Analysis of an outbreak of variably methicillin-resistant *Staphylococcus aureus* with chromosomal RFLPs and *mec* region probes

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Summary. An outbreak of infections with multi-resistant *Staphylococcus aureus* with unusual methicillin resistance at a Melbourne hospital was investigated by examining restriction fragment length polymorphisms (RFLPs) of total DNA digested with the rare-cutting endonuclease *Sma*I. The polymorphisms were identified by pulsed-field gel electrophoresis (PFGE) and were analysed numerically to give quantitative estimates of genetic distances between isolates. The majority of the isolates were found to belong to one group, with only minor genetic differences between the isolates that showed varying resistance to methicillin, thereby suggesting development of resistant variants from one clonal type during the outbreak. These results were confirmed by DNA hybridisation analysis with specific resistance gene probes for parts of a multi-resistance gene cluster (including methicillin) in the chromosome. Analysis of the RFLP patterns of *S. aureus* isolates is potentially a useful procedure in clinical epidemiology.

Introduction

Considerable effort has been applied to the analysis of epidemics of methicillin-(or multi-) resistant *Staphylococcus aureus* (MRSA), including the tracking of human carriers and strains or lineages of the bacterium by means of molecular, biochemical, serological and phage-typing methods.^{1–4} While individual genetic or biological markers (e.g., phage-type, antibiotic resistance, plasmid carriage) may be associated with particular strains of MRSA involved in epidemics or outbreaks, it is not known whether particular clones of the organism are responsible for the initiation and development of outbreaks of infection, or whether any type of *S. aureus* present in an environment can take advantage of susceptible hosts and favourable circumstances to develop as a pathogen. The first proposal, referred to in its original form as the single MRSA clone hypothesis,⁵ has not been tested rigorously, since to do so requires a method that samples and compares the overall genetic similarity of strains isolated during an outbreak. A molecular method for genomic analysis and comparison of MRSA isolates has now been described⁶ which appears to meet these requirements. It relies on the detection in the DNA of isolates of restriction fragment length polymorphisms (RFLPs) generated by digestion with a rare-cutting

endonuclease such as *Sma*I, and resolution of the resulting large genomic fragments by pulsed-field gel electrophoresis (PFGE). Its application to an outbreak of MRSA infection in Canberra hospitals in 1989 indicated that certain MRSA lineages have persisted in Australian hospitals since at least 1982, while others have arisen separately.⁶

The present paper describes the application of this method to a group of *S. aureus* strains with unusual methicillin-resistance characteristics which appeared at the Alfred Hospital in Melbourne. Isolates of this type test inconclusively with methicillin, giving an “intermediate” result rather than being clearly susceptible or resistant. Hyper-production of β -lactamase was not the cause of this borderline resistance. Such strains are referred to here as MISA (methicillin-intermediate *S. aureus*). The RFLP method was used to make quantitative comparisons of genetic distances between isolates. In addition, genomic DNA was probed with cloned sequences from the *mec* (methicillin resistance) region of the chromosome to compare the organisation of the cluster of resistance genes normally resident here.

Materials and methods

S. aureus isolates and standard strains

Clinical isolates of bacteria were collected from different areas of the Alfred Hospital, Melbourne, as

Table I. *S. aureus* isolates: epidemiological data

Isolate	Resistance; source; associated infections; date isolated; phage-type
<i>MISA (multi-resistant with intermediate resistance to Mc)</i>	
IBR	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r ; general medical unit; abdominal abscess, sinus swabs, sputum; Oct. 1989; 85/90/88
IFR	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r ; cardiothoracic ICU; blood cultures, intravenous catheter, sputum, throat swab; May 1990; 90
IHA	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r Ci ^r ; neuro-surgery unit; gastrostomy site; Jun. 1990; 83A/85/90/88
IMO	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r Ci ^r ; orthopaedic unit; leg cellulitis; May 1990; 83A/90
IPE	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r Ci ^r ; neurosurgery unit; bursa fluid; May 1990; 83A/90
IRO	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r Ci ^r ; general medical unit; urine, sacral swab; Jul. 1990; 83A/85/90/88
<i>MRSA (multi-resistant with high resistance to Mc)</i>	
RAN	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r ; infectious diseases unit; eye wound, sputum, brain tissue; Jun. 1990; nt*
RBR	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r ; Mc ^r subclone of IBR which appeared within zone of inhibition around Mc disk (see IBR above); 85/88
RMA	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r ; general surgery ICU; sputum; Jun. 1990; 52A/85/80
RNA	Mc ^r Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r Fu ^r ; cardiothoracic ICU; blood cultures; Jun. 1990; 88
<i>MSSA (Multi-resistant but sensitive to Mc)</i>	
SHO	Pc ^r Em ^r Su ^r Tp ^r Tc ^r Gm ^r Tb ^r Fu ^r ; general surgery unit; intravenous catheter; Nov. 1990; nt*
SPO	Pc ^r Em ^r Su ^r Tp ^r Tc ^r ; burns unit; burn swab; Jan. 1991; 52A/83A/85/88
<i>Miscellaneous isolates</i>	
ITA†	Pc ^r ; respiratory unit patient with cystic fibrosis; sputum (several isolates); Jan. 1988, Jan.–Nov. 1990, Mar.–Jun. 1991; 42E/47
RBL(Q)‡	Mc ^r Pc ^r Em ^r Su ^r ; Queensland isolate (not from Alfred Hospital); 6/42E/47/75/84/81
<i>Reference strains</i>	
ANS46	Mc ^r Hg ^r Tc ^r Cd ^r Km ^r Cm ^r Em ^r Tp ^r Sm ^r Nv ^r Rf ^r Vc ^r ; MRSA isolated at Royal Melbourne Hospital; no other epidemiological data available; 1982; 83A/95/90
ANS62	Mc ^r Hg ^r Tc ^r Cd ^r isogen of ANS46 generated by acriflavine treatment; ⁸ 83A/95/90

Cd, cadmium ion; Ci, ciprofloxacin; Cm, chloramphenicol; Em, erythromycin; Fu, fusidic acid; Gm, gentamicin; Hg, mercuric ion; Km, kanamycin; Mc, methicillin; Nv, novobiocin; Pc, penicillin G; Rf, rifampicin; Sm, streptomycin; Su, sulphathiazole; Tb, tobramycin; Tc, tetracycline; Tp, trimethoprim; Vc, vancomycin. Superscripts i, r and s denote intermediate resistance, high-level resistance, and sensitivity, respectively, as defined in *Materials and methods*.

*Not typable with standard phage typing set at 100 × RTD.

†Sensitive to all antibiotics tested except Pc.

‡Unusual strain that was resistant to methicillin but sensitive to trimethoprim and tetracycline, of a type recently isolated in Queensland hospitals.

indicated in table I. In the nomenclature adopted for these isolates, the first letter (I, R or S) indicates the level of methicillin resistance (as described below), followed by two letters uniquely encoding the individual patient. Resistance phenotypes are expressed by the abbreviations listed in table I.

Strain ANS46 was a standard MRSA strain isolated originally at Royal Melbourne Hospital in 1982, and studied extensively since then.^{7–12} Strain ANS62 is a Mc^s derivative of ANS46, generated by acriflavine treatment, from which the *mec* gene (which gives rise to Mc^r) and associated resistance genes (causing Hg^r, Tc^r, Cd^r) have been lost because of deletion of 41 kb of the chromosome.^{11,12} Canberra type-I and Canberra type-II strains are representative strains from an MRSA outbreak in Canberra hospitals in 1989.⁶

Antibiotic susceptibility testing

Agar dilution susceptibility tests¹³ were performed with the antibiotics indicated in table I with Iso-Sensitest Agar (Oxoid). Resistance to methicillin was measured at antibiotic concentrations of 4 and 8 mg/L, with an inoculum of *c.* 10⁷ cfu/spot and overnight incubation at 30°C. MICs for methicillin and oxacillin were determined by microdilution tests in IsoSensitest Broth (Oxoid), with inoculum sizes of 10⁴–10⁷ cells/ml to test the effect of inoculum size on the MIC, and incubation overnight at 30°C.

Resistance to antimicrobial agents encoded in the *mec* region of the chromosome was measured by a disk diffusion test⁷ on Tryptone Soya Agar (Oxoid) with disks containing(/disk): Mc 100 µg; Hg (mercuric chloride) 100 µg; Cd (cadmium chloride) 250 µg; and Tc 100 µg. Plates were incubated at 37°C for 24 h or at 30°C for 48 h before measuring zones of inhibition.

Preparation of cells and digestion of genomic DNA

Cells were suspended in agarose plugs and cell walls were digested *in situ* with lysozyme and lysostaphin, as described by Inglis *et al.*¹¹ After proteolytic removal of residual protein with proteinase K and SDS, the intact genomic DNA was cleaved with *Sma*I, a rare-cutting restriction endonuclease (recognition site 5'-CCCGGG-3') which cleaves the *S. aureus* chromosome into *c.* 20 fragments.^{11,14}

Pulsed-field gel electrophoresis (PFGE)

The digested plugs were inserted into wells in agarose 1% gels (12.5 cm × 14 cm), and PFGE was performed at 200 V for 27 h at 12–14°C in a BioRad CHEF apparatus with Tris-borate buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3). Electrode switching was as follows: single ramp; initial forward time of 1 s; final forward time of 40 s.¹⁵ When electrophoresis was complete, gels were stained for 1 h with ethidium bromide (0.5 mg/L in water), destained in water for 2–6 h, and photographed with UV trans-illumination.

Analysis of DNA fragment patterns on PFGE gels

DNA fragment alignments were determined for samples run on the same gels, with common and

Fig. 1. Map of the *mec* region in strain ANS46. Letters indicate endonuclease sites; shaded blocks show positions of insertion sequence IS257; hatched blocks show Tn554 (left) and ψ Tn554 (right); and bracketed regions show genes for resistance to spectinomycin (*spc*), macrolides, lincosamides and streptogramin B (*erm*), mercuric ion (*mer*), tetracycline (*tet*), methicillin (*mec*), and cadmium ion (*cad*). Cloned fragments used as probes are identified below the map. The endonuclease sites shown are: H, *Hind*III; E, *Eco*RI; and Sm, *Sma*I.

different fragments identified pairwise for all isolates, as described previously.⁶ Values of *p*, the estimate of genetic distance (fraction of nucleotides different between pairs of isolates), were calculated with numerical methods described previously.^{16,17} A matrix of *p* values for all pairwise comparisons of DNA from each isolate was constructed, from which clusters were generated by single linkage analysis, and fused by triangle overlap within clusters.¹⁸

Hybridisation analysis

DNA fragments resolved in agarose gels by PFGE were transferred to nitrocellulose filters by Southern blotting, after which the filters were probed with methods described previously.⁸ Three fragments of DNA cloned from the *mec* region of ANS46,^{8,11} which carries determinants for *Em*^r, *Sp*^r, *Hg*^r, *Tc*^r and *Cd*^r as well as *Mc*^r, were used as probes (fig. 1): (i) MA13, from the left end of the multi-resistance cluster (MA13 also hybridises with two copies of Tn554 located elsewhere in the chromosome of ANS46);¹⁹ (ii) MA11, which identifies fragments carrying the *mec* gene itself; and (iii) MA14, which identifies the right end of the cluster.¹²

Results

Measurement of resistance in isolates

Agar dilution tests differentiated between methicillin-sensitive, -resistant, and -intermediate types. Sensitive isolates (MSSA) were inhibited by 4 mg/L, resistant isolates (MRSA) were not inhibited by 8 mg/L, and intermediate isolates (MISA) were inhibited by 8 but not by 4 mg/L. Broth dilution tests revealed that methicillin and oxacillin MICs for MSSA and MRSA isolates (0.5 mg/L and > 32 mg/L, respectively) were relatively unaffected by inoculum size. However, MISA isolates showed considerable variation in MIC values of methicillin (2–16 mg/L) and oxacillin (8–> 32 mg/L), the MIC value being greater with larger inocula.

The disk diffusion method used to detect resistance

to antimicrobial agents encoded in the *mec* region (Cd, Hg, Mc and Tc), does not detect intermediate resistance to Mc as described for the agar dilution method. MISA isolates had zones of inhibition typically 34–36 mm in diameter at 37°C, whereas strain ANS62 (which has *mec* region genes deleted) and strain ANS46 (*mec* region intact) had zones of inhibition of 38 mm and 21 mm, respectively. Other MRSA isolates used in this study had zones of 12–22 mm. For the MRSA isolates and strain ANS46, these zone sizes were influenced strongly by temperature, decreasing to 0–9 mm at 30°C; strain ANS62 and the MISA isolates showed negligible changes in zone diameter when incubated at 30°C.

Strain RBR was not a primary clinical isolate, but was picked off a plate of strain IBR as a colony growing within the zone of inhibition of a methicillin disk. Strain RBR had MIC and disk inhibition values similar to those of the other MRSA isolates examined.

Epidemiology

All isolates tested were multi-resistant to antibiotics, with the exception of strain ITA which was only *Pc*^r and had intermediate resistance to methicillin; strains ITA and RBL(Q) were included in this analysis as control isolates likely to be unrelated epidemiologically to the other *S. aureus* isolates included in the study.

Of the seven MISA isolates, all but strains IBR and ITA appeared as a small outbreak lasting *c.* 2 months in mid-1990; strain IBR was identified > 6 months previously. The outbreak isolates came from a range of physically separate wards and units, and from different types of infections. Four different phage-typing patterns were represented within the outbreak group.

PFGE of DNA from the isolates

Gels stained with ethidium bromide showing the fragment patterns (RFLPs) generated by *Sma*I digestion of genomic DNA from each strain or isolate are shown in fig. 2. Fragment sizes were in the range *c.*

Fig. 2. PFGE of genomic DNA, digested with *Sma*I and stained with ethidium bromide, from clinical isolates SPO (track 1), SHO (2), IMO (3), IPE (4), IHA (5), IRO (6), IFR (7), ITA (9), IBR (10), RBR (11), RMA (12), RAN (13), RNA (14), RBL(Q) (15), and strain ANS46 (8). Size markers, derived from comparison of ANS46 fragments with λ concatemers in previous work,¹¹ are shown on the right of the figure.

7–700 kb. A general similarity of the RFLP patterns within the MRSA and MISA groups was observed, indicating that the *Sma*I sites were similar in number and location within the genome of isolates from these two groups, and thus that there was similarity of sequence throughout the genome.

A quantitative estimate of the extent of genomic similarity was obtained by counting the number of DNA fragments common to pairs of isolates, and calculating *p*, an estimate of the fraction of nucleotides different for that pair. A matrix of relationships between isolates was thus generated, from which a dendrogram summarising the relationships of all isolates was constructed (fig. 3). This showed that four of the five outbreak MISA isolates could not be separated (strains IHA, IMO, IPE and IRO), and that, with the exceptions of strains ITA, RBL(Q), RAN, RNA and ANS46/62, there was a general clustering of related types; this cluster will hereafter be referred to as the Alfred group. Strains RNA and RAN formed a loose cluster with strain ANS46/62, and the inde-

pendently isolated strains ITA and RBL(Q) had a distant relationship to each other and to the other isolates, both on epidemiological grounds (table I) and on the basis of RFLPs.

Arrangements of genes in the mec region of the chromosome of the isolates

Phenotypic testing of isolates for resistance genes known to map in the *mec* cluster^{8,9} showed variation among the isolates (table II). In contrast to strains ANS46 and RNA, none of the MISA or MRSA isolates of the Alfred group were Hg^r or Tc^r by disk sensitivity testing. It should be noted that this type of sensitivity testing gives a different outcome to agar dilution tests; thus, all isolates except strains ITA and RBL(Q) were resistant to Tc 5 mg/L in agar, and are recorded as Tc^r in table I. This low-level resistance is thought to result from a *tet* gene located elsewhere in the chromosome.¹⁴ Additional resistance is generated by an integrated copy of the Tc^r plasmid pT181 in the *mec* region of strains such as ANS46.⁸

The results of hybridisation experiments are shown in fig. 4. Strain ANS46 contained one fragment (145 kb) that hybridised with probes MA11 and MA14, and three fragments that hybridised with probe MA13—a 110-kb fragment carrying part of the *mec* region, and two other fragments (410 and 650 kb) which carry an additional copy of Tn554. Isolate RNA showed the same pattern of hybridisation. The Alfred group of MRSA and MISA isolates had one fragment (220 kb) that hybridised with probes MA11 and MA14, and two fragments (410 and 650 kb) that hybridised with probe MA13. The MSSA isolates of the Alfred group showed hybridisation only between the 410- and 650-kb fragments and probe MA13. Isolate RAN had the same pattern of hybridisation as the Alfred group. The two miscellaneous isolates

Fig. 3. Dendrogram calculated from the *p* values for genomic DNA of the isolates after RFLP analysis. The genetic distance (*p*) is represented by the vertical distance between isolates. Bar = *p* value 0.01.

Table II. Presence in *S. aureus* isolates of resistance determinants from the *mec* region of the chromosome

Isolate	Resistance*			
	Hg	Tc	Mc	Cd
<i>MISA</i>				
IBR	s	s	s	r
IFR	s	s	s	r
IHA	s	s	s	r
IMO	s	s	s	r
IPE	s	s	s	r
IRO	s	s	s	r
<i>MRSA</i>				
RAN	s	s	r†	r
RBR	s	s	r†	r
RMA	s	s	r†	r
RNA	r	r	r†	r
<i>MSSA</i>				
SHO	s	i	s	s
SPO	s	s	s	s
<i>Miscellaneous isolates</i>				
ITA	s	s	s	r
RBL(Q)	r	s	r†	r
<i>Reference strains</i>				
ANS46	r	r	r	r
ANS62	s	s	s	s

*Antimicrobial agents indicated are those for which resistance genes are clustered in the *mec* region of strain ANS46, and deleted from strain ANS62; s indicates sensitivity equal to or greater than that of ANS62, r indicates resistance equal to or greater than that of ANS46, and i indicates resistance intermediate between these by disk diffusion tests (this method of testing does not demonstrate intermediate levels of methicillin resistance).

†Resistance heterogeneity, detected as individual colonies growing within the zone of inhibition.

Fig. 4. DNA from the gel shown in fig. 2 after transfer to nitrocellulose filters and hybridisation with probes: (a) MA11; (b) MA13; and (c) MA14. **Fig. 5.** DNA from the isolates indicated was digested with *Hind*III, electrophoresed under constant field conditions, transferred to nitrocellulose, and hybridised with probes: (a) MA11; (b) MA13; and (c) MA14. Isolates were: RMA (track 1); IBR (2); IMO (3); IFR (4); SPO (5); SHO (6); Canberra Type-II (7); RAN (8); ITA (9); Canberra Type-I (10); RNA (13); RBL(Q) (14); strains ANS46 (12); and ANS62 (11). Molecular size markers from λ DNA digested with *Hind*III are shown on the right hand side.

showed quite different patterns. Strain RBL(Q) had one fragment (650 kb) that hybridised with probe MA13, and additional fragments that hybridised with probes MA11 (240 kb) and MA14 (80 kb). Isolate ITA hybridised only with probe MA14.

For more detailed comparisons, DNA from the isolates was digested with *Hind*III (which cuts staphylococcal DNA into at least several hundred fragments), electrophoresed, transferred to filters and hybridised with the same three probes. The results are shown in fig. 5, and a summary of the data is given in table III. The significant features were as follows. The MRSA and MISA isolates of the Alfred group were

Table III. *Sma*I and *Hind*III fragments of DNA from different isolates that hybridised with DNA probes for the *mec* region of the chromosome

Strain or isolate	<i>Sma</i> I fragments (kb) hybridising with			<i>Hind</i> III fragments (kb) hybridising with		
	MA11	MA13	MA14	MA11	MA13	MA14
ANS46	145	110,410,650	145	4	0·7,0·9,2·7,7,8†	2·2,2·4,2·5
RNA	145	110,410,650	145	4	0·7,0·9,2·7,7,8†	2·2,2·4,2·5
RAN	220	410,650	220	4	7	2·2,2·4,2·5
RNA						
RBR						
IBR						
IMO*						
ANS62	...	200,410,650	...	...	7,8†	...
SHO	...	410,650	...	...	7	...
SPO	...	...	180	...	...	2·0
ITA	240	650	80	4	7	4·5

DNA fragments used as probes (derived from the *mec* region of strain ANS46) are shown in fig. 1.
*Isolates IRO, IPE, and IHA gave hybridisation results identical to those of other members of this group when *Sma*I was used to digest the DNA; hybridisations were not done after *Hind*III treatment of the DNA for these three isolates.
†7-kb band comprises two fragments of identical size.

identical, with fragments that hybridised with probes MA11 (one fragment), MA13 (two identical fragments) and MA14 (three fragments). The MSSA members of the Alfred group had only the band that hybridised with probe MA13. Isolate RAN had the same hybridisation pattern as the Alfred MRSA and MISA isolates. Isolate RNA had the same hybridisation pattern in the *mec* region as the Alfred isolates but, like strain ANS46, differed from the Alfred isolates in having four additional fragments showing homology with probe MA13. Strain ANS62, the methicillin-sensitive variant of strain ANS46, had no bands that hybridised with probes MA11 or MA14, while probe MA13 showed that homology to Tn554 existed in two identical fragments, remote from the *mec* region, and a fragment adjacent to the *mec* region.

The miscellaneous isolate RBL(Q) had the same *Hind*III fragment that hybridised with probe MA11 as was found in all the MRSA isolates; it also carried the single MA13-hybridising band corresponding to a remote copy of Tn554, and a further single fragment that hybridised with probe MA14. The other miscellaneous isolate, ITA, was the only isolate that showed homology only to probe MA14. Therefore, the molecular features of the *mec* region revealed by DNA hybridisation experiments reflected closely the divisions identified by RFLP analysis with *Sma*I (fig. 2).

The MRSA and MISA isolates of the Alfred group had an identical pattern of hybridising fragments (fig. 5), and a similar *Sma*I-generated RFLP pattern (not shown), to the Canberra type-II strain which was involved in an outbreak of MRSA in the two public hospitals in Canberra in 1989. The Canberra type-I strain from that study shared an identical pattern of *Hind*III fragments (fig. 5) with the ANS46/RNA

group, but was shown to differ by comparison of RFLP patterns (not shown).

Discussion

In the past, MRSA isolates have occurred sporadically at the Alfred Hospital, usually as persistent types with high levels of methicillin resistance. The present study involved an outbreak of infections caused by *S. aureus* isolates characterised by intermediate resistance to methicillin (MISA), and therefore difficult to detect by routine methods. The first of these isolates (IBR) was identified some 6 months before other members of the MISA group and, because of its genetic similarity to the other MISA isolates, was possibly the pioneer of the group.

Analysis of genomic RFLPs from *Sma*I digests of total DNA permits an estimate of genetic similarity. Fragment differences occur where a difference in sequence within one of the two DNA sequences compared results in loss or gain of a *Sma*I site. For instance, a comparison of the RFLPs of strains ANS46 and ANS62 (a strain derived by the deletion from strain ANS46 of DNA from the *mec* region that contains a *Sma*I site), showed that two fragments in strain ANS46 were replaced by one fragment in strain ANS62.¹¹ A dendrogram, based on *p* values derived from RFLP analysis, showed the clustering of *S. aureus* strains into types.⁶ Within a type, isolates showed changes in up to three fragments, which corresponded to a *p* value of 0·01.⁶ Analysis of RFLPs in this study showed that there were only minor differences among the MISA isolates (four of the six were identical by this test), and between these and two MRSA isolates (strains RMA and RBR; the latter a

secondary isolate, apparently a genetic variant of strain IBR). Two multi-resistant, but Mc^s, isolates (strains SHO and SPO) were also part of this group on the basis of RFLP patterns. These MISA, MRSA and MSSA isolates together constituted the Alfred cluster.

DNA hybridisation analysis demonstrated that there was no detectable molecular difference between the MISA and MRSA isolates in this group, despite the large difference in expressed resistance to methicillin. Accordingly, the difference in resistance must be attributed to small undetected differences in the *mec* region, or in a part of the chromosome remote from the *mec* region. The regulatory *femA* gene, located > 1000 kb from *mecA*,²⁰ is one possible candidate. The appearance of the fully-resistant strain RBR, in a culture of strain IBR grown in the presence of methicillin, suggests that MISA strains may give rise to MRSA variants in infected patients, or in circumstances where MISA types encounter significant concentrations of methicillin or similar antibiotics. In any event, the presence of the difficult-to-detect MISA type should be taken as an indication of potential cognate MRSA types; thus, care in the interpretation of resistance measurements, particularly those of marginal significance, is indicated.

The two MSSA isolates from the Alfred group had lost the *mec* gene and sequences to the right of *mec* in the chromosome, as shown by their failure to hybridise with probes MA11 and MA14. They still carried sequences contained in probe MA13, but these were Tn554 (Em^r) sequences located remote from the *mec* region;¹⁹ they appeared to have lost parts of the *mec* region, and thus were similar to strain ANS62, a laboratory-derived deletion mutant of strain ANS46 whose *mec* region has been mapped in detail.^{11,12} That the MSSA isolates represent natural deletion homologues of the MRSA and MISA types in the Alfred group seems likely; they were isolated in Nov. 1990 and Jan. 1991, well after the main group of MISA and MRSA types (Oct. 1989–July 1990). The simultaneous appearance of the MRSA strains and their MSSA deletion homologues in an MRSA outbreak has been observed in three instances over a 10-year period.²¹

As noted, the Alfred group of isolates had an identical hybridisation pattern, and a similar *Sma*I-generated RFLP pattern, to the Canberra type-II group, which was involved in an outbreak of MRSA in 1989 in the two major public hospitals in Canberra.

However, the Alfred group differed significantly from the earlier Melbourne lineage represented by strain ANS46. This was shown by the absence from the Alfred isolates of a copy of Tn554 in or near the *mec* region, and their sensitivity to Hg and Tc, indicating that these resistance genes were also absent. The *Sma*I digestion patterns also separated the two Melbourne lineages. Accordingly, the Alfred and Canberra type-II groups may be representatives of a new lineage of MRSA/MISA, replacing older, though still extant, lineages represented by strain ANS46. The Alfred isolate RNA may be a modern representative of the older ANS46 lineage, as seems true of the Canberra type-I group described previously.⁶

Isolate RAN was interesting in having a *mec* region similar to that of the Alfred group, but chromosomal RFLPs which set it apart. It may be of a different chromosomal (i.e., clonal) background, with the Alfred type *mec* region inserted into a different chromosomal background following transfer by a phage or plasmid vector.

The other two isolates examined in this study—strains ITA and RBL(Q)—were confirmed to be different from the other MISA and MRSA isolates collected in the Alfred Hospital on the basis of their distinct RFLP patterns. Subsequent analysis has shown that strain ITA belongs to the same group as an MRSA type (representative strain R155) that has been found commonly in New Jersey, USA (unpublished results).

The use of RFLP analysis following digestion with rare-cutting endonucleases such as *Sma*I offers a useful approach to the analysis of *S. aureus* populations, and might be used to identify more precisely the origins and spread of new and old clones of staphylococci. Thus, even without the analysis of the *mec* region described here, conclusions could be drawn about the overall similarities and differences between the groups of isolates shown in fig. 2. With simplified procedures between bacterial isolation and resolution of genomic fragments on PFGE, the approach described here for comparisons on the basis of RFLP similarities and differences could become a useful method for general clinical application.

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