Acquisition of antibiotic resistance by *Staphylococcus aureus* in skin patients

JAY NAIDOO AND W. C. NOBLE

*From the Department of Bacteriology, Institute of Dermatology, Homerton Grove, London E9 6BX, UK*

**SUMMARY** Acquisition of resistance to neomycin, gentamicin, fusidic acid, or clindamycin has been observed in three strains of *Staphylococcus aureus* and data from three patients infected with these strains are presented in detail. Clindamycin resistance followed the expected pattern by appearing in a strain of *Staph. aureus* with dissociated resistance to erythromycin after treatment with erythromycin and clindamycin. Low-level resistance to fusidic acid appeared in two strains in the apparent absence of exposure to that antibiotic. Labile neomycin resistance was encountered in a previously sensitive strain after topical neomycin therapy. Gentamicin resistance appeared in all three strains after topical therapy. In all three strains, a labile resistance (presumably plasmid-mediated) occurred with minimum inhibitory concentrations (MICs) of 64-128 µg/ml but in one strain a stable resistance with MIC over 3000 µg/ml appeared.

*Staphylococcus aureus* infection in skin wards has been studied and reported previously (Wilson *et al.*, 1971; Noble and White, 1972). The large number of highly resistant staphylococci in such wards is more than probably due to the extensive use of topical and systemic antibiotics. Ayliffe *et al.* (1977) also surveyed staphylococcal carriage in dermatology and burns wards and found a high incidence of multiple antibiotic-resistant strains of *Staph. aureus* in both environments.

Multiple antibiotic-resistance in staphylococci is thought to arise from the acquisition of separate resistance genes (Dyke *et al.*, 1970). Neomycin and fusidic acid resistances are usually plasmid-mediated (Lacey, 1971; Lacey and Grinstein, 1972). Gentamicin resistance has until recently been rare in staphylococci, but there is now an increasing number of outbreaks (Porthouse *et al.*, 1976; Shanson *et al.*, 1976; Speller *et al.*, 1976; Bint *et al.*, 1977). In those strains that have been well documented the resistance has been plasmid-mediated (Soussey *et al.*, 1975; Wood *et al.*, 1977; Wyatt *et al.*, 1977). Clindamycin resistance normally occurs in strains already having erythromycin resistance of the dissociated type, reported by Garrod (1957).

In this study we describe gain of resistance to neomycin, fusidic acid, gentamicin, and clindamycin by three strains of *Staph. aureus* isolated from skin patients undergoing antibiotic and other therapy. The study is illustrated by reference to specific case histories and describes the nature of the genes' mediating resistance.

**Material and methods**

Strains of staphylococci were wild-type, clinical isolates from patients with diseases of the skin. All *Staph. aureus* isolates were coagulase-positive, DNase producers, fermented mannitol, and were markedly pigmented. *Staph. epidermidis* isolates were coagulase-negative, did not ferment mannitol, were not pigmented, and most did not produce DNase.

**GROWTH MEDIA**

Oxoid blood agar base, with or without antibiotic, was the solid growth medium. Mueller-Hinton agar was used for antibiotic sensitivity disc testing. Fluid growth medium was Oxoid nutrient broth.

**ANTIBIOTIC SENSITIVITY**

Initial testing was carried out by a modified Kirby-Bauer technique with Oxoid antibiotic sensitivity discs containing the following concentrations of antibiotics: penicillin 10 U (P); tetracycline 30 µg (T); neomycin 30 µg (N); erythromycin 15 µg (E); gentamicin 10 µg (G); clindamycin 2 µg (C); fusidic acid 10 µg (F); and methicillin 5 µg (M). One strain was also tested for resistance to streptomycin (S) by determination of the MIC. Incubation was at 37°C except for methicillin (30°C).

Minimum inhibitory concentrations (MIC) were
determined by inoculating a 1/1000 dilution of an overnight broth culture (c 10^4 cells/ml) on agar plates containing dilutions of the antibiotic. The MIC was the lowest concentration of antibiotic that prevented the formation of colonies on the agar.

**Bacteriophage-typing**

Typing was carried out with the International set of 24 Staph. aureus typing phages, plus the addition of a new phage (no. 90), kindly supplied by the Staphylococcal Reference Laboratory, Central Public Health Laboratories.

**Mitomycin-C Induction**

Cultures were grown to early logarithmic phase and exposed for 15 min to varying amounts of mitomycin-C in the range 0.1-5.0 µg/ml; the optimal amount for induction differed for each strain. Cultures were then centrifuged and resuspended in fresh warmed broth. After clearing, the lysate was centrifuged and the supernatant was stored with chloroform at 4°C.

**Plasmid Loss**

To induce loss of antibiotic resistance, resistant cultures were grown in broth containing 0.002% sodium dodecyl sulphate at 37°C. Dilutions were plated on nutrient agar, and sensitive variants were detected by replication on media containing the appropriate antibiotic.

**Results**

**Acquired Resistance During Antibiotic Therapy**

**Clindamycin**

Miss C was admitted with infected eczema. Before admission she had been receiving tetracycline cream, and on admission cloxacillin treatment (500 mg four times daily for 15 days) was started to eliminate a penicillin- and tetracycline-resistant strain already present. Unfortunately, this strain was not preserved for phage typing. Towards the end of cloxacillin therapy the patient became infected with a non-typing (NT) methicillin-resistant strain (strain 1) also resistant to high levels of streptomycin (MIC > 1000 µg/ml). This strain was isolated from eight other patients, including Mrs B referred to below; clindamycin resistance was not encountered in isolates from these other patients. Erythromycin resistance in this strain was of the dissociated type; first isolates had an intermediate resistance to erythromycin by disc test, but this was converted to full resistance after a course of therapeutic erythromycin (500 mg four times daily for 13 days) given towards the end of the cloxacillin treatment. The patient then received clindamycin (300 mg four times daily for 14 days), and the strain very quickly became resistant to clindamycin and its parent lincosycin, as determined by disc testing, providing a clear example of this form of resistance 'gain'. Gentamicin ear drops were applied for 29 days but no gentamicin resistance was encountered.

Strain 1 was not typable at RTD or RTD × 100 after growth of the cultures at 37°C or 42°C. Culture supernatants and mitomycin-C induced cultures yielded phages that lysed the Staph. aureus propagating strains PS6, PS47, PS75, PS77, and PS83 (Table 1). The induced phages were also lytic on the 77/84/90 strain discussed below.

**Fucidin and gentamicin**

Mrs B was infected with two distinct strains of Staph. aureus during her stay in hospital. Strain 2 was isolated from swabs taken on the day of admission (Fig. 1). The patient had been using neomycin ointment before admission. Strain 2 still persisted after erythromycin (250 mg four times daily for nine days), tetracycline (mouth wash), and cloxacillin (500 mg four times daily for 11 days) treatment. Fusidic acid resistance determined by disc test appeared after cloxacillin but before fucidin treatment (500 mg four times daily for six days); genta-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phage type</th>
<th>Source</th>
<th>Resistance pattern</th>
<th>Lytic pattern*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Staph. aureus</td>
<td>Mrs B and Miss C (infected eczema)</td>
<td>P T E M S</td>
<td>PS 6, 47, 75, 77, 83A</td>
</tr>
<tr>
<td>2</td>
<td>77/84/90</td>
<td>Mrs B (erythema multiforme)</td>
<td>P T N E</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>29/79/80/642E/54/85/90 Staph. epidermidis</td>
<td>Mr G (mycosis fungoides)</td>
<td>P T</td>
<td>PS 47, 75, 83A, 85</td>
</tr>
</tbody>
</table>

*International set of Staph. aureus phage propagating strains (PS) used as indicator.

NT = not typable at RTD and RTD × 100 after incubation at 37°C or 42°C
P = penicillin, T = tetracycline, N = neomycin, E = dissociated resistance to erythromycin, M = methicillin, S = high level streptomycin, and G = gentamicin.
Acquisition of antibiotic resistance by Staphylococcus aureus in skin patients

Gentamicin and neomycin

Figure 3 illustrates the change in resistance pattern of strain 3 colonising Mr G, who first received topical gentamicin (75 g of 0.3% cream) in November 1976. No gentamicin-resistant isolates were recovered after this therapy; all isolates were resistant to penicillin only (R/P) or to penicillin and tetracycline (R/PT). After further gentamicin treatment (30 g of cream) three months later, however, isolates resistant to penicillin and gentamicin were apparent. Treatment was changed to topical tetracycline (30 g of 3% ointment) preceding more gentamicin (60 g of cream). Consequently, the strain was found to be resistant to penicillin, tetracycline, and gentamicin. The patient then received neomycin sulphate ointment (215 g of 0.5% ointment in total) for four weeks, after which isolates

\[ \text{Fig. 1 Acquisition of resistance to fusidic acid and gentamicin in strain 77/84/90 (patient Mrs B):} \]
\[ P = \text{penicillin, } T = \text{tetracycline, } N = \text{neomycin,} \]
\[ E = \text{erythromycin, } M = \text{methicillin, } F = \text{fusidic acid,} \]
\[ G = \text{gentamicin, } C = \text{clindamycin.} + \text{indicates} \]
\[ \text{resistance to that antibiotic.} \]

\[ \text{Fig. 2 Acquisition of resistance to fusidic acid and gentamicin in strain NT (patient Mrs B). Symbols as for} \]
\[ \text{Fig. 1.} \]

\[ \text{Fig. 3 Acquisition of resistance to neomycin and gentamicin in strain 29/79/80/6/42E/54/85/90 (patient Mr G). Symbols as in Fig. 1.} \]
resistant to penicillin, gentamicin, and neomycin were recovered. After erythromycin treatment (250 mg four times daily for one week) only residual cells resistant to penicillin and gentamicin were isolated.

Throughout the study this patient's staphylococcus consistently typed with phages 29/79/80/6/42E/54/85/90 at 100 × RTD and was lysogenic. On induction it yielded phages lytic for PS47, PS75, PS83A, and PS85. After neomycin therapy Mr G was also found to carry a Staph. epidermidis strain R/PNG.

**GENTAMICIN-RESISTANCE CHARACTERISTICS**

The number of strains of gentamicin-resistant cocci isolated from the routine clinical samples increased during 1977. No gentamicin-resistant cocci were reported until 1976 when three strains were isolated; in 1977, nine apparently independent strains were isolated. Besides the pathogenic Staph. aureus strains infecting the skins of patients a number of gentamicin-resistant Staph. epidermidis strains have been found, often together with gentamicin-resistant Staph. aureus. The various antibiotic resistance patterns of strains isolated in 1977 are shown in Table 2.

<table>
<thead>
<tr>
<th>Staph. aureus</th>
<th>Phage type</th>
<th>Staph. epidermidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>P G *</td>
<td>3A</td>
<td>T G</td>
</tr>
<tr>
<td></td>
<td>29/79/80/6/42E/54/85/90</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>80/81/90/95</td>
<td>P T E G</td>
</tr>
<tr>
<td>P T E M G</td>
<td>NT</td>
<td>P T E M G</td>
</tr>
<tr>
<td>P T E N G</td>
<td>NT</td>
<td>P T E M C G</td>
</tr>
</tbody>
</table>

*Three other Staph. aureus strains had the resistance pattern R/P/G only; these strains were not available for phage typing but appeared epidemiologically distinct.

Gentamicin MICs for strains 1, 2, and 3 are given in Table 3. In strain 3 there were two markedly different levels of gentamicin resistance. The isolates R/P/G and R/PNG exhibited the extremely high-level resistance; only the R/PTG isolates had the lower MIC. The MICs of strains 1 and 2 were more difficult to determine accurately and tended to fluctuate between 64 and 128 μg/ml. This was probably due to inconsistent plasmid loss during growth of the culture (see below).

All strains acquiring resistance to gentamicin were cross-resistant to kanamycin and tobramycin. However, the high MIC isolates of strain 3 were, in addition, resistant to amikacin. It must be noted here that both the R/P/G and R/PTG isolates of this strain were sensitive to neomycin (MIC < 2 μg/ml) but resistant to kanamycin (MIC > 500 μg/ml). Similarly, strain 1 became resistant to kanamycin (MIC > 500 μg/ml) when gentamicin resistance was acquired, despite being sensitive to neomycin (MIC < 2 μg/ml) before and after acquisition of gentamicin resistance.

**LOSS OF RESISTANCE**

Loss of the acquired antibiotic resistances *in vitro* in response to sodium dodecyl sulphate treatment is given in Table 4. In each case a proportion of the sensitive variants were phage-typed and found to be the same as the parents.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Resistance tested</th>
<th>No. of isolates</th>
<th>No. sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. aureus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>77/84/90</td>
<td>G</td>
<td>712</td>
<td>348</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>3424</td>
<td>12*</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3878</td>
<td>0</td>
</tr>
<tr>
<td>NT</td>
<td>G</td>
<td>712</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>2607</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3509</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>2077</td>
<td>0</td>
</tr>
<tr>
<td>29/79/80/6/42E/54/85/90</td>
<td>(R/PTG)</td>
<td>G 1470</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>(R/PTG)</td>
<td>2421</td>
<td>0</td>
</tr>
<tr>
<td>Staph. epidermidis</td>
<td>G</td>
<td>847</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>(R/PNG) N</td>
<td>870</td>
<td>21</td>
</tr>
</tbody>
</table>

*All 12 variants were sensitive to penicillin but resistant to fusidic acid.

Loss of erythromycin resistance could not be demonstrated in the 2077 isolates of strain 1 tested. Fusidic acid resistances in both strains 1 and 2 were also stable and no sensitive variants have yet been obtained. The gentamicin resistances in these two strains, however, were extremely labile. Both cultures spontaneously gave rise to a high proportion of gentamicin-sensitive variants. Single colonies from gentamicin agar were also found to yield about 50% sensitive cells. In strain 3 the neomycin resistance was unstable; gentamicin resistance could be
lost from the R/PTG isolate but not from any of the high MIC isolates. Loss of gentamicin resistance could also be demonstrated in the Staph. epidermidis strain from Mrs B. Loss of kanamycin resistance accompanied loss of gentamicin resistance in strains 1, 2, and 3.

Discussion

The evolution of plasmids in Staph. aureus and the clinical importance of antibiotic resistance has been reviewed by Lacey (1975) and the specific role of the skin by Noble and Naidoo (1978). This study illustrates gain in resistance to clindamycin, fusidic acid, neomycin, and gentamicin in vivo in three clinical strains of Staph. aureus previously sensitive to the antibiotics. Appearance of resistance in each instance (except fusidic acid resistance) was coincident with the therapeutic use of antibiotics, topically applied in the case of neomycin and gentamicin.

The NT strain infecting Mrs B and Miss C was initially resistant to penicillin, tetracycline, erythromycin, methicillin, and streptomycin. The appearance of clindamycin resistance in this strain was typical of exposure of a strain with dissociated erythromycin resistance to erythromycin and then clindamycin (Weisblum et al., 1971).

Strains 1 and 2 acquired gentamicin resistance apparently simultaneously on one patient. The gentamicin-resistant isolate of strain 1 was sensitive to fusidic acid while that of strain 2 was resistant to fusidic acid, indicating that the two resistances were acquired independently. The gentamicin resistances in both strains were extremely labile and therefore probably plasmid-mediated. No linkage was observed with any of the other resistances. Fusidic acid resistance was stable in both strains and may be chromosomal; in any case it was of a very low level. MICs of the resistant and sensitive cultures of each strain were: 4 µg/ml and < 0.1 µg/ml (NT), 8 µg/ml and 0.8 µg/ml (77/84/90). There was no association of fusidic acid resistance with the penicillinase plasmid, that is, resistance to penicillin in strain 77/84/90 could be lost independently of fusidic acid resistance (Lacey and Grinstead, 1972). Why the resistance should arise in the absence of the antibiotic is open to speculation. No other patient in the ward had received fusidic acid during Mrs B’s period in hospital or for some months before her admission. Ayliffe et al. (1977) reported a high percentage of strains resistant to fusidic acid although use of this antibiotic had been restricted.

The neomycin resistance acquired by strain 3 was also apparently plasmid-governed. The first neomycin-resistant isolates picked up were very labile in nature when subcultured; in later isolates the resistance seemed to have stabilised but could still be lost with sodium dodecyl sulphate treatment. The gentamicin resistance acquired by various isolates of the same strain was found to consist of two types: a stable, abnormally high-level resistance and a presumably plasmid-mediated lower-level resistance. Interestingly, after Mr G had received his first treatment with gentamicin in November 1976, no gentamicin-resistant isolates were picked up. This seems to indicate that the gene responsible for the resistance was not present at this stage for the resistance would have been selected earlier. However, after the second application of gentamicin three months later, gentamicin-resistant isolates were apparent. Initial resistance patterns of the strain were R/P and R/PT. After gentamicin and neomycin treatment a variety of resistant isolates were also present— R/PG, R/PTG, and R/PNG. The lower gentamicin resistance was only present in the R/PTG isolate; other isolates possessed the stable high-level resistance. As yet, there is no explanation for this extraordinary high level of gentamicin resistance or why gentamicin resistance of the two types should appear simultaneously in the same strain. It may be pertinent that Mr G was receiving, in addition to antibiotic therapy, PUVA treatment (oral psoralen and UVA irradiation). However, the significance (if any) of this cannot yet be determined.

The source of the resistance plasmids has not been found. Transfer of antibiotic resistance between different strains of Staph. aureus is known to occur in vivo (Lacey and Richmond, 1974) and may well have occurred naturally between strains 1 and 2 infecting Mrs B since transfer between these strains occurs on skin under experimental conditions (Naidoo and Noble, 1978). But from where did these Staph. aureus strains acquire the plasmid? Lacey (1975) suggested that Staph. epidermidis strains of the normal microflora may act as reservoirs of resistance plasmids. In many of our patients infected with gentamicin-resistant Staph. aureus, we have also isolated gentamicin-resistant Staph. epidermidis. The characteristics of one Staph. epidermidis have been given. The strain isolated from Mrs B was found to harbour a gentamicin-resistant plasmid, which was equally labile and had a similar MIC to the other two strains colonising the patient. It is possible, of course, that this is not the source strain but was also lysogenized from elsewhere. In all of seven other Staph. epidermidis isolates from different patients the gentamicin resistance has been found to be labile and therefore probably plasmid-mediated. In vivo resistance transfer between Staph. epidermidis and Staph. aureus has been reported (Witte, 1977). The high incidence of gentamicin-resistant Staph.
epidermidis and Staph. aureus which we have isolated simultaneously from patients strongly supports the idea that plasmid transfer between these species may occur in vivo.

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References


Requests for reprints to: Dr W. C. Noble, Department of Bacteriology, The Institute of Dermatology, St John’s Hospital for Diseases of the Skin, Homerton Grove, London E9 6BX, UK.