ANTIBIOTIC SUSCEPTIBILITIES AND PLASMID PROFILES OF NOSOCOMIAL METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*: A RETROSPECTIVE STUDY

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SUMMARY. Methicillin-resistant *Staphylococcus aureus* strains isolated at a single Melbourne Hospital between 1969 and 1981 were examined for susceptibility to a range of antimicrobial agents and for the presence of plasmid DNA. Isolates obtained during 1969 possessed a plasmid of mol. wt 20×10^6 , encoding heavy metal resistance and penicillinase production, and a plasmid of mol. wt 2.8×10^6 , mediating tetracycline resistance. In the majority of isolates obtained after 1973, these functions were chromosomally encoded. Before 1980, both high- and low-level chromosomally-encoded gentamicin resistances were encountered, whereas isolates from 1980 and 1981 displayed low-level gentamicin resistance only; the latter phenotype was most commonly mediated by a plasmid of mol. wt 18×10^6 that also encoded resistance to tobramycin and kanamycin. Chloramphenicol resistance in strains isolated throughout the period was mediated by one of three plasmids, each of mol. wt $c. 3 \times 10^6$.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* strains were first detected in Australia at a Sydney hospital in October 1965 (Rountree and Beard, 1968) and by 1970, 18% of all *S. aureus* strains isolated at that hospital were resistant to methicillin (Rountree and Vickery, 1973). The majority of these isolates were also resistant to streptomycin and tetracycline and some demonstrated kanamycin and chloramphenicol resistance. Methicillin-resistant *S. aureus* were also isolated in other countries, including France (Courtieu *et al.*, 1964), Switzerland (Kayser and Mak, 1972) and the United States of America (Barrett, McGehee and Finland, 1968), during the 1960s. Following the increased usage of gentamicin in the early 1970s, gentamicin-resistant *S. aureus* were isolated from patients in hospitals in Melbourne (Perceval, McLean and Wellington, 1976) and Sydney (Rountree, 1978) during the mid-1970s. In 1978, strains resistant to gentamicin, chloramphenicol and methicillin comprised <2% of all *S. aureus* isolates at one teaching hospital in Victoria; but, by March 1979, 30% possessed this phenotype (Pavillard *et al.*, 1982). Such strains are now endemic in many Melbourne hospitals.

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This rapid spread of gentamicin and chloramphenicol resistance determinants among methicillin-resistant *S. aureus* strains was suggestive of plasmid-mediated resistance. Molecular analysis of plasmid DNA isolated from strains of *S. aureus* isolated in Melbourne has identified separate plasmids encoding resistance to chloramphenicol and to the aminoglycosides, gentamicin, tobramycin and kanamycin (Lyon *et al.*, 1982; Lyon, May and Skurray, 1983). However, the remainder of antibiotic-resistance determinants—including those for resistance to penicillin (mediated by penicillinase), erythromycin, streptomycin, tetracycline, kanamycin and neomycin—which have often been reported to be plasmid-encoded in strains isolated elsewhere (Lacey, 1975), were found to be chromosomally mediated in the Melbourne isolates.

We have, therefore, begun a retrospective investigation of the location and molecular relationships of resistance determinants in nosocomial strains of *S. aureus* isolated in Australia. In this paper we compare the antibiotic-resistance patterns and plasmid-DNA profiles of methicillin-resistant *S. aureus* isolated at a large Melbourne hospital.

MATERIALS AND METHODS

Strains of Staphylococcus aureus. Clinical isolates of methicillin-resistant *S. aureus* were collected and identified at the Royal Children's Hospital, Melbourne, between 1969 and 1981. A total of 353 isolates was obtained from patients and staff (the latter contributed 7% of the total number of isolates) located in burns, cardiac, diabetic, gastroenterology, general surgical, neonatal, oncology and orthopaedic wards and an isolation unit for patients bearing methicillin-resistant *S. aureus*. Organisms were isolated from blood, urine and various body sites, with 71% of the total from burns and nasal swabs. Isolates were stored at the Bacteriology Department, Royal Children's Hospital, either on agar slopes or vacuum-dried in ampoules, and subsequently made available to this laboratory. After recovery, 9% of cultures stored on slopes demonstrated an irreversible loss of methicillin resistance, similar to that reported by Lacey and Stokes (1979); all cultures that were vacuum-dried in ampoules retained phenotypic expression of methicillin resistance.

Strain SA113 (Iordanescu and Surdeanu, 1976), a restriction-deficient derivative of the plasmid-free strain NCTC8325, was kindly supplied by Dr S. Iordanescu, Institute Cantacuzino, Bucharest, Romania. A chromosomal mutant of SA113 (resistant to novobiocin 1 mg/L and rifampicin 10 mg/L) was selected in this laboratory and designated SK982 (Lyon, May and Skurray, 1984b).

General procedures. L-broth and L-agar media, and methods for determination of antibiotic and heavy metal resistance, penicillinase production and plasmid elimination were as described by Lyon *et al.* (1983). Resistance to ethidium bromide (Sigma Chemical Company, St Louis, MO 63178, USA) was determined at a concentration of 50 mg/L in L-agar. The incubation temperature employed throughout was 37°C unless otherwise stated. Minimum inhibitory concentrations (MIC) of antibiotics were determined by broth dilution (in L-broth) with an inoculum of *c.* 10^4 cfu/ml, or by agar dilution (in L-agar) with an inoculum of *c.* 10^4 cfu from an overnight culture; all were incubated for 48 h.

Plasmid DNA isolation. Plasmid DNA was routinely isolated by a rapid procedure previously described (Lyon *et al.*, 1983). Plasmid profiles of some strains were verified by an alternative DNA isolation procedure described to us by Dr R.P. Novick (Public Health Research Institute of the City of New York) and subsequently modified by B. Lyon, J. May and R. Skurray in this laboratory. In this alternative procedure, cells were harvested from 16–18 h, 50-ml shaken cultures and washed with 5 ml of TES (50 mM Tris, 5 mM EDTA, 50 mM NaCl at pH 8.0). The pelleted cells were resuspended in 100 μ l of a solution of lysostaphin (Sigma) 0.2 g/L in TE (10 mM Tris, 1 mM EDTA at pH 7.8), containing sucrose 25% w/v. The cell suspension was incubated for 45 min and, after addition of 4 ml of sodium dodecyl sulphate (SDS) 4% in TE

(pH 11.5), re-incubated for a further 20 min. The lysate was neutralised by the addition of 200 μ l of 2M Tris (pH 7.0) and 850 μ l of SDS 4% in TE (pH 8.0) followed by the addition of 1.2 ml of 5M NaCl. The solution was stored at 4°C overnight. The SDS was pelleted by centrifugation and the supernatant fluid was incubated with 60 μ l of RNAase type Ia (Sigma) 10g/L, for 30 min, after which 120 μ l of protease (Sigma) 2.5g/L was added, and the mixture was incubated for 60 min. The treated supernatant fluid was extracted with phenol, by addition of an equal volume of 50 mM Tris (pH 8.5), saturated with phenol; the phenol was then removed by double extraction with diethyl ether. DNA was precipitated by the addition of 360 μ l of 3M sodium acetate and two volumes of 95% ethanol, held at -70°C for 60 min, and then recovered by centrifugation; the pellet was air dried. The DNA, resuspended in 100 μ l of TES, was sufficient for five lanes in agarose gel and was amenable to restriction endonuclease digestion. Electrophoresis of isolated plasmid DNA and plasmids of known mol. wt in Tris-acetate-EDTA-buffered agarose gels and restriction endonuclease analysis with *EcoRI* (Boehringer Mannheim Australia Pty Ltd, North Ryde, 2113) were as previously described by Lyon *et al.* (1983).

Mixed culture transfer and transformation. Mixed culture transfer was performed as described by McDonnell, Sweeney and Cohen (1983) with strain SK982 as a recipient. Transformation was performed by a modification of the procedures of Lindberg and Novick (1973) and Lederberg and Cohen (1974). Recipient cells were grown in Trypticase Soy Broth (TSB; BBL, Division of Becton, Dickinson and Co., Cockeysville, MD 21030, USA), with shaking, to an extinction of 0.2 at 540 nm (Spectronic 20, Bausch and Lomb, Rochester, NY 14625, USA). The cells were rapidly chilled, pelleted and washed in 0.1M CaCl₂, and resuspended in ice-cold 0.1M CaCl₂ to give *c.* 10⁹ cfu/ml. Fifty μ l of transforming DNA prepared by the procedure of Pattee and Neveln (1975) were added to 1 ml of competent cells and allowed to adsorb at 0°C for 60 min. The transformation mixture was subjected to a heat pulse of 42°C for 2 min, incubated at 0°C for 30 min, diluted tenfold in TSB and then incubated for 2h, to allow phenotypic expression, before it was plated on to L-agar plates containing selective antibiotics.

RESULTS

Characteristics of S. aureus isolates

For each year, the percentage of isolates resistant to antistaphylococcal agents is presented in table I. All isolates were resistant to streptomycin and tetracycline; the majority were also resistant to erythromycin, kanamycin and neomycin. Penicillinase production was detected in 92% of isolates. Chloramphenicol resistance was

TABLE I
Antibiotic susceptibilities of methicillin-resistant S. aureus

Year of isolation	Number of isolates	Percentage of isolates resistant to								
		Sm	Tc	Em	Gm	Tm	Km	Nm	Ak	Cm
1969	3	100	100	0	0	0	0	0	0	67
1973	5	100	100	0	0	0	100	100	0	0
1974	76	100	100	100	0	0	99	99	76	0
1975	15	100	100	53	0	0	87	87	46	0
1976	20	100	100	15	75	75	80	80	75	0
1977	79	100	100	91	43	43	91	87	49	0
1978	91	100	100	99	52	52	97	83	95	4
1979	41	100	100	95	71	71	83	76	66	5
1980	14	100	100	86	86	86	86	7	0	71
1981	9	100	100	89	55	55	55	0	0	11

Sm, streptomycin; Tc, tetracycline; Em, erythromycin; Gm, gentamicin; Tm, tobramycin; Km, kanamycin; Nm, neomycin; Ak, amikacin; Cm, chloramphenicol.

TABLE II
Characteristics of selected methicillin-resistant S. aureus strains

Year of isolation	Strain number	Resistance to*	Penicillinase production	Bacteriophage type†
1969	SK1651	Sm, Tc, Cm, Asa, Asi, Cd, Hg	+	6/47/53/54/75/77/83A/84/85
	SK1654	Sm, Tc, Asa, Asi, Cd, Hg	+	6/47/53/54/77/83A/84/85
1973	SK1704	Sm, Tc, Km, Nm, Cd, Hg	+	NT
	SK1706	Sm, Tc, Km, Nm, Cd, Hg	+	84/85; 100 RTD
1974	SK1814	Sm, Tc, Em, Km, Nm, Cd, Hg	—	85; 100 RTD
	SK1821	Sm, Tc, Em, Km, Nm, Ak, Cd, Hg	+	85; 100 RTD
	SK1669	Sm, Tc, Em, Km, Nm, Ak, Cd, Hg	+	85; 100 RTD
1975	SK1696	Sm, Tc, Km, Nm, Cd, Hg	+	84/85
	SK1700	Sm, Tc, Km, Nm, Cd, Hg	+	84/85; 100 RTD
1976	SK1660	Sm, Tc, Em, Cd, Hg	—	NT
	SK1674	Sm, Tc	—	71
	SK1661	Sm, Tc, Gm, Tm, Km, Nm, Ak	+	84/85
	SK1665	Sm, Tc, Gm, Tm, Km, Nm, Ak	+	84/85; 100 RTD
1977	SK1766	Sm, Tc, Em	—	94/96
	SK1774	Sm, Tc, Gm, Tm, Km, Cd, Hg	—	85; 100 RTD
	SK1717	Sm, Tc, Em, Gm, Tm, Km, Nm, Ak, Cd, Hg	+	29/6/47/75/77/85/95
1978	SK1903	Sm, Tc, Em, Gm, Tm, Km, Cd, Hg	+	29/53/77/83A/85
	SK1734	Sm, Tc, Em, Gm, Tm, Km, Nm, Cm, Cd, Hg	+	29/6/47/75/85/95
	SK1728	Sm, Tc, Em, Gm, Tm, Km, Nm, Cd, Hg	+	75/84/85; 100 RTD
1979	SK1745	Sm, Tc, Em, Km, Nm, Cd, Hg	+	NT
	SK1795	Sm, Tc, Gm, Tm, Km, Cm, Cd, Hg	+	NT
	SK1783	Sm, Tc, Em, Gm, Tm, Km, Cm, Cd, Hg	+	NT
1980	SK18	Sm, Tc, Em, Gm, Tm, Km, Cm, Cd, Hg	+	85/95; 100 RTD
1981	SK456	Sm, Tc, Em, Gm, Tm, Km, Cd, Hg	+	29/53/83A/79/85; 100 RTD
	SK457	Sm, Tc, Em, Gm, Tm, Km, Cm, Cd, Hg	+	NT

* Abbreviations as in table I, with the addition of: Asa, arsenate; Asi, arsenite; Cd, cadmium; Hg, mercury.

† Unless otherwise indicated, bacteriophage types were determined at routine test dilution (RTD); NT = not typable at either RTD or 100 RTD.

demonstrated by two of the 1969 isolates but was not detected again until 1978. First observed in 1976, gentamicin-resistant *S. aureus* strains comprised 75% of isolates and persisted as a large percentage of the population in ensuing years. Resistance to fusidic acid, rifampicin and vancomycin was not encountered. The majority of isolates demonstrated cadmium and mercury resistance, with those from 1969 additionally resistant to arsenate and arsenite. The characteristics of 25 selected strains encompassing the period from 1969 to 1981 are shown in table II.

Of the 184 strains that have been typed with bacteriophages of the International typing set, the majority showed various group-III lytic patterns. Whereas the 1969 isolates showed an extensive lytic pattern, those from 1973 to 1976 were commonly of pattern 84/85 at either routine test dilution (RTD) or 100 RTD (table II). After 1976, isolates demonstrated various group-III or mixed group-I/III lytic patterns; however, many isolated during 1979 were untypable (table II).

Aminoglycoside resistance

Resistance to aminoglycosides could be divided into six distinct phenotypes (table III). Isolates from 1973 to 1975, inclusive, were generally resistant to kanamycin and neomycin (KN^R) or to kanamycin, neomycin and amikacin (KNA^R), but neither of these phenotypes was detected after 1979. Gentamicin-resistant strains, first noted in

TABLE III

Distribution of aminoglycoside-resistance phenotypes in S. aureus strains resistant to both methicillin and aminoglycosides.

Year of isolation	Number of isolates	Percentage of isolates with aminoglycoside-resistance phenotype*					
		KN ^R	KNA ^R	GTKNA ^R	GTKN ^R	GTKA ^R	GTK ^R
1969	0	...	...	...	...	...	...
1973	5	100	0	0	0	0	0
1974	75	23	77	0	0	0	0
1975	13	46	54	0	0	0	0
1976	16	6	0	94	0	0	0
1977	72	40.2	12.6	40.2	1.4	1.4	4.2
1978	81	0	39.5	50.6	2.5	0	7.4
1979	35	8.6	8.6	71.4	0	2.8	8.6
1980	12	0	0	0	7	0	93
1981	5	0	0	0	0	0	100

* In aminoglycoside-resistance phenotypes: A = amikacin; G = gentamicin; K = kanamycin; N = neomycin and T = tobramycin.

1976, were also resistant to tobramycin, kanamycin, neomycin and amikacin (GTKNA^R). This phenotype persisted as the dominant pattern of aminoglycoside resistance until 1980 when gentamicin, tobramycin and kanamycin resistance (GTK^R) became the prevalent phenotype (table III). The remaining patterns of gentamicin, tobramycin, kanamycin and neomycin resistance (GTKN^R) and gentamicin, tobramycin, kanamycin and amikacin resistance (GTKA^R) were infrequently encountered.

MICs of the five aminoglycosides for 40 isolates were determined in an attempt to relate these six resistance phenotypes (table IV). Two distinct MICs of kanamycin were found among kanamycin-resistant isolates: those that were additionally resistant to neomycin (i.e., KN^R, KNA^R, GTKNA^R and GTKN^R) had high MICs for kanamycin (up to 2048 mg/L) compared with those that exhibited the GTK^R and GTKA^R phenotypes, which had MICs of 128–256 mg/L (table IV). Gentamicin-resistant strains could also be divided into two distinct groups: those with the GTKNA^R phenotype displayed high-level resistance to gentamicin (> 4096 mg/L) and a variable degree of tobramycin resistance, whereas the remaining phenotypes (GTKN^R,

TABLE IV

MICs of aminoglycosides for S. aureus strains resistant to methicillin and aminoglycosides

Aminoglycoside resistant phenotype*	Period isolated	MICs (mg/L) of				
		Gm†	Tm	Km	Nm	Ak
KN ^R	1973–1979	...	...	512–2048	512–1024	...
KNA ^R	1974–1979	...	...	512–2048	512–1024	16–64
GTKNA ^R	1976–1979	> 4096	256–4096	2048	512–1024	64–256
GTKN ^R	1977–1980	32–64	32–256	512–2048	512–1024	...
GTKA ^R	1977–1979	32–64	32–256	128–256	...	16–64
GTK ^R	1977–1981	32–64	32–256	128–256	...	...

* See table III.

† See table I.

GTKA^R and GTK^R) displayed low-level resistance to gentamicin (32–64 mg/L) and tobramycin (32–256 mg/L).

Plasmid DNA profiles and location of resistance determinants

In total, 198 isolates were screened for the presence of extrachromosomal DNA. Agarose-gel electrophoresis of DNA isolated from chronologically-grouped strains from table II is shown in fig. 1 (1969–1976) and fig. 2 (1977–1981). Strains from 1973

FIG. 1.—Agarose gel (1.0% w/v) electrophoresis of *S. aureus* plasmid DNA from strains isolated 1969–1976; the chromosomal band is labelled Chr. Lanes contain (a) mol. wt standards with sizes ($\times 10^6$) shown on the left; (b) SK1651 and (c) SK1654—1969; (d) SK1704 and (e) SK1706—1973; (f) SK1814, (g) SK1821 and (h) SK1669—1974; (i) SK1696 and (j) SK1700—1975 (k) SK1660, (l) SK1674, (m) SK1661 and (n) SK1665—1976.

FIG. 2.—Agarose gel (1.0% w/v) electrophoresis of *S. aureus* plasmid DNA from strains isolated 1977–1981; the chromosomal band is labelled Chr. Lanes contain (a) mol. wt standards with sizes ($\times 10^6$) shown on the left; (b) SK1766, (c) SK1774 and (d) SK1717–1977; (e) SK1903, (f) SK1734 and (g) SK1728–1978; (h) SK1745, (i) SK1795 and (j) SK1783–1979; (k) SK18–1980 (l) SK456 and (m) SK457–1981.

to 1975 (fig. 1, lanes d-j) showed only a single plasmid species or were devoid of plasmid DNA, whereas plasmid DNA profiles from strains isolated after 1976 were complex (fig. 2), with many isolates containing more than one plasmid species.

Curing, mixed culture transfer and transformation procedures were employed to determine the phenotypes conferred by plasmids. The 1969 isolate SK1651, which demonstrated plasmid species of mol. wt 20, 2.9, 2.8 and 1.6×10^6 (fig. 1, lane b and fig. 3, lane a), transferred the three largest plasmids independently to strain SK982 in mixed culture experiments. Penicillinase production was cotransferred with resistance to arsenate, arsenite, cadmium and mercury; resistant transipients such as SK2312 (fig. 3, lane b), gained the 20×10^6 -mol. wt plasmid that we have designated pSK51. The acquisition of tetracycline resistance was accompanied by transfer of the 2.8×10^6 -mol. wt plasmid pSK52, as shown in strain SK1996 (fig. 3, lane c). The chloramphenicol-resistant transipient SK1997 (fig. 3, lane d) was found, on the basis of *Eco*RI restriction endonuclease analysis of plasmid DNA (data not shown), to possess the previously described plasmid pSK2 of mol. wt 2.9×10^6 (Lyon *et al.*, 1983). The phenotypes conferred by these plasmids have been confirmed by generating derivatives of strain SK1651 that separately lack penicillinase production and heavy metal resistance, tetracycline resistance and chloramphenicol resistance, and have concomitantly lost the plasmids pSK51, pSK52 and pSK2, respectively. Another 1969 strain, SK1657, possessed a plasmid profile identical to that of strain SK1651 and curing studies support the presence of plasmids conferring identical phenotypes to pSK2, pSK51 and pSK52 (data not shown). The plasmids identified in these and other strains in this investigation are summarised in table V.

Strain SK1654 (fig. 1, lane c), also isolated in 1969, contained three species of plasmid. The largest plasmid had the same size as pSK51 and, as with pSK51, was found by curing to encode linked penicillinase production and heavy metal resistance. Likewise, the next largest plasmid, which had the same size as pSK52, was found to encode tetracycline resistance. The smallest was identical in mobility to the plasmid of mol. wt 1.6×10^6 , pSK58; no phenotype could be attributed to the presence of either of these plasmids. Neither transfer, nor loss of resistance to methicillin or streptomycin, from any of the 1969 isolates was detected. Furthermore, in no strain examined were these resistances associated with plasmid DNA and we, therefore, assume that these resistances are chromosomally encoded.

Cadmium resistance in strain SK1700 (fig. 1, lane j) was found by curing to be mediated by the plasmid pSK53 of mol. wt 24×10^6 ; plasmid-free derivatives retained resistance to methicillin, streptomycin, tetracycline, kanamycin, neomycin and mercury and the ability to produce a penicillinase. These derivatives, which lacked extrachromosomal DNA but were able to produce a penicillinase, are similar to the isolates SK1704, SK1821, SK1696, SK1661 and SK1717 (fig. 1, lanes d,g,i,m and fig. 2, lane d); this result, coupled with the inability to associate penicillinase production with plasmid DNA on the basis of curing or transfer in isolates from 1973 onwards, suggests that this determinant is chromosomally encoded in the majority of strains. Plasmid DNA identical in size to pSK53 was detected in *c.* 60% of cadmium-resistant isolates from 1973 to 1975, inclusive, but was not found in isolates sensitive to cadmium. We have not been able to associate the cadmium resistance phenotype with any of these plasmids by transfer and, unexpectedly, loss of the 24×10^6 -mol. wt plasmids from some cadmium-resistant isolates, eg. SK1706 (fig. 1, lane e) and SK1669 (fig. 1, lane h),

FIG. 3.—Agarose gel (1.0% w/v) electrophoresis of *S. aureus* plasmid DNA. Lane a shows the plasmid profile of the 1969 isolate SK1651. Lanes b (SK2312), c (SK1996) and d (SK1997) show plasmid profiles of derivatives of recipient strain SK982 which have gained resistance to benzyl penicillin and heavy metals, tetracycline and chloramphenicol, respectively, in mixed culture transfers with the donor SK1651; the covalently closed circular (ccc) forms and open circular (oc) forms of plasmids pSK51 (a,b), pSK52 (a,c) and pSK2 (a,d) and the ccc form of pSK58 (a) are indicated at the left. The remaining lanes show plasmid profiles of chloramphenicol-resistant strains SK1795 (e) and SK1783 (g) and their respective cured derivatives SK1824 (f) and SK1967 (h); the ccc and oc forms of the plasmid pSK54 (e) and the ccc form of pSK55 (g) are indicated at the right. The oc form of pSK55, which can be seen in fig. 2 (lane j), is not visible. No phenotype could be ascribed to either the 2.9 or 1.7×10^6 -mol. wt plasmids seen in strain SK1795, nor to the 1×10^6 -mol. wt species present in strain SK1783.

TABLE V
Properties of plasmids detected in methicillin-resistant S. aureus

Plasmid	Resistance phenotype*	Size (mol. wt $\times 10^6$)	Years detected
pSK1	Gm ^R , Tm ^R , Km ^R , Eb ^R	18	1980–1981
pSK2	Cm ^R	2.9	1969, 1978–1981
pSK3	...	1.1	1980–1981
pSK4	Gm ^R , Tm ^R , Km ^R , Eb ^R	22	1981
pSK51	Pc ^R , Asa ^R , Asi ^R , Cd ^R , Hg ^R	20	1969
pSK52	Tc ^R	2.8	1969
pSK53	Cd ^R	24	1975
pSK54	Cm ^R	3.5	1979
pSK55	Cm ^R	2.4	1979
pSK58	...	1.6	1969

* Abbreviations as in table I and table II, with the addition of: Eb, ethidium bromide; Pc, benzylpenicillin.

did not alter the phenotype of these strains. However, *Eco*RI restriction endonuclease digestion of the plasmids of mol. wt 24×10^6 present in SK1706 and SK1669, generated seven fragments that were identical in electrophoretic mobility to those generated by *Eco*RI digestion of pSK53 DNA (data not shown), suggesting that these two plasmids may be identical to pSK53 and so encode resistance to cadmium. These strains are discussed further below.

Erythromycin resistance was first detected in isolates from 1974. These strains either lacked plasmid DNA entirely, as in strains SK1814 and SK1821 (fig. 1, lanes f and g), or possessed only a 24×10^6 -mol. wt plasmid (e.g., strain SK1669; fig. 1, lane h) which appeared identical to the cadmium-resistance plasmid pSK53; such plasmids are unlikely to encode erythromycin resistance because they were also present in the erythromycin-sensitive strains SK1706 and SK1700 (fig. 1, lanes e and j). Likewise, in later years, many erythromycin-resistant isolates lacked plasmid DNA (e.g., strains SK1660 and SK1717; fig. 1, lane k and fig. 2, lane d); even in the isolates SK1766 and SK1745 (fig. 2, lanes b and h), which possessed unique plasmid profiles, no relationship could be established between a plasmid and erythromycin resistance.

On the basis of curing, or mixed culture transfer, or both, tetracycline resistance was found to be encoded on a plasmid identical in size to pSK52 in only two isolates obtained after 1969—strains SK1674 (fig. 1, lane l) and SK1903 (fig. 2, lane e). Plasmid DNA of the same size was detected in one further isolate, SK1795 (fig. 2, lane i), although the tetracycline-resistance phenotype could not be assigned in this case.

As described above, chloramphenicol resistance in the 1969 strains SK1651 (fig. 3) and SK1657 (data not shown) was mediated by the plasmid pSK2. This plasmid was detected in a further 14 isolates, such as SK1734, SK18 and SK457 (fig. 2, lanes f, k and m), during the period 1978 to 1981. However, two strains, SK1795 (fig. 2, lane i) and SK1783 (fig. 2, lane j), were shown by curing studies (fig. 3) to carry chloramphenicol resistance on plasmids other than pSK2. Strain SK1824 (fig. 3, lane f), the chloramphenicol-sensitive derivative of strain SK1795 (fig. 3, lane e), lacked the 3.5×10^6 -mol. wt plasmid pSK54; whereas pSK55, the 2.4×10^6 -mol. wt plasmid present in strain SK1783 (fig. 3, lane g), was absent in the chloramphenicol-susceptible derivative SK1967 (fig. 3, lane h).

Resistance to the aminoglycosides kanamycin and neomycin was inferred to be chromosomally encoded in KN^R and KNA^R strains such as SK1704, SK1814, SK1821 and SK1696 (fig. 1, lanes d, f, g, and i, respectively) because these isolates lacked plasmid DNA; > 50% of KN^R and KNA^R strains were devoid of extrachromosomal DNA. Of the 15 high-level gentamicin-resistant isolates obtained during 1976, 14 (e.g., strain SK1661, fig. 1, lane m) lacked extrachromosomal DNA. Only in strain SK1665 (fig. 1, lane n) was plasmid DNA demonstrated but no phenotype could be assigned to this plasmid of mol. wt $c. 12 \times 10^6$. By the alternative plasmid DNA isolation procedure, extrachromosomal DNA was again detected in strain SK1665 but not in other 1976 gentamicin-resistant isolates. These results suggest that the determinants for resistance to gentamicin, tobramycin, kanamycin, neomycin, amikacin and streptomycin are chromosomally encoded in the majority of these isolates. Gentamicin resistance in isolates from 1977 to 1979 inclusive could not be associated with plasmids by curing or transfer procedures and 88% of gentamicin-resistant isolates were either devoid of extrachromosomal DNA (e.g., strains SK1774 and SK1717; fig. 2, lanes c and d) or only possessed plasmids of mol. wt $c. 1 \times 10^6$ (data not shown). The latter plasmids would be unlikely to encode gentamicin resistance when compared, on the basis of size, with previously reported gentamicin resistance plasmids (Cohen, Wong and Falkow, 1982; Jaffe *et al.*, 1982; Lyon *et al.*, 1984a; Townsend, Grubb and Ashdown, 1983). These results suggest, therefore, that gentamicin resistance is also most likely to be chromosomally encoded in most of the 1977-1979 isolates; however, we cannot exclude the possibility of plasmid-mediated resistance in instances where large plasmids were present.

Strain SK18 (fig. 2, lane k), isolated early in 1980, possessed the 18×10^6 -mol. wt plasmid pSK1 which has been shown, on the basis of curing, to encode GTK^R (Lyon *et al.*, 1982). This plasmid was also found in another 13 out of 16 gentamicin-resistant isolates from 1980 and 1981. In the remaining strains, GTK^R was encoded either on the plasmid pSK4 of mol. wt 22×10^6 , as in strain SK456 (fig. 2, lane l), or on the chromosome, as in strain SK457 (fig. 2, lane m); both locations have previously been reported by Lyon *et al.* (1983 and 1984a).

In order to characterise further the plasmid pSK1, DNA from strain SK18 was used to transform the recipient strain SA113, and independent transfer of the plasmid pSK1 was detected. In addition to GTK^R , these transformants also acquired ethidium bromide resistance (Eb^R), a character that has been reported to be encoded on some gentamicin-resistance plasmids (Jaffe *et al.*, 1982; McDonnell *et al.*, 1983). In contrast to these GTK^R - Eb^R transformants that carried pSK1, GTK^R transformants were obtained that lacked pSK1 and failed to gain Eb^R . Plasmid-bearing (Eb^R) and plasmid-free (Eb^S) transipients were also obtained from mixed culture transfers between strains SK18 and SK982; mixed culture transfers with other strains containing the GTK^R - Eb^R plasmids pSK1 and pSK4 have given similar results (B. Lyon, J. May and R. Skurray, unpublished data).

Small plasmid DNA of mol. wt 1.6×10^6 (such as pSK1651) was present in the 1969 isolates; however, DNA of low mol. wt was absent from isolates from 1973 until 1977 (fig. 1). In isolates from 1977-1979, plasmid DNA of mol. wt 0.7, 1.0, 1.1 or 1.7×10^6 was evident (e.g., fig. 2, lanes f,g,h,i and j) whereas isolates from 1980-1981 possessed the plasmid pSK3 of mol. wt 1.1×10^6 (Lyon *et al.*, 1983) as demonstrated in strain SK18 (fig. 2, lane k). No phenotype has yet been ascribed to these small plasmids.

DISCUSSION

Methicillin-resistant strains of *S. aureus* have become an increasing problem in metropolitan hospitals in Melbourne, Australia, largely because of their resistance to a wide range of antistaphylococcal agents; by 1979 such organisms had attained epidemic proportions (Pavillard *et al.*, 1982). A retrospective analysis of methicillin-resistant *S. aureus* isolated at a single hospital was, therefore, initiated to provide an insight into the nature and origin of the resistance determinants that have contributed to the clinical impact of these strains. In the study reported here, plasmid DNA analysis has permitted the locations of antibiotic-resistance determinants to be established in a series of methicillin-resistant *S. aureus* isolates obtained during the period from 1969 to 1981.

In none of the isolates could methicillin resistance be associated with a plasmid; indeed, a chromosomal location for the determinant would be in agreement with the findings of previous studies (Kuhl, Pattee and Baldwin, 1978). Although a penicillinase was produced by 92% of the isolates, only those from 1969 were found to possess penicillinase plasmids and these also encoded resistance to heavy metals (e.g., pSK51, table V). Erythromycin resistance, which is frequently plasmid-encoded, either alone (Iordanescu and Surdeanu, 1980) or together with penicillinase production and heavy metal resistance (Novick *et al.*, 1979), was found not to be associated with plasmid DNA in the strains we examined and is likely to be chromosomally encoded, as is penicillinase production in the majority of penicillinase-producing isolates.

Resistance to cadmium and mercury was demonstrated in the majority of isolates, whereas arsenate and arsenite resistance was only encountered in the 1969 isolates. Cadmium resistance was shown to be mediated by the plasmid pSK53 (table V) in the 1975 isolate, SK1700. Furthermore, plasmids having the same size and *Eco*RI restriction pattern as pSK53 were seen in a number of strains isolated from 1973 to 1975 and it is possible that these plasmids also encode resistance to cadmium. If so, then the observed loss of the plasmid from these isolates, without concomitant loss of cadmium resistance, indicates that resistance to this heavy metal is mediated both by a plasmid and by the chromosome in some strains. Subsequent to 1975, cadmium resistance was not associated with plasmid DNA, nor was mercury resistance in isolates from 1973 onwards and both are assumed to be chromosomally encoded, as has been reported for other Melbourne isolates (Lyon *et al.*, 1983).

Reports of tetracycline resistance frequently implicate a small plasmid (Lacey, 1973; Shafferman, Shalita and Hertman, 1978). In this investigation, however, resistance to this antibiotic was only associated with a 2.8×10^6 -mol. wt plasmid, such as pSK52 (table V), in five of the 198 tetracycline-resistant isolates subjected to plasmid DNA analysis; three of these were from 1969 and the remaining two from 1976 and 1978. Thus, among 97% of tetracycline-resistant isolates, a small tetracycline-resistance plasmid could not be detected and one third of these isolates lacked plasmid DNA entirely. Such results suggest that the tetracycline-resistance determinant is chromosomally encoded in the majority of isolates.

Chloramphenicol resistance was found to be exclusively plasmid-mediated. Two of the 1969 isolates, SK1651 and SK1657, possessed the plasmid pSK2 (table V) which was the most prevalent chloramphenicol-resistance plasmid detected in more recent

isolates both from this hospital and from hospitals throughout Australia (Lyon *et al.*, 1984a). Two additional chloramphenicol-resistance plasmids, pSK54 and pSK55 (table V), were present in 1979 isolates, whereas the 3.0×10^6 -mol. wt chloramphenicol-resistance plasmid pSK5, which is rarely detected in Australian *S. aureus* strains (Lyon *et al.*, 1984a), was not encountered in isolates from this hospital. The molecular relationship between these chloramphenicol resistance plasmids is currently under examination.

The six aminoglycoside-resistance phenotypes detected in the isolates from 1973 onwards may reflect a wide range of resistance mechanisms. Isolates whose phenotypes included resistance to kanamycin and neomycin (e.g., KN^R , KNA^R , GTKN^R and GTKNA^R) showed MICs equivalent to those of previously reported strains in which KN^R was due to a 3'-phosphotransferase (Courvalin and Fianndt, 1980). GTK^R isolates displayed a low-level resistance to gentamicin suggesting enzymic modification of these aminoglycosides by a 6'-acetyltransferase and a 2''-phosphotransferase (Shannon and Phillips, 1982); the GTKA^R phenotype may be attributed to overproduction of the 6'-acetylating enzyme (Graninger *et al.*, 1983). In contrast, the GTKNA^R isolates, first seen in 1976, displayed high-level resistance to gentamicin and showed similar MICs to those of recent isolates with that phenotype (Lyon *et al.*, 1984a). This high-level resistance may be due to altered permeability of these strains towards aminoglycosides similar to that observed in *Pseudomonas aeruginosa* (Shannon and Phillips, 1982), or to the modifying enzyme being either overproduced or unusual. The mechanisms involved in these six phenotypes will be further clarified by analysis of representative strains for the presence of aminoglycoside modifying enzymes.

Gentamicin resistance in *S. aureus* has most frequently been reported to be plasmid-mediated (Soussy *et al.*, 1975; Wood, Carter and Best, 1977; Cohen *et al.*, 1982; Jaffe *et al.*, 1982; McDonnell *et al.*, 1983), although chromosomally-encoded resistance has recently been observed in Australia (Lyon *et al.*, 1983; Townsend, *et al.*, 1983) and elsewhere (Kayser, Homberger and Devaud, 1981; Dowd, Cafferkey and Dougan, 1983). Among the strains isolated at this hospital, aminoglycoside resistance was suspected to be chromosomally encoded in the majority of isolates before 1980. However, early in that year methicillin-resistant *S. aureus* were isolated from infants in the Neonatal Unit (Gilbert *et al.*, 1982) and strain SK18, which possessed the GTK^R plasmid pSK1 (table V), was isolated from one such patient. Further isolates of methicillin-resistant *S. aureus* from the Neonatal Unit during 1980 and the Burns Unit during 1981 were similar to strain SK18 in their possession of pSK1. This plasmid was identified in 14 of the 17 gentamicin-resistant isolates from 1980 and 1981; the remaining isolates either possessed the larger GTK^R plasmid pSK4 (table V), which is closely related to pSK1 (Lyon *et al.*, 1983 and 1984a), or carried chromosomal GTK^R as seen in many of the earlier isolates from 1977 to 1979.

The natural distribution of the GTK^R determinant between plasmids of various size and the chromosome is suggestive of a transposon (Lyon *et al.*, 1984a). Such a transposable element would also account for the GTK^R progeny detected after transformation of strain SA113 with pSK1 DNA; transformants which lacked Eb^R and plasmid DNA probably arose by discrete integration of only the GTK^R determinant into the chromosome. In agreement with this hypothesis, recent physical and genetic evidence from this laboratory has demonstrated that GTK^R on pSK1 is

encoded on the 4.5 kilobase transposon Tn4001 (Lyon *et al.*, 1984b). If this transposon, residing at a chromosomal location, was responsible for GTK^R in the isolates from 1977 to 1979 it may have been transposed to a suitable plasmid vector, at some stage, in a strain at this hospital and hence provided a vehicle for more rapid dissemination of the GTK^R phenotype among the nosocomial population of *S. aureus*. Alternatively, a strain possessing pSK1, such as strain SK18, may have been introduced by transfer of a patient from another hospital (Gilbert *et al.*, 1982). The detection of a large number of isolates with plasmid profiles identical to strain SK18 at other hospitals, in Melbourne and elsewhere in Australia (Lyon *et al.*, 1983 and 1984a), provides support for the spread of such a strain.

The combined use of bacteriophage typing and plasmid DNA analysis has allowed the recognition of distinct groups of methicillin-resistant *S. aureus* strains during the study period. Those from 1969 were of extensive bacteriophage-group-III lytic patterns and possessed penicillinase-, chloramphenicol- and tetracycline-resistance plasmids; such plasmids were also commonly present in methicillin-sensitive *S. aureus* isolated during the 1960s at a second Melbourne hospital (M. Gillespie, J. May and R. Skurray, unpublished data). The second group of strains isolated from 1973 to 1979, in which the emergence of aminoglycoside resistance was noted, were of various bacteriophage-group-III or mixed group-I/III lytic patterns or were untypable. In the majority of isolates, the determinants for resistance to amikacin, erythromycin, gentamicin, kanamycin, neomycin, streptomycin, tetracycline, tobramycin, cadmium and mercury and for penicillinase production were chromosomally encoded, although plasmids encoding cadmium, tetracycline and chloramphenicol resistance were detected in some strains. Isolates from 1980 and 1981 also possessed an extensive array of chromosomally-encoded resistance determinants; however, they frequently demonstrated plasmid-mediated resistance to gentamicin, tobramycin, kanamycin and chloramphenicol.

Future identification of enzymes involved in mediating resistance phenotypes, and DNA-DNA hybridisation studies, planned with these strains will attempt to establish whether the resistance determinants for penicillin, tetracycline, the aminoglycosides and the heavy metals that are present in recent isolates are identical to or distinct from those found in isolates from earlier episodes. These results will provide evidence for or against the hypothesis that bidirectional rearrangements of pre-existing resistance determinants have occurred between plasmids and the chromosome during the evolution of contemporary *S. aureus* strains.

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