INFECTION AND IMMUNITY, Feb. 1985, p. 415–420
0019-9567/85/020415-06$02.00/0

Vol. 47, No. 2

A Conjugative Transposon (Tn*919*) in *Streptococcus sanguis*

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Received 12 October 1984/Accepted 14 November 1984

***Streptococcus sanguis* FC1, originally isolated from dental plaque, was found to be naturally resistant to tetracycline. Although no plasmid DNA could be detected, tetracycline resistance was transferable in filter matings to *Streptococcus faecalis* FA2-2. Again, no plasmid DNA was detectable in transconjugants, and the latter could donate tetracycline resistance to *S. faecalis*, *S. sanguis*, and *Steptococcus lactis*. The tetracycline resistance element was able to transpose to several sites on the *S. faecalis* hemolysin plasmid pAD1 and in each case resulted in a 15-kilobase insert. DNA filter blot hybridization studies showed that the element bears significant homology with the conjugative transposon Tn*916*. Designated Tn*919*, it was cloned into an *Escherichia coli* plasmid vector (pGL101) and, as has been shown for Tn*916*, excised readily in the absence of selective pressure.**

In the genus *Streptococcus*, plasmids determining drug resistance and other properties have been observed in a variety of species (3). However, there are numerous examples of drug-resistant clinical isolates that lack detectable plasmid DNA, yet in some of these cases the resistance determinant(s) was transferable by a process resembling conjugation (2, 8–10, 15, 16, 30, 31, 33). One of the best-documented systems involving nonplasmid resistance transfer is that of Tn*916*, a tetracycline resistance element originally identified on the chromosome of *Streptococcus faecalis* DS16 (8–11). This 15-kilobase (kb) "conjugative transposon" appears to encode information for transfer as well as for transposition (9, 10); tetracycline-resistant transconjugants can be obtained at frequencies of 10^{-8} to 10^{-5} per donor in overnight filter matings. Transfer is Rec-independent, and Tn*916* insertions into recipient DNA have been shown to occur in different sites (9). The variability in Tn*916* donor potential may reflect an influence from neighboring sequences, although changes within the transposon have not been ruled out. Tn*916* can transpose into several different conjugative hemolysin plasmids, often resulting in either hyperexpression or nonexpression of hemolysin genes (9). When residing on a conjugative plasmid, transfer of the plasmid to a new host results in a "zygotic induction" that gives rise to excision of the transposon; this leads to either loss of the element or insertion into the recipient chromosome (10). It was shown recently that Tn*916* could be cloned in *Escherichia coli* (11). Tetracycline resistance was expressed, but excision and loss of the transposon were observed in the absence of selective pressure. Since Tn*916* excision resulted in a splicing together of flanking DNA, Tn*916* offers a powerful approach for targeting and cloning DNA from gram-positive bacteria.

Streptococcus sanguis is one of several species of streptococci that reside in the human oral cavity. It is a major component of dental plaque and is frequently involved in subacute bacterial endocarditis. Hawley et al. (14) have reported significant increases in the frequency of tetracycline resistance among oral streptococci from patients undergoing tetracycline therapy. Analysis of resistant derivatives failed to detect plasmid DNA (14). However, one such strain (*S. sanguis* I141) was recently reported by Hartley et al. (13) to transfer tetracycline resistance to *S. faecalis* and then between *S. faecalis* strains. The absence of plasmid DNA plus evidence for insertion into more than one chromosomal site was strongly suggestive of a conjugative transposon.

In this report we describe the identification of a conjugative tetracycline resistance determinant in *S. sanguis* FC1. This strain was originally isolated from dental plaque (12) but was not known to be resistant to tetracycline. In efforts to make use of this strain as a recipient for Tn*916*, we observed that FC1, itself, was tetracycline resistant. An ensuing investigation, described here, revealed an element (designated Tn*919*) very similar but not identical to Tn*916*.

MATERIALS AND METHODS

Bacterial strains, media, and chemical reagents. The bacterial strains and their plasmid content are described in Table 1. All streptococci, with the exception of some of the *S. sanguis* strains, were grown in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.). In some cases the medium in the plates was supplemented with 5% Horse Blood (Colorado Serum Co.) to demonstrate the various hemolytic phenotypes of strains carrying plasmid pAD1 or its derivatives. The *S. sanguis* strains were grown in either THB or brain heart infusion (Difco) broth. The *E. coli* strains were routinely grown in LB medium (7). For solid media, agar (Difco) was added to the appropriate broth at a level of 1.5% before autoclaving. Antibiotics present in selective media were added at the following levels: ampicillin, 25 μg/ml; fusidic acid, 25 μg/ml; rifampin, 25 μg/ml; streptomycin, 1,000 μg/ml; spectinomycin, 500 μg/ml; and tetracycline, 10 or 4 μg/ml when either streptococci or *E. coli*, respectively, were involved. Spontaneous drug-resistant mutants of streptococcal strains were derived by plating 0.2 ml of a fully grown (late-stationary-phase) culture (10-fold concentrated) on the appropriate medium containing the relevant antibiotic. Restriction endonucleases, T4 DNA ligase, and lambda DNA were obtained from Bethesda Research Laboratories, Gaithersburg, Md., or New England Biolabs, Cambridge, Mass. Their use was according to the specifications of the manufacturers.

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TABLE 1. Bacterial strains and plasmids

Strain	Chromosome genotype	Plasmid content	Origin/derivation
S. faecalis			
OG1X(pAD1)	*str gel*	pAD1 (hem-bac)	18
JH2SS	*str spc*	None	36
OG1RF	*rif fus*	None	26
FA2-2	*rif fus*	None	5
GF590	*rif fus tet*	None	*S. sanguis* FC1SS × FA2-2
GF590(pAD1)	*rif fus tet*	pAD1	OG1X(pAD1) × GF590
JH2SS(pAM551)	*str spc*	pAM551 (pAD1::Tn*919* in *Eco*RI H; nonhemolytic)	GF590(pAD1) × JH2SS
JH2SS(pAM552)	*str spc*	pAM552 (pAD1::Tn*919* in *Eco*RI D; hyperhemolytic)	GF590(pAD1) × JH2SS
JH2SS(pAM553)	*str spc*	pAM533 (pAD1::Tn*919* in *Eco*RI B; typically hemolytic)	GF590(pAD1) × JH2SS
OG1RF(pAM551)	*rif fus*	pAM551	JH2SS(pAM551) × OG1RF
OG1RF(pAM552)	*rif fus*	pAM552	JH2SS(pAM552) × OG1RF
OG1RF(pAM553)	*rif fus*	pAM553	JH2SS(pAM553) × OG1RF
JH2-2(pAM210)	*rif fus*	pAM210	9
S. mutans			
6715-10RF	*rif fus*	None	Derived from 6715-10 (D. LeBlanc)
6715-10S	*str*	None	Derived from 6715-10
10T2RF	*rif fus*	Not tested	Derived from 10T2 (W. Loesche)
10T2S	*str*	Not tested	Derived from 10T2
GS5RF	*rif fus*	Not tested	Derived from GS5 (H. Kuramitsu)
Ingbritt RF	*rif fus*	Not tested	Derived from Ingbritt (20) (G. Westergren)
Ingbritt S	*str*	Not tested	Derived from Ingbritt
S. sanguis			
FC1SS	*str spc tet*	None	Derived from FC1 (12) (G. Jones)
Challis RF	*rif fus*	None	Derived from Challis (27) (F. Macrina)
G9BS	*str*	Not tested	Derived from G9B (29) (B. Rosan)
S. salivarius 186AS	*str*	Not tested	Derived from 186A (G. Westergren)
S. mitior 186JS	*str*	Not tested	Derived from 186J (G. Westergren)
S. lactis 4215S	*str*	Not tested	Derived from 4215 (L. Pearce)
E. coli			
HB101(pGL101)	F⁻ *hsdS20 galK2 recA13 ara-14 proA2 lacY1 rpsL20*(Smr) *xyll-5 mtl-1 supE44*	pGL101 (Ap)	C. Gawron-Burke
DH1	F⁻ *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*	None	C. Gawron-Burke
DH1(pAM554)	Same as DH1	pAM554 (pGL101 with H' [H::Tn*919*] fragment of pAM551)	DH1 transformed with pAM554 DNA

Genetic techniques. Filter matings and "cross-streak" matings were done as described previously (5, 9) except the medium used was THB in most cases. The membrane filters were type HA (0.45 μm), obtained from Millipore Corp., Bedford, Mass. Transformation of *E. coli* DH1 with plasmid DNA has been described previously (7).

Plasmid DNA isolation and characterization. Plasmid DNA was isolated from streptococci essentially as described elsewhere (6, 9). For non-*S. faecalis* strains, cells were treated with mutanolysin (final concentration, 100 μg/ml) at 37°C before the lysozyme treatment. Also, all streptococcal lysates were treated with RNase (final concentration, 50 μg/ml) before centrifugation on CsCl buoyant density gradients. Plasmid DNA was isolated from *E. coli* strains as described elsewhere (4). The rapid plasmid preparation protocol of Ish-Horowicz and Burke (19) was used in some

cases to obtain DNA from *E. coli* strains. All DNA preparations were purified through two cesium chloride-ethidium bromide gradients, by centrifugation in a VTi50 rotor and then in a VTi65 rotor with a Beckman L8-70 ultracentrifuge. For cloning experiments in which it was necessary to isolate and purify specific DNA fragments from agarose gels, the dialysis membrane method of Maniatis et al. (24) was used.

DNA fragments generated after restriction nuclease digestion were fractionated by agarose gel electrophoresis in Tris-borate buffer with a horizontal gel apparatus. DNA was visualized with short-wave UV light and photographed with a Polaroid MP4 Land camera with Polaroid type 667 film. DNA fragments of known size, generated by *Eco*RI single digests and *Eco*RI-*Hin*dIII double digests of lambda DNA, were used as size standards on all gels.

DNA-DNA hybridization. Transfer of DNA from agarose gels to nitrocellulose filters (Millipore Corp.) was done by the method of Southern (34) with the modification of Wahl et al. (37). DNA was radioactively labeled by the nick translation method of Rigby et al. (28) with a nick translation kit from New England Nuclear Corp., Boston, Mass. DNA-DNA hybridizations were done essentially as described by Maniatis et al. (24), and probed filters were exposed with Kodak X-Omat AR film in cassettes with Dupont Cronex intensifying screens at −70°C.

RESULTS

Transfer of tetracycline resistance from *S. sanguis* FC1SS. *S. sanguis* FC1SS is a derivative of strain FC1 resistant to streptomycin and spectinomycin as a result of independent spontaneous mutations. However, this strain is naturally resistant to tetracycline, exhibiting MICs of 10 μg/ml in AB3 broth and 20 μg/ml in THB. Plasmid DNA was not detected in strain FC1SS after analysis of cell lysates by cesium chloride-ethidium bromide buoyant density centrifugation.

Despite the absence of detectable plasmid DNA it was possible to transfer tetracycline resistance from strain FC1SS to a *S. faecalis* FA2-2 recipient at a low but consistent frequency by overnight filter matings (Table 2). Transfer to a number of other streptococcal strains (*Streptococcus mutans* and *S. sanguis*) could not be detected. *S. faecalis* FA2-2 tetracycline-resistant transconjugants could in turn retransfer the resistance trait to *S. faecalis* JH2SS, and ca. 10% could donate tetracycline resistance at a 10-fold elevated frequency (ca. 10^{-6}) compared with the others (ca. 10^{-7}). One of these higher-frequency donors, strain GF590, also free of detectable plasmid DNA, was found capable of transferring tetracycline resistance to *S. sanguis* G9BS and *Streptococcus lactis* 4125S. Transfer to *S. mutans*, *Streptococcus mitior*, and *Streptococcus salivarius* could not be detected (Table 2).

Association of tetracycline resistance with a transposable element. The absence of detectable plasmid DNA in both strains FC1SS and GF590 together with the ability of these strains to transfer tetracycline resistance in filter mating experiments suggested that the resistance determinant may be located on a transposable element similar to Tn*916*. This possibility was tested by introducing the 58-kb conjugative hemolysin-bacteriocin plasmid pAD1 into strain GF590, by mating it with *S. faecalis* OG1X(pAD1). Strain GF590 hemolytic transconjugants arose at a high frequency (8×10^{-3} per donor), and one such tetracycline-resistant transconjugant was used as a donor in filter matings with an *S. faecalis* JH2SS recipient. After selection on blood agar containing tetracycline, transconjugants were observed at a frequency of 5×10^{-5} per donor. These transconjugants exhibited three different hemolytic phenotypes: ca. 1% were nonhemolytic and another 1% were hyperhemolytic (i.e., the zones of hemolysis around these colonies were two to three times the normal size), whereas the remainder showed the typical hemolysis associated with pAD1. To test for linkage between tetracycline resistance and hemolysin expression, cross-streak matings were done with a representative from all three types of strain JH2SS transconjugants as donors and *S. faecalis* OG1RF as the recipient. In all cases, strain OG1RF transconjugants were obtained on THB blood agar containing tetracycline, implying a physical linkage between pAD1 and the tetracycline resistance element. (Unless linked to the plasmid, the resistance marker transfers at a frequency too low to be detected by the cross-streak mating technique.)

TABLE 2. Transfer of tetracycline resistance by conjugation[a]

Donor	Recipient	Frequency/donor
S. sanguis FC1SS	*S. faecalis* FA2-2	5×10^{-7}
	S. mutans 6715-10RF	$<5 \times 10^{-8}$
	S. mutans Ingbritt RF	$<5 \times 10^{-8}$
	S. mutans GS5RF	$<5 \times 10^{-8}$
	S. sanguis Challis RF	$<5 \times 10^{-8}$
S. faecalis GF590	*S. faecalis* JH2SS	6×10^{-6}
	S. sanguis G9BS	2×10^{-6}
	S. lactis 4125S	1×10^{-8}
	S. mutans 6715-10S	$<5 \times 10^{-8}$
	S. mutans Ingbritt S	$<5 \times 10^{-8}$
	S. mutans 10T2S	$<5 \times 10^{-8}$
	S. mitior 186JS	$<5 \times 10^{-8}$
	S. salivarius 186AS	$<5 \times 10^{-8}$

[a] The transfer experiments involved overnight filter matings (see the text).

Physical analysis of plasmid DNA isolated from transconjugants exhibiting different hemolytic phenotypes. The transfer data strongly suggested that the tetracycline resistance determinant was on an element which could insert into pAD1 and affect hemolysin expression. This was further tested by isolating plasmid DNA from the strain OG1RF transconjugants representing the three different phenotypes and comparing them with pAD1 by restriction enzyme analyses (Fig. 1). The results indicate that there is an insertion of ca. 15 kb in three different locations on pAD1, depending on the phenotype. The *Eco*RI digest of pAD1 resulted in eight visible fragments, A through H (lane 2). In the case of the plasmid isolated from the nonhemolytic transconjugant, OG1RF(pAM551), fragment H was no longer present but a new band, H′, which is ca. 15 kb larger than H, was evident (lane 4; H′ comigrates with the *Eco*RI A fragment). Similarly, pAM552, from a hyperhemolytic transconjugant, had an insertion in the D fragment, resulting in a new D′ band (lane 3). The insertion in the plasmid from the typically hemolytic transconjugant, OG1RF(pAM553), was in the B fragment, yielding a new B′ band (lane 5). These data agree with the previously published restriction map of pAD1 in which the hemolysin gene is known to be located in the region of the *Eco*RI D and H fragments (5). The restriction data also indicate that the tetracycline resistance element does not possess an internal *Eco*RI site, since only one new band was generated when pAD1 derivatives containing the insertion were restricted by this enzyme. The transposable element will henceforth be designated Tn*919*.

Comparison of Tn*919* with Tn*916*. Both the mating results and the restriction data indicate strong similarities between Tn*919* and the previously characterized Tn*916*. For further comparison, plasmids pAM552 and pAM210, carrying Tn*919*

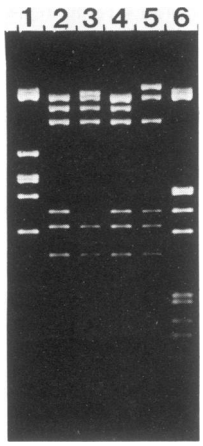

FIG. 1. Agarose gel (0.55%) electrophoresis of *Eco*RI-digested plasmids isolated from strain OG1RF transconjugants exhibiting different phenotypes. Lanes 2, pAD1 (fragments top to bottom are A to H); 3, pAM552 from a hyperhemolytic transconjugant (insertion in *Eco*RI D fragment of pAD1); 4, pAM551 from a nonhemolytic transconjugant (insertion in *Eco*RI H); 5, pAM553 from a typically hemolytic transconjugant (insertion in *Eco*RI B). Lanes 1 and 6 show size standards generated after digestion of lambda DNA with *Eco*RI and *Eco*RI-*Hin*dIII, respectively.

and Tn*916*, respectively, inserted in the *Eco*RI D fragment of pAD1 were examined by restriction endonuclease and Southern hybridization studies. *Hin*cII digests (Fig. 2A) confirmed the high degree of similarity between the two transposons. However, a 4.2-kb *Hin*cII fragment from the Tn*919* digest (lane 3, sixth band from top) is not apparent in Tn*916*, whereas a 4.8-kb fragment in Tn*916* (lane 4, fifth band from top) is absent in the Tn*919* digest. Mapping studies with Tn*916* have revealed that the tetracycline resistance determinant is located on this 4.8-kb fragment (C. Gawron-Burke, personal communication). Those bands present in the pAM552 and pAM210 digests but not in the pAD1 restriction pattern (lane 2) are unique to the respective transposons or are junction fragments containing both pAD1 and transposon DNA. A similarity in junction fragments suggests that both transposons inserted in the same site (or very close) in the *Eco*RI D fragment of pAD1; this is not surprising considering the similar hyperhemolytic phenotype exhibited by both derivatives. Conceivably, there is a "hot spot" for insertion in this region.

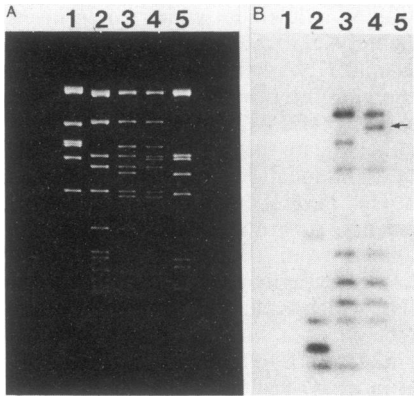

FIG. 2. (A) Agarose gel (0.9%) electrophoresis of *Hin*cII-digested derivatives of pAD1 containing Tn*919* or Tn*916* in the *Eco*RI D fragment. Lanes: 2, pAD1; 3, pAM552 (pAD1::Tn*919* in *Eco*RI D); 4, pAM210 (pAD1::Tn*916* in *Eco*RI D). Lanes 1 and 5 contain size standards generated after digestion of lambda DNA with *Eco*RI and *Eco*RI-*Hin*dIII, respectively. (B) Autoradiogram obtained after Southern transfer of DNA from agarose gel in panel A, followed by hybridization with a ^{32}P-labeled Tn*916* probe. The *Hin*cII fragments in lanes 3 (pAM552) and 4 (pAM210) which do not light up in lane 2 (pAD1) either are junction fragments or are unique to Tn*919* and Tn*916*, respectively. The 4.8-kb *Hin*cII band in the Tn*916* digest (lane 4, arrowed) contains the tetracycline resistance gene. It is noted that the photographs of the two geis are such that the lengths of A and B do not correspond exactly, with A appearing more compressed than B.

Hybridization studies with ^{32}P-labeled pAM170 (11) (i.e., the EcoRI D′ fragment of pAM210 cloned into the pBR322 derived vector pGL101 [22]) as a probe indicated significant sequence homology between the two transposons (Fig. 2B). Of interest was the fact that the unique 4.2-kb *Hin*cII fragment of Tn*919* hybridized strongly with the Tn*916* probe; this suggests a similarity with regard to the sequences of the tetracycline resistance determinants (see above).

Cloning of Tn*919* in *E. coli*. Tn*919*, present in the 1.0-kb *Eco*RI H fragment of pAD1 was isolated from an agarose gel and ligated with *Eco*RI-digested pGL101. After ligation the DNA was used to transform *E. coli* DH1; transformants were selected on ampicillin and tetracycline and arose at a frequency of 300 per μg of DNA. Individual transformants were picked, and their plasmid content was examined. Among the *Eco*RI digestion products of plasmid pAM554 (Fig. 3, lane 3) isolated from one such transformant are the pAD1 H′ fragment (*Eco*RI H::Tn*919* of pAM551) which comigrated with the *Eco*RI A band of pAD1 (lane 2; Fig. 1, lane 4) and a fragment representing the pGL101 vector. In addition, there is a third fragment present in lane 3 which comigrates with the *Eco*RI H fragment of pAD1 (lane 2). We believe that this was generated as a result of the genetic instability and subsequent excision of the transposon in *E. coli*, leaving just pGL101 vector DNA and the original *Eco*RI H fragment from pAD1. Such an occurrence has been observed with Tn*916* (11), whereby clones of this nature harbor a mixed population of plasmid DNA molecules. (Excision of the transposon occurs so readily that it can even be detected in cells grown in the presence of the drug.) This view is supported by the fact that when the stability of the Tn*919*-containing *E. coli* strain was examined by subculturing cells twice in the absence of selective pressure and then plating, 91% of the colonies arising were sensitive to tetracycline but still resistant to ampicillin.

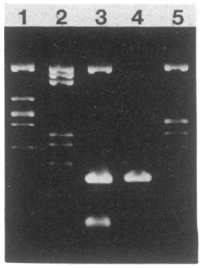

FIG. 3. Agarose gel (0.6%) electrophoresis of plasmid DNA isolated from an *E. coli* DH1 transformant after the cloning of the pAD1 *Eco*RI H::Tn*919* fragment into pGL101. Lane 2, pAD1: the smallest band is the 1.0-kb *Eco*RI H fragment. Lane 3, pAM554: the three bands, beginning with the largest, represent the H′ fragment (i.e., pAD1-*Eco*RI H::Tn*919*), pGL101, and pAD1-*Eco*RI H, respectively. Lane 4 shows linearized pGL101. Lanes 1 and 5 show *Eco*RI and *Eco*RI-*Hin*dIII digests, respectively, of lambda DNA.

DISCUSSION

A conjugative, nonplasmid, tetracycline resistance element has been identified in a strain of *S. sanguis* and has been designated Tn*919*. On the basis of size and extensive homology, Tn*919* closely resembles Tn*916*. The only detectable difference was in the size of one internal *Hin*cII fragment. This fragment, believed to contain the tetracycline resistance determinant, was smaller (4.2 versus 4.8 kb) in Tn*919*. In addition, Tn*919* was found to insert at multiple sites on pAD1, and a fragment of pAD1 containing a Tn*919* insert was cloned in *E. coli*. Like similar clones of Tn*916*, Tn*919* was found to be very unstable in the *E. coli* background. (This is in contrast to a high stability exhibited in *S. faecalis*.)

The identification in *S. sanguis* of a transposon (Tn*919*) closely resembling Tn*916* implicates a common origin for these elements. Together with the recent observation of a third transposon (Tn*918*) originating in *S. faecalis* RC73, again with properties similar to Tn*916* (D. B. Clewell, F. An, B. White, and C. Gawron-Burke, *in* D. Helinski, S. Cohen, D. Clewell, D. Jackson, and A. Hollaender, ed., *Plasmids in Bacteria*, in press), the data imply that Tn*916*-like elements are probably broadly distributed among strains of streptococci and have disseminated from a common ancestor. It is noteworthy that larger segments of DNA, carrying multiple resistance determinants, originating in *Streptococcus agalactiae* and *Streptococcus pyogenes* also have been observed to insert into pAD1 (23, 32), but a relationship to Tn*916* has not yet been established. Recent studies in our laboratory showing that Tn*916* can be transferred into *S. mutans*, *S. agalactiae*, and *S. lactis* (D. B. Clewell, G. Fitzgerald, L. Dempsey, L. Pearce, F. An, B. White, Y. Yagi, and C. Gawron-Burke, *in* S. Mergenhagen and B. Rosan, ed., *Molecular Basis of Oral Microbial Adhesion*, in press) and a report by Nida and Cleary (25) showing transfer into *S. pyogenes* attest to the broad host range exhibited by this type of element.

Burdett et al. (1) have reported that on the basis of hybridization studies the tetracycline resistance determinant of Tn*916* corresponds to the *tetM* class; it follows that the resistance determinant of Tn*919* (and Tn*918*) is similar. It is noteworthy that other chromosomal tetracycline resistance determinants observed in *Streptococcus pneumoniae* (33), *S. agalactiae* (33), *S. mutans* (35), and several *S. sanguis* strains (13), including the conjugative element described by Hartley et al. (13), fall within the *tetM* class.

The hyperhemolytic phenotype resulting from certain insertions of Tn*919* into pAD1 resembles a behavior now known to occur in several other systems. Tn*916* (9), Tn*918* (Clewell et al., *Plasmids in Bacteria*, in press), and an element conferring both erythromycin and tetracycline resistance originally identified in *S. pyogenes* (23) all have been observed to generate similar insertions into pAD1 when present on the chromosome of *S. faecalis*. In addition, a different tetracycline resistance element has been observed to generate similar insertions into a related hemolysin plasmid, pJH2 (22). The basis of the hyperhemolytic phenomenon is not known. If it represents the use of a transposon-related promoter, it would appear that similar promoters are present in many conjugative transposable elements. Further, the relative ease with which such insertions are generated suggests that the site in or near the hemolysin gene(s) represents a hot spot for recombination with such elements. It is noteworthy that this type of hyperhemolytic phenotype has never been observed as a result of insertions of the more conventional transposon Tn*917* (erythromycin), despite the fact that many inserts of the latter, spanning much of pAD1 (including the *Eco*RI D fragment), have been derived (5, 17).

ACKNOWLEDGMENTS

We thank our laboratory colleagues for their help and advice, notably R. Kessler, F. An, B. Erhenfeld, S. Flannagan, L. Pearce, J. Shaw, B. White, and R. Wirth. We also are grateful to C. Gawron-Burke for many helpful discussions and for sharing unpublished data.

This work was supported by Public Health Service grants DE02731 and AI10318 from the National Institutes of Health.

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