Transposon Tn5 mutagenesis in *Pseudomonas aeruginosa*: a study of B-lactamase expression

by

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To Judy

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ABSTRACT

The object was to identify genes regulating expression of the chromosomally-encoded, inducible class C B-lactamase gene of P. aeruginosa. Transposon mutagenesis was considered an effective method for the identification and subsequent cloning of such regulatory genes.

To facilitate identification of Tn5 insertions in P. aeruginosa a transposon Tn5-derivative, Tn5::gen, encoding gentamicin-resistance was constructed. Transposon Tn5::gen was transposed into a temperature-sensitive broad host-range plasmid, pMMB19. The resulting plasmid, pRTG1, was able to deliver Tn5::gen into the chromosome of E. coli. Attempts to introduce pRTG1 into P. aeruginosa were generally unsuccessful and recombination events were observed in most transconjugants tested. Spot-mating conjugation was capable of introducing pRTG1 into P. aeruginosa albeit at very low frequencies. Analysis of the resulting transconjugant plasmids showed pRTG1 was probably subject to a stepwise series of recombination events, sometimes resulting in total loss of plasmid within P. aeruginosa. One derivative, pRTG1a, retained temperature-sensitivity and was used to deliver Tn5::gen into the P. aeruginosa chromosome. After growth at the non-permissive temperature, B-lactamase hyperproducing mutants of P. aeruginosa were isolated. Transposon Tn5::gen was located in the same chromosomal position in these mutants. Kinetic analysis of B-lactamase from these hyperproducing mutants showed them to have different basal B-lactamase activities and it seemed unlikely that Tn5::gen had a direct effect on B-lactamase synthesis. However, the basal B-lactamase activities of these mutants indicated that there were possibly six different basal activities to which P. aeruginosa can mutate.

The following papers are in preparation:

The Restriction enzyme cleavage map of pRTG1: a broad host-range, temperature-sensitive vector of Tn5.

Evidence for multiple levels of basal B-lactamase expression from B-Lactamase hyperproducing mutants of Pseudomonas aeruginosa PA04069.

Possible stepwise degradation of a temperature-sensitive, broad host-range plasmid in Pseudomonas aeruginosa PA04069.

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Chemical and Physical Abbreviations and Terms

Chemical abbreviations:

ATP	Adenosine triphosphate
dATP	2´-Deoxyadenosine 5´-triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
MOPS	3-(morpholino)propanesulphonic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium d odecyl sulphate
Tris	2-amino-2-(hydroxy-methyl)-propane-1,3-diol

Physical abbreviations:

^{o}C	degrees Centrigrade
bp	base pairs
g	gravitational force
Kbp	Kilobase pair
$lbs.ins^{-2}$	pounds per square inch
M	Molar
Mbq	Megabecquerel
mg/ml	milligrams per millilitre
mM	millimolar
mm	millimetre
pI	Isoelectric point

OD	Optical Density
rpm	revolutions per minute
ug	microgram
ug/ml	microgram per millilitre
ul	microlitre
uM	micromolar
um	micrometre
UV	Ultra-violet
v/v	volume per volume
w/v	weight per volume

Terms:

CF	Cystic fibrosis
cfu	colony forming units
FP2	Fertility plasmid 2
hyperproducers	mutants producing more enzyme than would be produced under wild-type induction. These can be classified into inducible and constitutive hyperproducers; the inducible hyproducers can be further induced, the constitutive cannot be further induced.
IS	Insertion Sequence
MIC	Minimum inhibitory concentration
PBP	Penicillin-binding protein
semiconstitutive	inducible hyperproducers
Tn5	Transposon Tn5

CHAPTER 1

INTRODUCTION

1.1 Clinical Review of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen of man and has become more significant as an infectious agent since the general use of antibiotics and the introduction of cytotoxic and immunosuppressive therapy (for review, see Cryz 1984). Introduction of antibiotics against other, more overtly pathogenic bacteria (e.g. *Staphylococcus aureus*), which are sensitive to most antibiotics removes the competitors of the relatively antibiotic-resistant and very adaptable *P. aeruginosa* (for review, see Brown 1975, Doggett 1979). Anti-pseudomonal antibiotics have been developed (Slack 1981) and their use in prophylaxis and therapy has led to the selection of resistant strains (Livermore *et al.* 1982, Shannon *et al.* 1982, Then and Anghern 1982). Such mutants occur at high frequency and are of major concern in the control of infections caused by *P. aeruginosa*. The biochemical and genetic basis of the relatively high resistance of *P. aeruginosa* to antibiotics is unknown but a variety of mechanisms have been proposed and will be discussed after a summary of the clinical importance of *P. aeruginosa*.

1.1.1 *P. aeruginosa* as an infectious agent

P. aeruginosa is consistently associated with the highest case fatality rates of any of the bacteraemic, Gram-negative rod infections (Doggett 1979). *P. aeruginosa* can cause acute pneumonia, urinary,

renal and hepatic infections in immunocompromised hosts and is probably the major cause of morbidity and mortality in cystic fibrosis (CF) patients (Govan and Harris 1986, Simpson et al. 1988).

Colonization by P. aeruginosa is associated with length of stay in hospital, underlying disease and antibiotic therapy (Grogan 1966). P. aeruginosa has become a major contributor to hospital-acquired infections because it is able to survive in a variety of environments within the hospital and also because of its relative insensitivity to many antibiotics and disinfectants (Stephenson et al. 1985; Scheckelhoff et al. 1986). Reservoirs of P. aeruginosa include people carrying infection, contaminated food, especially fresh salad, as well as water decanters, drinking fountains, sinks and humidifiers. Hospital equipment found to be contaminated include nebulizers, "sterile" dialysis tubing, parenteral nutritional fluids and surgical equipment. Infection by P. aeruginosa arises from surface contamination or from translocation from the patient´s intestine and is aided by surgery that disrupts natural barriers, for example tracheostomies and urinary catheters.

Virulence factors of P. aeruginosa

Pili aid attachment to the host and subsequent infection, and prevent phagocytosis (Woods et al. 1980). Flagella provide motility; important in the rapid dissemination of the infection through the host (Drake and Montie 1988). The exopolysaccharide of mucoid strains of P. aeruginosa aids bacteria-epithelium attachment and bacteria-bacteria attachment, which protects cells from phagocytosis and muciliary clearance (Reynolds and Fick 1980, Simpson et al. 1988). Pyocyanin may also inhibit clearance by inhibiting the regular beating

of cilia (Wilson et al. 1987). Many virulence factors are synthesized by P. aeruginosa including exotoxin A, exoenzyme S, elastase, alkaline protease, leukocidin and haemolysin (for review see Cryz 1984, Pollack 1984).

Susceptible people

Patients who are immunosuppressed either by illness or by treatment (i.e. immunosuppressants used in treating organ transplant and cancer patients) are particularly prone to infection by P. aeruginosa. In the late 1960's P. aeruginosa was the most common infective pathogen in immuno-compromised hosts (Aduan and Reynolds 1979). Only 20% of infected patients with septiceamia responded to polymyxin treatment, the only anti-pseudomonal antibiotic then available, and the mortality rate was approximately 90% (Fainstein and Bodey 1985).

P. aeruginosa is found most frequently in patients suffering from CF and cancer. P. aeruginosa is found in 70-90% of sputum samples from CF patients and is probably the primary cause of death in CF with 90% of these patients dying as a result of progressive pulmonary failure complicated by P. aeruginosa infection (Aduan and Reynolds 1979). Intravenous drug abusers are also prone to septicaemia and endocarditis as a result of infection by P. aeruginosa (Rubenstein and Lev 1988).

Burns patients

P. aeruginosa rarely infects people with less than 30% burns (Pruitt and Lindberg 1979). People suffering burns greater than 30% of the body surface are immunosuppressed; reticuloendothelial system function is suppressed, neutrophil functions such as chemotaxis, phagocytosis

and bactericidal activity are suppressed and the T-lymphocyte population is reduced (Pruitt and Lindberg 1979). This results in P. aeruginosa colonization of the patient usually once the patient is in hospital. At the Institute of Surgical Research, 60% of burns patients had pseudomonas infections within 5 days of being put into hospital (Pruitt and Lindberg 1979) and mortality was high, with 60% of fatalities having invasive Pseudomonas burn wound sepsis. P. aeruginosa was the leading cause of death in burns patients who survived the initial phase (Pruitt 1974). In 1980, a major burn institute reported that P. aeruginosa was the second most important pathogen, after S. aureus (Rubenstein and Lev 1988).

Cancer patients

Cancer patients can become susceptible to P. aeruginosa either because of the nature of the disease or because of the chemotherapy used to treat the cancer itself. Leukaemics are predisposed to infection by P. aeruginosa due to the immunosuppressive effect of the disease, as are patients suffering from other neutropenic cancers. The increased infection rates in other cancer patients is a result of myelosuppressive chemotherapy causing immunosuppression and was especially prevalent in the 1950's and 1960's (Rubinstein and Lev 1988). In the absence of potent antipseudomonal antibiotics, fatality rates for cancer patients infected with P. aeruginosa were usually 80-100% with 30-40% dying within 24 hours of the onset of bacteraemia (Rodriguez and Bodey 1979). The introduction of carbenicillin and other anti-pseudomonal antibiotics initially resulted in cure rates greater than 70%. The use of antipseudomonal B-lactams led to an increase in the occurrence of resistant strains over the following years in a number of centres (Moody et al. 1972, Bodey and Rodriguez

1973, Schimpff et al. 1973).

In one study (Moody et al. 1972) the introduction of carbenicillin in 1968 reduced mortality in cancer patients from greater than 80% to less than 30%. Resistant mutants, however, were isolated over the next few years with increasing frequency. Only 1 of 197 pseudomonas isolates were carbenicillin-resistant from 1969 to 1971. After 9 months this increased to 14% of 141 isolates, and after a further 6 months it had increased to 23% of 142 isolates. The majority of these cases of resistance were acquired during antibiotic chemotherapy.

P. aeruginosa is still a frequent infectious agent in cancer units and was the major contributor to bacteraemia in one cancer institute; responsible for 21% of all cases of bacteraemia and was also the most frequent pathogen (Cross et al. 1983).

In summary, people who are immunosuppressed are susceptible to infection by P. aerurginosa. Mortality rates are mainly dependent on the severity of the underlying disease of the patient, the virulence factors associated with, and the intrinsic resistance to antibiotics of P. aeruginosa (Rubinstein and Lev 1988).

1.1.2 B-Lactam antibiotics

The increased frequency of P. aeruginosa as an infectious agent has led to the development of anti-pseudomonal antibiotics including those based on the B-lactam ring structure. Naturally occuring and semi-synthetic B-lactams have a four-membered lactam ring. In most B-lactams the nitrogen and adjacent tetrahedral carbon atoms are bound

to a secondary ring structure. Penicillins are based on the penam nucleus structure, whilst cephalosporins are based on the cephem nucleus structure (see Figure 1.1). Chemical modification of the side-chains on the B-lactam nucleus has led to the development of the second, third and fourth generation B-lactams, with varying antibacterial properties (Neu 1986).

Slack (1981) has categorized B-lactams into four groups based on their anti-pseudomonal activity and sensitivity to B-lactamase:

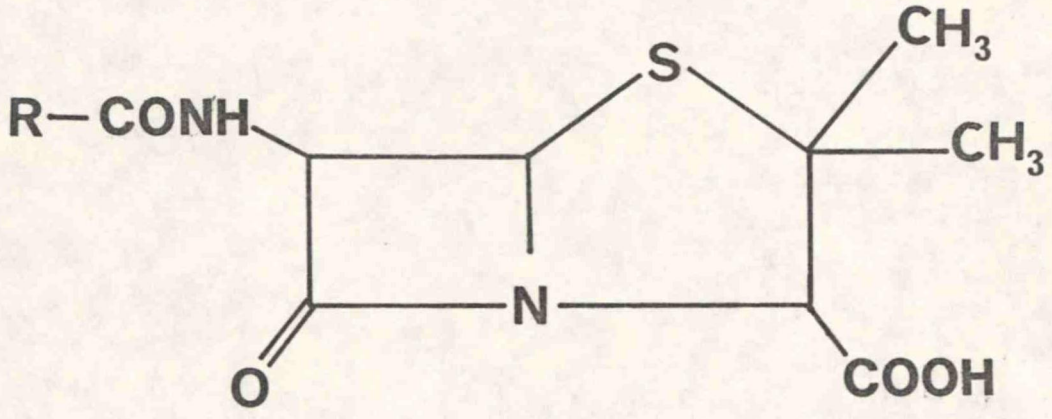
Group I. Compounds with moderate anti-pseudomonal activity and marked B-lactamase stability: cefotaxime, ceftioxime, cefatriaxone, moxalactam, cefmenoxime and nocardicin-A.

Group II. Compounds with moderate anti-pseudomonal activity which are susceptible to B-lactamases: cefoperazone, mezlocillin and ticarcillin.

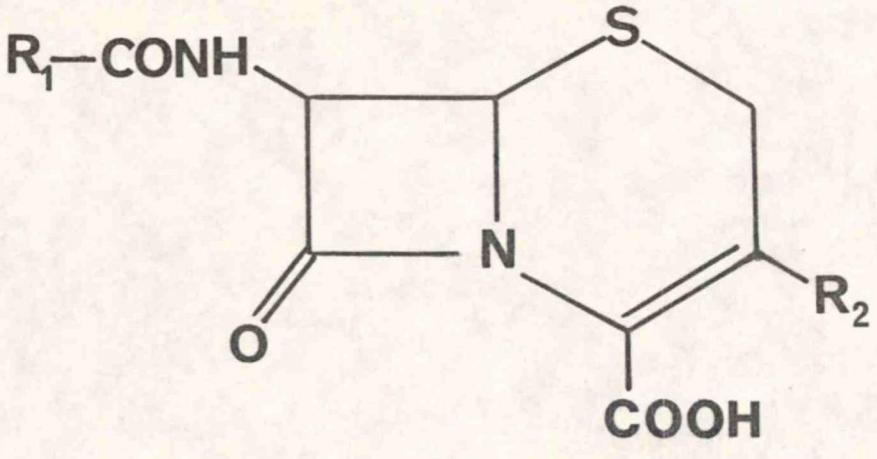
Group III. Compounds with marked anti-pseudomonal activity and B-lactamase stability: ceftazidime, cefsulodin and thienamycin.

Group IV. Compounds with marked anti-pseudomonal activity that are susceptible to B-lactamases: piperacillin, azlocillin, furazlocillin and apalicillin.

B-lactams with anti-pseudomonal activity have been widely prescribed for prophylaxis and therapy. Carbenicillin-resistant isolates were first reported in 1969 (Lowbury et al. 1969) and were shown to arise during therapy. Mutants resistant to these newer B-lactams occur at a



PENICILLINS



CEPHALOSPORINS

Figure 1.1: Generalized Structure of Penicillin and Cephalosporin B-lactams.

high frequency in P. aeruginosa and are prevalent in the hospital environment where people susceptible to P. aeruginosa infection are present. This is especially true of cancer institutes, burns units, cystic fibrosis centres and intensive care units (Sanders 1987). If the mechanism of such resistance could be elucidated it may be possible to improve the treatment of patients infected with P. aeruginosa. It may be possible to identify strains likely to mutate to B-lactam resistance and thereby use alternative antibiotics, for example, aminoglycosides in therapy. It may also be possible to affect the design of future B-lactams to overcome the current resistance mechanisms.

1.2 Mechanisms of Bacterial Resistance to Antibiotics

The use of antibacterial agents generates selective pressure for mutations conferring antibiotic resistance. Resistance mechanisms can be broadly categorized into three types:

1 Preventing the antibiotic reaching its site of antibacterial activity by

i) making the outer membrane impermeable to the antibiotic

ii) enzymic inactivation of the antibiotic

iii) actively transporting the antibiotic away from its site of action.

2 Modifying the target site to

i) reduce the affinity of the target site for the antibiotic

ii) increase the affinity of the target site for its "true" substrate.

3 Tolerating the antibiotic by

i) reducing the demand of the cell for the inhibited system

ii) using an alternative or less inhibited system

iii) diluting the inhibitory effect by increasing the number of target sites.

The effectiveness of any mechanism in conferring resistance to antibiotics is dependent upon the degree to which the above processes occur and the detrimental effects that the mechanism has upon the physiology of the cell. For example, in Gram-negative bacteria, decreased permeability of the outer membrane to antibiotics might reduce uptake of nutrients using that permeation pathway. As a result, in "natural" environments competition for resources may severely limit the survival of antibiotic resistant mutants with this particular altered cellular physiology.

It might be argued that any resistance mechanism which does not hinder the ability of the mutant to survive in the absence of antibiotic selection will be more likely to be maintained in the population than one that does. Antibiotic resistance genes that are additional to genes required for normal cellular function are advantageous because they can, in principle, leave the wild-type functions of the host cell unaltered. These genes probably increase genetic and physiological loading upon the host cell and it would therefore be advantageous for them to be dispensed with in the absence of the antibiotic. This may explain the important part played by plasmids in the maintenance and transmission of antibiotic resistance within a population (Campbell 1981). Plasmids are extra-chromosomal replicons and so the chromosome

of the bacterium does not have to be altered, allowing the cell to function as wild-type i.e. with no reduction of infectivity. As long as a sub-population maintains the plasmid there is a potential for rapid vertical dissemination, and less probably horizontal dissemination, of the plasmid through the population in the presence of selection (Grinsted and Bennett 1988).

In the absence of plasmids as carriers of resistance genes, chromosomal genes must confer resistance. A flexible response, where the resistance gene is switched on or off, depending on its´ function, only in the presence of the antibiotic may allow the cell to survive as wild-type in the absence of antibiotic.

Inactivating the antibiotic is a specific resistance mechanism and as the resistance gene itself encodes the protein that performs this specific function, there may be no requirement for other host genes to be modified and wild-type functions would remain unaffected. An example of such a resistance mechanism is that of the family of inactivating enzymes, B-lactamases, which inactivate B-lactam antibiotics by catalysing the hydrolysis of the amide bond in the B-lactam ring, producing inactive acidic derivatives (Ogawara 1981). In order to understand the genetic regulation of B-lactam resistance, it is helpful to describe the mechanism by which B-lactams function.

1.2.1 Mechanisms of action of, and resistance to, B-lactam antibiotics in Gram-negative bacteria

B-lactams constitute a broad family of antibiotics. The detailed mode of action of each one may be different, but eventually results in osmotic rupture of cells due to the weakening of the peptidoglycan structure in the cell wall (for a review see Tomasz 1986).

Cell wall synthesis and inhibition by B-lactams

Peptidoglycan is a major component of the cell wall and consists of a matrix of linear polysaccharide chains (glycan strands) cross-linked by short peptide bridges. In Escherichia coli, the amino acids of the peptide chain are L-alanine, D-glutamate, mesodiaminopimelic acid and D-alanine. Glycan strands consist of alternating units of N-acetyl-glucosamine and N-acetylmuramic acid joined by 1,4-B-glycosidic linkages. Peptidoglycan is anchored to the outer membrane by a covalent linkage between mesodiaminopimelic acid and the carboxy-terminal lysine of the lipoprotein.

In the first stage of the synthesis of peptidoglycan a precursor, UDP-N-acetylmuramic acid, is linked to a chain of five amino acids, the four previously mentioned and another D-alanine in position five. N-acetylglucosamine is added to this precursor in the cytoplasmic membrane. The cross-linking reaction is performed in the periplasm by a transpeptidase which recognises and binds the terminal D-alanine-D-alanine. The peptide bond between the D-alanine residues is cleaved and a new peptide bond is formed between the carboxyl group of the remaining D-alanine and the free epsilon amino group of mesodiaminopimelic acid of another glycan strand, in the growing

peptidoglycan (Figure 1.2). D,D-carboxypeptidases are able to cleave all 5-position D-alanine residues not used in crosslinking, and the relative activities of D,D-carboxy-peptidases and transpeptidases determine the level of cross-linking in peptidoglycan. Endopeptidases are able to cleave the cross-linking peptide bond, reversing the effect of transpeptidases allowing the incorporation of new glycan strands.

One hypothesis suggests that B-lactams inhibit transpeptidation, which is the final crosslinking step in the synthesis of peptidoglycan (Tipper and Strominger 1965). Evidence to support this lies in the similarity between the B-lactam ring structure of the antibiotic and the transformation-state conformation of D-alanyl-D-alanine in the final cross-linking reaction. B-lactam covalently binds to transpeptidases by acylating the hydroxyl group of a serine residue producing a penicilloyl-enzyme complex (Figure 1.3). The intermediate complex hydrolyses slowly, releasing the enzyme and producing an inactive antibiotic derivative, penicilloic acid (Tipper and Strominger 1965). The inhibition of proper enzyme function leads to improper cell growth and possibly activation of autolytic degradation of the peptidoglycan (for review, see Tomasz 1986).

Bacterial proteins capable of covalently binding B-lactam antibiotics, known as Penicillin-Binding Proteins (PBPs), are situated in the cytoplasmic membrane (for review, see Georgopapadakou 1988). Different PBPs have different enzyme activities including transpeptidase, D,D- carboxypeptidase, endopeptidase and B-lactamase activities, some of which are essential for cell wall synthesis, cell growth and cell division (Spratt 1983). For example, PBP-1a/1b, -2

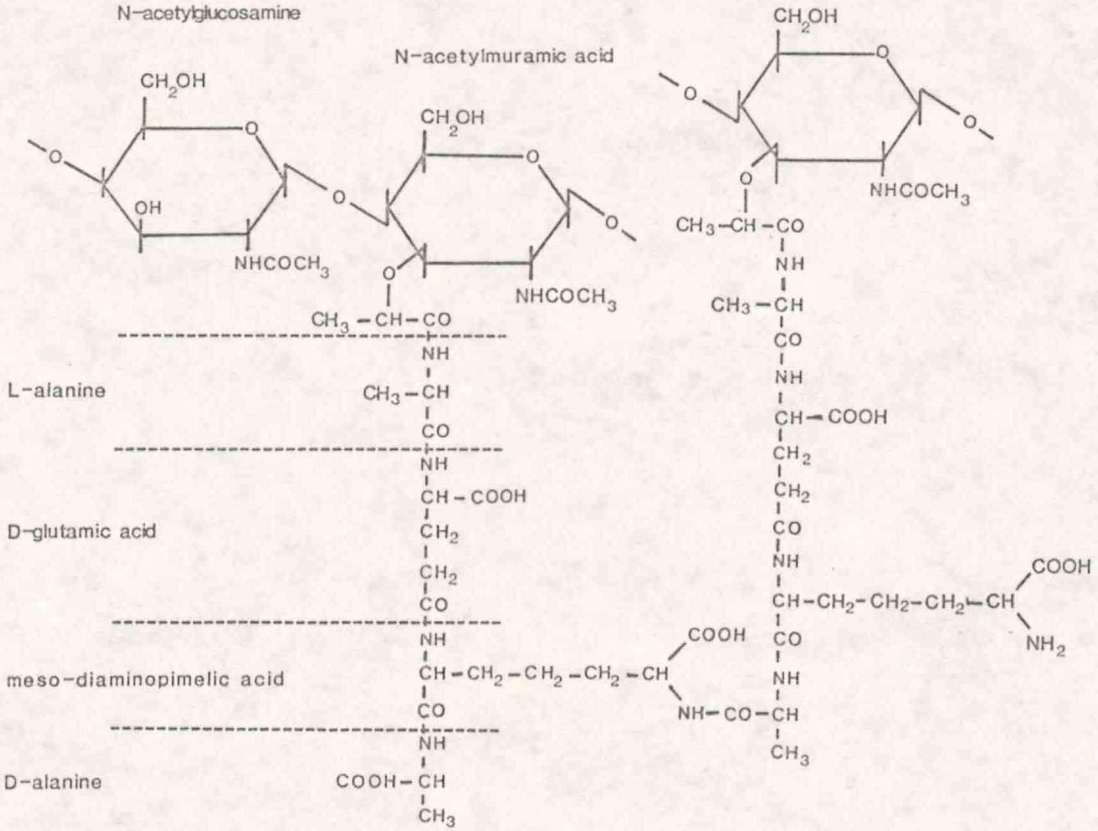


Figure 1.2: Structure of Peptidoglycan of Escherichia coli. Adjacent glycan strands are cross-linked by a peptide bond between mesodiaminopimelic acid and D-alanine. (Figure based upon Figures 1.3 and 1.4, Hammond et al. 1984).

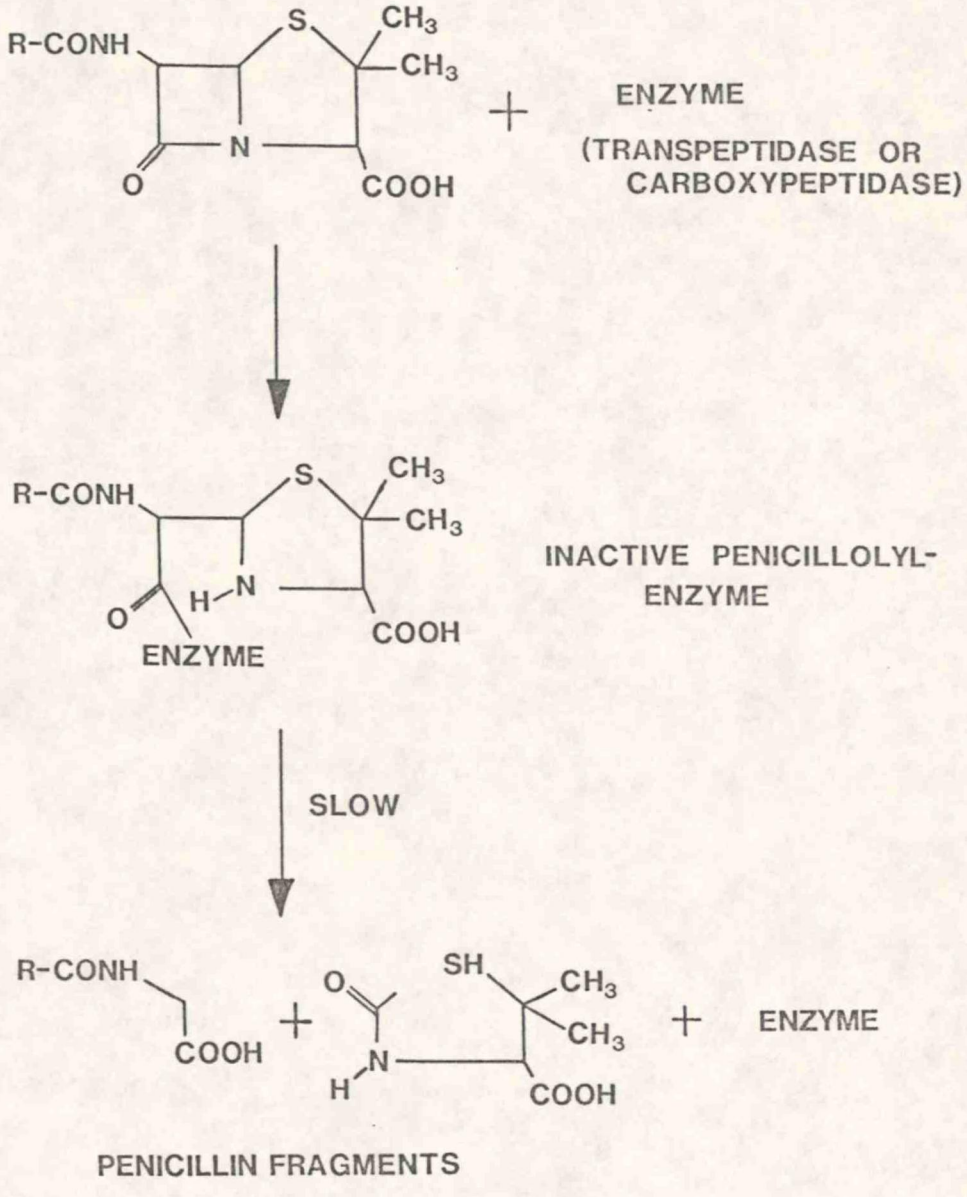


Figure 1.3: Inhibition of Transpeptidase by Penicillin.

and -3 of E. coli are involved with controlling cell elongation, shape and septation respectively. PBPs are the primary targets of B-lactam action with binding of B-lactams predominantly to PBP-1 causing cell lysis, to PBP-2, the formation of giant, spheroid cells and to PBP-3, filamentation (Spratt 1983). Support for the statement that B-lactams inhibit the function of PBPs comes from mutations that not only cause the loss of PBP function but also result in morphological consequences similar to those caused by B-lactams (Tomasz 1986).

Resistance of Gram-negative bacteria toward B-lactams is multifactorial, being dependent upon the permeability of the outer membrane to the B-lactam, the affinity of the B-lactam for the PBPs and the activity of inactivating enzymes in the periplasm (Waley 1987; Nikaido and Normark 1987; Nichols 1988). Increased resistance may develop by mutations or physiological changes affecting one or more of these factors.

Permeability mutations

B-lactams diffuse into the periplasm of Gram-negative bacteria via transmembrane protein channels formed by porin proteins extending through the outer membrane (Nikaido and Nakae 1979; Nikaido 1985; Nikaido and Vaara 1985). Any alteration in the number of functioning porins affects the entry of B-lactams into the periplasm (Harder et al. 1981) and the rate of diffusion into the periplasm depends upon the type of B-lactam. In E. coli, outer membrane porins are important in the uptake of cephalosporins but are less important in the entry of penicillins which can also enter through hydrophobic regions of the membrane (Yamaguchi et al. 1985).

Mutant strains of E. coli lacking the OmpF porin showed increased resistance to first- and second-generation cephalosporins, cefoxitin and moxalactam (Curtis et al. 1985). Susceptibility to aminothiazolyl cephalosporins was only slightly reduced suggesting that they are not as dependent on outer membrane porins for passage across the outer membrane. Porin mutants of other Gram-negative bacteria, however, do show an increased resistance to aminothiazolyl cephalosporins (Sanders and Sanders 1986b), so other uptake systems may not operate in these bacteria. The transfer of B-lactam through the outer membrane is reduced six-fold in porin F-deficient mutants of P. aeruginosa (Nicas and Hancock 1983). Resistance to aztreonam, ciprofloxacin, cefotaxime, ceftazidime, imipenem and moxalactam, along with other non-B-lactam antibiotics, was associated with alterations in outer membrane proteins in a clinical isolate of Serratia marcescens (Sanders and Watanakunakorn 1986). Permeability mutants of P. aeruginosa demonstrating slight changes in lipopolysaccharide composition are resistant to B-lactams probably due to a reduction in the number of functioning porins reducing the diffusion of B-lactams into the periplasm (Godfrey et al. 1984). Resistance is achieved if the rate of B-lactam entry by whatever mechanism is reduced such that the rate of inactivation by B-lactamase in the periplasm results in a substantially lower periplasmic B-lactam concentration than in the more permeable parent strain.

Permeability mutants of Gram-negative bacteria, however, are less prevalent than in Gram-positive and fastidious Gram-negative bacteria (Tipper 1985). This is possibly due to the greater variety of resistance mechanisms available to non-fastidious Gram-negative

bacteria. Furthermore, the level of resistance afforded by alterations in outer membrane proteins alone, is usually no greater than 8 times the Minimum Inhibitory Concentration (MIC) and is thought to be clinically unimportant (Jacoby 1985).

Penicillin-binding protein mutations

Resistance to B-lactams that bind to only one PBP, for example mecillinam, which specifically binds to PBP-2 can occur in a single step by altering the affinity of the PBP for the B-lactam (Spratt 1978). Most B-lactams however, bind to and inhibit more than one PBP, so a single step mutation is unlikely to confer resistance. However, Godfrey and Bryan (1982) identified single-step mutations in *P. aeruginosa*, resistant to carbenicillin in which all PBPs had reduced affinity for the B-lactam. A single mutation can therefore alter the affinity and/or the amounts of more than one PBP; a phenomenon also observed in *Streptococcus mutans*, *S. pneumoniae* and *S. sanguis* (Massidda and Daneo-Moore 1988). B-lactam-resistant clinical isolates of *P. aeruginosa* have also been shown to possess PBP-3 with reduced affinity for B-lactams (Godfrey *et al.* 1981). Resistance can also be achieved in *E. coli* and *P. aeruginosa* by both reducing the number of sensitive PBPs and using a compensator activity of an alternative PBP less inhibited by the B-lactam (Spratt 1978, Godfrey *et al.* 1981).

B-lactamase mutations

B-lactams must pass across the periplasm to bind to PBPs. Inactivation of B-lactams can occur by the action of B-lactamase present in the periplasm. In sensitive strains inactivation occurs at a low rate allowing the accumulation of B-lactam in the periplasm. B-Lactam molecules bind to PBPs in sufficient numbers to exert their

bactericidal effects. Some bacteria, for example E. coli, encode a chromosomal B-lactamase whose production is constitutive and low (Lindberg and Normark 1986). Such bacteria can mutate to resistance by increasing the activity of the enzyme. The rate of B-lactam inactivation can be increased by improving the catalytic activity of the enzyme or by producing more of the enzyme, however, the frequency of such mutations is low (Normark et al. 1977). Certain members of the Enterobacteriaceae and P. aeruginosa, however, are resistant to most first- and second- generation B-lactams because they encode an inducible chromosomal B-lactamase (Sykes and Matthew 1976). The increased expression of the gene in response to the presence of the B-lactam causes an increased B-lactamase activity capable of conferring resistance to the B-lactam. Furthermore, in these species mutation to constitutively high B-lactamase production is much more frequent (Lindberg and Normark 1986).

The major role of the chromosomally-encoded inducible B-lactamase in protecting Gram-negative bacteria against most first- and second-generation B-lactams has been demonstrated by an increased sensitivity to B-lactams in the presence of the non-antibacterial, non-competitive inhibitor of B-lactamase, cloxacillin (Then and Anghern 1982). Further support is that non-inducible B-lactamase mutants show increased sensitivity to these B-lactams with respect to the wild-type parent strain (Curtis et al. 1986). More importantly, bacteria encoding inducible B-lactamase can mutate at a high frequency to resistance to B-lactamase-stable B-lactams (e.g. extended-spectrum penicillins and third-generation cephalosporins) by hyperproduction of B-lactamase. B-Lactamase hyperproduction accounts for the resistance of some clinical isolates of P. aeruginosa previously treated with

these "anti-pseudomonal" antibiotics (Nichols and Milne 1986). The level of B-lactamase synthesis can be higher than that obtained by induction of the wild-type parent. I have categorized hyperproducing mutants as being either constitutive or inducible hyperproducers. Constitutive hyperproducers do not increase B-lactamase production in response to B-lactam inducers, whilst inducible hyperproducers can.

1.2.2 The chromosomal B-lactamases of Gram-negative bacteria

Many Gram-negative bacteria produce a chromosomally-encoded B-lactamase so it would seem that B-lactamase performs an important role in the cell. DNA homology for the chromosomally encoded class C B-lactamase exists in a diverse range of organisms including *E. coli*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Serratia marescens* and *P. aeruginosa* (Bergstrom *et al.* 1982). However, regulation of B-lactamase expression does vary between species.

E. coli encodes a B-lactamase gene (*ampC*) conferring a negligibly resistant phenotype (Burman *et al.* 1973). There is no increase in synthesis of B-lactamase in response to the presence of B-lactams (Sykes and Matthew 1976). The *ampC* B-lactamase is under the control of an inefficient promoter which is situated within the preceding fumarate reductase (*frd*) operon (Grundstrom and Jaurin 1982). An attenuator region has also been located between the promoter and the *ampC* structural gene (Jaurin *et al.* 1981) . This is the transcription terminator for the preceeding *frd* operon and controls B-lactamase synthesis with respect to growth rate (Grundstrom and Jaurin 1982).

Modest B-lactam resistance can be afforded by increasing the synthesis

of B-lactamase, for example, by reducing attenuation or by increasing promoter efficiency. Olsson *et al.* (1983) obtained B-lactam resistant clinical isolates of *E. coli* possessing a very efficient promoter for the *ampC* gene. This promoter was identified as being from *S. sonnei* and included the region immediately upstream of, and including the 5′ terminal end of, the *S. sonnei ampC* gene in which a single point mutation had occurred. Alterations in the *E. coli* promoter, however, may be less frequent than expected due to deleterious effects upon the expression of the fumarate reductase gene (Lindberg and Normark 1986).

Other high level B-lactam-resistant mutants of *E. coli* have been investigated. These mutants possess multiple tandem repeats of the B-lactamase gene showing that gene amplification is an alternative genetic mechanism for increasing resistance. Such mutations, however, are unstable due to deletion-recombination events occuring between homologous regions within the repeated segments (Normark *et al.* 1977). A constitutive but low expression of B-lactamase offers little resistance to B-lactams and mutations to high resistance are apparently infrequent and unstable (Normark *et al.* 1977).

In species encoding an inducible B-lactamase, little B-lactamase is expressed in the absence of B-lactam. B-Lactamase may either perform an unknown cellular role or may simply be due to leaky expression from the promoter site. Thus survival in the "normal" environment is achieved with no selective disadvantage with respect to other bacteria. In the presence of B-lactam, however, the level of expression of B-lactamase increases. One result of an inducible class C B-lactamase is the subsequent resistance to other B-lactam antibiotics. The effect of eight B-lactams on the susceptibility of

induced and non-induced cultures was investigated in five strains of P. aeruginosa and fifteen strains of Enterobacteriaceae (Murray and Niles 1986). Resistance was increased in all cases, but was not uniform; cefpirome being least affected by the increased level of B-lactamase on induction. B-Lactamase stability of cefpirome is, in part, due to reduced affinity class C B-lactamases have for cefpirome; $1x10^3$-$1x10^5$ fold lower with respect to other B-lactams tested (Cullmann 1985).

Increased production of B-lactamase alone or together with other resistance mechanisms, e.g. decreased permeability to B-lactams, may cause resistance to therapeutic dosage levels of many B-lactams. This results in the need to use alternative antibiotics that have to be administered at high, potentially toxic levels (e.g. aminoglycosides) (Smith et al. 1977, Smith et al. 1980). It has been a major aim of pharmaceutical companies to develop B-lactams that are inert to cleavage by B-lactamases; so even when B-lactamase is induced the rate of inactivation by the enzyme would be insufficient to prevent the successful inhibition of PBPs by the B-lactam. Such a development has been particularly important in infections caused by P. aeruginosa which is intrinsically resistant to a wide range of non-B-lactam antibiotics.

In the early 1970s, carbenicillin and, later, the third-generation cephalosporin B-lactam antibiotics were very successful in treating P. aeruginosa infections (Livermore et al. 1985). Success relied upon them being poor substrates for the inducible, chromosomally-encoded B-lactamase (Livermore 1983). Curtis et al. (1986) have also suggested that some of these B-lactams (the aminothi-azolyl-oxime

B-lactams e.g. ceftazidime) are poor inducers of B-lactamase; and so two factors, poor hydrolytic activity and poor induction of B-lactamase, could contribute to the effective inhibition of PBPs by these specific B-lactams. Some B-lactamase-stable cephalosporins, for example cefoxitin, are effective inducers of B-lactamase so B-lactam stability is entirely due to the poor hydrolytic activity of B-lactamase present (Gootz et al. 1982).

The effectiveness of B-lactamase-stable cephalosporin antibiotics (e.g. ceftazidime) has led to their considerable chemotherapeutic use especially against infections caused by P. aeruginosa and other species with inducible chromosomal B-lactamases. Mutants resistant to these antibiotics have been obtained from clinical samples many of which had either inducible or constitutive hyperproduction of chromosomal B-lactamase (Livermore et al. 1982; Shannon et al. 1982; Sanders 1983). Gutmann and Williamson (1983) showed that a crude preparation of Enterobacter cloacae B-lactamase was able to protect a culture of ceftriaxone-sensitive E. coli so resistance may be wholly dependent upon increased production of B-lactamase. Additional support for this theory was reported by Seeberg et al. (1983) who showed that the transfer of the B-lactamase structural gene from constitutive mutants of E. cloacae to E. coli also conferred the resistance phenotype upon the recipient.

Curtis et al. (1986) generated strains of C. freundii, E. cloacae, E. aerogenes, Morganella morganii, Providencia stuartii, P. aeruginosa and S. marcescens able to express the chromosomal B-lactamase constitutively at elevated levels and which were considered to be identical in all other respects to the parental strains. Constitutive

hyperproduction diminished the sensitivity to all the aminothiazolyloxime cephalosporins, aztreonam, cephamycins and the ureidopenicillins. The significance of this mulitiple resistance is the inability to treat a patient with other B-lactams. Elevated production of B-lactamase not only confers resistance to a widely used group of antibiotics but does not reduce the virulence of P. aeruginosa, as shown in a mouse infection model (Bryan et al. 1985).

In a national survey of third-generation cephalosporin-resistant P. aeruginosa, Williams et al. (1984) isolated 19 resistant strains which had elevated levels of B-lactamase activity. Seventeen had very high constitutive production of B-lactamase yet retained inducibility in the presence of inducer B-lactams. The other two strains were constitutive mutants with B-lactamase activity 1000-fold higher than a typical P. aeruginosa strain. It would seem that there are a variety of mechanisms by which P. aeruginosa can mutate to increase B-lactamase expression. However, the inducer potential of a B-lactam does not necessarily correlate with its ability to generate hyperproducing B-lactamase mutants (Sanders and Sanders 1986a).

Hyperproduction of B-lactamase may overcome both factors involved in the potency of third-generation cephalosporins; firstly, their low inducing ability, and secondly, low hydrolsis by B-lactamase. Two hypotheses have been presented to explain how elevated levels of B-lactamase could confer resistance to B-lactamase-stable B-lactams. Firstly, class C B-lactamases tightly bind B-lactams but hydrolyse them slowly, so it was suggested that the antibiotic is prevented from reaching the target PBPs by being tightly bound to B-lactamase present in the periplasm (Then and Anghern 1982; Sanders and Sanders 1983).

This was supported only in that the hydrolytic activity of B-lactamase was thought to be unable to inactivate all B-lactam present in the periplasm. B-lactam trapping is highly unlikely, however, since each B-lactam would have to be bound by one B-lactamase and the concentration of B-lactam used would require B-lactamase synthesis at unobtainable concentrations (Hewinson et al. 1989). Secondly, B-lactamase has a low activity for B-lactamase-stable B-lactams, hydrolysing them at a very low rate in B-lactamase inducible cells (Livermore 1983) resulting in B-lactam successfully inhibiting the PBPs. Mutants producing very high levels of B-lactamase may be able to hydrolyse B-lactam at a slow but significant rate and thereby maintain B-lactam at a sub-lethal concentration, so affording resistance. Sensitive assay techniques verify that the slow rate of hydrolysis that exists in hyperproducing mutants is capable of inactivating B-lactam at the rate of entry into the periplasm at typical elevated MICs (Livermore et al. 1985; Vu and Nikaido 1985; Hewinson et al. 1989).

1.2.3 Genetics of the inducible class C B-lactamase of *Citrobacter freundii*

The inducible chromosomal class C B-lactamase system of *C. freundii* is a well characterised system and the control of *ampC* expression in other members of the Enterobacteriaceae have so far been shown to be similar (Lindberg and Normark 1987, Honore et al. 1989). It would therefore be useful to discuss the system of induction operating in *C. freundii* in detail prior to discussing what is known to occur in *P. aeruginosa*.

C. freundii mutants producing B-lactamase semi-consitutively (inducible hyperproducers) have been isolated and investigated using molecular cloning techniques (Lindberg et al. 1985). The ampC gene encoding the B-lactamase is similar to that of E. coli, having a similar sequence and mapping closely to the frdD gene. Unlike E. coli, however, C. freundii (Lindberg et al. 1985) and E. cloacae (Lindberg and Normark 1987) have a gene, designated ampR, located between frdD and ampC.

Lindberg et al. (1985) described the cloning of the entire ampR-ampC region of C. freundii into E. coli. The introduction of this region confers inducible expression of B-lactamase upon the host, but it has no effect on the expression of the chromosomal ampC of E. coli (Lindberg et al. 1985). In the absence of an inducer, synthesis from the cloned ampC gene is repressed (2.5 fold) by AmpR. In the presence of an inducer, however, an 11-fold increase in expression of the cloned ampC is observed. Mutational inactivation of ampR causes loss of inducibility and constitutive production of B-lactamase at 2.5 times that expressed by the wild-type clone in the absence of inducer. Introduction of a functional ampR gene on a separate replicon restores inducibility showing that AmpR is active in trans (Lindberg et al. 1985). Protein AmpR therefore represses B-lactamase synthesis in the absence of B-lactam but also acts as an activator for the induction system when B-lactam is present.

B-Lactamase synthesis is increased within two minutes of the addition of B-lactam in E. coli mini-cells containing the ampR-ampC region of C. freundii indicating that the signalling mechanism is mediated by AmpR already synthesized (Lindberg et al. 1985). As AmpR does not

contain the Ser-X-X-Lys common to penicillin-binding proteins it is unlikely that induction of B-lactamase synthesis by B-lactams is due to binding of B-lactam to AmpR (Lindquist et al. 1989b). Furthermore, the 2.5-fold constitutive production of B-lactamase of ampR mutants cannot account for the high levels of resistance to B-lactams found in clinical strains that produce B-lactamase at a level 180 times that of the wild-type (Lindberg et al. 1985). No ampR mutations were identified as being able to confer this high level of resistance indicating that other regulatory factors exist.

Spontaneous resistant mutants of C. freundii hyperproducing B-lactamase occur at a frequency greater than $1x10^{-7}$ (Lindberg et al. 1985). Lindberg classifies them as being semi-constitutive because the addition of inducer is able to increase synthesis three-fold (Lindberg et al. 1987). Two such mutants were investigated and the mutations were located outside the frdD-ampC region suggesting that a further regulatory component exists. Similarly, cefotaxime-resistant mutants of E. coli containing the ampR-ampC region of C. freundii occur spontaneously at frequencies of approximately $1x10^{-7}$ to $3x10^{-7}$ (Lindberg et al. 1987). Such mutants constitutively produce B-lactamase at levels comparable to those of C. freundii resistant mutants (Lindberg et al. 1987). The mutations responsible in the E. coli strain were located on the chromosome and required a functional ampR gene present on the plasmid (Lindberg et al. 1987). The E. coli gene conferring high level resistance was designated ampD and has been located, cloned and sequenced (Lindberg et al. 1987, Lindquist et al. 1989a). Wild-type ampD is able to restore wild type B-lactamase synthesis and inducibility upon B-lactamase hyperproducing mutants of both E. coli

and C. freundii in trans (Lindberg et al. 1987). This indicates that E. coli and C. freundii hyperproducers have mutations in identical, or nearly identical, ampD genes. Since E. coli has no inducible B-lactamase because of the absence of ampR, ampD may have another function in the cell. Alternatively, evolutionary loss of ampR may have occurred and ampD is merely a remnant gene.

Lindquist et al. (1989b) have shown that AmpR acts as a transcriptional activator of ampC by binding to a region immediately upstream to the ampC promoter. However, there was no difference in the binding site of AmpR when extracted from induced and non-induced cultures or from ampD2 mutants. Lindquist et al. (1989b) suggested that no alteration in binding site is required and that an effector of AmpR leads to binding and/or open-complex formation between RNA polymerase and the ampC promoter. Alternatively, loss of an effector molecule may have occurred during the extraction procedure.

The ampR-ampC region of E. cloacae has great homology to that of C. freundii; the intercistronic region is highly conserved and the two AmpR proteins have an 81% amino acid sequence homology (Lindquist et al. 1989b). Furthermore, AmpR can regulate expression from the ampC of the heterologous host (Lindberg and Normark 1987) indicating a very similar system is in operation. This is supported further by the cloning and sequencing of ampD from E. cloacae (Honore et al. 1989).

1.2.4 Possible regulatory mechanisms in Enterobacteriaceae synthesizing inducible class C B-lactamase

Lindberg *et al.* (1987) showed that inactivation of *ampD* by insertion mutagenesis with Insertion Sequence 1 (IS1) gave almost the same induction level as addition of inducer. A likely explanation of this was that the inducer acts similarly to IS1 in that it inactivates AmpD. As *ampD* mutants produce B-lactamase semi-constitutively there must be an alternative induction system regulating B-lactamase synthesis. If AmpD had a role in cell wall synthesis as hypothesized by Lindberg *et al.* (1988) it may explain the presence of *ampD* in *E. coli* in that it has a role other than that of regulating B-lactamase production. Furthermore, if AmpD was a PBP the partial inducibility of B-lactamase in *ampD* mutants could be explained in terms of PBP compensatory activity. However, the genetic location of all PBPs has been determined in *E. coli* (Waxman and Strominger 1983) and none map in the *ampD* region (Lindberg *et al.* 1987). Moreover, *ampD* does not have a ser-X-X-lys nor does it covalently bind penicillin (Honore *et al.* 1989, Lindquist *et al.* 1989a).

Functional AmpR is required for AmpD to exert its' effects on B-lactamase synthesis and so it is likely that AmpR is an essential regulatory protein. A comparison can be drawn between this system and the *ara* operon where the regulatory protein in the absence of arabinose represses expression but in the presence of arabinose can induce expression. The putative molecule that binds to AmpR must presumably alter in concentration as a result of inactivation of AmpD by B-lactams or by mutation in *ampD*. One possible model shown in Figure 1.4a is that a rise in concentration as a result of the

presence of inducer enables an effector to bind AmpR and cause induction of B-lactamase. It is also possible that AmpR alone is an activator of ampC and that a co-repressor is bound to it in the absence of inducer (Figure 1.4b). If other proteins were able to partially compensate for inactive AmpD it could explain why ampD mutants are not fully constitutive.

Evidence for AmpR protein binding directly to DNA originally came from quantitative comparisons of homologous and heterologous complementation experiments in C. freundii and E. cloacae (Lindberg and Normark 1987). Protein AmpR acts similarly in both but expression from ampC is higher in both using the homologous ampR gene. Binding of AmpR to a region immediately upstream of the ampC promoter has now been shown and AmpR shows sequence homology to the nodD and lysR regulatory protein family (Lindquist et al. 1989b). Binding of AmpR to this region causes an increase in the expression of ampC and represses synthesis of AmpR itself probably because it binds to the putative ampR promoter. Autoregulation is also a feature of both LysR and NodD transcriptional activators. Transcription of ampR was not increased by induction with 6-APA (Lindquist et al. 1989b). This may have been an artefact due to the loss of an effector of AmpR during the extraction procedure, however, it was considered to be a true feature of the induction system as NodD also binds to DNA in the presence and absence of inducer (Lindquist et al. 1989b).

It is now known that IS1 insertion in ampD prevents transcription of another gene within the same operon (ampE) and that both genes must be inactivated to obtain high level semi-constitutive B-lactamase expression (Lindquist et al. 1989a). Analysis of the deduced amino

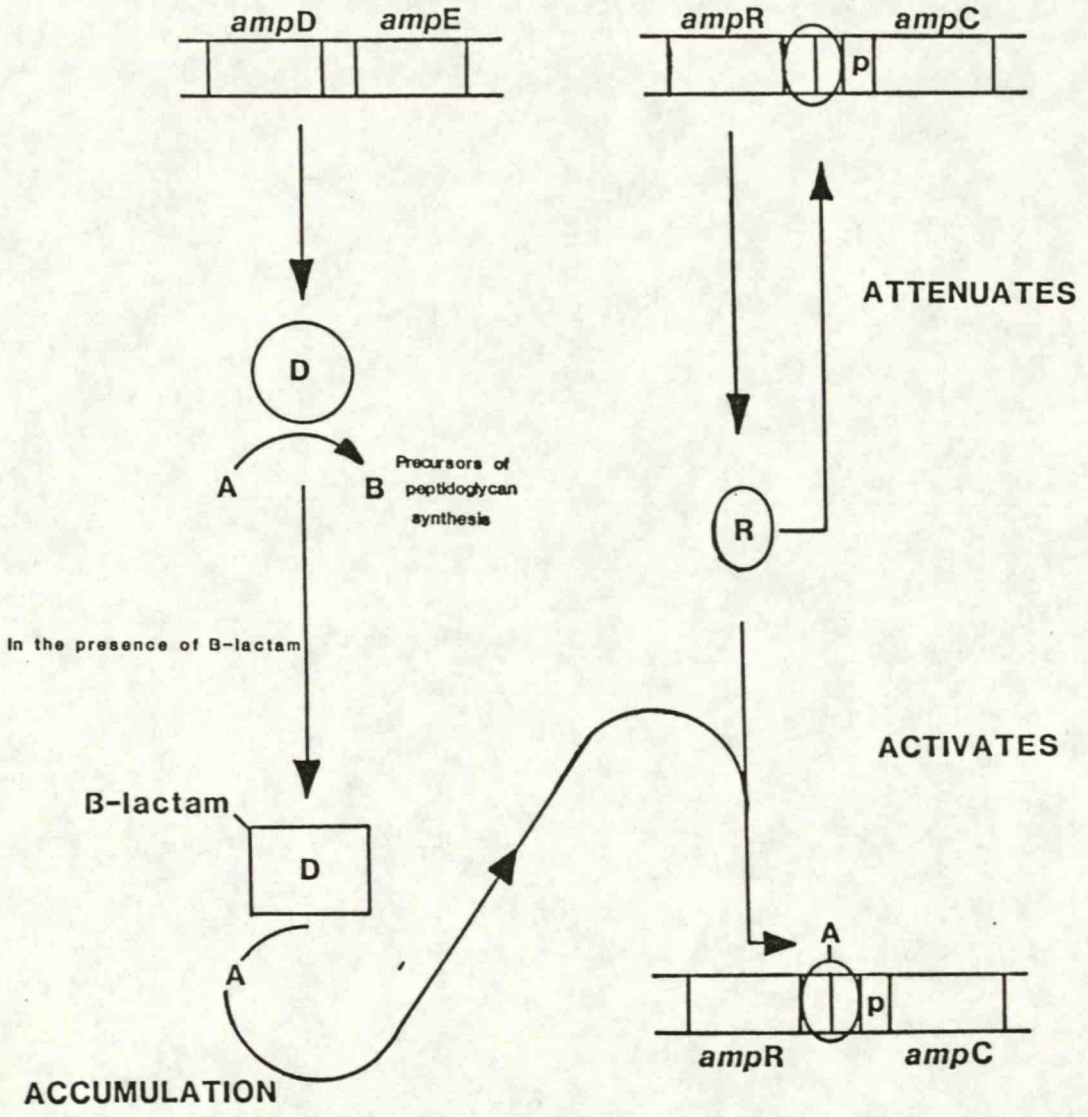


Figure 1.4: Possible Mechanisms of B-Lactamase Induction.

Figure 1.4a - AmpR as a native repressor. In this model AmpR is a native repressor of ampC. In the presence of B-lactam, however, AmpD is inhibited and "A" accumulates. Compound "A", possibly a precursor of peptidoglycan, acts as a co-activator of expression from ampC by binding to AmpR.

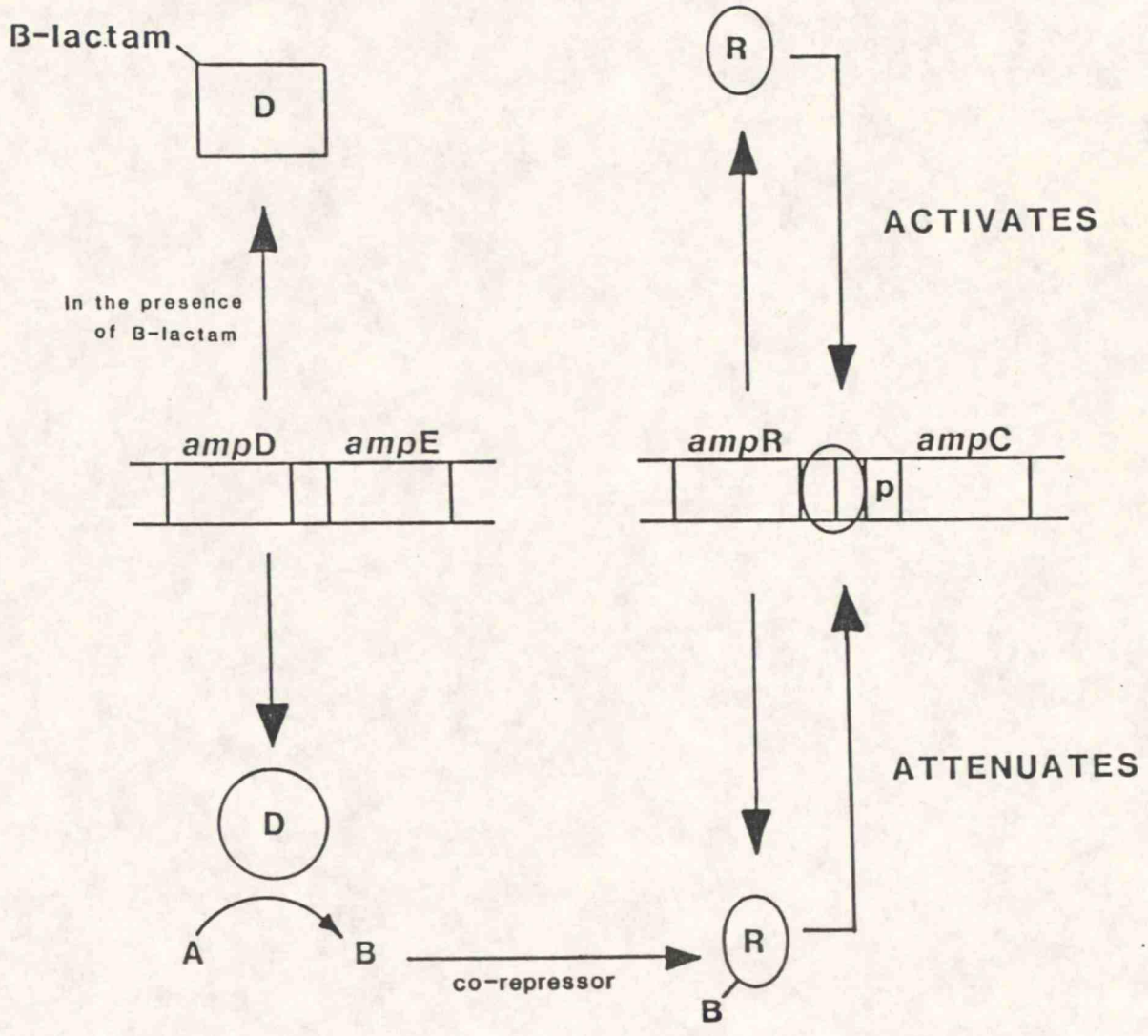


Figure 1.4b - AmpR as a native activator. In this model AmpR is a native activator of ampC. Active AmpD synthesizes a co-repressor, which binds AmpR and represses expression from ampC. Synthesis of co-repressor is inhibited in the presence of B-lactam and AmpR activates expression from ampC.

acid sequence of ampE from E. cloacae and C. freundii shows it to have four transmembrane domains and a Ser-X-X-Lys motif common to all PBPs present on the outer face of the cytoplasmic membrane (Honore et al. 1989, Lindquist et al. 1989a). Protein AmpE, however, is not a PBP, since it does not covalently bind penicillin (Honore et al. 1989, Lindquist et al. 1989a).

The precise effects of AmpD and AmpE on induction of B-lactamase are unclear as there appear to be anomalies. E. coli JRG582 has a deletion of nadC, ampD-ampE and aroP (Langley and Guest 1977). The presence of ampR and ampC from E. cloacae or C. freundii in E. coli JRG582 causes constitutive hyperproduction of B-lactamase (Honore et al. 1989, Lindquist et al. 1989a). Inactivation of ampD-ampE by IS1 in E. coli SNO302, however, causes inducible hyperproduction (Lindberg et al. 1987). Wild-type expression and induction of B-lactamase can be restored in E. coli JRG582 by ampD alone (Lindquist et al. 1989a). The absence of ampE alone appears to have no effect on induction in the presence of ampD (Lindquist et al. 1989a). However, the same authors show that ampE in the absence of ampD increases B-lactamase inducibility in response to cefoxitin more than ampD-ampE.

The fact that two genes, ampD and ampE controlling B-lactamase production are so closely linked indicates that an operon system exists. If a single signal system was in operation, containing two sub-units, then a mutation in one subunit should cause the total loss of the system. Mutations in one however may only cause semi-constitutive production indicating the two genes are not entirely interdependent. It may be that two systems, which are closely regulated, exist and the combined activities of these regulatory

system control B-lactamase production. As E. coli has these regulatory genes but does not have an inducible B-lactamase it is possible that they perform another role in the cell unrelated to B-lactam inactivation.

1.2.5 Implications for P. aeruginosa

Although the chromosomal B-lactamase of P. aeruginosa is inducible, little is known of the genetic basis of its regulation. P. aeruginosa synthesizes a class C B-lactamase evolutionarily similar, though showing little DNA homology, to those found in Enterobacteriaceae (Bergstrom et al. 1982). Hyperproduction of B-lactamase has been identified as a cause of ceftazidime-resistance in some mutants of P. aeruginosa (King et al. 1983). Hewinson and Nichols (1987) identified a regulatory mutant, 3-Post, showing sensitivity to third-generation cephalosporins at 20°C but resistance at 37°C. This was correlated with the B-lactamase gene (blaP) being inducible at 20°C but being constitutively expressed at 37°C. The temperature-sensitivity of this mutant supports the possibility that an alteration in protein conformation alters the regulatory control of blaP rather than hyperproduction being a result of an operator mutation. Williams et al. (1984) identified cephalosporin- resistant mutants of P. aeruginosa with high production of B-lactamase as either being semi-constitutive or fully constitutive. The former may be analogous to the ampD mutations of both C. freundii and E. cloacae.

In P. aeruginosa loss of penicillin-binding by PBP5 causes loss of B-lactamase induction by concentration-dependent B-lactam inducers (Martin et al. 1988). In E. coli loss of PBP2 binding to

6-aminopenicillanic acid (6-APA) causes loss of induction of the ampC B-lactamase of C. freundii (Oliva et al. 1989). Slight differences in the effect of antipseudomonal B-lactams on P. aeruginosa and E. coli exist. In P. aeruginosa they do not cause spheroplast formation, unlike in members of the Enterobacteriaceae. However, this difference in response to B-lactams does not necessarily indicate that different induction systems operate in P. aeruginosa and enteric bacteria. Various PBPs may be capable of causing induction within the same B-lactamase induction system. Active B-lactam is required for induction of blaP similar to the case in Enterobacteriaceae (P. Bennett, personal communication) since the presence of periplasmic TEM B-lactamase prevents induction of the chromosomal B-lactamase whilst cytoplasmic-bound TEM B-lactamase does not.

Matsumoto and Terawaki (1982) have mapped a number of genes affecting sensitivity to cephaloridine in P. aeruginosa PAO. At least five different loci have been identified as encoding genes which affect B-lactamase production and so regulation may be more complicated than in C. freundii. Genes blaP and blaJ are closely linked at 37′ and are probably the B-lactamase structural gene and promoter site respectively. Gene blaK at 37′ and blaL and blaI, closely linked at 57′ have controlling influences upon B-lactamase expression (Matsumoto and Terawaki 1982). Single mutations in either blaI or blaK cause inducible hyperproduction of B-lactamase whilst a double mutation in blaI-blaK causes non-inducible hyperproduction of B-lactamase. Lindberg et al. (1988) suggested that one of these genes may be analogous to ampD. It is now conceivable that the other gene is analogous to ampE.

Different control mechanisms may exist as there are several distinct levels of elevated expression (Gates et al. 1986, Sanders et al. 1988). Evidence that different systems may operate in P. aeruginosa derives from work performed in Sanders' laboratory. B-Lactam-resistant clinical isolates of P. aeruginosa were classified into four groups depending on their basal and induced B-lactamase activities (Sanders et al. 1988) whilst only three B-lactamase levels are known in C. freundii. The categories were based on analysis of the B-lactamase activities of 85 clinical isolates with resistance to B-lactams with or without induction by cefoxitin. The four phenotypes were those strains with:

low basal and inducible activity,

moderately elevated basal and inducible activity,

moderate basal and constitutive activity and

high basal and constitutive activity.

This classification suggests constitutive production of B-lactamase in two classes, however, the published data shows that B-lactamase activity of strains in these categories is still increased by on average at least two-fold on induction by cefoxitin. This discrepancy was not discussed and these classes are not distinct as shown by the wide variation of values in each class. Instead, B-lactamase activities may represent a gradual increase but unfortunately this cannot be discussed as a detailed breakdown of strain values was not published.

Another point to be made is that of these 85 isolates some were obtained from the same patient. The reason for this was that the second isolate had an altered B-lactamase activity. In fact, of these

85 isolates only 45 were derived from distinctly different strains. The second isolate from the same patient must have acquired a mutation affecting B-lactamase production. This second mutation may follow an initial mutation affecting B-lactamase production. Alternatively, it may follow an initial mutation to B-lactam resistance unrelated to B-lactamase production, for example reduced permeability. Taking all these points into consideration there may be too many variables to produce distinct categories of mutants with altered B-lactamase activity from clinically isolated strains. It would be desirable to investigate B-lactamase mutants generated within the laboratory.

Spontaneous mutants of P. aeruginosa resistant to third-generation B-lactams by hyperproduction of B-lactamase can be identified in the laboratory. Hewinson (1987) stated that at least two different classes of P. aeruginosa exist. This was concluded from the difference between two strains in their ability to mutate to resistance to cefsulodin by hyperproduction of B-lactamase.

Two chromosomal B-lactamases may exist in strains of P. aeruginosa hyperproducing B-lactamase (Gates et al. 1986). Gates described the novel B-lactamase as having better hydrolytic activity against third-generation B-lactams than the normal B-lactamase, and that this B-lactamase confers resistance to anti-pseudomonal B-lactams. If this second chromosomal B-lactamase was present only in strains capable of mutating at high frequencies to hyperproduction of B-lactamase it may explain the difference in frequencies of resistance in the two strains of Hewinson (1987).

It is also possible that this novel B-lactamase is an artefact of the

B-lactamase extraction procedure (as discussed by Hewinson 1987). Berks (1976) also observed the presence of a distinct band on isoelectric focusing of two strains constitutively producing B-lactamase, when purified by a similar crude method. The pIs of the two B-lactamases were 8.1 and 7.5 for Berks and 8.4 and 7.5 for Gates, the second pI value being that of the B-lactamase found only in hyperproducing strains. It seems probable that this novel B-lactamase is a breakdown product. Multiple bands of B-lactamase have been observed within strains of P. aeruginosa hyperproducing B-lactamase and were found to be the result of proteolysis or deamination of the original B-lactamase in crude B-lactamase preparations (Furth 1975, Berks 1976).

The two B-lactamase phenotypes of P. aeruginosa described by Hewinson (1987) may be explained within the C. freundii system. At least three genes are involved in the induction of B-lactamase from C. freundii; ampR, ampD and ampE (Lindquist et al. 1989a). Loss of ampD results in increased inducibility of B-lactamase but the absence of both ampD and ampE results in full constitutive hyperproduction of B-lactamase in E. coli JRG582 (Lindquist et al. 1989a). In P. aeruginosa, different strains have different basal levels of B-lactamase synthesis, respond differently to induction and have different mutation frequencies to cefsulodin resistance (Hewinson 1987). For example, P. aeruginosa 3-Pre has a relatively high basal B-lactamase activity and can mutate to cefsulodin-resistance with a frequency of $1x10^{-7}$. P. aeruginosa PAT210, however, has a lower basal B-lactamase activity and constitutive production cannot confer cefsulodin-resistance. This may be explained if P. aeruginosa 3-Pre already contains a mutation in a putative ampD, or conversely, ampE gene.

Mutation in the other gene results in full constitutive B-lactamase production at a frequency of ~$1x10^{-7}$. However, in P. aeruginosa PAT210 both genes are intact and to detect cefsulodin-resistance both would need to mutate simultaneously. The expected frequency of such an event is $1x10^{-14}$, which would explain the inability to detect such mutations in the laboratory. The classification of Sanders may be of use in this respect since under clinical conditions such double mutations could occur. For example, mutants in PAT210-like strains with B-lactam resistance through decreased B-lactam permeability may be observed as low basal, inducible strains. On a single or double mutation such strains may become moderate basal constitutive producers of B-lactamase. The class of moderately elevated basal and inducible B-lactamase producers could be similar to 3-Pre, with a mutation already in one regulatory protein controlling B-lactamase production. High basal constitutive B-lactamase producers could be the result of a mutation in another regulatory gene. This is all speculative and can only be tested by generating and investigating B-lactamase hyperproducers from a large sample using a number of strains. One of the aims of this project was to construct a transposon capable of generating mutations in regulatory genes of blaP.

1.3. Optimising Strategies for the Identification of Genes Controlling the Expression of B-Lactamase in Pseudomonas aeruginosa

The frequency of mutation can be increased by the use of mutagens. Transposon mutagens have several advantages over chemical mutagens. Generally a single-site mutation is generated in the target and

transposons carrying resistance determinants allow the genetic location of the transposon to be identified by complementation (Berg and Berg 1983). The resistance determinants of inserted transposons aid analysis of the mutant phenotype because mutations may subsequently be transferred by transduction, conjugation, or cloning selecting for the transposon marker (see 1.3.2).

A chromosomal region containing a transposon may be cloned in E. coli by selection for the transposon marker. This requires restriction enzyme digests with enzymes which have no recognition sites within the transposon. In some cases the transposon may encode an ori site and so no other vector is required (M.J. Woodward, personal communication). Probes to identify the location of the wild-type gene in a gene bank can be generated using the regions flanking the transposon. Furthermore, transposon insertion into a gene may disrupt the transcription of that gene and any other gene that uses the same promoter which lies downstream from the insertion site. Thus a series of transposon insertions can enable the sequence of genes within an operon to be determined (Berg et al. 1980, Luthi et al. 1986).

Once wild-type genes involved in regulating B-lactamase expression have been identified, problems of multiple copies in conventional cloning vectors can be overcome by cloning the gene into a transposon which can then integrate back into the pseudomonas chromosome as a single copy. Being in only two copies the gene is more likely to remain stable. Similarly, a gene in which a transposon is inserted, may be used for site-directed mutagenesis by gene replacement. Ohman et al. (1985) transduced fragments containing a Tn5-inactivated gene into a recipient strain to construct the analogous chromosomal

mutation in P. aeruginosa. The advantage of this method is that gene expression will be at the haploid level aiding investigations into the regulation of B-lactamase expression.

1.3.1 Transposons as tools for generating mutants in P. aeruginosa

Investigations of P. aeruginosa have been hindered by low frequencies of transposition into the chromosome and the site specificity of many transposons (e.g. Tn501, Tsuda et al. 1984). The intrinsic resistance of P. aeruginosa to a wide range of antibiotics and high spontaneous mutation rates to antibiotic resistance makes many broad host-range vectors and transposons unsuitable as genetic markers (Rella et al. 1985).

A suitable vector for transfer of transposons into P. aeruginosa would have a small size for increased transformation efficiency or be capable of conjugation into P. aeruginosa. It would have selectable markers in P. aeruginosa and lose replicative ability when required, with the subsequent loss of marker resistance allowing the identification of chromosomally-encoded transposon. An ideal transposon would have a marker selectable in P. aeruginosa and would transpose randomly into the chromosome at a high frequency. Transposon Tn5 is 5.7 Kilobase pairs (Kbp) and contains no chi sites which are the preferred sites for recA-dependent recombination events (Berg and Berg 1983).

Vector systems for transfer of Tn5 (e.g. pJB4JI, RP4::Mu, RK2.ColE1::Tn7::Tn5 and ColIdrd) from E. coli into other species transfer Tn5 at low frequency and retained portions of the vector occur at high frequency in the new host (Simon et al. 1983, Simon

1984, Rella et al. 1985). Narrow host-range plasmid vectors containing transfer genes have been constructed to obviate the problem of retention of vector sequences (Simon et al. 1983). These plasmids can enter P. aeruginosa but are unable to replicate in the new host (Simon et al. 1983). Transposition of Tn5 from such "suicide" plasmids into the chromosome should occur immediately as a result of zygotic induction (see later). Loss of vector and retention of Tn5 in the chromosome can be identified by the retention of the transposon resistance determinants and the loss of vector resistance determinants. The maximum theoretical frequency of transposition is equal to the frequency of plasmid transfer but this is unlikely to be attained. However, if a temperature-sensitive broad-host-range plasmid was used as a vector the frequency of transposition should be much greater. As the plasmid is capable of replication at permissive temperatures it should be possible to obtain a strain in which the plasmid is maintained at the permissive temperature. Growth of this strain at non-permissive temperatures would result in the loss of plasmid and transposition of Tn5 would occur as a result of derepression in the control of transposition. As all cells of the original strain contained Tn5, retention of Tn5 in the chromosome should be more frequent than that offered by narrow-host- range plasmids. The small (8.9KbP) broad-host-range incQ plasmid, pRSF1010, (Guerry et al. 1974) is capable of transfer from a donor strain when transfer factors are supplied in trans and temperature-sensitive derivatives exist (pMMB19, Scholz et al. 1985).

Transposon Tn5, which encodes resistance to kanamycin, bleomycin and streptomycin, has already been shown to insert into the P. aeruginosa chromosome and to be stably maintained there (Krishnapillai et al.

1986). P. aeruginosa PAO, however, has a high frequency of spontaneous mutation to kanamycin resistance (Bryan et al. 1980) and so wild-type Tn5 carries an unsuitable selectable marker. A Tn5-derivative encoding trimethoprim and kanamycin resistance (Tn5-751) has been made (Rella et al. 1985), and inserted into a broad host-range, temperature-sensitive vector (pME305). Selection of transpositions from pME305 at non-permissive temperatures in E. coli was made on media containing trimethoprim-kanamycin. Plasmid pME305 was unstable in P. aeruginosa PAO even at "permissive" temperatures and so had no advantage over other, narrow host-range vectors (Rella et al. 1985).

Detailed knowledge of the molecular biology of Tn5 (discussed later) allows the generation of derivatives containing alternative selection markers. Resistance to an anti-pseudomonal aminoglycoside, for example, tobramycin or gentamicin, is a potentially useful marker (Meyers and Hirschman 1972, Rubenstein and Lev 1988). The probable advantages of using a small broad host-range, temperature-sensitive vector carrying a Tn5-derivative with an aminoglycoside-resistance gene has led to the construction of pRTG1 in this work.

Tn5 as a tool for geneticists

Tn5 has been used to inactivate genes by insertional mutagenesis in a variety of Gram-negative bacteria, including Escherichia, Klebsiella, Agrobacterium, Rhizobium and Pseudomonas (for review, see Berg and Berg 1983). Transposon Tn5 is often used because it has an easily selectable marker (kanamycin and neomycin resistance), a relatively high frequency of transposition on entry into a cell as a result of

zygotic induction, and, once inserted, is very stable due to self-regulation of transposition (Johnson et al. 1982, Isberg et al. 1982). Tn5 has little site specificity for insertion and thus inserts "randomly" into the genome. Other transposons have shown preferential binding to specific DNA sequences in P. aeruginosa (e.g. Tn501; Tsuda et al. 1984, Tn1; Krishnapillai et al. 1981) and so have a reduced probability of inserting into genes of interest.

Transposon Tn5 (Figure 1.5a) was originally isolated as a kanamycin-neomycin determinant from Klebsiella (Berg et al. 1975) but also encodes resistance genes to bleomycin and streptomycin (Mazodier et al. 1985). The resistance determinants lie in a 2.7Kbp central region of Tn5 between two inverted repeats of insertion sequence IS50 (1.5Kbp), that differ from each other in function (Rothstein et al. 1980).

The right-hand repeat (IS50R) codes for two functional proteins (p1 and p2) involved in regulating the frequency of transposition of the element. The left-hand repeat (IS50L) contains a single mutation 93 bp from the inside end. This mutation causes premature termination of both protein transcripts and generates truncated, non-functional proteins (Rothstein and Reznikoff 1981). Transposition of Tn5 is then wholly dependent upon the presence of an intact IS50R element.

Proteins p1 and p2 are transcribed from different promoters but are "in frame" and consist of 461 and 421 amino acids respectively. Tn5 mutants unable to synthesize protein p1 are unable to transpose (Rothstein et al. 1980). Protein p1 is the transposase, or a component of the transposase. Since p2 is unable to complement

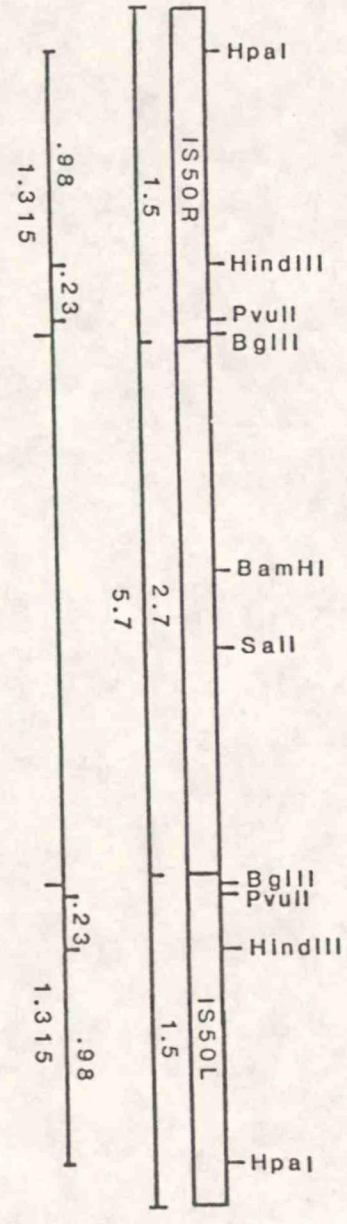


Figure 1.5a: Restriction Enzyme Map of Tn5. Based on the restriction enzyme map of Rothstein *et al.* 1980. Sizes are in Kilobase pairs.

mutants deficient in protein p1, the loss of transposability also indicates that the 40 amino acids of the N-terminal forms part of the transposase active site.

Transposition of p1-deficient mutant Tn5-derivatives (for example, Tn5-410) occurs only at a very low rate in the presence of a second, but wild-type, Tn5, on a separate replicon indicating that the transposase acts preferentially *in cis* (Isberg and Syvanen 1981 Berg and Berg 1983).

The transposition frequency of Tn5 is highest on entry into a cell. If an IS50R element is already present, however, transposition of any IS50R-based element in the cell is inhibited. The cell is then said to be "experienced" (Biek and Roth 1980). Protein p2 is able to provide the cell with this "experience". Protein p2 appears to be an inhibitor of transposition and is active *in trans* (Isberg *et al.* 1982).

Regulation of transposition

The regulation of transposition of transposon Tn10 is understood better than that of Tn5 and will be discussed in order to infer possible mechanisms that might be operating in Tn5. Transposon Tn10 predominately regulates transposition by controlling the amount of transposase present. The transposase gene promoter is very weak and adenine methylation within the promoter also decreases transcription of the transposase gene (Roberts *et al.* 1985). Translation of any transposase mRNA is regulated by antisense mRNA, where part of the transposase transcript overlaps a transciption unit read in the opposite direction. Complementary binding between such homologous

mRNA strands prevents translation of the transposase transcript (Simons and Kleckner 1983, Simons *et al.* 1983).

Within Tn5, the presumptive transcriptional start site of p1 (tss1) is 66 bp from the outside end of IS50R (see Figure 1.5b) (Krebs and Reznikoff 1986). The -10 region of this promoter site contains two recognition sites for the *dam* methylase of *E. coli* and a recognition site for *dcm* methylase is located just upstream of this region. In *dam*-deficient cells transcripts from this transcriptional start site accumulate (Krebs and Reznikoff 1986), and transposition of Tn5 is greater (Yin *et al.* 1988) supporting the hypothesis that regulation of transposition occurs at the transcriptional level by methylation within the promoter site of p1.

There are presumptive transcriptional start sites for p2 at 98 bp (tss2) and 109 bp (tss3) from the outside end (Krebs and Reznikoff 1986) which are in the same reading frame as the p1 transcriptional start site (Figure 1.5b). Transcripts initiated at tss2 are present in relatively high amounts with respect to those from tss1 and tss3. Increasing the production of p2 by insertion of the *lac*UV5 promoter between tss2 and tss3 demonstrated that inhibition of transposition parallelled the extent of derepression of transcription from this promoter (Johnson *et al.* 1982). Neither transcription nor translation of p1, however, is affected by p2, since the synthesis of transposase-B-galactosidase protein fusions was not affected by the presence of p2 (Isberg *et al.* 1982, Johnson *et al.* 1982). Thus p2 probably controls transposition by interacting with either transposase or with sites at which transposase acts. Expression of p2 from its natural promoters, however, may well interfere with the expression of

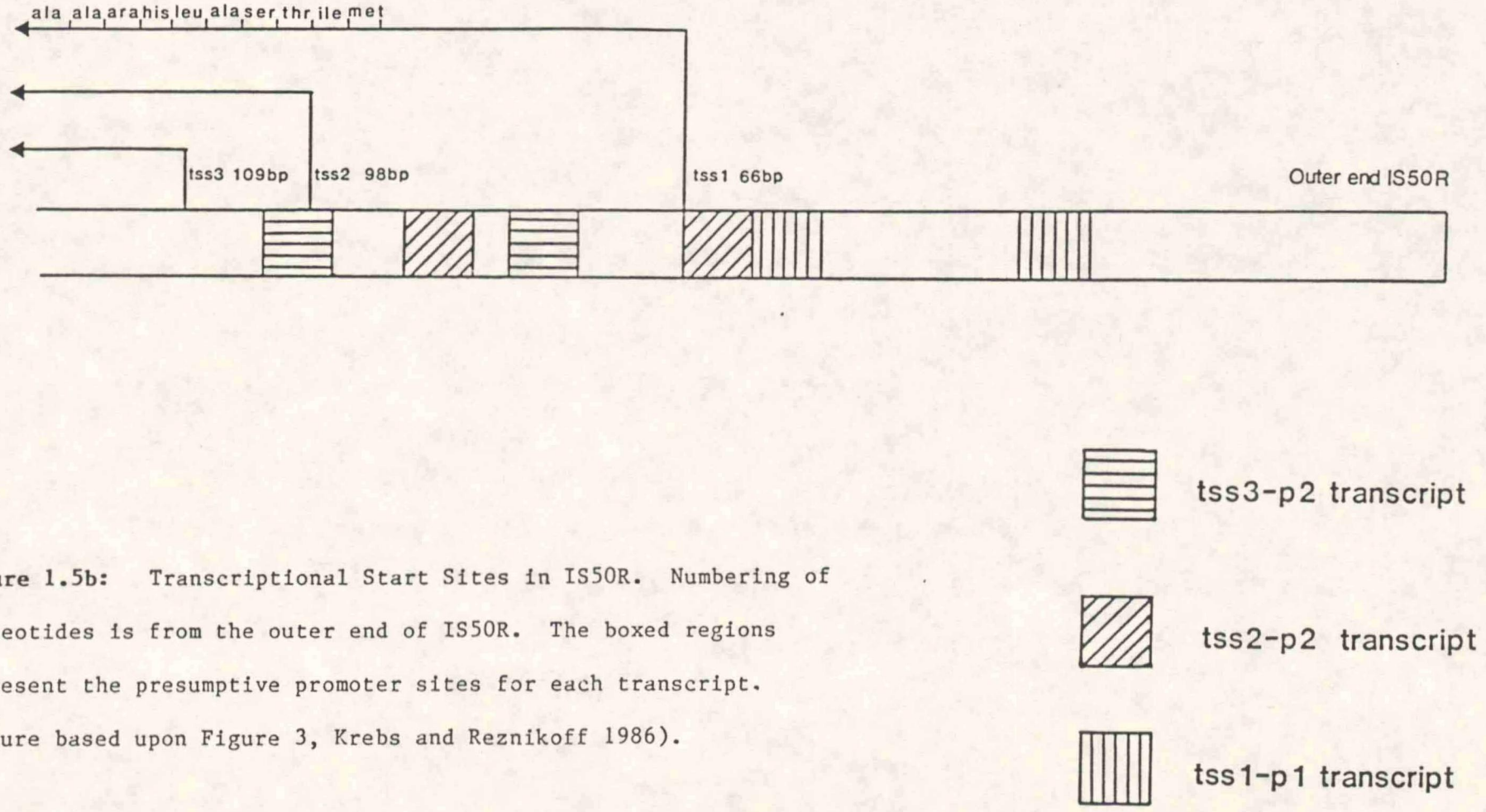


Figure 1.5b: Transcriptional Start Sites in IS50R. Numbering of nucleotides is from the outer end of IS50R. The boxed regions represent the presumptive promoter sites for each transcript. (Figure based upon Figure 3, Krebs and Reznikoff 1986).

the transposase gene since the promoter for tss2 lies over tss1. Similarly the promoter sites for tss2 and tss3 overlap resulting in a possibly complex system of regulation of p1 and p2. Protein p2 is synthesized approximately four times as frequently as p1 *in vivo* as determined by immunoprecipitation assays of right-repeat protein-*lacZ* fusion proteins, probably due to the different properties of their translational initiation signals (Johnson *et al.* 1982). The relative levels of p1 and p2 may contribute to control of transposition frequency, as could host-encoded proteins involved in the transposition process (Johnson and Reznikoff 1984).

Tn5 prevents expression of genes within it and distal to it from promoters upstream from the site of insertion (Berg *et al.* 1980) and read-through transcripts of p1 from promoter sites upstream of the site of insertion are not found. These observations may be a result of a protection mechanism that prevents over-expression of transposase and possible consequent cell death. It has been proposed that read-through transcripts contain secondary structures that are not present in transcripts initiated at tss1 and may reduce translational initiation (Krebs and Reznikoff 1986). Such a structure has already been proposed for Tn10 (Davis *et al.* 1985).

Transposition of Tn5 therefore seems to be regulated by a complex control system. Transcription of p1 is at a low level due to the poor promoter activity at tss1 and may be reduced further by methylation within the promoter site, similar to that control already found in Tn10. Also, the possible transcription of p1 from promoters upstream of insertion is prevented by some mechanism, thereby Tn5 maintains a stringent control of p1 transcription and translation. Transposition

is also regulated at the post-translational level, where p2 interferes with the transposition process.

Sites on Tn5 that are Essential for Transposition

Tn5 has certain internal sites that are required for transposition. Derivatives of Tn5 that contain only the external 186bp ends can transpose in the presence of an intact IS50R element elsewhere on the same replicon. The ends of insertion elements carry inverted repeat sequences that are precisely conserved and are believed to constitute transposase recognition sites. An inverted, hyphenated 8bp repeat in the first 9bp of each end of IS50 exists (Auerswald et al. 1980, Berg et al. 1982). Since transposition using pairs of outside ends (O/O) of IS50R is 100-1000 fold more efficient than using pairs of inside (I/I) ends (Berg et al. 1982, Isberg and Syvanen 1981) this sequence, common to both ends can only constitute part of the transposase recognition site. The entire 19bp region at each end of IS50 is essential for transposition (Sasakawa et al. 1983). Transposition of O/I elements, however, is nearly as efficient as that of O/O elements suggesting that once transposase is bound to a single O site, the structure of the second site is less important (Sasakawa and Berg 1982).

What determines the specificity of insertion into target DNA?

Neither the sequence of the vector, the O end of IS50, nor the activity of the transposase inhibitor affect the specificty of Tn5 insertion (Berg et al. 1984), which may only depend on transposase recognition of target sequences in the host DNA. The only sequence commonly found in target DNA is the presence of GC base pairs at the end of the 9bp target DNA duplicated during insertion (Berg et al. 1984). Since GCs with a 9bp spacing are not used as targets with

equal preference, other features must be important.

Supercoiled DNA is required as an efficient target for Tn5 insertion (Isberg and Syvanen 1982) which may explain the preference of Tn5 for insertion into plasmid DNA as opposed to chromosomal DNA. The degree of supercoiling of a replicon depends on the relative activities of DNA gyrase which generates supercoils, and DNA topoisomerases which relaxes supercoiling, so there are other host factors important in determining insertion sites apart from the GC sequence. Temperature-sensitive DNA gyrase mutants show a decrease in transposition frequency at non-permissive temperatures and this is reversed by the introduction of a DNA topoisomerase-deficient mutation which allows the accumulation of supercoiled DNA (Isberg and Syvanen 1982). DNA gyrase transiently generates single stranded DNA, and it has been proposed that the transposase binds to single-stranded DNA as part of the transposition process. IS50R of Tn5 often orientates itself closest to an active upstream promoter, which suggests that transposase-dependent insertion is affected by transcriptional activity and transposase may interact with RNA polymerase, or single-stranded DNA at, or near to, the site of insertion (Berg et al. 1982).

1.3.2 Strategies for identifying and analysing the Class C B-lactamase of the Pseudomonas chromosome

Mutations caused by transposition of Tn5 into the pseudomonal chromosome can be screened for resistance to anti-pseudomonal B-lactams. The transposon can be located by classical mapping of the genome using transduction, transformation or conjugation by selecting

for the transposons selectable marker. The flanking chromosomal region can be used to probe a gene library for the wild-type gene. Regulation of B-lactamase expression can then be investigated using cis/trans complementation studies by the generation of partial diploids in the mutant strains.

Conjugation

B-lactam resistant mutants of P. aeruginosa generated by chemical mutagenesis were used to map genes involved in regulating the chromosomal B-lactamase using R plasmids to mobilize the chromosome (Matsumoto and Terawaki 1982). R plasmids carrying selectable markers are capable of promoting the transfer of chromosomal DNA (Willets 1988). Insertion sequences carried by R plasmids can allow integration of the plasmid into the host chromosome if another copy of the insertion sequence is present there (Willets 1988). The plasmid mobilises the host chromosome at low frequency from the site of insertion (Willets 1988).

The most commonly used plasmids in conjugation experiments in P. aeruginosa are FP2, FP5 and R68.45. FP plasmids are able to mobilise 40 minutes of the pseudomonas chromosome but have the disadvantage of inserting at a single site in the chromosome (Matsumoto and Tazaki 1973). As genes regulating B-lactamase expression may be distributed widely through the chromosome it may be difficult to analyse all the genes controlling the production of B-lactamase. Plasmid R68.45 has multiple sites of insertion in the chromosome and can mobilise the pseudomonas chromosome from many origins and in either orientation. Transfer of chromosomal DNA occurs at a higher frequency than with FP2, however, R68.45 can transfer only short fragments of DNA, usually

less than 10 minutes (Haas and Holloway 1978) and the selectable marker of R68.45 is a carbenicillinase making it unsuitable in work concerned with B-lactamase structural and regulatory genes.

Transduction

Bacteriophages capable of propagation in P. aeruginosa are common and transduction systems for genetic analysis have been developed (for review, see Holloway et al 1979). Commonly used transducing phages are F116L and G101 which transduce markers at frequencies between $1x10^{-7}$ to $5x10^{-7}$ per plaque forming unit (Holloway and Krishnapillai 1975). Transduction phages are only useful to transfer closely linked genes which may be disadvantageous if regulatory genes are widely dispersed in the chromosome.

Transformation

Attempts to map the chromosome by transforming chromosomal DNA into P. aeruginosa have not been successful (Holloway et al. 1979).

1.3.3 Problems with these systems in Pseudomonas aeruginosa

The mapping of genes involved in B-lactamase expression requires suitable recipient strains. A problem with using mutant strains of P. aeruginosa as recipients is that P. aeruginosa is intolerant (with spontaneous DNA deletions or plasmid loss) to multiple copies of chromosomally homologous DNA (Morgan 1982, Jeenes et al. 1986, Krishnipillai et al. 1986). The instability of such regions due to reversion to haploidy occuring at high frequencies may hinder complementation studies. The use of E. coli as recipient is hindered by the very low frequency of R´ plasmid transfer (Morgan 1982).

Furthermore, expression of pseudomonas genes in E. coli is generally extremely poor (Jeenes et al. 1986; Luthi et al. 1986). Normal regulation of pseudomonas genes can be lost in E. coli even when the gene and its regulatory elements are still present and fully functional following reintroduction into P. aeruginosa (Luthi et al. 1986). Low levels of expression are probably due to poor recognition of pseudomonas promoters by the transcription system of E. coli (Minton and Clarke 1985, Jeenes et al. 1986). Selection in E. coli is complicated by the instability of large segments of pseudomonas DNA with deletions occurring in the cloned region (Coleman et al. 1983).

Ohman's group has bypassed some of the above problems by using the conjugative helper plasmid pRK2013 to transfer a pLAFR1 cosmid gene bank en masse from E. coli into suitable pseudomonal recipient strains for screening by complementation of a mutated strain. This allows the transfer of P. aeruginosa DNA from E. coli without requiring prior expression in E. coli (Goldberg and Ohman 1984). The stability of pLAFR1-derived cosmids carrying large fragments in P. aeruginosa however, was not discussed.

1.4 Aims of the Present Project

The regulatory control of the inducible class C B-lactamase of P. aeruginosa has yet to be elucidated. P. aeruginosa has a high frequency of mutation to resistance to anti-pseudomonal antibiotics and in some circumstances this is as a result of the loss of normal regulation of B-lactamase synthesis. As this loss of control is also seen in members of the Enterobacteriaceae which carry an inducible class C B-lactamase it would be of interest to see if the mechanisms for hyperproduction are similar.

The aims of this project were to generate and then investigate the genetic and biochemical nature of B-lactamase hyperproducing mutants in P. aeruginosa. Such mutants were to be produced by insertional mutagenesis using Tn5 derivatives. Initially the construction of transposon Tn5 derivatives carrying aminoglycoside-resistance markers and their subsequent transfer into P. aeruginosa on temperature-sensitive vectors were to be performed. Mutants resistant to anti-pseudomonal B-lactams as a result of insertional mutagenesis by Tn5-derivatives in the chromosome were then to be isolated and screened for B-lactamase hyperproduction.

Chromosomal DNA, digested with appropriate restriction enzymes, from mutants hyperproducing B-lactamase would then be cloned in E. coli. Clones with resistance to the Tn5-encoded resistance markers would then be further investigated. The cloned regions of chromosomal DNA which flank the transposon would be used to identify the wild-type gene which had been inactivated by insertional mutagenesis in the mutant. This would aid the isolation and analysis of wild-type genes important in the regulation of B-lactamase synthesis in P. aeruginosa.

CHAPTER 2

MATERIALS AND METHODS

2.1 General and Specific Reagents and Equipment

General reagents

General reagents were of Analar Grade unless otherwise stated and obtained from either BDH or Sigma. Commercially available media and and media components were supplied by Difco, Lab M or Oxoid and were made up following manufacturers recommendations.

Antibiotics

Antibiotics were bought from Sigma unless otherwise stated. Stock solutions of antibiotics used for *in vivo* genetic selection were made at a concentration of 10mg/ml in sterile distilled water and filter-sterilized using a sterile cellulose acetate membrane filter (pore-size 0.2um, Whatman or Flow Labs.) unless otherwise stated. Stock solutions were stored at $4^{o}C$ for up to one week. Antibiotics were added aseptically to liquid media or to cooled molten agar media just prior to pouring plates; because many antibiotics are thermolabile care was taken to add all antibiotics after the molten agar medium had cooled to about $50^{o}C$.

Antibiotic:	
ampicillin	
benzylpenicillin[1]	Glaxo
carbenicillin	Beecham
cefsulodin	Ciba-Geigy, gift
ceftazidime	Glaxo
cephalosporin C[1]	Glaxo, dissolved in MgMOPS buffer to 3mM
chloramphenicol	
gentamicin[2]	
kanamycin[2]	
nitrocefin[1]	Glaxo, dissolved in DMSO, then diluted to 1mM in sterile distilled water (DMSO 10% v/v final concentration).
piperacillin	
streptomycin[2]	
sulphonamide	dissolved in methanol
tetracycline	dissolved in ethanol and brought to 10mg/ml with an equal volume of distilled water, stored in a dark bottle.
tobramycin[2]	
trimethoprim	dissolved in ethanol

1 - not filter-sterilized

2 - soluble salt

Enzymes

Enzymes were stored at -20°C. Restriction enzymes were supplied by NBL, BRL or Amersham. Preparation of buffers is described in Appendix I.

Restriction enzymes:	**Reaction buffer:**
AvaII	Specific buffer
BamHI	Universal
BglII	"
EcoRI	"
HindIII	"
HpaI	Specific buffer
PstII	Universal
PvuII	Universal
SalI	High salt
Sau3A	Universal

Other enzymes:

DNA T4 Ligase: (BRL)
DNA T4 Ligase: Ligaid (P&S Biochemicals Ltd.)
DNase/DNA PolI Nick translation kit (BRL: S30-8160 SB)
Lysozyme: (Sigma) Lyophilized lysozyme was dissolved to 10mg/ml in GTE buffer.
Proteinase K (Sigma)

Nucleic acids

DNA Sodium salt Type III (from salmon testes) (Sigma)
Lambda DNA (for molecular weight markers) (BRL)
Lambda DNA HindIII (for molecular weight markers) (BRL)

Plasmids

Plasmid:	Antibiotic resistance and relevant genotypes:	Reference or source:
pMMB19	Sm, Su, temperature-sensitive pRSF1010	(Scholz *et al.* 1985)
pNU305	Tc, Ap, *ampC*$^+$,*ampR*$^+$	(Lindberg *et al.* 1985)
pPH1	Gm, Sp	(Hirsch *et al.* 1986)
pR1033	Ap, Cm, Gm, Km, Hg, Sm, Sp, Su, Tc	(Smith *et al.* 1975)
pR5	Tm	(Dr. Shannon, The London Hospital)
pR751	Tp, Sm, Su , *tra*$^+$	(M.J.Woodward)
pRSF1010	Sm, Su, *mob*$^+$	(Guerry *et al.* 1974)
pSUP2011	Cm, Km, *mob*$^+$	(Simon *et al.* 1983)
pVW6	Sm, Su, Gm, *mob*$^+$	(Hewinson *et al.* 1989)
pXS1	Gm	(Hirsch *et al.* 1986)
pRTG1	Gm, Sm, Su, *mob*$^+$	(this work)
pSG1	Ap, Cm, Gm, *mob*$^+$	"
PSG2	Ap, Cm, Gm, *mob*$^+$	"

Ap-ampicillin, Cm-chloramphenicol, Gm-gentamicin, Hg-mercury, Km-kanamycin, Sm-streptomycin, Sp-spectinomycin, Su-sulphonamide, Tc-tetracycline, Tm-tobramycin, Tp-trimethoprim.

mob$^+$ - mobilized in the presence of tra$^+$ donor

tra$^+$ - encodes transfer factors

Bacterial Strains used in this work

Strains: Genotype:

E. coli K12

C600 *thi*, *thr* *leu* (Maniatis *et al.* 1982)

HB101 F-, *thi*, *pro*, *met*, *recA*13, *rpsL*20, *hsd*S20r-m+, *supE* (Maniatis *et al.* 1982)

J62 *thi*, *trp*, *his*, *pro*, F-, rifampicin-resistant (PHLS Colindale)

SM10 :: pRP4 Km, *thi*, *thr*, *leu*, (Simon *et al.* 1983)

SNO3 *thi*, *ampA*1, *ampC*8, *recA*, *rpsL*, *pyrB* (Normark and Burman 1977)

SNO302 SNO3 *ampD* (Lindberg *et al.* 1985)

UB2272 *thi*, *trp*, *his*, *lys*, *polA* (P.M.Bennett, Univ. Bristol)

UB5201 *thi*, *pro*, *met*, *recA*56, *nalA* (P.M.Bennett, Univ. Bristol)

5K *thi*, *thr*, *leu*, *rpsL*, *hsd*RS14 (Colson *et al.* 1965)

P. aeruginosa

3-Pre (Slack and Pitt 1982)

PAO4069 *pro*A9024, *met*9020, (Matsumoto and Terawaki 1982)

PAO4082 *pro*A9024, *met*9020, *bla*J9111, (Matsumoto and Terawaki 1982)

PAO4095 *pro*A9024, *met*9020, *bla*K4097 (Matsumoto and Terawaki 1982)

PAO6520 *recA* (W.W. Nichols, J.Radcliffe Hospital, Oxford)

PAO6521 *recA* (W.W. Nichols, J.Radcliffe Hospital, Oxford)

PAT210 prototroph, FP^{+*} (NCIB 10549)

PAT216 prototroph (NCTC 10662)

P. putida KT2440 (R.Wilson, Brompton Hospital, London)

* - FP^{+} is analogous to F in *E. coli*

Radioisotopes and Autoradiographic Materials

^{32}P-labelled dATP (370MBq/ml)	(Amersham)
X-OMAT S Film	(Kodak)
X-ray film cassettes	(GRI)
Developer (LX24)	(Kodak)
Fixer (FX40)	(Kodak)

Specialized Equipment

Beckman L8-70 Ultracentrifuge, 70Ti centrifuge

Beckman J-6B Centrifuge

Cecil Spectrophotometer

Pharmacia Electrophoresis Power Supply EPS 500/400

Polaroid MP-4 Land Camera

Polaroid CVU5 Land Camera

Sonicator: Camlab Transsonic bath T420

Electroporation Apparatus: BRL Cell-Porator System II (1605 AF)

Bio-Rad Gene Pulser (165-2078)

Bio-Rad Capacitance Extender (165-2087)

Safety Codes

All work was performed in accordance with regulations of the following:-

Advisory Committee on Dangerous Pathogens

Advisory Committee on Genetic Manipulation

National Radiological Protection Board

Latex gloves were worn at all times when dealing with hazardous chemicals including chloroform, ethidium bromide and phenol.

2.2 Media

Media was usually made in 1 litre volumes and dispensed into 200ml volumes prior to autoclaving. All media were autoclaved at 10lbs.ins^{-2} for 15 minutes unless otherwise stated.

2.2.1 Complete broth

Tryptone	10g	(Oxoid)
Yeast extract	5g	(Lab M)
K_2HPO_4	3g	
KH_2PO_4	1g	
Glucose	2g	

Distilled water to 1000ml final volume.

The pH of the solution was adjusted to pH 7 - 7.2 using 1M NaOH and the solution autoclaved in the required volumes.

2.2.2 Luria broth (L broth)

Tryptone	10g	(Oxoid)
Yeast extract	5g	(Lab M)
NaCl	5g	
1M NaOH	2ml	

Distilled water to 1000ml final volume.

The pH of the solution was adjusted to 7 - 7.2 using 1M NaOH and the solution autoclaved in the required volumes.

2.2.3 Nutrient broth

Nutrient broth was made using Nutrient broth No. 2 (Oxoid) in distilled water and according to the manufacturers instructions.

2.2.4 Tryptone Soy broth

Tryptone Soy broth (Oxoid) was made in distilled water and according to manufacturers instructions.

2.2.5 Glucose minimal media

M9 salts (x4 concentrate)

Na_2HPO_4	28g
KH_2PO_4	12g
NaCl	2g
NH_4Cl	4g

Distilled water to 1000ml final volume.

Glucose minimal media

Distilled water	750 ml
M9 salts (x4 concentrate)	250 ml
1M $MgSO_4$	1 ml
0.1M $CaCl_2$	2.5 ml
2mg/ml thiamine	5 ml
20% glucose	10 ml

Stock solutions were autoclaved separately at 15 lbs.ins^{-2} for 15 minutes except glucose and thiamine solutions which were autoclaved as normal.

When amino acid supplements were required for bacterial growth they were added to a final concentration of 0.04mg/ml. Amino acids were made up as a stock solution of 4mg/ml, autoclaved separately (10lbs.ins.$^{-2}$ for 15 minutes) and stored at 4^{o}C.

2.2.6 **Chemically Defined Media (CDM)** (Anwar et al. 1983)

The final composition of the medium was:

		g / 2 litres
40.0	mM glucose	14.4
0.5	mM NaCl	0.0724
0.62	mM KCl	0.0746
3.2	mM $K_2HPO_4.3H_2O$	1.46
40.0	mM $(NH_4)_2SO_4$	10.56
0.062	mM $FeSO_4.7H_2O$	0.0246
0.4	mM $MgSO_4.4H_2O$	0.2
50.0	mM MOPS (3-morpholinolpropanesulphonic acid)	20.92

Glucose was dissolved in 937.5ml distilled water and autoclaved in 25x 37.5ml aliquots. $FeSO_4.7H_2O$ was dissolved in 60ml of distilled water, the pH adjusted to 1 using 1M H_2SO_4 and distilled water added to a final volume of 62.5ml; aliquots (2.5ml) were autoclaved. The remaining ingredients were added to distilled water (950ml), the pH adjusted to 7.8 using 1M NaOH and the volume made up to 1 litre with distilled water. The solution was dispensed in 40ml aliquots and autoclaved. The three solutions were stored separately at room temperature until just prior to use when they were added aseptically to give 80ml volumes of CDM (37.5ml glucose, 2.5ml $FeSO_4.7H_2O$, 40ml salts).

2.2.7 **Solid Media**

Pseudomomas Isolation Agar (PIA) (Difco) and **CN agar** (Oxoid) were made up in sterile distilled water according to manufacturers recommendations.

Liquid media was made up as described above. Agar was added to 1% (w/v) prior to autoclaving by using 1g Bacto-agar (Lab M) per 100 ml of liquid medium. For Glucose minimal agar 10g agar were added to 750ml distilled water to give the correct percentage agar (1%w/v) after addition of salts. All media containing agar was stored at room

temperature until required, when they were melted by autoclaving at 10lbs.ins^2 for 5-10 minutes. After the medium had set the plates were dried in an oven (37°C) and stored, inverted, at 4°C in plastic bags until required.

2.3 *In vivo* Techniques

2.3.1 Routine growth and maintenance of bacteria

Plasmid-free strains of *E. coli* were sub-cultured on complete or L-agar by streaking to single colonies. Plasmid-bearing strains were sub-cultured on complete or L-agar containing the appropriate antibiotic. Streak plates were inverted and incubated overnight at 37°C. All plates were stored at 4°C in a plastic bag for up to a month before subculturing. Only fresh streak plates of *E. coli* cultures were used to inoculate fresh cultures required for transformation or MIC analysis. Cultures were grown in L-broth with the appropriate antibiotic where necessary and incubated overnight at 37°C.

Strains of *P. aeruginosa* were sub-cultured as for *E. coli*. Plasmid-free strains, however, were sub-cultured on nutrient or PIA agar and plasmid-bearing strains were sub-cultured on nutrient agar containing the appropriate antibiotic selection. Streak cultures were stored inverted at room temperature in plastic bags. Only fresh streak plates were used to inoculate fresh cultures required for transformation or MIC analysis. Strains used for the extraction and assay of B-lactamase were grown in CDM broth.

For long term storage cultures were also stored at -70°C. The strain to be stored was grown at 37°C overnight on a fresh agar plate

containing appropriate antibiotics for plasmid maintenance. Using a Pasteur pipette an area of heavy growth was thoroughly resuspended in approximately 1ml of nutrient or Tryptone soy broth (Oxoid) containing 15% (v/v) glycerol. The thick suspension was transferred to a sterile 1.75ml glass vial (BDH) containing approximately 20 sterile embroidery beads (Ells and Farrier) and the culture thoroughly mixed with the beads. Excess culture broth was removed with a Pasteur pipette and the vial top replaced. The vial was coded and stored at -70°C. To sub-culture from bead stock a vial was taken in a paraffin wax container kept at -70°C to the work bench. A single bead was removed using a sterile but cool straight wire and transferred to 10ml nutrient broth for P. aeruginosa or complete broth for E. coli containing antibiotic when appropriate. The time the stock vial was at room temperature was kept to a minimum to prevent thawing.

2.3.2 MIC determination

Two methods for MIC analysis were used, a "rapid" and a "spread" method. For both methods, a range of inocula sizes was used to observe affects of population density on antibiotic resistance.

2.3.2.1 Rapid method

Individual colonies (four or five per strain) were used to inoculate separate 10ml volumes of L-broth for E. coli strains or Nutrient broth for P. aeruginosa strains. Cultures were grown with vigorous shaking overnight at 37°C. Ten-fold serial dilutions of the cultures were made and 20ul of each dilution placed on a single agar plate containing a known concentration of the antibiotic being tested. Plates were incubated at 37°C for 18-24 hours and numbers of colonies recorded.

2.3.2.2 Spread method

Individual colonies (five or six per strain) were pooled from a fresh sub-culture and used to inoculate 10ml broth. The cultures were grown with vigorous shaking overnight at 37°C. A ten-fold serial dilution of the culture was followed by spreading 100ul of each dilution on to agar plates (82mm diameter) containing the antibiotic being tested. Plates were incubated at 37°C for 18-24 hours and the number of colonies for each dilution recorded.

2.3.3 Nitrocefin drop test

Nitrocefin is a B-lactam antibiotic which changes in colour from orange to deep red upon hydrolysis. This change can be observed when nitrocefin (1mM) is applied to a colony with B-lactamase activity. The time taken for the colour change indicates the B-lactamase activity of the colony. As a comparison the time taken for a colony of the parental strain to hydrolyse nitrocefin was also measured. Nitrocefin (10ul) was dropped onto colonies from bacteria grown on nutrient agar for 18 hours at 37°C. This method is only useful in identifying B-lactamase hyperproducing mutants if colonies are not grown on agar containing B-lactam since induction of the parental B-lactamase might cause rapid hydrolysis of nitrocefin and prevent identification of hyperproducing mutants.

2.3.4 Plasmid transfer by conjugation

2.3.4.1 A filter-mating method

Donor strains containing plasmids to be transferred and recipient strains were grown separately in L-broth overnight at 37°C; the donor strain was grown in the presence of appropriate antibiotics for

plasmid maintenance. Samples of donor (0.5ml) and recipient (1.0ml) were gently mixed in an Eppendorf tube and pelleted by centrifugation for 15 seconds at 13000xg. The pellet was resuspended in pre-warmed L-broth (0.1ml) and placed on a filter placed aseptically on an L-agar plate. Incubation was for 2-18 hours at 37°C unless using temperature-sensitive plasmids in which case incubation was at 28°C unless stated otherwise. The filter was removed from the agar surface using alcohol-flamed forceps, placed in a Universal bottle containing M9 diluent (10ml) and the cells resuspended by vigorous vortexing. The filter was removed with sterile forceps and the Universal bottle containing the cell suspension centrifuged for 5 minutes at 2500rpm at room temperature. The supernatant was removed and the cells resuspended in 1ml of M9 diluent. A ten-fold serial dilution was made and 100ul of each dilution spread on selection plates. Controls of recipient and donor strains alone were treated similarly to the conjugal suspension and spread on selection media to ensure the absence of parental growth. Recipient cells were diluted to $1x10^{-6}$ and $1x10^{-7}$ and spread on glucose minimal media containing the required supplements for recipient growth to obtain the number of viable recipient cells. The plates were incubated at 37°C or 28°C where appropriate for up to three days. Presumptive transconjugant colonies were tested for phenotypic traits expected of a transconjugant before screening for the presence of plasmid.

For example, in a conjugation with *E. coli* SM10 (pVW6) as donor of pVW6 and *E. coli* UB5201 as recipient the following selection regime was used. *E. coli* SM10 requires thiamine, threonine and leucine for growth on glucose minimal medium. Plasmid pVW6 confers resistance to streptomycin, sulphonamide and gentamicin. Transconjugants of *E. coli*

UB5201, which requires thiamine, proline and methionine, were selected on glucose minimal medium containing thiamine, proline and methionine with gentamicin at 20ug/ml. Growth of E. coli SM10 was prevented due to the lack of threonine and leucine, whilst E. coli UB5201 lacking pVW6 was unable to grow in the presence of gentamicin at 20ug/ml. Presumptive transconjugants were tested for resistance to streptomycin at 100ug/ml in L-agar prior to plasmid analysis.

For filter-mating experiments with P. aeruginosa as the recipient, P. aeruginosa was grown stationary for 16-18 hours in nutrient broth at 43°C to reduce nuclease activity. Any specific modifications to this general method are discussed in the text.

2.3.4.2 A spot-mating method

P. aeruginosa was grown as described as above. A sample of culture (100ul) was spread on glucose minimal agar containing an appropriate antibiotic for the presence of plasmid and was incubated for about 15 minutes at 37°C until the agar surface was dry. An overnight culture of E. coli SM10 (pRTG1) was centrifuged at 4000xg for 5 minutes at room temperature. The supernatant was discarded and the cell pellet resuspended in 10ml M9 diluent. The cells were collected by centrifugation as above and the cell pellet gently resuspended in 1ml M9 diluent. Aliquots (20ul) were then dropped onto the plates previously seeded with the recipient culture and also onto control selection plates. Plates were incubated at room temperature for up to 3 days and presumptive transconjugants were streaked onto PIA agar.

2.3.5 Transformation of bacteria

2.3.5.1 Preparing competent *E. coli* using calcium chloride

An overnight broth culture (0.2ml) was used to inoculate a pre-warmed L-broth (20ml) in a conical flask which was incubated at 37°C with vigorous shaking (200rpm) for 2-3 hours to an OD_{650} of approximately 0.2 (0.5 for *recA* strains). Cultures were placed on ice for 10-15 minutes, transferred to chilled Universal bottles and centrifuged at 2500rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet gently resuspended in 10ml ice-cold 0.1M $CaCl_2$. The cell suspension was incubated on ice for 15-30 minutes prior to centrifugation at 2500rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet carefully resuspended in 2ml of ice-cold $CaCl_2$. Competent cells were used immediately or kept at 4°C and used within 24 hours. Any variations are discussed in the text.

2.3.5.2 Transformation of *E. coli* with plasmid DNA

Competent cells (200ul) were placed in pre-chilled Eppendorf tubes; one for each DNA sample to be introduced and one control sample. Approximately 0.2ug of plasmid DNA in TE buffer or neat ligation reaction mixture was added to competent cells and mixed gently. After incubation on ice for 15 minutes the tubes were transferred to a 42°C water bath (Grant Instruments) for 90 seconds. Tubes were reincubated on ice for a further 90 seconds and then again at 42°C for 90 seconds. Pre-warmed L-broth (0.8ml) was added to each tube and mixed by inversion. Cells were then incubated for 1-3 hours at 37°C without shaking to allow phenotypic expression prior to spreading aliquots (100ul) on selection media. Incubation was for 24 hours at 37°C. Putative transformants were picked from the selection plates, tested for other plasmid-encoded resistance phenotypes and those showing the

expected resistance phenotypes were screened for plasmid DNA. Growth incubations were at 30°C with a longer recovery period (3-6 hours) when transformation was with temperature-sensitive plasmids.

2.3.5.3 Transformation of P. aeruginosa with plasmid DNA

Transformation was attempted using a number of competence solutions using the same protocol as described above for E. coli. To inactivate endonuclease activity, cultures of P. aeruginosa were incubated stationary at 43°C overnight and 0.5ml used to inoculate prewarmed nutrient broth (50ml). Cells of P. aeruginosa were grown to the early log phase of growth by growing with vigorous shaking at 37°C for 2-3 hours until an OD_{650} of approximately 0.2 was obtained (~$5x10^8$ cfu/ml).

Harvesting and transformation was as described for E. coli above, except the incubation in ice-cold competence solution prior to DNA addition was generally 45-60 minutes. Transformation experiments specify which solution was used in each case. Competence solutions used in different experiments were 0.1M $CaCl_2$, 0.1M $MgCl_2$, 0.15M $MgCl_2$, 0.1M MOPS (pH6.5)/50mM $CaCl_2$/10mM RbCl and also 0.1M MOPS/0.1M $MgCl_2$/10mM RbCl.

In other experiments, to make P. aeruginosa competent conditions based on those of Drew (1984) were used. Cultures were grown and harvested as described above. Cells were resuspended in ice-cold sterile 0.1M $MgCl_2$ (20ml) and placed on ice for 20 minutes. After centrifugation and resuspension in 0.1M $CaCl_2$ (2ml). Cells were incubated on ice for 1 hour prior to adding DNA (0.2ug). Heat-shock, incubation and plating was performed as described above. A modification of this

method was also performed using 10mM MOPS, 0.1M $MgCl_2$ and 10mM RbCl in place of 0.1M $MgCl_2$ and 100mM MOPS, 0.1M $CaCl_2$ and 10mM RbCl in place of 0.1M $CaCl_2$.

2.3.5.4 Electroporation of P. aeruginosa

Cultures (40ml) were prepared as for transformation and harvested early in the log phase (OD_{650}~0.2) by centrifugation in a bench top centrifuge at 2500rpm at 4°C for 5 minutes. The pellet was suspended in electroporation buffer (20ml) and left on ice for 5 minutes. After recentrifugation the pellet was resuspended in ice-cold electroporation buffer (20ml), incubated on ice and used within 2 hours. Cuvettes containing treated culture (0.8 or 1.0ml, depending on the cuvette manufacturer) were used in all experiments. Electroporation was performed following the manufacturers recommendations (BRL Cell Porator System II and Bio-Rad "Gene Pulser"). After electroporation, a 100ul sample was serially diluted in M9 diluent (9.9ml). Cell counts were performed by spreading aliquots (100ul) from the serial culture on N-agar plates which were incubated at 37°C overnight.

Cells were incubated in the presence of DNA of varying concentrations (see Results) for 5-30 minutes prior to electroporation. A sample (200ul) of electroporated culture was inoculated in N-broth (0.8ml) and incubated stationary at 30°C for 3-4 hours to allow phenotypic expression of the plasmid-encoded resistance genes (streptomycin and gentamicin resistance). Selection for transformants was on nutrient agar containing gentamicin or streptomycin at known concentrations.

2.3.6 Dry weight determination of PAT210 and 3-Pre and correlation with optical density

The dry weight was determined by setting up a standard curve of dry cell weight per unit volume against optical density at 650nm. Cultures of P. aeruginosa PAT210 and 3-Pre were each grown in 80 mls CDM overnight at 37°C in baffled flasks. Cells were collected by centrifugation at 5,000 rpm for 5 minutes at 4°C in 50ml Sorvall centrifuge tubes. Pellets were resuspended and washed in 20mls ice-cold water and recentrifuged under the same conditions as above. Pellets were resuspended in ice-cold water to a final volume of 10ml.

A dilution series was set up by dilution in sterile water to measure the OD_{650}. The volume of the rest of the suspension was accurately measured and was then placed in a pre-weighed, dry, sterile Universal bottle. The cells were collected by centrifugation at 2,500 rpm for 10 minutes at room temperature. The supernatant was carefully decanted off without disturbing the cell pellet. The Universal bottle was then incubated in an oven at 94°C until there was no further weight loss over a period of an hour.

2.3.7 B-Lactamase extraction

Two 5ml CDM overnight cultures of each P. aeruginosa strain to be tested were inoculated into 80mls CDM (Anwar et al. 1983) and grown in baffled flasks with loose plugs at 37°C and 190rpm. Measurements at OD_{650} were taken periodically and the time taken to reach a log dry weight value of 2 (i.e. 0.1mg/ml) predicted. Two hours before the estimated time, 1 ml of benzylpenicillin (16mg/ml) in sterile water was added to a final volume of 200ug/ml to one culture and 1ml of sterile water added to the other culture. The cultures were harvested

2 hours after the addition of benzylpenicillin (or water). Harvesting consisted of measuring about 75mls of culture accurately into two 50ml Sorvall centrifuge tubes. Cells were collected by centrifugation for 5 minutes at 4°C and 5,000 rpm.

Pellets of the non-induced cultures were frozen at -20°C. Pellets of the induced cultures were resuspended into a single final volume of 30mls ice-cold 5mM $MgCl_2$/25mM MOPS (pH 7.4). Cells were collected by centrifugation at 5,000 rpm for 7 minutes. The supernatant was then carefully decanted and the inside walls of the centrifuge tube were dried with absorbant tissue. The pellets were then placed at -20°C.

Pellets were subjected to a total of four freeze/thaw cycles. Thawing was at room temperature and took approximately 15 minutes. Each freeze phase lasted for at least 4 hours. Following three freeze/thaw cycles, each pellet was resuspended in 3.5 ml ice-cold 5mM $MgCl_2$/ 25mM MOPS (pH7.4). Resuspension was thorough but foaming was avoided. The resultant suspensions were refrozen at -20°C and stored there until the enzyme activity was assayed. This process releases ~90% of cell-bound B-lactamase activity with respect to recovery using sonication (Berks 1976). Moreover, sonication can reduce the activity of B-lactamase from P. aeruginosa (W.W.Nichols, personal communication).

2.3.8 Analysis of B-lactamase activity

To prevent spectrophotometric interference from cell debris during the B-lactamase assay the suspension was thawed and 2ml of the sample was centrifuged at 12,000 rpm at 4°C for 30 minutes. The supernatant was used as the freeze/thaw supernatant. The remaining 1.5ml of sample

was similarly clarified and used in cases of very low B-lactamase activity.

The assay consisted of a sample of B-lactamase, the B-lactam substrate cephalosporin C and 5mM $MgCl_2$ / 25mM MOPS buffer (pH 7.4). The assay was performed in a quartz cuvette maintained at 37°C within an water jacket. The stock B-lactamase preparation was thawed and kept on ice throughout the experiment. Cephalosporin C was stored dessicated at 4°C. Prior to use it was allowed to come up to room temperature. A stock solution of 3mM cephalosporin C was made in 5mM $MgCl_2$/25mM MOPS (pH7.4) buffer and stored on ice through the experiment. Stock 5mM $MgCl_2$/25mM MOPS buffer was thawed and kept at 37°C through the experiment. An initial assay run was performed as follows: 30ul Cephalosporin C was added to 2.92ml MgMOPS in the cuvette. The absorbance at 260nm was measured over 2 minutes and shown to be stable, as observed on a chart recorder. B-lactamase (50ul) from the freeze/thaw supernatant was then added. After closing the spectrophotometer hood, the ´Continue´ key of the computer was hit and the computer took the first reading from the voltmeter. The computer was programmed to take a reading every 10 seconds and did so until the reaction had gone to completion. Completion was determined as being when there had been no reduction in absorbance over the last ten readings taken. The computer then performed the half-time kinetic analysis. The reaction assay was altered for the freeze/thaw sample from the non-induced cultures with 250ul of sample being added and the volume of buffer being reduced accordingly. V_{max} and K_m were also calculated from samples of the non-centrifuged suspensions.

If the B-lactamase activity was too fast or too slow then the amount

of sample was decreased or increased to yield progress curves lasting about 20 minutes. Once a suitable sample of B-lactamase had been determined the concentration of cephalosporin C was altered within the range 20-70uM and V_{max} and K_m determined for each concentration.

Occasionally, due to hardware fault, the computer stopped taking readings in the middle of the experiment. In these cases half-time analysis was performed by hand using the chart recording trace. The gradient and line of intercept were calculated using this data.

V_{max} (nmol/min/mg dry weight) was calculated as follows:

V_{max} = (1/2xslope) x 3 x (1/dry weight) x 60 where the slope was measured in units of $(uM/s)^{-1}$.

K_m (uM) was calculated as follows:

K_m = (Intercept/Slope)(1/2 ln2)

where the slope was measured in units of $(uM/s)^{-1}$, and the intercept in units of s.

2.4 *In Vitro* Genetic Methods

Preparations of solutions used in this section are described in Appendix II.

2.4.1 Rapid alkaline lysis extraction of plasmid DNA

Plasmids were extracted from bacteria using a modification of the rapid alkaline lysis (mini-prep) method described by Maniatis *et al.* (1982). Plasmid bearing cells were grown in L-broth (10ml) containing an appropriate antibiotic overnight at 37°C with agitation. Cells harbouring temperature-sensitive plasmids were grown at 28-30°C. Cells were harvested by centrifugation at 3000rpm for 5 minutes at room temperature and the supernatant discarded. The pellet was resuspended in GTE buffer (180ul) and 192ul was transferred to an Eppendorf microfuge tube. After 5 minutes at room temperature, lysozyme (8ul) was added and incubated for a further 5 minutes. SN buffer (400ul) was then added and mixed by inversion. Tubes were incubated on ice for 5 minutes and ice-cold KA buffer (300ul) was then added to each tube and mixed by inversion. Tubes were placed on ice again for a further 10 minutes. Cellular debris was removed by centrifugation at 13000xg for 5 minutes at room temperature using a MSE 'Micro Centaur' centrifuge. The supernatant (approximately 750ul) was transferred without disturbing precipitated protein to a fresh eppendorf tube. Isopropanol (450ul) was added and mixed by vortexing for 5 seconds and plasmid DNA was collected by centrifugation at 13000xg for 5 minutes at room temperature. The supernatant was carefully removed by aspiration and the pellet resuspended in TEN (200ul). Occasionally the DNA did not dissolve easily and so the tube was incubated at 37°C until the DNA had dissolved. Buffer-saturated

phenol (200ul) was added to the suspension and the tube was vortexed for 5 seconds. Chloroform (200ul) was added and the tube vortexed again. Organic and aqueous phases were separated by centrifugation at 13000xg for 2 minutes at room temperature. The aqueous phase was transferred to a fresh Eppendorf tube taking care to not transfer any precipitated protein at the interface. The phenol/chloroform extraction was repeated 2-3 times until no more protein accumulated at the interphase after centrifugation. The final aqueous phase (200ul) was transferred to a fresh Eppendorf tube containing ice-cold ethanol (500ul) and the tubes mixed by inversion. The solution was then stored for 30 minutes at -70°C. The precipitated DNA was collected by centrifugation at 13000xg for 5 minutes at room temperature. Ethanol was removed by careful aspiration and the pellet re-dissolved in TE (60ul) containing RNase (1ug/ml). The DNA preparation was then stored at -20°C until required.

Modifications of the rapid alkaline lysis method were used later in the project. The lysozyme step was removed and the cell suspension (200ul) was treated with SN buffer. After 5 minutes on ice, KA buffer (300ul) and RNase A (10ug/ml) were added and incubated for a further 10 minutes. This step made the later addition of RNase-A unnecessary. After centrifugation to remove insoluble protein, the supernatant was extracted immediately with phenol/chloroform prior to ethanol precipitation and resuspension in TE buffer (15ul). Precipitation of DNA with isopropanol and resuspension in TEN were then no longer required. Analysis of DNA was performed by electrophoresis of a sample of the DNA solution by agarose gel electrophoresis.

2.4.2 Large-scale purification of plasmid pRTG1

Plasmids were extracted from bacteria using a modification of the method of Maniatis et al. (1982). E. coli UB5201 transformed with pRTG1 was grown with shaking in 5ml L-broth containing streptomycin (100ug/ml) and gentamicin (20ug/ml) at 30°C for 8 hours. Of this culture, 2ml was used to inoculate each of two 200ml L-broth cultures each in 1 litre flasks. Cultures were incubated in an orbital shaker at 28°C for 18 hours. Plasmid was maintained by the presence of streptomycin at 100ug/ml and gentamicin at 20ug/ml. Cells were pelleted at 4°C in a 1 litre centrifuge bucket using a swing out rotor at 3000 rpm for 30 minutes in a Beckman J-6B Centrifuge. The supernatant was removed and the cell pellet resuspended in 15ml lysis buffer. Approximately 7.5ml of cell suspension was then dispensed into each of two 26ml Beckman centrifuge tubes and placed on ice. Lysozyme (1.5ml 10mg/ml) was added to each sample, mixed and the suspension incubated on ice for 5 minutes. RNase A (100ul 10mg/ml) and 3ml 0.25M Tris-0.25M EDTA were then added to each sample, which were incubated for a further 5 minutes. Triton (12.5ml 2%w/v) was added to each sample and mixed by rolling the tube gently. The solution was mixed approximately every 3 minutes for a 30 minute period until the suspension developed a white floccule. Samples were then centrifuged at 20000rpm for 60 minutes in a fixed angle rotor (Ti70 Beckman) at 4°C to remove cellular debris and chromosomal DNA. Supernatants were transferred to thick-walled glass Universal bottles and warmed to room temperature before adding 1/10 volume 10% (w/v) SDS. One half volume of buffer-saturated phenol was added and the solution vigorously shaken, followed by a half volume of chloroform. The aqueous and organic phases were separated by centrifugation in a bench top centrifuge at 3000rpm for 5 minutes at room temperature.

The upper aqueous phase was transferred into a new Universal tube and extracted again with phenol and chloroform. Each upper phase from two samples were then dispensed into a 50ml disposable tube and a 1/10 volume of 3M sodium acetate (pH6.0) added. Chilled absolute ethanol was added to a final volume of 50ml (approximately 30ml) and, after vortexing briefly, the solution was stored at -70°C for two hours. DNA was collected by centrifugation at 3000rpm at 4°C for 30 minutes. The supernatant was discarded and the DNA pellets dissolved in TE buffer to a final volume of 7.5ml.

The DNA was further purified by centrifugation through a caesium chloride density gradient. CsCl (8.0g) was dissolved in the DNA solution (7.5ml), ethidium bromide (50ul of a 10mg/ml solution) was added and the solution then transferred to a Beckman quick seal polyallomer tube using a syringe fitted with a 19G hypodermic needle. Care was taken to prevent the addition of air bubbles to the solution. To protect the rotor from CsCl an overlay of liquid paraffin was added to the neck of the tube. Tubes were balanced to within 10mg and centrifuged for 16 hours at 45000rpm (149000xg) at 20°C using a Beckman L8-70M Ultracentrifuge. Tubes were removed and placed over a tray. A scalpel blade was used to pierce the centrifuge tube near the neck to prevent a vacuum when the DNA band was extracted. The DNA band was harvested using a 19G hypodermic needle and syringe. The needle was placed immediately below the band with the aperture facing upward. DNA was withdrawn carefully in less than 1ml of solution. The DNA was transferred to a fresh Eppendorf tube and ethidium bromide removed by repeated extractions with butanol until the solution was no longer pink. The DNA solution was diluted with 3 volumes of distilled water, 3M sodium acetate (1/10 volume) and ice-cold absolute ethanol

(2 volumes). The solution was chilled for 30 minutes at -70°C and then centrifuged at 3000rpm for 30 minutes. The supernatant was discarded and the DNA pellet resuspended in 500ul TE buffer. Addition of 50ul 10% (w/v) SDS was followed by phenol/ chloroform extraction. After centrifugation the upper aqueous phase (500ul) was transferred to a fresh Eppendorf tube containing 3M sodium acetate (50ul). Approximately 1ml ice-cold absolute ethanol was added and DNA collected by centrifugation at 13000xg for 5 minutes. The supernatant was removed and the pellet washed in cold 75% alcohol twice. The alcohol was discarded and the DNA pellet air-dried before dissolving in TE buffer (150ul).

To estimate the DNA concentration, DNA (10ul) was first diluted in sterile distilled water (1.99ml) in a quartz cuvette and the absorbance measured at 260nm. The concentration was calculated using the following equation: DNA (ug/ml) = OD_{260} x 200 (dilution factor) x 50 (ug/ml). The sample was diluted to approximately 1mg/ml and the OD_{260} remeasured. The purity of DNA was then assessed by measuring absorbance at 280nm and calculating the $OD_{260/280}$ ratio, pure DNA having a ratio of 1.8.

2.4.3 Chromosomal DNA extraction from *P. aeruginosa* PAO4069

Strains were grown with shaking at 37°C in nutrient broth (40ml) to the mid-log phase (~3 hours). Bacteria were harvested by centrifugation in a benchtop centrifuge, washed in saline twice and centrifuged again. The pellet was resuspended in 10ml ice-cold ET buffer (10mM EDTA, 10mM Tris HCl (pH8.0). Lysozyme (1ml of 1mg/ml) was added and incubated for 15 minutes at 37°C. Sarkosyl-proteinase K (1.2ml) was added and the resulting solution incubated for 1 hour at

37°C. Phenol (10ml) was added and the solution vortexed. Waste phenol was removed after centrifugation in a benchtop centrifuge. Phenol extraction was repeated a further three times. Chloroform (10ml) was then added and mixed by vortexing. Chloroform was also removed after centrifugation and the chloroform purification stage was repeated twice. Ammonium acetate (1.2ml of 3M) was added to the aqueous layer. Isopropanol (20ml) was layered at room temperature and the phases mixed with a glass rod. DNA was spooled around the rod, blotted, air-dried and resuspended in TE (4ml). DNA was left at 4°C overnight to dissolve fully prior to gel electrophoresis.

2.5 Agarose Gel Electrophoresis

Electrophoresis of DNA was performed using horizontal agarose gels (Pharmacia and BRL) and DNA visualization was by staining with ethidium bromide and viewing under ultra-violet light.

Agarose (Type II from Sigma for routine use) was dissolved in TBE buffer to a final concentration of 0.7% (w/v) by heating over a bunsen flame with constant shaking or using a microwave. The solution was allowed to cool to 50-60°C prior to pouring into a mould containing a well-forming comb. The gel was allowed to set (15-30 minutes) and was transferred to the electrophoresis tank with the gel placed so that the comb was at the cathode end of the tank. TBE buffer was poured into the tank until the gel was immersed in 1-2mm buffer and the comb was removed. Samples of DNA solution were mixed with one quarter volume of loading buffer. Each DNA sample was then transferred to a single well of the gel. A well was also filled with a molecular weight marker standard, usually Lambda Hind III DNA. The tank lid was then placed in position and the power supply switched

on. After a 5-10 minute period at 40V samples were run at 60V for small gels (width 5 or 8cm) and 100V for large gels (width 11cm) until the tracking dye had migrated three-quarters of the way down the gel. The power was switched off, the gel removed from the electrophoresis tank and placed in an ethidium bromide (0.5ug/ml ethidium bromide in distilled water) for five minutes. The gel was then rinsed in distilled water prior to photography under ultra-violet light from a transilluminator (Ultraviolet Products Inc.). Gels were photographed using a Polaroid CUV5 Land camera fitted with an orange filter (Wratten 513). Later in the project photographs were taken with a Polaroid MP-4 Land camera.

2.6 Restriction Enzyme Digests

2.6.1 Single digests

Restriction digests were performed using 0.2-1ug of DNA (approximately 4-18ul of DNA prepared using the mini-prep method). Distilled water was added to give a final volume of 18ul and an appropriate buffer (2ul of 1x10 concentration) was added. Approximately 5 units of restriction enzyme was added and incubation was at 37°C for a minimum of two hours. The reaction was stopped either by storing at -20°C or by the addition of 5ul loading dye if agarose gel electrophoresis was to be performed immediately.

2.6.2 Double digests

Double digests were performed in Universal buffer when possible. If this was not possible, digestion with one enzyme was followed by dilution to 100ul in TE buffer followed by phenol/chloroform extraction and ethanol precipitation of DNA as described earlier. The

DNA pellet was redissolved in 18ul of sterile distilled water and 2ul of the appropriate restriction enzyme buffer added. The second enzyme was added and the reaction mix incubated for 2 hours at 37°C. The sample was then analyzed by agarose gel electrophoresis.

2.6.3 Partial digests

The restriction enzyme Sau3A recognises a four base sequence cleaving DNA on average every 256bp. By altering the incubation conditions for Sau3A, it was possible to obtain incompletely digested DNA with a desired average size for cloning.

Partial digestion of pR5 and pXS1 by Sau3A was performed to obtain aminoglycoside resistance genes which were to be cloned into Tn5. Plasmid DNA (~10ug) was diluted in 1x Universal Reaction Buffer (99.5ul), incubated in a water bath at 37°C for approximately 30 seconds and restriction enzyme Sau3A (4 units) added and mixed. Reaction mixture (20ul) was removed at 30 second intervals and added to an Eppendorf tube containing EDTA (0.5 mM in 1ul) which stopped further enzymic activity. Samples of DNA from each reaction time interval was analysed by electrophoresis and those showing DNA fragments with an average size of 2Kbp were pooled. The partially digested DNA was purified by phenol/chloroform extraction and ethanol precipitation as described in 2.4.1 and resuspended in 40ul TE buffer. This DNA sample was used as the source of the appropriate aminoglycoside resistance genes in ligation reactions with BglII-digested Tn5.

2.7 Ligation of DNA

Vector and insert DNA were prepared by digestion with relevant restriction enzymes as described above to give compatible cohesive ends. After phenol-chloroform extraction and ethanol purification the DNA was resuspended in sterile distilled water in an approximate 1:5 molar ratio. T4 DNA Ligase was used to covalently link insert and vector DNA.

Reaction mixture:

2ul 10x Ligation buffer
2ul 10mM ATP
xul vector DNA (20-50ng) cut with a suitable restriction enzyme
yul insert DNA (20-100ng) cut with a suitable restriction enzyme
sterile distilled water to a final volume of 20ul
0.5ul T4 DNA ligase (0.7 units)

Ligation reactions were performed for 16 hours at 9°C. The ligation mix was then used to transform competent bacterial cells.

Alternatively, Ligaid™ (P&S Biochemicals Ltd.) was used. Here, vector and insert DNA, cut with an appropriate restriction enzyme, were added to the same volume of Ligaid™ (final incubation volume 20-50ul) with incubation at 37°C for 2 hours. Transformation of competent *E. coli* with the ligate mixture was performed as described above.

2.8 DNA-DNA Hybridization

Preparation of buffers and solutions is described in Appendices I and III.

2.8.1 Isolation of specific DNA fragments for use as probes

Caesium chloride-purified pRTG1 DNA (15ug) was digested to completion with appropriate restriction enzymes (approximately 20 units) as described in Section 2.6. Fragments of pRTG1 DNA were obtained by excising separate DNA bands from a low melting point agarose gel (Seaplaque, Marine Colloids Inc. or Type VII, Sigma). DNA was visualized by UV light and the gel region containing the desired DNA fragment removed using a scalpel blade.

2.8.1.1 NACS column method

A gel segment containing the desired band was placed in an Eppendorf tube and heated at 70°C in a water bath until the agarose became molten. The volume of agarose was determined and four volumes of pre-warmed (40°C) Equilibration buffer added. After incubation for another 10 minutes the solution was transferred to a 40°C water bath for a further 10 minutes incubation. The sample was loaded onto a PREPAC NACS column which had been equilibrated following manufacturers recommendations. DNA samples flowed through the column under gravity. The column was washed with prewarmed (40°C) Equilibration buffer (5ml) to remove agarose and other impurities. DNA was eluted by adding Elution buffer (3x100ul) and eluate (300ul) was collected to which ice-cold absolute ethanol (0.6ml) was added. The sample was kept at -70°C for 30 minutes prior to centrifugation at 13000xg for 10 minutes. The supernatant was removed and the DNA pellet was washed in 75% ethanol and repelleted by centrifugation before being dissolved in

10ul TE buffer.

2.8.2 Generation of radio-labelled DNA probes

DNA Polymerase 1 was used to introduce ^{32}P-labelled dATP into double-stranded DNA from points at which DNase 1 had previously created nicks. Nick-translation was performed using a kit supplied by BRL. DNase 1/DNA Polymerase 1 (1ul) was added to a mixture of 1ul DNA (100-200ng DNA), 1ul nick translation buffer (x10 concentrated) containing dCTP, dGTP and dTTP and 7ul sterile distilled water. Finally 1ul of ^{32}P-dATP (specific activity: 3000 Ci/mmol, Amersham) was added and mixed. Stock ^{32}P-dATP was stored at -20^{o}C and thawed at room temperature in a lead pot. Normal precautions were taken when dealing with radioactive substances. The reaction mixture was incubated for 1-2 hours at 15^{o}C and stopped by dilution with 90ul TE buffer. Unincorporated dNTPs were removed by chromatography through a Sephadex-G50 column in TE buffer.

2.8.3 G-50 column chromatography under gravity

Sephadex G-50:medium (30g) was pre-equilibrated by adding 250ml TE buffer (pH8.0) and stirred for at least 18 hours. The TE buffer was then removed and replaced by 250ml of fresh TE buffer and the slurry stored at room temperature in a sealed conical flask.

A finely drawn-out Pasteur pipette was plugged with glass wool and packed with pre-equilibrated Sephadex G-50 slurry. The column was washed with TE buffer (5ml) and when the TE buffer meniscus was at the top of the sephadex bed, nick-translation reaction mix was loaded. Samples (100ul) were collected in Eppendorf tubes. When no more eluate was obtained the column was washed with consecutive 100ul

volumes of TE buffer. Each 100ul of eluate was monitored for radio-activity using a hand-held ^{32}P mini-monitor samples were chosen by their radioactivity as shown by the monitor readings. Labelled DNA was usually collected from four samples (5-9) whilst free ^{32}P-dATP was observed as an increased activity usually at the eleventh eluate sample. Samples containing bound radioactive probe were pooled and stored in lead pots at -20°C for upto three weeks.

2.8.4 Southern transfer

DNA samples for hybridization analysis were subjected to agarose gel electrophoresis in TBE buffer as described previously. After electrophoresis, staining with ethidium bromide and photography, the gel was soaked in 0.25M HCl for 15 minutes with gentle agitation at room temperature. The gel was then placed in a shallow tray filled with denaturation buffer which was agitated gently. After 30 minutes the buffer was replaced with fresh denaturation buffer and the gel incubated for a further 30 minutes. The buffer was replaced with neutralization buffer and incubated for a further 1-2 hours in neutralization buffer with a single change of buffer usually after an hour.

Capillary transfer of DNA to nylon membrane (Hybond-N, Amersham) was by a modification of the method of Southern (1975). The blotting platform consists of a glass plate with larger dimensions than the gel standing elevated in a shallow tray. Wicks were two pieces of Whatman 3MM paper cut to a size approximately 5cm greater than the glass plate along each side. The wicks were soaked in 20xSSC solution and placed separately on the glass plate. After each addition of paper to the glass plate a pipette was rolled over the surface to remove any air

bubbles that might have been trapped between the glass plate and the paper. The tray was then half-filled with 20xSSC solution ensuring that the buffer touched the paper wicks but did not flood the plate. The gel was placed upside down upon the paper and air bubbles removed by rolling a pipette across the gel surface. Nylon membrane was cut to the size of the gel. The nylon was completely wetted by flo tation on the surface of distilled water followed by immersion for 2 minutes. The nylon was then soaked in 2xSSC solution for 5-10 minutes and placed on the gel. Air bubbles were removed and the gel was surrounded by 5cm strips of parafilm to prevent buffer bypassing the gel. Two pieces of Whatman 3MM paper cut to the exact size of the nylon and presoaked in 2xSSC were placed on the membrane and air bubbles removed. A thick wad (5cm) of absorbent tissue was placed on the Whatman paper and care was taken to prevent buffer from soaking into the tissue from the tank directly but only via the gel. The system covered in cling film to prevent excessive evaporation of 20xSSC from the tray. A glass plate and a 1.5 kg weight were placed above the tissue. The blotting system was then left for approximately 16 hours to permit DNA transfer. After DNA transfer had occurred the system was dismantled to the nylon membrane. The orientation of the gel wells was marked on the membrane with a pencil and the membrane placed between two pieces of dry Whatman 3MM paper and incubated at 37^{o}C for 15 minutes prior to incubation at 80^{o}C for 2 hours. An An alternative method used to bind DNA to the nylon membrane was to place the nylon membrane in Saran wrap and place "DNA side" face down on the transilluminator (UV Products Inc.). The transilluminator was switched on for 3-5 minutes. Membranes were then stored damp in Saran wrap within a sealed plastic bag at room temperature.

2.8.5 Hybridization with radiolabelled probe DNA

The membrane was "prehybridized" in 1mM EDTA. The EDTA solution was brought to the boil in a shallow enamel dish. The membrane was floated on the surface and the solution allowed to simmer for a further 5 minutes, after which the membrane was placed in a plastic bag and used immediately for hybridization. This process replaces the traditional overnight pre-hybridization technique (L.A. King, personal communication).

The hybridization solution consisted of two solutions; a non-boil and a boil solution. The boil solution was mixed in a disposable polypropylene tube (Falcon) and placed in a boiling water bath for 5 minutes to denature DNA. After this incubation the tube was immediately transferred to ice and gently swirled for 3 minutes. The non-boil solution was then transferred to this tube and mixed.

The hybridization solution was added to the plastic bag containing the prehybridized membrane. The bag was then carefully heat-sealed and placed within another bag which was also heat-sealed. The bags were then placed in a "Tupperware" sandwich box and incubated at 37^{o}C in an orbital shaker for 16 hours.

After hybridization the membrane was washed to remove unbound probe DNA. Bags were cut and the hybridization solution disposed of down a liquid radioactive waste sink. The membrane was rinsed in 2xSSC, 0.1% SDS which had been preheated to 65^{o}C. This solution was then disposed of down the waste sink. The membrane was transferred to a sandwich box and washed three times in 2xSSC, 0.1% SDS (3x30 minutes), and finally in 0.1SSC, 0.1%SDS at 37^{o}C (1x30 minutes). The wash solutions

were preheated to 65°C prior to their addition and were discarded as radioactive waste after use. After washing, the membrane was wrapped in Saran wrap and exposed to X-ray film for radiography.

2.8.6 Autoradiography

Nylon membranes wrapped in Saran wrap were exposed to X-ray film (Kodak X-omat S) in an X-ray cassette with an intensifying screen (GRI). The cassette was stored at -70°C for 2-48 hours depending on the radioactivity associated with the membrane. The cassette was left on the bench to come to room temperature. Film was taken from the cassette (in the dark room with the safe lamp on) and immersed in developer and agitated gently for 3-5 minutes or until bands were visible. The film was rinsed in 2% glacial acid before immersion in fixer for 2-3 minutes, followed by rinsing in running tap water before air drying.

As membranes were often re-hybridized they were kept damp at all times and after autoradiography the ^{32}P-labelled DNA probe was removed by boiling in 1mM EDTA.

CHAPTER 3

ANTIBIOTIC-RESISTANCE ANALYSES AND THE INTRODUCTION OF TRANSPOSON Tn5 INTO *Pseudomonas aeruginosa*

Objectives: To determine the Minimal Inhibitory Concentrations (MICs) of various antibiotics toward *P. aeruginosa* in order to establish conditions for transfer and selection of Transposon Tn5 in *P. aeruginosa*.

3.1 Introduction

Detection of B-lactamase hyperproducing mutants of *P. aeruginosa* requires the use of a B-lactam antibiotic capable of selecting for such mutants within a wild-type population. Carbenicillin is a widely prescribed anti-pseudomonal B-lactam (Slack 1981) and mutants with increased B-lactamase activity have been isolated from clinical strains of *P. aeruginosa* after treatment with carbenicillin (Williams *et al.* 1984) and so may be considered suitable for this purpose. Carbenicillin, however, is a very poor substrate for the chromosomal B-lactamase and other resistance mechanisms, for example mutations which decrease permeability, often occur (Livermore and Pitt 1986, see Chapter 1). Identification of hyperproducing B-lactamase mutants may be hindered if their selection is dependent upon using carbenicillin because a significant number of mutations unrelated to changes in B-lactamase expression may arise. Hyperproduction of B-lactamase as a means of resistance is often associated with *P. aeruginosa* isolates from patients treated with third-generation B-lactams, for example cefsulodin and ceftazidime (Sanders 1983). In order to investigate the frequency of resistance to carbenicillin and cefsulodin, MICs were performed to determine intrinsic resistance levels to these

antibiotics.

A problem related to the intrinsic resistance of P. aeruginosa was the identification of suitable antibiotic-resistance genes for use as markers encoded by the transposon and transposon delivery vector. Transposon Tn5 (Figure 1.5a), encodes kanamycin-resistance and has been used to generate insertional mutations in P. aeruginosa (Krishnapillai et al. 1986). However, spontaneous kanamycin-resistant mutants are frequently obtained in P. aeruginosa (Bryan et al. 1980) hindering the investigation of bona fide transposon insertional mutants. As a result, other Tn5-derivatives have been constructed specifically for use in P. aeruginosa such as Tn5-751, which encodes trimethoprim and kanamycin resistance (Rella et al. 1985). As Tn5 was to be eventually introduced into clinical isolates of P. aeruginosa, many of which contain a chromosomally-encoded kanamycin-resistance AGPT gene (Okii et al. 1983), it was considered that other antibiotic-resistance genes would be more useful as markers. As the objective of the work was to obtain mutants resistant to B-lactams as a result of transposon mutagenesis, genes encoding resistance to B-lactams could not be used as markers of the transposon or vector. Aminoglycosides other than kanamycin have been used effectively in the treatment of P. aeruginosa infections supporting the possibility that genes conferring resistance to these antibiotics would be useful as selection markers for the transposon and vector. Tobramycin is the most potent aminoglycoside against P. aeruginosa in vitro (Meyers and Hirschman 1972) and gentamicin is another aminoglycoside often used to treat P. aeruginosa infections (Rubenstein and Lev 1988). Resistance to either antibiotic appears to be a useful selection marker. MICs were determined for gentamicin and tobramycin.

In order to generate transposon insertions in the chromosome of P. aeruginosa it was necessary to transfer a plasmid carrying the transposon into P. aeruginosa. Plasmid pSUP2011 (Figure 3.1) is conjugally transmissible into P. aeruginosa because it carries the mob+ site from the broad host-range incP plasmid, RP4 (Simon et al. 1983). Transfer occurs in the presence of incP tra+ genes in the donor strain, for example E. coli SM10 in which the tra+ genes of the incP plasmid RP4 are integrated in its chromosome (Simon et al. 1983). Regulation of Tn5 transposition dictates that transposition events are most likely to occur immediately upon introduction into the cell due to zygotic induction (see Chapter 1). Selection for transposon insertion into the host chromosome, as opposed to selection for the plasmid, requires loss of plasmid by preventing plasmid replication. Plasmid pSUP2011 has a narrow host-range and does not replicate in P. aeruginosa (Simon et al. 1983). Transposition of a Tn5-derivative (for example, carrying the tobramycin-resistance gene) from the plasmid into the host cell chromosome would be marked by retention of tobramycin-resistance and absence of plasmid DNA and plasmid-encoded resistances.

A laboratory strain of P. aeruginosa, PAT210, was used as a model in which initial transposition assays were to be performed prior to use of Tn5 or a Tn5-derivative in clinical strains of P. aeruginosa. MIC analyses were performed on both M9 glucose minimal and nutrient agar since Tn5 would be introduced by conjugation with selection on M9 glucose minimal medium or by transformation with selection on nutrient agar.

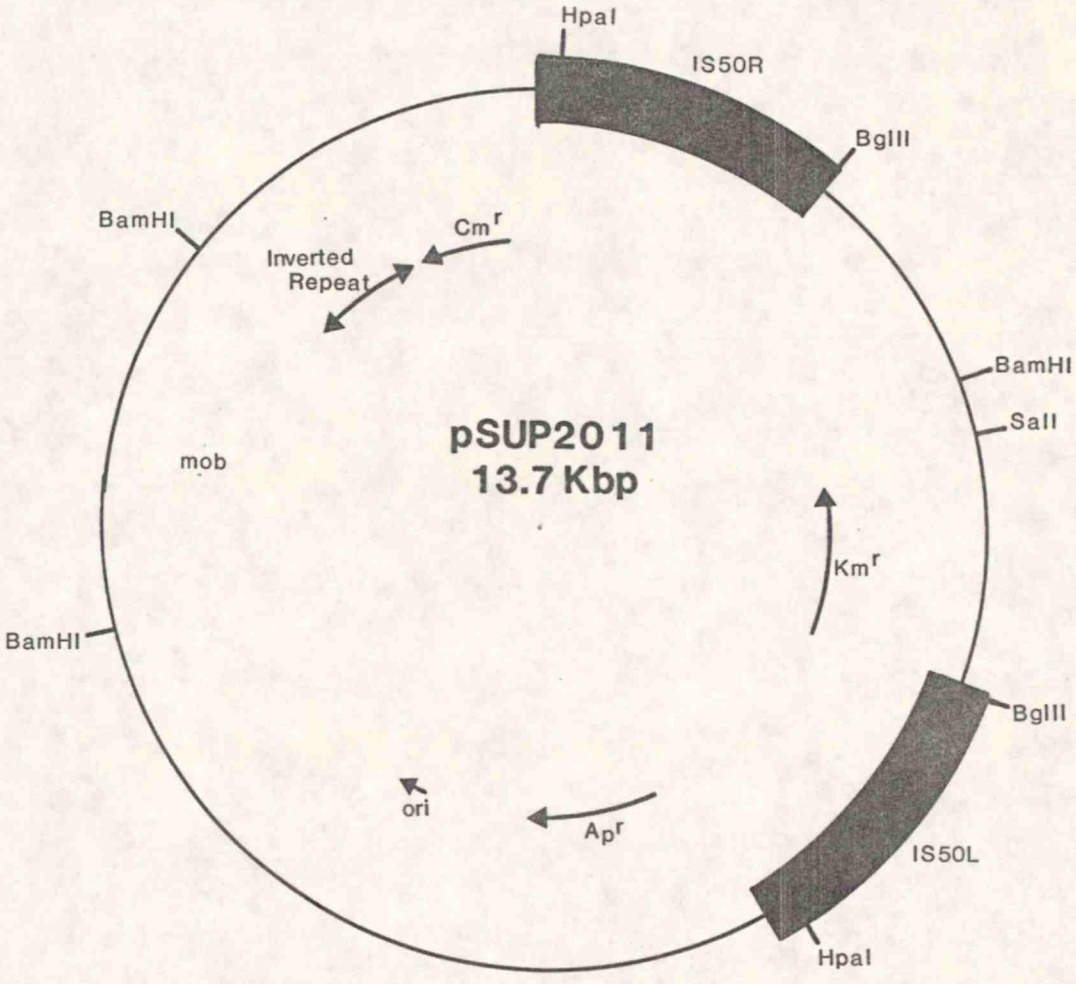


Figure 3.1: Restriction Enzyme Cleavage Map of pSUP2011. The plasmid was constructed by cloning the mob site required for conjugative transfer of the plasmid into the BamH! restriction enzyme site of pBR325 (Simon et al. 1983).

3.2 Results

3.2.1 MIC determinations in *P. aeruginosa*

Using a rapid analysis technique described in Methods, MIC analyses of two anti-pseudomomal B-lactam antibiotics, carbenicillin and cefsulodin, were performed to identify a suitable antibiotic for selecting mutants resistant to B-lactams. The results, shown in Table 3.1, demonstrated that cefsulodin was a more potent B-lactam than carbenicillin on both minimal and complex media.

MICs of kanamycin, tobramycin, gentamicin and chloramphenicol are also shown in Table 3.1. An example of the primary data obtained is given in Table 3.2. Both tobramycin and gentamicin were effective antibiotics against *P. aeruginosa* PAT210 thus genes conferring resistance to these antibiotics were considered as potential markers for modifying Tn5 for use in *P. aeruginosa* PAT210. The MIC of kanamycin, however, was low with respect to those found in the literature (Bryan 1979). It was considered that selection on kanamycin would probably select for wild-type Tn5 insertions in *P. aeruginosa* PAT210 due to the relatively low basal kanamycin-sensitivity of the strain. To test whether Tn5 was a suitable transposon for mutagenesis in *P. aeruginosa*, transfer of wild-type Tn5 by conjugation from *E. coli* into *P. aeruginosa* PAT210 was attempted.

TABLE 3.1 **Minimum Inhibitory Concentration Determination in *P. aeruginosa* PAT210 Using a Rapid Technique**

Antibiotic	MIC (ug/ml) M9 Glucose Minimal Agar	Nutrient Agar
Carbenicillin ($5x10^3$ cfu)	50	75
Cefsulodin ($5x10^4$ cfu)	2	8
Chloramphenicol ($5x10^4$ cfu)	320	320
Gentamicin ($5x10^4$ cfu)	4	2
Kanamycin ($5x10^5$ cfu)	40	20
Tobramycin ($1x10^7$ cfu)	1	1

TABLE 3.2 **An Example of Data Obtained in Determining the MIC of Gentamicin within *P. aeruginosa* PAT210**

	M9 Glucose minimal agar Gentamicin ug/ml					Nutrient agar Gentamicin ug/ml			
	0	1	2	4	8	0	1	2	4
Colony 1	C[a]	C	0	0	0	C	0	0	0
Colony 2	C	C	19[b]	0	0	C	0	0	0
Colony 3	C	C	4	0	0	C	7	0	0
Colony 4	C	C	3	0	0	C	1	0	0
PAT216[c]	C	C	15	2	0	C	0	1	0

a - C is confluent growth.
b - Number of colonies per 50ul drop (appoximate viability $5x10^4$ cfu/50ul).
c - Control strain, *P. aeruginosa* PAT2 (NCTC10662; PAT216))

3.2.2 Native Tn5 could not be selected in P. aeruginosa PAT210

In preliminary filter-mating experiments (see Methods), transfer of Tn5 with pSUP2011 (Figure 3.1) as vector (Simon et al. 1983) from E. coli into P. aeruginosa PAT210 was attempted. Two different donors were used for the transfer of pSUP2011; E. coli SM10 (pSUP2011) and E. coli HB101 (pR751, pSUP2011). E. coli HB101 (pR751, pSUP2011) acts similarly to E. coli SM10 but with tra+ genes located on the autonomous incP plasmid pR751.

After matings between either E. coli strain and P. aeruginosa PAT210 at 37°C for four hours selection was made for transconjugants on M9 glucose minimal agar containing kanamycin at 80ug/ml (2xMIC), 200ug/ml (5xMIC) and 320ug/ml (8xMIC). P. aeruginosa PAT210 grew to confluence on control plates irrespective of kanamycin concentration; supporting the previously held view that kanamycin was unsuitable as a selectable marker in P. aeruginosa PAT210 (Rella et al. 1985). An alternative antibiotic-resistance marker gene for the transposon was therefore sought.

3.2.3 Construction of Transposon Tn5-derivatives

MIC analyses demonstrated that tobramycin and gentamicin were potent antibiotics against P. aeruginosa PAT210. Resistance genes to both antibiotics have been expressed in P. aeruginosa (Smith et al. 1975, M.J. Woodward personal communication) and were considered suitable as markers of Tn5 in P. aeruginosa.

3.2.3.1 Construction of tobramycin-resistant Tn5 transposons

Plasmid pSUP2011 was used as the source of Tn5. Plasmid pR5 (a gift from Dr. P. Shannon, The London Hospital) was used as a source of the tobramycin-resistance gene.

DNA may be cloned into the BglII sites of Tn5 (Figure 1.5a) without inhibiting the transposition process (Berg and Berg 1983). Restriction enzymes Sau3A and BglII generate compatible cohesive ends and so a fragment encoding the tobramycin-resistance gene could be cloned into the BglII sites of Tn5. Plasmid pR5 was partially digested with Sau3A as described in Methods. BglII-digested pSUP2011 DNA was ligated with partial Sau3A-digested pR5 DNA and the ligation mixture used to transform competent E. coli 5K. Transformants were selected on L-agar containing tobramycin (10ug/ml) to select for recombinants encoding a tobramycin-resistance gene. Only two putative recombinant colonies grew. Both colonies, (designated TR1 and TR2), grew well on L-agar plates containing ampicillin (50ug/ml) and tobramycin (10ug/ml) indicating retention of both the tobramycin-resistance gene and the ampicillin-resistance marker gene of pSUP2011. Kanamycin-resistance was stably retained in TR1 but lost in TR2 indicating the loss of the NPTII gene of Tn5 in the latter.

To confirm that tobramycin-resistance was plasmid-encoded competent E. coli HB101 was transformed using plasmid extracted from the two tobramycin-resistant colonies. Transformants were not obtained when selecting on L-agar containing tobramycin (10ug/ml). However, transformants were obtained with DNA from TR1, at a frequency of $\sim 3 \times 10^{-4}$ with selection on L-agar containing kanamycin (25ug/ml) and L-agar containing ampicillin (50ug/ml).

Both the original transformants were also found to be unstable with resistance to tobramycin lost after sub-culturing. Transformants with stable resistance to both tobramycin and ampicillin were not obtained upon repeating the cloning experiment, indicating a problem in the method used to clone the tobramycin-resistance gene or problems relating to stable expression of tobramycin-resistance.

3.2.3.2 Construction of gentamicin-resistant Tn5 transposons

To clone the gentamicin-resistance gene into Tn5, the source plasmid pXS1 (Hirsch *et al.* 1986, see Figure 3.2), was partially digested with Sau3A. Digestion products were ligated with BglII-digested pSUP2011. The ligation mixture was used to transform competent *E. coli* 5K and transformants were selected by plating onto L-agar containing ampicillin (50ug/ml) selecting for the vector, and gentamicin (10ug/ml) selecting for the inserted gentamicin-resistance gene.

Eight ampicillin-gentamicin resistant, kanamycin-sensitive transformants were analysed. Recombinant plasmids were digested with HpaI to determine the size of the insert in each case (Figure 3.3a). Two such transformants contained plasmids with insert sizes of 1.5 and 3.3Kbp and were designated pSG1 and pSG2 respectively. Repeated streaking of transformants on selection agar plates showed these to retain resistance to both ampicillin and gentamicin. The restriction enzyme BamHI cut pSG1 and pSG2 in two sites present in pSUP2011, indicating that the insert did not extend beyond that of the pXS1 insert (Figure 3.3b). Transposons containing the gentamicin-resistance gene were designated Tn5::gen1 and Tn5::gen2.

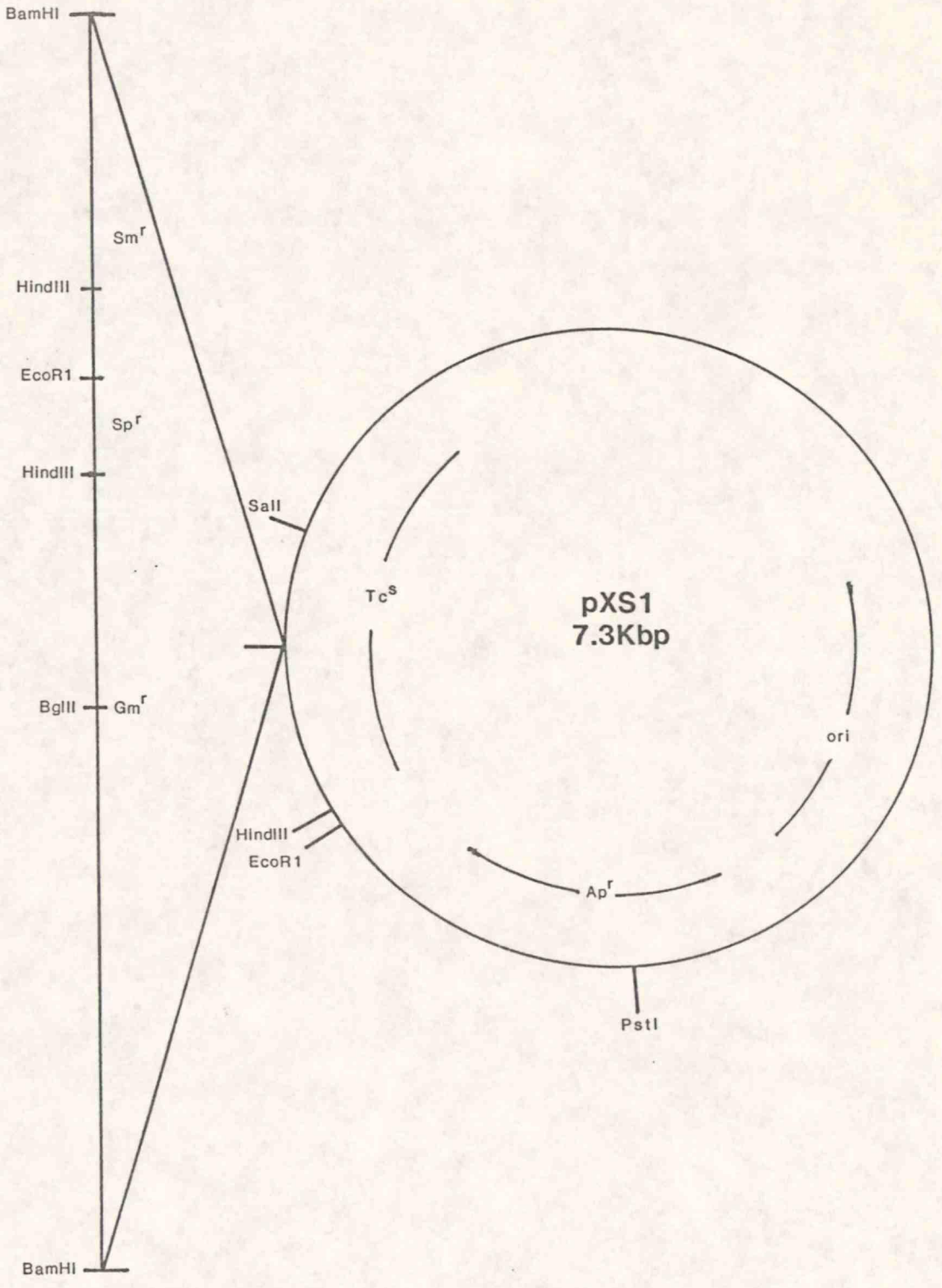


Figure 3.2: Restriction Enzyme Cleavage Map of pXS1. The gentamicin-resistance determinant lies within a 3.7Kbp BamH1 insert originally isolated from pR1033 and cloned into pAT153 (Hirsch et al. 1986).

Figure 3.3a: HpaI-digests of Plasmid DNA from Eight Transformants of E. coli 5K. E. coli 5K was transformed with pSUP2011 digested with BglII ligated with pXS1 partially digested with Sau3A. Lanes A-C - transformants 1-3 respectively; Lane D - lambda HindIII; Lane E - blank; Lanes F-J - transformants 4-8 respectively.

Transformants	Size of HpaI fragments (Kbp):	Insert size (Kbp):*	
1, 3, 6 and 8 -	8.2, 12.7	10.0	
2	8.2, 6.0,	8.1	
4	8.2, 6.5, 3.0	6.8	
5	8.2, 6.0	3.3	- **pSG2**
7	8.2, 4.2	1.5	- **pSG1**

* - Vector fragment (pSUP2011) was predicted as being 8.3Kbp (13.6-5.3), a similar sized fragment was observed in each transformant (8.2Kbp). The insert size was calculated by subtracting 2.7Kbp (size of IS50 HpaI-BglII, data from Auerswald et al. 1980) from the remaining fragment sizes. Fragments of incomplete digests are not shown in the above table.

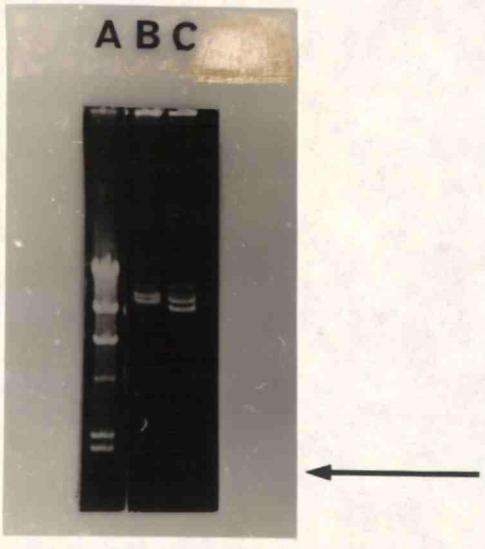


Figure 3.3b: BamH1-digests of pSG1 and pSG2 from E. coli 5K. Lane A - lambda HindIII; Lane B - pSG2 BamH1 (11.89, 1.86Kbp); Lane C - pSG1 BamH1 (10.12, 1.86Kbp). The smaller fragment (arrowed) is the mob insert of pSUP2011 (1.9Kbp, Simon et al. 1983). Fragment sizes of incomplete digests are not shown.

3.2.4. Assay of Tn5::gen transposition by transfer of pSG1 into *E. coli* UB2272 *polA* and *P. aeruginosa* PAT210

3.2.4.1 Conjugation

Conjugation was used to introduce pSG1 and pSG2 into *P. aeruginosa* PAT210. The *incP* plasmid, pR751, which encodes *tra*+ genes was mated from *E. coli* J62 (pR751) into *E. coli* 5K (pSG1) and 5K (pSG2). As a positive control for the transfer of the same gentamicin-resistance gene, the broad host-range plasmid pVW6 was mated from *E. coli* 5K (pVW6, pR751) into *P. aeruginosa* PAT210. *E. coli* 5K (pR751) containing pSG1, pSG2 or pVW6 was filter-mated in separate experiments with *P. aeruginosa* PAT210. Selection for gentamicin-resistant *P. aeruginosa* PAT210 was on glucose minimal medium containing gentamicin at 15ug/ml (4xMIC) and also at 30ug/ml (8xMIC). *E. coli* JC5466 was used as a recipient control selecting on glucose minimal medium containing thiamine, tryptophan, histidine, lysine and gentamicin at 15ug/ml.

Transfer frequencies are shown in Table 3.3 (lines 1 and 2). The broad host-range plasmid pVW6 transferred into *P. aeruginosa* PAT210 at a frequency of 2.0×10^{-4} at both gentamicin concentrations used. Transfer of pSG1 and pSG2 was not detected as similar numbers of spontaneous gentamicin-resistant mutations of *P. aeruginosa* PAT210 occurred on control plates as were found on selection plates. Plasmid transfer frequencies into *E. coli* JC5466 were 3.6×10^{-4}, 3.0×10^{-4} and 1.5×10^{-5} for pVW6, pSG1 and pSG2 respectively indicating transfer proficiency of both pSG plasmids. The inability to identify Tn5::gen in *P. aeruginosa* PAT210 may have been due to a low frequency of plasmid transfer into the recipient, a low frequency of transposition

of Tn5::gen from the vector into the recipient chromosome or an inability of a single copy of Tn5::gen to confer resistance to gentamicin at the concentration used for selection.

Plasmid pSG1 was introduced into E. coli UB2272 polA to test whether Tn5::gen was capable of transposition. E. coli 5K (pSG1, pR751) and E. coli 5K (pSG2, pR751) were mated with E. coli UB2272 with selection on glucose minimal medium containing tryptophan, histidine and lysine with gentamicin at 10ug/ml. E. coli UB2272 polA is deficient in DNA Polymerase I and ColE1-based plasmids such as pSG1 and pSG2 which require DNA Polymerase I in the early stages of replication, should not replicate in this host (Foster 1984). Retention of gentamicin-resistance should indicate the presence of Tn5::gen in the host chromosome. The transfer frequency was low in all cases ($<4x10^{-8}$, see Table 3.3, line 3).

TABLE 3.3 Frequencies of Transfer of Gentamicin-resistant Plasmids into P. aeruginosa PAT210, E. coli UB2272 and E. coli JC5466

Recipient Strain		Transferred Plasmid		
		pVW6	pSG1	pSG2
1	PAT210	$2.0x10^{-4}$	$<2x10^{-9}$	$<2X10^{-9}$
2	JC5466	$3.6x10^{-4}$	$3.0x10^{-4}$	$1.5x10^{-5}$
3	UB2272	$4.0x10^{-8}$	$4.0x10^{-8}$	$2.0x10^{-8}$

Gentamicin-resistant, ampicillin-sensitive transconjugants were not detected indicating that all were resistant to ampicillin (a vector trait). It would seem likely that selection on gentamicin identified polA revertants of E. coli UB2272 polA containing pSG plasmids, however, this was not tested by plasmid extractions.

Transposition of Tn5::gen from pSG plasmids into the chromosome of E. coli UB2272 polA did not occur at a detectable frequency as shown by the absence of gentamicin-resistant, ampicillin-sensitive strains. Assuming that the transfer frequencies of pSG plasmids were the same into E. coli UB2272 and JC5466 and that transposition into the chromosome occurs in 1% of all transconjugants I would have expected to obtain gentamicin-resistance at a frequency of $4x10^{-6}$ in E. coli UB2272. It seemed likely that pSG plasmids were able to transfer into non-permissive hosts but that transposition of Tn5::gen was infrequent. Poor transposition-proficiency may be as a result of either inserting DNA into the BglII-cloning site of Tn5 or because of the polA mutation decreasing the transposition frequency of Tn5::gen (Syvanen et al. 1982, Yin and Reznikoff 1987). Furthermore, the inability to detect transposition would prevent identification of Tn5::gen-mediated B-lactamase hyperproducing mutants in P. aeruginosa. A method was required to test for Tn5::gen transposition and also to increase the likelihood of obtaining transposition in P. aeruginosa. If the frequency of plasmid transfer was increased, an increased frequency of transposition may result within the population of putative recipient cells. The number of gentamicin-resistant colonies might thus increase to a level significantly higher than the spontaneous mutation rate.

3.2.4.2 Transformation

To test whether vectors carrying Tn5::gen could be introduced into P. aeruginosa PAT210 and E. coli UB2272 polA by transformation with subsequent transposition of Tn5::gen into the chromosome, P. aeruginosa PAT210 and E. coli UB2272 polA were made competent (see Methods). These strains were transformed using saturating concentrations of pSG1, pSG2 and pVW6 DNA. E. coli HB101 was used as a positive control strain. Selection was on Nutrient agar containing gentamicin (30ug/ml) for P. aeruginosa and on L-agar containing gentamicin (10ug/ml) for E. coli UB2272 polA and E. coli HB101.

No growth was observed on parental control plates. E. coli HB101 was transformed equally efficiently at a frequency of $>1x10^{-6}$ with all plasmids. P. aeruginosa PAT210 was transformed with pVW6 at a low frequency of $\sim1x10^{-8}$, and with pSG1 and pSG2 at $\sim4.2x10^{-9}$. No transformants of E. coli UB2272 were isolated with saturating concentrations of DNA indicating that the cells were not competent.

The presence of plasmid DNA was tested for in four gentamicin-resistant transformants of P. aeruginosa PAT210. Plasmid DNA was only observed in P. aeruginosa (pVW6) (Figure 3.4). Other transformants contained DNA but this was also observed in the parental control. The absence of plasmid DNA and the retention of gentamicin-resistance in DNA extracts of transformants indicates that transposition of Tn5::gen1 and Tn5::gen2 into the chromosome may have occurred. However, the frequency with which transformants were obtained suggested it unlikely that any mutants hyperproducing B-lactamase would be isolated by this method.

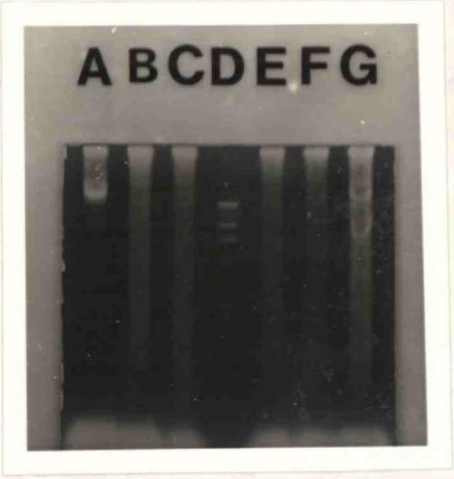


Figure 3.4: Plasmid Extracted from Gentamicin-resistant P. aeruginosa PAT210. Lane A - P. aeruginosa PAT210 parent; Lane B - pSG2 Isolate 1; Lane C - pSG2 Isolate 2; Lane D - Lambda HindIII; Lane E - pSG1 Isolate 1; Lane F - pSG1 Isolate 2; Lane G - pVW6. Plasmid DNA was only observed in Lane G.

To test whether reducing the concentration of gentamicin would increase the number of Tn5::gen transformants in P. aeruginosa PAT210 the experiment was repeated with selection on N-agar containing gentamicin (25 ug/ml). Similar frequencies of resistant colonies were obtained for the parental control as with either pSG plasmid. The frequency of transformation of pVW6 remained approximately $1x10^{-8}$.

3.3 Discussion

MIC analyses were required to determine the basal levels of resistance that P. aeruginosa had to two antipseudomonal B-lactams and to other antibiotics which might be of use as transposon markers. P. aeruginosa PAT210 was relatively resistant to carbenicillin. Carbenicillin is a poor substrate of the chromosomal class C B-lactamase and so selection at 300ug/ml (8xMIC) might be too high to select for mutants hyperproducing B-lactamase. Third-generation B-lactams such as cefsulodin are better substrates for class C B-lactamases and selection at 16ug/ml (8xMIC) should be capable of selecting for hyperproducing mutants (Towner and Massam 1985, Hewinson et al. 1986). Cefsulodin was considered to be a better antibiotic for selecting B-lactamase hyperproducing mutants.

Variation of resistance obtained when tested on two growth media may be due to a number of factors. A slowly growing cell on minimal medium is less susceptible to growth-dependent antibiotics (Tuomanen et al. 1986). This would suggest that MIC values for the aminoglycosides would be higher on glucose minimal agar than on nutrient agar; which they were. It is interesting to note that whilst

this was true for the aminoglycosides, the opposite was observed for the B-lactams. The physical nature of the media (for example, pH cation concentration, osmolarity) can also affect antibiotic potency and the physiological state of the cells. For example, Garrod and Waterworth (1969) showed that varying the magnesium concentration of the medium could alter the gentamicin MIC by as much as 32-fold; with increasing magnesium levels causing reduced sensitivity to gentamicin.

The technique employed to determine MIC values in P. aeruginosa PAT210 allowed a number of independent inocula, including a control strain, to be placed on one plate, permitting comparisons of MICs to be made between independent colonies and also between different population densities from the same culture. A disadvantage, however, was that confluence in a 50ul drop resulted from a wide range of population densities. For example, a drop containing $1x10^5$ cfu could give rise to 50 resistant colonies and would be observed as confluent growth yet this resistance frequency (0.05% of the population) indicates antibiotic sensitivity. This may have caused elevated MIC results to be recorded. As a result, the method of determining MICs was altered to spread plating inocula of similar densities to those obtained in conjugation experiments (see Methods).

In filter matings, in which P. aeruginosa PAT210 populations were determined as $1x10^9$, it appeared that PAT210 was resistant to kanamycin at 320ug/ml even though the MIC, established using a smaller inocula of $1x10^4$ per spot, was 40ug/ml. Selection at much higher concentrations of kanamycin might have killed putative transconjugants and increased the likelihood of obtaining multiple Tn5 insertions in the chromosome. Wild-type Tn5 was therefore considered unsuitable for

insertion mutagenesis in P. aeruginosa PAT210 and an alternative resistance-marker was required.

From the data in Table 3.1 it can be seen that tobramycin and gentamicin were highly effective against P. aeruginosa PAT210. The next objective then, was to clone a tobramycin-resistance gene, or as an alternative a gentamicin-resistance gene, into Tn5 and to introduce them into P. aeruginosa.

The lack of success with cloning the tobramycin-resistance gene confirms studies with this same gene in other vectors studied by M.J. Woodward (personal communication). Possible explanations for this may include poor expression or genetic instability due to recombination. There may be a Sau3A restriction enzyme site near to the tobramycin-resistance gene and cleavage here affects the expression of the gene. If this were true it should be possible to obtain the tobramycin-resistance gene on a larger Sau3A fragment. However, gentamicin-resistance transposons were successfully constructed and transposition studies were performed using two such Tn5::gen transposons. Further analysis of the tobramycin-resistance gene and its´ expression, although intriguing, was not pursued further.

Plasmids pSG1 and pSG2 being pSUP2011-derivatives should enter P. aeruginosa from a tra+ donor. Assuming that the transfer frequencies of pSG1 and pSG2 into P. aeruginosa were similar to that of pVW6 then the number of transpositions into the chromosome of P. aeruginosa PAT210 was low. The inability to identify transposition events in E. coli UB2272 polA after transfer of pSG vectors supports the view that the transposition frequency is

extremely low either as a result of cloning into the BglII sites of Tn5 (observed by Rothstein *et al.* 1980) or *polA* deficiency of the host strain (Syvanen *et al.* 1982, Yin and Reznikoff 1987). In retrospect, a temperate bacteriophage, for example P1 could have been used to test for transposition ability in *E. coli*. However, even if Tn5::gen was proven to transpose from pSG1 or pSG2 in *E. coli* the transposition frequency was insufficient to identify transposition events in *P. aeruginosa*. The frequency of transposition would rise if the vector could be stably maintained in *P. aeruginosa* before selecting for cells containing chromosomally-located transposons. The advantage would be that Tn5::gen would have the ability to undergo a transposition event within the entire population. I therefore decided to test for transposition of Tn5::gen from pSG1 into a temperature-sensitive broad host-range plasmid capable of replication in *P. aeruginosa*, pMMB19 (Scholz *et al.* 1985).

CHAPTER 4

CONSTRUCTION OF A BROAD HOST-RANGE, TEMPERATURE-SENSITIVE VECTOR CARRYING TRANSPOSON Tn5::GENTAMICIN

Objectives: To test transposition proficiency of Tn5::gen and to construct and characterize a temperature-sensitive vector for Tn5::gen.

4.1 Introduction

Selection for the rare event of a B-lactamase control mutant generated by transposition of a Tn5-derivative (Tn5::gen) from a suicide-plasmid into the chromosome of P. aeruginosa was considered too problematic (Chapter 3). An alternative delivery system was to be devised. One possible transposon delivery system would be a vector that could replicate stably in P. aeruginosa but could be lost when required. As the vector can be stably maintained in P. aeruginosa only one successful conjugation or transformation experiment would be necessary for its introduction. This avoids the problem of low frequencies of introduction and the dependence upon zygotic induction for transposition of Tn5::gen from suicide-plasmids such as pSG1 into the chromosome of P. aeruginosa.

Temperature-sensitive plasmids replicate at permissive temperatures but not at non-permissive temperatures. At non-permissive temperatures, as the plasmid and transposon copy number decrease with consequent dilution of the transposase inhibitor protein, p2, transposition would be expected to increase due to derepression

(Isberg et al. 1982). Bacteria with Tn5::gen insertions in the chromosome would be identified after incubation at the non-permissive temperature by selecting for gentamicin-resistance. Loss of plasmid could be determined by loss of vector resistance markers, loss of a plasmid band after DNA extraction and Southern hybridization. The aims were; to test whether Tn5::gen was transposase-efficient whilst simultaneously constructing a temperature-sensitive vector carrrying Tn5::gen and to show that such a system functions in E. coli prior to its introduction into P. aeruginosa. The broad host-range incQ plasmid pRSF1010 (Guerry et al. 1974, see Figure 4.1) has a derivative, pMMB19, which encodes a temperature-sensitive mutation in repA, an essential gene for replication of pRSF1010-derivatives (Scholz et al. 1985).

E. coli UB2272 is a polA mutant, deficient in DNA polymerase I, in which replication of ColE-derived plasmids such as pSG1 is severely restricted (Foster 1984). To test for transposition of Tn5::gen, pSG1 and pSG2 were introduced into E. coli UB2272 containing pMMB19 as a target plasmid. Such a protocol should result in the construction of suitable vectors of Tn5::gen for studies in P. aeruginosa. On mating pSG1 into E. coli UB2272 (pMMB19), the transposon may transpose from the restricted vector and insert into pMMB19. Plasmids carrying the transposon would be identified by DNA restriction enzyme digests and by transfer of the resistance phenotypes by transformation of competent E. coli. The construct would then be characterized with respect to temperature-sensitive kinetics of the vector prior to its introduction into P. aeruginosa.

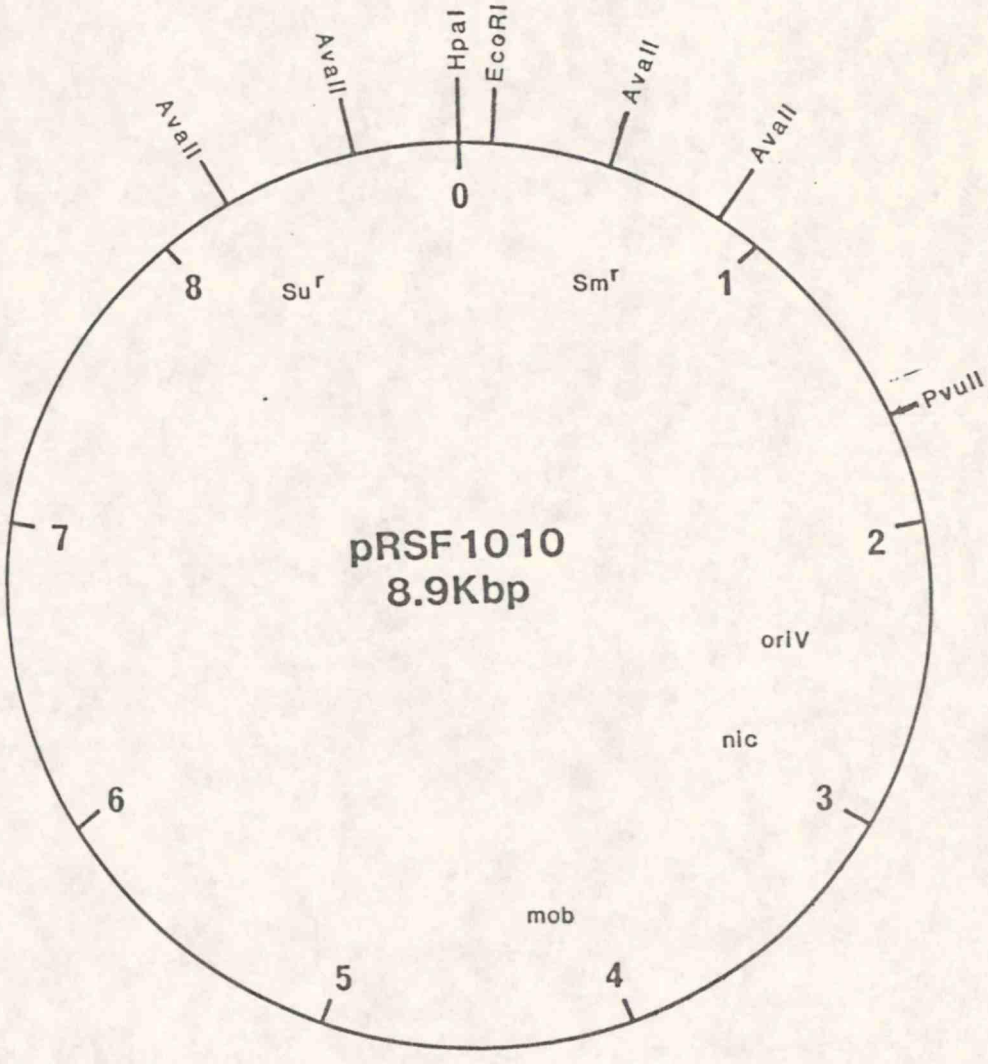


Figure 4.1: Restriction Enzyme Cleavage Map of pRSF1010. Figure based upon Figure 1, Bagdasarian et al. (1981).

4.2 Results

4.2.1 Construction of pMMB19::Tn5::gen

To test the temperature-sensitivity of pMMB19, *E. coli* C600 (pMMB19) was grown in L-broth at two different temperatures; 30°C and 42°C (permissive and non-permissive temperatures respectively). After initial growth overnight at 30°C in the presence of streptomycin (100ug/ml), *E. coli* C600 (pMMB19) (200ul) was used to inoculate two pre-warmed L-broths (20ml) lacking antibiotic selection. Cultures were grown for 28 hours at 30°C and 42°C. Samples were diluted (~100cfu/plate) and spread on L-agar and L-agar containing streptomycin (100ug/ml) and sulphonamide (200ug/ml). Plates were incubated at 30°C and results are shown in Table 4.1.

TABLE 4.1 Number of Resistant Colonies Counted of *E. coli* C600 Carrying a Temperature-sensitive Plasmid

		Number of colonies from time of inoculation (hours/minutes)			
		0	4	9.25	27.40
Plasmid	Medium				
pMMB19	L-agar	43	35	72	106
	L-agar Sm Su	38	19	6	0

Sm - streptomycin (100ug/ml), Su - sulphonamide (200ug/ml)

Plasmid pMMB19 was temperature-sensitive as observed by the loss of streptomycin and sulphonamide-resistance at the non-permissive temperature. Tn5::gen was to be transposed into the temperature-sensitive pMMB19. To transfer Tn5::gen from pSG1 into pMMB19, pSG1 was transferred from *E. coli* SM10 (pSG1) into *E. coli* UB2272 (pMMB19) at 30°C. Selection was made on glucose minimal media containing tryptophan, histidine, lysine and gentamicin (10ug/ml). The frequency of gentamicin-resistance transfer was $1x10^{-3}$. When replica-plated, all transconjugants were resistant to ampicillin

(50ug/ml) and chloramphenicol (40ug/ml), the resistance phenotypes of pSG1 indicating the presence of native pSG1. Either pSG1 and pMMB19 were retained as cointegrates or polA+ revertants were selected. E. coli UB2272 (pSG1) recipient control also retained resistance to gentamicin, ampicillin and chloramphenicol and so it most likely that the initial selection procedure identified polA+ revertants of E. coli UB2272 which had retained pSG1.

To remove the initial selection for polA+ revertants, the selection procedure was altered to select only for the retention of pMMB19 (streptomycin 100ug/ml and sulphonamide 200ug/ml). It was hoped that on entry into E. coli UB2272, pSG1 would fail to replicate and that the pMMB19 plasmid would act as a target for transposition of Tn5::gen. Replica-plating of the resulting streptomycin-sulphonamide-resistant colonies onto gentamicin (10ug/ml) and ampicillin (50ug/ml) identified those colonies containing the transposon but not pSG1. In such an experiment, twenty-five gentamicin-resistant colonies were obtained and when tested, six were ampicillin-sensitive.

Plasmid DNA was extracted by a mini-prep method from these six transconjugants (Figure 4.2). Unfortunately, very low concentrations of DNA were obtained in all cases. Two plasmid bands appeared to be present in lanes B-F. Lane A contained two distinct plasmids. As restriction enzyme analysis was not possible due to the low plasmid yield and no increase in plasmid yield was obtained on repeated extractions, phenotypic analysis was used to test for a suitable vector for Tn5::gen. To investigate the temperature-dependent nature of the resistance phenotypes, the six colonies showing gentamicin and streptomycin-resistance were grown overnight in Complete broth (20ml)

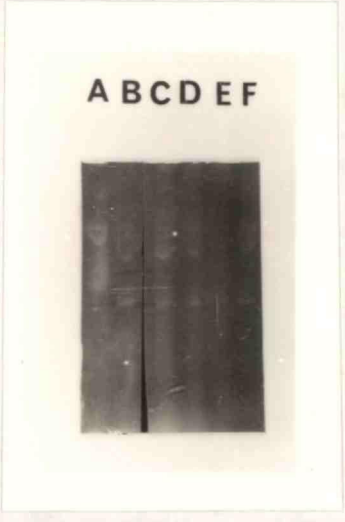


Figure 4.2: Plasmid DNA Extracts of Six Transconjugants of E. coli UB2272 polA (pMMB19, pSG1) Showing Gentamicin-resistance and Ampicillin-sensitivity.

containing gentamicin (15ug/ml) and streptomycin (100ug/ml) at 30°C. Each overnight culture (200ul) was used to inoculate two separate Complete broths (20ml) lacking antibiotic. One culture of each strain was incubated at 30°C, the other at 42°C for 10 hours. Each culture was diluted to ~500 cfu/ml and 100ul plated onto two of the following plates: on Complete agar (viability control), on Complete agar containing gentamicin (15ug/ml), sulphonamide (200ug/ml) and streptomycin (100ug/ml) (selecting for pMMB19::Tn5::gen), on Complete agar containing gentamicin (15ug/ml) alone (selecting for Tn5::gen) and also on Complete agar containing streptomycin (100ug/ml) and sulphonamide (200ug/ml) (selecting for pMMB19). One of each pair was incubated at 30°C, the other at 42°C (see Table 4.2).

TABLE 4.2 Number of Colonies of *E. coli* UB2272 Counted per Plate on Selection Media After Incubation at Two Different Temperatures

	Colony											
	SMT1		SMT2		SMT3		SMT4		SMT5		SMT6	
	Temp.°C.											
	30	42	30	42	30	42	30	42	30	42	30	42
Complete agar	32	53	43	38	58	44	35	11	35	41	32	42
Sm Su	34	0	47	0	68	0	49	0	53	0	38	0
Gm	40	59	32	1	57	1	31	16	51	3	58	3
Sm Su Gm	46	0	28	0	3	0	32	0	0	0	35	0

Sm - Streptomycin (100ug/ml), Su - Sulphonamide (200ug/ml), Gm - Gentamicin (15ug/ml)

Two of the six isolates (2 and 6) had the desired phenotype at 42°C (loss of streptomycin and sulphonamide resistance and retention of gentamicin resistance at a low frequency). Transformation of

competent E. coli UB5201 and E. coli SM10 with DNA from these two strains was performed to ensure all resistances were transferred en bloc. Selection was on L-agar containing gentamicin (15ug/ml) alone, and streptomycin (100ug/ml) and sulphonamide (200ug/ml), and all three antibiotics together. Transformants only grew on plates containing gentamicin (15ug/ml). Replica-plating of these colonies onto streptomycin (100ug/ml), sulphonamide (200ug/ml) plates and streptomycin (100ug/ml), sulphonamide (200ug/ml) and gentamicin (15ug/ml) plates showed all 88 colonies transformed with DNA from isolate 2 were resistant to the three antibiotics. None of the nineteen gentamicin-resistant colonies transformed with DNA from isolate 6 were resistant to streptomycin and sulphonamide as shown by replica-plating.

4.2.2. Restriction Enzyme analysis of pRTG1

Plasmid pMMB19 is a derivative of pRSF1010 (Figure 4.1). Tn5::gen encodes the two IS50 elements of Tn5 (Figure 1.5a), except for the internal ends beyond the BglII sites and the gentamicin-resistance gene insert from pXS1 (Figure 3.3). To test for the presence of Tn5::gen in pMMB19 restriction enzymes EcoR1, HindIII and HpaI were used to digest pRTG1 DNA (Figure 4.3a). Restriction enzyme EcoR1 generated a single band (~12.88Kbp) a size consistent with a single Tn5::gen (4.2Kbp) insertion into pMMB19 (8.9Kbp). In pXS1 (Figure 3.3), a EcoR1 site separates the two internal HindIII site and was not present in pRTG1. Restriction enzyme HindIII generated three fragments (9.44, 1.51 and .50 Kbp) within pRTG1. As pMMB19 does not contain any HindIII sites these exist within Tn5::gen. Each IS50 element contains one HindIII site and the other site indicates that the gentamicin-insert has one of the internal HindIII sites from pXS1

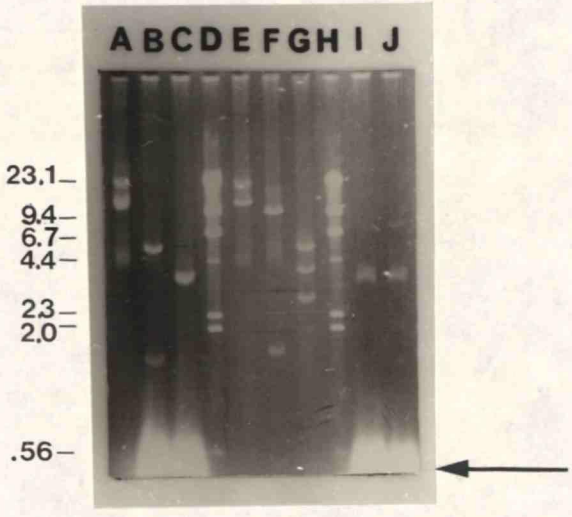


Figure 4.3a: Restriction enzyme digests of plasmid DNA extracted from E. coli UB2272 (pRTG1). Lane A - pRTG1 uncut; Lane B - pSG1 HindIII; Lane C - pSG1 HpaI; Lane D - lambda HindIII; Lane E - pRTG1 EcoR1; Lane F - pRTG1 HindIII; Lane G - pRTG1 HpaI; Lane H - lambda HindIII; Lane I - pMMB19 HindIII; Lane J - pMMB19 HpaI.

Estimated sizes of pRTG1 fragments (Kbp):

pRTG1 EcoR1	-	12.88		
pRTG1 HindIII	-	9.44, 1.51, 0.50	=	11.45Kbp total
pRTG1 HpaI	-	4.96, 3.94, 2.72	=	11.62Kbp total

(The 0.5Kbp HindIII fragment, Lane F is arrowed).

(Figure 3.3). Restriction enzyme digestion of plasmid DNA from E. coli UB5201 (pRTG1) transformants showed the expected pattern for pRTG1 (Figure 4.3b) and pRTG1 was used for further studies.

Plasmid pRTG1 was purified by Triton extraction and CsCl density gradient centrifugation (see Methods). Restriction enzyme digests using BglII, EcoRI, HpaI and PvuII were used to identify the size and position of Tn5::gen (Figure 4.4). Single sites existed for EcoRI and BglII. The EcoRI site is from pMMB19 and the BglII site is within the gentamicin resistance insert of Tn5::gen (see pXS1, Figure 3.3). The size of pRTG1 was estimated as being 13.18Kbp from the relative distances migrated with respect to HindIII-digested lambda DNA. The predicted size of Tn5::gen (4.2Kbp) into pMMB19 (8.9Kbp) was 13.1Kbp. The PvuII and EcoR1 digests show loss of restriction enzyme sites found in the gentamicin-resistance gene insert of pXS1 (Figure 3.3). Further digests and double digests of pRTG1 were performed (Figure 4.5a) and a restriction enzyme map constructed (Figure 4.5b). One AvaII restriction enzyme site expected from the restriction enzyme map of pRSF1010 was not present. Restriction enzyme AvaII digests of pMMB19 supported the possibility that one AvaII site had been lost prior to transposition of Tn5::gen (data not shown). Loss of the AvaII site may have resulted from a point mutation caused by the chemical mutagenesis used to generate temperature-sensitivity. A second, unexpected EcoR1 site was also observed, however, this was considered an artefact due to star activity. This was based upon the fact that this band was not always apparent on EcoR1 digestion (for example, see Figure 4.3a, 4.3b and 4.4) and that other minor fragments were also seen. Furthermore, the size of the smaller EcoR1 fragment positions the "EcoR1" site well within pMMB19 DNA supporting the view

Figure 4.3b: Restriction Enzyme Digests of pRTG1 DNA from E. coli UB5201 (pRTG1). Lane A - lambda HindIII; Lane B - pRTG1 HpaI; Lane C - pRTG1 HindIII; Lane D - pRTG1 EcoR1; Lane E - lambda HindIII.

Figure 4.4: Restriction Enzyme Digests of CsCl-purified pRTG1 DNA. Lane A - pRTG1 PvuII; Lane B - pRTG1 HpaI; Lane C lambda HindIII; Lane D - pRTG1 Bg1II; Lane E - pRTG1 EcoR1.

Estimated sizes of pRTG1 fragments (Kbp):

pRTG1 PvuII	-	6.38, 5.31, 1.95	=	13.64
pRTG1 HpaI	-	5.89, 4.47, 3.02	=	13.38
pRTG1 Bg1II	-	13.18		
pRTG1 EcoR1	-	13.18		

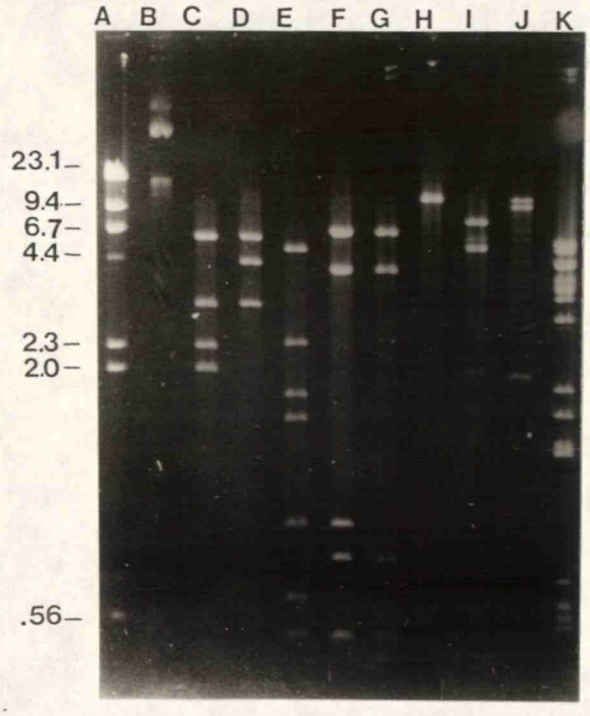


Figure 4.5a: Restriction Enzyme Digests of CsCl-purified pRTG1 DNA. Lane A - lambda HindIII; Lane B - pRTG1 uncut; Lane C - pRTG1 HpaI BglII; Lane D - pRTG1 HpaI; Lane E - pRTG1 HpaI AvaII; Lane F - pRTG1 AvaII; Lane G - pRTG1 AvaII BglII; Lane H - pRTG1 BglII; Lane I - pRTG1 BglII EcoR1; Lane J - pRTG1 EcoR1; Lane K - lambda HindIII EcoR1.

pRTG1 HpaI BglII	- 5.50, 3.00, 2.29, 1.85	12.64
pRTG1 HpaI	- 5.50, 4.27, 3.00	12.77
pRTG1 HpaI AvaII	- 4.87, 2.29, 1.77, 1.54, 0.91, 0.61, 0.50	12.49
pRTG1 AvaII	- 6.50, 4.01, 0.91, 0.72, 0.50	12.64
pRTG1 AvaII BglII	- 6.50, 4.01, 0.72, 0.50, 0.45	12.18
pRTG1 BglII	- 11.75	11.75
pRTG1 BglII EcoR1	- 7.59, 4.87*	12.46
pRTG1 EcoR1	- 11.75*	11.75

* - Star activity ignored (see text).

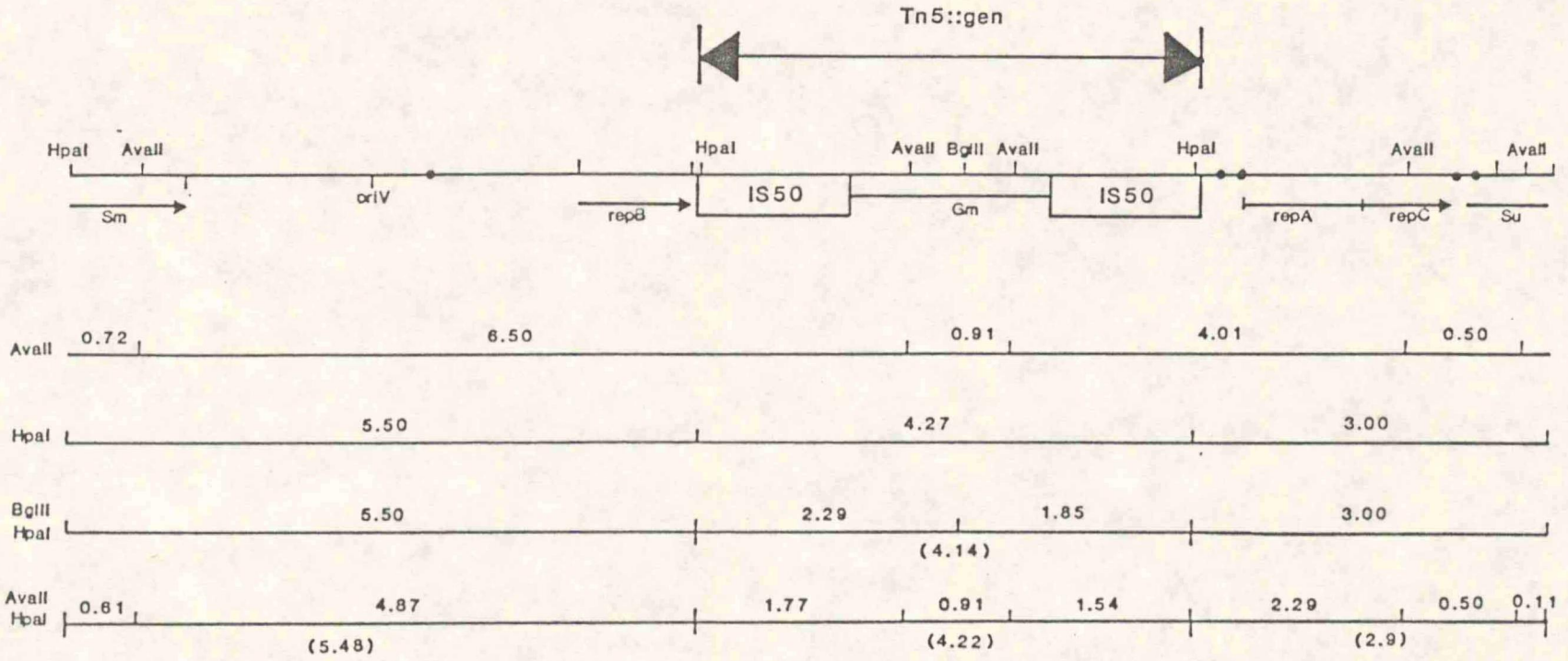


Figure 4.5b: Restriction Enzyme Cleavage Map of pRTG1. Filled circles represent RNA Polymerase binding sites of pRSF1010 (Scholz et al. 1985). The lower lines represent fragments (Kbp) generated from digestion with named restriction enzymes. Values in parentheses are the sizes of bordered regions calculated from restriction enzyme double digests.

that this site is not the EcoR1 site from the gentamicin-resistance gene insert of pXS1.

4.2.3 Plasmid pRTG1 as a vector for Tn5::gen

To test whether insertion of the transposon had interfered with any of the mobilization properties of the vector, pRTG1 was mated from E. coli SM10 (pRTG1) and E. coli UB2272 (pRTG1) into E. coli UB5201 in two separate experiments. Two different sources of tra+ genes were used to test whether the site of tra+ genes affected the transfer frequency of pRTG1. The tra+ genes were encoded by pR751 in E. coli UB2272 polA and from a chromosomally-inserted pRP4 fragment in E. coli SM10. Filter-mating at 30°C for 4 hours was followed by selection on glucose minimal agar containing proline and methionine with either gentamicin (15ug/ml) or gentamicin (15ug/ml) and streptomycin (100ug/ml). The transfer frequency with selection on gentamicin alone was $1x10^{-2}$ from E. coli SM10 (pRTG1) and $5x10^{-5}$ from E. coli UB2272 (pRTG1). The frequency of transfer selecting on gentamicin (15ug/ml) and streptomycin (100ug/ml) was $3x10^{-3}$ from E. coli SM10 (pRTG1) and was undetected from E. coli UB2272 (pRTG1). An inability to directly select for streptomycin-resistance was also found in transformation experiments. A prolonged incubation period may be required for the expression of streptomycin-resistance and initial selection for pRTG1 in a new host may have to be on gentamicin alone. Poor transfer frequencies from E. coli UB2272 may be related to its polA mutant. E. coli UB2272 was often poor growing, had a mucilagenous colony appearance and was less efficient both as donor and recipient of plasmid DNA than other E. coli strains.

E. coli SM10 and E. coli UB5201 transformed with pRTG1 and E. coli

UB5201 (pRTG1) derived from a conjugation experiment with E. coli SM10 (pRTG1) were used to test whether retention of antibiotic resistance was temperature-dependent. Cultures were grown for 24 hours at 30°C and at 42°C in L-broth in the absence of antibiotic selection, which prevented selection of the plasmid. A control culture incubated at 30°C was required to ensure any loss of resistance was due to temperature-sensitivity of the plasmid as opposed to plasmid instability due to the absence of antibiotic selection. Bacteria were diluted in M9 diluent and spread-plated at $3x10^3$ and 30 cfu onto two dry L-agar plates and two agar plates containing (i) gentamicin (20ug/ml) selecting for Tn5::gen, (ii) streptomycin (100ug/ml) and sulphonamide (200ug/ml), selecting for vector and (iii) the three antibiotics together, selecting for pRTG1 entire. One plate of each dilution was then incubated at 30°C and the other at 42°C for 24 hours. Results are shown in Table 4.3.

Table 4.3 A Comparison of the Number of Colonies of E. coli (pRTG1) Strains Counted on Various Selection Media After Incubation at Two Different Temperatures

Table 4.3a: Dilution - ~30cfu

	E. coli strain:					
	UB5201		UB5201*		SM10	
	TEMP. °C					
	30	42	30	42	30	42
Selection						
L-agar	23	17	29	30	24	30
(i) Gm	24	5	50	3	20	5
(ii) Sm Su	22	0	15	0	49	0
(iii) Sm Su Gm	19	0	23	0	23	0

* - A strain of E. coli in which pRTG1 was introduced by conjugation rather than by transformation.

Table 4.3b: Dilution - ~3000cfu

	E. coli strain:					
	UB5201		UB5201*		SM10	
	TEMP. °C					
	30	42	30	42	30	42
Selection						
L-agar	3,000	2,500	3,000	2,500	3,000	3,000
(i) Gm	3,000	220	3,000	140	3,000	500
(ii) Sm Su	3,000	0	3,000	0	3,000	0
(iii) Sm Su Gm	3,000	0	3,000	0	3,000	0

* - A strain of E. coli in which pRTG1 was introduced by conjugation rather than by transformation

Sm - Streptomycin (100ug/ml), Su - Sulphonamide (200ug/ml)
Gm - Gentamicin (15ug/ml)

After incubation at 42°C streptomycin-sulphonamide resistance was not maintained whilst gentamicin-resistance was maintained in approximately 10% of the population. The frequency of gentamicin-resistance retention was much higher than that found when using pSG plasmids. However, cells transformed with pRTG1 also did not grow when initially plated on streptomycin-sulphonamide plates but did after incubation in the presence of gentamicin. To ensure that the lack of streptomycin-resistant colonies in this experiment was due to the loss of plasmid as opposed to poor initial expression of streptomycin and sulphonamide resistance, colonies that had retained gentamicin resistance were tested for co-retention of streptomycin resistance.

Twenty gentamicin-resistant colonies grown in L-broth at 30°C overnight were then plated at $1x10^4$ cfu/plate onto L-agar containing gentamicin (15ug/ml) and L-agar containing streptomycin (100ug/ml). None were resistant to streptomycin whilst all retained gentamicin-resistance. I would suggest that pRTG1 is temperature-sensitive at

42°C in both transformed and transconjugant cells and gentamicin-resistance can be retained by Tn5::gen transposing into the host chromosome.

To test that the retention of gentamicin-resistance was due to the presence of Tn5::gen in the chromosome of E. coli UB5201 a plasmid, pR751, was passaged through this strain (Figure 4.6). As plasmid DNA acts as a preferential target for Tn5 transposition the transposon might transpose into pR751. Plasmid pR751 was transferred from E. coli HB101 into and then out of E. coli "UB5201::Tn5::gen" into E. coli C600. Selection was for trimethoprim (100ug/ml) and gentamicin (15ug/ml) in E. coli C600. The transfer frequency of pR751 with co-retention of gentamicin-resistance was $5x10^{-6}$. Plasmid extraction of 6 isolates showing resistance to gentamicin and trimethoprim are shown in Figure 4.7a. A single plasmid band was observed. To ensure that the two resistance phenotypes were co-retained rather than them existing on two separate plasmids, plasmid DNA from four isolates were used to transform competent E. coli UB5201 with selection on trimethoprim (100ug/ml) and gentamicin (15ug/ml). Transformants were obtained in all cases and single plasmids of identical size, slightly larger than pR751, were present in each (Figure 4.7b). HpaI digests of plasmid DNA from two such transformants, designated pR1 and pR2, and native pR751 were performed (Figure 4.7c). Plasmid pR751 had a single site. Plasmids pR1 and pR2 unlike pR751 contained three HpaI sites including a common sized fragment (~4.2Kbp) which is the size of the internal HpaI fragment of Tn5::gen. The presence of a fragment expected for Tn5::gen in plasmids of similar size but differing restriction enzyme profiles supports the possibility that pR1 and pR2 are the result of two different transposition events of Tn5::gen from

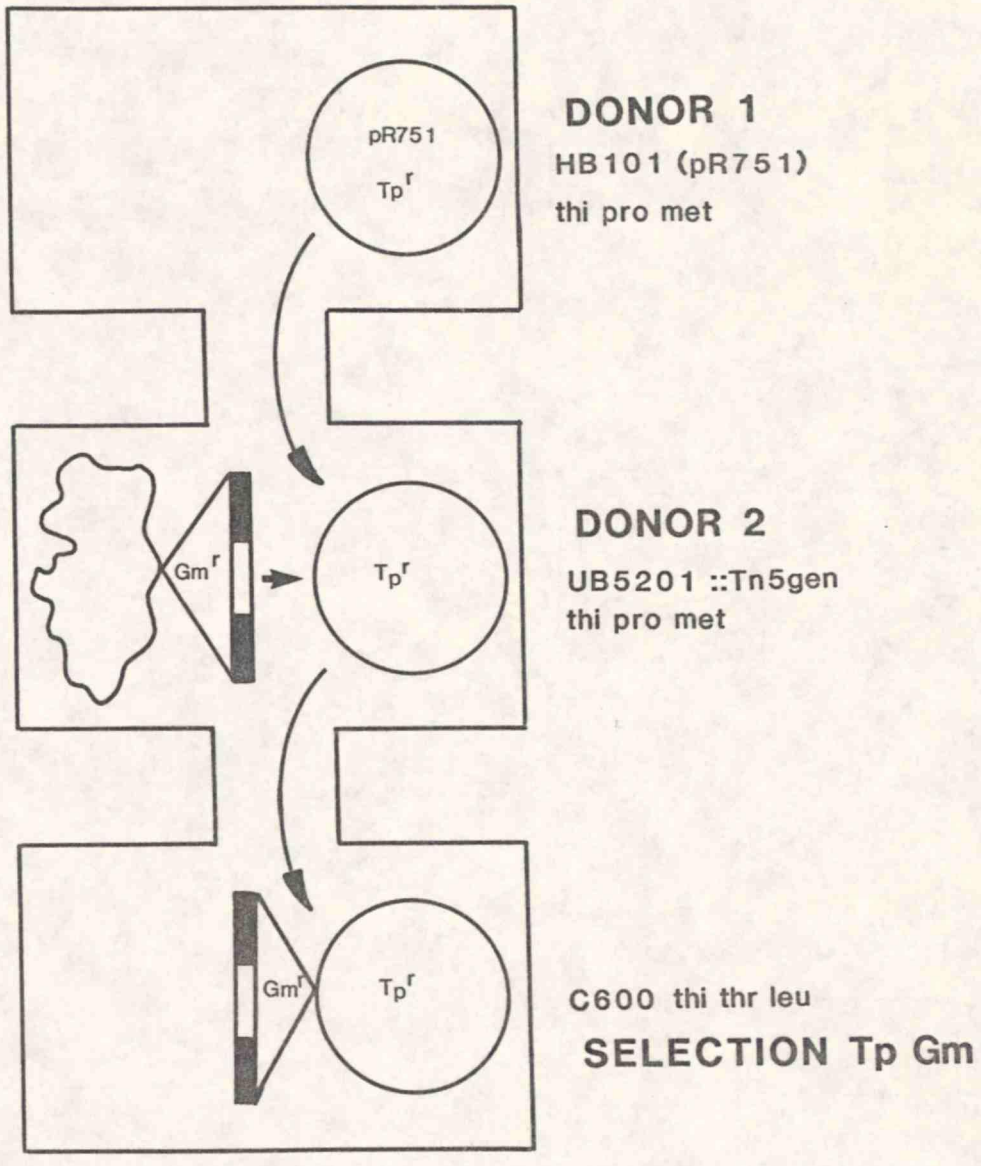


Figure 4.6: Passage of pR751 through E. coli UB5201::Tn5::gen. Plasmid pR751 was passaged through E. coli UB5201::Tn5::gen. Selection for transfer of trimethoprim and gentamicin-resistance into E. coli C600 indicated transposition of Tn5::gen from the E. coli chromosome into pR751. Glucose minimal medium containing thiamine, threonine, leucine, gentamicin (10ug/ml) and trimethoprim (100ug/ml).

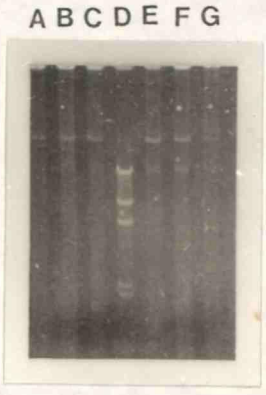


Figure 4.7a

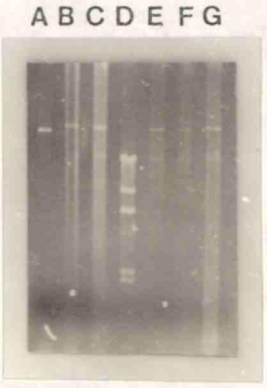


Figure 4.7b

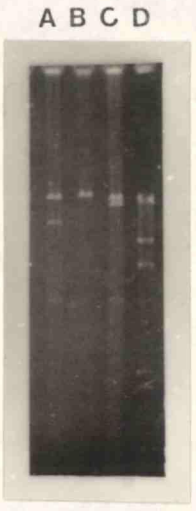


Figure 4.7c

Figure 4.7a: Plasmid DNA Extracted from Six Isolates of E. coli C600 (pR751::Tn5gen). Lanes A-C and Lanes E-G - Transconjugants 1-6 respectively; Lane D - lambda HindIII.

Figure 4.7b: Plasmid DNA Extracted from Transformants of E. coli UB5201 (pR751::Tn5gen). Lane A - E. coli HB101 (pR751), Lane B - pR751::Tn5gen isolate 3; Lane C - pR751::Tn5gen isolate 4; Lane D - lambda HindIII; Lane E - pR751::Tn5gen isolate 5; Lane F - pR751::Tn5::gen isolate 6; Lane G - E. coli C600 pR751::Tn5gen transconjugant 6.

Figure 4.7c: HpaI Restriction Enzyme Digests of pR751::Tn5:gen Plasmids. Lane A - E. coli UB5201 (pR1) HpaI; Lane B - E. coli HB101 (pR751) HpaI; Lane C - E. coli UB5201 (pR2) HpaI; Lane D - lambda HindIII.

the E. coli UB5201 chromosome into pR751.

4.2.4 Gene dosage and expression of gentamicin-resistance

Resistance conferred by the gentamicin-resistance gene derived from pR5 (Chapter 3) was unaffected by the copy number in E. coli, with the highest permissible concentration of gentamicin being 10ug/ml (Hirsch et al. 1986). Resistance to gentamicin (>10ug/ml) had been achieved in E. coli strains containing multiple copies of pRTG1. The reason for this increased resistance is unclear. However, it was necessary to test whether a single Tn5::gen could confer resistance to gentamicin at concentrations which would not select spontaneous gentamicin-resistant mutants in P. aeruginosa. To test whether a single copy of Tn5::gen could confer gentamicin-resistance upon E. coli at high gentamicin concentrations, temperature-sensitivity studies were performed on various selection media (see Table 4.4). Cultures of E. coli UB5201 (pRTG1) grown in L-broth overnight at 42°C were reinoculated in fresh L-broth and grown at 42°C for a further 5 hours before plating onto L-agar containing gentamicin at different concentrations and L-agar containing streptomycin (100ug/ml) and sulphonamide (200ug/ml). E. coli UB5201 (pRTG1) was also grown at 30°C to test whether pRTG1 could confer resistance to these gentamicin concentrations.

After incubation at 42°C in the absence of antibiotic selection for the transposon the population of transposon-bearing cells may become small. It may be advantageous to enrich the transposon-bearing population as long as this does not select a significant number of plasmid-bearing cells. To test whether reinoculation in the presence of gentamicin (15ug/ml) would enrich plasmid-bearing cells or

transposon-bearing cells, E. coli UB5201 (pRTG1) grown overnight at 42°C in L-broth was reinoculated in L-broth containing gentamicin (15ug/ml) and grown for 5 hours prior to being plated out on various selection media (see Table 4.4).

TABLE 4.4 Number of Colonies of E. coli UB5201 with Resistance to a Range of Gentamicin Concentrations at Two Temperatures

		L-agar containing antibiotic (ug/ml)			
		Gm (10)	Gm (20)	Gm (30)	Sm (100) Su (200)
Temp.	Inocula				
30°C	~4000	~4000	~4000	~4000	~4000
42°C	~4000	~1600	~1200	~400	0
"	~400	168	144	92	0
42°C (Gm[a])	~4000	~1600	~1200	~500	0
" (Gm[a])	~400	212	198	115	0

Gm - Gentamicin, Sm - Streptomycin, Su - Sulphonamide.

[a]- Gentamicin (15ug/ml) enrichment prior to plating cultures.

At 30°C there was no reduction in the number of gentamicin-resistant colonies with increasing gentamicin concentrations. However, at 42°C the number of gentamicin-resistant colonies declined with an increase in gentamicin concentration. Resistance to gentamicin (30ug/ml) at 42°C was most probably due to retention of Tn5::gen on the chromosome rather than retention of multicopy plasmid pRTG1 since no streptomycin- sulphonamide resistant colonies were observed. Similarly, the larger number of colonies resistant to gentamicin (10ug/ml) was not the result of spontaneous mutations within the population as no such mutants were observed in the parental E. coli UB5201 strain. 25-33% of colonies with a chromosomally encoded Tn5::gen were resistant to gentamicin (30ug/ml). It may be necessary to amplify this population prior to plating on selection media containing gentamicin (30ug/ml).

Enrichment of gentamicin-resistant cells after incubation at 42°C for 19 hours in the absence of selection did not select for retention of pRTG1 but neither did it increase significantly the population retaining the transposon. It would seem likely that enrichment would not be useful as a means to identify chromosomally inserted Tn5::gen.

4.3 Discussion

Plasmid pSG1 was introduced into E. coli UB2272 (pMMB19) in order to test whether Tn5::gen was transposition-proficient and to generate a suitable vector for introducing Tn5::gen into P. aeruginosa. Temperature-sensitivity of six cultures of E. coli UB2272 (pMMB19) which had retained gentamicin-resistance but lost ampicillin-resistance were investigated. Although replicates were not performed a number of points can be made. Firstly, all strains (SMT1-6) showed a loss of resistance to streptomycin and sulphonamide after incubation at 42°C, indicating these resistances were conferred by pMMB19 and not by a stable cointegrate of pRTG1-pSG1. SMT1 and SMT4 retained gentamicin-resistance in all viable cells. This is most easily explained if the transposon initially transposed into the host chromosome rather than pMMB19 when pSG1 entered the cell. One could argue that the transposon may have transposed from pMMB19 at a very high frequency at the non-permissive temperature but it is unlikely to occur in the entire population and survive in the absence of selection. Retention of gentamicin-resistance at 42°C in a small sub-population of SMT3 and SMT5 suggests that Tn5::gen is indeed located upon pMMB19 and that the loss of plasmid at the non-permissive temperature, as indicated by loss of streptomycin- and sulphonamide-resistance, leads to the identification of chromosomally-integrated

Tn5::gen at low frequencies. SMT3 and SMT5, however, did not express resistance to all three antibiotics simultaneously at 30°C. One possible explanation for this observ ation is that the position of the transposon in pMMB19 may have affected the transcription process initiated at the sulphonamide-streptomycin promoter when the gentamicin-resistance gene is itself being expressed. SMT2 and SMT6 show the expected phenotypes of a transposon being present on pMMB19: total loss of plasmid-mediated resistance at 42°C and retention of gentamicin-resistance in a minority of cells due to the transposition of Tn5::gen into the chromosome.

Tn5::gen was transposase-proficient in E. coli UB2272 polA when pMMB19 was present, supporting the possibility that pMMB19 offers a preferential target site for transposition to the chromosome of E. coli UB2272.

Plasmid extractions of the six strains were performed but no analysis was possible due to extremely low concentrations of plasmid present in every case. Plasmid extractions were repeated three times without substantial concentrations of plasmid being obtained. Plasmid DNA from SMT2 and SMT6 extractions were, however, able to transform E. coli UB5201 and plasmid DNA was extracted from this strain in higher yields than in E. coli UB2272 polA. I would suggest that the low yields of DNA from E. coli UB2272 polA was probably due to two factors; inefficient synthesis of plasmid DNA in the absence of DNA Polymerase I and inefficient activity of the temperature-sensitive RepA protein of pMMB19. The relatively low transfer frequency of pVW6 into E. coli UB2272 with respect to E. coli JC5466 is one observation which supports the view that all plasmid replication was inefficient.

Inability to express streptomycin- and sulphonamide-resistance on transformation of competent E. coli with pRTG1 and pRTG2 was an interesting observation. This was particularly noticeable for pRTG2 where gentamicin-resistance was conferred upon the host yet the marker genes of pRTG2 were not expressed even after replica-plating onto L-agar containing sulphonamide and streptomycin. Rather than investigate this problem I felt it peripheral to the main aim; the generation of a suitable vector for Tn5::gen. E. coli UB5201 and SM10 transformed with pRTG1 were resistant to gentamicin and, on replica-plating, to streptomycin and sulphonamide. The inability to select directly for the plasmid using streptomycin and sulphonamide initially may have been due to the poor expression of the resistance genes in newly transformed cells. Whether poor expression of these genes was due to the location of Tn5::gen in pMMB19 or was relevant to the problem of pRTG2 transformed cells is not known.

Using restriction enzyme profiles (Figure 4.5b), Tn5::gen maps just downstream of repB and just upstream of the promoter sites associated with repA and repC (Scholz et al. 1985). The location of Tn5::gen, however, did not inactivate any of these genes as they are all essential for replication of pRSF1010-derivatives (Scholz et al. 1985). The orientation of Tn5::gen is not known as the IS50 elements have identical restriction enzyme sites, however, IS50R is believed to orientate itself preferentially toward an active upstream promoter (Berg and Berg 1983) and so IS50R may be adjacent to repB.

Plasmid pRTG1 was mobilized in the presence of tra+ genes, showing that Tn5::gen had not interfered with plasmid transfer ability. It

seemed likely that E. coli SM10 was the best donor of pRTG1, not only because of the high transfer frequency, but also because no tra+ donor plasmid can co-transfer into the recipient host. Such a plasmid would act as a preferential site for transposition of Tn5::gen from pRTG1 at non-permissive temperatures because plasmid DNA is more supercoiled than chromosomal DNA (Isberg and Syvanen 1982). Retention of such a helper plasmid may also result in a stable cointegrate of pRTG1 with, for example, pR751 thus preventing the loss of vector.

Prior to introducing pRTG1 into P. aeruginosa the temperature-stability of pRTG1 was investigated in E. coli. Streptomycin-sensitivity and gentamicin-resistance phenotypically show loss of plasmid and retention of Tn5::gen. Spontaneous resistance to gentamicin was unlikely as parental E. coli UB5201 showed no such resistance. Transposon insertion in the chromosome was tested by passage of pR751 through E. coli "UB5201::Tn5::gen" and selecting for gentamicin-resistance in the recipient strain, E. coli C600. Transformation using plasmid DNA extracted from the recipient showed the plasmid encoded resistance to both gentamicin and trimethoprim resistance. Furthermore, restriction enzyme digests showed the presence of Tn5::gen in pR751. This data supports the possibility that Tn5::gen was present in the chromosome of E. coli UB5201 (pRTG1) after incubation at the non-permissive temperature. Southern transfer and hybridization of chromosomal DNA with Tn5::gen, however, would be required to confirm that the transposon was located in the chromosome.

Initially, experiments in the investigation of the temperature-sensitive nature of pRTG1 were performed in E. coli UB5201 and SM10. Plasmid pRTG1 was stable at 30°C in the absence of antibiotic

selection but was unstable at 42°C as shown by the total loss of streptomycin- and sulphonamide-resistance. Retention of gentamicin-resistance indicated transposition of Tn5::gen into the chromosome. The temperature-sensitive behavior of pRTG1 was the same in both strains tested.

Resistance conferred by Tn5::gen reduced with increasing gentamicin concentrations at 42°C but not at 30°C. Although other effects of temperature on resistance cannot be ruled out, I would suggest that the difference in resistance is due to the presence of Tn5::gen in multiple copies on pRTG1 at 30°C and single copies in the chromosome at 42°C. However, no gene dosage effect was observed for the same gentamicin-resistance gene when encoded by the multicopy pXS1 and the single copy pR1033 (Hirsch et al. 1986); in both cases the highest permissible concentration of gentamicin was reported as 10ug/ml. The gentamicin-resistance determinant may be under the control of a better, alternative promoter in Tn5::gen or is expressed better in the absence of adjacent promoters found in pR1033 and pXS1. Selection for retention of Tn5::gen in P. aeruginosa would require a concentration of gentamicin at 30ug/ml if spontaneous mutants are to be infrequent. In E. coli the number of colonies resistant to gentamicin at this concentration is approximately 25-50% of those resistant at 10ug/ml. The difference in resistance conferred may have been due to the specific position of transposon insertions in the chromosome affecting expression of the gentamicin-resistance gene or the presence of Tn5::gen in multiple copies in the chromosome. Enrichment did not significantly alter the number of resistant colonies obtained but may have caused clonal growth of specific mutants and may also have increased the number of spontaneous mutations and was considered

disadvantageous.

In summary, a temperature-sensitive plasmid carrying a Tn5 derivative has been constructed. The plasmid has been introduced into E. coli both by transformation and conjugation. The plasmid was lost from the population after incubation at the non-permissive temperature and gentamicin-resistance was retained within a sub-population (approximately 10%). Gentamicin-resistance implies the retention of Tn5::gen, and, although Southern transfer and hybridization of chromosomal DNA was not performed, was supported by the presence of Tn5::gen on pR751 after passaging pR751 through E. coli "UB5201::Tn5::gen". Transposon Tn5::gen can confer resistance to gentamicin at a concentration of 30ug/ml, required for selection in a population of P. aeruginosa if spontaneous mutants are to be infrequent. However, at this concentration there is a possibility that the majority of transposon- bearing cells are lost, and that only those specifically located to increase the expression of gentamicin-resistance or those strains with multiple copies of Tn5::gen can be isolated and observed. However, permeability of P. aeruginosa to antibiotics is different to that of E. coli and may increase the resistance conferred by the gentamicin- resistance gene accordingly. To test these possibilities the plasmid was introduced into P. aeruginosa.

CHAPTER 5

THE INTRODUCTION OF PLASMID pRTG1 INTO *Pseudomonas aeruginosa* AND THE GENERATION OF B-LACTAM-RESISTANT MUTANTS

Objectives: To introduce pRTG1 into *P. aeruginosa* and to detect B-lactam-resistant mutants generated by transposition of Tn5::gentamicin from pRTG1 into the chromosome.

5.1 Introduction

A vector, pRTG1, carrying a Tn5 transposon conferring gentamicin resistance has been constructed for use in *Pseudomonas aeruginosa* (Chapter 4). Originally the laboratory strain *P. aeruginosa* PAT210 was to be used to isolate hyperproducing B-lactamase mutants generated by transposon mutagenesis. However, kinetic analysis showed *P. aeruginosa* PAT210 B-lactamase to have a much lower maximal activity than a clinical isolate *P. aeruginosa* 3-Pre (Chapter 6). Furthermore, spontaneous mutation to cefsulodin-resistance has not been detected in *P. aeruginosa* PAT210 (Hewinson 1987). It was therefore considered that *P. aeruginosa* PAT210 was an unsuitable strain for selecting transposon-induced mutants hyperproducing B-lactamase. Mutations with altered expression of B-lactamase have been reported in *P. aeruginosa* PAO4069 and, furthermore, these mutations have been physically mapped using FP5 (Matsumoto and Terawaki 1982). *P. aeruginosa* PAO4069, therefore, seemed a useful strain for testing pRTG1, since hyperproducing B-lactamase mutants generated by transposon insertion could be compared biochemically and genetically with those previously characterized. *P. aeruginosa* 3-Pre was used as as an additional recipient of pRTG1 as it can give rise to B-lactamase hyperproducing mutants (Slack and Pitt 1982, Hewinson and Nichols 1987) and, being a

clinical isolate was pertinent to the study.

To select B-lactam-resistant mutants MICs of cefsulodin, ceftazidime and piperacillin were determined using a spread plate method. To establish suitable antibiotic concentrations to select for the transfer of pRTG1 MICs were also determined for streptomycin and gentamicin. Plasmid pRTG1 was to be transferred into each of P. aeruginosa PAO4069 and 3-Pre. Transposon Tn5 had been successfully introduced into P. putida KT2440 (R. Wilson, personal communication) and was used here as a control strain in transformation experiments.

5.2 Results

5.2.1 Minimum Inhibitory Concentrations

The spread method using various inocula sizes was used to determine MIC values, shown in Tables 5.1-5.5 and summarized in Table 5.6.

TABLE 5.1 Numbers of Colonies of Four Pseudomonad Strains Growing on Nutrient Agar Containing Streptomycin

Inoculum (cfu/plate)	Streptomycin (ug/ml) 0	40	80	160	320	640
PA04069						
4.4×10^4	Conf.	Conf.	~5000	217	0	0
4.4×10^6	Conf.	Conf.	Conf.	Conf.	0	0
PAT216[a]						
1.8×10^4	Conf.	Conf.	Conf.	Conf.	3[b]	0
1.8×10^6	Conf.	Conf.	Conf.	Conf.	50[b]	3
3-Pre						
5.0×10^4	Conf.	Conf.	Conf.	~4000	1	0
5.0×10^6	Conf.	Conf.	Conf.	Conf.	22	0
KT2440						
2.0×10^4	Conf.	5	1	0	0	0
2.0×10^6	Conf.	488	17	2	0	0

a - *P. aeruginosa* PAT216 was used as a control strain
b - micro-colonies present in background to those counted
Conf. - Confluent growth

TABLE 5.2 Numbers of Colonies of Four Pseudomonad Strains Growing on Nutrient Agar Containing Gentamicin

Inoculum cfu/plate	Gentamicin (ug/ml) 0	1	2	4	8
PAO4069					
$1.4x10^4$	Conf.	Conf.	5	0	0
$1.4x10^5$	Conf.	Conf.	62	0	0
$1.4x10^6$	Conf.	Conf.	~600	0	0
$1.4x10^7$	Conf.	Conf.	Conf.	189	3
PAT216					
$4.6x10^4$	Conf.	Conf.	Conf.	~1,500	4
$4.6x10^5$	Conf.	Conf.	Conf.	Conf.	85
3-Pre					
$1.1x10^4$	Conf.	Conf.	223	0	0
$1.1x10^5$	Conf.	Conf.	~2,000	22	0
$1.1x10^6$	Conf.	Conf.	~2,000	68	3
$1.1x10^7$	Conf.	Conf.	Conf.	>5000	103
KT2440					
$2x10^4$	Conf.	0	0	0	0
$2x10^5$	Conf.	0	0	0	0

Conf. - Confluent growth

TABLE 5.3 Numbers of Colonies of Four Pseudomonad Strains Growing on Nutrient Agar Containing Cefsulodin

Inoculum cfu/plate	Cefsulodin (ug/ml) 0	1	2	4	8	16
PAO4069						
4.4×10^4	Conf.	15	0	0	0	0
4.4×10^6	Conf.	>5,000	>2,000	2	1	1
PAT216						
1.8×10^4	Conf.	Conf.	4	0	0	0
1.8×10^6	Conf.	153[a]	150	6	0	0
3-Pre						
5.0×10^4	Conf.	Conf.	Conf.	>2,000	1[a]	2
5.0×10^6	Conf.	Conf.	Conf.	Conf.	30[a]	16[a]
KT2440						
2.0×10^4	Conf.	Conf.	9[a]	1	0	1
2.0×10^6	Conf.	Conf.	58[a]	6	3	1

a - micro-colonies present in background to those counted
Conf. - Confluent growth

TABLE 5.4 Numbers of Colonies of Two Strains of P. aeruginosa Growing on Nutrient Agar Containing Ceftazidime

Inoculum cfu/plate	Ceftazidime (ug/ml) 0	2	4	8	16
PA04069					
$1.5x10^7$	Conf.	Conf.	>5000	63	3
$1.5x10^4$	Conf.	>5000	0	0	0
3-Pre					
$3.2x10^7$	Conf.	Conf.	Conf.	Conf.	Conf.
$3.2x10^4$	Conf.	Conf.	Conf.	Conf.	>5000

Conf. - Confluent growth

TABLE 5.5 Numbers of Colonies of Two Strains of P. aeruginosa Growing on Nutrient Agar Containing Piperacillin

Inoculum cfu/plate	Piperacillin (ug/ml) 0	2	4	8	16	32	64
PA04069							
$1.5x10^6$	Conf.	Conf.	9	2	0	0	0
$1.5x10^4$	Conf.	>1000	0	0	0	0	0
3-Pre							
$7.5x10^3$	Conf.	Conf.	Conf.	Conf.	Feint	Feint	0

Conf. - Confluent growth
Feint - Confluent but severely restricted growth

TABLE 5.6 MICs of Various Antibiotics on Nutrient Agar Toward *P. aeruginosa* Strains Using a Spread Technique

Antibiotic	PA04069		3-Pre	
	Inoculum size (cfu/100ul)	MIC (ug/ml)	Inoculum size (cfu/100ul)	MIC (ug/ml)
Streptomycin	$4x10^4$	320	$5x10^4$	640
	$4x10^6$	320	$5x10^6$	640
Gentamicin	$1x10^4$	4	$1x10^4$	4
	$1x10^7$	8	$1x10^7$	8
Cefsulodin	$4x10^4$	2	$5x10^4$	>16
	$4x10^6$	4	$5x10^6$	>16
Ceftazidime	$1x10^4$	4	$3x10^4$	>16
	$1x10^7$	8	$3x10^7$	>16
Piperacillin	$1x10^4$	4	$8x10^3$	64
	$1x10^6$	8		

P. aeruginosa 3-Pre was generally more resistant to those antibiotics tested than *P. aeruginosa* PA04069. Cefsulodin was the most effective B-lactam against both *P. aeruginosa* strains but both ceftazidime and piperacillin might be considered useful alternatives for selecting B-lactam-resistant mutants in *P. aeruginosa* PA04069. In the first instance, the antibiotic concentration chosen to select for resistance was 8xMIC, in order to prevent the selection of a large number of spontaneous mutations. However, higher concentrations of B-lactams may be required at the population densities plated to detect resistant mutants ($\sim 1x10^7$) due to the effect of population density on resistance.

5.2.2 Conjugal Transfer of pRTG1 from *E. coli* SM10 to *P. aeruginosa*

Conjugal transfer of pRTG1 from *E. coli* SM10 (pRTG1) into *P. aeruginosa* PA04069 and 3-Pre was attempted in separate experiments. Filter-mating (see Methods) was carried out for 24 hours at 30°C. Selection for transconjugants was made on glucose minimal agar containing essential amino acids supplemented with varying antibiotic concentrations. In initial transfer experiments using selection media described in Table 5.7 (a), transconjugants were not detected. The transfer of pRTG1 may have been unsuccessful because pRTG1 was unable to confer resistance at the concentration of antibiotics in the selection medium. Previous transformation experiments in *E. coli* had shown that initial selection for pRTG1 with streptomycin was sometimes unsuccessful (Chapter 4). A second round of transfer experiments were performed where the streptomycin concentrations selecting for pRTG1 were reduced (Table 5.7 (b)).

TABLE 5.7 Media for Selection of Transfer of pRTG1 from *E. coli* SM10 (pRTG1) into *P. aeruginosa* PA04069 and 3-Pre

	Recipient	Selection conditions
a	PA04069	Glc. min. pro, met, Sm (400 ug/ml)
	"	" " " " Gm (20ug/ml)
	3-Pre	" " Sm (400ug/ml)
	"	" " Gm (32ug/ml)
b	PA04069	Glc. min. pro, met, Sm (200ug/ml)
	"	" " " " Sm (100ug/ml), Gm (20ug/ml)

Glc. min. - Glucose minmal medium
met - Methionine
pro - Proline
Gm - Gentamicin
Sm - Streptomycin

E. coli SM10 (pRTG1) did not grow on any medium, however, selection at lower streptomycin concentrations gave rise to small resistant

colonies of P. aeruginosa PA04069 on control plates. Larger colonies within this background of growth on selection plates may have been transconjugants. Eight large colonies from separate selection plates and two "resistant" micro-colonies from control plates were used to inoculate nutrient broth lacking antibiotic selection with incubation at 30°C for 4 hours. Samples ($1x10^3$/100ul) were spread onto selection media; nutrient agar containing streptomycin (200ug/ml) alone, gentamicin (20ug/ml) alone and both antibiotics together at 200ug/ml and 20ug/ml respectively. The two P. aeruginosa PA04069 micro-colony controls did not grow on selection media. Putative transconjugant colonies grew but with two colony types; one typically pseudomonad in size and colour and the other a smaller (<2mm), paler type. Oxidase tests on both colony types showed the presence of oxidase-positive cells in the "pseudomonad" type colony but no oxidase activity in the other colony type. It seemed likely that the oxidase-negative contaminant was E. coli SM10 (pRTG1). As nutrient deprivation appeared unable to select against E. coli SM10 (pRTG1) in the initial conjugation an alternative counter-selection mechanism was required.

P. aeruginosa is intrinsically resistant to many antibiotics to which E. coli is sensitive. Therefore, it seemed likely that selection against E. coli could be achieved using an appropriate antibiotic at a concentration which would kill all E. coli SM10 but would not affect P. aeruginosa. The MICs of tetracycline toward P. aeruginosa PA04069 and E. coli SM10 (pRTG1) were determined on glucose minimal medium containing essential amino acids in each case. P. aeruginosa PA04069 (MIC 50ug/ml) showed no loss of viability with tetracycline at 5ug/ml (Table 5.8) whilst E. coli SM10 was sensitive (MIC 5ug/ml) with no growth, even at high cell densities ($1x10^7$). Tetracycline (5ug/ml)

was chosen to inhibit growth of E. coli SM10 (pRTG1).

TABLE 5.8 Number of Colonies of P. aeruginosa PAO4069 on Glucose Minimal Agar Containing Tetracycline

Glucose minimal medium containing proline and methionine and tetracycline (ug/ml)			
Inoculum	5	15	50
~1.8×10^7	Conf.	10, 12	0, 0
176, 177	158, 195	0, 0	0, 0

A filter-mating between E. coli SM10 (pRTG1) and P. aeruginosa PAO4069 was performed as described above. Glucose minimal medium containing proline and methionine with tetracycline (5ug/ml) was used to select for P. aeruginosa PAO4069 with gentamicin (25ug/ml) and streptomycin (400ug/ml) selecting for pRTG1. Selection plates were incubated for 36 hours at 30°C. The results are shown in Table 5.9.

TABLE 5.9 Number of Colonies on Glucose Minimal Agar Containing Tetracycline (5ug/ml) and Other Antibiotics After a Conjugation Experiment Between E. coli SM10 (pRTG1) and P. aeruginosa PAO4069

	Antibiotic (ug/ml)		
Strain	Gm (25)	Sm (400)	Gm (25) Sm (400)
SM10 (pRTG1) Control	0	0	0
PAO4069 Control	0	Conf.	0
Conjugative mix			
1×10^8 a	>100	Conf.	>100
1×10^6	5	150	15
1×10^5	0	0	3

a - number of P. aeruginosa cfu present on control plates.

P. aeruginosa PA04069 control grew when selected on streptomycin (400ug/ml) alone but did not grow in the presence of gentamicin (25ug/ml). Putative transconjugant colonies were observed on selection plates containing gentamicin. To isolate P. aeruginosa from any E. coli contamination, 23 well-isolated colonies were streaked to single colonies on C-N agar plates. C-N agar is a pseudomonal-selective medium allowing growth of P. aeruginosa but preventing growth of any putative E. coli contaminant that may have survived the initial selection. Selection for pRTG1 was not used because of the possibility of P. aeruginosa protecting any E. coli SM10 (pRTG1) present.

Twenty-three well isolated colonies were streaked to single colonies on C-N plates. Single colonies from C-N plates were streaked onto nutrient agar containing gentamicin (32ug/ml) and nutrient agar containing streptomycin (400ug/ml). Any isolate found to be resistant to both antibiotics was considered indicative of the presence of pRTG1. A single colony was then streaked from an antibiotic plate to a C-N plate, to select for P. aeruginosa PA04069 again. Streaking of single colonies between C-N and nutrient agar with antibiotic selection was repeated once more with a final streaking onto nutrient agar containing both gentamicin (32ug/ml) and streptomycin (400ug/ml). Pseudomonal colonies are known to be pigmented on C-N agar (Difco Manual); pigmentation was observed, colonies were oxidase-positive and only a single colony form was observed on nutrient agar. At this point I considered that contamination with E. coli SM10 (pRTG1) was unlikely. Initially, five colonies resistant to both streptomycin and gentamicin were selected for further investigation by DNA analysis.

Electrophoresis of plasmid DNA from five putative pRTG1 transconjugants gave three different profiles (Figure 5.1). All five strains contained DNA bands (designated pRTG1a-pRTG1e) absent from a parental extract and assumed to have arisen from conjugation with E. coli SM10 (pRTG1). The presence of three different profiles indicated that pRTG1 had undergone alterations in P. aeruginosa. Plasmid pRTG1 was altered least in two transconjugants, pRTG1a and pRTG1e (Figure 5.2). Furthermore, in P. aeruginosa most plasmid DNA was present in a complex form. Of a further 21 transconjugants which had shown streptomycin and gentamicin resistance, 16 were unstable with loss of resistance on sub-culturing and 5 showed deletions within pRTG1 similar to those shown in Figure 5.1. Because of the apparent instability of pRTG1 in P. aeruginosa, alternative methods for introducing native pRTG1 were attempted with the aim of producing a collection of stable strains of P. aeruginosa (pRTG1) in which temperature-sensitivity studies could be performed (Appendix V). I also chose to analyze the behaviour of pRTG1 derivatives already introduced into P. aeruginosa in order to test whether any of the pRTG1-derivatives were still temperature- sensitive and able to generate hyperproducing B-lactamase mutants by transposition mutagenesis with Tn5::gen.

5.2.3 Temperature-sensitivity of pRTG1 in P. aeruginosa

Plasmid pRTG1a was tested for temperature-sensitivity as it appeared most similar in size to native pRTG1. To test whether pRTG1a was temperature-sensitive P. aeruginosa PAO4069 (pRTG1a) was first grown for 8 hours at 30°C in nutrient broth. Both vector and transposon DNA were selected by the presence of gentamicin (32ug/ml)

Figure 5.1: Plasmid DNA Extracted from Five Putative P. aeruginosa PA04069 (pRTG1) Transconjugants. Lane A - pRTG1e; Lane B - pRTG1d; Lane C - pRTG1c; Lane D - lambda HindIII; Lane E - pRTG1b; Lane F - pRTG1a; Lane G - P. aeruginosa PA04069 control.

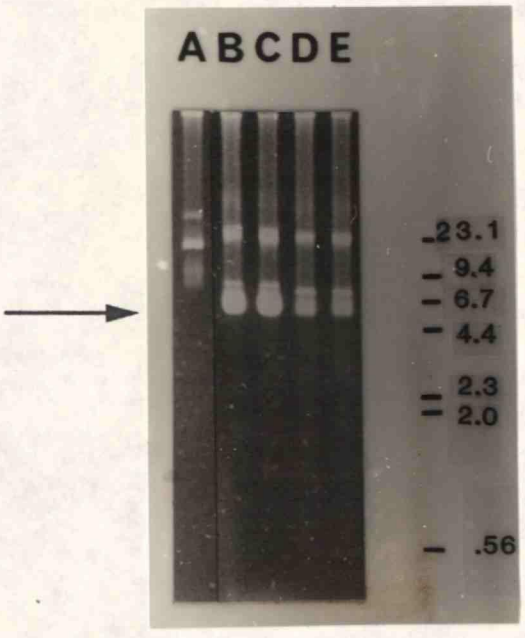


Figure 5.2: Relative Mobilities of Two P. aeruginosa PA04069 (pRTG1) Transconjugants against E. coli SM10 (pRTG1). Lane A - E. coli SM10 (pRTG1); Lanes B and C - pRTG1e; Lanes D and E; pRTG1a. Highly supercoiled DNA is arrowed.

and streptomycin (400ug/ml) in the growth medium. P. aeruginosa PA04069 (pRTG1a) (20ul) was used to inoculate two prewarmed nutrient broths (20ml); one incubated at 30°C and the other at 42°C. After maintaining log growth for 50 hours, samples (100ul) were plated onto nutrient agar containing gentamicin (32ug/ml) to test for the retention of Tn5::gen and also nutrient agar containing streptomycin (320ug/ml) for the retention of vector (Table 5.10).

TABLE 5.10 The Number of Resistant Colonies of P. aeruginosa PA04069 (pRTG1a) After Growth in the Absence of Antibiotic Selection at Two Temperatures

	Number of colonies on nutrient agar containing antibiotic (ug/ml)		
Temp. °C.	Nutrient agar	Gm (32)	Sm (320)
30	208, 203	137, 143	181, 116
30	24, 27	6, 14	24, 29
42	1.7×10^4	~800	~400
42	168	7, 1	1, 4

Plasmid pRTG1a appeared to be relatively stable in the absence of selection at 30°C with both gentamicin and streptomycin resistances being retained in approximately 75% of the population. At 42°C gentamicin-resistance was retained more often than streptomycin-resistance.

The experiment was repeated with inoculation from an overnight culture grown at 30°C as described above. Two prewarmed nutrient broths (20ml) were inoculated of which one contained gentamicin (32ug/ml) to continue selection for Tn5::gen. In this experiment, replication of pRTG1a was inhibited by incubation for 16 hours at 42°C and was followed by dilution and plating onto nutrient agar containing

gentamicin (32ug/ml) to test for the retention of Tn5::gen. Two prewarmed nutrient broths (20ml) one containing gentamicin (32ug/ml) were inoculated with an overnight culture of parental P. aeruginosa PAO4069 (20ul) and used as negative controls. Plates were incubated for a further 24 hours and the numbers of colonies recorded, as shown in Table 5.11.

TABLE 5.11 Numbers of Colonies of P. aeruginosa PAO4069 (pRTG1a) with Gentamicin-resistance After Incubation at 42°C

P. aeruginosa strain:	Nutrient agar containing antibiotic (ug/ml)	
	cfu/plate viability	Gentamicin (32)
PAO4069 Control[a]	$3x10^5$	0
(no gentamicin)	$3x10^4$	0
PAO4069 (pRTG1a)	$4x10^5$	~300, ~250
(no gentamicin)	$4x10^4$	17, 7
PAO4069 (pRTG1a)	$4x10^4$	~4000, ~4,000
gentamicin (32ug/ml)	$4x10^3$	~400, ~300

a - The parental control incubated in nutrient broth containing gentamicin (32ug/ml) did not grow.

Plasmid pRTG1 was very unstable as 90% of the population showed loss of gentamicin-resistance at 42°C even when grown in the presence of gentamicin (32ug/ml). To test whether any of the gentamicin-resistant colonies retained pRTG1, plates with distinct colonies were replica-plated onto streptomycin (400ug/ml). They were also replica-plated onto nutrient agar containing cefsulodin (32ug/ml) to identify any possible B-lactamase hyperproducing mutants. Plates were incubated at 37°C. Between 30-50% of colonies retained resistance to streptomycin (400ug/ml) and all were resistant to cefsulodin (32ug/ml). Population densities have been shown to affect the MIC

values of cefsulodin (see 5.2.1). To test whether the population density affected the results here, ten single colonies showing resistance to cefsulodin on the replica-plates were diluted in M9 minimal salts and replated at a density of $3x10^3$ cfu onto nutrient agar containing cefsulodin (32ug/ml). Bacteria were also spread onto nutrient agar containing streptomycin (400ug/ml). None were cefsulodin-resistant (32ug/ml) and five of the ten tested retained streptomycin-resistance (400ug/ml).

5.2.4 Generation of B-lactam-resistant mutants of P. aeruginosa

The effect of population density on the resistance to B-lactams meant that screening for B-lactam-resistance by replica-plating gentamicin-resistant colonies was unlikely to succeed. The protocol was altered so that after incubation at 42°C, cells were grown initially on nutrient agar containing cefsulodin (32ug/ml). Any cefsulodin-resistant colonies would be tested for the presence of Tn5::gen by replica-plating onto nutrient plates containing gentamicin (32ug/ml). Unfortunately, no resistant colonies were obtained at population densities less than $4x10^6$ cfu and above this population density parental resistance was observed. Ceftazidime (32ug/ml) was used as an alternative B-lactam to test whether a different B-lactam could generate B-lactamase mutants without generating spontaneous resistance mutations. Growth was not observed at population densisties of $3x10^7$ cfu per plate. The frequency of spontaneous mutation in P. aeruginosa PA04069 to cefsulodin at 8xMIC ($2.5x10^{-7}$) was similar to that obtained for 3-Pre ($1x10^{-7}$, Hewinson 1987). The frequency of spontaneous mutation to ceftazidime was $3x10^{-8}$, as opposed to $3x10^{-7}$ obtained for 3-Pre (Hewinson 1987). As the spontaneous frequency of resistance to ceftazidime was less common I decided to use it for selecting

transposon-mediated B-lactam resistant mutants.

I expected that B-lactam-resistant mutants produced as a result of transposon mutagenesis to be very rare. Postharvest selection for such resistance was going to require many plates and hyperproducing B-lactamase mutants due to Tn5::gen transposition being so rare as to be undetectable within the spontaneous background mutation rate. I therefore decided to select for B-lactam-resistance by adding ceftazidime to broth cultures at 42°C. Any ceftazidime-resistant mutant would be selected for and be amplified under such conditions. Although spontaneous resistance might occur it was hoped that enough transposon-induced resistant mutants would be obtained in order to characterize the mutation with respect to the location of Tn5::gen in the chromosome.

P. aeruginosa PAO4069 containing pRTG1a was incubated overnight at 30°C in nutrient broth containing gentamicin (32 ug/ml) and streptomycin (400 ug/ml) to maintain pRTG1. After growth at 42°C for 12 hours in the absence of antibiotic selection, samples (20ul) were used to inoculate nutrient broth (20ml) and nutrient broth containing gentamicin (32ug/ml) to enrich the population carrying Tn5::gen. After incubation at 42°C for 16 hours the sample grown in the absence of gentamicin was inoculated (1ml) into nutrient broth (100ml) containing ceftazidime (25ug/ml) and the sample grown in gentamicin was inoculated (1ml) into nutrient broth (100ml) containing gentamicin (32ug/ml) and ceftazidime (25ug/ml). After incubation at 42°C for 14 hours selection was made on nutrient agar containing a range of selective media as shown in Table 5.12.

TABLE 5.12 **Numbers of Colonies of *P. aeruginosa* PAO4069 (pRTG1a) Resistant to Ceftazidime at Different Concentrations After Incubation at 42°C**

Incubation conditions		Selection conditions Nutrient agar containing ceftazidime (ug/ml)			
First grown:	Subcultured into:	Viability cfu/plate	Ctz (8)	Ctz (25)	Ctz (64)
no antibiotic	Ctz (25)	~$2x10^6$	0	0 0	0 0
		~$2x10^5$	0	0	0
Gm (32)	Gm (32)	~$1x10^6$	55	53 84	49 52
	Ctz (25)	~$1x10^5$	-	5	11

Gm - Gentamicin Ctz - Ceftazidime

Figures in brackets refer to the concentration of antibiotic (ug/ml).

Ceftazidime-resistant mutants were generated in a culture where co-selection for gentamicin-resistance existed but not when ceftazidime-resistance was selected alone.

5.2.5 Investigating the stability of ceftazidime-resistant mutants

Putative ceftazidime-resistant mutants grown in nutrient broth lacking antibiotic selection were not ceftazidime-resistant when spread on selection plates. If ceftazidime-resistance could be retained in cultures grown in N-broth containing gentamicin (32ug/ml) I considered it an indication of Tn5::gen transposition into regulatory genes of *blaP*.

Thirty-six isolates from Nutrient agar containing ceftazidime (25ug/ml) were grown in N-broth containing gentamicin (32ug/ml) at 37°C for 18 hours and were then plated at various dilutions onto the selection plates shown in Table 5.13.

TABLE 5.13 Numbers of Colonies Growing on Various Selection Media to Test for the Retention of Ceftazidime-resistance

Strain:	1	2	3	4	5	6	7	8	9	10	11	12
Nutrient	112	129	119	95	88	131	94	112	109	97	132	178
Gm (32)	109	123	120	121	84	120	87	96	102	123	115	159
Cfz (16)	34	41	26	14	31	39	32	12	30	37	29	18
Strain:	13	14	15	16	17	18	19	20	21	22	23	24
Nutrient	27	20	18	13	22	28	32	12	23	12	16	22
Gm (32)	9	10	13	9	20	9	10	17	15	16	8	17
Cfz (16)	3	9	8	6	0	9	12	12	11	13	2	1
Strain:	25	26	27	28	29	30	31	32	33	34	35	36
Nutrient	17	16	72	22	66	35	28	2	13	16	27	18
Gm (32)	2	9	59	13	60	18	7	2	7	1	9	10
Cfz (16)	4	2	1	10	61	0	9	2	8	10	0	4

Strains 14, 15, 20, 21, 22, 28, 29 and 33 (designated M1-M8) were chosen for further analysis as they showed relatively stable retention of ceftazidime-resistance with gentamicin-resistance. Individual colonies of each strain grown on nutrient agar containing gentamicin (32ug/ml) were streaked onto nutrient agar containing ceftazidime (16ug/ml) and nutrient agar containing gentamicin (32ug/ml) and ceftazidime (20ug/ml). All eight strains were resistant to ceftazidime alone and in the presence of gentamicin.

The eight ceftazidime- and gentamicin- resistant strains were then tested for hyperproduction of B-lactamase using the nitrocefin drop test. Colonies from the gentamicin-ceftazidime agar plates were grown overnight in nutrient broth containing gentamicin (32ug/ml) and streaked onto nutrient agar containing gentamicin (32ug/ml). P. aeruginosa PAO4069 and P. aeruginosa PAO4069 (pRTG1a) were also

tested; P. aeruginosa PAO4069 was grown on nutrient agar and P. aeruginosa PAO4069 (pRTG1a) was grown on nutrient agar containing gentamicin (32ug/ml). There was very little colour change of nitrocefin by colonies of the two control strains even after 5 minutes after nitrocefin application. However, a total colour change occurred within 30 seconds after the addition of nitrocefin in all of the eight ceftazidime-resistant strains. Prior to detailed analysis of B-lactamase activities of these putative Tn5::gen-mediated ceftazidime-resistant mutants (see Chapter 6) the presence of Tn5::gen in the pseudomonas chromosome was determined.

5.2.6 Analysis of Chromosomally-inserted Tn5::gen in Ceftazidime-resistant P. aeruginosa

The objectives here were to use Southern blotting and hybridization techniques to locate Tn5::gen in the chromosome of eight strains of P. aeruginosa PAO4069 which had been shown to be resistant to gentamicin (32ug/ml), indicating the presence of Tn5::gen, and ceftazidime (32ug/ml). Tn5::gen probe consisted of the 4.27Kbp HpaI-fragment of pRTG1 and the vector probe was the 3.00Kbp HpaI fragment (Figure 5.3). Chromosomal DNA was extracted (see Methods, Figure 5.4) and digested with SalI (Figure 5.5a). As Tn5::gen does not contain any SalI sites, any hybridizing fragment should identify different transposon inserts in the chromosome. Following gel electrophoresis SalI-digested chromosomal DNA was denatured, transferred to a nylon membrane and hybridized to probe DNA (see Methods). An autoradiograph of SalI-digested chromosomal DNA after hybridization with Tn5::gen probe DNA is shown in Figure 5.5b. DNA from all strains except the parental control hybridized to Tn5::gen probe DNA. Although SalI-digestion of chromosomal DNA was incomplete, distinct hybridizing bands were seen

Figure 5.3: Purified pRTG1 HpaI Fragments. Lane A - DNA for Tn5::gen probe (pRTG1 HpaI fragment 4.27Kbp); Lane B - lambda HindIII; Lane C - DNA for vector probe (pRTG1 HpaI fragment 3.00Kbp).

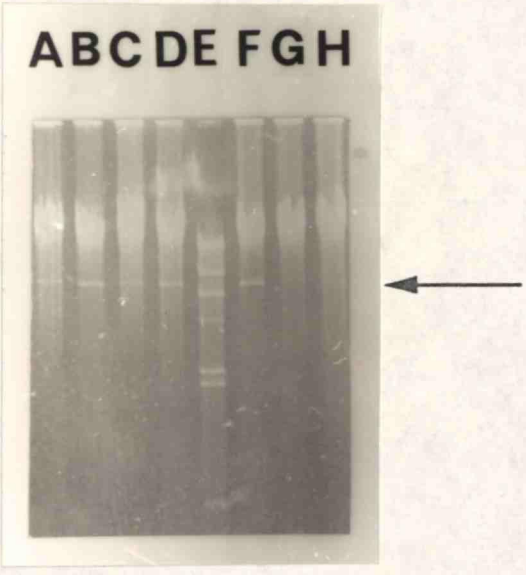


Figure 5.4: Chromosomal DNA Extracts of P. aeruginosa PA04069 (pRTG1a) Hyperproducing B-Lactamase. Lane A - M7; Lane B - M6; Lane C - M5; Lane D - M4; Lane E - lambda HindIII; Lane F - M3; Lane G - M2; Lane H - P. aeruginosa PA04069 control. In all ceftazidime-resistant strains a satellite band is present (arrowed) migrating with a relative mobility of 7.95Kbp against lambda HindIII DNA.

in each isolate. Three Tn5::gen binding regions were present in strains M1, M3, M5 and M7 and two were present in strains M2, M4 and M6. One band was present in strain M8 and had a similar mobility to that found in uncut DNA from strain M6.

DNA from all strains except the parental control and strain M8 hybridized to vector DNA probe (3.00Kbp) (Figure 5.5c). Two distinct hybridizing regions were present in the other strains (M1-M7). Two bands of similar mobilities to those described were also present in uncut DNA from strain M6 but not present in uncut pRTG1.

The experiment was repeated with chromosomal DNA which had been digested for a longer time with SalI (Figure 5.6a). Where DNA was completely digested only two hybridizing bands were observed with Tn5::gen as probe (strains M4, M6, M7) whilst three distinct hybridizing bands were apparent with Tn5::gen as probe in partially digested DNA (strains M1, M2, M3 and M5) (Figure 5.6b). Again vector probe hybridized to two distinct bands which migrated with differing mobilities to those bands binding to Tn5::gen DNA (Figure 5.6c).

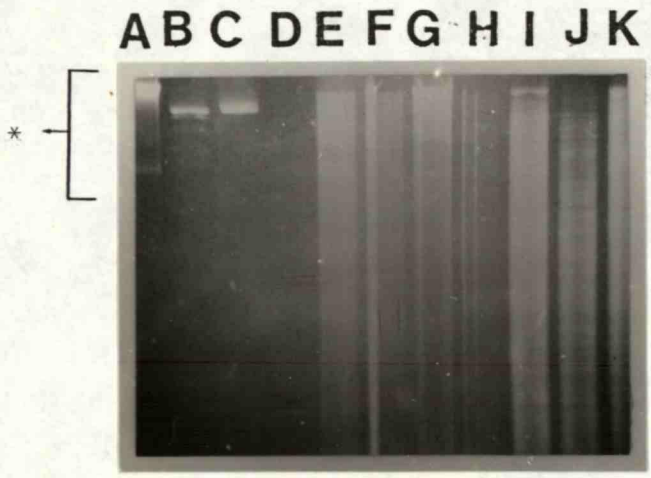


Figure 5.5a: SalI-digests of Chromosomal DNA from P. aeruginosa PA04069 (pRTG1a) Hyperproducing B-Lactamase. Lane A - M6; Lane B - pRTG1 EcoR1; Lane C - pRTG1 uncut; Lane D - M8 SalI; Lane E - M7 SalI; Lane F - M6 SalI; Lane G - M5 SalI; Lane H - M4 SalI; Lane I - M3 SalI; Lane J - M2 SalI; Lane K - M1 SalI; (P. aeruginosa PA04069 chromosomal DNA SalI digest is positioned adjacent to Lane K but was not photographed).

* - No homology to probe DNA was found in autoradiographs beyond the boundaries indicated.

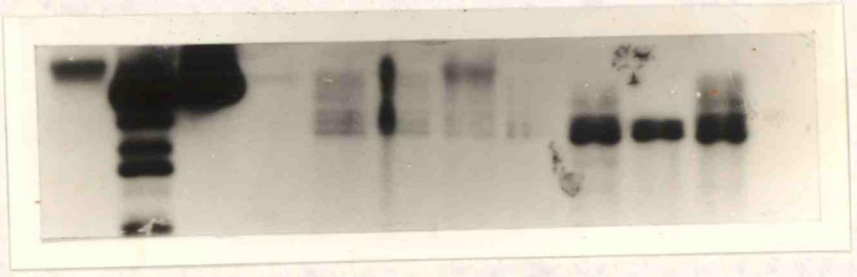


Figure 5.5b: Autoradiograph of SalI-digested Chromosomal DNA probed with ^{32}P-"Tn5::gen" DNA. Lanes as in Figure 5.5a.

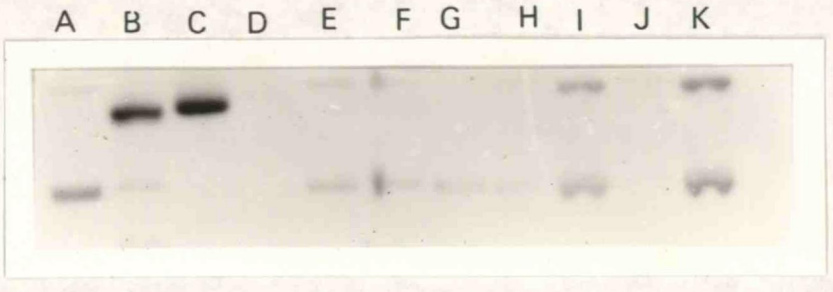


Figure 5.5c: Autoradiograph of SalI-digested Chromosomal DNA probed with ^{32}P-"vector" DNA. Lanes as in Figure 5.5a.

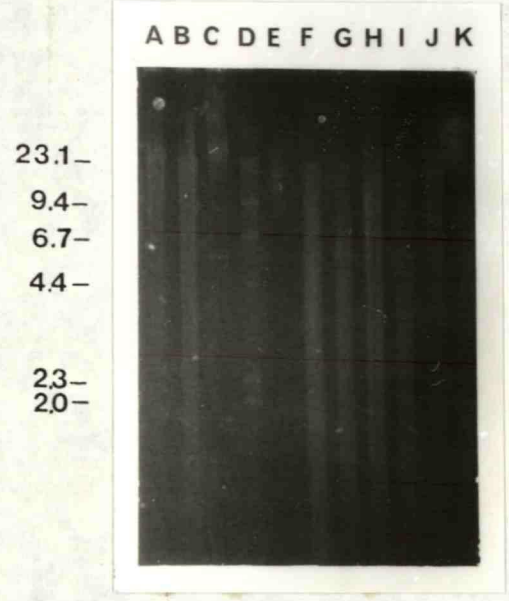


Figure 5.6a: SalI-digests of Chromosomal DNA from P. aeruginosa PA04069 (pRTG1a) Hyperproducing B-Lactamase. Lane A - M3 SalI; Lane B - M1 SalI; Lane C - M6 uncut; Lane D - Lambda HindIII; Lane E - pRTG1 BglII; Lane F - M7 SalI; Lane G - M6 SalI; Lane H - M5 SalI; Lane I - M4 SalI; Lane J - M2 SalI; Lane K - P. aeruginosa PA04069 SalI.

Figure 5.6b: Autoradiograph of SalI-digested Chromosomal DNA Probed with ^{32}P-"Tn5::gen" DNA. Lanes contain DNA described in Figure 5.6a.

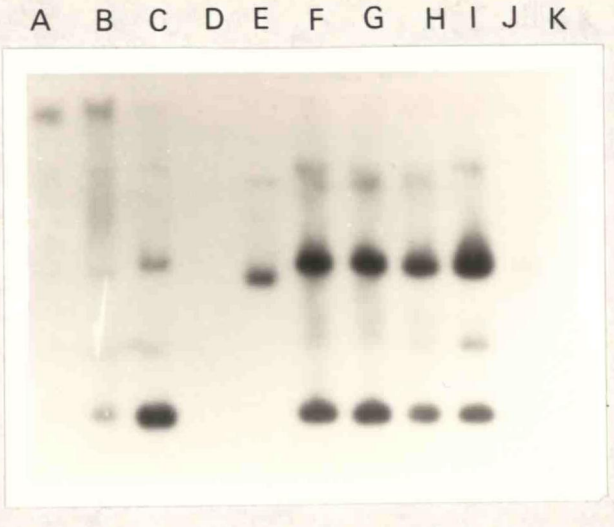


Figure 5.6c: Autoradiograph of SalI-digested Chromosomal DNA Probed with ^{32}P-"vector" DNA. Lanes contain DNA described in Figure 5.6a.

5.3 Discussion

Selection for transfer of pRTG1 into P. aeruginosa was unsuccessful in the initial experiments and it was possible that this was a result of poor expression of the pRTG1-encoded resistance genes in the new host. However, reducing the concentration of streptomycin gave rise to parental growth of P. aeruginosa PAO4069. E. coli SM10 (pRTG1) did not grow on parental control plates but contaminated transconjugant colonies, indicated that the original selection may have selected for a symbiotic relationship; with E. coli SM10 (pRTG1) providing antibiotic-resistance and P. aeruginosa PAO4069 providing essential nutrients for the survival of E. coli SM10 (pRTG1). Once selection against E. coli SM10 (pRTG1) had been removed, E. coli SM10 (pRTG1) was able to survive in the absence of P. aeruginosa PAO4069. Tetracycline was used to counter-select against E. coli SM10 (pRTG1) and transconjugants were also purified by three cycles of growth with alternate selection for P. aeruginosa and pRTG1.

There was good evidence for deletions of pRTG1 in P. aeruginosa from plasmid analysis and instability of resistance phenotypes in other transconjugants. Alterations of the multi-copy pRTG1 in P. aerginosa could have been due to a number of factors. P. aeruginosa PAO4069 is not a recA strain and may be intolerant to multiple copies of DNA as are many P. aeruginosa strains. The transposon also offers a site of homology within the plasmid and could act as a site for recombination events within pRTG1. Such alterations may have occurred during the isolation of P. aeruginosa on C-N plates. Transformation of P. aeruginosa PAO4069 with pRTG1 might offer a preferable strategy for the transfer of pRTG1 as there would be no donor contamination,

however, with the failure to improve the method of introducing pRTG1 (see later, Appendix V) I decided to use the pRTG1 derivatives already obtained from transconjugants to generate transposon-mediated B-lactam-resistant mutants.

P. aeruginosa PA04069 (pRTG1a) showed a loss of gentamicin- and streptomycin- resistance in the absence of antibiotic selection at both 30°C and 42°C. The number of streptomycin-resistant and gentamicin-resistant strains were approximately equal but accounted for only 75% of all viable cells grown at 30°C in the absence of selection. However, at 42°C streptomycin-resistance was retained in less than half the number of colonies that gentamicin-resistance was. This was expected if the plasmid was temperature-sensitive and Tn5::gen had transposed into the chromosome within a sub-population.

Strain P. aeruginosa (pRTG1a) was used to generate hyperproducing B-lactamase mutants. Identification of cefsulodin-resistant mutants generated by transposon mutagenesis by replica-plating gentamicin-resistant colonies onto nutrient agar containing cefsulodin was unsuccessful due to the high population density permitting all colonies to appear cefsulodin-resistant. Initial selection on nutrient agar containing cefsulodin followed by replica-plating onto nutrient agar containing gentamicin was also unsuccessful at obtaining mutants generated by transpositional mutagenesis, as the frequency of cefsulodin mutation was no greater than the spontaneous resistance frequency. Initial selection on ceftazidime was also unsuccessful at selecting Tn5::gen mutants, however, the spontaneous resistance frequency was less than that for cefsulodin and ceftazidime was used in further experiments. Initial selection for ceftazidime-resistance

and retention of gentamicin-resistance can not identify a causal link between transposition of Tn5::gen and B-lactam resistance. To identify any Tn5::gen-mediated B-lactamase hyperproducing mutants large numbers of ceftazidime- and gentamicin-resistant colonies would be required to screen for a consensus position of Tn5::gen in the chromosome. Using only one transconjugant had the disadvantage that transposition events into the chromosome prior to growth at the non-permissive temperature would generate a consensus location of Tn5::gen unrelated to B-lactam-resistance. As a result it was considered very important to obtain a reproducible method of transferring pRTG1 into P. aeruginosa and transfer experiments are described in Chapter 7 and Appendix V.

At the same time I considered that analysis of any B-lactam-resistant mutants generated in P. aeruginosa PA04069 (pRTG1a) after growth at a non-permissive temperature may still be of use in determining whether Tn5::gen could generate B-lactam resistant mutants by hyperproduction of B-lactamase. Identification of ceftazidime-resistant mutants by growing cultures in nutrient broth containing ceftazidime (25ug/ml) was considered one possible way of increasing the probability of obtaining Tn5::gen-mediated ceftazidime-resistant mutants. P. aruginosa PA04069 (pRTG1a) grown in nutrient broth containing ceftazidime (25ug/ml) alone did not generate ceftazidime-resistant mutants. Ceftazidime-resistant mutants were obtained, however, from the culture in which both ceftazidime and gentamicin were present. Gentamicin may have effected some form of ceftazidime-resistance in this culture but this possibility could not be tested in P. aeruginosa PA04069 as gentamicin prevented growth of control cultures. It was possible that selection of gentamicin-resistance was required for the

emergence of ceftazidme-resistance and identification of Tn5::gen in gentamicin and ceftazidime-resistant mutants was attempted.

In the absence of selection the loss of ceftazidime-resistance within the 36 isolates analysed showed the resistance-phenotypes were very unstable. In most cases ceftazidime-resistance was not retained by the presence of gentamicin either. I would suggest that these unstable B-lactam-resistant mutations could possibly be the result of spontaneous or transposon-induced mutations which were very detrimental to the host. When incubation in the presence of gentamicin caused retention of ceftazidime-resistance it indicated a possible causal link between the presence of Tn5::gen and ceftazidime-resistance. For this reason, isolates showing similar numbers of gentamicin and ceftazidime resistant colonies were considered those most likely to be ceftazidime-resistant as a result of transposon mutagenesis. The eight mutants chosen for further analysis on the basis of co-stability to ceftazidime and gentamicin resistance were all B-lactamase hyperproducers as shown by the nitrocefin drop test. The presence of gentamicin in the nutrient agar did not cause induction of B-lactamase in these strains since P. aeruginosa PAO4069 (pRTG1a) grown on nutrient agar containing gentamicin (32ug/ml) was shown to hydrolyse nitrocefin only very slowly.

The presence of Tn5::gen and pRTG1 in these mutants was tested for by Southern blotting and hybridization with probe DNA of Tn5::gen (4.27Kbp) and pRTG1 (3.00Kbp) obtained from HpaI-digested pRTG1. The HpaI fragment of pRTG1 (3.00Kbp) contained 185 bp of Tn5::gen but hybridization with Tn5::gen DNA was not observed here or in control

experiments. Probe Tn5::gen DNA did not hybridize to the chromosomal control P. aeruginosa PAO4069 DNA but did hybridize to uncut chromosomal DNA of the gentamicin-resistant strain M6 indicating the presence of Tn5::gen in the chromosome. No hybridization occured between Tn5::gen and the satellite band seen in uncut DNA of strain M6, indicating that Tn5::gen was present only in the chromosome. Hybridization of SalI-digested chromosomal DNA with Tn5::gen showed two distinct hybridizing regions to Tn5:gen DNA in similar positions in strains M2, M4, M6 and M7 and three in similar positions in DNA from strains M1, M3 and M5, which were presumably a result of partial SalI-digestion of chromosomal DNA. The single hybridizing site in strain M8 had similar mobility to that of uncut chromosomal DNA of strain M6 and may have been the result of inactivity of SalI. As no SalI sites are present in pRTG1 I would suggest that the two common hybridization bands were a result of transposition of two separate Tn5::gen transposons in the chromosome. Multiple insertions of Tn5::gen may not have been expected since only single insertions of Tn5 in P. aeruginosa PAO had been observed previously (Krishnipillai et al. 1986). Multiple insertions may have been selected for if single insertions were unable to confer resistance to high concentrations of gentamicin (30ug/ml) or less probably that two separate transposition insertions were required for ceftazidime-resistance. The similar mobilities of the insertions in each strain suggests that they are clonal in origin. Unfortunately, as no B-lactam-sensitive gentamicin-resistant strains from the same experiment were tested it was not possible to determine whether the transposon locations were specific to strains hyperproducing B-lactamase.

The HpaI-pRTG1 fragment used as vector probe did not hybridize to parental DNA but did hybridize to two bands of SalI-digested DNA found in strains M1-M7. In strain M8 the weak concentration of DNA may explain the lack of hybridization. DNA which hybridized to vector probe DNA did not hybridize to Tn5::gen probe DNA and I would suggest that the high molecular weight band with homology to vector DNA is not the result of transposon-mediated plasmid insertion into the chromosome. The satellite band that hybridized vector probe did not have the same mobility as uncut pRTG1 and was probably a derivative of pRTG1, which did not contain Tn5::gen DNA since it did not hybridize to Tn5::gen DNA. As the plasmid had survived prolonged growth at non-permissive temperatures for pRTG1, it must be a temperature-stable revertant of pRTG1.

In conclusion, pRTG1 was introduced into P. aeruginosa with difficulty, a problem investigated further in Appendix V. Both loss of resistance phenotypes and replication-stability were observed in transconjugants. However, after incubation of P. aeruginosa PA04069 (pRTG1a) at non-permissive temperatures, ceftazidime-resistant mutants were obtained from a culture pre-incubated with gentamicin and ceftazidime but not with ceftazidime alone. Ceftazidime-resistance was maintained by growth in gentamicin in some strains. In eight mutants selected for further analysis, DNA-DNA hybridization showed that Tn5::gen had transposed into the chromosome of P. aeruginosa PA04069 independently of the plasmid. The eight mutants were all B-lactamase hyperproducers and had Tn5::gen in similar if not identical positions in the chromosome. However, it was not possible to conclude that the transposon insertions had caused ceftazidime-resistance without repeating the experiment with other

transconjugants.

Attempts were made to clone Tn5::gen from genomic DNA from the eight ceftazidime-resistant mutants but were not successful. The inability to clone Tn5::gen from the chromosome may have been due to the large number of small chromosomal DNA fragments that were present in the ligation mixture. The method could have been improved by purifying SalI-digested DNA of the approximate size of the SalI fragments containing Tn5::gen. However, prior to repeating the cloning of Tn5::gen it was considered necessary to repeat the entire experiment again with other transconjugants in order to obtain a consensus sequence related to B-lactam-resistance. Further analysis of the B-lactamase activity of these ceftazidime-resistant mutants was also considered useful in order to quantify more accurately the B-lactamase activity in these strains and to identify whether any of these mutants had similar B-lactamase activity to mutants previously described by Matsumoto and Terawaki (1982) (Chapter 6).

CHAPTER 6

CHARACTERIZATION OF B-LACTAM-RESISTANT MUTANTS

Objectives: To analyse ceftazidime-resistant mutants of P. aeruginosa using kinetic analysis of B-lactamase activity.

6.1 Introduction

P. aeruginosa mutates to become resistant to antipseudomonal B-lactams (Brown 1975). One mechanism is increased production of the chromosomal B-lactamase (Livermore et al. 1985) and may involve mutations in genes controlling B-lactamase production (Matsumoto and Terawaki 1982). Strains with relatively greater basal activity which are induced to much greater activities in response to B-lactam inducers may become resistant to third-generation B-lactams more frequently than those strains with relatively low basal and induced B-lactamase activities (Hewinson 1987). It is possible that the difference between these two phenotypes is that those with relatively high basal B-lactamase activities already have a mutation in one regulatory gene of B-lactamase and only require a single mutation to become B-lactamase hyperproducers.

Initially P. aeruginosa PAT210 was to be used as the test strain for detecting B-lactamase hyperproducers as a result of transposon mutagenesis. The B-lactamase activity in this strain was assessed from induced and non-induced cultures to determine whether P. aeruginosa PAT210 was a suitable strain for selecting B-lactamase hyperproducers. The B-lactamase activity of P. aeruginosa PAT210 was compared with the B-lactamase activity of a strain, 3-Pre, known to be

capable of mutating to B-lactam resistance by increasing the production of the chromosomal B-lactamase, (Slack and Pitt 1982, Hewinson and Nichols 1987).

Kinetic analyses was performed using the automated half-time analysis apparatus (Nichols and Hewinson 1987). The technique is based on the half-time analysis of single progress curves of reaction (Wharton and Szawelski 1982, Wharton 1983). Equation 1 (Wharton and Szawelski 1982) was derived from the Michaelis-Menten equation:

Equation 1

$$t_{1/2} = \frac{S_o}{2.V_{max}(app)} + \frac{K_m(app)\ \ln 2}{V_{max}(app)}$$

S_o is the concentration of substrate at any given point on the progress curve and $t_{1/2}$ is the time taken for S_o to drop to $S_o/2$. $V_{max}(app)$ and $K_m(app)$ are values dependent on the kinetic mechanism of the reaction. It is assumed that the reaction here follows Michaelis-Menten kinetics and the values are equivalent to V_{max} and K_m.

The computer recorded the substrate concentration in terms of a voltage reading from the spectrophotometer which was usually taken every ten seconds. A number of half-time values were obtained from a single reaction progress curve and a graph plotted of half-time values against S_o. V_{max} was calculated from the slope of the line and K_m from the intercept at $S_o=0$. K_m was measured in uM and V_{max} was measured in nmol (cephalosporin hydrolysed)/min/mg dry weight.

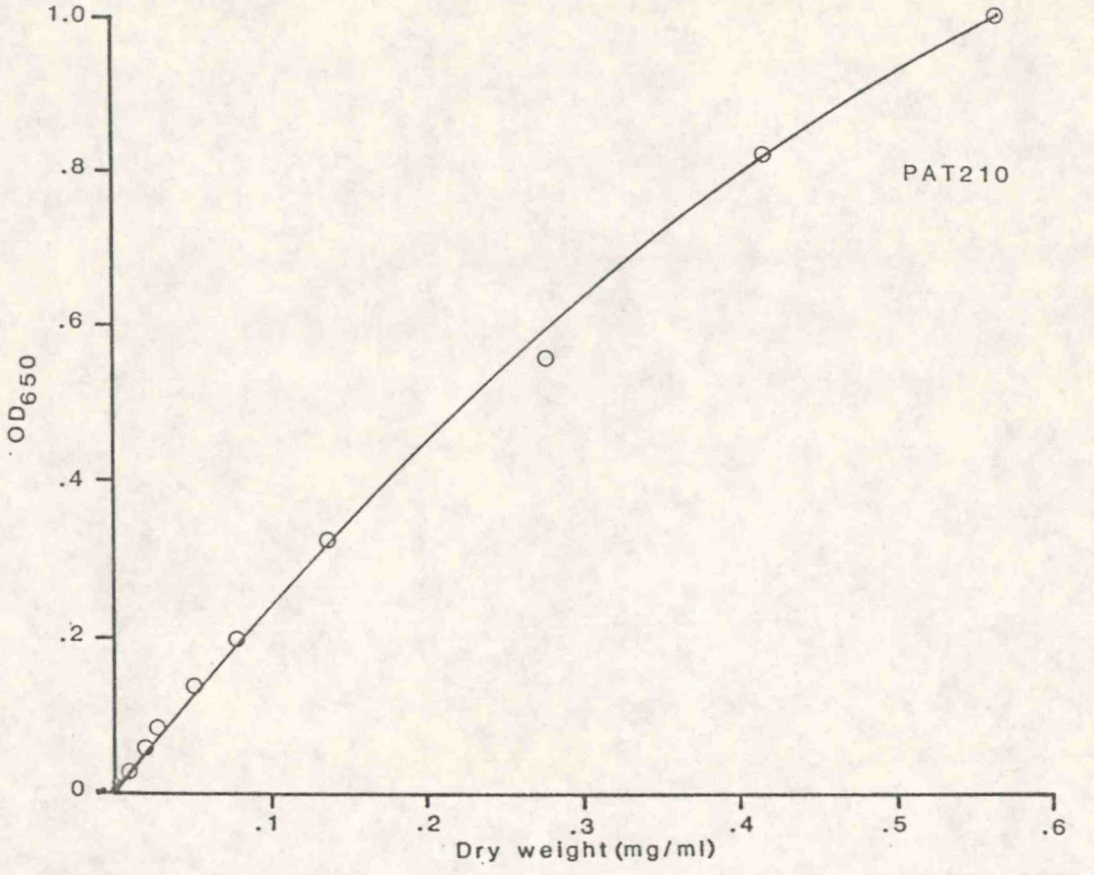
6.2 Results

6.2.1 Initial characterization of B-lactamase activity in *P. aeruginosa* PAT210 and 3-Pre

The dry weight needed to be determined in order to calculate the B-lactamase activity (see Methods). The dry weight obtained for *P. aeruginosa* PAT210 was 2.84mg/ml and for *P. aeruginosa* 3-Pre, 4.43 mg/ml. The cell density was plotted against dry weight in order to calculate growth curves with respect to dry weight of cells (Figure 6.1).

Growth curves of *P. aeruginosa* PAT210 and 3-Pre were determined in order to predict the time taken for a culture to obtain an OD_{650} of 0.2 (~0.1mg/ml); the measurement used to harvest cells for B-lactamase extraction (Figure 6.2). Cultures of *P. aeruginosa* PAT210 and 3-Pre were grown overnight in 5ml CDM at 37°C with orbital shaking at 190rpm. Each culture (0.8ml) was used to inoculate 80ml CDM in 250ml baffled flasks, the cultures were incubated at 37°C with shaking as above and OD_{650} was measured periodically.

Induced and non-induced cultures (80ml CDM) of *P. aeruginosa* PAT210 and 3-Pre were grown for B-lactamase extraction. Sterile benzylpenicillin (1ml of 16mg/ml) or 1ml of sterile water was added to one culture of each strain two hours prior to harvest. B-lactamase was extracted by a freeze-thaw method and analysed as described in Methods. Results are shown in Table 6.1.



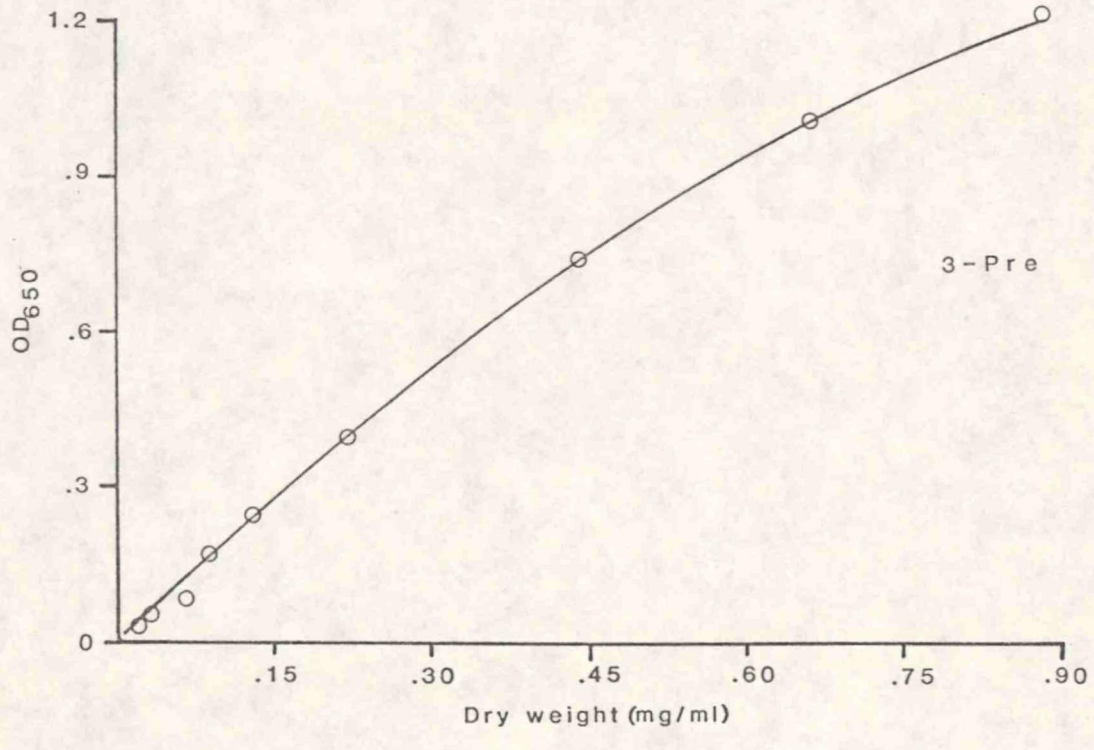


Figure 6.1

OD_{650} against dry weight (mg/ml) calibration curves for *P. aeruginosa* PAT210 and 3-Pre.

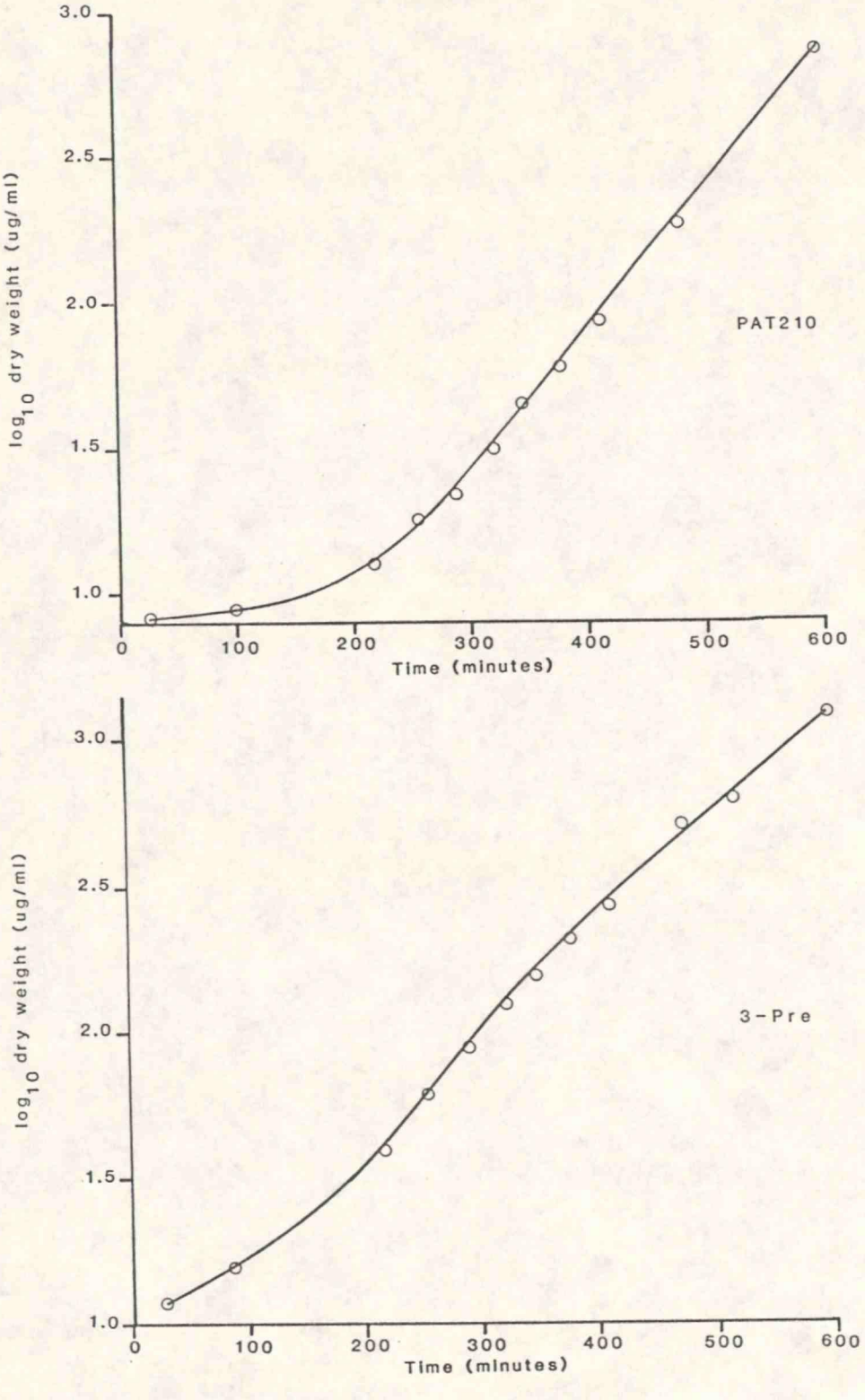


Figure 6.2

Growth curves of $\log_{10}$ dry weight (ug/ml) against time (minutes) for *P. aeruginosa* PAT210 and 3-Pre.

TABLE 6.1. Kinetic Analysis of Freeze/thaw Supernatant and Suspension Samples of Induced and Non-induced Cultures of P. aeruginosa PAT210 and 3-Pre

Sample	V_{max} nmol/min/mg dry wt	SEM	k_m uM	SEM	n
PAT210 induced	698	35.4	22.2	1.02	6
PAT210 non-ind	25.8	4.36	20.7	3.20	3
PAT210 induced suspension	272	13.5	15.5	1.06	3
3-Pre induced	1490	133	30.4	2.35	12
3-Pre non-ind[a]	852	310	44.8	9.45	3
3-Pre induced suspension	2010	130	24.2	1.51	3

[a] - all readings for non-induced supernatant of 3-Pre were done manually.

The values of B-lactamase activity in the suspension were prone to "noise" due to the presence of cell debris. This may partially explain the reduced V_{max} calculated for the PAT210 suspension. However, 3-Pre had slightly higher activity in the suspension sample as might be expected.

The data show that P. aeruginosa PAT210 has much lower basal and induced levels of B-lactamase activity than 3-Pre indicating that the likelihood of obtaining resisitant mutants will also be lower and the strain was not used further.

P. aeruginosa PA04069 was a strain known to generate different mutants affecting B-lactamase activity and these have been partially characterized (Matsumoto and Terawaki 1982). Further studies were performed with this strain.

6.2.2 Analysis of B-lactam resistant mutants in P. aeruginosa PAO4069

Eight ceftazidime-resistant mutants (M1-M8) derived from P. aeruginosa PAO4069 (pRTG1) were shown to hydrolyse a drop of nitrocefin (1mM) faster than the parent control (Chapter 5). Biochemical analyses were performed on five of these mutants and on three controls; the parental P. aeruginosa PAO4069; and two known mutants of PAO4069 which have increased basal B-lactamase activity, strains PAO4082 and PAO4095 (Matsumoto and Terawaki 1982). P. aeruginosa PAO4082 was considered to have a mutation in an operator region (blaJ) of the B-lactamase gene (blaP) and PAO4095 a mutation in a regulatory gene, blaI (Matsumoto and Terawaki 1982).

P. aeruginosa PAO4069 and its derivatives are auxotrophic for proline and methionine, so CDM was supplemented with these amino acids (40ug/ml). As the basal level of B-lactamase activity was to be determined, cells were not induced with benzylpenicillin prior to harvesting. Cells were harvested at an OD_{650} of 0.2 and B-lactamase extracted by the freeze/thaw method employed above. Following four freeze/thaw cycles the supernatant was used to measure the V_{max} and K_m of the B-lactamase. Results are shown in Table 6.2.

TABLE 6.2 **Table Showing the V_{max} and K_m of B-Lactamase from PAO4069-derived Strains as Determined by Half-time Kinetic Analysis on the Freeze/thaw Supernatant of Non-induced Cultures**

Strain	V_{max} nmol/min/mg dry wt	SEM	k_m mM	SEM	n
PAO4069	3.18	0.15	14.6	4.6	4
PAO4082	547	15.4	21.9	2.4	5
PAO4095	35	1.4	17.9	0.71	5
M1[a]	130	6.5	16.9	1.9	5
M2[a]	101	7.2	17.0	0.98	6
M4[a]	132	8.0	16.1	1.1	5
M6[a]	217	4.5	15.8	0.83	5
M8[a]	424	16	19.1	1.5	5

[a] - Mutant generated during this work (Section 5.4.2).

There were a variety of B-lactamase activities. Parental B-lactamase basal activity was very low (~3.18) when compared to that of *P. aeruginosa* 3-Pre (~852) or even PAT210 (~25.8). Basal B-lactamase activity of mutants ranged from ten-fold to two hundred-fold that of the parent strain, the two previously characterized mutants marking the boundaries (~35 and ~547). The mutants derived in this work seemed to have four different ranges of activity, approximately 100, 130, 220 and 420 nmol/min/mg dry weight.

To test whether the resistance of these mutants was dependent upon the B-lactamase activity the MIC of piperacillin was determined for each strain. Piperacillin was used as it is an antipseudomonal B-lactam susceptible to B-lactamase activity (Class IV, Slack 1981). Cultures were grown overnight in isosensitest broth at 37°C. Gentamicin was

added (30ug/ml) to maintain the transposon in those strains possessing it. Bacteria (~1x10^{4}cfu) were spread on and also dropped onto isosensitest agar containing a range of piperacillin concentrations (0-128ug/ml). Results are shown in Tables 6.3.and 6.4.

TABLE 6.3 Resistance of PAO4069-derived Strains to Piperacillin in Isosensitest Agar when Spread at an Inoculum of ~1x10^4

Strain	Inocula cfu/plate	Piperacillin concentration ug/ml											
		0	2	4	8	16	32	64	80	96	112	128	256
PAO4069	20000	C	0	0	0	0	0	0	0	0	0	0	0
PAO4082	18000	"	C	C	NC	0	0	0	0	0	0	0	0
PAO4095	13000	"	"	"	C	400	0	0	0	0	0	0	0
M1	67000	"	"	"	"	C	C	C	C	NC	2	0	0
M2	23000	"	"	"	"	"	"	NC	NC	112	1	0	0
M3	55000	"	"	"	"	"	"	C	NC	500	0	0	0
M4	21000	"	"	"	"	"	"	C	NC	73	3	0	0
M5	15000	"	"	"	"	"	"	NC	~500	164	0	0	0
M6	21000	"	"	"	"	"	"	C	C	134	2	0	0
M7	13000	"	"	"	"	"	"	C	C	212	4	0	0
M8	33000	"	"	"	"	"	"	C	C	C	C	21	0

C - Confluent growth
NC - Near-confluent growth

TABLE 6.4 Resistance of PA04069-derived Strains to Piperacillin in Isosensitest Agar when Dropped at an Inoculum of ~2.0×10^3

Strain	Piperacillin concentration (ug/ml)											
	0	2	4	8	16	32	64	80	96	112	128	256
PA04069	C	0	0	0	0	0	0	0	0	0	0	0
PA04082	"	C	C	C	NC	0	0	0	0	0	0	0
PA04095	"	"	"	"	C	0	0	0	0	0	0	0
M1	"	"	"	"	"	C	C	C	C	1.3	0	0
M2	"	"	"	"	"	"	"	7	1	0.7	0	0
M3	"	"	"	"	"	"	"	3	3	3	0	0
M4	"	"	"	"	"	"	"	5	8.7	2	0	0
M5	"	"	"	"	"	"	"	NC	9.3	0.3	0	0
M6	"	"	"	"	"	"	"	13	0	0	0	0
M7	"	"	"	"	"	"	"	C	C	0	0	0
M8	"	"	"	"	"	"	"	C	C	C	10	0

n = 3
C - Confluent growth
NC - Near-confluent growth

The parent strain was most sensitive to piperacillin (MIC 2ug/ml). The drop method showed the two control resistant strains P. aeruginosa PA04082 and P. aeruginosa PA04095 (MICs 32ug/ml) to be more sensitive to piperacillin than the mutants generated in this work, which all had similar sensitivities to piperacillin (MICs 96-128ug/ml) except M8 (MIC 256ug/ml). The MIC values obtained from the spread method were more variable. Parental P. aeruginosa PA04069 retained an MIC of 2ug/ml. The two control mutants had different MICs (16 and 32ug/ml). Strains M3 and M5 had identical MICs (112ug/ml), M8 had an MIC of 256ug/ml and the other mutant strains had an MIC of 128ug/ml. B-Lactamase activity of non-induced strains did not correlate with the MIC of piperacillin. It would appear that another resistance mechanism was present in the mutants generated in

this work.

As a further indication of piperacillin resistance, bacteria ($1-7x10^2$ cfu) were plated onto Isosensitest agar containing piperacillin (64ug/ml). As expected, none of the control strains grew. The percentage of viable cells with piperacillin resistance (64ug/ml) is shown in Table 6.5 and is compared with V_{max} (nmol/min/mg dry weight) and the MIC values obtained from Tables 6.3 and 6.4.

TABLE 6.5 Table Comparing Piperacillin-resistance of *P. aeruginosa* PA04069 Mutants with the Rate of Hydrolysis of Cephalosporin C by the Class C B-Lactamase (V_{max}, nmol/min/mg dry weight)

Strain	V_{max} (nmol/min/mg dry weight)	Piperacillin MIC (ug/ml)		Resistance to Piperacillin (64ug/ml) (% viability)
		Drop Method	Spread Method	
PA04069	3	2	2	0
PA04095	35	32	32	0
PA04082	547	32	16	0
M2	101	128	128	2
M3	-	128	112	7
M1	130	128	128	9
M4	132	128	128	12
M7	-	112	128	21
M5	-	128	112	23
M6	217	96	128	27
M8	424	256	256	37

As *P. aeruginosa* PA04082 had the largest basal B-lactamase activity but was not the most resistant strain no direct relationship between basal B-lactamase activity and MIC to piperacillin existed. However, within the mutants generated in this work, there appeared to be a

correlation between the B-lactamase activity and the proportion of the population resistant to piperacillin (64ug/ml).

6.2.3 *E. coli* SNO3 as a model system.

Introduction of pRTG1 into *P. aeruginosa* and identification of B-lactam resistant mutants as a result of Tn5::gen transposition without selection for spontaneous resistant mutants had proven insuperable. A control system was set up to test whether pRTG1 could function as required in a better understood and genetically sympathetic host.

E. coli SNO3 is a strain with negligble chromosomal B-lactamase activity (Lindberg *et al.* 1985). Plasmid pNU305 contains the class C B-lactamase and *amp*R regulatory gene of *Citrobacter freundii* (Lindberg *et al.* 1985, see Chapter 1). As a result of a spontaneous chromosomal mutation in the *amp*D gene, *E. coli* SNO3 (pNU305) can become resistant to B-lactams by increasing production of plasmid-encoded B-lactamase.

Incubation of *E. coli* SNO3 (pNU305, pRTG1) at a non-permissive temperature was predicted to cause the loss of pRTG1. Selection for retention of transposon Tn5::gen by growth on gentamicin (20ug/ml) could be followed by selection of B-lactam resistant mutants. Transposon insertions in the chromosome as opposed to pNU305 could be located by hybridization of whole cell DNA using a transposon-specific DNA probe.

Chromosomal DNA could be digested with a restriction enzyme known not to have recognition sites in Tn5::gen. Any DNA hybridizing to both

Tn5::gen and entire ampD probe DNA may indicate that a transposon insertion exists in the ampD region. To test whether the hybridization was due to insertion within ampD, the transposon could be cloned from the chromosome with chromosomal DNA flanking regions by digestion with the restriction enzyme SalI. Selection for gentamicin-resistance followed by hybridization of cloned DNA with an ampD probe containing all relevant DNA for expression of ampD would indicate the presence of Tn5::gen in ampD.

There should be no initial selection for B-lactam-resistance in detecting B-lactam resistant mutants in E. coli SNO3 (pNU305, pRTG1). After incubation at 42°C cells should be spread on nutrient agar containing gentamicin (20ug/ml) to select only for the retention of the transposon. These could be tested for resistance to a B-lactam, for example, piperacillin. However, replica-plating of gentamicin-resistant colonies onto nutrient agar containing piperacillin (64ug/ml) to detect resistant strains was not possible due to the effect of population density on increasing resistance to piperacillin. Instead, an assay was devised using the B-lactam nitrocefin to identify B-lactamase hyperproducers. This selection protocol would identify strains with both gentamicin-resistance and increased B-lactamase activity. A faster rate of nitrocefin hydrolysis would indicate B-lactamase hyperproduction without requiring prior selection for B-lactam resistance. An increase in frequency of B-lactamase hyperproducers with respect to E. coli SNO3 (pNU305, pRTG1) incubated at 30°C would indicate the presence of transposon-induced mutants. The advantage of this system was that those mutants identified would be identified without any previous selection with growth in the presence of B-lactam and would also be the result of

increased B-lactamase activity, preventing the selection of other mutants which may affect B-lactam sensitivity.

An assay system was devised to screen for increased B-lactamase production in gentamicin-resistant colonies of E. coli SNO3 (pNU305, pRTG1) after growth at 42°C. Nitrocefin (0.1mM), added to cool but molten 0.4% agarose in 5mM Tris-HCl (pH7.0), was poured over plates containing isolated colonies resistant to gentamicin (20ug/ml) isolated after incubation at 42°C. The time taken for nitrocefin to be hydrolysed was recorded to identify colonies with increased production with respect to the parent. To test the assay system colonies of E. coli SNO302, SNO3 (pNU305, pRTG1) and SNO3 (pNU305, pRTG1) mutants resistant to piperacillin were used. The piperacillin-resistant mutants of E. coli SNO3 (pNU305, pRTG1) had previously been selected on nutrient agar containing piperacillin (128ug/ml) and were shown to hydrolyse drops of nitrocefin (1mM) faster than the parent. These were suspected to be resistant due to a spontaneous mutation in the ampD region increasing the production of B-lactamase from pNU305. E. coli SNO302 is a derivative of E. coli SNO3 with a mutation in the ampD gene and should not hydrolyse nitrocefin.

Nitrocefin in cool but molten agarose was poured over nutrient plates each containing approximately 200 colonies of one of the strains tested. The time was taken at room temperature from adding nitrocefin to its complete hydrolysis, determined visually as a colour change from yellow to deep red. Hydrolysis of nitrocefin by E. coli SNO302 was not detected after 5 minutes. This was expected since E. coli SNO302 has negligible B-lactamase activity. E. coli SNO3 (pNU305,

pRTG1) hydrolysed nitrocefin usually in less than a minute, a similar time to that of piperacillin-resistant E. coli SNO3 (pNU305, pRTG1). The rapid hydrolysis of nitrocefin by the parent was probably due to the ampC B-lactamase being present on the high copy plasmid pNU305 rather than being in a single copy. Although having high B-lactamase activity as shown by the rapid hydrolysis of nitrocefin, this activity was unable to confer resistance to piperacillin (MIC 16ug/ml). In other words, nitrocefin at this concentration was unable to identify resistant from non-resistant colonies. Increasing the concentration of nitrocefin to 0.5mM in agarose did not detect the increased hydrolysis of nitrocefin by the piperacillin-resistant strains. Application of single drops of 1mM nitrocefin onto colonies of piperacillin-resistant and sensitive E. coli SNO3 (pNU305, pRTG1), however, did detect the increased production of B-lactamase by the resistant mutants. The time for complete hydrolysis of nitrocefin as determined visually was approximately 8 seconds for piperacillin-resistant strains and 15 seconds for parent E. coli SNO3 (pNU305, pRTG1). Screening of gentamicin-resistant colonies by single drop application of nitrocefin, however, would be too time-consuming considering the large number of colonies which would need to be screened.

Inability to identify hyperproducing B-lactamase mutants without selecting spontaneous mutations meant that selection would have to involve a B-lactam. It was hoped that the frequency of mutation due to transposition might be detectable above the spontaneous mutation rate. A consensus location of Tn5::gen in the chromosome of resistant colonies would identify insertion within ampD.

The frequency of E. coli SNO3 (pNU305, pRTG1) mutating to piperacillin-resistance at 4xMIC was not increased after incubation at 42°C, remaining at $2.0x10^{-7}$ (SEM $0.4x10^{-7}$ n=8). Retention of gentamicin-resistance was not tested. The problem remained that a significant number of B-lactamase hyperproducer mutants were not generated by transposition of Tn5::gen into regulatory genes of B-lactamase in E. coli SNO3 (pNU305). Identification of such insertions would require screening each B-lactamase hyperproducer to find a consensus location of transposon Tn5::gen within a background of spontaneous B-lactamase mutations; experiments which could also be carried out using P. aeruginosa.

6.3 Discussion

I had previously shown P. aeruginosa PAO4069 to spontaneously mutate to ceftazidime-resistance with a frequency of $2.5x10^{-7}$ (Chapter 5). As this frequency was similar to that of 3-Pre (Hewinson 1987) I expected P. aeruginosa PAO4069 to have a similar B-lactamase activity to P. aeruginosa 3-Pre and have a higher B-lactamase activity than P. aeruginosa PAT210. The low B-lactamase activity of non-induced P. aeruginosa PAO4069 was therefore surprising. Furthermore, since P. aeruginosa PAO4082 had the highest B-lactamase activity in vitro I would have expected it to have had the highest resistance to piperacillin in vivo but this was not so. All mutants in this work had lower basal B-lactamase activities but higher MIC values than P. aeruginosa PAO4082. Two possible reasons for this anomaly are that mutants generated in this work have other mutations also conferring piperacillin resistance, or induce

B-lactamase production more than P. aeruginosa PA04082 is able to. MICs of piperacillin within mutants generated in this work may indicate that the basal B-lactamase activity had some effect on the MIC obtained. Minor differences in MIC values should not be considered as being important and there are probably only negligible differences between the sensitivities of the two control strains and also between M1-M7. However, a correlation exists in mutants from this work between basal B-lactamase activity and the percentage viability of mutants in the presence of piperacillin (64ug/ml). How can the larger piperacillin MICs of mutants from this work be associated with the B-lactamase activities of these strains but not with P. aeruginosa PA04095 and PA04082?

Mutants from this work were all grown up in broth containing gentamicin prior to being tested for sensitivity to piperacillin and it is possible that the presence of gentamicin initiated some form of resistance to piperacillin. Any gentamicin-induced resistance mechanism is unlikely to have involved induction of B-lactamase since the nitrocefin drop test showed no difference in B-lactamase activity between P. aeruginosa PA04069 (pRTG1a) grown in the presence of gentamicin and P. aeruginosa PA04069 control which was not.

One possible reason why the twenty-fold increase in B-lactamase activity between PA04095 and PA04082 did not appear to increase the MIC values obtained was that the B-lactamase activities were basal B-lactamase activities whilst the MICs were from cultures induced by piperacillin within the agar. Both P. aeruginosa PA04082 and PA04095 were defined as semi-constitutive B-lactamase producers (Matsumoto and Terawaki 1982). It was concluded by these authors that

P. aeruginosa PA04082 had an operator mutation and PA04095 had a mutation in a regulatory gene. It is possible that P. aeruginosa PA04095 can induce B-lactamase production to a similar level as induced P. aeruginosa PA04082. However, induced B-lactamase production may be less than that achieved by mutants generated in this study.

It was hoped that mutants with similar B-lactamase activities to those of Matsumoto and Terawaki (1982) would have been generated so that these genes which have been partially characterized could be characterized further. However, none were similar but the presence of mutations from a single strain producing B-lactamase at possibly six different levels suggests a range of regulatory genes may exist in P. aeruginosa PA04069.

The ability to obtain mutants in this work resistant to piperacillin (64ug/ml) may be the result of greater induction of B-lactamase than that possible from either control mutant. For example, PA04082 was considered to have a mutation in the operator region of the B-lactamase gene (Matsumoto and Terawaki 1982). A mutation here may increase the basal B-lactamase activity by preventing binding of a repessor, however, an induced culture would produce similar amounts of B-lactamase as an induced culture of the PA04069 control. Data from Bryan et al. (1984) support this possibility: PA04082 produces 175-fold more B-lactamase than PA04069 in non-induced cultures, 5-fold more when induced with penicillin G and equal or only slightly more (1.3 fold) when induced with imipenem. No data was published for PA04095. It may be possible that on induction mutants generated in this study allowed greater B-lactamase production than that of

PAO4082.

Another possibility is that a second common resistance mutation exists in mutant strains generated in this work. For example, a spontaneous mutation increasing the impermeability of the outer membrane may in conjunction with increased B-lactamase activity increase the MIC of piperacillin above that of P. aeruginosa PAO4082. This would explain why basal B-lactamase production from M1-8 is less than those of PAO4082 or PAO4069 and yet still allow the frequencies of resistance to piperacillin (64ug/ml) to reflect the basal B-lactamase activities of M1-8. It was a known possibility that such multiple resistance mutations may have occurred during the selection and growth of mutant strains since the selection method employed to identify ceftazidime-resistant mutants could not differentiate mutations affecting B-lactamase expression alone from other resistant mutations. It is unfortunate that time did not allow an investigation into the B-lactamase activities of induced cultures. Rather than repeating the experiments in P. aeruginosa PAO4069 in which pRTG1 was unstable and from which Tn5::gen-mediated B-lactam-resistant mutants were difficult to select, I decided to test first whether pRTG1 could deliver Tn5::gen into B-lactamase regulatory genes of a more accessible host, E. coli SNO3.

E. coli SNO3 (pNU305) was not an ideal control system for the identification of hyperproducing B-lactamase mutants generated by transposon mutagenesis. Difficulties in selecting Tn5::gen-mediated B-lactam-resistant mutants within P. aeruginosa were also encountered in E. coli SNO3 (pNU305). Furthermore, the presence of pNU305 might have acted as a preferential site for transposition of

Tn5::gen and also interfered with the loss of regions of pRTG1 due to co-integration of plasmids. Investigations of the regulation of B-lactamase would continue within P. aeruginosa once pRTG1 could be transferred and maintained as desired.

CHAPTER 7

CHARACTERISATION OF *Pseudomonas aeruginosa* TRANSCONJUGANTS CONTAINING pRTG1 DERIVATIVES

Objectives: To analyse the behaviour and fate of pRTG1 in *P. aeruginosa* PAO4069.

7.1. Introduction

One aim of this thesis was to generate *P. aeruginosa* strains hyperproducing B-lactamase using transposon mutagenesis. To achieve this a vector, pRTG1, was constructed to deliver Tn5::gen into *P. aeruginosa* (Chapter 4). Many methods were used in an attempt to introduce pRTG1 into *P. aeruginosa* (Appendix V) and although difficulties were encountered, pRTG1 was introduced by conjugation into *P. aeruginosa* (Chapter 5). From initial screening of transconjugants it was apparent that pRTG1 in most if not all transconjugants had undergone recombination and/or deletion events as determined by plasmid DNA mobilities in agarose gels (Chapter 5). One transconjugant containing such a plasmid, designated pRTG1a, was temperature-sensitive with loss of streptomycin-resistance at 42°C (Chapter 5) and was used to test for transposition of Tn5::gen into the chromosome. Tn5::gen was identified in the chromosome of the eight B-lactamase hyperproducing mutants of *P. aeruginosa* PAO4069 (pRTG1a) which were investigated. Plasmid pRTG1a, however, did not behave as expected because a temperature-stable plasmid with homology to pMMB19 DNA but not Tn5::gen DNA was present in all of these mutants. Plasmid stability of another temperature-sensitive plasmid encoding a Tn5-derivative (pME109) had been reported previously in *P. aeruginosa* PAO (Rella *et al.* 1985). I therefore considered that a

more detailed analysis of the behaviour and fate of pRTG1 in P. aeruginosa PA04069 may give some indication of the problems associated with using temperature-sensitive vectors of Tn5::gen in P. aeruginosa. Furthermore, such information may be of use in constructing other, more suitable vectors of Tn5-derived transposons for use in P. aeruginosa. This chapter reports the results from analyses of several P. aeruginosa transconjugants obtained from separate conjugation events.

7.2 Results

7.2.1 Introduction of pRTG1 into P. aeruginosa using a spot-mating technique

A spot-mating technique (see Methods) was used to introduce pRTG1 into P. aeruginosa PA04069. Unlike filter-mating experiments each isolate was the result of a unique conjugation event and so many transconjugants could therefore be analysed from a single experiment.

Thirty-eight putative P. aeruginosa PA04069 (pRTG1) transconjugants were isolated from initial selection plates; minimal agar containing proline, methionine and gentamicin (20ug/ml). All grew when streaked onto PIA plates and onto nutrient agar containing streptomycin (320ug/ml) and gentamicin (30ug/ml). Loss of streptomycin and gentamicin resistance was observed in eighteen strains on streaking single colonies from PIA plates onto nutrient agar containing streptomycin (320ug/ml) or gentamicin (32ug/ml). Contamination of putative transconjugants by E. coli SM10 (pRTG1) was tested for using Hugh-Liefsen agar (see Methods). Growth was not observed in anaerobic cultures from any transconjugant.

The experiment was repeated using P. aeruginosa PAO4069, PAO6520 recA and PAO6521 recA as recipients of pRTG1. P. aeruginosa PAO6520 and PAO6521 are recA strains of P. aeruginosa which were used to test whether the low frequency of transfer obtained was a result of recA intolerance to multiple copies of DNA. Single colonies were isolated from the initial selection (as above) and streaked onto PIA agar. Single colonies were then streaked from the PIA plates onto nutrient agar containing streptomycin (320ug/ml) and gentamicin (30ug/ml); all but one of the sixteen colonies putative transconjugants grew on this medium. However, resistance to either gentamicin or streptomycin was lost from all sixteen recA recipients of pRTG1 within the three purification cycles. Four colonies of P. aeruginosa PAO4069 (pRTG1) were also isolated and purified by this method. Two retained resistance to streptomycin (320ug/ml) and gentamicin (30ug/ml) after three purification cycles and were not contaminated with E. coli SM10 (pRTG1) when tested using Hugh-Liefsen agar. Plasmid DNA was extracted from these two strains and also from six of the first spot-mating experiment. After electrophoresis, plasmid DNA was present in seven of the eight strains and the mobility of plasmid DNA varied between samples. Plasmid DNA was digested with PvuII restriction enzyme, which should have generated three fragments in pRTG1 DNA. However, PvuII only cleaved DNA from the two most recently isolated strains and the fragments obtained did not conform to that expected for pRTG1 PvuII (Figure 7.1). If this DNA was derived from pRTG1 the different fragment profiles and the larger sizes of plasmid from the most recently isolated transconjugants supported the possibility of systematic recombination/deletion events within pRTG1 in P. aeruginosa PAO4069 (pRTG1).

Figure 7.1: Plasmid DNA Extracted from Seven Independent Putative P. aeruginosa PA04069 (pRTG1) Transconjugants Digested with PvuII Restriction Enzyme. Lane A - pRTG1 PvuII; Lane B - lambda HindIII; Lane C-I - pRTG1-derivatives PvuII. A single PvuII site is present in DNA from Lanes C and D and other DNA was uncut by PvuII.

7.2.2 Analysis of pRTG1 from *P. aeruginosa* PAO4069 (pRTG1) transconjugants by agarose gel-electrophoresis

Introduction of pRTG1 from *E. coli* SM10 into *P. aeruginosa* PAO4069 was achieved using a spot-mating technique as described above. From such experiments fifty-five individual colonies were picked and donor contaminants removed by streaking to obtain single colonies on PIA and nutrient agar containing gentamicin (32ug/ml) and streptomycin (320ug/ml) sequentially as described previously. Plasmid DNA was extracted from fifty-five transconjugants of *P. aeruginosa* PAO4069 (pRTG1), electrophoresed and stained with ethidium bromide.

An example of plasmid extracts from *P. aeruginosa* PAO4069 (pRTG1) transconjugants is shown in Figure 7.2. Chromosomal DNA was present in the parental control extract but not plasmid DNA. Both transconjugants harboured plasmid DNA with a mobility similar to that of CsCl-purified pRTG1 and also what appeared to be a DNA complex. This DNA may be derived from plasmid or chromosomal DNA, and could be the result of degradation or complexing. However, this DNA was not present in all transconjugants. For example, DNA from transconjugant 7 appeared in two discrete bands (Figure 7.3). Discrete bands of plasmid DNA of varying mobilities were present in DNA from transconjugants 1, 2, 4 and 9.

Detection of specific pRTG1 fragments was by digestion with AvaII restriction enzyme, which cleaves pRTG1 into five fragments (Lane A, Figure 7.4). Following gel electrophoresis and ethidium bromide staining restriction fragments were compared to AvaII fragments of the

Figure 7.2: Plasmid DNA Extracted from Two Putative Transconjugants of P. aeruginosa PAO4069 (pRTG1). Lane A - Lambda BamH1; Lane 54 - Transconjugant 54; Lane 55 - Transconjugant 55; Lane B - P. aeruginosa PAO4069 parental control; Lane C - P. aeruginosa PAO6520 control; Lane D - pRTG1 uncut.

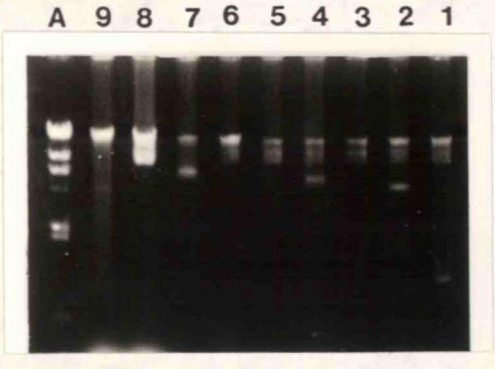


Figure 7.3: Plasmid DNA Extracted from Nine Putative Transconjugants of P. aeruginosa PAO4069 (pRTG1). Lane A - lambda HindIII; Lane 9 - Transconjugant 9; Lane 8 - Transconjugant 8; Lane 7 - Transconjugant 7; Lane 6 - Transconjugant 6; Lane 5 - Transconjugant 5; Lane 4 - Transconjugant 4; Lane 3 - Transconjugant 3; Lane 2 - Transconjugant 2; Lane 1 - Transconjugant 1.

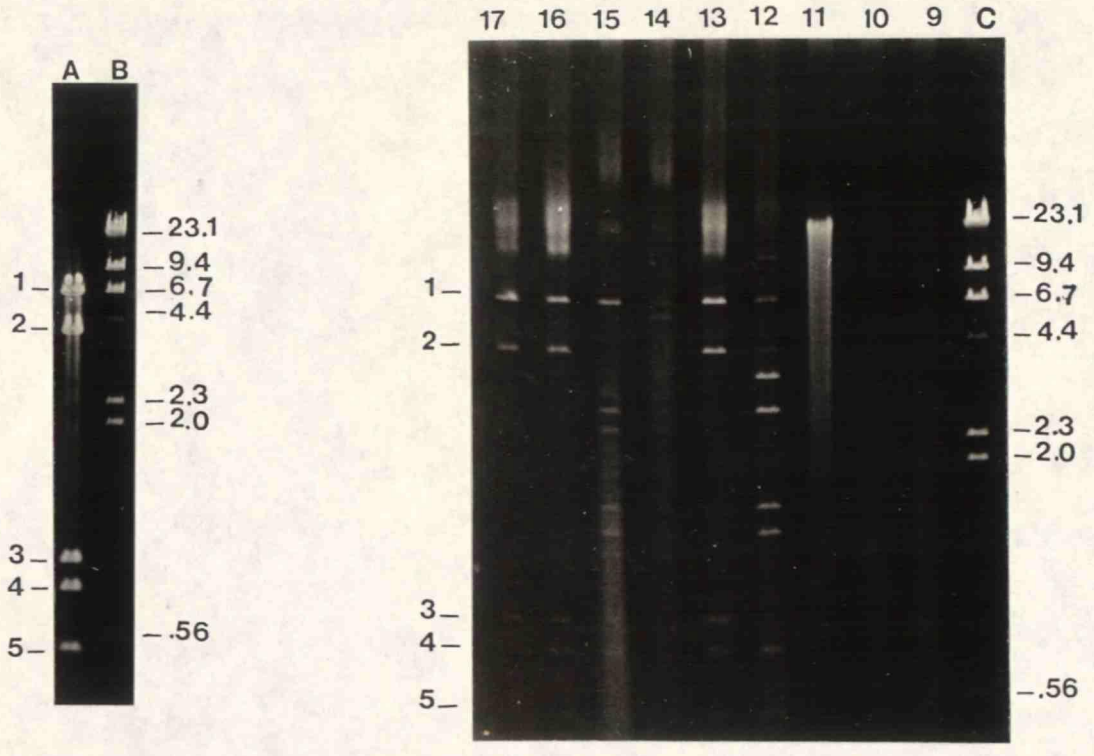


Figure 7.4: Plasmid DNA from Transconjugants 9-17 Digested with AvaII. Lane A - pRTG1 AvaII; Lane B - Lambda HindIII, Lane 17 - Transconjugant 17, Lane 16 - Transconjugant 16; Lane 15 - transconjugant 15; Lane 14 - transconjugant 14; Lane 13 transconjugant 13; Lane 12 - transconjugant 12; Lane 11 - transconjugant 11; Lane 10 - transconjugant 10; Lane 9 - transconjugant 9; Lane C - Lambda HindIII.

original construct, pRTG1. If a common host-intolerance response to a specific plasmid sequence existed, pRTG1 derivatives would be expected to have common AvaII restriction enzyme fragments. Unfortunately, the yield and purity of plasmid DNA was poor in some cases, resulting in low DNA intensities on gels and partial restriction enzyme digestion products. Interpretation of such results were therefore difficult and few conclusions were made in such instances. To overcome difficulties with restriction enzyme digestion, plasmid samples were extracted several times with phenol/chloroform and restriction enzyme digests were incubated overnight.

Plasmid DNA from eight transconjugants digested with AvaII overnight and separated by agarose gel electrophoresis are shown in Figure 7.4. DNA from transconjugants 9, 10 and 11 contained chromosomal DNA observed as a gradual smear down the gel. Transconjugant 9 also contained a single distinct DNA band migrating with a mobility of 4.16Kbp relative to lambda HindIII DNA, similar to that observed in the uncut DNA sample (Figure 7.3). Transconjugants 12, 14 and 15 contained some common discrete DNA bands but not of the expected sizes of pRTG1 AvaII whilst transconjugants 13, 16 and 17 contained plasmid DNA with fragment sizes expected for pRTG1. Complex plasmid DNA remained uncut in DNA from transconjugants 13, 16 and 17 yet partial digests of plasmid DNA were not seen. I would suggest that, if this DNA is derived from pRTG1, this complex was inaccessible to digestion by AvaII restriction enzyme.

After ethidium bromide staining plasmid DNA was transferred from gels to nylon membranes by Southern transfer and hybridized with various pRTG1-derived probes, as described below, to determine the regions of

homology between native pRTG1 and the derivatives in the various P. aeruginosa transconjugants. Probes were prepared from individual DNA fragments of pRTG1 (Figure 4.5b) after digestion with AvaII (Figure 7.5) except probe for Tn5::gen (Figure 5.3), which was isolated after digestion with HpaI (Fragment 2, 4.27Kbp). DNA fragments were excised from low-gelling temperature agarose and radio-labelled by nick-translation in the presence of ^{32}P-dATP (see Methods).

7.2.3 Hybridization Analysis of P. aeruginosa PAO4069 (pRTG1) transconjugants with pRTG1 probes:

Plasmid pRTG1 AvaII fragment 5 (0.50Kbp)

DNA from 21 of the first 25 transconjugants tested hybridized probe 5 (0.50Kbp) at the position expected for intact fragment 5 (0.50Kbp). DNA homologous with probe 5 was absent from three transconjugants (10, 21 and 24) and was present in an unusual position in transconjugant 18 (6.1Kbp).

Complex and partially cut DNA also shared homology to probe DNA. For example, an unexpected DNA fragment (1.26Kbp) with homology to probe 5 was observed in seven of eight transconjugants (Figure 7.6b) and was probably a result of incomplete digestion between fragments 4 (0.72Kbp) and 5 (0.50Kbp). Incomplete digestion was probably a result of the complex nature of the DNA and impurities in the DNA sample (CsCl-purified pRTG1 was totally digested within 2 hours of incubation with AvaII).

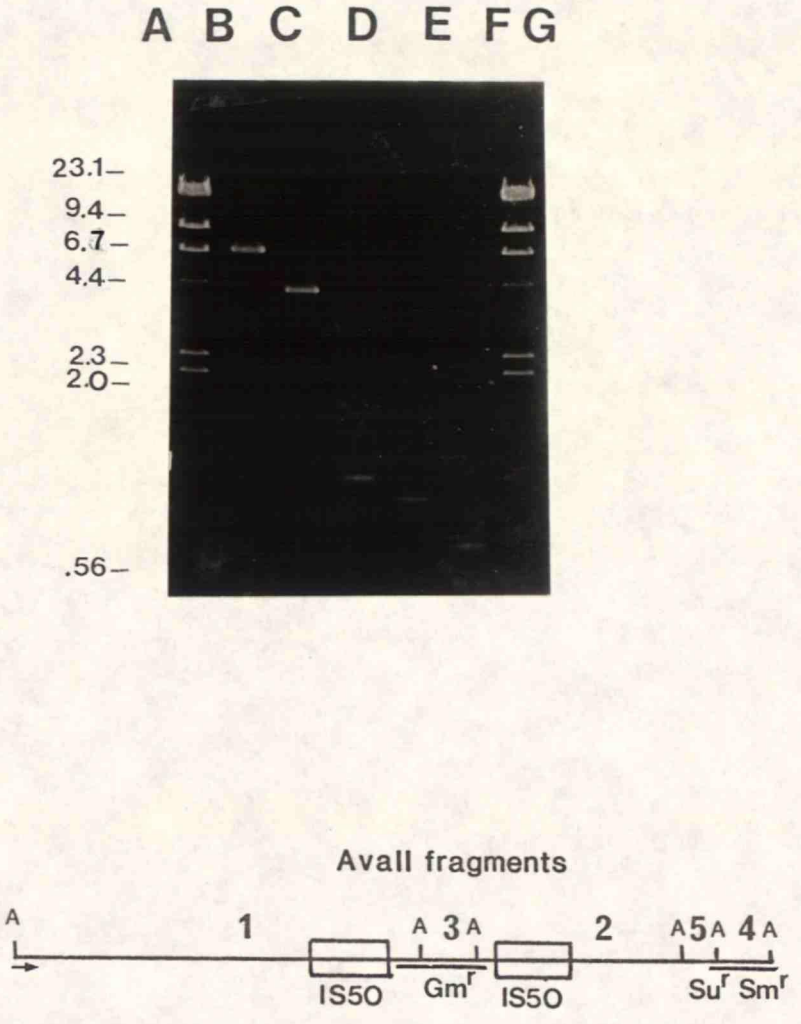


Figure 7.5: Purified AvaII Fragments of pRTG1. Lanes A and G - Lambda HindIII; Lane B - pRTG1 AvaII Fragment 1; Lane C - pRTG1 AvaII Fragment 2; Lane D - pRTG1 AvaII Fragment 3; Lane E - pRTG1 AvaII Fragment 4; Lane F - pRTG1 AvaII Fragment 5.

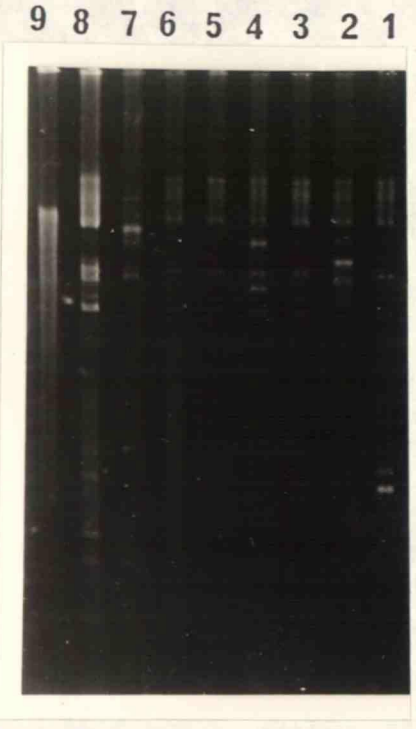


Figure 7.6a

Figure 7.6b

Figure 7.6a: Plasmid DNA from Transconjugants of P. aeruginosa PA04069 (pRTG1) (Transconjugants 1-9) Digested with AvaII Restriction Enzyme. Lane 9 - Transconjugant 9 AvaII; Lane 8 - Transconjugant 8 AvaII; Lane 7 - Transconjugant 7 AvaII; Lane 6 - Transconjugant 6 AvaII; Lane 5 - Transconjugant 5 AvaII; Lane 4 - Transconjugant 4 AvaII; Lane 3 - Transconjugant 3 AvaII; Lane 2 - Transconjugant 2 AvaII; Lane 1 - Transconjugant 1 AvaII.

Figure 7.6b: Autoradiograph Showing Homology between Fragment 5 of pRTG1 AvaII and Plasmids from Nine P. aeruginosa PA04069 (pRTG1) Transconjugants (Transconjugants 1-9) Digested with AvaII. Lanes as Lanes in Figure 7.6a (Lane A - pRTG1 AvaII).

Plasmid pRTG1 AvaII fragment 3 (0.91Kbp)

DNA from 22 of the first 25 transconjugants tested hybridized probe 3 (0.91Kbp) at the position expected for intact fragment 3 (0.91Kbp). Homology was not observed with DNA from transconjugants 18 and 21. DNA with homology to probe 3 was present in transconjugant 11 within a feint and gradual smear (~4.0-9.0Kbp, Figure 7.7) indicating the presence of this fragment within the chromosomal DNA of the transconjugant. Further evidence to support this possibility was that no plasmid DNA was observed in DNA extracted from transconjugant 11 and a chromosomal smear was present on digestion with AvaII at the position identified as having homology to fragment 3.

DNA from transconjugant 9 shared homology with probe 3 (0.91Kbp) consistent with the presence of intact fragment 3 and also to a fragment with mobility similar to that expected for fragment 5 (0.50Kbp, see Figure 7.7). Homology between fragment 5 and fragment 3 probe was not observed for pRTG1 nor DNA from other transconjugants and I would suggest that this fragment was a novel recombinant AvaII fragment containing a section of fragment 3 DNA.

Plasmid pRTG1 AvaII fragment 1 (6.50Kbp) and fragment 2 (4.01Kbp)

An IS50 element is present in both fragments 1 and 2 of pRTG1 AvaII and so both probe 1 (6.50Kbp) and probe 2 (4.01Kbp) hybridize to both DNA fragments (Figure 7.8, Lane A). Probe 1 (6.50Kbp) hybridized two AvaII fragments consistent with the presence of intact fragments 1 (6.50Kbp) and 2 (4.01Kbp) except with DNA from transconjugants 9 and 7 (Figure 7.8b). Complex DNA from transconjugants 1-8 also hybridized probe DNA (seen as a smear between the two heaviest molecular weight

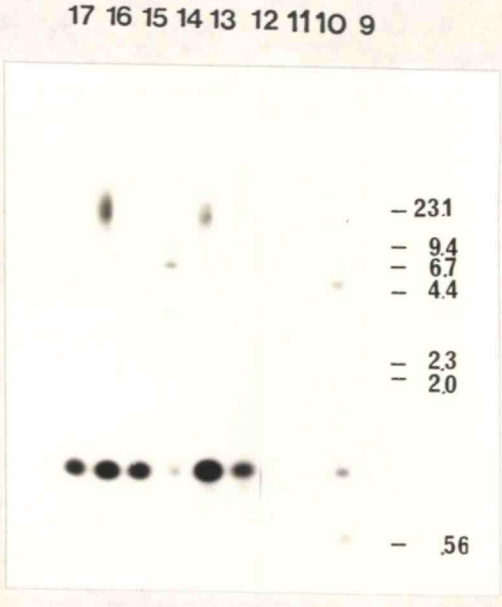


Figure 7.7: Autoradiograph Showing Homology between Fragment 3 of pRTG1 AvaII and DNA Digested with AvaII from Transconjugants 9-17. Lanes as Lanes in Figure 7.4.

markers) but supercoiled DNA from transconjugant 7 DNA had a unique mobility profile supporting the possibility that a derivative plasmid existed in this transconjugant. The inability to detect DNA homologous with probe 1 (6.50Kbp) from transconjugant 9 may have been due to the low concentration of plasmid DNA present.

Homology was shared by probe 1 and distinct DNA fragments of transconjugant 7 (11.75 and 2.48Kbp) as well as hybridizing to other uncut or partially cut DNA (Figure 7.8a and Figure 7.8b). As plasmid DNA was partially digested, the presence of a hybridizing band (11.75Kbp) may have been the result of incomplete digestion of pRTG1. However, the presence of a unique fragment (2.48Kbp) with homology to fragment 1 smaller than either intact fragment 1 (6.50Kbp) or fragment 2 (4.01Kbp) supports the possibility that recombination events within pRTG1 in transconjugant 7 had occurred.

Probe 1 hybridized DNA from transconjugant 1 consistent with the presence of fragment 1 (6.50Kbp) and fragment 2 (4.01Kbp) but two unexpected fragments with homology were also observed (with relative mobilities to lambda HindIII of 1.53Kbp and 1.73Kbp). Both fragments were smaller than either intact fragment and must be the result of recombination events within pRTG1. Furthermore, uncut DNA was observed in these regions (Figure 7.3) indicating the possiblity of an autonomous mini-pRTG1 recombinant plasmid with no AvaII cleavage sites.

Hybridization of AvaII-digested DNA from transconjugants 9-17 with probe 2 (4.01Kbp) (Figure 7.9) showed that only transconjugants 10 and 11 did not contain DNA with homology to probe 2. Transconjugants 12,

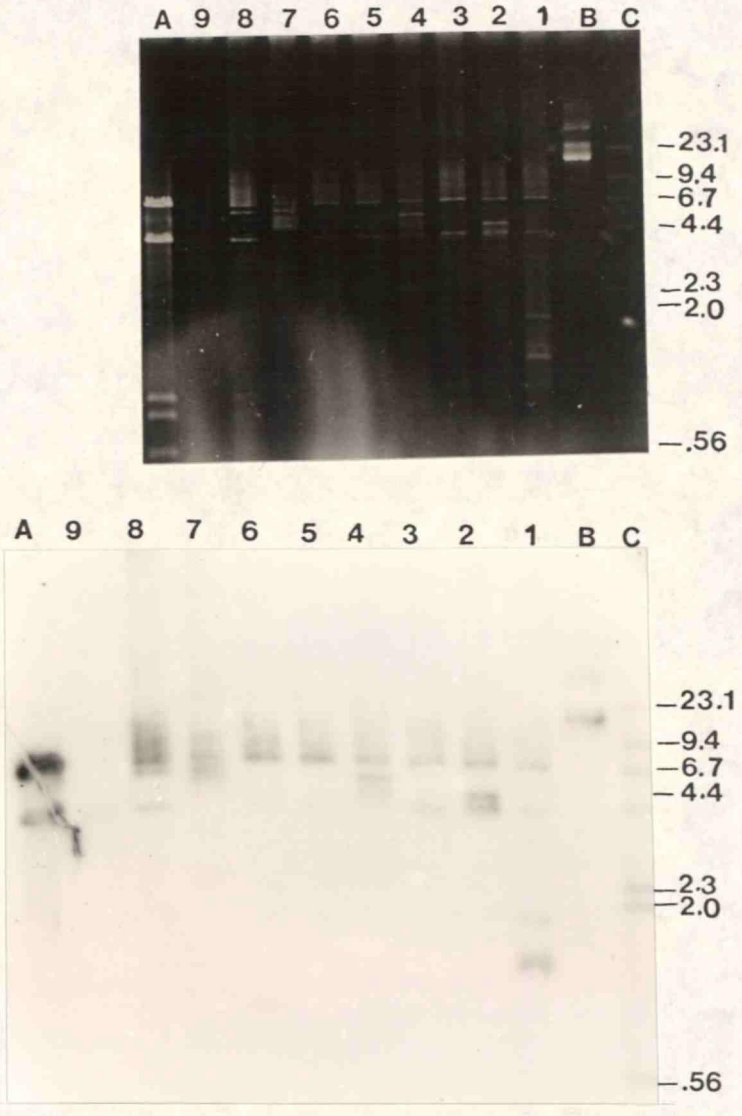


Figure 7.8a: Plasmid DNA from Nine Transconjugants of P. aeruginosa PAO4069 (pRTG1) (Transconjugants 1-9) Digested with AvaII Restriction Enzyme. Lane A - pRTG1 AvaII; Lane 9 - Transconjugant 9 AvaII; Lane 8 - Transconjugant 8 AvaII; Lane 7 - Transconjugant 7 AvaII; Lane 6 - Transconjugant 6 AvaII; Lane 5 - Transconjugant 5 AvaII; Lane 4 - Transconjugant 4 AvaII; Lane 3 - Transconjugant 3 AvaII; Lane 2 - Transconjugant 2 AvaII; Lane 1 - Transconjugant 1 AvaII; Lane B - pRTG1; Lane C - ^{32}-P lambda HindIII.

Figure 7.8b: Autoradiograph Showing Homology between Fragment 1 of pRTG1 AvaII and Plasmids from Nine P. aeruginosa PAO4069 (pRTG1) Transconjugants (Transconjugants 1-9) Digested with AvaII. Lanes as Lanes in Figure 7.8a.

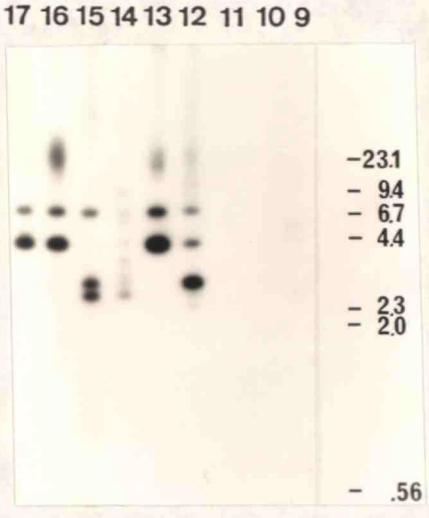


Figure 7.9: Autoradiograph Showing Homology between Fragment 2 of pRTG1 AvaII and DNA Digested with AvaII from Transconjugants 9-17. Lanes as Lanes in Figure 7.4.

13, 16 and 17 had homology to probe 2 with fragments 1 (6.50Kbp) and 2 (4.01Kbp) as expected (the two fragments share homologous sequences as both contain an IS50 element from Tn5::gen). Transconjugant 12 also contained three unexpected smaller fragments showing homology to probe 2 DNA; a major band (2.66Kbp) and two minor bands (2.33 and 2.14Kbp). Transconjugant 15 contained intact fragment 1 (6.50Kbp) as expected but not fragment 2 (4.01Kbp). However, two new fragments (2.66 and 2.33Kbp) with homology to probe 2 DNA were observed suggesting a recombination event within fragment 2 with the subsequent insertion of an AvaII site and flanking DNA (~0.98Kbp). Transconjugant 14 also contained two DNA fragments of the same size and homology profile as those found in transconjugant 15 (2.66 and 2.33Kbp). DNA from transconjugant 9 hybridized with probe 2 (4.01Kbp) at the same position found in transconjugants 12, 14 and 15 (2.33Kbp).

"Tn5::gen" pRTG1 HpaI fragment 2 (4.27Kbp)

To test whether any new AvaII-fragments (for example, from transconjugants 7 and 15) with homology to fragment 1 or fragment 2 DNA were derived from Tn5::gen or from pMMB19, hybridization with Tn5::gen as probe was performed (Figure 7.10). Probe Tn5::gen hybridized to both fragments 1 and 2 as expected in 16 of the 25 transconjugants. Homology was not observed in transconjugants 10, 11 and 24. Fragment 3 (0.91Kbp) hybridized to probe DNA as expected in 16 transconjugants but should have shown homology in a further 6 which had already shown homology to probe 3 (0.91Kbp). The reason for this discrepancy is not known but may be a result of a low concentration of probe DNA with homology to fragment 3.

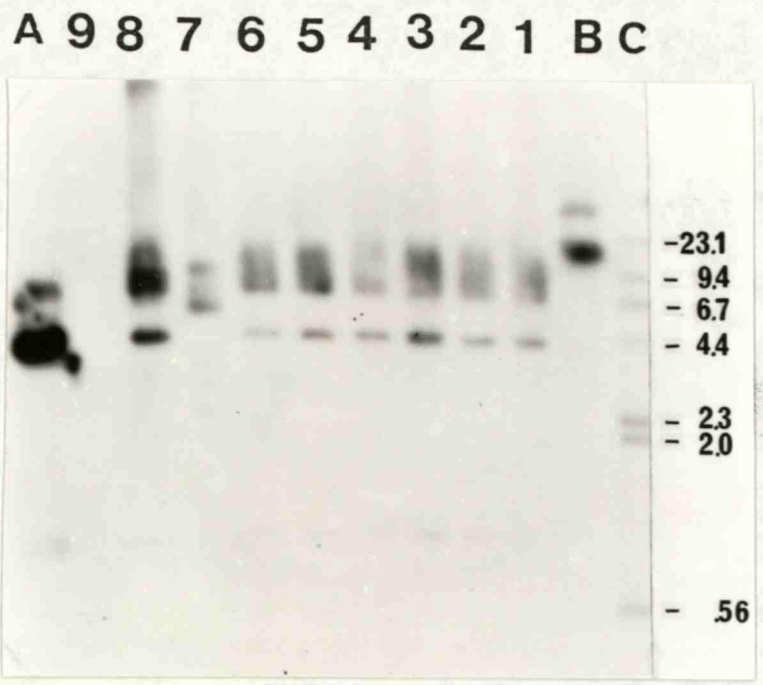
Two fragments from transconjugant 1 with mobilities relative to lambda

DNA HindIII of 1.53 and 1.73Kbp which showed homology to probe 1 did not hybridize to Tn5::gen probe (Figure 7.10a).

Four AvaII fragments of DNA from transconjugant 7 shared homology with Tn5::gen probe (Figure 7.10a). Fragment 3 (0.91Kbp) hybridized as expected but three other fragments were unexpected for pRTG1 AvaII (11.75, 7.41 and 2.48Kbp) and two of these fragments (11.75 and 2.48Kbp) also hybridized to probe 1. Although unlikely, the 11.75Kbp and 7.41Kbp fragments may be the result of incomplete digestion of pRTG1 and no conclusions should be drawn from these results. However, the 2.48Kbp fragment could not be due to incomplete digestion of pRTG1 as fragment 3 is situated between fragments 1 and 2 and the smallest partial digest fragment would be 5.97Kbp.

Tn5::gen probe (4.27Kbp) shared homology with the 2.33Kbp AvaII fragment observed to hybridize to fragment 2 (4.01Kbp) probe in transconjugants 12 (feint), 14, 15 and 25 (Figures 7.10b and 7.10c). The inability to detect hybridization to the 2.33Kbp fragment present in DNA from transconjugant 9 may be due to only a small region of Tn5::gen being present on this fragment or the low concentration of DNA in this sample. The 2.33Kbp fragment was the only region to hybridize to Tn5::gen probe DNA in transconjugant 14 whilst in transconjugants 15 and 25 Tn5::gen probe hybridized this fragment and also fragment 1 (6.50Kbp).

The 2.66Kbp fragment identified as having homology to probe 2 DNA in transconjugants 12, 14 and 15 lacked homology with Tn5::gen DNA. It is possible that the 2.33 and 2.66Kbp fragments were the result of recombination events which had occurred within fragment 2 generating



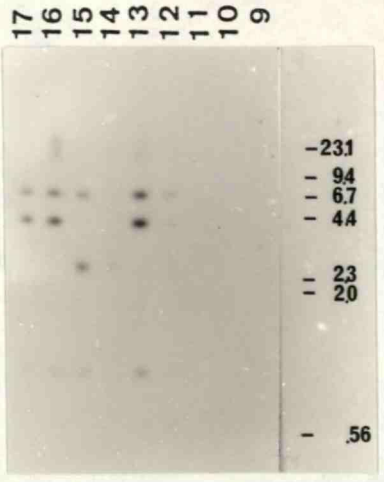
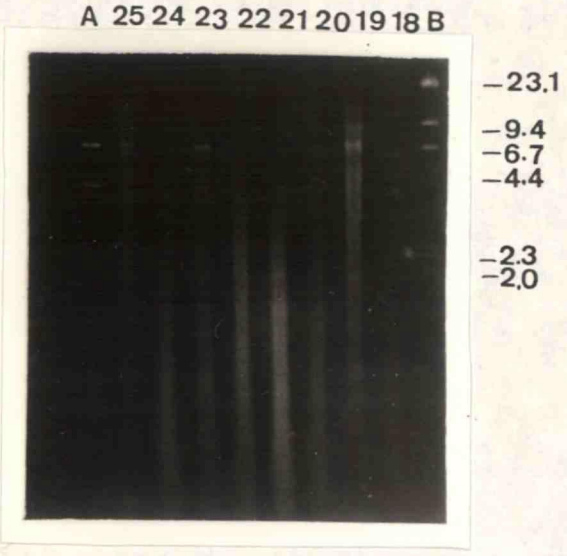


Figure 7.10a: Autoradiograph Showing Homology between "Transposon Tn5::gen" Probe (Fragment 2 of pRTG1 HpaI) and DNA Digested with AvaII from Transconjugants 1-9. Lanes as Lanes in Figure 7.6a.

Figure 7.10b: Autoradiograph Showing Homology between "Transposon Tn5::gen" Probe (Fragment 2 of pRTG1 HpaI) and DNA Digested with AvaII from Transconjugants 9-17. Lanes as Lanes in Figure 7.4.



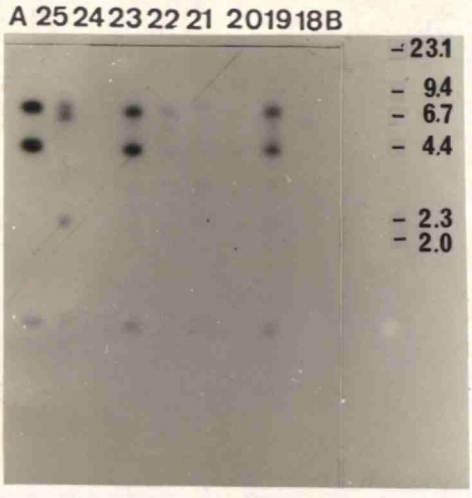


Figure 7.10c: AvaII Digests of Plasmid DNA Extracted from Putative P. aeruginosa PA04069 (pRTG1) (Transconjugants 18-25). Lane A - pRTG1 AvaII; Lane 25 - Transconjugant 25 AvaII; Lane 24 - Transconjugant 24 AvaII; Lane 23 - Transconjugant 23 AvaII; Lane 22 - Transconjugant 22 AvaII; Lane 21 - Transconjugant 21 AvaII; Lane 20 - Transconjugant 20 AvaII; Lane 19 - Transconjugant 19 AvaII; Lane 18 - Transconjugant 18 AvaII; Lane B - lambda HindIII.

Figure 7.10d: Autoradiograph Showing Homology between "Transposon Tn5::gen" Probe (Fragment 2 of pRTG1 HpaI) and DNA from Transconjugants 18-25 of P. aeruginosa PA04069 (pRTG1). Lanes as in Figure 7.10c.

an AvaII restriction enzyme cleavage site. As unexpected fragments were of similar origin from different transconjugants I considered a common activity may have caused instability of pRTG1 in P. aeruginosa.

7.2.4 Transformation of E. coli UB5201 and UB2272 with plasmid DNA from P. aeruginosa PA04069 (pRTG1)

Using DNA-DNA hybridization analysis, profiles expected for original pRTG1 were observed in fourteen P. aeruginosa PA04069 (pRTG1) transconjugants, although subtle changes were undetectable using this method. To test whether such plasmids encoded gentamicin, sulphonamide and streptomycin resistance and were autonomous replicons, plasmid DNA from transconjugants 4, 8, 13, 16 and 17 were used to transform E. coli UB5201 and UB2272. Transformants resistant to streptomycin (100ug/ml), sulphonamide (200ug/ml) and gentamicin (20ug/ml) were obtained in all cases. Plasmid extraction, digestion with AvaII and hybridization analyses confirmed that intact pRTG1 had been introduced into these transformants from DNA extracted from transconjugants of P. aeruginosa PA04069 (pRTG1) (data not shown).

7.2.5 Analysis of P. aeruginosa PA04069 (pRTG1) transconjugants after sub-culturing for three months

Apparently unaltered pRTG1 was obtained in 14 of 25 different transconjugants of P. aeruginosa as determined by AvaII restriction enzyme fragment and DNA-DNA hybridization analyses. However, there was evidence of recombination processes occurring within 11 transconjugants (for example, transconjugants 9, 12, 14, 15 and 25)

resulting in extensive losses of pRTG1 sequences in, for example, transconjugants 1, 11 and 18). Although not strictly relevant to the project I believed that information relevant to future developments may be obtained by investigating whether prolonged maintenance of pRTG1 in P. aeruginosa PAO4069 resulted in further genetic rearrangements of plasmid DNA and whether stable derivatives were eventually observed. Furthermore, I considered that analysis of stable derivatives may identify common deleted sequences which might have caused instability of pRTG1 in P. aeruginosa.

Twenty-eight transconjugants of P. aeruginosa PAO4069 (pRTG1) from a spot-mating experiment (Chapter 7.2.2) with E. coli SM10 as donor were sub-cultured fortnightly at room temperature over a period of three months on nutrient agar containing streptomycin (320ug/ml) and gentamicin (32ug/ml). It was hoped that further recombination events would occur during this time and comparisons could be made between these transconjugants and those previously analysed. Plasmid extraction, Southern transfer and hybridizations were carried out as previously described.

If any derivative of pRTG1 was present as a plasmid it would require the ori site located in pRTG1 AvaII fragment 1 (6.50Kbp). Probe 1 of pRTG1 AvaII was used to identify the presence of pRTG1-derived plasmid DNA. Probe 1 hybridized to plasmid DNA extracted from twenty of the twenty-eight transconjugants tested. However, the size of the homologous DNA varied from one transconjugant to another and none were that expected for pRTG1 (Figure 7.11b and 7.12b). Furthermore, only three of the twenty plasmids (transconjugants 30, 44, 45) had retained any AvaII restriction sites. As plasmid DNA remained uncut in

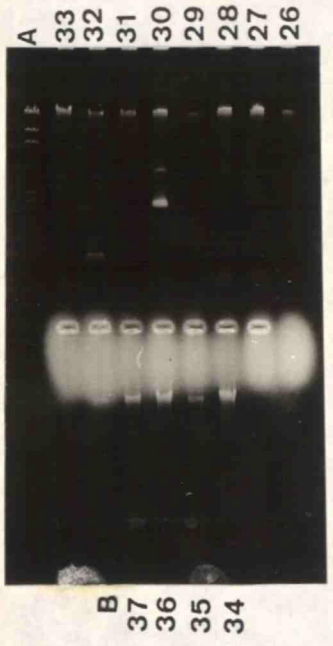


Figure 7.11a

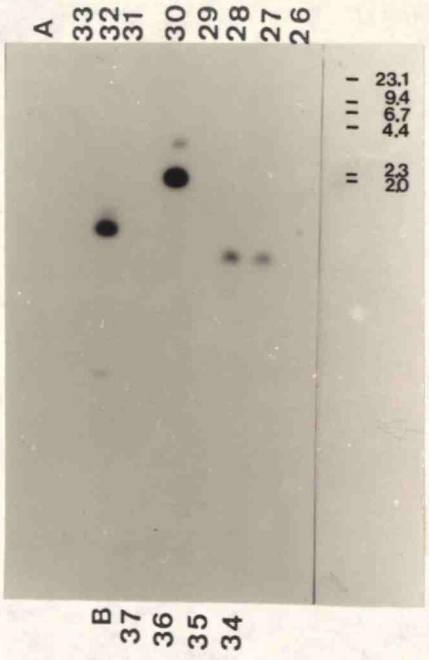


Figure 7.11b

Figure 7.11a: Plasmid DNA Extracted from Transconjugants 26-37 of P. aeruginosa PAO4069 (pRTG1). Lane A - lambda HindIII; Lanes 33-26 - Transconjugants 33-26 respectively; Lane B - pRTG1; Lanes 37-34 - Transconjugants 37-34 respectively.

Figure 7.11b: Autoradiograph Showing Homology between Fragment 1 of pRTG1 AvaII and DNA from Transconjugants 26-37 of P. aeruginosa PAO4069 (pRTG1). Lanes as lanes of Figure 7.11a.

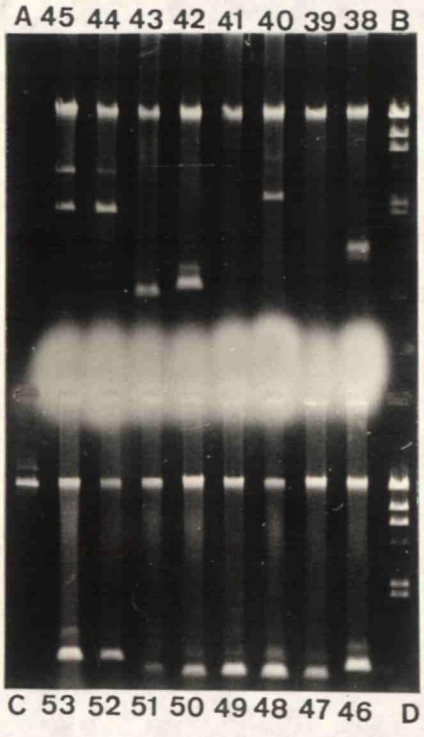


Figure 7.12a

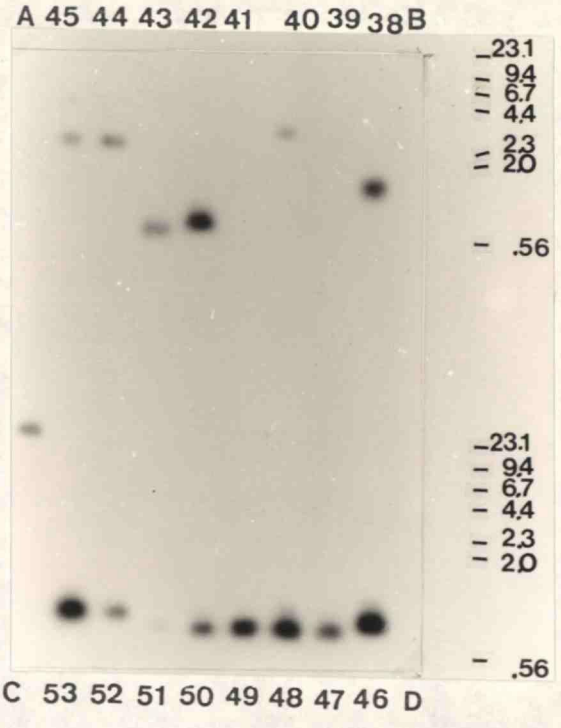


Figure 7.12b

Figure 7.12a: Plasmid DNA Extracts from Transconjugants 38-53 of P. aeruginosa PAO4069 (pRTG1). Lane A - Blank; Lanes 45-38 - Transconjugants 45-38; Lane B - lambda HindIII; Lane C - pRTG1; Lanes 53-46 - Transconjugants 53-46; Lane D - lambda HindIII.

Figure 7.12b: Autoradiograph Showing Homology between Fragment 1 of pRTG1 AvaII and DNA from Transconjugants 38-53 of P. aeruginosa PAO4069 (pRTG1). Lanes as Lanes in Figure 7.12a.

seventeen cases, the sizes shown in Table 7.1 are only relative mobilities to lambda HindIII DNA and are not intended to accurately determine plasmid size. The mobilities of closed circle plasmid DNA, however, does indicate that the plasmids were of small and varying sizes.

No hybridization was observed when fragments 2, 3, 5 and Tn5::gen were used as probes (not shown), with one exception, transconjugant 30, which contained DNA with homology to probe 3. The lack of regions homologous to 2, 3, 5 and Tn5::gen prevented determination of any possible common degradation pattern but proved extensive losses of pRTG1 had occurred in P. aeruginosa PAO4069. Furthermore, as the Tn5::gen probe did not show homology with plasmid DNA from any transconjugant, the fragments hybridizing to probe 1 could only have been derived from pMMB19 sequences from within probe 1 DNA.

Table 7.1 Comparitive mobilities of pRTG1-derivatives in P. aeruginosa PAO4069 (pRTG1) transconjugants relative to lambda HindIII

Transconjugant	26	27	28	30	32	37	38
Mobility (Kbp)	1.53	.59	.62	3.16 2.85	1.14	.75	1.15
Transconjugant	40	42	43	44	45	46	47
Mobility (Kbp)	2.41	.69	.63	2.97 1.97	2.97 2.79	.73	.67
Transconjugant	48	49	50	51	52	53	
Mobility (Kbp)	.67	.70	.70	.70	.87	.87	

AvaII only cut plasmid DNA from transconjugants 30, 44 and 45 and so restriction fragment size estimates were only possible for these three transconjugants. Other figures are not intended to be accurate assessments of plasmid sizes.

7.2.6 Temperature-sensitivity of pRTG1 in *P. aeruginosa* PAO4069 (pRTG1)

Plasmid pRTG1 encodes streptomycin and gentamicin resistance genes. Loss of resistance to streptomycin and gentamicin after growth at a non-permissive temperature would indicate that pRTG1 had retained temperature-sensitivity in *P. aeruginosa*. To test whether pRTG1 was temperature-sensitive in *P. aeruginosa* PAO4069, transconjugants 3, 8, 19, 23 and a recent transconjugant (transconjugant 54, Figure 7.2) were each inoculated from nutrient agar containing streptomycin (320ug/ml) and gentamicin (30ug/ml) into two separate nutrient broths (50ml) lacking antibiotics. One broth culture was incubated at 28°C and the other at 42°C for 24 hours. Samples ($1x10^2$cfu) were plated onto nutrient agar, nutrient agar containing gentamicin (30ug/ml) and nutrient agar containing streptomycin (320ug/ml). After 24 hours growth at the respective temperatures colonies were counted (Table 7.2).

Table 7.2 Number of colonies of antibiotic resistant *P. aeruginosa* PAO4069 (pRTG1) at two different temperatures

	Transconjugant									
	3		8		19		23		54	
	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
Nutrient	451	195	364	362	410	555	362	81	252	303
Sm (320)	245	106	134	96	385	290	332	5	226	157
% cfu	54	54	37	27	94	52	92	6	90	52
Gm (32)	253	78	70	0	174	45	153	0	222	101
% cfu	56	40	19	0	37	8	48	0	88	33

Temperature-sensitivity of both streptomycin and gentamicin resistance phenotypes was observed within all transconjugants except streptomycin resistance in transconjugant 3, which appeared stable within the population. Streptomycin and gentamicin-resistance

phenotypes of transconjugant 3 indicated that both resistance phenotypes were partially unstable within the population at the lower incubation temperature and that the gentamicin-resistance phenotype was also temperature-sensitive. Streptomycin and gentamicin resistance phenotypes for transconjugant 8 indicated plasmid instability at the permissive temperature too but a temperature-dependent effect was also observed for both resistance phenotypes. Streptomycin and gentamicin resistance phenotypes of transconjugant 19 were temperature-sensitive but a discrepancy existed between the phenotype retention frequencies at the permissive temperature. A similar discrepancy was also observed for transconjugant 23 yet both resistance phenotypes were almost entirely lost at the higher temperature. Transconjugant 54 showed stability of both plasmid resistance phenotypes at the permissive temperature and also temperature-sensitivity in approximately half the population at the elevated temperature.

7.3 Discussion

A spot-mating method successfully introduced pRTG1 into P. aeruginosa PA04069. An advantage of the spot-mating method was that each transconjugant colony was the result of an individual conjugation. Therefore any alteration of pRTG1 observed within these transconjugants would be the result of an independent event.

Selection for resistance to gentamicin (20ug/ml) was possible because of the reduction in the population density of P. aeruginosa. This may have helped identify more transconjugants because selection for resistance to streptomycin (320ug/ml) and gentamicin (30ug/ml) reduced the number of putative transconjugants isolated to 10% of those

resistant to gentamicin (20ug/ml). The number of transconjugants isolated from spot-matings was independent of the number of donor bacteria present (inocula ~$1x10^7$-$1x10^9$) and so conjugation success was probably limited by the number of viable recipient cells. Success of the spot-mating method was probably due to the extreme selection pressure for pRTG1. Any conjugation event would be immediately advantageous since recipients were plated onto media containing gentamicin prior to addition of donor. Filter-mating, however, may have been unsuccessful because it allowed time for selection against plasmid maintenance prior to plating onto selection media.

Each transconjugant was isolated and streaked to single colonies and subjected to three streakings between C-N and nutrient agar (containing streptomycin at 320ug/ml and gentamicin at 30ug/ml). Anaerobic fermenters (i.e. E. coli SM10) were not present in the purified isolates after this procedure.

During the purification procedure loss of resistance to gentamicin and streptomycin could have been a result of the loss of an E. coli SM10 (pRTG1) contaminant on PIA agar, deletions/loss of pRTG1 within P. aeruginosa, or loss of expression of resistance genes in the absence of selection. Lehrbach et al. (1984) noted plasmid instability in the absence of plasmid selection within P. aeruginosa. Putative pRTG1 transconjugants of P. aeruginosa recA strains also showed loss of resistance phenotypes, therefore instability was not entirely related to functional RecA. Jeenes et al. (1986) observed deletions of multi-copy plasmid DNA in both recA-efficient and deficient strains of P. aeruginosa whilst parent plasmids were totally stable in E. coli. Plasmid instability was not considered to be due to homologous

recombination. These comments are consistent with observations for pRTG1 in this work.

Eight strains were tested for the presence of plasmid DNA. Each strain was derived from a different conjugal event and plasmid DNA of various sizes were obtained. Assuming these plasmids were derivatives of pRTG1, pRTG1 was unstable in P. aeruginosa PAO4069 and recombination resulted in a number of recombinant plasmids. Furthermore, much smaller plasmids were isolated from more mature transconjugants indicating that a series of recombination events may occur within the same strain through time even in the continued presence of selection.

Deletions of pRTG1 in P. aeruginosa PAO4069 could have been due to a number of factors. Plasmid pRTG1 is a multi-copy plasmid and P. aeruginosa PAO4069 may be intolerant to multiple copies of DNA as many P. aeruginosa are (Jeenes et al. 1986). The transposon also offers a site of homology within the plasmid and could act as a site for recombination events within pRTG1. Such alterations may have occurred during the isolation of P. aeruginosa on C-N plates. Plasmid pRTG1 was obviously not functioning as desired in P. aeruginosa.

Considering the time left for further investigations and the problems related to identifying transposon-mediated B-lactam-resistant mutants I decided to investigate the instability of pRTG1 in P. aeruginosa in more detail.

Spot-mating conjugation was used to introduce pRTG1 from E. coli SM10 (pRTG1) into P. aeruginosa PAO4069. Hybridization analysis of plasmid

DNA from twenty-five recent transconjugants showed all but one (transconjugant 24) had DNA which shared homology with one or more probes from pRTG1. Furthermore, fourteen of the twenty-five transconjugants appeared to contain intact pRTG1 as observed by AvaII restriction enzyme digests and DNA-DNA hybridization with probes from pRTG1, although these methods were only able to detect gross changes in pRTG1.

It would appear that in all cases pRTG1 had been present, however, recombination/deletion within pRTG1-derived DNA was apparent in ten transconjugants including two (transconjugants 10 and 11) in which DNA homologous to pRTG1 regions may have been present only in the chromosome. The signal of probe DNA with DNA from these two transconjugants may have been weak due to partial homology with parental chromosomal DNA, however, no homology was observed with chromosomal DNA from the parental control nor other transconjugants. A more likely reason is that the weak signal was a result of a low concentration of homologous DNA. If plasmid DNA existed in transconjugants 10 or 11 it was dilute as none was observed. Furthermore, DNA with homology to probe 3 was observed within the chromosomal smear of DNA from transconjugant 11 and is indicative of the DNA being chromosomally located.

Few conclusions can be drawn concerning the derivation of DNA from transconjugant 18 due to the possibility of partial digestion (both Tn5::gen and probe 5 hybridized the 6.1Kbp fragment). However, fragments 1 and 2 of pRTG1 AvaII were not present.

Transconjugant 1 appeared to contain two different plasmids; one

plasmid had fragments expected for native pRTG1 whilst the other smaller plasmid shared homology with probe 1 but not Tn5::gen DNA. The absence of AvaII restriction enzyme sites in the smaller plasmid and homology to only probe 1 indicates that this plasmid is probably limited to DNA derived from within fragment 1. Furthermore, the small plasmid was a result of recombination events involving the loss of IS50 from fragment 1, as shown by the absence of homology to Tn5::gen probe. The presence of two plasmids within the same transconjugant culture may be the result of both being present in the same transconjugant or two sub-populations existing, each containing one plasmid. If the latter were true the population carrying the smaller plasmid would need rep gene functions to be conferred in trans by pRTG1 regions after insertion in the chromosome.

A number of AvaII restriction enzyme fragments of sizes not observed for original pRTG1 were observed in transconjugants 7, 9, 12, 14, 15 and 25. These were not the result of partial digestion by AvaII as the fragments were smaller than the AvaII fragment used as probe. These fragments are probably the result of a sequence of recombination/ deletion events within pRTG1. These transconjugants, however, contained intact fragments 3 and 5, which are required for expression of gentamicin and streptomycin resistance genes respectively within pRTG1.

Transconjugant 12 contained DNA with restriction enzyme fragments consistent with the presence of intact pRTG1. However, a number of additional AvaII fragments were seen and hybridization analyses showed these to be a result of recombination events rather than partial digestion.

One fragment (2.66Kbp) was common to DNA from transconjugants 12, 14 and 15 and had homology to probe 2 but not Tn5::gen probe. Another, smaller AvaII restriction enzyme fragment (2.33Kbp) was present in transconjugants 9, 12, 14, 15 and 25. This fragment had homology to both probe 2 (4.01Kbp) and Tn5::gen probe supporting the possibility that these two fragments (2.33 and 2.66Kbp) were a result of the introduction of an AvaII site within fragment 2 of pRTG1. If this is the case an insert of an extra 0.98Kbp also occurred within fragment 2 (4.01Kbp) to generate these two fragments. These novel fragments may result from plasmid cointegration generating a larger plasmid or from the presence of a smaller plasmid. It is unlikely to be the result of a chromosomally inserted region of pRTG1 due to the high concentration of DNA present in these positions.

The presence of plasmids from separate transconjugants with similar but not identical AvaII fragment profiles supports the possibility that a common host response to pRTG1 exists. For example, transconjugant 15 was similar to transconjugant 12 in containing fragment 1 (6.50Kbp) and the two fragments derived from fragment 2 (2.33 and 2.66Kbp). However, fragment 2 (4.01Kbp) was absent. Transconjugant 25 was similar to transconjugant 15; containing both fragment 1 (6.50Kbp) and the 2.33Kbp fragment but not containing fragment 2 (4.01Kbp).

Partial digestion of plasmid DNA from transconjugant 14 prevents accurate detailed analysis, however, it did contain the 2.66 and the 2.33Kbp fragments present in transconjugants 12 and 15. Probe 2 hybridized a number of fragments including some with mobilities

similar to those expected for fragments 1 and 2 but these fragments did not hybridize Tn5::gen probe. It is not possible to determine whether these are intact fragments 1 and 2 or whether they are partial digests of the derivative fragment (2.66Kbp).

Analysis of DNA from transconjugant 9 is difficult due to the low concentration of DNA present and differences between hybridization signals of probe 2 and Tn5::gen. DNA with homology to probe 2 indicated the presence of fragment 1 (6.50Kbp), fragment 2 (4.01Kbp) and one of the derivative fragments (2.33Kbp). Homology was not observed using Tn5::gen probe, however, probably because fragment 2 has greater homology with probe 2 than Tn5::gen probe. A comparison of the relevant autoradiographs shows that Tn5::gen probe generates a weaker signal than probe 2 in other lanes.

Probe 3 showed homology to three discrete DNA fragments in transconjugant 9; the uncut plasmid (4.2Kbp), the fragment 3 band (0.91Kbp) and another DNA band of a similar size to fragment 5 (0.50Kbp). Homology between fragment 3 and 5 was not seen elsewhere and it is possible that this DNA was a result of deletions within fragment 3. The presence of a fragment smaller than intact fragment 3 indicates that there are at least two fragment 3 regions in this transconjugant and that a recombination event may have occurred in one of them.

DNA from transconjugant 7 contained a fragment (2.48Kbp) which hybridized to both fragment 1 and Tn5::gen, a size similar to that of the fragment (2.33Kbp) which hybridized to fragment 2 and Tn5::gen probes found in transconjugants 9, 12, 14, 15 and 25. It is possible

that hybridization with fragment 1 probe was the result of homology with IS50, known to be present because of homology to Tn5::gen probe and I would suggest that this fragment is actually the result of recombination events within fragment 2. Furthermore, DNA from transconjugant 7 did not contain fragments 1 and 2 of pRTG1 and is therefore similar to transconjugant 14. Unfortunately, fragment 2 probe was not used in this instance to further characterize the pRTG1-derivative.

As all transconjugants were isolated from different conjugation events, the presence of similar, though not identical, patterns of pRTG1 derivatives (transconjugants 9, 12, 14, 15, 25 and possibly 7) indicates a common, though not necessarily unique, mechanism exists for recombination/deletion of pRTG1 in P. aeruginosa PA04069.

I consider the difficulty met in introducing pRTG1 was at least partly due to host factors and not just to low transfer efficiency. Such host factors might include modification on transfer of pRTG1 into the recipient and also post-transfer recombination/deletion. Intolerance to multicopy DNA has been reported in P. aeruginosa PAO (Krishnapillai et al. 1986). The presence of a complex supercoiled form of pRTG1 may indicate problems related to replication or expression of plasmid-encoded genes, possibly due to the presence of Tn5::gen open reading frames or the effect of Tn5::gen upon the structure of neighbouring regions on the plasmid. However, if such an effect existed it was specific to P. aeruginosa PA04069 since instability was not observed in E. coli UB5201 recA. Transposon Tn5::gen also offers a region of homology within the plasmid and could act as a site for recA-dependent recombination events within pRTG1. If Tn5::gen

transposed into the chromosome it could also offer an integration site for the plasmid in the chromosome and plasmid instability may be a result of this event if P. aeruginosa PAO is intolerant to the presence of multicopy DNA when that DNA is also present in the chromosome.

As native pRTG1 was transferred and capable of replication in P. aeruginosa PAO4069 it can be concluded that a recombination event is not concommitant with entry of pRTG1 into P. aeruginosa and also that recombination events within pRTG1 are not essential prerequisites for replication or expression of pRTG1 within P. aeruginosa. Furthermore, E. coli UB5201 transformants containing plasmid DNA from P. aeruginosa PAO4069 (pRTG1) transconjugants were resistant to streptomycin, sulphonamide and gentamicin. Restriction enzyme and hybridization analyses of transformants supported the view that pRTG1 from P. aeruginosa had not been altered, although changes at the base level may have occurred.

Plasmid pRTG1, however, was unstable in P. aeruginosa PAO4069 since a number of pRTG1 derivatives were identified. As independent transconjugants (12, 15, 14 and 9) carried plasmids with AvaII fragments of similar sizes and homologies, it would appear that specific regions of pRTG1 are sensitive to recombination/deletion events. Furthermore, DNA profiles from transconjugants 12, 15, 14 and 9 may form a sequence of recombination and deletion events (Table 7.3) although there is too little data to confirm this. Furthermore, analysis of fragment derivation has not yielded an explanation as to what specific events were involved. For example, analysis of DNA from transconjugant 15 showed fragments 1, 3, 4 and 5 were intact but

fragment 2 (4.01Kbp) was absent. Probe 2 hybridized to two AvaII fragments (2.66 and 2.33Kbp), one of which also shared homology with Tn5::gen probe DNA. It would appear that a 0.98Kbp fragment containing an AvaII restriction enzyme site had been inserted within fragment 2. As all other fragments have remained intact, insert DNA must have originated from another replicon. Furthermore, the generation of a novel fragment would require recombination between homologous but non-identical regions.

Insertion sequences have homologous ends and recombination between opposing ends of two separate IS50 elements may generate a new AvaII restriction enzyme cleavage site. Although this explains how a recombination event can generate a new fragment, the presence of more than one new fragment and the loss of fragment 2 from transconjugant 15 is not explained. The possibility that other events must have occurred is supported by analysis of DNA from transconjugant 12, which indicate that a further fragment (2.14Kbp) with homology to probe 2 may have existed previously. The 2.66Kbp fragment present in transconjugants 12, 14 and 15 is absent in DNA from transconjugant 9. Although the 2.66Kbp fragment may never have been present in transconjugant 9, its absence may indicate continued instability of pRTG1 derivatives.

Table 7.3 P. aeruginosa PA04069 (pRTG1) Transconjugants Showing a Possible Stepwise Sequence of Recombination/deletion Events within pRTG1

	Transconjugant			
	12	15	14	9
AvaII fragment 1 (6.50Kbp)	+	+	-	-
AvaII fragment 2 (4.01Kbp)	+	-	-	-
Homology to probe 2 at 2.66Kbp	+	+	+	-
Homology to probe 2/Tn5::gen at 2.33Kbp	+	+	+	+
Homology to probe 2 at 2.14Kbp	+	-	-	-

Analysis of transconjugant plasmids after three months sub-culturing supports the possibility that pRTG1 was subject to a series of recombination events. Of transconjugants 26-53 only eight contained no DNA homologous to pRTG1-derived probes used. The small and varying sizes of plasmid DNA obtained in the 20 transconjugants indicated that the majority of pRTG1 was unstable in P. aeruginosa PA04069. Homology to probe 1 but no AvaII cleavage sites indicated that the plasmid comprised of fragment 1 DNA only. These plasmids did not hybridize to Tn5::gen probe indicating loss of the IS50 and its flanking AvaII cleavage site from fragment 1. I propose that pRTG1 was gradually reduced to a size which must contain the ori site of pMMB19 but little else. Furthermore, the absence of plasmid DNA in the other eight transconjugants is probably due to the loss of pRTG1 derived plasmid rather than these transconjugants never having had the plasmid. This is supported by the fact that spontaneous mutations to streptomycin or gentamicin-resistance were not observed in the conjugation experiments from which these were isolated. However, the use of recA-mutant recipients, for example P. aeruginosa PA06521 did not alleviate the problem of instability since the resistance phenotypes were lost during the purification procedure.

Resistance to streptomycin and gentamicin in transconjugants carrying small pRTG1 derivatives may be due to integration of pRTG1-derived DNA into the chromosome and the failure to detect homology to pRTG1 probes may be due to the low concentration of chromosomal DNA in the DNA sample. Another possibility to explain the presence of the resistance phenotypes of pRTG1 when the majority of the plasmid has been deleted is that some resistant transconjugants may have been "trained" to resistance during the cyclic purification between PIA and pRTG1-selection media at the same time as plasmid DNA regions were being deleted. The fact that plasmid DNA was lost in the presence of antibiotic selection shows that antibiotic selection pressure was not enough to maintain the native plasmid. Genomic DNA extractions and hybridization analysis were not performed to test whether the streptomycin-resistance gene from pRTG1 was present in the chromosome. Support for the hypothesis that pRTG1 regions were present in the chromosome comes from unsuccessful attempts to reduce the size of pRSF1010 beyond 75% of its' original size (8.9Kbp) (Bagdasarian *et al.* 1981). The authors concluded that essential factors required for replication and maintenance of pRSF1010 were scattered over a large region of the plasmid. This was confirmed by Scholz *et al.* (1985) who showed mini-plasmid derivatives of pRSF1010 (1.2-3.3Kbp) encoding the *ori* site (2.6Kbp) required a separate helper plasmid encoding the three essential *rep* genes of pRSF1010 (4.2-8.7Kbp) (Scholz *et al.* 1985). As pRTG1 is based on a pRSF1010 replicon and plasmid derivatives were obtained which did not show homology with probe 2 (encoding two of the three essential *rep* genes, Scholz *et al.* 1985) it is likely that plasmid-encoded determinants for plasmid replication and maintenance were present in the chromosome of *P. aeruginosa*. As

rep genes lie either side of Tn5::gen in pRTG1 and lie adjacent to the sulphonamide-streptomycin gene operon, it is likely that the antibiotic resistance genes of pRTG1 are also present in the chromosome of P. aeruginosa and may confer resistance upon the cell.

Temperature-sensitivity of pRTG1 was investigated in five transconjugants of P. aeruginosa PAO4069 (pRTG1) which appeared to contain original pRTG1 as determined by restriction fragment and hybridization profiles. All five transconjugants showed some degree of temperature-dependent instability within the populations but variations in the temperature response were observed.

Transconjugant 54 was the most recently obtained transconjugant and may have encoded original pRTG1 (uncut plasmid migrated similarly to pRTG1, see Figure 7.2, and had homology to Tn5::gen probe, not shown). The plasmid appeared totally stable at the permissive temperature in the absence of selection and showed temperature-dependent loss of both streptomycin-resistance (90% to 52%) and gentamicin-resistance (88% to 33%) at the non-permissive temperature as expected if both resistance genes were present on pRTG1.

In transconjugant 23 streptomycin-resistance and gentamicin-resistance were both almost entirely lost from the population at the non-permissive temperature (94% and 100% loss respectively) indicating both resistances were encoded on pRTG1. At the permissive temperature, transconjugant 23 appeared resistant to streptomycin (92% of the population), indicating plasmid stability but gentamicin-resistance was limited to 48% of the population. The paradox may be explained if there were two plasmid populations of approximately equal

ratios. One population (48%) may carry native pRTG1 and the remaining resistance to streptomycin (44%) would be encoded upon a temperature-sensitive derivative of pRTG1 in which Tn5::gen had been deleted. Spontaneous loss of Tn5::gen from pRTG1 may be a result of transposon-mediated excision or recombination events within pRTG1.

Plasmid pRTG1 appeared to have a highly supercoiled structure in P. aeruginosa and this structure may interfere with the synthesis of, and expression from pRTG1, especially in the absence of antibiotic selection. Such a complex structure may be a result of the presence of the transposon on the plasmid. Plasmid derivatives in which Tn5::gen is deleted may be selected if the structure became more relaxed resulting in a plasmid more accessible to host replication factors. Such a plasmid being temperature-sensitive and encoding the streptomycin-resistance gene would confer the phenotype seen for transconjugant 23. No direct evidence for this is offered and furthermore, transconjugant 23 contained only pRTG1 when analysed previously. However, it is possible that recombination events occurred in the period between the DNA analysis and the temperature-senstivity analysis. DNA from transconjugants carrying pRTG1 was in a highly supercoiled form and this complex was less obvious in transconjugants which carried derivatives of pRTG1. Furthermore, two plasmids; one native pRTG1 and one derivative lacking Tn5:gen, had been observed previously within the same transconjugant (Transconjugant 1; this chapter).

Observations of other transconjugants may also be explained by the presence of two plasmids. Transconjugant 3 had similar losses of streptomycin-resistance at both temperatures (54%) indicating that it

could be a temperature-stable revertant but still showed temperature-independent instability. Transconjugant 8 also showed loss of streptomycin-resistance (67% loss) and gentamicin-resistance (81% loss) phenotypes at the permissive temperature indicating a similar temperature-independent plasmid instability. Furthermore, total loss of gentamicin-resistance at the non-permissive temperature indicated temperature-dependent instability. Assuming resistant phenotypes are plasmid-derived I would consider partial retention of streptomycin-resistance at the non-permissive temperature in the absence of gentamicin-resistance was due to the presence of a second plasmid encoding streptomycin-resistance only. The pRTG1-derivative would have to have lost Tn5::gen but unlike the plasmid of transconjugant 23, maintained the temperature-independent instability and reverted to temperature-stability. Such a series of events may appear unlikely but a pRTG1-derivative lacking Tn5::gen and showing temperature-stability had been observed in Chapter 5.

Transconjugant 19 had a similar phenotype to transconjugant 23 at the permissive temperature but a similar phenotype to transconjugant 8 at the non-permissive temperature. Therefore, native pRTG1 encoding streptomycin and gentamicin-resistance would have both temperature-dependent and temperature-independent instability. The pRTG1-derivative encoding streptomycin-resistance only would be stable irrespective of temperature and the observed loss of streptomycin-resistance at the non-permissive temperature would be due to the loss of native pRTG1. Subtracting the proportion of the population resistant to gentamicin (37%) from the proportion resistant to streptomycin (94%) gives a value (57%) fairly close to that observed (52%), supporting this possibility.

The explanation offered concerning the temperature-sensitive nature of the five transconjugants tested is speculative and requires a series of related events; supercoiling, interference with replication and repression and relief of this interference by recombination/deletion of regions within pRTG1 including Tn5::gen. The latter would require the presence of Tn5::gen and possibly other plasmid sequences either in intact pRTG1 or the chromosome.

In support of this hypothesis pRTG1 did appear in a complex, supercoiled form, which may have interfered with its replication (for example, in transconjugants 16 and 17). Furthermore, such supercoiled complexes were not present in recombinant derivatives of pRTG1 (for example, from transconjugants 14 and 15).

Retention of streptomycin-resistance at the non-permissive temperature, in particular for transconjugant 3, may be due to spontaneous resistance mutations, integration of pRTG1 regions into the chromosome or reversion to temperature-stability of pRTG1. In support of the last possibility a temperature-stable derivative of pRTG1 lacking Tn5::gen was observed in *P. aeruginosa* PA04069 (pRTG1a) (Chapter 5).

Mutant RepA protein is temperature-sensitive (Scholz *et al.* 1985) and may have reduced activity even at a permissive temperature. Revertants would be selected especially if plasmid replication was also hampered by supercoiling. The function of RepA has yet to be elucidated, however, it may function as an essential pRSF1010-specific substitute for DnaG, DnaB or DnaC of the *E. coli* chromosome since

these proteins are not required for pRSF1010 replication (Scholz *et al.* 1985). RNA primase is the product of *dnaG*, which along with DnaB and DnaC are components of the primosome required for the initiation of a new round of replication and replication along the lagging strand of the replication fork (Freifelder 1987).

If the activity of RepA is impeded at the permissive temperature in *P. aeruginosa* PAO4069 and results in inefficient plasmid replication, RepA may be responsible for the instability of pRTG1 and hence selection for stable revertants. If this is the case then pRTG1 may be of little use as a temperature-sensitive broad host-range vector in *P. aeruginosa*.

However, I have shown pRTG1 to be temperature-sensitive in *P. aeruginosa* PAO4069 and it is still possible that pRTG1 could be used successfully to introduce Tn5 into the chromosome of *P. aeruginosa*. In view of the problems encountered I would use pRTG1 as a suicide vector for two major reasons. Firstly, to prevent the production of temperature-stable plasmids (Chapter 5) and secondly, the use of a broad host-range plasmid could allow the accumulation of chromosomally-located transposons prior to growth at the non-permissive temperature, as was observed in *E. coli* (Chapter 4). The dangers here are that transposon-insertions may not be related to the mutation sought and furthermore may lead to clonal selection of specific transposon-bearing strains; both factors reducing the likelihood of identifying genes of interest by insertional mutagenesis.

CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

Each chapter contains a section in which results are discussed. In this chapter I will discuss the thesis as a whole and discuss work which I believe would help extend and explain results from this work. I will also discuss the regulation of the inducible, chromosomal class C B-lactamase within a wider context than that given in Chapter 1 and suggest possible experiments which could test certain hypotheses.

Transposon mutagenesis using Tn5 was considered a suitable method for identifying and cloning regulatory genes of the inducible, chromosomally-encoded class C B-lactamase (*bla*P) of *P. aeruginosa*. Selection for the presence of Tn5 in the chromosome followed by identification of B-lactam resistant mutants which hyperproduce B-lactamase may identify transposon insertional mutagenesis within regulatory genes of *bla*P. Selection for Tn5 in an initial cloning experiment would isolate flanking chromosomal regions and homology between cloned chromosomal regions flanking the transposon may identify the wild-type gene within a gene library.

Unfortunately, difficulties were encountered in nearly all stages of the development and use of the transposon. Initially, a suitable antibiotic resistance gene had to be cloned into Tn5 in order to identify Tn5 within a *P. aeruginosa* population. A tobramycin-resistance gene was inserted into Tn5 but appeared unstable with loss of resistance on sub-culturing. Loss of expression from the

tobramycin-resistance gene was observed in other constructs within the laboratory (M.J. Woodward, personal communication) and an alternative marker for Tn5 was made (Chapter 3). Plasmid pSG1 was a derivative of pSUP2011 (Simon *et al.* 1983) encoding gentamicin-resistance in Tn5 (Tn5::gen). Plasmid pSG1 expressed gentamicin-resistance on sub-culturing and was able to confer gentamicin-resistance when introduced into *E. coli* by transformation and conjugation.

Transfer of pSG1 into *P. aeruginosa* with concommitant transposition of Tn5::gen from pSG1 into the chromosome was not observed within the background of spontaneous mutation to gentamicin-resistance within the population. I considered it most unlikely that pSG1 would transfer Tn5::gen into *P. aeruginosa* at a frequency able to identify B-lactamase hyperproducing mutants resulting from Tn5::gen insertional mutagenesis into *blaP* regulatory genes.

A control system was unable to show transposition of Tn5::gen from pSG1 into *E. coli* UB2272 *polA*. This control system was used because it most closely followed the procedure for introducing Tn5::gen into *P. aeruginosa*. However, it would appear that transposition was undetectable because of the interference of the transposition process due to PolA-deficiency (Syvanen *et al.* 1982, Yin and Reznikoff 1987) rather than transposition-deficiency of Tn5::gen. On hindsight it would have been appropriate to test for transposition ability of Tn5::gen by other methods, for example, P1 transduction prior to the construction of other Tn5::gen-encoding plasmids. However, at the time I felt that transposition of Tn5::gen could be tested at the same time as constructing an alternative plasmid, better suited to introducing Tn5::gen into *P. aeruginosa*.

Transposon Tn5::gen was introduced into a broad host-range, temperature-sensitive plasmid, pMMB19, in order to remove the problem of poor transfer frequency reducing the maximal theoretical number of transpositions possible. Plasmid pRTG1 was temperature-sensitive in E. coli and transposition of Tn5::gen from pRTG1 into the E. coli chromosome was observed. However, transposition of Tn5::gen occurred at the permissive temperature for pRTG1 and was able to accumulate within the population. To minimise the accumulation of specific Tn5-bearing strains and spontaneous B-lactam resistance carrying Tn5::gen in the chromosome it was considered important to use transconjugants in which pRTG1 had recently been introduced.

Plasmid pRTG1 was transferred into P. aeruginosa by filter-mating conjugation. Plasmid instability was observed in all such transconjugants but a temperature-sensitive derivative, pRTG1a, was used to test whether transposition of Tn5::gen could generate hyperproducing B-lactamase mutants.

Difficulties were encountered when attempting to identify Tn5::gen-indced B-lactamase hyperproducing mutants because of the effects of population-dependent resistance and spontaneous resistance to B-lactams. After growth at the non-permissive temperature colonies retaining gentamicin-resistance were replica-plated to test for B-lactam resistance. Since B-lactams were not present in the initial selection procedure B-lactam-resistant mutants generated by Tn5::gen mutagenesis were expected to be more frequent than spontaneous resistant mutants. However, difficulties related to population-dependent resistance to B-lactams prevented this protocol from

identifying mutants generated by Tn5::gen mutagenesis. One method was able to generate B-lactam resistant mutants whilst spontaneous resistance was not observed in a control culture. Eight B-lactam resistant mutants showing co-retention of gentamicin and B-lactam resistance were chosen for further analysis. All eight mutants showed increased rates of nitrocefin hydrolysis with respect to the parent strain and furthermore, chromosomal DNA from these strains contained Tn5::gen in SalI fragments of similar size. These results were consistent with transposon mutagenesis of a blaP regulatory gene (Chapter 5).

Semi-automated analysis of B-lactamase activities from these mutants showed them to have different basal levels of B-lactamase production. P. aeruginosa PAO4082, a B-lactam-resistant mutant control strain had the highest basal production of B-lacatmase yet MIC analysis showed resistant mutants from this work were more resistant to piperacillin. It is therefore unlikely that these strains were resistant to B-lactams simply as a result of B-lactamase production. Other resistance mechanisms, for example decreased permeability to B-lactam, probably contributed to B-lactam resistance along with increased B-lactamase production. In conclusion, it is unlikely that Tn5::gen was present in a blaP regulatory gene. This thesis has not resulted in the identification of regulatory genes in P. aeruginosa and so has not contributed to the understanding of B-lactamase expression in P. aeruginosa.

A control system was devised to test whether Tn5::gen could identify ampD within E. coli SNO3 (pNU305). Using the B-lactam, nitrocefin, it was possible to identify mutants hyperproducing B-lactamase without

selecting for spontaneous B-lactam resistant mutants. However, expression of ampC from the high-copy number plasmid pNU305 interfered with the screening procedure. This system also posed problems with pNU305 acting as a preferential site for Tn5::gen mutagenesis and interference of pRTG1 instability due to the possible integration of plasmids. However, the nitrocefin hydrolysis method was able to identify hyperproducing B-lactamase mutants in P. aeruginosa and it could be used to screen for such mutants within P. aeruginosa PA04069::Tn5::gen.

Prior to screening for B-lactam hyperproducers a suitable method to generate P. aeruginosa PA04069::Tn5::gen strains had to be developed and this required an investigation into the instability of pRTG1 in P. aeruginosa PA04069 (pRTG1). The final chapter investigated the intolerance of P. aeruginosa to pRTG1 and showed that a specific recombination/deletion process may have occurred. However, the entire plasmid was unstable. In conclusion, pRTG1 may be limited to use as a suicide vector when attempting transposon mutagenesis of P. aeruginosa.

8.2. Future work: The short-term

It would be of interest to accurately observe the loss of plasmid sequences in P. aeruginosa and to ascertain whether this is specific to pRTG1, Tn5, the gentamicin-resistance gene or the host. Analysis of pRTG1 in recA P. aeruginosa was not possible, due to loss of plasmid during the purification procedure indicating instability may not be related to recombination events within homologous regions. Other workers have found instability of broad host-range,

temperature-sensitive vectors in P. aeruginosa (R. Wilson, personal communication) and I would suggest that this indicates a host-specific intolerance to the plasmid. It is possible that the host restriction system is effective in degrading hemi-methylated plasmid DNA present after DNA replication but other factors may also account for the continued reduction insize of plasmid DNA. The use of restriction-modification mutants as hosts of pRTG1 may not be successful as transposition of Tn5 is regulated by methylation (Yin et al. 1988).

Hewinson (1987) reported instability of the same gentamicin-resistance gene from vector DNA in P. aeruginosa. Instability as shown by recombination events within fragment 3, which encoded the gentamicin gene, was observed in one copy of the gene carried by transconjugant 9. Recombination/deletion events within other regions of pRTG1 were more common and so it is unlikely that the gentamicin-resistance gene is a major factor in the instability of pRTG1.

I was successful, however, in cloning the tobramycin gene from pR5 into Tn5 towards the end of this work. Two tobramycin-resistant transposon Tn5 derivatives on pSUP2011 were stable in E. coli. If these transposons are stable in P. aeruginosa they could be used as alternatives to Tn5::gen in generating mutants by insertional mutagenesis of the chromosome.

As well as the problems of introducing the transposon at a high frequency and the instability of pRTG1 in P. aeruginosa there was also the problem of trying to identify mutants of interest without identifying mutants which had generated B-lactam resistance

spontaneously with a coincidental insertion of the transposon in the chromosome. This problem would still remain but could be reduced by using freshly transformed strains for mutagenesis as these are less likely to contain a chromsomally-located transposon. Analysis of mutants from different transconjugants would also be advantageous as a common position of Tn5::gen in the chromosome of B-lactamase hyperproducers may be determined (transposon hot-spots should not be a problem for Tn5-derivatives).

The analysis of B-lactamase hyperproducing mutants is incomplete. Comparisons of induced and non-induced cultures of B-lactam-resistant mutants would be of interest, especially if induced cultures have higher induction ability than P. aeruginosa PAO4095 and PAO4082. These mutants may still be of use in mapping genes regulating B-lactamase synthesis.

Attempts to clone Tn5::gen and the flanking chromosomal regions from B-lactamase hyperproducing mutants of P. aeruginosa PAO4069 into pUC18 were unsuccessful in this thesis. This was probably because smaller chromosomal DNA fragments were present and were cloned preferentially. Purification of digested chromosomal DNA of the expected size for the transposon region obtained from DNA-DNA hybridizations would increase the probability of successful ligation. However, the presence of the transposon in the chromosome was not indicative of Tn5::induced B-lactam resistance. It is unlikely that transposon mutagenesis is a significantly sensitive tool for the identification of genes involved in controlling B-lactamase production in P. aeruginosa. A less cumbersome method may be using the genes already identified in controlling the inducible B-lactamase of C. freundii as probes in the

chromosome of P. aeruginosa. This assumes a similar mechanism exists in P. aeruginosa as in the inducible B-lactamase members of the Enterobacteriaceae. This possibility is supported by S. Cole, who has identified a gene in P. aeruginosa with homology to the ampD of E. coli (W. Nichols personal communication). However, as ampD is also present in E. coli where it performs no such function the presence of a gene with homology to ampD is not confirmatory evidence for its role in the induction of B-lactamase.

The use of Tn5 mutagenesis and hybridization analysis with known regulatory genes of other inducible ampC organisms would offer the best method of successfully identifying whether homologous genes have similar regulatory functions. For example, if the ampD probe bound to the same region as Tn5::gen in B-lactamase hyperproducing mutants but to a different region in the parent control it would suggest that Tn5::gen had transposed into or adjacent to ampD and had interfered with B-lactamase production.

8.3 Gene Organization and Regulation in P. aeruginosa

It is possible that blaP has a different regulatory mechanism to those of other inducible ampC bacteria and so comparisons between the control of gene organization of Pseudomonas and members of the Enterobacteriaceae may be of use. The size and and regulatory complexity of the pseudomonad gene pool are important aspects in the ability of P. aeruginosa to survive in a wide variety of often hostile environments (Govan and Harris 1986). Survival can be seen as a result of extensive genetic resources, physiological adaptation to the environment and mutation of existing genes. Gene organization of P.

aeruginosa is less clustered that of the Enterobacteriaceae (Holloway and Morgan 1986). The operon system is apparently less common in P. aeruginosa with all biosynthetic systems contained within an operon structure in enteric bacteria not being organized similarly in pseudomonads (Holloway and Morgan 1986). However, a system of supraoperons exists within some genes with related functions found in a number of clusters. For example, in P. aeruginosa there are four regions in which genes involved in the control and synthesis of tryptophan are organized; at 26 minutes (trpA-trpB), 34-35 minutes (trpC-trpD and trpE) and 54 minutes (trpF) (Holloway and Morgan 1986). In both E. coli and S. typhimurium, however, all the genes exist within a single operon system. Another difference in regulating expression of biosynthetic genes between enteric bacteria and P. aeruginosa is that repressors are more commonly used in enteric bacteria (Holloway and Morgan 1986).

An example of genetic regulation in response to an environmental stimulus in P. aeruginosa is the regulation of alginate production. A number of comparisons have been made between this and other regulatory systems which respond to environmental changes in other bacteria (for example, nitrogen limitation in E. coli, osmotic pressure changes in E. coli and conditions leading to sporulation in Bacillus subtilis, Ronson et al. 1987). Two regulatory proteins, AlgQ and AlgR, regulate the synthesis of AlgD, an enzyme involved in alginate production and may act as a two component regulatory system (Deretic and Konyecsni 1989). Protein AlgR is a transcriptional activator of algD and AlgQ appears to be a sensory protein which acts as a signal receptor and activator of AlgR. The mechanism of induction may be similar to that seen in the regulation of nitrogen-regulated promoters, where an

analogue of AlgR, NtrC, is activated by a protein kinase, NtrB, (Ronson et al. 1987). There is evidence of "cross-talk" between a sensor of one system and an activator of another system (Ronson et al. 1987). Superficially this appears to reduce the specificty of response to an environmental signal through a specific sensor-activator pair but it is important to consider that common responses are required to regulate cell metabolism in response to different environmental signals.

8.4 B-Lactamase Induction

Considering the variety of effects of different B-lactams on the target bacterium the concept of "cross-talk" is attractive. However, AmpD, AmpE and AmpR do not show homology to this sensor-activator family but show homology to another family of regulatory proteins (Lindquist et al. 1989a). Protein AmpR belongs to the group of LysR regulatory proteins, which includes NodD, and AmpE is similar to NodI.

Any system involved in B-lactamase induction of blaP would need to respond to B-lactams each of which may have different and sometimes very specific target site PBPs. Carbenicillin, ceftazidime, piperacillin and ticarcillin, for example, bind preferentially to PBP3 (Curtis et al. 1981, Noguchi et al. 1979) whilst imipenem and mecillinam bind PBP2 preferentially (Curtis et al. 1981). Furthermore, induction of chromosomal B-lactamase differs in response to different B-lactams. Differential induction by B-lactams may be due to B-lactams having different inhibitory effects on specific PBPs. Alternatively, they may act upon another system that is not the primary site of B-lactam antibacterial action. In either case,

regulation of B-lactamase production appears to be a highly complex system.

8.4.1 Inducer ability of B-lactams and other compounds

The potential of a B-lactam to act as an inducer is in part dependent upon the permeability of that B-lactam across the outer membrane and in part dependent upon the stability of the B-lactam against hydrolysis by B-lactamase (Furth 1979). The most successful inducers of the chromosomal B-lactamase of P. aeruginosa appear to be imipenem, cefoxitin and ceftazidime (Sanders and Sanders 1979, Farmer and Reading 1987) and these are particularly stable to hydrolysis by the chromosomal B-lactamase. B-Lactamase may be induced specifically in response to B-lactams or may have another, unknown function within the cell. For example, if the B-lactam acts as an analogue of D-ala-D-ala in binding to a PBP it is possible that excess D-ala-D-ala is the true "inducer" of B-lactamase.

There is little evidence that B-lactams enter the cytoplasm and directly effect B-lactamase synthesis. Peter Bennett (W. Nichols personal communication) has shown that TEM B-lactamase in the periplasm prevents induction of C. freundii ampC in E. coli SN03 yet cytoplasmic-bound TEM B-lactamase does not affect the normal induction of C. freundii ampC in E. coli SN03. Therefore, B-lactams probably exert their inductive effect from the periplasm via a receptor system. Furthermore, there is usually a lag phase between addition of B-lactam and induction of B-lactamase, which is dependent upon the permeability of the B-lactam. In spheroplasts of P. aeruginosa rapid induction of B-lactamase occurs when incubated with B-lactam (Nordstrom and Sykes 1974). Lindberg et al. (1985) identified induction of B-lactamase

synthesis within 2 minutes of B-lactam addition in mini-cells of E. coli containing a plasmid encoding the ampR-ampC region of C. freundii. Absence of chromosomal DNA in mini-cells and a rapid induction rate indicates no chromosomal genes are required to be activated and/or inhibited prior to induction of the ampC gene on the plasmid. Instead, induction of ampC functions through pre-existing proteins.

An accumulation of intermediates in the peptidoglycan synthesis pathway has been observed as a result of the presence of B-lactam in both B. cereus (Saz and Lowery 1965, Ozer et al. 1970), and S. aureus (Sachithandum et al. 1974). This supports the possibility that inhibition of PBPs causes the accumulation of intermediates of peptidoglycan which may induce synthesis of B-lactamase. However, this hypothesis has little support from studies in the Gram-negative C. freundii (Lindberg et al. 1985) nor in E. cloacae (Curtis et al. 1987), where non-B-lactam inhibitors of, and precursors and intermediates of, peptidoglycan synthesis do not induce B-lactamase synthesis. Furthermore, in P. aeruginosa the B-lactamase is not induced by other, non-B-lactam antibiotics that inhibit peptidoglycan synthesis (Nordstrom and Sykes 1974). Induction of B-lactamase by non-B-lactams has occurred under some conditions (P. Bennett personal communication). Though possibly an artefact it may also indicate B-lactamase induction is dependent upon unidentified physical or chemical factors. Although intermediates of peptidoglycan do not generally induce B-lactamase synthesis in the laboratory, inhibition of PBPs may still play a role in B-lactamase induction.

8.4.2 PBPs - a role in B-lactamase induction?

A protein, BlaR1, required for the induction of B-lactamase in *B. licheniformis* (Kobayashi *et al.* 1987) has been shown to contain many hydrophobic regions indicating that it may be a membrane-bound protein. Protein BlaR1 contains both the serine-X-X-lysine motif and the lys-thr-gly motif common to all known B-lactam binding proteins except one. It acts as an anti-repressor in the B-lactamase regulatory system and loss of this protein causes a low, constitutive production of B-lactamase. This last observation indicates a sensory function for the anti-repressor, unlike that of *ampD* where loss of function does not repress, but induces B-lactamase synthesis (Lindberg *et al.* 1987). Protein AmpE contains the ser-X-X-lys motif but not the lys-thr-gly found in most B-lactam binding proteins and does not bind B-lactam covalently (Lindquist *et al.* 1989a). Comparisons between the effect of BlaR1 and AmpE on B-lactamase induction are not possible due to the effect of AmpE-deficiency in different systems (Lindquist *et al.* 1989a, Honore *et al.* 1989).

A correlation seems to exist between induction by certain B-lactams (for example, ampicillin, cephloradine and latamoxef) and their ability to bind PBP5 in both *P. aeruginosa* and *E. cloacae* (Martin *et al.* 1988) irrespective of the primary PBP target site.

The possible importance of PBP5 in the induction system of B-lactamase production in *P. aeruginosa* is further supported by investigations of a temperature-sensitive strain of *P. aeruginosa*, hs-257 (Noguchi *et al.* 1980). This strain shows temperature-sensitivity with respect to both the penicillin-binding ability of PBP5 and the production of B-lactamase. At 40^{o}C no binding of penicillin to PBP5 was observed

and induction of B-lactamase was inhibited by at least 5-fold (Noguchi et al. 1985). PBP5 acts similarly to the anti-repressor of B. licheniformis in that in its´ absence B-lactamase is produced at a low, constitutive level. Binding of B-lactam to PBP5 may be the initial step in the induction of B-lactamase and it has been suggested that this initial event functions through ampD upon ampR-ampC (Martin et al. 1988). A paradox appears to exist; the absence of functional PBP5 causes low, constitutive production of B-lactamase yet a direct correlation exists between the percentage of PBP5 bound to B-lactam and the level of B-lactamase induction. The former implies that PBP5 must be functional for induction; the latter that inhibition of PBP5 activity by B-lactams causes induction. As the absence of PBP5 represses B-lactam synthesis yet inhibition of PBP5 induces B-lactamase synthesis, the enzymatic activity of PBP5 appears essential for induction of B-lactamase.

Comparisons between PBPs of E. coli and P. aeruginosa are possible due to the similarities observed in their profiles and morphological consequences of their inactivity (Georgopapadakou 1988). PBP5 is the most numerous PBP in E. coli and catalyses the major carboxypeptidase activity in vivo (Nicholas and Strominger 1988) PBP5 is non-essential but can, however, have compensatory activity for other inhibited PBPs. In response to certain "concentration-dependent inducer B-lactams" (Martin et al. 1988) PBP5 may compensate other, inhibited PBPs and also, directly or indirectly, induce B-lactamase production. The fact that loss of PBP5 activity causes loss of B-lactamase induction in response to specific B-lactams suggests an essential sensory role of PBP5 in the induction of B-lactamase. The possibility of regulatory ties between PBP5 and B-lactamase is particularly attractive

especially since PBP5 has weak B-lactamase activity and may be more closely related to B-lactamases than other PBPs (Spratt 1983).

B-Lactam binding to another PBP has also been suggested as an essential event in the induction of B-lactamase. Oliva et al. (1989) reported the requirement of PBP2 but not PBP3 for induction of the B-lactamase of C. freundii in E. coli in response to induction by 6-aminopenicillanic acid (6-APA). As B-lactam specifically binds to only PBP2 and PBP1a (Curtis et al. 1981) the involvement of PBP2 in signalling B-lactamase induction in response to other B-lactams can not be assumed. However, this observation may show the role of PBPs in an induction system is highly complex. Any inhibition of PBPs may be able to signal the presence of B-lactam. It is unlikely that a single signal system operates as B-lactams have different target sites and different inducer potentials. This system may act similarly to the adenylate cyclase system (as suggested by Oliva et al. 1989) with different receptors causing similar effects through a single secondary messenger. It is possible that a PBP bound to B-lactam is capable of binding to a single or PBP-specific transmembrane protein. A common linker protein may connect such a complex to a transducer protein which then directly or indirectly induce B-lactamase activity. However, PBP5 is anchored into the cytoplasmic membrane by only 10 amino acids (Nicholas and Strominger 1988) unlike receptor proteins of the adenylate cyclase system.

Two gene clusters are involved in cell envelope growth and division including peptidoglycan synthesis; at 2 minutes and also at 89 minutes on the E. coli chromosome (Spratt et al. 1980). It is therefore of interest to note that the ampD-ampE region has been mapped at 2.4

minutes on the map of E. coli (Lindberg et al. 1987), lying between the genes encoding PBP3 (1.8minutes) and PBP1b (3.3 minutes) (Waxman and Strominger 1983). However, the genes for PBP2 (pbpA) and PBP5 (dacA), which may be involved in the induction of B-lactamase, have been mapped at 14.4 minutes on the chromosome as has another gene (rodA) whose absence results in a morphological change similar to loss of PBP2 (Spratt et al. 1980, Waxman and Strominger 1983). As overproduction of PBP5 results in the same morphological consequence as the loss of pbpA and rodA it would appear that this is another gene cluster involved in co-ordinate regulation of peptidoglycan synthesis and cell shape determination (Spratt et al. 1980, Waxman and Strominger 1983). Moreover, ampC region was mapped just outside the second cluster; at 93.8 minutes on the E. coli chromosome (Bergstrom et al. 1983). The presence of ampD-ampE within a cluster of genes associated with peptidoglycan synthesis and cell envelope growth and division may indicate that these genes are involved in general cell wall metabolism. This possibility is supported by their presence in E. coli which does not encode an inducible B-lactamase.

The role of PBPs in the induction system may be variable, depending on the specific binding affinity of a B-lactam and compensatory abilities of various PBPs. The induction system must therefore be assumed to be able to respond to the inhibition of different PBPs and to respond selectively.

8.4.3 A role for B-lactamases in regulating peptidoglycan synthesis?

To understand the regulation of the inducible class C B-lactamase in *P. aeruginosa* it may be of use to understand the purpose of the system if a wild-type strain, for example 3-Pre, has a mutation in the regulation of B-lactamase synthesis. This may be the result of selection within the hospital environment where the mutation confers higher basal and inducible B-lactamase activities in the presence of B-lactam. Furthermore, what is the function of the *ampD-ampE* region in *E. coli* which does not have an inducible B-lactamase suggesting another role for these genes within the cell? As mutants of *E. coli* exist with a deletion of the *ampD-ampE* (Lindquist *et al.* 1989a) region the system is not essential for growth under laboratory conditions.

I believe that a system similar to that working in *C. freundii* is in operation in *P. aeruginosa*. This belief is partially based on the recurrent themes that exist in reported observations and also the homology between *ampD* and a region of the pseudomonal chromosome reported by Cole (W. Nichols, personal communication). Furthermore, since *ampD* of *E. coli* can control expression of the inducible *ampC* of *C. freundii* the presence of an homologous gene in *P. aeruginosa* suggests a role in the induction of the its' chromosomal B-lactamase.

Analysis of the evolution of B-lactamase may help in understanding certain features of its induction system. B-lactamases have become widespread as a result of selection pressure derived from chemotherapy in hospitals and indeed they have evolved as a result of such pressure. For example, the SVH 2 B-lactamase is a derivative of SVH 1 with an enhanced activity against third-generation cephalosporins

(Korfmann et al. 1986). However, recent man-made selection pressure can not explain the presence of what appears to be a complex induction system nor does it limit the role of B-lacatamase in the cell to B-lactam inactivation although B-lactamases may have evolved in response to B-lactam-producing fungi.

For example, both B-lactamase and PBP5 may have functions in sporulation of Bacillus species (Ozer et al. 1970, Todd et al. 1986). Other evidence suggests another possible role for B-lactamases in P. aeruginosa, Sabath (1984) was unable to identify non-B-lactamase producing mutants of P. aeruginosa after UV induced or chemical mutagensis. Chemical mutagenesis was used to mutate the chromosomal B-lactamase gene, blaP, (Matsumoto and Terawaki 1982) but their data shows mutant strains retained a B-lactamase activity which contributed to B-lactam resistance of the organism. Furthermore, B-lactamase activity has also been observed in these blaP-deficient strains by other workers (W. Nichols, personal communication). The inability to isolate non-B-lactamase producing strains of P. aeruginosa may indicate that the B-lactamase performs an essential role within the cell, unrelated to catalysis of B-lactams in the environment.

Inhibition of PBPs by B-lactams is due to the similarity that exists between the B-lactam ring and the D-alanyl-D-alanine substrate of PBPs. The active sites of PBPs and B-lactamases would be expected to be similar as suggested by Pollock (1971). Furthermore, B-lactamases may have evolved from PBPs (Tipper and Strominger 1965). The possible evolution of a PBP into a B-lactamase is supported by the observed weak B-lactamase activity by the low molecular mass PBPs of Gram-negative bacteria, for example PBP5 (Amanuna and Strominger 1980)

and similarities between PBP5 of E. coli with various carboxypeptidases from Bacillus sp. and class A B-lactamases (Waxman and Strominger 1983).

8.5 Induction of the class C B-lactamase: An Hypothesis

Any hypothesis of the mechanism of B-lactamase induction must explain a wide variety of observations. It must explain how B-lactams with different PBP targets can induce B-lactamase, how mutants hyperproducing B-lactamase can induce B-lactamase further, the significance of certain PBPs in the induction process and the possible roles of ampD-ampE.

An essential role in the induction process to specific B-lactams has been suggested for PBP2 in E. coli (Oliva et al. 1989) and PBP5 in P. aeruginosa (Martin et al. 1988). The action of these PBPs in the induction process must be through AmpD, AmpE and AmpR, otherwise induction via the AmpD-AmpR system would occur. Absence of PBP5 activity (at high temperatures) prevents B-lactamase induction in the presence of functional AmpD, which does not agree with the model proposed in Figure 1.4a. Inactivation of PBP5 in this model should mimic the presence of B-lactam and an accumulation of substrate would induce B-lactamase. PBP5 must catalyze a reaction where a product, or a derivative of a product, is the effector and is usually present since PBP5 is usually functioning. This would explain the requirement of PBP5 to be inhibited in the induction process and supports the model for induction as described in Figure 1.4b. Protein AmpR acts as an activator alone but in the presence of a co-repressor acts as an inhibitor of B-lactamase synthesis. Proteins AmpD and AmpE may act as

intermediates between PBP5 and AmpR, either converting a product of PBP5 to a co-repressor or binding to both the product and AmpR thereby preventing constitutive pr oduction of AmpC. Inhibition of PBP5 by B-lactam would cause a decrease in the product of PBP5, and thereby reduce the concentration of co-repressor.

Teleologically D-alanine would be ideal as the negative feedback effector since D-alanine is the common product of PBP enzyme activity and is specific to peptidoglycan synthesis. Different PBPs can have an effect on B-lactamase induction since each contributes to the amount of D-alanine present. The effect of PBP5 activity may be particularly important since PBP5 is the most common PBP and has a compensatory ability for other inhibited PBPs.

The presence of ampD-ampE in the cell envelope growth and division gene cluster around 2 minutes on the E. coli chromosome may indicate a role for them in monitoring cell wall metabolism and not just the presence of B-lactams. However, such a role is not essential under laboratory conditions. Furthermore, although AmpD and AmpE form an operon, their functions are not totally integrated since loss of one does not have the same effect as loss of both together (Lindquist et al. 1989a). It would appear that at least two pathways may affect B-lactamase induction. Multiple sensors or transducers would explain how different PBPs could induce B-lactamase synthesis and would also explain how a number of basal B-lactamase activities are possible within a single strain. However, such a complex induction system just for controlling B-lactamase synthesis in response to B-lactams would appear to be excessive. Proteins AmpD and AmpR, and presumably other proteins affecting B-lactamase induction, may have other roles.

This would explain the presence of AmpD and AmpE in E. coli where B-lactamase is not inducible. Protein AmpE shares homology to a family of transmembrane ATP-binding proteins, some of which are thought to function in transporting signal molecules across the membrane (Lindquist et al. 1989a). It may be possible that the ser-x-xlys site is required for binding D-alanine.

B-Lactams are analogues of peptidoglycan precursors. It may be possible that binding of B-lactam to PBPs mimics excess D-ala-D-ala and acts as a signal for new growth. Incorporation of new peptidoglycan requires partial breakdown of existing peptidoglycan (endopeptidase activity) and possibly removal of excess pentpeptide (carboxypeptidase activity). In E. coli PBP4 has endopeptidase and carboxypeptidase activities and PBP5 and PBP6 have carboxypeptidase activity (Noguchi et al. 1985). These PBPs may be induced to allow partial breakdown of peptidoglycan for new growth. Each PBP may act as a signal of improper peptidoglycan metabolism, working through similar induction systems co-ordinated by AmpR to cause breakdown of peptidoglycan via autolysins, endopeptidases and carboxypeptidases. As B-lactams continue to bind PBPs the regulation of peptidoglycan metabolism may continue degrading peptidoglycan precursors and mature peptidoglycan resulting in cell lysis.

I am unaware of reports that PBP4, PBP5 and PBP6 are produced as autolysins in response to B-lactams. However, overproduction of PBP4 can cause cell lysis (Korat and Keck 1988) and overproduction of PBP5 can cause the formation of spheroid cells, similar to mutants lacking PBP2 or the effects of mecillinam. Overproduction of PBP4 or PBP5 can therefore confer phenotypes observed when other PBPs are inhibited.

However, this could simply be seen as a loss of activities from opposing PBPs.

PBP5 has model transpeptidase activity *in vitro* (Amanuma and Strominger 1980) and overproduction of PBP5 can compensate for inhibited PBPs 1, 2 or 3. This would appear to conflict with the observation that overproduction of PBP5 causes spheroid cell formation. I believe the two observations are possible because overproduction of PBP5 could be a regulated compensatory effect possibly alongside changes in other PBP activities or it can be a non-regulated effect causing a spheroid cell morphology. Furthermore, in *P. aeruginosa* hs-257 PBP4 is overproduced when PBP5 activity is inhibtited by the non-permissive temperature (Noguchi *et al.* 1985). It would appear that the bacterium has a complex regulatory system able to resort to other PBPs when preferential PBPs are inhibited.

PBP5 acts as a B-lactamase in *E. coli* capable of conferring B-lactam resistance (Amanuma and Strominger 1980). I believe the induction of B-lactamase will not be wholly understood until further research into the general regulation of peptidoglycan synthesis, specifically the induction of PBPs and autolysins, is undertaken.

8.6 Future work

Testing D-alanine involvement in induction of B-lactamase

If D-alanine is a co-repressor, or a precursor of a co-repressor, of B-lactamase synthesis, excess D-alanine in the medium should prevent induction of B-lactamase in the presence of B-lactam inducer. However, if induction was inhibited it may not necessarily be a direct response to the presence of D-alanine.

Identifying the B-lactamase gene and genes regulating its' synthesis

The amino acid sequence around the active site and the N-terminal of the class C B-lactamase of P. aeruginosa are known. It may be possible to clone the intervening region of the B-lactamase gene using oligonucleotide primers with linkers from these two sites and the Polymerase Chain Reaction. The larger probe could then be used to identify blaP froma gene bank. Although regulatory proteins have not been identified in P. aeruginosa, sequence homology exists within ampR and also the ampD of E. cloacae and C. freundii (Lindquist et al. 1989b). Oligonucleotides of conserved regions within these proteins may help similarly, in identifying and cloning regulatory regions in P. aeruginosa.

The regulatory mechanism controlling B-lactamase production in P. aeruginosa may not be similar to that of those systems already investigated in which case the use of cloned genes to identify possible regulatory genes may be ineffective. Transposon mutagenesis may be the most satisfactory method for precisely locating regulatory genes. In this case it is essential to improve the methods of introducing the transposon into the host chromosome and also of identifying mutants hyperproducing B-lactamase.

Constructs of Tn5 for Use in Pseudomonas aeruginosa

Hewinson (1987) stated that there could be two classes of P. aeruginosa based on their ability to mutate to cefsulodin resistance by hyperproduction of B-lactamase. If the class in which resistant mutations were not detected are strains in which both putative regulatory units are intact then it should be possible to generate

resistant mutants by causing two sequential mutational events. Transposon Tn5 could be of use here. The knowledge of the regulation of, and essential sites for, Tn5 transposition, makes it a very versatile transposon, and many Tn5 derivatives have been made. The ability to generate and select for specific Tn5 deletion mutants may result in the development of transposase-deficient (one-shot) transposons, which require an external IS50R, on the same replicon to provide transposase activity. The use of such transposons as mutagens would aid in the detailed genetic analysis of a desired DNA sequence since the possibility of two transposition events occuring within the same cell would be greatly reduced and would allow subsequent transposition of other Tn5-derivatives for sequential mutational analysis.

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Appendix I

Buffers and Solutions for DNA manipulation

Restriction enzymes have different conditions for optimal endonuclease activity and buffers, if supplied by the manufacturer, were used or alternatively four general stock buffers were used. Three contained sodium salts of different concentrations (high, medium and low) whilst the fourth was based on an acetate salt and could be used with a wide range of restriction enzymes, Universal buffer, (Maniatis et al. 1982).

Stock solutions of 5M NaCl, 1M Tris-HCl (pH 7.5) and 1M $MgCl_2$ were made and autoclaved separately. Dithiothreitol (Sigma) was filter-sterilized at a concentration of 0.1M. These were added to sterile distilled water to obtain buffer at x10 working concentration and stored at -20°C.

Restriction enzyme buffers (x10 stock concentrations)

Buffer	NaCl (M)	Tris-HCl pH7.5 (M)	$MgCl_2$ (M)	Dithiothreitol (M)
x10 low salt	0	0.1	0.1	0.01
x10 medium salt	0.5	0.1	0.1	0.01
x10 high salt	1.0	0.5	0.1	0.01

Universal buffer (x10 stock solution)

0.33M Tris-acetate (pH7.9), 0.66M Potassium acetate, 0.1M Magnesium acetate.

The solution was autoclaved at 15lbs.ins.$^{-2}$ for 10 minutes to which filter-sterilized dithiothreitol (0.1M) was added to a final concentration of 5mM.

Low salt buffer - BglII, HpaI
Medium salt buffer - BamHI, HindIII, PvuII*, Sau3A
High salt buffer - EcoRI, SalI

* - Universal buffer unsuitable

Ligation Buffer was made as a 10x concentrated stock solution and stored at -20°C. The stock solution consisted of 0.5M Tris-HCl (pH7.4), 0.1M $MgCl_2$ and 0.1M Dithiothreitol in distilled water.

Buffers required for NACS Column (BRL) used in purifying DNA from low melting Point agarose gels:
Equilibration buffer: 0.5M NaCl in TE (10mM Tris-HCl, 1mM EDTA (pH7.4))
Elution Buffer: 2.0M NaCl in TE

Appendix II

Solutions Required for DNA Extraction and Gel Electrophoresis

GTE buffer
50mM Glucose
25mM Tris-HCl (pH8.0)
10mM EDTA (pH8.0)

The solution was autoclaved at 10lbs.ins.$^{-2}$ for 10 minutes and stored at 4^{o}C.

Lysozyme
Lyophilized lysozyme (Sigma) was dissolved in GTE buffer to 10mg/ml and stored at -20^{o}C.

SN Buffer
1% (w/v) SDS (Sodium dodecyl sulphate)
0.2M NaOH

This solution was prepared on the day from stock solutions of 10% (w/v) SDS and 1M NaOH and sterile distilled water. The stock solutions were made up in sterile distilled water and were not autoclaved. Stock solutions and Solution II were kept at room temperature.

KA Buffer - 3M Potassium acetate (pH 4.8)
11.5ml glacial acetic acid was added to 60ml 5M potassium acetate. The pH was adjusted to 4.8 with formic acid and distilled water was added to a final volume of 100ml. Solution III was autoclaved at 10lb.ins^{-2} for 10 minutes and stored at 4^{o}C. Alternatively, 5M potassium acetate (pH 4.8) was used.

TEN buffer
10mM Tris-HCl (pH 8.0)
1mM EDTA
1.5M NaCl

TEN was autoclaved at 10lb.ins^{-2} for 10 minutes and stored at room temperature.

TE buffer
10mM Tris-HCl (pH8.0)
1mM EDTA (pH8.0)

TE was autoclaved at 10lb.ins^{-2} for 10 minutes and stored at room temperature.

Buffer-saturated phenol
Crystaline phenol (Analar grade, BDH) was stored at 4^{o}C. Crystalline phenol was melted at 60^{o}C, poured into a sterile 500ml medical flat and an equal volume of sterile distilled water was added. The solution was shaken for a few minutes and then the phases were allowed to separate overnight. The solution was stored over a number of months at 4^{o}C. Prior to use water-equilibrated phenol (the lower phase) was mixed with an equal volume of 100mM Tris-HCl (pH8.0). The phases were allowed to separate and then stored at 4^{o}C in a dark bottle for up to two weeks.

Ethanol
Absolute ethanol was stored at -20°C.

RNase A
Stock RNase A (Sigma) was stored dessicated at -20°C. For use RNase A was dissolved in sterile distilled water at 5mg/ml and boiled for 15 minutes in a water bath to denature contaminating DNase activity. The solution was allowed to cool and was stored at -20°C.

Solutions required for large scale purification of pRTG-1:

Lysis buffer
50mM Tris-HCl (pH8.0)
25% (w/v) sucrose

The solution was autoclaved at 10lbs.ins^{-2} for 10 minutes and stored at 4°C.

Tris-EDTA solution
250mM Tris-HCl (pH8.0)
250mM EDTA (pH8.0)

The solution was autoclaved at 10lbs.ins^{-2} for 10 minutes and stored at 4°C.

RNase A
RNase A was dissolved at 10mg/ml in sterile distilled water and boiled for 15 minutes in a water bath. The solution was allowed to cool and was stored at -20°C.

Triton solution
50mM Tris-HCl (pH8.0)
10mM EDTA (pH8.0)
2% (v/v) Triton X-100 (Sigma)

The solution was autoclaved at 10lbs.ins^{-2} for 10 minutes, triton added and stored at 4°C.

Ethidium bromide solution
Ethidium bromide was dissolved at 10mg/ml in sterile distilled water and was stored at room temperature in a dark bottle.

Caesium chloride
Stock caesium chloride (BDH) was stored at room temperature.

Butanol
Analar grade butanol (BDH) was stored at room temperature.

Liquid paraffin
Analar grade liquid paraffin (BDH) was stored at room temperature.

3M Sodium acetate
3M sodium acetate was adjusted to pH6.0 with glacial acetic acid autoclaved at 10lbs.ins^{-2} for 10 minutes and stored at 4°C.

Solutions required for electrophoresis:

TBE buffer (x10 concentrated)

108 g Tris
55 g Boric acid
7.8g EDTA
Distilled water to 1 litre.
This was stored at room temperature.

Ethidium bromide
Stock ethidium bromide solution (10mg/ml) was diluted to 0.5ug/ml for use.

Loading buffer
40% (w/v) sucrose
0.2% bromophenol blue
0.2% xylene cyanol
in TE buffer and stored at 4°C

Appendix III

Solutions Required for Southern Hybridization

Denaturation solution: 1.5M NaCl, 0.5M NaOH
Neutralization solution: 1.5M NaCl, 0.5M Tris-HCl (pH7.5)
Sonicated salmon sperm DNA (Sigma):

DNA was dissolved in TE buffer (100ml) and sonicated in a water bath sonicator to generate fragments ranging between 500-2000bp as estimated from gel electrophoresis of DNA against Lambda HindIII DNA. The sample was then purified by two cycles of phenol/chloroform followed by ethanol precipitation. DNA was redissolved in TE buffer to 5mg/ml. The solution was stored at $4^{o}C$.

20xSSC: 3.0M NaCl, 0.3M Tri-sodium citrate
100x Denhardts solution: 2% (w/v) Bovine saline albumin V
2% (w/v) Ficoll
2% (w/v) Polyvinylpyrrollidone
Made in sterile distilled water and stored in 20ml volumes at $-20^{o}C$. Solutions from Sigma or BDH.

10% (w/v) SDS: 10g sodium dodecyl sulphate in 100ml sterile distilled waster. 10% (w/v) SDS stored at room temperature.
Deionized 100% formamide: 100ml formamide (BDH) was mixed with 5g mixed bed ion exchange resin (Bio-rad AG 501 - X8, 20-50 mesh). The solution was stirred at room temperature with a magnetic flea for approximately 30 minutes or until all the beads had turned yellow. The solution was filtered using Whatmann 3MM filter paper to remove the resin and formamide aliquoted in 20ml amounts in dark bottles at $-20^{o}C$.

1M phosphate buffer (pH 6.5)
2xSSC, 2xSSC, 0.1% SDS and **0.1%SSC, 0.1% SDS** solutions were prepared from stock solutions of 20xSSC and 10%SDS on the day of use. Radioactive waste was discarded as solid waste with the Pasteur pipette being placed in an Eppendorf tube to prevent the tip ripping of the radioactive disposal bag.

The non-boil solution was usually made to 18ml and consisted of:
10.0ml 100% deionized formamide
5.0ml 20x SSC stock
1.0ml 100x Denhardts solution stock
1.0ml 1M phosphate buffer (pH6.5)
1.0ml 10% (w/v) SDS

Solutions were mixed in a disposable Universal tube at room temperature with SDS being added last to prevent precipitation.
The boil solution consisted of:
0.1-0.2ml ^{32}P-labelled DNA probe
0.5ml 5mg/ml sonicated salmon sperm DNA
sterile distilled water to 2ml

The radioactive DNA probe had previously been thawed in a lead pot at room temperature.

Solutions required for Autoradiography:
Developer: 100ml of LX24 developer (Kodak) was diluted in 400ml tap

water. This solution would keep for 24 hours if left in an open tray in the dark. Later, however, the solution was stored in an upright tank with a lid and was changed monthly.

Fixer: 100ml of FX40 fixer (Kodak) was diluted in 400ml tap water. The solution was treated similarly to the developer, described above.

Appendix IV

Chemical Sources

General chemicals and reagents

BDH Chemicals Ltd., Fourways, Carlyon Industrial Estate, Atherstone, Warwickshire, UK.

Difco

Lab M, P.O. Box 19, Bury, Lancs., UK.

Oxoid Ltd., Basingstoke, Hampshire, UK.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, UK.

Antibiotics

Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, UK.

Ciba-Geigy Pharmaceuticals, Horsham, UK.

Glaxo Research Laboratories, Greenford, Middlesex, UK.

Sigma Cemical Company Ltd., Fancy Road, Poole, Dorset, U.K.

Enzymes

Amersham International Ltd., Lincoln Place, Green End, Aylesbury, Bucks., UK.

Gibco-BRL Ltd., P.O. Box 35, Trident House, Paisley, Scotland, UK.

NBL (Northumbria Biologicals Ltd.), South Nelson Industrial Estate, Cramlington, Northumberland.

P&S Biochemicals Ltd., Liverpool., U.K.

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, UK.

Equipment

Amersham International Ltd., Lincoln Place, Green End, Aylesbury, Bucks., UK.

BCL, Boehringer Mannheim House, Bell Lane, Lewes, Sussex, UK.

Bio-Rad Laboratories Ltd., Caxton Way, Watford Business Park, Watford, Herts., UK.

Cecil Instuments Ltd., Cambridge, U.K.

Ells and Farrier, 5, Princes St., Hanover Square, London, UK.

Gelman Sciences (UK) Ltd., 10, Harrowden Road, Brackmills, Northampton, UK.

GRI (Genetic Research Instrumentation Ltd.), Gene House, Dunmow Road, Felsted, Dunmow, Essex, UK.

Kodak (UK) Ltd., Acornfield Road, Knowsley Industrial Park North, Liverpool, UK.

Marine Colloids Inc.,

NBL (Northumbria Biologicals Ltd.), South Nelson Industrial Estate, Cramlington, Northumberland, UK.

Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Central Milton Keynes, Bucks., UK.

Polaroid (UK) Ltd., Ashley Road, Herts., UK.

APPENDIX V

ATTEMPTS TO INTRODUCE pRTG1 INTO *Pseudomomas aeruginosa*: METHODS USED

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V.i Introduction

Plasmid pRTG1 was constructed to introduce Tn5::gen into P. aeruginosa. Plasmid pRTG1 was transferred from E. coli SM10 into P. aeruginosa PAO4069 by conjugation albeit with low frequencies and after initial problems in counter-selecting against donor contaminants (Chapter 5). Transconjugants were generally unstable and although most transconjugants retained gentamicin and streptomycin-resistance, pRTG1 had undergone DNA deletions. Deletions may have occurred during conjugation and/or during isolation of transconjugants from donor contaminants.

Transformation of P. aeruginosa with pRTG1 bypassed the need for a donor and I considered that it would allow the isolation of P. aeruginosa carrying native pRTG1 if deletions were associated specifically with conjugation or the transconjugant isolation procedure. Transformation experiments are described in this appendix.

Tn5::gen was able to transpose into the host chromosome of E. coli UB5201 (pRTG1) prior to growth at the non-permissive temperature for pRTG1 (Chapter 4) and it was likely that such transpostion events would also occur in P. aeruginosa. It seemed necessary to develop a method capable of repeatedly introducing pRTG1 into P. aeruginosa rather than relying on a single transconjugant/transformant from which all mutants would be isolated. Furthermore, the most practical method of locating genes of interest was to obtain a consensus position of Tn5::gen in mutants hyper- producing B-lactamase isolated from different plasmid-bearing strains. Great emphasis was placed on obtaining a successful method for introducing pRTG1 into P. aeruginosa, many methods were attempted and these are described in this appendix.

Towards the end of this work spot-mating conjugations successfully transferred pRTG1 into P. aeruginosa strains. However, plasmid analysis of transconjugants showed pRTG1 to be generally unstable in P. aeruginosa (Chapter 7). The reader is referred to Chapter 7 for further explanation whilst this appendix deals with unsuccessful transfer experiments. Although this appendix describes experiments which did not advance the objectives of the thesis it highlights difficulties observed when working in P. aeruginosa.

V.ii Transformation of P. aeruginosa with Plasmid DNA

V.ii.a Introduction

Considering the problems encountered when selecting for conjugal transfer of pRTG1 from E. coli into P. aeruginosa I decided to attempt transformation of P. aeruginosa with purified pRTG1 DNA. Transformation removed problems related to alterations in pRTG1 which might have occurred between isolation and purification of P. aeruginosa transconjugants. P. putida KT2440 is a strain used to assess Tn5 transposition from suicide plasmids (R. Wilson, personal communication) and was used here as a positive control for transfer of pRTG1. P. aeruginosa 3-Pre (Slack and Pitt 1982) and P. aeruginosa PAO4069 (Matsumoto and Terawaki 1982) were also used as recipients.

V.ii.b **Results**

Recipient strains were treated with $CaCl_2$ and transformed with DNA (~0.2ug). Plasmid pVW6 was used as a control vector because both pRTG1 and pVW6 are based on the same replicon and contain the same gentamicin-resistance gene. If pVW6 DNA was also unsuccessful at transforming competent cells it may explain the difficulties experienced with pRTG1. Transformants were selected on nutrient agar containing streptomycin (400ug/ml), or gentamicin (40ug/ml) or streptomycin (400ug/ml) and gentamicin (30ug/ml) with P. aeruginosa 3-Pre as recipient or on nutrient agar containing gentamicin (20ug/ml) or gentamicin (15ug/ml) and streptomycin (400ug/ml) with P. aeruginosa PA04069 as recipient.

P. putida KT2440 was transformed successfully with both pRTG1 and pVW6 (Table V.i). P. aeruginosa 3-Pre ($8x10^8$cfu/plate) did not grow on any selection plates. P. aeruginosa PA04069 ($3x10^9$cfu/plate) did not grow in the presence of both gentamicin (15ug/ml) and streptomycin (400ug/ml) and spontaneous mutants were observed with a frequency of $5.7x10^{-8}$ when selecting on N-agar containing gentamicin (20ug/ml). The absence of transformants of P. aeruginosa may have been because bacteria were not competent, the restriction-modification system may have been more efficient in P. aerginosa, the selection conditions were unsuitable or the vectors themselves were unstable in these strains.

TABLE V.i Transformation Frequencies for P. putida KT2440 Using pRTG1 and pVW6

	Antibiotic (ug/ml)			
Plasmid	Sm (100)	Sm (400)	Gm (20)	Sm (100) Gm (20)
pVW6	$4.3x10^{-7}$	$3.9x10^{-7}$	$8.3x10^{-8}$	$8.3x10^{-8}$
pRTG1	$4.3x10^{-6}$	$1.1x10^{-6}$	$1.6x10^{-7}$	$8.3x10^{-8}$

In P. putida KT2440 the transformation frequency was poor in all cases but was no less frequent for pRTG1 than pVW6 supporting the possibility that temperature-sensitivity of, and expression of gentamicin-resistance in pRTG1 did not reduce the frequency of transformation in P. putida. The reduced transformation frequencies for both plasmids when selecting on gentamicin may have been because the gentamicin-resistance gene was poorly expressed whereas the streptomycin-resistance gene was better expressed. This observation contradicts the observations made with E. coli as host and may indicate differential promoter proficiencies. Furthermore, if gentamicin-resistance was poorly expressed in P. aeruginosa it may have contributed to the problem of selecting gentamicin-resistant colonies since the concentration of gentamicin has to be at least 20ug/ml if spontaneous mutations are to be infrequent.

To test the various possibilities for the lack of success in transforming P. aeruginosa PA04069, transformation experiments were repeated using various conditions. Prolonged incubation in ice

increases competence (Saunders and Saunders 1988) and extended incubation in $CaCl_2$ (0.1M) for one hour prior to addition of DNA (~0.2ug) was performed in an attempt to increase cell permeability to DNA. Selection conditions were also altered: streptomycin (400ug/ml), or gentamicin (15ug/ml), or streptomycin (200ug/ml) with gentamicin (20ug/ml), or streptomycin (400ug/ml) with gentamicin (15ug/ml). Growth was observed only on selection media containing gentamicin (15ug/ml) but parental controls of P. aeruginosa PA04069 also grew at this concentration. E. coli UB5201 was successfully transformed with pRTG1 under the same conditions on the following selection media; gentamicin (15ug/ml) and gentamicin (15ug/ml) with streptomycin (100ug/ml)) equivalent to a transformation frequency of approximately 2.5×10^{-4}.

To test whether the lack of success in obtaining transformants was due to the poor competence of the P. aeruginosa strains or whether it was specifically related to pRSF1010 derivatives, pR1033 encoding the same gentamicin-resistance gene, was used to transform P. aeruginosa PA04069. E. coli DH1 was used as a positive control for transformation. Three samples of E. coli DH1; transformed with either pRTG1, pVW6 or pR1033 were spread onto nutrient plates containing various concentrations of gentamicin to test whether pVW6 and pRTG1 were less able to express resistance to high concentrations of gentamicin than pR1033. Results are shown in Table V.ii.

TABLE V.ii Number of Colonies of P. aeruginosa PA04069 and E. coli DH1 Counted on Nutrient Agar with Antibiotic Selection for Different Gentamicin-resistant Plasmids

	Plasmid used to transform sample			
Antibiotic (ug/ml)	Control	pVW6	pRTG1	pR1033
PA04069[a]				
Gm (30)	4, 3	7, 3	4, 3	2, 1
Gm (40)	0, 0	1, 1	0, 0	0, 0
Sm (400)	0, 0	0, 0	0, 0	0, 0
DH1				
Gm (10)	0	360	220	15
Gm (20)	0	350	70	2
Gm (30)	0	88	15	0

a - duplicate plates

Two putative transformants of P. aeruginosa (pVW6) were isolated on nutrient agar containing gentamicin (40ug/ml). These were not analysed since even if they were successful the method was very inefficient.

Selection with higher gentamicin concentrations reduced the numbers of transformants obtained. More importantly, the number of gentamicin-resistant transformants selected were proportionately less with pRTG1 than with pVW6 at the higher gentamicin concentrations. Gentamicin-

resistance gene may have been less well expressed in the transposon than when cloned directly into pRSF1010. Another explanation was that, as a result of its' temperature-sensitive RepA protein, pMMB19 might have been present in a lower copy number which affected the resistance conferred by this resistance gene. The latter is supported by the observation that the low copy number plasmid pR1033 was also less able to confer resistance to gentamicin (30ug/ml). Low transformation frequencies of E. coli DH1 with pR1033 were probably a result of the large size of pR1033 (74Kbp).

$MgCl_2$ (0.1M) was used instead of $CaCl_2$ (0.1M) as the competence solution in subsequent experiments unless stated otherwise. To test whether the lack of transformation success was due to inefficient replication of pRTG1 in the new host and poor expression of the gentamicin-resistance gene of pRTG1 in P. aeruginosa PAO4069 a longer recovery period was allowed (6, 18 and 24 hours) prior to plating on selection media. Similar numbers of resistant colonies grew on P. aeruginosa PAO4069 control plates and so transformants were not obtained. In these experiments selection was on nutrient agar containing: gentamicin (20ug/ml), or gentamicin (30ug/ml), or gentamicin (20ug/ml) and streptomycin (400ug/ml).

Another possibility for the lack of success was an insufficient concentration of DNA (~0.2ug). Increasing the DNA concentration (0.4 and also 0.8ug) failed to give rise to pRTG1 or pVW6-derived transformants. Parental growth was observed on nutrient agar containing gentamicin (20ug/ml) with an inoculum of $5.0x10^8$ cfu/plate. No growth occurred when selection on nutrient agar was: streptomycin (400ug/ml), or streptomycin (400ug/ml) and gentamicin (15 ug/ml), or streptomycin (300 ug/ml) and gentamicin (15ug/ml), or streptomycin (300ug/ml) and gentamicin (20ug/ml).

To test whether the DNA prepared by the mini-prep method was too contaminated, for example with protein or phenol, to transform P. aeruginosa, CsCl-purified pRTG1 DNA was used to transform P. aeruginosa. Using 0.75ug, 1.5ug and 3.75ug of DNA, no transformants were observed with selection on streptomycin (400ugml), or gentamicin (30ug/ml), or gentamicin (20ug/ml) and streptomycin (320ug/ml).

In another attempt to make P. aeruginosa PAO4069 competent, a culture was harvested as described above but was incubated overnight in 150mM $MgCl_2$ prior to incubation with CsCl-purified DNA (0.75ug). No growth occurred with inocula of $\sim 1.4x10^6$ cfu when selecting for pRTG1 with streptomycin (320ug/ml) or gentamicin (30ug/ml) in nutrient agar. Plasmid pVW6 and pRTG1 extracted according to a mini-prep method (0.2ug) were also unsuccessful at generating transformants. Despite this, E. coli SM10 was transformed at an average frequency of approximately $2x10^4$ transformants per ug DNA.

In an attempt to make P. aeruginosa PAO4069 and 3-Pre competent two other competence solutions were used according to the method of Bagdasarian and Timmis (Saunders and Saunders 1988). Samples (200ul) were incubated on ice for 1 hour prior to the addition of CsCl-purified pRTG1 DNA (0.75ug) or mini-prep-purified pRTG1 (0.2ug) or pVW6 (0.2ug). Cultures were grown for 4 hours prior to plating samples (100ul) onto nutrient agar containing streptomycin (320ug/ml) and gentamicin (32ug/ml), or gentamicin (32ug/ml) alone. P. aeruginosa PAO4069 did not grow on any selection plates and parental

P. aeruginosa 3-Pre grew on both selection media. E. coli UB5201 was successfully transformed with the same DNA concentrations using $CaCl_2$ competence solution. Transformants were obtained with frequencies of $1.6x10^{-5}$ (CsCl-pRTG1), $1.2x10^{-5}$ (pRTG1) and $2.2x10^{-5}$ (pVW6) on nutrient agar containing gentamicin (20ug/ml). These results show that DNA was present in saturating concentrations.

Plasmid pRTG1a extracted from P. aeruginosa PAO4069 (pRTG1a) was also used to transform P. aeruginosa PAO4069. If the lack of success in introducing pRTG1 by transformation was due to the efficiency of the restriction system or other factors causing plasmid instability, it would be expected that pRTG1a should bypass these problems. Plasmid pRTG1a (~0.2ug) was used to transform P. aeruginosa PAO4069 and 3-Pre treated with 0.1M $MgCl_2$. Selection was on nutrient agar containing gentamicin (32ug/ml) and streptomycin (320ug/ml), and gentamicin (32ug/ml) alone with PAO4069 as recipient and gentamicin (40ug/ml) and streptomycin (400ug/ml) with 3-Pre as recipient. No growth was obtained on any selection except viability plates. E. coli SM10 (pRTG1a) was obtained with a frequency of $\sim 1x10^{-5}$ when made competent with $CaCl_2$. Selection was on L-agar containing gentamicin (20ug/ml) and streptomycin (200ug/ml). Restriction efficiency and plasmid instability were therefore not the only factors that may be involved in the lack of success in introducing pRTG1.

P. aeruginosa PAO6520 and PAO6521 are recA strains and were used to test whether RecA-dependent recombination events caused instability of pRTG1 in P. aeruginosa PAO4069. These recA strains were treated with 0.1M $MgCl_2$ and separate samples transformed with pRTG1 CsCl-purified pRTG1 (0.75ug), pRTG1a (~0.2ug) or pVW6 (~0.2ug). Parental controls grew on selection plates containing gentamicin (32ug/ml) or streptomycin (320ug/ml). The experiment was repeated using the competence solutions described by Drew (1984). No transformants were obtained on nutrient agar containing streptomycin (400ug/ml).

In conclusion, transformation of P. aeruginosa was unsuccessful. Different competence solutions, different incubation conditions on ice prior to addition of DNA, different recovery periods, different concentrations of DNA, different purification methods of DNA and different recipients were used. It was apparent that conventional means of introducing pRTG1 were unsuccessful and transformation by electroporation was attempted.

V.iii Attempts to Introduce pRTG1 into P. aeruginosa by Electroporation

V.iii.a Introduction

Transformation of bacteria by electroporation involves passing an electric current through a suspension of cells. At certain field strengths the cell membrane is disrupted, causing the cell to become transiently permeable. In this period it is possible for the cell to take up DNA. Electroporation has been used successfully to transform bacteria including E. coli and P. putida (Fiedler and Wirth 1988) with maximal transformation frequencies of between 10^4-10^5 transformants per ug DNA.

The important values in electroporation of bacterial cells are the initial electric field strength (E_o) and the time constant (tor). The

electric field experienced by a sample is the applied voltage divided by the distance between two parallel electrodes in the sample chamber. It may be changed by altering the voltage output or by altering the inter-electrode gap. The time constant is defined as the product of the total resistance (ohms) and capacitance (farads) and is measured in seconds. It is also a measure of pulse duration and can be adjusted by altering the capacitance or altering the resistance of the system.

Electric field strength and the time constant have compensatory effects. High field strengths of short duration (for example, 12.5kV for 2ms) and low field strengths of long duration (for example, 5.25kV for 26ms) can both achieve high transformation efficiencies; 0.4 to $1x10^6$ transformants/ug DNA (Miller et al. 1988).

The mortality rate due to electroporation gives an indication of the transformation efficiency with maximum efficiency being obtained between 30%-60% mortality. Transformation efficiencies of E. coli and Campylobacter jejuni increase if the samples are ice-cold during electroporation (Miller 1988). The ionic strength, osmolarity and pH of the electroporation medium may also effect the transformation efficiency. Various conditions, media, field strengths and electroporation apparatus (BioRad and BRL) were used to find suitable conditions for the successful transformation of P. aeruginosa.

V.iii.b **Results**

Electroporation was performed as described in Methods. The first experiments were to establish conditions which gave mortality values within the 30-60% range for P. aeruginosa PAO4069 in HEPES buffered saline. The electroporation equipment was obtained from BRL. Results are shown in Table V.iii. Significant cell death was not observed at these field strengths. This was either due to the field strength being unable to cause poration or due to a very high recovery ability.

To increase the field strength with this apparatus, cuvettes with an interelectrode gap of 2mm were used. Furthermore, lysozyme treatment was used to test whether weakening the cell wall would increase the effects of electroporation and thereby increase the mortality rate of lysed cells with respect to non-lysed cells, when treated with the same electroporation conditions. Cells of P. aeruginosa suspended in HEPES-buffered saline (pH 7.4) (HBS), were treated with lysozyme (final concentration 2mg/ml) for 30 minutes on ice prior to electroporation. The treatment was unsuccessful at causing lysis in distilled water as observed with a light microscope. Nonetheless treated and untreated samples of P. aeruginosa PAO4069, PAO4095, PAO6520 and PAO6521 were electroporated. Electroporation using the field strengths shown in Table V.iii was attempted using 200ng pRTG1/aliquot. After a recovery phase of 12 hours in Brain Heart Infusion broth at 28°C cells were plated at $\sim 2x10^8$ cfu/plate. No transformants were obtained selecting on nutrient agar containing gentamicin (32ug/ml) or streptomycin (320ug/ml).

The field strength could only be increased further using this equipment by giving a single sample multiple pulses in rapid succession. Double pulses were partially successful at increasing mortality but at only very high field strengths (Table V.iv). It had been shown, however, that multiple pulsing reduces the transformation ability of the sample (Miller 1988). It seemed that single pulses of

Table V.iiia Effect of different field strengths on the mortality of P. aeruginosa PA04069 in HEPES buffered saline

Capacitance (uF)	Number of colonies V/cm = 500	Number of colonies V/cm = 750
0	Conf.	Conf.
10	"	~60-Conf.
50	"	"
60	"	"
330	"	"
800	40-60	"
1180	Conf.	6[a]
1600	25-60	
1980	Conf.	~60-Conf.

Inoculum size ~230
a - Sample received two pulses

Table V.iiib Effect of different field strengths on the mortality of P. aeruginosa PA04069 in HEPES Buffer Saline

Capacitance (uF)	Electric field V/cm	Average number of colonies (n=3)	
0	0	Conf.[a]	20
10	975	Conf.	23
50	960	"	27
330	985	"	30
800	975	"	15
1180	985	"	2
1600	975	23	0
1980	970	30	0.6

a - Inoculum size ~2000

higher field strengths than those offered by the BRL equipment would be required to generate porated cells.

Using the Bio-Rad "Gene Pulser" higher field strengths were achieved. Initial viability studies of P. aeruginosa PAO4069 and PAO6520 are shown in Table V.v. From this data at a capacitance of 25uF and with the field strength ranging between 3.75 - 6.25 kV/cm, mortality was approximately 30-60% for P. aeruginosa PAO6520. These conditions are similar to the conditions of Fiedler and Wirth (1988) which gave optimum transformation efficiencies in E. coli and P. putida. No significant effect was noted for P. aeruginosa PAO4069. Plasmid pRTG1 was present during this electroporation experiment. After electroporation cells were kept on ice for 10 minutes prior to incubation in nutrient broth at 28°C. After a recovery period of 11 hours, cells were plated at ~$1x10^8$ on nutrient agar containing gentamicin (32ug/ml) or streptomycin (320ug/ml). No transformants were obtained under these conditions.

Higher field strengths can be obtained using a capacitance extender to increase the pulse duration and a electroporation cuvette with a narrower inter-electrode gap (0.2cm instead of 0.4cm which doubles the field strength). HBS and sucrose (300mM) were used as alternative conductance media and results are shown in Tables V.vi.a and b. Electroporation in HBS gave mortalities ranging between 4 - 40% and in 300mM sucrose ranging between 0.1 - 20%. After electroporation cells were left to recover in nutrient broth for 12 hours at 28°C and were then plated at ~$1x10^8$ onto nutrient agar containing gentamicin (20ug/ml), or gentamicin (32ug/ml) or streptomycin (320ug/ml) and incubated at 28°C. No transformants were observed using pRTG1 at 5ug/aliquot (~$1x10^7$ cells/ug) with confluent growth of parental controls and test samples on nutrient agar containing gentamicin (20ug/ml).

To test whether DNA concentrations were too dilute, electroporation was repeated with DNA (10ug/aliquot, ~$5x10^6$ cells/ug). Mortality results are shown in Tables V.viia and b. Similar mortalities are observed to those of Tables V.vi.a and b and no transformants were identified selecting on nutrient agar containing streptomycin (320 ug/ml) or gentamicin (32ug/ml).

To test whether the buffers were unsuitable electroporation was repeated using sucrose (300mM) and an alternative buffer containing sucrose (300mM), Na_2PO_4 (7mM) and $MgCl_2$ (1mM) (pH7.2) and DNA at 7.5ug/aliquot (~$7.5x10^6$ cells/ug). Bacteria were incubated for 30 minutes with DNA prior to electroporation. After electroporation, 10 hours recovery in nutrient broth and plating on selective media one colony grew on streptomycin (320ug/ml) (inocula ~$2x10^8$) but was not resistant to gentamicin (32ug/ml) on replica-streaking. Mortality figures are shown in Table V.viii.

To test whether pRTG1 was unable to replicate or unable to express gentamicin-resistance in P. aeruginosa PAO4069 and PAO6520, transformation by electroporation of these strains with alternative plasmids carrying gentamicin-resistance genes (pVW6 and pR1033) was attempted and was also unsuccessful. Similar numbers of gentamicin-resistant colonies were obtained with parental controls. Selection for plasmid was nutrient agar containing gentamicin (30ug/ml) and also nutrient agar containing streptomycin (320ug/ml) when selecting pRTG1

Table V.iv **Effect of a double-pulse of a set voltage/capacitance on the mortality of P. aeruginosa PA04069 in HEPES buffered saline**

Capacitance (uF)	Average numbers of colonies (n=3) (V/cm = 750)	
0	Conf.[a]	7
10	Conf.	6
50	"	12
330	"	7
800	"	3
1180	0.3	0
1600	30	1
1980	25	0.3

a - Inoculum size ~700

Table V.v **Viability of P. aeruginosa PA04069 and PA06520 after electroporation in HEPES buffered saline using the "Gene Pulser"**

Strain	Electric field (KV/cm)	Pulse length (ms)	Number of colonies
PA04069	0	0	37
	3.125	20.4	36
	3.75	22.5	22
	3.75	20.6	32
PA06520	0	0	97
	3.75	20.2	66
	3.75	21.7	60
	4.375	18.8	46
	5.0	17.5	50
	6.25	15.3	35

Capacitance = 25uF in all cases

Table V.vi.a Viability of P. aeruginosa after electroporation in HEPES buffered saline using the "Gene Pulser"

Capacitance (uF)	PA04069 Pulse duration (ms)	PA04069 Number of colonies	PA06520 Pulse duration (ms)	PA06520 Number of colonies
0	0	37, 45	0	113, 105
125	0.9	15, 18	0.9	69, 56
125	0.9	15, 10	0.9	13, 7
250	2.0	12, 14	1.9	8, 24
500	2.7	8, 7	2.6	8, 7
960	7.3	3, 2	7.3	4, 5

Electric field 2.25 KV/cm in all cases

Table V.vi.b Viability of P. aeruginosa after electroporation in 300mM Sucrose using the "Gene Pulser"

Capacitance (uF)	PA04069 Pulse duration (ms)	PA04069 Number of colonies		PA06520 Pulse duration (ms)	PA06520 Number of colonies	
			5000[a]			6200[a]
0	0	57, 42	Conf.	0	62, 62	Conf.
250	396	4, 2	301	446	15, 10	Conf.
250	57	1, 1	111	57	13, 7	651
500	70	1, 0	27	63	3, 5	381
960	79	0, 0	4	123	3, 1	253

Electric field 2.25 KV/cm in all cases
a - Inoculum (cfu/plate)

Table V.vii.a Viability of P. aeruginosa after electroporation in HEPES buffered saline using the "Gene Pulser"

Capacitance (uF)	PA04069 Pulse duration (ms)	PA04069 Number of colonies		PA06520 Pulse duration (ms)	PA06520 Number of colonies	
			730[a]			600[a]
0	0	73	-	0	56, 67	589
125	1.0	35	329	1.0	31, 37	299
125	1.0	39	249	1.0	29, 36	206
250	2.1	35	271	2.0	25	250, 224
500	2.8	25	234, 216	2.8	19	222, 259
960	6.5	2	21, 12	5.8	7	70, 65

Electric field 2.25 KV/cm in all cases except at 960 uF (12.5 KV/cm)
a - Approximate inoculum (cfu/plate)

Table V.vii.b Viability of P. aeruginosa after electroporation in 300mM Sucrose using the "Gene Pulser"

Capacitance (uF)	PA04069 Pulse duration (ms)	PA04069 Number of colonies		PA06520 Pulse duration (ms)	PA06520 Number of colonies	
		530[a]				560[a]
0	0	-	0	56, 56		
125	235	292, 291	236	22	287, 291	
125	223	293, 227	221	16	207, 218	
250	544	157	494	19	206, 211	
500	855	149	786	17	144, 140	
25	30	79	28	8	120, 94	

Electric field 2.25 KV/cm in all cases except at 25uF (12.5 KV/cm)
a - Approximate inoculum (cfu/plate)

Table V.viii.a Viability of P. aeruginosa after electroporation in 300mM Sucrose using the "Gene Pulser"

Capacitance (uF)	PA04069 Pulse duration (ms)	PA04069 Number of colonies		PA06520 Pulse duration (ms)	PA06520 Number of colonies	
			550[a]			640[a]
0	0	55	-	0	64	-
125	228	34	305	204	30	315
250	534	8	90	486	12	133
500	852	20	101	970	7	33
25	30	22	145	23.7	16	136
25	29	17	125	25	18	104

Electric field 2.25 KV/cm in all cases except at 25uF (12.5 KV/cm)
a - Approximate inoculum (cfu/plate)

Table V.viii.b Viability of P. aeruginosa after electroporation in buffered 300mM Sucrose using the "Gene Pulser"

Capacitance (uF)	PA04069 Pulse duration (ms)	PA04069 Number of colonies	PA06520 Pulse duration (ms)	PA06520 Number of colonies	
					660[a]
0	0	57	0	58, 73	-
125	20.9	46	21.1	26, 25	326
250	44	51	47	45, 34	373
500	64	17	67	29, 28	388
25	3.0	26	2.9	29	235
25	2.9	39	2.9	21	215

Electric field 2.25 KV/cm in all cases except at 25uF (12.5 KV/cm)
a - Approximate inoculum (cfu/plate)

Table V.ix **Viability of P. aeruginosa after electroporation in buffered 300mM saline using the "Gene Pulser"**

	Pulse duration (ms)	Number of colonies on nutrient agar containing antibiotic (ug/ml)		
		Viability	Gm (30)	Sm (320)
PAO4069		127[a]		
Control	2.7	31	2	3
pR1033	2.9	22	0	-
pRTG1	2.9	25	0	10
pVW6	2.9	29	2	5
PAO6520		82[a]		
Control	3.0	41	3	3
pR1033	2.9	31	4	-
pRTG1	2.9	26	3	4
pVW6	3.0	29	2	4

Electric field - 12.5 KV/cm in all cases
Capacitance - 25uF in all cases
a - Viability of non-electroporated samples

and pVW6. Table V.ix shows mortality figures and numbers of resistant colonies.

In conclusion, none of the strains of P. aeruginosa tested were transformed by the electroporation procedure. Bacteria were harvested in the early to mid-exponential phase of growth, bacteria and DNA concentrations were high and field strengths which gave a wide range of mortality values, including those optimal for other Gram-negative bacteria, were used. Various buffers were used to alter the field strength and selection was made only after a suitable recovery period to allow expression of the resistance-genes. From this evidence and that already obtained above using alternative methods for the introducion of plasmid it would seem that other more accessible strains (for example, restrictionless strains) or other suitable techniques (spheroplasting) would be required for the successful introduction of plasmid.

V.iv Testing Conjugation as a Means of Introducing pRTG1 into P. aeruginosa.

Plasmid pRTG1 was introduced from E. coli SM10 (pRTG1) into P. aeruginosa PAO4069 (Chapter 5). However, pRTG1 was unstable with deletions and loss of resistance phenotypes. P. aeruginosa PAO4069 (pRTG1a) was used to test whether ceftazidime-resistant mutants could be generated by Tn5::gen. However, transformation and further conjugation experiments were also performed in an attempt to obtain P. aeruginosa PAO4069 carrying native pRTG1 and to test whether the apparent instability of pRTG1 was plasmid-specific, rather than host strain-specific. Alternative vectors (pVW6 and pR1033) carrying gentamicin-resistance genes were used to test whether the instability of pRTG1 was because of multi-copy DNA, the transposon, the gentamicin gene or to pRTG1 specifically.

Selection regimes were required capable of differentiating transconjugants from spontaneous resistance mutants and to prevent contamination by E. coli SM10. Pseudomonas Isolation Agar (PIA) is similar to C-N (Chapter 5) in preventing growth of E. coli SM10. Selection on glucose minimal agar containing tetracycline (5ug/ml), streptomycin (400ug/ml) and gentamicin (30ug/ml) was compared with initial selection on PIA containing gentamicin (30ug/ml), or streptomycin (400ug/ml), or gentamicin (20ug/ml) and streptomycin (400ug/ml). No transconjugants of P. aeruginosa PAO4069 (pRTG1) were obtained on glucose minimal plates (inoculum 9.9×10^{8} cf/plate). However, on PIA selection media containing a single antibiotic, small colonies (<1mm) of parental P. aeruginosa were observed. Transfer of pRTG1 from E. coli SM10 (pRTG1) into E. coli UB5201 was performed to determine the frequency of pRTG1 transfer into E. coli using the same conjugation conditions used for P. aeruginosa. The transfer frequency of pRTG1 from E. coli SM10 into E. coli UB5201 was 5.4×10^{-2} with selection on glucose minimal agar containing proline, methionine, gentamicin (20ug/ml) and streptomycin (150 ug/ml).

Previously (Chapter 5), P. aeruginosa PAO4069 (pRTG1) transconjugants had been isolated from glucose minimal media containing tetracycline (5ug/ml), gentamicin (25ug/ml) and streptomycin (400ug/ml). This experiment was repeated without success. Furthermore, no transconjugants were observed with selection on glucose minimal plates containing tetracycline (5ug/ml) and gentamicin (25ug/ml), or gentamicin (25ug/ml) and streptomycin (200ug/ml).

Filter-matings were repeated with incubations of conjugal mixtures for 6 and 24 hour prior to selection on glucose minimal agar containing proline, methionine, and tetracycline (5ug/ml) with selection for pRTG1 as described in Table V.x.

Table V.x Number of Colonies Observed After Filter-matings Between *E. coli* SM10 (pRTG1) and *P. aeruginosa* PA04069

Glc. min. pro met containing antibiotic (ug/ml)	Number of colonies growing from mating mixtures incubated for 6 and 24 hours					
	SM10 (pRTG1)		PA04069		Conjugal mix	
	6	24	6	24	6	24
Tc(5) Gm (15)	0	0	33	~50	35	~100
Tc(5) Sm (300)	0	0	Conf.	Conf.	Conf.	Conf.
Tc(5) Gm (15) Sm (300)	0	0	0	0	2	0
Tc(5) Gm (15) Sm (200)	0	0	3	0	5	0
Tc(5) Gm (20) Sm (300)	0	0	0	0	0	0

PA04069 Inoculum - 6 hours - $3x10^9$, 24 hours - $7x10^9$

Glc. min. - Glucose minimal
met - methionine
pro - proline
Conf. - Confluent growth

Gm - Gentamicin
Sm - Streptomycin
Tc - Tetracycline

Prolonging the filter-mating did not generate a significant number of transconjugants if any at all, using these selection conditions.

Conjugation conditions described by Kokjohn and Miller (1987) were used in an attempt to increase the transfer frequency of plasmid into *P. aeruginosa* PA04069. In this experiment *P. aeruginosa* PA04069 was grown for 16 hours at 43°C and cells were concentrated five-fold by centrifugation prior to conjugation with *E. coli* SM10 (pRTG1). No growth was obtained when selecting on glucose minimal medium containing tetracycline (5ug/ml) and gentamicin (25ug/ml). However, when selecting on PIA containing streptomycin (400ug/ml) or gentamicin (32ug/ml) parental growth was obtained.

To test whether the gentamicin-resistance gene was not expressed in *P. aeruginosa* PA04069 similar conjugation conditions were used to transfer a control plasmid, pR1033, which encodes the same gentamicin-resistance gene. Within background parental growth, large, pigmented colonies were observed on PIA plates containing gentamicin (40ug/ml) plated with a mating mixture containing putative *P. aeruginosa* (pR1033). No such colonies were observed on plates containing a mating mixture containing putative *P. aeruginosa* PA04069 (pRTG1) even though pRTG1 transferred into *E. coli* UB5201 more frequently than pR1033. After isolation from conjugal plates, plasmid extraction

from three putative isolates was performed. On electrophoresis, the DNA banding was consistent with pR1033 obtained from E. coli SM10 (pR1033). Although restriction enzyme digests were not performed, major deletions of pR1033 was not observed in this small sample size.

V.v Discussion

Increasing the concentration of antibiotic present above concentrations allowing growth of spontaneous mutations did not select pRTG1 transconjugants. This may have been due to poor transformation and conjugation frequencies. Attempts to increase the plasmid transfer efficiency were unsuccessful. Possible explanations included poor expression of resistance genes in the new host, insufficient activity of the temperature-sensitive replication protein or host intolerance to pRTG1. To overcome the possibility of poor expression of resistance genes and poor activity of the temperature-sensitive replication protein, various recovery periods were tried but without success. P. aeruginosa PAO4069 (pRTG1a) and P. aeruginosa PAO4069 (Tn5::gen) were known to be resistant to gentamicin at 320ug/ml and P. aeruginosa PAO4069 (pRTG1) was also resistant to streptomycin at 1600 ug/ml. Moreover, there was no problem with initial selection for pRTG1 with gentamicin at a concentration of 20ug/ml in E. coli. It would seem unlikely that the poor expression of resistance genes were responsible for the lack of success in isolating P. aeruginosa PAO4069 (pRTG1). Most probably poor competence of P. aeruginosa combined with intolerance to plasmid DNA may have contributed to the inability to isolate P. aeruginosa PAO4069 (pRTG1).

A variety of methods and selection regimes were used but without reproducible success. The unpredictable behaviour of P. aeruginosa to selection conditions severely restricted the progress of the project. The inability to select against parental growth whilst trying to obtain transconjugants may have been hampered by the high population density of P. aeruginosa used in the filter-mating method, which concentrated the conjugate mix to increase the chances of contact between donor and recipient cells. This possibility is supported by the fact that using a spot-mating method transconjugants were isolated on selection media containing gentamicin (20ug/ml).