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High-Level Resistance to Gentamicin in Clinical Isolates of *Streptococcus (Enterococcus) faecium*

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During a 14-month period beginning in July 1986, three distinct clinical isolates of *Streptococcus (Enterococcus) faecium* demonstrating high-level resistance (MIC, >2,000 μg/ml) to gentamicin, kanamycin, tobramycin, and streptomycin were recovered from individual patients at one institution. Combinations of ampicillin with any of these agents failed to show bactericidal synergism. By filter-mating techniques, high-level gentamicin resistance could be transferred into a susceptible recipient of the same species at frequencies as high as 1×10^{-4}; transfer into *Streptococcus faecalis* JH2-7 occurred at lower frequencies ($<2 \times 10^{-7}$). Aminoglycoside substrate profile analysis of clinical isolates as well as of laboratory-derived cured strains and transconjugants revealed 2″-aminoglycoside phosphotransferase and 3′-aminoglycoside phosphotransferase (III) phosphorylating enzymes, AAC-6′ acetylating activity above that attributable to the intrinsic activity characteristic of *S. faecium*, and a streptomycin adenylylating enzyme. All three isolates carried a 51-megadalton plasmid. Curing of this plasmid or conjugative transfer into susceptible recipients was associated with the loss or acquisition of high-level gentamicin resistance, respectively. Loss of high-level gentamicin resistance was also observed when curing techniques resulted in a decrease in the size of this plasmid equivalent to a 10-megadalton deletion. Transferable, high-level resistance to gentamicin and other aminoglycosides, which was previously recognized in *S. faecalis*, has now emerged in clinical isolates of *S. faecium*, with the attendant concerns for possible spread.

High-level resistance to gentamicin among clinical isolates of *Streptococcus (Enterococcus) faecalis* was first reported in 1979 (8). The genetic determinants encoding the aminoglycoside-modifying enzymes responsible for high-level gentamicin resistance (MIC, >2,000 μg/ml) were found to be plasmid-mediated and transmissible in vitro into suitable recipient strains (3, 15). Since that time, isolates of *S. faecalis* with high-level resistance to gentamicin as well as to other clinically available aminoglycosides have been reported worldwide (2, 22) and currently make up a significant proportion of clinical enterococcal isolates at some centers (28). The importance of these observations rests in the fact that high-level aminoglycoside resistance confers resistance to bactericidal synergism between that aminoglycoside and cell wall-active antibiotics (3, 15).

The eventual discovery of high-level gentamicin resistance in isolates of *Streptococcus (Enterococcus) faecium* was predicted by Chen and Williams (2), who were able to transfer high-level gentamicin resistance determinants from *S. faecalis* into *S. faecium* in vitro by conjugation. We report here the recovery of three distinct clinical isolates of *S. faecium* which possess high-level resistance to gentamicin. We investigated the mechanism of aminoglycoside resistance in these strains and documented resistance to ampicillin-aminoglycoside synergism resulting from the acquisition of aminoglycoside resistance determinants.

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MATERIALS AND METHODS

Bacterial strains. Three clinical isolates of *S. faecium* with high-level resistance to gentamicin were recovered from individual patients who were hospitalized at Children's Hospital Medical Center, Boston, Mass., between July 1986 and August 1987 (Table 1). Strains were identified to the species level by using the API Rapid Strep system (Analytab Products, Plainview, N.Y.). The species of the organisms was confirmed by demonstration of penicillin-binding protein patterns characteristic of *S. faecium* (26). *S. faecium* GE-1, which was used as a recipient in mating experiments, is a rifampin- and fusidic acid-resistant laboratory mutant derived from a penicillin-hypersusceptible strain of *S. faecium*, designated 4379-S (5).

Susceptibility and synergism studies. Antimicrobial agent susceptibilities were determined by a broth macrodilution procedure (10) in dextrose phosphate broth (GIBCO Diagnostics, Madison, Wis.) by using inocula of ca. 2×10^5 to 5×10^5 CFU/ml. Tests of antimicrobial synergism were performed as described previously (18). Synergism was defined as a ≥100-fold killing by the aminoglycoside-penicillin combination at 24 h of incubation compared with the bactericidal effect of the penicillin alone.

Curing of resistance determinants. Initial attempts to cure strains of high-level (>2,000 μg/ml) resistance to gentamicin with novobiocin (14) were unsuccessful. Therefore, a method with ciprofloxacin was used (16). Parent isolates of *S. faecium* were incubated in serial twofold dilutions of ciprofloxacin in dextrose phosphate broth. After 18 h of incubation, tubes containing the highest subinhibitory concentration of ciprofloxacin were sampled onto antibiotic-free plates. Single colonies arising after 24 h of incubation were replica plated onto antibiotic-free and aminoglycoside-containing agar. Colonies failing to grow on these plates were

TABLE 1. Aminoglycoside susceptibilities of clinical enterococcal isolates and laboratory-derived strains

Strain	Source	MIC (μg/ml)				
		Gentamicin	Kanamycin	Tobramycin	Amikacin	Streptomycin
S. faecium 87-P1	Wound (July 1986)	>16,000	128,000	16,000	64,000	>128,000
S. faecium 87-P2	Urine (February 1987)	8,000	128,000	16,000	1,000	>128,000
S. faecium 87-P4	Urine (August 1987)	>8,000	128,000	16,000	4,000	>128,000
S. faecium 87-C1	Cured (strain 87-P2)	32	64,000	500	250	>128,000
S. faecium 87-C2	Cured (strain 87-P1)	32	64,000	1,000	125	128,000
S. faecium 87-C3	Cured (strain 87-P1)	500	64,000	16,000	1,000	>128,000
S. faecalis 87-T3	87-P1 × JH2-7	>8,000	64,000	16,000	2,000	128,000
S. faecium 87-T11	87-P1 × GE-1	8,000	64,000	16,000	>8,000	≤125
S. faecium 87-T24	87-P2 × GE-1	>8,000	64,000	16,000	2,000	4,000
S. faecalis JH2-7	Reference 9	32	250	250	250	250
S. faecium GE-1	See text	16	2,000	1,000	125	≤125

isolated, reidentified, and subjected to formal susceptibility testing.

Conjugation experiments. Transfer of high-level gentamicin resistance determinants into recipient strains of *S. faecalis* JH2-7 and *S. faecium* GE-1 was undertaken by two mating techniques. Cross-streak agar plate mating (7) was used to maximize detection of low-frequency events. To quantitate transfer frequencies, a filter-mating procedure was used (21). Donors and recipients were applied in ratios of 10:1, 1:1, and 1:10. Selective plates contained gentamicin (250 μg/ml), rifampin (100 μg/ml), and fusidic acid (25 μg/ml).

Plasmid detection. Plasmid DNA was prepared by the method of Birnboim and Doly (1) with the following modifications. Cells were grown at 35°C in dextrose phosphate broth (12 ml) to the mid-log phase, at which time glycine was added to a final concentration of 5% and incubation was continued for 1 h (23); lysozyme (5 mg/ml), sodium hydroxide (0.3 M), and sodium dodecyl sulfate (3%) were used for alkali lysis. Separation of plasmid bands was accomplished by horizontal agarose (0.7%) gel electrophoresis in 0.089 M Tris borate buffer (13). Digestion of plasmid DNA with restriction endonucleases *Eco*RI and *Hin*dIII was performed under the conditions recommended by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Aminoglycoside-modifying enzymes. Aminoglycoside-modifying enzyme activity in sonic extracts of bacterial cells was determined by previously described methods (4, 11, 20). Among the aminoglycoside analogs used were netilmicin, 6′-ethylnetilmicin, gentamicin C_1 and C_{1a}, sisomicin, and 2″-deoxysisomicin, which were generously provided by George Miller, Schering Corp., Bloomfield, N.J.

RESULTS

Strain characteristics and antimicrobial agent susceptibilities. The three high-level gentamicin-resistant clinical isolates resembled typical *S. faecium* strains in colony size and morphology and had strong alpha-hemolysis on horse blood agar. Sources of isolation and aminoglycoside susceptibilities are given in Table 1. MICs of ampicillin against strains 87-P1, 87-P2, and 87-P4 were 32, 32, and 128 μg/ml, respectively. All strains were resistant to erythromycin (MIC, >256 μg/ml), clindamycin (MIC, >256 μg/ml), and tetracycline (MIC, ≥16 μg/ml).

Curing experiments. Determinants of high-level resistance to gentamicin were cured after treatment with ciprofloxacin at rates of ca. 1% (1 of 103 colonies derived from strain 87-P1; 2 of 256 colonies derived from strain 87-P2). Two of the resulting strains (87-C1 and 87-C2) were inhibited by gentamicin at 32 μg/ml and also lost high-level tobramycin resistance, while a third strain (87-C3) demonstrated an intermediate level of resistance to gentamicin (MIC, 500 μg/ml) and retained high-level resistance to tobramycin (Table 1). None of the three strains was cured of high-level resistance to streptomycin or kanamycin or of resistance to macrolide antibiotics or tetracycline.

Mating experiments. Transfer of high-level gentamicin resistance to *S. faecalis* JH2-7 occurred at low frequencies ($<2 \times 10^{-7}$ per recipient) but was detected by the cross-streak mating technique. Resistance was transferred to the *S. faecium* recipient at frequencies of 6×10^{-6} to 1×10^{-4} per recipient, depending on the ratio of donors to recipients applied in the mating mixture. Properties of the resulting transconjugants are given in Table 1.

Synergism studies. When ampicillin was combined with any one of several aminoglycosides, no bactericidal synergism was seen against any of the clinical isolates. Curing of high-level resistance to gentamicin restored synergism between ampicillin and gentamicin, but not kanamycin, tobramycin, or amikacin. Transfer of high-level aminoglycoside resistance into *S. faecalis* JH2-7 or *S. faecium* GE-1 was accompanied by a loss of synergism between that aminoglycoside and penicillin or ampicillin.

Aminoglycoside-modifying enzymes. Aminoglycoside-modifying enzyme activities were studied in the first two clinical isolates and in their laboratory-derived strains (Table 2). In both clinical strains, 2″-aminoglycoside phosphotransferase (APH-2″), 3′-aminoglycoside phosphotransferase (APH-3′), and AAC-6′ activities were detected, as were streptomycin-adenylylating enzymes. 6′-Acetylating activity was recorded as positive only when it was greatly in excess of the low-level, chromosomally mediated enzyme activity characteristic of *S. faecium* (25) (Table 2). Substrate profiles of the phosphorylating and acetylating enzymes of representative strains are given in Table 3. The fact that netilmicin is a relatively poor substrate for the AAC-6′ enzyme, when compared with kanamycin (15), partially obscures the fact that acetylating activities of strains 87-P1 and 87-C3 against netilmicin were approximately 200 and 25 times, respectively, those against 6′-ethylnetilmicin, indicating that 6′-acetylating activity existed in both strains. These enzymes were also found in strain 87-P4.

TABLE 2. Relationship between high-level resistance and aminoglycoside-modifying enzymes in clinical isolates of *S. faecium* and laboratory-derived strains

Strain	High-level resistance to[a]:				Enzyme activity			
	Gentamicin	Kanamycin	Tobramycin	Streptomycin	APH-2″	APH-3′	AAC-6′[b]	ANT-SM[c]
87-P1	+	+	+	+	+	+	+	+
87-P2	+	+	+	+	+	+	+	+
87-C1	−	+	−	+	−	+	−	+
87-C2	−	+	−	+	−	+	−	+
87-C3	−[d]	+	+	+	−	+	+	+
87-T3	+	+	+	+	+	+	+	+
87-T11	+	+	+	−	+	−	+	−
87-T24	+	+	+	+	+	+	+	+

[a] MIC, >2,000 μg/ml for gentamicin, kanamycin, tobramycin, and streptomycin.
[b] Enzyme activity above low-level intrinsic activity of *S. faecium* (25).
[c] ANT-SM, Streptomycin adenylyltransferase.
[d] MIC, 500 μg/ml.

Plasmid analysis. Clinical isolates 87-P1 and 87-P2, which were isolated 7 months apart, demonstrated identical plasmid patterns consisting of a high-molecular-mass band of approximately 51 megadaltons (MDa) and two lower-molecular-mass bands of 10 and 6 MDa. Isolate 87-P4 also carried a 51-MDa plasmid but demonstrated a single 8-MDa low-molecular-mass band. Comparison of restriction fragments from plasmid DNA of the three clinical isolates suggested a high degree of genetic relatedness among the high-molecular-mass plasmids of these isolates. Curing of high-level gentamicin resistance from 87-P2 to yield 87-C1 was associated with a decrease in size of the high-molecular-mass plasmid to 41 MDa, which was consistent with a deletion of 10 MDa (Fig. 1). In cured strains 87-C2 and 87-C3, the high-molecular-mass plasmid seen in the parent strain was no longer evident. The smaller plasmid bands remained unchanged in each of the cured strains. Transfer of high-level gentamicin resistance by conjugation was associated with the acquisition of high-molecular-mass bands in strains 87-T11 and 87-T24. In strain 87-T24, the 51-MDa plasmid was transferred unchanged, while in strain 87-T11, the apparent molecular mass of the acquired plasmid was measured at approximately 58 MDa. Despite multiple attempts, plasmid DNA could not be demonstrated in strain 87-T3.

TABLE 3. Substrate profiles of aminoglycoside-modifying enzymes from selected strains

Aminoglycoside	Phosphorylation (%)[a]			Acetylation (%)[a]	
	87-P1	87-T11	87-C3	87-P1	87-C3
Kanamycin[b]	100	100	100	100	100
Gentamicin					
Complex	318	364	5		
C_{1a}				65	36
C_1				<1	<1
Tobramycin	108	90	2		
Amikacin	201	80	230	69	28
Neomycin	113	5	390		
Lividomycin	137	2	449		
Sisomicin	465	328	3		
2″-Deoxysisomycin	2	2	<1		
Netilmicin				9	3
6′-Ethylnetilmicin				<1	<1

[a] Activity relative to kanamycin, which was equal to 100%.
[b] Kanamycin A, 96 to 99% (4).

DISCUSSION

At a time when the increasing prevalence of highly gentamicin-resistant *S. faecalis* isolates has become widely appreciated (15, 28), we have documented here the emergence of high-level gentamicin resistance among clinical isolates of *S. faecium* as well. High-level resistance to aminoglycosides in these *S. faecium* strains could be attributed to the presence of aminoglycoside-modifying enzymes comparable to those seen in highly resistant *S. faecalis* strains (15). The presence of APH-3′ (III) in cured derivatives was deduced from differences in phosphorylation of kanamycin and tobramycin (3′-deoxykanamycin B) and by phosphorylation of amikacin

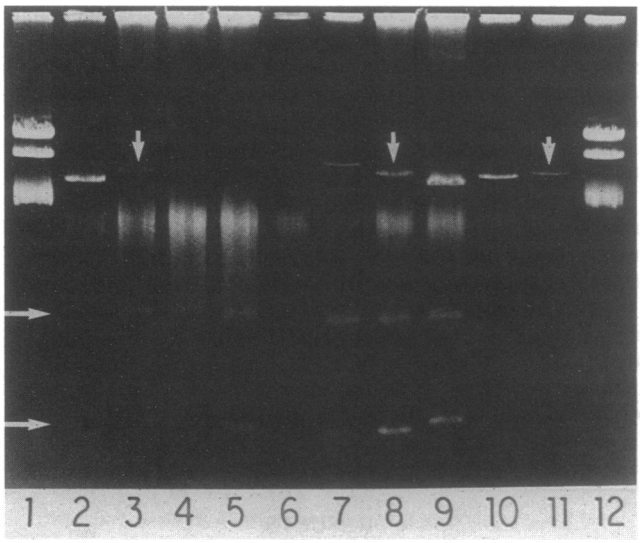

FIG. 1. Agarose gel electrophoresis of plasmid DNA from clinical isolates and laboratory-derived strains. *S. faecium* 87-P1 (lane 3), 87-P2 (lane 8), and 87-P4 (lane 11) were clinical isolates. Vertical arrows indicate the positions of high-molecular-mass plasmid bands. Horizontal arrows indicate low-molecular-mass bands in 87-P1, 87-P2, and derivative strains. Cured derivatives 87-C2 (lane 4) and 87-C3 (lane 5) were obtained from 87-P1, while 87-C1 (lane 9) was derived from 87-P2. Strains 87-T3 (lane 6), 87-T11 (lane 7), and 87-T24 (lane 10) represent transconjugants that were selected for the acquisition of high-level resistance to gentamicin. Molecular mass standards were as follows: Plac (101 MDa), R1 (62 MDa), RP4 (36 MDa), and R6K (26 MDa) (lanes 1 and 12); pDR1 (45 MDa) (lane 2); and Col E1 (4.2 MDa), which is not shown on this gel.

and lividomycin (3, 24). Phosphorylation of sisomicin but not 2″-deoxysisomycin pointed to the presence of an APH-2″ enzyme in parent strains. In two of three cured derivatives and in all three transconjugants, the presence or absence of APH-2″ activity predicted coexisting AAC-6′. This observation is consistent with the recent characterization that APH-2″–AAC-6′ activities of *S. faecalis* arise from a single bifunctional enzyme (6). Interestingly, strain 87-C3 demonstrated considerable acetylating activity in the absence of notable APH-2″ phosphorylation. It is not known whether this situation was the result of an event leading to hyperproduction of the intrinsic acetylating enzyme associated with curing of the bifunctional enzyme or to the dissociation of acquired AAC-6′ and APH-2″ activities, which are presumably fused under normal circumstances (6). As has been the case with highly gentamicin-resistant *S. faecalis*, there was no evidence of aminoglycoside adenylyltransferase-2″ (ANT-2″) activity in these strains of *S. faecium* (3, 15). Adenylylating activity against streptomycin was found in clinical isolates. Characterization of this activity as ANT-6 was inferred by the lack of effective adenylylation of spectinomycin (data not shown) (3, 15).

Loss of high-level resistance to gentamicin was associated with curing of a high-molecular-mass plasmid without a change in the content of smaller plasmid bands. In previous studies in *S. faecalis*, determinants for high-level gentamicin resistance have been localized to plasmids of approximately 45 to 60 MDa (8, 19, 27), which is comparable in size to the high-molecular-mass plasmids in our strains. That genetic determinants mediating production of APH-3′ and ANT-6 enzymes were not located on the high-molecular-mass plasmid was supported by experiments which demonstrated curing of the 51-MDa plasmid without loss of APH-3′ and ANT-6 activities (strain 87-C2) and conjugative acquisition of the 58-MDa plasmid without acquisition of these enzymes (strain 87-T11). Previous investigations of transferable resistance markers in *S. faecium* have associated conjugative transfer of high-level resistance to streptomycin and kanamycin with plasmids of various sizes, but such transfer has also occurred in the absence of detectable extrachromosomal DNA in either donor or recipient strains (12).

Because of the species-specific resistance of *S. faecium* to synergistic killing by combinations of penicillins with aminoglycosides that are susceptible to acetylation at the 6′ position (17) and the frequent occurrence of APH-3′ (III) in these isolates, gentamicin is often the only clinically available aminoglycoside that is capable of synergistically killing these bacteria when combined with a cell wall-active antibiotic. The clinical significance of high-level resistance to gentamicin is, therefore, that synergism between gentamicin and cell wall-active antimicrobial agents against such strains of *S. faecium* is precluded. Although strains of *S. faecium* possessing high-level resistance to gentamicin appear to be extremely uncommon, at least one clinical isolate, in addition to those described here, has been referred to the Centers for Disease Control (B. Metchock, R. Cooksey, B. Hill, and C. Thornsberry, 27th ICAAC, abstr. no. 1321, 1987). In assessing the potential importance of these observations, one can note that frequencies of conjugative transfer of high-level gentamicin resistance from the strains studied here (i.e., as high as 10^{-4}) are comparable to those described previously for *S. faecalis* strains (15). Less than 10 years after the isolation of the first highly gentamicin-resistant strains of *S. faecalis*, such organisms now account for more than 50% of clinical enterococcal isolates at one institution (28). In light of these facts, microbiologists and clinicians should remain alert to the worrisome possibility that highly gentamicin-resistant strains of *S. faecium* may emerge as an increasing clinical problem.

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