MOLECULAR EPIDEMIOLOGY OF MULTIRESISTANT *STAPHYLOCOCCUS AUREUS* IN AUSTRALIAN HOSPITALS

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SUMMARY. Antibiotic multiresistant isolates of *Staphylococcus aureus* from outbreaks of nosocomial infection throughout Australia were found to possess essentially similar patterns of antibiotic resistance. Plasmid DNA profiles from these isolates exhibited a common pattern of large plasmids, of $(15-22) \times 10^6$ mol. wt, associated with resistance to gentamicin, kanamycin and tobramycin, plasmids of 3×10^6 mol. wt, mediating resistance to chloramphenicol, and cryptic plasmids of 1×10^6 mol. wt. Restriction endonuclease digestion confirmed the presence of related plasmids in isolates from all the hospitals that were surveyed. The homogeneity of these organisms suggests the dissemination of a multiresistant, plasmid-bearing strain of *S. aureus*, or its derivatives, among geographically-separated hospitals in Australia.

INTRODUCTION

Strains of antibiotic multiresistant *Staphylococcus aureus* are currently implicated in an epidemic of nosocomial infections that has spread to hospitals in most major population centres in Australia. Initially isolated from hospital patients in Melbourne and Sydney during the late 1970's (McDonald, Hurse and Sim, 1981; King *et al.*, 1982; Pavillard *et al.*, 1982), these strains have subsequently been isolated in Adelaide, Brisbane, Darwin, Hobart and Perth. They persist as endemic pathogens in many of the large public hospitals and in some instances account for up to 50% of all *S. aureus* isolates (Pavillard *et al.*, 1982). The organisms involved pose a serious clinical problem because they are frequently resistant to almost all the available antibiotics, including penicillin, methicillin, streptomycin, erythromycin, clindamycin, tetracycline, gentamicin and kanamycin. The emergence of strains resistant to rifampicin and fusidic acid in addition has meant that vancomycin has often been the only effective antimicrobial agent (Pavillard *et al.*, 1982; Sorrell *et al.*, 1982).

The value of plasmid analysis in the epidemiological study of nosocomial infection with *S. aureus* is well established (McGowan *et al.*, 1979; El Solh *et al.*, 1980; Locksley *et al.*, 1982). We have examined previously strains of antibiotic multiresistant *S. aureus*, isolated from patients in Melbourne hospitals, for the presence of resistance

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plasmids (Lyon *et al.*, 1982; Lyon, May and Skurray, 1983). The plasmid profiles of these strains were basically similar, with three classes of plasmid present: large plasmids of 18 or 22×10^6 mol.wt, mediating resistance to gentamicin, kanamycin and tobramycin, plasmids of 3×10^6 mol.wt, mediating resistance to chloramphenicol, and small plasmids of 1×10^6 mol.wt, with no attributable function. The purpose of this investigation was to compare the plasmid profiles of isolates of multiresistant *S. aureus* from widely-separated Australian hospitals, in order to determine whether the present outbreak of nosocomial infections is due to the dissemination of a common epidemic strain of *S. aureus*, or to the emergence of unrelated local strains.

MATERIALS AND METHODS

Bacterial cultures. Clinical isolates (105) of *Staphylococcus aureus*, isolated during the period April 1981 to July 1982, were obtained from four hospitals in Sydney, two in Adelaide, and one each in Brisbane, Darwin, Hobart and Perth. To these were added four isolates from Melbourne hospitals. We are grateful to Dr J. Andrew and Dr D. Olden (St Vincent's Hospital, Melbourne), Dr V. Asche (Queen Victoria Hospital, Melbourne), Dr M. Guinness (Queen Elizabeth Hospital, Adelaide), Dr J. Harkness (St Vincent's Hospital, Sydney), Mr A. Hewstone (Royal Children's Hospital, Melbourne), Dr J. Hine (Royal Brisbane Hospital, Brisbane), Dr K. Karthigasu (Department of Microbiology, University of Western Australia), Ms V. Lennox (Royal North Shore Hospital, Sydney), Dr R. Munro (Institute of Clinical Pathology and Medical Research, Westmead, Sydney), Ms S. Savage (Casuarina Hospital, Darwin), Dr J. Spicer (Alfred Hospital, Melbourne), Dr T. Steele (Institute of Medical and Veterinary Science, Adelaide), Dr R. Tucker (Royal Hobart Hospital, Hobart) and Miss A. Vickery (Royal Prince Alfred Hospital, Sydney) for providing these isolates.

General methods. The media used, the methods for the determination of antibiotic and inorganic ion susceptibilities, minimum inhibitory concentrations (MICs) and penicillinase production, and the plasmid elimination procedures were described previously by Lyon *et al.* (1983).

Plasmid DNA isolation. The rapid plasmid DNA isolation technique previously described by Lyon *et al.* (1983) was modified to increase the yield of plasmid DNA. Cleared lysates from 30 ml (instead of 10 ml) of overnight bacterial cultures were prepared, with a five-fold increase in the volumes of the reagents. The concentration of lysostaphin (Sigma Chemical Company, St Louis, MO 63178, USA) was lowered to 0.1 g/L and a different TE-sucrose buffer (50 mM Tris, 50 mM EDTA, sucrose 15% w/v, pH 8.5) was used to enhance lysis. The cleared lysate was subjected to three successive potassium acetate and ethanol precipitations to remove contaminating sodium dodecyl sulphate and the DNA pellet was finally resuspended in 60 μ l of 10 mM Tris, 1 mM EDTA (pH 7.5) containing 10 mg/L RNAase IA (Sigma).

Restriction endonuclease digestion and agarose gel electrophoresis. The methods were as described by Lyon *et al.* (1983). DNA samples (12 μ l) were cleaved with restriction endonucleases *Eco*R1 (Boehringer Mannheim Australia Pty Ltd, North Ryde, 2113), *Hpa*II (New England Biolabs, Beverly, MA 01915, USA) or *Hind*III (Bethesda Research Laboratories Inc., Gaithersburg, MD 20760, USA). *Hind*III fragments of lambda viral DNA (Miles Laboratories Australia Pty Ltd, Mulgrave, 3170) were employed as standards; sizes in kilobases (kb) were as determined by Phillipsen, Kramer and Davis (1978, cited by Southern, 1979).

RESULTS

Characteristics of S. aureus isolates

Of the 109 *S. aureus* isolates from geographically separated Australian hospitals, >90% were resistant to penicillin, methicillin, streptomycin, tetracycline, erythromycin and clindamycin. Resistance to gentamicin, kanamycin and tobramycin was

TABLE
Characteristics of representative clinical isolates of S. aureus from Australian hospitals

Isolate no.	Source hospital	City	Bacteriophage type (100 RTD*)	Antibiotic sensitivity pattern*											
				Pc	Mc	Sm	Nm	Km	Gm	Tm	Ak	Cm	Cl	Em	Tc
SK456	Royal Children's Hospital	Melbourne	29/53/83A/79/85	R	R	R	S	R	R	R	S	S	R	R	R
SK634	Queen Elizabeth Hospital	Adelaide	80/85	R	R	R	S	R	R	R	S	S	R	R	R
SK568	Royal Brisbane Hospital	Brisbane	80	R	R	R	S	R	R	R	S	S	R	R	R
SK565	Royal Brisbane Hospital	Brisbane	80	R	R	R	S	R	R	R	S	S	R	R	R
SK529	St Vincent's Hospital	Melbourne	80/85	R	R	R	S	R	R	R	S	S	R	R	R
SK436	Royal North Shore Hospital	Sydney	80/85	R	R	R	S	R	R	R	S	S	R	R	R
SK625	Westmead Centre	Sydney	80/85	R	R	R	S	R	R	R	S	S	R	R	R
SK588	Royal Prince Alfred Hospital	Sydney	80	R	R	R	S	R	R	R	S	S	R	R	R
SK637	Queen Elizabeth Hospital	Adelaide	29/79/80	R	R	R	S	R	R	R	S	S	R	R	R
SK676	Institute of Medical and Veterinary Science	Adelaide	29/79/80†	R	S	R	S	R	R	R	S	S	R	R	R
SK569	Royal Brisbane Hospital	Brisbane	80	R	R	R	S	R	R	R	S	S	R	R	R
SK578	Casuarina Hospital	Darwin	77/80	R	R	R	S	R	R	R	S	S	R	R	R
SK963	QELI Medical Centre	Perth	84/85	R	R	R	S	R	R	R	S	S	R	R	R
SK604	Queen Victoria Hospital	Melbourne	77/80/84/85	R	R	R	S	R	R	R	S	S	R	R	R
SK572	Royal Brisbane Hospital	Brisbane	80	R	R	R	S	R	R	R	S	S	R	R	R
SK656	Royal Hobart Hospital	Hobart	NT	R	R	R	S	R	R	R	S	S	R	R	R
SK460	Alfred Hospital	Melbourne	83A	R	R	R	R	R	R	R	R	S	R	R	R
SK597	Royal Prince Alfred Hospital	Sydney	NT	R	R	R	R	R	R	R	R	R	R	R	R
SK597	Royal Prince Alfred Hospital	Sydney	NT	R	R	R	R	R	R	R	R	R	R	R	R

Pc = benzyl penicillin, Mc = methicillin, Sm = streptomycin, Nm = neomycin, Km = kanamycin, Gm = gentamicin, Tm = tobramycin, Ak = amikacin, Cm = chloramphenicol, Cl = clindamycin, Em = erythromycin, Tc = tetracycline; R = resistant, S = sensitive.

* Bacteriophage type at 100 × routine test dilution (RTD).

† Bacteriophage type at RTD.

NT = not typable by standard bacteriophages.

found in 72% of the strains, 36% were resistant to chloramphenicol, 16% to neomycin, and 11% to amikacin. No strain resistant to fusidic acid, rifampicin or vancomycin was encountered. These organisms also generally produced a penicillinase and were resistant to cadmium and mercury ions. Most strains could not be bacteriophage-typed at routine test dilution (RTD) and some remained untypable even at 100 RTD. Data for representative isolates are given in the table; two of these isolates (SK529 and SK456) have been described previously (Lyon *et al.*, 1983).

Minimum inhibitory concentration (MIC) determinations with selected isolates revealed two distinct types of aminoglycoside resistance. Strains resistant to gentamicin, kanamycin and tobramycin showed a low level of resistance, with MICs of 64–256 mg/L, 128–512 mg/L and 32–256 mg/L, respectively. In contrast, isolates resistant to gentamicin, kanamycin, tobramycin, neomycin and amikacin demonstrated high-level resistance with MICs of 4096 mg/L, 4096 mg/L, 256–4096 mg/L, 512–2048 mg/L and 16–32 mg/L, respectively.

The multiresistant *S. aureus* isolates from other parts of Australia, therefore, appear similar to their Melbourne counterparts already described (Lyon *et al.*, 1983), except that resistance to fusidic acid and rifampicin, noted among Melbourne isolates, was not observed.

Plasmid analysis of S. aureus isolates

Each of the 109 isolates of *S. aureus* examined above was screened for the presence of plasmid DNA. Agarose gel electrophoresis of DNA isolated from the 19 representative strains in the table is shown in fig. 1. For convenience, the order of strains across the figure corresponds to that down the table. The plasmid profiles of the 19 strains demonstrated a common pattern consisting of three distinct plasmid classes.

The representative strains all possessed a large plasmid, in the size range $(15-22) \times 10^6$ mol. wt, which ran more slowly than (or above) the band of chromosomal DNA (fig. 1). The first 16 of these strains exhibited the low-level resistance to gentamicin, kanamycin and tobramycin characteristic of the majority of clinical isolates. Loss of the large plasmid during plasmid elimination procedures was accompanied by a loss of this phenotype. Linked resistance to gentamicin, kanamycin and tobramycin is, therefore, encoded by a group of at least six plasmids: pSK4/16 with a mol. wt of 22×10^6 (fig. 1, lanes a–c); pSK15, mol. wt 19.5×10^6 (lane d); pSK1, mol. wt 18×10^6 (lanes e–m); pSK9, mol. wt 17×10^6 (lane n); and pSK14, mol. wt 15.5×10^6 (lanes o and p). Twenty-one percent of the isolates with this low-level resistance phenotype did not carry any large plasmids. Isolates bearing chromosomal resistance to these three aminoglycosides have been found in Melbourne hospitals (Lyon *et al.*, 1983), and it would appear that, elsewhere in Australia, low-level resistance to gentamicin, kanamycin and tobramycin is also mediated by chromosomally encoded determinants in some organisms.

The remaining three isolates in fig. 1 (SK460, SK592 and SK597) demonstrated high-level resistance to gentamicin, kanamycin, tobramycin, neomycin and amikacin. Loss of the plasmid pSK7 (mol. wt 15×10^6) from SK460 was not associated with any change in antibiotic-resistance pattern, which suggests that in this isolate, as with other clinical isolates with this phenotype that do not carry large plasmids, high-level

FIG. 1.—Agarose gel (1.1% w/v) electrophoresis of rapidly isolated *S. aureus* DNA: (a) SK456, (b) SK634, (c) SK568, (d) SK565, (e) SK529, (f) SK436, (g) SK625, (h) SK588, (i) SK637, (j) SK676, (k) SK569, (l) SK578, (m) SK963, (n) SK604, (o) SK572, (p) SK656, (q) SK460, (r) SK592, (s) SK597. Bands correspond to plasmid DNA (pSK) or chromosomal DNA (Chr). The covalently closed circular (CCC) and open circular (OC) forms of pSK2/5 are labelled.

aminoglycoside resistance is mediated by the chromosome. Plasmid-less or aminoglycoside-sensitive derivatives of strains SK592 and SK597 have not been obtained in curing attempts.

Resistance to chloramphenicol in Melbourne isolates of multiresistant *S. aureus* was shown to be encoded by plasmids of 3×10^6 mol. wt such as pSK2 and pSK5 (Lyon *et al.*, 1983). The 11 chloramphenicol resistant strains (table) exhibited two plasmid bands corresponding to the covalently closed circular (CCC) and open circular (OC) forms of such previously characterised plasmids (fig. 1).

The third class comprised small plasmids of approximately 1×10^6 mol. wt similar to the cryptic plasmids pSK3 and pSK6 previously described in Melbourne isolates (Lyon *et al.*, 1983). Such plasmids can be seen in 13 of the strains in fig. 1.

FIG. 2.—Agarose gel (1.2% w/v) electrophoresis of rapidly isolated *S. aureus* plasmid DNA cleaved with restriction endonuclease *EcoRI*: (a) SK456, (b) SK634, (c) SK568, (d) SK565, (e) SK529, (f) SK436, (g) SK625, (h) SK588, (i) SK637, (j) SK676, (k) SK569, (l) SK578, (m) SK963, (n) SK604, (o) SK572, (p) SK656, (q) SK460, (r) SK592, (s) SK597, (t) lambda DNA cleaved with *HindIII*; fragment sizes in kilobases (kb) are shown on the right. The single linear fragments formed by *EcoRI* digestion of pSK5 and pSK3 are labelled pSK5f1 and pSK3f1, respectively. Plasmids pSK2 and pSK6 are not cleaved by *EcoRI* and remain as CCC and OC forms. Other bands correspond to *EcoRI* fragments of the large plasmids.

Restriction endonuclease analysis

To compare the relatedness of the various plasmids, DNA from the 19 representative *S. aureus* isolates was separately digested with the restriction endonucleases *EcoRI*, *HpaII* and *HindIII* and analysed on agarose gels. The *EcoRI* and *HpaII* restriction patterns are shown in figs. 2 and 3, respectively.

These digests differentiated the seven large plasmids and revealed an underlying relationship between the members of this class. pSK7 was unexpectedly found to be related to the six plasmids (pSK1, 4, 9, 14, 15 and 16) described above as encoding

FIG. 3.—Agarose gel (1.0% w/v) electrophoresis of rapidly isolated *S. aureus* plasmid DNA cleaved with restriction endonuclease *Hpa*II: (a) SK456, (b) SK634, (c) SK568, (d) SK565, (e) SK529, (f) SK436, (g) SK625, (h) SK588, (i) SK637, (j) SK676, (k) SK569, (l) SK578, (m) SK963, (n) SK604, (o) SK572, (p) SK656, (q) SK460, (r) SK592, (s) SK597; (t) lambda DNA cleaved with *Hind*III; fragment sizes in kb are shown on the right. The two linear fragments formed by *Hpa*II digestion of pSK2/5 are labelled pSK2/5f1 and pSK2/5f2; the single linear fragment formed by *Hpa*II digestion of pSK3 is labelled pSK3f1. Plasmid pSK6 is not cleaved by *Hpa*II and remains as CCC and OC forms. Other bands correspond to *Hpa*II fragments of the large plasmids.

gentamicin, kanamycin and tobramycin resistance. The distinction between pSK4 (found in strains SK456 and SK568) and pSK16 (strain SK634), although not evident in *Eco*RI digests (fig. 2), was apparent in the *Hpa*II (fig. 3) and *Hind*III (data not presented) restriction patterns. These patterns also confirmed the prevalence of pSK1 (found in strains SK529, SK436, SK625, SK588, SK637, SK676, SK569, SK578 and SK963), and the presence of pSK14 in two isolates (SK572 and SK656) and pSK7 in three isolates (SK460, SK592 and SK597).

The *Eco*RI cleavage pattern of pSK1 (fig. 2, lanes e–m) had bands common to *Eco*RI digests of the other six plasmids, and four of the seven fragments in the *Hpa*II

cleavage pattern of pSK1 (fig. 3, lanes e-m) were common to *Hpa*II digests of all seven plasmids. Two of the *Eco*RI and six of the *Hpa*II fragments produced by digestion of pSK1 were common to digests of pSK4 (lanes a and c) and pSK16 (lane b). The sizes of the unique fragments were consistent with the insertion of an extra 6.5 kb of DNA within these plasmids. *Eco*RI and *Hpa*II digests of pSK9 (lane n) and pSK14 (lanes o and p) indicate that these plasmids have been produced by DNA deletion of a plasmid such as pSK1; but the cleavage patterns of pSK15 (lane d) suggest that this plasmid may possess both a DNA insert like pSK16 and a DNA deletion similar to pSK14. Finally, the *Eco*RI and *Hpa*II restriction patterns of pSK7 (figs. 2 and 3, lanes q-s) were identical to those of pSK1 except for a single smaller fragment in each case; we attribute this finding to the absence in pSK7 of a 4.5 kb segment of DNA present in pSK1.

A high degree of identity among the plasmids of 3×10^6 mol. wt mediating chloramphenicol resistance was indicated by the *Eco*RI (fig. 2), *Hpa*II (fig. 3) and *Hind*III (data not presented) restriction patterns. Ten isolates (SK568, SK565, SK529, SK436, SK625, SK676, SK578, SK604, SK572 and SK656) possessed pSK2, a chloramphenicol-resistance plasmid characterised by two *Hpa*II fragments, a single *Hind*III and no *Eco*RI site; one isolate (SK597) possessed a second chloramphenicol resistance plasmid, pSK5, characterised by two *Hpa*II fragments and single sites for *Hind*III and *Eco*RI (Lyon *et al.*, 1983).

Restriction endonuclease digestion (figs. 2 and 3) confirmed the presence of pSK3 in twelve isolates (SK634, SK565, SK529, SK436, SK625, SK569, SK578, SK572, SK656, SK460, SK592 and SK597) and pSK6 in one isolate (SK963); the single sites for *Eco*RI and *Hpa*II present in pSK3 are absent in pSK6.

DISCUSSION

Outbreaks of nosocomial infection caused by strains of multiresistant *S. aureus* have occurred in many Australian cities during recent years. We have compared clinical isolates of *S. aureus* from such episodes in Melbourne, Sydney, Adelaide, Brisbane, Darwin, Hobart and Perth. These organisms were found to possess similar antibiotic-resistance patterns and related plasmid DNA profiles. Homogeneity has also been noted in the biochemical characteristics of Australian isolates (Gedney and Lacey, 1982). Such findings are persuasive evidence for the dissemination of a multiresistant, plasmid-bearing strain of *S. aureus*, or its derivatives, among geographically-separated hospitals in Australia.

One of the most important characteristics of the strains of *S. aureus* responsible for these epidemics is their plasmid DNA profile. These organisms possess elements of only three basic classes of plasmid DNA: large plasmids of $(15-22) \times 10^6$ mol. wt associated with resistance to gentamicin, kanamycin and tobramycin, plasmids of 3×10^6 mol. wt mediating chloramphenicol resistance, and cryptic plasmids of approximately 1×10^6 mol. wt. The other resistance characters exhibited by these epidemic strains are invariably encoded by the chromosome.

Representatives of a family of at least six structurally related plasmids mediating gentamicin, kanamycin and tobramycin resistance were found in *S. aureus* isolates

from all the cities surveyed. Such isolates demonstrated the low-level resistance to these three aminoglycosides previously interpreted to indicate enzymic modification by a 6'-acetyltransferase and a 2''-phosphotransferase (Lyon *et al.*, 1983). The plasmid pSK1 of 18×10^6 mol. wt was deemed to be the prototype of the family because it was the most common species, with examples present in isolates from every city except Hobart. The other plasmids may have evolved from pSK1 through either the insertion (pSK4, pSK16) or the deletion (pSK9, pSK14) of DNA segments. Plasmid pSK15 appears to have been derived by a combination of these events, because restriction endonuclease analysis revealed similarities with both pSK16 and pSK14. This group of plasmids, therefore, provides further evidence for the evolution of antibiotic resistance plasmids in *S. aureus*, a process previously documented for plasmids encoding resistance to gentamicin (Jaffe *et al.*, 1982) and penicillin and metal ions (Shalita, Murphy and Novick, 1980).

On the basis of restriction endonuclease digests, the plasmid pSK7 of 15×10^6 mol. wt was found to be closely related to the family of gentamicin, kanamycin and tobramycin resistance plasmids, but all attempts to assign a phenotype to this plasmid have been inconclusive. pSK7 was only present in isolates that demonstrated high-level resistance to gentamicin, kanamycin, tobramycin, neomycin and amikacin, but apparently plays no role in determining that character. Thus, in these isolates, and in others that do not carry large plasmids, high-level aminoglycoside resistance appears to be encoded by the chromosome. In contrast, low-level resistance to gentamicin, kanamycin and tobramycin may be mediated by the same genes at either a plasmid or a chromosomal locus (Lyon *et al.*, 1983). We are now investigating the possibility that the 4.5-kb DNA segment, absent from pSK7 but present in pSK1 and the other gentamicin, kanamycin and tobramycin resistance plasmids, may be a transposon bearing the genes for low-level aminoglycoside resistance. The plasmid pSK7 may then be an example of pSK1 before acquisition of the transposon or after the excision of such an element.

Strains demonstrating the high-level resistance to aminoglycosides appear distinct from the epidemic *S. aureus* strains, which exhibit either low-level resistance or sensitivity to gentamicin, kanamycin and tobramycin. Although often possessing the same chloramphenicol and cryptic plasmid profiles, these high-level resistance strains are less widespread and may only constitute endemic varieties.

The archetypal epidemic strain of *S. aureus* may have had a plasmid complement consisting of pSK1, pSK2 and pSK3, because these plasmids are found throughout Australia; strain varieties would then have evolved by subsequent recombination events or plasmid loss. Plasmid DNA analysis has enabled an insight into the evolution of these strain varieties within individual institutions. For example, strains carrying four different plasmids (pSK1, pSK4, pSK14 and pSK15) mediating resistance to gentamicin, kanamycin and tobramycin were isolated from a Brisbane hospital. Furthermore, organisms carrying pSK9 were only found in two Melbourne hospitals where they coexisted with strains that carry pSK1.

Plasmid analysis has also proved to be an ideal technique for the study of epidemiology of these organisms because the relatedness of isolates in a given episode is readily demonstrated. This is exemplified by the situation in one Sydney hospital where resistance to gentamicin, kanamycin and tobramycin was exclusively chromosomally mediated in the isolates examined, and in the Perth outbreak, where the

examined isolates all possessed a plasmid profile containing pSK1 and pSK6; the latter was rarely isolated elsewhere. The method is especially useful when traditional epidemiological markers such as bacteriophage-type and antibiotic sensitivity pattern fail to distinguish between individual strain varieties.

The difficulty of eradicating multiresistant *S. aureus* from a hospital once it becomes endemic is recognised in Australia (King *et al.*, 1982) and overseas (Boyce, 1981). Molecular epidemiology promises to be very useful in the surveillance, and hence in the development of policies for the control, of nosocomial infection caused by these organisms.

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