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Tn*5381*, a Conjugative Transposon Identifiable as a Circular Form in *Enterococcus faecalis*

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We have identified two 19-kb conjugative transposons (Tn*5381* and Tn*5383*) in separate strains of multiply resistant *Enterococcus faecalis*. These transposons confer resistance to tetracycline and minocycline via a *tetM* gene, are capable of both chromosomal and plasmid integration in a Rec$^-$ environment, and transfer between strains in the absence of detectable plasmid DNA at frequencies ranging from $<1 \times 10^{-9}$ to 2×10^{-5} per donor CFU, depending on the donor strain and the growth conditions. Hybridization studies indicate that these transposons are closely related to Tn*916*. We have identified bands of ca. 19 kb on agarose gel separations of alkaline lysis preparations from *E. faecalis* strains containing chromosomal copies of Tn*5381*, which we have confirmed to be a circularized form of this transposon. This phenomenon has previously been observed only when Tn*916* has been cloned in *Escherichia coli*. Overnight growth of donor strains in the presence of subinhibitory concentrations of tetracycline results in an approximately 10-fold increase in transfer frequency of Tn*5381* into enterococcal recipients and an increase in the amount of the circular form of Tn*5381* as detectable by hybridization. These results suggest that Tn*5381* is a Tn*916*-related conjugative transposon for which the appearance of a circular form and the conjugative-transfer frequency are regulated by a mechanism(s) affected by the presence of tetracycline in the growth medium.

Conjugative transposons have been implicated in the dissemination of antimicrobial resistance among numerous gram-positive genera (4, 6). Tn*916*, a 16.4-kb conjugative transposon encoding resistance to tetracycline and minocycline, was first described for a strain of *Enterococcus faecalis* in 1980 (12). Since that time, numerous similar elements have been described elsewhere (1, 4, 9, 14). Common to all of these elements are a preference for integration into the bacterial chromosome, the ability to transfer between strains in the absence of detectable plasmid DNA, and the ability to alter the hemolytic properties of hemolysin- and bacteriocin-producing plasmids. With few exceptions (1, 21), conjugative transposons have encoded a *tetM* tetracycline and minocycline resistance determinant. In most cases, these elements have been shown to have a broad host range within gram-positive genera.

On the basis of the apparent precise excision and insertion without target duplication of Tn*916*, a novel mechanism of transposition involving a nonreplicative circular intermediate was proposed elsewhere (13). Evidence to support the hypothesis of this mechanism includes the identification of a supercoiled form of Tn*916* within an *Escherichia coli* strain which had been transformed with a Tn*916*-containing chimeric cosmid. This supercoiled molecule was able to transform *Bacillus subtilis* and subsequently transfer tetracycline resistance to *Streptococcus pyogenes* in the absence of detectable plasmid DNA (24). Although it is presumed that circular intermediates of conjugative transposons exist within the gram-positive genera from which they originate, such elements have not yet been identified.

We have recently described four strains of *E. faecalis* in which resistance to multiple antimicrobial agents (penicillin via production of β-lactamase, erythromycin, gentamicin, streptomycin, and tetracycline) is chromosomally encoded yet transferable in various combinations to an enterococcal recipient (22). The present paper further elucidates the mechanism of transfer of tetracycline resistance from two of these strains, implicating two 19-kb conjugative transposons (Tn*5381* and Tn*5383*), one of which has been identified as a closed circle independent of the host chromosome. To our knowledge, this is the first report of the identification of a conjugative transposon existing as a circular form in gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in these experiments are listed in Table 1. *E. faecalis* CH19 possesses a single detectable plasmid of approximately 50 kb in size which carries no known antimicrobial-resistance determinants. *E. faecalis* CH116 has the same antimicrobial-resistance profile as CH19 but contains no identifiable plasmids when alkaline lysis techniques are employed to isolate these elements. *E. faecalis* UV202 and OG1X and plasmids pAD1 and pAM211 were generously provided by Don B. Clewell (Ann Arbor, Mich.). Plasmid pTC1 was generously provided by Donald LeBlanc (San Antonio, Tex.).

Antimicrobial agents. All antimicrobial agents were purchased from Sigma Chemical Co., St. Louis, Mo.

Mating techniques. Conjugation experiments were performed by either cross-streak-mating (12) or filter-mating techniques. Cross-streak matings were used to rapidly determine transfer potential but did not allow precise calculation of transfer frequency. Filters matings were performed essentially as described by Christie et al. (3). Cells were grown overnight in brain heart infusion (BHI) broth at 37°C in the presence or absence of tetracycline (5 μg/ml) with mild agitation. All overnight cultures were washed twice in sterile 0.9% saline and resuspended in an equivalent volume of saline prior to placement of 100-μl aliquots of both donor and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Chromosomal or plasmid marker(s)[a]	Plasmid designation	Comment (reference)
Strains			
CH19	βla$^+$ Em^r Gm^r Sm^r Tet^r (Tn*5381*)	pLRM2	Clinical isolate (22)
CH116	βla$^+$ Em^r Gm^r Sm^r Tet^r (Tn*5383*)	None	Clinical isolate (22)
JH2-7	Fus^r Rif^r Thy$^-$	None	Recipient strain (17)
UV202	Fus^r Rif^r Rec$^-$	None	Rec$^-$ recipient strain (28)
OG1X	Sm^r	None	Recipient strain (15)
OG1X(pAD1)	Sm^r	pAD1	Plasmid donor (8)
CV2, CV3	Fus^r Rif^r Tet^r	pAD1	UV202 with chromosomal Tn*5383* (this study)
CV4, CV5, CV7	Sm^r	pCWR3, pCWR38, pCWR39	OG1X with Tn*5383* in the pAD1 *Eco*RI B fragment (this study)
CV6	Sm^r Tet^r	pAD1	OG1X(pAD1) with chromosomal Tn*5383* (this study)
CV8	Sm^r	pCWR4	OG1X with Tn*5383* in the pAD1 *Eco*RI D fragment (HHly, Tet^r) (this study)
CV9, CV10, CV13 to CV18	Fus^r Rif^r Tet^r	None	JH2-7 with various numbers of chromosomal copies of Tn*5381* (this study)
CV12	Fus^r Rif^r Tet^r	None	JH2-7 with five chromosomal copies of Tn*5381* (this study)
CV20	Sm^r Tet^r	None	OG1X with one chromosomal copy of Tn*5381* (this study)
CV23	Sm^r Tet^r	None	OG1X with seven chromosomal copies of Tn*5381* (this study)
CV30	Fus^r Rif^r Tet^r	pCWR21	JH2-7(pAD1) with Tn*5381* integrated into the pAD1 *Eco*RI D fragment (HHly, Tet^r) (this study)
Plasmids			
pTC1	Tet^r Amp^r		5-kb *Hin*cII fragment of Tn*916* in pUC8 (D. LeBlanc)
pAD1	Hly Tra$^+$		Transposon-mobilizing plasmid (8)
pCWR3, pCWR38, pCWR39	Hly Tet^r Tra$^+$		pAD1 with Tn*5383* integrated into the *Eco*RI B fragment (this study)
pAM211	Hly$^-$ Tet^r		Tn*916* integrated into the *Eco*RI F fragment of pAD1 resulting in a nonhemolytic phenotype (8)
pCWR4	HHly Tet^r		Tn*5383* integrated into the *Eco*RI D fragment of pAD1 resulting in a hyperhemolytic phenotype (this study)
pCWR21	HHly Tet^r		Tn*5381* integrated into the *Eco*RI D fragment of pAD1 resulting in a hyperhemolytic phenotype (this study)

[a] βla, β-lactamase producing; Amp^r, ampicillin resistant; Em^r, erythromycin resistant; Fus^r, fusidic acid resistant; Gm^r, high-level gentamicin resistant; Rif^r, rifampin resistant; Sm^r, high-level streptomycin resistant; Tet^r, tetracycline resistant; Hly, hemolytic; HHly, hyperhemolytic; Tra$^+$, conjugative; Thy$^-$, thymidine requiring.

recipient strains on sterile nitrocellulose filters. Matings were allowed to proceed either overnight (18 h) or for a period of 4 h at 37°C. Clinical isolates CH19 and CH116 (βla$^+$ Em^r Gm^r Sm^r Tc^r) (22) were originally mated with either *E. faecalis* UV202 or *E. faecalis* JH2-7 with selection for transconjugants on dextrose phosphate agar plates containing fusidic acid (25 μg/ml), rifampin (100 μg/ml), and tetracycline (10 μg/ml). Only transconjugants which acquired resistance to tetracycline alone were used for this study. Status as a transconjugant was confirmed by the expression of resistance to fusidic acid and rifampin and the absence of other antimicrobial-resistance characteristics found in the donor chromosome (see above). JH2-7 transconjugants were further confirmed by their inability to grow on antibiotic-free Mueller-Hinton agar, a result of JH2-7's Thy$^-$ phenotype. Subsequent conjugation experiments used transposon-containing derivatives of JH2-7 as donors and *E. faecalis* OG1X (Str^r) as a recipient with selection on plates containing streptomycin (2,000 μg/ml) and tetracycline (10 μg/ml). Each transfer experiment was performed multiple times, with colony counts for each mating done in triplicate. The transfer frequency reported for each mating was calculated as the rate per donor CFU (rates calculated per recipient CFU were found to be the same) and represents the average of the frequencies resulting from the filter-mating experiments.

DNA techniques. Plasmids were purified for restriction digestion by a modification of the method of Ehrenfeld and Clewell (10). In brief, individual colonies were streaked onto BHI agar plates containing either tetracycline (10 μg/ml) or no antibiotic, and the plates were incubated overnight at 37°C. Colonies were then taken from these plates and

inoculated into 5 ml of BHI broth, either with or without tetracycline (10 μg/ml), and the inoculations were incubated overnight at 37°C. Overnight cultures were centrifuged at 3,000 × *g* for 5 min and resuspended in 200 μl of 50 mM Tris-Cl (pH 8.0)–10 mM EDTA containing 10 mg of lysozyme per ml, and the suspension was incubated for 30 min at 37°C. The suspension was then transferred to a 1.5-ml Eppendorf tube, 300 μl of 0.2 N NaOH–1% sodium dodecyl sulfate (SDS) was added, and the mixture was mixed gently and incubated at room temperature for 5 min, followed by the addition of 200 μl of 30% potassium acetate (pH 5.0) and a second 5-min incubation at room temperature. The tubes were then centrifuged at room temperature for 5 min, and the supernatant was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with an equal volume of 100% isopropanol. Precipitated DNA was then centrifuged at room temperature for 15 min and resuspended in 100 μl of 125 mM Tris-Cl (pH 8.0) and 300 mM sodium acetate, and the suspension was precipitated a second time with 2 volumes of ice-cold 95% ethanol and centrifuged again at room temperature for 15 min. The final pellet was resuspended in 20 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0) containing 0.01 mg of DNase-free RNase per ml, 1 to 5 U of restriction enzyme, and the concentrated buffer appropriate for the enzyme used (Bethesda Research Laboratories, Gaithersburg, Md.). When DNA was prepared for comparison of the ability to visualize the circular form of Tn*5381* either with or without preincubation with tetracycline, the initial cell densities of overnight cultures were standardized so that preparations of a given strain with or without tetracycline were the same density before the initial centrifugation was begun. In order to obtain larger amounts of the circular form of Tn*5381* to facilitate comparison of *Hin*cII digestions, multiple preparations as described above were performed. These preparations were combined prior to the digestion step, digested with *Hin*cII, purified with a glass bead suspension (Geneclean; Bio 101, La Jolla, Calif.), and resuspended in 20 μl of TE buffer for separation on agarose gels. Although the efficiency of preparations varied somewhat from day to day, it generally required the combination of 5 to 10 routine preparations to yield an amount of circular transposon DNA equivalent to that obtained with a single preparation of single-copy plasmid pAD1, suggesting that a circular form was present (after growth in tetracycline) in approximately 10 to 20% of the cells in a given overnight culture grown in tetracycline-containing media.

Preparations were digested for 1 h at 37°C and then separated on 0.7 to 1% agarose gels. This procedure resulted in reproducible visualization of digestion fragments of single-copy plasmid pAD1. DNA was transferred to nylon filters (Nytrans; Schleicher and Schuell, Keene, N.H.) by using the Vacugene blotting system (Pharmacia LKB Biotechnologies, Uppsala, Sweden) for 1.5 h according to the specifications of the manufacturer. The filters were hybridized with digoxigenin-labelled probes under conditions of high stringency (68°C, followed by washes in 0.1× SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7.0) and hybridized fragments were detected with an anti-digoxigenin-alkaline phosphatase conjugate with a chromogenic enzyme substrate (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). DNA used as probes included the 5-kb *Hin*cII fragment of plasmid pTC1, which includes the *tetM* gene and surrounding transposon sequence, the circular form of Tn*5381* linearized by digestion with *Eco*RI, and the ca. 240-bp product resulting from the amplification of the region between the ends of Tn*5381* (see below). The probes were purified after restriction digestion or amplification by separation on agarose gels, excising the appropriate band from the gel, and removal of the agarose with the Geneclean kit. The probes were labelled with digoxigenin by a random primer method according to the specifications of the manufacturer (Boehringer Mannheim Biochemicals). Membranes to be rehybridized with a separate probe were decolorized in *N-N*-dimethylformamide (Fisher Scientific, Fairlawn, N.J.) at 55°C and stripped of probe by being incubated in 0.2 N NaOH–0.1% SDS at 37°C for 30 min, followed by prehybridization and hybridization according to the usual protocol.

DNA amplification. The polymerase chain reaction amplification was performed as previously described (23) with primers designed to amplify the joint region of the circularized form of Tn*1545* as described by Poyart-Salmeron et al. (20). The right-end oligonucleotide primer was CGT GAA GTA TCT TCC TAC AGT. The left-end oligonucleotide primer was GGA TAA ATC GTC GTA TCA AAG. Reactions were carried out for 25 cycles with a commercially available *Taq* polymerase (GeneAmp; Perkin-Elmer Cetus, Norwalk, Conn.). Amplification products were separated on 2% agarose gels and purified with the Geneclean kit before labelling.

RESULTS

Identification of Tn*5383*. Initial hybridization studies of genomic DNA digestions from clinical strains CH19 and CH116 revealed an identical restriction map surrounding the *tetM* gene in both strains. Since CH116 is plasmid free, pAD1 was introduced into this strain by cross-streak mating with selection for hemolytic strains on 4% horse blood plates containing gentamicin (500 μg/ml). CH116(pAD1) was then mated by cross-streak techniques with *E. faecalis* UV202. Two tetracycline-, rifampin-, and fusidic acid-resistant transconjugants resulted from this mating (CV2 and CV3). In both of these strains, plasmid analysis revealed the presence of pAD1 without insert, suggesting a chromosomal location for the tetracycline resistance determinant and the ability of this mobile element to integrate into the chromosome in a Rec^- environment. These two isolates were subsequently crossed with OG1X, resulting in five transconjugants: two from CV2 and three from CV3 (CV4 to CV8). Plasmid analysis of these five strains revealed the integration of an approximately 18- to 19-kb element in the *Eco*RI B fragment in three and into the *Eco*RI D fragment in one. The fifth strain showed pAD1 without an insert (Fig. 1A). Integration always resulted in the appearance of two new *Eco*RI fragments, suggesting the presence of an *Eco*RI site within the mobile element. Hybridization with the *tetM* gene probe revealed the presence of this gene on the plasmids with inserts (Fig. 1B). We designated this transposon Tn*5383*. Subsequent mating experiments between CH116 (without pAD1) and JH2-7 revealed that Tn*5383* was transferable in the absence of detectable plasmid DNA at a low frequency (7.4×10^{-9} per donor CFU), confirming that it is a conjugative element.

Identification of Tn*5381*. Matings between CH19, which possesses a single cryptic plasmid (pLRM2), and *E. faecalis* JH2-7 resulted in tetracycline-resistant transconjugants at a rate of 2×10^{-8} per donor CFU. Nine transconjugants (CV9, CV10, and CV12 to CV18) were randomly selected for plasmid analysis to exclude the presence of pLRM2. pLRM2 was not identified in any of these transconjugants. To our surprise, however, after digestion of the alkaline lysis preparations with *Eco*RI, a faint band was visible in a number of lanes; this band did not correspond to any of the *Eco*RI

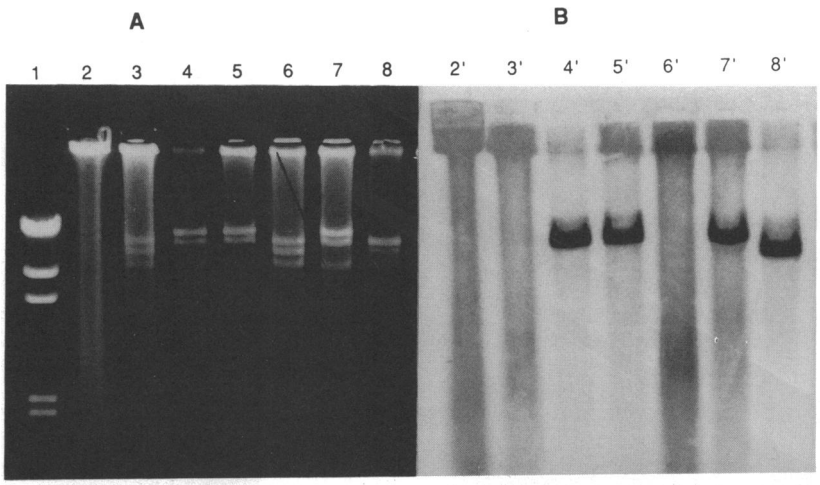

FIG. 1. (A) *Eco*RI restriction digestion of pAD1 with and without Tn*5383* inserted into different fragments. Lanes: 1, lambda DNA digested with *Hin*dIII (fragment sizes, 23.03, 9.4, 6.6, 4.3, 2.3, and 2.0 kb); 2, CH116 (plasmid free); 3, CH116(pAD1); 4, CV4(pCWR3); 5, CV5(pCWR38); 6, CV6(pAD1 without insert); 7, CV7(pCWR39); 8, CV8(pCWR4). (B) Hybridization of Southern blot of gel pictured in panel A with the 5-kb *Hin*cII fragment of pTC1 (containing the *tetM* gene) as a probe.

bands of pLRM2 and measured ca. 19 kb, a size approximating that of Tn*5383* (Fig. 2A). Hybridization of the blot resulting from the Southern transfer of this DNA with the *tetM* gene as a probe revealed the presence of this gene in all lanes, even lanes in which no band was visible in the gel (Fig. 2B). None of the visible bands of the *Eco*RI digestion of pLRM2 hybridized with this probe (although there is faint hybridization to a band not visible on the gel corresponding in size to hybridizations in the bands in the transconjugants), suggesting that they were not remnants of pLRM2 digestion. *Eco*RI digestion and hybridization of genomic DNA from these strains revealed the presence of the *tetM* gene in multiple locations within the chromosome (a phenomenon commonly seen with Tn*916* [5, 18]), with a rough correlation being noted between the number of chromosomal copies of the element and the ability to visualize the 18- to 19-kb band on the agarose gel in Fig. 2A (data not shown). We have designated the transposon originating in CH19 Tn*5381*.

To determine whether Tn*5381* was transferable from donors lacking detectable plasmid DNA, we performed filter matings between the strains shown in Fig. 2 (grown in the absence of tetracycline) and *E. faecalis* OG1X. The frequency of Tn*5381* transfer varied, depending on the donor, from $<1 \times 10^{-9}$ to 4×10^{-7}. Alkaline lysis preparations from all of the successful donor strains and a selected OG1X transconjugant from each (CV20 to CV25) revealed no detectable plasmid DNA but again revealed some faint

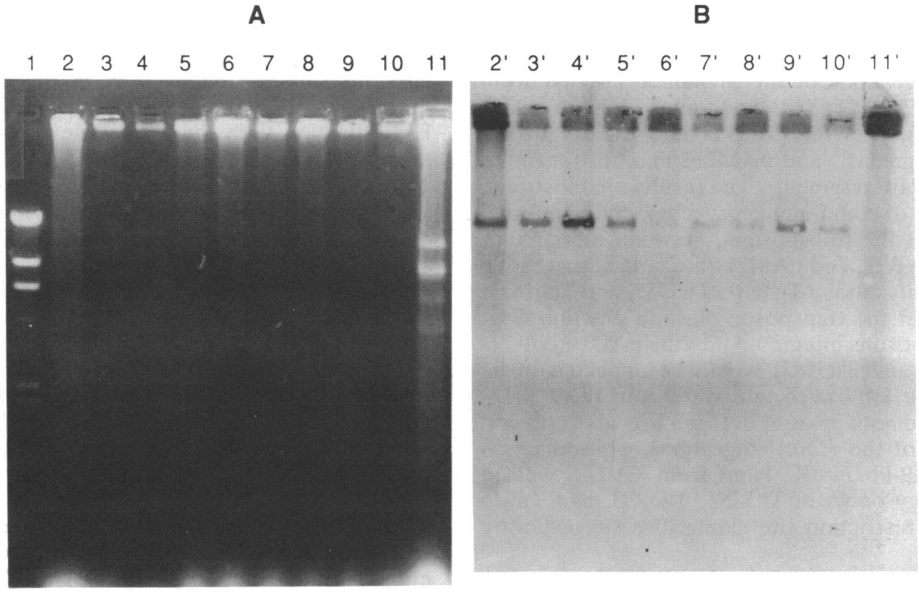

FIG. 2. (A) *Eco*RI digestion of alkaline lysis-derived DNA from transconjugants resulting from mating between CH19 and JH2-7. Lanes: 1, lambda DNA digested with *Hin*dIII (see Fig. 1); 2, CV9; 3, CV10; 4, CV12; 5, CV13; 6, CV14; 7, CV15; 8, CV16; 9, CV17; 10, CV18; 11, CH19. (B) Hybridization of Southern blot of gel pictured in panel A, with the 5-kb *Hin*cII fragment of pTC1 as a probe.

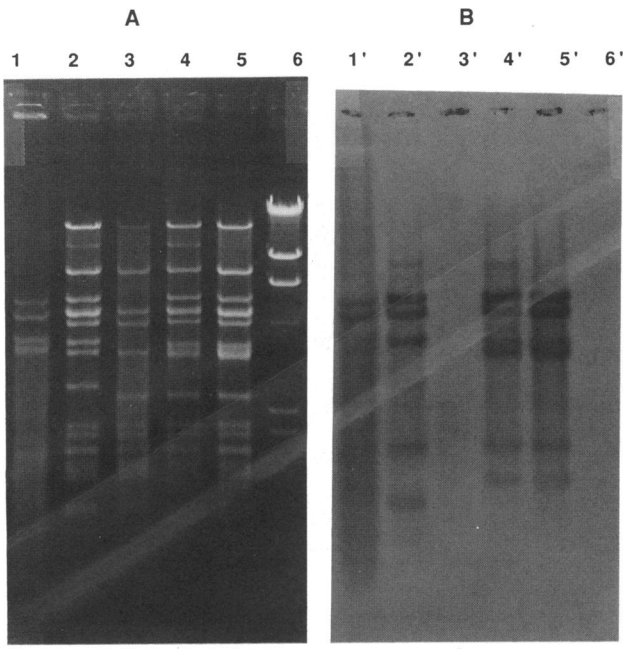

FIG. 3. (A) *Hin*cII digestions of DNA derived by alkaline lysis from enterococcal strains. Lanes: 1, CV12 (JH2-7 derivative containing five chromosomal copies of Tn*5381* and no identifiable plasmids); 2, pAM211 (Tn*916* integrated into the pAD1 *Eco*RI F fragment); 3, pAD1; 4, pCWR21 (Tn*5381* integrated into the pAD1 *Eco*RI D fragment); 5, pCWR4 (Tn*5383* integrated into the pAD1 *Eco*RI D fragment); 6, lambda DNA digested with *Hin*dIII (size standard). (B) Hybridization of Southern transfer of gel in panel A with labelled Tn*5381* as a probe.

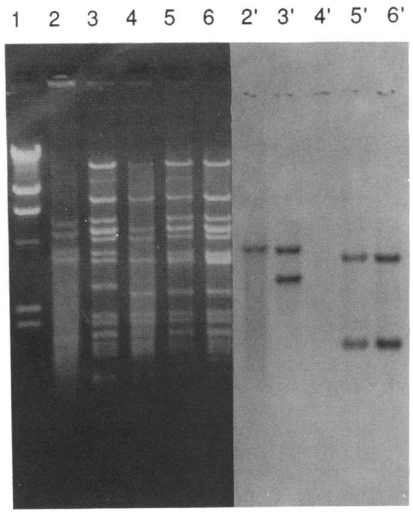

FIG. 4. (Left panel) *Hin*cII digestions of DNA derived by alkaline lysis of enterococcal strains (see Fig. 3). Lanes: 1, bacteriophage lambda digested with *Hin*dIII; 2, CV12; 3, pAM211; 4, pAD1; 5, pCWR21; 6, pCWR4. (Right panel) Hybridization of the Southern blot of the gel pictured in the left panel, with the amplified Tn*5381* joint fragment as a probe.

hybridization with the *tetM* probe in some lanes at approximately 19 kb (data not shown). Once again, multiple copies of the transposon were found in genomic restriction digestions of some of the recipients (data not shown).

In an effort to determine the degree of relatedness of Tn*5381* and Tn*5383* and similarities between these transposons and Tn*916*, we removed the 19-kb *Eco*RI band found in alkaline lysis preparations of CV12 and used this as a probe of *Hin*cII digestions of the presumed circular form itself (CV12), pCWR21 (Tn*5381* integrated into the pAD1 *Eco*RI D fragment), pCWR4 (Tn*5383* integrated into the pAD1 *Eco*RI D fragment), and pAM211 (Tn*916* integrated into the pAD1 *Eco*RI F fragment). The results are illustrated in Fig. 3. A number of common bands, representing internal *Hin*cII fragments of the transposons, between the circular form, pCWR4, pCWR21, and pAM211 are noted, suggesting a substantial similarity among Tn*5381*, Tn*5383*, and Tn*916*. It should be noted that the transposon-plasmid junction fragments for the transposons integrated into the pAD1 *Eco*RI D fragments (Tn*5381* and Tn*5383*) would be expected to be different from those for Tn*916* integrated into the F fragment. A 400-bp band not apparent in Fig. 3 was also noted to be common to all of the *Hin*cII digestions. Hybridization with the labelled 19-kb *Eco*RI band from CV12 revealed substantial homology between Tn*5381*, Tn*5383*, and Tn*916* and confirmed the restriction site similarities suggested by the gel analysis.

Confirmation of the presence of the closed circular transposon and identification of the *Hin*cII fragment containing the joined ends of the transposon. Using oligonucleotides designed to amplify the joint region of the closed circular form of Tn*1545* (over a region identical to the ends of Tn*916*) (20), we performed polymerase chain reaction amplification of crude alkaline lysis preparations of CV12 (five chromosomal copies of Tn*5381*). An approximately 240-bp product was identified (data not shown). This product was extracted from the gel, labelled, and hybridized with the stripped nylon filter shown in Fig. 3. The results of this hybridization are shown in Fig. 4. As expected, no hybridization is seen in the lane with pAD1 alone. In the lanes containing the transposons integrated into pAD1, two fragments are identifiable; these are the junction fragments of the ends of the transposons and pAD1. Only a single, 3.4-kb band is identified in the lane with the *Hin*cII digestion of the circular form. No corresponding band is seen in the *Hin*cII digestions of the integrated forms of Tn*5381* or Tn*5383*; this fact identifies this 3.4-kb band as the one containing the joint fragment and confirms that the bands seen in the first lane of the gel represent a *Hin*cIII digestion of the circular form of Tn*5381*. Hybridization with the *tetM* gene suggests that this resistance determinant lies within the 4.8-kb *Hin*cII fragments of Tn*5381* and Tn*5383*, just as in Tn*916* (data not shown). The sum of the sizes of the bands present in the *Hin*cII digestion of the circular form (5.5, 4.8, 3.4, 3.2, 1.7, and 0.4 kb) suggests that Tn*5381* is 19 kb in size.

Effect of incubation with tetracycline on transfer frequency. CV12, a JH2-7 derivative with five chromosomal copies of Tn*5381*, and CV23, an OG1X derivative with seven copies, were grown overnight in BHI broth in the presence and absence of tetracycline (5 μg/ml) and subsequently mated by the filter-mating technique with *E. faecalis* OG1X and JH2-7. The transfer rates from the different organisms grown in the presence or absence of tetracycline are listed in Table 2. Transfer from CV12 and CV23 into OG1X and JH2-7, respectively, was roughly 10-fold greater after preincubation with tetracycline. Four-hour matings were also performed to minimize any inhibitory effect of residual tetracycline on recipient organisms which did not acquire the *tetM* gene. Transfer frequencies were slightly less in the 4-h matings than in those performed overnight. However, transfer at 4 h

TABLE 2. Frequency of Tn*5381* transfer from plasmid-free and pAD1-containing strains after 4 and 18 h of growth in the presence and absence of tetracycline

Mating type	Growth condition (h)[a]	Transfer frequency (range)
CV12 × OG1X	+ (18)	2×10^{-6} (9×10^{-7}–4.5×10^{-6})
CV12 × OG1X	− (18)	1.5×10^{-7} (8.7×10^{-8}–2×10^{-7})
CV23 × JH2-7	+ (18)	1.9×10^{-5} (7×10^{-6}–3.2×10^{-5})
CV23 × JH2-7	− (18)	2×10^{-6} (7×10^{-7}–3.6×10^{-6})
CV12 × OG1X	+ (4)	8×10^{-7} (3×10^{-7}–1.3×10^{-6})
CV12 × OG1X	− (4)	2.3×10^{-8} (7×10^{-9}–3.9×10^{-8}
CV23 × JH2-7	+ (4)	5.4×10^{-6} (5×10^{-6}–5.8×10^{-6})
CV23 × JH2-7	− (4)	4×10^{-7} (4×10^{-7}–4×10^{-7})
CV12(pAD1) × OG1X	+ (18)	2×10^{-7} (2×10^{-8}–3×10^{-7})
CV12(pAD1) × OG1X	− (18)	3.8×10^{-7} (1×10^{-8}–1×10^{-6})

[a] + and −, presence and absence, respectively, of tetracycline (5 μg/ml). Numbers in parentheses are hours of growth.

was again found to be increased approximately 10-fold from that of CV23 exposed to tetracycline. Transfer from strain CV12 was increased 35-fold after preincubation with tetracycline (Table 2). Transfer of Tn*5381* from OG1X derivatives into chromosomal locations in *E. faecalis* UV202 was also noted to occur, confirming the ability of Tn*5381* to transpose in a Rec⁻ environment (data not shown). Interestingly, transfer of Tn*5381* from a CV12 strain into which pAD1 had been introduced by conjugation to *E. faecalis* OG1X was less after incubation with tetracycline than in strains grown without the antibiotic. This is in marked contrast to Tn*916*, which is reported to transfer at a 100-fold higher frequency from strains with coexisting pAD1 (7). Analysis of transconjugants resulting from matings between CV12(pAD1) and OG1X revealed integration of Tn*5381* into at least two distinct sites within pAD1. Integration into the *Eco*RI D fragment resulted in the expression of a hyperhemolytic phenotype.

Effect of incubation with tetracycline on the detectability of the circular form. It has been proposed that excision is the rate-limiting step in conjugative transposition (7). If this is the case, one plausible explanation for the increase noted above in transfer frequency after exposure to tetracycline would be that tetracycline stimulates excision of Tn*5381*. Such an increased rate of excision may be expected to result in an increase in the amount of circular form detectable in alkaline lysis preparations from strains grown in the presence versus the absence of tetracycline. We tested this hypothesis by performing alkaline lysis preparations on three strains with different numbers of chromosomal copies of Tn*5381* (CV20, one copy; CV12, five copies; CV23, seven copies) after overnight growth in the presence or absence of tetracycline (10 μg/ml) (see above). The results of the *Eco*RI digestion of these preparations are shown in Fig. 5. A band corresponding to the 19-kb transposon is readily visible in the lane corresponding to the preparation from CV12 grown in the presence of tetracycline. This has been the strain that has consistently yielded the largest amount of the circular form of the element. More striking results in the hybridization of the Southern transfer of this DNA with Tn*5381* as a probe were seen (Fig. 5). A less striking increase in the lanes containing CV23 was seen. One strain (CV20) demonstrated no detectable circular form of Tn*5381* even after incubation with tetracycline. This strain has only one chromosomal copy of Tn*5381* and exhibits very low rates of conjugative transfer. The final two lanes of Fig. 5 show *Eco*RI digestions of pCWR21 and are included as a control to demonstrate that preincubation with tetracycline does not increase the yield of plasmid DNA in the alkaline lysis preparations employed to demonstrate the circular form of Tn*5381* after preincubation with tetracycline.

DISCUSSION

Since Tn*916* was first described in 1980, numerous conjugative transposons varying widely in size and in incorporated resistance determinants have been reported elsewhere (1, 4, 9, 14, 21). Tn*5381* and Tn*5383*, the two transposons described in this paper, are clearly conjugative transposons by virtue of their ability to transfer among strains in the absence of detectable plasmid DNA and their ability to integrate in a Rec⁻ environment as well as into at least two sites in plasmid pAD1. The elements are homologous to Tn*916* and exhibit marked similarities in the sizes of four internal *Hin*cII fragments and in the location of the *tetM* gene within a 4.8-kb *Hin*cII fragment. Like Tn*916*, Tn*5381* can exist in multiple copies within a recipient strain chromosome (5, 18). Tn*5381* and Tn*5383* are also similar to other conjugative transposons in that integration into the *Eco*RI D fragment of plasmid pAD1 can result in a hyperhemolytic phenotype in an OG1X background, a phenomenon which has recently been attributed to variations in DNA sequences brought to the integration site by the transposon (16). Tn*5381* and Tn*5383* do exhibit clear physical differences from Tn*916* in their sizes (19 versus 16.4 kb) and in the existence of *Eco*RI sites near the end of the elements.

One feature of Tn*5381* which is unique among conjugative transposons is the ability to demonstrate a circular, presumably supercoiled form of the element in alkaline lysis preparations from enterococcal strains. The hybridization and amplification studies detailed in this paper conclusively demonstrate that the 19-kb band seen in digested alkaline

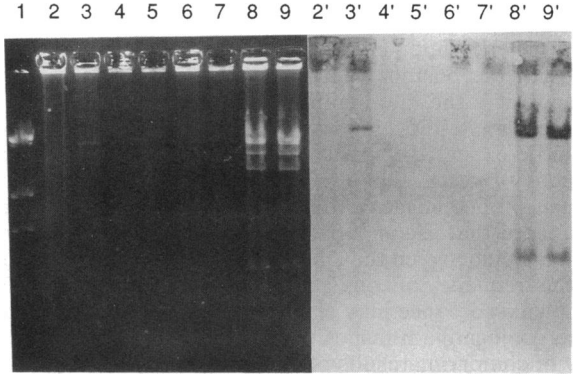

FIG. 5. (Left panel) *Eco*RI digestions of DNA derived by alkaline lysis from selected plasmid-free, Tn*5381*-containing JH2-7 and OG1X transconjugants after overnight growth with and without tetracycline (10 μg/ml). Lanes: 1, bacteriophage lambda digested with *Hin*dIII; 2, CV12 (five copies; without tetracycline); 3, CV12 (with tetracycline); 4, CV20 (one copy; without tetracycline); 5, CV20 (with tetracycline); 6, CV23 (seven copies; without tetracycline); 7, CV23 (with tetracycline); 9, CV30 (without tetracycline); 10, CV30 (with tetracycline). (Right panel) Hybridization of the Southern transfer of the gel in the left panel, with labelled Tn*5381* as a probe. Single bands in lanes 3′ and 7′ show hybridization to the circular form of the element linearized by digestion with *Eco*RI. In its integrated form (lanes 8′ and 9′), *Eco*RI digestion results in hybridization to two bands.

lysis preparations from these strains is in fact a circular form of Tn*5381* and that our ability to detect this form in some strains is increased by preincubation with tetracycline. Although it seems likely that this circular form of Tn*5381* represents an intermediate form in transposition, transformation studies with the circular form itself will be required for final confirmation. Regardless, these results lend further support to the validity of the circular intermediate mechanism originally proposed by Gawron-Burke and Clewell (13).

Tn*5381* is not unique in its ability to demonstrate enhanced conjugative transposition in response to incubation with tetracycline. Torres et al. recently reported that Tn*925*, a conjugative transposon that is in many ways indistinguishable from Tn*916*, transfers to an enterococcal recipient at a 10-fold higher frequency after preincubation with tetracycline (27). These investigators also noted the transfer of physically unrelated chromosomal markers between strains when a copy of Tn*925* was present in the donor strain (cotransfer of Tn*925* was not required), a process which they postulate resembles a cell fusion event (27). Conjugative transfer of Tn*916* has not been conclusively shown to be enhanced by exposure to tetracycline, and there is strong evidence that its transfer does not proceed via a process that involves cell fusion (2, 11). Because Tn*916* and Tn*925* are so closely related structurally, it has been proposed that these apparent differences may reflect a difference in the efficiency with which the transposons induce pore formation between mating cells (11). The strain from which Tn*5381* was derived (CH19) has been shown to transfer a number of chromosomal antimicrobial-resistance determinants in the absence of detectable plasmid DNA (22). These multiple-resistance transfers are always accompanied by the transfer of the *tetM* determinant, raising the possibility of a role for Tn*5381* in the transfer of a range of chromosomal antimicrobial-resistance genes. In this regard and because of the fact that tetracycline stimulates the conjugal transfer of the transposon, Tn*5381* appears to bear a greater resemblance to Tn*925* than to Tn*916*.

The ability to better demonstrate the circular form of Tn*5381* as well as an increase in the conjugative-transposition frequency after incubation with tetracycline is consistent with the postulate that the excision of a conjugative transposon is the rate-limiting step for transposition (7). In this scenario, tetracycline-stimulated excision of the element would be expected to result in an increase in transposition frequency overall and in a corresponding increase in the frequency of conjugative transposition. It should be noted in this regard that a comparison of CV12 and CV23 shows a discrepancy between the amount of detectable circular form and the frequency of conjugative transposition (Table 2; Fig. 5). This discordance may simply reflect the complex nature of the conjugative-transposition process as well as the difficulty of comparing results of conjugation experiments when different donor and recipient strains are used. The fact that our ability to demonstrate the circular form varies within a given strain with differences in the numbers of chromosomal copies of the element is consistent with the postulate that excision frequency (and therefore transposition frequency) is affected by the sequences surrounding the integrated transposon (13).

It is not immediately apparent why pAD1 does not enhance transfer of Tn*5381* from cells incubated without tetracycline or why preincubation of these strains with tetracycline results in a lower frequency of transfer than that seen for strains without pAD1. Conjugative transfer of Tn*916* is enhanced 10- to 100-fold when pAD1 is present, presumably as a result of a transient association between the transposon and the plasmid (7). It is possible that preincubation with tetracycline sets in motion processes which result in increased excision and/or decreased integration of Tn*5381*, thereby decreasing the likelihood that the presumed transient association between the transposon and the conjugative plasmid will occur. Alternatively, it is possible that for reasons yet to be elucidated, Tn*5381* is less likely to form a transient association with pAD1 than is Tn*916*.

Recent evidence suggests that the regulation of *tetM* production of Tn*916* and transposition functions may be related. The excisase (Xis-Tn) and integrase (Int-Tn) genes of Tn*1545* have been identified, sequenced, and found to exhibit significant deduced amino acid homology with site-specific recombinases and excisionases of lambdoid bacteriophages (Xis-Tn) and the highly conserved Int-related proteins (Int-Tn) (19, 25). These same (or closely related) genes have been identified near the left end of Tn*916*, lying between the end of the element and the *tetM* gene (5). Su et al. have noted that the transcription of the *tetM* gene in Tn*916* proceeds in the leftward direction and have identified a 3.2-kb transcript seen when Tn*916*-containing cells are exposed to higher concentrations of tetracycline, which they suggest may represent extension of transcription into the region of the transposition genes (26). The extensive homology between Tn*5381* and Tn*916* that we have demonstrated makes it likely that these transposons share genetic determinants responsible for transposition. It is conceivable that the structural differences between Tn*5381* and Tn*916* involve the region of the transposition genes, altering their regulation in ways that allow the identification of the circular form in a gram-positive background. We are currently performing more extensive structural and functional studies of Tn*5381* to better define the characteristics of this novel transposable element.

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