Characterization of the Left 4 kb of Conjugative Transposon Tn916: Determinants Involved in Excision

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The rate-limiting step in movement of the conjugative transposon Tn916, originally identified in Enterococcus faecalis, is believed to be an excision event that generates a non-replicative circular intermediate. When present on a plasmid vector in Escherichia coli, Tn916 generally excises at a high frequency. It was reported previously that insertion of Tn5 in a region near the left end of Tn916 eliminated the ability to excise; and the mutation could be complemented in trans. In this communication the nucleotide sequence of 4 kb of Tn916 DNA connecting the recently sequenced tet(M) determinant (Su et al., 1992; Burdett, 1990) with the left end of the transposon. Ten open reading frames (ORFs) were deduced, two of which (ORF3 and ORF4) were encoded in-frame within a third (ORF2). Mutants with Tn5 insertions in the ORF1 or ORF2 (ORF3 and ORF4) were defective in excision, but could be complemented in vivo by a co-resident plasmid containing the ORF1 or ORF2 determinant, respectively. The data support the view that both ORF1 and ORF2 are essential for excision. ORF1 and ORF2 are essentially identical to determinants designated xis-Tn and ini-Tn, respectively, in the closely related Tn1545. A Tn5 insertion in ORF5 eliminated conjugative transfer between E. faecalis strains. Functions for the remaining ORFs (ORF6 through ORF10) remain unknown; however, nucleotide sequences within ORF6 and ORF9 had significant homology with sequences downstream of other tet(M) determinants. © 1993 Academic Press, Inc.

Conjugative transposons are genetic elements characteristically able to promote their own intercellular transposition (reviewed by Clewell and Gawron-Burke, 1986; Clewell, 1990; Clewell and Flannagan, 1993; Scott, 1992). They frequently carry antibiotic resistance determinants, especially tet(M) (Burdett et al., 1982), and are commonly found in enterococci and streptococci. Their extremely broad host range and ability to express in both gram-positive and gram-negative bacteria has raised significant interest in their mechanism of transposition.

The most extensively studied conjugative transposons are the closely related Tn916 and Tn1545. Tn916 (16.4 kb) was originally identified on the chromosome of Enterococcus faecalis DS16 (Franke and Clewell, 1981) and encodes resistance to tetracycline. Tn1545 (25.3 kb) was initially found in Streptococcus pneumoniae BM4200 (Courvalin and Carlier, 1986) and confers resistance to tetracycline, kanamycin, and erythromycin. The two transposons move by an excision/insertion process (Gawron-Burke and Clewell, 1982, 1984; Flannagan and Clewell, 1991) that involves a non-replicative intermediate, and physical evidence for such a structure has been reported by Scott et al. (1988). Scrutiny of nucleotide sequences of junction regions and regenerated target sites, as well as the end-junctions within intermediates, revealed that: (i) excision probably results from staggered cleavages at the transposon termini with ligation of the ends generating the circular intermediates (Caparon and Scott, 1989; Poyart-Salmeron et al., 1989); and (ii) excision can restore the former target sequence precisely or imprecisely (Caillaud and Courvalin, 1987; Caparon and Scott, 1989; Clewell et al., 1988; Poyart-Salmeron et al., 1989). The analyses were conducted
using plasmid clones of the transposons in an *Escherichia coli* host where excision occurs at a relatively high frequency.

Genetic studies making use of Tn5 insertion mutagenesis (Senghas et al., 1988; Yamamoto et al., 1987) have revealed that the 10 kb region between tet(M) and the right end of Tn916 contains determinants necessary for intercellular (conjugal) transfer of the transposon. Tn916::Tn5 derivatives in this region could excise in *E. coli* and could be introduced back into *E. faecalis* by protoplast transformation where they were not able to undergo conjugative transposition. Some of these, however, could exhibit intracellular transposition to a resident plasmid (pAD1). Tn5 insertions near the left end of Tn916 identified a region required for excision in *E. coli*. In addition, these could not be introduced into *E. faecalis* via transformation. These mutations could be complemented by a plasmid clone containing the region in trans. Nucleotide sequence analyses of the equivalent region in Tn1545 (Poyart-Salmeron et al., 1989, 1990) revealed two open reading frames determining products designated Xis-Tn and Int-Tn based on an ability to facilitate excision in trans and the presence of local homology with Xis and Int proteins, respectively, of lambdoid bacteriophages. A preliminary report from our laboratory (Clewel et al., 1991), showed that Tn916 had essentially identical open reading frames. Storrs et al. (1991) subsequently reported that an insertion mutation in the Tn916 int determinant (Tn916-int1) could be complemented in trans by the int-Tn product of Tn1545. They also reported that successful conjugative transposition of the mutant Tn916-int1 required the presence of *int in trans* in both donor and recipient bacteria; although Bringel et al. (1992) subsequently reported int-Tn to be required only in donors.

Little is known about the region between int-Tn/xis-Tn and the tet(M) determinant, as Tn5 insertions in this 3 kb region were not observed. It is possible that mutations here are lethal to the element or to the bacterial host. In this communication we present nucleotide sequence data for a 4 kb sequence that links up the left end of Tn916 (Clewel et al., 1988) and the recently reported tet(M) sequence (Su et al., 1992; Burdett, 1990). We include additional genetic analyses of the region containing int-Tn and xis-Tn and also identify a determinant adjacent to xis-Tn that appears to be required for conjugative transfer.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Media**

Table 1 lists the bacterial strains and plasmids used in this study. The Tn916::Tn5 derivatives were generated from pAM620, which represents a pVA891 vector (derivative of pACYC184) carrying a cloned copy of Tn916 (Yamamoto et al., 1987). Some of the mutant plasmids were previously reported by Senghas et al. (1988); whereas others were generated in this study using essentially the same method.

The plasmids used in the complementation studies (Fig. 2) were chimeras using the vector pBDfo, a derivative of pBluescript II KS (−) (Su et al., 1992). pAM1005 was constructed as previously reported (Su et al., 1992) and was used to generate pAM1018, pAM1019, and pAM1020 by deletion from the HindIII site [within the 5' end of tet(M)] toward the left end of the transposon using exonuclease III and mung bean nuclease (Ausubel et al., 1987). pAM1006 was also constructed as previously described (Su et al., 1992); it was used to generate pAM1021 deleting (as above) from the XbaI site outside the left end of the transposon toward the right.

*E. faecalis* strains were grown in Todd-Hewitt broth (THB, Difco Laboratories), whereas *E. coli* strains were cultured in LB medium (Ausubel et al., 1987). Media and reagents used in protoplast transformation of *E. faecalis* OG1X were as described by Wirth et al. (1986). When present in selective plates, antibiotics were used at the following concentrations: tetracycline, 4 µg/ml for *E. coli* DH1
### Table 1

**Strains and Plasmids Used in This Study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype</th>
<th>Comments/reference</th>
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</thead>
<tbody>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td></td>
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</tr>
<tr>
<td>FA2-2</td>
<td><em>rif</em> fis</td>
<td>Clewell et al. (1982)</td>
</tr>
<tr>
<td>OG1X</td>
<td>str</td>
<td>Ike et al. (1983)</td>
</tr>
<tr>
<td>S11-1</td>
<td><em>str</em>, tet(M) (Tn916::Tn5)</td>
<td>This study</td>
</tr>
<tr>
<td>S11-2</td>
<td><em>str</em>, tet(M) (Tn916::Tn5)</td>
<td>This study</td>
</tr>
<tr>
<td>OG1X-669</td>
<td><em>str</em>, tet(M) (Tn916::Tn5)</td>
<td>Senghas et al. (1988)</td>
</tr>
<tr>
<td>OG1X-673</td>
<td><em>str</em>, tet(M) (Tn916::Tn5)</td>
<td>Senghas et al. (1988)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH1</td>
<td>F- recA1 endA1 gyrA96 thi-1 hsdR17 supE44 λ−</td>
<td>Manniatis et al. (1982)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAM620</td>
<td><em>erm</em>, tet(M)</td>
<td>pVA891 vector (derivative of pACYC184) carrying a cloned segment of Tn916 Yamamoto et al. (1987)</td>
</tr>
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<td>pAM669</td>
<td><em>erm</em>, tet(M), km (Tn5)</td>
<td>Yamamoto et al. (1987)</td>
</tr>
<tr>
<td>pAM673</td>
<td><em>erm</em>, tet(M), km (Tn5)</td>
<td>Yamamoto et al. (1987)</td>
</tr>
<tr>
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<td><em>erm</em>, tet(M), km (Tn5)</td>
<td>Yamamoto et al. (1987)</td>
</tr>
<tr>
<td>pAM676</td>
<td><em>erm</em>, tet(M), km (Tn5)</td>
<td>Senghas et al. (1988)</td>
</tr>
<tr>
<td>pAM696</td>
<td><em>erm</em>, tet(M), km (Tn5)</td>
<td>Yamamoto et al. (1987)</td>
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<td>pAM697</td>
<td><em>erm</em>, tet(M), km (Tn5)</td>
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</tr>
<tr>
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<td><em>erm</em>, tet(M), km (Tn5)</td>
<td>Senghas et al. (1988)</td>
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<td>pAM6010</td>
<td><em>erm</em>, tet(M), km (Tn5)</td>
<td>Senghas et al. (1988)</td>
</tr>
<tr>
<td>pBΔdeo</td>
<td>bla</td>
<td>Derivative of pBluescript II KS(−) (Su et al., 1992)</td>
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<td>Su et al. (1992)</td>
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<td>bla, xis-Tn, int-Tn</td>
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<td>This study</td>
</tr>
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<td>pAM1019</td>
<td>bla</td>
<td>This study</td>
</tr>
<tr>
<td>pAM1020</td>
<td>bla</td>
<td>This study</td>
</tr>
<tr>
<td>pAM1021</td>
<td>bla, xis-Tn</td>
<td>This study</td>
</tr>
</tbody>
</table>

containing Tn916 and 10 μg/ml for *E. faecalis* strains carrying Tn916: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; erythromycin, 150 μg/ml; fusidic acid, 50 μg/ml; and rifampin, 50 μg/ml.

**Enzymes and Reagents**

Restriction endonucleases, T4 DNA ligase, exonuclease III, mung bean nuclease, deoxyribonucleoside triphosphates, and dideoxyribonucleoside triphosphates were from Boehringer–Mannheim Biochemicals. A universal sequencing primer (catalog No. 1211) and an M13 primer (catalog No. 300304) were purchased from New England Biolabs and Stratagene Inc., respectively. Other primers were synthesized by the DNA core facility of the University of Michigan. Antibiotics, lysozyme and RNase A were from Sigma. Radioactive compounds and 14C-labeled “Rainbow” protein molecular weight markers (CFA.755) were from Amersham Corp. Sequenase kits were from United States Biochemical Corp.

**General DNA Techniques**

General procedures for isolation of plasmids and for DNA manipulations were as described elsewhere (Ausubel et al., 1987). The method for protoplast transformation was previously described (Wirth et al., 1986). Plasmids were introduced into *E. coli* cells by electroporation using the method of Dower et al. (1988). The *E. coli in vitro* transcription-
tion-translation studies utilized a kit produced by Amersham International.

**DNA Sequencing Analyses**

The DNA sequence in Fig. 1 was determined as previously described (Su et al., 1991, 1992) from templates consisting of pAM1005 and pAM1006 and their nested-deletion derivatives. The sequence was determined for both DNA strands. The sites of Tn5 within Tn916 were determined by use of the synthetic primers. Sequences were stored, matched, processed, and analyzed using a Macintosh computer and making use of MacVector 3.5 software and its GenBank data base (Release 71.0; IntelliGenetics Inc., Mountain View, CA). The nucleotide sequence GenBank accession number is L15633.

**Excision and Complementation Studies**

Excision frequencies of Tn916 and its Tn5 insertion mutants from plasmids in E. coli DH1 were determined as follows. Overnight cultures were grown in selective media. Fifty microliters of the overnight culture was used to inoculate 5 ml of fresh LB medium with erythromycin to select only the plasmid vector. This culture was incubated at 37°C for 72 h with a 100-fold dilution every 24 h. Appropriate dilutions of the 72-h culture were plated onto LB agar plates containing erythromycin. After 22 h of incubation at 37°C, plates with adequately separated colonies were replicated onto similar plates as well as LB plates containing either erythromycin and tetracycline to select Tn916 (pAM620) or erythromycin, tetracycline and kanamycin to select the Tn916::Tn5 derivatives. Excision frequencies of Tn916 and its Tn5 insertion mutants were determined based on a comparison of the number of colony forming units on the two kinds of plates.

For excision complementation analyses, the appropriate plasmids were introduced by electroporation into E. coli DH1 cells containing a plasmid bearing an excision-negative Tn916 mutant (Table 3 and Fig. 2). The newly introduced plasmid was selected for by ampicillin. The presence of the two different plasmids was confirmed by restriction enzyme digestion and agarose gel electrophoresis. Complementation of the excision function was measured by the emergence of colonies sensitive to tetracycline and kanamycin and confirmed by restriction analysis of plasmids isolated from representative susceptible colonies. It is noted that the level of LacI in the DH1 host is not able to efficiently repress expression from the lacZ promoter on the high copy plasmids tested for complementation (i.e. even in the absence of IPTG). Thus, IPTG was not used in the related experiments.

**Mating Experiments**

Filter mating experiments were performed as follows. A recipient E. faecalis FA2-2 and a donor OG1X containing Tn916 or Tn916::Tn5 insertion mutants were grown in THB media at 37°C shaking at 200 rpm for 22 h. Five microliters of the donor cells was then mixed with 50 µl of recipient bacteria. The mixture was dropped onto a membrane filter (type HA, 0.45, Millipore Corp.) which had been placed on a THB plate not containing antibiotics. After incubation at 37°C for 22 h, the cells were suspended in THB (0.1 ml) and then spread onto THB plates containing rifampin, fusidic acid, and tetracycline. The number of colony forming units, representing transconjugants, was recorded after 48 h of incubation.

**RESULTS**

**Sequence**

Figure 1 shows the nucleotide sequence (about 4 kb) of the region of Tn916 between the 3' end of the tet(M) determinant and the left end of the transposon. Deduced open reading frames are also shown and are diagrammed in Fig. 2. Information on properties of related potential protein gene products is listed in Table 2. (Although the diagram of Fig. 2 is shown in the commonly viewed ori-
orientation of the Tn916 map, the sequence of Fig. 1 is shown in the opposite orientation to enable most of the ORFs to be read in a 5' to 3' [left to right] direction. The position of potential promoter sequences that might relate to specific reading frames are underlined in Fig. 1.

ORF1 and ORF2 correspond to the xis-Tn and int-Tn determinants, respectively, previously identified in Tn1545. ORF1 is deduced to encode a 67-amino acid-residue protein of 8.1 kDa that is rich in basic amino acids (19.1%) and isoleucine (14.7%). ORF2, the largest open reading frame, corresponds to a 47 kDa protein with 405 amino acid residues. Interestingly, there are two open reading frames within ORF2, designated ORF3 and ORF4. They are both in the same reading frame as ORF2 but have translational start sites located 44 and 81 codons downstream, respectively, of the ORF2 start codon. ORF3 has a GTG start codon, whereas ORF4 begins with ATG. Both have potential ribosome binding sites. Like the ORF2 product, proteins corresponding to ORF3 and ORF4 are predictably basic. Potential sigma 70-type promoters are located upstream of ORF1 as well as between ORF1 and ORF2.

ORF5 is in the opposite orientation of ORF1, however the 5' ends of the two ORFs overlap by 17 nucleotides. Its predicted product would be a 9.2 kDa basic protein. With respect to the ATG start site the noted ribosome binding site is very close (within 1-2 nucleotides); although another potential binding site is more distally (20 nucleotides) located. A potential sigma 70-type promoter is observed upstream of the reading frame.

ORF6 corresponds to a basic protein whose translational start codon (ATG) is located 97 nucleotides downstream of the tet(M) determinant. The translational stop site is located within a region of dyad symmetry with inverted repeats designated as IR1 (Fig. 1). This region is believed to represent the transcription termination site for tet(M), based on previously reported northern blot analyses (Su et al., 1992). ORF6 may therefore be transcribed from the tet(M) promoter; however, the presence of a possible promoter just upstream (Fig. 1) suggests it might also be transcribed independently. Interestingly, the predicted ORF6 product contains 4 cysteine residues positioned in such a way as to generate a zinc finger consisting of 20 amino acid residues, nine of which are charged (6 positive and 3 negative).

ORF9 is adjacent to ORF6 and is in the opposite orientation; it corresponds to an acidic polypeptide. Its translational start codon is preceded by a good ribosome binding site, and a potential promoter (sigma 70-type) is located about 120 bp upstream.

The next reading frame, ORF10, is relatively short (23 codons) but has a strong ribosome binding site. It would encode a relatively basic protein (see Table 2). A sigma 28-type of promoter (Chater et al., 1989; Helmann et al., 1988) was noted 75 nucleotides upstream of ORF10; although, a possi-
FIG. 1
>BatXI

TTT CTG TCA AAA AAA CTG CTC TAT CTG CAA TAG ATAAAGCTACG GCAATTATATG ATCATATAGA TAGAATAACA GAAGCACATGTTAA
FLS KKV VYL Q>

               TR2

T3.2

CAGACCAAAATAATCTCTTTATGCTTTTATGCTATCTTTGTTTAGCATAAGAGTTATGCTAAAGATGATACTAAAGAA TCACTTTGCAACA AACCCCTTTCACATGGG ACTAA

               TR3

               IR2

GAGATUAAAGGAG ACA AAC GAG CAA GGC TCA CTT CCT TCC CTA GAG AGA AACG GGG GTG CAA AAG ATG AAA CCA TCT TCT YCT TAT CAG ACC
RBS MNE QGS LPL FLDR HKR GVR NKF SMS FSF Q>

               ORP7.2

ACA ATA GAA AAT CAG TTT GAC TAT ATC TGT AAA CAG GCT ATG GAA GAC GAG CGA AAG AAT TAT ATG CTT TAT CTT TCA AGG

TIENQFDYICKRAMEDERKNYMLYLSR>

ATT GCA AAG CGT GAG CTG TCC TCT CAG GAT TAT CTG GAG CTG AGT TTG CAG CAA ACA GAT AAC TAT TCA ACT
IARKREVSFSDSVGDLYLVSSQFAPTDNYST>

GAC TTT CAG ATT TTT ACA CTC AAT GGG TTA TCA GTA GGC GGT GAA AAT GAT TTG TTG AGT GAA GCA TTA GCT GAG TTG CCA
DFQIIFTNGLSVDGVENLDLSALSEALREL>

GAC AAG AAA CTT GAA ATT CTA CTG CTG TTT TAC TAT ATG ATC AGT GAC TGA CGT TCA GAA ATT GCA GAC CTG TTG AAA TTG AAC
DKKERILLTFFYFMDMSSDSEIADLKRLM>

CGT TCT ACT GTC TAT CGG CAT AGA ACC AGT GAA CTA GGC TTA ATT AAA AAG TTT ATG GAG GAA TTG GAA GAA TGA AA ACA CAA
RSTVYRGHRTSGGLALIKKFMEFEE>

               RBS

               ORP8

TAT CCT ATG ATT CCT CCT CTG ATT GTA AAG GCA ACA GAT GGC GAT ACC GAA GCG ATT AAC CAG ATT CTA CAT CAG TAC AGA
YPMNIPFPLIVKATDGDOETAEINQIILHRY>

GGT TAC ATG AGG CGT TCC CTA CGA CCT ATG AAA GAT GAA TAT GGC ATT CAA ACT ATG GTC GTT GAT GAA GTC TTA GCT GGA
GYITKRSLRLMKDKEYGNQMOSMVVDVLRG>

AGA ATG GAA ACC AGA CGT ATT ACA AAG ATT TTG TCA TTT GAA ATT AAA TATCTCTGTCT CTTTCTGTG AAGCGTGCTA AACCATTCCA
RMETFRLITKILSFEIK>

FIG. 1—Continued
FIG. 1—Continued
Fig. 1—Continued
Fig. 1—Continued
Table 2). It is noted that a frame-shift of one nucleotide would result in a fusion of ORF7 and ORF8 polypeptides. Between ORF8 and ORF1, there are no apparent factor independent transcription termination sites.

Between ORF7 and ORF10 there is a pair of inverted repeats, designated IR2 (13 b segments separated by 8 nucleotides). An RNA transcript could generate a stem–loop with a ΔG = −14.6 kcal/mole; it is followed by 6 Us. This site, designated T3,2 in Fig. 1, is positioned such that it corresponds nicely with a previously identified 3.2 kb transcript believed to be initiated from the tet(M) promoter (Su et al., 1992). The transcript is presumed to represent some degree of read-through of the earlier terminator T_{tet(M)}.

**Fig. 2.** Map generated from sequence data and plasmids used in genetic analyses. The open square indicates the very left end of Tn916. The single KpnI site of Tn916 (noted in the case of pAM1020) is not shown; it is located in the 3' end of the tet(M) determinant. Tn5 locations in Tn916 on corresponding plasmid derivatives are indicated. See Fig. 1 for the precise sites of Tn5 insertions. The black dots and arrowheads represent the start sites and the orientations of the corresponding ORFs. The open circle and the bold line in pAM1018, pAM1019, and pAM1020 represent the lacZ' promoter and the distance (100 bp) from the promoter to the Tn916 DNA. In the case of pAM1021, there is about 2.5 kb nucleotide sequence between the lacZ' promoter (not shown) and the ORF1. The plasmid vector (pBâÚ©) portion of these four clones is not shown.
TABLE 2

OPEN READING FRAMES (ORFs) DEDUCED FROM THE SEQUENCE IN FIG. 1

<table>
<thead>
<tr>
<th>ORFs</th>
<th>RBS*</th>
<th>Start codon</th>
<th>Stop codon</th>
<th>aa no.</th>
<th>Mol. wt.</th>
<th>pl</th>
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<td>ORF1</td>
<td>GGAGG</td>
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<td>67</td>
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<td>TAG</td>
<td>23</td>
<td>2,856</td>
<td>9.7</td>
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</table>

* RBS, ribosome binding site (Shine and Dalgarno 1975).

aa no., the numbers of the amino acid residues of the corresponding ORFs.

ORF1 and ORF2 Products Are Required for Excision of Tn916

Figures 1 and 2 show the location of several Tn5 insertions in Tn916. These were generated starting with pAM620 [a pVA891 vector ligated to a fragment carrying Tn916 (Yamamoto et al., 1987; Senghas et al., 1988)]; their precise locations have been determined by sequencing analyses (Fig. 1). Earlier studies (Senghas et al., 1988) indicated that Tn916::Tn5 mutants corresponding to pAM697, pAM673, and pAM669 exhibited the Tn916 excision typical for plasmid clones in an E. coli DH1 host [the Tn5 insertion of pAM669 (not shown) is to the right of tet(M)]. Table 3 shows that the excision frequencies in the case of pAM697 (Tn5 between ORF1 and ORF2) and pAM669 [Tn5 upstream of tet(M)] are 73 and 78%, respectively—essentially the same as for the "wild-type" pAM620. The excision frequency in the case of pAM673 was 23%—reproducibly about threefold lower than the others; conceivably this may be due to an effect on expression of ORF1 (xis-Tn).

Table 3 also illustrates the failure to detect excision in the case of the Tn5 insertions associated with plasmids pAM696, pAM674, and pAM676 (all in ORF2, 3, 4), as well for pAM6004 and pAM6010 (both in ORF1). To test for complementation of these mutations, the plasmids pAM1018, pAM1019, pAM1020, and pAM1021 were constructed (Fig. 2) and introduced by electroporation into E. coli DH1 cells containing specific Tn916::Tn5-carrying plasmids. (The vector portion of these plasmids was pBluescript, which is compatible with the Tn916::Tn5 plasmids.) The expression of ORF2, ORF3, and ORF4 (i.e., on pAM1018, pAM1019, and pAM1020, respectively) are presumed to be driven or at least significantly enhanced by the upstream lacZ promoter on pBluescript vector. However, in the case of pAM1021, ORF1 may depend on its own promoter, since there are about 2.5 kb of Tn916 DNA present upstream of the determinant.

As shown in Table 3, pAM1018 complemented the Tn916 defect in pAM696, pAM674, and pAM676; but pAM1019 and pAM1020 failed to complement in all cases. The data support the view that the ORF2 product, but not ORF3 or ORF4 products, were able to complement the Tn5 insertion mutations in ORF2, ORF3, and ORF4. The fact that proteins of a size corresponding to ORF3 (40 kDa) and ORF4 (36 kDa) could be detected in an in vitro E. coli transcription-translation system along with ORF2 (see Fig. 3) attests to a reasonable likelihood that they were synthesized in vivo in these experi-
<table>
<thead>
<tr>
<th>Tn916-containing plasmid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tn5 in Tn916</th>
<th>Excision of Tn916&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Excision complementation analysis&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAM696</td>
<td>Within int-Tn (ORF2)</td>
<td>0% (570)</td>
<td>14% (652)</td>
</tr>
<tr>
<td>pAM674</td>
<td>Within int-Tn</td>
<td>0% (414)</td>
<td>42% (111)</td>
</tr>
<tr>
<td>pAM676</td>
<td>Within int-Tn</td>
<td>0% (554)</td>
<td>27% (529)</td>
</tr>
<tr>
<td>pAM697</td>
<td>Between int-Tn and xis-Tn</td>
<td>73% (1581)</td>
<td>0% (134)</td>
</tr>
<tr>
<td>pAM6010</td>
<td>Within xis-Tn (ORF1)</td>
<td>0% (2651)</td>
<td>65% (1089)</td>
</tr>
<tr>
<td>pAM6004</td>
<td>Within xis-Tn</td>
<td>0% (2552)</td>
<td>64% (854)</td>
</tr>
<tr>
<td>pAM673</td>
<td>Within ORF5</td>
<td>23% (1812)</td>
<td></td>
</tr>
<tr>
<td>pAM669</td>
<td>Upstream of tet(M)</td>
<td>78% (1048)</td>
<td></td>
</tr>
<tr>
<td>pAM620</td>
<td>None</td>
<td>70% (1347)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> These plasmids are compatible with pBluescript derivatives, pAM1018, pAM1019, pAM1020, and pAM1021, used in the excision complementation analysis.

<sup>b</sup> Percentage of tetracycline sensitive colonies. The number of colonies tested is noted in the parentheses.

The data indicate that both ORF1 and ORF2 are required for excision of Tn916 and that the products of these determinants can complement (in trans) corresponding insertion mutations. Table 3 also shows that a mutation in ORF2 could not be compensated for by additional amounts of ORF1 (e.g., where pAM676 and pAM1021 were co-resident); and similarly, the presence of additional amounts of ORF2 did not overcome the dysfunction created by an insertion in ORF1 (e.g., where pAM6010 and pAM1018 were co-resident). It is assumed, in this regard, that additional ORF1 and ORF2 products are indeed synthesized due to an increased gene dosage. We cannot rule out the possibility, however, that some regulatory feature maintains these activities at a constant level, regardless of gene dosage.

**Tn5 Insertion in ORF5 Eliminates Conjugative Transfer of Tn916**

Senghas et al. (1988) showed that the Tn916::Tn5 derivative of pAM673, after insertion into the chromosome of *E. faecalis* (involves excision of the transposon from the plasmid), was unable to transfer conjugatively to another *E. faecalis* strain. Because the Tn5 insertion has now been positioned in
ORF5, and because independently generated Tn916 insertions (wild type) can vary significantly with respect to their conjugation frequencies (sometimes to <10^{-8}/donor), we believed it was important to confirm further the defect in conjugation. To do this, we used pAM673 DNA to transform protoplasts (Wirth et al., 1986) of E. faecalis OG1X generating two independent transformants S11-1 and S11-2. These, along with the previously generated OG1X-673 strain (Senghas et al., 1988), were used as donors in mating experiments. Transfer of the Tn916::Tn5 elements from these three strains to E. faecalis FA2-2 recipients could not be detected (<10^{-9} trans-conjugants per donor). In contrast, parallel mating experiments using E. faecalis OG1X-669 (Tn916::Tn5 DNA introduced via pAM669) as a donor resulted in transfer at a frequency of 3.8 × 10^{-7}/donor (average of three mating experiments).

Homology Analyses

Computer-assisted analyses comparing the ORF2-ORF1-ORF5 portion of the Tn916 sequence to the corresponding region of Tn1545 (Poyart-Salmeron et al., 1989) showed differences at only 3 nucleotides. These are noted in Fig. 1. One of these was in ORF5 (an additional C); the other two were in ORF2 and represent conserved differences in amino acid residues at position 9 (arginine vs. lysine) and 315 (valine vs alanine). The local homologies of ORF1 and ORF2 with Xis and Int of lambdoid bacteriophages have been previously noted (Poyart-Salmeron et al., 1989). Since the ORF2/Int homology is in the carboxyl terminal half of each, ORF3 and ORF4 share that homology as well. In the case of ORF6, significant homology was observed with nucleotide sequences downstream of tet(M) reported for Staphylococcus aureus (Nesin et al., 1990) and Ureaplasma urealyticum (Sanches-Pescador et al., 1988) and the tet(O) reported for Streptococcus mutans (LeBlanc et al., 1988). DNA within the adjacent and oppositely oriented ORF9 also exhibited significant homology with sequences downstream of tet(M) in the case of S. aureus (Nesin et al., 1990) and U. urealyticum (Sanches-Pescador et al., 1988).

Interestingly ORF7 shares local homology with a Streptococmyces coelicolor sigma factor (Chater et al., 1989) and sigma 28 of Bacillus subtilis (Helmann et al., 1989). The carboxyl terminal 61 residues of ORF7 exhibit 23% identity/48% similarity with the carboxyl terminus of the Streptomyces protein and 26% identity/48% similarity with that of the Bacillus protein. In the case of ORF5, ORF8 and ORF10, no significant homologies were found using a GenBank data base.

DISCUSSION

The nucleotide sequence between the tet(M) determinant and the left end of Tn916 has been found to contain at least 10 possible open reading frames corresponding to proteins consisting of 23 or more amino acid residues. ORF1 and ORF2 are essentially identical to similarly-located reading frames designated xis-Tn and int-Tn in Tn1545. However, we have noted two additional open reading frames, ORF3 and ORF4, which are within ORF2 and in the same frame. The appearance of protein bands having a size consistent with predicted products of ORF2, ORF3, and ORF4 in an E. coli in vitro coupled transcription-translation system suggests that all three proteins are expressed in vivo. Tn5 insertions into either ORF1 or ORF2 eliminated the ability of Tn916 to excise from a multicopy plasmid in an E. coli host. The defect in ORF1 could be restored by complementation in trans by a co-resident plasmid (pAM1021) carrying the corresponding region intact. Tn5 insertions disrupting ORF2, ORF3, and ORF4 also resulted in a failure to excise. These were complemented in trans by a DNA segment containing ORF2 (pAM1018) but not by plasmids carrying only ORF4 (pAM1020) or ORF3 and ORF4 (pAM1019).

In the case of Tn1545, Poyart-Salmeron et al. (1989) reported that the Int-Tn product alone was sufficient to allow excision in E.
coli. When Int-Tn was provided in trans with a "substrate" consisting of a plasmid carrying the Tn1545 ends but devoid of almost all but the kanamycin-resistance determinant in the internal portion of the transposon, they detected a very small amount of excision product (a restriction fragment from which the transposon excised). However, extended subculturing of cells (100 generations) did not result in detection of kanamycin-sensitive (i.e., transposon-free) derivatives—a result consistent with our Tn916 observations. We were not able to detect an excision product in the case of either our xis-Tn or in-Tn mutants (data not shown). In this regard, it is noteworthy that when our xis-Tn mutant (with an intact int-Tn) was provided with additional Int-Tn in trans, excision (based on generation of tetracycline-sensitive derivatives) still could not be detected. It should be kept in mind that transposition frequencies can differ dramatically depending on adjacent or junction sequences (Clewell and Grawron-Burke, 1986); conceivably, the required level of specific factors might therefore differ from one insertion to another.

The int-Tn/ORF3/ORF4 arrangement in Tn916 is in some ways analogous to a portion of Tn5, in which a 476-amino acid transposase (Tnp) and a 421-amino acid inhibitor (1nh) are encoded in the same frame within IS50R (Isberg et al., 1982; Johnson et al., 1982; Krebs and Reznikoff, 1986). It will be interesting therefore to determine whether ORF3 and/or ORF4 encode an inhibitor of Int-Tn; this possibility is currently being examined.

Tn5 insertion into ORF5 blocked conjugative transfer of Tn916 between E. faecalis strains. It also reduced excision by a factor of about 3 when present in E. coli. It is not clear whether these two phenomena are directly linked or whether they are independent effects resulting from the insertion. The conjugation assay is much more sensitive and represents a likely reduction by at least 2 orders of magnitude, compared to the only 3-fold reduction in excision. It is possible that while the Tn5 insertion may have knocked out expression of a key conjugative protein, it may also have affected the regulation of transcription of xis-Tn; the insertion is located only about 50 bp upstream of the postulated xis-Tn promoter. It is also noteworthy that since ORF5 overlaps opposingly with xis-Tn, expression of ORF5 might result in a countertranscript able to retard translation of xis-Tn and vice versa. Thus expression of one of the determinants might influence expression of the other. It is not clear, however, if this could relate to the dual effects of the Tn5 insertion.

The functions of ORFs identified between tet(M) and ORF5 remain unknown. ORF6 appears to be located within the same transcription unit as tet(M), and it is therefore conceivable that it relates to the expression of tetracycline resistance. It does, however, have a potential promoter of its own. Its apparent zinc finger and significant net positive charge suggests an interaction with DNA, and the fact that its translational stop site is within the first IR1 of the transcription termination site T$\text{e}_{\text{e}}$(M) raises the possibility that it could influence transcriptional readthrough. In this regard it was previously reported (Su et al., 1992) that during induction of Tet(M) synthesis there was some degree of transcriptional readthrough of T$\text{e}_{\text{e}}$(M) with termination occurring at a site resulting in transcripts that were 3.2 kb in size. We believe that termination of the latter occurs at the newly-identified potential factor independent transcription termination site designated $T_3_{\text{h}}$. The presence of significant DNA homology shared by the region corresponding to ORF6, as well as the opposingly oriented ORF9, with sequences reported downstream of other tet(M) [or tet(O)] determinants (e.g., in S. aureus and U. urealyticum, and S. mutans) suggests a frequent and possibly functional association with resistance determinants of this type. No significant homology was found in the GenBank database in the case of ORF5, ORF8, and ORF10.

ORF7 was interesting in the sense that it may have two possible translational start sites, and the one-base overlap between its stop codon and the start codon of ORF8 sug-
gests there could be frame-shift-generated fusions. It is also interesting that the deduced carboxyl terminus of the ORF7 product exhibits homology with the carboxyl termini of sigma factors of the sigma 28 variety. In this regard it may be of significance that a possible sigma 28-like promoter was observed upstream of ORF10.

There have been recent reports (Showsh and Andrews, 1992; Torres et al., 1991) that exposure of donor cells carrying Tn916 or similar transposons to tetracycline during overnight filter matings increases (e.g., 10- to 100-fold) the apparent frequency of transconjugants. This raises the question of whether induced transcriptional readthrough could be occurring from tet(M) all the way to the xis-Tn determinant. If this is the case, the level of transcription reaching that distance must be very small, as we previously could not detect such large transcripts by Northern blot analyses (Su et al., 1992). In our hands studies involving relatively short E. faecalis matings (4 h) in the presence of tetracycline did not show any significant increase in Tn916 transfer; whereas overnight (22 h) matings did indeed exhibit a 10- to 100-fold increase in transconjugants (unpublished data). The requirement for an extended mating period suggests that other factors including selection for secondary transfer events may be a factor (see Clewell and Flanagan, 1993). The fact that all member of the Tn916/Tn1545 family of conjugal transposons thus far would appear to have similarly positioned tet(M) determinants with respect to orientation to and distance from xis-Tn argues that there may indeed be some connection between expression of resistance and transposition functions. Additional studies will be required to reveal the molecular nature of any such linkage.

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REFERENCES


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