Characterization of a \textit{Bacteroides} Mobilizable Transposon, NBU2, Which Carries a Functional Lincomycin Resistance Gene

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The mobilizable \textit{Bacteroides} element NBU2 (11 kbp) was found originally in two \textit{Bacteroides} clinical isolates, \textit{Bacteroides fragilis} ERL and \textit{B. thetaitaomaicon} DOT. At first, NBU2 appeared to be very similar to another mobilizable \textit{Bacteroides} element, NBU1, in a 2.5-kbp internal region, but further examination of the full DNA sequence of NBU2 now reveals that the region of near identity between NBU1 and NBU2 is limited to this small region and that, outside this region, there is little sequence similarity between the two elements. The integrase gene of NBU2, intN2, was located at one end of the element. This gene was necessary and sufficient for the integration of NBU2. The integrase of NBU2 has the conserved amino acids (R-H-R-Y) in the C-terminal end that are found in members of the lambda family of site-specific integrases. This was also the only region in which the NBU1 and NBU2 integrases shared any similarity (28% amino acid sequence identity and 49% sequence similarity). Integration of NBU2 was site specific in \textit{Bacteroides} species. Integration occurred in two primary sites in \textit{B. thetaitaomaicon}. Both of these sites were located in the 3' end of a serine-rRNA gene NBU2 also integrated in \textit{Escherichia coli}, but integration was much less site specific than in \textit{B. thetaitaomaicon}.

Analysis of the sequence of NBU2 revealed two potential antibiotic resistance genes. The amino acid sequences of the putative proteins encoded by these genes had similarity to resistances found in gram-positive bacteria. Only one of these genes was expressed in \textit{B. thetaitaomaicon}, the homolog of \textit{linA}, a lincomycin resistance gene from \textit{Staphylococcus aureus}. To determine how widespread elements related to NBU1 and NBU2 are in \textit{Bacteroides} species, we screened 291 \textit{Bacteroides} strains. Elements with some sequence similarity to NBU2 and NBU1 were widespread in \textit{Bacteroides} strains, and the presence of \textit{linA}, in \textit{Bacteroides} strains was highly correlated with the presence of NBU2, suggesting that NBU2 has been responsible for the spread of this gene among \textit{Bacteroides} strains. Our results suggest that the NBU-related elements form a large and heterogeneous family, whose members have similar integration mechanisms but have different target sites and differ in whether they carry resistance genes.

\textit{Bacteroides} spp. are gram-negative obligate anaerobes that comprise 20 to 30% of the normal microbiota of the human colon. Some \textit{Bacteroides} species are opportunistic pathogens, and infections caused by them are becoming more difficult to treat successfully due to increasing antibiotic resistance in this genus. \textit{Bacteroides} spp. have been shown to carry a plethora of self-transmissible and mobilizable elements, which are probably responsible for the spread of antibiotic resistance genes. Antibiotic resistance genes have been found on conjugative and mobilizable plasmids (21, 34, 43), conjugative transposons (CTns) and integrated elements that are mobilized by CTns (33, 35, 37). In particular, a family of CTns, exemplified by CTnDOT and CTnERL, appears to be playing an important role in transferring resistance genes among \textit{Bacteroides} strains. CTns of this family not only transfer themselves but also mobilize coresident plasmids. In addition, proteins encoded on these CTns trigger trans the excision and circularization of mobilizable integrated elements called NBUs, and they mobilize these circular forms to \textit{Bacteroides} or \textit{Escherichia coli} recipients (35, 49, 57). They may also mobilize other integrated elements that have been given transposon designations, such as Tn4399 (14), Tn9555 (51), and Tn5520 (60). To distinguish mobilizable elements that have been given a transposon designation from nonmobilizable \textit{Bacteroides} transposons such as Tn4535 or Tn4551, we will designate them as MTns, e.g., Mtn5520.

So far, the MTns of \textit{Bacteroides} species seem to be falling into two distinct groups. Mtn5520 (60), the smallest of the MTns (5 kbp) and the only other MTn besides NBU1 (48) to be sequenced completely, integrates almost randomly and does not duplicate the target site. By contrast, the NBU1 integration was highly site specific, at least in \textit{Bacteroides} spp., and the target site was duplicated when NBU1 was inserted. We had noted that the integrase of NBU1, IntN1, was very different at the amino acid sequence level from the integrase of Mtn5520, although both were distantly related to the phage lambda integrase (46, 60). Since NBU2 seems to have the same general integration features of NBU1, we wanted to identify the integrase of NBU2 and determine whether it was more closely related to that of NBU1 than to that of Mtn5520.

Previously, we obtained the entire sequence of NBU1 (10.3 kbp) and identified its integrase gene (46). Subsequently, we have identified three other genes that appear to be essential for NBU1 excision (48). In earlier surveys of \textit{Bacteroides} clinical isolates for NBU1-like elements, we had identified a second NBU-type element that appeared at first to be very closely related to NBU1. A 2.5-kbp region of NBU2 that contained the transfer origin (oriT) and mobilization gene (mobN2) was sequenced and found to be >85% identical to a similarly sized segment from NBU1 (19, 20, 44). However, results of further hybridization experiments suggested that outside this region, NBU2 might be quite different from NBU1. We report here the complete DNA sequence of NBU2 and the characteristics of its integrase gene. NBU1 carried no antibiotic resistance.

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genes, but an MTn that seems to be related to NBU1, MTn5555, carries a cefoxitin resistance gene (28). Accordingly, we were interested in determining whether NBU2 carried any resistance genes.

Finally, we wanted to learn more about the distribution of NBU-type elements in Bacteroides strains. Virtually all work to date on CTns and MTns of Bacteroides species has focused on a small number of strains, most of which are clinical isolates. We have a collection of Bacteroides strains that includes a variety of clinical and community isolates, including strains of Bacteroides species has focused on the ability to integrate and R6K (10). Preparations of plasmid and total cellular DNA. Plasmid preparations from either Bacteroides or E. coli strains were done using the alkaline lysis procedure described previously (39). Total DNA was prepared by a modification of the method of Saito and Miura (32). A quick method for preparing total DNA from 2 ml of an overnight culture was used routinely. The cells were pelleted in a microfuge tube, washed either in 0.5 ml of saline-EDTA (0.5 M NaCl, 0.1 M EDTA; pH 8) or in 0.2 to 0.4 ml of TE (0.01 M Tris, 0.001 M EDTA; pH 8). The cells were resuspended in 0.5 ml of saline-EDTA and frozen in a –80°C freezer until the cells thawed and lysed completely. The lysate was mixed with 0.5 ml of phenol saturated with saline-EDTA, mixed well, and placed on ice for 20 min with occasional mixing. The mixture was centrifuged at 12,000 rpm in a microfuge for 10 min. The supernatant was removed and put into a fresh tube. Then, 0.8 ml of isopropanol was added, the tubes were inverted several times for complete mixing, and the DNA was allowed to precipitate at room temperature for at least 30 min. The tubes were centrifuged for 10 min at 12,000 rpm. The pellets were rinsed with 70% ethanol and then dried. The DNA was resuspended in 0.2 to 0.4 ml of TE (0.01 M Tris, 0.001 M EDTA; pH 8) containing 50 μg of RNase per ml.

Cloning and sequencing of NBU2. NBU2 was induced to excise from the chromosome of B. thetaiotaomicron BT4104N3-1 by growing the strain in medium containing tetracycline. Exposure to tetracycline induces the regulatory functions on the conjugative transposon required to induce the excision of NBU2 (55). The circular intermediate of NBU2 was isolated by using a plasmid preparation procedure described previously (44) and then digested with PstI and cloned into the PstI site of pBR328 (11). This clone was stable enough to allow subcloning for sequencing and for other analysis (Table 1). Various regions were subcloned into pUC19 (62) and sequenced using the Applied Biosystems model 373A version 2.0 dye terminator sequencing system.

Preparation of plasmid and total cellular DNA. Plasmid preparations from either Bacteroides or E. coli strains were done using the alkaline lysis procedure described previously (39). Total DNA was prepared by a modification of the method of Saito and Miura (32). A quick method for preparing total DNA from 2 ml of an overnight culture was used routinely. The cells were pelleted in a microfuge tube, washed one time in 0.5 ml of saline-EDTA (0.5 M NaCl, 0.1 M EDTA; pH 8). The cells were resuspended in 0.5 ml of saline-EDTA and frozen in a –80°C freezer until solid. Then, 0.5 ml of Tris-SDS (0.1 M Tris, 1% sodium dodecyl sulfate [SDS]; pH 9.0 to 9.3) was added to the frozen cells. The tubes were agitated constantly until the cells thawed and lysed completely. The lysate was mixed with 0.5 ml of phenol saturated with saline-EDTA, mixed well, and placed on ice for 20 min with occasional mixing. The mixture was centrifuged at 12,000 rpm in a microfuge for 10 min. The supernatant was removed and put into a fresh tube. Then, 0.8 ml of isopropanol was added, the tubes were inverted several times for complete mixing, and the DNA was allowed to precipitate at room temperature for at least 30 min. The tubes were centrifuged for 10 min at 12,000 rpm. The pellets were rinsed with 70% ethanol and then dried. The DNA was resuspended in 0.2 to 0.4 ml of TE (0.01 M Tris, 0.001 M EDTA; pH 8) containing 50 μg of RNase per ml.

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### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are shown in Table 1. The E. coli strains were grown aerobically in Luria broth (LB) or plated on Luria agar (LA) plates. The Bacteroides strains labeled BT (e.g., BT4001 and BT4004) are derivatives of the B. thetaiotaomicron 5482 strain (Virginia Polytechnical Institute [VPI] Anaerobe Laboratory, Blacksburg, VA). The source and time period of isolation of the Bacteroides strains used in the survey are described, along with the results of the NBU survey (see Table 4). The Bacteroides strains are grown anaerobically in preduced Trypticase-

<table>
<thead>
<tr>
<th>Bacterial strains or plasmid</th>
<th>Relevant phenotype*</th>
<th>Description and/or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α/MC, EM2ANR, BW19851</td>
<td>RecA</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>BT5482, BT4001, BT4004, BT4004N3, BT4004N6</td>
<td>Rif</td>
<td>Spontaneous rifampin resistant isolate of BT5482</td>
</tr>
<tr>
<td></td>
<td>Te</td>
<td>BT4001 containing the conjugative transposon, CTnERL</td>
</tr>
<tr>
<td></td>
<td>Ln</td>
<td>BT4004 with single copy of NBU2 in site 2</td>
</tr>
<tr>
<td></td>
<td>Tp</td>
<td>BT4004 containing two copies of NBU2, one in site 1 and one in site 2</td>
</tr>
</tbody>
</table>

### TABLE 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Relevant phenotype*</th>
<th>Description and/or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19</td>
<td>Ap'</td>
<td>E. coli cloning vector (62)</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Ap'</td>
<td>Promega PCR product cloning vector</td>
</tr>
<tr>
<td>pBR328::NBU2</td>
<td>Cm'</td>
<td>Circular intermediate of NBU2 digested with PstI and cloned into the PstI site of the ColEl replicon vector pBR328 (11), this study</td>
</tr>
<tr>
<td>pUC19::attN2</td>
<td>Ap'</td>
<td>1.2-kbp HincII of NBU2 containing the joined ends cloned into the HincII site of pUC19, this study</td>
</tr>
<tr>
<td>pUC19::LJ1</td>
<td>Ap'</td>
<td>A 5-kbp HindIII fragment containing the left junction of NBU2 in one of the chromosomal targets (site 1) in BT4004N6 cloned into the HindIII site of pUC19, this study</td>
</tr>
<tr>
<td>pEPE</td>
<td>Cm', R6KoriV</td>
<td>Cloning vector that requires pir product in trans for replication; contains the R4 oriT for mobilization (24)</td>
</tr>
<tr>
<td>pEPE::N2D</td>
<td>Cm', R6KoriV</td>
<td>1.8-kbp ermG PCR product from Ctn/753 (10) cloned into the NaiI site of pEPE1852 to create a suicide vector that is selectable in both Bacteroides and E. coli hosts, this study</td>
</tr>
<tr>
<td>pEPE::N2-J1 and pEPE::N2-J2</td>
<td>Cm'</td>
<td>1.8-kbp PCR product from NBU2 circular form containing the attN2 and intN2 region cloned into ApaI/XhoI site of pEPE (Fig. 1); integrates into the chromosomess of Bacteroides and E. coli hosts, this study</td>
</tr>
<tr>
<td>pEPE::N2D-1 and pEPE::N2D-2</td>
<td>Cm', R6KoriV</td>
<td>EcorV clone of both NBU2-chromosomal junctions from pEPEintN2 insertions in BT4001 chromosome in site 1 (11) and site 2 (12), this study</td>
</tr>
<tr>
<td>pNL1Y</td>
<td>Ap' Cm'</td>
<td>Bacteroides-E. coli shuttle vector (38)</td>
</tr>
<tr>
<td>pNL1Y-ML</td>
<td>Ap' Cm'</td>
<td>Bacteroides-E. coli shuttle vector (38)</td>
</tr>
</tbody>
</table>

* The phenotypes in parentheses are expressed Bacteroides hosts, and the phenotypes outside the parentheses are expressed E. coli. Abbreviations used for antibiotic resistances: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Ln, lincomycin; Nal, nalidixic acid; Rif, rifampin; Sm, streptomycin; Tp, trimethoprim. Other phenotype abbreviations: int’ or int- for the ability to integrate and R6KoriV for the pir-dependent replication origin of R6K. 

...
The resulting nucleotide sequences and the derived amino acid sequences of the potential open reading frames (ORFs) were used to search a variety of data bases for possible identification using Gapped BLAST and Psi-BLAST programs (1). The Southern blot and dot blot analysis. Southern blot analysis of restriction enzyme-digested DNA was performed as outlined in Sambrook et al. (39). The probes were made from isolated DNA fragments or purified PCR products labeled with fluorescein-dUTP using random primers according to the protocol in the Roche kit from Roche Diagnostics. The Southern blots were incubated with a chemiluminescence substrate for exposure to film as directed by the manufacturer. Dot blot analyses of bacterial strains were done by spotting 3 μl of a 2-mI (total DNA) preparation onto GeneScreen (NEN-Dupont) in a grid configuration. The spotted membranes were then treated as in colony or plaque hybridizations and were hybridized with NBU1- and NBU2-specific probes using the same protocol used for Southern blots. The NBU probes used for the dot blots were the 4.5-kbp HindIII fragment of NBU1 (C-terminal of IntegraseN1 to the N-terminal of MobN1), the PCR product of the prmN2, oriT-mobN2 (1.6-kbp Avai-PradI), intimN1-attN1 (2.4-kbp Pst-Scal), intimN2-attN2 (1.8-kbp PCR product), and a 1.3-kbp HindIII-EcoRV NBU2 fragment containing the C-terminal end of mefE,N2 and the N-terminal end of linK,N2. Primers used to produce specific PCR products to make the probes or for cloning are shown in Table 2.

Construction of minimal integration vector, pEPIntN2. A mobilizable vector which could be used to follow the integration of cloned regions of NBU2 in either Bacteroides or E. coli recipients was constructed using the pl-repressible Pir vector pEP185.2 (Fig. 1) (24). A 1.2-kbp gene from the conjugative transposon CTA7535 was PCR amplified (10) (Table 2) with PirI sites in the primers and then cloned into the PirI-compatible unique NruI site of pEP185.2 to produce pPE-E (Fig. 1). The sequences of the ends of the integrated form of NBU2 integrate using DNA from PirI-cut pEP185.2 into the E. coli chromosome. The potential open reading frames (ORFs) were used to search a variety of data bases for possible identification using Gapped BLAST and Psi-BLAST programs (1).

**NB2, A BACTEROIDES MOBILIZABLE TRANSPOSON**

<table>
<thead>
<tr>
<th>TABLE 2. Primers used for PCR amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequence</strong> (size [bp])</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td><strong>Joined ends of NBU2 (455)</strong></td>
</tr>
<tr>
<td><strong>Target site 1 in BT4001 (466)</strong></td>
</tr>
<tr>
<td>intN2-attN2 (1,882); minimal integrative region and probe (7,420-381)</td>
</tr>
<tr>
<td>ermG; erythromycin-clindamycin resistance marker for Bacteroides spp</td>
</tr>
<tr>
<td><strong>ermN1</strong> (974); NBU1 sequence (7,420-381)</td>
</tr>
<tr>
<td><strong>ermN2, AGT GGG A TA C</strong></td>
</tr>
<tr>
<td><strong>G</strong></td>
</tr>
<tr>
<td><strong>C</strong></td>
</tr>
</tbody>
</table>

*a The underlined sequences are restriction sites inserted in the primers: 1. PirI, 2. NruI, 3. BglII.

The construction of the minimal integration vector pEPIntN2 used DNA from two sources: a 2.5-kbp HindIII fragment of NBU2 (Fig. 1) (24) and a 0.6-kbp gene from the conjugative transposon CTA7535 was PCR amplified (10) (Table 2) with PirI sites in the primers and then cloned into the PirI-compatible unique NruI site of pEP185.2 to produce pPE-E. The 1.7-kbp fragment was first cloned into the pPE-E vector to form the plasmid TE18. The 2.5-kbp fragment was then cloned into the NruI site of pEP185.2 to produce TE21. The sequences of the ends of the integrated form of NBU2 integrate using DNA from PirI-cut pEP185.2 into the E. coli chromosome. The potential open reading frames (ORFs) were used to search a variety of data bases for possible identification using Gapped BLAST and Psi-BLAST programs (1). The Southern blot and dot blot analysis. Southern blot analysis of restriction enzyme-digested DNA was performed as outlined in Sambrook et al. (39). The probes were made from isolated DNA fragments or purified PCR products labeled with fluorescein-dUTP using random primers according to the protocol in the Roche kit from Roche Diagnostics. The Southern blots were incubated with a chemiluminescence substrate for exposure to film as directed by the manufacturer. Dot blot analyses of bacterial strains were done by spotting 3 μl of a 2-mI (total DNA) preparation onto GeneScreen (NEN-Dupont) in a grid configuration. The spotted membranes were then treated as in colony or plaque hybridizations and were hybridized with NBU1- and NBU2-specific probes using the same protocol used for Southern blots. The NBU probes used for the dot blots were the 4.5-kbp HindIII fragment of NBU1 (C-terminal of IntegraseN1 to the N-terminal of MobN1), the PCR product of the prmN2, oriT-mobN2 (1.6-kbp Avai-PradI), intimN1-attN1 (2.4-kbp Pst-Scal), intimN2-attN2 (1.8-kbp PCR product), and a 1.3-kbp HindIII-EcoRV NBU2 fragment containing the C-terminal end of mefE,N2 and the N-terminal end of linK,N2. Primers used to produce specific PCR products to make the probes or for cloning are shown in Table 2.

**Construction of minimal integration vector, pEPIntN2.** A mobilizable vector which could be used to follow the integration of cloned regions of NBU2 in either Bacteroides or E. coli recipients was constructed using the pl-repressible Pir vector pEP185.2 (Fig. 1) (24). A 1.2-kbp gene from the conjugative transposon CTA7535 was PCR amplified (10) (Table 2) with PirI sites in the primers and then cloned into the PirI-compatible unique NruI site of pEP185.2 to produce pPE-E. The 1.7-kbp fragment was first cloned into the pPE-E vector to form the plasmid TE18. The 2.5-kbp fragment was then cloned into the NruI site of pEP185.2 to produce TE21. The sequences of the ends of the integrated form of NBU2 integrate using DNA from PirI-cut pEP185.2 into the E. coli chromosome. The potential open reading frames (ORFs) were used to search a variety of data bases for possible identification using Gapped BLAST and Psi-BLAST programs (1).

**Sequence analysis of NBU2.** The 2.5-kbp mobilization region of NBU2, which has high sequence identity to NBU1, had been previously sequenced and characterized by Li et al. (20). This region had been cloned from the excised circular form of NBU2 using an NBU1-derived hybridization probe to detect it. To determine the sequence of the rest of NBU2, we first cloned the entire circular intermediate from plasmid preparations of a tetracycline-induced B. thetaiotaomicron strain that contained both the conjugative transposon CTnERL and NBU2. Sequence analysis of NBU2 revealed that the element was 11,123 bp in size, slightly larger than NBU1 (10,276 bp [48]). The sizes of the predicted ORFs and the sizes of the proteins they could encode are shown in Table 3. In Fig. 2A the location of the ORFs are shown on the circular intermediate form of NBU2. Genes on the integrated form of NBU2 are compared to genes on integrated NBU1 in Fig. 2B.
sons of these genes at the nucleic acid and amino acid sequence level showed clearly that, outside of the \( \text{prmN-oriT-mob} \) region, NBU2 differed appreciably from NBU1. Like NBU1, most of the NBU2 genes were transcribed in the same direction, but there was little sequence similarity to the other NBU1 genes. Examination of the sequences at the edges of the highly conserved internal \( \text{prmN-oriT-mob} \) region revealed that the transition from nearly identical sequences to very dissimilar

![FIG. 1. Construction of the integration vector, pEPIntN2. An insertional vector that could be used in either \textit{Bacteroides} or \textit{E. coli} was constructed from the \textit{pir}-dependent mobilizable pEP185.2 (24). The erythromycin-clindamycin resistance gene, \textit{ermG}, from CTn7853 was PCR amplified with \textit{Pst} sites encoded in the primers and cloned into the \textit{Pst}-compatible unique \textit{Nsi} site on pEP185.2 to form pEPE. pEPE, a Pir-dependent vector, has selectable markers for both \textit{E. coli} and \textit{Bacteroides} hosts. The NBU2 integration region, IntN2, consisting of the joined ends, \textit{attN2}, from the circular form of NBU2 and the adjacent gene, \textit{intN2}, was PCR amplified from the induced circular form of NBU2. The PCR product was first cloned into pGEM-T (Promega) and sequenced. The 1.8-kbp \textit{ApaI-SstII} fragment was isolated from pGEM-T and cloned into the \textit{ApaI} and \textit{SstII} sites of pEPE to form the NBU2 integration vector, pEPIntN2.]

<table>
<thead>
<tr>
<th>ORF</th>
<th>Range 5'-3' (bp)</th>
<th>% G+C</th>
<th>Length (aa)</th>
<th>Size (kDa)</th>
<th>pI</th>
<th>Search results and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>linAN2</td>
<td>769–237</td>
<td>40.5</td>
<td>171</td>
<td>19.8</td>
<td>4.5</td>
<td>52% identity and 70–72% similarity to LinA' from \textit{S. aureus} BM4611 and LinA on pIP855 in \textit{S. haemolyticus} (5, 6)</td>
</tr>
<tr>
<td>mefEN2</td>
<td>2,000–804</td>
<td>41.3</td>
<td>403</td>
<td>44.3</td>
<td>9.1</td>
<td>33–34% identity and 52–54% similarity to MefE of \textit{S. pneumoniae} and MefA of \textit{S. pyogenes} (8, 58)</td>
</tr>
<tr>
<td>attN2</td>
<td>2,000–2,400</td>
<td>41.3</td>
<td>403</td>
<td>44.3</td>
<td>9.1</td>
<td>Two target sites: 13-bp identity to \textit{attN2} at the 3' end of two Ser-tRNA(_{UGA}) genes, followed by inverted repeats</td>
</tr>
<tr>
<td>intN2</td>
<td>2,499–3,782</td>
<td>45.8</td>
<td>427</td>
<td>50.4</td>
<td>8.9</td>
<td>26–28% identity and 44–49% similarity to the C-terminal ends of MTn5520 Int(_{\text{MTn}}) and NBU1 IntN1 (46, 60); member of the lambda family of site-specific integrases</td>
</tr>
<tr>
<td>orf2</td>
<td>3,846–6,194</td>
<td>42.9</td>
<td>783</td>
<td>91.3</td>
<td>5.4</td>
<td>26% identity and 45% similarity to an \textit{E. coli} hypothetical protein</td>
</tr>
<tr>
<td>orf3</td>
<td>6,736–7,815</td>
<td>46.3</td>
<td>360</td>
<td>41.5</td>
<td>8.6</td>
<td>37% identity and 52% similarity to sigma 70 of \textit{Rhizobium} sp.</td>
</tr>
<tr>
<td>prmN2</td>
<td>8,028–8,984</td>
<td>48.1</td>
<td>319</td>
<td>36.8</td>
<td>9.1</td>
<td>&gt;85% sequence identity to \textit{prmN1} of NBU1 and sequence on MTn4399 (20); 30–33% identity and 52–57% identity to the N-terminal ends of DNA primases from \textit{L. monocytogenes} and \textit{B. subtilis dnaE}.</td>
</tr>
<tr>
<td>orT(_{N2})</td>
<td>880–910</td>
<td>44.0</td>
<td>469</td>
<td>55.1</td>
<td>9.3</td>
<td>Gram-positive family of nick sites also shared by NBU1, MTn455, pBI143 and pIP421 (53)</td>
</tr>
<tr>
<td>mobbN2</td>
<td>9,137–10,543</td>
<td>44.0</td>
<td>469</td>
<td>55.1</td>
<td>9.3</td>
<td>88% nucleotide identity to NBU1 mobN1 and 84% nucleotide identity to MTn555 mobA(<em>{\text{MTn}}); 44, 48, and 55% aa sequence similarity to pIP421 Mob(</em>{\text{E}}), pBI143 MobA(<em>{\text{BI}}), and MTn5520 Mob(</em>{\text{MTn}}), respectively (20, 52, 60)</td>
</tr>
</tbody>
</table>

\(^a\) aa, amino acids.
sequences was not an abrupt one, as might be expected if these
genes are on a gene cassette (Fig. 3).

There were some inverted repeat sequences that flanked
prmN on one side and mob on the other, but these were outside
the region of high identity. The fact that the inverted repeat
sequences seen on NBU1 were in approximately the same sites
relative to the region of identity as those on NBU2 could mean
that they play a role, either in the assembly of the NBUs or in
the current function of the intact element. However, there was
no indication that the region of identity was a gene cassette in
an integron or some other mobile gene cassette. MTn4555 has
pairs of inverted repeats flanking its oriT-mobATN region, a
region that exhibits sequence identity to the corresponding
region of NBU1 and NBU2, and the mobilizable Bacteroides
plasmid, pBI143, has 56-bp inverted repeats that separate its
NBU-related mobilization region and its replication region
(52, 54). Thus, the inverted repeats may prove to have some
significance in the future, but their role is not evident from
work done to date. Comparisons of the known Bacteroides
mobilizable transposons, including the NBUs, and mobilizable
Bacteroides plasmids suggest that these are modular elements
with mix-and-match components, but it is not clear how this
modular assembly was achieved.

Localization of the integration region (attN2) on NBU2.
DNA from strains of B. thetaiotaomicron that contained inser-
tions of NBU2 (V1NBU2) were probed on Southern blots and
the ends of the NBU2 were determined to be within the 1.2-
kbp HincII fragment indicated in Fig. 2 that is also called

FIG. 2. Circular and integrated forms of NBU2. (A) Partial restriction map of the excised circular form of NBU2. The location and orientation of the possible ORFs
derived from the NBU2 sequence are indicated. The region containing the joined ends of the NBU2 (attN2) is indicated. The attN2 is contained within a 1.2-kbp HincII
fragment. (B) The integrated form of NBU2 is compared to the integrated form of NBU1. The double-headed arrows indicate the region of high sequence identity
between NBU2 and NBU1 (see Fig. 3). The attN-left sequences of the integrated NBUs are the same sequence as the attN on the elements, and the attN-right se-
quences are the attBT sequences of the target sites. Both elements integrate site specifically into the 3' end of tRNA genes: Ser-tRNA<sub>UGA</sub> for NBU2 and Leu-tRNA<sub>CAA</sub>
for NBU1.
attN2. The Southern blots and previous pulse field analysis of 
*B. thetaiotaomicron* strains revealed that NBU2 had two pri-
mary target sites (3), and some of our isolates contained copies 
of VNBU2 in both sites (e.g., Fig. 4A). In Fig. 4A, the chro-
mosomal DNAs from BT4004N3 (lane 1), BT4004N6 (lane 2),
and BT4004N6 grown in tetracycline to induce the excision of 
NBU2 (lane 3) were digested with *Hinc*II. The Southern blot 
of the agarose gel was probed with the 1.2-kbp *Hinc*II NBU2 
fragment (Fig. 2). BT4004N3 has one ΩNBU2 (two junction 
bands) and BT4004N6 had two copies of ΩNBU2 in two dif-
f erent sites (four junction bands). One of the ΩNBU2 inser-
tions in BT4004N6 is the same as the ΩNBU2 in BT4004N3, as 
 is evident from the fact that two junction bands of the same 
size appear in both lanes 1 and 2. The arrow in lane 3 indicates 
an additional 1.2-kbp *Hinc*II band that runs just above one of 
the junction bands. This band is formed when tetracycline 
stimulation of the coresident CTnERL leads to excision of 
NBU2. No 1.2-kbp band is observed if the cells are not grown

FIG. 3. Sequence comparison of NBU2 and NBU1. The *prmN-oriT-mobN* regions of NBU1 and NBU2 have more than 85% identity. Outside of this mobilization 
region the sequence identity drops to <30%. Near the borders of the sequence identity both elements have inverted repeat sequences (underlined) of 11 to 12 bp 
indicated by arrows above the sequences for NBU1 (IR1) and dotted arrows below the sequences for NBU2 (IR2). The start and stop codons for the *prmN* (PrmN) 
and *mobN* (MobN) are indicated in boldface. The *oriT* nick sites as determined by sequence identity to MTn555 (53) are at the end of the TAG codon (Ω Stop) of 
the *prmN* genes and are indicated by the arrows. 

![](https://example.com/image.png)
in medium containing tetracycline (lanes 1 and 2). To determine the exact region on NBU2 where the crossovers occurred, a 5-kbp HindIII fragment containing one end of NBU2 in BT4004N6 was cloned and sequenced and compared to the sequence of the 1.2-kbp HindII fragment. The HindIII fragment was later determined to be the left junction of NBU2, as shown in Fig. 2B, and the chromosome from site 1. The single insertion of NBU2 in BT4004N3 (Fig. 4A, lane 1) was later shown to be in site 2.

The ORF immediately adjacent to the right end junction of the integrated NBU2 encoded a protein with 28% identity and 44 to 49% similarity to the C-terminal ends of the NBU1 integrase (IntN1) and the integrase of MTn5520 (IntM). All three of the Bacteroides MTn integrases have some sequence similarity to the lambda family of site-specific integrases. This similarity is confined to the C-terminal end of the proteins. C-terminal alignments of IntN2 to some of the closest sequences identified in BLASTP (1) searches are shown in Fig. 5. The three MTn integrases were 40 to 50 amino acids larger than other integrases, including the integrases of bacteriophage P21 and the E14 prophage of E. coli. The MTn integrases all contained the conserved amino acids in lambdoid integrase-transposase and with the first 200 amino acids for IntN1. This indicates that the N-terminal ends encoded the domains responsible for the element specific functions of the integrases. It was somewhat surprising that IntN2 had more sequence similarity in its N-terminal end to IntM than to IntN1, since NBU1 and NBU2 integrate site specifically, whereas MTn5520 inserts more randomly in AT-rich sites (3, 46, 60).

A simple unrooted tree depicting the relationship of the integrases included in Fig. 5 is shown in Fig. 6. The entire sequences of the integrases were used to form the tree and not just the conserved regions in the C-terminal ends. The three MTn integrases clustered with IntN2 closer to MTn5520 Int than to IntN1. From this, it is evident that there is quite a range of integrase sequences in Bacteroides MTns, including NBUs. Our earlier picture, based on traits of the MTns, which had the elements falling into two groups represented by NBU1 and MTn5520, is clearly too simplistic. The integrase-resolvase-like genes identified by sequence on the cyanobacterial plasmid, pDU1, and the plasmid, ece1, from the marine hyperthermophile Aquiflex aeolicus have not been characterized but were included because they were two of the closer relatives identified in the database searches. The similarity between these genes and proven integrases from NBU1 and NBU2 provides further evidence that these genes might well encode integrases. The integrases of pDU1 and ece1 clustered with the integrase from the site-specific Ctn5276 found in the gram-positive L. lactis (30). The bacteriophage integrases from the proteobacterial group of gram-negative bacteria formed the third cluster. The Bacteroides MTns are adding new branches to the lambda family of site-specific integrase tree. Note, however, that the branch lengths that separate the integrases of the NBUs and MTn5520 are, if anything, longer than those separating the integrases of the Ctn5276 group, which come from three different phylogenetic groups. This observation underscores the extent of the diversity within the NBU-MTN5520 group of integrases found in a single genus of gram-negative bacteria.
Use of a special vector to study integration and the targets sites of NBU2. An insertional shuttle vector, pEPE, was constructed to locate the integration gene of NBU2 and to clone chromosomal target sites (Fig. 1). This vector can be mobilized out of *E. coli* BW19851 to *Bacteroides* or *E. coli* recipients. In *E. coli* strains that contain the R6K *pir* gene the vector replicates but in *Bacteroides* recipients and *E. coli* recipients such as EM24NR, pEPE cannot replicate and transconjugants are only obtained if the vector contains regions that allow it to integrate (24, 47). Since we suspected that the first ORF at the right end of NBU2 was the integrase gene, we PCR amplified this gene together with the joined ends of the circular form and cloned it into pEPE to form pEPIntN2 (Fig. 1 and 2A).

In matings between BW19851(pEPIntN2) and BT4001, erythromycin-resistant transconjugants occurred at a frequency of $10^{-2}$ to $10^{-5}$ per recipient at the end of the mating. All of the transconjugants were contained insertions of pEPIntN2 (Fig. 1B). Since we suspected that the first ORF at the right end of NBU2 was the integrase gene, we PCR amplified this gene together with the joined ends of the circular form and cloned it into pEPE to form pEPIntN2 (Fig. 1 and 2A).

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strain not carrying a CTn as it did in one carrying a CTn. Thus, although NBU2 excision and mobilization require trans-acting CTn functions, integration is independent of CTn functions. To determine whether the NBU1 integrase might be able to act on the joined ends of NBU2, we transferred pEPIntN2D, which contained the joined ends but not the integrase of NBU2, into a strain that contained a copy of NBU1. No transconjugants were obtained, indicating that the NBU1 integrase could not replace NBU2 integrase in trans.

Junction regions of VpEPIntN2 integrated in both of the two target sites were cloned and sequenced. Analysis of the sequence of the junctions showed that integration had occurred via the ends of NBU2. Using this sequence information, we were also able to PCR amplify and sequence the integration site. The sequences of the two NBU2 integration sites are shown Fig. 7. There was a 13-bp sequence of identity between the attN2 formed by the joined ends of NBU2 and the two BT4001 target sites, attBT2-1 and attBT2-2. The integration event occurred within or adjacent to this 13-bp sequence, duplicating the 13-bp target site. Immediately downstream of the 13-bp region there were inverted repeats that contained a second region of partial identity between attN2 and the two attBT2 sites. In both attBT2 sites the 13-bp sequence was at the 3' end of a Ser-tRNA gene, Ser-tRNA<sub>UGA</sub>, and Ser-tRNA<sub>UGA</sub>. There was only one mismatch between the sequences of the two Ser-tRNA genes. Yet the regions outside the tRNA gene differed considerably.

The ability of NBU2 to integrate into the E. coli chromosome was tested by mating BW19851(pEPIntN2) with the non-permissive E. coli recipient, EM24NR. EM24NR is RecA deficient and lacks pir which is required for replication of the vector. Transconjugants were isolated at frequencies of 10<sup>26</sup> to 10<sup>27</sup> per recipient. Since transfer of pEPIntN2 to permissive hosts was 10<sup>21</sup> (24, 47), the efficiency of integration was lower (10<sup>25</sup>) than what was observed in the Bacteroides recipient, BT4001 (10<sup>21</sup>). The integration of pEPIntN2 in EM24NR was RecA independent, which was expected since the integration of NBU1 was previously shown to be RecA independent in both Bacteroides and E. coli recipients (9). A Southern blot analysis of EM24NR VpEPIntN2 transconjugants showed that the insertions E. coli were not site specific (data not shown). The sequence for one of the pEPintN2 insertion sites in E. coli (attBeC) is shown at the bottom. The insertion occurred in the 3' end of fecI. The crossover occurred adjacent to or within the triplet (CCT) at the beginning of a sequence with partial identity (8 of 13) to the 13-bp region on attN2. The crossover region for attBeC was followed by a set of IRs indicated by arrows which had no sequence identity to the NBU2 IR region.

![Crossover occurs within or adjacent to 13bp of identity](image-url)
vealed two ORFs whose derived amino acid sequences were related to those of known antibiotic resistance genes previously found in the gram-positive bacteria. The deduced amino acid sequence of \( \text{mef}_E \) had 34% identity and 54% similarity to \( \text{MefE} \) of \( \text{Staphylococcus aureus} \) (58), a protein that is thought to be a macrolide pump. Although the sequence identity was low, it extended throughout the protein. This is the first thought to be a macrolide pump. Although the sequence identity of \( \text{mef}_E \) and \( \text{linA} \) was low, it extended throughout the protein. This is the first time gene seen in \( \text{LinA} \) (5, 6) using the BLASTP search program. \( \text{LinA}(N2) \) has 52% identity and 70 to 72% identity to \( \text{LinA} \) from \( \text{S. aureus} \) and \( \text{LinA} \) from \( \text{pIP855} \) in \( \text{S. haemolyticus} \) (5.6) using the BLASTP search program. \( \text{LinA}(N2) \) has 52% identity and 70 to 72% identity to \( \text{LinA} \) and \( \text{LinA} \). The regions of identity are indicated in boldface. The total number of amino acids for each protein is indicated at the ends of the respective sequences. The accession numbers for \( \text{LinA} \) and \( \text{LinA} \) are X03947 and X25633, respectively.

Since the \( \text{mef}_E \) gene appeared to be nonfunctional in single copy, we cloned the 3.7-kbp \( \text{HindIII} \) fragment of \( \text{NBU2} \) that included \( \text{mef}_E \)-\( \text{linA}_2 \) into the shuttle vector \( \text{pNYL1} \) to provide 10 to 20 copies of the gene per cell. There was still no growth in erythromycin (3 \( \mu \)g/ml). Thus, the \( \text{mef}_E \) appears not to be capable of conferring macrolide resistance on \( \text{B. thetaiotaomicron} \) even if multiple copies are provided. We also checked for tetracycline resistance in BT4001 (pNYL-ML) since there was some similarity between \( \text{MeiEN2} \) and tetracycline resistance eflux proteins such as TetL (22, 29), but the strain remained susceptible (MIC, <1 \( \mu \)g/ml). The percent G+C content of \( \text{mefE} \) in \( \text{S. pneumoniae} \) is 38% and of \( \text{linA} \) in \( \text{S. aureus} \) is 31%, whereas the genes carried on \( \text{NBU2} \) had a G+C content of 41%, which is within the normal range of 40 to 45% G+C for \( \text{Bacteroides} \) spp. (16). Thus, the \( \text{linA}_2 \) and \( \text{mef}_E \) genes on \( \text{NBU2} \) probably did not come into the \( \text{Bacteroides} \) spp. from the low-GC gram-positive bacteria. Our results show that the \( \text{mefE} \) and \( \text{linA} \) type genes are not exclusively gram-positive resistances, as was once thought, but have a much wider distribution. \( \text{NBU2} \) is only the second \( \text{MTn} \) found that carries a functional antibiotic resistance gene. \( \text{MTn4555} \) carries a cefoxitin resistance gene (\( \text{cfxA} \) [28]), and there undoubtedly are other uncharacterized \( \text{MTn} \) elements that are carrying antibiotic resistance genes in the \( \text{Bacteroides} \) spp.
The integrase of NBU2, intN2, has been identified. This gene and the joined ends of NBU2 were all that were required for integration. The NBU2 integrase, like that of the related mobilizable element, NBU1, appears to be a member of the family of phage lambda recombinases, in the sense that it has the conserved C-terminal catalytic amino acids that are preserved on all members of the lambda Int family (27). The finding of lambda-type integrases in the NBUs and other nonphage integrating elements suggests that this mode of site-specific integration is more general than was previously realized. NBU2 and NBU1 both have some other phage-like traits. For one thing, they integrate site specifically via a 13-bp att sequence that is identical to the att site in the NBU2 joined ends. For another, they integrate into the 3' ends of tRNA genes. The number of integrating elements that share one or more of these properties is growing and now includes not only bacteriophages and the NBUs but also integrative Streptomyces plasmids, some pathogenicity islands, and integrative elements in Dichelobacter nodosus (4, 7, 31, 40, 61). The use of tRNA genes as targets for integration may increase the host range of an integrating element because tRNA genes are fairly highly conserved in different species. On the other hand, the use of tRNA genes as target sites could be considered as limiting their movement since such integration sites are unlikely to be found on plasmids or other self-transmissible elements. This limitation, however, does not seem to have prevented the NBUs from spreading extensively among different Bacteroides species in the human colon.

Transmissible elements that use tRNA genes as an integration site could be hazardous to a bacterial recipient if the structure of the tRNA gene was disrupted. In the case of NBU2, the crossover event exchanges the IR at the 3' end of the tRNA with the IR found in the attN site. This change seems not to be deleterious for the recipients, but such a change could possibly influence the processing of the tRNA transcript (61). The importance of the IRs in the attN or the attBT sites for either the integration or the excision of the NBUs is not yet known.

At present, there is no information available about Bacteroides host factors that might be involved in NBU integration or excision. Previous work has shown that NBU integration is independent of RecA (10), but whether integration requires a host factor-like IHF remains to be determined. Integration of NBU2 did not require any CTn functions, in contrast to the excision of NBUs, which requires functions provided by a CTn.

**DISCUSSION**

The integrase of NBU2, intN2, has been identified. This gene and the joined ends of NBU2 were all that were required for integration. The NBU2 integrase, like that of the related mobilizable element, NBU1, appears to be a member of the family of phage lambda recombinases, in the sense that it has the conserved C-terminal catalytic amino acids that are preserved on all members of the lambda Int family (27). The finding of lambda-type integrases in the NBUs and other nonphage integrating elements suggests that this mode of site-specific integration is more general than was previously realized. NBU2 and NBU1 both have some other phage-like traits. For one thing, they integrate site specifically via a 13-bp att sequence that is identical to the att site in the NBU2 joined ends. For another, they integrate into the 3' ends of tRNA genes. The number of integrating elements that share one or more of these properties is growing and now includes not only bacteriophages and the NBUs but also integrative Streptomyces plasmids, some pathogenicity islands, and integrative elements in Dichelobacter nodosus (4, 7, 31, 40, 61). The use of tRNA genes as targets for integration may increase the host range of an integrating element because tRNA genes are fairly highly conserved in different species. On the other hand, the use of tRNA genes as target sites could be considered as limiting their movement since such integration sites are unlikely to be found on plasmids or other self-transmissible elements. This limitation, however, does not seem to have prevented the NBUs from spreading extensively among different Bacteroides species in the human colon.

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In *E. coli*, integration was much more random than in *B. thetaiotaomicron*. This could be due to lack or a suitable primary integration site, because the ser-tRNA gene of *E. coli* is not identical to that of *B. thetaiotaomicron*. It is also possible, however, that the relative lack of NBU2 integration specificity in *E. coli*, together with the much lower frequency of integration, reflects the absence in *E. coli* of a host factor that aids accurate alignment of the att sequences and efficient formation of the protein-DNA contacts in *Bacteroides* species. Excision of NBU is proving to be a much more complex process than integration. The integrase is required, along with at least three other proteins and the oriT region (48). Moreover, excision requires trans action of CTn regulatory proteins, RteA and RteB. Thus, although the integration process of NBUs may resemble that of lambdoid phages, the excision process seems much different.

Integrated elements carrying genes that cross-hybridize with genes on NBU1 and NBU2 are very widespread in *Bacteroides* species. The fact that their incidence seems to be increasing is a good indication of the efficiency of their transfer and their stability once acquired. Our results suggest that this group of integrated elements is likely to be a very heterogeneous group. Not only were NBU1 and NBU2 quite different outside the *prmN-oriT-mob* region, but the integrases of NBU1, NBU2, and a related element, MTh5520, had substantially different amino acid sequences (Fig. 6). The differences between the integrases is evident at the functional as well as the amino acid sequence level because the integrase of NBU1 did not substitute for the integrase of NBU2 in an integration assay. Why the *prmN-oriT-mob* region of NBU1 and NBU2 is so highly conserved at the sequence level, whereas the remainder of the elements are so different, has yet to be determined. This region could be on a cassette but, if so, it has been in the NBUs long enough for its edges to be obscured by mutation. Another possible explanation is that this region of NBU1 is very important for excision (48). Yet, so are the integrase and genes downstream of the integrase, which are quite different on NBU1 and NBU2. Still another possible explanation for the conservation of the *prmN-oriT-mob* region is that one or more proteins or DNA segments in this region interact with functions supplied by the CTns. This is most likely in the case of the Mob protein, which must interact with transfer functions on encoded on the CTn, which mediate the transfer of the NBU circular form. Both NBU1 and NBU2 also interact in some with the CTn regulatory proteins that trigger excision (RteA, RteB), so the conserved region might also be involved in that interaction. In our survey of *Bacteroides* isolates, we noted a few isolates that hybridized with *prmN* but not with *mob*. It will be interesting to determine whether the NBU-like elements in such isolates are capable of excision and transfer.

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