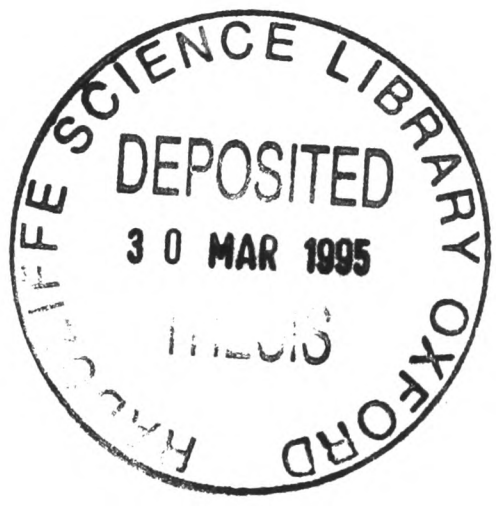
The basis of genetic rearrangements in mupirocin resistance plasmids

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Michaelmas, 1994.

The material contained herein has in no part been submitted or accepted for the award of any other qualification by any other university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference is made in the text.

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Christine Needham

For Rebecca Michelle

who arrived in the midst of this madness and folly

"Be happy, little girl, while you are young,
and let your heart give you joy in the days of your youth.
Follow the ways of your heart
and whatever your eyes see."

Ecclesiastes: 11v9.

"and I said,

'I am determined to be wise'

but this was beyond me.

Whatever wisdom may be,

it is far off

and most profound-

who can discover it?

So I turned my mind to understand,

to investigate

and to search out wisdom...

adding one thing to another

to discover the scheme of things."

Ecclesiastes: 7v23-25,27.

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Abstract

Abstract

Staphylococcus aureus is a Gram positive potentially pathogenic bacterium which has a propensity to gather resistance determinants. Mupirocin is a novel topical antibiotic active against many Gram positive species, including staphylococci and effective in the treatment and prevention of staphylococcal infections. Mupirocin acts by competitively inhibiting the charging of isoleucyl tRNA-synthetase (IRS) with isoleucine. Resistance has been observed in *Staphylococcus aureus* and coagulase negative staphylococci. Intermediate-level resistance (MIC >8μg ml^{-1} <512μg ml^{-1}) is thought to be due to spontaneous mutations in the native IRS. High-level resistance (>512μg ml^{-1}) is conferred by a second IRS protein, encoded by *mup*A which has a much lower affinity for mupirocin than isoleucine.

The mupirocin resistance gene (*mup*A) is usually found on a 4.05kb *Eco*RI fragment of plasmids of otherwise varied *Eco*RI restriction pattern which are easily transferred between strains by filter mating. Prior to the onset of these studies, mupirocin resistance had not been found linked to another resistance determinant. Initial investigations intended to identify mechanisms of gene flux resident on *mup*A plasmids revealed a family of related *mup*A plasmids, the p3356 family which includes three plasmid types: p3356, p3356D and p3358.

p3356 contains a single copy *mup*A flanked by direct repeats of the staphylococcal insertion sequence IS*257*. p3356D is identical to p3356 except for the duplication of a "*mup*A-IS*257*" cassette in tandem repeat. p3358 is related to p3356D by the insertion of a pT181-like plasmid (tetracycline resistant) accompanied by the duplication of an IS*257* in direct repeat to flank the inserted pT181; thus p3358 is the first documented example of linked resistance between mupirocin and another resistance determinant, namely tetracycline.

IS*257* has been implicated as the recombinogenic site in the gene duplication event involved in the evolution of p3356D from p3356. IS*257* co-integrative transposition has been demonstrated to allow the co-integration of pOX7 with p3356 to generate a p3358-type plasmid in which pT181 is replaced by pOX7. Therefore, it is concluded that IS*257* transposition and recombination is a mechanism by which staphylococcal replicons can evolve to form multiply resistant replicons.

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Abbreviations

International units of measurement and biology are used throughout.

Abbreviations for antibiotics and antibacterial agents are:
Ap: ampicillin; As: Arsenate; Cd: cadmium; Cm: chloramphenicol; Em: erythromycin; EtBr: ethidium bromide; Fd: fusidic acid; Gm: gentamicin; Hg: mercury; Mu: mupirocin; Pc: penicillin; Rp: rifampicin; Sb: antimony; Tc: tetracycline; Tm: trimethroprim. x^R denotes resistance to the substance *x*.(N.B. Occasionally M and T refer to mupirocin and tetracycline.)

Genes referred to are: MLS: macrolide/lincosamide/streptogramin resistance
amp: ampicillin resistance; *erm*: erythromycin resistance; *mec*: methicillin resistance; *mup*A: mupirocin resistance; *ori*: origin of replicatio; *rec*: the entire homologous recombination gene complement; *rec*A: homologous recombination protein gene A; *tet*: tetracycline resistance.
ori : origin of replication
Restriction enzyme sites are given in the figure legends.

Other abbreviations used are:

bp: base pair
chr: chromosomal
dH_2O: distilled water
DR: direct repeat
Fig. figure
h: hour
IC_{50}: 50% inhibitory concentration
IRS: isoleucyl tRNA synthetase
IS: insertion sequence
kb: kilobase
MIC: minimum inhibitory concentration
min: minute
no.: number
OD_x: optical density at x nanometers wavelength
ORF: open reading frame
'phage: bacteriophage
RepC: protein for the initiation of replication of pT181
rpm: revolutions per minute
Tn: transposon
ts: temperature sensitive
vol: volume
::: co-integrate with

A black box always refers to the mupirocin resistance gene; a hatched box arrow always refers to IS*257*, pointing in the direction of the ORF.

ethicillin resistant
standard convention.

Introduction

Chapter One

Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a non-motile, Gram positive, facultatively anaerobic bacterium of the family of *Micrococcaceae*. Its name perfectly describes the appearance of these bacteria: grape-like clusters of spherical cells with a golden hue,[1] the clustering is due to the coagulase produced by this species of staphylococci, absent from coagulase negative strains of staphylococci.

S.aureus has a single circular chromosome, which has been mapped for laboratory strain NCTC8325 and is approximately 2.78 x10^3kb; the prophage and plasmid population is strain dependent. The plasmids found in *S.aureus* have been classified into four groups. Class I plasmids are small, multicopy plasmids (15-60 per cell), ranging in size from 1-5kb and often carrying a single resistance determinant, (e.g. pT181, Tc^R; pE194, Em^R) and replicating via a rolling circle mechanism. Class II plasmids are slightly larger, ranging from 15-30kb, with a lower copy number than Class I , 4-6 per cell, often carrying one or more resistance determinants but replicating via the theta intermediate. Class III are larger still, 30-60kb, and harbour the *tra* gene for conjugative transfer as well as one or more resistance determinants, and also replicate via the theta intermediate. Class IV plasmids are the misfits not falling into categories I-III, and includes a number of plasmids which are "relaxed" rather than supercoiled. Plasmids similar to *S.aureus* plasmids are found in coagulase negative staphlyococci. (For a review see Novick, 1989.)

Although normally a commensal organism, *Staphylococcus aureus* is a potential pathogen, and can lead to infection particularly in the immunocompromised. Staphylococci are

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[1] The intensity of the colouring is dependent upon both the strain and the conditions of growth, indeed some laboratory strains have lost this colouring and appear white, e.g.RN4220.

responsible for 20% of nosocomial infections and c.1200 fatalities per year in the UK (Borriello, 1994). The main resident site of *S.aureus* on normal, healthy individuals is the anterior nares, which acts as a reservoir for *S.aureus* in c.35% of the population; *S.aureus* is rarely found on the skin of normal healthy individuals. The main site of infection in non-hospitalised patients is the skin (Hoeger *et al*, 1992), such infections are more proliferate in eczema sufferers, which is thought to be due to access to subcutaneous tissue within the eczema lesions where micoflora are more common than on intact skin. Infections of the skin are both localised subcutaneous infections, e.g. boils, the superficial infection impetigo and more severe infections classified as scalded skin syndrome. Specific toxins produced by different strains of *S.aureus* can lead to specific infections, e.g. toxic shock syndrome, by TSST-1 and food poisoning by enterotoxin B. Deep-seated infections of *Staphylococcus aureus* occur most frequently in the hospitalised and immunocompromised. The route of access may be a natural lesion or a surgical wound, particularly if the wound contains a prosthetic instrument, e.g. a catheter, artificial bone, or heart valve. Such a deep seated infection can lead to endocarditis, septicaemia, pneumonia (particularly in the newborn using incubators[2]) and hence often fatality. (For reviews see Noble and White, 1983; Wheat *et al*, 1983).

1.2 Antibiotic resistance and *Staphylococcus aureus*

Antibiotic resistance is a great cause of concern both to the scientific and lay community (Neu, 1992; Lemonick, 1994) Antibiotic therapy against infections of *S.aureus* can be thwarted due to the many resistance determinants carried both on the chromosome and plasmids of various strains (for a review see Lyon and Skurray, 1987). Although *S.aureus* contains the occasional chromosomal mutation which confers resistance to an antibiotic (e.g. N^6 dimethylation of the 23S ribosomal rRNA, leading to resistance to the macrolides, lincosamides and streptogramins; mutation of DNA gyrase to give resistance to quinolones (Nakanashi *et al*, 1991; Cambau and Gutmann, 1993); and, mutation of the elongation factor-G gene to reduce its affinity for fusidic acid), by far the majority of resistance is due to the

[2] Coagulase negative staphylococci are becoming increasingly significant in such pneumonia

product of an additional gene, considered to have evolved elsewhere in the bacterial population, and "hi-jacked" by *S.aureus*. Such resistance determinants can be either chromosomally encoded (e.g. methicillin resistance, *mec*, Gillespie *et al*, 1986) or be plasmid-bo rne (e.g. tetracycline resistance, Iordanescu *et al*, 1978). *S.aureus* also carries resistance determinants to inorganic ions (Silver and Laddaga, 1990), intercalating agents (Sasatsu *et al*, 1992), quaternary ammonium salts and antiseptics (Littlejohn *et al*, 1990).

Moreover, *S.aureus* appears to have a propensity to gather resistance determinants producing multiply resistant strains (MRSA) which are a great clinical threat and have often caused epidemics (Gillespie and Skurray, 1986; Maple *et al*, 1989). Chromosomally encoded resistances include methicillin (*mec*), tetracycline (*tmn*), MLS, macrolide / lincosamide / streptogramin, erythromycin and spectinomycin, and gentamicin (in European strains). Several plasmids which harbour many resistance determinants are known in *S.aureus*. Plasmid pUW3626, a 54.6kb plasmid, confers resistance to the aminoglycosides (tobramycin, kanamycin, neomycin and gentamicin), the intercalating agents (acriflavine and ethidium bromide), quaternary ammonium compounds, and penicillins (Gillespie and Skurray, 1986); this plasmid belongs to a family of related plasmids (pSK1 family) which includes pSK4 which also encodes resistance to trimethroprim, but not neomycin. Plasmid pI258 belongs to a different family of related plasmids, it harbours the resistance determinants to mercury, cadmium, penicillins, arsenate, antimony and MLS (Novick *et al*, 1979). Small plasmids, encoding only one resistance determinant, are also found in MRSA strains, e.g. the chloramphenicol resistance plasmids pSK2, and pC221 (Projan and Novick, 1988). The presence of a few such plasmids in a strain with several resistance determinants on the chromosome, can produce a multiply resistant strain, susceptible to only one or two therapeutic antibiotics: in many cases, infections of such a strain can only be treated by the slightly toxic antibiotic vancomycin, an undesirable situation. Unfortunately, vancomycin resistance is proliferate in streptococci (Uttley *et al*, 1989), due to its existence on a Class I type transposon, Tn*1546* (Arthur and Courvalin, 1993; Arthur *et al*, 1993); this is of particular concern as it is expressed in *S.aureus* to which it has been shown to transfer by filter mating (Noble, 1992).

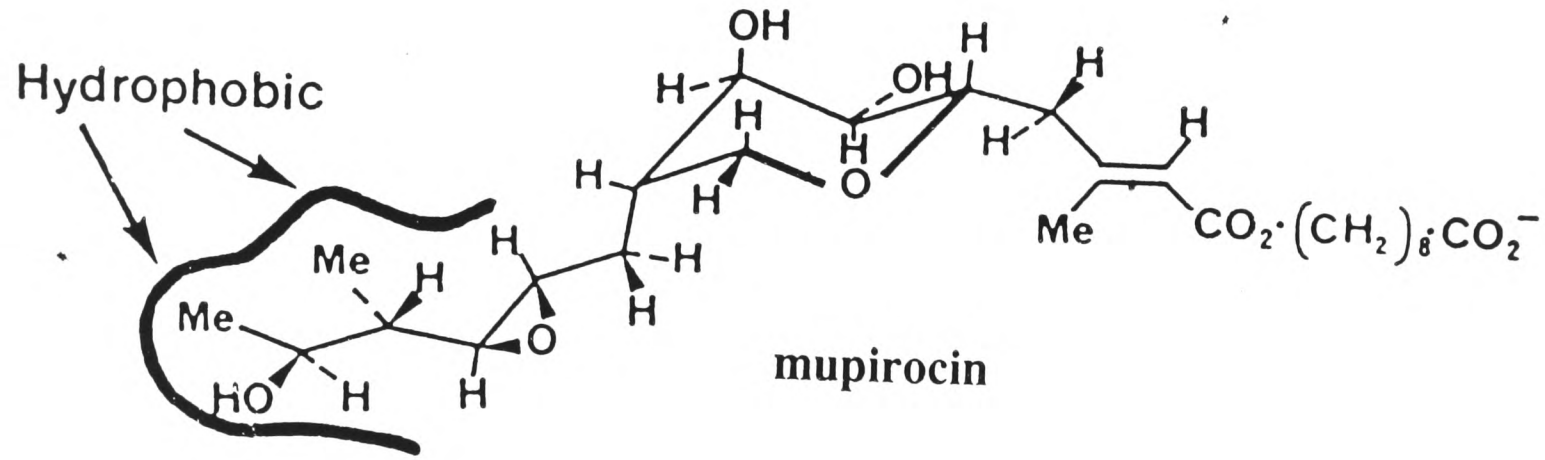
1.3 Mupirocin: a topical antibiotic

The introduction of mupirocin (Bactroban®, SmithKline Beecham) into clinical practice in the UK (1985) was heralded as a major advance in antibiotic therapy; indeed it merited the "Queen's Award for Industry".

Mupirocin (pseudomonic acid A) is a natural product of submerged fermentation of the soil bacterium *Pseudomonas fluorescens* (NCIB10586)(Fuller *et al*, 1971). It is the active component of Bactroban ointment and nasal preparations, present at 2% (Mellows, 1984). Mupirocin has a novel mode of action for an antibiotic and therefore it is not cross-resistant with other therapeutic antibacterial agents. Its uptake into the cell is by passive diffusion, upon entry it binds to the isoleucyl tRNA synthetase (Capobianco *et al*, 1989). It inhibits protein synthesis by reducing the cellular concentration of charged Ile-tRNA^{Ile}, which it achieves by competitive inhibition of isoleucyl tRNA synthetase (IRS) with respect to isoleucine (Fersht and Kaethner, 1976; Hughes and Mellows, 1978 and 1980). A comparison of the structures of mupirocin and isoleucine is given in Figure 1.1 from which it is easy to visualise how mupirocin may replace isoleucine in the active site of the IRS of *S.aureus*. *In vitro* studies using a mutant *E.coli* IRS, resistant to mupirocin suggest that this inhibition is bifunctional, displaying inhibition of both isoleucine and ATP binding (Yanagisawa *et al*, 1994); indeed, a *Methanobacterium thermoautotrophicum* mutant with only a single base change near the ATP-binding KMSKS motif is more effectively inhibited (x10) by mupirocin than its native counterpart, and this inhibition affects both isoleucine and ATP binding (Jenal *et al*, 1991). The only other compound known to inhibit this reaction is furanomycin, which is not suitable for clinical application (Tanaka *et al*, 1969, Kohno *et al*, 1990). In clinical trials, mupirocin was shown to be highly effective against most Gram-positive cocci (staphylococci and streptococci, except *Enterococcus faecalis*), but ineffective against many Gram-negative bacilli, although it was effective against *Haemophilus influenzae* and *Neisseria* spp. (White *et al*, 1984). Mupirocin was equally effective against coagulase negative staphylococci as *S.aureus*, including MRSA strains, and was bacteriocidal at concentrations exceeding 0.25μg ml^{-1}. The antibiotic was more active in slightly acidic conditions (pH6) than at alkaline pH (8.0).

® Bactroban is a SmithKline Beecham registered trademark

Figure 1.1 The structure of mupirocin resembles that of isoleucine



The structures of mupirocin and isoleucine are shown , with a sketch line to demonstrate the potential active site interface. (Taken from Mellows, 1984.)

As mupirocin has a narrow antibacterial spectrum, is most active at pHs resembling those found on the skin and is degraded rapidly when used systemically, it has been exploited for its ideal clinical use as a topical antibiotic against Gram positive cocci infections, e.g. *S.aureus*. Initial studies, using the ointment on the site of skin infection, were successful in the eradication of many Gram positive species from the infected area (Wuite *et al*, 1983). As infections of S.aureus can be so difficult to treat, as well as antibiotic therapy against a present infection, topical antibiotics are often used prophylactically, to prevent the acquisition of a nosocomial infection and the spread of an epidemic. Initial studies of nasal application of mupirocin both to eradicate a strain of MRSA and prevent its further spread were successful (Dacre *et al*, 1983).

Approximately forty percent of the population are healthy carriers of *S.aureus*.

Carriers of *S.aureus* are known to be more susceptible to infection with *S.aureus* when immunocompromised than non-carriers. The strain of *S.aureus* isolated from the site of infection is often the same strain as that found in the anterior nares of the patient. Therefore one recommended use of a topical antibiotic is to eradicate bacteria from the anterior nares prior to treatment. Intranasal application of mupirocin has been successful in the reduction of infection in patients undergoing haemodialysis (Boelart *et al*, 1993) and continuous ambulatory peritoneal dialysis (Perez-Fontan *et al*, 1993). Generalised infection of patients with severe burn injuries has also been reduced by this method (Mackie *et al*, 1994). Another approach is to prevent the "nose-hand-hand-etc" spread via health care workers, thus controlling the potential spread of an infection within a hospital (Reagan *et al*, 1991); such cases have been successful when used in response to a particular infection (Doebbeling *et al*, 1993 and 1994). (For a review of the prophylactic use of mupirocin through intranasal application see Hudson, 1994.)

1.4 Mupirocin resistance

Within two years of the introduction of mupirocin into clinical practice in the UK, resistant strains (MIC>512μg ml^{-1}) of *S.aureus* were documented (Baird and Coia, 1987; Kavi *et al*, 1987; Rahman *et al*, 1987; Smith *et al*, 1987). Since then, more strains of both coagulase positive and negative staphylococci with high-level resistance to mupirocin (MIC >512μg

ml^{-1}) have been reported (Cookson *et al*, 1990; Connolly *et al*, 1993); in addition a lower level of resistance has been observed (MIC >8, <512μg ml^{-1}), named intermediate resistance (Rahman *et al*, 1993). Sensitive strains are susceptible to 2μg mupirocin ml^{-1}. *In vitro* studies had demonstrated the slow emergence of a low level of resistance to mupirocin when *S.aureus* were subcultured in mupirocin (40μg ml^{-1}), but this did not exceed 40 μg ml^{-1} (Casewell and Hill, 1984). The isoleucyl tRNA synthetase (IRS) of the *Pseudomonas fluorescens* NCIB 10586, has a much lower affinity for mupirocin than the IRS *E.coli*, and thus escapes the antibacterial action of its fermentation product.

Intermediate-level resistance is thought to be due to spontaneous point mutations of the native IRS. Point mutations in and near the KMSKS-motif of the native IRS of *Methanobacterium thermoautotrophicum* and *E.coli* IRSs show increased inhibition of isoleucine and ATP binding by mupirocin (Jenal *et al*, 1991; Yanagisawa *et al*, 1994). Screening of intermediate-resistant *S.aureus* strains of staphylococci has identified two mutations in the KMSKS region of the native IRS which differ from those observed in *E.coli* and *M.thermoautotrophicum*, and are thought to be involved in the resistance; the details of these mutations are not available for publication (J.Ward, personal communication).(Fig.1.2)

High-level resistance appeared to be associated with a plasmid-bo rne gene, found in all high-level resistant strains (Rahman *et al*, 1990). Many of these plasmids appear to be conjugative, and are transferred between strains of *S.aureus* at high frequency in filter mating experiments, the high-level mupirocin resistance transferred with the plasmid (Rahman *et al*, 1989). The plasmids had very different *Eco*RI restriction patterns (Rahman *et al*, 1990). The gene encoding high-level mupirocin resistance, *mup*A, was cloned on a 4.05kb *Eco*RI fragment (Dyke *et al*, 1991), a fragment found to be common to most *mup*A plasmids (Rahman *et al*, 1993), found on plasmids from coagulase negative staphylococci as well as *S.aureus* including plasmids isolated in Nigeria as early as 1965 (Connolly et al, 1993; Rahman *et al*, 1993).

The *mup*A gene has been sequenced and encodes an isoleucyl tRNA synthetase (*ile*S), the amino acid code of which is given in Fig.1.2 (Dyke *et al*, 1991; Hodgson *et al*, 1994). High-

Figure 1.2 The amino acid sequence of *mup*A-isoleucyl tRNA synthetase

MTKKYLNTQNEISAFWNTQKIFKKSIDNRKGQESFVFY
DGPPTANGLP**HAGH**VLGRVIKDLVARLKTMQGFYVERK
A**GWD**THGLPVELEVEKKIGIKGKQDIEKYGIENFINEC
KKSVFNYEKEWRDFSKDLGYWVDMDSPYITLENNYIES
VWNILSTFHKKGLLYKGHKVTPYCTHDQTALSSHEVAQ
GYKNVKDLSAVVKFQLTNSKDTYFLSWTTTPWTLPANV
ALAINKDLNYSKIRVENEYYILATDLINSIITEKYEII
DTFSGSNLINLKYIPPFESDGLVNAYYVVDGEFVTNSE
GTGIVHIAPAHGEDDYQLVLERDLDFLNVITREGVYND
RFPELVGNKAKNSDIEIIKLLSKKQLLYKKQKYEHNYP
HCWRCGNPLIYYAMEGWFIKTTNFKNEIINNNNNIEWF
PSHIKEGRMGNFLENMVDWNIGRNRYWGTPLNVWI**CND**
CNHEYAPSSIKDLQNNSINKIDEDIELHRPYVDNITLS
CPKCNGKMSRVEEVIDVWFDSGSMPFAQHHYPFDNQKI
FNQHFPADFIAEGVDQTRGWFYSLLVISTILKGKSSYK
RALSLG***H***ILDSN***G***K**KMSKS**KGNVINPTELINKYGADSL
RWALISDSAPWNNKRFSENIVAQTKSKFIDTLDNIYKF
YNMYNKIDHYNPNNEITKSRNTLDNWALSRLNTLIKES
NIYVNNYDFTSAARLINEYTNTISNWYIGDSRGRFWEQ
GISNDKKDAYNTLYEILTTLSRLVAPFVPFISEKIHYN
LTGKSVHLQDYPQYKESFINQALEDEMHTVIKIVELSR
QARKNADLKIKQPLSKMVIKPNSQLNLSFLPNYYSIIK
DELNIKNIELTDNINDYITYELKLNFSSVGPKLGNKTK
NIQTLIDSLSEYDKKSLIESNNFKSLSSDAELTKDDFI
IKTLPKDSYQLSEDNDCVILLDKNLSPELIREGHAREL
IRLIQQLRKKKNLPINQRIDIYIGVTGELLESIKTNKN
MFKENFVIKNIHLNVIDEYENTIHFNNKEIKISLLYZ

The consensus motif sequences are highlighted in bold and underlined. The amino acids which when mutated were found to decrease the IRS's affinity for mupirocin (in *E.coli* and *M.thermoautotrophicum)* are given in bold and italics.

level mupirocin resistant strains were shown to contain two proteins with isoleucyl-tRNA synthetase (IRS) activity separated by ion-exchange affinity chromatography, one with a low 50% inhibitory concentration of mupirocin, IC_{50} (0.7-3.0ng ml^{-1}), thought to be the native *S.aureus* host enzyme; the other IRS had a high IC_{50} (7-10µg ml^{-1}) and is thought to be the product of *mup*A. Intermediate resistant strains however contained only one IRS protein with an IC_{50} of 19-43ng ml^{-1} (Gilbart *et al*, 1993), considered to be a slightly mutated version of the native host IRS. Therefore, the different levels of resistance appear to be due to two very different mechanisms: the intermediate level of resistance is caused by a mutation to the host IRS which slightly reduces its susceptibility to mupirocin; the high-level resistant strains maintain an unaltered host IRS, but have acquired a second IRS, the product of *mup*A, which is not susceptible to mupirocin. The origins of this second IRS gene, *mup*A, are the cause of much speculation: sequence homology searches alone distinguish little between archaebacteria, yeast, and *S.aureus* itself, ranging from 50-58% similarity and 27-36% identity (Hodgson *et al*, 1994); searches for known aminoacyl tRNA synthetase motifs, e.g. HIGH and KMSKS consensus sequences (Fig.1.2), which are involved in amino acid activation and ATP hydrolysis, demonstrate this second IRS to be a Class I aminoacyl tRNA synthetase (Moras, 1992; Delarue and Moras, 1993); the GWD motif is involved in isoleucine binding (Fig.1.2), whilst the presence and N-terminal location of the two CxxC Zn^{2+} binding motifs (Fig.1.2) indicate that it is more closely related to the archaebacterial enzyme than Gram negative bacterial, yeast or even *S.aureus* enzymes which have this motif in the C-terminal region (Chalker *et al*, 1994).

Intermediate-level resistant staphylococci can be effectively treated with mupirocin (Hudson, 1994). Strains harbouring the high-level resistance gene, *mup*A, however are clinically resistant to mupirocin, and the cause of the reports of resistance which began in 1987. As high-level resistance is the only one which causes clinical resistance in terms of treatment, hereafter, mupirocin resistance will only refer to the high-level resistance mediated by *mup*A gene product. The incidence of mupirocin resistance is low, the most recent national survey showed that of isolates taken in hospitals, only 0.3% *S.aureus* and 3% coagulase negative staphylococci were resistant to mupirocin (Cookson *et al*, 1990). However, mupirocin resistance has been observed in a retrospective study of methicillin-resistant MRSA which caused an epidemic in Western Australia in 1987 (Udo *et al*, 1994), and also more recently

in a strain isolate in the UK which had previously been sensitive to mupirocin (Dawson *et al*, 1994). Moreover, the incidence of mupirocin resistant strains of *S.aureus* in long-term care facilities have been reported as much higher than in the normal population. The use of mupirocin to decrease colonisation of methicillin resistant MRSA of the nares and wounds was successful at eradicating many infections at first but led to a patient harbouring mupirocin resistant MRSA after long-term treatment in a Veterans Affairs facility (Kauffman *et al*, 1993)[3]. Long-term use of mupirocin to reduce infections in eczema lesions of children in a specialist school in the UK led to high incidence of three strains of *S.aureus* resistant to mupirocin in staff and untreated children as well as the eczema sufferers (89% of children with eczema, 65% of children without eczema and 5% of staff harboured a mupirocin resistant strain) (Chatfield *et al*, 1994). Such reports have led to the general opinion that mupirocin ought not to be used as a long term measure in the eradication and prevention of spread of *S.aureus* infection, but rather should be reserved for effective measures against the outbreak of occasional infections (Bradley, 1993; Hudson, 1994; Muder, 1994).

1.5 Tetracycline resistance

Tetracycline is a broad range antibiotic, active against both Gram positive and Gram negative bacteria, used systemically in clinical application. It acts by binding to the 30S ribosomal subunit, which sterically hinders the transfer of aminoacyl tRNAs to the ribosome, thus inhibiting protein synthesis with a bacteriostatic effect. (For a review see Foster, 1983.) Tetracycline resistance in *S.aureus* has both inducible and constitutive forms (Chopra *et al*, 1974). The constitutive resistance is active against both tetracycline and the semi-synthetic form, minocycline and is chromosomally encoded at the *tmn* locus (Asheshov, 1975); the mechanism of resistance mediated by the *tmn* locus is unknown. Inducible resistance is usually plasmid bo rne, on one of several of a family of small, Class I plasmids (Iordanescu, *et al* 1978). One of these plasmids, pT181, has been particularly well characterised. It encodes the tetracycline resistance determinant, *tet*K, which has been sequenced (Khan and Novick, 1983). Analysis of a slightly corrected sequence of *tet*K indicates that the protein

[3] Initial studies suggested that this rate was higher, at 10.8% of patients in the unit, but further analysis showed that only one of these was a high-level mupirocin resistant strains.

it encodes contains 14 transmembrane helices and is highly related to other tetracycline resistance products (Guay *et al*, 1993). This homology extends also to other resistance genes, (e.g. *qac*A, the resistance determinant to intercalating dyes and quaternary ammonium compounds) and to sugar transport proteins, which act via energy-dependent export mechanisms (Rouch *et al*, 1990; Paulsen and Skurray, 1993); thus *tet*K is assumed to mediate resistance to tetracycline by providing a membrane protein which allows an energy-dependent efflux of intracellular tetracycline (Guay *et al*, 1993). Although these small plasmids are normally extrachromosomal, pT181 has been found integrated into the chromosome, at a site distinct from *tmn* (Gillespie *et al*, 1986).

1.6 Genetic flux in *Staphylococcus aureus*

The existence of multiply resistant *S.aureus* is no doubt the result of inter- and intra-specific genetic transfer, via conjugation, transduction and transformation and intracellular linkage mechanisms, via both homologous and site specific recombination. (For a review of genetic flux see Amabile-Cuevas and Chicurel, 1992.) Little is known as to the significance of transformation *in vivo*, but should it be an active mechanism for genetic transfer, it is most likely to occur early in the exponential growth prior to the production of the variety of extracellular proteins. Transduction was the first observed mechanism of genetic flux in staphylococci. Many strains of *S.aureus* carry multiple lysogenised 'phage and 'phage typing is a common mechanism for classification of *S.aureus*. The 'phage packing size limits transfer to pieces of DNA smaller than 40kb.

Conjugation is another mechanism for intercellular transfer of plasmids. (For a review see Archer and Thomas, 1990.) The conjugative genes are encoded on Class III plasmids, e.g. pGO1 and pSK41, which also carry resistance determinants (Thomas and Archer, 1989). The main region required for conjugation (*tra/trs*) has been cloned and sequenced (Firth 1993; Morton *et al*, 1993) and encodes 15 genes including its own transcriptional regulator (Sharma *et al*, 1994); there is a second smaller region required in addition to the main operon which has been identified on pJE1 (Evans and Dyke, 1988). The mechanism of transfer is not known, however it appears that, unlike for *E.coli* the formation of pili is not required.

However, one of the gene products of *tra*(H) encodes a protein which acts as a pheromone in *Enterococcus faecalis* (Firth *et al*, 1994) and thus the mechanism of conjugation in *S.aureus* may be found to share elements of the sex-pheromone induced conjugation of *E.faecalis* (reviewed in Clewell, 1993). Indeed there are many examples of transfer of plasmids from *E.faecalis* to *S.aureus* (Engel *et al*, 1980; Jones *et al*, 1987; Noble *et al*, 1992). Conjugative plasmids are able to mobilise small staphylococcal plasmids which carry the *mob* genes for plasmid relaxation, e.g. pC221 (Projan and Archer, 1989). Mobilisation of chromosomal genes has been demonstrated by a mechanism involving co-integrate formation facilitated by Tn*551* (Stout and Iandolo, 1990). The phenomenon of phage-mediated conjugation has been seen in *S.aureus*, but this is poorly understood (Lacey, 1980).

Intracellular genetic recombination can lead to the rearrangement of DNA. Many plasmid families have been observed in *S.aureus*. The members of the families are often identical but for the presence or absence of one or more sections of DNA. Analyses of these families conclude that these families have evolved from common ancestors and that various recombination events have been mechanistic in this evolution. Generalised homologous recombination is well characterised for *E.coli* (for a review see Kowalczykowski *et al*, 1994), however, generalised homologous recombination is poorly understood in *S.aureus*; mutants deficient for homologous recombination have been identified (Wyman *et al*, 1974), but it is only recently that the *rec*A gene has been cloned and shown to be homologous to *rec*A of *Bacillus subtilis* (Bayles *et al*, 1994). Generalised homologous recombination is involved in the phage-mediated recombination between small staphylococcal plasmids at the RS_A and RS_B sites (Novick *et al*, 1984). Site-specific recombinases have been found at various plasmid sites. The pI524 plasmid family has two recombinase genes (*bin*R and *bin*L), flanking the invertible region (Rowland and Dyke, 1989). Another potential recombinase, encoded by *sin*, is present on plasmid pS1 parent as well as members of the pSK1 family (Paulsen *et al*, 1994).

Transposition is another specialised form of site-specific recombination. There are three well characterised non-composite transposons in *S.aureus*: Tn*551*, which harbours an erythromycin resistance gene, is identical to Tn*917* of *Enterococcus faecalis*, and has been demonstrated to be active for transposition (Novick *et al*, 1979), Tn*552* (alias Tn*4002*) which expresses

resistance to the β-lactams (Dyke and Rowland, 1990) and Tn*554*, which harbours MLS resistance and differs from Tn*551* and Tn*552* in that it only integrates into one specific site in the chromosome and does not cause a target site duplication (Murphy and Lofdahl, 1984). Several composite transposons have been proposed in *S.aureus*, although only Tn*4001*, flanked by inverted repeats of IS*256* and expressing resistance to gentamicin, kanamycin and tobramycin, has been demonstrated to be active for transposition (Lyon *et al*, 1984). Tn*4003*, a potential trimethroprim resistance transposon (Rouch *et al*, 1989), Tn*4004*, a potential mercury resistance transposon (Gillespie *et al*, 1987) and *tra*, the conjugative transposon (Firth *et al*, 1993) have been designated composite transposons by virtue of the IS*257*s which flank the resistance determinants in direct repeat.

IS*256* has never been shown to transpose independently, but the diverse locations of its multiple copies on the chromosomes of many clinical isolates of *S.aureus* suggest that it is able to do so (Dyke *et al*, 1992). IS*257* is a putative insertion sequence, sometimes referred to as IS*431* (Barberis-Maino *et al*, 1987) which has not been shown to transpose either independently or as part of a composite transposon although its structure and putative transposase ORF suggest that it ought to be capable of transposition (Fig.1.3). It occurs at many, diverse locations of plasmids and chromosomes of *S.aureus* and coagulase negative staphylococci (Byrne *et al*, 1990; Gillespie *et al*, 1986; Schwarz, personal communication). Comparison of the structure of plasmids containing IS*257* with other family members, suggests that IS*257* has been active in the evolution of these plasmids, either by transposition or by recombination leading to co-integrate formation (Byrne *et al*, 1990 and 1991; Leelaporn *et al*, 1994); indeed, IS*257* has been implicated in the integration of plasmids into the chromosome by recombination (Gillespie *et al*, 1986; Stewart *et al*, 1994). A third insertion element of *S.aureus*, IS*1181*, has recently been isolated from three distinct regions of the chromosome (Derbise *et al*, 1994). It is distinct from both IS*256* and IS*257* and appeared in multiple copies of the chromosome of many strains tested. (For a review of transposable elements in *S.aureus*, see Murphy, 1989.) As well as being active in transposition insertion sequences have the potential to act as sites of homologous recombination, in fact this was thought to be the role of IS*257* in the formation of co-integrates (Galas and Chandler, 1989).

Figure 1.3 Sequence of IS257L

GGTTCTGTTGCAAAGTTGAATTTATAGTATAATTTTAACAAAAAGGAGTCTTCTGTATGAACTATTTCAGATATAAACAA 80

TTTAACAAGGATGTTATCACTGTAGCCGTTGGCTACTATCTAAGATATGCATTGAGTTATCGTGATATATCTGAAATATT 160

AAGGGGACGTGGTGTAAACGTTCATCATTCAACGGTCTACCGTTGGGTTCAAGAATATGCCCCAATTTTATATCAAATTT 240

GGAAGAAAAAGCATAAAAAAGCTTATTACAAATGGCGTATTGATGAGACGTACATCAAAATAAAAGGAAAATGGAGCTAT 320

TTATATCGTGCCATTGATGCAGAGGGACATACATTAGATATTTGGTTGCGTAAGCAACGAGATAATCATTCAGCATATGC 400

GTTTATTAAACGTCTCATTAAACAATTTGGTAAACCTCAAAAGGTAATTACAGATCAGGCACCTTCAACGAAGGTAGCAA 480

TGGCTAAAGTAATTAAAGCTTTTAAACTTAAACCTGACTGCCATTGTACATCGAAATATCTGAATAACCTCATTGAGCAA 560

GATCACCGTCATATTAAAGTAAGAAAGACAAGGTATCAAAGTATCAATACAGCAAAGAATACTTTAAAAGGTATTGAATG 640

TATTTACGCTCTATATAAAAAGAACCGCAGGTCTCTTCAGATCTACGGATTTTCGCCATGCCACGAAATTAGCATCATGC 720

TAGCAAGTTAAGCGAACACTGACATGATAAATTAGTGGTTAG**CTATATTTTTTACTTTGCAACAGAACC** 789

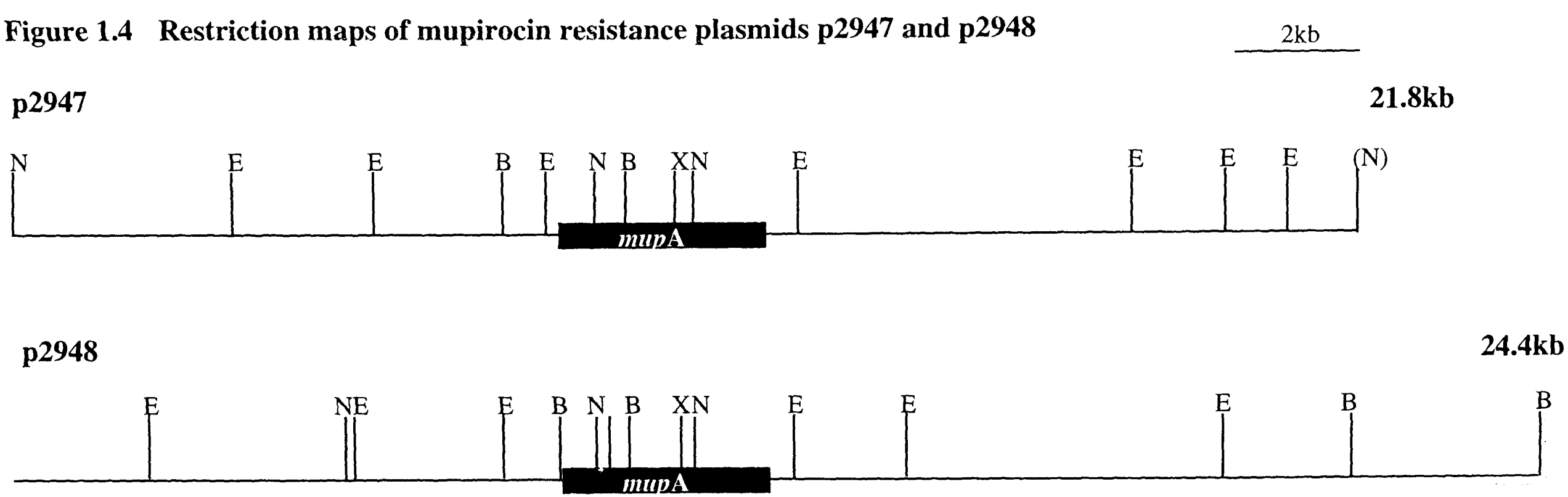
The sequence of IS257L is given (Byrne *et al*, 1990). The terminal inverted repeats are highlighted in bold; the open reading frame of the putative transposase is printed in italics; the promoter sequences are underlined, and the Shine-Dalgarno sequence is double underlined.

1.7 Scope of the research proposal

The emergence of mupirocin resistance provides an ideal opportunity to follow the molecular epidemiology of a resistance determinant within the genome of *S.aureus*. Studies on the evolution of MRSA have been retrospective and conducted many years after the antibiotic has been released into clinical practice. Mupirocin is a recent addition to the range of clinical antibiotics and, although resistance determinants were present in the plasmid population prior to its clinical application, the effect of the use of mupirocin can be followed by monitoring changes in the genomes of the mupirocin resistance population from the inception of its application. Knowledge of the rate and mech nisms of spread of the resistance gene may be gained from such a study; this is important not only for appropriate guidelines for the clinical application of mupirocin, but also for predictions as to the potential for the spread of resistance to any possible variant of mupirocin.

To date, the resistance gene *mup*A has always been found to be plasmid-bo rne, which would allow rapid dissemination of the gene both to other sites within the cell or in horizontal transfer, either inter or intraspecifically. That the gene was located on plasmids of varied restriction patterns suggested that it may be resident on a transposable element, which has spread amongst different plasmids, but not demonstrably to the chromosome. That these plasmids were large and transferred by filter-mating at high frequencies suggested that they were conjugative. The known mechanisms for transposition and conjugation in *S.aureus* are outlined above. The initial remit of this work was to define the nature of *mup*A plasmids particularly to seek evidence of potential mechanisms of genetic transfer and thus the propensity of *mup*A to spread around the genome of *S.aureus*. Two mupirocin resistance plasmids had been sketchily mapped (Fig.1.4), and the sequence of *mup*A obtained from one of them by other workers prior to the onset of these studies. Investigation of several *mup*A plasmids identified a family of plasmids, the first related *mup*A plasmids observed. This family was characterised and the possible mechanisms leading to its evolution probed further.

Figure 1.4 Restriction maps of mupirocin resistance plasmids p2947 and p2948



Plasmids p2947 and p2948 as mapped in the laboratory previous to the commencement of these studies. Restriction enzyme sites given are: B-*Bgl*II, E=*Eco*RI, N=*Nco*I and X=*Xho*I. The 4.05kb *Eco*RI fragment of p2947 had been sequenced, revealing the *Xho*I site and several others not shown.

Materials and Methods

Chapter Two

Materials and Methods

2.1 Chemicals

2.1.1 General

A.R. grade and Molecular Biology grade chemicals were used throughout. General chemicals were obtained from Sigma (Dorset), BDH (Poole), and Oxoid (Basingstoke).

2.1.2 Antibiotics

All antibiotics were obtained from Sigma (Dorset) except for mupirocin which was donated by SmithKline Beecham Pharmaceuticals (Brockham Park, Surrey).

2.1.3 Radioactivity

[α^{35}S] dATPαS (>1000 Ci/mmol) was obtained from Amersham International plc (Aylesbury).

2.1.4 Restriction endonucleases

Restriction enzymes were obtained with appropriate buffers from Amersham International (Aylesbury), BDH (Poole), Gibco Bethesda Research Laboratories (Cambridge), New England Biolabs (Bishops Stortford) or Northumbria Biologicals (Cramlington).

2.1.5 Other enzymes

Lysostaphin, lysozyme (grade 1) and bovine serum albumin (BSA) were obtained from Sigma (Dorset). T4 DNA ligase and Klenow fragment were obtained from Gibco BRL (Cambridge). Bovine pancreatic ribonuclease A and ribonuclease T1 (grade 3) were obtained from Boehringer-Mannheim.

2.1.6 Size markers

1kb ladder and High Molecular Weight Markers and 123bp ladder were obtained from Gibco BRL (Cambridge). Raoul ladder was obtained from Appligene (Co.Durham).

2.1.7 Oligonucleotides

Universal "forward" and "reverse" primers to sequence into the polylinker of pUC18 were purchased from USB (Amersham International, Aylesbury) and Promega (Southampton).

Designed oligonucleotides were synthesised by Valerie Cooper (Dyson Perrins, Oxford). The sequences of these oligonucleotides, 5'-3' are:

Name	Sequence	Sequence obtained
J1IS*257*	TAG-TTC-ATA-CAG-AAG-ACA-CC	Out of N-terminus of IS*257*
J2IS257	CAG-ATC-TAC-GGA-TTT-TCG-CC	Out of C-terminus of IS*257*
UPIN	GGG-TAT-TGT-AAA-TGT-TAG-G	Into upstream IS*257* of p3356
UPOUT	GCC-GAT-TCA-TTA-TTG-CTC-TCT-CC	Out of *mup*A *Eco*RI fragment into the sequences upstream
DNOUT	GGA-TGG-ATA-ACC-TAT-GTT-CTG	Out of *mup*A *Eco*RI fragment into the sequences downstream
DNIN	CGA-GAA-AAG-AGT-TAG-ATA-CG	Into downstream IS*257* of p3356
ISCN1	CGT-GCC-ATT-GAT-CCA-GAG-GG	Internal sequences of IS*257*
ISCN2	GCC-ATT-GCT-ACC-TTC-GTT-GC	Internal sequences of IS*257*
PT181REPC	CAG-TAT-GTG-CGT-CCA-ACC	Into IS*257* of pT181-IS from *rep*C
PT181ORI	GGA-GTC-GCT-CAC-GCC-CTG	Into IS*257* of pT181-IS from *ori*
$DR6_2POX7$	CGC-AAA-AGA-CAT-AAT-CG	Into IS*257* from pOX7 of 6_2
$DR6_5POX7$	GGG-TTG-CCA-GAG-TTA-AAG-G	Into IS*257* from pOX7 of 6_5

2.2 Media

2.2.1 Media

2xTY

Tryptone	16g
Yeast extract	10g
Sodium Chloride	5g

to one litre with dH_2O
pH7.0

2xHB

Sucrose	479.2g
Maleic acid	4.64g
Magnesium chloride (dihydrate)	8.13g

to one litre with dH_2O
pH6.5

CY

Yeast extract	10g
Casein hydrolysate	10g
Magnesium sulphate (heptahydrate)	0.2465g
Trace elements	40µl

to one litre with dH_2O, pH7.5

HBM

2xHB	500ml
Antibiotic Medium 3	35g

to one litre with dH_2O
(allowed to settle prior to use)

Trace elements

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

ψ-broth

Tryptone	20g
Yeast extract	5g
$MgSO_4.7H_2O$	4.93g
NaCl	0.585g
KCl	0.375g

to one litre with dH_2O
pH7.5

2xTY agar
as for broth with 1.5% agar

CY agar

Yeast extract	5g
Casein hydrolysate	5g
$MgSO_4.7H_2O$	0.2465g
Trace elements	40µl
agar	1.5%

to one litre with dH_2O, pH7.5

TBAB/HB overlay

2xHB	500ml
Tryptose blood agar base	16.5g

to one litre with dH_2O

G-min agar

10*x*M9 salt solution	100ml
1M $MgSO_4.7H_2O$	1ml
0.1M $CaCl_2$	1ml
1M thiamine HCl	1ml
20% glucose	10ml

10xM9 salt agar

Na_2HPO_4	60g
KH_2PO_4	30g
NH_4Cl	10g
NaCl	5g
agar	1.5%

to one litre with dH_2O

DM3 agar

1M sodium succinate, pH7.3	500ml
Yeast extract	5g
Casein hydrolysate	5g
agar	2%

to 850ml with dH_2O

once DM3 agar has cooled to 60°C add:

3.5% K_2HPO_4/1.5% KH_2PO_4	100ml
1M $MgCl_2.6H_2O$	20ml
20% glucose	25ml
BSA, 5%, filter sterilised	2ml

All media was stored at room temperature. Agar plates were stored at 4°C.

2.2.2 Additions to media

Antibiotic	**Stock concentration**	**Final concentration**
Ampicillin	50mg ml^{-1}	50µg ml^{-1}
Chloramphenicol	40mg ml^{-1}	40µg ml^{-1}
Erythromycin	20mg ml^{-1}	40µg ml^{-1}
Mupirocin	5mg ml^{-1} and 200mg ml^{-1}	5µg ml^{-1} and 200µg ml^{-1}
Tetracycline	10mg ml^{-1}	10µg ml^{-1}

for blue/white selection of β-galactosidase:

IPTG	40µg ml^{-1} final concentration
X-gal	2µl 2% (w/v) in DMF ml^{-1}
10M NaOH	1-2 drops

All stored at -20°C, except NaOH which is kept at room temperature.

2.3 Buffers and solutions

For "mini-prep" DNA:

Lysis buffer I		**Lysis buffer II**		**Lysis buffer III**	
0.5M EDTA, pH8.0	2ml	10% SDS	1ml	5M potassium acetate	60ml
1M Tris-HCl, pH8.0	2.5ml	2MNaOH	1ml	glacial acetic acid	11.5ml
20% glucose	5ml	to 10ml with dH_2O		to 100ml with dH_2O	
to 100ml with dH_2O		(freshly prepared)			

For large scale DNA preparations:

1xTE	
1M Tris HCl, pH8.0	1ml
0.5M EDTA, pH 8.0	0.2ml
to 100ml with dH_2O	

For electrophoresis:

10xTBE	
Tris HCl	108g
Boric Acid	55g
0.5M EDTA	40ml
to one litre with dH_2O, pH 8.0	

For electrophoresis:

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

For phenol/chloroform extraction:

Phenol/chloroform	
Phenol, equilibrated in TE	25ml
Chloroform	24ml
Isoamyl alcohol	1ml

For competent *E.coli*:

TFB-I	
KCl	30mM
RbCl	100mM
$MnCl_2$	50mM
$CaCl_2$	10mM
glycerol	15%(w/v)
pH5.8	

TFB-II	
RbCl	10mM
MOPS	10mM
$CaCl_2$	75mM
glycerol	15% (w/v)
pH6.8	

All stored at 4°C.

For transduction of *S.aureus*:

Phage-buffered medium	
Sodium β-glycerophosphate	18.9g
$MgSO_4.7H_2O$	0.25g
NaCl	4.96g
Gelatin	1g
to one litre with dH_2O	

CY-citrate	
CY broth	95ml
1M sodium citrate	5ml
Freshly prepared	

For Southern Blot:

Depurination buffer
0.25M HCl

Denaturation buffer
2M NaOH 250ml
5M NaCl 300ml
to one litre with dH_2O

Neutralisation buffer
1.5M Tris, pH7.5 667ml
5M NaCl 300ml
to one litre woth dH_2O

20xSSC
NaCl 175.3g
Sodium citrate 88.2g
to one litre with dH_2O
pH7.0

20xSSPE
NaCl 174g
NaH_2PO_4 27.6g
EDTA 7.4g
5M NaOH 13ml
to one litre with dH_2O
pH7.4

10xPBS
Na_2HPO_4 107g
NaH_2PO_4 13.9g
NaCl 58.44g
to one litre with dH_2O
pH7.4

For "Flash" detection:

Pre-hybridisation buffer
Deionised formamide 5ml
5M NaCl 2ml
10% SDS 1ml
50% dextran sulphate 2ml

Wash solution I
20xSSC 120ml
Tween-20 0.4ml
to one litre with dH_2O

Blocking buffer
Dissolve 2 capsules of blocking powder in 280ml dH_2O
add 20xSSC 120ml (solution clears) and 0.4ml Tween-20 (blocking powder provided).

1xSTET
5M NaCl 2ml
1M TrisHCl 2ml
EDTA 2ml
Tween-20 0.1ml
to 100ml with dH_2O

Wash solution II
10xPBS 100ml
Tween-20 3ml
to one litre with dH_2O

Conjugate buffer
10xPBS 25ml
to 250ml with dH_2O
add 1 capsule of casein and heat to 70°C for 10min.
Cool to room temperature
(casein provided in kit).

Assay Buffer
Diethanolamine 0.96ml
1M $MgCl_2$ 0.5ml
to 100ml with dH_2O
pH10 with HCl

For "ECL" detection:

Prehybridisation buffer
20xSSC 25ml
Blocking agent 0.5g
10% SDS 1ml
50% dextran sulphate 10ml
to one litre with dH_2O

Antibody wash buffer
1M Tris HCl, pH7.5 100ml
5M NaCl 300ml
to one litre with dH_2O

For sequencing:

Acrylamide mix (6%)
Urea 84g
10xTBE 20ml
to 170ml with dH_2O - water bath to dissolve
acrylamide, 40% 30ml

Fixer
Methanol 100ml
Acetic acid 100ml
to one litre with dH_2O

2.4 Bacterial strains and plasmids

Strains:	Properties	Reference
Escherichia coli		
JM107	*rec*A$^+$	Yanisch-Perron *et al*, 1985
JM109	*rec*A$^-$	Yanisch-Perron *et al*, 1985
DH5α	*rec*A$^-$	Hanahan, 1983.
Staphylococcus aureus		
NCTC8325	*rec*A$^+$	Iordanescu and Surdeanu, 1976.
RN4220	*rec*A$^+$, recipient of *E.coli* transformation	Novick, 1990.
RN981	*rec*A$^-$	Wyman *et al*, 1974.
Standard vectors		
JM107 (pUC18)	Ap^R, blue/white selection with X-gal	Yanisch-Perron *et al*, 1985.
JM107 (pOX7)	Ap^R, blue/white selection with X-gal	Yanisch-Perron *et al*, 1985.
RN4220 (pOX7)	Em^R, blue/white selection with X-gal shuttle vector *E.coli/S.aureus*	Dyke *et al*, 1991 (=pOX300).
DH5α (pMAK705)	Cm^R, blue/white selection with X-gal temperature sensitive.	Hamilton *et al*, 1989.
Antibiotic resistant strains and hybridisation controls		
8325 (pT181)	Tc^R	Khan and Novick, 1983.
RN4220 (pC194)	Cm^R	**Iordanescu *et al*., 1978**
8325 (pI258)	As^R, Cd^R, Hg^R, Pc^R, Sb^R	Novick *et al*, 1979.

Mupirocin resistant strains[1]		
8325 (p2710)	Mu^R, chr Rp^R, Fd^R	Rahman *et al*, 1990, Mu^R plasmid, strain 2.
8325 (p2795)	Mu^R, chr Rp^R, Fd^R	Rahman *et al*, 1990, Mu^R plasmid strain 1.
RN2677 (p2947)	Mu^R, chr Rp^R, Fd^R	Rahman *et al*, 1993, Mu^R plasmid strain 5.
RN2677 (p2948)	Mu^R, chr Rp^R, Fd^R	Rahman *et al*, 1993, Mu^R plasmid strain 8.
8325 (p2993)	Mu^R, chr Rp^R, Fd^R	Rahman *et al*, 1990, Mu^Rplasmid strain 13.
8325 (p3274)	Mu^R, chr Rp^R, Fd^R	Rahman *et al*, 1990, Mu^Rplasmid strain 18.
8325 (p3355)	Mu^R, chr Rp^R, Fd^R	Needham *et al*, 1994.
8325 (p3356)	Mu^R, chr Rp^R, Fd^R	Needham *et al*, 1994.
8325 (p3357)	Mu^R, chr Rp^R, Fd^R	Needham *et al*, 1994.
8325 (p3358)	Mu^R, Tc^R ,chr Rp^R, Fd^R	Needham *et al*, 1994.
Derivatives of mupirocin resistant strains		
RN4220 (p3356)	Mu^R	This study, chapter 4.
RN4220 (p3356D)	Mu^R	This study, chapter 4.
RN4220 (p3358)	Mu^R, Tc^R	This study, chapter 5.
RN4220 (pMT)	Mu^R, Tc^R	This study, chapter 5.
RN4220 (pT181-IS)	Tc^R	This study, chapter 5.
Clones of p3356		
JM107 (pCN1.1)	pOX7 clone, 1.6kb *Bgl*II	This study, chapter 3.
JM107 (pCN4.2)	pOX7 clone, 3.6kb *Bgl*II	This study, chapter 3.
JM107 (pCN7.1)	pUC18 clone, 5.2kb *Hin*dIII	This study, chapter 3.
JM107 (pCN11.1)	pUC18 clone, 5.2kb *Hpa*II/*Cla*I	This study, chapter 3.
DH5α (pID2.1)	pUC18 clone, 6.5kb *Eco*RV	This study, chapter 3.
DH5α (pRE6.1)	pUC18 clone, 6.3kb *Eco*RI	This study, chapter 3.
Clone of p3356D		
JM107 (pCN9.1)	pOX7 clone, 1.3kb *Eco*RI (=pOX-IS)	This study, chapter 4.
Clones of pMT		
DH5α (pMTN1.1)	pUC18 clone, pMT *Nhe*I	This study, chapter 6.
DH5α (pMTN2.1)	pUC18 clone, pMT *Nhe*I	This study, chapter 6.
Clones for hybridisation probes		
JM107 (pMZ2)	pOX7 clone, 1.6kb *Nco*I p2947	Dyke *et al*, 1991.
JM107 (pSK397.Δ*Xba*I)	pUC18 clone of *tra*	Gift from Prof.R.A. Skurray.
JM107 (pSK397.2.4)	pUC18 clone of *tra*	Gift from Prof.R.A. Skurray.
JM107 (pIP1551)	pUC18 clone 491bp of IS*256*	Dyke *et al*, 1992.
JM107 (pIP1644)	pUC18 clone 629bp of IS*257*	Gift from V.Loncle.

[1] The recipient strains for the mupirocin resistance plasmids had been made resistant to both rifampicin and fusidic acid for selection purposes.

2.5 Maintenance and growth of cultures

Long term storage was at -70°C in CY broth containing 30% glycerol. Short term (up to 1 month) storage was at 4°C on CY-agar for *S.aureus* strains and 2xTY-agar for *E.coli* strains, the agar contained the appropriate antibiotic except where otherwise stated in the text (e.g. mupirocin resistant strains were stored on antibiotic-free agar from an early date in the studies). Plates for short term storage were renewed each month.

Inoculation to agar or broth was with either a sterile toothpick or a sterilised wire loop, if the culture was from a plate or glycerol stock; liquid inocula were 100-200µl pipetted and spread with a glass spreader which had been sterilised in ethanol and flamed immediately prior to use. Liquid cultures were prepared in a sterile flask containing broth of 0.1-0.2xvolume of the flask, flasks were shaken vigorously unless otherwise stated. Cultures grown on agar plates were inverted for incubation. Incubation was at 30°C for strains harbouring temperature sensitive plasmids and otherwise at 37°C unless stated to the contrary.

2.6 Sterilisation

Solutions, media and plastic-ware were autoclaved at 121°C, 15 lb in^{-2} for 20min, except for solutions containing sugars which were autoclaved at the reduced pressure of 10 lb in^{-2}. Glassware was heated at 160°C for 1h. Solutions that would be destroyed by autoclaving were sterilised by passage through a "Millipore" filter with pore-size 0.45µm

2.7 Plasmid DNA Isolation

2.7.1 Rapid small scale preparation of *E.coli* plasmid DNA ("mini-prep")

This method is based on that of Birnboim and Doly (1979). 1.5ml of an overnight culture of cells in 2xTY was transferred to an Eppendorf tube and microfuged for 1min (12000rpm). The supernatant was removed and the pellet resuspended in 100µl ice-cold lysis solution I by vortexing. After a 5min incubation at room temperature, 200µl of lysis solution II was added and mixed by inversion, this was transferred to ice for 5min. 150µl of lysis solution III was then added, mixed by inversion and returned to ice to incubate for a further 5min. The cell debris was then pelleted by microfuging for 5min, and the supernatant was decanted into a fresh Eppendorf containing 400µl phenol/chloroform which was capped tightly and inverted to produce an emulsion and immediately microfuged for 3min. The upper aqueous DNA layer was removed by pipette and transferred to a fresh Eppendorf. 1ml ethanol was added and mixed by inversion, and the DNA precipitated at -20°C for 20min. The DNA was then pelleted by microfuging for 5min, the ethanol was decanted away and the Eppendorf left inverted to dry in the air. Pellets were then resuspended in 50µl 2mM Tris HCl pH8.

2.7.2 Rapid small scale preparation of *S.aureus* plasmid DNA ("mini-prep")

The method used to isolate plasmid DNA from *E.coli* was modified in the following ways:

a) The overnight culture was grown in CY broth,

b) Incubation with lysis buffer I was at 37°C for 45min and with 3µl of 10mg lysostaphin ml^{-1},

c) Incubations with lysis buffers II and III were for 15min each,

d) In place of phenol/chloroform, proteins were extracted using StrataResin: 12µl resin was added to the supernatant, mixed by inversion, left at room temperature for 2min and microfuged for 3min; the supernatant was transferred to a fresh Eppendorf by pipette and extracted for a second time with 8µl resin in the same manner,

e) Ethanol precipitation at -20°C was continued for 1h.

2.7.3 Quick preparation of plasmid DNA using the "Wizard" kit

The "Wizard Quick Prep" kit produced by Promega (Southampton) was employed as according to the manufacturer's instruction for plasmids isolated from *E.coli*. The kit was also used to purify plasmid DNA from *S.aureus*, with the modification of the addition of 3µl 10mg lysostaphin ml^{-1} to the lysis buffer, and incubation at 37°C for 15min at this stage.

2.7.4 Large scale plasmid preparation from *E.coli* by caesium chloride centrifugation (based on the method of Birnboim and Doly, 1979)

100ml of an overnight culture in 2xTY was transferred to a centrifuge bottle and the cells pelleted by centrifugation for 10min at 7000rpm using a JA14 Beckman rotor in a J2-21 Beckman centrifuge. The pellet was resuspended in 4ml lysis buffer I and incubated at room temperature for 15min, after which 8ml lysis buffer II was added and the tube inverted to mix. After 15min incubation at room temperature, 6ml lysis buffer III was added and the tube inverted to mix. Following a 15min incubation on ice, cell debris was pelleted by centrifugation at 19000rpm in a JA20 Beckman rotor in a J2-21 Beckman centrifuge, for 20min at 4°C and the supernatant decanted off to a Corex tube. DNA was precipitated by addition of 0.6vol isopropanol which was inverted to mix, incubated at -20°C for 30min and centrifuged at 10000rpm in a JA20 Beckman rotor in a J2-21 Beckman centrifuge, for 20min at 4°C; the supernatant was decanted off and the DNA pellet left to air dry. DNA was resuspended in 3.2 ml TE buffer and 2.97g CsCl was dissolved in the DNA solution. The DNA/CsCl solution was added to a Beckman "QuickSeal" tube containing 100µl ethidium bromide ($5mgml^{-1}$), and the tube then filled with a TE/CsCl solution at the same concentration as the DNA/CsCl. The tube was sealed and spun at 45000rpm for 16h at 19°C in a VTi65.2 Beckman rotor in an L7 Beckman ultracentrifuge when the lower plasmid band was removed by syringe and transferred to a stoppered test tube. The ethidium bromide was removed from the DNA by equilibration with 2vol of CsCl saturated isopropanol added and mixed by inversion, the upper "pink" layer pipetted off, and the process repeated until both

layers were colourless. The lower layer, containing the DNA was then transferred to a section of dialysis tubing which was sealed by clips, placed in 2mM EDTA pH8 and spun dialysed at 4°C for 2h, the dialysis buffer replaced with fresh and dialysis continued for another 2h. DNA was then transferred to a Corex tube, 2.5vol ethanol and 0.1vol 3M sodium acetate were added, mixed by inversion and DNA precipitated on a dry ice/ethanol mix for 30min, and then pelleted by centrifugation at 10000rpm in a JA20 Beckman rotor in a J2-21 Beckman centrifuge, for 30min at 4°C. The supernatant was decanted and the DNA pellet left to air dry before resuspending in 200µl 2mM Tris-HCl pH8.

2.7.5 Large scale plasmid preparation from *S.aureus* by caesium chloride centrifugation (based on the method of De Buyser *et al*, 1989)

From an overnight culture, 20ml of stationary phase culture was diluted in 200ml CY and incubated at 37°C with shaking for 2-2.5h until the cells were in mid-log phase when they were harvested by centrifugation at 5000rpm for 5min at 4°C in a JA14 Beckman rotor in a J2-21 Beckman centrifuge. The cells were then washed in 200ml ice-cold 10mM EDTA pH8 and re-harvested by a 7000rpm spin at 4°C. The pellet was then resuspended in 16ml 0.1M NaCl/0.05M EDTA pH7, transferred to a fresh tube containing 20µl lysostaphin ($10mgml^{-1}$), and incubated at 37°C with agitation for 30min or until lysis was observed.

When the cells had lysed, the tubes were transferred to ice and 1.85ml 0.5M EDTA added, mixed by inversion and incubated on the ice for 15min, when 17ml 2% SDS/0.7M NaCl was added, inverted to mix and incubated on ice for a further 15min before adding 2.85ml 5M NaCl again mixed by inversion, and transferred to 4°C for overnight incubation. The following day, the cell debris was pelleted by centrifugation at 19000rpm in a JA20 Beckman rotor in a J2-21 Beckman centrifuge for 45min at 4°C. The supernatant was transferred to a fresh centrifuge tube and the DNA precipitated by the addition of 2.5vol ethanol and 0.1vol 3mM sodium acetate, incubation at -20°C for 30min and centrifugation for 30min at 10000rpm in a JA20 Beckman rotor in a J2-21 Beckman centrifuge,at 4°C to pellet the DNA. Once air dried the pellet was resuspended in 3.2ml 0.1M NaCl/0.05M EDTA pH7, and the remainder of the protocol was as for *E.coli*.

2.8 Purification of DNA

2.8.1 Phenol/chloroform extraction

In order to remove proteins from DNA, including modifying enzymes which are not inactivated by heat (65°C), phenol/chloroform extraction was used. The volume of the DNA solution was made up to 300µl with MilliQ dH_2O and an equal volume of phenol/chloroform was added and thoroughly mixed to thick emulsion by shaking. The phenolic and aqueous layers were separated by spinning in a microfuge (12000rpm) for 3min at room temperature. The upper aqueous layer was removed to a fresh Eppendorf by pipette, and then precipitated with ethanol.

2.8.2 Ethanol precipitation

To the DNA solution, 2.5vol of ethanol and 0.1vol 3M sodium acetate were added and mixed by inversion, and then the tubes were incubated at -20°C for 30min before pelleting the DNA by centrifugation in a microfuge (12000rpm) for 15min at 4°C. The ethanol was then decanted away and DNA pellets were left to air dry and then resuspended in 2mM Tris-HCl pH8.

2.8.3 Recovery of DNA from an agarose gel

The required band was cut out of the gel with a scalpel whilst being visualised using UV light. The gel fragment was then placed in a well of an electroeluter (IBI) containing 0.5xTBE so that the gel fragment was just immersed in the buffer. 75µl of 3M sodium acetate (or 7.5M ammonium acetate for small fragments,<500bp) containing bromophenol blue to colour, was pipetted into the "V"-channel, and the interface sharpened by pipetting off 50µl of the overlying solution. The electroeluter was connected to a voltage supply of 80V for 45min (small fragments) to 1h (large fragments), so that the DNA would migrate out of the gel into the "high-salt"solution where it would be retained. The blue salt/DNA solution was then removed by pipette (c. 200µl) and the DNA precipitated with ethanol.

2.9 Modification of DNA

2.9.1 Restriction endonuclease digestion

Single restriction enzyme digestions were carried out using 1-5 units per 1μg of DNA in buffers and temperatures as recommended by the manufacturers' instructions in volumes of 10-40μl When more than one enzyme was to be used, and a compatible buffer was not available, the enzyme requiring the lower salt concentration was used to restrict the DNA first, and then the salt concentration raised for the second enzyme; if this was not possible then the DNA was phenol extracted and ethanol precipitated between enzyme digestions.

2.9.2 Klenow "fill-in" to convert cohesive to blunt ends

For DNA which had been restricted to leave a 5'-overhang to be ligated to a blunt-ended DNA, Klenow was used after appropriate inactivation of restriction enzymes. 1 unit of Klenow and 5μl of a 0.125mM dNTP mix were added to the restriction digest per 1μg DNA, and the tube incubated at 37°C for 1h.

2.9.3 Inactivation of enzymes

When necessary, enzymes were inactivated by incubation at 65°C for 20min and/or removed by phenol/chloroform extraction followed by ethanol precipitation.

2.9.4 Ligation of linear DNAs

After appropriate restriction and enzyme inactivation, samples of the insert and vector DNA were run on an agarose gel to determine relative DNA concentration. Then 1*x* vector (10-200ng) and 5*x* insert DNA (50-1000ng) were added to 4μl of 5x ligation buffer and 1unit

T4 DNA ligase and H_2O added to a 20µl final volume. Controls for ligation were (a) lacking insert, (b) lacking insert and ligase, (c) using only uncut vector DNA without ligase, and (e) adding only ligase buffer and MilliQ H_2O: MilliQ H_2O made up the volume in each case. Each mix was incubated at 4°C for 16h and then used to transform *E.coli* competent cells.

2.10 Transformation

2.10.1 Preparation of stocks of competent *E.coli* (based on Cohen *et al*,1972)

JM107 was used for most purposes, and DH5α or JM109 if a recA$^-$ strain was desired. The cells were grown on a G-min plate and a single colony from a freshly grown plate used to inoculate 10ml 2xTY which was then grown overnight at 37°C. 4ml of the overnight culture was used to inoculate 200ml pre-warmed (37°C) ψ broth and this was further incubated for 1.5h at 37°C with agitation, after which the mid-log phase culture was cooled rapidly on ice. The cooled cells were transferred to a sterile centrifuge tube and spun at 5000rpm for 5min at 4°C using a JA14 Beckman rotor in a J2-21 Beckman centrifuge, the supernatant decanted away and the pellet resuspended in 40ml ice-cold TFB-I, maintaining sterility. This was incubated on ice for 30min before harvesting the cells by a second centrifugation at 5000rpm for 5min at 4°C, as before. The cells were then resuspended in 8ml ice-cold TFB-II and incubated on ice for 15min, after which the cells were aliquoted into pre-chilled sterile Eppendorfs. Cells were stored at -70°C until required. Relative transformation efficiency was tested using c.10ng uncut plasmid DNA.

2.10.2 Transformation of *E.coli* (based on the method of Hanahan, 1983)

Up to 20µl of DNA (10ng uncut plasmid or an entire ligation mix of ligated DNA) was placed in a fresh Eppendorf and the tubes incubated on ice. Competent cells were removed from the -70°C freezer and transferred to ice~until just thawed. 25-50µl of competent cells were added to each DNA sample, the exact quantity dependent upon the efficiency of transformation of the stock of cells being used. The tubes were incubated on ice for 15min and then heat-shocked by incubation at 42°C for 3min, after which they were returned to ice.

1ml 2xTY was added to each tube and they were incubated at 37°C for 1h to allow expression of the newly acquired genes before being plated onto selective agar: 100, 200, 300 and 400µl aliquots were plated onto 2xTY plates containing the appropriate concentration of the selective antibiotic. The plates were then incubated overnight at 37°C, or 30°C for temperature sensitive plasmids, after which colonies were selected and "mini-prepped" to test for clones.

2.10.3 Transformation of *S.aureus* (adapted from Chang and Cohen, 1979)

Competent *S.aureus* cannot be maintained and therefore were made fresh for each transformation. A 20ml overnight culture of the appropriate strain was transferred to a sterile 50ml polypropylene screw-cap centrifuge tube and harvested by a 5min spin at 5000rpm in a JA14 Beckman rotor in a J2-21 Beckman centrifuge, at room temperature. The supernatant was decanted off and the cells gently resuspended in 10 ml HBM. To this a further 10ml HBM, containing 4mg lysozyme and 0.1mg lysostaphin per ml, was added and gently mixed by inversion. The tube was incubated in a 37°C water bath without agitation for 2-3h until lysis was observed. Then any remaining un-protoplasted cells were pelleted by a 7000rpm spin for 15min at 4°C in a JA14 Beckman rotor in a J2-21 Beckman centrifuge. The protoplasts were decanted off to a fresh sterile 50ml screw-cap centrifuge tube, which was spun at 19000rpm for 15min at 4°C to harvest the protoplasts. The supernatant was discarded and the protoplast pellet gently resuspended in HBM by pipette (200µl per transformation). 200µl of protoplasts were added to each sterile 50ml centrifuge tube containing the DNA (200-500ng) and 20µl 2xHB, after which 1.8ml 50% PEG 6000 (in HB) was immediately added and the tubes gently rocked to mix. After a 2min incubation at room temperature, 10ml HBM was added and the transformed protoplasts pelleted by centrifugation at 19000rpm for 30min at 4°C. The protoplasts were then resuspended in 0.4ml HBM and 100µl placed onto 4 plates: 3 DM3 and 1 CY (as a control for regeneration of the protoplast) all of 20ml, and the protoplasts were spread over the plate by agitation of the plate rather than using any "spreader" which might break open the fragile protoplasts. The plates were then incubated at 30°C to allow regeneration of the cell wall and expression of the newly acquired genes (3h for RN4220 and 5h for RN981). Once the cells had been allowed to regenerate, 5ml of a soft TBAB/HB agar overlay (containing the appropriate selective

antibiotic at 5x the "normal" plate concentration) was poured gently over the plates from a test tube and allowed to set. The plates were then incubated for 3-7 days (30°C for temperature sensitive strains and 37°C for others) until transformant colonies were visible below the overlay. Transformants were picked and "mini-prepped" to test the DNA.

2.11 Transduction of *S.aureus*

2.11.1 Preparation of stock bacteriophage

An overnight culture of the propagating strain (containing the appropriate plasmid) was grown in CY broth, and 100µl used to inoculate 15ml phage-buffered medium. This was allowed to grow at 30°C with gentle agitation for 30min after which 200µl 'phage were added and incubation continued for 5-8h until the solution cleared due to lysis by the 'phage. The 'phage were purified by passing through sterile filters of pore size 0.45µm. 'Phage were then stored at 4°C until required. The concentration of 'phage was determined by placing 20µl drops of 10-fold dilutions onto dry plates prepared to grow lawns of the propagating strain (containing no plasmid) and the number of plaques counted.

2.11.2 Transduction

An overnight culture of the strain to be transduced was prepared and 0.8ml used to inoculate 23.5ml of phage-buffered medium. This was incubated at 30°C for 1h with gentle shaking to allow the cells to reach early-log phase. 1.4ml was transferred into each of four sterile Eppendorfs and 0, 100, 150 and 200µl aliquots of 'phage containing the appropriate plasmid added. The tubes were then returned to 30°C with gentle agitation for 30min to allow the 'phage DNA to enter the cells but not allow cell lysis. Immediately the cells were pelleted by microfugation (12000rpm) and resuspended in 1ml freshly prepared CY-citrate. The resuspension was repeated three times in order to dilute free 'phage away and to inhibit lysis. The final resuspension volume was 300µl which was plated onto selective agar, and the colonies which grew tested by "mini-prep".

2.12 Electrophoresis

A 0.5-1% agarose gel (made in 1xTBE containing 25µl ethidium bromide (10mg ml^{-1})) was loaded with 10-15µl DNA, containing 0.1vol "sinker" per lane. The gel was placed in the electrophoresis unit and submerged in 1xTBE (containing 25µl ethidium bromide (10mg ml^{-1})). The gels were run at voltages between 80 and 120V, for the appropriate length of time to allow visualisation of the bands using a UV transilluminator.

2.13 Southern blotting and hybridisation with labelled probes

2.13.1 Southern Blotting (Southern, 1975)

Restricted DNA was run in a 0.5-1.0% agarose gel and a photograph taken. Excess buffer was rinsed off with dH_2O. The backbone of the DNA in the gel was broken by shaking in 3vol depurination buffer for 30min after the bromophenol blue dye in the "sinker" had turned yellow. The buffer was decanted away and the gel and box rinsed three times in dH_2O. DNA was then made single stranded by two successive 15min washes in 3vol denaturation buffer after which it was rinsed three times in dH_2O and neutralised by a 30min wash in 3vol neutralisation buffer. All washes were at room temperature. The gel was then transferred onto the wick of a blot stack soaked in 20xSSC. The membrane ("FLASH" nylon, Stratagene), of similar size to the gel, was soaked in 2xSSC and placed on top of the gel. A similarly sized piece of 3MM paper soaked in 20xSSC was placed on top followed by two more dry pieces and a 10cm stack of blotting tissues. Onto all this a glass plate was balanced and all weighed down with a 0.5kg weight. The blot stack was left to transfer for at least 16 h after which the top papers were removed and the membrane lifted onto blotting paper (DNA side up) where it was left until dry. The dry membrane was fixed by exposure to UV transilluminator for 3min (a time found to be appropriate for the transilluminator used). Blots were then stored wrapped in SaranWrap at 4°C until used.

2.13.2 Preparation of probe DNA using the "Flash" (Stratagene)

DNA to be used as a probe was linearised. 50-500ng were diluted to a final volume of 29µl with dH_2O. 10µl of the oligonucleotide mix provided was added and the whole denatured by boiling in a water bath for 5min. The DNA was left at room temperature for 10min to encourage annealing of the primers to the DNA template. 10µl 5xPrimer buffer, which contained biotin labelled dUTP and 2units of T7 DNA polymerase was then added and this was incubated at 37°C for 20min to allow extension from the primers and thus probe synthesis. The reaction was stopped by the addition of 2µl of the stop solution provided. The probe was then purified from the unincorporated nucleotides by the addition of 5µl 1% Tween-20 and 15µl 1xSTET buffer and passage through a "NucTrap Push Column" (Stratagene) which had been wetted with 70µl 1xSTET buffer, the DNA remaining in the column was flushed out by a further 70µl 1xSTET buffer. The probe was then stored at -20°C until required.

2.13.3 Hybridisation of probe to the membrane using the "Flash" kit

The membrane to be hybridised was wetted in 2xSSPE and then pre-hybridised at 42°C for at least 30min in 0.1vol pre-hybridisation buffer with agitation. The probe to be used was denatured by boiling for 5min and direct transfer to ice to prevent re-annealing before being added to the pre-hybridisation solution (not directly onto the membrane). The hybridisation was then continued for at least 16h at 42°C with agitation.

2.13.4 Detection of hybridisation - "Flash"

Hybridisation buffer was decanted off the membrane which was then briefly washed in 2xSSPE/0.1%SDS to dilute the remaining traces. In order to rid any associated or loosely bound probe, two low stringency washes in 2xSSPE/0.1%SDS for 15min at room temperature and then two high stringency washes in 0.2xSSPE/0.1%SDS for 30min at 60°C were performed. The detection process was then all at room temperature with agitation

consisting of two 5min washes in 100ml Wash buffer I, followed by a 30min wash in blocking buffer (100ml) and a 30min wash in 15ml conjugate buffer containing avidin-conjugate to the dilution as directed. This was then further blocked by a 5min wash in blocking buffer (100ml) in a new container band then excess blocking buffer removed by four 5min washes in Wash buffer II (100ml each). The membrane with bound avidin-conjugate was then prepared for detection by a 5min wash in 50ml assay buffer, which was decanted off before adding 7.5ml assay buffer containing 45µl "Lumigen". The membrane was washed in the Lumigen buffer by agitating by hand to ensure that the membrane was adequately covered at all times for 10min. Excess buffer was shaken off the membrane and the membrane wrapped in SaranWrap before exposing it to X-ray film for 30min. Further exposure lengths were determined from the intensity of bands on the initial developed film.

2.13.5 Preparation of probe DNA using the "ECL" kit (Amersham)

DNA to be used as a probe was digested so as to separate the vector and insert sequences. 50-500ng of probe DNA was then diluted to a final volume of 34µl with H_2O. This was then denatured by heating for 5min in a boiling water bath, after which the Eppendorf was removed to ice immediately to prevent denaturation. 10µl nucleotide mix, 5µl primers and 1µl Enzyme mix were added (provided in the kit) and mixed by pipette. The Eppendorf was then incubated at 37°C for 1h. There was no requirement to separate the probe from the nucleotide mix.

2.13.6 Hybridisation of probe to blot using the "ECL" kit (Amersham)

The membrane to be hybridised was wetted in 20xSSC before placing in pre-hybridisation buffer. 0.25ml pre-hybridisation buffer was used per cm^2 membrane. Pre-hybridisation was continued in a sealed a plastic box at 60°C for 1h with shaking, after which the probe was denatured (5min in boiling water bath) and added to the buffer away from the membrane. Hybridisation was continued for 16h at 60°C with shaking.

2.13.7 Detection of hybridisation using the "ECL" kit (Amersham)

The membrane was removed from the hybridisation mix and briefly rinsed three times in dH_2O and transferred to a fresh container. Then the membrane was rinsed in 1xSSC/0.1%SDS prior to two 15min washes at 60°C in 100 ml aliquots of the same solution which had been pre-heated to 60^0C. The membrane was then briefly rinsed in 0.5xSSC/0.1%SDS prior to two 15min washes at 60°C in 100 ml aliquots of it which had been pre-heated to 60^0C. The membrane was next briefly rinsed in antibody wash buffer, prior to a 1h room temperature, shaking incubation in antibody wash buffer containing blocking agent (0.5ml per cm^2 of membrane). After this the membrane was rinsed in antibody wash buffer prior to a 1h room temperature, shaking incubation in antibody wash buffer containing 0.5% BSA and 0.001vol anti-fluorescein-HRP conjugate (0.25ml per cm^2 of membrane). (Both the blocking agent and the anti-fluorescein-HRP conjugate were provided in the kit.) Unbound conjugate was removed by two 10min and two 5min washes at room temperature, shaking in antibody wash buffer containing 0.1% Tween-20. The membrane was then drained of excess wash solution and transferred to a fresh container. Equal volumes of detection solutions I and II (provided in the kit) were added to a final volume of 0.125ml per cm^2 of membrane, and the solutions manually shaken over the membrane, gently for 1min. Excess detection solution was drained from the membrane, which was briefly placed DNA side up onto blotting paper to remove further traces, and then the membrane wrapped in Saran-Wrap and the DNA side placed next to autoradiography film. After 1min exposure the film was developed and further exposure lengths determined from the strength of the signal produced.

2.14 Sequencing DNA (based on the method of Sanger *et al*, 1977)

2.14.1 Purification of the oligonucleotide

The oligonucleotide was provided in ammonia solution which was vapourised by leaving the tube with the lid partially unscrewed inside a tightly closed bottle at 55°C overnight. The remaining DNA solution was aliquoted into sterile Eppendorfs, ethanol precipitated; one aliquot was resuspended in 100µl sterile MilliQ, the remaining were stored at -20°C as pellets. The resuspended oligonucleotide concentration was determined by using the formula: OD_{260}=1 is the equivalent of 20µg oligonucleotide ml^{-1}. The oligonucleotide was then further diluted in sterile MilliQ so as to give a final concentration of 1pmol μl^{-1}.

2.14.2 Sequencing by dideoxy chain termination using the Sequenase kit (United States Biologicals)

Template DNA (prepared by either large scale caesium chloride or quick "wizard" methods) was linearised at a site not in the sequence of interest and 5µg diluted in MilliQ to 7µl final volume. This was then denatured by boiling for 5min in a water bath, with direct transfer to ice to prevent re-annealing. To the denatured template 2µl RxBuffer and 1µl of primer oligonucleotide (1pmol μl^{-1}) were added and the tube incubated at 37°C for 30min to allow annealing of the primer to the template. During this incubation, 2.5µl of termination mixes were added to the base of four labelled tubes (one per dideoxy) which were incubated on ice. After the primer had annealed to the template, the following were added: 1µl DTT, 2µl 1xlabelling mix, 1µl manganese buffer, 0.5µl ^{35}S-ATP and 2µl T7 polymerase diluted 8-fold in the buffer provided. This was then mixed by pipette and during a 5min incubation at room temperature 3.5µl aliquots were placed on the sides of the tubes containing the termination mixes, taking care not to mix the two; these tubes were then placed in a microfuge and spun to collect and mix at the end of the 5min incubation. After the mixing of the DNA with the termination mixes, the tubes were transferred to a 37°C water bath for a 5min incubation during which 4µl stop solution was placed onto the side of the tube taking care not to mix

with the DNA solution. At the end of the 5min incubation, the tubes were quickly transferred to a microfuge and spun to mix and stop the reactions. The DNA solutions were stored at -20°C until required.

2.14.3 Preparation of the sequencing gel

Glass plates were cleaned with "Fairy Liquid" which was removed with excess dH_2O, then rinsed with acetone and again with dH_2O, then rinsed with ethanol and this removed by rinsing in dH_2O, and the plates were finally rinsed with chloroform and allowed to air dry. The side of the "eared" plate to come into contact with the gel was treated by pouring 5ml 2% dimethyldichlorosilane onto it and wiping it evenly over the surface with a tissue until all had evaporated, this was to ease separation of the gel from this plate later. The plates were then placed on top of one another with wedged spacers in between them and taped in position whilst being held fast with 3 "bulldog" clips along each side. The acrylamide mix was then poured in between the plates. The flat end of the comb was then placed in the opening and held in place by "bulldog" clips. This was left as near to horizontal as possible overnight to ensure that the gel had set.

2.14.4 Running and developing the sequencing gel

The gel was run for an appropriate length of time on a standard sequencing rack in 1xTBE at 1200-1500V after a 30min pre-run. The gel was then removed and the "eared" plate prised away from the gel. The gel was transferred to a container containing enough fixer to cover the gel, and left for 30min. The fixer was then aspirated away and a piece of 3MM paper placed on top of the gel, which transferred to it. This was then covered in SaranWrap and dried under vacuum at 80°C. The dried gel was then exposed to autoradiography film.

2.15 Comparison of the relative resistances of strains by the "disc method"

An overnight culture of the strain was diluted 1:10 and incubated with aeration at 37°C until mid-log phase when 10^6 cells, as determined by OD_{675}, were added to 10ml CY broth and mixed by vortex. The culture was then poured onto a 20ml CY agar plate the excess pipetted off and the plate allowed to dry for 1h at room temperature. A sterile 5mm diameter blotting paper disc inoculated with 20µl of the appropriate antibiotic and dried was placed centrally onto the dried plate and the plates then incubated for 20h at 37°C. Relative resistances were determined by comparison of the halo sizes.

2.16 Serial dilution of bacteria

6.25µl of an overnight culture were used to inoculate 6.5ml of broth containing the appropriate concentration of the selective antibiotic. The remainder of the culture was used to prepare "mini-prep" DNA. The freshly inoculated broth was allowed to grow overnight to stationary phase, allowing 10 generations of growth. This overnight culture was then used as previously until the required number of generations had been achieved.

Characterisation of
High-level Mupirocin Resistance plasmids
of Staphylococcus aureus

Chapter Three

Characterisation of High-level Mupirocin Resistance Plasmids of *Staphylococcus aureus*

3.1 Introduction

Mupirocin is a novel topical antibiotic (Mellows, 1984) that was introduced onto the UK market in 1985, and onto the wider European markets in 1987. Its major applications are for the treatment and prevention of spread of Gram positive bacterial infections (White *et al.*, 1984). Within only two years of its use as a clinical antibiotic, strains of *Staphylococcus aureus* were isolated that were resistant to high concentrations of mupirocin (>2mg ml^{-1}) (Baird and Coia, 1987[o]; Kavi *et al.*, 1987[o]; Rahman *et al.*, 1987; Smith *et al.*, 1987[o]; Cookson *et al.*, 1990). Strains isolated in Nigeria as early as 1965 express such high levels of resistance to mupirocin, demonstrating that the resistance determinant was present before the onset of use of mupirocin in clinical practice. The resistance determinant (*mup*A) was found to be plasmid-borne (Rahman *et al.*, 1990; Dyke *et al.*, 1991) and many of these plasmids are transferable to other staphylococci by filter mating (Rahman *et al.*, 1989). *mup*A has been cloned (Dyke *et al.*, 1991) and sequenced (Dyke *et al.*, 1991; Hodgson *et al.*, 1994) and encodes an isoleucyl-tRNA synthetase (Dyke *et al.*, 1991; Hodgson *et al.*, 1994). The mupirocin resistance (Mu^R) plasmids are very diverse (Rahman *et al.*, 1990), but the resistance determinant is always *mup*A, and in plasmids from both *Staphylococcus aureus* and coagulase negative strains, *mup*A is usually located on a 4.05kb *Eco*RI fragment (Connolly *et al.*, 1993; Rahman *et al.*, 1993).

Mupirocin resistance plasmids were to be further investigated both to identify features which may explain their present diversity, in light of the constancy of the resistance determinant itself, and also to look for characteristics which may lead to future dissemination of *mup*A within the bacterial gene pool.

[o] Although the data herein refers to the lower level, intermediate resistance, reference is made to the high-level resistance documented in Rahman *et al*, 1987.

3.2 Determination of the antimicrobial agent resistance profile of high-level mupirocin resistance plasmids

High-level mupirocin resistance (Mu^R) plasmids were provided as transconjugants to RN2677 or NCTC8325[1] (W.C.Noble by the method in Naidoo and Noble, 1987). The resistances of the Mu^R transconjugants 8325(p2710), 8325(p2795), RN2677(p2947), RN2677(p2948), 8325(p3355), 8325(p3356), 8325(p3357) and 8325(p3358) to various antibiotics and other antibacterial agents were compared to those of known resistant staphylococcal strains by the disc method (Section 2.15). The diameter of the halo was measured and a comparison of the size of the test halo with that of the positive and negative controls made to determine the relative resistance of the test strain. RN4220, a close relative of NCTC8325, the recipient strain of many of the transconjugants, was used as a negative control; the positive controls are given with the results in Table 3.1.

All transconjugants were confirmed to express resistance to mupirocin as demonstrated by the lack of halo as compared to the substantial halos of RN4220 and other negative controls. None of the eight plasmids were found to harbour resistance determinants for the antibiotics gentamicin (Gm), chloramphenicol (Cm), erythromycin (Em), penicillin (Pc), trimethroprim (Tm) or streptomycin (Sm) nor for the antibacterial agents arsenate (As), antimony (Sb), mercury (Hg), cadmium (Cd) or ethidium bromide (EtBr) as demonstrated by their apparently similar sensitivity (halo size) to these substances as RN4220. However, when subject to tetracycline, strain 8325(p3358), containing plasmid p3358, had a similar halo size to 8325(pT181) (19 and 21mm respectively), which was much smaller than those of the other Mu^R strains and RN4220 (34-41mm). Thus p3358 was shown to confer resistance to tetracycline to a similar level as pT181, as well as to confer high level mupirocin resistance. This is the first example of linkage of high level mupirocin resistance with another resistance determinant.

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[1] The NCTC8325 strain used had been made resistant to rifampicin and fusidic acid prior to conjugation to allow selection.

Table 3.1 Antibiotic Resistance Profile of Mupirocin Resistance Plasmids

Strain	Control for:	Antibiotic in disc (20µl, x mg/ml): nh = no halo; nt = not tested; halo (mm)													Resistance
		Mu 0.1	Tc 0.5	Gm 0.5	Cm 0.5	Em 0.5	Pc 0.25	Tm 0.5	Sm 0.5	As 0.5	EtBr 0.5	Sb 0.5	Hg 0.1	Cd 6.65	
8325(pJE1)	Gentamicin, Trimethroprim	20	nt	<6	nt	nt	nt	nh	12	20	nh	28	18	nt	
8325(pI258)	Arsenate, Sb, Hg, CdAc, Penicillin	20	nt	17	12	nt	10	12	13	nh	11	nh	12	11	
RN4220	Negative control	22	40	13	10	22	32	10	19	20	11	25	21	18	
8325(pT181)	Tetracycline	nt	21	rt	13	21	44	nt	14	nt	nt	nt	nt	22	
RN4220(pC194)	Chloramphenicol	nt	nt	nt	nh	22	nt	nt	nt	nt	nt	nt	nt	34	
RN4220(pE194)	Erythromycin	nt	nt	nt	12	nh	nt	nt	nt	nt	nt	nt	nt	34	
RN4220(pS194)	Streptomycin	nt	nt	nt	12	nt	41	16	9	nt	nt	nt	nt	38	
8325(p2710)		nh	49	15	10	18	39	17	15	12	11	15	20	28	mupirocin
8325(p2795)		nh	40	15	10	20	38	16	11	12	12	16	19	28	mupirocin
RN2677(p2947)		nh	41	12	12	19	38	17	12	13	11	16	19	28	mupirocin
RN2677(p2948)		nh	34	13	12	20	39	16	13	15	10	14	22	26	mupirocin
8325(p3355)		nh	40	12	10	20	45	17	15	14	9	17	18	25	mupirocin
8325(p3356)		nh	41	13	12	19	42	17	12	12	9	18	18	26	mupirocin
8325(p3357)		nh	41	13	12	13	42	16	12	12	8	16	20	26	mupirocin
8325(p3358)		nh	19	13	12	17	41	16	14	10	8	13	18	25	mupirocin and tetracycline

3.3 Hybridisation of mupirocin resistance plasmids with *mup*A gene probe

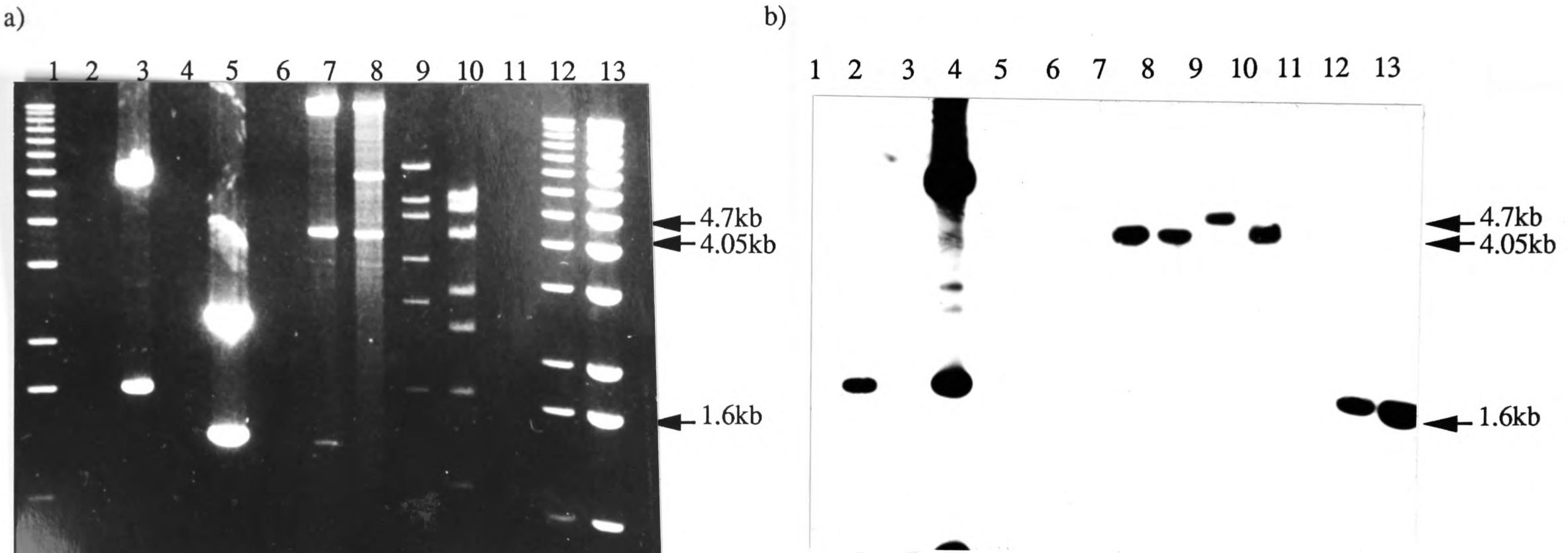
Restriction enzyme digests of eight Mu^R plasmids (p2710, p2795, p2991, p3274, p2947, p2948, p3356 and p3358) were Southern blotted and hybridised with pMZ2, a pOX7 (identical to pOX300 in Dyke *et al.*, 1991) clone of the internal 1.6kb *Nco*I fragment of the *mup*A gene, demonstrated to be a faithful probe for *mup*A (Rahman *et al.*, 1993; Gilbart *et al.*, 1993). Hybridisation in six of eight plasmids was to a 4.05kb *Eco*RI fragment, as found previously (Figs.3.1 and 3.2). However hybridisation was to a 4.7kb *Eco*RI fragment of plasmid p2948 and to an *Eco*RI fragment c.11kb of plasmid p2795, confirming previous data (Rahman *et al.*, 1993). Plasmids p2710, p2991 and p3274 all contained 4.05kb *Eco*RI fragments to which the probe hybridised, however the sizes of the *Bgl*II fragments to which the probe hybridised were varied: c.16kb, 5.0kb, and 9.5kb respectively. This is consistent with previous observations that *mup*A is usually located on a 4.05kb *Eco*RI fragment (Rahman *et al.*, 1993; Connolly *et al.*, 1993) which is found on plasmids of a very diverse restriction enzyme pattern (Rahman *et al.*, 1990).

3.4 Restriction enzyme mapping of mupirocin resistance plasmids p2947, p2948 and p3356.

Rahman *et al* (1990) had found that the *Eco*RI restriction patterns of Mu^R plasmids varied considerably; this was confirmed in Section 3.3, and shown to extend to *Bgl*II restriction patterns also. Preliminary restriction enzyme mapping of p2947 and p2948 had been undertaken in the laboratory previously and had demonstrated that these two plasmids were dissimilar, although similar in size (c.22kb and c.24kb respectively). This mapping was continued and p3356, again of similar size (c.24kb) was also mapped.

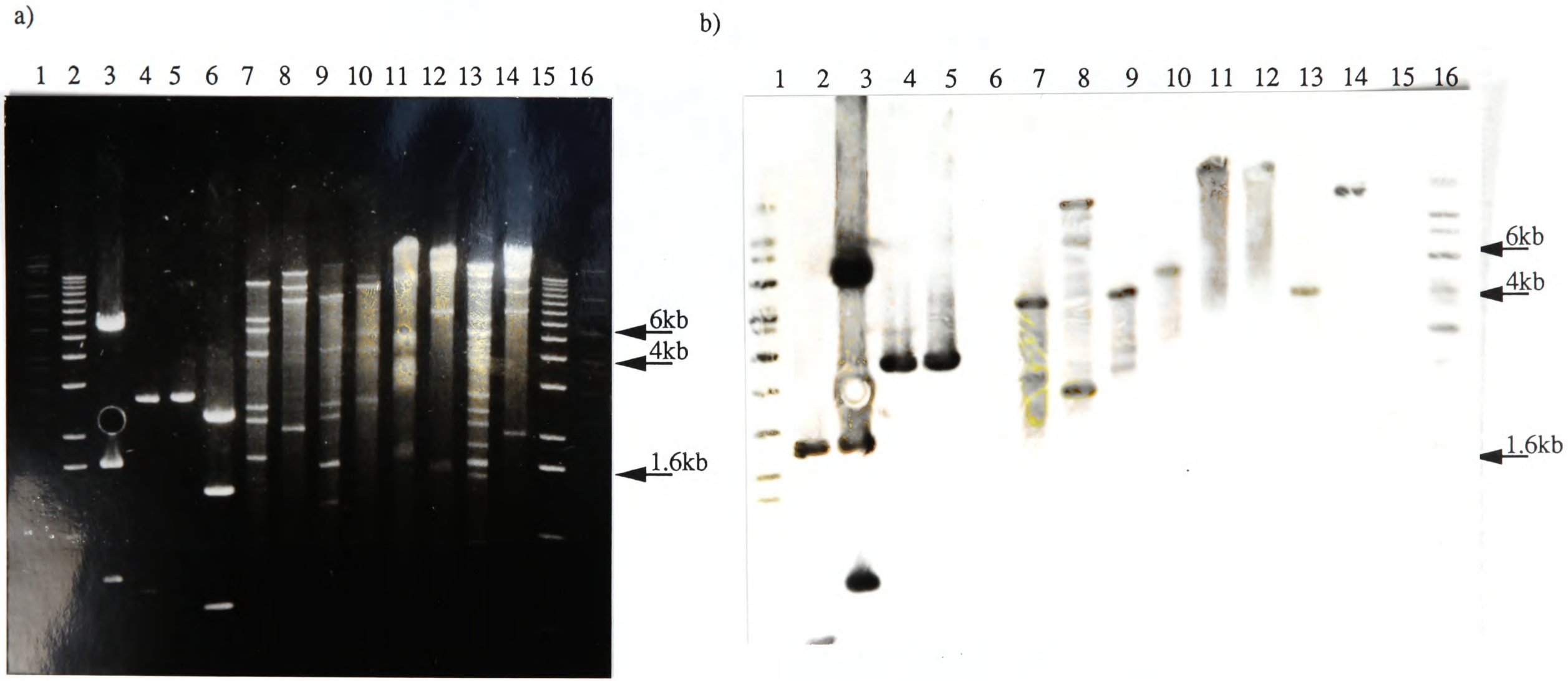
DNA was made by the full scale CsCl method for restriction mapping purposes. Digestions were both singular and double for both the whole staphylococcal plasmids and for clones of those plasmids. A typical gel of such digests is shown in Fig.3.3, and such gels were used

Figure 3.1 A Southern blot of mupirocin resistance plasmids, hybridised with the *mup*A probe



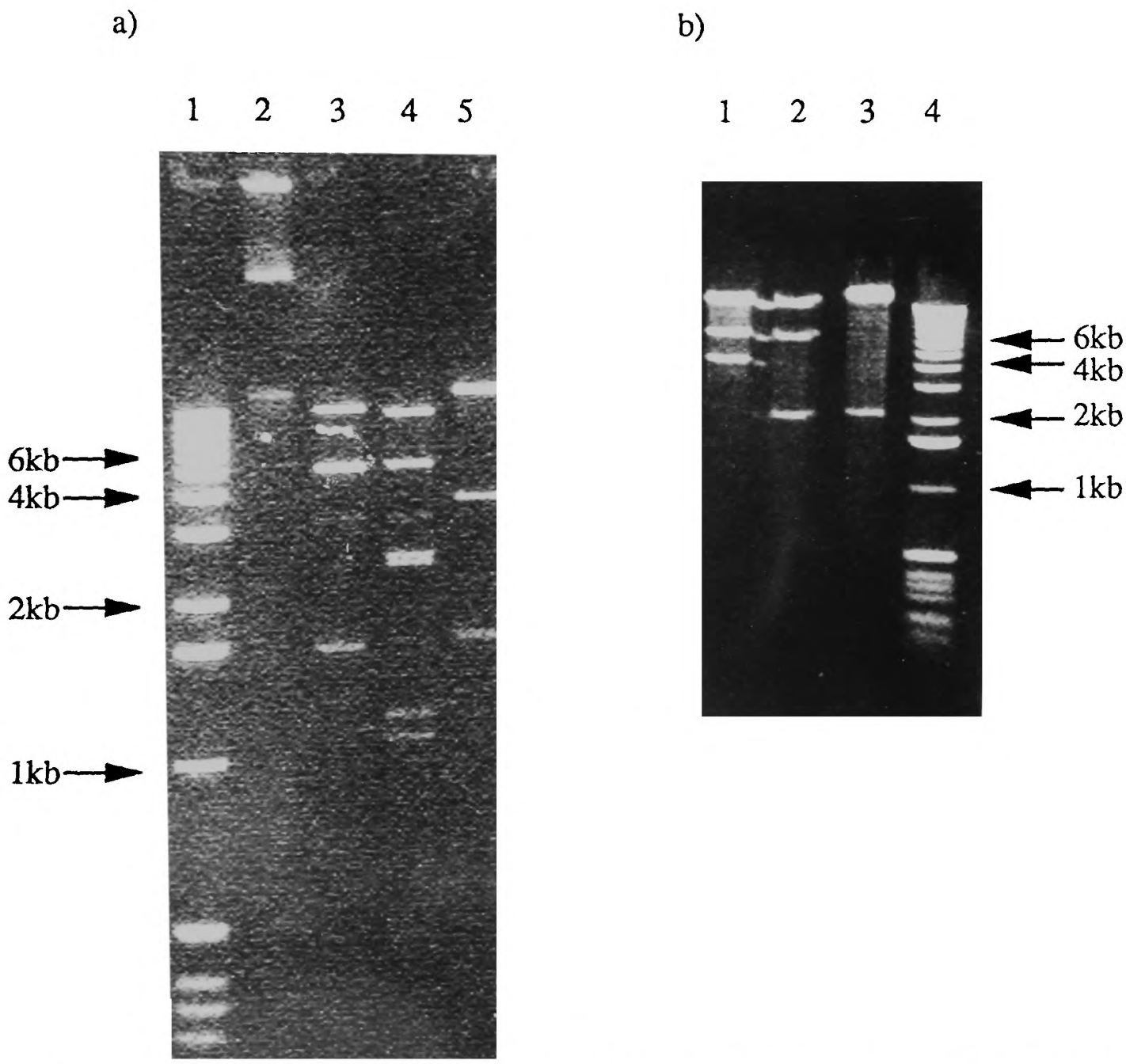
a) Digested plasmid DNA: Lanes 1, 12 & 13, 1kb ladder; lanes 2, 4, 6, & 11 empty; lane 3, pMZ2 digested with *Eco*RI and *Pst*I; lane 5, pT181 digested with *Hin*dIII; lane 7, p3358; lane 8, p3356; lane 9, p2948; lane 10, p2947; lanes 7-10 are digested with *Eco*RI.
b) Autoradiograph of gel in (a) probed with pMZ2 which contains the 1.6kb *Nco*I fragment internal to *mup*A and pUC18 which has homology to some fragments in the 1kb ladder.

Figure 3.2 A Southern blot of mupirocin resistance plasmids hybridised with the *mup*A probe



a) Digested plasmid DNA: lanes 1 & 16, Raoul ladder; lanes 2 & 15, 1kb ladder; lane 3, pMZ2 digested with *Eco*RI and *Pst*I; lane 4, pIP1644 digested with *Eco*RI and *Pst*I; lane 5, pUC18 digested with *Eco*RI; lane 6, pT181 digested with *Hin*dIII, as negative control; lanes 7 & 8, p3274; lanes 9 & 10, p2993; lanes 11 & 12, p2795; lanes 13 & 14, p2710; lanes 7, 9, 11, & 13 are digested with *Eco*RI; lanes 8, 10, 12, & 14, digested with *Bgl*II.
b) Autoradiograph of the gel in (a) hybridised with pMZ2, a pOX7 clone of the 1.6kb *Nco*I fragment internal to *mup*A: pOX7 contains pUC18 which has homology with some fragments of both 1kb and Raoul ladders.

Figure 3.3 Example gels of single and double digests of p3356 DNA from which the restriction map was calculated



a) Digested DNA: lane 1, 1kb ladder; lanes 2-5, p3356; lane 2, undigested; lane 3, digested with *Nco*I; lane 4, digested with *Nco*I and *Bgl*II; lane 5, digested with *Bgl*II.
b) Digested DNA: lane 4, 1kb ladder; lanes 1-3, p3356; lane 1, digested with *Eco*RI; lane 2, digested with *Cla*I and *Eco*RI; lane 3, digested with *Cla*I.

to generate the maps shown in Fig.3.4 which are aligned for *mup*A. *Bam*HI and *Sma*I failed to restrict all three plasmids; *Kpn*I failed to restrict only p2947; in addition p3356 was not restricted by *Aat*II, *Bgl*I, *Mlu*I, *Nar*I, *Pst*I, *Pvu*I, *Sal*I, *Sac*II, *Sca*I, *Sna*BI, *Sph*I and *Stu*I.

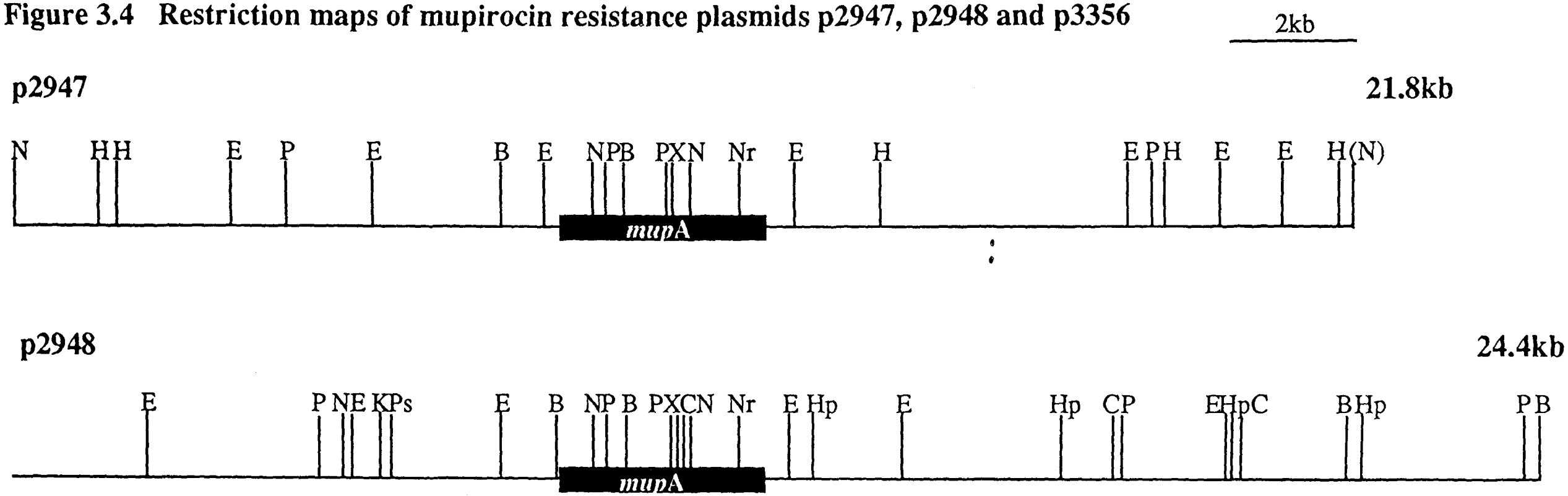
The restriction maps shown in Fig.3.4 confirm that although the mupirocin resistance determinant is the same (*mup*A), interplasmid homology extends very little outside of *mup*A and although the plasmids are of similar size, they are quite diverse except for *mup*A.

3.5 Hybridisation of mupirocin resistance plasmids with conjugative probes

The Mu^R plasmids had been transferred to the laboratory strains by filter mating conjugation (Rahman *et al.*, 1990). The frequencies of transfer had been high: $1x10^{-6}$ to $1x10^{-7}$, suggesting that the plasmids encoded genes for conjugation. A common region (*tra* or *trs*) is shared by many conjugative plasmids of *S.aureus* (Byrne *et al.*, 1990; Thomas and Archer, 1989a). This *tra* region (c.13.6kb) is found on large plasmids and has been cloned and sequenced (Firth *et al.*, 1993; Morton *et al.*, 1993) and, along with a second smaller region (Evans and Dyke, 1988), has been shown to be sufficient for conjugation.

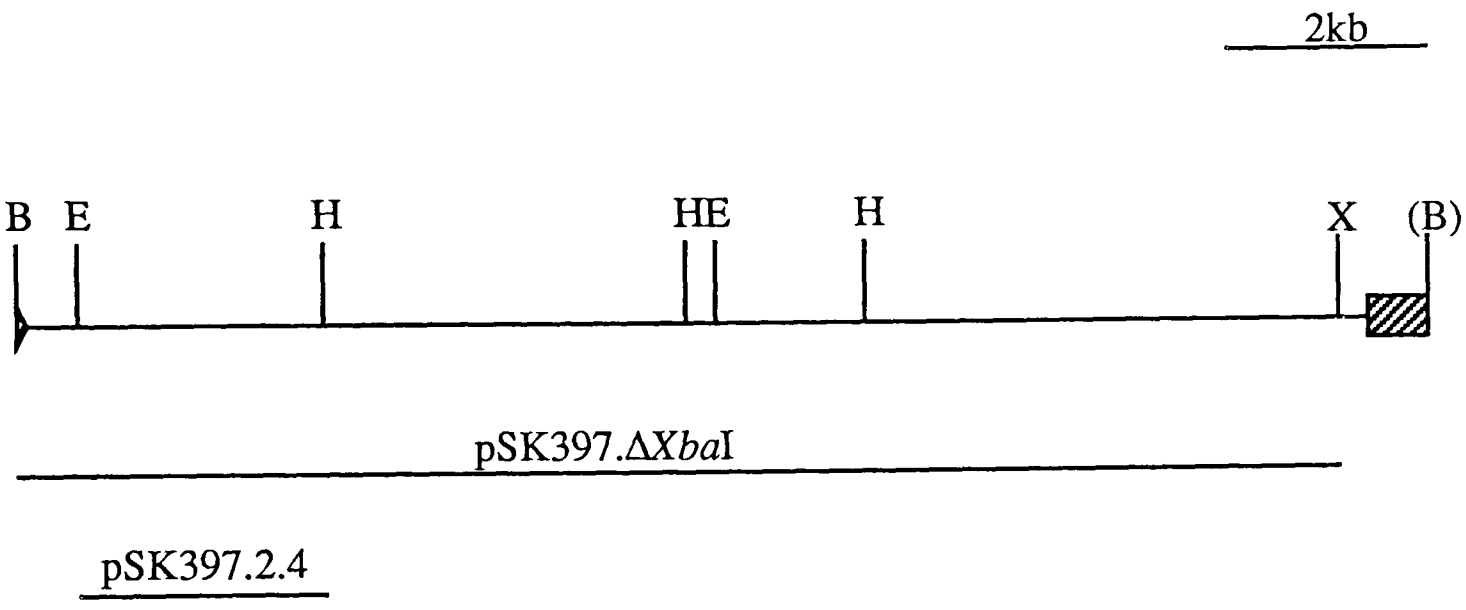
Clones of the *tra* region were provided by R.A.Skurray (Fig 3.5). The clone which includes most of the *tra* region (pSK397.Δ*Xba*I) was used to probe a Southern blot of *Eco*RI restriction digests of p2947, p2948, p3356 and p3358 (Fig.3.6). No fragments of p3356 or p3358 hybridised. Hybridisation was only to the 5.0kb fragment of p2947 and the 6.7kb fragment of p2948. As the probe itself is c.14kb, if the whole *tra* region were present, more bands than this would be expected to hybridise, thus only partial *tra* sequences are present on p2947 and p2948. A smaller probe from within the *tra* region, pSK397.2.4, was used to probe digests of these two plasmids (Fig 3.7). Only p2948 hybridised, even on long exposures, no fragments of p2947 showed up on the autoradiograph. Therefore the section of *tra* present on p2948 included the pSK397.2.4 region, but that of p2947 did not.

Figure 3.4 Restriction maps of mupirocin resistance plasmids p2947, p2948 and p3356



Plasmid DNAs are linearised so as to allow alignment of *mup*A (black box). Restriction enzyme sites are:
Av=*Ava*II, B=*Bgl*II, Bc=*Bcl*I, C=*Cla*I, E=*Eco*RI, EV=*Eco*RV, H=*Hin*dIII, Hp=*Hpa*II, K=*Kpn*I, N=*Nco*I, Nr=*Nru*I,P=*Pvu*I, Ps=*Pst*I and X=*Xho*I.

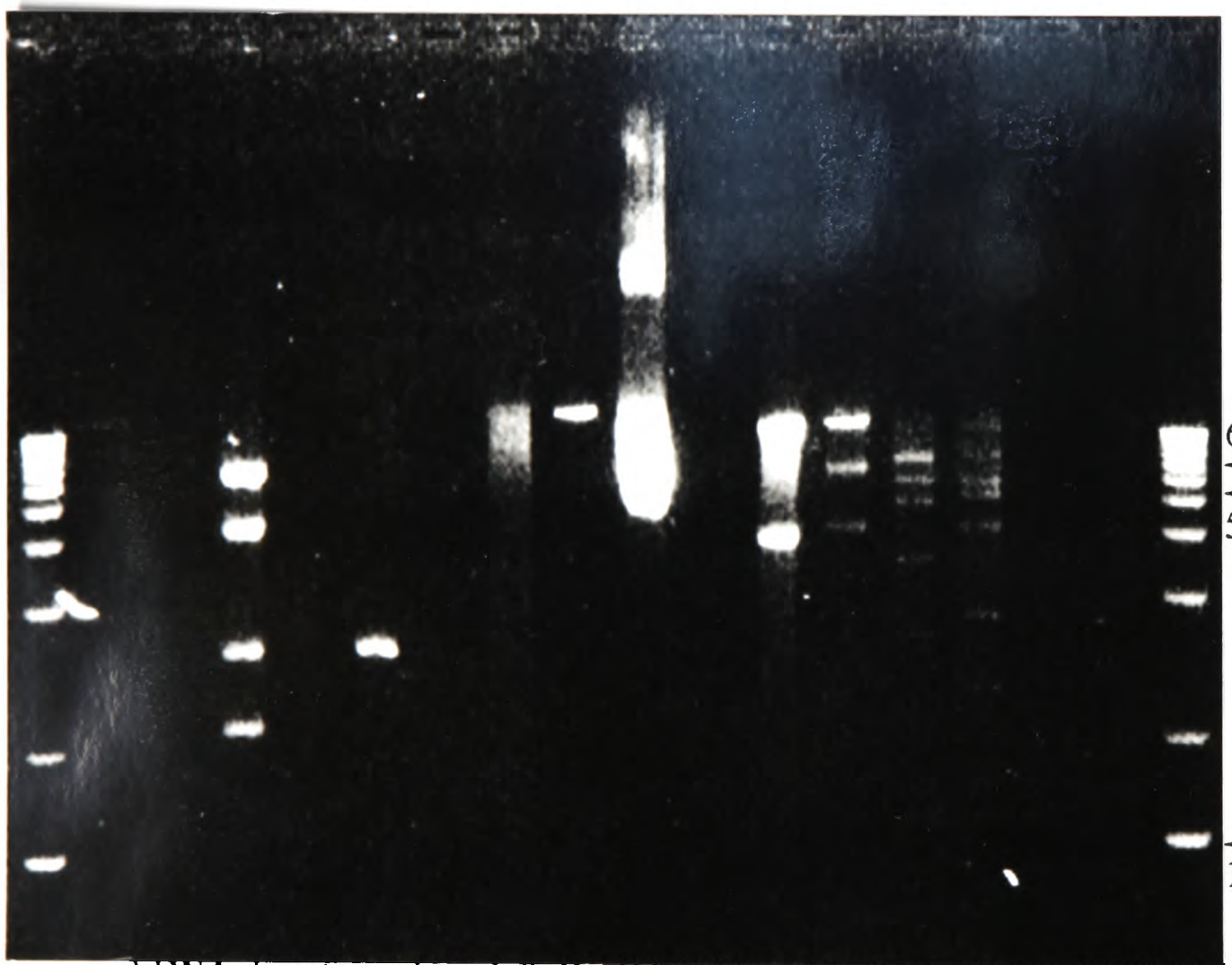
Figure 3.5 The *tra* region of pSK41



The *tra* region of pSK41, and portions of the flanking IS*257*s (hatched boxes) are shown, the positions of the cloned fragments in pSK397.Δ*Xba*I and pSK397.2.4 are given. (These clones are in pUC18.) B=*Bgl*II; E=*Eco*RI; H=*Hin*dIII; X=*Xba*I.

Figure 3.6 A Southern blot of plasmids p2947, p2948, p3356 and p3358 probed with the pSK397.Δ*Xba*I clone of the conjugation *tra* region

a)

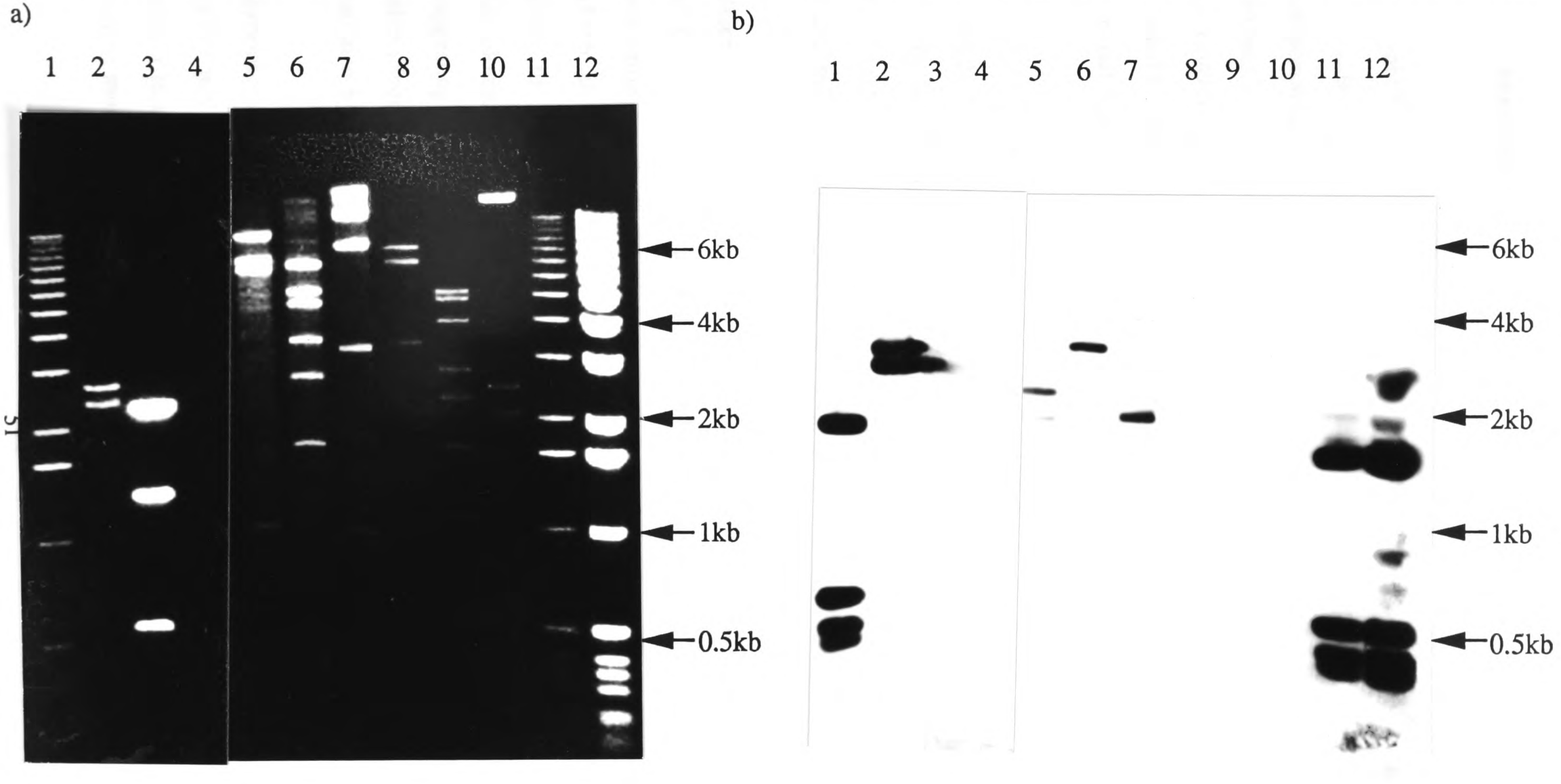


b)



a) DNA digestions: lane 1 & 18, 1kb ladder; lanes 2 & 17, Raoul ladder; lanes 3, 5, 7, 11 & 16 are empty; lane 4, pSK397.Δ*Xba*I digested with *Eco*RI and *Hin*dIII; lane 6, pUC18 digested with *Eco*RI; lane 8, 8325 chromosomal DNA; lane 9, pJE2 (a conjugative plasmid deleted for *tra*); lanes 8 & 9 are digested with *Eco*RI; lane 10, pT181 undigested; lanes 8-10 act as negative controls; lane 12, p3358; lane 13, p3356; lane 14, p2948; lane 15, p2947; lanes 12-15 are digested with *Eco*RI.
b) Autoradiograph of the gel in (a) hybridised with pSK397.Δ*Xba*I, which contains the majority of the *tra* conjugative region and pUC18 which has homology to fragments in both 1kb and Raoul ladders.

Figure 3.7 A Southern blot of plasmids p2947 and p2948 hybridised with the conjugative probe pSK397.2.4



a) Digested DNA: lanes 1, 11 & 12 1kb ladder; lane 2, pSK397.2.4 digested with *Eco*RI and *Pst*I; lane 3, pT181 digested with *Hin*dIII (negative control); lane 4 is empty; lanes 5-7 contain p2948, digested with *Bgl*II, *Eco*RI and *Pvu*II respectively; lanes 8-10 contain p2947 digested with *Bgl*II, *Eco*RI and *Pvu*II respectively.
b) Autoradiograph of photograph in (a) hybridised with pSK397.2.4, which contains sequences from within the *tra* region and pUC18 which has homology with fragments of the 1kb ladder.

3.6 Hybridisation of mupirocin resistance plasmids with insertion sequences

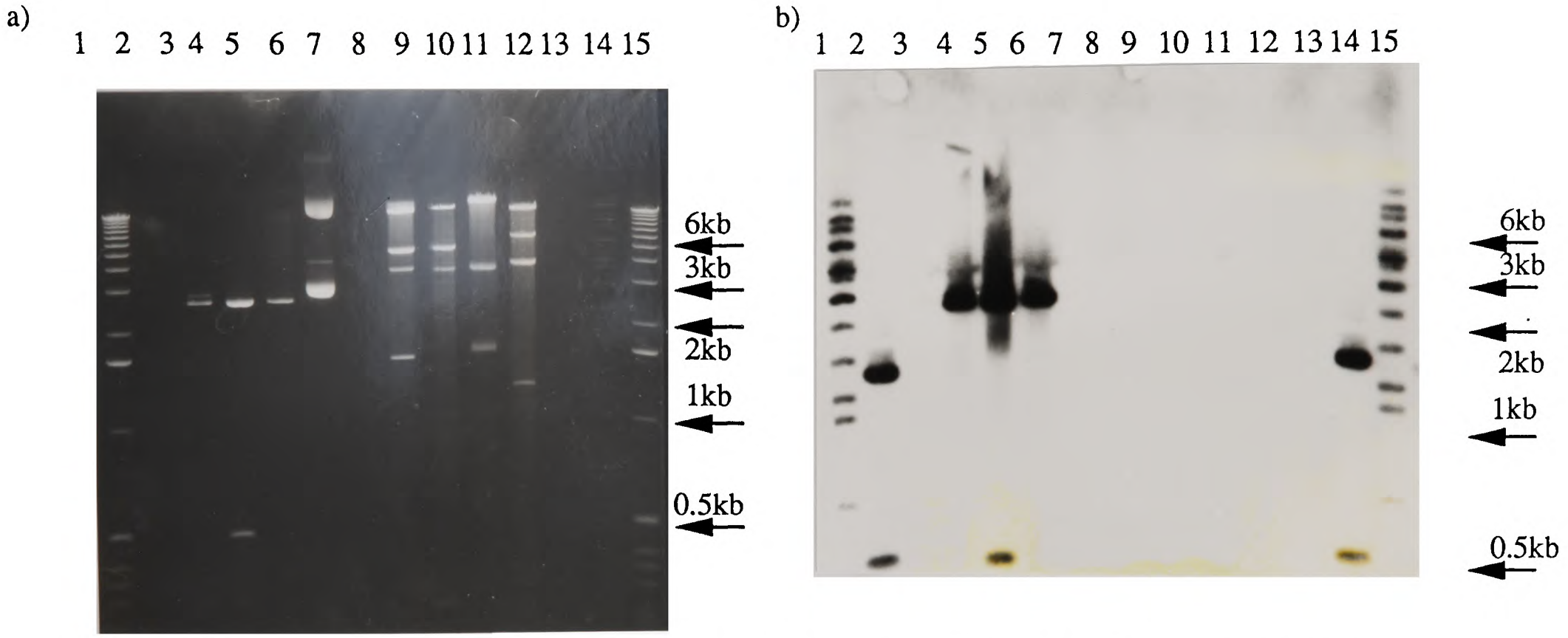
Many antibiotic resistance determinants in staphylococci appear to be on transposons, both composite and not (for review see Lyon and Skurray, 1987). Many are described as putative transposons as they have not been demonstrated to transpose. Only two insertion sequences had been found to be associated with (putative) staphylococcal composite transposons: IS*256* (eg Tn*4001*, Byrne *et al.*,1990) and IS*257* (e.g. Tn*4003*, Rouch *et al.*, 1989). Therefore it was decided to probe Mu^R plasmids for homology to these insertion sequences to investigate the potential for *mup*A to be associated with transposition.

A pUC18 clone of 491bp internal to the putative transposase of IS*256* (pIP1551, Dyke *et al.*,1992) was digested with *Eco*RI and *Pst*I to separate the cloned region from the vector, and the whole digest used to probe Southern blots of restriction digests of the plasmids p2947, p2948 and p3356. IS*256* failed to hybridise to any of the plasmids, the results of the p3356 hybridisation are shown in Fig.3.8.

Using an *Eco*RI/*Pst*I digest of a pUC18 clone of 629bp from within the putative transposase of IS*257* (pIP1644, V.Loncle personal communication) to probe of Southern blots of restriction digests of the Mu^R plasmids p2710, p2795, p2991, p3274, p2947, p2948 and p3356, all seven plasmids hybridised (Figs.3.9 & 3.10). Fragment sizes of the digests are given in Table 3.2 and the fragments which hybridised are highlighted. Fragments homologous to the IS*257* transposase were of varied sizes for both *Eco*RI and *Bgl*II digests, suggesting that they were on the sections of the plasmids which were diverse from each other (Section 3.4). However in some cases the bands which hybridised to the IS*257* probe had also hybridised to the *mup*A probe (Section 3.3), also highlighted in Table 3.2.

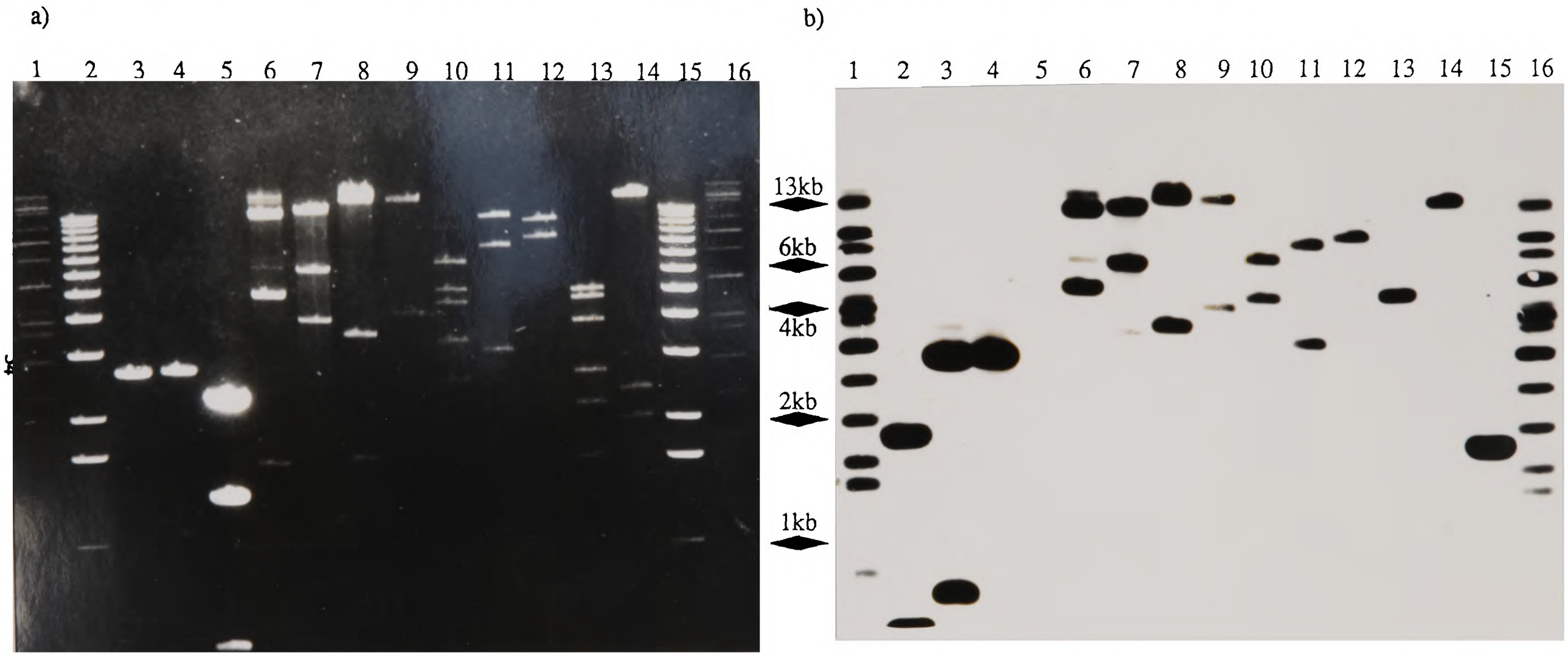
Hybridisation data allowed the positioning of the IS*257*s on the restriction enzyme maps of p2947, p2948 and p3356 (Fig.3.11). The positions of the IS*257*s on the plasmids varied with respect to *mup*A. Plasmid p2947 had only one copy of IS*257* and that was mutated in at least as much as it had lost its *Bgl*II restriction site. Plasmid p2948 however had two IS*257*s

Figure 3.8 A Southern blot of p3356 hybridised with a probe of IS*256*



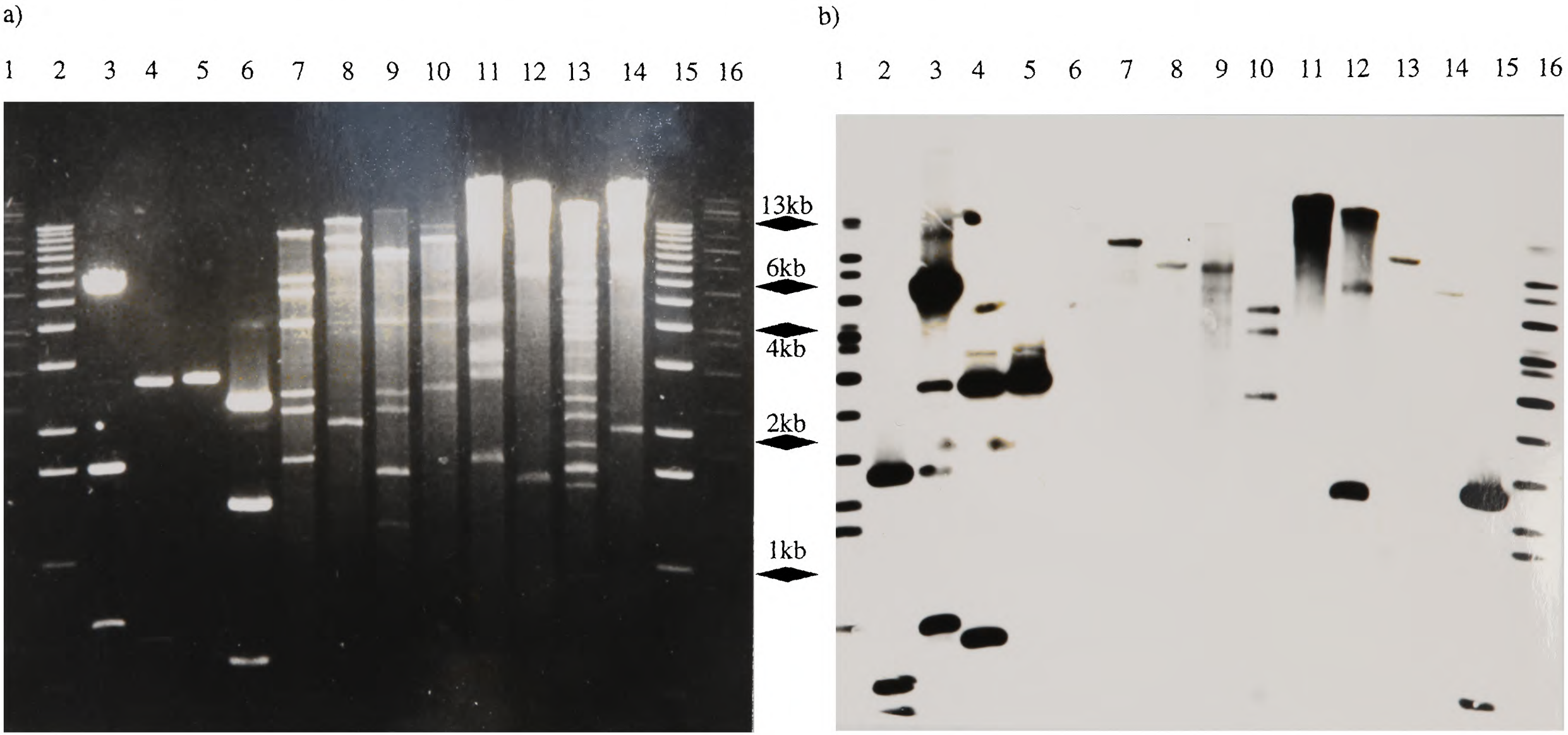
a) Digested DNA: lanes 1 & 15, Raoul ladder; lanes 2 & 14, 1kb ladder; lanes 3, 8, & 13 empty; lane 4, pIP1644 digested with *Eco*RI and *Hin*dIII; lane 5, pIP1551 digested with *Eco*RI and *Hin*dIII; lane 6 pUC18 digested with *Eco*RI; lane 7, pT181 uncut as negative control; lanes 9-12 contain p3356, digested with *Nco*I, *Hin*dIII, *Bgl*II and *Eco*RI respectively.
b) Autoradiograph of the gel in (a) probed with pIP1551, whih contains IS*256* sequences and pUC18 which has homology to both 1kb and Raoul ladders.

Figure 3.9 A Southern blot of mupirocin resistance plasmids hybridised with the IS*257* probe



a) Digested DNA: lanes 1 & 16, Raoul ladder; lanes 2 & 15, 1kb ladder; lane 3, pIP1644 (clone of IS*257* transposase internal sequences) digested with *Eco*RI and *Pst*I; lane 4, pUC18 digested with *Eco*RI; lane 5, pT181 digested with *Hin*dIII (negative control); lanes 6-8 contain p3356; lanes 9-11 contain p2948; lanes 12-14 contain p2947; lanes 6, 9, & 12 are digested with *Nco*I; lanes 7, 10, and 13 are digested with *Eco*RI; lanes 8, 11, & 14 are digested with *Bgl*II.
b) Autoradiograph of the gel in (a) hybridised with pIP1644 (which contains sequences internal to the IS*257* transposase and pUC18 sequences which have homology to fragments in both the 1kb and Raoul ladders.

Figure 3.10 A Southern blot of mupirocin resistance plasmids hybridised with the IS*257* probe



a) Digested DNA: lanes 1 & 16, Raoul ladder; lanes 2 & 15, 1kb ladder; lane 3, pMZ2 (clone of *mup*A) digested with *Eco*RI and *Pst*I; lane 4, pIP1644 (clone of internal transposase sequences of IS*257*) digested with *Eco*RI and *Pst*I; lane 5, pUC18 digested with *Eco*RI; lane 6, pT181 digested with *Hin*dIII (negative control); lanes 7 & 8 contain p3274; lanes 9 & 10 contain p2991; lanes 11 & 12 contain p2795; lanes 13 & 14 contain p2710; lanes 7, 9, 11 & 13 are digested with *Eco*RI; lanes 8, 10, 12 & 14 are digested with *Bgl*II.
b) Autoradiograph of the gel in (a) hybridised with pIP1644 (which contains sequences internal to the IS*257* transposase and pUC18 sequences which have homology to fragments in both the 1kb and Raoul ladders.

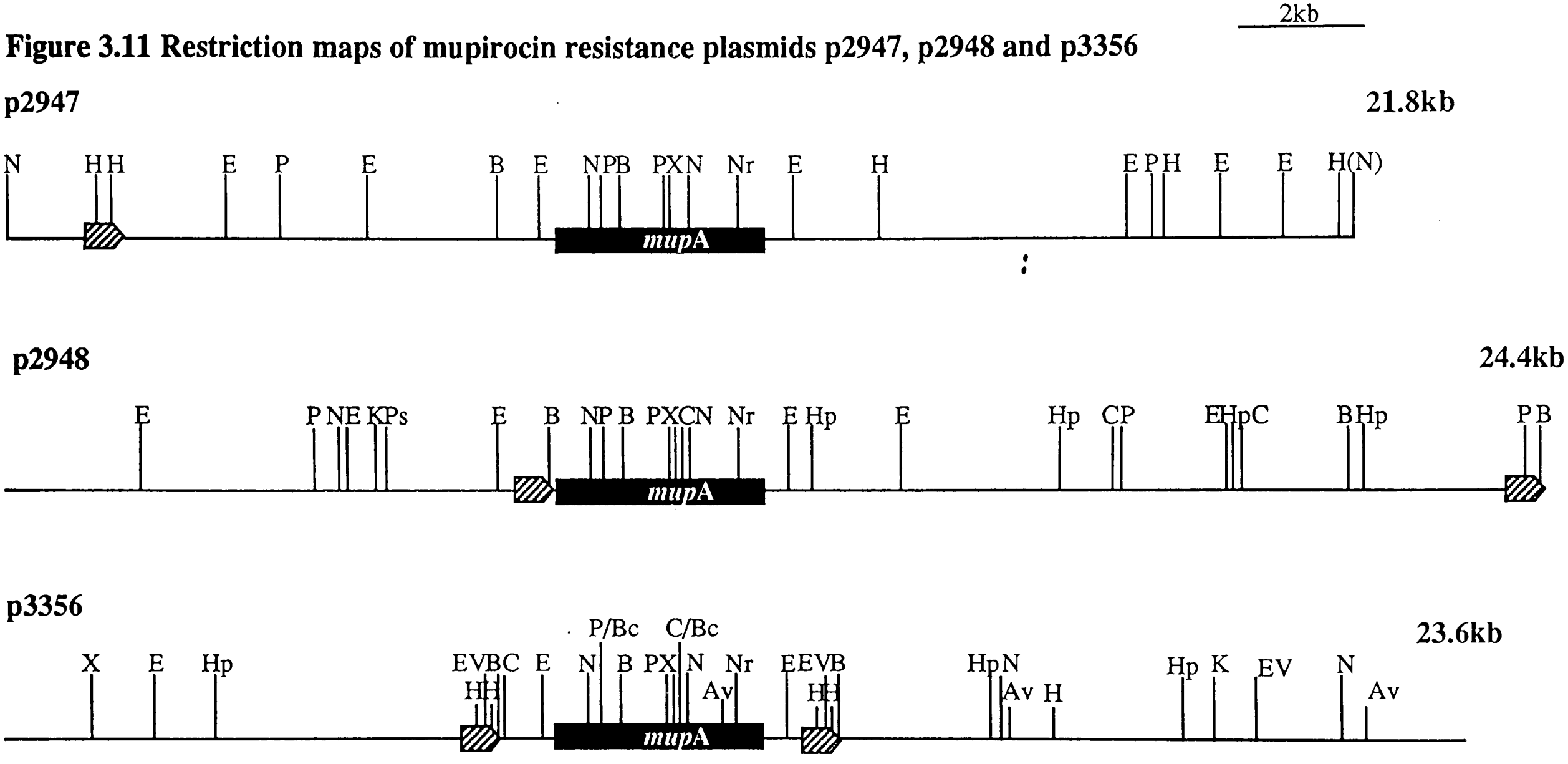
Table 3.2 Fragment sizes of restriction enzyme digestions of mupirocin resistance plasmids.

Plasmid	p2947		p2948		p3356	
Enzyme	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II
Fragment sizes (kb)	5.7 **5.0** 4.05 2.9 2.3 1.8 (1.0)	**>18** 2.65 2.0	**6.7** 5.1 **4.7** 3.4 2.65 (1.7)	c.11 **7.7** **3.2**	**c.13** **6.3** 4.05	**>18** **3.6** 1.6

Plasmid	p2710		p2795		p2991		p3274	
Enzyme	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II	*Eco*RI	*Bgl*II
Fragment sizes (kb)	>18 c.14 **c.10** 6.2 5.2 5.1 4.5 4.05 3.9 3.6 2.7 2.4 2.1 1.8 1.65 1.6 1.5 (0.9)	>18 c.15 c.14 9.5 **7.0** 2..0	**>18** **c.11** 4.8 3.6 3.4 3.0 1.7	**≥18** c.18 c.18 **6.5** **1.6**	**8.0** 4.05 2.5 2.3 1.6 1.6 1.3	9.5 **5.0** **4.0** **2.6**	**9.5** 6.2 5.4 4.05 2.5 2.3 1.6	c.12 9.0 **7.5** 2.1

Sizes (kb) are a consensus from several gels and are estimated within 0.1kb for fragments <7kb, within 0.3kb for those 7-10kb, and within 0.5kb for those over 10kb (indicated as c.*x*kb). Fragments which hybridised to *mup*A probe, pMZ2, are underlined, n.b. the *Bgl*II digests of p2947, p2948 and p3356 were not probed with pMZ2 (Figs.3.1 & 3.2); those in bold type hybridised to the IS*257* probe, pIP1644 (Figs. 3.9 & 3.10). Faint fragments are shown in brackets.

Figure 3.11 Restriction maps of mupirocin resistance plasmids p2947, p2948 and p3356



Plasmid DNAs are linearised so as to allow alignment of *mup*A (black box). IS*257*s are denoted by hatched box arrows.
Restriction enzyme sites are:
Av=*Ava*II, B=*Bgl*II, Bc=*Bcl*I, C=*Cla*I, E=*Eco*RI, EV=*Eco*RV, H=*Hin*dIII, Hp=*Hpa*II, K=*Kpn*I, N=*Nco*I, Nr=*Nru*I, P=*Pvu*I, Ps=*Pst*I and X=*Xho*I.

one on either side of *mup*A. IS*257*s also flanked *mup*A in p3356 in direct repeat but differently spaced from it than the IS*257*s in p2948.

Should the IS*257*s be part of a composite transposon with *mup*A, it would be expected that their positions would be constant with respect to *mup*A in the various plasmids; this is clearly not the case for p2947, p2948 and p3356 and thus it is not likely that *mup*A has spread amongst the staphylococcal plasmid population as part of a composite transposon with IS*257*. However, the possibility that the "IS*257*-*mup*A-IS*257*" cassette of a plasmid such as p3356 may be able to act as a composite transposon cannot be ruled out should at least one of the IS*257*s be active.

3.7 Nucleotide sequence of the regions flanking the IS*257*s of p3356

Transposition of an insertion sequence is usually accompanied by the duplication of the target site of insertion so that the newly transposed IS is directly flanked by short direct repeats (DRs), the length of which is characteristic of the IS (Grindley and Reed, 1985). Although transposition had not been demonstrated for IS*257*, some copies of IS*257* flanked by 8bp direct repeats had been observed and thus assumed to have arrived at their present location relatively recently by transposition (Rouch *et al.*, 1989; Matthews *et al.*, 1990; Byrne *et al.*, 1991).

The lack of a constant arrangement of the IS*257*s with respect to *mup*A in the various Mu^R plasmids (Section 3.6) suggested that *mup*A had not spread as part of a composite transposon. However this does not preclude the possibility of either such a composite transposon forming as a result of IS transposition to positions flanking *mup*A, or of the IS*257*s themselves transposing into or out of the plasmid. It was decided to sequence the flanking regions of the IS*257*s of p3356 to look for 8bp direct repeats as evidence of recent transposition.

The sequences of several IS*257*s had been published (Barberis-Maino *et al.*, 1987; Byrne *et al.*, 1990). Therefore oligonucleotides (J1IS257 and J2IS*257*) were designed from regions of

IS*257* homology close to the ends of the IS and pointing out of the IS (Fig.3.18, Section 3.9) to be used as primers to sequence out of the ISs and into their flanking regions. However, as there are two copies of IS*257* on p3356, it was not possible to use the whole plasmid as a substrate for these sequencing reactions as there would be two different sites of primer annealing (one in each of the upstream and the downstream IS*257*s) and thus two sequences would be generated in the extension reactions, giving indecipherable gels. Thus instead, clones of each IS*257* or sections of them, containing only one site for each primer to bind, were used as substrates for the sequencing reactions. The clones generated for this and the direction of sequencing from each one are given in Fig 3.12.

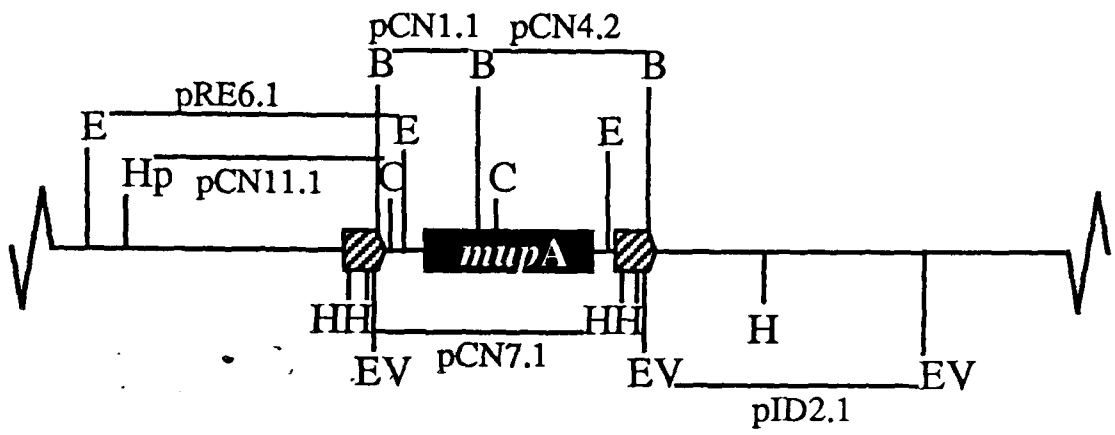
The flanking sequences generated are given in Fig.3.13. These sequences were used to design oligonucleotides in the opposite orientation (i.e. pointing in towards the IS*257* from the flanking regions) to allow sequencing of the opposite strand, thus confirming the initial sequence data. The positions of these oligonucleotides (UPIN, UPOUT, DNOUT and DNIN) are given in Fig.3.13.

There were no 8bp direct repeats found flanking either the putative composite transposon or either of the IS*257*s individually. The sequences of each of the four 8bp flanking the IS*257*s are sufficiently different from each other to show that no recent transposition involving the IS*257*s had occurred to generate the plasmid observed (Fig 3.13).

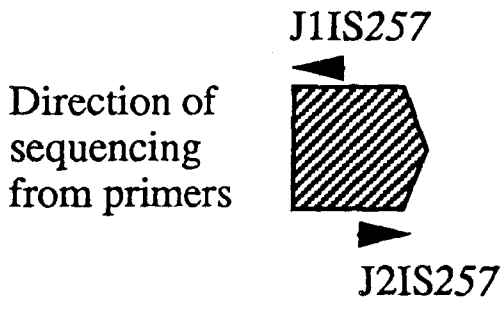
3.8 Nucleotide sequence of the regions flanking IS*257*s of p2947 and p2948

Oligonucleotides J1IS*257* and J2IS*257* (Fig 3.18) were used as primers to sequence out from the 4.55 and 4.65kb *Eco*RI clones of the IS*257*s of p2947 and p2948 respectively (these clones had been made previously in the laboratory). As with the ISs of p3356, there was no evidence of an 8bp direct repeat flanking either IS and thus no evidence of duplication of the target site (Fig.3.14).

Figure 3.12 Schematic diagram of the region of p3356 from which clones were derived for sequencing the IS*257*s of p3356



Clone	Fragment	Vector	Vector site	Primers used
pCN1.1	1.6kb *Bgl*II (B)	pOX7	*Bam*HI	J2IS*257*
pCN4.2	3.6kb *Bgl*II (B)	pOX7	*Bam*HI	J1IS*257*
pRE6.1	6.3kb *Eco*RI (E)	pUC18	*Eco*RI	J1IS*257* & J2IS*257*
pCN11.1	5.2kb *Hpa*II/*Cla*I (Hp/C)	pUC18	*Acc*I	J1IS*257*
pCN7.1	5.2kb *Hin*dIII (H)	pUC18	*Hin*dIII	J1IS*257* & J2IS*257*
pID2.1	6.5kb *Eco*RV (EV)	pUC18	*Sma*I	J2IS*257*



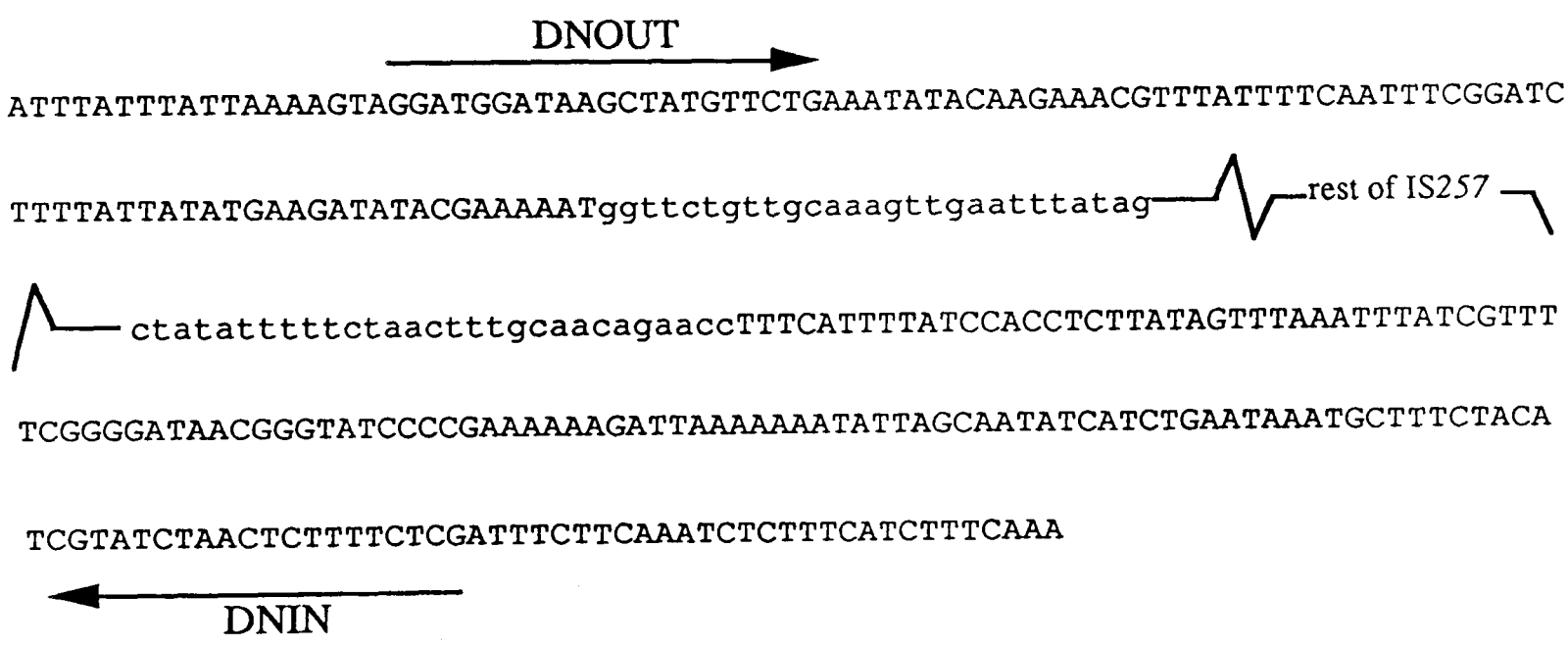
Positions of cloned fragments are given by horizontal lines between the enzyme sites at the ends of each clone, the names given above and below the lines, and the sites used are given in the table. The primers are given in Fig.3.18. Hatched boxed arrows show the positions and direction of IS*257*; a black box denotes *mup*A.

Figure 3.13 Sequences flanking the IS*257*s of p3356

(i) Sequences flanking the IS*257* upstream of *mupA* in p3356



(ii) Sequences flanking the IS*257* downstream of *mupA* in p3356



(iii) Schematic diagram of the "IS*257*-*mupA*-IS*257*" region of p3356, showing each 8bp directly flanking the IS*257*s are given



(i) Shows the flanking sequences of the IS*257* upstream of *mupA* in capitals and the inverted repeats of IS*257* in lower case, the positions of the primers UPIN and UPOUT used to check the sequence of the opposite strand, are given by arrows along the sequence (the strand shown is the one in the direction of transcription of the putative transposase). (ii) as (i) but for the IS*257* downstream of *mupA* (iii) summarises the 8bp which flank the IS*257*s clearly demonstrating that they are NOT direct repeats.

Figure 3.14 Sequences flanking the IS*257*s of p2947 and p2948

(i) 8bp sequences flanking the IS*257* of p2947



(ii) 8bp sequences flanking the IS*257* of the 4.7kb *Eco*RI fragment of p2948



The 8bp sequences which flank the IS*257*s (hatched box arrows) of the plasmids p2947 and p2948 are shown not to be direct repeats, with only one base pair in identical positions on each side (denoted by an arrow).

Unlike in most Mu^R plasmids where *mup*A is on a 4.05kb *Eco*RI fragment (Rahman *et al.*, 1993; Connolly *et al.*, 1993), *mup*A of p2948 lies on a 4.7kb *Eco*RI fragment. Hybridisation studies had shown that this 4.7kb fragment shared homology with IS*257* sequences (Section 3.6). Nucleotide sequencing of the 4.7kb *Eco*RI clone of this fragment using oligonucleotides J1IS*257* and J2IS*257* (Fig. 3.18) showed that both ends of the IS*257* were intact within this piece (Further sequencing and restriction mapping suggests that the whole IS*257* is intact, Fig. 3.18, Section 3.9). A comparison of the flanking sequences of this IS*257* with the published sequence of the smaller 4.05kb *Eco*RI fragment of p2947 (Hodgson *et al.*, 1994) showed identity of the p2948 sequence downstream of the IS*257* to *mup*A with the 157 bases upstream of *mup*A in the p2947 4.05kb *Eco*RI clone (Fig. 3.15). Thus it appeared that IS*257* had inserted between bases 319 and 320 of an *Eco*RI fragment such as that of p2947 to generate the *Eco*RI fragment of p2948. However, the sequence upstream of the IS*257* on the 4.7kb p2948 fragment showed no homology with the first 319bp of the 4.05kb *Eco*RI fragment of p2947 (Fig. 3.15). Therefore the evolution of p2948 from its precursor involved more than a mere insertion of an IS into a plasmid containing the "typical" 4.05kb *Eco*RI fragment of p2947. As the DNA downstream of this IS*257* is identical to sequences of p2947, whilst the DNA upstream of the IS*257* is so divergent, it is most likely that the DNA either side of that IS*257* in p2948 is from different sources.

Insertion sequences and transposons can have a polar effect on the expression of the genes downstream of the site of integration by providing (-35) sequences which form a hybrid promoter with (-10) sequences downstream of the transposon (Galas and Chandler, 1989; Charlier *et al.*, 1982; Dodd *et al.*, 1994; Prentki *et al.*, 1986; Wood *et al.*, 1991). IS*257* contains within its N-terminal inverted repeat partial promoter sequences (the "-35" region) for the expression of the IS's putative transposase. Due to the inversion of this sequence, the C-terminal IR also contains these sequences but directed out of the IS towards the sequences downstream of it. It has been suggested that this -35 sequence can act in conjunction with potential -10 sequences appropriately spaced downstream of the IS and thus form a hybrid promoter responsible for transcription of downstream sequences (Rouch *et al.*, 1989; Leelaporn *et al.*, 1994).

Figure 3.15 A comparison of the sequences flanking the IS*257* upstream of *mup*A in p2948 with the sequence upstream of *mup*A in p2947



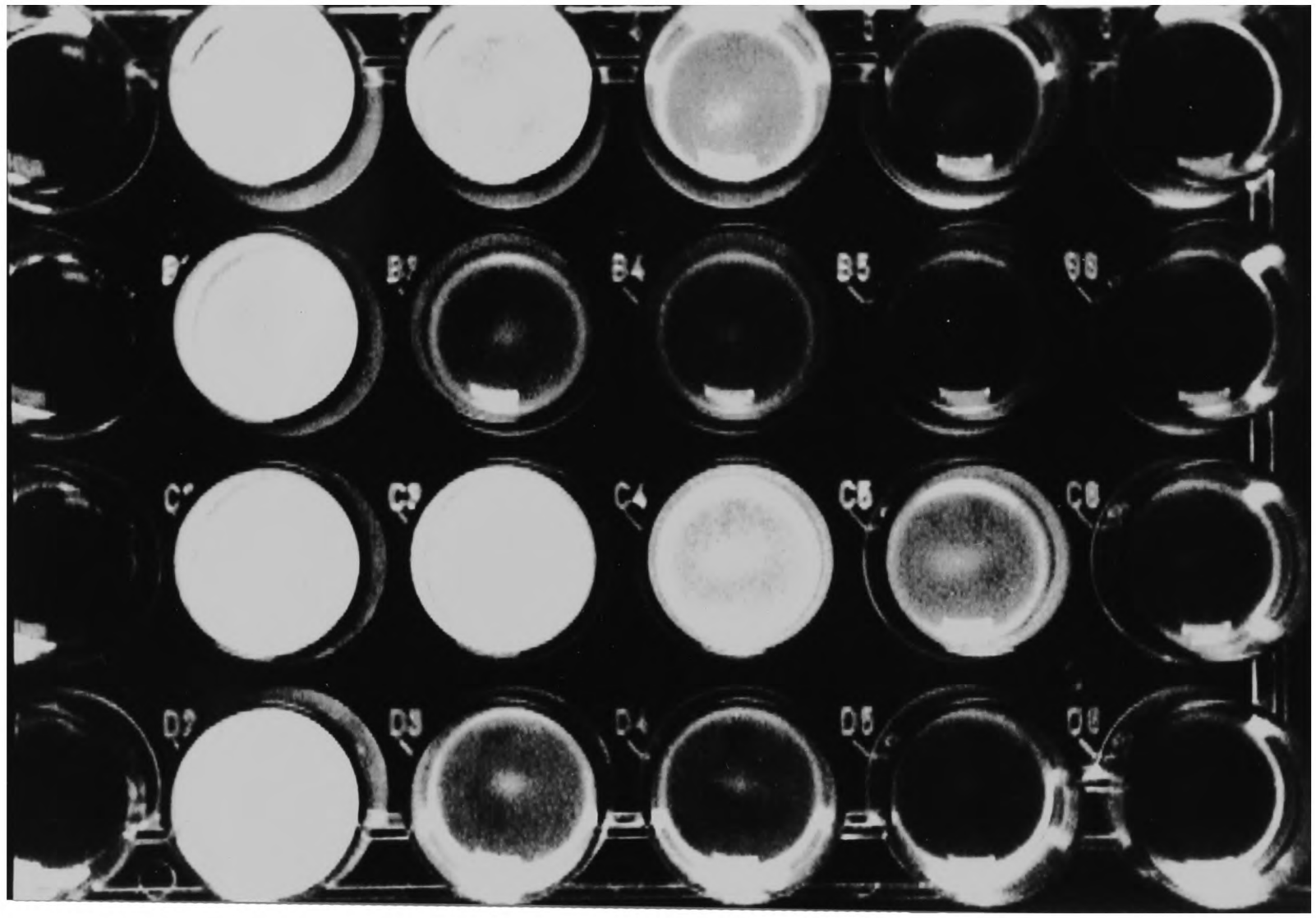
The DNA sequences from this region of p2947 (upper row) are aligned with those of p2948 (lower row) by leaving a gap at the apparent point of integration (between 319 & 320) in p2947. Sequences of the inverted repeats of the IS*257* are given in lower case, but the rest of the IS*257* omitted for simplicity. Numbering refers to the 4.05kb *Eco*RI fragment of p2947 (Hodgson *et al.*, 1994). The positions of potential promoter sequences are given: -35/-10(IS) show the putative hybrid promoter between the IS*257* and the downstream sequences of p2948. If the -10 of this putative hybrid promoter were active in p2947, an approximately placed -35 sequence would be needed upstream of it, labelled [-35(IS)]. The suggested promoter of Hodgson *et al.*, 1994 is shown as -35/-10(H).

The IS*257* integrated just upstream of *mup*A in p2948 is oriented so that a potential -35 sequence faces out toward the start of *mup*A. This opened the possibility that the IS played a role in promoting the expression of *mup*A in p2948 using a promoter such as that postulated in Fig.3.15 (-35(IS) and -10(IS)), and therefore possibly increasing the expression of *mup*A from this plasmid relative to wild type. As it was not possible to compare the MICs of the two strains as they are resistant to the highest concentrations of mupirocin obtainable in solution, it was decided to compare the relative mupirocin resistances of RN2677(p2947) and RN2677(p2948) in growing cultures. Overnight cultures were diluted 10-fold and their OD_{675} followed until early log-phase when a known quantity of cells (1.375×10^8 and 2.75×10^8) was used to inoculate broth of varied concentrations of mupirocin in a multidish, to give a final volume of 1ml in each case. The multidish was then incubated with gentle shaking for 24h at 37°C. The density of cell growth at each concentration was then compared, Fig.3.16. It appeared that RN2677(p2948) was able to grow in approximately four times the concentration of mupirocin as RN2677(p2947) under these conditions, although after a further 24h incubation, cultures had grown in even the highest mupirocin concentration wells. The result was repeatable on four separate occasions. As both plasmids were resident in the same strain, the apparent difference in mupirocin resistance was inherent to the plasmids, either due to a difference in the levels of expression of *mup*A on the two plasmids or due to a difference in the number of copies of *mup*A in the two strains, i.e. a difference in copy number of the two plasmids.

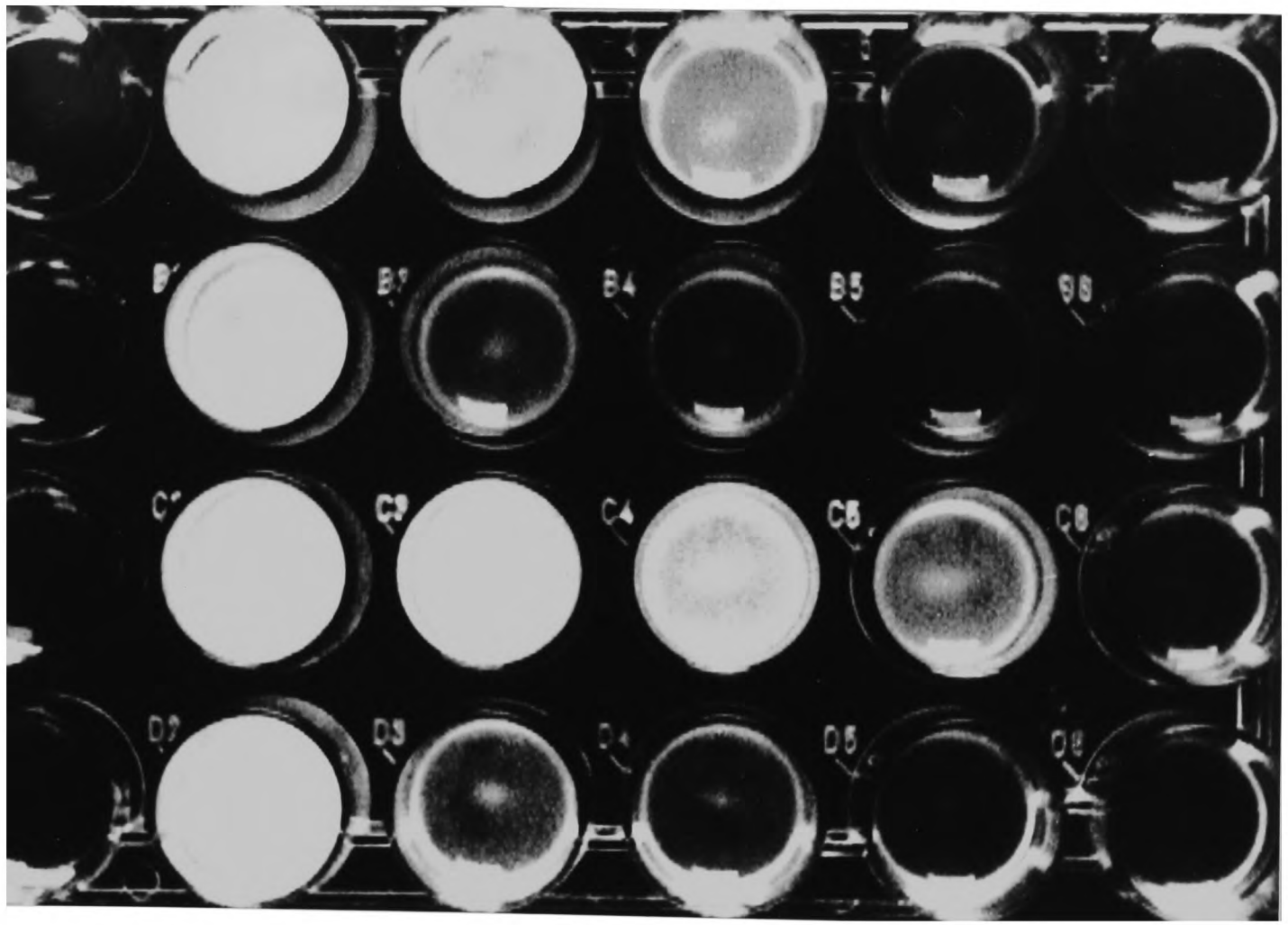
In order to determine relative copy number of the two plasmids, an equal quantity of early log-phase cells of each strain was taken (as determined by OD_{675}) and used to prepare total DNA. The concentration of the DNAs was measured by OD_{260} (purity of the two samples was approximately the same as determined by OD_{280}). An equal quantity of each DNA sample and dilutions of them were digested with *Nco*I and electrophoresed on an agarose gel which was subsequently Southern blotted. The blot was then hybridised with a pUC18 clone of the 1.6kb *Nco*I fragment internal to *mup*A. The original photograph and the autoradiograph can be seen in Fig.3.17. Unfortunately a glance shows that the method used to determine DNA concentration was not sufficiently accurate; it is probable that the chromosomal DNA was not entirely dissolved and thus false readings were obtained. However, the concentration of DNA from the strain containing p2947 was greater than that

Figure 3.16 **A comparison of the relative resistances of RN2677(p2947) and RN2677(p2948) to mupirocin**

Culture added	Quantity of cells by OD_{675}	Concentration of mupiroin (mg ml^{-1})					
		0 (no cells)	0	4	8	16	32
RN2677(p2948)	$1.375x10^8$						
RN2677(p2947)	$1.375x10^8$						
RN2677(p2948)	$1.375x10^8$						
RN2677(p2947)	$1.375x10^8$						



Culture added	Quantity of cells by OD_{675}	Concentration of mupirocin (mg ml^{-1})				
		0 (no cells)	0	4	8	16	32
RN2677(p2948)	$1.375x10^8$						
RN2677(p2947)	$1.375x10^8$						
RN2677(p2948)	$1.375x10^8$						
RN2677(p2947)	$1.375x10^8$						

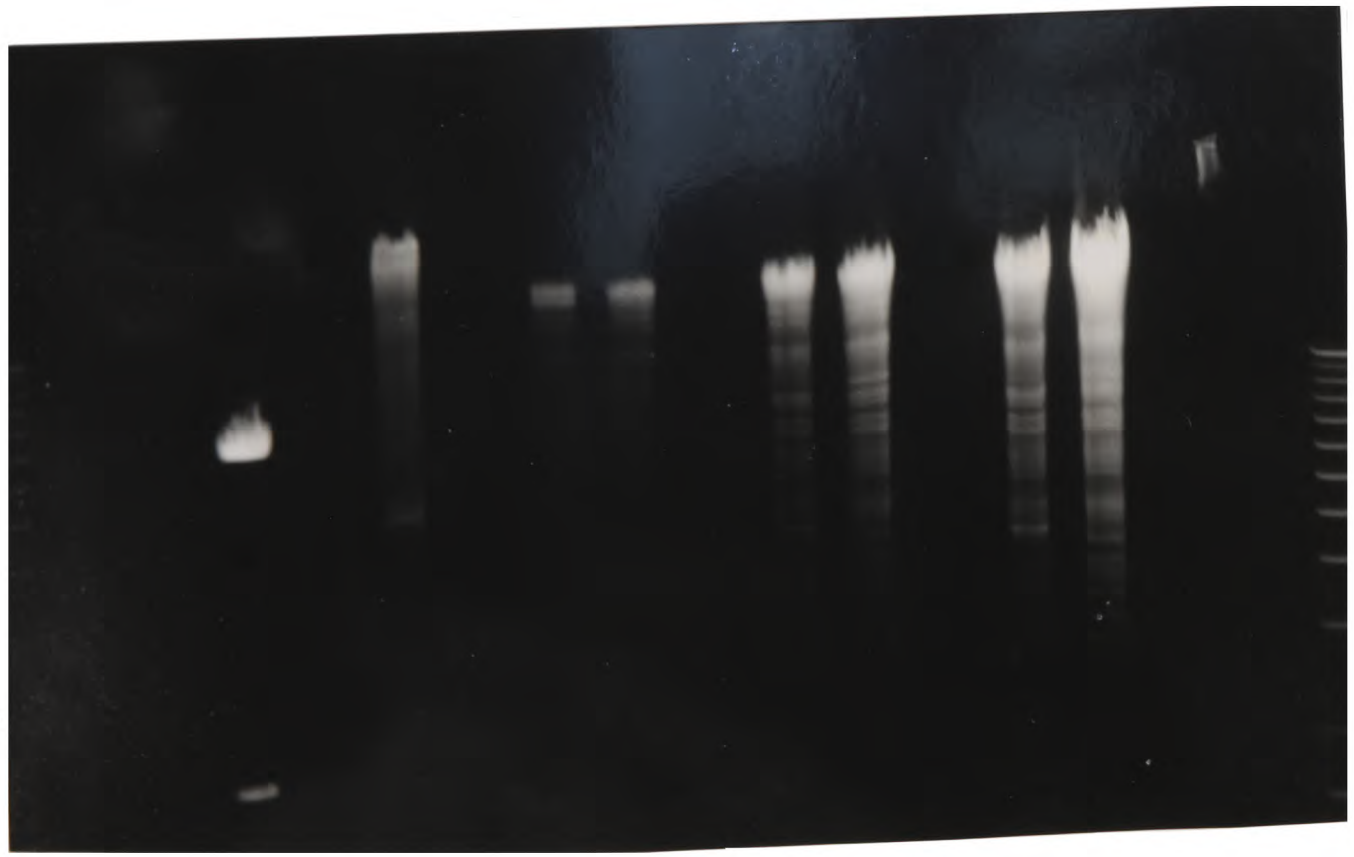


Photographs of the two sets of cultures after 24h growth as described in Section 3.8. No cells were added to the first well of each row.

Figure 3.17 A comparison of the relative copy-numbers of plasmids p2947 and p2948 by Southern blot

a)

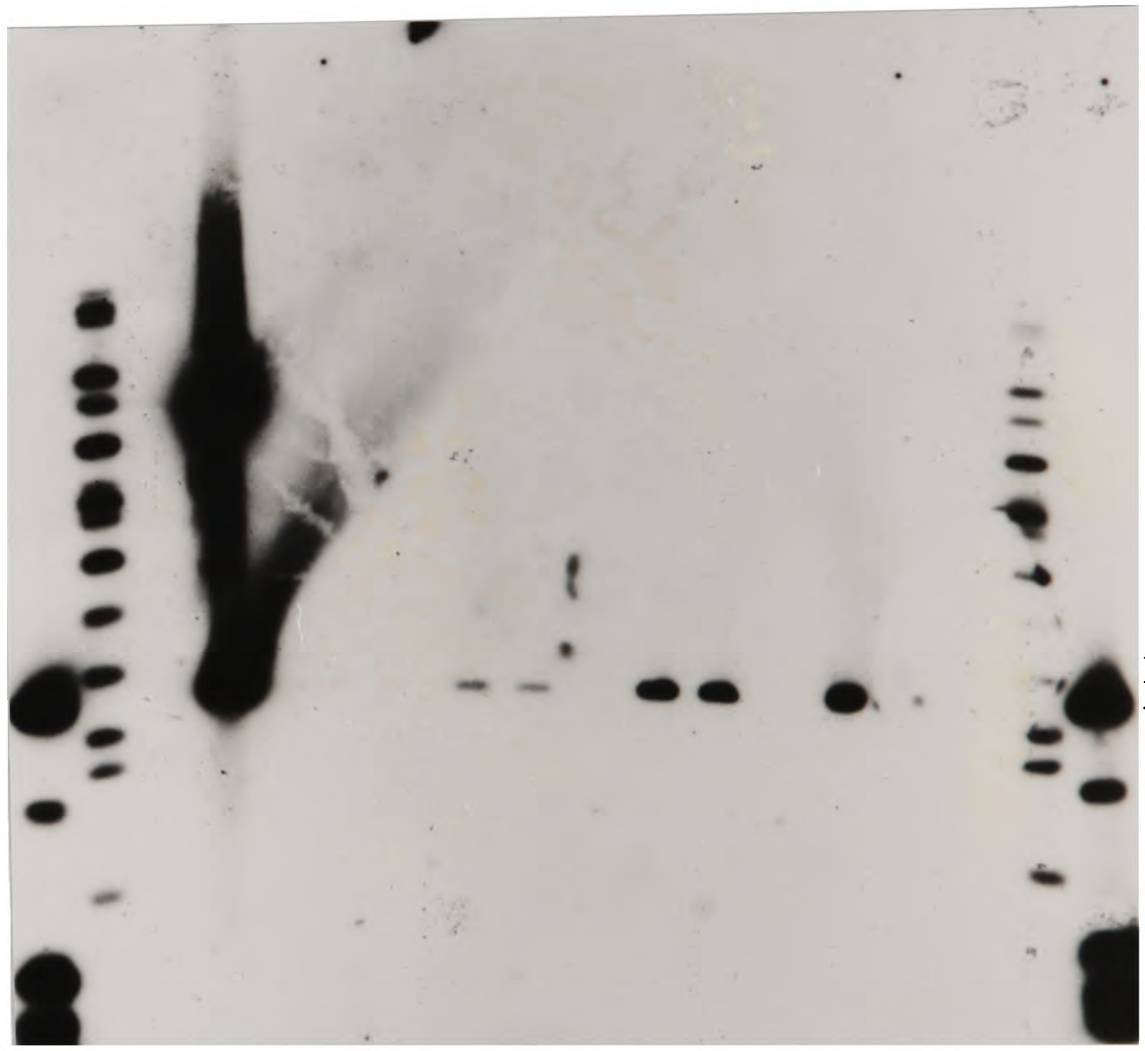
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



1.6kb

b)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



1.6kb

a) Digested DNA: lane 1, 1kb ladder; lane 2, Raoul ladder; lanes 3, 5, 7 & 10 are empty; lane 4, pMZ2 digested with *Eco*RI and *Pst*I; lane 6, 8325 chromosome digested with *Eco*RI; lanes 8, 11 & 14 contain p2948; lanes 9, 12 & 15 contain p2947; all p2947 and p2948 digestions are with *Eco*RI.
b) Autoradiograph of the gel in (a) hybridised with pMZ2, which contains the 1.6kb *Nco*I fragment internal to *mup*A and pUC18 which has homology to fragment of both 1kb and Raoul ladders.

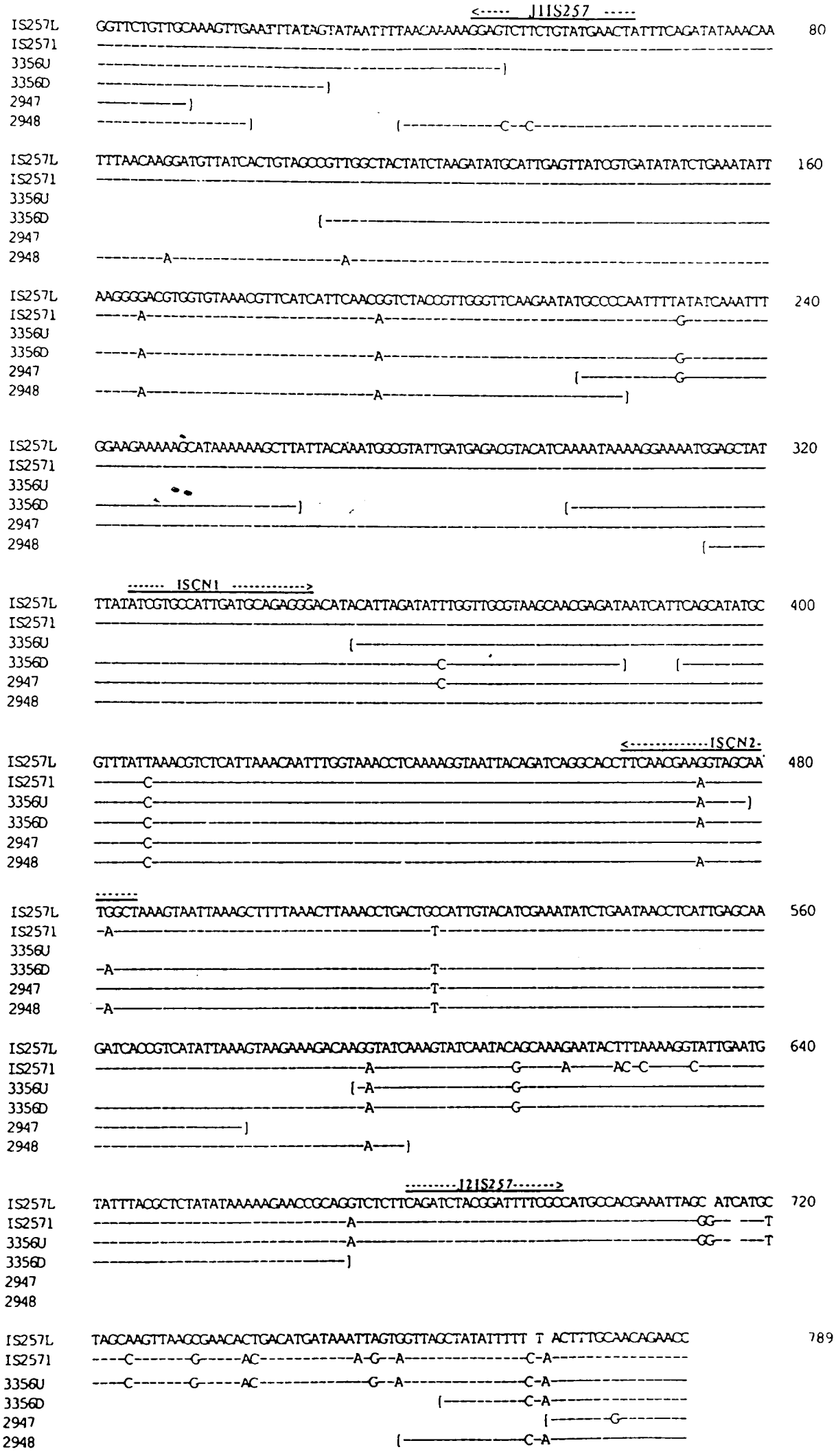
from the strain containing p2948, whilst the probe hybridised more strongly to p2948 DNA than that of p2947. Thus, the apparent increase in expression of *mup*A in p2948 than p2947 is likely to be due to a higher plasmid copy number of p2948 as compared to p2947, rather than a difference in expression of the gene itself. Thus even if IS*257* is providing promotion for *mup*A, it is not greatly different to the wild type level of promotion. It should also be noted that the point of integration of IS*257* in the 4.7kb *Eco*RI fragment of p2948, and thus any -10 sequence of a hybrid promoter is different to the suggested position of the wild type promoter (Fig.3.15; Hodgson *et al.*, 1994).

3.9 Nucleotide sequences of IS*257*s of mupirocin resistance plasmids

In the course of searching for sequence data of the regions which flank the IS*257*s in Mu^R plasmids, some of the sequences internal to the IS*257*s have been generated. Additional information as to these sequences has been gained from the use of two oligonucleotides designed from the central region of the putative transposase (ISCN1 and ISCN2, see Fig 3.18). The IS*257* *Eco*RI clones of p2947 and p2948 (Section 3.8) and the clones of p3356 given in Fig 3.12, were used as substrates for sequencing. The sequences are aligned with IS*257*L and IS*2571* (Byrne *et al.*, 1990) in Fig.3.18.

As the sequences are not complete, no comment can be made as to the possible activity of the putative transposase. However it is interesting to note that many of the differences in sequence which distinguish IS*2571* from IS*257*L are in the sequences of these Mu^R plasmid ISs. Indeed all the sequence available for p3356 ISs is the equivalent of either that of IS*257*L or IS*2571* except for the replacement of nucleotide 362 (ATT_{362} to ATC) in the downstream IS which maintains codon usage as Ile. This base change is also seen in the IS*257* of p2947, where again it is the only one known to differ from either IS*257*L or IS*2571* within the ORF, although there is another change in the C-terminal IR. As for the partially sequenced IS*257* from p2948, two base changes within the ORF are known which differ from both IS*257*L and IS*2571* but they still maintain codon usage (AAG_{89} to AAA, Lys; GTT_{110} to GTA, Val). That nucleotide changes are such that codon usage is maintained

Figure 3.18 Aligned sequences of the IS*257*s of mupirocin resistance plasmids



The sequences of the IS*257*s of p3356, that of p2947, and the 4.65k *mupA* fragment of p2948 are aligned. The standard ACGT four-letter code is used. Where the sequences differ from IS*257*L, the base is noted. When sequences are identical to IS*257*L, a dash is given. The ends of the known sequences are bracketed[]. The positions of primers designed from the IS*257*L sequence are given by arrows above the sequence.

further supports the idea that the ORF codes for an active transposase, as predicted by sequence homologies.

3.10 The search for potential ORFs flanking *mup*A in p3356

The gene product of *mup*A is an isoleucyl-tRNA synthetase (Dyke *et al.*, 1991; Hodgson *et al.*, 1994). Prokaryotic genes including some aminoacyl tRNA synthetases are often found in operons (Grunberg-Manago, 1983). In Gram negative bacteria the isoleucyl-tRNA synthetase gene is found in an operon with lipoprotein signal peptidase, although this is not the case for *Staphylococcus aureus, Methanobacterium thermoautotrophicum* or yeast (Chalker *et al.*, 1994). It is possible that the original genes that flanked *mup*A, and may have been part of an operon with it, still flank it either in part or entirety.

Sequence analysis of the initial 4.05kb *Eco*RI clone of *mup*A derived from p2947, pOX301 (Dyke *et al*, 1991), had revealed open reading frames flanking *mup*A in the same direction of transcription as *mup*A (ORFB and ORFC, Fig.3.19), but no significant homologies of these ORFs to other proteins had been found by database searches (Hodgson *et al.*, 1994). The IS*257*s of p3356 are close (within 0.5kb) to the 4.05kb *Eco*RI fragment common to p2947 and p3356. Therefore the sequences flanking the IS*257*s of p3356 were searched for ORFs which may be continuations of the ORFs found in the 4.05kb *Eco*RI clone of p2947.

The sequence flanking the C-terminus of the IS*257* upstream to *mup*A (X in Fig.3.19) contained two open reading frames which remained open to the end of the known sequence, and therefore could be the start of the ORF found upstream of *mup*A in the *Eco*RI clone (B in Fig. 3.19). Likewise, the sequence flanking the N-terminus of the IS*257* downstream of *mup*A (Y in Fig. 3.19) contained three ORFs open from the beginning of the known sequence, and therefore could be the continuation of the ORF found downstream of *mup*A in the *Eco*RI clone (C in Fig. 3.19).

The predicted protein translated product of these sequences was used in the GCG.TFASTA package to search the GenEMBL protein database. No significant homologies were found.

Figure 3.19 A schematic diagram of the "IS*257*-*mup*A-IS*257*" region of p3356 showing the regions flanking the IS*257*s which were tested for ORFs with the potential to form operons with *mup*A



Regions B & C were shown to contain ORFs B & C respectively (Hodgson *et al.*, 1994). Regions X & Y were tested for ORFs which may be continuations of ORFs B & C. Hatched boxed arrows represent IS*257*s; the black box represents *mup*A.

Neither were any DNA homologies found using the GCG.FASTA package to search the GenEMBL DNA database. Therefore no potential proteins or RNAs have been found that can be inferred as being part of an operon with *mup*A. Due to the diverse nature of the plasmids within a short distance from *mup*A, it appeared futile to look for ORFs further away from *mup*A than the IS*257*s of p3356.

3.11 Summary

Initial characterisation of several Mu^R plasmids has confirmed that resistance to mupirocin is due to the product of one gene, *mup*A, which nearly always exists on a constant 4.05kb *Eco*RI fragment on otherwise very diverse plasmids which range in size from c.20-90kb. These plasmids appear conjugative in filter mating, and some of them have homologies to known staphylococcal transfer genes; those which do not are thought to conjugate by an alternative, as yet unidentified mechanism being investigated by other groups. Sequence analysis has failed to identify potential genes which may have formed the basis of an operon with *mup*A in its original location.

The first example of linkage of *mup*A with another resistance determinant, tetracycline resistance, was found in p3358. The clustering of resistance determinants is a great problem in *Staphylococcus aureus* leading to multiply resistant strains which respond to few antibacterial therapies. IS*257* had been implicated as one possible route by which the resistance determinants gather in multiply resistant *Staphylococcus aureus* (Rouch and Skurray, 1989).

All the Mu^R plasmids tested carry one or more copies of IS*257*, including those that were isolated in Nigeria as early as 1965 (p2991 and p3724). Although there is no obvious composite transposon, nor evidence for recent transposition of the individual ISs, it is possible that the IS*257*s have played a role in dispersing *mup*A onto such a broad range of plasmids. Support for this comes from the comparison of p2948 with p2947, which suggests that the IS*257* just upstream of *mup*A in p2948 lies at the junction of DNA from two different sources.

It was to investigate the role of IS*257* in the evolution of Mu^R plasmids that research was directed.

Duplication of the Mupirocin Resistance Gene

Chapter Four

Duplication of the mupirocin resistance gene

4.1 Introduction

Initial studies had demonstrated that IS*257* was frequently associated with mupirocin resistance plasmids. Insertion sequences are mobile genetic elements and have a great capacity to elicit genetic rearrangements such as transposition, gene duplication, replicon fusion and adjacent deletions (for review see Galas and Chandler, 1989). IS*257* (alias IS*431*) had attained the status of an "insertion sequence" by virtue of the fact that it occurred ubiquitously in plasmids and chromosomes of *Staphylococcus aureus* and *Staphylococcus epidermidis* and had the structure of an insertion sequence, with an ORF which encoded a putative transposase (Barberis-Maino *et al.*, 1987). IS*257* had never been demonstrated to transpose or cause other rearrangements typical of insertion sequences, although it had been implicated in replicon fusion by the occurrence of an integrated plasmid flanked by IS*257* in direct repeat in the chromosome of *Staphylococcus aureus* (Gillespie *et al.*, 1986) and in gene amplification of the *mec* region of the chromosome of methicillin resistant *S.aureus*. Copies of IS*257* are also found flanking genes so as to form potential composite transposons (Byrne *et al.*, 1990; Rouch *et al.*, 1989). In both these examples IS*257* was merely inferred to have been involved in past events rather than demonstrated to be active.

Routine investigations of *mup*A plasmids revealed that IS*257* was involved in gene duplication.

4.2 Duplication of the "IS*257*-*mup*A" cassette *in vitro*

The restriction map of p3356 is given in Section 3.4, Fig.3.4. Whilst mapping p3356, a complication had arisen. Later preparations of DNA from 8325(p3356) appeared to contain larger plasmids than the earlier preparation had displayed: this often appeared as extra restriction fragments in digestions of the later DNA preparation as compared to that of the

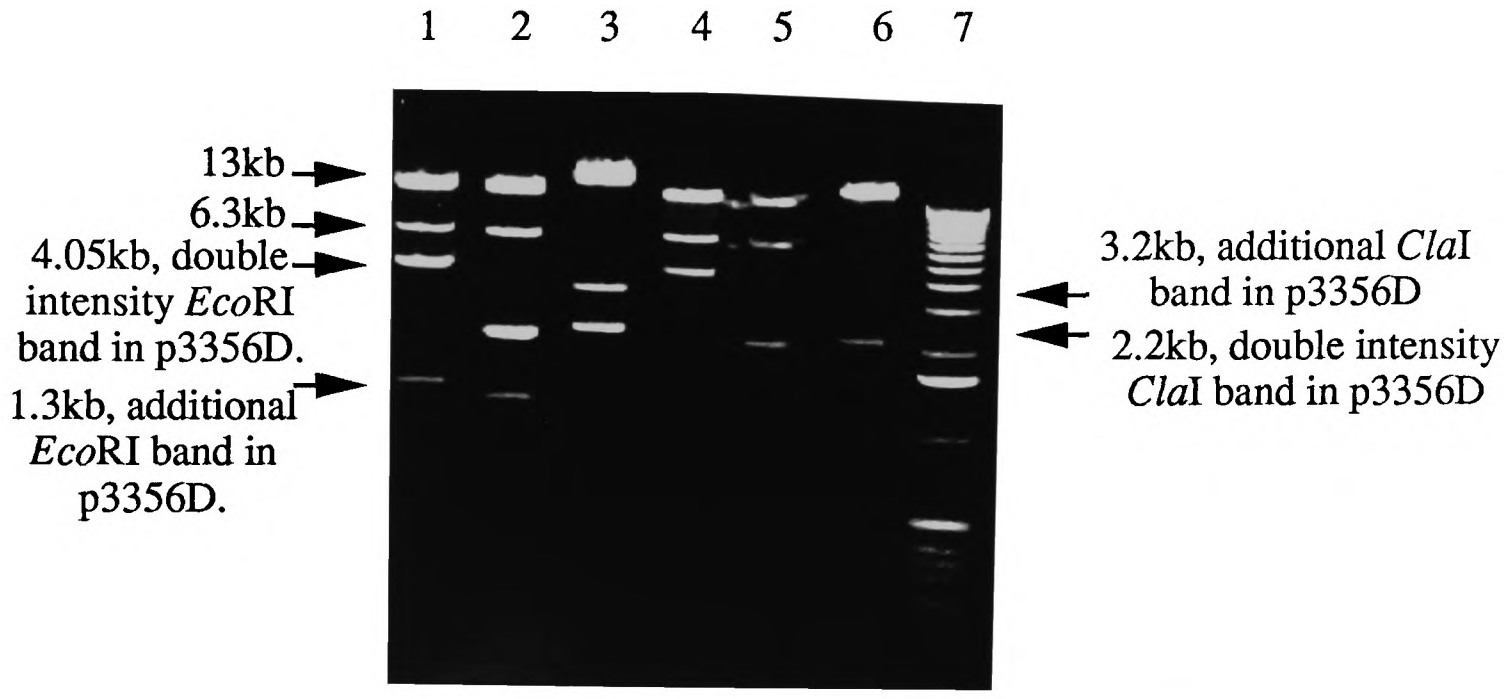
earlier one. A comparison of the restriction products of earlier and later preparations is given in Fig.4.1. Whereas *Eco*RI digestion of the earlier preparation (p3356) had yielded fragments of sizes 4.05, 6.3 and c.13kb, only, *Eco*RI digestion of later preparations yielded an extra 1.3kb fragment in addition to these. Likewise *Cla*I digestion of the earlier preparation had given only two fragments (2.2kb and a very large fragment), but an extra 3.2kb fragment was generated by *Cla*I restriction of later preparations.

Considering that no fragments were missing from digestions of later preparations as compared with those of the earlier preparation, and as prolonged (24h) digestions of the earlier preparation with excess enzyme did not yield the extra fragments observed in digestions of later preparations, it was concluded that the DNA made later had somehow changed relative to the DNA made earlier, although ostensibly the cells from which the DNA was prepared came from the same stock. Therefore the plasmid of later preparations was designated as the derivative of p3356, p3356D.

Further digestions of the two preparations were compared (Fig.4.2a). Superficially, there was no difference in *Bgl*II digestions of p3356 and p3356D. However, p3356D *Nco*I digestions had a 3.8kb fragment missing from p3356 digestions. The additional fragments of p3356D compared to p3356 were not of equal size in the various digestions: *Cla*I digestions of p3356D had an extra 3.2kb band as compared to p3356, the extra *Eco*RI band was only 1.3kb, whereas that of *Nco*I was 3.8kb, moreover *Bgl*II digestion rendered no additional bands. As p3356D had to be larger than p3356 by a constant size it appeared that DNA was "missing" in some digestions.

Closer inspection of the digestions revealed that some fragments of p3356D were of greater intensity than would be observed if they were equimolar quantities with all bands on that gel. For instance in Fig 4.1, the 2.2kb band of *Cla*I digest of p3356D is more intense than the "new" 3.2kb band. Similarly, the 4.05kb *Eco*RI band of p3356D was more intense than the 6.3kb band; this was not so in the p3356 digestions, where the 4.05kb band was less bright than the 6.3kb band as would be expected if the bands were equimolar. Thus it was concluded that some of the restriction fragments had duplicated. With these duplicated bands included, the "extra DNA" in p3356D digestions as compared with those of p3356, was now

Figure 4.1 Restriction enzyme digestions of two preparations of DNA from 8325 (p3356)



Lanes 1-3 contain digests of DNA from the later preparation (named p3356D) and lanes 4-6 contain digests of DNA from the earlier preparation (p3356); lane 7 contains 1kb ladder. Lanes 1 & 4 are digested with *Eco*RI; lanes 2 & 5 are digested with both *Eco*RI and *Cla*I; lanes 3 & 6 are digested with *Cla*I.

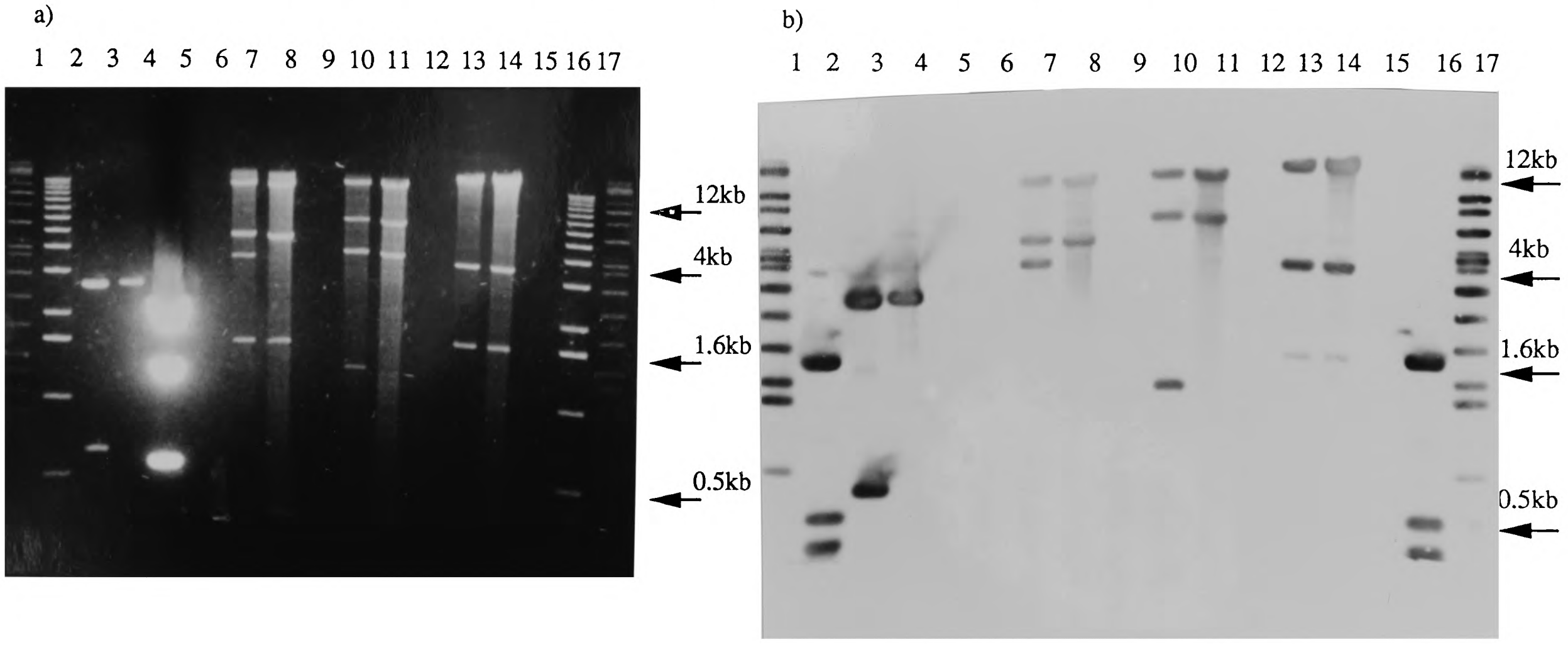
approximately the same whichever enzyme was used: *Eco*RI, 4.05 + 1.3 = 5.35; *Nco*I, 3.8 + 1.6 = 5.4; *Cla*I, 2.2 + 3.2 = 5.4 and even *Bgl*II where two bands had duplicated, 1.6 + 3.6 = 5.2.

It appeared that a duplication had arisen in p3356 to give p3356D. This duplication included *mup*A (present on the duplicated 4.05kb *Eco*RI fragment) and maintained a constant *Bgl*II restriction pattern, but varied *Eco*RI, *Nco*I and *Cla*I patterns. That the *Bgl*II pattern remained constant implied that the ends of the duplication were within the IS*257*s flanking *mup*A (the flanking *Bgl*II sites are within IS*257*). Hybridising a Southern blot of digestions of p3356 and p3356D with pIP1644 (a probe for IS*257*, Section 3.6) showed that the extra 3.8kb *Nco*I and 1.3kb *Eco*RI bands had homology to IS*257* (Fig 4.2). Further digestions allowed p3356D to be mapped (Fig 4.3) and showed the p3356D had arisen from p3356 by duplication of an "IS*257*-*mup*A" cassette in tandem repeat to the original. The IS*257*s mark the extremes of the duplicated region. This duplicated region lies within a *Hpa*II fragment 12.4kb in the unduplicated form and 17.8kb when duplicated; the relative differences of the size of the *Hpa*II bands of p3356 and p3356D are shown by hybridisation with the *mup*A probe in Fig 4.4, lanes 10 & 11. The increased size of the *Hpa*II band is diagnostic of the duplication.

4.3 Sequences flanking the IS*257*s of p3356D

The sequences flanking the IS*257*s of p3356 had been determined (Section 3.7, Fig 3.13). Comparison of these sequences with those flanking the IS*257*s of p3356D would give clues as to the mechanism of the duplication. The oligonucleotides designed to sequence into the IS*257*s from the flanking regions of p3356 (UPIN, UPOUT, DNOUT and DNIN, Section 3.7, Fig.3.13) were used to sequence in from the flanking regions of p3356D. The oligonucleotides UPIN and DNIN only have one binding site in plasmid p3356D, whereas UPOUT and DNOUT have two (Fig 4.5a). If the duplication had created any differences in the sequences generated from primers UPIN and DNIN, then a comparison of the sequences determined from p3356D with those of p3356 would reveal this difference. As UPOUT and DNOUT will each bind to two sites, a difference in the sequence generated from one of these sites would lead to two bands at each position on the gel thereafter.

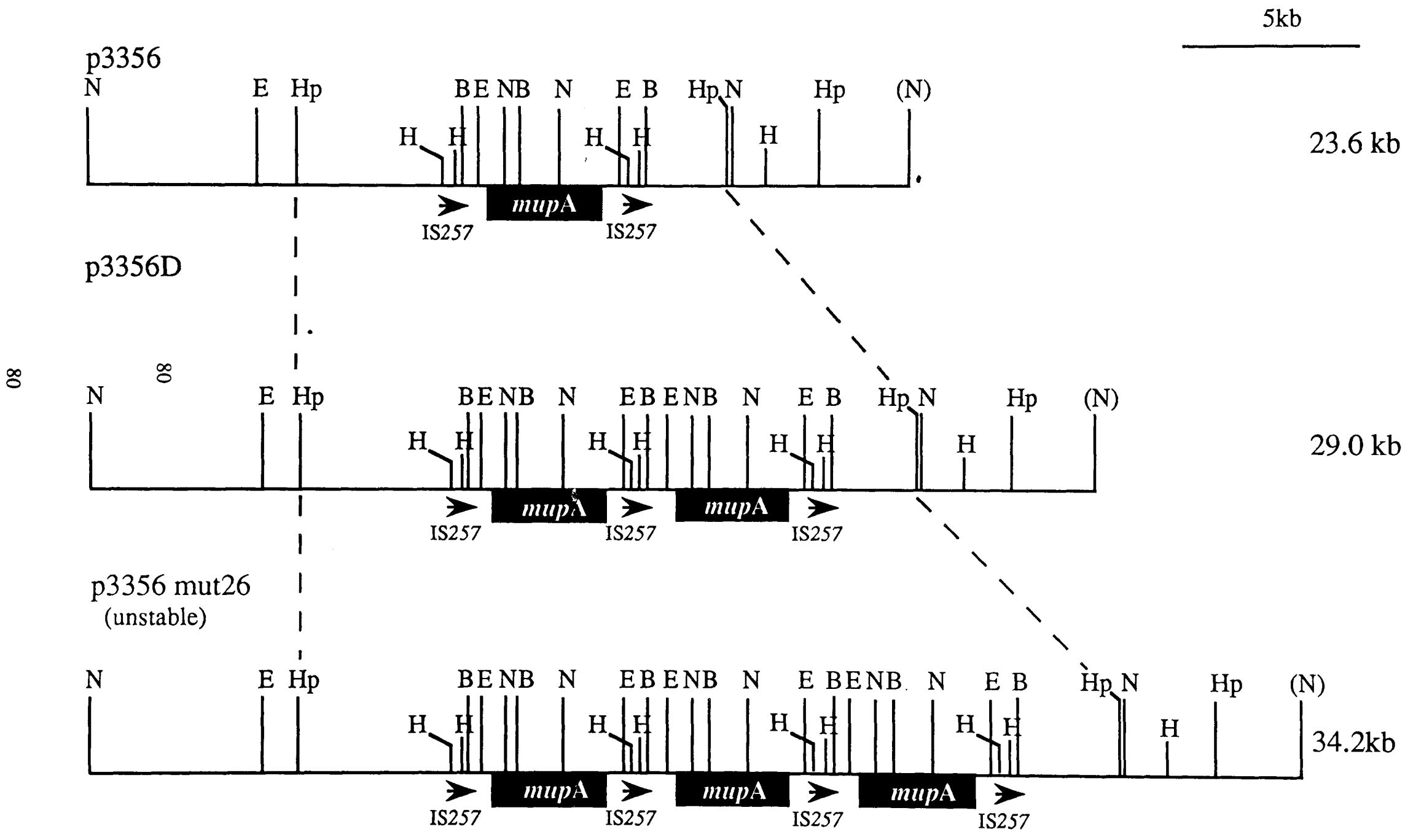
Figure 4.2 A Southern Blot of p3356 and p3356D hybridised with IS*257* sequences



a) DNA digestions: lanes 1 & 17, Raoul ladder; lanes 2 & 16, 1kb ladder; lane 3, pIP1644 digested with *Eco*RI and *Pst*I; lane 4, pUC18 digested with *Eco*RI; lane 5, pT181 digested with *Hin*dIII as a negative control; lanes 6, 9, 12 & 15 are empty; lanes 7, 10 & 13 contain p3356D; lanes 8, 11 & 14 contain p3356; lanes 7 & 8 are digested with *Nco*I, lanes 10 & 11 are digested with *Eco*RI, lanes 13 & 14 are digested with *Bgl*II.

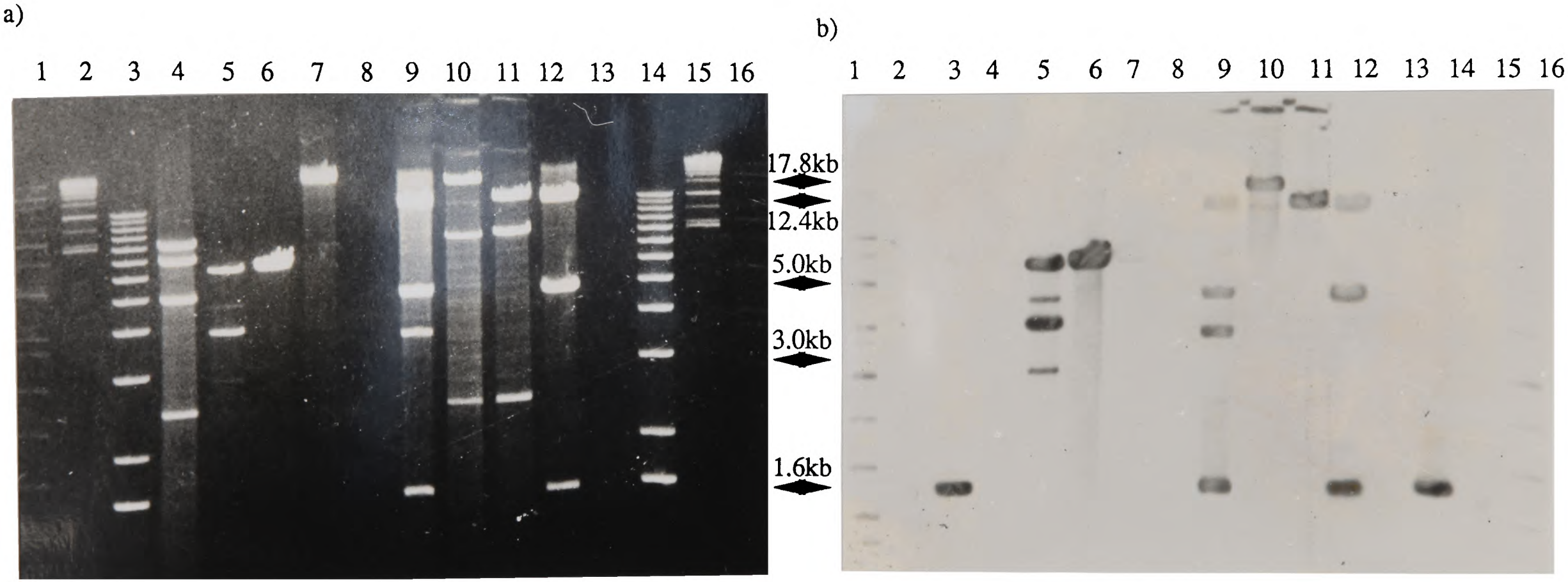
b) Auoradiograph of the gel in (a) hybridised with pIP1644 which contains sequences of the IS*257* putative transposase and pUC18 which has homology to fragments of both 1kb an Raoul ladders.

Figure 4.3 Restriction maps of p3356, p3356D and p3356mut26, plasmids related by tandem duplication



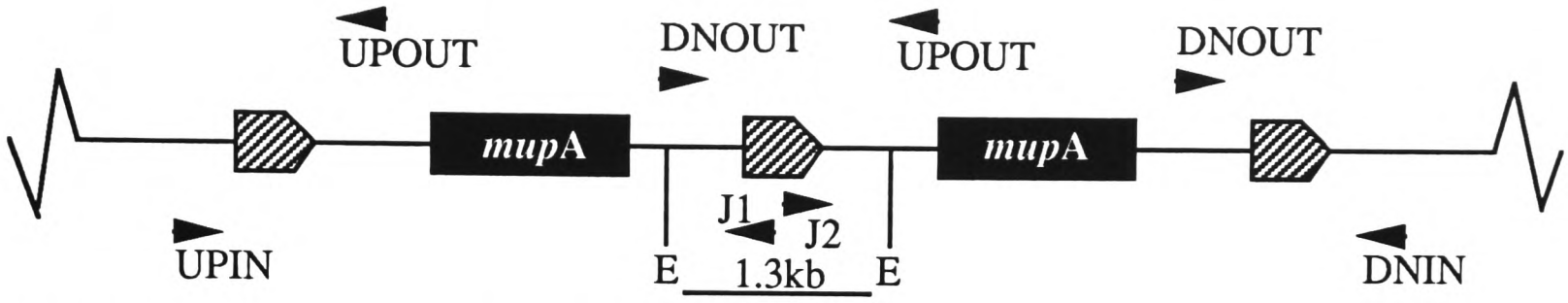
Plasmids p3356, p3356D and p3356mut26 are related by tandem duplication of the 5.4kb "*mup*A-IS*257*" cassette. The duplication between the plasmids is easily visualised by the increased size of the *Hpa*II fragment (see Fig.4.3b) emphasised here by dashed lines. The *mup*A gene is represented by a black box; arrows show the positions of the IS*257*s. Restriction enzyme sites shown: B=*Bgl*II, E=*Eco*RI, , H=*Hin*dIII, Hp=*Hpa*II and N=*Nco*I.

Figure 4.4 A Southern blot of p3356 and p3356D hybridised with the *mup*A probe

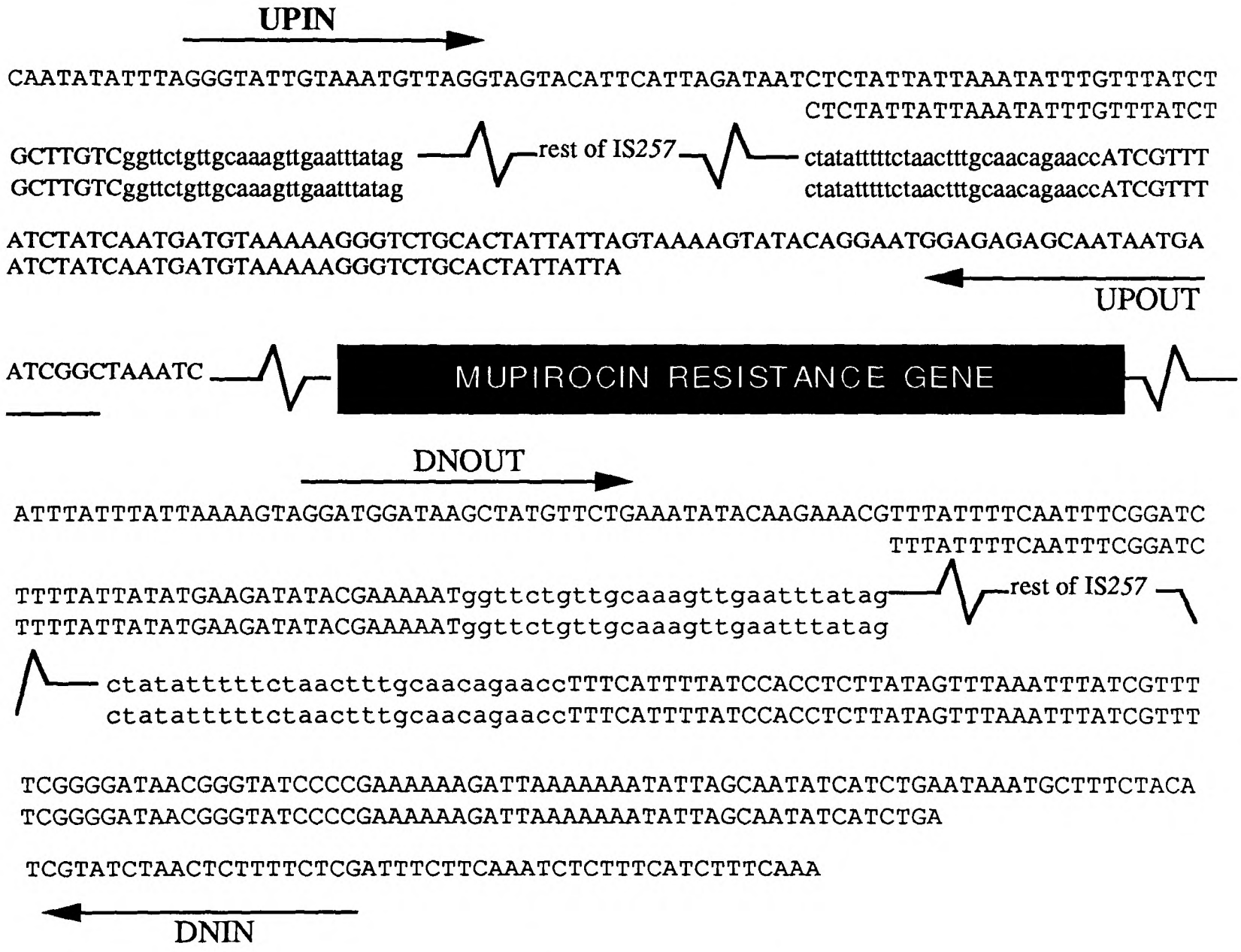


a) Digested DNA: lanes 1 & 16, Raoul ladder; lanes 2 & 15, High Molecular Weight marker; lanes 3 & 14, 1kb ladder; lane 4, p3626; lane 5, pCN5.1 digested with *Eco*RI; lane 7, pJE1 digested with *Eco*RI; lanes 8 & 13 are empty; lanes 9 & 10 contain p3356D; lanes 11 & 12 contain p3356; lanes 9 & 12 are digested with *Nco*I; lanes 10 & 11 are digested with *Hpa*II.
b) Autoradiograph of the gel in (a) hybridised with pCN5.1, which contains the 4.05 *Eco*RI *mup*A fragment of p3356 and pUC18 sequences which have homology to fragments of both 1kb and Raoul ladders.

Figure 4.5 Sequencing the flanks of the IS*257*s of p3356D



(a) Diagramatic representation of the region of p3356D containing *mup*A (black box) and IS*257* (hatched boxed arrows) sequences showing the positions of the primer binding sites as determined by homology with p3356. UPIN and DNIN only have one binding site each; UPOUT and DNOUT have two each. The position of the 1.3kb *Eco*RI clone, pCN9.1, is given: J1IS*257* and J2IS*257* primers were used to sequence from this clone.



(b) Sequences generated from p3356D (lower row) using the primers shown in (a) are aligned with those of p3356 (upper row). As there is no difference in sequence, only one "IS*257*-*mup*A-IS*257*" cassette is represented, this can be taken to be either the upstream or the downstream cassette of p3356D.

The sequences obtained from all four primers were single sequences which were the same as those determined for p3356 (Fig 4.5b). Therefore no change of DNA sequence had occurred during the duplication of p3356 to p3356D in the terminal regions of the IS*257*s and their flanks.

4.4 Duplication of the "IS*257-mup*A" cassette *in vivo*

Plasmid p3356 had been isolated from a mupirocin resistant strain of *Staphylococcus aureus* taken from a patient. Three other Mu^R plasmids had been isolated from strains of other patients of the same ward, p3355, p3357 and p3358 (Needham *et al.*, 1994). Restriction enzyme digestion patterns of p3355 were indistinguishable from those of p3356, and thus the two plasmids were considered the same. The restriction enzyme digestion profile of p3357 was identical to that of p3356D, concluding that these two plasmids were also fundamentally the same and that p3357 contained the tandem repeat duplication of the "IS*257-mup*A" cassette. The plasmid profile of the original strain from which p3357 had been transferred (J3332) did not contain a plasmid of the size of p3356 (23.6kb) but did contain a plasmid of the size of p3356D (29.0kb); p3357 had never restricted to give patterns found from p3356, suggesting that p3357 had contained the duplicated "IS*257-mup*A" cassette in the original clinical strain (Needham *et al.*,1994).

4.5 Selection for duplication of the "IS*257-mup*A" cassette

As mentioned in Section 4.2, the earlier (p3356) and later (p3356D) DNA preparations were made from cells of the same original stock. However the cells had been maintained at 4°C on CY agar containing 5μg mupirocin ml^{-1} with monthly subculture to fresh plates[1] for five months in between the two preparations. Cells taken from the original -70°C glycerol stock, after this five month period, grown overnight in broth devoid of mupirocin, rendered DNA which gave restriction enzyme fragments of the p3356 type. The difference in maintenance of the cells had made a difference to the structure of the plasmids within the cells. There

[1] Each subculture entailed a 24h period of growth at 37°C.

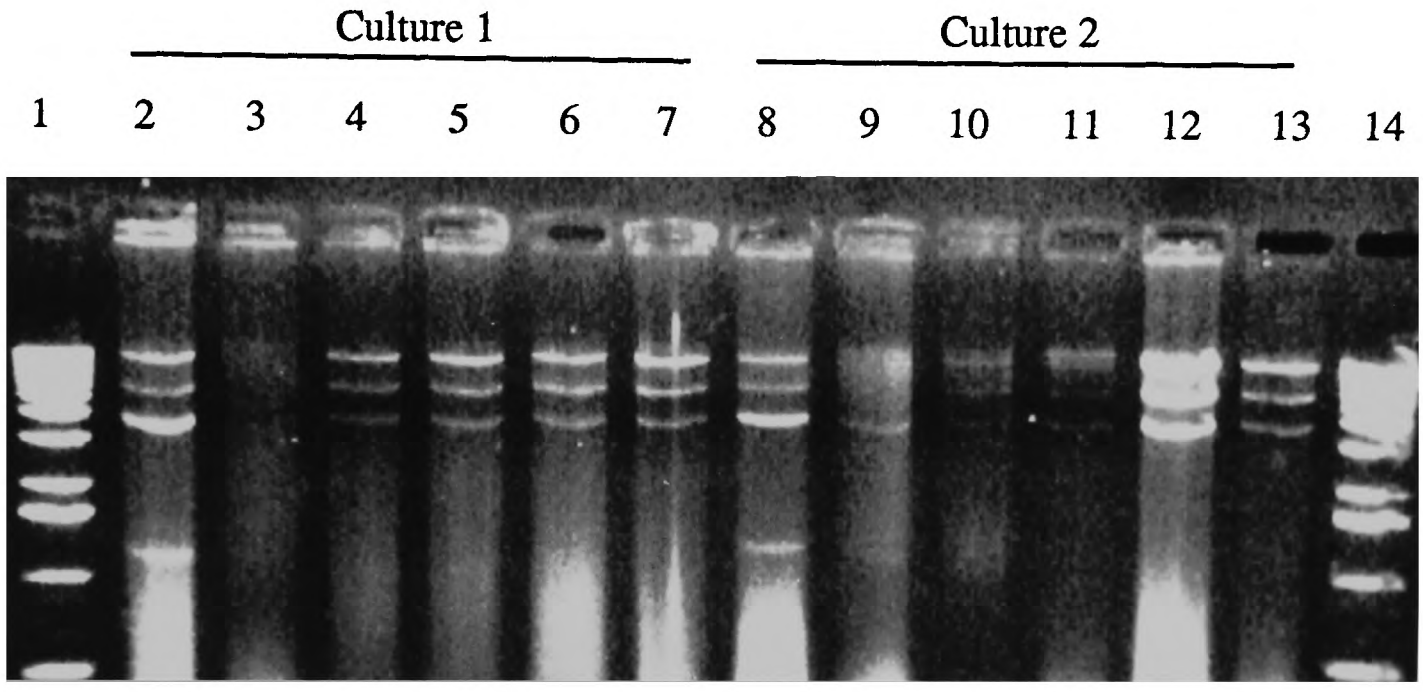
were two main differences in the maintenance procedures. Firstly the temperature: those cells kept at -70°C were not allowed to grow, whereas those at 4°C required intermittent growth at each monthly subculture[1], and thus DNA replication. Secondly, those maintained at 4°C were continually in the presence of mupirocin, but not those maintained at -70°C.

DNA replication would have been essential for the stable inheritance of any "new" DNA forms. That it alone may not be sufficient to cause the duplication within p3356 to give p3356D was demonstrated by serial dilution of cells taken from the original -70°C stock of 8325(p3356) in the absence of mupirocin (method of Section 2.16). DNA preparations made after each 10 successive generations for 50 generations were digested with *Eco*RI; the restriction patterns of all preparations were the same: no extra 1.3kb band, diagnostic of p3356D, was observed demonstrating that replication alone did not cause the duplication of the "IS*257*-*mup*A" cassette and its stable inheritance within 50 generations.

It seemed likely that the presence of mupirocin had encouraged the duplication observed and allowed its stable inheritance. Serial dilution of 8325(p3356) in the same manner, except in the presence of mupirocin confirmed this. The 1.3kb *Eco*RI band, indicative of duplication, was seen in the DNA preparation taken after 20 generations serial dilution in 200µg mupirocin ml^{-1} (Fig.4.6, lane 7). However, at the lower mupirocin concentration of 5µg ml^{-1}, no evidence for duplication was seen even after 50 generations (Fig.4.6, lanes 10 & 11). These results were reproduced on five separate occasions. As the duplication had been seen when cells had been maintained on agar containing only 5µl mupirocin ml^{-1}, it was apparent that duplication did occur in the presence of such low concentrations of mupirocin, but not as quickly as at higher concentrations. Thus it appeared that mupirocin selected for the duplication of the "IS*257*-*mup*A" cassette.

RN981 is a derivative of NCTC8325 and is the most stringent *rec*A$^-$ mutant available, with a frequency of homologous recombination reduced by a factor of 10^4 in comparison to NCTC8325 (Wyman *et al.*, 1974). Attempts were made to transform RN981 with p3356 to allow study of the requirement of *rec*$^+$ in the duplication events. Selection was made on both 1 and 5µg mupirocin ml^{-1}, and protoplast regeneration was allowed for 6h before challenging with mupirocin. After mupirocin challenge, protoplast regeneration was continued for 8 days

Figure 4.6 Restriction digests of DNA preparations taken during the serial dilution of p3356 in the presence of various concentrations of mupirocin



Lanes 1 & 14, 1kb ladder; lanes 2-7 contian DNA prepared after 50 generations of serial dilution; lanes 8-13 contain DNA prepared after 20 generation serial dilution. Cells used to prepare DNA in lanes 2, 3, 8 & 9 were serially diluted in broth containing 200μg mupirocin ml^{-1}; cells used to prepare DNA in lanes 4, 5, 10 & 11 were serially diluted in broth containing 5μg mupirocin ml^{-1}; cells used to prepare DNA in lanes 6, 7, 12 & 13 were serially diluted in the absence of mupirocin.The results of two separate cultures are displayed.

at both 30 and 37°C, colonies that grew were streaked onto CY-agar plates containing 5µg mupirocin ml^{-1}. Many of the potential transformants did not grow on the mupirocin-agar plate; the few that did were used to inoculate CY-broth and growth occurred within 24h indicating that the host strain was not RN981 which is a slow grower and requires at least 48h to produce such a culture. RN4220 was successfully used to make protoplasts, and as a recipient for the same plasmid preparation, in parallel with the failed attempts in RN981. Attempts to transduce RN981 with 'phage 53 which had been propagated on 8325(p3356) selecting on 5µg mupirocin ml^{-1} were unsuccessful, whereas transduction of RN4220 with the same 'phage rendered RN4220(p3356). Due to the lack of a *rec*A$^-$ (p3356) strain, studies of *rec* requirement in the duplication of the "IS*257*-*mup*A" cassette of p3356 were not possible.

As mupirocin appeared to encourage the duplication, mupirocin resistant strains were maintained and grown in the absence of mupirocin from this point onwards, unless otherwise stated. It remains possible that the duplication may occur in the absence of mupirocin, but this has not been observed in the laboratory to date.

4.6 Are the duplicated forms more resistant to mupirocin than the unduplicated forms?

The MIC of *mup*A strains is greater than 2mg mupirocin ml^{-1}, thus the unduplicated form would be resistant to the concentrations of mupirocin which elicit the duplication. However, as duplication was selected for by serial dilution in the presence of mupirocin, it was postulated that the strains containing duplicated plasmids may be more resistant to mupirocin than those with the unduplicated forms. The advantage selected for need not be a higher overall level of resistance but a higher immediate level of resistance, when first subject to mupirocin: initial translation from two genes would be more immediately effective than that from only one, should the expression of *mup*A not be constitutive.

As *mup*A strains are resistant to the highest concentrations of mupirocin readily obtainable in solution, a comparative measure was required. The relative resistances of the strains were

displayed as the differences in halo size of each culture when plated with a disc containing 20µl of 200mg mupirocin ml^{-1} (method of Section 2.15).

A comparison of strains containing plasmids with one or two copies of *mup*A displayed no difference in their halo sizes (all were 22mm diameter) and therefore no difference in their relative resistances to mupirocin. Single colonies grew within the halos; these were thought to be more resistant, but there was no correlation between the number of colonies in the halo and the number of *mup*A copies in the plasmid of the strain plated (Table 4.1). It was postulated that the exposure to mupirocin had "activated" (multiple) duplications of *mup*A in some cells, whether duplicated originally or not, and that being more resistant these had grown into colonies within the halo. If this was the case then pre-exposure to mupirocin would "activate" more cells and thus more would grow in the halo than those not pre-exposed.

8325(p3356) was serially diluted in the presence of 200µg mupirocin ml^{-1} (Section 2.16), cells were taken after each 10 generations and plated with a disc inoculated with 20µl of 200mg mupirocin ml^{-1} (Section 2.15). Indeed, those cells taken from later generations gave plates with more colonies within the halo (Fig.4.7) indicating that pre-exposure to mupirocin did increase the number of colonies apparently "more resistant" to mupirocin. Some of these colonies at known distance from the disc were selected, grown in CY broth and plated with a disc inoculated with 20µl of 200mg mupirocin ml^{-1} (Section 2.15). The halo diameters were 22mm and the number of colonies within the halos were similar to the plates from which the colonies had been selected. The population grown from an apparently more resistant colony was consistently as varied as the original, with a similar halo size and number of individual colonies within that halo, rather than the smaller halo size that would be expected if the population had maintained the apparent increased resistance.

Therefore, the halo size and the number of colonies growing within the halo are independent of the copy number of *mup*A in the original culture. Pre-exposure to mupirocin increases the number of colonies within the halo. Continued incubation of these plates for a further 24h at 37°C, allowed growth up to the disc for all plates, whether pre-exposed to mupirocin or not. Therefore, it appears that rather than being intrinsically more resistant to mupirocin,

Table 4.1 Summary of the relative mupirocin resistance of p3356 and its derivatives with "IS*257-mup*A" duplications

Culture	No. copies of "IS*257-mup*A" cassette+	Number of colonies in the halo	Average no.colonies in the halo
8325(p3356)	**1**	7 16 9 7 53	**18**
8325(p3356D)	**2**	57 61 4 7 39	**34**
8325 (p3356mut17)*	**4**	17 55 24 27 16	**28**

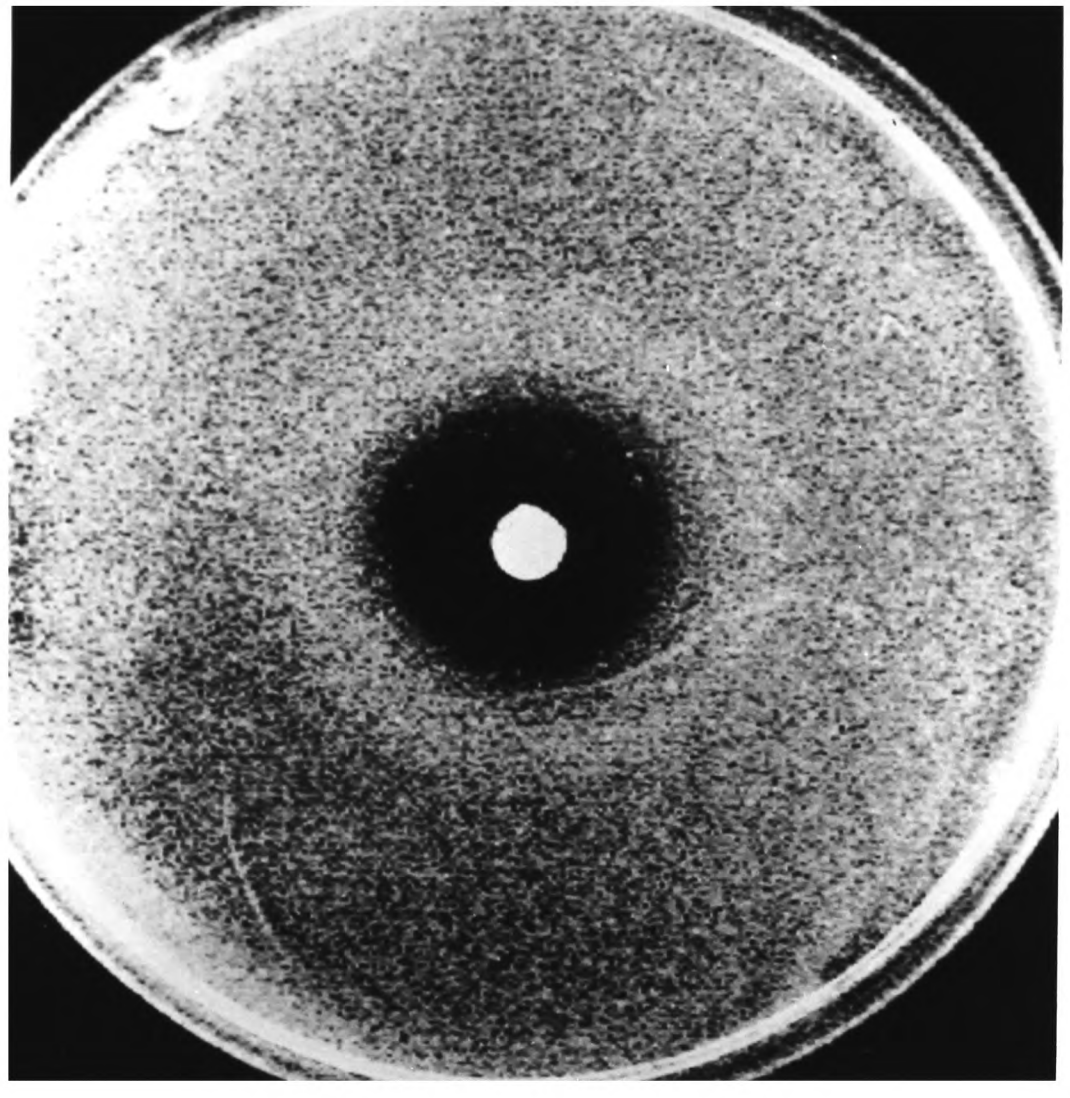
*colony number 17 selected from the gradient plate.

+refers to initial plasmid, prior to plating.

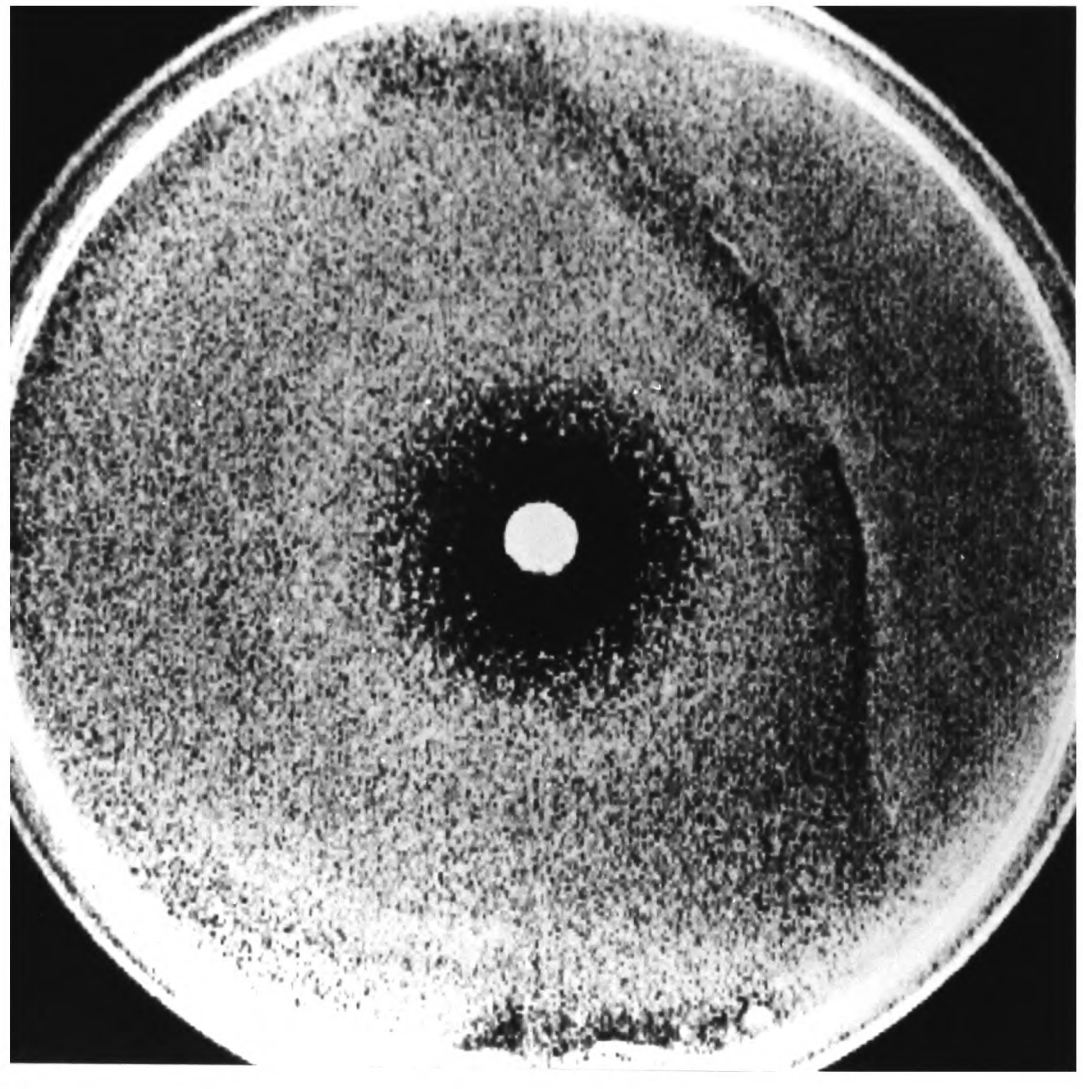
All 5 plates for each culture were prepared and tested independently.

Figure 4.7 Comparison of the relative resistances of 8325 (p3356) cells after serial dilution in mupirocin for varied times

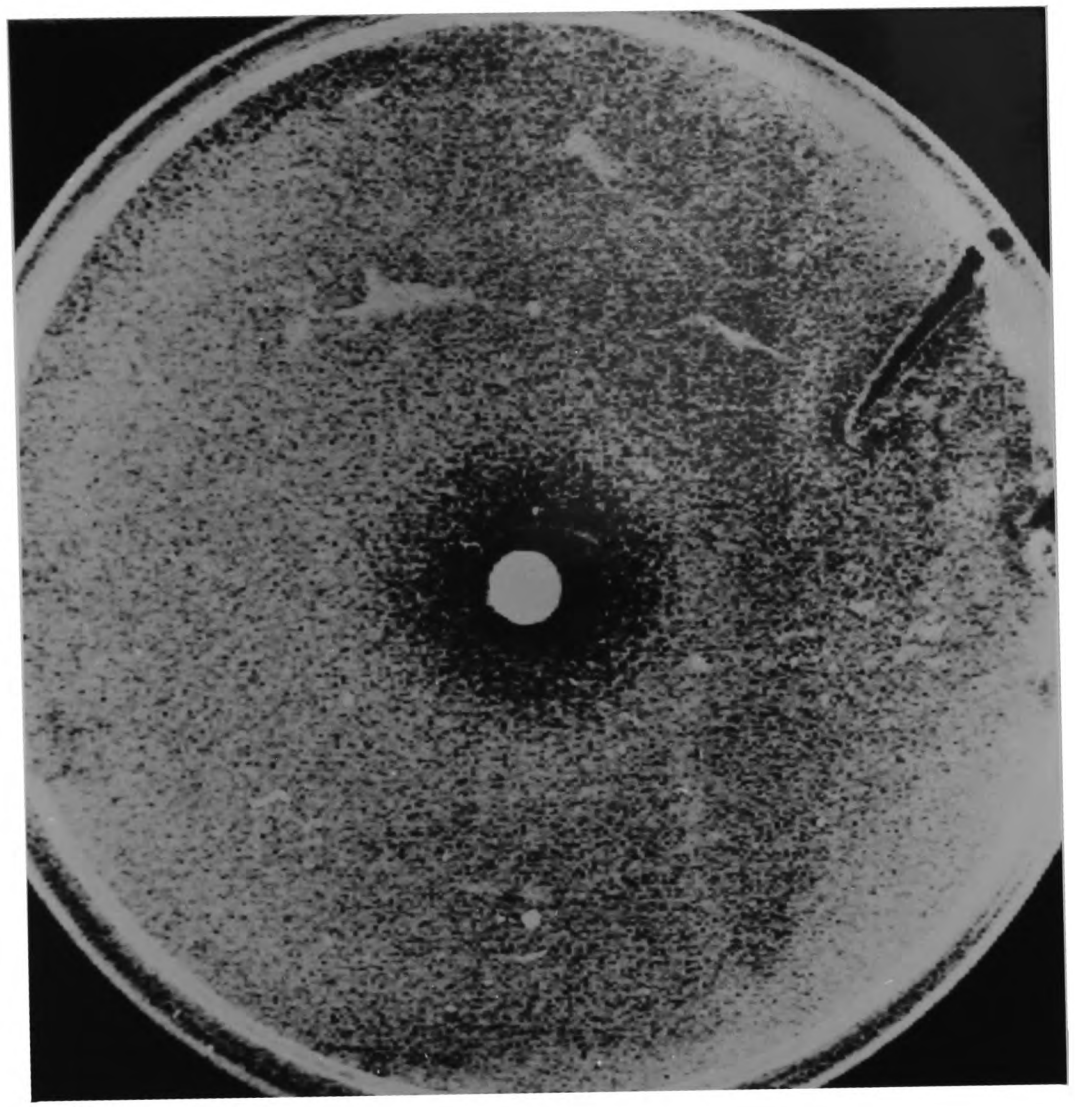
a)



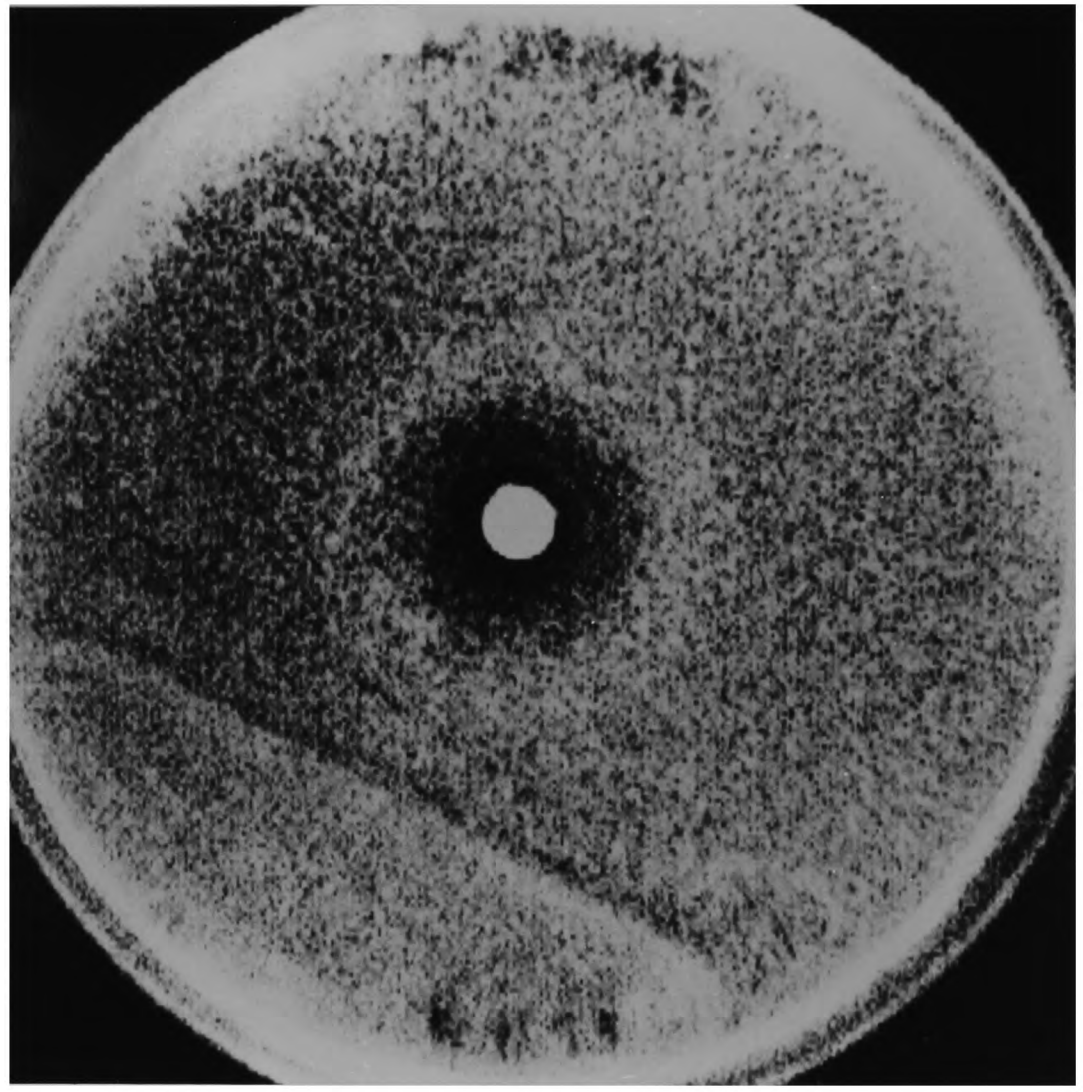
b)



c)



d)



a) cells plated with no prior exposure to mupirocin; (b) cells serially diluted in 200µg mupirocin ml^{-1} for 10 generations prior to plating; (c) cells serially diluted in 200µg mupirocin ml^{-1} for 20 generations prior to plating; (d) cells serially diluted in 200µg mupirocin ml^{-1} for 30 generations prior to plating; all cells were taken from the same serial dilution. The halo size remained constant (a-d), but the number of colonies within the halo increased with longer exposure to mupirocin prior to plating. Note: if serial dilution is in the absence of mupirocin then plates of type (a) are found even after 40 generations.

colonies which grow up within the halo within the first 24h are immediately more resistant. This would explain why cultures pre-exposed to mupirocin in serial dilution before plating are likely to yield more colonies growing within the halo (and hence a greater number do grow) than cultures not previously exposed, as those pre-exposed have needed to express *mup*A prior to plating.

4.7 Attempts to select for multiple duplication of the "IS*257-mup*A" cassette

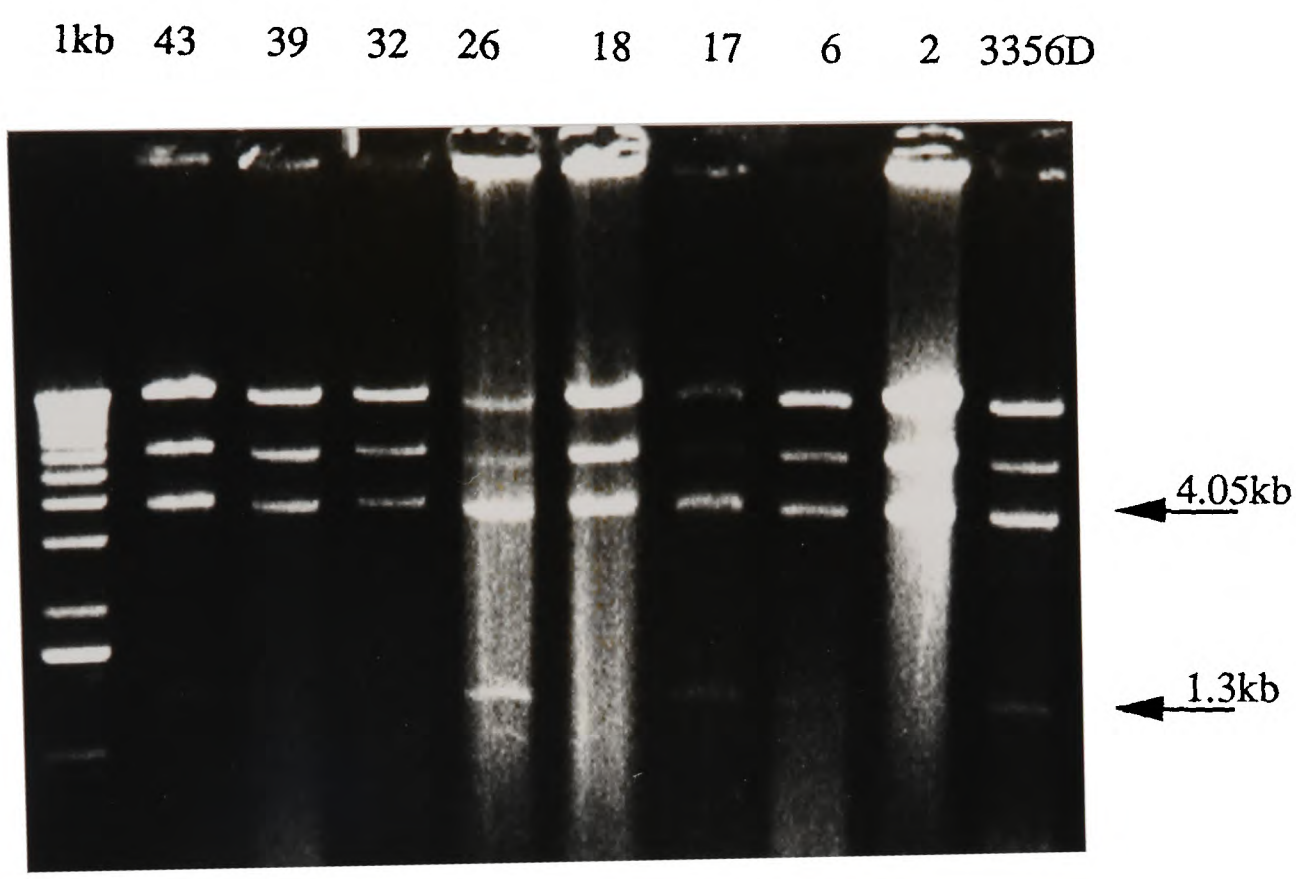
As higher concentrations of mupirocin in serial dilution (Section 4.5) caused observable levels of the duplication more rapidly than lower concentrations of mupirocin, it was proposed that longer exposure to these higher concentrations, or exposure to even higher concentrations of mupirocin may select for plasmids in which the "IS*257-mup*A" cassette has duplicated multiple times (Fig.4.3).

To test this hypothesis, 8325(p3356D) was inoculated onto a plate containing a concentration gradient of mupirocin (0-10mg ml^{-1}). Colonies which grew at the highest mupirocin concentrations were picked and plasmid DNA prepared from them. The *Eco*RI digests of these plasmids had two profiles: one indistinguishable from that of p3356D (the plasmid in the strain used as the inoculum) and the other the same as for p3356 (Fig 4.8a). The majority of the *Hpa*II digests were also of either p3356 (2.2, 8.6 & 12.4kb) or p3356D (2.2, 8.6 & 17.8kb) type (Fig 4.8b, e.g.numbers 39 and 50 respectively); although those of p3356D type often had additional faint bands larger than 17.8kb at c.23 and c.28kb (Fig 4.8b, number 26), suggesting that a small proportion of the plasmids had three or four copies of "IS*257-mup*A" (Fig 4.3), but that their inheritance was unstable as compared to those with only one or two copies of *mup*A[2]. In one case, the majority form of the plasmid contained *Hpa*II bands of sizes 2.2, 8.6 and c.28kb (Fig. 4.8b, number 17) indicating that in this case, the plasmid was stable with four copies of "IS*257-mup*A" in tandem repeat.

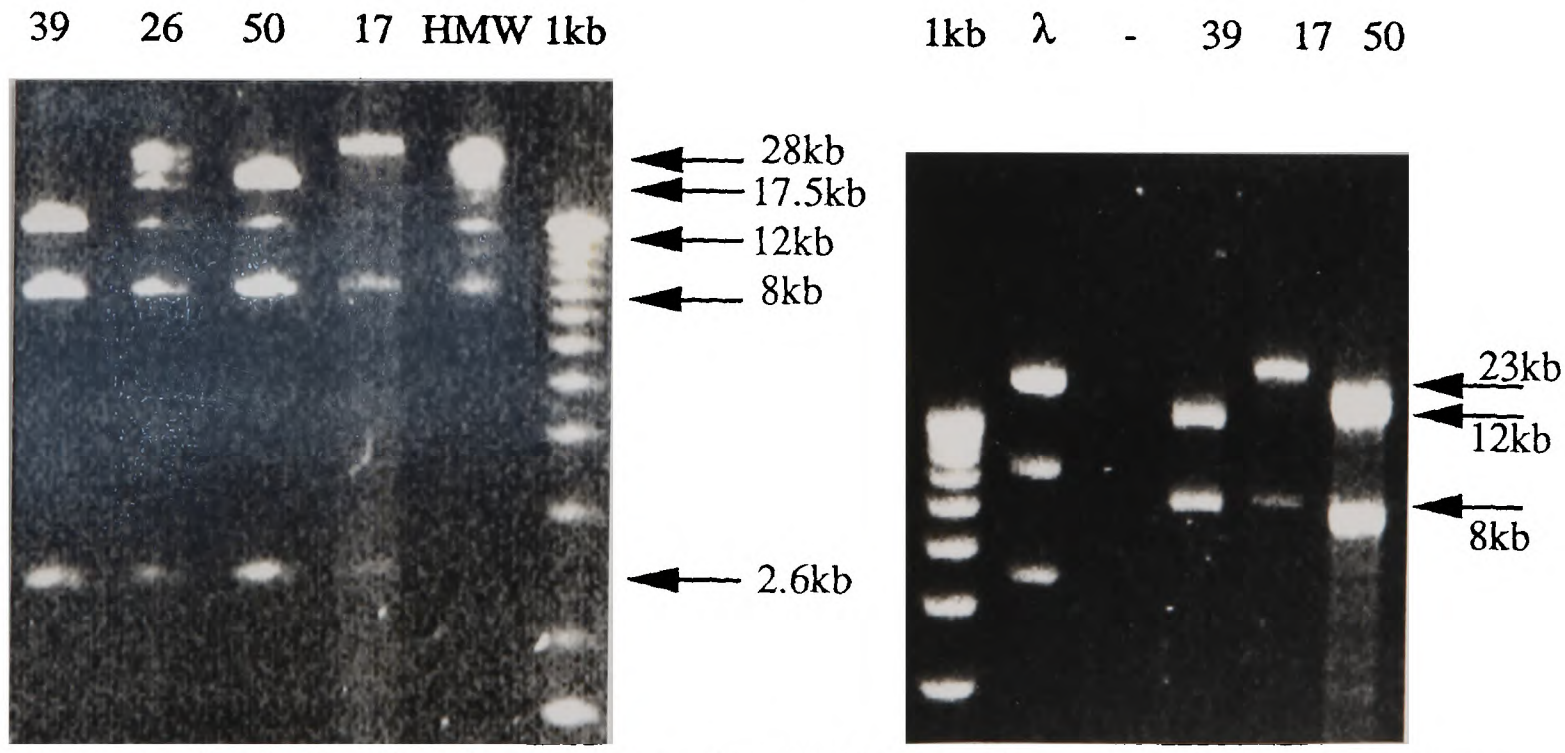
[2] These faint bands were also observed in p3356D, and shown to contain *mup*A by hybridisation (Fig.4.4). The consistency of their appearance further supports the claim that multiple copies of "IS*257-mup*A" are unstable.

Figure 4.8 Restriction digests of DNA prepared from colonies selected from the gradient plate.

a) *Eco*RI digests, numbers refer to selected colony number.



b) *Hpa*II digests, selected colony numbers are given



Colonies 2, 6, 18, 32, 39 and 43 contain p3356-type plasmids; colony 50 contains a p3356D-type plasmid; colony 26 contains a p3356D-type plasmid and larger unstable forms; colony 17 contains a p3356-based plasmid with four copies of *mup*A which are stable. 1kb, High Molecular Weight and lambda *Hin*dIII (λ) ladders are used.

That colonies grown on very high concentrations of mupirocin contained plasmids with less "IS*257*-*mup*A" cassettes than the inoculum cells, indicated that although the rearrangements causing duplication of "IS*257*-*mup*A" and loss of the duplicated products were encouraged by the presence of mupirocin, the extra copies were not required for growth at higher concentrations of mupirocin. This supports the data presented in Section 4.6 which failed to demonstrate a difference in the level of resistance to mupirocin of strains containing plasmids with one and two copies of *mup*A. (The same tests on the strain containing four copies also failed to show a difference in resistance levels.)

4.8 Discussion

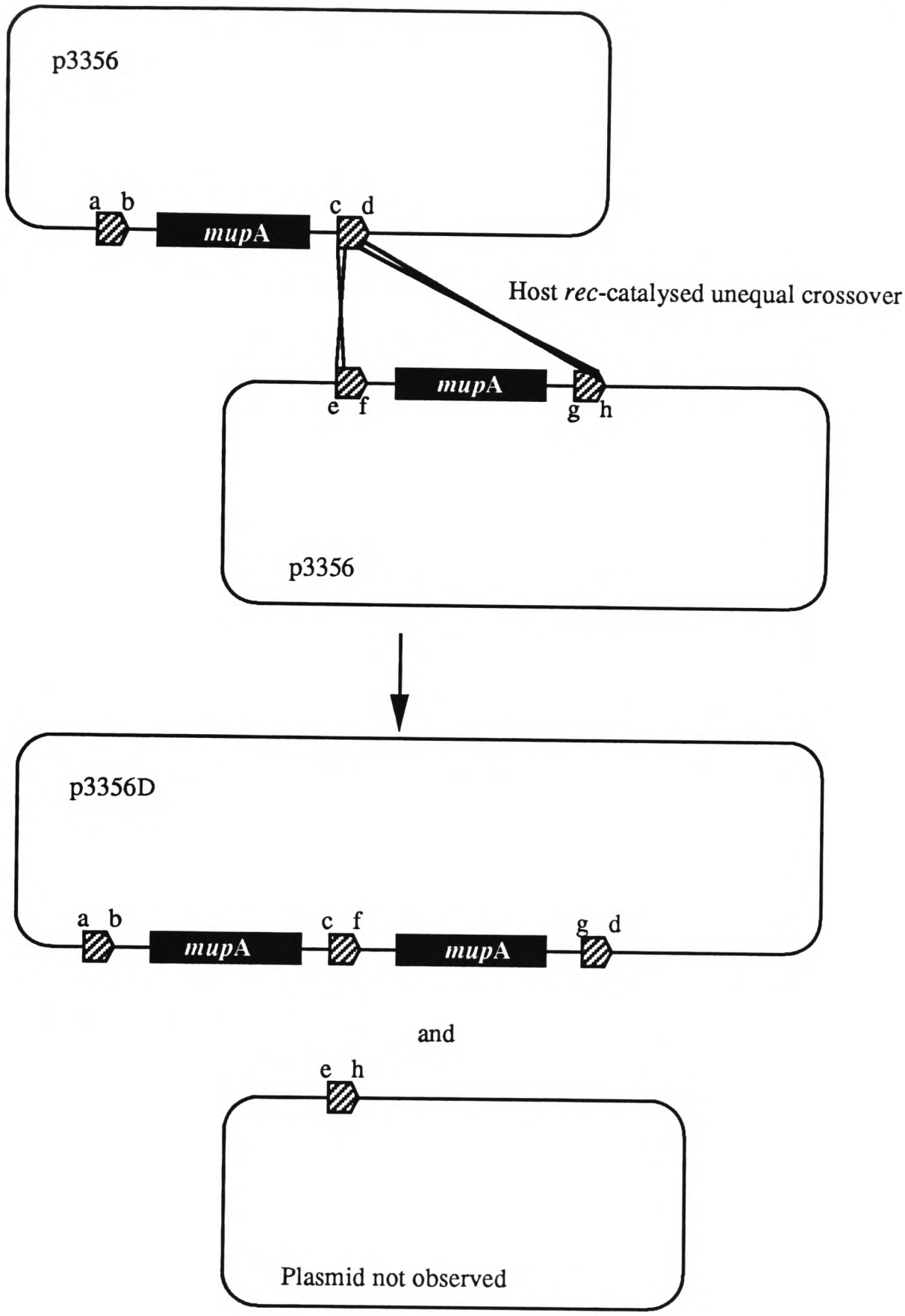
The "IS*257*-*mup*A" cassette of p3356 has been shown to duplicate in the laboratory, and appears also to have duplicated in a similar plasmid in "nature". Although IS*257* has been implicated in gene duplicatior(Matthews *et al*, 1990)this is the first clear demonstration of this role for IS*257*. Duplication of genes in tandem repeat along with insertion sequences has been observed previously. (For a review of gene duplication see Anderson and Roth, 1977.) Tn*9*, consisting of direct repeats of IS*1* flanking the chloramphenicol resistance gene on a plasmid isolated from *E.coli* will undergo tandem duplication in *Proteus mirabilis* (although not in *E.coli*); this duplication is recA dependent (Chandler *et al.*, 1979). Both IS*1* and Tn*9* flank multiple tandem repeats of pBR322 when a fusion plasmid of pOX38::Tn*9* is made with pBR322 in *Proteus mirabilis*, again this is dependent on functional *rec*A and *pol*A products; the tandem duplication has been shown not to be linked to the formation of the co-integrate plasmid (Chandler and Galas, 1983). The kanamycin resistant transposon Tn*2680* forms tandem duplications which can be selected for by kanamycin (Iida *et al.*, 1982). Tn*1525*, from *Salmonella panama*, consists of a kanamycin resistant determinant flanked by direct repeats of IS*15* (an insertion sequence with high homology to IS*257*, both in the putative transposase and its inverted repeats, Rouch and Skurray, 1989); it will undergo tandem duplication upon selection for kanamycin in a *rec*A$^+$ *E.coli* host (Labigne-Roussel *et al.*, 1983). These are all examples of "IS-resistance gene" cassette duplications which occur in *rec*A$^+$ hosts in response to a requirement for the resistance product. The IS*257*-*mup*A duplication observed occurred in a *rec*A$^+$ host apparently in response to mupirocin selection. There was no change to the sequences flanking the "IS*257*-*mup*A-

IS*257*" cassette upon duplication. Due to the requirement for homologous recombination in these parallel systems (not demonstrated for IS*257-mup*A), the model proposed for the amplification of the "IS*257-mup*A" cassette is that of unequal-crossover between the IS*257*s using the host's recombination machinery (Fig.4.9).

The duplicated cassette also deleted to give a plasmid with only one "IS*257-mup*A" cassette, as shown by the appearance of p3356-type plasmids (one cassette) in colonies taken from the gradient plate which had originally been inoculated with 8325(p3356D) (two cassettes). The loss of duplicated product by intramolecular recombination between the IS*15*s had been observed in plasmids containing amplified Tn*1525* upon the removal of kanamycin (Labigne-Roussel *et al.*, 1983). Loss of amplification of the *mec* region of methicillin resistant *S.aureus* has also been demonstrated: a region containing *mec* with IS*257* in close proximity to its left end and an unknown right end amplifies when the strain in challenged with high concentrations of methicillin; this amplification is lost with the removal of the methicillin (Matthews and Stewart, 1988). That the IS*257-mup*A duplication deletes upon exposure to mupirocin at first appears contrary to these examples which delete upon the removal of the appropriate antibiotic. However, unduplicated *mup*A strains are resistant to the levels of antibiotic which elicited duplication, and therefore there is no requirement for duplicated *mup*A in the long term; this is unlike the examples given above where the duplication is necessary to increase the resistance of the host cells to allow growth in the high levels of antibiotic with which they were challenged to create the duplication. Therefore for deletion of the duplication of Tn*1525* or the *mec* region with cell survival, the antibiotic challenge must be removed. This is not the case for *mup*A, which remains resistant upon deletion of the duplication and thus deletion is observed without the requirement for removal of the antibiotic.

The degree of amplification of the "IS*257-mup*A" cassette varied between experiments. The plasmids isolated from the colonies grown on the highest mupirocin concentrations of the gradient plate contained different numbers of the cassette. Strains containing plasmids of one, two or four copies gave disc plates with similar numbers of (more resistant) colonies growing within the halo suggesting that the number of copies of *mup*A present originally

Figure 4.9 A model of "IS*257*-*mup*A" duplication by unequal crossover



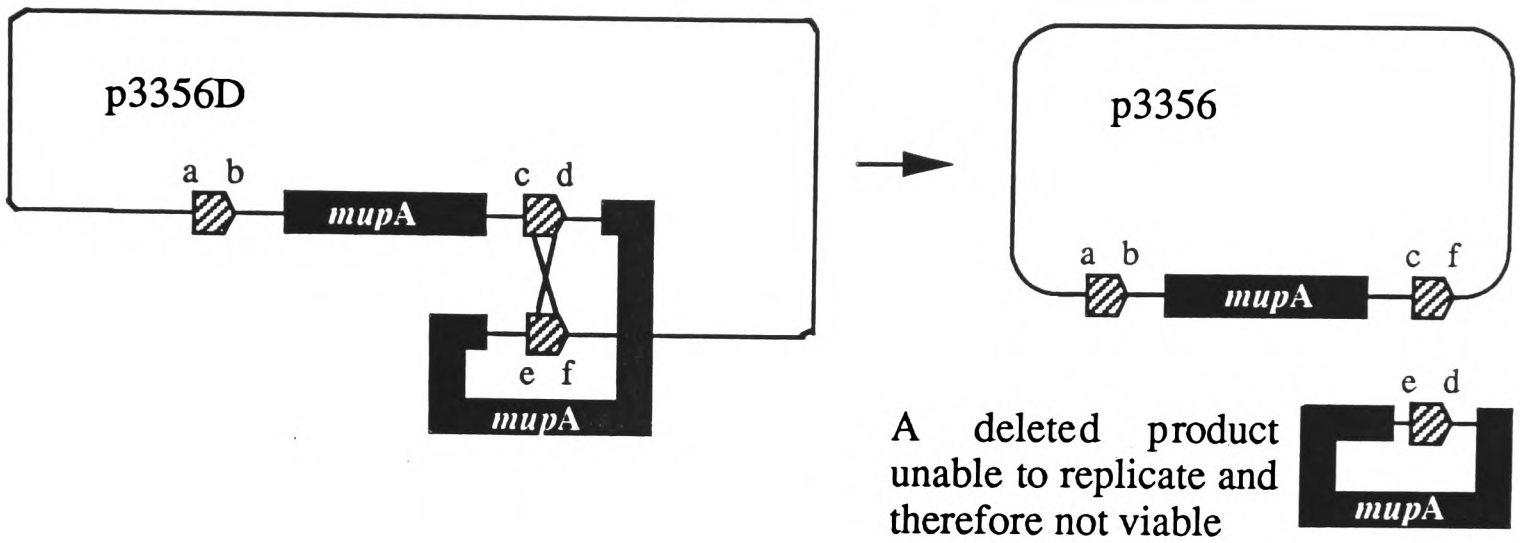
Two identical p3356 plasmids align so as to allow unequal crossover between homologous IS*257*s. The ends of the IS*257*s are labelled only so as to allow their original positions and final destinations to be traced, rather than to denote any difference between them. It is suggested that homologous recombination between the IS*257*s of two p3356s gives a p3356D type plasmid (i.e. a plasmid with a tandem duplication of the "IS*257*-*mup*A" cassette) and a plasmid deleted for *mup*A and an IS*257*. The IS*257*s at the junctions of the deletions and duplications would be hybrids of the original IS*257*s, but as the known sequences of the two IS*257*s of p3356 are identical, this could not be used as a method of validation of the model.

does not determine the final level of duplicated product when cells are plated with a mupirocin disc. These observations suggested that the duplication of the "IS*257-mup*A" cassette (and deletion of the duplicated product) was a random event. The frequency of duplication of Tn*2680* was not constant, both in the degree of amplification and the proportion of genomes displaying the amplification after serial dilution in chloramphenicol (Iida *et al.*, 1982). It is suggested for Tn*2680* that the duplication is a spontaneous event and that the duplicated product gives a selective advantage to the host cell in selective media. If there is no long term selective advantage for the duplication, as suggested for *mup*A, a deleted product will be maintained equally to a duplicated one, explaining how the gradient plate could render strains containing plasmids of the p3356-type from an original p3356D inoculum. A lack of selective advantage would also explain why many multiple duplications appeared labile (Section 4.7). A lack of selective advantage to gene duplication/deletion would allow a random plasmid population, as observed.

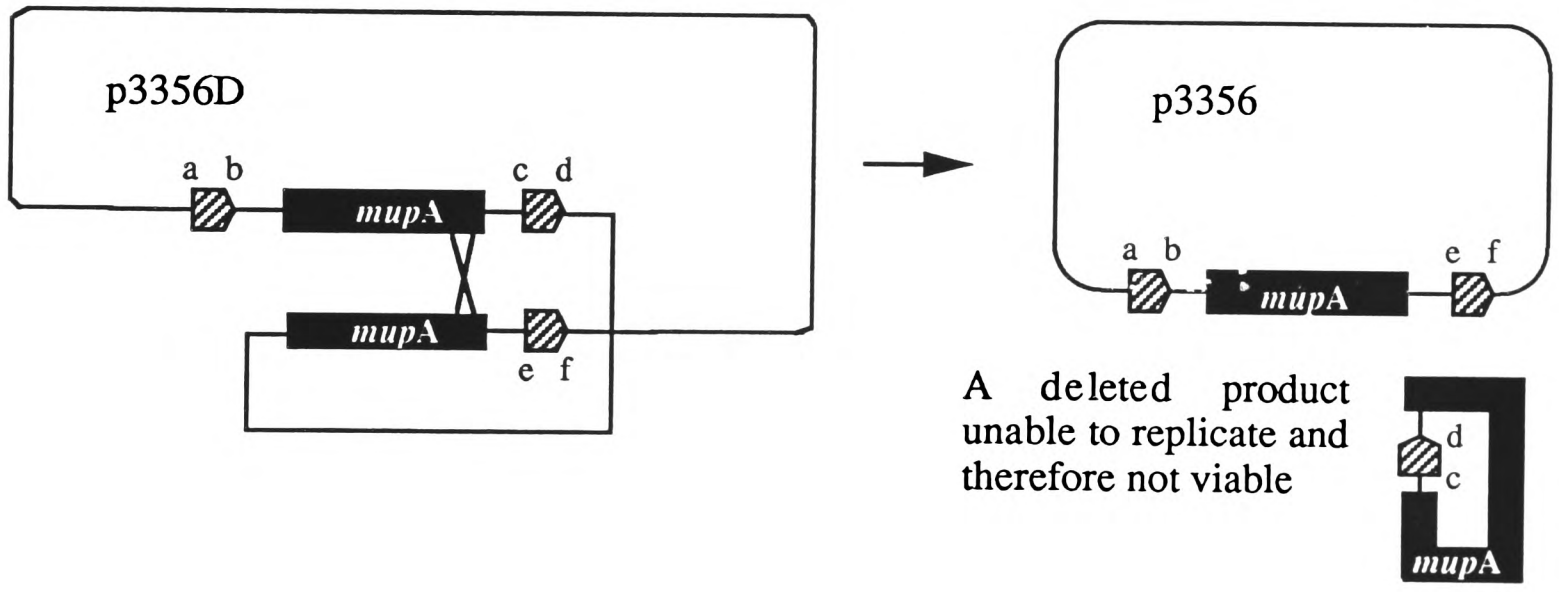
That IS sequences flanking tandem repeats are not required for gene amplification was demonstrated by Peterson and Rownd (1983). A plasmid was constructed containing neomycin and kanamycin resistance genes flanked by homologous portions of a chloramphenicol resistance gene. Selection for increased neomycin resistance yielded plasmids with tandem duplications of the "partial-chloramphenicol, neomycin^R, kanamycin^R" cassette. The removal of neomycin in the subcultures led to loss of the duplications. Other non-IS homologous regions have been implicated in gene amplification: rRNA genes in the flanking regions of chromosomal gene amplifications are often implicated as such a source of homology (eg in *Bacillus subtilis*, Wilson and Morgan, 1985); the *coa* locus of *S.aureus* was used to flank a tetracycline resistance gene on a plasmid integrated into the chromosome of *S.aureus* (M^{c}Devitt *et al.*, 1993) and it was shown to act as a site of homology for the amplification of this gene. When non-IS sequences form the homologous sites to allow gene amplification of an antibiotic resistance determinant, the level of amplification can be controlled by the level of antibiotic requirement (e.g. Young, 1984). As non-IS sequences are able to form sites of homologous recombination in gene duplication/deletion, it is possible that the deletion of the duplicated "IS*257-mup*A" cassette occurs by homologous recombination at any site within the cassettes rather than simply at the IS*257*s (Fig.4.10).

Figure 4.10 A model for the deletion of the "IS*257*-*mup*A" duplication

(a) Unequal crossover homologous recombination between two IS*257*s



(b) Unequal crossover homologous recombination between two *mup*As

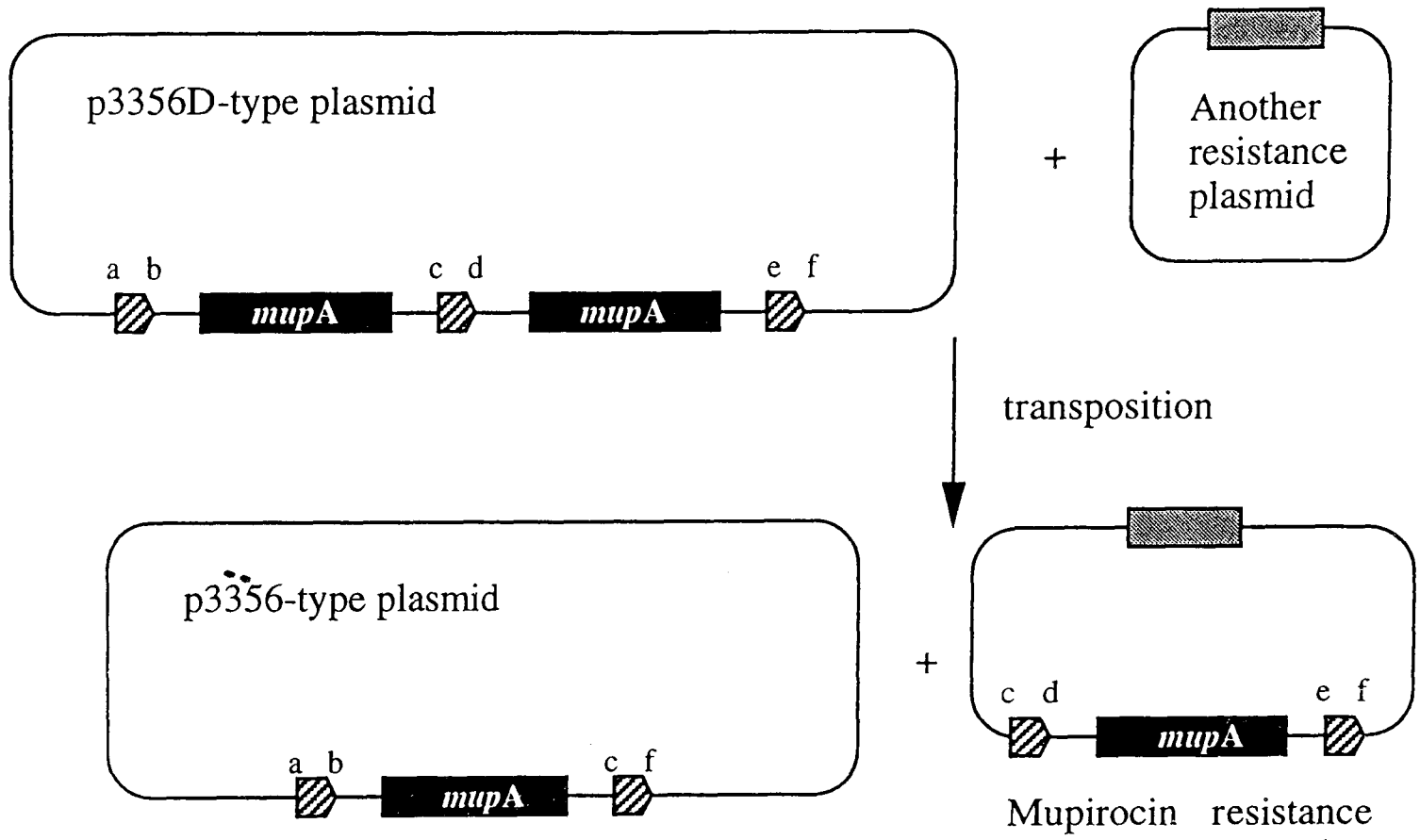


Deletion of p3356D to give p3356 could occur by homologous recombination within any part of the "IS*257*-*mup*A" cassette. (a) If recombination occurred between the IS*257*s (box hatched arrows), the IS*257*s at the junction of the deletion would be a hybrid (although untraceable due to sequence identity); (b) if recombination was between another part of the cassette, such as *mup*A (black box), no such hybrid would form. Although the model shows intramolecular deletion, it is feasible that the deletion could be intermolecular, leading to the amplification in one plasmid alongside deletion of a second.

To conclude, the phenomenon of gene duplication/amplification is well documented in many species both for plasmid-borne and chromosomal genes. It is generally accepted to be caused by unequal-crossover between flanking regions of homology, by homologous recombination. Often the duplication can be manipulated by requirement for the expressed gene. The example of "IS*257*-*mup*A" cassette duplication fits this model. The IS*257*s supply the flanking sites of homology. Although there is no long term requirement for increased *mup*A copy number it is suggested that there is an immediate requirement for the duplication when each cell is first challenged with mupirocin. Therefore duplication will be initially selected, but the random process of duplication/deletion thereafter will lead to a varied plasmid population, even in the presence of high concentrations of mupirocin.

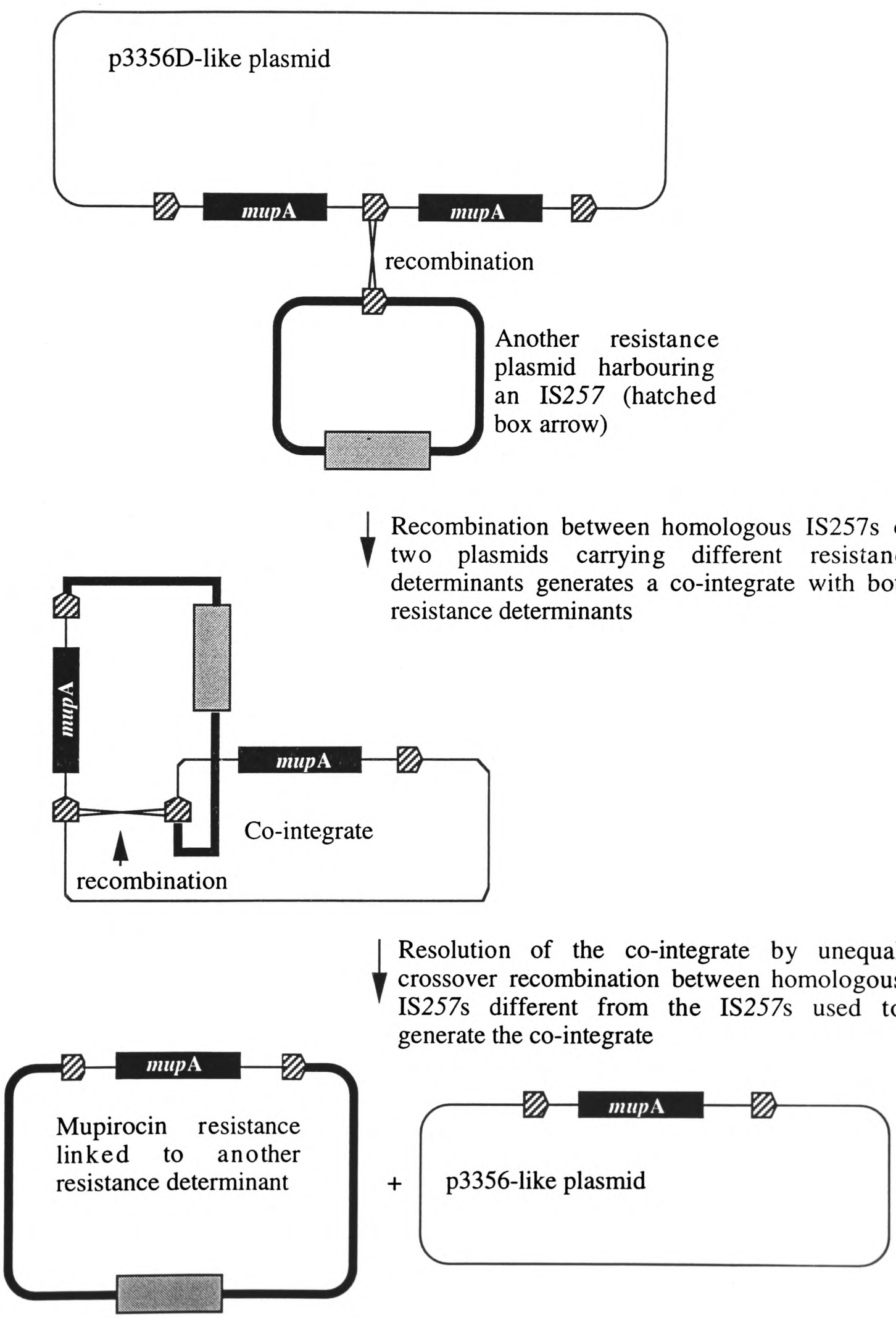
The significance of the duplication in the nosocomial strain 8325(p3357) is not one of increased resistance, as the unduplicated forms, 8325(p3355) and 8325(p3356), are resistant to much greater concentrations of mupirocin than those clinically treatable with mupirocin. It is possible that the duplication may be of significance due to the increased numbers of *mup*A on the plasmid. There are many possibilities as to the further dissemination of *mup*A. Although the known conjugative genes of *S.aureus* were not found on p3356 (Section 3.5), the high frequency of filter-transfer suggests that it, and therefore p3357, are conjugative plasmids. Should the potential "IS*257*-*mup*A-IS*257*" composite transposon be active, the second copy may transpose to a different plasmid, and potentially therefore create a multiply resistant plasmid (Fig.4.11). Another possibility is that the IS*257* between the two copies of *mupA* is involved in replicon fusion and that subsequent resolution leads to two mupirocin resistant plasmids instead of only one, again the linkage to other resistance determinants may be of significance (Fig.4.12). Additionally, a recent review outlines the possibilities for mutation of the second copy of a gene after its duplication to provide a more appropriate product (Hughes, 1994). Although at present *mup*A is sufficient for resistance to mupirocin, this need not always be so: should an analogue of mupirocin be introduced onto the market which can act as a substrate for *mup*A's IRS-like protein, mutation of a second copy of *mup*A may yield a product not susceptible to the mupirocin analogue whilst maintenance of the original copy provides continued resistance to the present form of mupirocin. Therefore although clinically insignificant *per se*, the possibility of *mup*A duplication in plasmids such as p3356/p3357 may provide a wider range of resistant strains in the future.

Figure 4.11 Transposition of one copy of the "IS*257*-*mup*A" tandem repeat



Replicative transposition of one copy of the "IS*257*-*mup*A" tandem repeat from a p3356D-type plasmid to a plasmid containing another resistance determinant would lead to a novel linkage of resistance determinants in a new *mupA* plasmid and would also give a p3356-type plasmid

Figure 4.12 Replicon fusion and resolution between IS*257*s of plasmids carrying different resistance determinants



Mupirocin and Tetracycline Resistances are linked

Chapter Five

Mupirocin and tetracycline resistances are linked

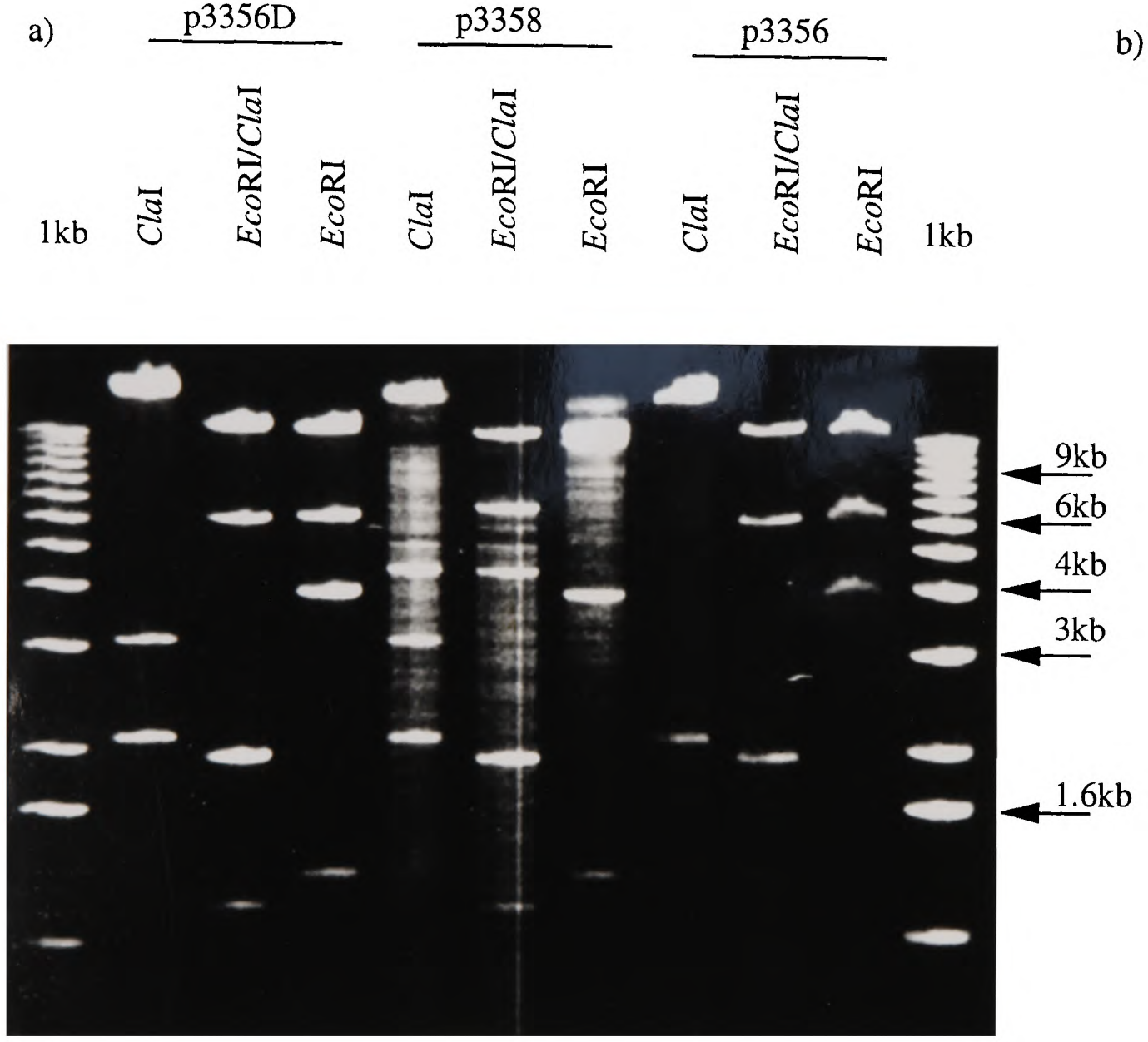
5.1 Introduction

The first linkage of *mup*A with another resistance determinant was cited in Section 3.2: 8325(p3358) expresses both mupirocin and tetracycline resistances. As 8325(p3358) is a transconjugant of the plasmid p3358 to the tetracycline sensitive strain NCTC8325, the tetracycline resistance must be plasmid-borne. The only known tetracycline resistance determinant found on plasmids of *Staphylococcus aureus* is on the family of plasmids typified by pT181 (Mojumdar and Khan, 1987; Iordanescu *et al.*, 1978). The resistance is due to a protein which allows the efflux of the intracellular accumulation of tetracycline (Guay *et al.*, 1993). pT181 has not been reported to be linked to other resistance determinants in larger plasmids, although a pT181-like plasmid is reported to be integrated into the chromosome of methicillin resistant *S.aureus*, the site of integration is identical in all clinical isolates examined (Gillespie *et al.*, 1986). p3358 was investigated for its tetracycline resistance determinant in order to establish a model for the evolutionary mechanisms which had led to the linkage of tetracycline and mupirocin resistances.

5.2 The mupirocin and tetracycline resistance plasmid p3358 belongs to the p3356 plasmid family

Several restriction enzyme digestions of p3358 share common bands with p3356 and p3356D. Example gels are given in Fig.5.1, the data of which are summarised in Table 5.1. *Cla*I digestions of p3358 contain bands corresponding to all three fragments of p3356D (2.2kb, 3.2kb and a very large band); *Cla*I/*Eco*RI double digestion of p3358 yields similar bands to all six fragments of *Cla*I/*Eco*RI digestion of p3356D (the small fragments have run off the end of the gel). Many digestions yield an extra fragment from p3358 than from p3356D digestions, e.g. a 5.2kb band in *Bgl*II digestion, and an extra 4.4kb band in both *Cla*I and *Cla*I/*Eco*RI digestions. In some cases, one of the fragments created in p3356D

Figure 5.1 Restriction enzyme digestions of p3358, p3356D and p3356



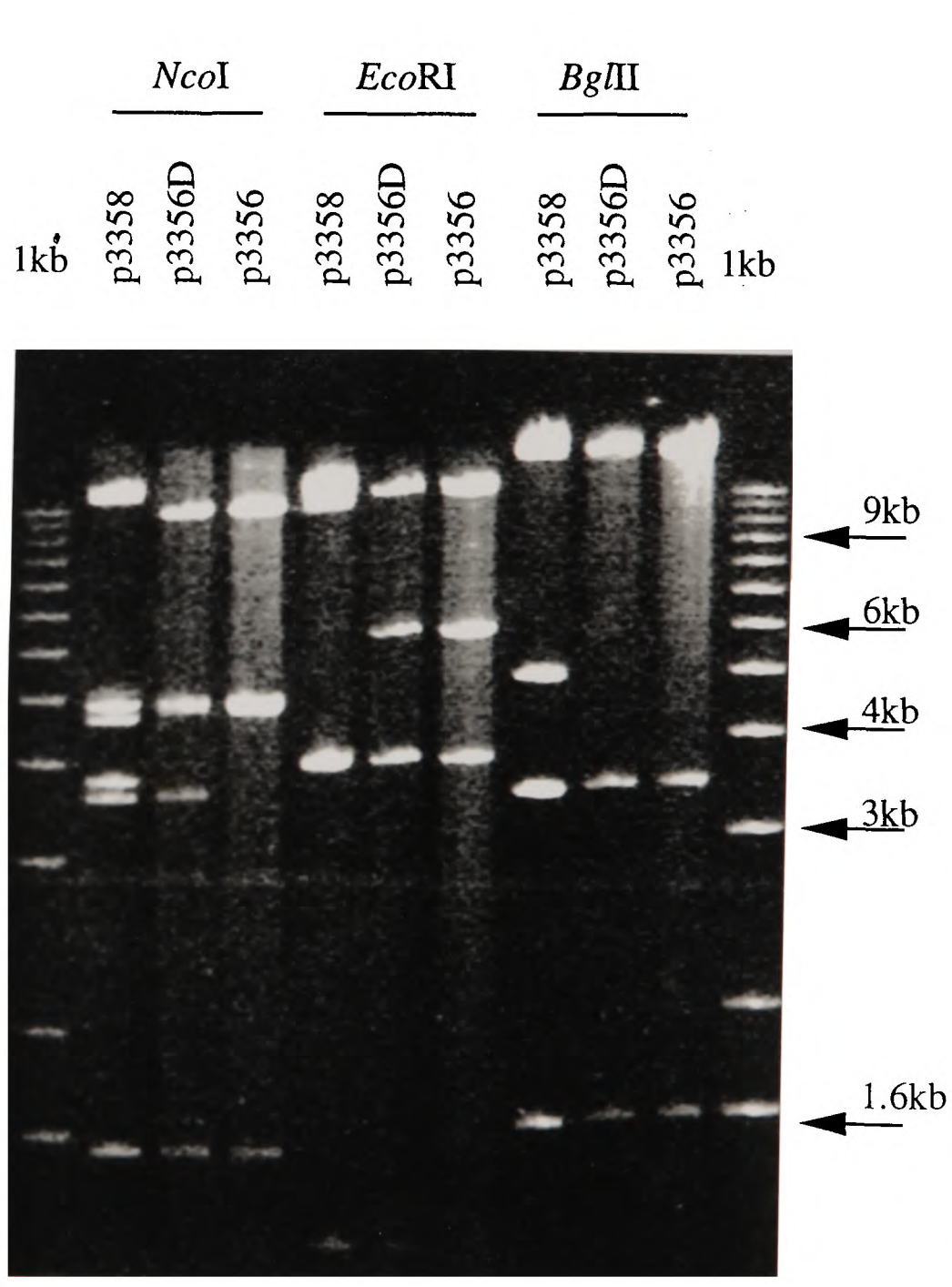


Table 5.1 Fragment sizes of restriction enzyme digestions of p3358, p3356D and p3356

Enzyme	*Bgl*II			*Nco*I			*Eco*RI			*Cla*I			*Eco*RI /*Cla*I		
Plasmid	3358	3356D	3356	3358	3356D	3356	3358	3356D	3356	3358	3356D	3356	3358	3356D	3356
Size (kb)	c.18	c.18	c.18	c.13			c.13	c.13	c.13	c.21			c.13	c.13	c.13
	5.2				12	12	11.5				c.20	c.20	6.9		
	3.6	3.6	3.6	5.0	5.0	5.0		6.3	6.3	**4.4**				6.1	6.1
	3.6	3.6			5.0	5.0	4.05	4.05		3.2	3.2		**4.4**		
	1.6	1.6	1.6	4.8*			4.05	4.05	4.05	2.2	2.2	2.2	2.0	2.0	2.0
	1.6	1.6		**3.9**			1.3	1.3		2.2	2.2		2.0	2.0	2.0
				3.8	3.8								2.0	2.0	
				1.6	1.6	1.6							2.0	2.0	
				1.6	1.6								1.2	1.2	
				(0.2)									(0.2)	(0.2)	(0.2)

Table 5.1 summarises the data of Figure 5.1:

The additional fragments found in p3358 but not in p3356D or p3356 are highlighted in **bold** type; the fragments of p3356D and p3356 replaced by larger fragments in p3358 are underlined as are the replacement bands; the *Nco*I fragment with an asterisk is thought to have been created by a point mutation not involved in the additional DNA of p3358 relative to p3356D; fragments which have run off the gel in Fig 5.1 are (bracketed).

digestions (e.g.*Eco*RI 6.3kb) is missing from p3358 digestions where another larger band is present (*Eco*RI 11.5kb), or two replacement bands are seen (*Nco*I 12kb replaced by 3.9kb & c.14kb)[1]. As p3358 yielded restriction enzyme patterns with great similarities to those of p3356D for many different enzymes, p3358 was concluded to be related to p3356D (and hence p3356).

p3358 contained more fragments than p3356D, and was larger by 5.2kb. Comparison of the restriction fragment patterns of p3356D and p3358 showed that the difference between them was upstream of the "IS*257*-*mup*A" tandem repeat, in the vicinity of the 6.3kb *Eco*RI fragment of p3356D which is missing from p3358. Due to the similarity of the rest of the fragments (except two *Nco*I bands[1]) it appeared that p3358 was virtually identical to p3356D except for a 5.2kb insertion upstream of the *mup*A genes. Further digestions allowed p3358 to be mapped confirming this structure (Fig.5.3).

It was interesting to note that there was a *Bgl*II restriction site close to the upstream end of the "inserted" region. Considering the association of this family of plasmids with IS*257*, which contains a *Bgl*II site, it was decided to probe p3358 for homology to IS*257*. Indeed IS*257*-homologous sequences were found in fragments containing this region (Fig 5.2).

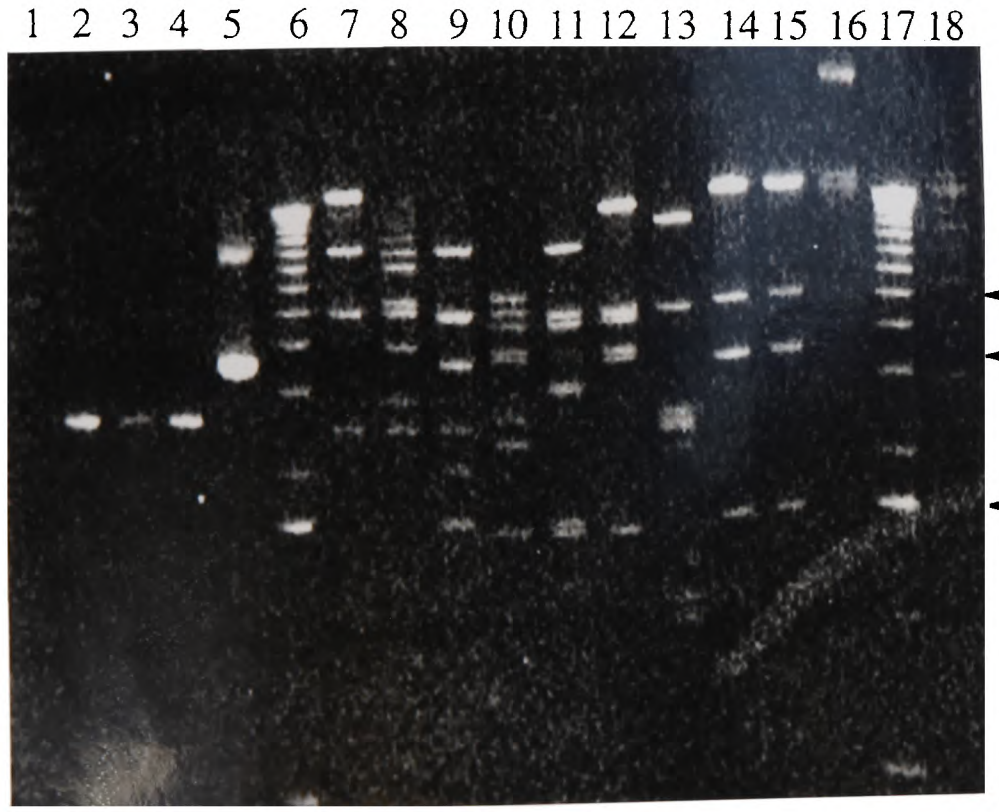
Placing of this "new" IS*257* onto the map of p3358 revealed that p3358 was related to p3356D by the insertion of an extra 4.4kb DNA, with no restriction map homology to p3356D, upstream of the "IS*257*-*mup*A" tandem repeat and flanked by an additional IS*257* in direct repeat with those of p3356D (Fig 5.3). The duplication of the IS*257* in direct repeat inferred a role for IS*257* in the integration process.

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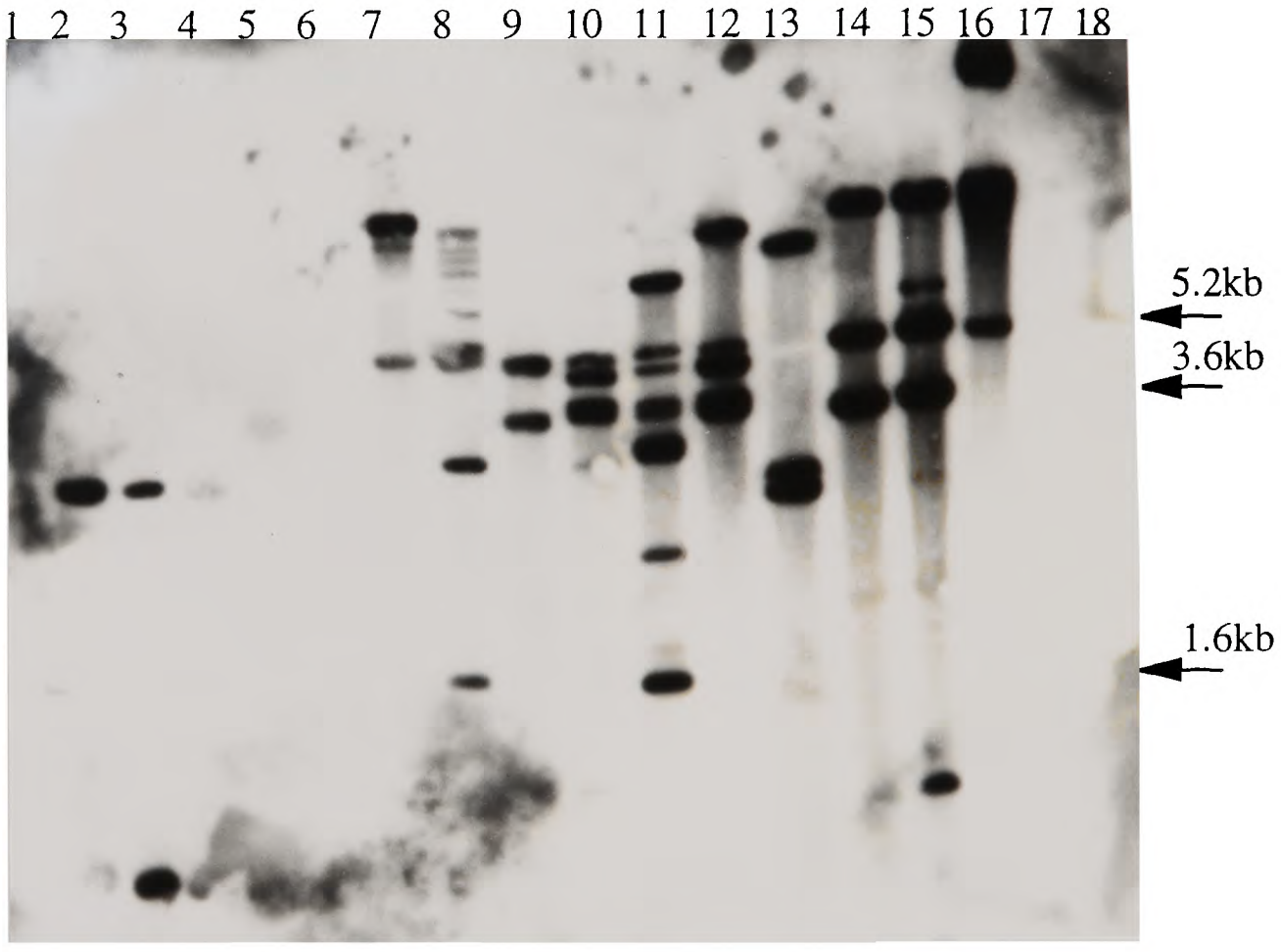
[1] There are three extra fragments in the *Nco*I digestion of p3358 than p3356D, two of these are thought to be due to a point mutation internal to one of the *Nco*I fragments (5.0kb in p3356D), giving 0.2kb and 4.8kb bands in p3358, the 0.2kb fragment has run off the end of the gel.

Figure 5.2 A Southern blot of p3358 hybridised with a probe of IS*257*

a)

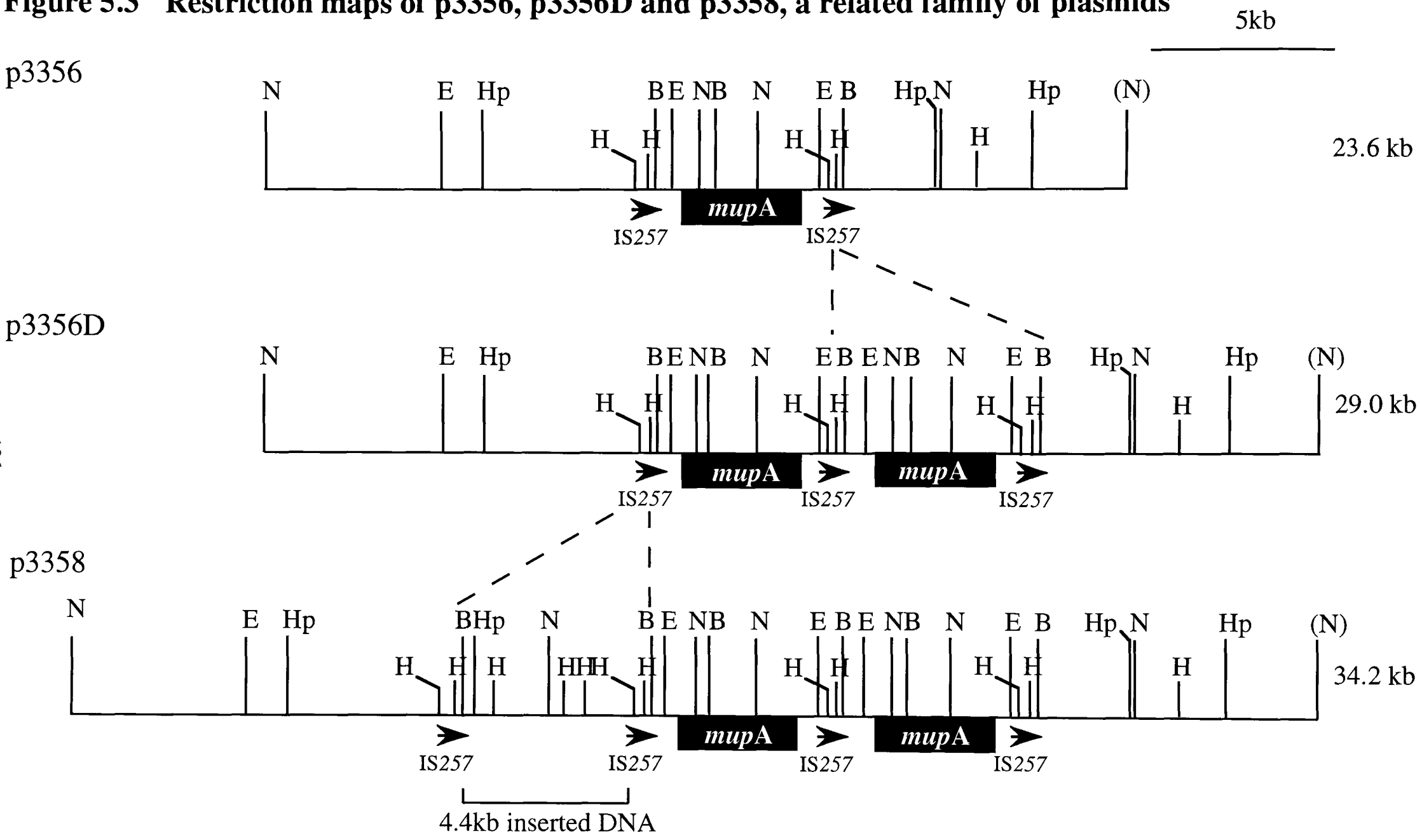


b)



a) DNA digestions: lanes 1 & 18, Raoul ladder; lane2, pUC18; lane 3, pIP1644; lane 4, pIP1551; lane 5, pT181 as negative control; lanes 2-5 digested with *Eco*RI and *Pst*I; lanes 6 & 17, 1kb ladder; lanes 7-16, p3358: digested with *Hpa*II, *Hpa*II/*Eco*RI; *Hpa*II/*Bgl*II; *Hpa*II/*Nco*I, *Nco*I/*Eco*RI, *Nco*I, *Nco*I/*Bgl*II, *Bgl*II and *Bgl*II respectively, lane 16 is undigested.
b) Autoradiograph of gel in (a) hybridised with PCR product of the transposase sequences internal to pIP1644, which contains IS*257* and pUC18 sequences, 1kb and Raoul ladders contain fragments which have homology to pUC18.

Figure 5.3 Restriction maps of p3356, p3356D and p3358, a related family of plasmids



Plasmids p3356, p3356D and p3358 are related, dashed lines highlight the differences. The duplication of the "IS257-mupA" cassette in p3356D relative to p3356 is also present in p3358. p3358 has a 4.4kb insertion relative to p3356D, and is also duplicated for one of its IS257s. The arrows represent IS257s and the black boxes represent the mupA gene. Plasmids are linearised at one of the NcoI sites.

5.3 p3358 is the co-integrate of a p3356D-like plasmid with a small pT181-like tetracycline resistance plasmid

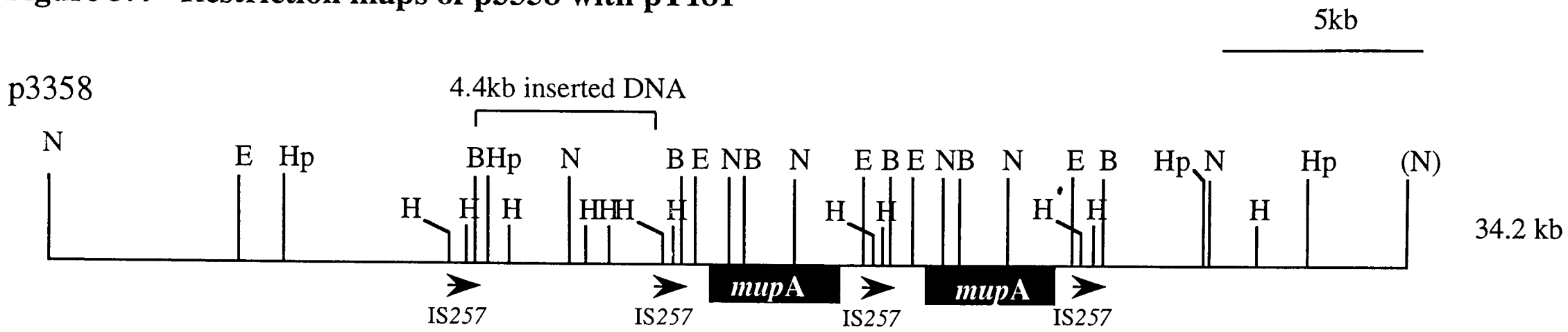
As 8325(p3358) expresses tetracycline resistance, whereas 8325(p3356) and 8325(p3357) (a homologue of 8325(p3356D)) do not, the additional DNA inserted upstream of the *mup*A genes must contain the tetracycline resistance gene(s). Mary Rahman had already probed with a probe internal to the *tet* gene of the small staphylococcal tetracycline resistance plasmid pT181 (Khan and Novick, 1983), and had shown homology of this extra DNA to the *tet* probe (Needham *et al.*, 1994). Indeed comparison of the restriction map of pT181 with the "inserted" DNA of p3358, showed restriction homology of the entire insertion (c.4.4kb) with pT181 (4437bp). It appears that a pT181-like plasmid linearised within its 1.5kb *Hin*dIII fragment has integrated into a p3356D-like plasmid (Fig.5.4). As the pT181-like insertion is flanked by a duplicated IS*257* in direct repeat, it is likely that the replicon fusion of the two plasmids was mediated by IS*257*s.

5.4 Selection for smaller plasmids from p3358

p3358 DNA was used to transform RN4220, a well characterised staphylococcal recipient of transformant DNA, derived from NCTC8325 (Novick, 1990) with selection on tetracycline (10μg ml^{-1}). Forty of the transformants were selected and streaked onto CY-agar plates containing either mupirocin (200μg ml^{-1}) or tetracycline (10μg ml^{-1}) to check for both resistances. Only 21 selected transformants were resistant to both tetracycline and mupirocin, the remaining 19 were resistant only to tetracycline. The transformation was repeated with similar results: 20 of 40 transformants selected were resistant to both mupirocin and tetracycline, and the remaining 20 were resistant to tetracycline only. Mupirocin resistance was being lost in 50% of the transformations.

"Mini-prep" DNA was prepared from transformants of the initial transformation. Restriction enzyme digestion demonstrated that the loss of mupirocin resistance was concurrent with the loss of DNA: the plasmids yielded from $Mu^{S}Tc^{R}$ transformants were much smaller than the 34.2kb size of p3358; in fact, they were all of one type and 5.2kb. Twenty of the $Mu^{R}Tc^{R}$

Figure 5.4 Restriction maps of p3358 with pT181



The restriction map of the inserted DNA in p3358 (as compared to p3356D, Fig.5.3) is the same as that of pT181 if pT181 is linearised in the 1.5kb *Hin*dIII fragment. Black boxes represent the *mup*A gene. Arrows indicate the positions of the IS*257*s. N.B. the sequence of pT181 contains two *Hpa*II sites not distinguishable by restriction enzyme mapping.

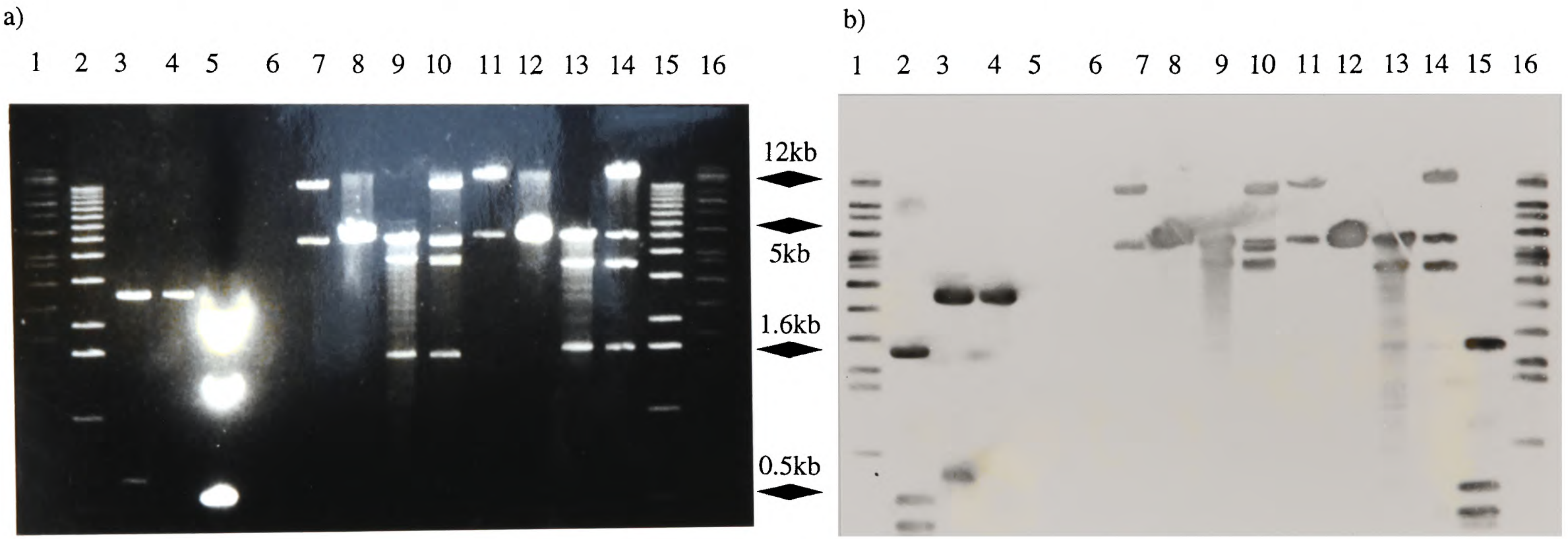
transformants mapped as p3358. The remaining transformant contained two much smaller plasmids, of only 10.6kb and 5.2kb.

During filter mating with strain J3347 (the strain from which p3358 had been transferred to NCTC8325), W.C.Noble had found that selection for tetracycline rendered various transconjugants: most were $Mu^{R}Tc^{R}$, but nine were $Mu^{S}Tc^{R}$. The seven $Mu^{R}Tc^{R}$ plasmids tested were all the size of p3358. The nine $Mu^{S}Tc^{R}$ transformants gave a variety of plasmid profiles: five contained a 4.4kb plasmid, four contained a 4.4kb plasmid along with a larger plasmid of c.20kb, and one contained a 5.2kb plasmid along with a c.20kb plasmid (Needham *et al.*, 1994). Filter transfer, as well as transformation had selected a 5.2kb $Mu^{S}Tc^{R}$ plasmid, although the frequency of a 5.2kb transconjugant was much lower than that of a 5.2kb transformant (c.1 in 10^{3} and 1 in 2 respectively). In addition, filter transfer had selected for 4.4kb and c.20kb plasmids.

One of the c.20kb transconjugant plasmids, p3626 (transferred by filter mating to NCTC8325 by W.C.Noble), and the 5.2kb and 10.6kb transformant plasmids were digested; Southern blots of these digestions were hybridised with pIP1644 (a probe internal to the transposase of IS*257*, Section 3.6). The results of this hybridisation (Fig 5.5) and restriction enzyme digestions allowed these plasmids to be mapped; the restriction maps are aligned with that of p3358 in Fig.5.6.

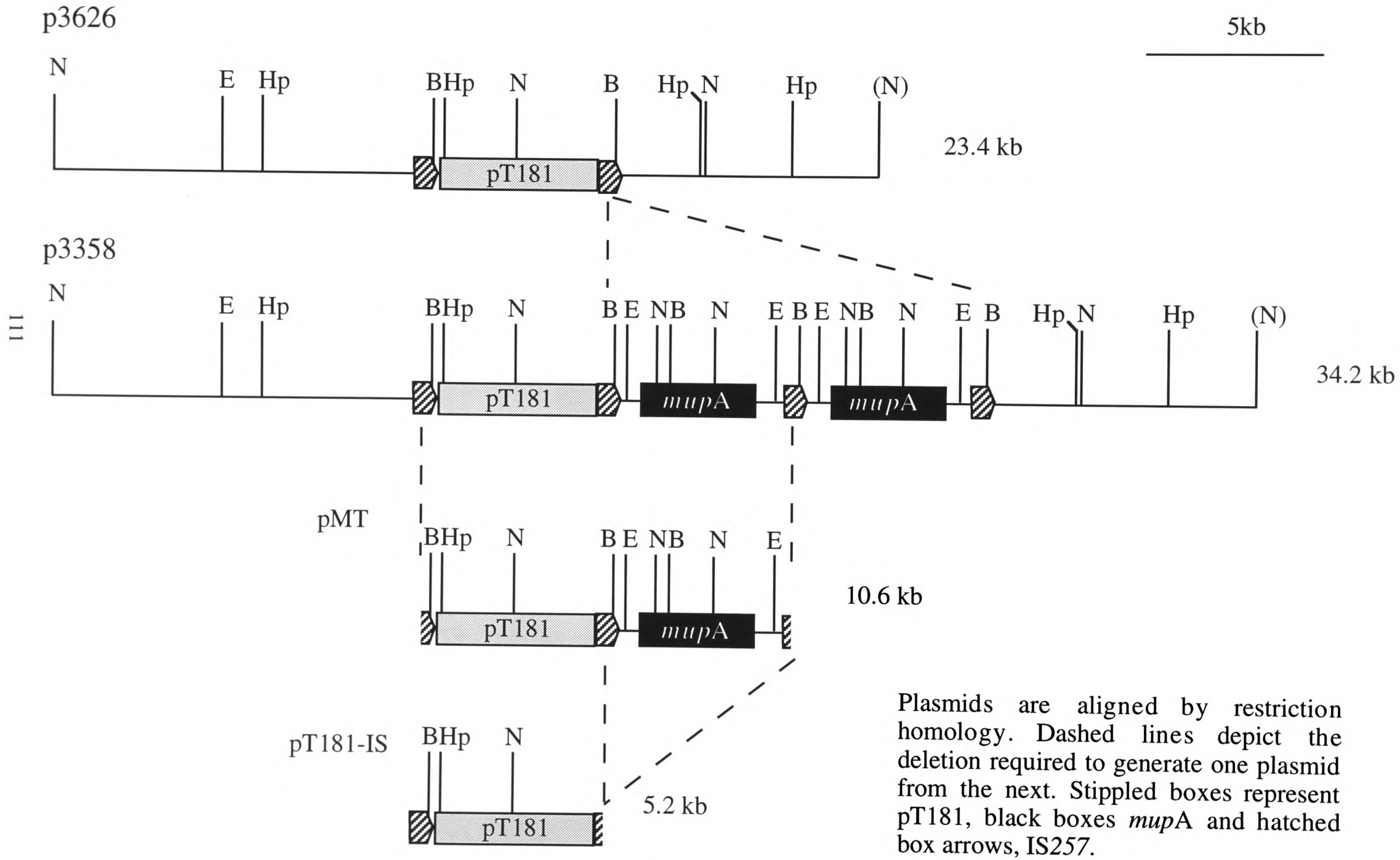
The transconjugant plasmid, p3626, is equivalent to p3358 deleted for two copies of *mup*A and two IS*257*s, but retaining the pT181-like sequences flanked by IS*257*s in direct repeat and the original "backbone" of the p3358/p3356 family of plasmids. The 10.6kb transformant plasmid, named pMT, is a deleted version of p3358 retaining only two copies of IS*257* interspersing the *mup*A region and the pT181-like insertion. The 5.2kb transformant plasmid (pT181-IS) is a pT181-like plasmid with an IS*257* integrated into the 346bp *Mbo*I fragment (Fig.5.7), as found for the 5.2kb transconjugant (Needham *et al.*, 1994) and the 5.2kb plasmid found in the same transformant as the 10.6kb plasmid. In all cases the ends of the deletion were in the vicinity of the IS*257*s, and the number of IS*257*s reduced, implicating a role for IS*257* in the deletion.

Figure 5.5 A Southern blot of the transformant and transconjugant plasmids generated from p3358



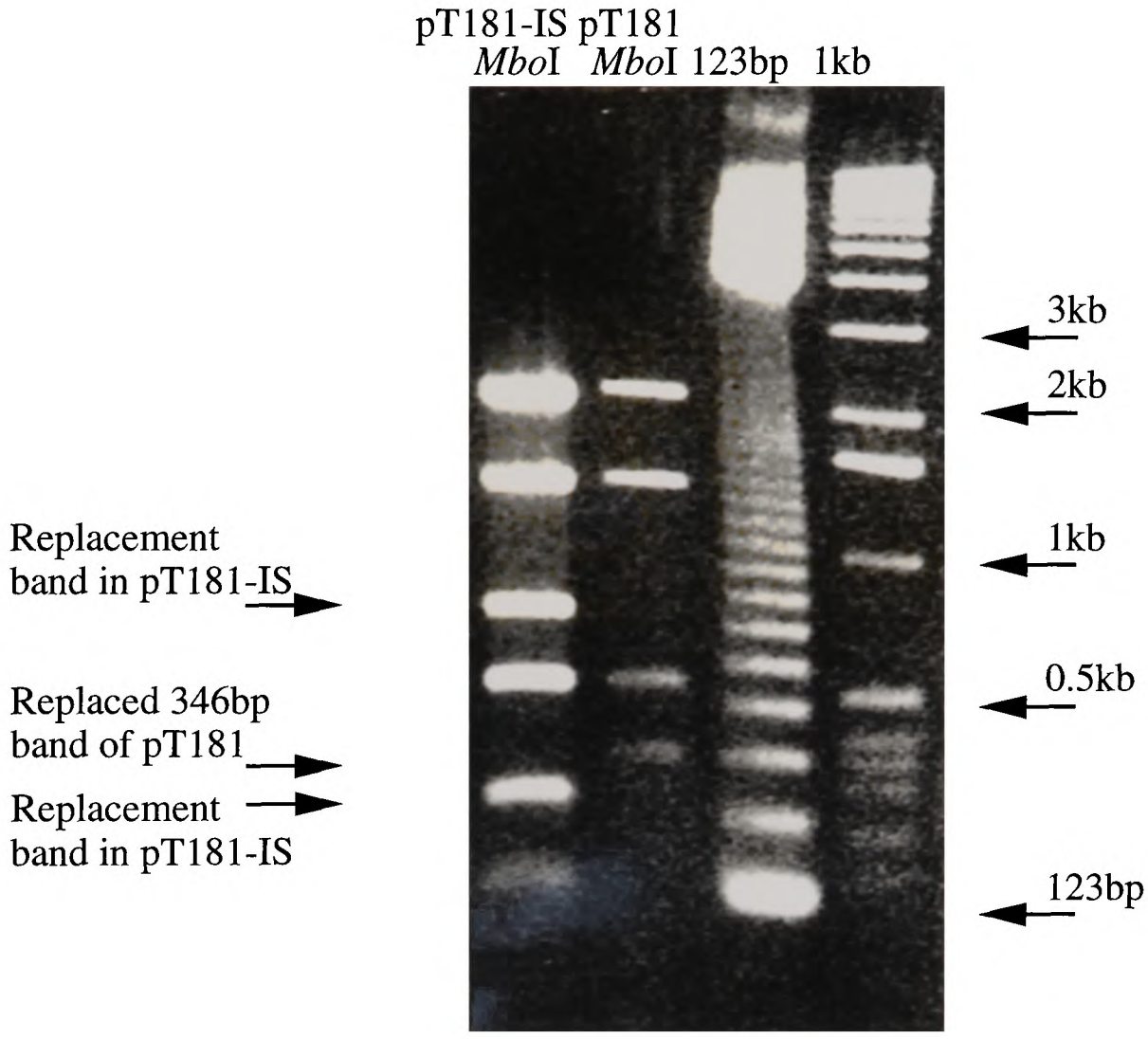
a) Digested DNA: lanes 1 & 16, Raoul ladder; lanes 2 & 15, 1kb ladder; lane 3, pIP1644 digested with *Eco*RI and *Pst*I; lane 4 pUC18 digested with *Eco*RI; lane 5, pT181 digested with *Hin*dIII as a negative control; lane 6, empty; lanes 7 & 11, c.20kb plasmid (p3626); lanes 8 & 12, 5.2kb plasmid (pT181-IS); lanes 9 & 13, 10.6kb plasmid (pMT); lanes 10 & 14, p3358; lanes 7-10 digested with *Nco*I; lanes 11-14 digested with *Bgl*II.
b) Auoradiograph of the gel in (a) hybridised with pIP1644, which contains sequences internal to the transposase of IS*257* and pUC18 which has homology to bands of both 1kb and Raoul ladders.

Figure 5.6 Restriction maps of p3358 and deletion plasmids p3626, pMT and pT181-IS derived from p3358



Plasmids are aligned by restriction homology. Dashed lines depict the deletion required to generate one plasmid from the next. Stippled boxes represent pT181, black boxes *mup*A and hatched box arrows, IS*257*.

Figure 5.7 *Mbo*I restriction enzyme digestion of pT181 and pT181-IS

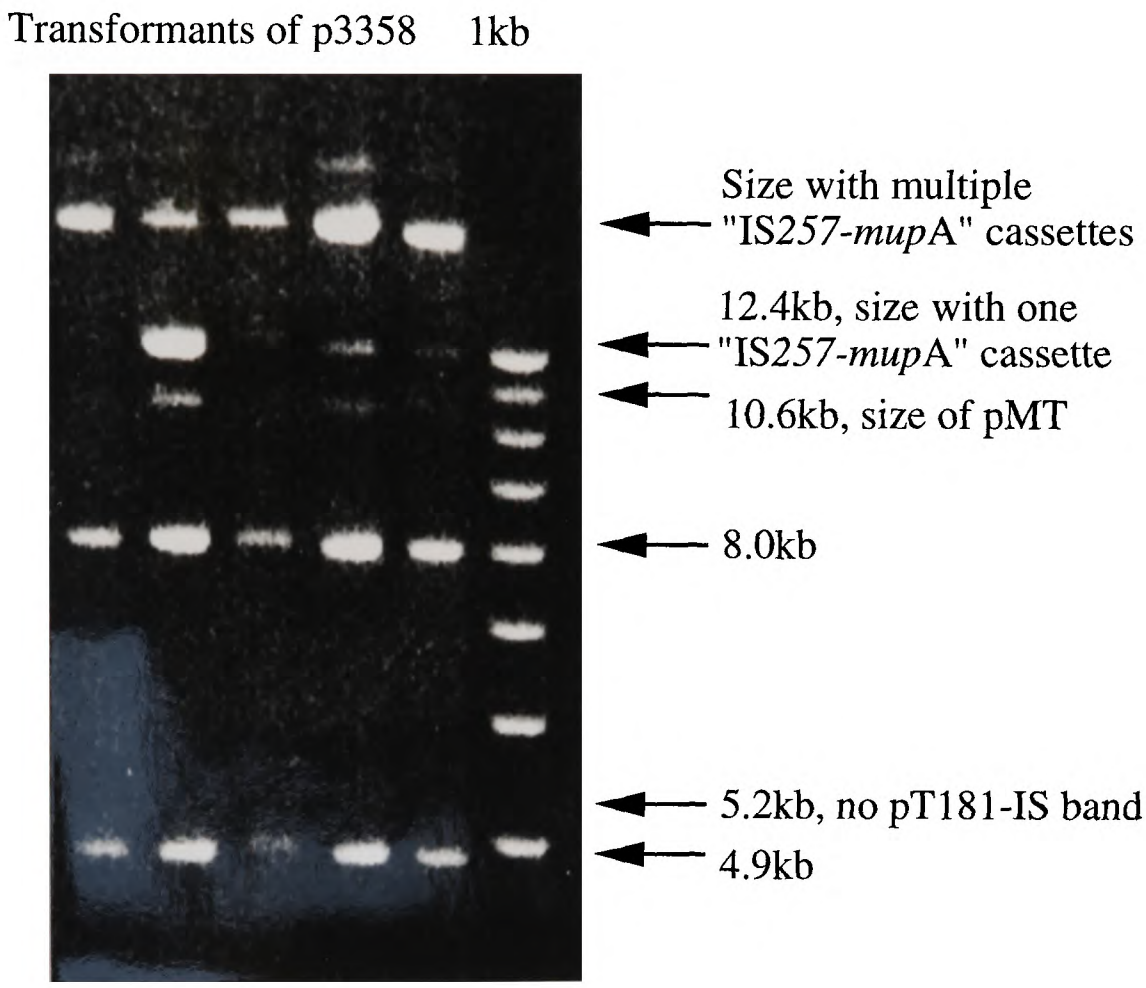


Transformation with mupirocin selection only generated Mu^RTc^R transformants containing plasmids with p3358-type restriction patterns, although the varied *Hpa*II fragment sizes suggested that the degree of "IS*257*-*mup*A" tandem duplication varied between the transformants (Fig.5.8). The variation could be caused by a difference in either the level of duplication originally, or in the deletion after the duplication. That small quantities of a pMT-sized band were visualised in *Hpa*II digestions of two transformants, not seen on gels of uncut DNA, suggesting that deletion has occurred in a fraction of the plasmids post transformation. Likewise W.C.Noble had found only Mu^RTc^R transconjugants when selecting for mupirocin resistance (Needham *et al.*, 1994).

5.5 Plasmid pMT is unstable

The transformant containing the 10.6kb plasmid, pMT, also contained the smaller 5.2kb plasmid, pT181-IS. It is commonly accepted that transformation selects for one plasmid only, whereas in this case it appeared that two plasmids had entered concomitantly. It was possible that inadvertently two colonies containing different plasmids had been selected together, and the resulting DNA preparation therefore appeared mixed. Cells were sonicated to separate the aggregates of staphylococci, streaked for singles on CY-agar and "mini-prep" DNA made from twenty of them. Undigested DNA was electrophoresed and demonstrated that two plasmids, of 5.2kb and 10.6kb were present in DNA from all twenty single colonies. Bacteriophage 53 was grown on the initial transformant, and the resultant 'phage used to infect RN4220; "mini-prep" DNA from single transductants, selected on CY-agar containing 5μg mupirocin ml^{-1}, contained the two plasmids. Should the initial transformant have selected two separate plasmids, neither sonication nor transduction would have separated them. As the uptake of two plasmids by transformation is such a rare event, it was likely that a second round of transformation using DNA from the initial transformant with selection on mupirocin (5μg ml^{-1}) would select only for the 10.6kb plasmid, as the 5.2kb plasmid does not carry *mup*A. A second round of transformation to RN4220 was performed but of the twenty resultant Mu^RTc^R transformants used to prepare DNA, all contained the two plasmids, 10.6kb and 5.2kb. It was concluded that the larger Mu^RTc^R 10.6kb plasmid, pMT, was unstable, readily losing 5.4kb to generate the smaller 5.2kb plasmid, pT181-IS. Both pMT

Figure 5.8 *Hpa*II restriction enzyme digests of DNA of transformants of p3358, selected on mupirocin



and pT181-IS were able to co-exist in the same cell even in the absence of antibiotic selection.

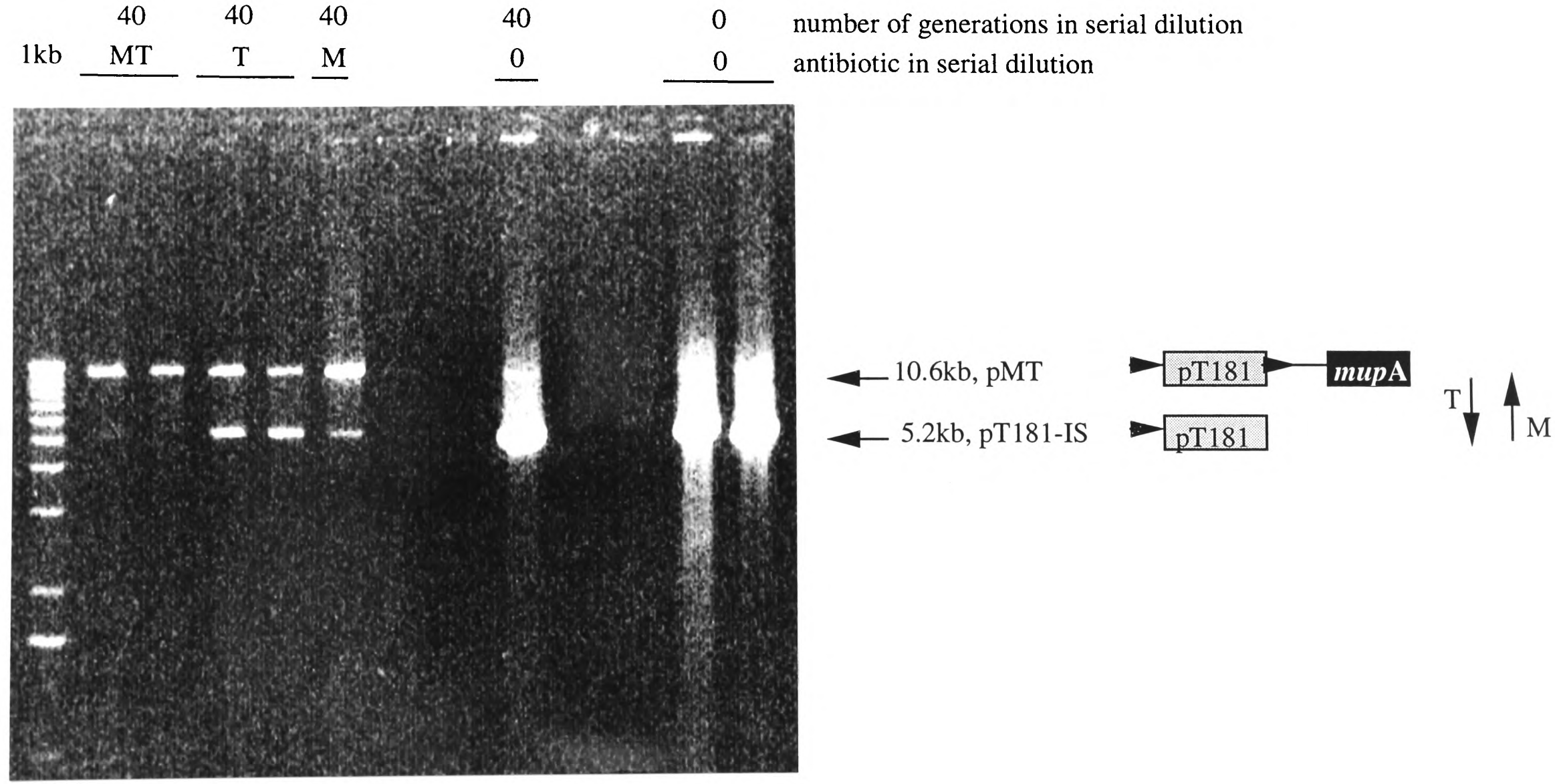
5.6 Manipulation of the plasmid population by antibiotic selection

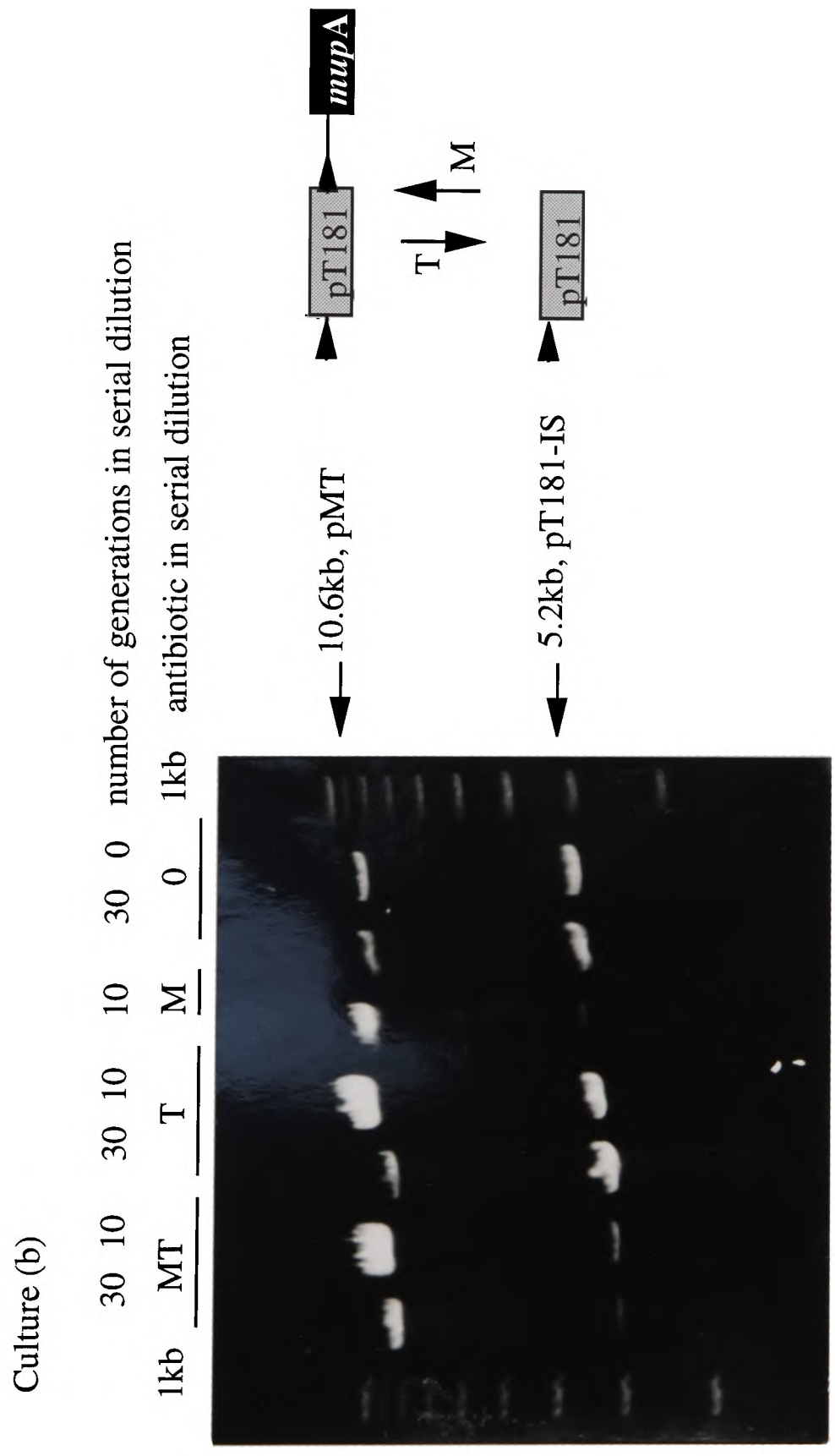
Duplication of the "IS*257*-*mup*A" cassette of p3356 was elicited by mupirocin (Section 4.2). Selection with tetracycline created deleted forms of p3358 upon transformation (Section 5.4). Thus it appeared that antibiotics were selecting for rearrangements in plasmids of the p3356 family.

pMT was unstable, and always found to co-exist with pT181-IS, a smaller plasmid deleted for the *mup*A region and an IS*257*. The relative proportions of the two plasmids varied between preparations made from cultures grown in the absence of antibiotics, suggesting that the rate of rearrangement was not constant. Two separate cultures, each containing different proportions of the two plasmids, were serially diluted (as Section 2.16) in the absence of antibiotics, in the presence of only mupirocin (200μg ml^{-1}), tetracycline only (10μg ml^{-1}) and in both mupirocin and tetracycline (concentrations as when used alone). DNA was prepared from cells both prior to the dilution, and after a known quantity of generations; DNA was digested with *Hpa*II, which restricts both pMT and pT181-IS plasmids within the *ori* of pT181 (Fig.5.9). The plasmid population was stable over 40 generations in the absence of antibiotics, the initial proportions of the two plasmids being maintained for each culture. Serial dilution with mupirocin selected for the larger Mu^RTc^R plasmid pMT in preference to the smaller Mu^STc^R plasmid pT181-IS. Subculture in the presence of tetracycline appeared to select for both plasmids, most noticeable by the reduction in the proportion of pT181-IS in Fig.5.9 example (a) after serial dilution in tetracycline. Dilution with both antibiotics selected for the larger plasmid, which expresses both resistances. Thus, although the laboratory manipulation had selected for a rearranged *mup*A plasmid of higher copy number than the naturally occurring *mup*A plasmids, this plasmid is unstable in the absence of antibiotic selection.

Figure 5.9 ***Kpn*I digestions demonstrating the change in plasmid population in RN4220 (pMT/pT181-IS) upon serial dilution in various antibiotics**

Culture (a)





The proportions of plasmid pMT (10.6kb) and pT181-IS (5.2kb) are constant in the absence of antibiotics.The proportion of pMT increases upon selection with mupirocin (M), and also when both mupirocin (M) and tetracycline (T) are used to select. Results of two different experiments are given.

It was possible to select for rearrangements of the unstable plasmid pMT by antibiotic manipulation. The possibility of selecting for rearrangements of a stable plasmid by the use of antibiotics was investigated by serial dilution of p3626 in the presence of both 10 and 50μg tetracycline ml^{-1} and in its absence. Within 10 generations at either concentration, the smaller 5.2kb pT181-IS plasmid was present in the DNA population in high proportions (as seen by the 5.2kb fragment of the *Eco*RI/*Kpn*I digestion in Fig.5.10). Tetracycline had selected for rearrangement of the stable plasmid p3626 by the loss of all but the pT181-like sequences and one of the IS*257*s.

Likewise antibiotic selection of p3358 generated rearrangements: serial dilution in 200μg mupirocin ml^{-1} generated versions of p3358 with higher numbers of the "IS*257*-*mup*A" tandem repeat; subculture in 10μg tetracycline ml^{-1} selected for the deleted plasmid forms pMT and pT181-IS, but greater proportions of pT181-IS; and dilution in both antibiotics selected for both pMT and pT181-IS equally (Fig.5.11).

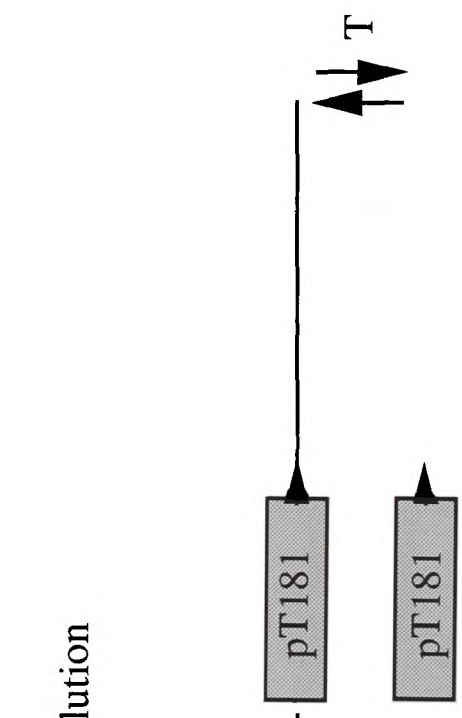
The plasmid population of cultured cells could be manipulated by the use of both antibiotics mupirocin and tetracycline. It appeared that the rearrangements all involved IS*257* as the ends of the deletions and duplications were within the IS*257*s (Fig.5.6).

5.7 Sequencing the flanks of the IS*257*s of the plasmids p3358, p3626, pMT and pT181-IS

The deletions in the plasmids p3626, pMT and pT181-IS relative to p3358 appear from mapping to end in close proximity to the ends of IS*257*s. The insertion of a pT181-like plasmid, into a p3356D-like plasmid to give p3358 appears to have involved IS*257* as the inserted plasmid is flanked by direct repeats of IS*257*. The sequences of the flanks of the IS*257*s at the ends of these deletions and insertions were generated in order that any differences could provide clues as to the mechanisms involved in these rearrangements.

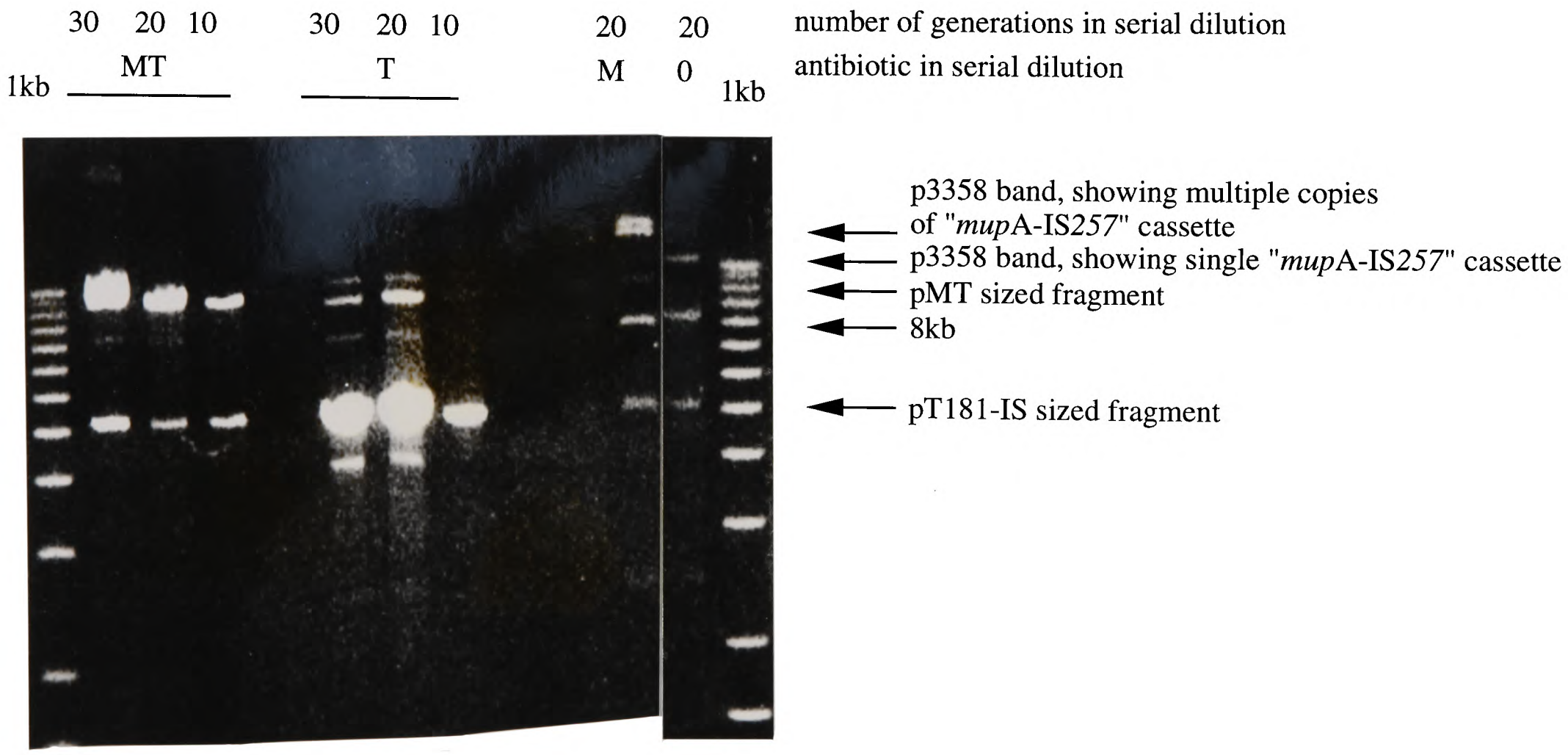
As pT181-IS only contains one IS*257*, the oligonucleotides J1IS*257* and J2IS*257*, designed to sequence out from IS*257* (Fig.3.18, Section 3.9), were used to sequence the flanking

Figure 5.10 *Kpn*I/*Eco*RI digestions demonstrating the tetracycline selection for pT181-IS from p3626



Plasmid p3626 gives fragment sizes 7.0, 7.5, and 8.4kb upon *Eco*RI and *Kpn*I digestion, the 5.2kb band is from pT181-IS which only contains a *Kpn*I site.

Figure 5.11 *K*pnI restriction digests to demonstrate the change in plasmid population upon serial dilution of RN4220 in mupirocin and tetracycline



regions of pT181-IS. The sequence verified the restriction enzyme based homology of pT181-IS to pT181 (Fig.5.7). The IS*257* was flanked by 8bp direct repeats of pT181 sequence (bp 4252-4259, numbering from the unique *Pvu*I site, against the direction of transcription, and opposite to Khan and Novick, 1983) (Fig.5.12).

Due to homologies with p3356, the oligonucleotides UPIN, UPOUT, DNOUT and DNIN (Fig.3.13, Section 3.7), used to sequence into the IS*257*s of p3356 from the flanking regions could be used to sequence many of the flanking regions of the IS*257*s of p3358, p3626 and pMT (Fig.5.13). There were no differences in these sequences as compared to the corresponding sequences of p3356 and p3356D (Fig.4.5, Section 4.3). The rearrangements had left the remaining flanking sequences of the IS*257*s intact.

The sequences of the flanks of the IS*257* in pT181-IS had been determined, and therefore primers (pT181ORI and pT181REPC) could be designed to sequence out of the pT181-like sequences of plasmids pMT, p3626 and p3358 into the flanking IS*257*s (Fig.5.12 and Fig.5.13). There was no difference in these sequences from those of pT181-IS: the duplication of the 8bp of pT181 directly adjacent to the IS*257*s was present in all plasmids which contained the pT181-like plasmid (Fig.5.13).

5.8 Discussion

Plasmid p3358 is a member of the p3356 family of plasmids. It differs from p3356D (Section 4.2) in that it has a pT181-like plasmid integrated upstream of the "IS*257*-*mup*A" tandem repeat, and that the integrated plasmid is flanked by IS*257* in direct repeat. The integration of a pT181-like plasmid into the chromosome of *S.aureus* has been documented previously (Gillespie *et al.*, 1986; Matthews *et al.*, 1990), the site at which pT181 is linearised in the chromosomal insertion (between *rep*C and *tet* control region) differs from that in p3358. Although pT181 has not been reported as a co-integrate with another plasmid, pUB110, a small plasmid encoding kanamycin, neomycin, paromomycin and tobramycin (aminoglycoside) resistances, has been reported integrated into both the chromosome (Dubin *et al.*, 1991) and plasmids pSK41 and pUW3626 (Byrne *et al.*, 1991). In all three of these

Figure 5.12 Sequences of the flanks of the IS*257* on pT181-IS

(a) Sequence of pT181-IS, showing the 8bp direct repeat flanking the IS*257*



The hatched boxed arrow represents the IS*257*. The 8bp direct repeat flanking the IS*257* is highlighted in bold type. The sequences in uppercase are taken directly from pT181-IS, and are identical to pT181 published sequence except for the 8bp duplication; the sequences in lowercase are taken from the published sequence of pT181 (Khan & Novick, 1983). The positions of the oligonucleotides, PT181ORI & PT181REPC designed to sequence back into the IS*257* from the surrounding pT181, are given by arrows.

(b) Autoradiographs of the sequences of pT181-IS generated using the oligonucleotides J1IS*257* and J2IS*257*. The 8bp direct repeats are highlighted in bold type. The order of dideoxy termination mixes in the lanes is A, C, G, T.



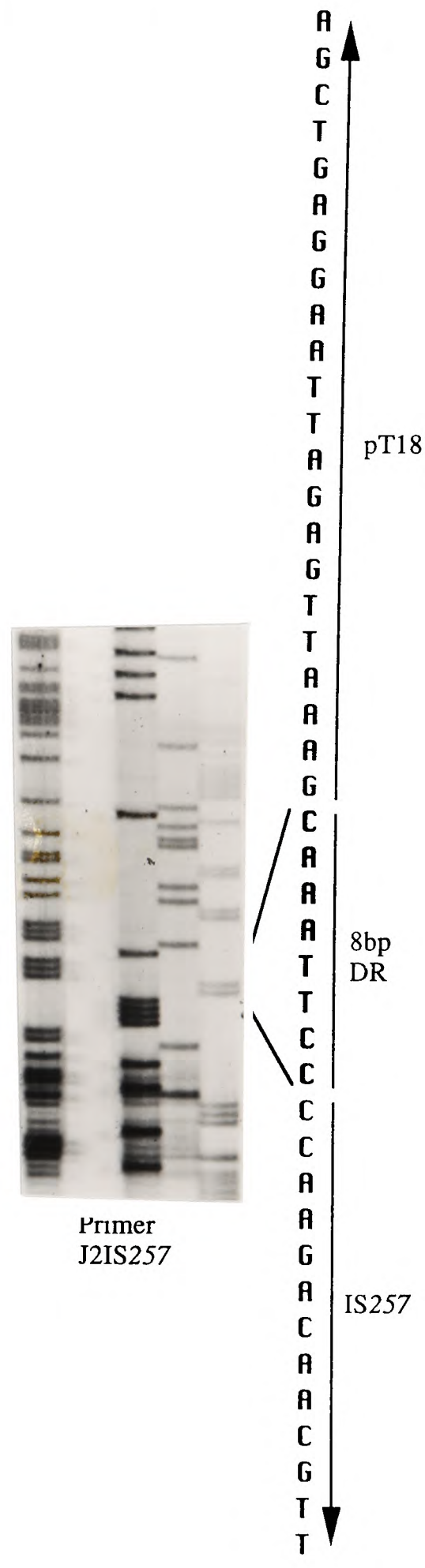
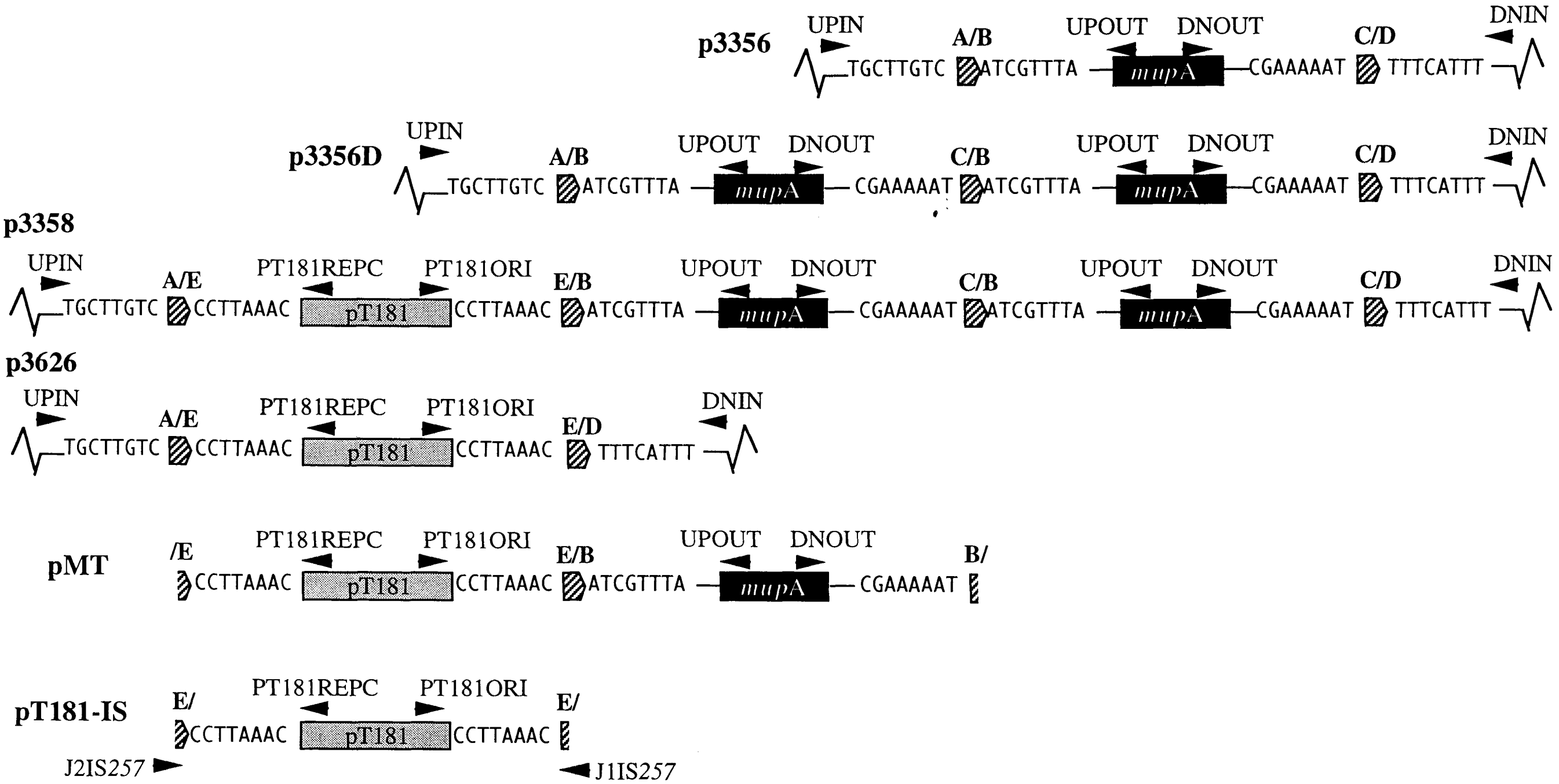


Figure 5.13 Sequences of the junctions of the IS*257*s in the p3356 family of plasmids and deletion plasmids generated from them



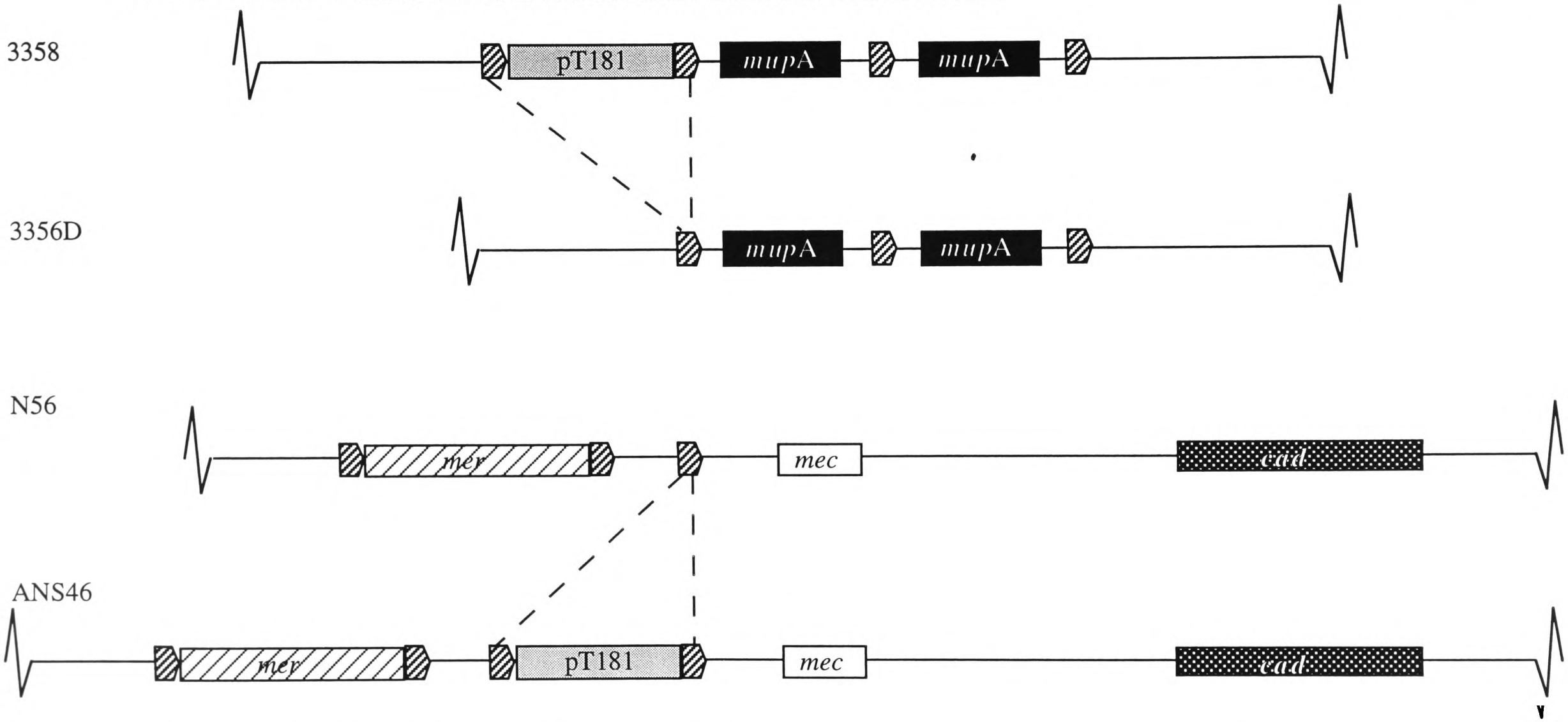
Sketch diagrams of the p3356 family of plasmids and deletion plasmids generated from them. The sequences of the 8 bases flanking the junctions of the IS*257*s are given, demonstrating that the sequences have not changed, but remained intact upon deletion/rearrangement. The only direct repeat (indicative of a transposition event) is internal to the pT181 and remains after deletion of larger plasmids to smaller plasmids. Only pMT and pT181-IS are shown as complete, and are linearised in one of the IS*257*s. The positions of binding sites of the primers used for sequencing are depicted by arrows. Letter codes are given above the IS*257*s to facilitate recognition of the origin of the sequence.

cases, the integrated plasmid is flanked by direct repeats of IS*257* and the sequences at the internal flanks of the IS*257* are 8bp duplications of sequences of the integrated plasmid. Likewise, the 8bp direct repeats (duplicated with respect to pT181) internal to the IS*257*s flanking the integrated pT181-like plasmid in p3358 are sequences inherent to pT181, and are interpreted to be the result of a transposition event involving an IS*257*.

Studies of methicillin resistant *S.aureus* (mc-MRSA) showed that there was a unique site of integration of pT181 in all Tc^R mc-MRSA (e.g.ANS46). Comparison of the *mec* region of the mc-MRSAs ANS46 and N56 showed that N56 was related to ANS46 in all but the presence of the integrated pT181: instead of the pT181 flanked by direct repeats of IS*257*, it contained only an IS*257* upstream of *mec* (Stewart *et al.*, 1994). Therefore the *mec* chromosomal regions ANS46 and N56 parallel p3358 and p3356D in their relationship to each other (Fig.5.14). The sequences flanking the junctions of the lone IS*257* upstream of *mec* in N56 are identical to the external flanks of the IS*257*s flanking the integrated pT181 of ANS46; the same is true of p3356D, of which the flanks of the most upstream IS*257* of the tandem repeats are identical in sequence to the external flanks of the IS*257*s flanking the integrated pT181 of p3358.

Two models were proposed for the evolution of both the pUB110 integrated plasmids and the Tc^R mc-MRSA. The first is based on the observation of Ohtsubo *et al* (1980) that plasmids which contain an insertion sequence are able to act as transposons. This model suggests that an IS*257* had first transposed into the small plasmid (pUB110 or pT181) and the resulting plasmid had then integrated into the chromosome or target plasmid by homologous recombination between this IS*257* and a pre-existing IS*257* on the target DNA. The second model is based on that of Shapiro (1979) which suggests that in replicative transposition, an intermediate co-integrate molecule is formed with the transposable element interspersing the two replicons in direct repeat; for this model to be correct, the process of transposition must have stopped after the formation of the co-integrate and resolution not have occurred. For the cases previously cited (summarised in Stewart *et al.*, 1994), the former model is preferred as it appears that the sites of the integrated IS*257* in the proposed intermediate plasmids (pUB110-IS*257* and pT181-IS*257*, different to pT181-IS) would all

Figure 5.14 Schematic diagrams of the relationship of p3358 with p3356D and the parallel relationship of tetracycline resistant MRSA (ANS46) with tetracycline sensitive MRSA (N56)



Sections of plasmids p3358 and p3356D are represented, with the insertion of pT181, and duplication of IS*257* highlighted by dashed lines. The *mec* region of the chromosome of the MRSA strains ANS46 and N56 are given, with the inserted pT181 and duplicated IS*257* also highlighted by dashed lines. The two situations are very similar, but the point of linearisation of pT181 differs in each case.

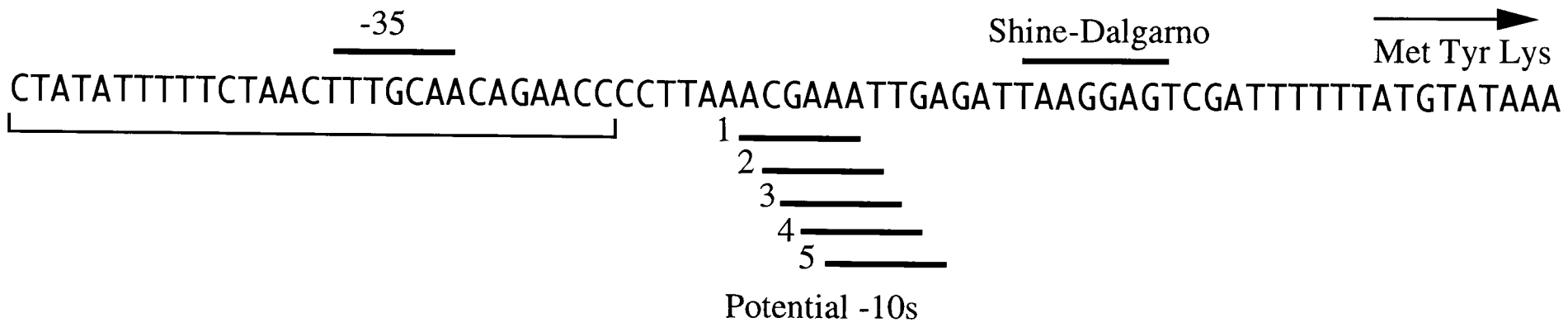
allow independent replication of the intermediate plasmid, although such plasmids have not been isolated.

The proposed intermediate plasmid in the transposition/homologous recombination model (Ohtsubo *et al.*, 1980) of the evolution of p3356D to p3358, pT181-IS, has been isolated and is capable of independent replication. It is not possible to state that the plasmid pT181-IS existed prior to p3358 as pT181-IS has been shown to be a deletion product of p3358 and other related plasmids. Therefore the mere existence of pT181-IS does not confirm the IS-transposition/homologous recombination model. However, the independent existence of pT181-IS is of note: the IS*257* in pT181-IS has integrated into the N-terminus of the RepC mRNA transcript coding region which would be expected to cause termination of transcription from *rep*C promoter at the transcriptional terminator of the transposase gene. RepC is required for initiation of pT181 replication (Manch-Citron *et al.*, 1986). Obviously, *rep*C is transcribed as pT181-IS is capable of independent replication. It is proposed that the IS*257* is providing a hybrid promoter with downstream *rep*C sequences to provide a transcript including the entire ORF and allow translation of full length *rep*C (Fig 5.15). Such an IS*257*-hybrid promoter has recently been demonstrated to be active by S1 nuclease mapping in trimethroprim resistance plasmids (Leelaporn *et al.*,1994). Although the potential -10 promoter sequences have low homology to the consensus, TATAAT, other promoters used in pT181 also have weak homology (Novick, 1990). A cause to doubt a hybrid promoter being used is the proximity of the promoter sequences to the Shine-Dalgarno sequence (Fig.5.16d).

It is of interest to consider the control of pT181-IS replication. pT181 is a member of a family of plasmids which replicate via a uni-directional rolling circle (Novick, 1989; Gruss and Erlich, 1989). There are three main mechanisms controlling pT181 replication. The more minor of these is the cis-acting enhancer nucleotide sequence *cmp* which remains active in plasmids containing only *cmp* and *ori* (Gennaro and Novick, 1988). RepC is the main target for replication control (Manch-Citron *et al.*, 1986). RepC initiates plasmid replication by creating a nick in the leading strand origin and covalently attaching to its 5' terminus (Thomas *et al.*, 1990). In order to prevent activity of the RepC for a second round of replication, the active homodimer is converted to an inactive heterodimer by the covalent

Figure 5.15 Potential hybrid promoters in pT181-IS

(a) Potential hybrid promoters using -35 of IS*257*

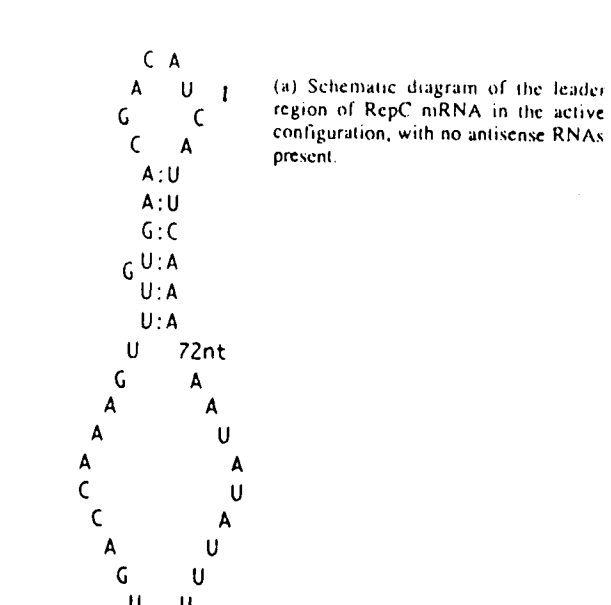


(b) Comparison of real and potential promoters of pT181 and pT181-IS, with the consensus sequences

Gene	-35	-10	Matches of -10 with consensus
pT181*repC*	ATTAGA	TGTTAT	4
pT181*cmp*	TCTTGA	TTTAAT	5
pT181*pre*	TATTAA	TAGACT	4
pT181*tet*	AGCGAA	AACAAA	3
Hybrids	TTGCAA	CGAAAT	3
	TTGCAA	GAAATT	3
	TTGCAA	AAATTG	1
	TTGCAA	AATTGA	2
	TTGCAA	ATTGAG	2
Consensus	TTGACA	TATAAT	

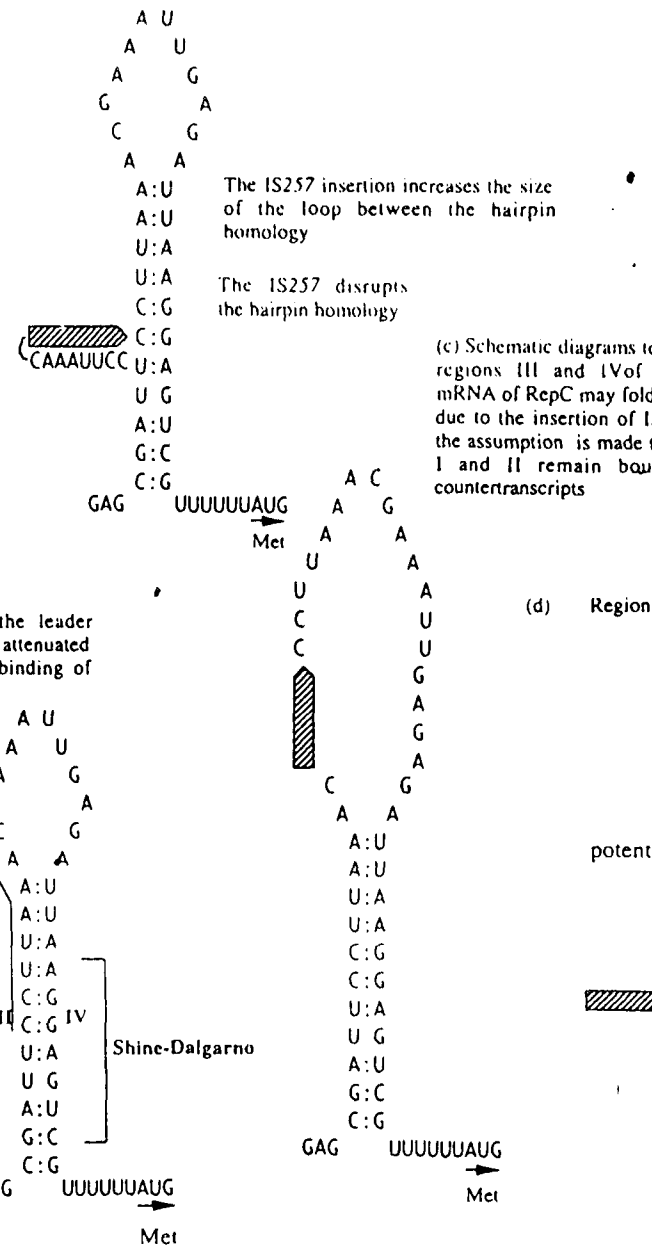
Although the -10 sequences of the potential hybrid promoters of pT181-IS have low homology with the consensus -10, so does the -10 of pT181*tet*

Figure 5.16 Schematic representation of the predicted folding structures of the leader region of RepC mRNA



(a) Schematic diagram of the leader region of RepC mRNA in the active configuration, with no antisense RNAs present.

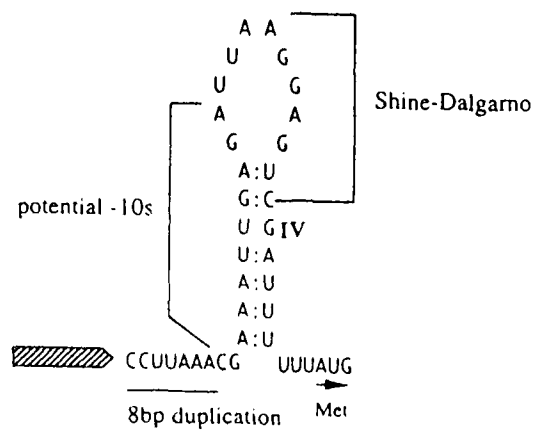
(b) Schematic diagram of the leader region of RepC mRNA in the attenuated conformation caused by the binding of homologous antisense RNAs



(c) Schematic diagrams to show how regions III and IVof the leader mRNA of RepC may fold differently due to the insertion of IS257 (N.B. the assumption is made that regions I and II remain bound to the countertranscripts

IS257 is represented by a hatched box arrow

(d) Region IV of the RepC leader sequence



As region IV contains the potential -10 sites, regions I, II, and III and part of region IV would not be transcribed if the hybrid promoter were active. Also the proximity of the Shine-Dalgarno sequence suggests that an alternative may be used should a hybrid promoter be used.

attachment of a short oligonucleotide to one RepC molecule (Rasooly and Novick, 1993; Rasooly *et al.*, 1994a and b). Neither of these mechanisms would be affected by the IS*257* insertion in pT181-IS. However, these are the more minor control mechanisms.

The main control of the replication of pT181 is inhibition of the synthesis of RepC (Novick *et al.*, 1984a). Two overlapping countertranscripts in the 5' non-coding region of *rep*C mRNA are responsible for this control (Kumar and Novick, 1985). In the absence of these anti-sense RNAs, RepC mRNA will fold (to give hairpin loop I:III, Fig 5.16a) so as to allow recognition of the Shine-Dalgarno sequence and hence translation of RepC. The antisense RNAs bind to the complementary mRNA (regions I and II) preventing the usual conformation and allowing an alternative hairpin loop to form (III:IV) which sequesters the Shine-Dalgarno sequence (Fig 5.16b). This alternative structure acts as a transcriptional attenuator (Novick *et al.*, 1989). The IS*257* in pT181-IS has integrated into the region of the mRNA (III) which would form this attenuation structure (Fig.5.16 a & b). Copy number mutations have been mapped to this region (Novick *et al.*, 1985). In fact, a spontaneous point mutation (*cop623*) in the first base of the 8bp duplication in pT181-IS, causes a very high-copy number phenotype, predicted to be due to a lower free energy of formation of the transcriptional attenuator. Although it is impossible to predict the precise conformation of any mRNA generated from this region in pT181-IS in the presence of the countertranscripts (suggestions are given in Fig.5.16c), its presence is predicted to reduce the free energy of formation of the usual transcriptional attenuator, therefore it is not surprising to find that pT181-IS has a very high copy-number, rendering DNA concentrations nearer to mutant *cop-623* than pT181 in all types of DNA preparation. It is also possible that these interactions may be irrelevant: if indeed the IS*257* does provide both a transcriptional terminator and a hybrid promoter, the RepC mRNA generated will not be subject to the control of the countertranscripts as it would only include region IV (Fig 5.16d).

pT181-IS is one of several smaller plasmids generated by tetracycline selection of p3358 in transformation and serial dilution (and filter transfer by W.C.Noble). These smaller plasmids also gave deletion products on tetracycline selection, each resulting in pT181-IS eventually. As the sequences of the junctions flanking the IS*257*s of the larger plasmids are retained upon deletion of intervening sequences give identical junction sequences in the smaller

plasmids, it is proposed that the deletions occur due to unequal crossover recombination between the homologous IS*257*s which are present in direct repeat. All the reactions occurred in *rec*A$^+$ strains, the dependency of these rearrangements on host *rec*$^+$ functions is not known. The selective advantage of a smaller plasmid may be two-fold. A smaller plasmid would be a reduced biological load on its host which is undergoing the stress of antibiotic selection. The very small plasmid (pT181-IS), to which all the larger plasmids delete upon continued selection, is of much higher copy-number than the larger plasmids thus producing more *tet* product, which may increase the rate of efflux of tetracycline, and thus increase the resistance of the host. That mupirocin selection of p3358 as well as p3356D and p3356 (Section 4.2) leads to increased plasmid size suggests that the non-*mup*A sequences in the "IS*257*-*mup*A-IS*257*" cassette do not contain the replication requirements of this plasmid family, which if present would allow the deletion of a "*mup*A-IS*257*" plasmid. Additionally, that both antibiotics elicit rearrangements which involve unequal crossover between the IS*257*s of p3358 indicates that these rearrangements are in no way caused by the antibiotic, but rather occur due to the selection by the antibiotic of a cell containing a rearranged plasmid which confers a selective advantage to its host.

It is interesting to note that all the deleted plasmids are stable in the absence of antibiotics except for pMT. The instability of pMT is thought to be due to the mechanism of plasmid replication. There are four groups of staphylococcal plasmid, small Class I plasmids (1-5kb) and large plasmids (>15kb, Classes II, III and IV) (Novick, 1989). Small staphylococcal plasmids replicate via a rolling circle mechanism. This process generates large amounts of single-stranded DNA which is thought to be more unstable in larger plasmids (Riele H.T., *et al.*, 1986; Murray *et al.*, 1989); this is often cited as a reason for the discontinuity of plasmid size. pMT is 10.6kb, which is above normal size for the group of small Class I plasmids, but it is thought to be using the replication system of pT181. It is possible that it is inherently unstable due to the large single-stranded intermediate of replication, and that any deleted forms would be selected for by default. A more favourable explanation is that the occurrence of the single stranded intermediate of replication increases the probability for mispairing of the IS*257* direct repeats, concurrently in a single-strand during replication, thus increasing the frequency of unequal crossover between them and selecting for the deleted form, pT181-IS. Larger plasmids (Classes II, III and IV) use the bi-directional theta mode

of replication (Novick, 1989). The large plasmids (p3358 and p3626) will be more stable than pMT, as there is no large single-stranded intermediate of replication encompassing both IS*257*s. (For a review of plasmid replication, see Kornberg and Baker, 1992.)

Rearrangements of plasmids of the p3356 family have occurred in nature: plasmids p3356, p3356D and p3358 were originally found in clinical isolates, as was pT181-IS in very small quantities detectable only by hybridisation. Although the duplications observed in both p3356D and p3358 may have occurred in routine selection of these clinical isolates[2] the integration of pT181 into a p3356D-type plasmid to generate p3358 must have occurred in the natural environment. It is not known whether the patient had been treated with tetracycline, which may have led to the selection of this linked resistance plasmid. Whatever the cause, it appears that two types of rearrangement involving IS*257*s have occurred in the p3356 family of plasmids: recombination, leading to both deletion and duplication of DNA between the IS*257* direct repeats, and transposition involving IS*257*, perhaps linked to a recombination event. Both of these types of rearrangement have parallels in other systems, e.g. the rearrangements inferred in the *mec* region of the chromosome of mc-MRSA strains discussed above and in Section 4.8. The recombination events had been repeated in the laboratory, whereas the transposition event had not, in fact Galas and Chandler (1989) had suggested that IS*257* was no longer active in transposition. Research continues to investigate the mechanisms of both the recombination and the transposition rearrangements.

[2] In order to identify mupirocin resistant strains, cells were subject to mupirocin in routine screening.

Investigation of rearrangements involving IS257s

Chapter Six

Investigation of rearrangements involving IS*257*s

6.1 Introduction

Investigations of *mup*A plasmids have identified several rearrangements involving duplication, integration and deletion of DNA which appeared to be caused by crossover between copies of IS*257*, a putative insertion sequence (Rouch *et al*, 1989). Although transposition of IS*257* had not been demonstrated, its ubiquitous nature in staphylococcal DNA, implied that at some stage it had been active in transposition; however, current opinion was that it was no longer active in this way (Galas and Chandler, 1989). Likewise, comparison of related plasmids had implicated a role for IS*257* in replicon fusion and gene duplication (Gillespie *et al*, 1986) but such functions had not been formally demonstrated.

The rearrangements discussed in this volume so far, infer a role for IS*257* as the site of recombination which allows the rearrangement observed. It is possible that the IS*257* merely provides a site of homology, and that the rearrangements are catalysed wholly by host recombination machinery (*rec*). Alternatively, the putative transposase may itself act as the catalyst for the recombination events, dispensing the need for active *rec* functions. On the other hand, both systems may be required. It is also possible that the various types of rearrangements utilise different combinations of the two. Experiments were designed to elucidate this issue.

6.2 Replicon fusion *in vitro*

p3358 is a co-integrate of pT181-IS and p3356D-type plasmids. As pT181 is a small self-replicating plasmid, it is most likely that p3358 evolved from p3356D rather than *vice versa.* pT181-IS is a deletion product of p3358 and therefore, although it has been found in J3347 (the clinical strain from which p3358 was isolated), it is not known whether pT181-IS existed as an independent plasmid prior to the formation of p3358 or only as a deletion

product of it. Two models exist for the formation of p3358: firstly that IS*257* had transposed into pT181 to produce pT181-IS which then recombined with p3356D to give p3358 (Shapiro, 1979); and secondly that p3358 is a product of replicative transposition from p3356D into pT181, a process which does not include pT181-IS as an intermediate (Ohtsubo *et al.*, 1980). The requirement for a pT181-IS intermediate easily distinguishes the two models, this distinction was exploited in initial attempts to dissect the mechanism of replicon fusion.

It was decided to attempt to "repeat" nature by creating heteroplasmid strains containing p3356 and a small staphylococcal plasmid both with and without a copy of IS*257*, recombination would then be encouraged between them. The erythromycin resistance plasmid pOX7 (pOX300 in Dyke *et al.*, 1991) was an ideal such small plasmid as it contains the multiple cloning site of pUC18 to allow a copy of IS*257* to be cloned into it, and also it is temperature sensitive for replication in staphylococci allowing selection on erythromycin at restrictive temperature to encourage recombination and thus rescue the strain.

RN4220, a strain of *S.aureus* which can be made competent for transformation with DNA made in *E.coli* (Novick, 1990), was transformed with p3356 with selection on mupirocin (5μg ml^{-1}). A transformant was then made competent and transformed with either pOX7 or the pOX7 clone, pCN9.1 (a clone of the 1.3kb *Eco*RI fragment of p3356D which contains a copy of IS*257*, hereafter referred to as pOX7-IS), selection was on erythromycin (20μg ml^{-1}) at 30°C. Transformants were streaked onto CY-agar containing mupirocin (5μg ml^{-1}) and CY-agar containing erythromycin (20μg ml^{-1}); "mini-prep" DNA was prepared from $Em^{R}Mu^{R}$ transformants to check for the presence of both plasmids expected: pOX7-based plasmids were present in higher copy number than p3356 as determined by ethidium bromide staining. Two heteroplasmid strains of each type (RN4220 $(p3356/pOX7)_{6\ and\ 7}$; RN4220 $(p3356/pOX7\text{-}IS)_{2\ and\ 4}$) were used to inoculate CY-broth containing 20μg erythromycin ml^{-1} and cultures were grown overnight at permissive temperature (30°C). 200μl aliquots of each overnight culture were used to inoculate 2.5ml CY-broth which were then grown in the absence of antibiotics until mid-log phase. The OD_{675} of each culture was used to determine the concentration of cells. The culture was then serially diluted in CY-broth to allow specific numbers of cells to be plated on CY-agar containing 40μg erythromycin ml^{-1}. Plates were

incubated for 20h at both permissive (30°C) and restrictive (42°C) temperatures. The colonies which grew up on each plate were counted and the frequency of co-integration of the pOX7-based plasmid into a temperature stable replicon was determined (Table 6.1). Twenty colonies were selected from plates of each strain and streaked onto CY-agar containing mupirocin (5μg ml^{-1}) and CY-agar containing erythromycin (20μg ml^{-1}): all retained resistances to both mupirocin and erythromycin. "Mini-prep" DNA was made from colonies of each type, and electrophoresed to demonstrate that only one plasmid was present in each co-integrate.

That the colonies grew at restrictive temperature and retained erythromycin resistance, indicated that the pOX7-based plasmids had integrated into a temperature stable replicon: either another plasmid or the chromosome. To consider the DNA preparations of the co-integrates of p3356 and pOX7-IS: *Bgl*II and *Eco*RI digestions contained the same fragments as the two comprising plasmids, but all bands were approximately equimolar (Fig.6.1a). It appeared that the IS*257* of pOX7-IS had recombined with one the IS*257*s of p3356. *Xho*I digestions and further mapping revealed that the integration occurred with either of the two IS*257*s of p3356, with equal preference (Figs.6.1b & 6.2)[1].

Colonies grew from the heteroplasmid strains RN4220(p3356/pOX7) indicating that at least the Em^R determinant of pOX7 had integrated into a temperature stable replicon. Only one of the initial plasmids in the strain carried IS*257*, and so the integration could not merely have occurred between IS*257*s already resident on the two plasmids as with RN4220(p3356/pOX7-IS). *Bgl*II digestions of all these plasmids gave the same pattern containing all the fragment sizes of p3356 plus an extra band, 7.0kb, larger than pOX7, (6.3kb) but smaller than pOX7-IS (7.6kb) (Fig.6.1a). The additional *Bgl*II site indicated IS*257* duplication, and the size of the extra band suggested that it had duplicated to flank an integrated pOX7 in direct repeat, in the same way that the IS*257*s of p3358 flank the integrated copy of pT181 (Fig.5.6, Section 5.4); *Xho*I digestion showed that the integration had occurred in either the upstream or the downstream IS*257* of p3356, with equal

[1] Although it is possible that the recombination had occurred between the DNA flanking the IS*257* on the clone and its homologue on p3356, this was impugned by the outcome of the same experiment on the RN4220(p3356/pOX7) strains.

Table 6.1 Frequencies of replicon fusion between p3356 and either pOX7 or pOX7-IS

Heteroplasmid strain	No. cfu plated	Temp. (°C)	Number colonies grown		Frequency fusion[1]
RN4220 (p3356/pOX7)$_6$	10^5	42	++	++	$6.45x10^{-3}$ to $7.49x10^{-3}$
	10^4	42	201	73	
	10^3	42	20	1	
	10^3	30	++	++	
	10^2	30	267	105	
	10^1	30	31	11	
RN4220 (p3356/pOX7)$_7$	10^5	42	++	++	$6.90x10^{-3}$ to $8.66x10^{-3}$
	10^4	42	259	80	
	10^3	42	20	0	
	10^3	30	++	++	
	10^2	30	299	88	
	10^1	30	29	12	

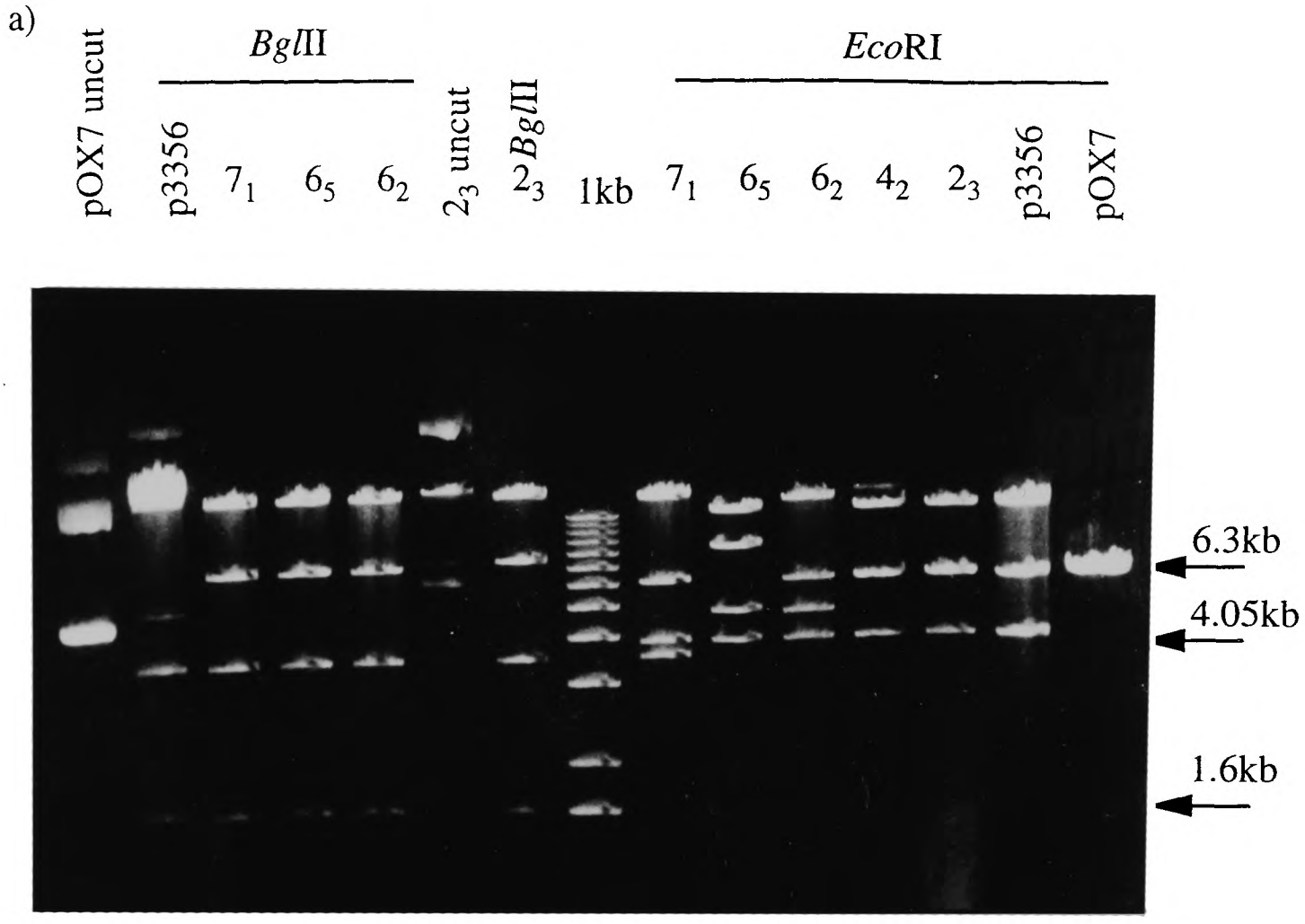
[1] Frequency of replicon fusion is given as the ratio of the average number of colonies grown per colony forming unit (cfu) in the inoculum on plates incubated at 42°C, with those incubated at 30°C. Figures are given for two experiments. ++ denotes confluent growth.

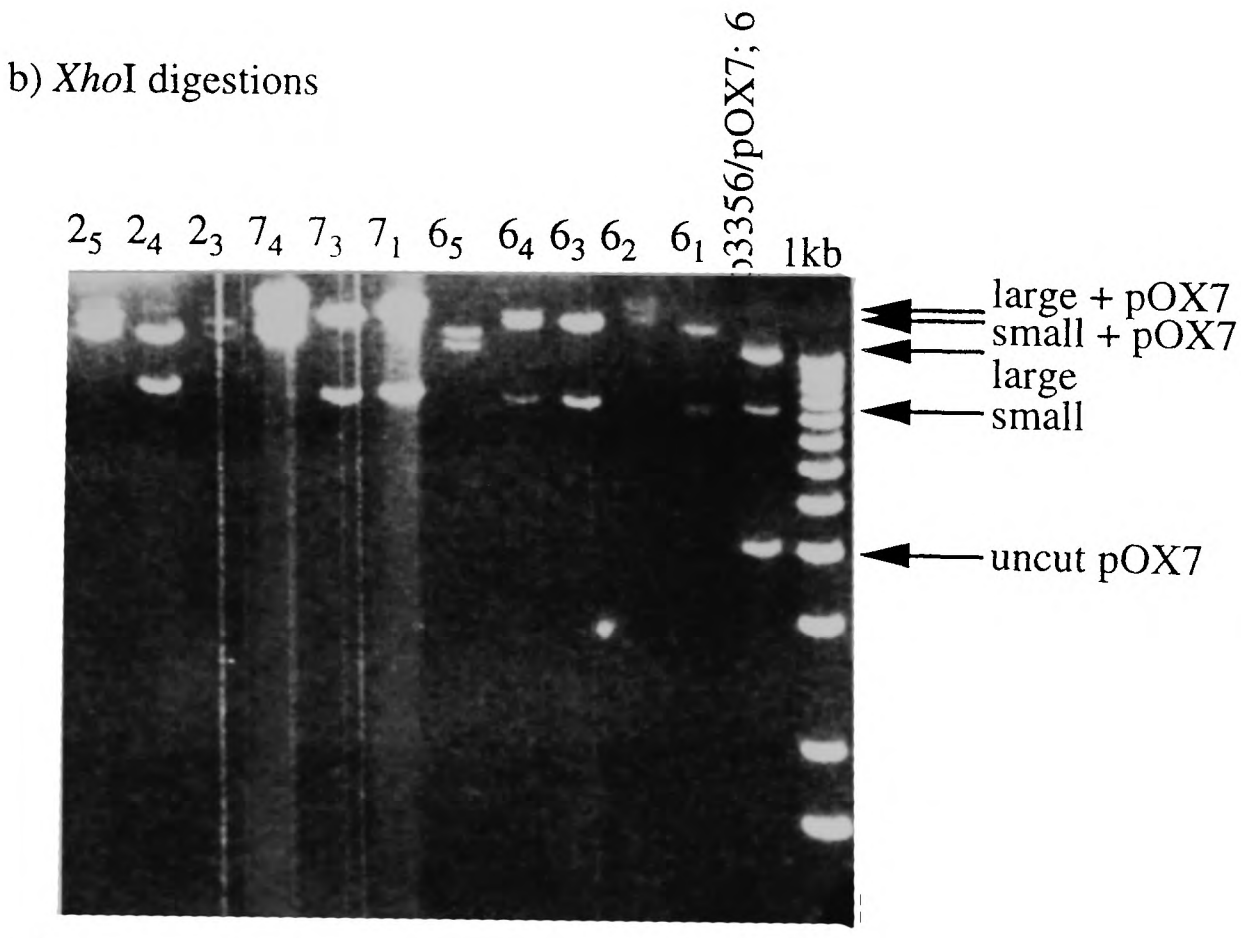
Table 6.1 continued **Frequencies of replicon fusion between p3356 and either pOX7 or pOX7-IS**

Heteroplasmid strain	No. cfu plated	Temp. (°C)	Number colonies grown		Frequency fusion[1]
RN4220	10^5	42	++	++	$8.52x10^{-3}$ to $1.01x10^{-2}$
$(p3356/pOX7\text{-}IS)_2$	10^4	42	269	189	
	10^3	42	23	17	
	10^3	30	++	++	
	10^2	30	269	170	
	10^1	30	27	19	
RN4220	10^5	42	++	++	$8.40x10^{-3}$ to $8.97x10^{-3}$
$(p3356/pOX7\text{-}IS)_4$	10^4	42	231	153	
	10^3	42	26	14	
	10^3	30	++	++	
	10^2	30	275	161	
	10^1	30	29	15	

[1] Frequency of replicon fusion is given as the ratio of the average number of colonies grown per colony forming unit (cfu) in the inoculum on plates incubated at 42°C, with those incubated at 30°C. Figures are given for two experiments. ++ denotes confluent growth.

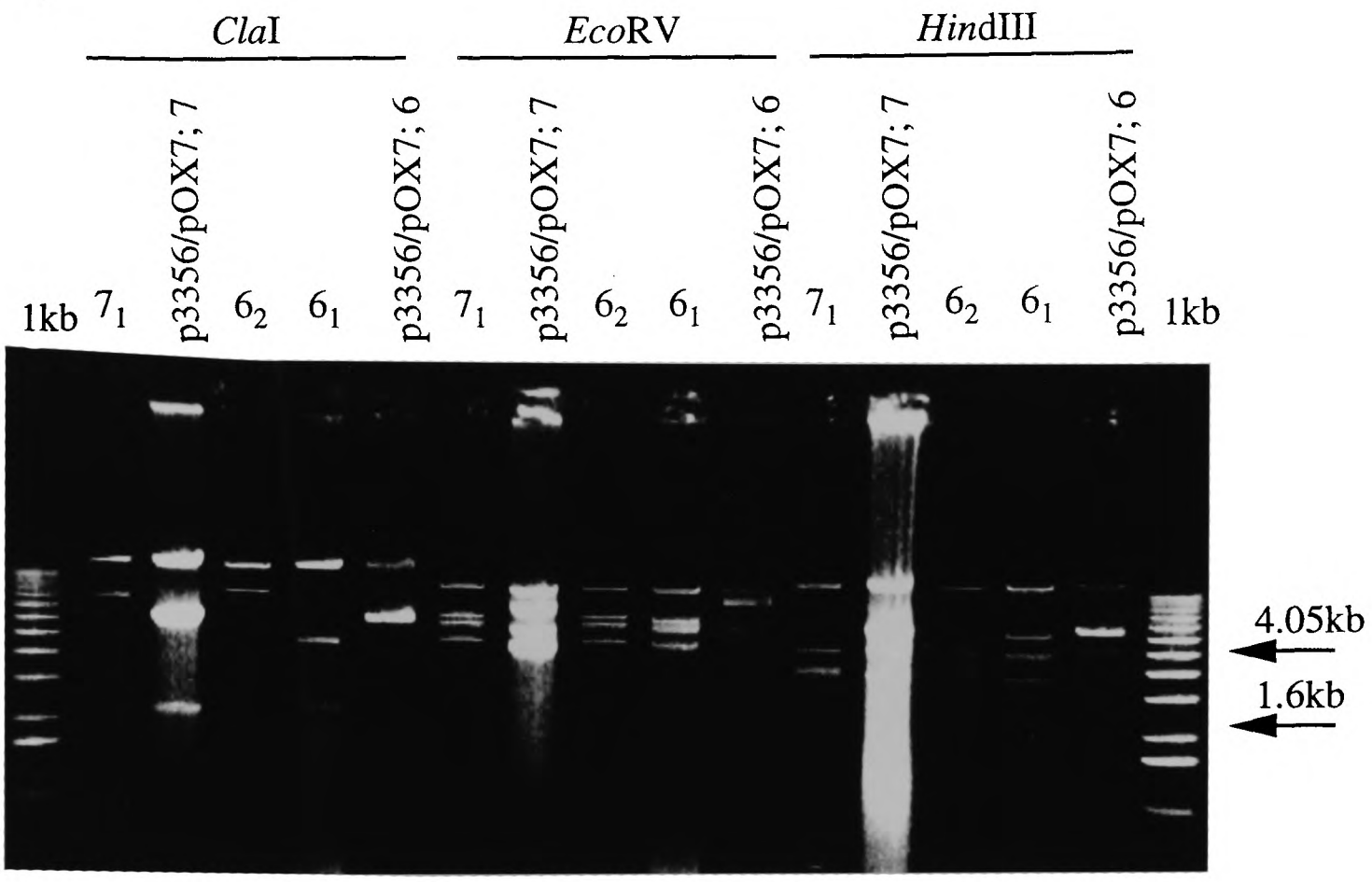
Figure 6.1 Restriction digests of the co-integrates of p3356 with both pOX7 and pOX7-IS





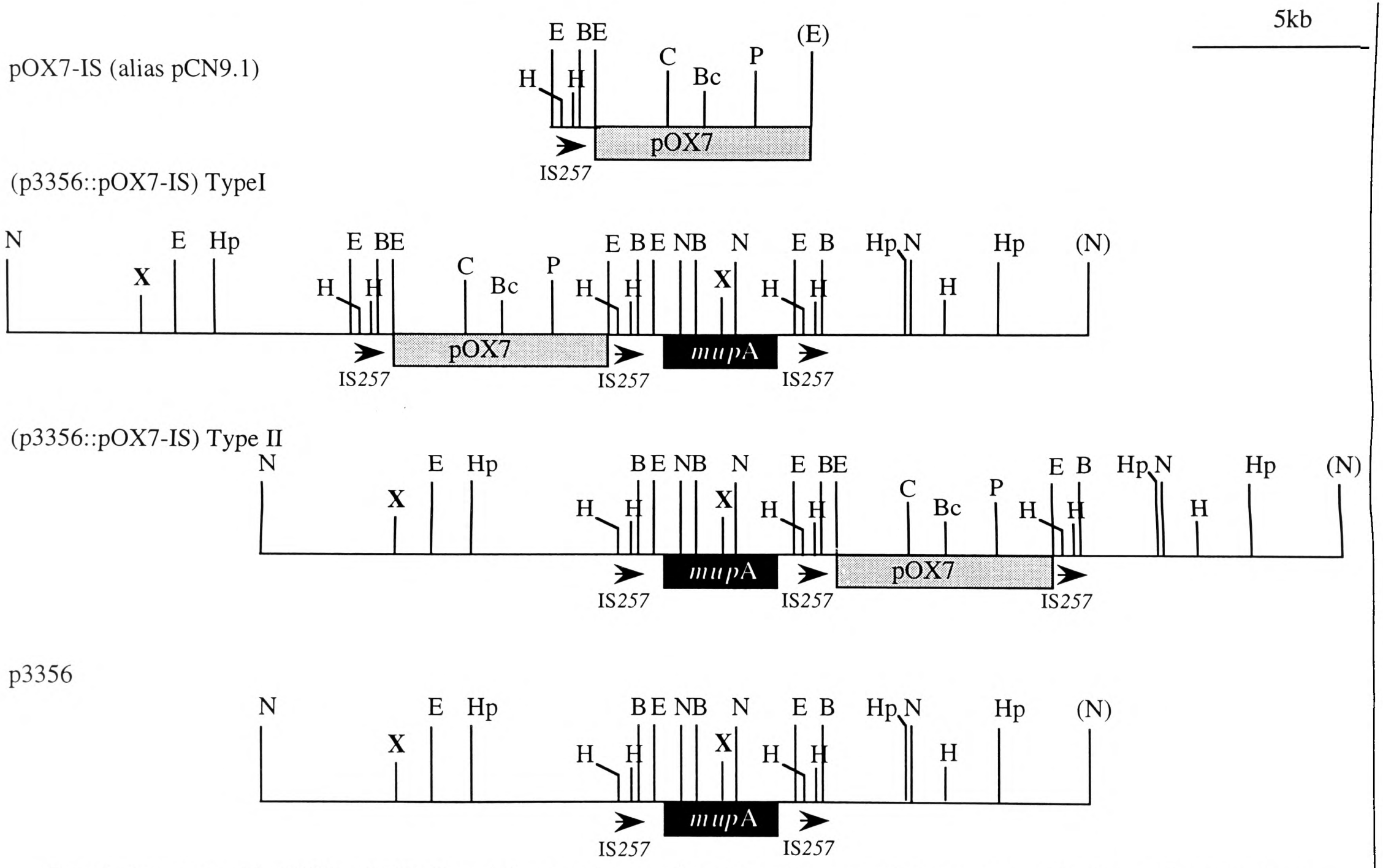
Large/small refer to the relative sizes of the *Xho*I fragments of p3356. "+pOX7" indicates increased size upon pOX7 integration.

c)



Numbers refer to the heteroplasmid number, the subscript refers to the co-integrate number produced after co-integration of the two plasmids.

Figure 6.2 Restriction maps of p3356, pOX7-IS and co-integrates formed from them



Restriction maps of p3356, pOX7-IS and the two types of co-integrate generated are given. Co-integration has been observed in both the upstream and the downstream IS*257*s of p3356, easily visualised by *Xho*I (**X**) digestion. Nine examples of each have been mapped. All plasmid maps are linearised at an *Nco*I site except for pOX7-IS which is linearised at an *Eco*RI *site.*

preference, as for pOX7-IS/p3356 co-integration (Fig.6.1b). The *Eco*RI restriction fragment patterns of these co-integrate plasmids were varied (Fig.6.1a) as were those for several other enzymes including *Cla*I and *Hind*III (Fig.6.1c). Further mapping demonstrated that the point of linearisation of the pOX7 in the co-integrate varied. Integration appeared to have occurred in non-functional regions of pOX7 (Fig.6.3a) to give co-integrate plasmids of varied restriction pattern (Fig.6.3b). Co-integrates were stable over 50 generations of serial dilution (as Section 2.16) in the absence of antibiotic selection.

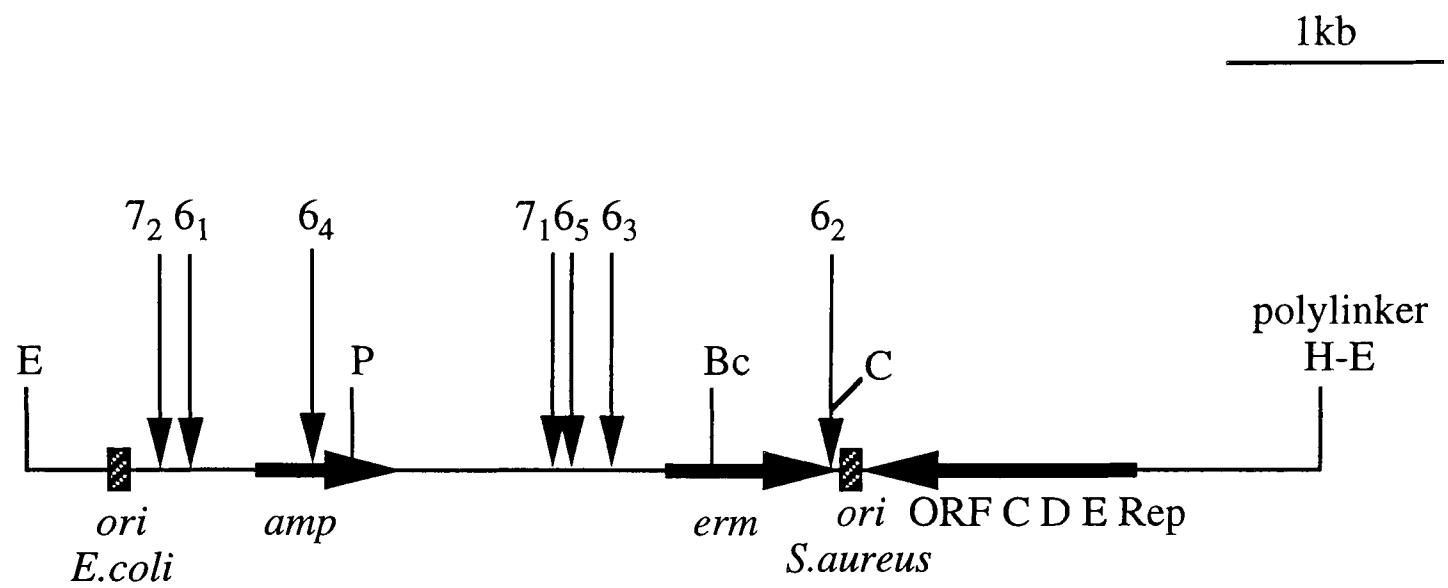
6.3 Sequencing the flanks of the IS*257*s of the co-integrates

The maps of the pOX7-IS::p3356 co-integrates were all the same; the plasmids had recombined between the IS*257*s and there was no duplication of DNA detectable by restriction mapping. The flanks of the IS*257*s internal to the pOX7-IS clone insertion were sequenced using the universal "forward" and "reverse" primers homologous to the pUC18 sequences of pOX7. The flanks were the same in these two co-integrates as the flanks of the IS*257* in the pOX7-IS clone, pCN9.1. The lack of alteration of sequence supported the view that the replicon fusion had occurred by recombination; the enzymes catalysing the recombination could be host encoded (*rec*) or the putative transposase acting as a recombinase.

The maps of the pOX7::p3356 co-integrates differed. The backbone of p3356 remained intact; there was a duplicated copy of IS*257* flanking the insertion in all cases; the point of linearisation of the inserted pOX7 plasmid varied. The IS*257* duplication and apparently random site of co-integration[2] implicated a transposition event in the co-integration process. It had been suggested that transposition of IS*257* created an 8bp direct repeat (Rouch *et al*, 1989). Two co-integrates (p3356::pOX7;6_2 and p3356::pOX7;6_5) with very different sites of fusion were selected to look for such a duplication. Due to the multiple IS*257*s in the co-integrates, one of the IS*257*s of each plasmid was cloned to allow the precise site of co-

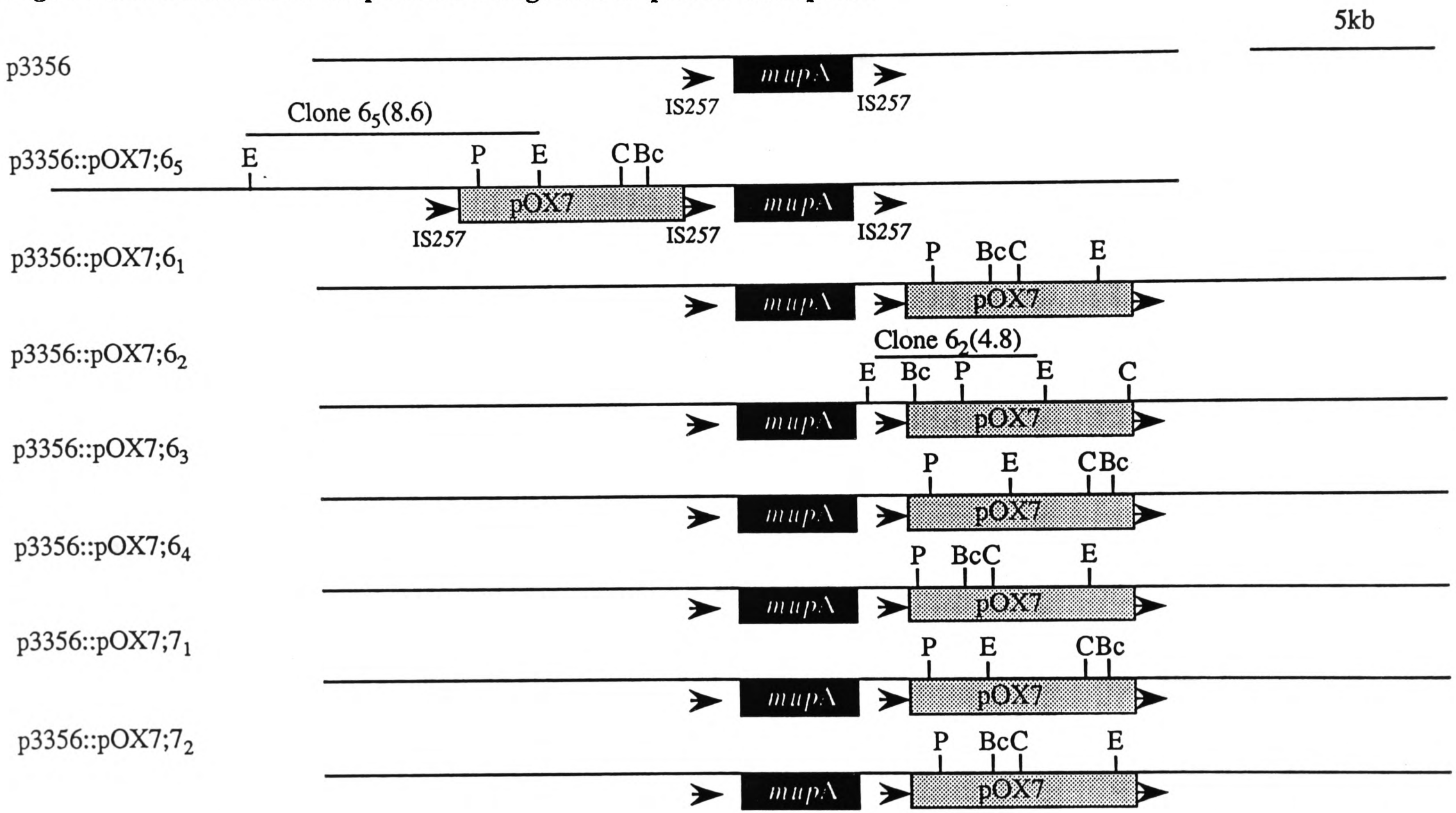
[2] The site of linearisation of pOX7 in the co-integrates varied and occurred in all the regions not required to be intact for gene expression, and thus apparently without site preference

Figure 6.3a Sites of integration of p3356 into pOX7



The map of pOX7 is given showing the structural genes. The only genes required for growth of the co-integrate are those of pE194, as the pUC18 sequences are active in *E.coli* not in *S.aureus*. The random site of integration of p3356 into pOX7 (shown by arrows pointing down from the numbers of the co-integrate plasmids tested), is demonstrated. The sites of integration have been mapped to within 100bp of the site given. The numbers refer to the heteroplasmid strains from which the co-integrates were made and the subscript numbers refer to the number of the selected co-integrate. For maps of the co-integrates and enzyme nomenclature, see Fig.6.3b.

Figure 6.3b Restriction maps of co-integrates of p3356 with pOX7



Restriction maps of the co-integrates of p3356 with pOX7 are aligned for *mup*A. For clarity the maps show only the restriction sites for the enzymes *Bcl*I, *Cla*I, *Eco*RI and *Pvu*I in the pOX7 region of the maps: the variety of positions of these sites relative to the flanking IS*257*, demonstrates the random site and orientation of co-integration. Although there are more examples given of integration of pOX7 within the downstream IS*257* of p3356 than within the upstream IS*257*, restriction digests of a larger sample size with *Xho*I demonstrated there to be no preference in the IS*257* used. The positions of the *Eco*RI clones of the co-integrates numbers 6_2 and 6_5, used for sequencing, are marked.

integration to be determined. As the inserted plasmid was active as a shuttle vector between *E.coli* and *S.aureus*, each integrated plasmid contained an *ori* active in *E.coli*, this *ori* was exploited in the cloning strategy: a restriction enzyme (*Eco*RI) with sites flanking the *ori* was used to digest DNA of each of the co-integrates; this DNA was then re-ligated and the ligation mix used to transform DH5α (a *rec*A$^-$ strain of *E.coli*); transformants were selected on 2xTY-agar containing 50μg ampicillin ml^{-1}. The flanks of the IS*257*s of the two clones 6_2(4.8) and 6_5(8.6) (Fig.6.3b) were sequenced using the primers designed to sequence out from IS*257* (J1IS*257* and J2IS*257*, Fig.3.18). The sequence generated demonstrated the precise site of linearisation of pOX7 in the known sequence (pOX7 contains sequences of pE194 (Horinouchi and Weisblum, 1982) and pUC18 (Yanisch-Perron *et al*, 1985)), allowing the design of oligonucleotides with binding sites in pOX7 on the other side of the point of linearisation. These new oligonucleotides ($DR6_2$pOX7 and $DR6_5$pOX7) were used to sequence the other flank of the IS*257* on the internal side of the inserted plasmid (Fig.6.4b); the remaining flank was sequenced using either UPOUT or DNIN as appropriate (Fig.6.4b). The flanks of the IS*257*s external to the inserted pOX7 were as those flanking the upstream IS*257* of p3356[†]. The flanks of the IS*257*s internal to the inserted plasmid were pOX7 sequence; however the sequence was duplicated for 8bp with respect to pOX7 in direct repeat at the junctions of the IS*257*s with pOX7 (Figs.6.4a & b). The duplicated bases in the two co-integrates were not the same (ATTTATAG and GAATAAAA). This 8bp duplication verified that the co-integration of pOX7 and p3356 involved a transposition event, and the variety of the restriction maps of the co-integrates and lack of homology of the duplicated bases in co-integrates 6_2 and 6_5 confirmed this by showing that the transposition target site was random. However, it was not possible to distinguish whether there was only a replicative transposition event or both a conservative transposition and a recombination event, and if a recombination reaction, whether this was catalysed by *rec* or by the transposase itself.

6.4 Co-integration in *E.coli*

As it proved impossible to transform RN981 (a *rec*A$^-$ strain of *S.aureus*) with p3356 (Section 4.5), the requirement for *rec* could not be studied directly. *E.coli rec* mutants are

† This reference is made to the 6_5 co-integrate; read downstream in place of upstream for 6_2.

Figure 6.4a Sequences flanking the IS*257*s flanking pOX7 in the co-integrates of pOX7 with p3356

a) Autoradiographs of sequences generated from the co-integrate p3356::pOX7;6_2

Sequence from DR6_2POX7 primer



Sequence from J2IS*257* primer

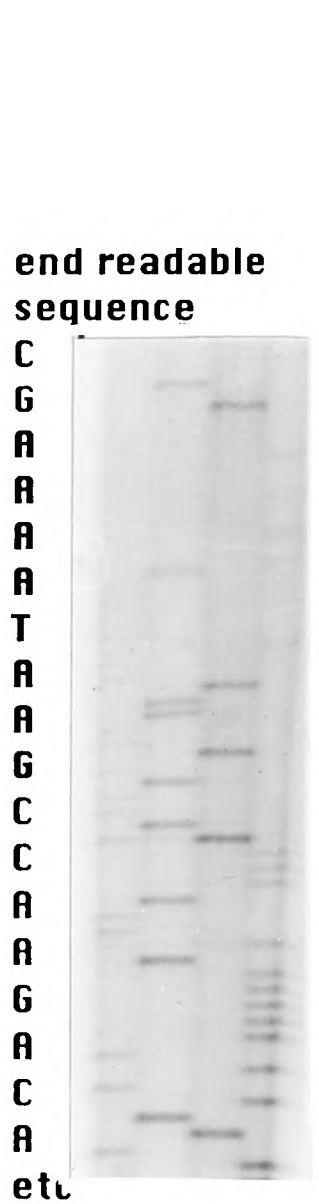


b) Autoradiographs of sequences generated from the co-integrate p3356::pOX7;6_5

Sequence from DR6_5POX7 primer



Sequence from J2IS*257* primer



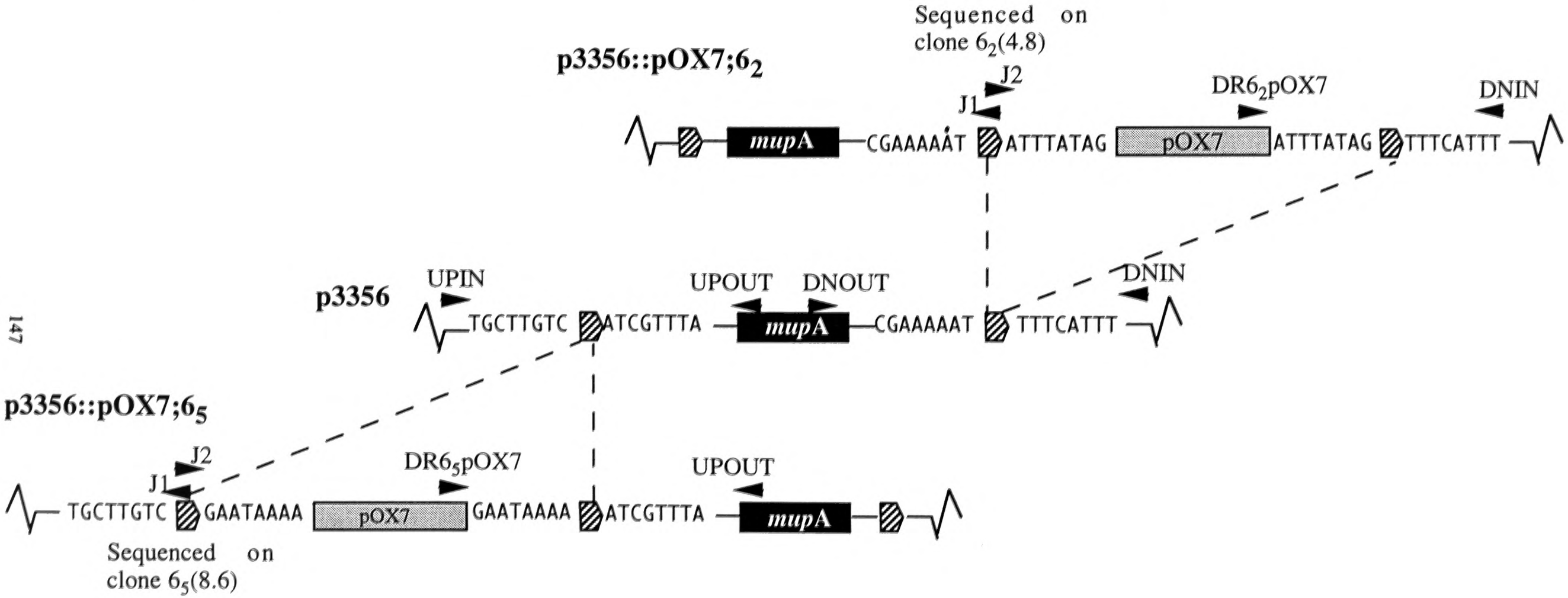
well characterised (Smith, 1987), and thus the possibility of using such mutants to study the transposition process was investigated.

The co-integration experiment between pOX7 or pOX7-IS and p3356 in *S.aureus* (Section 6.3) had failed to distinguish the pathway of co-integration as either replicative transposition terminating at the co-integrate or conservative transposition of the IS*257* followed by interplasmid recombination. It was decided to perform a similar experiment in *E.coli* to allow *rec* mutants and transposase mutants to be used, and thus to investigate the requirement of each type of catalysis at each stage in the co-integration.

The temperature sensitive, chloramphenicol resistance plasmid pMAK705 (Hamilton *et al*, 1989) was used as the recipient of the transposed element (i.e. in the place of pOX7 in experiments conducted in *S.aureus*). Plasmid pCN9.1 (pOX7-IS in Section 6.3) containing IS*257* from p3356D was used as the donor of the transposable element (i.e. in the place of p3356 experiments conducted in *S.aureus*). "Mini-prep" DNA from JM107(pCN9.1) was used to transform JM109, a *rec*A$^-$ strain of *E.coli*, selection was on 50μg ampicillin ml^{-1}. Competent cells were prepared from cultures of both JM107(pCN9.1) and JM109(pCN9.1) which had been grown in the presence of 50μg ampicillin ml^{-1}. pMAK705 was provided as DNA and used to transform JM107(pCN9.1) and JM109(pCN9.1), with selection at 30°C on 2xTY-agar containing 40μg chloramphenicol ml^{-1}. Transformants were streaked onto 2xTY-agar plates containing either 50μg ampicillin ml^{-1} or 40μg chloramphenicol ml^{-1}, and Ap^{R}Cm^R transformants used to make "mini-prep" DNA to test for both plasmids.

Heteroplasmid strains JM107(pCN9.1/pMAK705) and JM109(pCN9.1/pMAK705) were grown overnight at 30°C in 2xTY broth containing 40μg chloramphenicol ml^{-1}. A 1:10 dilution was made of the overnight culture in 2xTY broth (40μg chloramphenicol ml^{-1}) and grown to mid-log phase, when the cell density was determined from the OD_{675}. Serial dilution of the culture was made in 2xTY broth to allow specific numbers of cells to be used to inoculate 2xTY-agar plates containing 40μg chloramphenicol ml^{-1}; plates were incubated at both 30°C and 42°C. The colonies which grew were counted to determine the frequency of replicon fusion (Table 6.2). The frequency of replicon fusion was slightly lower than that in the *S.aureus* experiments (c.$1x10^{-3}$ per cell plated as compared to c.$1x10^{-2}$ per cell plated,

Figure 6.4b Sequences of the junctions of the IS*257*s in the p3356 and co-integrates of p3356 and pOX7



Schematic diagrams of p3356 and the co-integrates of p3356 with pOX7, p3356::pOX7;6_2 and p3356::pOX7;6_5 are given showing the 8bp flanks of the IS*257*s flanking pOX7. The positions of the primers used for sequencing are given. The internal flanks of the IS*257*s are 8bp direct repeat duplications of pOX7 sequence; the other sequences flanking the IS*257*s of the co-integrates are the same as the corresponding flanks of the IS*257*s of p3356.

Table 6.2 Frequencies of replicon fusion between pCN9.1 and pMAK705

Heteroplasmid strain	No. cfu plated	Temp. (°C)	Number colonies grown		Frequency fusion[1]
JM107	10^7	42	++	++	$2.38x10^{-3}$ to $6.4x10^{-3}$
(pCN9.1/pMAK705)	10^6	42	1076	nt	
	10^5	42	147	128	
	10^4	42	16	17	
	10^3	30	++	1	
	10^2	30	228	147	
	10^2	30	260	108	
	10^1	30	38	14	
	10^1	30	40	13	
JM109	10^7	42	nt	nt	$0.79x10^{-3}$ to $3.78x10^{-3}$
(pCN9.1/pMAK705)	10^6	42	104	242	
	10^5	42	25	27	
	10^4	42	4	3	
	10^3	30	76	110	
	10^2	30	16	14	

[1] Frequency of replicon fusion is given as the ratio of the average number of colonies grown per colony forming unit (cfu) in the inoculum on plates incubated at 42°C, with those incubated at 30°C. Figures are given for two experiments. ++ denotes confluent growth. nt indicates that this plating was not performed.

respectively). Colonies grew from inocula of both strains even at 42°C indicating that the temperature sensitive replicon (pMAK705) had integrated into a temperature stable replicon. "Mini-prep" DNA was made from 10 colonies of both types of co-integrate for analysis of plasmid content. Both JM107(pCN9.1::pMAK705) and JM109(pCN9.1::pMAK705) co-integrates contained only one plasmid. However, the plasmid contained only one *Bgl*II site, indicating that the fusion had not created a duplication of the IS*257*, indeed the plasmid co-integrate was 11.7kb, the cumulative size of the plasmids pMAK705 and pCN9.1. Moreover the co-integrate plasmids all had the same restriction patterns with both *Eco*RI and *Pst*I. Further restriction mapping of one of these plasmids (and pMAK705 for which only a vague map had been provided) clearly showed that although the two plasmids had fused, the point of fusion was distal from the IS*257* and sites of specific recombination in pE194[3] (Novick *et al*, 1984; Gennaro *et al*, 1987) (Fig.6.5). This recombination had occurred in both *rec*A$^+$ and *rec*A$^-$ strains, indicating that it was independent of host *rec* functions. Unfortunately, a site-specific recombination event had masked any transposition activity which may have been observed, thus making this an unsuitable system in which to study the transposition process.

6.5 Replicon fusion in *S.aureus*

As it was not possible to study the effect of transposase mutation in *rec*A$^-$ *E.coli* with the system used, it was decided to return to *S.aureus* for these studies. Both RN4220(pT181) and RN4220(pT181-IS) were transformed with pOX7, pOX7-IS* or pOX7-IS DNAs (pOX7-IS* is pOX7-IS deleted for the 237bp *Hin*dIII fragment internal to the transposase). These six heteroplasmid strains were grown and plated on CY-agar containing 40μg erythromycin ml^{-1} and incubated at 42°C to select for Tc^{R}Em^R recombinants, as in Section 6.3. "Mini-prep" DNA prepared from six colonies of each type was prepared and restricted. Each of the six examples of all six types contained one plasmid only which had the restriction map of pT181 or pT181-IS (whichever had been in the original strain). It was concluded that the

[3] pCN9.1, a clone in pOX7, contains pE194 sequences which are known to contain two sites at which site specific recombination is known to have occurred.

pOX7-based plasmid had fused with the chromosome[4]. Hence it was not possible to study transposition in this system.

6.6 Recombination between IS*257*s in *E.coli*

Attempts to study interplasmid transposition of IS*257* had proved unfruitful. However, results to date had implicated IS*257* as the site for intraplasmid recombination. It was not possible to study this in *S.aureus* due to the inability to transform RN981 with the IS*257*-plasmids, therefore the possibility of using *E.coli rec*A$^-$ strains was investigated.

pMT contains two IS*257*s interspersing the *mup*A region and pT181 (Fig.5.6, Section 5.4). The plasmid is unstable with recombination between the IS*257*s giving rise to the smaller plasmid pT181-IS. Recombination between these IS*257*s can be encouraged by providing antibiotic stress of one of the two antibiotics for which resistance is encoded in pMT, tetracycline or mupirocin (Section 5.6). pMT lacks an origin for replication that is active in *E.coli*.

pMT was linearised at its single *Nhe*I site between the *tet* and *pre* genes of pT181. This was ligated to *Xba*I linearised pUC18, the ligation mix used to transform *E.coli* JM107 (*rec*$^+$), with selection on 2xTY-agar containing 50µg ampicillin ml^{-1}. "Mini-prep" DNA was made from transformants, and two clones with appropriate restriction maps selected, pMTN1.1 and pMTN1.2 (Fig.6.6b). Unfortunately *E.coli* is naturally resistant to mupirocin and the *tet* gene of these clones is not expressed in *E.coli*, and thus only ampicillin could be used to select further. Overnight cultures of the two clones were used to inoculate 2xTY broth containing 250µg ampicillin ml^{-1}, and serial dilution of these continued for 20 generations (as Section 2.16). Comparison of the restriction digests of DNA made from cells before the serial dilution and after twenty generations, showed that the original plasmid (13.2kb) had been replaced by a much smaller plasmid (7.8kb) (Fig.6.6a). The deletion was

[4] It is thought that the lack of co-integration between these plasmids is due to the instability of the larger plasmid which would be formed, as with pMT (Sections 5.5 & 5.8).

5.4kb the size of the *mup*A region and an IS*257*; further restriction mapping confirmed this to be the deletion (Fig.6.6b). Subculture in ampicillin had selected for a smaller plasmid, generated by unequal recombination between the IS*257*s. Thus inter-IS*257* intraplasmid recombination was active in *E.coli*. Of interest, the clones pMTN1.1 and pMTN1.2 were stable in serial dilution for 50 generations in the absence of antibiotic selection, unlike plasmid pMT[5].

"Mini-prep" DNA of the plasmids pMTN1.1 and pMTN1.2 were used to transform both DH5α and JM109, two *rec*A$^-$ strains of *E.coli*. The transformants were subject to serial dilution in ampicillin as above. Again, deletion of the *mup*A-region by unequal crossover between the IS*257*s occurred within 20 generations (Fig.6.6a). The recombination process was as active in a *rec*A$^-$ host as it was in a *rec*A$^+$ host, indicating that the recombination was not dependent upon host *rec* catalysis. Therefore the transposase of IS*257* is implicated as the recombinase catalysing the intraplasmid IS*257* deletions.

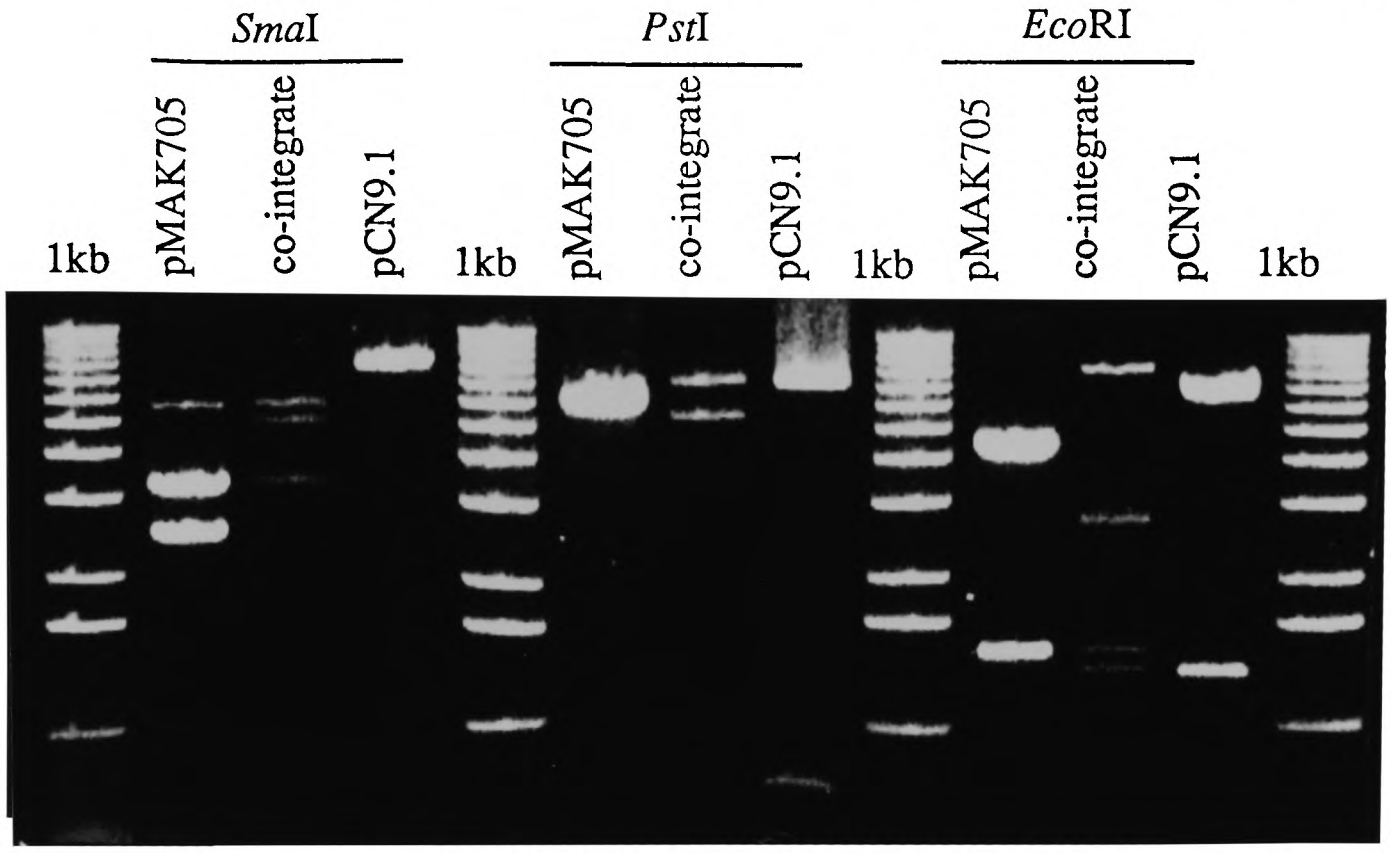
6.7 Discussion

Contrary to the predictions of Galas and Chandler (1989), IS*257* has been shown to be currently active for transposition: forced recombination between p3356 and pOX7 demonstrated various sites of co-integration and the duplication of 8bp flanking the duplicated active IS*257* confirmed that a transposition event had occurred. The mechanism of the transposition process could follow the models of both Shapiro (1979) and Ohtsubo (1980). That replicon fusion also occurred between p3356 and pOX7-IS by recombination between the IS*257*s does not help to distinguish between these models. The frequency of recombination between the IS*257*s of p3356 and pOX7-IS (c.$1x10^{-3}$) was several orders of magnitude higher than that expected if the recombination was catalysed by the host *rec* enzymes (c.$1x10^{-6}$ to $1x10^{-9}$), suggesting that the recombination was being catalysed by the transposase of the IS*257* rather than *rec* general homologous recombination. *rec*A is an

[5] This further supports the idea postulated in Section 5.3, that pMT is unstable due to the large single-stranded intermediate of replication, which would not be present in a replication intermediate of a plasmid utilising the pUC18 *ori*, which replicates by the theta bi-directional mode (Kornberg, 1992).

Figure 6.5 Co-integration of pMAK705 with pCN9.1 at a specific site

(a) Restriction digests of pMAK705, pCN9.1 and the co-integrate formed between them



(b) Restriction maps of pMAK705, pCN9.1 and the co-integrate formed between them

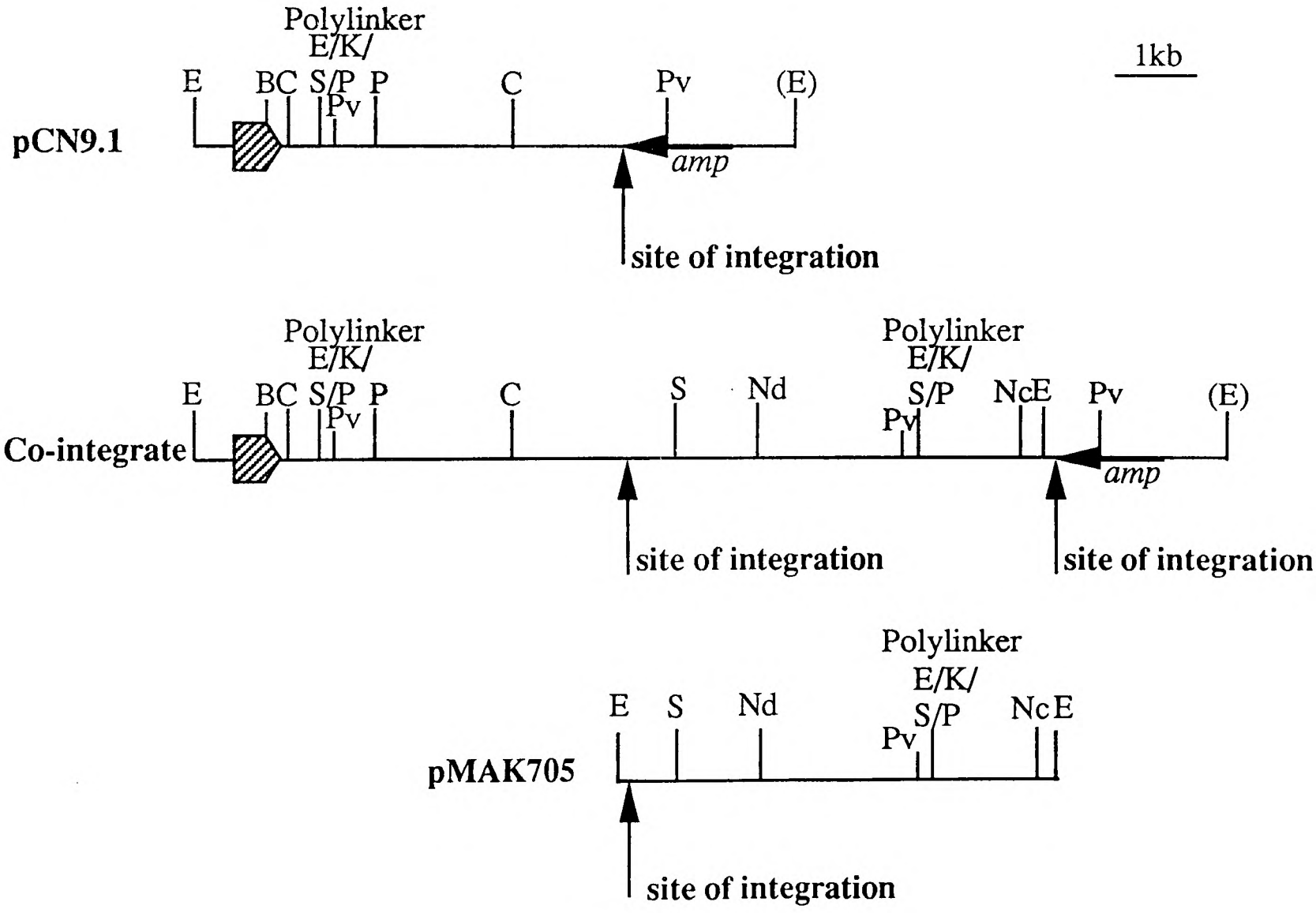
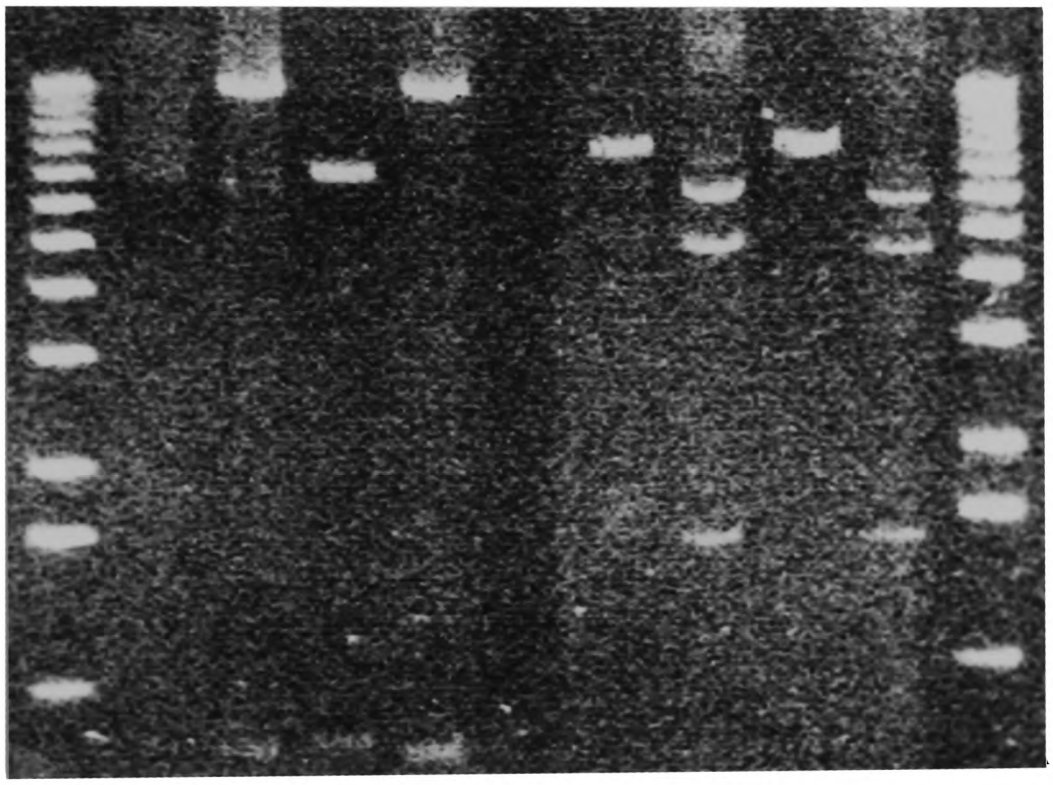


Figure 6.6 Deletion of the "IS*257*-*mup*A" cassette of pMT clones in *E.coli*

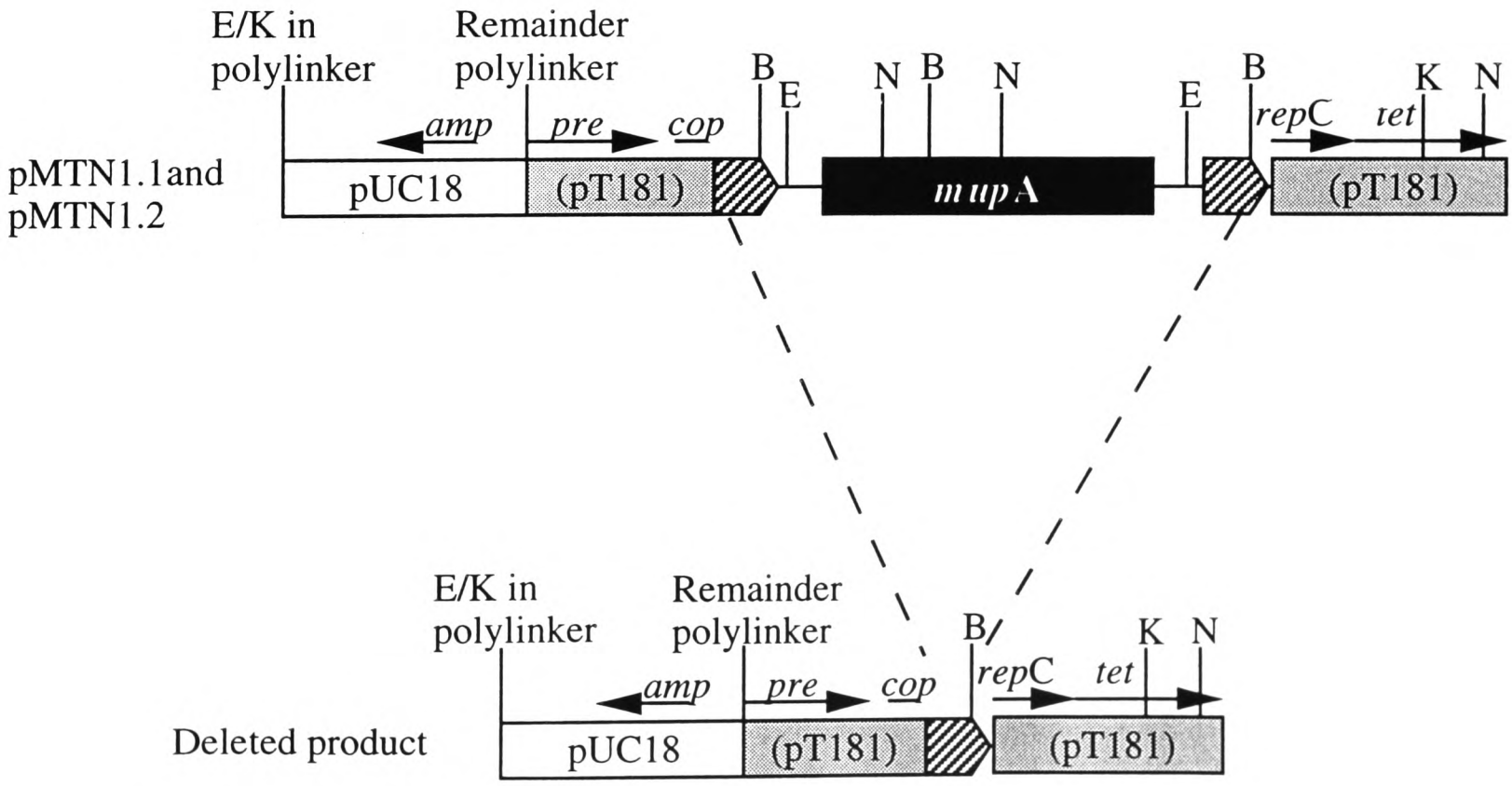
(a) Restriction digests of pMTN1.1 and deletion products generated from them by serial dilution in ampicillin

	*Nco*I					*Kpn*I				
	J	J	D	D		J	J	D	D	
1kb	20	0	20	0	--	20	0	20	0	1kb



Strain D=DH5α, J=JM107

Number of generations in serial dilution



Selection of pMTN1.1 or pMTN1.2 on ampicillin gave a smaller product deleted for the "IS*257*-*mup*A" cassette, as indicated by the dashed lines.

essential requirement for the various pathways of homologous recombination in *E.coli* (Smith, 1987; Smith, 1989). That efficient intraplasmid deletion between the IS*257*s of pMTN1.1 and pMTN1.2 occurred in two different *rec*A⁻ *E.coli* strains, demonstrated that homologous recombination was not required for this recombination. Hence it is concluded that IS*257* is an active recombinase for intraplasmid recombination between IS*257*s in *E.coli*. That IS*257* has been demonstrated to be active for inter-IS*257* recombination in *E.coli* supported the view that IS*257* is able to catalyse the interplasmid inter-IS*257* recombination observed at high frequency in *S.aureus*. However, as the co-integrates p3356::pOX7 and p3356::pOX7-IS and indeed the plasmids pMTN1.1 and pMTN1.2 are stable upon serial dilution in the absence of antibiotic selection, such an intraplasmid recombinogenic role appears to be dormant under non-selective conditions.

Insertion sequences which act as the site of replicon fusion have been observed previously. The R plasmid, R100 containing two IS*1* elements in direct repeat flanking the resistance determinant has been shown to fuse with the IS*1* resident on the genome of bacteriophage P1 (Iida, 1980), this parallels the observation of the fusion of p3356 with pOX7-IS; the co-integrate in both cases maintains the full complement of DNA of each replicon, which are separated at each fusion point by an insertion sequence in direct repeat. IS*1*-containing plasmids, and indeed plasmids containing the IS*1*-flanked compound transposon Tn*9*, have also been shown to form co-integrates with plasmids devoid of IS*1*: the plasmid pOX38::Tn*9* will co-integrate with pBR322 with direct repeats of either IS*1* or Tn*9* at the points of fusion, here the transposable element is duplicated in the co-integrate relative to the original plasmid (Galas and Chandler, 1982). The co-integrate pOX38::Tn*9*::pBR322 plasmids are stable; this parallels the co-integrates formed between p3356 and pOX7, which are duplicated for IS*257* at the points of fusion in the various co-integrates. Indeed it was based on the observations of studies of co-integration of plasmids bearing IS*1* that Ohtsubo (1980) designed the model for transposition which views insertion sequence-bearing plasmids as potential transposons. Co-integration has also been observed between the IS*140*-bearing plasmids, pWP12a and pWP14a, and the genome of the bacteriophage P1Cm, which has no homology to IS*140* (Brau and Piepersberg, 1983) with the concomitant increase in IS*140* copy number, separating the fused plasmids in direct repeat.

IS*1* has been observed to be the site of resolution of DNAs separated by direct repeats of IS*1*: the R100.1 plasmid chloramphenicol resistance determinant is flanked by direct repeats of IS*1* in the composite transposon Tn*9*, recombination between these flanking IS*1*s gives a deleted form of this plasmid with only one IS*1* remaining (Chandler *et al*, 1977). Indeed, it is suggested that Tn*9* is the product of an IS*1* mediated deletion of such a resistance determinant element, without the capacity to self-replicate (Chandler *et al*, 1979). However, in these cases the resolution is dependent upon the host recombination system. Resolution of the pWP12a::P1Cm co-integrate was observed, releasing both pWP12a, indistinguishable from the original plasmid, and P1Cm-IS*140*, a P1Cm plasmid with a copy of IS*140* integrated into the site at which the co-integration had occurred; this process was not tested for dependence on *rec*A (Brau and Piepersberg, 1983). Recombination between the direct repeats of IS*15* flanking the kanamycin resistance determinant in the transposon Tn*1525* of the ampicillin resistance plasmid pIP1087 to give the deleted plasmid pIP1088, containing only one copy of IS*15*, occurs upon transformation with selection for ampicillin; this occurs at high frequency when transformation is to a *rec*A$^+$ recipient (1 in 2), but at a lower frequency ($1x10^{-3}$) when *rec*A$^-$ recipients are used (Labigne-Roussel *et al*, 1983). However the frequency upon transformation to *rec*A$^-$ strains is higher than expected if the recombination was fully dependent upon host *rec* systems, suggesting that the IS*15* may provide a recombinase function in the absence of *rec*A, as observed for pMTN1.1 and pMTN1.2 deletion of the IS*257*-*mup*A cassette.

Thus investigations using the IS*257*s derived from mupirocin resistance plasmids have shown that IS*257* shares properties in common with other insertion sequences: the ability of IS*257* to transpose to an apparently random location causing the duplication of a short region of the target sequence has been clearly demonstrated, although this has only been observed in transposition events resulting in co-integrates; IS*257* is also able to act as a recombinase in a *rec*A$^-$ strain of *E.coli*, demonstrating not only another feature typical of insertion sequences but also the broad host range of that function, suggesting close evolutionary links of insertion sequences across the species.

General Discussion

Chapter Seven

General Discussion

7.1 A family of mupirocin resistance plasmids

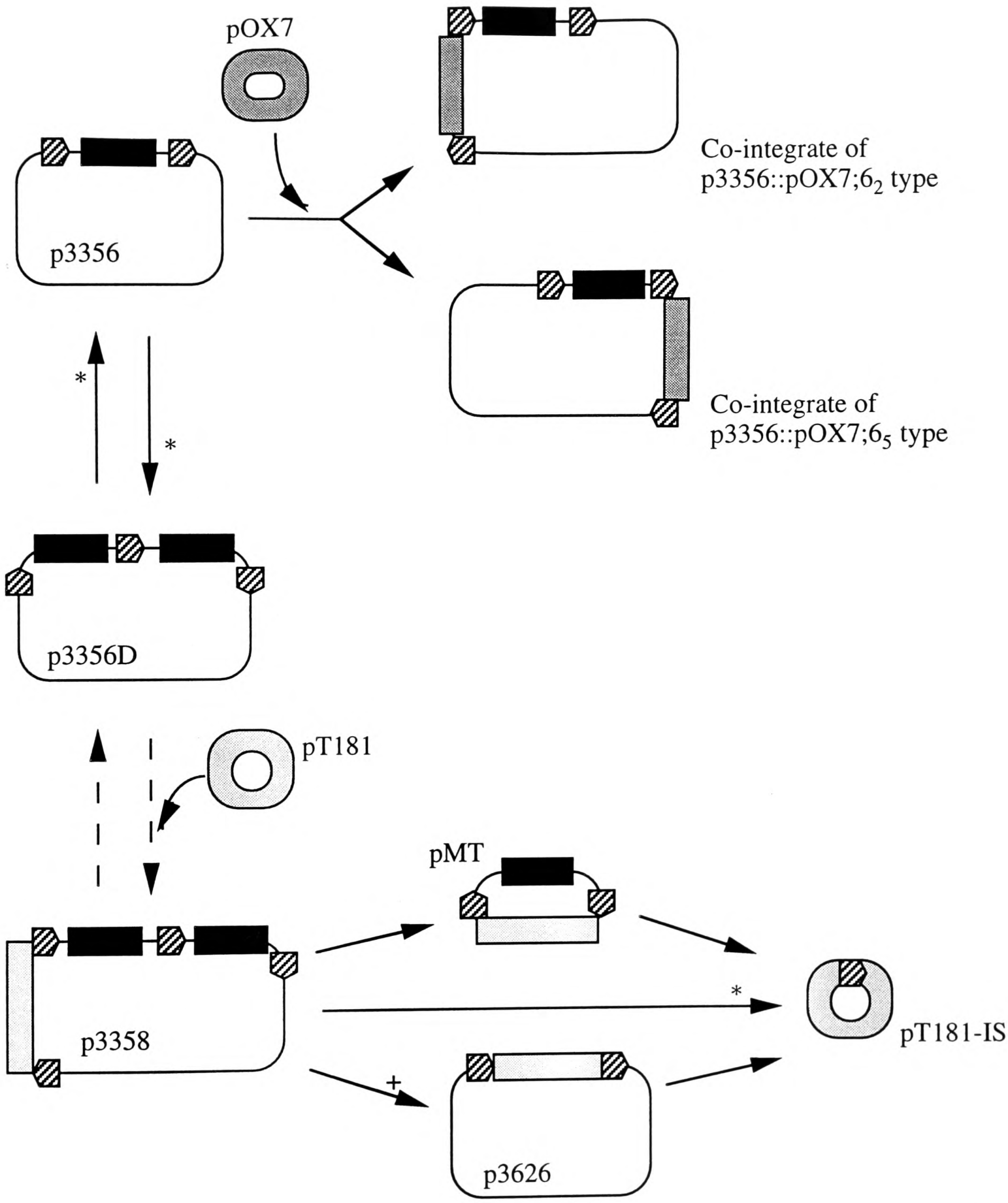
Studies in the preceding chapters of this volume have concentrated on a family of plasmids isolated from clinical strains of *Staphylococcus aureus* which confer high-level resistance to the antibiotic mupirocin. The prototype of the family, p3356, is 23.6kb and contains one copy of the mupirocin resistance gene, *mup*A, flanked by copies of IS*257* in direct repeat. Another member of the family, p3356D[1], is related to p3356 by duplication of the "IS*257*-*mup*A" cassette; whilst a third plasmid, p3358, is related to p3356D by the co-integration of the small tetracycline resistance plasmid, pT181, into the site of an IS*257* with concomitant duplication of the IS*257*. A summary of the relationships of these plasmids is given in Fig.7.1.

The family of plasmids were all isolated from clinical strains of *Staphylococcus aureus* taken from patients on the same hospital ward and represent an example of past events of plasmid evolution mediated by the transposable element IS*257*: specifically tandem gene duplication and replicon fusion. Previous citations of IS*257* in plasmids and chromosomes of *S.aureus* have pointed to such rearrangements: the possibility of IS*257* acting in duplication of the *mec* region of the chromosome of methicillin resistant *S.aureus* was documented by Gillespie *et al* (1986); fused replicons with direct repeats of IS*257* at the inter-replicon boundaries had been observed in the integrated small tetracycline resistance plasmid, pT181 in the *mec* region of the chromosome (Gillespie *et al*, 1986) and the integrated small aminoglycoside resistance plasmid, pUB110 in the large plasmids pSK41 and pUW3626 (Byrne *et al*, 1991) and into the *mec* region of the chromosome, at a different site to the pT181 integration (Dubin *et al*, 1991). However, although IS*257* was ubiquitous and often found in multiple

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[1] p3356D is the laboratory-derived version of the clinical isolate p3357; as most of the work was on p3356D, it is quoted here and is assumed identical to p3357.

Figure 7.1 Relationships of the p3356 family of plasmids and their derivatives



The p3356 family of plasmids are represented with arrows representing the direction of plasmid evolution: plain arrows are used for changes which have been observed in the laboratory, dashed arrows represent the evolution assumed to have occurred in nature, starred arrows represent changes which have been observed in the laboratory for which there is some evidence that they have also occurred in nature. (The + marked arrow was observed only by W.C.Noble). A black box denotes *mup*A, a lightly stippled box denotes pT181 and a heavily stippled box represents pOX7. Boxed, hatched arrows represent IS*257*s.

copies in direct repeat (Rouch *et al*, 1989; Byrne *et al*, 1990; Stewart *et al*, 1994), all these observations had been of past events. Thus, although IS*257* had the structure of an insertion element (Barberis-Maino *et al*, 1987) and appeared to have spread in a manner typical of an insertion element at some point, there was no direct evidence of its present activity as a transposable element.

7.2 IS*257*: an active insertion element

As the family of *mup*A plasmids was a compact example of the different types of rearrangement implicated from previous observations, it proved an ideal system in which to investigate the present ability of IS*257* to act as a transposable element. Gene duplication has been achieved in the laboratory by exposure of p3356 to serial dilution in high concentrations of mupirocin (Chapter 4). Recombination between IS*257*s has been demonstrated in *S.aureus*, by the products of transformation and serial dilution with appropriate antibiotic selection (Chapter 5); although the pathway for recombination in *S.aureus* is unknown, such interplasmid inter-IS*257* recombination has been shown to occur via *rec*A$^-$ pathways in *E.coli* (Chapter 6). Replicon fusion has been demonstrated between the temperature sensitive, erythromycin resistant plasmid pOX7 and p3356 by selection for erythromycin resistance at restrictive temperature, and this co-integrate formation must have involved the transposition of one of the IS*257s* of p3356, either independently (followed by a recombination event) or along with the whole of p3356 in a co-integrational transposition event (Chapter 6). These interactions are summarised in Fig.7.1. IS*257* has clearly been shown to be active as a transposable element.

It should be noted that although IS*257* has not been shown to transpose independently or indeed as part of a composite transposon, the experiments described in this volume did not select for these events. However, previous studies have also failed to demonstrate the independent transposition of IS*257*.

7.3 Insertion elements homologous to IS*257*

The sequence of IS*257*[2] is particularly homologous to those of the insertion sequences IS*15* (Trieu-Cuot and Courvalin, 1984), IS*15*Δ (Labigne-Roussel and Courvalin, 1983), IS*26* (Mollet *et al*, 1983), IS*46* (Brown *et al*, 1984), and IS*S1* (Polzin and Shimizu-Kadota, 1987); IS*140* has also been shown to be homologous by Southern blot (Brau and Piepersberg, 1983)(For a summary see Rouch and Skurray, 1989). The homology is greatest within the open reading frame of the transposases and the inverted repeats, and all create 8bp repeats at the target site of transposition (Trieu-Cuot *et al*, 1983; Mollet *et al*, 1985; Huang *et al*, 1992; Stewart *et al*, 1994). As the sequences themselves are homologous, it is appropriate to consider the similarities in their modes of action, as compared to less homologous insertion elements represented by IS*1* and IS*903*, which generate 9bp duplication of the target site upon transposition.

Gene amplification of the "IS*257*-*mup*A" cassette has been demonstrated (Chapter 4). Tandem gene duplication of the composite transposons Tn*1525* and Tn*2680*, (flanked in direct repeat by IS*15* and IS*26* respectively) has been observed (Iida *et al*, 1982; Labigne-Roussel *et al*, 1983). Gene duplication of Tn*9* flanked by direct repeats of the less homologous element IS*1* has also been demonstrated (Chandler *et al*, 1979) as has the tandem amplification of other sequences flanked by direct repeats of IS*1* along with a copy of IS*1* (Meyer and Iida, 1979; Chandler and Galas, 1983). Tandem gene amplification has also been seen between homologous, non-insertion element sequences. As all the gene duplications have been observed in *rec*$^+$ host cells, it is likely that it is not due the inherent structure of the elements but rather a general homologous recombination event catalysed at sites of homology, provided by the insertion elements.

Transposition of the composite transposons Tn*1525* and Tn*2680* has been observed (Meyer and Iida, 1979; Chandler and Galas, 1983), as has the independent transposition of IS*15* and IS*46* (Brown *et al*, 1984; Trieu-Cuot and Courvalin, 1984). IS*257* has failed to transpose

[2] As the IS*257*s of the p3356 family are most homologous to IS*2571* (Byrne *et al*, 1990), this is the IS*257* referred to unless otherwise stated.

independently or as part of a composite transposon, despite all attempts (Thomas and Archer, 1989). Although this appears to be a discrepancy of function between these homologous insertion sequences, the frequencies of the observed transposition events are very low, compared to those of IS*1* and IS*903*. A slight reduction in this very low frequency of transposition would lead to an undetectably low frequency of transposition, this may explain the lack of observed transposition of the deleted form of IS*15*, IS*15*Δ as well as IS*257*.

Co-integrational transposition has been observed for IS*15*, IS*15*Δ (Trieu-Cout and Courvalin, 1985), IS*26* (Iida *et al*, 1984), IS*46* (Brown *et al*, 1984), IS*140* and IS*S1*. For IS*15*, IS*26* and IS*46*, the frequencies of co-integrational transposition are far higher than simple insertion transposition. Deletion of the duplicated transposase of IS*15* to give the naturally occurring IS*15*Δ, leads to loss of simple insertion transposition function, whilst maintaining co-integrational transposition activity (Labigne-Roussel and Courvalin, 1983). IS*1* also has been observed to be active in co-integrational transposition, but at low frequencies compared to simple transposition, and usually only generated if the co-integrate is specifically selected for (Galas and Chandler, 1982). One of the IS*903*s of Tn*903* will give co-integrates involving Tn*903* when the other IS*903* of Tn*903* is deleted for the transposase region, whereas normally Tn*903* undergoes transposition to give a simple insertion (Grindley and Joyce, 1981). The preference for the product of transposition (co-integrate or simple insertion) may reflect a fundamental difference in the activities of the ISs: it is proposed that IS*257* homologous ISs prefer the co-integrate product, whilst IS*1* and IS*903* prefer simple insertion transposition. Although experiments in this volume have selected for the co-integrate, it is proposed that this is the usual end-product of the IS*257* transposition process, with simple transposition being a much less common occurrence. This proposal is supported by the high frequencies of co-integration obtained in this volume, by the existence of co-integrates found in naturally occurring plasmids with IS*257*s at the points of fusion (Gillespie *et al*, 1986; Byrne *et al*, 1991; Stewart *et al*, 1994) and by the lack of 8bp direct repeats flanking individual IS*257*s. Also, recently pT181 and pSK108 small staphylococcal plasmids have been found resident in larger plasmids which appear to have arisen by co-integrational transposition of the IS*257* downstream of the trimethroprim resistance determinant of pSK639 (R.A.Skurray, personal communication). Although IS*1* elements do

occur at the junctions of co-integrates in naturally occurring plasmids (Iida, 1980; Iida and Arber, 1980) they are often found in active composite transposons also (Chandler *et al*, 1977; Chandler *et al*, 1979; Clerget *et al*, 1980; for review). Moreover, some insertion sequences do not appear to be involved in replicon fusion at all, except when catalysed by *rec* homologous recombination, e.g. IS*10* (Harayama *et al*, 1984) and IS*50* (Berg *et al*, 1981; Hirschel *et al*, 1982; Berg, 1983).

IS*1* and IS*903*-based fused replicons do not occur often, but when formed are stable, their resolution is wholly dependent upon *rec* homologous recombination (Grindley and Joyce, 1981; Galas and Chandler, 1982). Sequences flanked by direct repeats of IS*1* within the same replicon are also stable, although appropriate antibiotic selection will lead to *rec* dependent recombination between the ISs and loss of the intervening sequences along with one copy of IS*1* (Chandler *et al*, 1977; Clerget *et al*, 1980; Chandler and Galas, 1983). IS*15* and IS*15*Δ-based fused replicons are also stable in *rec*A$^+$ cells requiring antibiotic selection for detectable levels of resolution (Trieu-Cuot and Courvalin, 1985). Likewise, co-integrates with IS*26*, IS*46*, IS*140* or IS*S1* at the fusion junctions are all stable in *rec*A$^+$ hosts, and require antibiotic selection for resolution to be seen (Brau and Piepersberg, 1983; Brown *et al*, 1984; Polzin and Shimizu-Kadota, 1987; Huang *et al*, 1992). The transposon Tn*1525* was deleted for all but one copy of IS*15* upon antibiotic selection (Labigne-Roussel *et al*, 1983), likewise recombination between the IS*26*s of Tn*2680* deleted all but one copy of IS*26* upon selection (Iida *et al*, 1982). Sequences encoding tetracycline resistance flanked by direct repeats of IS*46* were spontaneously lost along with one copy of IS*46* (Hall, 1987). IS*257* co-integrates and plasmids containing direct repeats of IS*257* were also stable in *rec*A$^+$ hosts over several generations of serial dilution in the absence of selection. Thus, as for IS*1* and IS*903* based co-integrates, once formed plasmids containing IS*257* and IS*257* homologous ISs in direct repeat are generally stable. However, unlike IS*1* co-integrates and direct repeats which are dependent upon *rec* for their resolution, sequences flanked by direct repeats of IS*257* were lost by recombination between the flanking IS*257*s upon antibiotic selection in both *rec*A$^+$ and *rec*A$^-$ strains of *E.coli*. Resolution of co-integrates with IS*15* or IS*15*Δ at the junctions also occurred in *rec*A$^-$ cells, although at reduced frequency in comparison to the frequency in *rec*A$^+$ cells ($x10^{-3}$) (Labigne-Roussel *et al*, 1983). It was concluded that the transposase of IS*257* was able to provide the necessary recombinational activity, as was the

transposase of IS*15* although with reduced activity. It is proposed that this is another area of difference between the reactions of IS*257*- homologous insertion sequences and reactions of IS*1* and IS*903*. Moreover, there appears to be a spectrum of activity within the IS*257*-homologous ISs, each having differing abilities to catalyse homologous recombination between copies of themselves.

7.4 Implications for IS*257* transposition

Two differences are postulated in the activities of IS*257*-like insertion sequences compared to IS*1* and IS*903*. Firstly that IS*257*-like ISs will tend to form co-integrates as a product of transposition rather than the simple insertions favoured by IS*1* and IS*903*. Secondly that although co-integrates with IS*257*-like ISs, IS*1* or IS*903* at the inter-replicon boundaries are all stable in *rec*A$^+$, in the absence of antibiotic selection for segregation, their stabilities vary in *rec*A$^-$ hosts; IS*1* and IS*903* based co-integrates will not recombine in *rec*A$^-$, demonstrating their dependence upon *rec* for segregation, whereas the transposases of IS*257* and IS*15* demonstrate a degree of recombinase activity in the resolution of co-integrates.

It is generally accepted that the Class II transposons (e.g. Tn*3*; γδ, alias Tn*1000*) usually transpose via a pathway which involves a co-integrate as its intermediate (Arthur and Sherratt, 1979), whereas Class I transposons (ISs and composite transposons including ISs) transpose via a mechanism which allows the choice of a co-integrate product of resolution, or a simple insertion (Galas and Chandler, 1981). (For a review see Kleckner, 1981.) The apparent differences in the preferred products of IS*1* and IS*903* transposition (simple insertions) and IS*257*-like IS transposition (co-integrates) represent the diversity of the Class I transposons in the use of this mechanism. Indeed, there is a possible difference in emphasis of the preferred product of transposition of the IS*257*-homologous family of insertion sequences, reflecting the minor differences within the broader spectrum of transposition mechanisms of the insertion sequences in general. As the IS*257*-like transposases appear to favour replicon fusion, a mechanism is required for resolution of those replicons, should they become a burden to the host. It is proposed that the recombinase activity, demonstrated for IS*257* and IS*15*, provides this mechanism: the recombinase has such a low activity that

under non-selective conditions plasmids containing direct repeats of these ISs are stable, however under great selective pressure the recombinase activity leads to deletion products which are then selected due to their reduced load on the host.

7.5 Implications for MRSA

The corollary of IS*257* transposition leading to the formation of co-integrates is that IS*257*s are found in direct repeat, and will rarely be flanked by the 8bp direct repeat of target sequence expected from the independent insertion of IS*257*. Indeed this is the case (Gillespie *et al*, 1987; Stewart *et al*, 1994), the only cited example of an IS*257* in inverted repeat with respect to other IS*257*s on the same replicon is one of the IS*257*s (2) which interrupt the incomplete IS*256*s flanking the *aac*A-*aph*D genes in the truncated Tn*4001* of the pSK41 family of plasmids (Byrne *et al*, 1990), and this IS*257* is mutated so as not to produce a functional transposase.

The frequency of transposition of composite transposons decreases with increasing distance between the flanking ISs, thus ISs which prefer the simple insertion product in favour of a co-integrate are limited in the size of DNA piece that they can transfer to another replicon. However, by following the pathway to co-integrate end products, IS*257*s can link large pieces of DNA together, conditional upon one of the replicons containing an *ori* which is able to replicate large plasmids. Combined with the potential to act as a site of homologous recombination, the ability to join replicons places IS*257* in an ideal situation to gather DNAs of various sorts onto one large replicon. Should these DNAs code for antibiotic resistance determinants, the result is a plasmid or chromosome containing many antibiotic resistance determinants: such replicons are seen in the MRSA strains which are able to proliferate with such devastating results (Gillespie and Skurray, 1986). That direct repeats of IS*257* are stable in the absence of undue selection for smaller plasmid size, allows such multiple resistance plasmids to be maintained. Moreover, should an extreme situation arise which would select for a smaller form, it appears that IS*257* may code for the recombinase activity to allow the generation of smaller forms by the specific recombination between IS*257*s. Overall, IS*257* appears to have evolved as a transposable element so as to be an ideal

mechanism via which resistance determinants can be gathered on the same replicon, and therefore it appears not accidental that direct repeats of IS*257* do intersperse many of the resistance determinants on plasmids and areas of the chromosome which contain many such genes. To postulate further, it has been suggested that general homologous recombination (*rec*) of *S.aureus* is not very efficient (Novick, 1990); it is not impossible that *rec* and insertion sequences of *S.aureus* have evolved in concert so as to allow stable co-integrates by the reduced *rec* activity between homologous direct repeats on the same replicon.

7.6 Implications for antibiotic therapy against staphylococcal infection

Although the plasmids used for the basis of the studies in this volume are mupirocin resistance plasmids, the rearrangements discussed concern the IS*257*s on those plasmids and the resistance determinant is of little significance. For instance, the naturally occurring co-integrate plasmid, p3358, encodes resistance to both mupirocin and tetracycline, whereas the co-integrates created in the laboratory were mupirocin and erythromycin resistant: the two plasmids pT181 and pOX7 were interchangeable. Likewise, the products of recombination between the IS*257*s, whether in *rec*A$^+$ *S.aureus* or in *rec*A$^+$ or *rec*A$^-$ *E.coli*, were selected for by the very different antibiotics tetracycline and ampicillin. Hence, the significance of the gathering and dissolution of resistance determinants by IS*257* lies in the very nature of the IS*257* not the antibiotic: IS*257* will catalyse the co-integration of many different replicons, whatever the DNA content of those replicons.

The significance of the antibiotic resistance determinants is one of selection. For instance, in the co-integration of the temperature sensitive erythromycin resistance plasmid pOX7 with the mupirocin resistance plasmid p3356 (Chapter 6), IS*257* provided a means of linking the required erythromycin resistance gene to a temperature stable replicon, to allow maintenance of the erythromycin resistance determinant. It is probable that such interactions occur at a much reduced frequency in non-selective conditions. Once two resistance determinants are linked, even a low level of selection for one resistance determinant automatically selects for the other, and thus maintenance of a replicon coding for multiple resistances. Although laboratory selection for one resistance determinant has often led to the resolution of the co-

integrate, and maintenance of a plasmid with only one resistance determinant (Chapter 5 & 6), it is thought that this is due to the extremely high pressure of selection. The existence of stable co-integrate plasmids, often at very diverse geographical locations (Gillespie and Skurray, 1986) suggests that co-integrates are stably maintained. It appears important therefore that the resistance profile of any infection of *S.aureus* is known prior to the onset of antibacterial therapy, in an attempt not only to prevent selection of resistance to one antibiotic, but also potentially to many more.

To add perspective to this discussion, it is interesting to note that the mupirocin resistance gene has been in the staphylococcal population for at least 30 years, on plasmids which also harbour a copy of IS*257*. At present *mup*A is found on a wide variety of plasmids, and only one family has been identified which relates any of these diverse plasmids to each other: the p3356 family. Mupirocin has been used therapeutically for 10 years and only one example of linkage has been observed: that cited in this volume, p3358, in which mupirocin resistance is linked to tetracycline resistance. Therefore, although it is apparent that these rearrangements do occur and are of great clinical significance when they lead to the linkage of resistance determinants, it appears that such events are very rare. Although this does not negate the necessity for appropriate monitoring of resistances within the bacterial population, the prospects for the efficacy of the antibiotics are not greatly diminished by the possibility of linkage by such mechanisms. However, it should also be noted that co-integrational transposition of IS*257* is only one mechanism of horizontal transfer of DNA of a plethora of potential mechanisms available, and that any one mechanism which leads to an increase in resistance determinants, particularly those which link otherwise unlinked resistance determinants, has the potential to decrease the usefulness of any one antibiotic to fight infection.

7.7 Further experiments

IS*257* has undergone co-integrational transposition when encouraged to rescue the temperature sensitive plasmid by antibiotic selection of that plasmid. Using a similar selection method, the possibility of simple insertion transposition should be investigated. In order to look for transposition of the IS itself, a visible selection system for transposition would be ideal to assist in the screening of large numbers of colonies, such as the interruption of a β-galactosidase gene in the presence of X-gal, with selection of white colonies. Attempts to clone the putative transposon of p3356 have not been successful to date; therefore, to investigate transposition of potential compound IS*257*-based transposons, it is suggested that a "transposon" be constructed from two IS*257*s and an easily selectable resistance determinant on a temperature sensitive plasmid, selection for the resistance determinant should demonstrate transposition.

To provide conclusive evidence that the transposase of IS*257* is providing the co-integrational transposition and recombinase catalysis, the transposase mutant should be used in other systems which do not fall foul of the site specific recombination and chromosomal integration observed in these studies. It may be possible to use the *E.coli* system described (Chapter 6) by deleting one or both of the plasmids of the specific site of recombination or using a plasmid containing only the polylinker of pUC18. It may be possible to conduct investigations in *S.aureus* by placing the pOX7 clones of IS*257* and its mutant into a strain containing a large plasmid.

The studies describe the role of IS*257* in the dissemination of genetic information within the genome and plasmids of *S.aureus*, it would be enlightening to have a broader knowledge of the distribution of IS*257* throughout the genome and plasmids of both *S.aureus* and the coagulase negative staphylococci, believed to access the same plasmid pool and becoming an increasing clinical threat.

Other more separate issues have been raised by this study including the role of IS*257* as a portable promoter, the possibility of an altered mechanism for conjugation between staphylococci, and a site specific recombination between two plasmids in *E.coli*, all these areas may prove interesting avenues to explore further.

Bibliography

Bibliography

Amabile-Cuevas C.F. & Chicurel M.E. (1992) Bacterial plasmids and gene flux. *Cell*; **70**: 189-199.

Anderson R.P. & Roth J.R. (1977) Tandem genetic duplications in phage and bacteria. *Ann Rev Microbiol*; **31**: 473-505.

Archer G.L. & Thomas W.D. (1990) Conjugative transfer of antimicrobial resistance genes between staphylococci. In *"Molecular Biology of the Staphylococci"*. pp. 1-40. Edited by R.P.Novick. VCH, New York, NY.

Arthur A. & Sherratt D.J. (1979) Dissection of the transposition process: a transposon-encoded site-specific recombination system. *Mol Gen Genet*; **175**: 267-274.

Arthur M. & Courvalin P. (1993) Genetics and mechanisms of glycopeptide resistance in enterococci. *Antimicrob Agents Chemother*; **37**: 1563-1571.

Arthur M., Molinas C., Depardieu F. & Courvalin P. (1993) Characterisation of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol*; **175**: 117-127.

Asheshov E.H. (1975) The genetics of tetracycline resistance in *Staphylococcus aureus*. *J Gen Microbiol*; **88**: 132-140.

Baird D. & Coia J. (1987) Mupirocin-resistant *Staphylococcus aureus*. *Lancet*; **II**: 387-388.

Barberis-Maino L., Berger-Bachi B., Weber H., Beck W.D. & Kayser F.H. (1987) IS*431*, a staphylococcal insertion sequence-like element related to IS*26* from *Proteus vulgaris*. *Gene*: **59**: 107-113.

Bayles K.W., Brunskill E.W., Iandolo J.J., Hruska L.L., Huang S., Pattee P.A., Smiley B.K. & Yasbin R.E. (1994) A genetic and molecular characterisation of the *rec*A gene from *Staphylococcus aureus*. *Gene*; **147**: 13-20.

Berg D.E., Egner C., Hirschel B.J., Howard J., Johnsrud L., Jorgensen R.A. & Tlsty T.D. (1981) Insertion, excision and inversion of Tn*5*. *Cold Spring Harbour Symp Quant Biol*; **45**: 115-123.

Berg D.E. (1983) Structural requirement for IS*50*-mediated gene transposition. *Proc Natl Acad Sci USA*; **80**: 792-796.

Birnboim H.C. & Doly J. (1979) A rapid alkaline extraction for screening recombinant plasmid DNA. *Nucleic Acids Res*; **7**: 1513-1523.

BOELAERT J.R., VAN LANDUYT H.W., GODARD C.A., DANEELS R.F., SCHURGERS M.L., MATTHYS E.G., DE BAERE Y.A., GHEYLE D.W., GORDTS B.Z. & HERWALDT L.A. (1993) Nasal mupirocin ointment decreases the incidence of *Staphylococcus aureus* bacteraemias in haemodialysis patients. *Nephrol Dial Transplant*; **8**: 235-239.

BORIELLO P. (1994) Bacterial infection - still thriving after all these years. *MRC News*; **58**: 31-33.

BRADLEY S.F. (1993) Effectiveness of mupirocin in the control of methicillin-resistant *Staphylococcus aureus*. *Infections in Medicine*;**10**: 23-31.

BRAU B. & PIEPERSBERG W. (1983) Cointegrational transduction and mobilisation of gentamicin resistance plasmid pWP14a is mediated by IS*140*. *Mol Gen Genet*; **189**: 298-303.

BROWN A.M.C., COUPLAND G.M. & WILLETTS N.S. (1984) Characterisation of IS*46*, an insertion sequence found on two IncN plasmids. *J Bacteriol*; **159**: 472-481.

BYRNE M.E., GILLESPIE M.T. & SKURRAY R.G. (1990) Molecular analysis of a gentamicin resistance transposonlike element on plasmids isolated from North American *Staphylococcus aureus* strains. *Antimicrob Agents Chemother*; **34**: 2106-2113.

BYRNE M.E., GILLESPIE M.T. & SKURRAY R.A. (1991) 4',4" adenyltransferase activity on conjugative plasmids isolated from *Staphylococcus aureus* is encoded on an integrated copy of pUB110. *Plasmid*; **25**: 70-75.

CAMBAU E. & GUTMANN L. (1993) Mechanisms of resistance to quinilones. *Drugs*; **45** Suppl.3: 15-23.

CAPOBIANCO J.O., DORAN C.C. & GOLDMAN R.C. (1989) Mechanism of mupirocin transport into sensitive and resistant bacteria. *Antimicrob Agents Chemother*; **33**: 156-163.

CASEWELL M.W. & HILL R.L.R. (1984) The laboratory assessment of the antistaphylococcal activity of mupirocin (pseudomonic acid). In *Bactroban (mupirocin) Proceedings of an International Symposium (Current Clinical Practice Series 16)*, pp37-45. Edited by R.L. Dobson, J.J. Leyden, W.C. Noble & J.D.Price. Amsterdam: Excerpta Medica.

CHALKER A.F., WARD J.M., FOSBERRY A.P. & HODGSON J.E. (1994) Analysis and toxic overexpression in *Escherichia coli* of a staphylococcal gene encoding isoleucyl-tRNA synthetase. *Gene*; **141**: 103-108.

CHANDLER M., ALLET B., GALLAY E., BOY DE LA TOUR E. & CARO L. (1977) Involvement of IS*1* in the dissociation of the r-determinant and RTF components of the plasmid R100.1. *Mol Gen Genet*; **153**: 289-295.

CHANDLER M., BOY DE LA TOUR E., WILLEMS D. & CARO L. (1979) Gene properties of the chloramphenicol resistance transposon Tn*9*. *Mol Gen Genet*; **176**: 221-231.

CHANDLER M. & GALAS D.J. (1983) IS*1*-mediated tandem duplication of plasmid pBR322. Dependence on *rec*A and DNA polymerase I. *J Mol Biol*; **165**: 183-190.

CHANG S. & COHEN S.N. (1979) High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Mol Gen Genet*; **168**: 111-115.

CHARLIER D., PIETTE J. & GLANSDORFF N. (1982) IS*3* can function as a mobile promoter in *E.coli*. *Nucleic Acids Res*; **10**: 5935-5948.

CHATFIELD C.A., O'NEILL W.A., COOKE R.P.D., M^{C}GHEE K.J., ISSACK M., RAHMAN M. & NOBLE W.C. (1994) Mupirocin-resistant *Staphylococcus aureus* in a specialist school population. *J Hosp Infect*; **26**: 273-278.

CHOPRA I., LACEY R.W. & CONNOLLY J. (1974) Biochemical and genetic basis of tetracycline resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother*; **6**: 397-404.

CLERGET M., CHANDLER M. & CARO L. (1980) Isolation of an IS*1* flanked kanamycin resistance transposon from R1*drd*19. *Mol Gen Genet*; **180**: 123-127.

CLEWELL D.B. (1993) Bacterial sex pheromone-induced plasmid transfer. *Cell*; **73**: 9-12.

COHEN S.N., CHANG S.C.Y. & HSU L. (1972) Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli*. *Proc Natl Acad Sci*; **69**: 2110.

CONNOLLY S., NOBLE W.C. & PHILLIPS I. (1993) Mupirocin resistance in coagulase-negative staphylococci. *J Med Microbiol*; **39**: 450-453.

COOKSON B.D., LACEY R.W., NOBLE W.C., REEVES D.S., WISE R. & REDHEAD R.J. (1990) Mupirocin-resistant *Staphylococcus aureus*. *Lancet*; **II**: 1095-1096.

DACRE J.E., EMMERSON A.M. & JENNER E.A. (1983) Nasal carriage of gentamicin and methicillin resistant *Staphylococcus aureus* treated with topical pseudomonic acid. *Lancet*; **II**: 1036.

DAWSON S.J., FINN L.F., M^{C}CULLOCH J.E., KILVINGTON S. & LEWIS D.A. (1994) Mupirocin-resistant MRSA. *J Hosp Infect*; **28**: 75-78.

DEBUYSER M.-L., MORVON A., GRIMONT F. & ELSOLH N. (1989) Characterisation of staphylococcal species by rRNA gene restriction patterns.. *J Gen Microbiol*; **133**: 989-999.

DELARUE M. & MORAS D. (1993) The aminoacyl-tRNA sythetase family: modules at work. *BioEssays*; **15**: 675-687.

DERBISE A., DYKE K.G.H. & EL SOLH N. (1994) Isolation and characterisation of IS*1181*, an insertion sequence from *Staphylococcus aureus*. *Plasmid*; **31**: 251-264.

DODD H.M., HORN N. & GASSON M.J. (1994) Characterisation of IS*905*, a new multicopy insertion sequence identified in lactococci. *J Bacteriol*; **176**: 3393-3396.

DOEBBELING B.N., BRENEMAN D.L., NEU H.C., ALY R., YANGCO B.G., HOLLEY H.P., MARSH R.J., PFALLER M.A., M^{C}GOWAN J.E., SCULLY B.E., REAGAN D.R., WENZEL R.P. & THE MUPIROCIN COLLABORATIVE STUDY GROUP (1993) Elimination of *Staphylococcus aureus* nasal carriage in health care workers: analysis of six clinical trials with calcium mupirocin ointment. *Clin Infect Dis*; **17**: 466-474.

DOEBBELING B.N., REAGAN D.R., PFALLER M.A., HOUSTON A.K., HOLLIS R.J. & WENZEL R.P. (1994) Long-term efficacy of intranasal mupirocin ointment. A prospective cohort study of *S.aureus* carriage. *Arch Intern Med*; **154**: 1505-1508.

DUBIN D.T., MATTHEWS P.R., CHIKRAMANE S.G. & STEWART P.R. (1991) Physical mapping of the *mec* region of an American methicillin-resistant *Staphylococcus aureus* strain. *Antimicrob Agents Chemother*; **35**: 1661-1665.

DYKE K.G.H. & ROWLAND S.-J. (1990) The β-lactamase transposon Tn*552*. In *"Molecular Biology of the Staphylococci"*. pp. 1-40. Edited by R.P.Novick. VCH, New York, NY.

DYKE K.G.H., CURNOCK S.P., GOLDING M. & NOBLE W.C. (1991) Cloning of the gene conferring resistance to mupirocin in *Staphylococcus aureus*. *FEMS Microbiol Letts*; **77**: 195-198.

DYKE K.G.H., AUBERT S. & ELSOLH N. (1992) Multiple copies of IS*256* in staphylococci. *Plasmid*; **28**: 235-246.

ENGEL H.W.B., SOEDIRMAN N., ROST J.A., VAN LEEUWEN W.J. & VAN EMBDEN J.D.A. (1980) Transferability of macrolide, lincomycin and streptogramin resistances between group A, B and D streptococci, *Streptococcus pneumoniae* and *Staphylococcus aureus*. *J Baceteriol*; **142**: 407-413.

EVANS J.E. (1986) The conjugation system of *Staphylococcus aureus*. DPhil thesis, University of Oxford.

EVANS J.E. & DYKE K.G.H. (1988) Characterisation of the conjugation system associated with the *Staphylococcus aureus* plasmid pJE1. *J Gen Microbiol*; **134**: 1-8.

FERSHT A.R. & KAETHNER M.M. (1976) Mechanism of aminoacylation of tRNA. Proof of the aminoacyl adenylate pathway for the isoleucyl- and tyrosyl-tRNA synthetases from *Escherichia coli* K12. *Biochem*; **15**: 818-823.

FIRTH N., RIDGWAY K.P., BYRNE M.E., FINK P.D., JOHNSON L., PAULSEN I.T. & SKURRAY R.A. (1993) Analysis of a transfer region from the staphylococcal conjugative plasmid pSK41. *Gene*: **136**: 13-25.

FIRTH N., FINK P.D., JOHNSON L. & SKURRAY R.A. (1994) A lipoprotein signal peptide encoded by the staphylococcal conjugative plasmid pSK41 exhibits an activity resembling that of *Enterococcus faecalis* pheromone cAD1. *J Bacteriol*; **176**: 5871-5873.

FOSTER T.J. (1983) Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol Rev*; **47**: 361-409.

FULLER A.T., BANKS G.T., MELLOWS G., BARROW K.D., WOOLFORD M. & CHAIN E.B. (1971) Pseudomonic acid: an antibiotic produced by *Pseudomonas fluorescens. Nature*; **234**: 416.

GALAS D.J. & CHANDLER M. (1981) On the molecular mechanisms of transposition. *Proc Natl Acad Sci USA*; **78**: 4858-4862.

GALAS D.J. & CHANDLER M. (1982) Structure and stability of Tn*9*-mediated co-integrates. Evidence for two pathways of transposition. *J Mol Biol*; **154**: 245-272.

GALAS D.J. & CHANDLER M. (1989) Bacterial Insertion Sequences. In "*Mobile DNA* " pp109-162. Edited by D.E. Berg & M.M.Howe. Am Soc Microbiol, Washington D.C..

GENNARO M.L., KORNBLUM J. & NOVICK R.P. (1987) A site-specific recombination function in *Staphylococcus aureus* plasmids. *J Bacteriol*; **169**: 2601-2610.

GENNARO M.L. & NOVICK R.P. (1988) An enhancer of DNA replication. *J Bacteriol*; **170**: 5709-5717.

GILBART J., PERRY C.R., & SLOCOMBE B. (1993) High-level mupirocin resistance in *Staphylococcus aureus*: evidence for two distinct isoleucyl-tRNA synthetases. *Antimicrob Agents Chemother*; **37**: 32-38.

GILLESPIE M.T. & SKURRAY R.A. (1986) Plasmids in multiresistant *Staphylococcus aureus. Microbiol Sci*; **3**: 53-58.

GILLESPIE M.T., MAY J.W. & SKURRAY R.A. (1986) Detection of an integrated tetracycline resistance plasmid in the chromosome of methicillin-resistant *Staphylococcus aureus. J Gen Microbiol*; **132**: 1723-1728.

GILLESPIE M.T., LYON B.R., LOO L.S.L., MATTHEWS P.R., STEWART P.R. & SKURRAY R.A. (1987) Homologous direct repeat sequences associated with mercury, methicillin, tetracycline and trimethroprim resistance determinants in *Staphylococcus aureus. FEMS Microbiol Letts*; **43**: 165-171.

GRINDLEY N.D.F. & JOYCE C.M. (1981) Analysis of the structure and function of the kanamycin-resistance transposon Tn*903*. *Cold Spring Harbour Symp Quant Biol*; **45**: 125-133.

GRINDLEY N.D.F. & REED R.R. (1985) Transpositional recombination in prokaryotes. *Ann Rev Biochem*; **54**: 863-896.

GRUNBERG-MANAGO M. (1983) Regulation of the expression of aminoacyl-tRNA sythetases and translation factors. In "Escherichia coli *and* Salmonella typhimurium*: Cellular and Molecular Biology.*" pp 1386-1409. Edited by: F.C. Neidhardt, J.L.Ingraham, K.B.Low, B.Magasanik, M.Schaechte & H.E.Umbarger. Am Soc Microbiol, Washington D.C..

GRUSS A. & EHRLICH S.D. (1989) The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol Rev*; **53**: 231-241.

GUAY G.G., KHAN S.A. & ROTHSTEIN D.M. (1993) The *tet*(K) gene of plasmid pT181 of *Staphylococcus aureus* encodes an efflux protein that contains 14 transmembrane helices. *Plasmid*: **30**: 163-166.

HALL R.M. (1987) pKM101 is an IS*46*-promoted deletion of R46. *Nucleic Acids Res*; **15**:5479.

HAMILTON C.M., ALDEA M., WASHBURN B.K., BABITZKE P. & KUSHNER S.R. (1989) New method for generating deletions and gene replacements in *Escherichia coli. J Bacteriol*; **171**: 4617-4622.

HANAHAN D. (1993) Studies on transformation of *Escherichia coli* with plasmids. *.J Mol Biol*; **166**: 557-**580.**

HARAYAMA S., OGUCHI T. & IINO T. (1984) Does Tn*10* transpose via the co-integrate molecule? *Mol Gen Genet*; **194**: 444-450.

HIRSCHEL B.J., GALAS D.J. & CHANDLER M. (1982) Co-integrate formation by Tn*5*, but not transposition, is dependent on *rec*A. *Proc Natl Acad Sci USA*; **79**: 4530-4534.

HODGSON J.E., CURNOCK S.P., DYKE K.G.H., MORRIS R., SYLVESTER D.R. & GROSS M.S. (1994) Molecular characterisation of the gene encoding high-level mupirocin resistance in *Staphylococcus aureus* J2870. *Antimicrob Agents Chemother*; **38**: 1205-1208.

HOEGER P.H., LENZ W., BOUTONNIER A. & FOURNIER J.-M. (1992) Staphylococcal skin colonisation in children with atopic dermatitis: prevalence, persistence and transmission of toxigenic and nontoxigenic strains. *J Infect Dis*; **165**: 1064-1068.

HORINOUCHI S. & WEISBLUM B. (1982a) Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. *J Bacteriol*; **150**: 804-814.

HORINOUCHI S. & WEISBLUM B. (1982b) Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. *J Bacteriol*; **150**: 815-825.

HUANG B.C., NOVEL M., HUANG X.F. & NOVEL G. (1992) Nonidentity between plasmid and chromosomal copies of IS*S1*-like sequences in *Lactococcus lactis* subsp. *lactis* CNRZ270 and their possible role in chromosomal integration of plasmid genes. *Gene*; **118**: 39-46.

HUDSON I.R.B. (1994) The efficacy of intranasal mupirocin in the prevention of staphylococcal infections: a review of recent experience. *J Hosp Infect*; **27**: 81-98.

HUGHES A.L. (1994) The evolution of functionally novel proteins after gene duplication. *Proc R Soc Lon* B; **256**: 119-124.

HUGHES J. & MELLOWS G. (1978) Inhibition of isoleucyl-transfer ribonucleic acid synthetase in *Escherichia coli* by pseudomonic acid. *Biochem J*; **176**: 305-318.

HUGHES J. & MELLOWS G. (1980) Interaction of pseudomonic acid A with *Escherichia coli* B isoleucyl-tRNA sythetase. *Biochem J*; **191**: 209-219.

HUGHES J., MELLOWS G. & SOUGHTON S. (1980) How does *Pseudomonas fluorescens*, the producing organism of the antibiotic pseudomonic acid A, avoid suicide? *FEBS Letts*; **122**: 322-324.

IIDA S. (1980) A co-integrate of the bacteriophage P1 genome and the conjugative R plasmid R100. *Plasmid*; **3**: 278-290.

IIDA S. & ARBER W. (1980) On the role of IS*1* in the formation of hybrids between the bacteriophage P1 and the R plasmid NR1. *Mol Gen Genet*; **177**: 261-270.

IIDA S., MEYER J., LINDER P., GOTO N., NAKAYA R., REIF H-J. & ARBER W. (1982) The kanamycin resistance transposon Tn*2680* derived from the R plasmid Rts1 and carried by phage P1Km has flanking 0.8kb-long direct repeats. *Plasmid*; **8**: 187-198.

IIDA S., MOLLET B., MEYER J. & ARBER W. (1984) Functional characterisation of the prokaryotic mobile genetic element IS*26*. *Mol Gen Genet*; **198**: 84-89.

IORDANESCU S. & SURDEANU M. (1976) Two restriction and modification systems in *Staphylococcus aureus* NCTC8325. *J Gen Microbiol*; **96**: 277-281.

IORDANESCU S., SURDEANU M., LATTA P.D. & NOVICK R.P. (1978) Incompatibility and molecular relationships between small staphylococcal plasmids carrying the same resistance marker. *Plasmid*; **1**: 468-479.

JENAL U., RECHSTEINER T., TAN P.-Y., BUHLMANN E., MEILE L. & LAISINGER T. (1991) Isoleucyl-tRNA synthetase of *Methanobacterium thermoautotrophicum* Marburg. *J Biol Chem*; **266**: 10570-10577.

JONES J.M., CONNOLLY YOST S. & PATTEE P.A. (1987) Transfer of the conjugal tetracycline resistance transposon Tn*916* from *Streptococcus faecalis* to *Staphylococcus aureus* and identification of some insertion sites in the staphylococcal chromosome. *J Bacteriol*; **169**: 2121-2131.

KAUFFMAN C.A., TERPENNING M.S., HE X., ZARINS L.T., RAMSEY M.A., JORGENSEN K.A., SOTTILE W.S. & BRADLEY S.F. (1993) Attempts to eradicate methicillin-resistant *Staphylococcus aureus* from a long-term-care facility with the use of mupirocin ointment. *Am J Med*; **94**: 371-378.

KAVI J., ANDREWS J.M. & WISE R. (1987) Mupirocin-resistant *Staphylococcus aureus*. *Lancet*; **II**: 1472.

KHAN S.A. & NOVICK R.P. (1983) Complete nucleotide sequence of pT181, a tetracycline resistance plasmid from *Staphylococcus aureus*. *Plasmid*; **10**: 251-259.

KLECKNER N. (1981) Transposable elements in prokaryotes. *Ann Rev Genet*; **15**: 341-404.

KOHNO T., KOHDA D., HARUKI M., YOKOYAMA S. & MIYAZAWA T. (1990) Nonprotein amino acid furanomycin, unlike isoleucine in chemical structure, is charged to isoleucine tRNA by isoleucyl-tRNA synthetase and incorporated into protein. *J Biol Chem*; **265**: 6931-6935.

KORNBERG A. & BAKER T.A. (1992) "DNA Replication". W.H.Freeman, New York.

KOWALCZYKOWSKI S.C., DIXON D.A., EGGLESTON A.K., LAUDER S.D. & REHRAUER W.M. (1994) Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol Rev*; **58**: 401-465.

KUMAR C.C. & NOVICK R.P. (1985) Plasmid pT181 replication is regulated by two countertranscripts. *Proc Natl Acad Sci USA*; **82**: 638-642.

LABIGNE-ROUSSEL A. & COURVALIN P. (1983) IS*15*, a new insertion sequence widely spread in R plasmids of Gram-negative bacteria. *Mol Gen Genet*; **189**: 102-112.

LABIGNE-ROUSSEL A., BRIAUX-GERBAUD S. & COURVALIN P. (1983) Tn*1525*, a kanamycin R determinant flanked by two direct copies of IS*15*. *Mol Gen Genet*; **189**: 90-101.

LACEY R.W. (1980) Evidence for two mechanisms of plasmid transfer in mixed cultures of *Staphylococcus aureus*. *J Gen Microbiol*; **119**: 423-435.

LEELAPORN A., FIRTH N., BYRNE M.E., ROPER E. & SKURRAY R.A. (1994) Role of insertion sequence IS*257* in the dissemination and expression of high- and low-level trimethroprim resistance in staphylococci. *Antimicrob Agents Chemother* **38**: 2238-2244.

LEMONICK M.D. (1994) The killers all around. New viruses and drug-resistant bacteria are reversing human victories over infectious disease. *Time*; **144**: 50-57.

LITTLEJOHN T.G., DIBERARDINO D., MESSEROTTI L.J., SPIERS S.J. & SKURRAY R.A. (1990) Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. *Gene*; **101**: 59-66.

LYON B.R., MAY J.W. & SKURRAY R.A. (1984) Tn*4001*: a gentamicin and kanamycin resistance transposon in *Staphylococcus aureus*. *Mol Gen Genet*; **193**: 554-556.

LYON B.R. & SKURRAY R.A. (1987) Antimicrobial resistance of *Staphylococcus aureus*: Genetic basis. *Microbiol Rev*; **51**: 88-134.

MACKIE D.P., VAN HERTUM W.A.J., SCHUMBERG T., KUIJPER E.C., KNAPE P. & MASSARO F. (1994) Reduction in *Staphylococcus aureus* wound colonisation using nasal mupirocin and selective decontamination of the digestive tract in extensive burns. *Burns*: **20** Suppl 1: 14-18.

MANCH-CITRON J.N., GENNARO M.L., MAJUMDER S. & NOVICK R.P. (1986) RepC is rate limiting for pT181 plasmid replication. *Plasmid*; **16**: 108-115.

MAPLE P.A.C., HAMILTON-MILLER J.M.T. & BRUMFITT W. (1989) World-wide antibiotic resistance in methicillin-resistant *Staphylococcus aureus*. *Lancet*; **I**: 537-540.

MATTHEWS P.R. & STEWART P.R. (1988) Amplification of a section of chromosomal DNA in methicillin-resistant *Staphylocooccus aureus* following growth in high concentrations of methicillin. *J Gen Microbiol*; **134**: 1455-1464.

MATTHEWS P.R., INGLIS B. & STEWART P.R. (1990) Clustering of resistance genes in the *mec* region of the chromosome of *Staphylococcus aureus*. In *"Molecular Biology of the Staphylococci"*. pp. 69-83. Edited by R.P.Novick. VCH, New York, NY.

M^{c}DEVITT D., WANN E.R. & FOSTER T.J. (1993) Recombination at the coagulase locus in *Staphylococcus aureus*: plasmid integration and amplification. *J Gen Microbiol*; **139**: 695-706.

MELLOWS G. (1984) The mode of action of mupirocin. In *London Royal Society of Medicine, Mupirocin, a Novel Topical Antibiotic. International Congress and Symposium Series No. 80*, pp 23-29. Edited by D.S. Wilkinson & J.D.Price. London.

MEYER J. & IIDA S. (1979) Amplification of chloramphenicol resistance transposons carried by phage P1Cm in *Escherichia coli. Mol Gen Genet*; **176**: 209-219.

MOJUMDAR M. & KHAN S.A. (1988) Characterisation of the tetracycline resistance gene of plasmid pT181 of *Staphylococcus aureus. J Bacteriol*; **170**: 5522-5528.

MOLLET B., IIDA S., SHEPHERD J. & ARBER W. (1983) Nucleotide sequence of IS*26*, a new prokaryotic mobile genetic element. *Nucleic Acids Res*; **11**: 6319-6330.

MOLLET B., IIDA S.& ARBER W. (1985) Gene organisation and target specificity of the prokaryotic mobile genetic element IS*26*. *Mol Gen Genet*; **201**: 198-203.

MORAS D. (1992) Structural and functional relationships between aminoacyl-tRNA synthetases. *Trends Biochem Sci*; **17**: 159-164.

MORTON T.M., EATON D.M., JOHNSTON J.L. & ARCHER G.L. (1993) DNA sequence and units of transcription of the conjugative transfer gene complex (*trs*) of *Staphylococcus aureus* plasmid pGO1. *J Bacteriol*; **175**: 4436-4447.

MUDER R.R. (1993) Mupirocin and MRSA: current status. *Infect Med*; **10**: 21-22.

MURPHY E. & LOFDAHL S. (1984) Transposition of Tn*554* does not generate a target duplication. *Nature*; **307**: 292-294.

MURPHY E. (1989) Transposable elements in Gram-positive bacteria. In "*Mobile DNA*" pp269-288. Edited by D.E.Berg & M.M.Howe. Am Soc Microbiol. Washington D.C..

MURRAY R.W., KOEPSEL R.R. & KHAN S.A. (1989) Synthesis of single-stranded plasmid pT181 DNA *in vitro*. *J Biol Chem*; **264**: 1051-1057.

NAIDOO J. & NOBLE W.C. (1987) Skin as a source of transferable antibiotic resistance in coagulase-negative staphylcocci. *Zentrabl Bakteriol Mikrobiol Hyg Suppl*; **16**: 225-234.

NAKANISHI N., YOSHIDA S., WAKEBE H., INOUE M., YAMAGUCHI T. & MITSUHASHI S. (1991) Mechanisms of clinical resistance to fluoroquinolones in *Staphylococcus aureus. Antimicrob Agents Chemother*; **35**: 2562-2567.

NEEDHAM C., RAHMAN M., DYKE K.G.H. & NOBLE W.C. (1994) An investigation of plasmids from *Staphylococcus aureus* that mediate resistance to mupirocin and tetracycline. *Microbiol*; **140**: 2577-2583.

NEU H.C. (1992) The crisis in antibiotic resistance. *Science*; **257**: 1064-1073.

NOBLE W.C. & WHITE M.I. (1983) Staphylococcal skin infections in man. In "*Staphylococci and staphylococcal infections. Volume I.* ", pp.165-192. Edited by: C.S.F.Easmon & C.Adlam. Academic Press, London.

NOBLE W.C., VIRANI Z. & CREE R.G.A. (1992) Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC12201 to *Staphylococcus aureus. FEMS Letts*; **93**: 195-198.

NOVICK R.P., EDELMAN I., SCHWESINGER M.D., GRUSS A.D., SWANSON E. C. & PATTEE P.A. (1979) Genetic translocation in *Staphylococcus aureus. Proc Natl Acad Sci*; **76**: 400-404.

NOVICK R.P., MURPHY }., GRYCZAN T.J., BARON E. & EDELMAN I. (1979) Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. *Plasmid*; **2**: 109-129.

NOVICK R.P., ADLER G.K., PROJAN S.J., CARLETON S., HIGHLANDER S.K., GRUSS A., KHAN S.A. & IORDANESCU S. (1984a) Control of pT181 replication I. The pT181 copy control function acts by inhibiting the synthesis of a replication protein. *EMBO J*; **3**: 2399-2405.

NOVICK R.P., PROJAN S.J., ROSENBLUM W. & EDELMAN I. (1984b) Staphylococcal plasmid cointegrates are formed by host- and phage- mediated general *rec* systems that act on short regions of homology. *Mol Gen Genet*; **195**: 374-377.

NOVICK R.P., PROJAN S.J, KUMAR C.C., CARLETON S., GRUSS A., HIGHLANDER S.K. & KORNBLUM J. (1985) Replication control for pT181, an indirectly regulated plasmid. In *Plasmids in Bacteria.* pp299-320. Edited by D.R.Helinski, S.N.Cohen, D.B.Clewell, D.A.Jackson and A.Hollaender. Plenum Press, New York, NY.

NOVICK R.P. (1989) Staphylococcal plasmids and their replication. *Ann Rev Microbiol*; **43**: 537-565.

NOVICK R.P., IORDANESCU S., PROJAN S.J., KORNBLUM J. & EDELMAN I. (1989) pT181 plasmid replication is regulated by a countertranscript-driven transcriptional attenuator. *Cell*; **59**: 395-404.

NOVICK R.P. (1990) The staphylococcus as a molecular genetic system. In *"Molecular Biology of the Staphylococci"*. pp. 1-40. Edited by R.P.Novick. VCH, New York, NY.

OHTSUBO E., ZENILMAN M. & OHTSUBO H. (1980) Plasmids containing insertion elements are potential transposons. *Proc Natl Acad Sci USA*; **77**: 750-754.

PAULSEN I.T. & SKURRAY R.A. (1993) Topology, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes - an analysis. *Gene*; **124**: 1-11.

PAULSEN I.T., GILLESPIE M.T., LITTLEJOHN T.G., HANVIVATVONG O., ROWLAND S.-J., DYKE K.G.H. & SKURRAY R.A. (1994) Characterisation of *sin*, a potential recombinase-encoding gene from *Staphylococcus aureus*. *Gene*: **141**: 109-114.

PEREZ-FONTAN M., GARCIA-FALCON M., ROSALES M., RODRIGUEZ-CARMONA A., ADEVA M., RODRIGUEZ-LOZANO I. & MONCALIAN J. (1993) Treatment of *Staphylococcus aureus* nasal carriers in continuous ambulatory peritoneal dialysis with mupirocin: long-term results. *Am J Kidney Dis*; **22**: 708-712.

PETERSON B.C. & ROWND R.H. (1983) Homologous sequences other than insertion elements can serve as recombination sites in plasmid drug resistance gene amplification. *J Bacteriol*; **156**: 177-185.

POLZIN K.M. & SHIMIZU-KADOTA M. (1987) Identification of a new insertion element, similar to Gram-negative IS*26*, on the lactose plasmid of *Streptococcus lactis* ML3. *J Bacteriol*; **169**: 5481-5488.

PRENTKI P., TETER B., CHANDLER M. & GALAS D.J. (1986) Functional promoters created by the insertion of transposable element IS*1*. *J Mol Biol*; **191**: 383-393.

PROJAN S.J. & NOVICK R.P. (1988) Comparative analysis of five related staphylococcal plasmids. *Plasmid*; **19**: 203-221.

PROJAN S.J., MOGHAZEH S. & NOVICK R.P. (1988) Nucleotide sequence of pS194, a streptomycin-resistance plasmid from *Staphylococcus aureus*. *Nucleic Acids Res*; **16**: .

PROJAN S.J. & ARCHER G.L. (1989) Mobilisation of the relaxable *Staphylococcus aureus* plasmid pC221 by the conjugative plasmid pGO1 involves three pC221 loci. *J Bacteriol*; **171**: 1841-1845.

RAHMAN M., NOBLE W.C. & COOKSON B. (1987) Mupirocin-resistant *Staphylococcus aureus*. *Lancet*; **II**: 387.

RAHMAN M., NOBLE W.C. & COOKSON B. (1989) Transmissable mupirocin resistance in *Staphylococcus aureus*. *Epidemiol Infect*; **102**: 261-270.

RAHMAN M., CONNOLLY S., NOBLE W.C., COOKSON B. & PHILLIPS I. (1990) Diversity of staphylococci exhibiting high-level resistance to mupirocin. *J Med Microbiol*; **33**: 97-100.

RAHMAN M., NOBLE W.C. & DYKE K.G.H. (1993) Probes for the study of mupirocin resistance in staphylococci. *J Med Microbiol;* **39**: 446-449.

RASOOLY A. & NOVICK R.P. (1993) Replication-specific inactivation of the pT181 plasmid initiator protein. *Science*; **262**: 1048-1050.

RASOOLY A., PROJAN S.J. & NOVICK R.P. (1994) Plasmids of the pT181 family show replication-specific initiator protein modification. *J Bacteriol*; **176**: 2450-2453.

RASOOLY A., WANG P.-Z. & NOVICK R.P. (1994) Replication-specific conversion of the *Staphylococcus aureus* pT181 initiator protein from an active homodimer to an inactive heterodimer. *EMBO J*; **13**: 5245-5251.

REAGAN D.R., DOEBBELING B.R., PFALLER M.A., SHEETZ C.T., HOUSTON A.K., HOLLIS R.J. & WENZEL R.P. (1991) Elimination of coincident *Staphylococcus aureus* nasal and hand carriage with intranasal application of mupirocin calcium ointment. *Annals Internal Med*; **114**: 101-105.

RIELE H.T., MICHEL B. & EHRLICH S.D. (1986) Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylococcus aureus*. *Proc Natl Acad Sci USA*; **83**: 2541-2545.

ROUCH D.A. & SKURRAY R.A. (1989) IS*257* from *Staphylococcus aureus*: member of an insertion sequence superfamily prevalent among Gram-positive and Gram-negative bacteria. *Gene*; **76**: 195-205.

ROUCH D.A., MESSEROTTI L.J., LOO L.S.L., JACKSON C.A. & SKURRAY R.A. (1989) Trimethoprim resistance transposon Tn*4003* from *Staphylococcus aureus* encodes genes for a dihydrofolate reductase and thymidylate synthetase flanked by three copies of IS*257*. *Mol Microbiol*; **3**: 161-175.

ROUCH D.A., CRAM D.S., DIBERARDINO D., LITTLEJOHN T.G. & SKURRAY R.A. (1990) Efflux-mediated antiseptic resistance gene *qac*A from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. *Mol Microbiol*; **4**: 2051-2062.

ROWLAND S.-J. & DYKE K.G.H. (1989) Characterisation of the staphylococcal β-lactamase transposon Tn*552*. *EMBO J*; **8**: 2761-2773.

SANGER F., NICKLEN S. & COULSON A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA*; **74**: 5463-5467.

SASATU M., SHIBATA Y., NOGUCHI N. & KONO M. (1992) High-level resistance to ethidium bromide and antiseptics in *Staphylococcus aureus*. *FEMS Microbiol Letts*; **93**: 109-114.

SHAPIRO J.A. (1979) Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. *Proc Natl Acad Sci USA*; **76**: 1933-1937.

SHARMA V.K., JOHNSTON J.L., MORTON T.M. & ARCHER G.L. (1994) Transcriptional regulation by *trs*N of conjugative transfer genes on staphylococcal plasmid pGO1. *J Bacteriol*; **176**: 3445-3454.

SILVER S. & LADDAGA R.A. (1990) Molecular genetics of heavy metal resistance systems of staphylococcal plasmids. In *"Molecular Biology of the Staphylococci"*. pp. 1-40. Edited by R.P.Novick. VCH, New York, NY.

SMITH G.R. (1987) Mechanism and control of homologous recombination in *Escherichia coli. Ann Rev Genet*; **21**: 179-201.

SMITH G.R. (1989) Homologous recombination in *E.coli*: multiple pathways for multiple reasons. *Cell*; **58**: 807-809.

SMITH M.D., SANGHRAJKA M. & LOCK S. (1987) Mupirocin-resistant *Staphylococcus aureus. Lancet*; **II**: 1472-1473.

SOUTHERN E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol*; **98**: 503-517.

STEWART P.R., DUBIN D.T., CHIKRAMANE S.G., INGLIS B., MATTHEWS P.R. & POSTON S.M. (1994) IS*257* and small plasmid insertions in the *mec* region of the chromosome of *Staphylococcus aureus. Plasmid*; **31**: 12-20.

STOUT V.G. & IANDOLO J.J. (1990) Chromosomal gene transfer during conjugation by *Staphylococcus aureus* is mediated by transposon-facilitated mobilisation. *J Bacteriol*; **172**: 6148-6150.

TANAKA K., TAMAKI M. & WATANABE S. (1969) Effect of furanomycin on the synthesis of isoleucyl-tRNA. *Biochim Biophys Acta*; **195**: 244-245.

THOMAS C.D. BALSON D.F. & SHAW W.V. (1990) *In vitro* studies of the initiation of staphylococcal plasmid replication. *J Biol Chem*; **265**: 5519-5530.

THOMAS W.D. & ARCHER G.L. (1989a) Identification and cloning of the conjugative transfer region of *Staphylococcus aureus* plasmid pGO1. *J Bacteriol*; **171**: 684-691.

THOMAS W.D. & ARCHER G.L. (1989b) Mobility of gentamicin resistance genes from staphylococci isolated in the United States: identification of Tn*4031*, a gentamicin resistance transposon from *Staphylococcus epidermidis. Antimicrob Agents Chemother*; **33**: 1335-1341.

TRIEU-CUOT P., LABIGNE-ROUSSEL A. & COURVALIN P. (1983) An IS*15* insertion generates an

eight-base-pair duplication of the target DNA. *Gene*; **24**: 125-129.

TRIEU-CUOT P. & COURVALIN P. (1984) Nucleotide sequence of the transposable elemet IS*15*. *Gene*; **30**: 113-120.

TRIEU-CUOT P. & COURVALIN P. (1985) Transposition behaviour of IS*15* and its progenitor IS*15*Δ: are co-integrates exclusive end products? *Plasmid*; **14**: 80-89.

UDO E.E., PEARMAN J.W. & GRUBB W.B. (1994) Emergence of high-level mupirocin resistance in methicillin-resistant *Staphylococcus aureus* in Western Australia. *J Hosp Infect*; **26**: 157-165.

UTTLEY A.H.C., GEORGE R.C., NAIDOO J., WOODFORD N., JOHNSON A.P., COLLINS C.H., MORRISON D., GILFILLAN A.J., FITCH L.E. & HEPTONSTALL J. (1989) High-level vancomycin-resistant enterococci causing hospital infections. *Epidem Infect*; **103**: 173-181.

WHEAT L.J., KOHLER R.B & WHITE A. (1983) Diagnosis and management of deep seated infection. In "*Staphylococci and staphylococcal infections. Volume I.* ", pp.121-148. Edited by: C.S.F.Easmon & C.Adlam. Academic Press, London.

WHITE A.R., BEALE A.S., BOON R.J., GRIFFIN K.E., MASTERS P.J. & SUTHERLAND R. (1984) Antibacterial activity of mupirocin. In *Bactroban (mupirocin) Proceedings of an International Symposium (Current Clinical Practice Series 16)*, pp19-36. Edited by R.L. Dobson, J.J. Leyden, W.C. Noble & J.D.Price. Amsterdam: Excerpta Medica.

WOOD M.S., BYRNE A. & LESSIE T.G. (1991) IS*406* and IS*407*, two gene-activating insertion sequences from *Pseudomonas cepacia. Gene*; **105**: 101-105.

WILSON C.R. & MORGAN A.E. (1985) Chromosomal-DNA amplification in *Bacillus subtilis. J Bacteriol*; **163**: 445-453.

WUITE J., DAVIES B.I., GO M., LAMBERS J., JACKSON D. & MELLOWS G. (1983) Pseudomonic acid: a new topical antimicrobial agent. *Lancet*; **II**: 394.

WYMAN L., GOERING R.V. & NOVICK R.P. (1974) Genetic control of chromosomal and plasmid recombination in *Staphylococcus aureus. Genetics*; **76**: 681-702.

YANAGISAWA T., LEE J.T., WU H.C. & KAWAKWMI M. (1994) Relationship of protein structure of isoleucyl-tRNA synthetase with pseudomonic acid resistance of *Escherichia coli. J Biol Chem*; **269**: 24304-24309.

YANISCH-PERRON C., VIEIRA J. & MESSING J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*; **33**: 103-

YOUNG M. (1984) Gene amplification in *Bacillus subtilis. J Gen Microbiol;* **130**: 1613-1621.

'My child, there is something else to watch out for:

there is no end to the writing of books

and too much study will wear you out."

Ecclesiastes: 12 v12.