ANTIMICROBIAL RESISTANCE

Heterogeneous location of the *mupA* high-level mupirocin resistance gene in *Staphylococcus aureus*

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Epidemiologically unrelated clinical isolates of *Staphylococcus aureus* with high-level resistance to mupirocin (MIC $\geq$ 512 mg/L) were studied to determine the location of the *mupA* resistance gene. The gene was carried on plasmids of variable size, some of which were transferable *in vitro*. DNA hybridisation of genomic DNA from 85 isolates showed that *mupA* was located on *EcoRI* fragments of seven different sizes; the most frequently observed fragments were 7 kb (46 isolates) or 4.1 kb (21 isolates). All isolates retained a 1.6-kb *NcoI* fragment that hybridised with *mupA* probes, but showed heterogeneous hybridisation patterns after digestion with *HincII*. These data suggested that *mupA* may be conserved, but that variation occurs in the flanking DNA proximal to it. Amplification of spacer regions between *mupA* and closest proximal copy of IS257 yielded products of variable size and was consistent with the presence of IS257 in either orientation. It is proposed that IS257-mediated events are responsible for the heterogeneity observed. The location of *mupA* varied between epidemiologically unrelated isolates of the same strain, including isolates of EMRSA-16 – one of the two predominant methicillin-resistant strains in UK hospitals at the present time – and this correlated with variations in the digestion patterns of the mupirocin resistance plasmids. The variable location of *mupA* should be evaluated further as a potential epidemiological tool with which to monitor the spread of high-level mupirocin resistance in EMRSA-16 or other strains of *S. aureus*.

Introduction

Mupirocin is an antibiotic used topically for the treatment of staphylococcal and streptococcal skin infections and for the eradication of carriage of *Staphylococcus aureus*, including strains resistant to methicillin (MRSA). High-level, transferable resistance to mupirocin (MIC $\geq$ 512 mg/L) [1] was first reported among clinical isolates of *S. aureus* from a London hospital [2] and abrogates the effective clinical use of the agent. This form of resistance is encountered increasingly in the UK, particularly in the south-east of England, and is exhibited by both methicillin-sensitive and -resistant *S. aureus*. In particular, its association with various epidemic strains of MRSA (EMRSA) may cause serious infection control problems [3].

High-level mupirocin resistance results from the acquisition of new DNA which encodes production of a mupirocin-resistant isoleucyl tRNA synthetase [1]. In the presence of mupirocin, this enzyme takes over the function of the sensitive chromosomal enzyme. The resistance gene has been cloned [4], sequenced [5] and probes have been used to investigate its distribution. It has been designated variously *ileS* [5], *ileS-2* [6] or *mupA* [7]. Hereafter, it will be referred to as *mupA*. This gene is carried on diverse plasmids, although plasmids in epidemiologically related isolates may appear similar [8]. However, despite this diversity of plasmids, the *mupA* gene is located on an *c.* 4-kb *EcoRI* DNA fragment in all but three high-level mupirocin-resistant isolates of *S. aureus* and coagulase-negative staphylococci examined previously [9–12]. This homogeneous pattern of hybridisation in staphylococci from diverse sources is consistent with the dissemination of a conserved resistance transposon of which the *mupA* gene is a part [10, 11]. The present study examined the location of the *mupA*

mupirocin resistance gene in *S. aureus* isolated in the UK since 1990.

Materials and methods

Bacterial isolates

Eighty-five clinical isolates of *S. aureus* with high-level resistance to mupirocin ($MIC \geq 512$ mg/L) were included in this study. In the initial investigations, 15 isolates from different centres were chosen to represent various strains causing current problems in UK hospitals (Table 1). These were five epidemiologically distinct isolates of EMRSA-16 (including the first high-level mupirocin-resistant isolate of this strain from 1993), two isolates of EMRSA-3, two other non-epidemic MRSA strains (OMRSA) and six isolates of a *mecA*-negative strain with intermediate methicillin resistance (MIC 4–8 mg/L, IMRSA) [3]. In addition, isolates 'STH 1' and 'Bristol 3', both of which had been shown previously to hybridise with a mupirocin resistance probe [10, 11], were included as controls for hybridisation and PCR studies. Subsequently, studies were extended to include 16 isolates of EMRSA-16 referred from different UK hospitals during 1995 and 52 *S. aureus* isolates referred from a single UK centre since 1991. Isolates from this latter centre represented numerous phage types and were mainly methicillin-sensitive isolates from patients prescribed mupirocin in the community and referred by their general practitioners to outpatient clinics.

Transfer studies and plasmid analysis

Transfer of mupirocin resistance from 17 isolates (Table 1) was investigated by a semi-quantitative filter mating technique. Overnight cultures of potential donor isolates and the *S. aureus* recipient strain WG3358 [13] were suspended in nutrient broth. Equal volumes (100 μ l) of the suspensions of each donor and the recipient were mixed on a sterile filter (0.45- μ m pore

size) on a nutrient agar plate and incubated overnight. Growth on the filters was harvested into 1 ml of nutrient broth and 100- μ l samples were plated on to Brain Heart Infusion (BHI; Unipath) Agar containing rifampicin (Sigma) 25 mg/L and novobiocin (Sigma) 5 mg/L, together with either mupirocin (SmithKline Beecham Pharmaceuticals) 50 mg/L or gentamicin (Sigma) 4 mg/L. All donor and recipient cultures were also plated on to this selective medium as controls and incubated at 37°C for 24–48 h. Putative transconjugant colonies were subcultured on selective agar once before further non-selective passages. Plasmids were extracted from donors and transconjugants by Brij lysis [14] or, when digestion with restriction endonucleases was required, with cetyltrimethylammonium bromide (CTAB) [13]. DNA was separated on agarose 0.8% gels at 90 V for 2–3 h in $0.5 \times$ TBE buffer (1 $\times$ TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8) and stained with ethidium bromide (1 mg/L) before being viewed under UV light. The plasmids were transferred to Hybond-N membrane (Amersham) by a VacuGene blotter (Pharmacia-LKB) and fixed at 80°C for 2 h; these blots were stored at room temperature until required for hybridisation studies.

Extraction of genomic DNA

Genomic DNA was prepared from isolates of *S. aureus* with guanidium thiocyanate [15] and 5- μ g samples were digested overnight with 10 U of *Eco*RI, *Hinc*II or *Nco*I (Life Technologies). Digested DNA was electrophoresed through agarose 0.8% gels at 25 V for 16 h in $0.5 \times$ TBE buffer and blotted to nylon membrane as above.

DNA probes for *mupA* and hybridisation studies

Two digoxigenin-labelled probes specific for the *mupA* high-level mupirocin resistance gene were used in this investigation (Fig. 1). Probe A consisted of an intragenic

Table 1. High-level mupirocin-resistance in diverse *S. aureus* isolates from 17 UK hospitals

Isolate	Year of isolation	Strain	Size of Mu ^r Plasmid (MDa)	Transfer of Mu ^r <i>in vitro</i>	Size of <i>mupA</i> -bearing <i>Eco</i> RI fragment (kb)
1	1987	'STH 1'	10*	+	18.0
2	1987	'Bristol 3'	25	—	4.1
3	1994	EMRSA-16	30	+	7.0
4	1993	EMRSA-16	30	+	4.1
5	1994	EMRSA-16	30	+	7.0
6	1994	EMRSA-16	30	+	7.0
7	1994	EMRSA-16	30	+	7.0
8	1994	EMRSA-3	30	+	7.0
9	1990	EMRSA-3	25	—	5.5
10	1993	OMRSA	22	—	10.0
11	1994	OMRSA	25	+	7.0
12	1994	IMRSA	35	—	10.0
13	1994	IMRSA	35	—	10.0
14	1994	IMRSA	35	—	10.0
15	1994	IMRSA	40	+	12.0
16	1994	IMRSA	35	—	10.0
17	1994	IMRSA	35	—	7.0

*See text for details.

Fig. 1. Schematic representation to show the positions of the open reading frame of *mupA* (solid block), probes A and B and restriction sites for *EcoRI* (E), *HincII* (H) and *NcoI* (N) on the 4013-bp sequenced fragment [5] of staphylococcal mupirocin resistance plasmid pJ2947.

1.6-kb *NcoI* fragment of recombinant plasmid pMZ-1. This plasmid consists of a 4.05-kb *EcoRI* fragment of mupirocin resistance plasmid pJ2947 [4] cloned into *Escherichia coli*-*S. aureus* shuttle vector pOX7 [7]. The recombinant plasmid was extracted from the *E. coli* host by alkaline lysis [16] and digested with *NcoI* (Life Technologies). The resulting 1.6-kb fragment was excised from an agarose 0.8% gel, purified with a Prep-a-Gene kit (BioRad) and labelled with digoxigenin by random priming. Probe B, which was also intragenic, was generated by PCR and consisted of a 190-bp fragment amplified with forward and reverse primers *mupA*_F (5'-TGA CAA TAG AAA AGG ACA GG-3') and *mupA*_R (5'-CTC TAA TTC AAC TGG TAA GCC-3') and equivalent to nucleotide positions 554-743 of the published sequence [5]. Strain 'STH 1' [10, 11] was used as the source of template DNA. The method for preparation of template and the amplification conditions were the same as those described previously for amplification of sections of the *mecA* and 16S rRNA genes of *S. aureus* [17]. All amplifications were achieved with Touchdown (Hybaid) or Genius (Techne) thermal cyclers. Labelling of the 190-bp product was carried out in a second-round PCR reaction to which digoxigenin-11-dUTP was added [18]. Probe A was predicted from sequence data [5] to hybridise with c. 1.6-kb *NcoI* and 2.3-kb *HincII* intragenic fragments of the *mupA* gene. In contrast, probe B was predicted to hybridise with a *HincII* fragment of 863 bp that extended beyond the 5' end of the resistance gene (Fig. 1) and was included to provide information about flanking DNA proximal to *mupA*. Hybridisations with all probes were performed at 68°C under stringent conditions as recommended by Boehringer Mannheim.

Amplification of IS257-mupA spacer regions and an internal IS257 fragment

The spacer regions between *mupA* and the closest proximal copy of IS257 were amplified with primers *mupA*_R and IS257 forward primer 1234 (IS257_F; 5'-GGC ATG GCG AAA ATC CGT AG). To investigate the presence of a proximal copy of IS257 in the reverse orientation, primer *mupA*_R was also used with IS257 reverse primer 1235 (IS257_R; 5'-TGG CGT ATT GAT GAG ACG TAC ATC). Amplification of these spacer

regions used cycling conditions described previously [17]. A 407-bp fragment internal to IS257 was amplified with primers 1234 and 1235, labelled with digoxigenin by PCR as above and used in hybridisation studies to confirm the presence of IS257 sequences in the IS257-*mupA* spacer regions. The protocol for amplifying this probe fragment consisted of five cycles of 94°C for 40 s, 55°C for 30 s, 72°C for 30 s, followed by 25 cycles of 92°C for 30 s, 52°C for 30 s and 72°C for 30 s with a final cycle at 25°C for 30 s. The sequences of primers 1234 and 1235 and the amplification conditions for the 407-bp product were kindly provided by Dr W. Grubb [19].

Results

Plasmids encoding mupirocin resistance

Initial studies of 15 clinical isolates and two control strains showed that the *mupA* resistance gene was located on plasmids of variable size in all except control strain 'STH 1' (Table 1). For this particular strain, hybridisation always occurred with DNA that co-migrated with residual chromosomal DNA. A vague band of c. 10 MDa (possibly a plasmid) was visible at this position in some preparations from this strain. In filter mating experiments, high-level mupirocin resistance was transferred readily to *S. aureus* recipient strain WG3358 from nine of the 17 isolates, although transfer frequencies could not be determined with the semi-quantitative method used (Table 1). Importantly, all five isolates of EMRSA-16 tested were able to act as donors in these experiments. One isolate of EMRSA-16 was also resistant to gentamicin but, while mupirocin resistance was carried on an c. 30 MDa plasmid, the aminoglycoside resistance transferred separately on a plasmid of c. 40 MDa. Furthermore, mupirocin resistance transferred from this isolate at c. 100-fold greater efficiency than gentamicin resistance (data not shown).

Hybridisation of genomic DNA with mupirocin resistance probes

Many of the 85 clinical isolates of *S. aureus* used in this study contained plasmids in addition to the

mupirocin resistance plasmid and, as initial studies indicated that some isolates were unable to transfer mupirocin resistance *in vitro* (Table 1), it was not possible to overcome this problem by generating transconjugants that contained single plasmids. Hence, direct digestion and comparison of these mupirocin resistance plasmids was not possible. In view of the large number of isolates studied, the location of the *mupA* gene was therefore investigated further with digests of genomic DNA, the inference being that fragments hybridising with the probe would probably be derived from the resistance plasmids rather than from chromosomal DNA.

The *mupA* resistance gene was located on seven different sized *EcoRI* fragments of genomic DNA among the 85 isolates studied. These were designated A–G in order of increasing size (Table 2). The patterns of hybridisation observed for control strains 'Bristol 3' (group A) and 'STH 1' (group G) were consistent with the findings of previous studies [10, 11]. DNA fragments of two sizes accounted for the majority of the isolates; a 7-kb *EcoRI* fragment in 46 (54%; group D) isolates and a fragment of 4.1 kb in 21 (25%; group A) isolates (Table 2). Probes A and B gave identical results with *EcoRI*-digested DNA. Despite the heterogeneous *EcoRI* hybridisation patterns, all isolates of all groups had a 1.6-kb *NcoI* fragment that hybridised with probe A (Fig. 2a); this was consistent with predictions made from sequence data [5]. However, heterogeneity was again observed when *HincII*-digested DNA was hybridised with probe B (Fig. 2b). This probe was designed to investigate variation in the flanking DNA sequences proximal to *mupA* and was predicted to hybridise with an 863-bp *HincII* fragment that extended beyond the 5' end of the resistance gene (Fig. 1). However, the actual sizes observed for the *HincII* fragments hybridising with probe B varied from *c.* 850 bp to >10 kb (Fig. 2b). Bands of *c.* 850 bp were assumed to correspond to the predicted 863-bp fragment and were obtained from isolates of *EcoRI* groups A, B and E. Although good

correlation was observed between the hybridisation patterns obtained with *HincII*- and *EcoRI*-digested DNA for isolates of groups A (4.1-kb *EcoRI* fragment) and D (7-kb *EcoRI* fragment), the isolates of other *EcoRI* groups showed greater diversity in their *HincII* hybridisation patterns.

Variable location of the mupA gene within a strain

This heterogeneity of *EcoRI* and *HincII* hybridisation patterns was even observed among epidemiologically unrelated isolates of the same strain (as confirmed by phage typing and pulsed-field gel electrophoresis, data not shown). Two isolates of EMRSA-3 and six isolates of a *mecA*-negative IMRSA strain had two and four *EcoRI* hybridisation patterns, respectively (Table 1). Twenty-one isolates of EMRSA-16 had six *EcoRI* hybridisation patterns (Table 1 and Fig. 3) and, in 16 isolates studied further, this heterogeneity correlated with variations in the digestion pattern of the mupirocin resistance plasmids (Fig. 3).

Preliminary analysis of proximal IS257-mupA spacer regions

The *mupA* gene is flanked by direct repeats of IS257 in diverse isolates of *S. aureus* [7, 20]. Hybridisation studies with *HincII* digests and probe B suggested that the variable location of *mupA* might result from alterations in the flanking DNA proximal to the resistance gene. To explore this possibility further, the study attempted to amplify the spacer region between the closest proximal copy of IS257 and *mupA* from isolates chosen to represent each of the seven *EcoRI* hybridisation patterns. Three of these isolates, belonging to hybridisation groups A, E and G, yielded amplicons of variable size with primers IS257_F and *mupA*_R (Table 2). However, variably sized amplicons were obtained from isolates representing groups C, D and F, when primer IS257_R was used with *mupA*_R.

Table 2. Location of *mupA* in *EcoRI*-digested DNA of 85 clinical isolates of high-level mupirocin-resistant *S. aureus* and amplification of proximal flanking DNA

Pattern designation	<i>EcoRI</i> fragment bearing <i>mupA</i> (kb)	Number of isolates	Amplification of proximal IS257- <i>mupA</i> spacer*	
			IS257 _F + <i>mupA</i> _R	IS257 _R + <i>mupA</i> _R
A	4.1	21 [†]	2000 bp	—
B	5.5	4	—	—
C	6.2	3	—	950 bp
D	7.0	46	—	950 bp
E	10.0	6	2000 bp	—
F	12.0	3	—	1050 bp
G	18.0	2 [‡]	1200 bp	—

—, No amplicon detected.

*One isolate tested in each category.

[†]Including strain 'Bristol 3'.

[‡]Including strain 'STH-1'.

Fig. 2. **a**, Conserved *Nco*I fragments; **b**, heterogeneous *Hinc*II fragments of genomic DNA carrying *mupA* (hybridisation with probe B) among clinical isolates of high-level mupirocin-resistant *S. aureus*. Lanes 1–7 correspond to isolates of *Eco*RI groups A–G, respectively. *Hind*III digests of phage λ DNA are shown as size markers.

Confirmation that these amplicons were indeed spacer regions was checked by hybridisation with both probe B (for *mupA*) and the IS257 internal probe. No spacer region was amplified with either pair of primers from the tested isolate of pattern group B. Only the *c.* 2-kb IS257_F-*mupA*_R amplicons from isolates of groups A and E contained internal *Eco*RI target sites; both yielded two products of *c.* 1200 bp and 800 bp.

Discussion

In the present study a small panel of clinical isolates were chosen to represent strains causing current problems in UK hospitals to confirm previous reports [9–11, 19] that high-level mupirocin resistance (MIC $\geq$ 512 mg/L) in *S. aureus* is usually encoded on plasmids that vary both in size and in their ability to transfer on filters to suitable recipient strains. Therefore, it must be inferred that this resistance may also be transferable *in vivo* to other strains of *S. aureus*. The

present study was unable to detect a mupirocin resistance plasmid in control strain 'STH 1', as probes appeared to hybridise with residual chromosomal DNA. This strain has been shown previously to contain an atypical resistance plasmid that co-migrates with chromosomal DNA, but which may be separated by pulsed-field gel electrophoresis [9].

In the 85 isolates studied, the *mupA* gene was located on seven different *Eco*RI fragments of genomic DNA, although in most isolates (54%) it was on a 7-kb fragment. This finding contrasts with all previous large-scale studies of *S. aureus* or coagulase-negative staphylococci [9–11] in which *mupA* was located on a 4.1-kb *Eco*RI fragment in all except two isolates examined; a similar sized fragment was the second most common among the isolates in the present study. Despite the use of digested genomic DNA for hybridisation studies, it was assumed that the DNA fragments carrying *mupA* were derived from mupirocin resistance plasmids, although the possibility that

Fig. 3. **a**, Digestion of mupirocin resistance plasmids from 16 epidemiologically unrelated isolates of EMRSA-16 with *Eco*RI; **b**, the corresponding Southern blot hybridised with a *mupA* probe.

some isolates also possessed chromosomal copies of the *mupA* gene cannot be excluded. This assumption did not confuse the analysis, because none of the 85 isolates showed two bands of hybridisation – indicating that, if chromosomal and plasmid copies were indeed present in some isolates, both copies must have been located on DNA fragments of identical size. Furthermore, the only isolates of *S. aureus* presently shown to have chromosomal copies of *mupA* expressed only low-level mupirocin resistance [12]. Interestingly, in this latter study one isolate with high-level resistance and two of three isolates with low-level resistance each had two *Eco*RI fragments of genomic DNA (4.1 and >12 kb) that hybridised with a *mupA* probe, but no explanation for this was provided [12]. It should be noted that probes A and B used in the present study did not react with DNA prepared from *S. aureus* sensitive to mupirocin (MIC ≤ 4 mg/L), showing low-level resistance (MIC 8–256 mg/L) or trained to high levels of resistance

(MIC ≥ 512 mg/L) by growth on agar containing increasing gradients of mupirocin (data not shown). Furthermore, primers *mupA*_F and *mupA*_R described herein have been used routinely in this laboratory since April 1996 to detect the *mupA* gene in staphylococci, combined in the multiplex assay for *mecA* and 16S RNA described previously [17]. To date, *mupA* has not been detected in any isolates showing low-level mupirocin resistance. Taken together, these data do not support the recent suggestion that *S. aureus* isolates showing high- and low-level mupirocin resistance differ only in the location (plasmid *versus* chromosomal) or copy number of *mupA* [12].

In the present study, the heterogeneous location of *mupA* was also indicated by *Hinc*II-digested DNA, although a conserved *Nco*I fragment was present in all isolates. As the two *Nco*I target sites are intragenic (Fig. 1), these data suggest that *mupA* itself may be

conserved. However, as the *EcoRI* and *HincII* fragments predicted to hybridise with the probes extend beyond the *mupA* open reading frame, it seemed probable that the variation might result from alterations in the flanking DNA proximal to the resistance gene. Although the heterogeneity might have resulted from mutations affecting *EcoRI* and *HincII* target sites, *mupA* has been shown to be flanked by direct repeats of IS257 (which has no internal *EcoRI* or *HincII* target sites) and recombination [7, 19] or transposition [20] events involving these elements could also account for the differences. By amplifying the spacer regions, this study has shown that the copy of IS257 proximal to *mupA* can be present in either orientation and that the sizes of these spacer regions (with IS257 in either orientation) vary; a further indication of proximal heterogeneity. On the basis of these observations it is suggested that IS257-mediated events are responsible, at least in part, for the variable location of *mupA* in *S. aureus*. The molecular basis for the heterogeneity observed requires further detailed characterisation and studies are under way to sequence the flanking DNA proximal to *mupA* in selected isolates. Further hybridisation studies with new probes, amplification of *mupA*-IS257 spacer regions and sequencing are also necessary to determine whether these isolates show similar heterogeneity in the flanking DNA distal to *mupA*.

It is tempting to speculate that the variable location of *mupA* in epidemiologically unrelated isolates of certain strains of *S. aureus* might warrant evaluation as an epidemiological tool. Although high-level mupirocin resistance is present in diverse strains of *S. aureus*, its emergence in EMRSA-16 has caused particular problems. This epidemic strain is currently causing major infection control problems in many UK hospitals [3]. High-level mupirocin resistance was readily transferable *in vitro* from each of five EMRSA-16 isolates used in mating experiments in this study, which suggests that it will become far more widely disseminated in the future, mediated both by the spread of resistant EMRSA-16 and also by the horizontal transmission of resistance to other staphylococci, including MRSA. *mupA* was found on six different DNA fragments in isolates of EMRSA-16, and these differences correlated with variations in the digestion pattern of the mupirocin resistance plasmids. Hence, although the low numbers of EMRSA-16 isolates in this study did not permit correlation between fragment size and geographical area, this heterogeneity could potentially be used to monitor the dissemination of high-level mupirocin resistance within EMRSA-16 and other strains of *S. aureus*.

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