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Mapping of *Streptococcus faecalis* Plasmids pAD1 and pAD2 and Studies Relating to Transposition of Tn*917*

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Plasmids pAD1 (37.8 megadaltons) and pAD2 (17.1 megadaltons) of *Streptococcus faecalis* strain DS16 have been mapped with restriction enzymes. The location of a hemolysin-bacteriocin determinant on the conjugative pAD1 plasmid was derived from analyses of transposon insertions. Electron microscope and hybridization analyses located Tn*917*(Em) and the streptomycin (Sm) and kanamycin (Km) resistance determinants on the nonconjugative pAD2 plasmid. It was shown previously that the erythromycin (Em) resistance associated with Tn*917* is inducible and that transposition from pAD2 to pAD1 is also stimulated by exposure of cells to low concentrations of Em. Here we show that inducing concentrations of Em also increase the conjugative transfer potential of pAD1; this is possibly related to a mild and short-lived inhibitory stress placed on the cells before full induction of resistance. Selection of Em-resistant transconjugants arising from matings between DS16 and a plasmid-free recipient gave rise to transconjugants which primarily harbor stable pAD1::pAD2 cointegrates. A 30-min exposure of donors to Em (0.5 μg/ml) before mating resulted in a severalfold increase in the number of such transconjugants. However, a small fraction (e.g., 3 of 40) of these Em^r Sm^r Km^r transconjugants harbored pAD1::Tn*917* and pAD2 molecules. Since we believe pAD2 is incapable of being mobilized by pAD1 without being covalently linked, it is likely that transfer in these cases involved cointegrates representing structural intermediates in the transposition of Tn*917* from pAD2 to pAD1. It follows that such intermediates probably had two copies of Tn*917* and readily resolved after transfer. (These cointegrates are different from the stable cointegrates which were shown to have only a single copy of Tn*917*; the latter are assumed not to be related to transposition.) Two variants with altered Tn*917* transposition properties were derived. One of them transposed at an elevated frequency, whereas the other showed no detectable transposition. In neither case was transposition influenced by Em exposure; however, both remained inducible for Em resistance.

Recent reports from our laboratory have dealt with a multiple-drug-resistant, hemolytic strain of *Streptococcus faecalis* (designated DS16) harboring two plasmids (4, 30, 31). One of the plasmids, pAD1 (37.8 megadaltons [Mdal]), determines a hemolysin-bacteriocin and a conjugative response to the sex pheromone cAD1. The other plasmid, pAD2 (17.1 Mdal), is nonconjugative and encodes resistance to streptomycin (Sm), kanamycin (Km), and the MLS antibiotics (macrolides, lincosamides, and streptogramin Bα). The MLS resistance determinant is located on a 3.3-Mdal transposon designated Tn*917* (29, 30). MLS resistance is inducible; however, exposure to low concentrations of erythromycin (Em) also induces transposition. The latter is detectable through the acquisition of the transposon by the coresident, highly conjugative pAD1 (30).

Strain DS16 also harbors a second transposon, Tn*916*, located on the chromosome. This 10-Mdal element determines tetracycline resistance and has been shown to insert into pAD1 and other hemolysin plasmids (12, 13), in some cases giving rise to a hyperhemolytic phenotype. Tn*916* also has the intriguing ability to undergo plasmid-independent conjugal transfer (13).

In this report restriction maps of pAD1 and pAD2 are presented along with the locations of key markers. In addition, we provide further information on the nature of Tn*917* transposition

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as well as pAD1::pAD2 cointegrate structures which transfer at elevated frequencies after exposure to inducing concentrations of Em.

MATERIALS AND METHODS

Bacteria, media, and reagents. The *S. faecalis* strains used in this study are listed in Table 1. Antibiotic medium no. 3 (Difco Laboratories) was used for the broth matings and in selective agar plates. Cross-streak matings were carried out on antibiotic medium no. 3 agar. Hemolysin production was observed on Todd-Hewett (Difco) agar plates containing 4% horse blood (Colorado Serum Co.). In the preparation of plasmid DNA for analyses by agarose gel electrophoresis or sucrose density gradient centrifugation, the cells were grown in M9-yeast extract medium (33). Broth cultures were grown at 37°C and monitored with a Klett-Summerson colorimeter (no. 54 filter).

When present in selective plates, antibiotics were used at the following concentrations: Em, 50 μg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; Sm, 1,000 μg/ml; Km, 500 μg/ml; and spectinomycin, 500 μg/ml.

The sources of most of the reagents used in this study were as previously described (6, 10, 13, 30). Spectinomycin was a gift from The Upjohn Co.; novobiocin was from Sigma Chemical Co.; and φX174 DNA, lambda DNA, and all restriction enzymes were from Bethesda Research Labs.

Mating procedures. Broth matings were performed as previously described (10), using a ratio of 1:10 (donors/recipients). In matings between DS16 and JH2-2, pAD1 transfers at 10^{-3} to 10^{-1} per donor; determinants on pAD2 are usually mobilized at 10^{-8} to 10^{-7}. In some cases cross-streak matings were performed to determine whether specific resistance determinants transferred at high frequency. Donor cells to be tested were streaked across a perpendicular streak of recipient cells. After growth overnight on drug-free agar, cells in the intersection were streaked onto selective plates. If the determinant being monitored was linked to the conjugative pAD1 plasmid, many colonies were readily apparent, whereas few or no colonies implied an absence of linkage.

Transposition experiments. To obtain derivatives of pAD1 which had received Tn*917* from pAD2, DS16 cells grown in an inducing concentration of Em (generally 0.5 μg/ml) for 4 h were washed and mated with JH2-2 cells for 4 h. After selection on plates containing rifampin, fusidic acid, and Em, transconjugants were purified and tested for sensitivity to Km or Sm or both. Strains which were hemolytic, resistant to Em, and sensitive to Km and Sm were candidates for harboring pAD1::Tn*917* derivatives. Generally, these strains would donate Em resistance at high frequency in subsequent matings. Plasmid DNA used in restriction enzyme analyses was isolated from the JH2-2 transconjugants or, in some cases, from JH2SS or OG1SS transconjugants derived from subsequent matings. Cointegrate structures were obtained similarly, except that exposure of DS16 cells to Em was for only 30 min.

In cases in which quantitative results were desired,

TABLE 1. *S. faecalis* strains used in this study

Strain	Chromosomal genotype[a]	Plasmid content	Construction	Reference
DS16	*tet*	pAD1 (Hem-Bac),[b] pAD2 (Em^r Km^r Sm^r)	Clinical isolate	31
DS16C1	*tet*	pAD2	Cured of pAD1	13
DS16C2	*tet*	pAD1	Cured of pAD2	13
DS16C3	*tet*	None	Cured of pAD1 and pAD2	13
JH2-2	*rif fus*	None	Derivative of JH2	20
FA2-2	*rif fus*	None	Derivative of JH2	This study
JH2SS	*str spc*	None	Derivative of JH2	30
OG1SS	*str spc*	None	Derivative of OG1	13
FA301	*rif fus*	pAD1::Tn*917* (pAM301)	DS16 × JH2-2	This study
FA307	*rif fus*	pAD1::Tn*917* (pAM307)	DS16 × JH2-2	This study
FA308	*rif fus*	pAD1::Tn*917* (pAM308)	DS16 × JH2-2	This study
FA310	*rif fus*	pAD1::Tn*917* (pAM310)	DS16 × JH2-2	This study
FA330	*rif fus*	pAD1::pAD2 (pAM330)	DS16 × JH2-2	This study
FA332	*rif fus*	pAD1::pAD2 (pAM332)	DS16 × JH2-2	This study
FA333	*rif fus*	pAD1::pAD2 (pAM333)	DS16 × JH2-2	This study
FA334	*rif fus*	pAD1::pAD2 (pAM334)	DS16 × JH2-2	This study
FA7	*rif fus*	pAD1::Tn*917* (pAM311), pAD2	DS16 × JH2-2	This study
FA13	*rif fus*	pAD1::Tn*917* (pAM312), pAD2	DS16 × JH2-2	This study
FA16	*rif fus*	pAD1::Tn*917* (pAM313), pAD2	DS16 × JH2-2	This study
FA330S	*str spc*	pAM330	FA330 × JH2SS	This study
FA334S	*str spc*	pAM334	FA334 × JH2SS	This study
FA3714	*str spc*	pAD1::Tn*917* (pAM714)	OG1RF (pAM714) × JH2SS	From Y. Ike
FA3001	*tet*	pAD1, pAD2	DS16 variant with elevated transposition	This study
FA3002	*tet*	pAD1, pAD2	DS16 variant with reduced level of transposition	This study

[a] *tet*, Tetracyline; *rif*, rifampin; *fus*, fusidic acid; *str*, streptomycin; *spc*, spectinomycin.
[b] Hem-Bac, Hemolysin-bacteriocin.

the extent of transposition was expressed as a percentage based on the number of Em^r Km^s Sm^s transconjugants divided by the total number of Em^r transconjugants (30).

Restriction enzyme analyses. Digestion with restriction enzymes was carried out under conditions specified by the manufacturer. Physical mapping made use of double and partial digestions with appropriate restriction enzymes. The general strategy for mapping pAD1 has been outlined in detail elsewhere (A. Franke, Ph.D. thesis, University of Michigan, Ann Arbor, 1980), and the approach used for pAD2 was essentially the same. Electrophoresis on agarose gels has also been described previously (13). Analyses of pAD1 and pAD2 were performed on plasmid DNA isolated from strains DS16C2 and DS16C1, respectively. In some cases pAD1 was introduced into OG1SS before isolation.

Other procedures involving the isolation and analyses of plasmid DNA. The isolation of plasmid DNA (radioactively labeled or otherwise) was by equilibrium centrifugation of crude Sarkosyl lysates in ethidium bromide-CsCl gradients essentially as described elsewhere (6). In cases where plasmid DNA was used for restriction enzyme analyses, pooled satellite (plasmid) DNA was centrifuged a second time in dye-buoyant density gradients. The analysis of plasmid DNA on alkaline sucrose density gradients was also as previously described (5), as was the examination of DNA fragments by electron microscopy (26, 33). In the latter procedure, single-stranded circular molecules of φX174 molecules were used as a standard. Cloning of pAD2 determinants in the *S. sanguis* vector system was as described by Macrina et al. (22). Filter-blot hybridization was as described by Southern (28), as modified by Wahl et al. (32).

Curing experiments involved the overnight growth of cells in the presence of novobiocin (32 μg/ml).

RESULTS

Physical map of pAD2 and location of Tn*917*. From analyses of pAD2 with several restriction enzymes the map shown in Fig. 1 was deduced. Tn*917* was located on the basis of electron microscopic analyses (Fig. 2) of self-annealed restriction fragments (the single *Bam*HI fragment and *Eco*RI fragment A). On the basis of contour lengths of fragments similar to those shown in Fig. 2, Tn*917* measured 3.28 ± 0.25 (standard deviation) Mdal (5.1 kilobases), with the inverted repeats estimated at 0.20 ± 0.07 Mdal (0.31 kilobase) (nine molecules measured; the masses correspond to those of double-stranded structures). These measurements are in close agreement with earlier reported measurements (30). The larger pair of inverted repeats, occurring on the opposite side of the molecule, measured 0.94 ± 0.09 Mdal (1.29 kilobases) (nine molecules measured), a value also close to that previously observed (30). These repeats flank a segment measuring about 1.77 kilobases.

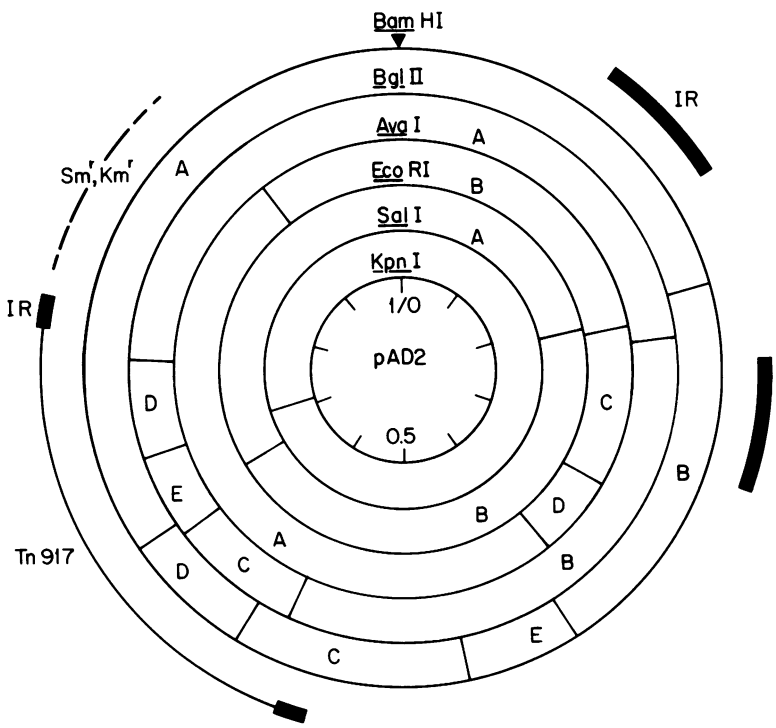

FIG. 1. Physical map of pAD2. *Bam*HI, *Bgl*II, *Ava*I, *Eco*RI, *Sal*I, and *Kpn*I sites were mapped on the basis of double and partial digestion analyses with appropriate enzymes. The locations of Tn*917* and the Sm^r and Km^r determinants were derived from electron microscopic analyses (Fig. 2) and hybridization studies (Fig. 3).

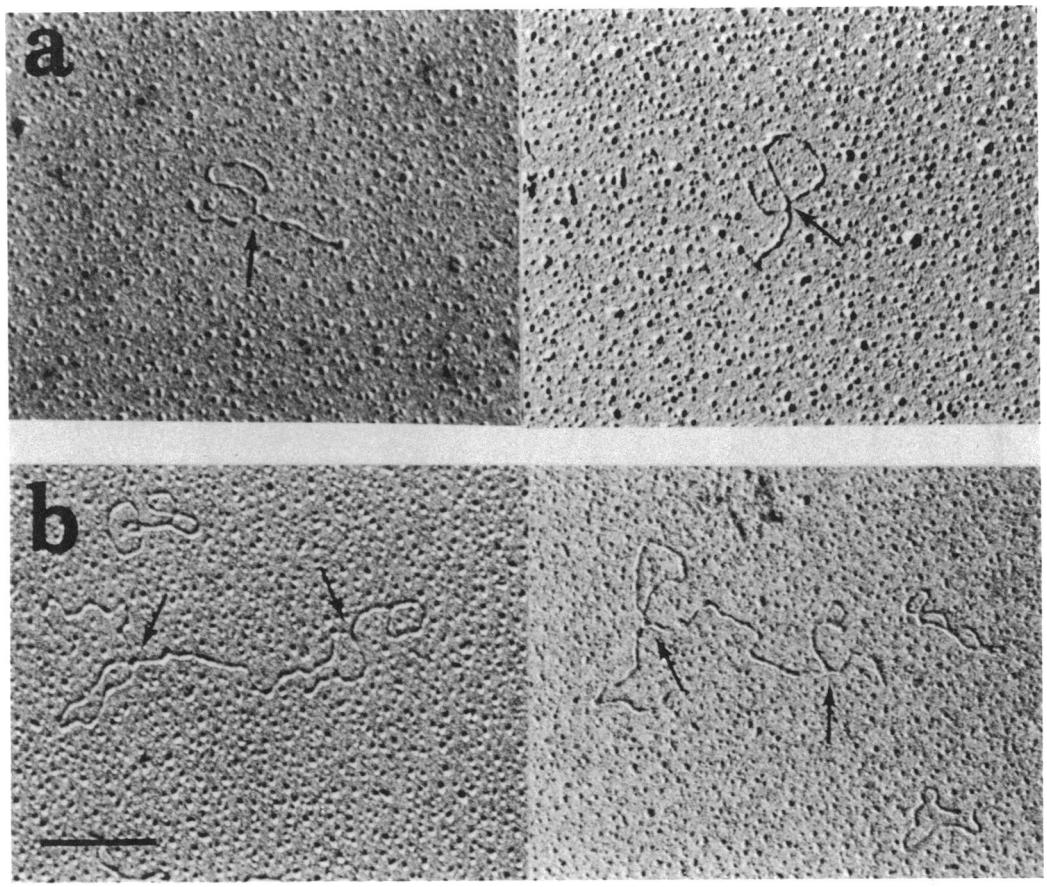

FIG. 2. Electron micrographs of self-annealed restriction fragments of pAD2. (a) *Eco*RI fragment A. (b) *Bam*HI fragment. The small circular molecules represent single-stranded ϕX174 molecules used as a standard. The arrows point to the "stems" corresponding to annealed inverted repeats. The short stem is associated with Tn*917*. Bar, 1 μm.

Whereas previous observation of self-annealed pAD2 molecules detected two small loops (1.7 and 0.47 kilobases) coming from this stem (30), only a single loop was observed here. Insofar as the present study involved a different single-colony isolate of DS16C1, it is possible that a spontaneous deletion of DNA corresponding to the smaller loop had occurred.

The location of the Sm^r and Km^r determinants followed the generation of recombinant clones specifying these markers, using the *S. sanguis* vector pVA736(Em^r) (5.0 Mdal) (22). Cleavage of pAD2 and pVA736 with *Eco*RI (pVA736 has a single *Eco*RI site) followed by ligation and transformation into *S. sanguis* strain Challis, selecting in one case for Em and Sm resistance and in another case for Em and Km resistance, resulted in two useful chimeric plasmids. One of them, pAM510 (6.2 Mdal), contained the Sm resistance determinant (but did not exhibit Km resistance), whereas pAM511 (6.5 Mdal) contained the Km resistance determinant but did not exhibit Sm resistance. Plasmid transformation of *S. sanguis* readily results in deletions (2, 22, 23); thus, retransformation with pAM510 and pAM511 gave rise to derivatives with deletions of the Em^r determinant. Selected isolates were called pAM5101 (5.2 Mdal) and pAM5111 (5.6 Mdal), respectively. Using the latter plasmids as probes in filter-blot hybridizations to pAD2 DNA cleaved with *Bgl*II, *Eco*RI, and *Ava*I, we obtained the data of Fig. 3. Hybridization only to *Bgl*II fragment A, *Ava*I fragment A, and *Eco*RI fragment A was observed for both probes. The Sm^r and Km^r determinants must therefore be located in the region between 0.8 and 0.9 on the pAD2 map shown in Fig. 1. With the pVA736 vector as a probe, no significant homology with pAD2 was observed (data not shown), implying that the Em^r determinant of Tn*917* and that of pVA736 are quite different. The Em^r determinant of pVA736 originally came from pAMβ1 (22).

Physical map of pAD1 and insertions of Tn*917*.

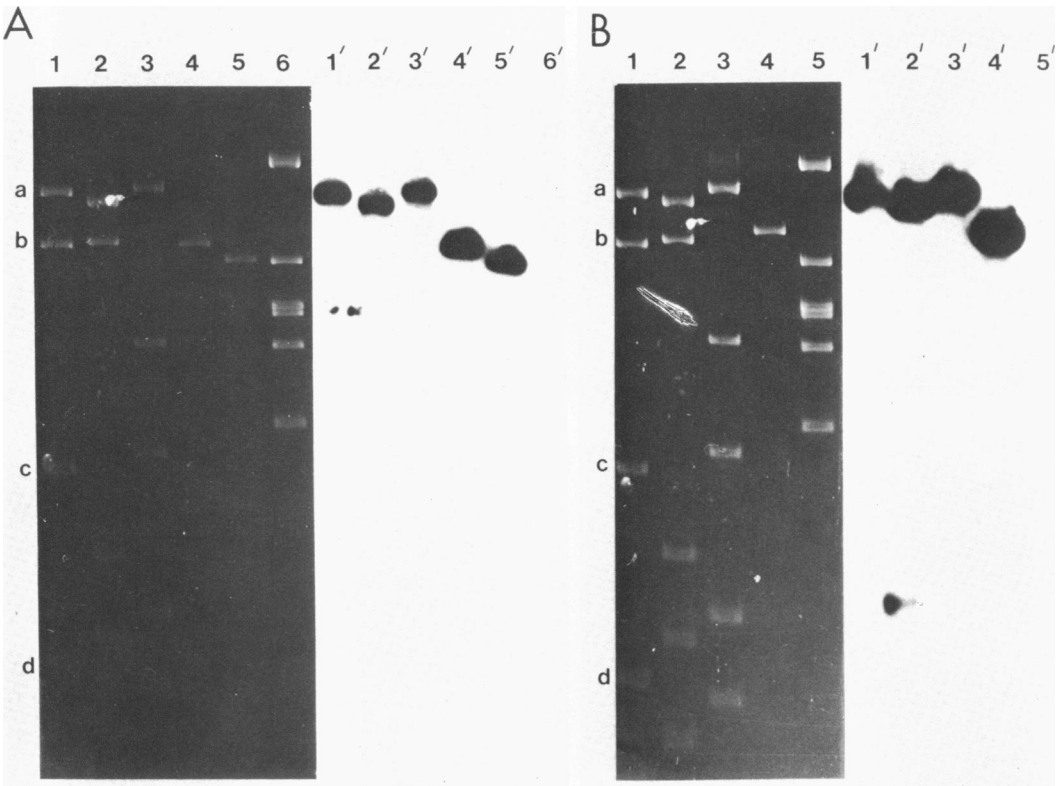

FIG. 3. Localization of the Sm and Km resistance determinants on the physical map of pAD2. (A) Lanes 1 to 6 depict agarose gel electrophoresis of plasmid DNA digests as visualized by ethidium bromide stain. Lanes 1 to 3 contain pAD2 DNA digested with *Eco*RI, *Ava*I, and *Bgl*II restriction endonucleases, respectively. Lanes 4 to 6 contain *Eco*RI digests of pAM5101, pVA736, and lambda DNA, respectively. The *Eco*RI fragments (A to D) of pAD2 are marked in lane 1. Lanes 1′ to 6′ illustrate the autoradiogram obtained from a Southern blot of this gel after its hybridization with ^{32}P-labeled pAM5101 (Sm^r Em^s) DNA. Note that the pAD2 *Eco*RI-A, *Ava*I-A, and *Bgl*II-A fragments hybridized to probe as did the positive controls of pAM5101 and pVA736 plasmid DNAs. (B) Lanes 1 to 5 depict agarose gel electrophoresis of plasmid DNA digests as visualized by ethidium bromide stain. Lanes 1 to 3 contain pAD2 DNA digested with *Eco*RI, *Ava*I, and *Bgl*II, respectively. Lanes 4 and 5 contain *Eco*RI-digested pAM5111 and lambda DNA, respectively. Lanes 1′ to 5′ illustrate the autoradiogram obtained from a Southern blot of this gel after its hybridization with ^{32}P-labeled pAM5111 (Km^r Em^s) DNA. Note that the *Eco*RI-A, *Ava*I-A, and *Bgl*II-A fragments of pAD2 as well as the positive control of pAM5111 DNA show significant hybridization.

Figure 4 shows a physical map of pAD1 with an approximate positioning of the hemolysin-bacteriocin determinant at about 0.54 to 0.62. The location of the latter was based on effects exhibited by insertions (Fig. 5; Table 2) of both Tn*917* and Tn*916* (from reference 13). Insertion of Tn*917* into *Eco*RI fragment H and Tn*916* into *Eco*RI fragment F resulted in no expression of hemolysin. Insertion of Tn*916* into *Eco*RI fragment D frequently (but not always) resulted in hyperexpression of hemolysin (13; Franke, unpublished data). An insertion derivative designated pAM113 lost (deleted) *Eco*RI fragments F and H, whereas fragments C and D were fused (data not shown). This derivative did not express hemolysin, and Tn*917* (or at least the Em^r determinant) is believed to be located on the C-D fusion fragment.

Whereas Tn*917* was previously reported to insert into several different plasmids (30), we had not shown whether it would insert at more than one site on pAD1. The data of Fig. 5 show that Tn*917* can insert into at least four different *Eco*RI restriction fragments. In each case, a new fragment appeared with a size approximately corresponding to that of the "missing" fragment plus the size of Tn*917*. Of 10 independent isolates examined, 7 harbored Tn*917* in the *Eco*RI-B fragment, whereas one insertion into each of fragments D, G, and H was observed.

Em-induced enhancement of resistance transfer. As reported previously (30), exposure of

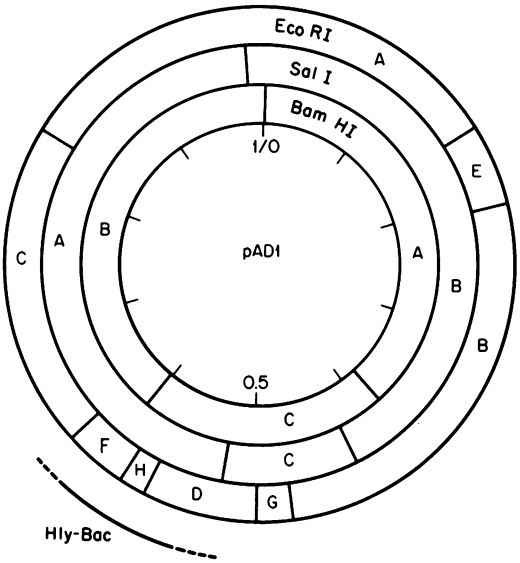

FIG. 4. Physical map of pAD1. *Eco*RI, *Bam*HI, and *Sal*I sites were mapped on the basis of double and partial digestion analyses with appropriate enzymes. Hly-Bac, Hemolysin-bacteriocin determinant.

DS16 cells to an inducing concentration of Em for 30 or 60 min before a mating with the plasmid-free strain of JH2-2 resulted in a six- to sevenfold increase in the frequency (per donor) of linked transfer of the Em^r, Sm^r, and Km^r determinants; the transconjugants harbored cointegrate structures. After a 4-h induction, however, the frequency of Em-resistant transconjugants (a significant proportion of which are now sensitive to Sm and Km) was usually not significantly higher than the case for donors induced for only 30 min. We therefore examined the kinetics of transfer enhancement during the first 30 min of induction. An increase in the potential to transfer Em resistance was found to occur almost immediately after Em exposure and appeared to reach the six- to sevenfold maximum within about 20 min. Results of a typical experiment are shown in Fig. 6.

On the structure of the cointegrate plasmid DNA induced to transfer by Em. Previously, we suspected that the cointegrate structures that transferred after a 30- to 60-min exposure to Em might relate to the formation of intermediates in the transposition of Tn*917* (30) which, for reasons not clear, failed to resolve. The involvement of cointegrate intermediates in transposition had been reported in other systems (1, 14, 18, 24, 25), and such intermediates were shown to contain two copies of the transposon (with the same orientation) located at the two plasmid-plasmid junction sites. As shown below, this proved not to be the case for the structures which establish as stable cointegrates in recipient cells.

The data of Fig. 7 show *Eco*RI digests of four cointegrate structures obtained from four independent induction experiments and show that in three cases (lanes c, d, and e) cointegration involved the B fragment of pAD2 and the G fragment of pAD1. The sum of two new fragments (marked with arrows) corresponds to the

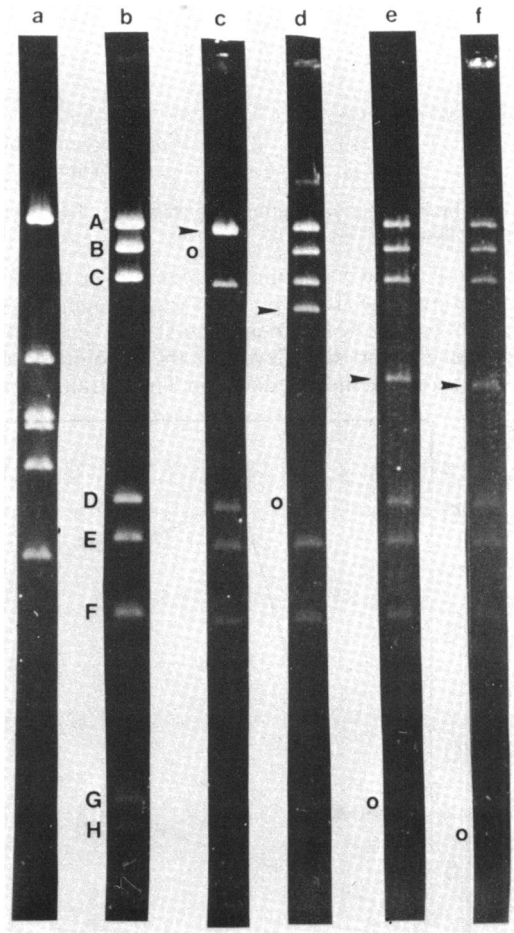

FIG. 5. Agarose gel electrophoresis of fragments of pAD1::Tn*917* DNA generated by *Eco*RI endonuclease digestion. (A) Lambda DNA standard cut with *Eco*RI. (b) pAD1 fragments A through H with corresponding molecular masses of 12.5, 10.0, 7.8, 2.57, 2.15, 1.57, 0.7, and 0.5 Mdal. (c) pAM301; the B fragment is missing and a new band was found superimposing the A fragment. (d) pAM308; the D fragment is missing and replaced by a 6.0-Mdal fragment. (e) pAM310; the G fragment is missing and replaced by a 4.2-Mdal fragment. (f) pAM307; the H fragment is missing and replaced by a 4.0-Mdal fragment. The arrows point to the "new" band; "o" indicates the absent fragment. (pAM308 and pAM310 were isolated after transfer into JH2SS and OG1SS hosts, respectively; pAM301 and pAM307 were isolated from JH2-2 hosts.)

TABLE 2. Effect of insertions on pAD1 hemolysin expression

Transposon	Representative derivative	*Eco*RI fragment inserted into:	Phenotype
Tn*917*	pAM301	B	Hemolytic
	pAM308	D	Hemolytic
	pAM310	G	Hemolytic
	pAM307	H	Nonhemolytic
	pAM113	C-D fusion fragment (F and H missing)	Nonhemolytic
Tn*916*[a]	pAM250	B	Hemolytic
	pAM210	D	Hyperhemolytic
	pAM211	F	Nonhemolytic

[a] The information relating to Tn*916* was taken from reference 13.

sum of the two missing fragments. The fourth structure (lane f) also involves the B fragment of pAD2 but the C fragment of pAD1. These data do not support the view that these cointegrate structures are intermediates in Tn*917* transposition. Tn*917* is located in the *Eco*RI-A fragment; if the cointegrate structures represented transposition intermediates, this fragment should have been absent from these structures. This was clearly not the case. Furthermore, since Tn*917* contains a single *Sal*I site, an additional

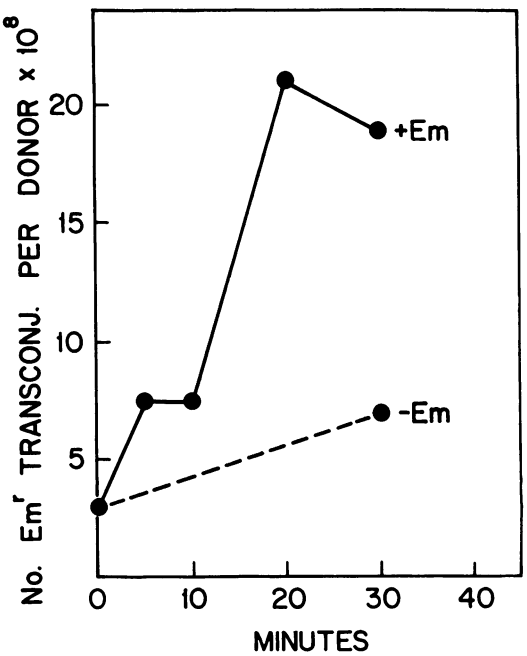

FIG. 6. Enhancement of potential to transfer resistance during the first 30 min of exposure to Em. A 40-ml mid-log culture of DS16 cells was divided into 30- and 10-ml portions. Em (0.5 μg/ml) was added to the 30-ml portion; the 10-ml portion served as a control. Samples (5 ml) were removed and placed on ice at 0, 5, 10, 20, and 30 min. (The sample not exposed to Em was put on ice after 30 min.) The cells were washed and mated with strain JH2-2 for 4 h, after which the culture was plated on selective medium containing rifampin, fusidic acid, and Em.

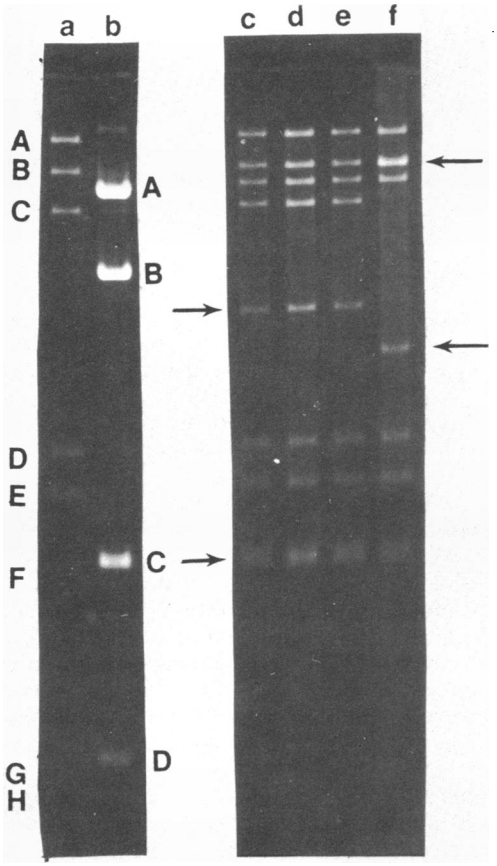

FIG. 7. Agarose gel electrophoresis of *Eco*RI-generated fragments of cointegrate structures transferred to JH2-2 from DS16 cells exposed to Em (0.5 μg/ml) for 30 min. (a) pAD1; (b) pAD2 (fragments A through D correspond to 9.0, 5.7, 1.7, and 0.7 Mdal); (c, d, e, f) stable cointegrates pAM330, pAM332, pAM333, and pAM334, respectively. The arrows left of lane c point to the position of the new bands (at 4.4 and 1.7 Mdal) appearing in the cointegrates of lanes c, d, and e. One of these bands is part of a "triplet" also containing fragment C of pAD2 and fragment F of pAD1. The two new bands replaced fragment G of pAD1 and fragment B of pAD2. (Note that the D fragment of pAD2 migrates in the same place as the G fragment of pAD1.) The arrows to the right of lane f point to the two new bands (about 10.0 and 3.8 Mdal) of pAM334 which replace fragment C of pAD1 and fragment B of pAD2. (Note that the higher-molecular-weight new band superimposes pAD1 fragment B. Fragments G and H are difficult to see here because of the low concentrations of DNA; their existence is seen in Fig. 5.)

*Sal*I site would be predicted in the cointegrate structures if indeed two copies of Tn*917* were present. This was not the case (data not shown).

On the stimulation of cointegrate transfer by Em. In an effort to explain the basis of Em-enhanced transfer of stable cointegrate structures, we considered the possibility that inhibiting effects of the drug could in some way be related. We note, in this regard, that whereas 0.5 μg of Em per ml quickly induces a resistance to >2 mg of the drug per ml, the initial exposure to this low concentration results in some decrease in growth rate; growth reaches its original rate within 45 min.

Two strains, FA330S and FA334S, harboring the stable cointegrate structures pAM330 and pAM334, respectively, strain FA3714 harboring pAM714 (pAD1::Tn*917*), and strain DS16 were exposed to 0.5 μg of Em per ml for 60 min. The cells were then washed and used as donors in matings with strain FA2-2 (plasmid-free). Exposure to Em gave rise to a >10-fold increase in the transfer of plasmid DNA (Table 3). It is, therefore, likely that the observed enhancement of cointegrate transfer afforded by Em exposure in the case of strain DS16 represented a transfer enhancement of rare pAD1::pAD2 cointegrate structures already present before exposure to the drug. That the transfer frequencies in the case of DS16 donors are about three orders of magnitude lower than for the donors harboring only cointegrates (Table 3) suggests that the cointegrates are present in about 0.1% of the DS16 population.

TABLE 3. Em-induced conjugal transfer of cointegrate structures and pAD1::Tn*917*[a]

Strain	Plasmid content	Em concn (μg/ml) (exposed for 1 h)	No. of Em-resistant transconjugants per donor after mating with FA2-2
FA330S	pAM330 (pAD1::pAD2)	0	2.5×10^{-4}
		0.5	5.0×10^{-3}
FA334S	pAM334 (pAD1::pAD2)	0	3.5×10^{-4}
		0.5	3.8×10^{-3}
FA3714	pAM714 (pAD1::Tn*917*)	0	4.1×10^{-4}
		0.5	5.4×10^{-3}
DS16	pAD1, pAD2	0	1.8×10^{-7}
		0.5	2.4×10^{-6}

[a] Cultures of mid-log cells were divided into two equal portions. One portion was exposed to Em, whereas the other served as a parallel control. After 1 h the cells were washed by centrifugation, mated with recipients for 2 h, and plated on media containing Em, rifampin, and fusidic acid.

On the nature of Tn*917* transposition. Although the above-noted stable cointegrates do not appear to be intermediates in the transposition process, it is possible that short-lived intermediates with two copies of Tn*917* are indeed involved in the generation of pAD1::Tn*917* derivatives and could transfer, as such, before resolution in the recipient. In a search for strains that may have resolved such cointegrates into two plasmids, 40 transconjugants from two independent 30-min induction experiments were used as donors in cross-streak matings with the plasmid-free JH2SS (resistant to Sm and to spectinomycin). Selection was carried out with plates containing spectinomycin and either Km or Em. (The Sm^r determinant of JH2SS is a mutational marker which masks the Sm^r determinant of pAD2.) The purpose here was to detect donors which readily transfer the Em^r determinant, but transfer the Km^r determinant poorly or not at all. Although this approach is not quantitative, it worked well in distinguishing cases where transferable Em resistance was no longer linked to Km resistance. Three strains (FA7, FA13, and FA16) were found to transfer Em resistance without transferring Km resistance. Analysis by alkaline sucrose density gradient centrifugation demonstrated the presence of two plasmids in each of these three strains. One of the plasmids sedimented slightly faster than pAD1, whereas the other sedimented exactly the same as pAD2 (Fig. 8A). This was the case for all three strains. (Note difference from Fig. 8B showing a cointegrate structure.) Examination of an Em-resistant (but Km-sensitive) transconjugant which had acquired DNA from FA7 revealed a single plasmid, and restriction analysis indicated that Tn*917* had inserted into the *Eco*RI-B fragment of pAD1 (data not shown). By exposing strains FA7, FA13, and FA16 to novobiocin and screening for hemolytic negative derivatives, it was possible in all cases to obtain strains cured of pAD1::Tn*917*; the latter were still resistant to Em and maintained the smaller of the two plasmids. Analysis of this plasmid with *Eco*RI revealed the expected fragment profile of pAD2. Thus, Tn*917* was still located on pAD2.

It appears, therefore, that in the case of strains FA7, FA13, and FA16 transfer may have occurred as a cointegrate intermediate which subsequently resolved into two plasmids, each with a copy of Tn*917*. Alternatively, resolution may have occurred before transfer, with pAD2 being mobilized through a re-cointegration with pAD1::Tn*917* (afforded by the Tn*917* homology). This seems less likely, however, since without selection for the Km^r or Sm^r determinant detec-

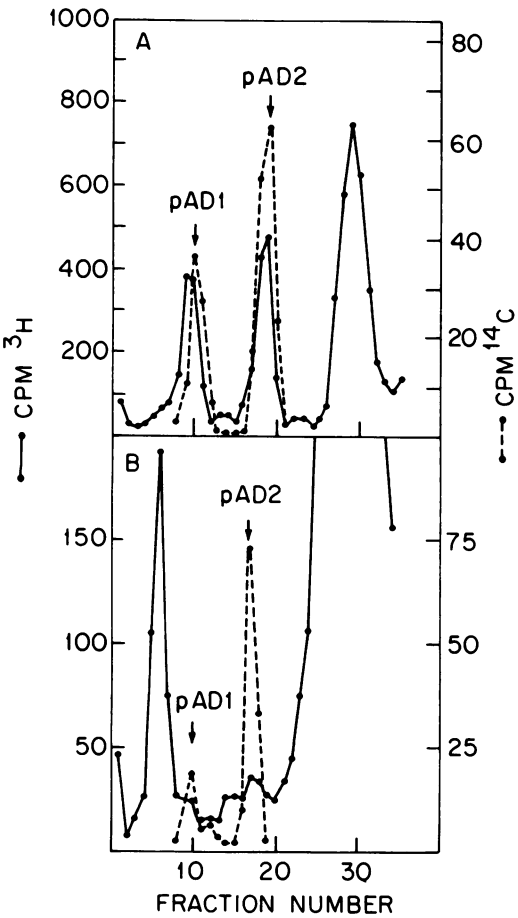

FIG. 8. Sucrose density gradient sedimentation of plasmid DNA isolated from strains FA16 and FA332. Sedimentation (from right to left) was through a 5 to 20% alkaline sucrose density gradient in an SW50.1 (Beckman) rotor at 45,000 rpm for 30 min at 15°C. ^{3}H-labeled plasmid DNAs were from strains FA16 (A) and FA332 (B); the ^{14}C-labeled DNA was isolated from DS16. The large peak near the top of the gradient represents nicked (denatured) forms of pAD1 and pAD2.

tion of transconjugants containing pAD2 and pAD1::Tn*917* should be less frequent than transconjugants receiving pAD1::Tn*917* alone. In the above experiment which randomly selected 40 Em-resistant transconjugants from the DS16 × JH2-2 matings, all were resistant to Sm and Km.

Whereas 3 of the 40 transconjugants harbored pAD1::Tn*917* and pAD2, the other 37 strains maintained stable cointegrates; examination by cross-streak analysis of 50 subisolates from each of two stable strains (FA330 and FA332) after several passages failed to reveal any cases in which Em resistance could be transferred without Km resistance. Transfer of the cointegrate structures of FA332 and FA333 into DS16C3 (i.e., a DS16 background) did not lead to resolution. Examination of 20 such transconjugants in subsequent matings with JH2-2 showed that Em resistance remained linked to Sm and Km resistance.

Variants with altered transposition behavior. In carrying out numerous experiments involved with the induction of Tn*917* transposition by Em, we picked up an occasional isolate of DS16 which exhibited aberrant behavior. In one case, a variant designated FA3001, a high level of transposition was observed without exposure to Em, whereas another derivative, designated FA3002, exhibited no detectable transposition even after exposure to Em. These properties are seen in Table 4, where they are contrasted with the more typical behavior of DS16. The properties of FA3001 and FA3002 were still exhibited in isolates derived from subcultures and are, thus, relatively stable. Interestingly, both strains remained inducible (30) with regard to Em resistance (data not shown).

The frequency at which the above variants appear is difficult to estimate. The nontransposable type appeared in 1 of 20 colonies of DS16 selected for transposition induction experiments. Variants exhibiting the high level of transposition in the absence of Em were seen more frequently. Colonies selected after several weeks of storage on agar medium at 4°C gave rise to this behavior more often than not, whereas colonies picked within 48 h of plating rarely exhibited this trait. When it became evident that "aging" of cells at 4°C might be selecting for the mutant genotype, we routinely used cells freshly derived from our frozen stock. The latter exhibited a consistent behavior with respect to inducibility of transposition.

TABLE 4. Isolates of DS16 with aberrant transposition behavior

Strain	Expt	% of Em-resistant JH2-2 transconjugants sensitive to Sm and Km[a]	
		No Em	Em (0.5 μg/ml for 4 h)
DS16	1	4.6	63
	2	<2	36
FA3001	1	64	71
	2	80	93
FA3002	1	<2	<2
	2	<2	<2

[a] In each case, at least 50 Em-resistant transconjugants were tested for sensitivity to Km and Sm after mating with DS16 cells (see text).

DISCUSSION

The studies presented above have included the derivation of physical maps of both pAD1 and pAD2. Analyses of transposon insertions into pAD1 have pointed to the approximate location of the hemolysin-bacteriocin determinant, whereas electron microscope and hybridization analyses have localized Tn*917* and the Sm^r and Km^r determinants on pAD2.

Exposure of DS16 cells to 0.5 μg of Em per ml resulted in an increase in the potential to donate all three of the resistance determinants associated with the nonconjugative pAD2. Within 20 min this increase reached a maximum at about sevenfold higher than for cells not exposed to drug. (Significant accumulation of pAD1::Tn*917* structures does not occur until after 2 to 4 h of drug exposure [30].) Transconjugants arising from such matings were found to harbor stable cointegrate structures with a single copy of Tn*917*. These structures do not appear to be involved in the induced transposition of Tn*917*, and their acquisition probably reflected a response to a perturbation of protein synthesis imparted by Em before the time resistance became fully induced. Evidently, the pAD1 conjugation system becomes enhanced under stress, and transfer of rare cointegrate structures in the population is also affected. The basis of stable cointegrate formation is not known; the involvement of *Eco*RI fragment B of pAD2 raises the question of whether the large inverted repeat could be involved.

Whereas the above-noted cointegrate structures were not related to the transposition of Tn*917* from pAD2 to pAD1, the involvement of transient cointegrate intermediates in the transposition process remains a likely possibility. Reports dealing with certain other transposons have involved a rigorous characterization of cointegrate intermediates containing two copies of the insertion element (1, 14, 18, 25), and a number of reports have implicated transient cointegrate structures as the basis of mobilization of nonconjugative plasmids (3, 7, 8, 15, 16, 21). Upon examination of 40 transconjugants receiving plasmid DNA from DS16 donors exposed to Em for 30 min, 3 were found to simultaneously harbor pAD1::Tn*917* and pAD2. It is quite possible that transfer occurred in these cases as a cointegrate intermediate which resolved after transfer; the intermediate would probably have had two copies of Tn*917*, unlike the case for the stable cointegrates.

The mechanism by which Em induces transposition of Tn*917* remains unclear. A regulatory gene that affects the expression of Em resistance as well as a transposase is not difficult to imagine. (A *trans*-acting regulatory gene [encoding a repressor] for transposition has been implicated on Tn*3* [14, 17].) It is interesting, however, that two transposition variants of DS16 (one with a high level of spontaneous transposition; the other failing to exhibit transposition at all) seemed to be normal with regard to inducibility of resistance. This suggests that the two functions (i.e., transposition and Em resistance) are controlled separately or at least can be readily unlinked. Horinouchi and Weisblum (19) have recently sequenced an Em resistance determinant on the staphylococcal plasmid pE194. The sequence strongly suggests an induction mechanism for Em resistance based on post-transcriptional changes in message conformation. Whether or not expression of Tn*917*-related Em resistance and perhaps a transposition function(s) are of a similar nature remains to be determined.

To our knowledge, Tn*917* is still the only antibiotic resistance transposon reported to undergo drug-induced transposition. However, transposition of a mercury resistance transposon (Tn*501*) in *Escherichia coli* has been shown to be enhanced by exposure to mercury ions (27).

The characterization of plasmid and transpositional elements in *S. faecalis* DS16 provides a framework from which additional studies on these elements can ensue. The pAD1 plasmid is currently being investigated with regard to its sex pheromone-related conjugation functions (4, 9, 11), and the two transposons are proving to be useful as insertional mutagens in these studies (Y. Ike, personal communication). In the future, Tn*917* along with Tn*916* (13) may be of general value in genetic studies in streptococci and perhaps other gram-positive genera. Tn*917* has recently been shown to undergo transposition in *Bacillus subtilis* (P. Youngman, personal communication).

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LITERATURE CITED

1. **Arthur, A., and D. Sherratt.** 1979. Dissection of the transposition process: a transposon encoded site-specific recombination system. Mol. Gen. Genet. **175**:267–274.
2. **Behnke, D.** 1981. Plasmid transformation of *Streptococcus sanguis* occurs by circular and linear molecules. Mol. Gen. Genet. **183**:490–497.
3. **Clark, A. J., and G. J. Warren.** 1979. Conjugal transmission of plasmids. Annu. Rev. Genet. **13**:99–125.
4. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.
5. **Clewell, D. B., and D. R. Helinski.** 1970. Properties of deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochemistry **9**:4428–4440.

6. **Clewell, D. B., Y. Yagi, G. M. Dunny, and S. Schultz.** 1974. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. J. Bacteriol. **117**:283–289.
7. **Crisona, N., and A. J. Clark.** 1977. Increase in conjugational transmission frequency of nonconjugative plasmids. Science **196**:186–187.
8. **Crisona, N. J., J. A. Nowak, H. Nagaishi, and A. J. Clark.** 1980. Transposon-mediated conjugational transmission of nonconjugative plasmids. J. Bacteriol. **142**:701–713.
9. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. U.S.A. **75**:3479–3483.
10. **Dunny, G. M., and D. B. Clewell.** 1975. Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of a noninfectious drug resistance plasmid. J. Bacteriol. **124**:784–790.
11. **Dunny, G. M., R. A. Craig, R. L. Carron, and D. B. Clewell.** 1979. Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. Plasmid **2**:454–465.
12. **Franke, A. E., and D. B. Clewell.** 1980. Evidence for conjugal transfer of a *Streptococcus faecalis* transposon (Tn*916*) from a chromosomal site in the absence of plasmid DNA. Cold Spring Harbor Symp. Quant. Biol. **45**:77–80.
13. **Franke, A. E., and D. B. Clewell.** 1981. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145**:494–502.
14. **Gill, R., F. Heffron, G. Dougan, and S. Falkow.** 1978. Analysis of sequences transposed by complementation of two classes of transposition-deficient mutants of Tn*3*. J. Bacteriol. **136**:742–756.
15. **Goebel, W., W. Lindenmaier, F. Pfeifer, H. Schrempf, and B. Schelle.** 1977. Transposition and insertion of intact, deleted and enlarged ampicillin transposon Tn*3* from mini-R1 (Rsc) plasmids into transfer factors. Mol. Gen. Genet. **157**:119–129.
16. **Guyer, M.** 1978. The γδ sequence of F is an insertion sequence. J. Mol. Biol. **126**:347–365.
17. **Heffron, F., P. Bedinger, J. Champoux, and S. Falkow.** 1977. Deletions affecting the transposition of an antibiotic resistance gene. Proc. Natl. Acad. Sci. U.S.A. **74**:702–706.
18. **Heffron, F., B. J. McCarthy, H. Ohtsubo, and E. Ohtsubo.** 1979. DNA sequence analysis of the transposon Tn*3*: three genes and three sites involved in the transposition of Tn*3*. Cell **18**:1153–1163.
19. **Horinouchi, S., and B. Weisblum.** 1980. Post transcriptional modification of mRNA conformation: mechanism that regulates erythromycin-induced resistance. Proc. Natl. Acad. Sci. U.S.A. **77**:7079–7083.
20. **Jacob, A., and S. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. **117**:360–372.
21. **Kopecko, D., and S. Cohen.** 1975. Site-specific *rec*A-independent recombination between bacterial plasmids: involvement of palindromes at the recombinational loci. Proc. Natl. Acad. Sci. U.S.A. **72**:1371–1377.
22. **Macrina, F., K. Jones, and P. Wood.** 1980. Chimeric streptococcal plasmids and their use as molecular cloning vehicles in *Streptococcus sanguis* (Challis). J. Bacteriol. **143**:1425–1435.
23. **Macrina, F. L., J. Tobian, K. Jones, and R. P. Evans.** 1982. Molecular cloning in the streptococci, p. 195–210. *In* A. Hollaender, R. DeMoss, S. Kaplan, J. Konisky, D. Savage, and R. Wolfe (ed.), Genetic engineering of microorganisms for chemicals. Plenum Publishing Corp., New York.
24. **Meyer, R., G. Bach, and J. Shapiro.** 1979. Transposition of DNA inserted into deletions of the Tn*5* kanamycin resistance element. Mol. Gen. Genet. **171**:7–13.
25. **Shapiro, J.** 1979. A molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. Proc. Natl. Acad. Sci. U.S.A. **76**:1933–1937.
26. **Sharp, P., M. Hsu, E. Ohtsubo, and N. Davidson.** 1972. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. I. Structure of F prime factors. J. Mol. Biol. **71**:471–497.
27. **Sherratt, D., A. Arthur, and M. Burke.** 1980. Transposon-specified site specific recombination systems. Cold Spring Harbor Symp. Quant. Biol. **45**:275–281.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
29. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1978. A transposon (Tn*917*) in *Streptococcus faecalis* which exhibits enhanced transposition during induction of drug resistance. Cold Spring Harbor Symp. Quant. Biol. **43**:1217–1221.
30. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1980. Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **141**:1366–1374.
31. **Tomich, P. K., F. Y. An, S. P. Damle, and D. B. Clewell.** 1979. Plasmid-related transmissibility and multiple-drug resistance in *Streptococcus faecalis* subsp. *zymogenes* strain DS16. Antimicrob. Agents Chemother. **15**:828–830.
32. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
33. **Yagi, Y., and D. B. Clewell.** 1976. Plasmid-determined tetracycline resistance in *Streptococcus faecalis*: tandemly repeated resistance determinants in amplified forms of pAMα1 DNA. J. Mol. Biol. **102**:583–600.