TRANSFERABLE RESISTANCE AND AMINOGLYCOSIDE-MODIFYING ENZYMES IN ENTEROCOCCI

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SUMMARY. Ten isolates of *Streptococcus faecalis* and two isolates of *S. faecium* were studied together with an NCTC strain of each species. Antibiotic susceptibility tests showed different patterns of high-level resistance to aminoglycosides. Three *S. faecalis* strains were highly resistant to gentamicin and other aminoglycosides. By means of a filter membrane technique, transfer of high-level resistance to aminoglycosides was demonstrated from *S. faecalis* to *S. faecalis*, from *S. faecium* to *S. faecalis* and from *S. faecalis* to *S. faecium*, the last of which has not previously been described.

Aminoglycoside-modifying enzymes were assayed with radio-labelled cofactors. High-level resistance to gentamicin and other aminoglycosides could be attributed to the production of 2''-O-phosphotransferases, 3'-O-phosphotransferases, 6-O-adenylyltransferases and 6'-N-acetyltransferases. Other resistance mechanisms accounted for resistance in two strains of *S. faecalis* and one strain of *S. faecium* that were highly resistant only to streptomycin and one *S. faecalis* strain that was moderately resistant to all aminoglycosides. A low level of 6'-N-acetyltransferases was detected in the three strains of *S. faecium* but this did not confer high-level resistance to aminoglycosides and this trait could not be transferred.

INTRODUCTION

Synergy between penicillins and aminoglycosides against enterococci has been observed *in vitro* and *in vivo* (Hunter, 1947; Watanakunakorn, 1971; Sapico *et al.*, 1972) and combination treatment has commonly been used in enterococcal infections (Moellering *et al.*, 1971; Weinstein and Moellering, 1973). The emergence of enterococci with high-level resistance to aminoglycosides—minimum inhibitory concentration (MIC) > 1000 mg/L—has given rise to treatment problems because bactericidal synergy of penicillins and aminoglycosides cannot be achieved (Standiford *et al.*, 1970; Watanakunakorn, 1971; Calderwood *et al.*, 1977). Wild strains of *Streptococcus faecalis* with high-level resistance to gentamicin were first reported in

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France (Horodniceanu *et al.*, 1979; Combes *et al.*, 1983) and, more recently, in the USA, Thailand, Chile and England (Mederski-Samoraj and Murray, 1983; Murray *et al.*, 1983; Birch *et al.*, 1984). *S. faecium* strains highly resistant to gentamicin have not previously been reported.

In this paper we describe experiments to transfer aminoglycoside and other antibiotic resistance between enterococci and, in particular, to transfer high-level aminoglycoside resistance from *S. faecalis* to *S. faecium*. Aminoglycoside-modifying enzymes were also assayed in resistant enterococci and their transconjugants.

MATERIALS AND METHODS

Bacterial strains. Ten isolates of *S. faecalis* and two isolates of *S. faecium* were studied and compared with NCTC reference strains. Two of the three *S. faecalis* strains with high-level resistance to gentamicin were received from Parma, Italy. The other isolates were collected from the London Hospital, London. The identities of all the test strains were confirmed by a series of biochemical tests (Cowan, 1974). The media used for culture were obtained from Oxoid.

Antibiotics and isotopes. The aminoglycoside-aminocyclitol antibiotics used were: streptomycin (Glaxo Laboratories Ltd, Greenford); neomycin (Biorex Lab Ltd, London); kanamycin (Winthrop Laboratories, Surrey); gentamicin (Roussel Laboratories, Wembley Park); netilmicin, 2'-N-ethyl-netilmicin and 6'-N-ethyl-netilmicin (Schering Corporation Bloomfield, USA); tobramycin (Eli Lilly and Co. Ltd, Basingstoke); spectinomycin (Upjohn Ltd, Sussex); rifampicin (Gruppo Lepetit SpA, Milan, Italy); and fucidin (Leo Laboratories Ltd, Bucks). Erythromycin, chloramphenicol and tetracycline were obtained from Sigma Chemical Co. Ltd, USA. The concentrations of the relevant antibiotics used for selecting resistance markers in the mating experiments were: rifampicin 256 mg/L, gentamicin and kanamycin 512 mg/L, erythromycin 4 mg/L, chloramphenicol 32 mg/L, tetracycline 4 mg/L and fucidin 25 mg/L. Abbreviations of antibiotics and given in table III.

The radiolabelled cofactors—(γ - ^{32}P) ATP, (8- ^{14}C) ATP and (1- ^{14}C) acetyl coenzyme A—were purchased from Amersham International PLC, Bucks. Unlabelled cofactors were obtained from Sigma.

Sensitivity tests. Sensitivity tests with eight aminoglycoside-aminocyclitol and three other antibiotics were performed by the conventional plate dilution method with an inoculum of 10^4 cfu on Isosensitest Agar (Oxoid). The MIC was taken as the lowest concentration at which no growth was seen after overnight incubation at 37°C.

Mating experiments. *S. faecalis* strains G1, G2 and G3 and *S. faecium* strain 37 were used as donors. The rifampicin-resistant mutants (MIC > 500 mg/L) of *S. faecalis* NCTC 775 and *S. faecium* NCTC 7171 were selected from agar plates containing increasing concentrations of rifampicin. These two strains and *S. faecalis* JH2-2, which were resistant to rifampicin and fucidin (Jacob and Hobbs, 1974), were used as recipients.

Donors and recipients were grown in Brain Heart Infusion Broth (BHI) and 0.2 ml of a mixture of late-exponential-phase cultures of the donor and the recipient (1:4) was spread on to a filter membrane (type GS, 0.22 μm , 47 mm, Millipore Corp, Bedford, MA, USA) which was placed on BHI agar containing horse serum 5%. The cells were washed off with 0.5 ml of broth after incubation for 24 h at 37°C and 0.1-ml volumes of appropriate dilutions of the cell suspension were spread on to the selective media incorporating rifampicin and another test antibiotic. The plates were incubated for 48 h. The presumptive transconjugants were subcultured on the fucidin-containing agar for intraspecies transfers or on tellurite 0.04% and tetrazolium 0.01% agar for interspecies transfers and they were checked on the agar containing individual antibiotics or with antibiotic disks to observe the resistance markers carried over from the donor. The MICs of relevant antibiotics were then determined. Transfer frequencies were expressed as the number of the transconjugants per donor cell placed on the membrane.

Preparation of modifying enzyme extracts for aminoglycoside-antibiotics. Thirteen test strains, three recipients and three transconjugants were used for detection of enzymes. The organisms were subcultured into 10 ml of BHI broth containing an appropriate aminoglycoside (10 mg/L) to which the strain was resistant. Of this overnight culture, 2 ml was inoculated into

200 ml of BHI broth to obtain heavy growth. Cells were harvested, washed with 10 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.4, and resuspended in 6 ml of 10 mM Tris-HCl buffer containing 10 mM MgCl₂ and 10 mM dithiothreitol (DTT), pH 7.8. The cell suspension was disintegrated in a Mickle disintegrator (H. Mickle Laboratory Engineering Co., Surrey) for short intervals totalling 10 min at 4°C and most of the cells were broken. The supernate, which was separated from debris by centrifugation at 4000 *g* for 15 min at 4°C, was then centrifuged at 100 000 *g* for 2 h. The supernate extracts were stored in small volumes at -70°C until used.

Assay of modifying enzymes. The cellulose phosphate paper binding method was used to detect the modifying enzymes (Drasar, 1978; Shannon and Phillips, 1983).

For phosphotransferases (APH) and adenylyltransferases (AAD) the following mixtures were incubated for 45 min at 37°C: 25 µl of 0.2 M Tris-HCl buffer (pH 8.1) containing 32 mM MgCl₂ and 4 mM DTT, 25 µl of a solution containing antibiotic 200, 80 or 20 mg/L, 25 µl of 1 mM (γ-³²P) ATP for APH (specific activity 12.5 Ci/mol) or 0.4 mM (8-¹⁴C) ATP for AAD (5 Ci/mol), and 25 µl of enzyme extract.

For acetyltransferases (AAC) the following mixture was incubated for 30 min at 37°C: 25 µl of 0.12 M citrate buffer (pH 5.7) containing 12 mM MgCl₂ and 4 mM DTT, 20 µl of a solution containing antibiotic 200, 80 or 20 mg/L, 10 µl of 0.3 mM (1-¹⁴C) acetyl coenzyme A (10 Ci/mol), and 25 µl of enzyme extract.

The selected antibiotics used as substrates were: gentamicin, neomycin and streptomycin for phosphorylation; gentamicin for adenylylation; kanamycin, amikacin, netilmicin, 2'-N-ethyl-netilmicin (2'-Net) and 6'-N-ethyl-netilmicin (6'-Net) for acetylation, in order to infer the sites of modification (Reynolds and Smith, 1979; Shannon and Phillips, 1983; tables I and II). Gentamicin and neomycin were also used to observe acetylation by enzyme extracts.

The reaction mixture (70 µl) was transferred to the cellulose phosphate paper (2 × 2 cm).

TABLE I

Specificity of modifying enzymes inferred from their reactions with various aminoglycoside-aminocyclitol antibiotics

Reaction	Antibiotic	Inferred specificity
Phosphorylation	Gentamicin	APH(2'')
	Neomycin	APH(3'')
	Streptomycin	APH(6) or APH(3')
Adenylylation	Gentamicin	AAD(2'')
	Neomycin	AAD(4'')(4'')
	Streptomycin	AAD(6) or AAD(3'')(9)
	Spectinomycin	AAD(9) or AAD(3'')(9)
Acetylation	Gentamicin	AAC
	Neomycin	AAC
	Kanamycin	AAC(3) or AAC(6')
	Amikacin	presumable AAC(6')
	Netilmicin	See table II
	2'-N-ethyl-netilmicin 6'-N-ethyl-netilmicin	

TABLE II

Site specificity of acetyltransferases in S. faecium as determined by reactions with netilmicin and its derivatives

Acetylation of			Inferred acetylation site
Netilmicin	2'-N-ethyl-netilmicin	6'-N-ethyl-netilmicin	
+	+	+	3
+	-	+	2'
+	+	-	6'

After washing with 5 mM Tris-HCl buffer (pH 7.4), the paper squares were dried under an infrared lamp and soaked in 5 ml of toluene containing PPO 3 g/L and POPOP 0.3 g/L.

When APH was detected the cellulose phosphate paper was pre-soaked with 20 mM ATP in a saturated solution of sodium pyrophosphate and dried to reduce the non-specific binding of ATP (Drasar, 1978).

Radioactivity on paper was counted in a liquid scintillation spectrometer (SL30 Inter technique Ltd, Middlesex). Counts which were at least twice those of the controls without antibiotics, were regarded as positive. The sites of modification were inferred from substrate profiles and from their chemical structures.

RESULTS

Susceptibility to antibiotics

The antibiotic susceptibility of the test strains in comparison with the reference strains is shown in table III. *S. faecalis* NCTC 775, *S. faecium* NCTC 7171, their rifampicin-resistant mutants and *S. faecalis* JH2-2 possessed low-level resistance to aminoglycosides (MIC $\leq$ 512 mg/L). Three isolates of *S. faecalis* were highly resistant to gentamicin but had different antibiograms. *S. faecalis* G1 had low-level resistance to streptomycin and neomycin. *S. faecalis* strains G2 and G3 were highly resistant (MIC $\geq$ 1024 mg/L) to all the test aminoglycosides except that the MIC of netilmicin was 512 mg/L for *S. faecalis* G3. Three different resistance patterns were observed in the other enterococci which were not highly resistant to gentamicin. *S. faecalis* strains 4, 45, 55 and 56 and *S. faecium* 37 were highly resistant to streptomycin, neomycin and kanamycin. *S. faecalis* strains 10 and 11 and *S. faecium* 44 were highly resistant only to streptomycin. *S. faecalis* 14 was moderately resistant to all the aminoglycosides with high-level resistance to neomycin and amikacin. Nine out of ten *S. faecalis* strains

TABLE III
Susceptibility of enterococci to antibiotics

Strain	MIC (mg/L) against the given strain of										
	Sm	Neo	Kan	Gen	Net	Tob	Ami	Spe	Ery	Cm	Tc
<i>S. faecalis</i>	G1	128	256	> 2048	> 2048	> 2048	> 2048	64	1	128	16
	G2	> 2048	> 2048	> 2048	> 2048	> 2048	> 2048	64	$\leq$ 0.25	8	64
	G3	> 2048	> 2048	> 2048	> 2048	512	> 2048	1024	64	0.5	128
	4	> 2048	> 2048	> 2048	16	8	32	512	64	> 128	64
	45	> 2048	> 2048	> 2048	16	8	16	128	128	> 128	64
	55	> 2048	> 2048	> 2048	16	8	32	256	64	> 128	64
	56	> 2048	> 2048	> 2048	16	8	32	512	64	> 128	64
	10	> 2048	256	64	16	16	64	256	64	1	8
	11	> 2048	256	64	16	8	64	256	64	1	8
	14	256	2048	256	32	16	64	1024	64	$\leq$ 0.25	8
NCTC 775	128	64	64	8	8	16	128	64	1	4	1
<i>S. faecium</i>	NCTC 7171	32	16	256	4	64	128	64	64	2	8
	37	> 2048	> 2048	> 2048	8	128	256	128	64	> 128	32
	44	> 2048	16	128	8	128	128	64	32	> 128	8
<i>S. faecalis</i>	JH2-2	128	256	256	16	16	32	512	32	0.5	4
<i>S. faecalis</i>	775R	128	128	64	8	8	16	128	64	1	4
<i>S. faecium</i>	7171R	32	16	128	4	64	64	64	64	4	4

Figures in bold type indicate high-level resistance to antibiotics.

Sm, Streptomycin; Neo, neomycin; Kan, kanamycin; Gen, gentamicin; Net, netilmicin; Tob, tobramycin; Ami, amikacin; Spe, spectinomycin; Ery, Erythromycin; Cm, chloramphenicol; Tc, tetracycline.

carried tetracycline resistance markers. Three strains of *S. faecium* were slightly more resistant to netilmicin and tobramycin than the *S. faecalis* NCTC 775.

All the enterococci tested were of similar susceptibility to spectinomycin (MIC 90 c. 64 mg/L).

Transfer of resistance

The results of mating experiments are shown in table IV. Selected resistance was transferred from *S. faecalis* strains G1, G2 and G3 to *S. faecalis* JH2-2, from *S. faecium* 37 to *S. faecalis* 775R and from *S. faecalis* G3 to *S. faecium* 7171R with other resistance markers. The transfer frequencies varied with different donors and recipients. All the resistance markers to aminoglycosides were transferred from *S. faecalis* G3 to *S. faecium* 7171R but not the resistance to chloramphenicol and tetracycline.

Detection of modifying enzymes

The aminoglycoside-modifying enzymes detected in enterococci and their transconjugants are shown in table V. There were no appreciable modifying-enzyme activities in *S. faecalis* strains 775R, JH2-2, 10, 11 and 14. High activities of two distinct phosphotransferases APH(2'') and APH(3'), which modified gentamicin and neomycin respectively, were detected in three highly gentamicin-resistant *S. faecalis* strains. APH(3') was found in *S. faecalis* strains 4, 45, 55 and 56 and *S. faecium* 37 and a low activity of APH (2'') was also found in *S. faecalis* 56 and *S. faecium* 37. Streptomycin-modifying adenylyltransferases were found in these five strains but not in *S. faecalis* strains 10 and 11 and *S. faecium* 44. The adenylyltransferases failed to modify gentamicin, neomycin and spectinomycin.

The three highly gentamicin-resistant *S. faecalis* strains produced acetyltransferases which could modify gentamicin, kanamycin, amikacin, neomycin and netilmicin, but the latter two compounds seemed to be poor substrates for enzymes from *S. faecalis* G2. All the three *S. faecium* strains were acetyltransferase-producers which could acetylate netilmicin and 2'-Net but not 6'-Net (table II).

Enzyme activity was also assayed in the three recipients and three transconjugants (table V). The low-level AAC(6') was not affected by the acquisition of rifampicin resistance in *S. faecium* 7171R. All the traits of aminoglycoside-modifying enzymes were transferred from the three donors to the corresponding transconjugants except for the AAC(6') in *S. faecium* 37.

DISCUSSION

All the test isolates were generally resistant to aminoglycoside-aminocyclitol antibiotics and most MICs were above clinically attainable serum levels. The isolates with low-level MICs were susceptible to the combinations of penicillins and aminoglycosides but bactericidal synergy between penicillins and aminoglycosides could not be obtained for strains with high-level MICs of aminoglycosides (≤ 1024 mg/L) (Standiford *et al.*, 1970; Zimmermann *et al.*, 1971; Krogstad *et al.*, 1978*a* and *b*). Because combinations of penicillins and gentamicin are widely used and highly gentamicin-resistant *S. faecalis* strains have emerged, the determination of susceptibility to aminoglycosides, especially gentamicin is of clinical importance in predicting the results of treatment of enterococcal infections with combinations of these antibiotics.

TABLE IV
Transfer of resistance and its frequency among enterococci

Donor	Resistance of donor*	Recipient	Selected marker	Phenotype of transconjugants*	Transfer frequency (per donor)
<i>S. faecalis</i> G1	Kan, Gen, Net, Tob, Ami, Cm, Tc	<i>S. faecalis</i> JH2-2	Gen	Kan, Gen, Net, Tob, Ami, Cm	3.0×10^{-2}
<i>S. faecalis</i> G2	Sm, Neo, Kan, Gen, Net, Tob, Ami, Tc	<i>S. faecalis</i> JH2-2	Kan	Sm, Neo, Kan, Gen, Net, Tob, Ami	5.6×10^{-7}
<i>S. faecalis</i> G3	Sm, Neo, Kan, Gen, Net, Tob, Ami, Cm, Tc	<i>S. faecalis</i> JH2-2	Gen	Sm, Neo, Kan, Gen, Net, Tob, Ami	1.5×10
<i>S. faecium</i> 37	Sm, Neo, Kan, Ery, Cm, Tc	<i>S. faecalis</i> 775R	Kan	Sm, Neo, Kan, Ery, Cm	2.0×10^{-3}
<i>S. faecalis</i> G3	Sm, Neo, Kan, Gen, Net, Tob, Ami, Cm, Tc	<i>S. faecium</i> 7171R	Gen	Sm, Neo, Kan, Gen, Net, Tob, Ami	8.4×10^{-6}

* For abbreviations, see table III.

TABLE V

Presence of aminoglycoside-modifying enzymes in enterococci and their transconjugants

Strains		Modification sites for		
		APH	AAD	AAC
<i>S. faecalis</i>	G1 Donor	2'', 3'	—	6'*
	G2 Donor	2'', 3'	6	6'*
	G3 Donor	2'', 3'	6	6'*
	4	3'	6	...
	45	3'	6	...
	55	3'	6	...
	56	2'', 3'	6	6...
	10	...	...	...
	11	...	...	...
	14	...	...	...
	775R Recipient	...	...	...
	JH2-2 Recipient	...	...	...
<i>S. faecium</i>	37	2'', 3'	6	6'†
	44	...	...	6'†
	NCTC 7171	...	...	6'†
	7171R Recipient	...	...	6'†
Transconjugants				
<i>S. faecalis</i>	G1XJH	2'', 3'	...	6'*
<i>S. faecalis</i>	37X775R	2'', 3'	6	...
<i>S. faecium</i>	G3X7171R	2'', 3'	6	6', 6'†

* Amikacin-modifying subgroup of AAC(6');

† a different subgroup of AAC(6');

‡ names of transconjugants are expressed as working numbers of donor × recipient

High-level resistance to aminoglycosides in enterococci was attributed mainly to the production of aminoglycoside-modifying enzymes although defective uptake of the aminoglycoside has been reported (Moellering *et al.*, 1980) and some high-level resistance to streptomycin was ribosomal (Zimmermann *et al.*, 1971). Elipoulos *et al.* (1984) studied the mechanism of high-level resistance to streptomycin in 12 isolates of *S. faecalis* resistant only to streptomycin. Six were streptomycin-adenylyltransferase producers. Three enzyme-negative strains tested showed ribosomal resistance.

In our study, streptomycin-modifying enzymes could not be detected in *S. faecalis* strains 10 and 11 and *S. faecium* 44 which were highly resistant only to streptomycin and this resistance was presumably ribosomal. It was still not certain whether the ribosomal resistance co-existed with the enzymatic resistance in the enzyme-producing strains.

The sites of the modifications could be inferred from the selected substrates (tables I and II). Phosphotransferases were detected in the three gentamicin-resistant *S. faecalis* strains and other neomycin- and kanamycin-resistant strains. The presence of APH(2'') was inferred from the modification of gentamicin (gentamicin C) which possesses a 2''-OH group but no other target sites for known phosphotransferases. For the same reason, APH(3') was inferred from the modification of neomycin (neomycin B and C) (Davies and Smith, 1978; Courvalin *et al.*, 1980; Shannon and Phillips, 1983). Because gentamicin lacks a 3'-OH group and neomycin lacks a 2''-OH group, modifications of both antibiotics indicated the possibility of co-existence of two distinct enzymes. Adenylyltransferases for streptomycin were found but no enzyme activity could be detected when spectinomycin, gentamicin or neomycin were used as

substrates. Therefore, these enzymes were presumably AAD (6) (Davies and Smith, 1978; Shannon and Phillips, 1983). Acetyltransferases which acetylated gentamicin, kanamycin and amikacin were found in the three gentamicin resistant *S. faecalis* strains. Amikacin does not possess an amino group at the 2' position and is not attacked by the AAC(3) described so far; therefore the 6'-NH₂ group was likely to be the target site for acetylation.

The enzyme from *S. faecalis* G2 could not acetylate netilmicin and neomycin as efficiently as that from *S. faecalis* strains G1 and G3 (data not shown). Its enzymatic activity may have been insufficient for these two compounds or the enzyme may have belonged to a different subgroup.

Although the MICs of aminoglycosides for the three *S. faecium* strains were different, similar enzymes which acetylated gentamicin, neomycin, netilmicin and 2'-Net but not 6'-Net or amikacin, were found. They were likely to be in a subgroup of AAC(6') different from that found in *S. faecalis*. The genes coding for this AAC(6') could not be transferred from *S. faecium* 37 to *S. faecalis* 775R like those coding APH(2''), APH(3') and AAD(6). This finding was consistent with the report that AAC(6') in *S. faecium* could not be transferred nor the phenotypic resistance cured (Wennersten and Moellering, 1980). The genes appeared to be located on the chromosome. Although this AAC(6') had low-level activity against gentamicin, the MICs for *S. faecium* remained 4-8 mg/L. The MICs of netilmicin and tobramycin for *S. faecium* are higher than those for *S. faecalis* with low level resistance (Moellering *et al.*, 1979), and AAC(6') might account for the difference.

Low-level activity of phosphotransferases against gentamicin was found in *S. faecalis* 56, *S. faecium* 37 and its transconjugant *S. faecalis* 37 × 775R which acquired this characteristic from *S. faecium* 37. These strains were still relatively susceptible to gentamicin. In *S. faecalis* G1, there were APH(3') and AAC(6') which modified neomycin but they did not confer high-level neomycin resistance to the strain. Indeed, some modified aminoglycosides retain substantial antibiotic activities. Moreover, susceptibility also depends on the balance between the rate of uptake and the rate of inactivation of the aminoglycosides (Brayan and Van Den Elzen, 1977).

Three concentrations of substrate antibiotics were used in the enzyme assay. In phosphorylation and acetylation experiments, the scintillation counts were in direct proportion to the substrate concentrations. Enzyme activity fell to low levels when the concentrations of substrates fell to 20 mg/L. Higher concentrations of substrate antibiotics led to increased phosphorylation and acetylation. In most adenylation experiments, activity appeared to be in inverse proportion to concentrations when streptomycin was used as substrate. Supplementary experiments on the relationship between streptomycin concentration and scintillation counts showed that the adenylation was inhibited by increased concentrations of streptomycin. The optimum concentration of streptomycin for adenylation in these laboratory conditions was 20 mg/L.

No enzymes were found in *S. faecalis* 14 which was less susceptible to all the aminoglycosides. It was presumed that the resistance was due to other mechanisms such as the cell wall barrier, defective uptake or ribosomal resistance.

Transfer of resistance in enterococci is mediated by plasmids which are not always detectable (Jacob and Hobbs, 1974; Horodniceanu *et al.*, 1979; Le Bouguenec and Horodniceanu, 1982). Buu-Hoi *et al* (1984) reported the transfer of resistance to

macrolides, lincosamides and streptogramin B from *S. faecalis* to *S. faecium*. In the present study, resistance to aminoglycosides was transferred from *S. faecalis* G3 to *S. faecium* 7171R. *S. faecium* 7171R presumably acquired the genes coding for APH(2''), APH(3'), AAD(6) and AAC(6') from the donor despite the pre-existence in it of a low-level of AAC(6'). The expression of the acquired AAC(6') which modified amikacin, obscured the original AAC(6') which did not modify amikacin. In this case, it was difficult to detect the production of the underlying enzymes by their substrate profiles. This *S. faecium* transconjugant with high resistance to gentamicin suggests that resistant strains of *S. faecium* may emerge in the future.

Infections caused by the multiresistant enterococci can be very refractory because synergy can not be obtained by the combination of penicillins and aminoglycosides. The three *S. faecalis* strains and the transconjugants with high resistance to gentamicin were not resistant to all the antibiotics. For instance, *S. faecalis* G1 did not produce streptomycin-modifying enzymes and had only low-level resistance to streptomycin, *S. faecalis* G2 was sensitive and *S. faecalis* G3 was moderately susceptible to erythromycin. The transconjugant, *S. faecium* G3X7171R, was slightly susceptible to tetracycline. Sensitivity tests with a wider range of antibiotics and more combination tests may be useful. Adenylyltransferases for spectinomycin were not found and the majority of the test strains were inhibited by spectinomycin 64 mg/L. However, antagonism between penicillin G and spectinomycin has been reported in two *S. faecalis* strains including JH2-2 (Combes *et al.*, 1983). Because synergy between penicillins and gentamicin cannot be achieved with enterococci highly resistant to gentamicin, the use of other aminoglycosides resistant to modifying enzymes should be explored.

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