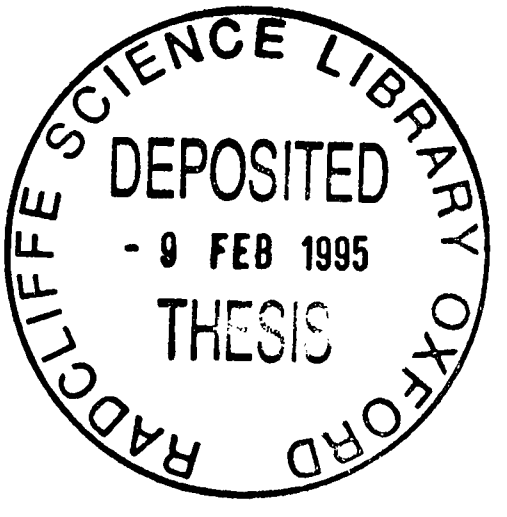
The study of plasmid-plasmid and plasmid-chromosome interactions in Staphylococcus aureus

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A dissertation submitted to the Faculty of Biological Sciences for the degree of Doctor of Philosophy at the University of Oxford

Trinity Term, 1994
Linacre College



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Abstract

Experiments designed to study the movement of genes between the plasmids and the chromosome of *Staphylococcus aureus* are described.

Two derivatives of the plasmid pI9789::Tn*552*, that are temperature-sensitive for replication, pS1 and pΔD, are used as a model system for this investigation. The plasmids carry a gene conferring resistance to cadmium salts and so the number of cadmium-resistant colonies appearing on selective plates at a restrictive temperature is used as a means of measuring the frequency of suppression of the temperature-sensitivity. The frequency depends on the nature of the host, the structure of the plasmid and in some cases is enhanced by the presence of a cloned site-specific recombinase gene, *binR*. One explanation for suppression is the integration of the plasmid into the chromosome. The presence of Tn*551* on the chromosome also greatly enhances the number of cadmium-resistant colonies.

The chromosome of *S. aureus* strain PS80N has been shown by hybridization to carry at least most of Tn*552* on a single *Eco*RI fragment. In several cases following selection for cadmium-resistance at the restrictive temperature, the plasmid pS1 has been shown to interact with the *Eco*RI fragment at a particular plasmid site. The plasmid is presumed to have integrated into the chromosome and subsequent growth of bacteriophage followed by its use in transduction has revealed different types of recovered plasmids often with deletions commencing at a specific site. If the host chromosome carries Tn*551* then on transduction of the presumed integrated plasmid, a copy of Tn*551* is found inserted into the transductant plasmid. For pS1 the site of insertion of Tn*551* is into the Tn*552* transposase gene, whereas for pΔD insertion occurs into different sites for different transductants.

pS1 and pΔD can form co-integrates with the resident cryptic plasmid pOX1054 of *S. aureus* 1054. The recombination occurs by a novel method. The data show that pS1 or pΔD contribute the site for recombination and that the gene(s) for the protein(s) involved in recombination are encoded on pOX1054 or the 1054 chromosome. Integration of the plasmids into the chromosome of 1054 was not detected.

Acknowledgements

I am short of words to thank Dr. KGH Dyke for his guidance, kindness and unlimited patience throughout the course of this work.

I thank Professors Paul Nurse and David Sherratt for allowing me the opportunity of working in the Microbiology Unit.

I also thank the Ministry of Science and Technology, Government of Pakistan for the financial assistance during the course of this study.

I also thank my friends and colleagues and many past and present members of the Microbiology Unit for their encouragement and help during my stay in the Unit, especially the *Bacillus* Group. My special thanks to Steve Curnock for his "**skillful**" technical and other assistances. I also thank Arshad, Arif, Bilal, Kehkashan, Khalid, Mujahid, Rizwan and other members of OUPS for many "PROGRAMMES". I also owe a great deal to my relatives and friends in London for their support and encouragement.

I find no words to express my deepest gratitude and very special thanks to my grandparents, Dad, Mum, brothers and sisters whose infinite love, prayers and best wishes have been a source of energy in the completion of this work.

IN THE NAME OF GOD

Abbreviations and symbols

amp	gene conferring resistance to ampicillin
Ap	ampicillin
Asa	arsenate
bla	gene conferring resistance to β-lactams
bp	base-pair
BSA	bovine serum albumin
cad	gene conferring resistance to cadmium
CBAP	2-(2'-carboxyphenyl)-benzoyl-6-amino-penicillanic acid
Cd	cadmium
CTAB	cetyltrimethyl-ammonium bromide
Δ	deletion
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetate
Em	erythromycin
erm	gene conferring resistance to erythromycin
Eth Br	ethidium bromide
ϕ	phage
Hg	mercury
hr	hour
IPTG	isopropylthio-β-D-galactoside
IS	insertion sequence
kb	kilobase-pair
λ	lambda
min	minute
MSO	minus strand origin

NCTC	National Collection of Type Cultures
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PSO	plus strand origin
r	resistant (resistance)
Rev	revertant
RNA	ribonucleic acid
RNAase	ribonuclease
rpm	revolutions per minute
rs	recombination site
s	sensitive
SDS	sodium dodecyl sulphate
sec	second
TBAB	tryptose blood agar base
Td	transductant
Tn	transposon
Tris	tris (hydroxymethyl) aminomethane
tsr	temperature-sensitive for replication
UV	ultraviolet
Ω	insertion
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Chapter One:

General Introduction

Chapter One: General Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, coagulase-positive, facultatively anaerobic and non-motile bacterium. Cells occur singly or in pairs and cell division in more than one plane gives rise to irregular clusters of the bacteria. *S. aureus* is an important pathogen and can cause a wide variety of infections in both humans and animals. It is commonly found on the surface of animals and humans as well as on nasal membranes and to some extent on the perineum and gastrointestinal and genital tracts. The bacterium can invade directly through breaks in skin and mucous membrane leading to a variety of soft tissue infections. Infections of deep skeletal structures can lead to primary osteomyelitis and septic arthritis. Some other important clinical conditions caused by *S. aureus* include endocarditis, meningitis, pneumonia, bacteraemia, enterocolitis and urinogenital infections. *S. aureus* may also produce enterotoxins which are involved in staphylococcal food poisoning. Toxigenic strains of *S. aureus* may cause toxic shock syndrome (TSS); an illness involving many organs and of acute onset. (see Sneath *et al.*, 1986 and Sheagren, 1984a &b).

The staphylococcal genome like many other procaryotic genomes consists of a single linkage group referred to as the chromosome. The estimated size of the *Staphylococcus* chromosome ranges from 2.7×10^3 kb to 6×10^3 kb. In addition to the chromosome, *Staphylococcus* also contains a group of variable accessory genetic elements including prophages, plasmids and transposons. The G + C content ranges from 30 - 39

percent (Pattee *et al.*, 1990; also see Novick, 1990 and Sneath *et al.*, 1986). Some one hundred loci on the chromosome of *S. aureus* strain NCTC8325 have been mapped by Pattee *et al.* (1990) using both physical and genetic methods. The overall size of the chromosome is 2.78 x 10^3 kb.

Extrachromosomal DNA in the form of plasmids which range in size from ~10^3 base-pairs to ~60 x 10^3 base-pairs is present in most of the naturally occurring *S. aureus* strains. *S. aureus* plasmids have been classified into four major classes on the basis of their size and into thirteen incompatibility groups (Novick, 1989; Poston and Naidoo, 1983). Plasmids falling in Class-I range in size from 1-5kb. They are high copy number (15-50) plasmids and are either cryptic or carry a single resistance determinant. Rarely, they carry two resistance determinants. They replicate by asymmetric rolling circle mechanism and their copy number is regulated at the level of initiator protein (Rep) synthesis. Most of the staphylococcal cloning vectors currently in use are derived from this class of plasmids. Class-II plasmids range in size from 15-30kb with a copy number of 4-6 per cell. They carry resistance to the β-lactam or macrolide types of antibiotics and, in addition, contain a variety of metal-ion resistance determinants, such as those for cadmium, lead, mercury, arsenate and bismith. This group of plasmids also encodes a replication initiator protein but replicates by a theta type of mechanism. Class-III contains a family of large plasmids ranging from 30-60kb. They carry determinants for antibiotic resistance and, in addition, carry genes specifying a conjugation system. Replication of these plasmids is not well understood. Class-IV plasmids consist of a group of plasmids which do not seem to belong to any of the above three classes. They have only been cursorily studied and have been provisionally placed in a separate group. Staphylococcal plasmids and their replication have been reviewed by Novick (1989).

1.2 Epidemiology and genetics of antimicrobial resistances

S. aureus is a problem organism in the treatment of nosocomial and community acquired infections because it often possesses multiple antimicrobial resistances. This has prompted many investigators to carry out research on the evolution and epidemiology of antimicrobial resistances and the nature of the resistance determinants. Staphylococcal infections resulted in nearly 80% mortality in the pre-antibiotic era. The problem was to some extent controlled by the use of penicillin-type antibiotics. Many clinical strains of *S. aureus* isolated in the 1940's were susceptible to penicillin but strains resistant to the antibiotic very quickly emerged (Wise *et al.*, 1989; Sheagren, 1984a & b). The pattern of emergence of antimicrobial resistant strains was repeated with successive introductions of new antimicrobial agents. This resulted in selection in favour of organisms resistant to multiple antimicrobial compounds (Lacey, 1975). Staphylococcal isolates carrying multiresistance phenotypes including resistance to a combination of two or more of erythromycin, clindamycin, chloramphenicol, tetracycline, gentamicin, trimethoprim, rifampin, quinolones, heavy metals and quaternary ammonium compounds, have been responsible for outbreaks of nosocomial infections in recent years in countries all around the world (Udo and Grubb, 1991; McGowan, 1991; Watanabe *et al.*, 1990; Inglis *et al.*, 1990; Dornbusch, 1990; Wise *et al.*, 1989; Chang *et al.*, 1989; Schaefler *et al.*, 1984; Espersen *et al.*, 1982; Price *et al.*, 1980 and others). McGowan (1991) recorded abrupt increases in the prevalence of antimicrobial resistances to drugs, such as, amikacin, ciprofloxacin and β-lactamase production among bacteria (*e.g.*, *S. aureus* and gonococci) isolated from both hospital- and community-acquired infections and postulated four

probable mechanisms for development of resistances; (i). resistant organisms may be introduced into a previously susceptible population, (ii). previously susceptible strains may acquire resistance through genetic mutation or by transfer of genetic material from another species or genus, (iii). a dormant resistance mechanism already present in the population may emerge, (iv). resistant sub-populations of organisms may emerge due to selective pressure applied to bacterial populations by the use of a drug. Antimicrobial resistance determinants in *S. aureus* and their genetics have been reviewed by Lyon and Skurray (1987).

Antimicrobial resistances in staphylococci and other bacteria were initially believed to be solely the result of mutation and selection. This suggestion was based on the observation that, spontaneously, bacterial mutants resistant to certain antibiotics can be generated at frequencies of 10^{-6} to 10^{-8} per cell in the laboratory and it was assumed that analogous events may have occurred in natural populations of bacteria to give rise to resistant strains. Although, some resistant determinants, like those for novobiocin and fusidic acid resistance, are thought to be derived by chromosomal mutation, such mutations may also lead to creation of less virulent forms of the organism. Nevertheless, acquisition of new traits in this manner without affecting the fitness of the organism would be expected to occur over a substantial period of time. The dispersal of bacterial resistances to antibiotics has been an extraordinarily rapid phenomenon, and in evolutionary terms antimicrobial resistances have emerged over a very short span of time (some 50 years). For this reason the accumulation of such chromosomal mutations as an explanation of the rapid emergence of resistant phenotypes appears unsatisfactory and other pathways must be involved in the evolution of multiresistant strains (for example see Saunders, 1984 and Lacey, 1984). This view was supported by the discovery in Japan in 1959 of extra-

chromosomal or plasmid DNA carrying resistances to antibiotics, involved in bidirectional transfer of multiple resistances between *Shigella* and *E. coli* during infection. Such bi-directional transfer may result in the conversion of non-pathogens into a resistance gene pool available to pathogens during colonization of the infected organism. For example, *Staphylococcus epidermidis* may serve as a reservoir of a resistance gene pool available to pathogenic members of this genus (see Amábile-Cuevas and Chicurel, 1992 and, Lacey, 1984). Ample evidence has now accumulated that resistance to many antimicrobials in *S. aureus* is due to the presence of plasmids carrying the genetic determinants for resistance (Lyon and Skurray, 1987). It is also known that in staphylococci and other bacteria, resistance determinants may be encoded on genes carried by structurally defined and genetically discrete units called transposons. Transposons are segments of DNA capable of moving from one genome to another without any obvious homology with the recombining DNA and have been found in almost all species surveyed since their discovery in the 1940s (see Capy *et al*, 1994 and, Grinsted *et al.*, 1990). Transposons may be found on both the bacterial chromosome and the plasmids. Due to their ability to insert into any replicon, transposons are also believed to have played an important role in the development of multiresistant replicons. Transposons are not replicons and the primary route for them to move to a different bacterial host is by insertion into a plasmid which can serve as a 'delivery vehicle' for the transposon (see later). Several transposons may transpose to various sites on the same plasmid thus generating a plasmid carrying multiple resistance determinants. Plasmids may also contribute to the development of chromosomal resistances, *e.g.*, by in part or *in toto* integration into the chromosome. Antimicrobial resistances carried on plasmids can be translocated from a plasmid to a chromosomal site or to another plasmid, *e.g.*, by transposition of a transposable resistance determinant. (Sayre

and Miller, 1991; Skurray *et al.*, 1988; Gillespie and Skurray, 1986; Cohen, 1976). It may, therefore, be suggested that the antimicrobial resistances seen in the present day nosocomial ecosystems have largely resulted from the process of clonal selection operating on bacteria carrying plasmids containing antimicrobial resistance genes possibly as part of transposons.

1.3 Origins and evolution of resistance determinants

There is some evidence to suggest that resistance genes may have existed within the bacterial gene pool prior to man's introduction of chemotherapeutic antimicrobials. For example, resistant *E. coli* have been isolated from the faeces of a human from the pre-antibiotic era (see Saunders, 1984). Some enterobacteria, isolated and lyophilized prior to the antibiotic era (between mid 1930s and mid 1940s), have been found to be resistant to antimicrobials that had not been introduced at the time of storage, for example, to spectinomycin and tetracycline (Smith, 1967). However, a more recent survey, conducted by Hughes and Datta (1983), of over 400 hundred enterobacteria isolated and stored between 1917 and 1954 (Murray's collection) suggests that resistance was very uncommon in the pre-antibiotic era. Twenty-four percent of these strains were found to carry conjugative plasmids which were able to mobilize other non-conjugative test plasmids. A similar percentage of *E. coli* isolates from faecal samples of healthy humans from the post-antibiotic era were found to contain conjugative plasmids. Such findings suggest that the human microflora not only had the genes for resistance but also the potential to transfer genes before resistance became a problem (see Saunders, 1984).

A large number of the known antimicrobial resistances are encoded on extra-chromosomal plasmid DNA, there is, however, no evidence that the resistance genes carried on plasmids themselves originated in human pathogens or commensals. The ultimate source of many resistance genes appears to be the soil microflora (*e.g.*, *Streptomyces*) that actually produce most of the antibiotics or the soil microflora that would be competing with such producers. Antibiotics are potentially toxic to the producers, these microorganisms thus must carry resistance determinants (Cundliffe, 1984; also see Amábile-Cuevas and Chicurel, 1992). For example, *Streptomyces erythraeus* which produces erythromycin protects itself from the antibiotic by methylation of its 23S rRNA. There is evidence to support the hypothesis that some resistance determinants in staphylococci are derived from the resistance genes present in antibiotic producers. For example, the plasmid-mediated erythromycin resistance specified by *ermC* in *S. aureus* is apparently determined by a 23S rRNA methylase similar to that specified by *ermE* in *S. erythraeus* (*e.g.*, Graham and Weisblum, 1979; Weisblum *et al.*, 1979), the two genes also share amino acid sequence homology. In *Bacillus licheniformis*, the *ermD* gene encodes a methylase and shows some amino acid sequence homology to both *ermC* and *ermE*. The *ermD* gene is flanked by sequences that are homologous to the *S. erythraeus* chromosome which suggests that there may be a common evolutionary origin for genes encoding this resistance. There is also some evidence to suggest the exchange of resistance determinants and replicons between staphylococci and soil bacteria. Comparison of the amino acid sequences of the β-lactamases produced by *B. licheniformis* and *S. aureus* suggests that they might have evolved from a common ancestral gene (see Lyon and Skurray, 1987).

It is also believed that the members of the genus *Staphylococcus* share certain gene pools. For example, there is similarity between plasmids carrying tetracycline,

chloramphenicol and neomycin resistances isolated from *S. aureus* and *S. epidermidis* (see Lyon and Skurray, 1987). Homology has been shown between *S. xylosus* arsenate resistance plasmid pSX267 and *S. aureus* heavy-metal (and β-lactamase) resistance plasmid pI258 (Götz *et al.*, 1983). The transfer of gentamicin resistance plasmids has also been demonstrated from a clinical isolate of *S. hominis* to several different *S. aureus* strains (Naidoo and Noble, 1981). The dynamic nature of the resistance gene pool and the homologies shared in this by various microorganisms may suggest a common ancestry for many of the resistance determinants rather than a need for each species or genus to develop its own resistance gene pool.

1.4 Modes of *inter*cellular movement of resistance determinants

The horizontal or lateral transfer of resistance genes, *i.e.*, from one microorganism to another, of the same or different species, is crucial to the emergence and evolution of resistant microflora (Amábile-Cuevas and Chicurel, 1992). As in other bacteria, several mechanisms for lateral gene exchange exist in staphylococci. These include the processes of gene transfer like conjugation, transduction and transformation and, in addition, a process called 'phage-mediated conjugation' which is known to occur only in *Staphylococcus* (*e.g.*, Lacey, 1980). Although these gene transfer mechanisms have been shown to occur in experiments in the laboratory, their precise *in vivo* role and the part played by each mechanism in the evolution of antimicrobial resistances is, however, unknown.

Transfer of DNA by **conjugation** or **mobilization** demands cell-to-cell contact. The genes for conjugative transfer are normally found on plasmids and less commonly also on transposons (*e.g.*, Tn*916*: Clewell *et al.*, 1988; Tn*1545*: Courvalin and Carlier, 1987). Plasmids that encode the apparatus for their own transfer by conjugation are termed self-transmissible. In *Staphylococcus*, such self-transmissible (Tra^+) plasmids range in size from 38kb to 57kb (Lyon and Skurray, 1987) and belong to class III of staphylococcal plasmids (Novick, 1989). Many staphylococcal conjugative plasmids carry gentamicin resistance and many also encode for resistance to other aminoglycosides, ethidium bromide and quaternary ammonium compounds, penicillin and trimethoprim (Lyon and Skurray, 1987). Conjugation is efficient in mixed cultures under laboratory conditions in *Staphylococcus* (Novick, 1990), but it has also been demonstrated among staphylococci on human skin (Jaffe *et al.*, 1980) and between strains of *E. coli* in the intestines of both humans and animals albeit at lower frequencies (for example, see Saunders, 1984). Conjugative mobilization is a widespread method of DNA transfer and has been observed in both Gram-negative and Gram-positive bacteria. It may also occur between members of two groups, *e.g.*, conjugal transfer of plasmid DNA has been reported from *E. coli* to *Streptococcus*, *Staphylococcus*, *Bacillus*, *Listeria* (Trieu-Cuot *et al.*, 1987) and to *Corynebacterium* (Schäfer *et al.*, 1990). Conjugal transfer of genes has also been reported to occur from *E. coli* to *Saccharomyces cerevisiae* in the laboratory (Heinemann and Sprague, 1989). The conjugative mechanisms of Gram-negative bacteria are best understood and appear to be more sophisticated than in Gram-positive bacteria. In Gram-negative bacteria, the conjugal transfer requires some 24 genes spanning nearly 33kb (see Willetts and Wilkins, 1984) compared to nearly 15kb in *Staphylococcus* (for example, Firth *et al.*, 1993; Evans and Dyke, 1988). Conjugative plasmids are also able to mobilize

other co-resident plasmids that carry a set of mobility genes, *mob*, and mobilization may occur at a 100-fold greater frequency than the transfer of the conjugative plasmid itself. Mobilization is characterised by lack of co-valent linkage between the participating conjugative and non-conjugative mobilizable plasmid and suggests a further significant role for plasmid-mediated conjugation in horizontal transfer of resistance genes (Novick, 1990; Lyon and Skurray, 1987; Saunders, 1984).

Transduction is the transfer of genes in which bacteriophage particles carry DNA from donor to recipient bacteria only when they share appropriate cell surface receptors for the transducing phage. This method of gene transfer is therefore limited and is relatively ineffectual as a means of genetic transfer and is very unlikely to occur between different genera. Calcium ions are required for the attachment of some types of phage particles to the cell surface and so transduction can be inhibited by the presence of chelating agents such as citrate ions. Nevertheless, the majority of clinical isolates of *Staphylococcus* possess one or more temperate phages integrated into the chromosome and for many years transduction was believed to be the main mechanism of *in vivo* genetic exchange in staphylococci. There is evidence that transduction plays a significant role in the natural transmission of resistance genes and plasmids between strains of *S. aureus* and between strains of *Streptococcus pyogenes* (Amábile-Cuevas and Chicurel, 1992; Lyon and Skurray, 1987; Saunders, 1984) However, it may be difficult to carry out transduction between natural isolates of *S. aureus* in the laboratory due to restriction barriers (Novick, 1990). The staphylococcal transducing phages have genome sizes of approximately 45kb and this may explain why high transduction frequencies are obtained with plasmids of 30kb to 40kb and that majority of staphylococcal plasmids are smaller than 45kb. Plasmids larger than the phage genome must be truncated before they can be carried by the phage and smaller plasmids probably

form linear multimers that are inserted into the phage (Novick, 1990; Lyon and Skurray, 1987). The co-transduction of small staphylococcal resistant plasmids (*e.g.*, pE194 and pT181) has also been shown and may result in the formation of new linkage groups by stable co-integration of the participating plasmids (*e.g.* Novick *et al.*, 1984).

Phage-mediated conjugation is a poorly characterized method of gene transfer. It requires the presence of a prophage in either the donor or the recipient together with a high cell density and calcium ions. However, unlike transduction, the donor cell need not be lysogenic and resistance determinants encoded on plasmids may be transferred at a frequency as high as 10^{-1} resistant recipients per donor cell (Lacey, 1980). The non-transduction-like nature of this transfer is supported by a number of observations: little phage activity is found in broth cultures and filtrates do not necessarily contain phage particles. Transfer proceeds even when either the donor or the recipient is lysogenised with a ϕ11 mutant unable to form phage heads and hence viable phage particles (see Lyon and Skurray, 1987). Lacey (1980) has postulated that the phage may alter the adhesiveness of the cell surface, thereby enhancing the clumping of cells and enabling plasmid DNA to pass directly from donor to recipient.

Transformation is generally referred to as the process whereby bacteria are able to take up naked DNA. The process occurs naturally only in about 15 bacterial genera (Stewart and Carlson, 1986) and must be induced by artificial treatment in others. Generally, bacteria do not discriminate against foreign DNA during uptake. Thus transformation is an ideal mechanism for DNA transfer across generic boundaries (see Saunders, 1984). Naked DNA is, however, exposed to degradation by nucleases and this may seriously limit the role of this process in gene transfer in the natural environment. Transformation of *S. aureus* can be achieved with both plasmid and chromosomal markers

in the laboratory using competent cells in the presence of calcium ions or using protoplasts prepared with lysostaphin. Higher transformation frequencies may be achieved by the use of nuclease- and restriction-negative mutants (see Novick, 1990; Lindberg, 1981) or by heating the recipient culture to inactivate nucleases (see Lyon and Skurray, 1987).

Both transduction and transformation have been considered to be of less evolutionary importance than conjugation because they are restricted to a fairly small group of bacteria and the conditions required by these processes are less frequently met in nature than those for conjugation (Amábile-Cuevas and Chicurel, 1992).

1.5 Genetic recombination: modes of *intra*cellular movement of resistance determinants - an overview

Successful DNA transfer requires, not only a system for the DNA to enter the recipient, but also, if the DNA is not present as a complete replicon, stabilization by recombination into a recipient replicon. Genetic recombination can be broadly divided into *recA*-dependent or homologous and *recA*-independent.

General or **homologous recombination** occurs between segments of DNA that carry substantial sequence homology and is of great importance for the maintenance of genetic diversity and genomic integrity. Homologous recombination has been reviewed by Smith (1988) and its biochemical nature reviewed by Kowalczykowski *et al.* (1994). Homologous recombination also appears to be important in the evolution of multiresistant plasmids and chromosomes. For example, a plasmid may integrate reversibly or irreversibly into the chromosome by recombination at a region of homology that can be

provided on the recombining genomes by the presence of a transposon or insertion sequence. Recombination may occur at any point within the region of homology although particular DNA sequences may influence the frequency of exchange (Craig, 1988; Saunders, 1984; Cohen, 1976). Homologous recombination between chromosome and plasmid may also occur at comparatively very short regions of sequence homology (Khasanov *et al.*, 1992). Recombination between short regions of homology may also be carried out among small staphylococcal plasmids by host- and phage-mediated general recombination systems (Novick *et al.*, 1984). Such recombination events can result in irreversible co-valent linkage of two or more resistance determinants and may have played some role in the evolution of multiresistant genomes in the natural environment. The chromosomal fragment transferred into a recipient bacterium may recombine with the host chromosome by local homologous recombination. Such events may not generate novel linkage groups but are important in producing allelic variations (see Amábile-Cuevas and Chicurel, 1992).

The ***recA*-independent recombination** may be classified into **conservative site-specific recombination** and **transposition.**

Conservative site-specific recombination can occur in the absence of the host's general recombination machinery but requires short regions of homology which are identified by the catalytic enzymes - the site-specific recombinases - to bring about recombination. Strand exchange occurs by precise breakage and joining events without the loss or gain of any nucleotides. The sequence-specific recombinases are generally divided into two families: the integrase family and the resolvase-invertase family. The integrase family recombinases can mediate co-integration of two replicons and resolution of the co-integrate (both *inter* and *intra*molecular recombination). This family of recombinases is

exemplified by the lambda integrase 'Int' which brings about both integration of phage lambda into the *E. coli* chromosome and excision of the prophage. The resolvase-invertase family acts on DNA sequences called '*res*' (or the 'resolution site') and performs only *intra*molecular recombination. The resolvase members of this family can catalyse resolution of DNA placed between two *res* sites which are in the same orientation with respect to each other. The resolvases can also carry out inversion of DNA flanked by two *res* sites in opposite orientation but very inefficiently. The invertases can bring about reciprocal inversion of a DNA fragment which is flanked by two inversely placed *res* sites. However, very inefficiently, they can also resolve DNA between two directly repeated *res* sites. Both the integrase family recombinases and the resolvase members of the resolvase-invertase family seem to be of more evolutionary significance than the invertases because of their ability to produce changes in the linkage groups of a genome (reviewed by Sherratt, 1993; Sadowski, 1993; Landy, 1989; Berg and Howe, 1989; Craig, 1988). The conservative sequence-specific recombination systems of *S. aureus* are not very well-characterized. Although systems similar to those reported in other microorganisms for site-specific recombination have been shown to occur in *S. aureus* (*e.g.*, Rowland and Dyke, 1990; 1989; 1988), the others appear to be different (Gennaro *et al.*, 1987; Novick *et al.*, 1984). The site-specific recombination system encoded on small staphylococcal plasmids like pT181 and pE194 can mediate recombination between two *inter* or *intramolecularly* placed recombination sites called RS_A in a *recA*-independent fashion and is mediated by the site-specific recombinase 'Pre'. This recombination system however appears not be totally independent of the host general recombination system. In a recombination proficient host, the frequency of Pre-mediated recombination between two RS_A sites is slightly enhanced and co-integrates between two plasmids having RS_A can be obtained in a Pre$^-$

recombination-proficient host. Another recombination site called RS_B occurs more frequently on small staphylococcal plasmids but recombination at this site is apparently mediated by a phage-encoded recombination system and not by Pre (Gennaro *et al.*, 1987).

Transposition is the recombination mechanism where the protein involved are encoded on structurally defined DNA segments, the transposable genetic elements. Transposable DNA is generally divided into two major groups: insertion sequences (ISs) and transposons (Tns). An insertion sequence solely carries genes encoding transposition functions and have short terminal inverted repeats that are sites where transposase (the transposition enzyme) acts. Transposons carry genes for transposition and in addition may also carry other genes, for example those encoding antimicrobial resistances. Transposons can be 'composite' in which case the central region is flanked by IS elements or 'non-composite' where the central region is flanked by short inverted repeats of DNA like in IS elements. Transposable elements generally do not require extensive homology in order to insert into a target molecule and transposition can occur in the absence of the host's general recombination system. Transposons are not replicons and therefore they must be maintained as a part of a functional replicon, such as, plasmid, bacteriophage or chromosome. Transposition can be replicative or non-replicative. In replicative transposition, the whole transposable element is duplicated and the donor molecule retains a copy of the transposon. In non-replicative transposition, the transposon is excised from the donor DNA molecule and is integrated into a site on the recipient replicon. Although transposons generally do not show specificity for an insertion site, they may show regional specificity. The *S. aureus* transposon Tn*554* is a site-specific transposon and transposes specifically into a sequence called *att554*. (for reviews see Sayre and Miller, 1991;

Kleckner, 1990; Berg and Howe, 1989; Grindley and Reed, 1985). Murphy (1989) has reviewed the transposable elements in Gram-positive bacteria.

Although transposons are not directly involved in the horizontal gene transfer, they can incorporate into other transmissible replicons involved in gene flux and made accessible to other organisms (see Amábile-Cuevas and Chicurel, 1992). The *Streptococcus faecalis* transposon Tn*917* and the *S. aureus* transposon Tn*551* are very similar and may have originated from a common ancestor (Shaw and Clewell, 1985). Some transposons like Tn*916* (Clewell *et al.*, 1988) and Tn*1545* (Courvalin and Carlier, 1987) also code for a conjugal transfer system and may play a direct role in horizontal gene transfer. Genes for several clinically important antimicrobial resistances are encoded on transposons and their existence provides an explanation for the emergence of multiply-resistant bacteria. Since, transposons generally do not require a specific site for insertion, it is therefore possible for a replicon to acquire many different resistance genes by a series of transposition events into non-essential sites (see Saunders, 1984).

1.6 Evolution of multiresistant plasmids and chromosome

Mechanisms of both *inter* and *intra*cellular movement of genes seem to play important roles in the evolution of novel combinations of the genetic material. The evolution of resistant plasmids in *S. aureus*, as in other bacteria, is thought to occur by *recA*-dependent general recombination and *recA*-independent site-specific recombination and transposition or translocation of structurally defined DNA segments (Lyon and Skurray, 1987). The resultant plasmids are then spread by the mechanisms of lateral gene

transfer. The characterization of families of *S. aureus* plasmids, *e.g.*, those which confer resistance to penicillin and metal-ions (Shalita *et al.*, 1980), and gentamicin and other aminoglycosides (*e.g.*, Gillespie and Skurray, 1986) reveals that members of each family are largely homologous. However, members of each group also possess some differences which could be explained by deletion or insertion of discrete segments of DNA like transposons. For example, a penicillin and metal-ion resistant carrying pI9789::Tn*552*-like plasmid seems to be at the branch point for further evolution of two families of staphylococcal plasmids, the α and the γ family. Further development of the two families of plasmids seems to have occurred as a result of defined deletions or insertions of DNA fragments (Shalita *et al.*, 1980). The pSK1 family of plasmids, the members of which may encode resistance to antiseptics, disinfectants, trimethoprim, aminoglycosides and penicillin, provides an example of the role played by transposons in the evolution of multiresistant plasmids. The multiple-resistance-carrying members of this group possibly emerged by insertions of the transposons, like Tn*4001* (carries resistance to gentamicin, tobramycin and kanamycin) and Tn*4002* (carries genes for β-lactamase production) into a pSK7-type plasmid. The relationship of members of the pSK1 family of plasmids and genetic determinants have been reviewed by Skurray *et al.* (1988).

The mechanisms of homologous recombination, site-specific recombination and transposition have also played key roles in the evolution of a multiresistant chromosome in *S. aureus*. Accumulation of multiple resistances on the chromosome may have involved transposition as well as integration of a part or the whole of the plasmids carrying resistance determinants into the bacterial chromosome. This plasmid integration may be accomplished through insertion sequences/transposons that reside on both the plasmid and the chromosome, and provide regions of DNA sequence homology or sites for

recombination (Sayre and Miller, 1991; Lyon and Skurray, 1987; Cohen, 1976). Although some chromosomally specified resistance determinants may arise by mutation (*e.g.*, resistance to nalidixic acid and rifampicin; Saunders, 1984), not all chromosome-borne resistances are due to mutation. Evidence suggests that some of the resistance determinants, which were originally known to be present on plasmids, have now been accumulated on the chromosome. In the majority of recently isolated multiresistant *S. aureus* strains, the determinants for resistance to erythromycin, tetracycline, streptomycin, spectinomycin, penicillin via a β-lactamase, *etc.*, and to cadmium and mercury may be found on chromosome (Skurray *et al.*, 1988). Evidence from some more recent Australian epidemic staphylococcal strains suggests that the chromosomally located determinants for tetracycline, penicillin and mercury resistances are homologous to determinants which were plasmid-borne in isolates prior to 1970. In some cases there is evidence for transposition from plasmid to chromosome. For example, the transposons Tn*4002* (β-lactamase) and Tn*4004* (mercury resistance), which were originally found on β-lactamase/metal-ion resistance plasmids may also be found on the chromosome of the recently isolated staphylococcal strains (Skurray *et al.*, 1988; Lyon and Skurray, 1987). Although accumulation of the resistance determinants on the chromosome is disadvantageous in the way that the genes occur in a lower copy number in the population and may be less easily accessible to other bacteria, such accumulation, however, provides a greater stability to the resistance determinants in the absence of selective pressure.

1.7 Introduction to the plasmid pI9789 and its derivatives

Plasmid pI9789 is a large, naturally occurring plasmid of *Staphylococcus aureus* placed in the γ-family of heavy-metal-resistance plasmids (Shalita *et al.*, 1980). It carries genes for resistance to ions, such as, cadmium, arsenate, arsenite, mercury, bismuth and lead. Also, it has acquired resistance to β-lactam antibiotics through acquisition of the β-lactamase transposon, Tn*552*, (carrying an α-family *bla* determinant) from the chromosome during storage (Rowland, 1989), the plasmid now is called pI9789::Tn*552* (abbreviated to pS1 parent).

Johnston and Dyke (1971) mutagenised pI9789::Tn*552* with EMS and isolated a mutant of the plasmid which is thermosensitive in replication and is called pI9789::Tn*552seg*-1 (abbreviated to pS1). This plasmid does not replicate at 42°C so that a strain originally containing this plasmid is unable to form colonies at 42°C on an appropriate selective medium (*e.g.*, containing cadmium acetate). Johnston and Dyke (1971) grew phage 80 on the strain containing the plasmid pS1 and irradiated the phage preparation with UV-light prior to its use in transduction. One cadmium-resistant transductant carries a plasmid that has deletions in pS1 and was labelled pI9789::Tn*552seg*-1ΔD (abbreviated to pΔD). The sequence of pΔD (Rowland, 1989) revealed that this plasmid has two closely placed deletions rather the one suggested by Browne (1985). One 3,225bp deletion removes nearly half of the terminal inverted repeat of the transposon, Tn*552*, and extends to just before the *res* site which is located inside the transposon (called *resR*), removing *blaI*, *blaRI* and *blaZ* but retaining the *inv* region with two flanking *res* sites (*resL* and *resR*). A second deletion of 671bp, just outside the transposon, removes *binR* - a putative site-specific recombinase on the plasmid pI9789. The relationships

between the various derivatives of the plasmid pI9789 are shown in Figs.1.1 and 1.2. For a more detailed account of the plasmid pI9789 and its derivatives see Rowland (1989).

1.8 Previous work

Johnston (1971) observed that on plating out 10^6 colony forming units of the PS80N strain of *S. aureus* containing the plasmid pS1, on average 33 colonies grew on cadmium acetate-containing agar plates (at 13.3μg/ml) at 42°C after 18hrs. This suppression of the thermosensitive replication was suggested to operate largely by integration of the plasmid pS1 into the host chromosome, thereby relying on the chromosomal thermostable replication system. Johnston (1971) was able to transduce Cd^r from any one of these 33 colonies to a new PS80N host and show that the resulting transductant contained the Cd^r determinant-carrying plasmid that was still thermosensitive in replication and indistinguishable from pS1. The thermostability was, therefore, due to suppression and not reversion. The frequency of the suppression was enhanced greatly when a gene conferring erythromycin resistance was present on the host chromosome. He also showed co-transduction of Cd^rEm^r phenotypes from such temperature-stable revertants and reasoned that this co-transduction was the result of imprecise excision of the integrated pS1 from the chromosome during transduction. This argument was weakened by the subsequent finding that the Em^r is on a transposon, Tn*551*, (Pattee *et al.*, 1977; Khan and Novick, 1980) and so the co-transduction could have been a result of transposition and not of imprecise excision (Warne, 1982). This, however, suggests that the structure of the chromosome is also important in the suppression phenomenon. The plasmid pΔD showed

Fig.1.1 Schematic representation of the relationships of pI9789 and its derivatives plasmids, pS1 parent, pS1 and pΔD. The plasmid pS1-parent has Tn*552* inserted at site '**E**' and the plasmid pS1 was generated by deletion of Tn*552* followed by insertion at site '**F**'. Two closely placed deletions, one in the *blaZ* region and the other in the *binR* region give rise to the plasmid pΔD (after Rowland, 1989 and Browne, 1985). *binL, binR* and *sin* are the genes for three putative site-specific recombinases. The transposon, Tn*552*, is flanked by two terminal inverted repeat structures (TIR_R and TIR_L) and carries the genes for resistance to β-lactam antibiotics. The region flanked by *resL* and *resR* can invert.

Fig.1.1

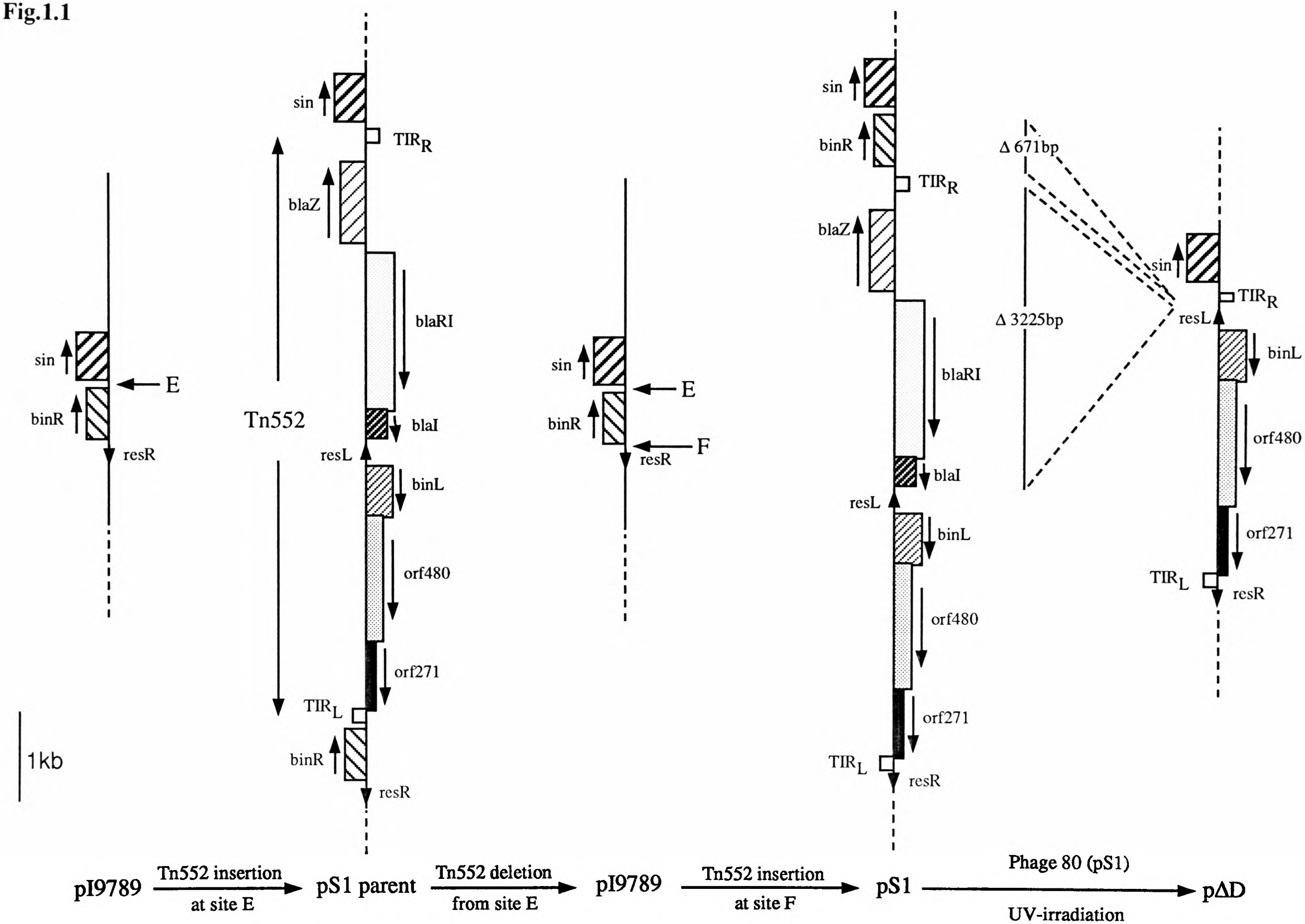
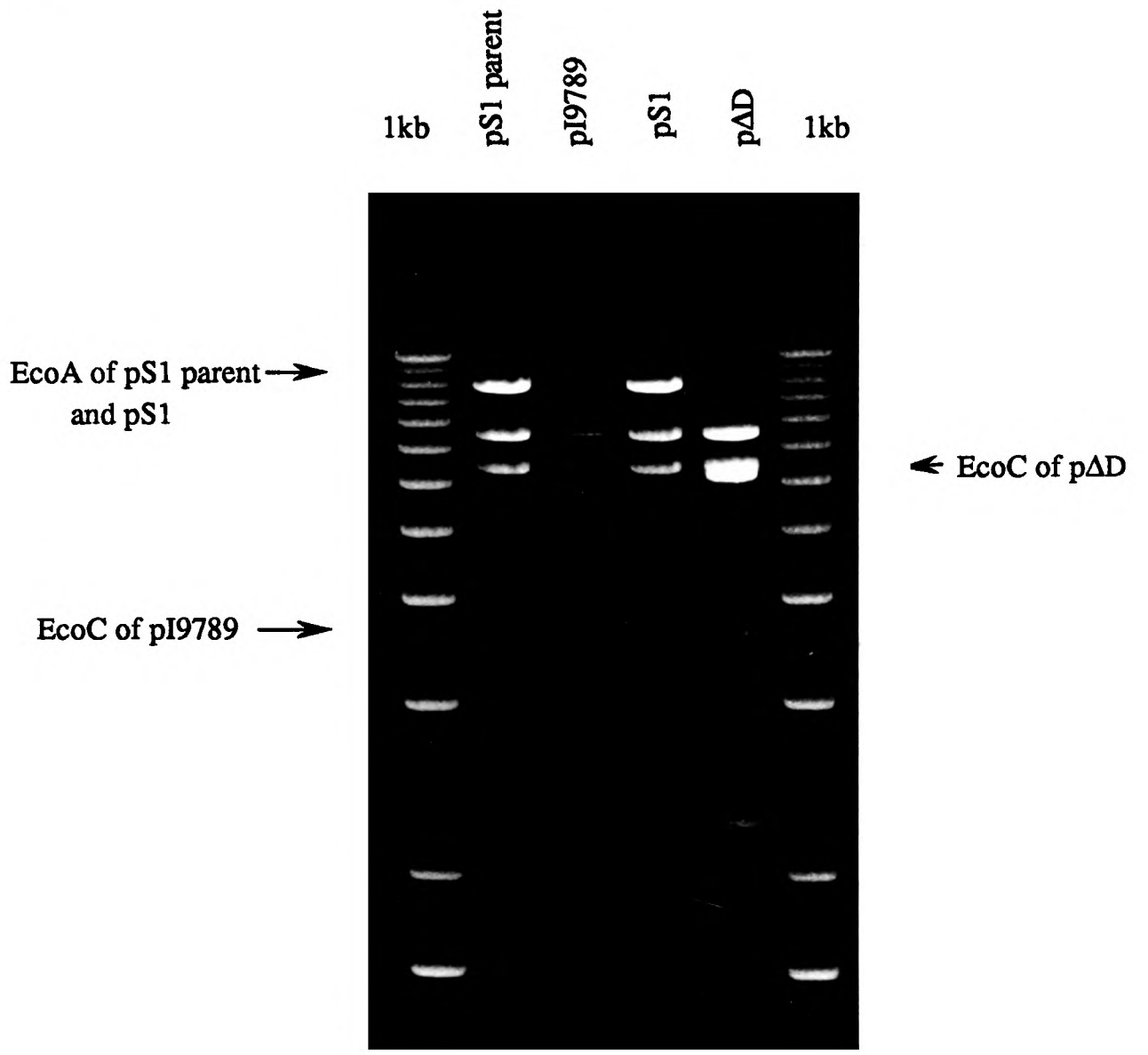

sin
E
binR
resR
Tn552
sin
TIR$_R$
blaZ
blaRI
blaI
resL
binL
orf480
orf271
TIR$_L$
binR
resR
1kb
sin
E
binR
F
resR
sin
binR
TIR$_R$
blaZ
blaRI
blaI
resL
binL
orf480
orf271
TIR$_L$
resR
Δ 671bp
Δ 3225bp
sin
TIR$_R$
resL
binL
orf480
orf271
TIR$_L$
resR
pI9789
Tn552 insertion
at site E
pS1 parent
Tn552 deletion
from site E
pI9789
Tn552 insertion
at site F
pS1
Phage 80 (pS1)
UV-irradiation
pΔD

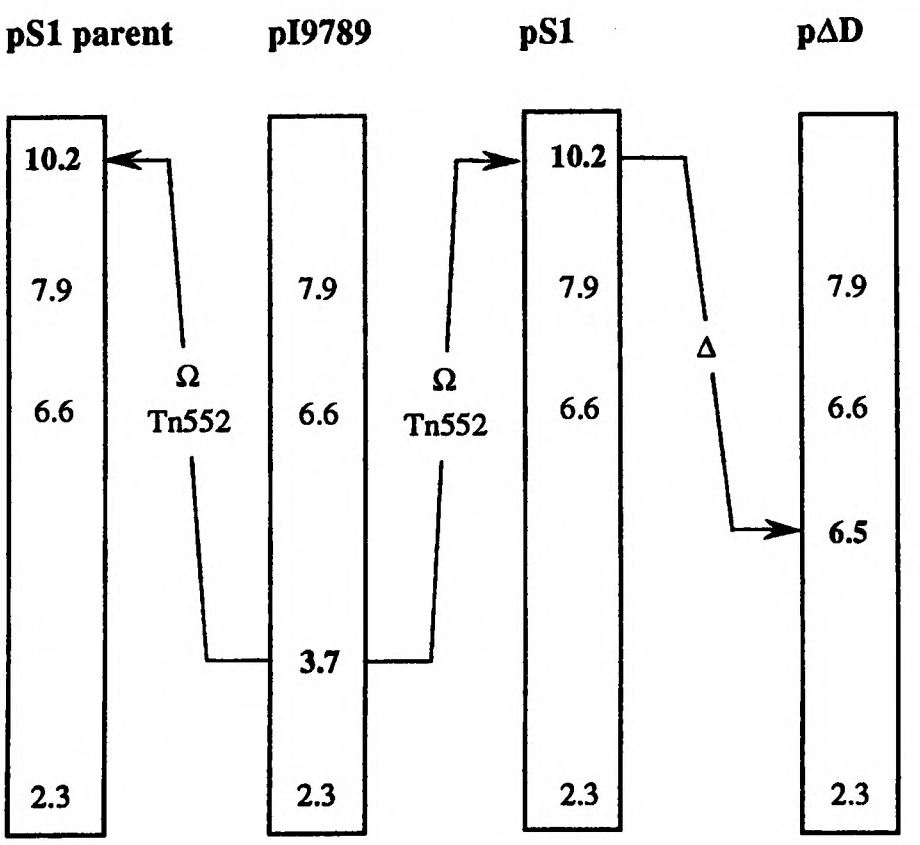
Fig.1.2 The gel shows *Eco*RI digests of the plasmid pI9789 and its derivatives pS1 parent, pS1 and pΔD. The 3.7kb *Eco*C fragment of pI9789 gives rise to the 10.2kb *Eco*A fragment of both pS1 parent and pS1 on Tn*552* insertion at two differet locations (see Fig.1.1). The *Eco*A fragment of pS1 gives rise to the 6.5kb *Eco*C fragment of the plasmid pΔD on deletion. The relationships of these fragments are indicated in the diagram.

(KEY: Δ = deletion; Ω = insertion)

Fig.1.2



Fragment sizes:



a lower frequency of thermosensitivity suppression (9 colonies on average for pΔD compared to 33 for pS1). Johnston (1971) regarded pΔD as "integration deficient". Warne (1982) carried out preliminary mapping of the plasmids pS1 and pΔD using *Eco*RI and *Bgl*II restriction enzymes. Johnston's integration hypothesis was investigated further by Browne (1985) who isolated a number of pS1 plasmid-derivatives that had been forced to integrate into the host chromosome and subsequently been transduced to a new host. She found that, whenever different from the original plasmid, the difference always resided in the *inv* or *blaZ* region. Browne (1985) also reported that the insertion of the erythromycin transposon, Tn*551*, into pS1 was always site- and orientation-specific but that insertion into pΔD was random. The specific site of insertion of Tn*551* in pS1 was shown to be at or near to *res*R. These analyses were based on restriction enzyme mapping (Browne, 1985). As yet, no direct evidence has been presented in support of the integration hypothesis.

The available evidence suggests the involvement of the β-lactamase transposon Tn*552* and the flanking region in the thermosensitivity suppression phenomenon. The transposon Tn*552* and some of its flanking regions have been sequenced by Rowland (1989). The presence of the two putative site-specific recombinase genes (*binR* and *sin*) on one side of the transposon and a *res* site on the other side suggest that this region has been a hot-spot for transposition and that these structures possibly are remains of previous transposition events. Understanding the role of such regions of DNA is not only important from the evolutionary viewpoint of the antibiotic resistances among clinically significant bacteria, but also, form the viewpoint of the evolution of the genomes in general.

1.9 The Aims of research

The aims of the present study were to investigate:

(1) The role played by the host chromosome in the suppression phenomenon or "integration" and to find direct evidence for the integration of pI9789 into the host chromosome.

(2) The role of Tn*552* and its flanking region in the suppression phenomenon.

(3) The role of site-specific recombination in the *intra*cellular movement of genes and plasmids with particular reference to the spread of antibiotic resistances in this organism. Such studies would be important, in the long term, to develop methods to prevent the spread of resistance to an antibiotic when a new antibiotic is introduced into clinical environment.

Chapter Two:

Materials and Methods

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2.1 Materials

2.1.1 Chemicals

Analytical grade or molecular biology grade chemicals were used. All chemicals were purchased from Sigma (Dorset), BDH Ltd. (Poole), Oxoid Ltd. (Basingstoke), Calibiochem Corporation (La Jolla, USA), Life Technologies Ltd. (Renfrewshire), Fluka Chemicals Ltd. (Derbyshire) or Rose Chemicals Ltd. (UK). SEAPLAQUE® GTG® agarose and NUSIEVE® GTG® agarose were from FMC BioProducts (Rockland, USA).

2.1.2 Radioactivity

[α^{35}S] dATPαS (37TBq/mmol) was obtained from Amersham International plc., Aylesbury, UK.

2.1.3 Antibiotics

Tetracycline (hydrochloride) was obtained from Cyanamid Ltd. (Gosport, Hants.). All other antibiotics were obtained from Sigma.

2.1.4 Enzymes

Lysostaphin (from *S. staphylolyticus [9011-93-2]*), lysozyme (from chicken egg whites, cat# L-6876) and bovine albumin (fraction V powder, cat# A-7906; 5% solution, cat# A-4628) were obtained from Sigma. T4 DNA Ligase, bovine pancreatic ribonuclease A, ribonuclease T1 (from *Aspergillus oryzae*), proteinase K were from Boehringer-

Mannheim. Klenow fragment was from Pharmacia Ltd., Milton Keynes, Bucks. $Vent_R^{TM}$ (exo^-) DNA Polymerase was bought from New England Biolabs Ltd. (Hertfordshire).

2.1.5 Restriction endonucleases

These were obtained from Amersham International, Bethesda Research Laboratories (UK) Ltd. (Cambridge), New England Biolabs Ltd. Pharmacia, Promega Corporation (Southampton), Stratagene Ltd. (Cambridge) or Northumbria Biologicals Ltd. (Cramlington).

2.1.6 Kits

2.1.6.1 DNA sequencing kits

Either SEQUENASE® Version 2.0 DNA Sequencing Kit form United States Biochemical Corporation (Ohio, USA) or CircumVent™ Thermal Cycle Dideoxy DNA Sequencing Kit from New England Biolabs Ltd. was used.

2.1.6.2 DNA purification kits

Magic™/Wizard™ Minipreps DNA Purification System and Magic™/Wizard™ PCR Preps DNA Purification System were purchased from Promega Corporation. Strataclean™ Resin was from Stratagene Ltd.

2.1.6.3 Southern blotting kits

Either Flash™ Detection System (Stratagene Ltd.) was used, in which case probe was labelled using Flash™/Prime-it™ Random Primer Labelling Kit (Stratagene Ltd.) or ECL™ random primer labelling and detection systems form Amersham International plc.

(Buckinghamshire). In both cases neutral Flash™ Nylon Membrane from Stratagene Ltd. was used.

2.1.7 X-ray films

These were bought from Eastman Kodak Company (Rochester, New York, USA).

2.1.8 DNA

E. coli plasmid vectors pUC18, pBR322, pTZ19R and *E. coli* - *S. aureus* shuttle vector pOX7 were all gifts from S.P. Curnock. *Sma*I digested and bacterial alkaline phosphatase treated pUC18 DNA was purchased from Pharmacia. M13 Universal and Reverse sequencing primers were both obtained from Pharmacia Ltd. All other oligonucleotides were obtained from V. Cooper, Dyson Perrins Laboratory, Oxford. A list of oligos used in this study is given in Table 2.1.

One kb DNA ladder was from Bethesda Research Laboratories Inc. Raoul™ DNA ladder was obtained from Appligene, France.

2.1.9 Media

2.1.9.1 2 x TY medium (per litre)

Tryptone	16g
Yeast extract	10g
NaCl	5g
12g agar was added for solid medium	

2.1.9.2 ψ broth (pH 7.5) (per litre)

Tryptone	20g
Yeast extract	5g
$MgSO_4$	4.93g
NaCl	0.585g
KCl	0.375g

Table 2.1 List of oligonucleotides.

Name	Sequence (5' - 3')	Comment
JR551 (20-mer)	CGGTTGCGAACTGTACCACC	Chapter 5
JL551 (22-mer)	GCATGTATCTCCATTAATTCGG	Chapter 5
HIN3005 (19-mer)	CAGTAACCTTTAGGTGATG	Chapter 6
BGL3001 (24-mer)	CCCTAACGTATATCTATAAAAATC	Chapter 6
J1ECR1 (24-mer)	CCATTGTCGACTCATCATGCAAGC	Chapter 7
J2BGL2 (21-mer)	GTATTACTCACTTATTTCGGC	Chapter 7
J1OX54 (18-mer)	AAGGTTAACTCGCGATGC	Chapter 7
J2OX54 (19-mer)	CAATGTTGGGACTAGTTAG	Chapter 7

2.1.9.3 CY medium (per litre)

Yeast extract	10g	Trace elements contained (per litre)-	
Casein hydrolysate	10g	$ZnSO_4.7H_2O$	2.5g
$MgSO_4.7H_2O$	0.25g	$FeSO_4.7H_2O$	2.5g
trace element solution	40ml	$MnCl_2.4H_2O$	1.0g
12g agar was added for solid		$CuSO_4.5H_2O$	2.5g
medium (and 6g for soft agar)		conc. HCl	50ml

2.1.9.4 Glucose minimal agar (per litre)

10 x M9 salt solution	100ml	10 x M9 salts contained (per litre)-	
1M $MgSO_4$	1ml	Na_2HPO_4	60g
0.1M $CaCl_2$	1ml	KH_2PO_4	30g
1M thiamine HCl	1ml	NH_4Cl	10g
20% glucose	10ml	NaCl	5g
minimal agar	15g		

2.1.9.5 DM3 agar (per litre)

Solutions were made separately, autoclaved and mixed before use. Glucose and bovine albumin solutions were filter sterilized.

4% agar	200ml
1M sodium succinate (pH 7.3)	500ml
5% casein hydrolysate	100ml
10% yeast extract	50ml
3.5% K_2HPO_4 + 1.5% KH_2PO_4	100ml
1M $MgCl_2.6H_2O$	20ml
20% glucose or 20% glycerol	25ml
2% bovine serum albumin	5ml

2.1.9.6 1 x HB (pH 6.5)

Sucrose	0.7M
Maleic acid	0.02M
$MgCl_2$	0.02M

2.1.9.7 HB/TBAB

1 x HB	1000ml
Tryptose blood agar base	16.5g

2.1.9.8 HBM

1 x HB	1000ml
Penassay broth powder	35g

2.1.9.9 40% PEG for *S. aureus* transformation

Polyethylene glycol 6000	8g
2 x HB	10ml
H_2O	3.6ml

2.1.10 Additions to media

Addition	Stock solution	Final concentration
Ampicillin	50mg/ml in H_2O (+0.1%NaOH)	50µg/ml
Erythromycin	100mg/ml in 50% ethanol	100 - 200µg/ml for *E. coli* 20 - 50µg/ml for *S.aureus*
X-gal	2% (w/v) in DMF	0.04% (w/v)
IPTG	40mg/ml in H_2O	40µg/ml
Cadmium acetate	26.6mg/ml in H_2O	26.6µg/ml for *S.aureus*
$CaCl_2$	0.4M	0.04M (to CY-agar)
Starch		0.045% (w/v, to CY-agar)

2.1.11 Buffers and chemical solutions

2.1.11.1 10 x TBE

Tris-HCl pH 8.3	500mM
Boric acid	500mM
EDTA	100mM

Stored at room temperature

2.1.11.2 10 x TAE

Tris	400mM
Sodium acetate pH 8.0	200mM
EDTA	10mM

Stored at room temperature

2.1.11.3 Lysis buffer

Glucose	50mM
EDTA	10mM
Tris-HCl pH 8.0	25mM

Stored at room temperature

2.1.11.4 Alkaline lysis solution

NaOH	200mM
SDS	1% (w/v)

Stored short term at room temperature

2.1.11.5 High-salt buffer

5M potassium acetate	60ml
Glacial acetic acid	11.5ml
H_2O to	100ml

Solution is 3M with respect to K^+ ions and 5M with respect to acetate ions.

2.1.11.6 Phenol

Phenol (chromatography grade) was saturated with TE buffer and contained a few grains of 8-hydroxy quinoline to prevent oxidation.

2.1.11.7 Phenol/chloroform

Phenol, chloroform and iso-amyl alcohol were mixed in a ratio of 1:1:1/24 (v/v), respectively.

2.1.11.8 10 x TE buffer (pH 8.0)

Tris-HCl pH 8.0	100mM
EDTA	10mM

2.1.11.9 Enzyme stock solutions

i- Proteinase K was dissolved in 1 x TE buffer (pH 8.0) at 10mg/ml, incubated at 37°C for 1 - 2 hours and stored at -20°C.

ii- Pancreatic RNAase A was dissolved in 10mM Tris-HCl (pH 7.5), 15mM NaCl at 10mg/ml, incubated at 100°C for 15 minutes and stored at -20°C.

iii- Lysozyme was dissolved in distilled water at 10mg/ml and stored at -20°C.

iv- Lysostaphin was dissolved in distilled water at 10mg/ml and stored at -20°C.

2.1.9.10 CTAB solution (50ml)

NaCl	2.05g
Cetyl trimethyl ammonium bromide	5g

Dissolved in distilled water at 65°C to final volume of 50ml

Stored at room temperature

2.1.11.11 Bacterial turbidity standards

E. coli OD_{600nm}	= 1 = ~8 x 10^8 cells per ml
S. aureus OD_{675nm}	= 1 = ~5 x 10^8 cells per ml

2.1.11.12 *E. coli* transformation solutions (all stored at 4°C)

Solution 1

10ml of each of the following stock solutions:

NaCl	50mM
$MnCl_2$	500mM
CH_3COONa, pH 5.6	100mM

were mixed and made upto 100ml with distilled water.

Solution 2

$CaCl_2$	70mM
$MnCl_2$	100mM
CH_3COONa, pH 5.6	100mM

TFB I (pH 5.8)		*TFB II (pH 6.8)*	
NaCl	30mM	RbCl	10mM
RbCl	100mM	MOPS	10mM
$MnCl_2$	50mM	$CaCl_2$	75mM
$CaCl_2$	10mM	glycerol	15% (v/v)
glycerol	15% (v/v)		

2.1.11.13 Phage buffer (per litre)

Sodium β-glycerophosphate	18.9g
$MgSO_4.7H_2O$	0.25g
NaCl	5g
Gelatin	1g

2.1.11.14 20 x SSC (per litre)

NaCl	175.3g
NaCitrate, trisodium salt	88.2g

Adjusted pH to 7.0 with HCl

Stored at room temperature

2.1.11.15 20 x SSPE (per litre)

NaCl	174g
$NaH_2PO_4.H_2O$	31.74g
EDTA	7.4g
5M NaOH	13ml

Adjusted pH to 7.4 with alkali

Stored at room temperature

2.1.11.16 Southern blot depurination solution

HCl	0.25M

Stored at room temperature

2.1.11.17 Southern blot denaturation solution

NaCl	1.5M
NaOH	0.5M

Stored at room temperature

2.1.11.18 Southern blot neutralization solution

NaCl	1.5M
Tris-HCl pH 8.0	1M

Stored at room temperature

2.1.11.19 Acrylamide sequencing gel (per 200ml)

40% (w/v) acrylamide*	30ml
Urea	84g
10 x TBE	20ml

*Obtained from National Diagnostics Inc.

Filtered through a Whatman No.1 and stored at room temperature

2.1.11.20 Gel loading buffer (for agarose gel electrophoresis)

Ficoll	20% (w/v)
Xylene cyanol FF	0.025% (w/v)
Bromophenol blue	0.025% (w/v)

2.1.11.21 Bacteriophages

Staphylococcus phages 53 (Group III) and 80 (Group I) of the International Phage Typing Set (Sneath *et al.*,1986) were used for transduction.

2.1.11.22 Bacterial strains and plasmids

A list of *E. coli* strains is given in Table 2.2 and of *S. aureus* strains in Table 2.3. The letter 'N' following the *S. aureus* strain number indicates the absence of a known plasmid in that strain. Plasmids are listed in Table 2.4.

Table 2.2 List of *E.coli* strains

Strain	Genotype	Remarks	Reference
JM107	*supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*) F' [*traD*36 *proAB*$^+$ *lac*^{q} *lacZ*ΔM15]	host for pUC, pTZ and pOX7 clones	Sambrook *et al.*, 1989
DH5α	*supE*44 Δ*lacU*169 (ϕ80*lacZ*ΔM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	host for pUC clones	Sambrook *et al.*, 1989

Table 2.3 List of *S. aureus* strains

Strain	Remarks	Reference
RN4220	modified NTCC 8325N strain which acts as an acceptor of *E. coli* DNA	Kreiswirth *et al.*, 1983
PS9789	chromosome carries genes involved in β-lactamase production	Asheshov, 1966
PS80N	β-lactamase negative derivative of PS9789	Johnston and Dyke, 1971
PS80::Tn*551*	PS80N with Tn*551* (*erm*) on the chromosome; also called PS80*d.ero*	Johnston, 1971
1054	propagating strain of phage 53 and 80	Dyke and Richmond, 1967

Table 2.4 List of plasmids

Plasmid	Remarks	Reference
Staphylococcal plasmids:		
pI9789	genes for resistance to cadmium, arsenate and mercury	Asheshov, 1969
pI9789::Tn*552* (pS1 parent)	derivative of pI9789 with genes for β-lactamase production	Asheshov, 1969
pI9789::Tn*552seg*-1 (pS1)	derivative of pI9789::Tn*552* with temperature sensitive replication	Johnston, 1971
pI9789::Tn*552seg*-1ΔD (pΔD)	β-lactamase negative deletion derivative of pS1	Johnston, 1971
pBB33	derivative of pS1 with Tn*551* insertion	Browne, 1985
pTd1	derivative of pS1 with Tn*551* insertion	Browne, 1985
pDB51	derivative of pS1 with Tn*551* insertion	Browne, 1985
pRA1	derivative of pΔD with Tn*551* insertion	Browne, 1985
pRDII	derivative of pΔD with Tn*551* insertion	Browne, 1985
pRE56	derivative of pΔD with Tn*551* insertion	Browne, 1985
pWE180	confers Ap^r in *E.coli* and Em^r in *S. aureus*; replicates in *E. coli* but not in *S. aureus*	Walters, 1991
pOX1054	cryptic plasmid of the strain 1054	This study
E. coli plasmids:		
pBR322	*tet, amp*	Sambrook et al.,1989
pTZ18R	*amp*	Sambrook et al.,1989
pTZ19R	*amp*	Sambrook et al.,1989
pUC18	*amp*	Sambrook et al.,1989
E. coli - S. aureus shuttle plasmid:		
pOX7	confers Ap^r in *E. coli* and Em^r in *S. aureus*; replication is temperature sensitive in *S. aureus*	Curnock (Personal communication)

2.2 Methods

2.2.1 Maintenance and growth of bacterial cultures

Bacterial strains used routinely were maintained on plates containing appropriate antibiotic or metal ion. *E. coli* cultures were grown on 2 x TY agar plates and *S. aureus* cultures on CY agar plates. Plates were incubated overnight at 37°C (or at 30°C for cultures containing a plasmid that is temperature sensitive for replication), stored at 4°C and renewed every 4-5 weeks. A stock of all bacterial strains was maintained at -70°C. The stocks were prepared by mixing 1ml of an overnight culture with an equal volume of sterile 25%glycerol in appropriate broth (2 x TY-broth for *E. coli* and CY-broth for *S. aureus*). The 2ml sample was divided into two sterile screw-capped vials and stored at -70°C.

Solid culture: Plates were used to store, screen and select bacterial strains and were inoculated using a sterile wire loop, sterile tooth-picks or by spreading 100 - 200µl of liquid culture with a sterilized glass spreader. Plates were inverted and incubated overnight at 37°C or 30°C, as appropriate.

Liquid culture: A sterile flask or test-tube containing broth was inoculated by transferring either a single colony (or a sample of thick growth) from a plate or an aliquot of liquid culture, and was shaken vigorously at 37°C or 30°C.

Bacterial lawn: To make a lawn of bacteria, an overnight culture was diluted 1/100 in 2 x TY or CY broth. The diluted culture was then poured onto the agar plate and the excess liquid removed. The plates were dried in a laminar flow hood for 1/2hr before use.

2.2.2 Sterilization

Solutions, media, microfuge tubes and plastic tips were autoclaved at 121°C, 15lb/in^2 for 20 minutes (10lb/in^2 for 10 minutes for solutions containing sugar). Glassware

was heated at 160°C for 1hr. Solutions of antibiotics, bovine serum albumin, lysozyme, lysostaphin and others which would be damaged by autoclaving were filter sterilized through a 'Millipore' filter (0.45µm pore size).

2.2.3 Small-scale preparation of plasmid DNA from *E. coli*

The method described in Sambrook *et al.* (1989) was modified as below:-

Cells were harvested in a sterile microfuge tube from 1.5ml of an overnight culture, grown in 2 x TY broth with aeration and appropriate antibiotic, by centrifuging at 12,000g for 1min. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended in 200µl of lysis buffer by vigorous vortexing. To this bacterial suspension, 200µl of freshly prepared alkaline lysis solution was added and the contents of the tube were mixed by inverting the tube a number of times till the liquid was nearly clear. A 200µl volume of the high salt buffer was added to this tube and the contents of the tube were mixed by gentle inversions. The tube was then centrifuged at 12,000g for 5min to pellet the debris. The supernatant was transferred to a fresh sterile microfuge tube and extracted with 400µl of phenol/chloroform. Plasmid DNA was precipitated by adding 1ml of ice-cold absolute ethanol and incubating at room temperature for 5min, and then pelleted by microfuging for 5min at 12,000g. The pellet was rinsed with 1ml of ice-cold 70% (v/v) ethanol. DNA was resuspended in 50µl of sterile distilled water. This DNA was sufficiently clean for digesting with restriction enzyme. For the preparation of plasmid DNA of high-grade purity for DNA sequencing, cloning, *etc.*, either the 'Magic™/Wizard™ Minipreps DNA Purification System' kit of Promega Corporation was used, or the large-scale method, as described in the following section, was used. The kit was used according to manufacturer's instructions.

2.2.4 Large-scale preparation of plasmid DNA from *E. coli*

The method of Sambrook *et al.* (1989) was used. A 100ml overnight culture, grown in 2 x TY broth with the appropriate antibiotic was centrifuged (7,000rpm, 10min, JA-14 rotor, Beckman J2-21 centrifuge) and the cell pellet was resuspended in 4ml of lysis buffer by vortexing. To this 10mg of lysozyme was added and mixed by vortexing for a few seconds. This was incubated at room temperature for 15min, then 4ml of freshly prepared alkaline lysis solution was added and mixed by gentle inversions. After incubation at 4°C for 15min, 4ml of the high salt buffer was added to the lysate and this was mixed by inversions, followed by incubation at 4°C for another 15min. Cell debris was pelleted by centrifugation (19,000rpm, 20min, JA-20 rotor, Beckman J2-21 centrifuge). Plasmid DNA was precipitated by addition of 0.6vol of isopropanol to the supernatant and incubation at room temperature for 30min and pelletted by centrifugation (10,000rpm, 20min, JA-20 rotor, Beckman J2-21 centrifuge). The DNA pellet was rinsed with 70% (v/v) cold ethanol, dried and resuspended in 4ml TE buffer. To this 4g CsCl and 200µl of ethidium bromide (10mg/ml) was added. The solution was sealed in a 4ml Beckman polyallomer 'Quick-seal' tube and centrifuged at 45000rpm for 16hrs at 19°C (VTi65.2 rotor, Beckman L7 ultracentrifuge). The lower (plasmid) band, visualized under UV-light (or sometimes visible even without UV-light), was removed by puncturing the tube with a syringe needle. Ethidium bromide was removed by 3-4 extractions with CsCl-saturated isopropanol and CsCl by exhaustive dialysis at 4°C against 2mM Tris-HCl, pH 8.0. Plasmid DNA was re-precipitated by addition of 0.1vol of 3M sodium acetate, 2.5vol of cold ethanol, incubation at -20°C for 2hr and pelleted by centrifugation (10,000rpm, 20min, JA-20 rotor, Beckman J2-21 centrifuge). Plasmid DNA was resuspended in 200ml of sterile distilled water and stored at 4°C.

2.2.5 Small-scale preparation of plasmid DNA from *S. aureus*

The method described in Section 2.2.3 was used with a few modifications as below:-

i- After the addition of lysis buffer and resuspension of the cell pellet, 7µl of lysostaphin (10mg/ml) was added to the suspension and incubated at 40°C for 7min.

ii- Phenol/chloroform extraction step was not carried out. Instead, two extractions with the StrataClean Resin (from Stratagene) were done. The resin was used according to manufacturer's instructions.

The rest of the steps were compeleted exactly as described in Section 2.2.3.

2.2.6 Large-scale preparation of plasmid DNA from *S. aureus*

The method of Novick and Bouanchaud (1971) was employed. A single colony of the required strain was inoculated into 50ml of CY broth in a 1 litre sterile flask and grown for 18hrs at 37°C or 30°C with vigorous shaking. The culture was then diluted with pre-warmed 200ml of CY broth and grown for another 90min with shaking to ensure that the culture was in mid-log phase of growth so as to optimize the action of lysostaphin. The cells were harvested by centrifugation (7,000rpm, 10min, JA-14 rotor, Beckman J2-21 centrifuge). The cell pellet was washed by resuspension in ~200ml of cold 10mM EDTA, pH 8.0. The cells were re-sedimented and resuspended in 4ml of 10mM EDTA by vortexing. Two volumes of 1:1 acetone:ethanol (8ml) were added and the cells were held on ice for 20min. Ten volumes of cold 10mM EDTA, pH 8.0, (80ml) were added and the cells were held on ice for a further 20min. The cells were centrifuged (7,000rpm, 10min, JA-14 rotor, Beckman J2-21 centrifuge) and acetone/ethanol was removed with air. The pellet was resuspended in 16ml of 0.1M NaCl,0.05M EDTA, pH 7.0, to give a cell density

of ~3 x 10^{10} bacteria/ml. Lysostaphin was added to give a final concentration of 15mg/ml and the suspension dispensed into 50ml screw-cap polypropylene tubes suitable for the JA-20 rotor, Beckman J2-21 centrifuge. The sample was then incubated at 37°C for 1hr. After lysis the following three solutions were added and the tube was inverted gently after each addition.

5M NaCl	2.85ml
0.5M EDTA, pH 8.0	1.85ml
2% (w/v) SDS in 0.7M NaCl	17ml

The samples were then kept at 4°C overnight. Unbroken cells, protoplasts, membranes and a substantial quantity of chromosomal DNA were sedimented by centrifugation (19,000rpm, 45min, JA-20 rotor, Beckman J2-21 centrifuge). The supernatant was retained and and treated with heat-treated (80°C for 30min, - in order to destroy DNAase activity) pancreatic RNAase A at final concentration of 5µg/ml and T1 RNAase at a final concentration of 1u/ml and incubated at 37°C for 1hr. Proteinase K was then added to a final concentration of 50µg/ml and the sample incubated at 37°C for 1hr. DNA was precipitated by adding 4ml of 3M sodium acetate, 80ml of ice-cold absolute ethanol and incubation in an ethanol/dry ice bath. The plasmid DNA was sedimented by centrifugation (10,000rpm, 20min, JA-14 rotor, Beckman J2-21 centrifuge) the pellet resuspended in 3.2ml of 0.1M NaCl, 0.05M EDTA, pH 7.0. To this was added 2.97g of Caesium chloride and 200µl of ethidium bromide (10mg/ml). The solution was sealed in 4ml Beckman polyallomer 'Quick-seal' tube and centrifuged at 45000rpm for 16hrs at 19°C (VTi65.2 rotor, Beckman L7 ultracentrifuge). The procedure was completed exactly as in Section 2.2.4.

2.2.7 Large-scale isolation of total DNA from *S. aureus*

The method of De Buyser *et al.* (1989) was used with some modifications:

Cells, harvested from 8ml of an overnight broth culture (7,000 rpm, 10min, JA-20 rotor, Beckman centrifuge JA14-21), were resuspended in 4ml TE (containing heat treated pancreatic RNAase A at 10µg/ml) and 18µl of lysostaphin (10mg/ml) was added. The mixture was incubated at 37°C for 30min with occasional gentle shaking. To this were added 210µl of 10% SDS (w/v) and 15µl of proteinase K (10mg/ml) and incubated for another 30min at 37°C. Seven hundred µl of 5M NaCl and 560µl of CTAB solution were added to this and the sample was incubated at 65°C for 10min. The resultant lysate was cooled slowly and extracted twice with phenol/chloroform and once with chlorofom/isoamyl alcohol (24/1, v/v). DNA was precipitated with 0.6vol of isopropanol at 4°C for 2hrs and pelleted by centrifugation (12,000 rpm, 20min, JA-20 rotor, Beckman centrifuge JA14-21). The DNA pellet was rinsed with cold 70% ethanol (v/v) and dissolved in 400µl of sterile distilled water for 2hrs at 4°C.

2.2.8 Small-scale isolation of total DNA from *S. aureus*

The method of De Buyser *et al.* (1989) was modified (this study):

Cells were harvested from 1.5ml of an overnight culture and resuspended in 400µl of TE buffer (containing heat-treated pancreatic RNAase A at 10µg/ml). Six µl of lysostaphin (10mg/ml) was added and the mixture incubated at 37°C for 7min. This was followed by addition of 20µl of 10% SDS (v/v), 2µl of proteinase K (10mg/ml) and another incubation at 37°C for 7min. Seventy µl of 5M NaCl and 56µl of CTAB solution were then added and the mixture incubated at 65°C for 3min. After incubation, the sample was cooled slowly and extracted twice with phenol/chloroform and once with

chloroform/isoamyl alcohol (24/1, v/v). DNA was precipitated with 0.6vol of isopropanol at room temperature for 15min, pelleted by centrifugation at 12,000g for 10min and washed with cold 70% ethanol (v/v). The DNA pellet was suspended in 50µl of sterile distilled water.

2.2.9 Transformation of *S. aureus*

The method of Lindberg (1981) was used as below:-

A single colony was used to inoculate 20ml of CY broth and grown at 30°C/37°C. Bacteria were harvested by centrifugation (7,000rpm, 10min, JA-20 rotor, Beckman J2-21 centrifuge) at room temperature and resuspended in 10ml of HBM. To 10ml of HBM, separately, were added 40µl of lysostaphin stock (10mg/ml) and 40mg of lysozyme (2mg/ml final). This medium filter-sterilized and added to the cell suspension to give a final volume of 20ml. The sample was incubated at 37°C for 2 - 2½hrs. The un-protoplasted cells were pelleted by centrifugation (7,000rpm, 10min, JA-20 rotor, Beckman J2-21 centrifuge, 4°C) and the protoplasts-containing supernatant retained. Protoplasts were then harvested by centrifugation at 19,000rpm (JA-20 rotor, 20min, Beckman J2-21 centrifuge, 4°C) and gently resuspended in 1ml of HBM. Two hundred ml of this suspension was mixed with ~100ng of DNA in a 5ml volume and 20ml of HBM. To this was added 1.8ml of 40% PEG6000 or PEG8000 and incubated at room temperature for two minutes. Ten ml of HBM was added to this and the protoplasts pelleted at 19,000rpm (JA-20 rotor, 30min, Beckman J2-21 centrifuge, 4°C). The protoplasts were resuspended in 500µl of HBM and plated onto DM3 plates. Samples of untransformed protoplasts were also spread onto DM3 and CY plates. The plates were incubated at 30°C for 3hrs before 5ml of a soft TBAB/HB agar overlay, containing the appropriate antibiotic (at 5 x 'normal' plate concentration), was

poured onto the plate and allowed to set. Incubation was at 30°C/37°C for 5 - 7 days, by which time colonies were visible under the soft agar overlay.

2.2.10 Transformation of *E. coli*

Transformation of *E. coli* was performed by either of the following two methods:

1. Method-I This was a modification of the method of Fort and Piggot (1984). A single colony was used to inoculate 2 x TY medium. After overnight growth the culture was diluted 1:100 in pre-warmed fresh 2 x TY medium (5ml per transformation) and grown for a further 100min. The cells were harvested by centrifugation (7,000rpm, 10min, JA-20 rotor, Beckman J2-21 centrifuge, room temperature). The pellet was chilled on ice, resuspended in 100µl of ice-cold 'Transformation Solution 1' and incubated on ice for 20min. The cells were transferred to a cold microfuge tube, pelleted by microfuging for 30sec and resuspended in 100µl of ice-cold 'Transformation Solution 2'. Competent cells were added to 10 - 50ml of pre-chilled DNA solution. The sample was incubated on ice for 40min and then transferred to 42°C for three minutes. After the heat shock, the cells were transferred back to ice and 1ml of 2 x TY was added to the tube. The tube was incubated at 37°C for 30min. The cells were then pelleted by microfuging for 1min and resuspended in 200µl of 2 x TY. The transformants were plated onto selective plates.

2. Method II *E. coli* (JM107 or DH5α) were streaked onto a glucose minimal agar plate and incubated overnight at 37°C. A single colony from the plate was used to inoculate 10ml of ψ-broth and was grown at 37°C, overnight. Four ml of the overnight culture was diluted with 200ml of pre-warmed ψ-broth and grown for another $1^1/2$hr with shaking. The culture was cooled rapidly on ice, harvested by centrifugation (6,000rpm, 10min, JA-14 rotor, Beckman J2-21 centrifuge, 4°C), resuspended in 40ml ice-cold TFB I

and incubated on ice for 30min. The cells were harvested again, resuspended in 8ml of ice-cold TFB II and incubated on ice for 15min. Four hundred µl aliquots were then dispensed into pre-chilled 1.5ml screw-cap tube using pre-chilled tips and immediately frozen in a dry-ice/ethanol bath. Competent cells then could be stored indefinitely at -70°C.

Competent cells from the frozen stock were thawed on ice and 50µl were added to the DNA solution (20µl). The sample was incubated on ice for 20min and then transferred to a 42°C water bath for 3min (or 37°C for 5min). Five hundred ml of pre-warmed 2 x TY was added and incubated at 37°C for 30min. The cells were then plated onto selective agar.

2.2.11 Propagation of bacteriophages

Phages 53 and 80 were propagated by either of the following two methods:

i. **Broth propagation** To a 50ml flask were added 10ml of phage buffer, 5ml of CY-broth, $CaCl_2$ to 0.004M, 0.1ml of a 16hr-culture of the propagating strain (donor strain) and phage to a multiplicity of infection of 0.5. The mixture was incubated at 30°C with gentle shaking until complete cell lysis had occurred. Time periods taken by various phages to lyse the cell suspensions are given below:-

Phage	Strain	Time taken for complete lysis
80	PS80	7-8 hours
80	1054	7-8 hours
53	RN4220	6-7 hours
53	1054	5-7 hours

The Phage suspension was filtered through a 'Millipore' filter (0.45µm pore size) into a sterile bottle and stored at 4°C.

ii. **Soft-agar method** Soft-agar was melted and cooled to 42°C. To 10ml of the soft-agar were added 100µl of $CaCl_2$ (0.4M), 100µl of a 16hr-culture of the propagating strain (donor strain) and 200µl of the phage suspension (10^8 - 10^9 pfu/ml) and mixed

gently. The mixture was then poured onto a CY-agar plate containing $CaCl_2$ at 0.04M and the plates incubated at 30°C for 16-18hrs. The soft-agar layer was broken and mixed with 10ml of the phage buffer by vigorous vortexing. The agar and unlysed cells were pelletted by centrifugation (7,000rpm, 10min, JA-20 rotor, Beckman J2-21 centrifuge). The supernatant was filtered through a 'Millipore' filter (0.45µm pore size) into a sterile container and stored at 4°C.

2.2.12 Titration of phages

The phage preparations were titred by spotting 10µl of various dilutions (ranging from 10^{-2} - 10^{-8} in phage buffer containing 0.004M $CaCl_2$) onto a lawn of bacteria on CY-agar plates containing $CaCl_2$ at 0.04M and incubated at 30°C overnight. The bacterial lawn was prepared as follows. An overnight culture of the propagating strain was diluted 1/10 in phage buffer containing 0.004M $CaCl_2$ and poured onto the agar plate. The excess liquid was poured off and the plates were dried in a laminar flow hood for 1/2hr.

The number of plaques forming units (pfu) per ml was calculated by multiplying with appropriate dilution factor.

2.2.13 Transduction of *S. aureus*

The method described by Dyke *et al.* (1970) was modified (this study):

To a 50ml flask were added 8ml of phage buffer, 3.6ml of CY-broth, $CaCl_2$ to 0.004M and 0.4ml of an overnight culture of the recipient strain. The sample was incubated at 30°C for 1hr with shaking. A 1.3ml of this culture was dipensed into four sterile microfuge tubes. To three of these tubes 100µl, 150µl and 200µl of the phage suspension grown on the donor strain (10^8 - 10^9 pfu/ml) was added, respectively, and the fourth was kept as a no

phage control. The tubes were incubated at 30°C for 1/2hr and then microfuged to pellet the cells. The pellet was resuspended in 1ml of CY-broth containing 0.1M sodium citrate and microfuged again. This was repeated at least three time. The pellet was finally resuspended in 200ml of CY/sodium citrate and then plated onto the selective agar.

2.2.14 Agar plate test for β-lactamase production

An ovenight culture was diluted 1/100 and 5µl of this suspension was spotted onto a CY-agar plate containing 0.045% starch (w/v). The plate was incubated at 30°C/37°C overnight and then flooded with 20ml of benzyl penicillin solution (30mg/ml in sodium phosphate buffer, pH 7.0) and 5ml of iodine solution (40.6g iodine and 200g of potassium iodide per litre of distilled water) and the excess was poured off. The presence of iodine turned the starch-agar blue/black. Hydrolysis of penicillin produced penicilloic acid which reduced the iodine to iodide. The production of β-lactamase was thus indicated by the presence of white haloes around the bacterial colonies.

2.2.15 Determination of antimicrobiol and metal-ion resistance

Various resistances were detemined using filter paper discs (6mm) containing an appropriate amount of the antibiotic or metal salt. A bacterial lawn was prepared as described in the Section 2.2.12 and the plates were dried in the laminar flow hood. The discs were then palced onto the lawn of bacteria under sterile conditions. Sensitivity to an antibiotic or metal-ion was shown by a zone of inhibition after an overnight incubation of the plate at 30°C/37°C. Antibiotic discs were usually prepared at three different concentrations (i.e., 20µg, 40µg and 80µg per disc). Metal salts were used at the following concentrations:-

Metal salt	Stock solution	Volume/disc	Final amount
Arsenate, sodium salt.$7H_2O$	62.4mg/ml (0.2M)	10µl	600µg/disc
Cadmium acetate	6.6mg/ml	10µl	66µg/disc
Mercury, phenyl acetate	0.2mg/ml	10µl	2µg/disc

2.2.16 Reversion experiment

The method of Browne (1985) and Johnston (1971) was used as below:

A single colony from a one-day old selective-agar plate was used to inoculate 10ml of CY-broth and grown for 18hrs at 30°C, with aeration. One ml of this culture was dilute with 9ml of CY-broth and the OD_{675nm} measured. This sample was diluted further to give 5 x 10^6 cells per ml and 200µl were plated onto freshly prepared CY-Cd (26.6µg/ml) plates. The plates were incubated at 42°C and the number of colonies counted after 18hrs.

2.2.17 The "Disc Method"

This method was devised to test thermosensitivity of a plasmid that carries gene(s) for resistance to antibiotics or metal-ions (this study). The plasmid-containing bacterial strain was grown in CY-broth at 42°C for 18hrs with shaking. The culture was diluted to give 10^8 cells/ml in CY-broth and 500µl of this were then plated onto CY-agar plates. The plates were dried in laminar flow hood for 1/2hr and discs containing appropriate selective compound were placed onto the lawn. The plates were incubated at 42°C for 18hrs. The results were interpreted as follows. The strain carrying a plasmid that was temperature sensitive in replication gave a halo of a certain size and behaved as if sensitive, whereas, a strain carrying a temperature stable plasmid gave no halo under identical conditions. The

strains carrying plasmids, such as, pS1 or pΔD, showed resistant temperature-stable revertant colonies within the haloes.

2.2.18 Purification of sequencing and PCR oligodeoxynucleotides

The oligodeoxynucleotides, that were obtained from V. Cooper, Dyson Perrins Laboratories, were incubated overnight at 55°C, precipitated with 1/10vol of 3M CH_3COONa and 3vol of ethanol at -20°C for one hour and pelletted by microfuging for 15min at 4°C. The pellet was resuspended in 200µl deionized H_2O. The concentration was determined by measuring OD_{260nm} and applying the formula:

N = # of bases
$\varepsilon_{260} \cong 10{,}000 \times N(M^{-1}\ cm^{-1})$
Molecular weight = 330 x N

$$\frac{OD_{260}}{\varepsilon_{260}} \times 10^6 = \text{concentration } (\mu M)$$

concentration (mM) x molecular weight = concentration (ng/ml)
(reference: USB Molecular Biology Reagents/Protocols, 1992; p. 610)

The oligodeoxynucleotide solution was diluted to 100µM and 1/10 dilution was used in each sequencing or PCR reaction.

2.2.19 Restriction enzyme analysis

Plasmid DNA was digested with restriction enzymes in an appropriate volume (usually 10 - 50µl) for analysis by agarose gel electrophoresis or for cloning. Genomic DNA for cloning was digested in 100µl volume. Enzymes reactions were carried out as

recommended by the manufacturer in the buffers supplied and at recommended optimal temperature, or as described in Sambrook *et al.*(1989). For double digestions with two or more enzymes which were not active in the same buffer, either the DNA was digested with the restriction enzymes requiring the lowest salt concentration first, and then the salt concentration raised before the addition of the second restriction enzyme, or the universal restriction buffer was used. If restriction enzymes required completely incompatible buffers, phenol extraction and ethanol precipitation of DNA was performed following the first digestion and a second reaction with the next restriction enzyme was set up under optimal conditions for its digestion.

2.2.20 Agarose gel electrophoresis

Plasmid and genomic DNA were analysed by agarose gel electrophoresis. Agarose gels were prepared in TBE buffer containing 0.5µg ethidium bromide per ml as described in Sambrook *et al.* (1989). All gels were run in Scie-plas gel tank (Scie-plas Ltd., Long Itchington, UK), electrophoresis apparatus GNA-200 or GNA-100 (Pharmacia Ltd.) with a Pharmacia Electrophoresis Power Supply EPS 500/400.

BRL 1kb ladder and/or Raoul ladder were also run on all agarose gels to size DNA fragments. The DNA bands were observed and photographed on an UV-transilluminator (Ultra-violet Products Inc., California, USA). Gels were photographed through a red filter (Wratten No. 25) using an UVP vidio camera fitted with a Cosmi-car television lens (70mm) and the image printed using a Mitsubishi Video Processor model K65HM.

2.2.21 Phenol/chloroform extraction

Phenol chloroform extractions were used to remove proteins from DNA and to inactivate restriction enzymes that were not inactivated by heat. Restriction enzymes were inactivated by making the volume of completed digestion up to 200µl with distilled water and adding 200µl of phenol/chloroform, followed by vigorous shaking and then microfugation for 5min at 4°C. The top aqueous layer was removed into a fresh microfuge tube and the DNA was precipitated with ethanol.

2.2.22 Ethanol precipitation

To the DNA solution, in a microfuge tube or in a 50ml 'Corex' tube, were added 1/10vol of 3M sodium acetate and 2½ - 3vol of ice-cold absolute ethanol. The mixture was incubated at -20°C or in an ethanol/dry-ice bath for 10 - 60min. The precipitated DNA was pelletted by centrifuging the microfuge tube in a microfuge for 10 - 15min and Corex tube in a JA-20 rotor of Beckman J2-21 centrifuge at 10,000rpm for 20min. The pellet was washed with ice-cold 70% ethanol, dried and then suspended in deionized H_2O or TE.

2.2.23 Isopropanol precipitation

Ethanol was replaced with isopropanol to precipitate DNA from a larger volume of the DNA solution. Zero point six volume of isopropanol was added to the DNA solution, incubated at room temperature for 10 - 60min and then centrifuged to pellet DNA. The pellet was washed with cold 70% ethanol, dried and suspended in deionized H_2O or TE.

2.2.24 Klenow fragment 'fill-in' reaction

This reaction was used to fill in 3' recessed ends of double-stranded DNA to allow blunt-end ligations. A reaction mixture was set up using 1µg of DNA, 2µl of 10 x Klenow reaction buffer, 2µl of a solution of 0.25mM dNTPs, 1 unit of Klenow fragment and sterile H_2O was added to 20µl. After incubation at 37°C for 30min, DNA was extracted with phenol/chloroform, precipitated with ethanol and resuspended in suitable volume of deionized H_2O or TE.

2.2.25 Ligation reaction

The vector and the insert DNA to be ligated were digested with appropriate restriction enzymes which were then inactivated by heating at 65°C for 10min or by phenol/chloroform extraction. Before setting-up the ligation reaction, the extent of digestion of DNA and its concentration were checked by running a small sample of the digestion mix on an agarose gel. For ligating compatible cohesive DNA ends, the 20µl reaction mix contained:

a) vector DNA (usually 30 - 40ng)

b) insert DNA (4 - 8 x molar concentration of vector DNA)

c) 4µl of 5 x ligation buffer

d) 1 unit of T4 DNA ligase

e) deionized H_2O to 20µl

The ligation reaction was carried out at 4°C for 12-16hrs. For ligation involving blunt-ended DNA fragments, in addition to the conditions described above, the amount of T4 DNA ligase was increased to 2 units and the incubation time to 24hrs. Also, 2µl of 50%

PEG6000 or PEG8000 were added to the ligation mix. The mixture was used to transform competent *E. coli.*

2.2.26 Extraction of DNA fragments from agarose gels

For purification of DNA fragments from agarose gels, low-melting point agarose was used. For smaller DNA fragments (*e.g.*, 100bp - 1kb) 3%NuSieve® GTG® agarose (FMC) and for larger fragments (>8kb) 1%SeaKem® GTG® agarose (FMC) was used. Gels were prepared in TAE buffer and electrophoresed as described earlier. The relevant band was excised from the agarose gel using a sterile scalpel knife under UV light and transferred to a microfuge tube. The slice of gel was melted by incubating the tube at 65°C for 10 - 12min. DNA was purified using the PCR cleaning kit (Promega Corporation) as recommended by the manufacturer and resuspended in an appropriate volume of deionized H_2O or TE. A small sample of DNA was electrophoresed on an agarose gel to determine its concentration before proceeding with further manipulations.

2.2.27 Polymerase chain reaction (PCR)

Each polymerase chain reaction was carried out in a 100µl volume and in a 0.6µl microfuge tube. dNTPs and the 10 x PCR reaction buffer were purchased from Perkin Elmer Cetus, Norwalk, CT, USA. $Vent_R^{TM}$ (exo^-) DNA Polymerase (New England Biolabs Ltd.) was used to synthesize DNA. The PCR reaction was done in MiniCycler™ (Genetic Research Instruments Ltd., Essex). Appropriate volume of each component was added to the microfuge as below:

Component	Volume	Final concentration
10mM dATP	2µl	0.2mM
10mM dCTP	2µl	0.2mM
10mM dGTP	2µl	0.2mM
10mM dTTP	2µl	0.2mM
10 x PCR reaction buffer	10µl	1 x
$Vent_R^{TM}$(exo$^-$) Polymerase (2 units/µl)	1µl	2 units
Template DNA		~10^8 -10^9 targets
10µM primer#1	1µl	~10-15pmol
10µM primer#2	1µl	~10-15pmol
Deionized H_2O to	100µl	

The reaction mixture was overlaid with 75µl mineral oil to prevent evaporation and then transferred to the thermocycler. The following temperature profile was used and 30 cycles of PCR were performed.

Step-1	*Denaturation*	94°C	60sec
Step-2	*Primer annealing*	40°C	60sec
Step-3	*Primer extension*	72°C	180sec

The reaction was stopped by addition of EDTA to 10mM and then stored at -20°C.

2.2.28 Southern blotting

DNA samples were electrophoresed on an agarose gel in TBE buffer and the gel photographed as described earlier. DNA in the gel was depurinated by gentle shaking in 0.25M HCl for 15min at room temperature. The gel was washed in distilled water briefly, transferred to denaturation solution and left with gentle shaking for 2 x 15min at room

temperature. The gel was rinsed briefly with water again and transferred to neutralization solution for 15min with gentle shaking at room temperature. DNA was transferred to the nylon membrane for 12 - 18hrs as described by Sambrook *et al.* (1989) and the membrane air dried for 2 - 3hrs. DNA was covalently bound to the membrane by exposure to UV light (UV-transilluminator , Ultra-violet Products Inc., California) for three minutes.

Southern blotting was performed using, either Flash™ Detection System (Stratagene Ltd.), in which case probe was labelled using Flash™/Prime-it™ Random Primer Labelling Kit (Stratagene Ltd.), or ECL™ random primer labelling and detection systems form Amersham International plc. (Buckinghamshire). In both cases DNA was transferred to neutral Flash™ Nylon Membrane (Stratagene Ltd.). Protocols described by the manufacturers were followed without any modification.

The signal was detected by exposing the membrane to an X-ray film for from a few seconds to 30min, depending upon the strength of the signal.

2.2.29 Colony Hybridization

Either single colonies were patched onto selective agar-plate and grown for 12 - 14hrs at 37°C, or if the colonies were well dispersed on the plate, the plate was used as such. The colonies were transferred to Flash™ Nylon Membrane by placing the membrane on the plate and leaving at room temperature for 1min.The membrane was gently peeled off and transferred, colony side up, to a series of 3MM papers soaked in one of the following solutions, one by one, for 5min in each as below:-

a) 10% SDS (w/v) b) 0.5N NaOH, 1.5M NaCl

c) 1.5M NaCl, 0.5M Tris, pH 7.4

d) 2 x SSC

The membrane was air dried for 30min, DNA fixed to the membrane by exposure to UV light for three minutes and then processed as Section 2.2.28.

2.2.30 DNA sequencing

The method of Sanger *et al.* (1977) was employed. Commercially available kits were used. Double-stranded DNA, prepared from *S. aureus* or *E. coli*, was used in sequencing and labelled with [α^{35}S] dATPαS.

SEQUENASE® Version 2.0 DNA Sequencing Kit form United States Biochemical Corporation (Ohio, USA) was used as described by the manufacturer without any modification. Where CircumVent™ Thermal Cycle Dideoxy DNA Sequencing Kit was used, the reactions were performed in the MiniCycler™ (Genetic Research Instruments Ltd., Essex) and overlaid with 50µl of mineral oil. The following temperature profile was generally used, except for the oligonucleotides with a lower T_m.

Step-1	*Denaturation*	95°C	60sec
Step-2	*Primer annealing*	50°C	60sec
Step-3	*Primer extension*	70°C	80sec

Where the T_m was ≤54°C, the following temperature profile was used:

Step-1	*Denaturation*	95°C	60sec
Step-2	*Primer annealing*	43°C	70sec
Step-3	*Primer extension*	66°C	80sec

The rest of the steps were completed exactly as described by the manufacturer.

2.2.31 Polyacrylamide gel electrophoresis for DNA sequencing

Gels were formed using two glass plates (gel dimensions: 47cm x 15cm) separated by wedge spacers (0.25mm - 0.75mm; Bio-Rad Laboratories Ltd.) and held in place with sealing tape and fold-back clips. The DNA samples were heated to 100°C for 3min in a water bath. Electrophoresis was performed in a Scie-Plas Vertical Gel Rig (for 20cm x 50cm plates) and TBE buffer, pH 8.3, at 1500V using LKB BROMMA 2197 Power Supply. On completion of electrophoresis, the gel was transferred to to 3MM Whatman chromatography paper, covered in Saran cling film and dried for ~2hrs at 80°C under vacum (Bio-Rad Dual Temperature Slab Dryer, Modal SE1 125B). The cling film was removed and the gel was exposed to X-ray film in a Kodak X-Omatic cassette for 1-10 days.

2.2.32 Computer handling and manipulation of DNA sequence data

The Macintosh compatible programme DNA Strider™ version 1.0 was run on a **Macintosh SE** or **Macintosh SE FDHD** and was used to analyse sequence data for restriction enzyme sites, open reading frames and translation of DNA sequence into amino acid sequence.

DNA nucleotide sequence was compared to the EMBL database using the FASTA computer programme and the derived amino acid sequence using TFASTA, both available on the Oxford University Molecular Biology VAX. All additional work was carried out using GCG package (Devereux *et al.*, 1984) available on the Oxford University Molecular Biology VAX.

Reference searches were carried out using an IBM compatible reference compilation, Med-Line, made available in Oxford University, Biochemistry Department Library.

Chapter Three:

Interaction of the pI9789-derivatives With the Host Chromosome

Chapter Three: Interaction of the pI9789-derivatives With the Host Chromosome

3.1 Introduction

Browne (1985) and Johnston (1971) have shown that suppression of the thermosensitivity of replication of the plasmid pS1 is approximately three times greater than that of the plasmid pΔD. Thus investigation of the role of the regions deleted in the plasmid pΔD may provide clues as to the mechanism of the suppression. Rowland (1989) defined the deletions on the plasmid pΔD and showed two closely placed deletions: one in the *bla* region; the other in the region specifying a putative site-specific recombinase, BinR. Browne (1985) and Johnston (1971) also showed that suppression is influenced by the composition of the chromosome of the PS80 strain of *S. aureus*. The presence of the erythromycin transposon, Tn*551*, on the chromosome greatly enhances the frequency of suppression and Browne (1985) used the host strain PS80*blaIblaZ* (with the β-lactamase transposon, Tn*552*, on the chromosome) to investigate the effect of homology on the frequency of suppression. She showed that homology did not detectably increase the frequency of suppression for pS1 or pΔD. Although from the available data, the most plausible explanation seems integration of the plasmid into the chromosome, for ease of reference, the phenomenon will be called 'reversion' and the thermosensitivity suppression frequencies will be labelled 'reversion frequencies' in this thesis. The following two questions will be addressed:-

1- Is the DNA deleted from the plasmid pΔD important in reversion?

2- Is reversion strain specific?

3.2 Cloning of *binR*

If reversion is the result of integration, then, of the two deletions of pS1 that gave rise to pΔD, the 671kb deletion which removed the site-specific recombinase gene, *bin*R, seems a stronger candidate for the investigation of an effect on reversion. To evaluate this, *bin*R was cloned to see whether such a clone could increase the reversion frequency of pΔD, *in trans*.

binR had already been cloned in *E. coli* in a pBR322 vector to give pSJB44. It was now necessary to sub-clone the *binR* so that it could be transferred back into *S. aureus*. In pSJB44, the 0.435kb *Ssp*I - *Pvu*I piece of pBR322 (in the *amp* region) has been replaced by a 1.133kb *Eco*RV - *Pvu*I DNA fragment containing *binR* from the plasmid pS1. The *Eco*RV site on one end of this fragment was lost during cloning (Rowland, 1989). DNA containing *bin*R was obtained as a 1.65kb *Pvu*I - *Bam*HI fragment from pSJB44, the protruding ends filled in by using Klenow polymerase and deoxynucleotide triphosphates and then ligated into the *Sma*I site of a shuttle vector for *E. coli* and *S. aureus*, called pOX7 (see Fig.3.1(a) for cloning strategy). pOX7 has the blue/white selection system of the plasmid pUC18 and has selectable markers for both *E. coli* (Ap^r) and *S. aureus* (Em^r). The ligation mixture was transformed into competent *E. coli* JM107 and the transformants were plated onto 2 x TY plates containing X-gal/IPTG/ampicillin at standard concentrations. Plasmid DNA was prepared from the white colonies and digested with *Eco*RI + *Hind*III to

Fig.3.1 (a) Cloning strategy for *bin*R. The drawings are not accurately to scale. A pBR322 based plasmid, pSJB44, was the source of *bin*R. *bin*R was cloned into an *E.coli* - *S.aureus* plasmid vector, pOX7, and the resultant clone of *bin*R was named pOX86. The restriction enzyme sites lost during cloning are shown in brackets.

Fig.3.1 (b) shows restriction enzyme digests of the plasmids.

Fig. 3.1:(a)

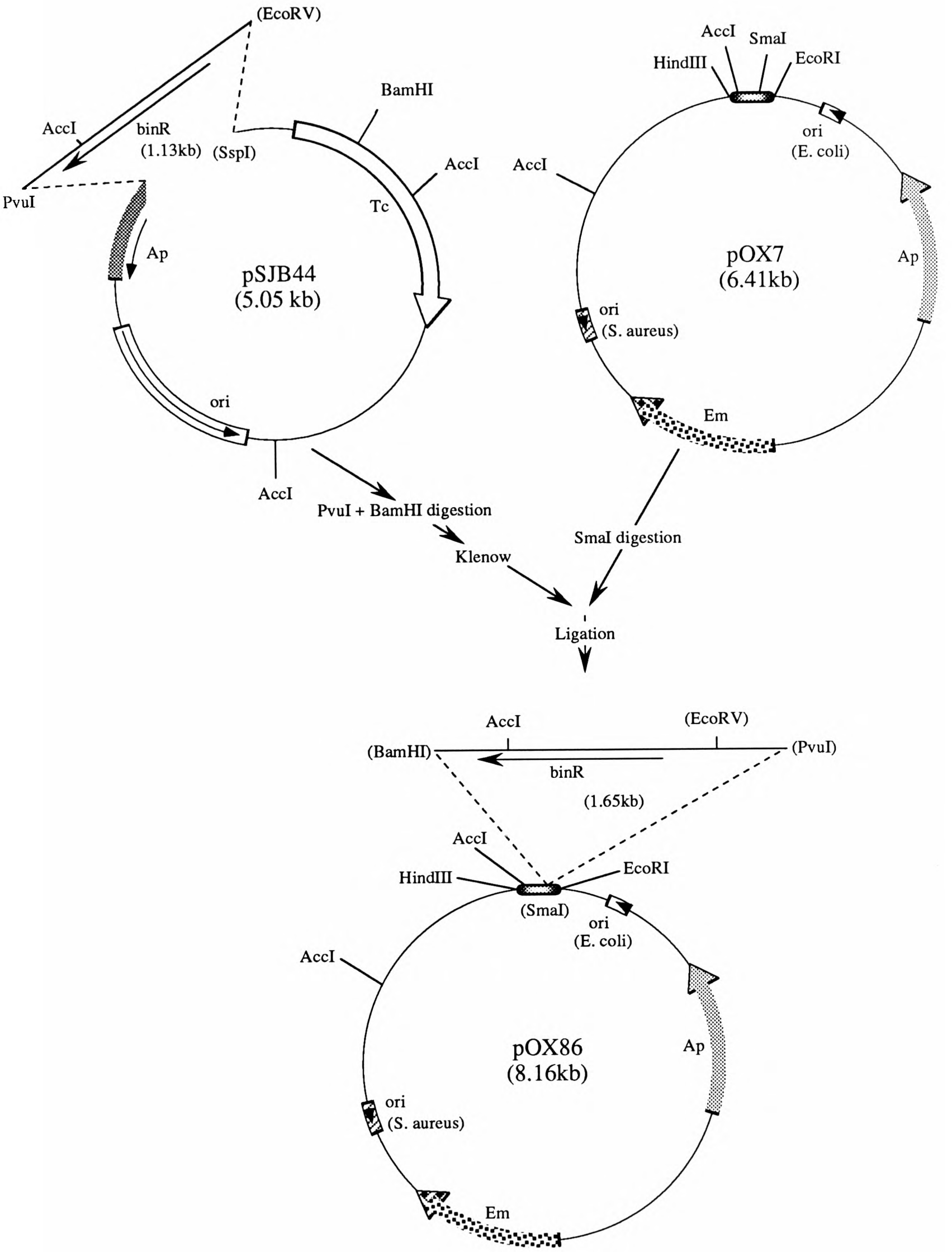

(EcoRV)
AccI
binR
(1.13kb)
(SspI)
BamHI
AccI
Tc
PvuI
Ap
pSJB44
(5.05 kb)
ori
AccI
PvuI + BamHI digestion
Klenow
AccI
SmaI
HindIII
EcoRI
ori
(E. coli)
AccI
pOX7
(6.41kb)
Ap
ori
(S. aureus)
Em
SmaI digestion
Ligation
AccI
(EcoRV)
(BamHI)
(PvuI)
binR
(1.65kb)
AccI
HindIII
EcoRI
(SmaI)
ori
(E. coli)
AccI
pOX86
(8.16kb)
Ap
ori
(S. aureus)
Em

Fig. 3.1(b)


pOX7/SmaI
pOXR/
EcoRI+HindIII
1KB ladder
pSJB44/
PvuI+BamHI
pOX7 (6.4kb)
pBR322 (3.36kb)
1.7kb
1.65kb
binR

confirm the presence of the insert. The orientation of the insert was determined by *Acc*I digestion and one colony was selected. The construct was named pOX86.

3.3 Construction of heteroplasmid strains

To investigate the impact of the composition of the chromosome on the reversion phenomenon, the reversion frequencies of the plasmids pS1 and pΔD were studied in *S. aureus* strains 1054 and RN4220 in addition to PS80N. These experiments were conducted both in the presence and the absence of the cloned *bin*R. The experiments to test the reversion frequencies of various plasmids will be referred to as 'reversion experiments' and the bacteria exhibiting the thermosensitivity suppression will be called 'revertants'. *Eco*RI digestion profiles of all the heteroplasmid strains constructed are shown if Figs.3.2, 3.3 and 3.4. The constructions of the heteroplasmid strains of *S. aureus* are summarised in Fig.3.5.

3.3.1 Construction of PS80N heteroplasmid strains

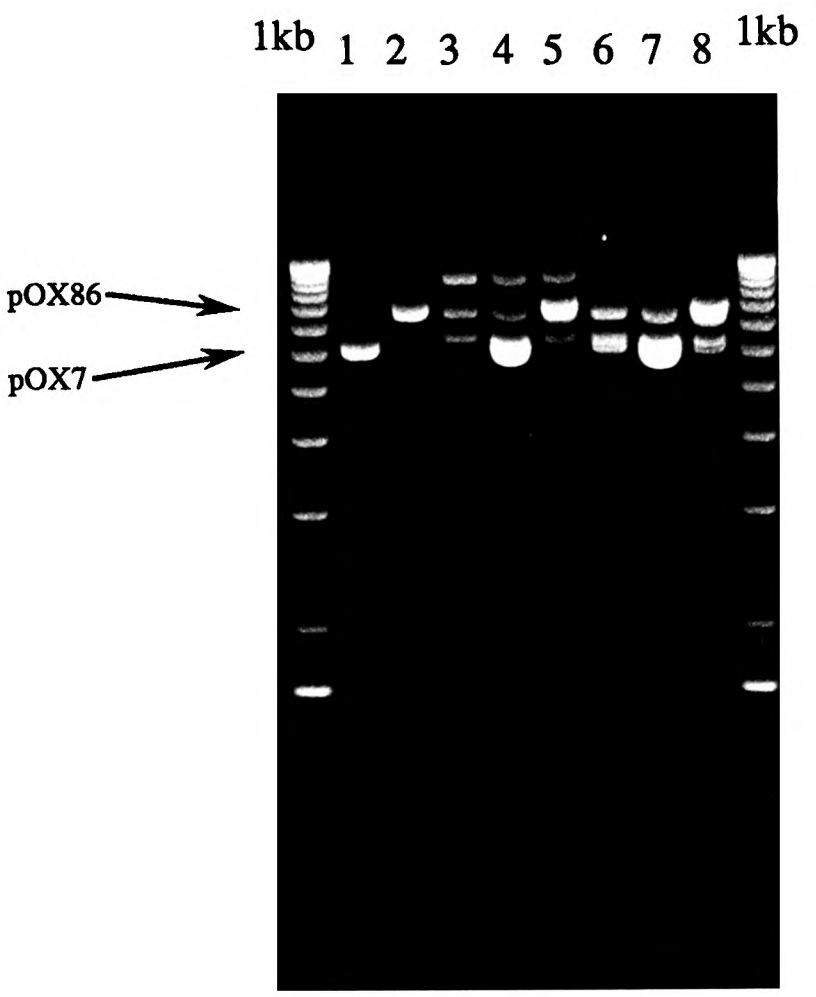
PS80N is refractory to transformation and electroporation and several attempts to transform pOX86 into PS80N failed. Another strain of *S. aureus*, RN4220, has been successfully used in transformation. The clone pOX86 was, thus, initially transformed into RN4220 using protoplast transformation. The transformants were selected on DM3-agar plates overlaid with TBAB/HB containing appropriate amount of erythromycin to give a final concentration of 20μg/ml. Plasmid DNA was isolated from four transformants and suitable restriction enzymes were used to verify the presence of the correct plasmid. The

strain was called RN4220(pOX86). The plasmid pOX86 was then transduced to PS80N. Two phages, phage 53 and phage 80, were used in the transductions, since no transducing phage was available which could grow on both RN4220 and PS80N. *S. aureus* 1054, which is susceptible to both phage 53 and phage 80, was used as an 'intermediate' in the transductions. Phage 53 was grown on RN4220(pOX86) and used to transduce pOX86 to 1054 with selection on CY-Em (20μg/ml)-agar plates. Plasmid DNA was prepared from four transductants and digested with appropriate restriction enzymes to verify the presence of pOX86. The profile showed the expected bands but, in addition, a number of other bands were also seen (Fig.3.4). These bands represent a cryptic plasmid in 1054, named pOX1054. The presence of both of the plasmids ((pOX86 and pOX1054) in the bacteria suggests that they are compatible (*i.e.*, belong to different incompatibility groups). Phage 80 was then grown on 1054(pOX1054/pOX86) and the phage used to transduce pOX86 into PS80N, PS80(pS1) and PS80(pΔD). Transductants were selected on CY-Em (20μg/ml)-agar plates. Plasmid DNA from four colonies from each transduction was analysed by restriction enzymes to verify successful transduction of pOX86. The resultant strains were named PS80(pOX86), PS80(pS1/pOX86) and PS80(pΔD/pOX86). As a control the vector, pOX7, was also transferred to the PS80 plasmid containing strains (Fig.3.5).

3.3.2 Construction of RN4220 heteroplasmid strains

To perform the reversion experiments in hosts other than PS80, heteroplasmid strains having the plasmids pOX86 and pS1 or pΔD were constructed in RN4220. Phage 80 was grown on PS80(pS1) and PS80(pΔD). The phage preparations were used to transduce plasmids pS1 and pΔD to 1054(pOX1054). Transductants were selected on CY-

Fig. 3.2 *Eco*RI digests of the plasmid DNA of the constructs in PS80N.



KEY:

1- pOX7

2- pOX86

3- pS1

4- pS1 + pOX7

5- pS1 + pOX86

6- pΔD

7- pΔD + pOX7

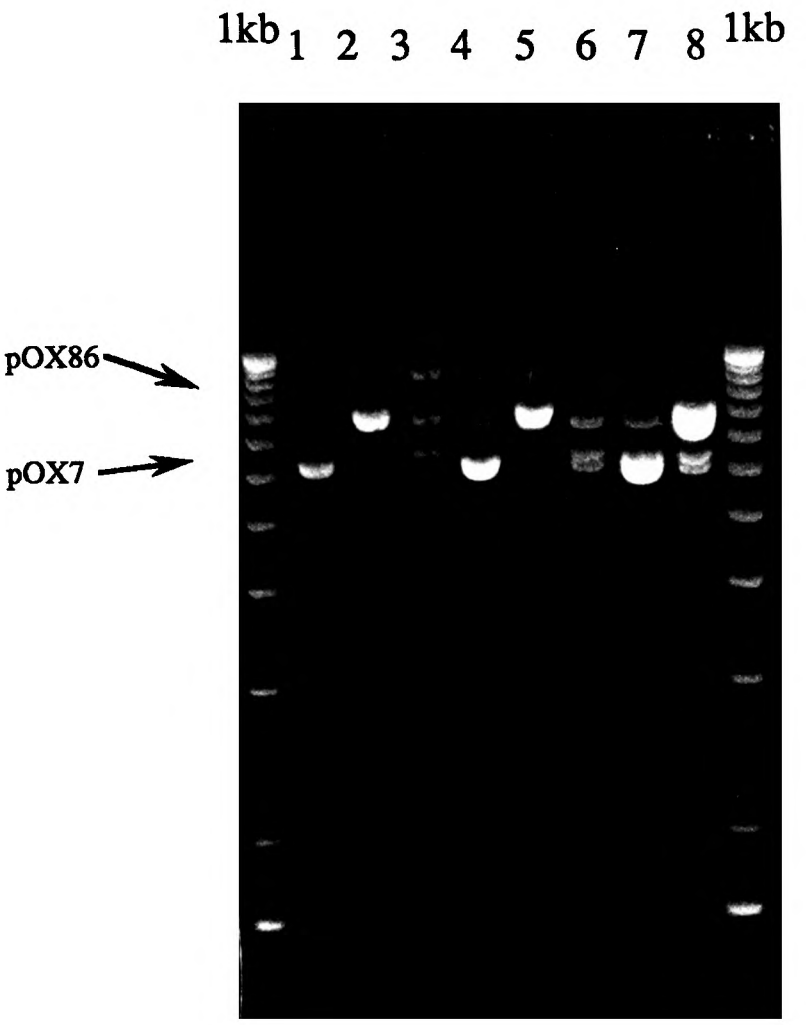
8- pΔD + pOX86

Cd (26.6μg/ml)-agar plates. Plasmid DNA from four colonies from each transduction was prepared and analysed using restriction enzymes to verify successful transduction. The transductants were also tested for some phenotypic markers (*e.g.*, β-lactamase production for pS1; mercury resistance for both pS1 and pΔD). Also, the plasmids were tested for thermosensitivity using the Disc Method. The resultant strains were named 1054(pOX1054/pS1) and 1054(pOX1054/pΔD), respectively. Plasmid DNA profiles show the co-existence of the plasmid pOX1054 with both pS1 and pΔD (Fig.3.4). A transductant, containing a derivative of the plasmid pΔD with the mercury resistance determinant deleted, was isolated during this transduction. This plasmid was named pΔDmer^{s}. The restriction enzyme map of this plasmid is shown in Fig.3.6. A deletion of part of the plasmid pΔD, identical in restriction enzyme pattern to pΔDmer^{s}, was also found following transductions involving phage 53. No such deletions were observed with the plasmid pS1. The plasmid pΔDmer^{s} was transduced back to PS80N using phage 80 and its reversion frequency was determined. It was found to be indistinguishable from that for pΔD (*i.e.*, 9±3 revertants per 10^{6} bacteria). Phage 53 was grown on both 1054(pOX1054/pS1) and 1054(pOX1054/pΔD) and used to transduce the plasmids pS1 and pΔD to RN4220, RN4220(pOX86) and RN4220(pOX7). The transductants were selected on CY-Cd (26.6μg/ml)-agar plates and found to contain the correct plasmids as measured by studying phenotypic expression and by plasmid profiles (Fig.3.3). The thermosensitivity of the plasmids was retained as determined by the Disc Method.

3.3.3 Construction of 1054 heteroplasmid strains

The reversion experiments were also performed in the 1054 strain of *S. aureus* to study the effect of this host chromosome. The effect of *bin*R, if any, could also be studied

Fig. 3.3 *Eco*RI digests of the plasmid DNA of the constructs in RN4220.



KEY:

1- pOX7

2- pOX86

3- pS1

4- pS1 + pOX7

5- pS1 + pOX86

6- pΔD

7- pΔD + pOX7

8- pΔD + pOX86

if pOX86 can co-exist with pOX1054 and pS1 or pΔD. Strains 1054(pOX1054/pOX86), 1054(pOX1054/pS1), 1054(pOX1054/pΔD) and 1054(pOX1054/pOX7) had already been obtained (see Sections 3.3.1 and 3.3.2). The phage 80, grown on PS80(pS1) and PS80(pΔD) was used to transduce the plasmids pS1 and pΔD, respectively, to 1054(pOX1054/pOX86) and 1054(pOX1054/pOX7). The transductants were selected on CY-Cd (26.6μg/ml)-agar plates. Plasmid DNA was prepared and the profile showed the co-existence of plasmids pOX86 and pOX7 with the others (*i.e.,* pOX1054 plus pS1 or pΔD). The new heteroplasmid strains were designated 1054(pOX1054/pS1/pOX86), 1054(pOX1054/pΔD/pOX86), 1054(pOX1054/pS1/pOX7) and 1054(pOX1054/pΔD/pOX7).

3.4 Reversion experiments

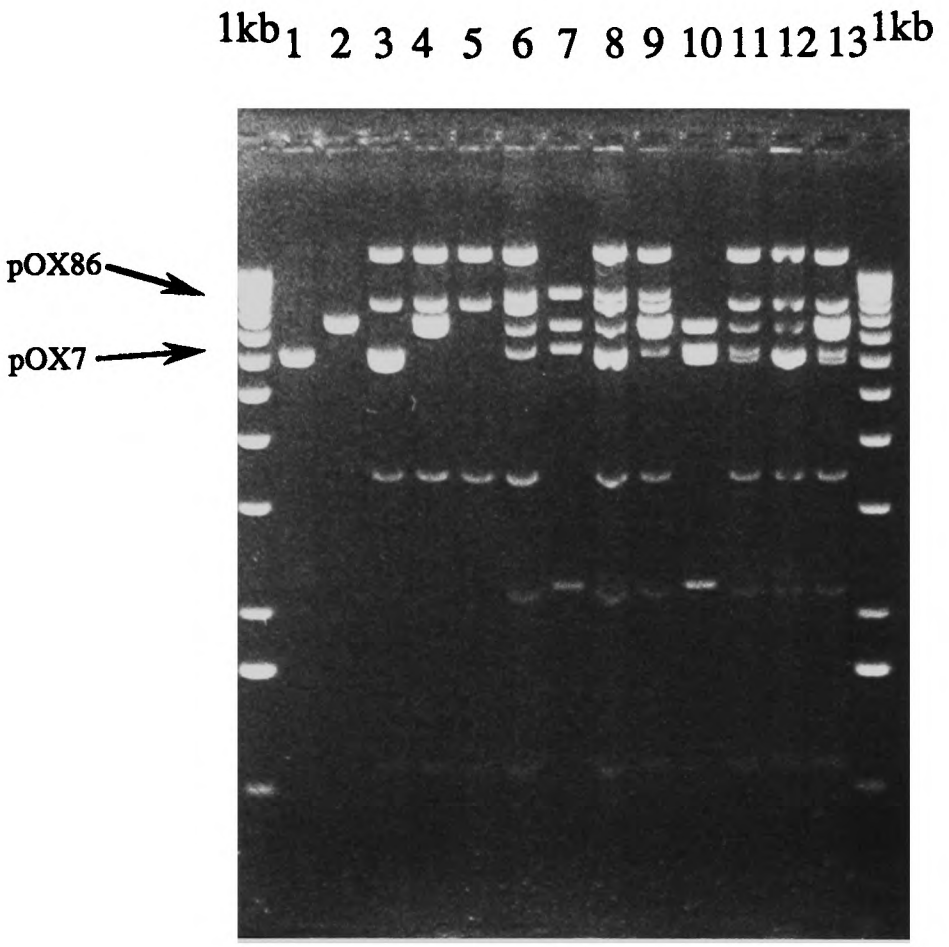
This section describes the reversion frequencies of the plasmids pS1 and pΔD in the three different hosts of *S. aureus* with and without other plasmids.

3.4.1 Reversion experiments in PS80

3.4.1.1 Reversion experiments in PS80N

The method of Browne (1985) and Johnston (1971) was used to determine the reversion frequencies and was standardized. The initial overnight culture was set in 10ml broth for 18hrs and from a one-day old selective-agar plate for all the reversion experiments. An initial inoculum from an old plate gave a higher number of revertants (*e.g,* 64±6 revertants for PS80N(pS1) and 21±5 for PS80N(pΔD)) than an inoculum from a

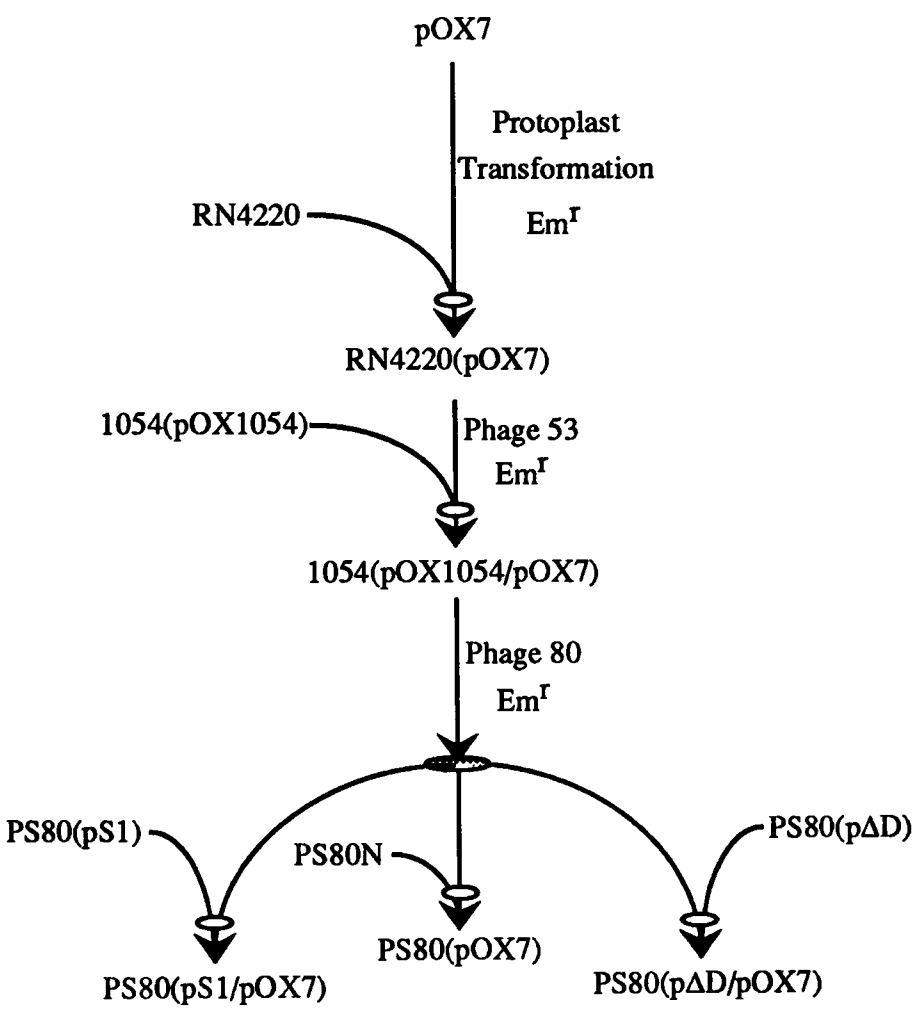
Fig. 3.4 *Eco*RI digests of the plasmid DNA of the constructs in 1054.

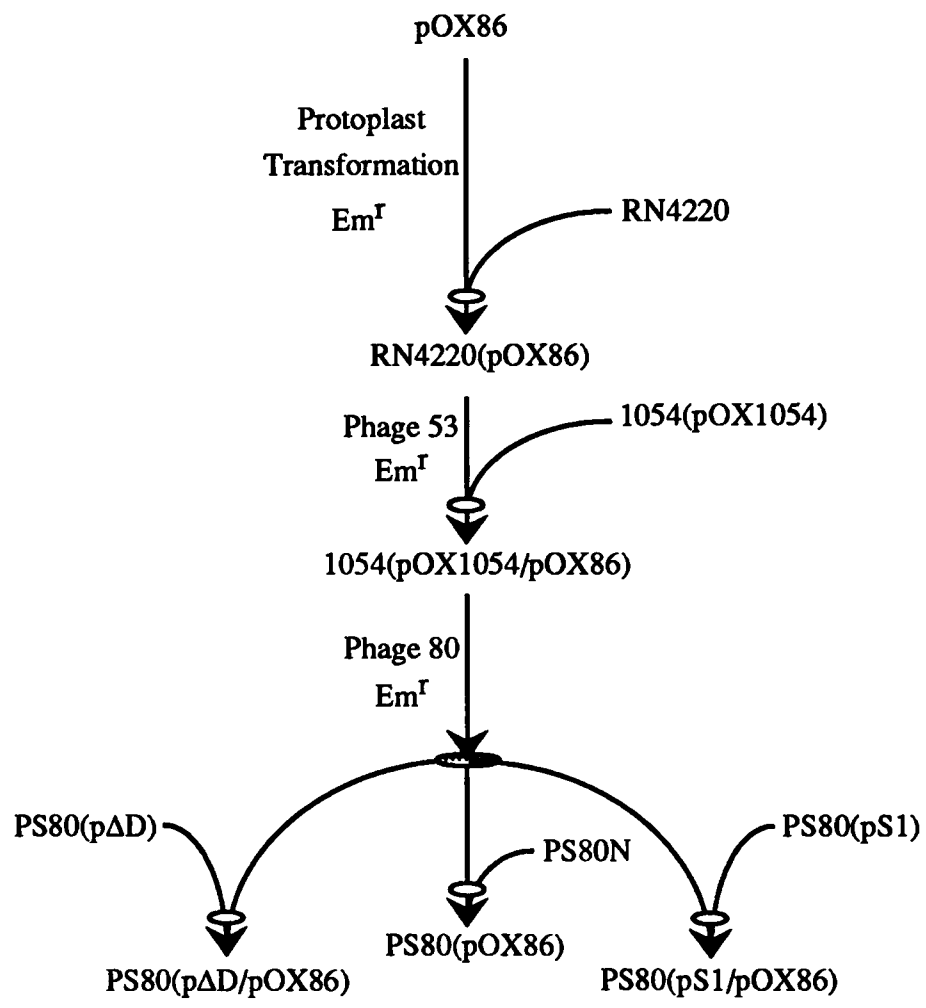


KEY:

1- pOX7
2- pOX86
3- p1054 + pOX7
4- p1054 + pOX86
5- p1054
6- p1054 + pS1
7- pS1
8- p1054 + pS1 + pOX7
9- p1054 + pS1 + pOX86
10- pΔD
11- p1054 + pΔD
12- p1054 + pΔD + pOX7
13- p1054 + pΔD + pOX86

Fig.3.5 Construction of various heteroplasmid strains of *S. aureus*.





(contd.)

(contd.)

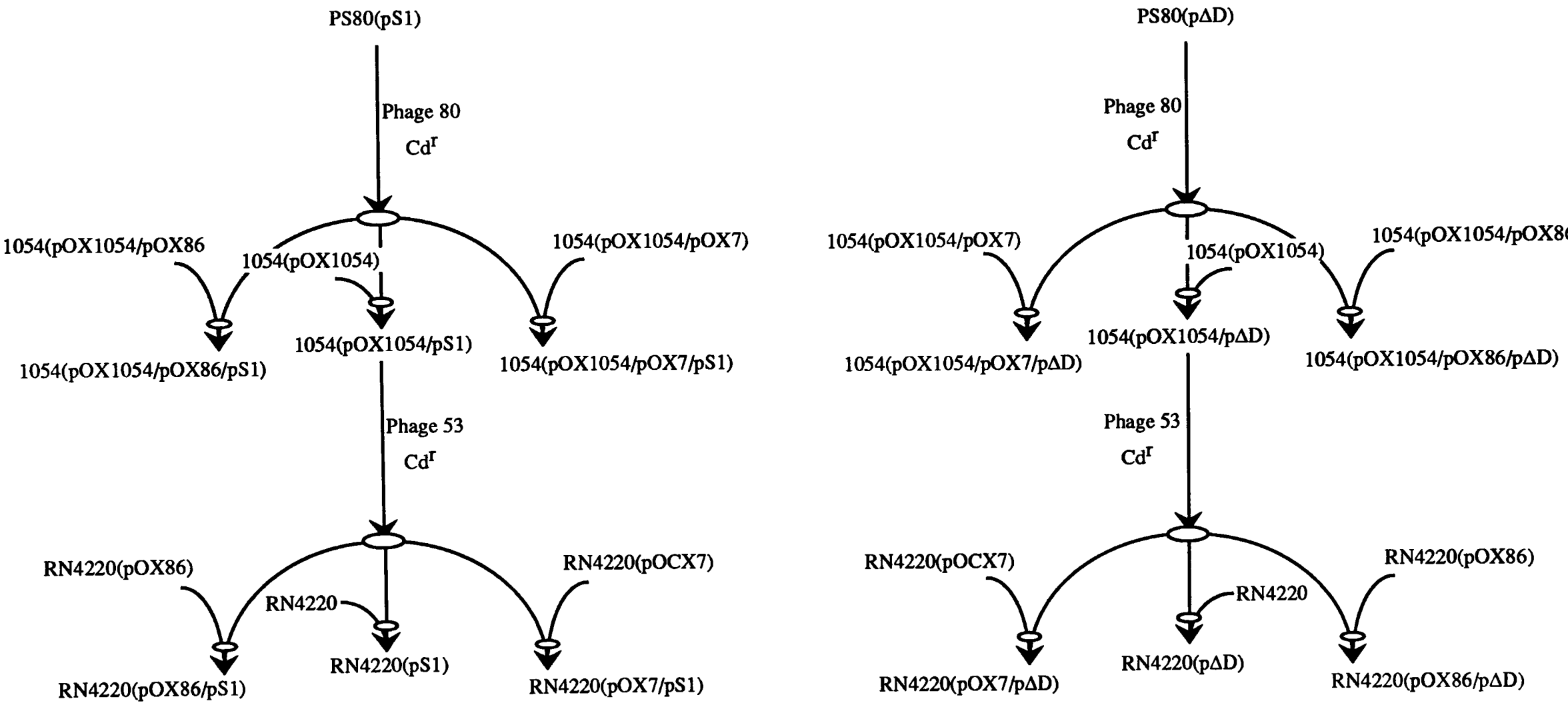
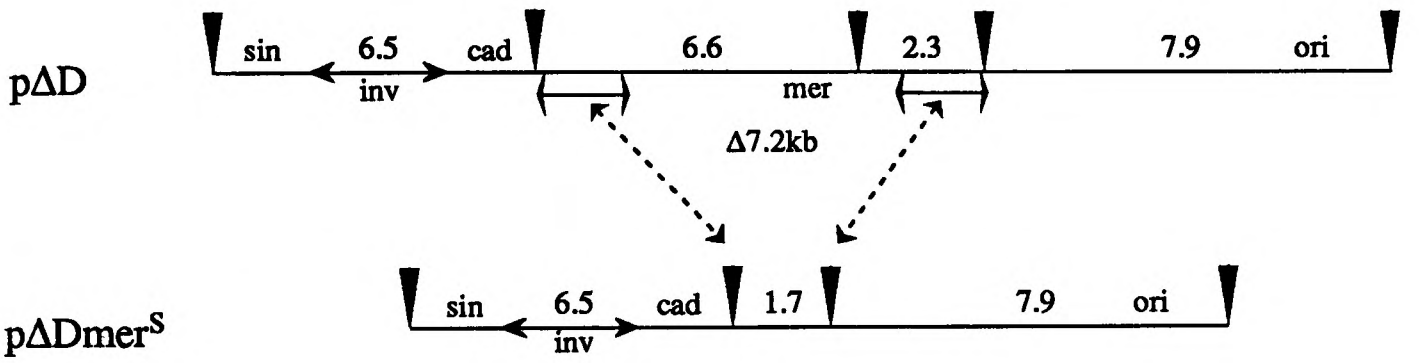
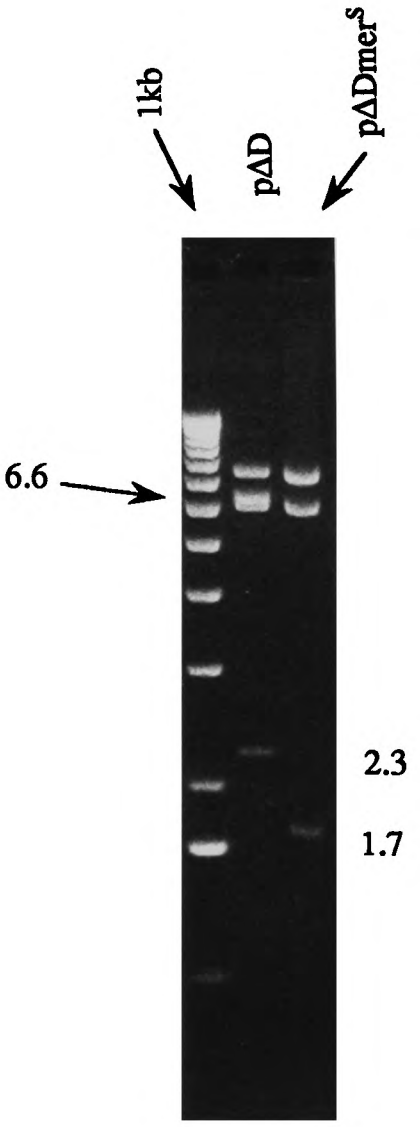

PS80(pS1)
Phage 80
Cd^r
1054(pOX1054/pOX86
1054(pOX1054)
1054(pOX1054/pOX7)
1054(pOX1054/pOX86/pS1)
1054(pOX1054/pS1)
1054(pOX1054/pOX7/pS1)
Phage 53
Cd^r
RN4220(pOX86)
RN4220
RN4220(pOCX7)
RN4220(pOX86/pS1)
RN4220(pS1)
RN4220(pOX7/pS1)
PS80(pΔD)
Phage 80
Cd^r
1054(pOX1054/pOX7)
1054(pOX1054)
1054(pOX1054/pOX86
1054(pOX1054/pOX7/pΔD)
1054(pOX1054/pΔD)
1054(pOX1054/pOX86/pΔD)
Phage 53
Cd^r
RN4220(pOCX7)
RN4220
RN4220(pOX86)
RN4220(pOX7/pΔD)
RN4220(pΔD)
RN4220(pOX86/pΔD)

Fig.3.6 Restriction enzyme map of the mercury-sensitive derivative of the plasmid pΔD (pΔDmer^S). A ~7.2kb deletion has occurred in the adjacent 6.6kb and 2.3kb *Eco*RI pieces to give a 1.7kb *Eco*RI piece. *Eco*RI fragment sizes and a gel showing *Eco*RI digests of the plasmids pΔD and pΔDmer^S are also shown.



Gel: *Eco*RI digests



KEY:

Δ: Deletion

EcoRI

***Eco*RI Fragment sizes (kb):**

pΔD:	7.9, 6.6, 6.5, 2.3
pΔDmer^S:	7.9, 6.5, 1.7

fresher culture. The latter gave a lower number for each type (e.g, 20±4 revertants for PS80N(pS1) and 7±3 for PS80N(pΔD)). To eliminate background growth, the concentration of cadmium acetate was increased from 13.3μg/ml to 26.6μg/ml. The results were recorded after precisely 18hrs, since the number of revertant colonies increases progressively. The longer the incubation the larger the number of the revertants obtained. Strictly identical conditions of plating, incubation time and temperature, cadmium concentration, etc., are necessary to get reproducible results (also see Browne, 1985 and Johnston, 1971). Onto each plate, 10^6 colony forming units were plated and each reversion experiment was done at least in triplicate. The number of colonies obtained after 18hrs incubation at 42°C on CY-Cd (26.6μg/ml) from an original inoculum of 10^6 bacteria is given in Table. 3.1. The results show that the presence of pOX86 but not pOX7 increased the number of revertants for pS1 but not for pΔD. Plasmid DNA was prepared from 10 colonies each of PS80(pS1) revertants, PS80(pS1/pOX86) revertants and PS80(pΔD) revertants. None of the thirty revertants tested contained detectable plasmid DNA.

3.4.1.2 Reversion experiments in PS80::Tn*551*

PS80::Tn*551* carries the erythromycin transposon, Tn*551*, on the chromosome. Johnston (1971) and Browne (1985) reported an increase in the reversion frequency of the plasmids pS1 and pΔD in this variant. No such increase was found when Tn*552* was present on the chromosome. Browne (1985) also reported restriction enzyme data supporting site- and orientation- specific insertion of Tn*551* into the plasmid pS1 when this plasmid is transduced out of such revertants, whereas, this site of insertion is apparently random for pΔD. PS80::Tn*551* was used in the reversion experiments not only to provide a different chromosomal background for the purpose of experiment but also to measure

Table 3.1 Results of the reversion experiments in PS80.

Plasmid(s)	No. of Cd^r colonies per plate			
	Exp. 1	**Exp. 2**	**Exp. 3**	**Overall average**
No plasmid	0,0,0	0,0,0	0,0,0	0
pOX86	0,0,0	0,0,0	0,0,0	0
pS1	36,39,40	26,29,31	30,33,35	33±7
pΔD	11,13,16	7,10,10	8,10,12	11±5
pS1+pOX86	108,116,122	96,104,110	100,110,115	108±14
pΔD+pOX86	12,12,14	9,11,12	7,7,11	11±4
pOX7	0,0,0	0,0,0	0,0,0	0
pS1+pOX7	35,41,42	29,30,35	30,35,36	35±6
pΔD+pOX7	10,10,14	5,9,11	11,12,14	10±5
PS80::Tn*551*	0,0,0	0,0,0	ND	0
PS80::Tn*551*(pS1)	~300(x3)	~300(x3)	ND	~300
PS80::Tn*551*(pΔD)	~1000(3)	~1000(x3)	ND	~1000

Notes: The Table above shows results of three independent reversion experiments done on separate days. Variability in the number of revertants is seen from day to day, but the same general pattern is observed. 10^6 colony forming units from an overnight culture were plated onto freshly prepared CY plates containing 26.6µg of cadmium acetate per ml. Plates were incubated at 42°C and the number of colonies visible after 18 hours recorded. (ND= not done)

quantitatively this effect and to investigate further the reported phenomenon. More revertants were obtained than in any previous experiments. The results for PS80::Tn*551*(pS1) and PS80::Tn*551*(pΔD) are given in Table 3.1 and show that there is a significant increase in frequency of reversion for both pS1 and pΔD.

3.4.2 Reversion experiments in RN4220

The experimental procedures were the same as those in Sections 3.4.1.1 and 3.4.1.2. The numbers of revertants obtained are given in Table 3.2. The number of revertants is lower than for PS80 strain and the frequency is not affected by the presence of pOX86 or pOX7. Plasmid DNA was prepared from 10 colonies each of RN4220(pS1) revertants and RN4220(pΔD) type revertants. No plasmid DNA was detected in the RN4220(pΔD) revertants or nine RN4220(pS1) type revertants, but for one of the latter, a plasmid identical to pS1 in *Eco*RI restriction pattern was found. This plasmid was further analysed using other restriction enzymes to compare with pS1 and was found to be indistinguishable from pS1 in *Bgl*II, *Sna*BI, *Eco*RV, *Acc*I, *Pvu*I and *Msc*I restriction enzyme patterns. This temperature-stable pS1 was named as pTST.

3.4.3 Reversion experiments in 1054

The number of revertant colonies is given in Table 3.3. The results show that the number of revertants is quite high for pS1 and somewhat lower for pΔD. In neither case did the presence of pOX86 or pOX7 significantly influence the frequency of reversion. Plasmid DNA was prepared form 10 each of 1054(pOX1054/pS1) revertants and 1054(pOX1054/pΔD) revertants. A plasmid, larger than either pS1 or pΔD, was found in every example. The plasmid DNA was digested with *Eco*RI and analysed by agarose gel

Table 3.2 Results of the reversion experiments in RN4220.

Plasmid(s)	No. of Cd^r colonies per plate			
	Exp. 1	**Exp. 2**	**Exp. 3**	**Overall average**
No plasmid	0,0,0	0,0,0	0,0,0	0
pOXR	0,0,0	0,0,0	0,0,0	0
pS1	4,4,5	4,4,6	3,5,5	4±2
pΔD	2,4,4	2,5,6,	3,3,6	4±2
pS1+pOX86	2,2,3	3,4,6	3,4,5	4±2
pΔD+pOX86	2,3,4	4,5,6	3,3,5	4±2
pOX7	0,0,0	0,0,0	0,0,0	0
pS1+pOX7	2,3,3,	4,4,4	3,5,6	4±2
pΔD+pOX7	2,3,4	3,3,6	1,3,4	4±3

Table 3.3 Results of the reversion experiments in 1054.

Plasmid(s)	No. of Cd^r colonies per plate			
	Exp. 1	**Exp. 2**	**Exp. 3**	**Overall average**
p1054	0,0,0	0,0,0	0,0,0	0
p1054+pOX86	0,0,0	0,0,0	0,0,0	0
p1054+pS1	145,157,164	133,137,144	141,147,158	147±17
p1054+pΔD	50,54,63	59,65,82	58,62,66	62±20
p1054+pS1+pOX86	152,164,167	145,152,157	134,139,146	151±17
p1054+pΔD+pOX86	45,52,57	53,60,68	48,50,61	55±13
p1054+pOX7	0,0,0	0,0,0	0,0,0	0
p1054+pS1+pOX7	137,144,149	141,145,156	146,154,155	147±10
p1054+pΔD+pOX7	57,65,71	55,62,63	50,58,65	61±11

electrophoresis. The results are shown in Fig.3.7 and the restriction fragment sizes are given in Table 3.4. The plasmids pS1 and pΔD has integrated into the large cryptic plasmid, pOX1054, instead of the chromosome in all the examples examined. No plasmid-free bacteria were detected among the examples tested, so it is not possible to judge whether any 'integration' of these plasmids into the chromosome has occurred.

3.5 Discussion

3.5.1 pOX7/pOX86 and the construction of the heteroplasmid *S. aureus*

The putative site-specific recombinase gene of pS1, *binR*, (a Hin family site-specific recombinase from *S. aureus*; Rowland and Dyke, 1988) which is deleted in pΔD, was cloned in a shuttle vector for *S. aureus* - *E. coli* called pOX7 (the clone named pOX86). The replication of pOX7 is temperature sensitive (*e.g.*, at 42°C) in *S. aureus*. Such a plasmid vector was chosen deliberately to avoid any problems that might arise in the reversion frequencies by the integration of the plasmids pS1 and pΔD into the vector or into the cloned fragment. Any co-integration of these plasmids would not confer temperature-stable phenotypes (*e.g.*, cadmium resistance). Both pOX7 and pOX86 were transducible into and from PS80, RN4220 and 1054 using appropriate phage (80 or 53). pOX7/pOX86 are compatible with the plasmids pS1 and pΔD and both appear to have a similar high copy number compared to pS1 and pΔD in all three strains. This is important since in all of the *S. aureus* strains used in the reversion experiments, the gene dosage (*i.e.*, the copies of the *binR* genes) is similar and presumably the protein (BinR) is expressed to the same level.

Plasmids that are unable to co-exist stably within a cell-line are said to be incompatible. Members of the same incompatibility group are closely related (Scott, 1984).

Fig.3.7 *Eco*RI digests of the co-integrates between the plasmid pOX1054 and pI9789 derivatives (pS1 and pΔD). The co-integrates were obtained in the reversion experiments (see section 3.4.2). The 10.2kb *Eco*RI piece of pS1 or the related 6.4kb *Eco*RI piece of pΔD has recombined with the 9.5kb piece of the plasmid pOX1054. Two new *Eco*RI pieces of 3.9kb and 15.8kb are generated for a co-integrate between pS1 and pOX1054, whereas, the co-integrate between pΔD and pOX1054 gives 3.9kb and 12kb *Eco*RI pieces.

KEY:

1 & 9- 1kb ladder

2- pOX1054::pS1

3- pOX1054 + pS1

4- pS1

5- pOX1054

6- pΔD

7- pOX1054 + pΔD

8- pOX1054::pΔD

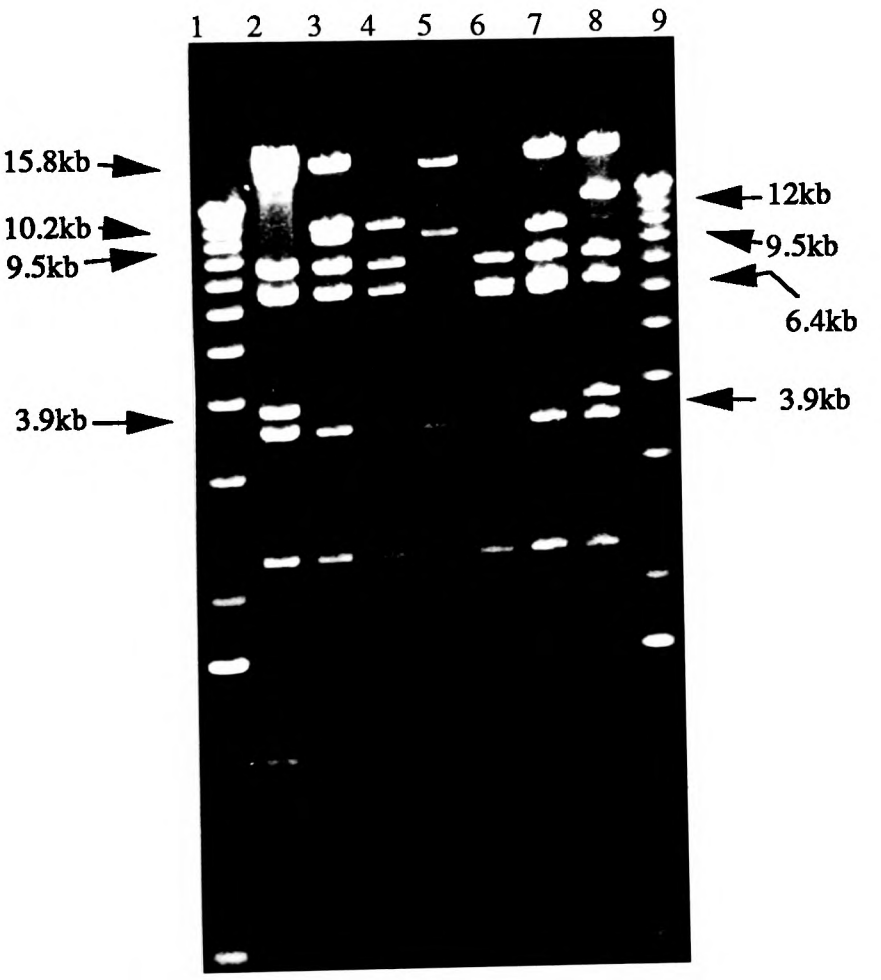


Table 3.4 *Eco*RI fragment sizes of the plasmids that resulted form the co-integration of pOX1054::pS1 and pΔDpOX1054::pΔD.

Plasmid	Fragment sizes (kb)								[Total]
pS1		**10.2**	7.9	6.5			2.3		[26.9]
pΔD			7.9	6.5	**6.4**		2.3		[23.1]
p1054	~20	**9.5**				3.7	1.2	.55	[~35]
pOX1054::pS1	~20	***~15.8***	7.9	6.5	***3.9***	3.7	2.3	1.2 .55	[~62]
pOX1054::pΔD	~20	***12***	7.9	6.5	***3.9***	3.7	2.3	1.2 .55	[~58]

Notes: The fragment sizes given in this Table are derived from Fig.3.7. The fragments involved in the recombination are given in **bold** text, whereas, the fragments that resulted from recombination are **bold** as well as in ***italics***. For details of the experiment please refer to Section 3.4.3.

The plasmid pOX1054 of the 1054 strain of *S. aureus* is compatible with pS1 or pΔD and pOX7 or pOX86 which suggests that pOX1054 is unrelated to either pI9789 or pOX7 and that is in some other compatibility group of the staphylococcal plasmids. Staphylococcal plasmids have been classified into 13 incompatibility groups by Poston and Naidoo (1983) and it would be of interest to discover whether or not pOX1054 is in an as yet undescribed group. However, the lack of any phenotype associated with pOX1054 would make such experiments very time-consuming.

Increase of the genetic load on the *S. aureus* strains by introducing new plasmids did not have any detectable effect on their growth rate (data not shown) which otherwise could have made it difficult to interpret the results of the reversion experiments.

A deletion of the mercury resistance determinant from the plasmid pΔD (resulting in pΔDmer^{s}) was observed during transduction of this plasmid from one strain to another. Mercury resistance on the alpha family (*e.g.*, pSK74) and the gamma family plasmids (*e.g.*, pI258) is encoded on a transposon called Tn*4004* which is flanked by direct repeats of the IS*257*-like insertion element, IS*431*. All of the plasmids carrying genes for mercury resistance examined, with the exception of the alpha family plasmid pUB101, share identity in the region of mercury resistance (Lyon and Skurray, 1987; Witte *et al.*, 1986). The plasmid pI9789 (and hence pΔD) is a gamma family plasmid (Shalita *et al.*, 1980) and presumably the mercury resistance is encoded on a Tn*4004*-like element on this plasmid. The loss of a similar ~7.2kb DNA from the plasmid pΔD, which resulted in the loss of the *mer*^{r}-determinant from the plasmid, may correspond to the deletion of the *mer*^{r}-transposon from the plasmid (by a homologous cross-over between the flanking IS*431*s, catalysed by the host general recombination system or by a resolution system or by a phage recombination system) or may be due to the non-replicative transposition of the *mer*^{r}-

transposon to the host chromosome. The transposition to the host chromosome, however, would not be detected because the plasmid-carrying donor cells are lysed prior to transduction. This kind of a deletion was not observed in the very closely-related plasmid pS1, although an equal number of transductants was analysed for both plasmids.

3.5.2 Growth-state and the reversion frequencies

For the reversion frequency experiments, rigorous attempts were made to standardize the experimental conditions (*e.g.*, concentration of cadmium; freshness and thickness of the CY-Cd-agar in the plates; temperature of the incubator, *etc.*). The concentration of cadmium acetate was increased from 13.3µg/ml (Browne, 1985; Johnston, 1971) to 26.6µg/ml, since, the culture showed an intrinsic resistance to the lower cadmium concentration and a background growth was seen on the plates which interfered with the experimental results.

The growth state of the initial inoculum from a plate, which was used to set-up the overnight (18hrs) broth culture for the use in determining the reversion frequencies, was found to cause variation in the reversion frequencies. The older the initial inoculum the higher the number of revertants. A possible explanation is that the older culture when introduced into broth had a longer lag phase than a newer culture and so at the end of 18hrs, most cells would still be in the exponential phase of growth, whereas the newer culture may already be entering stationary phase. The longer lag with the older culture could simply be the result of a much lower proportion of live cells.

3.5.3 *binR* and strain-specificity of the reversion frequencies

3.5.3.1 Reversion frequencies in PS80

Table 3.5 gives a summary of the reversion frequencies for the plasmids pS1 and pΔD in various *S. aureus* strains in the presence or absence of pOX86. A nearly three fold increase in the number of revertants for PS80N(pS1/pOX86) compared to PS80(pS1) is indicative of the, *in trans*, activity of *binR*. This gives indirect evidence for the transcription and translation of *binR* into a functional protein, as predicted (Rowland and Dyke, 1988). A gene dosage effect is observed, since pS1 itself has one copy of *binR* and pOX86 which provides additional copies increases the reversion frequency of the plasmid pS1. Although, the plasmid pOX86 is diluted out from the culture at 42°C (as it is temperature sensitive for replication and could not be isolated from any of the revertants tested), enough protein (BinR) has presumably already been synthesized to produce a detectable increase in the reversion frequency. Enhancement of the reversion frequency of the plasmid pS1 by a putative site-specific recombinase, BinR, suggests that for at least some revertants there is the involvement of site-specific recombination in the reversion phenomenon and it is likely that the reversion of pS1 to temperature-stability in PS80N is caused by recombination between the plasmid and the chromosome catalysed by BinR. BinR has already been shown to be active in catalysing specific recombination between the *res* sites, both resolution and inversion, in artificial plasmid systems (Rowland, 1989). It is noteworthy, however, that the presence of *binR* does not detectably enhance the reversion frequency of pΔD, *in trans*, although the sequence evidence shows that the *res* site is present. There are at least two possible explanations for this; either, another *cis-acting* factor (*e.g.*, a DNA sequence) is involved that acts in conjunction with BinR. This is absent from pΔD (for example, the half of the TIR_R of the transposon Tn*552* that is deleted

Table 3.5 Summary of the reversion frequencies

	Reversion frequencies			
Plasmid(s)	**PS80N**	**PS80::Tn*551***	**1054**	**RN4220**
pS1	33	300	147	4
pΔD	11	1000	62	4
pS1 + pOX86	108	-	151	4
pΔD + pOX86	11	-	55	4

from pΔD along with *blaI*, *blaRI* and *blaZ* could be important); Or, the reversion in this case may be governed by a completely different phenomenon from that for pS1 and is also catalysed by integration of the plasmid into the chromosome but involves other putative site-specific recombinases that are present on the plasmid pΔD, *e.g.*, *sin* and *binL*. The roles of the products of these genes in plasmid-chromosome interaction remain to be investigated. The reversion frequency for pΔDmer^S is the same as that for pΔD so that the deletion of the mercury resistance determinant appears to have no effect on the reversion.

In PS80::Tn*551*, which carries Tn*551* on its chromosome, the reversion frequencies are much higher and the frequency for pΔD is higher than that for pS1. This appears not to be related to the general homologous recombination system of the host, since no homology between the plasmid pS1 and Tn*551* was found on hybridization analysis (Oldridge, 1993). Browne (1985) investigated the effect of the increase in homology between the plasmid and the chromosome. She used the *S. aureus* strain PS80*blaIblaZ*, that has the transposon Tn*552* on the chromosome which is also present on pS1 (and in part on pΔD) and showed that the reversion frequencies of pS1 and pΔD in this strain are very close to those in PS80N so that homology apparently has little if any effect on the reversion. Investigation of the impact of the general homologous recombination system on reversion is hampered by the unavailability of a suitable recombination-deficient (Rec$^-$) strain of *S. aureus*. The only Rec$^-$ strain of *S. aureus* available is RN981 (a derivative of the *S. aureus* NCTC 8325) which has a long generation time and grows poorly. Wyman *et al.* (1974) reported that only 10% of bacteria of this strain are viable. The use of such a strain would give results that could not be directly comparable to those given by any of the Rec$^+$ strains used.

Previously, Browne (1985) has suggested that a protein active, *in trans*, and specified by the transposon, Tn*551*, is involved in the enhancement of the reversion frequency of the plasmids pS1 and pΔD in the strain PS80::Tn*551*. However, different results were obtained by Oldridge (1993). He used the plasmids pS1::Tn*551* and pΔD::Tn*551* and determined their reversion frequencies and showed that the presence of Tn*551*, on any one of the plasmids pS1 or pΔD, does not affect their reversion frequency. This suggests that when Tn*551* is present on the chromosome, it provides a hot-spot for recombination and hence increases the integration frequency of the plasmids pS1 and pΔD into the chromosome. Data, similar to these, have been reported by other workers. For example, hot-spots for recombination have been reported in the transposon Tn*21*; in a recA$^-$ *E. coli*, integration of antibiotic resistance genes from *Klebsiella pneumoniae* and *Serratia marcescens* into specific sites on Tn*21* (hs1 and hs2) resulted in replicon fusion (Nücken *et al.*, 1991). Presence of the ϕ11 prophage in *S. aureus* enhances the frequency of reversion of thermosensitive, replication-defective-mutant penicillinase plasmids by integration into the chromosome some 10^3-fold and it has been suggested that the integration is promoted at or near the attachment sites of ϕ11 on the chromosome (Schwesinger and Novick, 1975). In the case of Tn551, however, involvement of both a *cis* active recombinase protein and a hot-spot for recombination by the transposon Tn*551* cannot be ruled out.

3.5.3.2 Reversion frequencies in RN4220 and 1054

All of the experiments reported so far (Oldridge, 1993; Browne, 1985 and Johnston, 1971) to investigate the effect of the host chromosome on the reversion frequencies were in the PS80 strain of *S. aureus* and the chromosome differed only in the presence or absence of one of the transposons (*e.g.*, Tn*551* or Tn*552*). It was decided to

investigate whether different strains would give different results and so RN4220 and 1054 were used. The results of the reversion experiments obtained with both RN4220 and 1054 strains of *S. aureus* (see Table 3.5 for a summary) differ from those obtained in PS80N and the reversion frequencies are apparently not influenced by the presence of extra copies of *binR*.

The reversion frequencies are low and very similar in RN4220 for both pS1 and pΔD. None of the ten RN4220(pΔD) revertants, nor nine of the ten RN4220(pS1) revertants analysed showed any detectable plasmid so that presumably at least the cadmium resistance gene and probably the whole plasmid has integrated into the chromosome (see later). One of the examples, tested for RN4220(pS1), was found to contain a plasmid indistinguishable from pS1 by restriction enzyme pattern but is temperature-stable for replication. This plasmid presumably carries either a true reversion mutation or a suppressing mutation. It is also conceivable that a mutation on the host chromosome is causing the suppression of the temperature-sensitive phenotype. Further investigation of this mutant is in progress.

If reversion is the result of recombination, then the most plausible explanation for the low reversion frequencies of the two plasmids in RN4220 seems to be the absence of the site for recombination on the host chromosome for the integration of the plasmid. The integrative mechanism, if any, involved in the reversion of the plasmids pS1 and pΔD in RN4220, appears however, to be different from that in PS80N at least the following ways:

1- There is no evidence of the involvement of *binR*.

2- The reversion frequencies are lower than those in PS80N.

3- There is no detectable difference in the reversion frequencies of pS1 and pΔD.

4- More than one mechanisms seems to be involved in reversion. This may also be true for PS80N but has not been observed.

The reversion frequencies for the plasmids pS1 and pΔD in 1054 (Table 3.5) are higher than those in PS80N and RN4220. The reversion is not detectably influenced by *binR*, is lower for pΔD than that for pS1 and at least in some cases is the result of the integration of the plasmids into the resident cryptic plasmid pOX1054. Site-specific recombination in *S. aureus* has been shown to be responsible for the formation of stable plasmid co-integrates (Iordanescu, 1975). Most reports on co-integrate formation are on small plasmids and various mechanisms have been proposed that govern the formation of the co-integrates, such as, site-specific recombination (Gennaro *et al.*, 1987), host-mediated general recombination, phage-mediated recombination (Novick *et al.*, 1984 and, Murphy and Novick, 1980) and illegitimate recombination (Bashkirov *et al.*, 1988; Bashkirov *et al.*, 1987). Novick *et al.* (1984) showed that co-integrate formation between small Gram-positive plasmids at specific sites, RS_A and RS_B, is carried out by phage- or host-mediated general recombination. Transposons/insertion sequences can also serve as 'portable regions of homology' which allow homologous recombination to occur between DNA elements which would not otherwise share DNA homology and such recombinational events can lead to the formation of co-integrates as well as other DNA rearrangements (Kleckner, 1989; Cohen, 1976). Formation of co-integrates of the plasmid pOX1054 with pS1 or pΔD could conceivably have involved any of the known mechanisms or a new one. Preliminary analysis using the restriction enzyme *Eco*RI suggests that the recombination occurs within defined regions on the plasmids. In the case of the plasmid pS1, the site is on the large EcoA (10.2kb) fragment and for pΔD, the site is on the related EcoC (6.4kb) fragment. For both pS1 and pΔD, the recombination site on the plasmid pOX1054 is

within its EcoB (9.5kb) fragment and recombination generates a 3.9kb fragment together with a larger fragment (Table 3.4) for both pS1 and pΔD.

At this stage it is not possible to be precise about what is happening in the different situations. The reversion phenomenon differs in all four strains of *S. aureus* studied. *binR* is expressed in PS80N and enhances the reversion of pS1. However, the inability of *binR* to produce any detectable increase in the reversion frequency of pS1 in RN4220 and 1054 (inability to enhance the 'integration' of the plasmids into the host chromosome) may be due to the absence of the site for recombination on their chromosomes but could also be due to the lack of expression of *binR* in these strains.

The possible ways of obtaining temperature-stable cadmium resistance are:-

1. Reversion of the mutation.

2. Suppression of the mutation by a second mutation.

3. Suppression by integration into:

(a). The chromosome by	i, Site-specific recombination.
	ii, Homologous recombination.
(b). A second plasmid by	i, Site-specific recombination.
	ii, Homologous recombination.

For PS80N, it is believed that 3(a)i is the most common event.

For RN4220, it is not possible to say but 1, 2 and 3(a) are all possibilities.

For 1054N, 3(b)i seems most common.

For PS80::Tn*551* 3(a)i is the most likely.

Some of these possibilities with be examined in future chapters.

Chapter Four:

Interaction of pS1 With the PS80N Chromosome

Chapter Four: Interaction of pS1 With the PS80N Chromosome

4.1 Introduction

Previous data in Chapter 3 have indicated that the reversion phenomenon depends not only upon the structure of the plasmid pI9789 but also on the structure of the chromosome and/or on the nature of the host bacterium. One of the putative site-specific recombinases of pS1, BinR, enhances the reversion, *in trans,* suggesting the involvement of a site-specific recombination between plasmid and chromosome in PS80. There is no evidence for the involvement of BinR in reversion in RN4220 and 1054.

If an integrated plasmid is excised from the chromosome, linkage between the plasmid DNA and some chromosomal DNA may be expected in the similar way to the production of λ*dgal* or λ*bio* phage following integration of the phage λ into the *E. coli* chromosome. Johnston (1971) cited linkage of plasmid (*cad*) and chromosomal (*erm*) genes in support of the integration hypothesis. Johnston (1971) argued that this linkage was due to imprecise excision of pS1 from the chromosome, thereby picking up the chromosomal *erm* gene. Since, Em^r was subsequently found to be encoded on the transposon Tn*551* this linkage could be regarded as an artifact generated by transposition of Tn*551* before, during or after excision. The demonstration of the linkage of non-transposable chromosomal DNA to plasmid DNA would support the integration hypothesis. Browne (1985) obtained results similar to those of Johnston, but she could not show the linkage of any chromosomal DNA to pS1 other than Tn*551*. Both Johnston

(1971) and Browne (1985) did the integration experiments in PS80 with Tn*551* on the chromosome. In the present study, PS80N without Tn*551* was used for these experiments for simplification. Evidence for linkage between the plasmid and chromosomal DNA would not only support the integration hypothesis but would also provide data for any specific sites of recombination between the plasmid pI9789 and the chromosome. In this Chapter, experiments are reported that were designed to discover whether or not the plasmid can be co-valently linked to chromosomal DNA.

4.2 Generation of temperature-stable revertants

PS80(pS1) and PS80(pS1/pOX86) gave different reversion frequencies (Chapter 3) and reversion may involve different phenomena in the two strains. Temperature-stable revertants for both PS80(pS1) and PS80(pS1/pOX86) were thus used to investigate linkage between plasmid and chromosomal DNA and were obtained as in section 3.4.1. Bacteria were grown in CY-broth at 30^{o}C overnight and then 10^{6} colony forming units of this culture were plated onto CY-Cd (26.6μg/ml)-agar plates. The plates were incubated at 42^{o}C for 18hrs and twenty temperature-stable revertant colonies were picked from the PS80(pS1) revertants and twenty from the PS80(pS1/pOX86) revertants.

4.3 Recovery of plasmid from temperature-stable revertants by transduction

To test whether the gene conferring the cadmium resistance present on the original plasmid had become linked to chromosomal DNA, transduction was used. Such transduction had also been used by Browne (1985) and Johnston (1971) to recover plasmid from PS80(pS1) revertants. Phage 80 was grown separately on each of the 40 colonies and was then used to transduce Cd^r to PS80. These transductants were called 'revertant-derived transductants' and two transductants from each transduction were picked and labelled 'tdA' and 'tdB' (*e.g.*, for revertant colony-1, the two derived transductants were labelled as Rev1tdA and Rev1tdB). Thus, a total of 80 transductants were selected.

4.4 DNA analysis of the revertant-derived transductants

In the first instance, plasmid DNA was prepared from all the eighty revertant-derived transductants by the small-scale method and was electrophoresed uncut on 0.7% (w/v) agarose gels to determine the size of the plasmids. Three different sizes were recognized and eighteen transductants derived from both PS80(pS1) and PS80(pS1/pOX86) revertants were selected for further analysis as follows :

Set-I: A set of eleven transductants was picked out of the sixty-three [28 from PS80(pS1) and 35 from PS80(pS1/pOX86) revertants] that had the plasmid DNA of the same size. Five of them (Rev2tdA, Rev3tdB, Rev10tdB, Rev16tdB and Rev19tdB)

originated from PS80(pS1) type revertants and the other six (Rev30tdB, Rev32tdB, Rev33tdB, Rev36tdA, Rev40tdA and Rev40tdB) came from PS80(pS1/pOX86) type revertants.

Set-II: A second set of six transductants was picked out of the sixteen [10 from PS80(pS1) and 6 from PS80(pS1/pOX86) revertants] that showed plasmid DNA of similar size. Four of these (Rev5tdA, Rev14tdB, Rev18tdA and Rev20tdA) came from the PS80(pS1) type revertants and the other two (Rev24tdA and Rev25tdA) from PS80(pS1/pOX86) type revertants.

Set-III: Rev8tdA showed plasmid DNA of a unique size and was also picked for further analysis.

Plasmid DNA was prepared from these eighteen selected colonies using the CsCl method and analysed after digestion with *Eco*RI. The transductants were grouped into four types on the basis of *Eco*RI digestion (Types I, II, III and IV). Six examples of Type I (originally from Set-II), seven of Type II, four of Type III (both from Set-I) and one of Type IV (from Set-III) were obtained out of the total eighteen analysed. The *Eco*RI digests of the four types of revertant-transductants' plasmid DNA are shown in Fig.4.1 and the fragment sizes are given in Table 4.1. The plasmid DNA was also digested with *Bgl*II. All examples of the same *Eco*RI type gave indistinguishable *Bgl*II digestion patterns. Fig.4.2 shows *Bgl*II digests of the plasmid DNA of one example of each of the four types of the revertant-derived transductants. The fragment sizes of the *Bgl*II digestions are given in the Table 4.2. On the basis of this tentative maps of the plasmids were produced (Fig.4.3). Type I is indistinguishable from pS1, and the other three types have deletions within the EcoA fragment (10.2kb) of pS1 that includes Tn*552* and the invertible region. Only Type I

Fig.4.1 *Eco*RI digests of the plasmids of four types of the revertant-derived transductants.

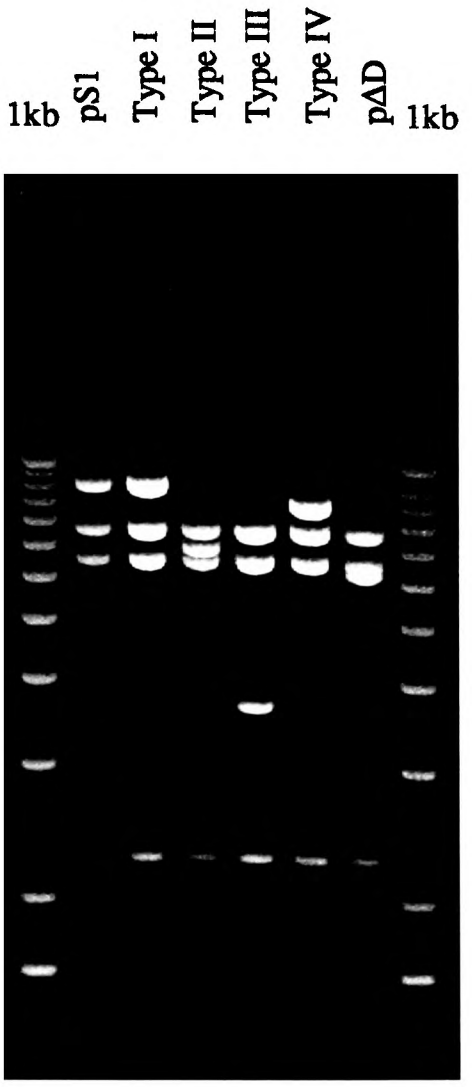


Table 4.1 *Eco*RI restriction fragment sizes of the pS1 revertant-derived transductants.

Plasmid	(Examples)	Fragment sizes (kb)						Total
pS1		**10.2**	7.9		6.5		2.3	26.9
Type I	(6)	10.2	7.9		6.5		2.3	26.9
Type II	(7)		7.9	***7.0***	6.5		2.3	23.7
Type III	(4)		7.9		6.5	***3.7***	2.3	20.4
Type IV	(1)	***9.0***	7.9		6.5		2.3	25.7

Notes: The fragment sizes given in this Table refer to Fig.4.1. The fragments of the revertant-derived transductants, that are in **bold** as well as in ***italics,*** are thought to be derived from the **10.2**kb *Eco*RI fragment of the plasmid pS1.

Fig.4.2 *Bgl*II digests of the plasmids of four types of the revertant-derived transductants.

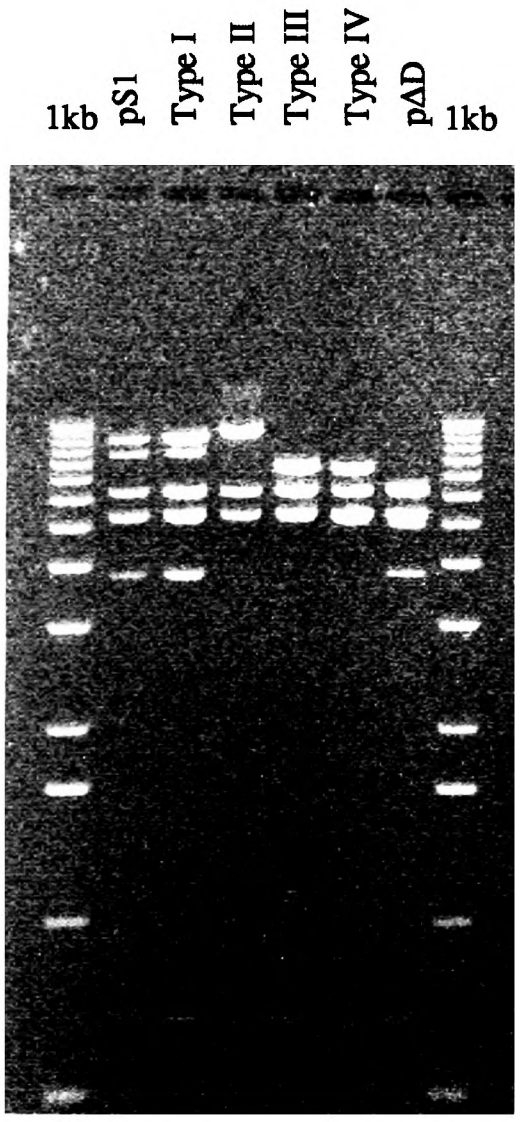
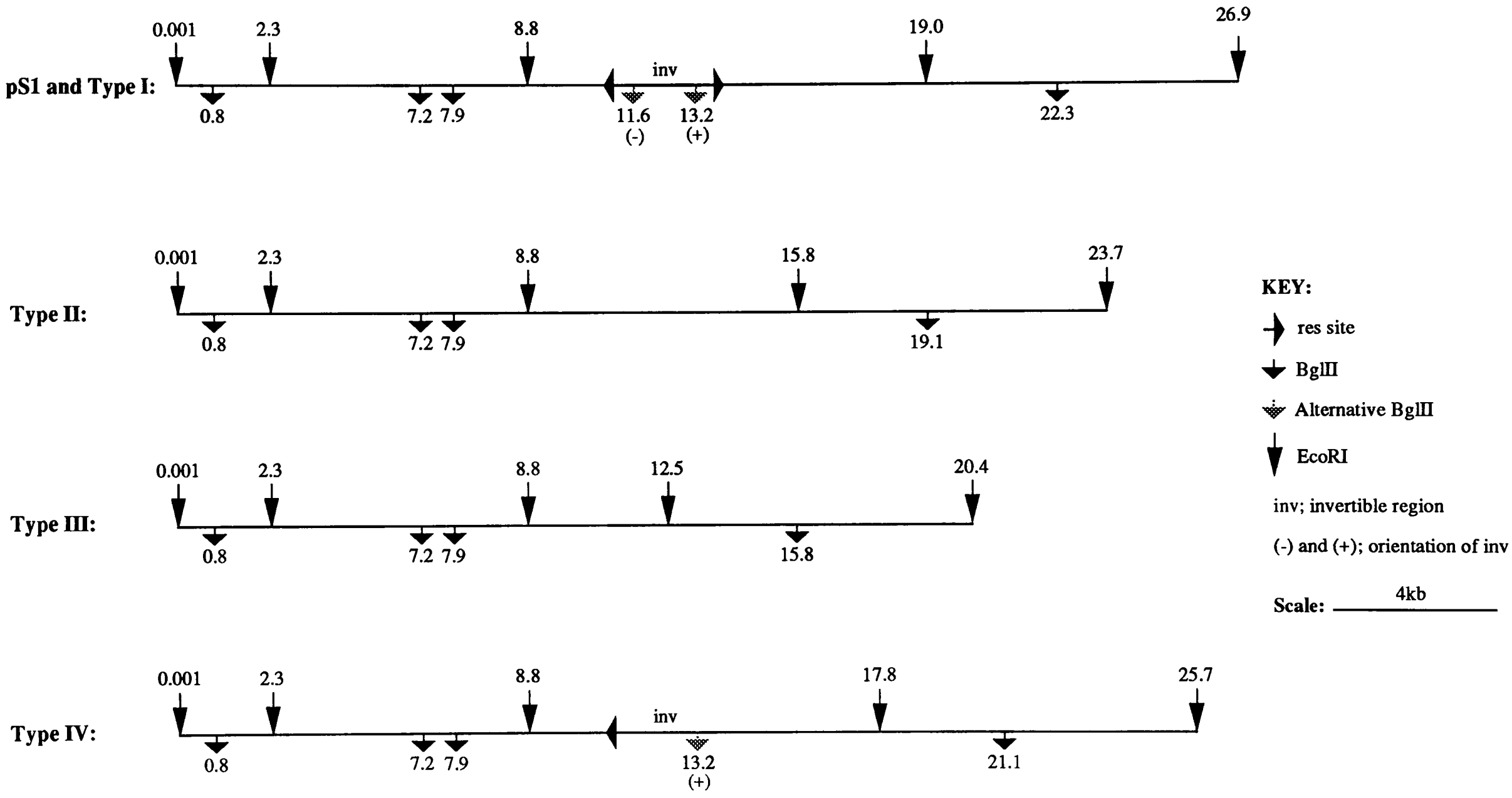


Table 4.2 *Bgl*II restriction fragment sizes of the pS1 revertant-derived transductants.

Plasmid	Fragment sizes (kb)					Total
pS1	**(10.7)**	6.4	5.4	**(3.7)**	0.7	26.9
	(9.1)			**(5.3)**		
Type I	**(10.7)**	6.4	5.4	**(3.7)**	0.7	26.9
	(9.1)			**(5.3)**		
Type II	***11.2***	6.4	5.4		0.7	23.7
Type III	***7.9***	6.4	5.4		0.7	20.4
Type IV	***7.9***	6.4	5.4	***5.3***	0.7	25.7

Notes: The fragment sizes given in this Table refer to Fig.4.2. The fragments of the plasmid pS1 and the Type I revertant-derived transductants that are given in the **brackets** correspond to the bands that appear on the gel in half molar concentrations due to the asymmetric location of the *Bgl*II site in the invertible region (see Fig.4.3). The fragments of the revertant-derived transductants, that are in **bold** as well as in ***italics***, are the derived fragments of the plasmid pS1 that are thought to have been generated by deletion within the **10.2**kb *Eco*RI fragment.

Fig.4.3 Restriction enzyme maps showing *Eco*RI and *Bgl*II restriction sites on the plasmids of the four types of the revertant-derived transductants. The plasmids have been linearized at one of the *Eco*RI sites.



shows inversion so it seems that at least one *res* site has been deleted in Type II, Type III and Type IV.

4.5 Thermosensitivity of Cd^r in the revertant-derived transductants

To find out whether or not the revertant-derived transductant plasmids are thermosensitive for replication, and that the thermostability was not due to a back mutation in the plasmid or to a thermosensitivity-suppressor mutation on the chromosome, the thermosensitivity was investigated by measuring the reversion frequencies of some of the revertant-derived transductants (Table 4.3).

The cultures were grown at 30°C overnight and then 10^6 bacteria were plated onto CY-Cd (26.6μg/ml) agar plates, in triplicate. The plates were incubated at 42°C for 18 hours before the results were recorded. The results for two out of the three Type I revertant-derived transductants tested are similar to those for PS80(pS1). The third Type I transductant gave an almost three times higher reversion frequency than did PS80(pS1). Type II and Type III transductants gave frequencies close to those for PS80(pΔD), whereas, the Type IV transductant gave a frequency intermediate of those for PS80(pS1) and PS80(pΔD). The results are summarized in Table 4.3 and suggest that the temperature-sensitive phenotype of the plasmid pS1 is not lost during reversion and can be recovered.

Table 4.3 Reversion frequencies of the four types of revertant-derived transductants and control plasmids.

Plasmid	Reversion frequency@	Deletion analysis†
PS80(pS1)	37±5	
PS80(pΔD)	13±4	
Type-I		
rev5tdA	103±10	YES
rev14tdB	44±7	YES
rev18tdA	47±5	ND
Type-II		
rev2tdA	14±3	YES
rev16tdB	8±4	YES
rev19tdB	11±4	ND
Type-III		
rev3tdB	6±3	YES
rev32tdB	10±3	YES
rev33tdB	7±4	ND
Type-IV		
rev8tdA	20±4	YES

@Number of cadmium resistant colonies per 10^6 cfu (Section 4.5).
†Sections 4.6.
ND = not done.

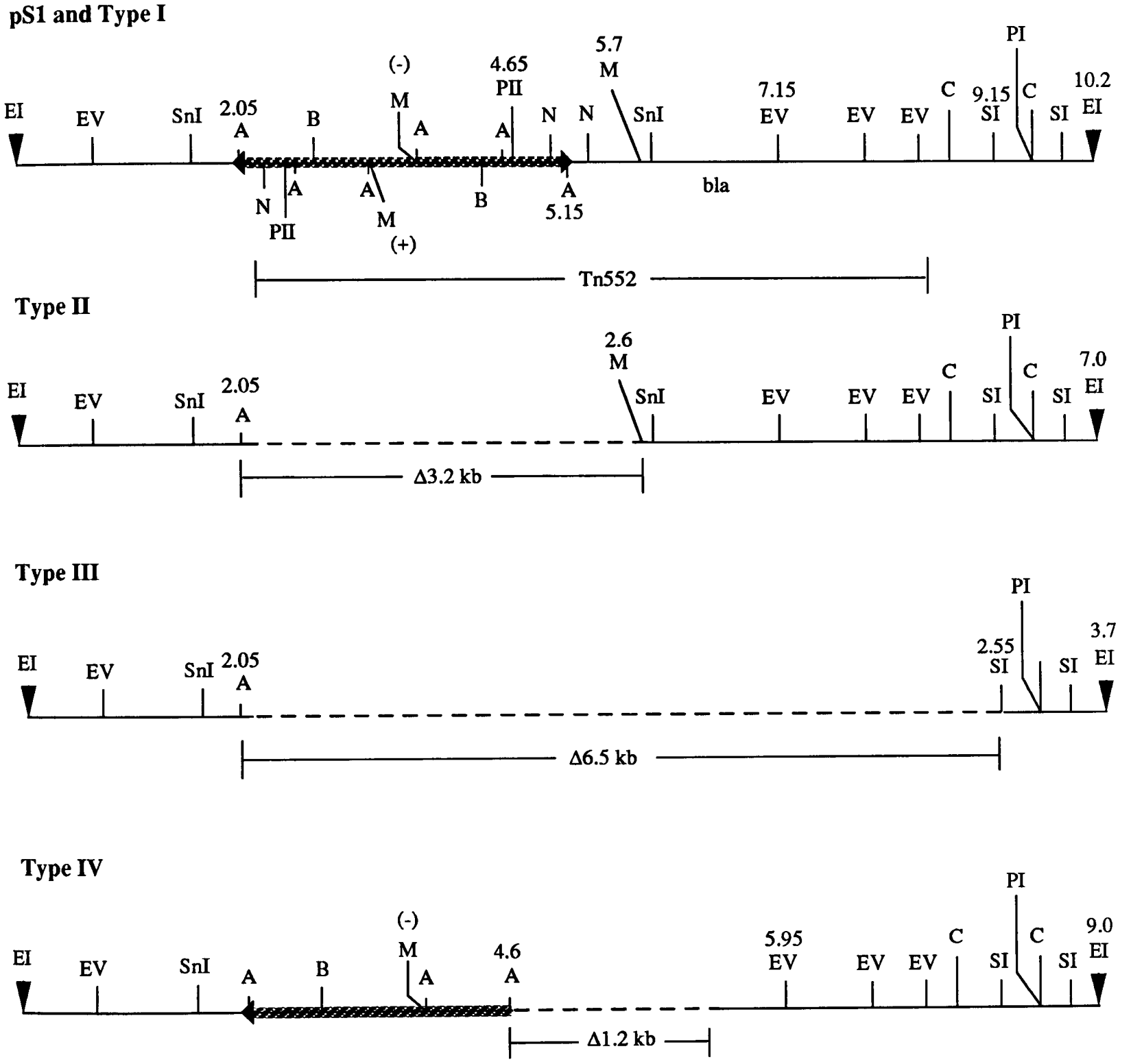
4.6 Mapping of the deletions on the revertant-derived transductants' plasmids

The positions of the deletions were investigated. Digestion with *Eco*RI and *Bgl*II gave the same restriction enzyme fragments for all plasmids within a particular type. It was, therefore, decided to map only two examples of each type (one for Type IV). In every case, the deletion had been shown to be within the EcoA fragment and so restriction enzymes known to cut within this fragment were chosen. The following were used singly and in pairs:- Rev5tdA and Rev14tdB (Type I); Rev2tdA and Rev16tdB (Type II); Rev3tdB and Rev32tdB (Type III); Rev8tdA (Type IV).

The restriction enzyme maps of the plasmids of the four types of revertant-derived transductants are shown in Fig.4.4. Type I transductant plasmids showed a restriction enzyme pattern indistinguishable from that of pS1. For both Type II and Type III, the deletions have removed the entire invertible region, *inv*, and seem to start at or near one *res* site. For Type II, the 3.2kb deletion has deleted DNA between the *Acc*I site (2.05) and the *Msc*I site (5.7) that is situated in the *bla* region of Tn*552*. For Type III, the 6.5kb deletion has removed DNA between the *Acc*I site (2.05) and the proximal *Sal*I site (9.15) situated outside the transposon, Tn*552*, resulting in the deletion of the whole of the Tn*552*. In the case of Type IV, the deletion starts at a point, probably, within the *inv* region and has involved the *res* site (*resR*) with the result that the *inv* is frozen in the negative orientation. The deletion is of 1.2kb somewhere between the *Acc*I site (4.6) and the proximal *Eco*RV (7.15) but situated towards the *Acc*I site as suggested by the absence *Pvu*II site (4.65).

The results show that the deletions start at or near one of the *res* sites at one end. Although, in most of the cases the *inv* has been involved, yet none of the deletions appear

Fig.4.4 Restriction enzyme maps showing deletions within the EcoA fragment in three types of pS1 revertant-derived transductants. The restriction enzyme map for the Type I transductant is indistinguishable from that of pS1 and is not shown separately. The region flanked by *res* sites on the plasmid pS1 is capable of 'flipping' and can occur in either of the two orientations. [KEY: A, *Acc*I; B, *Bgl*II; C, *Cla*I; EI, *Eco*RI; EV, *Eco*RV; M, *Msc*I; N, *Nde*I; PI, *Pvu*I; PII, *Pvu*II; SI, *Sal*I; SnI, *Sna*BI]. All the sizes are in kilobase pairs.



KEY:

◆ res

Δ Deletion

(-) Negative orientation

(+) Positive orientation

to be a result of precise excision of the *inv* from the plasmid. Also, for the Type III plasmids, the deletion does not correspond to a precise excision of the transposon Tn*552*, since the *Cla*I site (8.8) of pS1 is absent and it is not within Tn*552*.

4.7 Hybridization of the total DNA of the revertants

To provide direct evidence for the integration of the plasmid pS1 into the host chromosome, it was decided to hybridize the chromosomal DNA of the revertants using suitable probes derived from pS1. These experiments should yield results that could answer the following questions:-

1. Is there evidence for integration? For example, this would be indicated if new restriction enzyme fragments hybridizing to the pS1 probe are present.

2. If integration occurs:-

    a) Is there a specific site on the plasmid?

    b) Is there a specific site on the chromosome?

    c) Are there multiple sites of insertion?

    d) Is a single copy of the plasmid integrated?

4.8 Design of the hybridization probes

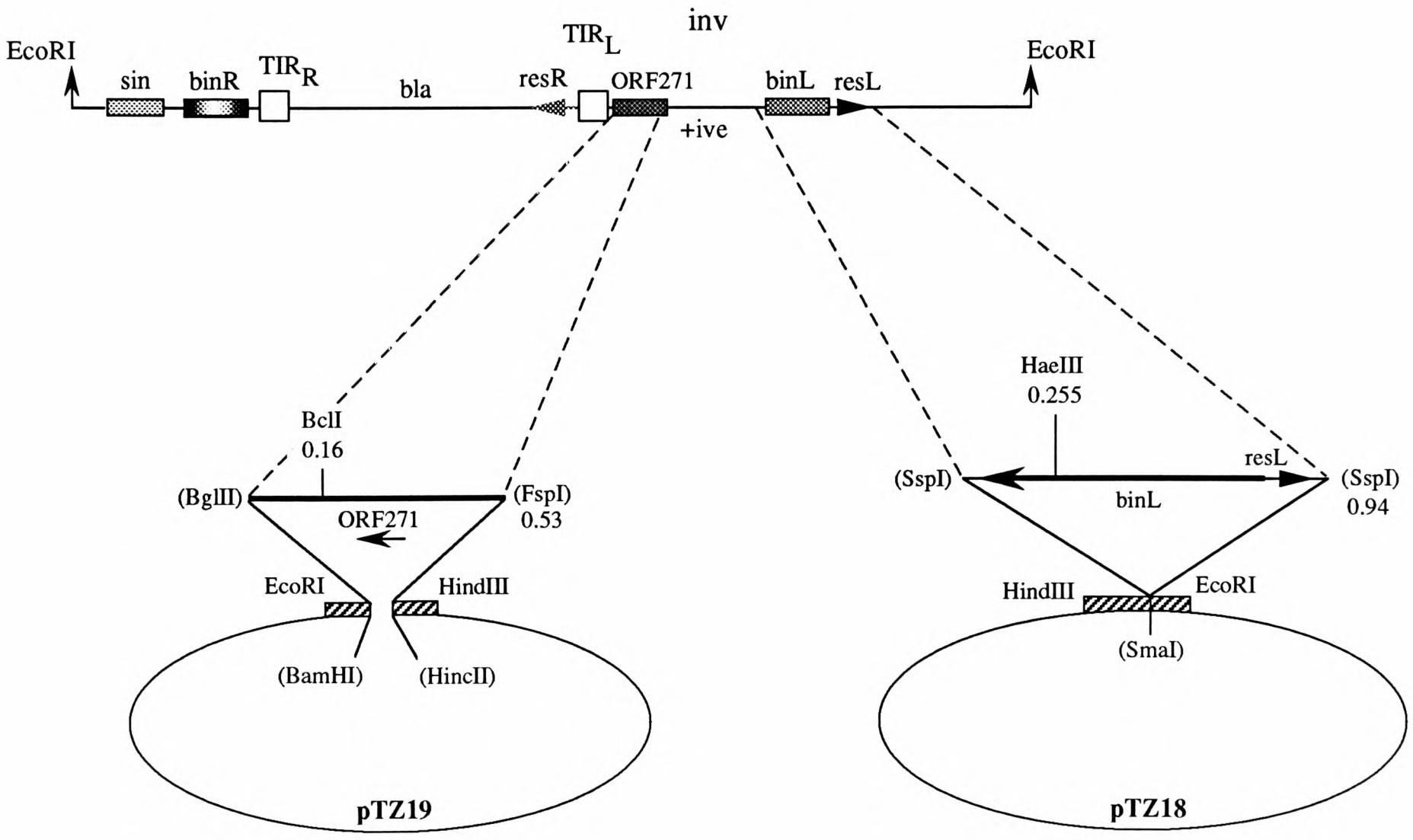
The plasmids pSJZ17 and pSJZ50 (Rowland, 1989), which are pTZ based clones, were chosen as probes. Neither pTZ18 nor pTZ19 hybridize to the chromosomal DNA of PS80 or to the plasmid pS1 (this study; data not presented).

The plasmid pSJZ17 is a pTZ19 based clone of a 0.53kb internal *Fsp*I - *Bgl*II fragment of the ORF271 of the plasmid pS1. The map of this plasmid is shown in Fig.4.5. The plasmid pSJZ50 is a pTZ18 based clone of a 0.94kb *Ssp*I - *Ssp*I fragment from the plasmid pS1 containing *binL* and *resL* (the 0.94kb fragment is generated when *inv* is in positive orientation). The map of this plasmid is also shown in Fig.4.5. The probable sites for hybridization of these probes on the plasmid pS1 are shown in Fig.4.6. The probe pSJZ17 is specific and should bind only to the ORF271 (a part of Tn*552*) on the plasmid pS1. The probe pSJZ50 is less specific and will hybridize not only to its specific sequence on pS1 (*i.e.*, *binL* and *resL*) but also, to *resR*, which is approximately 80% homologous to *resL*, and to the putative site-specific recombinase genes, *binR* and *sin*, which show considerable homology to *binL* (Rowland, 1989).

4.9 Results of the hybridization of the total DNA of revertants to pSJZ17 and pSJZ50

Total DNA was prepared from the revertants, Rev2 (parent of Type II rev2tdA), Rev3 (parent of Type III rev3tdB) and Rev8 (parent of Type IV rev8tdA). Total DNA of

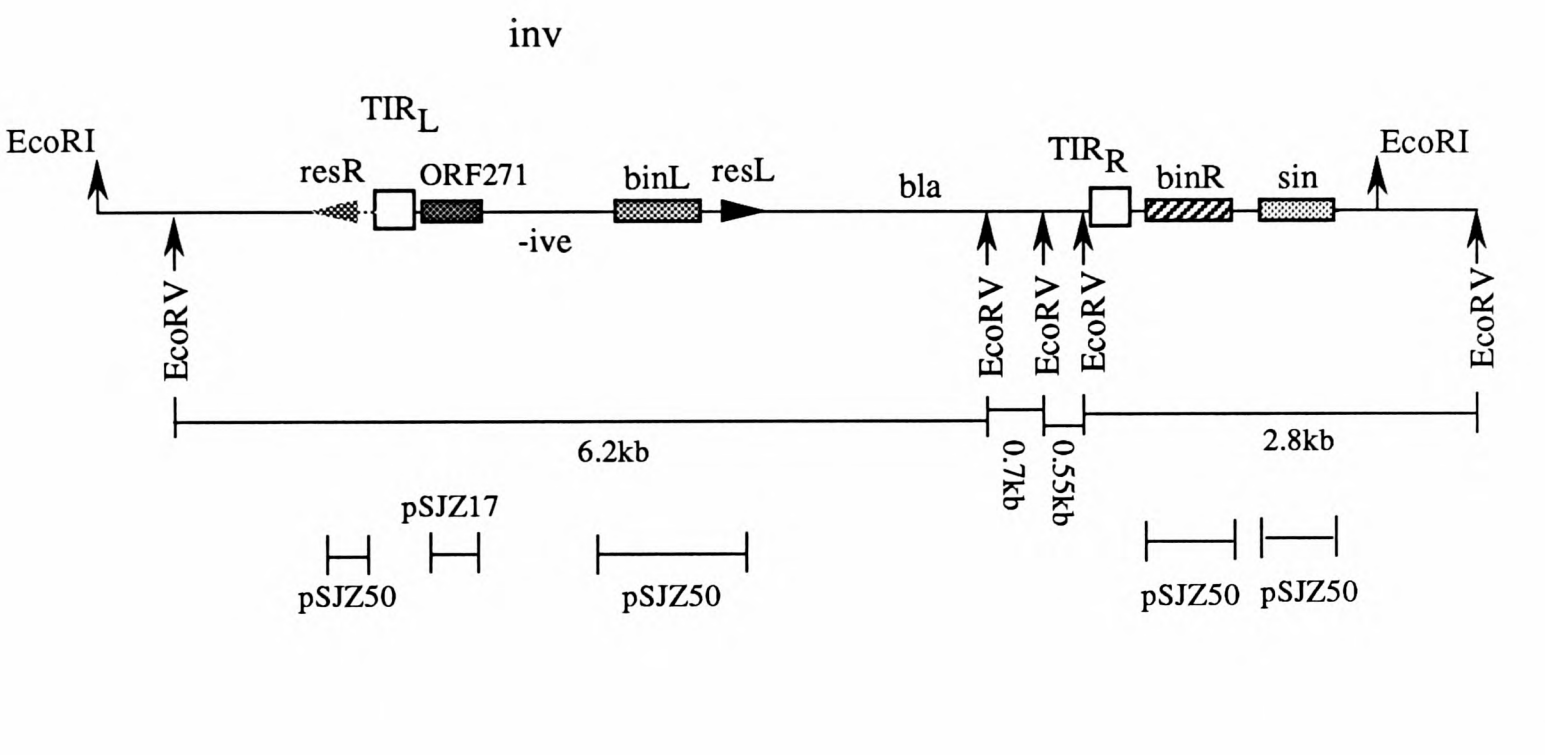
Fig.4.5 Restriction enzyme maps of the plasmids pSJZ17 and pSJZ50, and the regions from which they were derived on the EcoA fragment of the plasmid pS1 are shown. The sites that are lost during cloning are given in brackets. The drawings are not to scale. (▨ = multiple cloning site)



pSJZ17:

pSJZ50:

Fig.4.6 The regions on the EcoA fragment of the plasmid pS1 where the probes pSJZ17 and pSJZ50 would be expected to hybridize are shown. An *Eco*RV site outside the EcoA fragment on pS1 (on the right-hand side of the map) is also shown. Various ORFs are shown. TIR_R and TIR_L are the terminal inverted repeats of the transposon Tn*552*.



PS80N, RN4220 and the plasmid pS1 were used as controls. The DNA of all the samples was digested with *Eco*RV and electrophoresed on two 0.7% (w/v) agarose gels. Southern blots were taken of the two gels and one hybridized to pSJZ17 digested with *Eco*RI and the other to similarly digested pSJZ50 and then developed using the Stratagene Flash System.

The probe pSJZ17 hybridized to a 6.5kb fragment of the DNAs of PS80N and Rev2, and to 6.5kb and 5.1kb fragments of Rev8 but not to RN4220 DNA. On the plasmid pS1 and Rev3 DNA, the probe hybridized to a 6.2kb fragment (Fig.4.7). The sizes of the fragments that hybridized are given in Table 4.4.

The plasmid pSJZ50 hybridized to two fragments (6.2kb and 2.8kb) of the plasmid pS1, to a 6.5kb fragment of PS80N but not to RN4220. Three fragments hybridized to the probe for each of Rev2 (*i.e.*, 6.5kb, 3.2kb and 2.8kb) and Rev8 (*i.e.*, 6.5kb, 5.1kb and 2.8kb), whereas, only one fragment (6.2kb) from the DNA of Rev3 (Fig.4.8). The sizes of the fragments that hybridized are given in Table 4.5.

In the case of plasmid pS1, the hybridization of both of the probes to a 6.2kb *Eco*RV fragment was as expected, since this is the fragment from which both probes originated. The 2.8kb *Eco*RV fragment that hybridizes to pSJZ50 and not to pSJZ17 is the one which contains *binR* and *sin* (Fig.4.6). Hybridization of the probes to a fragment on the PS80N chromosome is noteworthy and suggests the presence of structures similar to ORF271 and *binL/resL* on the chromosome and that despite the fact that PS80N does not produce β-lactamase, at least a part of Tn*552* found on PS80 is still present. The results also show that Tn*552* is not present on the chromosome of RN4220.

For Rev2 (which gives rise to a Type II transductant plasmid with a 3.2kb deletion within the 6.2kb *Eco*RV fragment - see Fig.4.4), the chromosomal 6.5kb *Eco*RV band is not affected but the 6.2kb *Eco*RV band from pS1 is absent, although the 2.8kb *Eco*RV,

Fig.4.7 (A). Photograph of the agarose gel stained with ethidium bromide. Total DNA was digested with *Eco*RV and electrophoresed on a 0.7% agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pSJZ17 probe: (B). An autoluminograph of the membrane showing hybridization of the plasmid pSJZ17 to the total DNA of PS80 revertants.

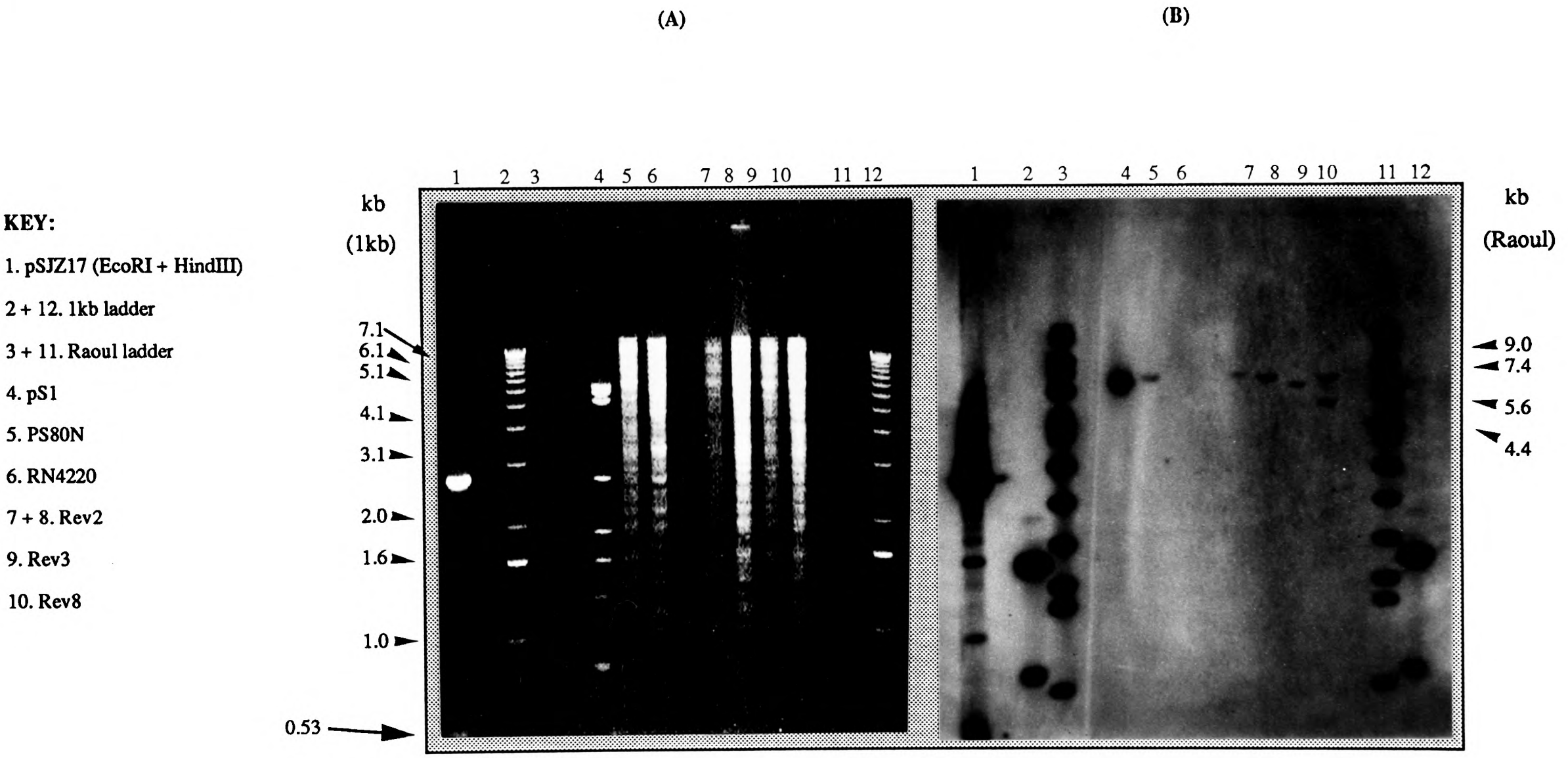


Table 4.4 *Eco*RV fragment sizes of the of the total DNA of the revertants that hybridize to the plasmid pSJZ17.

DNA	Fragment sizes (kb)		
pS1	6.2		
PS80N	6.5		
RN4220	--None--		
Rev2	6.5		
Rev3	6.2		
Rev8	6.5	5.1	

Fig.4.8 (A). Photograph of the agarose gel stained with ethidium bromide. Total DNA was digested with *Eco*RV and electrophoresed on a 0.7% agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pSJZ50 probe: (B). An autoluminograph of the membrane showing hybridization of the plasmid pSJZ17 to the total DNA of PS80 revertants.

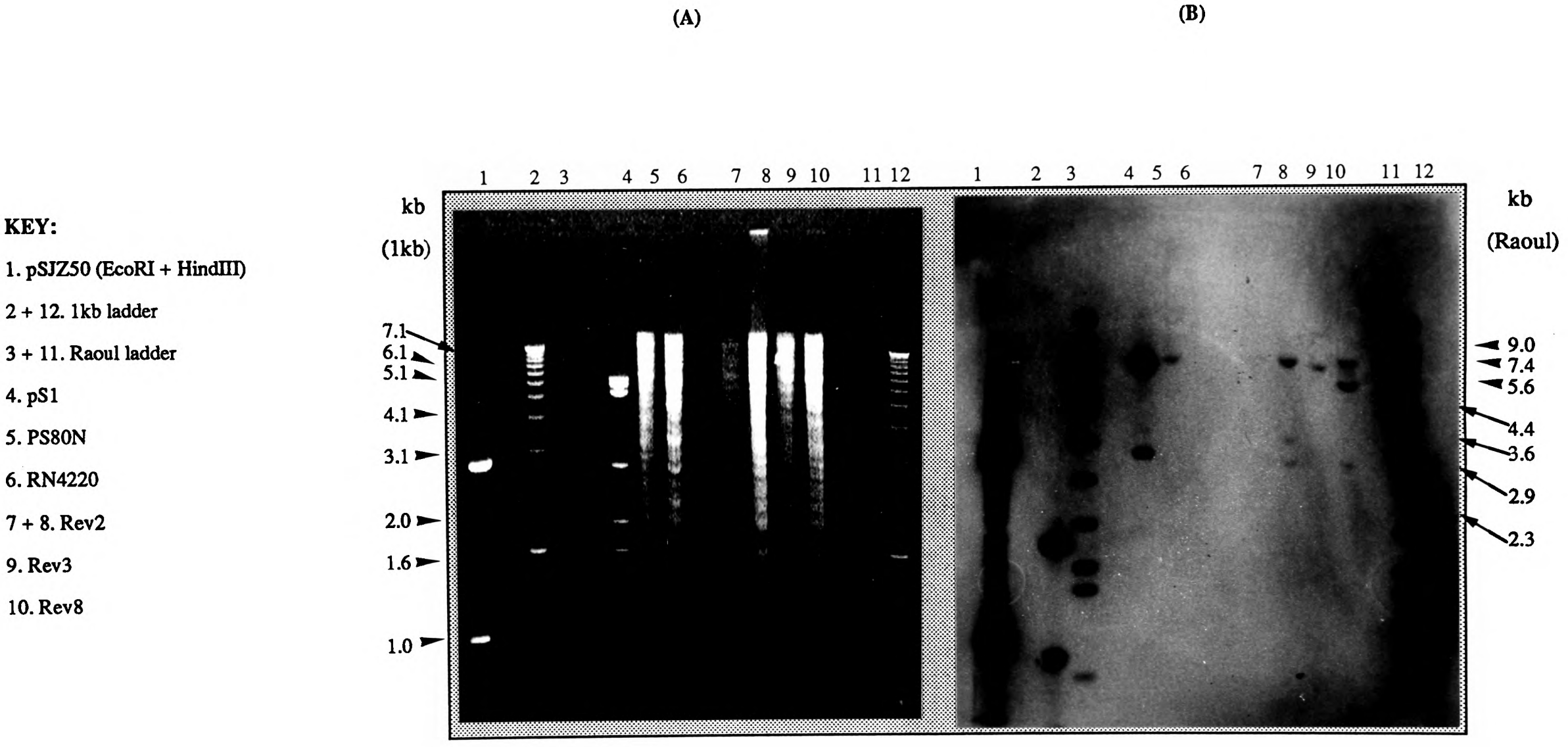


Table 4.5 *Eco*RV fragment sizes of the of the total DNA of the revertants that hybridize to the plasmid pSJZ50.

DNA	Fragment sizes (kb)				
pS1		6.2			2.8
PS80N	6.5				
RN4220	--None--				
Rev2	6.5			3.2	2.8
Rev3		6.2			
Rev8	6.5		5.1		2.8

that hybridizes to pSJZ50 in the DNA of pS1, is present together with a new 3.2kb band not containing ORF271 DNA, but carrying *binL* or *binR* or *sin* or *res*. The origin of this 3.2kb band is not clear. It is, however, possible that this fragment is a result of deletion in the 6.2kb EcoRI fragment of pS1. The results with Rev2 do not provide proof of integration of pS1 into the chromosome in this revertant.

For Rev3, the 6.5kb chromosomal band of PS80N that hybridizes to ORF271 DNA, is no longer present and the 2.8kb band of pS1 is absent. This argues for change in both the chromosomal and the plasmid in this case although it does not provide proof of integration, it does provide proof of interaction between the chromosome and the plasmid, with probable deletion both from the chromosome and subsequently from the plasmid.

Rev8 still carries the chromosomal 6.5kb bands and the 2.8kb band of pS1, but in addition has a 5.1kb fragment that corresponds with the 1.2kb deletion from within the 6.2kb pS1 band, shown for the plasmid in the transductant derived from Rev8 (see Fig.4.4). Thus it is concluded that in this case the deletion has occurred prior to the preparation of transducing phage.

Overall, the results are not conclusive but do suggest interactions between pS1 and the chromosome during the production of revertants. To see whether the whole of the plasmid is present in the revertants and to try to identify within which *Eco*RI piece of pS1 integration (if any) occurs, it was decided to use the complete pS1 as a probe.

4.10 Use of the plasmid pS1 as probe

Total DNA was prepared from revertants Rev5 (parent of Type I Rev5tdA), Rev14 (parent of Type I Rev14tdB), Rev24 (parent of Type I Rev24tdA), Rev2 (parent of Type II Rev2tdA), Rev16 (parent of Type II Rev16tdB), Rev26 (parent of Type II Rev26tdB), Rev3 (parent of Type III Rev3tdB), Rev32 (parent of Type III Rev32tdB), Rev33 (parent of Type III Rev33tdB) and Rev8 (parent of Type IV Rev8tdA). Total DNA of PS80N, RN4220 and the plasmid pS1 were used as controls. Plasmid DNA of one example of each type of the revertant-derived transductant was also used. All DNA samples were digested with *Eco*RI and electrophoresed on 0.7% (w/v) agarose gel slowly to prevent smearing of the chromosomal DNA (40 volts for ~14hrs in TBE buffer). The DNA was transferred and fixed to nylon membrane and hybridized to the plasmid pS1 digested with *Eco*RI (and with pUC18 to illuminate the Raoul ladder) and the hybridization detected using the Stratagene Flash System. The results are given in Fig.4.9 and the fragments that hybridize to the plasmid pS1 are given in the Table 4.6.

The pS1 probe hybridizes to a single *c.*16kb *Eco*RI fragment of PS80N (Lane 3) but not to the chromosome of RN4220, even though a larger amount of the latter was electrophoresed (Lane 5). The whole of pS1 (27kb) cannot be present on the chromosome and the 16kb piece must contain the 6.5kb *Eco*RV fragment.

The pS1 and Type I plasmids are very similar in their hybridization pattern and show all the predicted bands (Lane 4 and 6). In addition there are fainter hybridizing bands at 13.5kb and 7kb that do not correspond to any band seen in the corresponding lane on the ethidium bromide stained photograph. These bands have been ignored as either artifacts or very minor contaminants.

Fig.4.9 (A). Photograph of the agarose gel stained with ethidium bromide. Total DNA was digested with *Eco*RI and electrophoresed on a 0.7% agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pS1 probe (plus pUC18 to illuminate the Raoul ladder): (B). An autoluminograph of the membrane showing hybridization of the plasmid pS1 to the total DNA of PS80 revertants.

KEY:

1. 1kb ladder
2 + 20. Raoul ladder
3. PS80N
4. pS1
5. RN4220
6. Type I plasmid
7. Rev5
8. Rev14
9. Rev24
10. Type II plasmid
11. Rev2
12. Rev16
13. Rev26
14. Type III plasmid
15. Rev3
16. Rev32
17. Rev33
18. Type IV plasmid
19. Rev8

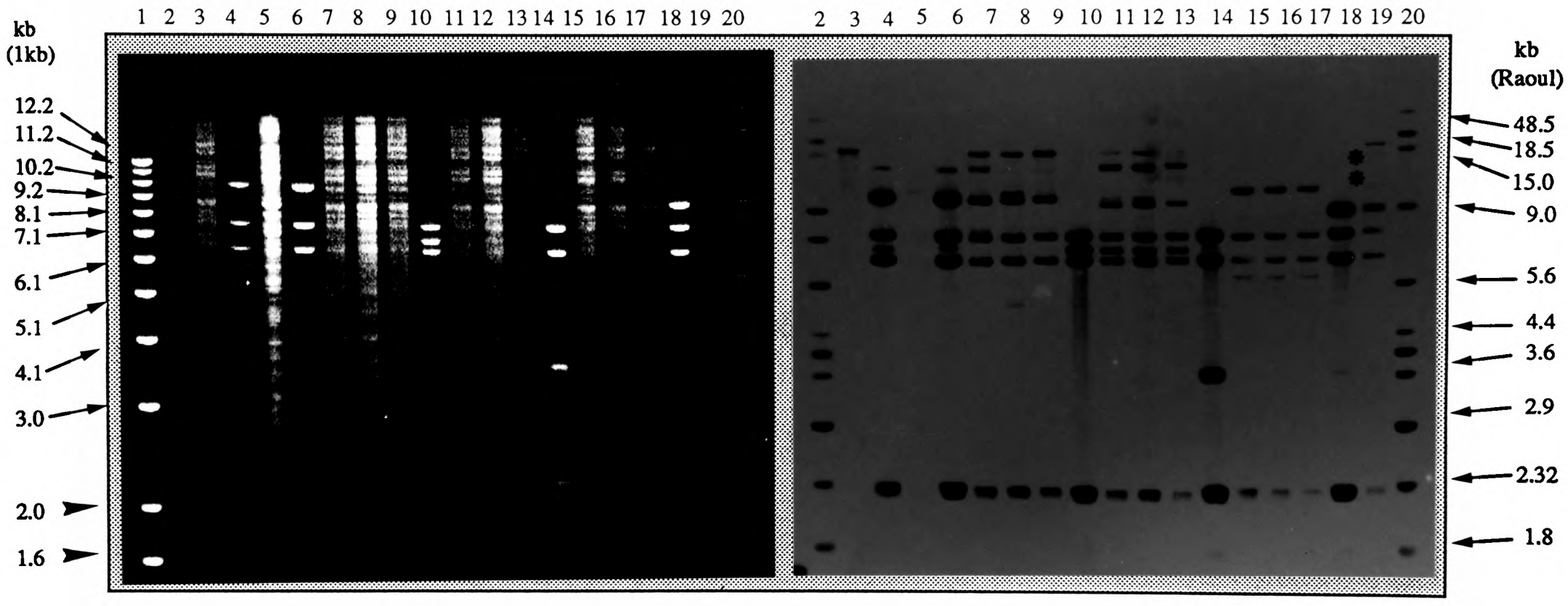


Table 4.6 *Eco*RI fragment sizes of the of the total DNA of the revertants and their derived plasmids that hybridized to the plasmid pS1.

DNA	Fragment sizes (kb)								
PS80N	16								
pS1			10.2		7.9		6.5		2.3
Type I			10.2		7.9		6.5		2.3
rev5	16	*13.5*	10.2		7.9		6.5		2.3
rev14	16	*10.6*	10.2		7.9		6.5	*(5.1)*	2.3
rev24	16	*(13.5)*	10.2		7.9		6.5		2.3
Type II (plasmid)					7.9	7.0	6.5		2.3
rev2	16	*13.5*	*10.2*	*9.7*	7.9	7.0	6.5		2.3
rev16	16	*13.5*	*10.2*	*9.7*	7.9	7.0	6.5		2.3
rev26	(16)	*13.5*	*(12.2)*	*9.7*	7.9	7.0	6.5		2.3
Type III (plasmid)				7.9		6.5		3.6	2.3
rev3		*11*		7.9		6.5	*5.7*		2.3
rev32		*11*		7.9		6.5	*5.7*		2.3
rev33		*11*		7.9		6.5	*5.7*		2.3
Type IV (plasmid)	16			9.0	7.9	6.5			2.3
rev8	16	*(14.5* 13.5 12* 11)*		9.0	7.9	6.5			2.3

Note: The fragments that are not shown in the *Eco*RI-hybridization to pS1 of either pS1 or PS80N are given in *italics*. The fragments that are very faint are given in (brackets).

For the three revertants that gave rise to Type I plasmids which are indistinguishable from pS1 by restriction enzyme analysis, it is clear that all four bands equivalent to those of pS1 are present. It is not possible to see any alteration in the size of these bands, so possible conclusions are: (i). that the plasmid has not integrated, (ii). that there are multiple tandem copies of the plasmid integrated in such a way that the junction between tandem copies is near to an *Eco*RI site, (iii). that a single copy has integrated at a site close to an *Eco*RI site. However, additional hybridizing bands are present (for example, for Rev14; Lane 8), although it seems that the 16kb chromosomal band of PS80N is unaffected. For Rev14, a band of 10.6kb and a faint band at 5.1 are seen but it is not possible to draw conclusions about the nature of these bands, and hence not possible to conclude that the plasmid pS1 is integrated in the three revertants.

For the revertants that gave rise to the Type II plasmids, they all retain the 16kb hybridizing chromosomal fragment and all possess the four bands corresponding to those found in the Type II plasmid, so suggesting that the deletion found in the plasmids has occurred before preparation of the transducing phage. All three revertants also show bands at 13.5kb and 9.7kb which one can speculate to be equivalent to 16kb (from the chromosome) and 7.0kb from the plasmid. Thus, a possible derivation is that growth at 42°C first caused or selected a deletion of 3.2kb from within the EcoA (10.2kb) fragment of pS1 to give rise to the 7.0kb piece. The plasmid continued to replicate to produce multimeric tandem repeats of the plasmid and this then integrated into the 16kb chromosomal piece at some site in the 7.0kb piece, still homologous to Tn*552* in the chromosome. The integration may be unstable and reversible so producing a mixture of bacteria, some with intact 16kb piece and others without. There is a slightly lower intensity

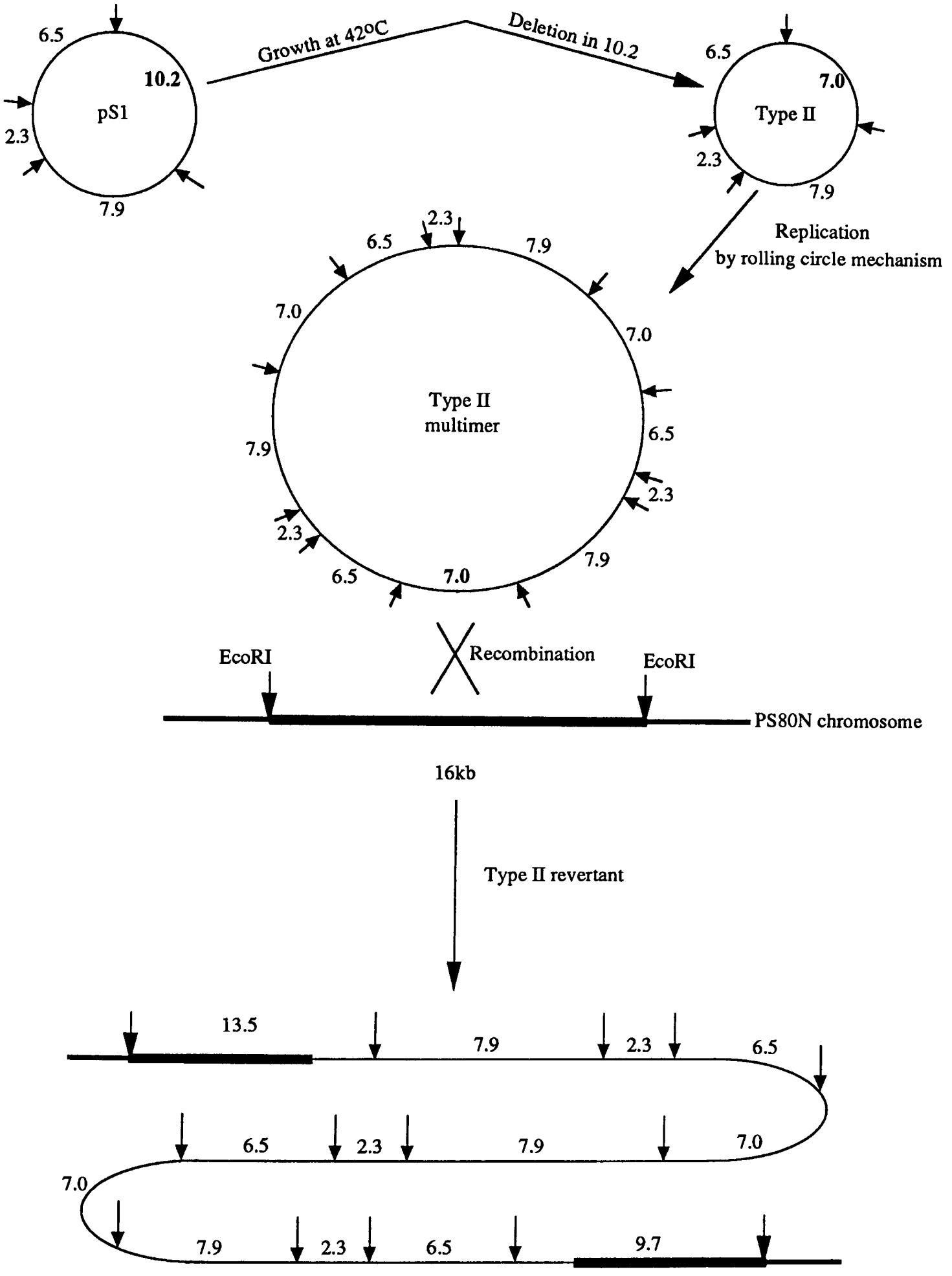
of the hybridization to the 7.0kb piece as compared to the 7.9kb and the 6.5kb pieces which is consistent with the hypothesis (see Fig.4.10).

The hybridization results for all three revertants that gave rise to the Type III transductant plasmids are very similar and show probable recombination between the plasmid pS1 and the PS80N chromosome. Two fragments - one each from the *Eco*RI digests of the plasmid pS1 (10.2kb) and the chromosome (16kb) are replaced by two new fragments of 11kb and 5.7kb. The two new fragments may be a result of recombination between pS1 and the chromosome accompanied by a probable deletion in both the plasmid and the chromosome, since, 16kb + 10.2kb (=26.2kb) generates 11kb + 5.7kb (=16.7kb). Moreover, the Type III plasmid has neither the 11kb nor the 5.7kb piece and these are replaced by a 3.6kb piece. Thus excision of the plasmid as a result of propagating phage 80 on the revertants has resulted in a recombination presumably between sites within the 11kb and the 5.7kb pieces. Again, formal proof of integration is not provided by this result.

In the case of the revertant that generated Type IV transductant plasmids, in addition to the hybridization fragments that are of the sizes corresponding to the Type IV transductant plasmid and to the 16kb of PS80N, four very faint fragments (14.5kb, 13.5kb, 12kb and 11kb) can be seen. The faintness could either be due to half molar concentrations of the fragments resulting from the generation of an invertible system on the chromosome on integration of the plasmid, or due just to poor homology to the probe.

The data with *Eco*RI digestion are difficult to interpret precisely and so it was decided to repeat the hybridization experiment but in this case to use *Eco*RV as the enzyme since it might be possible to identify within which of the four *Eco*RV pieces from the chromosome that hybridize to pS1 is any putative integration event.

Fig.4.10 A possible route for the generation of Type II revertants. See text for details. The drawings are not to scale.



4.11 Hybridization of the *Eco*RV and the *Eco*RI + *Eco*RV digested total DNA of the revertants to pS1

The revertants Rev5 (parent of Type I Rev5tdA), Rev14 (parent of Type I Rev14tdB), Rev24 (parent of Type I Rev24tdA), Rev2 (parent of Type II Rev2tdA), Rev26 (parent of Type II Rev26tdB), Rev3 (parent of Type III Rev3tdB) and Rev8 (parent of Type IV Rev8tdA) were used. Total DNA of PS80N, RN4220 and the plasmid pS1 were used as controls.

The total DNA of the revertants was digested, separately, with *Eco*RV, and *Eco*RI + *Eco*RV and electrophoresed on a 0.7% (w/v) agarose gel at 40V for ~14hrs in TBE buffer. DNA was transferred to nylon membrane, hybridized to pS1 cut with *Eco*RI and the hybridization detected using Amersham's ECL System. The results of this hybridization are given in Fig.4.11. The fragment sizes of the *Eco*RV and *Eco*RI + *Eco*RV digestions are given in Tables 4.7 and 4.8.

On digestion with *Eco*RI + *Eco*RV, the hybridization pattern of the PS80N chromosome to pS1 is indistinguishable from that with *Eco*RV digestion and the four *Eco*RV fragments (6.5kb, 1.25kb, 0.7kb and 0.55kb) thus have no internal *Eco*RI restriction sites and all came from within the *Eco*RI 16kb fragment. If Tn*552* is present on the chromosome then the 6.5kb and the 1.25kb *Eco*RV fragments will also contain non-Tn*552* DNA.

The data with pS1 are difficult to interpret although the predicted major bands are present. The problem is that there are many additional bands that hybridize and that could be due either to incomplete digestion by *Eco*RI or by non-specific "star" activity. Only the expected strongly hybridizing bands are shown in Table 4.7. The three revertants that gave

Fig.4.11 (A). Photograph of the agarose gel stained with ethidium bromide. Total DNA was digested with *Eco*RV and *Eco*RI + *Eco*RV, separately, and electrophoresed on a 0.7% agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pS1 probe (plus pUC18 to illuminate the Raoul ladder): (B). An autoluminograph of the membrane showing hybridization of the plasmid pS1 to the total DNA of PS80 revertants.

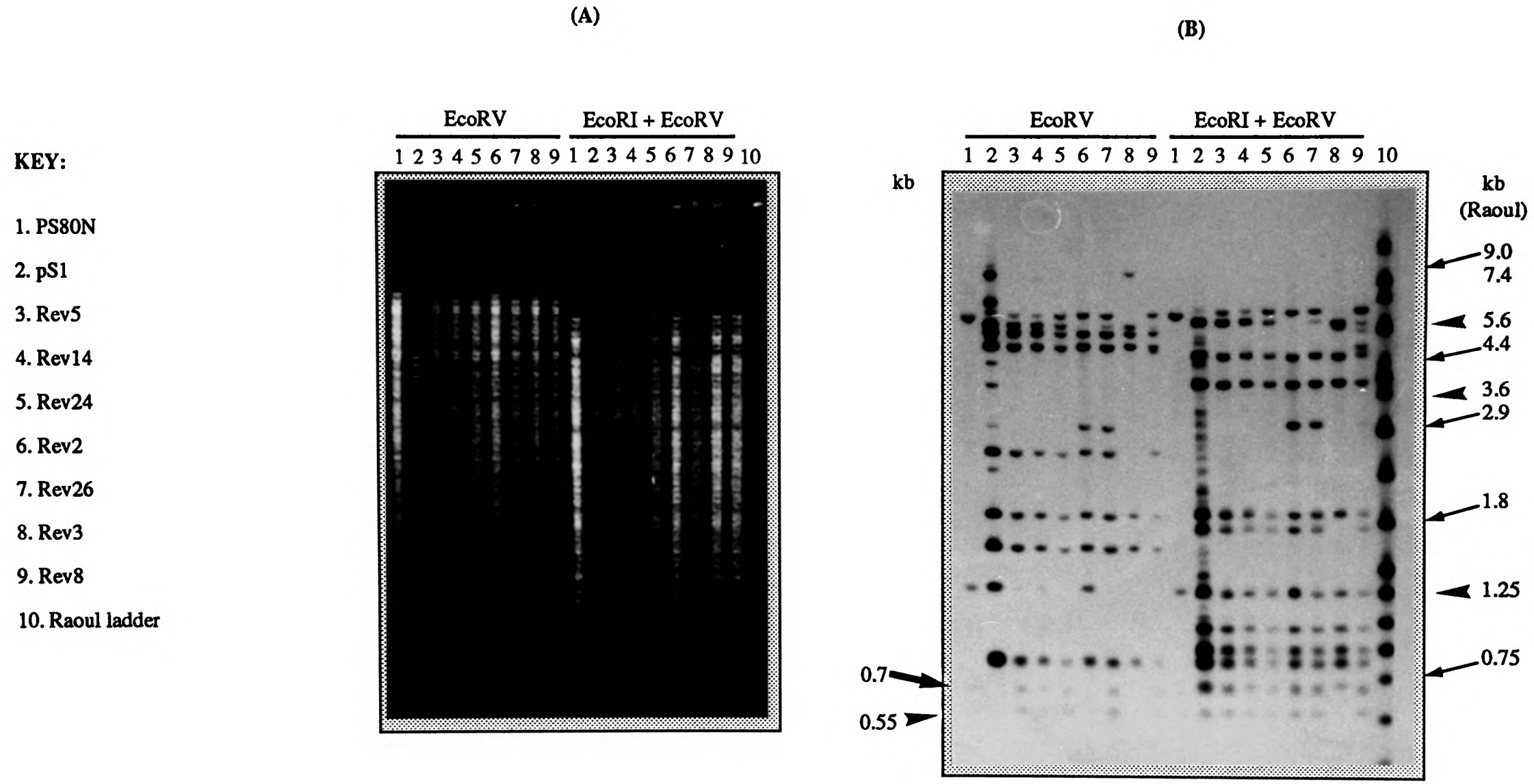


Table 4.7 *Eco*RV fragment sizes of the total DNA of the revertants that hybridize to pS1.

DNA	Fragment sizes (kb)										
PS80N	6.5							1.25		0.7	0.55
pS1		6.2	5.8	5.1	2.7	1.9	1.55	1.25	0.85(x2)	0.7	0.55
rev5	(6.5)	6.2	5.8	5.1	2.7	1.9	1.55	(1.25)	0.85	0.7	0.55
rev14	(6.5)	6.2	5.8	5.1	2.7	1.9	1.55	(1.25)	0.85	0.7	0.55
rev24	6.5	6.2	5.8	5.1	2.7	1.9	1.55		0.85	0.7	0.55
rev2	6.5		5.8	5.1	3.1	2.7	1.9	1.55	1.25	0.85	
rev26	6.5	(6.2)	5.8	5.1	3.1	2.7	1.9	1.55		0.85	0.7	0.55
rev3	(10.6)	6.2	5.8	5.1			1.9	1.55		0.85	
rev8	6.5	(6.2)	5.8	5.1	(5.0)	2.7	1.9	1.55		0.85	0.7	0.55

Notes: The fragments that are faint are given in (brackets).

Table 4.8 *Eco*RI + *Eco*RV fragment sizes of the total DNA of the revertants that hybridize to pS1.

DNA	Fragment sizes (kb)											
PS80N	6.5						1.25				0.7	0.55
pS1		6.2	4.7	3.8	1.9	1.7	1.25	1.0	0.9	0.85	0.7	0.55
rev5	6.5	6.2	4.7	3.8	1.9	1.7	1.25	1.0	0.9	0.85	0.7	0.55
rev14	(6.5)	6.2	4.7	3.8	1.9	(1.7)	1.25	1.0	0.9	0.85	0.7	0.55
rev24	6.5	6.2	4.7	3.8	1.9	1.7	1.25	1.0	0.9	0.85	0.7	0.55
rev2	6.5		4.7	3.8	3.1	1.9	1.7	1.25	1.0	0.9	0.85	0.7	0.55
rev26	6.5	(6.2)	4.7	3.8	3.1	1.9	1.7	1.25	1.0	0.9	0.85	0.7	0.55
rev3		6.2	4.7	3.8		1.9		1.25	1.0	0.9	0.85	0.7	
rev8	6.5	(6.2)	(5.8)	(4.9)	(4.7)	3.8		1.9	1.7	1.25	1.0	0.9	0.85	0.7	0.55

Notes: The fragments that are faint are given in (brackets).

rise to Type I plasmids gave very similar results to each other (Fig.4.11, Lanes 3,4 and 5) and all contain the expected *Eco*RV and *Eco*RI + *Eco*RV bands from both the chromosome and the plasmid with no good evidence of interactions.

The Rev2 and the Rev26 are similar except that for Rev2 (Fig.4.10, Lane 6), the 1.25kb *Eco*RV fragment is present, whereas, for the other revertant that gave rise to a Type II plasmid this band appears absent, although when cut with *Eco*RI + *Eco*RV, it is present. A fainter 6.2kb fragment, present in Rev26 (Fig.4.11, Lane 7), is absent from Rev2. The relative intensities of the bands are difficult to interpret in that for example, the 0.7kb and the 0.55kb bands are absent in Lane 6 for the *Eco*RV digestion but apparently present in Lane 6 for *Eco*RI + *Eco*RV digestion.

Overall, the results are very difficult to interpret and there seemed little likelihood of obtaining definitive evidence for integration from these experiments. It was, therefore, decided to clone the section of the chromosome possibly involved in any recombination and then study the recombination process with the cloned piece. First, however, it was decided to check on the presence of the relevant *Eco*RI and *Eco*RV pieces in PS80N and to see how they correspond to various plasmids.

4.12 Hybridization analysis of the PS9789 total DNA

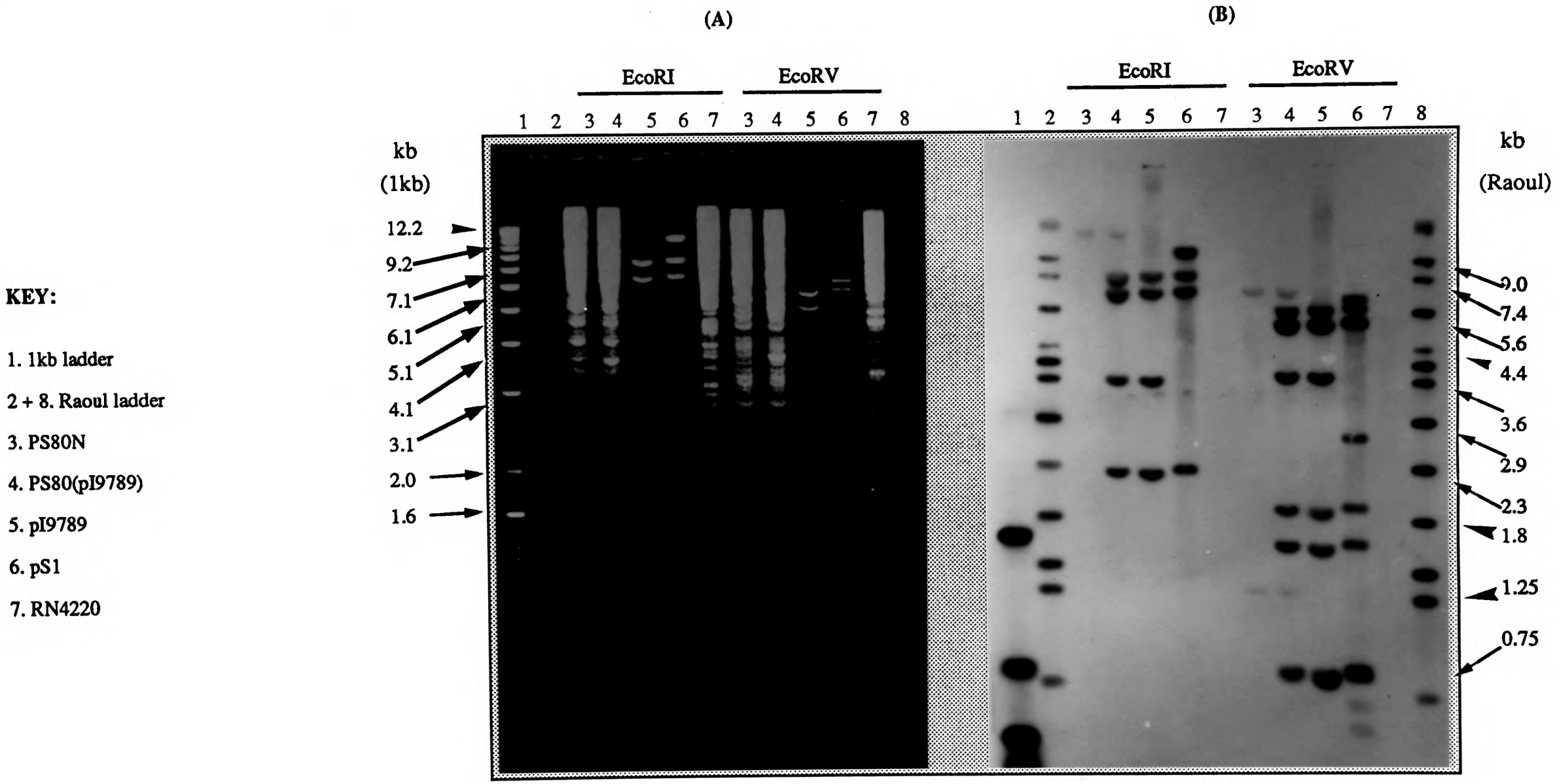
The original NCTC 9879 (PS80) strain had been shown to carry a plasmid conferring resistance to mercury salts and that a gene specifying β-lactamase could sometimes also be found on this plasmid (Asheshov, 1969). Thus PS80(pI9789) is the strain with the plasmid conferring mercury and cadmium resistance and

PS80(pI9789::*blaZ*) is a strain with a plasmid carrying genes specifying β-lactamase, Cd^r and Hg^r. Subsequent investigation has shown that pI9789::*blaZ* is more correctly designated pI9789::Tn*552*, since the pI9789 plasmid is converted to pI9789::*blaZ* by the acquisition of Tn*552* (Rowland and Dyke, 1989). PS80(pI9789) can be made to lose its plasmid but it still retains the ability to produce β-lactamase and was designated PS80::Tn*552*. This latter strain was mutagenized with EMS by Johnston (1971) and a variant was obtained that does not produce β-lactamase and is designated PS80N. The nature of the change between PS80::Tn*552* and PS80N is not known but was assumed to be a deletion. It was, therefore, decided to check some of the above assumptions by hybridization of relevant DNA with pS1 as probe.

Total DNA was prepared from PS80N and PS80(pI9789). Plasmid pS1 and the plasmid pI9789 (the resident plasmid of PS80) were used as controls. The DNA was digested with restriction enzymes *Eco*RI and *Eco*RV (*Eco*RV was chosen since it has sites in Tn*552*) separately, and electrophoresed on a 0.7% (w/v) agarose gel at 40 volts for ~10hrs in TBE buffer. DNA was then transferred to nylon membrane and hybridized to pS1 digested with *Eco*RI and hybridization detected using the Stratagene Flash System.

The results (Fig.4.12) confirm that pS1 does not hybridize to RN4220 DNA and that it hybridizes to a single *Eco*RI fragment of about 16kb and to four *Eco*RV fragments of PS80N. Strain PS80(pI9789) has the same hybridization band as PS80N and in addition has bands hybridizing to the pS1 probe that are indistinguishable from those hybridizing to purified pI9789 plasmid. Plasmid pS1 is similar to pI9789 but the 3.6kb *Eco*RI band of pI9789 is now 10.2kb, consistent with the insertion of the 6.6kb Tn*552* into the plasmid. The *Eco*RV results confirm these observations.

Fig.4.12 (A). Photograph of the agarose gel stained with ethidium bromide. Total DNA was digested with *Eco*RI and *Eco*RV, separately, and electrophoresed on a 0.7% agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pS1 probe: (B). An autoluminograph of the membrane showing hybridization of the plasmid pS1 to the total DNA of PS80 and PS80N.



It was thus decided that it was probable that PS80N carries an almost intact Tn*552* on its chromosome (although it does not express β-lactamase) and that this is likely to be the site of possible integration of the plasmid pS1. It was, therefore, decided to clone from the chromosome of PS80N, the piece of DNA carrying the Tn*552*.

4.13 Structural analysis of the 16kb *Eco*RI fragment of PS80N that hybridizes to pS1

4.13.1 Cloning of the 16kb *Eco*RI and *Eco*RV fragments

Total DNA of PS80N was digested with *Eco*RI and electrophoresed in 1.0% FMC's SeaPlaque GTG low melting agarose. The relevant region (16kb) of the gel was cut out and DNA was purified using the Promega's Wizard PCR Preps DNA purification Kit. This DNA was ligated separately to *Eco*RI digested pUC18 and pTZ19R vectors. The ligation mixes were transformed into competent JM107 and the transformants were plated onto 2 x TY agar plates containing ampicillin/Xgal/IPTG at standard concentrations. Only the re-ligated plasmid vector was revealed by the few white colonies obtained in these clonings. The cloning was abandoned because of the difficulty of cloning of pieces of 16kb DNA in these vectors and so it was decided to clone the *Eco*RV fragments of the PS80N chromosome that hybridize to pS1, since they are much smaller. In particular the 6.5kb and the 1.25kb *Eco*RV pieces that contain both parts of Tn552 and pieces of the chromosome would be sought.

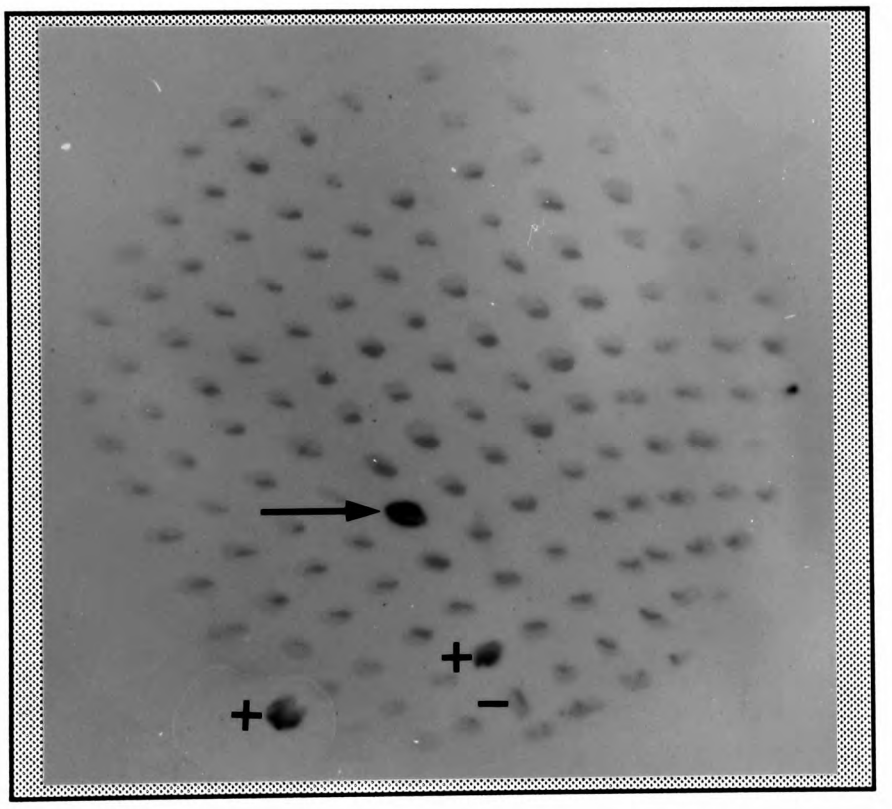
Total DNA of PS80N was completely digested with *Eco*RV and since neither of the two derived pieces contain an *Eco*RI site, the DNA was also digested with *Eco*RI to reduce the number of fragments with ligatable ends. The *Eco*RV/*Eco*RI digested PS80N DNA was

ligated to *Sma*I digested and phosphatased pUC18 DNA and used to transform *E. coli* JM107 to ampicillin resistance. Approximately, 5000 - 6000 white transformants were obtained. Plasmid DNA was prepared from 20 colonies and fifteen of these had insertions ranging in size from 0.2kb to ~8.0kb. Colony hybridization was then employed to identify the desired clones using the plasmid pS1 as probe. The transformants were picked and patched onto 2 x TY agar plates (140mm diameter). JM107(pOX450), a pUC18 based clone that contains Tn*552* (Curnock, personal communication) was used as a positive control and JM107(pUC18) was used as negative control on each plate. More than 5000 transformants were screened by this method and the DNA of only one colony gave a positive hybridization. A picture of the plate with the positively hybridizing colony is shown in Fig.4.13. The colony was labelled "P7" (the plasmid as pP7) and plasmid DNA was prepared from it for further analysis. On *Eco*RI + *Hind*III digestion, the DNA revealed a ~4.1kb insert that corresponds to neither of the four expected *Eco*RV fragments. Nevertheless, It was decided to investigate the hybridization of this clone with pS1. The plasmid DNA was digested with *Eco*RI + *Hind*III, electrophoresed on a 0.7% (w/v) agarose gel in TBE buffer, transferred to a nylon membrane and then hybridized to pS1 (plus pUC18 that hybridizes to the Raoul ladder) and the hybridization detected using Amersham's ECL System. The result of this hybridization are not shown separately, since, this clone (pP7) was later used in a hybridization to pS1 (see Fig.4.16). It was then decided to analyse this clone further using restriction enzymes and DNA sequencing.

4.13.2 Structural analysis of pP7

Based on the data obtained in the previous sections, the enzymes were chosen from the known restriction enzyme map of the transposon Tn*552* (Rowland, 1989), which were

Fig.4.13 An autoluminograph of a nylon membrane showing results of a colony hybridization experiment. The *E. coli* colonies carrying potential chimeric plasmid DNA (pUC18 + *Eco*RI/*Eco*RV chromosomal library of PS80N) were patched onto 2 X TY agar plate. The colonies were transferred to a nylon membrane and subsequently lysed to release their DNA contents. The DNA was fixed to the nylon membrane by exposure to UV-light and then used in hybridization to the pS1 probe. The hybridization was detected using Stratagene's Flash Detection System. One colony gave a positive signal and is labelled with an arrow. The two positive (+) and one negative (-) controls are also labelled.



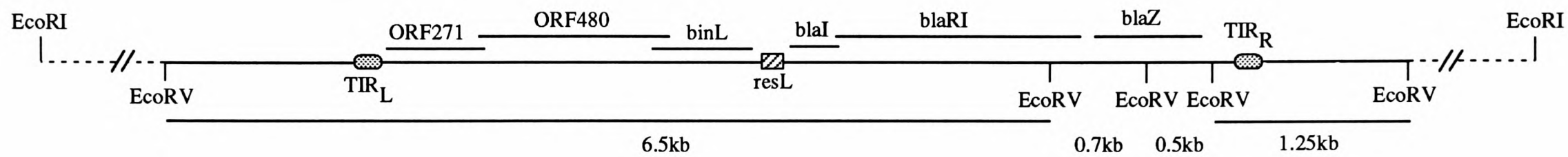
as follows:- *Sna*BI, *Nde*I, *Pvu*I, *Acc*I, *Hpa*I, *Xba*I and *Bgl*II. The results show the presence of restriction site for all the enzymes listed above except for *Bgl*II. A restriction map of the 4.1kb fragment of pP7 was constructed and a comparison of this with Tn*552* and the 16kb *Eco*RI fragment from the chromosome of PS80N is given in Fig.4.14. The restriction analysis suggests that at least, a part of Tn*552* has been cloned from the chromosome of PS80N. The 4.1kb *Eco*RV fragment was probably generated by a non-specific break between the *Bgl*II and *Xba*I sites or because of non-specific activity of the restriction enzymes used to digest the chromosomal DNA.

DNA sequencing was, therefore, employed for precise mapping of the ends of the 4.1kb fragment of the plasmid pP7. Forward and reverse M13 primers were used. The result shows that, using the forward primer, the sequence on the 4.1kb fragment was read from the polylinker region of pUC18 into the *Eco*RV site (proximal to the *resL*) at position 5013 in *blaRI* gene of Tn*552* (Rowland and Dyke, 1990). At the other end, using the reverse primer, the sequence was read from the polylinker of pUC18 into the 4.1kb fragment from the base No. 847 within the ORF p271 into the transposon Tn*552*. The relevant sequences are shown in the Fig.4.15 and are compared with the corresponding sequences on Tn*552* (Fig.4.16). The data suggest that the sequence at the region of the non-specific break (No. 847 within the ORF 271) is very similar to the restriction site for *Eco*RI and the break might have resulted from a non-specific "star" activity of *Eco*RI. These results not only present sequence of the 4.1kb fragment at its ends but also strongly support the presence of Tn*552* on the chromosome of PS80N. Unfortunately, the insert contains no non-Tn*552* chromosomal DNA.

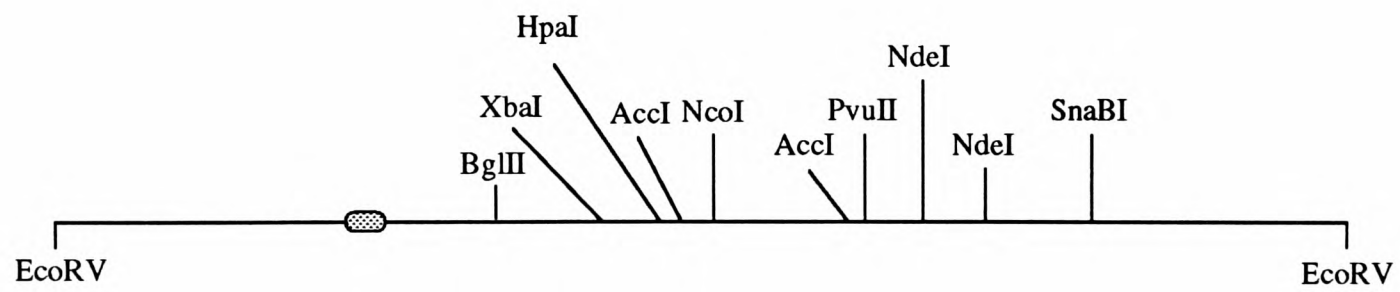
It was decided to repeat the cloning but with the *Eco*RV-digested DNA of PS80N rather than the *Eco*RI + *Eco*RV-digested DNA to avoid any repetition of the problem with

Fig.4.14

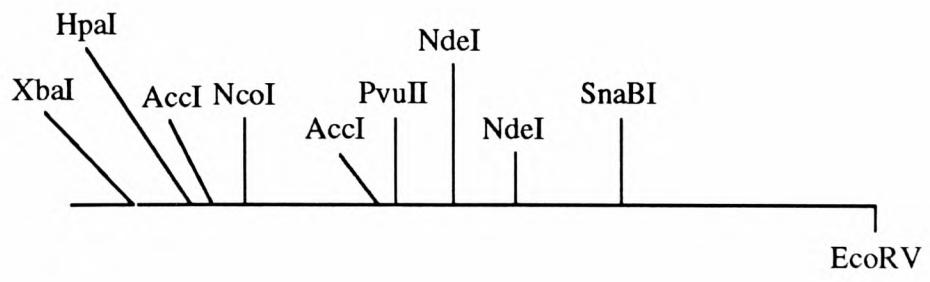
(A) Presumed 16kb EcoRI fragment of PS80N chromosome



(B) Restriction enzyme map of the presumed 6.5kb EcoRV fragment of PS80N chromosome



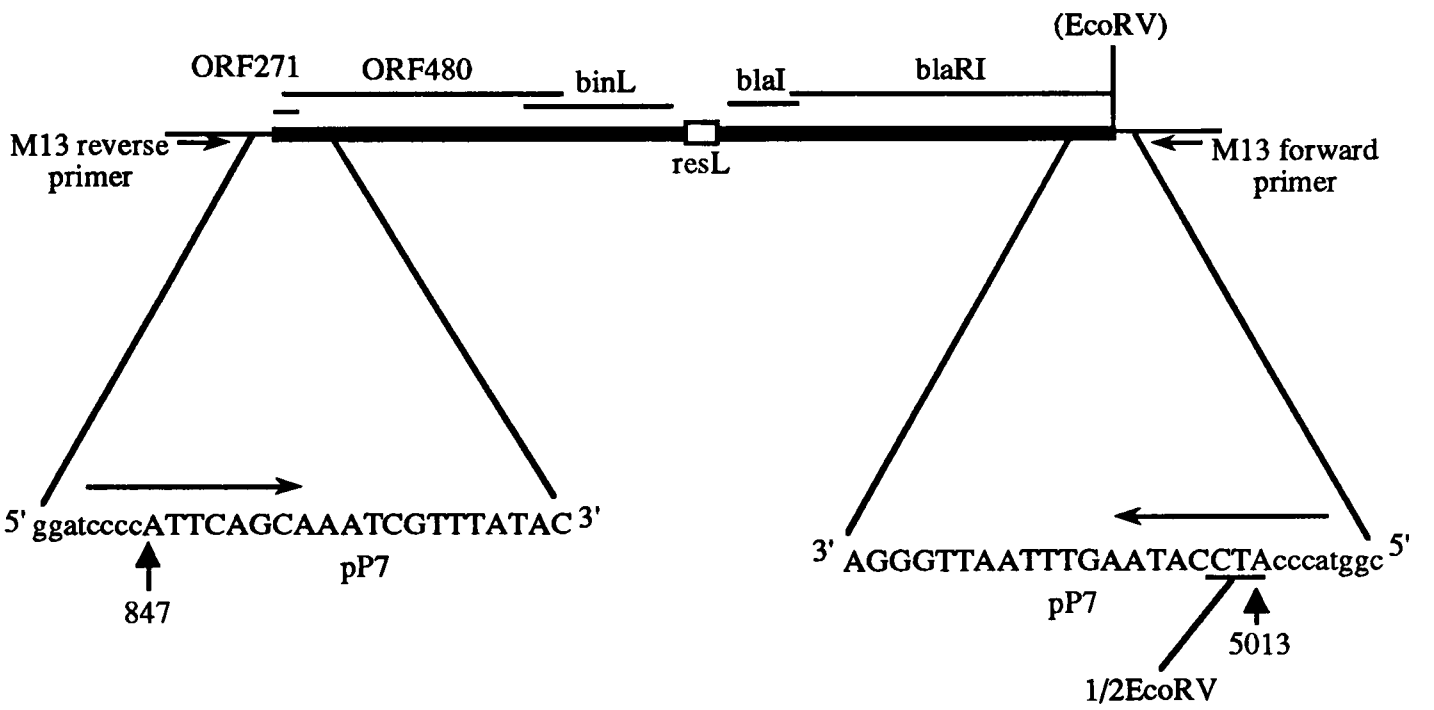
(C) Restriction enzyme map of the 4.1kb insert of pP7



KEY: 500bp

unknown

Fig.4.15 The sequencing strategy used to sequence the ends of the 4.1kb fragment of the plasmid pP7 and the sequence at the ends of the 4.1kb fragment. The M13 forward and reverse primers were used in sequencing. The pUC18 sequence which is in theregion of the multiple cloning site is given in lower case, whereas, the sequence of pP7 is given in CAPITALS. Various putative genes of Tn*552* are also shown. The numbers (847 and 5013) refer to the Tn*552* sequence (Rowland and Dyke, 1990). The *Eco*RV restriction site at one end is also marked. The direction of sequencing is indicated by arrows (⟶).

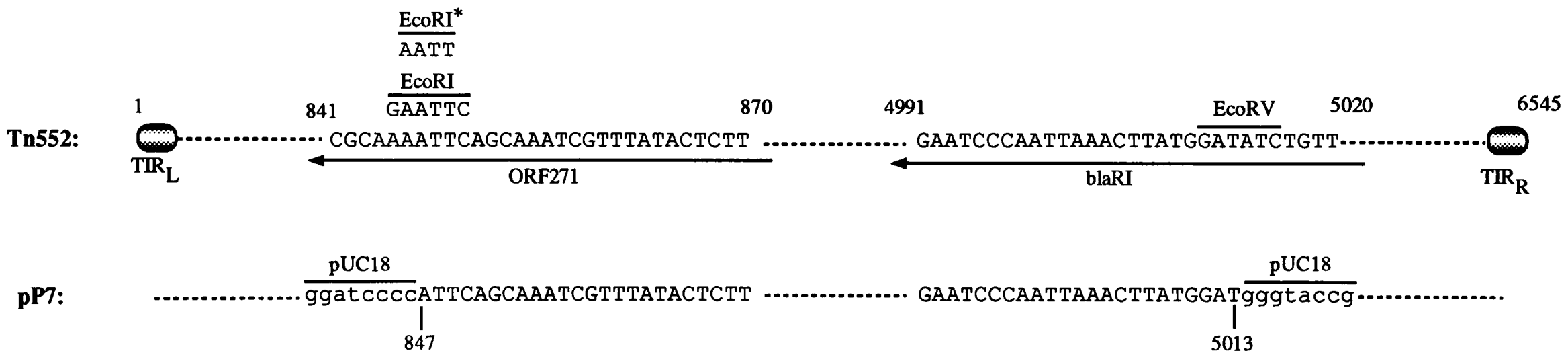


KEY: ▬ The 4.1kb fragment

— pUC18

Scale: ——— 500bp

Fig.4.16 Sequence obtained at the ends of the 4.1kb fragment of the plasmid pP7 is compared with the Tn*552* sequence in the region of interest. The numbers correspond to the Tn*552* sequence (Rowland and Dyke, 1990). The arrows represent the direction of transcription ORF271 and *blaRI* and not their size. The region of non-specific strand break/digestion is compared with the *Eco*RI restriction site and the four base-pair sequence that would be restricted by *Eco*RI*-activity (Old and Primrose, 1991) has also been shown. The sequence of the pUC18-multi-cloning-site at the ligation points with the 4.1kb fragment in pP7 is shown in small letters.

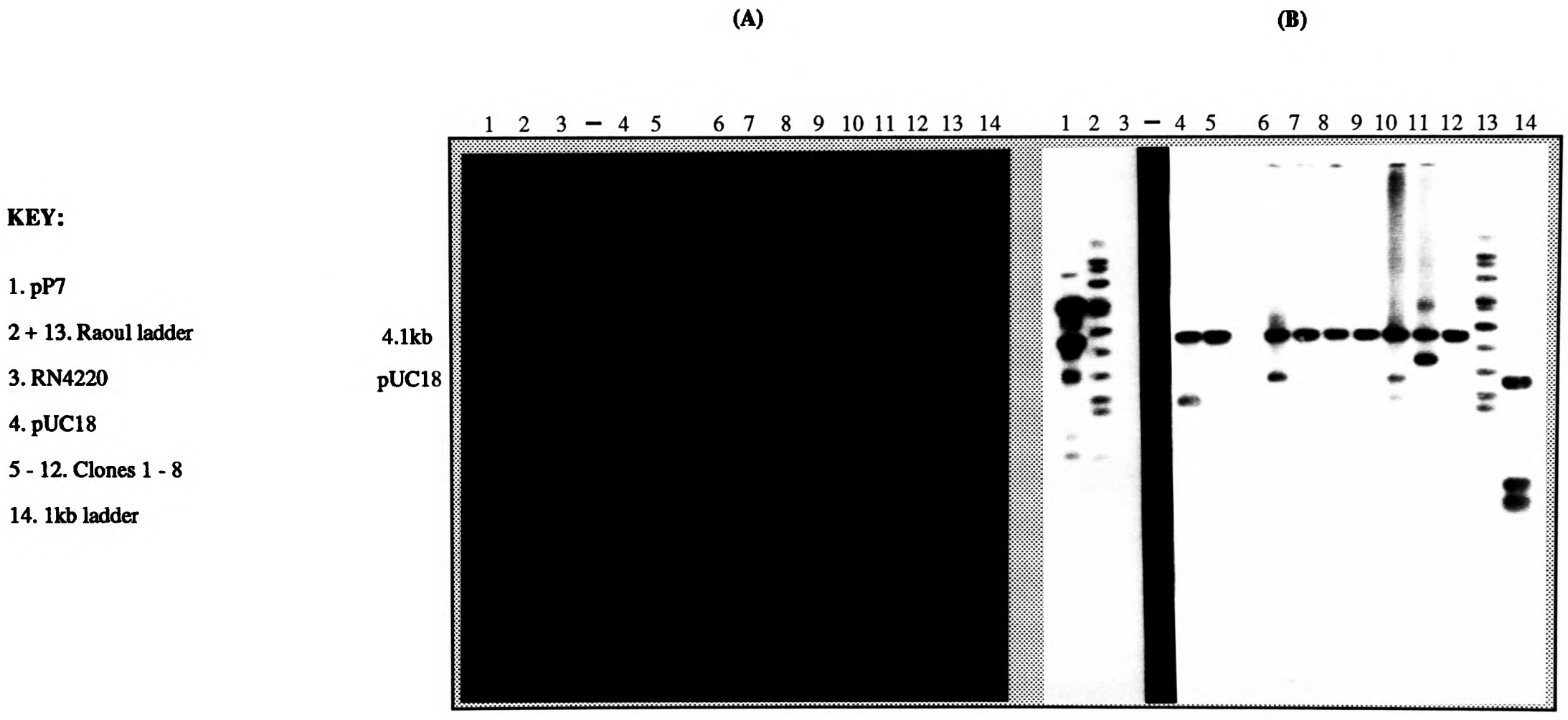


pP7. Also, the *recA*$^-$ *E. coli* DH5α was used as host, instead of JM107 which is recombination proficient, to clone the possibly recombinogenic fragments of the chromosome.

4.14 Cloning of the *Eco*RV fragments from PS80N chromosome

The chromosomal DNA of PS80N was digested with *Eco*RV and used to ligate into the Pharmacia's *Sma*I digested and bacterial alkaline phosphatase (BAP) treated pUC18 cloning vector. The ligation mixture was used to transform competent DH5α on fifty 2 x TY agar plates containing ampicillin/Xgal/IPTG at standard concentrations. DNA was prepared from 20 colonies and showed the insertion of foreign DNA into the pUC18 vector of sizes ranging from 0.2kb to ~7.0kb. Colony hybridization was employed to identify the desired clones using the plasmid pS1 as probe. Replica plates were prepared and colonies from the original plates were transferred to nylon membrane. The colonies were lysed, hybridized to pS1 probe and the hybridization detected using Amersham's ECL System. Thirty-seven plates were screened (on average 150-200 white colonies on each plate). On eight of the plates positive signals were detected. The eight colonies were identified on the plates and plasmid DNA was prepared from them. The DNA was digested with *Eco*RI + *Hind*III in order to cut the insert out from the vector. The DNA was then used in hybridization analysis to pS1 (*Eco*RI cut pUC18 was added to hybridize to the Raoul DNA ladder). The plasmid DNA of pP7 and the chromosomal DNA of RN4220 were used as controls. The results are shown in Fig.4.17 and show that there is no insert corresponding

Fig.4.17 (A). Photograph of the agarose gel stained with ethidium bromide. Plasmid DNA was prepared from eight *Eco*RV-library clones of the PS80N chromosome in pUC18 and digested with *Eco*RI + *Hind*III. The DNA samples were then electrophoresed on a 0.7% (w/v) agarose gel. The DNA was transferred to a nylon membrane for hybridization to the pS1 probe: (B). An autoluminograph of the nylon membrane showing hybridization of the plasmid DNA to the pS1 probe.



to 6.5kb, 1.25kb, 0.7kb or 0.55kb that hybridizes to the pS1 probe, although all the clones contain small inserts. The reason for the positive hybridization of the clones with pS1 in the primary screen is not known. Reluctantly, it was decided to abandon the cloning of a piece of PS80N chromosome adjacent to the Tn*552*.

4.15 Discussion

4.15.1 Interaction between the EcoA fragment of pS1 and the PS80N chromosome

Plasmid integration into the host chromosome has been proposed as a mechanism to overcome thermosensitive replication and incompatibility barriers (Khasanov *et al.*, 1992; Conrad *et al.*, 1992; Pattee *et al.*, 1977; Schwesinger and Novick, 1975; Wyman and Novick, 1974; Johnston and Dyke, 1974). Although, the most likely mechanism to attain thermostable replication by the thermosensitive plasmid pS1 in PS80N, from the data reported by some previous workers (Browne, 1985; Warne, 1982; Johnston, 1971) and enhancement of the reversion frequencies by the site-specific recombinase *binR* (Chapter 3), seems to be integration of the plasmid into the host chromosome, no direct evidence for this has as yet been provided. Johnston (1971) proposed the linkage of the chromosomally-borne *erm* gene to the plasmid-borne *cad* gene in support of the integration. The *erm* gene was, however, found to be on transposon Tn*551* (Pattee *et al.*, 1977) later and the linkage of the chromosomal and plasmid genes seen in this case could simply be a result of the transposition of the Em^r-determinant to the plasmid. Due to unavailability of any other useful chromosomal markers, no chromosomal DNA other then *erm* was shown to be linked to the plasmid. With the advent of restriction endonucleases, it is now possible to

detect the linkage of phenotypically cryptic chromosomal DNA to the plasmid DNA (see Conrad *et al.*, 1992).

Absence of any detectable plasmid DNA in the PS80N(pS1) revertants supports the integration hypothesis as does the fact that intact temperature-sensitive plasmids can be recovered by transduction with phage grown on the revertants. The recovered plasmids sometimes have deletions but no evidence was obtained of chromosomal DNA becoming associated with the recovered plasmids. Deletions were reported to occur in the plasmid pE194 when chromosomally integrated pE194 is excised from the chromosome. Chromosomal DNA, ranging from 1kb to 6kb, was also found linked to these excised plasmids (Conrad *et al.*, 1992). The deletions recorded in pS1 during this study could be regarded as a result of imprecise excision during transduction, although, no chromosomal DNA was found linked to the plasmids. However, since extensive plasmid deletions have been observed following transduction (McDonnell *et al.*, 1983; Gray *et al.*, 1983), this result cannot be used as evidence for integration. The deletions in pS1 occur only when the plasmid is transduced from a revertants. No such deletions were observed when pS1 was transduced from cells grown at 30^{o}C (see Chapter 3; construction of heteroplasmid stains). Although, the deletions in the plasmid pS1 occur at a number of places to give rise to the three distinct types, the process seems somewhat specific, since, more than one example was obtained for some of the deletion types (*e.g.*, II and III). Also, the deletions appear to start at the same site (at or near *resR*) in most of the cases and occur within a defined 7.1kb region [between *Acc*I (2.05) and *Sal*I (9.15); Fig.4.4] on the EcoA fragment.

The deletion analysis suggests that the *inv* region is involved in the reversion. Supposing, that reversion occurs by integration of the plasmid into the chromosome within this region, it is possible that recombination is influenced by the orientation of the *inv*. The

analysis of the deletions in the four types of the transductants suggests that the deletion occurred when the *inv* was in negative orientation - the orientation in which a full copy of the transposon Tn*552* (Rowland and Dyke, 1990; Rowland and Dyke, 1989) is on the plasmid pS1. In the case of the Type I transductant Rev5TdA, a higher reversion frequency was observed than other member of this group (Table 4.3), although they are indistinguishable on restriction enzyme analysis. It is possible that the excision of the integrated plasmid from the chromosome produced an invertible region (*inv*) with a higher or a lower than the normal rate of inversion, and so, more plasmids with an *inv* in the orientation that favoured integration (possibly negative orientation) were present.

The reversion frequencies obtained for the four types of revertant-derived transductant plasmids (I, II, III and IV) suggest that reversion might also be related to the size of Tn*552* on pS1. pS1 and Type I plasmids have a full copy of Tn*552*. Nearly half of Tn*552* is deleted from Type II plasmids (Δ3.2kb) and most of it is probably deleted from Type III plasmids (Δ6.5kb). Type IV (Rev8tdA) plasmid has the smallest deletion of all (Δ1.2kb). The frequency of reversion was the highest for pS1 and Type I plasmids. The transductant Rev8tdA gave 20 revertant colonies on average, which is lower than pS1 and the Type I plasmids but higher than pΔD, Type II and Type III plasmids. It seems likely that the mechanism (probably integrative) which operates in PS80(pΔD) to produces revertant colonies is different from that which operates in Type I and Type IV plasmids and probably similar to that which functions in Type II and Type III plasmids.

4.15.2 The PS80N chromosome

Hybridization analysis of the *Eco*RI-digested PS80N chromosome using specific probes derived from the 10.2kb EcoA fragment of pS1 (Section 4.9), as well as using the

whole of pS1 as probe (Sections 4.10), revealed that a 16kb fragment of the chromosome shows homology to pS1. The hybridization pattern of the *Eco*RV-digested chromosome to the probes pSJZ17 and pSJZ50, that of the *Eco*RV and *Eco*RI + *Eco*RV (Section 4.11) digested chromosome to the pS1 probe, and the ancestry of the strain PS80N suggest the presence of a Tn*552*-like structure on the 16kb *Eco*RI fragment of the chromosome. The endonuclease and the sequence analysis of the 4.1kb fragment derived from the chromosome (in the plasmid pP7) of PS80N shows that at least some of the Tn*552* (containing *resL*) is present on the chromosome of PS80N. The generation of the β-lactamase negative phenotype in PS80N may have resulted from a point mutation or a small deletion rather then deletion of the whole or most of the *bla* region (*penIpenZ*) from the chromosome (Johnston and Dyke, 1971).

Browne (1985) used the strain PS80::Tn*552* to show the effect of an increase in the homology between pS1 and the host chromosome on reversion of the plasmid pS1 to temperature stability. She reported that the presence of Tn*552* on the chromosome had no detectable effect on the reversion frequency of pS1. The results obtained in this study show that the chromosomes of both PS80N and PS80::Tn*552* probably contain copies of Tn*552* and so Browne's result do not allow one to conclude that Tn*552* is not involved in reversion. The chromosome of the two strains, RN4220 and 1054, show no detectable homology to pS1 (see Chapter 7 for 1054) and the frequency of reversion of pS1 by 'integration' into the chromosome is very low in these strains compared to PS80N (and PS80::Tn*552*; Browne, 1985). The presumed amount of homology of the Type IV plasmid (Rev5tdA) to the PS80N chromosome is higher (by the presence of more of Tn*552*) than that of the Type II and III plasmids and so the higher reversion frequency of the Type IV

plasmid than the other two could be attributed to the difference in the levels of homology of these plasmids to the host chromosome.

4.15.3 Interaction between the EcoA fragment of pS1 and the 16kb *Eco*RI fragment of the PS80N chromosome which shows homology to pS1

A stronger piece of evidence for the interaction between the 10.2kb EcoA fragment of pS1 and the PS80N chromosome was provided by the hybridization analysis of the total DNA of the revertants.

Hybridization of the *Eco*RV-digested total DNA of the revertants to the pSJZ17 and pSJZ50 probes (Section 4.9) although does not provide a proof for integration of the plasmids into the chromosome on reversion, however, a change occurs in both the plasmid and the chromosome. The data suggest that the deletions which were observed in the three types of the revertant-derived transductants occur prior to transduction of these plasmids and not during the preparation of transducing phage or transduction (*e.g.*, Rev8). Deletions not only occur in the plasmid but may also occur in the chromosome (*e.g.*, Rev3).

Hybridization of the *Eco*RI-digested total DNA of revertants with pS1 shows that a change has occurred in the 16kb *Eco*RI fragment of the chromosome in some cases (*e.g.*, Rev3, Rev32, Rev33) and in the 10.2kb EcoA fragment of pS1 in many cases (*e.g.*, Rev26, Rev3, Rev8). In the others, the 16kb *Eco*RI fragment of the chromosome and the EcoA fragment of pS1 appear to be indistinguishable from the parental DNA (*e.g.*, for Type I plasmids; especially Rev24). It is possible that the 16kb *Eco*RI fragment is involved in the recombination in some cases. No obvious change in the other *Eco*RI fragments of pS1 can be observed. This suggests that the interaction of the plasmid with the

chromosome (*e.g.*, integration) probably involves the EcoA fragment only and not the others.

Interpretation of the hybridization data is, however, made difficult by the presence of all four *Eco*RI fragments of the transductant plasmids in the *Eco*RI digests of many of the revertants. On integration, the one involved in recombination out of the four *Eco*RI fragments would be expected to be absent in the revertants. The 10.2kb, 7.0kb, 3.7kb and the 9.0kb fragments of the Type I, II, III and IV plasmids, respectively, which are related to the 10.2kb EcoA fragment of pS1 and are apparently derived from it, are presumably involved in recombination and would not be expected to be present in the *Eco*RI digests of the revertants. One possibility is that multimeric tandem repeats of the plasmid are integrated into the chromosome at a site which is located within the the EcoA-related fragment of these plasmids. Only in the Type III revertants, the EcoA-related fragment (3.7kb) is absent and show probable recombination with the PS80N chromosome. Two fragments - one each from the *Eco*RI digests of the plasmid pS1 (10.2kb) and the chromosome (16kb) are replaced by two new fragments of 11kb and 5.7kb. Probably, deletion occurs in the 10.2kb fragment of pS1 during recombination. On transduction, excision of the plasmid from the chromosome results in the 3.7kb fragment of the Type III transductants.

In Rev8, the four fragments of very low intensity (14.5kb, 13.5kb, 12kb and 11kb) appear to be a result of inversion of a DNA segment containing an asymmetrically located *Eco*RI site (in the fashion similar to the *inv* of pS1). In one orientation, the 14.5kb and the 11kb fragments and in the other the 13.5kb and the 12kb are generated. It is possible that one of the *res* sites involved in the inversion is provided by the integrated plasmid and the second is already present on the chromosome. These fragments apparently

are not a result of partial digestion or non-specific 'star' activity of the restriction enzyme, since they were observed on other occasions as well when the *Eco*RI-digested total DNA of Rev8 was hybridized to pS1 probe (data not presented).

Overall, the results can be summarized as below:-

1- The thermosensitive replication-defective plasmid pS1 and the PS80N chromosome interact during reversion of the plasmid-borne phenotypes to temperature stability. The nature of this interaction is probably a recombination between the plasmid and the chromosome.

2- The EcoA fragment of pS1 appears to be involved in recombination with the chromosome. More than one mechanisms of recombination may operate.

3- The deletions observed in the transductant plasmids are not phage-mediated and occur on integration of the plasmid into the chromosome. The deletions always reside in the EcoA fragment of pS1 and so probably this fragment is involved in recombination.

4- The 16kb *Eco*RI fragment of PS80N chromosome which hybridizes to pS1 is apparently a target for recombination in many cases. However, there appear to be more than one site for recombination on the chromosome. During integration of the plasmid, deletion may also occur in the chromosome.

- No linkage of chromosomal DNA to the plasmid DNA was detected.

Chapter Five:

Interaction of Chromosomally-borne Tn*551* With the Resident Plasmids

Chapter Five: Interaction of Chromosomally-borne Tn*551* With the Resident Plasmid

5.1 Introduction

Data presented in Chapter 3 show that the presence of the transposon Tn*551* on the chromosome of *S. aureus* PS80N (*i.e.*, in PS80::Tn*551*), enhances the reversion frequencies of the plasmids pS1 and pΔD. Browne (1985) reported that when the plasmids pS1 and pΔD are transduced out of the PS80::Tn*551* revertants (*i.e.*, the strains in which the plasmids confer temperature stable Cd^r), the transposon Tn*551* showed linkage to and co-transduced with both of the plasmids. Restriction enzyme analysis demonstrated that the transposon had integrated in a site and orientation specific fashion into pS1 but randomly into pΔD. In this Chapter, further experiments on the reversion phenomenon in PS80:Tn*551* will be described. The following questions will be addressed:-

1. What is the frequency of Cd^rEm^r co-transduction? This will arise if Tn*551* (conferring Em^r) integrates into pS1 (conferring Cd^r) or pΔD (also Cd^r).

2. What is the exact site of Tn*551*-integration into the plasmids pS1 and pΔD?

In *S. aureus* RN4220, the reversion frequencies of the plasmids pS1 and pΔD are lower than in the two other *S. aureus* strains tested. Absence of any detectable plasmid DNA in the majority of revertants suggests that the reversion in this strain might also be a result of integration. One example out of the ten temperature-stable revertants tested was shown to contain a plasmid indistinguishable from pS1 on restriction enzyme analysis

(discussed in Chapter 6). This Chapter describes experiments designed to discover whether or not reversion in RN4220 can involve integration.

5.2 Cd^rEm^r co-transduction frequencies

5.2.1 Cd^rEm^r co-transduction frequency for pΔD

The frequency of co-transduction of plasmid-borne Cd^r and the Tn*551*-borne Em^r was determined. An overnight broth culture of the strain of PS80::Tn*551* containing pΔD was diluted and plated onto CY-Cd (26.6μg/ml)-agar plates. The plates were incubated at 42°C for 18hrs to obtain revertants. Ten revertant colonies were picked at random and tested for the presence of plasmid DNA. No plasmid DNA could be detected in any of the revertants using the CsCl method. Phage 80 was grown on each of the revertants, separately, and used to transduce Cd^r to a PS80N host. The transductants were selected on CY-Cd (26.6μg/ml)-agar plates at 30°C. For each revertant, >100 transductants were obtained. One hundred transductants were picked at random and tested for Em^r phenotype by patching individual colonies onto CY-Em (20μg/ml)-agar plates. The plates were incubated overnight at 30°C and the results recorded. Complete linkage of Cd^r and Em^r was found for the transductants derived from nine of the revertants, whereas, for the tenth, the linkage was 97%.

5.2.2 Cd^rEm^r co-transduction frequency for pS1

The frequency of Cd^rEm^r co-transduction for the plasmids pS1 was also investigated using the strain PS80::Tn*551*(pS1).

The revertants were obtained by growing PS80::Tn*551*(pS1) at 42°C for 18hr on CY-Cd (26.6µg/ml)-agar plates as above. Ten revertants were picked at random and tested for the presence of plasmid DNA using the CsCl gradient method. None of the ten revertants revealed any detectable plasmid DNA. Phage 80 was grown on each revertant separately and used to transduce Cd^r to PS80N. The transductants were selected on CY-Cd (26.6µg/ml)-agar plates at 30°C. The number of transductants obtained was lower than for pΔD, particularly in three cases (Table 5.1). The frequency of Cd^rEm^r co-transduction was determined by patching individual transductant colonies onto CY-Em (20µg/ml)-agar plates. Only 100 transductants were tested when the total number of the transductants was >100. The results of this experiment are summarized in Table 5.1.

It is noteworthy that the frequency of Cd^rEm^r linkage was 100% when the number of transductants was greater than 100 but very low or 0% when the number of transductants was low under similar conditions of transduction (*e.g.*, cell density of the recipient culture, plaque forming units, *etc*). The results also suggest that, overall, the frequency of transduction and Cd^rEm^r-phenotype-linkage was lower for the plasmid pS1 than for pΔD.

5.3 Plasmid DNA analysis of the Cd^rEm^r transductants

Plasmid DNA was prepared from five examples each of pS1::Tn*551* (Cd^rEm^r) and pΔD::Tn*551* (Cd^rEm^r) and analysed after digestion with *Eco*RI. The results show a 5.3kb insertion (Tn*551*) into the large 10.2kb *Eco*RI fragment of pS1 in all five cases. For pΔD two such insertions are into the 7.9kb *Eco*RI fragment, two are into the 6.5kb *Eco*RI

Table 5.1 The number of transductants and the frequency of Cd^rEm^r linkage among the transductants for each of the ten PS80::Tn*551*(pS1) type revertants. The transductants were selected on cadmium.

Revertant#	Total No. of Transductants	Cd^rEm^r	Cd^rEm^s
1	>100	100	0
2	>100	100	0
3	40	2	38
4	51	0	51
5	2	0	2
6	>100	100	0
7	6	1	6
8	>100	100	0
9	2	0	2
10	>100	100	0

fragment and one into the 6.4kb *Eco*RI fragment. *Eco*RI digests of the one example of pS1::Tn*551* and all five of pΔD::Tn*551* are shown in Fig.5.1. The results are indicative of specific insertion of Tn*551* into the large EcoA fragment of pS1, whereas, into the plasmid pΔD, the Tn*551*-insertions are apparently at random, reproducing the results of Browne (1985).

5.4 DNA sequence analysis of the pS1::Tn*551* and pΔD::Tn*551* plasmids

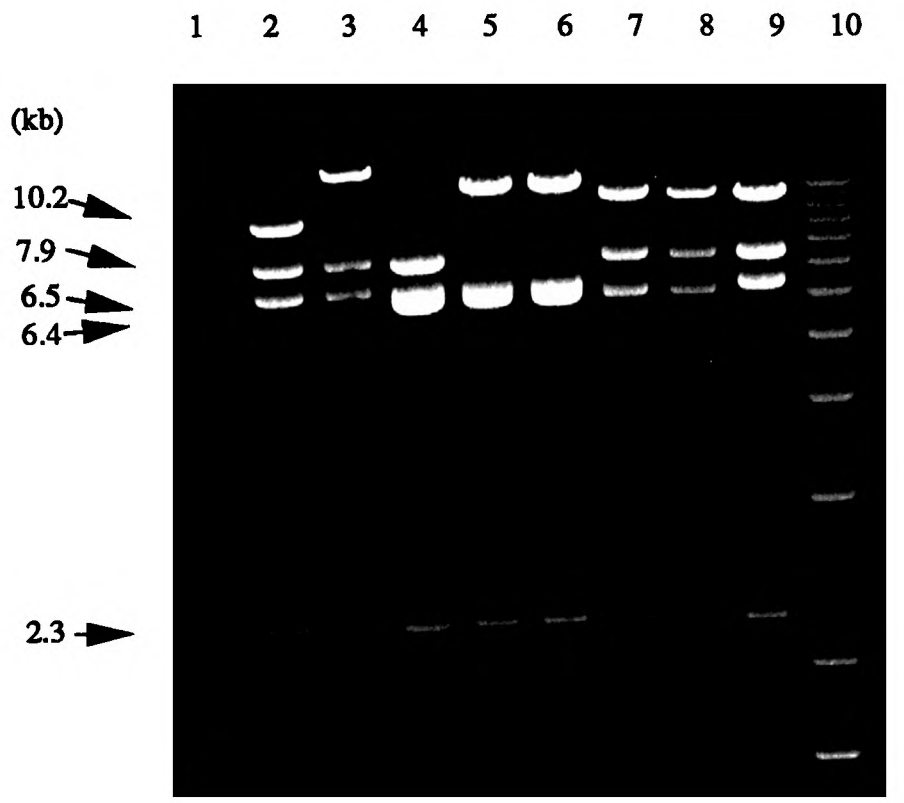
The exact site of insertion of Tn*551* into the plasmids pS1 and pΔD was investigated using nucleotide sequencing. The aims of this experiment were as follows:-

i. To discover any non-plasmid DNA at the ends of the transposon Tn*551* (attached by an imprecise excision during transduction) that might reflect on the site of integration of the plasmids pS1 and pΔD into the chromosome.

ii. To locate the preferred site(s) of integration of Tn*551* on pS1 and the sites of insertion on pΔD.

The plasmids used in this investigation were those in which the regions of Tn*551*-insertion have already been mapped using restriction enzymes (Oldridge, 1993; Browne, 1985). Two oligonucleotides were designed in order to sequence out of both of the termini of Tn*551* into the flanking DNA to determine the sequence at the two junctions. The pS1::Tn*551* type plasmids used were pBB33, pDB51 and pTD1 and the pΔD::Tn*551* type plasmids were pRA1, pRD11, and pRE56 (Browne, 1985). The restriction enzyme maps of these plasmids are shown in Figs.5.2 and 5.3, respectively.

Fig.5.1 *Eco*RI digests of the pS1::Tn*551* and pΔD::Tn*551* plasmids. The *Eco*RI fragments of pS1 and pΔD into which the Tn*551* insertion (5.3kb) has occurred are given in brackets.



KEY:

1 & 10. 1kb ladder

2. pS1

3. pS1::Tn551 (EcoA; 10.2::Tn551)

4. pΔD

5. pΔD::Tn551 (EcoA; 7.9::Tn551)

6. pΔD::Tn551 (EcoA; 7.9::Tn551)

7. pΔD::Tn551 (EcoB; 6.5::Tn551)

8. pΔD::Tn551 (EcoB; 6.5::Tn551)

9. pΔD::Tn551 (EcoC; 6.5::Tn551)

Fig.5.2 Site- and orientation-specific insertion of Tn*551* into the plasmid pS1, as predicted by Browne (1985), is shown. The transposon, Tn*551*, can be found at two positions due to the 'flipping' of the invertible region, *inv*. [Re-drawn after Browne (1985)]

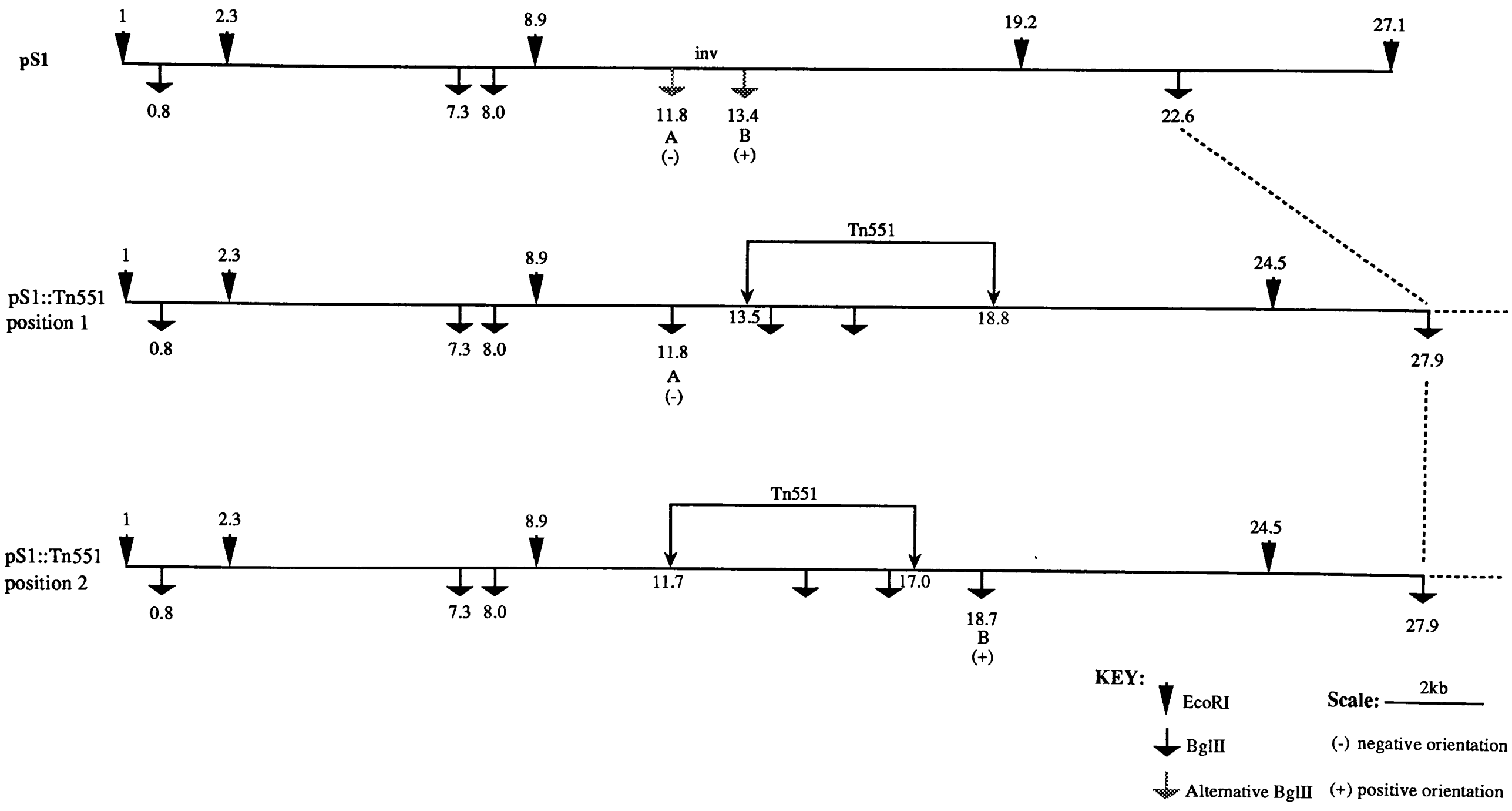
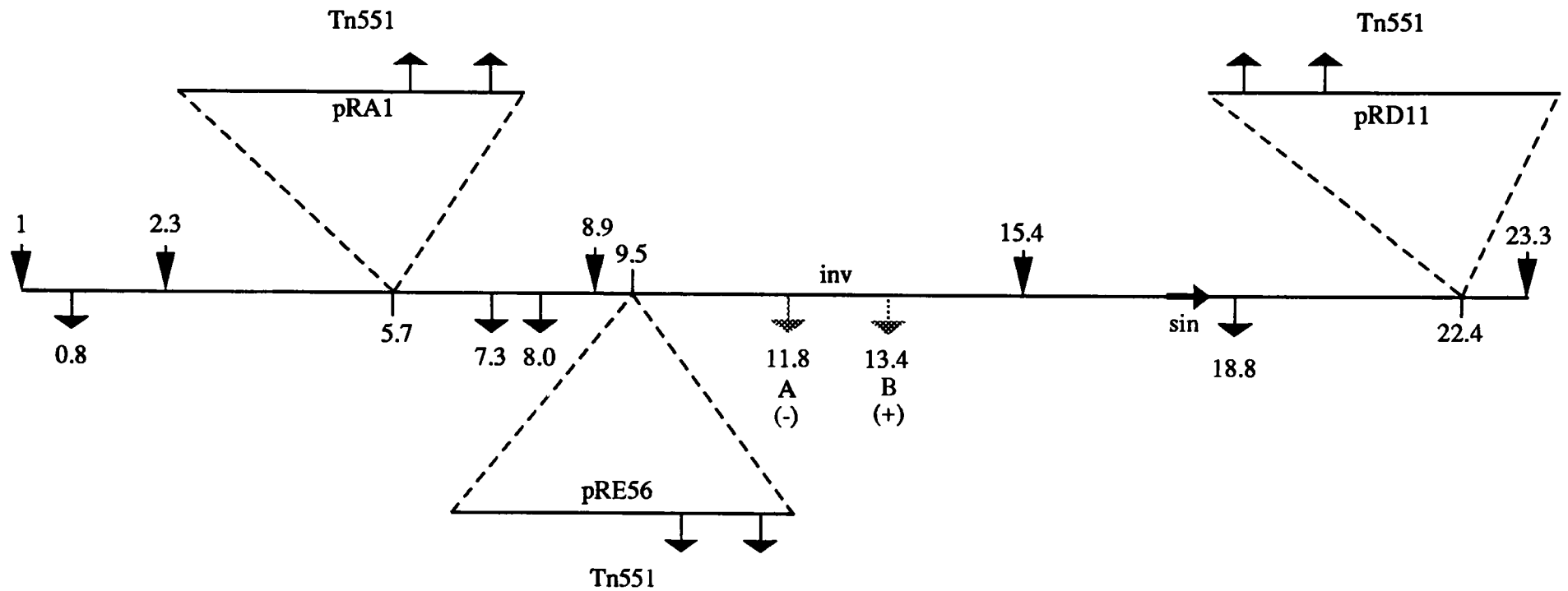
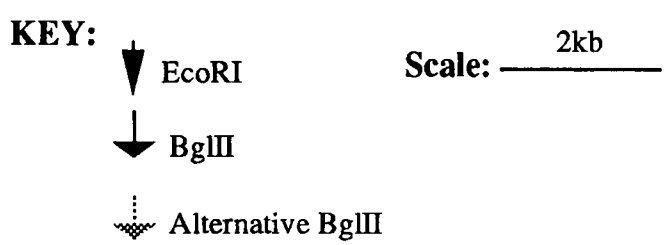


Fig.5.3 A composite linear restriction map of pΔD revertant transductants showing three insertion sites of Tn*551*. The plasmid has been opened up at one of the *Eco*RI sites [drawn after Oldridge (1993) and, Browne (1985)]. The invertible region, *inv*, can occur in either of the two orientations, *i.e.*, negative (-) or positive (+). The gene, *sin*, has also been labelled for reasons discussed later.





5.4.1 Design of the sequencing oligonucleotides

The terminal inverted repeats (TIRs) of the transposon Tn*551* have been sequenced by Khan and Novick (1980). A sequence in the interior of the transposon but close to the TIRs was required in order to design the sequencing oligonucleotides. The required sequence was obtained from Dr. Saleem Khan (unpublished, personal communication). Two oligonucleotides, JR551 and JL551, the binding sites of which on Tn*551* are shown in Fig.5.4, were designed in order to sequence out from TIR_R and TIR_L, respectively.

5.4.2 Sequence analysis of pBB33

The two oligonucleotides, JR551 and JL551, were used to sequence the Tn*551* junctions on pBB33. A very faint sequence was obtained after 24hrs autoradiography only for the right junction and a readable sequence was obtained only after 15 days autoradiography. The data show the insertion of Tn*551* into the putative transposase gene (ORF480; Rowland and Dyke, 1990) of Tn*552*. The sequence on Tn*551* was found to be different at many places from the previously published (Khan and Novick, 1980) and the unpublished (Khan, personal communication) one. This probably suggests the presence of a variant of the transposon, Tn*551*, in the Em^r *S. aureus* strains from this laboratory.

An autoradiograph of the gel showing the sequence at the right junction between Tn*551* and the plasmid pS1 is given in Fig.5.5.

A comparison of the sequence of the right terminus of Tn*551* as read on pBB33 with the previous sequence data (Khan, personal communication and, Khan and Novick, 1980) is given in Fig.5.6. The sequence at the site of insertion of Tn*551* into Tn*552* on the plasmid pBB33 (pS1::Tn*551*) is also shown in Fig.5.6. It was, then, decided to sequence the terminal region at the right junction of Tn*551* on the plasmid pI258 - the plasmid on

Fig.5.4 A simplified diagram to show the oligonucleotides JR551 and JL551 and their binding sites on the transposon Tn*551*. The drawings are not to scale. The distance of the oligonucleotides from the start of each TIR is shown as number of bases. [KEY: TIR_R, right terminal inverted repeat; TIR_L, left terminal inverted repeat].

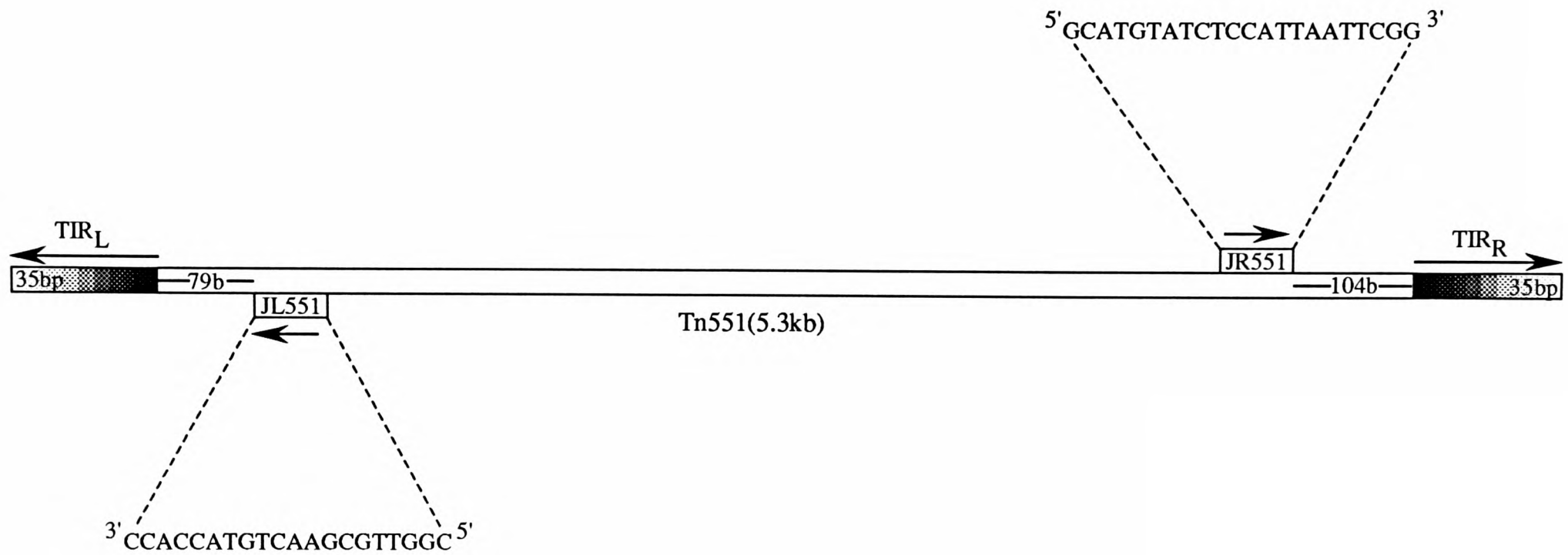


Fig.5.5 An autoradiograph of the sequencing gel of pBB33 using the oligonucleotide 'JR551'. The nucleotide sequence at the junction between the TIR_R of Tn*551* and pS1 is also given adjacent to the gel.

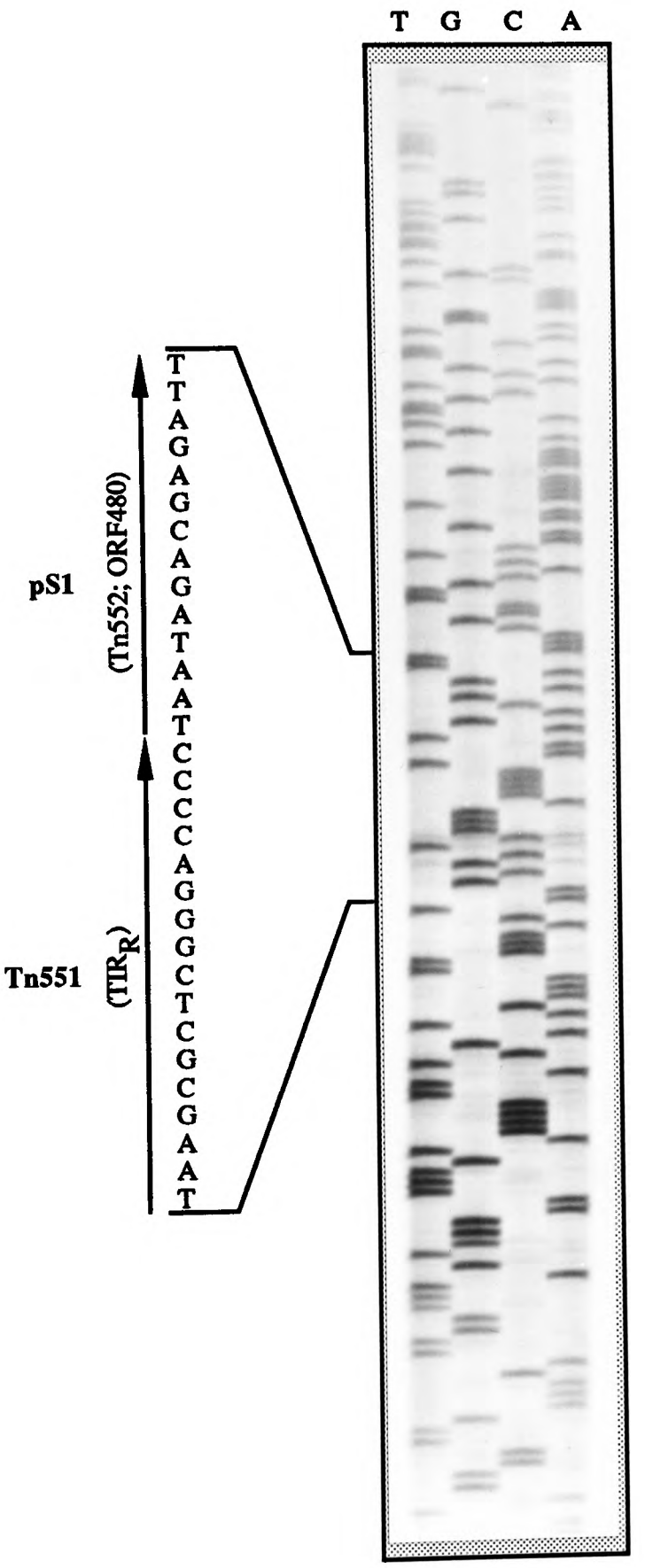


Fig.5.6 The sequence obtained on pBB33 (pS1::Tn*551*) using the oligonucleotide JR551 is shown. The sequence is compared with the Tn*552* sequence (in *italics*) within ORF480 to show the site of insertion of Tn*551* on pS1 (pBB33). The sequence is also compared with the previously reported sequence data on Tn*551* (Khan, personal communication and, Khan and Novick, 1981). The putative direct repeat (DR) at the right junction of Tn*551* derived from the Tn*552* DNA is *underlined*. The numbers correspond to the Tn*552* sequence (Rowland and Dyke, 1990). Gaps have been introduced to maximize homology. (TIR_R: Right terminal inverted repeat of Tn*551*). The differences are shown in lower case.

pBB33: (JR551)TTTGACTTaTctaT-TCCTAAACACTTAAGAGAATTGaATATaAAAaaTAAATA--GGCCTTGAAAtATTGGTTTA

Tn*551*: TTTGACTTgTtacTATCC--AACAC---------TTGgAT-TgA--ttTAAATAATGGCC--GAAAcATTGGTTTA

(2125) (2093) (2065)

Tn*552*: (ORF480) *TTGATAAAGGTGAGCATAAATTAAAACAAAACA TAATAGACGAGATTAAACGCCTTGCACT*

pBB33: GTGGGAATTTGTACCCCTTATCGATACAAATT-CcCACTAAGCGCTCGGGACCCC *TAATA*GACGAGATTAAACGCCTTGCACT

DR

Tn*551*: GTGGGAATTTGTACCCCTTATCGATACAAATTTCtC-GT--GCGCTC-GGACCCC

TIR_R

which the initial sequencing of the Tn*551*'s TIRs was performed (Khan and Novick, 1980). The sequencing of the left terminus was not followed due to unavailability of sequence data for the designing of a sequencing oligonucleotide.

5.4.3 Sequence of the right terminus of Tn*551* on the plasmid pI258 and its comparison with other relevant sequences

The oligonucleotide JR551 was used to sequence the terminal region at the right junction of Tn*551* on the plasmid pI258. An alignment of the sequence data obtained with the following sequences of relevance is given in Fig.5.7.

i. sequence at the right terminus of Tn*551* on pBB33,

ii. Khan and Novick's data, and

iii. terminal sequence of Tn*917*.

The results show that the terminal region of the transposon Tn*551* from this laboratory is almost identical to the *Streptococcus faecalis* transposon Tn*917* (Shaw and Clewell, 1985) and is much more diverse from the Tn*551* sequence data reported by Khan and Novick.

5.4.4 Sequence analysis of pDB51 and pTD1

Only the right terminal region was sequenced to find out the junction of Tn*551* on these plasmids. The results obtained in this sequencing were identical to those obtained for pBB33 and are, thus, not reported separately. The Tn*551* has integrated site- and orientation-specifically into the putative transposase (ORF480) of Tn*552* in all these cases. The exact site of insertion of Tn*551* into all three pS1::Tn*551* plasmids is shown in Fig.5.8.

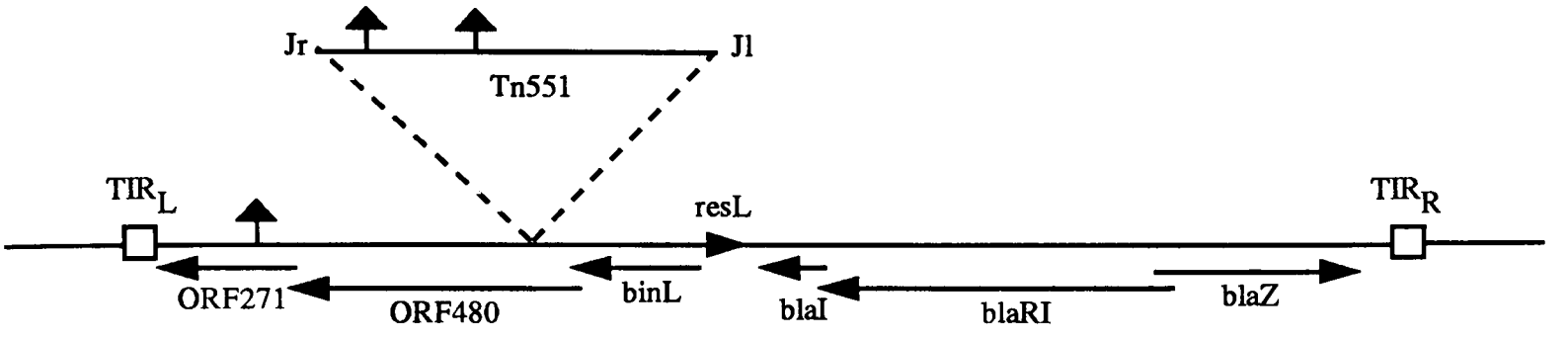
Fig.5.7 The DNA sequence of the right terminus of Tn*551*, obtained on the plasmid pI258. The sequence is compared with the Tn*551*'s terminal sequences from the plasmid pBB33 and Khan and Novick's (K&N's) data (Khan, personal communication and, Khan and Novick, 1980). The sequence is also compared with the Tn*917* sequence (Shaw and Clewell, 1985). Gaps have been introduced to maximize homology. The terminal inverted repeats of the transposons have been underlined. The differences are given in small letters.

```
Tn551: (pI258)TATCACTTTTCTTGGAGAGTATAAA
Tn917:        TATCACTTTTCTTGGAGAGTATAAA
Tn551: (K&N's)TATCACTTTTCTTGGAGAGTATAA-

Tn551: (pI258)TTTGACTTGTTATCTAT-TCCTAAACACTTAAGAGAATTGaATATgAAAaaTAAATA--GGCCTTGAAAtATTGGTTTA
BB3.3: (JR551)TTTGAC---TTATCTAT-TCCTAAACACTTAAGAGAATTGaATATgAAAaaTAAATA--GGCCTTGAAAtATTGGTTTA
Tn917:        TTTGACTTGTTATCTAT-TCCTAAACACTTAAGAGAATTGaATATgAAAaaTAAA-A--GGCCTTGAAAcATTGGTTTA
Tn551: (K&N's)TTTGACTTGTTA-CTA--TCC--AACAC---------TTGgAT-TgA--ttTAAATAATGGCC--GAAAcATTGGTTTA

Tn551: (pI258)GTGGGAATTTGTACCCCTTATCGATACAAATT-CcCAc-AAGCGCTCGGGACCCC
BB3.3: (JR551)GTGGGAATTTGTACCCCTTATCGATACAAATT-CcCAcTAAGCGCTCGGGACCCC
Tn917:        GTGGGAATTTGTACCCCTTATCGATACAAATT-CcCAcTAAGCGCTCGGGACCCC
Tn551: (K&N's)GTGGGAATTTGTACCCCTTATCGATACAAATTTCtC-gT--GCGCTC-GGACCCC
```

Fig.5.8 Site of Tn*551*-integration in Tn*552* on pS1.



KEY: ↑ BglII

5.4.5 DNA sequence analysis of the pΔD::Tn*551* plasmids and the comparison to the GenEMBL databases

The oligonucleotide JR551 was used to sequence the region adjacent to the right hand side of Tn*551* on the plasmids pRA1, pRD11 and pRE56, derived from pΔD.

5.4.5.1 pRA1

pRA1 is a derivative in which the transposon, Tn*551*, has been inserted into the 6.6kb EcoB fragment of pΔD (Browne, 1985). Sequencing was carried out at the right junction of Tn*551* and the results are shown in Fig.5.9. The sequence of 118 bases, adjacent to the TIR_R of Tn*551* on pΔD, is 98.3% homologous to 119 bases (1217 - 1336) from the sequence of ORF2 in the mercury-resistance operon (Fig.5.10) on the plasmid pI258 (Laddaga *et al.*, 1987). No function has yet been assigned to ORF2. However, the strain PS80(pRA1) was found to be Hg^s. The site of insertion is consistent with the restriction enzyme enzyme map of pRA1 which predicted the site of Tn*551* insertion to be the *mer* region of pΔD (Oldridge, 1993 and, Browne, 1985).

5.4.5.2 pRD11

pRD11 is a derivative in which the the transposon, Tn*551*, has been inserted into the 7.9kb EcoA fragment of pΔD (Browne, 1985). The sequence obtained at the right junction of the transposon on the plasmid using the oligonucleotide JR551 is given in Fig.5.11. The sequence of 98 bases, adjacent to the TIR_R of Tn*551* on pΔD, thus obtained shows no homology to those in the GenEMBL databases. However, it shows homology to the nucleotide sequence in a region called "the Earthquake Region" on a Cd^r plasmid, pOX6 (Dyke *et al.*, unpublished, personal communication). The trivial name "the

Fig.5.9 An autoradiograph of the sequencing gel of pRA1 using the oligonucleotide 'JR551'. The nucleotide sequence at the junction between the TIR_R of Tn*551* and pΔD is also given adjacent to the gel.

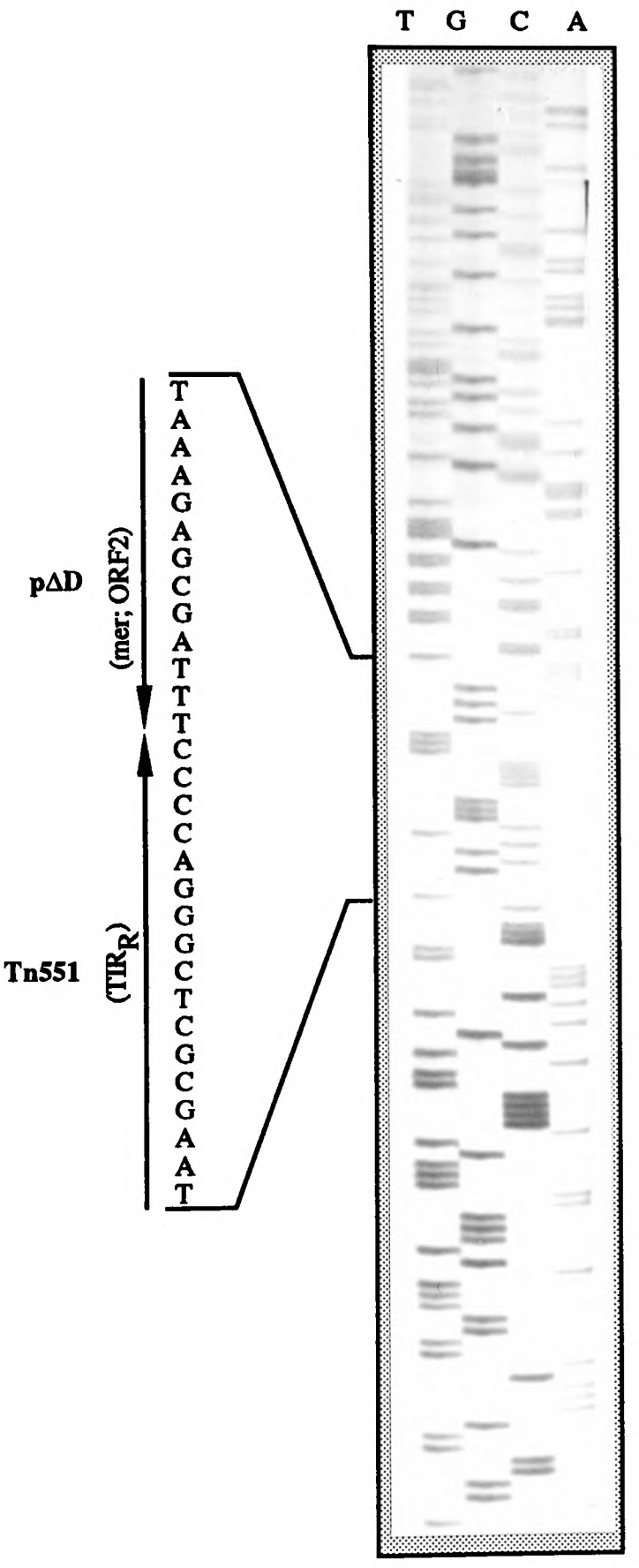


Fig.5.10 The DNA sequence of the right terminus of Tn*551*, obtained on the plasmid, pRA1, is given. The sequence at the site of insertion of Tn*551* is compared with the ORF2 sequence from the mercury-resistance operon on the plasmid pI258 (Laddaga *et al.*, 1987). The right terminal inverted repeat (TIR_R) of Tn*551* has been marked (overlined) and is given in *italics*. The putative direct repeat is underlined. The sequence at the site of insertion on pΔD and that of ORF2 is in plain text. The base number (1217), where the homology of the sequence of pΔD at the site of Tn*551*-insertion to ORF2 starts, is given on the ORF2.

TIR_R

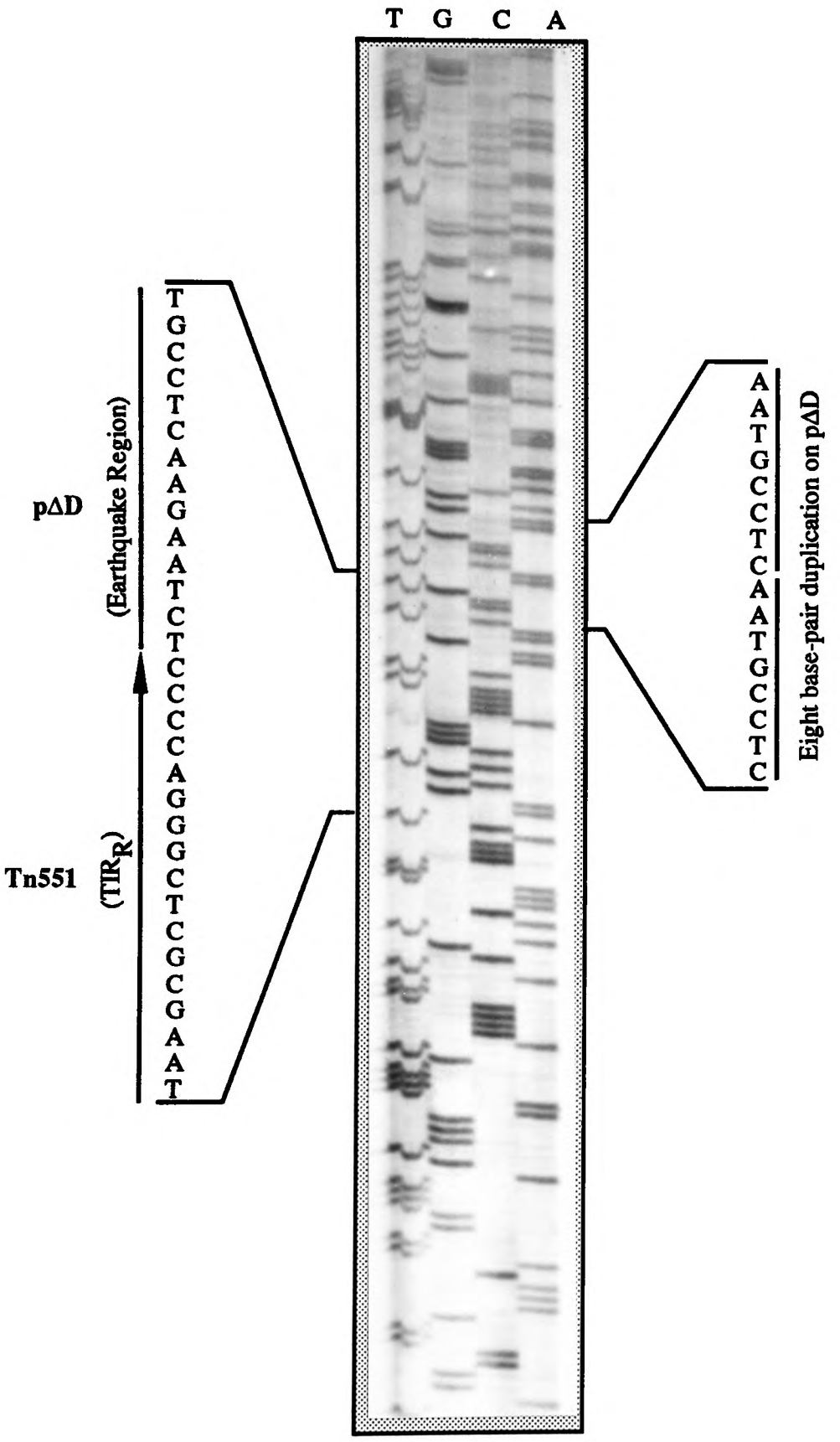
RevA1: (Tn*551*) *CTTATCGATACAAATTCCCACTAAGCGCTCGGGACCCCTTTAG*CGAGAAATCCAATTCCTTCATTCGTTTAATAAACCG

ORF2: (*mer*) CATCTTGATCAACCACACCAAACAACAGGTGGATTTCCTTTAGCGAGAAATCCAATTCCTTCATTCGTTTAATAAACCG
1217

RevA1: TACCCGATCTGCTGTTTCCTCTGAATATATTCGATACCCTGATTCGTTTCAGGGAGGCC-GGCTATCAATCCTTTCC

ORF2: TACCCGATCTGCTGTTTCCTCTGAATATATTCGATACCCTGATTCGTTTCAGGGAGGCCGGGCTATCAATCCTTTCC
1336

Fig.5.11 An autoradiograph of the sequencing gel of pRD11 using the oligonucleotide 'JR551'. The nucleotide sequence at the junction between the TIR_R of Tn*551* and pΔD is also given adjacent to the gel.



Earthquake Region" is assigned to the nucleotides from 1 to 900 on the plasmid, pOX6, and there are no large open reading frames in this region. This region undergos spontaneous rearrangements and the homologies of the small pieces of this sequence to the sections of others (*e.g.*, transposase of the *E. coli* transposon, Tn*2501*) suggests that this region may be a result of many recombination events (Dyke *et al.*, personal communication). A comparison of the sequences is given in Fig.5.12. An eight base-pair direct repeat close to the insertion site of Tn*551* on pΔD is present only once in the plasmid pOX6. The eight base-pair duplication could be the result of the insertion of Tn*551* or could be a feature of pΔD alone. The sequence of pΔD at this point is not known.

5.4.5.3 pRE56

pRE56 is a derivative in which Tn*551* has been inserted into the 6.5kb EcoC fragment of pΔD (Browne, 1985). The sequence obtained at the right junction of the transposon on the plasmid using the oligonucleotide JR551 is given in Fig.5.13. The 117bp sequence shows 100% homology to the *bin3* gene (also called *sin*) of the plasmid pI9789 (Rowland and Dyke, 1989). The homology is also shown to the *bin3* sequence on the plasmid pI258 (100% homology; *bin3* partially sequenced by Ji and Silver, 1992) and the *sin* sequence of the plasmid pSK1 (Paulsen *et al.*, 1994). A sequence comparison is given in Fig.5.14. The sequence data, does not agree with the restriction map of pRE56 (Oldridge, 1993 and, Browne, 1985). In which the Tn*551* insertion has been predicted to be in the *cad* region on pΔD. The insertion was predicted to be at a distance of ~0.6kb from the *Eco*RI site (see Fig.5.3) on the 6.5kb *Eco*RI fragment (EcoC) fragment. The sequence data show the insertion to be at approximately the same distance from the *Eco*RI site but on the other end. This result is illustrated in Fig.5.15.

Fig.5.12 The DNA sequence of the right-hand junction of Tn*551* in pRD11 is given. The sequence at the site of insertion of Tn*551* is compared with "the Earthquake Region" of the plasmid pOX6 (Dyke *et al.*, unpublished). The right terminal inverted repeat (TIR_R) of Tn*551* has been marked (overlined). The putative direct repeat (DR) is underlined. The eight base-pair direct repeat on pΔD is given in *italics*. Gaps have been introduced to maximize homology. The differences in the region of homology are shown in lower case.

TIR_R DR

RevD11: (Tn*551*)CTTATCGATACAAATTCCCACTAAGCGCTCGGGACCCCTCTAAGAA*CTCCGTAACTCCGTAA*GAGCATAAGGGgAAA

pOX6: GGGTCTTTTTATCAAAAATCGAGAAATTTCAATTTTTCTCTAAGAA*CTCCGTAA*--------GAGCATAAGG-aAAA

RevD11: TtTGACCCCATgTATAcAT--GGTATCTGGCAAAGCgA-AcAaTTCAAAG-CTTCaaCaAcaT

pOX6: TaTGACCCCATcTATAgATGAGGTATCTGGCAAAGCcAGAtAtTTCAA-GACTTCgtCgAccT

Fig.5.13 An autoradiograph of the sequencing gel of pRE56 using the oligonucleotide 'JR551'. The nucleotide sequence at the junction between the TIR_R of Tn*551* and pΔD is also given adjacent to the gel.

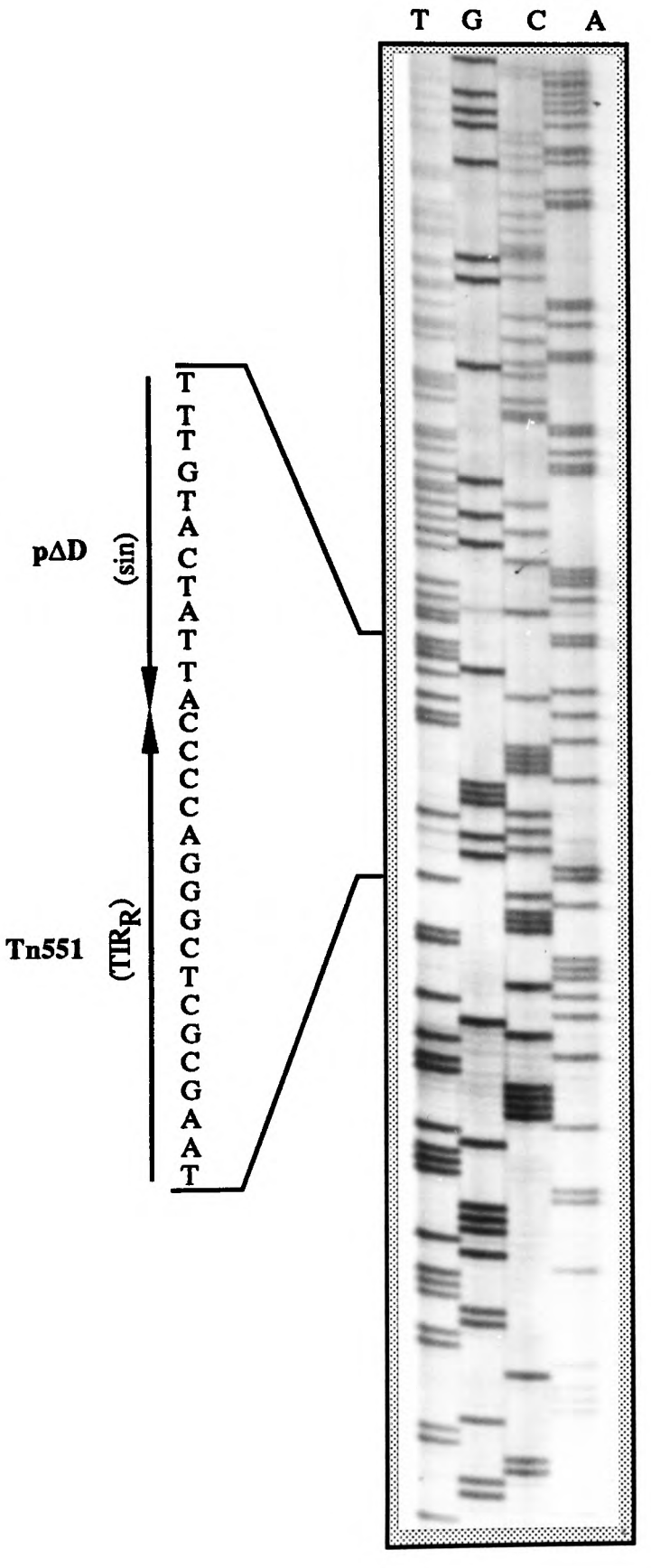


Fig.5.14 The DNA sequence at the right-hand junction of Tn*551* on pRE56 (i). The sequence at the site of insertion of Tn*551* is compared with the *sin* (*bin3*) sequence (ii. Paulsen *et al.*, 1994; iii. Ji and Silver, 1992 and, iv. Rowland and Dyke, 1989). The right terminal inverted repeat (TIR_R) of Tn*551* has been marked (overlined). The putative direct repeat next to the TIR_R of Tn*551* is underlined. The sequence at the site of insertion on pΔD and that of *sin* (*bin3*) is in plain text. The base numbers, where the homology of the sequence of pΔD at the site of Tn*551*-insertion to *sin* (*bin3*) starts, is given on the *sin* sequence. Non-matching bases in the region of homology are in lower case. Colons (:) represent identity of the sequence of Rowland and Dyke (1989) to that of Ji and Silver (1992).

TIR_R

i. CTTATCGATACAAATTCCCACTAAGCGCTCGGGACCCCaTTATCATGTTTAATTCTATAAACTGTcTGTCTTGTaATATTAACc

ii. GCTCATTTGCATTGACAATCtATTTATCAAGATAATCCcTTATCATGTTTAATTCTATAAACTGTtTGTCTTGTgATATTAACt
985

iii. GCTCATTTGCATTGACAATCcATTTATCAAGATAATCCaTTATCATGTTTAATTCTATAAACTGTcTGTCTTGTaATATTAACc
2492

iv. ::
619

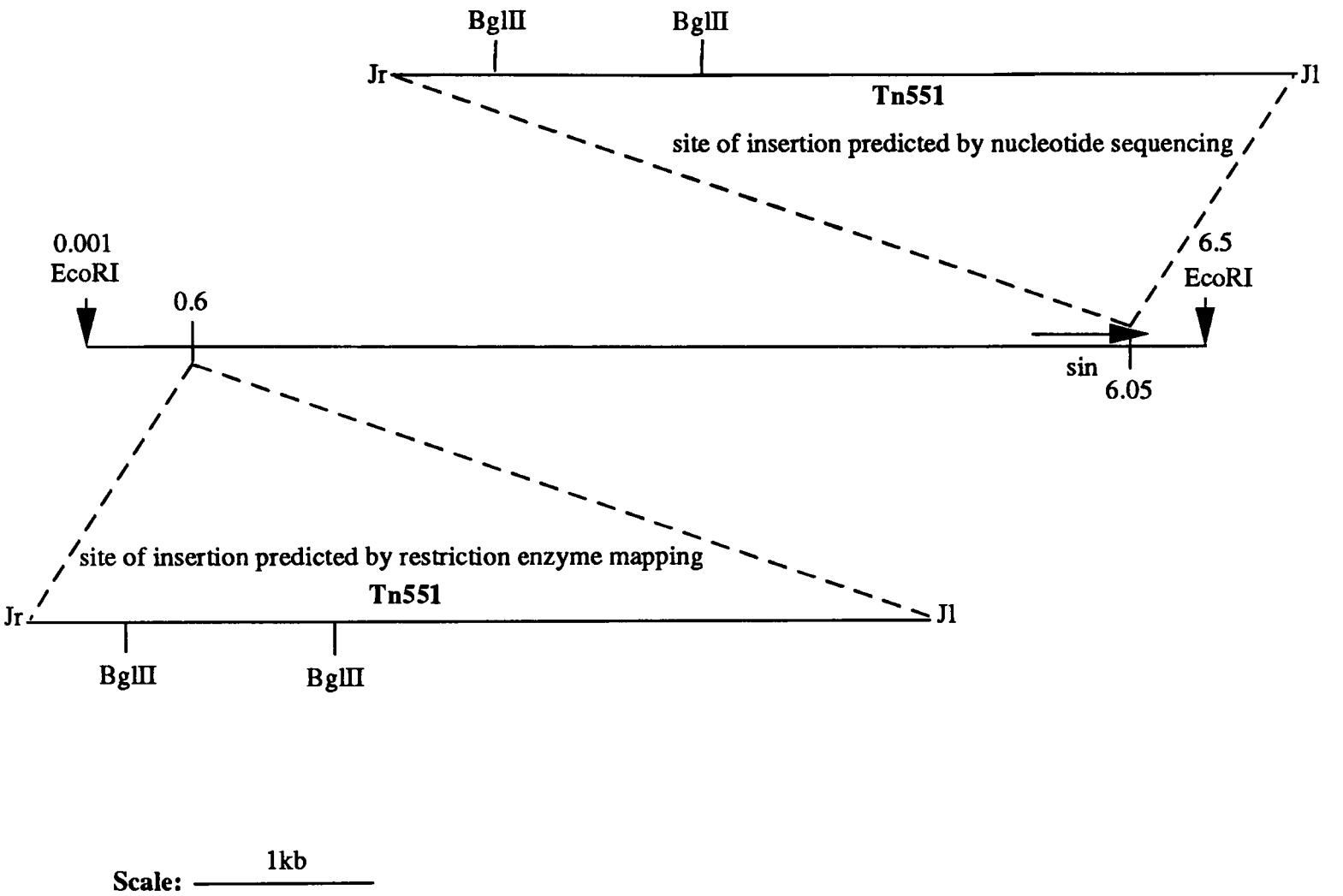
i. TCTTTCGCAATCTTACTAATtGCTTgCCCTTCTTCTAACATTTCgACaACtCGATGATAgATAACACG

ii. TCTTTCGCAATCTTACTAATcGCTTtGCCTTCTTCTAACATTTCcACcACgCGATGATAaATAACACG

iii. TCTTTCGCAATCTTACTAATtGCTTgCCCTTCTTCTAACATTTCgACaACtCGATGATAgATAACACG

iv. ::

Fig.5.15 The putative insertion site of Tn*551* into the plasmid pΔD in pRE56, as deduced by restriction endonuclease mapping (Oldridge, 1993 and, Browne, 1985) as well as that by nucleotide sequencing (this study) is shown. Only the relevant EcoC fragment of pΔD is shown. [KEY: Jr, right junction; Jl, left junction].



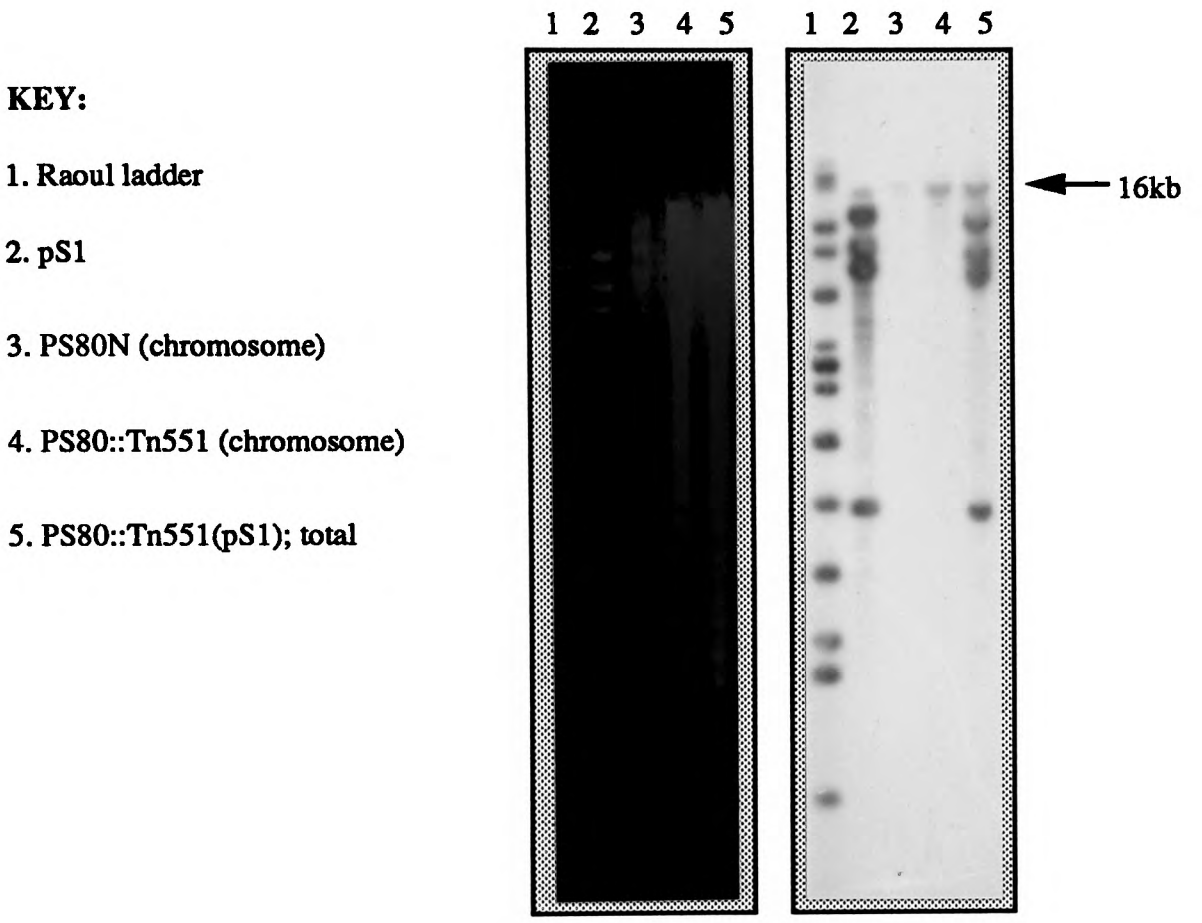
5.5 Hybridization analysis of the chromosome of PS80::Tn*551*

The presence of Tn*551* on the chromosome of *S. aureus*, PS80, increases the reversion frequency of the plasmids pS1 and pΔD, suggesting an interaction between Tn*551* and the plasmids. A 16kb *Eco*RI fragment of the chromosome of PS80N was shown to be involved in the reversion phenomenon (Chapter 4). The location of Tn*551* on the PS80 chromosome is not known and it was, thus, decided to investigate whether Tn*551* is present at or near to the part of Tn*552* shown to be involved in reversion in PS80N. The *Eco*RI digested chromosomal DNA of PS80::Tn*551* and PS80N was used to hybridize to pS1. The results are shown in Fig.5.16. For both strains, a 16kb *Eco*RI fragment hybridizes and, since there is no *Eco*RI site in Tn*551*, it is concluded that in PS80::Tn*551*, the Tn*551* has not inserted into the 16kb *Eco*RI fragment of PS80N but at some other location.

5.6 Hybridization analysis of the chromosome of the RN4220(pS1) revertants

The chromosomal DNA of the RN420(pS1) revertants was analysed using Southern blotting to discover whether or not the reversion involves integration. The chromosomal DNA was digested with *Eco*RI and electrophoresed on a 0.7% (w/v) agarose gel. The DNA was transferred and co-valently bound to a nylon membrane. The

Fig.5.16 An autoluminograph of the membrane showing hybridization of the plasmid pS1 to the genomic DNA of PS80::Tn*551*. A photograph of the agarose gel is also given. All DNA samples were digested with *Eco*RI and electrophoresed on 0.7% (w/v) agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pS1 probe.



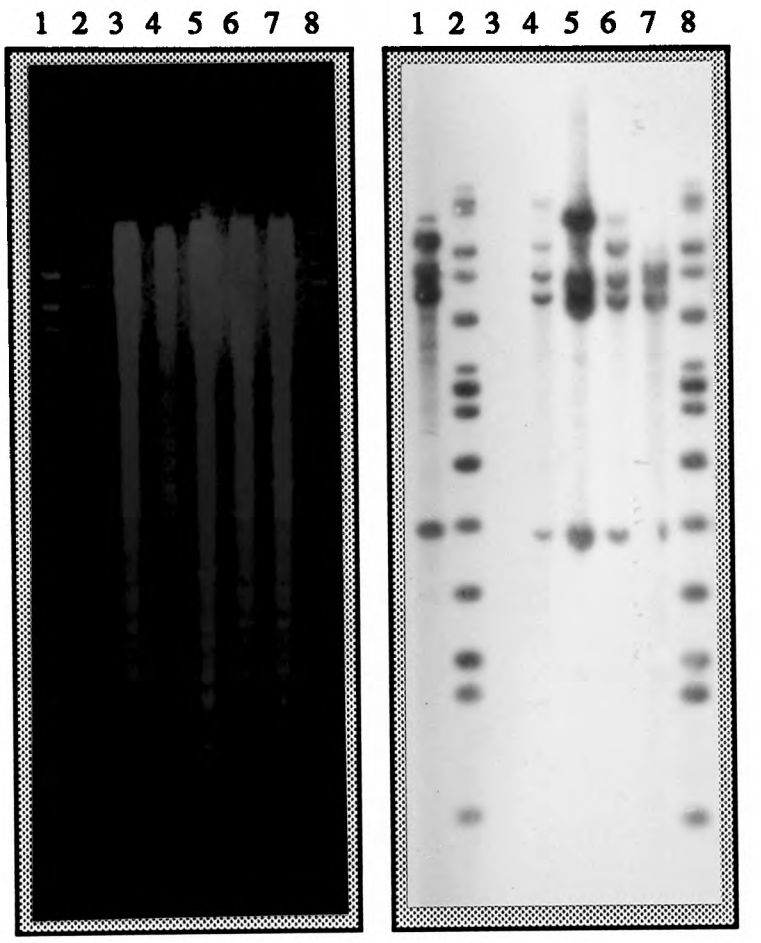
hybridization to the pS1 probe was detected using Stratagene's Flash System. The results are shown in Fig.5.17. and show that, for all four revertants tested, the EcoB, EcoC and EcoD bands are not changed but that for Rev2 and Rev4, the EcoA bands have disappeared.This can be interpreted as showing that there is recombination between chromosome and plasmid involving the EcoA band on pS1 and an unidentified site on the chromosome. The chromosomal site is different in Rev2 from that in Rev4. The Rev1 and Rev3 results can be interpreted as showing tandem repeats of the plasmid being integrated into sites on the chromosome with the result that the 10.2kb EcoA fragment is present in smaller amounts than the other three bands and that a new larger band consisting of both chromosomal DNA and at least part of the 10.2kb pS1 EcoA band has been created. It is tentatively concluded that for RN4220, at least some of the revertants are the result of integration into the chromosome but that there is no specific chromosomal site but possibly a plasmid specific site within the EcoA fragment.

5.7 Discussion

5.7.1 Role of Tn*551* in the reversion phenomenon

The presence of the transposon Tn*551* (conferring Em^r) on the chromosome of PS80N (*i.e.*, in PS80::Tn*551*) enhances the reversion frequencies of pS1 and pΔD (Chapter 3 - this study; Browne, 1985; Johnston, 1971). The absence of any detectable plasmid DNA in the Cd^r-revertants suggests that the plasmids have integrated into the host chromosome. Tn*551* presumably provides a site for integration to the plasmids pS1 and pΔD to enhance the reversion frequency (Oldridge, 1993). When the plasmids pS1 and pΔD are transduced to a new PS80N host from such revertants, with selection on cadmium

Fig.5.17 An autoluminograph of the membrane showing hybridization of the plasmid pS1 to the total DNA of RN4220(pS1) revertants. A photograph of the agarose gel is also given. All DNA samples were digested with *Eco*RI and electrophoresed on 0.7% (w/v) agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pS1 probe.



KEY:

1. pS1
2. Raoul ladder
3. RN4220 (chromosome)
4. RN4220(pS1) Rev1; total
5. RN4220(pS1) Rev2; total
6. RN4220(pS1) Rev3; total
7. RN4220(pS1) Rev4; total
8. Raoul ladder

(Cd^r encoded on both plasmids), linkage of Cd^r and Em^r phenotypes was seen. Browne (1985) reported that the transposon Tn*551* was found on the Cd^rEm^r plasmids. In the case of the plasmid pS1, Tn*551* was found inserted into a specific location in the invertible region, *inv*, of the plasmid. The Tn*551* insertion was into several different sites in the Cd^rEm^r plasmids found originating from pΔD.

Data presented in Section 5.2 show that the Cd^rEm^r linkage frequency is higher on transduction of Cd^rEm^r pΔD plasmids (*i.e.*, pΔD::Tn*551*) from PS80::Tn*551*(pΔD) revertants and lower in the case of pS1 (*i.e.*, pS1::Tn*551*). In the five pS1::Tn*551* examples tested, the transposon Tn*551* was found inserted specifically into the EcoA fragment of pS1 and in the five pΔD::Tn*551* examples the Tn*551* was found inserted into several different sites on pΔD (Section 5.3). This agrees with the data reported by Browne (1985).

The data obtained so far suggest the likelihood of the existence of two mechanisms of reversion of the plasmids pS1 and pΔD in PS80::Tn*551*:-

I- A mechanism which drives the integration of the plasmids pS1 and pΔD into the chromosome of PS80N, *i.e.*, the PS80N-mechanism. This mechanism operates in PS80::Tn*551* as well. The integration by this mechanism gives rise to 33 and 11 revertants per 10^6 cfu for pS1 and pΔD, respectively. No Cd^rEm^r plasmids are generated on transduction from such revertants and the transduced plasmids are Cd^rEm^s. The Type II Cd^rEm^s plasmids derived from PS80::Tn*551*(pS1) revertants reported by Browne (1985) and the Cd^rEm^s revertant-derived transductants from PS80::Tn*551*(pS1) and PS80::Tn*551*(pΔD) obtained in this study probably resulted from those revertant colonies in which the plasmids integrated into the chromosome by the PS80N-mechanism.

II- A second mechanism which involves Tn*551* and which is more active in forming plasmid-chromosome co-integrates than the native PS80N-mechanism. It generates a large number of the Cd^r revertants at 42°C. The integration involving Tn*551* somehow (see later) links the Em^r of Tn*551* to the integrated plasmid. The revertant-derived transductants arising from PS80::Tn*551*(pS1) revertants or PS80::Tn*551*(pΔD) revertants, in which the plasmid integrated into the chromosome using Tn*551*-mechanism would, therefore, be Cd^r as well as Em^r.

The number of revertants obtained for pS1 in PS80::Tn*551* is nearly three times less than that for pΔD. The revertants were picked at random to produce transductants. So on the CY-Cd-agar plates containing the revertant colonies, the probability of picking a revertant to produce transductants, generated by the PS80N-mechanism is higher for PS80::Tn*551*(pS1) revertants than for PS80::Tn*551*(pΔD) revertants. In the same way the probability of picking a revertant to produce transductants, generated by the Tn*551*-mechanism is higher for PS80::Tn*551*(pΔD) revertants than for PS80::Tn*551*(pS1) revertants. Therefore, more Cd^rEm^s pS1 transductants are obtained than for Cd^rEm^s pΔD transductants.

The integrative mechanism, if any, in PS80::Tn*551* producing Cd^rEm^r plasmids on transduction could operate in either of the following ways.

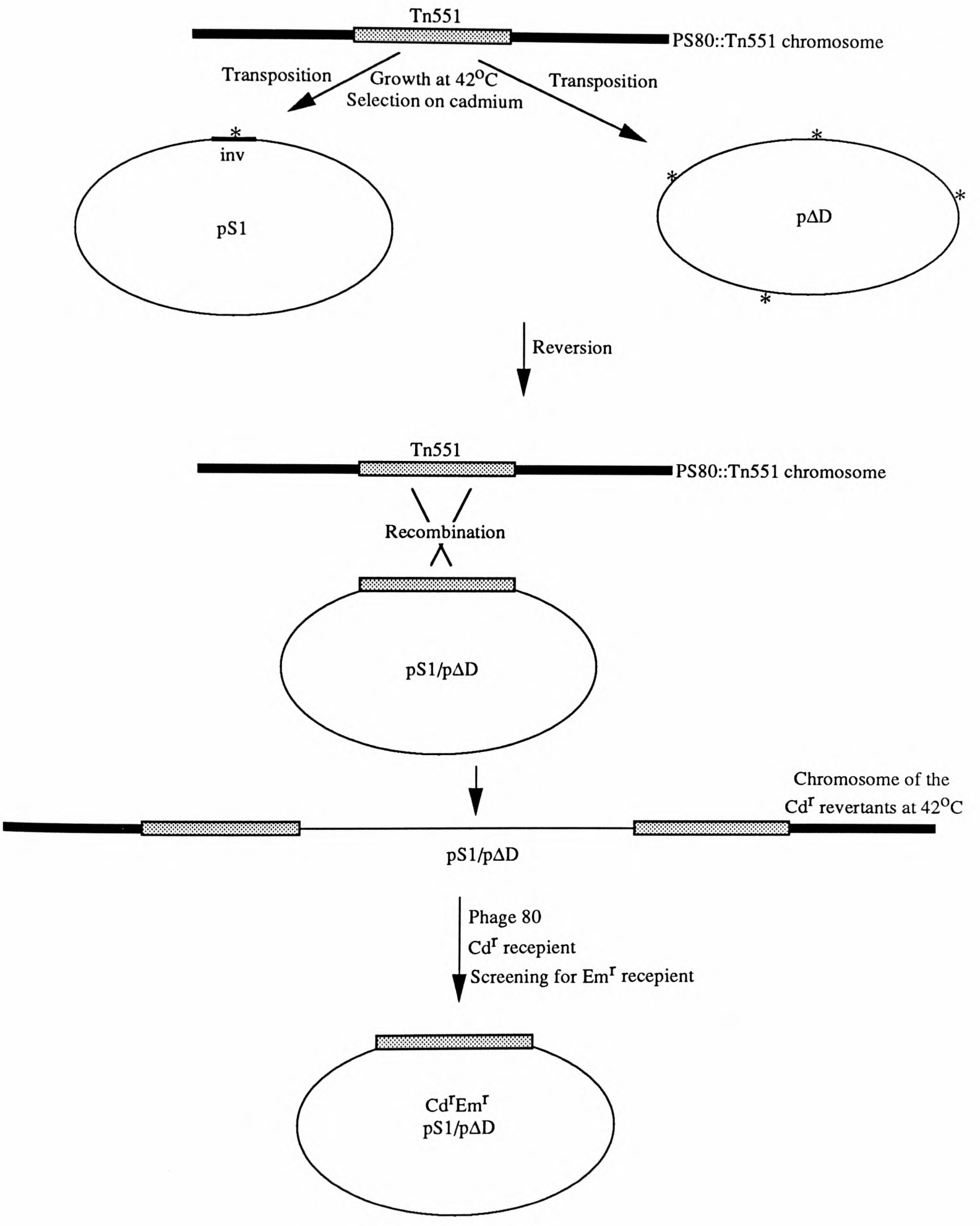
i. During growth at 42°C with selection for Cd^r the plasmids pS1 and pΔD are inserted into the chromosome of PS80::Tn*551* at a site close to the transposon Tn*551*. The site of integration for the plasmid pS1 into the chromosome is specific but is random for pΔD. During transduction, an imprecise excision of the plasmid occurs and results in the linkage of the plasmid and Tn*551* and hence co-transduction of Cd^r and Em^r phenotypes. Since, the integration involves one specific site for pS1 and many for pΔD, if the rate of

integration reaction is proportional to the availability of recombining sites, the chance of the occurrence of the recombination reaction involving pS1 will be lower than that for the recombination between pΔD and the chromosome. As a result of this, the total number of PS80::Tn*551*(pΔD) revertants generated by both the PS80N-mechanism and the Tn*551*-mechanism exceeds the number of revertants of PS80::Tn*551*(pS1). If the excision is imprecise then it may be expected that the transductant plasmids will contain some DNA derived from the chromosome.

ii. A second explanation is that more than one copy of Tn*551* is present on the PS80::Tn*551* chromosome and a copy of Tn*551* transposes from the chromosome into the plasmid during growth of the strain at 42°C. The plasmid carrying Tn*551* then recombines with the chromosome, either by homologous recombination (the region of homology for which is provided by the presence of Tn*551* on both the plasmid and the chromosome), a mechanism by which insertion sequences and transposons have been reported to generate genomic co-integrates (Kleckner, 1989; Cohen, 1976), or by site-specific recombination at a site, within Tn*551*, on both the plasmid and the host chromosome (Fig.5.18). Since, the integration of the plasmid occurs at Tn*551* on the chromosome, when such integrated plasmid is excised during transduction, a copy of the transposon Tn*551* is also excised with the plasmid resulting in a plasmid coding for both Cd^r and Em^r. The location of Tn*551* on the chromosome of PS80::Tn*551* and whether it carries one or more copies of Tn*551* is, however, not known, except that the transposon is not inserted into the 16kb *Eco*RI fragment of PS80N that hybridizes to the plasmid pS1 (Section 5.5).

The generation of a lower number of the revertants for the plasmid pS1 than pΔD in PS80::Tn*551*, if the transposition frequency is considered to increase with the availability of more sites for transposition, could be explained. Since, the transposition of Tn*551* is

Fig.5.18 A possible route to the generation of Cd^rEm^r pS1 and pΔD. The transposon Tn*551* first transposes to the plasmid (pS1 or pΔD) during growth at 42°C. The transposition occurs to a specific site (*) on the plasmid pS1 and into many random sites (*) on pΔD. The plasmid carrying Tn*551* then recombines with the chromosome. During transduction the plasmid is excised from the chromosome such that Tn*551* is co-excised with the plasmid resulting in a Cd^rEm^r plasmid.



into a specific site of the plasmid pS1 and into many sites on pΔD, therefore, the probability of the occurrence of the transposition reaction into pS1 becomes lower than that for pΔD and so a smaller number of revertants for pS1 than pΔD is obtained in PS80::Tn*551*.

In the case where both Cd^rEm^r and Cd^rEm^s transductants were produced by the same PS80::Tn*551*(pS1) or PS80::Tn*551*(pΔD) revertant colonies [*e.g.*, from PS80::Tn*551*(pS1) revertants # 3 and 7 - Table 5.1], presumably the plasmid integrated into two different location of the chromosome in the same host - in one case using the PS80N-mechanism and in the other case the Tn*551*-mechanism. On transduction both Cd^rEm^r and Cd^rEm^s transductants were obtained. Or, in the Cd^rEm^s transductants, the transposon transposed to a different location during transduction. It is, however, also possible that the revertant colony producing two types of transductants resulted from two independent revertant bacteria growing at the same location on the plate - one bacterium using the PS80N-mechanism of reversion and the other using the Tn*551*-mechanism. Thus on transduction both Cd^rEm^r and Cd^rEm^s transductants were produced.

Although the transductions were carried out under similar conditions, in the absence of the quantitative measurement of the transduction frequencies, it is not possible to draw any conclusion about the lower number the transductants obtained from some of the revertants [*e.g.*, from PS80::Tn*551*(pS1) revertants # 5 - Table 5.1]. The lower number of the transductants appears to be related somehow to the Cd^rEm^s phenotype.

5.7.2 The site of Tn*551* insertion in pS1 and pΔD

For all five examples, insertion of Tn*551* into pS1 is site- and orientation-specific, whilst into pΔD it seems to occur at random and without orientation-specificity. The reason

for the loss of this site- and orientation-specificity of insertion of Tn*551* into pΔD is not clear and is probably related to the features of pS1 missing from pΔD (*e.g.*, *binR*, one half of the TIR_R of Tn*552*, *etc.*).

Although, the nucleotide sequence at only the right junction of Tn*551* was obtained on the plasmids pS1 and pΔD, the nucleotide sequence at the site of Tn*551* insertion can be deduced from the known sequences of these regions on the plasmids pS1 and pΔD. Table 5.2 shows the nucleotide sequences on pS1 and pΔD at the sites of insertion of Tn*551*. Neither, the sequence of pS1, where the Tn*551* integration is site- and orientation-specific, nor on pΔD reveal any obvious structure except that these regions are AT-rich. Also, these regions do not show good homology. Some of the nucleotides in the 5 base pair sequence, duplicated on insertion of Tn*551*, are conserved, *e.g.*, the sequence 5' TTA is present in both pRA1 and pRE56; 5' CTA is present in pRD11 instead. The first 'T' nucleotide is conserved in pS1::Tn*551*, pRA1 and pRD11.

No DNA, foreign to Tn*551*, pS1 or pΔD (*i.e.*, chromosomal), was found at the right junction of Tn*551* with the plasmids pS1 or pΔD in any of the six cases sequenced, suggesting that if reversion in this strain involved integration of the plasmids into the chromosome, the excision during transduction was precise and involved only the plasmid and the Tn*551* DNA.

Table 5.2 The nucleotide sequence at the site of insertion of Tn*551* on the plasmids pS1 and pΔD. The regions showing conservation are boxed. The nucleotide sequence which is presumably duplicated on insertion of Tn*551* is underlined. The arrows indicate inverted repeats in the pRA1 sequence.

Plasmid	**Nucleotide sequence at the site of Tn551 insertion**
pBB33 (pDB51 & pTD1)	5' ATTAAAACAAAACATAATAGACGAGATTAAACG
pRA1	5' ACAGGTGGATTTCCTTTAGCGAGAAATCCAATT
pRD11	5' AATTTCAATTTTTCTCTAAGAACTCCGTAACTC
pRE56	5' CCATTACTAGATAAATTATCATGTTTAATTCTA

Site of Tn551 insertion

Chapter Six:

Investigation of the Thermosensitivity-suppressor Mutation in pS1

Chapter Six: Investigation of the Thermosensitivity-suppressor Mutation in pS1

6.1 Introduction

A locus on the plasmid pS1 that is capable of conferring autonomous replication in *S. aureus* was isolated and characterized by Walters (1992). This locus was called 'autonomously replicating region' (ARR) and is present as a 2.1kb *Bgl*II - *Hin*dIII fragment in the plasmid pOX3000. The equivalent 2.1kb *Bgl*II - *Hin*dIII fragment from the temperature-stable variant pS1-parent is present in the plasmid pOX4000. It was reported that the temperature sensitivity of replication of the plasmid pS1 is due to a single nucleotide difference within the minus strand origin (MSO) of replication of pS1 and the related temperature-stable plasmid pS1-parent. The complete nucleotide sequences of the 2.1kb *Bgl*II - *Hin*dIII fragments of both pOX3000 and pOX4000 were found identical except for pOX4000 having a 'C' residue at position 592 and that was missing in pOX3000. The complete sequence of the 2.1kb *Bgl*II - *Hin*dIII fragment of pOX4000 is given in Fig.6.1.

A variant of the plasmid pS1 called pTST, that is temperature-stable for replication, was isolated in *S. aureus* strain RN4220 after exposure of the strain RN4220(pS1) to 42°C in the presence of cadmium ions. It was decided to investigate the nature of the mutation responsible for the temperature-stable phenotype of pTST. The following questions were addressed:-

1. Is the mutation located on the plasmid pS1 or on the host chromosome?

Fig.6.1 The complete nucleotide sequence of the 2.1kb *Bgl*II - *Hin*dIII fragment of the plasmid pOX4000. The 'C' residue at position 592 is in **bold** text. The putative MSO is shown enclosed. The *Bgl*II and the *Hin*dIII sites are shown.

BglII

```
   1  AGATCTAAAT CATGTGTTAT GACAATCATA GTTTTATTTT CATCAAGTAG
  51  ATTTAATAAT AAATCAATGA CTATTTTTCC GTTTTTCGTA TCTAATGACC
 101  CAGTAGGTTC ATCAGCTAAA ATGATTTTCG GATCTTTTAA TATAAGTCTC
 151  GCCAACGCAA CTCTTTGCTG TTCACCACCA CTTAGAGAAT TAACAATTCT
 201  CTTGATATCT AAATCTAGGC TGACCTTTTT TAATACTTCT TTCTTTAAGT
 251  TCATATTATC TTTTTTATTC TTTTTGAACT TTAGGGCTAA ATCTAAATTT
 301  TGACTGATTG TTTGATTTTC AAGTAAAGCA AAATTTTGAA ACAAATACCC
 351  TAACGTATAT CTATAAAAAT CGACTTTCTT TTTTATTAAC TTATCTTCAA
 401  TATATATGCT TCCTTCTTTT TTAAAATTTT ACCATAGTTG CTTTATTCCA
 451  ATTGCTTTAT TGATGTTGAG ACGCGGACCC CTTAACAATC CCAAAACTTG
 501  TCGAATGGTC GGCTTAATAG CTCACGCTAT GCCGACATTC GTCTGCAAGT
 551  TTAGTTAAGG GTTCTTCTCA ACGCACAATA AATTTTCTCG GCATAAATGC
 601  GTGTCTATGC GTAGACATGG AGACACTTCA GAATTATGGA GTGATTACAT
 651  GAGTTTAACT ATTTTTCTTT ATTAAGTTTT AGGAAGTTTA TTAGCAAATT
 701  ATATCTACGA AAAATCTTCG AAAAGAAGTT CAGCTCATGA TAACGAGTTT
 751  ACCTATGATG AATGAAGTCA TTGGAAATCC ACTACTAGAT AAATTTATGA
 801  AAGATTTAAT TATTCAAATT TTAGCAATGA TTTCAGAACA AGAAAGAAAT
 851  GAAAGTAAAC GTCGACAAGC TCAAGGGATT CAAGTCGCCA AAGAAAAAGG
 901  TATATATAAA GGGAGACCTG TTCTGTATTC TCCCAATGCT AAAGACCCAC
 951  AAAAGCGTTT AGTTTATTAC CGAGTTGTTG AATTACTTGA ACAGGGTAAA
1001  TCTATAAGTA CTATAGCTAA AGAAGTTGGT ATTACACGTC AAACTATATA
1051  TAGAATAAAA AATAGTAAAT AAAATAACAT AGACAATAAT CTATATAGAT
1101  GTTAATCTAT TTAACATCTA GGAGGTTTAT TCATGTCTTA TAAAGAACTA
1151  TCAACAATAT TAAAAATTTT ATCAGATTCA AGTAGGTTAG AAATATTAGA
1201  TTTACTTTCT TGTGGTGAGC TATGCGCTTG TGACTTATTA GAACACTTTC
1251  AATTCTCACA ACCTACACTA AGTCATCATA TGAAGTCATT AGTAGATAAT
1301  GAATTAGTTA CAACACGAAA AGACGGCAAT AAACATTGGT ATCAACTTAA
1351  TCATGCTATT TTAGATGATA TTATCCAAAA CTTGAACATC ATTAATACAT
1401  CTAATCAAAG ATGTGTATGT AAAAATGTGA AATCAGGTGA CTGTTGATGA
1451  CTATTTTAGC AATTGTAATT TTTCTTTTAA CTTTAACCTT TGTGATTTGG
1501  CAACCAAAAG GTTTAGATAT TGGTATTACA GCTTTAATTG GAGCTGTTGT
1551  TGCTATCATT ACAGGAGTCG TAAGTCTTTC TGATGTATTA GAAGTAACAG
1601  GTATTGTTTG GAATGCAACT TTAACTTTTG TGGCTGTTAT TCTTATTTCA
1651  TTAATATTAG ATGAAATTGG TTTTTTTGAA TGGTCTGCGA TACATATGGT
1701  CAAGGCTTCA AACGGTAATG GCTTAAAAAT GTTTGTTTTT ATTATGTTAC
1751  TTGGGGCAAT TGTAGCAGCA TTTTTCGCAA ATGATGGTGC AGCTTTAATC
1801  TTAACGCCTA TTGTATTAGC GATGGTAAGG AATCTAGGAT TTAATCAAAA
1851  AGTGATTTTC CCCTTTATTA TTGCCAGTGG TTTTATTGCT GATACTACAT
1901  CACTTCCCTT AATTGTAAGT AACTTAGTTA ATATCGTTTC TGCAGATTAC
1951  TTCGATATTG GATTTATTGA ATATTTTAGC CGAATGATCA TTCCTAATAT
2001  ATTCTCTCTG ATTGCTAGTA TTCTCGTTTT ATGGTTATAT TTCAGAAAAT
2051  CCATACCTAA AACGTTCGAT ACAGAAAATC TATCAGATCC TAAAAGTGCA
2101  ATTAAAGATT CTAAGTTATT TAAGCTT
                            HindIII
```

2. If on pS1, is the mutation located in minus strand origin (MSO)?

6.2 Where is the mutation located?

Whether the putative mutation in pTST is on the plasmid or on the host chromosome was investigated. Phage 53 was grown on RN4220(pTST) and used to transduce Cd^r to RN4220 at 30°C with selection for Cd^r. Plasmid DNA was prepared from five transductants that had been grown at 42°C overnight in CY-broth before DNA preparation. All five examples contained a plasmid that was found to be indistinguishable from the plasmid pS1 on restriction enzyme analysis with *Eco*RI, *Eco*RV, *Bgl*II, *Acc*I and *Nde*I. All five transductants were further tested in a reversion experiment and, after 18hrs at 42°C on CY-Cd (26.6µg/ml)-agar plates, a lawn of bacteria was obtained for all five examples and so it was concluded that the mutation or suppression was present on pTST.

6.3 Cloning of the 2.1kb *Bgl*II - *Hin*dIII fragments from the plasmids pTST and pS1

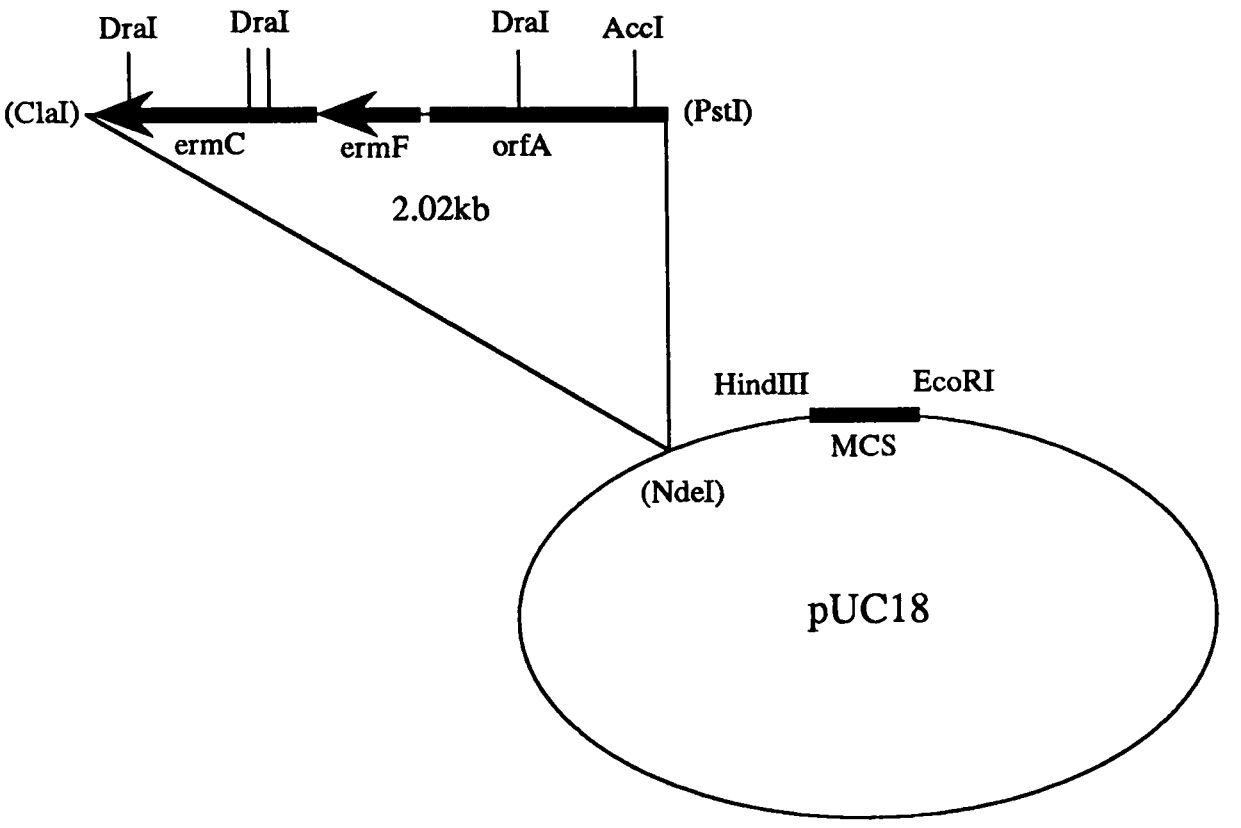
It was decided to investigate whether or not the mutation resides within the 2.1kb *Bgl*II - *Hin*dIII fragment of pTST.

The plasmid pWE180 is a screening vector and was constructed by Walters (1992). pWE180 is based on the *E. coli* plasmid vector pUC18 and consists of the 2.02kb *Pst*I -

*Cla*I fragment from the staphylococcal plasmid pE194 cloned into the unique *Nde*I site of pUC18. The 2.02kb fragment contains the erythromycin operon (*ermF* and *ermC*) plus part of orfA thought not to be involved in replication. This 4.7kb plasmid vector confers Ap^r and Em^r to *E. coli* but cannot replicate in *S. aureus*, although it contains an expressible system conferring Em^r in *S. aureus*. The multiple cloning site and the blue/white selection system of pUC18 have been retained by pWE180 (Walters, 1992). The restriction enzyme map of the plasmid pWE180 is shown in Fig.6.2.

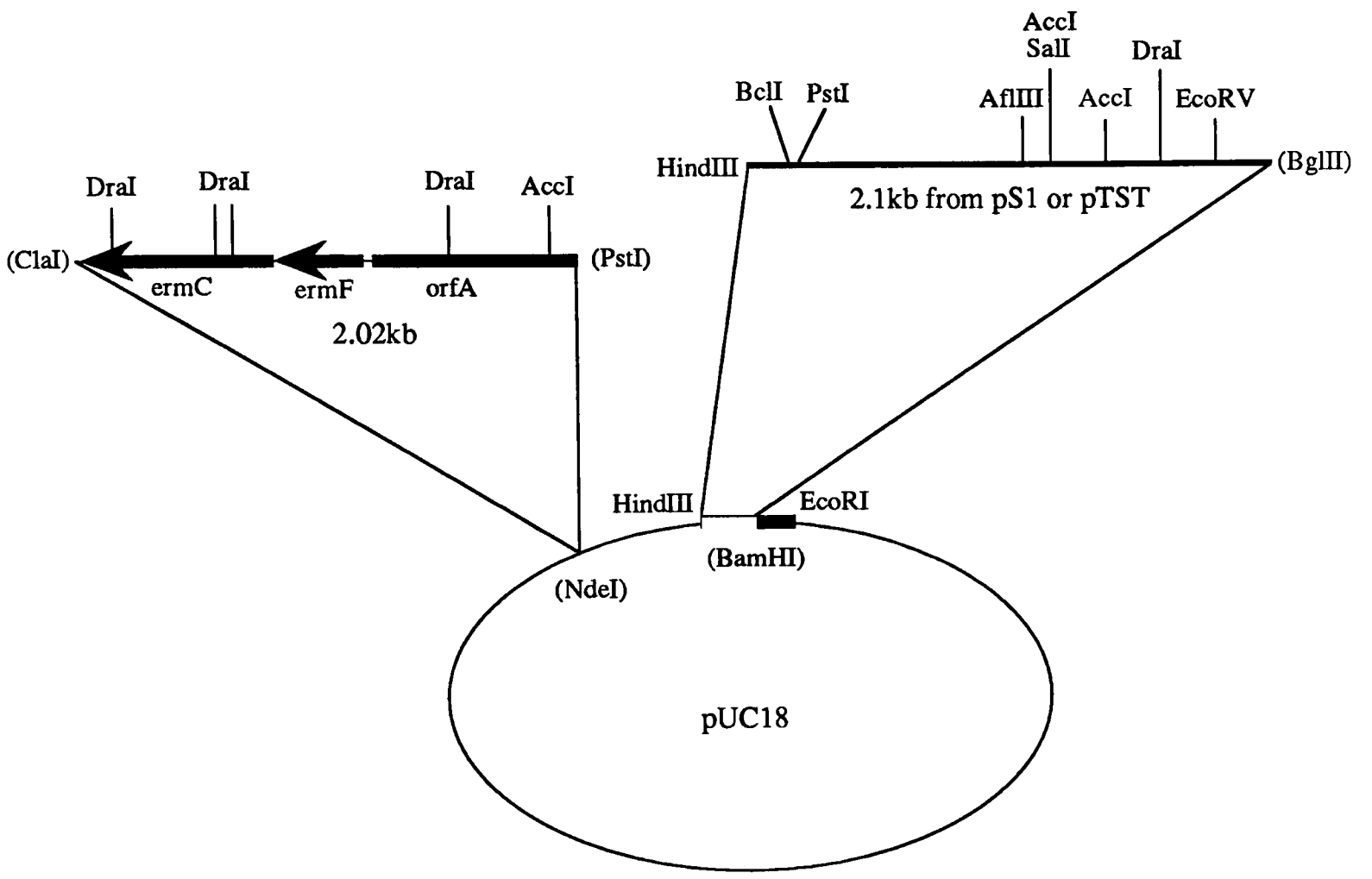
The pTST plasmid DNA was digested with *Bgl*II and *Hin*dIII together and was then ligated into pWE180 DNA digested with both *Bam*HI and *Hin*dIII. The restriction enzymes in the digestion were removed by two extractions with the StrataClean Resin™. The ligation mixture was used to transform competent JM107 to Ap^r. Plasmid DNA was prepared from twenty white colonies and suitable restriction enzymes were used to verify the presence of the correct 2.1kb fragment inserted into pWE180. Four colonies were found to contain the plasmid of interest and in one specific orientation due to the selectivity of the ligating ends of the two DNAs (*Bgl*II/*Bam*HI and *Hin*dIII). The plasmid was named pMS4000. The corresponding 2.1kb *Bgl*II - *Hin*dIII fragment from the plasmid pS1 was also cloned into pWE180 in the similar fashion and the resultant plasmid was named pMS3000. A restriction enzyme map of the plasmids pMS3000 and pMS4000, which is same for both, is shown in Fig.6.3.

Fig.6.2 Restriction enzyme map of the plasmid pWE180. MCS is the multiple cloning site of pUC18. The restriction enzyme sites lost during cloning are given in brackets. The 2.02kb *Cla*I - *Pst*I fragment of pWE180 is from the plasmid pE194 and is thought not to carry DNA involved in replication but confers resistance to erythromycin. This fragment was cloned into the *Nde*I site of the plasmid pUC18.



Plasmid pWE180

Fig.6.3 Restriction enzyme map of the plasmids pMS3000 and pMS4000. The 2.1kb *Hin*dIII - *Bgl*II fragment of the plasmids pS1 and pTST was cloned into the *Hin*dIII - *Bam*HI sites of the MCS of pUC18 to obtain the plasmids pMS3000 and pMS4000, respectively. The restriction enzyme sites lost during cloning are given in brackets.



pMS3000 & pMS4000

6.4 Transformation of pMS3000 and pMS4000 into *S. aureus*

Plasmid DNAs of pMS3000 and pMS4000 were prepared and purified from their respective JM107 strains on CsCl gradients and approximately 1μg DNA of each plasmid was used to transform the *S. aureus* strain RN4220 by the protoplast transformation method. The transformants were selected for Em^r. Plasmids pOX7 and pE194 (1μg DNA of each) were used as controls in the transformation. After incubating at 30°C for four days, approximately 10^6 transformants were obtained for both pOX7 and pE194 but none for the plasmids pMS3000 and pMS4000. The transformation was repeated using 0.5μg, 1μg and 2μg DNA for each plasmid and the plates were incubated for a longer duration (7 days) but no transformants could be obtained for either pMS3000 or pMS4000.

It was decided to use electroporation to transform the plasmid DNA instead of transformation. Approximately, 1μg of each plasmid DNA was used in the electroporation. Plasmids pOX7 and pE194 were used as controls. The transformants were plated onto CY-Em (20μg/ml)-agar plates. Some 12-15 colonies were obtained for pOX7 and pE194 and none for either pMS3000 or pMS4000.

6.5 The nucleotide sequence analysis of the 2.1kb fragments from pS1 and pTST

It was decided to sequence the region on the 2.1kb *Bgl*II - *Hin*dIII fragments of the plasmids pS1 and pTST that has been reported to contain the single nucleotide difference between pS1 and pS1-parent. Two oligonucleotides HIN3005 and BGL3001, that had been used by Walters (1992), were used to sequence both strands in this region. The oligonucleotide HIN3005 hybridizes to position (5' to 3') 785 - 767 and BGL3001 hybridizes to position (5' to 3') 347 - 371 on the 2.1kb fragment. The sequencing strategy is shown in Fig.6.4. The relevant region on the autoradiographs of the sequencing gels of both strands of the plasmids pMS3000 and pMS4000 are shown in Fig.6.5 and Fig.6.6. The 'C' residue at position 592 on the plus strand (Fig.6.5) and the complementary 'G' residue on the minus strand were found to be present on the 2.1kb fragments of both pMS3000 and pMS4000 and the previously reported results (Walters, 1992) thus could not be verified.

6.6 Discussion

The experiments on pTST reported in Section 6.2 suggested that the mutation which confers temperature-stable replication to this plasmid is on the plasmid itself and not on the chromosome, because the temperature-stable phenotype was co-transferred with the plasmid pTST. Walters (1992) discovered a minus strand origin (MSO), similar to that of

Fig.6.4 The binding sites of the oligos HIN3005 and BGL3001 on the 2.1kb *Bgl*II - *Hind*III fragment are shown.

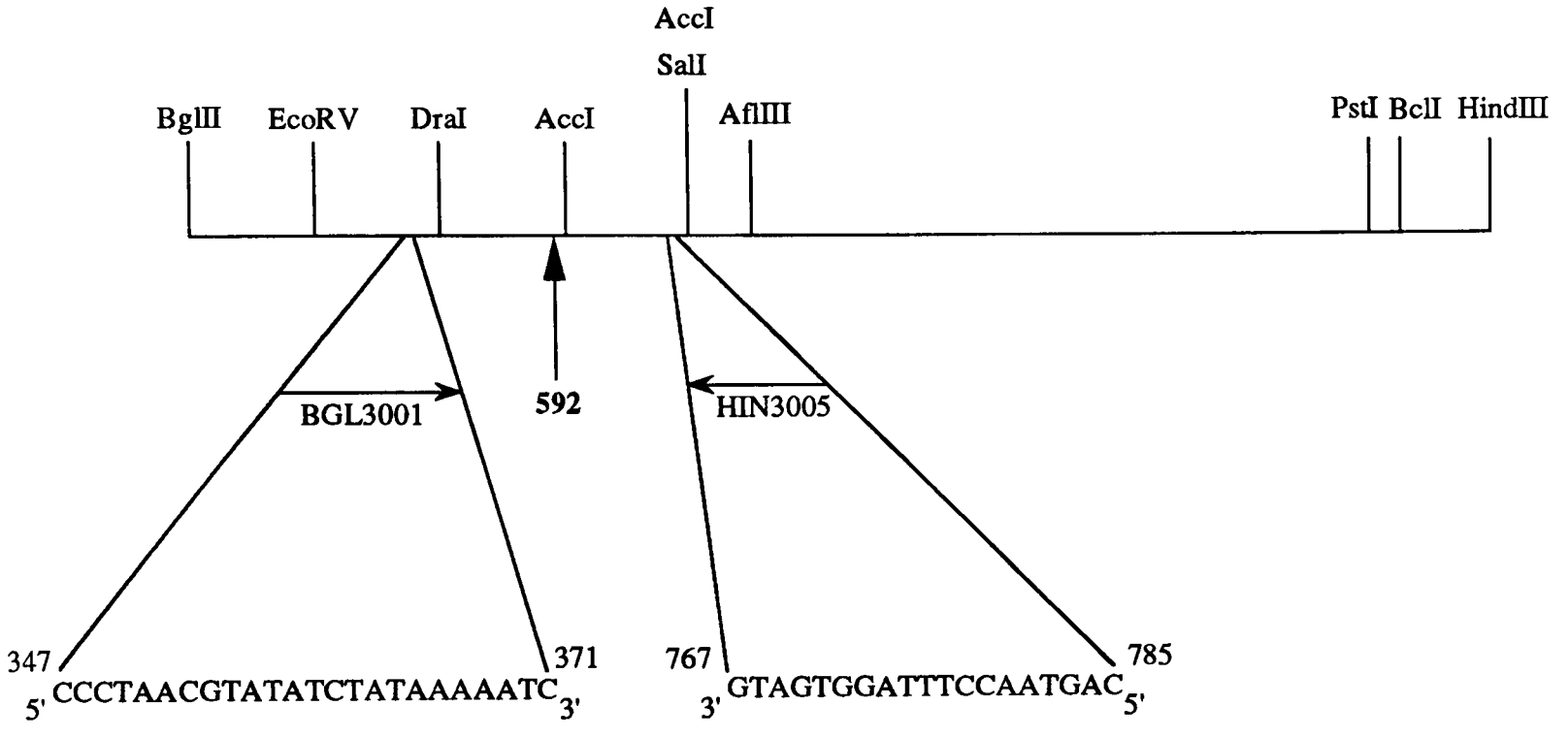


Fig.6.5 Autoradiograph of the sequencing gels of the plus strands of pMS3000 and pMS4000 are compared. The 'C' residue at position 592 is marked with an arrow. Some of the nucleotide sequence of this region is also given adjacent to the gels.

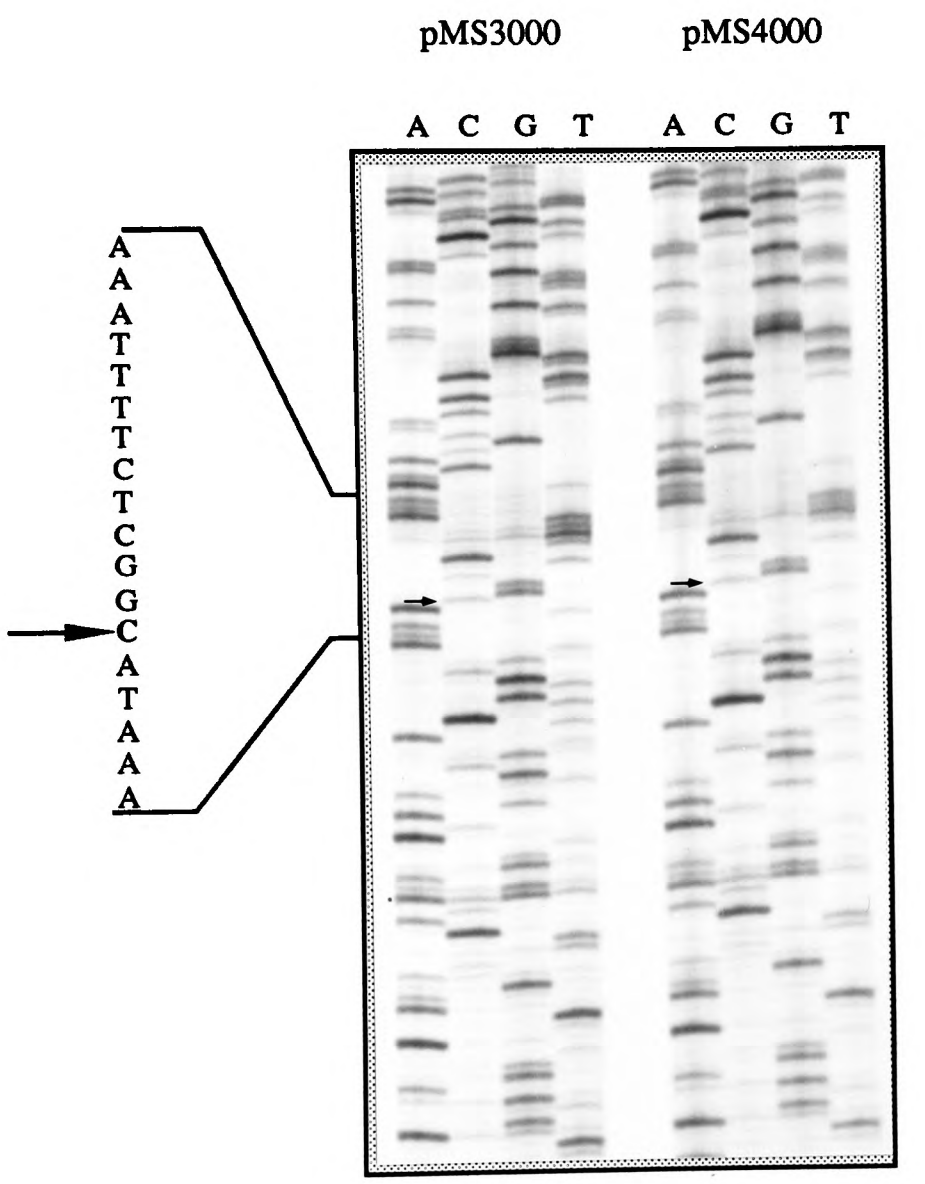
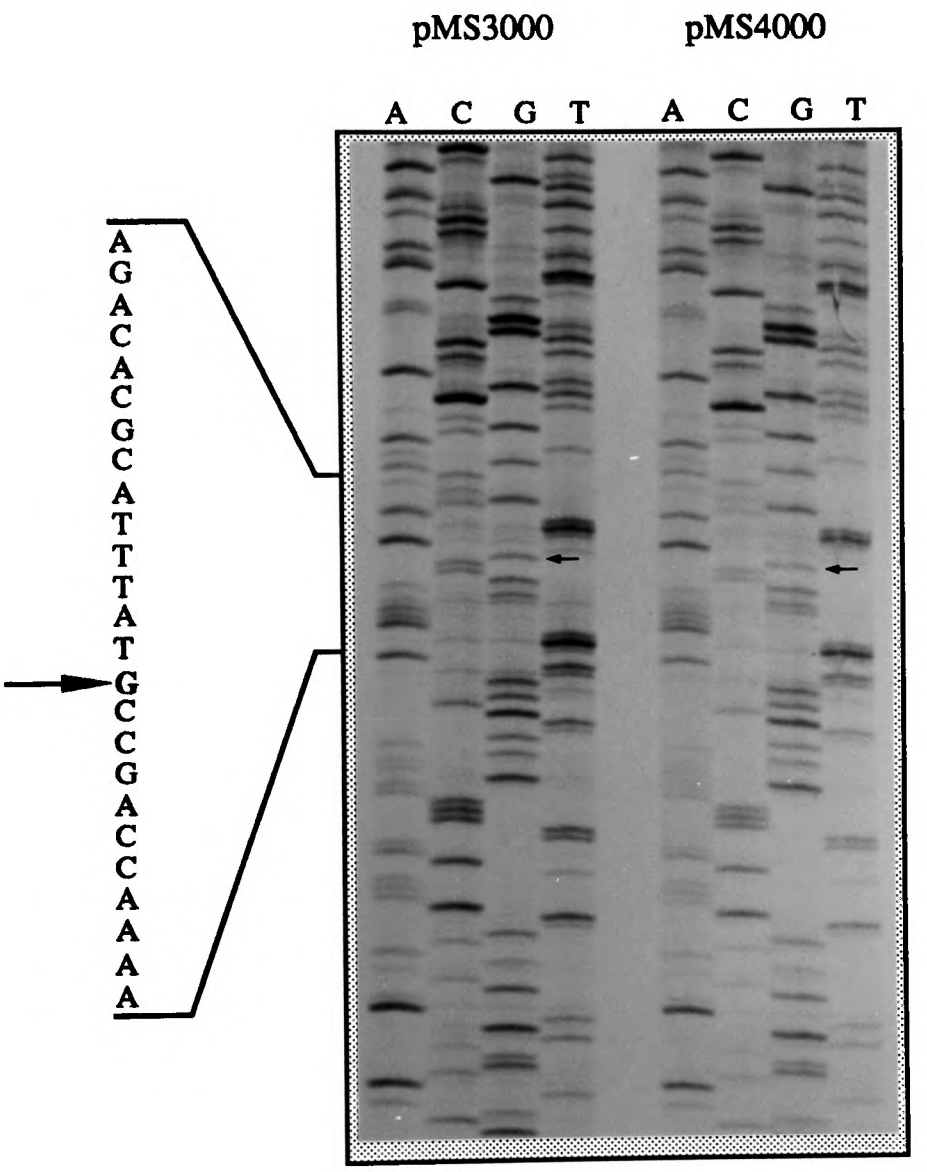


Fig.6.6 Autoradiograph of the sequencing gels of the minus strands of pMS3000 and pMS4000 are compared. The 'G' residue on the minus strand, that is complementry to the 'C' residue at position 592 on the plus strand, is marked with an arrow. Some of the nucleotide sequence of this region is also given adjacent to the gels.



the Type I plasmid pOX6, in a 2.1kb *Bgl*II - *Hin*dIII fragment of DNA from pI9789 which confers autonomous replication in *S. aureus* [see Walters (1992) for a comparison of the putative MSO of pOX4000 with other Type I MSOs]. He suggested that the absence of the 'C' residue at position 592 of MSO of pS1 causes the replication to be temperature-sensitive. The plasmid pTST is a revertant derived from the plasmid pS1 and the location of the mutation reverting the plasmid to temperature-stability was thus sought. Neither the 2.1kb *Bgl*II - *Hin*dIII fragment derived from pTST nor that derived from pS1, when ligated to pWE180 plasmid, were able to result in transformation of *S. aureus* RN4220 to Em^r. This result for pS1 differs from that found by Walters (1992) and so despite the inability to repeat the experiment, it was decided to check the sequence of the MSO region of both pS1 and pTST. The sequences were identical but differ from that reported for pS1 in that the reported 'C' deletion was not found. Further investigation is in progress to resolve this discrepancy. However, it should be noted that neither the plasmid pWE180 nor the 2.1kb *Bgl*II - *Hin*dIII insert carry a sequence that shows homology to one capable of specifying a replication protein for *S. aureus*. All staphylococcal plasmids that have been investigated for replication contain at least one open reading frame that specifies a rep-type protein (Novick, 1989). Thus there is some doubt about the results obtained by Walters (1992) and investigation of this in this thesis was not continued.

Chapter Seven:

Investigation of the Co-integration Between the pI9789-derivatives and pOX1054

Chapter Seven: Investigation of the Co-integration Between the pI9789-derivatives and pOX1054

7.1 Introduction

The temperature-sensitive derivatives of the plasmid pI9789 (pS1 and pΔD) can form co-integrates with the plasmid pOX1054, as reported in Chapter 3. The co-integrate formation was observed in the 1054N strain of *S. aureus* and involves the large 10.2kb *Eco*RI fragment of pS1 (6.4kb EcoC in the case of pΔD) and the 9.5kb *Eco*RI fragment of pOX1054. The mechanisms of formation of this co-integrate will be investigated in this Chapter and the following questions will be asked.

1- Where on the plasmid pI9789 does the recombination occur?

2- Where on pOX1054 does co-integration occur?

3- Is a site-specific or a homology-dependent recombination involved?

4- If site-specific, does the formation of co-integrate involve *res* sites?

5- If site-specific, is the site-specific recombinase catalysing this recombination on the plasmid pI9789 or pOX1054 or the 1054 chromosome?

7.2 Characterization of the strain 1054 and the plasmid pOX1054

1054 is a β-lactamase producing strain of *S. aureus*, isolated from the hospital environment and the gene for β-lactamase activity is known to be plasmid-borne. A β-lactamase-negative variant was obtained, named 1054N and since it had also lost resistance to cadmium acetate, was presumed to be plasmid-negative (Dyke *et al.*, 1970; Dyke and Richmond, 1967). To check for the absence of plasmid in 1054N, DNA was prepared from 1054 and 1054N and analysed uncut and also after digestion with *Eco*RI and *Bam*HI. The results (Fig.7.1) show the presence of two plasmids in 1054 but only one in 1054N.

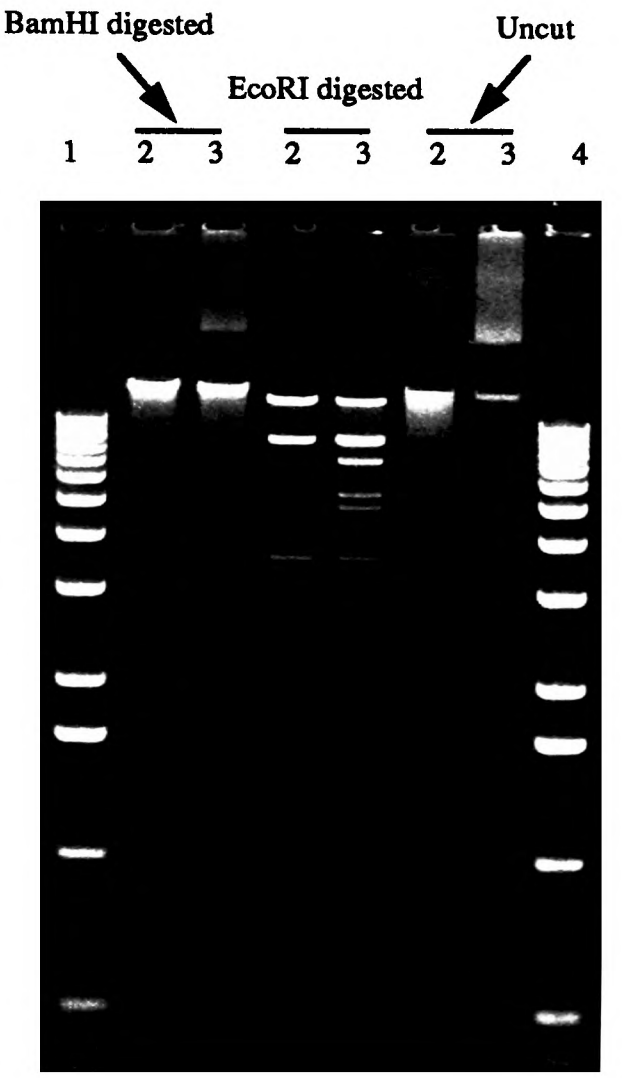
7.2.1 Resistance to metal ions

The strains 1054 and 1054N were tested for resistance to various metal ions by the standard disc method. The results (Fig.7.2) show that both cultures are equally sensitive to phenylmercuric acetate and sodium arsenate but that 1054N is more sensitive than 1054 to cadmium acetate. It is tentatively concluded that 1054N has lost a plasmid that carries the determinants for β-lactamase production and resistance to cadmium acetate and that the second plasmid (named pOX1054) does not carry genes for resistance to any of the three metal ions tested.

7.2.2 Resistance to antimicrobial agents and other compounds

The strain 1054N is sensitive to erythromycin (Chapter 3; Section 3.3). 1054N was further tested for resistance to other antimicrobial drugs by the disc method. The zones of inhibition for each drug were measured and are given in Table 7.1. The results show that

Fig.7.1 An agarose gel showing the plasmid DNA of the *S. aureus* strains 1054N and the 1054.



KEY:

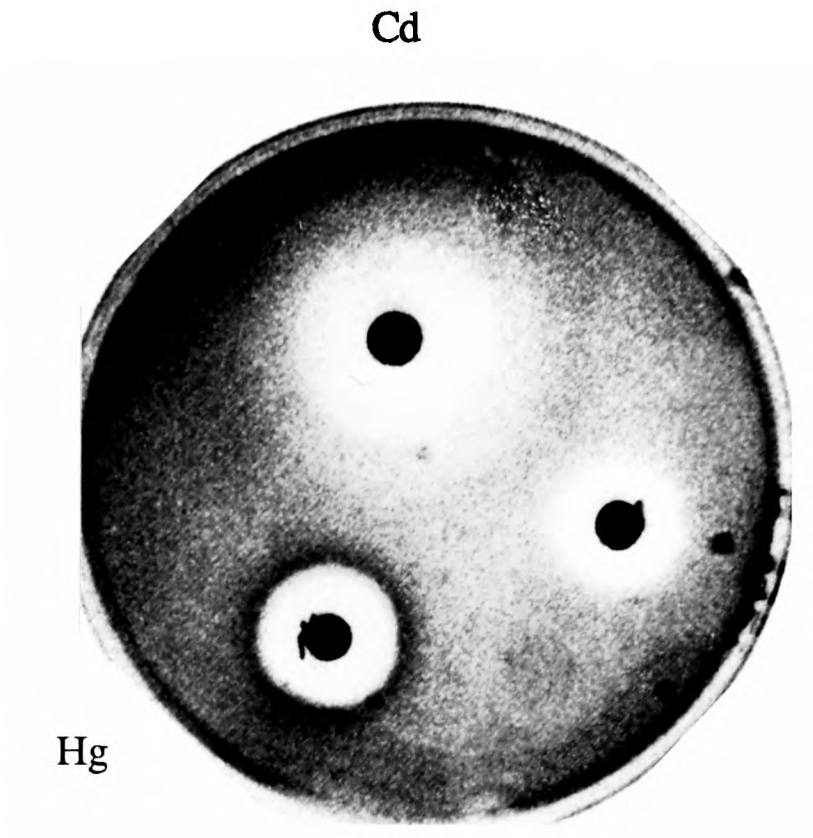
1&4. 1kb ladder

2. 1054N

3. 1054

Fig.7.2 Zones of inhibition for 1054 and 1054N {Cd: Cadmium acetate; Asa: Sodium arsenate; Hg: Phenylmercuric acetate}.

1054N:



1054:

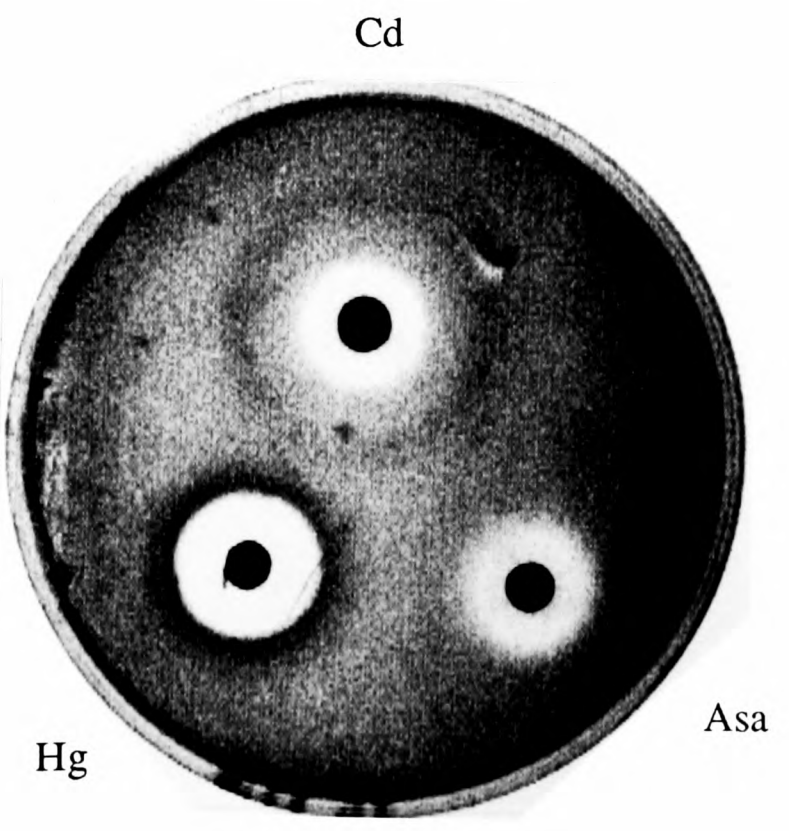


Table 7.1 The zones of inhibition produced by various *Staphylococci* around antibiotic discs (s = sensitive; r = resistant)

Antibiotic / Strains	Zones of inhibition (mm) 20µg/disc	40µg/disc	80µg/disc	Phenotype
Chloramphenicol (Cm)				
1054N	2.9	3.1	3.6	s
1054	2.7	3.0	3.5	s
RN451(pC194)	1.0	1.1	1.2	r
RN451	2.7	3.2	3.6	s
Gentamicin sulfate (Gm)				
1054N	2.4	2.6	2.9	s
1054	2.4	2.6	3.0	s
WN8325-5Gm^r	0	0	0	r
8325	2.5	2.6	3.0	s
Kanamycin monosulfate (Km)				
1054N	1.8	2.2	2.7	s
1054	1.9	2.1	2.9	s
HM3	0	0	0	r
RN4220	1.6	1.7	2.6	s
Novobiocin: sodium salt (Nv)				
1054N	3.6	3.8	4.1	s
1054	3.6	3.8	4.1	s
8325Nv^{r}N	2.0	2.2	2.3	r
8325	3.6	3.8	4.1	s
Tetracycline hydrochloride (Tc)				
1054N	2.9	3.5	3.6	s
1054	3.0	3.3	3.5	s
RN2424(pT181)	1.0	1.1	1.2	r
RN2424	3.0	3.4	3.7	s
Straeptomycin sulfate (St)				
1054N	2.4	2.6	2.9	s
1054	2.4	2.6	2.9	s
RN4220	2.6	3.0	3.2	s
Trimethoprim (Tm)				
1054N	3.0	3.2	3.5	s
1054	3.5	4.0	4.5	s
RN4220†	3.5	4.0	4.5	s

the strain 1054N is sensitive to chloramphenicol, gentamicin, kanamycin, novobiocin, tetracycline, streptomycin and trimethoprim. The plasmid pOX1054, thus, does not carry genes for resistance to these antimicrobial drugs.

1054N was also tested for resistance to ethidium bromide [0.04μg/disc; 2.0cm zone of inhibition: no zone of inhibition for Eb^r 8325(pJE1); Evans and Dyke, 1988)] and quaternary ammonium [cetyltrimethylammonium bromide (CTAB) 15μg/disc; 2.6cm zone of inhibition: 2.5cm zone of inhibition for RN4220] and was found sensitive.

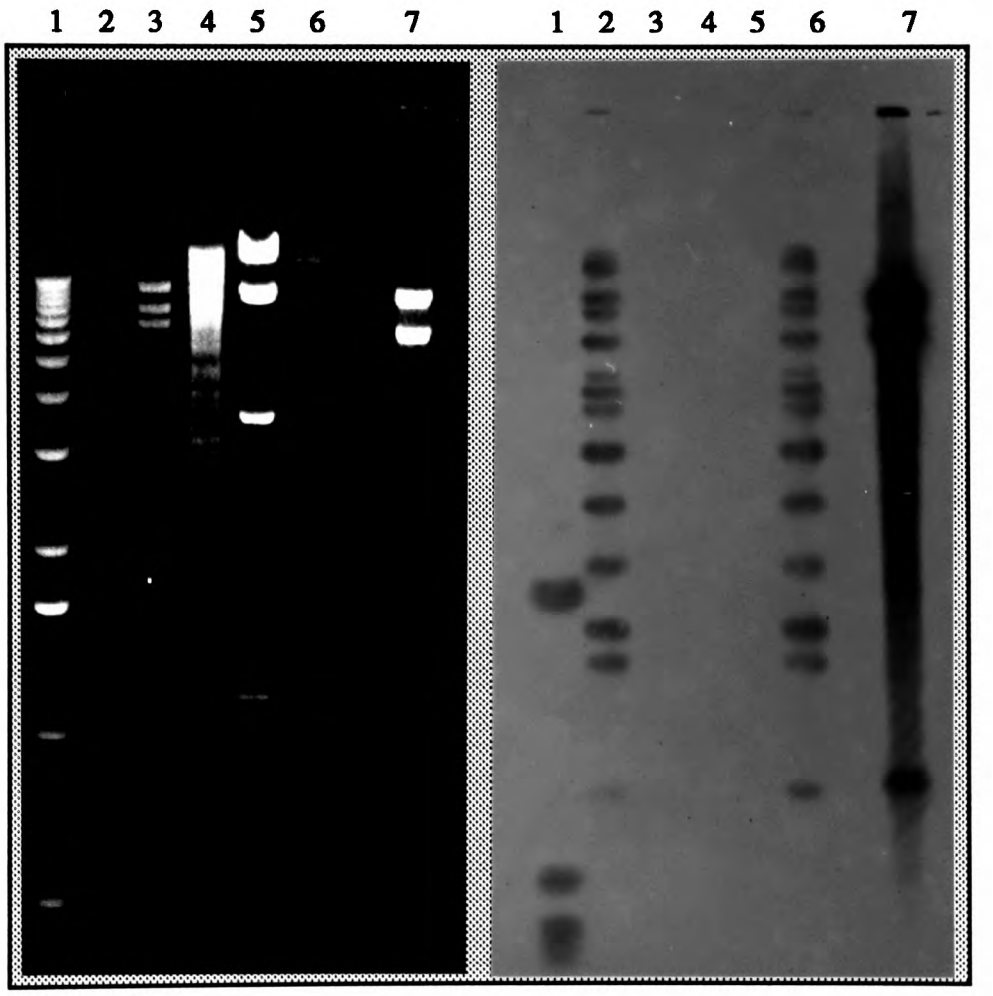
7.2.3 Hybridization analysis of pOX1054 to a conjugation-specific probe

Conjugative plasmids have been identified in the natural populations of different staphylococcal species from the nosocomial environment and usually they also carry genes for resistance to antibiotics. The set of genes involved in conjugation (the transfer genes or *tra*) spans approximately 15kb (Firth *et al*, 1993; Archer and Thomas, 1989; Evans and Dyke, 1988). The plasmid pOX1054 was, thus, investigated for the presence of transfer genes by hybridization to a conjugation-specific DNA probe. A ~14kb *Bam*HI - *Xba*I fragment containing conjugative transfer genes from a conjugative plasmid pSK397 has been cloned into pUC18 (named pSK397*Xba*Δ) and was a gift from Dr. R. Skurray. The plasmid DNA of pOX1054 was digested with *Eco*RI and electrophoresed on a 0.7% (w/v) agarose gel. Plasmids pS1 and pSK397*Xba*Δ and the chromosomal DNA of RN4220 were used as controls. The DNA was transferred to a nylon membrane and the hybridization to the plasmid pSK397*Xba*Δ was detected using the ECL System. The result (Fig.7.3) suggests that the plasmid pOX1054 does not show detectable homology to pSK397*Xba*Δ and so it is concluded that it does not have the genes for conjugal transfer found in some other staphylococci.

Fig.7.3 (A). Photograph of the agarose gel stained with ethidium bromide. All DNA samples were digested with *Eco*RI and electrophoresed on 0.7% (w/v) agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pSK397*Xba*Δ probe: (B). An autoluminograph of the membrane showing the results of the hybridization of the plasmid pSK397*Xba*Δ to pOX1054.

KEY:

1. 1kb ladder
2. Raoul ladder
3. pS1
4. RN4220
5. pOX1054
6. Raoul ladder
7. pSK397XbaΔ



7.2.4 Hybridization analysis of pOX1054 to pS1

The plasmid pOX1054 was investigated for homology to the plasmid pS1. The plasmid DNA of pOX1054 was digested with *Eco*R1 and electrophoresed on a 0.7% (w/v) agarose gel. Total DNAs of 1054N and RN4220 and the plasmid pS1 were used as controls. The DNA was transferred to a nylon membrane and the hybridization to the plasmid pS1 was detected using the ECL System. The results (Fig.7.4) show that at a high stringency, there is no detectable homology between the plasmids pOX1054 and pS1. Since, the plasmid pS1 is known to carry the insertion element IS*257* (C. Needham, personal communication), this result also suggest that pOX1054 does not carry IS*257*. The result also shows that the replication systems of the two plasmids are not very similar.

7.3 Stability of the co-integrates

To establish whether or not the formation of the co-integrate between pOX1054 and pS1 or pΔD is reversible, the ability of co-integrates to resolve was studied.

The *S. aureus* strains, 1054(pOX1054::pS1) and 1054(pOX1054::pΔD) were inoculated into 10ml of the medium and then incubated for 18hrs as follows:-

Culture-i: In CY-broth; at 42°C

Culture-ii: In CY-broth; at 30°C

Culture-iii: In CY-Cd (26.6μg/ml)-broth; at 42°C

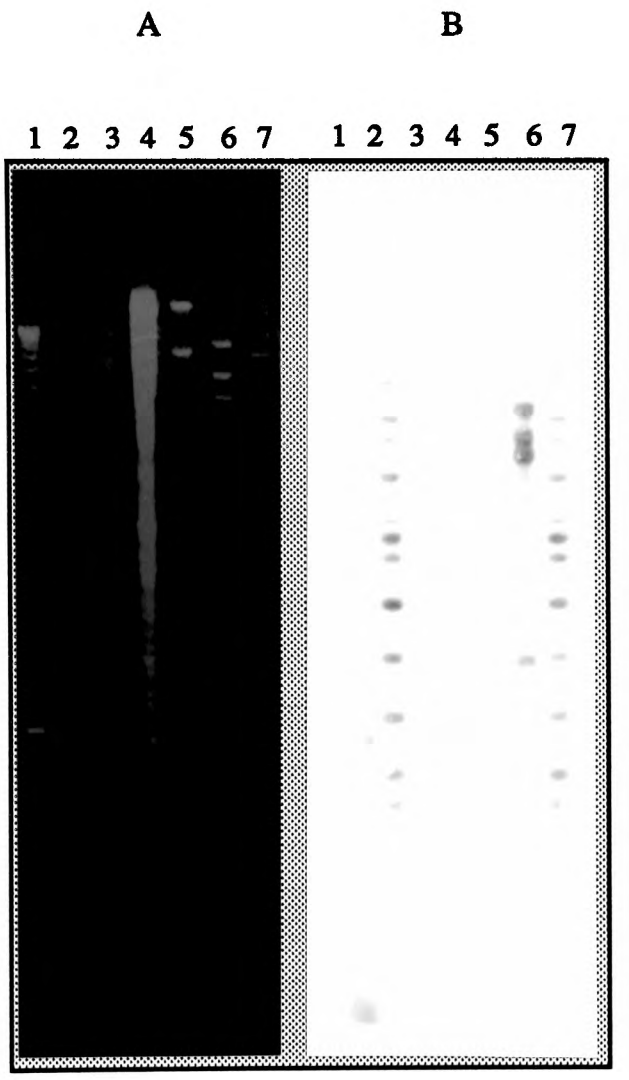
Culture-iv: In CY-Cd (26.6μg/ml)-broth; at 30°C

Then 5μl of each culture was re-inoculated into fresh medium, as above. This step was repeated 10 times (*c.* 75 generations). Plasmid DNA was prepared at the end of each

Fig.7.4 (A). Photograph of the agarose gel stained with ethidium bromide. All DNA samples were digested with *Eco*RI and electrophoresed on 0.7% (w/v) agarose gel. The DNA was transferred to a nylon membrane and hybridized to the pS1 probe (plus pUC18 to illuminate the Raoul ladder): (B). An autoluminograph of the membrane showing lack of hybridization of the plasmid pS1 to pOX1054.

KEY:

1. 1kb ladder
2. Raoul ladder
3. RN4220 chromosome
4. 1054N total DNA
5. pOX1054
6. pS1
7. Raoul ladder



step from 1.5ml of the culture by the mini-prep method and analysed after digestion with *Eco*RI. However, no resolution products could be detected in any of the cultures (at 30°C or 42°C) and only the co-integrates could be isolated, suggesting that the co-integrates are stable under these conditions.

7.4 Cloning and restriction endonuclease analysis of the junctions between pOX1054 and pS1, and pOX1054 and pΔD in the co-integrates

The formation of co-integrates of pOX1054 with pS1 and pΔD was detected by the *Eco*RI-band-displacement (Chapter 3). The 9.5kb *Eco*RI fragment of pOX1054 and the 10.2kb *Eco*RI fragment of pS1 (6.4kb *Eco*RI fragment for pΔD) were replaced by a 3.9kb and a 15.8kb (12kb for pΔD) *Eco*RI fragment in the co-integrates. The junction between the two plasmids which is contained in the 3.9kb *Eco*RI fragment for both pOX1054::pS1 and pOX1054::pΔD was labelled 'J1' and the junction contained in the 16kb *Eco*RI fragment of pOX1054::pS1 and in the 12.1kb *Eco*RI fragment of pOX1054::pΔD was named 'J2'.

7.4.1 Cloning of J1

The junction J1 was cloned from two independent examples each of pOX1054::pS1 and pOX1054::pΔD. The plasmid DNA was digested with *Eco*RI, ligated to *Eco*RI digested pUC18 and used to transform JM107. The predicted (3.9kb) inserts were cloned for both examples of pOX1054::pS1 but not from pOX1054::pΔD. Two clones were

selected from each cloning and the orientation of the insert was found to be the same for all four. One example of each type was kept for further analysis and were labelled pMS101 and pMS102, respectively.

Two samples of *Eco*RI-cut pOX1054::pΔD DNA were used to clone into the *Eco*RI site of pTZ19R in place of pUC18. On analysis by *Eco*RI digestion, one transformant containing pTZ19R with a 3.9kb *Eco*RI insert was obtained for each of the two examples. The orientation of the insert was determined and was found to be the same for both cases. The resultant chimeric plasmids were named pMD101 and pMD102.

7.4.2 Restriction enzyme mapping of the insert in the plasmids, pMS101, pMS102, pMD101 and pMD102

Restriction enzymes were chosen from the the known restriction enzyme map of the 10.2kb *Eco*RI fragment of the plasmid pS1. On restriction enzyme analysis, the 3.9kb fragments in all four plasmids (pMS101, pMS102, pMD101 and pMD102) were found to be indistinguishable which suggests not only that the co-integrate formation might have occurred at the same site on both pS1 and pΔD but also at a single site on pOX1054. A comparison of the restriction map of the 3.9kb fragment with that of the corresponding region of the plasmid pS1 (or pΔD; shown later in Fig.7.6) indicates that the recombination might have involved the DNA within or near the invertible region, *inv*. It follows that the restriction enzyme map of the 3.9kb fragment is indistinguishable from that of pS1 between the *Eco*RI site at co-ordinate 1 to the *Sna*BI site at co-ordinate 1.6, just outside of the *inv*, on the map and is different beyond this. No enzyme sites were found for *Bam*HI, *Pst*I, *Sph*I, *Xba*I, *Cla*I, *Sal*I and *Nde*I on the 3.9kb insert.

7.4.3 Cloning and restriction enzyme mapping of J2

The junction, J2, is present in the 15.8kb (or 12kb) *Eco*RI fragment of the co-integrates, which is difficult to clone into pUC or pTZ vectors. It was thus decided to use an enzyme that would generate smaller and more easily clonable fragments containing the junction J2. The plasmids pOX1054::pS1 and pOX1054::pΔD were digested with a range of restriction enzymes to discover a suitable enzyme for this purpose. Digestion with *Bgl*II gave two new fragments (1.75kb and 3.2kb), for both pOX1054::pS1 and pOX1054::pΔD, that are not seen in digests of pS1, pΔD or pOX1054 (Fig.7.5). These two fragments are present in about half-molar concentration, which suggests the presence of an alternative *Bgl*II site due to the inversion of the DNA. This inversion was tentatively interpreted as due to the involvement of the invertible region, *inv*, of pS1 (or pΔD), having an asymmetrically located internal *Bgl*II site. The 1.75kb *Bgl*II fragment is generated when the *inv* is in negative orientation and the 3.2kb *Bgl*II fragment is generated when the *inv* is in positive orientation (Fig.7.6).

The junction J2 was, thus, cloned as 3.2kb and 1.75kb *Bgl*II fragments into the compatible *Bam*HI site of pUC18 from two independent examples each of pOX1054::pS1 and pOX1054::pΔD. The correct recombinant plasmids were identified by digestion with *Eco*RI + *Hind*III on a 0.7% (w/v) agarose gel. The resultant chimeric plasmids containing the 1.75kb *Bgl*II fragment from the two pOX1054::pS1 plasmids were named pBSs201 and pBSs202 and those from pOX1054::pΔD as pBDs201 and pBDs202. The recombinants containing the 3.2kb *Bgl*II fragment from the two pOX1054::pS1 plasmids were labelled pBSl201 and pBSl202 and those from pOX1054::pΔD as pBDl201 and pBDl202.

Fig.7.5 *Bgl*II digests of the co-integrates pOX1054::pS1 and pOX1054::pΔD to show the 3.2kb and the 1.75kb *Bgl*II fragments. See text for details.

KEY:

1 & 9. 1kb ladder

2. pOX1054::pS1 #1

3. pOX1054::pS1 #2

4. pS1

5. pOX1054

6. pΔD

7. pOX1054::pΔD #1

8. pOX1054::pΔD #2

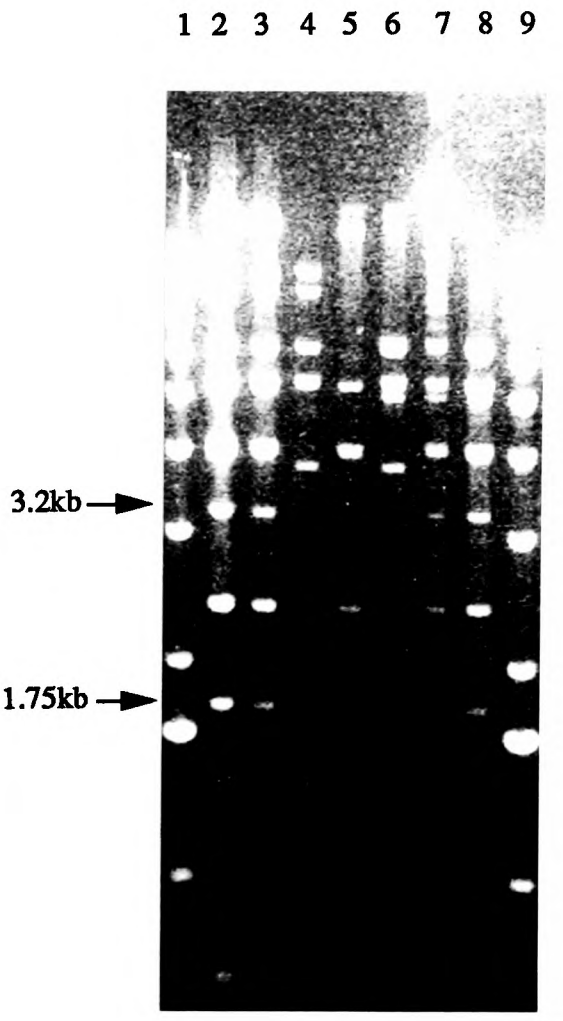
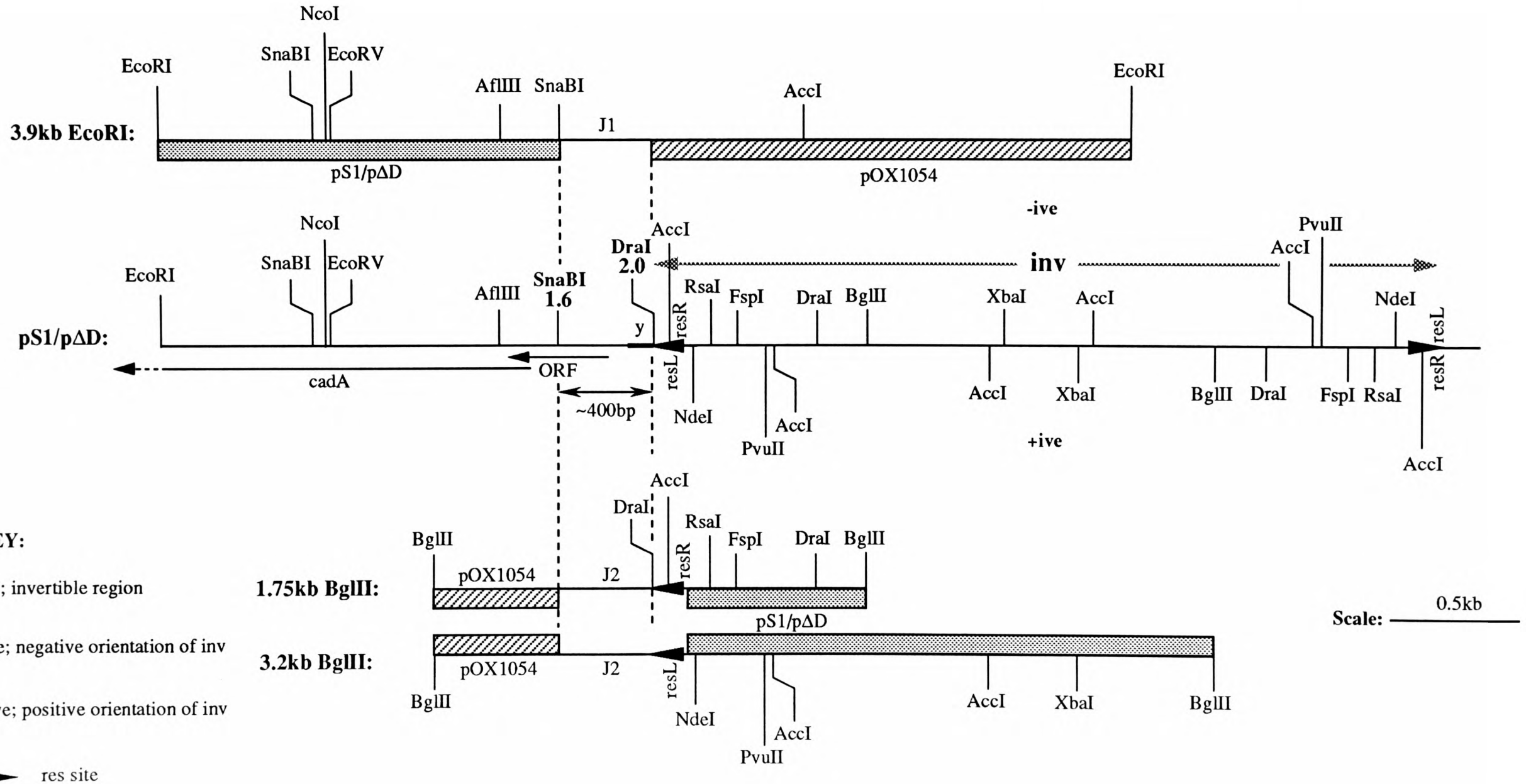


Fig.7.6 An alignment of the junction clones, containing the junctions J1 and J2, with the corresponding region on the plasmid pS1/pΔD. The alignment was made in order to identify the region on the pS1 and pΔD involved in recombination to form co-integrate with pOX1054. Various restriction enzyme sites are shown. For the invertible region, the restriction enzyme sites in both orientations are shown. The potential 400bp DNA between the *Sna*BI (1.6) and the *Dra*I (2.0), involved in the recombination, is labelled ['y': the region on pS1 labelled as 'y' by Rowland (1989); ORF and *cadA*: cadmium resistance; J1 and J2: junctions between pS1/pΔD and pOX1054].



On restriction enzyme analysis, the 1.75kb *Bgl*II fragments of all four plasmids were found to be indistinguishable as were the 3.2kb *Bgl*II fragments. Restriction enzyme maps of both the 1.75kb and 3.2kb *Bgl*II fragments are given in Fig.7.6.

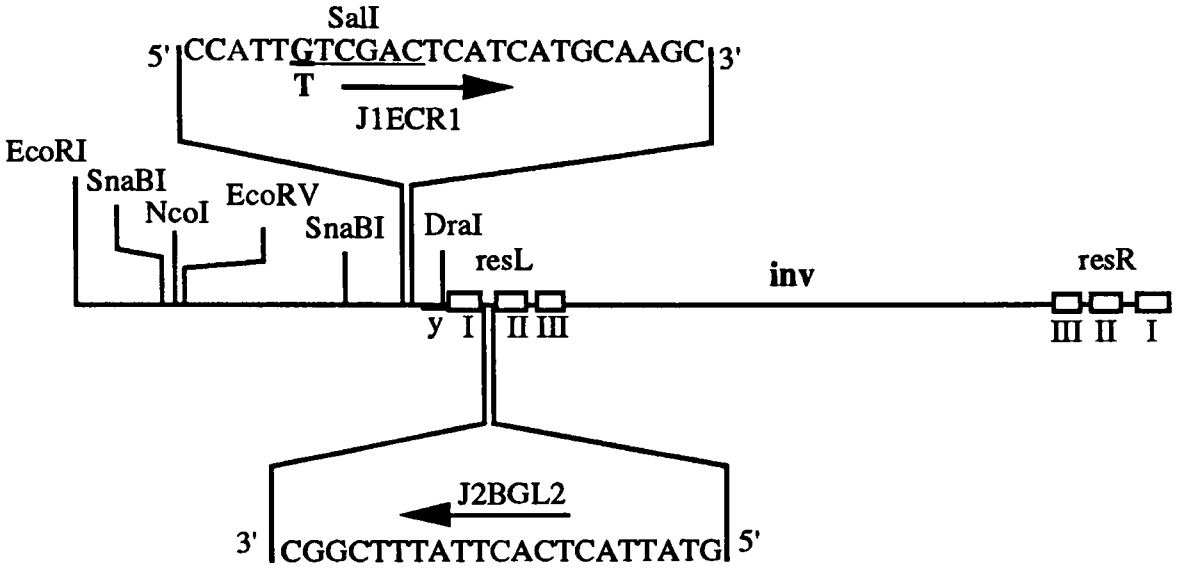
7.4.4 Putative site of recombination on the plasmids pS1 and pΔD

The potential region on the plasmids pS1 and pΔD, involved in recombination to form a co-integrate with the plasmid pOX1054, was identified by using the restriction endonuclease data obtained in the previous sections. The restriction endonuclease maps of the clones containing J1 and J2 were aligned with the known restriction enzyme map of the 10.2kb fragment of the plasmid pS1 in order to achieve this (Fig.7.6). The alignment demonstrates that the recombination has occurred close to the *res* site on both of the plasmids pS1 and pΔD. However, the inversion activity of the *inv* region is retained suggesting that the *res* site remains functionally intact. The potential recombinogenic region on the plasmids pS1 and pΔD involved in the formation of co-integrate with pOX1054 was thus identified as within the 400bp between the *Sna*BI site at co-ordinate 1.6 and the *Dra*I site at co-ordinate 2.0 on the pS1/pΔD map.

7.5 Nucleotide sequence analysis of the junctions J1 and J2

In order to find out precisely, the sites of recombination on the plasmids pS1 (and pΔD) and pOX1054, DNA sequencing was carried out across the two junctions, J1 and J2. The sequencing strategy for both junctions is shown in Fig.7.7.

Fig.7.7 Binding sites of the oligonucleotide primers J1ECR1 and J2BGL2 on the plasmid pS1/pΔD are shown. A 'T' nucleotide at position 6 from the 5' end of the oligonucleotide, J1ECR1, was replaced with a 'G' in order to introduce a *SalI* site. This site was later used to digest and clone a PCR product of this region. The restriction enzyme site on the left-hand side of *resL* of the invertible region, *inv*, are shown for reference. The drawings are not to scale.



7.5.1 Sequence analysis of J2

An oligonucleotide (J2BGL2) was designed, to act as a sequencing primer, within the *resL* site of the *inv* region to sequence the junction J2 on the cloned 3.2kb *Bgl*II fragment (a suitable sequence on the pS1/pΔD DNA in the 1.75kb fragment, for designing an oligonucleotide, was not available and so the 1.75 fragment was not used to sequence J2). The plasmids pBS1201 and pBS1202 (for the junction J2 between the plasmids pS1 and pOX1054 in pOX1054::pS1), and pBD1201 and pBD1202 (for J2 between plasmids pΔD and pOX1054 in pOX1054::pΔD) were used in the sequence analysis. The sequence obtained using the oligonucleotide, J2BGL2, on all four plasmids was identical and is given and compared with the sequence in the corresponding region of pS1/pΔD (Rowland, 1989) in Fig.7.8. The results suggest that the recombination with the plasmid pOX1054 has occurred at the same site on both the plasmids pS1 and pΔD. This site is situated outside the *resR* and next to the region labelled 'y' (Fig.7.6) on the plasmid pI9789 (Rowland, 1989).

7.5.2 Sequence analysis of J1

An oligonucleotide (J1ECR1) was designed in order to sequence the junction J1, which binds in the region just outside the region 'y' (Fig.7.6) on pS1/pΔD directed towards the region 'y'. The plasmids, pMS101 (for J1 in pOX1054::pS1) and pMD101 (for J1 in pOX1054::pΔD), were chosen for the sequence analysis. The sequence data obtained and an alignment of this sequence with the corresponding region of the plasmid pS1/pΔD is given in Fig.7.9.

The sequences thus obtained at the two junctions, J1 and J2, were aligned with each other and with the known sequence on pS1/pΔD in order to find out the sequence at

Fig.7.8 The nucleotide sequence, obtained using the oligonucleotide J2BGL2, on the 3.2kb *Bgl*II fragments of the plasmids pBSl201, pBSl202, pBDl201 and pBDl202 is compared with the corresponding region on the plasmid pI9789 (Rowland, 1989). The pOX1054 sequence is underlined. Restriction sites for the enzymes *Dra*I and *Ssp*I on the pI9789 sequence are shown in **bold** text. Colons (:) represent the sequence of pI9789 identical to that of the 3.2kb *Bgl*II fragments.

DraI

i. 3.2kb *Bgl*II: AGT**TTTAAA**TATCAAAAACATTATTTACATTTTACGTTTAAGAACTAATGGTTAAAAATTTCATTTTGTCAAAAACGTT

ii. pI9789: ::

i. CGTTTTTGA<u>AATGAATATAAATAACTCTAAGGCTAATATAAAAGTTAAAAGACTATATACCATGATATCAATTACTAAAGGTCTAAGAAATTTT</u>

SspI

ii.:::::::::CTAGAATTTTATGCCCTACTTACTTCTTTTATTTTCATTCA**AATATT**TGCTTGCATGATGAGTCGAAAATGGTTATAATAC

i. <u>TAAATATAAATACTAACTAGTCCCAACATTGATATTAATAACAATAAT</u>

ii.ACTCAAATAAATATTTGAATGAAGATGGGATGATAATATGAAAAAGAA

Fig.7.9 The nucleotide sequence obtained using the oligonucleotide, J1ECR1, on the 3.9kb *Eco*RI fragment of the plasmids pMS101 and pMD101 is compared with the corresponding region on the plasmid pI9789 (Rowland, 1989). The putative pOX1054 sequence is underlined. Restriction site for the enzyme *Dra*I on the pI9789 sequence is shown in **bold** text. Colons (:) represent the sequence of pI9789 identical to that of the 3.9kb *Eco*RI fragments. The nucleotide sequence of pI9789 continues into the site I of *resL* or the *resR* of the invertible region, *inv*, depending on the orientation of the *inv*.

i. 3.9kb *Eco*RI: AGTAAGTAGGGCATAAAATTCTAGTCTCAAAAACGAACGTTTTTGACAAAATCCCCTAATGATAAATTAAGAAGATAGA

ii. pI9789: ::GAAATTTTTAACCATTAGTTCTTAAAC

i. TTAAGAAATTTATCCTTGAATTTAAAGGGGATTTTATTTTGGCCATAATTCCTTTAACTTGTGTTATAATCTATGTATCATAAAATTCAAAA

DraI

ii.GTAAAATGTAAATAATGTTTTTGATA**TTTAAA**ACTG (*resL/R* site I)

i. TATACGAGTTTTTAGACGATGAATGGACTTGTTTATTTGCATCGAGAGTTAACCTTAGATG

the site of recombination on these plasmids. The alignment revealed the nucleotide sequence at the junction for both plasmids pS1 and pΔD in the co-integrates to be indistinguishable and the presence of a sequence of 26bp, at both of the junctions, in direct-repeat in the co-integrates (Fig.7.10). This 26bp sequence is apparently derived from the nucleotide sequence of the plasmid pI9789. However, the possibility of the 26bp sequence being derived from the plasmid pOX1054 cannot be ruled out. The nucleotide sequence at the site of recombination on the plasmid pOX1054 was, therefore, investigated.

7.6 Nucleotide sequence analysis of the site of recombination on pOX1054

In order to obtain the nucleotide sequence of the site of recombination on the plasmid pOX1054, two oligonucleotides, J1OX54 and J2OX54 were designed to act as sequencing primers against the sequence of pOX1054 obtained in the Section 7.5. The two oligonucleotides were directed so as to sequence into the recombination site from both sides.

The base number "12" from the 5' end of the oligonucleotide J1OX54 was changed from a 'T' to a 'G' in order to introduce a *Hae*II site which could later be used to digest and clone a PCR product of this region if required. The sequence of J1OX54 is 5'AAGGTTAACTCGCGATGC and that of J2OX54 is 5'CAATGTTGGGACTAGTTAG. Overlapping nucleotide sequence was obtained at the site of recombination using these two oligonucleotides. The sequence of 340 base-pairs thus obtained is given in Fig.7.11 and reveals that a 27bp sequence of pOX1054 (boxed in Fig.7.11), which has sequence

Fig.7.10 (A). Relevant regions of the nucleotide sequences obtained using the oligonucleotides J1ECR1 and J2BGL2 on the 3.9kb *Eco*RI and 3.2kb *Bgl*II fragments containing junctions J1 and J2, respectively, are compared with the corresponding region on pS1/pΔD (Rowland, 1989). The nucleotide of both strands of pS1/pΔD is presented. The putative pOX1054 sequences have been underlined. The 26 base-pair direct repeat at the junctions between pS1/pΔD and pOX1054 is in **bold** text: (B). The nucleotide sequence at the two junctions, J1 and J2, in the co-integrates (pOX1054::pS1 and pOX1054::pΔD). The 26 base-pair direct repeat (DR) at the junctions is labelled and is in **bold** text. The sequnces derived from pS1/pΔD and pOX1054 are labelled.

(A):

1. Junction 1: 5'AGTAAGTAGGGCATAAAATTCTAGTC**TCAAAAACGAACGTTTTTGACAAAAT**CCCCTAATGATAAATTAAGAAGATAGA 3'

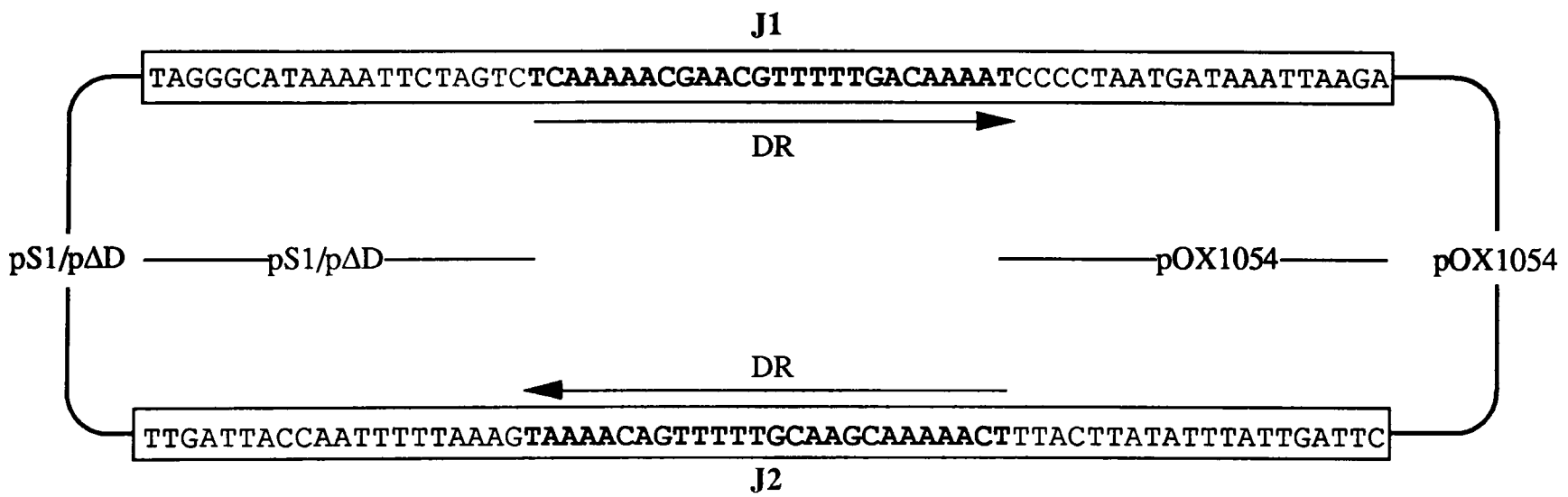
2. pS1/pΔD: 5'AGTAAGTAGGGCATAAAATTCTAGTC**TCAAAAACGAACGTTTTTGACAAAAT**GAAATTTTTAACCATTAGTTCTTAAAC 3'
(Top strand)

(Bottom strand)

2. pS1/pΔD: 3'TCATTCATCCCGTATTTTAAGATCAG**AGTTTTTGCTTGCAAAAACTGTTTTA**CTTTAAAAATTGGTAATCAAGAATTTG 5'

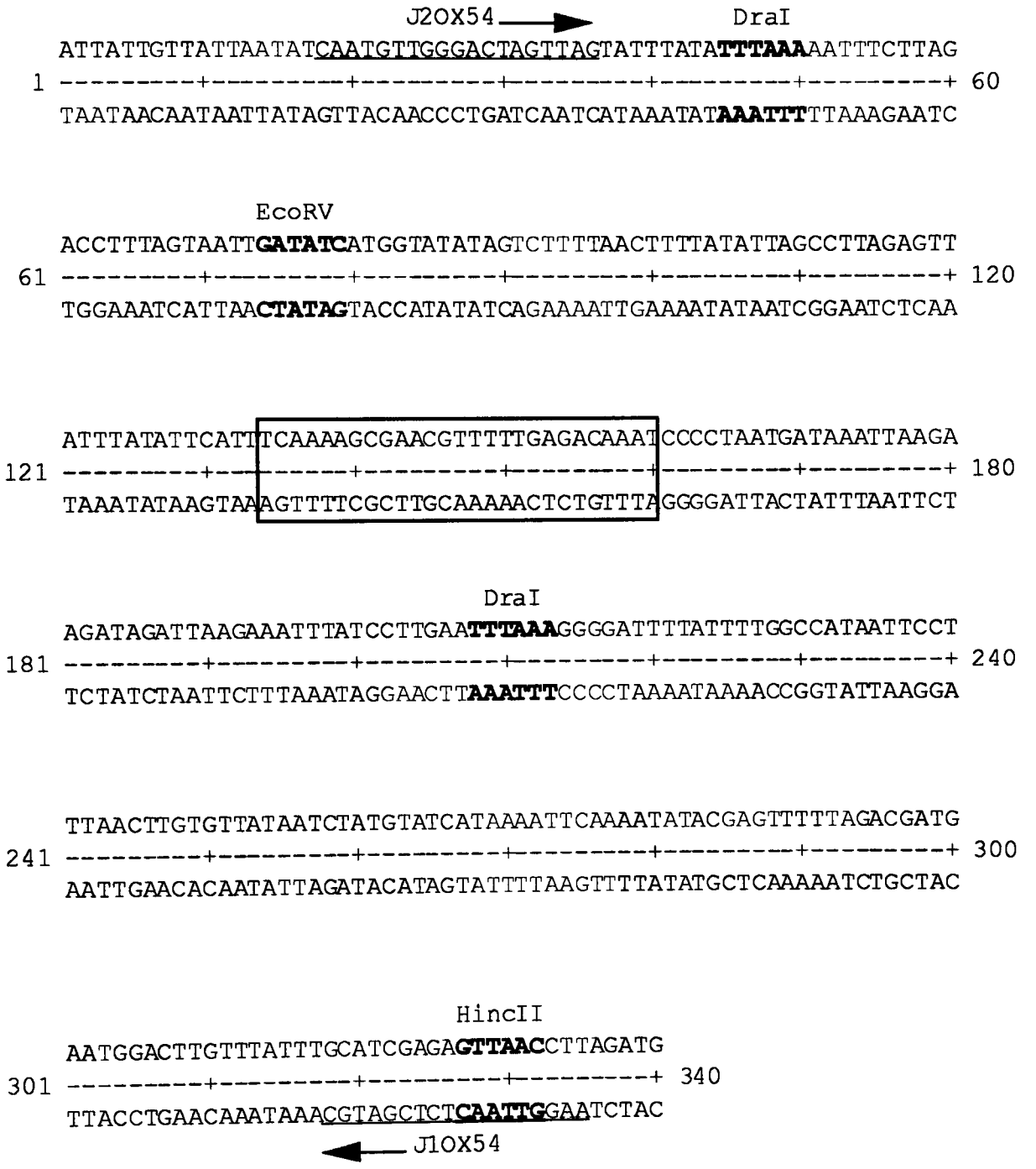
3. Junction 2: 3'TAATCGGAATCAATAAATATAAGTAA**AGTTTTTGCTTGCAAAAACTGTTTTA**CTTTAAAAATTGGTAATCAAGAATTTG 5'

(B):



pOX1054::pS1/pOX1054::pΔD

Fig.7.11 Nucleotide sequence of both strands of pOX1054, obtained using the oligonucleotides J1OX54 and J2OX54 to prime pOX1054 together with the sequence obtained on the co-integrates using oligonucleotides J1ECR1 and J2BGL2. Restriction sites for the enzymes *Dra*I, *Eco*RV and *Hinc*II are shown. The oligonucleotides J1OX54 and J2OX54 are labelled. The 27bp sequence of pOX1054 which shows similarity to the 26bp direct repeat present in the co-integrates is boxed.



similarity to the 26bp direct repeat present in the co-integrates, appears to have been deleted at least partially in the formation of the co-integrates.

Overall, the sequence analysis suggests that the formation of the co-integrates between pS1 or pΔD and pOX1054 is the result of a site- and orientation-specific recombination between the plasmids. The recombination involves a probable duplication of a nucleotide sequence (within the 26bp) derived from the plasmid pS1/pΔD accompanied by deletion of a nucleotide sequence (within the 27bp) from the plasmid pOX1054. The DNA duplicated in pS1/pΔD and the DNA deleted in pOX1054 presumably are recombination points between these plasmids. The 26bp sequence of the plasmid pS1 was named *rs9789* and the 27bp sequence of pOX1054 as *rs1054*. A comparison of *rs9789* and *rs1054* is given in Fig.7.12.

7.7 Computer analysis of the sequence at the recombination site on pOX1054

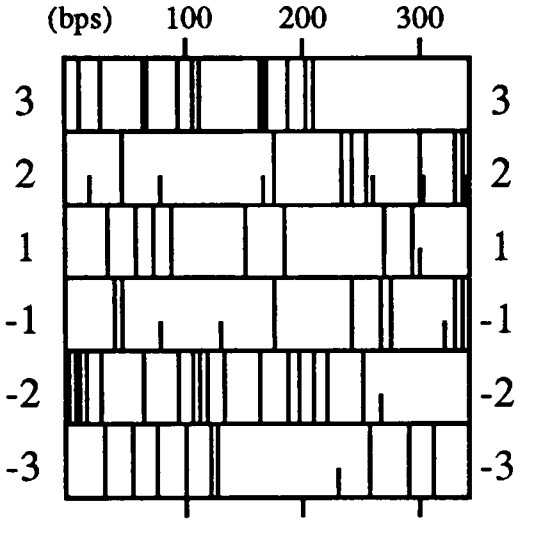
Analysis of the 340bp sequence of pOX1054 showed several short open reading frames (ORFs: Fig.713). Comparison of the DNA sequence and the predicted amino acid sequence of the ORFs with the GenEMBL databases revealed no obvious homologies. Specific search for comparison with the conserved domains at the N-terminus of integrase family recombinases (Sherratt, 1993) also revealed no homology. No good candidates for either promoter sequences or a ribosomal binding sites upstream of ORFs directed out of the sequence were found. It, thus, seems that there are probably no proteins specified by this region.

Fig.7.12 A comparison of *rs9789* to *rs1054*. The terminal base sequence which is identical in the recombination sites is given in **bold** text. The differences are shown in lower case.

rs1054: **TCAAAA**gCGAACGTTTTTGAgaC-**AAAT** (27bp)

rs9789: **TCAAAA**aCGAACGTTTTTGA--Ca**AAAT** (26bp)

Fig.7.13 ORF map of the 340bp sequence of the plasmid pOX1054. A complete vertical bar across any phase represents a stop codon, whereas a partial bar represents a '*met*' codon (ATG). The ORF map was constructed using the Macintosh compatible programme, DNA Strider (bps; base-pairs).



7.8 Cloning of *rs9789* and *rs1054*

The two recombination sites, *rs9789* and *rs1054*, were cloned in order to study their role in the formation of the co-integrates between pS1/pΔD and pOX1054. PCR was employed to amplify DNA segments of interest on the plasmids pS1 and pOX1054.

7.8.1 PCR amplification, cloning of the PCR product and sequence analysis of the clone containing *rs9789*

rs9789 and its flanking DNA was amplified using the polymerase chain reaction (PCR). The oligomers used previously to sequence the junctions between the plasmids pS1/pΔD and pOX1054, J1ECR1 (24-mer) and J2BGL2 (21-mer), were used here to allow amplification of a 222bp segment of pS1 containing *rs9789*. The regions on the plasmid pS1 to which these oligonucleotides bind have been previously shown in Fig.7.7.

The polymerase chain reaction was carried out using $Vent_R$™ DNA polymerase in a 100μl volume as described earlier. The expected single band of 222bp was obtained and purified.

The PCR product was then digested with *Sal*I and *Dra*I simultaneously to obtain a 150bp fragment and used to ligate to *Sal*I + *Sma*I digested pUC18 DNA. The ligation mix was used to transform *E. coli* JM107. Plasmid DNA was prepared from some of the transformants and analysed after digestion with *Eco*RI + *Hind*III. The plasmid DNA of a transformant containing the correct-sized insert into pUC18 was analysed by DNA sequencing. The M13 forward and reverse primers were used to sequence the whole of the insert. A single base pair change was found close to the putative site of recombination *rs9789* in this clone and it was thus decided to sequence the plasmid DNA of another clone

in order to discover a correct insert. pUC18 DNA of the second clone was found to contain the DNA fragment of correct sequence. A 150bp fragment from the plasmid pS1 containing the *rs9789* has been cloned. The plasmid was named pUCRS9789.

7.8.2 PCR amplification, cloning of the PCR product and sequence analysis of the clone containing *rs1054*

The site of recombination, *rs1054*, on the plasmid pOX1054 was also cloned. The oligonucleotides J1OX54 (18-mer) and J2OX54 (19-mer) were used to amplify a 318bp segment containing *rs1054*. The binding sites of the oligomers are shown in Fig.7.11.

PCR was performed in a 100µl volume using the Vent$_R$™ DNA polymerase. An expected single band of 311bp was obtained and purified.

The purified PCR product was digested with *Dra*I, which generated a 163bp fragment (see Fig.7.11) which was then used to ligate to *Sma*I digested pUC18 and the ligation product was transformed into competent JM107. Plasmid DNA was prepared from some of the transformants and analysed after digestion with *Eco*RI + *Hin*dIII. The M13 forward and reverse primers were used to sequence the whole of the insert in a clone of the predicted size and found to contain the correct DNA fragment. This plasmid was named pUCRS1054.

7.8.3 Cloning of the fragments containing *rs9789* and *rs1054* into a shuttle vector for *E. coli* - *S. aureus*

In order to carry out a functional analysis of the fragments containing *rs9789* and *rs1054* in *S. aureus*, the two fragments were cloned into the shuttle vector, pOX7, for *E. coli* - *S. aureus*.

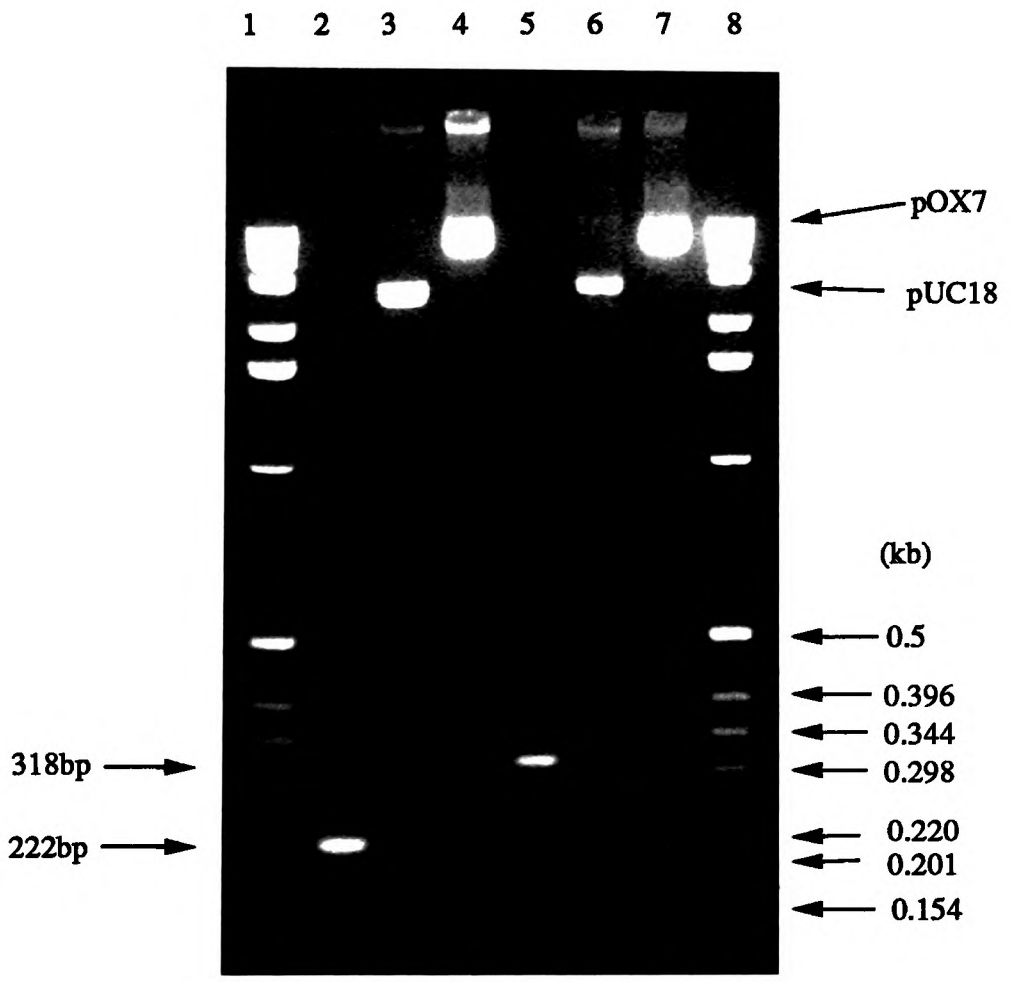
The fragments containing *rs9789* and *rs1054* were excised from their respective pUC18-based clones by digestion with *Eco*RI + *Hin*dIII. In addition, the DNA samples were also digested with *Dra*I in order to cleave the pUC18 backbone. Subsequently, the restriction enzymes were heat-inactivated and the digestion products were used to ligate to *Eco*RI + *Hin*dIII digested pOX7 DNA, separately. The two ligation mixes were used to transform JM107. Plasmid DNA was prepared from some of the transformants obtained in both clonings. The plasmids showing correct-sized inserts into pOX7 were identified by *Eco*RI + *Hin*dIII digestion. The desired clones were selected and were named pOXRS9789 and pOXRS1054, respectively.

The gel in Fig.7.14 shows the original PCR products and their constructs in pUC18 and pOX7.

7.8.4 Transformation of pOXRS9789 and pOXRS1054 into *S. aureus* RN4220

The plasmids pOXRS9789 and pOXRS1054 were then transformed into *S. aureus* RN4220 using the protoplast transformation. The transformants were selected on plates with transformation-medium containing erythromycin (20μg/ml) at 30°C. The plates were incubated for three days before the transformants could be obtained. Some of the potential transformants were picked and their plasmid DNA analysed. The presence of the correct clones in the transformants was identified by *Eco*RI + *Hin*dIII digestion of their plasmid DNA. The plasmids pOXRS9789 and pOXRS1054 were thus obtained in RN4220. The resultant strains were named RN4220(pOXRS9789) and RN4220(pOXRS1054), respectively.

Fig.7.14 An agarose gel to show the PCR products from the plasmids pI9789 and pOX1054 and *Eco*RI + *Hin*dIII digests of the plasmids pUCRS9789, pOXRS9789, pUCRS1054 and pOXRS1054. See text for details.



KEY:

1&8. 1kb ladder

2. PCR product from pI9789

3. pUCRS9789

4. pOXRS9789

5. PCR product from pOX1054

6. pUCRS1054

7. pOXRS1054

7.9 Construction of heteroplasmid *S. aureus*

To study the ability of the plasmids pOXRS9789 and pOXRS1054 to form co-integrates with the plasmids pS1/pΔD and pOX1054, heteroplasmid strains of RN4220, 1054 and PS80 were constructed. The plasmids pOXRS9789 and pOXRS1054 are temperature-sensitive for replication, since they are based on a temperature-sensitive plasmid vector, pOX7. The plasmid pI9789 was used instead of the plasmids pS1 and pΔD. The plasmid pI9789 is indistinguishable from pS1 and pΔD in the region on these plasmids that is involved in the formation of co-integrates with pOX1054. Furthermore, the plasmid pI9789 is thermostable for replication and would allow the detection of co-integrate formation with the plasmids pOXRS9789 and pOXRS1054 at 42°C when the cultures are grown on appropriate selective medium.

7.9.1 Construction of heteroplasmid RN4220

The ability of the plasmids pOXRS9789 and pOXRS1054 to form co-integrates with the plasmid pI9789 was investigated in RN4220. The plasmid pI9789 was transferred from PS80(pI9789) to RN4220 using transduction. Phage 80 was propagated on the strain PS80(pI9789) and was used to transduce pI9789 to 1054 with selection on CY-Cd (26.6μg/ml)-agar plates. Plasmid DNA was prepared from the transductants and digested with appropriate restriction enzymes to verify the presence of pI9789. The resultant strain was named 1054(pOX1054/pI9789). Phage 53 was then grown on 1054(pOX1054/pI9789) and used to transduce pI9789 to RN4220, RN4220(pOXRS9789) and RN4220(pOXRS1054). The transductants were selected on CY-Cd (26.6μg/ml)-agar plates. Plasmid DNA was prepared form the transductants and

was digested with appropriate restriction enzymes to verify the presence of correct plasmids. The resultant strains were named as RN4220(pI9789), RN4220(pOXRS9789/pI9789) and RN4220(pOXRS1054/pI9789).

7.9.2 Construction of heteroplasmid 1054

The ability of the plasmid pOXRS9789 and pOXRS1054 to form co-integrates with the plasmid pOX1054 was also investigated. In order to perform this, the plasmids pOXRS9789 and pOXRS1054 were transferred to the 1054 strain using bacteriophage. Phage 53 was grown on the strains RN4220(pOXRS9789) and RN4220(pOXRS1054), separately, and was used to transduce the plasmids pOXRS9789 and pOXRS1054 to 1054. The transductants were selected on CY-Em (20μg/ml)-agar plates. Plasmid DNA was prepared from some of the transductants and digested with appropriate restriction enzymes to verify the presence of correct set of plasmids. The resultant strains were named 1054(pOX1054/pOXRS9789) and 1054(pOX1054/pOXRS1054).

7.9.3 Construction of heteroplasmid PS80

The plasmids pOXRS9789 and pOXRS1054 were transferred to PS80 strain of *S. aureus* using transduction. Phage 80 was grown on strains 1054(pOX1054/pOXRS9789) and 1054(pOX1054/pOXRS1054), separately, and was used to transduce the plasmids pOXRS9789 and pOXRS1054 to PS80N and PS80(pI9789). The transductants were selected on CY-Em (20μg/ml)-agar plates. Plasmid DNA was prepared from some of the potential transductants and digested with appropriate restriction enzymes to verify the presence of the correct set of plasmids. The resultant strains were named

PS80(pOXRS9789), PS80(pOXRS1054), PS80(pI9789/pOXRS9789) and PS80(pI9789/pOXRS1054), respectively.

7.10 Recombination experiments

The ability of the various plasmids to form co-integrates was studied. All recombination experiments were performed at least twice.

7.10.1 Recombination experiments in RN4220

The strains RN4220(pOXRS9789/pI9789) and RN4220(pOXRS1054/pI9789) were used in the experiment. RN4220, RN4220(pOXRS9789), RN4220(pOXRS1054) and RN4220(pI9789) were used as controls. The method of Browne (1985) and Johnston (1971) was used as described in Section 3.4. The initial overnight cultures were set in 10ml broth for 18hrs and 10^6 cfu of this culture were plated in triplicate onto CY-Em (50μg/ml)-agar plates and incubated at 42°C for 18hrs. No discrete colonies were observed on any of the plates. The absence of Em^r colonies may be the result of the inability of the resident plasmids to form co-integrates. This can be interpreted as, either due to the absence of the relevant recombinase in the cells, or that the fragments of DNA cloned in pOX7 are too small.

7.10.2 Recombination experiments in 1054

The experimental procedures were the same as in the previous section. Discrete revertant colonies were obtained for both 1054(pOX1054/pOXRS9789) and

1054(pOX1054/pOXRS1054) at 42°C, when 10^6 cfu were plated onto CY-Em (50μg/ml)-agar plates. The number of revertant colonies obtained in two independent experiments is given Table 7.2. The results show that the number of revertants is five times greater for the strain 1054(pOX1054/pOXRS9789) than for 1054(pOX1054/pOXRS1054). No Em^r colonies were obtained for 1054(pOX1054/pOX7) under the same conditions. It is tentatively concluded that co-integration has occurred between pOX1054 and both pOXRS9789 and pOXRS1054 but this has to be checked by examining the plasmids within the revertants.

7.10.2.1 Plasmid DNA analysis of the revertants

Twenty revertant colonies were picked at random for both types of the revertants and their plasmid DNA analysed. A plasmid, larger than pOX1054, was found in every example. The plasmid DNA was digested with *Eco*RI and analysed by agarose gel electrophoresis. The results (Fig.7.15) suggest that the plasmid found in the revertants is a result of the co-integration of the plasmids pOXRS9789 or pOXRS1054 with pOX1054. The fragment sizes are given in Table 7.3. The recombination in both cases is site- and orientation-specific. However, the orientation of recombination for the pOX1054 - pOXRS9789 pair is opposite to that for the pOX1054 - pOXRS1054 pair of plasmids. This is illustrated in Fig.7.16.

All 20 bacteria of each type were the same and thus no plasmid-free bacteria were detected among the 40 examples tested. Thus, co-integration is the commonest event under these conditions and any integration of the plasmids pOXRS9789 or pOXRS1054 into the chromosome is rarer.

Table 7.2 Results of the recombination experiments in 1054

Plasmid(s)	**No. of Em^r colonies per plate at 42°C**		
	Exp. 1	**Exp.2**	**overall average**
pOX1054	0,0,0	0,0,0	0
pOX1054+pOXRS9789	168, 179, 183	194, 197, 209	188
pOX1054+pOXRS1054	26, 31, 36	37, 44, 46	37
pOX1054+pOX7	0,0,0	0,0,0	0

Fig.7.15 Results of the recombination experiments in *S. aureus* strain 1054. The agarose gel shows *Eco*RI digests of the plasmid DNA of the Em^r colonies of 1054 at 42°C containing the plasmids pOX1054 and pOXRS9789 or pOX1054 and pOXRS1054. The resultant recombinant plasmids are shown.

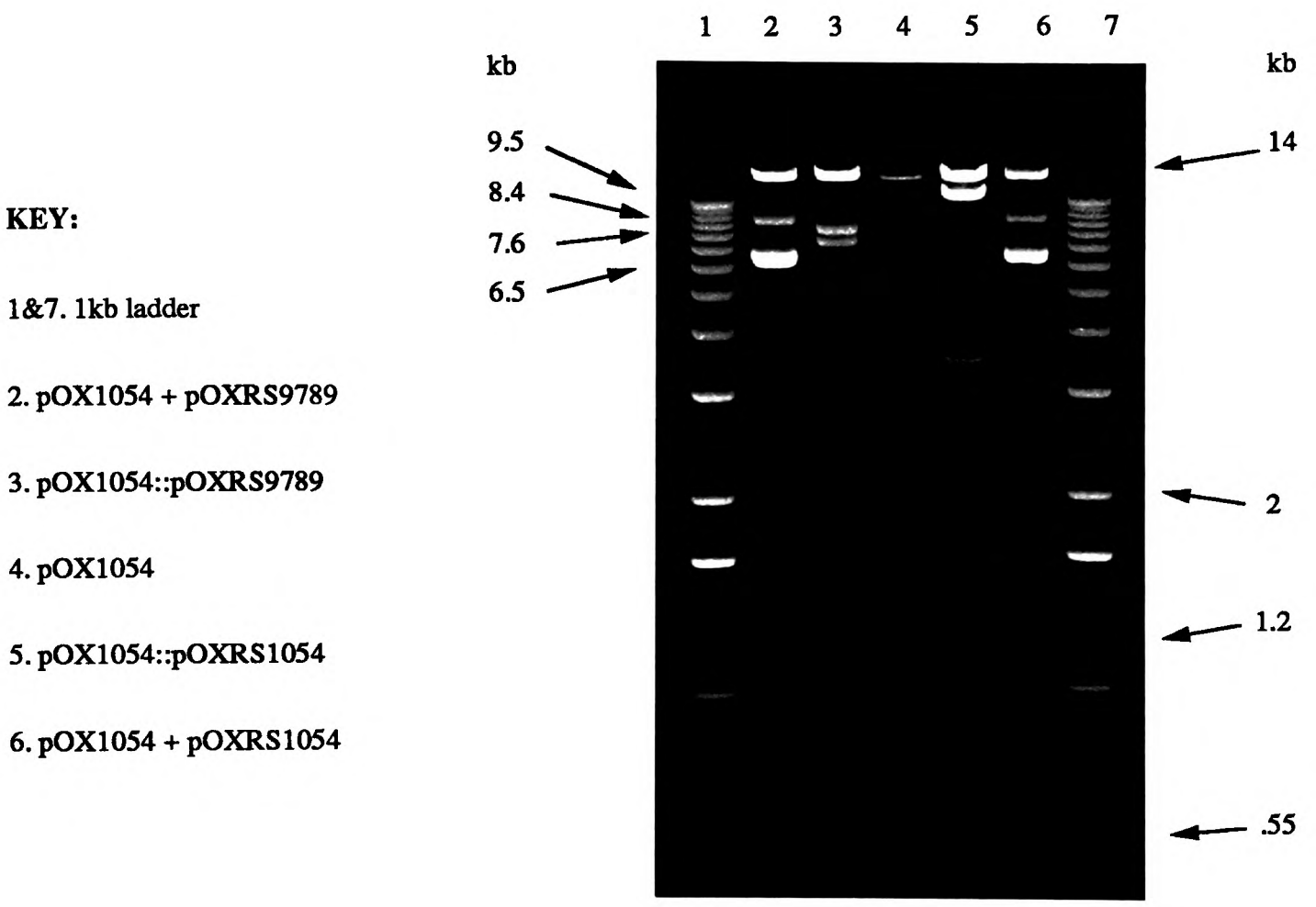


Table 7.3 *Eco*RI fragment sizes of pOX1054::pOXRS9789 and pOX1054::pOXRS1054 and the control plasmids. The bands involved in the recombination are in *italics* and the bands resulting from the recombination are in **bold**.

Plasmid(s)	**Fragment sizes (kb)**									
pOX1054	20		*9.5*				3.7		1.2	0.55
pOXRS9789					*6.5*					
pOXRS1054					*6.5*					
pOX1054::pOXRS9789	20			**8.4**	**7.6**		3.7		1.2	0.55
pOX1054::pOXRS1054	20	**14**					3.7	**2.0**	1.2	0.55

Fig.7.16 Proposed recombination of the plasmids pOXRS9789 and pOXRS1054 with the plasmid pOX1054. (a). Recombination between the plasmids pOX9789 and pOX1054. The recombination between the two plasmids is site- and orientation-specific. It occurs between the site labelled *rs9789* on the plasmid pOXRS9789 and the site labelled *rs1054* (at co-ordinate 1.9) on the 9.5kb *Eco*RI fragment of the plasmid pOX1054. In the diagram, only the relevant 9.5kb *Eco*RI fragment of pOX1054 is shown. Some of the known restriction enzyme sites on the 9.5kb fragment are shown. On *Eco*RI digestion of the co-integrated plasmids, an 8.4kb *Eco*RI and a 7.6kb *Eco*RI fragment replace the 6.5kb pOXRS9789 and the 9.5kb fragments of pOX1054. The rest of the *Eco*RI fragments of pOX1054 remain unchanged. (b). Mechanism of recombination between the plasmids pOXRS1054 and pOX1054. The recombination between the two plasmids is site- and orientation-specific. The orientation of *rs1054* in the plasmid pOXRS1054 is opposite to that of *rs9789* in the plasmid pOXRS9789. The recombination occurs between the site labelled *rs1054* on the plasmid pOXRS1054 and the site labelled *rs1054* (at co-ordinate 1.9) on the 9.5kb *Eco*RI fragment of the plasmid pOX1054 but in the orientation opposite to that between the plasmids pOXRS9789 and pOX1054. Thus, on *Eco*RI digestion of the co-integrated plasmids, a 14kb *Eco*RI and a 2.0kb *Eco*RI fragment replace the 6.5kb pOXRS1054 and the 9.5kb of pOX1054. The rest of the *Eco*RI fragments of pOX1054 remain unchanged.

Fig.7.16(a):

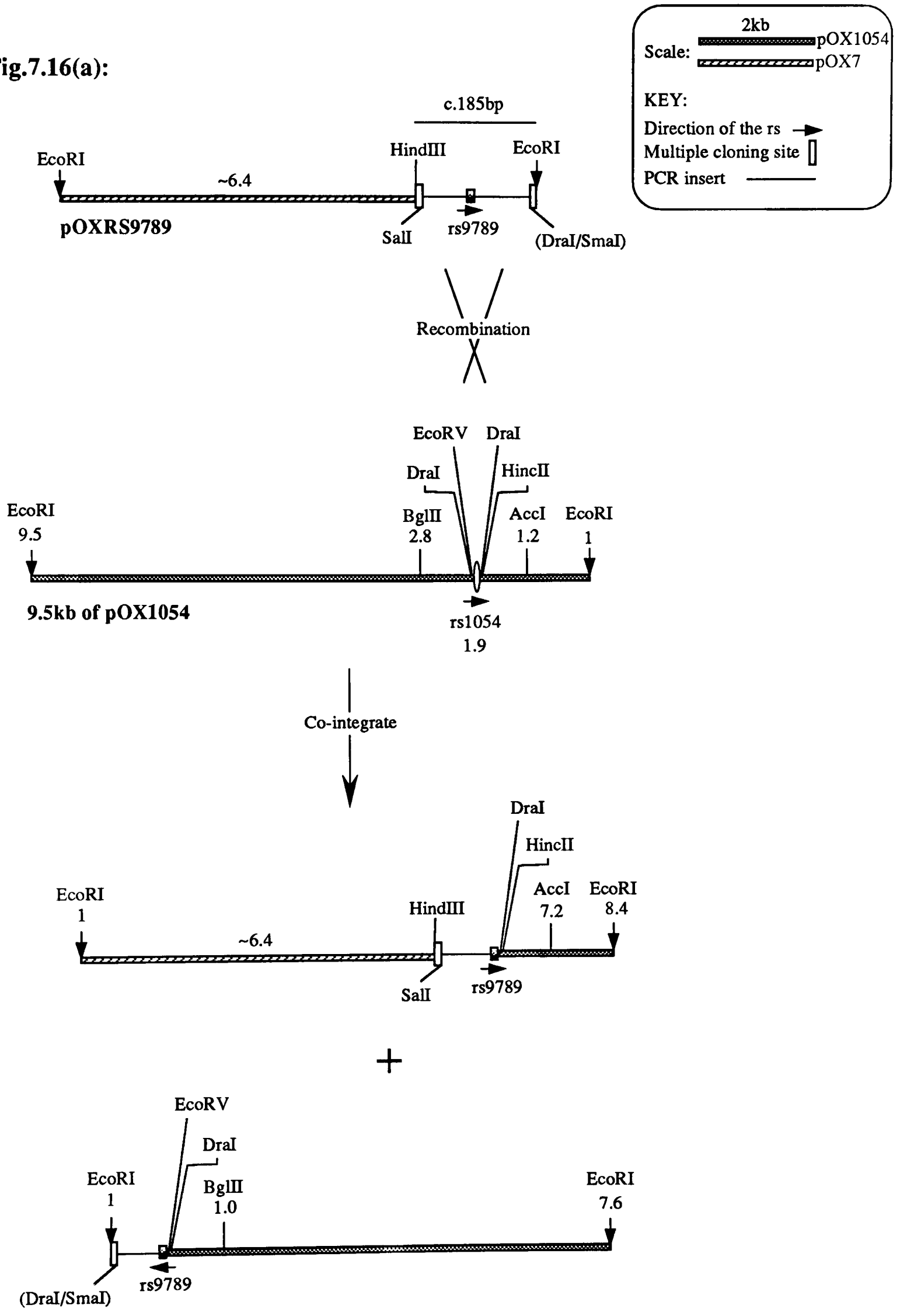
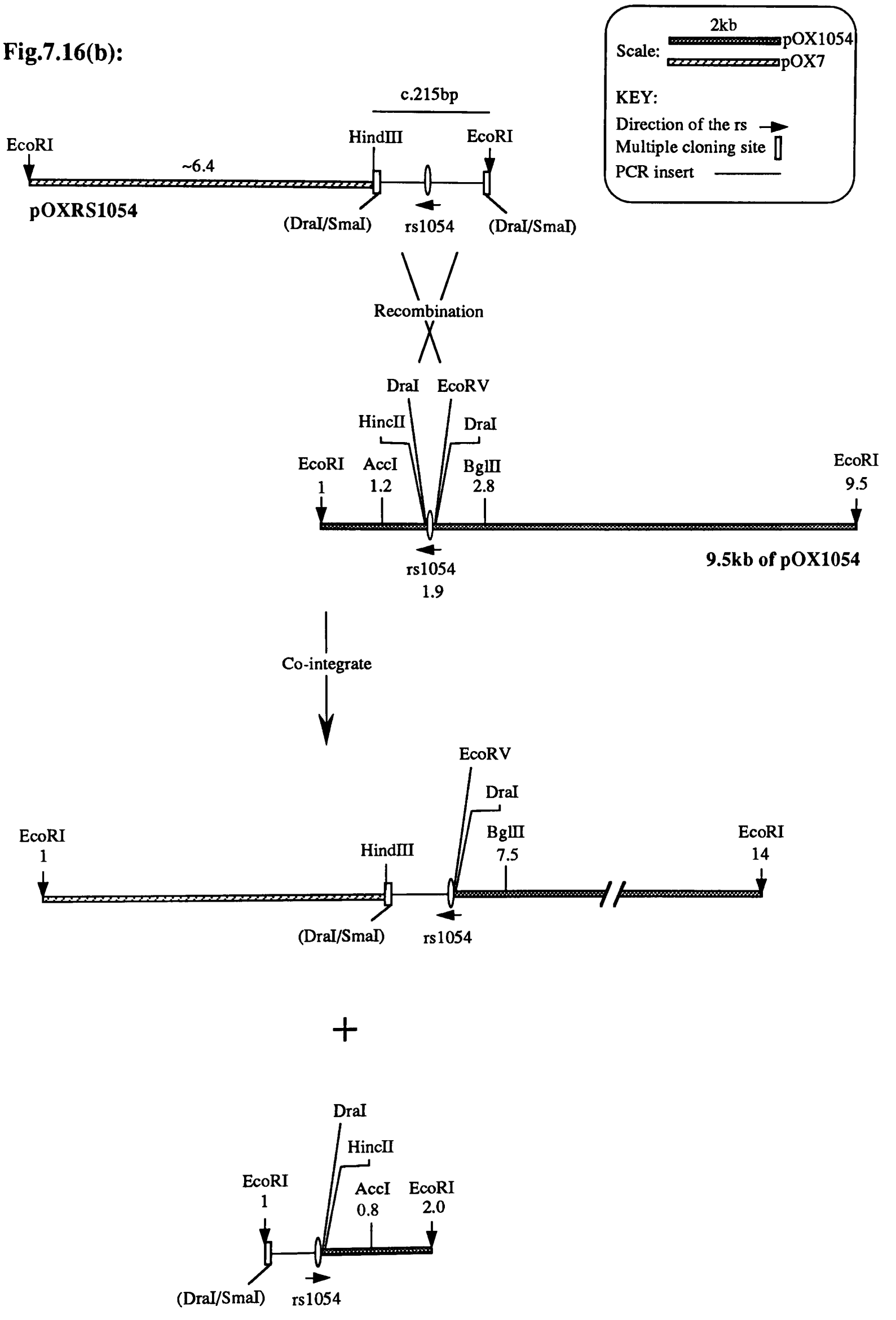

2kb
Scale:
pOX1054
pOX7
KEY:
Direction of the rs
Multiple cloning site
PCR insert
c.185bp
EcoRI
HindIII
EcoRI
~6.4
pOXRS9789
SalI
rs9789
(DraI/SmaI)
Recombination
EcoRV
DraI
DraI
HincII
EcoRI
9.5
BglII
2.8
AccI
1.2
EcoRI
1
9.5kb of pOX1054
rs1054
1.9
Co-integrate
DraI
HincII
EcoRI
1
AccI
7.2
EcoRI
8.4
HindIII
~6.4
SalI
rs9789
+
EcoRV
DraI
EcoRI
1
BglII
1.0
EcoRI
7.6
(DraI/SmaI)
rs9789

Fig.7.16(b):


2kb
Scale:
pOX1054
pOX7
KEY:
Direction of the rs
Multiple cloning site
PCR insert
c.215bp
EcoRI
HindIII
EcoRI
~6.4
pOXRS1054
(DraI/SmaI)
rs1054
(DraI/SmaI)
Recombination
DraI
EcoRV
HincII
DraI
EcoRI
1
AccI
1.2
BglII
2.8
EcoRI
9.5
rs1054
1.9
9.5kb of pOX1054
Co-integrate
EcoRV
DraI
EcoRI
1
HindIII
BglII
7.5
EcoRI
14
(DraI/SmaI)
rs1054
+
DraI
HincII
EcoRI
1
AccI
0.8
EcoRI
2.0
(DraI/SmaI)
rs1054

7.10.2.2 Nucleotide sequence analysis of the junctions of the plasmids in the co-integrates

In order to investigate precisely the sites of recombination between various plasmids in the co-integrates, nucleotide sequencing was carried out. Two independent examples (from two different experiments) of each type of co-integrate were taken for the nucleotide sequence analysis.

The oligonucleotides J1OX54 and J2OX54 (see Fig.7.11) were chosen to act as sequencing primers. The sequencing results of both examples of the pOX1054::pOXRS9789, for both junctions were indistinguishable and so were the results for the two examples of pOX1054::pOXRS1054 (Figs.7.17 and 7.18).

The co-integrate formation between the plasmids pOX1054 and pOXRS9789 (containing *rs9789*) was similar to that between the plasmids pOX1054 and pS1/pΔD. A specific sequence (17 - 27bp; *rs1054*) on the plasmid pOX1054 has been deleted accompanied by a duplication of a specific sequence (16 - 26bp; *rs9789*) of the plasmid pOXRS9789. The recombination between the plasmids pOX1054 and pOXRS1054 has involved a site within the 162bp *Dra*I fragment of pOXRS1054 and a site within the corresponding 162bp *Dra*I fragment of pOX1054. However, it is not possible to discover the exact site of recombination between these plasmids from these data.

7.10.3 Recombination experiments in PS80

Erythromycin resistant colonies were not found when PS80(pOXRS9789/pI9789) or PS80(pOXRS1054/pI9789) were incubated at 42°C with selection for Em^r. The co-integration is presumed not to occur in this strain. The results also show that under these

Fig.7.17 The nucleotide sequences at one of the co-integration sites for pOX1054::pOXRS9789 and pOX1054::pOXRS1054. Restriction sites for the enzymes *Dra*I, *Eco*RV and *Ssp*I are in **lower case** as are the *Sal*I and, *Sma*I, and *Dra*I half sites. The pI9789 sequence is <u>underlined</u>. The pOX7 sequence is in *italics*. The 26bp and the 27bp *rs* sequences are in **bold**.

DraI

i. **pOX1054::pOXRS9789:** TATTTTGAATTTTATGATACATAGATTATAACACAAGTTAAAGGAATTATGGCCAAAATAAAATCCCC**tttaaa**

ii. **pOX1054::pOXRS1054:** TATTTTGAATTTTATGATACATAGATTATAACACAAGTTAAAGGAATTATGGCCAAAATAAAATCCCC**tttaaa**

i. TTCAAGGATAAATTTCTTAATCTATCTTCTTAATTTATCATTAGGGG**ATTTTGTCAAAAACGTTCGTTTTTGA**GACTAGAATTTTATGCCCTACTTA

ii. TTCAAGGATAAATTTCTTAATCTATCTTCTTAATTTATCATTAGGGG**ATTTGTCTCAAAAACGTTCGCTTTTGA**AATGAATATAAATAACTAAGGCT

SspI SalI

i. <u>CTTCTTTTATTTTCATTCA**aatatt**TGCTTGCATGATGA**gtcgac***CTGCAGGCAT*</u>(pOX7)

ii. AATATAAAAGTTAAAAGACTATATACCAT**gatatc**AATTACTAAAGGTCTAAGAAATT**ttt*ggg*TACCG**(pOX7)

EcoRV DraI/SmaI

Fig.7.18 The nucleotide sequences at one of the co-integration sites for pOX1054::pOXRS9789 and pOX1054::pOXRS1054. The restriction site for the enzyme *Eco*RV on the pOX1054 sequence and for the half sites of *Dra*I and *Sma*I are in **lower case**. The pI9789 sequence is underlined. The pOX7 sequence is in *italics*. The 26bp and the 27bp *rs* sequences are in **bold**.

EcoRV

i. **pOX1054::pOXRS9789:** **gatatc**ATGGTATATAGTCTTTTAACTTTTATATTAGCCTTAGTTATTTATATTCATT**TCAAAAACGAAC**

ii. **pOX1054::pOXRS1054:** ATGGTATATAGTCTTTTAACTTTTATATTAGCCTTAGTTATTTATATTCATT**TCAAAAGCGAAC**

DraI/SmaI

i. **GTTTTTGACAAAAT**GAAATTTTTAACCATTAGTTCTTAAACGTAAAATGTAAATAATGTTTTTGATA**ttt*ggg***TACCGAGCTCGAATTC*(pOX7)

ii. **GTTTTTGAGACAAAT**CCCCTAATGATAAATTAAGAAGATAGATTAAGAAATTTATCCTTGAA**ttt*ggg***TACCGAGCTCGAATTC*(pOX7)

DraI/SmaI

conditions the *erm* gene from neither pOXRS9789 nor pOXRS1054 integrates into the chromosome and so it is presumed that neither plasmid integrates.

7.11 Discussion

7.11.1 pOX1054 is cryptic

A plasmid which is not known to confer any phenotypic traits upon its host is said to be 'cryptic'. Such plasmids of varying sizes are wide-spread among Gram-negative and Gram-positive bacteria isolated from diverse niches, *e.g.*, *Campylobacter hyointestinalis* (Waterman *et al.*, 1993), *Corynebacterium diphtheriae* (Archer and Sinskey, 1993), *Bacillus stearothermophilus* (Narumi *et al.*, 1993), *Acinetobacter calcoaceticus* (Minas and Gutnick, 1993), *Lactobacillus curvatus* (Klein *et al.*, 1993), *Neisseria gonorrhoeae* (Grimholt *et al.*, 1993), *Chlamydia trachomatis* (Fahr *et al.*, 1992), *Pasteurella multocida* (Price *et al.*, 1993). Most of the cryptic plasmids reported are small, ranging in size from ~1.5kb to 8.0kb. However cryptic plasmids of 38kb (from *Lactococcus lactis* subsp. *lactis*; which can form co-integrates with other plasmids to generate fusion plasmids as large as 75kb - 115kb; see Hayes *et al.*, 1993) and 450kb from *Agrobacterium tumefaciens* (Allardet-Servent *et al.*, 1993) have also been reported. Some small cryptic plasmids have been sequenced and found to contain genes required for their own replication together with some uncharacterised DNA. A few reports are available on cryptic plasmids of *Staphylococcus* (Walters and Dyke, 1990; Dyke and Curnock, 1989).

The plasmid pOX1054 is large (~35kb) and apparently cryptic. Tests failed to reveal genes for resistance to any of a wide variety of antibiotics, metal ions, ethidium bromide and quaternary ammonium (CTAB) compounds.It does not carry genes for

conjugal transfer found on some staphlylococcal plasmids, such as pSK41 (Firth *et al.*, 1993). For pOX1054, it is likely that there are functional genes present and one way to discover their function might be by DNA sequencing followed by data base searches.

It has been suggested that small cryptic plasmids might act as a transposon 'hitch-hiking' station (Walters and Dyke, 1990; Dyke and Curnock, 1989). Cryptic plasmids, thus, may have played some role in the evolution of multiresistant plasmids and might have provided a starting replicon, which, by acquisition of transposons carrying resistance genes and/or by forming co-integrates with plasmids carrying resistance genes (*e.g.*, pOX1054::pS1, pOX1054::pRS1054, *etc.*) resulted in multiresistant plasmids. Cryptic plasmids may also integrate into the host chromosome in part or *in toto* - a phenomenon which could have evolutionary implications. For example, in the human pathogen *Neisseria gonorrhoeae* (Grimholt *et al.*, 1993), parts of a cryptic plasmid were found in the chromosome of both the plasmid-containing and plasmid-free isolates. Imprecise excision of such integrated plasmids and subsequent transfer to other bacteria will result an exchange of genetic material.

7.11.2 pOX1054 can form stable co-integrates with other plasmids containing *rs9789* or *rs1054*

The co-integrates of the plasmid pOX1054 with pS1 and pΔD are stable under the conditions used as are the co-integrates of pOX1054 with pOXRS9789 and pOXRS1054. Thus, this site-specific recombination system differs from that catalysed by the Integrase Family of site-specific recombinases, which may catalyse both integration and resolution (Thompson and Landy, 1989; Argos *et al.*, 1986). It is, however, possible that the resolution frequency of these co-integrates is very low and could not be detected in the

system used. It is known that site-specific recombination systems can generate stable co-integrates between Gram-positive plasmids in *S. aureus* (Novick *et al.*, 1984; Novick *et al.*, 1981; Iordanescu, 1975). A site-specific recombination system encoded on small Gram-positive plasmids involving "Pre-protein" [such as Pre(T) of pT181 and Pre(E) of pE194 can mediate the formation of stable co-integrates between these plasmids if the recombination site RS_A is available (Gennaro *et al.*, 1987). It is, thus, conceivable that a site-specific recombination system similar to that of small plasmids may exist in 1054 (presumably on pOX1054) which generates stable co-integrates. This recombination, however, appears to be independent of BinR.

Thus, it is possible that the recombinase involved in the formation of co-integrates catalyses recombination only intermolecularly. To function intramolecularly it may require the recombination sites to be in a specific orientation that is absent from the pOX1054 co-integrates tested here.

7.11.3 *rs9789* and *rs1054*

A comparison of the recombination sites, *rs9789* and *rs1054*, with a number of other DNA sequences, involved in site-specific recombination, *e.g.*, RS_A, RS_B, *λatt*, *etc.*, is provided in Fig.7.19 and shows that while *rs9789*, *rs1054* and *rs258* are homologous, the other sequences do not resemble them.

The sequence labelled *rs258* in Fig.7.19 was identified as a potential recombination site (similar to *rs9789*) by inspection and its biological activity has not been determined. The presence of these related sequences on pI9789 and pI258 is of interest due to their location, since, plasmids pI9789 has been proposed as a progenitor of pI258 (see Fig.7.20; Shalita *et al.*, 1980; also see Dyke and Rowland, 1990 and Rowland, 1989). The

Fig.7.20 Comparison of the part of the restriction maps of pI258 and pI9789. The region labelled 'x' on pI258 has no sequence homology to the region labelled 'y' on pI9789 (Rowland, 1989). The two recombination sites *rs9789* and *rs258* are labelled. Restriction enzyme sites within the part of Tn*552* are not shown.

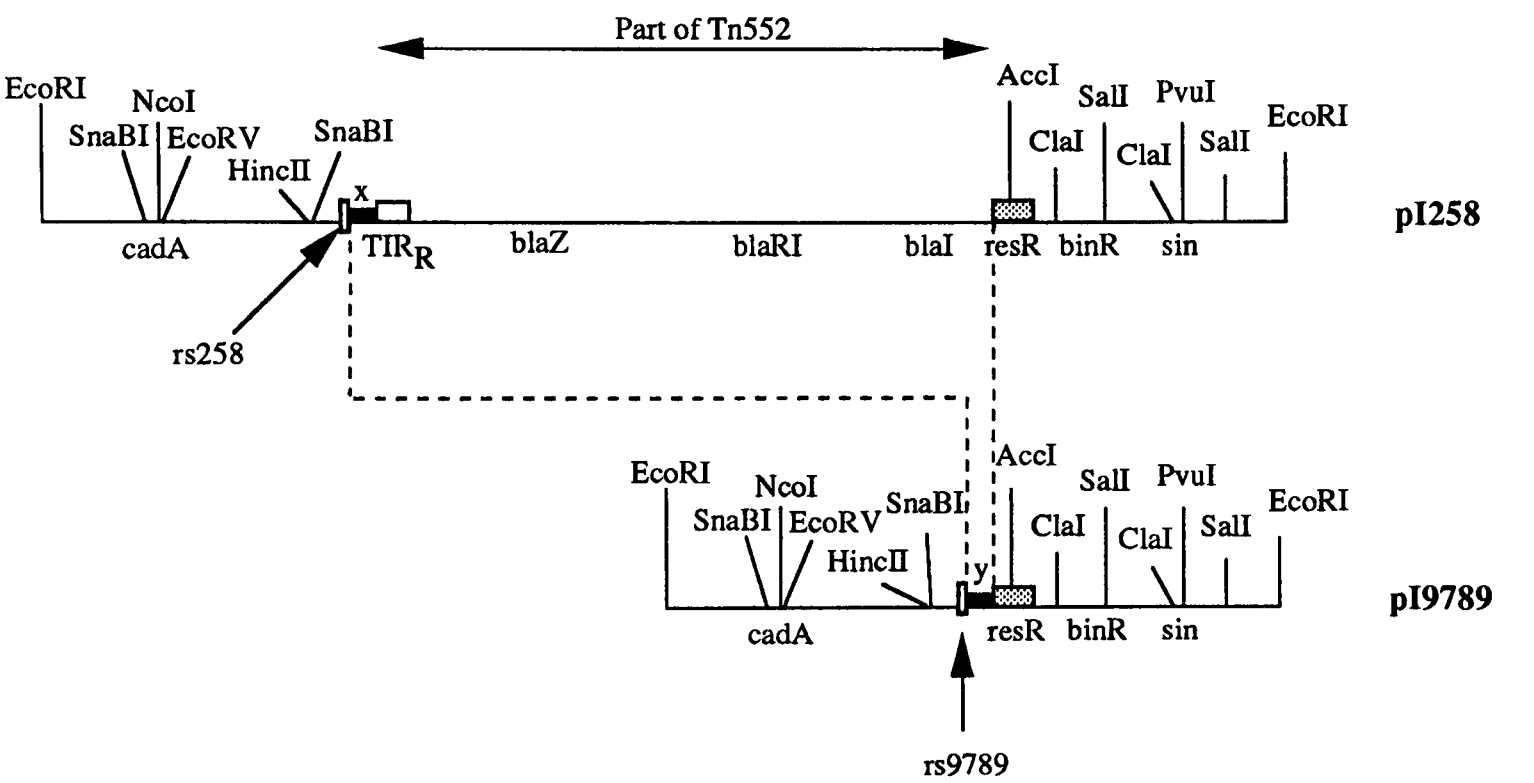


Fig.7.19. The recombination sites *rs9789* and *rs1054* are compared with a number of other recombination sites found on small Gram-positive plasmids, and in E.coli λ*att*, B*att* (the core sequences only; Freifelder, 1986) and hs2 of Tn*21* (Nücken *et al.*, 1991). For RS_A (Gennaro *et al.*, 1987), the central highly conserved region is given in capitals and a part of the flanking less conserved sequence is given in lower case. The RS_B sequence is from Novick *et al.* (1984). The sequence labelled *rs258* is from the plasmid pI258 (see text for details) and was taken from Rowland (1989). hs2 is a sequence in the Tn*21* transposon into which specific insertions of various antibiotic genes are found. The sequence labelled pE194 (Conrad *et al.*, 1992) is from this plasmid and is thought to be involved in integration into the chromosome of *Bacillus subtilis*. A region of homology within the *rs* sites of pI9789, pOX1054 and pI258 is in **bold**. The complementary strand of *rs9789* is also given. All sequences are 5' to 3'.

rs9789	ATTTTG--**TCAAAAACGTTCGTTTTTGA**
rs1054	ATTT-GTC**TCAAAAACGTTCGCTTTTGA**
rs258	CCCTGG--**TCAAAACCGTTCGTTTTTGA**
pE194	GGGGAGAAAACAT
hs2	CTAAAACAAAGTTA
RS_A(T181)	gtttAAGTCTAACACACTAGACTTATTTtt
RS_A(E194)	cataAAGTCTAACACACTAGACTTATTTag
RS_B(E194)	CAAAAAGTTTTCTCGGCATAAAAGTACCTCTA
RS_B(T181)	CAATAAGTTTTCTCGGCATAAATGCATGTTCTA
RS_B(C194)	CAATAAATTTTCTCGGCATAAATGCGTGGTCTA
RS_B(SN2)	CAAAATGTTTTCTCGGCATAAATGCTTAATTAA
λ*att*	TTAGTATAAAAAAGC
B*att*	TTAGTATAAAAAAGC

rs9789	**TCAAAAACGAACGTTTTTGA**--CAAAAT

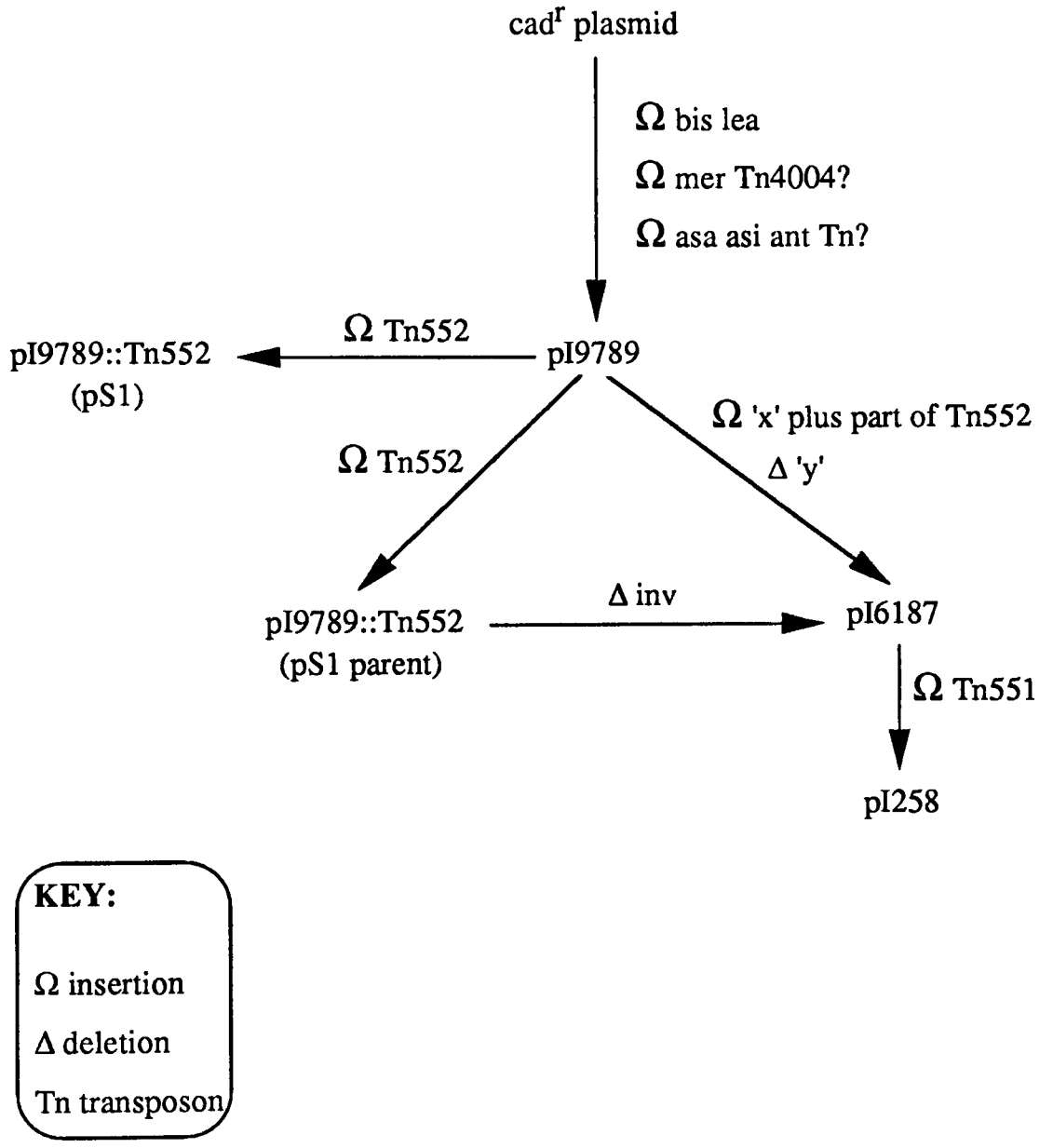
sequences *rs9789* and *rs258* make one end of the boundary of homologous sequences with *resR* at the other boundary. The section between *rs9789* and *resR* in pI9789 labelled 'y' is substituted by a sequence 'x' plus part of Tn*552* in pI258 (Fig.7.20). Detailed sequence analysis of pI6187 is not available, so it is not possible to judge whether the proposal of Shalita *et al.* (Fig.7.21) that pI6187 arose by insertion of a complete Tn*552* into pI9789 followed by deletion of the invertible region is correct. An alternative could be that a recombination happened in which part of Tn*552* plus 'x' were substituted for 'y' using the *rs* sequence and *resR*. In either case Tn*551* subsequently inserted into pI6187 to create pI258.

The discovery of the recombination site *rs* on pI9789 and the potential recombination site on pI258 adds to the elements present on these plasmids (*e.g.*, *resR*, *binR* and *sin*). Site-specific recombination seems important in the development of multiresistant plasmids.

7.11.4 The recombination between *rs9789* and *rs1054* occurs by a novel site- and orientation-specific reaction

Restriction endonuclease analysis of the co-integrates pOX1054::pS1 and pOX1054::pΔD using *Eco*RI suggested that the recombination between pOX1054 and pS1/pΔD occurs within defined regions on both plasmids and in one specific orientation. DNA sequence analysis of the junctions in the co-integrates revealed that the recombination is both site- and orientation-specific and occurs between a 27bp segment (called *rs1054*) on pOX1054 and a 26bp segment (called *rs9789*) on pS1/pΔD. The end product of this reaction is a co-integrate plasmid in which apparently the 27bp *rs1054* is deleted and the 26bp *rs9789* occurs as direct-repeat at the two junctions (see Fig.7.10). A comparison of

Fig.7.21 Possible evolutionary pathway for the plasmid pI258 (modified from Rowland, 1989 and Shalita *et al.*, 1980). See text for details.



rs9789 with *rs1054* (Fig.7.12) shows that these two sites are homologous and the terminal 6 nucleotides at one end and 4 at the other are identical. The central region which shows some differences consists of 16bp in *rs9789* and 17bp in *rs1054*. It is, therefore, not possible to suggest whether the whole of *rs1054* is deleted and the whole of *rs9789* is duplicated. However, the data suggest that at least the middle 17bps of *rs1054* are deleted and the middle 16bps of *rs9789* are duplicated.

This recombination demonstrates a combination of features which is novel and is not exhibited in the recombinations carried out by any known group of site-specific recombinases. The site-specific recombinases are classified into two well-characterized families, the "integrase family (Int)" and the "Tn*3*/Hin" family. The recombination catalysed by these families of enzymes is conservative and occurs at specific sites within a short region of sequence identity shared by the participating DNA segments. Strand exchange occurs by precise breakage and joining events in the absence of DNA loss or synthesis and recombination is reciprocal (reviewed by Thompson and Landy, 1989; Sherratt, 1989; Glasgow *et al.*, 1989; Cox, 1989; Craig, 1988). **The Int family** recombinases represent a class of conservative site-specific recombinases (exemplified by the phage lambda integrase) which may catalyse integration, excision (*e.g.*, integration and excision of phage lambda from the chromosome) and inversion (*e.g.*, *fimA* inversion reaction to allow alternate expression of different fimbriae of *E. coli*; Dorman and Higgins, 1987). **The Tn3/Hin family** of site-specific-recombinases includes both DNA resolvases and DNA invertases. They catalyse recombination reactions only between *intra*molecularly placed recombination site, the *res* sites, and strictly require supercoiled substrate. **DNA Resolvases** are highly specialized in catalysing deletions between two directly oriented *res* sites and perform inversions between two inversely oriented *res* sites

but very inefficiently. **DNA invertases** have the opposite characteristics. They are efficient in promoting inversions between two inversely orientated *res* sites but perform resolution of the DNA between two directly placed *res* sites at a very low frequency. Target duplication is a characteristic exhibited by the recombination reactions carried out by a family of non-specific recombinases, **the transposases**, which are encoded on transposons (Tn) and insertion sequences (IS). In transposition, no homology between the recombination sites is required. Breakage and rejoining is accompanied by DNA synthesis and recombination is non-reciprocal. A target duplication of between 3bp to 13bp may occur during transposition (reviewed by Kleckner, 1990; Grindley, 1985).

The recombination between pOX1054 and pS1/pΔD differs from any of the classes stated above. The pOX1054 - pS1/pΔD-co-integration is an *inter*molecular reaction (as carried out by Int family and transposases, but not by Tn*3*/Hin family) but as opposed to a reaction catalysed by a member of Int family, DNA sysnthesis and deletion of one of the recombining sites accompanies strand exchange. It also differs from transposition; although, the target site is duplicated as in transposition, but, (i) it is a site-specific recombination, whereas, transposition is generally non-specific. The recombining sites show very good homology, a feature not required for many transpositions. Although, the transposon Tn*554* transposes into a specific site (see Murphy *et al.*, 1991) and Tn*551* transposes specifically into a site in ORF480 of Tn*552* (Chapter 5), no homology is found between the recombining sites, (ii) one of the recombining sites is apparently deleted; such deletions are not found on transposition, though deletions in the neighbouring DNA may be observed, (iii) the duplication is larger than any known for transposase-mediated recombinations (*e.g.* 11-13bp for IS*4*; Galas and Chandler, 1989).

The site- and orientation-specificity of recombination between *rs9789* and *rs1054* is also supported by the results obtained in Section 7.10.2 (also see Fig.7.16). Recombination between the plasmids pOXRS9789 (a clone of *rs9789* in pOX7) and pOX1054 is in one specific orientation and occurs at *rs9789* and *rs1054*. The plasmid pOXRS1054 has the closely-related recombination site *rs1054* but in an orientation opposite to that of *rs9789* in pOXRS9789. This plasmids recombines with pOX1054 in an orientation which is opposite to that between pOXRS9789 and pOX1054.

7.11.5 The recombination between *rs9789* and *rs1054* is strain-specific.

The results obtained in Section 7.10 suggest that the co-integrate formation is strain specific and occurs only in 1054 or in the presence of more of pOX1054 than is present in the clones of *rs1054*. The recombination probably does not involve the host general recombination system. Co-integration does not happen in RN4220 and PS80 and both strains are recombination-proficient. The recombinase that catalyses the formation of these co-integrates is contributed either by the plasmid pOX1054 or by the 1054 chromosome. The 340bp sequence obtained from pOX1054 does not provide any evidence of a recombinase type protein. To find out whether the protein involved in this recombination is on the plasmid pOX1054 or on the chromosome, it would be necessary to mobilize pOX1054 to a different *S. aureus* strain or cure 1054 of this plasmid, which is difficult in the absence of a marker. Failure of the plasmids pOXRS9789 or pOXRS1054 to form a co-integrate with pI9789 in either PS80 or RN4220 indicates that the plasmid pI9789 (pS1 or pΔD) contributes the recombination site but not the recombination enzyme.

7.11.6 A possible recombination mechanism

A possible model for recombination between pI9789 and pOX1054 is presented in Fig.7.22. The recombination is characterized by duplication of the whole or at least a part of *rs9789* and deletion of the whole or a part of *rs1054*. The reverse reaction (*i.e.*, deletion of *rs9789* and duplication of *rs1054*) was not seen and this suggests some degree of specificity of the recombinase catalysing this recombination, to recognize and to cleave the sequences involved in the recombination in a particular fashion. The model presented in Fig.7.22 demands a protein which can specifically cleave both strands of pOX1054 at two sites in order to delete *rs1054* but cleaves each strand of pI9789 only once. It is possible that for site- and orientation-specificity and for recognition, a highly specific sequence is not required (*i.e.*, both *rs9789* and *rs1054* can be recognized) but cleavage is introduced sequence-specifically; one DNA strand is cleaved when *rs9789* is found but both when *rs1054* is recognized. The recombination sites, *rs9789* and *rs1054*, may also form secondary structures (Fig.7.23). The energies of formation of the secondary structures for both *rs* sites are such that these topological forms may not exist under normal physiological conditions. However, such structures may be introduced by the recombinase in order to cleave the recombining DNA molecules in a specific fashion by a difference in their free energies (ΔG*rs9789* - ΔG*rs1054* = -1.0kcal/mol).

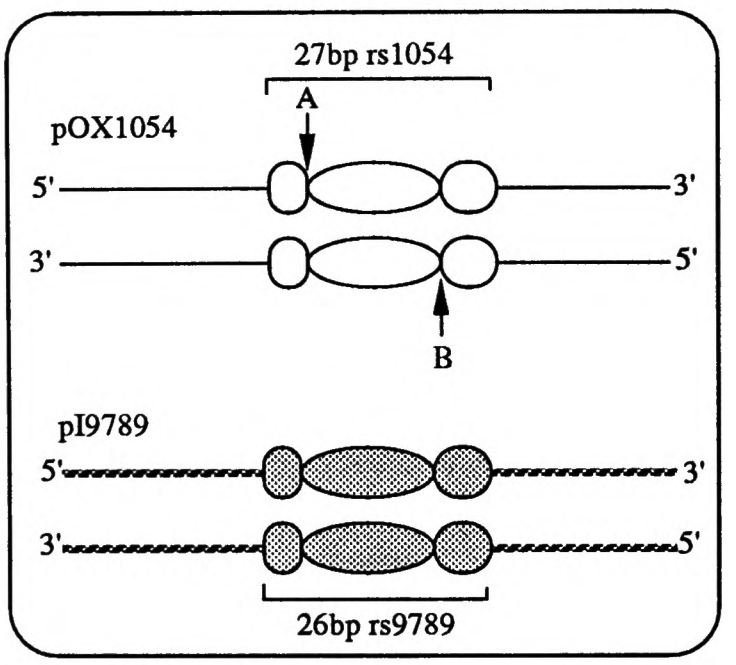
The lower recombination frequency observed between *rs1054* and *rs1054* (*i.e.*, pOXRS1054 and pOX1054) than between *rs9789* and *rs1054* (*i.e.*, pOXRS9789 and pOX1054) may be due to a difference in processivity or ability of the recombinase to bring about recombination between a particulr pair of the *rs* sites.

Fig.7.22 One possible model for recombination between pI9789 and pOX1054.

The recombination site rs1054 and rs9789 are represented by ovals. Small ovals on either side of the large central oval represent the region of sequence identity between rs1054 and rs9789 (see Fig.7.12). It is not possible to judge at what site occur within the small ovals but for simplicity it is assumed that they occur at the 3' end.

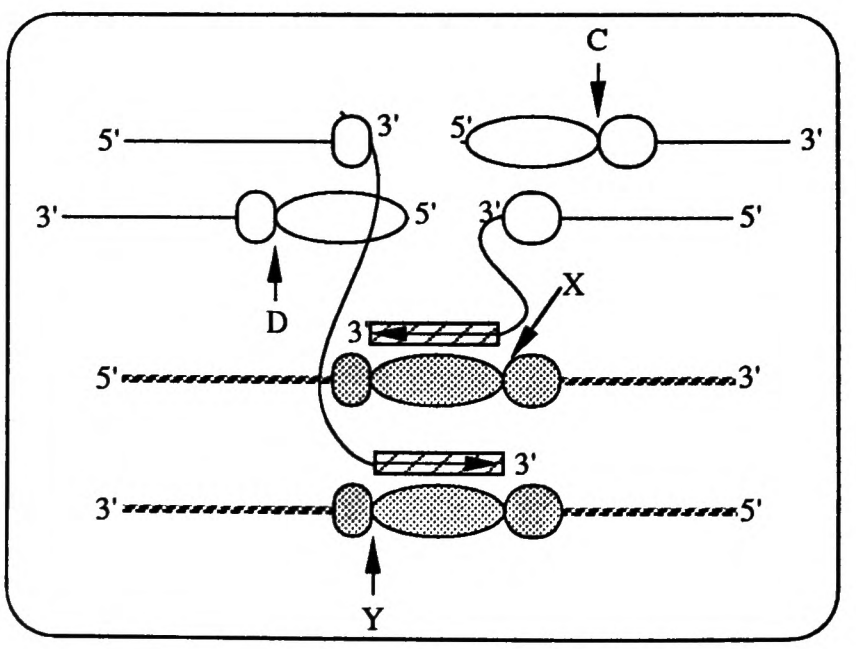
(a):

pOX1054 is cleaved by an endonuclease (recombinase) at positions A and B to generate two free 3'OH ends.



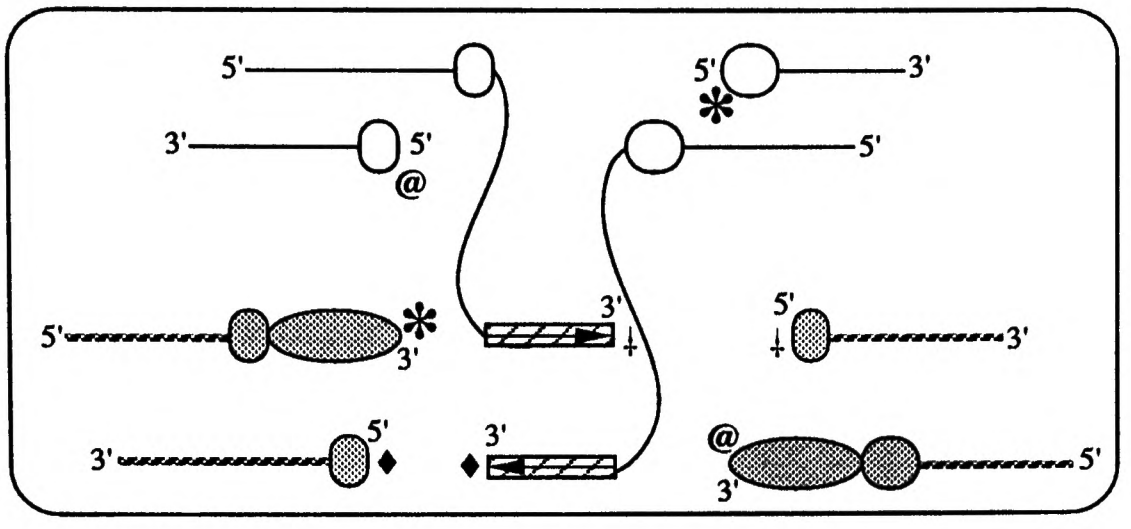
(b):

The free 3'OH ends of the nicked strands are extended using rs9789 as template. Nicks are generated at sites X and Y in pI9789 and at sites C and D in pOX1054.



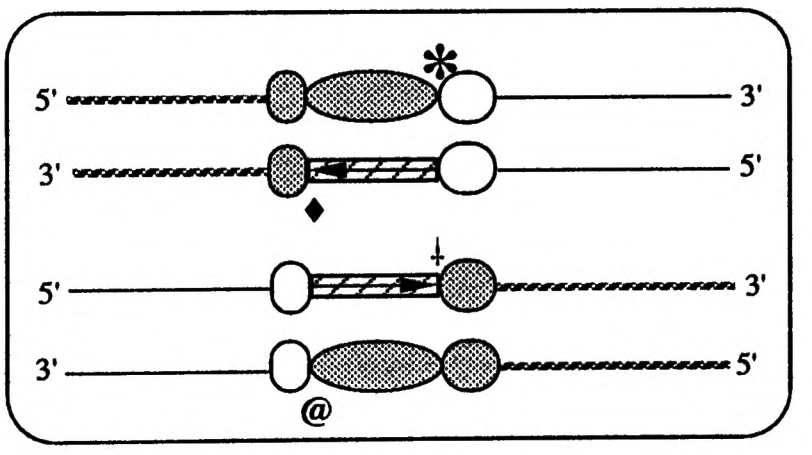
(c):

Free ends are generated.



(d):

Free-ends with the same symbols are ligated to produce co-integrate with rs9789 duplicated and rs1054 deleted in the co-integrate.



The system needs:-

1- Recombinase that cuts both ends of pOX1054 but only one of pI9789.
2- Helix-unwinding activity.
3- DNA polymerase to copy rs9789.
4- DNA ligase activity (which may be performed by the recombinase).
5- Other host co-factors?
6- DNA gyrase?

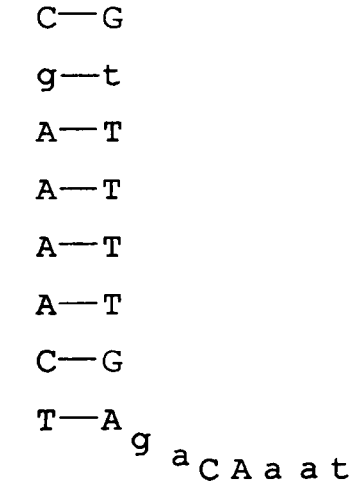
Alternative models could need 5' exonuclease activity.

Fig.7.23 (A). A comparison of *rs1054*, *rs9789* and *rs258*. The differences are shown in lower case: (B). The recombination sites show imperfect dyad-symmetry and may form stem-loop stuctures. The free energies (ΔG at 25°C) for the loop formation were calculated according to Lewin (1994) and Tinoco *et al.*, (1973).

(A):

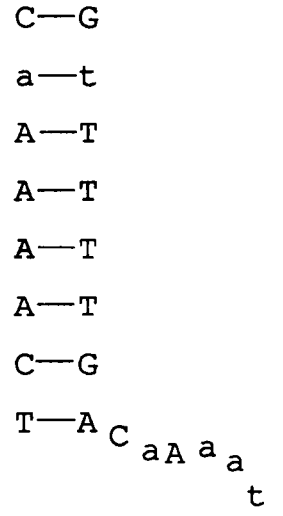
```
rs1054:    TCAAAAgCGAACGtTTTTGAgaC-Aaat (27bp)
rs9789:    TCAAAAaCGAACGtTTTTGA--CaAaat (26bp)
rs258:     TCAAAAaCGAACGgTTTTGA--CcAggg (26bp)
```

(B):



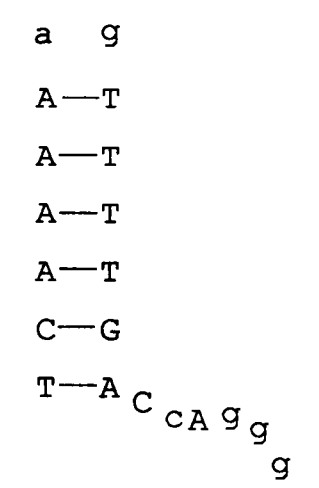
rs1054

ΔG = -4.1Kcal/mol



rs9789

ΔG = -5.1Kcal/mol



rs258

ΔG = -2.7Kcal/mol

Chapter Eight:

General discussion

Chapter 8: General Discussion

8.1 Gene flux

Genome stability is of considerable significance in the maintenance of a species in an ecosystem. However, genome stability is not absolute due to the limits set by various mechanisms of mutagenesis which together with a continuous but controlled flux of genetic information form the bases of evolutionary development (*e.g.*, Arber, 1991). The spread of multiple resistance to antimicrobial drugs among clinically important bacteria is seen as an extraordinarily rapid evolutionary development. Apparently, the mechanisms of both the mutagenesis and the gene flux have participated in the development of multiresistant bacterial genomes. Most genetic studies on the mechanisms of the evolution of antimicrobial resistances are laboratory-based and although the precise *in vivo* role played by each mechanism is unknown, it is likely that the mechanisms similar to those observed in the laboratory may also exist in the nature. The main focus of the present study was the role of the mechanisms of mutagenesis, such as site-specific recombination and transposition, in the spread of resistance determinants in *S. aureus*. Several examples of intermolecular gene transfer were seen.

Two-way gene transfer between the staphylococcal chromosome and the plasmids and the formation of new plasmid linkage groups was demonstrated. The chromosome of the *S. aureus* strain PS80 was seen to acquire resistance determinants encoded on a resident temperature-sensitive plasmid by forced integration. The data thus obtained give the integration frequencies under the controlled laboratory conditions and may not reflect upon

the *in vivo* integration frequencies. Nevertheless, alternative restrictive conditions can be speculated to exist in nature, for example, those met by the transfer of a plasmid to a plasmid-containing bacterium such that the transferred and the resident plasmids belong to the same incompatibility group, or those when the transferred plasmid is unable to replicate in the recipient bacterium. The mechanisms of site-specific recombination and transposition are thought to be significant in evolution by providing means for gene acquisition and by causing DNA rearrangements and set examples of genetic variety generators (Arber, 1993; Syvanen, 1984). Transposable genes can provide regions of homology for recombination between two replicons. They may also provide a specific site for recombination (*e.g.*, Birkenbihl and Vielmetter, 1989; Cohen, 1976). It was observed that the recombination of the model plasmid pS1 with the *S. aureus* chromosome is influenced by a number of chromosome- or plasmid-borne factors particularly those involved in site-specific recombination and transposition. For example, the presence or absence and the copy number of the plasmid-borne gene, *binR*, affects the integration of the plasmid into the chromosome. A visible consequence of this integration is the accumulation of resistance determinants on the chromosome. The presence of the transposon Tn*551* on the chromosome of the *S. aureus* strain PS80 also enhances the recombination between the plasmid and the chromosome [*e.g.*, in PS80::Tn*551*(pS1)]. Tn*551* can be co-transferred with the integrated plasmid. Some important features of this interaction are, the transfer of a resistance determinant (*erm*) from the chromosome to the plasmid by the method of transposition and/or site-specific recombination, and the site-specific insertion of Tn*551* into the transposase gene of plasmid-borne Tn*552*. The specificity of insertion of Tn*551* may involve the DNA deleted from pΔD but present in pS1, possibly *binR*. These data show an unusual insertional inactivation of the gene encoding the transposition protein of

one transposon (Tn*552*) by another transposon (Tn*551*) and may set an example of intermolecular competition (*e.g.*, for transposition function), and region-specific accumulation of antibiotic resistance genes on pS1. The recombination mechanisms to integrate the plasmid into the chromosome may also involve homology between the recombining replicons (*e.g.*, Luchansky *et al.*, 1989). Such an example was obtained in the case of PS80N(pS1). The homology between the plasmid and the chromosome is apparently in the region of the transposon Tn*552*. In the absence of homology, alternative mechanisms or isomeric forms of the same mechanism may be active [as in the case of RN4220(pS1)]. It is possible that in PS80N the plasmid pS1 integrates into specific recombination sites on the chromosome which are presumably absent from the chromosome of RN4220. In the absence of the substrate crossover sites, the site-specific recombinases usually do not carry out recombination. In some instances, however, the site-specific recombinases may perform recombination at crossover sites showing sequence divergence from the primary site (called the secondary crossover sites) but at a much reduced rate (Lida and Hiestand-Nauer, 1987; 1986). It is possible that the low integration frequency of pS1 in RN4220 results from recombination at secondary crossover sites on the RN4220 chromosome. Replicon fusion may result in the generation of an invertible fragment by the presence of primary or secondary crossover sites on both genomes and thus causing local DNA rearrangements (see Chapter 4). Such events occurring in nature may result in the activation of a gene conferring selective advantage to the host (*e.g.*, kanamycin resistance; Lida and Hiestand-Nauer, 1987) and the host thus maintained and enriched in the population. The relative frequencies of recombination mediated by various mechanisms recorded though differ from each other in the laboratory, this however does not necessarily reflect on their *in vivo* role.

Exchange of genetic information between plasmids was also observed though not in a modular fashion. New plasmid linkage groups were generated by massive and stable plasmid co-integration (*e.g.*, that between pS1 and pOX1054 to form a ~65kb plasmid). Formation of such large co-integrates seems disadvantageous in terms of intercellular mobility *in vivo*. It has been suggested that the plasmids larger than the phage genome (>~45kb) will be truncated during transductional transfer due to packaging constraints (Parker, 1983) and may result in the loss of important genetic information. The transfer would probably be dependent on the conjugal mechanisms. However, a complete loss of genetic information from the gene pool would possibly be prevented. Formation of such co-integrates may be speculated to occur in the nature between two incompatible plasmids [for example see Murphy and Novick (1980)]. The interaction of the plasmid pS1 with the chromosome of 1054, if any in the form of recombination, remains cryptic. Overall, the data suggest that replicon co-integration can be an important mechanism to overcome instability problems.

Several examples of the horizontal gene transfer were also obtained. Genes were moved between chromosomes by phage (*e.g.*, transduction of integrated pS1 from the chromosome of one PS80 host to a new host and integration of the transferred genes into the chromosome of new host - Sections 4.3 and 4.5). Chromosomal Tn*551* was co-transferred with the plasmids to new host cells by phage. Plasmid-borne resistance genes like those on pS1 and on pOX7 were moved by phage among various strains of *S. aureus*. The phage successfully mobilized the plasmids of sizes between ~6.5kb (pOX7) and ~32kb (pS1::Tn*551*).

Barriers are presumably posed on both *inter* and *intra*cellular gene flux. For example, the site-specific recombination systems do not allow recombination at secondary

crossover site frequently enough to endanger genetic stability (Arber, 1993). Other examples are those of the mechanisms of phage-virulence-specificity shown by staphylococcal strains and the occurrence and the specificity of the recombination mechanisms. A delicate balance between the control and the flux mechanisms would be needed in nature to ensure both genome stability and evolution.

8.2 Rapid evolution of antimicrobial resistances: is hindrance of undesirable gene flux a solution?

The rapid emergence of the antimicrobial resistances is a serious threat to human health and a challenge to medicine and pharmacology. Some of the solutions sought to overcome this problem include a search for new antimicrobials and temporary suppression in the use of certain antibiotics (see Amábile-Cuevas and Chicurel, 1992), it however seems that the necessary genetic weapons against antimicrobial drugs already exist in bacteria. New antimicrobials may provide a temporary solution to cure an infection but they are not a solution to the rapid emergence of resistances. Conversely, a serious consequence of this therapy is the accumulation of multiple resistances in the clinical gene pool. The rationale for suppression in the use of an antibiotic is to dilute the resistance determinants from a bacterial population by spontaneous loss of the resistant plasmids in the absence of selective pressure. The drawback of this approach is that the chromosomal resistance determinants will prevail and those borne on multiresistant plasmids will be retained in the gene pool due to the selective pressure of another antibiotic. Another possible approach to prevent the spread of resistance genes could be by the use of drugs that inhibit

transposition, the mechanisms of recombination or replication - the phenomena potentially involved in the spread of resistance determinants. In the presence of several mechanisms of gene transfer, a careful evaluation of the role of each mechanism in the spread of resistance genes *in vivo* will be needed. The potential undesirable consequences of such drugs/methods will have to be realized before their widespread usage.

8.3 Conclusions

In *S. aureus*, gene transfer from chromosome to plasmid, from chromosome to chromosome and from plasmid to chromosome occurs by the methods similar to those in other procaryotes, although they may differ in finer details from other procaryotes. Such gene transfer can lead to the accumulation of resistance determinants on a replicon. An assessment of the role of recombinational mechanisms in the spread of resistance genes in nature is needed and such data may be useful to design drugs or methods selectively to prevent the spread of the resistance genes.

8.4 Future Work

The work reported in this thesis has produced more questions than answers. Some of the questions could be answered by the following investigations:-

[1] Construction of the PS80N chromosome library using an appropriate non-blunt enzyme and the use of selected clones for recombination studies with pI9789-derivative to find out the precise role of integrative mechanism in thermosensitivity suppression.

[2] Investigation of the mechanism of thermosensitivity suppression in pΔD, and the Type II and III revertant-derived transductants.

[3] Precise role of *binR* in the thermosensitivity suppression phenomenon.

[4] Role of the deleted DNA in pΔD, especially of *binR*, in the site- and orientation specific insertion of Tn*551* into the putative transposase gene of Tn*552*.

[5] Role of Tn*551* in the enhancement of the suppression phenomenon.

[6] Replication mechanisms of large staphylococcal plasmids and the use of these data to design drugs to inhibit replication of multiresistant plasmids as a tool to cure antimicrobial resistances.

[7] Investigation of the recombinase in 1054 and other host factors involved the recombination between *rs1054* and *rs9789* and mechanistic studies.

[8] Investigation of the recombinational potential of *rs258* and search for the presence of similar recombination sites on other large multiresistant plasmids.

[9] Design of methods to assess the *in vivo* role of various recombination mechanisms in the spread of antimicrobial resistance in *S. aureus* and other bacteria.

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