An Unexpected Effect of Tetracycline Concentration: Growth Phase-Associated Excision of the Bacteroides Mobilizable Transposon NBU1

Bo Song, Gui-Rong Wang, Nadja B. Shoemaker, and Abigail A. Salyers*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 7 May 2008/Accepted 15 October 2008

Early studies of the Bacteroides mobilizable transposon NBU1 established that excision, the first step in NBU1 transfer, requires exposure of the cells to tetracycline. More recently, we found that excision is also associated with growth phase; even after exposure to tetracycline, excision is detectable only after the cells enter late exponential phase. The tetracycline effect is mediated by a two-component regulatory system, RteA and RteB, which is provided in trans by an integrated self-transmissible element, CTnDOT. The rteA and rteB genes are part of a three-gene operon that also contains the tetracycline resistance gene tetQ. We report here that neither transcription nor translation of the tetQ-rteA-rteB operon is affected by growth phase. Moreover, RteA is not required for the growth phase effect, because a mutant form of RteB that does not require phosphorylation by RteA did not make excision independent of growth phase. Two conditions made NBU1 excision independent of growth phase. One was reducing the tetracycline concentration from an inhibitory concentration (1 μg/mL) to a subinhibitory level (0.05 μg/mL). Independence of growth phase also occurred when rteA and rteB were placed under the control of a heterologous maltose-inducible promoter, P\textsubscript{malt}. Our results suggest that at low concentrations of tetracycline, ribosomes are capable of translating enough RteA and RteB for excision to occur. At higher tetracycline concentrations, however, TetQ is needed to protect enough ribosomes to allow the translation of excision genes, and this protection takes time to develop. Thus, subinhibitory concentrations of tetracycline may increase the probability of gene transfer because, in contrast to inhibitory concentrations, excision can occur at all phases of growth.

NBU1 was the first mobilizable transposon (MTn) to be discovered (16). It was found originally in a strain of Bacteroides uniformis, but integrated mobilizable elements related to NBU1 have since been found in over 50% of Bacteroides strains surveyed (10). This observation has given rise to the question of why NBU1-related elements have been so successful in transferring to so many different Bacteroides species. Since the discovery of NBU1, a variety of unrelated MTns have been found in proteobacteria, such as Salmonella enterica serovar Typhimurium, and in gram-positive bacteria, such as Clostridium difficile and Clostridium perfringens (5, 30). It is not clear how widely these other MTns have moved in natural isolates.

MTns carry genes responsible for excision, integration and mobilization functions, but they require a coresident self-transmissible element to provide the mating apparatus through which the excised MTn circular form transfers. NBU1 requires the Bacteroides conjugative transposon (CTn) CTnDOT. Investigation of NBU1 excision also led to the discovery of a novel interaction between CTnDOT and NBU1. Not only does NBU1 require transfer genes carried by CTnDOT, but it also requires two regulatory proteins encoded by CTnDOT, RteA and RteB (22). RteA and RteB form a two-component regulatory system that is required for both CTnDOT and NBU1 excision. More recent studies have shown that this type of regulatory interaction occurs with other integrated elements (Fig. 1). RteA and RteB also control the expression of excision genes carried by the NBU1-related element NBU2 and genes that are essential for the excision and transfer of several cryptic Bacteroides CTns (23).

The rteA and rteB genes are part of a three-gene operon that contains the ribosomal protection type tetracycline resistance gene tetQ (18, 19, 22). Production of proteins encoded by the tetQ-rteA-rteB operon is dependent on exposure of the cell to tetracycline, an effect that is mediated by a translational attenuation mechanism (28, 29). Recently, we made the surprising observation that excision of NBU1 not only is controlled by tetracycline stimulation but is also dependent on growth phase (27). Excision is seen only when cells exposed to tetracycline enter late exponential stage (27). CTnDOT excision is also associated with growth phase, presumably due to the same mechanism (N. Shoemaker, unpublished observations). Growth phase-associated excision has also been noted in the case of the gram-positive interleukin-converting enzymes Tn916 and ICEB1 (1, 2), but the mechanism is unknown.

In this report, we examine the mechanism of growth phase-associated excision of NBU1 and, by extension, of CTnDOT and related CTns. Initially, we had expected that the growth phase association might be due to quorum sensing involving RteA and RteB. We show here, however, that quorum sensing is not involved and that the growth phase-associated excision of NBU1 and CTnDOT excision is due
Escherichia coli needed to introduce a plasmid. One antibiotic resistance gene makes it useful for constructions in which ermF it lacks a 13-kbp segment that carries ermF. Experiments to determine the effect of growth phase on transcription and translation of CTnDOT excision genes. RteB also acts directly to activate NBU1 excision genes and genes of unknown function on some cryptic Bacteroides CTns (12).

Instead to the concentration of tetracycline to which the cells are exposed.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. The *Bacteroides thetaiotaomicron* strain used in many of these experiments was BT4001, a spontaneous rifampin-resistant strain of *B. thetaiotaomicron* 5482, the type strain of the species. *Bacteroides* strains were grown anaerobically at 37°C in prereduced Trypticase-yeast-extract-glucose broth (7) or agar plates incubated in BBL GasPak jars. A derivative of BT4001, which contained a single copy of CTnERL, was also used in experiments to determine the effect of growth phase on transcription and translation of the tetQ operon. CTnERL is virtually identical to CTnDOT except that it lacks a 13-kbp segment that carries ermF. The fact that CTnERL carries only one antibiotic resistance gene makes it useful for constructions in which ermF is needed to introduce a plasmid. *Escherichia coli* strains were grown aerobically at 37°C in Luria-Bertani medium broth or on agar plates. The following antibiotic concentrations were used in this study: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; erythromycin, 10 μg/ml; tetracycline, 1 μg/ml.

**DNA manipulation and conjugation procedures.** Chromosomal DNA was prepared by a modification of a method described by Saito and Miura (13). Plasmids were isolated by the Qiagen miniprep kit. Oligonucleotides were obtained from Integrated DNA Technologies and are listed in Table 2. Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were purchased from New England BioLabs, and Fisher Scientific. The manufacturers’ instructions were followed for restriction digestion and ligation. The vectors used in this study were constructed in *E. coli* and then transferred to *Bacteroides* strains by using filter mating, as described previously (14, 21, 25).

**qRT-PCR.** To determine whether the transcription of the tetQ operon is growth phase dependent, quantitative reverse transcriptase PCR (qRT-PCR) analysis of *rteB* mRNA was performed on RNA obtained from a strain containing NBU1 and CTnERL at different growth phases (optical densities [ODs] of 0.1, 0.2, 0.4, and 0.8 and overnight culture). An OD of 0.7 to 0.8 corresponded to late exponential phase. RNA was purified using an RNasy kit (Qiagen), treated with DNase, and checked for DNA contamination by PCR. With the purified RNA, cDNA was generated using 1 μg of RNA in a total volume of 20 μl plus random hexamers (New England Biolabs [NEB]) as primers and the Moloney murine leukemia virus reverse transcriptase (NEB). Primers used in these experiments are shown in Table 2. The *Bacteroides* sigma 70 gene (*rpoD*) was used as an internal standard to allow quantification (12).

**Cloning of *rteB* without *rteA* and *tetQ* under the control of the *tetQ* promoter** (*PQ*). BT4007 genomic DNA (containing a copy of CTnDOT) was used as the template to amplify the *rteB* gene. Pfu polymerase was used for high-fidelity amplification. The forward primer contained an NcoI restriction enzyme site, and the reverse primer contained an SstI site. The PCR amplicon was digested with NcoI and SstI and then cloned into the NcoI-SstI sites of pGFK59 (12) to place the *rteB* gene under the control of the *tetQ* promoter, *P*<sub>Q</sub> (pRteB). The cloning was done so that the start codon of *rteB* was fused to the start codon of tetQ.

---

**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Description (source or reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> S17-1</td>
<td>RecA Tp&lt;sup&gt;+&lt;/sup&gt; RP4</td>
<td>Contains the transfer functions of RP4 integrated in the chromosome (20)</td>
</tr>
<tr>
<td>BT4001</td>
<td>Rif&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Spontaneous Rif-resistant mutant of <em>B. thetaiotaomicron</em> 5482</td>
</tr>
<tr>
<td>BT4007</td>
<td>Rif&lt;sup&gt;+&lt;/sup&gt; Te&lt;sup&gt;+&lt;/sup&gt; Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td><em>B. thetaiotaomicron</em> 4001 that contains wild-type CTnDOT</td>
</tr>
<tr>
<td>BT4100N1-S1</td>
<td>Thy&lt;sup&gt;+&lt;/sup&gt; Tp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>BT4100 containing an integrated copy of NBU1 (19)</td>
</tr>
<tr>
<td>BT4104N1-3</td>
<td>Thy&lt;sup&gt;+&lt;/sup&gt; Te&lt;sup&gt;+&lt;/sup&gt;</td>
<td>BT4104 containing an integrated copy of NBU1 and CTnERL (19)</td>
</tr>
<tr>
<td>BT4004</td>
<td>Rif&lt;sup&gt;+&lt;/sup&gt; Te&lt;sup&gt;+&lt;/sup&gt;</td>
<td>BT4001 containing a copy of CTnERL on the chromosome</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGFK59.3</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (Cc&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>0.2-kb Spl-SstI PCR product containing the wild-type tetQ promoter with an Ncol site cloned into Spl-SstI-digested pLYL02 (12)</td>
</tr>
<tr>
<td>pNJR12</td>
<td>Te&lt;sup&gt;+&lt;/sup&gt; (Km&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>RSF1010-based shuttle vector that replicates in <em>B. thetaiotaomicron</em> (22)</td>
</tr>
<tr>
<td>pRteB</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (Cc&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>pGFK59.3 containing <em>rteB</em> under the control of the tetQ promoter <em>P</em>&lt;sub&gt;Q&lt;/sub&gt; (this study)</td>
</tr>
<tr>
<td>pRteB&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (Cc&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>pGFK59.3 containing <em>rteB</em> with a D55E mutation (this study)</td>
</tr>
<tr>
<td>pTUG3</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt; (Ap&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>An in-frame fusion plasmid constructed by cloning 423 bp of the tetQ upstream region into pMIF2 so that the sixth codon of tetQ is fused to the start codon of <em>uidA</em> (28)</td>
</tr>
<tr>
<td>pSUSAB</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt; (Ap&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>The <em>rteA</em> and <em>rteB</em> genes cloned into the shuttle vector pNYL1 under the control of the <em>susA</em> promoter, <em>P</em>&lt;sub&gt;susA&lt;/sub&gt; (4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Phentotypes in parentheses are expressed only in *Bacteroides*, and phenotypes not in parentheses are expressed in *E. coli*. Abbreviations used for antibiotic resistance: Tc, tetracycline; Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Rif, rifampin; Thy, thymidine; Km, kanamycin; Cef, cefoxitin; Tp, trimethoprim.
terQ. This approach provided a good ribosome binding site and the terQ leader region so that terQ was expressed in the same way as in the wild-type terQ operon.

**Excision assay.** NBU1 excision was detected by PCR amplification of the joined ends using primers NBU1-LJ and NBU1-RJ, and the amplified product was about 410 bp. To test the growth phase effect on excision, Bacteroides strains were grown overnight in Trypti-case-yeast extract-glucose broth plus tetracycline (1 μg/ml or 0.05 μg/ml) and then inoculated into the same medium and removed at different OD values. For early growth phases, a larger volume of culture was used as the template to amplify for the mutagenesis reaction. The mutagenesis reaction was performed by using the QuickChange Site-directed mutagenesis kit (Stratagene). Primers carrying the specific mutations are shown in Table 2. The plasmid pRteB was used as the template for PCR amplification of NBU1-joined ends and sigma 70 region so that a small but significant increase beginning in late exponential phase that brought the specific activity to 700 U/mg. The β-glucuronidase specific activity associated with cells not exposed to tetracycline was barely detectable (<1 U/mg).

**Contribution of different portions of the terQ operon to the growth phase effect.** The results of experiments to identify genes or sequences that contributed to the growth phase effect are summarized below and in Table 3. Some typical results of the NBU1 excision experiments are shown in Fig. 2 to illustrate the growth phase-associated (regulated) and growth phase-independent (unregulated) profiles referred to in Table 3. Since RteA appears to function as the sensor component of the RteA/RteB two-component system, we considered the possibility that a signal sensed by RteA might be responsible for the growth phase-associated excision. RteA is essential for excision, so to delete rteA and still have excision occur, we constructed a mutated form of RteB that is independent of RteA. This was done by aligning the RteB amino acid sequence with its closest relatives, NtrC and LuxO, and by locating the as-

---

**TABLE 2. PCR primers used in this study**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma 70 Fw</td>
<td>AGGACCTGATTACAGTGAGGAAGAGGTG</td>
</tr>
<tr>
<td>Sigma 70 Rev</td>
<td>ATTTTCTCAGCAAGAAGACATCTAACCAGA</td>
</tr>
<tr>
<td>NBU1-LJ</td>
<td>GATTACTTTACCTAGCTTC</td>
</tr>
<tr>
<td>NBU1-RJ</td>
<td>GATTCTGACCCAGATTGTTG</td>
</tr>
</tbody>
</table>

---

**RESULTS AND DISCUSSION**

**Transcription and translation of the terQ operon do not differ with growth phase.** qRT-PCR analysis of transcription of the terQ operon showed that the concentration of terB mRNA remained at about 50% (±5%) of that of the internal control, rpoD, at all stages of growth. Translation of the terQ operon was determined using an in-frame fusion of terQ with the uidA gene (pTUG3) (28). A single chromosomal copy of CtnERL, a Ctn that is virtually identical to CtnDOT except that it lacks ermF, provided the terQ operon in trans. The β-glucuronidase specific activities of this strain (BT4004/pTUG3) were similar at different phases of growth (600 U/mg), with the exception of a small but significant increase beginning in late exponential phase that brought the specific activity to 700 U/mg. The β-glucuronidase specific activity associated with cells not exposed to tetracycline was barely detectable (<1 U/mg).

**TABLE 3. Results of growth phase experiments with various combinations of terQ, rteA, and terB and different concentrations of tetracycline**

<table>
<thead>
<tr>
<th>Element(s) carried by BT4001</th>
<th>Tc concn (μg/ml)</th>
<th>Growth phase effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{rteQ}-rteA-rteB</td>
<td>1.0</td>
<td>Regulated</td>
</tr>
<tr>
<td>P_{rteQ}-rteA-rteB</td>
<td>0</td>
<td>No excision</td>
</tr>
<tr>
<td>P_{rteQ}, P_{rteB}</td>
<td>1.0</td>
<td>Regulated</td>
</tr>
<tr>
<td>P_{rteQ}, P_{rteB}</td>
<td>0.05</td>
<td>Unregulated</td>
</tr>
<tr>
<td>CtnERL (in BT4001N1-3)</td>
<td>1.0</td>
<td>Regulated</td>
</tr>
<tr>
<td>CtnERL</td>
<td>0.05</td>
<td>Unregulated</td>
</tr>
<tr>
<td>P_{rteQ}-rteA-rteB</td>
<td>0 (grown on maltose)</td>
<td>No excision</td>
</tr>
<tr>
<td>P_{rteQ}-rteA-rteB</td>
<td>0 (grown on glucose)</td>
<td>No excision</td>
</tr>
</tbody>
</table>

*a* rteB is the activated mutant form of terB.

*b* BT4001 containing CtnERL has been called BT4004 in previous publications.

"Regulated" means that there was a growth phase-associated pattern (Fig. 2, panel A), and "Unregulated" means that excision was independent of growth phase (Fig. 2, panel B). Cases in which excision did not occur are indicated by "No excision."

Downloaded from http://jb.asm.org/ on July 9, 2017 by guest
particulate residue on RteB that corresponded to the phosphorylation site on NtrC and LuxO. We changed this residue (D55) to a glutamate, a change that partially activates NtrC and LuxO even in the absence of the corresponding sensor, RteB* (6, 8). The mutated rte gene was fused to the start codon of tetQ, cloned on a plasmid (pRteB*). The tetQ gene was provided on another plasmid (pNJR12). The resulting strain was able to excise NBU1, although at a level that was about one-fifth of the level of excision obtained with wild-type RteB (data not shown). Nonetheless, the excision level was high enough to monitor growth phase-associated excision. Excision was still growth phase associated (Table 3, row 3), a result that shows that RteA is not required for growth phase-associated excision.

To determine whether TetQ was needed for growth phase-associated excision, pRteB* was introduced into a Bacteroides strain which did not contain a copy of tetQ. Since this strain was not resistant to the inhibitory tetracycline concentration normally used in excision experiments (1 μg/ml), we used a subinhibitory concentration of tetracycline (0.05 μg/ml) to cause elevated production of RteB. This lower concentration of tetracycline was previously shown to be capable of triggering production of RteA and RteB in the absence of TetQ (28). Unexpectedly, excision of NBU1 became growth phase independent under these conditions (Table 3, rows 4 and 5). This result showed that RteB alone was not mediating growth phase-associated excision and that either TetQ or the low tetracycline concentration alone might be important for the growth phase effect.

To test the effect of tetracycline concentration on excision, a strain which exhibited growth phase-associated excision at 1 μg/ml of tetracycline (pNJR12, pRteB*, and NBU1) was grown at a lower concentration of tetracycline (Table 3, row 5). Under these conditions, excision occurred at all growth phases. To confirm the result, the same low concentration of tetracycline was added to the culture for BT4104N1-3, which contains both CTPoERL and NBU1 on the chromosome. At 0.05 μg/ml of tetracycline, NBU1 excision in BT4104N1-3 was independent of growth phase (Table 3, row 7). Thus, the TetQ protein is not required for the growth phase-associated phenotype, and the concentration of tetracycline was the major factor.

To obtain further confirmation that the tetracycline concentration is critical for growth phase-associated excision, we placed the rteA and rteB genes under the control of the B. thetaotaomicron susA promoter (P-susA), a promoter that controls the expression of a starch utilization gene (susA) and is regulated by maltose (3). The wild-type rteA and rteB genes were used rather than tetA and mutant rteB* to maximize the level of excision, because P-susA is somewhat weaker than the P-susA promoter and might thus make excision triggered by the mutant RteB more difficult to detect. P-susA is one of the strongest promoters we have encountered in Bacteroides strains, so it is unlikely that low production of TetQ, RteA, and RteB is a factor in the growth phase effect. The strain containing the tetQ operon under the control of P-susA was grown in medium that contained maltose but not tetracycline. The pattern of NBU1 excision in this strain was dependent on maltose but independent of growth phase (Table 3, row 8). As expected, no excision was detected if the same strain was grown on glucose rather than maltose. These results further support the contention that the concentration of tetracycline is the main factor responsible for growth phase-associated excision.

Conclusions. Based on our results, growth phase-associated excision occurs only at inhibitory concentrations of tetracycline. In our model, the lack of excision in early growth phases when the tetracycline concentration is inhibitory (1 μg/ml) is due to the fact that as the cells begin to divide more slowly in a concentration of tetracycline that is high enough to partially inhibit growth, it takes time for TetQ to protect enough ribosomes to allow increased expression of NBU1 excision genes due to enhanced production of RteA and RteB and increased translation of excision genes. During the late exponential phase, when more ribosomes are protected by TetQ, a high enough concentration of excision proteins can be produced to initiate excision. The reason the growth phase effect is still seen after stationary-phase cells are diluted in fresh tetracycline-containing medium may be that TetQ turnover reduces the amount of protection available for cells diluted in new medium, and thus, it takes time for levels of TetQ to increase enough to keep up with production of ribosomes. At a subinhibitory concentration of tetracycline, the necessary level of TetQ is achieved much earlier. The same argument explains why CTnDOT excision is also associated with later phases of growth at the inhibitory concentration of tetracycline we have used in earlier studies.

Our findings show that growth phase regulation can occur outside the quorum-sensing paradigm. An effect of an antibiotic on ribosomes, an effect that is dependent on the concentration of the antibiotic, is sufficient to achieve a growth phase-associated effect. This phenomenon has practical implications as well. Subinhibitory concentrations of an antibiotic can select for resistant strains over the long term if the low concentration of antibiotic gives a slight selective advantage to resistant cells. We report here for the first time a growth phase effect. Our results suggest that low concentrations of tetracycline might increase the likelihood of horizontal gene transfer of integrated mobile elements, such as CTnDOT and NBU1. This occurs on two levels. We showed previously that a low concent-
tration can trigger production of RtcA and RteB, central regulators of excision genes. We show here that low concentrations of tetracycline, in contrast to higher concentrations of tetracycline, allow excision to occur at all phases of growth.

In nature, subinhibitory concentrations are probably encountered by bacteria more often than inhibitory concentrations. Evidence is mounting that some antibiotics, such as tetracycline, survive in the environment and make their way into groundwater and possibly even tap water. Subinhibitory concentrations of tetracycline and other antibiotics are used in animal production as growth promoters. Finally, in the colon of a person taking tetracycline, subinhibitory concentrations of tetracycline would be experienced by colonic bacteria during the initial phase of treatment and during the phase at the end of treatment.

It is interesting to note that the spread of CTnDOT and NBU1 has been more successful among Bacteroides spp. than the spread of CTns whose excision and transfer are not regulated (e.g., CTnBST, CTnGERM) (17). Possibly, cells that carry a regulated CTn are more likely to survive in the competitive environment of the colon, or exposure to tetracycline is more ubiquitous than previously appreciated, or both.

A similar effect of antibiotic concentration may prove to give rise to growth phase-associated excision of other integrated elements besides NBU1 and CTnDOT. It is unlikely, however, that this mechanism explains the growth phase dependence of Tn916 and ICEBs1. Tn916 carries a copy of a tetracycline resistance gene, tetM, that like tetQ encodes a ribosome protection type resistance protein (24). However, there is some debate about whether tetracycline stimulation is involved in Tn916 excision (2, 11). Celli et al. (2) stated that the presence of tetracycline did not have any effect on Tn916 excision at different growth phases, although the data to support this conclusion were not shown. ICEBs1 contains no resistance genes, so the mechanism of growth phase-associated excision is likely to be different from that of CTnDOT and NBU1, which is dependent on CTnDOT for excision and transfer. The excision of ICEBs1 has been shown to be controlled at the transcriptional level by DNA damage or overproduction of Ralp (9). In this case, stress on the host cell might be involved in this growth phase-associated excision.

ACKNOWLEDGMENT

This work was supported by grant AI/GM 22383 from the National Institutes of Health.

REFERENCES


