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The *Bacteroides* Mobilizable Insertion Element, NBU1, Integrates into the 3′ End of a Leu-tRNA Gene and Has an Integrase That Is a Member of the Lambda Integrase Family

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NBU1 is a 10.3-kbp integrated *Bacteroides* element that can be induced to excise from the chromosome and can be mobilized to a recipient by *trans*-acting functions provided by certain *Bacteroides* conjugative transposons. The NBU1 transfer intermediate is a covalently closed circle, which is presumed to be the form that integrates into the recipient genome. We report here that a 2.4-kbp segment of NBU1 was all that was required for site-specific integration into the chromosome of *Bacteroides thetaiotaomicron* 5482. This 2.4-kbp region included the joined ends of the NBU1 circular form (*attN1*) and a single open reading frame, *intN1*, which encoded the integrase. Previously, we had found that NBU1 integrates preferentially into a single site in *B. thetaiotaomicron* 5482. We have now shown that the NBU1 target site is located at the 3′ end of a Leu-tRNA gene. The NBU1 integrase gene, *intN1*, was sequenced. The predicted protein had little overall amino acid sequence similarity to any proteins in the databases but had limited carboxy-terminal similarity to the integrases of lambdoid phages and to the integrases of the gram-positive conjugative transposons Tn*916* and Tn*1545*. We also report that the *intN1* gene is expressed constitutively.

A family of mobilizable insertion elements called NBUs (nonreplicative *Bacteroides* units) has been found in human colonic *Bacteroides* species. Four members of the NBU element family (NBU1, NBU2, NBU3, and Tn*4555*) have been described (3, 22, 24, 26). All of these elements have in common that they are about 10 to 12 kbp in size and their transfer intermediate is a covalently closed circle (13, 15). This circle does not replicate but can be transferred by conjugation, starting from an internal transfer origin (*oriT*). Recent studies have shown that the NBU-type elements all share a region of high sequence similarity that includes *oriT*, a mobilization gene (*mob*) that lies immediately downstream of *oriT*, and a 900-bp region upstream of *oriT* (15, 24).

NBUs are not self-transmissible, nor can they excise on their own. Both excision and mobilization of NBUs require *trans*-acting functions provided by a *Bacteroides* conjugative transposon (CT) (22, 27, 28). *Bacteroides* CTs are large (>60 kbp) self-transmissible integrated elements that are usually located in a part of the chromosome different from the location of the NBU they excise and mobilize (3). A regulatory protein provided by the CT, RteB, is needed to stimulate excision and circularization (27). This stimulation is assumed to occur via activation of one or more NBU genes by RteB, but the precise nature of the interaction between RteB and genes on the NBU is not known. Expression of *rteB* is induced by low levels of tetracycline (27). Thus, both the presence of a CT carrying *rteB* and exposure of the cells to tetracycline are necessary for excision and mobilization of the NBU circular form. Mobilization of the NBU circular form requires not only RteB but also mating apparatus proteins provided by the CT (14). Since the same 20-kbp segment of the CT is required for self-transfer of the CT and for mobilization of the NBU circle form, the NBU circle form is probably transferred via the same mating apparatus used by the CT itself (14, 27, 28). Although NBUs rely on CTs for excision and transfer, the NBUs are not simply miniature, transfer-defective forms of the CTs. So far, no sequence similarity between an NBU and a CT has been reported.

Previous studies have defined and characterized the mobilization region of NBU1 and NBU2 (13, 15). In this study, we define and characterize the minimal region necessary for integration of NBU1 in *Bacteroides thetaiotaomicron*. We had shown previously that NBUs integrate site specifically in *B. thetaiotaomicron*. We report here that the primary insertion site of NBU1 in *B. thetaiotaomicron* is in the 3′ end of a *Bacteroides* Leu-tRNA gene, which is highly conserved in different *Bacteroides* species. We also report the sequence of the NBU1 integrase gene and show that the deduced amino acid sequence of the integrase gene product has limited similarity to integrases of lambdoid phages and the gram-positive conjugative transposons Tn*916* and Tn*1545*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were grown in Luria broth (LB) or on LB agar plates. *Bacteroides* strains were grown in prereduced Trypticase (BBL Microbiological Systems, Cockeysville, Md.)-yeast extract-glucose (TYG) (9) medium under an atmosphere of 80% nitrogen–20% carbon dioxide or on TYG agar plates in BBL GasPak jars.

DNA extractions and Southern blot analysis. Plasmids were isolated by using the Ish-Horowitz modification of the alkaline lysis procedure (19). Total DNA was isolated by using a modification of the method of Saito and Miura (18). Restriction enzyme digests of the DNA and cloning procedures were done according to the manufacturers' suggestions (GIBCO-BRL; New England Biolabs; or Promega, Madison, Wis.) or as described by Sambrook et al. (19). Southern blot analysis was done as previously described (19, 23). Under low-stringency conditions, the hybridization temperature was 42°C with no formamide and the washes were done at room temperature. The probes were gel-purified DNA fragments which were labelled with [^{32}P]dCTP by using the Random Prime-It II kit (Stratagene, La Jolla, Calif.).

Filter matings. The conditions for filter matings have been described previously (20, 30). The mating filters (GN-6; Gelman) were incubated at 37°C aerobically on TYG agar, and the donor-to-recipient ratio was 1:3. *Bacteroides* transconjugants were selected on TYG agar plates containing gentamicin (200 μg/ml) and erythromycin (10 μg/ml).

Construction of minimal NBU1 insertion vectors. To determine the minimum region of NBU1 needed to insert in *B. thetaiotaomicron*, the insertion vector

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Description and/or source (reference)
E. coli strains		
DH5αMCR	RecA	GIBCO-BRL
S17-1	RecA Tra^+ Tp^r Sm^r	IncP RP4 inserted in chromosome (ΩRP4-Tc::Mu-Kn::Tn*7*); furnishes transfer functions (25)
B. thetaiotaomicron 5482 strains		
BT4001	Rif^r	Spontaneous Rif^r mutant of BT5482
BT4004	Rif^r Tc^r	BT4001 containing Tc^r ERL
BT4100	Thy^- Tp^r	Thymidine-requiring spontaneous mutant of BT5482
BT4100N1-S1	Thy^- Tp^r	BT4100 containing NBU1 in *attB*, BT1-1 (24)
BT4104	Thy^- Tp^r Tc^r	BT4100 containing CT, Tc^r ERL
BT4104N1-3	Thy^- Tp^r Tc^r	BT4104 containing NBU1 in *attB*, BT1-1 (24).
Plasmids and vectors		
R751	Tp^r Tra^+	IncP plasmid used to mobilize vectors in conjugal matings (20)
pUC19	Ap^r	*E. coli* cloning vector (31)
pVAL1	Ap^r Tc^r Mob^+ (Em^r Mob^+ Rep^+)	*E. coli-Bacteroides* shuttle vector (30)
pNV19	Ap^r Mob^+ (Em^r Mob^+ Rep^-)	IS′ *ermF*-pB8-51Mob region of pVAL1 on a 5.2-kbp *Ava*I-to-*Eco*RV fragment cloned into *Nde*I site of pUC19 (Fig. 1) (this study)
pNVIN3	Same as pNV19 (Int^+ Exc^-)	5.6-kbp *Pvu*II-*Pst*I fragment of NBU1 in pNV19 (Fig. 1) (this study)
pIA_{46}	Same as pNVIN3	Deletion clone of pNVIN3 (this study)
Y5D and Y11D	Ap^r Tc^r Mob^+ (Em^r Mob^+ Rep^- Int^+ $Exc^{+/-}$)	pEG920::NBU1 cointegrates, Y5 and Y11, with the pB8-51 replicon removed. Integrate and excise in *Bacteroides* hosts (21, 24)
Y11DP	Same as Y11D except Exc^-	3-kbp *Pst*I deletion of Y11D (24).
pMJF2	Ap^r Mob^+ (Em^r Rep^+ Mob^+ GUS^-)	Replicative GUS (*uidA*) transcriptional fusion vector (8)
pCQW1	Ap^r Mob^+ (Em^r Rep^- GUS^-)	Insertional GUS (*uidA*) transcriptional fusion vector (8)
pMJF::P*intN1*	Same as pMJF2	850-bp region of NBU1 containing promoter region of *intN1* cloned in correct orientation in front of GUS reporter gene in pMJF2 (this study)
pCQW::*intN1*	Same as pCQW1	346-bp fragment in N-terminal end of IntN1 cloned in correct orientation in front of GUS to measure activity of P*intN1* in inserted NBU1 form (this study)

[a] Phenotypes in parentheses are expressed only in *Bacteroides* spp., and phenotypes not in parentheses are expressed in *E. coli*. Abbreviations: Ap^r, ampicillin resistant; Rif^r, rifampin resistant; Sm^r, streptomycin resistant; Tp^r, trimethoprim resistant; Thy^-, thymidine requiring; Tra, conjugal transfer functions; Mob, mobilizable by Tra function of IncP plasmids or (if in parentheses) by *Bacteroides* conjugative transposons; Rep, replication; Int, integration; Exc, excision.

pNVIN3 was constructed. Steps in the construction of pNVIN3 are shown in Fig. 1. pNVIN3 was mobilized by IncP plasmids (R751 or RP4 in S17-1) from *E. coli* to *B. thetaiotaomicron* BT4001. BT4001 transconjugants that contained the selectable marker carried on pNVIN3 (erythromycin resistant) (Em^r) were checked by Southern analysis and in some cases by DNA sequence analysis to determine whether pNVIN3 had inserted in the NBU1 primary site, BT1-1. Unidirectional deletions of pNVIN3 were made by using the exoIII Erase-a-Base system from Promega, and the resulting (Erase-a-Base) deletion vectors were tested for their ability to insert in BT4001. All of the deletion vectors which successfully integrated into the BT4001 chromosome were considered to contain both the NBU1 integrase (IntN1) and the NBU1 *att* site (*attN1*). The smallest deletion vector isolated was pIA_{46}. Transconjugants containing pIA_{46} insertions were checked to make sure that the plasmid had inserted via the ends of NBU1 and into the NBU1 primary site.

Cloning and sequencing of the NBU1 primary and secondary insertion sites, BT1-1 and BT1-2. The cloning and sequencing of the primary *attB* site for the insertion of NBU1 in BT4001 (*attB*), BT1-1, have been previously described (24). In the present study, more extensive sequencing of the chromosomal region adjacent to the left end of the integrated NBU1 was done to determine the identity of the locus that contained the primary integration site. The only observed secondary *attB* site for NBU1 in BT4001, BT1-2, was cloned as described for BT1-1 (24). The sequences of the junctions were obtained by using the left and right junction primers from NBU1 (24). The chromosomal sequence obtained was used to design primers for PCR amplification of the intact secondary NBU1 integration site, BT1-2. The resulting 370-bp fragment was cloned into pUC19 (pUC::BT1-2) and sequenced.

DNA sequencing and sequence analysis. Double-stranded template sequencing was performed on plasmids by using the dideoxy-chain-terminating reaction with T7 polymerase as provided in the Sequenase 2.0 kit (U.S. Biochemical, Cleveland, Ohio). More extensive sequencing was performed at the University of Illinois Biotechnology Genetic Engineering Facility in Urbana with synthesized primers and the Applied Biosystems model 373A version 2.0.1S dye terminator automated sequencer. Computer analyses of the nucleotide and amino acid sequences were done by using the Genetics Computer Group software system (7). The amino acid sequence alignment of the integrases of NBU1, Tn*1545*, and lambda was done by using the BESTFIT program. The BLAST program (1) was used to search databases for nucleotide and amino acid sequence similarities.

Transcriptional fusions to the NBU1 integrase gene, *intN1*. To determine if the NBU1 integrase was regulated at the transcriptional level, while in the circular transfer intermediate form or in the inserted preexcision form, two transcriptional fusion constructs were made. To test the circular form of the promoter region of *intN1*, a segment containing the joined ends of the circular form of NBU1 (bp 1 to 840) was cloned upstream of the reporter group, *uidA* (β-glucuronidase) (GUS), on pMJF2 to form pMJF2::P*intN1*. pMJF2 is an *E. coli-Bacteroides* shuttle plasmid that replicates in *Bacteroides* spp. as well as in *E. coli* (8). A GUS fusion to the *intN1* gene in the inserted form of NBU1 was made by cloning an internal 346-bp region near the N terminus of *intN1* (bp 767 to 1113) in the correct orientation relative to the GUS gene in plasmid pCQW1 (8),

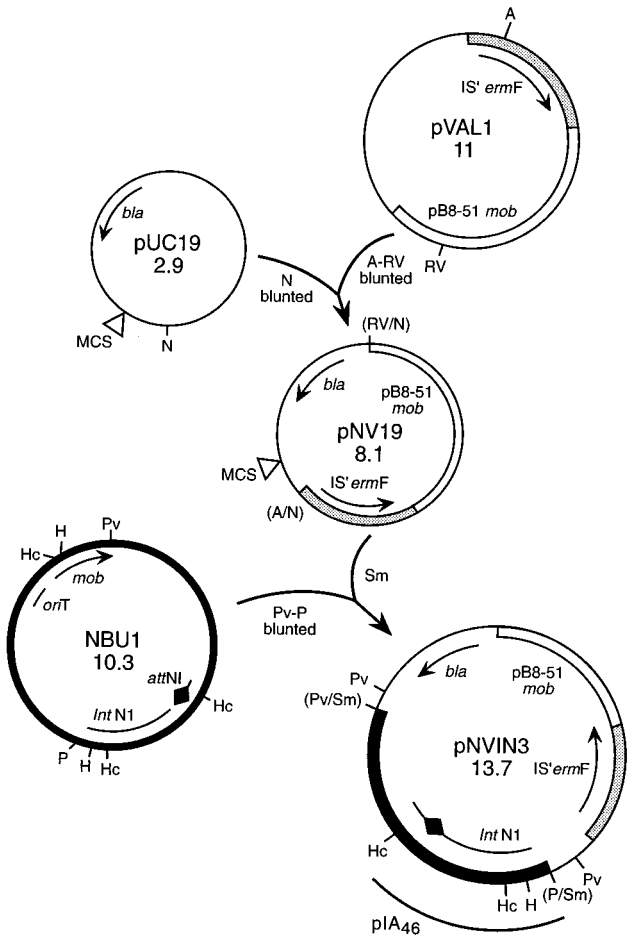

FIG. 1. Construction of the NBU1 insertion vector in *Bacteroides* recipients, pNVIN3. The 5.2-kbp *Ava*I (A)-to-*Eco*RV (RV) fragment from the *Bacteroides-E. coli* shuttle vector pVAL1 (30) was blunted and cloned into the blunted *Nde*I (N) site on pUC19 (31). This fragment contains the promoter region of IS*4351* and the *Bacteroides ermF* (IS′*erm*F) and the mobilization region (*mob*) but not the replication region of the *Bacteroides* plasmid pB8-51. The resultant vector, pNV19, is a mobilizable suicide vector in *Bacteroides* strains. The integration region of NBU1, including the integrase (IntN1) and crossover region (*attN1*), on a 5.6-kbp *Pvu*II (Pv)-to-*Pst*I (P) fragment was blunted and cloned into the *Sma*I (Sm) site in the multiple cloning sites (MCS) of pNV19 for integration studies with *B. thetaiotaomicron*. The region defining the smallest site-specific, integration-positive deletion vector, pIA46, is indicated. Other restriction site abbreviations are as follows: H, *Hin*dIII; Hc, *Hin*cII.

to produce pCQW1::*intN1*. pCQW1 replicates in *E. coli* but not in *Bacteroides* spp. This plasmid was transferred by conjugation from *E. coli* S17-1 to *B. thetaiotaomicron* recipients with selection for Gen^r (gentamycin-resistant) Em^r transconjugants. Three types of recipients were used. Both fusion plasmids were transferred to a *B. thetaiotaomicron* strain containing an integrated copy of NBU1 (BT4100N1-S1) and to a strain containing an integrated copy of NBU1 plus an integrated copy of the CT tetracycline-resistant (Tc^r) ERL (BT4104N1-3). pMJF::*intN1* was also transferred to a strain that contained the CT Tc^r ERL but no integrated NBU1 (BT4104). To determine whether RteB (from the CT Tc^r ERL) was involved in expression of the NBU1 *intN1* gene, *B. thetaiotaomicron* transconjugants that contained Tc^r ERL and either pMJF2::P*intN1* or pCQW1::*intN1* integrated into NBU1 were grown on 1 μg of tetracycline per ml to induce synthesis of RteB (27). GUS assays were done as described by Feldhaus et al. (8).

Excision assay. A Southern blot approach was used to determine whether NBU1 or portions of it were capable of excision from the *B. thetaiotaomicron* chromosome. This was done as described previously by Stevens et al. (27, 28) except that the probe used was one that hybridized only with the ends of NBU1, not to the entire NBU1.

Nucleotide sequence accession numbers. The GenBank accession numbers are as follows: 2,446-bp NBU1 *attN1-intN1* region, no. 451917; primary NBU1 integration region of BT4001 including BT1-1 and the *Bacteroides* Leu-tRNA gene, no. 451915; and secondary *attB* site BT1-2, no. 451916.

RESULTS AND DISCUSSION

NBU1 primary and secondary integration sites in *B. thetaiotaomicron*. Previously we found that NBU1 inserts site specifically in *B. thetaiotaomicron*, in an *attB* site designated BT1-1. A comparison of the sequence in the region containing the joined ends of NBU1 (*attN1*) with the sequence of BT1-1 revealed that *attN1* and BT1-1 had two regions of high similarity, region I and region II (Fig. 2A). Region I was a 14-bp sequence that was identical in both the NBU1 *attN1* and BT1-1. Region II was a 10-bp sequence found in *attN1* that had partial identity to a corresponding region in BT1-1 and was located within a region that contained inverted repeats (Fig. 2A). Therefore, when the circular intermediate of NBU1 inserts into BT1-1, as shown in Fig. 2B, there is a copy of the 14-bp region I sequence and a copy of a 10-bp region II sequence at both ends of the inserted NBU1. The inverted-repeat region, within which the 10-bp region II structure is located, is depicted as a stem-and-loop structure in Fig. 2B. In only one instance has NBU1 been seen to insert into a secondary site (24). This site has been designated BT1-2. BT1-2 was cloned from the BT4001 chromosome and sequenced. This site had identity of 10 of 14 bp in region I but lacked region II of *attN1* (Fig. 2A).

The primary site in *B. thetaiotaomicron* lies at the 3′ end of a tRNA gene. To determine the identity of the locus into which NBU1 was integrating, an extended region of chromosomal DNA adjacent to *attL* and *attR* was sequenced. This sequence was compared with GenBank sequences, by using the BLAST program (1). An 86-bp stretch of the sequence in the left junction region, including the 14-bp sequence in region I of BT1-1, was 60 to 68% identical to Leu-tRNA sequences from a variety of organisms, including the Leu-tRNA4 (TAA) of *E. coli* K-12 (12). The *E. coli* Leu-tRNA4 had 63% identity along its entire length with the Leu-tRNA sequence of *B. thetaiotaomicron*. Eleven of the 14 bp in region I and 9 of the 10 bp in region II of *attN1* were identical to the corresponding regions in the *E. coli* Leu-tRNA sequence. However, the 10-bp sequence of region II of the *E. coli* gene was not within a region containing inverted repeats. The sequence of the putative *B. thetaiotaomicron* Leu-tRNA is shown in Fig. 3, along with its *E. coli* Leu-tRNA4 homolog. The anticodon of the *Bacteroides* Leu-tRNA (CAA) differs from that of the *E. coli* Leu-tRNA4 (UAA). The 14-bp region I sequence was in the acceptor stem, within 2 nucleotides of the 3′ ends of the Leu-tRNAs (shown boxed in Fig. 3). The 10-bp region II sequence of BT1-1 (GAAAAGCTAAG) started 1 nucleotide from the 3′ CA of the *B. thetaiotaomicron* Leu-tRNA. In *E. coli* Leu-tRNA, the region II sequence (GAAAGATAAG) started 3 nucleotides after the 3′ CA. Several bacteriophages are known to integrate into the 3′ ends of tRNA genes (6). tRNA genes are also the targets of the actinomycete plasmids that integrate into the chromosomes of some hosts (4, 5). Thus, NBU1 falls into this family of elements that integrate into tRNA genes.

The overall DNA-DNA homologies among different *Bacteroides* species are 45% or lower, so that sequences similar enough to be detected by Southern blot analysis (>70%) are the exception rather than the norm (10). Southern hybridization with a probe derived from the *attL* region demonstrated that the tRNA gene into which NBU1 was integrating was found in all *Bacteroides* spp. tested (data not shown). Thus, the NBU1 target is found widely in *Bacteroides* spp., as expected from the fact that NBU-hybridizing elements have been found

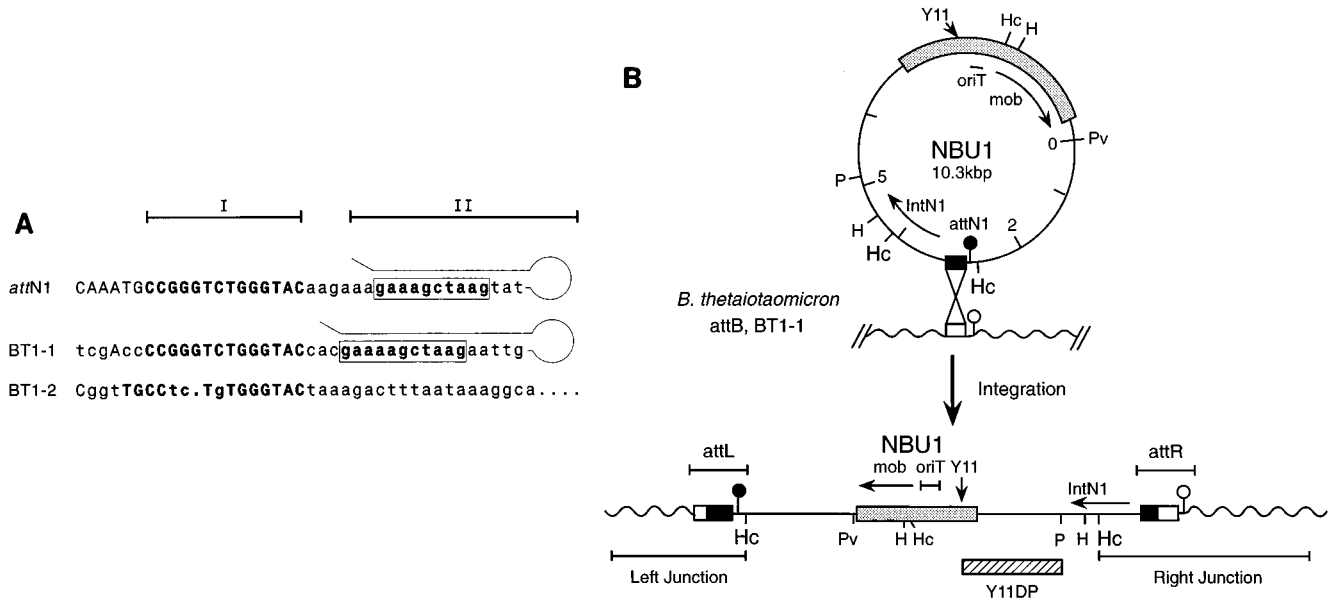

FIG. 2. (A) Comparison of NBU1 *attN1* and the primary and secondary *attB* sites in *B. thetaiotaomicron*. The NBU1 *attN1* is shown at the top with the 14-bp sequence of region I in bold capital letters, and the 10-bp region is shown, boxed, in a region containing inverted repeats, which are depicted as a stem-and-loop structure. The primary NBU1 *attB* site in *B. thetaiotaomicron*, BT1-1, has 100% identity to the 14-bp sequence of region I and partial identity to the 10-bp sequence of region II. BT1-2, the only known secondary *attB* site in *B. thetaiotaomicron*, had partial identity to region I but no region II sequence or inverted repeats. The identical nucleotides in the target sequences in region I are shown as capital letters. The bold capital letters indicate where the crossover occurred to give the hybrid *attR* and *attL* sequences at the NBU1-target junctions shown in panel B. The crossover occurred in BTI-2 within the first four bases, TGCC. (B) Integration of NBU1 into the primary *attB* site, BT1-1, in *B. thetaiotaomicron* 5482. NBU1 is shown in the excised circular intermediate form which can either be mobilized from an internal origin of transfer (*oriT*) and mobilization (*mob*) region by a conjugative transposon (13, 24) or integrate into an *attB* site. For integration of NBU1 into its primary *B. thetaiotaomicron attB* site, BT1-1, the crossover occurs within or at either end of the 14-bp region of identical sequence contained on both *att* sites shown as filled (NBU1 *attN1*) and open (*attB*, BT1-1) rectangles. Depending on the location of the actual crossover in the 14-bp region, the 14-bp sequences at the left junction, *attL*, and the right junction, *attR*, would be hybrid sequences with part of the sequence coming from *attB* and the remainder coming from *attN1*. These hybrid sequences are identical for *attN1* and BT1-1, but they would be different if the insertion was in a secondary site with only partial sequence identity, as seen for the secondary *attB*, BT1-2 (see panel A). There is a second region on both *attN1* and BT1-1 which includes a 10-bp sequence of partial identity within an inverted-repeat region. The inverted repeats are represented by the corresponding closed (*attN1*) and open (*attB*, BT1-1) lollipop symbols. The location of the site of insertion of the shuttle vector pEG920 into NBU1 to form Y11 (21, 24) is indicated by the arrows in both the circular and integrated forms of NBU1, and the *Pst*I deletion in Y11D derivative Y11DP is also indicated. Y11 and its derivatives were used to monitor integration and transfer of NBU1 in *Bacteroides* hosts (24). The NBU1 gene required for integration, *intN1*, is also indicated. The highlighted (grey) portion of NBU1 is the region that contains sequences homologous to other NBU elements (15, 24). The enlarged *Hin*cII (Hc) sites flank a 1.5-kbp fragment in the NBU1 circular intermediate which includes *attN1* and part of the IntN1 region. In the integrated form of NBU1 the same Hc sites were used to clone NBU1-chromosomal right and left junction fragments to use as probes on Southern blots. The *att* sequence at the left junction, *attL*, was identical to *attN1*, and the sequence at the right end, *attR*, was identical to the respective *attB* sequence, BT1-1 or BT1-2. Restriction site abbreviations are as follows: H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; Pv, *Pvu*II.

widely in the *Bacteroides* group. The DNA sequence of the *attR* region had no similarity to any known genes, and a probe from this region did not detect any cross-hybridizing segments in *Bacteroides* spp. other than *B. thetaiotaomicron* (data not shown).

When the secondary site, BT1-2, was used as a probe in Southern blot experiments, it hybridized only to *B. thetaiotaomicron* DNA (data not shown). Approximately 370 bp of sequence was obtained from the right and left chromosomal regions adjacent to BT1-2. No significant homologies to genes in the database were seen when a BLAST search was performed with these sequences. Thus, BT1-2, in contrast to BT1-1, is not located at the end of a conserved gene.

The minimal region required for integration of NBU1 consists of the joined ends plus 2 kbp of the right end of the element. Generally, integrases are located near an end of the element that carries them (6). In our initial investigations of NBU1 integration, we had found that clones of NBU1 that lacked NBU1 sequences between the Y11 insertion site and the *Pst*I site (Y11DP, Fig. 2B) were still able to integrate (24), but a clone with a deletion to the *Hin*dIII site was not able to integrate. These results suggested that the NBU1 integrase was located near the right end of the element, as depicted in Fig. 2B. To test this hypothesis and to determine what sequences near the left end of NBU1 may be required, a 5.6-kbp *Pst*I-*Pvu*II fragment of NBU1, which contained the closed ends of the circular form of NBU1 plus a sizable portion of the left end of NBU1, was cloned into the *Sma*I site of the insertional vector, pNV19, to produce pNVIN3 (Fig. 1). pNV19 replicates in *E. coli* and is mobilizable by IncP plasmids from *E. coli* donors to *Bacteroides* recipients, but it does not replicate in *Bacteroides* spp. pNVIN3 was mobilized into BT4001 or BT4004 recipients with selection for the erythromycin resistance (*ermF*) marker on pNVIN3. pNVIN3 inserted into the chromosome of BT4001 at a frequency of about 10^{-6} transconjugants per recipient. BT4004 differs from BT4001 in that BT4004 carries the conjugative transposon, Tc^r ERL. BT4004 was used as a recipient in some experiments to determine whether the presence of a CT would stimulate integration. pNVIN3 integrated into the BT4004 chromosome at the same frequency as it did in BT4001 (10^{-6} transconjugants per recipient). Thus, integration of NBU1, unlike excision and mobilization, was not dependent on functions provided by Tc^r ERL.

In all of the transconjugants tested, pNVIN3 integrated into the primary *attB* site, BT1-1. Integrated pNVIN3 could not be induced to excise and transfer out of the BT4004 transconjugants to *E. coli* recipients, even when the transconjugants were grown in the presence of tetracycline to induce functions on

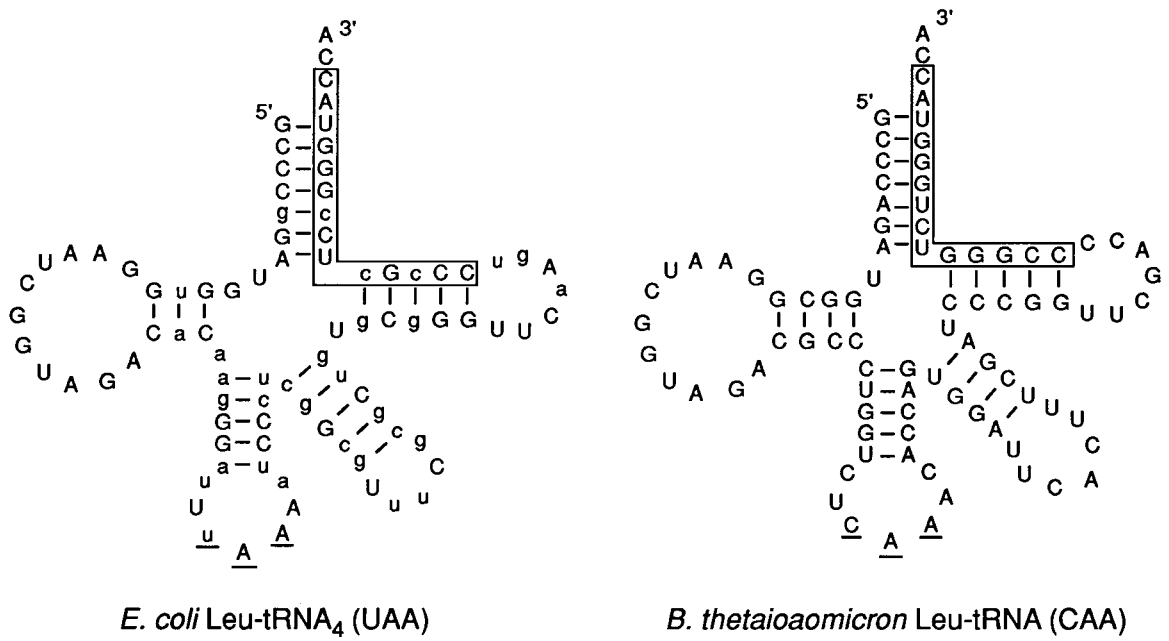

FIG. 3. NBU1 primary *attB* site in *Bacteroides* spp. identified as the 3′ end of a Leu-tRNA gene. The sequence adjacent to *attL* had 67% identity with the Leu-tRNA4 gene of *E. coli* K-12 (12). The nucleotides in the Leu-tRNA4 gene that were identical to the putative *Bacteroides* Leu-tRNA gene are shown as capital letters. The boxed sequences on the acceptor and psi stem regions correspond to the 14 bp of region I on *attN1* and BT1-1 and the corresponding sequence in the *E. coli* Leu-tRNA4. Immediately 3′ of the ACCA-3′ end of the Leu-tRNA4 is the 10-bp sequence (GAAAGATAAG) that has a high degree of identity to the corresponding 10-bp sequences in region II of *attN1* (GAAAGCTAAG) and BT1-1 (GAAAAGCTAAG), but it is not within an inverted-repeat region. Sequence differences are indicated by underlined bases.

Tc^r ERL ($<10^{-9}$ transconjugants per recipient). A possible explanation for the failure of integrated pNVIN3 to excise was that the large segment of plasmid DNA inside the integrated NBU1 ends of pNVIN3 somehow interfered with excision, making the distance between the NBU1 ends too great for efficient excision. This was not the case, however, because in previous experiments we had shown that the NBU::plasmid chimeras Y11D and Y5D, which had integrated into the BT4004 chromosome, were capable of excision and transfer out of BT4004 (24). Both Y11D and Y5D carry a segment of non-NBU1 DNA as large as the plasmid DNA segment in pNVIN3. Thus, the failure of the integrated pNVIN3 to transfer out of BT4004 suggests that the portion of NBU1 cloned on pNVIN3 is unable to excise and transfer because it is missing one or more genes necessary for excision. This was confirmed by the finding that no circular intermediate of pNVIN3 could be detected on Southern blots of DNA from tetracycline-induced BT4004 hosts or by PCR amplification of the joined ends of NBU1 (data not shown). The excised circular forms of Y11D and Y5D were detectable under the same conditions, either by Southern blotting or by PCR amplification. Thus, although pNVIN3 contained all of the DNA necessary for integration, it lacked one or more genes necessary for excision. pNVIN3 contained about 2.5 kbp of DNA from the right end of the element, as well as 2 kbp of DNA from the left end. To localize the integration region further, unidirectional deletions were made from the *Pvu*II end of the NBU1 fragment in pNVIN3 and tested for the ability to integrate site specifically in *B. thetaiotaomicron* 4001. The smallest integration-proficient deletion clone was pIA$_{46}$. pIA$_{46}$ contained both region I and region II, plus 200 bp upstream of the inverted repeat of region II and about 2 kbp of DNA downstream from region I. The integration frequency for pIA$_{46}$ was the same as that for Y11D, which carried the entire NBU1 sequence (about 10^{-6} transconjugants per recipient), and insertion occurred only in the primary site, BT1-1 (data not shown).

NBU1 integrase is in the lambda integrase family. A segment of NBU1 which extended from the *Hin*cII site (61 bp upstream of the end of pIA$_{46}$) to the *Pst*I site at the other end of the DNA cloned in pIA$_{46}$ (bp 2446) was sequenced. In this sequence, the inverted-repeat region containing the 10-bp sequence of region II was located at bp 269 to 308 and the 14-bp sequence of region I was located at bp 314 to 327. There was only one complete open reading frame (*orf*) on this fragment. This *orf* was designated *intN1* (for integrase of NBU1). The first possible ATG start codon in *intN1* was at bp 533, and the termination codon was at bp 1866. *intN1* could encode a 445-amino-acid (53-kDa) protein. The *Hin*dIII site at bp 1802 was located in the C-terminal portion of the putative IntN1 protein. Deletions between this *Hin*dIII site and the *Pst*I site resulted in plasmids that were unable to integrate in *B. thetaiotaomicron* (24). Thus, IntN1 was clearly essential and sufficient for NBU1 integration but is not sufficient for excision, as seen for both Y11DP and pNVIN3.

The nucleotide sequence of the IntN1 gene did not show significant homology to any sequences in the database, but the amino acid sequence could be aligned with the C-terminal domains I and II of the lambda integrase family (2). The BESTFIT (7) amino acid alignment between IntN1 and lambda integrase in the C-terminal conserved domains, I and II, is shown in Fig. 4. IntN1 has the conserved arginine (R) of lambda integrase domain I and the conserved amino acids of lambda integrase domain II, which have been defined by Argos et al. (2) as essential for integrase function. In particular, IntN1 has the catalytic tyrosine residue (Y) that mediates the integration reaction. Integrases of many bacteriophages, insertional actinomycete plasmids, and gram-positive CTs have similar lambda integrase motifs (for example, see references 4–6,

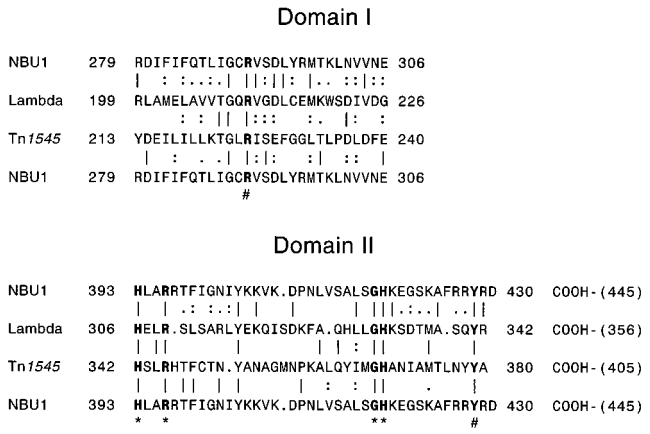

FIG. 4. Similarity of NBU1 integrase to lambda integrase. NBU1 integrase has low overall similarity to lambda and related integrases, but it had 26% identity and 48% similarity to lambda Int in the conserved C-terminal domains identified by Argos et al. (2). NBU1 integrase contained the conserved arginine (R) in domain I and the tyrosine (Y) in domain II, indicated by the # symbol. The other highly conserved amino acids shared by most of the lambda family integrases in domain II are in bold letters and are indicated by * symbols. The sequence of the Tn*1545* Int, which is nearly identical to Tn*916* Int (16, 29), is shown at the bottom for comparison with the gram-positive conjugative transposons. Identical amino acids are indicated by a | symbol, and amino acid similarities are indicated by a period or a colon.

11, 16, 17, and 29). The integrase of the gram-positive CT, Tn*1545*, is shown in Fig. 4 for comparison, and the additional amino acids shared by the Tn*1545* Int and IntN1, besides the conserved lambda sequences, are indicated. Although the Tn*1545* Int and the NBU1 IntN1 had some amino acid sequence similarity in this region, they were otherwise unrelated at the amino acid sequence level. Thus, NBU1 is clearly not closely related to the Tn*1545*-Tn*916* family of CTs, which were found initially in gram-positive bacteria but have now been found in many gram-negative bacteria as well. NBU1 may, however, prove to have a mechanism of integration similar to that of members of the Tn*1545*-Tn*916* family.

Genes essential for excision are located downstream of *intN1*. An unusual feature of the *Bacteroides* NBU1 *intN1* gene is that it is transcribed away from the nearest end of NBU1. All of the lambda family integrase genes described to date, and the *int* genes of Tn*916* and other gram-positive CTs, are transcribed toward the nearest end of the element that carries them. We do not yet know the significance of the direction of transcription of the *intN1* gene. The genes encoding excision proteins (Xis) of bacteriophages and gram-positive CTs are usually located immediately adjacent to the integrase gene. In the NBU1 sequence we report here, the *Pst*I site was in a second *orf*, *orf2*, which began 7 nucleotides from the end of *intN1*. This *orf* appeared to continue beyond the *Pst*I site. Preliminary searches for DNA or protein sequences similar to this segment of *orf2* identified no similar genes or proteins. Xis proteins of lambdoid phages and Tn*916* have no amino acid sequence similarity but are usually basic proteins (4, 5, 11, 16, 17, 29). The pI of the N-terminal fragment of Orf2 protein was acidic, around 4.8. The function of the *orf2* gene product is not known.

Some of our results suggest that the entire 3.5-kbp region downstream of *intN1* may be involved in excision. For example, Y11D, a form of NBU1 with an insertion about 3.5 kbp from the end of *intN1*, was defective in the ability to excise. The excision frequency of Y11D, detected by the mating-out assay, was down at least 100-fold in comparison with the estimated values for the wild type. Y11D was not completely deficient in excision, however, because a small number of transconjugants were detected in the assay and low levels of the circular form were detected on Southern blots. Y11DP, a derivative of Y11D which contained a deletion of the region between the Y11 insertion and a *Pst*I site close to the end of *IntN1*, was completely deficient in excision as measured by the mating-out assay, and no circular form could be detected on Southern blots. Further sequence analysis of the region, which is currently under way, suggests that there are at least three open reading frames in this region. The function of the open reading frames in the region of NBU1 downstream of *intN1* remains to be determined. Also, it is not yet clear whether there are still other genes on the NBU1, e.g., between the 3′ end of the *mob* region and the left end of the NBU1, which are required for excision.

Expression of *intN1* is constitutive. Integration of NBU1 in *B. thetaiotaomicron* appeared to be independent of functions provided by Tc^r ERL because pNVIN3 could integrate into a *B. thetaiotaomicron* strain that did not contain Tc^r ERL or any other *Bacteroides* CT. Nonetheless, we have shown previously that the regulatory protein RteB from Tc^r ERL is essential for excision and circularization of NBU1. This finding suggested that RteB was regulating expression of one or more excision-integration genes on NBU1. Since *int* genes of Tn*916*-Tn*1545* and lambdoid phages are essential for both excision and integration, it was possible that expression of the NBU *intN1* gene was regulated at some level by RteB.

To determine whether *intN1* was expressed constitutively or was regulated by RteB, we constructed two types of transcriptional GUS fusions to *intN1*. In one type, a segment from the circular form of *attN1* was fused to GUS on a plasmid that replicates in *B. thetaiotaomicron* (pMJF1:P*intN1*). This plasmid was introduced into BT4100N1-S1 (containing a single copy of NBU1), BT4104 (containing Tc^r ERL but no NBU1), and BT4104N1-3 (containing NBU1 and Tc^r ERL). In the second type of fusion, the GUS gene was inserted into the *intN1* gene of an integrated NBU1 to produce BT4100N1-S1 (ΩCQW1::*intN1*) or BT4104N1-3 (ΩCQW1::*intN1*). These two strains differed in that BT4100N1-S1 had no CT and BT4104N1-3 carried Tc^r ERL. The GUS specific activity in extracts from strains containing the multicopy plasmid pMJF2:P*intN1* was 50 to 60 U/mg of protein regardless of whether the strain contained Tc^r ERL and regardless of whether the strain containing Tc^r ERL was grown in medium containing low levels of tetracycline (Table 2). GUS expression from fusions to *intN1* in the integrated form of NBU1 (ΩCQW::*intN1*) was also constitutive and was about eightfold lower than in strains containing pMJF2:P*intN1*, as expected from the fact that the copy number of pMJF2 is approximately 10 per cell (8).

We also considered the possibility that proteins encoded on NBU1 itself might affect expression of *intN1*. In pMJF2:P*intN1*, none of the NBU1 genes was present on the plasmid. With the insertion into the *intN1* of the chromosomal copy of NBU1, *intN1* was disrupted but the insertion might have exerted a polar effect on downstream genes. To test the hypothesis that proteins encoded on NBU1 might affect expression of *intN1*, we introduced pMJF2:P*intN1* into a strain that contained an integrated copy of NBU1 as well as the CT Tc^r ERL. The GUS specific activity in extracts from this strain was the same as that in extracts from the other strains tested. Taken together, these results suggest that *intN1* is expressed constitutively and that neither the CT regulatory protein, RteB, nor proteins encoded by NBU1 have any effect on expression of the gene. The requirement for RteB in the NBU1 excision process

TABLE 2. GUS activities of NBU1 *intN1*::GUS transcriptional fusions in *Bacteroides* recipients

NBU1 *intN1*::GUS fusion	GUS activity (U/mg of protein) in *Bacteroides* recipient:				
	BT4100N1-S1	BT4104[a]		BT4104N1-3[a]	
		−TC	+TC	−TC	+TC
pMJF::P*intN1*	56	50	43	41	58
ΩCQW::*intN1*[b]	7	NA[c]	NA[c]	7	5
None	<0.2[d]		<0.2		<0.2

[a] Contains a conjugative transposon Tc[r] ERL. Cells were grown either with tetracycline induction of functions (+TC) or without induction (−TC).

[b] pCQW::*intN1* integrated into a resident integrated NBU1 element by homologous recombination placing the GUS gene under the control of the NBU1 *intN1* promoter, ΩCQW::*intN1*.

[c] NA, not applicable because strain does not contain an NBU1, required for recombination.

[d] Background GUS activity in *B. thetaiotaomicron* strains is <0.2 U/mg of protein.

must reflect either the interaction of RteB with the promoter of some other gene or a posttranscriptional effect of RteB.

The integrated NBU1 that had the GUS gene inserted into *intN1* was unable to excise. Results presented above suggested that *intN1* was not sufficient for excision. This result suggests that *intN1* is essential for excision or that the disruption in *intN1* is having a polar effect on an essential excision gene downstream of *intN1*. Work to identify the gene(s) necessary for excision of NBU1 is currently under way.

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