ANTIMICROBIAL RESISTANCE

Co-transfer of plasmids in association with conjugative transfer of mupirocin or mupirocin and penicillin resistance in methicillin-resistant *Staphylococcus aureus*

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Two distinct strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from patients in a dermatology ward were also resistant to mupirocin. The mupirocin resistance plasmids from both strains were indistinguishable by *EcoRI* and *HindIII* restriction digest analysis, except for the presence of genes apparently mediating penicillinase production in some transconjugants. Conjugative transfer of the plasmid mediating mupirocin resistance from one of these strains to a recipient *S. aureus* was accompanied in some cases by co-transfer of plasmids mediating resistance to tetracycline or erythromycin; in some instances a plasmid which possessed no apparent resistance markers was also transferred. The second strain demonstrated conjugative transfer of penicillin and mupirocin resistance as well as transfer of a plasmid mediating gentamicin resistance, but transfer of erythromycin resistance was not apparently plasmid-mediated.

Introduction

In the late 1970s and early 1980s, studies on the transfer of plasmids mediating gentamicin resistance between strains and between species of staphylococci firmly established conjugative transfer as a mechanism whereby resistance genes borne on plasmids could be disseminated in this genus [1–4]. In some instances, conjugative transfer was also found to encompass the co-transfer of other, smaller, plasmids which transferred at extremely low or undetectable frequencies in the absence of the conjugative plasmid [4, 5]. Conjugative transfer of plasmids mediating mupirocin resistance has followed the same course [6–12] except that co-transfer was not reported until early 1997 [13].

This report describes mupirocin resistance plasmids in isolates from three patients in a single hospital ward.

Materials and methods

Five isolates of mupirocin- and methicillin-resistant *Staphylococcus aureus* were received from routine clinical sources in an on-going study of mupirocin resistance in staphylococci. All were re-tested for resistance to the following antibiotics by disk diffusion tests on Oxoid blood agar base medium (BAB; Oxoid) or on Direct Sensitivity Test Agar (Difco) containing lysed horse blood at 37°C except for methicillin which was tested at 30°C: amoxycillin 25 µg, chloramphenicol 10 µg, ciprofloxacin 1 µg, clindamycin 2 µg, erythromycin (Em) 15 µg, fusidic acid 10 µg, gentamicin (Gm) 10 µg, methicillin 5 µg, minocycline (Mn) 30 µg, mupirocin (Mu) 200 µg, neomycin 30 µg, penicillin (Pc) 10 units, streptomycin 10 µg, tetracycline (Tc) 10 µg, trimethoprim 5 µg and vancomycin 30 µg. Recipient strains of *S. aureus* were B111 and NCTC8325 made resistant to fusidic acid and rifampicin by serial culture on increasing amounts of these antibiotics; these strains have been shown previously to act as good recipients [1, 2].

Conjugative transfer was performed by a variation of filter mating, in which recipient strains grown overnight on BAB containing rifampicin 80 µg/ml and donor strains grown overnight on BAB containing mupirocin

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80 µg/ml were suspended in broth, mixed and centrifuged to form a pellet. The supernate was removed and the pellet was resuspended in *c.* 0.25 ml of sterile distilled water and inoculated as a dense spot on BAB. After overnight incubation, the growth was removed and resuspended in *c.* 0.5 ml of sterile distilled water; this suspension and a 1 in 10 dilution were then inoculated on to BAB containing both rifampicin 80 µg/ml and mupirocin 100 µg/ml. After incubation, presumed transconjugant colonies were subcultured to purity and tested for resistance to antibiotics as described above.

Plasmid profiles were established by a method modified from that of Naidoo [5]. Briefly, cultures from BAB plates containing mupirocin 80 µg/ml were suspended in 2.5 M NaCl containing lysostaphin (AMBI, Wilts) 50 µg/ml and incubated at 37°C for 80 min; 2 volumes of Brij 58 solution containing (g/L) Brij 58 10 g, deoxycholic acid 4 g, EDTA 20 g, pH8–9, were added to give a viscous solution. Cell debris was pelleted by centrifugation and the supernate was removed; RNAase A (Sigma) 50 µg/ml was then added to the supernate and the mixture was incubated at 37°C for 30 min. Proteinase K (Boehringer Mannheim, Sussex) was added to a final concentration of 100 µg/ml and the mixture was incubated at 37°C for 45 min. One volume of cold absolute ethanol was added and the mixture was stored overnight at –20°C. Precipitated DNA was centrifuged, washed in cold ethanol and the tubes were allowed to dry. DNA was then resuspended in 60 µl of TE buffer (10 mM Trizma base, 1 mM EDTA, pH 8) for 1 h at room temperature and the mixture was stored at –20°C until required.

Restriction endonuclease digests were made with *Eco*RI (Pharmacia, Herts) and *Hind*III (BRL, Paisley) according to the manufacturers' instructions. Plasmids from *Escherichia coli* V517 were used as size markers and were extracted by standard methods [14]. Other mol. wt markers were λ phage cut by *Hind*III (Gibco BRL).

Hybridisation was performed as described by Rahman *et al.* [15]. Briefly, *Eco*RI digests were transferred to a nylon membrane. The probe comprising the 4.05-kb *Eco*RI fragment of the mupirocin gene was labelled by the BioNick labelling system (Life Technologies, Scotland). Biotinylated DNA was detected with the

BluGene non-radioactive detection kit (Life Technologies) according to the manufacturer's instructions. Except where stated, all chemicals were the appropriate grade from Sigma.

Whole-cell restriction digests with *Sma*I (Pharmacia) and pulsed-field gel electrophoresis (PFGE) were performed according to the method of Khambaty *et al.* [16]. Briefly, cells grown in Brain Heart Infusion Broth (Oxoid) overnight at 37°C in an orbital incubator were pelleted by centrifugation. After being washed, the pellet was resuspended in TES buffer (10 mM Trizma base, 50 mM EDTA, 1 M NaCl, pH 8.0); the suspension was mixed with an equal volume of low-melting temperature agarose (BioRad, Herts) and the mixture was set in plastic moulds. When set, the agarose plugs were placed in a container with TE buffer containing lysostaphin 50 µg/ml for 4 h at 37°C. Lysostaphin solution was replaced by proteinase K in NDS buffer (10 mM Tris, 0.5 M EDTA, laurylsarcosine 1%, NaOH 2%) and incubated overnight at 50°C. This was followed by two washes with phenylmethylsulphonylfluoride in TE buffer and three washes in TE buffer alone, each of which was allowed to remain in contact with the plugs for 45 min. Restriction with *Sma*I was done according to the manufacturer's instructions. Electrophoresis was in a Pharmacia hexagonal array system with a 5-s pulse time for 18 h at 12°C with agarose 1% gels and TBE (89 mM Tris, 2.5 mM EDTA, 32 mM boric acid) buffer.

Results

The MRSA isolates exhibited two different antibiotic resistance patterns as shown in Table 1. PFGE of *Sma*I digests showed that isolates 4516, 4528, 4529 and 4538 were indistinguishable in pattern but isolate 4515 differed by at least seven major bands and was, therefore, considered to represent a distinct strain (data not shown). Furthermore, this latter isolate (designated strain A) was typical 'aureus' in colony colour whereas the remainder (designated strain B) were white. Plasmids >30 kb were seen in all isolates. Strain A possessed one large plasmid but also possessed plasmids of 6.7 kb, 3.8 kb and 2.7 kb. The four strain B isolates possessed no small plasmids but did contain two or three large plasmids which were difficult to resolve by gel electrophoresis (data not shown).

Table 1. Characteristics of MRSA strains resistant to mupirocin

Patient	Isolate No.	Date isolated	Resistance pattern	Colony colour
A strain A	4515	11 March	Pc, Tc, Em, Me, Mu	Aureus
B strain B	4516	15 March	Pc, Em, Gm, Me, Mu	White
	4529	15 March	Pc, Em, Gm Me Mu	White
C strain B	4538	15 March	Pc, Em, Gm, Me, Mu	White
	4528	17 March	Pc, Em, Gm, Me, Mu	White

All isolates were tested for susceptibility to penicillin (Pc), tetracycline (Tc), erythromycin (Em), gentamicin (Gm), methicillin (Me), mupirocin (Mu) and other antibiotics as described in the text. Only resistances are recorded in the table.

Conjugative transfer occurred from all isolates, with diverse results (Table 2). Plasmid profiles of a selection of the transconjugants indicated that two features could be detected. The first feature was that in some instances genes governing penicillinase production were apparently located on the same plasmid as the gene for mupirocin resistance, as only one large plasmid was transferred into 11 strain B transconjugants, six exhibiting resistance to mupirocin (Mu^R) and five exhibiting resistance to mupirocin and penicillin ($Mu^R Pc^R$). Two large plasmids were seen in the transconjugant exhibiting resistance to gentamicin in addition to mupirocin and penicillin ($Gm^R Mu^R Pc^R$).

The second feature was that, in transconjugants of strain A, co-transfer of plasmids mediating resistance to erythromycin and tetracycline had occurred. Thirteen transconjugants of strain A into BIII were examined for plasmid profile. In addition to the single large plasmid, six possessed the 6.7-kb plasmid and were the only tetracycline-resistant (Tc^R) transconjugants; 12 possessed the 2.7-kb plasmid and were the only erythromycin-resistant (Em^R) transconjugants, and 10 possessed the 3.8-kb plasmid. No function could be assigned to this 3.8-kb plasmid on the basis of antibiotic resistance. This plasmid appeared in all six $Mu^R Em^R Tc^R$ transconjugants and in three of the six $Mu^R Em^R$ transconjugants, but not in the only Mu^R transconjugant tested.

When cut by restriction endonuclease *EcoRI*, the plasmid associated with resistance to mupirocin only (Mu^R plasmid transferred from strain A isolate 4515) and that associated with mupirocin resistance and penicillinase production ($Mu^R Pc^R$ plasmid from strain B isolate 4528) had six fragments in common; the Mu^R plasmid also yielded a fragment of 7.2 kb and the $Mu^R Pc^R$ plasmid one of 13.2 kb (Fig. 1). *HindIII* digests had 14 fragments in common with a fragment of 5.3 kb in Mu^R and fragments of 6.1 kb and 5.9 kb in $Mu^R Pc^R$ plasmids. Double digests with these two enzymes yielded 17 visible fragments in common with a fragment of 1.82 kb in Mu^R and fragments of 6.1 kb and 2.1 kb in $Mu^R Pc^R$ plasmids. Both plasmids hybridised with the *mupA* probe to the 10.6 kb *EcoRI* fragment and to a 4.3 kb *HindIII* fragment; this latter was also the fragment size of the chief hybridisation band in the double digests. Plasmids from another nine strain B transconjugants (five resistant to mupirocin

Fig. 1. *EcoRI* restriction patterns of plasmids associated with resistance to mupirocin and mupirocin + penicillin. Lane 1; λ DNA *HindIII*; 2, Mu^R transconjugant from 4515 3, $Mu^R Pc^R$ transconjugant from 4528.

only and four to penicillin and mupirocin) were cut with *EcoRI* only and gave the same patterns as described above.

Repeated incubation in broth of the transconjugant resistant to gentamicin as well as mupirocin and penicillin gave isolates resistant to gentamicin only (data not shown). The plasmid from an isolate that was only Gm^R gave seven visible bands on *EcoRI*

Table 2. Resistance patterns of transconjugants from MRSA to recipients and number of transconjugants recovered

Isolate code	Number of transconjugants with resistance pattern						
	Mu^R only	$Mu^R Pc^R$	$Mu^R Tc^R$	$Mu^R Em^R$	$Mu^R Tc^R Em^R$	$Mu^R Pc^R Gm^R$	$Mu^R Gm^R$
4515	43	0	2	22	17	0	0
4516	13	1	—	0	—	1	0
4529	9	44	—	0	—	0	0
4538	15	52	—	4	—	0	1
4528	31	12	—	2	—	0	0

—, Not applicable.

restriction, only two of which appeared to be similar to bands in the Mu^R and Mu^RPc^R plasmids, and was, therefore, considered distinct from these latter plasmids.

Discussion

It is apparent that two distinct strains of mupirocin-resistant MRSA were present in the ward within a few days; one (strain A) was typical 'aureus' in colony colour, was resistant to erythromycin and tetracycline by plasmid-borne genes and also carried a plasmid with no apparent resistance function; the other (strain B) was white in colony colour and had no small plasmids. In PFGE of *Sma*I digests, these two strains were also quite distinct. Nevertheless, these strains apparently shared a plasmid (or plasmids) of *c.* 60 kb in which the *MupA* gene was encoded on an *Eco*RI fragment of *c.* 10.6 kb and a *Hind*III fragment of *c.* 4.3 kb with all restriction fragments generated by *Eco*RI, *Hind*III and *Eco*RI + *Hind*III in common except for a *c.* 13.2-kb *Eco*RI fragment presumably encoding a penicillinase production gene. The Mu^R plasmid in strain A might have transferred to strain B or both might have had a common (undetected) origin. None of the 84 transconjugants from strain A exhibited penicillin resistance, in contrast to 75 of 110 transconjugants from strain B, which might suggest that penicillinase production was chromosomally located in strain A. However, it should be noted that the distribution of Mu^R versus Mu^RPc^R differed between the transconjugants from the four strain B isolates (Table 2). Rowland and Dyke [17] have reported a penicillinase transposon (Tn552) of 6.5 kb which might suggest that the penicillinase genes were an addition to the putative 'mupirocin resistance' plasmid of strain A. In contrast, Udo *et al.* [12] have suggested that a mupirocin gene was incorporated into a plasmid 'at the expense of the penicillinase gene' and Morton *et al.* [18] have reported the apparent exchange of gentamicin and other resistance determinants for a *mupA* gene. The presence of the insertion element IS257 has been implicated in the integration of a tetracycline plasmid into one possessing *mupA* and with the doubling of the *mupA* gene [18] and with the integration of *mupA* into a pre-existing plasmid [19], but insertion elements were not sought in this study.

The 6.7-kb plasmid associated with Tc^R in strain A is unusual, as Tc^R plasmids from staphylococci of human origin are more usually *c.* 4.2 kb; however, Tc^R plasmids between *c.* 6 and 10 kb are found in staphylococci from the skin of pigs [20].

No small plasmids were observed in the four strain B isolates or in Em^R transconjugants from these isolates and it is presumed that a transposon such as Tn917 mediated this resistance, but this possibility was not investigated here. Transposon transfer in conjugation experiments has been well documented and transposons

mediating Em^R have been reported upstream of the *mec* gene in MRSA [21, 22].

Co-transfer of small plasmids by conjugative Gm^R plasmids has been well documented [4, 5] but was not encountered amongst the nearly 50 distinct Mu^R plasmids investigated in earlier studies [8, 9]. However, in early 1997 Udo *et al.* [13] reported co-transfer of a 3.5-kb chloramphenicol resistance plasmid by Mu^R plasmid from *S. haemolyticus*. This present report confirms that co-transfer of small plasmids, some of which encode other antibiotic resistances, may occur in strains bearing Mu^R plasmids as well as those with plasmids mediating Gm^R.

References

1. Naidoo J, Noble WC. Transfer of gentamicin resistance between strains of *Staphylococcus aureus* on skin. *J Gen Microbiol* 1978; **107**: 391–393.
2. Naidoo J, Noble WC. Transfer of gentamicin resistance between coagulase-negative and coagulase-positive staphylococci on skin. *J Hyg* 1981; **86**: 183–187.
3. Forbes BA, Schaberg DR. Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. *J Bacteriol* 1983; **153**: 627–634.
4. McDonnell RW, Sweeney HM, Cohen S. Conjugational transfer of gentamicin resistance plasmids intra- and interspecifically in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob Agents Chemother* 1983; **23**: 151–160.
5. Naidoo J. Interspecific co-transfer of antibiotic resistance plasmids in staphylococci in vivo. *J Hyg* 1984; **93**: 59–66.
6. Udo EE, Love H, Grubb WB. Intra- and inter-species mobilisation of non-conjugative plasmids in staphylococci. *J Med Microbiol* 1992; **37**: 180–186.
7. Rahman M, Noble WC, Cookson B. Mupirocin-resistant *Staphylococcus aureus*. *Lancet* 1987; **2**: 387.
8. Rahman M, Noble WC, Cookson B. Transmissible mupirocin resistance in *Staphylococcus aureus*. *Epidemiol Infect* 1989; **102**: 261–270.
9. Rahman M, Connolly S, Noble WC, Cookson B, Phillips I. Diversity of staphylococci exhibiting high-level resistance to mupirocin. *J Med Microbiol* 1990; **33**: 97–100.
10. Moy JA, Caldwell-Brown D, Lin AN, Pappa KA, Carter DM. Mupirocin-resistant *Staphylococcus aureus* after long-term treatment of patients with epidermolysis bullosa. *J Am Acad Dermatol* 1990; **22**: 893–895.
11. Layton MC, Perez M, Heald P, Patterson JE. An outbreak of mupirocin-resistant *Staphylococcus aureus* on a dermatology ward associated with an environmental reservoir. *Infect Control Hosp Epidemiol* 1993; **14**: 369–375.
12. Udo EE, Pearman JW, Grubb WB. Emergence of high-level mupirocin resistance in methicillin-resistant *Staphylococcus aureus* in Western Australia. *J Hosp Infect* 1994; **26**: 157–165.
13. Udo EE, Jacob LE, Mokadas EM. Conjugative transfer of high-level mupirocin resistance from *Staphylococcus haemolyticus* to other staphylococci. *Antimicrob Agents Chemother* 1997; **41**: 693–695.
14. Macrina FL, Kopecko DJ, Jones KR, Ayers DJ, McCowen SM. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* 1978; **1**: 417–420.
15. Rahman M, Noble WC, Dyke KGH. Probes for the study of mupirocin resistance in staphylococci. *J Med Microbiol*; **39**: 446–449.
16. Khambaty FM, Bennett RW, Shah DB. Application of pulsed-field gel electrophoresis to the epidemiological characterization of *Staphylococcus intermedius* implicated in a food-related outbreak. *Epidemiol Infect* 1994; **113**: 75–81.
17. Rowland S-J, Dyke KGH. Characterization of the staphylococcal beta-lactamase transposon Tn552. *EMBO J* 1989; **8**: 2761–2773.

18. Morton TM, Johnstone JL, Patterson J, Archer GL. Characterization of a conjugative staphylococcal mupirocin resistance plasmid. *Antimicrob Agents Chemother* 1995; **39**: 1272–1280.
19. Needham C, Rahman M, Dyke KGH, Noble WC. An investigation of plasmids from *Staphylococcus aureus* that mediate resistance to mupirocin and tetracycline. *Microbiology* 1994; **140**: 2577–2583.
20. Schwarz S, Noble WC. Tetracycline resistance genes in staphylococci from the skin of pigs. *J Appl Bacteriol* 1994; **76**: 320–326.
21. Matthews PR, Inglis B, Stewart RP. Clustering of resistance genes in the *mec* region of the chromosome of *Staphylococcus aureus*. In: Novick RP (ed) *Molecular biology of the staphylococci*. New York, VCH Publishers. 1990: 69–83.
22. Chikramane SG, Matthews PR, Noble WC, Stewart PR, Dublin DT. Tn554 inserts in methicillin-resistant *Staphylococcus aureus* from Australia and England; comparison with an American methicillin-resistant group. *J Gen Microbiol* 1991; **137**: 1303–1311.