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Analysis of Plasmids in Nosocomial Strains of Multiple-Antibiotic-Resistant *Staphylococcus aureus*

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Nosocomial infections caused by *Staphylococcus aureus* strains resistant to methicillin and multiple antibiotics have reached epidemic proportions in Melbourne, Australia, over the past 5 years. Plasmid analysis of representative clinical isolates demonstrated the presence of three classes of plasmid DNA in most strains. Resistance to gentamicin, kanamycin, and tobramycin was usually mediated by an 18-megadalton plasmid but could also be encoded by a related 22-megadalton plasmid. Two distinguishable plasmids of 3 megadaltons each endowed resistance to chloramphenicol, and the third class consisted of small plasmids, each approximately 1 megadalton in size, with no attributable function. An extensive array of resistance determinants, including some which have usually been associated with a plasmid locus, were found to exist on the chromosome. Evidence that resistance to gentamicin, kanamycin, and tobramycin is chromosomally encoded in some clinical isolates suggests that this determinant may have undergone genetic translocation onto the staphylococcal chromosome.

The incidence of nosocomial infections caused by *Staphylococcus aureus* strains resistant to methicillin and multiple antibiotics has steadily increased in recent years. Although generally restricted to small outbreaks (5, 16, 37) strains of multiply resistant (MR) *S. aureus* have shown a propensity for spread and persistence (2, 35), and some reports suggest that the appearance of these organisms is more than episodic (10, 32).

Over the past 5 years, the occurrence of MR *S. aureus* in metropolitan hospitals in Melbourne, Australia, has reached epidemic proportions. A survey conducted during 1979 found that more than 2,500 patients in 31 hospitals were known to be infected or colonized (or both) by these organisms (30). The current incidence of MR *S. aureus* in some major hospitals is as high as 50% of all *S. aureus* strains isolated, and the number of patients infected, as opposed to those colonized, has climbed to approximately half of all cases, with a corresponding increase in morbidity and mortality. Patients most at risk from MR *S. aureus* bacteremia appear to be those with some form of predisposing condition such as a surgical wound, burns, or an intravascular device. Additionally, prior therapy with multiple or broad-spectrum antibiotics is often associated with acquisition of the organism (24).

A preliminary report has been made on the genetic nature of the multiple-antibiotic resistance expressed by Melbourne strains of *S. aureus* (22). The purpose of this investigation was to expand these findings in an attempt to establish whether dissemination of common antibiotic resistance plasmids was involved in the apparent epidemic spread of MR *S. aureus* throughout Melbourne hospitals.

MATERIALS AND METHODS

Bacterial strains and plasmids. Clinical isolates of *S. aureus* were obtained from Melbourne metropolitan hospitals and other Victorian hospitals during the period from 1979 to 1982. We are grateful to J. Andrew and D. Olden (St. Vincent's Hospital), V. Asche (Queen Victoria Medical Centre), P. Carson (Repatriation and General Hospital), R. Pavillard (Royal Melbourne Hospital), A. Hewstone (Royal Children's Hospital), J. Spicer (Alfred Hospital), and F. Tosolini (Austin Hospital) for providing these isolates. Representative strains, together with their derivatives, are listed in Table 1.

Strains carrying plasmids for use as molecular weight standards were kindly provided by R. N. Novick, Public Health Research Institute of the City of New York [RN1465(pII147), RN453(pI258), RN3135(pSA4500), RN1305(pC221), RN2425(pC194)] and M. L. Cohen, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga. [L3626(pUW3626)].

Strain SA113 (12), a restrictionless derivative of *S. aureus* 8325, was kindly provided by S. Iordanescu, Institute Cantacuzino, Bucharest, Romania.

Media. L broth contained 1% (wt/vol) tryptone (Oxoid Ltd., London, England), 0.5% (wt/vol) yeast extract (Oxoid), and 1% (wt/vol) sodium chloride (pH 7.2). L agar contained 1.25% (wt/vol) Davis New Zealand agar in L broth.

TABLE 1. Characteristics of representative *S. aureus* strains

Strain	Hospital source or derivation	Phage type[a]		Antibiogram[b]															
		RTD	100 RTD	Pc	Mc	Sm	Nm	Km	Gm	Tm	Ak	Cm	Cl	Em	Fa	Rf	Su	Tc	Tp	Vm
SK529	St. Vincent's	NT	80/85	R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK553	Derivative of SK529	NT	80/85	R	R	R	S	S	S	S	S	R	R	R	S	S	R	R	R	S
SK554	Derivative of SK553	NT	80/85	R	R	R	S	S	S	S	S	S	R	R	S	S	R	R	R	S
SK526	St. Vincent's	NT	80/85	R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK539	St. Vincent's	NT	79/80/53/85	R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK427	Austin	NT	79/80/85	R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK462	Alfred	83A/85		R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK608	Queen Victoria	29		R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK429	Repatriation/ General	NT	80/85	R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK497	Derivative of SK429	NT	80/85	R	R	R	S	R	R	R	S	S	R	R	S	S	R	R	R	S
SK484	Royal Melbourne	NT	80/85/96	R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK480	Royal Melbourne	NT	80/85	R	R	R	S	R	R	R	S	R	R	R	R	S	R	R	R	S
SK665	Derivative of SK480	NT	80/85	R	R	R	S	R	R	R	S	R	R	R	R	S	R	R	R	S
SK478	Royal Melbourne	NT	79/80/85	R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK18	Royal Children's	NT	80/85	R	R	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK240	Royal Children's	NT	85	R	S	R	S	R	R	R	S	R	R	R	S	S	R	R	R	S
SK456	Royal Children's	NT	29/53/83A/79/85	R	R	R	S	R	R	R	S	S	R	R	S	S	R	R	R	S
SK700	Derivative of SK456	NT	29/53/83A/79/85	R	R	R	S	S	S	S	S	S	R	R	S	S	R	R	R	S

[a] RTD, Routine test dilution; NT, nontypable.

[b] Pc, Penicillin; Mc, methicillin; Sm, streptomycin; Nm, neomycin; Km, kanamycin; Gm, gentamicin; Tm, tobramycin; Ak, amikacin; Cm, chloramphenicol; Cl, clindamycin; Em, erythromycin; Fa, fusidic acid; Rf, rifampin; Su, sulfathiazole; Tc, tetracycline; Tp, trimethoprim; Vm, vancomycin; R, resistant; S, susceptible.

Susceptibility tests. Bacterial susceptibility to a number of antimicrobial agents and inorganic ions was ascertained by suspending single colonies in L broth in separate wells of a microtiter tray and, using a multipoint inoculator (23), inoculating L agar plates containing one of the antibiotics or inorganic salts (28) at the following concentrations: benzylpenicillin (Glaxo Australia, Pty. Ltd., Boronia, Australia), 20 μg/ml; methicillin (Beecham Australia, Moonabbin, Victoria, Australia), 8 μg/ml; streptomycin (Glaxo Australia), 10 μg/ml; neomycin (The Upjohn Co., Kalamazoo, Mich.), 10 μg/ml; kanamycin (Bristol Laboratories, Syracuse, N.Y.), 10 μg/ml; gentamicin (Schering Corp., Bloomfield, N.J.), 4 μg/ml; tobramycin (Eli Lilly & Co., Indianapolis, Ind.), 4 μg/ml; amikacin (Bristol Laboratories), 8 μg/ml; chloramphenicol (Boehringer Mannheim Australia, Pty. Ltd., North Ryde, New South Wales, Australia), 16 μg/ml; clindamycin (Upjohn), 10 μg/ml; erythromycin (Abbott Laboratories, Pty. Ltd., Kurnell, New South Wales, Australia), 5 μg/ml; fusidic acid (Leo Pharmaceuticals, Ballerup, Denmark), 1 μg/ml; rifampin (Lepetit, ●●●●●), 1 μg/ml; sulfathiazole (Sigma Chemical Co., S.p.A., Milan, Italy), 100 μg/ml; tetracycline (Sigma), 5 μg/ml; trimethoprim (Sigma), 50 μg/ml; vancomycin (Eli Lilly & Co.), 4 μg/ml; sodium arsenate, 7×10^{-4} M; sodium arsenite, 2×10^{-4} M; cadmium nitrate, 5×10^{-5} M; and mercuric nitrate, 5×10^{-6} M. Susceptibility was determined after incubation for 16 to 18 h at 30°C (methicillin) or 37°C (all others).

MICs. Minimal inhibitory concentrations (MICs) were determined by inoculating serial twofold dilutions of an antibiotic in L broth with a 0.0005 volume of an 18-h bacterial culture and incubating at 37°C for 48 h.

Penicillinase assay. Penicillinase production was detected by inoculating resuspended colonies on 0.1 brain heart infusion agar (BBL Microbiology Systems, Cockeysville, Md.), followed by treatment with *N*-phenyl-α-napthylaminocarboxybenzene and 20 mg of benzylpenicillin per ml as described previously (27).

Plasmid elimination. Plasmid elimination was achieved by growth in L broth at 44°C for 16 to 18 h (1) or by growth in L broth containing various concentrations of ethidium bromide (EtBr) at 37 or 44°C for 16 to 18 h (3). Single colonies from such cultures were picked and screened to determine phenotypic changes.

Rapid plasmid DNA isolation. A rapid plasmid DNA isolation technique (7) has been modified for general use in this laboratory (R. Jackson, J. Tennent, B. Lyon, and R. Skurray, unpublished data). The procedure specific for staphylococcal DNA is as follows. Cultures in 10 ml of L broth containing the relevant antibiotic(s) were incubated with shaking at 37°C for 16 to 18 h. Cells were centrifuged, resuspended in 1 ml of TES buffer (50 mM Tris, 5 mM EDTA, 50 mM NaCl [pH 8.0)], transferred to a 1.5-ml Eppendorf tube, and pelleted in an Eppendorf centrifuge. The pellet was resuspended in 50 μl of lysostaphin (0.2 mg/ml in TE-sucrose—10 mM Tris, 1 mM EDTA, 25% [wt/vol] sucrose [pH 7.8]; Sigma), and a further 450 μl of TE-sucrose was added to the tube, which was then incubated at 37°C for 45 min. Nucleases were inactivated by the addition of 5 μl of diethyl pyrocarbonate (Sigma), and 75 μl of lysis buffer (10% [wt/vol] sodium dodecyl sulfate, 50 mM Tris, 10 mM EDTA [pH 8.0]) was added and mixed by rolling before incubation at room temperature for 10 min. The addition of 400 μl of potassium acetate (5 M) was followed by incubation on ice for at least 30 min, centrifugation in an Eppendorf centrifuge at 4°C for 20 min, and incubation of the decanted clear lysate with 5 μl of RNase type I-A (10 mg/ml in sterile distilled water; Sigma) for 30 min at 37°C. DNA was precipitated by filling the tube with room-temperature absolute ethanol and centrifuging in an Eppendorf centrifuge at room temperature for 10 min. The DNA pellet was air dried and dissolved in 40 μl of TE (pH 7.5). DNA prepared by this method is sufficient for two gel tracks, can be cleaved by restriction endonucleases, and can be stored at 4°C for at least 10 days after incubation at 65°C for 5 min.

Isolation of purified plasmid DNA. Cleared lysates, from 1-liter aerated L broth cultures shaken at 37°C for 16 h, were prepared by the rapid DNA procedure as described above, with proportional increases in reagents, and concentrated by polyethylene glycol precipitation (11). Covalently closed circular (CCC) plasmid DNA was separated from chromosomal DNA by cesium chloride-EtBr (CsCl-EtBr) equilibrium density centrifugation. CsCl-EtBr gradients were formed by centrifugation (40,000 rpm, 40 h) with a Ti70.1 rotor in an L8-70 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Plasmid bands were visualized under UV light and collected with needle and syringe, and the EtBr was removed by double extraction with cold *n*-butanol. To remove CsCl, DNA was dialyzed (16 to 18 h, 4°C) against 20 mM Tris–1 mM EDTA (pH 7.8) containing Dowex 50W-X8 (BDH, Poole, England) with one change of buffer.

Agarose gel electrophoresis. Whole plasmid DNA samples were analyzed by submerged horizontal electrophoresis (1.8 V/cm, 16 h) in an 0.9% (wt/vol) agarose (Sigma, Type II) gel buffered with TAE (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA [pH 7.8]) (J. Watson, Australian National University, Canberra, personal communication). After staining with EtBr (5 μg/ml) and destaining with distilled water, the DNA was visualized with a 302-nm-wavelength UV transilluminator (Ultra-Violet Products, San Gabriel, Calif.) and photographed through combined 2A/15 Kodak Wratten filters with an MP-4 Polaroid Land camera and Kodak Tri-X film. Plasmid sizes were calculated from the migration of DNA bands with molecular weight standards and the relationship log molecular weight versus mobility (38). Plasmid standards were as follows: pUW3626, 32 megadaltons (Md) (4); pII147, 21.4 Md (29); pI258, 18.6 Md (29); pSA4500 5.4 Md (13); pC221, 3.0 Md (29); and pC194, 1.8 Md (29).

Restriction endonuclease digestion. Rapidly isolated DNA samples were cleaved with restriction endonucleases *Eco*RI, *Hin*dIII, and *Sal*I (Boehringer Mannheim) and *Hpa*II and *Pvu*II (New England Biolabs, Beverly, Mass.) over 2 h, as described previously (6), and analyzed by 1.0 to 1.4% (wt/vol) agarose gel electrophoresis as described above. *Hin*dIII and *Hin*dIII-*Eco*RI double digests of λ viral DNA (Miles Laboratories, Inc., Elkhart, Ind.) were employed as standards. Sizes of the fragments in kilobases (kb) were taken from Phillipsen et al. (31).

RESULTS

Characteristics of *S. aureus* isolates. A total of 152 strains of MR *S. aureus* isolated from 14

hospitals over the period from July 1979 to February 1982, were examined in this study. Almost all of these strains were resistant to penicillin, streptomycin, and tetracycline; 93% were resistant to erythromycin and clindamycin; and 89% were resistant to methicillin. Linked resistance to gentamicin, kanamycin, and tobramycin was demonstrated by 79% of the strains, whereas 28% exhibited apparently unlinked resistance to neomycin. Resistance to chloramphenicol was seen in 37% of the organisms. Resistance to amikacin, fusidic acid, and rifampin was sometimes encountered, but all isolates appeared to be susceptible to vancomycin. Production of a penicillinase; resistance to sulfonamide, trimethoprim, and cadmium and mercury ions; and susceptibility to arsenate and arsenite were also characteristic of the hospital strains surveyed.

Representative MR *S. aureus* strains and their characteristics are shown in Table 1. Most strains could not be phage typed at routine test dilution; however, phage types were obtained at 100 routine test dilution. Several different phage types from groups I and III were present, with the 80/85 complex being the most common.

Plasmid composition of *S. aureus* isolates. Plasmid DNA from all 152 clinical isolates of *S. aureus* was analyzed to determine the location of genetic determinants for antibiotic resistance in these strains. Agarose gel electrophoresis of DNA isolated from the twelve representative strains in Table 1 is shown in Fig. 1. CsCl-EtBr-purified CCC plasmid DNA from strain SK529 (Fig. 1, lane b) indicated the presence of three plasmids with approximate molecular sizes of 18, 3, and 1 Md. Rapidly isolated DNA of the same strain (Fig. 1, lane c) showed these three plasmid bands together with a band of chromosomal DNA and a band corresponding to the putative open circular (OC) form of the 3-Md plasmid (see below). This plasmid pattern was common in other strains with antibiograms similar to that of SK529 (e.g., SK427, SK462, SK429, SK484, SK18; Fig. 1) and was also found in methicillin-susceptible isolates with otherwise identical antibiograms, such as SK240 (Fig. 1, lane n). In some of these lanes, putative OC forms of the 18-Md plasmid are evident just below the wells.

These three plasmids were not always found together, however, and many strains were shown to possess only one or two of the plasmid types (e.g., SK526, SK608, SK478; Fig. 1). Overall, 51% of the strains possessed an 18-Md plasmid, 37% a 3-Md plasmid, and 69% a 1-Md plasmid. In addition, 9% of the strains, such as SK480 (Fig. 1, lane k), possessed a large plasmid other than the 18-Md variety, and a plasmid species smaller than the 1-Md plasmid was found in two strains (e.g., SK539; Fig. 1, lane e). Only 7% of the isolates examined were shown to be devoid of plasmid DNA.

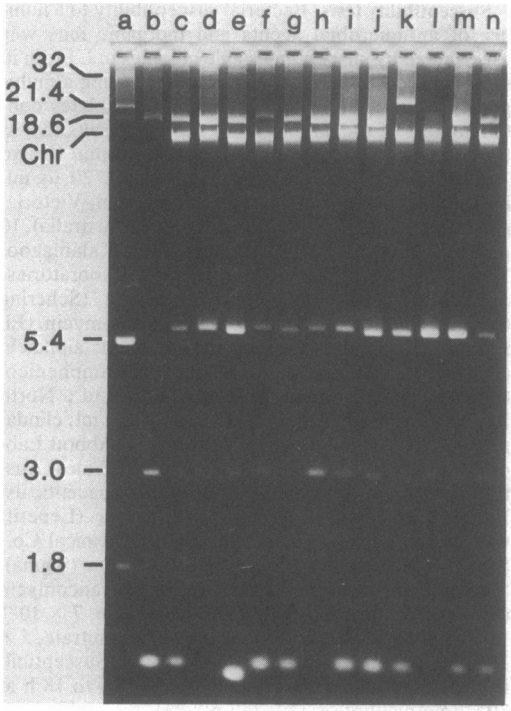

FIG. 1. Agarose gel (0.9% [wt/vol]) electrophoresis of *S. aureus* DNA. Lanes a and b contain purified CCC plasmid DNA, and lanes c through n contain rapidly isolated plasmid DNA from which OC plasmid DNA and chromosomal (Chr) DNA have not been removed. Lane a, molecular weight standards as described in Materials and Methods; sizes (in Md) are shown at left. Lane b, SK529; lane c, SK529; lane d, SK526; lane e, SK539; lane f, SK427; lane g, SK462; lane h, SK608; lane i, SK429; lane j, SK484; lane k, SK480; lane l, SK478; lane m, SK18; lane n, SK240.

Determination of plasmid phenotypes. Strain SK529, with a common antibiogram and plasmid pattern, was selected for plasmid elimination studies as described in Materials and Methods. After overnight incubation at 44°C, up to 40% of cells were found to be susceptible to gentamicin, kanamycin, and tobramycin. Plasmid DNA isolated from susceptible variants, such as SK553 (Fig. 2, lane c), demonstrated loss of the 18-Md plasmid species seen in SK529 (Fig. 2, lane b). Curing studies with a further 14 clinical isolates also demonstrated the association between the 18-Md plasmid, which has been named pSK1, and resistance to gentamicin, kanamycin, and tobramycin.

Incubation (44°C) of cultures of strain SK456 (Fig. 2, lane i), which possessed a large 22-Md plasmid, resulted in the production of plasmid-less derivatives, such as SK700 (Fig. 2, lane j),

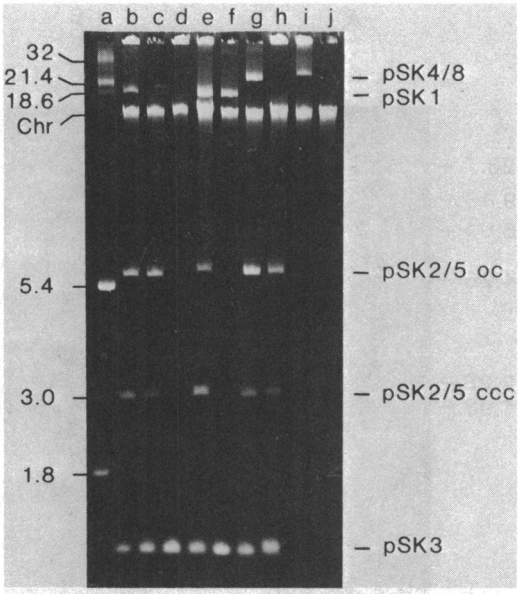

FIG. 2. Agarose gel (0.9% [wt/vol]) electrophoresis of rapidly isolated *S. aureus* DNA. Lane a, molecular weight standards as described in Materials and Methods; sizes (in Md) are shown at left. Lane b, SK529; lane c, SK553, a derivative of SK529 cured of gentamicin, kanamycin, and tobramycin resistance; lane d, SK554, a derivative of SK553 cured of chloramphenicol resistance; lane e, SK429; lane f, SK497, a derivative of SK429 cured of chloramphenicol resistance; lane g, SK480; lane h, SK665, a derivative of SK480 with the same phenotype; lane i, SK456; lane j, SK700, a derivative of SK456 cured of gentamicin, kanamycin, and tobramycin resistance.

which were now susceptible to gentamicin, kanamycin, and tobramycin. Therefore, in this strain, resistance to these aminoglycosides is mediated by a 22-Md plasmid (pSK4). Strain SK480 (Fig. 2, lane g) also possessed a 22-Md plasmid (pSK8), but incubation at 44°C failed to yield antibiotic-susceptible variants among more than 300 colonies screened from several curing attempts. Six colonies so treated were chosen for plasmid DNA analysis, and all, like SK665 (Fig. 2, lane h), demonstrated loss of the 22-Md plasmid while retaining gentamicin, kanamycin, and tobramycin resistance. Twenty-eight gentamicin-, kanamycin-, and tobramycin-resistant strains, such as SK478 (Fig. 1, lane l), which also lack any form of large plasmid, have been isolated from clinical sources. All attempts to cure aminoglycoside resistance in strains SK665 and SK478 have been unsuccessful. Furthermore, use of an alkaline-lysis plasmid DNA isolation procedure (R. P. Novick, personal communication), which we have also employed successfully for the isolation of large plasmid DNA from staphylococci, failed to reveal any new plasmid DNA in those strains tested. These results suggest that resistance to gentamicin, kanamycin, and tobramycin in strain SK665 and the clinical isolates represented by SK478 is mediated by the chromosome.

MICs of the various aminoglycosides have been used to deduce which aminoglycoside-modifying enzymes are present in resistant bacteria (34). MICs for MR *S. aureus* strains were therefore determined to compare the mechanism of aminoglycoside resistance mediated by the plasmid-borne resistance determinants with that apparently encoded by the chromosomes of strains SK665 and SK478. The MICs of gentamicin, kanamycin, and tobramycin for strain SK529 were 64, 128 and 32 μg/ml, respectively, as compared with 0.5 μg/ml for SK553, the derivative of SK529 cured of pSK1. The MIC of neomycin was ≤1 μg/ml for both SK529 and SK553; however, the MIC of amikacin was 4 μg/ml in SK529 but only 1 μg/ml in SK553. These results suggest that pSK1 mediates an effect on amikacin, although not enough to confer full resistance on the host. The other plasmid-carrying strains, SK456(pSK4) and SK480 (pSK8), and those strains in which aminoglycoside resistance appears to be chromosomally encoded (SK665 and SK478) all demonstrated MICs identical to those for SK529 over the same range of aminoglycosides. These results can be interpreted to indicate the presence of determinants for a 6′-acetyltransferase and a 2″-phosphotransferase (34), not only where plasmid-borne resistance occurs, but also in those strains with chromosomal aminoglycoside determinants.

The mixed culture techniques described by Lacey (17) and Jaffe et al. (14) were used in an attempt to transfer the determinants encoding gentamicin, kanamycin, and tobramycin resistance to plasmidless recipients. However, strains carrying plasmids pSK1, pSK4, and pSK8 all proved to be unsuccessful as donors. Such results are in agreement with those from similar experiments with Australian strains reported by Gedney and Lacey (9). Transformation with DNA isolated from derivatives of strain SK18 carrying pSK1, by a modification of the method of Lindberg and Novick (21) did, however, achieve transfer of linked gentamicin, kanamycin, and tobramycin resistance to other SK18 derivatives cured of pSK1 and also into the restrictionless strain SA 113. Although pSK1 plasmid DNA could be isolated from most of these transformants, some strains with the appropriate phenotype did not demonstrate this plasmid (M. Gillespie, J. May, and R. Skurray, unpublished data).

Strain SK553, the derivative of SK529 cured of pSK1, when subjected to incubation over-

night at 44°C in the presence of EtBr (15 μg/ml), produced up to 35% chloramphenicol-susceptible derivatives. Plasmid DNA isolated from such derivatives as SK554 (Fig. 2, lane d) showed loss of both the 3-Md plasmid species (pSK2) and its putative OC form. Absence of the putative OC form in CsCl DNA preparations immediately after isolation, its progressive appearance in the stored preparation, and its removal after denaturation of the DNA by treatment at 90°C are indicative that this band is produced by the OC form of pSK2. A plasmid marginally larger than pSK2 was found in strain SK429 (Fig. 2, lane e). Curing studies (Fig. 2, lane f) confirmed that this plasmid, pSK5, also carries a chloramphenicol resistance determinant. Curing data from other strains, including SK539, SK480, and SK18 (Table 1), and the 100% correlation seen in clinical isolates between chloramphenicol resistance and the presence of a plasmid of approximately 3 Md in size provide strong evidence that chloramphenicol resistance is exclusively plasmid mediated in these strains.

Loss of the small 1-Md plasmid (pSK3) was only rarely detected after plasmid elimination procedures. Three strains, including SK529 and SK480 (Table 1), were cured of this plasmid with no evident change in phenotype, and comparisons of strains with and without pSK3 confirmed its lack of association with the antibiogram. Screening for the loss of resistance determinants other than for the aminoglycosides and chloramphenicol proved unsuccessful. This result, combined with the absence of any further classes of uncharacterized plasmid DNA, leads to the conclusion that the remaining resistance determinants in these strains, including penicillinase production and resistance to methicillin, streptomycin, neomycin, clindamycin, erythromycin, fusidic acid, rifampin, sulfonamide, tetracycline, trimethoprim, and cadmium and mercury ions, are encoded on the chromosome.

Restriction endonuclease analysis of plasmid DNA. To examine the relatedness of phenotypically similar plasmids found in these *S. aureus* strains, rapidly isolated plasmid DNA was subjected to *Eco*RI and *Hin*dIII digestion and analyzed on agarose gels. Comparison of the *Eco*RI restriction patterns of strain SK529 (Fig. 3A, lane b) and its gentamicin-kanamycin-tobramycin-susceptible derivative SK553 (Fig. 3A, lane c) revealed that pSK1 is cleaved into three fragments of 13, 9, and 4.5 kilobases (kb); pSK1 thus has a total length of approximately 26.5 kb. A diagrammatic summary of these three *Eco*RI fragments (labeled A, B, and C, respectively) is shown in Fig. 4. Restriction analyses of nine other strains which possess plasmids equivalent in size to pSK1 (SK429 and SK539 [Fig. 3A, lanes f and g]; SK526, SK427, SK462, SK608, SK484, SK18, and SK240 [data not shown]) display identical patterns of these three fragments. Digestion of plasmid DNA from strains SK456 and SK480 with *Eco*RI (Fig. 3A, lanes d and e) indicated that an identical 22-Md (33-kb) plasmid was present in both strains. Each plasmid was cleaved into three fragments of 13, 11, and 9 kb, the largest and smallest of these being equivalent in size to fragments A and B of pSK1, respectively (Fig. 4). It would appear that pSK4 and pSK8 are related to pSK1, differing by the presence of an additional 6.5 kb of DNA in *Eco*RI fragment C′ (11 kb) not found in fragment C (4.5 kb) of pSK1.

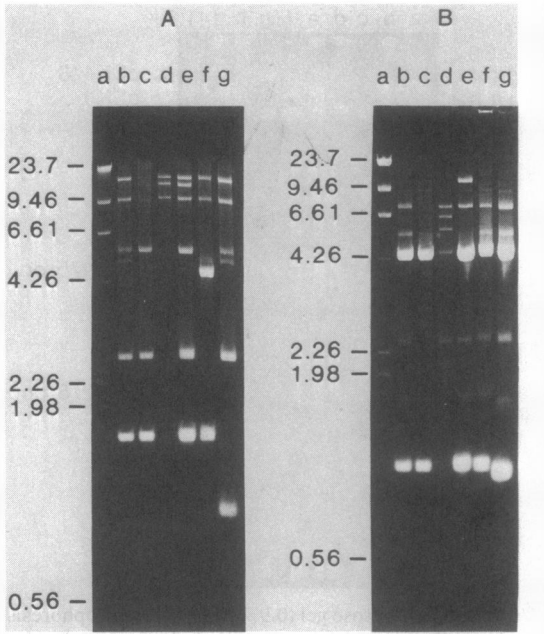

FIG. 3. *Eco*RI (A) and *Hin*dIII (B) restriction digests of rapidly isolated plasmid DNA from *S. aureus* strains after electrophoresis in 1.2% (A) and 1.4% (B) (wt/vol) agarose gels. Lane a, λ DNA (cleaved with *Hin*dIII; fragment sizes (in kb) are shown at left of each gel. Lane b, SK529; lane c, SK553; lane d, SK456; lane e, SK480; lane f, SK429; lane g, SK539.

Comparison of the *Hin*dIII restriction patterns for SK529 and SK553 (Fig. 3B, lanes b and c) revealed that pSK1 is cleaved into at least seven fragments which are also visible in digests of SK429 and SK539 (Fig. 3B, lanes f and g). Electrophoresis of *Hin*dIII digests of SK529 DNA in agarose gels of different concentrations and *Hin*dIII digests of separated pSK1 DNA (data not shown) revealed that pSK1 is cleaved into an extra two fragments that are masked by other plasmid forms in Fig. 3B. The pattern of the nine *Hin*dIII fragments of pSK1 (labeled a

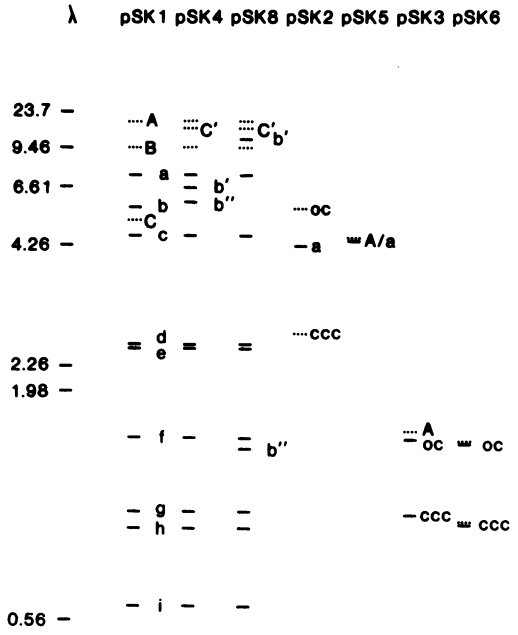

FIG. 4. Diagrammatic representation of the agarose gel electrophoresis pattern of the seven *S. aureus* plasmids digested with *Eco*RI (dashed lines and capital letters) and *Hin*dIII (solid lines and lowercase letters). Plasmids uncleaved by these enzymes remain as CCC and OC forms. λ *Hin*dIII standard fragments (in kb) are shown at left. The interpretation is based on data from Fig. 3A and B and other gels (not shown).

through i) is presented diagrammatically in Fig. 4. Digestion of plasmid DNA from strains SK456 and SK480 with *Hin*dIII (Fig. 3B, lanes d and e) revealed some differences between pSK4 and pSK8. The additional DNA in both pSK4 and pSK8 contains a *Hin*dIII site which leads to the production of two *Hin*dIII fragments (b' and b'') from each plasmid in place of *Hin*dIII fragment b in pSK1 (Fig. 4). The other eight *Hin*dIII fragments in pSK1 (a, c, d, e, f, g, h, and i) are also common to pSK4 and pSK8.

Analysis of the *Eco*RI restriction pattern of strain SK529 (Fig. 3A, lane b) indicates that pSK2, the plasmid which mediates chloramphenicol resistance in this strain, retains its CCC and OC forms and therefore must lack an *Eco*RI site. Restriction analysis of strain SK429 (Fig. 3A, lane f) indicates that pSK5, a second chloramphenicol resistance plasmid slightly larger than pSK2, produces a single fragment when treated with *Eco*RI (Fig. 4). Digestion with other restriction endonucleases revealed further differences as well as an underlying relatedness between these two plasmids. Cleavage with *Hin*dIII (Fig. 3B, lanes b and f) produced single linear fragments from both plasmids, with the fragment from pSK2 (4.3 kb) appearing marginally smaller than that from pSK5 (4.4 kb). This difference in size was again noticed when DNA isolated from the two strains carrying these plasmids was digested with *Hpa*II (Fig. 5, lanes b and c). Each of these plasmids produced two *Hpa*II fragments; the smaller fragments (1.7 kb) derived from pSK2 and pSK5 appeared to be identical, but the larger fragment from pSK2 (2.6 kb) differed from that produced with pSK5 (2.7 kb). Finally, digestion of SK529 with *Pvu*II and *Sal*I independently had shown that pSK2 possesses a single site for each of these endonucle-

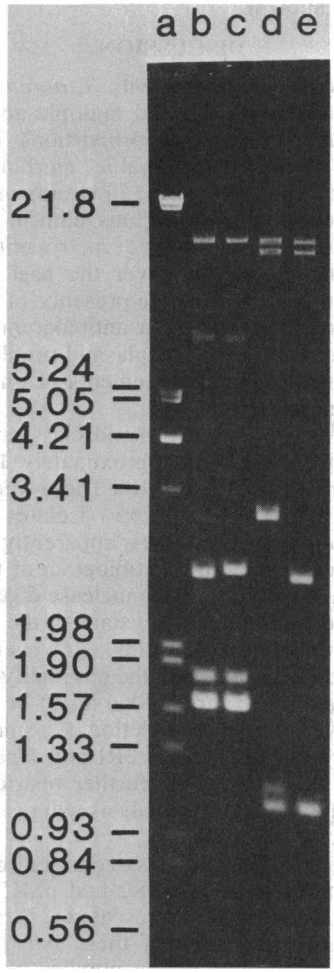

FIG. 5. Agarose gel (1.0% [wt/vol]) electrophoresis of restriction digests of rapidly isolated plasmid DNA from *S. aureus* strains. Lane a, λ DNA (double digest with *Hin*dIII and *Eco*RI); fragment sizes (in kb) are shown at left. Lane b, SK529 cleaved with *Hpa*II; lane c, SK429 cleaved with *Hpa*II; lane d, SK529 double digest with *Pvu*II and *Sal*I; lane e, SK429 double digest with *Pvu*II and *Sal*I.

ases (data not shown). Double digestion of SK529 and SK429 DNA by *Pvu*II and *Sal*I (Fig. 5, lanes d and e) therefore produced two fragments (3.2 and 1.1 kb) from pSK2; in contrast, pSK5 retained the CCC and OC forms, not possessing sites for either enzyme.

Restriction analysis of strain SK529 (Fig. 3A, lane b) showed that the cryptic plasmid pSK3 is cleaved by *Eco*RI at a single site, whereas the smaller plasmid pSK6 in strain SK539 (Fig. 1, lane e) lacked this site (Fig. 3A, lane g; Fig. 4). Whereas both pSK3 and pSK6 lack sites for *Hin*dIII (Fig. 3B, lanes b and g), a further difference between the two is the single site for *Hpa*II present in pSK3, producing a 1.6-kb fragment (Fig. 5, lane b), but absent from pSK6 (data not shown).

DISCUSSION

Nosocomial infections with *S. aureus* strains resistant to methicillin and multiple antibiotics have reached epidemic proportions in Melbourne, causing considerable morbidity and mortality (30). More than 150 strains of MR *S. aureus* received from various clinical sources throughout the Melbourne metropolitan and Victorian country area over the past 3 years have been screened for the presence of plasmid DNA. Isolates with similar antibiograms generally demonstrated similar plasmid profiles, with each profile comprising elements of only three basic plasmid classes.

The most prevalent plasmid class contained the small plasmids of approximately 1 Md in size, such as pSK3 and pSK6. These were found in 70% of the MR *S. aureus* isolates but, as evidenced by curing studies, apparently play no part in determining the antibiogram of the host organism. Restriction endonuclease digestion of pSK3 and pSK6 failed to establish the relatedness of these two plasmids. However, these limited studies leave open the possibility that the smaller and much rarer pSK6 might be derived from pSK3 through the deletion of a small piece of DNA containing the *Eco*RI and *Hpa*II sites found in the latter. Only further restriction digests and hybridization analysis will resolve this question.

The second plasmid class consisted of the 3-Md plasmids, such as pSK2 and pSK5, which encode chloramphenicol resistance. Restriction endonuclease digestion of these two plasmids revealed similarities in the number of *Hin*dIII and *Hpa*II sites. The slightly larger size of pSK5 (4.4 kb) as compared with pSK2 (4.3 kb) could be attributed to the deletion of a small piece of DNA containing the *Eco*RI site found in pSK5 but absent from pSK2. However, if these plasmids are related, more evolutionary change than this has occurred, since pSK2 possesses single sites for *Pvu*II and *Sal*I which are absent in pSK5. It is interesting to note that the restriction pattern for pSK5 is similar to that published for pC221 (39), a chloramphenicol plasmid carried by an *S. aureus* strain isolated in France some 20 years ago.

The final class of plasmids comprised those responsible for mediating gentamicin, kanamycin, and tobramycin resistance. Of the strains resistant to these aminoglycosides, 65% possessed an 18-Md plasmid, for which pSK1 is the prototype. A further 12% of the resistant strains possessed other large plasmids exemplified by pSK4 and pSK8. These two plasmids differ from pSK1 in only one *Eco*RI and two *Hin*dIII restriction fragments, and it is tenable that pSK4 and pSK8 evolved from the more common pSK1 through the insertion of DNA within the region of *Eco*RI fragment C. The fact that the *Eco*RI and *Hin*dIII patterns of pSK4 and pSK8 are closely related suggests that pSK8, like pSK4 and pSK1, encodes resistance to the three aminoglycosides, although this has not been proven by curing or transfer.

The phenotype of strains carrying plasmids pSK1, pSK4, and pSK8, demonstrating resistance to gentamicin, kanamycin, and tobramycin and susceptibility to amikacin and neomycin, implies the complicity of two aminoglycoside-modifying enzymes, a 6′-acetyltransferase and a 2″-phosphotransferase (34). The resistance determinants from the Melbourne plasmids may therefore be similar to ones characterized elsewhere (20, 36); DNA-DNA hybridization and enzymatic studies are required to establish the involvement of a like gene(s).

In 23% of the gentamicin-, kanamycin-, and tobramycin-resistant clinical isolates, we were unable to detect plasmid DNA capable of encoding this determinant (e.g., SK478), suggesting that the gene(s) for resistance have a chromosomal location. MIC testing of strains with chromosomally borne resistance determinants (e.g., SK478 and SK665) showed a pattern across a range of five aminoglycosides identical to that demonstrated for plasmid-borne resistance (e.g., SK529 and SK456). Hence, it seems probable that the same resistance determinants occur at chromosomal and extrachromosomal sites, a trait which is usually indicative of a transposon (26). Further evidence for the involvement of a transposon is seen in the transformation data in which a plasmid locus could not always be demonstrated for the transferred resistance determinants.

By inference from the above, strains such as SK480 may be in a transition state whereby copies of the transposon are found on both a plasmid and the chromosome in the one cell (25). Alternatively, the gentamicin-kanamycin-tobra-

mycin resistance determinant located on pSK8 may have transposed onto the chromosome of SK480 during the procedure which cured the strain of the plasmid, thus producing derivatives such as SK665. The high proportion of clinical isolates with chromosomal resistance to these aminoglycosides could similarly result from a trend to dispense with the plasmid replicon, which had earlier aided the dissemination of the transposon within the staphylococcal population, while still retaining the resistance gene(s) it carried.

Melbourne strains of MR *S. aureus* are endowed with an impressive array of resistance determinants associated with the chromosome. These include resistance to penicillin, methicillin, streptomycin, neomycin, clindamycin, erythromycin, fusidic acid, rifampin, tetracycline, sulfonamide, trimethoprim, and some heavy-metal ions. Many of these determinants are thought to be of chromosomal origin (18), and some occur as such in other recent MR *S. aureus* isolates (4, 32). The Melbourne isolates differ from these, however, with respect to the total number of resistance determinants carried by the chromosome, being only rivalled, perhaps, by recently described strains from France (8), and by the addition to the chromosome of genes for penicillinase production and resistance to heavy-metal ions, which are frequently reported as being located on plasmids (4, 15, 19, 40). It is possible that these loci, which may be transposable (33), have undergone translocation to the chromosome in these recent Melbourne strains, and our evidence suggests that a similar process might now be occurring with the aminoglycoside determinant(s). *S. aureus* strains isolated in Melbourne therefore seem capable of both accruing chromosomal resistance markers and seconding resistance genes from plasmids transiently existent in the cell.

The homogeneity of the clinical isolates from Melbourne hospitals, with regard to their antibiograms, phage types, and plasmid profiles, provides substantial evidence for their common origin. Although the transfer of plasmids between organisms must have accounted for the original emergence of multiple-antibiotic resistance, and indeed may be the rationale for the presence of plasmid DNA in these strains, we believe that the current epidemic of MR *S. aureus* in Melbourne is more likely to have arisen via the spread of a single strain or its derivatives from hospital to hospital. Subsequent evolutionary events, such as mutation and DNA insertion and deletion through transposition or recombination, have lead to the production of the strain variants that are currently being isolated.

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