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Infections Due to Gentamicin-Resistant *Staphylococcus aureus* Strain in a Nursery for Neonatal Infants

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An apparently homogeneous strain of *Staphylococcus aureus* resistant to gentamicin (Gm^r), kanamycin, tobramycin, and sisomicin, but susceptible to amikacin and netilmicin, caused multiple infections in neonatal infants in a special care nursery. Nasal cultures revealed a high rate of carriage of the Gm^r staphylococcus in infants without clinical infection. Segregating patients according to a modified cohort system and use of careful aseptic techniques led to apparent elimination of the Gm^r strain. The resistance to aminoglycosides in this strain was mediated by an aminoglycoside 6′-*N*-acetyltransferase and a gentamicin phosphotransferase. Genetic determinants for these enzymes were borne on a circular covalently closed plasmid of approximately 11 megadaltons. These resistance determinants closely resemble those found in isolates of *S. aureus* that have caused nosocomial infections in patients in Europe.

Until recently, *Staphylococcus aureus* has been thought to be almost regularly susceptible to gentamicin. However, within the past few years, strains of *S. aureus* resistant to gentamicin and other related aminoglycoside antibiotics have appeared in European countries, where they have caused infections, notably in patients in hospitals (1, 6, 11, 13–16, 19). As might have been expected, similarly resistant *S. aureus* strains have appeared in the United States and have been responsible for hospital-acquired infections (M. J. Carter, G. P. Greenhood, R. E. Dixon, W. P. Kanto, E. Aziz, V. Ashline, and J. Galbraith, Abstr. Annu. Meeting Am. Soc. Microbiol. 1977, C123, p. 56). In this paper we report the epidemiological and microbiological aspects of a small epidemic of neonatal infections caused by a strain of *S. aureus* that was resistant to gentamicin, tobramycin, kanamycin, and sisomicin.

MATERIALS AND METHODS

Bacteria and plasmids. Clinical isolates of *S. aureus* and *S. epidermidis* are described in the Results. RN450 is a plasmid-free, phage-"cured" derivative of *S. aureus* 8325 (9). pSH2 is a staphylococcal kanamycin-neomycin resistance plasmid which has a sedimentation coefficient (s_{20w}) of 32*s* and a molecular mass of 10 or 11 megadaltons (3, 18).

Media and culture methods. Specimens were cultured for *S. aureus* by streaking on 5% sheep blood agar or mannitol salt agar (BBL) petri plates. The plates were examined after incubation for 18 to 24 h at 36°C. *S. aureus* colonies were identified by colonial morphology, Gram stain, and positive coagulase tests with rabbit plasma. For survey cultures, specimens were taken regularly from swabs that were passed through the nose as far as the nasopharynx.

A single colony of each *S. aureus* isolate was submitted to the Public Health Laboratories, State of Illinois Department of Public Health, for phage typing.

Susceptibility tests. Qualitative susceptibility tests were performed by a standard disk diffusion (Kirby-Bauer) method (12). Susceptibility to methicillin was determined with use of a disk containing 5 μg of methicillin, after overnight incubation at 32°C. For quantitative assays we used an inoculum of a 10^{-2} dilution of an overnight culture in Mueller-Hinton broth deposited by a Steers-Foltz replicator on Mueller-Hinton agar plates containing serial twofold dilutions of the antibiotic under test. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic that prevented visible growth after incubation for 18 h at 36°C. Tests for susceptibility to Hg^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, AsO_4^{4-}, and AsO_3^{3-} were performed by a disk diffusion method (10).

Plasmid elimination. A single colony was picked from growth on a brain heart infusion agar plate containing 100 μg of kanamycin per ml and was inoculated into a few milliliters of Trypticase soy broth. After 2 h at 37°C, this culture was used to provide inocula of 10^4 colony-forming units per ml into tubes of Trypticase soy broth containing graded concentrations of ethidium bromide from 0.5 to 3.0 μg/ml and into broth without ethidium bromide. After incubation for 48 h at 30, 37, or 42°C, appropriate dilutions of cultures without ethidium bromide were isolated on Trypticase soy agar. Similar platings were made from tubes with the highest concentration of ethidium bromide that allowed macroscopic growth. After 18 h at

37°C, the resultant colonies were replica plated on agar plates containing 100 μg of kanamycin/ml or 10 μg of gentamicin/ml.

Other methods. Transduction of resistance determinants was performed by published methods (2), with typing phage 53 at a multiplicity of 0.07 and a selective medium of brain heart infusion agar containing 100 μg of kanamycin/ml. We allowed a posttransductional incubation for 2.5 h in brain heart infusion broth without antibiotics to permit expression of transduced genes.

Staphylococcal deoxyribonucleic acid (DNA) was labeled with [^{3}H]thymidine in young log-phase cells at 30°C for 2 h in casein hydrolysate yeast extract broth (17). DNA was extracted and analyzed on isopycnic ethidium bromide-CsCl gradients and on neutral and alkaline sucrose gradients (17, 18).

Assays for aminoglycoside adenylyltransferase, acetyltransferase, and phosphotransferase were performed by published methods (5).

RESULTS

Clinical epidemiology and control measures. We detected our first gentamicin-resistant (Gm^r) strains of *S. aureus* in cultures from infants in the Neonatal Special Care Nursery, a unit for infants with low birth weight or other medical or surgical problems that require intensive care. This nursery was comprised of two rooms: one room was an intensive care unit with a capacity of 12 bassinets, and the other was an intermediate care unit with a capacity of 18 bassinets. Babies were transferred in either direction between these two units on the basis of clinical improvement or relapse. In addition, there were two adjacent separate isolation rooms for one to three babies each. The babies were housed in incubators or open bassinets with or without an overhead warmer. Standard nursery technique included a hand scrub for 2 min with an iodophor or hexachlorophene soap on entering the unit and a soap and water scrub for 15 s between patients. Personnel wore gowns, but these were not changed between patients. Kanamycin and ampicillin were administered frequently to patients in this nursery in attempts at the early treatment of potential bacterial infection in this highly susceptible population. Our earlier experience has shown that this practice may lead to a high prevalence of kanamycin-resistant *S. aureus* strains in the babies. After recent reports of the isolation of Gm^r staphylococci in England and France, we tested isolates of *S. aureus* for susceptibility to gentamicin and found a Gm^r *S. aureus* strain in an endotracheal specimen obtained 31 December 1976 from a baby in the Special Care Nursery. During the following 3 weeks, similar strains were isolated from six additional premature infants in the same nursery. These included three cases of bacteremia, one case of umbilical infection, one case of dermatitis at the site of a pleural drainage tube, and one case of nasal colonization.

To determine whether we were dealing, at this time, with widespread colonization of the nursery population with Gm^r staphylococci, we performed a series of weekly nasal survey cultures on all patients in the nursery (about 25 in number), beginning 18 January. We detected seven other colonized patients from such cultures obtained through 7 February 1977. Two of these babies developed clinical infections due to the Gm^r strain of *S. aureus* within 1 month of their first positive survey culture.

A modified cohort system of distribution of patients in the nursery was instituted on 28 January 1977. Infants were classified into three groups: those that had been culture positive for Gm^r staphylococci through 24 January, those that had been admitted to the nursery before 28 January but had not been culture positive, and new admissions after 28 January. Culture-positive infants were housed initially in the isolation rooms and the intensive care unit. Newly admitted infants were housed in a separate section of the intermediate care unit. After culture-positive infants had been discharged from the intensive care unit, the remaining babies in that unit were transferred to separate sections of the intermediate care unit, one section for each of the three classifications of patients. Each section had its separate nursing staff, although there was no physical barrier between the sections. The intensive care unit was cleaned thoroughly. Personnel were instructed in the importance of hand washing when entering the nursery and after handling babies. A survey of personnel working in the Special Care Nursery by nasal swab cultures revealed no carriers of the Gm^r staphylococcus. In February, the four remaining culture-positive infants were transferred to the two isolation rooms, and the Special Care Nursery reverted to normal operation.

From 7 February through 22 April, nine surveys failed to detect any additional colonized patients among approximately 70 new patients admitted to the nursery during this period. Some patients previously colonized or infected with the Gm^r staphylococcus had intermittently or persistently positive cultures until 8 March, but these patients were discharged by 24 March. No Gm^r staphylococci were detected in five surveys from 8 March to 22 April.

Two babies who had been hospitalized in the Special Care Nursery during the outbreak, but had been discharged before the nasal culture surveys were instituted, were readmitted subsequently to another nursing unit, one with nasal colonization and the other with conjunctivitis

due to the Gm^r strain. In addition, one baby housed in another newborn nursery developed conjunctivitis and infection of the umbilical stump with the Gm^r *S. aureus*. We assumed that this baby acquired the organism from one of the index cases who was housed for a short time in this newborn nursery after transfer from the Special Care Nursery. The infected baby was discharged within a few days. A survey of the remaining babies in the newborn nursery by nasal and unbilical swabs disclosed no other carriers of the Gm^r staphylococcus.

After the ninth negative survey, on 22 April, the next survey of infants in the Special Care Nursery was performed on 24 May. This revealed two new babies colonized with the Gm^r staphylococcus. These babies were isolated and discharged without clinical evidence of infection. A final survey on 2 June 1977 showed no other colonized infants. In the absence of further cases of clinical infection by the Gm^r staphylococcus, we conducted no other surveys. Routine cultures from babies in the Special Care Nursery were negative for Gm^r staphylococci.

During this study, 37 isolates of gentamicin-susceptible (Gm^s) *S. aureus* were obtained from 22 patients, one from a blood culture and the remainder from survey cultures. Among these, there were 23 isolates from 11 patients, including the blood culture isolate, that were phenotypically comparable to the Gm^r strain in all tests employed as detailed below, except that they were susceptible to aminoglycoside antibiotics. Nine of these 11 patients also harbored the Gm^r strain concurrently or at other times.

From 18 January to 7 February, the period during which all of the original Gm^r strains were detected, 12 of 26 (46%) staphylococcal isolates obtained from the survey cultures were Gm^r. From 7 February through 28 February, the period during which the original Gm^r strains persisted in the environment, 5 of 13 (39%) isolates were Gm^r. From 8 March to 22 April, none of the isolates was Gm^r. The survey of 24 May showed two of eight (25%) isolates to be Gm^r.

Although we did not screen routinely for Gm^r strains of *S. epidermidis*, we detected, incidentally, two isolates of *S. epidermidis* that had the same aminoglycoside resistance pattern as the Gm^r *S. aureus*.

Microbiological observations. Some phenotypic properties of the Gm^r isolates are listed in Table 1. The resistant strains formed a relatively homogeneous group. Their phage types were, for the most part, similar, albeit not identical. Successive Gm^r isolates from each of four patients had phage types that varied within the range listed in Table 1. All Gm^r strains had identical patterns of susceptibility to antibiotics and inorganic ions. They exhibited an intermediate degree of resistance to Cd^{2+} characterized by a zone of inhibition 15 mm in diameter around the Cd^{2+} disk. Staphylococci that harbor a penicillinase plasmid usually have no zone of inhibition, whereas staphylococci lacking a penicillinase plasmid exhibit a zone of inhibition measuring 22 mm in diameter. This relatively unusual property reinforced the probability that the Gm^r *S. aureus* isolates present in the Special Care Nursery were derived from a single clone.

Broth cultures of each of three isolates of Gm^r *S. aureus* accumulated readily detectable Gm^s clones when they were grown at 37 or 42°C and much higher proportions of Gm^s clones after growth at 37°C with ethidium bromide (Table 2). Gm^s clones did not revert to resistance when tested by plating 10^9 colony-forming units on brain heart infusion agar containing 15 μg of gentamicin/ml or 100 μg of kanamycin/ml. The Gm^r property was distinctly more stable at 30°C than at 37°C. After strain Bell was subcultured 12 times serially in Trypticase soy broth at 37°C,

TABLE 1. *Properties of gentamicin-resistant S. aureus isolates from patients in a premature nursery*

Property	Result[a]
Antibiotic susceptibility	
Penicillin	R
Streptomycin	S
Tetracycline	S
Chloramphenicol	S
Methicillin	S
Gentamicin	R
Tobramycin	R
Kanamycin	R
Sisomicin	R
Amikacin	S
Netilmicin	S
Inorganic ion susceptibility	
Cd^{2+}	I
Hg^{2+}	S
Pb^{2+}	R
Zn^{2+}	R
AsO_4^{3-}	S
Bacteriophage type	
42E/53//81	11
29//42E	2
(42E)	10
(42E)/53	1
Nontypable	3
29/(3C)/6/42E	1
29/42E/(47)/81	1
(29)	1
(29)/42E/(81)	2
6	1

[a] R, Resistant; S, susceptible; I, intermediate. Numbers represent the number of isolates of each phage type.

the culture contained 40% Gm^s clones, whereas after similar cultures at 30°C there were 1% Gm^s clones. These observations suggested that aminoglycoside resistance in the Gm^r strains might be borne on a plasmid. Aminoglycoside resistance was eliminated similarly from one of the Gm^r *S. epidermidis* isolates.

The aminoglycoside resistance pattern of the Gm^r strains was transduced by phage 53 at frequencies of 5×10^{-6} to 4×10^{-8} from *S. aureus* strain Bell to two other *S. aureus* isolates of similar phage type and into the standard laboratory strain 8325(p1524). The higher rate of transduction was obtained with recipient strains of the same bacteriophage type as the donor.

TABLE 2. *Elimination of aminoglycoside resistance from S. aureus strain Bell*

Conditions of growth		Aminoglycoside-susceptible clones[a]
Temp (°C)	Ethidium bromide	
37	−	1.6
37	+	33
42	−	2.3

[a] Percentage of total population.

Exposure of the transducing phage for 2 min to ultraviolet irradiation from a General Electric germicidal lamp (18 ergs/s per mm^2) reduced the frequency of transduction to one-third of that with unirradiated phage. A similar dose of ultraviolet has been shown to reduce the transduction of staphylococcal plasmids, whereas it increased the transduction of chromosomal genes (2). The aminoglycoside MICs for the transductants were virtually the same as those for the naturally occurring Gm^r strain (Table 3). No other resistance determinants were cotransduced.

Crude extracts of strain Bell, obtained by grinding the staphylococci with alumina (4), were examined for aminoglycoside-modifying activity. These extracts catalyzed the acetylation and phosphorylation of gentamicin and other aminoglycosides (Table 4). There was no adenylyltransferase activity. The profile of substrate modification by acetylation (i.e., activity against gentamicin C_{1a}, gentamicin C_2, and neomycin B, and lack of activity against gentamicin C_1 and paromomycin) was compatible with the presence of an aminoglycoside 6′-*N*-acetyltransferase. The substrate profile of the phospho-

TABLE 3. *MICs (μg/ml) of antibiotics for various strains of S. aureus*

Strain	Gentamicin	Tobramycin	Amikacin	Kanamycin	Netilmicin	Sisomicin
Bell	40	10	5	>80	1.2	10
Bell (cured)	0.15	0.3	1.2	1.2	0.3	0.3
8325	0.07	<0.3	0.15	0.15	<0.03	<0.03
8325 (Gm^r)[a]	40	20	2.5	>80	2.5	40

[a] A Gm^r transductant from strain Bell to strain RN450.

TABLE 4. *Aminoglycoside-modifying activities of extracts of gentamicin-resistant S. aureus strains Bell and 8325 (Gm^r) and S. epidermidis strain rod*

Substrate	Acetyltransferase activity[a]			Phosphotransferase activity[b]		
	Bell	8325 (Gm^r)	Rod	Bell	8325 (Gm^r)	Rod
Gentamicin	112[c]	124	94	100	100	100
Gentamicin C_1	2	3	3	93	114	119
Gentamicin C_{1a}	130	105	101	104	114	129
Gentamicin C_2	47	45	40	77	104	79
Kanamycin A	53	61	66	36	74	60
Kanamycin B	100	100	100	7	26	22
Amikacin	135	124	80	10	16	11
Netilmicin	126	81	70	83	95	63
Sisomicin	172	154	133	122	132	96
Tobramycin	96	70	84	11	34	28
Neomycin B	139	114	52	7	20	15
Paromomycin	9	5	4	7	4	6
Streptomycin	1	0	1	1	2	1

[a] The results of enzymatic modification of kanamycin B are set at 100%, and other values are reported proportionately. The 100% value reflected at least 400 cpm compared with background counts of about 40 cpm.

[b] Expressed similarly to acetyltransferase activity except that the 100% value was set with gentamicin as substrate.

[c] Results are means of two to four determinations except for acetyltransferase activity of 8325 (Gm^r), which represents a single series of determinations.

transferase activity was compatible with the presence of a gentamicin phosphotransferase similar to that reported by others (4, 7). Similar enzymatic activities were present in strain 8325 transduced to kanamycin resistance from strain Bell (Table 4). The cotransduction of these aminoglycoside-modifying activities suggested that they were determined by genes on a single plasmid. The combined presence of an aminoglycoside 6′-*N*-acetyltransferase and phosphotransferase in aminoglycoside-resistant staphylococci has been reported (4). *S. epidermidis* strain Rod has similar acetylating and phosphorylating activities. Strains of *S. aureus* or *S. epidermidis* that had been "cured" of their aminoglycoside resistance had no aminoglycoside-modifying activity.

The DNA of strain Bell and that of its Gm^r transductant in strain 8325 were analyzed by isopycnic centrifugation in solutions of CsCl-ethidium bromide. In each case a plasmid band was present (data not shown). Zonal centrifugation of the plasmid bands in neutral 20 to 30% sucrose solutions showed the DNA of strain Bell to contain two plasmid peaks with s_{20w} of 48*s* and 35*s* by comparison with pSH2 as a standard (Fig. 1A). The 35*s* plasmid was absent from a Gm^s derivative of strain Bell (Fig. 1B) and was present in a Gm^r transductant of strain RN450 (Fig. 1C). Accordingly, we concluded that the 35*s* plasmid was the genetic determinant for gentamicin resistance in strain Bell. The 48*s* plasmid was presumably a penicillinase plasmid. Freezing and thawing the DNA from a Gm^r transductant of strain Bell caused a discontinuous shift of label from the 35*s* peak to a new peak at ~20*s*, as would be expected if the 35*s* peak was a closed circular plasmid and the 20*s*

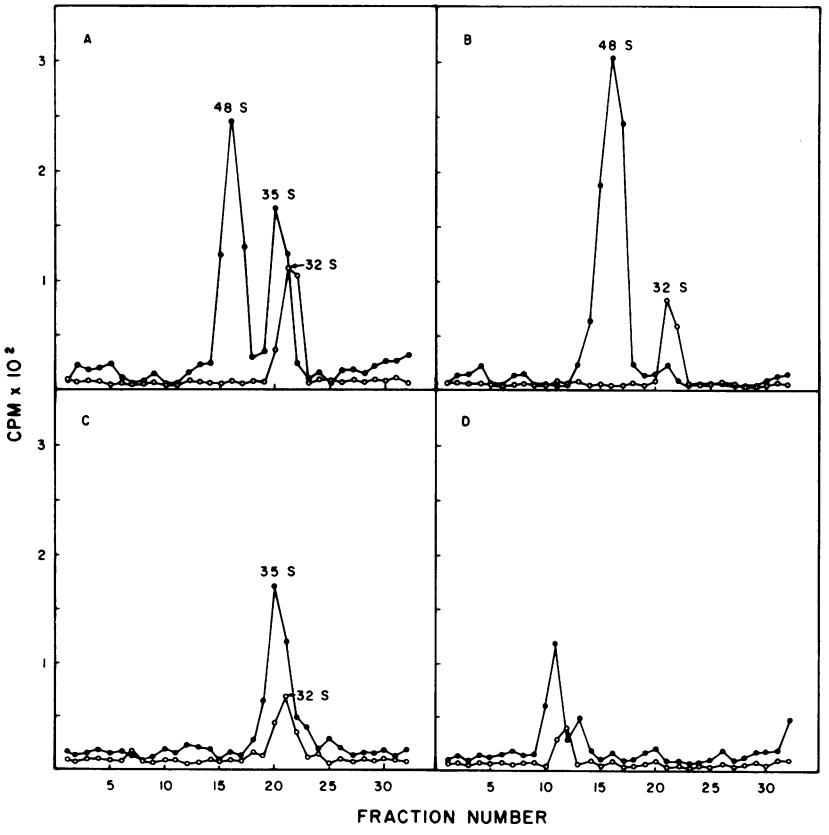

FIG. 1. *Sedimentation analysis of* ^{3}*H-labeled aminoglycoside resistance plasmid (●) from S. aureus strain Bell in sucrose gradients. In each experiment* ^{14}*C-labeled pSH2 (○) was cosedimented as a marker. (A) Sedimentation of plasmid DNA from strain Bell in a 20 to 30% neutral sucrose gradient. (B) Sedimentation in a 20 to 30% neutral sucrose gradient of plasmid DNA from a derivative of strain Bell that had been "cured" of aminoglycoside resistance. (C) Sedimentation in a 20 to 30% neutral sucrose gradient of plasmid DNA obtained from a strain of S. aureus RN450 that was derived by transduction of aminoglycoside resistance from strain Bell. (D) Same as (C) except that plasmids were sedimented in a 20 to 30% alkaline sucrose gradient.*

peak was an open circular derivative thereof (data not shown). In an alkaline sucrose gradient the sedimentation coefficient of the Gm^r plasmid increased to 61s, in accordance with the expected behavior of a covalently closed circular plasmid (Fig. 1D). Based upon cosedimentation with pSH2 in the sucrose gradients, the Bell Gm^r plasmid had a molecular mass of 11 if one takes 10 as the molecular mass of pSH2.

DISCUSSION

In recent years Gm^r *S. aureus* strains have caused infections in hospital patients in England and France. Our experience, in addition to that of others (Carter, et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C 123, p. 56), indicates that aminoglycoside-resistant staphylococci are present in the United States. As might be expected, an environment of frequent treatment of patients with aminoglycoside antibiotics has preceded some Gm^r staphylococcal infections (1, 11). However, treatment with aminoglycosides is not a regular prerequisite for such occurrences (6, 14). In our Special Care Nursery, the frequent prescription of kanamycin and ampicillin probably gave the Gm^r staphylococcus a competitive advantage over susceptible strains. Pediatricians frequently prescribe kanamycin or gentamicin plus ampicillin as the initial therapy of suspected infections in neonates before culture data are available. This therapy is aimed primarily at infection by gram-negative bacilli and streptococci, but in addition is thought to provide some initial protection against the possibility of staphylococcal infection which is ordinarily infrequent at this age. However, when staphylococcal infection is the primary suspect or is proven by culture, therapy should consist of a penicillinase-resistant penicillin, or vancomycin for organisms resistant to these agents. Our experience indicates that the physician can no longer rely on kanamycin or gentamicin to provide even partial protection by their antistaphylococcal activity, nor would tobramycin or sisomicin be suitable substitutes. For the present, either amikacin or netilmicin (not yet available for general use) may be a satisfactory replacement for kanamycin, since the MICs of these agents for our isolates were within the susceptible range, albeit four- to eightfold higher than those for the corresponding fully susceptible strains. However, it is to be expected that such use of amikacin or netilmicin will lead to the advent of staphylococci and other bacteria resistant to these aminoglycosides.

Although the Gm^r strain of *S. aureus* was apparently eradicated from the Special Care Nursery, we have isolated other Gm^r staphylococci, differing in Cd^{2+} resistance and colonial color, from a few adult patients with no discernible contacts with the newborn nurseries and from two patients in the Special Care Nursery. These isolates resemble the Gm^r strain from the Special Care Nursery in their pattern of resistance to the aminoglycoside antibiotics. They have not been examined for their genetic or biochemical mechanism of resistance, but their occurrence indicates that Gm^r staphylococci are not limited to a single strain in our hospital.

The genetic determinants of aminoglycoside resistance in the *S. aureus* strain Bell (and presumably in the other similar isolates) were evidently borne on a circular covalently closed DNA plasmid. The two demonstrated mechanisms of resistance, acetyltransferase and phosphotransferase, were mediated by a single plasmid. This was shown by the conjoint transduction of both enzymatic activities at the relatively low multiplicity of 0.07 phage particles to each recipient staphylococcus and by the conjoint elimination of these enzymes by plasmid "curing" procedures.

The efficiency of the acetyltransferase activity in extracts of strain Bell for different aminoglycoside antibiotics was generally similar to that reported by Dowding (4) and by Le Goffic et al. (7) and, as in their strains, is presumably due to an aminoglycoside 6′-*N*-acetyltransferase. However, extracts of strain Bell acetylated amikacin, gentamicin, and neomycin B to a greater extent than kanamycin B, whereas the reverse was true of extracts of isolates examined by Dowding (4) and Le Goffic et al. (7). The acetyltransferase activity of extracts of *S. epidermidis* strain Rod resembled that of strain Bell, with the exception of relatively less acetylation of amikacin and neomycin B. Phosphorylation of aminoglycoside antibiotics by extracts of strain Bell was most efficient with gentamicin or sisomicin and slight to negligible with neomycin B and paromomycin. These results indicate that the principal phosphotransferase activity in strain Bell was a gentamicin phosphotransferase, very likely similar to the 2″-*O*-phosphotransferase described by others (7). Our studies do not exclude the possible additional presence of a weak aminoglycoside 3′-phosphotransferase activity, as was the case with one of Dowding's strains (4). The substrate specificity of the phosphotransferase in strain Bell resembled that in the strain studied by Le Goffic et al. (7) but differed from that reported by Dowding (4) in exhibiting lesser activity toward kanamycin B and amikacin. Evidently, the enzymatic mechanisms of aminoglycoside resistance in our Gm^r strain resembled those found in the European isolates. The differences in substrate profiles obtained with our

crude extracts probably are not great enough to warrant an assertion of structural differences in the enzymes of strain Bell, *S. epidermidis* strain Rod, and the European strains of Gm^r *S. aureus*. Closer comparison of the properties of the purified enzymes of each set of organisms is necessary to determine this point. Such studies might be important in weighing the possibility that these Gm^r organisms reflect the widespread dissemination of a single resistance plasmid or multiple acquisitions of different but related determinants of resistance.

Our frequent isolation of a strain of *S. aureus* that was susceptible to aminoglycosides but otherwise phenotypically similar to the Gm^r strain suggests the possibility of ready in vivo acquisition or loss of the Gm^r aminoglycoside resistance plasmid. This hypothesis is supported by the easy in vitro plasmid loss from the Gm^r strain. Evidence for in vitro loss of other resistance plasmids in *S. aureus* has been presented recently (8).

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