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Evidence that Coupling Sequences Play a Frequency-Determining Role in Conjugative Transposition of Tn*916* in *Enterococcus faecalis*

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The conjugative transposon Tn*916* (encodes resistance to tetracycline), originally identified in *Enterococcus faecalis*, moves by an excision-insertion process in which the rate-limiting step is believed to be excision. Individual transposon-containing strains exhibit characteristic mating frequencies which range over several orders of magnitude; the basis of this phenomenon is addressed in the present study. We were able to generate independent single-copy insertions in identical target locations and with similar orientations within a plasmid hemolysin determinant (*cylA*); however, transposition from this site occurred at very different frequencies (10^{-8} to 10^{-4} per donor) depending on the individual isolate. DNA sequencing analyses showed that the coupling (junction) sequences differed between isolates and thus appeared to be responsible for differences in excision frequencies. Other experiments showed that inducible transcription into either end of the transposon had no significant effect on transfer.

Conjugative transposons are genetic elements that characteristically exhibit intercellular transposition, primarily among gram-positive bacteria (for reviews, see references 5, 8, 27, and 28). Tn*916* (16.4 kb; encodes resistance to tetracycline), which was originally identified in *Enterococcus faecalis* DS16 (13), and Tn*1545* (25.3 kb; encodes resistance to tetracycline, erythromycin, and kanamycin) from *Streptococcus pneumoniae* BM4200 (10), are two such elements that have been studied in some detail. These transposons exhibit significant homology, have imperfect terminal inverted repeats (20 of 26 nucleotides), and, unlike many other transposons, do not generate duplications of the target DNA upon insertion (2, 6). Movement is via an excision-insertion mechanism somewhat analogous to that of the lambdoid phages (24, 25), with the intermediate in the process being a nonreplicative circular molecule (5, 29). Excision of Tn*916* or Tn*1545* is facilitated by the products of two open reading frames which are located near one end of the transposon (7, 31, 33). These determinants encode the proteins Xis-Tn and Int-Tn. The amino-terminal portion of the Xis-Tn protein shows similarity with the Xis proteins of bacteriophage P22 and, to a lesser extent, lambda and φ80. Int-Tn exhibits homology with respect to the highly conserved carboxy-terminal domains of Int-related site-specific recombinases (24), including the lambda integrase.

Independent Tn*916*-containing derivatives exhibit widely differing donor potentials (transfer frequencies) which are characteristic for the particular isolate. The excision event, believed to be the rate-limiting step in transposition, occurs by a reciprocal site-specific recombination between nonhomologous, 6-bp junction ("coupling") sequences (3, 5, 6, 25, 27, 28). Staggered nicks are introduced in the coupling sequences (i.e., 6 bp apart), and the overlapping, single-stranded ends are joined in a RecA-independent (13, 15) recombination event, yielding the circular intermediate. Since the two junctions are not generally homologous, a heteroduplex is formed (3). The joining of the flanking (nontransposon) DNA also results in a heteroduplex, with replication or repair leading to daughter molecules having one or the other junction sequence. Integration of the transposon into its target is viewed as a reversal of this process, with either none, one, or both of the coupling sequences appearing as junctions at the new insertion location (5). Although insertion does not target a specific site, as in the case of the lambdoid bacteriophages, it does show a preference for particular target sequences. Preferential insertion sites are A-T-rich regions that exhibit short regions of homology with the ends of the element (3, 5, 6, 24, 37).

The transfer potentials for individual Tn*916* isolates range from less than 10^{-8} to greater than 10^{-5} per donor (5, 14). Although a strain may carry multiple insertions of the transposon (7, 14, 15), this does not necessarily result in higher transfer frequencies. Given that Tn*916* insertions located at multiple sites in the bacterial genome might not exhibit similar donor potentials, it is likely that the insertion with the greatest transfer potential would excise first, and there is evidence that this in turn results in a *trans* activation of the other elements (12). It has been suggested that the different conjugation efficiencies observed with various isolates might be attributable to differences in homology between the ends of the transposon and adjacent sequences or to effects of outside promoters generating different levels of transcription into one or the other end of the inserted transposon, triggering events leading to the initiation of transposition (6).

In the study presented here, we have attempted to reveal the molecular basis of the differences observed in Tn*916* conjugation potential. The construction and examination of transposon insertions which then transferred with widely differing frequencies has provided evidence that the nucleotide content of the coupling sequences is intimately involved. In contrast, transcription into either end of the transposon appeared to have no effect.

MATERIALS AND METHODS

Bacteria, media, and reagents. The bacterial strains used and the plasmids they contain are listed in Table 1. Strains

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. faecalis[a]		
OG1RF	*rif fus*	22
OG1SS	*str spc*	13
CG110	*rif fus tet* (Tn*916*)	14
OG1X	*str*	17
Plasmids		
pAM717	*hem*$^+$ Em^r (Tn*917*) *tra*$^-$ (ca. 60% deletion of pAD1)	16
pAM2005K	Pheromone-inducible miniplasmid of pAD1; growth inhibited by cAD1	39
pAM2516KT	Tn*916*-derived pheromone (cAD1)-resistant derivative of pAM2005K	39
pAM2524KT	Tn*916*-derived pheromone (cAD1)-resistant derivative of pAM2005K	39
pAM5100	pAM717::Tn*916*	This study
pAM5101	pAM717::Tn*916*	This study
pAM5104	pAM717::Tn*916*	This study
pAM5105	pAM717::Tn*916*	This study
pAM5106	pAM717::Tn*916*	This study
pAM5107	pAM717::Tn*916*	This study
pAM5109	pAM717::Tn*916*	This study
pAM5110	pAM717::Tn*916*	This study
pAM5111	pAM717::Tn*916*	This study
pAM5112	pAM717::Tn*916*	This study

[a] None of the strains contained a plasmid.

were grown in Todd-Hewitt broth (THB) (BBL Microbiology Systems) supplemented with 0.5 to 1% glycine for preparation of plasmid and genomic DNAs. For broth matings involving exposure to pheromone, strains were grown in N2GT medium (Nutrient Broth no. 2 [Oxoid Ltd., London, United Kingdom] supplemented with 0.1 M Tris buffer [pH 7.5] and 0.2% glucose). Solid medium was prepared by adding 1.5% agar (Difco) to the broth. Filter and broth matings not involving pheromone induction were performed with either THB agar plates or THB, respectively. Hemolysis was detected with the use of solid THB medium containing 4% horse blood (Colorado Serum Co., Denver, Colo.). Unless otherwise specified, antibiotics were added at the following concentrations: streptomycin, 1 mg/ml; spectinomycin, 500 μg/ml; tetracycline, 5 μg/ml; erythromycin, 10 μg/ml; rifampin, 25 μg/ml; and fusidic acid, 25 μg/ml. Synthetic sex pheromone cAD1 was prepared by the University of Michigan peptide facility.

Plasmid extraction procedures. Plasmid DNA minipreparations from *E. faecalis* were extracted by an alkaline lysis method essentially as described by Weaver and Clewell (38). Plasmid DNA for sequencing was isolated by isopycnic CsCl-ethidium bromide density gradient centrifugation as described by Franke and Clewell (13).

Manipulation of DNA. Restriction endonucleases were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and were used under conditions suggested by the manufacturer. Agarose gel electrophoresis was typically carried out in 0.7% agarose in Tris-borate buffer (0.045 M Tris-borate, 0.001 M EDTA [pH 8.0]). DNA transfer to nitrocellulose and subsequent Southern hybridization experiments were performed as described previously (14, 15) with Tn*916* DNA (pAM170) to probe *E. faecalis* DNA cleaved with *Hin*dIII or *Eco*RI. Plasmid DNA was nick translated by using a nick translation kit (Bethesda Research Laboratories) and [α-^{32}P] dATP (Amersham). Sequencing of double-stranded DNA templates was carried out as described previously (34) with Sequenase 1.0, using a Sequenase kit (United States Biochemical, Cleveland, Ohio). Primers used for sequencing and PCR analysis, i.e., TnRO3 (5′TGAGTGGTTTTGACC3′), TnLO4 (5′GACATGAGGAAATATGCAAAGAAACGTGAAG3′), BamRO (5′CACGGATCCTTCATAAGTTCACTTCCTTTC3′), and SalLO (5′GTGGGTCGACAATGGCAGAAATGAAA CGCT3′), were synthesized by the DNA core facility at the University of Michigan. Sequences were stored and analyzed by using MacVector 3.5 software. PCR amplification of DNA segments from minipreparations (see above) was carried out with a PCR kit obtained from Perkin-Elmer (Norwalk, Conn.) and a Perkin-Elmer 480 thermal cycler.

Mating procedures. Donor and recipient cells were grown separately overnight in 5 ml of THB containing the appropriate selective antibiotic(s) for both chromosomal and plasmid markers. For filter matings, a 1:10 donor/recipient ratio (based on culture volume) was prepared, and cells were pelleted from a 500-μl portion of the mixture. The supernatant was decanted, and the cells were resuspended in 25 μl of THB. The cell suspension was dropped onto a 0.45-μm-pore-size HA filter (Millipore Corp., Bedford, Mass.) which was placed on nonselective THB agar. After incubation at 37°C for the designated length of time, the filter was suspended and vortexed in 1 ml of THB, and 100-μl aliquots of the cell suspension were plated on THB agar containing the appropriate selective antibiotic(s). Transconjugants were scored after 1 to 2 days at 37°C.

For matings involving pheromone (cAD1) exposure, donor and recipient cells were grown individually overnight in 5 ml of N2GT broth. Donors were diluted 1:10 in fresh medium and after 1 h were exposed to 40 ng of synthetic cAD1 per ml for 90 min to induce transcription into transposon DNA downstream. Donors (0.05 ml) and recipients (0.5 ml) were then mixed, brought to 2 ml in THB broth, and filtered onto a 0.45-μm-pore-size HA filter. The filter was placed on an N2GT plate (also containing 40 ng of cAD1 per ml in the case of the cells previously exposed to cAD1) and incubated at 37°C for 2 to 6 h. The filter was then washed in 1 ml of N2GT broth, and 100-μl aliquots were plated on selective agar.

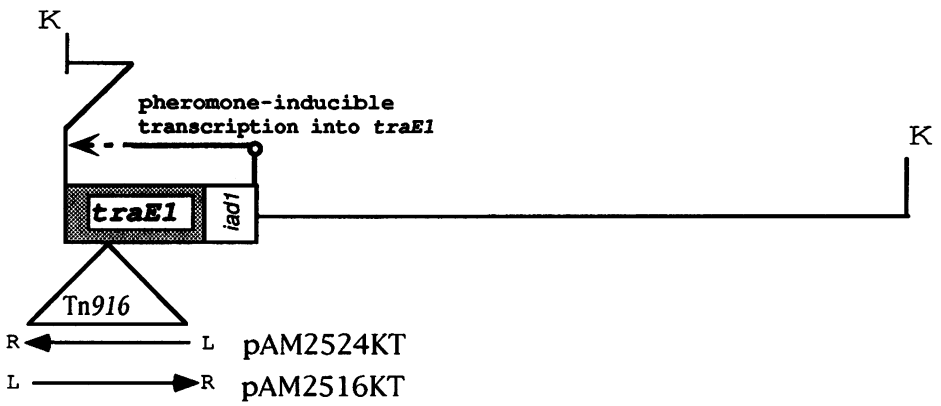
Broth matings were performed essentially as described previously (4) with the following modifications: the volume of the mating mix was 2 ml, and the mating time was as designated. Rapid screening for plasmid isolates exhibiting high transfer abilities was done via cross-streak matings as described previously (4).

Transformation of *E. faecalis*. The introduction of plasmid DNA into *E. faecalis* was accomplished by electroporation according to the protocol of Cruz-Rodz and Gilmore (11).

RESULTS

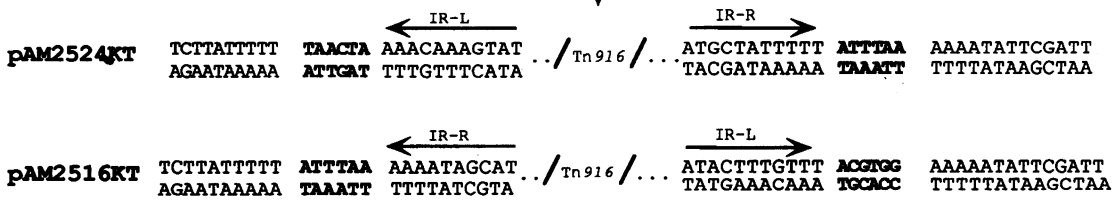
Determination of whether variable expression of an outside promoter affects Tn*916* conjugative transposition. To determine whether the different conjugation efficiencies observed with different isolates can be attributed to transcriptional effects of an outside promoter reading into one or the other end of the inserted transposon, we made use of two previously constructed derivatives of the peptide sex pheromone (cAD1)-inducible, hemolysin/bacteriocin plasmid pAD1 (39). The derivatives, designated pAM2516KT and pAM2524KT, are miniplasmids containing Tn*916* within the cAD1-inducible, positive regulatory gene *traE1* (23, 38, 39) (Fig. 1A). The two miniplasmids, which are also devoid of structural genes necessary for conjugation, do not exhibit plasmid-specific transfer.

A



B

traE1 target: AAACAACGTGTTGCATGGCTCTTATTTTTATTTAAAAAATATTCGATTGGGGGAATGTAAA
TTTGTTGCACAACGTACCGAGAATAAAAATAAATTTTTTATAAGCTAACCCCCTTACATTT



C

cAD1 Exposure	Mating frequency per donor ±S.D. pAM2516KT	pAM2524KT
-	$5.2 \pm 2.2 \times 10^{-7}$	$7.5 \pm 3.1 \times 10^{-5}$
+	$6.0 \pm 2.8 \times 10^{-7}$	$6.0 \pm 1.1 \times 10^{-5}$

FIG. 1. Tn*916* insertions in the *traE1* gene of a mini-pAD1. (A) Schematic representation of *Kpn*I fragment representing pAM2516KT and pAM2524KT. The pheromone-inducible promoter upstream of the *traE1* gene is depicted by the open circle, and the direction of transcription is indicated by the arrow. The site(s) of insertion of Tn*916* into the pAD1 miniplasmid pAM2005K to yield pAM2516KT and pAM2524KT is depicted by the large triangle, and the orientation of the transposon within the *traE1* gene is shown underneath by the arrows. R, right end of Tn*916*; L, left end of Tn*916*. (B) Insertion site(s) of Tn*916* for pAM2516KT and pAM2524KT. The double-stranded portion of the *traE1* nucleotide sequence is shown, with the target region present beneath the bracketed line and the coupling sequence present within the boxed area. Shown beneath the target sequence are the nucleotide junction region sequences for the insertion of Tn*916* in pAM2516KT and pAM2524KT. The last 11 bases of the inverted repeats of the element (IR-L, left inverted repeat; IR-R, right inverted repeat) are shown under the arrows. The boldface sequences represent the coupling sequences present between the ends of the element and the flanking sequence of the insertion site. Note that the right inverted repeat of the transposon in pAM2516KT is representative of a four-T transposon, and the right inverted repeat of the transposon in pAM2524KT is a five-T transposon. (C) Frequencies of tetracycline resistance transfer from OG1X(pAM2516KT) and OG1X(pAM2524KT) donors to an OG1RF recipient. Donors were exposed or not exposed to cAD1 (40 ng/ml for 90 min) prior to the mating. Preexposed cells also had cAD1 present in the plates during the mating. Frequencies are the averages for at least three matings and are based on transfer per donor cell.

Restriction analyses of these constructs with *Hin*dIII had previously shown that the two Tn*916* insertions were in opposite orientations (39). This allowed us to look at the effects of inducible *traE1* transcription into either the right end (in pAM2516KT) or the left end (in pAM2524KT) of the transposon. The exact locations of the Tn*916* insertions within *traE1* were identified by sequencing using oligonucleotide primers which read outward from the ends of the transposon; the results are shown in Fig. 1B. It was confirmed that the inserts were indeed present in opposite orientations, and, in addition, they were found to be in identical positions in *traE1*. (The insertions were within a 22-bp segment that is 100% A+T, with the specific target sequence viewed as ATTTAA; in one case the right end of the transposon has five T residues, while in the other case it has four T residues [5].)

We then examined the effect of transcriptional "read-in" to

either end of the similarly located transposons from the same external pheromone-inducible promoter. Inducible transcription has been shown to occur initially from the upstream *iad* promoter and later from an independent *traE1* promoter (35). The insertion derivatives were originally selected on the basis of their prevention of a cAD1-induced lethality caused by transcription through *traE1* and into a replication-related region of the miniplasmid (39).

E. faecalis OG1X strains harboring either pAM2516KT or pAM2524KT were exposed to 40 ng of cAD1 per ml for 90 min; parallel controls were incubated in the absence of pheromone. The cells were then mixed with OG1RF recipients, placed on filter membranes, and incubated on nonselective agar (still in the presence of cAD1 in the case of the cells previously exposed) for a period of 6 h. The cells were then suspended and plated, with selection for tetracycline-resistant transconjugants. The results are summarized in Fig. 1C. As is evident, the induction of the upstream promoter assumed to occur in the presence of cAD1 did not affect the efficiency with which Tn*916* was transferred. With or without exposure to cAD1, the transposon on pAM2516KT transferred at a frequency of approximately 5×10^{-7}, while the transposon on pAM2524KT transferred at a frequency approximately 100-fold higher. It is noted that, as previously observed (7), the transfer of the tetracycline resistance determinant was not accompanied by the transfer of the erythromycin resistance determinant located on the plasmid outside the transposon (<1% for the transposon on pAM2516KT and <0.2% for the transposon on pAM2524KT). These experiments provide good evidence that transcriptional read-in from an outside promoter does not significantly influence the frequency of conjugative transposition.

Although induced transcription had no apparent effect, the significant difference (100-fold) in transfer frequencies between the two strains was interesting. The junction sequence ATTTAA, which corresponds to the *traE1* target, is located on the element's right end in both derivatives. The junctions at the opposite ends, however, were different for the two insertions, reflecting differences in sequences brought in by the transposon.

Generation of independent Tn*916* insertions on a different nonconjugative pAD1 derivative. To further investigate the basis of the wide range of transfer efficiencies exhibited by independent transposon insertions, we elected to construct independently generated insertions with as widely differing conjugation potentials as possible. To do this we exploited the characteristics of a derivative of pAD1 designated pAM717 (16). The pertinent characteristics of the plasmid that made it useful for this study are that (i) it confers erythromycin resistance (because of to a Tn*917* insertion), (ii) it carries the hemolysin/bacteriocin determinant, and (iii) it is deleted for a major portion (60%) of pAD1 that includes most of the conjugation-related functions. Previous work showed that the hemolysin determinant includes a hot spot for Tn*916* insertion (13, 18). Thus, insertion of Tn*916* into this target plasmid could be monitored by screening for alterations of the hemolysin phenotype.

The generation of independent insertions in the plasmid was accomplished via the introduction of Tn*916* from an *E. faecalis* donor strain in which transfer is known to occur at a relatively high frequency. *E. faecalis* CG110 (14), containing approximately six copies of Tn*916* (as judged by Southern hybridization data [7]), was used as a donor with recipient *E. faecalis* OG1RF harboring the target plasmid, pAM717. Filter matings (4 h) were carried out with selection for transconjugants that acquired resistance to tetracycline. Horse blood was included in the selection plates to monitor the hemolysin phenotypes of the transconjugants.

Over half of the transconjugants exhibited a hyperhemolytic phenotype, implying that a majority of the insertions of Tn*916* occurred near the 3′ end of *cylA* (18), the determinant for the hemolysin/bacteriocin activator protein on the plasmid (30). The selection plates were flooded with TES buffer containing 25% sucrose (pH 8.0) to remove the cells; plasmid DNA was prepared from the mixture of transconjugants by CsCl-ethidium bromide buoyant density centrifugation. The plasmid DNA was used to transform OG1RF cells by electroporation, with selection for both the *tet* and *erm* determinants. Plasmid DNA from the electroporants was purified by the miniprep-aration method (see Materials and Methods) and digested with *Eco*RI. The results showed that of 100 isolates examined, 95 contained an insertion of Tn*916* in a 4-kb fragment, previously designated the *Eco*RI D fragment (9), which carries the *cylA* determinant (29). Among these, not all the derivatives were hyperhemolytic. Some were nonhemolytic, and some would express hemolysin only in the presence of tetracycline. These differences in phenotype have been previously noted for insertions at an identical site in *cylA* (18).

Determination of transfer efficiencies. The transposon transfer efficiencies of the pAM717::Tn*916*-containing OG1RF donors were then examined. Cross-streak matings of 94 potential donors with OG1SS(pAM717) recipients were performed. (The presence of pAM717 in the recipient was to provide immunity to the bacteriocin [hemolysin] of the donor.) Donors possessing a relatively high transfer potential would be expected to yield detectable colonies (transconjugants) from this rather inefficient method of conducting matings, whereas strains with low donor potential would not yield detectable transconjugants. Seven strains representative of those with high transfer potentials (40 gave rise to detectable transfer), and three strains representative of those with lower transfer potentials (no transconjugants detected in cross-streak matings) were chosen and subsequently examined quantitatively by filter matings. The resulting transfer frequencies are shown in Table 2. As is evident, the transfer frequencies exhibited by the two groups differed significantly, ranging from $<10^{-8}$ to 10^{-7} for the lower-proficiency group to ca. 10^{-4} for the higher-potential group, a difference of as much as 10,000-fold. Southern hybridization experiments using total genomic DNA confirmed that Tn*916* in these donors was located only on the plasmid (data not shown).

The high- and low-frequency donors were then tested for their abilities to transfer Tn*916* in broth matings. Previous work in our laboratory had shown that conjugative transposition was usually not detectable in broth or was, at best, marginal (unpublished data). The results of 8-h broth matings (Table 2) indicate that the higher-potential group is able to conjugate at a relatively high frequency, while in the case of the lower-efficiency group, transfer was not detectable. Observation of tetracycline resistance transfer generally required at least 4 h of mixed growth, with 6 to 8 h needed for consistent detection. When cells were incubated together for 3 h, no transfer was detected.

Primary transconjugants as donors of Tn*916*. A possible interpretation of the above-described differences in transfer frequencies is that there was a mutation in one of the transposons in the original donor, CG110, resulting in an increase in the excision rate. This point was addressed by using primary transconjugants arising from high-frequency donors as donors themselves. If a mutation in Tn*916* caused an elevated transfer frequency, then the primary transconjugants which harbor this transposon would also be expected to exhibit

TABLE 2. Intraspecific mating frequencies of independent Tn*916* insertions in filter and broth matings

Donor plasmid	Mating frequency (mean ± SD)[a]	
	Filter	Broth[b]
pAM5100	$(4.1 \pm 1.9) \times 10^{-4}$	$(2.0 \pm 1.2) \times 10^{-5}$
pAM5101	$(8.9 \pm 1.7) \times 10^{-5}$	$(6.2 \pm 1.9) \times 10^{-6}$
pAM5104	$(9.5 \pm 1.0) \times 10^{-5}$	$(3.2 \pm 0.9) \times 10^{-6}$
pAM5105	$(1.7 \pm 2.2) \times 10^{-4}$	$(6.7 \pm 2.1) \times 10^{-6}$
pAM5109	$(2.5 \pm 6.1) \times 10^{-5}$	ND[c]
pAM5110	$(3.2 \pm 5.6) \times 10^{-5}$	ND
pAM5112	$(2.3 \pm 6.7) \times 10^{-4}$	ND
pAM5106	$(2.0 \pm 2.1) \times 10^{-7}$	$<10^{-8}$
pAM5107	$<10^{-8}$	$<10^{-8}$
pAM5111	$(1.3 \pm 4.5) \times 10^{-7}$	ND

[a] The frequency of mating events was calculated as the number of tetracycline-resistant transconjugants divided by the number of donor cells observed (in the final 1-ml suspension) at the end of the mating period. The mating was allowed to proceed for 8 h. The numbers represent the averages for three mating experiments. The lowest limit of transconjugant detection was 10^{-8} transconjugants per donor.
[b] Broth matings were done with slow shaking for 8 h.
[c] ND, not determined.

elevated transfer frequencies. From three of the high-frequency donors (those containing either pAM5100, pAM5101, or pAM5105), four primary tetracycline-resistant transconjugants were in each case selected and used as donors of tetracycline resistance in filter matings as described above. Table 3 shows that different tetracycline-resistant transconjugants exhibited different transfer frequencies in these secondary matings. The frequencies observed were over a 100-fold range in each case. It should be noted that Southern hybridization analysis of all donors was done to confirm that only one copy of the transposon was present; on the basis of the sizes of the homologous fragments, the transposon was located at different sites (data not shown).

High-frequency donors contain more excised circular intermediate. To determine if there was a difference between high- and low-potential donors with respect to excision of the transposon, the amount of circular intermediate was determined by PCR analysis. (Rice and colleagues [26] recently reported on the detection of intermediate molecules of a Tn*916*-like conjugative transposon in *E. faecalis*.) Oligonucleotide primers BamRO and SalLO (see Materials and Methods), which read out the ends of the transposon, were used to amplify a segment (about 500 bp) containing the joint of the intermediate from plasmid preparations of high- and low-frequency donors. The results are shown in Fig. 2. Preparations utilizing the high-frequency donors clearly gave rise to 500-bp products (Fig. 2, lanes 3, 4, and 5), whereas preparations from the low-frequency donors yielded no detectable bands.

TABLE 3. Filter mating frequencies of independent primary transconjugants from high-frequency donors

Original donor plasmid	Isolate no.	Mating frequency (mean ± SD)[a]
pAM5100	1	$(3.1 \pm 1.9) \times 10^{-4}$
	2	$(3.6 \pm 2.1) \times 10^{-5}$
	3	$(8.7 \pm 2.4) \times 10^{-5}$
	4	$(1.1 \pm 1.1) \times 10^{-6}$
pAM5101	1	$(7.9 \pm 1.5) \times 10^{-5}$
	2	$(3.4 \pm 2.3) \times 10^{-5}$
	3	$(4.2 \pm 3.2) \times 10^{-6}$
	4	$(5.4 \pm 2.1) \times 10^{-7}$
pAM5105	1	$(1.2 \pm 1.3) \times 10^{-4}$
	2	$(3.3 \pm 2.1) \times 10^{-4}$
	3	$(4.5 \pm 2.3) \times 10^{-6}$
	4	$(8.9 \pm 3.5) \times 10^{-7}$

[a] The frequency of mating events was calculated as the number of tetracycline-resistant transconjugants obtained divided by the number of donor cells at the end of the mating period. The mating was allowed to proceed for 8 h. The numbers represent the averages for three mating experiments.

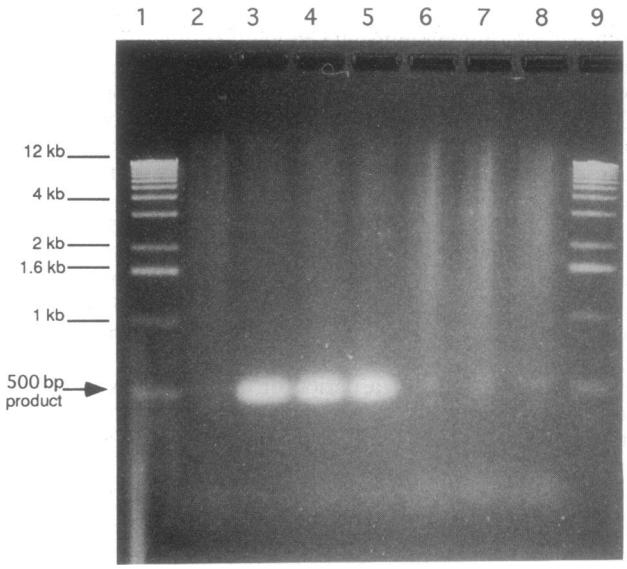


FIG. 2. PCR analysis of the joint region of the circular intermediate in high- and low-frequency donors. PCR was performed with alkaline lysis plasmid preparations from pAM717::Tn*916* high- and low-frequency donors. The primers utilized were BamRO and SalLO (see Materials and Methods). If the circular intermediate is present in the population, a 500-bp PCR product should be revealed. Lane 1, Bethesda Research Laboratories kilobase ladder; lane 2, DS16C3 (plasmid-free derivative of original Tn*916*-containing clinical isolate); lanes 3 to 8, PCR products generated from preparations carrying pAM5100, pAM5101, pAM5105, pAM5106, pAM5107, and pAM717, respectively; lane 9, kilobase ladder. The faint band appearing at 500 bp in lanes 2, 6, 7, and 8 is most likely an artifactual band relating to the PCR procedure, since it was also generated from a strain not carrying Tn*916* (i.e., lane 8).

Determination of the coupling sequences. DNA sequences that flank the above-described Tn*916* inserts in pAM717 were determined by using primers (TnRO3 and TnLO4; see Materials and Methods) directing DNA synthesis out the ends of the transposon. The results are shown in Fig. 3. In all cases, the insertion of Tn*916* occurred in the same position within *cylA*, which is consistent with the recent study of Ike et al. (18) showing that effects on hemolysin expression were associated with Tn*916* insertions at this site. In addition, the orientation of the transposon was identical in each case, with the right end of the transposon directed toward the 5′ end of *cylA*. The only differences between the insertions were the coupling sequences. In every case, at least one of the coupling sequences (at either the right or left end) was from the sequence ATAGAT in the target. The other coupling sequence, brought in by the transposon, was different in several cases. It is noteworthy that the coupling sequence corresponding to the target appeared at either end of the transposon among isolates of both high and low donor potential. However, high- and low-transfer-potential derivatives differed from each other at

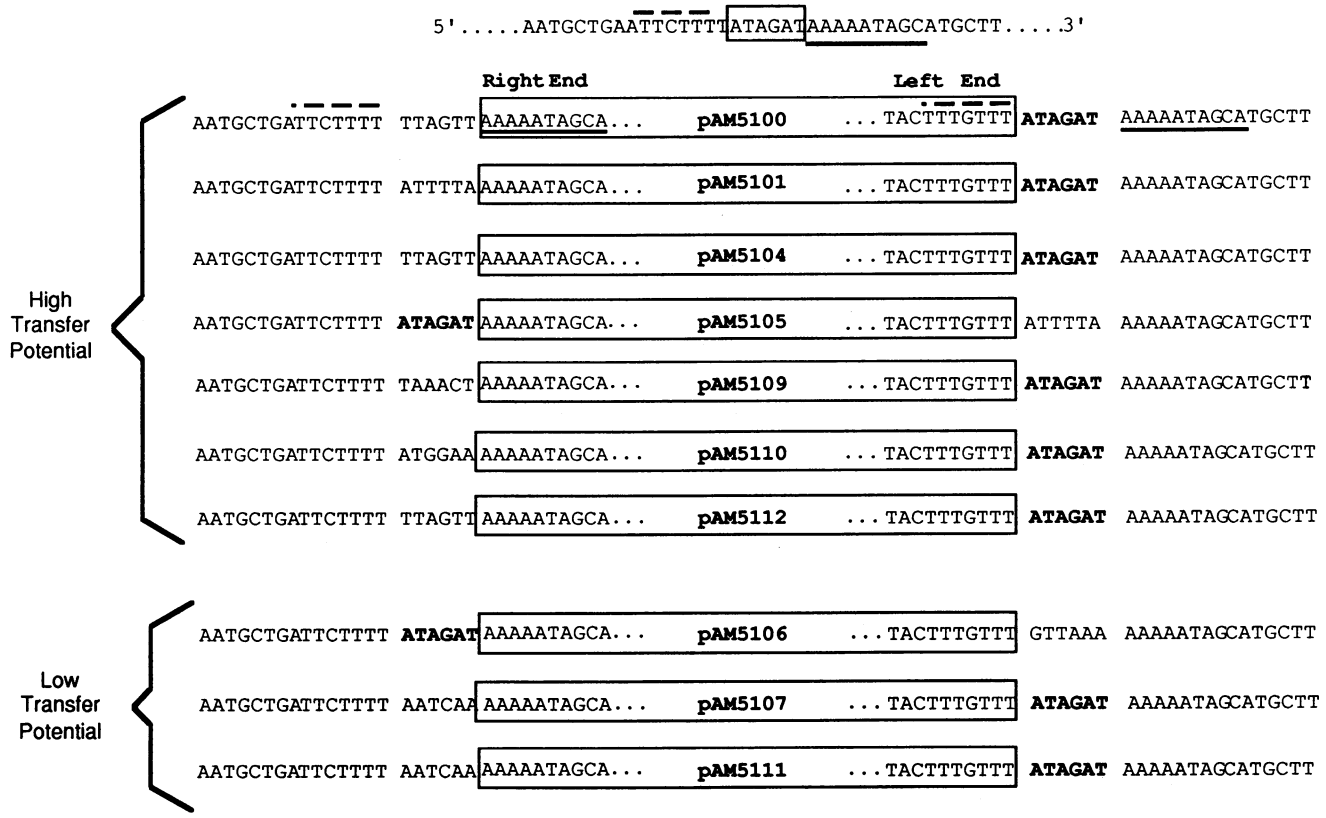


FIG. 3. Nucleotide sequence of the DNA that flanks the insertions of Tn*916* within the *cylA* gene. The target area sequence within the *cylA* determinant is shown in the 5′-to-3′ (left to right) direction. The sequence that becomes associated with most of the insertions as a coupling sequence is ATAGAT (shown within the boxed area), and the underlined and overlined regions flanking this hexamer are sequences which have identity or similarity with the right and left ends, respectively, of the excised circular form of the element. Insertion of the transposon, in all cases, was such that the right end of the inserted element is toward the 5′ end. The coupling sequences are shown separated from the flanking DNA, and the sequences in boldface are the coupling sequences derived from this particular insertion site. The transposons are grouped into two categories: those that transfer at high frequencies (pAM5100, pAM5101, pAM5104, pAM5105, pAM5109, pAM5110, and pAM5112) and those that transfer at low frequencies (pAM5106, pAM5107, and pAM5111).

one of the ends. For example, the difference between pAM5100 and pAM5107 is only at the similarly positioned coupling sequences TTAGTT and AATCAA, respectively, yet there is a 10,000-fold difference in the mating potentials. The derivatives pAM5105 and pAM5106 differ only by the similarly positioned sequences ATTTTA and GTTAAA, respectively, and again these derivatives differ greatly in transfer potential. For one pair, pAM5100 and pAM5107, the differences were at the transposon's right end, whereas for the other pair, pAM5105 and pAM5106, the difference was at the left end of the transposon. (Note that the transposon's right and left ends are oppositely oriented in Fig. 3.) The insertions of pAM5100, pAM5104, and pAM5112 had identical coupling sequences and gave rise to similar high transfer potentials. It is important to note that these derivatives were originally generated from independent matings using different single-colony isolates of CG110 as donors. Similarly, pAM5107 and pAM5111 had identical sequences but low transfer potentials; these two derivatives were also generated from independent matings. It is also noteworthy that the two high-transfer derivatives pAM5101 and pAM5105 had the same two coupling sequences but located at opposite ends of the transposon.

The coupling sequences of additional derivatives corresponding to high transfer potential (i.e., detectable transfer in cross-streak matings) and to low transfer potential (10 of each) were determined; however, these strains did not arise from matings independent of those described above. The sequences among high-donor-potential derivatives were not different from those of any of the high-frequency donors shown in Fig. 3, with the exception of one that had an apparent 5-bp coupling sequence at one end and a 7-bp sequence at the other. Similarly, the sequence of the low-frequency derivatives corresponded to one or the other of the two independent variants (i.e., pAM5106 or pAM5107) shown in Fig. 3.

DISCUSSION

The data presented here provide evidence that differences in Tn*916* transfer potential are related to differences in excision frequencies influenced by the nucleotide content of the coupling sequences. In contrast, transcription into either end of the transposon did not appear to affect transfer.

Independent transposon insertions in the same orientation in identical locations within the plasmid (pAM717)-borne *cylA* determinant exhibited widely differing conjugation potentials. The coupling sequence corresponding to the target was present at either end of the inserted element, depending on the particular derivative, whereas those brought in with the transposon were at the opposite end. The fact that the donor strain (CG110) from which the transposon was acquired had six

copies (7) of Tn*916* (at different locations) accounts for the diversity of coupling sequences in the various transconjugants. Although the six copies doubtless had different conjugation potentials, the transfer of one (i.e., that with the greatest potential) is likely to have *trans* activated the others (12). The disparity in donor potentials of transconjugants of the original mating (i.e., that in which CG110 had been the donor) did not appear to be due to a high-excision mutation within one of the Tn*916* elements, since the high-frequency trait was not always propagated in secondary matings. Moreover, the fact that derivatives with an identical pair of coupling sequences generated from independent mating experiments (i.e., transfer from different CG110 donor isolates) exhibited similar donor potentials adds further support for these junctions playing a determining role.

Among the pAM717::Tn*916* derivatives compared were those that were distinguishable only in their left-end coupling sequences or only in their right-end sequences. In both cases, conjugative transposition frequencies differed by as much as 10,000-fold. Interestingly, two derivatives had identical coupling sequences but at reversed positions, and both exhibited a high-frequency transfer potential. This suggests that while one of the two sequences may be of greater importance in affecting the excision frequency, that sequence can be located at either end. Differences in transfer did not relate to homology between opposite coupling sequences that might contribute to the overall homology between the transposon ends and the corresponding opposite-end DNA outside the element. That is, the coupling sequences associated with the high-frequency donors did not provide additional homology that could be viewed as contributing to a better alignment of the ends of the transposon prior to excision.

In the case of lambda recombination between *att* sites, there are data supporting the view that initial single-strand cleavage and transfer resulting in the formation of a Holliday structure leads to branch migration through the 7-bp overlap region where the second cleavage and strand transfer then occurs (19); homology between the overlap regions appears to be important for the process to occur efficiently. For Tn*916*, heterology of the overlap (coupling) sequences would be expected to retard such a branch migration mechanism. But one might anticipate that coupling sequence pairs in derivatives with high donor potential would reveal closer degrees of homology than in the case of derivatives with low donor potential. However, inspection of our paired sequences did not reveal any suggestion of this and would appear, therefore, to argue against a mechanism requiring branch migration—or at least one dependent on sequence homology.

The insertion and excision of Tn*916* and Tn*1545* circular intermediates into and from their A-T-rich target sites may be more analogous to the integration and excision of lambdoid bacteriophage DNA with respect to secondary target sites (i.e., when the 15-nucleotide core *attB* sequence that is homologous with *attP* is not available). The efficiency with which excision (induction) of such prophage occurs varies significantly. It has been reported that the curing frequencies of these secondary-site lambda lysogens vary by more than 3,000-fold (32). Some of these secondary *att* sites have been sequenced and found to have heterologous base pairs within the 15-bp core regions (21) compared with that of the lambda *attP*. The basis for such differences in excision efficiency in the case of members of the Tn*916*/Tn*1545* family may therefore resemble that of the induction of prophage located at different secondary sites in the *Escherichia coli* chromosome. The Int-Tn and Xis-Tn proteins encoded near the end of the transposon are believed to act at or near the junctions (5, 28). Integration host factor and possibly other host proteins might also bind here. Indeed the presence of potential binding sites for integration host factor near the left end of Tn*916* has been noted (5), and Bringel et al. (1) have reported on the possible requirement for a host factor that was missing in lactococci. The host factors IHF and FIS, as well as Int and Xis, are known to act at or very close to the *att* sites of the lambdoid systems, where DNA curvature and specific bending play an important role in the overall process (20, 36). In this context, it can be envisioned that the differences in coupling sequences in the Tn*916*/Tn*1545*-related systems could affect the binding and/or activity of any one or several of the proteins involved in the excision-insertion cycle.

Finally, it was of interest that strains carrying Tn*916* insertions with high-frequency donor potential were able to transfer the element in broth matings. Previously, conjugative transposition had been observed only on solid surfaces (e.g., on filter membranes). As we have now shown that transfer in broth occurs at 1 to 2 orders of magnitude lower than that on solid surfaces, it is not surprising that the earlier studies which dealt with conjugation frequencies at 10^{-7} or lower (solid surfaces) were not able to demonstrate significant conjugation in broth.

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