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Plasmid-Borne High-Level Resistance to Gentamicin in *Enterococcus hirae*, *Enterococcus avium*, and *Enterococcus raffinosus*

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***Enterococcus hirae*, *E. avium*, and *E. raffinosus* isolated in Romania, Tunisia, and Portugal harbored plasmids pICC8, pIP1700, and pIP1701, respectively, encoding resistance to high levels of gentamicin (Gm^r). The Gm^r marker was carried on pIP1700 by a Tn*4001*-like element and on pICC8 and pIP1701 by Tn*4001*-truncated structures. pICC8 carried, in addition to Gm^r, chloramphenicol, erythromycin, and tetracycline-minocycline (TetM) resistance determinants. The gene *tetM* of pICC8 was carried on a Tn*916*-like element.**

Enterococci are commonly isolated from diverse human infections. *Enterococcus faecalis* and *Enterococcus faecium* are found in about 85 and 10% of cases, respectively, whereas the other species are implicated in only about 5% of human enterococcal diseases. Severe infections with enterococci are treated with an association between a cell wall-active agent and an aminoglycoside, usually gentamicin. Enterococcal strains resistant to high-levels of gentamicin have been so far found among *E. faecalis* (14, 20), *E. faecium* (6), *Enterococcus gallinarum*, *Enterococcus raffinosus*, *Enterococcus casseliflavus* (22), and *Enterococcus avium* (23, 27).

In the present study we report high-level resistance to gentamicin (Gm^r) in one *Enterococcus hirae* strain, one *E. avium* strain, and one *E. raffinosus* strain. For the three strains, we investigated the conjugative transfer and the location of the Gm^r marker and compared the structure of the genetic elements that carried the Gm^r determinant with that of Tn*4001* or Tn*5281*, originally found in *Staphylococcus aureus* (18) or *E. faecalis* (12) strains, respectively.

The enterococcal strains, isolated from urine and blood cultures, were *E. hirae* VB8, *E. avium* BM6807, and *E. raffinosus* BM6808, obtained in Romania, Tunisia, and Portugal, respectively. Species were identified by conventional tests, according to the criteria reported by Facklam and Collins (8). Antibiotic resistance was tested by the disk diffusion method (250 μg of gentamicin per disk) and, only for gentamicin, by MIC: serial concentrations from 250 to 8,000 μg of gentamicin per ml were incorporated in brain heart infusion agar with 5% horse blood. Mating experiments were carried out on membrane filters as described previously (13). Plasmid-free *E. faecalis* JH2-2 (16) and BM133 (15) were used as recipients. Final concentrations of antibiotics (in micrograms per milliliter) used for selection of transconjugants were 1,000, 8, 25, and 4 for gentamicin, tetracycline, chloramphenicol, and erythromycin, respectively, and those for counterselection of the donors were 25, 100, and 2,000 for fusidic acid, rifampin, and streptomycin, respectively. Plasmid DNA isolation, digestion by restriction enzymes (Table 1), agarose gel electrophoresis, DNA blotting, DNA-DNA hybridization (under stringent conditions), and labeling of the probes with [α-^{32}P]dCTP were done as reported earlier (17). The probes used for hybridization experiments were pSF815A (Gm^r probe) (9), pAM170 (Tn*916* probe) (11), pAT101 (*tetM* probe) (19), pIP1551 (IS*256* probe) (5), and pIP1644 (IS*257* probe) (7) containing the gene *aac*6-*aph*2, the conjugative transposon Tn*916*, the gene *tetM*, 468-bp DNA fragment internal to IS*256*, and 629-bp DNA fragment internal to IS*257*, respectively. In addition, the genes *cat* of pIP501 (26), *ermB* of Tn*917* (24), and *tetL* of pMV158 (3), amplified by PCR in our laboratory (26), were also used as probes.

The three enterococcal strains were mated with JH2-2. When selection was done with gentamicin, no transconjugants were detected for the cross BM6807 × JH2-2 or BM6808 × JH2-2 (transfer frequency, $<3 \times 10^{-9}$ transconjugants per donor cell). In contrast, the Gm^r marker of VB8 transferred into JH2-2 at a frequency of 10^{-8}. When selection was done with tetracycline, the frequency of transfer of the tetracycline-minocycline resistance marker (Tc^r Mn^r) from VB8 was 10^{-6}. Analysis of the unselected markers of at least 100 transconjugant clones for each selection (done by replica plating) showed that all the antibiotic resistance markers of VB8 (Table 1) transferred en bloc. One of these transconjugants, IC1080, was mated with BM133, and selection was done with chloramphenicol, erythromycin, gentamicin, or tetracycline. The frequency of retransfer was 10^{-6} for all the selection agents used. Analysis of at least 100 transconjugants for each selection showed that, again, all the resistance markers of IC1080 transferred en bloc into BM133.

Plasmid DNA was detected in all three wild-type strains studied here as well as in IC1080. The Gm^r marker was located, by DNA-DNA hybridization using pSF815A as a probe, on a plasmid of about 90.0 kb in VB8 and plasmids of about 70.0 kb in BM6807 and BM6808 (not shown); the Gm^r plasmids harbored by these strains were designated pICC8, pIP1700, and pIP1701, respectively (Table 1). The profiles of pICC8 (isolated from IC1080), pIP1700, and pIP1701, obtained by digestion of plasmid DNA with different restriction endonucleases were further analyzed. DNA-DNA hybridization experiments, performed with the probes described above, revealed that the three plasmids carried both the gene *aac6-aph2* and the insertion sequence IS*256* and that pIP1701 also carried IS*257*. In addition, sequences homologous to the genes *cat* of pIP501, *ermB*, and *tetM* were detected in pICC8 on 12.5-, 0.9-, and 4.8-kb *Hin*cII fragments, respectively. Strain VB8 also harbored a smaller plasmid on which the gene *tetL* was located.

The hybridization patterns, shown in Table 1, allowed us to map the structures that carried the Gm^r marker in each plasmid (Fig. 1). An element resembling Tn*4001* or Tn*5281* was detected only in pIP1700; in the two other plasmids we found elements having "Tn*4001*-truncated" structures. The truncated

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TABLE 1. Plasmids and DNA-DNA hybridization profiles

Plasmid (wild-type strain) and relevant marker[a]	Probe	Fragment size (kb)					
		*Hae*III	*Hin*dIII	*Hin*cII	*Sca*I	*Sca*I + *Hin*dIII	*Eco*RI
pICC8 (*E. hirae* VB8)	Gm^r	3.4	5.0	2.5, 1.2	45.0, 1.8	3.7, 1.4	≈28.0
Tra$^+$ Cm^r Em^r Gm^r-Km^r Tc^r-Mn^r	IS*256*	3.4	11.2	1.2	1.8, 1.1	0.35	≈28.0
	IS*257*	NT[b]	—[c]	—	NT	—	—
pIP1700 (*E. avium* BM6807)	Gm^r	3.9	2.5	2.2, 1.2	1.8, 1.5	1.4, 1.1	≈40.0
Tra$^-$ Em^r Gm^r-Km^r Sm^r Tc^r-Mn^r	IS*256*	3.9	4.9	2.2, 1.2	1.8, 1.5	0.35	≈40.0
	IS*257*	NT	—	—	NT	—	—
pIP1701 (*E. raffinosus* BM6808)	Gm^r	5.8	8.6	3.8, 2.2	11.2, 1.5	7.2, 1.1	11.2
Tra$^-$ Cm^r Em^r Gm^r-Km^r Pc^r Tc^r-Mn^r	IS*256*	5.8	4.4	2.2	4.8, 1.5	4.0, 0.35	11.2
	IS*257*	NT	4.4	8.6	NT	4.0	11.2

[a] Cm^r, chloramphenicol resistance (*cat* gene); Em^r, erythromycin resistance (*ermB* gene); Gm^r-Km^r, high-level gentamicin-kanamycin resistance (*aac6-aph2* gene); Pc^r, penicillin resistance; Sm^r, high-level streptomycin resistance; Tc^r-Mn^r; tetracycline-minocycline resistance (*tetM* gene); Tra, conjugative transfer. For this analysis, pICC8 was isolated from IC1080. MIC of gentamicin was 8,000 μg/ml.

[b] NT, not tested.

[c] —, no homology detected with IS*257*.

part of IS*256* involved either the left-hand or the right-hand flanking region of the *aac6-aph2* gene in pICC8 and in pIP1701, respectively. Tn*4001*-truncated elements, like that in pIP1701, have recently been detected in 9 of 16 Gm^r plasmids harbored by *E. faecalis* strains isolated in Romania (25).

pICC8 (isolated from both VB8 and IC1080) as well as the genomic DNAs of VB8, BM6807, and BM6808, digested with *Hin*cII, *Hin*dIII, and *Eco*RI, were probed with Tn*916* and the gene *tetM*. Analysis of the DNA-DNA homologies obtained revealed the presence in pICC8, whether isolated from VB8 or IC1080, of an element resembling the conjugative transposon Tn*916* (11). Interestingly, the bacterial genome of VB8 carried, in addition to the Tn*916*-like structure similar to that in pICC8, another fragment hybridizing with the gene *tetM* for each digestion tested. These results suggest that VB8 carries either two Tn*916*-like elements, one situated on pICC8 and the other on the chromosome, or a single Tn*916*-like element on pICC8 and an additional chromosomal *tetM* gene. Sequences homologous to the gene *tetM*, but not to Tn*916*, were detected on the bacterial DNA of BM6808, but not on that of BM6807, on *Hin*cII, *Hin*dIII, and *Eco*RI fragments of 10.3, 7.3, and 10.0 kb, respectively.

pICC8 is the seventh plasmid to be reported that carries the gene *tetM*. The six other conjugative or nonconjugative plasmids, carrying either *tetM* or both *tetM* and *tetL* genes, are pCF10 (4), pIP1440 (21), and pIP614 (1) in *E. faecalis*; pKQ1 (10) and pIP1534 (2) in *E. faecium*; and pIP1549 (1) in *E. hirae*.

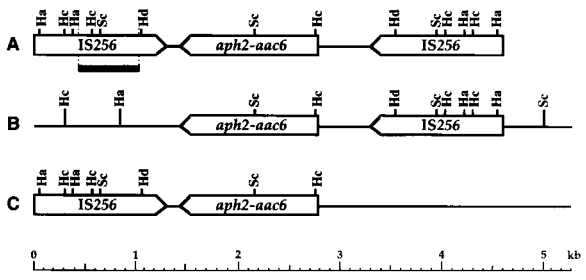

FIG. 1. Restriction maps of the Tn*4001*-like and Tn*4001*-truncated elements. (A, B, and C) Structures carried by pIP1700, pICC8, and pIP1701, respectively. The location of the probe IS*256* is indicated (solid bar). The maps were constructed from DNA-DNA hybridization data presented in Table 1. Ha, *Hae*III; Hc, *Hin*cII; Hd, *Hin*dIII; Sc, *Sca*I.

The gene *tetM* is borne on the seven plasmids on elements resembling Tn*916* (11) or on Tn*916*-modified structures (1). pICC8 is, to our knowledge, the sole plasmid described so far that carries the genes *cat* (of pIP501), *ermB*, and *aac6-aph2*, in addition to *tetM*.

High-level gentamicin resistance is found so far in almost all enterococcal species implicated in human infections. In all these cases, except in one *E. casseliflavus* strain (23), DNA-DNA homology is detected with the gene *aac6-aph2*, which is carried either by structures resembling Tn*4001* (18), such as Tn*5281* (12), or by Tn*4001*-truncated elements such as those found in the strains studied here, as well as in different *E. faecalis* strains (25). These reports demonstrate the genetic diversity of high-level resistance to gentamicin in enterococci.

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