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Strategies for molecular characterisation of methicillin- and gentamicin-resistant *Staphylococcus aureus* in a Canadian nosocomial outbreak

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Summary. Sixteen methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, from a single nosocomial outbreak, were tested for molecular and phenotypic relationships. Two of the 16 outbreak strains were gentamicin resistant (Gm^r) and the plasmids that they carried were characterised by reverse field electrophoresis, restriction endonuclease analysis and gene hybridisation. The gentamicin-resistant (Gm^r) strains harboured two plasmids, a Gm^r plasmid of 36·5 kb and a cryptic plasmid of 25·4 kb, whereas the other 14 isolates contained only the cryptic plasmid. Gentamicin resistance was encoded by a 2·5-kb *Hin*dIII fragment of the 32·8-kb plasmid and is similar to the 2·5-kb *Hin*dIII fragment also described for *S. aureus* Gm^r plasmids from Australia and the USA. The Gm^r plasmid was non-conjugal and was cured by ethidium bromide at a frequency of 4%. Two MRSA strains isolated subsequently from the same hospital were also Gm^r and had identical plasmid and restriction endonuclease profiles to the two Gm^r strains studied initially. Two other *S. aureus* isolates from the original carrier detected in this study and from his son were methicillin and gentamicin susceptible and had novel profiles. Since large plasmids show anomalous migration in agarose gels, more definitive analyses than simple plasmid identification should be considered when studying nosocomial outbreaks.

Introduction

During the late 1960s to early 1970s, methicillin-resistant *Staphylococcus aureus* (MRSA) strains were reported in many countries (Borowski *et al.*, 1964; Rountree and Beard, 1968; O'Toole *et al.*, 1970; Parker and Hewitt, 1970; Kayser and Mak, 1972). Nosocomial outbreaks caused by gentamicin-resistant (Gm^r) *S. aureus* were first observed in 1975 (Buckwold *et al.*, 1979). Strains resistant to both gentamicin and methicillin, as well as to a wide range of other antibiotics, were first reported in Australia (Perceval *et al.*, 1976) and England (Shanson *et al.*, 1976). Multiresistant *S. aureus* strains have subsequently resulted in many other nosocomial outbreaks worldwide (Price *et al.*, 1980; King *et al.*, 1982; Wenzel 1982; Varaldo *et al.*, 1984).

Gentamicin resistance in *S. aureus* has been shown to be mediated primarily by plasmids (18–57 kb) which specify two aminoglycoside-modifying enzymes, aminocyclitol-2″-phosphotransferase and aminocyclitol-6′-acetyl-transferase [APH (2″)-AAC (6′)I] (Shannon and Phillips, 1982). These enzymes also specify tobramycin and kanamycin resistance (Lyon and Skurray, 1987). The genes for the enzymes mediating gentamicin, tobramycin and kanamycin resistance were mapped to a 2·5-kb *Hin*dIII fragment on gentamicin-resistance plasmids. This *Hin*dIII fragment was sequenced and found to encode a single bifunctional protein of 59 Kda (Rouch *et al.*, 1987). Both the gentamicin-resistance genes and methicillin-resistance determinants in some *S. aureus* strains have been located on transposons (Lyon *et al.*, 1984; Trees and Iandolo, 1988).

Because plasmid profiles and methicillin resistance in *S. aureus* have not been well characterised or documented in Canada, we investigated 18 MRSA isolates from a Canadian outbreak. Their plasmid content and the restriction endonuclease patterns of their plasmids were determined and compared with *S. aureus* plasmids reported from

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other outbreaks. The nature of gentamicin resistance in these strains was also examined with a specific DNA probe because, apart from a recent study (Kreiswirth *et al.*, 1988), Gm^r plasmids in MRSA have not been investigated in Canadian strains.

Materials and methods

Bacterial strains and growth

Eighteen clinical methicillin-resistant strains of *S. aureus* (Royal Victoria Hospital, Montréal, Québec) were analysed. Sixteen of the strains (SA1–SA16) were isolated in an initial outbreak, and as a follow-up several months later, two further strains (SA17 and SA18) from the same hospital were investigated. We also examined two methicillin-susceptible strains (SA19 and SA20) isolated from the original carrier and his son.

Other strains used in mating and hybridisation studies are listed in table I. Chromosomal antibiotic resistance markers for strain RN450 were selected by spontaneous mutation on Tryptone Soya Agar (TSA) (Oxoid Ltd, Nepean, Ont., Canada) containing rifampicin 1000 mg/L.

S. aureus isolates were routinely subcultured on TSA. Strains for mating studies were grown on Brain Heart Infusion (BHI) agar or in BHI Broth (Difco Laboratories, Detroit, MI, USA). Antibiotics used for mating experiments were purchased from Sigma (St Louis, MO, USA).

Identification and antimicrobial susceptibility testing

Antimicrobial susceptibilities of *S. aureus* strains were determined by the disk diffusion method outlined by Thornsberry (1984). The plates were incubated at 35°C and, after 24 h, the zones of inhibition were measured. Standard disks (General Diagnostics, Warner-Lambert Co., Morris Plains, NJ, USA) for the following antibiotics were used: penicillin, erythromycin, oxacillin, methicillin, clindamycin, amikacin, gentamicin, cephalothin, vancomycin, chloramphenicol, tetracycline, and tobramycin. Strains were classified as resistant according to the recommendations of the National Committee for Clinical Laboratory Standards (1984).

Isolation of plasmid DNA and gel electrophoresis

Rapid lysis and extraction of plasmid DNA from isolates was accomplished by the method of Birnboim and Doly (1979) with the modification that cells were incubated initially for 30 min at 37°C in lysostaphin (Sigma) 0·5 g/L and ribonuclease A (Boehringer Mannheim Canada Ltd, Dorval, Qué., Canada) 10 g/L.

Plasmid DNA for restriction endonuclease analysis was obtained by pooling cells from four TSA plates and by lysing them according to a scaled-up modification of the Birnboim and Doly procedure (Ng *et al.*, 1987). DNA was then purified on caesium chloride density gradients (Maniatis *et al.*, 1982); the plasmid band was isolated and ethidium bromide was extracted with isobutanol as described by Dillon *et al.* (1985).

Screening of plasmid DNA was performed on agarose (BIORAD, Mississauga, Ont., Canada) 0·7% gels in either tris-acetate (TA) (40 mM Tris, pH 7·8, 20 mM sodium acetate, 1·8 mM EDTA) or tris-borate (TBE) (89 mM Tris, pH 8·3, 89 mM boric acid, 25 mM Na_2 EDTA) buffer. Gels were electrophoresed for approximately 3 h at 80 V.

Field inversion electrophoresis (Carle *et al.*, 1986) was performed with a PP1-100 programmable power inverter (MJ Research Inc., Cambridge, MA, USA). Program 2 (5–6 volts/cm) was employed on agarose 0·8% gels in 0·5 × TBE at room temperature for approximately 5 h. Gels

Table I. Bacterial strains used in mating and hybridisation studies

Species	Strain	Phenotype	Plasmids	Source
S. aureus	RN450	nonlysogenic derivative of *S. aureus* NCTC 8325 phage-free recipient	none	Dr R. P. Novick (Novick, 1967)
S. aureus	RN450SR	Sm^r and Rif^r mutant of RN450 recipient	none	This laboratory
S. epidermidis	UM899	$Gm^rEm^rTc^rPc^rCc^r$ Clinical isolate, control donor	PAM899-1 PAM899-2 PAM899-3	Dr D. Schaberg (Forbes and Schaberg, 1983)
S. aureus	879R4RF	Phage-free Rif^r, Fus^r Restriction deficient, modification deficient recipient	none	Dr D. Schaberg (Forbes and Schaberg, 1983)
E. coli	RN6189	Gm^rPc^r	pH13	Dr B. Kreiswirth (Dickgiesser and Kreiswirth, 1986)

Rif, rifampicin; Sm, streptomycin; Gm, gentamicin; Em, erythromycin; Tc, tetracycline; Pc, penicillin; Cc, clindamycin; Fus, fusidic acid.

were stained with ethidium bromide (Sigma) 0·1 mg/L and DNA bands were visualised on a UV transilluminator (Fotodyne, New Berlin, WI, USA).

Restriction endonuclease analysis

Restriction endonuclease digestions of DNA were performed as recommended by the supplier (Boehringer Mannheim Canada Ltd). Restricted DNA was resolved on horizontal agarose 0·7% gels for 18 h at 40 V with TBE buffer.

DNA hybridisation

DNA from agarose gels was transferred to nitrocellulose sheets by the method of Southern (1975). The Southern blot was hybridised with the 2·1-kb *Dde*I fragment of pH 13 (Dickgiesser and Kreiswirth, 1986) which was used as a probe for gentamicin resistance and was extracted from low melting-point agarose (Gibco BRL, Burlington, Ont., Canada) after gel electrophoresis (Ogden and Adams, 1987). The DNA fragment was precipitated in 2 volumes of 95% ethanol at −20°C.

Hybridisation was performed at 68°C for 18–24 h as described by Zoller *et al.* (1985). DNA (gentamicin resistant; Gm^r probe) was labelled by nick translation (Maniatis *et al.*, 1982; Dillon *et al.*, 1985) with [α-32P] dATP (ICN Radiochemicals, Irvine, CA, USA) that had a specific activity of 4500 Ci/mmol. Approximately 1×10^6 cpm was used for each blot.

Mating and curing procedures

Filter matings were performed by the method of Forbes and Schaberg (1983). The ratio of donor: recipient was adjusted to 1:1 or 1:2, and filters (Gelman GA-6, Ann Arbor, MI, USA) were incubated for 24 h. Putative donors SA1 (Pc^r) and SA2 (Gm^r) were each mated with recipients RN450SR and 879R4rf (table I). Transconjugants were selected on BHI agar containing the appropriate antibiotics: rifampicin 25 mg/L, fusidic acid 25 mg/L, gentamicin 10 mg/L, streptomycin 1000 mg/L and penicillin G 10 mg/L (Forbes and Schaberg, 1983).

Curing procedures were performed by inoculating an overnight culture of *S. aureus* SA1 or SA2 (0·2 ml) into 5·0 ml of Tryptone Soya Broth (Oxoid) containing various concentrations of ethidium bromide (50, 100, 150, 200, 300 or 400 mg/L) and incubating them at 37°C, overnight. Growth from the highest concentration of ethidium bromide which produced visible turbidity was plated on TSA and incubated at 37°C for 18–24 h. One hundred colonies were randomly selected and inoculated on to TSA, and TSA containing the appropriate antibiotics (penicillin for SA1, gentamicin for SA2). The plates were then incubated for 24 h at 37°C. The rate of cure was calculated as the percentage of antibiotic-sensitive colonies in the total colonies screened. Gentamicin-sensitive (Gm^s) strains were lysed and their plasmid profiles were compared to those of the Gm^r strains.

Results

Antimicrobial susceptibilities

Antimicrobial susceptibility patterns of the MRSA strains are shown in table II. Strains SA1, SA3–7 and SA9–16 had the same resistance pattern, which differed from SA2 and SA8 only by the additional presence of oxacillin and gentamicin resistance determinants. Resistance to oxacillin was present only in the pattern represented by strain SA1 and resistance to gentamicin only in the pattern represented by strain SA2. The patterns of the two Gm^r strains which had been isolated subsequently (SA17, SA18) were similar to the SA2 pattern except that both were resistant to tobramycin; strain SA17 carried an oxacillin resistance determinant. Strains SA19 and SA20 had unique patterns. As with all of the other strains studied, SA19 and SA20 were resistant to penicillin but, unlike the other strains, SA19 was susceptible to all other antibiotics tested and SA20 was the only strain resistant to tetracycline.

Plasmid analysis with different buffers and electrophoresis conditions

Plasmid profiles of MRSA strains obtained by gel electrophoresis in TA and TBE buffer systems were different. These results are summarised with *S. aureus* strain SA1, representative of a Gm^s MRSA (fig. 1, lanes 1A and 2A), and with strain SA2, representative of a Gm^r MRSA (fig. 1, lanes 1B and 2B). With TBE buffer, only one plasmid band was visualised in each strain (fig. 1, lanes 2A and 2B). Electrophoresis in TA buffer produced two and four bands for Gm^s and Gm^r strains respectively (fig. 1, lanes 1A and 1B). In addition, the plasmid bands ran differently with respect to

Table II. Susceptibility patterns of representative *S. aureus* strains from outbreak

Strain nos.	Resistance
SA1, SA3–7, SA9–16	Pc, Em, Ox, Mec, Cc, Ak
SA2, SA8	Pc, Em, Mec, Cc, Ak, Gm
SA17	Pc, Em, Ox, Mec, Cc, Ak, Gm, Tm
SA18	Pc, Em, Mec, Cc, Ak, Gm, Tm
SA19	Pc
SA20	Pc, Tc

Pc, penicillin; Em, erythromycin; Ox, oxacillin; Mec, methicillin; Cc, clindamycin; Ak, amikacin; Gm, gentamicin; Tm, tobramycin

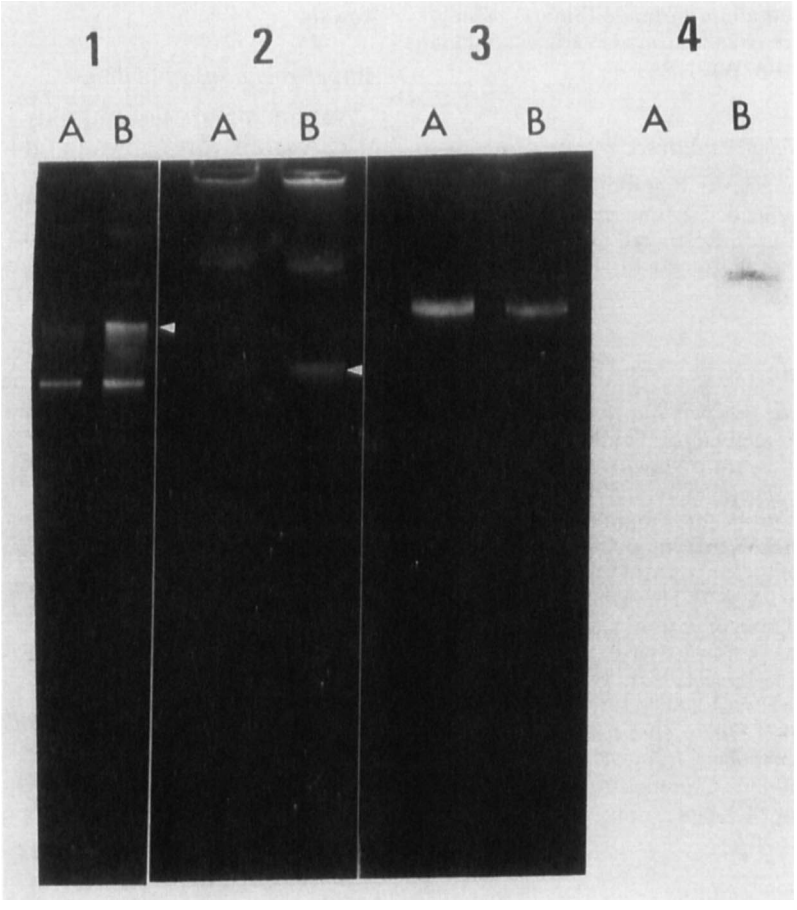

Fig. 1. Agarose gel electrophoresis of Gm^s and Gm^r MRSA strains. Lane A, Gm^s strain SA1; lane B: Gm^r strain SA2. Gel 1, agarose 0·75% gel in TA buffer; gel 2, agarose 0·75% gel in TBE buffer; gel 3, agarose 0·8% gel in TBE buffer with reverse field electrophoresis; gel 4, autoradiograph of gel 3 blotted to nitrocellulose and probed with 32P-labelled 2·1-kb *Dde*I fragment of pH13 coding for gentamicin resistance. White triangles represent chromosomal DNA.

chromosomal DNA (fig. 1). These anomalous results were resolved by reverse field electrophoresis and by restriction endonuclease analysis. Plasmid DNA was prepared in caesium chloride gradients and subsequent reverse field gel electrophoresis revealed one band for the Gm^s strain SA1 (fig. 1, lane 3A) and two for Gm^r strains SA2 (fig. 1, lane 3B). Plasmids resolved by reverse field electrophoresis (fig. 1, lanes 3A and 3B) were blotted on to nitrocellulose and probed with the 2·1-kb *Dde*I fragment of pH 13 which carried gentamicin-resistance determinants. Only the larger plasmid band for Gm^r strain SA2 hybridised with the probe (fig. 1, lane 4B) indicating that it was responsible for gentamicin resistance as well as eliminating the possibility that it (fig. 1, lane 3B) represented the open circular form of the lower band.

Restriction endonuclease analysis of total plasmid DNA with *Hin*dIII and *Pst*I confirmed that the plasmid profiles of Gm^s and Gm^r strains were different (fig. 2) and also allowed definitive sizing of the two plasmids. These results showed that 18 MRSA strains, including the four Gm^r strains, shared a common cryptic plasmid of 25·4 kb. The four Gm^r strains also carried a larger plasmid (32·8 kb). The plasmid size was determined by averaging the sum of the *Hin*dIII fragment sizes of four total plasmid DNA digests (table III). The *Pst*I fragments were not used because the largest fragment could not be accurately sized with our markers (table III). In lanes with both the 25·4- and 32·8-kb plasmids, the small cryptic plasmid could be distinguished visually from the 32·8-kb Gm^r plasmid by the higher intensity of its restriction endonuclease fragments (see fig. 2).

Plasmids from methicillin-susceptible and Gm^s strains SA19 and SA20 had restriction profiles distinct from all other strains examined (fig. 2a). It

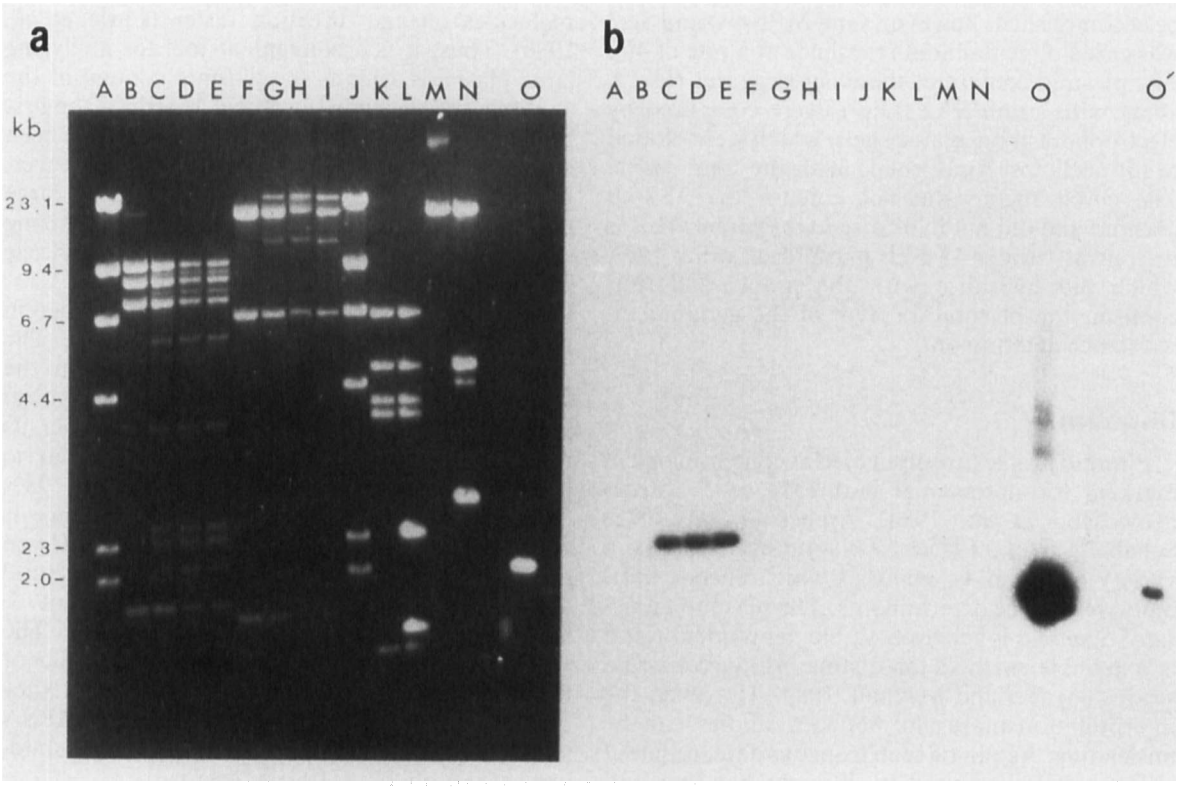

Fig. 2. (**a**) Agarose 0·7% gel electrophoresis of *Hin*dIII (lanes B–E) and *Pst*I (lanes F–I) restriction endonuclease digests of purified plasmids from designated strains. Lanes A and J, λ *Hin*dIII; lanes B and F, strain SA1; lanes C and G, strain SA2; lanes D and H, strain SA17; lanes E and I, strain SA18; lanes K and M, strain SA19; lanes L and N, strain SA20; lane 0, 2·1-kb *Dde*I fragment of pH13. (**b**) Gel **a** blotted to nitrocellulose and probed with nick-translated 2·1-kb *Dde*I fragment of pH13 coding for APH(2″)–AAC(6′)I. A–0—2-h exposure of autoradiograph; 0′—15 min exposure of autoradiograph.

appeared from the restriction endonuclease analysis that they shared a common plasmid of 21·1 kb. SA20 also contained a small unique plasmid of 4·61 kb.

The DNA fragments from restriction endonuclease digests (fig. 2a) were also probed with the 2·1-kb *Dde*I fragment of pH 13. A 2·5-kb *Hin*dIII fragment of the Gm^r plasmids hybridised with the probe (fig. 2b, lanes C, D and E), as did a 10·8-kb *Pst*I fragment (fig. 2B, lanes G, H and I). The Gm^s plasmids showed no homology with the 2·1-kb *Dde*I probe (fig. 2b, lanes B, F, K, L, M and N).

Table III. Fragments generated by restriction endonuclease digestion of *S. aureus* strains SA1 (Gm^s) and SA2 (Gm^r)

Restriction enzyme	Strain	Size of fragments (kb)*
*Hin*dIII	SA1	9·27, 8·2, 7·17, 1·76
	SA2	9·27, **8·93**, 8·2, **7·77**, 7·17, **5·8**, **3·67**, **2·43**, **2·24**, **2·0**, 1·76
*Pst*I	SA1	17·0, 6·66, 1·58
	SA2	**29·5**, 16·7, **11·34**, 6·66, 1·58

*Sizes in bold represent fragments from gentamicin resistance plasmids.

Plasmid transfer and curing

Transfer of gentamicin or penicillin resistance in this study could not be accomplished by the method of Forbes and Schaberg (1983), despite the successful transfer in a control mating between *S. aureus* UM899 and recipient *S. aureus* 879R4RF of gentamicin resistance as reported by Forbes and Schaberg (1983). Altering the ratio of recipient: donor or employing an overnight incubation of the mating mixture in filters did not result in a successful mating.

Curing of Pc^r from MRSA strain SA1 could not

be accomplished; however, Gm^r MRSA strain SA2 was cured of gentamicin resistance at a rate of 4%. The plasmid content of these cured strains (Gm^s), along with strain SA2 (Gm^r), were compared by electrophoresis on agarose gels, which were blotted to nitrocellulose and probed with the Gm^r probe. The cured strains did not contain the 32·8-kb plasmid and did not hybridise to the probe. This is in contrast to the 32·8-kb plasmid of strain SA2, which did hybridise with the probe, and thus confirms the plasmid location of the gentamicin-resistance determinant.

Discussion

Plasmid profiles are often used as epidemiological markers for nosocomial outbreaks of *S. aureus* (Townsend *et al.*, 1984; Archer *et al.*, 1985; Schaberg *et al.*, 1985). *S. aureus* strains carry a variety of plasmids, many of which encode antibiotic resistance determinants. The plasmid analysis of *S. aureus* is generally stable, reproducible and is a reliable method for distinguishing *S. aureus* strains (Archer and Mayhall, 1983). However, the interpretation of results between studies can be misleading. As can be seen from our data, different buffer systems resulted in different banding patterns. With TA buffer more than one molecular form of each plasmid was evident, while with TBE buffer the individual plasmid bands co-migrated and were, therefore, obscured. Several workers have used either tris-borate buffers (Archer and Johnston, 1983; Goering and Ruff, 1983; Lyon *et al.*, 1983; Dunkle and Sippel, 1984) or tris-acetate buffers (Kozarsky *et al.*, 1986). Tris-acetate is a higher ionic strength buffer and seems to result in better separation of large molecules, but determination of the number of different plasmids is difficult with this buffer. Researchers who have employed plasmid patterns to differentiate *S. epidermidis*, have reported similar difficulties (Archer *et al.*, 1984; Parisi *et al.*, 1986); these have included problems with chromosomal DNA obscuring plasmid bands and with the variable conversion of plasmid DNA to open circular, closed circular and linear forms. These difficulties confirm the need for careful analyses when comparing plasmid profiles. The mobility of large molecules (<20 kb) in agarose gels is independent of size (Degennes, 1971). Molecules of this size no longer sieve through the gel pores during electrophoresis but travel along their long axes at the same speed. Reverse or pulsed field gel electrophoresis involves cycles of forward and reverse electrical field pulses which result in size separation because small molecules change direction faster (Carle *et al.*, 1986). Thus, it is a convenient tool for analysing large plasmids such as the plasmid content of the *S. aureus* in this study (fig. 1, gel 3). This is the first time that reverse field electrophoresis has been employed to analyse *S. aureus* plasmids. However, because of the anomalous migration of large plasmids on agarose gels, accurate plasmid sizing should be completed by restriction endonuclease analysis.

Gentamicin resistance in *S. aureus* is encoded in plasmids which vary in size considerably. The 36·5-kb Gm^r plasmid in this study falls within the size ranges previously reported (18–57 kb) (Lyon *et al.*, 1983; Lyon and Skurray, 1987), however its restriction endonuclease profile is not similar to those previously reported (Jaffe *et al.*, 1982; Schaberg *et al.*, 1985). Gentamicin resistance is encoded on a transposon in some instances (Lyon *et al.*, 1984), thereby explaining the structural diversity of plasmids in gentamicin-resistant *S. aureus* strains (Lyon and Skurray, 1987). The similarity of the restriction endonuclease profiles of the Gm^r strains in this study indicates that they originated from a common source. The Gm^s MRSA strain, which carried a common cryptic plasmid, were also probably of similar origin.

The 36·5-kb Gm^r plasmid examined in this study appears to bear no similarity with respect to size or restriction endonuclease profile to those studied extensively in the USA (Jaffe *et al.*, 1982) or Australia (Lyon *et al.*, 1983).

Transfer of the Gm^r plasmid could not be accomplished by a filter mating technique. Either the Gm^r plasmid from strain SA2 lacks the *tra* phenotype or has a mutated *tra* gene. Another explanation could be that the transfer is donor or host strain dependent, a phenomenon which has been reported previously (Goering and Ruff, 1983). American Gm^r *S. aureus* plasmids have been transferred from *S. aureus* to *S. aureus* and from *S. epidermidis* to *S. aureus* (McDonnell *et al.*, 1983). Some Australian Gm^r plasmids (Lyon *et al.*, 1983, 1984) are non-conjugal, whereas others could be routinely transferred by mixed culture matings (Townsend *et al.*, 1984). Conjugal plasmid transfer among MRSA strains seems to be a variable trait as far as Gm^r plasmids are concerned and the Canadian plasmids described in this study were nontransferable.

As transfer was not achieved, identification of our specific Gm^r plasmid and the resistance determinant on the plasmids was accomplished by probing with the APH(2″)–AAC(6′)I sequence which was carried on a 2·1-kb *Dde*I fragment from

pH 13. A 2·5-kb *Hin*dIII fragment of the Gm^r plasmids in our study hybridised with this probe. A similar sized *Hin*dIII fragment was associated with gentamicin resistance in both American (Jaffe *et al.*, 1982) and Australian (Lyon *et al.*, 1983) Gm^r plasmids. This fragment has also been characterised on a 4·7-kb transposon (Lyon *et al.*, 1984) in Australian isolates but not American isolates (Archer and Johnston, 1983), possibly because the inverted repeats flanking the Gm^r determinant were shorter than those in Australian strains (Lyon and Skurray, 1987). The 2·5-kb *Hin*dIII fragment has been characterised on different Gm^r *S. aureus* plasmids around the world, thereby inferring an evolutionary relationship between these plasmids with respect to the acquisition of gentamicin resistance. Although the plasmids carrying the Gm^r determinant varied among MRSA strains, the mechanism of Gm^r in this study is similar to that observed in other parts of the world.

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