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The Mobilization Regions of Two Integrated *Bacteroides* Elements, NBU1 and NBU2, Have Only a Single Mobilization Protein and May Be on a Cassette

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***Bacteroides* conjugative transposons can act in *trans* to excise, circularize, and transfer unlinked integrated elements called NBUs (for nonreplicating *Bacteroides* units). Previously, we localized and sequenced the mobilization region of one NBU, NBU1, and showed that this mobilization region was recognized by the IncP plasmids RP4 and R751, as well as by the *Bacteroides* conjugative transposons. We report here that the single mobilization protein carried by NBU1 appears to be a bifunctional protein that binds to the *oriT* region and catalyzes the nicking reaction that initiates the transfer process. We have also localized and sequenced the mobilization region of a second NBU, NBU2. The NBU2 mobilization region was 86 to 90% identical at the DNA sequence to the *oriT-mob* region of NBU1. The high sequence similarity between NBU1 and NBU2 ended abruptly after the stop codon of the *mob* gene and about 1 kbp upstream of the *oriT* region, indicating that the *oriT-mob* regions of NBU1 and NBU2 may be on some sort of cassette. A region on NBU1 and NBU2 which lies immediately upstream of the *oriT* region had 66% sequence identity to a region upstream of the *oriT* region on a mobilizable transposon, Tn*4399*, an element that had previously appeared to be completely unrelated to the NBUs.**

Many *Bacteroides* clinical isolates carry large (>70-kbp) integrated self-transmissible elements, called conjugative transposons (2, 11). These elements have also been called Tc^r elements because most of them carry a tetracycline resistance (Tc^r) gene, *tetQ*. *Bacteroides* Tc^r elements have an unusual activity not reported for any other known conjugative transposon. They act in *trans* to excise and mobilize smaller unlinked DNA elements called NBUs (nonreplicating *Bacteroides* units) (Fig. 1) (1, 12, 13, 16, 17). Two NBUs, NBU1 and NBU2, have been characterized (12, 13). These NBUs are 10 to 12 kbp in size and appear to be unrelated to the conjugative transposons that excise and mobilize them (12). During transfer, the NBUs are excised from the chromosome to form a double-stranded circle, which is the transfer intermediate (Fig. 1) (7, 13). The circular form of the NBU then uses the transfer pore provided by the conjugative transposon to move into a recipient cell (7). The *Bacteroides* conjugative transposons also enhance, by at least 1000-fold, the transfer frequency of another integrated element, the mobilizable transposon Tn*4399* (5, 8). Results of previous studies had indicated that Tn*4399* was not related to the NBUs. For example, Tn*4399* has a different mode of integration than that of the NBUs, and the mobilization region of Tn*4399* had no significant sequence similarity to the mobilization region of the NBUs (6, 7, 13). In this report, we provide the first evidence that Tn*4399* and the NBUs do in fact share some sequence similarity.

The *oriT-mob* region of NBU1 allows NBU1 to be mobilized not only by *Bacteroides* conjugative transposons but also by the IncP plasmids R751 and RP4 (7). Thus, mobilization of the NBU1 circle form by IncP plasmids presumably occurs by the same process as conjugal transfer of coresident plasmids. That is, a relaxosome complex forms at the *oriT* region of the NBU1 circle form and nicks one strand to initiate transfer of a single copy of NBU1 to a recipient. Previously, we had localized and sequenced the minimal region of NBU1 that was required for mobilization by IncP plasmids and by the *Bacteroides* conjugative transposons. This region contained a 220-bp *oriT* segment and a single open reading frame (*mob*), which encoded a 54-kDa mobilization protein (7). The finding that only one open reading frame was required for mobilization of NBU1 was surprising, because all of the conjugative plasmids studied to date have proved to require at least two mobilization proteins for nicking at the *oriT*. In the case of RP4, for example, TraJ binds at the *oriT* region. Then the relaxase, TraI, binds to form the relaxosome complex. The relaxosome nicks at the *oriT* nick site and binds covalently to the 5′ end of the nicked DNA to initiate the transfer process (1, 4, 10). Even the mobilizable *Bacteroides* transposon, Tn*4399*, has two mobilization genes, *mocA* and *mocB* (5, 8). The single Mob protein encoded by NBU1 could be a bifunctional protein, which mediates both *oriT* binding and nicking, or it could be an *oriT*-binding protein that is recognized by the relaxase of RP4. In this report, we present evidence that the NBU1 Mob protein is a bifunctional protein with both *oriT*-binding and nicking activities.

NBU1 and NBU2, the two NBUs studied most extensively to date, have different restriction maps, but they cross-hybridize on high-stringency Southern blots in a small region that includes the *oriT-mob* region of NBU1 (12). We have now localized and sequenced the *oriT-mob* region of NBU2. We report that this *oriT-mob* region is virtually identical to that of NBU1 but that the sequence similarity falls off abruptly after the stop codon of the *mob* gene and about 900 bp upstream of the end of the *oriT* region. The mobilization region may thus be located on a cassette.

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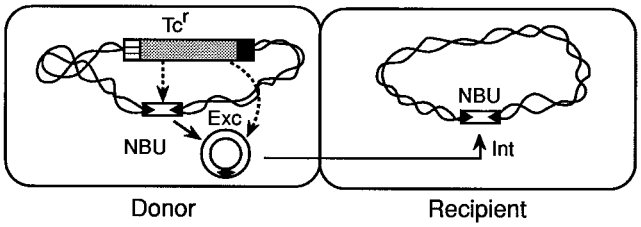

FIG. 1. Excision and mobilization of NBUs by *Bacteroides* conjugative transposons (large shaded bar denoted by Tc^r). Action in *trans* is denoted by dashed lines with arrowheads. Solid lines with arrowheads indicate the movement of DNA. RteB, provided by the *Bacteroides* conjugative transposon, stimulates NBU excision and circularization (Exc), presumably by activating an excision gene(s) on the NBU. The NBU circle form is mobilized in *trans* by the conjugative transposon, which provides the mating pore proteins. The NBU provides the Mob protein that cuts at its *oriT* region and initiates its transfer through the mating pore. In the recipient, the circular form of the NBU integrates into the recipient genome (Int).

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria broth or Luria broth agar plates. *Bacteroides* strains were grown in prereduced trypticase-yeast extract-glucose broth or agar (11, 17).

Mobilization by *traI* mutant plasmid RP4-12m. The TraI$^-$ derivative of RP4, RP4-12m, is a TraI Δ(I11-G211) deletion derivative of RP4. This deletion was constructed in a pMB1 replicon, with a MURF1 linker (translational termination codons in all three frames) inserted at the I11 codon (1). The pMB1 derivative was recombined into RP4, and the cointegrate was resolved as described previously (3). Mutant plasmid RP4-12m and pLYL21 (a plasmid carrying the *oriT-mob* region of NBU1) were both introduced into *E. coli* DH5α by transformation. Mobilization of pLYL21 from *E. coli* DH5αMCR (containing RP4-12m) to *E. coli* HB101 was done as described previously (7).

Cloning the NBU2 *mob-oriT* region. The NBU2 circle form can be seen as a faint band in large-scale plasmid preparations from a strain of *Bacteroides thetaiotaomicron* (BT4104N3-1), which carries both the conjugative transposon Tc^r ERL and a chromosomal copy of NBU2 and which has been pregrown in medium containing low concentrations of tetracycline (1 μg/ml). We cloned a large internal segment of NBU2 by first digesting the NBU2 circle form with *Pst*I, which cuts once in NBU2, and then ligating the digested NBU2 with *Pst*I-digested pUC19 and introducing the ligation mixture into *E. coli* DH5αMCR. None of the resulting chimeric plasmids contained intact NBU2, but some contained large portions of the element. A plasmid containing the largest insert (approximately 9 kbp of NBU2) was chosen for further study. Matings were done as described previously (7, 17). Various subclones of the cloned region were tested for mobilization by the IncP plasmids R751 and RP4.

Locating the *oriT* region of NBU2. $oriT_{NBU2}$ was localized by subcloning segments of the 3.2-kbp *oriT-mob*$_{NBU2}$ region into pUC19. To provide Mob$_{NBU2}$ in *trans*, the 3.2-kbp *oriT-mob*$_{NBU2}$ region was recloned into pACYC184 to produce pLYL23. pACYC184 is compatible with pUC19 and carries a different selectable marker. Triparental matings were done with *E. coli* JS53 (R751) and *E. coli* DH5αMCR (carrying pLYL23 [*mob*$_{NBU2}$] plus deletion derivatives of the 3.2-kbp *oriT-mob* region cloned in pUC19) as donors. *E. coli* HB101 was the recipient. Selection was for Ap^r (ampicillin resistance) on pUC19. Once the *oriT* region was identified, the ability of integrated copies of NBU1 or NBU2 in *Bacteroides* to mobilize plasmids carrying the *oriT* region of NBU1 or the *oriT* region of NBU2 was determined in the following way. The *oriT* regions from pLYL20 (NBU1) or pLYL45 (NBU2) were first cloned into pLYL7, an *E. coli-Bacteroides* shuttle vector which is not mobilized by *Bacteroides* conjugative transposons (7). These clones were mobilized into BT4104N1-3 (which contains an integrated copy of NBU1) or BT4104N3-1 (which contains an integrated copy of NBU2). The resulting *B. thetaiotaomicron* strains were then used as donors in transfer experiments with *E. coli* HB101 as the recipient, to determine whether the *oriT*-containing plasmid was transferred from *B. thetaiotaomicron* to *E. coli.*

Locating the ends of NBU2. From previous work, we had reason to believe that the ends of NBU2 lay within the small *Hin*dIII fragment of NBU2 (12). To locate the ends more precisely, an improved restriction map of this region of NBU2 was obtained by using cloned regions of NBU2. Various restriction fragments within the small *Hin*dIII segment were used as probes in Southern blots of DNA from BT4104N3-1, a strain with an integrated copy of NBU2, as described previously for NBU1 (12, 13). Probes that cross-hybridized with two bands of BT4104N3-1 DNA contained the ends of NBU2, whereas probes that cross-hybridized with only one band contained internal regions of NBU2.

DNA sequence analysis. For DNA sequence analysis, nested deletions of the *oriT-mob* region were created by using the Erase-a-base system of Promega Corp. DNA sequencing of the *oriT* region was done by using the Sequenase 2.0 kit (U.S. Biochemicals, Cleveland, Ohio). Sequencing of the NBU2 *mob* gene and the region upstream of the *oriT* region was done at the University of Illinois DNA sequencing facility.

Nucleotide sequence accession number. The DNA sequence of this region of NBU2 has been deposited in GenBank (accession number L42370).

RESULTS AND DISCUSSION

The *mob* gene of NBU1 encodes a bifunctional protein that enables NBU1 to be mobilized by RP4. Mobilization of a plasmid carrying the *oriT* region of NBU1 occurred only if the NBU1 Mob protein was supplied in the donor and did not occur if the Mob protein was supplied in the recipient (data not

TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Relevant phenotype(s)[a]	Description (reference or source)
Plasmids		
RP4-12m	TraI$^-$	RP4 with TraI transcriptionally terminated (this study)
pLYL7	Ap^r Mob$^+$ (Ap^r Mob$^-$)	*E. coli-Bacteroides* shuttle vector that is nonmobilizable in *Bacteroides* spp. (7)
pNBU1$_{oriT}$	Ap^r *oriT*$^+$ (Ap^r *oriT*$^+$)	718-bp *Dde*I fragment of pLYL11EABacc90 (7) cloned into the *Sma*I site of pLYL7 (this study); contains *oriT* but not *mob* of NBU1
pLYL21	Cm^r Mob$^+$	1.9-kbp NBU1 *mob* gene cloned into pACYC184 (7)
pLYL23	Cm^r Mob$^+$	3.2 *Sph*I-*Sma*I fragment of pLYL43 (Fig. 3), cloned into pACYC184, which had been digested with *Sph*I and *Eco*RV (this study)
pLYL24	Ap^r Mob$^+$ (Ap^r Mob$^+$)	3.2 *Sph*I-*Sma*I fragment of pLYL43 (Fig. 3) cloned into pLYL7, which had been digested with *Sph*I and *Sma*I (this study)
pNBU2$_{oriT}$	Ap^r *oriT*$^+$ (Ap^r *oriT*$^+$)	1.2-kbp *Ava*I fragment of pLYL45 cloned into pLYL7 (this study)
pLYL40s	Ap^r	*Pst*I fragment of NBU2 cloned into the *Pst*I site of pUC19 (this study)
pLYL40-28	Ap^r Mob$^+$	Mob$^+$ spontaneous deletion of pLYL40s (this study)
pLYL40L	Ap^r Mob$^+$	*Eco*RI deletion of pLYL40-28 (Fig. 3) (this study)
Bacteroides strains		
BT4001	(Rif^r)	Rif^r derivative of *B. thetaiotaomicron* 5482A (13)
BT4100	(Thy$^-$ Tp^r)	Thy$^-$ Tp^r derivative of *B. thetaiotaomicron* 5482A (13)
BT4104	(Thy$^-$ Tp^r Tc^r)	BT4100 carrying a single copy of the *Bacteroides* conjugative transposon, Tc^r ERL (13)
BT4104N1-3	(Thy$^-$ Tp^r Tc^r NBU1)	BT4104 carrying a single copy of NBU1 (13)
BT4104N3-1	(Thy$^-$ Tp^r Tc^r NBU2)	BT4104 carrying a single copy of NBU2 (13)

[a] *Bacteroides* phenotypes are shown in parentheses, and *E. coli* phenotypes are shown without parentheses. Abbreviations: Mob$^+$, mobilizable by RP4 or R751 (in *E. coli*); (Mob$^+$), mobilizable by Tc^r ERL (in *B. thetaiotaomicron*); *oriT*$^+$, *oriT* activity; Cm, chloramphenicol; Rif, rifampicin; Tp, trimethoprim; Thy$^-$, thymidine auxotroph.

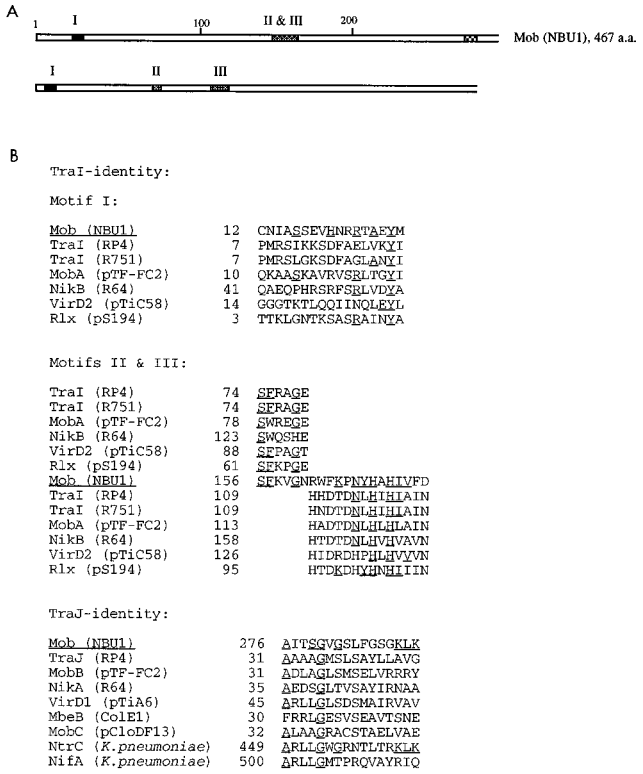

FIG. 2. Amino acid sequence similarities between the Mob protein of NBU1 and mobilization proteins of conjugal plasmids. (A) The placement of regions of similarity along the NBU1 Mob is indicated by shaded regions in the top bar. Motifs I, II, and III of TraI (relaxase of RP4) are indicated by the shaded regions in the bottom bar. Regions on the NBU Mob that have similarity to these motifs are labeled with the RP4 motif number. A region of similarity to TraJ and other DNA-binding proteins is indicated on the NBU1 bar by a checkered segment. (B) Specific alignments between the NBU1 Mob protein and plasmid relaxases and between the NBU1 Mob carboxy terminus and DNA-binding proteins. Amino acid residues of the NBU1 Mob protein, which are found in other mobilization or DNA-binding proteins, are underlined. The numbers represent the positions of the first amino acid residues of each sequence. *K. pneumoniae*, *Klebsiella pneumoniae.*

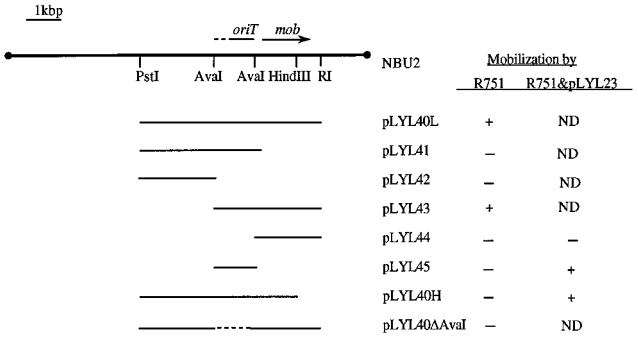

FIG. 3. A partial restriction map of the portion of NBU2 containing the *oriT-mob* region is shown. The locations of the *oriT* and *mob* regions of NBU2 are indicated by horizontal lines above the restriction map. The sizes and locations of different cloned regions tested for Mob or *oriT* activity are shown below the restriction map of NBU2. Clones were tested for mobilization by R751 (*mob* and *oriT* activity). Some clones were tested for mobilization by R751 and pLYL23 (*oriT* activity), where pLYL23 provided *mob* function in *trans*. The dashed line in pLYL40Δ*Ava*I indicates a deletion of the *Ava*I fragment. pLYL43, region cloned in pLYL23 and pLYL24. pLYL45, region cloned in pNBU2$_{oriT}$. ND, not determined.

shown). Thus, the NBU1 Mob protein must act at an early stage in NBU1 transfer. The first step in plasmid mobilization, nicking at the *oriT* region, is normally catalyzed by a multiprotein complex, which has *oriT*-binding activity as well as nicking activity (4, 10). Mob$_{NBU1}$ is likely to have *oriT*-binding activity, because the *oriT* regions of NBU1 and RP4 are too different at the DNA sequence level for the RP4 *oriT*-binding protein TraJ to recognize the NBU1 *oriT*. The specificity of *oriT*-binding proteins is evident from the fact that TraJ of RP4 does not act as an *oriT*-binding protein for IncP plasmid R751 and vice versa, despite the fact that these two plasmids have virtually identical sequences around their nick site (10). If Mob$_{NBU1}$ is an *oriT*-binding protein, what protein functions as the relaxase? One possibility is that Mob$_{NBU1}$ binds to the NBU1 *oriT* region and interacts with the relaxase of RP4 (TraI) to form the relaxosome complex. To determine whether TraI is required for the mobilization of NBU1, we tested the ability of a TraI$^-$ derivative of RP4, RP4-12m (1), to mobilize pLYL21, a plasmid carrying the *oriT-mob* region of NBU1. RP4-12m was able to mobilize pLYL21 at a frequency of 10^{-2} to 10^{-3} transconjugants per recipient. This frequency was comparable to that seen with wild-type RP4 or R751 (7). This result suggests that the NBU1 Mob protein has both *oriT*-binding and nicking activities (9).

The NBU1 Mob protein has some amino acid similarity to TraI and TraJ of RP4. Our earlier computer searches for similarities between the NBU1 Mob and TraI or TraJ of RP4 had revealed no regions of similarity (7). A more careful visual search of the amino acid sequence of the NBU1 Mob has now revealed that some of the essential residues on TraI and TraJ appear to be present on the NBU1 Mob (Fig. 2). Three motifs which are essential for nicking activity and covalent binding have been identified on TraI (Fig. 2) (1). Similarities between essential amino acids in these regions and amino acids in the corresponding amino-terminal regions on the NBU1 Mob protein were found (Fig. 2B), but the spacing of the motifs on the NBU1 Mob was not the same as on TraI. Y-22, the tyrosine of TraI that forms a covalent link with the nicked DNA, was one of the conserved motif I residues that was found on the NBU1 Mob protein. On the NBU1 Mob, this tyrosine was near the amino terminus of the protein (Y-27), just as it is on TraI and other members of the TraI family. It should be noted, however, that there were a number of residues shared by TraI and other plasmid relaxases that were not conserved in the NBU1 Mob sequence. There was also a short region near the carboxy terminus of the NBU1 Mob protein that had limited sequence similarity to those of NtrC and other DNA-binding proteins (Fig. 2A). Thus, the carboxy-terminal region of the NBU1 Mob protein could mediate initial binding to the *oriT*. We attempted to demonstrate nicking by using crude extracts of *E. coli* containing the NBU1 Mob protein, as described previously for RP4 (4, 9), but we did not detect any nicks in the NBU1 *oriT* region.

The *oriT-mob* region of NBU2 is similar in sequence to that of NBU1. Previous work had shown that although NBU1 and NBU2 had different restriction maps, a portion of NBU2 cross-hybridized with NBU1 DNA (12). To compare the *oriT-mob* regions of NBU1 and NBU2, we localized and sequenced the *oriT-mob* region of NBU2. To localize the NBU2 mobilization region, subclones of NBU2 were inserted into the nonmobilizable plasmid pUC19, and the resulting constructs were tested for mobilization by both RP4 and R751. The smallest clone that was mobilizable by RP4 and R751 contained a 3.2-kbp segment of NBU1 (pLYL43; Fig. 3). This segment clearly contained the *oriT-mob* region recognized by the IncP plasmids, but since the *Bacteroides* conjugative transposons are so

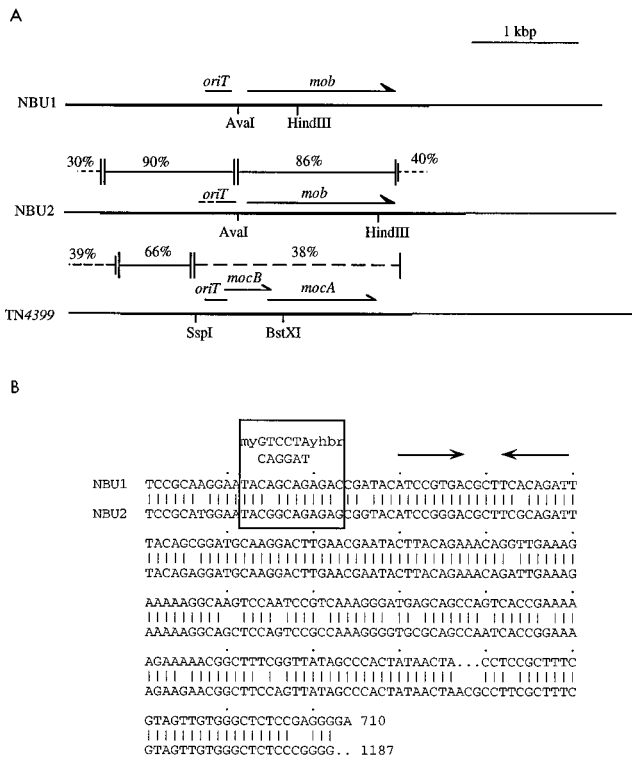

FIG. 4. Summary of DNA sequence comparisons of the *oriT-mob* regions of NBU1, NBU2, and Tn*4399*. (A) The gene organization and location of *oriT* are indicated above the line representing each element. Horizontal lines between each pair of elements indicate the regions of high DNA sequence similarity (solid line) and low sequence similarity (dashed line). The percentage of DNA similarity for each segment of the elements being compared is also shown. (B) An alignment of the portion of the $oriT_{NBU1}$ and $oriT_{NBU2}$ sequences thought to contain the nick site. The sequence of $oriT_{NBU1}$ is the first sequence. The letters in the boxed region show both strands of a consensus nick site obtained by comparing the nick sites of the IncP and other plasmids (8). The arrows show the location of an inverted repeat sequence in $oriT_{NBU1}$, another feature of *oriT* regions that is highly conserved on transmissible plasmids. Abbreviations in the consensus sequence: m stands for A or C; y stands for C or T; h stands for A, C, or T; b stands for C, G, or T; r stands for A or G.

distant genetically from the IncP plasmids, it was possible that the *oriT-mob* region recognized by the IncP plasmids might not be the one recognized by the *Bacteroides* conjugative transposons. To test this, the 3.2-kbp *oriT-mob* region was recloned into pLYL7, a plasmid that replicates in *Bacteroides* spp. and carries a *Bacteroides* selectable marker but is not mobilized by the *Bacteroides* conjugative transposons. The 3.2-kbp NBU2 segment cloned into pLYL7 was called pLYL24 (Table 1). pLYL24 was transferred into a *Bacteroides* recipient carrying the conjugative transposon Tc^r ERL (*B. thetaiotaomicron* 4104 [BT4104]), and the resulting strain was tested for the ability to transfer pLYL24 back to *E. coli*. pLYL24 was mobilized from BT4104 to *E. coli* HB101 at a frequency of 10^{-2} to 10^{-3} per recipient. Thus, the region of NBU2 that allowed the plasmid to be mobilized by IncP plasmids was also recognized by the *Bacteroides* conjugative transposon, Tc^r ERL.

To localize the *oriT* region of NBU2, portions of the 3.2-kbp region from pLYL43 were cloned into pUC19 (Fig. 3). To provide the mobilization protein in *trans*, the entire 3.2-kbp NBU2 *oriT-mob* region was cloned into pACYC184, a plasmid that is compatible with pUC19. This helper plasmid was called pLYL23. The smallest region of NBU2 that made pUC19 mobilizable by a combination of pLYL23 (mob_{NBU2}) and R751 was a 1.2-kbp *Ava*I fragment (Fig. 3). Deletion of this fragment from pLYL43 (the original NBU2 *oriT-mob* clone) rendered it nonmobilizable by R751. This confirmed that the *Ava*I fragment was essential for mobilization of pLYL43 by R751.

We sequenced the entire 3.2-kbp *oriT-mob* region of NBU2 and compared this sequence with the sequence of the *oriT-mob* region from NBU1. The sequences of $oriT_{NBU2}$ and $oriT_{NBU1}$ were 89% identical in the 200-bp region that contained the minimal $oriT_{NBU1}$ (Fig. 4). A single open reading frame was found in the sequenced region and was thus assumed to be the NBU2 mobilization gene, mob_{NBU2}. The *mob* genes of NBU1 and NBU2 shared an overall DNA sequence identity of 86%, but sequence differences were not distributed uniformly over the length of the gene. Most of the differences occurred in the last third of the gene.

The Mob protein of NBU1 acts on the *oriT* region of NBU2 and vice versa. The high sequence similarity between the *oriT* regions and *mob* genes of NBU1 and NBU2 suggested that the Mob protein of one might be able to act on the *oriT* region of the other. In triparental *E. coli*-to-*E. coli* matings, with *E. coli* HB101 as the recipient, a plasmid carrying mob_{NBU1} (pLYL21) mobilized a plasmid carrying $oriT_{NBU2}$ (pLYL45) at the same frequency as a plasmid carrying its cognate $oriT_{NBU1}$ (pEAB96; Table 2). Similarly, the NBU2 Mob mobilized the NBU1 *oriT* region as well as its cognate NBU2 *oriT* region (Table 2). In *Bacteroides* spp., the NBUs are located in the chromosome and are not present in multiple copies as in the triparental *E. coli* matings just described. To determine whether the Mob protein of NBU1 could act in *trans* on the *oriT* region of NBU2 and vice versa in *B. thetaiotaomicron*, we tested the ability of BT4104N1-3 (single chromosomal copy of NBU1) or BT4104N3-1 (single chromosomal copy of NBU2) to mobilize a plasmid carrying $oriT_{NBU2}$ or a plasmid carrying $oriT_{NBU1}$ from *B. thetatioaomicron* donors to *E. coli* recipients. Since pLYL45, the $oriT_{NBU2}$ plasmid used for *E. coli-E. coli* matings, did not have a *Bacteroides* replication origin or selectable marker, the 1.2-kbp *Ava*I fragment from pLYL45 was cloned into pLYL7 (a nonmobilizable plasmid that replicates in *Bacteroides* spp.), and the resulting plasmid ($pNBU2_{oriT}$)

TABLE 2. Mobilization of NBU1 and NBU2 *mob-oriT* regions by the IncP plasmid R751 from *E. coli* donors to *E. coli* recipients[a]

Donor strains[b]	Mobilizing plasmid(s)	Plasmid mobilized	Transfer frequency[c]
E. coli JS53(R751), *E. coli* DH5α(pEAB96)	R751	pEAB96[d] ($oriT_{NBU1}$)	$<10^{-9}$
E. coli JS53(R751), *E. coli* DH5α(pLYL21, pEAB96)	R751 and pLYL21 (mob_{NBU1})	pEAB96 ($oriT_{NBU1}$)	10^{-2}–10^{-3}
E. coli JS53(R751), *E. coli* DH5α(pLYL21, pLYL45)	R751 and pLYL21 (mob_{NBU1})	pLYL45 ($oriT_{NBU2}$)	10^{-2}–10^{-3}
E. coli JS53(R751), *E. coli* DH5α(pLYL23, pEAB96)	R751 and pLYL23 (mob_{NBU2})	pEAB96 ($oriT_{NBU1}$)	10^{-2}–10^{-3}
E. coli JS53(R751), *E. coli* DH5α(pLYL21, pLYL45)	R751 and pLYL23 (mob_{NBU2})	pLYL45 ($oriT_{NBU2}$)	10^{-2}–10^{-3}

[a] The *E. coli* recipient in all cases was *E. coli* HB101.
[b] Triparental mating.
[c] Frequencies are expressed as transconjugants per recipient.
[d] pEAB96 was called pLYLEAB96 in reference 7.

TABLE 3. Mobilization of plasmids containing the *oriT* regions of NBU1 and NBU2 by the *Bacteroides* conjugative transposon Tc^r ERL and an integrated NBU[a]

Donor strain[b]	Mobilizing element(s)	Plasmid mobilized	Transfer frequency[c]
BT4104(pNBU2$_{oriT}$ [or pNBU1$_{oriT}$])	Tc^r ERL	pNBU2$_{oriT}$ or pNBU1$_{oriT}$	$<10^{-9}$
BT4104N1-3(pNBU2$_{oriT}$)	Tc^r ERL and NBU1	pNBU2$_{oriT}$	1×10^{-2}–2×10^{-2}
BT4104N1-3(pNBU1$_{oriT}$)	Tc^r ERL and NBU1	pNBU1$_{oriT}$	1×10^{-3}–2×10^{-3}
BT4104N3-1(pNBU2$_{oriT}$)	Tc^r ERL and NBU2	pNBU2$_{oriT}$	1×10^{-2}–2×10^{-2}
BT4104N2-3(pNBU1$_{oriT}$)	Tc^r ERL and NBU2	pNBU1$_{oriT}$	2×10^{-5}–3×10^{-5}

[a] The *E. coli* recipient in all cases was *E. coli* HB101.
[b] All *B. thetaiotaomicron* donors carry the conjugative transposon Tc^r ERL, and all were pregrown in medium containing tetracycline. No transfer was seen ($<10^{-9}$ per recipient) if the donors were pregrown in medium that did not contain tetracycline. BT4104N1-3 also carries a chromosomal copy of NBU1. BT4104N3-1 carries a chromosomal copy of NBU2.
[c] Frequencies are expressed as transconjugants per recipient.

was transferred into BT4104N1-3 or BT4101N3-1. A similar plasmid carrying the *oriT* of NBU1 (pNBU1$_{oriT}$) was also transferred into these two *Bacteroides* strains. The chromosomal copies of NBU1 and NBU2 were both able to join with Tc^r ERL to mobilize pNBU2$_{oriT}$ from *Bacteroides* spp. to *E. coli* HB101 (Table 3). No transfer of pNBU2$_{oriT}$ was obtained if the *Bacteroides* donor carried Tc^r ERL but lacked a chromosomal copy of NBU1 or NBU2 (BT4104) (Table 3). Similarly, both NBU1 and NBU2 were able to mobilize the plasmid carrying the *oriT* region of NBU1 (pNBU1$_{oriT}$), although mobilization occurred at lower frequencies compared with the case for pNBU2$_{oriT}$ (Table 2). These results show that the *oriT-mob* regions of NBU1 and NBU2 are functionally equivalent in *B. thetaiotaomicron* as well as in *E. coli* and that the naturally occurring form of the NBUs could act in *trans* to give high-level mobilization. Since NBUs do not exclude each other, more than one of them can enter the same strain. Our results suggest that two NBUs in the same strain might stimulate each other's mobilization.

The fact that the Mob proteins of NBU1 and NBU2 were cross-functional suggested that *oriT* nick site sequences should be absolutely conserved between NBU1 and NBU2. Previously, we had identified a region of *oriT*$_{NBU1}$ (7) that had a limited similarity to the 12-bp consensus sequence deduced for nick sites of RP4-related plasmids (9). This sequence is indicated by a box in Fig. 4 (CAGGAT). Adjacent to this region was a possible stem-loop structure, similar to that found adjacent to nick sites on conjugal plasmids. A comparison of the sequences of NBU1 and NBU2 in this region revealed that there were two differences between NBU1 and NBU2 within the boxed region. One of these fell within the CAGGAT core consensus region. In NBU1 the sequence was CAGCAG, and in NBU2 the sequence was CGGCAG, an even worse match to the RP4 consensus, CAGGAT. This raises questions about whether the similarity between the consensus nick sequence of the IncP plasmids and this sequence of the NBU *oriT* region is significant.

The *oriT-mob* region of the NBUs may be located on a cassette. Immediately downstream of the *mob* gene, the sequence identity between NBU1 and NBU2 fell abruptly, from over 80 to about 40% (Fig. 4). Upstream of the 200-bp *oriT* region of NBU1, the near identity between NBU1 and NBU2 sequences continued for over 900 bp and then fell abruptly to about 30% (Fig. 4). These results suggest that the *oriT-mob* regions of NBU1 and NBU2 might be located on a cassette. Previously, when we searched the databases for sequences similar to that of the *oriT-mob* region of NBU1, the only significant similarity was to an open reading frame adjacent to a cefoxitin resistance gene on Tn*4555*, a mobilizable integrated *Bacteroides* element (15). When we searched the databases for sequences similar to the region upstream of *oriT*, however, we found significant sequence similarity to a region upstream of the *oriT* region on the mobilizable transposon Tn*4399*. Tn*4399* has two mobilization genes, *mocA* and *mocB*. A forced pairwise alignment between the NBU2 *oriT-mob* region and the *oriT-mocA-mocB* region of Tn*4399* revealed less than 40% sequence identity, with numerous gaps required to attain this level of similarity, but in the upstream region the sequence identity between Tn*4399* and the two NBUs was 61 to 68% over a 900-bp region. Since the region of similarity between Tn*4399* is outside the region required for mobilization of NBU1 and NBU2, this region is clearly not essential for mobilization. Nonetheless, the high sequence similarity in this region and the fact that it is located immediately upstream of the *oriT* regions on both the NBUs and Tn*4399* suggest that this region has some function in the transfer process. A search of the databases, using just this conserved 900-bp region, revealed sequence similarity between this region and several plasmid primases. Thus, the 900-bp region might play a role in regeneration of the double-stranded form of the transfer intermediate after transfer, a function that could have been supplied in the experiments described in this paper by the plasmid into which the *oriT-mob* region was cloned or by the recipient.

Further evidence that the *oriT-mob* region of the NBUs might be on a cassette came from experiments designed to determine the location of the NBU2 *oriT-mob* region relative to the ends of the element. On NBU1, the *oriT-mob* region is near the middle of the element. Using Southern analysis of integrated and excised forms of NBU2, we located the ends of NBU2. The joined ends of the circular form of NBU2 are located within an 0.8-kbp *Hin*cII-*Hin*dIII fragment on NBU2. The *oriT-mob* region of NBU2 ends about 1.5 kbp from one end of NBU2, whereas the *oriT-mob* region of NBU1 ends about 3 kbp from the nearest end of NBU1. The different locations of the *oriT-mob* regions on NBU1 and NBU2 are consistent with the hypothesis that the *oriT-mob* region is carried on a cassette, which has inserted into different places on NBU1 and NBU2.

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