JOURNAL OF BACTERIOLOGY, Apr. 1997, p. 2731–2739 Vol. 179, No. 8
0021-9193/97/$04.00+0

The *Bacteroides* Mobilizable Transposon Tn*4555* Integrates by a Site-Specific Recombination Mechanism Similar to That of the Gram-Positive Bacterial Element Tn*916*

GENA D. TRIBBLE, ANITA C. PARKER, AND C. JEFFREY SMITH*

Department of Microbiology and Immunology, East Carolina University School of Medicine, Greenville, North Carolina 27858-4354

Received 18 November 1996/Accepted 6 February 1997

The *Bacteroides* mobilizable transposon Tn*4555* is a 12.2-kb molecule that encodes resistance to cefoxitin. Conjugal transposition is hypothesized to occur via a circular intermediate and is stimulated by coresident tetracycline resistance elements and low levels of tetracycline. In this work, the ends of the transposon were identified and found to consist of 12-bp imperfect inverted repeats, with an extra base at one end. In the circular form, the ends were separated by a 6-bp "coupling sequence" which was associated with either the left or the right transposon terminus when the transposon was inserted into the chromosome. Tn*4555* does not duplicate its target site upon insertion. Using a conjugation-based transposition assay, we showed that the coupling sequence originated from 6 bases of genomic DNA flanking either side of the transposon prior to excision. Tn*4555* preferentially transposed into a 589-bp genomic locus containing a 207-bp direct repeat. Integration occurred before or after the repeated sequence, with one integration site between the two repeats. These observations are consistent with a transposition model based on site-specific recombination. In the bacteriophage lambda model for site-specific recombination, the bacteriophage recombines with the *Escherichia coli* chromosome via a 7-bp "crossover" region. We propose that the coupling sequence of Tn*4555* is analogous in function to the crossover region of lambda but that unlike the situation in lambda, recombination occurs between regions of nonhomologous DNA. This ability to recombine into divergent target sites is also a feature of the gram-positive bacterial transposon Tn*916*.

It is becoming increasingly clear that conjugative transposons are major mediators of prokaryotic genetic exchange. The first conjugative transposon discovered, Tn*916*, has become the paradigm for a family of related elements in gram-positive bacteria (4, 30). These self-transmissible elements have a broad host range, with members of this family detected in several different species. Conjugative transposons also have been discovered in the phylogenetically ancient anaerobes, the gram-negative *Bacteroides* spp. The genus *Bacteroides* is host to a family of conjugative elements called tetracycline resistance elements (Tc^r elements) (20, 31). These large transposons (≈70 kb) encode all functions necessary for their own conjugation; nearly all members of this family encode resistance to tetracycline, but some do not encode known antibiotic resistance phenotypes (32). Conjugative transfer of these elements is thought to be responsible for the widespread resistance of *Bacteroides* to tetracycline (26, 29). Interestingly, the efficiency of Tc^r element conjugation is enhanced by pretreatment of donor cells with tetracycline. This induction phenomenon has been linked to the TetQ-Rte operon of the transposons (44), but the exact mechanism of induction is unknown.

Tc^r and related elements are also able to mediate the conjugative transfer of independent, unlinked elements. These elements include plasmids (40, 45), NBUs (34), and mobilizable transposons (11, 39). The ability of these smaller genetic units to parasitize the conjugative machinery of Tc^r elements has been linked to the presence of an *oriT* and mobilization proteins. The Mob proteins of four elements, pBI143, NBU1, NBU2, and Tn*4555*, appear to be related and to exist as a mobilization module within each of these elements (15, 16, 38). The ability of these divergent genetic elements to utilize Tc^r element transfer pathways is a significant discovery, and this pathway is apparently one mechanism by which *Bacteroides* species rapidly disseminate antibiotic resistance genes.

Previously, transfer of cefoxitin resistance between *Bacteroides* species had been linked to the mobilizable transposon Tn*4555* (39). In addition to *mobA* and *oriT*, this 12.2-kb element encodes a β-lactamase gene, *cfxA* (24). Tn*4555* is not self-mobilizing, but in the presence of a chromosomal Tc^r element or other mobilizing element, the transposon can be transferred into and subsequently transpose in a new host strain. A covalently closed circular molecule which is thought to be the transposition intermediate can be readily purified from tetracycline-treated cultures. Tn*4555* is normally integrated in the chromosome; the ability of the Tc^r elements to enhance the appearance of the circular form while simultaneously increasing transfer efficiency suggests that the circular molecule is the transfer intermediate. While the mobilization properties of Tn*4555* are becoming clearer, little is known of the element's mechanism of transposition. Introduction of Tn*4555* into *Bacteroides fragilis* by either electroporation or conjugation results in both single and multiple insertions within the chromosome (39). The enzymes responsible for this integration have not been identified, nor have the transposon terminal sequences and integration sites been characterized. The current report describes the identification of the Tn*4555* termini and begins to elucidate the events involved in their recombination with genome integration sites.

* Corresponding author. Mailing address: East Carolina University, School of Medicine, Department of Microbiology and Immunology, Greenville, NC 27858-4354. Phone: (919) 816-3127. Fax: (919) 816-3535. E-mail: jsmith@brody.med.ecu.edu.

TABLE 1. Bacterial strains and plasmids

Bacterial strains and plasmids	Characteristics[a]	Source
B. fragilis strains		
638R	Rf^r	25
132	Fus^r	40
246C	Rf^r Tc^r Fx^r, progeny of 638×CLA341 mating, one cryptic plasmid	38
Plasmids		
pFD280	Sp^r Ap^s derivative of pUC19, does not replicate in *B. fragilis*	38
pFD428	Tn*4555* cloned into *Pst*I site of pFD280	This study
pFD600	2.3-kb *Hin*dIII-*Nde*I fragment from pFD428 cloned in pUC18	This study
pFD635	589-bp Tn*4555* primary target from *B. fragilis* in pGEM-T	This study

[a] Antibiotic resistance phenotypes: Tc^r, tetracycline; Fx^r, cefoxitin; Rf^r, rifampin; Fus^r, fusidic acid; Sp^r, spectinomycin; Ap^r, ampicillin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *Bacteroides* species were grown in supplemented brain heart infusion broth (35) in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.). The following antibiotic concentrations were used unless otherwise noted: tetracycline, 5 μg/ml; rifampin, 20 μg/ml; cefoxitin, 20 μg/ml; gentamicin, 25 μg/ml; and fusidic acid, 25 μg/ml.

Escherichia coli DH5α (*recA hsdR17 lac*), HB101 (*recA rpsL*), and DH10B (*recA endA1 mcrA*) were used for subcloning and library construction, and DH5α was also used as a recipient in *Bacteroides*/*E. coli* matings. These strains were grown aerobically in L-broth (or agar) supplemented with 60 μg of ampicillin or 40 μg of spectinomycin/ml when appropriate (19).

Bacterial conjugation and transformation. Standard filter mating procedures were used for conjugal transfer between *Bacteroides* and *E. coli* strains (39). The mating conditions were chosen to favor the donor, i.e., anaerobic for *Bacteroides* and aerobic for *E. coli*. Matings with *E. coli* as the donor used RK231 as the conjugal helper plasmid. Matings with *B. fragilis* donors employed either the Tc^r element, 341TET (39), or the cryptic mobilizing element present in strain 638R (41; unpublished observations). Transformation of *Bacteroides* cells was done by the electroporation method described by Smith et al. (37), and *E. coli* plasmid transformation was by the Hanahan method (10) or by electroporation (9).

DNA isolation and analysis. Small-scale *Bacteroides* chromosome preparations were prepared as described previously (36). Purified *Bacteroides* plasmid and chromosomal DNAs were obtained by CsCl-ethidium bromide density gradient centrifugation of phenol-chloroform-treated alkaline lysates (6, 35). The circular, supercoiled transposon was isolated by CsCl-ethidium bromide ultracentrifugation from cultures of IB246C grown overnight in medium supplemented with 5 μg of tetracycline per ml (39). A restriction map of the purified circular form of Tn*4555* was derived by using standard techniques and DNA sequence data (U38243; 19, 39). Plasmid purification from *E. coli* was done as described previously (1). Plasmids were analyzed by agarose gel electrophoresis with Tris-borate or Tris-acetate buffers and staining with ethidium bromide. Restriction endonucleases were supplied by Gibco-BRL (Gaithersburg, Md.), with enzymatic digests done according to the manufacturer's directions. Other routine DNA manipulations were done as previously described (19).

DNA analyzed by Southern filter blot hybridization was restricted, electrophoresed in 0.8% agarose gels with Tris-acetate buffer, and then transferred to nylon membranes (Hybond N; Amersham Corp., Arlington Heights, Ill.) by capillary action (42). Tn*4555* was detected in genomic DNA with the 12.2-kb *Pst*I fragment from pFD428, which contains the sequence of the entire transposon, as the probe. All hybridization probes were labeled by the random primer method with a commercial kit (Pharmacia LKB Inc., Piscataway, N.J.). Membranes were prehybridized for 6 h in 3× SSC (1× SSC is 0.15 M NaCl and 15 mM sodium citrate [pH 7]), 4× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 1 mg of sheared salmon sperm DNA per ml at 65°C and then hybridized to the probe overnight under identical conditions. Membranes were washed twice for 20 min per wash at 65°C in each of the following buffers: 5% SSC–1% SDS, 1% SSC–0.1% SDS, and 0.1% SSC.

Plasmid construction. Tn*4555* was cloned from the purified circular form isolated from a *B. fragilis* 638R tetracycline-, cefoxitin-resistant transconjugant. The DNA was digested with *Pst*I and cloned into pUC19. Next, a *Bam*HI/*Sph*I fragment containing the entire transposon was subcloned into the *Bacteroides* suicide vector pFD280, creating pFD428. The plasmid pFD600 was created by excising the 2.3-kb *Hin*dIII-*Nde*I fragment from pFD428 and cloning it into pUC18. The plasmid pFD635 was created by PCR amplifying the Tn*4555* primary target from 638R genomic DNA and cloning the 589-bp product into pGEM-T (Promega Corp., Madison, Wis.).

Cloning of H3 transposon-chromosome junction fragments. Genomic DNA from Tn*4555* transformant H3 was digested with *Sau*3A and separated on a 0.6% Tris-acetate agarose gel. Regions containing the appropriate size fragments were excised, and the DNA from these regions was purified using Glassmilk (Bio101, Inc., La Jolla, Calif.). These fragments were ligated into the positive selection vector pEco251, electroporated into *E. coli* HB101, and plated on LB agar with 60 μg of ampicillin/ml. Transformant colonies containing DNA homologous to Tn*4555* were identified by colony blot hybridization (19). Clones corresponding to the two junctions were identified by restriction analysis of plasmids from the hybridizing clones.

Inverse PCR of transposon/chromosome junctions. Genomic DNAs from strains containing single insertions of Tn*4555* were digested with *Sau*3A and then self-ligated at concentrations of 3 μg/ml to preferentially form monomeric circles (5). Outward-oriented PCR primer pairs (alpha and gamma primers; see Table 2) were used to amplify the flanking chromosomal sequences from the monomeric circles (22, 23). Cycling conditions were as follows: (i) preheat for 5 min at 95°C, and add *Taq* polymerase; (ii) 45 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C; (iii) one cycle of 10 min at 72°C. PCR products were either sequenced directly or cloned into the vector pGEM-T (Promega) for plasmid sequencing.

Plasmid and genomic PCR. Amplification with plasmid or chromosomal templates was carried out as described previously (14, 28). The coupling region of the circular intermediate was amplified with primers p10a and p2 (Table 2). The chromosomal target sites of selected transformants and transconjugants were amplified from *B. fragilis* 638R DNA by using primers deduced from the junction fragment sequences (Table 2). PCR products were cloned into pGEM-T (Promega) for sequencing.

DNA sequencing. PCR products were sequenced directly from gel-purified templates using the ΔTaq Cycle Sequencing System (Amersham LIFE SCIENCE, Inc.) as recommended by the manufacturer. PCR sequencing reactions were chased with terminal transferase (U.S. Biochemical Corp., Cleveland, Ohio) as described previously (7). Plasmid DNA sequencing was done using Sequenase version 2.0 and a Sequenase Quick Denature Plasmid Sequencing kit (U.S. Biochemical). Templates for plasmid sequencing were purified with Qiagen columns (QIAGEN, Inc., Chatsworth, Calif.). The 2.3-kb *Hin*dIII-*Nde*I fragment of Tn*4555* was sequenced from a set of nested deletions created using exonuclease III as described previously (13). Each deletion clone was sequenced using the pUC18 reverse primer. The complementary strand was sequenced using primers deduced from the primary strand sequence. The Tn*4555* primary target in pFD635 was sequenced using pUC19 forward and reverse primers and internal primers deduced from the transposon-chromosome junction sequences of Tn*4555* insertions. Transposon-chromosome junction clones were sequenced using primers designed to obtain the sequences of the transposon ends and the adjacent regions (Table 2). DNA sequence data were analyzed using the University of Wisconsin Genetics Computer Group DNA analysis software (8).

Nucleotide sequence accession numbers. The primary target sequence from pFD635 and the *Hin*dIII-*Nde*I insert sequence from pFD600 were submitted to GenBank and assigned accession numbers U74490 and U75371, respectively.

RESULTS

Identification of the ends of Tn*4555*. To identify the ends of the transposon, a library of chromosomal insertions was created in *B. fragilis* (Fig. 1A). This was done by electroporation of the purified circular intermediate into competent 638R or by conjugation of the transposon from IB246C into the isogenic strain IB132. Transformed cells were selected for cefoxitin resistance, and strains containing single insertions were identified by Southern blot hybridizations of genomic DNA digested with *Eco*RI and *Sau*3A and probed with the 12.2-kb transposon fragment derived from pFD428. Detailed restriction maps were created for each insertion, and these maps were compared to the circular restriction map of Tn*4555* (Fig. 2A). In each case, the intact region of the transposon consisted

TABLE 2. Primers for PCR and nucleotide sequencing of Tn*4555*

Primer	Sequence (5′-3′)	Use
Upper alpha	CCC CAA TCC ATC CAC ACC AAC AAC CA	Inverse PCR
Lower alpha	AAA CAA GCT TCG CAA ATT CTC CA	Inverse PCR
Upper gamma	AAG CCT AAT AAA CGA GTT CTT ACT G	Inverse PCR
Lower gamma	CCA AGT TGA TGT CCT TTT TGA GTT A	Inverse PCR
1980	GCA ACC GAA AAG TGT TA	Sequencing (α)
p2	CAC CTT TTA GAA TGT GC	Sequencing (γ)
p10a	CGG ATA TTC TGA TTA G	Sequencing (α)
A1 upper	TGT GGT AGT CTT GCT GAT GTA	Target PCR
A1 lower	GCN ACT GTT ATT TAG CGT TG	Target PCR
K1 upper	AGT TAG TAT TGG AGT TTC TGC	Target PCR
K1 lower	ATA AGA TTT CCA CAT TCA	Target PCR
H3 upper	GAT CAC ATT CCC TAA AAG CA	Target PCR
H3 lower	CTA GTT TCG GTT GAG ATT CC	Target PCR
DD3 upper	CAT GCT GTT CGA TGG TTC ACG	Target PCR
DD3 lower	CAG TCC CTG AGC CTG TAC CCG	Target PCR
DD1 upper	CAG CAG CCG GAT TGA CCA GGA	Target PCR
DD1 lower	CCG ATT TAC CCC GTT TCT NC	Target PCR
DD10 upper	ATC AAC TCT ATA ACT CTG TCC	Target PCR
DD10 lower	TGA GCC TGT ACC CGA ATC ACC	Target PCR
A1 seq	GGA ATG TGA TGG TTT GTC	Target sequencing
K1 seq	CAG GAG TCT GAC TTC GG	Target sequencing
DD1 seq	AAA CGC CAA CTA TCA GGA GC	Target sequencing
DD3 seq	GTC AGA ACA GGC GGT CA	Target sequencing
DD10 seq	ACC TTT ATT AAA TGT AC	Target sequencing

of a 9.8-kb fragment from *Hin*dIII (bp 1103) to *Nde*I (bp 3411), suggesting that the 2.3-kb *Hin*dIII-*Nde*I fragment in the circular form must contain the ends of the transposon (Fig. 2A). In order to simplify analysis of the 2.3-kb *Hin*dIII-*Nde*I region, the DNA fragment was isolated from plasmid pFD428 and cloned into pUC18, resulting in pFD600.

To precisely locate the ends of the transposon, the transposon-chromosome junction fragments from one Tn*4555* insertion strain, H3, were cloned and sequenced. The sequences from the two clones were aligned with the sequence of the 2.3-kb *Hin*dIII-*Nde*I fragment (Fig. 3A). The ends of Tn*4555* were located 202 bp from the *Nde*I restriction site and consisted of imperfect inverted repeats 12 bp in length with an additional A residue at one end. The end nearest the *Hin*dIII site containing the additional A residue will be referred to as the alpha end, and the end nearest the *Nde*I site will be referred to as the gamma end (Fig. 3A). Each transposon end contained a putative integration host factor binding site: one was 37 bases from the alpha end, and the other was 83 bases from the gamma end. Each binding site had one mismatch with the *E. coli* consensus in the third position (data not shown). Located between the alpha and gamma ends in the circular form were 6 bases of sequence that could not be readily accounted for in the sequence of the H3 junction fragments.

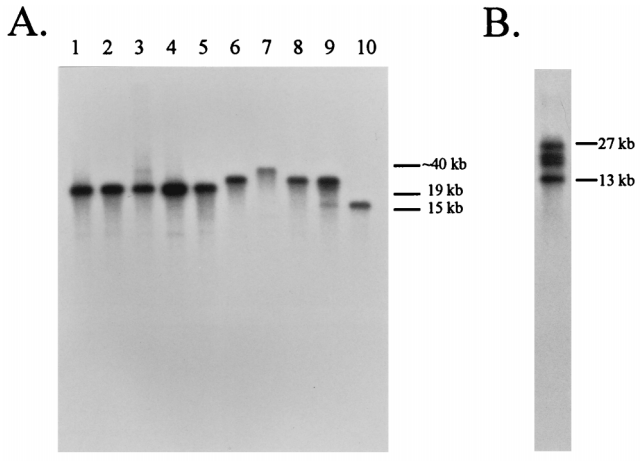

FIG. 1. Hybridization analysis of Tn*4555* insertions in *B. fragilis*. (A) Autoradiograph showing the Tn*4555* single-insertion library. Genomic DNA was digested with *Eco*RI and probed with Tn*4555*. Approximate fragment sizes are indicated at right. Insertions were obtained either from transformations (single-letter designations) with circular Tn*4555* purified from IB246C or from conjugations (double-letter designations) with IB246C as the donor. Insertions from strictly independent transformations or matings were given different letter designations, but all insertions were deemed independent on the basis of restriction analysis (*Sau*3AI) and subsequent sequence analysis. Lanes: 1, A1; 2, H3; 3, K1; 4, K20; 5, AA20; 6, DD1; 7, DD3; 8, DD10; 9, A12; 10, K17. (B) Autoradiograph of Tn*4555* insertions in IB246C.

To determine the origin of the 6 bases, the sequence for the H3 target site before insertion was obtained as follows. The naive target site was amplified from the chromosome of *B. fragilis* 638R by using PCR primers designed from the sequence of the H3 junction fragments (Table 2). The PCR product was cloned, sequenced, and then compared to the H3 junction fragments (Fig. 3B). The comparison revealed that the H3 insertion had six additional bases of DNA inserted with the alpha end of the transposon. These 6 bases differed from the sequence observed between the ends of the circular form seen in pFD428. A similar observation was noted previously for the conjugative transposon Tn*916*, which uses a variable 6-bp coupling sequence to covalently close the circular form of the transposon. These bases are also used to link the transposon to its target site during integration (2, 3).

Analysis of Tn*4555* insertions. Tn*4555* insertions derived from either conjugation or transformation were analyzed in order to document the presence of a coupling sequence associated with integration. Inverse PCR of the genomic DNA templates was performed using upper/lower alpha and upper/lower gamma primers to amplify the respective junction fragments (Fig. 2B). Once the junction sequence was known, the native chromosomal target sites were PCR amplified and com-

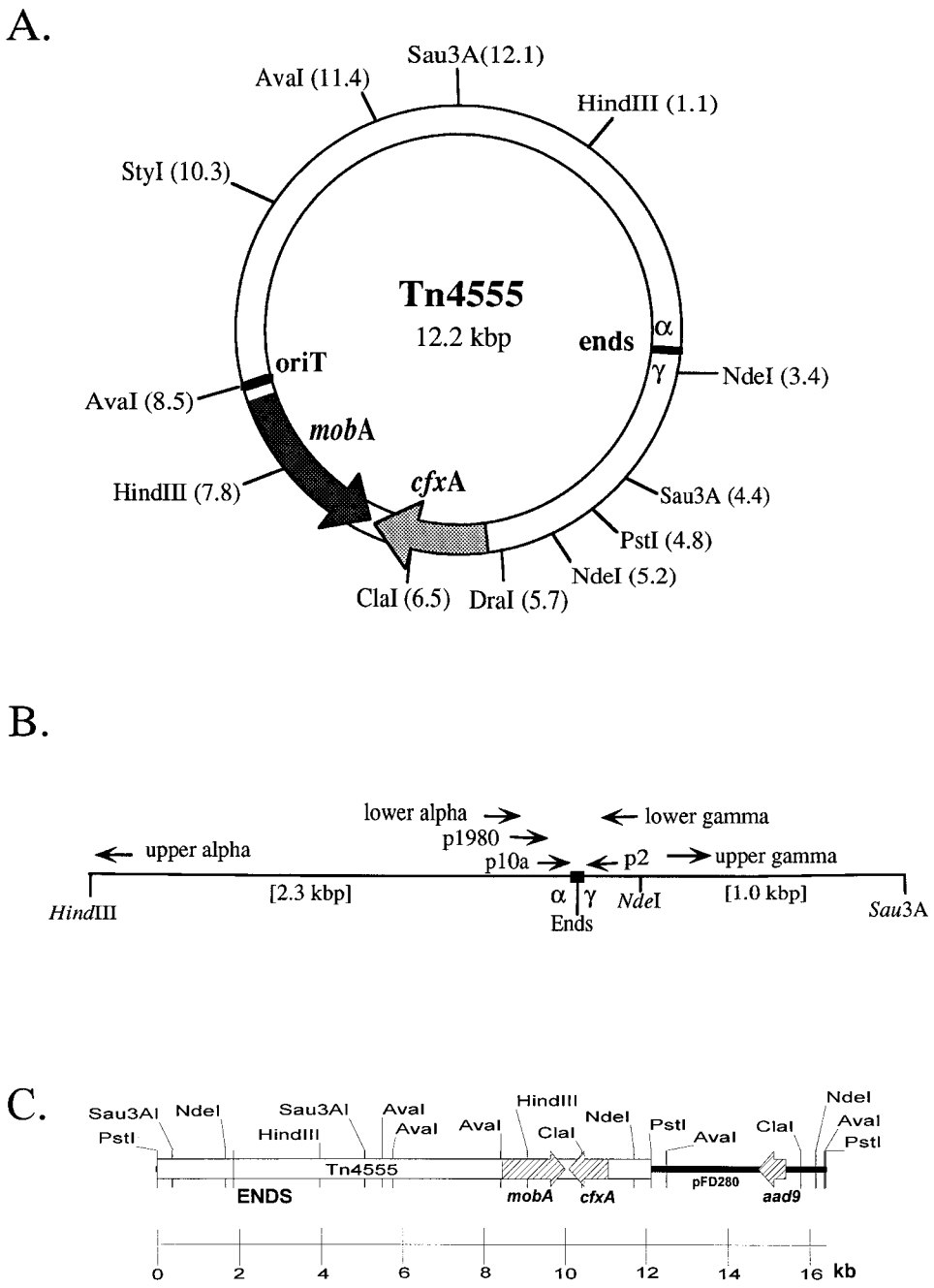

FIG. 2. Genetic and structural features of Tn*4555*. (A) Restriction endonuclease map of the circular form of Tn*4555*. The covalently closed circular form of the transposon is shown, with the alpha and gamma ends indicated. Open reading frames are shown for *mobA*, which is required for mobilization, and *cfxA*, which encodes a β-lactamase. Restriction sites are based on nucleotide sequence data and standard mapping techniques. (B) Linear restriction map showing primer locations relevant to the Tn*4555* ends. (C) Partial restriction and functional map of pFD428. The pFD280 replicon (Table 1) is shown by the thick black line, and Tn*4555* cloned into the *Pst*I site of pFD280 is shown by the open box. The *mobA*, *cfxA*, and *aad9* (spectinomycin resistance) genes are shown by the hatched marked arrows on the map, and the Tn*4555* termini are indicated by a vertical line designated ENDS. For the sake of clarity, the *Sau*3AI sites in the vector are not shown.

pared to those of the junction fragments. This allowed us to determine which 6 bases of flanking DNA were inserted with the transposon and which bases were part of the original target site. Of the seven target sites sequenced, five had the coupling sequence inserted with the alpha junction, and two had it inserted with the gamma junction (Fig. 4). The coupling sequence was observed to be inserted with either the alpha or the gamma end but never with both simultaneously. Interestingly, three different coupling sequences were seen in these insertions: CTCTTA, TGTGTA, and TATGTA.

Identification of coupling sequences from IB246C. The observation of more than two coupling sequences associated with the insertion of Tn*4555* suggested the possibility that the initial population of Tn*4555* molecules was heterogeneous. Although IB246 originally had a single insertion (39), Southern blot analysis of the strain after storage revealed that there were

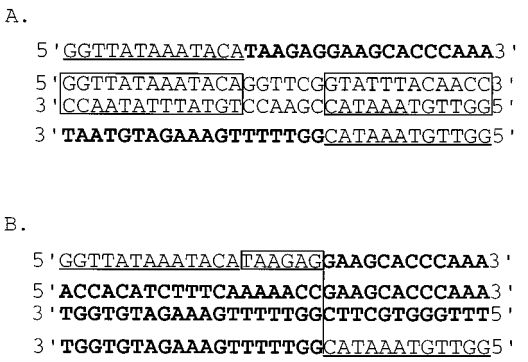

FIG. 3. Detection of Tn*4555* ends and coupling sequence by nucleotide sequence alignment. (A) Alignment of Tn*4555* circular-form ends and H3 transposon junction fragments. The nucleotide sequence of the H3 junctions is shown single stranded above and below the sequences of the closed ends. The alpha junction is above and the gamma junction is below the sequence of the circular form. Residues homologous between the junction sequences and the circular form are underlined and represent the ends of the transposon. DNA not forming the transposon ends is shown in boldface type. Imperfect inverted repeats at the transposon ends are shown by the boxed sequence. The 6 bases between the ends in the closed sequence are not homologous to the chromosomal DNA of either junction fragment. (B) Alignment of the H3 naive target and the H3 junction fragments. The H3 junction fragments are shown above and below the H3 target site. The ends of the transposon are underlined; genomic DNA is in boldface type. The 6 bases in the alpha junction not present in the target are boxed. The transposon insertion site within the target sequence is indicated by the vertical line.

currently at least four insertions in the genome; the new strain was designated IB246C (Fig. 1B). To analyze the coupling sequences, CsCl-purified covalently closed circular DNA from strain IB246C was used as the template, and the coupling sequence of Tn*4555* was PCR amplified with primers p2 and p10a. Nineteen clones were sequenced, yielding a total of seven different coupling sequences (Fig. 5). Coupling sequence CTCTTA or TATGTA was the predominant sequence in Tn*4555* insertions. The coupling sequence from DD1, TGTG TA, could represent an error in the TATGTA sequence intro-

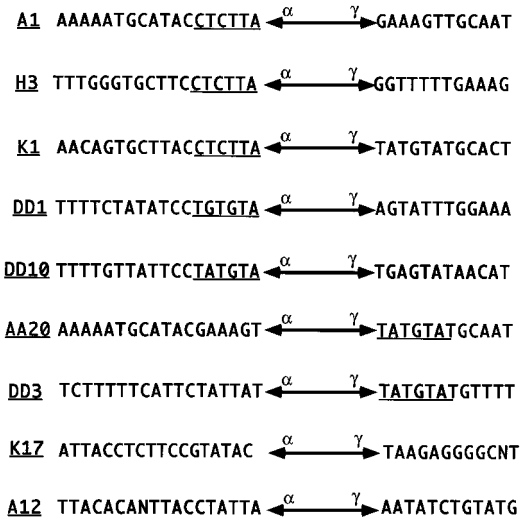

FIG. 4. Junction fragments and coupling sequences from the single insertion library. Tn*4555* is represented by the double-headed arrows, with the alpha and gamma ends labeled. Sequence data represent the DNA immediately flanking the terminal inverted repeats. Coupling sequences inserted with the transposon are underlined, but coupling sequences for insertions A12 and K17 were not determined. See the legend to Fig. 1 for strain designations.

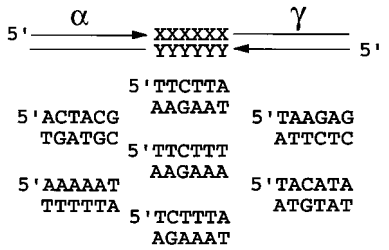

FIG. 5. Coupling sequences amplified from 246C. A schematic diagram of the ends and coupling sequence of Tn*4555* is shown. Coupling sequences are shown below the diagram.

duced by *Taq* polymerase or may represent a coupling sequence not detected from IB246C. None of the coupling sequences from IB246C were the same as the sequence from the clone pFD428. However, pFD428 was constructed by using Tn*4555* DNA isolated from another *B. fragilis* transconjugant. These results confirmed the variation in the coupling sequence and implied that the variation seen in the circular form was derived from the multiple chromosomal insertion sites of the transposon in strain 246C.

Transposition assays using pFD428. In order to observe transposition of Tn*4555* in a defined system, we utilized the unusual properties of pFD428 (Fig. 2C). This plasmid was based on the suicide vector pFD280 and contains the entire transposon cloned from the purified covalently closed circular form via the unique *Pst*I site (Fig. 2A and C). pFD428 replicates as a plasmid in *E. coli*, but there is no functional *Bacteroides* replicon. Thus, when conjugated into *B. fragilis* 638R, pFD428 behaves as a transposon and inserts into the chromosome via the intact Tn*4555* ends. The integrated form of pFD428 can subsequently excise from the *Bacteroides* chromosome and be transferred back into *E. coli*, where it regains its plasmid function. This system allowed us to begin with the known coupling sequence of pFD428 and follow it through one round of insertion, excision, and conjugation.

After transfer of pFD428 from *E. coli* into *Bacteroides*, several transconjugants from independent mating experiments were isolated and screened by Southern blot analysis to identify the insertion sites. Approximately half of the transconjugants appeared to be inserted in the same *Eco*RI fragment. Two of these transconjugants were selected for further analysis, along with one other independent insertion. The junction fragments of the integrated element were obtained by inverse PCR and sequenced. Two of the transconjugants were inserted in target sites already sequenced during analysis of the insertions, as discussed above. The integrated transposon/plasmid from each transconjugant was then mobilized back into *E. coli*, and several coupling regions were sequenced from each mating. The results of these transposition assays are shown in Table 3. The original coupling sequence from pFD428 (α 5′GGTTCG γ) was detected in all three insertions associated with either the alpha or the gamma junction. After transfer of the transposon/plasmid back into *E. coli*, the assays showed a mixture of coupling sequence products. Two coupling sequences were detected for each assay, representing the 6 bases flanking either transposon end. These results clearly indicate that the transposon derives its coupling sequence from the 6 bases of genomic DNA flanking the ends.

Analysis of the Tn*4555* primary target site. To determine if the transposon had a preferred target site sequence, the insertion site sequences were aligned using the Wisconsin Package programs Bestfit and Gap. It was discovered that for four of

TABLE 3. Identification of junction fragments and coupling sequences resulting from the Tn*4555* transposition assay

Strain	*B. fragilis* junction fragments[a]	*E. coli* plasmid coupling sequences[b]
TA2	α 5′GGTTATAAATACATACATAGTAAGAA	αGGTTCGγ (5)
	γ 5′GGTTGTAAATACCGAACCTGCACTAA	αTACATAγ (2)
TA9	α 5′GGTTATAAATACAGGTTCGNAAAGTA	αGGTTCGγ (5)
	γ 5′GGTTGTAAATACTATTTATGTTTTTG	αTAAATAγ (1)
TA10	α 5′GGTTATAAATACAGGTTCGGTATGCA	αGGTTCGγ (5)
	γ 5′GGTTGTAAATACGAAAGTTGCAATAC	αACTTTCγ (4)

[a] Tn*4555* ends are underlined, with the specific junction fragments indicated by alpha or gamma. The inserted coupling sequences from pFD428 are indicated in boldface type.

[b] The coupling sequences identified from *E. coli* plasmid progeny are shown, with the orientation relative to the ends indicated by alpha and gamma. Numbers in parentheses represent total number sequenced for each mating.

the insertions, large regions of sequence were identical, overlapping in a way to suggest that these insertions were within the same fragment of DNA. This information was consistent with data from Southern blot hybridizations, which indicated that insertions A1, H3, K1, and AA20 were in similar-size *Eco*RI fragments (Fig. 1A). The naive target region was amplified by using PCR primers that mapped to the outermost insertions, H3 and K1. PCR reactions with these primers amplified a 589-bp fragment of the *B. fragilis* genome, which was subsequently cloned and sequenced. Based on the original target sequence overlaps, these primers were expected to produce a product approximately 600 bp long.

Further analysis of the primary target, designated PT, revealed two large regions of directly repeated sequence. The two repeats were adjacent and consisted of 207 bp with 78% identity. Tn*4555* insertions occurred within two general locations within these repeats (Fig. 6): either at the start or at the end of a repeat. One insertion region was in fact precisely between the two repeated copies. Two sites within the PT had multiple insertions of the transposon; in each case, there were two insertion sites exactly 6 bases apart. The orientation of the insertions relative to that of the target was the same: within the PT as shown in Fig. 6, insertions were oriented with the alpha end to the left and the gamma end to the right. Two insertions from the transposition assays were also found to be within the PT; these insertions sites are indicated in Fig. 6.

FASTA comparison of the PT nucleotide sequence to the GenBank database revealed no significant homology with other known sequences. Translation of the sequence into open reading frames revealed one large open reading frame. The putative protein was 101 amino acids in length, with a high (12%) leucine content, but it did not share significant homology with any other protein in the database.

Southern blot hybridization analysis of the genomic DNA of a variety of *Bacteroides* species revealed the PT to exist only in *B. fragilis* (Fig. 7). Within *B. fragilis*, four bands hybridized to the probe, two strongly and two weakly. This indicates that a PT-like sequence exists in at least three additional sites in the chromosome, with various levels of sequence conservation.

Analysis of Tn*4555* secondary targets. Based on Southern blot hybridization results, the majority of Tn*4555* insertions are in the PT *Eco*RI fragment. Besides these preferred sites, there are other less commonly utilized targets. These secondary targets are represented in the single-insertion library by clones A12, K17, DD1, DD3, and DD10 and by transposition assay strain 9. The secondary sites had no homology with the PT direct repeats and no nucleotide or translational homology with any database sequences. Nucleotide alignment of the secondary targets using Pileup showed limited sequence conservation among the target sites (data not shown).

During screening of Tn*4555* insertions for selection of the transformant and transconjugant single-insertion libraries, a total of 94 different strains were analyzed by Southern blot hybridization of *Eco*RI-restricted genomic DNA. On the basis of migration rates of the hybridizing bands, we were able to determine which insertions were in the PT versus the secondary targets. Although not all of the insertions were independently obtained, we estimate that approximately 50% of the insertions were single insertions in the PT. In addition, another 30% of strains had insertions in the PT as well as at a second site, similar to our previous observations (39). The final 20% of strains had insertions in secondary targets only. According to these estimates, Tn*4555* appears to favor insertions into the PT but is also able to effectively utilize secondary target sites.

DISCUSSION

In our study, we have analyzed the transposition behavior of the mobilizable transposon Tn*4555*. While not a true conjugative transposon, Tn*4555* is part of a family of elements that are transferred by the *Bacteroides* Tc^r transposons. Through development of a model of transposition for this element, we hope to gain a better understanding of the evolution of this gene transfer network in these anciently diverged gram-negative bacteria.

We have shown that the ends of Tn*4555* are short inverted

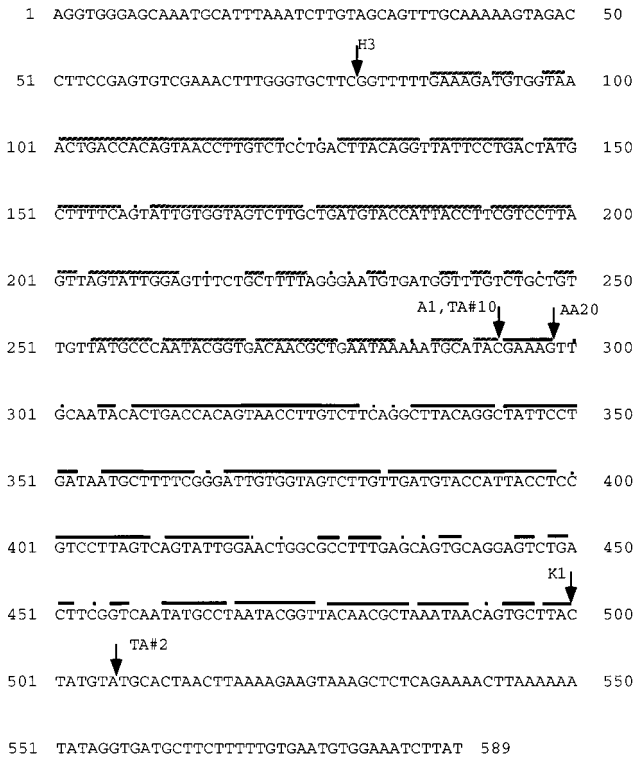

FIG. 6. Nucleotide sequence of the Tn*4555* primary target. Insertion sites within the PT are shown by arrows above the sequence. The first and second imperfect direct repeats are represented by the hatched and solid bars, respectively. The bars are superimposed over regions of sequence identity.

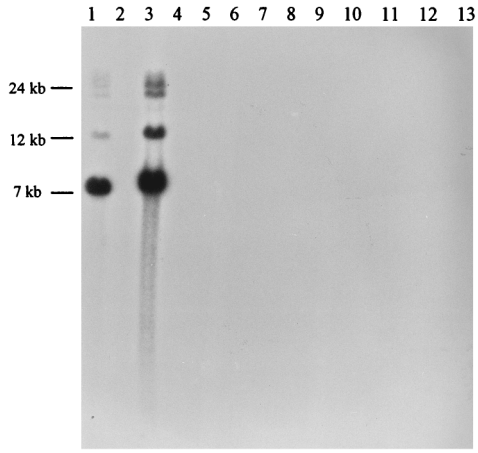

FIG. 7. Southern blot analysis of *Bacteroides* species. Genomic DNA was digested with *Eco*RI, and filter blots were prepared. These were probed with the 589-bp primary target fragment under high-stringency conditions. The PT has no internal *Eco*RI sites. Lanes: 1, ATCC 25285; 2, *B. fragilis* 20656-2-1; 3, *B. fragilis* 638R; 4, *B. uniformis*; 5, *B. ovatus*; 6, *B. thetaiotaomicron*; 7, *B. vulgatus* CLA341; 8, *B. vulgatus* WAL7062; 9, *B. distasonis* CLA348; 10, *B. eggerthii*; 11, *B. merdae*; 12, *B. stercoris*; 13, *E. coli* DH5α.

repeats, a characteristic common to many classic transposons and insertion sequences, including another mobilizable transposon from *Bacteroides*, Tn*4399* (12). Unlike most of these elements, Tn*4555* does not duplicate its target site upon insertion. Instead, a 6-bp sequence of transposon origin is inserted at the integration site. This appears to be identical to the mechanism used by Tn*916* (2), which uses a 6-bp coupling sequence to recombine with the integration site, and is reminiscent of conservative site-specific recombination used by lambda and other phages to achieve integration. In fact, preliminary results indicate that the integrase of Tn*4555* is related to the XerC recombinase of *E. coli*, a member of the lambda family of site-specific recombinases (unpublished data). The coupling sequence of Tn*4555* may therefore be functionally analogous to the crossover region of lambda, with one significant difference. The crossover region of lambda must be identical to *attB* for efficient recombination to occur, while the coupling sequence of Tn*4555* is not homologous to its site of insertion. Previous models of conservative site-specific recombination concluded that the crossover regions must be homologous for efficient branch migration and subsequent recombination to occur. A recently proposed "strand-swapping" model of site-specific recombination suggests that this homology may not be an absolute requirement and that efficient recombination can occur between nonhomologous sequences (21).

Our observation of a heterogeneous population of coupling sequences within the circular form can be explained by recombination between nonhomologous target sites and coupling sequences. Sequence analysis of the junction fragments and target sites indicated that the coupling sequence was always inserted with the transposon, with either the alpha or the gamma end. The pFD428 transposition assays showed that subsequent excision of the transposon generated a new coupling sequence from the DNA flanking the transposon inverted repeats. Excision of the transposon by joining the previously inserted coupling sequence with nonhomologous target DNA would result in a new coupling sequence of heteroduplex DNA. This heteroduplex region could be resolved by mismatch repair or by replication of the single-stranded conjugation product. This resolution would result in two potential coupling sequences for each insertion. While we do not have direct physical evidence for the existence of a heteroduplex coupling sequence, this idea is supported by the mixed populations produced by the transposition assays.

Based on the current observations and previous models for Tn*916* transposition, a proposed model for Tn*4555* transposition can be summarized as follows. Excision of the molecule occurs by recombination between the 6 bases of DNA flanking the transposon, resulting in a covalently closed circular molecule with a heteroduplex coupling sequence. At this point the coupling sequence could be converted to homoduplex DNA by mismatch repair enzymes, or the transposon could be transferred immediately to a recipient cell. Although little is known about the mechanism of conjugative transfer in *Bacteroides*, the similarity of the Tn*4555* MobA protein to TraI of RP4 and the recent identification of a nicked circular intermediate of Tn*4555* transfer (39a) suggests a conventional single-stranded DNA transfer mechanism. Once in the recipient, the single-stranded molecule is copied and ligated to produce a double-stranded circular molecule with a homoduplex coupling sequence. The circular intermediate is then aligned with the insertion site and integrated by site-specific recombination. This results in an integrated transposon with one strand of the coupling sequence to the left and one to the right on the opposite strand, each overlapped with 6 bases of the genomic DNA of the target site. These heteroduplex overlaps are resolved by replication, resulting in two daughter cells, one with the coupling sequence to the left, and one with the coupling sequence to the right. This model explains the observations of Tn*4555* transposition documented in this report: lack of target site duplication, insertion of the coupling sequence to either the left or the right, and generation of multiple coupling sequences from the DNA flanking the terminal repeats.

Analysis of the primary target sequence reveals additional evidence for integration of Tn*4555* by site-specific recombination. The first step for site-specific integration is synapsis, the alignment of the DNA element with its insertion site (27, 43). The second step is simultaneous nicking of the analogous 5′ strands of the overlap regions followed by strand swapping and Holliday junction resolution. Two sets of PT insertion sites are separated by 6 bp, the same number of bases as the coupling sequence contains. These double-insertion sites could each represent one synaptic site, with strand nicking at each of the 5′ ends of the crossover region resulting in two possible transposon insertions 6 bases apart.

Tn*4555* use of the PT target site is intriguing, as the duplication of this genomic locus is indicative of previous recombinational activity in this region. The selection of the periphery of the repeat as the site of integration shows that these regions may have some inherent characteristics favorable for recombination. Tn*4555* does not require the PT sequence for recombination; the transposon can integrate in other sites on the *B. fragilis* chromosome and in other *Bacteroides* species that lack the PT. The nucleotide sequences of targets in other species have not been determined, but they may be similar to the secondary targets in *B. fragilis*. The alignment of the secondary targets shows some limited sequence homology between the six sites (data not shown), but the targets have no homology to the ends of the transposon. Tn*916* target selection has been shown to be determined in part by the binding affinity of the integrase for target DNA (17, 18). This binding affinity may be dependent on the secondary structure, as Tn*916* targets have been shown to have intrinsic curvature, a feature also detected for lambda *att*B. Curvature of DNA has been attributed to stretches of poly(A) and poly(T) within the sequence; all of the Tn*4555* secondary targets have AT-rich regions surrounding

the integration sites. The sequence homology between secondary target sites may be a reflection of secondary structure. Further analysis will be necessary to determine the exact basis of target selection for Tn*4555*.

Comparison of the features of Tn*4555* transposition with those of other *Bacteroides* mobilizable elements reveals them to be dissimilar. Tn*4399* creates a 3-bp duplication of the target site upon insertion and inserts a 5-bp fragment with the right end only (12). NBU1 selects its target site based on homology between the ends and the target sequence (34), apparently integrating via a site-specific recombination mechanism more similar to that of lambda than to that of Tn*916*. The preferred target for NBU1 in *Bacteroides thetaiotaomicron* is the 3′ end of the leucine tRNA gene (33); many phage also insert at a similar location in other tRNA genes. While all of these mobilizable elements are able to utilize the Tc^r element genetic transfer pathway, they each have their own distinctive mechanisms of transposition.

In conclusion, the mobilizable element Tn*4555* appears to use the same mechanism of transposition as Tn*916*. These elements are integrated by site-specific recombinases but have a relaxed requirement for crossover site homology. This could represent an evolutionary advantage for conjugative elements, as it releases them from integration into one specific target sequence. For effective transposition to occur between different genera or species, an integration mechanism that can utilize a variety of target sites is preferred over one with stringent sequence requirements, as the specific sequence required may not be present in the new host. Bacteriophages and NBU1 have circumvented this difficulty by selecting a target site that is highly conserved from one genus to another. The advantage provided by this new subclass of site-specific recombination can be seen in the separate evolution of two transposons utilizing the same basic mechanism of transposition: Tn*4555* and Tn*916* have no significant homology at the DNA sequence level and thus appear to have evolved by separate pathways. Future studies of Tn*4555* will focus on the specific proteins required for integration and excision. These studies should help further our understanding of the role of conjugative and mobilizable transposons as mediators of intercellular genetic exchange.

ACKNOWLEDGMENTS

This work was supported in part by grant PHS AI-28884 (C.J.S.).

We thank E. R. Rocha for helpful technical advice and J. P. Coleman and D. J. Smalley for critical reading of the manuscript.

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