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Conjugal Transfer of Antibiotic Resistance Factors in *Bacteroides fragilis*: the *btgA* and *btgB* Genes of Plasmid pBFTM10 Are Required for Its Transfer from *Bacteroides fragilis* and for Its Mobilization by IncPβ Plasmid R751 in *Escherichia coli*

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Transferable plasmids play an important role in the dissemination of clindamycin-erythromycin resistance in *Bacteroides fragilis*. We previously described the isolation and properties of pBFTM10, a 14.9-kb ClnR transfer factor from *B. fragilis* TMP10. We also reported the isolation of a transfer-deficient deletion derivative of pBFTM10 contained in the *B. fragilis-Escherichia coli* shuttle vector pGAT400. In the present study we used pGAT400 and a similar shuttle vector, pGAT550, to characterize and sequence a region of pBFTM10 required for its transfer from *B. fragilis* to *B. fragilis* or *E. coli* recipients and for its mobilization by the broad-host-range plasmid R751 from *E. coli* donors to *E. coli* recipients. Deletion of certain *BglII* restriction fragments from pBFTM10 resulted in partial or complete loss of transfer ability. Tn*1000* insertions into this same region also resulted in altered transfer properties. We used the sites of Tn*1000* insertions to determine the DNA sequence of the transfer region. Two potential open reading frames encoding proteins of 23.2 and 33.8 kDa, corresponding to two genes, *btgA* or *btgB*, were identified in the sequence. Tn*1000* insertions within *btgA* or *btgB* or deletion of all or portions of *btgA* or *btgB* resulted in either a transfer deficiency or greatly reduced transfer from *B. fragilis* donors and alterations in mobilization by R751 in *E. coli*. A potential *oriT* sequence showing similarity in organization to the *oriT* regions of the IncP plasmids was also detected. Thus, pBFTM10 encodes and requires at least two proteins necessary for efficient transfer from *B. fragilis*. These same functions are expressed in *E. coli* and are required for mobilization by R751.

There is increasing awareness that the occurrence and dissemination of drug resistance determinants in bacteria of medical importance play a significant role in the virulence of these organisms. Indeed, for *Bacteroides fragilis*, one of the major obligately anaerobic bacteria involved in infections of humans, resistance to tetracycline, once rare, is now seen in more than 60% of all clinical isolates (25). Tetracycline transfer elements (TET elements [21]) located on the chromosomes of several *Bacteroides* species of clinical importance may be responsible for the spread of the tetracycline resistance determinant. There have been reports of outbreaks of clindamycin-resistant (ClnR) *B. fragilis* strains in hospital settings (2). Several plasmids carrying a determinant for clindamycin-erythromycin resistance (*ermF* [21]) have been isolated from these *B. fragilis* strains; indeed, >90% of all clindamycin-resistant *B. fragilis* strains show homology in either their chromosomal DNA or within their autonomous plasmids when tested with the *ermF* probe (12). Three of these plasmids, pBF4 (29), pBFTM10 (pCP1) (26), and pBI136 (24), can transfer by conjugation from the plasmid-containing cells to *B. fragilis* recipient cells and convert the transconjugants to ClnR.

At 14.9 kb, pBFTM10 is considerably smaller than the other *B. fragilis* transfer factors pBF4 (41 kb) (14) and pBI136 (82 kb) (24) and other gram-negative transfer factors (for reviews, see references 7 and 30). The clindamycin-erythromycin resistance genes of pBFTM10 are contained within transposon Tn*4400* (20), which is 5.4 kb in size; in addition, a region of approximately 4 kb that is essential for autonomous replication in *B. fragilis* has been localized (6). This leaves less than 5 kb for all remaining plasmid functions, including transfer, although it is possible that sites or functions involved in transposition and/or replication may also play a role in transfer. For this reason others have hypothesized that pBFTM10 cannot truly be self-transferable but instead must be mobilized by as yet uncharacterized products from the bacterial chromosome (21).

pBFTM10 has been fused to the *Escherichia coli* plasmid pDG5, a pBR322 derivative containing the *oriT* region from the broad-host-range plasmid RP4 (6), to form the shuttle vectors pGAT400 and pGAT550 (10). Transfer of these plasmids from *B. fragilis* donor cells to *B. fragilis* and *E. coli* recipients can be easily detected, although the transfer efficiency is low. The transfer from *B. fragilis* donor cells does not require a coresident plasmid or transfer element; for this reason we have designated pBFTM10 and its derivatives as self-transferable (11). In *E. coli* cells containing pGAT400 or pGAT550 and a coresident RP4 transfer factor, mobilization of the shuttle plasmids to *E. coli* or *B. fragilis* recipient cells occurs in trans, with RP4 supplying the necessary transfer functions to act at the *oriT* site within pDG5 (6, 10).

Two transfer factors have been shown to increase the efficiency of transfer of pBFTM10. In *B. fragilis* donors containing TET elements, pretreatment with subinhibitory levels of tetracycline (10, 11, 17, 18) greatly increases the transfer efficiency of shuttle vectors containing pBFTM10. In *E. coli*, Salyers and coworkers have demonstrated mobi-

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TABLE 1. Bacterial strains, plasmids, and transfer factors[a]

Strain, plasmid, or factor	Relevant phenotype	Source
B. fragilis		
TM4000	Rif^r	M. Sebald, Pasteur Institute, Paris, France, as 638rfm
TM4.23	Tet^r Rif^r	Hecht and Malamy (10)
TM429	Trs^r Rif^r	Hecht and Malamy (10)
E. coli		
HB101	Sm^r	Sambrook et al. (22)
DW1030	Sp^r	Robillard et al. (20)
Plasmids		
pGAT400	Tra$^+$, *E. coli-B. fragilis* shuttle vector, Cln^r Amp^r	Hecht and Malamy (10)
pGAT400ΔBglII	Tra$^-$, deletion derivative of pGAT400	Hecht and Malamy (10)
pGAT550	Tra$^+$, *E. coli-B. fragilis* shuttle vector, Cln^r Amp^r	Robillard et al. (20)
pBFD1	Tra$^\pm$, deletion derivative	This study
pBFD2	Tra$^+$, deletion derivative	This study
pBFD3	Tra$^+$, deletion derivative	This study
pBFD4	Tra$^\pm$, deletion derivative	This study
pBFD7	Tra$^-$, deletion derivative	This study
pGAT400Δ2.8	Tra$^+$, deletion derivative	This study
pDHL1	Tra$^-$, linker insertion	This study
pTGD1	Tra$^+$, Tn*1000* insertion	This study
pTGD4	Tra$^+$, Tn*1000* insertion	This study
pTGD5	Tra$^+$, Tn*1000* insertion	This study
pTGD74	Tra$^\pm$, Tn*1000* insertion	This study
pTGD75	Tra$^-$, Tn*1000* insertion	This study
pTGD83	Tra$^\pm$, Tn*1000* insertion	This study
pTGD94	Tra$^-$, Tn*1000* insertion	Hecht and Malamy (10)
pTGD558	Tra$^-$, Tn*1000* insertion	This study
pTGD555	Tra$^+$, Tn*1000* insertion	This study
Transfer factors		
R751	Tra$^+$ IncPβ Tmp^r	Meyer and Shapiro (13)
pRK231	Tra$^+$ IncPα Kan^r	Guiney et al. (6)
F′ *lac*	Tra$^+$ IncFI	Pasteur Institute

[a] Amp^r, Cln^r, Kan^r, Rif^r, Tmp^r, Tet^r, Trs.^{r}, Sm^r, and Sp^r indicate resistance to ampicillin, clindamycin, kanamycin, rifampicin, trimethoprim, tetracycline, trospectomycin, streptomycin, and spectinomycin, respectively.

lization of pDP1 (containing pDG5 and pCP1 [pBFTM10]) in the presence of the broad-host-range plasmid R751 (IncPβ) (23). R751 is similar to RP4 (IncPα) but contains its own specific *oriT* region that does not cross-react to any considerable extent with the *oriT* of RP4 in pDG5 (3, 23). The mechanisms of enhanced transfer by TET elements and mobilization by R751 are unknown but are dependent on an intact transfer region of pCP1 and pBFTM10.

We have previously described two transfer-deficient derivatives of pGAT400: pGAT400ΔBglII has a 4-kb deletion within pBFTM10 and is unable to transfer from *B. fragilis* to *B. fragilis* or to *E. coli*; a Tn*1000* insertion mutation (pTGD94) within the region deleted to form pGAT400ΔBglII is also incapable of transfer (10). The deletion and Tn*1000* insertion establish that pBFTM10 must supply some sites and/or functions involved in its own transfer. To further characterize the transfer region of pBFTM10, we isolated a set of mutants with Tn*1000* insertions within the pBFTM10 portion of pGAT400 and pGAT550, and we engineered partial deletions of the transfer region. These derivatives were tested for their ability to transfer from *B. fragilis* donor cells in the absence or presence of the chromosomal TET element and for their ability to be mobilized by R751 in *E. coli*. We localized and sequenced a region of pBFTM10 necessary for transfer, and we identified two genes and two potential proteins that are involved in transfer.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids are listed in Table 1. The medium requirements for *B. fragilis* and *E. coli* strains and the anaerobic growth conditions and antibiotic concentrations were as previously described (10). To select for the presence of plasmid R751 in donor cells and in transconjugants, Meuller-Hinton plates containing 10 μg of trimethoprim per ml were used.

Plasmid constructions. The characteristics of the *E. coli-B. fragilis* shuttle vectors pGAT400 and pGAT550 were previously described (10). Deletion mutants of pGAT400 were obtained by partial digestion of purified DNA with *Bgl*II restriction endonuclease followed by ligation with T4 DNA ligase (New England BioLabs, Beverly, Mass.). Ligated plasmid DNA was used to transform *E. coli* HB101 to ampicillin resistance. Tn*1000* insertion mutations of pGAT 400 and pGAT550 were isolated by using the F′ *lac* mobilization protocol as previously described (10).

Plasmid transfer experiments. Quantitative filter matings were performed as previously described (26). When *B. fragilis* was the donor, 2.5 ml of an exponential donor culture was mixed with 2.5 ml of the recipient culture and then filtered on a Nalgene filter (0.45 μm), which was then removed to the surface of a BHIS plate (10) and incubated

anaerobically overnight at 35°C. When *E. coli* donors containing R751 were used to mobilize pGAT400 derivatives, 0.5 ml of the donor culture and 4.5 ml of the recipient culture were mixed, filtered onto Nalgene filters, placed on the surface of an L-agar plate, and then incubated for 90 min at 37°C.

Tetracycline induction was achieved by preexposing exponentially growing *B. fragilis* donor cells to 1 μg of tetracycline per ml 5 h before the mating. Transfer frequencies from *B. fragilis* donors were calculated by dividing the number of antibiotic-resistant transconjugants by the number of input donor cells. Transfer frequencies from *E. coli* cells containing R751 were calculated as the number of transconjugants receiving the mobilized plasmid divided by the number receiving R751.

Preparation of plasmid DNA, restriction endonuclease analysis, and DNA ligation. Preparation of plasmid DNA and restriction endonuclease analysis were as described previously (1, 22). T4 DNA ligase was obtained from New England BioLabs and was used as recommended by the supplier.

Double-stranded plasmid DNA sequencing. Nucleotide sequences were determined with double-stranded plasmid DNA by using the Sequenase method (U.S. Biochemical Corp., Cleveland, Ohio) according to the supplier's instructions. Primers of 17 to 20 bp were synthesized by using a Milligen/Biosearch (Medford, Mass.) DNA synthesizer. Specific primers to the ends of Tn*1000* were 5′ TCAATAA GTTATACCAT 3′ for the gamma end and 5′ GAATTATC TCCTTAACG 3′ for the delta end (27). Additional primers were synthesized as needed to fill gaps between Tn*1000* insertions, to extend sequencing into adjacent regions, or to determine complementary sequences. Figure 3 indicates the locations and sizes of many of these primers. The sequence of the double-stranded linker containing the *Bam*HI site used for insertional mutagenesis is given in Fig. 4A.

R751 mobilization of plasmids with Tn*1000* insertions. Plasmids containing Tn*1000* insertions were restricted with *Xho*I, religated, and transformed into HB101. This procedure produces a 3.3-kb internal deletion within Tn*1000* removing sequences required for transposition (8). The resulting plasmids were tested for mobilization by R751 as described above.

Nucleotide sequence accession number. The sequence data reported have been submitted to GenBank and assigned accession no. M77806.

RESULTS

As a first step in defining the regions of pBFTM10 required for plasmid transfer from *B. fragilis* donor cells, we mutagenized the shuttle vectors pGAT400 and pGAT550 with Tn*1000* in *E. coli* (8). The Tn*1000*-containing plasmids were then transferred via the RP4 mobilization system to two *B. fragilis* recipient strains, TM4000 and TM4.23. Quantitative transfer experiments were carried out from these *B. fragilis* donor strains to *B. fragilis* and *E. coli* recipients to compare the transfer frequencies of the Tn*1000*-containing plasmids with those of the parental plasmids.

Transfer from TM4000. TM4000 is the standard plasmid-free, tetracycline-sensitive strain used for genetic experiments. Transfer of pGAT400 and pGAT550 from *B. fragilis* TM4000 to *E. coli* HB101 and *B. fragilis* TM429 (a trospectomycin-resistant derivative of TM4000) occurs at a low frequency (Tables 2 and 3). Transfer of the deletion plasmid pGAT400ΔBglII to either recipient was below the level of detectability. Although the difference in transfer frequency of the parental plasmids pGAT400 and pGAT550 and the transfer-deficient deletion derivative pGAT400ΔBglII is at best only 2 orders of magnitude, we were nevertheless able to use the transfer assay to assign the Tn*1000* insertions into three categories. In the major category, of which TGD555, TGD1, TGD4, and TGD5 are examples, the Tn*1000* insertion probably lies outside of the transfer region, since these plasmids could be transferred to both recipients at frequencies close to those of the parental plasmids. In contrast,

TABLE 2. Transfer of shuttle vectors pGAT400 and pGAT550, Tn*1000* insertion derivatives, and deletion derivatives from *B. fragilis* TM4000 and TM4.23[a] to *B. fragilis* TM429

Donor plasmid	Mean frequency of transfer (±SE) from strain:	
	TM4000	TM4.23
pGAT400	$8.3 (\pm 0.8) \times 10^{-8}$	$5.9 (\pm 1.3) \times 10^{-5}$
pGAT400ΔBglII	$<10^{-9}$	$<10^{-9}$
pGAT550	$2.8 (\pm 0.7) \times 10^{-7}$	$6.3 (\pm 1.0) \times 10^{-5}$
pTGD75	$<10^{-9}$	$<10^{-9}$
pTGD94	$<10^{-9}$	$<10^{-9}$
pTGD74	$2.4 (\pm 1.6) \times 10^{-8}$	$<10^{-9}$
pTGD83	$1.0 (\pm 3.5) \times 10^{-8}$	$7.8 (\pm 1.9) \times 10^{-8}$
pTGD558	$<10^{-9}$	$<10^{-9}$
pTGD1	$6.0 (\pm 0.6) \times 10^{-8}$	$2.5 (\pm 1.4) \times 10^{-8}$
pTGD4	$8.2 (\pm 0.7) \times 10^{-8}$	$9.0 (\pm 0.9) \times 10^{-8}$
pTGD5	$7.4 (\pm 0.9) \times 10^{-8}$	$6.2 (\pm 1.0) \times 10^{-8}$
pTGD555	$5.9 (\pm 0.5) \times 10^{-8}$	$5.0 (\pm 0.5) \times 10^{-5}$
pDHL1	$<10^{-9}$	$<10^{-9}$
pBFD1	$2.5 (\pm 0.6) \times 10^{-8}$	$1.5 (\pm 0.3) \times 10^{-6}$
pBFD2	$3.0 (\pm 0.4) \times 10^{-8}$	$2.5 (\pm 0.5) \times 10^{-5}$
pBFD3	$8.3 (\pm 4.8) \times 10^{-8}$	$1.8 (\pm 0.6) \times 10^{-5}$
pBFD4	$2.0 (\pm 0.6) \times 10^{-8}$	$1.1 (\pm 0.7) \times 10^{-7}$
pBFD7	$<10^{-9}$	$<10^{-9}$
pGAT400Δ2.8	$3.9 (\pm 0.1) \times 10^{-8}$	$1.5 (\pm 0.1) \times 10^{-4}$

[a] All matings from the TM4.23 background were with tetracycline induction.

TABLE 3. Transfer of shuttle vectors pGAT400 and pGAT550, Tn*1000* insertion derivatives, and deletion derivatives from *B. fragilis* TM4000 and TM4.23[a] to *E. coli* HB101

Donor plasmid	Mean frequency of transfer (±SE) from strain:	
	TM4000	TM4.23
pGAT400	$4.5 (\pm 0.5) \times 10^{-8}$	$2.0 (\pm 1.0) \times 10^{-5}$
pGAT400ΔBglII	$<10^{-9}$	$<10^{-9}$
pGAT550	$4.8 (\pm 1.0) \times 10^{-8}$	$5.8 (\pm 2.7) \times 10^{-5}$
pTGD75	$<10^{-9}$	$<10^{-9}$
pTGD94	$<10^{-9}$	$<10^{-9}$
pTGD74	$<10^{-9}$	$2.0 (\pm 1.5) \times 10^{-8}$
pTGD83	$<10^{-9}$	$1.5 (\pm 0.8) \times 10^{-8}$
pTGD558	$<10^{-9}$	$<10^{-9}$
pTGD1	$1.6 (\pm 0.2) \times 10^{-8}$	$1.1 (\pm 0.6) \times 10^{-5}$
pTGD4	$3.1 (\pm 0.5) \times 10^{-8}$	$1.3 (\pm 0.5) \times 10^{-5}$
pTGD5	$1.7 (\pm 0.2) \times 10^{-8}$	$1.2 (\pm 0.1) \times 10^{-5}$
pTGD555	$1.1 (\pm 0.2) \times 10^{-8}$	$3.0 (\pm 0.6) \times 10^{-5}$
pDHL1	$<10^{-9}$	$<10^{-9}$
pBFD1	$3.2 (\pm 1.7) \times 10^{-8}$	$5.2 (\pm 1.3) \times 10^{-7}$
pBFD2	$3.1 (\pm 0.7) \times 10^{-8}$	$4.4 (\pm 1.7) \times 10^{-5}$
pBFD3	$2.1 (\pm 1.3) \times 10^{-7}$	$3.4 (\pm 1.0) \times 10^{-5}$
pBFD4	$6.9 (\pm 1.7) \times 10^{-9}$	$9.8 (\pm 2.7) \times 10^{-8}$
pBFD7	$<10^{-9}$	$<10^{-9}$
pGAT400Δ2.8	$4.6 (\pm 1.3) \times 10^{-8}$	$6.5 (\pm 0.8) \times 10^{-5}$

[a] All matings from the TM4.23 background were with tetracycline induction.

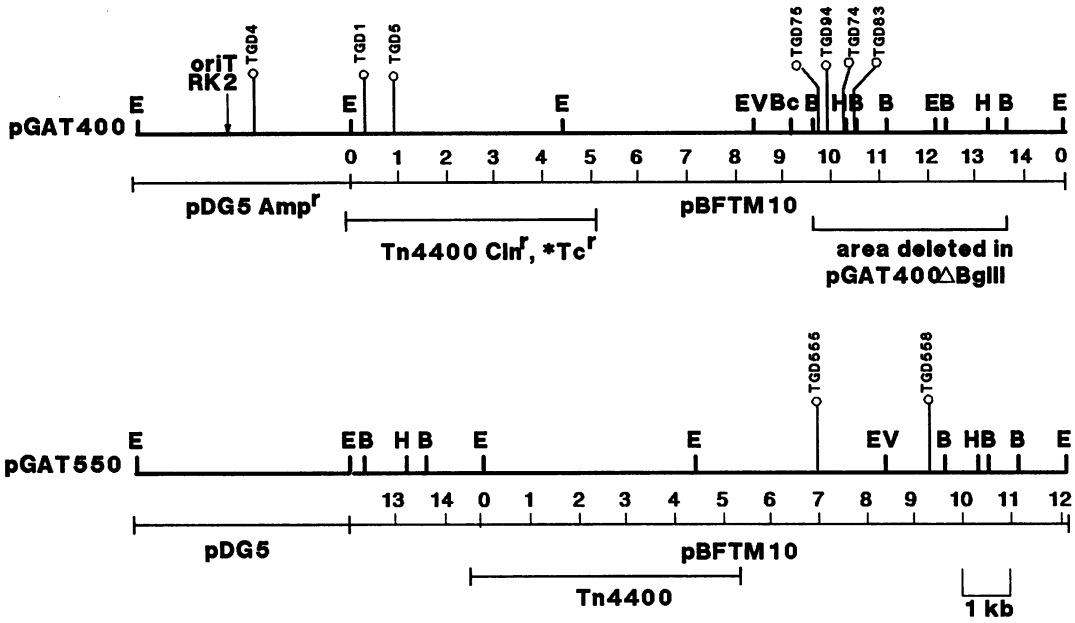

FIG. 1. Maps of shuttle plasmids pGAT400 and pGAT550 with locations of Tn*1000* insertions. Numbers 1 through 14 denote kilobase coordinates of pBFTM10 starting at the second *Eco*RI site located in the left direct repeat of Tn*4400* (19). pGAT550 differs from pGAT400 by the insertion point of pDG5 at the *Eco*RI site at approximately 12.2 kb of pGAT400. Vertical lines labeled TGD1 through TGD558 indicate locations of Tn*1000* insertions in either pGAT400 or pGAT550. Abbreviations: Amp^r, ampicillin resistance; *Tc^r, aerobic tetracycline resistance; Cln^r, clindamycin resistance; E, *Eco*RI; B, *Bgl*II; EV, *Eco*RV; H, *Hin*dIII; Bc, *Bcl*I.

transfer of Tn*1000*-containing plasmids TGD94, TGD75, TGD74, and TGD83 in pGAT400 and TGD558 in pGAT550 to *E. coli* HB101 could not be detected. Plasmids in the third category, TGD74 and TGD83, showed very low transfer to TM429.

Mapping the Tn*1000* insertions. Restriction enzyme analysis was used to determine the location of the Tn*1000* insertions within pGAT400 and pGAT550 (Fig. 1). Six of the seven insertions that led to undetectable or reduced transfer mapped within the region deleted from pGAT400 to create pGAT400ΔBglII. One completely Tra$^-$ insertion, TGD558, mapped to the left of the *Bgl*II site at 9.3 kb, within a region of DNA retained by pGAT400ΔBglII. Plasmids TGD1, TGD5, and TGD555, which showed no alteration in transfer properties, were mapped at 0.2, 0.9, and 7.0 kb, respectively. Only TGD555 mapped within the 5-kb region to the left of TGD558 known to contain genes required for pBFTM10 replication in *B. fragilis* (6). TGD4 mapped within the pDG5 vector.

Transfer from *B. fragilis* TM4.23. TM4.23 is a derivative of TM4000 that contains a TET transfer element derived from *B. fragilis* TMP230. The TET element promotes efficient transfer of a chromosomal tetracycline resistance gene to *B. fragilis* recipients and increases the frequency of transfer of pGAT400 and pGAT550 after tetracycline pretreatment (Tet induction) (10). We tested for transfer of Tn*1000* insertion plasmids from TM4.23 to *B. fragilis* and *E. coli* recipients after Tet induction of the donor cells. Under these conditions pGAT400 and pGAT550 transferred to both *E. coli* and *B. fragilis* at a frequency that was 3 orders of magnitude higher than that for TM4000; transfer of pGAT400ΔBglII to either recipient was not detected (Tables 2 and 3). The transfer frequencies of TGD555, TGD1, TGD4, and TGD5, plasmids in which the transfer from TM4000 was essentially unaltered, were similar to those of the parental plasmids. Insertions TGD75, TGD94, and TGD558 remained completely transfer deficient. In contrast to the TM4000 donor, the insertions TGD74 and TGD83 from TM4.23 transferred at low frequency to HB101. Compared with that from TM4000, the transfer frequency of TGD83 from TM4.23 to TM429 was only slightly higher.

Transfer properties of pGAT400 deletion derivatives. To define what other regions of pBFTM10 may be required for plasmid transfer, we isolated a series of deletion derivatives of pGAT400 by removing *Bgl*II restriction fragments from the transfer region (Fig. 2). An additional deletion derivative of pGAT400 was formed by dropping out the 2.8-kb *Eco*RI restriction fragment to create pGAT400Δ2.8. pBFD1, pBFD2, pBFD3, pBFD4, and pGAT400Δ2.8 transferred from TM4000 to TM429 at frequencies within a fivefold range of pGAT400 (Table 2). Transfer of pBFD7, which contains the inverted *Bgl*II fragment 2 of pBFD1, to TM429 was not detected. With *E. coli* as the recipient (Table 3), pBFD1, pBFD2, and pGAT400Δ2.8 transferred at similar low frequencies, whereas the frequency of pBFD3 transfer was 1 order of magnitude higher. pBFD4 transfer occurred at a frequency lower than that observed for pBFD1 and pBFD2, whereas transfer of pBFD7 to *E. coli* could not be detected.

Transfer of deletion derivatives of pGAT400 from TM4.23. To assess the interaction of the TET element with the deletion plasmids, we tested for transfer of the deletion derivatives from TM4.23 with Tet induction to *B. fragilis* and *E. coli*. The transfer frequency of pBFD2 and pBFD3 to TM429 was similar to that of pGAT400, whereas the transfer frequencies of pBFD1 and pBFD4 were 10- and 100-fold lower, respectively (Table 2). pBFD2 and pBFD3 again transferred to *E. coli* at frequencies similar to that of pGAT400, whereas the transfer frequencies of pBFD1 and pBFD4 were 2 and 2.5 orders of magnitude lower, respec-

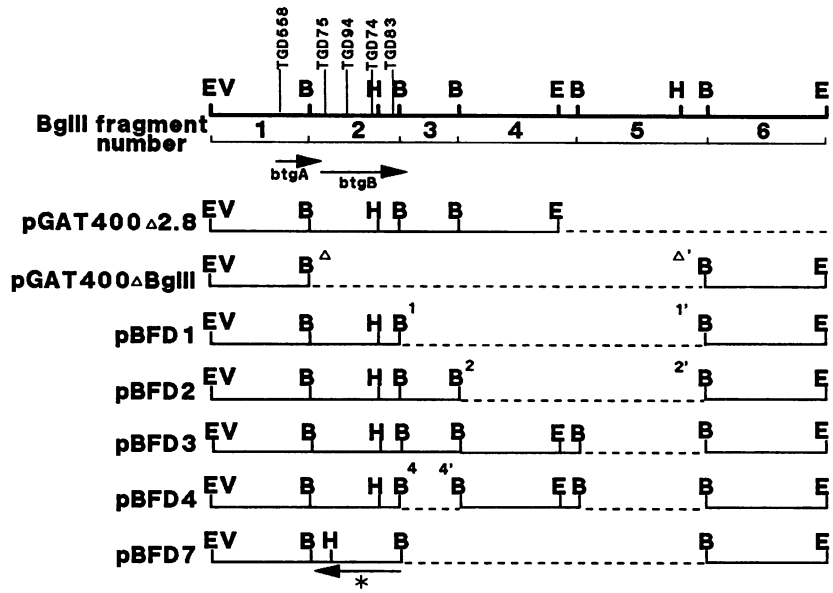

FIG. 2. Maps of pGAT400 deletion derivatives. Heavy lines indicate the addition of *Bgl*II or *Eco*RI fragments to pDG5 and the portion of pBFTM10 from pDG5 (at 0 kb) to the *Eco*RV (EV) site (at 8.4 kb) shown in Fig. 1. The *Bgl*II fragment numbers correspond to *Bgl*II fragments 1 through 6, with the exception of pGAT400Δ2.8, which contains fragments 1, 2, and 3 and a portion of fragment 4 up to the *Eco*RI site. Dotted lines indicate missing fragments. Vertical lines indicate location of Tn*1000* insertions as shown in Fig. 1. Horizontal arrows labeled *btgA* and *btgB* indicate location of ORFs (see the text). The horizontal arrow labeled with an asterisk in pBFD7 indicates that this plasmid has an inversion of fragment 2. The superscripts Δ, Δ', 1, 1', 2, 2', 4, and 4' adjacent to B sites in pGAT400ΔBglII, pBFD1, pBFD2, and pBFD4 correspond to fusion joints. Abbreviations are as in Fig. 1.

tively (Table 3). The transfer frequency of pGAT400Δ2.8 to *B. fragilis* and *E. coli* was slightly higher than that of pGAT400. pBFD7 did not transfer from TM4.23 to *B. fragilis* or *E. coli*.

DNA sequence analysis of the transfer region of pBFTM10. The DNA sequence of a large segment of pBFTM10 including the transfer region was determined by utilizing the ends of Tn*1000* inserted throughout the plasmid as sites of primer annealing. Oligonucleotide primers complementary to derived DNA sequences were used to fill gaps and extend the sequence. The DNA sequence of the 2,162-bp sense strand, location of Tn*1000* inserts, and important restriction sites are shown in Fig. 3. Examination of the sequence for potential polypeptide coding regions reveals two open reading frames (ORFs); ORF1 (bp 612 through 1217) and ORF2 (bp 1215 through 2092) could potentially code for polypeptides of 23,277 and 33,795 Da, respectively. With the *E. coli* consensus sequences as a guide, a potential −35 promoter region for ORF1 occurs at positions 571 through 575 and a −10 promoter region at positions 597 through 602. A potential Shine-Dalgarno translation initiation sequence is located at positions 605 through 610. ORF2 is preceded by a well placed SD sequence at positions 1206 through 1211 and contains a possible −35 promoter region at positions 1153 through 1159 and a −10 promoter region at positions 1168 through 1173.

Location of Tn*1000* insertion sites in the DNA sequence. All Tn*1000* insertions with altered transfer phenotypes were located within either ORF1 or ORF2. TGD558 is inserted in ORF1, whereas TGD75, TGD94, TGD74, and TGD83 are inserted in ORF2. Since insertions in ORF1 and ORF2 are associated with an altered transfer phenotype, we have designated the corresponding genes as *btgA* (*Bacteroides* transfer gene) and *btgB*. TGD558 in *btgA* and TGD75 and TGD94 in *btgB* are associated with a transfer-deficient (Tra$^-$) phenotype, whereas TGD74 and TGD83 in *btgB* are associated with a phenotype (Tra$^\pm$) of low transfer frequency in *B. fragilis*-to-*E. coli* and *B. fragilis*-to-*B. fragilis* matings.

Correlation of the pGAT400 deletion derivatives with the DNA sequence. Figure 4 shows the deletion or segment rearrangement of the *btgA* and *btgB* genes in the deletion derivatives of pGAT400 based on the DNA sequences. The sequences of the fusion joints were determined directly by using synthetic primers immediately upstream of the respective *Bgl*II sites used for fusion. pGAT400ΔBglII contains only a portion of the *btgA* gene, which would lead to the synthesis of a *btgA* protein truncated by the loss of 34 amino acids at its C terminus as a result of fusion to *Bgl*II fragment 6; the *btgB* gene is completely missing. pBFD1 contains all of the *btgA* gene and has a portion of the *btgB* gene, which would lead to the formation of a *btgB* protein truncated 12 amino acids from its C terminus and with 24 new amino acids added by fusion to fragment 6. The fusion site in pBFD1 is downstream of the TGD74 and TGD83 insertion sites. pBFD4 contains all of the *btgA* gene and a portion of the *btgB* gene, leading to the formation of a *btgB* protein truncated 12 amino acids from its C terminus and with 8 amino acids from fragment 4 added. pBFD2 and pBFD3 contain both the *btgA* and *btgB* genes. pBFD7 contains only a portion of the *btgA* gene, leading to the synthesis of a *btgA* protein truncated 34 amino acids from its C terminus but fused to the 3' end of *Bgl*II fragment 2 to give an additional 34 amino acids as the C terminus of the fusion product. The *btgB* gene of pBFD7 is fused to the 3' end of *Bgl*II fragment 1 to give a potential protein truncated 12 amino acids from its original C terminus and with 50 amino acids added.

Linker insertion mutagenesis of *btgA*. The above analysis suggests that there are at least two genes and proteins required for transfer of pBFTM10. However, the insertion

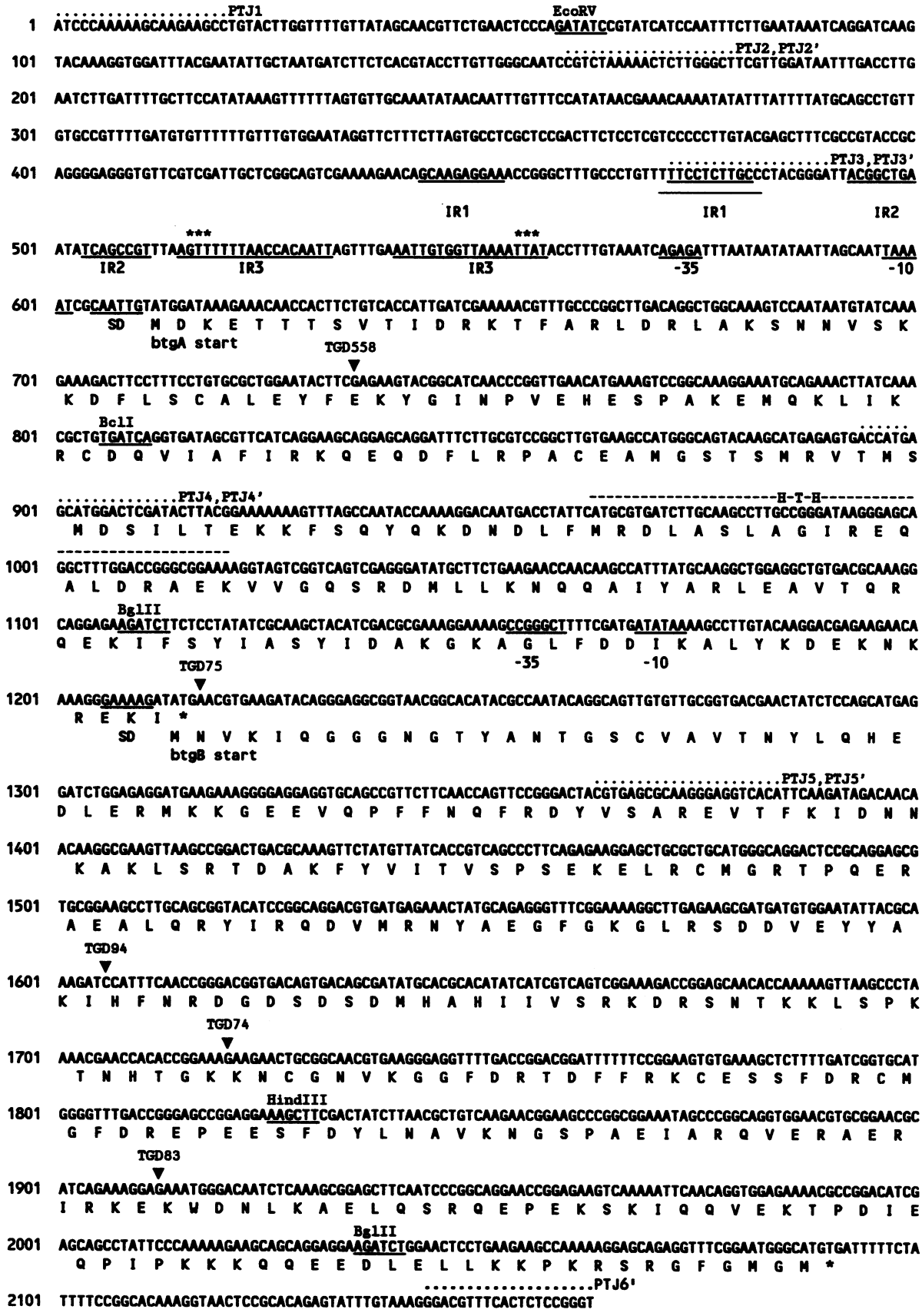
PTJ1
EcoRV
PTJ2,PTJ2'
PTJ3,PTJ3'
IR1
IR1
IR2
IR2
IR3
IR3
-35
-10
BclI
PTJ4,PTJ4'
H-T-H
BglII
TGD558
TGD75
-35
-10
btgA start
SD
btgB start
PTJ5,PTJ5'
TGD94
TGD74
HindIII
TGD83
BglII
PTJ6'

FIG. 3

FIG. 3. Sense-strand DNA sequence of the transfer region. The sequence starts near the *Eco*RV site shown in Fig. 1 and corresponds to bp 59. Translation of two ORFs with start codons are shown and labeled *btgA* and *btgB*. Putative Shine-Dalgarno, −10, and −35 promoter regions for each ORF are underlined and labeled. The locations of the Tn*1000* insertion sites are shown. The TGD558 insertion is located in *btgA*, whereas TGD75, TGD94, TGD74, and TGD83 are located in *btgB*. The location of the *Bam*HI linker inserted into *btgA* in pDHL1 is at the indicated *Bcl*I site. Other important restriction sites are labeled. Inverted repeats upstream of *btgA* are underlined in pairs and labeled IR1, IR2, and IR3. Asterisks above IR3 indicate mismatched nucleotides. The double-underlined region starting at position 471 indicates the location of a possible consensus sequence for a nick site similar to that described by Waters et al. (28) and Panseqrau and Lanka (15). The region identified by the dashes above the sequence indicates the possible helix-turn-helix sequence in *btgA*. Synthetic primers used to sequence gap regions are shown by dots above sense strand and labeled pTJ1-6. The prime (′) designation indicates that a complementary primer was used to determine upstream sequences.

mutant TGD558 in *btgA* may only be exerting a polar effect on *btgB* expression. To test directly whether *btgA* is essential for transfer, we synthesized a 36-bp double-stranded DNA linker and ligated it into a unique *Bcl*I site near the middle of the *btgA* gene (Fig. 1). This linker (Fig. 4A) could code for 12 amino acids (1′ through 12′) and contains a *Bam*HI site to assist in verifying its presence. The linker was designed to alter the primary structure of the *btgA* protein but does not alter the reading frame or lead to translation termination. Thus, the effect of this insertion would be to disrupt the function of *btgA* without altering potential transcription of *btgB*. A plasmid containing this linker, pDHL1, was tested for transfer from TM4000 and TM4.23. pDHL1 could not transfer from either TM4000 or TM4.23 with Tet induction to *B. fragilis* or *E. coli* (Tables 2 and 3). This result is the same as that seen with the *btgA* insertion mutant TGD558 and the mutant with the complete transfer region deletion, pGAT400ΔBglII.

R751 mobilization of pGAT400, deletion, and Tn*1000* insertion derivatives. *E. coli* strains containing the shuttle vector pGAT400 or pGAT550 are unable to transfer these plasmids to *E. coli* or *B. fragilis* recipients. The transfer factor R751 has been previously shown to mobilize in *trans* shuttle vectors containing pBFTM10 from *E. coli* donors to *E. coli* recipients (23). We therefore tested the ability of R751 to mobilize our shuttle vectors pGAT400 and pGAT550, the Tn*1000* insertion mutants, and the pGAT400 deletion derivatives from R751 containing derivatives of *E. coli* HB101 to *E. coli* DW1030. However, before the Tn*1000* insertion mutants were tested for interactions with R751, the transpo-

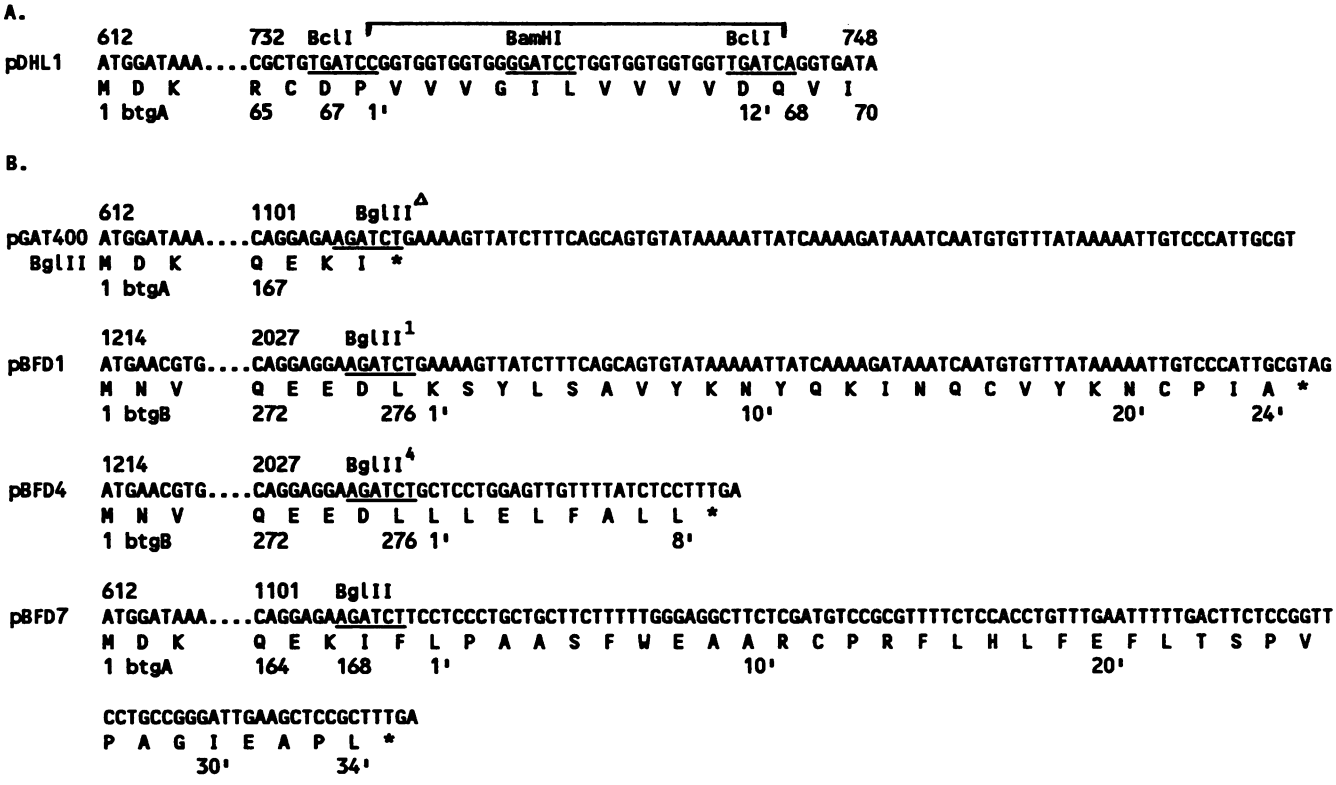

FIG. 4. *btgA* and *btgB* fusion proteins created by linker insertion and fragment deletions. (A) Sense-strand DNA sequence of the *Bam*HI linker inserted into the *Bcl*I site in *btgA*. The bracketed region indicates the additional amino acids inserted 1′ to 12′. The numbers above the sequence indicate sequence locations of insertions, and the numbers below the sequence indicate amino acid locations. (B) DNA and amino acid sequence of fusion proteins from deletion derivatives. Only deletion plasmids with altered BtgA or BtgB proteins are shown. The DNA sequence with the location of the *Bgl*II fusion joint is shown above, and the corresponding amino acid sequence is shown below. The superscripts Δ, 1, and 4 adjacent to the *Bgl*II restriction sites correspond to the fusion joints shown in Fig. 2. Amino acids corresponding to the wild-type protein are numbered. Amino acids added as a result of the fusion are shown by the prime (′) designation.

TABLE 4. Mobilization by R751 of shuttle vector pGAT400 and Tn*1000* insertions or deletion derivatives from *E. coli* HB101 to *E. coli* DW1030

Donor plasmid	Mean frequency of transfer (±SE)
pGAT400	$9.2 (\pm 0.89) \times 10^{-3}$
pGAT400ΔBGLII	$1.5 (\pm 0.5) \times 10^{-5}$
pBFD1	$7.4 (\pm 3.0) \times 10^{-4}$
pBFD2	$3.3 (\pm 0.63) \times 10^{-3}$
pBFD3	$6.0 (\pm 0.55) \times 10^{-3}$
pBFD4	$3.0 (\pm 0.1) \times 10^{-4}$
pBFD7	$1.5 (\pm 0.5) \times 10^{-5}$
pTGD1	$8.3 (\pm 0.9) \times 10^{-3}$
pTGD4	$3.0 (\pm 1.5) \times 10^{-2}$
pTGD5	$7.2 (\pm 0.8) \times 10^{-3}$
pDHL1	$3.1 (\pm 0.86) \times 10^{-5}$
pTGD83	$1.4 (\pm 0.25) \times 10^{-5}$
pTGD94	$1.2 (\pm 0.21) \times 10^{-5}$
pTGD75	$1.9 (\pm 0.57) \times 10^{-5}$
pTGD74	$4.5 (\pm 1.0) \times 10^{-5}$

sition properties of the Tn*1000* transposon were inactivated by deleting a transposon internal *Xho*I segment (8) that contains a portion of the *tnpA* gene and *res* site. Thus the inactivated Tn*1000* could not cause cointegrate formation with R751. Mobilization of pGAT400 by R751 occurs at a frequency per input donor of 9.2×10^{-3}, whereas that of pGAT400ΔBglII occurs at least 3 orders of magnitude less frequently (Table 4). Restriction enzyme analysis of plasmid DNA from transconjugants of each mating demonstrated that the pGAT400 plasmids were unaltered; however, all transconjugants from the pGAT400ΔBglII mobilization were altered, probably as a result of cointegrate formation with R751 (Fig. 5). Representative examples of transconjugants of R751 mobilization are shown in Fig. 5. These results allow us to distinguish between transfer events occurring in *trans* and those occurring by cointegrate formation. Transfer-defective Tn*1000*-containing derivatives of pGAT400 (TGD74, TGD75, TGD83, TGD94) were mobilized at frequencies

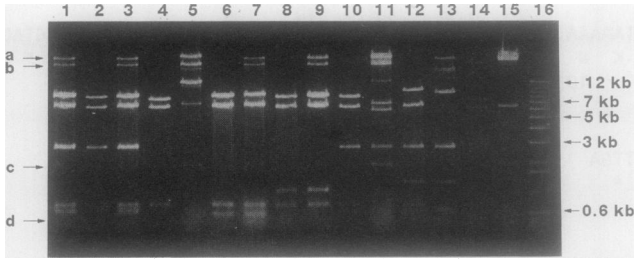

FIG. 5. Analysis by agarose gel electrophoresis of *Hin*dIII digests of pGAT400, pGAT400 deletion derivatives, pGAT400:: Tn*1000*, and R751 plasmids and their transconjugant products after R751 mobilization. Lanes: 1, pGAT400 and R751 donor; 2, pGAT 400 parental plasmid; 3, pGAT400 transconjugant (unaltered); 4, pGAT400ΔBglII parent; 5, pGAT400ΔBglII transconjugant showing cointegrate formation; 6, pBFD1 parent; 7, pBFD1 transconjugant (unaltered); 8, pBFD2 parent; 9, pBFD2 transconjugant (unaltered); 10, pDHL1 parent; 11, pDHL1 transconjugant (cointegrate); 12, pTGD74 parent; 13, pTGD74 transconjugant (cointegrate); 14, pBFD7 parent; 15, pBFD7 transconjugant (cointegrate); 16, size standard (1-kb ladder; BRL Laboratories, Grand Island, N.Y.). The four arrows labeled a, b, c, and d indicate parental R751 bands and correspond to fragments sizes of 29.2, 20.0, 1.6, and 0.5 kb respectively (12). The arrows on the right indicate the size standards.

similar to that of pGAT400ΔBglII; in each case analysis of transconjugant plasmids revealed that the original plasmid had been altered. The deletion derivatives pBFD2 and pBFD3 were mobilized only slightly less frequently than was pGAT400, whereas mobilization of pBFD1 and pBFD4 was at least 10-fold less frequent. Analysis of plasmid DNA from the transconjugants involving pBFD2 and pBFD3 showed that all were unaltered (mobilized in *trans*); 90% of pBFD1 transconjugants were also unaltered, whereas 10% were altered, probably from cointegrate formation. The frequency of pDHL1 mobilization by R751 was similar to that of pGAT400ΔBglII, and all transconjugants were altered.

DISCUSSION

Although the conjugal transfer factors such as the F and R factors of the family *Enterobacteriaceae* have been intensively studied over the last 30 to 40 years, attention has only recently been focused on conjugal elements of the *Bacteroides* spp., the obligately anaerobic majority component of the gastrointestinal flora. The 15-kb *B. fragilis* plasmid pBFTM10 can transfer by a conjugation process from *B. fragilis* donor cells to *B. fragilis* or *E. coli* recipients (26). We have characterized and sequenced a 2,162-bp region of pBFTM10 that contains genes and sites required for transfer of the plasmid from *B. fragilis* donor cells.

The DNA sequence of the pBFTM10 transfer region revealed the presence of two ORFs; the Tra^- insertion TGD558 is located in ORF1, and the Tra^- insertions TGD75 and TGD94 are located in ORF2. ORF1 could code for a 23.2-kDa protein that is interrupted by TGD558, corresponding to amino acid 42, 159 amino acids from the N terminus. No other Tn*1000* insertions were located in ORF1; however, the in vitro insertion of a 36-bp linker within ORF1, 133 amino acids from its C terminus, also resulted in a Tra^- phenotype. The gene corresponding to ORF1 has been designated *btgA*. The predicted polypeptide structure gives no indication that it is a membrane protein. Visual inspection of amino acid sequence of BtgA reveals a potential helix-turn-helix region located at amino acids 118 through 137 (9). The identified sequence shows strong similarities to the helix-turn-helix region of the phage 434 Cro protein. David Sanford of the Department of Biochemistry at Tufts University has built a model of the Cro molecule from the X-ray crystal structure coordinates (structure 2CRO in the Brookhaven Protein Data Bank) by using the molecular modeling program Quanta (Polygen Corp., Waltham, Mass.). He then replaced the residues in the helix-turn-helix region of 434 Cro with the corresponding residues from the BtgA protein. No unfavorable steric interactions were introduced by these substitutions, indicating that the sequence differences between 434 Cro and BtgA do not preclude the adoption of the helix-turn-helix conformation.

ORF2 could code for a 33.8-kDa protein. Of the four Tn*1000* insertions located in this ORF, the transfer defective TGD75 and TGD94 inserts are located near the N terminus (amino acid 4, TGD75) and the middle of the ORF (amino acid 131, TGD94). The other two insertions, TGD74 and TGD83, are located near the C terminus (amino acid 169, TGD74; amino acid 233, TGD83) and show low but detectable transfer to the *B. fragilis* recipient. These results indicate that the product of ORF2 is necessary for transfer but that a truncated product may have a partial function. The gene for ORF2 has been designated *btgB*. The predicted polypeptide structure gives no indication that BtgB is a membrane protein.

The coding regions of the *btgA* and *btgB* genes overlap; the initiation codon for *btgB* translation is located 2 bp before the stop codon of *btgA*. Translation of *btgB* is therefore in a different reading frame from that of *btgA*. It may be that *btgA* and *btgB* are part of an operon. Thus transposon insertion mutations in *btgA* could lead to inactivation of *btgB* by a polarity effect. By inserting the 36-bp fragment into the unique *Bcl*I site of *btgA* without introducing transcriptional or translational stop signals and conserving the proper reading frame, we have demonstrated that the *btgA* product is necessary for transfer.

The region just upstream of the *btgA* gene may contain an origin of transfer (*oriT*). Three sets of inverted repeats (Fig. 3) occur within a 115-bp region 170 bp from the start of the *btgA* gene. The largest inverted repeat (IR3) is 18 bp long with a 15- out of 18-bp match. A perfect 8-bp inverted repeat (IR2) and a perfect 10-bp inverted repeat (IR1) are located 9 and 39 bp, respectively, from IR3.

The *oriT* region of IncPα plasmid RP4 has a similar organization; there is a 19-bp perfect inverted repeat located adjacent to two genes, *traI* and *traJ*, that are involved in binding and nicking at the *oriT* site to initiate DNA transfer (4, 16, 31). The nick site in the RP4 *oriT* is located 8 bp from the right end of the inverted repeat. Recently, Waters et al. reported a consensus sequence that occurs at the nick sites in the *oriT* regions of RP4 and R751 and in the Ti (tumor-inducing) plasmids of *Agrobacterium tumefaciens* (28). Pansegrau and Lanka have also published common sequence motifs in DNA relaxases and nick regions of several DNA transfer systems (15). We can identify a sequence in the 115-bp region of pBFTM10 at position 480 upstream of the *btgA* gene that contains an 9- out of 12-bp match to the nick consensus sequence. Whether this represents the nick site of pBFTM10 remains to be determined. Preliminary experiments with the relaxosome methods of Guiney et al. (4) have allowed us to localize the nick site of pBFTM10 to this region (unpublished data).

In light of the overall similarity in the organization of the transfer region of pBFTM10 to RP4 and the closely related R751, we conducted a computer analysis comparing *btgA* and *btgB* with *traI*, *traJ*, and *traK* of RP4. The analysis with the Compare and BestFit programs of GCG failed to detect any significant similarities at either the DNA or amino acid level. In addition, no significant similarities were found for any other DNA segments or proteins in GenBank release 67.0. We did, however, locate a potential *Cro*-like DNA binding domain in *btgA* (helix-turn-helix, Fig. 3) by visual inspection; this feature was not found with the Motifs or Profile program of the Genetics Computer Group. A DNA binding motif in *btgA* is consistent with its possible role in transfer initiation.

There is strong evidence that the *btgA* and *btgB* transfer genes of pBFTM10 are expressed in *E. coli* as well as in *B. fragilis*. The presence of R751 is required for mobilization of the parental plasmid pGAT400 from *E. coli* donor cells to *E. coli* recipient cells. The inactivation of *btgA* in TGD558 or pDHL1 and the inactivation of *btgB* in TGD75 and TGD94 not only result in a Tra$^-$ phenotype from *B. fragilis* donors but also the inability to be mobilized in *trans* by R751 in *E. coli* donors. The *btgB* C-terminal insertions TGD74 and TGD83 were not mobilized in *trans* by R751, although they showed lowered efficiency of transfer from *B. fragilis* donors. In contrast, the *btgB* deletion of pBFD1 was still mobilizable by R751. This suggests that an intact C terminus of *btgB* is required for R751 mobilization but that the altered C terminus of pBFD1 supplied by *Bgl*II fragment 5 (Fig. 2) can also function in this system. pBFD4 has a different but also partially functional *btgB* fusion product. It is interesting to note that partial function of a truncated *traJ* protein from RK2(RP4) has also been reported (5). Thus, the expression of both *btgA* and *btgB* in *E. coli* is required for efficient transfer in the presence of R751.

Previous work from Salyers' laboratory (23) implicated the same transfer region defined in this paper in R751-promoted mobilization of shuttle vectors containing pBFTM10. Salyers et al. found that subclones of the pBFTM10 portion divided at the *Hin*dIII site located at 10.2 kb (Fig. 1) had lost the ability to interact with R751. We now know that the *Hin*dIII site is in the *btgB* gene, which is required for pBFTM10 transfer in both *B. fragilis* and *E. coli*.

The roles of the *btgA* and *btgB* gene products in pBFTM10 transfer from *Bacteroides* donor cells are unknown. It is likely that these genes and the potential *oriT* sequence located just upstream function in the initiation of DNA transfer to the recipient cell by the mechanism described for the IncP plasmids (7). Indeed, a possible DNA binding domain is present in *btgA*. However, the functions required for the association of donor and recipient cells to establish effective mating contacts are probably not coded for by pBFTM10. It may be that *Bacteroides* cells are intrinsically able to interact to provide the contacts that allow transfer of conjugal elements, or possibly the chromosome of *B. fragilis* TM4000 contains a cryptic transfer element that encodes functions for effective contact formation.

The role of the chromosomal TET element of TM4.23 in the increased transfer efficiency of pGAT400 and pGAT550 also remains unknown. Although it could be suggested that the TET element supplies all transfer functions that act in *trans* to promote plasmid transfer, none of the Tra$^-$ plasmids was complemented by the TET element. In addition, the *btgB* insertion mutations TGD74 and TGD83 were able to transfer at very low frequency from TM4.23 but not TM4000; this most likely reflects the limits of detectability for these mutants in interspecies matings and not complementation. Therefore, in addition to whatever function the donor cell is contributing to the transfer, the role of the TET element of TM4.23 may be to create a greater number of effective contacts or to increase the efficiency of the overall mating process.

In *E. coli*, although *btgA* and *btgB* are clearly required for efficient transfer of pGAT400, an IncP transfer factor is also required. Despite the similarity of the organization of the DNA processing portions of pBFTM10 to those of the *oriT* activation regions of R751 and RP4, the *traI* and *traJ* functions of R751 cannot complement *btgA* or *btgB* mutants or deletions. We presume that the effective contacts formed by using the R751 transfer apparatus, including the P pilus, are sufficient to allow the DNA transfer initiation and processing functions of pBFTM10 to carry out plasmid transfer to the recipient cells.

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