An investigation of plasmids from *Staphylococcus aureus* that mediate resistance to mupirocin and tetracycline

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Plasmids conferring mupirocin resistance were prepared from isolates of *Staphylococcus aureus* obtained from four patients in the same ward. The plasmids are related and in all of them the gene conferring mupirocin resistance (*mupA*) is flanked by copies of IS257 in direct repeat. In two plasmids *mupA* and IS257 have been duplicated and in one of these plasmids (pJ3358) a small pT181-like plasmid conferring tetracycline resistance is present flanked by copies of IS257. Filter mating with a strain containing pJ3358 as donor and selection on tetracycline sometimes resulted in transfer of the pT181-like plasmid containing a copy of IS257. Analysis showed that the pT181-like plasmid with the insertion of IS257 is present in high copy number and that the IS257 element is inserted in the copy number control region of the plasmid.

Keywords: *Staphylococcus aureus*, plasmids, mupirocin resistance, tetracycline resistance, IS257

INTRODUCTION

Staphylococcus aureus is an important pathogen that is frequently found to be resistant to a large number of antibiotics. There is therefore a continuing search for new anti-staphylococcal antibiotics and mupirocin (Bactroban ointment from SmithKline Beecham) (Fuller *et al.*, 1971) is such a new antibiotic that has been available for topical use since 1985 (White *et al.*, 1985). Mupirocin has a novel action in that it inhibits the binding of isoleucine to isoleucyl tRNA synthetase, so preventing protein synthesis (Hughes & Mellows, 1980; Mellows, 1984). Strains of *S. aureus* with a reduced sensitivity to a high concentration of mupirocin were reported in 1987 (Rahman *et al.*, 1987; Kavi *et al.*, 1987; Smith *et al.*, 1987; Cookson *et al.*, 1990; Baird & Coia, 1987) and resistance has been found to be present in bacteria isolated as early as 1965 (Rahman *et al.*, 1990). Despite widespread use of mupirocin, only isolated instances of resistance have been detected and the clinical significance of resistance has not been established. The mupirocin resistance (Mu^R) is due to the acquisition of an isoleucyl tRNA synthetase, additional to and distinct from the chromosome-encoded enzyme, which is much less affected by mupirocin and whose gene, *mupA*, is plasmid-borne (Noble *et al.*, 1988; Dyke *et al.*, 1991; Gilbert *et al.*, 1993; Hodgson *et al.*,

1994). These Mu^R plasmids are variable in size and many can be transferred by filter mating (Rahman *et al.*, 1993).

A total of 33 apparently epidemiologically independent strains of *S. aureus* and 15 strains of coagulase-negative staphylococci have yielded 41 Mu^R plasmids that differ on the basis of restriction enzyme digests (Rahman *et al.*, 1993). All these plasmids hybridized with probes made from the resistance gene and, in 39 of the 41, this hybridization was to a 4.05 kb *Eco*R1 fragment of the plasmid (Dyke *et al.*, 1991). Some 27 of the 48 strains carried a Mu^R plasmid which was easily transferred to other staphylococci by filter mating (Rahman *et al.*, 1993). Transconjugant colonies were checked for transfer of resistances other than Mu^R. In only one instance did the plasmid-bearing Mu^R genes mediate any other antibiotic resistance. The ability to conjugate is specified by the plasmid since transferred plasmids have been shown to undergo a second round of transfer.

Tetracycline resistance (Tc^R) is specified by a gene frequently found on small plasmids, for example pT181 (Khan & Novick, 1983). Resistance is due to the acquisition of an efficient efflux system for tetracycline (Guay *et al.*, 1993). However, it has been shown that a pT181-like plasmid can integrate into a large plasmid where it is flanked by copies of the staphylococcal

Table 1. Strains used in this study

Strain	Antibiotic resistance*	Plasmids present (kb)†	Designation of plasmid in Mu ^R transconjugant
NCTC 8325 derivative	Rf Fd	None	—
NCTC 8325 (pT181)	Tc	4.4	—
B111	Rf Fd	None	—
J3347	Mu Pc Tc	34.2, 33, (5.2), 4.4, 1.8	pJ3358
J3332	Mu Pc	33, 29.0, 17, 6.3, + +	pJ3357
J3352	Mu Pc Tc Em	33, 23.6, 6.3, 3.5, 3.1	pJ3355
J3350	Mu Pc Tc	33, 23.6, 6.3, 4.4, + +	pJ3356

* Abbreviations: Rf, rifampicin; Fd, fusidic acid; Tc, tetracycline; Pc, penicillin; Em, erythromycin; Mu, mupirocin.

† Parentheses around the plasmid size indicates detection only by hybridization. + +, small plasmids present.

insertion element IS257 (Rouch & Skurray, 1989; Gillespie *et al.*, 1986).

This paper reports the results of detailed studies on the Mu^R plasmids found in isolates from four patients in the same hospital ward. One of these plasmids transferred both Mu^R and Tc^R.

METHODS

Bacterial strains and media. The characteristics of all strains used in this study (including the four Mu^R strains isolated from the same ward) are shown in Table 1. Strains used routinely were maintained on CY-agar or Oxoid Blood Agar Base (Unipath) for *S. aureus* and 2 × TY (Sambrook *et al.*, 1989) for *Escherichia coli*. Long-term storage was at -70 °C in broth containing 30% (v/v) glycerol. Antibiotograms were obtained as described previously (Rahman *et al.*, 1993). Minimal inhibitory concentrations (MIC) of tetracycline were determined by incorporating tetracycline in Oxoid Blood Agar Base. Curing of plasmids was achieved by growth at 42 °C or in the presence of ethidium bromide (4 µg ml⁻¹).

Transfer of plasmids from wild-type to reference strains and subsequent transfer to other strains were performed by filter mating (Naidoo & Noble, 1987).

Plasmid DNA preparation. The alkaline lysis method (Sambrook *et al.*, 1989) was used for both small- and full-scale caesium chloride gradient preparation of plasmid DNA from *S. aureus* except that lysis was for 30 min at 37 °C with lysostaphin (50 µg ml⁻¹) and Stratagene Resin was used for protein precipitation.

DNA manipulations. Restriction enzymes were obtained from Gibco BRL, and used according to the manufacturer's instructions. DNA fragments were separated by electrophoresis in 0.8% or 2.0% (w/v) agarose run in Tris/borate buffer, pH 8.0, with appropriate size markers. DNA probes were prepared by purifying plasmid DNA using Qiagen tips (Hybaid) according to the manufacturer's instructions. A *HincII* fragment of 871 bp from within the *tet* gene of pT181 (Khan & Novick, 1983) was extracted from a low melting point agarose gel (Biorad) and purified using the GeneClean Kit (Stratagene). Plasmids carrying fragments cloned from within the transposase genes of IS256

(pIP1551, Dyke *et al.*, 1992) and IS257 (pIP1644, V. Loncle, personal communication) were digested with *EcoRI* and *HindIII*, and fragments of 491 and 705 bp, respectively, were purified as above. A pOX7 (identical to pOX300 in Dyke *et al.*, 1991) clone of the 4.05 kb *EcoRI* fragment of pJ3356 was cut with *EcoRI* and the whole plasmid digest used as a probe (pOX7 has been shown not to hybridize to p3356 or p3356D, data not shown). Probes were labelled using the Enhanced Chemiluminescence (ECL) direct nucleic acid labelling (Amersham) or with the FLASH random priming kit (Stratagene) and purified with NucTrap push columns (Stratagene).

Southern blotting to nylon membranes was as described in Sambrook *et al.* (1989) and detection was carried out by the ECL or FLASH method according to the manufacturers' instructions.

DNA sequencing was performed by the method of Sanger *et al.* (1977) using the Sequenase kit (USB) according to manufacturer's instructions. The oligonucleotide 5' CAGTATGTGC-GTCCAACC 3' was used as a sequencing primer (provided by SmithKline Beecham).

RESULTS

Mu^R plasmids which can undergo duplication of a segment encoding *mupA*

The antibiotic resistance patterns and the plasmid profiles of four Mu^R strains of *S. aureus* isolated from four patients in the same ward are shown in Table 1. In the transfer experiments, *S. aureus* 8325 or B111 were used as recipients; when selection was for Mu^R, a single plasmid transferred in each case. The frequencies of transfer of Mu^R from J3347, J3352 and J3350 were about 1 × 10⁻⁵, whereas that for J3332 was 1 × 10⁻⁷. The plasmid from J3347 (pJ3358) was 34.2 kb and mediated resistance to Tc and Mu, whilst that from J3332 (pJ3357) was 29 kb and those from J3352 or J3350 (pJ3355) were 23.6 kb and these mediated only Mu^R.

Plasmids pJ3358, pJ3355, pJ3357 and pJ3356 and a derivative of this last plasmid, pJ3356D, were mapped by

Fig. 1. Restriction enzyme maps of plasmids pJ3355, pJ3356, pJ3356D, pJ3357 and pJ3358. The black boxes represent the *mupA* gene, the hatched box the *tet* gene of the integrated pT181 plasmid. The position and orientation of the IS257 elements are shown. N, *Nco*I; E, *Eco*RI; Hp, *Hpa*II; B, *Bgl*II; H, *Hind*III (sites within pT181 not shown).

restriction enzyme analysis and shown to be related (Fig. 1). Strain J3350 at first was shown to contain a plasmid, pJ3356, that was indistinguishable from pJ3355 by restriction enzyme analysis (Fig. 2a). However, the transconjugant 8325(pJ3356) was maintained at 4 °C on CY-agar containing 5 µg mupirocin ml⁻¹, with monthly subcultures to fresh plates (each subculture entailed a 24 h incubation at 37 °C). After 5 months of this practice, the plasmid contained in the strain on the plate was found to have altered; the derivative plasmid was shown to possess *Eco*RI, *Hind*III, *Bgl*II and *Nco*I restriction enzyme fragments indistinguishable from those of pJ3357. The derivative plasmid pJ3356D had an extra piece of DNA compared to pJ3356 which appeared as a double intensity 4.05 kb *Eco*RI band and a 1.3 kb *Eco*RI band (Fig. 2a, lane 6). Plasmid pJ3358 was very similar to pJ3356D but had additional DNA that increased the size of the second largest (6.5 kb) *Eco*RI fragment to 11.7 kb (Fig. 1; Fig. 2, lane 8).

Restriction enzyme digests of all five plasmids were hybridized after Southern blotting to various probes. None of the plasmids hybridized to a probe consisting of the internal part of IS256. All five hybridized to a probe comprising the internal part of the transposase of IS257 (V. Loncle, personal communication), as well as to probes comprising the 4.05 kb *Eco*RI fragment containing *mupA* and to the 1.6 kb *Nco*I or the 751 bp *Xho*I-*Bgl*II fragments derived from within the gene conferring Mu^R (Rahman *et al.*, 1993). Only plasmid pJ3358 hybridized to the probe from within the Tc^R gene of pT181. Fig. 2 shows an example of hybridization with IS257 and demonstrates

that pJ3357 and pJ3358 both possess an additional 1.3 kb *Eco*RI fragment, as compared to pJ3355 and pJ3356, and that this fragment contains IS257 sequences. Plasmid pJ3356D also contains this 1.3 kb *Eco*RI fragment (data not shown). This additional IS257-containing sequence has been acquired by the duplication of *mupA*-IS257 (Fig. 1). This is also suggested by the replacement of the approximately 12 kb *Hpa*II band of pJ3356 with a band of approximately 17 kb in pJ3356D (Fig. 3, lanes 4 and 5), and also by the acquisition of an extra 3.8 kb *Nco*I fragment (Fig. 3, lanes 3 and 6). The 12 kb, 17 kb and 3.8 kb all hybridized to the 4.05 kb *Eco*RI *mupA* clone derived from pJ3356. The faint *Hpa*II band of 12 kb in the DNA prepared from pJ3356D (Fig. 3, lane 5) is thought to be a deleted form where pJ3356D has reverted to a pJ3356-type plasmid in a small proportion of the population.

Selection for higher resistance gives further amplification which is unstable

As strain 8325 containing the plasmid pJ3356 with one copy of *mupA* had altered after long-term exposure to low concentrations of mupirocin to give the derivative pJ3356D with two copies of *mupA*, it was thought that the presence of the mupirocin may have selected for the duplication. It was further postulated that exposure of the strain 8325(pJ3356D) to higher concentrations of mupirocin may select plasmids with more copies of *mup* in tandem. Hence, 8325(pJ3356D) was inoculated onto a plate containing a concentration gradient of mupirocin (0–10 mg ml⁻¹) and colonies which grew at the highest

Fig. 2. (a) Digested plasmid DNA. Lanes: 1 and 5, pJ3356; 2 and 6, pJ3357; 3 and 7, pJ3355; 4 and 8, pJ3358; 9, λ DNA digested with *Eco*T141; 10, 1 kb ladder. DNA in lanes 1–4 was digested with *Hind*III and in lanes 5–8 with *Eco*RI. (b) Autoradiograph of the gel in (a) probed with pIP1644, which contains a fragment from within the IS257 transposase gene (see Methods).

concentrations were analysed for their plasmid content. Two types of plasmid profile were found: one which had an *Eco*RI restriction enzyme map identical to pJ3356D and the other which was smaller than pJ3356D and that now had a restriction map indistinguishable from that of pJ3356 with a single copy of *mupA*. It should be noted that although the plasmids of the pJ3356-type gave *Hpa*II fragments of 2.6, 8.6 and 12.4 kb (see Fig. 3, lane 6), the pJ3356D-type plasmid consistently gave very faint bands of approximately 23 and 28 kb in addition to the more intense 2.6, 8.6 and 17.8 kb fragments. This suggests that a small proportion of plasmids with three or four *mupA* genes in tandem occur in the pJ3356D population. Since

this restriction enzyme profile is reproducible it is thought that the larger forms may be unstable.

Mu^R can be lost to give a variety of plasmids conferring Tc^R only

Filter mating studies with strain J3347 revealed that when selection was for transfer of Mu^R, all of the 871 transconjugants tested possessed both Mu^R and Tc^R. When selection was for transfer of Tc^R, however, 1382 of 1391 transconjugants were both Mu^R and Tc^R but nine were Tc^R only. All seven Mu^RTc^R transconjugants tested contained a single 34.2 kb plasmid, whereas the nine

Fig. 3. (a) Digested plasmid DNA. Lanes: 1, 1 kb ladder; 2, empty; 3 and 4, pJ3356; 5 and 6, pJ3356D. DNA in lanes 3 and 6 was digested with *Nco*I, and in lanes 4 and 5 with *Hpa*II. (b) Autoradiograph of the gel in (a) probed with pOX7 containing the 4.05 kb *Eco*RI fragment of pJ3356.

Mu^STc^R transconjugants showed a variety of plasmid profiles. Five showed only a single plasmid of about 4.4 kb, two had plasmids of 4.4 kb together with plasmids of about 20 kb (not mapped) and one had a 5.2 kb plasmid together with a plasmid of about 20 kb (not mapped).

To cure plasmids from strain J3347, cultures were grown in the presence of ethidium bromide and after plating colonies were obtained from which either Mu^R or Tc^R or both had been lost. Loss of Tc^R correlated with the loss of the 4.4 kb plasmid together with a reduction in size of the 34.2 kb plasmid to 29 kb. This latter plasmid had a restriction enzyme map indistinguishable from that of pJ3356D. Loss of Mu^R was sometimes accompanied by the appearance of a 5.2 kb plasmid which, from the intensity of its band, was present in high copy number. Also the strain carrying it had an MIC for tetracycline of 256 µg ml⁻¹ compared to 128 µg ml⁻¹ of both the original J3347 strain and the transconjugants carrying the 4.4 kb plasmid.

Further investigation of the plasmids by Southern blotting of plasmid DNA with the *tet* gene probe revealed that the gene was present on the 34.2 kb, 5.2 kb and 4.4 kb plasmids, but not on the 29 kb or 23.6 kb plasmids (data not shown). The IS257 probe revealed the presence of IS257 on the 5.2 kb, but not on the 4.4 kb plasmid. Restriction enzyme analysis of the 5.2 kb plasmid that arose during selection of Tc^R transfer in filter mating experiments showed that it is related to pT181 but with

Fig. 4. Copy number control region of pT181 showing point of insertion of IS257. The numbering is in base pairs starting at the *Pvu*I site in pT181. RNA I and RNA II are antisense RNAs.

IS257 inserted into the 1.6 kb *Taq*I fragment and the 0.4 kb *Mbo*I fragment (Fig. 4). The precise site of this insert has been determined by sequencing the relevant part of the 5.2 kb plasmid (Fig. 5).

DISCUSSION

The plasmids carrying *mupA* in the four strains of *S. aureus* isolated within a single ward are clearly related. The plasmids of 23.6 kb found in J3350 and J3352 each carry a single copy of the *mupA* gene flanked by two copies of IS257 in direct repeat. The Mu^R plasmids of 29 kb derived from J3332 or J3350 contain an additional copy of the *mupA* gene and IS257. However, the plasmids seem to be

unstable and can readily lose a 5.4 kb fragment which is the size of the duplication. The mechanism of this deletion is most likely homologous recombination between the two copies of IS257 resulting in loss of the sequence between them. This process is being investigated further. It is not clear that the presence on the plasmid of two copies of *mupA* increases the resistance of the strain, because even with only one copy the strain is resistant to the highest concentration of mupirocin readily obtainable in agar. The data indicate that it may be possible to convert the plasmid containing a single *mupA* gene to one carrying more than two copies of the *mup* gene each flanked by a copy of IS257 which again may be the result of homologous recombination between copies of IS257 on the two recombining plasmids.

The 34 kb plasmid contains a copy of a pT181-like plasmid flanked by copies of IS257. It has been shown that a deletion of this latter plasmid can occur to give rise to a plasmid of about 20 kb which now no longer carries Tc^R. Gillespie *et al.* (1986) reported a pT181-like plasmid integrated into the chromosome of methicillin-resistant *S. aureus* flanked by direct repeats of IS257. They did not observe deletion of the integrated pT181 but their results, together with those reported here, suggest that it is possible for pT181 to integrate intact into the chromosome or into a plasmid and that this process somehow involves IS257.

In some instances these interactions result in the production of a 5.2 kb plasmid that is a pT181-like plasmid with an integrated copy of IS257. The integrated IS257 is flanked by 8 bp direct repeats which suggests that it arrived at this site as the result of a transposition event. Presumably the insertion of the IS257 preceded the probable integration of the 5.2 kb plasmid into a pJ3357-like plasmid. Hybridization of the *tet* probe to the DNA of intact plasmid profiles revealed that in *S. aureus* J3347 hybridization occurred to a 5.2 kb plasmid not visible under UV/ethidium bromide detection suggesting that the plasmids are labile under non-selective conditions. Thus, this is another example of the involvement of IS257 in the evolution of staphylococcal plasmids.

The site in pT181 into which IS257 transposed is different from that found by Gillespie *et al.* (1986). In the case of the 5.2 kb plasmid described here, the site is between the start of the translation of the RepC messenger RNA and the promoter for RNAs I and II that are believed to be involved in copy number control of pT181. It is therefore of interest to note that the copy number of plasmid DNA is very much higher for the 5.2 kb plasmid than for pT181 itself in the same host as judged by the intensity of staining. This is supported by the higher MIC of tetracycline in the former. In its location in the 5.2 kb plasmid, the IS257 would be expected to interrupt synthesis of the *repC* mRNA since it is between the promoter and the translational start site (Fig. 4). If this assumption is correct, then it is assumed that the IS257 itself provides promoter sequences at least in part to allow the synthesis of RepC. Whatever the promoter sequences used, it is of note that the IS257 has integrated into the stem-loop structure predicted to be involved in the

Fig. 5. Sequence of the copy number control region of pT181 showing the point of insertion of IS257 into pT181. The 8 bp direct repeat flanking IS257 is shown in bold.

attenuation of the *repC* transcript (Projan *et al.*, 1990). This may go some way to explaining the high copy number of the 5.2 kb plasmid as compared with the 4.4 kb plasmid, since the insertion into the proposed stem will render the attenuation structure much weaker and therefore the level of attenuation would be reduced in the larger plasmid. In Fig. 5 the stem-loop is shown as that formed by a single strand of DNA since it is not clear whether this structure will be present as RNA when IS257 is present.

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