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Characterization of the Left 4 kb of Conjugative Transposon Tn*916*: Determinants Involved in Excision

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The rate-limiting step in movement of the conjugative transposon Tn*916*, 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*, Tn*916* generally excises at a high frequency. It was reported previously that insertion of Tn*5* in a region near the left end of Tn*916* eliminated the ability to excise; and the mutation could be complemented *in trans*. In this communication the nucleotide sequence of 4 kb of Tn*916* 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 Tn*5* 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 *int-Tn*, respectively, in the closely related Tn*1545*. A Tn*5* 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.

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 Tn*916* and Tn*1545*. Tn*916* (16.4 kb) was originally identified on the chromosome of *Enterococcus faecalis* DS16 (Franke and Clewell, 1981) and encodes resistance to tetracycline. Tn*1545* (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

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using plasmid clones of the transposons in an *Escherichia coli* host where excision occurs at a relatively high frequency.

Genetic studies making use of Tn*5* 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 Tn*916* contains determinants necessary for intercellular (conjugal) transfer of the transposon. Tn*916*::Tn*5* 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). Tn*5* insertions near the left end of Tn*916* 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 Tn*1545* (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 (Clewell *et al.*, 1991), showed that Tn*916* had essentially identical open reading frames. Storrs *et al.* (1991) subsequently reported that an insertion mutation in the Tn*916 int* determinant (Tn*916-int1*) could be complemented *in trans* by the *int-Tn* product of Tn*1545*. They also reported that successful conjugative transposition of the mutant Tn*916-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 Tn*5* 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 Tn*916* (Clewell *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 Tn*916*::Tn*5* derivatives were generated from pAM620, which represents a pVA891 vector (derivative of pACYC184) carrying a cloned copy of Tn*916* (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 pBΔfo, 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 *Hin*dIII 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 *Xba*I 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

Strain/plasmid	Relevant genotype	Comments/reference
Enterococcus faecalis		
FA2-2	*rif fus*	Clewell *et al.* (1982)
OG1X	*str*	Ike *et al.* (1983)
S11-1	*str, tet*(M) (Tn*916*::Tn*5*)	This study
S11-2	*str, tet*(M) (Tn*916*::Tn*5*)	This study
OG1X-669	*str, tet*(M) (Tn*916*::Tn*5*)	Senghas *et al.* (1988)
OG1X-673	*str, tet*(M) (Tn*916*::Tn*5*)	Senghas *et al.* (1988)
Escherichia coli		
DH1	F- *recA1 endA1 gyrA96 thi-1 hsdR17 supE44* λ–	Manniatis *et al.* (1982)
Plasmids		
pAM620	*erm, tet(M)*	pVA891 vector (derivative of pACYC184) carrying a cloned segment of Tn*916* Yamamoto *et al.* (1987)
pAM669	*erm, tet(M), km* (Tn*5*)	Yamamoto *et al.* (1987)
pAM673	*erm, tet(M), km* (Tn*5*)	Yamamoto *et al.* (1987)
pAM674	*erm, tet(M), km* (Tn*5*)	Yamamoto *et al.* (1987)
pAM676	*erm, tet(M), km* (Tn*5*)	Senghas *et al.* (1988)
pAM696	*erm, tet(M), km* (Tn*5*)	Yamamoto *et al.* (1987)
pAM697	*erm, tet(M), km* (Tn*5*)	Yamamoto *et al.* (1987)
pAM6004	*erm, tet(M), km* (Tn*5*)	Senghas *et al.* (1988)
pAM6010	*erm, tet(M), km* (Tn*5*)	Senghas *et al.* (1988)
pBΔfo	*bla*	Derivative of pBluescript II KS(−) (Su *et al.*, 1992)
pAM1005	*bla, xis-Tn, int-Tn*	Su *et al.* (1992)
pAM1006	*bla, xis-Tn, int-Tn*	Su *et al.* (1992)
pAM1018	*bla, int-Tn*	This study
pAM1019	*bla*	This study
pAM1020	*bla*	This study
pAM1021	*bla, xis-Tn*	This study

containing Tn*916* and 10 μg/ml for *E. faecalis* strains carrying Tn*916;* 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 ^{14}C-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* transcrip-

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 Tn*5* within Tn*916* 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 Tn*916* and its Tn*5* 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 Tn*916* (pAM620) or erythromycin, tetracycline and kanamycin to select the Tn*916*::Tn*5* derivatives. Excision frequencies of Tn*916* and its Tn*5* 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 Tn*916* 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 Tn*916* or Tn*916*::Tn*5* 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 Tn*916* 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-

entation of the Tn*916* 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 Tn*1545*. 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 (ATT) 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 preceeded 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. Nucleotide sequence of the left 4 kb of Tn*916*. The deduced amino acid sequences of 10 ORFs are given in single-letter codes under the nucleotide sequence. ORF3 and ORF4 are in frame within the ORF2 (*int-Tn*) open reading frame, and the translational start sites of ORF3 and ORF4 are indicated. ">" at the end of each line of amino-acid sequences indicates the corresponding sequence is encoded in the nucleotide strand shown; whereas, "<" indicates the corresponding ORF is encoded in the complementary strand. Ribosome binding sights (RBS) and possible sigma 70-like promoters and one sigma 28-like promoter are underlined. ">" in front of Tn*5* (with corresponding plasmid derivative indicated) means that the site of insertion is to the right of the nucleotide indicated by a vertical line. The DNA segments of Tn*916* in the plasmids pAM1018, pAM1019, and pAM1020 (see Fig. 2) are those from the position indicated by the horizontal arrows to the 3′ end of the sequence presented here, whereas the fragment in pAM1021 is that from the indicated site to the single *Kpn*I site in Tn*916* (see Fig. 2). The sequence from position 90 to the 3′ end was compared with the published sequence of Tn*1545*. "–" in the parentheses above the second line of the sequence indicates a "missing" nucleotide in Tn*1545*. Nucleotide differences in the Int-Tn coding region are also indicated in the parentheses above the corresponding residues; these correspond to two amino acid differences as indicated in the parentheses under the corresponding residues.

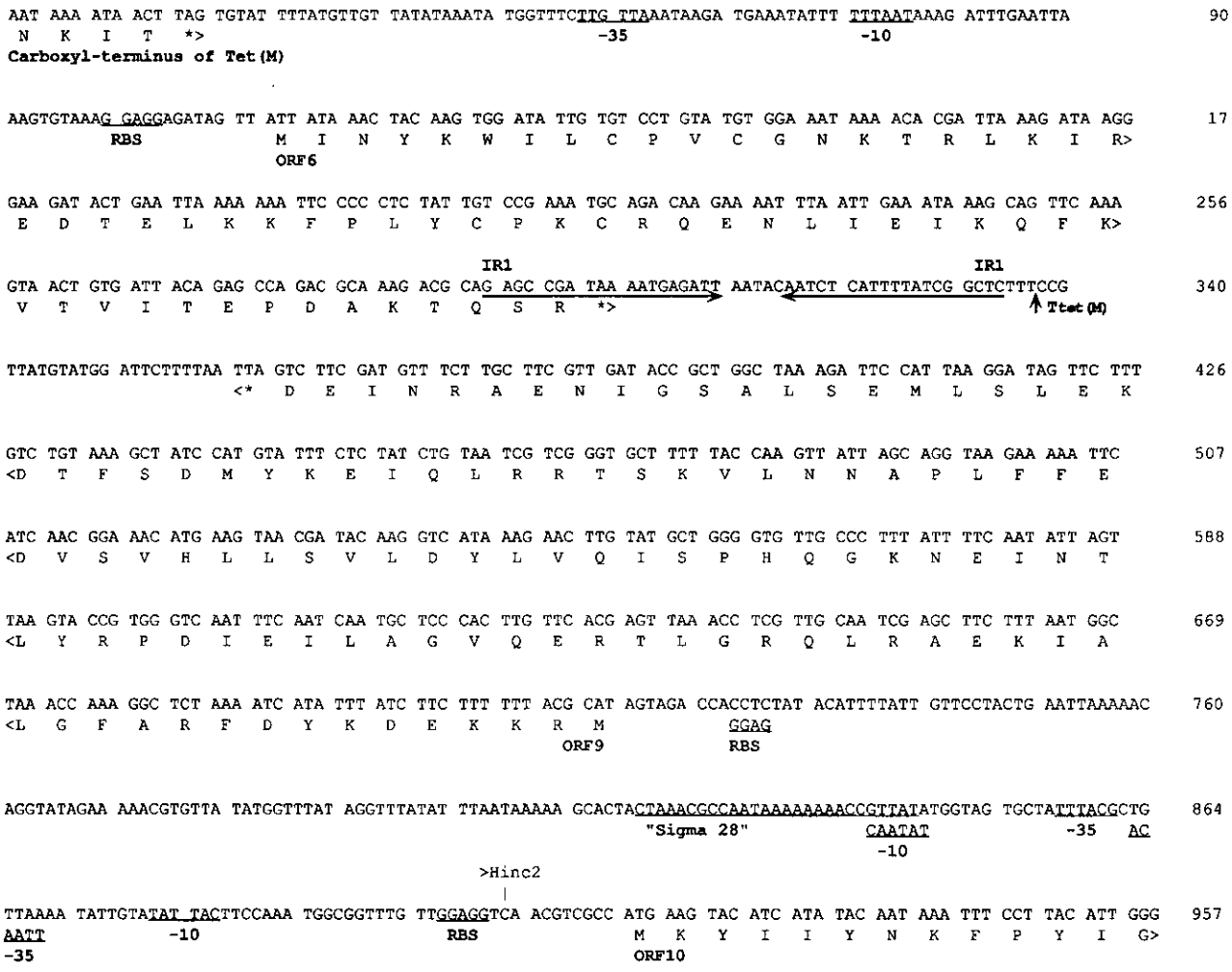
AAT AAA ATA ACT TAG TGTAT TTTATGTTGT TATATAAATA TGGTTTCTTG TTAAATAAGA TGAAATATTT TTTAATAAAG ATTTGAATTA 90
N K I T *>
-35
-10
Carboxyl-terminus of Tet(M)
AAGTGTAAAG GAGGAGATAG TT ATT ATA AAC TAC AAG TGG ATA TTG TGT CCT GTA TGT GGA AAT AAA ACA CGA TTA AAG ATA AGG 17
RBS
M I N Y K W I L C P V C G N K T R L K I R>
ORF6
GAA GAT ACT GAA TTA AAA AAA TTC CCC CTC TAT TGT CCG AAA TGC AGA CAA GAA AAT TTA ATT GAA ATA AAG CAG TTC AAA 256
E D T E L K K F P L Y C P K C R Q E N L I E I K Q F K>
IR1
IR1
GTA ACT GTG ATT ACA GAG CCA GAC GCA AAG ACG CAG AGC CGA TAA AATGAGATT AATACAATCT CATTTTATCG GCTCTTTCCG 340
V T V I T E P D A K T Q S R *>
Ttet(M)
TTATGTATGG ATTCTTTTAA TTA GTC TTC GAT GTT TCT TGC TTC GTT GAT ACC GCT GGC TAA AGA TTC CAT TAA GGA TAG TTC TTT 426
<* D E I N R A E N I G S A L S E M L S L E K
GTC TGT AAA GCT ATC CAT GTA TTT CTC TAT CTG TAA TCG TCG GGT GCT TTT TAC CAA GTT ATT AGC AGG TAA GAA AAA TTC 507
<D T F S D M Y K E I Q L R R T S K V L N N A P L F F E
ATC AAC GGA AAC ATG AAG TAA CGA TAC AAG GTC ATA AAG AAC TTG TAT GCT GGG GTG TTG CCC TTT ATT TTC AAT ATT AGT 588
<D V S V H L L S V L D Y L V Q I S P H Q G K N E I N T
TAA GTA CCG TGG GTC AAT TTC AAT CAA TGC TCC CAC TTG TTC ACG AGT TAA ACC TCG TTG CAA TCG AGC TTC TTT AAT GGC 669
<L Y R P D I E I L A G V Q E R T L G R Q L R A E K I A
TAA ACC AAA GGC TCT AAA ATC ATA TTT ATC TTC TTT TTT ACG CAT AGTAGA CCACCTCTAT ACATTTTATT GTTCCTACTG AATTAAAAAC 760
<L G F A R F D Y K D E K K R M
GGAG
ORF9
RBS
AGGTATAGAA AAACGTGTTA TATGGTTTAT AGGTTTATAT TTAATAAAAA GCACTACTAAACGCCAATAAAAAAAACCGTTATATGGTAG TGCTATTTACGCTG 864
"Sigma 28"
CAATAT
-10
-35
AC
>Hinc2
TTAAAA TATTGTATAT TACTTCCAAA TGGCGGTTTG TTGGAGGTCA ACGTCGCC ATG AAG TAC ATC ATA TAC AAT AAA TTT CCT TAC ATT GGG 957
AATT
-10
RBS
M K Y I I Y N K F P Y I G>
-35
ORF10

FIG. 1

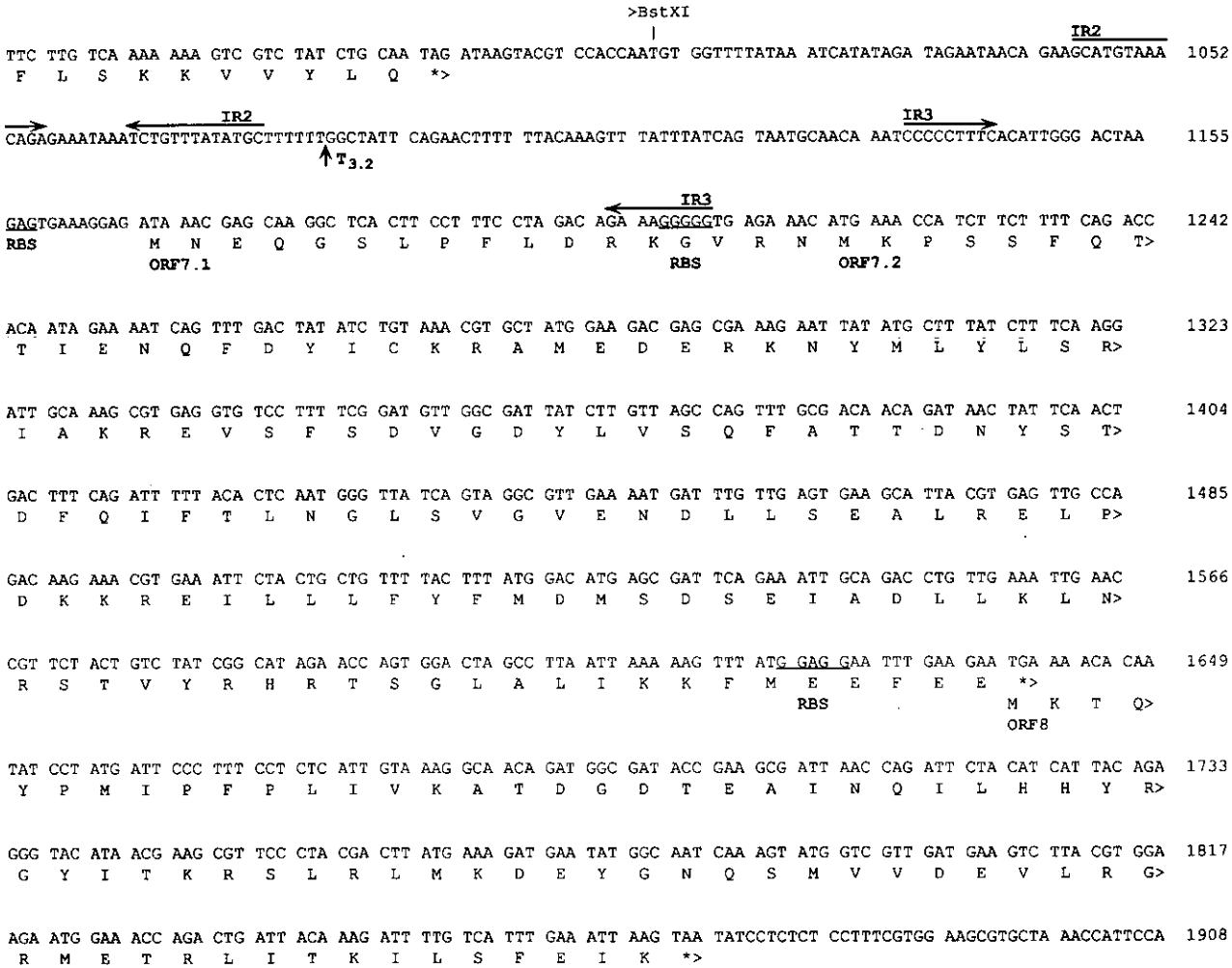

FIG. 1—*Continued*

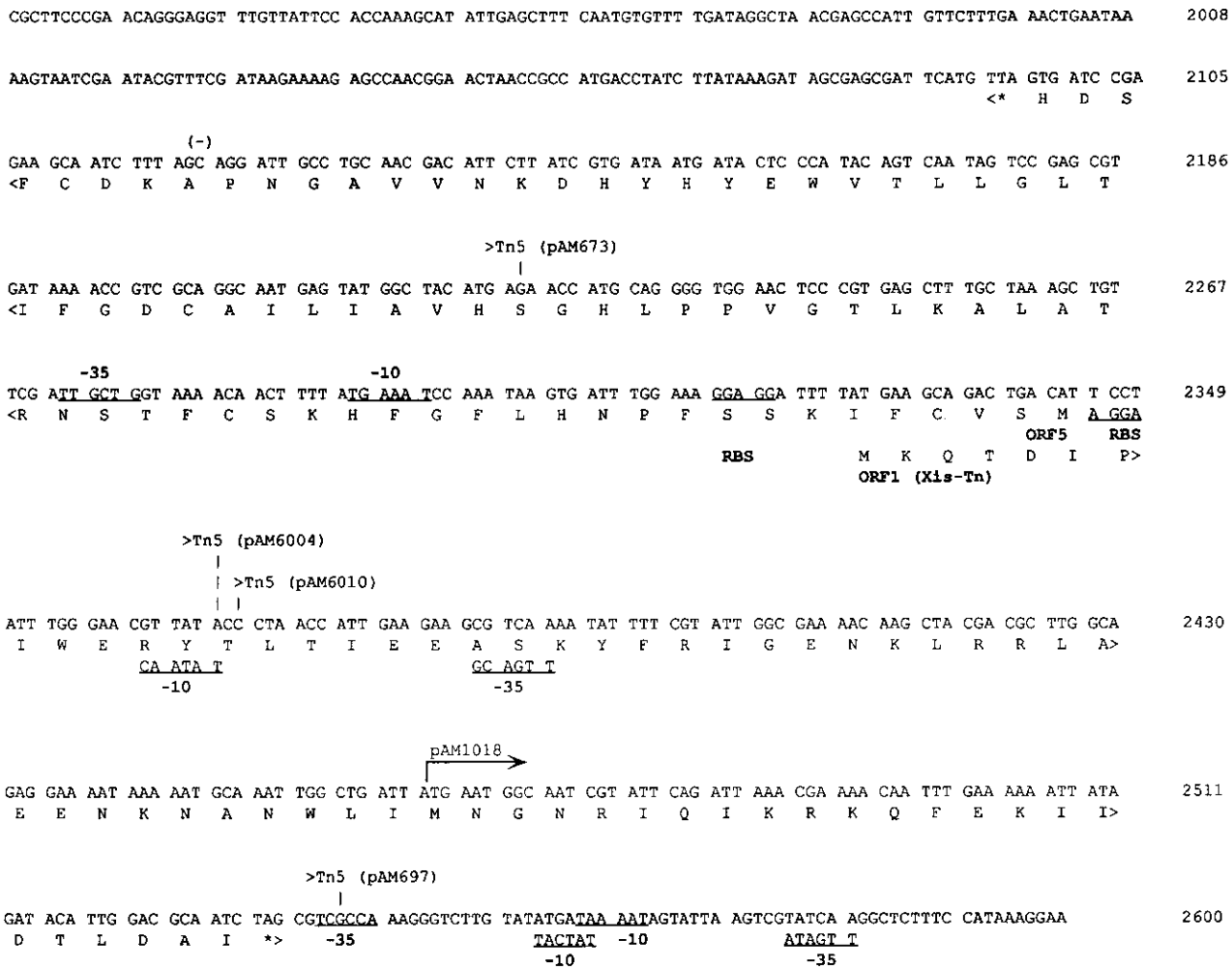

FIG. 1—*Continued*

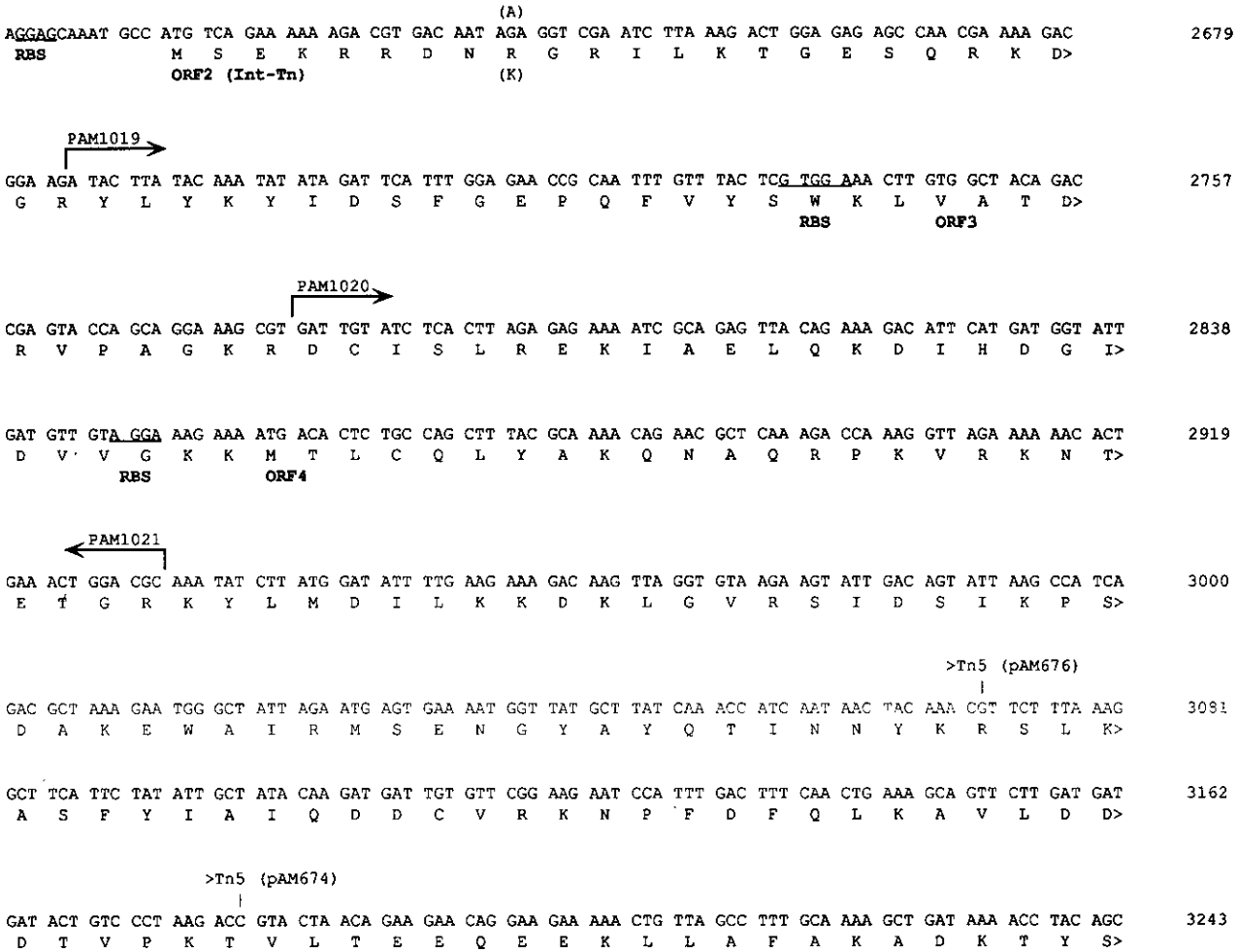

FIG. 1—*Continued*

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AAA AAT TAT GAT GAA ATT CTG ATA CTC TTA AAA ACA GGT CTT CGT ATT TCA GAG TTT GGT GGT TTG ACA CTT CCA GAT TTA      3324
 K   N   Y   D   E   I   L   I   L   L   K   T   G   L   R   I   S   E   F   G   G   L   T   L   P   D   L>

GAT TTT GAG AAT CGT CTT GTC AAT ATA GAC CAT CAG CTA TTG AGA GAT ACT GAA ATT GGG TAC TAC ATT GAA ACA CCA AAG      3405
 D   F   E   N   R   L   V   N   I   D   H   Q   L   L   R   D   T   E   I   G   Y   Y   I   E   T   P   K>

ACC AAA AGT GGC GAA CGT CAA GTT CCT ATG GTT GAA GAA GCC TAT CAA GCA TTT AAG CGA GTG TTA GCG AAT CGA AAG AAT      3486
 T   K   S   G   E   R   Q   V   P   M   V   E   E   A   Y   Q   A   F   K   R   V   L   A   N   R   K   N>

                                                                                    (C)
GAT AAG CGT GTT GAG ATT GAT GGA TAT AGT GAT TTC CTC TTT CTT AAT AGA AAG AAC TAT CCA AAA GTG GTA AGT GAT TAC      3567
 D   K   R   V   E   I   D   G   Y   S   D   F   L   F   L   N   R   K   N   Y   P   K   V   V   S   D   Y>
                                                                                    (A)

AAC GGC ATG ATG AAA GGT CTT GTT AAG AAA TAC AAT AAG TAT AAC GAG GAT AAA TTG CCA CAC ATC ACT CCA CAT AGT TTG      3648
 N   G   M   M   K   G   L   V   K   K   Y   N   K   Y   N   E   D   K   L   P   H   I   T   P   H   S   L>

CGA CAT ACA TTC TGT ACC AAC TAT GCA AAT GCA GGA ATG AAT CCA AAG GCA TTA CAG TAC ATT ATG GGA CAT GCT AAT ATA      3729
 R   H   T   F   C   T   N   Y   A   N   A   G   M   N   P   K   A   L   Q   Y   I   M   G   H   A   N   I>

              >Tn5 (pAM696)
                  |
GCC ATG ACG CTG AAC TAT TAC GCA CAT GCA ACA TTC GAT TCT GCA ATG GCA GAA ATG AAA CGC TTG AAT AAA GAG AAG CAA      3810
 A   M   T   L   N   Y   Y   A   H   A   T   F   D   S   A   M   A   E   M   K   R   L   N   K   E   K   Q>

CAG GAG CGT CTT GTT GCT TAG TAGTACAA ATGAATTTAC TACTTATTTA CCACTTCTGA CAGCTAAGAC ATGAGGAAAT ATGCAAAGAA          3899
 Q   E   R   L   V   A   *>

ACGTGAAGTA TCTTCCTACA GTAAAAATAC TCGAAAGCAC ATAGAATAAG GCTTTACGAG CATTTAAGAA AATATAAAAA GATAATTAGA AATTTATACT  3999
                                                                                    IRL

TTGTTTAGAT                                                                                                     4009
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FIG. 1—*Continued*

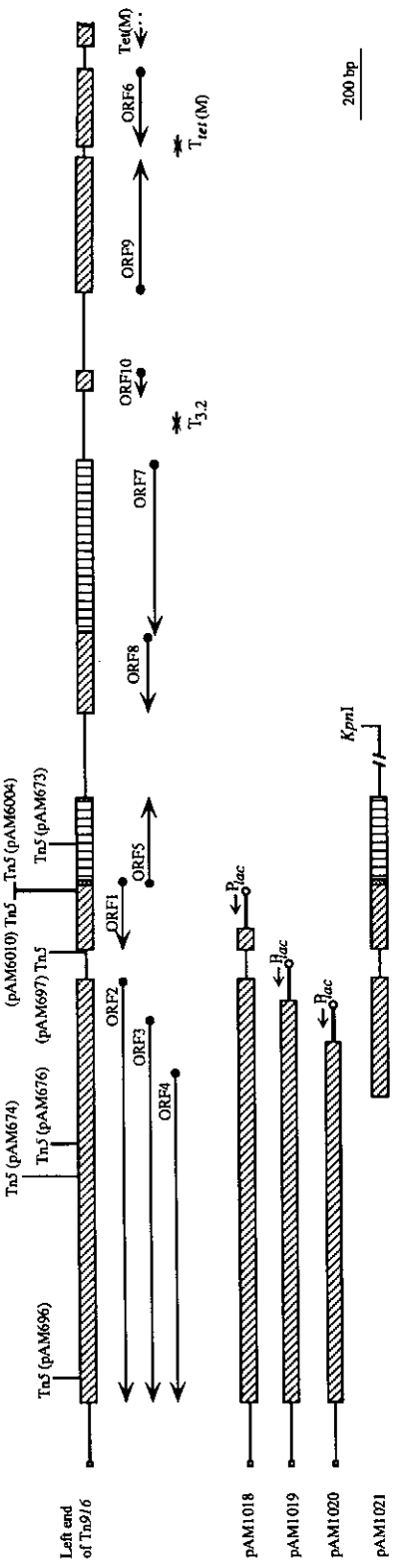

FIG. 2. Map generated from sequence data and plasmids used in genetic analyses. The open square indicates the very left end of Tn*916*. The single *Kpn*I site of Tn*916* (noted in the case of pAM1020) is not shown; it is located in the 3' end of the *tet*(*M*) determinant. Tn*5* locations in Tn*916* on corresponding plasmid derivatives are indicated. See Fig. 1 for the precise sites of Tn*5* 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 Tn*916* 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 (pBΔfo) portion of these four clones is not shown.

ble sigma 70-type is also present about 35 nucleotides upstream.

ORF7 has two possible translational start sites—one located 17 codons downstream from the other; the related reading frames are designated ORF7.1 and ORF7.2. As seen in Fig. 1, inverted repeats (9 b repeats separated by 59 nucleotides) designated IR3 flank the ORF7.1 start codon.

ORF8 corresponds to a basic protein with a start site (ATG) that overlaps the stop codon of ORF7 (TGA). The "junction," ATGA is preceded by a strong ribosome binding site 10 b upstream of the ORF8 start codon. In contrast to the predicted ORF7 products which are acidic, a protein determined by ORF8 would be basic (see 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 $\Delta G = -14.6$ kcal/mole; it is followed by 6 Us. This site, designated $T_{3.2}$ 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 readthrough of the earlier terminator T_{tet}(M).

TABLE 2

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

ORFs	RBS[a]	Start codon	Stop codon	aa no.[b]	Mol. wt.	pI
ORF1	GGAGG	ATG	TAG	67	8,110	9.3
ORF2	AGGAG	ATG	TAG	405	47,074	9.4
ORF3	GtGG	GTG	TAG	361	41,689	9.2
ORF4	AGGAa	ATG	TAG	324	37,633	9.2
ORF5	AGGAa	ATG	TAA	83	9,132	8.5
ORF6	GGAGG	ATT	TAA	62	7,400	9.8
ORF7.1	aGAGt	ATA	TGA	157	18,385	4.8
ORF7.2	GGgGG	ATG	TGA	140	16,459	4.7
ORF8	GGAGG	ATG	TAA	76	8,943	9.3
ORF9	aGAGG	ATG	TAA	117	13,543	5.6
ORF10	GGAGG	ATG	TAG	23	2,856	9.7

[a] RBS, ribosome binding site (Shine and Dalgarno 1975).

[b] 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 Tn*5* insertions in Tn*916*. These were generated starting with pAM620 [a pVA891 vector ligated to a fragment carrying Tn*916* (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 Tn*916*::Tn*5* mutants corresponding to pAM697, pAM673, and pAM669 exhibited the Tn*916* excision typical for plasmid clones in an *E. coli* DH1 host [the Tn*5* insertion of pAM669 (not shown) is to the right of *tet*(M)]. Table 3 shows that the excision frequencies in the case of pAM697 (Tn*5* between ORF1 and ORF2) and pAM669 [Tn*5* 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 Tn*5* 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 Tn*916*::Tn*5*-carrying plasmids. (The vector portion of these plasmids was pBluescript, which is compatible with the Tn*916*::Tn*5* 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 Tn*916* DNA present upstream of the determinant.

As shown in Table 3, pAM1018 complemented the Tn*916* 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 Tn*5* 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 3

EXCISION AND COMPLEMENTATION ANALYSIS IN *E. coli* DH1 CELLS

Tn*916*-containing plasmid[a]	Tn*5* in Tn*916*	Excision of Tn*916*[b]	Excision complementation analysis[b]			
			pAM1018	pAM1019	pAM1020	pAM1021
pAM696	Within *int-Tn* (ORF2)	0% (570)	14% (652)	0% (2073)	0% (2109)	
pAM674	Within *int-Tn*	0% (414)	42% (111)	0% (851)	0% (707)	
pAM676	Within *int-Tn*	0% (554)	27% (529)	0% (890)	0% (720)	0% (130)
pAM697	Between *int-Tn* and *xis-Tn*	73% (1581)				
pAM6010	Within *xis-Tn* (ORF1)	0% (2651)	0% (134)			65% (1089)
pAM6004	Within *xis-Tn*	0% (2552)				64% (854)
pAM673	Within ORF5	23% (1812)				
pAM669	Upstream of *tet(M)*	78% (1048)				
pAM620	None	70% (1347)				

[a] These plasmids are compatable with pBluescript derivatives, pAM1018, pAM1019, pAM1020, and pAM1021, used in the excision complementation analysis.

[b] Percentage of tetracycline sensitive colonies. The number of colonies tested is noted in the parentheses.

ments. The defect due to the Tn*5* insertion in ORF1 in the case of pAM6004 and pAM6010 was complemented by pAM1021.

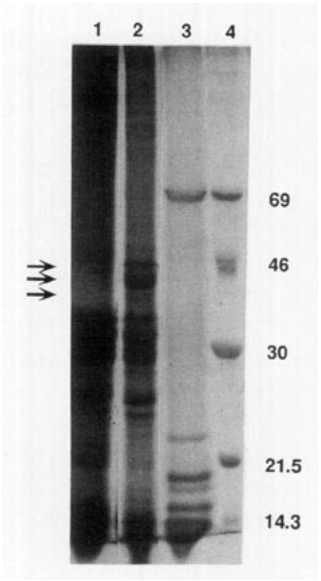

FIG. 3. Autoradiogram of SDS-polyacrylamide (10%) gel containing [^{35}S]L-methionine labeled polypeptides specified in vitro by the vector pBΔfo (lane 1) and pAM1018 (lane 2). Lane 3 was a control reaction conducted in the absence of DNA. Lane 4 shows the molecular markers; the sizes of the markers are indicated. The three arrows (close to the 46 kDa marker) indicate the polypeptide bands believed to correspond to ORF2, ORF3, and ORF4.

The data indicate that both ORF1 and ORF2 are required for excision of Tn*916* 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 Tn*916*::Tn*5* 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 Tn*5* insertion has now been positioned in

ORF5, and because independently generated Tn*916* 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 Tn*916*::Tn*5* elements from these three strains to *E. faecalis* FA2-2 recipients could not be detected ($<10^{-9}$ transconjugants per donor). In contrast, parallel mating experiments using *E. faecalis* OG1X-669 (Tn*916*::Tn*5* 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 Tn*916* sequence to the corresponding region of Tn*1545* (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 *Ureaplasama 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* (Sanchez-Pescador *et al.*, 1988).

Interestingly ORF7 shares local homology with a *Streptocmyces 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 Tn*916* 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 Tn*1545*. 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*. Tn*5* insertions into either ORF1 or ORF2 eliminated the ability of Tn*916* 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. Tn*5* 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 Tn*1545*, 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 Tn*1545* 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 Tn*916* 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 Gawron-Burke, 1986); conceivably, the required level of specific factors might therefore differ from one insertion to another.

The *int-Tn*/ORF3/ORF4 arrangement in Tn*916* is in some ways analogous to a portion of Tn*5*, in which a 476-amino acid-transposase (Tnp) and a 421-amino acid-inhibitor (Inh) are encoded in the same frame within IS*50R* (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.

Tn*5* insertion into ORF5 blocked conjugative transfer of Tn*916* 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 Tn*5* 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 counter-transcript 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 Tn*5* 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_{tet}(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_{tet}(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 $T_{3.2}$. 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 optional 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 Tn*916* or similar transposons to tetracycline during overnight filter matings increases (e.g., 10-100 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 Tn*916* 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 Flannagan, 1993). The fact that all member of the Tn*916*/Tn*1545* family of conjugative 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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