Plasmid profiles and restriction enzyme fragmentation patterns of plasmids of methicillin-sensitive and methicillin-resistant isolates of *Staphylococcus aureus* from hospital and the community.

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Summary. The number, frequency distribution and restriction enzyme fragmentation patterns of plasmids harboured by 163 methicillin-sensitive isolates of *Staphylococcus aureus* (MSSA) and 53 methicillin-resistant isolates (MRSA) were compared. Plasmids were demonstrated in less than half of the MSSA isolates; their frequency distribution did not differ from that predicted by a simple model of plasmid distributions. In contrast, all the MRSA isolates harboured plasmids, their distribution suggesting dissemination of a limited number of clones within the hospital. Among 72 MSSA isolates harbouring plasmids, 38 different restriction patterns were identified. There were fewer patterns among MRSA isolates; 11 were observed, and two predominant patterns accounted for 68% of those identified. These restriction patterns correlated with the presence or absence of aminoglycoside resistance. A multicopy plasmid of 2.6 kb was present in both MSSA and MRSA isolates that harboured more than one plasmid; it had the same restriction pattern irrespective of its source. The importance of these results in choosing a method of studying the spread of staphylococci is discussed.

Introduction

Staphylococcus aureus is a major human pathogen that produces a wide spectrum of disease ranging from superficial skin lesions to severe systemic infections (Shanson, 1986). Widespread outbreaks of nosocomial infection caused by methicillin-resistant isolates have renewed interest in this species.

Methicillin-resistant *S. aureus* (MRSA) were first reported in the early 1960s shortly after the introduction of methicillin into clinical practice, and by the late 1960s constituted a small but significant proportion of all *S. aureus* isolates. Their incidence declined in the early 1970s (Jepsen, 1986), but in the last decade there has been a resurgence of MRSA isolates often resistant to many antibiotics. Since the early 1980s significant outbreaks due to such isolates have been reported from Australia, North America, South Africa, Greece, the Middle East and much of Europe, including the United Kingdom (Casewell, 1986). In Glasgow Royal

Infirmary, MRSA at present account for 3–5% of all isolates of *S. aureus* (D. R. Baird, personal communication).

The genetic basis of antibiotic resistance in staphylococci has long been a subject of interest and speculation amongst microbiologists (Lacey, 1972, 1975; Shalita *et al.*, 1980), and new techniques in molecular biology, e.g., restriction enzyme fragmentation patterns, nucleic acid hybridisation and molecular cloning, have recently been applied to study it. Individual plasmids mediating resistance in MRSA isolates have been studied by Townsend *et al.* (1984, 1985a, 1985b), and the plasmids carried by resistant isolates by Dowd *et al.* (1983) and Kayser *et al.* (1986). However, a clearer understanding of the ecology of staphylococcal plasmids in strains causing disease requires first that the relative abundance and distribution of plasmids within defined collections of bacteria is determined (Platt *et al.*, 1984, 1986a).

In this study, the plasmid profiles and plasmid DNA restriction enzyme fragmentation patterns of a collection of MRSA isolates and of methicillin-sensitive isolates representative of those in the

hospital and the community have been examined and compared.

Materials and methods

Bacterial strains

A series of 216 isolates of *S. aureus* was studied, comprising (from Glasgow Royal Infirmary): 53 MRSA isolates; 50 consecutive methicillin-sensitive isolates (MSSA) from blood cultures (BC); 63 MSSA isolates from other routine diagnostic specimens (RD); and 50 MSSA isolates from the noses of patients attending a general practitioner's surgery (GP). All were identified as *S. aureus* on the basis of their colony morphology and positive results in coagulase and latex agglutination (Staphaurex, Wellcome) tests. Multiple isolates from the same patient were excluded. Three isolates of epidemic MRSA (EMRSA) from London, two from St Bartholomew's Hospital and one from St Thomas's Hospital were also examined.

Antibiotic sensitivity testing

Sensitivity to methicillin, streptomycin, kanamycin, gentamicin, vancomycin, trimethoprim, mupirocin, penicillin, erythromycin, clindamycin, fucidin, tetracycline, chloramphenicol and teicoplanin was determined by disk-diffusion tests in which the radii of inhibition zones were compared with critical radii (R_c) previously determined for a fully sensitive strain of *S. aureus* (strain GRI 22686). The sizes of critical radii were calculated from $R_c = \bar{x} - 3\sigma$, where $\bar{x}$ was the mean radius obtained from 12 determinations and σ was the standard deviation. If an antibiotic produced an inhibition zone less than R_c , the isolate was deemed to be resistant to it (Platt *et al.*, 1986a).

Determination of plasmid profiles

About 25% of the growth from overnight cultures on nutrient agar at 37°C was washed in 500 µl of a solution containing 50 mM tris, 50 mM NaCl and 5 mM disodium EDTA, pH 8.0 (TES), and suspended in 400 µl of TES containing 50 mM sucrose to which 50 µl of a solution of lysostaphin (1 mg/ml) and 50 µl of a solution of lysozyme (40 mg/ml) were added. After incubation for 10 min at 37°C, 400 µl of a solution of sodium dodecyl sulphate, 10% w/v (SDS) was added to complete lysis. The lysates were centrifuged for 15 min at 12 000 rpm, and the plasmids in the supernate were separated by electrophoresis in an agarose gel (0.7% w/v) as described by Platt and Sommerville (1981) and Platt *et al.* (1984). The molecular sizes of the plasmids were estimated by comparison with standard plasmids of known size. The size of small plasmids was estimated by comparing their mobility with that of a set of low-mol. wt standard supercoiled plasmids (Supercoiled Ladder, Gibco-BRL, Paisley), as described by Platt and Taggart (1987).

Preparation of purified plasmid DNA and restriction enzyme fragmentation

Cultures incubated overnight in 10 ml of brain heart infusion broth (BHI) were centrifuged at 4000 rpm for 5 min, the cell pellet was washed in TES buffer, resuspended in 200 µl of TES containing 50 mM sucrose, treated with lysozyme and lysostaphin as described above and the cells then lysed with alkaline SDS. Plasmid DNA was extracted, purified and digested with restriction enzymes following the sequence described by Platt *et al.* (1986b); the enzymes used were *Pst*I, *Hae*III and *Alu*I (Gibco-BRL). In addition, the enzymes *Ava*II, *Hind*III, *Sma*I, *Eco*RI, *Bsp*1286 and *Alu*I were employed in attempts to cleave low-mol. wt plasmids present in some of the isolates. The size of restriction fragments was estimated from a standard curve produced by fitting a robust modified hyperbola to the observed mobilities of fragments of known mol. wt of bacteriophage λ digested by the enzyme *Pst*I (Plikaytis *et al.*, 1986).

Statistical analysis

The prevalence of plasmids in the different collections of strains was compared by means of the X²-squared test (Siegel, 1956). Plasmid distributions were compared with values predicted from mathematical models based on the Poisson and modified Poisson curves as previously described by Platt (1987). The relatedness of plasmids was determined by calculation of their coefficients of similarity, (S_p) (Dice, 1945).

Results

Resistance to antibiotics

The percentage of isolates in each group resistant to none, one, two or three or more of the antibiotics tested is shown in table 1. Penicillin resistance accounted for most of the resistance of MSSA isolates whereas, as expected, all the MRSA isolates were resistant to three or more antibiotics and could be further divided into two equal groups on the basis of their sensitivity to aminoglycosides. One of the aminoglycoside-sensitive MRSA isolates was mupirocin resistant (Baird and Coia, 1987).

Plasmid profiles

Plasmids were detected in 42% of the BC isolates, 48% of the GP isolates and 41% of the RD isolates. All the MRSA isolates harboured plasmids. The frequency distributions of plasmids in the BC, GP and RD groups did not differ significantly ($p > 0.5$). When the BC, GP and RD isolates were combined to form a methicillin-sensitive group their plasmid distribution differed significantly

Table I. Distribution of resistance determinants in isolates from different sources

Number of resistance determinants/isolate	Percentage of isolates resistant to the given number of antibiotics			
	BC	GP	RD	MRSA
0	6	32	11	0
1	78	54	53	0
2	10	10	24	0
≥3	6	4	12	100

BC = blood cultures; GP = general practitioners; RD = routine diagnostic; MRSA = methicillin-resistant *S. aureus*.

($p < 0.001$) from that of the MRSA isolates (table II).

Almost all of the plasmids identified were either 14–51 kb or 2.9–5.8 kb in size. In isolates with a single plasmid, it was always of the larger size, but when there was more than one plasmid both sizes were invariably present. Typical plasmid profiles are shown in fig. 1.

Restriction enzyme fragmentation patterns

Among the plasmids from 125 isolates, 49 different restriction patterns were found. Eleven representative patterns resulting from digestion with *Hae*III are shown in fig. 2; in all cases the *Hae*III restriction patterns correlated well with those obtained by digestion with *Pst*I and *Alu*I. Eleven patterns were identified among the plasmids from the MRSA isolates; these fell into two groups, and correlated with the presence or absence of

Fig. 1. Typical *S. aureus* plasmid profiles. Lane 1—Supercoiled ladder standards; lanes 2 and 9—52 kb plasmid standards; lanes 3, 4, 5 and 6—MSSA; lane 7—aminoglycoside-sensitive MRSA; lane 8—aminoglycoside-resistant MRSA.

Fig. 2. Examples of *Hae*III restriction patterns of *S. aureus* together with the sources of strains, the number of plasmids harboured, and number of strains showing identical fingerprints. M(R) and M(S) represent aminoglycoside-resistant and -sensitive isolates respectively.

aminoglycoside resistance. The patterns from the plasmids of 26 of the 28 aminoglycoside-resistant isolates were identical (pattern 1, fig. 2), and the S_D values of the remainder were $>80\%$. The 25 patterns from plasmids from the aminoglycoside-sensitive isolates varied more: 11 were identical (pattern 3, fig. 2), and the S_D values of a further seven were $>80\%$ (pattern 4, fig. 2). The remainder of the aminoglycoside-sensitive MRSA isolates shared a number of fragments with the predominant pattern ($S_D > 40\%$), and the single isolate which carried four plasmids and was also the mupirocin-resistant isolate (pattern 11, fig. 2), shared fragments with both the major patterns from plasmids of MRSA isolates.

The patterns from plasmids from MSSA isolates were much more heterogeneous and, although six patterns accounted for more than 40% of the strains (patterns 5, 6, 7, 8, 9, and 10, fig. 2), 38 different patterns were identified. None of the patterns from MRSA isolates was present amongst those from MSSA isolates although some restriction fragments were common to both; e.g., a fragment of 2.7 kb after digestion with *Hae*III was present in all the digest patterns from the MRSA isolates and in the patterns from more than 70% of the MSSA isolates. Particularly noteworthy were small, multicopy plasmids present in those MRSA and MSSA isolates that harboured two plasmids. These were not cleaved by *Pst*I, *Hae*III, *Ava*II, *Hind*III, *Sma*I,

Table II. Frequency distribution of plasmids in isolates from different sources

Number of plasmids isolate	Number (%) of isolates with the given number of plasmids				
	BC (n=50)	GP (n=50)	RD (n=63)	All MSSA (n=163)	MRSA (n=53)
0	29 (58)	26 (52)	37 (59)	92 (56)	0
1	19 (38)	22 (44)	22 (35)	63 (39)	6 (11)
2	2 (4)	2 (4)	4 (6)	8 (5)	44 (83)
3	0	0	0	0	2 (4)
4	0	0	0	0	1 (2)

EcoRI or *Bsp1286* enzymes as was shown by their comparative mobility in profile and digest gels, and by evidence of open circle and dimer formation. However, digestion with *AluI* enzyme produced five fragments, the total molecular size of which (2.6 kb) agreed well with estimates from profile gels, and confirmed the homogeneity of this small plasmid. The observed differences between patterns 1 and 2, between 3 and 4, between 5 and 6, and between 7 and 8 reflect the presence or absence of this plasmid. Restriction fragmentation patterns that demonstrate some of the above features, including examples of those from plasmids of EMRSA isolates are shown in fig. 3.

Considering together the mol. wts of the fragments obtained by digestion and the plasmid profiles, it appears that the aminoglycoside-resistant MRSA isolates carried a highly conserved plasmid of 18 kb, whereas more than 70% of the aminoglycoside-sensitive MRSA isolates carried a plasmid of 23 kb. The rest of the aminoglycoside-sensitive MRSA isolates harboured a variety of plasmids with sizes in the range 17–26 kb, but which on the basis of restriction patterns were less closely related, either to each other or to the other plasmids in MRSA isolates. The plasmids of MSSA isolates were more heterogeneous; more than 30 different plasmids with sizes in the range 14–51 kb were identified. Those MRSA and MSSA isolates that carried more than one plasmid harboured a small plasmid of 2.6 kb. These plasmids were indistinguishable regardless of their origin. The commonest plasmids from the MSSA isolates, as judged by restriction patterns, were present in both hospital and community isolates.

Discussion

The results of this study show that the plasmids of isolates of MSSA and MRSA differ in their prevalence, number, distribution, and restriction

patterns. Although plasmid-free MRSA have been described by Gelmi *et al.* (1987), all the MRSA isolates examined in this study carried plasmids, in sharp contrast to the MSSA isolates of which more than half were plasmid-free. The frequency distribution of the plasmids in each group (table II) also differed markedly. Among MSSA isolates, the observed frequency distribution of plasmids did

1 2 3 4 5 6 7 8 9 10 11 12

Fig. 3. Typical *HaeIII* restriction patterns of *S. aureus*. Lanes 1–5 and 6–12 are from two different gels; lanes 5 and 6—bacteriophage λ /*PstI* fragments as calibration standards from each gel; lanes 1–3—EMRSA; lanes 7 and 9—aminoglycoside-resistant MRSA; lanes 8 and 10—aminoglycoside-sensitive MRSA; lanes 4, 11 and 12—MSSA. Arrows indicate small multicopy plasmids present in MRSA and MSSA and their larger size with respect to those found in EMRSA.

Table III. Comparison of observed plasmid distributions in MSSA and MRSA with distributions predicted by a modified Poisson model

Number of plasmids/isolate	Number of isolates with the given number of plasmids			
	MSSA		MRSA	
	observed	predicted	observed	predicted
1	62	63	6	25
2	10	8	44	17
3	0	0	2	8
4	0	0	1	3

not differ from that predicted by the modified Poisson model developed to analyse the distribution of plasmids among representative collections of enterobacteria (Platt, 1987). This suggests, firstly, that the substantial proportion of plasmid-free MSSA isolates are poor plasmid recipients and, secondly, that accumulation of plasmids by gram-negative and gram-positive genera may be subject to the same variables. By contrast, the frequency distributions of plasmids from MRSA isolates differed significantly ($p < 0.001$) from that predicted by the model (table III), and were consistent with the hypothesis that the MRSA isolates represent dissemination of a limited number of clones within the hospital.

This view is supported by the limited number of restriction patterns among the plasmids carried by MRSA isolates, in marked contrast to the diversity of patterns among the plasmids from the MSSA isolates. Although this suggests an exogenous source of the MRSA with their subsequent spread consequent upon selection pressure, the findings with respect to small multicopy plasmids can be interpreted as support for the evolution of MRSA *de novo* from indigenous hospital strains. The small number of MSSA strains collected that harboured two plasmids does not preclude the existence of MRSA progenitors amongst MSSA in the hospital. It is interesting that amongst the MSSA isolates the commonest larger plasmids were present in both hospital and community isolates. It is likely that

this reflects interchange of strains, although it may represent exchange of plasmids.

Many of the strains of MRSA isolated from patients in different hospitals in England and which have been described as epidemic MRSA (EMRSA) (Cookson *et al.*, 1986) carry small plasmids similar in size to those of local MRSA as described here. However, their restriction patterns (fig. 3) differ markedly from those of local MRSA. The small plasmids harboured by MRSA and MSSA from this hospital are similar but differ from the plasmids of EMRSA; this suggests evolution *de novo* of local MRSA from local methicillin-sensitive strains. However, MSSA strains harbouring two plasmids are rare; if MRSA strains have evolved from them, the plasmids have played at best an indirect role in such evolution as methicillin resistance is not plasmid-mediated.

The diversity of restriction patterns found amongst strains with apparently similar plasmid profiles, particularly MSSA, suggest that, if such patterns are stable, increased strain discrimination is possible with this technique. Clearly, this has important implications for the value of such analysis in studying the spread of strains in the community.

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