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Genetic and Functional Analyses of the *mob* Operon on Conjugative Transposon CTn*341* from *Bacteroides* spp.▽†

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Bacteroides **are Gram-negative anaerobes indigenous to the intestinal tract of humans, and they are important opportunistic pathogens. Mobile genetic elements, such as conjugative transposons (CTns), have contributed to an increase in antibiotic resistance in these organisms. CTns are self-transmissible elements that belong to the superfamily of *i*ntegrative and *c*onjugative *e*lements (ICEs). CTn*341* is 52 kb; it encodes tetracycline resistance and its transfer is induced by tetracycline. The mobilization region of CTn*341* was shown to be comprised of a three-gene operon, *mobABC*, and the transfer origin, *oriT*. The three genes code for a nicking accessory protein, a relaxase, and a VirD4-like coupling protein, respectively. The Mob proteins were predicted to mediate the formation of the relaxosome complex, nick DNA at the *oriT*, and shuttle the DNA/protein complex to the mating-pore apparatus. The results of mutational studies indicated that the three genes are required for maximal transfer of CTn*341*. Mob gene transcription was induced by tetracycline, and this regulation was mediated through the two-component regulatory system, RteAB. The *oriT* region of CTn*341* was located within 100 bp of *mobA*, and a putative *Bacteroides* consensus nicking site was observed within this region. Mutation of the putative nick site resulted in a loss of transfer. This study demonstrated a role of the mobilization region for transfer of *Bacteroides* CTns and that tetracycline induction occurs for the *mob* gene operon, as for the *tra* gene operon(s), as shown previously.**

Bacteroides are Gram-negative anaerobes that colonize the gastrointestinal (GI) tract of humans and other animals. Although symbionts under normal conditions, they also are significant opportunistic pathogens comprising nearly two-thirds of the anaerobic isolates obtained from clinical specimens (13). Treatment of these infections requires the use of appropriate antibiotics, but the choices have become more limited as the rate of antibiotic resistance has increased in the *Bacteroides*. The driving force behind this increase is horizontal gene transfer mediated by transmissible genetic elements. In one study, Shoemaker et al. (31) showed that tetracycline resistance in *Bacteroides* has increased dramatically in the past 30 years due to the dissemination of resistance genes that are nearly identical homologs of *tetQ*. In *Bacteroides*, conjugative transposons (CTns) are considered the most important contributors to horizontal gene transfer, and most encode a TetQ. These CTns are on the order of 50 to 100 kb in size and are generally defined as self-transmissible, integrating elements that cannot be maintained extrachromosomally. The CTns are part of the larger family of ICEs (*i*ntegrative and *c*onjugative *e*lements) that have recently been described in a variety of other organisms, where they are also associated with antibiotic resistance (6). There are two important factors that have contributed to the success of CTns in the spread of antibiotic resistance among the *Bacteroides*. First, the transfer of most *Bacteroides* CTns is induced 1,000- to 10,000-fold by exposure to low concentrations of tetracycline (27, 40). Second, CTns contribute to the spread of resistance genes by their ability to mediate the transfer of coresident elements, such as plasmids and mobilizable transposons (38, 39). These coresident elements also have been shown to carry resistance genes for many clinically relevant antibiotics (25, 44).

There has been considerable interest in the tetracycline regulation, integration, and excision of the CTns, but fewer studies have focused on the mechanism of conjugative transfer (21, 29, 47). The available evidence suggests that the integrated transposon excises from the chromosome and forms a circular intermediate which is the substrate for conjugation. Nucleotide sequence analysis of the predominant CTnDOT family has shown that the genes required for transfer are organized in two divergently transcribed regions of about 18 kb (1, 4, 5). Interestingly, of the 21 *tra* genes in CTn*341*, only three, $traG_{341}$, $mobC_{341}$, and $mobB_{341}$, have obvious homology to known conjugation genes in other organisms (1, 11). $TraG_{341}$ is similar to one of the most highly conserved transfer proteins in Gram-negative and Gram-positive bacteria, VirB4. $MobC_{341}$ has homology to the coupling protein VirD4, and $MobB_{341}$ has a distinct relaxase motif. These are key proteins in the conjugative type IV secretion systems (T4SS), suggesting that the *Bacteroides* transfer mechanism is related to this conserved conjugation system.

The conjugative T4SS has evolved from two fundamental cellular processes: rolling circle replication and protein secretion systems. Enzymes involved in the processing of DNA during conjugation are termed DNA transfer and replication (DTR) proteins, and these resemble those involved in rolling

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
BT5482	*B. thetaiotaomicron*, fecal isolate; Rf^r	2
BTΩpFD699	Independent transconjugant from pFD699 × BT5482 mating; Rf^r Tc^r Em^r	35
638R	*B. fragilis* clinical isolate; Rf^r	27
IB132	638R spontaneous fusidic acid-resistant mutant; Rf^r Fu^r	37
IB255	Independent transconjugant from 638R × *Bacteroides vulgatus* strain CLA341 mating, contains CTn*341*; Rf^r Tc^r	33
BT-Jo36	BT5482 containing derivative of pFD699 with mutation in $mobB_{341}$; Rf^r Tc^r Em^r	1
BF-Jo82	638R containing derivative of pFD699 with mutation in $mobC_{341}$; Rf^r Tc^r Em^r	1
Plasmids		
pFD340	8.8 kb, expression, shuttle vector; (Ap^r) Em^r	36
pFD351	11.1 kb, shuttle vector; (Sp^r) Fx^r Em^r	24
pFD1112	8.6 kb, mobilization-deficient derivative of pFD340; (Ap^r Em^r) Em^r	This study
pFD1113	8.9 kb, derivative of pFD1112 containing 270-bp *oriT* region; (Ap^r Em^r) Em^r	This study
pMob	15.8 kb, derivative of pFD351 with 4.7-kb mob region and upstream untranslated sequences; (Sp^r) Fx^r Em^r	This study
pMobAB	13.6 kb, derivative of pFD351 with 2.5-kb $mobAB_{341}$ gene fragment and upstream untranslated sequences; (Sp^r) Fx^r Em^r	This study
pMobCΔN	10.4 kb, derivative of pFD340 with 1.6-kb $mobC_{341}$ gene fragment lacking transmembrane domains; (Ap^r) Em^r	This study

[a] Antibiotic resistance phenotypes shown in parentheses are expressed in *E. coli* strains, and resistance phenotypes without the parentheses are expressed in *Bacteroides* strains. Abbreviations for antibiotics are described in Materials and Methods.

circle replication (46). In the cytoplasm, the relaxase, a DTR protein, binds and nicks the transfer strand (T-strand) at the origin of transfer (*oriT*). The relaxase remains bound to the T-strand and interacts with the membrane-bound coupling protein, which is a receptor for the protein/DNA complex to be transported through the mating pore apparatus (11). The proteins responsible for assembly of the membrane mating pore, through which the DNA passes, belong to the T4SS family. Since the majority of the *Bacteroides* Tra proteins have little homology to any known proteins in the NCBI databases, the mechanism of transfer can only be hypothesized.

In this paper, we describe for the first time the mobilization region of a *Bacteroides* CTn, CTn*341*. The region is comprised of three genes, $mobA_{341}$, $mobB_{341}$, and $mobC_{341}$, and evidence is presented that suggests the genes encode proteins with similarity to T4SS DTR components. The genes are shown to be organized in an operon that is induced transcriptionally by subinhibitory levels of tetracycline, and this induction is controlled by the regulatory genes *rteA* and *rteB*. An *oriT* region was located within 100 bp of $mobA_{341}$.

MATERIALS AND METHODS

Bacterial strains and growth. The relevant bacterial strains and plasmids used in this study are described in Table 1. For many of the studies described in this report, the pFD699 model system was used (1, 35). Plasmid pFD699 is a chimera of an *E. coli* plasmid and CTn*341*, and it is a fully functional conjugative transposon in *Bacteroides* strains but a stable plasmid in *E. coli*. The complete sequence of CTn*341* was derived from work with pFD699 and has the GenBank accession number AY515263.1. *Bacteroides* strains were grown in BHIS medium (per liter: 37 g Bacto brain heart infusion broth, 1.0 g cysteine, 5 mg hemin, 2.0 g $NaHCO_3$) in an anaerobic chamber (32), and *Escherichia coli* strains were grown aerobically in L broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract). The following antibiotics were used for selection in *E. coli*: ampicillin (Ap, 100 μg/ml), spectinomycin (Sp, 50 μg/ml), nalidixic acid (50 μg/ml), erythromycin (Em, 250 μg/ml), kanamycin (35 μg/ml), and chloramphenicol (25 μg/ml). The antibiotics used for selection of *Bacteroides* were rifampin (Rf, 20 μg/ml), tetracycline (Tc, 5 μg/ml), erythromycin (Em, 10 μg/ml), gentamicin (50 μg/ml), and fusidic acid (Fu, 25 μg/ml).

Bacterial matings. Standard filter-mating protocols were used to mobilize plasmids between strains (16). To mobilize plasmids from *E. coli* donors into *Bacteroides* recipients, the mating plates were incubated aerobically and the selection plates anaerobically. To mobilize plasmids from *Bacteroides* to *E. coli* ("mating-out" assays), the mating plates were incubated anaerobically and the selection plates aerobically. Multiple attempts were made to demonstrate activity of the CTn*341* Mob region in *E. coli*-based systems, but all were unsuccessful. Thus, for many of the assays designed to measure the transfer frequency of mutant derivatives of CTn*341*, we used pFD699 (Ap^r in *E. coli*) mutants in mating-out assays which measured transfer to *E. coli* recipients. In the mating-out assays, *Bacteroides* donors were induced with 1 μg of tetracycline per ml and *E. coli* transconjugants were selected aerobically on medium containing Ap (1). The transfer frequency was calculated as the mean of the number of transconjugants per input donor cell in three independent experiments.

Plasmid construction and DNA manipulations. In order to test the mobilization activities of the CTn*341 oriT* and *mob* genes, a mobilization-deficient plasmid was constructed. This plasmid, designated pFD1112, was formed by deletion of the *Bacteroides mob* region and *ermF* gene from the vector pFD340 (36) and insertion of the *ermA-ermF* resistance cassette for selection in both *E. coli* and *Bacteroides* (15). PCR-amplified sequences from CTn*341* were cloned into the PstI and BamHI sites of pFD1112 to assess transfer as described below.

The entire *mob* region and the *mobAB* genes were cloned into shuttle vector pFD351 for complementation experiments. PCR was used to amplify fragments (Fig. 1; also see Table S1 in the supplemental material) with restriction sites for PstI and BamHI, and these were then directionally cloned into pFD351. A plasmid containing a truncated derivative of $MobC_{341}$ [pMobCΔN] (Fig. 1) was constructed by directional cloning of the deletion fragment into the BamHI and SstI sites of the *Bacteroides* expression vector pFD340. The deletion fragment containing the $mobC_{341}$ ribosome binding site and lacking the bases for codons 2 to 145 was constructed by PCR using the primers described in Table S1 in the supplemental material [primers mobC)TM-N and mobC)TM-C].

Site-directed mutagenesis (SDM) of the suspected *oriT* sequences was performed using a QuikChange II site-directed mutagenesis kit (Stratagene) and mutagenic primers (see Table S1 in the supplemental material for the SDM primers). The region to be mutated was cloned into pUC19, mutated, and then subcloned into pFD1112 for analysis. All mutations were verified by nucleotide sequence analysis.

RNA isolation and cDNA synthesis. *Bacteroides* strains were grown to mid-log phase (A_{550} of 0.3 to 0.5) in BHIS with or without tetracycline. Chloramphenicol was added to a final concentration of 100 μg/ml, and total cellular RNA was extracted using the hot acid phenol method previously described (28). Fifty micrograms of RNA was precipitated with 100% ethanol, treated with Turbo DNA-free DNase (Ambion) according to the manufacturer's instructions, and then checked for contaminating DNA by PCR. The RNA concentration was calculated by the absorption at A_{260}/A_{280}. cDNA was synthesized with a Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) using either gene-specific primers for reverse transcription (RT)-PCR (Table S1 in the supplemen-

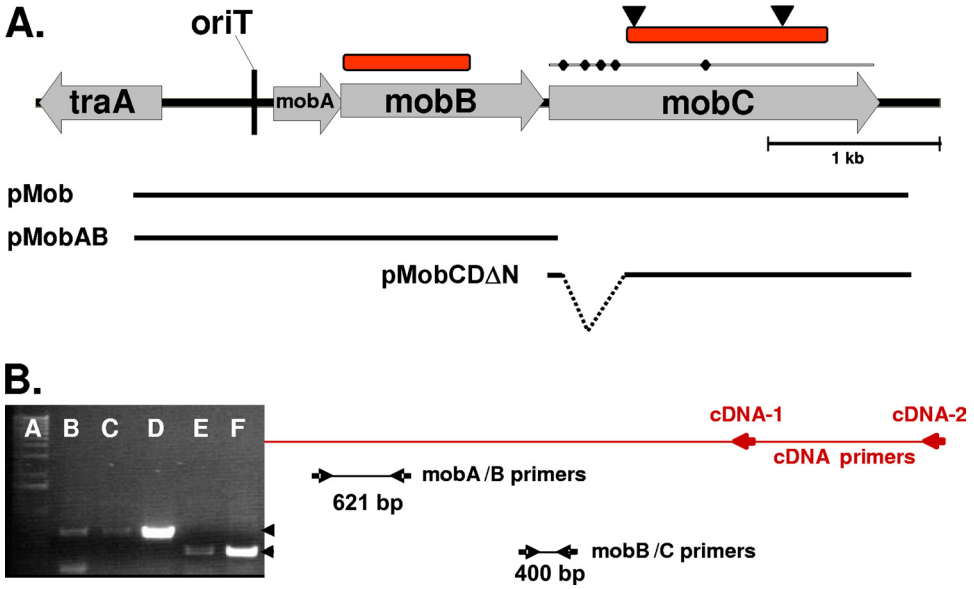

FIG. 1. Genetic map of the *mob* operon and RT-PCR evidence showing linkage between genes. (A) Map showing *mob* genes and functional domains of the Mob proteins. The regions used for the construction of complementing plasmids are shown below the map. The functional domains of the Mob proteins are shown above the map. The red bar above MobB indicates the location of the relaxase/mobilization nuclease domain (Pfam accession number 03432), and the red bar above MobC shows the TraG/TraD superfamily domain (Pfam accession number 02534). The Walker boxes are shown by the inverted triangles above the red bar, and the five MobC transmembrane domains are indicated by black diamonds above the map. (B) Design and results of RT-PCR experiment, showing the locations of the primers used and the agarose gel of the amplified products. The cDNA (shown in red) was generated by reverse transcription of the *mob* mRNA using primers located toward the end of the message in the *mobC* gene. The arrowheads to the right of the gel indicate the sizes of the expected 621- and 400-bp products. Lane A, 1-kb-ladder molecular-size marker; B, cDNA-1 plus primer set mobA/B; C, cDNA-2 plus primer set mobA/B; D, pFD699 DNA-positive control with primer set mobA/B; E, cDNA-1 plus primer set mobB/C; F, pFD699 DNA-positive control with primer set mobB/C. All primers are listed in Table S1 in the supplemental material.

tal material lists the RT-PCR primers) or 200 ng/μl random hexamers for quantitative RT-PCR.

Quantitative PCR. Quantitative RT-PCR was performed essentially as described previously, using a Bio-Rad iCycler with the real-time PCR detection system (Bio-Rad, Hercules, CA) (41). The PCR products were approximately 125 bp in size (see the quantitative RT-PCR primers in Table S1 in the supplemental material) and were verified by agarose gel electrophoresis and by melting point analysis according to the Bio-Rad iCycler software. The reaction mixture contained 12.5 μl 2× iQ SYBR green Supermix, 1.5 μl of 5 μM forward primer, 1.5 μl of 5 μM reverse primer, 8.5 μl H2O, and 1 μl cDNA template (diluted 1/10) per well. All samples were run in triplicate, and RNA without reverse transcriptase was run as a control for DNA contamination. Relative expression values were calculated using the method of Pfaffl (26). Fold induction was determined by comparing the results for uninduced cells to those for cells induced with 1 μg/μl tetracycline, using the sigma-54 modulation protein gene as the reference gene. The sigma-54 modulation protein gene was selected because its expression is not changed in the presence of tetracycline and its expression level is similar to that of the genes being examined.

Northern hybridization. Northern blotting was performed as described previously (28), using RNA isolated from uninduced strains and from those induced with 1 μg/ml tetracycline. The gene-specific probes were generated by PCR amplification of strain BTSpFD699 genomic DNA (see Table S1 in the supplemental material for the primers used) and labeled with [^{32}P]dCTP by using the Prime-a-Gene labeling system (Promega, Madison, WI). The resulting blots were exposed to a phosphorimaging cassette and visualized using a Typhoon 9410 (Amersham, Piscataway, NJ).

RESULTS

The *mob* genes are essential for CTn*341* transfer. In a previous study, the 18-kb locus that mediates conjugative transfer of CTn*341* was described (1). Mutational analysis of the locus indicated that most of the genes tested were required for the transfer of both CTn*341* and coresident plasmids or the mobilizable transposon Tn*4555*. However, null mutations in two genes, $mobA_{341}$ and $mobB_{341}$, resulted in the loss of CTn*341* but not of the coresident elements. As shown in Fig. 1, these genes are part of an apparent *mob* operon that is divergently transcribed from the *tra* region, and database searches suggested that these genes code for proteins involved in DTR reactions. Multiple sequence alignments of the $MobA_{341}$ protein (GenBank accession number AAS83498) revealed that this protein is highly conserved in nearly all of the *Bacteroidetes* phylum, including all *Bacteroides* species, and in many *Flavobacteria*, *Porphyromonas*, and *Prevotella* species. In most cases, the $MobA_{341}$ homologs are adjacent to putative relaxase proteins, such as $MobB_{341}$. $MobA_{341}$ has similarity to a newly described family of nicking accessory proteins referred to as the ribbon-helix-helix family which are found in conjugative systems in diverse bacteria (42) (see Fig. S1 in the supplemental material). These proteins have a role in helping the relaxase gain access to the nick site during DNA-processing reactions.

$MobB_{341}$ (GenBank sequence accession number AAS83499) was also highly conserved in *Bacteroides* species, usually with multiple homologs per genome. These homologs generally ranged between 40% and 99% identity and were usually associated with a $MobA_{341}$-like partner. $MobB_{341}$ has similarity to relaxase proteins and contains several conserved regions (Fig. 1; also see Fig. S2 in the supplemental material) (23). To verify a role for $MobB_{341}$, we tested the $mobB_{341}$ mutant Jo36 (1) and two complemented strains for transfer in mating-out assays. The data from these experiments, shown in Table 2 (assays 1 to 4), indicate that $mobB_{341}$ is essential for CTn*341* trans-

TABLE 2. Transfer frequencies of CTn*341* mutant derivatives and complemented strains

Assay[a]	Donor[b]	Complementing Plasmid	Recipient	Transfer Frequency
Mating out				
1	BTSpFD699 (wt)	None	DH10B	4.4×10^{-4}
2	BT-Jo36 ($mobB_{341}$)	None	DH10B	$<10^{-9}$
3	BT-Jo36 ($mobB_{341}$)	pMob	DH10B	1.9×10^{-4}
4	BT-Jo36 ($mobB_{341}$)	pMobAB	DH10B	1.1×10^{-4}
Bacteroides-Bacteroides matings				
5	IB255 (wt)	None	IB132	3.7×10^{-4}
6	IB255 (wt)	pMobCΔN	IB132	5.9×10^{-5}
7	BF-Jo82 ($mobC_{341}$)	None	IB132	1.7×10^{-6}
8	BF-Jo82 ($mobC_{341}$)	pMobCΔN	IB132	8.6×10^{-8}
9	IB255 (wt)	pMobCΔN	None[c]	$<10^{-9}$
10	BF-Jo82 ($mobC_{341}$)	pMobCΔN	None	$<10^{-9}$

[a] Mating-out assays measure transfer of CTn*341* mutant derivatives from *Bacteroides* to *E. coli* recipients. *Bacteroides-Bacteroides* matings measure transfer of CTn*341* mutant derivatives from *B. fragilis* donors to *B. fragilis* recipient IB132 (Fu^r).
[b] The specific mutation or wild-type (wt) status of the donor strain is given in parentheses next to the strain number.
[c] Matings with no recipients were performed to determine the level of background conversion of the donor strain to fusidic acid resistance.

fer and that this defect can be complemented in *trans* when $mobB_{341}$ is present on a plasmid.

The third *mob* gene, $mobC_{341}$, codes for a protein ($MobC_{341}$, GenBank sequence accession number AAS83500) with significant homology to the VirD4 family of coupling proteins which mediate interactions between the relaxosome and the secretion pore. Multiple sequence alignments showed that the conserved Walker A and B motifs were present, as were predicted transmembrane domains (Fig. 1; also see Fig. S3 in the supplemental material). Previously and in the experiments whose results are shown in Table 2, we found that null mutations in $mobC_{341}$ were still able to mediate the transfer of CTn*341* (1). This was unexpected, since the coupling proteins are essential in other T4SS conjugation systems (10). We reasoned that the lack of a phenotype might be due to complementation of the mutated $mobC_{341}$ by one or more of the MobC homologs in the chromosome. Four MobC homologs with greater-than-70% amino acid identity to $MobC_{341}$ are present in each of the commonly used *Bacteroides* strains (see Table S2 in the supplemental material). In order to determine whether $MobC_{341}$ is important for transfer, an N-terminally truncated $MobC_{341}$ protein lacking the first four membrane-spanning regions was constructed by the deletion of amino acid codons 2 to 145 (Fig. 1). This mutated protein's gene, cloned into an expression shuttle vector, pMobCΔN, lacked the regions needed to insert into the membrane but maintained the relaxase interaction motif. It was predicted that the $MobB_{341}$ relaxase would bind to the mutated $MobC_{341}$ in the cytoplasm but, since it could not associate with the T4SS in the membrane, CTn*341* would not transfer. When the plasmid was tested in a CTn*341* strain that was wild type with respect to $mobC_{341}$, there was about a 10-fold decrease in transfer frequency relative to the transfer frequency in the control (Table 2). This compared to about a 100-fold decrease for the strain with a $mobC_{341}$ null mutation. When pMobCΔN was expressed in the CTn*341* $mobC_{341}$ mutant, transfer was nearly abolished and approached the background level (Table 2). These results suggest that $MobC_{341}$ preferentially interacts with the CTn*341* conjugation apparatus but that MobC homologs can compensate somewhat for a loss in functionality.

The *mob* operon is regulated by tetracycline induction. The genetic organization of the *mob* genes suggested that they would be transcribed as an operon. This linkage was first examined by RT-PCR, as shown in Fig. 1B. The cDNA for these reactions was synthesized from *Bacteroides fragilis* strain IB255, containing CTn*341* and induced with tetracycline, using primers located in the *mobC* coding region or at the end of the gene (primers cDNA-1 and cDNA-2, in Table S1 in the supplemental material). Then, using this cDNA as the template, specific primers were used to amplify the intergenic regions between *mobA* and *mobB* and between *mobB* and *mobC* as shown in Fig. 1B (primer sets mobA/B and mobB/C, in Table S1 in the supplemental material). In all cases, the intergenic regions were amplified and the size of the amplicons corresponded to the predicted sizes of 400 or 621 bp. In no case were there amplification products in control reaction mixtures lacking reverse transcriptase (data not shown).

Northern hybridization analyses were then used to further examine *mob* regulation. The transfer of CTn*341* is known to be controlled by tetracycline; thus, RNA from tetracycline-induced and noninduced cultures was compared in a Northern blot analysis in order to determine if the *mob* genes were controlled at the transcriptional level (Fig. 2A). The results readily showed significant induction of *mob*-specific mRNA in the presence of tetracycline, but much of the RNA was degraded and it was difficult to visualize the 4-kb *mob* mRNA. In order to optimize the levels of the intact 4-kb message, the *mob* operon was cloned onto a plasmid (Fig. 1, pMob). When this plasmid was introduced into a strain containing CTn*341*, a more distinct 4-kb mRNA was observed in tetracycline-induced cultures (Fig. 2B).

To confirm tetracycline control of the operon, quantitative RT-PCR analyses were performed with primers for $mobA_{341}$ and $mobB_{341}$ and for *traA* as a positive control. Previous studies using transcriptional fusions showed an increase in *traA* transcription under tetracycline induction conditions (5). The results of the quantitative RT-PCR analyses showed more than a 100-fold-increase in the mRNA levels of $mobA_{341}$, $mobB_{341}$, and *traA* under tetracycline induction conditions (Fig. 3). Tetracycline induction of the *tra* genes is controlled by the two-

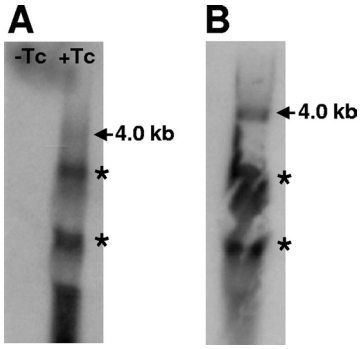

FIG. 2. Northern blot analysis of *mob* operon region. Fifty micrograms of RNA was separated on formaldehyde agarose gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled probes from the *mob* operon as indicated. (A) RNA samples from *B. fragilis* cells containing CTn*341* were either induced (+Tc) or not induced (−Tc) and probed with a 4-kb probe containing the entire *mob* operon. (B) RNA sample from tetracycline-induced *B. fragilis* containing CTn*341* plus a multicopy plasmid with the cloned *mob* operon. Lanes are labeled to indicate induction where appropriate, and the full-length 4-kb mRNAs are shown by the arrows to the right of the blots. The apparent bands (*) at about 1.5 and 2.5 kb are a commonly observed compression artifact caused by the 16S and 23S rRNA. These artifacts are exacerbated by the extensive RNA degradation.

component regulatory system RteA/RteB (45). Quantitative RT-PCR was performed using *rteA* and *rteB* mutants to determine if they played a role in *mob* regulation. The results were very similar for both mutants and showed that the majority of tetracycline induction was mediated by RteAB, although there

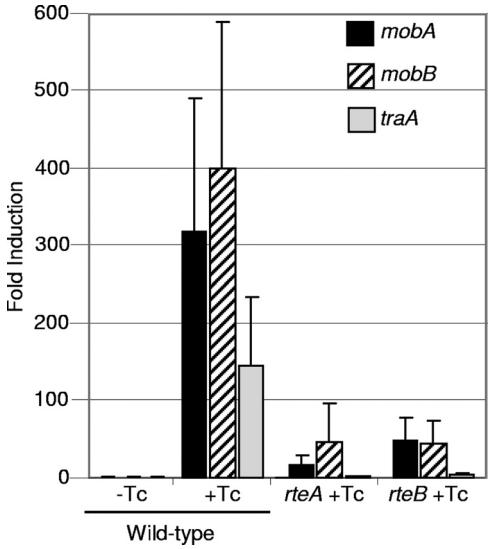

FIG. 3. Quantitative RT-PCR analysis of the *mobA*, *mobB*, and *traA* genes in *rteA* and *rteB* mutants. Overnight cultures of the wild-type (BTSpFD699), *rteA* (BTSJo68), and *rteB* (BTSJo69) strains were subcultured and grown to mid-log phase either with or without 1 μg/μl tetracycline (1). RNA was isolated from the cultures, and two independent sets of RNA were used for analysis, using primers for the *mobA*, *mobB*, or *traA* gene as indicated. Each PCR measurement was performed in triplicate. The sigma-54 modulation protein gene was used as the reference gene. The results are expressed as the fold induction relative to the results with no tetracycline. Error bars represent standard deviations.

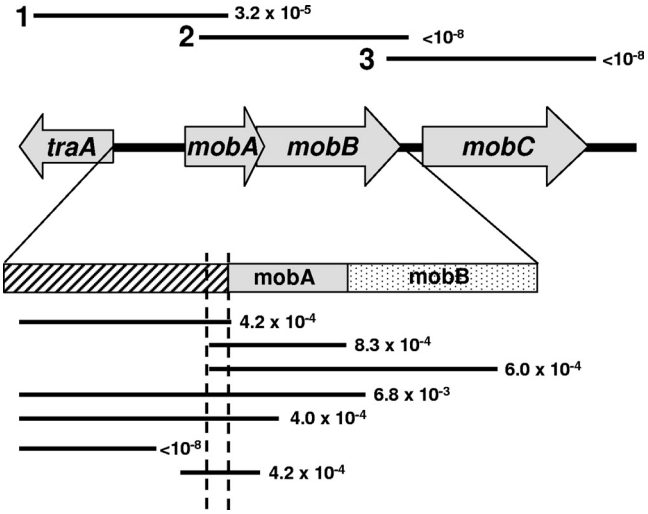

FIG. 4. Identification of the CTn*341 oriT* region. Three fragments from the *mob* operon region (shown by the numbered lines over the map) were PCR amplified, inserted into the mobilization-deficient plasmid pFD1112, and tested for the ability to mediate transfer of the plasmid in mating-out assays, as described in the text. Transfer frequencies were determined and are shown to the right. Based on these results, subfragments (shown below the map) were amplified and tested in the mating-out assay, and the results showed that there was a common 120-bp region just upstream of *mobA* (see parallel dashed vertical lines). Transfer frequencies are the number of transconjugants per input donor cell (means of the results of triplicate experiments).

was still some weak induction in the mutants (Fig. 3). Interestingly, *traA* was more tightly controlled by RteAB.

The CTn*341 oriT* and nick site are located upstream from the *mob* operon. The transfer origin, *oriT*, of many conjugative elements is located adjacent to the transfer genes (14). It was hypothesized that the *oriT* of CTn*341* would be located in the intergenic region between the *tra* genes and the *mob* operon. To test this, we inserted three DNA fragments that spanned the region from *traA* to *mobC* into a mobilization-deficient shuttle plasmid, pFD1112, and then measured its transfer in mating-out assays with *E. coli* DH10B as the recipient. There was no transfer of the pFD1112 control, but a construct that contained the region between *traA* and $mobA_{341}$ was able to mediate transfer in the mating-out assays (Fig. 4). This region was then further subcloned into a series of overlapping fragments, and transfer was observed in all but one of these constructs. It was determined that these fragments all shared a common 120 bp directly upstream of $mobA_{341}$. In order to confirm the *oriT* location, a 270-bp fragment that encompassed the 120-bp region was tested in pFD1112. The results showed that this plasmid mediated transfer at a frequency similar to the transfer frequencies of all other constructs (Fig. 4). In fact, the transfer frequencies for all constructs were comparable to the transfer frequency of the parent fragment, suggesting that no *cis*-acting regions were lost during subcloning.

In order to identify the presumed *oriT* nick site, a combination of bioinformatics and site-directed mutagenesis was used. Previously, consensus nick sites were determined for several *Bacteroides*-transmissible elements, and these were found to cleave between guanine and cytosine residues (34, 43). Using a pattern-matching homology search, we examined the 120-bp

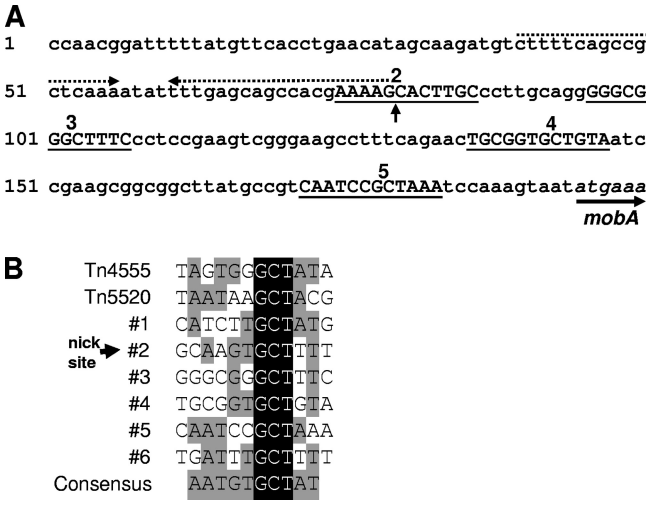

FIG. 5. Identification of the CTn*341* nick site. (A) Location of the *oriT* region between *mobA* and the divergently transcribed *traA*. DNA sequence showing four of the putative nick sequences (underlined and in upper case) that were mutated. The number 2 nick site shows the putative GC cleavage nick site with the arrowhead under the sequence, and the imperfect inverted repeat sequence is shown by the dashed arrows over the sequence. (B) Alignment of *Bacteroides* nick sites and generation of consensus. The Tn*5520* nick site, Tn*4555* nick site, and nick site homologues in the CTn*341 oriT* region are shown. Site-directed mutagenesis of the GC of homologue 2 resulted in a loss of transfer, indicating that it is the nick site of CTn*341*. Mutations of the other sites did not result in loss of the transfer phenotype.

region for similarity to the consensus sequence. The results revealed six potential homologs with GC cleavage sites, and of these, four were located in proximity to the *mobA* gene (Fig. 5). Site-directed mutagenesis was used to change the GC to AT at four of the sites. Three of the four mutated sites mediated normal levels of transfer, but mutation of homolog 2 resulted in a complete loss of transfer, implying that it is the CTn*341* nick site. In addition, this site was adjacent to a large imperfect inverted repeat which has the potential to fold into a stable structure (Fig. 5; also see Fig. S4 in the supplemental material). These secondary structures are typical of the relaxase cleavage sites, and none of the other putative cleavage sites possessed significant secondary structure.

DISCUSSION

The mobilization region plays an important role in the transfer of CTn*341*. Transfer experiments with mutants lacking $mobA_{341}$ or $mobB_{341}$ showed a loss of transfer, indicating that these genes are essential for the transfer of CTn*341* (1) (Table 1). These results are consistent with results in the literature which show that the relaxases and, often, the accessory nicking proteins are necessary for the transfer of conjugal elements. An active tyrosine is the major component of motif I in relaxase proteins. TrwC, the relaxase of conjugal plasmid R388, was found to be required for transfer, and its Tyr18 was essential for nicking activity (20). The relaxase for plasmid RSF1010 was also was required for transfer, and the active tyrosine, Tyr24, was shown to be covalently linked to the nick site (30). $MobB_{341}$ contains motif I, including a tyrosine at position 17, and it has a histidine-rich metal binding site, which is typical of most relaxase enzymes (see Fig. S2 in the supplemental material).

Coupling proteins are essential components of the conjugative apparatus, acting as adaptors between the relaxosome and the type IV secretion system. Thus, it was of interest when we found that a $mobC_{341}$ mutant was still transmissible. In this report, we showed that this was probably due to the ability of the CTn*341* relaxosome to interact with multiple homologs of the proteins that are present on the chromosomes of *B. fragilis* and *Bacteroides thetaiotaomicron* (see Table S2 in the supplemental material). These homologs are probably remnants of ancient transposons and were adjacent to transfer and mobilization genes at each chromosomal site. Other researchers have demonstrated that some coupling proteins can interact with relaxases from multiple elements, resulting in productive transfer reactions (7, 8), but our finding of multiple functional homologs in the genome is novel. The results of experiments designed to demonstrate preferential interaction between $MobC_{341}$ and CTn*341* transfer indicated that $MobC_{341}$ is the preferred coupling protein for CTn*341* (Table 2). This is consistent with the results of experiments using TraG (RP4) family homologs to transfer different mobilizable plasmids, which demonstrated that the most efficient mobilization occurred with the cognate coupling proteins (8).

The CTn*341* mobilization proteins are encoded in an operon which is similar to plasmid R388, where the genes *trwABC*, encoding an accessory relaxosome protein, a coupling protein, and a relaxase, are all transcribed as a single message (3). However, many different genetic organizations of the Mob protein genes are seen in nature. Plasmid pIP501 contains 11 open reading frames (ORFs) related to transfer that are transcribed on a single operon, including *orf1*, encoding a relaxase, and *orf10*, encoding a coupling protein (17). In RP4, the relaxase and the coupling-protein genes are in different operons (12, 22, 49). In the case of CTn*341*, this genetic organization is probably important because of the tetracycline-regulated expression of the transfer genes.

Tetracycline-induced transfer is common among most *Bacteroides* CTns encoding the ribosome protection protein, TetQ. The excision and transfer of CTn*341*, CTnERL, and CTnDOT have been shown to be tetracycline induced (1, 9, 29). The results of CTn*341* Northern blot experiments indicated a dramatic increase in *mob* mRNA after tetracycline induction, with no detectable mRNA in the noninduced samples (Fig. 2). Tetracycline induction of *mob* gene transcription had not been previously shown in a *Bacteroides* CTn. The tetracycline induction of $mobA_{341}$, $mobB_{341}$, and *traA* was confirmed using quantitative RT-PCR. The transcript levels of $mobA_{341}$ and $mobB_{341}$ increased as much as 300-fold, but this was greatly decreased in the RteA and RteB mutants, indicating that this two-component regulatory system controls *mob* expression but that other factors may also be involved (Fig. 3).

In many conjugative elements, the *oriT* region is often located in close proximity to the *tra* gene cluster and is oriented so that the *tra* genes are the last to transfer to a recipient cell. In addition, this region often contains a promoter for the *tra* genes that is transcribed in a direction opposite from that of the transfer (18). In the present study, the *oriT* of CTn*341* was identified directly upstream from the $mobA_{341}$ gene and downstream from *traA*, consistent with most described conjugative

elements. This was different than the *oriT* described for the related elements, CTnDOT and CTnERL. In these elements, the *oriT* region was tentatively located downstream from the regulatory gene *rteC*, about 5 kb distant from the *tra* gene cluster. This location was hypothesized when fragments of CTnDOT were cloned into a plasmid and tested for transfer. The two smallest regions sufficient for mobilization overlapped by 267 bp, and this region was termed the putative *oriT*. However, when the 267-bp region was cloned onto a plasmid, it was not mobilizable (19, 48). We did not specifically test this site in CTn*341*, but it is possible that there is more than one *oriT* on these large elements.

Previous studies had identified a *Bacteroides* consensus nick site with cleavage between adjacent guanine and cytosine residues (34, 43). Site-directed mutations of potential cleavage sites allowed the identification of the CTn*341* nick site within the 120-bp *oriT* region and showed that it was related to several other known *Bacteroides* nick sites. Unfortunately, it was not possible to biochemically verify this nick site since CTn*341* Mob proteins purified from *E. coli* were not functional. Furthermore, CTn*341* does not transfer between *E. coli* cells, thus preventing other approaches, such as its isolation of *in vivo*-nicked DNA templates.

In summary, this study identified three genes that play an important role in the transfer of CTn*341*. Evidence suggests that $MobB_{341}$ functions as a relaxase and that $MobA_{341}$ is a nicking accessory protein. $MobC_{341}$ was the coupling protein, but chromosomal homologs could partially restore transfer in $mobC_{341}$ mutants. The genetic organization of the genes was established as an operon, and the *oriT* and nick site were located adjacent to the *tra* genes of the CTn. It was shown that *mob* transcription is tetracycline induced either directly or indirectly through the regulatory genes *rteAB*. Although there is still much to learn regarding the regulatory and transfer regions of CTn*341*, this study has contributed to the characterization of the mobilization region.

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