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Self-Transmissible Plasmids in Staphylococci That Encode Resistance to Aminoglycosides

GORDON L. ARCHER* AND J. LINDA JOHNSTON

Department of Medicine, Division of Infectious Diseases, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

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High-level resistance to gentamicin, tobramycin, and kanamycin was transferred between staphylococci of the same and different species by filter mating. Resistance and transfer proficiency were mediated by plasmids ranging from 38 to 54 kilobases in size. All of the plasmids encoded intermediate resistance to amikacin and netilmicin and resistance to ethidium bromide; some encoded beta-lactamase production. None of these plasmids carried resistance to other antibiotics or heavy metals. Transfer of antibiotic resistance occurred by a mechanism similar to that of conjugation, because it was DNase resistant, required cell-to-cell contact, and did not appear to involve phage. The participation of phage in transfer appeared to be unlikely because mitomicin C-induced lysates of donor isolates did not mediate transfer, filter mating transfer proceeded at high frequency between nonlysogenic donor and recipient cells, and transfer of the aminoglycoside resistance plasmid mobilized the transfer of as many as five additional plasmids. All 17 gentamicin-resistant *Staphylococcus aureus* and all 6 *Staphylococcus epidermidis* isolates obtained from an outbreak of staphylococcal infections in a newborn nursery contained conjugative plasmids, as did all 6 gentamicin-resistant *S. aureus* isolates from bacteremic adults. However, only 3 of 10 gentamicin-resistant *S. epidermidis* isolates from colonized cardiac surgery patients and 1 of 2 *S. epidermidis* isolates from patients with prosthetic valve endocarditis transferred gentamicin resistance by filter mating. The recent increase in nosocomial infections caused by gentamicin-resistant staphylococci may be partially explained by the evolution of self-transmissible plasmids in these isolates.

Strains of both *Staphylococcus epidermidis* and *Staphylococcus aureus* resistant to aminoglycoside antibiotics have been seen with increasing frequency among hospital isolates (1, 2a, 24). Although aminoglycosides are not first-line therapy for staphylococcal infections, the rapid increase in strains resistant to these agents is important for several reasons. First, aminoglycoside resistance is often associated with resistance to more commonly used antistaphylococcal antibiotics, including semisynthetic penicillinase-resistant penicillins, cephalosporins, lincosamides, macrolides, and chloramphenicol (1, 2a). This leaves only such antibiotics as vancomycin and rifampin as therapeutic agents for serious staphylococcal infections (11). Second, gentamicin resistance in staphylococci has been shown to be plasmid mediated (7, 9, 15, 16, 23). These aminoglycoside resistance plasmids can reside in avirulent, colonizing *S. epidermidis* strains which are selected by antibiotics as the predominant skin flora in ill patients (2a, 9, 24). Transfer of aminoglycoside resistance to more virulent *S. aureus* strains can then occur on human skin (9, 17). Aminoglycoside resistance allows staphylococci to survive in areas of the hospital where aminoglycoside usage is high.

Transfer of R-plasmids among staphylococci traditionally has been thought to occur only by transformation or transduction (9, 13). However, the rapid emergence and spread of aminoglycoside resistance among staphylococci in hospitals and the transfer of aminoglycoside resistance plasmids among staphylococci in mixed culture (9) and on human skin (9, 17) suggested that another means of aminoglycoside resistance transfer may be involved. Two publications appeared recently which documented conjugal transfer of aminoglycoside resistance plasmids among staphylococci (7, 16). In the following study we present evidence confirming the transfer of plasmids encoding resistance to aminoglycosides between staphylococcal species by cell-to-cell contact. These plasmids ap-

pear to have genes which encode their own transfer and the mobilization of other plasmids. We also present epidemiological data confirming the importance of plasmid transfer in vivo.

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MATERIALS AND METHODS

Isolates. Clinical isolates of staphylococci resistant to gentamicin came from four sources. First, isolates of *S. aureus* and *S. epidermidis* were recovered from neonates during epidemiological investigations of outbreaks of *S. aureus* infections in a neonatal intensive care unit which began in 1980 and continued into 1983. Gentamicin-resistant *S. epidermidis* strains were found to be colonizing infants infected with gentamicin-resistant *S. aureus* strains. Second, sporadic gentamicin-resistant *S. aureus* isolates which caused documented infections were obtained from the clinical microbiology laboratory from 1977 to 1981. Third, gentamicin-resistant *S. epidermidis* isolates obtained from colonized cardiac surgery patients were obtained in culture surveys conducted yearly from 1977 to 1980. Finally, there were two gentamicin-resistant *S. epidermidis* isolates which caused prosthetic valve endocarditis in patients at the Medical College of Virginia hospitals in 1979 and 1980. The wild isolates studied were chosen only on the basis of their resistance to gentamicin and susceptibility to antibiotics to which recipient strains were chromosomally resistant (novobiocin, rifampin, and fusidic acid). Staphylococcal isolates were identified as *S. aureus* by their production of coagulase and fermentation of mannitol. Staphylococci not producing coagulase were identified as *S. epidermidis* by the method of Kloos and Schleifer (12).

Isolates used as recipients in transfer experiments were chosen on the basis of three characteristics. First, all were staphylococci known to be free of plasmids. Second, chromosomal antibiotic resistance markers were present which were not found in donor staphylococci. Third, the presence of prophage and susceptibility to lysis by transducing phages $\phi 11$ and $\phi 80\alpha$ were established. This was either known from previous studies for the standard isolates RN450, RN27, and RN2677 (kindly supplied by R. Novick, New York City Public Health Research Institute, New York, N.Y.) or determined in our laboratory for wild *S. epidermidis* MG1 (see below).

Antimicrobial susceptibility. Antimicrobial susceptibility was determined for all antimicrobial agents by disk diffusion in Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) and for aminoglycosides by both disk diffusion and broth dilution. Zone diameters in the disk diffusion assay were read after 24 h of incubation at 37°C, and broth dilution susceptibility was determined in Mueller-Hinton broth by the microtiter method previously described (2). Antimicrobial disks impregnated with the indicated drug concentrations were obtained from BBL: penicillin (2 U), gentamicin (10 μg), amikacin (30 μg), kanamycin (30 μg), netilmicin (10 μg), tobramycin (10 μg), erythromycin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg), rifampin (5 μg), and novobiocin (10 μg). The manufacturer's zone size criteria were used to establish susceptibility and resistance. Known susceptible *S. aureus* strains (RN2677 and RN450) were always included as standards.

Disks were impregnated with heavy metals or ethidium bromide by adding 20 μl of one of the following solutions: cadmium nitrate (10^{-2} M), lead nitrate (10^{-3} M), mercury nitrate (10^{-3} M), phenylmercuric nitrate (10^{-4} M), sodium arsenate (10^{-1} M), silver nitrate (10^{-1} M), or ethidium bromide (1 mg/ml). Resistance to heavy metals and ethidium bromide was determined by comparing the zone diameters of test isolates with those of known susceptible (RN2677 or RN450) or resistant (RN2677 containing pI6187 [18]) strains of *S. aureus*. Zone diameters had to fall within 2.0 mm of that for the control strain for the test strain to be considered susceptible or resistant. When no resistant control strain was available (resistance to ethidium bromide and silver nitrate), the zone diameter was required to be ≤50% that of the susceptible control to be considered resistant. All bacteria tested were easily and reproducibly assigned susceptibility on the basis of these criteria.

Bacteria were determined to be susceptible by broth dilution testing when their minimal inhibitory concentrations (MICs) were within one twofold dilution of the susceptible standard (0.05 μg/ml for gentamicin, tobramycin, and netilmicin; 0.8 μg/ml for amikacin and kanamycin). Bacteria were considered resistant to gentamicin, tobramycin, or netilmicin when their MICs were ≥6.3 μg/ml and to amikacin and kanamycin when their MICs were ≥25 μg/ml. Strains with MICs between susceptible and resistant were called intermediate.

Susceptibility to sulfamethoxazole and trimethoprim was determined by standard agar dilution techniques with 20 μg of sulfamethoxazole per ml and 3 μg of trimethoprim per ml incorporated individually into agar. Resistance was defined as growth on agar after 24 h of incubation at 37°C.

Penicillinase production was determined for each donor and recipient pair by the starch-iodine method (19). Bacteria were grown on agar containing 1 μg of methicillin per ml to induce enzyme production.

The identification of specific aminoglycoside-modifying enzymes produced by wild isolates and transcipients was performed at Bristol Laboratories, Syracuse, N.Y., by David Bobey. Cell-free extracts of staphylococci to be assayed for enzyme activity were prepared by lysostaphin lysis as described by Scott et al. (22). Enzyme analysis was performed by a modification of the phosphocellulose paper binding procedure of Haas and Dowding (8).

Phage. Transducing phages $\phi 11$ and $\phi 80\alpha$ (kindly donated by R. Novick) were used to produce lysis on lawns of selected organisms. The organism to be tested was swabbed on the surface of Mueller-Hinton agar, and 10 μl of phage (10^{10} PFU) was added. Phage sensitivity was defined as complete clearing after 24 h of incubation at 37°C.

Lysogeny of individual isolates was identified by lysis of growing cultures after treatment with 1 μg of mitomycin C per ml. The cleared culture was filtered through a 0.45-μm filter, and the filtrate was used for spotting lawns of susceptible *S. aureus* strains RN450

and RN2677 and for transduction experiments (see below). Mitomicin C-treated cultures which did not clear were also filtered, and the filtrate was used as described above. Some growing cultures were spotted directly on lawns of phage-susceptible *S. aureus* strains without filtering to detect any cell-bound phage.

Plasmid transfer. Transfer of gentamicin resistance was attempted by filter mating, generalized transduction, and mixed culture. Filter mating was performed as described by Schaberg et al. (20) on 0.22-μm nitrocellulose filters. Filter mating was also performed after the addition of 0.01 M sodium citrate or DNase I (50 μg/ml; Sigma Chemical Co., St. Louis, Mo.) with 0.1 M $MgCl_2$ to brain heart infusion broth (BBL) containing donor and recipient bacteria. Selection was made on Mueller-Hinton agar with antibiotics at the following concentrations: gentamicin, 5 μg/ml; novobiocin, 5 μg/ml; rifampin, 5 μg/ml; or fusidic acid, 20 μg/ml.

Generalized transduction was performed by incubating 10^8 recipient bacteria in phage buffer with phage-containing culture filtrates at various phage/bacteria ratios. Recipient bacteria were plated on selective agar and incubated at 37°C for 24 to 48 h. Mixed-culture transfer was performed as described by Jaffe et al. (9) in broth containing $CaCl_2$ (400 μg/ml) and lysozyme (20 μg/ml).

All bacteria growing on selective agar were confirmed as the recipient strain by toothpicking colonies to plates containing each selective antibiotic and determining the specific lytic pattern to transducing phages φ11 and φ80α. When necessary, other phenotypic characteristics of recipient bacteria were sought as well. These included production of pigment and coagulase and resistance to nonselected antibiotics. All recipient colonies were identified by at least two phenotypic traits.

Purification of plasmid DNA. Donor and recipient staphylococci were screened for the presence of plasmid DNA by the following rapid lysis procedures. *S. aureus* strains were grown overnight on agar, and the growth from one-quarter of an agar plate was transferred by swabbing it to 10 ml of a solution containing 0.1 M NaCl and 0.05 M EDTA at pH 6.9. The bacteria were washed twice and resuspended in 1 ml of this salt solution to which lysostaphin (Sigma) was added to a final concentration of 70 μg/ml. After 30 min of incubation at 37°C, the solution of lysed bacteria was spun at high speed (40,000 × *g*) for 45 min, and the cleared supernatant was removed and treated with 10 μg of pancreatic RNase A (Sigma) per ml for 1 h at 37°C, followed by treatment with 10 μg of proteinase K (Sigma) per ml for 1 h at 37°C. A 40-μl portion of the cleared lysate was then electrophoresed at 80 V (35 mA) for 3 h on a vertical slab gel through 0.7% agarose in Tris-borate buffer as previously described (4). The gel was washed, stained with ethidium bromide, transilluminated with UV light, and photographed. This lysis procedure produced a lysate containing abundant plasmid DNA and very little chromosomal DNA and was the preferred lysis method. However, *S. epidermidis* isolates were relatively insensitive to lysostaphin and would not lyse by this method. A previously described rapid lysis method which uses a detergent (sodium dodecyl sulfate) and does not remove chromosomal DNA was required for *S. epidermidis* lysis (4). Electrophoresis was carried out at 10 V for 18 h to avoid the smearing of chromosomal DNA which occurred at higher voltage.

Plasmid DNA was purified for restriction endonuclease digestion by ethidium bromide-cesium chloride buoyant density gradients as previously described (4). Digestion with restriction endonucleases *Eco*RI and *Bgl*II (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was done by the manufacturer's specifications. The molecular size of the plasmids was estimated by comparing the gel migration distance of restriction endonuclease-generated fragments of unknown plasmids against that of fragments of known size generated by cleaving lambda phage DNA.

RESULTS

Transfer of aminoglycoside resistance. All 18 wild aminoglycoside-resistant *S. epidermidis* isolates chosen for study contained multiple plasmid bands (range, 2 to 11), produced beta-lactamase, and were resistant to ethidium bromide, heavy metals, and multiple antibiotics, including methicillin, erythromycin, clindamycin, and tetracycline. Wild aminoglycoside-resistant *S. aureus* isolates contained either one (13 isolates) or two (10 isolates) plasmid bands, produced beta-lactamase, and were susceptible to heavy metals and other antibiotics, but resistant to ethidium bromide.

Wild isolates were screened for the transfer of gentamicin resistance to *S. aureus* RN2677 by filter mating (Table 1). Transfer was most efficient from *S. aureus* to *S. aureus* recipients, with 100% of isolates transferring resistance at frequencies ranging from 5×10^{-5} to 5×10^{-6}. Only 9 of 18 (50%) *S. epidermidis* isolates transferred aminoglycoside resistance, at frequencies varying from 1×10^{-6} to 4×10^{-7}. Those isolates failing to transfer resistance were consistently transfer negative after three matings. Recipient staphylococci were analyzed for the acquisition of plasmid DNA. Large plasmids (38 to 54 kilobases) were present in all recipients; donors having more than one plasmid transferred the large plasmid plus from zero to six smaller plasmids. Purified plasmid DNA was

TABLE 1. Transfer of gentamicin resistance from clinical staphylococcal isolates to *S. aureus* RN2677

Donor isolates	Source	No. transferring resistance/ no. tested (%)
S. aureus	Newborns	17/17 (100)
S. epidermidis	Newborns	6/6 (100)
S. aureus	Random clinical isolates	6/6 (100)
S. epidermidis	Cardiac surgery patients	3/10 (30)
S. epidermidis	Patients with prosthetic valve endocarditis	1/2 (50)

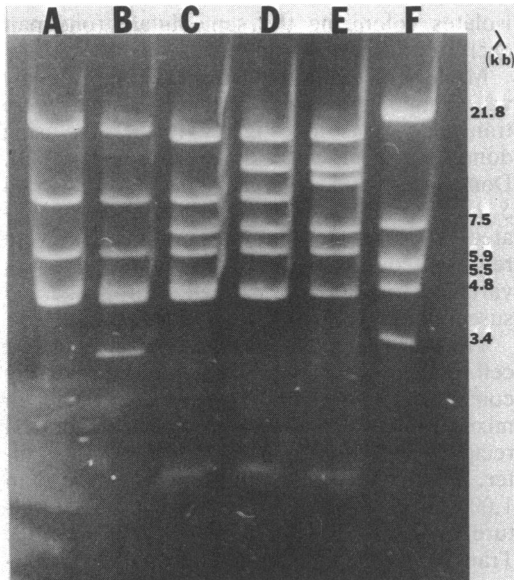

FIG. 1. Agarose gel electrophoresis of plasmid DNA digested with restriction endonuclease *Eco*RI. This gel shows the five different restriction patterns found among the self-transmissible aminoglycoside resistance plasmids. All of these plasmids were obtained by dye buoyant density gradient centrifugation after they had been transferred from wild isolates to recipient *S. aureus* RN2677. Lanes A through E, Plasmids representative of classes 1 through 5, respectively. Lane F, lambda phage DNA digested with *Eco*RI as a molecular size standard, with sizes indicated in kilobases (kb) to the right.

obtained from donors and recipients having single plasmids and digested with restriction endonucleases. All donors having a single plasmid transferred this plasmid intact to recipients; *Eco*RI and *Bgl*II restriction digests of donor and recipient plasmids were identical. There were, however, five similar but different restriction digests among gentamicin resistance plasmids. Screening recipient colonies from each of 33 successfully mated donors with a rapid lysis technique yielded at least one gentamicin-resistant transconjugant which contained a single large plasmid from each mating. Only transconjugants containing single plasmids were used as sources of purified plasmid DNA (Fig. 1). Five different restriction patterns were also seen among the same plasmids after *Bgl*II digestion (data not shown).

Aminoglycoside-modifying enzyme activity and the transfer of nonselected antimicrobial resistance was assessed in gentamicin-resistant transconjugants. All of the resistant transconjugants contained the same aminoglycoside-inactivating enzymes, which encoded high-level resistance to gentamicin, kanamycin, and tobramycin (Table 2). Resistance to netilmicin and amikacin was intermediate; the recipient was more resistant than before the transfer but still clinically susceptible. The MICs for aminoglycosides conferred by the transmissible plasmids were as follows: gentamicin, 12.5 to 50 (median, 25) μg/ml; tobramycin, 25 to 50 (median, 25) μg/ml; kanamycin, 25 to 100 (median, 50) μg/ml; amikacin, 1.7 to 6.3 (median, 3.1) μg/ml; and netilmicin, 1.7 to 6.3 (median, 3.1) μg/ml. The substrate specificity of the identified aminoglycoside-modifying enzymes was consistent with the susceptibility pattern of the transconjugants. The aminoglycoside nucleotidyltransferase [ANT(4')] modifies kanamycin, tobramycin, and gentamicin B, but not gentamicins C_1, C_{1a},

TABLE 2. Characteristics of aminoglycoside resistance plasmids transferred by filter mating

Plasmid class[a]	Size (kilobases)	Source (no.) of isolates[b]	Median MIC (μg/ml) for aminoglycoside:	Additional resistance markers[c]	Aminoglycoside-modifying enzymes[d]
1	38.4	Nursery: *S. epidermidis* (5), *S. aureus* (11)	Gentamicin, 25; tobramycin, 25; kanamycin, 50; netilmicin, 3.1; amikacin, 3.1	EB^r	AAC(6')-I, ANT(4')-II, APH(2")
2	41.6	Clinical: *S. aureus* (3)	As for class 1	EB^r	As for class 1
3	46.7	SEP (1), SEC (2)	As for class 1	EB^r	As for class 1
4	53.2	Nursery: *S. aureus* (2), *S. epidermidis* (1); clinical: *S. aureus* (2)	As for class 1	EB^r, β-L^+	As for class 1
5	53.5	Nursery: *S. aureus* (4); clinical: *S. aureus* (1); SEC (1)	As for class 1	EB^r, β-L^+	As for class 1

[a] EcoRI digests defining each plasmid class are shown in Fig. 1.

[b] SEC, *S. epidermidis* cardiac surgery colonizing isolates; SEP, *S. epidermidis* prosthetic valve endocarditis isolates.

[c] Resistance to 20 μg of ethidium bromide on a disk (EB^r) and beta-lactamase production (β-L^+).

[d] Roman numerals indicate an isozymic form of each enzyme. Detailed nomenclature of aminoglycoside-modifying enzymes can be found in reference 5.

or A, amikacin, or netilmicin (15, 21); the aminoglycoside acetyltransferase [AAC(6')] modifies kanamycin, tobramycin, and gentamicins B and C_{1a}, but neither amikacin nor netilmicin (15, 23); and the aminoglycoside phosphotransferase [APH(2")] modifies predominantly gentamicin, with poor specificity for kanamycin, tobramycin, and amikacin (15), and presumably it has poor activity against netilmicin (23). In addition, it was found that although all the transmissible plasmids encoded the same aminoglycoside-modifying enzymes, one enzyme [APH(3')-IV] was produced in some gentamicin-resistant *S. epidermidis* isolates that did not transfer resistance which was different from the enzyme [APH(2")] found in staphylococci that did.

Resistance to ethidium bromide was found to be associated with plasmid transfer in all, and beta-lactamase production in some, transconjugants. No resistance to macrolides, lincosamides, chloramphenicol, tetracycline, sulfonamide, trimethoprim, or heavy metals was transferred from either *S. aureus* or *S. epidermidis* donors. The molecular sizes, distribution of the five restriction patterns among staphylococcal isolates, and phenotypic characteristics are shown in Table 2. Plasmid classes correspond to the restriction patterns shown in Fig. 1. Plasmid classes 1, 4, and 5 contained plasmids from both *S. aureus* and *S. epidermidis* strains, whereas class 2 contained only plasmids found in *S. aureus* strains and class 3 contained only plasmids found in *S. epidermidis* strains. In two instances, identical plasmids were found in gentamicin-resistant *S. aureus* and *S. epidermidis* isolates colonizing the same infant (one pair each from classes 1 and 4).

Mechanism of plasmid transfer. The means by which aminoglycoside resistance plasmids were transferred was investigated further by using donor and recipient staphylococci (Table 3). Donor staphylococci were chosen so that both *S. aureus* and *S. epidermidis* strains were evaluated, four of the five plasmid classes were represented (classes 2 through 5), and there was variation in both the presence of prophage and susceptibility to transducing phage.

Transfer was DNase I resistant and required cell-to-cell contact. The latter was assessed by comparing transfer on filters versus transfer in mixed-cell culture and by separating donor and recipient cells with a 0.45-μm nitrocellulose filter. The frequency of transfer was more than 1,000-fold greater on filters than in mixed culture for donor *S. aureus* strains 661 and 913. Transfer did not occur in mixed culture (frequency of transfer, $<10^{-9}$) with donor *S. epidermidis* strains Wr and Tr. There was no transfer when donor and recipient cells were separated by a filter.

Transfer was interspecific. *S. aureus* strains 661 and 913 transferred aminoglycoside resistance plasmids into both *S. aureus* and *S. epidermidis* recipient strains by filter mating. *S. epidermidis* strains Wr and Tr transferred resistance by filter mating into *S. aureus* strains RN2677 and RN27(FA), but not into *S. aureus* RN450(NR) or *S. epidermidis* Mg(NR).

Transfer did not appear to involve phage. First, the transfer frequency was the same in

TABLE 3. Characteristics of staphylococci used in the study of transfer of aminoglycoside resistance plasmids by filter mating

Species	Strain designation	Relevant markers[a]	Lysogenic[b]	Susceptibility to transducing phage:	No. of plasmids (size)	Additional characteristics[c]
Donor						
S. aureus	661	Gm^r Nov^s Rif^s	–	φ11	1 (41.6 kilobases)	EB^r
S. aureus	913	Gm^r Nov^s Rif^s	+		1 (53.2 kilobases)	β-L, EB^r
S. epidermidis	Wr	Gm^r Nov^s Rif^s	+		3	M^r, Cc^r, Er^r, Te^r, EB^r
S. epidermidis	Tr	Gm^r Nov^s Rif^s	–		10	M^r, Cc^r, Er^r, Te^r, EB^r
Recipient						
S. aureus	RN450(NR)	Gm^s Novr Rif^r	–	φ11, φ80α	None	None
S. aureus	RN2677	Gm^s Novr Rif^r	+ (φ11)	φ80α	None	Restriction deficient
S. aureus	RN27(FA)	Gm^s $FusA^r$	+ (φ80)		None	None
S. epidermidis	Mg(NR)	Gm^s Novr Rif^r	–		None	Clinical isolate

[a] Gm, Gentamicin; Nov, novobiocin; Rif, rifampin; FusA, fusidic acid.

[b] Lysis induced by treatment with 1 μg of mitomycin C per ml.

[c] β-L, Beta-lactamase positive; M, methicillin; Cc, clindamycin; Er, erythromycin; Te, tetracycline; EB, ethidium bromide.

broth with or without the addition of sodium citrate to chelate the Ca^{2+} ions required for transduction. Second, the frequency of transfer was not affected by the lysogenic state or the susceptibility to phage lysis of the donor or recipient cells. The frequency of transfer of gentamicin resistance plasmids from each of the four donor staphylococcus strains into the restriction-deficient, lysogenic recipient strain RN2677 was similar (1×10^{-5} to 3×10^{-6} per recipient cell), and nonlysogenic donor strain *S. aureus* 661 transferred gentamicin resistance to both nonlysogenic recipient strains RN450(NR) and *S. epidermidis* Mg(NR). In addition, resistance was serially transferred by filter mating from the nonlysogenic recipient strain RN450(NR) to the nonlysogenic strain RN450, which was made resistant to fusidic acid. Third, filtrates of three of the four mitomicin C-treated donor cells did not transfer aminoglycoside resistance to recipient strain RN2677 (frequency, $<10^{-9}$ per recipient cell). Aminoglycoside resistance could be transferred by generalized transduction with transducing phage ϕ11 from strain 661 to strain 2677 of *S. aureus* at a frequency of 10^{-7}, but transfer by transduction resulted in the deletion of plasmid DNA in eight of nine transductants. In contrast, the transfer of aminoglycoside resistance by filter mating from *S. aureus* 661 to strain 2677 or 450(NR) resulted in the transfer of intact plasmid DNA in the 10 recipients examined by restriction endonuclease analysis.

Fourth, an experiment to further rule out generalized transduction in the filter mating transfer was carried out by constructing a strain which contained both the self-transmissible plasmid from *S. aureus* 661 and a smaller plasmid, RN3208. This latter plasmid is a 28.2-kilobase derivative of pI258 which encodes resistance to multiple heavy metals, including cadmium, and contains Tn*551*, an erythromycin resistance transposon (18). Each plasmid was introduced into strain RN2677 by generalized transduction with phage ϕ11 and could be transduced out of strain RN2677 independently into strain RN27 with phage $\phi 80\alpha$ at a high frequency (10^{-6}). In contrast, filter mating resulted in transfer of the self-transmissible aminoglycoside resistance plasmid from *S. aureus* 661 at high frequency (2×10^{-5} per recipient cell), but the smaller, nontransmissible plasmid did not transfer at all (no colonies per 10^9 recipients). If transduction were involved in filter mating transfer, the smaller plasmid should have transferred at a frequency similar to that of the larger plasmid.

Fifth, transfer of the aminoglycoside resistance plasmid mobilized the transfer of from one to five additional plasmid bands from wild *S. epidermidis* isolates Wr and Tr to *S. aureus* 2677. The transfer of additional plasmids was random. No specific R-plasmids were identified among the plasmids mobilized and cotransferred from the wild isolates.

Conditions for transfer. Transfer frequency, expressed as transfers of gentamicin resistance per recipient cell, was determined after manipulating the components of the transfer system. The standard for transfer was donor and recipient cells in log phase, with the filter inverted on the surface of agar and incubated at 37°C for 24 h. The standard frequency of transfer of gentamicin resistance from *S. aureus* 661 into strain RN2677 varied from 5×10^{-5} to 3×10^{-6}. The following manipulations did not alter transfer frequency: decreasing the mating time to 6 h or increasing it to 48 or 72 h, mating cells in stationary phase, incubating filters at 30°C, and mating under anaerobic conditions (using agar overlay). The highest frequencies were obtained with the restriction-deficient recipient strain RN2677, compared with frequencies for all other isolates with intact restriction modification systems (S. aureus strains RN450 and RN27 and *S. epidermidis* Mg). The frequency of transfer into these latter recipients was 10- to 100-fold lower.

DISCUSSION

R-plasmids that are self-transmissible between gram-positive cocci are being reported with increasing frequency. Plasmids encoding macrolide, lincosamide, and streptogramin resistance were initially identified in group D streptococci and found to be interspecifically self-transmissible to other streptococci and to *S. aureus* strains (6). The transfer process required cell-to-cell contact and mobilized non-self-transmissible plasmids, as is seen with conjugal plasmid transfer in gram-negative bacteria. Plasmid transfer between staphylococci, however, has traditionally been believed to require the participation of phage (13). Recently, transfer of aminoglycoside resistance between staphylococci of the same and different species was shown in mixed culture and on human skin (9), and Lacey showed a requirement for cell-to-cell contact for the transfer of a tetracycline resistance plasmid between *S. aureus* strains (14). However, he felt that phage participation for this transfer was necessary on the basis of a requirement for Ca^{2+} or Mg^{2+} ions and called the transfer process "phage-mediated conjugation." The first demonstration that self-transmissible aminoglycoside resistance plasmids may occur in staphylococci and that transfer may proceed without phage was made by Forbes and Schaberg (7) and independently confirmed by McDonnell et al. (16). We have extended these observations by identifying a group of related self-transmissible

plasmids found in both *S. epidermidis* and *S. aureus* isolates from our hospital which encode resistance to aminoglycosides and, in some instances, the production of beta-lactamase.

We felt that these plasmids were self-transmissible because of the need for cell-to-cell contact and because the traditional transfer mechanisms of transformation and transduction were ruled out. However, although these mechanisms were excluded, it could be argued that phage DNA may have participated in some way in the transfer process. Cells that are not induced to lyse by treatment with mitomycin C or UV light may still contain defective prophage in their genome which may facilitate transfer by unknown mechanisms. However, documentation of the serial transfer of apparently unaltered plasmid DNA in all of the transcipients and mobilization of the transfer of as many as five additional plasmids by the aminoglycoside resistance plasmid suggest that this plasmid contains genes which encode its transfer by a mechanism which is similar to that of conjugation.

We identified plasmids with five different restriction endonuclease digestion patterns which were all self-transmissible. Jaffe et al. (10) also found five classes of aminoglycoside resistance plasmids among the staphylococci in their hospital which had extensive homology when tested by Southern blot hybridization. Although we did not perform Southern blot hybridization, homology among our plasmids was suggested by the common sizes of many restriction endonuclease digest fragments. This fact and the similar phenotypic profiles of the plasmids (Table 2) suggest that these plasmids are related. The production of the same aminoglycoside-inactivating enzymes by all of the plasmids suggested either their evolutionary similarity or the possibility that gentamicin resistance genes were present on a transposable element. Attempts to induce the transposition of gentamicin resistance to another plasmid or to the chromosome have so far been unsuccessful (G. Archer, unpublished data).

Conjugative transfer of gentamicin resistance between staphylococci may explain several observations about the epidemiology of aminoglycoside-resistant staphylococci in our hospital. First, there has been a recent dramatic increase in the incidence of gentamicin-resistant staphylococci in our hospital. This increase occurred without any change in gentamicin use or dosage in the areas where resistant staphylococci were found. Although only 20% of cardiac surgery patients were colonized with gentamicin-resistant *S. epidermidis* strains in 1977–78 (3), 70% were colonized in 1980–81 (2a). Of the 10 isolates which we examined for the ability to transfer aminoglycoside resistance, 4 were from the 1977–78 group. None of these isolates transferred resistance by filter mating. In contrast, three of the six gentamicin-resistant *S. epidermidis* isolates from the 1980–81 period and the single *S. epidermidis* PVE isolate recovered in 1980–81 contained transmissible plasmids. Therefore, the rapid increase in aminoglycoside resistance among the staphylococci seemed to coincide with the acquisition or evolution of a plasmid which was transmissible by cell-to-cell contact. Our inability to transfer the aminoglycoside resistance plasmid from wild gentamicin-resistant *S. epidermidis* strains to an *S. epidermidis* recipient strain may reflect the difficulty that we had in finding a suitable plasmid-free recipient. The isolate (*S. epidermidis* Mg) that we selected was the only suitable gentamicin-susceptible, plasmid-negative *S. epidermidis* isolate that we found after screening more than 80 clinical isolates. This isolate was, in fact, a recipient for the low-frequency transfer of aminoglycoside resistance from both *S. aureus* donor strains, but from neither of the two *S. epidermidis* donor strains.

Second, although gentamicin resistance among *S. aureus* strains did not increase dramatically in the hospital between 1978 (7% of all isolates resistant) and 1981 (11% resistant), there was an outbreak of gentamicin-resistant *S. aureus* infections in the newborn nursery beginning in 1980 and continuing into 1983. All of the *S. aureus* and *S. epidermidis* isolates from babies in this unit transferred gentamicin resistance to an *S. aureus* recipient (Table 1), and *S. aureus* isolates recovered from two babies contained aminoglycoside resistance plasmids with restriction endonuclease digests identical to those of *S. epidermidis* isolates recovered from the same baby. Similar data were presented by Weinstein et al. (24), who showed that gentamicin resistance plasmids which were identical by restriction endonuclease fingerprinting occurred in both *S. aureus* and *S. epidermidis* isolates and suggested that interspecific transfer occurred in vivo. Experimental in vivo transfer was shown by Jaffe et al. (9), who inoculated donor and recipient *S. aureus* strains onto human skin and observed transfer frequencies as high as 10^{-7} per donor input cell. Our data showing that isolates of different species with the same plasmid were present in the same babies and that the gentamicin resistance plasmid was self-transmissible further strengthen the case for in vivo transfer. Furthermore, the findings that gentamicin resistance among *S. epidermidis* isolates in our hospital is common, is self-transmissible from this species to *S. aureus*, and preceded the outbreak of gentamicin-resistant *S. aureus* infections in the neonatal nursery suggest that, as postulated by Jaffe et al. (10), *S. epidermidis* is a

genetic reservoir for gentamicin resistance among hospital-acquired staphylococci.

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LITERATURE CITED

1. **Allen, J. R., A. W. Hightower, S. W. Martin, and R. E. Dixon.** 1981. Secular trends in nosocomial infections: 1970–1979. Am. J. Med. **70**:389–392.
2. **Archer, G. L.** 1978. Antimicrobial susceptibility and selection of resistance among *Staphylococcus epidermidis* isolates recovered from patients with infections of indwelling foreign devices. Antimicrob. Agents Chemother. **14**:353–359.

2a. **Archer, G. L., and B. Armstrong.** 1983. Alteration of staphylococcal flora in cardiac surgery patients receiving antibiotic prophylaxis. J. Infect. Dis. **147**:642–649.

3. **Archer, G. L., and M. J. Tenenbaum.** 1979. Antibiotic-resistant *Staphylococcus epidermidis* in patients undergoing cardiac surgery. Antimicrob. Agents Chemother. **17**:269–272.
4. **Archer, G. L., N. Vishniavsky, and H. G. Stiver.** 1982. Plasmid pattern analysis of *Staphylococcus epidermidis* isolates from patients with prosthetic valve endocarditis. Infect. Immun. **35**:627–632.
5. **Davies, J., and D. I. Smith.** 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32**:469–518.
6. **Engel, H. W. B., N. Soedirman, J. A. Rost, W. J. van Leeuwen, and J. D. A. van Embden.** 1980. Transferability of macrolide, lincomycin, and streptogramin resistances between group A, B, and D streptococci, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. J. Bacteriol. **142**:407–413.
7. **Forbes, B. A., and D. R. Schaberg.** 1983. Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. J. Bacteriol. **153**:627–634.
8. **Haas, M. J., and J. E. Dowding.** 1975. Aminoglycoside modifying enzymes. Methods Enzymol. **43**:611–628.
9. **Jaffe, H. W., H. M. Sweeney, C. Nathan, R. A. Weinstein, S. A. Kabins, and S. Cohen.** 1980. Identity and interspecific transfer of gentamicin-resistance plasmids in *Staphylococcus aureus* and *Staphylococcus epidermidis*. J. Infect. Dis. **141**:738–747.
10. **Jaffe, H. W., H. M. Sweeney, R. A. Weinstein, S. A. Kabins, C. Nathan, and S. Cohen.** 1982. Structural and phenotypic varieties of gentamicin resistance plasmids in hospital strains of *Staphylococcus aureus* and coagulase-negative staphylococci. Antimicrob. Agents Chemother. **21**:773–779.
11. **Karchmer, A. W., G. L. Archer, and W. E. Dismukes.** 1983. *Staphylococcus epidermidis* causing prosthetic valve endocarditis: microbiologic and clinical observations as guides to therapy. Ann. Intern. Med. **98**:447–454.
12. **Kloos, W. E., and K. H. Schleifer.** 1975. Simplified scheme for the routine identification of human *Staphylococcus* species. J. Clin. Microbiol. **1**:82–88.
13. **Lacey, R. W.** 1975. Antibiotic resistance plasmids of *Staphylococcus aureus* and their clinical importance. Bacteriol. Rev. **39**:1–32.
14. **Lacey, R. W.** 1980. Evidence for two mechanisms of plasmid transfer in mixed cultures of *Staphylococcus aureus*. J. Gen. Microbiol. **119**:423–435.
15. **Le Goffic, F., A. Martel, N. Moreau, M. L. Capmau, C. J. Soussy, and J. Duval.** 1977. 2″-O-phosphorylation of gentamicin components by a *Staphylococcus aureus* strain carrying a plasmid. Antimicrob. Agents Chemother. **12**:26–30.
16. **McDonnell, R. W., H. S. M. Sweeney, and S. Cohen.** 1983. Conjugal transfer of gentamicin resistance plasmids intra- and interspecifically in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **23**:151–160.
17. **Naidoo, J., and W. C. Noble.** 1978. Transfer of gentamicin resistance between strains of *Staphylococcus aureus* on skin. J. Gen. Microbiol. **107**:391–393.
18. **Novick, R. P., I. Edelman, M. D. Schwesinger, A. D. Gruss, E. C. Swanson, and P. A. Pattee.** 1979. Genetic translocation in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. **76**:400–404.
19. **Perret, C. J.** 1954. Iodometric assay of penicillinase. Nature (London) **174**:1012–1013.
20. **Schaberg, D. R., D. B. Clewell, and L. Glatzer.** 1982. Conjugative transfer of R-plasmids from *Streptococcus faecalis* to *Staphylococcus aureus*. Antimicrob. Agents Chemother. **22**:204–207.
21. **Schwotzer, V., F. H. Kayser, and W. Schwotzer.** 1978. R-plasmid mediated aminoglycoside resistance in *Staphylococcus epidermidis*: structure determination of the products of an enzyme nucleotidylating the 4′- and 4″-hydroxyl group of aminoglycoside antibiotics. FEMS Microbiol. Lett. **3**:29–33.
22. **Scott, D. F., D. O. Wood, G. H. Brownell, M. J. Carter, and G. K. Best.** 1978. Aminoglycoside modification by gentamicin-resistant isolates of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **13**:641–644.
23. **Vogel, L., C. Nathan, H. M. Sweeney, S. A. Kabins, and S. Cohen.** 1978. Infections due to gentamicin-resistant *Staphylococcus aureus* strain in a nursery for neonatal infants. Antimicrob. Agents Chemother. **13**:466–472.
24. **Weinstein, R. A., S. A. Kabins, C. Nathan, H. M. Sweeney, H. W. Jaffe, and S. Cohen.** 1982. Gentamicin-resistant staphylococci as hospital flora: epidemiology and resistance plasmids. J. Infect. Dis. **145**:374–382.