

Tetracycline-Related Transcriptional Regulation of the CTnDOT Mobilization Region

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CTnDOT is a 65-kb conjugative transposon (CTn) in *Bacteroides* spp. that confers resistance to the antibiotics erythromycin and tetracycline (Tc). Conjugative transfer of CTnDOT is regulated upon exposure of cells to Tc. In the absence of Tc, no transfer is detectable; however, a cascade of regulatory events results in the conjugative transfer of CTnDOT upon Tc induction. Previous studies addressing regulation of CTnDOT conjugative transfer focused primarily on the 13-kb transfer (*tra*) operon, which encodes the proteins required for assembly of the mating apparatus. We report here that the *mob* operon that encodes the relaxase and coupling proteins required for mobilization of CTnDOT are regulated at the transcriptional level upon Tc induction. The Xis2d and Exc excision proteins are required for the upregulation of *mob* transcription upon Tc induction, and yet a deletion of *xis2c* has no effect. We also show preliminary evidence suggesting that the integrase, IntDOT, may play a regulatory role, as pLYL72 transfer is not detectable when *intDOT* is provided in *trans*.

Bacteroides spp. are Gram-negative obligate anaerobes that are primarily found in the human colon. *Bacteroides* are abundant members of the gut microbiota and play an important role by aiding in processes such as nutrient acquisition and pathogen exclusion (1–3). However, *Bacteroides* spp. can be detrimental to the host if they escape the colon, causing opportunistic infections that may be difficult to treat. These infections may not respond well to antibiotic therapy due to the increasing prevalence of antibiotic resistance within *Bacteroides*, which is often linked to mobile genetic elements (3–5). One such element is CTnDOT, a 65-kb conjugative transposon that confers resistance to the antibiotics erythromycin (*ermF*) and tetracycline (*tetQ*).

An interesting feature of CTnDOT is that excision and conjugative transfer of CTnDOT are dependent upon tetracycline exposure (6–8). In the absence of tetracycline (Tc), there is no detectable transfer of CTnDOT (9). Upon exposure to low (<1 µg/ml) levels of Tc, a regulatory cascade (Fig. 1A) initiates through the *tetQ-rteA-rteB* operon, which is regulated by a translational attenuation mechanism. RteB activates the transcription of *rteC*, and RteC in turn activates transcription of the excision operon that contains *xis2c*, *xis2d*, *orf3*, and *exc* (8, 10). Not only do Xis2c, –2d, and Exc function to excise CTnDOT from the chromosome, but Xis2c and –2d also activate transcription of the *tra* operon, which encodes the mating apparatus (11, 12). We have yet to identify any regulatory or structural role for Orf3. An overview of the steps involved in the regulation of CTnDOT conjugative transfer is summarized in Fig. 1.

Many studies have described transcriptional regulation of both excision and transfer of CTnDOT (10, 11, 13, 14), but thus far we have yet to characterize a very important set of genes involved in CTnDOT conjugative transfer: the mobilization region. The CTnDOT *mob* genes encode proteins that are involved in formation of the relaxosome that is required to nick the *oriT* and for coupling the CTnDOT circular intermediate to the mating bridge (15).

We report here that the *mob* genes of CTnDOT are part of a 4-kb operon that is divergently transcribed from the transfer (*tra*) operon (Fig. 1B). Like the *tra* operon, the *mob* genes are also regulated at the transcriptional level by Tc induction. CTnDOT *mob*

mRNA is detectable from the element in the chromosome only when cells have been exposed to Tc. However, when detected from the self-transmissible plasmid pLYL72 (containing the 18-kb region of CTnDOT shown in Fig. 1B), a transcript was seen whether or not Tc was present, demonstrating that the *mob* operon is actually transcribed constitutively with respect to Tc exposure. This observation suggests that CTnDOT encodes transcriptional activators that are enhancing the level of transcription upon Tc induction and that a repressor of *mob* transcription is also present on CTnDOT that prevents *mob* transcription in the absence of Tc. Xis2d and Exc are required for enhancement of *mob* transcription upon Tc induction. Xis2c, which is required for activation of the neighboring *tra* genes, does not appear to play a role in *mob* activation as a deletion of *xis2c* shows no detectable defect in *mob* transcription. This observation was surprising to us given the close proximity between the *mob* and *tra* promoters (Fig. 1B). Nonetheless, this further demonstrates the complex nature of coordinating excision and transfer of CTnDOT.

Not only did we characterize the transcriptional regulation of the *mob* operon, but we also wanted to confirm the location of the *oriT*, which is the DNA site nicked by the relaxases to initiate mobilization of CTnDOT. We had previously identified an *oriT* that was downstream of *mobC*; however, the *oriT* on a CTnDOT-like element (CTn341) is in fact upstream of *mobA* (15). The upstream *mobA* regions of CTnDOT and CTn341 are approximately 94% identical, which prompted us to evaluate whether this region on CTnDOT also contained an *oriT*. We report that the upstream *mobA* region from CTnDOT does contain an *oriT*, similar to CTn341. However, the CTnDOT-like element CTnERL, which is

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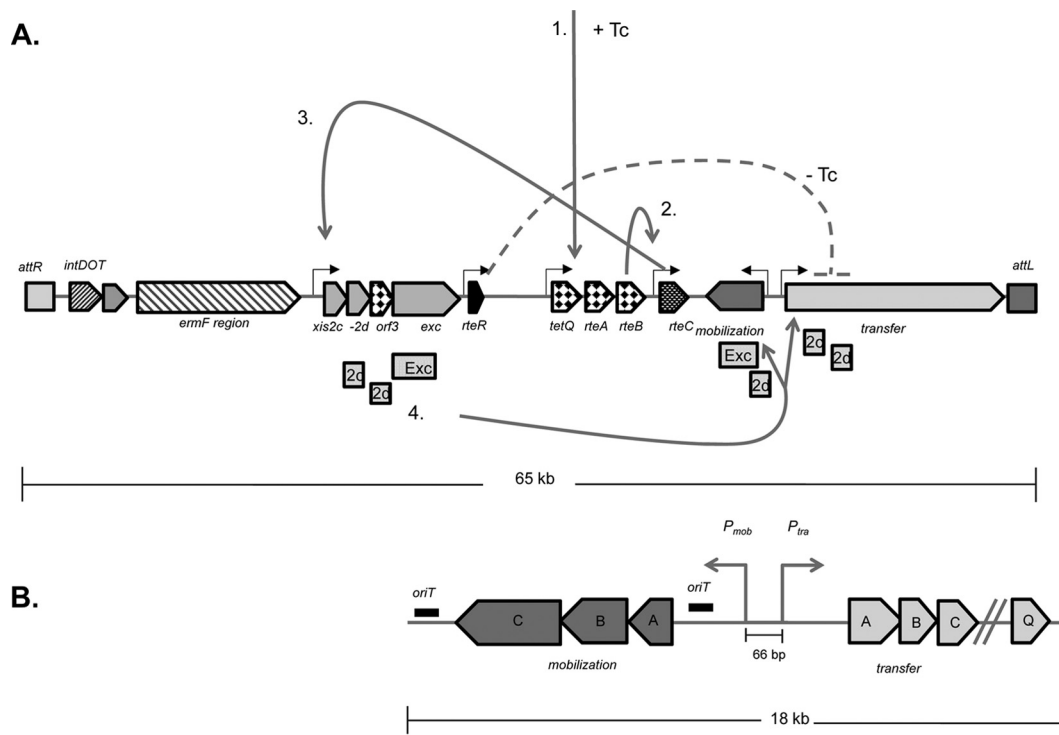


FIG 1 Tetracycline-dependent regulation of CTnDOT transfer. (A) Upon tetracycline (Tc) induction (solid gray lines), translation of TetQ, RteA, and RteB resumes, as this region is regulated by a translational attenuation mechanism (step 1). In step 2, RteB activates the transcription of *rteC*, which activates (step 3) transcription of the excision operon. In step 4, the proteins encoded in the excision operon then activate transcription of the transfer and mobilization operons, thereby inducing conjugative transfer of CTnDOT. In the absence of Tc (dashed gray line), no transfer is detectable due to RteR-mediated premature transcription termination in the *tra* operon. (B) The 18-kb region of CTnDOT containing the mobilization and transfer regions is present on the pLYL72 self-transmissible plasmid. The *mob* and *tra* promoters are 66 bp apart.

remarkably similar to CTnDOT, does not recognize the upstream *mobA oriT* region (16, 17). We have confirmed that CTnDOT does possess a second *oriT* that is located in a 900-bp region downstream of *mobC* and that CTnERL was able to recognize this *oriT* region. It is unusual that CTnDOT appears to harbor two *oriT* regions, as most elements have a single *oriT*.

Last, we report that the enhancement of *mob* transcription upon Tc induction does not appear to directly correspond with the frequency of conjugative transfer. A deletion of *exc*, which is required for *mob* transcriptional enhancement but has no role in *tra* regulation, shows no substantial defect in the transfer frequency of pLYL72. Further, we show that the *mob* genes are transcribed at a higher level on CTnDOT than on CTnERL and yet these two CTns possess the same frequency of conjugative transfer.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains and plasmids used in this study are summarized in Table 1. Unless otherwise noted, all *Bacteroides* strains were initially cultured in chopped meat broth (Remel) and then transferred to anaerobic Trypticase-yeast extract-glucose (TYG) medium (18) for overnight growth with antibiotics when appropriate. Subculturing of *Bacteroides* strains was also done in TYG liquid medium, and all *Bacteroides* culturing was performed anaerobically at 37°C. *Escherichia coli* was grown aerobically in Luria-Bertani (LB) broth at 37°C with antibiotics when appropriate. Unless otherwise noted, antibiotic concentrations used for culturing were as follows: ampicillin at 100 µg/ml; cefoxitin at 10 µg/ml; chloramphenicol at 10 µg/ml; erythromycin at 10 µg/ml; gentamicin at 200 µg/ml; kanamycin at 100 µg/ml; rifampin at 2 µg/ml; streptomycin at 100 µg/ml; and tetracycline at 1 µg/ml.

Bacterial mating assays. *E. coli* strain HB101 containing the RPI IncPα plasmid was used to mobilize plasmids from an *E. coli* donor strain to *Bacteroides* recipients, as described previously (18).

Mating-out assays measuring the frequency of transfer of pLYL72 from a *Bacteroides* donor to recipient *E. coli* (HB101) were performed as described previously (19). The transfer frequency of pLYL72 is represented as the ratio of the number of transconjugants per recipient.

RNA isolation. *Bacteroides* strains were grown in anaerobic TYG medium to an optical density at 650 nm (OD₆₅₀) of 0.3 to 0.5. RNA was then isolated using a QIAgen RNeasy kit. Removal of contaminating genomic DNA was performed using Ambion Turbo DNA-free reagents, and RNA samples were subsequently tested to confirm that no DNA was remaining by performing PCR. RNA concentrations were measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific).

Qualitative RT-PCR. Reverse transcription-PCR (RT-PCR) was performed using an Access RT-PCR system (Promega). Reactions that omitted reverse transcriptase were tested to ensure that all genomic DNA had been adequately removed. Samples were electrophoresed on a 1.2% to 1.5% agarose gel for analysis. Amplification of *rpoD* was used as a control to ensure that equal concentrations of RNA were present in all samples tested. Sequences of the primers used for qualitative RT-PCR are shown in Table 2.

RT-quantitative PCR (RT-qPCR). Synthesis of cDNA was performed using a Bio-Rad iScript kit per the manufacturer's protocol, with the addition of 200 ng random pentadecamers (Integrated DNA Technologies) per reaction. Approximately 1 µg of DNase-treated RNA was used for each reaction. Quantitative PCR was then performed using a Realplex2 Mastercycler (Eppendorf), with Bio-Rad Ssofast EvaGreen as a signal reporter. Amplification of *mobB* from various strains was used as a marker to measure the effect on transcription of various CTnDOT regions in

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relative phenotype(s) ^a	Description and/or reference
Strains		
<i>B. thetaiotaomicron</i> BT4001	(Rif ^r)	Spontaneous <i>B. thetaiotaomicron</i> Rif ^r mutant
<i>B. thetaiotaomicron</i> BT4001 ΩQABC	(Thy ⁻ , Tp ^r , Tc ^r)	BT4100 with a site-specific insertion of the regulatory region <i>tetQ-rteA-rteB-rteC</i> (9)
<i>B. thetaiotaomicron</i> BT4001 ΩQABC Ω2.6pGERM	(Rif ^r , Tc ^r)	BT4001 ΩQABC containing an insertion of pDJE2.3, which contains <i>intDOT</i> , integrated into the chromosome (26)
<i>B. thetaiotaomicron</i> BT4004	(Rif ^r , Em ^r)	BT4001 containing CTnERL integrated into the chromosome
<i>B. thetaiotaomicron</i> BT4007	(Rif ^r , Tc ^r , Em ^r)	BT4001 containing CTnDOT integrated into the chromosome
<i>B. thetaiotaomicron</i> BT4104	(Thy ⁻ , Tp ^r , Tc ^r)	BT4100 containing a chromosomal copy of CTnERL (27)
<i>E. coli</i> DH5αMCR	RecA	Gibco BRL
<i>E. coli</i> HB101	RecA, Str ^r	<i>E. coli</i> strain used as a conjugative transfer recipient (28)
<i>E. coli</i> HB101 RP1	RecA, Str ^r	HB101 containing the IncPα plasmid RP1 (18)
Plasmids		
pAFD1	Ap ^r (Em ^r)	<i>E. coli-Bacteroides</i> shuttle vector containing <i>ermF</i> (A. M. Stevens, unpublished data)
pGRW53Δ2C	Ap ^r (Em ^r)	pGW46 insert containing a 114-aa in-frame deletion of <i>xis2c</i> , ligated into pAFD1 (13)
pGRW53Δ2D	Ap ^r (Em ^r)	pGW46 insert containing an in-frame deletion of <i>xis2d</i> from aa 3 through the end of <i>orf3</i> , ligated into pAFD1 (13)
pGRW53ΔExc	Ap ^r (Em ^r)	pGW46 insert containing a 670-aa in-frame deletion of the C-terminal end of <i>Exc</i> , ligated into pAFD1 (13)
pGRW54	Ap ^r (Cf)	A 900-bp fragment containing the region between <i>rteC</i> and <i>mobC</i> from CTnDOT, cloned into pLYL7oriT _(RK2) (this study)
pGRW55	Ap ^r (Cf)	A 200-bp fragment containing the putative CTnDOT <i>oriT</i> region upstream of <i>mobA</i> , cloned into pLYL7oriT _(RK2) (this study)
pGW45	Ap ^r (Cf)	A 1.6-kb fragment containing <i>orf2b</i> , <i>xis2c</i> , <i>xis2d</i> , <i>exc</i> , <i>orf2e</i> , and <i>rteR</i> cloned into pLYL05 (9)
pGW46	Ap ^r (Cf)	A 1.3-kb fragment containing <i>xis2c</i> , <i>xis2d</i> , <i>exc</i> , <i>orf2e</i> , and <i>rteR</i> cloned into pLYL05 (9)
pHopp1	Ap ^r (Em ^r)	pAFD1 containing <i>xis2c</i> , <i>xis2d</i> , <i>orf3</i> , <i>exc</i> , and <i>rteR</i> (13)
pKS04	Ap ^r (Cf)	A 4.9-kb fragment containing <i>orf2a</i> , <i>orf2b</i> , <i>orf3</i> , and a truncation of <i>exc</i> where a 840-bp sequence was deleted from the 3' end (26)
pLYL05	Ap ^r (Cf)	<i>E. coli-Bacteroides</i> shuttle vector (L. Y. Li, unpublished data)
pLYL72	Kn ^r , Cm ^r (Cm ^r)	Self-transmissible plasmid containing <i>mob</i> genes, <i>tra</i> operon, and OriT from CTnDOT (19)
pLYL7oriT _{RK2}	Ap ^r (Cf)	<i>E. coli-Bacteroides</i> shuttle vector that is mobilizable in <i>E. coli</i> by the IncP plasmid RP4 but is not mobilizable in <i>Bacteroides</i> (29)
pYS41	Ap ^r (Cf)	A 1.5-kb fragment ligated into pLYL05 containing <i>orf2a</i> , <i>orf2b</i> , <i>exc</i> , and <i>rteR</i> (Y. Sutanto, unpublished data)

^a *Bacteroides* phenotypes are shown in parentheses, whereas *E. coli* phenotypes are shown without parentheses. Abbreviations: aa, amino acid; Ap, ampicillin; Cf, cefoxitin; Cm, chloramphenicol; Em, erythromycin; Kn, kanamycin; Rif, rifampin; Str, streptomycin; Tc, tetracycline; Thy, thymidine auxotroph; Tp, trimethoprim.

trans to the *mob* operon. Amplification of *rpoD* was used as a reference marker to normalize for variations in the quantity of RNA template, as *rpoD* is a single-copy gene and the expression of *rpoD* is not altered by Tc induction (14). The sequences of the primers used are listed in Table 2. The final levels of reaction components were as follows: 1 μM primers, 5 μl cDNA, and 10 μl Ssofast EvaGreen Supermix (Bio-Rad). The reaction conditions were as follows: initial denaturation for 2 min at 98.0°C and amplification and quantification at 98.0°C for 5 s and 55°C for 10 s for 40 cycles. A melting curve was performed at 95.0°C for 15 s, followed by 55.0°C for 15 s, with subsequent heating to 95.0°C over the course of 20 min with continuous fluorescence measurement, followed by a final incubation at 95.0°C for 15 s. Each measurement was performed in triplicate, and relative quantification determinations were performed using the Pfaffl equation $N = 2^{-\Delta\Delta C_T}$, where N is the relative fold difference. ΔC_T represents the C_T (cycle threshold) value difference within a sample between the target gene (*mob*) and the reference marker (*rpoD*). $\Delta\Delta C_T$ then represents the difference in ΔC_T values between tetracycline-induced and noninduced samples (20).

Localization of the *oriT* regions recognized by CTnDOT and CTnERL. A 900-bp region containing the intergenic region between *rteC* and *mobC* was PCR amplified using primers GRW54 fwd and rev (Table 2) from BT4007 and cloned into pLYL7oriT_(RK2), which is mobilizable by the IncP plasmid RP4 but cannot be mobilized by *Bacteroides*. The resulting plasmid, pGRW54, was then introduced into both BT4007 and

BT4004. Mating assays were performed to evaluate mobilization of pGRW54.

Similarly, a 200-bp region containing the region upstream of *mobA* was PCR amplified from BT4007 using primers GRW55 fwd and rev (Table 2). The resulting product was then subcloned into pLYL7oriT_(RK2), and the resulting pGRW55 plasmid was introduced into BT4007 and BT4004 via a triparental mating, as described above. Mating assays were then performed to determine whether CTnERL (BT4004) and/or CTnDOT (BT4007) could mobilize pGRW55. Tetracycline (1 μg/ml) was added to the donor growth medium, as it is required for conjugative transfer from these strains.

RESULTS

The CTnDOT *mob* genes are regulated by tetracycline induction and are assembled in an operon. A recent study of a CTnDOT-like element, CTn341, revealed that the CTn341 *mob* genes comprise an operon that is induced at the transcriptional level by tetracycline (15). To determine if the CTnDOT *mob* genes are regulated in response to tetracycline (Tc) exposure, we performed qualitative RT-PCR detecting *mobA*, *-B*, and *-C* from BT4007, a *B. thetaiotaomicron* strain that contains a single copy of CTnDOT integrated into the chromosome (Table 1). BT4007 cultures were grown in the absence of Tc or with low levels of Tc (1 μg/ml). As

TABLE 2 Primers used in this study

Primer function and name	5' to 3' sequence
Cloning	
GRW54 fwd	GATGACTGCAGGGAATCACAAT
GRW54 rev	TCTTGATAAGAGCTCTAGAAAGTCATT
GRW55 fwd	TGAAAGGTATCTGCAGTCCGT
GRW55 rev	GGATCGAGCTCGGGATTCTT
RT-PCR	
mobA fwd	TCAAGGTGCTGAAGGTGGACAAGA
mobA rev	TCTGACGGCTCAGTTTCACGAGTT
mobB fwd	TTTCCGTGCTGCAAGGCACTTATG
mobB rev	ATGAAAGCCTCTTCTCGGCATCT
mobC fwd	TGAACCGCGCTACGAGGATATTT
mobC rev	TCGGGATTGTTGATGTCCAGCGTA
mobA-B fwd	TGGACTACTACACCAAGCTATCGG
mobA-B rev	TTTCCAGATACTCGGGGGC
mobB-C fwd	ACAACCGCGATACCGTTATCCAT
mobB-C rev	CCCTTTCGTACCCAGACAGGACA
RpoD 67 fwd	TTTAATCTGACGAACGGCTCACG
RpoD 548 rev	ACGCTGTATGGTGGATTTCGTCAGT
RT-qPCR	
mobA fwd	ACAACCGTTTCTCGCCATGTTTG
mobA rev	TCTTGTCACCTTCAGCACCTTGA
mobA-B fwd	AACTCGTGAAGACTGAGCCGTCAGA
mobA-B rev	TGTAGATGCGGTTGGTGGTGAGAA
mobB fwd	ACAACCGCGATACCGTTATCCAT
mobB rev	ATGAAAGCCTCTTCTCGGCATCT
RpoD 467 fwd	ACCTGATCAACGGGAGACGACA
RpoD 548 rev	ACGCTGTATGGTGGATTTCGTCAGT

shown in Fig. 2, *mobA*, -*B*, and -*C* transcripts are detectable only when cells are exposed to Tc, demonstrating that the CTnDOT *mob* genes are in fact directly or indirectly regulated by Tc exposure.

To establish whether the *mob* genes are assembled in an operon, we tested for transcriptional linking, where the intergenic regions of *mobA* and *mobB* (*mobA-B*) and *mobB-C* were amplified using qualitative RT-PCR. If these genes are transcriptionally linked, and thus organized in an operon, we would expect to detect these intergenic transcripts. Conversely, if each *mob* gene was transcribed from an independent promoter, no transcript pertaining to the intergenic region would be detected. An amplifica-

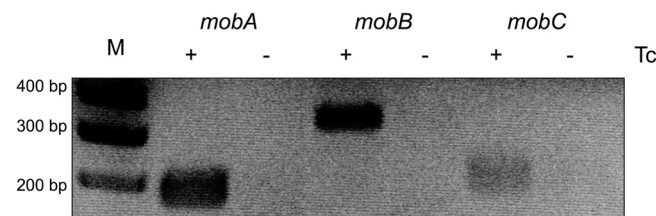


FIG 2 The *mob* genes are detected from CTnDOT only upon Tc induction. To establish whether the transcription of the *mob* genes is regulated by Tc induction, RT-PCR was performed, detecting *mobA*, *mobB*, and *mobC* from BT4007, which contains a copy of CTnDOT integrated into the chromosome. RT-PCR was performed on BT4007 RNA samples from cells that were noninduced or induced with (1 μ g/ml) tetracycline (Tc). An amplicon pertaining to each of the *mob* genes was detected only upon Tc induction, thus confirming that the transcription of the *mob* genes is dependent upon exposure of cells to Tc.

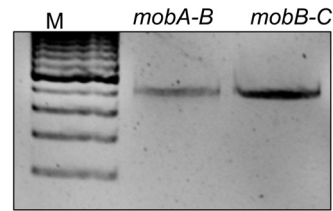


FIG 3 The CTnDOT *mob* genes are transcriptionally linked. To address whether the *mob* genes form a polycistronic transcript, RT-PCR was performed using primers that amplified the regions of the *mobA-B* and *mobB-C* intergenic junctions from BT4001 pLYL72 pGW45 induced with tetracycline (1 μ g/ml). Amplicons for both of these primer sets were observed, thus indicating that the *mob* genes are transcriptionally linked and organized as an operon.

tion product was detected for both the *mobA-B* and *mobB-C* transcripts, which suggests that the *mob* genes are in an operon (Fig. 3). Further supporting these findings is the recent demonstration by Peed and colleagues that the *mob* genes of a CTnDOT-like element, CTn341, are also in an operon that is regulated by Tc induction (15).

CTnDOT appears to harbor two *OriT* regions that are located upstream of *mobA* and downstream of *mobC*. Prior studies of CTnDOT suggested that the *oriT* of CTnDOT was within the approximately 1-kb region that is located between *rteC* and *mobC*. However, a recent study characterizing the Mob proteins on the CTn341 CTnDOT-related element suggested that the *oriT* of CTn341 was just upstream of *mobA*. The previous studies that localized the CTnDOT *oriT* used subcloned regions in *trans* to another element, CTnERL, which is also considered to be virtually identical to CTnDOT with the exception that CTnERL lacks the 13-kb *ermF* region (17, 21). Collectively, these observations prompted us to re-evaluate the location of the *oriT* regions on CTnDOT.

A 900-bp region located between *rteC* and *mobC*, previously reported to contain the CTnDOT *oriT*, was cloned onto a vector that cannot be mobilized from *Bacteroides*. The resulting plasmid, pGRW54, was then introduced into a *Bacteroides* strain that contained either CTnDOT (BT4007) or CTnERL (BT4004) integrated into the chromosome. Mating assays were performed using both of these *Bacteroides* strains to see whether pGRW54 was able to transfer to an *E. coli* recipient (HB101). If transconjugants were detected, this would suggest that an *oriT* region was present on pGRW54 and could be mobilized by the conjugative transposon present in the donor *Bacteroides* strain. Plasmid pGRW54 was able to transfer from both BT4004 and BT4007, demonstrating that an *oriT* downstream of *mobC* is mobilized by both CTnERL and CTnDOT (Table 3).

A 200-bp region upstream of *mobA* that is almost identical to the CTn341 *oriT* region was cloned into a vector that is not mo-

TABLE 3 Mobilization of putative *oriT* regions

Donor strain	Transfer frequency ^a
BT4004 pGW54	1.3×10^{-6}
BT4007 pGRW54	6.3×10^{-4}
BT4004 pGRW55	$<10^{-9}$
BT4007 pGRW55	4.2×10^{-3}

^a The transfer frequency is represented as the average ratio of the number of transconjugants per recipient cell.

bilizable in *Bacteroides*. Transfer of the resulting pGRW55 plasmid was detected only from BT4007 and not from BT4004, thus suggesting that an *oriT* was present upstream of *mobA* that is mobilizable by CTnDOT and yet not by CTnERL (Table 3). While these data strongly indicate that CTnDOT contains two *oriT* regions as opposed to the single *oriT* region on CTnERL, future studies performing mutational analysis on these regions would further support the notion that they are true origins of transfer.

Due to this observation that the *oriT* recognition patterns may differ between CTnDOT and CTnERL, we wanted to investigate whether the CTnERL *mob* genes are also regulated at the transcriptional level in response to Tc induction. We performed both qualitative and quantitative RT-PCR to detect the *mob* genes from CTnERL (BT4014) and CTnDOT (BT4007). The *mob* genes from both CTnERL and CTnDOT were regulated by exposure to Tc (Fig. 4A). However, much more transcript was observed from CTnDOT than from CTnERL upon Tc induction. CTnERL showed an approximately 55-fold induction of detectable *mob* transcript upon Tc induction, whereas CTnDOT had an over 130-fold increase in transcript (Fig. 4B). Despite the difference in *mob* transcription results, the two elements are reported to transfer at similar rates, suggesting that the level of *mob* expression does not directly affect the frequency of conjugative transfer (21, 22).

Providing the excision operon in *trans* to the mobilization region is sufficient for enhancement of *mob* transcription upon Tc induction. After observing that the *mob* genes from CTnDOT are detected only upon exposure to Tc, we predicted that proteins encoded within the excision operon are acting as regulators of the *mob* operon. We have previously reported that when the excision operon is provided in *trans* with the self-transmissible plasmid pLYL72, there is transcriptional activation of the *tra* genes as well as enhanced pLYL72 transfer (9, 11, 13). Because of the close proximity of the *tra* promoter and the putative *mob* promoter, we predicted that the interactions that regulate the *tra* operon could also be mediating *mob* transcriptional activation. Using both qualitative and quantitative RT-PCR, we first amplified the *mob* genes from BT4001 Ω QABC pLYL72 pHopp1 both with Tc (1 μ g/ml) and without Tc. As shown in Fig. 5, an increase in *mob* transcript was observed upon Tc induction, which suggests that proteins encoded within the excision operon can act as transcriptional activators of the *mob* operon.

We then wanted to see if we could determine which excision proteins were necessary for enhancement of *mob* transcription. Various subclones of the excision region were provided in *trans* to pLYL72 to detect the regulation of *mob*. As shown in Fig. 5, a plasmid containing *xis2c xis2d exc rteR* (pGW45) still resulted in *mob* activation, demonstrating that *orf3* is not required. However, a deletion of either *xis2d* or *exc* decreased the detectable *mob* transcript to constitutive levels in the absence of Tc, suggesting that Xis2d and Exc are required for the upregulation of *mob* transcription. A deletion of *xis2c* had no noticeable effect on the *mob* transcriptional activation. This was unexpected, as we predicted that a deletion of *xis2c* would greatly diminish *mob* activation based on the observation that *xis2c* is required for *tra* operon activation (13). Further, a deletion of *exc* had no effect on the *tra* operon and yet brought *mob* activation down to the constitutive levels in the absence of Tc. These observations suggest that the patterns of transcriptional regulation of the *mob* and *tra* operons are not as similar as we had predicted based on their close proximity.

A deletion of *xis2d* abolishes conjugative transfer of pLYL72. Mating assays were performed to detect the frequency of transfer of pLYL72 from BT4001 Ω QABC containing various excision operon deletions on a second plasmid provided in *trans* to pLYL72. When the complete excision operon (pGW45) or a plasmid with a deletion of *orf3* (pGW46) was present, both enhancement and repression of pLYL72 transfer were observed with and without the addition of Tc, respectively. When we investigated the ability of pGRW53 Δ Exc to regulate pLYL72 transfer, the transfer frequency was similar to that seen with a strain containing the fully intact excision operon, where both enhancement and repression were observed in response to Tc induction. This observation suggests that Exc is not required for enhanced transfer of pLYL72.

A deletion of *xis2c* (pGRW53 Δ 2c) resulted in a loss of enhancement, and pLYL72 transferred at a rate similar to that seen with the empty vector control upon Tc induction, where transconjugants were detected at a frequency of approximately 10^{-5} to 10^{-6} transconjugants per recipient. However, a deletion of *xis2d* (pGRW53 Δ 2d) resulted in no pLYL72 transfer even with the induction of Tc. Similarly, a deletion of both *xis2c* and *xis2d* (pYS41)

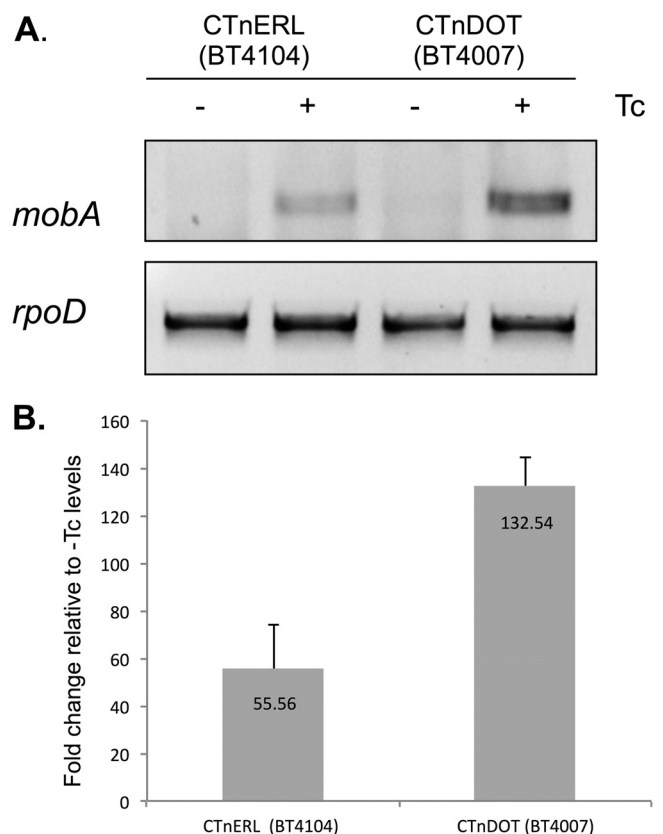


FIG 4 More *mob* transcript is detectable from CTnDOT than from CTnERL. (A) Qualitative RT-PCR was performed using primers to detect *mobA* from a strain containing CTnERL (BT4104) and CTnDOT (BT4007) both with and without Tc induction. No transcript is detectable in the absence of Tc, and yet, upon exposure to Tc, a *mob* transcript is detected. However, more *mobA* transcript is seen in cells containing CTnDOT than in cells containing CTnERL. *rpoD* is a reference marker used to ensure equivalent RNA concentrations in samples. (B) RT-qPCR was performed using primers to detect *mobA*. RNA content was normalized to the reference marker *rpoD*, and the $\Delta\Delta C_T$ was calculated comparing induced (+) to noninduced (-) Tc levels.

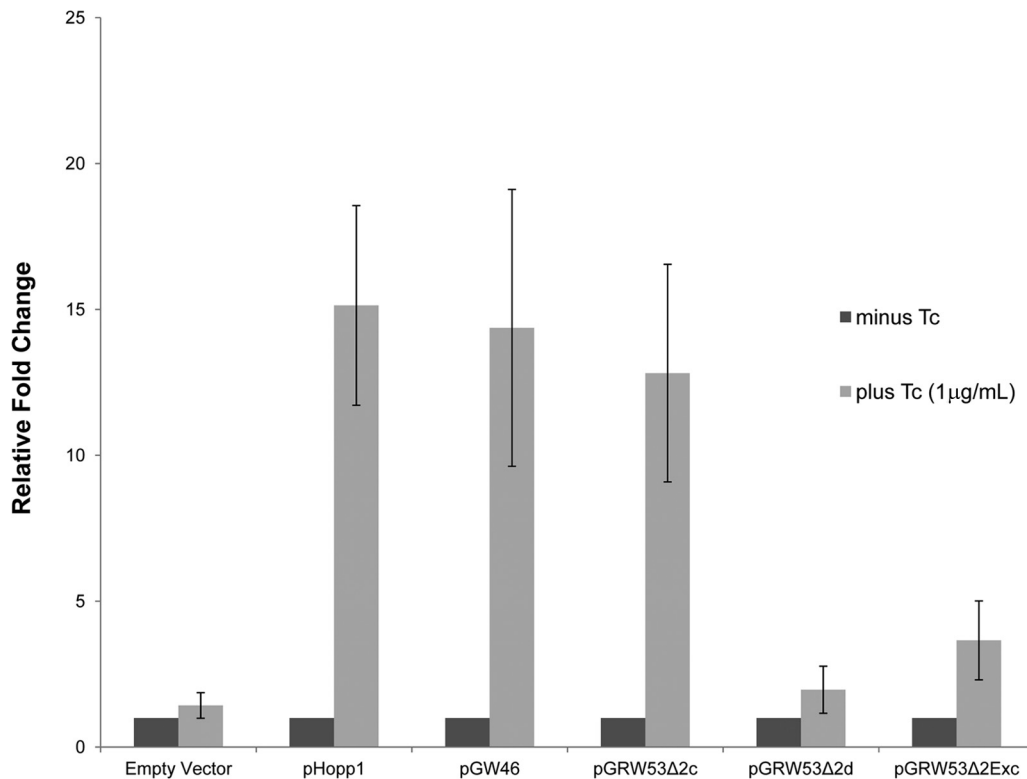


FIG 5 The *mob* genes are transcribed constitutively from pLYL72, and the excision proteins Xis2d and Exc are required for sufficient enhancement of transcription upon Tc induction. RT-qPCR was performed to localize potential regulators of *mob* transcription; the relative fold changes in transcription measuring *mobB* relative to the levels in the absence of tetracycline (–) are shown. Fold changes were calculated using the Pfaffl $\Delta\Delta C_T$ equation, and samples were normalized using the reference marker *rpoD* (20). The observations described above were also confirmed using qualitative RT-PCR (not shown). The *mob* genes were transcribed constitutively from pLYL72, and the excision operon in *trans* was sufficient for transcriptional activation. A deletion of *xis2C* appears to have no deleterious effect on *mob* transcription, whereas a deletion in either *xis2d* or *exc* drops transcription to the levels determined for constitutive empty vector.

resulted in no detectable pLYL72 transfer, likely due to the requirement for Xis2d. For each of the plasmids described above, no transconjugants were detectable without Tc added to the growth medium due to RteR-mediated negative regulation.

We also tested the effect of the CTnDOT integrase, IntDOT, on the transfer of pLYL72. We hypothesized that IntDOT could be a candidate for the negative regulator of *mob* transcription in the absence of Tc since the integrase of Tn916 is thought to bind the *oriT* in an effort to prevent premature conjugative transfer (23). Our results demonstrated that the *mob* genes are transcribed constitutively from pLYL72 when *intDOT* is in *trans* (Fig. 6). RT-qPCR confirmed that the *mob* genes are transcribed constitutively from this strain (data not shown). However, no transfer of pLYL72 was detected with or without Tc induction with *intDOT* present. This suggests that IntDOT may play a role in preventing premature conjugative transfer, although IntDOT does not appear to do so by preventing transcription of the *mob* operon.

DISCUSSION

Coordinating the events of CTnDOT excision, mobilization, transfer, and integration results in a mobile element whose transfer is very tightly regulated. Such coordination is crucial, as it is important to delay the initiation of mobilization and transfer functions until CTnDOT has properly excised from the chromosome. If transfer were to happen prematurely, an Hfr-like transfer would be the consequence, which could be rather costly to the

donor cell. Many studies have detailed the coordinated regulation of excision and transfer of CTnDOT (10, 13, 14, 24), while this report is the first to characterize the transcriptional regulation of CTnDOT mobilization functions.

We have shown that the *mob* genes of CTnDOT are part of an operon that is transcribed upon induction with low levels of the antibiotic tetracycline (Tc), while no transcript is detectable in the absence of Tc. This tetracycline-dependent *mob* regulation is sim-

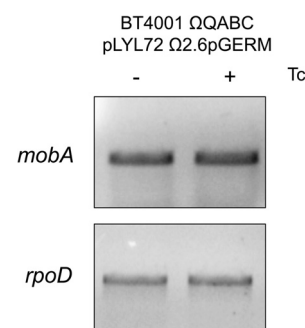


FIG 6 The *mob* genes are transcribed when *intDOT* is present. Qualitative RT-PCR was performed detecting *mobA* from strain BT4001 ΩQABC pLYL72 Ω2.6pGERM, which contains a copy of *intDOT* integrated into the chromosome. *Mob* transcript was detectable whether or not Tc was present, which suggests that IntDOT was not preventing transcription of the *mob* operon.

TABLE 4 Effect of excision protein deletions on the transfer frequency of pLYL72^a

Plasmid used with donor strain BT4001 ΩQABC pLYL72	Transfer frequency of pLYL72		Regulation of transfer ^b	Source or reference
	+Tc	-Tc		
pLYL05	10 ⁻⁵ -10 ⁻⁶	10 ⁻⁵ -10 ⁻⁶	E-, R-	5
pKS04	10 ⁻⁵ -10 ⁻⁶	10 ⁻⁵ -10 ⁻⁶	E-, R-	5
pGW46	5.5 × 10 ⁻³	<10 ⁻⁹	E+, R+	This study
pGRW53Δ2c	1.8 × 10 ⁻⁴	<10 ⁻⁹	E-, R+	This study
pGRW53Δ2d	<10 ⁻⁹	<10 ⁻⁹	R+, R+	This study
pGRW53ΔExc	1.2 × 10 ⁻³	<10 ⁻⁹	E+, R+	This study
pYS41	<10 ⁻⁹	<10 ⁻⁹	R+, R+	This study
Ω2.6pGERM	<10 ⁻⁹	<10 ⁻⁹	R+, R+	This study

^a The transfer frequency of pLYL72 is expressed as the average ratio of the number of transconjugants per recipient cell. +, present; -, absent.

^b E, enhancer; R, repressor.

ilar to observations made with respect to a related conjugative transposon, CTn341. Peed et al. reported that the *rteA* and *rteB* genes were required in *trans* for transcriptional activation of the *mob*_{CTn341} operon, a fact that prompted us to further investigate the regulation of the CTnDOT *mob* operon (15).

From what we know about the CTnDOT regulatory cascade initiated upon Tc induction (Fig. 1), RteA and RteB are required for the activation of a regulatory protein, RteC, which acts as a transcriptional activator of the excision operon (8, 10, 14, 25). Because RteA and RteB are not known to directly regulate any regions of CTnDOT other than the *rteC* regulatory protein-encoding gene, we predicted that the genes located in the excision region could be acting as transcriptional activators of the *mob* genes. We report here that the Xis2d and Exc excision proteins are required for the activation of *mob* transcription upon Tc induction (Fig. 5). Although Exc is required for enhancement of *mob* expression, a deletion of *exc* has no obvious effect on pLYL72 transfer (Table 4). This suggests that there is no direct correlation between the level of *mob* expression and the frequency of conjugative transfer. This idea is further supported by the observation that although there is a large difference between CTnERL and CTnDOT in *mob* expression levels, they are reported to transfer at similar rates (Fig. 5 and Table 4).

A deletion of *xis2c* has no detectable effect on *mob* expression but decreases the transfer of pLYL72 to a rate of 1.8 × 10⁻⁴ transconjugants per recipient. A deletion of *xis2d*, on the other hand, results in no detectable transfer of pLYL72 upon Tc induction. Previous studies have demonstrated that a deletion of *xis2c* or *xis2d* abolishes the enhancement of *tra* transcription upon Tc induction, and yet the different effects these deletions have on pLYL72 conjugative transfer suggest that a deletion of *xis2d* has greater consequences than deletion of *xis2c*. This intimates that there may be a greater requirement of Xis2d for transcriptional regulation and that Xis2c is possibly acting more as an accessory protein that is facilitating Xis2d-mediated regulation.

Our findings have also demonstrated that a negative regulator prevents transcription of the *mob* operon when no Tc is present. The *mob* genes are transcribed constitutively from the self-transmissible plasmid pLYL72, and yet a transcript is detected from CTnDOT only upon Tc induction. We investigated the possibility that the CTnDOT integrase (IntDOT) was a potential repressor, as the integrase from Tn916 can bind the *oriT* of Tn916 and is thought to prevent premature conjugative transfer (23). RT-PCR demonstrated that the *mob* genes were transcribed constitutively when *intDOT* was provided in *trans*; however, no pLYL72 transfer

was detectable (Fig. 6 and Table 4). Although IntDOT is not directly acting as the negative regulator of the *mob* operon, this finding suggests that IntDOT plays a regulatory role in preventing conjugative transfer. Nevertheless, this observation is preliminary and warrants further investigation.

Concluding remarks. In summary, our findings have added another layer of complexity to understanding the already intricate regulation of CTnDOT. These observations further support the idea of the importance of highly coordinated regulation of excision and conjugative transfer. It would be advantageous to initiate these steps as soon as possible so that antibiotic resistance determinants could readily disseminate throughout the population but not prematurely to the disadvantage of the donor cell.

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