The Bacteroides fragilis BtgA Mobilization Protein Binds to the oriT Region of pBFTM10

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Received 15 January 1998/Accepted 13 July 1998

Bacterial conjugation is a major mechanism for the transfer of chromosomal or plasmid DNA between prokaryotic cells. As a result, dissemination of antibiotic resistance determinants can occur, significantly influencing the virulence of various pathogens such as Escherichia coli, enterococci, staphylococci, streptococci, streptomycetes, and the Bacteroides fragilis group (2, 4, 10, 22, 33, 34). The initial reactions during conjugal DNA transfer require multiple complex protein-DNA and protein-protein interactions that result in the formation of a relaxosome at the cis-acting origin of transfer (oriT). Relaxosomes are stable nucleoprotein complexes resulting in site- and strand-specific nicking at a site (the nic site) in the oriT region (24). The relaxase, or DNA strand transferase, cleaves at this nic site and attaches covalently to the 5′ terminus. DNA is transmitted unidirectionally as a single strand into a recipient cell with 5′-to-3′ polarity in a rolling-circle manner, followed by complementary-strand synthesis in the new host (for detailed reviews of conjugative transfer involving R and F factors, see references 25 and 44).

Much attention has been focused on the biochemical processes involved in the initiation of DNA transfer of the IncP plasmid RP4. The generation of the single DNA strand that is transferred requires formation of the initiation complex, or DNA relaxosome, in a cascade-like fashion. The first step in relaxosome formation is binding of an RP4 protein, TraJ, to a 19-bp inverted repeat within the oriT region and a 10-bp palindrome, sr (24, 43). In the second step, TraI, another RP4 protein, binds TraJ by both protein-protein interactions and the recognition of a 6-bp sequence, sri, in the nic region. Subsequently, site- and strand-specific cleavage of a unique phosphodiester bond at the nic site of the transfer origin occurs, followed by covalent attachment of TraI to the 5′ terminus of the DNA strand involved in transfer. The binding and nicking events are thought to be independent processes (16, 26). TraI is also able to induce a second cleavage reaction proposed to terminate the rolling-circle model of transfer DNA replication (27). Following strand transfer to a new host, TraI catalyzes the recombination of two single-stranded DNA molecules at the RP4 nic site (27). A third protein, TraH, stabilizes the TraI and TraJ nucleoprotein complex by specific protein-protein interactions and does not bind DNA itself (24, 45). A fourth protein, TraK, interacts with oriT by binding DNA as a tetramer over a range of approximately 180 nucleotides, downstream of nic (46). Binding of TraK increases the formation of relaxation complexes in vitro and in vivo, possibly by influencing DNA topology to expose the nic site for more efficient cleavage by TraJ (43).

Transferable antibiotic resistance plasmids of the B. fragilis group have also been described. They include the clindamycin-resistant plasmids pBFTM10 (12), pBF4 (40), and pB136 (31) and the metronidazole-resistant plasmids pIP417, pIP419, and pIP421 (11, 37, 38). pBFTM10 is a 14.9-kb plasmid that is transferable within B. fragilis. When pBFTM10 is fused to the E. coli replicon pDG5 to form pGAT400, it can also transfer from B. fragilis to E. coli. Further, pGAT400 is also mobilizable within E. coli when coresident with the IncP plasmid R751, but it requires an intact pBFTM10 transfer region (12). Characterization of the pBFTM10 transfer region has demonstrated that only two genes, btgA and btgB, are necessary for transfer within B. fragilis and mobilization by R751 in E. coli. DNA sequence analysis of btgA revealed a helix-turn-helix DNA binding motif, suggesting that it was a DNA binding protein (12). In addition, the identification of three sets of inverted repeats (IRI, IRII, and IRIII) and a putative nic site in the region upstream of btgA suggested that this was the pBFTM10 oriT (12, 39).

In this paper, we report the cloning of the pBFTM10 oriT and the expression and purification of the BtgA protein, and we demonstrate specific binding of BtgA to its cognate oriT as determined by mobility shift assays and footprinting analyses.
TABLE 1. E. coli strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW1030</td>
<td>E. coli Sp' recA</td>
<td>29</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>E. coli F' (r' m') (DE3)</td>
<td>Novagen</td>
</tr>
<tr>
<td>HB101</td>
<td>E. coli Sm' recA</td>
<td>29</td>
</tr>
<tr>
<td>pGAT400</td>
<td>Tra+ Amp' Cm'</td>
<td>12</td>
</tr>
<tr>
<td>R751</td>
<td>Tra' Tmp'</td>
<td>19</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Tra' Cm' Tet'</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pGEM-7Zf(+)</td>
<td>Amp' cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pJA-7ZT</td>
<td>pBFTM10 ortI cloned into pGEM-7Zf(+)</td>
<td>This study</td>
</tr>
<tr>
<td>pJAYC1</td>
<td>pBFTM10 ortI cloned into BamHI site of pACYC184</td>
<td>This study</td>
</tr>
<tr>
<td>pET-19b</td>
<td>Amp', expression vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pDHBA</td>
<td>big4 in pET-19B BamHI site</td>
<td>This study</td>
</tr>
</tbody>
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*MATERIALS AND METHODS*

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Media, antibiotic concentrations, and E. coli growth conditions were as previously described (1, 20). Bacto Agar and tryptone were obtained from Difco Co. (Detroit, Mich.). For strains requiring antibiotic selection, cells were grown at 37°C overnight in Luria broth (LB) supplemented with 200 μg of ampicillin per ml. E. coli XL1-Blue was used for routine cloning procedures (29). The putative ortI region was cloned into the BamHI site of pGEM-7Zf(+). The BtgA protein was purified by an affinity binding procedure using His-Tag resin as specified by the manufacturer (Perkin-Elmer Cetus Co., Norwalk, Conn.) was used according to the manufacturer's specifications for all PCR amplifications, which were performed on a Perkin-Elmer Cetus GeneAmp PCR System 2400 thermal cycler with plasmid pGAT400 (12) as the template. Two primers, arot5B (5'-GGTGTGATGCGGTCCCGGCTTCTTCTTGGC-3') and arot3B (5'-CGCGGGATCCGAAGGTTCTTGTC-3'), were used to introduce BamHI restriction endonuclease sites at both ends of the ortI region cloned into the pGEM-7Zf(+) (Fig. 1) to give pLA-7ZT. Primer arot5B is complementary to nucleotides 334 to 351 upstream of the inverted repeats, and primer arot3B is complementary to nucleotides 621 through 638 immediately downstream of the inverted repeats (12). Similarly, primers arbaSN (5'-CGCGGATCCGAGAAGAAGTGGTTGTTGTC-3') and arba3B (5'-CGATGAGTCTACCCTGCTTCTATTTAT-3') were used to introduce BamHI restriction endonuclease site at the 5' and 3' ends of the B. fragilis btgA gene for cloning in pET-19B/BamHI to give pDHBA. All PCR products were electrophoresed through 1.3% agarose gels. The appropriate bands (646 bp for btgA and 304 bp for the ortI region) were excised and purified by using GeneClean II (Bio 101) prior to restriction endonuclease treatment and cloning.

DNA sequence determination. Sequencing was performed by the Sanger dideoxy-chain termination method, using a Sequenase version 2.0 reagent kit as specified by the manufacturer (Amersham Co., Arlington Heights, Ill.).

Protein expression and purification. The BtgA protein was purified by an affinity binding procedure using His-Tag resin as specified by the manufacturer (New England Biolabs). Bacterial growth was monitored by measuring the optical density at 600 nm, and the culture was harvested when the absorbance was 0.6. The bacterial cells were washed once in phosphate-buffered saline and then suspended in 100 mM Na3HPO4, 15 mM Na2HPO4, 145 mM NaCl [pH 6.9]). Resuspended cells were then spotted onto sterile 25-mm, 0.45-μm-pore-size Gelman GN-6 cellulose nitrate filters (Nalge Co., Rochester, N.Y.) on Luria agar plates and incubated at 37°C for 180 min. Transconjugants were enumerated by plating on selective media, and the mobilization frequencies of plasmids were normalized to the number of R751 transconjugants from the same experiment. The mobilization frequency of R751 was calculated as the mean ± standard error of the number of R751 transconjugants divided by the number of viable R751 donor cells.

**FIG. 1.** Schematic representation of the pBFTM10 transfer region. Base pair numbers correspond to those used by Hecht et al. (12). Open rectangles represent the positions of the DNA oligonucleotides used for PCR amplification of the ortI region and the btg4 gene: a, arbaSN; b, arba3B; c, arot5B; and d, arot3B. The expanded view of the ortI region illustrates the positions of IRI, IRII, and IRIII relative to the start codon (ATG) of the btg4 gene.
soluble protein. Therefore, the following dialysis steps were carried out to reduce the urea, salt, and imidazole concentrations: the first dialysis, to 100 mM imidazole, 2 M urea, and 200 mM NaCl; the second, to 10 mM imidazole, 0.5 M urea, and 100 mM NaCl; the third, to 100 mM urea and 100 mM NaCl; and the fourth, to store at 4°C. The third dialysis buffer containing 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 15% glycerol. With this method, approximately 85% of purified protein was recovered in soluble form. The protein concentration was determined with the Bradford reagent (Bio-Rad Co., Hercules, Calif.), aliquoted, and stored at −80°C. Aliquots of BigA were treated with an equimolar amount of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 5 min, and subjected to SDS-PAGE on 10% gels (15). Gels were stained with 0.5% Coomassie brilliant blue (Boehringer Mannheim) in 25% methanol-10% acetic acid.

### RESULTS

**Cloning the oriT region of pBFMT10.** Previous DNA sequence analysis of the pBFMT10 mobilization region in pGAT400 revealed three sets of inverted repeats and a possible nic site, based on consensus sequence immediately upstream of big4 (12). In addition, preliminary DNA relaxosome isolation studies also demonstrated that single-stranded DNA nicking occurred in or near this region (unpublished data). To determine if this region served as a functional oriT and a likely binding region for big4, a 304-bp fragment encompassing the three inverted repeats and the putative nic site was initially amplified by PCR using plasmid pGAT400 as a template. Figure 1 illustrates the transfer region of pBFMT10 and the locations of the PCR primers (see Materials and Methods). Following cloning into the pGEM-Zf(+) BamH I site, DNA sequence analysis confirmed that the amplified oriT region was properly cloned without any PCR-induced errors. The 304-bp oriT fragment was then excised, cloned again into the unique BamH I site of pACYC184 to form pJAYC1, and used in bacterial conjugation experiments.

**Demonstration of oriT function by bacterial conjugation.** Previously, pGAT400 was shown to mobilize between E. coli mating pairs when coresident with R751. pGAT400 contains big4 and big5, both necessary for its own transfer, while R751 is presumed to provide the machinery necessary for transfer between E. coli cells (12, 17, 19, 30). To determine if the cloned putative oriT region was functional, pJAYC1 was transformed into E. coli HB101 containing pGAT400 and R751, or R751 alone, and used as a donor in bacterial conjugation experiments. HB101 cells containing either pGAT400, R751, pJAYC1, or pACYC184 were used as negative controls.

Donor cells containing pGAT400 and R751 together with either pJAYC1 or pACYC184 were mixed with E. coli recipient strain DW1030 in mating experiments, and the transfer frequencies of R751, pGAT400, pJAYC1, and pACYC184 were determined. Throughout all mating experiments, the transfer frequencies of R751 ([1.87 ± 0.23]×10⁻³) and pGAT400 ([5.72 ± 0.64]×10⁻³, normalized to the level for R751) were similar to previously observed values (12). When coresident with both pGAT400 and R751, pJAYC1 was mobilized to DW1030 at a frequency of (4.29 ± 1.18)×10⁻² (normalized to the level for R751), similar to that of pGAT400. However, when coresident with R751 alone or when present with no coresident plasmid, pJAYC1 was not mobilized, indicating the requirement for pGAT400. In addition, when the donor harbored pJAYC1 and pGAT400 in the absence of R751, pJAYC1 was not mobilized, indicating the requirement for R751 for mobilization in E. coli. As expected, pACYC184 did not mobilize when coresident with either R751 or pGAT400 or with both.

To ensure that pJAYC1 transfer was not the result of coincident mobilization by pGAT400, transconjugants were plated on selective media and assayed for cotransfer of pGAT400 and pJAYC1 (see Materials and Methods). Only 15% of 200 Sp⁻ Cm⁺ pJAYC1 transconjugants contained pGAT400 (Amp⁺), while only 1% of 100 Sp⁺ Amp⁺ pGAT400 transconjugants contained pJAYC1 (Cm⁺). Restriction endonuclease treatment of plasmid DNA isolated from 20 Sp⁻ Cm⁺ Amp⁺ or Sp⁻ Cm⁺ Amp⁺ pJAYC1 transconjugants confirmed that pJAYC1 and
pGAT400 were not altered, and cointegration of pJAYC1 with either pGAT400 or R751 did not occur (data not shown). In addition, R751 was found in 100% of pGAT400 transconjugants but only 85% of pJAYC1 transconjugants. These findings indicated that pGAT400 and pJAYC1 cotransfer was rare and not the result of cointegrate formation. Thus, the 304-bp pBFTM10 DNA fragment cloned in pJAYC1 contained a functional oriT, and pJAYC1 mobilization required the btgA and bigB genes.

Cloning and expression of BtgA. The predicted amino acid sequence of BtgA revealed a helix-turn-helix DNA binding motif (amino acids 118 to 137) (12). This protein, therefore, seemed a likely candidate for binding to the oriT region. To test this possibility, btgA was cloned into the pET-19b expression vector to give pDHBA and overexpressed in E. coli BL21 (DE3).

Cloning and expression of BtgA. Lane 1, Promega mid-range protein molecular mass marker (masses in kilodaltons are shown at the left); lane 2, proteins that did not bind to the His-Tag binding resin following the addition of crude extract; lane 3, BtgA protein eluted from the His-Tag column following multistep dialysis (see Materials and Methods).

Gel mobility shift assay. The BtgA-oriT complex was analyzed in an electrophoretic mobility shift assay to demonstrate noncovalent interaction of BtgA with the oriT (1, 8). Purified BtgA protein ranging from 10 to 300 ng and labeled 304-bp pBFTM10 oriT fragment were incubated with a binding buffer and electrophoresed in 5% polyacrylamide gels at 4°C (Fig. 3; see Materials and Methods). Increasing concentrations of BtgA (10, 50, and 100 ng [Fig. 3, lanes 2 to 4, respectively]) demonstrated a concentration-dependent manner of binding with oriT. At 300 ng (lane 5), BtgA demonstrated the maximum shift of oriT DNA (position A). The presence of poly(dI-dC) at 1 and 3 $\mu$g failed to disrupt the interaction between the oriT region and BtgA (lanes 6 and 7, respectively). To demonstrate the specificity of binding, specific competitor pJA-7ZT (containing pBFTM10 oriT) plasmid DNA was added in a 50-fold excess, resulting in partial inhibition of BtgA binding, while 100- and 300-fold excess competitor completely inhibited the binding reaction (lanes 8 to 10). Of note, storage of BtgA at −80°C resulted in some loss of binding activity after a few days, likely the result of precipitation.

DNase I footprinting. DNase I protection experiments were used to localize the precise binding sites of BtgA on the pBFTM10 oriT. The oriT fragment was specifically labeled on the upper or lower strand with [γ-32P]ATP and then incubated in the presence of increasing amounts of purified BtgA. These DNA-protein complexes were subjected to partial DNase I digestion to define the binding site(s) of BtgA. The resulting products were separated on 8% polyacrylamide sequencing gels and visualized by autoradiography (Fig. 4). Three distinct regions of protection were visualized on the lower strand. Two regions, consisting of 36 nucleotides (462 to 498 [Fig. 4A]) and 35 nucleotides (501 to 535 [Fig. 4A]), demonstrated the strongest protection after the addition of 0.5 $\mu$g of BtgA, while a third region, containing three identifiable nucleotides (536 to 558 [Fig. 4A]), demonstrated weaker protection at higher protein concentrations. In addition, 3 nucleotides, 498 to 500, showed weaker protection and were located between the strongly protected regions. The third protected region overlaps the right half of IRI (which contains a consensus sequence for the nick site) and the left half of IRII (Fig. 4 and 5), while the second protected region overlaps the right half of IRII and the left half of IRIII. The third, weaker region of protection overlaps 10 nucleotides of the right half of IRIII. Bands at 458 to 461 and 498 to 500 demonstrated possible minimal protection. No regions of protection were found in the remaining sequence. We detected no regions of protection on the upper strand that would correspond to the protected zones on the lower strand (Fig. 4B).

DISCUSSION

The conjugative mechanisms required for transfer of plasmids in Bacteroides spp. are not well understood but are presumed to include initiation processes, similar to those of RP4 or the F plasmid (6, 9, 27, 44). Thus, it is presumed that relaxosomes are formed prior to transfer of a single strand of DNA. For RP4, this requires an oriT region, two DNA binding proteins, a stabilizing protein, and a nickase (16, 43). pBFTM10 has only two transfer genes, both of which are necessary for DNA transfer.
To further our understanding of the processes required for mobilization of plasmid DNA in *Bacteroides* spp., we first determined the location of the oriT region by testing for mobilization in *E. coli*. A 304-bp fragment that contained the three sets of inverted repeats identified previously as likely targets for protein binding was chosen. The putative promoter of *btgA* is immediately downstream of IRIII and was also included in the cloned fragment. Mobilization of the oriT region in *E. coli* was demonstrated only when both *btgA* and *btgB* were provided in trans, although R751 must also be coresident. It is presumed that the role of R751 is to provide a transfer apparatus for mobilization of *Bacteroides* transfer factors, including pGAT400, pLV22a, and the conjugal transposon Tn4399, in *E. coli* (12, 13, 21, 25, 32). However, intermediate mobility shifts were seen at protein concentrations ranging from 10 to 100 ng (Fig. 3). These findings could represent the oligomerization of BtgA either before or during binding. Alternatively, this could represent a concentration-dependent increase in binding to additional regions within the oriT fragment, as seen for TraK from RP4 (46), and
may be supported by the results of DNase I footprinting experiments (see below). Assays to determine if oligomers are involved in binding should resolve this observation.

DNase I protection assays localized BtgA binding to three regions on the lower strand of the pBFTM10 oriT (Fig. 5). The most prominent zones of protection were visualized at nucleotides 462 to 497 and 501 to 535. Taken together, these two regions span 71 nucleotides that cover parts (or all) of the three inverted repeats within the oriT region. The first region of protection toward the 5’ terminus of the oriT region includes the right half of IRI, which also contains the consensus nick site sequence -TTCCTCTTG/C- (39). Results of preliminary experiments using labeled double-stranded linear oriT DNA combined with BtgA support this observation (unpublished data). A weaker zone of protection was visualized on nucleotides 536 to 558, which was visualized only with higher concentrations of protein. No large regions of protection were visualized on the upper strand of the pBFTM10 oriT, although individual bases could be protected in more compressed areas but not seen. This apparent asymmetric binding of BtgA protein to oriT could be due to a conformational change in the DNA as a result of wrapping or bending during BtgA-oriT interaction. This is not unusual, having been previously observed for single-stranded DNA binding proteins involved in replication (protection over 70 nucleotides on one strand only [5]) and the E. coli Fpg zinc finger protein (tested by using high-resolution hydroxyl radical footprinting analysis [35]), as well as several other bacterial and eukaryotic DNA binding proteins (7, 14, 18, 36, 41, 42, 47).

The location of BtgA binding strongly supports its role in DNA processing functions necessary for transfer initiation. Binding to recognition sequences within the oriT region is necessary for efficient transfer in RP4 and other plasmids. For RP4, TraJ binds to the right arm of a 19-nucleotide inverted repeat recognizing a 10-bp sequence within 8 nucleotides of the downstream nick site (45). TraJ binding to supercoiled DNA is required for binding and nicking activity of the relaxase TraI, presumably by conformational change of the nick site. TraK is not required for RP4 transfer but increases the efficiency of relaxosome formation. It binds downstream of the nick site on an approximate 180-nucleotide region, which requires as many as 15 to 20 monomers of TraK to form the complex resulting in bending of DNA (46). The precise role of BtgA in DNA processing has not been defined, but it is required for transfer of pBFTM10 (12). The relatively large region of protection overlapping the inverted repeats, and possible nick site, raises the possibility that BtgA is multifunctional.

ACKNOWLEDGMENTS

This work was supported by Veterans Administration Medical Research Service Merit Review grant 001. We thank J. Nawrocki, A. Wolfe, S. Baker, C. Hofmann, and V. Bublys for providing various materials or equipment necessary for completion of this project. We thank B. Wakim for synthesis and sequencing the amino terminus of BtgA. We thank M. Malamy and C. Murphy for valuable discussions regarding R751 and pDG5 mobilization in E. coli.

REFERENCES


FIG. 5. Double-stranded nucleotide sequence of the oriT region of pBFTM10. The dark gray boxed regions on the lower strand indicate regions of BtgA binding, as determined by DNase I protection assays. The light gray box indicates the region of weak binding. The location of a putative nic site based on the consensus sequence (12, 39) is shown, as are the left (L) and right (R) arms of the three inverted repeats and the start codon for bg4.


