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Identification of a Circular Intermediate in the Transfer and Transposition of Tn*4555*, a Mobilizable Transposon from *Bacteroides* spp.

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Transmissible cefoxitin (FX) resistance in *Bacteroides vulgatus* CLA341 was associated with the 12.5-kb, mobilizable transposon, Tn*4555*, which encoded the β-lactamase gene *cfxA*. Transfer occurred by a conjugation-like mechanism, was stimulated by growth of donor cells with tetracycline (TC), and required the presence of a *Bacteroides* chromosomal Tc^r element. Transconjugants resistant to either FX, TC, or both drugs were obtained, but only Fx^r Tc^r isolates could act as donors of Fx^r in subsequent matings. Transfer of Fx^r could be restored in Fx^r Tc^s strains by the introduction of a conjugal Tc^r element from *Bacteroides fragilis* V479-1. A covalently closed circular DNA form of Tn*4555* was observed in donor cells by Southern hybridization, and the levels of this circular transposon increased significantly in cells grown with TC. Both the *cfxA* gene and the Tn*4555* mobilization region hybridized to the circular DNA, suggesting that this was a structurally intact transposon unit. Circular transposon DNA purified by CsCl-ethidium bromide density gradient centrifugation was used to transform Tc^s *B. fragilis* 638, and Fx^r transformants were obtained. Both the circular form and the integrated Tn*4555* were observed in transformants, but the circular form was present at less than one copy per chromosomal equivalent. Examination of genomic DNA from Fx^r transformants and transconjugants revealed that Tn*4555* could insert at a wide variety of chromosomal sites. Multiple transposon insertions were present in many of the transconjugants, indicating that there was no specific barrier to the introduction of a second transposon copy.

Studies of the transmissible antibiotic resistance determinants in *Bacteroides* species have uncovered a variety of unique conjugal elements. These include the *erm* plasmids pBF4 (48), pBFTM10 (pCP1 [18, 45]), and pBI136 (35); the mobilizing transposon Tn*4399* (13); the large (>70-kb) chromosomal elements encoding tetracycline (TC) resistance or Tc^r and *erm* (TET elements [1, 9, 29]); and the cryptic, nonreplicating *Bacteroides* units (32). The TET elements are among the most widespread of these conjugal elements and are likely responsible for the high rate of *Bacteroides* Tc^r (67% [44]) that has arisen in the past few decades. The first studies of Tc^r transfer described the unusual effect of TC pretreatment on conjugation frequency, and it was shown that growth of donor cells with TC for five or more generations enhanced transfer frequency severalfold (23). Subsequent studies documented the lack of plasmid involvement in the transfer process (17, 19, 41), but it became apparent that these chromosomal elements could promote or increase the transfer of plasmids such as pBFTM10, several classes of the small cryptic *Bacteroides* plasmids, and the nonreplicating *Bacteroides* units (19, 31, 32). Thus, the ability of TET elements to enhance gene transfer may play a central role in the dissemination of antibiotic resistance in these organisms.

The mechanisms of gene transfer and mobilization in *Bacteroides* species are not well characterized, and the way in which TC treatment affects these processes is not understood. Very low TC concentrations (0.1 μg/ml) did not enhance Tc^r transfer in *Bacteroides fragilis* V479-1 (41), and in *Bacteroides uniformis*, the production of the nonreplicating *Bacteroides* units was detected after overnight growth with 0.3 μg but not 0.1 μg of TC per ml (32). At the drug concentrations used in these experiments, it is not clear whether the TC effects are due to a true induction or a selection. In this regard, experiments focusing on the temporal expression of TC-enhanced functions have not been done, but two genetic loci, *rteA* and *rteB*, responsible for controlling TC-dependent transfer of a TET element, have been identified elsewhere (43). These genes also seem to be required for the TC-dependent appearance of the nonreplicating *Bacteroides* units, and they appear to be part of a TC signal transduction pathway with homology to two-component regulatory systems.

Another *Bacteroides* conjugation system that has been studied is pBFTM10. Two genes required for efficient transfer of the plasmid, *btgA* and *btgB*, were identified and sequenced (12). The protein encoded by *btgA* possessed a DNA-binding motif, but overall the *btgA* and *btgB* gene products had no homology to known transfer proteins from other bacterial systems. However, a region containing a putative nick site and general structural similarity to the *oriT* of IncP plasmids was identified (12). In contrast to the transmissible Tc^r and *erm* systems, there is no detailed information available on the genetic elements encoding transmissible β-lactam resistance. In previous reports, plasmid-free transfer of cefoxitin (FX) resistance was observed, but there was no further characterization of the determinants responsible for transfer (6, 24, 26).

Recently, we described the cloning and nucleotide sequence analysis of a novel, widely disseminated β-lactamase gene (*cfxA*) encoding Fx^r in *Bacteroides* species (21). This resistance gene is not plasmid encoded, but preliminary evidence showed that the resistance phenotype was transmissible when donor cells were grown in the presence of TC

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TABLE 1. Plasmids and *Bacteroides* strains[a]

Strain or plasmid	Relevant properties	Source or reference
Strains		
B. vulgatus CLA341	Fx^r Tc^r Cc^r; four cryptic plasmids	P. Appelbaum
B. fragilis 638	Rf^r	22
B. fragilis IB220	Rf^r Cc^r; Tn*4551* insertion in 638	40
B. fragilis IB143	Rf^r Tc^r; derivative of 638; 2.7-kb cryptic plasmid (pBI143)	34
B. fragilis V479-1	Tc^r Cc^r; pBF4; 41 kb	48
B. uniformis V528	Rf^r; derivative of 0061	47
B. fragilis IB245	Rf^r Fx^r; progeny from 638 × CLA341 mating; one cryptic plasmid	This report
B. fragilis IB246	Rf^r Fx^r Tc^r; progeny from 638 × CLA341 mating; one cryptic plasmid	This report
B. uniformis IB247	Rf^r Fx^r Tc^r; progeny from V528 × CLA341 mating; one cryptic plasmid	This report
Plasmids		
pFD288	Cc^r Sp^r *oriT*; 8.8-kb shuttle vector	36
pFD280	Sp^r *oriT*; an Ap^s derivative of pUC19; 4 kb	This report
pBFTM10	Cc^r 14-kb *erm* plasmid	45
pFD410	Cc^r Sp^r *oriT*; 15-kb chimera of pFD280 and pBFTM10Δ*Bgl*II; mob^- in *Bacteroides* strains	This report; 12
pFD418	Cc^r Sp^r Kn^r; 3.5-kb *Hin*dIII-*Sau*3A *cfxA* fragment from Tn*4555* cloned into pFD410	This report
pFD420	Cc^r Sp^r Kn^r; 4-kb *Hin*dIII-*Sau*3A mob fragment from Tn*4555* cloned into pFD410	This report
pABU3	Ap^r; 4.3-kb NBU1 *Hin*dIII fragment cloned into pBR328	A. Salyers; 32
pJST61.cfx	Ap^r Fx^r Cc^r; 7.5-kb *Sau*3A *cfxA* fragment from Tn*4555* cloned into pJST61	21

[a] Sp^r, Kn^r, and Ap^r are *E. coli* antibiotic resistance determinants not expressed in *Bacteroides* strains. The Fx^r and Cc^r *Bacteroides* antibiotic resistance determinants are not expressed in *E. coli*.

(38). We now report that *cfxA* is located on a discrete 12.5-kb segment of DNA that can be mobilized by resident conjugal TET elements. Circular intermediates of the *cfxA* element were isolated by density gradient centrifugation and could transform *Bacteroides* recipients to Fx^r. Analysis of Fx^r transformants or transconjugants showed numerous chromosomal insertions of *cfxA*; on the basis of these data, we have designated the *cfxA* element a mobilizable transposon, Tn*4555*.

MATERIALS AND METHODS

Bacterial strains and growth. The primary *Bacteroides* strains and plasmids used in this study are described in Table 1. *Bacteroides* cells were cultured in supplemented brain heart infusion broth (BHIS) and grown in an anaerobic chamber as described previously (35). *Escherichia coli* DH5α (*recA hsdR17 lac*) was used as the cloning host and as a recipient in matings of *Bacteroides* strains to *E. coli*. These strains were grown aerobically in L broth (agar [16]) supplemented with the appropriate antibiotics. The following antibiotics and concentrations were used, unless otherwise noted in the text: clindamycin (CC), 10 μg/ml; TC, 5 μg/ml; rifampin (RF), 20 μg/ml; gentamycin, 25 μg/ml; kanamycin (KN), 25 μg/ml; FX, 20 μg/ml; ampicillin (AP), 50 μg/ml; and spectinomycin (SP), 40 μg/ml.

Bacterial conjugation and transformation. Standard filter-mating protocols (41) were used in conjugation experiments, with the following modifications. For the transfer of plasmids from *E. coli* donors to *Bacteroides* recipients, triparental matings with RK231 as a helper plasmid were used (10) and the mating plates were incubated aerobically as described previously (30). For analysis of transfer between *Bacteroides* strains or from *Bacteroides* strains to *E. coli*, mating plates were incubated in the anaerobic chamber to favor growth of the donor. In all cases, after 18 h of growth, cells were washed from the filters with BHIS broth and then plated on selective media. When indicated, donor cells were treated with TC by growing cultures overnight in BHIS with 1 or 2 μg of TC per ml, and then they were diluted 1/50 in fresh BHIS broth and grown to early logarithmic phase for mating. Transfer frequencies are reported as the number of transconjugants per input donor cell.

Plasmid transformation of *E. coli* was performed with frozen competent cells by the method described by Hanahan (11). *Bacteroides* cells in 10% glycerol–1 mM $MgCl_2$ buffer were transformed with plasmid DNA by electroporation (5 ms at 8.75 kV/cm) in 100-μl reaction mixtures (39).

DNA isolation and analysis. *Bacteroides* strains were screened for plasmid content by a high-salt–sodium dodecyl sulfate (SDS) lysis method (48). Purified *Bacteroides* plasmid DNAs were obtained by CsCl-ethidium bromide density gradient centrifugation of lysates prepared by alkaline denaturation and phenol extraction (7, 35). Plasmid screening and purification from *E. coli* transformants were performed as described previously (2). Plasmids were analyzed by agarose gel electrophoresis with Tris-borate or Tris-acetate buffers containing ethidium bromide. Restriction endonuclease digestions were done according to the suppliers' instructions, and other routine DNA manipulations, such as ligations and Klenow reactions, have been described elsewhere (16).

For Southern filter blot hybridizations, genomic or plasmid DNA samples were electrophoresed in 0.8% agarose gels in Tris-acetate buffer, and the DNA was transferred to nitrocellulose filters by capillary action (42). DNA probes were purified from agarose gels and labeled with ^{32}P by the random primer reaction with a commercial kit (Pharmacia LKB Inc., Piscataway, N.J.). The following Tn*4555* probes were used: *cfxA* was a 0.6-kb *Hae*III-*Pvu*II fragment contained within the β-lactamase structural gene (GenBank accession no. M72418); mob was a 4-kb *Hin*dIII-*Sau*3A fragment from pJST61.cfx; and *Sau*3A-B was the 4.8-kb *Sau*3A-B fragment from Tn*4555* (also see Fig. 1). Hybridiza-

tions were performed overnight at 67°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 4× Denhardt's solution, and 1 mg of sheared salmon sperm DNA per ml. Filters were washed under the following stringent conditions: three washes for 30 min at 52°C in 0.1× SSC–0.1% SDS followed by three 30-min washes in 0.1× SSC at 52°C.

In some experiments, autoradiographs and photographs of ethidium bromide-stained agarose gels were scanned on a Hewlett-Packard flatbed scanner. Images were analyzed on a personal computer with the Collage software (Fotodyne, New Berlin, Wis.).

Plasmid constructions. The plasmid pFD288 (see Fig. 1 [36]) is a shuttle vector composed of the *Bacteroides* replicon pBI143 (34) and pUC19 (20). The pUC19 β-lactamase was replaced by a Sp^r gene (14) for selection in *E. coli*, and an *ermF* gene fragment from Tn*4351* (25) was inserted for a selective marker (Cc^r) in *Bacteroides* strains. The *oriT* from RK231 (0.75-kb *Hae*II fragment) has been added to ensure a high frequency of mobilization by RK231 (10). The plasmid pFD280 is similar to pFD288, except that it lacks both the pBI143 replicon and the *ermF* gene fragment.

The vector pFD410, which is transfer deficient in *Bacteroides* species, was constructed by insertion of the 10.5-kb *Bgl*II fragment of pBFTM10 into the *Bam*HI site of pFD280. Previous work has shown that this *Bgl*II fragment is sufficient for replication, stable maintenance, and Cc^r (*erm*) in *Bacteroides* species but that it does not mediate conjugation or mobilization (12). However, because of the presence of the RK2 *oriT* (see Fig. 1), pFD410 could be mobilized from *E. coli* to *Bacteroides* species by the helper plasmid RK231. The pFD410 derivatives, pFD418 and pFD420, were constructed by the following strategy. The appropriate Tn*4555* *Hin*dIII-*Sau*3A fragment was isolated from pJST61.cfx and cloned into pBR322 previously digested with *Hin*dIII and *Bam*HI. Next, a 1.5-kb *Cla*I Kn^r fragment (46) was inserted into the unique pBR322 *Cla*I site. This construct was digested with *Eco*RI and *Sal*I to produce a single fragment containing the Tn*4555* fragment linked to the Kn^r cassette. This DNA fragment was made blunt ended with Klenow and then inserted into the *Sma*I site of pFD410.

RESULTS

Properties of Fx^r transfer. *Bacteroides vulgatus* CLA341 is resistant to CC, TC, FX and most other β-lactam antibiotics except for carbapenems. Resistance to β-lactams is mediated by an Ambler class A β-lactamase encoded by *cfxA* (21, 38). The ability to transfer the drug resistance phenotypes by conjugation was examined by using filter matings with the recipient strains *B. fragilis* 638 or *B. uniformis* V528 (Table 2). When donor cells were untreated, Tc^r was transferred at a low frequency to the *B. fragilis* recipient, but there was no detectable transfer of Fx^r. However, donor cultures grown overnight with TC (1 or 2 μg/ml) were able to transfer both Fx^r and Tc^r at 100- and 1,000-fold higher frequencies, respectively. Treatment of donors with CC did not affect Fx^r or Tc^r transfer, and the transfer of Cc^r was not observed for any condition tested. Control experiments were all consistent with Fx^r transfer being mediated by a conjugation-like mechanism. These were performed as described previously (41) and included DNase treatment of mating mixtures, separation of donor and recipient by a 0.45-μm-pore-size filter, and use of donor cell-free filtrates in matings.

The Fx^r and Tc^r determinants were transferred at different frequencies, and this suggested that they were not physically linked on the same mobile genetic element. This was confirmed by testing 909 transconjugants for resistance to both drugs. The results showed that regardless of the drug used for initial selection of the transconjugants, only 7% were resistant to both TC and FX. Experiments with *B. uniformis* V528 were similar to those with strain 638, except that Fx^r transfer frequencies were lower and that no Tc^r transfer was observed in the absence of TC treatment (Table 2 [experiments 4 and 5]).

Although Fx^r and Tc^r were not physically linked, transfer of Fx^r in the primary matings was observed only if donor cultures were pregrown with TC. Evidence suggesting that the Tc^r element was required for Fx^r transfer was obtained from secondary matings (Table 2 [experiments 9 to 12]). IB246, an Fx^r Tc^r transconjugant, was able to transfer drug resistance to an isogenic *B. fragilis* recipient. With this donor strain, there was a detectable but very low level of Fx^r transfer in the absence of TC pretreatment, and the frequency increased nearly 500-fold in cultures treated with TC. However, when IB245, an Fx^r Tc^s transconjugant, was mated with the same *B. fragilis* recipient, no Fx^r transconjugants were recovered. We were able to restore the transmissible phenotype by constructing an IB245 derivative containing the V479 conjugal TET determinant. This strain, IB245.TET, transferred both Fx^r and Tc^r at frequencies comparable to those with TC treated IB246.

Fx^r transfer also seemed to be affected by the concentration or type of β-lactam used for selection of transconjugants (Table 2 [experiments 6 through 8]). When high levels of FX were used for selection, transfer frequency decreased significantly, and recovery of transconjugants was sporadic when selection was on 90 μg of FX per ml. Transconjugants selected on AP (100 μg/ml) were also Fx^r, and the frequency of transfer was equal to that observed for FX (20 μg/ml). The FX MICs and β-lactamase activities were determined for representative Fx^r transconjugants from experiments 1 to 8 (Table 2). MICs ranged from 64 to 256 μg/ml, and β-lactamase activities were 3.6 to 12.5 U/mg of protein. These compared with 8 μg/ml and 0.009 U/mg of protein for *B. fragilis* 638. Transconjugants originally selected on high levels of FX (e.g., FX at 90 μg/ml) tended to have β-lactamase activities toward the high end of the range, but there was no strict correlation between the selection conditions and the level of β-lactamase in the transconjugant.

Identification of the Fx^r mobilization region. The strategy devised to locate the mobilization locus employed a vector that was not transmissible in *Bacteroides* strains (pFD410; Fig. 1). Fragments from the Fx^r element were inserted into the vector, and the resulting plasmids were transferred into *B. fragilis* IB143 containing a conjugal TET element (34). These plasmid constructs were then evaluated in filter matings with *E. coli* DH5α as the recipient. The vector was derived from transfer-deficient pBFTM10Δ*Bgl*II (12), and as seen in Fig. 1, pFD410 did not transfer from either TC-treated or untreated *Bacteroides* cultures. The positive control, pFD288 (mobilizable cloning vector), readily transferred from IB143, and about a 30-fold increase was observed when cultures were pretreated with TC. Transfer assays with pFD418, a recombinant plasmid containing the 3.5-kb *Hin*dIII *Sau*3A-*cfxA* fragment, indicated that this region of the element was not capable of mediating transfer. However, when the adjacent 4-kb *Hin*dIII-*Sau*3A fragment was inserted into pFD410, transfer frequencies increased dramatically to 1.1×10^{-5}, and there was a further 10-fold increase in transfer when cultures were pretreated with TC (see pFD420 in Fig. 1). The Tn*4555* *Sau*3A-B fragment was

TABLE 2. Transfer of Fx^r and Tc^r determinants from *B. vulgatus* CLA341 and transconjugants

Expt	Donor	Recipient[a]	Treatment[b]	Selection[c]	Transfer frequency[d]	
					FX	TC
Primary matings						
1	341 (Fx^r Tc^r Cc^r)	638	None	FX20 or TC5	$<10^{-8}$	$4.7 \times 10^{-7} \pm 2.7 \times 10^{-7}$
2	341	638	TC	FX20 or TC5	$3.6 \times 10^{-6} \pm 1.5 \times 10^{-6}$	$3.3 \times 10^{-4} \pm 3.1 \times 10^{-4}$
3	341	638	CC	FX20 or TC5	$<10^{-8}$	$<10^{-8}$
4	341	V528	None	FX20 or TC5	$<10^{-8}$	$<10^{-8}$
5	341	V528	TC	FX20 or TC5	$3.9 \times 10^{-8} \pm 2.7 \times 10^{-8}$	$6.2 \times 10^{-4} \pm 0.6 \times 10^{-4}$
6	341	638	TC	FX60	$3.8 \times 10^{-8} \pm 3.0 \times 10^{-8}$	
7	341	638	TC	FX90	$1.7 \times 10^{-8} \pm 1.7 \times 10^{-8}$	
8	341	638	TC	AP100	2.2×10^{-5}	
Secondary matings						
9	IB246 (Fx^r Tc^r Rf^s)[e]	IB220	None	FX20 or TC5	$7.4 \times 10^{-7} \pm 5.5 \times 10^{-7}$	$<10^{-8}$
10	IB246	IB220	TC	FX20 or TC5	$3.3 \times 10^{-4} \pm 2.9 \times 10^{-4}$	$5.5 \times 10^{-4} \pm 3.6 \times 10^{-4}$
11	IB245 (Fx^r Rf^s)	IB220	None	FX20	$<10^{-8}$	
12	IB245.TET (Fx^r Tc^r Rf^s)[f]	IB220	TC	FX20 or TC5	$1.2 \times 10^{-5} \pm 1.1 \times 10^{-5}$	$6.6 \times 10^{-6} \pm 5.4 \times 10^{-6}$

[a] Recipients 638 and IB220 were isogenic *B. fragilis* strains resistant to RF. IB220 also was resistant to CC because of a chromosomal insertion of Tn*4551* (40).
[b] Cultures were treated prior to mating by growth overnight in 1 μg of each antibiotic indicated.
[c] Mating mixtures were plated on BHIS agar containing RF (20 μg/ml) and either FX (20, 60, or 90 μg/ml [FX20, FX60, and FX90, respectively]), AP (100 μg/ml [AP100]), or TC (5 μg/ml [TC5]), depending on the experiment. In experiments 9 to 12, CC was also included to select for IB220.
[d] Transfer frequencies (± standard errors) were determined from at least three independent trials and are given as the numbers of transconjugants per input donor cell.
[e] IB246 and IB245 were independent transconjugants obtained from experiment 2.
[f] IB245.TET contains the chromosomal TET element from V479-1 and was obtained from a V479-1 × IB245 mating.

also cloned onto pFD410; however, this fragment did not mediate conjugal transfer (37).

Circular intermediate of the Fx^r element. We had shown previously (21) that the Fx^r determinant was not plasmid encoded in the original CLA341 parent strain, although one or two of the small CLA341 cryptic plasmids are usually transferred in matings. During subsequent studies with the transconjugant IB246, we noted the occurrence of a weak, rapidly migrating *cfxA* hybridization signal in Southern blots of undigested genomic DNA. This result suggested the existence of a free circular form of the Fx^r element, and we examined this possibility in the experiments shown in Fig. 2 by using density gradient-purified, covalently closed circular DNA (cccDNA) from untreated cultures (lanes 1 to 3) or TC-treated cultures (lanes 5 to 7). Figure 2B shows an ethidium bromide-stained agarose gel, and the small cryptic plasmids of CLA341 (donor), IB143 (negative control), and IB246 (transconjugant) are clearly seen. There was no obvious difference in the plasmid patterns of TC-treated or untreated cultures of CLA341 or IB143, but careful examination of the transconjugant (see arrow) revealed a new cccDNA of about 12.5 kb in the TC-treated cultures. Southern blots prepared from this gel were then probed with an internal *cfxA* gene fragment. The result in Fig. 2A shows that in untreated cultures of CLA341 and IB143, there were no hybridizing bands but that when these cultures were grown with TC, a 12.5-kb *cfxA* hybridizing band was observed in CLA341 but not in the IB143 control. In the transconjugant, there was a *cfxA*-homologous 12.5-kb plasmid band present in cultures grown without TC, and the intensity of this signal increased significantly in TC-grown cells. Two or three high-molecular-weight-hybridizing bands estimated to be up to 22 kb in size also were observed in the TC-treated cultures of CLA341 and IB246. These may be multimeric forms, open circular forms, or differences in the amounts of supercoiling of the 12.5-kb circle, because digestion of the plasmid samples with a restriction enzyme resulted in just a single hybridizing fragment (37).

A similar experiment was performed with the Fx^r mob region as a probe, and the autoradiograph is shown in Fig. 2C. With regard to the 12.5-kb cccDNA, the results were identical to those described above in that the *cfxA* and mob probes hybridized to the same 12.5-kb plasmid band. Results with the mob probe showed that the 12.5-kb circle was present in untreated IB246 cells but that with TC treatment there was a significant increase in the amount of hybridization. The appearance of the Fx^r circular form in CLA341 was strictly controlled by TC treatment. An unexpected observation was that the mob probe also had homology to the small cryptic plasmids present in each of the strains tested, including IB143. In all cases, hybridization to the closed and nicked circular forms of the 2.7-kb plasmids in both TC-treated and untreated cultures was observed. The three larger cryptic plasmids of CLA341 and the *E. coli* V517 plasmids did not hybridize to the mob probe.

Origin of Fx^r circles. Circular DNA molecules were observed in CLA341 only when cultures were grown in the presence of TC. This suggested that the Fx^r element was normally integrated in a chromosomal location. To verify this, three probes covering the entire 12.5-kb length of the element were hybridized to total CLA341 genomic DNA samples obtained from a culture grown in the absence of TC (Fig. 3). The chromosomal DNAs were digested either with *Sst*I or *Eco*RI, which has no recognition sites within the Fx^r circular element. The hybridization patterns for each of the three probes were identical. The mob probe (A), *cfxA* probe (B), and the *Sau*3A-B probe (C) all appeared to hybridize to the same genomic fragments, and these homologous fragments were much larger than the circular form of the element (Fig. 3, arrowhead).

These results, together with the hybridization analysis of the CLA341 circles, indicate that the entire Fx^r element was encoded on the 12.5-kb cccDNA molecule. Therefore, a restriction map of the element based on restriction endonuclease digestions of the purified circular form of the DNA was constructed. The linearized version of this map is shown

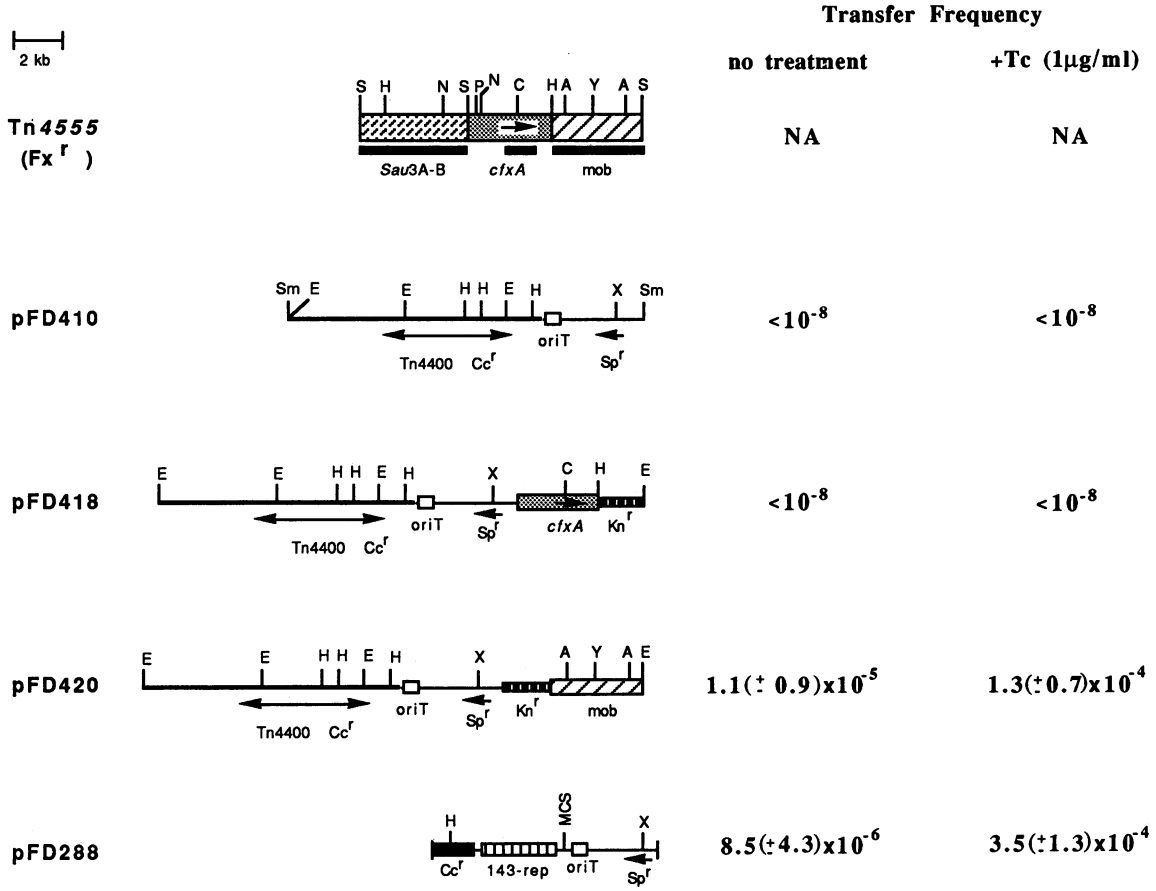

FIG. 1. Location of the Fx^r (Tn*4555*) mobilization region. Restriction fragments from Tn*4555* were cloned into pFD410, and the plasmids were tested for mobilization from *B. fragilis* IB143 to *E. coli* DH5α. Shown are restriction endonuclease cleavage site maps for Tn*4555* and the pFD410 derivatives. Transfer frequencies obtained with untreated or TC-treated donor cultures are shown, along with the standard errors. Abbreviations: A, *Ava*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; MCS, multiple cloning site; N, *Nde*I; P, *Pst*I; S, *Sau*3A; Sm, *Sma*I; X, *Xba*I; Y, *Sty*I. The black bars under the map of Tn*4555* show the locations of the *Sau*3A-B restriction fragment probe, the *cfxA* internal probe, and the mob probe utilized for the experiments depicted in Fig. 2, 3, 4, and 5. On the restriction maps, the thick black lines are pBFTM10Δ*Bgl*II sequences, and the thin lines designate pFD280 sequences. The *oriT* regions of pFD280 and pFD288 are shown as the open boxes, and the Sp^r is shown by the arrow. The *ermF* gene (Cc^r) and *Bacteroides* replicon (143-rep) on pFD288 are also shown. The map of Tn*4555* was derived from analysis of cccDNA obtained from IB246 and does not represent the orientation of the element in the chromosome.

at the top of Fig. 1, but this is not meant to indicate the specific orientation of the element when integrated in the chromosome.

Evidence for Fx^r transposition. Total genomic DNA isolated from untreated cultures of independent Fx^r transconjugants was examined in Southern hybridizations with the internal *cfxA* probe. To differentiate between the circular and integrated forms of the Fx^r element, genomic DNAs were digested with *Eco*RI, which does not cut within the element. The results show that the element was clearly integrated into the chromosome and that this integration had the potential to occur at many different sites, as indicated by the variation in the sizes of the hybridizing fragments (Fig. 4). However, several of the strains shared hybridizing fragments with similar sizes (arrow p), suggesting that there might be a common insertion site. The autoradiograph shown in Fig. 4 was purposely overexposed so that both the integrated and the circular forms could be observed. The circular form (Fig. 4, arrow c) was difficult to observe under these conditions, indicating that there was a low copy (less than one per chromosome equivalent) in cells that were not treated with TC.

More than one insertion of the Fx^r element was observed in some strains. The multiple insertions were often found in transconjugants that were initially selected on high concentrations of FX or AP (Fig. 4, lanes g to k), but there was no strict correlation between the selection conditions and the number of *cfxA*-hybridizing bands. These results and those discussed above have been confirmed in experiments in which the DNAs were digested with other restriction enzymes.

A second approach used to demonstrate the transposition potential of the Fx^r element was to transform 638 cells with Fx^r cccDNA. Density gradient-purified cccDNA was prepared from TC-treated cultures of the transconjugant, IB246 (which contains one cryptic plasmid), and from CLA341 cultures grown without TC. The DNA was electroporated into 638 cells, and transformants were selected on media containing either 15 or 20 μg of FX or 5 μg of TC per ml. The results in Table 3 show that Fx^r transformants were obtained readily when DNA from the TC-treated cells was used, and about twice as many transformants were observed on selection plates with the lower FX concentration. Although the frequency of transformation was not very high, the amount

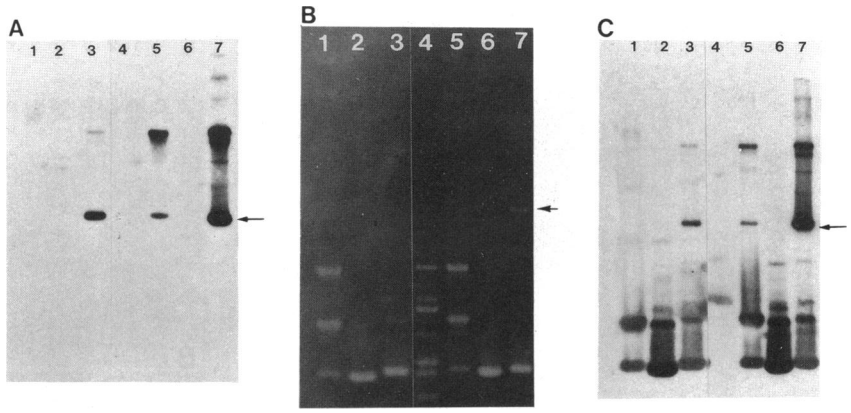

FIG. 2. Identification of the Fx^r element (Tn*4555*) circular intermediate in cells grown with and without TC. Circular DNA was purified by CsCl-ethidium bromide density gradient centrifugation from overnight cultures grown without TC, (lanes 1 to 3) or with 1 μg of TC per ml (lanes 5 to 7). The following strains were used: CLA341 (lanes 1 and 5), IB143 (lanes 2 and 6), and IB246 (lanes 3 and 7). Lane 4 is the plasmid size marker *E. coli* V517 (15). The arrows to the right of the photographs indicate the locations of the 12.5-kb circular DNA. (A) Autoradiograph of Southern blot from the agarose gel shown in panel B and probed with the internal *cfxA* probe. (B) Agarose gel of undigested cccDNA stained with ethidium bromide. Each lane contains 1 μg of DNA. (C) Autoradiograph of Southern blot from an agarose gel identical to the one in panel B and probed with the 4.0-kb mob region of Tn*4555*.

of Fx^r circular DNA was estimated to be only 73 ng/μg of total plasmid DNA. Thus, the low recovery of transformants accurately reflects the low concentration of Fx^r element used in transformations. When CLA341 DNA (from untreated cells) was used, no transformants were detected even when the total cccDNA concentration in the transformation reaction mixture was increased to 50 μg/ml.

To determine the fate of the Fx^r-transforming DNA, transformants were screened for both the circular and the integrated forms of the Fx^r element. By a high-salt–SDS plasmid enrichment technique, the circular form was not readily visible on agarose gels, but filter blots of the gels probed with *cfxA* revealed the 12.5-kb cccDNA as well as higher-molecular-weight forms of this element (Fig. 5A). It also was apparent that a significant portion of the total hybridization signal was associated with chromosomal DNA

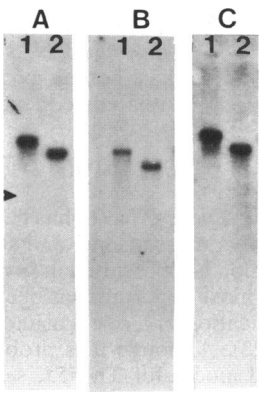

FIG. 3. Hybridization analysis of the Fx^r element from CLA341 cells grown without TC treatment. Total genomic DNA was digested with either *Eco*RI (lane 1) or *Sst*I (lane 2) and electrophoresed on a 0.8% agarose gel. The DNA was blotted onto nitrocellulose filters and hybridized to the ^{32}P-labeled DNA probes described in the legend to Fig. 1. (A) Tn*4555* mob region; (B) Tn*4555 cfxA* probe; (C) Tn*4555 Sau*3A-B fragment probe. The arrowhead indicates approximately where the circular form of the Fx^r element would migrate.

that was not removed by the plasmid enrichment. Subsequently, total genomic DNAs were isolated from five of the transformants, digested with *Eco*RI, and examined by Southern hybridization with the *cfxA* probe (Fig. 5B). These results clearly indicate that the Fx^r element had integrated into the chromosome and that most of the transformants had insertions into different *Eco*RI fragments. The circular form of the DNA was not visible in the exposures used for these autoradiographs, suggesting that the copy number of the transposon circles was less than one per chromosome equivalent. These results and those above show that the Fx^r element is composed of a specific DNA segment that has the ability to insert into a variety of different chromosomal locations. Therefore, the Fx^r element is designated a mobilizable transposon, Tn*4555*.

Homology of Tn*4555* to NBU1. The TC-regulated appearance of Tn*4555* circles in *B. vulgatus* CLA341 was similar to the response seen with the nonreplicating *Bacteroides* units previously described with *B. uniformis* (32). To look at the relationship between these elements, complementary Southern hybridizations were performed with either the 7.5-kb Tn*4555 Sau*3A fragment (from pJST61.cfx) as a probe or a 4.3-kb NBU1 *Hin*dIII fragment (from pABU3 [32, 33]) as a probe. The results from these experiments showed that there was some homology between the two elements, although the

TABLE 3. Transformation of *B. fragilis* 638 with plasmid DNAs from Fx^r strains

Plasmid source[a]	Amt of DNA (μg)[b]	Selection[c]	No. of transformants/μg
IB246 (+TC)	0.6	FX15	239
IB246 (+TC)	0.6	FX20	122
IB246 (+TC)	0.6	TC5	0
CLA341 (NTC)	5	FX15	0

[a] DNAs used in transformations were density gradient-purified from cultures grown either in the presence of 1 μg of TC per ml (+TC) or without TC (NTC).

[b] The amounts of DNAs added to the transformation reaction mixtures.

[c] Transformants were selected on BHIS containing FX (15 μg/ml [FX15] or 20 μg/ml [FX20]) or TC (5 μg/ml [TC5]).

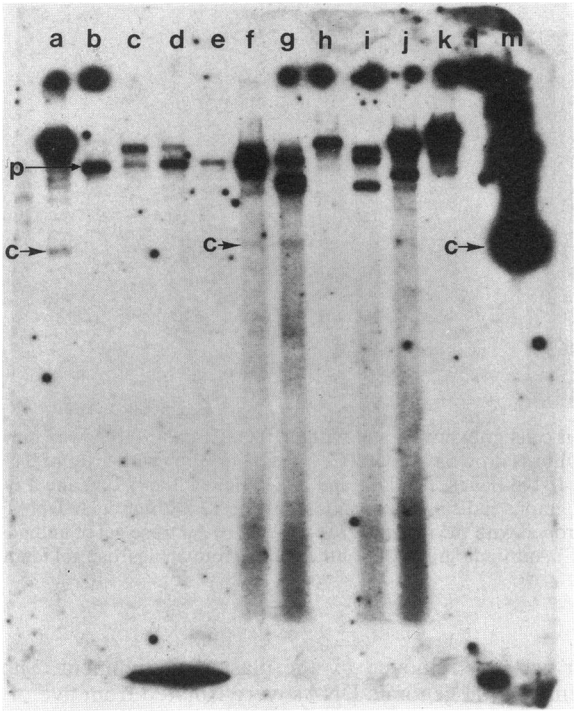

FIG. 4. Insertion of Tn*4555* in the chromosome of Fx^r transconjugants. Total genomic DNAs from 11 independent Fx^r transconjugants and the recipient *B. fragilis* 638 were digested with *Eco*RI, electrophoresed, and blotted to a nitrocellulose filter. The filter was probed with the internal *cfxA* fragment, and the resulting autoradiograph was exposed for 36 h. The arrow marked p indicates a common site of Tn*4555* insertion, and the arrows marked c indicate the locations of the 12.5-kb closed circular form of Tn*4555*. Lanes a to k are transconjugants obtained from the following experiments listed in Table 2: a, Fx^r (experiment 2); b, Fx^r Tc^r (experiment 2 [IB246]); c, Fx^r (experiment 2); d, Fx^r Tc^r (experiment 2); e, Fx^r (experiment 2); f, Fx^r Tc^r (experiment 2); g, Fx^r (experiment 8); h, Fx^r (experiment 6); i, Fx^r Tc^r (experiment 8); j, Fx^r (experiment 6); k, Fx^r (experiment 7). Lane 1, 638; lane m, purified cccDNA from IB246.

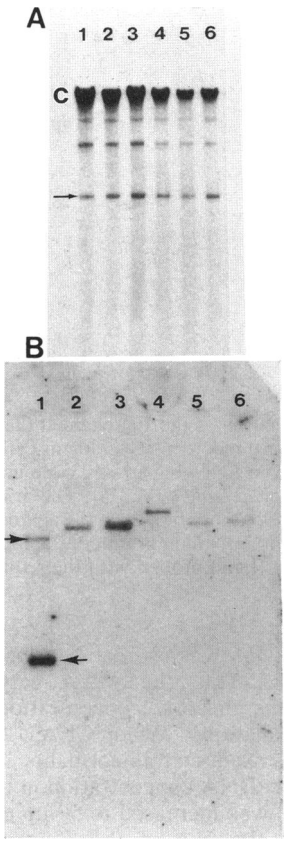

FIG. 5. Analysis of Fx^r *B. fragilis* 638 cells transformed with cccDNA from TC-treated IB246. (A) Autoradiograph of plasmid-enriched DNA samples from Fx^r transformants electrophoresed in 0.6% agarose gel, blotted, and probed with the internal *cfxA* gene fragment. The arrow shows the location of the 12.5-kb Tn*4555* circle, and c shows the chromosomal DNA. (B) Autoradiograph of *Eco*RI-digested total genomic DNAs from five Fx^r transformants hybridized to the *cfxA* probe. Lanes: 1, purified plasmid sample from TC-treated IB246; 2 to 6, Fx^r transformants. The arrows indicate the locations of the 12.5-kb Tn*4555* closed and nicked circular forms.

specific location of the homology was not determined (37). To see whether homology with NBU1 affected integration of Tn*4555*, genomic DNAs from CLA341, IB246 (*B. fragilis* transconjugant), and IB247 (*B. uniformis* transconjugant) were examined in Southern blots with the NBU1 probe (Fig. 6). The results showed that NBU1 homologous sequences were present in both recipient strains: *B. fragilis* 638 and *B. uniformis* V528. In addition to these indigenous sequences, the Fx^r transconjugants possessed an additional DNA fragment that had weak homology to the probe. This fragment comigrated with the 7.5-kb Tn*4555* *Sau*3A fragment containing *cfxA*. None of the indigenous NBU1 fragments of the recipients appeared to be disrupted in the transconjugants, and this is especially clear in IB247, in which the three high-molecular-weight parental fragments remained intact. A purified plasmid sample from non-TC-treated CLA341 did not hybridize with the NBU1 probe (Fig. 6, lane 5), but the CLA341 chromosomal sample had two hybridizing fragments (lane 1). One of these fragments comigrated with Tn*4555* (arrowhead) and the other, more homologous fragment was probably an indigenous NBU1-like chromosomal element present in this strain. From these results and similar experiments with *Hin*dIII-digested DNAs (data not shown), it is clear that Tn*4555* has homology with but did not integrate within NBU1 or related sequences.

DISCUSSION

During the past few years, Tn*916* has become the standard model for conjugative transposons, and studies of its mechanism of transposition have resulted in several novel findings (5, 27). One of the most important has been the proposal and subsequent identification of a circular intermediate in Tn*916* transposition (4, 28). Although it is probably premature to propose a detailed model for Tn*4555*, several lines of evidence described in the present paper suggest that it is a novel chromosomal element that transfers and transposes via a circular intermediate. Transfer of Tn*4555* from *B. vulgatus* CLA341 was detected only when donor cultures were grown with TC prior to mating. The same TC treatment also controlled the appearance of Tn*4555* cccDNA in CLA341. However, in the *B. fragilis* 638 strain background, Tn*4555* transferred at a low but detectable frequency without TC

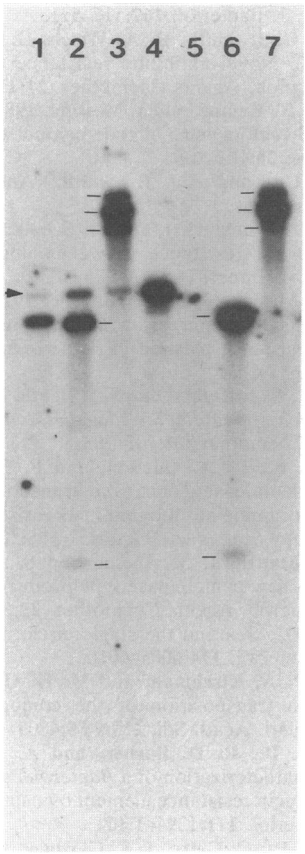

FIG. 6. Relationship between NBU1 and Tn*4555* in *B. fragilis* and *B. uniformis* Fx^r transconjugants. Total genomic DNA or plasmid DNA was digested with *Sau*3A, and Southern hybridizations were performed with the 4.3-kb *Hin*dIII NBU1 fragment (gel purified from pABU3) as a probe. Lanes: 1, CLA341 genomic DNA; 2, IB246 genomic DNA; 3, IB247 genomic DNA; 4, pJST61.cfx plasmid DNA; 5, CLA341 plasmid DNA (from non-TC-treated cells); 6, 638 genomic DNA; V528 genomic DNA. The arrowhead shows the location of the 7.5-kb, Tn*4555* *Sau*3A fragment containing *cfxA*, and indigenous 638 and V528 sequences homologous to NBU1 are shown by the ticks.

treatment, and in these untreated cells we also detected low levels of the circular transposon. When the Tn*4555*-containing *B. fragilis* cells were grown with TC, there was a significant increase in both the concentration of transposon circles and in the Fx^r transfer frequency. These results establish a close link between the appearance of the Tn*4555* circles and the ability of Tn*4555* to transfer, suggesting that the transposon excises from the chromosome and circularizes prior to transfer.

When circular transposon DNA was introduced into the Tc^s *B. fragilis* 638 strain by electroporation, it integrated into the chromosome, and several different Tn*4555* insertions were identified. In addition, transposon circles were observed in these transformants, indicating that TC treatment per se is not required for circularization of the transposon. Alternatively, it is possible that the circular transposon can replicate to some extent in *B. fragilis* transformants and that TC treatment somehow selects for these cells. However, this seems unlikely since the copy number of the circular form is less than one per chromosome equivalent (Fig. 4 and 5). Taken together, these results suggest that covalently closed circular Tn*4555* can serve as a transposition intermediate which can integrate and subsequently excise from the chromosome. However, the transposon must be dependent on conjugal TET elements to supply the conjugation functions as indicated by the inability of Fx^r Tc^s transconjugants to act as donors in matings (Table 2 [experiment 11]).

Conjugation of Tn*4555* also resulted in its integration within the recipient genome. A notable difference between Fx^r transconjugants and transformants was the identification of multiple insertions in the transconjugants. It is not known whether the multiple copies resulted from a fundamental difference in the transposition mechanism during conjugation or whether they resulted from multiple transfer events which could occur during the overnight matings with these TC-treated donor cells. This is similar to Tn*916*, in which multiple insertions are often observed following conjugation (8) but have not been seen in strains transformed with the supercoiled form of the transposon (28). Thus, as with Tn*916*, there does not seem to be any significant barrier or immunity to subsequent transposition events in strains containing Tn*4555*.

The transfer of Tn*4555* was different from those of the *Bacteroides* TET elements or Tn*916* because it was not self-transmissible, as indicated by the inability of Fx^r Tc^s transconjugants to act as donors in matings. The transfer properties more closely resembled those of Tn*4399* (13) in that the element could impart mobilization properties on a transfer-deficient replicon in *cis*. However, an important difference is that a circular form of Tn*4399* has not been observed. The mobilization region of Tn*4555* was located on a 4-kb restriction fragment, and when this was cloned onto pFD410, it mediated transfer in both TC-treated and untreated cells. Transfer frequencies for the untreated cells carrying pFD420 were relatively high and increased only about 10-fold with TC treatment. This would be the expected result if transposon circles are intermediates in conjugation, because if the element was already circularized and in high copy, as in the case of pFD420, it would be primed for transfer even when overall conjugation activity was low. The end result would be a TC-enhanced transfer frequency of a magnitude lower than that expected.

DNA hybridization studies showed that Tn*4555* has some homology with the small, cryptic *Bacteroides* plasmids and with the nonreplicating *Bacteroides* unit, NBU1. These elements also share the common feature that they are readily mobilized by conjugal TET elements but do not seem to be self-transmissible (1, 19, 31). The homology that we observed was found in regions associated with mobilization. For example, it was the Tn*4555* mob probe which hybridized to the 2.7-kb cryptic plasmids (Fig. 2C), and the NBU1 probe used in Fig. 6 covered the entire NBU1 mobilization region (33). The small *Bacteroides* cryptic plasmids are widely disseminated in nature; they are found in more than 30% of all isolates (3). In a limited survey, DNA sequences with homology to the nonreplicating *Bacteroides* units also seemed to be broadly distributed (32). The success of these plasmids and nonreplicating units may be attributed to their ability to be mobilized by the TET elements, and our results suggest that they may share critical components in the mobilization regions with the transposon described here. A logical evolutionary step in the spread of antibiotic resistance would be to make use of these highly successful TC-stimulated gene transfer pathways, and this appears to be the strategy adopted by Tn*4555*.

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