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Transferability and Genetic Relatedness of High-Level Gentamicin Resistance among Enterococci

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Gentamicin resistance in six enterococcal species was investigated. Transfer of resistance was observed for the donors *E. faecium* UC 79, *E. avium* CC 54, and *E. gallinarum* B 51, but not for *E. raffinosus* UC 78 or *E. casseliflavus* UC 73. Except for *E. casseliflavus* UC 73, homology was observed between the *E. faecalis aac6'-aph2"* gene and DNA from other species. Whereas 2.6-kb *Hin*dIII fragments encoded resistance in *E. faecalis* UC 244, its transconjugant, and *E. raffinosus* UC 78, 3.4-kb fragments encoded resistance in *E. faecium* UC 79, *E. gallinarum* B 51, and their transconjugants. A 3.4-kb fragment encoded resistance in *E. avium* CC 54, but 2.6-kb fragments encoded resistance in the transconjugants. Although many similarities were found among the strains, the heterogeneity in gentamicin resistance exhibited by some isolates indicates diversity among these determinants.

The bifunctional enzyme 6'-acetyltransferase–2"-phosphotransferase that mediates high-level gentamicin resistance in *Enterococcus faecalis* is encoded by a fused gene (*aac6'-aph2"*) that has been cloned and sequenced (6). Recently, Hodel-Christian and Murray (7) have reported the genetic determinant in *E. faecalis* to be on a mobile element, Tn*5281*. Although gentamicin resistance in *E. faecalis* has been well characterized, the same cannot be said of resistance in other enterococcal species (3, 5, 8, 13, 14). In the present study, we used clinical isolates to investigate the transfer of this resistance among various enterococcal species and we also determined the genetic relatedness between gentamicin resistance in *E. faecalis* and that in other species.

The enterococcal strains studied were clinical isolates that had been identified and characterized as described previously (14–16). Transfer of resistance was investigated by using a previously described filter mating technique with *E. faecalis* JH2-2 and rifampin- and fusidic acid-resistant strains of other species obtained by selection for spontaneous mutants (4, 9). Intragenic probes for *aph2"-aac6'* were provided by J. J. Ferretti, Oklahoma University Health Sciences Center, as two fragments. The *aph2"* portion was a 770-bp *Bam*HI-*Pst*I fragment of pSF815AP, and the *aac6'* portion was an 800-bp *Eco*RI-*Pst*I fragment of pSF815AC (6). Probes ([^{32}P]dCTP; Amersham) labeled with an oligolabeling kit according to the manufacturer's recommendations (Pharmacia LKB, Uppsala, Sweden) were used to hybridize DNA from colony lysates, which were prepared essentially as described previously (11). Undigested and digested plasmid DNAs, obtained by a modification of the procedure reported by Weaver and Clewell (18), were separated by electrophoresis and were transferred for hybridization by using the *aph2"* probe by the method of Southern (17).

The MICs of gentamicin for the resistant strains, which served as donors in subsequent mating experiments, were ≥8,000 μg/ml, and none of the isolates produced a zone of inhibition around the high-content gentamicin disk (Table 1). Although DNA preparations from *E. casseliflavus* UC 73 repeatedly failed to hybridize with either probe, DNA from colony lysates and plasmid preparations of the other donors hybridized with both probes. DNAs from the gentamicin-susceptible recipients did not hybridize.

Intraspecies transfer of gentamicin resistance was detected for *E. faecalis*, *E. faecium*, *E. avium*, and *E. gallinarum* mating pairs, but not among strains of *E. raffinosus* or *E. casseliflavus* (Table 2). Intraspecies transconjugants *E. faecalis* TUC 244, *E. faecium* TUC 79, *E. avium* TCC 54, and *E. gallinarum* TB 51 hybridized with both *aph2"* and *aac6'* probes; the MICs of gentamicin for these strains were comparable to those for the donor strains. Interspecies transfer of resistance was evaluated by mating *E. faecalis* UC 244 with each recipient species listed in Table 1 and by mating the other enterococcal donors with *E. faecalis* JH2-2. Except for *E. faecalis* UC 244, the same strains that demonstrated intraspecies transfer of resistance also showed interspecies transfer of resistance, but transfer frequencies tended to be lower than those observed in intraspecies mating experiments (Table 2). The MICs of gentamicin for interspecies transconjugants were comparable to those for the respective donors, and interspecies transconjugants hybridized with both *aph2"* and *aac6'* probes. Neither *E. raffinosus* UC 78 nor *E. casseliflavus* UC 73 detectably transferred resistance to *E. faecalis* JH2-2 (frequency, $<5 \times 10^{-9}$).

Intraspecies transfer of gentamicin resistance has been reported for *E. faecalis* and *E. faecium*, as has interspecies transfer between these two organisms (3, 5, 8, 12, 19). Results from the present experiments demonstrate the capacity for the intra- and interspecies transfer of high-level gentamicin resistance in two additional enterococcal species, *E. avium* and *E. gallinarum*. Additionally, hybridization results indicate that the high-level gentamicin resistance expressed in the *E. faecium*, *E. gallinarum*, *E. avium*, and *E. raffinosus* strains is encoded by a gene that is homologous to the fused *aac6'-aph2"* gene found in *E. faecalis* (6), extending the observations by Kaufhold et al. (10) and Woodford et al. (19).

Analysis of plasmid DNA revealed hybridization between

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TABLE 1. Characteristics of donor and recipient enterococcal strains

Strain[a]	Gentamicin		Colony hybridization		Plasmid content and size (kb)[b]
	MIC (μg/ml)	Disk inhibitory zone (mm)	*aph2″*	*aac6′*	
Donors					
E. faecalis UC 244	>16,000	6	+	+	**61**
E. faecium UC 79	>16,000	6	+	+	**65**, 7.4, 4.1
E. gallinarum B 51	>16,000	6	+	+	**62**, 10.1, 6
E. avium CC 54	>16,000	6	+	+	**65**, 40
E. raffinosus UC 78	8,000	6	+	+	**62**
E. casseliflavus UC 73	>16,000	6	−	−	77, 62, 7.4, 3.9
Recipients					
E. faecalis JH2-2	≤16	14	−	−	
E. faecium UC 1R	≤16	19	−	−	60
E. avium UC 84R	≤16	14	−	−	
E. gallinarum UC 55R	≤16	17	−	−	
E. raffinosus UC 77R	≤16	18	−	−	
E. casseliflavus UC 65R	≤16	16	−	−	

[a] All donors were susceptible to rifampin and fusidic acid; all recipients were resistant to rifampin and fusidic acid.
[b] Plasmid sizes are approximate. Boldface type indicates the plasmids that hybridized with the *aph2″* probe.

the *aph2″* probe and the 2.6-kb *Hin*dIII fragments of plasmid DNA from donor *E. faecalis* UC 244, its intraspecies transconjugant *E. faecalis* TUC 244, and donor *E. raffinosus* UC 78. This 2.6-kb fragment appears to be the same as that encoded on pBEM10 (7). For donors *E. gallinarum* B 51 and *E. faecium* UC 79 and their intra- and interspecies transconjugants, the probe hybridized with 3.4-kb *Hin*dIII plasmid fragments. Interestingly, a 3.4-kb fragment from the donor *E. avium* CC 54 hybridized with the *aph2″* probe, but only 2.6-kb *Hin*dIII DNA fragments from the intraspecies and interspecies transconjugants *E. avium* TCC 54 and *E. faecalis* TCC 54 hybridized with the *aph2″* probe.

The observation of larger (3.4-kb) *Hin*dIII fragments encoding gentamicin resistance in *E. faecium* UC 79, *E. gallinarum* B 51, and their respective transconjugants suggests that the determinants within these strains may be more closely related to one another than to those found in *E. faecalis* UC 244 or *E. raffinosus* UC 78. This implies that the broad dissemination observed may not be directly to or directly from *E. faecalis*. Also, a relationship appears to exist between the higher-order organization of the gentamicin resistance determinant most commonly borne on a 2.5- to 2.6-kb *Hin*dIII fragment in *E. faecalis* (1, 7) and that observed to occur on 3.4-kb *Hin*dIII fragments in the *E. faecium*, *E. gallinarum*, and *E. avium* strains examined in the present study. Transfer of the *E. avium* CC 54 gentamicin resistance determinant consistently resulted in conversion of the donor's 3.4-kb probe-positive *Hin*dIII fragment to a 2.6-kb fragment.

To further investigate this observation, we repeated the Southern transfer and hybridization experiments using *Cla*I-restricted donor and transconjugant plasmid DNAs, and the findings were identical to those generated with *Hin*dIII-restricted DNA. This observation supports the prospect that rearrangement occurred internal to the *Hin*dIII and *Cla*I restriction sites found in copies of IS*256* that commonly flank the *aac6′-aph2″* gene (2). The precise nature of the DNA rearrangements and the mechanisms of zygotic induction of these rearrangements are the subjects of continuing study.

TABLE 2. Intra- and interspecies transfer of high-level gentamicin resistance

Mating pair	Transfer frequency[a]	Transconjugant	Colony hybridization		Plasmid content and size (kb)[b]
			aph2″	*aac6′*	
Intraspecies					
E. faecalis UC 244 × JH2-2	5×10^{-5}	*E. faecalis* TUC 244	+	+	**61**
E. faecium UC 79 × UC 1	4×10^{-5}	*E. faecium* TUC 79	+	+	**65**, 7.4, 4.1
E. avium C 54 × UC 84	3×10^{-4}	*E. avium* TCC 54	+	+	**65**
E. gallinarum B 51 × UC 55	1×10^{-3}	*E. gallinarum* TB 51	+	+	**62**
E. raffinosus UC 78 × UC 77	$<4 \times 10^{-8}$				
E. casseliflavus UC 73 × UC 65	$<2 \times 10^{-9}$				
Interspecies					
E. faecium UC 79 × *E. faecalis* JH2-2	3×10^{-7}	*E. faecalis* TUC 79	+	+	**65**, 7.4, 4.1
E. avium CC 54 × *E. faecalis* JH2-2	5×10^{-3}	*E. faecalis* TCC 54	+	+	**65**
E. gallinarum B 51 × *E. faecalis* JH2-2	8×10^{-6}	*E. faecalis* TB 51	+	+	**62**
E. raffinosus UC 78 × *E. faecalis* JH2-2	$<5 \times 10^{-8}$				
E. casseliflavus UC 73 × *E. faecalis* JH2-2	$<9 \times 10^{-8}$				

[a] Frequency per donor; each mating was performed in triplicate at a donor-recipient ratio of 1:10.
[b] Plasmid sizes are approximate. Boldface type indicates the plasmids that hybridized with the *aph2″* probe.

The discrepancy between hybridization results and phenotypic expression of high-level gentamicin resistance, evidenced by MIC data and time-kill synergy studies (data not shown), prompted us to characterize the resistance profile further. The MIC of gentamicin for *E. casseliflavus* UC 73 (>32,000 μg/ml) was the same as that which we obtained for five enterococcal strains whose DNAs did hybridize with the *aph2″* probe. *E. casseliflavus* UC 73 also exhibited high-level resistance to kanamycin and tobramycin (MICs, >2,000 μg/ml), but not to streptomycin (MIC, 32 μg/ml) or amikacin (MIC, 64 μg/ml). A similar profile was obtained with another *E. casseliflavus* strain whose DNA hybridized with the fused *aac6′-aph2″* gene (6a), indicating that the mechanism of resistance expressed in *E. casseliflavus* UC 73 is not necessarily a species-specific characteristic. Studies are under way to further investigate the underlying resistance mechanism in this strain.

In conclusion, genes homologous to the fused *aac6′-aph2″* gene of *E. faecalis* were demonstrated in four additional enterococcal species, and transfer of these determinants between and among various species was shown. Many similarities exist between gentamicin resistance in *E. faecalis* and gentamicin resistance in other species, but the heterogeneity in gentamicin resistance evidenced by our findings indicates that measurable divergence exists among these determinants. This divergence may be of value in characterizing the natural course of dissemination. From a clinical perspective, these results demonstrate that enterococcal isolates from infections requiring gentamicin as part of the therapeutic regimen should be screened for high-level resistance, regardless of the species.

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