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Location of Antibiotic Resistance Markers in Clinical Isolates of *Enterococcus faecalis* with Similar Antibiotypes

KAREN PEPPER, THEA HORAUD,* CHANTAL LE BOUGUÉNEC, AND GILDA DE CESPÉDÈS

Laboratoire des Staphylocoques et des Streptocoques, Institut Pasteur, 75724 Paris Cedex 15, France

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Eight wild-type strains of *Enterococcus faecalis*, resistant to chloramphenicol (Cm^r), erythromycin (Em^r), tetracycline (Tc^r), and minocycline (Mn^r), were examined for the genetic basis of their antibiotic resistance. Five of the strains transferred all of their antibiotic resistance markers by conjugation, while the other three strains transferred only Tc^r and Mn^r. Cm^r and Em^r determinants were localized by DNA-DNA hybridization experiments, in which the Cm^r gene of plasmid pIP501, of group B *Streptococcus* origin, and the Em^r gene of transposon Tn*917*, of *E. faecalis* origin, served as probes. A chromosomal location was found for the nonconjugative Cm^r and Em^r markers of one wild-type strain. In two strains these markers were carried by nonconjugative plasmids, and in the other strains they were carried by plasmids that transferred by conjugation. Plasmids isolated from three transconjugants resistant to tetracycline but susceptible to minocycline bore nucleotide sequences homologous to the *tetL* gene. Nucleotide sequences homologous to conjugative transposon Tn*916*, of *E. faecalis* origin, were detected by hybridization in the tetracycline–minocycline-resistant transconjugants. Three of these transconjugants were plasmid free, while four harbored conjugative cryptic plasmids. Sequences homologous to Tn*916* were also found on two conjugative plasmids, one of which appeared to be a conjugative cryptic plasmid that had acquired chromosomal Tc^r Mn^r markers during transfer.

About half of the *Enterococcus faecalis* clinical isolates identified since 1978 at the National Reference Center of Streptococci, Institut Pasteur, Paris, France, are resistant to more than one antibiotic (27). The markers most frequently carried by these strains are those encoding resistance to tetracycline (Tc^r), chloramphenicol (Cm^r), and erythromycin (Em^r), the last of which is associated with resistance to other macrolides, to lincosamides, and to streptogramin B. Most of these strains have also been tested for minocycline resistance (Mn^r), and this marker almost invariably accompanies Tc^r (T. Horaud and F. Delbos, unpublished data).

In *E. faecalis*, Tc^r determinants are located either on the chromosome or on plasmids. Chromosomal Tc^r Mn^r determinants may be carried by conjugative transposons, which are able to transfer from chromosome to chromosome in the absence of a plasmid intermediary (13). Tn*916* (20), the most intensively studied conjugative transposon, carries a gene encoding resistance to both tetracycline and minocycline, which has been designated *tetM* (8). The other conjugative transposons so far identified that encode resistance to tetracycline and minocycline are Tn*918* in *E. faecalis* (12) and Tn*919* in *Streptococcus sanguis* (19). Two conjugative plasmids of *E. faecalis* origin, pIP614 (8, 17) and pCF-10 (10), bear homology to Tn*916*. Another Tc^r determinant, *tetL*, which does not encode Mn^r, has been found on plasmids isolated from group B streptococci and from *E. faecalis* (8).

Determinants encoding resistance to antibiotics other than the tetracyclines are generally plasmid borne in *E. faecalis*, and many of these antibiotic resistance (R) plasmids transfer by conjugation (11, 22). Two Em^r transposons of *E. faecalis* origin have been described: Tn*917* is situated on nonconjugative plasmid pAD2 (41), and Tn*3871* is found on conjugative plasmid pJH1 (1). Structures similar to these transposons and carrying homologous Em^r determinants are widely disseminated among group D streptococci of various origins (37).

We thought it of interest to investigate the genetic basis of antibiotic resistance of *E. faecalis* clinical isolates having similar antibiotypes. Eight wild-type *E. faecalis* strains resistant to tetracycline, minocycline, chloramphenicol, and erythromycin were studied with respect to the conjugative transfer of their antibiotic resistance markers. Two of these strains were also resistant to high levels of streptomycin. Antibiotic resistance determinants were localized by DNA-DNA hybridization experiments in which the probes were the conjugative transposon Tn*916* (21), which carries the *tetM* gene (8), and DNA fragments corresponding to the *tetL* gene (8) and to genes encoding Cm^r (34) and Em^r (14, 38).

MATERIALS AND METHODS

Bacterial strains. The eight wild-type *E. faecalis* strains used in this study (Table 1) were independent isolates; most of them were obtained from individuals with urinary tract infections. The years and the geographical areas in which they were isolated are as follows: D394 in 1983 in Orleans, France; D405, D406, and D412 in 1984, 1984, and 1983, respectively, in Broussais Hospital, Paris, France; D410 in 1978 in Hôtel-Dieu Hospital, Paris, France; D397 in 1983 in Perugia, Italy; D338 in 1976 in Alençon, France; and D434 in 1985 in St. Joseph Hospital, Paris, France. The transconjugant strain JH2-2(pMV120), resistant to tetracycline and minocycline (6), was also studied. The wild-type strains were resistant to at least 64 μg of chloramphenicol per ml, to at least 512 μg of erythromycin per ml, and to tetracycline and minocycline. The MICs of tetracycline and minocycline for the wild-type strains are presented in Table 1. Strains D410 and D397 were also resistant to 4,000 and 128,000 μg of streptomycin per ml, respectively. All MICs were determined as described by Chabbert (9).

The plasmid-free recipient strains used here were resistant to rifampin and fusidic acid, to streptomycin, or to pe-

* Corresponding author.

TABLE 1. Wild-type strains and conjugative transfer of antibiotic resistance markers into JH2-2

Strain[a]	MIC (μg/ml)		Conjugative transfer		Transconjugants		
	Tetracycline	Minocycline	Selective donor marker	Frequency of transfer	Resistance markers[b] (%)	Designation	Plasmid(s)
D394	64	16	Tc^r	6×10^{-9}	Tc^r Mn^r (100)	BM5463	None
			Cm^r or Em^r	$<1 \times 10^{-9}$			
D405	32	4	Tc^r	2×10^{-7}	Tc^r Mn^r (100)	BM5465	None
			Cm^r or Em^r	$<1 \times 10^{-9}$			
D410	64	16	Tc^r	7×10^{-9}	Tc^r Mn^r (100)	BM5467	None
			Cm^r or Em^r	$<1 \times 10^{-9}$			
D397	128	16	Tc^r, Mn^r, or Sm^r	3×10^{-4}	Tc^r Mn^r Sm^r (100)	BM5471	pIP1440
			Cm^r or Em^r	1×10^{-5}	Cm^r Em^r (100)	BM5472	pIP1438
D406	32	16	Tc^r or Mn^r	7×10^{-7}	Tc^r Mn^r (100)	BM5469	pIP1442[c]
			Cm^r or Em^r	5×10^{-6}	Cm^r Em^r (100)	BM5470	pIP1435, pIP1442[c]
D412	64	8	Tc^r or Mn^r	2×10^{-7}	Tc^r Mn^r (100)	BM5473	pIP1502[c]
			Cm^r or Em^r	9×10^{-5}	Cm^r Em^r (100)	BM5474	pIP1503, pIP1502[c]
D338	128	16	Tc^r	2×10^{-4}	Tc^r Cm^r Em^r (95.7)	BM5476	pIP1321
					Tc^r Em^r (1.4)	BM5483	pIP1327
					Tc^r (0.5)	BM5481	pIP1507
					Tc^r Mn^r (2.4)	BM5475	pIP1506[c]
			Mn^r	6×10^{-6}	Tc^r Mn^r (100)		
			Cm^r	2×10^{-4}	Tc^r Cm^r Em^r (99.5)		
					Cm^r (0.5)	BM5484	pIP1130
			Em^r	2×10^{-4}	Tc^r Cm^r Em^r (99.5)		
					Cm^r Em^r (0.5)	BM5485	pIP1328
D434	128	16	Tc^r	1×10^{-5}	Tc^r Cm^r Em^r (98)	BM5480	pIP1127, pIP1141[c]
					Tc^r Mn^r (2)	BM5478	pIP1129
			Mn^r	3×10^{-8}	Tc^r Mn^r (100)	BM5479	pIP1141[c]
			Cm^r or Em^r	1×10^{-5}	Tc^r Cm^r Em^r (99.5)		
					Cm^r Em^r (0.5)	BM5486	pIP1128

[a] All wild-type strains are resistant to tetracycline, minocycline, chloramphenicol, and erythromycin (includes resistance to other macrolides, to lincosamides, and to streptogramin B); D410 and D397 are also resistant to 4,000 and 128,000 μg of streptomycin, respectively, per ml.
[b] All transconjugant strains are also resistant to rifampin and fusidic acid.
[c] These conjugative plasmids are designated cryptic Tra^+ plasmids.

floxacin. The rifampin- and fusidic acid-resistant strains were JH2-2 (28), BM137 (26), BM132 (25), BM138, BM140 (26), and BM90 (this study), and the streptomycin-resistant strains were BM133 (25), BM105 (31), BM134 (25), BM107, BM106 (31), and BM120 (26), which belong to the species *E. faecalis* and to streptococcal groups A, B, C, G, and H (strain Challis), respectively. *E. faecalis* BM108 (pefloxacin resistant) (16) was also used as recipient.

Media and antibiotics. Media, growth conditions, and antibiotics used for selection and counterselection in mating experiments have been described previously (24, 25). The final concentration of pefloxacin (Roger-Bellon, Neuilly-sur-Seine, France) was 10 μg/ml (16).

Mating conditions. Broth matings (28) and filter matings (32) were performed as previously described. The time of contact was 18 h for both types of matings. The cell suspension on the filter, obtained by washing the filter with 0.5 ml of broth, either was homogeneous or contained visible clumps (cell aggregates) that did not disintegrate after vortexing (see Fig. 5). Cell suspensions from control filters, on which donors or recipients were grown alone, were always homogeneous. We have previously described the appearance of cell aggregates (3). Transconjugants were scored on selective media after 48 h of incubation at 37°C. Matings in which transconjugants served as donors are designated as retransfers. The frequency of transfer, measured at the end of the mating time, is expressed as the number of antibiotic-resistant recipients per donor CFU.

Curing procedures. Curing by novobiocin (33) was carried out at 45°C.

Isolation of plasmid and cellular DNA. Conjugative *E. faecalis* plasmids were isolated from transconjugants (JH2-2 as host); nonconjugative plasmids were isolated from their wild-type hosts. pMV158 was isolated from *E. faecalis* JH203 (6), and pAD2 was isolated from JH2-2 (14). The technique of Klaenhammer et al. (29) was used with the following modifications. To 100-ml overnight cultures were added 10 g of glycine (Prolabo, Paris, France) and 100 ml of brain heart infusion. After 1 h of incubation at 37°C with agitation, cultures were centrifuged and washed in 0.1 times the original volume of a buffer containing 30 mM Tris, 5 mM EDTA, and 50 mM NaCl. Cleared lysates were not diluted before being centrifuged on dye-buoyant density gradients.

Chromosomal DNA was obtained by collecting the upper band in cesium chloride gradients (the lysis procedure was the same as that used for plasmid isolation). Chromosomal DNA was extensively dialyzed against a buffer consisting of 10 mM Tris (pH 8) and 1 mm EDTA and concentrated 10-fold by ethanol precipitation before being digested with restriction endonucleases.

The *Escherichia coli* plasmids pIP1322 (34), pAM170, and pAM170-LT (21) were prepared in large quantities by the

method of Birnboim and Doly (4) scaled up to an initial culture volume of 250 ml. The pellet obtained after alcohol precipitation was suspended in sterile water and centrifuged to equilibrium in dye-buoyant density gradients.

Restriction endonucleases. DNA was digested with the following enzymes as specified by the manufacturer: *Bst*EII (New England BioLabs, Beverly, Mass.), *Ava*I, *Eco*RI, *Hha*I, *Hin*cII, *Hin*dIII, and *Hpa*II (Amersham International, Amersham, England).

Gel electrophoresis, molecular size determination, and extraction of DNA from agarose gels. Agarose (Sigma Chemical Co., St. Louis, Mo.) gels (0.8% agarose) were used for the electrophoresis of plasmid and chromosomal DNA. Plasmid sizes were measured by comparing the electrophoretic migration of restriction fragments with that of bacteriophage λ digested by *Hin*dIII and *Eco*RI; each value represents the mean of at least three independent determinations. The sizes of hybridizing chromosomal fragments were determined in the same manner. For small plasmids having no *Hin*dIII restriction site, sizes were determined by comparing the migration of covalently closed circular molecules with that of covalently closed circular forms of plasmids pIP1322 (34), pHV33 (36), pMV158 (6), pC221 (39), pBR322 (5), and pC194 (23), with sizes of 10.6, 7.2, 5.2, 4.5, 4.3, and 2.9 kilobases (kb), respectively. Specific restriction fragments were extracted from 1 or 1.2% low-melting-point agarose (Sigma) gels, as we described previously (34).

Probes for DNA-DNA hybridization. The following probes were used in hybridization experiments: (i) the 1.6-kb *Bst*EII-*Hpa*II fragment of pIP1322 (34), which includes most of the Cm^r gene of pIP501 (2), as previously described (34) (Cm^r probe); (ii) the 1.7-kb *Ava*I fragment of pAD2 (14), which contains the Em^r gene of Tn*917* (38) (Em^r probe); (iii) the 1.1-kb *Hha*I fragment of pMV158, which includes most of the *tetL* gene (8) (*tetL* probe); and (iv) pAM170 (21), which carries the conjugative transposon Tn*916*. pAM170-LT (21), which is pAM170 from which Tn*916* has excised spontaneously, was used as a control probe to verify that hybridization with pAM170 was the result of sequence homology with Tn*916* and not with either the vector or pAD1 sequences present in pAM170.

DNA blotting and hybridization. DNA was transferred from agarose gels to nitrocellulose filters by the method of Southern (40). The probes were labeled with ^{32}P by nick translation with Amersham kit 5500 (Amersham International). Hybridization was carried out under stringent conditions, as previously reported (31). In each experiment involving the hybridization of chromosomal DNA, the plasmid and the chromosome from the same strain were tested side by side on the same filter.

RESULTS

Conjugative transfer of antibiotic resistance markers and plasmid isolation. Filter matings were carried out between each of the eight wild-type *E. faecalis* strains listed in Table 1 and the *E. faecalis* recipient JH2-2. In these matings, resistance to each of the antibiotics carried by the wild-type strains, including minocycline, was selected independently. At least 100 clones from each selection were analyzed for the transfer of unselected markers. The frequencies of conjugative transfer, the antibiotypes of the transconjugants, and the plasmids they harbored are presented in Table 1. For each cross and for each transconjugant antibiotype, at least one clone was examined for the presence of plasmids. Plasmid sizes are reported in Table 2.

Wild-type strains D394, D405, and D410 transferred Tc^r Mn^r at low frequencies (10^{-9} to 10^{-7} transconjugants per donor) but did not detectably transfer either Cm^r or Em^r. Plasmids were isolated from each of the wild-type strains, but none was found in any of the tetracycline-minocycline-resistant transconjugants examined.

Wild-type strains D397, D406, and D412 transferred all of their resistance markers into JH2-2. The transconjugants were resistant to chloramphenicol and erythromycin, or to tetracycline and minocycline, or, for D397, to tetracycline, minocycline, and streptomycin. Each of the tetracycline–minocycline-resistant transconjugants harbored one plasmid, as did BM5472, the chloramphenicol- and erythromycin-resistant transconjugant derived from wild-type strain D397. The chloramphenicol- and erythromycin-resistant transconjugants derived from D406 and D412 each harbored two plasmids. These strains (BM5470 and BM5474) could be cured of their Cm^r and Em^r markers. The cured derivatives (Cm^s Em^s) each harbored a plasmid having a *Hin*dIII profile indistinguishable from that of the plasmids in the tetracycline–minocycline-resistant transconjugants derived from the corresponding wild-type strains. Since these plasmids, pIP1442 and pIP1502, did not appear to alter the antibiotypes of their hosts (Fig. 1, lanes A and B), they are referred to throughout this paper as cryptic Tra$^+$ plasmids.

D338 and D434 transferred all their antibiotic resistance markers by conjugation. These transfers yielded a variety of transconjugant antibiotypes: from both wild-type strains, transconjugants carrying the combinations of markers Tc^r Cm^r Em^r, Cm^r Em^r, and Tc^r Mn^r were obtained; from D338, transconjugants with the antibiotypes Tc^r Em^r, Tc^r, or Cm^r were also obtained. The transconjugants derived from D338 each harbored a single plasmid; similarity of the *Hin*dIII profiles of some of these plasmids suggested that they resulted from molecular rearrangements and did not therefore represent distinct plasmid types (pIP1506, pIP1507, pIP1130, and pIP1321; Fig. 1, lanes C to F). One plasmid was also found in each of the transconjugants derived from D434, except BM5480 (Tc^r Cm^r Em^r), in which two plasmids were detected. One of these plasmids had a *Hin*dIII profile identical to that of pIP1141, harbored by the tetracycline–minocycline-resistant transconjugant BM5479. Another tetracycline–minocycline-resistant transconjugant, BM5478, derived from the same wild-type strain, harbored a plasmid, pIP1129, which had a restriction profile differing from that of pIP1141 only by the presence of several additional bands whether digested by *Hin*dIII (Table 2) or by *Hin*cII (Fig. 1, lanes G and H). The difference in size between pIP1129 and pIP1141 was about 16 kb (Table 2).

Localization of chloramphenicol and erythromycin resistance determinants. The plasmid and chromosomal DNA of wild-type strains, D394, D405, and D410 and the plasmids harbored by the erythromycin- and chloramphenicol-resistant transconjugants derived from the five other wild-type strains were tested for nucleotide sequence homology with the Cm^r and Em^r probes.

Nucleotide sequences homologous to both probes were detected in all the strains tested. In D405, sequences homologous to the Cm^r and Em^r probes were located on chromosomal *Hin*dIII fragments of 3.5 and 4.0 kb, respectively (data not shown). pIP1142, the larger plasmid isolated from D394, hybridized with both probes (Table 2). No homology was detected with either of these probes and the small plasmids harbored by D405 and D394. pIP1143, harbored by D410, hybridized with both probes (Fig. 2A; Table 2).

Plasmids isolated from the chloramphenicol- and erythro-

TABLE 2. Location of antibiotic resistance markers on plasmids

Wild-type strain	Plasmid[a]	Plasmid size (kb)	Size (kb) of *Hin*dIII fragment hybridizing with:			
			Cm^r probe	Em^r probe	*tetL* probe	Tn*916*
D394	pIP1142	38.6	10.3	3.6	–[b]	–
	pIP1159	3.3	–	–	–	–
D405	pIP1155	4.8	–	–	–	–
D410	pIP1143	22.3	6.4	3.9	–	–
D397	pIP1440	91.0	NA[c]	NA	8.0	10.9, 8.0, 2.7[d]
	pIP1438	57.2	8.2, 3.1	8.2, 4.1	NA	NA
D406	pIP1442[e]	56.3	NA	NA	–	–
	pIP1435	38.5	6.3	4.8	NA	NA
D412	pIP1502[e]	56.8	NA	NA	–	–
	pIP1503	23.0	6.3	6.7	NA	NA
D338	pIP1321	83.3	6 or 6.3	13.2	13.2	–
	pIP1507	68.8	NA	NA	11.9	–
	pIP1328	29.1	6.3	7.1	NA	NA
	pIP1130	64.3	7.4	–	NA	NA
	pIP1506[e]	62.7	NA	NA	–	–
D434	pIP1127	26.4	7.5	11.2	11.2	–
	pIP1141[e]	57.7	NA	NA	NT[c]	–
	pIP1129	73.6	NA	NA	–	24.0, 8.2[f,g]

[a] All plasmids were isolated from transconjugants (JH2-2 host), except pIP1142, pIP1155, and pIP1143, which were isolated from wild-type strains.
[b] –, No homology detected.
[c] NA, Not applicable; NT, not tested.
[d] Sequences homologous to Tn*916* were also detected on *Hin*cII fragments of 5.6, 4.2, 1.5, and 1.1 kb.
[e] Cryptic Tra$^+$ plasmid.
[f] These are the additional fragments not observed in the *Hin*dIII profile of pIP1141.
[g] Sequences homologous to Tn*916* were also detected on *Hin*cII fragments of 11.8, 5.6, 4.8, 3.8, 2.0, 1.5, and 1.1 kb.

mycin-resistant transconjugants derived from D397, D406, D412, D338, and D434 also hybridized with both probes. The sizes and the *Hin*dIII profiles of these plasmids are presented in Table 2 and Fig. 2. Sequences homologous to the Cm^r probe were carried by a *Hin*dIII fragment of 6.3 kb in about half of the plasmids, while sequences homologous to the Em^r probe were located on *Hin*dIII fragments of different sizes. The sequences homologous to the Cm^r and Em^r probes were not located on the same *Hin*dIII fragment in any of the plasmids, except in pIP1438, in which sequences homologous to each probe were detected on two *Hin*dIII fragments (Fig. 2B).

Hybridization experiments with probes bearing resistance to tetracycline. Plasmid and chromosomal DNA isolated from the transconjugants listed in Table 1 that carried either Tc^r or Tc^r Mn^r, as well as from the wild-type strains D394, D405, and D410, was probed with the *tetL* probe and with pAM170. In addition, the chromosomal DNA of JH2-2(pMV120) was probed with pAM170. In control experiments no sequence homology was detected between pAM170 and the chromosomal DNA of tetracycline-susceptible *E. faecalis* BM133 or between pAM170-LT and any of the plasmids or chromosomes which carried sequences homologous to pAM170. To facilitate comparison with Tn*916*, DNA was digested with either *Hin*dIII or *Hin*cII. Tn*916* has a single *Hin*dIII site and five internal *Hin*cII fragments, 5.6, 4.8, 1.5, 1.1, and 0.4 kb in size. The 4.8-kb *Hin*cII fragment bears the Tc^r determinant (13).

The plasmids isolated from transconjugants resistant to tetracycline but susceptible to minocycline (pIP1321, pIP1507, and pIP1127) hybridized with the *tetL* probe but not with pAM170. The homologous sequences were situated on the largest *Hin*dIII fragment of each of these plasmids (Table 2; Fig. 3A to C).

pIP1440 bore sequence homology both to the *tetL* probe and to pAM170. pAM170 hybridized intensely with a 10.9-kb *Hin*dIII fragment and slightly with both an 8.0-kb fragment and a 2.7-kb fragment. The *tetL* probe hybridized with a single *Hin*dIII fragment, of 8.0 kb (Table 2; Fig. 3D). When

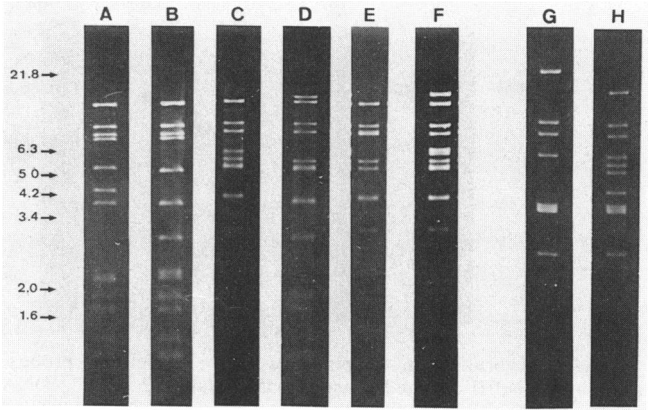

FIG. 1. Restriction profiles of cryptic Tra$^+$ plasmids and their derivative resistance plasmids. *Hin*dIII-digested plasmids: lane A, pIP1442; lane B, pIP1502; lane C, pIP1506; lane D, pIP1507 (Tc^r); lane E, pIP1130 (Cm^r); lane F, pIP1321 (Tc^r Cm^r Em^r). *Hin*cII-digested plasmids: lane G, pIP1141; lane H, pIP1129 (Tc^r Mn^r). Molecular size standards (in kilobases) are shown at left.

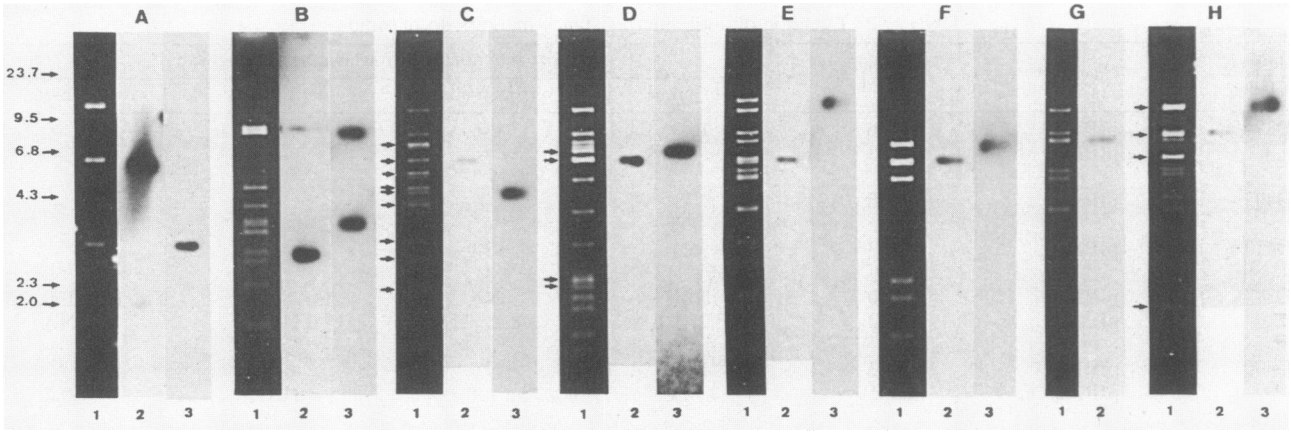

FIG. 2. Plasmids isolated from chloramphenicol- and erythromycin-resistant transconjugants. Lanes 1, *Hin*dIII-digested plasmid DNA; lanes 2, the same DNA transferred to nitrocellulose filters and probed with the ^{32}P-labeled Cm^r gene of pIP501 (Cm^r probe); lanes 3, the same DNA probed with the ^{32}P-labeled *Ava*I D fragment of pAD2 (Em^r probe). (A) pIP1143; (B) pIP1438; (C) pIP1435; (D) pIP1503; (E) pIP1321; (F) pIP1328; (G) pIP1130; (H) pIP1127. Molecular size standards (in kilobases) are shown at left. The *Hin*dIII fragments of pIP1435, pIP1503, and pIP1127 are marked with arrows to distinguish them from the cryptic Tra$^+$ plasmids (pIP1442, pIP1502, and pIP1141, respectively) present in the same strains.

pIP1440 was digested with *Hin*cII and then probed with pAM170, sequence homology was detected on four fragments, three of which were equivalent in size to internal *Hin*cII fragments of Tn*916* (Table 2). Of the plasmids isolated from the tetracycline–minocycline-resistant transconjugants derived from D434, pIP1129 and pIP1141, only pIP1129 hybridized with pAM170. Sequence homology was detected on two *Hin*dIII fragments and on seven *Hin*cII fragments, four of which had sizes equal to those of Tn*916* internal *Hin*cII fragments. No homology was detected between pIP1129 and the *tetL* probe (Table 2). In the chromosomal DNA of the strains harboring pIP1440 and pIP1129 (BM5471 and BM5478, respectively), sequences homologous to pAM170 were detected only on restriction fragments that aligned on autoradiograms with hybridizing plasmid fragments; this hybridization was considered to represent homology between the probe and plasmid DNA nicked during lysis which cosedimented with the chromosome (data not shown).

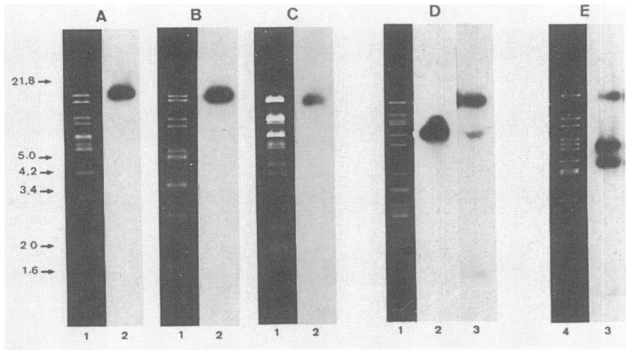

FIG. 3. Hybridization of plasmids with Tc^r-bearing probes. Lanes 1, *Hin*dIII-digested plasmid DNA; lanes 2 and 3, DNA transferred to nitrocellulose filters and probed with the ^{32}P-labeled *tetL* gene of pMV158 (*tetL* probe) (lanes 2) or with ^{32}P-labeled pAM170 (lanes 3). Lane 4, *Hin*cII-digested plasmid DNA. (A) pIP1321; (B) pIP1507; (C) pIP1127; (D) pIP1440; (E) pIP1129 (additional hybridizing fragments were observed on overexposed radiograms [Table 2]). Molecular size standards (in kilobases) are shown at left.

pIP1442, pIP1502, and pIP1506 hybridized neither with the *tetL* probe nor with pAM170 (Table 2). However, the chromosomes of the strains harboring these plasmids, as well as that of BM5479, harboring pIP1141, hybridized with pAM170 (Table 3), suggesting that the location of the determinants specifying Tc^r Mn^r in these strains was chromosomal and confirming that pIP1442, pIP1502, pIP1506, and pIP1141 were cryptic Tra$^+$ plasmids. The *Hin*dIII profiles of these plasmids were similar (Fig. 1A to C).

Hybridization of pAM170 with the *Hin*cII-digested chromosomal DNA of the strains harboring cryptic Tra$^+$ plasmids revealed considerable sequence homology with Tn*916* (Table 3). Similar results were obtained with the *Hin*cII-digested chromosomal DNA of the wild-type strain D405 and of the plasmid-free transconjugants BM5463 and BM5467. Following the reasoning of Fitzgerald and Clewell (19), we considered the number of *Hin*cII fragments hybridizing with pAM170 and equivalent in size to the internal *Hin*cII fragments of Tn*916* to be an index of structural similarity with

TABLE 3. Homology between Tn*916* and the chromosomal DNA of tetracycline- and minocycline-resistant strains

Strain (wild-type origin)	Size (kb) of restriction fragments hybridizing with pAM170[a]:	
	*Hin*cII digestion	*Hin*dIII digestion
D394	NT[b]	21.8, 6.8
BM5463 (D394)	5.6, 4.8, 4.3, 2.3[c], 1.5, 1.1	NT
D405	5.6, 4.8, 4.4, 1.5	17.0, 8.5, 7.6
D410	NT	17.0, 7.2
BM5467 (D410)	5.6, 4.8, 4.3, 2.5[c], 1.5, 1.1	NT
BM5469 (D406)	5.6, 4.8, 4.6, 3.7, 3.4, 1.5, 1.1	14.2, 12.6, 11.2, 7.7, 6.5
BM5473 (D412)	5.6, 4.8, 3.6, 1.5, 1.3, 1.1	14.5, 11.4
BM5475 (D338)	5.6, 4.8, 2.2[c], 1.5, 1.1	18.1, 7.2
BM5479 (D434)	7.8, 5.6, 4.8, 3.5, 2.2[c], 1.5, 1.4, 1.1	20.5, 17.0, 14.8, 8.6

[a] Tn*916* has internal *Hin*cII fragments of approximately 5.6, 4.8, 1.5, 1.1, and 0.4 kb (13).
[b] NT, Not tested.
[c] Weak hybridization.

TABLE 4. Retransfer of antibiotic resistance markers

Donor strain	Transferring markers[a]	Frequency of conjugative transfer into various recipients[b]						
		E. faecalis		Streptococci[c]				
		Broth mating	Filter mating (cell aggregates)[d]	Group A	Group B	Group C	Group G	*S. sanguis*
BM5482[e]	Tc^r Mn^r Sm^r (pIP1440)	3×10^{-6}	5×10^{-2} (+)	$<1 \times 10^{-9}$	1×10^{-5}	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
BM5472	Em^r Cm^r (pIP1438)	1×10^{-5}	2×10^{-1} (+)	2×10^{-8}	1×10^{-4}	1×10^{-7}	2×10^{-5}	3×10^{-6}
BM5470[f]	Em^r Cm^r (pIP1435)	3×10^{-8}	5×10^{-6} (+)	$<1 \times 10^{-9}$	3×10^{-9}	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
BM5474[f]	Em^r Cm^r (pIP1503)	1×10^{-8}	3×10^{-4} (+)	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
BM5476	Tc^r Em^r Cm^r (pIP1321)	3×10^{-7}	5×10^{-4} (+)	1×10^{-9}	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	2×10^{-9}
BM5481	Tc^r (pIP1507)	2×10^{-7}	7×10^{-3} (+)	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
BM5485	Em^r Cm^r (pIP1328)	4×10^{-8}	4×10^{-4} (+)	4×10^{-9}	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	2×10^{-9}	2×10^{-9}
BM5484	Cm^r (pIP1130)	2×10^{-2}	7×10^{-2} (+)	NT[g]	NT	NT	NT	NT
BM5480[f]	Tc^r Em^r Cm^r (pIP1127)	1×10^{-8}	1×10^{-4} (+)	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
BM5478	Tc^r Mn^r (pIP1129)	2×10^{-6}	3×10^{-1} (+)	3×10^{-7}	5×10^{-9}	2×10^{-8}	4×10^{-7}	$<1 \times 10^{-9}$
BM5463[h]	Tc^r Mn^r	$<1 \times 10^{-8}$	2×10^{-8} (−)	1×10^{-8}	3×10^{-9}	7×10^{-9}	1×10^{-8}	2×10^{-9}
BM5465[h]	Tc^r Mn^r	1×10^{-8}	1×10^{-7} (−)	1×10^{-7}	3×10^{-9}	3×10^{-9}	4×10^{-8}	3×10^{-9}
BM5467[h]	Tc^r Mn^r	$<1 \times 10^{-8}$	3×10^{-7} (−)	1×10^{-6}	5×10^{-8}	8×10^{-8}	3×10^{-7}	1×10^{-8}
BM5469[f]	Tc^r Mn^r	$<1 \times 10^{-8}$	1×10^{-5} (+)	7×10^{-7}	2×10^{-8}	2×10^{-8}	1×10^{-7}	1×10^{-8}
BM5473[f]	Tc^r Mn^r	$<1 \times 10^{-8}$	1×10^{-4} (+)	4×10^{-6}	2×10^{-7}	1×10^{-7}	9×10^{-7}	4×10^{-8}
BM5475[f]	Tc^r Mn^r	$<1 \times 10^{-8}$	3×10^{-6} (−)	7×10^{-9}	1×10^{-9}	$<1 \times 10^{-9}$	1×10^{-9}	$<1 \times 10^{-9}$
BM5479[f]	Tc^r Mn^r	$<1 \times 10^{-8}$	2×10^{-6} (+)	1×10^{-7}	1×10^{-8}	4×10^{-8}	1×10^{-7}	$<1 \times 10^{-9}$
JH2-2 (pMV120)	Tc^r Mn^r	$<1 \times 10^{-8}$	2×10^{-6} (+)	2×10^{-8}[i]	4×10^{-9}[i]	3×10^{-8}[i]	4×10^{-8}[i]	6×10^{-8}[i]

[a] Plasmid-borne markers are followed by the plasmid in parentheses; other markers are chromosomal. Plasmids were in a JH2-2 host (Table 1) unless otherwise indicated.
[b] All recipients were streptomycin resistant (see Materials and Methods) unless otherwise indicated.
[c] The matings with streptococci of groups A, B, C, and G and with *S. sanguis* were done on filter membranes.
[d] +, Cell aggregates observed; −, no cell aggregates observed.
[e] Since pIP1440 encodes streptomycin resistance, it was first transferred from BM5471 into *E. faecalis* BM108 (pefloxacin resistant), and the resulting strain, BM5482, was used as donor for retransfer into recipients resistant to fusidic acid and rifampin (see Materials and Methods).
[f] Strains harboring cryptic Tra^+ plasmids.
[g] NT, Not tested.
[h] Plasmid-free strains.
[i] These results are taken from reference 26.

Tn*916*. Sequences homologous to Tn*916* were carried by four *Hin*cII fragments equivalent in size to internal *Hin*cII fragments of Tn*916* and by one or more additional fragments in the chromosomes of the strains tested, except that of D405, in which only three *Hin*cII fragments equivalent in size to internal Tn*916* fragments were detected. The additional fragments might represent junctions between an element resembling Tn*916* and the chromosome, particularly since some of them hybridized only weakly.

The conjugative plasmid pMV120, originally isolated from a group B *Streptococcus* strain (6), bears sequence homology neither to *tetL* nor to *tetM* (8). A third determinant, *tetN*, was reported to be carried by this plasmid (8). The transfer of the Tc^r Mn^r marker from JH2-2(pMV120) was not detected in broth matings, and, as we reported previously (26), occurred at low frequency in filter matings (Table 4). For these reasons we thought pMV120 might be a cryptic Tra^+ plasmid, the transfer of which accompanies the transfer of chromosomal resistance genes. The *Hin*cII-digested chromosomal DNA of JH2-2(pMV120) was probed with pAM170, and a hybridization profile similar to that of Tn*916* was observed (Fig. 4).

The *Hin*dIII-digested chromosomal DNA of strains D394, D405, D410, BM5469, BM5473, BM5475, and BM5479 was also probed with pAM170; at least two fragments hybridized in each case (Table 3). Since for a single copy of Tn*916* two *Hin*dIII fragments would be expected to hybridize, the strains in which more than two hybridizing *Hin*dIII fragments were detected (D405, BM5469, and BM5479) may carry more than one copy of a Tn*916*-like element. The nonconjugative plasmids harbored by D394, D405, and D410

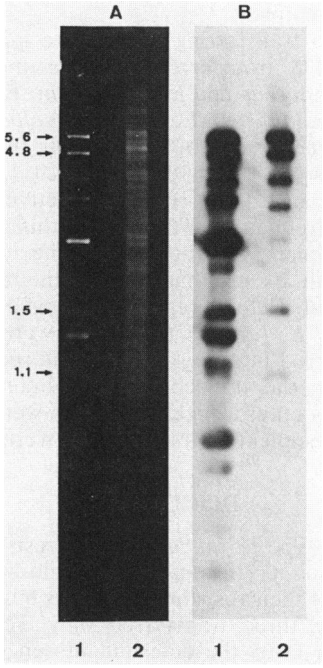

FIG. 4. Hybridization of chromosomal DNA of JH2-2(pMV120) with pAM170. Lanes 1, pAM170; lanes 2, JH2-2(pMV120). (A) *Hin*cII-digested DNA. (B) The same DNA, transferred to a nitrocellulose filter and probed with ^{32}P-labeled pAM170. Molecular sizes (in kilobases) of internal Tn*916* fragments are shown at left.

did not hybridize with either of the Tc^r-bearing probes (Table 2).

Retransfer and host range of antibiotic resistance markers. The various transconjugants obtained in the mating experiments described above, except BM5471, were mated with the streptomycin-resistant *E. faecalis* recipient strain BM133 both in broth and on filter membranes. They were also mated on filter membranes with streptomycin-resistant streptococcal recipients of groups A, B, C, G, and H. Since BM5471 carried pIP1440, which encodes resistance to streptomycin, it was first mated with a pefloxacin-resistant *E. faecalis* strain (BM108), and the resulting transconjugant, BM5482, was used as donor in matings with various recipients resistant to rifampin and fusidic acid. The results of these mating experiments are presented in Table 4.

The R plasmids studied here transferred into the *E. faecalis* recipient in both broth and filter matings; in each case the frequency of transfer in broth was lower than that in the filter mating. For the chromosomal Tc^r Mn^r markers, practically no retransfer was detected in broth, and the frequencies of transfer on filter membranes were lower than those of the R plasmids. The R plasmids, except pIP1438 and pIP1129, had narrow host ranges, as do other R plasmids isolated from *E. faecalis* (26). Cell aggregates (Fig. 5) were observed in filter matings when *E. faecalis* served as the recipient for the R plasmids but not with the streptococcal recipients. For BM5470, BM5474, and BM5480, which harbored both resistance and cryptic Tra$^+$ plasmids, it is not clear which plasmid elicited the formation of cell aggregates. Cell aggregates, however, were observed in three of the four matings in which strains harboring only cryptic Tra$^+$ plasmids served as donors (BM5469, BM5473, and BM5479) and were not observed in the retransfer of chromosome-borne markers from plasmid-free transconjugants (BM5463, BM5465, and BM5467). Cell aggregates also appeared during the transfer of the Tc^r Mn^r marker of JH2-2(pMV120) into the *E. faecalis* recipient.

In addition to transferring into the recipients shown in Table 4, pIP1438 transferred on filter membranes into *Staphylococcus aureus* and into *E. avium*, *E. casseliflavus*, *E. faecium*, *E. gallinarum*, *E. hyrae*, *E. malodoratus*, and *E. mundtii*. The frequencies of transfer for the enterococcal recipients, 1.5×10^{-6}, 7×10^{-7}, 1.5×10^{-4}, 5×10^{-8}, 2×10^{-7}, 9×10^{-7}, and 1.5×10^{-7}, respectively, were much lower than the frequency of transfer of this plasmid into an *E. faecalis* recipient. Furthermore, none of the transfers occurred in broth, except that in which the recipient was an *E. faecalis* strain. Cell aggregates, observed when pIP1438 transferred into *E. faecalis* (Table 4), were not observed when it transferred into any other of the recipients tested. However, when the transconjugants obtained in matings with the various enterococcal recipients were crossed with an *E. faecalis* recipient, cell aggregates were observed.

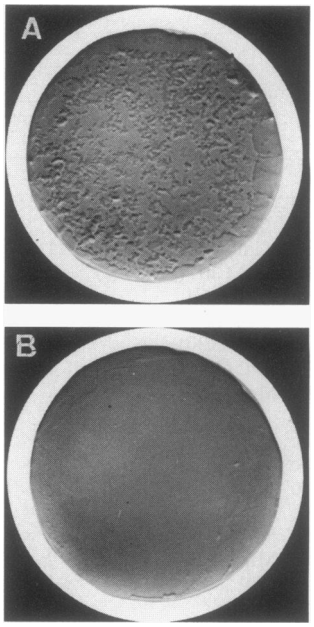

FIG. 5. Filter mating experiment. Following 18 h of mating contact, filters were washed with 0.5 ml of broth. (A) Cell aggregates. (B) No cell aggregates.

DISCUSSION

Of eight wild-type *E. faecalis* strains resistant to tetracycline, minocycline, erythromycin, and chloramphenicol, five transferred all of their resistance markers into an *E. faecalis* recipient, while three transferred only Tc^r Mn^r. In the wild-type strain D405 the determinants encoding Cm^r and Em^r, which were not conjugative, were found to be located on the chromosome. Cm^r and Em^r were carried by nonconjugative plasmids in two of the wild-type strains and by plasmids that transferred by conjugation in the other strains. In the transconjugants harboring two plasmids, it was not clear whether the R plasmid was itself conjugative or whether it was mobilized by the accompanying cryptic Tra$^+$ plasmid.

All of the transconjugant strains resistant to tetracycline and minocycline bore considerable sequence homology to Tn*916*. The presence of a hybridizing fragment of 4.8 kb suggested that these strains carry the gene *tetM*. The wild-type strains D397, D338, and D434 apparently carried both *tetM* and *tetL*; the MICs of tetracycline were slightly higher for these strains than for those in which homology with only one Tc^r probe was detected (Table 1). The presence of two Tc^r determinants in the same *E. faecalis* strain (JHI) was reported previously (30).

The occurrence of Tn*916* sequences on enterococcal plasmids is rare; the only naturally occurring *E. faecalis* plasmids previously reported to carry such sequences are pIP614 (8) and pCF-10 (10). pIP1440, derived from D397, is similar to these plasmids insofar as it shares sequence homology with Tn*916*, but differs from them in that it also carries *tetL* and encodes streptomycin resistance.

Since nucleotide sequences homologous to Tn*916* were detected in the chromosomal DNA of BM5469, BM5473, BM5475, and BM5479, but not in the conjugative plasmids they harbored, these plasmids (pIP1442, pIP1502, pIP1506, and pIP1141) apparently do not alter the antibiotypes of their hosts. The similarity of their *Hin*dIII profiles would suggest a common core structure (Fig. 1). Cell aggregates (Fig. 5), observed during the conjugative transfer of three of these plasmids, may be considered a plasmid marker; we suppose them to be indicative of a sex pheromone-mediated transfer mechanism (18).

The appearance of cryptic Tra$^+$ plasmids in transconjugants may be misleading, since antibiotic resistance markers that transfer by conjugation are not necessarily plasmid borne. We would suggest that such was the case with the Tc^r Mn^r determinant carried by JH2-2(pMV120). Several lines of evidence suggest that pMV120 is a cryptic Tra$^+$ plasmid,

similar to those we describe here. The Tc^r Mn^r marker of JH2-2(pMV120) transferred by conjugation in the same manner as the chromosomal Tc^r Mn^r markers of strains harboring cryptic Tra$^+$ plasmids: no transfer was detected in broth, and a low transfer frequency and cell aggregates were observed in filter matings. Hybridization experiments indicated the presence of an element similar or identical to Tn*916* in the chromosome of JH2-2(pMV120) and suggested that the gene encoding Tc^r Mn^r in JH2-2(pMV120) is *tetM*. In fact, to our knowledge, the only evidence so far presented for the existence of the gene *tetN* is the absence of homology between pMV120 and either *tetM* or *tetL* (8). Moreover, *tetN* cannot be distinguished from *tetM* on the basis of the mechanism by which it confers resistance (7). In our previous report, the absence of plasmid DNA in transconjugants of streptococcal groups A, B, C, G, and H which had received the Tc^r Mn^r marker associated with pMV120 was interpreted as the result of an integration of pMV120 into the recipient chromosome (26). In light of the results obtained in the present study, a simpler explanation may be proposed: the chromosomal Tc^r Mn^r marker transferred, but the plasmid, which may have a more limited host range, did not. We would suggest that cotransfer of pMV120 with the chromosomal Tc^r Mn^r marker may have led to a misinterpretation.

The conjugative transposon Tn*918*, which encodes resistance to tetracycline and minocycline, can insert into the cryptic plasmid pAM373 in the course of conjugation (12). In the present study, we observed a similar phenomenon with the element encoding Tc^r Mn^r of the wild-type strain D434. In one of the Tc^r Mn^r transconjugants derived from this strain the marker was on the chromosome, and in another transconjugant it was on the plasmid (pIP1129), suggesting that pIP1129 was the result of a transposition of an element resembling Tn*916* from the chromosome to the cryptic Tra$^+$ plasmid pIP1141 (T. Horaud et al., manuscript in preparation). The frequency of retransfer of the Tc^r Mn^r determinant from BM5478 was 5 orders of magnitude greater than that from BM5479 (Table 4). Of 30 other phenotypically identical transconjugants derived from D434, none displayed this increased frequency of retransfer (data not shown). The transfer frequency of pIP1129 may be the same as that of the cryptic Tra$^+$ plasmid pIP1141, although the insertion itself may have affected the plasmid transfer frequency. The broad host range observed for pIP1129 raises some doubt as to whether this plasmid was stably maintained in the streptococcal hosts or whether the Tc^r Mn^r element it bears resumed a chromosomal location upon retransfer. That its host range and retransfer frequency were similar to those of the other chromosomally located Tc^r Mn^r markers reported here would support the latter possibility; the plasmid content of the various streptococcal transconjugants has not, however, been examined.

Modifications of the cryptic Tra$^+$ plasmid pIP1506 appear to have given rise to pIP1130 (Cm^r) and to pIP1507 (Tc^r) (Fig. 1). The *Hin*dIII profiles of the last two plasmids were the same as that of pIP1506, except that in each case the 5.8-kb fragment had been replaced by a larger fragment on which the antibiotic resistance determinant was located. pIP1507 may have been formed by the legitimate recombination of pIP1506 with a small plasmid present in the wild-type strain. It is interesting in this regard that the *E. faecalis* plasmid pAMα1 (15), which carries the *tetL* determinant (8), actually consists of two smaller plasmids joined by recombination (35).

The results of this study attest to the genetic flexibility of multiply resistant *E. faecalis* strains. In the clinical isolates that we examined, resistance to tetracycline, minocycline, chloramphenicol, and erythromycin would seem to be the outcome of the dispersal of a few genes, rather than of the acquisition of many genes from diverse sources or a convergent evolution. In particular, elements similar to Tn*916* appear to be widespread in *E. faecalis* clinical isolates. Rapidly evolving antibiotic resistance plasmids, such as those described in the present report, may serve as a model for certain evolutionary processes. It is possible that conjugation itself enhances the likelihood of certain plasmid rearrangements, such as those by which pIP1506 gave rise to pIP1507 and pIP1130. Once a cryptic Tra$^+$ plasmid acquires antibiotic resistance genes, its subsequent transfer may serve as an efficient means of dissemination of such genes, at least within the species *E. faecalis*. The manner in which new genes are acquired and the structural changes such acquisitions may merit further study.

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