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High-Level, Plasmid-Borne Resistance to Gentamicin in *Streptococcus faecalis* subsp. *zymogenes*

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Each of three isolates of *Streptococcus faecalis* subsp. *zymogenes* harbored three R plasmids and a hemolysin-bacteriocin plasmid. The plasmids carried by one of these strains were physically characterized after their conjugative transfer. In each strain one of the plasmids carried genetic markers for resistance to gentamicin, kanamycin, sisomicin, netilmicin, and tobramycin.

Enterococci are etiological agents of numerous human infections, such as urinary and genital tract infections and, less often, serious subacute bacterial endocarditis. They are less susceptible to penicillins (minimal inhibitory concentration, 2 to 8 U/ml) than are other streptococci (minimal inhibitory concentration, 0.002 to 0.5 U/ml), and as a result penicillin alone is not always effective in the therapy of enterococcal infections. The aminoglycoside antibiotics are relatively inactive against all naturally occurring streptococcal strains (minimal inhibitory concentration, 5.0 to 125 μg/ml), and they are not used for the treatment of streptococcal infections. However, combinations of penicillins with aminoglycosides are synergistically bactericidal against enterococci in vitro and are effective in treating severe enterococcal endocarditis. The mechanism of this synergy has been explained by the enhanced uptake of aminoglycosides in the presence of penicillins or other agents which inhibit cell wall synthesis (13).

An increased frequency of high-level resistance to aminoglycoside antibiotics (MIC > 8,000 μg/ml) in clinical isolates of enterococci has been reported in recent years for streptomycin and kanamycin (1, 2, 12). These strains were also resistant to synergism with the penicillins (2, 10, 12). Recently, there are several reports demonstrating that high-level resistance to streptomycin and kanamycin is carried by conjugative plasmids (9, 10) and that the aminoglycoside resistance is mediated by both streptomycin adenylyltransferase and aminoglycoside phosphotransferase (3).

High-level, plasmid-borne resistance to gentamicin in streptococci has not yet been reported. The present report describes the isolation of three strains of *Streptococcus faecalis* subsp. *zymogenes* resistant to gentamicin, kanamycin, sisomicin, netilmicin, and tobramycin, to macrolide antibiotics, chloramphenicol, and tetracycline, as well as the transferability and the molecular weights of the R plasmids carried by these strains.

The bacteria used in this study are listed in Table 1. The isolates were identified as group D streptococci by serogrouping and were classified as strains of *S. faecalis* subsp. *zymogenes* by the criteria of Facklam (6). Media, drugs, and growth conditions have been described previously (7, 8). In addition to these drugs, gentamicin and kanamycin were used for R-plasmid selection and analysis of transconjugants. Abbreviations and final concentrations of all drugs are given in Table 1. Determination of high-level resistance to aminoglycosides was performed according to Moellering et al. (12), with the following final drug concentrations: spectinomycin, streptomycin, lividomycin, neomycin, netilmicin, and amikacin at 1,000 μg/ml; kanamycin (Kan) and gentamicin (Gen) at 1,000 to 16,000 μg/ml; tobramycin at 1,000 to 8,000 μg/ml; and sisomicin at 1,000 to 4,000 μg/ml. Bacteriocin production (Bcn) was tested as described by Dunny and Clewell (5), using strain JH2-2 as an indicator. For mating experiments donor and recipient strains were grown under the conditions described previously (8), and 0.1 ml of each was directly spread on a sterile membrane filter (type HA, 0.45 μm; Millipore Corp.), which was placed on a brain heart infusion-5% horse serum agar plate and allowed to incubate at 37°C for 1 or 2 h. The cells were then suspended in 0.5 ml of brain heart infusion, and dilutions were spread on appropriate selective media. Frequencies of transfer are expressed as the number of resistant recipients per donor organism. Plasmid deoxy-

ribonucleic acid (DNA) isolation and sedimentation analysis have been described previously (7, 8).

The results of the frequency of transfer of markers harbored by strains D362, D366, and D367 into JH2-2 are shown in Table 2. Selection was carried out for all donor markers (except Bcn). A low transfer frequency (1×10^{-5} to 1×10^{-6}) was obtained when selection was made for tetracycline (Tc), and a high transfer frequency (1×10^{-1} to 3×10^{-3}) was obtained for the other markers used for selection. The markers carried by strain D367 were transferred at frequencies 10 to 100 times higher than those of strains D362 and D366. This increase of transfer frequency is probably related to an increase in mating time (2 h for D367). The transconjugants obtained in the crosses shown in Table 2 were analyzed (100 clones for each selection) for the nonselected markers. The relevant findings were (i) whatever selection was made (except Tc), all of 1,800 transconjugants analyzed were susceptible to tetracycline; (ii) in each selection and for all donor strains, three groups of linked markers

TABLE 1. *Bacterial donor and recipient strains*

Group D strain designation (species)	Clinical isolate source	Antibiotic treatment before sampling	Relevant markers[a]	Mating type	Origin or reference
D362[b] (*S. faecalis* subsp. *zymogenes*)	Subphrenic abscess	Gentamicin	(Cm, Em, Lm, Gen, Kan, Tc, Hly, Bcn)	R^+	Paris, May 1978 (Hotel-Dieu Hospital)
D366 (*S. faecalis* subsp. *zymogenes*)	Urine culture	Amikacin	(Cm, Em, Lm, Gen, Kan, Tc, Hly, Bcn)	R^+	Paris, June 1978 (Boucicaut Hospital)
D367 (*S. faecalis* subsp. *zymogenes*)	Urine culture	Gentamicin	(Cm, Em, Lm, Gen, Kan, Tc, Hly, Bcn)	R^+	Paris, July 1978 (Hotel-Dieu Hospital)
JH2-2 (*S. faecalis*)			(Fus, Rif)	R^-	(9)
BM133 (*S. faecalis*)			Str	R^-	(8)

[a] Abbreviations (final concentration used for selection): Cm, chloramphenicol (25 μg/ml); Em, erythromycin (4μg/ml); Lm, lincomycin (40 μg/ml); Gen, gentamicin (1,000 μg/ml); Kan, kanamycin (1,000 μg/ml); Tc, tetracycline (4 μg/ml), Hly, hemolysin; Bcn, bacteriocin production; Fus, fusidic acid (25 μg/ml); Rif, rifampin (100 μg/ml); Str, streptomycin (8,000 μg/ml). Parentheses indicate linked markers.

[b] This strain is also designated BM4100.

TABLE 2. *R-plasmid transfer frequency from strains of S. faecalis subsp. zymogenes into strain JH2-2*

Donor strain (mating time)	Selective marker[a]	Plasmid transfer frequency/donor cell	Markers transferred[b]	Strain designation of transconjugants	Plasmid designation	Molecular weight ($\times 10^6$)
D362 (1 h)	Em[c]	8×10^{-3}	(Em, Lm, Hly, Bcn)	BM6206	pIP654	
	Gen[d]	3×10^{-3}	(Cm, Gen, Kan)	BM6207	pIP655	
	Tc	1×10^{-6}	Tc	BM6208	pIP656	
D366 (1 h)	Em[c]	6×10^{-2}	(Em, Lm, Hly, Bcn)	BM6210	pIP684 + pIP718	30 + 37
	Gen[d]	2.5×10^{-2}	(Cm, Gen, Kan)	BN6211	pIP683	44
	Tc	1×10^{-5}	Tc	BM6212	pIP685	20
D367 (2 h)	Em[c]	1×10^{-1}	(Em, Lm, Hly, Bcn)	BM6214	pIP688	
	Gen[d]	2×10^{-2}	(Cm, Gen, Kan)	BM6215	pIP687	
	Tc	1×10^{-5}	Tc	BM6216	pIP689	
JH2-2 (pIP655) (1 h)[e]	Kan[f]	1×10^{-4}	(Cm, Gen, Kan)		pIP655	

[a] Counterselection was done on fusidic acid (25 μg/ml) and rifampin (100 μg/ml).

[b] At least 100 transconjugants were analyzed by replica plating. Parentheses indicate linked markers.

[c] The same results were obtained when selection was done on lincomycin or hemolysin (for Hly selection brain heart infusion-horse blood agar with fusidic acid and rifampin was used).

[d] The same results were obtained when selection was done on kanamycin or chloramphenicol.

[e] The recipient strain was BM133.

[f] The same results were obtained when selection was done on gentamicin or chloramphenicol.

were observed: (Cm, Gen, Kan);(Em, Lm, Hly, Bcn); and Tc alone. The (Cm, Gen, Kan) markers (pIP655) were transferred en bloc from the host JH2-2 to strain BM133.

The DNAs of five transconjugant clones (BM6210, BM6211, BM6212, BM6207, and BM6215) were analyzed by centrifugation in cesium chloride-ethidium bromide density gradients and showed the presence of satellite DNA bands. The molecular weights of the plasmid DNA molecules of BM6210, BM6211, and BM6212 were measured by velocity sedimentation in neutral sucrose gradients. They were found to be 44×10^6 and 20×10^6 for pIP683 (BM6211) and pIP685 (BM6212), respectively; two plasmids (pIP684 and pIP718) of different weights (30×10^6 and 37×10^6, respectively) were present in strain BM6210 (see Table 2). The same results were obtained for these plasmid DNA molecules by using the agarose gel electrophoresis technique (11) (data not shown). Moreover, the molecular weights of plasmid DNAs obtained from pIP655 (BM6207) and pIP687 (BM6215) measured by agarose gel electrophoresis were similar to that of pIP683 (data not shown).

We investigated the patterns of high-level resistance to 10 aminoglycoside antibiotics for the initial isolate strains D362, D366, and D367; for strain JH2-2 harboring pIP655, pIP683, and pIP687 plasmids; and for the control strain JH2-2. These strains were susceptible to 1,000 μg/ml of amikacin, lividomycin, neomycin, spectinomycin, and streptomycin. In contrast, all the strains (except the control) were resistant to >16,000 μg/ml of gentamicin and kanamycin, >4,000 μg/ml of sisomicin, 4,000 μg/ml of tobramycin, and >1,000 μg/ml of netilmicin. These results explain the lack of synergism between gentamicin, sisomicin, netilmicin, and tobramicin with penicillin (Y. A. Chabbert, personal communication).

Recently, the resistance to gentamicin, kanamycin, netilmicin, sisomicin, and tobramicin of *S. faecalis* strain BM4100 (designated as D362 in the present study) was reported to be mediated by two aminoglycoside-modifying enzymes: AAC6′ (acetyltransferase 6′) and APH2″ (phosphotransferase 2″) (P. Courvalin, C. Carlier, and E. Collatz, *in* Mitsuhashi et al., ed., Medical and Biological, Aspects of Resistant Strains, in press).

The results presented in this study demonstrate that the high-level gentamicin resistance of these *S. faecalis* strains is carried by high-frequency conjugative plasmids and mediated by the production of modifying enzymes (4). Two of the three strains (D362 and D367) were isolated from patients after they received gentamicin therapy. The occurrence of these gentamicin resistance plasmids could be explained, at least in part, by the antibiotic selective pressure. The third patient (D366) was not treated with gentamicin; the appearance of the (Gen, Kan), plasmid here may reflect either a nosocomial spread of such gentamicin-resistant strains or a transfer in vivo of gentamicin resistance plasmids carried by the intestinal flora.

The prevalence of bacterial strains resistant to a given antimicrobial agent is related, at least in part, to the selective pressure resulting from the clinical or other use of the antibiotic. Despite extensive use of gentamicin, there have been no reports where high-level resistance to this agent is demonstrated in enterococci (1, 2, 12). High-level, plasmid-borne resistance to gentamicin in streptococci was not described earlier probably because the screening of high-level aminoglycoside resistance is usually done only in severe enterococcal infections (subacute endocarditis). It is likely that the existence of high-level gentamicin resistance could occur at a higher frequency among common isolates. The results presented here also suggest that the determination of a high-level aminoglycoside profile should be performed routinely, and combined therapy with penicillin and gentamicin should no longer be considered as the treatment of choice in enterococcal infections.

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