The Conjugation System of *Staphylococcus aureus*

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ABSTRACT

A conjugation system in *Staphylococcus aureus* has been investigated and shown to be determined, at least in part, by genes carried on plasmids. Conjugation required cell-to-cell contact but not calcium ions. The frequency of conjugation depended on the recipient used and on the incubation conditions.

Two conjugative plasmids were mapped by restriction enzyme analysis but experiments to clone the conjugation-determining region were unsuccessful although separate regions specifying gentamicin resistance, ethidium bromide resistance and cadmium resistance were cloned. The gentamicin resistance determinant was probably part of Tn*4001*. Deletion of various sized pieces of DNA from one of the plasmids resulted in reduction of its ability to specify conjugation but no specific part of this plasmid could be implicated in the process. Further experiments led to the conclusion that this particular plasmid (p8325-4) is probably not self-transmissible but transferred by a phage-mediated system.

Strains of *Staphylococcus aureus* produced a pheromone-like substance that elicited a clumping response in *Streptococcus faecalis* but no evidence was found for the involvement of staphylococcal conjugative plasmids in this.

The conjugative plasmid, p8325-2, mobilized a small plasmid (pT181) but not a chromosomal gene.

Insertion of transposon Tn*551* was used to produce mutants of the conjugative plasmid p8325-2. Some twenty-six mutants were studied and the position of Tn*551* in them mapped. There were preferred regions of insertion for Tn*551* and twenty out of the twenty-six mutants had altered ability to conjugate. One showed a significantly higher frequency of conjugation and the other nineteen, all with substantially lower frequencies of conjugation, were mapped to two well-separated regions of the plasmid. Similarity between the locations of these putative regions and those reported for some other conjugative plasmids from staphylococci is striking and suggests a common origin.

p 8325-4 is different from the strain 8325-4 referred to in Novick, R.P., 1967. Properties of a cryptic high-frequency transducing phage in Staphylococcus aureus. Virology 33, 155-166.

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ABBREVIATIONS

AAC	acetyltransferase
AAD	adenyltransferase
Amp	ampicillin (phenotypically sensitive or resistant)
APH	phosphotransferase
AS	aggregation substance
asa	gene conferring resistance to arsenate
Asa	arsenate (phenotypically sensitive or resistant)
ATP	adenosine 5′-triphosphate
BHI	brain heart infusion
bla	gene conferring the ability to produce β-lactamase
bp	base pairs
BS	binding substance
BSA	bovine serum albumin
cad	gene conferring resistance to cadmium ions
Cad	cadmium (phenotypically sensitive or resistant)
cat	gene conferring resistance to chloramphenicol
CIA	clumping-inducing agent
CIP	calf intestinal alkaline phosphatase
Cjt	transconjugant
Cm	chloramphenicol (phenotypically sensitive or resistant)
cop	copy control region
CY	casein hydrolyase yeast extract
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
drd	derepressed for conjugal transfer
DTT	dithiothreitol
Eb	ethidium bromide (phenotypically sensitive or resistant)
EDTA	diaminoethane tetra-acetic acid
EMRSA	epidemic methicillin-resistant *Staphylococcus aureus*
ero	gene conferring resistance to erythromycin
Ero	erythromycin (phenotypically sensitive or resistant)
Gm	gentamicin (phenotypically sensitive or resistant)
HB	hypertonic buffer
λH3	phage lambda DNA digested with restriction endonuclease *Hin*d III
Int$^-$	integration deficient
IS	insertion sequence
kbp	kilobase pairs
Km	kanamycin (phenotypically sensitive or resistant)
Mcr	region mediating in plasmid maintenance, compatability and replication
min	minute
MLS	resistance to macrolides, lincomycin and streptogramin B
MRSA	methicillin-resistant *Staphylococcus aureus*
mob	gene/s involved in mobility
Nov	novobiocin (phenotypically sensitive or resistant)
*ori*T	transfer origin of replication
*ori*V	vegetative origin of replication
Pc	penicillin G (phenotypically sensitive or resistant)
PEG	polyethylene glycol
penPC	gene for β-lactamaseI
Pm	paromomycin (phenotypically sensitive or resistant)
ϕ	phage
Qa	quaternary ammonium compounds (phenotypically sensitive or resistant)
rec	region involved in recombination functions

rep	region specifying replication functions
Rev	revertant to temperature sensitivity
RNA	ribonucleic acid
RNAase	ribonuclease
rom	gene specifying the repressor of modulator protein
SDS	sodium dodecyl sulphate
Tc	tetracycline (phenotypically sensitive or resistant)
tet	gene conferring resistance to tetracycline
Tn	transposon
Tp	trimethoprim (phenotypically sensitive or resistant)
Tra/tra	mediating conjugation
Tris	tris (hydroxymethyl)aminomethane
ts	temperature sensitive
U.V.	ultraviolet
- - -	indicates disruption of a gene

Chapter 1

Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a member of the Micrococcaceae family of bacteria. It is a non-motile, Gram-positive, facultative anaerobe whose cocci are usually arranged in clusters. It is distinguished from other staphylococcal species in having the capability of producing coagulase. Most strains contain Protein A in their cell wall. This immunologically active substance has the ability to bind and aggregate immunoglobulin IgG molecules (see Sheagren, 1984). A variety of extracellular enzymes are produced by *S.aureus,* many participating in its pathogenic capabilities (see Arvidson, 1983). Six enterotoxins (A to F) have also been described and are best known as the causative agents of staphylococcal food poisoning.

In the absence of a useful serological typing system, phage typing has been employed to classify *S.aureus* strains. Most coagulase-positive staphylococci carry phages that can be detected by cross culture of pairs of strains and propagated on the sensitive member of the pair. Lysis by these phages is strain-specific. Certain combinations of lytic reactions occur much more often than others and it is possible to divide the phages into lytic groups, members of which appear together in typing patterns and to divide staphylococci into corresponding phage groups. Three main phage groups have been characterized and are numbered I, II, and III (see Parker, 1983).

The molecular genetics of *S.aureus* is not very well developed but some basic facts are known. The amount of DNA carried by the staphylococcal cell has been estimated at 2 to 4×10^9 daltons assuming one chromosome per cell. The estimate is based on between 3.5×10^{-14} and 12×10^{-14} g of DNA per cell (Novick and Bouanchaud, 1971; Lacey, 1975). The value is similar to that estimated for other bacteria such as *Escherichia coli* (2.5×10^9 daltons) and *Bacillus subtilis* (1.3×10^9 daltons). For staphylococci this represents about 3×10^3–6×10^3 kbp of DNA, enough theoretically to code for about 3000–6000 proteins.

Transformation has been used to establish three major linkage groups of chromosomal genes in *S.aureus* strain NCTC 8325 (Pattee and Neveln, 1975; Pattee, 1976) including nutritional markers,

antibiotic resistance loci, phage attachment sites and pigment production. Protoplast fusion has been used to identify linkage between the three groups of genes and establish a circular linkage map in *S.aureus* 8325N (Stahl and Pattee, 1983).

1.2 Transfer of antibiotic resistance in staphylococci

Staphylococcus aureus is a normal inhabitant of the skin and anterior nares of man and infection represents an uncommon migration from these habitats. Its biochemical mechanisms enable it to invade through minor breaks in skin and mucous membranes causing a variety of soft tissue infections such as cellulitis and lymphangitis and infections of the bones and joints leading to such diseases as septic arthritis. It frequently reaches the blood stream and once blood-borne is likely to seed to organs already injured by another process. Acute bacterial endocarditis is perhaps one of the most dramatic syndromes which can result (for review, see Sheagren, 1984). In the pre-antibiotic era, staphylococcal bacteremia resulted in 80% mortality. This was initially controlled by antibiotics but their introduction into clinical use has been accompanied by the emergence of resistant strains within the bacterial population and *S.aureus* continues to be a major pathogen. As a result of antibiotic use, resistant strains are selected to form a resistant population.

The rapidity with which resistance to novel antibiotics can develop has been attributed to the observation that most resistance determinants are borne on extrachromosomal pieces of DNA such as plasmids. These are genetic elements which replicate independently of the host chromosome. They generally exist in the form of supercoiled covalently-closed, circular DNA molecules, ranging in size from about one kilobase (1 or 2 genes) to over 300 kilobases. Large (> 35 kbp) plasmids are maintained at about 1–4 copies per chromosome whereas smaller plasmids are usually present in multiple (10–40) copies. The minimum requirement for independent replication of a plasmid is that it contains at least one origin of replication (*ori*V), the site at which vegetative replication is initiated. Different plasmids make varying contributions to their own replication, but in all cases, ordered replication and segregation is essential if they are to be maintained in the host cell line. Plasmids are maintained at widely differing growth rates by the coupling of plasmid replication to the host's growth rate via the initiation at the origin. Regulation of replication and hence of copy number is related to plasmid incompatibility (the failure of two different plasmids to co-exist in the same host cell). This allows the classification of plasmids into incompatibility (Inc) groups, useful in epidemiological studies of resistance (see Saunders, 1984).

Resistance genes may also be organized as part of a transposon which can transfer between bacterial replicons to build up complex groups of genes. Unlike plasmids, transposons are not able to replicate independently but must be maintained as part of a functional replicon (self-replicating DNA molecule). The existence of resistance-conferring transposons could account for the emergence of multiply resistant bacteria due to the insertion of such transposons into non-essential sites on resident plasmids (see Saunders, 1984).

The plasmid pSK1 was the first staphylococcal plasmid shown to carry a transposable aminoglycoside resistance determinant. This 4.7 kbp transposon specifies resistance to gentamicin, tobramycin and kanamycin and can undergo *rec*-independent transposition to the chromosome from pSK1 (Lyon *et al,* 1984). The presence of Tn*4001* sequences on all gentamicin resistance plasmids in Australian isolates as well as aminoglycoside resistance plasmids from the USA (Gillespie and Skurray, 1986) has been shown, demonstrating its wide geographical distribution.

The success of plasmid-encoded antibiotic resistance could be due to a number of factors:

a) genetic load on a host strain is reduced by the maintenance of a limited number of resistance plasmids. These are selected for by antibiotic use.
b) plasmid genes evolve independently of the chromosome and there is therefore no pressure to maintain chromosome integrity.
c) plasmids are able to transfer freely between or within strains so their genes are more mobile than those on the chromosome.
d) transposons often reside on plasmids.

Plasmids can be released or extracted from a donor cell and incorporated into a recipient by transduction, transformation or conjugation (see Lacey, 1980).

i) Transduction.

Lysogenic bacteriophages are able to integrate into the plasmid genome by recombination where they can exist as a prophage, replicating in synchrony with the host. Transduction occurs when the phage incorporates the plasmid genome into its protein coat, infects a new host cell and so inserts the plasmid DNA into the recipient. Bacteriophages extracted from cultures of *S.aureus* are well known to mediate generalized transduction in this species (Novick, 1963) and these transduction techniques have been used in the elucidation of the genetics of antibiotic resistance (Novick, 1963; Dornbusch *et al,* 1969; DeSaxe and Porthouse, 1979).

Townsend *et al* (1985) reported that a representative of the most common aminoglycoside resistance plasmids in Australian isolates of methicillin-resistant *S.aureus* was non-conjugative and transferred by a bacteriophage-mediated system to a lysogenic recipient. With one exception, all conjugative plasmids could also be transferred by transduction.

The possibility that antibiotic resistance in *S.aureus* might spread between bacteria in nature by transduction was strengthened by the construction of an element that contained DNA of both plasmid and phage origin (Novick, 1967). The extent to which spontaneous transfer by generalized transduction accounts for the spread of resistance in nature is unknown but antibiotic resistance does spread rapidly between strains in a particular phage group whilst the spread of resistance between phage groups is much slower, suggesting that phage is involved in the transmission.

ii) Transformation.

Transformation involves the uptake and expression of free DNA by a recipient strain and has been shown for both chromosomal and plasmid genes in *S.aureus* (Lindberg and Novick,1973; Lindberg, 1981). The competence of a strain (the ability to be transformed) is dependent upon a number of factors including a high concentration of divalent cations. The presence of phage enhances competence, possibly due either to the involvement of an early phage gene (Sjostrom and Philipson, 1974) or the modification of the cell surface by incomplete phage virions (Tompson and Pattee, 1977). Nuclease production by the recipient or the presence of a host restriction system can prevent transformation making it difficult to assess the extent to which transformation plays a role ***in vitro***.

iii) Mixed broth transfer.

It has been shown that antibiotic resistance can be transferred between strains in mixed broth culture (Lacey, 1980; Lacey and Lord, 1980; Fouace, 1981). This process could account for intraspecific transfer of resistance genes ***in vivo*** since staphylococci are often found in close proximity to each other under natural conditions.

Lacey (1980) has provided evidence for a type of spontaneous transfer in mixed culture, distinct from conventional transduction. He describes this process as "phage-mediated conjugation" since transfer requires direct cell-to-cell contact as well as the presence of lysogenic transducing phage in either the donor or the recipient. That the process is similar to transfer by transduction is

suggested by the requirement for calcium ions (about 0.001 to 0.01M) and the higher frequency of plasmid gene transfer compared with transfer of chromosomal genes. In contrast to transduction, however, transfer of resistance can occur from a non-lysogenic donor to a recipient, the frequency of transfer is often much higher than that of conventional transduction and transducing phage are not detectable in the supernatant. It is possible that the transducing vector may be a short-lived or cell-bound phage particle that causes cell-to-cell adhesion by the alteration of surface proteins. The precise mechanism of this "phage-mediated conjugation" and its similarity to conventional transduction, remains obscure.

iv) Conjugation.

Conjugation is a process requiring cell-to-cell contact whereby DNA is transferred from a donor to a recipient bacterium. The ability to conjugate is normally encoded by conjugative plasmids although a conjugal process has recently been described in which no plasmid DNA could be detected (El Solh *et al,* 1986a). Conjugation has been extensively studied in Gram-negative bacteria but is also known to occur in Gram-positive bacteria such as clostridia and streptococci (Jacob and Hobbs, 1974). There is now substantial evidence for conjugation between *Staphylococcus aureus* strains (Archer and Johnson, 1983; Goering and Ruff, 1983; McDonnell *et al,* 1983), between *Staphylococcus epidermidis* and *Staph. aureus* (Forbes and Schaberg, 1983; McDonnell *et al,* 1983) and between *Streptococcus faecalis* and *Staph. aureus* (Engel *et al,* 1980; Schaberg *et al,* 1982).

In 1982, Schaberg *et al* reported the self-transfer of plasmid pAMβ1 from *Streptococcus faecalis* JH2-2 to *Staphylococcus aureus* via conjugation on membrane filters. A non-conjugative plasmid (pAMα1) was mobilized by pAMβ1 and once in *S.aureus,* the conjugative plasmids could be transferred to a second *S.aureus* or back into *S.faecalis.* Leblanc and Lee (1984) have located at least one conjugative function on pAMβ1 within an 8 kbp region.

Several groups have now independently demonstrated the conjugative transfer of staphylococcal gentamicin resistance conferring plasmids between staphylococci.

Forbes and Schaberg (1983) showed that plasmid pAM899-1 was able to direct its own transfer in filter matings between non-lysogenic donors and recipients. A number of factors proved that the process was a conjugative one. Firstly, direct cell-to-cell contact was necessary since transfer failed to occur in broth or when filters of donor and recipient were placed back-to-back. The process was energy dependent and required viable donor cells. It was resistant to DNAase and occurred in the

presence of chelating agents such as citrate and EDTA. Such evidence is contrary to the theory that either transduction or transformation are involved. Plasmid pAM899-1 was able to mobilize plasmid pAM899-2 (a small tetracycline resistance conferring plasmid) and plasmid pAM899-3 (an erythromycin resistance conferring plasmid).

Mc Donnell *et al* (1983) characterized a series of gentamicin resistance conferring plasmids from *S.aureus* and *S.epidermidis* and demonstrated inter- and intra-specific transfer both in mixed broth matings (required 0.01M Ca^{2+}) and on filters (no Ca^{2+} requirement). Donors and recipients were both non-lysogenic. The conjugative plasmids studied (pSH8 and pSH9) were large (46 and 58 kbp respectively) and encoded resistance to ethidium bromide and tobramycin as well as gentamicin. During the transfer process, deletions often formed, allowing a functional map to be constructed. Jaffe *et al* (1982) reported the transfer of a single gentamicin resistance conferring plasmid between *S.aureus* and *S.epidermidis* in a neonatal special care nursery. Restriction endonuclease analysis results showed that the two plasmids were indistinguishable and that therefore plasmid transfer may occur between the two species in nature.

Archer and Johnson (1983) reported that all 17 gentamicin resistant *S.aureus* and all 6 *S.epidermidis* isolates from an outbreak of staphylococcal infections in a nursery for neonates contained conjugative plasmids. Again, the transfer mechanism did not involve phage (mitomycin C-induced lysates of donor did not mediate transfer which occurred at high frequency between non-lysogenic donor and recipient cells) but required cell-to-cell contact. They postulated that the increase in nosocomial infections caused by gentamicin-resistant staphylococci could be partially explained by the evolution of self-transmissible plasmids in these isolates.

Goering and Ruff (1983) demonstrated that certain gentamicin resistance conferring self-transmissible plasmids of unrelated geographical origin gave similar restriction endonuclease patterns, suggesting the significant contribution of common conjugal plasmids to the emergence of gentamicin resistance in *S.aureus* populations.

Asch *et al* (1984) analysed plasmid deletion mutants from the self-transmissible gentamicin-resistant plasmid pCRG1600 (52.9 kbp). The transfer region was located within a 14.6 kbp region whilst an 8.5 kbp deletion was associated with an increased conjugal transferability (100-fold), similar to derepressed (*drd*) plasmid mutants in Gram-negative bacteria.

In vivo, staphylococci accumulate in large numbers in pathological situations such as boils, abscesses and on the skin of dermatology patients subjected to antibiotic therapy (Naidoo and Noble,

1978a; Noble and Naidoo, 1978b). Such close contact of cells would provide an ideal environment for conjugation as a means of transferring resistance.

1.3 Gentamicin resistance

Gentamicin is an aminoglycoside-aminocyclitol antibiotic containing 2-deoxystrepamine substituted at positions 4 and 6 (Figure 1.1). Aminoglycoside antibiotics are often used for the treatment of serious outbreaks of infection. They form a large and diverse group but all contain cyclic alcohols (aminocyclitols) in glycosidic linkage with amino-substituted sugars. Uptake into the cell is thought to occur in at least two phases (for review see Phillips and Shannon, 1984). The first energy-independent phase involves binding to the cell exterior. The second phase is energy-dependent in which the aminoglycoside associates with membrane "transporters", and is driven across the cytoplasmic membrane by a difference in electric potential.

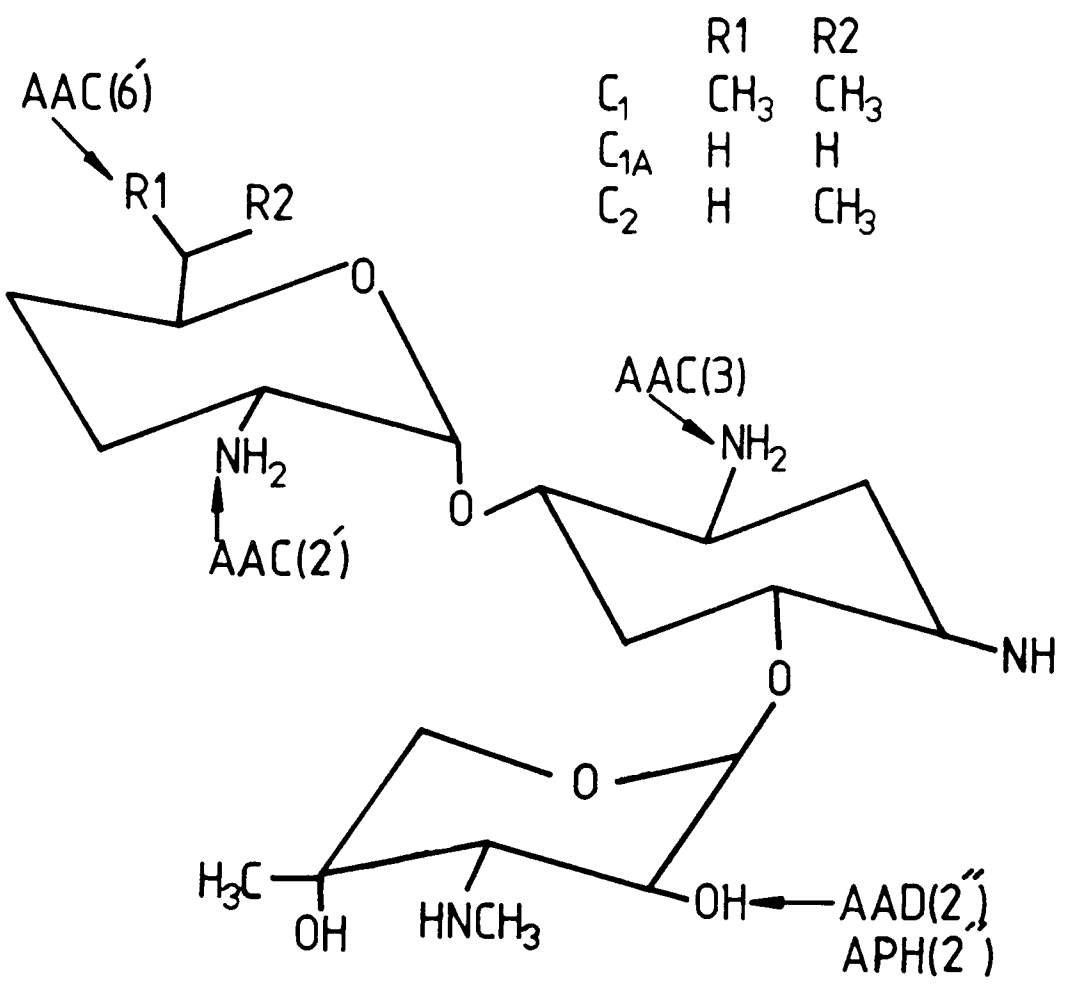
The mode of action of aminoglycosides has been studied most extensively using streptomycin which inhibits protein synthesis via its interaction with the bacterial ribosome resulting in the formation of aberrant initiation complexes. It is thought that lethality results from a disorganization of the cytoplasmic membrane during the transport of aminoglycosides. This leads to a loss of permeability control and hence cell death (see Bryan, 1984).

Gentamicin was used for almost a decade before, in 1976, outbreaks of hospital infection due to gentamicin-resistant strains of *S.aureus* occurred in Britain (Shanson *et al,* 1976). The epidemic strains were all multiply antibiotic resistant. The dissemination of gentamicin resistance in different strains of *S.aureus* during the 1970's was probably due to topical gentamicin therapy used mainly on dermatology patients. During the 1970's and 1980's, outbreaks of hospital infection in different countries were being increasingly caused by *S.aureus* strains resistant to at least five antibiotics including gentamicin and methicillin (Shanson *et al,* 1976).

Methicillin-resistant *S.aureus* strains (MRSA) are now a serious problem; in the South East of England for example, there has been a slow but uninterrupted spread of epidemic MRSA (EMRSA) between hospitals (Cooke and Marples, 1985). Antibiotic-resistance studies showed that EMRSA sent to Colindale during 1983 and 1984 were resistant to tetracycline, minocycline and stretomycin. Some 70% were resistant to gentamicin. They were constitutively resistant to macrolide antibiotics (Marples and Cooke, 1985).

Figure 1.1

Structure of Gentamicin



The arrows indicate the sites of attack by aminoglycoside-modifying enzymes

Methicillin-resistant *S.aureus* strains are also currently endemic in many Australian hospitals (Gillespie *et al,* 1986). They frequently encode resistance to antiseptics and disinfectants, such as quaternary ammonium compounds and to ethidium bromide. Resistance to such agents is encoded by a common determinant on a group of structurally related plasmids, many of which carry the transposon Tn*4001* and also encode resistance to trimethoprim. The recognition of antiseptic resistance in *S.aureus* is of particular significance in terms of the organism's potential for survival in the hospital environment. To establish the nature and distribution of the determinant in the staphylococcal population, it has been cloned into an *E.coli* vector system (Gillespie *et al,* 1986).

Methicillin-resistant *S.aureus* (MRSA) are now a serious problem in Australian hospitals and are difficult to treat due to their general resistance to many other chemotherapeutic agents (Townsend *et al,* 1983). Although vancomycin is often used (Marples and Cooke, 1985) this is an expensive and toxic drug so alternatives and drug combinations must be assessed.

In *S.aureus,* gentamicin resistance is due to specific adenyltransferases (AAD), acetyltransferases (AAC) and phosphotransferases (APH) (Scott *et al,* 1978). The site of modification is provided by the number of the position modified given in parenthesis following the enzyme designation. Enzymes with a distinctly different substrate profile are numbered by roman numerals following the designation of the type of enzyme activity and the site of modification (see Bryan, 1984). The assay often employed in the detection and identification of aminoglycoside-modifying enzymes is based on the firm binding of modified and unmodified aminoglycosides to phosphocellulose paper (Ozanne*et al,* 1969). This assay uses radiolabelled acetate, acetyl-coenzyme A or ATP and measures the transfer of the labelled acetate. Definitive conclusions require identification of the structure of its product. Site determination is made by the combination of a selection of substrates which possess or lack a specific OH or NH_2 group, exclusion and by characteristic reactions of certain enzymes.

In staphylococci, the most common enzymes modifying deoxystreptamine aminoglycoside are the APH (2″)- AAC (6″) dual function enzyme, AAD (4′, 4″), and APH (3′) III (Gray*et al,* 1983). All aminoglycoside modifying enzymes that have been studied are constitutively expressed, but their activity can in some cases be enhanced to a small extent by growth in sub-inhibitory concentrations of a substrate.

Several enzymes have been partially purified and characterized and sequence analysis of cloned DNA specifying some of the aminoglycoside-modifying enzymes is well underway. Chromosomal

DNA fragments from *S.aureus* of 1.5 and 1.95 kbp carrying *aph*A and *aac*A genes respectively (specifying the aminoglycoside-modifying enzymes APH3′ III and an AAC6′-APH2′) have been cloned and successfully introduced into *E.coli* and *B.subtilis* (El Solh *et al,* 1986b).

Gentamicin resistance (mediated by APH (2″) and AAC (6′)) has been localized to two small adjacent *Hin*d III fragments on the plasmid pSH8 (McDonnell *et al,* 1983) which also carries the gene encoding the AAD (4′) enzyme which inactivates tobramycin and paromomycin. Extensive homology between the amino acid sequences for APH (3′) from Tn*903,* Tn*5* and *Streptomyces fradiae* and the APH (3′) II genes of *Staphylococcus aureus* has been found suggesting a common evolutionary origin for these genes. A survey of a series of plasmids specifying aminoglycoside resistance has been made by Gray (Gray *et al,* 1983) leading to the tentative hypothesis that aminoglycoside resistance determinants in *S.aureus* may reside on transposable elements. This conclusion been supported by Gillespie and Skurray (1986) who detected Tn*4001* sequences in all gentamicin resistance plasmids in Australian isolates in addition to aminoglycoside resistance plasmids from the USA.

1.4 Conjugation in other bacterial species

i) *Escherichia coli*

The first extrachromosomal genetic element identified in *E.coli* was the F plasmid (or F sex factor) which is a covalently-closed circular molecule of DNA of about 100 kbp. The importance of F lies in the fact that it is a conjugative plasmid: it exhibits the property of DNA transmission between bacterial cells. F can replicate as an autonomous plasmid or as part of the host genome. Cells that carry the F plasmid (F^+ bacteria) are morphologically distinct from their F^- counterparts. In addition to the large number of small "common" pili often present, there are a few long, thin proteinaceous appendages called F (or sex) pili. These thread-like appendages (up to several micrometres in length and about 8 nm in diameter) are primarily made up of subunits of a phosphorylated glycoprotein, F pilin. F pili are male specific, that is, they are encoded by the F plasmid.

In addition to its property of self-transfer, the F plasmid also mediates the transfer of other DNA sequences. This process of F-duction has two forms; mobilization of the bacterial chromosome (an Hfr $\times$ F^- cross) or transfer of small pieces of chromosome (an $F'\times$ F^- cross).

Mobilization of donor chromosomal DNA is due to insertion of the F plasmid into the chromosome. Bacteria harbouring the F plasmid in this integrated state are called Hfrs since they give a high frequency of recombination of donor markers. The integration of the F plasmid into the host chromosome is a reversible process and excision restores the genome to its original structure. Occasionally however, aberrant removal of the F plasmid occurs forming a genetic element carrying F and some bacterial DNA. This results in the formation of a hybrid called an F-prime (F′) which has gained genes from the bacterial chromosome but may have lost some of its own DNA in the process.

Organization of the F plasmid.

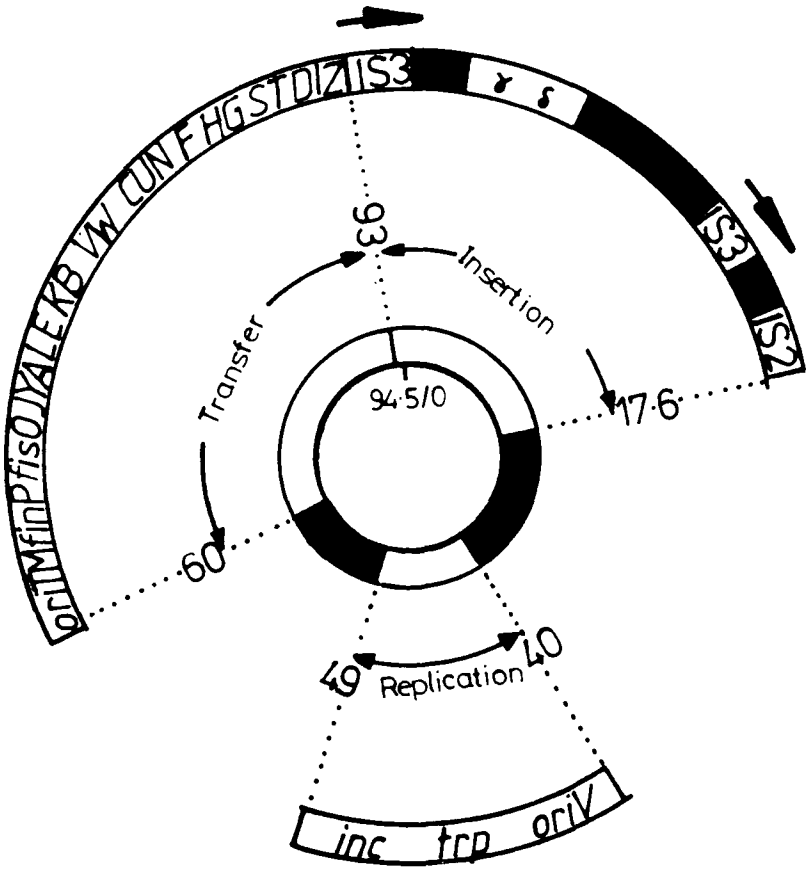
Despite its relatively large size, the F plasmid makes use of host functions for the majority of its needs such as transcription, translation and replication. It does however, encode information for transfer, vegetative replication and site-specific recombination represented by three main gene clusters on the circular genetic map (Figure 1.2). Comprehensive reviews on F-conjugation can be found in Lewin, 1977; Broda, 1979; Willetts and Skurray, 1980; Glass, 1982 and Willetts and Wilkins, 1984.

The number and organization of genes in the *tra* operon (responsible for transfer) was produced by allocating *tra* mutations to particular genes by complementation tests. The order of genes was determined from complementation tests between deletion mutants and point mutants; their organization into a single operon was determined from the effects of polar mutations, especially those produced by insertion of bacteriophage Mu. Complementation tests had to be carried out between compatible plasmids; this involved the use of R100.1 (an F-like R plasmid), the formation of "transient heterozygotes" or *in vitro* recombination of plasmid or phage with F.

The *tra* operon consists of a 33 kbp contiguous DNA sequence of at least 22 different genes situated between positions 60 and 93 on the F map (Figure 1.2). The *traJ* product is required for transcription of all *tra* genes other than M and is itself regulated by *fis*O. The *tra* functions are naturally derepressed; a negative control system exists whereby a repressor acts at an operator to inhibit transcription. This was discovered by analysing plasmid mutants de-repressed for conjugal transfer which expressed donor ability constitutively. The transfer origin of replication (*ori*T) is located to the left of *tra*M. The majority of genes identified to date are implicated in pilus production, including *traJ*, A, L, E, K, B, V, W, C, U, F, H and the promoter-proximal part of *tra*G. The *traJ* gene product resides in the outer membrane and may have both a regulatory and

Figure 1·2

Organization of the F plasmid



The three main gene clusters on the 94·5kb F plasmid are concerned with insertion, replication and transfer. The *tra* operon consists of atleast 22 different genes. The arrowhead represents the direction of transfer from *ori* T

Taken from Glass(1982)

a structural role. Mating aggregate formation, triggering of DNA transfer and transfer itself, are mediated by *tra*G/*tra*N, *tra*I/M and *tra*D respectively, the *tra*YZ endonuclease is responsible for nicking at *ori*T whilst the *tra*S and *tra*T genes are responsible for surface exclusion.

A model for F-conjugation is given in Figure 1.3 (Willetts and Skurray, 1980). Cell-cell interaction between the F^+ donor and F^- recipient results in the formation of a mating-pair. The tip of the pilus is probably involved, inter-acting with a specific recognition site on the surface of the F^- cell. It may then either contract or be reabsorbed. It is, as yet unknown whether DNA is actually transferred through the pilus (a conjugation tube) or whether this merely serves to bring the bacteria close together to form wall-to-wall contact upon pilus contraction, and it has been suggested that the term "conjugation bridge" is more appropriate.

Transfer of F DNA is initiated from a unique site (*ori*T) at which a specific plasmid-encoded endonuclease (the *tra*YZ gene products) is triggered to act by pair formation. Transfer is orientated and unidirectional; a single linear strand of F DNA enters the F^- cell, 5′-end first, leaving behind the complementary strand in the F^+ cell. Transfer is completed within one to two minutes. For transfer to occur, there is a requirement for donor F plasmid replication (probably via the rolling-circle mode of replication). Normally, the transferred strand is also replicated.

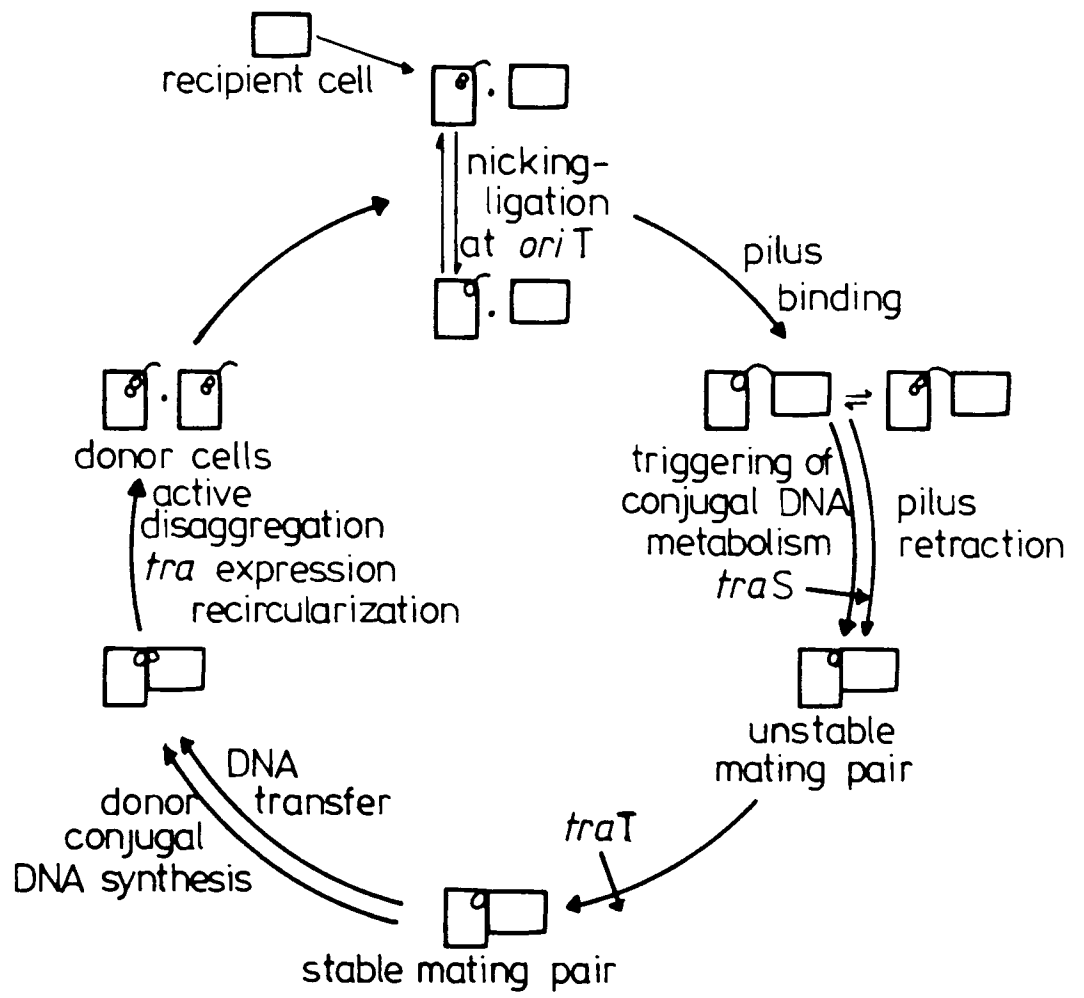
The region implicated in F insertion into the chromosome contains four insertion sequences (Figure 1.2), three of which are non-identical (IS2, IS3 and $\gamma\delta$). As IS sequences lie at the junctions of plasmid and chromosomal DNA, it is likely that F integration is via these transposable elements. Although there are hotspots at which insertion occurs, integration is not restricted to certain positions since the IS elements are capable of translocation.

F-like R plasmids.

F-like R plasmids encode both drug resistance and conjugative properties. They possess a transfer region showing great homology with the F *tra* operon and produce F-like pili allowing co-polymerization of the F-like and F pilin subunits. F-like R plasmids are generally repressed for transfer however. The presence of an F-like R plasmid in an F^+ cell reduces the frequency of F transfer. A model for this transfer inhibition has been postulated involving three loci, an operator site, *fis*O and two repressor genes, *fin*O and *fin*P. The *tra* operon is subject to two regulatory circuits: positive control through *tra*J and negative control of *tra*J through the *fin*OP-*fis*O system. That F-like R plasmids possess a closely related transfer system to F is demonstrated by homology

Figure 1·3

A model for the mating cycle of E.coli.



Taken from Willets and Skurray, 1980.
δ and o represent the superhelical and nicked circular forms of plasmid DNA respectively.

between the *tra* regions and the fact that two systems interact when the two genetic elements co-exist in one cell.

ii) Streptococci.

Three main types of conjugative systems have been found in the streptococci, two of which are mediated by plasmids (see Brunton, 1984). The first type of plasmid-mediated conjugation requires cell-to-cell contact which can be achieved on the surface of solid agar or on filters. Plasmids mediating this type of transfer (such as pAMβ1) have a broad host range.

The second type of plasmid-requiring transfer system occurs in broth culture but only in *Streptococcus faecalis*. The recipient secretes a heat-stable peptide (a pheromome) called clumping-inducing agent (CIA) which induces the donor cells (harbouring the pheromone responsive plasmid) to produce a protein called aggregation substance. This adheres to the recipients facilitating the formation of mating aggregates. The aggregation substance is believed to adhere to a binding substance located on the surface of both donor and recipient cells (Dunny *et al,* 1978). Clumping-inducing-agent (a sex pheromone) also promotes the subsequent transfer of plasmids from the donor to the recipient (Clewell and Brown, 1980) via the initiation of DNA metabolism. Examples of plasmids exhibiting this type of transfer are pAD1 and pAMγ1. The genetics of these plasmids have been studied in some detail and will be discussed in a later chapter. For a general review see Clewell, 1981.

The third type of transfer occurs in the absence of extrachromosomal DNA. Examples of this are Tn*916* in *S.faecalis* and Ω*cat tet* in *S.pneumoniae*.

1.5 Mobilization

Many plasmids are not conjugative themselves but are nevertheless spread much more rapidly between strains of bacteria than are chromosomal genes due to their mobilization by conjugative plasmids. For example, more than 90% of recipients of the F plasmid from a donor harbouring both F and the non-conjugative plasmid ColE1 also receive ColE1 and about 5% of recipients receive ColE1 but remain F^-. Vegetative replication of ColE1 has recently been reviewed by Sherratt (1986). The plasmid is known to specify at least one protein which acts *in trans;* the *mob*5 protein that nicks at *ori*T. A 555 bp primer (RNAII) initiates DNA synthesis at *ori*V. It is negatively controlled by a *trans*-acting RNA repressor (RNAI) encoded by the DNA strand complementary to that which encodes RNAII. Complexes between RNAI and II prevent the processing needed to produce an active primer. The *rom* protein (repressor of modulator) is required for repression by RNAI; it enhances the initial interaction between RNAI and II (Tomizawa and Som, 1984).

Mobilization is important in the transmission of drug resistance; resistance determinants to streptomycin, sulphonamide, ampicillin and tetracycline could be transferred by conjugation from *Salmonella* although none was present on a conjugative plasmid. Their transfer produced strain *Salmonella typhimurium* type 29, responsible for the outbreak of salmonellosis during 1963-66 (Anderson, 1968).

1.6 Plasmids as cloning vehicles

The ideal properties for cloning vectors include: low molecular weight, the ability to confer readily selectable phenotypic traits on host cells and single sites for a large number of restriction endonucleases, preferably in genes with an easily recognizable phenotype. Plasmids are widely used as cloning vehicles.

Basic techniques in gene cloning and recombinant DNA technology can be found in Maniatis *et al,* 1982; Old and Primrose, 1985 and Brown, 1986. The vectors available for cloning in *S.aureus* are discussed in detail in Chapter 5.

Shuttle vectors provide a means whereby genes from one organism can be introduced and expressed in another. The vector is constructed by *in vitro* genetic manipulation; it consists of DNA from the species from which DNA is to be cloned in addition to DNA from the species into

which the cloned fragment is to be introduced. By carrying genetic determinants, at least one of which must be selectable in each species, it can be transferred between the species. To enable it to replicate and express its resistances in both organisms, it must have a replication origin functional in both species, or, two origins, one of which is expressed in each species.

1.7 Transposon mutagenesis

Transposons are transposable genetic elements that confer a heritable property on the host bacterium (see Bennett, 1985). The most common transposon-carried genes confer antibiotic resistance. There are two types of transposon. Class I (compound) transposons have a resistance determinant flanked by copies of an insertion sequence either as direct or indirect repeats. Examples are Tn*5* and Tn*10*. Class II (complex) transposons such as Tn*1* are flanked by short inverted repeats. Examples of staphylococcal transposons include Tn*551* (5.3 kbp) and Tn*554* which both encode resistance to erythromycin. Transposon Tn*551* has been used to locate the methicillin resistance determinant on the chromosome of *S.aureus* (Berger-Bach, 1983) and by Luchansky and Pattee (1984) to map the staphylococcal chromosome. It was used in this survey (Chapter 7) to locate regions of a gentamicin resistance conferring plasmid involved in conjugal transfer.

Transposons are useful genetic tools which can be used to mutate and locate genes. Their ability to integrate at random in two opposing orientations provides a means for the generation of non-leaky polar mutations at any site. Their drug resistance genes provide convenient markers linking the insertion and the mutation, simplifying genetic analysis. The location of the transposon can be determined by restriction endonuclease analysis, providing a means of physically locating the gene into which the transposon has inserted. The presence of the inserted DNA in a coding region is usually polar on distal genes: transcription often terminates within the transposon resulting in the inhibition of expression of genes within the same operon, downstream from the site of insertion.

There are practical restrictions to the technique. Firstly, DNA sequences may differ in their readiness to act as target site for a particular transposon and secondly transposons often transpose at low frequency into plasmids already containing another copy of the transposon. This is known as transposon immunity (Robinson *et al,* 1977). There are a number of ways in which the plasmid could affect the host genome. It may affect the expression of more than one gene as transcription could terminate within the transposon or it could increase transcription of a particular gene due to the presence of a high level promoter within the transposon itself. Alternatively, the transposon may

have no effect. The orientation of insertion is also important; termination and/or promoter signals may be present in one or both orientations. Care must therefore be taken in the interpretation of data derived from transposon mutagenesis. The technique is described in detail in Chapter 7.

1.8 Aims of the research

The aim of the research was to study the newly described conjugation system of *Staphylococcus aureus*, at least part of which is specified by genes present on plasmids that also carry determinants for gentamicin resistance. Firstly the mechanism of transfer would be investigated and a suitable plasmid selected with which to pursue genetic analysis of the system. The regions of this plasmid involved in conjugation would be located and analysed with the longer term possibility of studying the products of the genes. This research would provide the preliminary analysis for future work into devising methods of limiting the transfer of such plasmids and hence resistance.

Chapter 2

Materials and Methods

2.1 Chemicals

Standard chemicals used were obtained from Fisons Ltd, Loughborough, Leics., B.D.H. Ltd, Poole, Dorset, or Oxoid Ltd, Basingstoke, Hants. These were of analytical grade when available. The sources of other chemicals are listed below:

Agarose type I (low electroendosmosis)	Sigma Chemical Company, St. Louis, USA.
Adenosine triphosphate	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	May and Baker Ltd, Dagenham, UK.
Difco agar	Difco laboratories, Detroit, USA.
Dithiothreitol	Sigma
Ethidium bromide	Sigma
Fusidic acid (sodium salt)	Sigma
Sodium dodecyl sulphate	Sigma
Tris (hydroxymethyl amino methane)	Sigma

2.2 Enzymes

Lysostaphin, lysozyme (Grade I), T4 DNA ligase, ribonuclease-A from bovine pancreas, ribonuclease T1 (Grade III), protease purified Type XIV and calf intestinal alkaline phosphatase were all obtained from Sigma.

Solutions of RNAase were heated at 80°C for 30 minutes to inactivate any contaminating DNAase.

Solutions of proteases were heated at 30°C for 30 minutes for activation.

2.3 Restriction Endonucleases

These were obtained from Amersham International, Amersham, Bucks., Bethesda Research Laboratories (UK) Ltd, Cambridge or New England Biolabs (CP Laboratories Ltd, Herts.) and were used with the restriction buffers shown in Table 2.1. The *Sma* I buffer was obtained from Amersham International.

2.4 Antibiotics

Table 2.2 describes the use of antibiotics for selection of resistant bacteria. Antibiotics were dissolved in the solvent described and filter sterilized (except for ethanol solutions). The stocks were stored at $-20°C$. The stock solutions were added to sterile medium or agar which had been autoclaved and cooled to 50°C to produce selective plates or media. The final concentrations of antibiotics used are also listed in Table 2.2.

2.5 Media

i) Brain Heart Infusion (BHI) medium.

37g of BHI powder was dissolved in one litre of deionised water.

ii) BHI-agar.

20g of Difco agar was dissolved in one litre of BHI medium.

iii) Casein Hydrolyase Yeast Extract (CY) medium.

Amounts per litre:

Casein hydrolysate (acid)	10g
Yeast extract	10g
$MgSO_4.7H_2O$	0.25g
Trace elements solution	0.04ml.

One litre of trace element solution contained:

$CuSO_4.5H_2O$	2.5g
$ZnSO_4.7H_2O$	2.5g
$FeSO_4.7H_2O$	2.5g
$MnCl_2.4H_2O$	1.0g
Conc. HCl	50ml.

Before use, 10g glucose was added per litre of CY medium.

Table 2.1

Buffers used for restriction enzyme analysis

Enzyme	Type of buffer used.
Acc I *Ava* I *Bcl* I *Bgl* I *Bgl* II *Cla* I *Eco* RI *Eco* RV *Hae* II *Hinc* II *Hind* III *Hpa* I *Mlu* I *Pst* I *Pvu* II *Sac* II *Sca* I *Sst* I *Xho* I	core buffer (medium salt)
Bam HI *Hgi* AI *Nco* I *Sal* I *Sph* I *Xba* I	high salt buffer
Dra I *Kpn* I *Nar* I *Sac* I	low salt buffer
Sma I	*Sma* I buffer

Table 2.2

Antibiotics used for the selection of resistant bacterial strains

Antibiotic	Solvent	Stock concentration (mg/ml)	Working concentration (μg/ml)	Source
Ampicillin	H_2O (+0.1%NaOH)	25	50	Sigma
Chloramphenicol	Et.OH	5	20	Sigma
Erythromycin	H_2O	5	10	Sigma
Gentamicin (sulphate)	H_2O	10	20	Sigma
Kanamycin (sulphate)	H_2O	5	50	Sigma
Novobiocin (sodium salt)	H_2O	5	5	Sigma
Paromomycin	H_2O	5	100	Parke-Davis Research Labs.
Penicillin-G (benzyl penicillin)	H_2O	—	8000	Sigma
Sulphanilamide (p-Aminobenzene-sulphonamide)	H_2O	5	100	Sigma
Tetracycline (hydrochloride)	H_2O	5	5	Cyanamid Ltd, Gosport, Hants.

iv) 2×TY medium.

Amounts per litre:

Bacto-tryptone	15g
Bacto-yeast extract	10g
NaCl	5g

The pH was adjusted to 7.5 with NaOH.

v) DM3-agar

As described by Chang and Cohen (1979).

vi) Starch plates

4g of starch was dissolved in 1 litre of BHI agar and autoclaved.

vii) TBAB (tryptone broth agar base).

33g of TBAB powder was dissolved in one litre of deionised water.

2.6 Chemical solutions

i) 10×TBE

500mM	Tris
500mM	boric acid
100mM	EDTA

The pH was adjusted to 8.3 with HCl

ii) 10×core buffer

50mM	Tris-HCl (pH8.0)
10mM	$MgCl_2$
50mM	NaCl

Stored at 4°C

iii) High salt buffer

6mM	Tris-HCl (pH7.9)
6mM	$MgCl_2$
150mM	NaCl

Stored at 4°C

iv) Low salt buffer

6mM Tris-HCl (pH7.5)

6mM NaCl

6mM β-mercaptoethanol

6mM $MgCl_2$

v) 2×CIP reaction buffer (calf intestinal alkaline phosphatase)

20mM Tris-HCl pH9.2

0.2mM EDTA

vi) 10×ligation buffer

100mM Tris-HCl (pH 7.5)

500mM NaCl

100mM $MgCl_2$

vii) TE buffer

10mM Tris-HCl

1mM EDTA

Adjust to pH8.0 with HCl

viii) Tf buffer

50mM $CaCl_2$

15% glycerol

10mM Pipes-NaOH pH6.6

ix) Phage buffer

10mM Tris-HCl (pH7.4)

100mM NaCl

10mM $MgCl_2$

1mM $CaCl_2$

0.7g gelatin per litre.

The gelatin was dissolved in deionized water by gently heating before being added to the other solutions and then autoclaved.

x) TCG solution

50mM glucose

10mM CDTA (trans 1,2 diaminocyclohexane N,N,N^1,N^1, tetraacetic acid)

25mM Tris-HCl (pH8.0)

xi) High salt buffer (*E.coli* plasmid prep.)

3M potassium acetate

1.8M formic acid

xii) TES solution

50mM Tris

5mM EDTA

100mM NaCl pH 7.2

xiii) 2×HB (hypertonic buffer)

1.4M sucrose

0.04M maleate

0.04M $MgCl_2$

xiv) HBM (hypertonic buffered medium)

43g of Penassay broth (antibiotic medium 3) was dissolved in 1 litre of HB solution.

xv) Sinker/dye solution

20% Ficoll

0.025% bromophenol blue

0.025% xylene cyanol

2.7 Sterilization

In general, media and solutions were sterilized by autoclaving for 20 minutes at a pressure of 15lb/in^2. Aqueous stock solutions of sugars were sterilized at a pressure of 10lb/in^2 for 10 minutes. Bacteriophage suspensions, solutions of bovine serum albumin (BSA) and of antibiotics were sterilized by passage through a sterile 25mm millipore filter (0.45μm pore size) into a previously sterilized container. Glassware was generally sterilized in an oven at 160°C for 1 hour. Plastic Eppendorf tubes and plastic pipette tips used in DNA manipulation procedures were also autoclaved.

2.8 Bacterial strains

The convention of Novick *et al* (1976) has been used for the plasmid nomenclature. The phenotypes of naturally occurring strains and their plasmids are shown, together with reference to their origins in Table 2.3. The letter "N" following the strain number indicates the absence of a known plasmid in that strain. The *E.coli* strain MC1061 was used in cloning steps because cells may be frozen in a competent state and then defrosted for use in transformation.

2.9 Maintenance of cultures

a) Frozen stock.

A stock of all bacterial strains was kept at −70°C. To add a strain to the long term stock, 1ml of an overnight culture of the strain (in BHI medium) was added to a 2ml barrel jar containing 1ml sterile glycerol/BHI medium (30%/70%).The jar was tightly capped, inverted to mix and placed immediately at −70°C.

b) Plate stock.

For normal use, bacterial strains were stored on BHI-agar plates at 4°C, containing appropriate antibiotics. Stock plates were prepared by removing a frozen aliquot of the desired strain from the −70°C stock, sterilizing a wire loop in a flame, inserting it into the frozen culture and streaking a fresh plate for single colonies. The jar was immediately returned to the freezer and the plate was incubated at 37°C overnight before being stored at 4°C. Stock plates were subcultured onto fresh agar every 6–8 weeks.

2.10 Bacterial growth

a) Solid culture.

Plates of BHI-agar containing appropriate antibiotics were used to store, select and to screen bacterial strains. Plates were inoculated either by streaking with bacteria from a sterile platinum loop or a toothpick or by spreading 0.1–0.2mls of a liquid culture over the surface with a sterile bent glass rod. Plates were inverted and incubated at 37°C. Colonies generally grew overnight.

b) Liquid culture.

BHI medium was transferred to a conical flask or test tube of at least five times the volume of the medium. Appropriate antibiotics were added. The medium was inoculated either by transferring bacteria from a single colony on a stock plate or by pipetting bacteria from another liquid culture. The culture was grown at 37°C with vigorous shaking unless otherwise stated.

Table 2.3

Table of Bacterial Strains and Plasmids

Strain/Plasmid	Antibiotic Resistances	Comment, Source and Reference
Staphylococcus aureus **strains**		
8325N	none	A naturally occurring strain Novick and Roth, 1968
A53	gentamicin erythromycin tetracycline neomycin	German strain, phage-type 85. Contains a Gm^r plasmid. Donated by Dr. J. Naidoo.
8325 Nov^r	novobiocin	A novobiocin resistant mutant of 8325N. Donated by Dr. J. Naidoo.
B111 Nov^r	novobiocin penicillin	A novobiocin resistant mutant of B111 (a wild type strain isolated at the Institute of Dermatology of phage type 3A/53/85). Naidoo *et al,* 1983. Donated by Dr. J. Naidoo.
PS80d.*ero* [pI9789,seg-1.Int^-A] RevE.17	cadmium erythromycin	A strain containing the transposon Tn551. Made and donated by Browne, D.Phil thesis, 1985. This laboratory
PS80d.*ero*	erythromycin	Johnston, D.Phil thesis, 1971. This laboratory.

p 8325-4 is different from the strain 8325-4 referred to in

Novick, R.P., 1967. Properties of a cryptic high-frequency transducing phage in Staphylococcus aureus. Virology 33, 155-166.

Table 2.3 (cont.)

Strain/Plasmid	Antibiotic Resistances	Comment, Source and Reference
Staphylococcus aureus plasmids (all in 8325N)		
p8325–2 (strain 8325–2)	gentamicin ethidium bromide paromomycin	Constructed by transfer of the Gm^r plasmid from A118 (German-strain, phage-type 29/52/79/95/6/42E/47/53/54/77/84).†
p8325–3 (strain 8325–3)	gentamicin	Constructed by transfer of the Gm^r plasmid from A53.†
p8325–4	gentamicin penicillin cadmium arsenate arsenite	Constructed by transfer of the Gm^r plasmid from A102 (German strain, phage-type 77).†
p8325–5 (strain 8325–5)	gentamicin	Constructed by transfer of the Gm^r plasmid from *S. hominis*.†
pC194	chloramphenicol	Horinouchi and Weisblum, 1982a.
pCW3	tetracycline	Transformed from *S.aureus* JB48827 Wilson and Baldwin, 1978. Donated by Ms. S.J. Harris
pCW7	chloramphenicol	Transformed from *S.aureus* JB48827 Wilson and Baldwin, 1978. Donated by Ms. S.J. Harris
pCW59	chloramphenicol tetracycline	Wilson *et al,* 1981.
pC221	chloramphenicol	Natural isolate. Novick and Bouanchaud, 1971. Donated by Prof. W.V. Shaw.
pCW41	chloramphenicol	Wilson *et al,* 1981. Donated by Prof. W.V. Shaw.

Table 2.3 (cont.)

Strain/Plasmid	Antibiotic Resistances	Comment, Source and Reference
pCE10	chloramphenicol erythromycin	Keller *et al*, 1983 Donated by Dr. F. Gotz.
pT181	tetracycline	Natural isolate. Khan *et al*, 1981.
pT181 *cop-608*	tetracycline	A mutant of pT181 with a high copy number. Khan *et al*, 1981.
Staphylococcus hominis	gentamicin tetracycline penicillin erythromycin	A coagulase-negative *Staphylococcus* isolated at the Institute of Dermatology, London. Donated by Dr. J. Naidoo.
Staphylococcus epidermidis	none	Naidoo and Noble, 1978b. Donated by Dr. J. Naidoo.
Streptococcus faecalis strains		
JH2-2	rifampicin fusidic acid	Plasmid-free strain. Jacob and Hobbs, 1974. Donated by Dr. A. Jacob.
FA2-2	rifampicin fusidic acid	A plasmid-free derivative of JH2-2. Clewell *et al*, 1982. Donated by Dr. D.B. Clewell.
FA373	rifampicin fusidic acid tetracycline (Tn*918*)	An FA2-2 strain harboring pAM373. Clewell *et al*, 1985. Donated by Dr. D.B. Clewell.

Table 2.3 (cont.)

Strain/Plasmid	Antibiotic Resistances	Comment, Source and Reference
Escherichia coli **strains**		
JM107	none	A restriction minus, modification plus strain. Yanisch-Perron *et al,* 1985.
MC1061	none	Casadaban and Cohen, 1980.
RR1 [pRWY21]	ampicillin chloramphenicol tetracycline	Donated by Mr. B. Tidor. (personal communication).
Bacillus subtilis **strains**		
IE21 BD426[pBD8]	chloramphenicol kanamycin streptomycin	Obtained from the *Bacillus* Genetic Stock Centre, Columbus, Ohio.
MY2016	rifampicin sulphanamide	Donated by Dr. M. Yudkin

† All four strains were made and donated by Dr. J. Naidoo (personal communication). Dept. of Bacteriology, Institute of Dermatology, London E9 6BX.

2.11 Determination of antibiotic resistance

In addition to the use of selective BHI plates, strains were screened for antibiotic resistance by the use of multodisks. A lawn of the bacteria to be tested was made by flooding a BHI-agar plate with 3mls of a 6 hour culture. Excess liquid was removed and the plate allowed to dry for 1 hour at 37°C. A sterile Oxoid multodisk was laid on the lawn of cells and incubation continued for 16 hours at 37°C. The zones of inhibition of growth around the various antibiotics were compared with a control (normally 8325N). Growth up to the disk was taken to indicate resistance. The disks carried the antibiotics as shown below:

Concentration of antibiotics in multodisks.

Antibiotic	Amount per disk
Chloramphenicol	10μg
Erythromycin	10μg
Penicillin G	1.5units
Tetracycline	10μg
Streptomycin	5μg
Novobiocin	5μg
Oleandomycin	5μg
Sulphafurazole	100μg

For antibiotics not on the disks (such as gentamicin) and for cadmium resistance, agar plates containing antibiotic/chemical to the required concentration were made. Bacteria to be tested were streaked over the plate using a sterile loop.

2.12 Agar plate test for β-lactamase production

Colonies to be tested were grown on BHI-agar plates containing 0.4% starch. The plates were flooded with a solution made up of:

0.2M $Na_2HPO_4.12H_2O$	10ml
0.2M $NaH_2PO_4.2H_2O$	10ml
0.5M iodine solution	5ml (40.6g I_2 and 200g KI per litre of distilled water)
Penicillin G	0.2g

Excess liquid was poured off. The presence of iodine turned the starch agar blue/black. Hydrolysis of penicillin produced penicilloic acid which reduced the iodine to iodide. The production of β-lactamase was thus indicated by the appearance of white halos around colonies.

2.13 Test for arsenate resistance

Resistance to arsenate was tested by applying a small paper disc containing 20μl of 0.1M sodium arsenate to the centre of a horizontal inoculum streak of the strain to be tested on a BHI-agar plate. Resistant colonies grew right up to the disc whereas sensitive colonies grew only at some distance from the disc.

2.14 Replica plating

Individual colonies were transferred to a "master" BHI-agar plate using sterile wooden tooth-picks and grown overnight. A grid pattern was used beneath the plate to ensure even spacing of the colonies. The grid of colonies was then transferred in a single step to the surface of a piece of sterile velvet, stretched tightly over a cylindrical block by pressing the inverted master plate onto it. The colonies were transferred by placing a new agar plate over the velvet. Overnight incubation followed.

2.15 Full scale preparation of plasmid DNA from *Staphylococcus aureus*

The method of Novick and Bouanchaud (1971) was used to purify plasmid DNA from *S.aureus* for cloning, transformation and restriction endonuclease analysis. The appropriate *S.aureus* strain was grown overnight in 50mls of CY + glucose medium at 37°C with aeration. A further 200mls of the same medium was added and incubation continued for 90 minutes. The culture was centrifuged for 10 minutes at 5,000 rpm (Sorvall RC-5B, GSA rotor) and the pellet resuspended in 250mls of cold 10mM EDTA (pH 7.0). Following re-centrifugation, the pellet was resuspended in 4mls of cold 10mM EDTA. To this, 8mls of acetone : ethanol (1:1 by volume) was added and the mixture incubated on ice for 20 minutes, with occasional shaking. After a further addition of 40mls EDTA, cells were pelleted by centrifugation as before, then drained well and all remaining acetone/ethanol removed by evaporation under compressed nitrogen. The pellet was resuspended in 16mls of 0.1M NaCl + 0.05M EDTA (pH7.0) and lysostaphin added to a final concentration of 15μg/ml. Lysis occurred after incubation for 1 hour at 37°C whereupon,

2.85ml 5M NaCl

1.85ml 0.5M EDTA

17ml 2% SDS in 0.7M NaCl

were added sequentially. The mixture was inverted once after each addition and the lysate held overnight at 4°C. Unbroken cells and other large debris were removed from the lysate by centrifugation at 19,000 rpm (Sorvall RC-5B, SS34 rotor) for 45 minutes. The supernatant containing the

DNA was retained. RNAase (previously heat treated at 80°C for 30 minutes to remove DNAase activity) was added to 50μg/ml and T_1 RNAase to 1 unit/ml. After incubation for 1 hour at 37°C, pronase was added to a final concentration of 50μg/ml and incubation continued for a further hour. Sodium acetate was added to a final volume of 0.3M followed by 2 volumes of cold ethanol (96%). After incubation in a dry ice/ethanol bath for 1 hour, the DNA was pelleted by centrifugation at 10,000 rpm (Sorvall RC-5B, GSA rotor) for 20 minutes. The pellet was drained well and resuspended in 3.2ml 0.1M NaCl + 0.05M EDTA in preparation for caesium chloride density centrifugation. Solid caesium chloride (2.97g) was dissolved in the resuspension and 0.2ml of ethidium bromide solution (10mg/ml) added. This solution was sealed in a polyallomer Beckman Quick Seal tube and centrifuged at 45,000 rpm for 16 hours (Beckman L5 50B ultracentrifuge, VTi65 rotor). Two flourescent bands were visible under U.V. light. The lower plasmid band was removed with a syringe after side puncture of the tube. Ethidium bromide was removed by two extractions with 2 volumes of propan-2-ol and CsCl by exhaustive dialysis against 2mM Tris-HCl (pH7.2) at 4°C. DNA was precipitated as before and resuspended in 2mM Tris-HCl (pH7.2).

Storage of DNA.

Plasmid DNA was stored in 2mM Tris-HCl (pH7.2) at 4°C in Eppendorf tubes.

2.16 Rapid purification of plasmid DNA from *S.aureus*

This method allows the rapid preparation of plasmid DNA from many cultures simultaneously. The product, although crude, can be used for restriction enzyme analysis. The method is based upon that of Birnboim and Doly (1979). A sample of liquid culture (1.0 to 1.5ml) was placed in a 1.5ml Eppendorf tube and centrifuged for 1 minute in a Beckman Microfuge. The supernatant was removed using a fine aspirator and the pellet was resuspended in 0.1ml of lysis buffer (50 mM glucose, 25mM Tris-HCL pH 8.0, 10mM EDTA) containing 50μg lysostaphin/ml. After 30 minutes at room temperature, 0.2ml of alkaline SDS solution (0.2 M NaOH, 1% (w/v) SDS) was added and the tube contents were mixed thoroughly by inversion. The tube was maintained at 0°C for 5 minutes and then 0.15ml of 3M sodium acetate (pH 4.8) was added. When the tube contents were mixed gently, a clot of DNA formed. After a further 1 hour at 0°C (when most of the protein, high molecular weight RNA and chromosomal DNA precipitated) the tube was centrifuged for 5 minutes and 0.4ml of the supernatant transferred to a second tube. Cold ethanol (1ml) was added and the tube held at 20°C for 30 minutes. The precipitate was collected by centrifugation for 2 minutes and the supernatant removed by aspiration. The pellet was dissolved in 0.1ml of 0.1M sodium acetate, 50 mM Tris-HCl (pH 8.0) and re-precipitated with 0.2ml of cold ethanol. After 20 minutes at 20°C, the precipitate was collected by centrifugation as before. The pellet was dissolved in 40μl of 2 mM

Tris and 20μl of sinker/dye solution (20% Ficoll, 0.025% bromophenol blue, 0.025% xylene cyanol) was added. Samples of 25μl were loaded into the wells of an agarose gel slab for electrophoretic analysis. The remaining sample was stored at 4°C.

2.17 Preparation of plasmid DNA from *E. coli*

The method is based on that of Birnboim and Doly (1979) modified by Maniatis *et al* (1982). Appropriate strains were grown overnight in 100mls 2×TY medium with antibiotic if desired. The culture was centrifuged at 5,000 rpm for 5 minutes (Sorvall RC-5B, GSA rotor) and the pellet resuspended in 2ml TCG in a 50ml polypropylene tube. To each tube, 10mg lysozyme was added. The tube was vortexed briefly. After standing at room temperature for 15 minutes, 4ml alkaline SDS (0.2N NaOH, 1% SDS) was added and mixed by inversion. Incubation on ice for 15 minutes was followed by addition of 3ml high salt buffer. Tubes were vortexed for 20 seconds and placed on ice for 15 minutes. Chromosomal DNA and protein complexes were pelleted by centrifugation for 20 minutes at 18,000 rpm (Sorvall SS-34 rotor, 4°C). The supernatant was carefully decanted into a 30ml corex tube and 5.5ml of propan-2-ol added. The tubes were carefully inverted to mix the contents then incubated at room temperature for 10 minutes. The RNA+DNA precipitate was collected by centrifugation for 20 minutes at 10,000 rpm (Sorval SS-34 rotor). The supernatant was removed and the pellet rinsed in 80% ice-cold ethanol, dried under compressed nitrogen and resuspended in 4mls TC buffer. Solid caesium chloride (4.0g) was added followed by 0.2ml ethidium bromide (10mg/ml). The solution was sealed in a polyallomer Beckman Quick Seal tube and centrifuged at 45,000 rpm for 16 hours (Beckman L5 50B ultracentrifuge, VTi65 rotor). The procedure was completed as given for the preparation of plasmid DNA from *S.aureus* (Section 2.15).

2.18 Preparation of plasmid DNA from *Bacillus.subtilis*

The appropriate strain was grown overnight at 30°C in 100 mls of 2XTY medium. The following morning, 120ml of fresh medium was added and the incubation continued for 2-3 hours. The cells were harvested by centrifugation at 5,000 rpm for 5 minutes (Sorvall GSA rotor, 4°C), washed twice in ice-cold TES solution and resuspended in 10ml of TES. To this was added 10mg lysozyme and 5mg heat-shocked RNAase I. The sample was incubated with gentle shaking for 30 minutes at 37°C after which time, 10ml fresh TES was added with 5mg of self-digested (37°C, 90 min) pronase. Following an incubation at 37°C for 30 minutes, the solution was centrifuged for 20 minutes at 15,000 rpm (Sorvall SS34 rotor, 4°C). The slightly viscous supernatant was tipped into a clean tube, holding the viscous mass at the bottom. The supernatant was ethanol precipitated. The procedure was completed as given for the preparation of *S.aureus* (Section 2.15). The final sample of DNA was resuspended in 2mM Tris-HCl.

2.19 Conjugation of *S.aureus* strains

Bacterial matings were performed by a modification of the procedure described by Forbes and Schaberg (1983). Donor and recipient cells were grown overnight on selective BHI-agar plates. Cells were scraped off the plates and adjusted in BHI medium (containing 0.01M $CaCl_2$) to an optical density of 2.5 at 675nm. Suspensions (2 or 3mls) of donor and recipient cells were combined, mixed well and collected by membrane filtration (nitrocellulose, 0.45μm). The filter was immediately placed on a BHI-agar plate, with the bacterial cells touching the surface. After an overnight incubation at 37°C, each filter was transferred to 10mls of 0.9% saline and vortexed thoroughly. Mating mixtures, with dilutions where necessary, were spread onto plates containing appropriate selective antibiotics. Colonies were counted after overnight incubation at 37°C.

2.20 Restriction enzyme analysis

The amount of plasmid DNA solution required for each digestion was estimated by running 2μl of the stock solution on an agarose minigel. The DNA to be restricted was incubated with 2μl of the appropriate 10×concentration reaction buffer and 1–10 units of restriction enzyme was added. The final volume of the reaction mixture was made up to 20μl with d.H_2O. Incubations were at 37°C for up to 6 hours. In reactions involving digestion by two restriction enzymes requiring different buffer systems, DNA was first digested by the enzyme with the lower salt requirement. When digestion was complete, the salt concentration was raised to that required by the second enzyme which was then added. Reactions were terminated by heating at 65°C for 10 minutes. If the sample was to be loaded onto a gel, 10μl of sinker/dye solution was also added.

2.21 Agarose gel electrophoresis

Plasmid DNA was analysed by agarose slab gel electrophoresis. Agarose powder was added to 150ml of 1×TBE buffer to a concentration of 1% (w/v) and dissolved by boiling. Once dissolved, the agarose was allowed to cool to around 45°C and ethidium bromide was added to a concentration of 500μg/l. For vertical gel electrophoresis, the gel mould consisted of two glass plates separated by 3mm spacers producing a gel of dimensions 15 × 15 × 0.3cm. Before pouring the gel, a small amount of moulten agarose was run along the inside edges of the spacers and allowed to harden. A sample comb giving 12 wells was inserted and the gel allowed to set for one hour. Before running the gel, the bottom spacer was removed and replaced by a porous polythene strip. The gel was then clamped to the electrophoresis apparatus and the reservoirs filled with 1×TBE buffer containing 500μg ethidium bromide per litre. Sinker/dye solution (10μl) was added to each 20μl of DNA sample. The comb was removed and the samples (30μl) loaded into the wells. The gel was run at 30V (6mA) for 16 hours from a constant D.C. voltage supplied by a power pack. At the end of the run, the gel was removed from the plates and the DNA bands visualised on a U.V. transilluminator (Ultra-Violet Products Inc., California USA.) The gel was photographed through a red filter (Wratten no.25) using either 35mm Ilford FP4 or Polaroid type 667 films. Where appropriate, negatives were printed onto Ilfospeed photographic paper.

For small gels, a minigel electrophoresis apparatus (workshop produced) was used, giving a gel of dimensions 5 × 7 × 2mm. The gel was run at 50V for 30 minutes in 1×TBE buffer containing ethidium bromide at 500μg/l.

2.22 Dephosphorylation of DNA

The terminal 5' phosphates were removed from DNA by treatment with calf intestinal phosphatase (CIP). The method was taken from the Amersham cloning and sequencing handbook.

A sample (1-2 μg) of the plasmid DNA to be de-phosphorylated was ethanol precipitated and resuspended in 19μl d.H_2O, to which 20μl of 2×CIP buffer was added. The sample was incubated with 1μl calf intestinal phosphatase (1 unit) for 30 minutes at 45°C. A further 1μl of enzyme was added and the reaction was re-incubated for a further 30 minutes and then terminated by phenol extraction (Section 2.24). The phosphatased sample was stored at -20°C in TE buffer.

2.23 Phenol extraction

The DNA to be phenol extracted was mixed with 50μl of phenol saturated with TE buffer in an Eppendorf tube and vortexed to form an emulsion. The sample was then centrifuged for 4 minutes in a microfuge and the upper aqueous layer removed to a clean tube with a micropipette. Another 50μl of phenol was added to this aqueous phase and the procedure repeated. The aqueous phase from the second reaction was mixed with 500μl of diethyl ether and centrifuged briefly. The lower (aqueous) phase was removed and placed in a clean tube. The ether extraction was repeated twice, after which, the solution was ethanol precipitated.

2.24 Ethanol precipitation

The DNA to be ethanol precipitated was mixed carefully with one tenth volume 3M sodium acetate and 2 volumes cold ethanol. After incubation in a dry ice/ethanol bath (15 minutes for samples in Eppendorf tubes, up to 1 hour for larger samples) the DNA was pelleted (microcentrifuge for 5 minutes or 20 minutes at 10,000rpm in a Sorvall RC-5B centrifuge, GSA/SS34 rotor for larger samples). The pellet was dried well (under compressed nitrogen) and resuspended in the desired buffer solution.

2.25 Ligation

The vector and insert DNA to be ligated were restricted with appropriate enzymes. After a minigel had been run to ensure digestion was complete, the restriction enzymes were inactivated by heat treatment (65°C/10 minutes) and the digests diluted to the required concentration. Vector DNA (usually 10–30ng) and insert DNA (usually 5 times molar excess of vector) were combined with the ligation mixture shown below:

5μl 10×ligation buffer

2μl DTT (500mM)

1μl ATP (50mM)

1μl T4 DNA ligase (1 unit)

The volume was adjusted to 50μl with d.H_2O and the mixture incubated at 12–16°C for 12 hours. The reaction was terminated by heating at 65°C for 10 minutes and subsequently stored on ice.

2.26 Transformation of *S.aureus* protoplasts

The procedure is based upon that by Lindberg (1981). A culture of the strain to be protoplasted was grown overnight in 10mls BHI. The cells were harvested (bench centrifuge, 10 minutes no.10) and resuspended in 5mls HBM containing 100μg lysostaphin and 10mg lysozyme. The culture was incubated at 37°C and the O.D. taken every half hour until it remained constant (approximately 3 hours). The protoplast suspensions were centrifuged at 7,000 rpm (Sorvall RC-2B, SS34 rotor, 4°C) which removed cell debris and most of the non- protoplasted bacteria. The supernatants were collected and centrifuged again at 19,000 rpm for 17 minutes. The pelleted protoplasts were resuspended in 0.5–1.0ml HBM and divided into 0.2ml aliquots. A 50μl sample of DNA (about 150ng) was added. After gentle mixing, 1.8ml of 40% PEG 6000 was added followed 2 minutes later by 10ml HBM. The suspension was centrifuged at 19,000 rpm for 30 minutes (Sorvall RC-5B, SS34 rotor) and the pellet resuspended in 0.4mls HBM. Samples of 0.1ml were gently plated onto DM3 agar plates and phenotypic expression allowed for 3 hours at 37°C before 5mls of soft agar, containing an appropriate amount of antibiotic was added. Transformed colonies were picked for purification after 3–4 days incubation at 37°C.

2.27 Transformation of *E.coli* cells

The *E.coli* strain MC1061 was used to prepare competent cultures which were frozen at −70°C in aliquots and defrosted when needed for transformation. Competent cells were prepared by inoculating 5mls of 2×TY medium with a single colony of *E.coli* from a fresh stock plate. The culture was grown with shaking at 37°C until the $O.D._{600}$nm was 0.2–0.3 (usually 4 hours). A 1ml sample of the culture was used to inoculate 100ml of pre-warmed 2×TY medium and grown to an $O.D._{600}$ of 0.2 (usually 2–3 hours). The culture was chilled for 10 minutes in ice water then centrifuged (RC-5B, SS34 rotor) for 10 minutes at 5,000 rpm (4°C). The pellet was resuspended in half the original volume of cold Tf buffer, incubated for 20 minutes on ice then centrifuged as before. The pellet was resuspended in 0.05 times the original volume of cold Tf buffer and 0.15ml aliquots were dispensed into Eppendorf tubes and frozen at −70°C. The cells remained competent for several months. For transformation, an aliquot of competent cells was removed from the freezer, thawed rapidly in a water bath (1–2 minutes, room temperature) and incubated for 5 minutes on ice. A 50μl sample of DNA (about 150ng) was added, the tube mixed by gentle inversion and then placed for 5 minutes at 37°C. Pre-warmed 2×TY medium (0.15ml) was added and the tube re-incubated for 1 hour at 37°C. Selective BHI plates were spread with 0.1–0.2ml and grown overnight at 37°C.

2.28 Sex pheromone testing

The method is based upon that of Dunny *et al* (1979).

a) Filtrate production.

Strains to be tested for their ability to produce clumping-inducing agent (CIA) were grown overnight in 2mls BHI medium. Each strain was diluted (1 in 10) in BHI medium and grown for about 5 hours at 37°C with shaking. Cells were pelleted by centrifugation (bench centrifuge, no. 10, 10 minutes) and the supernatant was filtered through a Millipore filter (0.45μm pore size). The resulting filtrates were autoclaved for 20 minutes at 15lb/in^2 to "stabilize" the CIA activity and stored at 4°C.

b) CIA assay.

Responder cells (*Streptococcus faecalis* FA373) were grown overnight in 2mls BHI medium and diluted the following morning (1 in 10) in fresh BHI medium. The culture was grown to early stationary phase (usually 5–6 hours) with shaking, then diluted to O.D.$_{660}$nm of 0.5 in BHI. A microtitre system was used for the quantitation of CIA. Filtrates were serially diluted two-fold into BHI medium (in sterile Eppendorf tubes) and added to Inter Med "U"-shaped plates (100μl into each well). A control using BHI medium in the place of a filtrate sample was also used. A 100μl sample of responder cells was then added to each well. The titre was read after approximately 5 hours incubation at 37°C on a shaker. The maximum dilution at which clumping was visible represented the titre of that particular filtrate and was used to express the CIA activity. The "endpoints" were reasonably well defined.

2.29 Phage assaying

a) Growing phage.

The bacterial strain upon which the phage was to be grown was cultured overnight in 2mls of BHI. The following morning, 0.1ml of this culture was added to 5mls of BHI and 10mls of phage buffer containing calcium to a final concentration of 4mM. The phage to be grown was added (usually 0.1mls of a stock solution) and the mixture incubated for 4–8 hours at 30°C, gently shaking. After lysis (the solution suddenly becomes clear) the phage was collected by centrifugation for 15 minutes (bench centrifuge, no. 10). The supernatant was filtered through a Millipore filter (0.45μm pore size) and then titred.

b) Titration.

The stock solution of phage was serially diluted 1 in 10 in phage buffer. A lawn of the bacteria upon which the phage was to be titrated was made on a BHI-agar plate containing calcium and allowed to dry for one hour. A sample (20μl) of each dilution of the phage was carefully dropped

onto an appointed position on the lawn of bacteria. Plates were incubated overnight a 30°C. The resulting plaques were counted and the titre determined, taking into account the dilution factor. The phage titre is expressed as the number of phage per millilitre of solution.

Chapter 3

The Mechanism of Conjugation

3.1 Introduction

Recent reports have demonstrated the conjugal transfer of gentamicin resistance plasmids in *Staphylococcus aureus* (Schaberg *el al,*1982; Archer and Johnson, 1983; Forbes and Schaberg, 1983; McDonnell *et al,* 1983). That the process is one of true conjugation and not transduction has been proved by Forbes and Schaberg (1983) in a number of experiments. For example, no transfer of resistance was observed when cell-free or mitomycin C-induced filtrates of donor and recipient cells were incubated and transconjugants showed no plaque-forming ability. The chelation of calcium ions by the addition of EDTA or citrate resulted in no change in the transfer frequency whereas cultures treated with chloroform showed no transfer. It is therefore probable that a chloroform resistant phage was not involved. Archer and Johnson (1983) also showed that involvement of phage was unlikely as mating proceeded at high frequency between non-lysogenic donor and recipient cells, and transfer of the gentamicin resistance conferring plasmid mobilized the transfer of as many as five additional plasmids.

Transformation was eliminated as a potential mechanism for this transfer after a crude lysate of donors failed to transfer resistance and DNAase treatment showed no effect. The DNAase treatment would eliminate transformation due to the digestion of unprotected DNA as it was transferred. Cell-to-cell contact was an absolute requirement since transfer did not occur in broth or when filters were incubated back-to-back so that donor and recipient cells were not touching (Forbes and Schaberg, 1983).

Since substantial evidence for conjugal transfer in *S.aureus* has already been provided by other workers in this field, this aspect of the work was not pursued. It was decided instead to direct the initial series of investigations towards the actual mechanism by which conjugal transfer occurs in this system. Firstly a suitable experimental transfer method had to be established. It was also important to select a suitable recipient with which to work. Once these factors had been achieved, variations on the basic method were employed in order to gain a better understanding of the mechanism of transfer.

After consultation with Dr.J.Naidoo, who had much experience in the routine conjugation of staphylococcal cells, it was decided to base the experimental method for mating *S.aureus* cells upon that of Forbes and Schaberg (1983). This method, described in detail in Section 2.19, involves the forcing together of donor and recipient cells onto a nitrocellulose membrane filter.

Finally, a second type of transfer system was analysed involving smaller plasmids and their related phages. This latter system is distinct from the newly described conjugation system of *S.aureus* and occurs at a much lower frequency (verified in the initial series of experiments).

3.2 Establishing the conjugation method

The method of conjugation used is described in detail in Section 2.19 and illustrated in Figure 3.1. It is a modification of the procedure used by Forbes and Schaberg (1983), the main difference being that donor and recipient cells were grown initially on agar plates rather than in broth. The transfer frequency is expressed as the number of transconjugants per recipient cell, which seems to be a standard method of expression in such filter mating experiments (Forbes and Schaberg, 1983).

3.2.1 Selection and analysis of recipient strains.

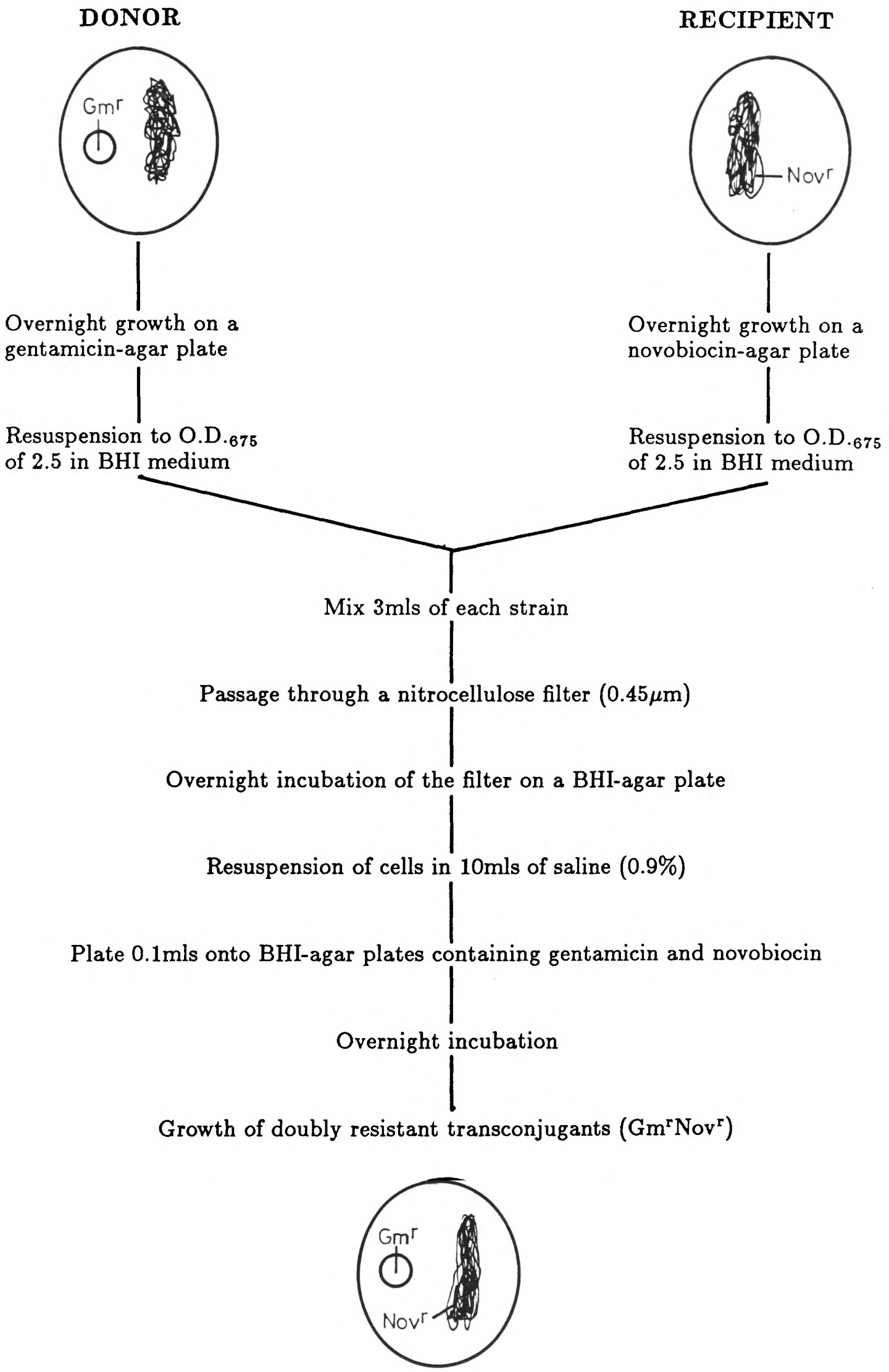
Two possible *S.aureus* strains (BIII Nov^r and 8325 Nov^r) suitable as recipients for the study of gentamicin resistance transfer were kindly donated by Dr.J.Naidoo. Both were resistant to the antibiotic novobiocin but sensitive to other antibiotics and to ethidium bromide (Table 2.3). The novobiocin resistance marker was chromosomal in both cases and was not present in any of the gentamicin resistant donor strains.

To compare the two recipients, a basic conjugation experiment was performed using the donor strains *S.hominis* and A53 (Table 2.3). Gentamicin-resistant, novobiocin-resistant transconjugants were selected.

There was found to be no significant difference in the number of transconjugants obtained between *S.hominis* and A53. However, ten times the number of transconjugants were counted when B111 Nov^r was used as the recipient compared to 8325 Nov^r. The strain B111 Nov^r was subsequently used in all experiments to characterize the conjugation system, unless otherwise indicated.

Figure 3.1

Diagrammatic representation of the method for conjugation.



3.2.2 Selection and analysis of donor strains.

The gentamicin resistant strains of *S.aureus* used are listed in Table 2.3. In order to compare their rates of plasmid transfer, bacterial matings were carried out using B111 Nov^r as the recipient. Gentamicin resistant, novobiocin resistant transconjugants were selected. Results are shown in Table 3.1 which shows that the rates of transfer ranged from 4.00×10^{-7} to 5.33×10^{-3} transconjugants per recipient cell.

With reference to Table 3.1, it can be seen that 8325-4 failed to transfer its plasmid to B111 Nov^r at all. In order therefore to confirm that the strain containing 8325-4 was actually able to transfer its plasmid by conjugation, bacterial matings were carried out using 8325 Nov^r as the recipient. Again gentamicin resistant, novobiocin resistant transconjugants were selected. Results are shown in Table 3.2. This experiment confirmed that the gentamicin-resistance plasmid p8325-4 was transferred to 8325 Nov^r during conjugation. There is therefore a variation in the frequency with which donors transfer their plasmids to different recipients.

3.3 Variation in the basic conjugation method

To gain a more detailed understanding of the precise mechanism by which staphylococci transfer gentamicin-resistance conferring plasmids, conditions in the basic conjugation method were varied. In all experiments, the strain harboring plasmid p8325-2 was used as the donor and the strain B111 Nov^r as the recipient.

3.3.1 Conjugation without filtration.

An experiment was set up to determine if transfer of gentamicin resistance occurred without the donor and recipient cells being artificially forced together on a nitrocellulose filter. High concentrations (3.75×10^9 cells) of both donor and recipient cells were mixed in BHI medium and plated directly onto selective agar plates. Cells were also mixed together, again in high concentrations, on the surface of the selective agar plates. No transconjugants were detected in either experiment even after several nights incubation at 37°C. Enforced cell-to-cell contact via membrane filtration was therefore a requirement for transfer.

Table 3.1

Comparison of the transfer frequencies from gentamicin-resistant strains with B111 Nov^r as recipient

Donor Strain	Frequency of Conjugation		
	Range	No. of Expts.	Mean
S.hominis	1.39×10^{-5}—2.67×10^{-4}	6	1.17×10^{-4}
8325-2	3.71×10^{-5}—5.33×10^{-3}	40	5.12×10^{-4}
8325-3	4.00×10^{-7}—2.37×10^{-5}	6	9.84×10^{-6}
8325-4	no conjugation	6	$< 2.67 \times 10^{-8}$
8325-5	5.33×10^{-6}—3.33×10^{-5}	6	1.80×10^{-5}
Mean Frequency	—	—	1.64×10^{-4}

Table 3.2

Comparison of the transfer frequencies from gentamicin-resistant strains with 8325 Nov^r as recipient

Donor Strain	Frequency of Conjugation		
	Range	No. of Expts.	Mean
S.hominis	2.00×10^{-5}—2.27×10^{-5}	4	2.13×10^{-5}
8325-2	7.20×10^{-7}—1.04×10^{-5}	10	4.49×10^{-6}
8325-3	1.33×10^{-6}—2.53×10^{-5}	4	1.28×10^{-5}
8325-4	2.27×10^{-6}—1.29×10^{-4}	22	3.93×10^{-5}
8325-5	1.33×10^{-6}—2.93×10^{-6}	4	2.21×10^{-6}
Mean Frequency	—	—	1.60×10^{-5}

3.3.2 Incubation time prior to filtration.

Results from the experiment described above suggest that transfer occurs only on the filter. To verify this and to determine whether any plasmid transfer occurred in solution prior to filter incubation, donor and recipient cells were incubated together in BHI medium for various times before filtration. Equal numbers of donor and recipient cells were mixed well by vortexing and left to stand at room temperature for the appropriate time. They were then filtered as normal. Two such experiments were carried out and the results for each shown in Table 3.3. Although different time courses were used in each experiment, the results are comparable. The frequency of conjugation was found not to be proportional to the incubation time prior to filtration, suggesting that little, if any, conjugation occurred in solution.

3.3.3 Filter size.

The initial experiments established the importance of the filter in the transfer process. Its involvement was therefore studied further. Firstly, the effect of varying the size of the nitrocellulose filter, by which cell-to-cell contact is normally established, was investigated. Two sizes of nitrocellulose filter (0.45μm pore size) were used:

a) 25mm diameter, 491mm area

b) 47mm diameter, 1735mm area.

The ratio of the area sizes is 1 : 3.5.

The same number of donor and recipient cells (3.75×10^9 cells) were mixed and forced through the filters. Results are given in Table 3.4. The frequency of transfer was about four times higher when the smaller filter was used. It is interesting that this corresponds to the difference in filter area. In all subsequent experiments, the smaller filter was used, as it had been in all previous experiments.

3.3.4 Duration of filter incubation.

In the standard method of conjugation (Section 2.19) the nitrocellulose filter was incubated overnight before the amount of conjugation which had occurred was determined by plating on selective media. To determine whether conjugation actually occurred on the filter whilst it was incubating, the duration of filter incubation was varied and the number of resulting transconjugants

Table 3.3

Conjugation frequencies after various incubation times prior to filtration

Length of incubation (mins)	Frequency of Conjugation	
	Experiment 1	**Experiment 2**
0	5.44×10^{-4}	1.31×10^{-3}
5	—	1.15×10^{-3}
15	—	7.55×10^{-3}
30	9.12×10^{-4}	1.08×10^{-3}
60	4.03×10^{-4}	2.40×10^{-3}
120	5.44×10^{-4}	—
180	1.44×10^{-4}	—
240	5.52×10^{-4}	—

Table 3.4

Comparison of the transfer frequencies using small and large filters

Area of filter (mm^2)	Frequency of Conjugation
491 (small)	1.22×10^{-3}
1735 (large)	3.15×10^{-4}

determined after various times. The incubation time was varied between 0 and 8 hours and results compared with overnight incubation (16 hours). The total number of cells recovered after filter incubation from each conjugation was also determined. This allowed the total number of cells recovered from the filter to be compared with the number of transconjugants recovered. The results given in Table 3.5 demonstrate that although the frequency of conjugation increased with the length of filter incubation, the total number of cells recovered varied and was not proportional to the final number of transconjugants. This leads to the speculation that any one donor cell can donate its plasmid to more than one recipient. That is, there is more than one round of conjugation in any one generation time. The following experiment was carried out to pursue this theory in greater depth.

3.3.5 Investigating the donor to recipient ratio.

In all previous experiments, the ratio of donor to recipient cells mixed together prior to filtration was 1 : 1 (using 3mls of each). Varying the ratio of donor to recipient cells, whilst keeping the total number of cells constant should indicate the number of transfer processes which occur in any one generation time. That is, to how many recipient cells any one donor will transfer its gentamicin resistance plasmid before it divides. The ratios used were 1:5, 1:2, 1:1, 2:1 and 5:1 but the total number of cells (that is, donor plus recipients) held on the filter was 7.5×10^9 in each mating. The number of cells recovered after each filter incubation is also given. The results given in Table 3.6 show that an equal ratio of donor and recipient cells results in the highest frequency of conjugation. The total number of cells recovered after filter incubation varies very little.

3.3.6 Investigating the calcium requirement.

Various reports have demonstrated that the transfer of resistance by *S.aureus* in mixed broth culture is dependent on the presence of calcium chloride (Naidoo and Noble, 1981; Lacey, 1980). Since many phages require calcium for successful infection, this suggests that transfer in mixed broth involves phage participation, as the process is abolished by addition of sodium citrate. The conjugation process, however, does not appear to require calcium ions. Forbes and Schaberg (1983) pre-treated donor and recipient cells with EDTA and tri-sodium citrate which inactivated calcium cations as a cofactor for the adsorption of phage. The presence of these chelating agents during the incubation of mating filters had no effect on resistance transfer, providing evidence against the involvement of phage in the conjugation process. McDonnell *et al* (1983) also showed that addition of calcium cations did not appreciably alter the frequency of transfer. To verify that

Table 3.5

Conjugation frequencies after varying durations of filter incubation

Duration of filter incubation (hours)	No. of cells after filter incubation	Frequency of Conjugation
0	5.95×10^{9}	$< 2.67 \times 10^{-8}$
2	6.50×10^{9}	1.07×10^{-7}
4	3.70×10^{8}	9.07×10^{-7}
6	5.75×10^{8}	6.67×10^{-6}
8	7.25×10^{8}	2.08×10^{-5}
16	1.16×10^{10}	1.65×10^{-3}

Table 3.6

Conjugation frequencies after variation in the ratio of donor to recipient cells

Ratio of donor to recipient cells	No. of cells after filter incubation	Frequency of Conjugation
1 : 5	9.75×10^{9}	1.57×10^{-4}
1 : 2	1.25×10^{10}	7.49×10^{-4}
1 : 1	1.92×10^{10}	8.32×10^{-4}
2 : 1	1.36×10^{10}	4.75×10^{-4}
5 : 1	1.57×10^{10}	1.63×10^{-4}

the conjugation system in *S.aureus*, requiring cell-to-cell contact (via filter matings) is a distinct process to the "phage-mediated process" postulated by Lacey (1980) the calcium ion requirement was investigated.

The method by which mating experiments were performed (Section 2.19) involves the addition of calcium chloride to a final concentration of 0.01M. This method was established on the basis of reports by other workers (Forbes and Schaberg, 1983; McDonnell *et al*, 1983; Dr. J. Naidoo, personal communication). To determine whether calcium ions were an absolute requirement in the conjugation process, their involvement was studied by varying the concentration of calcium chloride added to the incubation medium into which donor and recipient cells were suspended prior to filtration. The conjugation method was performed as usual and the resulting transconjugants used to determine the level of conjugation. The results given in Table 3.7 demonstrate that there is no direct correlation between the calcium ion concentration and the frequency of transfer. Indeed, transfer occurred in the absence of the added cations.

3.3.7 Addition of antibiotics to the filter incubation plates.

In all previous conjugation experiments, the filters had been incubated on BHI-agar plates overnight. In general, this seems to be the common procedure employed in the investigation of transfer via filter matings (Schaberg *et al*, 1982; Archer and Johnson, 1983; Forbes and Schaberg, 1983; Goering and Ruff, 1983). Although McDonnell *et al* (1983) reported similar transfer frequencies whether nutrient agar or brain heart infusion plates were used, to date, no report has investigated the effect of incubating the filter on selective agar plates. An experiment was therefore set up to study the effect of plating the filters on BHI-agar plates containing various antibiotics. Transfer frequencies between matings placed on the antibiotic-agar plates were compared with those on BHI-agar. All filters were placed, as usual, with the bacterial cells touching the surface of the agar.

Firstly, plates containing either one or both of the antibiotics used for the selection of transconjugants (gentamicin and novobiocin) were prepared. The concentration of antibiotic used were the same as those used in the selection of transconjugants (20 and 5μg/ml respectively). Results are given in Table 3.8.

The addition of gentamicin and novobiocin obviously influenced the frequency of transfer to a large extent. Transfer occurred most readily when gentamicin and novobiocin were added together,

Table 3.7

Conjugation frequencies after the addition of various concentrations of calcium ions

Concentration of CaCl added. (M).	Frequency of Conjugation
0	2.80×10^{-5}
0.001	6.29×10^{-5}
0.01	3.71×10^{-4}
0.05	6.61×10^{-5}
0.1	1.12×10^{-4}
0.5	2.85×10^{-5}
1.0	2.08×10^{-4}

Table 3.8

Conjugation frequencies after the addition of the antibiotics gentamicin and novobiocin to the filter incubation agar plates

Medium for filter incubation	No. of cells after filter incubation	Frequency of Conjugation
BHI-agar only	1.02×10^{10}	4.59×10^{-4}
BHI-agar + gentamicin	1.49×10^{10}	1.87×10^{-6}
BHI-agar + novobiocin	1.28×10^{10}	5.33×10^{-5}
BHI-agar + gentamicin and novobiocin	5.35×10^{9}	$> 2.67 \times 10^{-3}$

Table 3.9

Conjugation frequencies after the addition of chloramphenicol to the filter incubation plates

Medium upon which filter was incubated.	Frequency of Conjugation
BHI-agar only	6.67×10^{-4}
BHI-agar + chloramphenicol	2.13×10^{-3}
BHI-agar + gentamicin	2.19×10^{-5}
BHI-agar + novobiocin	2.69×10^{-4}
BHI-agar + gentamicin and novobiocin	2.40×10^{-3}

Table 3.10

Conjugation frequencies after filter incubation on de-ionised water-agar plates supplemented with antibiotics

Medium for filter incubation	No. of cells after filter incubation	Frequency of Conjugation
BHI-agar only	9.00×10^{9}	2.93×10^{-5}
Water-agar only	1.70×10^{9}	1.33×10^{-6}
Water-agar + chloramphenicol	1.70×10^{9}	3.73×10^{-6}
Water-agar + gentamicin	1.05×10^{9}	$< 2.67 \times 10^{-8}$
Water-agar + novobiocin	1.55×10^{9}	$< 2.67 \times 10^{-8}$
Water-agar + gentamicin and novobiocin	1.35×10^{9}	$< 2.67 \times 10^{-8}$

but was reduced when either was added alone. It was possible therefore, that growth on the filters was affecting the rate of transfer in some way. The investigation was pursued further by the use of BHI-agar plates containing the antibiotic chloramphenicol at a concentration of 5 μg/ml. Chloramphenicol inhibits protein synthesis by its action upon bacterial ribosomes. Plating filters on chloramphenicol should therefore eliminate growth. The results in Table 3.9 show that the frequency of conjugation was indeed increased when chloramphenicol was added to the plates. In fact, transfer occurred at a similar rate to when gentamicin and novobiocin were added together. It is possible therefore, that growth inhibits transfer in some way. To verify this theory, agar plates were made up using only deionised water and agar (no BHI) and supplemented with the antibiotics as before. This ensured that there were no traces of contaminating elements or compounds which were interfering with the conjugation process in any way and that there were no nutrients available for growth. Filters were placed, as before, on the surface of these plates and incubated overnight. Results are given in Table 3.10. Transfer was higher when the filter was placed on BHI-agar compared to de-ionised water-agar platings and failed to occur at all when gentamicin and/or novobiocin were added. The addition of chloramphenicol to water-agar plates did increase the transfer frequency slightly compared with that using water-agar plates only. This is contrary to the theory that growth inhibits transfer but suggests that chloramphenicol affects the process in another way. These results are discussed in more detail in Section 3.5.2.

3.3.8 Orientation of the filter on the agar surface.

In all previous experiments, the nitrocellulose filter had been placed, on the BHI-agar plate with the bacterial cells in direct contact with the agar surface, allowing the direct absorption of nutrients. An experiment was carried out to determine if transfer occurred when filters were placed with the bacteria uppermost (not therefore, in contact with the agar surface). This reduces the availability of nutrients. The results given in Table 3.11 show that transfer was 40 times higher when growth was allowed to take place. Again, this is contrary to the growth inhibition theory.

Table 3.11

Conjugation frequencies with filters incubated in opposite orientations

Orientation of filter	No. of cells after filter incubation	Frequency of Conjugation
Bacteria-side-down	1.13×10^{10}	1.02×10^{-3}
Bacteria-side-up	1.03×10^{10}	2.67×10^{-5}

3.4 The involvement of bacteriophage in transfer processes

The involvement of phage in plasmid transfer in *S.aureus* was studied to gain a better understanding of the process and to detect differences between this type of transfer and that of true conjugation. The plasmids used in this study are listed in Table 2.3, but are presented in more detail in Table 3.12.

3.4.1 Basic transfer involving phage.

A conjugation experiment was set up to compare the frequencies of conjugation between strains containing the small plasmids pCW59, pCW3, pCW7 and pC194. Before the conjugations were performed, all the small plasmids were transformed into the *S.aureus* strain 8325N using the protoplast regeneration method (Section 2.26). Accurate comparisons between the rates of transfer could then be made. To examine whether the host donor strain in which the small plasmid resided had any effect on the transfer frequency a conjugation was also performed using the original strain in which pCW59 was obtained, that is, RN4220[pCW59]. The conjugation experiment was performed in the standard way and the number of resulting transconjugants taken as a measure of the frequency of conjugation. The two strains, B111 Nov^r and 8325 Nov^r, were used as recipients. Results are shown in Table 3.13 and are discussed in detail in Section 3.5.3. The antibiotic resistance markers carried by the small plasmids were transferred to 8325 Nov^r in all cases. The frequency of transfer however was considerably lower (up to 2000 times) than that of the transfer of gentamicin resistance plasmid, p8325-4.

3.4.2 Transfer with a high copy number mutant.

The effect of increased copy number was studied using plasmid pT181 (copy number of about 20 per cell) compared with its deletion mutant, *cop-608* (copy number of 800–1000 per cell). The increased copy number is due to a deletion of 179 base pairs (Khan *et al*, 1981). The strains are described in Table 2.3 and results are given in Table 3.13.

3.4.3 Investigation of the involvement of relaxation complexes.

The involvement of relaxation complexes was then investigated using plasmids pC221, pCW41, pT181, pC194 and pCW59. Plasmid pC221 is a chloramphenicol resistant isolate from *S.aureus* which has been reported to form unstable relaxation complexes. That is, the DNA is covalently

Table 3.12 **Description of plasmids**

Plasmid Name	Size (kbp)	Phenotype	Description	Reference
pC194	2.9	Cm	R plasmid from *S.aureus,* incompatibility group 8. Replicates in *Bacillus subtilis* and *Escherichia coli.* Specifies chloramphenicol-induced resistance to chloramphenicol, mediated by the enzyme chloraanphenicol acetyl transferase. Its complete nucleotide sequence is known.	Horinouchi and Weisblum (1982a)
pCW3	4.5	Tc	Isolated from *S.aureus* JB48827.	Wilson and Baldwin (1978)
pCW7	4.2	Cm	Isolated from *S.aureus* JB48827.	Wilson and Baldwin (1978)
pCW59	5.3	Cm Tc	*Hin*d III fragment A from pCW3 inserted into the *Hin*d III site of pCW7; spontaneous deletion of 1.2kbp of pCW7 DNA.	Wilson *et al* (1981)
pC221	4.4	Cm	Natural isolate of *S.aureus.*	Novick and Bouanchaud (1971)
pCW41	1.8	Cm	*Mbo*I fragment A from pC221	Wilson *et al,* (1981)
pT181	4.5	Tc	Naturally occuring *S.aureus* plasmid encoding inducible resistance to tetracycline. Has a copy number of about 20 per cell and belongs to the incompatibility group *inc* 3. The complete nucleotide sequence is known. (Khan and Novick, 1983).	Kahn *et al* (1981)
pT181 *cop-608*	4.3	Tc	A mutant of pT181 with an increased copy number (800–1000 per cell). This phenotype is due to a deletion of 179 base pairs.	Kahn *et al* (1981)
pCE10	5.3	Cm Em	Chimeric plasmid from a *Hin*d III digestion of pE12 and pCT20, the latter being derived from pC194.	Keller *et al* (1983)

Table 3.13

Table showing the transfer frequencies of small plasmids

Plasmid residing in 8325N	Selection antibiotics	Transfer frequency to: 8325 Nov^r	B111 Nov^r	"Background growth"
p8325-4	Gm + Nov	3.93×10^{-5}	$< 2.67 \times 10^{-8}$	none
pCW59	Tc + Nov	1.06×10^{-6}	$< 2.67 \times 10^{-8}$	none
pCW59	Cm + Nov	1.13×10^{-6}	$< 2.67 \times 10^{-8}$	some
pCW3	Tc + Nov	3.29×10^{-6}	$< 2.67 \times 10^{-8}$	none
pCW7	Cm + Nov	6.00×10^{-7}	$< 2.67 \times 10^{-8}$	much
pC194	Cm + Nov	$< 2.67 \times 10^{-8}$	$< 2.67 \times 10^{-8}$	much
pT181	Tc + Nov	1.80×10^{-6}	$< 2.67 \times 10^{-8}$	none
pT181*cop-608*	Tc + Nov	4.32×10^{-5}	$< 2.67 \times 10^{-8}$	none
RN4220[pCW59]	Tc + Nov	6.86×10^{-5}	$< 2.67 \times 10^{-8}$	none
pCE10	Cm + Nov	3.30×10^{-6}	$< 2.67 \times 10^{-8}$	some

closed but is nicked by a disturbance of the complex possibly due to bound protein (Novick, 1976). There is considerable variability between plasmids with respect to their ability to form these complexes. Plasmids pC194 and pT181, for example, are described as non-relaxable. Plasmid pCW41 consists of the *Mbo*I fragment A (1.8 kbp) from pC221. It is also non-relaxable (Wilson *et al*, 1981). The five small plasmids (pC221, pCW41, pCW59, pC194 and pT181) were conjugated with 8325 Nov^r and the frequency of transfer determined by the number of transconjugants produced. Results are shown in Table 3.14 which also indicates their ability to form a complex. All plasmids had been transformed into 8325N before conjugation so that an accurate comparison could be made.

3.4.4 Isolation of "lytic activity" from a conjugation plate.

After the conjugation between pC221 and 8325 Nov^r had been performed and the plates incubated overnight, it was observed that there were some plaques (absence of bacterial growth) on the conjugation plates. These were observed within the "background growth" (a faint lawn of bacterial growth covering the plate through which distinct transconjugant colonies could be seen). Initially it was thought that these plaques were due to the presence of phage, participating in some type of low frequency "phage-mediated conjugation" as postulated by Lacey (1980). To analyse the role of these phage, they were isolated from the selective agar plates by the method in Section 2.29 and then titered on 8325 Nov^r. A 20μl suspension of either undiluted or a 1 in 10 dilution of phage produced complete clearing. Further dilutions (1 in 10^2 to 1 in 10^8), however, resulted in no lysis of the bacteria. Although phage may have been present in the stock solution, the titre could not be calculated. The ability of this phage (originally isolated from 8325 Nov^r[pC221] transconjugant plates) to infect and lyse various plasmid strains and their corresponding transconjugants (with 8325 Nov^r) was investigated. The procedure is the same as that used to titre the phage except that several bacterial host strains were used (Section 2.29). The results given in Table 3.15 show, in general, either complete lysis or the absence of lysis at all. The titre could only be calculated in two examples. This leads to the speculation that the plaques may not have been due to the presence of phage but to a type of staphylococcin which was lysing the cells. This is discussed in more detail in Section 3.5.3.

Table 3.14

The effect of relaxability on the frequency of transfer

Plasmid	Ability to form a relaxation complex	Frequency of Conjugation
pC221	yes	1.20×10^{-7}
pCW41	no	$< 2.67 \times 10^{-8}$
pC194	no	$< 2.67 \times 10^{-8}$
pT181	no	1.74×10^{-6}
pCW7	no	6.00×10^{-7}

Table 3.15

The ability of "phage" isolated from 8325 Nov^r[pC221] transconjugant plates to lyse various plasmid strains and their corresponding transconjugants

Test strain	Dilution of phage used for infection 10^{-1}	10^{-2}	10^{-3}	10^{-4}
8325N	√	√	×	×
8325 Nov^r	√	√	3.5×10^5	×
8325 [pC221]	×	×	×	×
8325 Nov^r [pC221]	×	×	×	×
8325 [pCW59]	√	√	√	×
8325 Nov^r [pCW59]	√	√	√	×
8325 [pCW41]	√	√	×	×
8325 Nov^r [pCW41]	√	√	√	5×10^6
8325 [pT181]	√	√	√	×
8325 Nov^r [pT181]	√	√	√	×

√ indicates complete lysis

× indicates no lysis

3.5 Discussion

3.5.1 Conjugation between various strains of *S.aureus*.

The procedure employed by Forbes and Schaberg (1983) was established as being suitable for the analysis of the conjugation system in *S.aureus*. The method involves the forcing of bacterial donor and recipient cells onto a nitrocellulose membrane filter followed by incubation of the filter on BHI-agar plates and then resuspension of the resulting growth the following day into saline solution. The number of transconjugants found in this solution was taken as a measure of the conjugation frequency between the two strains. Although it was decided that this was the most suitable method for analysis of the conjugation system, various problems and inaccuracies were encountered.

Firstly, it often proved difficult to force all the medium in which donor and recipient cells were suspended through the filter. Presumably the high concentration of mating cells required in the method blocked the pores in the filter. It was not possible in every mating experiment therefore, to force all the mixture onto the membrane. This reduced the number of bacteria available for conjugation on the filter in certain cases. Another limiting factor is that when the filter was removed from the agar plate and added to saline solution, some bacterial cells remained on the surface of the agar plate. Although as much as possible was removed, varying amounts remained in each mating.

Although the conjugation method used throughout the investigation does result in a number of inaccuracies, it is suitably effective for the comparison of conjugation between various strains of staphylococci and for the analysis of the conditions under which conjugation takes place. The conjugation frequencies (expressed as the number of transconjugants per recipient cell) were in general agreement with those of other workers (Schaberg *et al,* 1982; Forbes and Schaberg, 1983; McDonnell *et al*, 1983). The mean frequencies of conjugation for each of the five gentamicin-resistant strains studied in this survey are given in Tables 3.1 (with recipient B111 Nov^r) and 3.2 (8325 Nov^r) together with the range in frequency and the number of experiments carried out for each particular strain. The highest mean frequency ever obtained was 5.12×10^{-4} transconjugants per recipient and the lowest was 2.21×10^{-6} transconjugants per recipient in the initial series of basic conjugations.

When B111 Nov^r was used as a recipient (Table 3.1) the mean frequency of conjugation

varied between 5.12×10^{-4} and 9.84×10^{-6} transconjugants per recipient (for 8325-2 and 8325-3 respectively). There was a complete absence of transconjugants when 8325-4 was mated with B111 Nov^r. The frequency of conjugation is therefore given as less than 2.67×10^{-8} transconjugants per recipient which would be the frequency of transfer if only one transconjugant had been found.

When 8325 Nov^r was used as the recipient (Table 3.2) the mean frequency of conjugation varied between 3.93×10^{-5} and 2.21×10^{-6} transconjugants per recipient cell (for 8325-4 and 8325-5 respectively).

In general, B111 Nov^r is a better recipient than 8325 Nov^r (mean conjugation frequencies of 1.64×10^{-4} and 1.60×10^{-5} respectively).

It is interesting to note that donors transfer at different rates to different recipients. For example, p8325-2 is transferred to B111 Nov^r at high frequency (5.12×10^{-4}) but at a much lower frequency to 8325 Nov^r (4.49×10^{-6}).

The rate of transfer to B111 Nov^r (in decreasing order) is :

8325-2 > *S. hominis* >8325-5 > 8325-3 > 8325-4

and to 8325 Nov^r :

8325-4 > *S.hominis* >8325-3 > 8325-2 > 8325-5

Although therefore p8325-2 is transferred more efficiently than any other plasmid to B111 Nov^r, it is one of the plasmids transferred least effectively to 8325 Nov^r. The reason for this can only be speculated upon until the precise mechanism by which transfer occurs is known. No sex pili have ever been observed in *S.aureus* and it is therefore possible that the mating process involves some type of transient protoplast fusion event. It is possible that the gentamicin-resistant donor produces a membrane degrading enzyme which acts on the recipient cell wall. If different donors produced slightly different enzymes, these could act with varying degrees of effectiveness upon the various recipients. Recipient cells may vary in their resistance to enzymatic attack, some may even be totally resistant and hence not conjugate with donors which secrete that particular enzyme as in the case, for example, of 8325-4 and B111 Nov^r.

It is possible that the non-transferability of p8325-4 to B111 Nov^r is not at the level of entry into the cell but is due to the failure of the donor DNA to replicate and express its gentamicin resistance once it has entered the cell. For example, a restriction system may exist in the recipient which degrades the donor DNA. Certain donor plasmids may be resistant to attack, due perhaps to a modification type of system. Others may be susceptible due to the lack of such a system.

The mechanism of conjugation in *S.aureus* must be analysed in more detail before the variation in conjugation frequencies between various strains can be understood.

3.5.2 The mechanism of conjugation.

It was found (Section 3.3.1) that membrane filtration was an important requirement for gentamicin resistant plasmid transfer in the strain studied. No transconjugants were detected when cells were mixed in broth or on the surface of an agar plate. Also, increasing the length of incubation in broth did not result in any significant increase in the frequency of transfer. As can be seen from Table 3.3, the frequency of conjugation was virtually unchanged after an incubation of 240 minutes (5.52×10^{-4}) compared to that following no incubation (5.44×10^{-4}). Conjugation does not, therefore, appear to occur to any significant extent in suspension. This suggests that direct cell-to-cell contact is needed and the process is one of conjugation and not transduction or transformation. Further evidence for this is exhibited by the use of small and large filters (Table 3.4). The frequency of conjugation was four times higher when small filters were used compared with large filters whilst the ratio of small filter surface area to large filter surface area is 1 to 3.5. This suggests that the small filters increase the rate of transfer by bringing the donor and recipient cells into closer proximity, increasing the probability of cells meeting and forming a mating pair. The extent to which cells are brought into such close proximity in nature must be limited. However, Naidoo and Noble (1978a) report the increase in the transfer frequency of gentamicin resistance after inoculation onto human skin compared with the transfer in laboratory culture media.

The results in Table 3.5 show the effect of varying the filter incubation time. The figures in the second column represent the number of cells resuspended in saline solution after filter incubation. These figures do not increase linearly with time. The total number of cells recovered from the filter therefore, does not correspond to the number of transconjugants detected. Indeed, multiplication and growth may not even be a requirement for transfer as the frequency of conjugation was higher after a filter incubation of 8 hours (2.08×10^{-5}) when 7.25×10^8 cells were resuspended than after 2 hours (1.07×10^{-7}) when 6.50×10^9 cells were resuspended. It should be noted that the total

number of cells mixed prior to filtration was 7.5×10^9 in each experiment. On this basis there appears to have been no growth during filter incubation at all. However, as discussed in Section 3.5.1, some of the bacteria never reach the filter and some remain on the filter incubation plates after the filter has been shaken in saline solution.

Filter incubation itself is essential for conjugation as no transfer was detected when the filter was not incubated (Table 3.5). As growth seems to play a small, if any, part in the process, the role of the filter is probably in providing a suitable surface on which the mating cells can come into close contact and actually transfer their plasmids. The frequency of transfer increases exponentially with time, supporting this theory. It is interesting to speculate upon the number of rounds of transfer which occur. That is, whether a donor cell replicates and donates its plasmid to more than one recipient. After overnight filter incubation, conjugation using 3.75×10^9 donor cells resulted in the production of a total of 6.20×10^6 transconjugants resuspended from the filter. This suggests that only about two-thirds of the available donors have transferred their plasmid or that even fewer donors have conjugated, each a number of times. Evidence for only one round of transfer is provided by the results obtained from varying the donor to recipient ratio (Table 3.6). Transfer frequencies show that an equal number of donor and recipient cells favours conjugation. Divergence from the equal ratio decreases the frequency of transfer; the greater the divergence, the lower the frequency of conjugation. The interpretation that in any one generation time, a donor cell donates its plasmid to only one recipient seems plausible. The total number of cells resuspended after filter incubation was similar for each of the donor to recipient ratios. It is therefore probable that donor and recipient cells grow and divide on the filter to the same extent.

The effect of varying the calcium ion concentration was studied to demonstrate that transduction (which has a requirement for such ions) was not the method by which the gentamicin resistance plasmids were being transferred. The results shown in Table 3.7 indicate that transfer via filter matings occurred at a reasonable rate (2.80×10^{-5}) when calcium ions were not added to the incubation medium. The frequency of transfer did not increase in proportion to the increase in calcium ion concentration but fluctuated as the concentration was raised. This suggests that calcium ions play little, if any, role in the conjugation process although their presence was not totally eliminated in any sample due to the minute traces which are probably present in the BHI medium itself.

The study of the mechanism of conjugation was concluded by investigating the involvement of growth and division on the filters. Results from the study into the effect of increasing the duration

of filter incubation (Section 3.3.4, Table 3.5) suggested that there was little, if any growth on the filter. However, due to the limitations of the conjugation method, in that particular experiment it was difficult to determine whether growth had actually occurred. Further work was carried out by experimentally varying the filter incubation plates, firstly by the addition of various antibiotics. To date, no report has discussed the use of selective filter incubation in the mating process.

Initially, BHI-agar plates containing gentamicin and novobiocin together and separately, were used. There was a significant increase in the frequency of transfer when filters were placed on double selection plates (gentamicin plus novobiocin) compared with normal BHI-agar plates with no additions (Table 3.8). The frequency of conjugation after filter plating on double selection plates was extremely high indeed ($> 2.67 \times 10^{-3}$). These results could be interpreted by postulating that selection for early transconjugants occurs and such cells divide and produce further doubly resistant progeny without competition from donor or recipient cells. Plating on singly selective plates however (gentamicin or novobiocin) resulted in a decrease in the transfer frequency. A possible explanation for this is that growth of either donor or recipient is inhibited, leading to an unequal ratio of donor to recipient cells and hence a lower transfer rate. This is in agreement with the results obtained after varying the donor to recipient ratios (Section 3.3.5). Again, the final number of cells resuspended in saline from the filter appears to have little effect on the final number of transconjugants produced. There were fewer bacterial cells in the final saline suspension for the sample which was placed on double selective plates (5.35×10^{9}) than when the filter was placed on either gentamicin (1.49×10^{10}) or novobiocin (1.28×10^{10}) alone. Although there were more cells in the latter two samples, there were far fewer transconjugants.

The role of growth in the process was studied further by plating filters onto BHI-agar plates containing chloramphenicol. This antibiotic prevents cell growth by inhibiting protein synthesis. Reference to Table 3.9 shows that the chloramphenicol increased the transfer frequency compared to the BHI-agar control (2.13×10^{-3} and 6.67×10^{-4} respectively) and occurred at a similar rate to that obtained when gentamicin and novobiocin were added together (2.40×10^{-3}). The chloramphenicol prevents all cells from growing, including the transconjugants, therefore the increase in transfer frequency may not be due to a selection for early transconjugants. It is possible that cell growth actually inhibits conjugal transfer and that chloramphenicol, by preventing cell growth, increases the transfer frequency. To study this further, filter incubation plates were made using de-ionised water and agar with added antibiotics as before. Such plates provided absolutely no nutrients for growth. Transfer was compared with filter incubation on BHI-agar plates. By reading the optical density after resuspension in saline, it was confirmed that growth had been prevented in all samples

except for that on BHI-agar (Table 3.10). On average there were about six times as many cells after incubation on BHI-agar plates (9.0×10^9) compared with incubation on de-ionised water-agar plates (1.7×10^9). By comparing these figures obtained with those from Table 3.6 it is probable that a certain amount of growth normally takes place on the filters.

Considering again Table 3.10, it can be seen that transfer was considerably higher when BHI-agar plates were used compared with water-agar plates. Conjugal transfer did occur to a limited extent however, in the latter case. This leads to a number of possibilities. Although not an absolute requirement for conjugation, a certain amount of cell growth may increase the transfer frequency. For example, although perhaps the cells are not able to move once on the filter, if a donor cell is within close proximity to a recipient, by dividing a few times it may be able to touch the recipient, form a contact and transfer its plasmid. The probability of this occurring may be relatively small compared to the overall transfer events which occur when cells are in direct contact initially on the filter. It is possible that the water-agar plates provide a surface on which the osmotic pressure differs from that of the BHI-agar plates. This may provide protection for the conjugating cells in some way, especially if the event involves some type of transient protoplast formation. The results in Table 3.11 lead further support to the fact that allowing growth to occur on the filters does not inhibit transfer. Plating the filter bacteria-side-down on the agar surface (allowing growth) gave a higher frequency of transfer than when the cells were uppermost (no nutrients available). It appears from Table 3.11 that similar levels of growth took place in both platings as the number of cells recovered after filter incubation is similar (1.13×10^{10} and 1.03×10^{10} cells respectively). Many cells were not recovered from the surface of the agar however when the filter was placed bacteria-side-down whereas most were resuspended when the filter was placed bacteria-side-up. Considerably more growth did, therefore, take place when the filter was placed bacteria-side-down than was accounted for from the optical density reading.

Conjugation was increased about three times by the addition of chloramphenicol to water-agar plates compared with water-agar plates alone (Table 3.10). Addition of gentamicin plus novobiocin, to such plates however, did not result in any transfer. It is probable therefore that growth on the filter is required for gentamicin plus novobiocin to increase the transfer frequency. This may be because the selection of early transconjugants by doubly selective plates only increases the final number of transconjugants if there is subsequent growth and division of such cells. In the absence of growth, the selection of transconjugants has no effect. The increase in transfer frequency due to addition of chloramphenicol, does not appear to require growth. The effect of this antibiotic may therefore, be due to a completely different phenomenon. For example, chloramphenicol is known

to increase the rate of cell wall synthesis (Mandelstam and Rogers, 1958) and this may explain its ability to increase transfer. Although the mechanism of the conjugation process in *S.aureus* is not yet established, it is possible that some type of transient protoplast formation takes place. The incorporation of amino acids into the staphylococcal cell wall is chloramphenicol resistant. Therefore, by preferentially utilizing the cell's resources of aminoacids, addition of chloramphenicol may increase the rate of cell wall synthesis after transfer has occurred. This, in conjunction with the selection of early transconjugants on doubly selective plates (gentamicin plus novobiocin) and the favourable effect of growth, could explain the observed results. Further experiments must be performed before these speculations can be proved or disproved. A variety of filter incubation plates could be made containing inhibitors of protein synthesis (for example erythromycin or puromycin). Cell wall synthesis could also be investigated using amino acid starvation. The role of osmotic lysis could be studied using agar plates with a range of osmotic potentials.

3.5.3 The involvement of bacteriophage in transfer in *S.aureus*.

The frequencies of plasmid transfer between strains containing small plasmids was compared with the frequency of transfer of the gentamicin resistance plasmid from 8325-4. This strain was chosen due to its ability to conjugate with 8325 Nov^r but, like the strains containing smaller plasmids, it failed to transfer to B111 Nov^r. It can be seen from Table 3.13 that the frequency of transfer for the 8325-4 plasmid is higher than that of all the smaller plasmids except pT181 *cop-608*. This shows that strains harboring small, non-gentamicin resistance plasmids, are able to transfer to a recipient via a type of "conjugal-mating". The inability of the small plasmids to transfer to the recipient BIII Nov^r lends support to the theory that phage is involved and that this type of transfer only occurs between strains of certain phage typing patterns. The process is distinct from that of conjugation described above however and occurs at a much lower rate. It is possible that it is a type of "phage-mediated conjugation," similar to that described by Lacey (1980).

To determine if this "low frequency transfer" system was indeed due to the presence of phage, it was decided to isolate them from plaques observed in the "background growth" on the selection plates of 8325 Nov^r[pC221] transconjugants. This background growth was often observed when chloramphenicol was used as one of the selective antibiotics. The reason for this faint background lawn of growth is unclear. Approximately the same number of doubly resistant colonies were observed whether selection occurred on chloramphenicol plus novobiocin or tetracycline plus novobiocin plates (pCW59 transfer, Table 3.13). This suggests that the type of selection used does not

affect the observed result, doubly resistant strains being clearly visible as distinct colonies through the faint background "lawn" of growth.

After the "phage" had been isolated by growth on 8325 Nov^r, it was found that a titre (the number of phage per millilitre of solution) could not be calculated: the phage either caused complete lysis or no lysis at all. Some factor had been isolated however which was causing lysis. It was possible that it was not phage but some type of extracellular bacteriolytic enzyme.

The investigation was pursued by testing the ability of this "lytic activity factor" to lyse other strains. The results in Table 3.15 demonstrate that all strains except the donor strain containing pC221 and the transconjugant 8325 Nov^r[pC221] were lysed. It is possible therefore that phage had indeed been isolated and that this phage was lysogenic for 8325[pC221], that is, it was either integrated into and replicated in association with the host's chromosome or it existed as an autonomously replicating circular piece of DNA. Its lytic (lethal) functions would have been repressed by specific phage-encoded regulatory elements. This blockage of the lytic cycle also imparts immunity to the lysogen against superinfection by the same phage (or phage of the same immunity type). The isolated phage was unable therefore to superinfect 8325[pC221]. That the phage was able to infect and lyse 8325N and 8325 Nov^r but not the 8325 Nov^r[pC221] transconjugant, may have been because the phage was associated with the plasmid pC221 and not with the strain 8325N and that it was not present in 8325 Nov^r but was transferred together with pC221 from the donor strain.

The ability of the phage associated with pC221 to lyse strains containing other plasmids (pT181, pCW59, pCW41) suggests that such plasmids have phages of a different immunity type associated with them (they were all in the strain 8325N) and with their transfer. The absence of transfer to B111 Nov^r may be due to the presence of prophage within the chromosome sharing the same immunity type as the "donor phages".

Alternatively, an extracellular bacteriolytic enzyme may have been isolated. It is well known that strains of *S.aureus* release a large number of proteins into their environment when grown *in vitro* or *in vivo* (see Easmon and Adlam, 1983). It is possible that the donor strain containing plasmid pC221 produced a specific enzyme involved in the degradation of cell wall material. It was not lysed itself by this activity but, like the transconjugant 8325 Nov^r[pC221] was resistant to attack. As the strain 8325 Nov^r was susceptible to attack, it is possible that resistance was transferred with the plasmid. The plasmid involved (pC221) is extremely small however (4.4 kbp)

so this explanation seems unlikely. As most of the strains tested were susceptible to the activity at high concentrations but resistant at lower ones, the enzymatic activity must have been diluted in the same manner as phage.

That the lysis was due to phage seems the more plausible explanation. To confirm their involvement, a number of studies could be carried out. Firstly, a sample of the solution containing the activity could be dried down, stained appropriately and examined under the electron microscope. If phage were present, they should be seen. Secondly, the size of the agent involved could be determined. Filter systems would separate phage and protein on this criteria. Finally the two could probably be distinguished immunologically using samples of all known staphylococcal phage antisera.

With reference to Table 3.15, it can be seen that it was only possible to calculate the amount of lytic activity in two of the titrations. This was 3.5×10^5 "activity units" per ml for 8325 Nov^r and 5.0×10^6 per ml for 8325 Nov^r[pCW41]. Comparisons between strains cannot therefore be drawn. All other samples showed either complete lysis or no lysis at all.

The plasmid pT181*cop-608* (a high copy number mutant of pT181) gave a transfer frequency 25 times that of pT181. The mutant contains 800-1000 copies of the plasmid, that is, about 40 times the number of DNA molecules to that of pT181. This is comparable with the increase in transfer frequency suggesting that the more plasmid DNA molecules there are in the cell, the more transconjugants are produced possibly due to the transfer of a number of plasmid molecules from one donor to a number of recipients.

Many of the plasmids found in enteric organisms are isolated as site-specific protein-DNA relaxation complexes in which attempts to remove the bound protein *in vitro* usually convert the supercoiled plasmid DNA to a relaxed form by the introduction of a scission in one strand at the site of the bound protein. Novick (1976) reported protein-DNA relaxation complexes for six *Staphylococcus aureus* strains. Plasmid pC221 is described as having a very unstable complex (present as approximately 40% supercoiled and 60% open circular molecules) whereas pC194 and pT181 were nonrelaxable (present as essentially 100% supercoils). Plasmids pCW41 and pCW7 are also nonrelaxable (Wilson *et al,* 1981). Novick has also reported that pC221 was transferred 10^3 times as effectively as pT181.

Plasmids pT181 and pCW7 which form no relaxation complex were transferred at a higher rate than pC221 which forms an unstable complex. The reason for the difference in these results

compared with those of Novick could be due to the host strain in which the plasmids reside. Before conjugation was performed, all the small plasmids, including pC221 and pT181, were transformed into strain 8325N. This transformation process may have eliminated phage in some way. In connection with this, is the finding that the host strain in which the plasmid resides does indeed influence the frequency of that plasmid transfer (Table 3.13). The transfer frequency of RN4220[pCW59] was 65 times higher than 8325[pCW59]. The plasmid pCW59 had been transformed from RN4220 to 8325N. The transformation process itself may have altered the phage content (or other lytic activity) in some way (probably a reduction) resulting in a lower frequency of subsequent transfer.

3.6 Conclusion

A procedure has been established for analysing the mechanism of conjugation in *S.aureus*. This method, employing filtration onto nitrocellulose filters, allowed comparisons to be made between the conjugation frequencies of various *S.aureus* strains. Frequencies of conjugation, expressed as the number of transconjugants per donor cell, varied between 5.33×10^{-3} and 4.00×10^{-7} (Table 3.1). In general, frequencies were of the same order of magnitude as those reported by other workers, but were found to vary between donor and recipient strains.

By varying certain factors in the basic method of conjugation, the actual mechanism involved was analysed. It was concluded that:

1) Membrane filtration (and therefore cell-to-cell contact) was an absolute requirement.
2) A ratio of 1 : 1 donor to recipient cell favoured conjugation which may suggest that there is only one round of transfer in any one generation time.
3) There was no absolute calcium requirement although addition of such cations to a final concentration of 0.01M favoured transfer.
4) Addition of the antibiotics gentamicin and novobiocin together in the transconjugant plates, greatly increased number of transconjugants. Either antibiotic added alone however, reduced transfer.
5) Addition of chloramphenicol raised the level of transfer possibly by increasing the rate of cell wall synthesis following some type of protoplast formation.
6) Growth on the filters favours conjugation.

A second transfer process, distinct from that of conjugation, may occur in *S.aureus*. This process possibly involves phage and occurs at a much reduced frequency compared to the high frequency transfer observed associated with gentamicin resistance plasmids.

Chapter 4

Construction of Restriction Enzyme Maps and Analysis of Transfer Functions by Deletion for Two Conjugative Plasmids

4.1 Introduction

To characterize the conjugation system of *S.aureus* in more detail it was decided to investigate the genes involved in the process. Initially this involved the identification of the approximate areas of the gentamicin resistance conferring plasmid DNA that affected the frequency of transfer. Subsequent analysis would involve the more detailed study of the genes and other controlling elements in these areas.

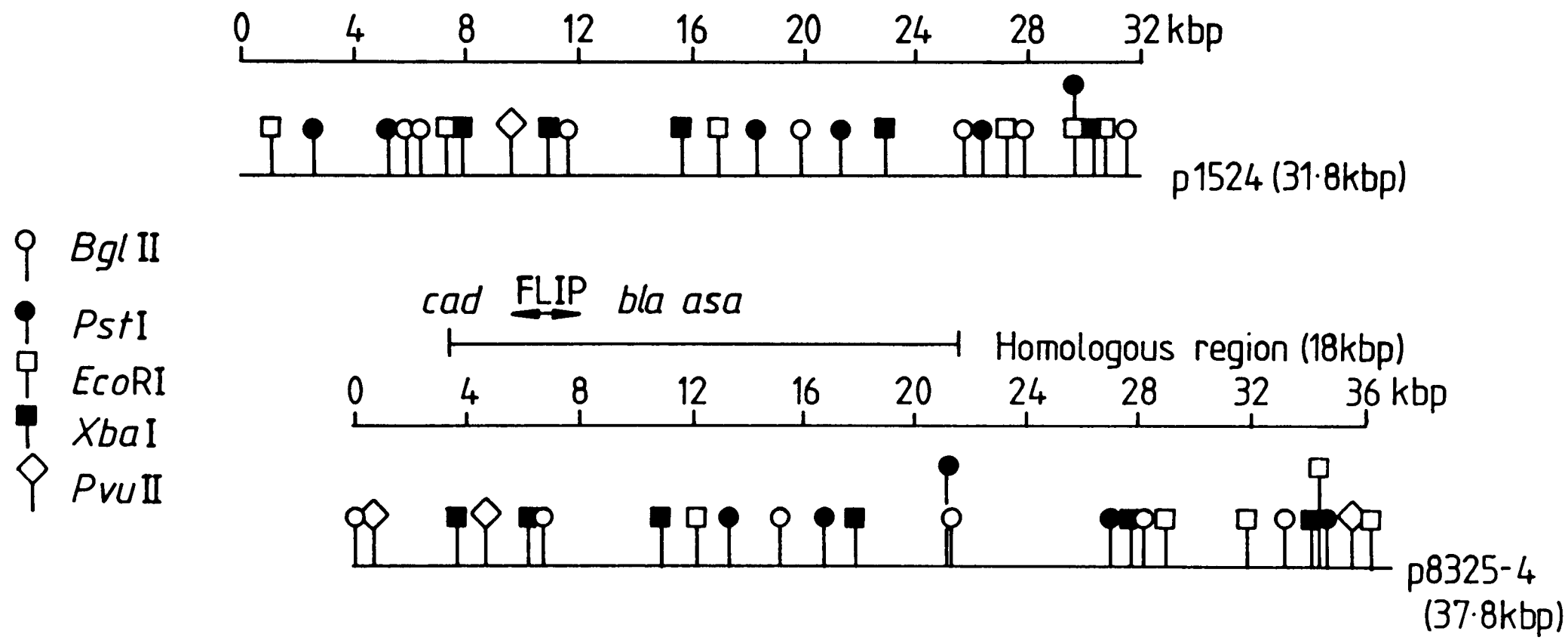
It was decided to concentrate this analysis on two particular gentamicin resistance conferring plasmids: p8325-2 (50 kbp) and p8325-4 (38 kbp) since some preliminary mapping data was available (Curnock and Dyke, unpublished). The approximate map positions of the restriction enzymes *Bam* HI, *Bcl* I, *Bgl* II, *Eco* RI, *Pvu* II and *Xba* I were known for p8325-2 and approximate positions of *Bgl* II, *Eco* RI, *Pst* I, *Pvu* II, *Sal* I and *Xba* I were known for p8325-4. In addition, Curnock and Dyke (unpublished data) had shown that a section of about 18kbp of the map of plasmid p8325-4 very closely resembled a part of the plasmid pI524 (Figure 4.1). It is possible therefore that p8325-4 is a recombinant of part or all of a gentamicin resistance conferring plasmid with a substantial part related to pI524.

It is known that the 18kbp piece of pI524 specifies β-lactamase, resistance to cadmium, arsenate and arsenite and includes the putative origin of replication (Curnock and Dyke, unpublished data). The similarity between pI524 and p8325-4 is useful in that the former is not known to conjugate and strains containing the plasmid are sensitive to gentamicin. It can therefore be assumed that the genes involved in the gentamicin resistance specified by p8325-4 and that the plasmid's ability to conjugate will be located, at least in part, to regions outside the apparently homologous 18kbp section. When tested, the plasmid p8325-4 was indeed found to specify β-lactamase synthesis and resistances to cadmium, arsenate and arsenite.

Much less was known about the plasmid p8325-2 (50 kbp). It was known however to specify resistance to ethidium bromide as well as to gentamicin.

Figure 4.1

Comparison between the restriction enzyme cleavage map of the plasmids p1524 and p8325-4



For both plasmids the preliminary maps were checked and further restriction enzymes were used to construct more detailed maps. This was a necessary prerequisite to the analysis of the transfer functions by deletion.

4.2 Restriction enzyme mapping of the plasmids

a) Plasmid p8325-2.

In addition to those enzymes previously used, the sites cut by *Eco* RV, *Nco* I and *Sph* I were mapped by analysis of the results from digests with two enzymes relative to all the other enzymes (Figure 4.2). The restriction enzymes *Pst* I, *Sac* II, *Sma* I, *Sst* I and *Xho* I were found not to have any sites within the plasmid. Restriction enzyme *Hind* III cut p8325-2 into a large number of fragments and it has not proved possible to locate some of the smaller pieces unequivocally on the map. The results are therefore incomplete and hence not shown.

To illustrate the method of restriction enzyme mapping, the positioning of *Eco* RV sites within p8325-2 will be described (Figure 4.3).The table below Figure 4.3 shows the calculated sizes of the fragments from the appropriate digestion.

The enzyme *Eco* RV cleaves p8325-2 into fragments of 28.1, 15.8, 3.5 and 1.8 kbp. The smallest two are not cut by *Eco* RI. The *Eco* RI fragments of 16.4 and 7.0 kbp are cut by *Eco* RV into pieces of 7.6, 4.4, 4.0, 3.5, 1.8 and 0.8 kbp (from the double digest). The best arrangement of sizes occurs if the 16.4 kbp piece is cut by *Eco* RV into 7.6, 4.0 and 3.5 kbp fragments, of which the latter is an *Eco* RV fragment not cut by *Eco* RI. The 7.0 kbp piece is cut by *Eco* RV into 4.4, 1.8 and 0.8 kbp fragments, the 1.8 kbp piece being uncut by *Eco* RI. If the 3.5 and 1.8 kbp *Eco* RV fragments lie within the 16.4 and 7.0 kbp *Eco* RI fragments respectively, the largest 28.1 kbp *Eco* RV fragment must lie between them, spanning the *Eco* RI fragments of 9.3, 4.6 and 4.2 kbp. By trial and error, the best fit is obtained if the 4.4 and 4.0 kbp fragments from the double digest come from the 28.1 kbp piece, and the 0.8 and 7.6 kbp double digest fragments from the 15.8 kbp piece. This places *Eco* RV fragments of 3.5 and 1.8 kbp about 4 kbp from the end of the *Eco* RI fragments of 4.2 and 4.6 kbp. The most probable coordinates are given in Table 4.1.

This method is then applied to mapping with respect to the enzymes *Bcl* I, *Pvu* II and *Xba* I, and the probable coordinates from each double digest correlated to give the best fit (Table 4.1).

Figure 4·2

Restriction enzyme cleavage map of the 8325-2 plasmid.

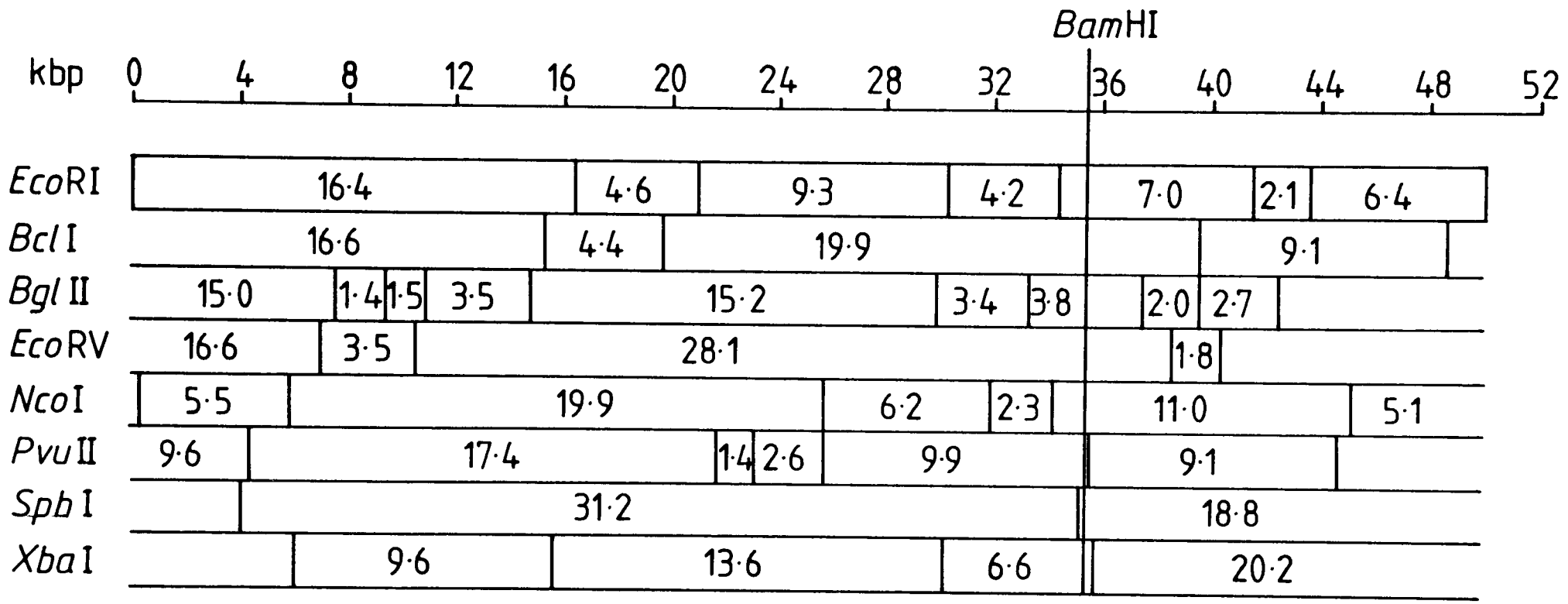


Figure 4.3

Restriction mapping gel of p8325-2

with respect to *Eco* RV

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	p8325-2/*Eco*RV	28.1 15.8 3.5 1.8
3	p8325-2/*Eco*RV/*Eco*RI	9.3 7.6 6.3 4.6 4.4 4.2 4.0 3.5 2.1 1.8 0.8
4	p8325-2/*Eco*RI	16.4 9.3 7.0 6.3 4.6 4.2 2.1
5	p8325-2/*Eco*RV/*Bcl*I	19.6 9.2 9.1 4.4 3.7 3.5 1.8 0.1
6	p8325-2/*Bcl*I	21.2 16.6 9.1 4.4
7	p8325-2/*Pvu*II	17.9 9.9 9.7 9.1 2.6 1.4
8	p8325-2/*Eco*RV/*Pvu*II	11.5 9.9 9.7 3.7 3.5 3.5 2.9 2.6 1.8 1.4
9	p8325-2/*Xba*I	20.8 13.6 9.6 6.6
10	p8325-2/*Eco*RV/*Xba*I	15.0 13.6 6.6 4.3 3.6 3.5 1.8 0.8

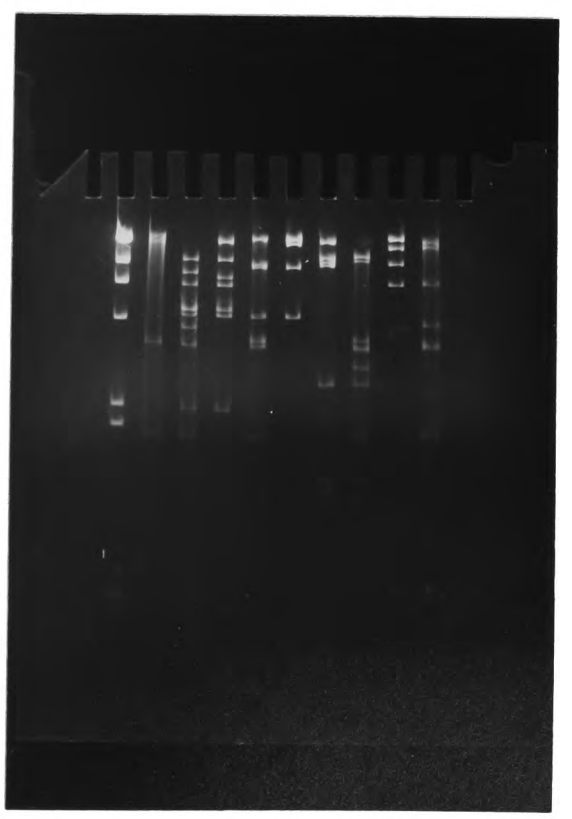


Table 4.1

Mapping position coordinates for p8325-2 with respect to *Eco*RV

Restriction Enzyme.				Probable coordinate
***Eco*RI**	***Bcl*I**	***Pvu*II**	***Xba*I**	
7.6	6.8	6.8	6.8	7.0
11.1	10.3	10.3	10.3	10.5
38.9	38.6	39.2	39.0	38.9
40.7	40.4	41.0	40.8	40.7

b) Plasmid p8325-4.

As shown in Figure 4.4, the plasmid p8325-4 has been mapped with respect to 12 restriction enzymes, including six whose position was not even approximately known previously. The enzyme *Hin*d III was found to have a large number of sites whose positions have not been accurately placed. Although digestion with *Eco* RI results in six fragments it has not been possible to locate the smallest (1.0kbp) piece accurately. Similarly, digestion with *Cla* I results in eight fragments but the position of the 500 bp piece remains undetermined. The enzymes *Bam* HI, *Sac* I, *Sac* II, *Sst* I and *Xho* I do not have a site within the plasmid.

4.3 Deletion analysis of the plasmids

The overall strategy was to digest plasmid DNA to completion with a suitable restriction enzyme, to re-ligate the DNA and to use the mixture to transform a recipient (*S.aureus* 8325N) with selection for a suitable resistance determinant.

a) Plasmid p8325-2.

Unfortunately it proved impossible to design conditions for the selection of plasmids by ethidium bromide so that only gentamicin was available as a selective agent for this plasmid. The following restriction enzymes were used:*Eco* RI, *Eco* RV, *Hae* II, *Nco* I, *Pvu* II, *Sac* I, *Sph* I and *Xba* I. These enzymes were selected for use because they were found to cut the plasmid into a suitable number of fragments (between 2 and 8).

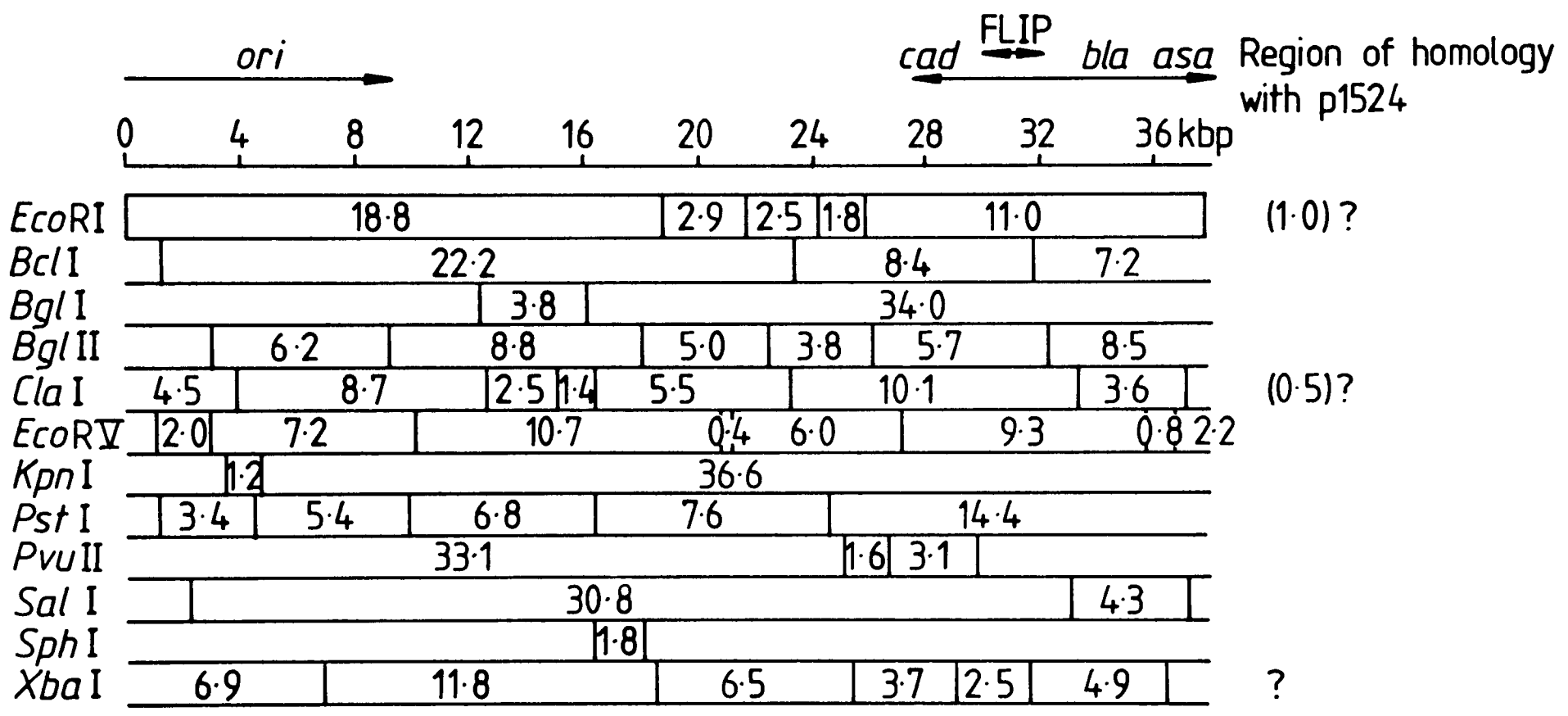
Although each digestion and transformation was repeated at least twice, no gentamicin-resistant transformants were obtained.

b) Plasmid p8325-4.

Again, only gentamicin was used as a selective agent for this plasmid because it proved impossible to select for transformation of cadmium resistance. The following enzymes were used separately: *Ava* I, *Bcl* I, *Bgl* II, *Cla* I, *Eco* RI, *Eco* RV, *Hae* II, *Kpn* I, *Nco* I, *Pst* I, *Pvu* II, *Sph* I and *Xba* I. Again, these enzymes were chosen because they digested the plasmid into a suitable number of fragments. Although p8325-4 had not been mapped with respect to *Ava* I and *Hae* II,

Figure 4.4

Restriction enzyme cleavage map of the 8325-4 plasmid



these were both found to cleave the plasmid into three fragments (a suitable number for deletion analysis). If deletion plasmids had been obtained, the sites would have been mapped.

The five enzymes *Cla* I, *Eco* RI, *Eco* RV, *Pst* I and *Sph* I gave pieces of DNA that on ligation gave rise to gentamicin-resistant transformants. Table 4.2 shows the number of transformants obtained on each initial selection plate together with the number of true deletion mutants finally selected for each of the five enzymes listed above. It will be noted that the gentamicin selection employed sometimes allowed a few gentamicin-sensitive bacteria to grow.

The transformants were screened and found to contain plasmids that were smaller than the parental plasmid. These plasmids were designated pJE1-5 and characterized by analysis with restriction enzymes in comparison to the parental plasmid. Examples of agarose gels of the digested DNA are shown in Figures 4.5-4.8 together with tables giving the sizes of the fragments determined. A summary is given in Table 4.3. Plasmids created by any one particular restriction enzyme were found to contain the same restriction fragments.

Each of the plasmids pJE1-5 was tested for its ability to promote the transfer of resistance of gentamicin to 8325 Nov^r. The results (Table 4.3) are the means of at least two experiments. They indicate that all five of the mutant plasmids were transferred to the recipient but at a lower frequency than the parental plasmid. In general, the values for the conjugation frequencies of the deletion plasmids was very low. For this reason, the cells recovered from overnight incubation were suspended in only 3mls (rather than the normal 10mls) of saline. The lowest detectable frequency of transfer would be 8.0×10^{-9} transconjugants per recipient (the frequency of transfer if only one transconjugant is found). An example of a transconjugant was chosen for each conjugation and plasmid DNA prepared from each. The plasmid DNA was digested and compared with the parental plasmid (Figures 4.9-4.11). In all cases, the transfer of gentamicin resistance correlates with the transfer of a plasmid of the expected size and with the predicted restriction enzyme sites.

Table 4.2

Table showing the total number of deletion plasmids obtained from p8325-4

Restriction enzyme digestion	No. of transformants per selection plate	No. of deletion mutants
Ava I	136	0
Bgl II	112	0
Cla I	72	1
Eco RI	210	3
Eco RV	131	1
Hae II	246	0
Kpn I	252	0
Nco I	114	0
Pst I	134	2
Sph I	243	5
Xba I	257	0

Figure 4.5

Gel of pJE1

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	uncut pJE1	closed circular DNA
3	8325-4/*Eco*RI	18.8 11.0 2.9 2.5 1.8 (1.0)
4	pJE1/*Eco*RI	18.8
5	8325-4/*Eco*RI/*Pst*I	11.0 6.8 5.4 3.4 2.9 2.5 2.4 1.3 1.1 0.3
6	pJE1/*Eco*RI/*Pst*I	6.8 5.4 3.4 2.4 1.1
7	8325-4/*Eco*RI/*Bgl*II	8.8 6.2 5.7 3.8 3.0 2.9 2.5 1.6 0.8 0.8 0.5
8	pJE1/*Eco*RI/*Bgl*II	8.8 6.2 3.0 0.8
9	8325-4/*Eco*RI/*Xba*I	11.8 6.8 4.9 2.9 2.5 2.5 1.3 1.2 1.2 0.4 0.2
10	pJE1/*Eco*RI/*Xba*I	11.8 6.8 0.2

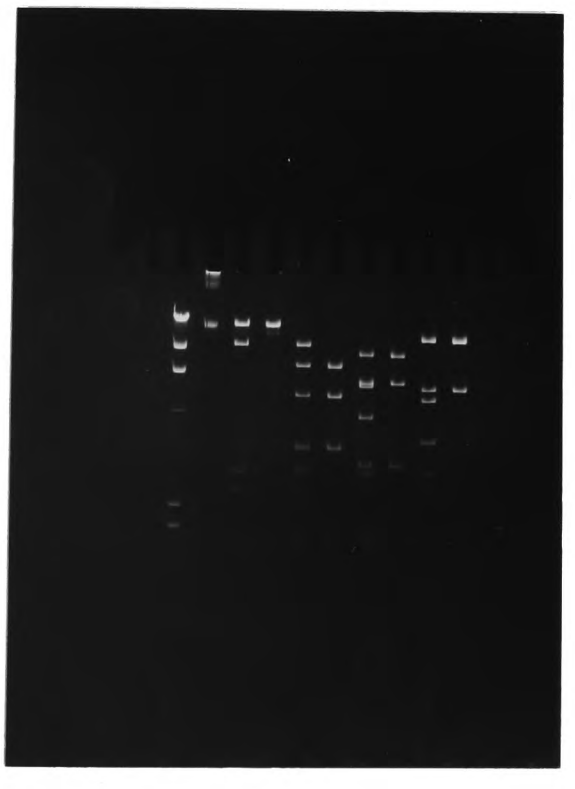


Figure 4.6

Gel of pJE2

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	8324-4/*Pst*I	18.0 7.6 6.8 5.4 3.4
3	pJE2/PstI	6.8 5.4
4	8324-4/*Pst*I/*Eco*RI	11.0 6.8 5.4 3.4 2.9 2.5 2.4 1.3 1.1 0.3
5	pJE2/*Pst*I/*Eco*RI	6.8 5.4
6	8325-4/*Pst*I/*Bgl*II	6.8 5.7 4.7 4.5 3.8 3.0 2.1 1.8 1.6 1.5 0.7
7	pJE2/*Pst*I/*Bgl*II	6.8 4.7 0.7
8	8325-4/*Pst*I/*Cla*I	8.8 5.5 5.4 3.6 2.8 2.7 2.5 1.4 1.3 1.1 0.8 0.6
9	pJE2/*Pst*I/*Cla*I	5.4 2.7 2.5 1.4
10	pJE2/*Pst*I	6.8 5.4

Figure 4.7

Gel of pJE3 and pJE4

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	uncut pJE3	closed circular DNA
3	8325-4/*Eco*RV	10.7 9.3 7.2 6.0 2.2 2.0 0.8 0.4
4	pJE3/*Eco*RV	10.7 7.2
5	uncut pJE4	closed circular DNA
6	8325-4/*Cla*I	10.1 8.7 5.5 4.5 3.6 2.5 1.4
7	pJE4/*Cla*I	8.7 2.5
8	pJE4/*Cla*I/*Eco*RI	8.7 2.5
9	pJE4/*Cla*I/*Pst*I	5.4 2.7 2.5 0.6
10	pJE3/*Eco*RV/*Eco*RI	8.7 7.2 2.0
11	pJE3/*Eco*RV/*Pst*I	6.3 5.4 4.4 1.6 0.2

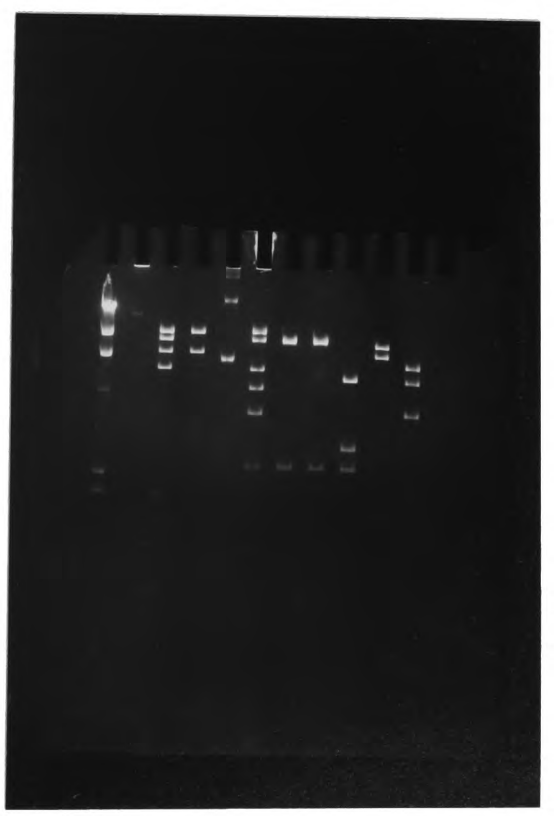


Figure 4.8

Gel of pJE5

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	p8325-4/*Sph*I	36.1 1.7
3	pJE5/*Sph*I	36.1
4	p8325-4/*Eco*RI/*Sph*I	16.3 11.0 2.9 2.5 1.8 1.7 (1.0) 0.7
5	pJE5/*Eco*RI/*Sph*I	16.3 11.0 2.9 2.5 1.8 (1.0) 0.7
6	p8325-4/*Pst*I/*Sph*I	13.2 6.8 6.5 5.4 3.4 1.3 0.4
7	pJE5/*Pst*I/*Sph*I	13.2 6.8 6.5 5.4 3.4
8	p8325-4/*Bgl*II/*Sph*I	8.5 8.0 6.2 5.7 5.0 3.8 1.1 0.6
9	pJE5/*Bgl*II/*Sph*I	8.5 8.0 6.2 5.7 5.0 3.8

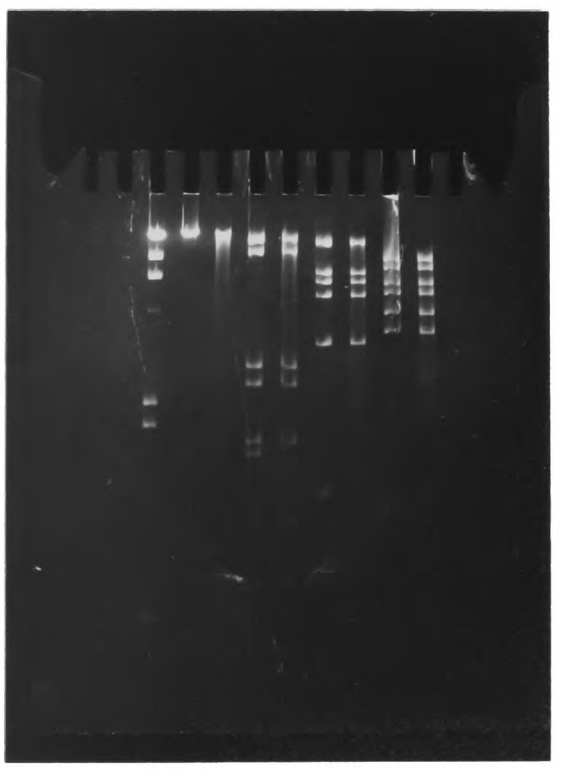


Table 4.3

Summary of the deletion mutants obtained from p8325-4

Restriction enzyme used for digestion	Name of Gm^r plasmid produced	Size of plasmid (kbp)	Fragment/s involved	Frequency of conjugation
*Eco*RI	pJE1	18.8	*Eco*RI.A	3.2×10^{-8}
*Pst*I	pJE2	12.2	*Pst*I.C (6.8 kbp) + *Pst*I.D (5.4 kbp)	8.0×10^{-9}
*Eco*RV	pJE3	17.9	*Eco*RV.A (10.7 kbp) + *Eco*RV.C (7.2 kbp)	7.2×10^{-8}
*Cla*I	pJE4	11.2	*Cla*I.B (8.7 kbp) + *Cla*I.F (2.5 kbp)	3.2×10^{-8}
*Sph*I	pJE5	36.2	*Sph*I.A	3.3×10^{-7}
Parental plasmid	p8325-4	37.8	—	8.2×10^{-7}

Figure 4.9

Gel of pJE1 and pJE2 transconjugant DNA

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	p8325-4/*Eco*RI	18.8 11.0 2.9 2.5 1.8 (1.0)
3	p8325-4Nov Cjt/*Eco*RI	18.8 11.0 2.9 2.5 1.8 (1.0)
4	pJE1/*Eco*RI	18.8
5	pJE1Nov Cjt/*Eco*RI	18.8
6	pJE2/*Pst*I	6.8 5.4
7	pJE2Nov Cjt/*Pst*I	6.8 5.4
8	p8325-4/*Pst*I	14.4 7.6 6.8 5.4 3.4

Cjt stands for transconjugant in this and all subsequent tables.

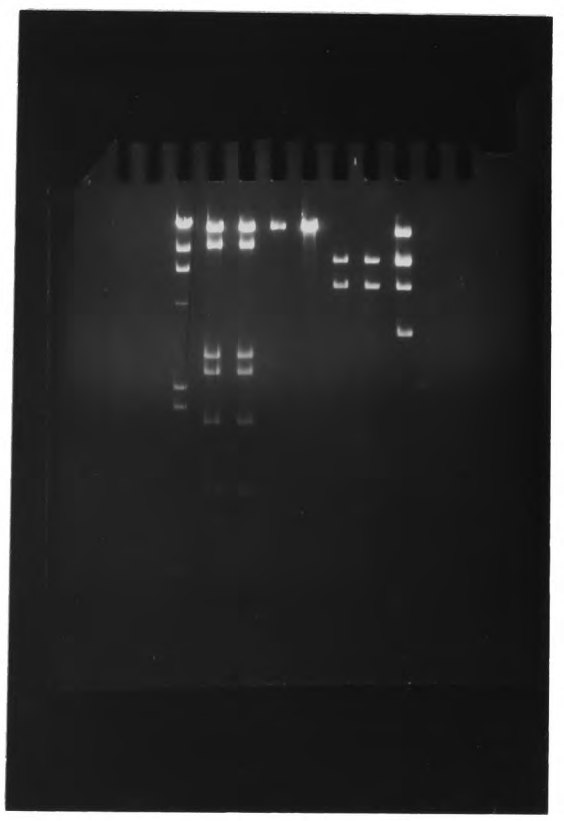


Figure 4.10

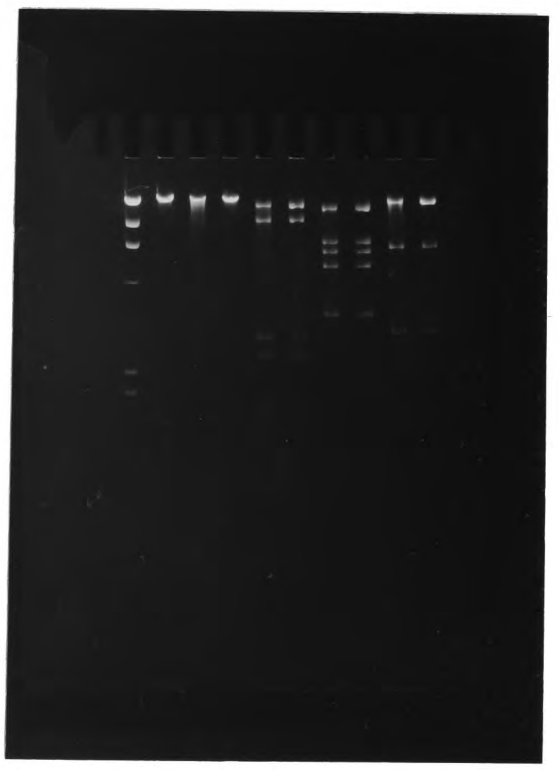
Gel of pJE3 and pJE4 transconjugant DNA

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	p8325-4/*Eco*RV	10.7 9.3 7.2 6.0 2.2 2.0 0.8 0.4
3	pJE3/*Eco*RV	10.7 7.2
4	pJE3Nov Cjt/it EcoRV	10.7 7.2
5	p8325-4/*Cla*	10.1 8.7 5.5 4.5 3.6 2.5 1.4 (0.5)
6	pJE4/*Cla*I	8.7 2.5
7	pJE4Nov Cjt/*Cla*I	8.7 2.5
8	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56

Figure 4.11

Gel of pJE5 transconjugant DNA

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	p8325-4/*Sph*I	36.1 1.7
3	pJE5/*Sph*I	36.1
4	pJE5Nov Cjt/*Sph*I	36.1
5	pJE5/*Eco*RI/*Sph*I	16.3 11.0 2.9 2.5 1.8 (1.0) 0.7
6	pJE5Nov Cjt/*Eco*RI/*Sph*I	16.3 11.0 2.9 2.5 1.8 (1.0) 0.7
7	pJE5/*Pst*I/*Sph*I	13.2 6.8 6.5 5.4 3.4
8	pJE5Nov Cjt/*Pst*I/*Sph*I	13.2 6.8 6.5 5.4 3.4
9	pJE5/*Pvu*II/*Sph*I	> 20 7.1 3.1 1.6
10	pJE5Nov Cjt/*Pvu*II/*Sph*I	> 20 7.1 3.1 1.6



4.4 Discussion

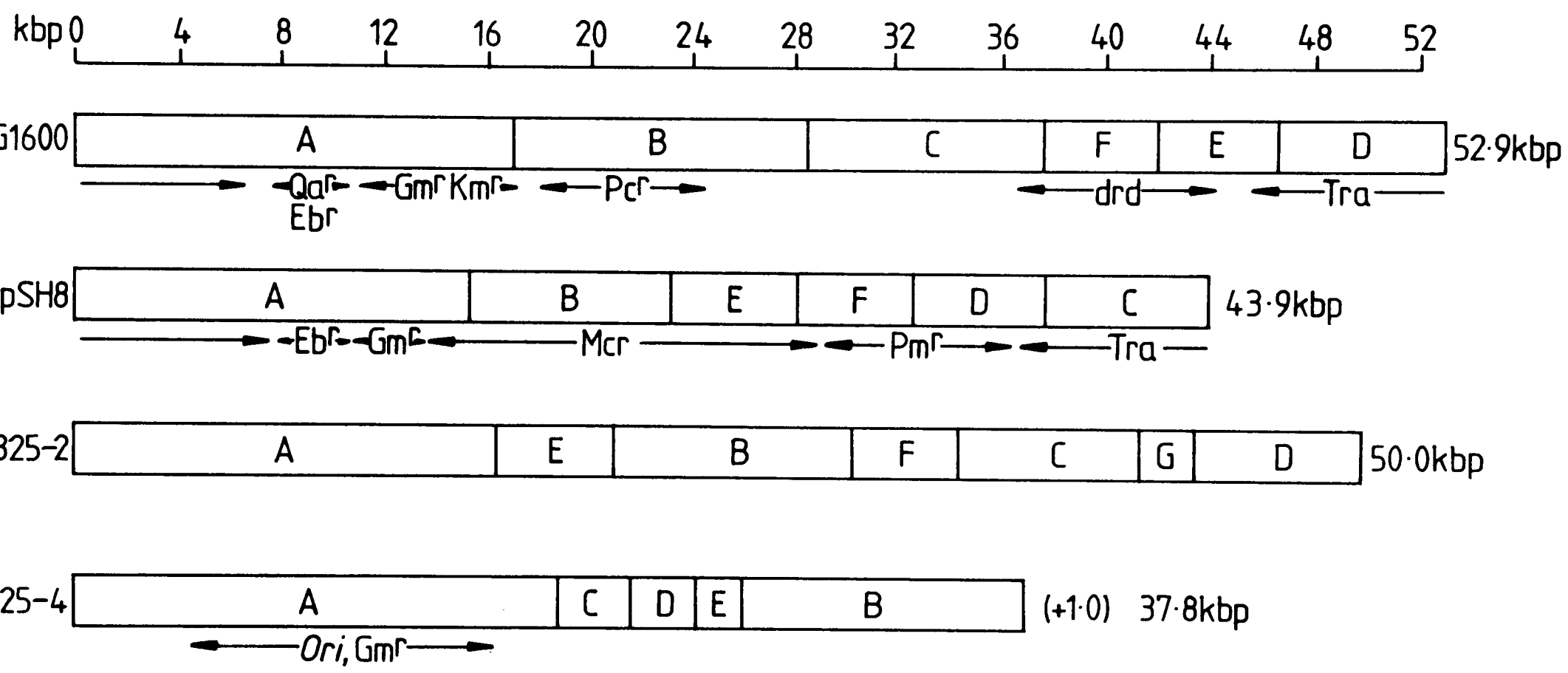
Mapping the two plasmids p8325-2 and p8325-4 proved difficult for several reasons. Firstly, as the plasmids are so large (50 and 38 kbp respectively) even restriction enzymes which only cleave at specific six base pair sequences often have as many as eight or nine sites. A double digest results in an even larger number of fragments, increasing the number of permutations of fragment positions. Also, due to their large size, the copy number of the plasmids is comparatively low, allowing only small quantities of plasmid DNA to be produced in any one DNA preparation (typically about 10μg). The mapping procedure requires relatively large amount of DNA (about 400ng per digestion) necessitating the preparation of large stocks of DNA. Finally, certain enzymes (notably *Bcl* I, *Pvu* II and *Xba* I) often failed to cleave effectively even under carefully controlled incubation conditions (Table 2.1). This often resulted in partial fragments, which although useful at times, were often misleading. Inefficient cleavage is probably due to protein which has remained bound to the DNA even after overnight centrifugation in a caesium chloride gradient. Phenol extraction is often useful but does result in the loss of large amounts of DNA.

The plasmids p8325-2 and p8325-4 have now been mapped with respect to eight and twelve enzymes respectively (Figures 4.2 and 4.4). The sites for nine of these enzymes had been previously unknown and all results were re-checked and altered where necessary. To date, it has not been possible to position the smallest *Eco* RI and *Cla* I fragments (1.0 and 0.5 kbp respectively) on the p8325-4 map. All other enzyme sites on this plasmid are considered to be quite accurately placed. The sites of a couple of enzymes in the p8235-2 map remain uncertain. As the largest *Bgl* II fragments (15.2 and 15.0 kbp) are very similar in size, it is possible that their positioning is the opposite of that shown. Similarly, three of the *Pvu* II fragments are very similar in size (9.9,, 9.6 and 9.1 kbp) possibly resulting in their misplacement between themselves.

The fairly extensive maps now available of the two plasmids allows comparisons to be made between them and other gentamicin resistance conferring plasmids. Comparison between Figures 4.2 and 4.4 shows no obvious similarity between p8325-2 and p8325-4. Also, aligning the *Eco* RI maps of plasmids pCRG1600 (Asch *et al*, 1984) and pSH8 (McDonnell *et al*, 1983) fails to demonstrate obvious similarities (Figure 4.12). The plasmid pCRG1600 (52.9 kbp) was obtained from a clinical isolate of *S.aureus*. It is a self-transmissible plasmid encoding for resistance to aminoglycoside antibiotics, including gentamicin (McDonnell *et al*, 1983). The plasmid pSH8 is also a transferable gentamicin resistance conferring plasmid, again from a clinical isolate of *S.aureus*

Figure 4.12

Comparison of the *Eco*RI maps of five gentamicin resistance conferring plasmids



(Asch *et al*, 1984). As the regions involved in transfer have been approximately located on these plasmids, they will be discussed in relation to the plasmids studied in this survey in Chapter 8.

The only similarity observed between the *Eco* RI maps is that the gentamicin resistance determinant resides on the largest fragment in each case. However, the plasmids p8325-4 and pCRG1600 both have three *Bcl* I, two *Kpn* I and six *Xba* I sites whilst p8325-2 and pCRG1600 both have a single *Bam* HI site. The plasmids p8325-2 and pSH8 each have four *Xba* I sites. It would be interesting to compare the restriction maps in more detail if such information could be made available and to use DNA hybridization to check for apparent homologies.

The maps of p8325-4 and pI524 are very similar over the 18 kbp "homologous" region (Figure 4.1). Plasmid pI524 however, includes an invertible region of about three kbp within this 18 kbp piece. Analysis of the restriction digests of p8325-4 demonstrates that this invertible region is present but in a single orientation. It is interesting that the invertible region is in close proximity to the junction of the region of similarity between p8325-4 and pI524. As the determinant for cadmium resistance is also in this area on pI524, it would be interesting to know whether the same gene is present at this position on p8325-4. The plasmid pI524 contains an origin of replication towards the other end of the similarity region. The plasmid p8325-4 may use this, or it may possess a second origin of replication. The F factor of *E.coli* contains two origins of replication (*ori* V and *ori* S) whilst R6K (38 kbp) has three potential replication origins (Sherrett, 1986).

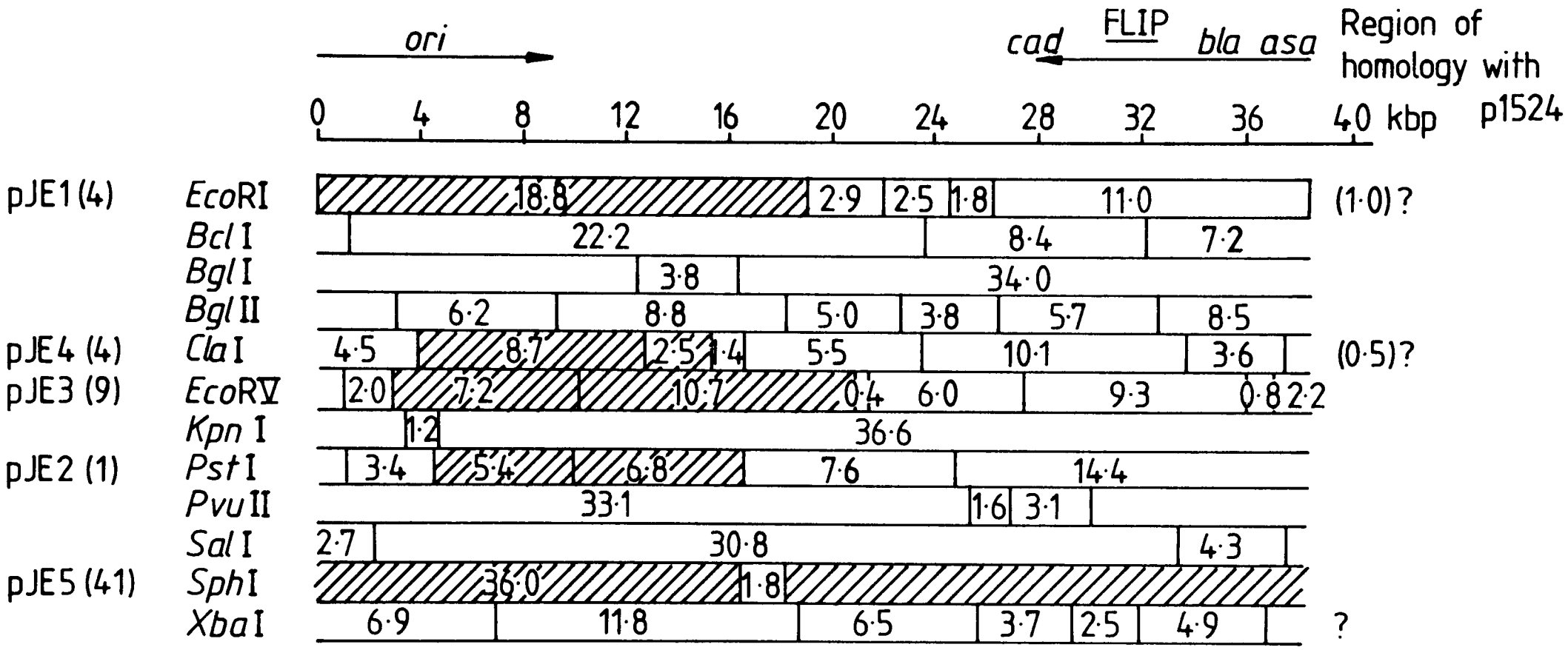
As pI524 does not encode resistance to gentamicin, this determinant probably lies outside the region of homology, that is, between about 9.0 and 27.0 kbp on the *Eco* RI map of p8325-4. As pI524 is not self-transmissible, the region specifying the ability to conjugate must also lie, at least in part, within the region of 9.0 to 27.0 kbp.

More extensive mapping may be achieved by the use of partial digestions, by hybridizations between plasmids or by isolating individual fragments from agarose gels for further characterization.

Deletion analysis of the plasmid p8325-4 resulted in the formation of five deletion mutants. Each must contain at least one origin of replication and the gentamicin resistance determinant. This necessitates the positioning of these determinants between 4.5 and 15.1 kbp on the p8325-4 map (Figure 4.13) which is consistent with the known positions of the gentamicin resistance determinant on the maps of pCRG1600 and pSH8 (Figure 4.12).

Figure 4.13

Restriction enzyme cleavage map of the 8325-4 plasmid showing the deletion mutants constructed (shaded)



The number of transconjugants obtained after a standard conjugation with 8325 Nov^r is given in brackets for each deletion plasmid

The three deletion plasmids pJE2, pJE3 and pJE4 were created from partial digestions of p8325-4 with the enzymes *Pst* I, *Eco* RV and *Cla* I respectively. As no mutants composed of single fragments were obtained from these enzymes, it is probable that an origin and the gentamicin determinant lie on separate *Pst* I, *Eco* RV and *Cla* I fragments. These mutants are probably formed from partial fragments and not from the religation of digested pieces as the probability of the latter occurring is relatively small and also in each example the two fragments are adjacent on the map.

The formation of pJE1-pJE5 was also useful in that it confirmed the restriction map. The deletion mutants were not mapped in both orientations due to the eventual lack of success in locating the transfer system by this method.

All five deletion plasmids promoted transfer at a lower frequency than the parental plasmid. The mean number of transconjugants produced by each mutant is given in Figure 4.13. The plasmid pJE5 produced significantly more transconjugants than any of the other mutants. It is therefore possible that pJE5 (36.0 kbp) contains a virtually intact conjugation system but that one or both of the *Sph* I sites are close to the border of this region. Cleavage may reduce transfer but not abolish it. The remaining mutants (pJE1-pJE4) did not possess enough of the "transfer promoting region" to establish conjugation to any significant extent. Therefore, such a region must lie outside the area of 0-20.8 kbp (the total area encompassed by all four plasmids) or more than one area is involved, at least one of which lies outside the 0-20.8 kbp region. If p8325-4 is similar to pCRG1600 and pSH8, it is possible that the transfer region spans across the *Eco* RI fragments A and B (Figure 4.12).

It was noted that the frequency of conjugation of p8325-4 was lower than that obtained in the initial series of conjugation experiments (Section 3.2.2). This interesting observation will be discussed in detail in Chapter 8.

The small number of transconjugants produced by the deletion plasmids did contain intact plasmids (Figures 4.9-4.11). These may have been due to the low level phage transfer system described in Chapter 3.

The enzymes which did not result in deletion plasmids such as *Bcl* I, *Kpn* I, *Pvu* II and *Sal* I may have produced plasmids which were too large to be effectively transformed (22.2, 36.6, 33.1 and 30.8 kbp respectively). Evidence against this is that pJE5 is 36.0 kbp in size although this

may have been anomalous. Another possibility is that the blunt-ended enzymes (such as *Pvu* II) did not re-ligate effectively.

These problems may have prevented the formation of deletion plasmids from p8325-2 although other reasons are also possible. The origin of replication and gentamicin resistance determinant may be well separated on p8325-2, not residing together on any one particular restriction fragment. This would necessitate the formation of partial fragments or the re-ligation of two separate fragments in the correct orientation. Although the former occurred in the case of p8325-4, all digestions of p8325-2 may have gone to completion. The probability of the latter occurring is relatively small. As was suggested for p8325-4, certain fragments, such as the 31.2 kbp *Sph* I piece, may have been too large for effective transformation. It is also possible that either the origin or the gentamicin resistance determinant (or even both) were cut by one of the enzymes in every case. Again the probability of this happening is very small. Finally, the heat inactivation of the restriction enzymes may have been ineffective in some cases. Certain enzymes are known to be more efficiently inactivated by this method. Such enzymes however, include *Cla* I and *Eco* RI which formed deletion plasmids from p8325-4, whereas enzymes such as *Nco* I and *Sac* I (used unsuccessfully) should be almost fully inactivated. The lack of deletion plasmids may have been the result of a combination of these problems.

Ethidium bromide and cadmium were not suitable selective agents for transformants, possibly because protoplasts are more sensitive to these agents than whole cells. Gentamicin was also not fully effective as a selective agent. It allowed the growth of approximately 100-200 colonies per plate, of which only one or two were derived from true gentamicin resistant transformants (Table 4.2).

This line of work could be pursued by the construction of deletion plasmids using a cloned origin of replication, possibly by ligating pCW59 to restricted gentamicin resistance conferring plasmids. This would prevent the necessity of the two determinants (origin and gentamicin resistance determinant) lying in the same restriction fragment.

It was decided to pursue the analysis of the conjugation system by attempting to clone the complete transfer region into a suitable vector molecule.

4.5 Conclusion

The gentamicin resistance plasmids, p8325-2 and p8325-4 have been mapped with respect to eight and twelve restriction endonucleases respectively. Their restriction-cleavage maps are given.

No deletion plasmids were constructed from p8325-2 although eight different enzymes were used, each at least twice.

Five gentamicin-resistant deletion plasmids were constructed from the parental plasmid p8325-4 using the enzymes *Eco* RI, *Pst* I, *Eco* RV, *Cla* I and *Sph* I, creating the mutants pJE1-pJE5 respectively. This allowed the gentamicin resistance determinant and at least one origin of replication to be located between 4.5 and 15.1 kbp on the map of p8325-4. Only pJE5 (36.0 kbp) was transferred to recipient 8325 Nov^r to any significant extent. This deletion plasmid may, therefore, contain a virtually intact conjugation system, the boundaries of which are close to either one or both of the *Sph* I sites in p8325-4. The smallest four deletion plasmids failed to conjugate to any significant extent: transfer of intact deletion mutants may have been due to the involvement of phage.

The technique of deletion analysis did not isolate the complete "transfer region" and it was therefore decided to try a different approach.

Chapter5

Cloning Restriction Fragments from Two Conjugative Plasmids

5.1 Introduction

Since deletion analysis had produced inconclusive results in defining the region involved in the transfer process of the two conjugative plasmids, p8325-2 and p8235-4, it was decided to pursue the investigation by cloning restriction fragments into suitable vectors. Only a very limited number of such vectors are available for cloning with *S.aureus* and their use is made more difficult due to the poor and variable efficiency with which this organism can be transformed.

Available vectors included the chloramphenicol resistance plasmid, pC194 (Horinouchi and Weisblum, 1982a) which has a single *Hin*d III site outside the gene conferring resistance to chloramphenicol, pE194 (Horinouchi and Weisblum, 1982b) which has a suitable *Xba* I site and pCW59 (Wilson and Baldwin, 1978) which confers resistance to both tetracycline and chloramphenicol. The latter vector is particularly useful because the insertion of DNA into its unique *Bgl* II site enables the selection of tetracycline-resistant colonies that can be screened for their sensitivity to chloramphenicol.

5.2 Cloning using the plasmids pC194, pE194, pBD8 and pCE10

Plasmid DNA was isolated from a *S.aureus* strain containing pC194 and both undigested and *Hin*d III digested and re-ligated DNA were successfully used to transform *S.aureus* 8325N to chloramphenicol resistance via the protoplast system. The transformation efficiencies were low: 50 and 2 transformants per microgram of DNA for uncut and cut and re-ligated DNA respectively.

Plasmid DNA was prepared from 8325-2 and 8325-4 and 5μg of each was cut with *Hin*d III. Following the phenol extraction of all three samples to inactivate the reactions, digested DNA from p8325-2 and p8325-4 was separately ligated to the vector. This ligated DNA was used to transform 8325N to chloramphenicol resistance. Approximately 50 transformants were obtained and all were tested for the presence of plasmid DNA by the rapid purificatio n method (Section 2.16). All transformants contained DNA that was indistinguishable from pC194.

Similar experiments were performed with *Xba* I digested pE194 and p8325-2 and p8325-4 DNA. Again, all samples were phenol extracted before ligation. The same quantities of DNA were used as in the experiment with pC194. Following transformation, erythromycin-resistant colonies were obtained at a similar frequency to the chloramphenicol-resistant transformants. Preparation of DNA from 55 transformants showed no evidence of any insertion within pE194.

It was decided to dephosphorylate the vector DNA before ligation to prevent self-ligation and hence reduce the number of transformants containing only re-ligated vector molecules. The enzyme calf intestinal alkaline phosphatase (CIP) was used which removes the 5′-phosphate groups from the cleaved molecule. The DNA will then only re-ligate when a fragment with intact 5′-phosphate groups is inserted so that the only circular molecules formed are recombinants. A stock preparation (2μg) of dephosphorylated vector was made. Phosphatase treatment (Section 2.22) was followed by phenol extraction (Section 2.23) and ethanol precipitation (Section 2.24). Two control transformations were initially carried out to assess the value of the phosphatase treatment. In both cases, both dephosphorylated and untreated vector DNA (20ng) was used to transform 8325N to chloramphenicol/erythromycin resistance. The average number of transformants obtained was 50 per plate for untreated vector and 2 per plate for dephosphorylated vector. To obtain 20ng of dephosphorylated vector for transformation (the recommended quantity by Maniatis *et al*, 1982) about 400ng of stock DNA had been used. In all subsequent transformations therefore, phosphatase treatment was not used as it was decided that the advantage of producing a higher proportion of recombinants did not compensate for the large quantities of DNA needed.

The screening of transformant colonies for the presence of DNA however, even using the method of rapid purification for *S.aureus* (Section 2.16) was a time consuming operation. In all, more than 100 colonies had been examined in this way. It was decided therefore, that a plasmid containing two resistance determinants, one of which was inactivated by DNA insertion, would be a more suitable vector with which to pursue the cloning.

The *Bacillus subtilis* strain IE21[pBD8] carries the determinants for chloramphenicol, streptomycin and kanamycin resistance. It has a single *Eco* RI site which inactivates the resistance to streptomycin and was therefore thought useful for cloning.

Plasmid DNA was prepared from IE21[pBD8] and about 1μg digested with *Eco* RI. Plasmid DNA from p8325-2 and p8325-4 was separately cut with *Eco* RI. Samples of vector (20-40ng) and insert (1-5μg) DNA were ligated and used to transform *S.aureus* 8325N and *S.aureus* SA113 to either chloramphenicol or kanamycin resistance. Selection by kanamycin was not effective and continually resulted in heavy growth by sensitive colonies on the transformation plates. Chloramphenicol selection was also ineffective at low concentration (8μg/ml) but prevented any growth, even of cells transformed by uncut vector (and therefore chloramphenicol resistant) at higher concentrations (10μg/ml).

The chimeric plasmid pCE10 (5.3 kbp) was constructed for cloning in *S.aureus* and *S.carnosus* (Keller*et al*, 1983) and was kindly donated by Dr.F.Gotz. The plasmid carries resistance determinants to both chloramphenicol (inactivated by insertion of DNA at the single *Hae* III site) and erythromycin (inactivated by insertion of DNA at the single *Bcl* I site). Plasmid DNA was prepared from SA113[pCE10] and 1μg was digested with *Bcl* I. Plasmid DNA from both p8325-2 and p8325-4 (1μg of each) was also digested with *Bcl* I. The samples of DNA were ligated (50ng vector, 1μg insert) and used to transform both 8325N and SA113 to chloramphenicol resistance. For cloning using p8325-2, 24 chloramphenicol-resistant colonies were obtained and for the p8325-4 transformation, 61 chloramphenicol-resistant colonies were obtained. All 85 transformants were screened for sensitivity to erythromycin. As all colonies were resistant to this antibiotic, it was concluded that the transformants contained only re-ligated vector DNA.

Work with these cloning systems was not pursued at this stage as cloning into the vector pCW59 was proving more productive.

5.3 pCW59 as a cloning vector for p8325-2

The ligation of p8325-2 to pCW59 and subsequent transformation into 8325N was performed a total of 10 times with varying amounts of insert (1-2.5μg) and vector (20-100ng) DNA. The maximum number of clones from any one experiment (20) was obtained when 1μg of *Bgl* II digested insert DNA was ligated to 20ng of similarly digested vector DNA. The reactions were judged to be complete by running samples on agarose gels.

The ligation mixture was used to transform *S.aureus* 8325N to tetracycline resistance. A total of 2600 tetracycline resistant colonies were picked and screened for their sensitivity to chloramphenicol. From the 48 tetracycline-resistant, chloramphenicol-sensitive colonies found, plasmid DNA was prepared, digested with *Bgl* II and compared with *Bgl* II-digested vector and p8325-2 DNA. Examples of 11 clones are shown in Figures 5.1 and 5.2.

Of the nine possible fragments, seven had been cloned. The expected sizes of the cloned fragments (in kbp) with the number of clones obtained for each size shown in parenthesis are:

15.2 (0); 15.0 (0); 3.8 (6); 3.5 (6); 3.4 (6); 2.7 (9); 2.0 (6); 1.5 (9) and 1.4 (6).

The orientation of each insert was not determined.

To clone the two larger fragments, it was decided to purify them from agarose gels by electrophoretic elution onto DEAE cellulose paper (Dretzen *et al,* 1981). Since the two fragments are of very similar size, no attempt was made to separate them from each other. On six occasions the larger bands were successfully extracted. A total of 600 tetracycline-resistant transformants were obtained but every one was chloramphenicol resistant.

5.3.1 Characterization of the cloned fragments.

Plasmid p8325-2 carries determinants for resistance to paromomycin, ethidium bromide and gentamicin. The 48 colonies were therefore tested for their resistance and the results are shown for the 11 examples given in Figures 5.1 and 5.2.

A representative example of each of the seven different clones was tested for its ability to conjugate with BIII Nov^r. Tetracycline-resistant, novobiocin-resistant transconjugants were selected in each case but in addition, gentamicin-resistant, novobiocin-resistant transconjugants were selected

Figure 5.1

Gel of six *Bgl* II clones from p8325-2 inserted into the vector pCW59

Lane	Name of clone/digestion	Size of fragments observed (kbp)	Resistance carried
1	λH3	23.1, 9.42, 6.68, 4.36, 2.32, 2.03, 0.56	—
2	undigested pCW59	open circular form	Tc, Cm
3	Clone 2.A	5.3, 3.8	—
4	Clone 2.B	5.3, 3.5	Gm
5	Clone 2.C	5.3, 3.5	Gm
6	Clone 2.D	5.3, 2.0	Eb
7	Clone 2.E	5.3, 1.5	—
8	Clone 2.F	5.3, 2.7	—
9	pCW59 / *Bgl* II	5.3, —	Tc
10	p8325-2 / *Bgl* II	15.2, 15.0, 3.8, 3.5, 3.4 2.7, 2.0, 1.5, 1.4	Gm, Eb, Pm
11	λH3	23.1, 9.42, 6.68, 4.36, 2.32, 2.03, 0.56	—

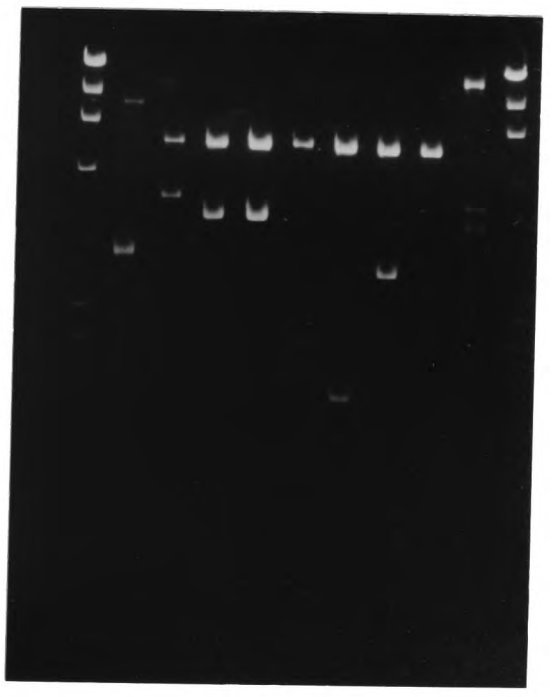
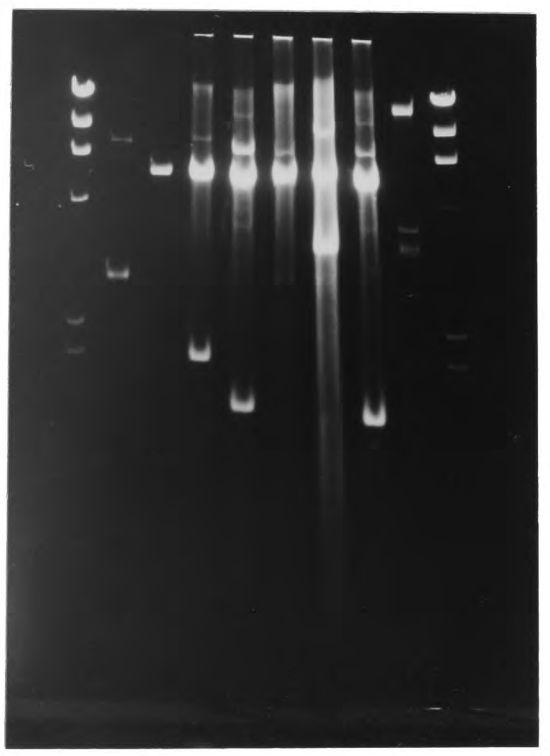


Figure 5.2

Gel of five *Bgl* II clones from p8325-2 inserted into the vector pCW59

Lane	Name of clone/digestion	Size of fragments observed (kbp)	Resistance carried
1	λH3	23.1, 9.42, 6.68, 4.36, 2.32, 2.03, 0.56	—
2	undigested pCW59	open circular form	—
3	pCW59 / *Bgl* II	5.3, —	—
4	Clone 2.G	5.3, 2.0	Eb
5	Clone 2.H	5.3, 1.5	—
6	Clone 2.I	5.3, 2.7	—
7	Clone 2.J	5.3, 3.4	—
8	Clone 2.K	5.3, 1.4	—
9	p8325-2 / *Bgl* II	15.2, 15.0, 3.8, 3.5, 3.4 2.7, 2.0, 1.5, 1.4	Gm, Eb, Pm
10	λH3	23.1, 9.42, 6.68, 4.36, 2.32, 2.03, 0.56	—



for the clone conferring resistance to gentamicin. In no case was there any evidence of conjugation promoted by one of the clones although the parental plasmid p8325-2 transferred at its normally high frequency (5×10^{-4} transconjugants per recipient).

5.3.2 Cloning *Bcl* I fragments from p8325-2 into pCW59.

The restriction endonuclease *Bcl* I produces ends that are compatible with *Bgl* II cut DNA. Therefore, 400ng, 1μg and 2μg of *Bcl* I digested p8325-2 DNA were separately ligated to 20ng of *Bgl* II cut pCW59 DNA. The three samples were used to transform 8325N to tetracycline resistance and transformants screened for sensitivity to chloramphenicol. The experiment was repeated four times and a total of 1000 transformants were screened. All were found to be resistant to chloramphenicol.

5.4 Cloning *Bgl* II fragments from p8325-4 into the vector pCW59

The cloning strategy employed was the same as that for the plasmid p8325-2. Plasmid DNA from p8325-4 (1μg) was digested to completion with *Bgl* II and ligated to *Bgl* II cut DNA from pCW59 (20ng). The mixture was used to transform 8325N to tetracycline resistance. From a total of 1000 transformants picked, 15 were sensitive to chloramphenicol. Plasmid DNA was prepared from these clones and the size of the insert DNA was determined. Of the six possible *Bgl* II fragments, five had been cloned, the uncloned fragment being the second largest piece of 8.5 kbp. The numbers of each fragment cloned are given below in parenthesis after the size of the insert (in kbp):

8.8 (1): 8.5 (0): 6.2 (3): 5.7 (4): 5.0 (4): 3.8 (3):.

An example of each of the cloned fragments is shown in Figure 5.3.

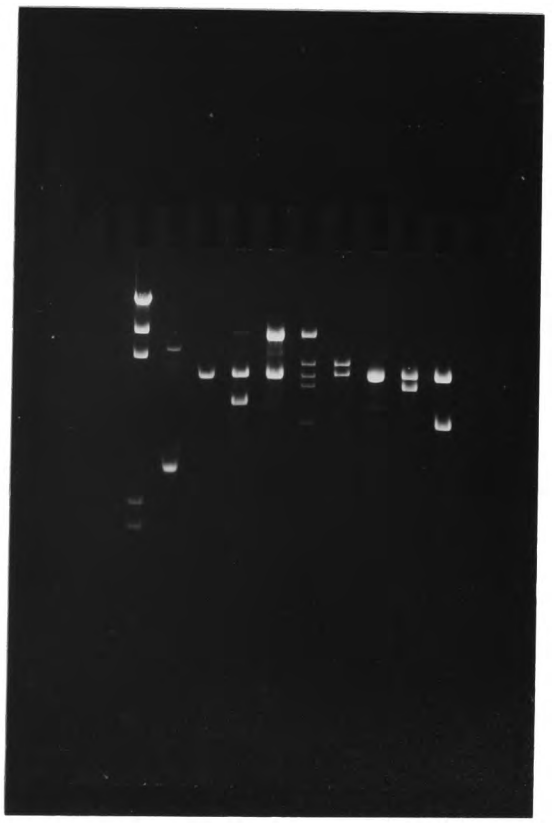
Clone 4.A is of particular interest since it contains, in addition to the expected size of DNA fragment from the vector (5.3 kbp), fragments of about 8.8 and 4.2 kbp but not in equimolar amounts. The smaller fragment has a more intense band and therefore is present in a larger amount than the 8.8 kbp band.

As it was not possible to be sure whether the larger fragment of clone 4.A was the largest (8.8 kbp) or second largest (8.5 kbp) fragment from p8325-4 from Figure 5.3, further digestion of the

Figure 5.3

Gel of six *Bgl* II clones from p8325-4 inserted into the vector pCW59

Lane	Name of clone/digestion	Size of fragments observed (kbp)	Resistance carried
1	λH3	23.1, 9.42, 6.68, 4.36, 2.32, 2.03, 0.56	—
2	undigested pCW59	open circular form	—
3	pCW59 / *Bgl* II	5.3, —	—
4	Clone 4.A	8.8, 5.3, 4.2	Gm
5	Clone 4.A.Gm	8.8, 5.3, 4.2	Gm
6	p8325-4 / *Bgl* II	8.8, 8.5, 6.2, 5.7, 5.0, 3.8	Gm, Cad Pc, Asa
7	Clone 4.C	6.2, 5.3,	—
8	Clone 4.D	5.7, 5.3	Cad
9	Clone 4.E	5.3, 5.0	—
10	Clone 4.F	5.3, 3.8	—



DNA was necessary. With reference to the general restriction map of p8325-4 (Figure 4.4) it can be determined that endonuclease *Sal* I cuts the 8.5 kbp *Pst* I fragment into four pieces but should not cut the 8.8 kbp fragment. The DNA from clone 4.A was therefore digested with *Bgl* II and *Sal* I together and the digest analysed on an agarose gel. *Sal* I did not cut clone 4.A and it was therefore concluded that it contained the larger 8.8 kbp piece. To confirm this, clone 4.A was then digested with *Bgl* II and *Pst* I together. The insert was cut into three fragments of 6.8, 1.5 and 0.5 kbp as expected for the 8.8 kbp piece (the 8.5 kbp piece would have been digested into only two fragments of 6.7 and 1.8 kbp). The *Bgl* II fragment inserted into clone 4.A was therefore the 8.8 kbp piece.

5.4.1 Characterization of resistance determinants.

Plasmid p8325-4 is known to carry genes for conferring resistance to gentamicin, cadmium, arsenate and penicillin. All 15 clones were therefore tested to determine whether they carried these resistances. One clone was resistant to gentamicin, three were resistant to cadmium but none were resistant to arsenate and none produced β-lactamases (Figure 5.3).

5.4.2 Characterization of clone 4.A.

It cannot be concluded that the gentamicin resistance is specified by the 8.8 kbp fragment present in clone 4.A since there is also a 4.2 kbp piece present. To characterize these fragments further, the strain was subcultured six times onto BHI-agar plates containing gentamicin at 20μg/ml. Plasmid DNA was then prepared from a single colony. When digested with *Bgl* II and analysed on an agarose gel, fragments of 8.8 and 4.2 kbp were again seen together with vector DNA (5.3 kbp) but now the 8.8 kbp piece was present in a much higher proportion (Figure 5.3, lane 5). This interesting observation was followed up by further analysis of the 4.2 kbp fragment.

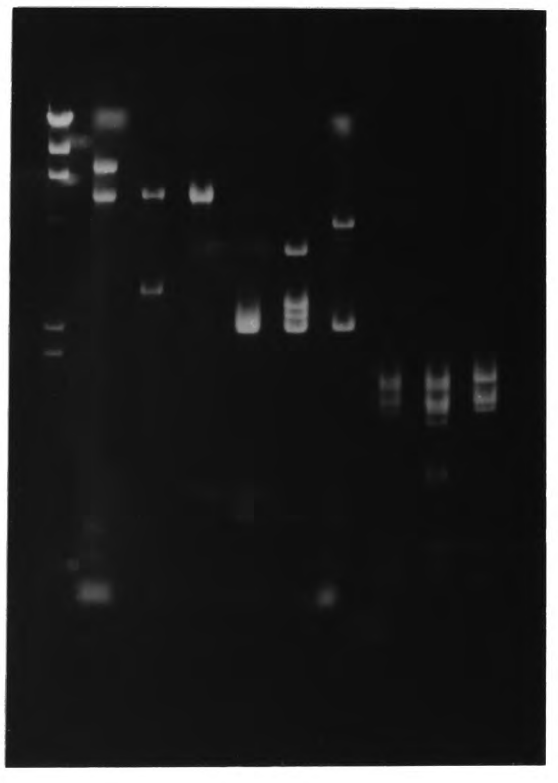
Firstly both clone 4.A and 4.A.Gm (the latter being the 4.A clone subcultured onto gentamicin) were digested with *Bgl* II and *Pst* I. The results are shown in Figure 5.4. Comparison of the relative amounts of DNA present led to the conclusion that the 8.8 kbp piece was digested by *Pst* I into three fragments of 6.8, 0.9 and 0.6 kbp (lane 2). The 4.2 kbp piece was digested by *Pst* I into three pieces of 2.6, 0.9 and 0.6 kbp. Digestion with *Hin*d III resulted in the cleavage of the 8.8 kbp piece into three pieces of 3.6, 2.9 and 2.5 kbp (lane 6), whilst the 4.2 kbp piece was uncleaved by *Hin*d III (lane 7). The *Sau* 3A digestions confirmed that the 8.8 and 4.2 kbp fragments were related.

Figure 5.4

Gel showing the insertion of Tn*4001* within the clone 4.A

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	Clone 4.A.Gm/*Bgl*II/*Pst*I	6.8 5.3 0.9 0.6
3	Clone 4.A/*Bgl*II/*Pst*	8.8(*p*) 5.3 4.4(*p*) 2.6 0.9 0.6
4	pCW59/*Bgl*II/*Pst*I	5.3
5	pCW59/*Bgl*II/*Hin*dIII	2.7 1.9 0.7
6	Clone 4.A.Gm/*Bgl*II/*Hin*dIII	3.6 2.9 2.7 2.5 1.9 0.7
7	Clone 4.A/*Bgl*II/*Hin*dIII	4.2 3.9(*p*) 3.7(*p*) 2.7 1.9 0.7
8	Clone 4.A/*Bgl*II/*Sau*3A	—
9	Clone 4.A.Gm/*Bgl*II/*Sau*3A	—
10	pCW59/it BglII/*Sau*3A	—

(p) represents a partial fragment



The vector, pCW59 was not cut by *Pst* I (lane 4) but was cleaved by *Hin*d III into fragments of sizes 2.7, 1.9 and 0.7 kbp (lane 5).

Finally, both clones were digested with *Bgl* II and *Hae* III (results not shown). The 8.8 kbp piece was digested into four pieces of 3.8, 2.5, 1.6 and 0.4 kbp, whilst the 4.2 kbp fragment was cut into three fragments of 2.5, 1.6 and 0.2 kbp. Following the analysis of these results (Section 5.6) it was concluded that the gentamicin resistance on plasmid p8325-4 was carried on the transposon Tn*4001*. The 8.8 kbp fragment carries the 4.7 kbp transposon Tn*4001* which has been deleted in the 4.2 kbp fragment.

5.4.3 Localization of the transfer region.

The plasmid p8325-4 directs transfer to 8325 Nov^r but not to BIII Nov^r (Tables 3.1 and 3.2). The ability of an example from each of the clones to transfer tetracycline resistance to 8325 Nov^r was therefore tested. The results given in Table 5.1 show that none of the clones transferred tetracycline resistance at a frequency significantly higher than that for transfer by pCW59 alone. It was concluded that the complete transfer region had not been cloned.

5.5 Cloning *S.aureus* plasmid DNA into a shuttle vector

Due to the availability of only a limited number of *S.aureus* plasmid vectors, it was decided to use an *E.coli* vector, of which there are many. Cloning into an *E.coli* vector would also be an advantage in that the frequency of transformation is considerably higher in this organism (typically about 10^7-10^8 transformants per microgram of vector DNA) than it is in *S.aureus*.

To determine if the functional transfer region from one of the gentamicin resistance conferring plasmids had been cloned into *E.coli*, it was necessary to be able to transform the clones back into *S.aureus*. The vector used had therefore, to be a shuttle vector, capable of replication and expression of its resistance determinants in *E.coli* and *S.aureus*.

5.5.1 Strategy for the construction of the shuttle vector pACT.

It was decided to construct a shuttle vector containing a single *Pst* I site as a suitable *E.coli* plasmid was readily available. This plasmid, called pRWY21, contained a functional origin of

Table 5.1

Conjugation frequencies of *Bgl* II clones of p8325-4 in the vector pCW59

Name of clone	Size of *Bgl* II fragment cloned	Frequency of Conjugation
8325N[pCW59]	—	1.2×10^{-6}
Clone 4.A	8.8 and 4.2	3.0×10^{-6}
Clone 4.A.Gm	8.8 and 4.2	3.1×10^{-6}
Clone 4.C	6.2	5.6×10^{-6}
Clone 4.D	5.7	7.2×10^{-6}
Clone 4.E	5.0	6.0×10^{-6}
Clone 4.F	3.8	2.1×10^{-6}
p8325-4	—	4.2×10^{-6}

replication in *E.coli* (being derived via pRW33 from pBR322) as well as two resistance determinants (ampicillin and tetracycline). The former is inactivated by *Pst* I cleavage. To enable the shuttle vector to replicate in *S.aureus*, the origin of replication from the *S.aureus* plasmid pC194 was ligated to pRWY21. Plasmid pC194 contains two *Cla* I sites. The origin of replication is known to be located on the same *Cla* I fragment (the larger, 1.7 kbp fragment) as the chloramphenicol resistance determinant (Horinouchi and Weisblum, 1982a). By ligating the two origins of replication from pRWY21 and pC194 together, a plasmid theoretically capable of replication in both *E.coli* and *S.aureus* could be constructed. A diagram outlining the method of construction of pACT is given in Figure 5.5.

5.5.2 Construction of the shuttle vector.

Plasmid DNA was prepared from the *E.coli* strain RRI[pRWY21] and the *S.aureus* strain 8325[pC194]. The endonuclease *Cla* I was used to digest plasmid pRWY21 into its linear form (8.9 kbp) and pC194 into two fragments of 1.7 and 1.2 kbp. Ligation was followed by transformation into *E.coli* MC1061. Transformants resistant to chloramphenicol, tetracycline and ampicillin resulted in the selection of a shuttle vector consisting of the 1.7 kbp *Cla* I fragment from pC194 ligated into the single *Cla* I site of pRWY21. Sixteen such transformants were actually obtained. One was chosen, tested using a multodisk for correct resistance patterns and named pACT. Further verification of the shuttle vector was carried out by digesting the plasmid DNA with appropriate enzymes and running the resulting fragments on an agarose gel. The results of one such gel are shown in Figure 5.6 and verify that pACT is indeed the correct shuttle vector of about 10.6 kbp.

5.5.3 Cloning plasmid DNA from p8325-4 into pACT.

A diagrammatic representation of the cloning strategy is given in Figure 5.7.

To clone plasmid DNA from p8325-4 into pACT, 1μg of p8325-4 DNA and 20ng of pACT DNA were separately digested to completion with *Pst* I and then ligated. Following transformation into *E.coli* MC1061, chloramphenicol-resistant, tetracycline-resistant colonies were selected and screened for ampicillin sensitivity. In total, 587 Cm^r Tc^r transformants were obtained of which, 138 were sensitive to ampicillin. Plasmid DNA was initially prepared from 15 clones and analysed after *Pst* I digestion for the DNA insert.

The results from a selection of five pACT clones are shown, together with *Pst* I digestions of

Figure 5.5

A synopsis of the construction of a shuttle vector for cloning *Pst* I restriction fragments.

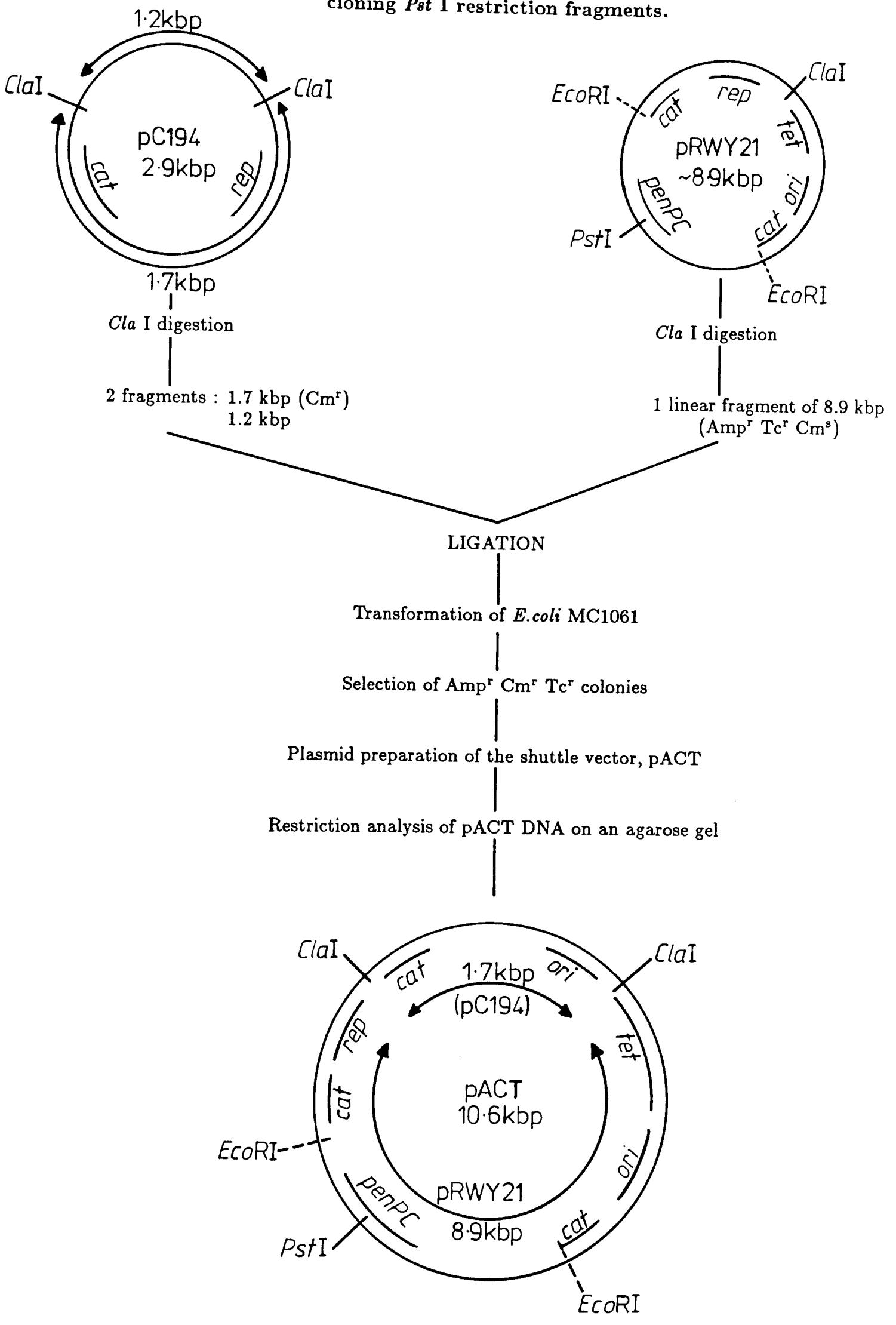


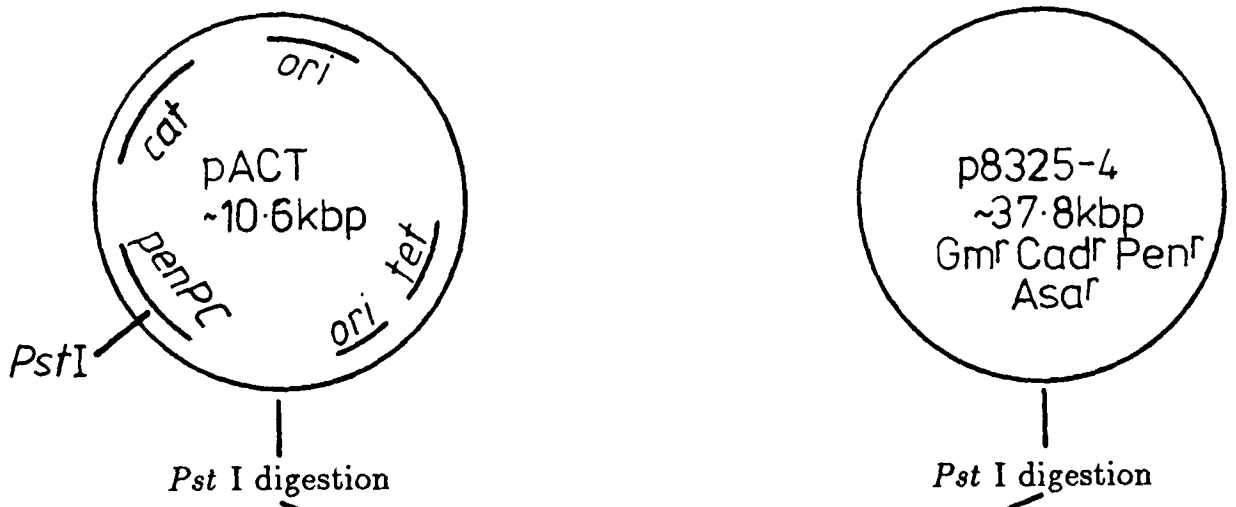
Figure 5.6

Gel showing that pACT is the required shuttle vector

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	pACT undigested	closed circular DNA
3	pACT*Pst*I	10.6
4	pACT/*Pst*I/*Cla*I	6.5 3.4 1.7
5	pACT/*Cla*I	8.9 1.7
6	pACT/*Eco*RI	6.3 4.3
7	pACT/*Eco*RI/*Cla*I	4.3 3.4 1.7 1.2
8	pRWY21/*Eco*RI	4.6 4.3
9	pRWY21/*Cla*I	8.9
10	pACT/*Hin*dIII	6.0 4.8
11	pRWY21/*Hin*dIII	6.1 3.2
12	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56

Figure 5.7

A synopsis of the strategy for cloning restriction fragments from p8325-4 into the shuttle vector pACT.



Pst I digestion

Pst I digestion

LIGATION

Transformation of *E.coli* MC1061

Selection of Cm^r Tc^r colonies

Screen for Amp^s colonies

Plasmid preparation of clones

Sizing (by agarose gel electrophoresis) of the cloned *Pst* I fragment

Transformation of clones into *S.aureus* (selection of Cm^r Tc^r colonies)

Test for the conjugal transfer of tetracycline or chloramphenicol resistance.

p8325-4 and pACT itself in Figure 5.8. The size of the inserted *Pst* I fragment is given for each clone.

Of the five possible *Pst* I fragments, three were cloned into pACT (from the first 15 samples analysed). The number of times each fragment was cloned into pACT is given in parenthesis after the size of the fragment (in kbp) below:

14.4 (0): 7.6 (0): 6.8 (1): 5.4 (8): 3.4 (6).

The clone pACT.10 was resistant to gentamicin.

As it was difficult to distinguish between the *Pst* I fragments of 7.6 and 6.8 kbp from Figure 5.8, clone pACT.10 was digested with suitable enzymes to verify that it did indeed contain the 6.8 kbp fragment. The result is shown in Figure 5.9.

The clone pACT.10 had no sites for *Eco* RI or *Sph* I but was cleaved by *Cla* I into three fragments of about 2.7, 2.5 and 1.6 kbp. If it had contained the larger 7.6 kbp fragment the inserted *Pst* I fragment would have been cleaved by *Eco* RI into fragments of 2.8, 2.4, 2.3 and 0.2 kbp, by *Sph* I into two fragments of 6.4 and 1.2 kbp and by *Cla* I into two fragments of 5.5 and 2.1 kbp. In addition, as expected for the 6.8 kbp fragment, the cloned *Pst* I fragment was not cut by *Bcl* I, *Bgl* II or *Xba* I enzymes (results not shown). The larger 7.6 kbp fragment would have been cleaved by all three enzymes. The slight variation in fragment sizes between these results and those shown on Figure 4.4 are due to errors inherent in restriction mapping.

5.5.4 Localization of the transfer region.

To determine if the complete transfer region had been cloned on any one of the *Pst* I fragments, it was necessary to transform the clones back into *S.aureus* to perform a conjugation experiment.

Plasmid DNA was therefore prepared from the clones pACT.1, pACT.5 and pACT.10 as well as from pACT itself and transformed into *S.aureus* 8325N. Although tetracycline and chloramphenicol were used for selection, no transformants were obtained even after 12 attempts had been made. varying conditions such as antibiotic concentration (5-20μg of either antibiotic) or quantity of DNA (20ng-1μg) each time. To eliminate the possibility that the *S.aureus* strain 8325N was restricting the DNA from the shuttle vector, a restriction minus strain of *S.aureus* was also used (SA113/83A). Again, no transformants were obtained. As the clones could not be transformed into *S.aureus,* their

Figure 5.8

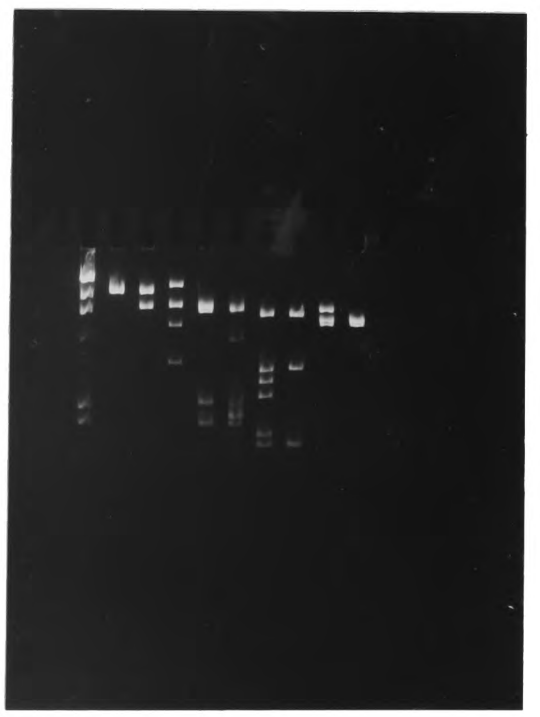
Gel of five pACT/p8325-4 clones

Lane	Name of clone/digestion	Size of fragments observed (kbp)	Resistance carried
1	pACT/*Pst*I	10.6	
2	Clone pACT.5/*Pst*I	10.6, 3.4	—
3	Clone pACT.3/*Pst*I	10.6, 5.4	—
4	Clone pACT.2/*Pst*I	10.6, 5.4	—
5	p8325-4/*Pst*I	14.4, 7.6, 6.8, 5.4, 3.4	Gm, Cad Pc, Asa
6	Clone pACT.14/*Pst*I	degraded	
7	Clone pACT.10/ *Pst*I	10.6, 6.8	Gm
8	pACT/*Pst*I	10.6	—
9	λH3	23.1, 9.42, 6.68, 4.36, 2.32, 2.03, 0.56	—

Figure 5.9

Gel of clone pACT.10 showing it contains *Pst*I fragment C from p8325-4

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	pACT /*Pst*I	10.6
3	Clone pACT.10/*Pst*I	10.6 6.8
4	p8325-4/*Pst*I	14.4 7.6 6.8 5.4 3.4
5	Clone pACT.10/*Pst*I/*Eco*RI	6.9 6.8 2.3 2.1 1.9
6	pACT/*Pst*I/*Eco*RI	6.9 2.3 2.1 1.9
7	Clone pACT.10/*Pst*I/*Cla*I	6.3 2.8 2.7 2.5 1.6 1.5
8	pACT/*Pst*I/*Cla*I	6.3 2.8 1.5
9	Clone pACT.10/*Pst*I/*Sph*I	6.8 5.5 5.3
10	pACT/*Pst*I/*Sph*I	5.5 5.3



ability to promote conjugation between strains of *S.aureus* could not be tested. It was therefore unnecessary to prepare DNA from the remaining 123 clones merely to determine their insertion.

5.6 Discussion

Molecular cloning techniques have been employed with great success for many years for the analysis of chromosomal and plasmid DNA of *Escherichia coli,* whilst genetic analysis of *S.aureus* has been hampered by a poorly defined and low level transformation system (Wilson and Baldwin, 1978). This may be due to the high extracellular nuclease activity characteristic of *S.aureus* (Rudin *et al,* 1974). The transformation system employed in this study was that reported by Lindberg (1981) involving the uptake of plasmid DNA by bacterial protoplasts in the presence of polyethylene glycol.

The maximum number of clones was obtained when 1μg of insert DNA (p8325-2) was ligated to 20ng vector DNA (pCW59). This resulted in 1000 transformants of which, 20 contained suitable insertions The transformation efficiency, calculated as the number of transformants per microgram of plasmid DNA would be 20.

Phosphatase treatment of the vector DNA (pC194 and pE194) was carried out to prevent self-ligation and hence reduce the number of transformants which had to be screened for suitable insertions. The treatment was successful as it reduced the number of transformants containing re-ligated vector molecules from 50 to 2 per plate. The treatment resulted in a loss of DNA however, possibly due to the phenol extraction and ethanol precipitation techniques which were necessary to remove the phosphatase. The technique was not therefore applied routinely as it was considered more practical to use the vector pCW59 (which carries a resistance determinant inactivated by DNA insertion) to screen a higher number of tetracycline-resistant transformants for chloramphenicol sensitivity than to pursue the time consuming process of phosphatase treatment, phenol extraction and ethanol precipitation, especially as the two colonies obtained after phosphatase treatment still contained no insertion.

The selection of suitably resistant transformants proved to be a problem in the use of the vector pBD8. Kanamycin was of no selection value at all whilst the concentration of chloramphenicol was critical. This system might have been of use if more time had been spent in varying the experimental

conditions. As the cloning of *Bgl* II fragments into pCW59 was proving successful however, work was continued with this system in preference to other vector systems (such as pBD8 and pCE10).

The cloning of *Bgl* II fragments into pCW59 was highly successful in that a total of 63 clones were constructed from p8325-2 and p8325-4.

In general, large fragments were not easily ligated into pCW59. Even after the two largest *Bgl* II fragments from p8325-2 had been purified, they were not incorporated. The effective concentration (j) of one end of a DNA molecule in the volume/vicinity of the other end of the same molecule is an important parameter in the ligation of DNA molecules (Maniatis *et al,* 1982). The value of j is based upon the assumption that duplex DNA behaves as a random coil and is inversely proportional to the length of the DNA molecule (as the DNA becomes larger, the ends of a given molecule are less likely to interact).

The frequency with which the two ends of the molecule approach each other is given by the equation:

$$j = \left(\frac{3}{2\pi \ell b}\right)^{\frac{3}{2}} \qquad \text{ends/ml}$$

where ℓ is the length of DNA in centimeters and b is the length of a randomly coiled segment of DNA (dependent upon the ionic strength of the buffer).

The value i is a measure of the concentration of all complimentary termini in the solution. For duplex linear DNA with self- complementary cohesive ends:

$$i = 2 \times N \times M \times 10^{-3} \qquad \text{ends/ml.}$$

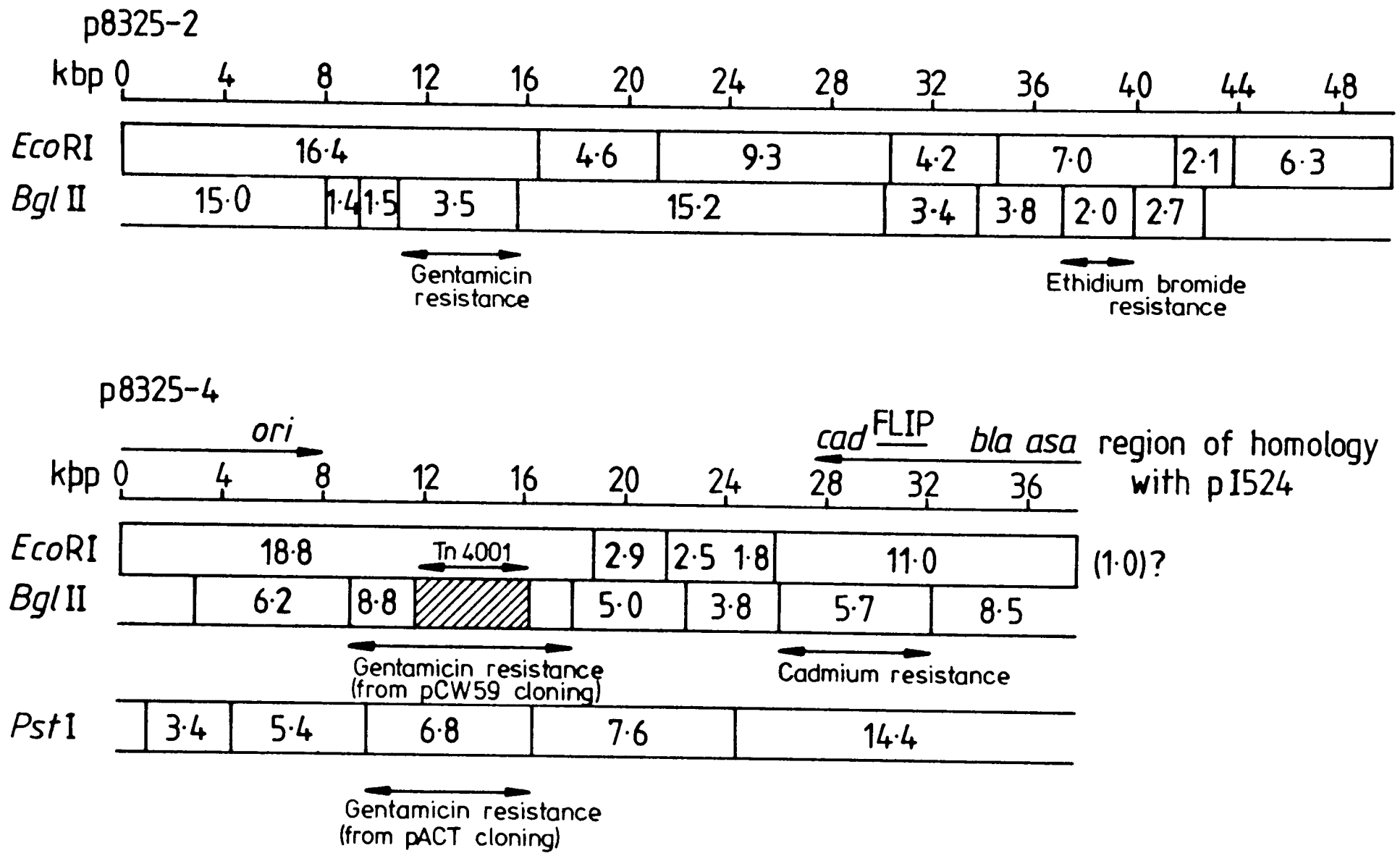
where N. is Avagadro's number and M is the molar concentration of the DNA.

Insertion of DNA is favoured when $j/i > 1$.

Large fragments of DNA are also more likely to be destroyed by cleavage when handling the mixtures. Once cloned, there is more chance that the larger circular molecules will be disrupted. This may explain the inability to clone *Bcl* I fragments from p8325-2 which are relatively large (19.1, 16.6, 9 .1 and 4.4 kbp). Given that the largest fragment cloned into pCW59 was 8.8 kbp (clone 4.A from p8325-4) it is possible that only the smallest, 4.4 kbp fragment was of suitable size and that this, by chance, was not ligated.

Figure 5·10

Diagram showing the resistance determinants located after cloning restriction fragments from p8325-2 and p8325-4



Although only 11 examples are shown for the clones of p8325-2, all six containing the 3.5 kbp insertion are resistant to gentamicin and all six containing the 2.0 kbp fragment are resistant to ethidium bromide. The position of these determinants is shown on Figure 5.10. No clone was resistant to paromomycin, the determinants of which must lie either on one of the uncloned fragment or may have been spanned by a *Bgl* II site.

The 8.8 kbp *Bgl* II fragment from p8325-4 carried the gentamicin resistance determinant whilst all four clones containing the 5.7 kbp fragment were resistant to cadmium. In addition, the gentamicin resistance determinant was present on the 6.8 kbp *Pst* I fragment cloned into pACT. This data is shown on Figure 5.10. The location of the gentamicin resistance determinant on the *Bgl* II and *Pst* I fragments correlates well and places it between 9.9 and 16.4 kbp on the general map (Figure 4.4). This is outside the region of homology with pI524. The cadmium resistance determinant lies just within the 5.7 kbp *Bgl* II fragment. It is therefore probable that p8325-4 and pI524 carry the same cadmium resistance determinant. Both the arsenate resistance and the gene specifying β-lactamase production for pI524 lie within the uncloned 8.5 kbp *Bgl* II fragment, explaining why no clone was resistant to arsenate or ampicillin.

The location of the gentamicin resistance determinant within the 6.8 kbp *Pst* I and 8.8 kbp *Bgl* II fragments (9.9-16.4 kbp on Figure 5.10) correlates with the results from deletion analysis, when the determinant was placed between 9.0 kbp (the boundary of the region of p8325-4/pI524 homology) and 15.1 kbp (the boundary of the *Cla* I fragment) (Figure 4.13).

There are several possible reasons why the complete transfer region was not located on any one *Bgl* II fragment for either plasmid. Firstly, the transfer region may be spanned by a *Bgl* II site (and by a *Pst* I site for p8325-4). For p8325-2 it is also possible that the transfer region lies on one of the larger, uncloned *Bgl* II pieces which together represent about 60% of the plasmid genome. Another possibility is that the region was cloned in the opposite orientation for expression. Finally, several regions, widely separated on the genome, may be needed to promote conjugal transfer.

To pursue the technique of cloning to isolate the transfer region, partial fragments could be generated from the plasmids and cloned into pCW59. This would increase the chance of isolating the complete transfer region if it was positioned across a *Bgl* II site although the cloning of larger fragments may be a problem.

It may also be useful to clone restriction fragments created by other enzymes into plasmid vectors. Keller *et al* (1983) constructed a series of chimeric plasmids suitable as molecular cloning

vehicles in *S.aureus* and *S.carnosus*. The vector pCE10 is one such plasmid but others include pCT20 (Cm^r Tc^r) with a single *Ava* II site, or pTE15 (Tc^r Em^r) with single sites for *Bcl* I, *Eco* RI and *Kpn* I.

With reference to Table 5.1 showing the rates of transfer for the p8325-4/pCW59 clones, it should be noted that the conjugation frequency given for p8325-4 (4.2×10^{-6}) is actually lower than values for some of the clones. The value is also lower than the one given in Table 3.2 (3.93×10^{-5}) which was obtained during the initial series of conjugation experiments. This result was noted with interest and will be discussed in detail in Chapter 8.

To determine the frequencies of conjugation for the various clones of p8325-4, selection was made on tetracycline plus novobiocin plates for all the clones and for pCW59, whereas gentamicin plus novobiocin plates were used for p8325-4 itself. The final conjugation frequency value seems to be independent of the selection system employed however, since the conjugation of clone 4.A (Tc^r Nov^r Gm^r) resulted in the same number of transconjugants with either selection system.

The plasmid p8325-2 was transferred to BIII Nov^r at its usual high frequency (5.12×10^{-4}) whereas pCW59 and all the corresponding clones were not transferred.

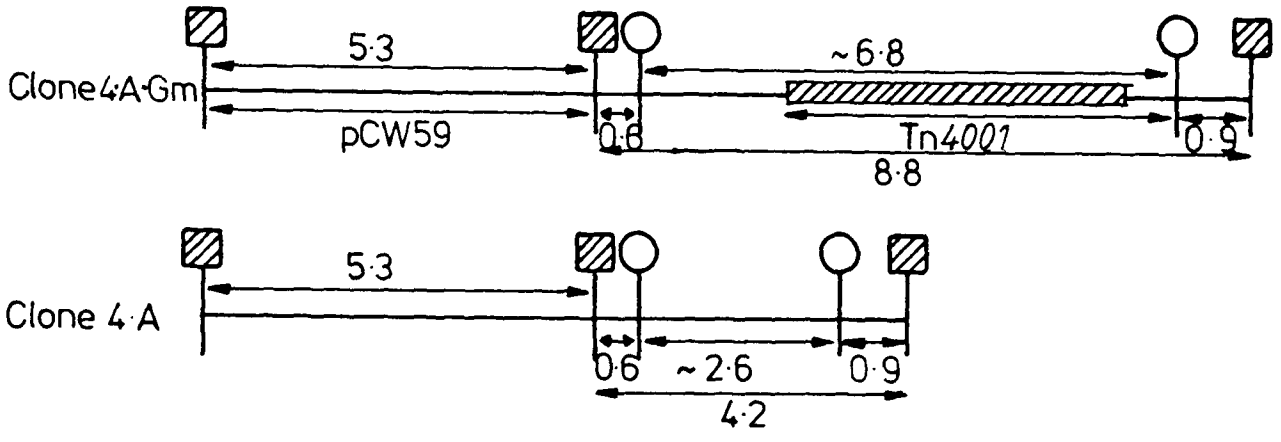
An interesting observation was made when clone 4.A from the ligation of p8325-4/pCW59 was analysed on a gel. It contained an insertion of 4.2 kbp as well as the 8.8 kbp *Bgl* II fragment. Although the smaller fragment was initially present in larger amounts, following subculture onto gentamicin, the larger piece became more dominant. Results of further restriction analysis on these fragments led to the conclusion that the smaller fragment was the result of a deletion of about 4.5 kbp from the central region of the larger fragment. The deleted piece is probably the transposon Tn*4001* (Figure 5.11).

The 4.5 kbp transposon, Tn*4001* mediates resistance to gentamicin, tobramycin and kanamycin in *S.aureus* and was present on all gentamicin resistance plasmids in a survey of Australian isolates (Gillespie and Skurray, 1986). Figure 5.11 helps to explain the results of the restriction digests shown in Figure 5.4.

The fragments of 8.8 and 4.2 kbp, when digested with *Pst* I, produce fragments of 6.8 and 2.6 kbp respectively. This can be explained if there is a fragment of approximately 4.2 kbp deleted from between the two *Pst* I sites in the 8.8 kbp piece. This 4.2 kbp deleted fragment represents the

Figure 5·11

Diagram showing the insertion of Tn*4001* within the 8·8kbp *Bgl* II fragment of p8325-4



transposon Tn*4001*. The slight variation in the fragment sizes is inherent in restriction mapping. In fact, the size of Tn*4001* has recently been reported as being 4.7 kbp (Gillespie and Skurray, 1986) rather than 4.5 kbp as was originally thought.

*Hin*d III cuts the 8.8 kbp fragment into three pieces of 3.6, 2.9 and 2.5 kbp. The two *Hin*d III sites within the 8.8 kbp piece lie within the transposon Tn*4001,* each about 1.1 kbp from each of its ends, giving a characteristic 2.5 kbp fragment which encompasses the central coding region (Gillespie and Skurray, 1986). The 4.2 kbp fragment has lost Tn*4001* (and hence the two *Hin*d III sites) and therefore appears as an uncleaved 4.2 kbp piece.

Finally, the *Hae* III cut the 8.8 kbp fragment into four pieces (3.8, 2.5, 1.6 and 0.4 kbp) and the 4.2 kbp fragment into three pieces (2.5, 1.6 and 0.2 kbp) (results not shown). The transposon must therefore contain a single *Hae* III site with the two sites in the 8.8 kbp piece lying close to the boundaries of the Tn*4001* insertion so that, after deletion, fragments of 2.5, 1.6 and 0.2 kbp are obtained. The distance between the two *Hae* III sites would be 0.2 kbp. The position of Tn*4001* within p8325-4 is between 11.8 and 16.3 kbp (Figure 5.10).

The transposon Tn*4001* appears to be stable in p8325-4, it is stably inherited when transferred via conjugation to the recipient 8325 Nov^r. Once cloned into pCW59 however, it becomes unstable and the tendency is for it to delete out of the vector system. The 8.8 kbp gentamicin resistance conferring fragment was selected for by plating on gentamicin which selected those colonies in which the deletion had not yet occurred. It is possible that there is a region present on p8325-4 required to maintain the stability of Tn*4001*. Such a region would lie outside the 8.8 kbp *Bgl* II fragment and would not be present on pCW59.

There is evidence that Tn*4001* can insert at various sites within the *S.aureus* chromosome, perhaps even being responsible for the global spread of gentamicin resistance among *S.aureus* (Gillespie and Skurray, 1986).

The use of the shuttle vector pACT increased the number of clones obtained in any one transformation (138 true clones from 587 transformants compared with 20 clones from 1000 transformants for the cloning of *Bgl* II fragments from p8325-2 into pCW59).

It is interesting that it was possible to select clones on the basis of either chloramphenicol or tetracycline resistance in *E.coli*. This is a little surprising as the chloramphenicol resistance was

derived originally from the *S.aureus* plasmid pC194. Goze and Ehrlich (1980) reported that the pC194 chloramphenicol resistance gene was not adequately expressed for it to be used as a selectable marker in transformation although it has been shown to replicate and express its chloramphenicol resistance in *B.subtius* (Ehrlich, 1977). The plasmid was also shown to replicate in *E.coli* although less efficiently than in *S.aureus* (Goze and Ehrlich, 1980). It is to be expected that tetracycline resistance would be expressed in *E.coil* as pRWY21 was originally derived from the *E.coli* plasmid pBR322 via pRW33. The β-lactamase gene was derived from *B.cereus* 569/H (Mezes *et al*, 1983). It would be interesting to determine if the tetracycline and ampicillin resistances are expressed in *S.aureus*. Their expression in *S.aureus* was not a requirement however as transformants could be selected on the basis of chloramphenicol resistance.

Although the experimental conditions such as amount of vector (20ng-1μg) and antibiotic concentration (5-20μg/ml of either chloramphenicol or tetracycline) under which the transformation was carried out were varied extensively, it was not possible to transform pACT into *S.aureus*.

The reason for the lack of replication and/or expression of chloramphenicol resistance from pC194 is not understood. As it was originally a natural isolate from *S.aureus*, it was able to replicate and to express its resistance prior to its incorporation into the shuttle vector. No portion of these determinants had been deleted (Figure 5.6) (Horinouchi and Weisblum, 1982a). After transformation into *E.coli*, however, one or both of these functions was suppressed. There are two possible explanations for this. The *E.coli* strain MC1061 may have modified the shuttle vector, possibly by some type of restriction or methylation process which prevented the subsequent expression of chloramphenicol resistance or ability to replicate in *S.aureus*. If the modification process involved were known, it may be possible to use a modification deficient mutant strain of *E.coli*.

Another possibility is that the *S.aureus* restriction system may be degrading the "foreign" DNA from the pRWY21 plasmid. Although a restriction deficient strain of *S.aureus* was used (SA113) to eliminate this possibility, it is possible that there is a second restriction system in *S.aureus* still functional in SA113.

The use of shuttle vectors in *E.coli* is well known. Leblanc and Lee (1984) have reported the cloning of a 5.0 kbp fragment from the *Streptococcus faecalis* plasmid pAMβ1 into *E.coli* HB101 and used this to transform *Streptococcus sanguis* Challis with concurrent expression of the *E.coli* kanamycin resistance determinant. Chang and Cohen (1974) reported the replication of hybrid plasmids constructed from *S.aureus* plasmid DNA (pI258) covalently joined to the *E.coli* plasmid

pSC101. Although the plasmids could be transferred among *E.coli* strains, they were not transferred into *S.aureus*. Goze and Ehrlich (1980) have also combined *S.aureus* and *E.coli* plasmids, again showing that some plasmids from a Gram-positive bacterium (*S.aureus*) can replicate in a Gram-negative one (*E.coli*). More recently, El Solh *et al,* (1986b) cloned the chromosomal genes for two aminoglycoside-modifying enzymes into *E.coli* and *B.subtilis* strains using the shuttle vector pHV33 which consists of pBR322 and pC194 joined at their *Hind* III sites (Primrose and Ehrlich, 1981). In none of these examples were the vectors transformed from *E.coli* back into *S.aureus* although it has been possible to transform hybrids between *E.coli* and *B.subtius* (Primrose and Ehrlich, 1981). It may be possible therefore to transform pACT into *S.aureus* via *B.subtilis*. There are no examples in the literature of this having been achieved to date.

Finally, the problem may be associated with the nuclease activity of *S.aureus*. The shuttle vector may be susceptible to nuclease attack by extracellular enzymes excreted by this organism. One way of testing this theory would be to use a nuclease deficient mutant of *S.aureus* (Omenn and Friedman, 1970).

5.7 Conclusion

Cloning restriction fragments into the limited number of *S.aureus* vectors available is made even more difficult by the low level transformation system available.

The successful cloning of *Bgl* II digested fragments however from p8325-2 into pCW59 located the gentamicin and ethidium bromide resistance determinants to between 10.9-14.4 and 37.5-39.5 kbp respectively on the general restriction enzyme map (Figure 4.2).

The gentamicin resistance of p8325-4, carried by the transposon Tn*4001,* lies between 11.8 and 16.3 kbp which agrees with deletion analysis data as well as from the cloning of *Pst* I fragments. This transposon is stable in p8325-4 but becomes unstable and deletes out of the system when cloned into the pCW59 vector. It is possible that a region on p8325-4, outside the 8.8 kbp *Bgl* II fragment, is required to maintain the transposon's stability. The cadmium-resistance determinant is probably the same as that on pI524, residing between 26.2 and 32.3 kbp.

Cloning restricted DNA from *S.aureus* into an *E.coli* system proved to be considerably more efficient. It was not possible however, to transform the shuttle vector into *S.aureus*. Reasons for

this include the modification by *E.coli* of the DNA, preventing its replication or expression of the resistance determinants in *S.aureus*, or degradation of the DNA by the *S.aureus* strain.

The complete transfer system was not isolated on any of the cloned restriction fragments. Whether this was due to its cleavage by a *Bgl* II/*Pst* I site, its incorrect orientation within the vector or because there is more than one region involved is unknown.

Chapter 6

Mobilization of Genes by Conjugative Plasmids and Production of Pheromones by Staphylococci

6.1 Introduction

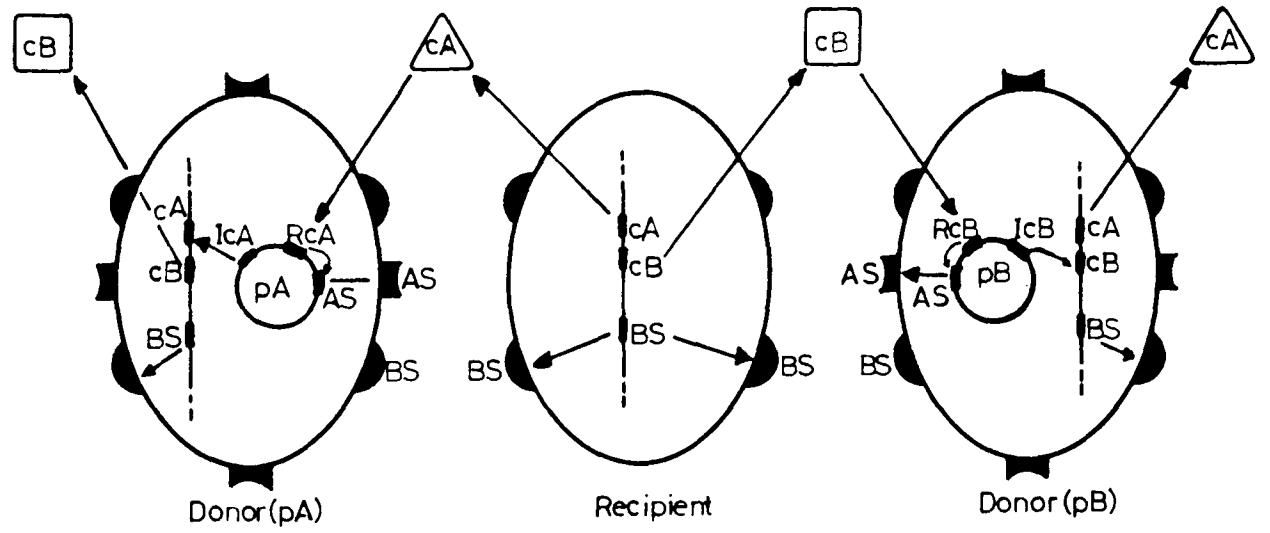
Two types of conjugative plasmids are found in *Streptococcus faecalis* (Clewell, 1981). The first type are transferred very poorly in broth (usually $< 10^{-6}$ per donor cell) but more efficiently (10^{-4} – 10^{-2} per donor cell) when matings are carried out on filter membranes. The best studied example of a plasmid exhibiting this forced-cell-contact type of transfer is pAMβ1, originally found in a clinical isolate of *S.faecalis*. This type of plasmid has been shown to have a broad host range. In addition to its ability to transfer to virtually every species of streptococci (Clewell, 1981) it transfers to *Staphylococcus aureus* (Engel *et al,* 1980; Schaberg *et al,* 1982), *Bacillus subtilis* (Landman *et al,* 1980) and *Lactobacillus casei* (Gibson *et al,* 1979). At least one conjugative function on pAMβ1 has been located within an 8 kbp region (Leblanc and Lee, 1984).

The other type of conjugative plasmid, observed to date only in *Streptococcus faecalis,* transfers at high frequency (10^{-3} – 10^{-1} per donor) in broth matings. The transfer is associated with visible clumping of donor and recipient cells (Dunny *et al,* 1978). The recipient cells constitutively produce small (1000–2000 Md) heat-stable proteins called clumping-inducing-agent (CIA) (Dunny *et al,* 1978). These mating signals (sex pheromones) induce the synthesis of a proteinaceous adhesin that coats the donor cell surface (Yagi *et al,* 1983). The adhesin is called aggregation substance (AS) and is believed to facilitate the formation of mating aggregates upon random collision between the non-motile donors and recipients.

The aggregation substance is believed to adhere to a binding substance which is located on the surface of both donor and recipient cells. The different pheromones are designated by their relationship to the plasmid system originally used to resolve them. For example, cAD1 and cAMγ2 refer to the pheromones to which specific responses occur by strains respectively harbouring the plasmids pAD1 and pAMγ2. These phenomena are summarized by the model proposed by Dunny *et al,* (1979) shown in Figure 6.1.

Figure 6·1

A model showing various donor and recipient relationships with respect to the synthesis of and response to sex pheromones.



Taken from Dunny et al, 1979
See text for details.

This model schematically shows a plasmid-free recipient strain that produces two different pheromones, cA and cB; two isogenic donor strains harbouring the conjugative plasmids pA and pB are also present. All three strains have the chromosomally determined binding substance. Plasmid pA determines the ability to respond to cA and, at the same time, through an IcA gene (inhibitor of cA) prevents production of endogenous cA (alternatively inactivation of cA could be involved). Plasmid pB allows its host to respond to cB and prevents the production of endogenous cB via gene IcB. The response of the donor cell to the pheromone is shown as an interaction of the latter with a responding substance determined by gene RcA or RcB which activates aggregation substance synthesis. Aggregation substance, which could actually be plasmid or chromosomally determined, locates itself on the cell surface where it recognizes binding substance.

Clewell and Brown (1980) have shown that in addition to the production of surface substances, pheromones are also involved in the actual transfer of plasmid DNA. It is possible that they induce a polycistronic operon which contains determinants related to transfer as well as to aggregation. This may be analogous to the transfer operon of certain conjugative plasmids in Gram-negative bacteria (Willetts and Skurray, 1980).

Once a copy of plasmid DNA has been acquired by the recipient, the production of the related pheromone ceases; however, different pheromones specific for donors harbouring different classes of plasmids continue to be excreted (Dunny *et al,* 1979). Pheromone inactivation is thought to be via a chemical addition to the pheromone itself (Ike et al, 1983). A phosphodiester bond is generated and the modified pheromone inhibits exogenous pheromone activity probably by competition for a pheromone receptor site (Clewell *et al,* 1985).

Insertional inactivation of the pheromone-responsive plasmid pAD1 with Tn*917* produced mutants that constitutively expressed AS and exhibited self-clumping in the absence of pheromone (Clewell *et al,* 1982). The insertions were mapped to two regions, designated *tra* A (1.5 kbp) and *tra* B (1.3 kbp). The two clusters were spanned by 1.7 kbp.

The naturally occurring conjugative pheromone-dependent resistance plasmid pCF-10 (Dunny *et al,* 1981) has now been mapped with restriction enzymes and mutagenesis with Tn*917* has been used to determine several regions involved in the conjugative transfer. Immunoblot analyses and enzyme-linked immunoassays have revealed that cells carrying pCF-10 derivatives with Tn*917* insertions into the *tra* 5 region are defective in synthesis of pheromone-induced antigens (Christie and Dunny, 1986).

Streptococcus faecalis RC73 harbours a conjugative plasmid, (pAM373) which confers a mating response to a sex pheromone, cAM373 excreted by plasmid -free members of the same species. This pheromone was also detected in 23 *Staphylococcus aureus* strains but in only 2 of 22 coagulase-negative staphylococci strains. The activity was also produced by *Streptococcus faecium* 9790, *Streptococcus sanguis* Challis and G9B but not by 10 other *Streptococcus* strains (Clewell *et al,* 1985).

In this survey, all available *S.aureus* strains, including those carrying gentamicin resistance plasmids were tested for pheromone production. Other bacterial species were also tested.

The first part of the chapter however, describes the mobilization of small, non-conjugative plasmids by the large, conjugative gentamicin resistance plasmids and provides evidence for second round transfer of gentamicin resistance by conjugation.

Many non-conjugative R-plasmids are spread more rapidly between strains of bacteria than are chromosomal genes due to their mobilization by conjugative plasmids (see Hardy, 1986). The transmissible drug-resistance of a strain is sometimes the result of it harbouring several R plasmids, only some of which are conjugative. For example, *Salmonella typhimurium* type 29, responsible for the outbreak of salmonellosis between 1963-66 carried resistance determinants for sulphonamide, streptomycin, ampicillin and tetracycline on non-conjugative plasmids. All these determinants could be transferred from *Salmonella* via conjugation due to a conjugative plasmid called Δ (see Hardy, 1986).

In *E.coli,* more than 90% of recipients of the F plasmid from a donor harbouring both F and the non-conjugative plasmid, Col E1, also receive Col E1 and about 5% of recipients receive Col E1 but not F.

The extent to which mobilization occurs depends both on the conjugative and non-conjugative plasmids involved. For example, the *S.aureus* plasmid pSH8 mobilized pC22.1, pSH5 and pI258 at frequencies of 1.1×10^{-4}, 8.7×10^{-6} and 3.1×10^{-9} respectively. It was transferred itself at about 1.4×10^{-4} (McDonnell *et al,* 1983). There was no detectable mobilization by pSH8 of a chromosomal methicillin resistance marker.

The co-transfer frequency of the non-conjugative *S.aureus* plasmid pWG612 ranged between less than 0.5 to 83%. depending on the culture conditions and transfer method (Townsend *et al,*

1985). There was no detectable co-transfer however between the conjugative plasmid pWG53 and pWG3.

Finally, the ability of a conjugative, erythromycin resistance conferring streptococcal plasmid (pAMβ1) to mobilize a non-conjugative tetracycline resistance conferring plasmid (pAMα1) from a streptococcal strain, JH2-2 to *S.aureus* 879R-4S was examined (Schaberg *et al,* 1982). Mobilization of pAMα1 occurred at a frequency of 10^{-8}, representing 14% of the transferred erythromycin resistant strains. The conjugative erythromycin resistance plasmid was present in all tetracycline-resistant strains, therefore the mobilization of the non-conjugative plasmid had only occurred with concomitant transfer of the conjugative plasmid.

6.2 Mobilization of plasmid and chromosomal genes by conjugative plasmids

A series of conjugation experiments were set up to determine whether the conjugative plasmids conferred the ability to mobilize genes present on a separate plasmid or on the chromosome. As four different recipients were available, comparison could also be made of the effect of the recipient on the frequency of transfer of the plasmid and chromosomal genes. For ease of selection, the chromosomal gene chosen was the erythromycin resistance determinant present on Tn*551* although it was realized that transposition might complicate the interpretation of the result.

To construct suitable strains, the gentamicin-resistant strains 8325[p8325-2] and 8325[p8325-4] were conjugated with the recipients 8325[pT181] and PS80d.*ero*. The former recipient contains a plasmid of 4.4 kbp which confers resistance to tetracycline. The latter carries the transposon Tn*551* on the chromosome. As a control experiment both donors were also conjugated with the recipients B111 Nov^r and 8325 Nov^r.

The transfer frequencies for each conjugation were calculated as the number of transconjugants obtained per recipient. These values were broadly classified into four classes:

none (less than 2.7×10^{-8})

low (5.3×10^{-8} — 4.5×10^{-7})

medium (1.7×10^{-6} — 1.2×10^{-5})

high (1.1×10^{-4} — 1.4×10^{-4})

The results (Table 6.1) confirm that B111 Nov^r is a good recipient for p8325-2 but a poor one for p8325-4. The presence of pT181 in the recipient enhances the apparent conjugation when 8325[p8325-2] is the donor (the frequency of conjugation to 8325[pT181] is high whereas that to 8325 Nov^r is only medium). No transconjugants were obtained after mixing PS80d.*ero* and 8325[p8325-4] so it was not possible to study the mobilization of the chromosomal erythromycin determinant by this conjugative plasmid.

In a control experiment it was surprisingly found that pT181 was transferred at high frequency to 8325 Nov^r. This may be due to the presence of phage and may explain the very high frequency of conjugation when 8325[pT181] and 8325[p8325-2] are mixed (Table 6.1). Some of these transconjugants may be the result of transfer by phage of pT181 from 8325[pT181] to 8325[p8325-2] and not conjugal transfer. This control result makes it difficult to conclude much about mobilization of plasmids when 8325 Nov^r is the recipient.

When 8325[pT181][p8325-2] is used as donor, the frequency of conjugal transfer of p8325-2 to 8325 Nov^r is less than that obtained in the absence of pT181 when gentamicin is used to select but is very similar when selection is by tetracycline. In the former case, none of the transconjugants are resistant to tetracycline whereas about two-thirds of those selected as tetracycline resistant are also resistant to gentamicin.

Plasmid DNA was prepared from an example of each class of transconjugant, ie,Gm^{r}Nov^{r}Tc^r colonies (containing p8325-2 and pT181); Gm^{r}Nov^{r}Tc^s colonies (containing only p8325-2) and Gm^{s}Nov^{r}Tc^r colonies (containing only pT181). Restriction enzyme digests of the plasmid(s) present in each sample were compared with plasmid DNA prepared from 8325[p8325-2], 8325pT181] and 8325[pT181][p8325-2] (Figure 6.2). In each case, the expected plasmid was present.

With B111 Nov^r as recipient there was a high frequency of conjugation of p8325-2 but the frequency of transfer of pT181 was quite low. However, since pT181 is not transferred at all in the absence of p8325-2, this is evidence that pT181 has been mobilized by p8325-2. A small proportion of the transconjugants selected on tetracycline (and therefore carrying pT181) were gentamicin sensitive; p8325-2 had mobilized pT181 in the absence of its own conjugal transfer (or the p8325-2 plasmid had been transferred but not maintained).

For 8325[pT181][p8325-4] only 8325 Nov^r acts as a recipient. In almost every case, transconjugants that are gentamicin-resistant are also tetracycline-resistant whether the selection is on

Table 6.1

Frequencies of transfer to recipient strains

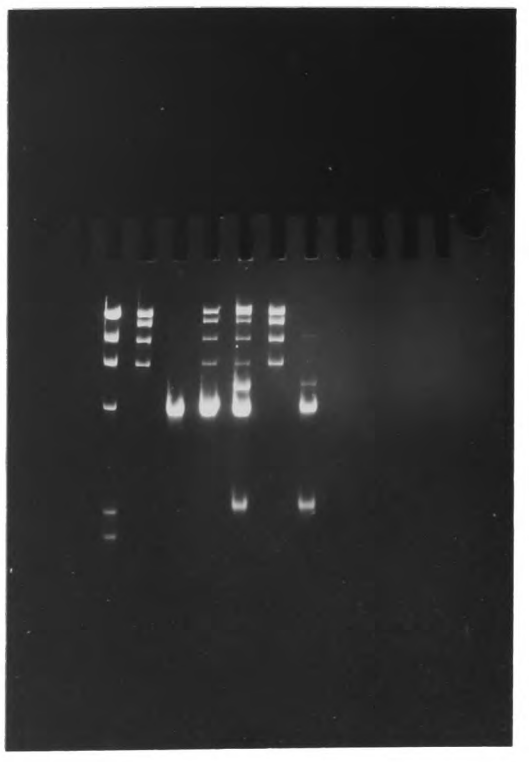
Donor	Selection system	Recipient			
		8325[pT181]	**8325 Nov^r**	**B111 Nov^r**	**PS80d*ero*[N]**
8325 [p8325-2]	Gm + Tc Gm + Nov Gm + Em	high — —	— med —	— high —	— — low
8325 [p8325-4]	Gm + Tc Gm + Nov Gm + Em	med — —	— med —	— none —	— — none
8325 [pT181]	Tc + Nov Tc + Em		high —	none —	— none
8325 [pT181] [p8325-2]	Gm + Nov Gm + Em Tc + Nov Tc + Em		low ($^{0}/_{34}$ Tc^r) — med ($^{50}/_{76}$ Gm^r) —	high ($^{0}/_{194}$ Tc^r) — med ($^{58}/_{60}$ Gm^r) —	— none — none
8325 [pT181] [p8325-4]	Gm + Nov Gm + Em Tc + Nov Tc + Em		med ($^{210}/_{211}$ Tc^r) — med ($^{54}/_{56}$ Gm^r) —	none — none —	— none — none
PS80d*ero*[N]	Em + Tc Em + Nov	none —	— none	— none	
PS80d*ero*[N] [p8325-2]	Gm + Nov Gm + Tc Em + Nov	— med ($^{0}/_{80}$ Em^r) —	none — none	high — low	

Figure 6.2

Analysis of plasmid DNA prepared from transconjugants of 8325[p8325-2]

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
	Donor	
2	8325[p8325-2]/*Xba*I	20.2 13.6 9.6 6.6
	Recipient	
3	8325[pT181]/*Xba*I	4.4
	First round transconjugant	
4	8325[pT181][p8325-2]/*Xba*I	20.2 13.6 9.6 6.6 4.4
	Second round transconjugants	
5	8325Nov^r[pT181][p8325-2]/*Xba*I (Nov^{r}Tc^{r}Gm^r)	20.2 13.6 9.6 6.6 4.4
6	8325Nov^r[p8325-2]/*Xba*I (Nov^{r}Tc^{s}Gm^r)	20.2 13.6 9.6 6.6
7	8325Nov^r[pT181]/*Xba*I (Nov^{r}Tc^{r}Gm^s)	4.4

Note: open circular and supercoiled forms of pT181 can be seen in lanes 5 and 7.



gentamicin or tetracycline. Again, plasmid DNA was prepared from an example of each class of transconjugant, that is, $Gm^rNov^rTc^r$ colonies; $Gm^rNov^rTc^s$ colonies and $Gm^sNov^rTc^r$ colonies. Restriction enzyme digests of the plasmid(s) present in each sample were compared with plasmid DNA prepared from 8325[p8325-4], 8325[pT181] and 8325pT181][p8325-4] (Figure 6.3). The plasmids present in each case were of the expected size; a 4.4 kbp plasmid (pT181) if resistant to tetracycline; a 50 kbp plasmid (p8325-2) cut into the expected sizes by *Xba* I if resistant to gentamicin.

The donor PS80d.*ero*[p8325-2] transferred gentamicin resistance to both recipients 8325[pT181] and B111 Nov^r. When selection was for gentamicin resistance in the former transfer, none of the transconjugants had become resistant to erythromycin. However, when B111 Nov^r was the recipient, two erythromycin-resistant transconjugants were selected by erythromycin; they were both also resistant to gentamicin. Analysis of the plasmid DNA present in one of these transconjugants (Figure 6.4) showed that it contained a single plasmid very similar to p8325-2 except that it had increased in size by 5.3 kbp. The smallest *Eco*RI fragment of 2.1 kbp had been replaced by a band of 7.4 kbp. It is concluded that in this case, Tn*551* has transposed into the 2.1 kbp *Eco*RI fragment of p8325-2 prior to transfer. There is thus no evidence that p8325-2 or p8325-4 can mobilize a chromosomal gene.

6.3 Establishing a system for detecting sex pheromone activity

The method used to detect sex pheromone activity was based upon that of Dunny *et al* (1979) who reported that the production of pheromone closely paralleled cell growth. An experiment was therefore set up to determine if the level of sex pheromone activity depended upon the extent to which the cells had been grown prior to filtrate production. The strain chosen for analysis was *S.aureus* B111 Nov^r which had been shown in a preliminary series of studies to show a sex pheromone response to the donor *S.faecalis* strain FA373.

Six cultures of B111 Nov^r were grown overnight, diluted 1 in 10 in BHI medium the following morning and grown for five hours at 37°C. The cultures were diluted appropriately to produce a series of six cultures with $O.D._{675}$ values of 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0. A 10 ml sample of each culture was then filtered, autoclaved and later tested for its pheromone activity. The results showed that all six cultures produced the same sex pheromone activity, each having a CIA titre of 4. The control filtrates of *Strep. faecalis* FA2-2 had CIA titres of 8 whilst the filtrate from

Figure 6.3

Analysis of plasmid DNA prepared from transconjugants of 8325[p8325-4]

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
	Donor	
2	8325[p8325-4]/*Xba*I	11.8 6.9 6.5 4.9 3.7 2.5
	Recipient	
3	8325[pT181]/*Xba*I	4.4
	First round transconjugant	
4	8325[pT181][p8325-4]/*Xba*I	11.8 6.9 6.5 4.9 4.4 3.7 2.5
	Second round transconjugants	
5	8325Nov^r[pT181][p8325-4]/*Xba*I (Nov^{r}Tc^{r}Gm^r)	11.8 6.9 6.5 4.9 4.4 3.7 2.5
6	8325Nov^r[p8325-4]/*Xba*I (Nov^{r}Tc^{s}Gm^r)	11.8 6.9 6.5 4.9 3.7 2.5
7	8325Nov^r[pT181]/*Xba*I (Nov^{r}Tc^{r}Gm^s)	4.4

Note: open circular and supercoiled forms of pT181 can be seen in lane 7.

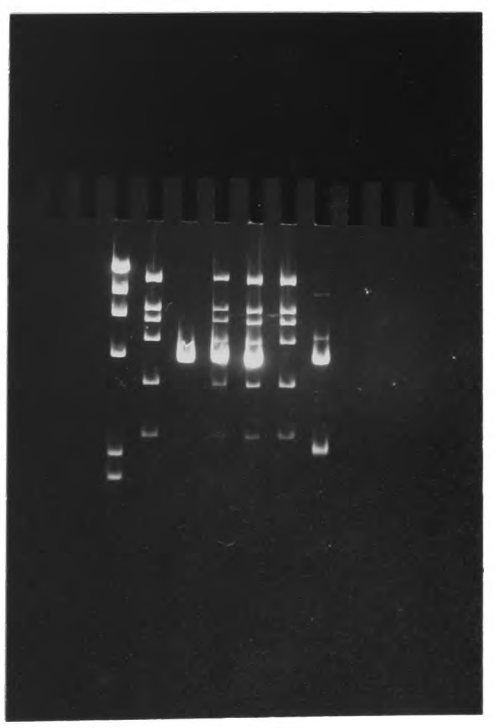
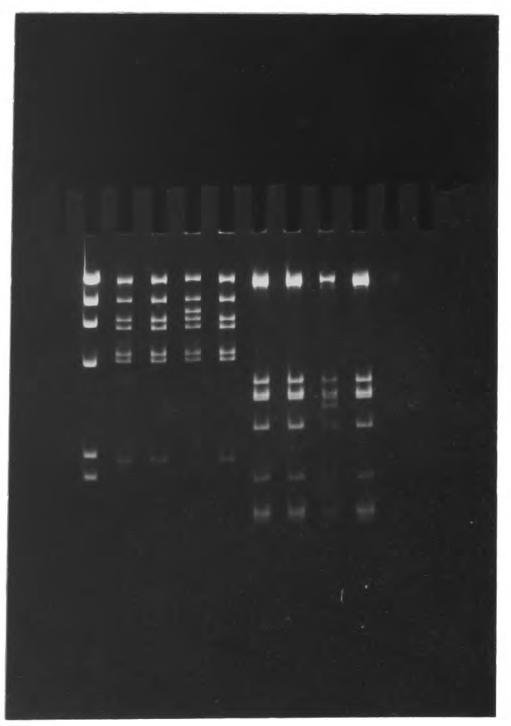


Figure 6.4

Analysis of plasmid DNA prepared from transconjugants of 8325[p8325-2] with recipients PS80d.*ero* and B111Nov^r

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
	Donor	
2	8325[p8325-2]/*Eco*RI	16.4 9.3 7.0 6.4 4.6 4.2 2.1
	First round transconjugant	
3	PS80d.*ero*[p8325-2]/*Eco*RI	16.4 9.3 7.0 6.4 4.6 4.2 2.1
	Second round transconjugant	
4	B111Nov^r[p8325::Tn*551*]/*Eco*RI	16.4 9.3 7.4 7.0 6.4 4.6 4.2
5	B111Nov^r[p8325-2]/*Eco*RI	16.4 9.3 7.0 6.4 4.6 4.2 2.1
	Donor	
6	8325[p8325-2]/*Bgl*II	15.2 15.0 3.8 3.5 3.4 2.7 2.0 1.5 1.4
	First round transconjugant	
7	PS80d.*ero*[p8325-2]/*Bgl*II	15.2 15.0 3.8 3.5 3.4 2.7 2.0 1.5 1.4
	Second round transconjugant	
8	B111Nov^r[p8325-2::Tn*551*]/*Bgl*II	15.5 15.2 3.8 3.5 3.4 3.0 2.7 2.0 1.8 1.5 1.4
9	B111Nov^r[p8325-2]/*Bgl*II	15.2 15.0 3.8 3.5 3.4 2.7 2.0 1.5 1.4



E.coli JM107 failed to respond. It was therefore concluded that the slight variation in cell growth made no significant difference to the CIA titre.

To determine whether the length of incubation of donor and recipient cells affected the final CIA titre, incubations were set up between *Strep. faecalis* FA2-2, *Staph. aureus* 8325[p8325-2] and *E.coli* JM107 and *Strep. faecalis* FA373 and analysed after 2, 4, 6, 12, 24 and 48 hours. To prevent desiccation, the Inter-Med plates were incubated in a sealed sandwich box with a small amount of water in the bottom.

The results are shown in Table 6.2. After 12 hours incubation the samples which had clumped after 6 hours had lysed whereas the samples which had showed no clumping after 6 hours resulted in a visible white "clot" at the bottom of the well. These results remained constant even after 48 hours incubation. The results could therefore be read after 12 hours.

6.4 Survey of sex pheromone activity in *Staphylococcus aureus* strains and other bacterial species.

The pheromome assay system, as developed in the previous section was used to determine the sex pheromone activity present in filtrates obtained from a wide range of variants of *Staph. aureus* and some other bacterial species. In every case, the test responder organism was *Streptococcus faecalis* FA373. Each experiment was repeated at least twice (in some cases ten times) and the results of duplicate experiments always agreed.

The results (Table 6.3) are interpreted to show that all strains of *Staph. aureus* produced a clumping response in *Streptococcus faecalis* FA373 whereas, at least for the strains tested, *Staph. epidermidis, Staph. hominis, Bacillus subtilis* and *E.coli* did not produce a factor that promoted clumping of *Strep. faecalis* FA373. As expected, FA373 did not produce a pheromone response to itself since it contains the plasmid pAM373.

The strength of the response elicited depends on the strain of *S.aureus* used and is not apparently affected to any great extent by the presence of plasmids.

Derivatives of 8325 gave rise to filtrates that had to be very concentrated to produce a response whereas strain B111 Nov^r apparently produces a more active pheromone. In certain cases, there is a slight increase in the activity of the response if p8325-2 or p8325-4 is present in *S.aureus* 8325N.

Table 6.2

Table showing the CIA titres of bacterial strains after various incubation times with FA373

Strain	Highest dilution at which clumping is visible after time indicated (hrs)					
	2	4	6	12	24	48
Strep. faecalis FA2-2	—	—	8	8*	8*	8*
Staph. aureus 8325[p8325-2]	—	—	2	2*	2*	2*
E. coli JM107	—	—	—	—*	—*	—*

— no clumping observed.

* indicates highest dilution at which clearing was observed. All other wells contained a white globular mass.

—* white globular mass.

Table 6.3

Clumping-inducing agent titres of culture filtrate from different species†

No detectable clumping	Highest dilution at which a filtrate prepared from the bacterium gave a clumping response with *Streptococcus faecalis* FA373			
	1	2	4	8
Strep. faecalis FA373 *Staph. epidermidis* *Staph. hominis* *Bacillus subtilis* MY2016 *E.coli* JM107	8325N 8325[pT181] 8325[pT181*cop608*] 8325[pCW59] 8325[pC194] 8325Nov^r 8325Nov^r[p8325-2](2) 8325Nov^r[pT181](2) 8325Nov^r[pT181][p8325-2](2) 8325Nov^r[p8325-4](2) 8325Nov^r[pT181][p8325-4](2) 8325[pT181][p8325-4](1)	8325[p8325-2] 8325Nov^r[p8325-2](1) 8325[pT181][p8325-2](1) 8325[pJE1] 8325[pJE2] 8325[pJE3] 8325[pJE4] 8325[pJE5] 8325[p8325-4] 8325Nov^r[p8325-4](1) PS80d.*ero* PS80d.*ero*[p8325-2] PS80d.*ero* [p9789Int$^-$ARevE.17] RN450 A53	B111Nov^r B111Nov^r[p8325-2] B111Nov^r[pT181] B111Nov^r[pT181][p8325-2] B111Nov^r[p8325-2::Tn*551*]	*Strep. faecalis* FA2-2 *Strep. faecalis* JH2-2

(1) strain from a first round transfer

(2) strain from a second round transfer

† The values represent the reciprocal of the highest serial (twofold) dilution which still exhibited a detectable clumping responce.

6.5 Conjugation between *Staphylococcus aureus* and *Streptococcus faecalis*

The *Streptococcus faecalis* strains available (FA2-2, FA373 and JH2-2) were all found to be resistant to gentamicin, therefore selection for transconjugants between these strains and 8325[p8325-2] or 8325[p8325-4] could not be made using this antibiotic. As ethidium bromide (to which 8325[p8325-2] is resistant) was not a suitable selective agent, it was not possible to determine if conjugal transfer occurred between 8325[p8325-2] and *Strep. faecalis*.

It was possible however, to test for conjugal transfer between *Staph. aureus* 8325[p8325-4] and *Strep. faecalis* FA2-2 and FA373 by selecting transconjugants using cadmium (to which 8325[p8325-4] is resistant) and fusidic acid. The standard conjugation experiment was carried out twice, selection for transfer being made on cadmium and fusidic acid plates at 120 and 25 μg/ml respectively. After 48 hours incubation no transconjugants were obtained on any of the selection plates.

The *Strep. faecalis* strain FA373 carries the pheromone-responding conjugal plasmid pAM373 (Clewell *et al,* 1985). To determine if this plasmid was able to promote its own transfer to *Staph. aureus,* a conjugation experiment was performed in the normal manner between *Strep. faecalis* FA373 and *Staph. aureus* PS80d.*ero*. Selection of transconjugants was made using tetracycline and erythromycin at 10 and 20 μg/ml respectively. The experiment was performed twice but in neither case were transconjugants visible even after five days incubation. It was not possible to use the *Staph. aureus* recipients B111 Nov^r or 8325 Nov^r as *Strep. faecalis* FA373 was resistant to novobiocin.

6.6 Discussion

6.6.1 Mobilization

The conjugative plasmid p8325-2 was able to transfer to all the four recipients B111 Nov^r, PS80d.*ero*, 8325 Nov^r and 8325[pT181]. The conjugative plasmid p8325-4 was not transferred to B111 Nov^r or PS80d.*ero*. This result is significant as in a control experiment it was found that the plasmid pT181 was transferred from 8325[pT181] to 8325 Nov^r at high frequency. As no known conjugative plasmid is present in 8325[pT181] it is possible that transfer is due to transducing phage. The transfer of p8325-2 and p8325-4 to recipients 8325[pT181] and 8325 Nov^r cannot therefore be defined as being solely due to a conjugative system although this may play a second role. That the plasmid p8325-2 was transferred to B111 Nov^r and PS80d.*ero* at high frequency whereas no transfer was detected between 8325[pT181] and these recipients, provides substantial evidence for conjugal transfer of p8325-2. The strain PS80d.*ero* was a poor recipient but a good donor, transferring p8325-2 to B111 Nov^r at high frequency.

The system used to select transconjugants seems to affect the resistance patterns of the selected colonies. For example, when 8325[pT181][p8325-2] was conjugated with B111 Nov^r and selection made on tetracycline, a medium conjugation frequency was observed and from 60 Tc^r colonies tested, 58 were also Gm^r. When selection was made on gentamicin, none of the transconjugants were resistant to tetracycline. A possible explanation is that gentamicin prevents establishment of pT181.

There was no evidence for the mobilization of chromosomal genes although transposon Tn*551* was transferred to B111 Nov^r in two examples. This was probably due to transposition to p8325-2 from the chromosome of PS80d.*ero* (Figure 6.4).

There is evidence that pT181 can be mobilized by p8325-2 from 8325[pT181][p8325-2] to the recipient B111 Nov^r because, although pT181 was not transferred to B111 Nov^r from 8325[pT181], it was transferred from 8325[pT181][p8325-2]. Transfer of pT181 in this system only occurred when selection was made on tetracycline and was usually associated with concomitant transfer of p8325-2. The plasmid pT181 was transferred in two cases without the simultaneous establishment of p8325-2 in the transconjugant. It is possible that in these cases transfer of p8325-2 had not occurred. This is not entirely unexpected since the *E.coli* plasmid ColE1 is mobilized by *tral* mutants of the conjugative F plasmid which cannot transfer copies of themselves (see Hardy, 1986).

The mobilization of ColE1 requires a specific transfer origin and mobility genes that specify proteins necessary for ColE1 conjugal DNA metabolism (Sherratt, 1986). The *mob*5 protein (mobility protein specified by gene 5) of 20 kd is thought to nick the DNA specifically at *ori*T (Figure 6.5). Comparison between this system and that of pT181 is interesting. The complete nucleotide sequence of pT181 (4437 bp) has now been determined and found to contain four open reading frames (Khan and Novick, 1983). Polypeptide A encodes the *repC* protein required for replication whereas polypeptides B and D are involved in tetracycline resistance (Figure 6.6). No function has, as yet, been assigned to polypeptide C although deletion of 1161 bp (including most of the C reading frame) had no effect on replication, stability, copy control or tetracycline resistance. It is interesting to speculate on the possibility of polypeptide C (23 kd) being involved in mobility; it may even correspond to the *mob*5 protein of ColE1 since it is of similar size. To confirm this hypothesis, the deletion mutant plasmid could be tested for its mobilization.

It is possible that pT181 is not being mobilized by p8325-2 but is using the plasmid to "hitch-hike" into B111 Nov^r. This would involve it combining transiently with p8325-2 by site specific intermolecular recombination due to the presence of homologous sequences on the two plasmids providing hotspots for recombination. Hitch-hiking is a phenomenon of phage transfer (Novick *et al,* 1981) and considering the possibility of phage involvement in the 8325[pT181] system, the co-transduction of these two independent plasmids may provide an alternative explanation to mobilization.

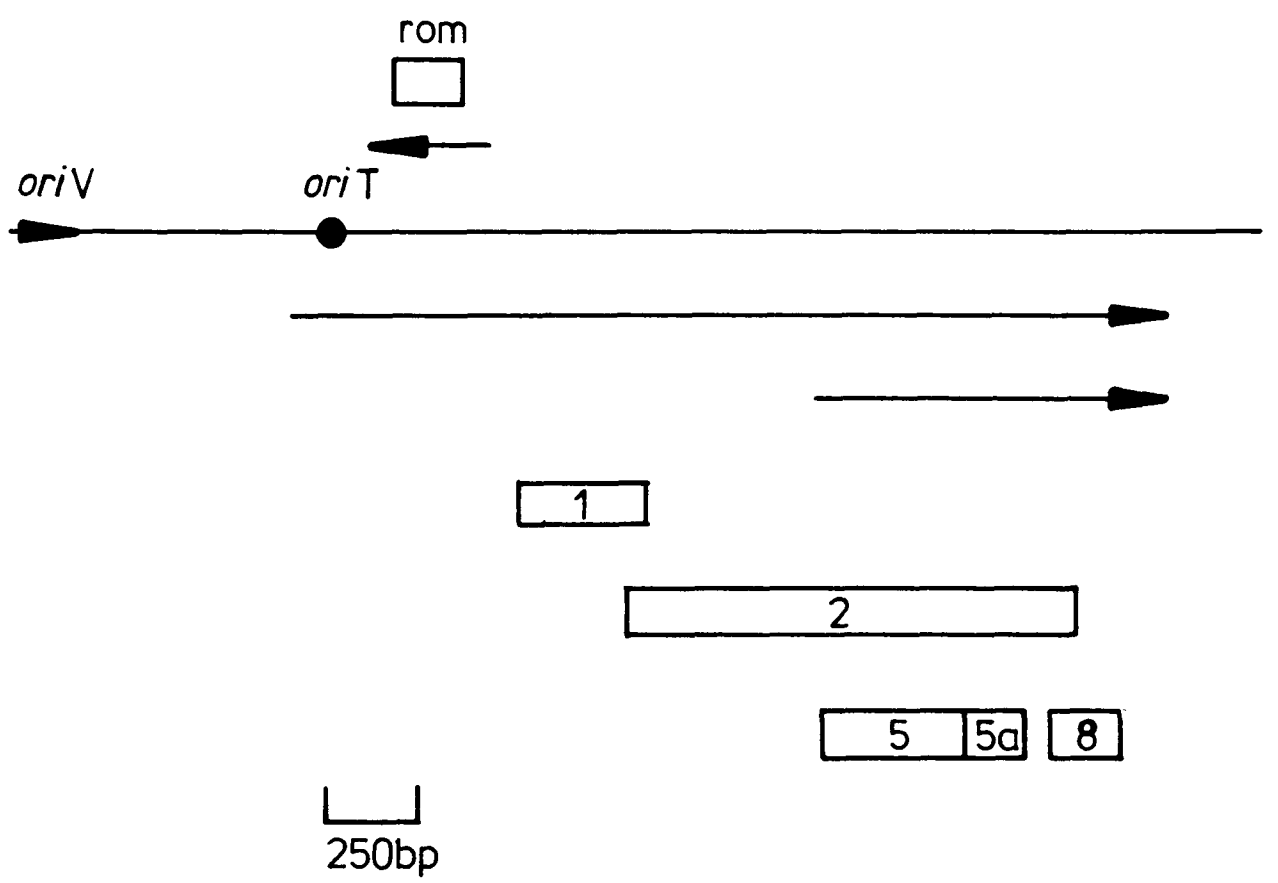
More evidence is needed for the mobilization of small elements by p8325-2. This could involve the construction of a range of strains carrying p8325-2 together with other plasmids, perhaps with more than one different plasmid in the cell. Tests could be made for the mobilization of a wide range of plasmids.

It would also be interesting to locate the region(s) of the p8325-2 plasmid involved in mobilization. This could involve the construction of deletion mutants lacking the mobilization function which could then be mapped. It is thought in the case of the F plasmid that initiation of ColE1 conjugal DNA synthesis results from an activation signal (Sherratt, 1986). Whether such a signal exists in p8325-2 is unknown.

Plasmid analysis of the three types of transconjugant strains ($Gm^rNov^rTc^r$, $Gm^rNov^rTc^s$ and $Gm^sNov^rTc^r$) showed that pT181 and/or p8325-2 had been transferred without detectable alteration in the DNA.

Figure 6·5

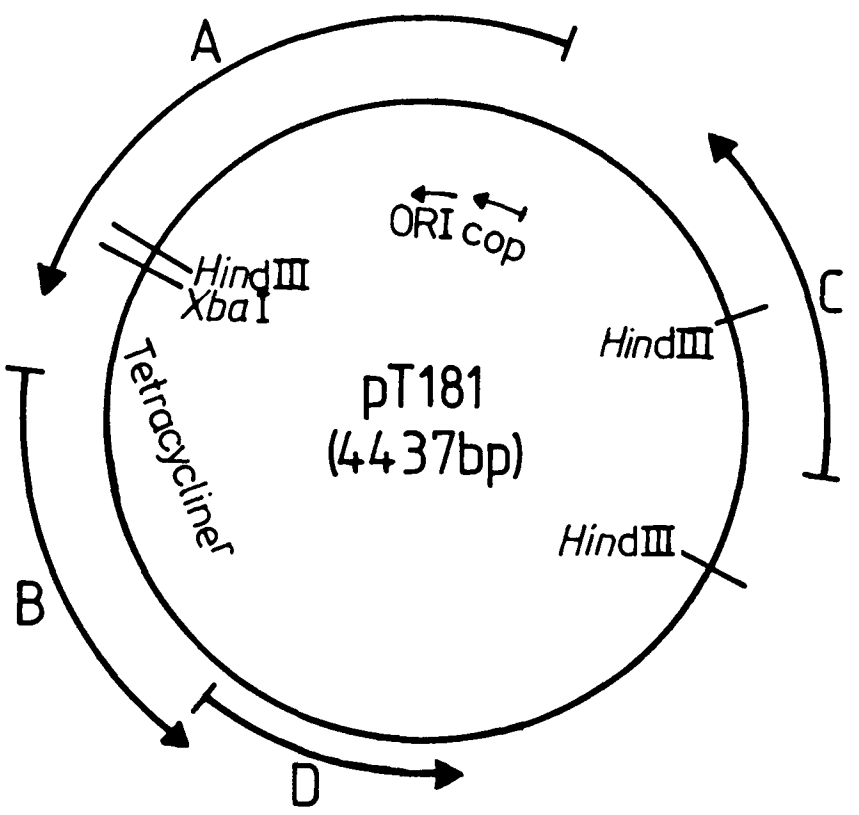
The ColE1 mobility region.



The five presumptive mobility proteins and the transcripts that give rise to them are indicated. (Sherratt, 1986)

Figure 6·6

Diagrammatic representation of pT181



Location of the origin and direction of replication are shown. The copy control region and four open reading frames coding for polypeptides A,B,C and D are also indicated. From Khan and Novick, 1983.

The pattern of transfer to recipients is a function of the plasmid itself and is carried through a second round transfer. This was shown by the transfer of 8325[p8325-2]. The plasmid p8325-2 was transferred to B111 Nov^r at very high frequency but to PS80d.*ero* at low frequency. The transconjugant strain PS80d.*ero*[p8325-2] was transferred to B111 Nov^r at a high frequency; the frequency is therefore carried with the plasmid through a second round. Even if phage is involved, it must be carried with the p8325-2 plasmid through several rounds of transfer; the ability to conjugate is certainly associated with p8325-2.

6.6.2 Conjugation to *Streptococcus faecalis*.

It has been shown that the broad host range plasmids such as pAMβ1 from *Streptococcus faecalis* can be transferred to *Staphylococcus aureus* via filter membrane conjugation experiments (Engel *et al,* 1980; Schaberg *et al,* 1982). These plasmids can mobilize non-conjugative plasmids into *Staph. aureus* and can be transferred back to *Strep. faecalis* or to a second *Staph. aureus* strain (Schaberg *et al,* 1982). There is no evidence to date that sex pheromone responsive plasmids such as pAMγ2 can be transferred in the same way. The *Strep. faecalis* plasmid pAM373 was reported not to be able to establish in *Staph. aureus* (Clewell *et al,* 1985). The results of this survey are in agreement; no transfer was detected between *Staph. aureus* 8325[p8325-4] and *Strep. faecalis* in either direction, although it is possible that pAM373 and p8325-4 were transferred but were not able to become established in the new host

6.6.3 Sex pheromone activity.

Dunny *et al* (1979) found that the production of pheromone paralleled cell growth during the exponential phase but reached a maximum as the cells approached stationary phase. This activity then remained constant for several hours. It could be further stabilized by autoclaving after which no subsequent loss in activity was found even after storage for several weeks at 4°C. Autoclaving probably inactivates degradative proteolytic enzymes.

The results obtained in this survey similarly show no loss in pheromone-like activity after storage at 4°C and once the cells had reached exponential phase, slight variations in optical density made little difference to the activity of the filtrate. A reproducible test system was established in which the filtrate and responder cells were incubated together for 12 hours.

The sex pheromone system was investigated to determine if it was a feature of the conjugative *Staph. aureus* strains being studied. The ability to produce pheromone-like molecules seems to be

a feature of *Staph. aureus* strains and is unrelated to their ability to conjugate and to whether or not they contain plasmids.

The finding that strains of *Staph. aureus* produce a substance that elicits a clumping response in *Strep. faecalis* is interesting and may be of significance in that streptococci and staphylococci are known to co-exist in some environments such as wounds. This phenomenon may be part of a much larger system of communication between staphylococci and a thorough investigation would constitute a major project in itself.

Filtrates from the strain B111 Nov^r produce a stronger reaction in *Strep. faecalis* than filtrates from strain 8325 but it is not known if this is due to the production of a different molecule or if more of the same molecule is present. Nevertheless, strain B111 Nov^r could be the best available starting point for an investigation of chemical signalling between strains of *Staph. aureus*. A filtrate of B111 Nov^r could be used to investigate the response of a large number of strains of *Staph. aureus*. In particular, it would be of interest to determine if the presence of any plasmids (such as p8325-2) altered the response of the bacteria since it is possible that in *Strep. faecalis* the determinant for aggregation substance is plasmid-borne (Dunny *et al,* 1979). The experiments could be repeated with filtrates from other strains of *Staph. aureus*.

Other questions that could be answered include:

1) Does *Staph. aureus* produce pheromone-like substances that can elicit responses from *Strep. faecalis* sensitive to other pheromones?

2) Does pAM373 regulate the production of the cAM373-like substance in *Staph. aureus*? Although attempts to transfer *Staph. aureus* by conjugation were not successful, transformation should be tried.

3) Does *Staph. aureus* produce a binding substance related to that of *Strep. faecalis* described by Dunny *et al* (1979)?

4) Is there a network of communication between strains and species of staphylococci? If so, how far does it extend to other bacteria and what is its function?

Because the work to be reported in the next chapter was progressing well and because the questions to be answered would require extensive investigation, the study of sex pheromone activity was set aside as a separate project.

Chapter 7

Transposon Mutagenesis as a Method for Locating Parts of Plasmid DNA Involved in Transfer

7.1 Introduction

The techniques of deletion analysis (Chapter 4) and cloning of restriction fragments (Chapter 5) had failed to identify the region(s) of plasmid DNA involved in conjugation. The results from these studies however, led to the speculation that more than one region of the genome was involved. It was therefore decided to pursue the investigation by using the technique of transposon mutagenesis.

Integration of a transposon into a coding region often results in a polar effect because transcription (and translation) can terminate within the transposable genetic element resulting in the inhibition of expression of genes within the same operon downstream from the site of insertion. Deletion mutants can often be generated since the transposon can promote DNA loss adjacent to the site of integration and there is also the possibility of transposon-induced rearrangements. Care is therefore necessary in the interpretation of the data from mutants derived in this way.

There is an increasing number of reports describing the use of transposon mutagenesis to localize determinants. Lyon *et al* (1986) employed the technique to locate the pSK1-mediated resistance to trimethoprim in *S.aureus* using Tn*5* whilst Hartley *et al* (1985) used the erythromycin resistance transposon Tn*917* to map the conjugative transfer gene(s) of pVA797, a streptococcal plasmid. Mutagenesis of pCF-10 from *Streptococcus faecalis* with Tn*917* was also used to localize a tetracycline resistance determinant and several regions involved in conjugal transfer (Christie and Dunny, 1986).

The transposon chosen for use in this study was Tn*551,* a 5.3 kbp class II transposon encoding resistance to erythromycin (Khan and Novick, 1980). The ends of the transposon consist of an inverted repeat of 40 bp flanked by a direct repeat of 5, placing it in the same class as Tn*3*. The *S.aureus* transposon Tn*551* occurs naturally on plasmid pI258 and is capable of *rec*-independent insertion into nonhomologous sites in the host chromosome or other plasmids (Novick *et al,* 1979). It causes insertional inactivation and local rearrangements (Novick *et al,* 1979).

Tn*551* has been used by Berger-Bachi (1983) to locate the methicillin resistance determinant on the chromosome of *S.aureus* and by Luchansky and Pattee (1984) to provide linkage data and map locations for markers on the *S.aureus* chromosome.

7.2 Construction of transposon mutants

Transposon mutagenesis was carried out on the gentamicin resistance conferring plasmids p8325-2 and p8325-4. The aim was to define the region(s) involved in transfer by forcing Tn*551* to integrate into the plasmid genome and determining the conjugation frequency of resulting mutants. Insertion into regions involved in conjugation should affect the transfer frequency. The location of such regions could then be determined by analysing the position of Tn*551* insertion.

The donor of transposon Tn*551* was strain PS80d.*ero*[pI9789,seg-1.Int$^-$A]. Revertant E, number 17 (Browne, 1985). She constructed this strain by transduction of PS80d.*ero* (a *S.aureus* strain carrying Tn*551* on the chromosome) to cadmium resistance by ϕ80 grown on PS80[pI9789,seg-1,Int$^-$A]. This latter strain contains a plasmid (derived from pI9789) that is temperature sensitive for replication, carries a deletion of 7.4 kbp and confers resistance to cadmium ions (Browne, 1985). A transductant, designated PS80d.*ero*[pI9789,seg-1, Int$^-$A] was chosen, grown in CY-agar containing cadmium ions (cadmium acetate to 13μg/ml). After growth at 42°C, only very few colonies grew. One of these was picked and called PS80d.*ero*[pI9789,seg-1,Int$^-$A] Revertant E. Phage 80 was grown on this revertant and used to transduce erythromycin resistance to PS80N. A transductant designated PS80d.*ero*[pI9789, seg-1, Int$^-$A] Rev.E.17 was chosen. It is resistant to erythromycin, cadmium ions and was shown to carry a plasmid that is temperature sensitive for replication.

Analysis of the plasmid DNA from this transductant showed it to have the restriction map shown in Figure 7.1. Thus it is a plasmid, temperature-sensitive for replication, that carries Tn*551*.

The temperature sensitivity of the Int$^-$A plasmid was useful as it allowed selection for the translocation of Tn*551* into the gentamicin resistance conferring plasmids by heat inactivation of pInt$^-$A. A diagrammatic representation of the method used is given in Figure 7.2.

Plasmid DNA was prepared from PS80d.*ero*,[pI9789,seg-1,Int$^-$A].RevE.No.17 and transformed into 8325N[p8325-2]. Erythromycin-resistant transformants were selected at 30°C and screened for

Figure 7·1

Restriction endonuclease map of pS80d. *ero*[pI9789, seg-1. Int⁻A] Revertant E. Number 17 containing the transposon Tn*551*

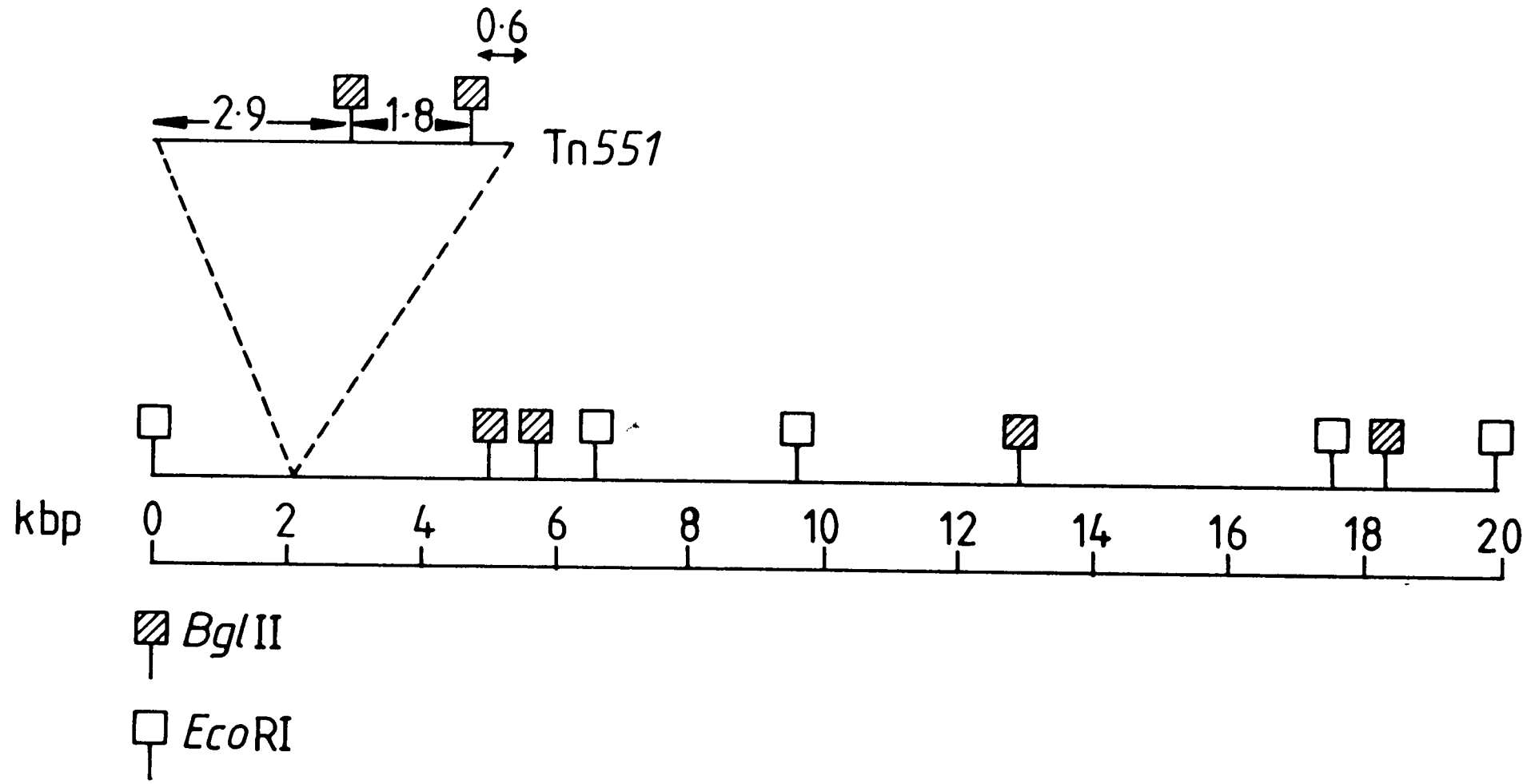
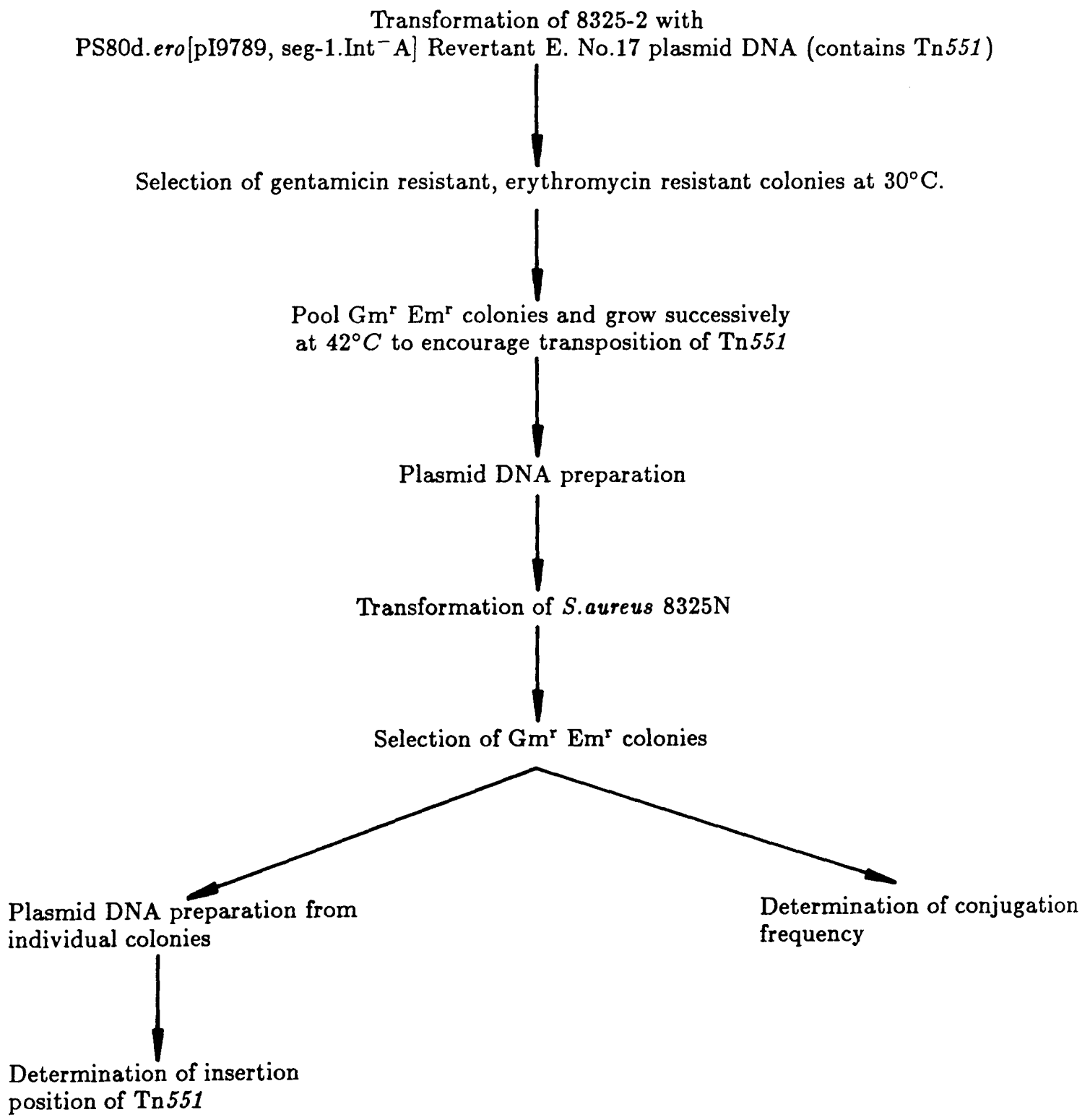


Figure 7.2

Strategy for Transposon Mutagenesis.



gentamicin resistance. A total of 33 colonies were obtained, all of which were resistant to both gentamicin and erythromycin.

These 33 colonies were pooled, grown up and plasmid DNA prepared from the culture. The plasmid DNA was analysed on agarose gels after digestion with various restriction enzymes and compared with p8325-2 and pInt$^-$A.RevE[::Tn*551*] DNA (Figure 7.3). The pooled colonies did indeed contain the two plasmids although from the intensity of the DNA bands, the gentamicin resistance plasmid appeared to be present in greater amounts. Whether this was due to its higher copy number per cell or whether the smaller plasmid was not present at all in a proportion of transformants cannot be determined.

The *Bgl* II digestion is interpreted to show that Tn*551* is inserted into pInt$^-$A.RevE in the orientation shown in Figure 7.1 and that there is a 1.8 kbp *Bgl* II fragment entirely from within the transposon.

The pooled 33 colonies were grown in CY-medium at 42°C, diluted 1 in 100 into CY medium containing 20μg/ml gentamicin and 10μg/ml erythromycin and grown for 5 hours at 42°C. The dilution and growth at 42°C were repeated six times. Since the plasmid carrying Tn*551* is temperature sensitive for replication, this procedure should select for transposition of Tn*551* to some other replicon. This could be the chromosome or p8325-2.

Plasmid DNA was prepared from the resulting culture and used to transform *S.aureus* 8325N to erythromycin resistance. In the first experiment, 112 erythromycin-resistant transformants were selected and of these, 12 were also resistant to gentamicin. From a repeat transformation, using the same stock of plasmid DNA, 53 erythromycin-resistant transformants were obtained, of which 14 were also resistant to gentamicin.

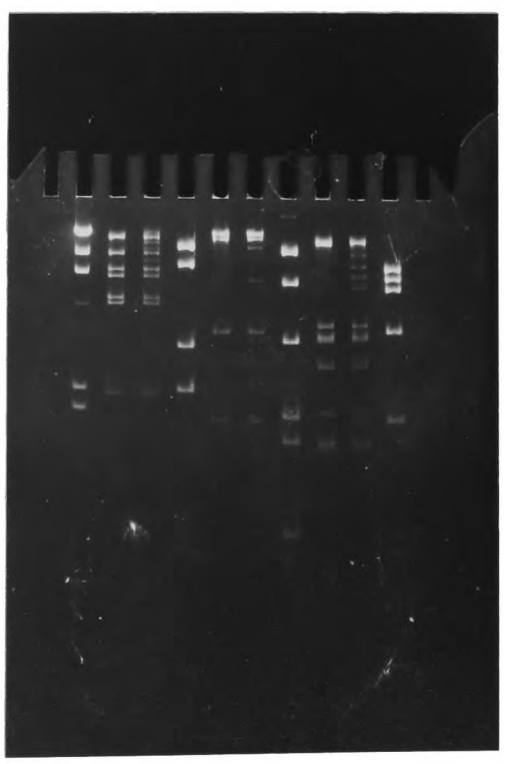
It is unlikely that both plasmids would be transformed into 8325N. It is more probable that each transformants contains only one plasmid (p8325-2[::Tn*551*]), that is, p8325-2 with Tn*551* inserted into it. Transformants that contained both plasmids (p8325-2 and pInt$^-$A.RevE[::Tn*551*]) would be expected to be resistant to cadmium ions. All 26 were found to be sensitive to cadmium acetate and were therefore investigated to test whether they did indeed contain p8325-2[::Tn*551*].

The transformation of plasmid DNA from PS80d.*ero* [pI9789, seg-1, Int$^-$A].Rev.E.No.17 into 8325N[p8325-4] was carried out six times without success. Two conjugation experiments were then

Figure 7.3

Gel showing the presence of both plasmids p8325-2 and pInt⁻A.RevE[::Tn551] in pooled transformants

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	p8325/*Eco*RI	16.4 9.3 7.0 6.4 4.6 4.2 2.1
3	p8325pInt⁻A.RevE [::Tn*551*]/*Eco*RI	16.4 11.9 9.3 7.9 7.0 6.4 4.6 4.2 3.0 2.3 2.1
4	pInt⁻A.RevE [::Tn*551*]/*Eco*RI	11.9 7.9 3.0 2.3
5	p8325-2/*Eco*RI	28.1 16.6 3.5 1.8
6	p8325-2pInt⁻A.RevE [::Tn*551*]/*Eco*RV	28.1 16.6 10.5 6.2 3.5 3.3 1.9 1.8 1.6 0.8
7	pInt⁻A.RevE [::Tn*551*]/*Eco*RV	10.5 6.2 3.3 1.9 1.6 0.8
8	p8325/*Bgl*II	15.2 15.0 3.8 3.5 3.4 2.7 2.0 1.5 1.4
9	p8325Int⁻A.RevE [::Tn*551*]/*Bgl*II	15.2 15.0 7.2 6.6 5.4 3.8 3.5 3.5 3.4 2.7 2.0 1.8 1.5 1.4 0.7
10	pInt⁻A.RevE [::Tn*551*]/*Bgl*II	7.2 6.6 5.4 3.5 1.8 0.7



carried out between these strains producing a total of 700 and 917 transconjugants respectively (representing conjugation frequencies of 2.8×10^{-6} and 3.7×10^{-6} transconjugants per recipient). The transconjugants from each conjugation were pooled separately and grown successively at 42°C as described above for p8325-2. Plasmid DNA was prepared from the resulting growth and each used to transform 8325N to erythromycin resistance in two separate transformation experiments. Although 23 erythromycin-resistant transformants were obtained from the first experiment, none were resistant to gentamicin. No transformants were obtained from the second transformation. Plasmid DNA from pInt$^-$A.RevE[::Tn*551*] was again transformed into p8325-4, resulting this time in 48 erythromycin-resistant transformants of which 15 were also resistant to gentamicin. These transformants were pooled, grown successively at 42°C and plasmid DNA prepared from the resulting growth. This DNA was used on two separate occasions to transform 8352N but no erythromycin-resistant transformants were obtained.

7.3 Characterization of the mutants

Plasmid DNA was prepared from each of the 26 mutants and analysed for the position of Tn*551* insertion by digestion with each of the restriction enzymes *Eco* RI, *Bgl* II, *Nco* I, *Pvu* II and *Xba* I. Fragments were sized by agarose gel electrophoresis and compared with similar digests of the parental plasmid. The mutant plasmids have been designated 2-1 to 2-26.

Initially the particular restriction enzyme fragment into which Tn*551* had inserted was determined for each enzyme. The results are given in Table 7.1. The fragments have been named alphabetically in decreasing order of size, thus fragment A is the largest fragment in each case. Comparison with the map for p8325-2 (Figure 4.2) and correlation of the limits of insertion for each enzyme allows a more precise limitation to be placed upon the site of each insertion (given in the last column of Table 7.1). Insertions with the same limits have been grouped together and the mutants ordered by their map unit limits.

Each mutant was then mapped more precisely by sizing each restriction fragment resulting from its digestion by each of the three restriction enzymes; *Bgl* II, *Nco* I and *Xba* I. As Tn*551* contains no *Eco* RI or *Pvu* II sites, digestion with these enzymes only identifies the fragment into which the transposon is inserted.

The position of the two *Bgl* II sites within Tn*551* are known to be 2.8 kbp and 0.5 kbp from the ends giving a middle fragment of 2.0 kbp (Browne, 1985). The analysis of Tn*551* reported

Table 7.1

Table showing the particular restriction fragment into which Tn*551* has inserted in each mutant

Name of mutant	Restriction fragment affected					Limits (map units)
	*Eco*RI	*Pvu*II	*Bgl*II	*Nco*I	*Xba*I	
2-18	A	C	B	D	A	0.3–4.3
2-3,2-16,2-20	A	A	B	D	A	4.3–5.8
2-4	A	A	B	D	A	
2-24	A	A	I	A	C	7.5–8.9
2-13	A	A	D	A	C	10.9–14.4
2-5	E	A	A	A	B	16.4–21.0
2-19	E	A	A	A	B	
2-26	E	A	A	A	B	
2-6 (Δ 3.7 kbp)	B,E	A,E,F	A	A	B	16.4–25.7
2-7, 2-12	B	A	A	A	B	21.0–21.7
2-21	B	A	A	A	B	
2-15	B	A	A	A	B	
2-1, 2-17 2-22, 2-23	B	F	A	A	B	21.7–23.1
2-14	B	E	A	A	B	23.1–25.7
2-2	B	E	A	A	B	
2-11	B	B	A	C	—	25.7–29.9
2-8, 2-10	F	B	E	C	D	30.3–31.9
2-9	D	C	B	E	A	45.2–50.0
2-25	D	C	B	E	A	

here gives the central piece a size nearer to 1.8 kbp and so this value will be used. The overall size of Tn*551* is 5.3 kbp so the *Bgl* II sites are taken as 2.9 kbp and 0.6 kbp from the ends of the transposon.

7.3.1 Mapping Tn*551*

a) *Nco* I site.

In every case where Tn*551* has inserted into p8325-2, analysis shows that one of the *Nco* I derived bands disappears when the digested DNA is analysed and two new bands appear. There is therefore a single *Nco* I site within Tn*551*.

For mutant 2-21 it is known that the insertion is between map units 21.0 and 21.7 and within the *Nco* I A band (Table 7.1). Digestion of 2-21 plasmid DNA results in two new *Nco* I derived bands of 17.4 and 7.8 kbp. The *Nco* I A band extends from map units 5.8 to 25.7. Therefore the minimum distance from the left-hand end of the transposon is:

17.4 (a band derived from digestion of mutant 2-21) minus the sum of 15.2 (the distance between map units 5.8 and 21.0) and 0.7 (the distance between map units 21.0 and 21.7)= 1.5.

The *Nco* I site is at least 1.5 kbp from the left-hand end of Tn*551*. Similarly, the minimum distance from the right-hand end can be calculated as 7.8 (the other band derived from the digestion of 2-21 plasmid DNA) minus the sum of 4.0 (the distance between 21.7 and 25.7) and 0.7 (the distance between 21.0 and 21.7)=3.1. The *Nco* I site in Tn*551* is at least 3.1 kbp from the right-hand end of the transposon.

For mutant 2-21, the position of the insertion o Tn*551* is between 21.0 and 21.7 map units. Digestion of mutant 2-12 plasmid DNA results in two "new" fragments of 18.3 and 6.9 kbp. The *Nco* I A band lies between 5.8 and 25.7 map units. Therefore, as before, the minimum distance of the *Nco* I site from the ends of Tn*551* are:

$$18.3 - (15.2 + 0.7) = 2.4$$

and

$$6.9 - (4.0 + 0.7) = 2.2$$

Comparison of the minimum figures for mutant 2-21 of 3.1 and 1.5 with those for mutant 2-12 of 2.4 and 2.2 allows the conclusion that the 5.3 kbp transposon Tn*551* is cut by *Nco* I at a site 3.1 kbp from one end and 2.2 kbp from the other.

The same calculations have been done for all the other mutants and are all consistent with the stated *Nco* I map position.

b) *Xba* I site.

Again, analysis of the mutant digestions indicates that there is a single *Xba* I site within Tn*551*.

For mutant 2-21 in which Tn*551* is inserted within the *Xba* I B fragment (map coordinates 15.6-29.2), *Xba* I cuts the mutant plasmid DNA to give new pieces of 12.1 and 6.8 kbp. It is known that the site of Tn*551* insertion must lie between 21.0 and 21.7 map units (Table 7.1), therefore the *Xba* I site within the transposon must lie at least:

$$6.8 - (5.4 + 0.7) = 0.7$$

from one end and

$$12.1 - (7.5 + 0.7) = 3.9$$

from the other end.

For mutant 2-3 in which Tn*551* is within the *Xba* I A fragment (coordinates 35.8 and 6.6) *Xba* I cuts the mutant plasmid DNA to give pieces of 19.2 and 6.3 kbp. For this mutant, the site if insertion is between 4.3 and 5.8 map units so that the *Xba* I site must be at least:

$$6.3 - (0.2 + 1.5) = 4.6 \text{ kbp} \qquad \text{from one end.}$$

From the results with mutants 2-12 and 2-3, it is possible to predict that the *Xba* I site is 4.6 kbp from one end of the transposon and 0.7 kbp from the other end.

All remaining mutants were analysed and the results are consistent with the predicted *Xba* I site within Tn*551*.

c) Orientation of the sites.

Using the data from mutant 2-21, it is known that the *Nco* I site is 2.2 kbp from the left-hand end of the transposon and the *Xba* I site is 0.7 kbp from the same end. This allows a restriction map of Tn*551* to be constructed (Figure 7.4). The orientation in which Tn*551* has been drawn has been designated orientation A. The opposite orientation is termed B.

This data was then used to determine more accurately the positions of the insertions and, in most cases, the orientation of the transposon. The results from this detailed analysis of each mutant are given in Table 7.2. All possible positions of Tn*551* insertion within each mutant are shown for each restriction enzyme. In each case, the values corresponding to the two orientations have been separated. The values for Tn*551* in orientation B are given first in each case and these are aligned across the table for each enzyme. The orientations are all in agreement with the restriction map of Tn*551* (Figure 7.4).

7.3.2 Characterization of mutants 2-3 and 2-24.

Examples of gels from two mutants (2-3 and 2-24) are shown, together with the fragment sizes, in Figures 7.5 and 7.6. these two mutants have been chosen to illustrate the method by which the position of insertion was determined. Diagrammatic representations of the method of calculation of the points of insertion are given in Figures 7.7 and 7.8 (for 2-3 and 2-24 respectively).

In mutant 2-3 (Figure 7.5 and 7.7) Tn*551* is inserted into *Bgl*II.B (15.0 kbp) to give pieces of 12.5, 6.0 and 1.8 kbp. The map positions of the ends of *Bgl*II.B in p8325-2 are 42.5 and 7.5. From the *Bgl*II digestion, the ends of the transposon can be at map positions $42.5 + (6.0 - 0.6) = 47.9$ or $[42.5 + (12.5 - 0.6)] - 50 = 4.4$ if Tn*551* is in one orientation (designated orientation B). Alternatively, if Tn*551* is inserted in the opposite orientation (orientation A) then the map positions can be $[42.5 + (12.5 - 2.9)] - 50 = 2.1$ or $42.5 + (6.0 - 2.9) = 45.6$.

Mutant 2-3, is inserted into *Nco* I.D (map positions 0.3 to 5.8 kbp). Digestion results in "new" fragments of 4.4 and 6.4 kbp. Thus the possible sites of Tn*551* insertion are $0.3+(4.4-2.2) = 2.5$ or $0.3+(6.4-2.2) = 4.5$ if Tn*551* is in one orientation and $0.3+(4.4-3.1) = 1.6$ or $0.3+(6.4-3.1) = 3.6$ if Tn*551* is inserted in the opposite orientation.

The transposon is inserted into *Xba* fragment.A (map positions 35.8 to 6.0). Digestion results in "new" fragments of 19.2 and 6.3 kbp. As the *Xba* I site is 0.7 and 4.6 kbp from the ends of

Figure 7·4

Restriction enzyme map of the transposon Tn*551*

<u>*Orientation A*</u> (0⟶50kbp on p8325-2 map)

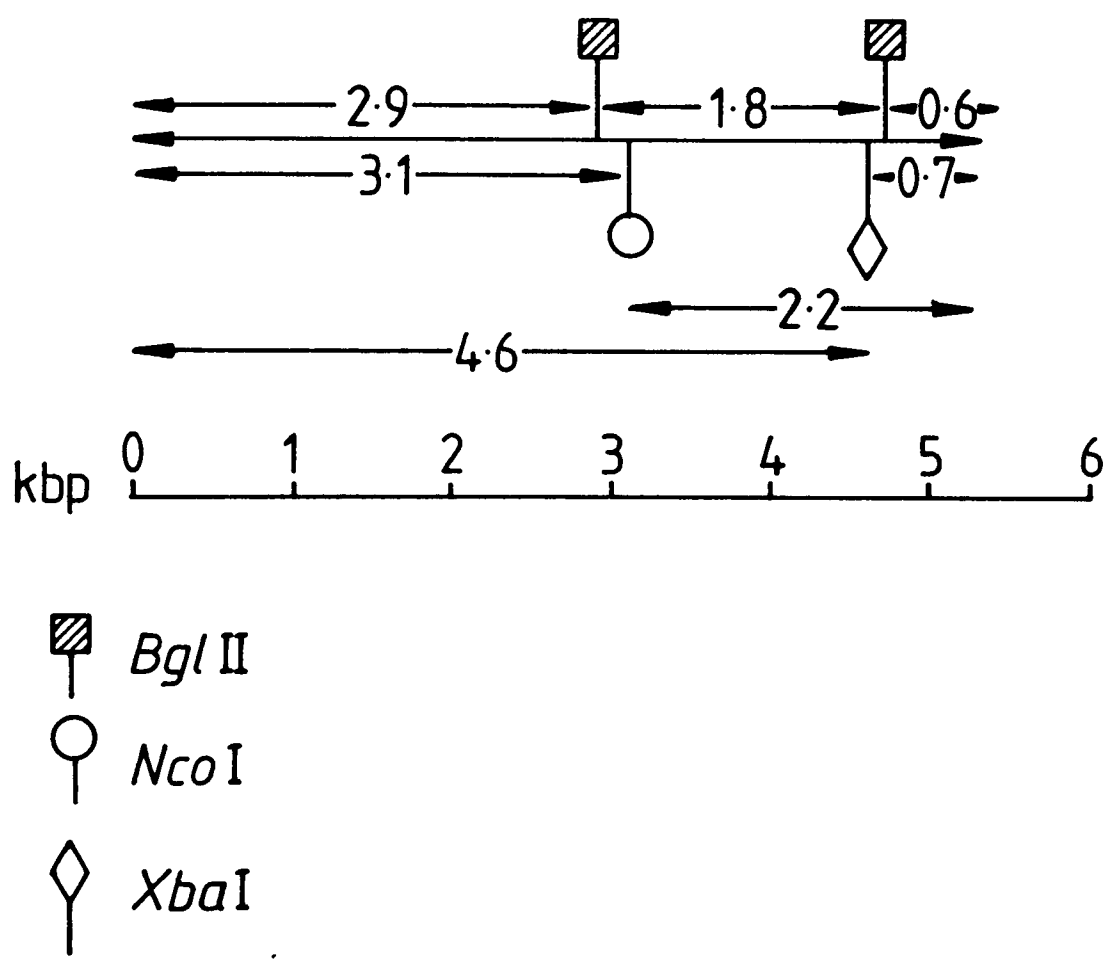


Table 7.2 Table showing all possible positions of Tn*551* insertion within each mutant (in map units)

Name of Mutant	Restriction enzyme used for digestion.				
	*Eco*R1	*Bgl*II	*Nco*I	*Xba*I	*Pvu*II
2-18	0–16.4	4.8 47.5 2.5 45.2	3.5 2.6	5.8 39.9 1.9 36.0	44.7–4.3
2-3, 2-16, 2-20	0–16.4	4.4 47.9 2.1 45.6	2.5 4.5 1.6 3.6	4.3 41.4 0.4 37.5	4.3–21.7
2-4	0–16.4	5.8 46.7 3.5 44.4	1.4 5.6 0.5 4.7	4.6 41.1 0.7 37.2	4.3–21.7
2-24	0–16.4	8.7 7.7	9.1 23.3 8.2 22.4	11.4 14.1 7.5 10.2	4.3–21.7
2-13	0–16.4	11.5 13.8	14.1 18.3 13.2 17.4	7.2 14.4	4.3–21.7
2-5	16.4–21.0	22.9 24.4 20.6 22.1	11.5 20.9 10.6 20.0	24.3 24.5 20.4 20.6	4.3–21.7
2-19	16.4–21.0	22.3 25.0 20.0 22.7	11.3 21.1 10.4 20.2	23.8 24.9 19.9 21.0	4.3–21.7
2-26	16.4–21.0	21.8 27.0 19.5 24.7	12.2 20.2 11.3 19.3	20.5 28.2 16.6 24.3	4.3–21.7
2-6 (Δ 3.7 kbp)	16.4–30.3	19.9 23.7 17.6 21.4	10.1 19.6 8.2 18.7	20.2 24.8 16.3 20.9	4.3–25.7

Table 7.2 (cont.)

Name of Mutant	Restriction enzyme used for digestion.				
	*Eco*R1	*Bgl*II	*Nco*I	*Xba*I	*Pvu*II
2-7, 2-12	21.0–30.3	23.6 23.7 21.3 21.4	10.3 22.1 9.4 21.2	23.7 25.0 19.8 21.1	4.3–21.7
2-21	21.0–30.3	21.8 25.5 19.5 23.2	11.4 21.0 10.5 20.1	21.7 27.0 17.8 23.1	4.3–21.7
2-15	21.0–30.3	19.9 27.4 17.6 25.1	10.3 22.1 9.4 21.2	21.5 27.2 17.6 23.3	4.3–21.7
2-1, 2-17, 2-22, 2-23	21.0–30.3	23.0 24.3 20.7 22.0	9.8 22.6 8.9 21.7	22.7 26.0 18.8 22.1	21.7–23.1
2-14	21.0–30.3	22.2 25.8 19.9 23.5	8.3 24.1 7.4 23.2	21.3 27.4 17.4 23.5	23.1–25.7
2-2	21.0–30.3	21.8 25.5 19.5 23.2	8.4 24.0 7.5 23.1	21.5 27.2 17.6 23.3	23.1–25.7
2-11	21.0–30.3	15.4 29.6	28.1 30.4 27.2 29.5	no data available	25.7–35.6
2-8, 2-10	30.3–34.5	31.8 31.4	26.8 31.7 25.9 30.8	31.8 34.2	25.7–35.6
2-9, 2-25	43.6–50.0	3.7 48.6 1.4 46.3	48.0 48.4 47.1 47.5	46.1 49.6 42.2 45.7	44.7–4.3

Note: The figures corresponding to orientation B are given first for each mutant.

Figure 7.5

Gel showing the restriction enzyme digestions of mutant 2-3

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	p8325-2/*Eco*RI	16.4 9.3 7.0 6.4 4.6 4.2 2.1
3	2-3/*Eco*RI	21.7 9.3 7.0 6.4 4.6 4.2 2.1
4	p8325-2/*Bgl*II	15.2 15.0 3.8 3.5 3.4 2.7 2.0 1.5 1.4
5	2-3/*Bgl*II	15.2 12.5 6.0 3.8 3.5 3.4 2.7 2.0 1.8 1.5 1.4
6	p8325-2/*Nco*I	19.9 11.0 6.2 5.5 5.1 2.3
7	2-3/*Nco*I	19.9 11.0 6.4 6.2 5.1 4.4 2.3
8	p8325-2/*Xba*I	20.2 13.6 9.6 6.6
9	2-3/*Xba*I	19.2 13.6 9.6 6.6 6.3
10	p8325-2/*Pvu*II	17.4 9.9 9.6 9.1 2.6 1.4
11	2-3/*Pvu*II	22.7 9.9 9.6 9.1 2.6 1.4

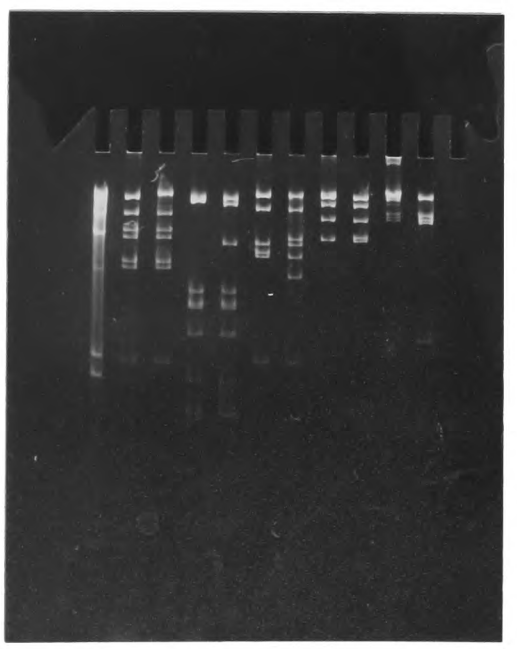


Figure 7.6

Gel showing the restriction enzyme digestions of mutant 2-24

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	p8325-2/*Eco*RI	16.4 9.3 7.0 6.4 4.6 4.2 2.1
3	2-24/*Eco*RI	21.7 9.3 7.0 6.4 4.6 4.2 2.1
4	p8325-2/*Bgl*II	15.2 15.0 3.8 3.5 3.4 2.7 2.0 1.5 1.4
5	2-24/*Bgl*II	15.2 15.0 3.8 3.5 3.4 3.1 2.7 2.0 1.8 1.8 1.5
6	p8325-2/*Nco*I	19.9 11.0 6.2 5.5 5.1 2.3
7	2-24/*Nco*I	19.7 11.0 6.2 5.5 5.5 5.1 2.3
8	p8325-2/*Xba*I	20.2 13.6 9.6 6.6
9	2-24/*Xba*I	20.2 13.6 8.8 6.6 6.1
10	p8325-2/*Pvu*II	17.4 9.9 9.6 9.1 2.6 1.4
11	2-24/*Pvu*II	22.7 9.9 9.6 9.1 2.6 1.4

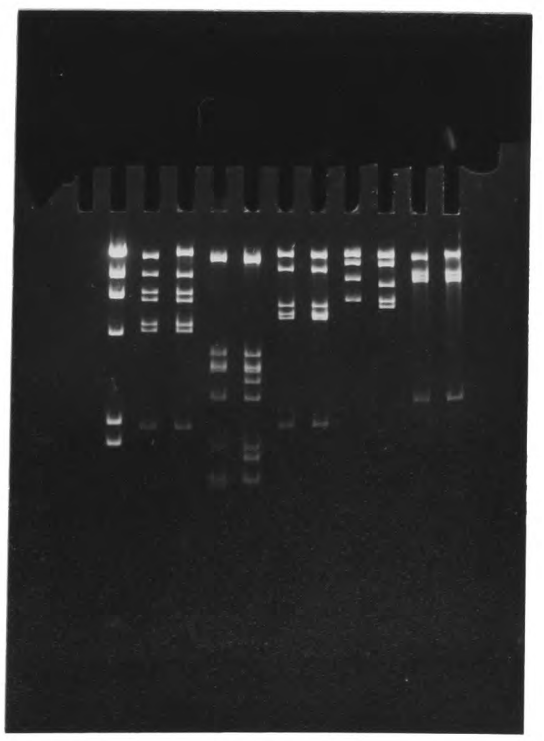


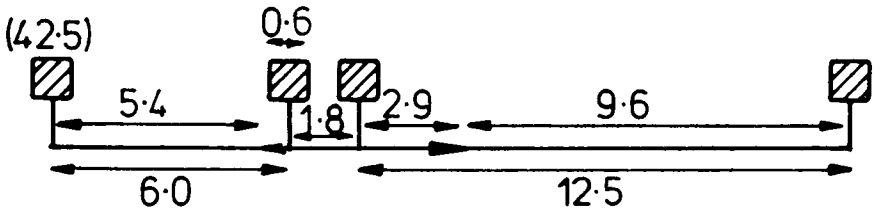
Figure 7·7

Diagram showing all possible insertions of Tn*551* within mutant 2-3

(a) *Eco*RI A (16·4) ⟶ 21·7

(b) *Pvu*II A (17·4) ⟶ 22·7

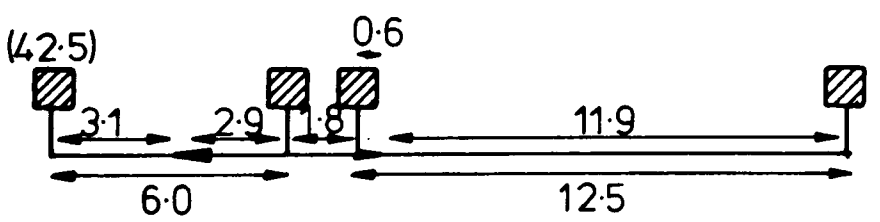
(c) *Bgl* II B (15·0) ⟶ 12·5 +6·0+1·8



Orientation B

Positions: 42·5+(6·0−0·6)= 47·9

or [42·5+(12·5−0·6)]−50=4·4



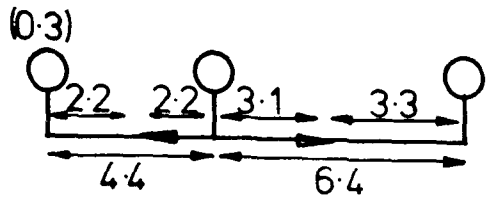
Orientation A

Positions: 42·5+(6·0−2·9)=45·6

or [42·5+(12·5−2·9)]−50=2·1

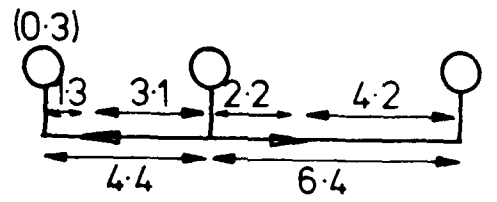
Figure 7·7(cont.)

(d) *Nco*I D (5·5) ⟶ 6·4+4·4



Orientation B

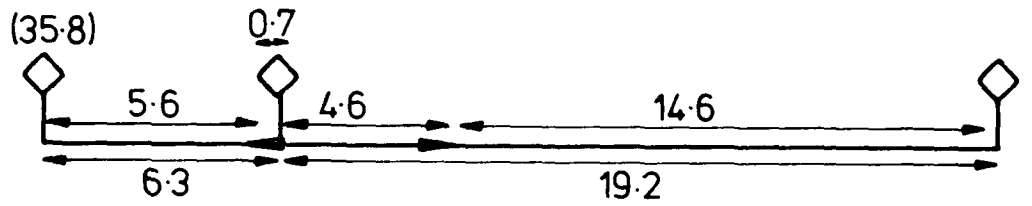
Positions: 0·3+(4·4−2·2) = 2·5
or 0·3+(6·4−2·2) = 4·5



Orientation A

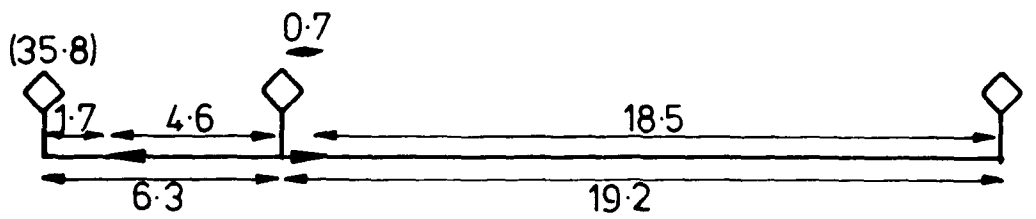
Positions: 0·3+(4·4−3·1) = 1·6
or 0·3+(6·4−3·1) = 3·6

(e) *Xba*I A (20·2) ⟶ 19·2+6·3



Orientation B

Positions: 35·8+(6·3−0·7) = 41·4
or [35·8+(19·2−0·7)]−50 = 4·3



Orientation A

Positions: 35·8+(6·3−4·6) = 37·5
or [35·8+(19·2−4·6)]−50 = 0·4

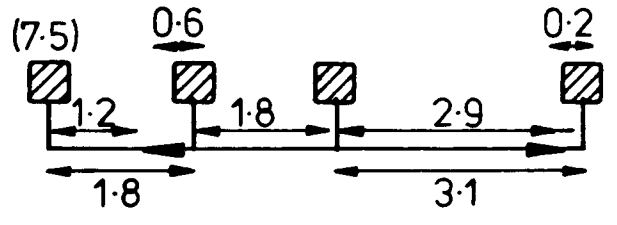
Figure 7·8

Diagram showing all possible insertions of Tn*551* within mutant 2-24

(a) *Eco*RI A (16·4) ⟶ 21·7

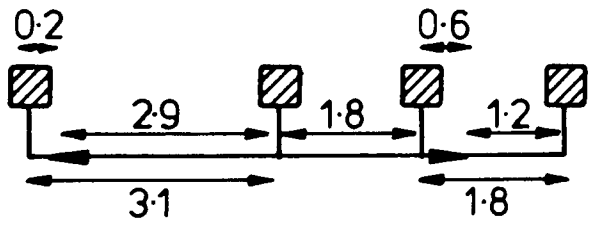
(b) *Pvu*II A (17·4) ⟶ 22·7

(c) *Bgl*II I (1·4) ⟶ 3·1+1·8+1·8



Orientation B

Position: 7·5+(1·8−0·6)=8·7

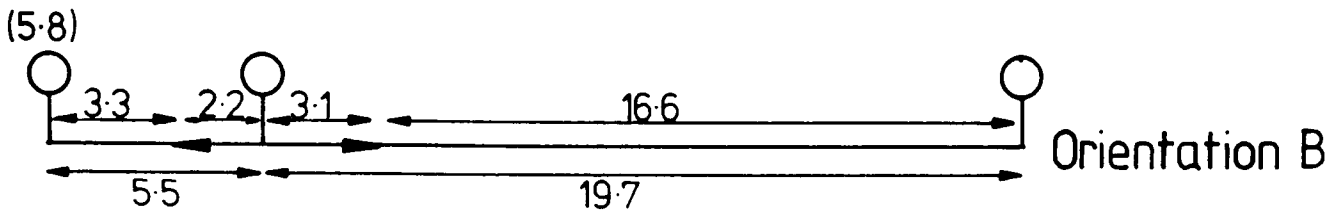


Orientation A

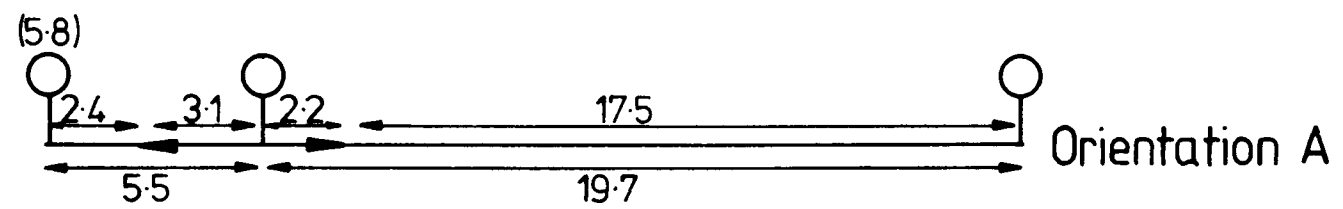
Position: 7·5+(3·1−2·9)=7·7

Figure 7·8 (cont.)

(d) *Nco* IA (19·9) ⟶ 19·7 + 5·5

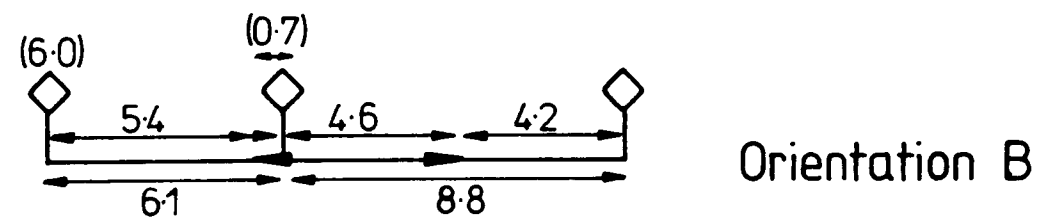


Positions: 5·8 + (5·5 − 2·2) = 9·1
or 5·8 + (19·7 − 2·2) = 23·3

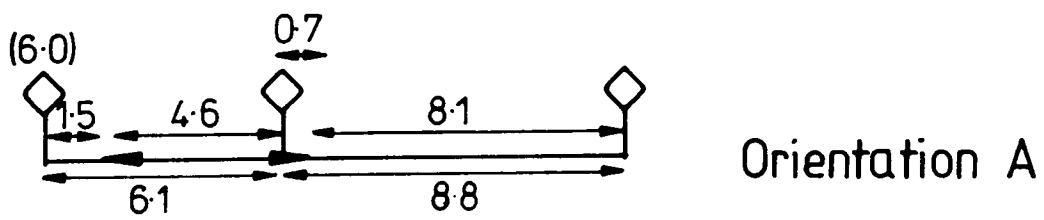


Positions: 5·8 + (5·5 − 3·1) = 8·2
or 5·8 + (19·7 − 3·1) = 22·4

(e) *Xba* Ic (9·6) ⟶ 8·8 + 6·1



Positions: 6·0 + (6·1 − 0·7) = 11·4
or 6·0 + (8·8 − 0·7) = 14·1



Positions: 6·0 + (6·1 − 4·6) = 7·5
or 6·0 + (8·8 − 4·6) = 10·2

Tn*551*, the possible sites of insertion are $[35.8 + (19.2 - 0.7)] - 50 = 4.3$ or $35.8 + (6.3 - 0.7) = 41.4$ if Tn*551* is in one orientation and $[35.8 + (19.2 - 4.6)] - 50 = 0.4$ or $35.8 + (6.3 - 4.6) = 37.5$ if Tn*551* is inserted in the opposite orientation.

From *Eco* RI and *Pvu* II data, the insertion must lie between map units 4.3 and 16.4 (*Eco*.A and *Pvu A*). The only possible *Xba* I position is therefore at 4.3 map units. This corresponds to orientation B (arbitrarily assigned) with the *Xba* I site 0.7 kbp from the left-hand end of Tn*551*. The *Nco* I and *Bgl* II insertion sites which most closely fit this data are at map positions 4.5 and 4.4 respectively. These correspond to site at 2.2 and 0.6 kbp from the left-hand end of Tn*551*. These positions can therefore be assigned as orientation B and confirm the map of Tn*551* shown in Figure 7.4. The orientation of Tn*551* insertion was designated for as many mutants as possible and the data used to confirm the map of the transposon.

The gel used for the analysis of Tn*551* insertion within mutant 2-24 is given in Figure 7.6 and the calculation shown diagrammatically in Figure 7.8.

For each mutant, the most probable site of transposon insertion for each enzyme was chosen and from these three results, an average value was calculated. The results are given in Table 7.3 together with the range of insertion for each mutant and its orientation. These results are also plotted under a map of p8325-2, shown in Figure 7.9.

7.4 Conjugation of the mutants

A conjugation experiment was performed between each of the 26 mutants and the recipient BIII Nov^r. A total of 11 experiments were performed (referred to as A-K) and in each case the conjugation frequency between 8325[p8325-2] and BIII Nov^r was calculated as a control. The results of these experiments are given in Table 7.4 and also on Figure 7.9. The experiment number in which each transfer was performed is given together with the actual number of transconjugants counted on two selection plates. This is followed by a column showing the conjugation frequencies. The fifth column lists the actual number of transconjugants from p8325-2 in the same experiment and this enables the ratio of the control conjugation to the mutant conjugation to be calculated (sixth column). In certain cases, the conjugation was performed several times so an average ratio is given.

The conjugation results have been classified into five groups according to the level of conjugation:

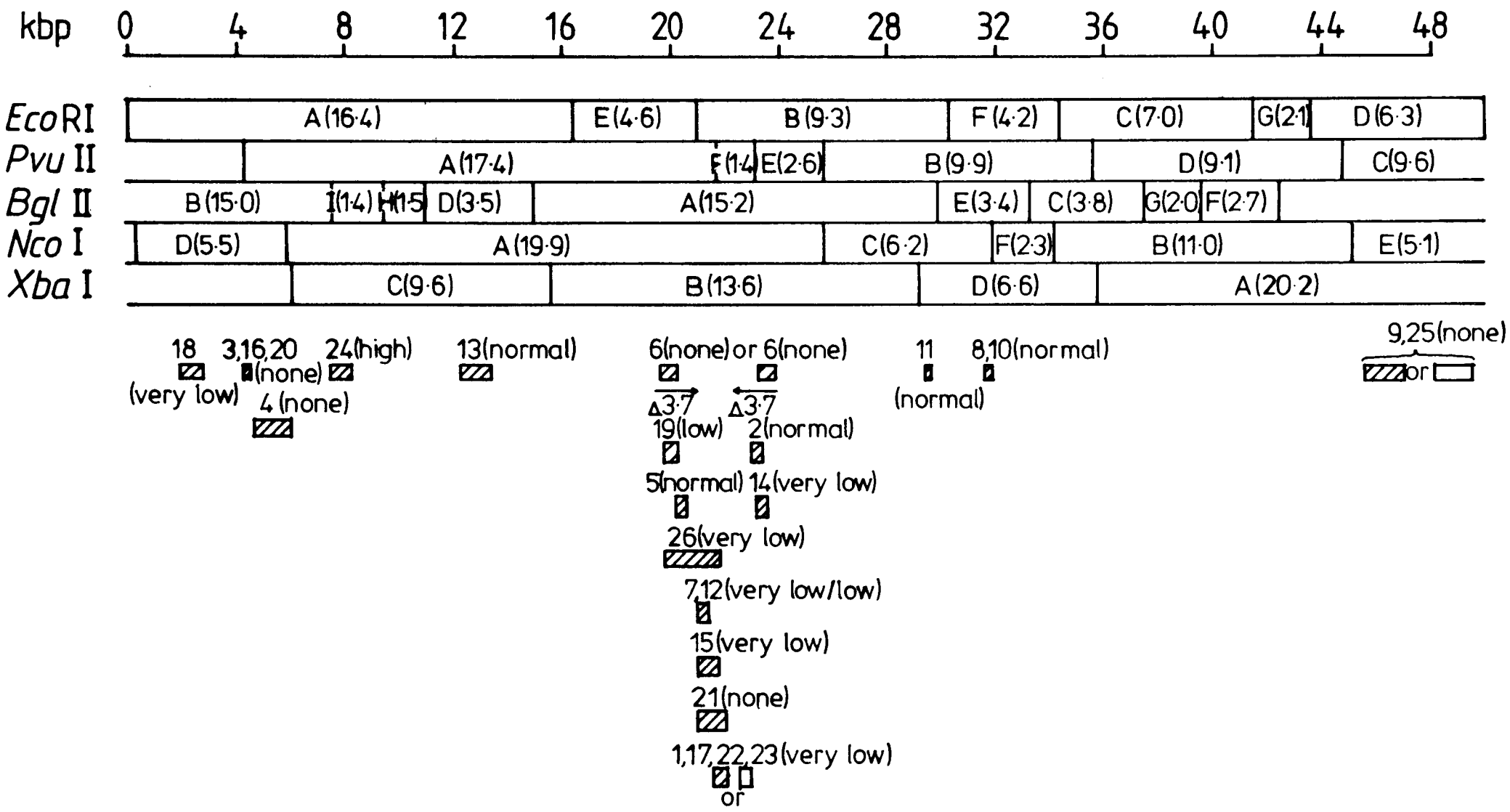
Table 7.3

Table showing the most probable points of insertion and orientation for each mutant

Name of mutant	Orientation	Insertion position (map units)			Average value and range
		*Bgl*II	*Nco*I	*Xba*I	
2-18	A	2.5	2.6	1.9	2.3 ± 0.4
2-3, 2-16, 2-20	B	4.4	4.5	4.3	4.4 ± 0.1
2-4	B	5.8	5.6	4.6	5.3 ± 0.7
2-24	A	7.7	8.2	7.5	7.8 ± 0.4
2-13	A	13.8	13.2	14.4	13.8 ± 0.6
2-6	B	19.9	19.6	20.2	19.9 ± 0.3 23.6 ± 0.3
2-19	A	20.0	20.2	19.9	20.0 ± 0.2
2-5	A	20.6	20.0	20.6	20.4 ± 0.2
2-26	B	21.8	20.2	20.5	20.8 ± 1.0
2-7, 2-12	A	21.3	21.2	21.1	21.2 ± 0.1
2-15	B	19.9	22.1	21.2	21.2 ± 1.3
2-21	B	21.8	21.0	21.7	21.5 ± 0.5
2-1, 2-17, 2-22, 2-23	A	22.0	21.7	22.1	21.9 ± 0.2
	B	23.0	22.6	22.7	22.8 ± 0.2
2-2	A	23.2	23.1	23.3	23.2 ± 0.1
2-14	A	23.5	23.2	23.5	23.4 ± 0.2
2-11	A	29.6	29.5	—	29.6 ± 0.1
2-8, 2-10	B	31.8	31.7	31.8	31.8 ± 0.1
2-9, 2-25	A	46.3	47.1	45.7	46.4 ± 0.7
	B	48.6	48.4	49.6	48.9 ± 0.7

Figure 7·9

Diagram showing the position and ranges of insertion for the mutants created by insertion of Tn*551* into the genome of p8325-2



The conjugation frequencies are given in brackets

Table 7.4 Table showing the conjugation frequencies of all p8325-2 mutants

Mutant No.	Expt. No.	No. of transconjugants	No. of transconjugants with p8325-2 in the same experiment	Frequency of conjugation	Ratio of conjugation p8325-2 : conjugation of mutants	Average ratio	Classification	Average insertion site	Orientation
2-3	A	0	1400	$< 8 \times 10^{-9}$	∞	∞	none	4.4	B
2-16	F	0	1500	$< 4 \times 10^{-8}$	∞	∞	none		
2-20	G	0	377	$< 4 \times 10^{-8}$	∞	∞	none		
2-4	B	0	1400	$< 8 \times 10^{-9}$	∞	∞	none	5.3	B
2-6	B	0	1400	$< 8 \times 10^{-9}$	∞	∞	none	19.9	B
2-21	G	0	377	$< 4 \times 10^{-8}$	∞	∞	none	21.5	B
	J	0	1319	$< 4 \times 10^{-8}$	∞				
2-25	I	0	688	$< 4 \times 10^{-8}$	∞	∞	none	46.4	A/B
2-9	C	0	1400	$< 8 \times 10^{-9}$	∞	∞	none	48.9	
2-14	E	1	3000	3.0×10^{-8}	3000	3000	very low	23.4	A
2-17	F	2	1500	8.0×10^{-8}	750	750	very low	21.9 22.8	A/B
2-15	E	9	3000	2.4×10^{-7}	333	333	very low	21.2	B
2-26	I	3	688	1.2×10^{-7}	229	229	very low	20.8	B
2-7	A	7	1400	6.0×10^{-8}	183	183	very low	21.2	A
2-23	H	11	990	4.4×10^{-7}	90	90	very low	21.9 22.8	A/B
2-18	I	9	688	3.6×10^{-7}	76	76	very low	2.3	A
2-1	A	47	1400	3.8×10^{-7}	29	68	very low	21.9	A/B
	D	13	1400	1.0×10^{-7}	107			22.8	
2-22	H	18	990	7.2×10^{-7}	55	55	very low	21.9 22.8	A/B

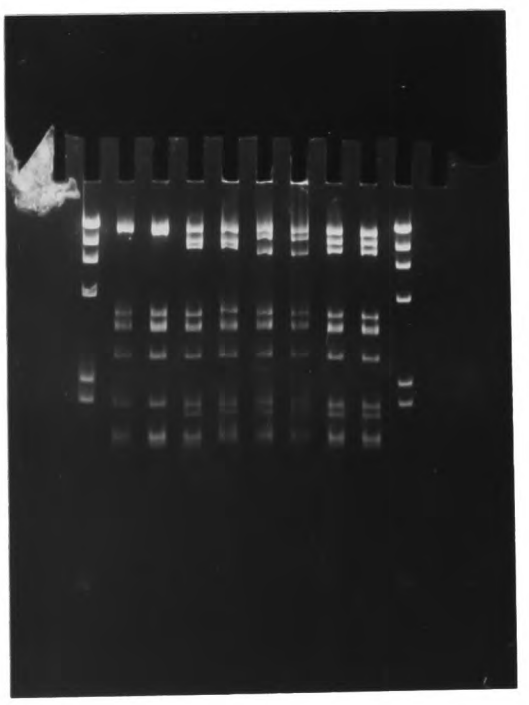
Table 7.4 (cont.)

Mutant No.	Expt. No.	No. of transconjugants	No. of transconjugants with p8325-2 in the same experiment	Frequency of conjugation	Ratio of conjugation p8325-2 : conjugation of mutants	Average ratio	Classification	Average insertion site	Orientation
2-12	C	146	1400	1.2×10^{-6}	9.3	9.3	low	21.2	A
2-19	G	81	377	3.2×10^{-6}	4.7	4.7	low	20.0	A
2-13	E	1500	3000	4.0×10^{-5}	2.0	2.0	normal	13.8	A
2-5	B	668	1400	5.3×10^{-6}	2.1	1.5	normal	20.4	A
	D	1400	1400	1.1×10^{-5}	1.0				
2-2	A	1400	1400	1.1×10^{-5}	1.0	1.2	normal	23.2	A
	D	1400	1400	1.1×10^{-5}	1.0				
	J	790	1319	3.2×10^{-5}	1.6				
2-11	C	1400	1400	1.1×10^{-5}	1.0	1.2	normal	29.6	A
	K	862	1308	3.4×10^{-5}	1.5				
2-8	B	1400	1400	1.1×10^{-5}	1.0	1.0	normal	31.8	B
2-10	C	1400	1400	1.1×10^{-5}	1.0	1.0	normal		
2-24	H	2398	990	9.6×10^{-5}	0.41	0.37	high	7.8	A
	K	4304	1308	1.7×10^{-4}	0.30				

Figure 7.10

Gel showing mutant plasmid transfer to BIII Nov^r

Lane	Digestion	Fragment sizes (kbp)
1	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56
2	8325[p8325-2]/*Bgl*II	15.2 15.0 3.8 3.5 3.4 2.7 2.0 1.5 1.4
3	8325Nov^r[p8325-2]/*Bgl*II	15.2 15.0 3.8 3.5 3.4 2.7 2.0 1.5 1.4
4	8325[p2-1]/*Bgl*II	15.0 10.0 8.7 3.8 3.5 3.4 2.7 2.0 1.8 1.5 1.4
5	8325Nov^r[p2-1]/*Bgl*II	15.0 10.0 8.7 3.8 3.5 3.4 2.7 2.0 1.8 1.5 1.4
6	8325[p2-2]/*Bgl*II	15.0 11.2 7.5 3.8 3.5 3.4 2.7 2.0 1.8 1.5 1.4
7	8325Nov^r[p2-2]/*Bgl*II	15.0 11.2 7.5 3.8 3.5 3.4 2.7 2.0 1.8 1.5 1.4
8	8325[p2-5]/*Bgl*II	15.0 10.1 8.6 3.8 3.5 3.4 2.7 2.0 1.8 1.5 1.4
9	8325Nov^r[p2-5]/*Bgl*II	15.0 10.1 8.6 3.8 3.5 3.4 2.7 2.0 1.8 1.5 1.4
10	λH3	23.1 9.42 6.68 4.32 2.32 2.03 0.56



none (ratio ∞)

very low (ratio of 50-3000)

low (ratio of 4-10)

normal (ratio of 1-2) and

high (ratio of <1).

Plasmid DNA was prepared, where possible, from a selected transconjugant from each of the conjugations. Each was digested with *Bgl* II and run alongside similarly digested parental mutant DNA to determine if exact transfer had occurred. The results of one such gel showing the transfer of 2-1, 2-2 and 2-5 are given in Figure 7.10. The transfer of mutant plasmids occurred without detectable alterations of the DNA in every case.

7.5 Discussion

Mapping of the Tn*551* insertion mutants within p8325-2 helped confirm the previously derived restriction enzyme map with respect to the enzymes *Bgl* II, *Eco* RI, *Nco* I, *Pvu* II and *Xba* I. The position of insertion could be best defined using the enzymes *Bgl* II, *Nco* I and *Xba* I as these contain sites within TN*551* itself. Although the two *Bgl* II sites within the transposon were already known (Browne, 1985) it was possible to map the single *Nco* I and *Xba* I sites on the basis of the data obtained in this survey.

As Tn*551* can insert in one of two possible orientations and because some fragments are large, there are often four possible insertion sites. If insertion is near the end of a fragment however, there are only two possible sites. The results from *Eco* RI and *Pvu* II digests were used, where possible, to discard any of the four sites from the other enzymes. The orientation of the transposon could be determined in all but six insertions. These were used to determine the most probable insertion position on the basis of the positions which most closely correlated from each of the enzymes *Bgl* II, *Nco* I and *Xba* I. It must be emphasized that the map position given for each mutant is an average value only. Certain discrepancies will occur due to errors inherent in restriction mapping by agarose gel electrophoresis and for this reason, a range has been given for each mutant.

Considering the relatively large size of the restriction fragments to be sized, the limits of insertion are relatively narrow.

Insertion of Tn*551* into certain positions within p8325-2 affected the conjugation frequency and from this it can be tentatively concluded that such regions are involved in conjugal transfer. More than one region of the p8325-2 genome is involved in the process.

Insertion of Tn*551* into the area between map units 20 and 23 either abolished or considerably reduced the conjugation frequency. On the basis that this region is involved in conjugation it has been designated region "*Tra* I". Another area of the genome into which certain insertions abolished conjugation and others reduced it to a minimum was between 45.7 and 6.0 map units (10.3 kbp). This region has therefore been designated "*Tra* II". These regions are separated by areas in which insertions caused no apparent effect on conjugation. The regions *Tra* I and *Tra* II are therefore most probably separate operons.

It is possible of course that there are more distinct operons involved but that no insertions occurred within them. It is also possible that the area designated "*Tra* II" is infact two or more operons as mutants 2-9 and 2-25 are relatively well separated from mutants 2-3, 2-4, 2-16 and 2-20. Christie and Dunny (1986) obtained 50 insertions of Tn*917* within the *Streptococcus faecalis* plasmid pCF-10 (58 kbp) and were able to map nine distinct *Tra* regions.

The conjugation frequencies obtained with the 26 mutants could be broadly classified into five classes. In eight mutants transfer was abolished but in nine transfer was extremely low and in two (2-12 and 2-19) transfer was slightly higher but still greatly reduced compared with that of the parental plasmid. It is possible that these insertions are close to the boundary of the actual region involved in transfer and affect the transfer to varying extents, depending upon their distance to the operon.

It is interesting that when Christie and Dunny (1986) used Tn*917* to localize transfer regions in pCF-10, they also reported partial loss of transfer at the boundaries of a *Tra* region. Mutations within such regions however resulted in a completely transfer-negative phenotype. The area enclosed by the *Tra* region and its boundary was less than 1.5 kbp and the partial effect on transfer extended about the same distance each side of the transfer region, perhaps due to two similar or identical operons transcribed in opposite directions from within the *Tra* region.

From the data obtained during this survey, it is not possible to predict whether a similar system exists in p8325-2; a far greater number of insertions would have to be mapped to confirm such a hypothesis.

Although transposon mutagenesis is a valuable technique in the determination of regions involved in any one event, insertion of a transposon into an operon may affect the expression of more than one gene as transcription can be terminated within the transposon, therefore due to the pleiotropic effect of the insertion mutants it is difficult to interpret the changes in phenotype and to ascribe an effect to inactivation of a particular gene. Results from this study can therefore only be interpreted on the basis that at least two operons are involved in the conjugal transfer process.

There are several effects that insertion of Tn*551* into the p8325-2 genome may have on transcription/translation:

1) Tn*551* may have a stop signal in one or both orientations

2) the transposon may have a promotor in one or both orientations

3) there may be no effect on transcription/translation.

Unfortunately little is known about the genomic structure of Tn*551* so its effect in this respect is unknown. It is possible that it possesses a low level promoter, transcription from which results in the low level of transfer obtained from certain mutants especially within the *Tra* II region. Whether the orientation in which it has inserted is important is also speculative. It is possible that it has inserted in one particular orientation (designated B) in all mutants in which conjugation was abolished and in the opposite orientation (designated A) in most examples where transfer occurred at a normal rate. It has not however, inserted in any one particular orientation in the mutants where there is a very low transfer frequency.

Mutant 2-2 (map position 23.2 ± 0.1) resulted in a normal transfer frequency whilst mutant 2-14 (map position 23.4 ± 0.2) produced a very low transfer rate. It is possible therefore that these mutants lie very close to the boundary of the transfer region; mutant 2-2 may be inserted at 23.3 whilst 2-14 is inserted at 23.2. If this mapping was correct, this would define the boundary of the *Tra* I region as between map units 23.2 and 23.3.

Mutant 2-5 which exhibits a normal conjugation phenotype has inserted within the *Tra* I region between other mutants which greatly reduce the conjugation frequency. The region designated *Tra* I may actually consist of more than one operon or mutant 2-5 may have inserted into an intercistronic region, that is, into a non-essential coding region of the operon, between genes. This region could be very small.

Insertion of Tn*551* into map position 7.8 ± 0.4 resulted in a mutant (2-24) which gave an enhanced frequency of conjugation compared with the parental plasmid. In this example, Tn*551* may have inserted into a control region for conjugation. Such a region would normally depress transfer; insertion into the gene/s involved abolishes or reduces the control. Asch *et al* (1984) also report such a region on the self-transmissible *S.aureus* plasmid pCRG1600. Christie and Dunny (1986) found six separate regions on the *Streptococcus faecalis* plasmid pCF-10, insertion into which lead to enhanced levels of transfer. Cells carrying these mutant plasmids differed in colony morphology or growth in broth from cells carrying the parental plasmid. No such change was observed for mutant 2-24. It is possible that Tn*551* has not inserted into a control region but is using a high level promotor of its own to activate an operon of p8325-2 involved in the conjugal process.

Insertion of Tn*551* at map position 19.9 ± 0.3 was accompanied by a deletion of about 3.7 kbp extending towards the right-hand end of p8325-2. As drawn in Figure 7.9, it is also possible that Tn*551* has inserted at $(19.9+3.7) = 23.6$ map units creating a deletion of 3.7 kbp, towards the left. However, as mutant 2-2 results in a normal transfer frequency (mutant 2-6 results in no transfer) and would lie to the left of 2-6 if the deletion were to the left, the most likely position of insertion into 2-6 would be at 19.9 kbp with the deletion to the right.

Although deletions are known to be a consequence of the excision of transposons it is not thought that they occur as a concomitant of insertion (Novick *et al,* 1979). The insertion of Tn*551* into p8325-2 reported in this study appears to be one such example. It is possible that p8325-2 normally carries a transposon of 3.7 kbp within this region. The ends of this transposon may have homology with those of Tn*551* making them recombinogenic. In the case of mutant 2-6, a recombination event between the two transposons may have caused the insertion of Tn*551* accompanied by the deletion of the original transposon.

It is evident (Figure 7.9) that the insertion points of the 26 mutants are not randomly distributed around the plasmid genome but that the transposon has inserted into "preferred sites"or

"hotspots". One such site lies between 19.9 and 23.4 map units, within which, the transposon has inserted 14 times. Although this area represents only 7 per cent of the total genome, it contains 54 per cent of the total insertions. A second "preferred insertion area" appears between 2.3 and 7.8 map units, representing 11 per cent of the genome, into which 23 per cent of the insertions have occurred. There are no insertions however, between 31.8 and 46.4 map units (29 per cent of the genome) or between 13.3 and 19.9 map units (13 per cent of the genome).

Cloning experiments (Chapter 5) revealed that the gentamicin resistance determinant/s lies on *Bgl* II fragment D (between 10.9 and 14.4 map units). Since selection was made for gentamicin-resistant transformants, insertions into the gentamicin resistance determinant would not be screened for. Therefore insertions within this area would not be detected. Any insertions which occurred within the *ori* V region (position unknown) would also not be selected for, as the plasmid could not be maintained without a functional origin of replication. These regions are probably comparatively small however compared to at least 42 per cent of the p8325-2 genome that contains no insertions. It is known that only a small region of ColE1 (about 580 bp) is essential for replication (see Hardy, 1986) and not more than 3.5 kbp are needed to confer gentamicin resistance (Chapter 5).

DNA sequencies differ in thir readiness to act as target sites for a particular transposon so although a transposon can usually be inserted at one of a large number of positions, such sites may not be randomly distributed at the nucleotide level. For example, in the transposition of Tn*9* into the terminal 160 bases of the *E.coli lac Z* gene, 28 independent transpositions were detected; 16 positions observed but five were represented by multiple occurrences of insertion.

It is interesting that the clustering of Tn*551* insertions into p8325-2 appears to be within the two regions involved in conjugation. This transposon may have a hotspot within the operons involved in transfer. As discussed above, p8325-2 may carry a transposon inserted within the *Tra* 1 area (19.9 to 23.4 map units). If this transposon had homologous ends to Tn*551* this would provide a region of homology for transposition creating a hotspot for insertion as well as explaining the 3.7 kbp deletion in mutant 2-6 if insertion was accompanied by deletion of the original transposon.

Certain mutants were indistinguishable. For example, mutants 2-3, 2-16 and 2-20 all mapped at 4.4 ± 0.1 map units. Similarly, mutants 2-1, 2-17, 2-22 and 2-23 all mapped at either 21.9 ± 0.2 or22.8 ± 0.2 map units. From the 26 mutants obtained, 13 mapped at the same site as another, providing more evidence for the clustering of insertions. Each mutant was created by an independent insertion of Tn*551* into p8325-2. It would not have been possible in the time available during the

transformation reaction for insertion into a particular site to be followed by cell division prior to the plating out and selection for transformants. Also, two transformation experiments were carried out, therefore, many of the mutants containing the same insertion sites were created on different occasions.

There are several possible reasons for the inability to obtain insertion within p8325-4. Firstly, it is now known that the activity of a transposable element can be directly interconnected with the activity of its host. For example, the loss of methylation can increase transposition (Kleckner, 1986). A similar event may reduce transposition. It is also known that Tn*A*, a transposon related to Tn*551*, transposes only at low frequency into plasmids already carrying another copy of the transposon. This inhibition is known as "transposition immunity". It has been shown (Chapter 5) that p8325-4 carries the transposon Tn*4001* and it is possible that this is reducing or even preventing transposition of Tn*551*. This explanation would be less likely if p8325-2 carried a transposon itself as discussed above.

Finally, it has been reported that the rate of transposition per element for Tn*10* actually decreases when the element is present at high copy number (Kleckner, 1986). The transposon Tn*551* may have been present in too high amounts for suitable transposition to occur into p8325-4.

7.6 Conclusion

The technique of transposon mutagenesis proved successful in the analysis of those parts of the genome of the gentamicin resistance plasmid p8325-2 involved in the conjugal transfer process.

Insertion of Tn*551* into p8325-2 produced 26 mutants. The position of transposon insertion in each were mapped and their conjugation frequency determined. From these results it was possible to construct a more detailed restriction map of Tn*551*. It was also possible to conclude that:

1) there are at least two regions involved in the conjugal process

2) there appear to be preferred sites of insertion on the p8325-2 genome for Tn*551*

3) there is probably a region involved in the control of the conjugal process.

Chapter 8

General Discussion

8.1 Transfer systems in *Staphylococcus aureus*

The two gentamicin resistance conferring plasmids chosen for the study of the conjugal transfer system in this species (p8325-2 and p8325-4) could both be transferred via a filter mating experiment to 8325 Nov^r at quite high frequency (4.5×10^{-6} and 3.9×10^{-5} transconjugants per recipient respectively) (Table 3.2) whereas only p8325-2 was transferable to B111 Nov^r (5.12×10^{-4} transconjugants per recipient) (Table 3.1). Further analysis of p8325-4 and its conjugative properties lead to the conclusion that these findings are more significant than was originally thought.

Conjugation experiments performed subsequent to those reported in Chapter 3 resulted in lower conjugation frequencies between 8325[p8325-4] and 8325 Nov^r: 8.2×10^{-7} (Table 4.3) and 4.2×10^{-6} (Table 5.1). In addition, these values were not significantly higher than controls carried out at the same time (1.2×10^{-6} for 8325[pCW59]) (Table 5.1). Further evidence indicating that p8325-4 may not be transferred by conjugation in this system was provided by its inability to transfer to PS80d.*ero* or B111 Nov^r whilst plasmid p8325-2 could transfer to both recipients (Table 6.1). The plasmid p8325-4 contains an 18 kbp region homologous with pI524, presumably not involved in transfer. This leaves only about 20 kbp for conjugative functions compared with almost 50 kbp for p8325-2. Although 20 kbp would certainly be adequate to code for a conjugation system, in general, conjugative plasmids are larger than this.

During the investigation into mobilization (Chapter 6) it was found that pT181 (a small non-conjugative plasmid) was transferred from 8325[pT181] to 8325 Nov^r at high frequency(1.1×10^{-4}). This process was probably not conjugation but could be bacteriophage-mediated transfer between *S.aureus* strains. Circumstantial evidence for this is that transfer of p8325-4 and pT181 was only to the Phage Group III strain 8325 and its derivatives but not to B111 Nov^r and PS80d.*ero* which are in different Phage Groups. The nature of the bacteriophage involved is not known and was not investigated but it is tentatively concluded that, under the conditions used, there is no evidence that p8325-4 is a self-transmissible plasmid. It is more likely that the transfer of p8325-4 and pT181

is by phage-mediated conjugation (Lacey, 1980) in which there is a requirement for both phage and cell-to-cell contact.

What then, is the evidence for conjugation in *Staphylococcus aureus*?

Archer and Johnson (1983), Forbes and Schaberg (1983) and McDonnell *et al* (1983) have concluded that there is a transfer system from the evidence that:

1) Cell-to-cell contact is an absolute requirement since transfer fails to occur in broth or when filters are placed back-to-back.

2) The process is energy dependent and requires viable donor cells.

3) It is resistant to DNAase.

4) There is no calcium requirement; transfer occurs in the presence of chelating agents.

5) Transfer occurs between non-lysogenic donor and recipient cells.

Although similar experiments have not been rigorously repeated for the p8325-2 system, it is concluded that this plasmid, at least in part, specifies a conjugation system of similar kind because:

1) Membrane filtration (and therefore presumably cell-to-cell contact) was an essential pre-requisite for transfer.

2) There was no evidence for a requirement for calcium ions which are essential for the adsorption of many staphylococcal transducing phages.

3) Transfer of p8325-2 to B111 Nov^r was at similar high frequencies whether 8325[p8325-2] or PS80d.*ero*[p8325-2] was the donor whereas PS80d.*ero* was a much poorer recipient than B111 Nov^r. Thus the frequency of transfer to a particular recipient is a property of the plasmid itself and not of the host bacterium.

4) The mutants resulting from insertion of Tn*551* that reduce the rate of transfer are in at least two distinct locations. Although it is possible that the mutants are the results of insertion into prophage DNA, it seems unlikely that two phages are involved or, alternatively, that two parts of the prophage are inserted into different sites. Similarly it is

difficult, although not impossible, to explain the mutant with an elevated frequency of conjugation on the basis of a lysogenic phage model. If phage is involved in the transfer system then at least part of the phage genome or its control must be located on p8325-2. It still remains a possibility that transfer of p8325-2 is the result of a combination of a phage-mediated system and non-phage mediated conjugation. For example, transfer to 8325 may be phage-mediated and transfer to B111 Nov^r "true" conjugation.

The conjugative transfer of resistance markers between *S.aureus* strains in the absence of detectable plasmids has recently been reported (El Solh *et al,* 1986a). Evidence for the chromosomal location of these genes included their low frequency of transduction and the absence of extrachromosomal DNA in the transconjugants. Although such transfer is known to occur in streptococci (Franke and Clewell, 1981); *Bacillus licheniformis* (Docherty *et al,* 1981); *Pseudomonas aeruginosa* (Ingram *et al,* 1972) and *Clostridium innocuum* (Magot, 1983), reports of conjugal transfer in staphylococci have so far been limited to plasmid-borne determinants (Archer and Johnson, 1983; Forbes and Schaberg, 1983; Goering and Ruff, 1983; McDonnell *et al,* 1983; Asch *et al,* 1984; Townsend *et al,* 1984).

The elements responsible for this non-plasmid mediated transfer have not yet been identified. It is possible that they are very large plasmids and the techniques used for isolation were not sensitive enough to detect them; plasmids larger than 60 kbp have not been isolated from staphylococci (Lyon *et al,* 1984). The transfer could be mediated by chromosomally integrated conjugative plasmids or transposons or even by chromosomally determined conjugation genes.

8.2 Transposition of gentamicin resistance

Cloning restriction fragments from p8325-4 revealed the presence of the transposon Tn*4001* on this plasmid (Chapter 5). That the gentamicin resistance was encoded on a transposable element in 8325[p8325-4] was hitherto unknown. Tn*4001* was the first transposon encoding aminoglycoside resistance determinants to be found on a staphylococcal plasmid (Lyon *et al,* 1984) and was first found on pSK1, the most frequently detected plasmid in methicillin-resistant *S.aureus* isolates from Australian hospitals (Gillespie *et al,* 1986). It is known to contain two *Hind* III sites, each about 1.1 kbp from the ends. In this study, the characteristic 2.5 kbp *Hin*d III fragment which encompasses the central coding region (Gillespie and Skurray, 1986) was found but, in addition, it was mapped with respect to a single *Hae* III site and found to contain no *Pst* I site (Figure 5.11).

The 2.5 kbp *Hind* III fragment has been cloned and used as a probe in DNA-DNA hybridizations, demonstrating the presence of Tn*4001* sequences on gentamicin-resistance conferring plasmids isolated from many Australian strains as well as strains from the USA such as pSH6 and pUW3626 (Gillespie and Skurray, 1986). As the latter is known to be self- transmissible, this could provide means for the rapid spread of gentamicin resistance.

The discovery of Tn*4001* on p8325-4 extends its known geographical location to Europe (p8325-4 was originally isolated from *S.aureus* A102, a strain from Germany) making it a possible means by which the worldwide spread of gentamicin resistance has occurred.

8.3 Comparisons between conjugative plasmids

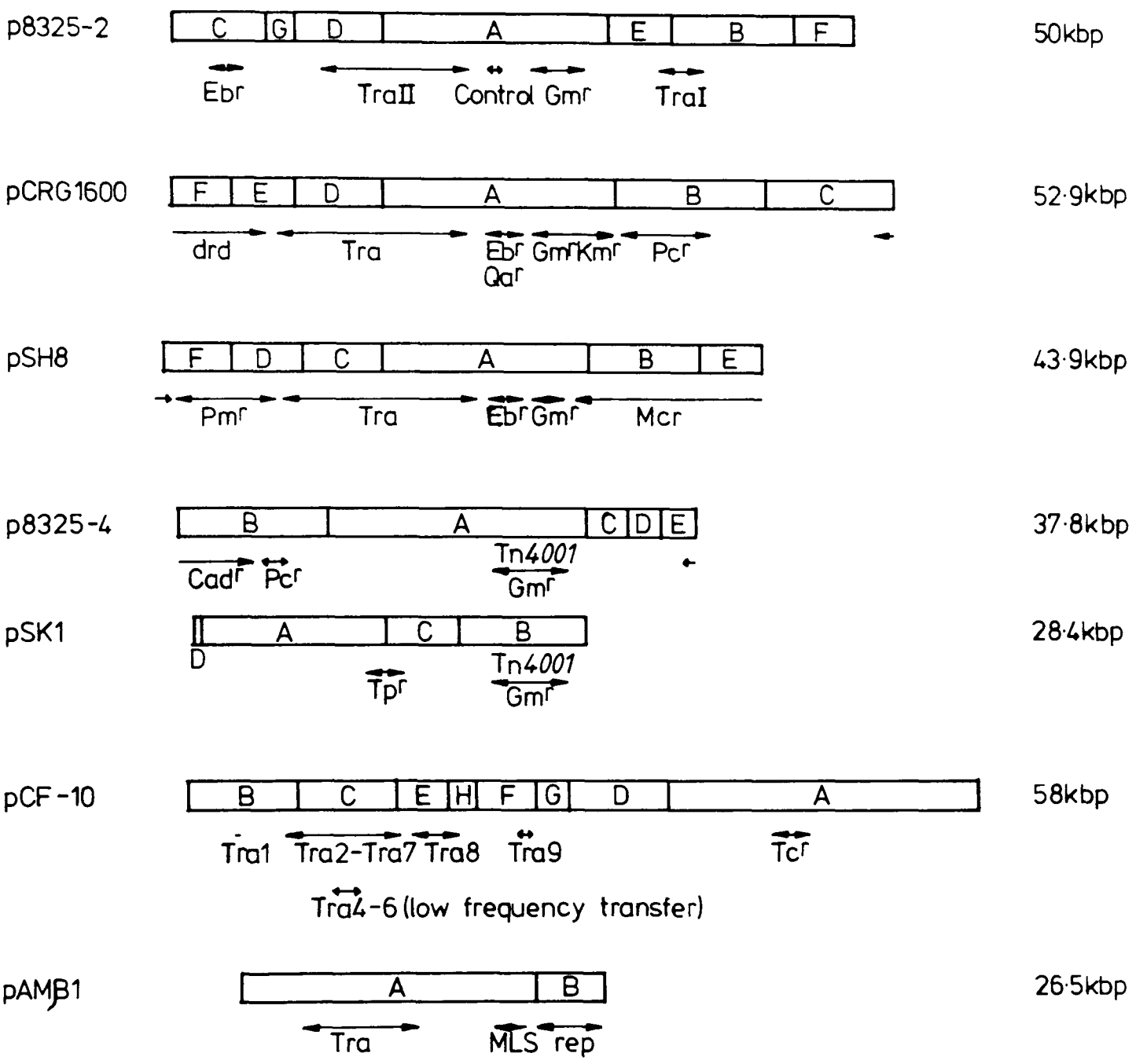
The *S.aureus* plasmids pCRG1600 and pSH8 are self-transmissible and encode gentamicin resistance. Regions involved in transfer have been located on both by the mapping of deletion mutants. These were readily obtained after mixed culture transfer in the case of pSH8 McDonnell *et al,* 1983) and after transduction in the case of pCRG1600 (Asch *et al,* 1983). The regions of each plasmid thought to be involved in transfer are shown in Figure 8.1.

The similarity between the locations of the *Tra* regions is striking. In all three examples (p8325-2, pCRG1600 and pSH8) a *Tra* region extends across *Eco* RI fragment C or D about one-third of the way into *Eco* RI fragment A. Plasmid p8325-2 contains at least two *Tra* regions whereas only one has been found on pCRG1600 and pSH8. A second region should now be sought in the latter two plasmids. Mutant plasmids of pSH8 and pCRG1600 carried relatively large deletions, ranging from 2 to 14 kbp for pSH8 (McDonnell *et al,* 1983) and from 7 to 32 kbp for pCRG1600 (Asch *et al,* 1983). Since, for p8325-2, the two regions are less than 15 kbp apart, one *Tra* region may have been deleted in some of the deletion mutants of pCRG1600 and a second in some other mutants. Some mutants may have both regions deleted. Transposon mutagenesis is a more accurate method of defining the regions.

The Mcr region of pSH8 (Figure 8.1) is thought to mediate plasmid maintenance, compatability and replication. If deletions occurred within this region the plasmid would be unable to replicate and hence would not be detected. From Figure 8.1, this region corresponds to the *Tra*I region of p8325-2. Hence it is possible that there is a *Tra* region in pSH8 corresponding to the *Tra*I region of p8325-2 which could only be located by deletion analysis with great difficulty.

Figure 8·1

Comparison of funtional maps of staphylococcal and streptococcal plasmids.



The plasmids are drawn with respect to their *Eco*RI digests. Fragments are listed alphabetically in order of decreasing size.

An alternative is that *Tra*I is really the vegetative replication system for p8325-2 but that it appears as a transfer region because Tn*551* interferes with the synthesis of a component that is, for example, required in only small amounts for vegetative replication but in much greater quantities for the conjugative transfer.

The location of possible *Tra* regions on the three plasmids correlates well and suggests divergence from a common basic structure for the transfer regions of these conjugative gentamicin resistance conferring plasmids.

The gentamicin resistance determinant present within a large *Eco* RI fragment in all five staphylococcal plasmids is, in all cases, about 3 kbp from an *Eco* RI site. The distance of the Tn*4001* transposon to the nearest *Eco* RI site is very similar for p8325-4 and pSK1. Thus, if it turns out that gentamicin resistance is carried by Tn*4001* in all five cases it could be that there is only one site into which Tn*4001* has integrated. These possibilities should be studied by further restriction enzyme mapping and hybridizations but, if true, will lead to the conclusion that these plasmids acquired Tn*4001* a long time ago and have subsequently undergone considerable re-arrangement. Another explanation would be if there was a very strong "hotspot" for Tn*4001* insertion at the position on the five plasmids.

The streptococcal and staphylococcal plasmids show little similarity (Figure 8.1). The conjugative streptococcal plasmid pCF-10 is reported to have nine regions involved in transfer (Christie and Dunny, 1986). Inactivation of only three (*Tra* 4, 5, 6) leads to lower frequencies of transfer. Insertional inactivation of the remaining six regions leads to an increased frequency of conjugal transfer. Whether this is due to a relatively large number of control regions or whether the increase is due to the presence of an efficient promoter in Tn*917* is not known.

Cells carrying mutant plasmids with insertions into six *Tra* regions (1-3 and 7-9) differed in colony morphology or growth in broth culture from cells carrying the wild-type plasmid. No such change was detected in the cells carrying transfer-deficient mutants of p8325-2 or those carrying the mutant plasmid with an elevated frequency of transfer. It is possible that whereas cell surface proteins are being inactivated in pCF-10, intra-cellular proteins have been inactivated in the p8325-2 mutants. On the other hand, the transfer-deficient mutants of pCF-10 did not have altered morphology.

The ability of the mutant pCF-10 plasmids with increased transfer ability to respond to sex pheromone was reduced whereas mutant plasmids with insertions into *Tra* regions 4 and 6 (low

frequency of transfer mutants) responded to sex pheromones. Mutations within the *Tra* 5 region gave two subclasses of cells; the first failed to transfer even when exposed to pheromone, the second responded to pheromone. It would be extremely interesting to determine if the ability of the *S.aureus* mutant strains to respond to sex pheromone was also altered. If so, this would be evidence for plasmid determination of a substance, possibly binding substance, involved in the response.

On the evidence available, pCF-10 appears very different from p8325-2 although more *Tra* regions may be present on the latter. Christie and Dunny (1986) were able to map 500 insertion mutants whereas in this survey, only 26 were located. Further insertions may reveal more *Tra* regions.

The streptococcal plasmid pAMβ1, unlike pCF-10, is not a sex pheromone responsive plasmid but is self-transmissible (Leblanc and Lee 1984). Although a *Tra* region was localized to a single 8 kbp fragment, the results were based on analysis of only three deletion mutants. More extensive analysis is needed before proper comparisons can be made.

To determine the number and precise location of *Tra* regions, extensive mapping and complementation analysis is needed as *Tra* operons could lie within only a few base pairs of each other. Confirmation of distinct *Tra* regions and their sizing requires cloning and sequence analysis for specific promoter and termination signals.

The F plasmid of *E.coli* K-12 has been studied most extensively and hence far more is known about it than conjugative staphylococcal plasmids. Until some evidence of homology between the two systems is produced, it is not likely to be productive to compare them in detail. Superficially they seem very different.

8.4 Future investigations

In future investigations, plasmid p8325-2 should be used and not p8325-4. The experiments which could be undertaken are:

1) The isolation and characterization of more mutants of p8325-2 by transposon mutagenesis, employing a variety of transposons perhaps with different "hotspots" for insertion.

2) The cloning of parts of p8325-2 into suitably compatible vectors (such as pCW59) that would permit the construction of diploids of parts of p8325-2 and p8325-2 with Tn*551* inserted. Complementation tests could be carried out and suitably complementing plasmids used, with the aid of restriction enzymes and the endonuclease Bal31, to define more precisely the regions of DNA involved in conjugation. The genetics of the conjugation system could then be determined; *Tra* mutants could be allocated to specific genes, the number of genes involved calculated and their order determined. Regulatory genes (identified by mutations resulting in an increased rate of conjugation) can be studied by constructing diploids and determining whether the high rate of conjugation can be repressed in ***trans***.

3) Site-directed mutagenesis may be helpful in constructing mutants whose ability to transfer can be determined. This method would produce single base pair changes, allowing mutations, and their effect on the conjugative properties, to be mapped precisely. Analysis of deletion mutants obtained spontaneously after transduction could also be carried out although the mapping of *Tra* regions would not be as accurate by this method.

4) Visualization and analysis of conjugating staphylococci by electron microscopy could provide substantial evidence for conjugation, determine whether pili were involved and whether direct intracellular connections of the sort seen between cells of *Streptococcus faecalis* (Krogstad *et al,* 1980) also occur in *Staphylococcus aureus*.

5) The phenotypes of structural and regulatory conjugative genes could then be studied. The presence of pili could be sought in "derepressed" strains and enzyme activities, such as nucleases, analysed. Surface phenomena, especially aggregation and colony morphology should be studied in mutant strains. The ability of mutants to mobilize non-conjugative plasmids should also be tested.

6) Regions involved in conjugative transfer could be cloned and used in Southern blot hybridizations to investigate relationships with other conjugative plasmids. This could provide evidence for the origin and evolution of these plasmids.

7) Long term research would include experiments designed to interfere with the process of conjugation to prevent its occurrence and possible role in the emergence of supermultiply-resistant strains. Conjugation may be more sensitive to antibiotics that interfere with DNA synthesis so an investigation into the sensitivity of the process to nalidixic acid and novobiocin may be informative. Conjugating bacteria may be more sensitive to lytic agents. If pheromones are important in staphylococcal conjugation, it may be possible to synthesize an analogue of the peptide pheromone that prevents the response of the strain carrying the conjugative plasmid.

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