocon formation (Penfold et al. 2004), it could suggest that the entire colicin translocates across the OM through the lumen of OmpF or OmpC. The results of chapter 4 showed through the fluorescent labelling of the T-domain and R-domain that after ColE9 translocation, fluorescence could be visualised following trypsin treatment. This suggests that the whole colicin translocates into the cell across the OM. In addition, plate killing assays showed that fluorescent labelling of the T-domain and R-domain affected ColE9 cytotoxicity, raising the question of why would translocation be affected if these domains remained at the cell surface and did not cross the OM?

Once the T-domain threads through OmpF, the PMF-energised Tol system could be responsible for pulling the entire unfolded colicin through the lumen of OmpF, from the N- terminal IUTD, T-domain, R-domain through to the C-terminal cytotoxic DNase domain. The C-domain of ColE3, essentially identical in the T-domain and R-domain to ColE9, has been shown to undergo large unfolding and a decreased stability following release of the immunity protein, the isolated unfolded C-domain was capable of occluding the lumen of OmpF in planar lipid bilayers (Zakharov et al. 2006). Taken together, this suggests that the pore of OmpF could support the insertion of the N-terminal T-domain and followed by the unfolded C-domain when pulled through from N- to C-terminus. Chapters 3 and 6 showed using several assays, that in the absence of porins, cytotoxicity and therefore translocation was inhibited. Cells expressing both OmpF and OmpC porins showed killing by all constructs, however the *in vivo* cytotoxicity and kinetics of entry for fluorescently-labelled constructs were reduced. Cells expressing only OmpF had dramatically improved kinetics of killing for all constructs and showed by microscopy a higher number of translocated ColE9*^{AF647} molecules. This is compared to cells expressing only OmpC, in which the kinetics of entry of wild-type ColE9 was reduced and the fluorescently-labelled constructs were severely reduced or completely inhibited from translocating. This therefore suggests that the route of entry of ColE9 across the OM into cells is through the lumen of OmpF or OmpC.

The number of colicins needed for cell death

The literature has reported that colicin action has single-hit kinetics. Meaning that although thousands of colicin molecules bind to receptors on the cell surface, they each act non-cooperatively to translocate and kill the cell (Nomura 1963). It is also reported that although 2000-3000 colicins can bind to the surface, only a small proportion of colicins translocate to kill, which can correspond to as little as 40-300 colicins (Shannon and Hedges 1967). First-order kinetics for killing by colicins, has been suggested to mean that a single molecule could be sufficient to kill a cell, although this has yet to be formerly demonstrated.

The work of this thesis has visualised for the first-time single-colicin molecules diffusing within the cytoplasm, with between 1 and 10 molecules per cell in 30 minutes of incubation. This is likely an underestimation of the number of colicins per cell, due to the different stages of cell division, a difference in the number of receptor or translocator proteins between cells, a function of the length of cell treatment during the experiment, or the lack of visualisation and detection of the molecule due to its diffusion, location in the cytoplasm or overlap with other molecules. The numbers suggest that although potentially thousands of colicins bind to the surface via BtuB, only a very small proportion of those molecules actually translocate into the cell.

There could be multiple reasons to explain why only a few molecules translocate. One reason could be to ensure rapid cell death before cell division; several colicins are translocated. Another reason could be that as the construct used in microscopy is cytotoxically inactive, several colicins can still translocate because of the presence of the PMF in living cells. One reason to explain why the number of molecules was limited could be related to the biology of the cell and the availability of the Tol system and PMF for translocation of the colicins. Or it could be due to the additional step of translocation of the DNase domain across the IM, reducing the number that translocates into the cytoplasm within a given period of time. This could be reflected by the observation of a higher number of ColB molecules observed in the periplasm. Or it could be a combination of several of these factors.

It could also be that it is possible to visualise individual molecules inside the cell due to the choice of fluorophore used, restricting the number of colicins that translocate within the same period of time as wild-type ColE9. Comparison of cytotoxicity of wild-type ColE9 and ColE9^{AF647} by plate killing assays using JM83 cells (OmpF⁺ and OmpC⁺) in chapter 3, showed that cytotoxicity of ColE9^{AF647} was reduced by at least ~81-fold. CFU kinetic experiments in chapter 6 showed using JM83 cells that translocation of ColE9^{AF647} was 6-fold slower, whereas in JW2203

(OmpF⁺) translocation of ColE9^{AF647} was 12-fold slower than wild-type ColE9. This could reflect the reduction in the number of ColE9*^{AF647} molecules that translocate compared to wild-type ColE9. If this were the case, the number of molecules reported for ColE9*^{AF647} would not represent the true number of molecules of wild-type ColE9 that would translocate into the cell. However, as performed under laboratory conditions, this may also not reflect the number of molecules of ColE9 that would translocate within its environmental niche. Regardless of this, visualisation of single-molecules allowed identification of two populations of ColE9 molecules that may not have otherwise been possible if large numbers of molecules had translocated.

The mobile and immobile colicin populations

There could be multiple reasons to explain what the two populations of ColE9 molecules represent, the majority of which appeared immobile. Firstly, immobile molecules might be bound to the chromosome, in addition as they are unable to cleave DNA due to the active site mutation they could remain bound. The mobile population could be diffusing in the cytoplasm ready to bind non-specifically to its target DNA and therefore a function of the time-point at which the cells were imaged. This would reflect the observations of other cytoplasmic DNA binding proteins like RNAP (Stracy et al. 2015). This would be unlikely, as ColE9 DNase binds to the minor groove of dsDNA with μM affinity. In addition, preliminary microscopy experiments using Im9-eGFP, which formed a complex with ColE9 DNase*^{AF568} *in vitro*, indicated that both mobile and immobile populations were still present.

Secondly, the immobile molecules could be translocation intermediates; for example, due to the size of the fluorophore, they could be retarded in the lumen of OmpF and OmpC. The microscopy performed in chapter 6 using *E. coli* strains expressing OmpF⁺ or OmpC⁺, showed that ColE9*^{AF647} fluorescence remained after trypsin treatment using OmpF⁺ cells, with a higher number of translocated molecules, suggesting their entry and protection from trypsin treatment. Whereas using OmpC⁺ cells, >55 % of cells had no fluorescence and the remaining

cells had very few molecules after trypsin treatment. The lack of fluorescence in OmpC⁺ cells, suggests that the AF⁶⁴⁷ fluorophore is exposed to the surface for trypsin treatment and therefore not within the lumen of OmpC. This is in agreement with the cytotoxicity assays performed in chapters 3 and 6, where fluorescently-labelled constructs had their cytotoxicity reduced in the presence of OmpC with OmpF, or inhibited using OmpC alone. The presence of immobile molecules using cells with OmpF only, suggests that the AF⁶⁴⁷ fluorophore is able to pass through OmpF, but has its rate of entry reduced due to steric hinderance. This would explain why using JM83 cells containing both OmpF and OmpC, that fewer molecules are able to translocate inside compared to using OmpF only expressing cells.

In addition, ColE9^{AF568} construct was shown in chapter 3, to be very poor at killing by plate killing and bioluminescence assays. However, when microscopy was performed with ColE9^{AF568} in chapters 3 and 5, fluorescence remained after trypsin treatment and immobile molecules were present. This showed entry into the cell for protection from trypsin treatment, however lack of DNA damage. This suggests that the AF⁵⁶⁸ fluorophore was getting stuck in the lumen of OmpF and unable to translocate further into the cell to kill. Based on the microscopy performed with AF⁶⁴⁷ fluorophore and its lack of presence in OmpC⁺ expressing cells, it is unlikely AF⁵⁶⁸ dye is able to enter the lumen of OmpC, which is reflected in its complete inhibition of killing OmpC⁺ expressing cells by plate killing assay in Chapter 6. In agreement with this proposal, is the presence of immobile molecules using ColE9^{AF488} (data not shown), which plate killing and bioluminescence assays in chapter 3, has shown to be the best fluorescently-labelled construct to kill cells and therefore expected to be the least hindered in translocation. This therefore suggests that the most likely explanation for the immobile population, is the fluorophore getting restricted within the lumen of OmpF porin when translocating across the OM.

Thirdly, the immobile molecules could be translocation intermediates with their rate of entry across the IM reduced. Perhaps the presence of the fluorophore affects the unfolding and

insertion of the DNase domain in the IM to form short-lived non-voltage-gated channels. This may also affect its recognition and proteolytic cleavage by FtsH. Labelling of the DNase domain or the 6-residue R-domain fragment that translocates across the IM, could affect retro-translocation across the IM. This was investigated in chapter 4 by the fluorescent labelling of the R-domain of ColE9 before and after the FtsH cleavage site. Plate killing assays showed that labelling of ColE9 before the cleavage site (S420C) slightly reduced the cytotoxicity in comparison to wild-type ColE9, however labelling of ColE9 after the cleavage site (S451C) dramatically reduced cytotoxicity which was nearly completely inhibited. This could suggest that the fluorescent label affects retro-translocation across the IM. However, this is unlikely, as when plate killing assays were repeated using OmpF⁺ cells the cytotoxicity of each of the constructs was restored to near wild-type level killing. This again suggests that the rate-limiting step for translocation is through the porins of OmpF and OmpC and that the location of the cysteine and fluorescent dye can impact translocation. The reason for the two populations could also be a combination of these different factors.

The generation of spheroplasts still showed immobile molecules of ColE9*^{AF647} (data not shown), but this does not address if the immobile molecules are retarded in the lumen of OmpF and OmpC, as the OM is only perforated during spheroplasting and OMPs have been observed to remain in the OM (Hobb et al. 2009) (Tefsen et al. 2005). In addition, it does not address if the immobile molecules are within the IM as the IM is also still present and intact during spheroplast formation.

The rate of cell death by ColE9

The rate of cell death on addition of wild-type ColE9 is quick, with $t_{1/2}$ values of 13-18 minutes reported (Walker et al. 2007). The faster rate of killing by wild-type ColE9 using OmpF over OmpC has also been reported by liquid killing assays (Housden et al. 2010). The work of this thesis has characterised the difference in rate of killing by wild-type ColE9 using OmpF and OmpC through

CFU kinetic experiments, with wild-type ColE9 having a $t_{1/2}$ of ~8.5 minutes using actively growing cells containing both OmpF and OmpC, which is reduced to a $t_{1/2}$ of ~2.5 minutes using growth-inhibited cells containing only OmpF, thereby demonstrating just how quickly colicins can translocate and kill *E. coli* cells.

The findings of this thesis can therefore be used to confirm and expand on our current understanding of how ColE9 binds and translocates into *E. coli* to kill cells, as described in Introduction Section 1.6. These findings and use of this fluorescence microscopy method can thereby aid in the progress and development of colicins for testing in clinical settings for the implementation of colicins as potential narrow-spectrum antibiotics in the fight against antibiotic-resistance.

7.2 Future directions

The work presented in this thesis describes the development of a fluorescence-based microscopy assay, as a tool to visualise the mechanism of colicin binding and translocation into *E. coli in vivo*. However, there are still a lot of outstanding questions to be answered.

Investigation of the energy requirements for ColE9 translocation

The results of chapter 4 demonstrated through the use of CCCP and ColB, that the PMF is required for the translocation of ColE9 across the OM. In addition, translocation was shown to be dependent on the electrical potential of the PMF at neutral pH. The electrical potential is dominant at neutral pH (Borisov et al. 2011) (Padan, Zilberstein, and Rottenberg 1976), which could explain the requirement of it for ColE9 entry in these experiments performed at neutral pH. In addition, all of the energy dependence experiments performed using ColE9 and Im9 have been performed at neutral pH under laboratory conditions (Vankemmelbeke et al. 2009) (Rassam et al. 2018). Experiments could be repeated at different pH values, to investigate if translocation is dependent only on the electrical potential or if the energy requirements change in different pH environments. It is also unknown if translocation across the IM is also dependent

on the PMF, as the current experiments only test for initial entry across the OM of the cell. It would be interesting to investigate if entry across the IM is also PMF-dependent, as has been seen with CDIs (Ruhe et al. 2014), or if energy is provided by FtsH ATPase, which could be investigated using the FtsH ATPase mutant strain once the abhorrent cell phenotype has been addressed.

Identification of how much of the colicin translocates into the cell

Another question to address is if the entire colicin translocates across the OM into the cell, or if only parts of the T-domain and R-domain translocate along with the C-domain, and the rest remains in contact with the cell surface. There is evidence using other colicins like ColE2 and ColA, to suggest that some part of the colicin remains bound to the cell surface after translocation (Duche 2007) (Benedetti et al. 1992). Work in this thesis provided *in vivo* evidence to suggest that at least part of the T-domain and R-domain of ColE9 translocates across the OM through the fluorescent labelling of the T-domain and R-domain and remaining fluorescence after trypsin treatment. This could be further investigated by fluorescently-labelling multiple locations of the T-domain and R-domain and investigating how much of the colicin enters the cell. Further to this, spheroplasting experiments could be performed to investigate the location of these domains, present in the periplasm or cytoplasm.

In addition, based on the microscopy data for ColE9*^{AF647} translocation in chapters 3 and 4, a high level of OM labelling is observed, followed by the translocation of only a few molecules after trypsin treatment. To investigate if the small fraction of molecules that translocate remain bound to the cell surface, the OM of cell division inhibited cells could be saturated with unlabelled ColE9*, followed by ColE9*^{AF647} and measure fluorescence to identify if any receptors become unoccupied during ColE9* translocation. It is also possible that the fluorescently-labelled T-domain and R-domain have not entered the cell and fragments have remained on the surface bound to BtuB or OmpF, somehow protected from trypsin treatment. This could be

investigated through saturation of the OM of cells with unlabelled ColE9*, followed by trypsin then labelling with ColE9*^{AF647} to see if fluorescent colicin can bind to free receptors. It is unlikely that ColE9 could remain on the surface after trypsin treatment, due to the high level of trypsin used and presence of multiple trypsin cleavage sites on ColE9.

Investigating the role of FtsH in ColE9 translocation by fluorescence microscopy

FtsH was shown to play an essential role in the retro-translocation of ColE9 DNase domain across the IM into the cytoplasm for cytotoxicity to occur, using a series of FtsH deletion and mutation strains (Walker et al. 2007). As discussed above, one of the possible reasons for the immobile ColE9 population observed in this thesis, was the unfolding and insertion of ColE9 in the IM and proteolytic processing by FtsH. Hence the interest in studying FtsH by fluorescence microscopy. In this thesis, through the use of these FtsH deletion and mutation strains and imaging by widefield microscopy, previously unobserved cell phenotypes were seen for the FtsH deletion and mutation strains used, which suggested that a functional FtsH was also necessary for cell growth and division. However, these phenotypes meant that visualisation of the involvement of FtsH for ColE9 translocation was not possible through fluorescence microscopy. The reason for these phenotypes was unclear and may be due to an imbalance in the LPS and PL ratio, or loss of proteolytic cleavage of protein substrates due to the lack of a functional FtsH. Further work needs to be done to identify the reason behind these cell phenotypes and the best experimental conditions to observe normal cell phenotypes. This could then be used to visualise the involvement of FtsH in the retro-translocation across the IM and help to identify if it is the location of the immobile population. In addition, if the fluorophore is affecting the unfolding and insertion of the DNase domain into the IM, the secondary and tertiary structure could be investigated by CD and the formation of short-lived non-voltage-gated channels in the IM by the DNase by planar lipid bilayer experiments.

Identification of the role of the mobile and immobile population

As discussed above, through the direct visualisation of colicin molecules by fluorescence microscopy, two populations of translocated ColE9 molecules were observed, a mobile and immobile population. It is unclear what these two ColE9*^{AF647} populations represent.

Future work requires optimisation of the fluorescence microscopy detection and analysis technique to more accurately count both the number of single-molecules observed per cell and to count how many of those are mobile and immobile. The current issue encountered, is that by widefield microscopy using AF⁶⁴⁷ dye, the molecules bleach quickly and are visualised at the resolution limit. This makes it difficult to detect and count the number and determine how many are mobile and immobile with only a very short number of frames before photobleaching. Use of TIRFM allows the molecules to be observed for much longer periods of time before photobleaching, but predominately images at the basal membrane where fewer molecules were present. The current method is a useful technique to identify what conditions allow translocation to occur and overall cell fluorescence intensity allows comparison between conditions. However, a more accurate determination of the number of single-molecules and the proportion of mobile and immobile molecules would contribute to our understanding of the translocation mechanism and identify what the two populations of molecules represent by identifying what conditions change their proportion.

To address if the immobile population is the DNase domain bound to its target DNA or if the DNase remains bound to DNA because of the inactive construct, this could be further investigated through development of the technique using Im9-eGFP. By confirming complex formation of Im9-eGFP and DNase^{AF568} in the cytoplasm *in vivo* by performing SM-FRET, this could then be used to identify if it affects the presence of immobile colicins. Microscopy could also be repeated using active ColE9 to see if this affects the two populations. To address if the immobile colicins are due to their insertion in the IM prior to retro-translocation by FtsH, the

proportion of colicins within the IM could be investigated through isolation of the IM through density gradient centrifugation.

Another possibility to explain the two populations, may be a result of timing of imaging. ColE9 binds to dsDNA randomly, with a preference for thymine-rich regions (Pommer et al. 2001). It is unknown how long a nuclease is bound to DNA to cause hydrolysis and if it remains bound or it dissociates and diffuses in the cytoplasm to bind and hydrolyse elsewhere on the DNA until cell death occurs. In the experiments performed in this thesis, cells are incubated with ColE9 for 30 minutes, before washing and treating with trypsin for 1 hour. Cells are then imaged as quickly as possible before cell division occurs. Therefore, a series of time-course experiments could be performed to look at the effect over time. An initial time-course could grow and label cells with colicin as per the current method, but after trypsin treatment, treat cells with chloramphenicol to prevent further cell division. Cells can then be imaged over much longer periods of time without the issue of cell division to see if the ratio of the two populations of molecules changes. Another time-course that could be performed is the incubation of cells with colicin for shorter amounts of time, for example from 5 minutes up to 30 minutes, to look earlier in the incubation and translocation process to see if it affects the ratio of mobile and immobile populations.

It could also be worth investigating the proportion of the two populations in different growth medias. The findings of Stracy et al. showed that the proportion of mobile and immobile RNAP molecules changed between LB and M9 minimal media, due to the rate of cell growth and nucleoid organisation. This could also be a factor for colicin organisation and proportion of the two populations (Stracy et al. 2015), however conditions that alter the OmpF and OmpC ratios will need to be controlled between the different medias.

One observation from the results of this thesis, was that fluorescently-labelled colicins had reduced ability to translocate across the OM due to the different sizes and dimensions of

the fluorescent dyes. This also indicated that the colicins, in particular the DNase domains, were translocating through the lumen of OmpF and OmpC and thereby getting stuck. To investigate if the labelled colicins are getting stuck, OmpF or OmpC can be isolated from the OM after incubation with the colicin, to identify if the DNase is present within the lumen of the porins. In addition to this, as immobile molecules are still present after spheroplasting, the presence of OmpF and OmpC porins in spheroplasted cell OMs could be probed by western blotting, or fluorescently-labelled ColN.

The data in this thesis demonstrated that different dyes had a clear impact on the effects of translocation of ColE9. Three different commercially available dyes were used, AF⁴⁸⁸, AF⁵⁶⁸ and AF⁶⁴⁷. Dyes with an absorbance of <500 nm were complicated by the issue of autofluorescence, so two far-red dyes were chosen with different fluorescent lifetimes and molecular weights. However, there are many other AF and other commercial maleimide dyes available, that could be investigated for their role and effect on ColE9 translocation and the identity of the two populations.

The wider aspect of future directions from the work presented in this thesis, would be the implementation and establishment of these fluorescence microscopy tools as a common technique, along with the arsenal of existing *in vivo* and *in vitro* techniques, to better understand and elucidate the translocation mechanisms of other less well understood colicins and colicin-like bacteriocins. A more detailed dissection of the requirements for binding and translocation would aid in the development of colicins and bacteriocins as potential narrow-spectrum species-specific antibiotics in the fight against antibiotic resistance.

In addition to the development as potential antibiotics, the study of bacteriocins are also of biological interest. They are one of the few proteins that can enter bacteria, where the majority of proteins that are studied are released from bacteria. Therefore, their entry mechanism is of interest as a bacterial import system. Bacteriocins are also being used as tools

to aid in the elucidation of the organisation and properties of proteins in both the inner and outer membrane of the widely used model organism *E. coli*.

7.3 Overall summary and conclusions

In summary, the aim of this thesis was to develop a fluorescence-based assay to investigate the requirements for binding and translocation of ColE9 molecules in live *E. coli* cells for the first time. ColE9 was fluorescently-labelled within its cytotoxic domain (K469C) with AF⁴⁸⁸, AF⁵⁶⁸ and AF⁶⁴⁷. The fluorescent labels did not affect nuclease activity *in vitro*, but did affect translocation and therefore *in vivo* cytotoxicity. AF⁴⁸⁸ had the smallest effect on reducing entry and cytotoxicity compared to wild-type ColE9, but AF⁵⁶⁸ and AF⁶⁴⁷ had a larger effect, with the cytotoxicity of ColE9^{AF568} nearly completely inhibited, most likely due to the large bulky structure of the dyes.

ColE9*AF⁶⁴⁷ bound specifically to BtuB on the cell surface and translocation across the OM was dependent on the presence of the PMF and a series of protein interactions with the Tol system, which was independent of the fluorophore used. This was shown through chemical inhibition using CCCP and protein ionophore inhibition using ColB. PMF-dependent translocation was also seen using ColB for its entry into the periplasm. Translocation of ColE9 at neutral pH was dependent on the electrical potential ($\Delta\Psi$) of the PMF. ColE9*AF⁶⁴⁷ and ColB were shown to translocate to the cytoplasm and periplasm, respectively, through dual-labelling spheroplast experiments. A mean of 4-5 ColE9*AF⁶⁴⁷ molecules/cell were observed translocated within 30 minutes of incubation by widefield microscopy.

The involvement of FtsH in ColE9 translocation was observed through repetition of previously reported plate killing assays. However, experiments to show the involvement of FtsH by microscopy, was impossible due to abhorrent cell growth of the FtsH deletion and mutation strains. Fluorescence microscopy also suggested that the T-domain and R-domain of ColE9

translocates across the OM along with the DNase domain, through protection from trypsin of the fluorescently-labelled domains.

Single-molecules of ColE9*AF647 were observed in the cytoplasm for the first-time through TIRF microscopy, by the ability of the fluorescently-labelled molecules to photobleach in a single-step. The single-molecules were visualised as two populations; mobile and immobile, with the vast majority immobile. The mobile population was tracked *in vivo* and gave an average apparent diffusion coefficient of $6.2 \mu\text{m}^2/\text{s}$ displaying Brownian diffusion.

Finally, this thesis showed that ColE9 can use OmpF or OmpC to translocate across the OM, but that the rate of translocation and thereby killing by ColE9 was faster in cells using only OmpF by kinetic experiments. This was reflected in the microscopy, where a larger number of molecules of ColE9*AF647 translocated in OmpF⁺ expressing cells, and very few molecules in OmpC⁺ expressing cells. The reduced rate of entry into cells using fluorescently-labelled constructs, suggested that their route of translocation was through the central lumen of the porin of OmpF and OmpC. A proposed model for the translocation of ColE9, based on the experimental data obtained in this thesis, is shown in Figure 7. 1.

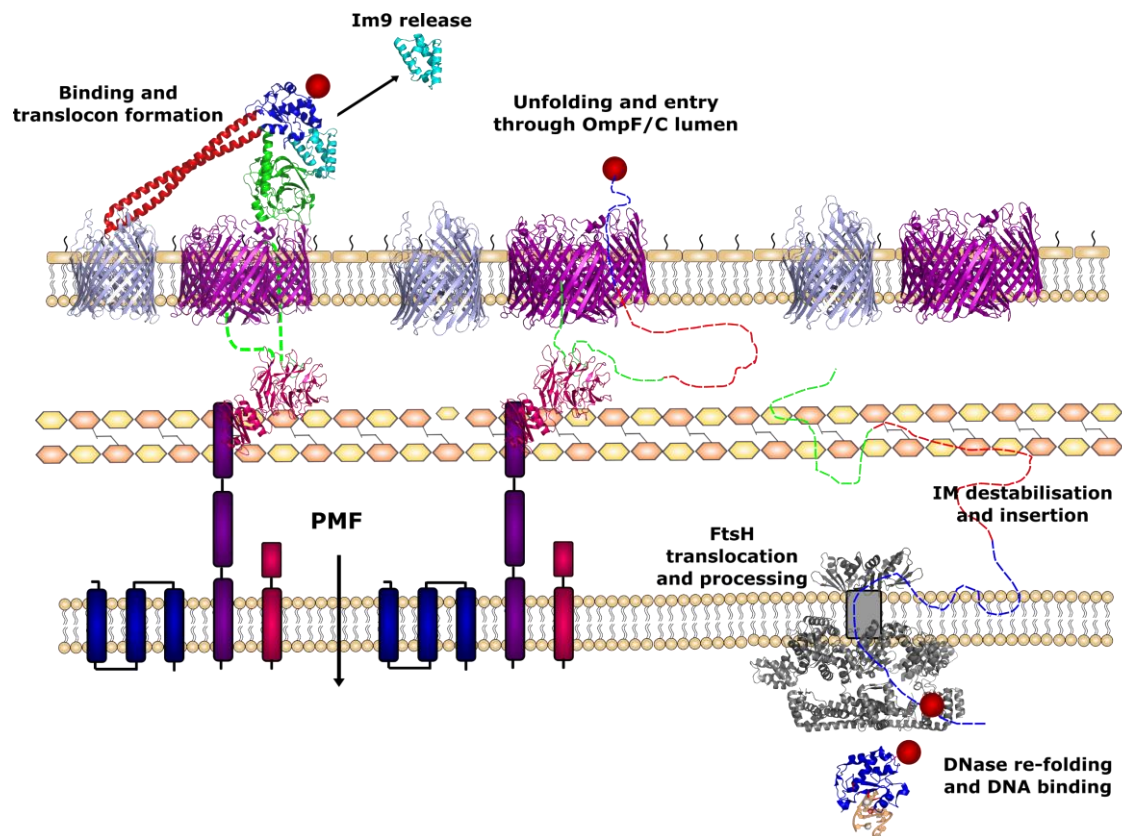


Figure 7. 1 - Proposed model for the translocation of ColE9 into *E. coli* cells based on experimental data.

ColE9 binds BtuB as its receptor via its R-domain, and OmpF as its translocator via its T-domain. The IUTD of the T-domain threads through the lumen of OmpF to present the TBE epitope to TolB, which makes contact with TolA. This allows communication of the PMF to ColE9, which unfolds at the cell surface, causing dissociation of Im9 and the PMF-dependent translocation of ColE9 through the lumen of OmpF. The DNase domain interacts with the IM through electrostatically-driven interactions, which leads to its destabilisation and unfolding. The unfolded DNase domain is then recognised by FtsH where it gets dislocated, translocates across the IM and proteolytically-cleaved from the R-domain. The DNase domain spontaneously refolds where it diffuses by Brownian diffusion to bind and cleave dsDNA in the cytoplasm.

The results of this thesis have demonstrated that the method developed in this work of fluorescently-labelling ColE9, can be used to confirm and expand on previous findings for ColE9 translocation that have used other *in vitro* and *in vivo* techniques. This technique can be used as an additional method to determine the requirements for the binding and translocation of ColE9, and in the future for other colicins and colicin-like bacteriocins, in live cells *in vivo* for the first time and address some of the many outstanding questions for the mechanism of translocation.

Appendix I

Protein sequences used in this work

pCS4 ColE9 WT

MSGGDGRGHNTGAHSTSGNINGGPTGIGVSGGASDGSWSENPNWGGGSGSGIHWGGGSGRGNGG
GNGNSGGGSGTGGNLSAVAAPVAFGFPALSTPGAGGLAVSISASELSAAIAGIIAKLKKVNLKFTPFQVVLSS
LIPSEIAKDDPNMMSKIVTSLPADDITESPVSSLPLDKATVNVNVRVDDVKDERQNISSVSGVPMSPVPVD
AKPTERPGVFTASIPGAPVLNISVNDSTPAVQTLSPGVTNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHN
AVYVSVSDVLSPDQVKQRQDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAV
QVYNSRKSELDAANKTLADAIAEIKQFNRFADHPMAGGHRMWQMAGLKAQRAQTDVNNKQAAFDAAA
KEKSDADAALSAAQERRKQKENKEKDAKDKLAMESKRNPKGKATGKGKPVGDKWLDDAGKDSGAPIPDRI
ADKLDRDKEFKSFDDFRKAVWEEVSKDPELSKNLNPSNKSSVSKGYSPFTPKNQVVGGRKVYELHHDKPISQ
GGEVYDMDNIRVTTPKRHIDIHRGK

pBH29NK3 ColE9 Y324C L447C K469C

MSGGDGRGHNTGAHSTSGNINGGPTGIGVSGGASDGSWSENPNWGGGSGSGIHWGGGSGRGNGG
GNGNSGGGSGTGGNLSAVAAPVAFGFPALSTPGAGGLAVSISASELSAAIAGIIAKLKKVNLKFTPFQVVLSS
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AVYVSVSDVLSPDQVKQRQDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAV
QVYNSRKSELDAANKTLADAIAEIKQFNRFADHPMAGGHRMWQMAGLKAQRAQTDVNNKQAAFDAAA
KEKSDADAALSAAQERRKQKENKEKDAKDKCAMESKRNPKGKATGKGKPVGDCWLDDAGKDSGAPIPDR
IADKLDRDKEFKSFDDFRKAVWEEVSKDPELSKNLNPSNKSSVSKGYSPFTPKNQVVGGRKVYELHHDKPISQ
GGEVYDMDNIRVTTPKRHIDIHRGK

pCS4 ColE9 H551A

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LIPSEIAKDDPNMMSKIVTSLPADDITESPVSSLPLDKATVNVNVRVDDVKDERQNISSVSGVPMSPVPVD
AKPTERPGVFTASIPGAPVLNISVNDSTPAVQTLSPGVTNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHN
AVYVSVSDVLSPDQVKQRQDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAV
QVYNSRKSELDAANKTLADAIAEIKQFNRFADHPMAGGHRMWQMAGLKAQRAQTDVNNKQAAFDAAA
KEKSDADAALSAAQERRKQKENKEKDAKDKLAMESKRNPKGKATGKGKPVGDKWLDDAGKDSGAPIPDRI
ADKLDRDKEFKSFDDFRKAVWEEVSKDPELSKNLNPSNKSSVSKGYSPFTPKNQVVGGRKVYELHADKPISQ
GGEVYDMDNIRVTTPKRHIDIHRGK

pMLF07 ColE9 K469C

MSGGDGRGHNTGAHSTSGNINGGPTGIGVSGGASDGSWSENPNWGGGSGSGIHWGGGSGRGNGG
GNGNSGGGSGTGGNLSAVAAPVAFGFPALSTPGAGGLAVSISASELSAAIAGIIAKLKKVNLKFTPFQVVLSS
LIPSEIAKDDPNMMSKIVTSLPADDITESPVSSLPLDKATVNVNVRVDDVKDERQNISSVSGVPMSPVPVD
AKPTERPGVFTASIPGAPVLNISVNDSTPAVQTLSPGVTNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHN
AVYVSVSDVLSPDQVKQRQDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAV
QVYNSRKSELDAANKTLADAIAEIKQFNRFADHPMAGGHRMWQMAGLKAQRAQTDVNNKQAAFDAAA
KEKSDADAALSAAQERRKQKENKEKDAKDKLAMESKRNPKGKATGKGKPVGDCWLDDAGKDSGAPIPDRI
ADKLDRDKEFKSFDDFRKAVWEEVSKDPELSKNLNPSNKSSVSKGYSPFTPKNQVVGGRKVYELHHDKPISQ
GGEVYDMDNIRVTTPKRHIDIHRGK

pMLF08 ColE9 H551A K469C

MSGGDGRGHNTGAHSTSGNINGGPTGIGVSGGASDGSWSENPNPWGGGSGSGIHWGGGSGRGNGG
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ADKL RDKEFKSFDDFRKAVWEEVSKDPELSKNLNPSNKSSVSKGYSPFTPKNQVGGGRKVYELHADKPISQG
GEVYDMDNIRVTTPKRHIDIHRGK

pMLF09 pBAD24-lm9-eGFP

HHHHHHNMELKHSISDYTEAEFLQLVTTICNADTSSEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDSPSG
IVNTVKQWRAANGKSGFKQIEGRHMSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLT LKFI
CTTGKLPVPWPPTLVTTTAYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD
TLVNRIELKGIDFKEDGNILGHKLEYNYN SHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIG
DGPVLLPDNHYLSTQSALS KDPNEKRDHMLLEFVTAAGITHGMDELYKASMTGGQQMGRGS

pRJ353 ColE9 DNase WT

RNKP GKATGKGKPVGDKWLDDAGKDSGAPIPDRIADKL RDKEFKSFDDFRKAVWEEVSKDPELSKNLNPS
NKSSVSKGYSPFTPKNQVGGGRKVYELHHDKPISQGGEVYDMDNIRVTTPKRHIDIHRGK

pBC1 ColE9 DNase K469C

RNKP GKATGKGKPVGDCWLDDAGKDSGAPIPDRIADKL RDKEFKSFDDFRKAVWEEVSKDPELSKNLNPS
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pREN94 pQE2-lm9-eGFP

HHHHHHNMELKHSISDYTEAEFLQLVTTICNADTSSEELVKLVTHFEEMTEHPSGSDLIYYPKEGDDDSPSG
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CTTGKLPVPWPPTLVTTTAYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD
TLVNRIELKGIDFKEDGNILGHKLEYNYN SHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIG
DGPVLLPDNHYLSTQSALS KDPNEKRDHMLLEFVTAAGITHGMDELYKASMTGGQQMGRGS

pREN113 ColE9-K298C

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GGEVYDMDNIRVTTPKRHIDIHRGK

pREN115 ColE9-S420C

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pREN116 ColE9-S451C

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GNGNSGGGSGTGGNLSAVAAPVAFGFPALSTPGAGGLAVSISASELSAAIAGIIAKLKKVNLKFTPFVVLSS
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GGEVYDMDNIRVTTPKRHIDIHRGK

pREN118 ColE9-W39A K469C

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GGEVYDMDNIRVTTPKRHIDIHRGK

pPW13 ColB TR-Cys

MSDNEGSVPTEGIDYGDTMVVWPSTGRIPGGDVKPGGSSGLAPSMPPGWGDYSPQGIALVQSVLFPGIIR
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VTQQLEQVTDIPASQLPAAGNNVPVKYRLTDLMQNGTQYMAIIGGIPMTVPVVDVAVVPDRSRPGTNIKD
VYSAPVSPNLPDLVLSVGQMNTPVRSNPEIQEDGVISETGNYVEAGYTMSSNNHDVIVRFPEGSGVSPLYIS
AVEILDSNLSQRQEAENNAKDDFRVKKEQENDEKTVLTKTSEVIISVGDKVGEYLGK

pEE01 ColB WT

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VTQQLEQVTDIPASQLPAAGNNVPVKYRLTDLMQNGTQYMAIIGGIPMTVPVVDAVPVPDRSRPGTNIKD
VYSAPVSPNLPDLVLSVGQMNTPVRSNPEIQEDGVISETGNYVEAGYTMSSNNHDVIVRFPEGSGVSPLYIS
AVEILDSNSLSQRQEAENNAKDDFRVKKEQENDEKTVLTKTSEVIISVGDKVGEYLGDKYKALSREIAENINNF
QGKTIRSYDDAMSSINKLMANPSLKINATDKEAIVNAWKAFNAEDMGNKFAALGKTFKAADYAIKANNIRE
KSIEGYQTGNWGPLMLEVESWVISGMASAVALSLSLTLGSALIAFGLSATVVGVGVVIAGAIGAFIDDKFV
DELNHKIIK

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      1      10      20      30      40      50      60
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ColE3 MSGGDGRGHNTGAHSTSGNINGGPTGLGVGGASDGSWSENPNPWWGGSGSGIHWGGGS
ColE4 .....
ColE5 MSGGDGRGHNTGAHSTSGNINGGPTGLGVGGASDGSWSENPNPWWGGSGSGIHWGGGS
ColE6 MSGGDGRGHNTGAHSTSGNINGGPTGLGVGGASDGSWSENPNPWWGGSGSGIHWGGGS
ColE7 MSGGDGRGHNSGAHNTGGNINGGPTGLGNGGASDGSWSENPNPWWGGSGSGVHWGGGS
ColE8 MSGGDGRGHNTGAHSTSGNINGGPTGIGVSGASDGSWSENPNPWWGGSGSGIHWGGGS
ColE9 MSGGDGRGHNTGAHSTSGNINGGPTGIGVSGASDGSWSENPNPWWGGSGSGIHWGGGS

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1

T-domain

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      70      80      90     100     110     120
ColE2 GHGNGGGNGNSGGGSGTGGNLSA AAPVAFGFPALSTPGAGGLAVSISAGALSAAIADIM
ColE3 GHGNGGGNGNSGGGSGTGGNLSA AAPVAFGFPALSTPGAGGLAVSISAGALSAAIADIM
ColE4 .....
ColE5 GHGNGGGNGNSGGGSGTGGNLSA AAPVAFGFPALSTPGAGGLAVSISAGALSAAIADIM
ColE6 GHGNGGGNGNSGGGSGTGGNLSA AAPVAFGFPALSTPGAGGLAVSISAGALSAAIADIM
ColE7 GHGNGGGNSNSGGGSGN.....SSVAAPMAFGFPALAAPAGTGLGISVSGEALSAAIADIF
ColE8 GRGNGGGNGNSGGGSGTGGNLSA AAPVAFGFPALSTPGAGGLAVSISASELSAAIAGII
ColE9 GRGNGGGNGNSGGGSGTGGNLSA AAPVAFGFPALSTPGAGGLAVSISASELSAAIAGII

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     130     140     150     160     170
ColE2 AALK.GPFFKGLWGVALYGVLPQIAKDDPNMMSKIVTSLPADDITESPVSSLPDKATV
ColE3 AALK.GPFFKGLWGVALYGVLPQIAKDDPNMMSKIVTSLPADDITESPVSSLPDKATV
ColE4 .....
ColE5 AALK.GPFFKGLWGVALYGVLPQIAKDDPNMMSKIVTSLPADDITESPVSSLPDKATV
ColE6 AALK.GPFFKGLWGVALYGVLPQIAKDDPNMMSKIVTSLPADDITESPVSSLPDKATV
ColE7 AALK.GPFFKSAWGIALYGLPSEIAKDDPNMMSKIVTSLPAETVTNVQSTLPDLQATV
ColE8 AKLKKVNLKFTPFVGVLSLIPSEIAKDDPNMMSKIVTSLPADDITESPVSSLPDKATV
ColE9 AKLKKVNLKFTPFVGVLSLIPSEIAKDDPNMMSKIVTSLPADDITESPVSSLPDKATV

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     180     190     200     210     220     230
ColE2 NVNVRVDDVKDERQNISVSVGVPMSVPVVDKPTERPVGFTASIPGAPVLNISVNNSTP
ColE3 NVNVRVDDVKDERQNISVSVGVPMSVPVVDKPTERPVGFTASIPGAPVLNISVNNSTP
ColE4 .....
ColE5 NVNVRVDDVKDERQNISVSVGVPMSVPVVDKPTERPVGFTASIPGAPVLNISVNNSTP
ColE6 NVNVRVDDVKDERQNISVSVGVPMSVPVVDKPTERPVGFTASIPGAPVLNISVNNSTP
ColE7 SVTKRVTDVVKDTRQHIAVVAAGVPMSVPVVDKPTRTPGVFHASFPVPSLTVSTVKGFLP
ColE8 NVNVRVDDVKDERQNISVSVGVPMSVPVVDKPTERPVGFTASIPGAPVLNISVNNSTP
ColE9 NVNVRVDDVKDERQNISVSVGVPMSVPVVDKPTERPVGFTASIPGAPVLNISVNDSTP

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     240     250     260     270     280     290
ColE2 EVQTLSPGVTNNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHNAVYVSVSDVLSPDQVKQR
ColE3 AVQTLSPGVTNNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHNAVYVSVSDVLSPDQVKQR
ColE4 .....
ColE5 AVQTLSPGVTNNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHNAVYVSVSDVLSPDQVKQR
ColE6 AVQTLSPGVTNNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHNAVYVSVSDVLSPDQVKQR
ColE7 VSTLPRGITEDKGRTAVPAGFTFGGGSHEAVIRFPKESGQKPVVSVTVLTPAQVKQR
ColE8 AVQTLSPGVTNNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHNAVYVSVSDVLSPDQVKQR
ColE9 AVQTLSPGVTNNNTDKDVRPAGFTQGGNTRDAVIRFPKDSGHNAVYVSVSDVLSPDQVKQR

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     300     310     320     330     340     350
ColE2 QDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAVQVNSRKSELD
ColE3 QDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAVQVNSRKSELD
ColE4 .....
ColE5 QDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAVQVNSRKSELD
ColE6 QDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAVQVNSRKSELD
ColE7 QDEEKRLLQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAVQVNSRKSELD
ColE8 QDEENRRQQEWDATHPVEAAERNYERARAELNQANEDVARNQERQAKAVQVNSRKSELD
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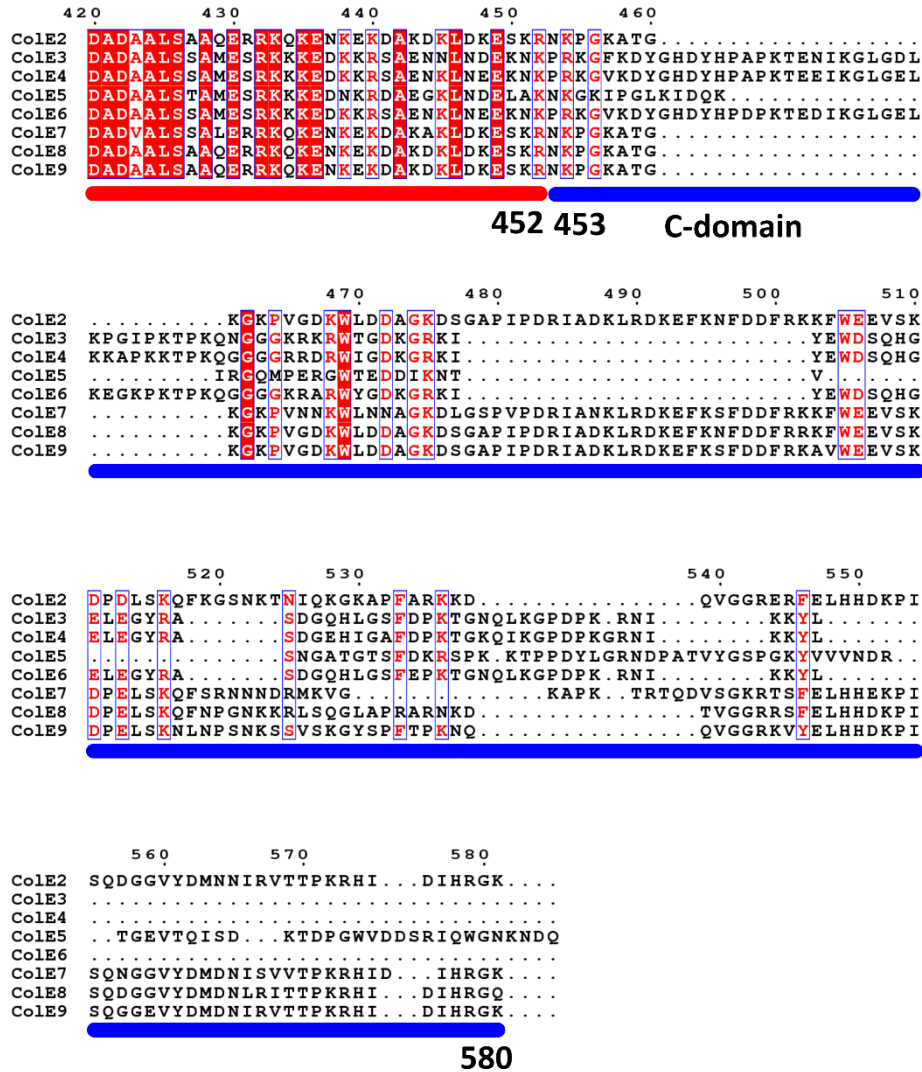
316 317

R-domain

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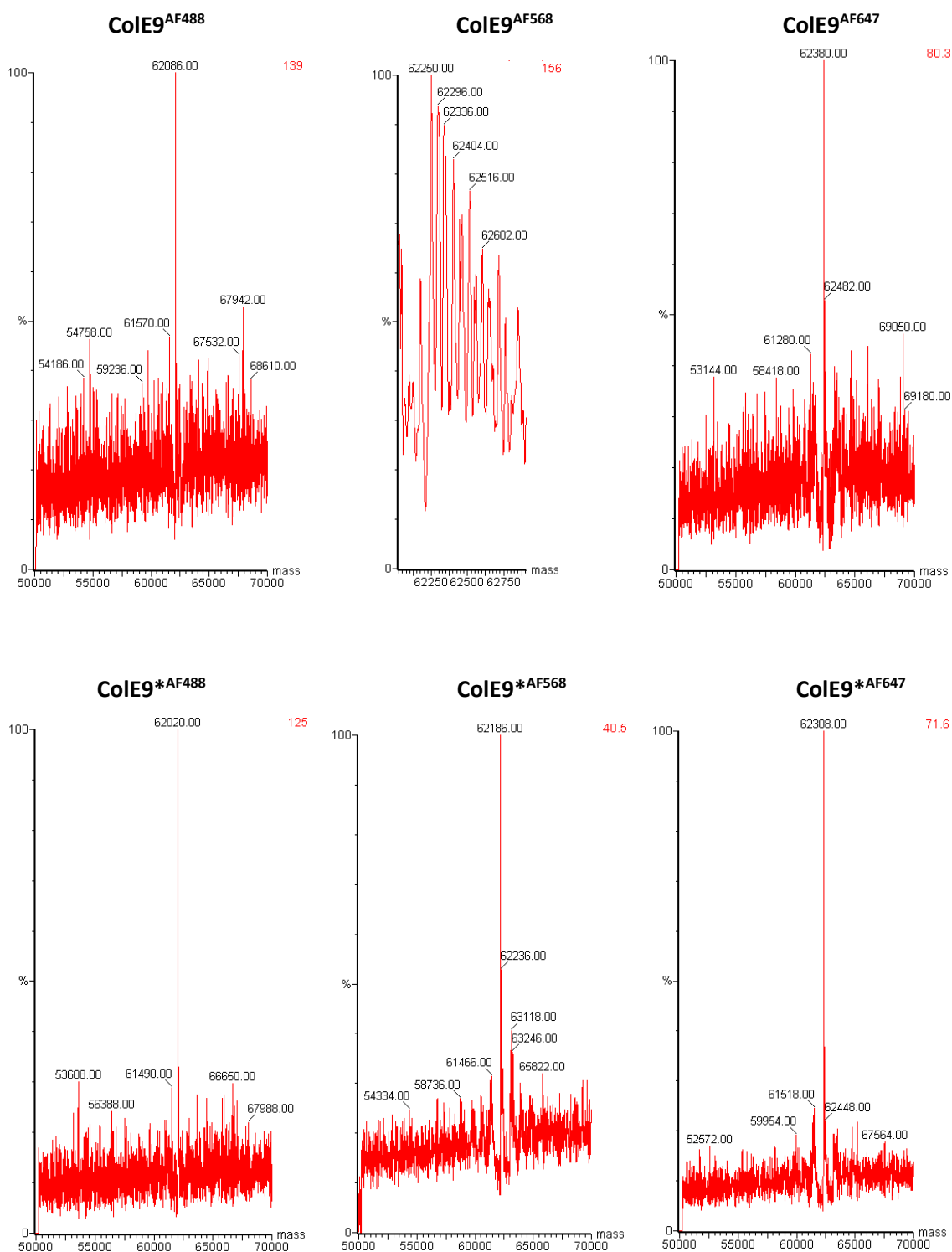
     360     370     380     390     400     410
ColE2 AANKTLADAIAEIKQFNRFADHPMAGCHRMWQ MAGLKAQRAQTDVNNKQAAFDAAAKEKS
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ColE4 .....ERFAREPMAAGCHRMWQ MAGLKAQRAQTDVNNKQAAFDAAAKEKS
ColE5 AANKTLADAIAEIKQFNRFADHPMAGCHRMWQ MAGLKAQRAQTDVNNKQAAFDAAAKEKA
ColE6 AANKTLADAIAEIKQFNRFADHPMAGCHRMWQ MAGLKAQRAQTDVNNKQAAFDAAAKEKS
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ColE8 AANKTLADAIAEIKQFNRFADHPMAGCHRMWQ MAGLKAQRAQTDVNNKQA.....A
ColE9 AANKTLADAIAEIKQFNRFADHPMAGCHRMWQ MAGLKAQRAQTDVNNKQAAFDAAAKEKS

```



Appendix I Figure 1 - Sequence alignment of ColE2-Cole9.

Sequence alignment of ColE2-Cole9, residues coloured in red and framed within the blue box have more than 70 % identity, residues highlighted in red have 100% identity. Residues have been underlined and numbered based on the domain organisation of ColE9, with residue 1-316 within the T-domain (green), residues 317-452 within the R-domain (red) and residues 453-580 within the C-domain (blue) (Robert 2014).



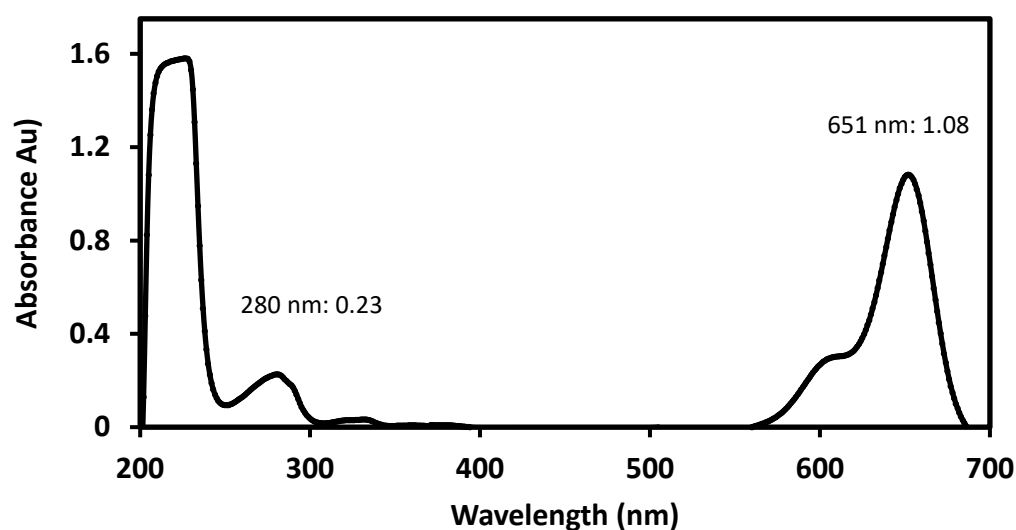
Appendix I Figure 2 - ESI-MS of CoIE9 with fluorescent labels.

ESI-MS gave observed molecular weights consistent with the expected molecular weights with the various AlexaFluor dyes. (*) represents H551A inactivation mutation.

Appendix I Table 1 - Molecular weights by ESI-MS of colicins used in this study.

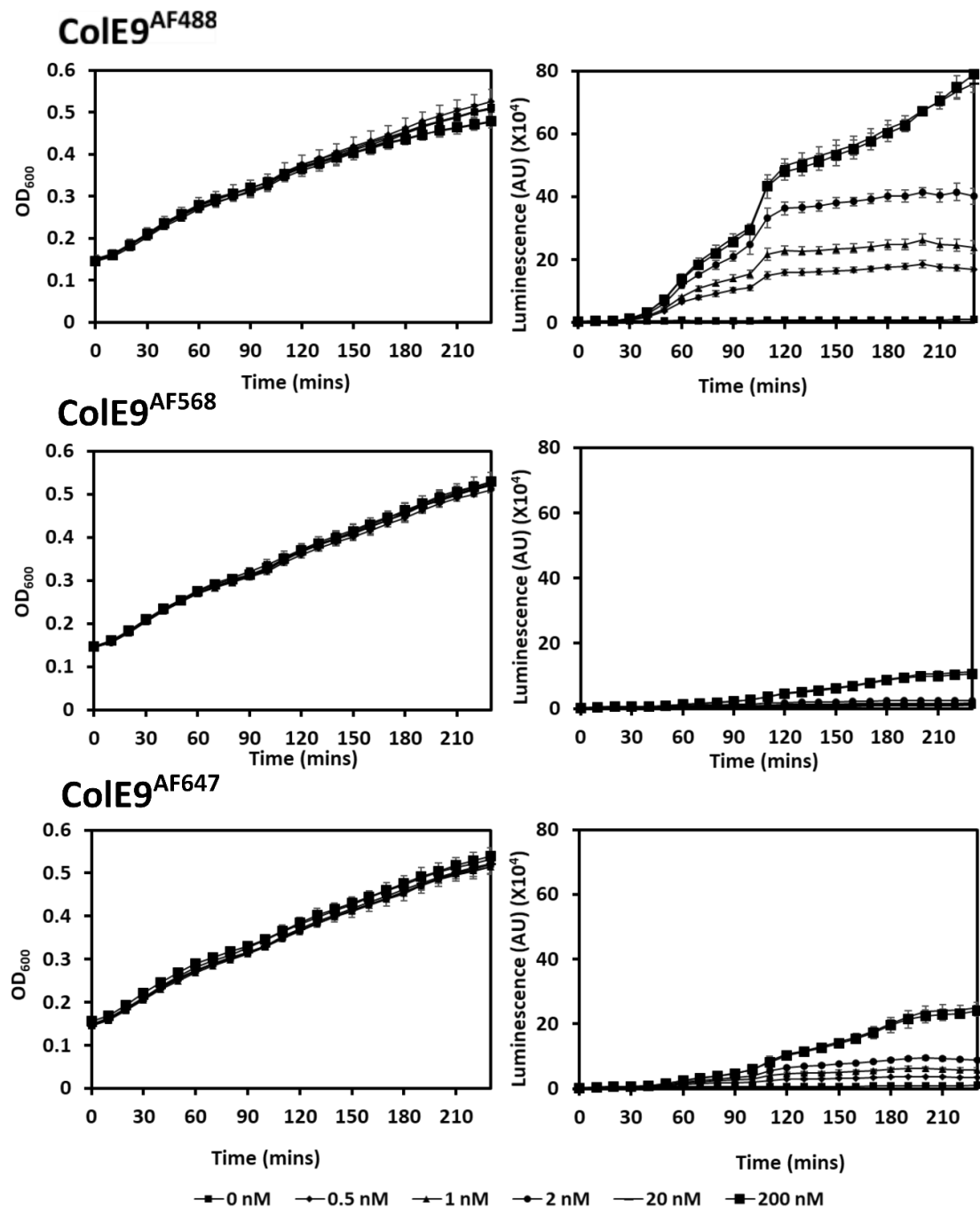
(*) represents H551A inactivation mutation. Expected molecular weights are calculated in the absence of the initial methionine residue.

Colicin	Expected Molecular weight (kDa) (-Met)	Observed Molecular weight (kDa)
ColE9 ^{AF488}	62,087	62,086
ColE9 ^{AF568}	62,247	62,250
ColE9 ^{AF647}	62,616	62,380
ColE9* ^{AF488}	62,021	62,020
ColE9* ^{AF568}	62,181	62,186
ColE9* ^{AF647}	62,550	62,308



Appendix I Figure 3 - UV VIS spectrophotometry profile for calculating ColE9 concentration and labelling efficiency.

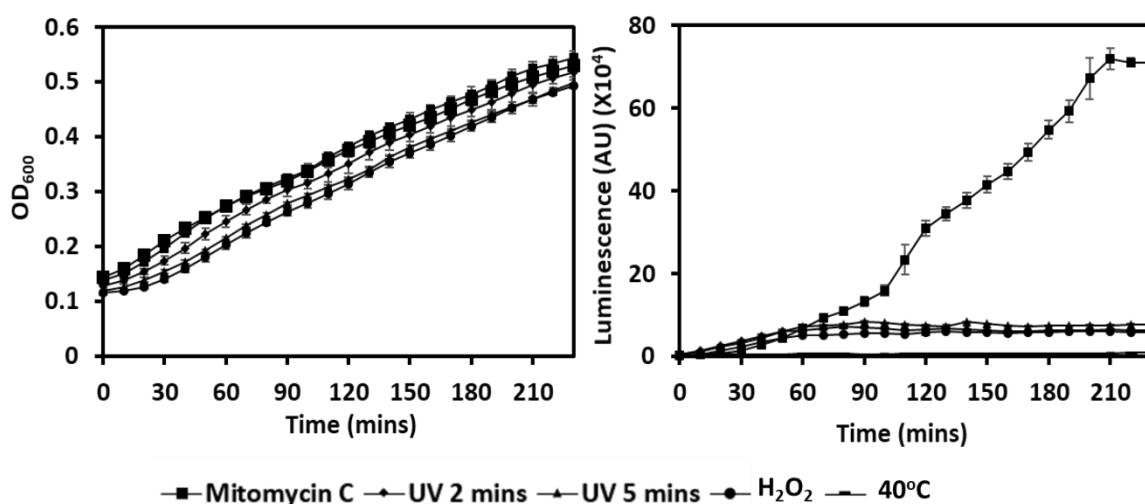
An example spectrophotometry profile of ColE9^{AF647} absorbance at 280 nm and 651 nm, used to calculate the concentration and labelling efficiency, using the protein and fluorophores molar extinction coefficients (ColE9 K469C; 47,510 M⁻¹ cm⁻¹, AF647; 265,000 M⁻¹ cm⁻¹). Protein concentration is 4.16 μM and labelling efficiency is 98 %.



Appendix I Figure 4 - Comparison of the effect of CoIE9 constructs on bioluminescence emission and cell density.

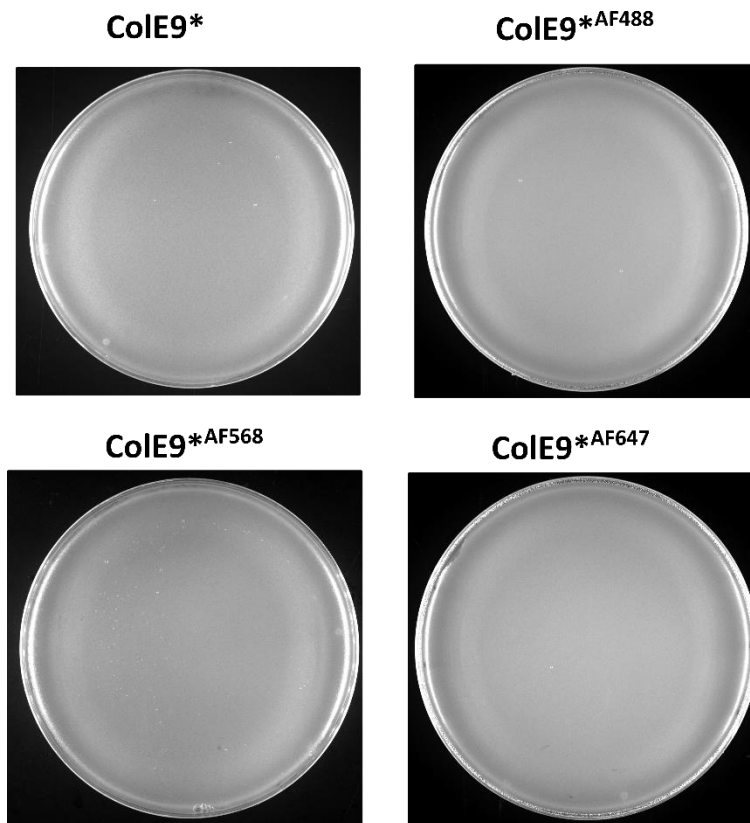
E. coli DPD1718 cells were used to measure the effect of cell growth and DNA damage through the emission of bioluminescence, using CoIE9^{AF488} on OD₆₀₀ (top left) and bioluminescence (top right), CoIE9^{AF568} on OD₆₀₀ (middle left) and bioluminescence (middle right) and CoIE9^{AF647} on OD₆₀₀ (bottom left) and bioluminescence (bottom right). Constructs were used at concentrations of 0 (■), 0.5 (◆), 1 (▲), 2 (●), 20 (—) and 200 nM (■).

DNA damage inducing agents



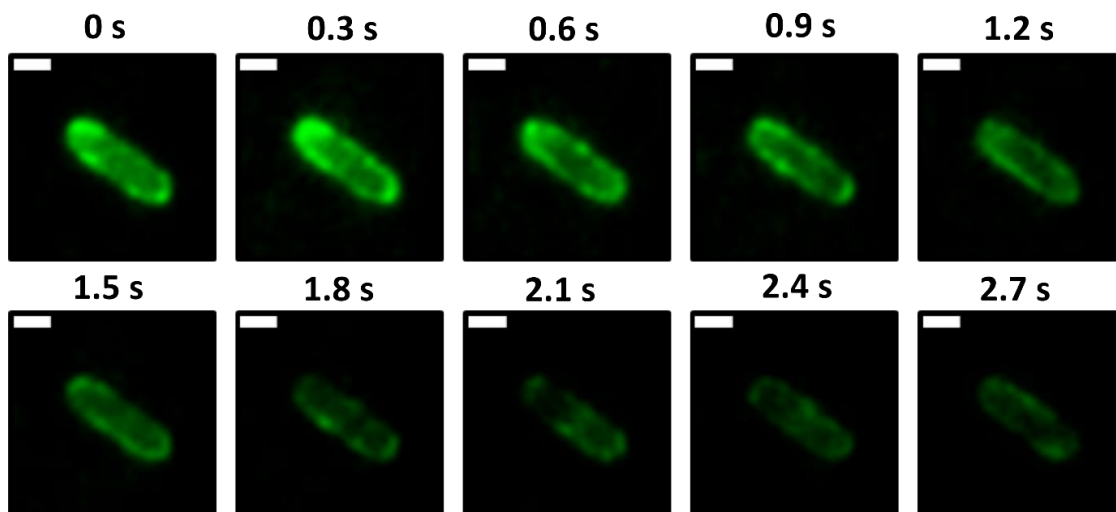
Appendix I Figure 5 - Effect of DNA damage inducing agents on bioluminescence emission and cell density.

E. coli DPD1718 cells were used to measure the effect of cell growth and DNA damage by a variety of DNA damaging agents through the effect on OD₆₀₀ (left) and emission of bioluminescence (right). Cells were treated with 0.6 µg/ml Mitomycin C (■), UV exposure for 2 (◆) and 5 minutes (▲), 2 mM H₂O₂ (●) and exposure to 40°C for 10 minutes (—). On rapid addition of the different agents, the plate was read every 10 minutes for a total of 4 hours and reported on both the cell density at OD₆₀₀ and the emission of bioluminescence at OD₄₉₀.



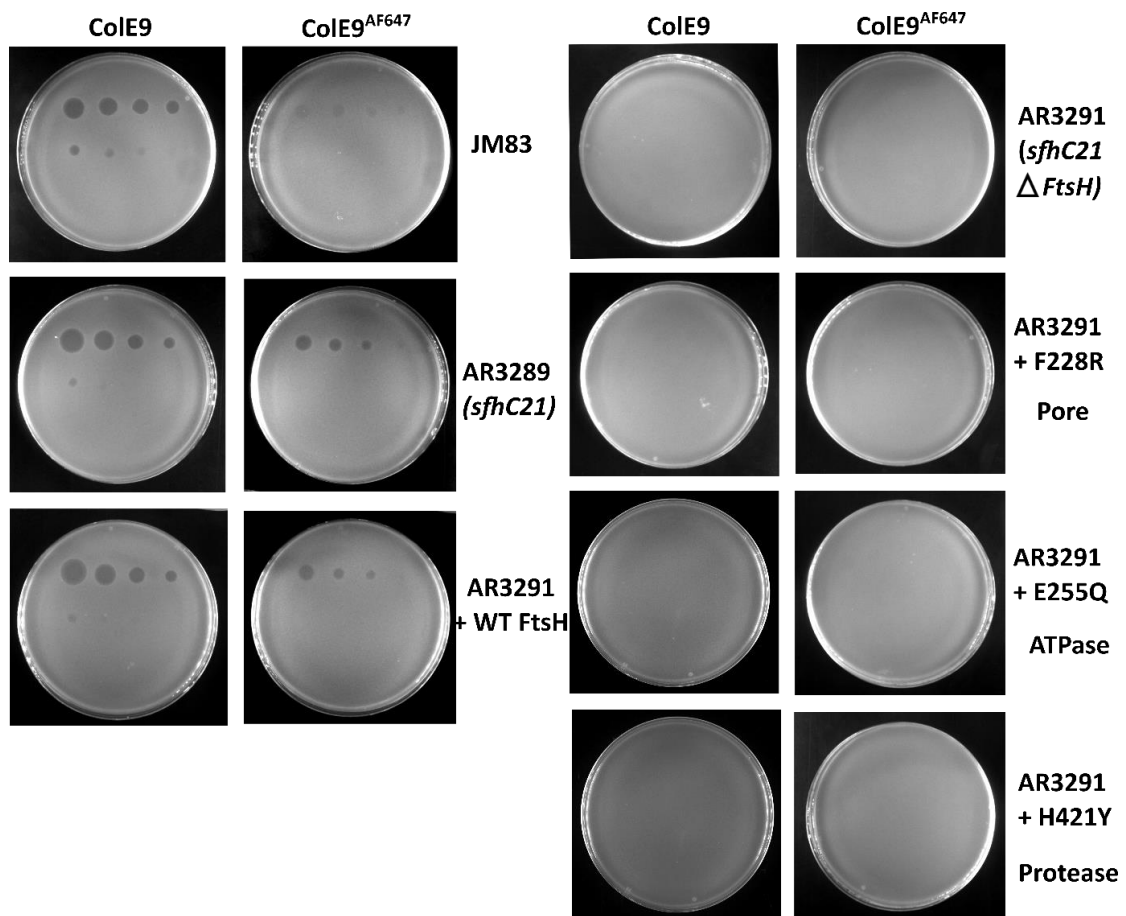
Appendix I Figure 6 - *In vivo* plate killing assay comparison of ColE9*, ColE9*AF488, ColE9*AF568 and ColE9*AF647.

E. coli JM83 cells were used to perform plate killing assays, using ColE9* or fluorescently-labelled ColE9*, from 10 μ M in 3-fold serial dilutions to 57 pM (Section 2.6.1). ColE9 H551A (*) mutation abolished colicin activity, regardless of the fluorophore used.



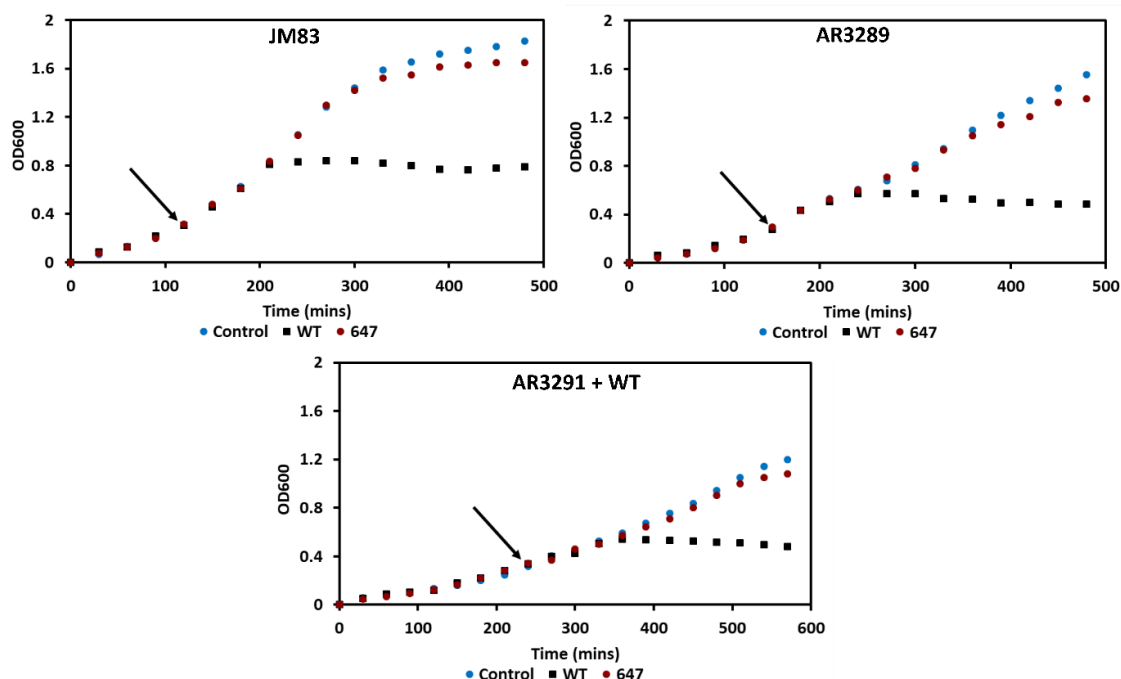
Appendix I Figure 7 - Widefield microscopy video frames of *E. coli* JM83 cells treated with ColB*AF488 and trypsin.

E. coli JM83 cells were labelled with 1.5 μ M ColB*AF488 before treating with trypsin and imaged using widefield fluorescence microscopy (Section 2.5). The fluorescence of ColB*AF488 is seen (green). There was remaining internal fluorescence observed after trypsin treatment. The autofluorescence was seen throughout the cell, but the most intense fluorescence appeared confined to the perimeter of the cell, which moved in a track-like motion. Images were collected every 30 ms, with every 10 frames or 0.3 seconds (s) shown above. Scale bar is 1 μ m.



Appendix I Figure 8 - *In vivo* plate killing assay comparison of wild-type ColE9 and ColE9^{AF647} on FtsH strains in M9.

E. coli FtsH strains were grown in M9 and spotted with wild-type ColE9 or ColE9^{AF647} from 10 μ M in 3-fold serial dilutions to 57 pM (Section 2.6.1). Colicin induced cell killing is indicated as zones of clearance in the bacterial lawn. JM83, AR3289 (*sfhC21*) and AR3291 + WT FtsH (plasmid-encoded) showed killing by both wild-type ColE9 down to 4.6 nM and by ColE9^{AF647} down to 0.37 μ M. AR3291 (*sfhC21* Δ *FtsH*) showed no killing by wild-type ColE9 or ColE9^{AF647} in the absence of FtsH. AR3291 + F228R, + E255Q or + H421Y mutant FtsH plasmids showed no killing by ColE9 WT or ColE9^{AF647}.

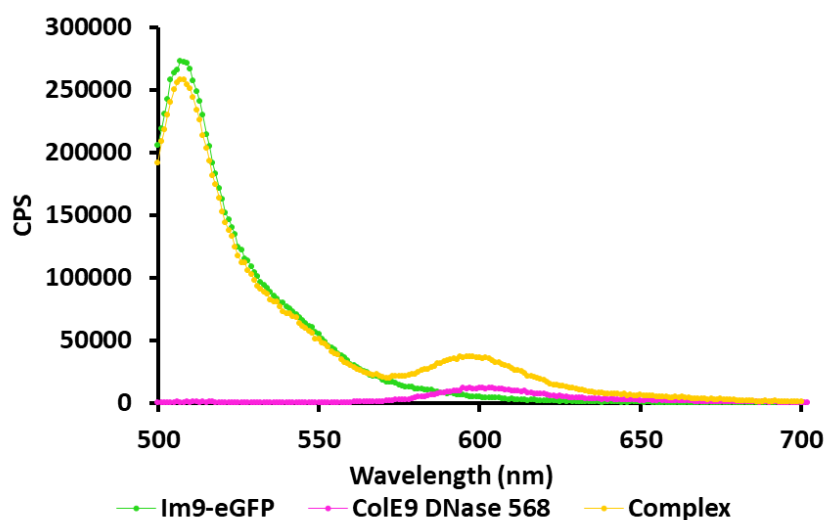


Appendix I Figure 9 - In vivo liquid culture killing assay comparison of wild-type ColE9 and ColE9^{AF647} on JM83, AR3289 and AR3291 + WT FtsH strains in M9.

E. coli cells JM83, AR3289 (*sfhC21*) and AR3291 + WT FtsH (plasmid-encoded FtsH), were grown in M9 minimal media to OD₆₀₀ ~0.4, in the presence of 100 µM IPTG for AR3291 + WT to induce plasmid expression. 80 nM wild-type ColE9 (WT) (black square) and ColE9^{AF647} (red circle) was added (as highlighted by the arrow) and the growth of each culture by OD₆₀₀ was monitored every 30 minutes for ~5 hours after addition of colicin alongside a control (blue circle). Time-courses showed the delay in cell growth to reach OD₆₀₀ 0.4 in comparison to JM83, by ~30 minutes and ~2 hours for AR3289 and AR3291 + WT, respectively. All strains showed rapid killing by wild-type ColE9 (WT) and poor killing by ColE9^{AF647} in comparison to the control in the absence of ColE9. There is a longer delay of ~30 minutes for observing killing by ColE9 in AR3291 + WT than the other strains.

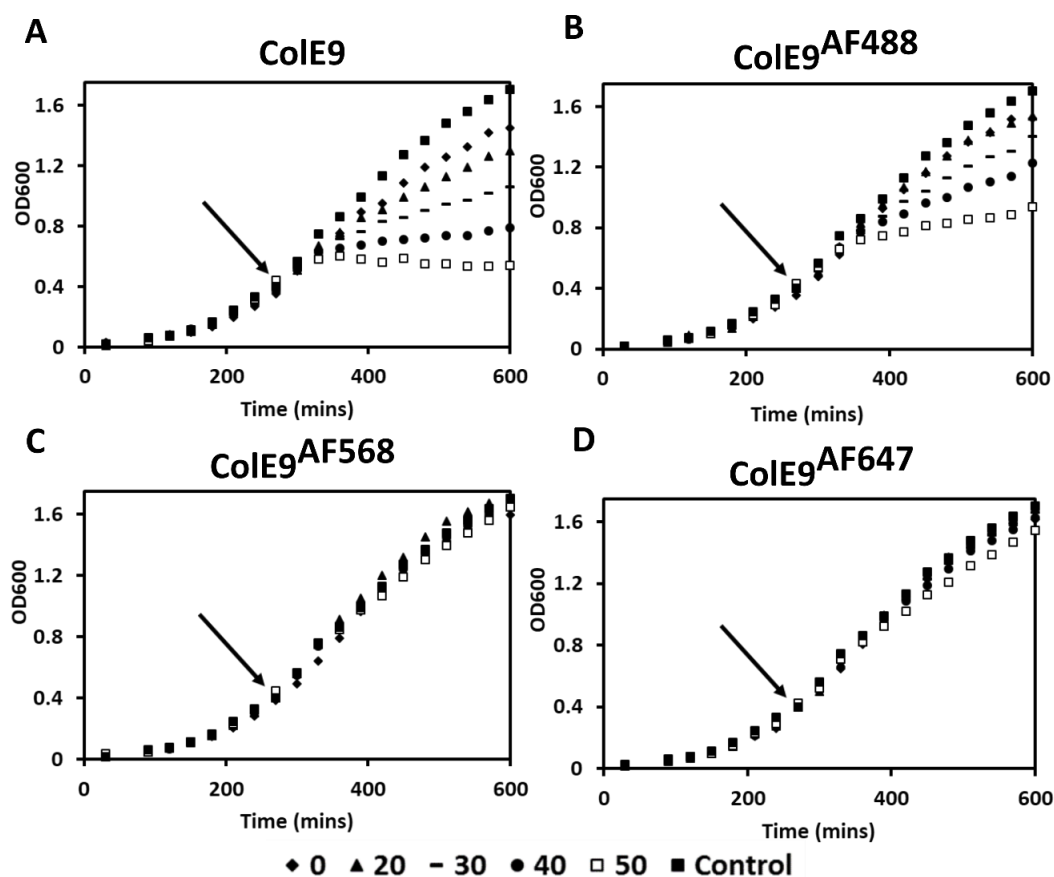
Appendix I Table 2 - Rate of growth of FtsH strains and mutants in LB and M9 minimal media.

Strain/ mutant	Time taken to reach OD 0.4 in LB (hrs)	Time taken to reach OD 0.4 in M9 (hrs)
JM83	1.5	3
AR3289	1.5	3
AR3291 Δ	4	7
AR3291 + WT	2.5	4.5
AR3291 + F228R	4	7
AR3291 + E255Q	4	7
AR3291 + H421Y	4	7



Appendix I Figure 10 - Emission spectra of Im9-eGFP, ColE9 DNase^{AF568} and the complex by fluorimetry.

Fluorimetry was performed using Im9-eGFP, ColE9 DNase^{AF568} and the complex (Section 2.4.2). Figure shows an overlay of the emission of Im9-eGFP (green), ColE9 DNase^{AF568} (pink) and the complex (yellow) between 500-700 nm after excitation at 488 nm. There is slight overlap of the emission of ColE9 DNase^{AF568} alone at 600 nm with the emission of Im9-eGFP alone. However, the emission of the complex at 600 nm does increase, suggesting low levels of FRET.



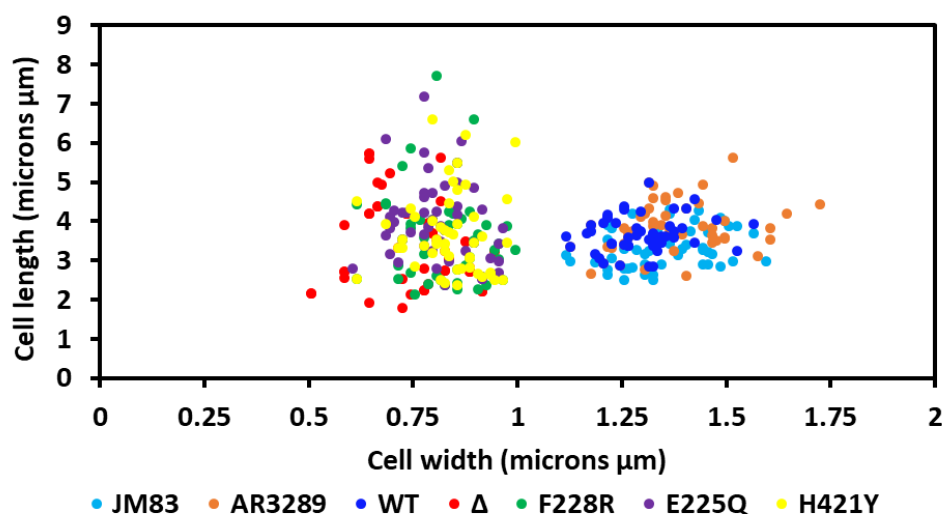
Appendix I Figure 11 - Effect of IM charge on the rate of translocation of wild-type ColE9, ColE9^{AF488}, ColE9^{AF568} and ColE9^{AF647} at each IPTG concentration.

E. coli HDL11 cells were used to look at the effect of IM charge on translocation, using wild-type ColE9, ColE9^{AF488}, ColE9^{AF568}, ColE9^{AF647} and a control with no added colicin (Section 2.6.5). A-D shows wild-type ColE9, ColE9^{AF488}, ColE9^{AF568}, ColE9^{AF647} using IPTG concentrations of 0 (◆), 20 (▲), 30 (-), 40 (●) and 50 (□) μM IPTG and control (■) of no IPTG or colicin. For ColE9 WT and ColE9^{AF488}, on the addition of colicin but no IPTG (0), there was a marked decrease in OD₆₀₀. As the concentration of IPTG was increased to 20, 30, 40 and 50 μM IPTG, there was a correlative decrease in the OD₆₀₀. For ColE9^{AF568} and ColE9^{AF647}, there was no real change in the OD₆₀₀ in the absence of colicin or absence of IPTG. There was only a slight emergence of OD₆₀₀ when 50 μM IPTG was used compared to the controls.

Appendix II

Widefield microscopy was performed to investigate the binding and translocation of ColE9*^{AF647} using the various *E. coli* FtsH strains. *E. coli* strains AR3291 (*sfhC21* Δ FtsH), in the absence of FtsH, and complemented with the mutant FtsH plasmids (+F228R/E255Q/H421Y), were grown in M9 minimal media to an OD₆₀₀ 0.4 with 1 mM IPTG and incubated with ColE9*^{AF647}, before treating with trypsin and visualising by widefield microscopy (Section 2.5).

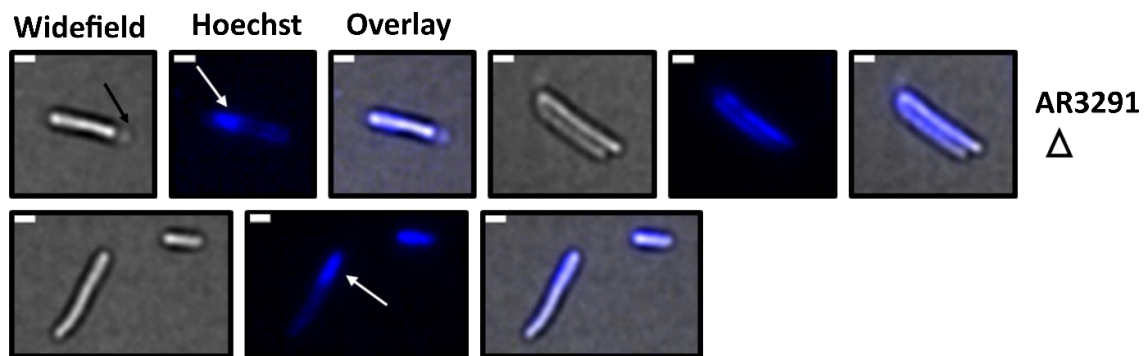
One interesting common observation for the FtsH deletion and mutation cells, was the reduced cell width and increased length compared to wild-type strains JM83, AR3289 or AR3291 + WT FtsH (plasmid-encoded). These cell dimensions were present as mixed populations ~3-8 μ m long (Appendix II Figure 1). Wild-type FtsH strains JM83, AR3289 and AR3291 + WT FtsH, showed similar cell width and length and are clustered together to the right of the graph (Appendix II Figure 1). AR3289 deletion (Δ) and the mutants + F228R, + E255Q, + H421Y, are clustered together to the left of the graph with ~ ½ the cell width and up to twice the cell length.



Appendix II Figure 1 - Cell dimensions for the *E. coli* FtsH strains.

Cells length and width were measured for wild-type FtsH strains JM83 (light blue), AR3289 (chromosomal FtsH, orange) and AR3291 + WT (WT) (plasmid FtsH, dark blue), for the AR3291 deletion strain (Δ , red) and mutants AR3291 + F228R (F228R, green), + E255Q (E255Q, purple) and + H421Y (H421Y, yellow). Measurements were taken using 50 cells from the widefield microscopy images.

All of the FtsH deletion and mutation strains had common abnormalities and unique features observed by widefield microscopy. One feature unique to the AR3291 FtsH deletion strain, was curving and folding of the cells which contained DNA (Appendix II Figure 2). Another feature was uneven distribution of the Hoechst DNA stain; some cells had Hoechst present throughout the cell but tended to accumulate at one pole (white arrows) (Appendix II Figure 2). Some cells had cell membrane extensions that usually only appeared in one cell pole and contained DNA (black arrow), they had much less contrast to their walls than the rest of the cell. The majority of cells were not fluorescently labelled by ColE9*AF647 and the few that were showed very poor labelling that was difficult to observe and therefore not shown here.

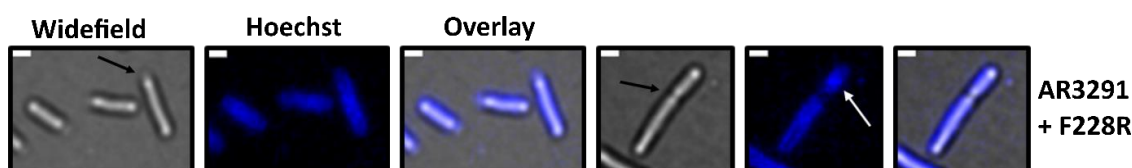


Appendix II Figure 2 - *E. coli* AR3291 cells incubated with ColE9*AF647 by widefield microscopy.

E. coli AR3291 (*sfhC21 ΔftsH*) cells were labelled with 1.5 μ M ColE9*AF647 and 20 μ M Hoechst and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey) and the DNA stained by Hoechst (blue). Overlays of the cell and DNA are also shown. Cells showed phenotypes of curving and folding, uneven Hoechst DNA stain distribution to the pole and cellular membrane extensions. Fluorescent labelling by ColE9*AF647 was very poor or absent and therefore not shown here. Scale bar is 1 μ m.

Strain AR3291 + F228R (pore), had a higher proportion of cells with polar cell membrane extensions containing DNA, (black arrow in left cell images) (Appendix II Figure 3). Another feature of these cells was the location of the septum cell division sites, apparent by the faint line across the cell (black arrow in right cell images) and the separation of the Hoechst DNA stain (white arrow), that the septum was no longer central to the cell. This suggested that the MinCDE system, responsible for controlling the location of the cell division site, had been affected by the absence of wild-type FtsH. The majority of cells were not fluorescently labelled by ColE9*AF647

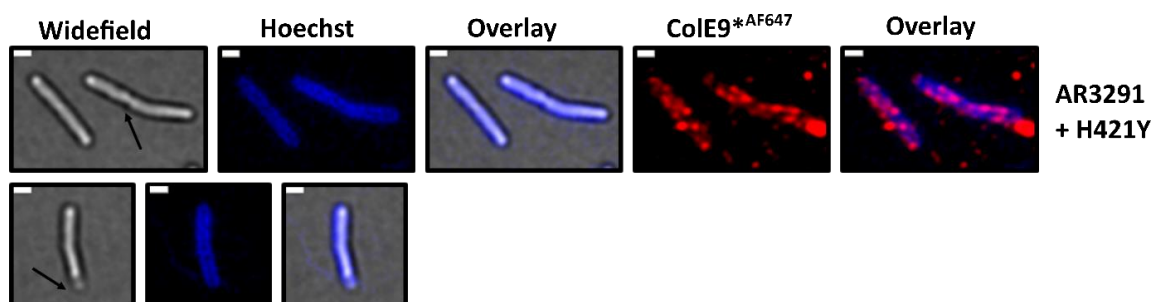
and the few that were showed very poor labelling that was difficult to observe and therefore not shown here.



Appendix II Figure 3 - *E. coli* AR3291 + F228R cells incubated with ColE9*AF647 by widefield microscopy.

E. coli AR3291 (*sfhC21 ΔftsH*) +F228R cells were labelled with 1.5 μM ColE9*AF647 and 20 μM Hoechst and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey) and the DNA stained by Hoechst (blue). Overlays of the cell and DNA are also shown. Cells showed phenotypes of cellular membrane extensions and polar septum formation. Fluorescent labelling by ColE9*AF647 was very poor or absent and therefore not shown here. Scale bar is 1 μm.

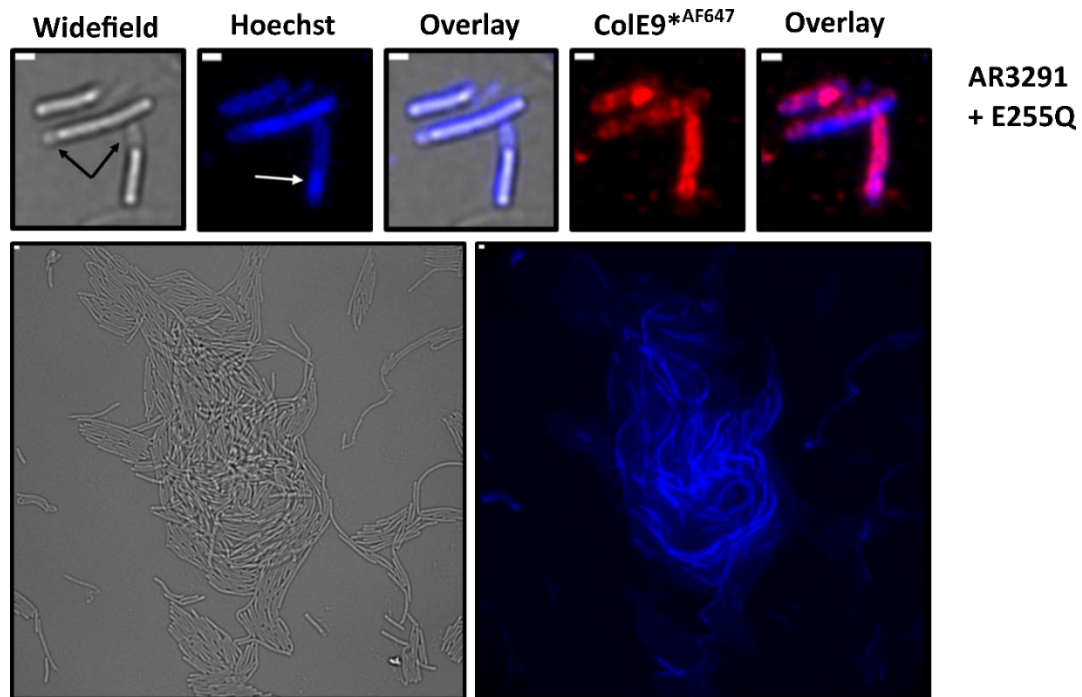
Strain AR3291 + H421Y (protease), also had a higher proportion of cells with the polar cell membrane extensions containing DNA (black arrow in the bottom cell figure) and polar septum formation (Appendix II Figure 4). This strain showed some fluorescent labelling by ColE9*AF647 however the fluorescent background was quite high and noisy and the cell labelling was not uniform around the OM, which possibly showed islands or puncta. This may be due to the occurrence of a similar number of OMPs but spaced out on elongated cells.



Appendix II Figure 4 - *E. coli* AR3291 + H421Y cells incubated with ColE9*AF647 by widefield microscopy.

E. coli AR3291 (*sfhC21 ΔftsH*) +H421Y cells were labelled with 1.5 μM ColE9*AF647 and 20 μM Hoechst and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the cell, DNA and ColE9*AF647 are also shown. Cells showed phenotypes of cellular membrane extensions and polar septum formation. Cells showed fluorescent labelling by ColE9*AF647 in islands or puncta dispersed around the OM. Scale bar is 1 μm.

Strain AR3291 + E255Q (ATPase), had a higher proportion of cells with polar cell membrane extensions containing DNA, (black arrow) (Appendix II Figure 5) and uneven distribution of Hoechst DNA stain at the poles of cells (white arrow). The main difference in phenotype of this strain was the propensity of very long large masses of cells to aggregate. This strain showed the highest fluorescent labelling by ColE9*AF647.



Appendix II Figure 5 - *E. coli* AR3291 + E255Q cells incubated with ColE9*AF647 by widefield microscopy.

E. coli AR3291 (*sfhC21 ΔftsH*) +E255Q cells were labelled with 1.5 μM ColE9*AF647 and 20 μM Hoechst and imaged using widefield fluorescence microscopy (Section 2.5). The cell is seen in DIC (grey), the DNA stained by Hoechst (blue) and the fluorescence of ColE9*AF647 (red). Overlays of the cell, DNA and ColE9*AF647 are also shown. Cells showed phenotypes of cellular membrane extensions, polar accumulation of Hoechst, a higher proportion of elongated cells and their propensity to aggregate as large masses. Cells showed fluorescent labelling by ColE9*AF647. Scale bar is 1 μm.

Due to the lack of observable OM fluorescent labelling for the FtsH deletion and several mutation strains and the reduced cell width, it was not possible to investigate ColE9*AF647 translocation by widefield microscopy.

The impact of FtsH on ColE9^{AF647} translocation

Each of the single mutations used do not have an effect on one particular function of FtsH, like ATPase or protease activity inhibition or blocking entry to the pore, all FtsH functions are linked, without ATPase activity there is no energy for protease activity. Therefore, interpreting the observed phenotype to a functional region of FtsH is not possible. There are several possible biological explanations for why these cell phenotypes could have occurred in these strains.

FtsH controls the tightly regulated balance in LPS and PL synthesis (Ogura et al. 1999). The suppressor mutation allows cells survival in the absence of FtsH, by increasing the amount of PL to keep the balance with levels of LPS (Ogura et al. 1999). One possibility may be that the ratio of LPS:PL are similar, but without FtsH to cleave and regulate the two pathways, the levels of both LPS and PL are much higher than usual and without increased rates of cell division, this could elongate cells.

It has been observed previously by microscopy, that overexpression of LpxC, a protein involved in the LPS pathway whose gene product is involved in the first committed step in Lipid A biosynthesis, leads to the formation of filamentous elongated cells. Due to overproduction, LPS accumulates in the cell and leads to abnormal membrane stacks in the periplasm (Ogura et al. 1999) and severe growth defects (Langklotz, Schakermann, and Narberhaus 2011). These abnormal membranes were reported to be suppressed in the presence of *sfhC21* (Ogura et al. 1999). Lack of cell elongation or effect on KDO levels in cells overexpressing LpxC have been reported, however this was performed using overexpression of plasmid-encoded LpxC in strain AR3289 cells containing FtsH (Schakermann, Langklotz, and Narberhaus 2013). It is possible that the suppressor mutation and hyperactive FabZ do not balance the amount of LPS. LPS levels measured by the amount of KDO for AR3289 and AR3291 were 7.8 and 10.3 µg/mg protein, respectively (Ogura et al. 1999), which might explain the filamentous elongated cells and polar cell membrane extensions in FtsH deletion strain.

PLs in bacteria determine cell integrity and function. *E. coli* IM and the inner leaflet of OM contains ~ 75 % PE, ~20 % PG and ~5 % CL. Altering these PLs leads to temperature sensitivity, envelope disorders, defective chemotaxis, cell division, osmoregulation and energy metabolism, issues with OMP synthesis and the activation of envelope stress response pathways (Rowlett et al. 2017). Alterations in the PLs in minus-PE and minus-CL *E. coli* cells leads to higher proportions of elongated cells observed by microscopy. In addition, cell elongation phenotype and slower growth rate was further enhanced when the media was changed from LB to supplemented M9 minimal media.

A higher PL:LPS ratio is reported to provide an environment that promotes assembly of OMPs, possibly through altering the fluidity. This was shown using OmpF315 assembly defect mutant and a mutation in LpxC which lowered its activity and thereby LPS levels. Higher PL levels, suppresses the assembly defect of OmpF315 and permits the assembly conversion to stable trimers (Kloser, Laird, and Misra 1996) (Kloser et al. 1998). In addition, cells in the absence or low levels of PE and CL PLs, showed impairment in OmpF folding and the overall levels of OmpF (Rowlett et al. 2017). Lack of cell death by ColE9 in the absence of a functional FtsH may be a direct result of its absence, alternatively a lowering or change in the composition of the PLs of the cell could lower the levels of OmpF. This in turn, would reduce the number of colicins that could translocate.

Another possibility to explain these phenotypes is the Min system, which is responsible for positioning the cell-division plane in the centre of the cell in rod-shaped bacteria. It is composed of MinCDE, which oscillate between the two cell poles to prevent assembly of the FtsZ ring at the poles. Changes in growth conditions, protein levels and the cell cycle vary the Min system oscillation. During later stages of cell division, Min proteins pause at the midcell when the septum is forming, to aid in cell division and formation of two daughter oscillating Min systems. The location of pausing is dependent on cell length and geometry, and in elongated

cells, pole-to-pole Min oscillations no longer occur and instead two or more oscillating Min patterns occur throughout the cell. In addition, the membrane curvature and/or PL composition is a physical cue for Min protein localisation to the poles (Rowlett and Margolin 2015). This may explain why in elongated cells I observe polar accumulation of DNA and loss of central septum formation occurs.

Finally, YgfM is a periplasmic substrate of FtsH (Westphal et al. 2012). Once a stress signal is sensed and communicated to RcsB, cytoplasmic response regulator of the Rcs signal transduction system phosphorelay that coordinates adaptation to stresses, it activates expression of a series of genes related to acid and osmotic tolerance, cell division and stationary phase adaptation. YgfM is a negative regulator of RcsB and therefore of expression of genes for these functions. Without cleavage of YgfM by FtsH, the negative regulation may continue, thereby affecting cell division, which may contribute to the elongated cell phenotype observed (Bittner, Westphal, and Narberhaus 2015).

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