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Excision, Transfer, and Integration of NBU1, a Mobilizable Site-Selective Insertion Element

NADJA B. SHOEMAKER,* GUI-RONG WANG, ANN M. STEVENS,† AND ABIGAIL A. SALYERS

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

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The *Bacteroides* species harbor a family of conjugative transposons called tetracycline resistance elements (Tc^r elements) that transfer themselves from the chromosome of a donor to the chromosome of a recipient, mobilize coresident plasmids, and also mediate the excision and circularization of members of a family of 10- to 12-kbp insertion elements which share a small region of DNA homology and are called NBUs (for nonreplicating *Bacteroides* units). The NBUs are sometimes cotransferred with Tc^r elements, and it was postulated previously that the excised circular forms of the NBUs were plasmidlike forms and were transferred like plasmids and then integrated into the recipient chromosome. We used chimeric plasmids containing one of the NBUs, NBU1, and a *Bacteroides-Escherichia coli* shuttle vector to show that this hypothesis is probably correct. NBU1 contained a region that allowed mobilization by both the Tc^r elements and IncP plasmids, and we used these conjugal elements to allow us to estimate the frequencies of excision, mobilization, and integration of NBU1 in *Bacteroides* hosts to be approximately 10^{-2}, 10^{-5} to 10^{-4}, and 10^{-2}, respectively. Although functions on the Tc^r elements were required for the excision-circularization and mobilization of NBU1, no Tc^r element functions were required for integration into the recipient chromosome. Analysis of the DNA sequences at the integration region of the circular form of NBU1, the primary insertion site in the *Bacteroides thetaiotaomicron* 5482 chromosome, and the resultant NBU1-chromosome junctions showed that NBU1 appeared to integrate into the primary insertion site by recombining within an identical 14-bp sequence present on both NBU1 and the target, thus leaving a copy of the 14-bp sequence at both junctions. The apparent integration mechanism and the target selection of NBU1 were different from those of both XBU4422, the only member of the conjugal Tc^r elements for which these sequences are known, and Tn*4399*, a mobilizable *Bacteroides* transposon. The NBUs appear to be a distinct type of mobilizable insertion element.

Large (>70-kbp) conjugative transposons have been found in many clinical isolates of *Bacteroides* species from the human colon. These conjugative transposons have been called Tc^r elements because most of them carry a tetracycline resistance (Tc^r) gene, *tetQ*, but some members of this family of conjugative transposons are cryptic (Tc^S), e.g., XBU4422 (13). NBUs were originally detected as 10- to 12-kbp covalently closed circles (plasmidlike forms) in a *Bacteroides uniformis* 0061 derivative (BU1004) which carried a Tc^r element. The circular forms of the NBUs were seen only when the strain was grown in medium containing tetracycline. Subsequently, we found that the NBUs are normally integrated in the chromosome and that *trans* action by a Tc^r element is required to excise and circularize them (12, 15).

The circular forms of the NBUs appeared not to replicate and were designated nonreplicating *Bacteroides* units to distinguish them from plasmids. Two lines of evidence supported the hypothesis that the NBU circle forms do not replicate. First, the copy number of the NBUs was estimated to be less than one per cell. Second, tetracycline induction of functions on a coresident Tc^r element was needed to produce the circle forms, and the circle forms disappeared when tetracycline was removed (14, 15). However, it is still possible that the apparent low copy number was due to an artifact of the procedure used to isolate the DNA (12, 15). It is also possible that the disappearance of the circle forms after the removal of tetracycline reflected tetracycline regulation of NBU replication functions. In this paper, we present evidence that one of the NBUs, NBU1, does not replicate in *Bacteroides* hosts.

Three NBUs have been partially characterized to date: NBU1, NBU2, and NBU3. Previously, we reported that NBU2 and NBU3 sometimes cotransfer with a Tc^r element to *Bacteroides* recipients. In the recipient the NBU and the Tc^r element integrate site selectively and into separate *Not*I fragments of the chromosome (1). Since there are no selectable markers on these NBUs, the cotransfer events were scored by probing colony blots of recipients that had received a Tc^r element with an NBU probe, taking advantage of an early observation that although the NBUs are not identical, they share a region of homology (1, 12). We did not, however, determine whether NBUs could transfer and integrate independently of a Tc^r element. Also, we could not distinguish between *trans* mobilization of the NBUs, presumably the circular forms, by the Tc^r elements and NBU transfer mediated by a cointegrate formed between an NBU and a Tc^r element followed by resolution of the cointegrate in the recipient. In this paper and an accompanying paper (6), we present evidence that the NBU circle forms are mobilized in *trans* in *Bacteroides* hosts by the Tc^r elements and in *Escherichia coli* hosts by the IncP plasmids, similar to the case of mobilizable plasmids. Additionally, we show that the integration of the NBUs in the recipient, unlike excision and mobilization, does not require *trans* action of a Tc^r element.

The excision and transfer of the NBUs are regulated by tetracycline-inducible functions on the conjugal Tc^r elements, which leaves the question of whether the NBUs are small versions of the Tc^r elements or a completely different type of element. In this paper we show that the DNA sequences of the integration region on NBU1, the primary NBU1 insertion site

* Corresponding author.
† Present address: Department of Microbiology, University of Iowa, Iowa City, IA 52242.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid[a]	Relevant phenotype[b]	Reference, description, and/or source
E. coli		
HB101	RecA Str^r	2, 8
EM24NR	RecA Str^r Nal^r Rif^r	16
DH5αMCR	RecA	BRL-GIBCO
B. uniformis 0061		VPI, species type strain; contains XBU4422 and NBU1 (2, 13)
BU1001	Rif^r	Rif^r mutant of 0061
BU1100	Thy^- Tpm^r	Thy^- mutant of 0061
BU1004	Rif^r Tc^r	BU1001 Tc^r ERL, NBU2 transconjugant from BF-ERL Thy (9, 12)
BU1006	Rif^r Tc^r Em^r	BU1001 Tc^r Em^r DOT transconjugant from BT-DOT Thy (12)
B. thetaiotaomicron 5482		
BT4001	Rif^r	Rif^r mutant of 5482
BT4100	Thy^- Tpm^r	Thy^- mutant of 5482
BT4104	Thy^- Tpm^r Tc^r	BT4100 Tc^r ERL transconjugant from BU1004 (this study)
BT4007N2	Rif^r Tc^r Em^r	BT4001 Tc^r Em^r DOT, NBU2 cotransconjugant from BT-DOT Thy; this paper
Bacteroides clinical isolates		
BF-ERL Thy	Thy^- Tpm^r Tc^r Em^r	Thy^- mutant of *B. fragilis* ERL; contains Tc^r Em^r ERL, Tc^r ERL, and NBU2 (1)
BF-12256 Thy	Thy^- Tpm^r Tc^r Em^r Ap^r	Thy^- mutant of *B. fragilis* 12256; contains Tc^r Em^r 12256 and NBU3 (1)
BT-DOT Thy	Thy^- Tpm^r Tc^r Em^r Ap^r	Thy^- mutant of *B. thetaiotaomicron* DOT; contains Tc^r Em^r DOT and NBU2 (1)
Plasmids		
R751	Tp^r Tra^+ (Rep^-)	IncPβ plasmid; mobilizes vectors from *E. coli* to *Bacteroides* spp. (9, 16)
pEG920	Ap^r *Tc^r $Mob^{+/-}$ (Em^r $Mob^{+/-}$)	Mobilization-deficient shuttle vector; contains site for XBU4422 insertion and NBU1 cointegration (9, 10, 13)
pEG920::NBU1		
Y5, Y11	Ap^r *Tc^r Mob^+ Rep^+ (Em^r Mob^+ Rep^+)	pEG920-NBU1 cointegrates Isolated in *E. coli* as Mob^+ insertions in pEG920 (6, 10)
Y17	Ap^r *Tc^r Mob^- Rep^+ (not known)	Mob^- pEG920::NBU1 (6, 10)
Y5D, Y11D	Ap^r *Tc^r Mob^+ (Em^r Rep^-)	Deletion of pB8-51 from Y5 and Y11; suicide vectors in *Bacteroides* spp. (this study)
Y11DP	Same as Y11D	2.7-kbp *Pst*I deletion of Y11D; used to clone NBU1-BT4001 junctions (this study)
pABU3	Cm^r Ap^r	4.6-kbp *Hin*dIII fragment of NBU1 with homology to NBU2 and NBU3 cloned in *Hin*dIII site of pBR328. (this study)
R751 INS1	Tp^r	R751 containing a cointegrate of NBU1::NBU2 (11, 12)

[a] The *Bacteroides* type strains and BF-12256 are from the VPI Anaerobe Laboratory, Blacksburg, Va. BF-ERL and BT-DOT were isolated in Mercy Hospital, Urbana, Ill. All of the *Bacteroides* strains are naturally resistant to gentamicin.

[b] Phenotypes in parentheses are expressed in *Bacteroides* spp. Phenotypes not in parentheses are expressed in *E. coli*. Abbreviations used for antibiotic resistances (r) or other phenotypes: Ap^r, ampicillin; Cm^r, chloramphenicol; Em^r, erythromycin; Tc^r, tetracycline; *Tc^r, *tetX* tetracycline; Tp^r, trimethoprim; Nal^r, nalidixic acid; Rif^r, rifampin; Str^r, streptomycin; Thy^- Tpm^r, thymidine requiring and therefore trimethoprim resistant; Rep, replication; Mob, mobilization.

in the *Bacteroides thetaiotaomicron* 5482 chromosome, and the resultant NBU1-chromosome junctions are all very different from those of the representative Tc^r element, XBU4422 (1).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study are shown in Table 1. *B. uniformis* 0061 and *B. thetaiotaomicron* 5482 are the type strains for their respective species. *B. thetaiotaomicron* 5482 has no known inserted conjugal or mobilizable elements and consequently was used as a presumably clean background for the NBU and Tc^r element transfer studies. The *Bacteroides* strains were grown on prereduced Trypticase (BBL Microbiological Systems, Cockeysville, Md.)-yeast extract-glucose (TYG) medium under an atmosphere of 80% nitrogen–20% carbon dioxide or on TYG agar plates in a BBL GasPak jar, unless otherwise indicated (5). *E. coli* strains were grown in Luria broth (LB) or on LB agar plates.

Isolation of plasmid and total cellular DNAs. The Ish-Horowitz modification of the alkaline lysis procedure of Birmboim and Doly (8) was used for plasmid purification from both *Bacteroides* and *E. coli* strains. Total cellular DNA was isolated by the method of Saito and Miura (7). Plasmid and total cellular DNAs were digested by restriction enzymes according to the conditions suggested by the manufacturers (Bethesda Research Laboratories, New England Biolabs, and Boehringer Mannheim Biochemicals). Digested or undigested DNA samples were loaded on 0.8 to 1% agarose slab gels and electrophoresed in 4× GGB (0.16 M Tris, 0.01 M sodium acetate, 8 mM EDTA [pH 8.3]) for 15 to 18 h at 50 V or in 1× GGB for 2 to 3 h at 200 V. Gels were stained with ethidium bromide and photographed.

Southern hybridization. The procedure we use for Southern blot analysis has been described previously (8, 13). The DNA was transferred to either BAS-NC (Schleicher & Schuell, Keene, N.H.) or Millipore (Marlborough, Mass.) Immobilon-N membrane by capillary action. Plasmid and DNA fragments of >5 kbp were labelled with [^{32}P]dCTP by using a nick translation kit (BRL Life Technology Inc., Gaithersburg, Md.). Smaller DNA fragments were labelled by using the random primer Prime-It II kit (Stratagene, La Jolla, Calif.). Approximately 10^7 cpm of the labelled probes or 10^5 cpm of labelled lambda DNA was used for each hybridization.

Filter matings. The conditions for filter matings have been described previously (9, 16). The mating filters were incubated aerobically if the donors were *E. coli* and anaerobically if the donors were *Bacteroides* strains. *E. coli* transconjugants were selected aerobically on LB agar plates containing the appropriate antibiotics. pEG920 and pEG920::NBU1 derivative transconjugants from HB101(R751) or DH5αMCR(R751) donors to EM24NR recipients were selected on LB agar containing either 100 μg of nalidixic acid per ml and 10 μg of tetracycline per ml (tet10) or 10 μg of rifampin per ml and tet10, respectively. *Bacteroides* pEG920 and pEG920::NBU1 transconjugants from HB101(R751) or DH5αMCR(R751) donors were selected anaerobically on TYG agar plates containing 200 μg of gentamicin, 10 μg of erythromycin, and either 10 μg of rifampin or 100 μg of thymidine plus 200 μg of trimethoprim per ml, depending on the recipient. For matings between *Bacteroides* strains, the donors were grown in TYG or TYG containing tet1 when necessary to induce functions on the resident Tc^r elements. The selection medium for the transconjugants contained either 10 μg of rifampin or 100 μg of thymidine plus 200 μg of trimethoprim per ml, depending on the recipient (usually BT4001 or BT4100, respectively), and 10 μg of erythromycin per ml for pEG920 and pEG920::NBU1 derivatives or tet3 for Tc^r element transconjugants. Frequency of transfer is defined as the number of transconjugants per the number of recipients at the end of the mating period.

Natural transfer of the NBUs. Since the NBUs, (NBU1, NBU2, and NBU3) do not contain any known selectable markers, their transfer was detected by colony hybridization. The recipient for all the NBU transfers was either *B. thetaiotaomicron* 5482 Thy^- (BT4100) or *B. thetaiotaomicron* 5482 Rif^r (BT4001). To determine the frequency of cotransfer of an NBU with a Tc^r element, the Tc^r transconjugants were isolated as indicated above and patched onto TYG plates. After overnight incubation of the plates, the bacteria were transferred to Millipore HATF nitrocellulose 0.45-μm filters and hybridized with [^{32}P]dCTP-labelled pABU3, which contained the 4.6-kbp *Hin*dIII fragment of NBU1 (see Fig. 1) cloned into the *Hin*dIII site of pBR328. This fragment includes the region of NBU1 which hybridizes to NBU2 and NBU3. The insertion and the location of the NBU in the *B. thetaiotaomicron* chromosome were determined by Southern blot analysis of *Hin*dIII- or *Hin*cII-digested total cellular DNA on regular agarose gels or of *Not*I-digested DNA from pulsed field gels (1). [^{32}P]dCTP-labelled pEG920::NBU1 (Y11) or R751 INS1 containing both NBU1 and NBU2 (11, 12) was used to probe the blots. Independent isolates are defined as transconjugants isolated from different filter matings.

Transfer of NBU1 without cotransfer of a Tc^r element was detected by mating a *Bacteroides* donor (BU1006) containing Tc^r Em^r DOT and NBU1 with BT4100. The donor was grown in TYG containing tet1 to induce functions on the Tc^r element that are required for the excision and mobilization of NBU1. For selection of NBU1 transconjugants, the mating mixture was plated to give approximately 50,000 recipient CFU per plate on TYG agar containing 100 μg of thymidine plus 200 μg of trimethoprim per ml. The plates were incubated anaerobically at 37°C for 24 h. The colonies were transferred to HAFT filters as described above, and the plates were stored anaerobically at room temperature until after the colony hybridizations were analyzed. pABU3 or R751 INS1 (NBU1::NBU2) was labelled and used as the probe. Cells from the areas on the plates that showed dark hybridization regions were restreaked, and 50 to 100 isolated colonies from each region were reprobed. Several isolates from each filter mating that hybridized to the probe were streaked for purity and checked by Southern blot analysis of total cellular DNA for insertion and location of NBU1 and/or NBU2 in the BT4100 chromosome.

Isolation of the NBU1 insertion region and the NBU1-chromosomal junctions. Total cellular DNA from *B. uniformis* 0061 derivative BU1001, which contains one copy of NBU1 (12), was digested with *Hin*dIII, and DNA fragments of the size determined (by Southern blot analysis) to contain NBU1-chromosomal junctions were isolated from agarose gels. The isolated fragments were cloned into the *Hin*dIII site of pBR328, and colony hybridizations of the clones probed with R751 INS1 were done to detect the positive clones. Only the right junction, J_R, was successfully cloned into pBR328 (to give pABU7).

Both junction regions between NBU1 and the *B. thetaiotaomicron* chromosome from the predominant insertion site were cloned by using a suicide derivative of pEG920::NBU1 (Y11). A *Pst*I deletion of Y11 removed 2.7 kbp of NBU1, pB8-51, and most of the pUC19 multiple cloning region, yielding the insertional vector Y11DP (Table 1; see Fig. 2). Y11DP was mobilized into BT4104 from DH5αMCR(R751, Y11DP), and Y11DP inserted into the *B. thetaiotaomicron* 5482 chromosome at frequencies (10^{-6}) similar to those of Y11. The transconjugants were isolated, and cellular DNA was extracted. The insertion site locations were determined by Southern blot analysis. All 10 of the Y11DP insertions tested were in the preferred NBU1 insertion site (see Fig. 3). The DNA from two of the transconjugants was digested to completion with several enzymes that did not cut within the Y11DP inserted element. The DNA was diluted to about 1 μg/ml, ligated, and used to transform competent DH5αMCR. Several Tc^r transconjugants were isolated from the *Acc*I, *Eco*RV, and *Kpn*I digests, and all were shown to contain 8 to 10 kbp of chromosomal DNA. Although the plasmids were 22 to 24 kbp, there was at least 100 to 150 bp of readable sequence across both junctions. Fortunately, more than one restriction enzyme was used to isolate the junction clones, because there was a *Kpn*I site created at J_R and an *Acc*I site just 47 bp from J_L. The third enzyme, *Eco*RV, covered both junctions.

The 1.5-kbp *Hin*cII fragment of NBU1, which contained the insertion region, and the 2.4-kbp *Hin*dIII-*Pvu*II fragment from pABU7, which contained the NBU1-*B. uniformis* right junction, were cloned into pUC19. Progressive unidirectional deletions were made by using the Exonuclease III Erase-a-Base system (Promega, Madison, Wis.).

DNA sequence analysis and isolation and sequencing of the primary chromosomal target site. Double-stranded template sequencing was performed by using the dideoxy-chain-terminating reaction with T7 polymerase as provided in the Sequenase 2.0 kit (U.S. Biochemical, Cleveland, Ohio). The sequences of the NBU1 insertion region and the NBU1-*B. uniformis* J_R clone were compared. Primers for the region near the NBU1 insertion sequence were designed to sequence both the J_R and the J_L NBU1-*B. thetaiotaomicron* junctions contained in the Y11DP junction clones. Reverse primers were designed from the chromosomal sequences obtained from the Y11DP junction clones. These primers were designed with *Eco*RI sites near the 5′ end and were used in a polymerase chain reaction with BT4001 chromosomal DNA to produce a 370-bp fragment containing the primary NBU1 target site for insertion. The primers were synthesized at the University of Illinois Biotechnology Genetic engineering Facility, Urbana. The amplification procedure was performed with *Taq* polymerase and reagents from Promega; 100 to 200 ng of each primer and approximately 50 ng of undigested chromosomal DNA were in each reaction mixture. The cycle consisted of 5 min at 94°C before addition of the *Taq* polymerase followed by

TABLE 2. Natural transfer of NBU elements by conjugal Tc^r elements.

Donors			Recipient	Frequency of cotransfer of Tc^r element and NBU[a]
Strain	Tc^r element	NBU(s)		
B. uniformis 1006	Tc^r Em^r DOT	NBU1	BT4100	0.13–0.27 (4)
BU1004[b]	Tc^r ERL	NBU1, NBU2	BT4100	<0.001–0.14 (6)
BT-DOT Thy	Tc^r Em^r DOT	NBU2	BT4001	0.13–0.04 (10)
BF-12256 Thy	Tc^r Em^r 12256	NBU3[c]	BT4001	0.15–0.37 (18)
BF-ERL Thy[d]	Tc^r ERL	NBU2	BT4001	0.03–0.37 (6)
	Tc^r Em^r ERL	NBU2	BT4001	0.08–0.30 (6)
BT4007N2	Tc^r Em^r DOT	NBU2	BT4001	0.17–0.60 (6)

[a] The frequency of Tc^r-NBU cotransfer is the ratio of Tc^r transconjugants that also received an NBU to the total number of Tc^r transconjugants. The recipients for all of these matings were derivatives of *B. thetaiotaomicron* 5482. The range for the frequencies of transfer for each mating pair is shown. The minimum number of Tc^r transconjugants tested for cotransfer of an NBU was 100. However, for the Tc^r ERL donors 500 to 1,000 transconjugants often were tested. The number in parentheses is the number of filter matings done. The transfer frequency for the Tc^r elements was 2×10^{-7} to 1×10^{-6} per recipient at the end of the mating period.

[b] BU1004 has both NBU1 and NBU2. The cotransfer frequency given here is the frequency for both NBU1 and NBU2 determined by colony hybridizations.

[c] NBU3 was detected by homology to NBU1 probes. NBU3 inserts into a single *Not*I fragment of BT4001 (1).

[d] BF-ERL contains two Tc^r elements, Tc^r ERL and Tc^r Em^r ERL, which transfer independently. Cotransfer of the two elements, separable by Em^r selection, has not been detected.

25 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. The reaction ended with 5 min at 72°C and 20 min to several hours at 24°C. The resulting 372-bp product was purified, digested with *Eco*RI, and cloned into the *Eco*RI site of pUC19 for sequencing as described above. Three clones were sequenced.

RESULTS AND DISCUSSION

Natural transfer of NBUs by Tc^r elements. Previously, we had found cases in which two NBUs, NBU2 and NBU3, cotransferred with Tc^r elements (1). Cotransfer frequencies for the three known NBUs and different Tc^r elements are given in Table 2. Generally, the frequency of NBU-Tc^r element cotransfer from the same donor was quite reproducible. The exception was Tc^r ERL. Cotransfer of Tc^r ERL and NBU1 or NBU2 varied 10-fold from the original *B. fragilis* donor, BF-ERL, and more than 100-fold from the *B. uniformis* 0061 transconjugant donor, BU1004, which had received both Tc^r ERL and NBU2 from *B. fragilis* ERL. The reason for this variation is not known. The location of the Tc^r ERL insertion site relative to the NBU1 and NBU2 insertion sites could be important. Both BF-ERL and BU1004 have a second coresident Tc^r element, Tc^r Em^r ERL in BF-ERL and XBU4422 in BU1004, which could interfer with the Tc^r ERL transfer functions. However, even in the case of Tc^r ERL, fairly high cotransfer frequencies are sometimes observed. In the examples in Table 2, the cotransfer was close to 10% for most of the mating pairs. Since the transfer frequencies for the Tc^r elements were 10^{-7} to 10^{-6}, the frequencies of transfer of the NBUs to the recipients that received a Tc^r element were in the range of 10^{-8} to 10^{-7}.

All of the strains that have been shown to contain an NBU (Tables 1 and 2) also contain a member of the conjugal Tc^r element family. The type strain *B. uniformis* 0061, which has NBU1, is Tc^S, but it contains the cryptic or Tc^S member of the family, XBU4422 (13). To determine if NBUs ever integrated in a recipient without the cointegration of a Tc^r element, we used an NBU probe to screen recipients that had been selected only for the recipient marker and not for the presence of a Tc^r element (see Materials and Methods). The donor used in these matings was BU1006, which contained Tc^r Em^r DOT, a Tc^r element which cotransferred NBU1 to BT4100 at a consistently high frequency of about 20% (Table 2). We found a few BT4100 transconjugants which acquired NBU1 without also acquiring Tc^r Em^r DOT. The frequency of these transconjugants was roughly estimated to be about 10^{-6} per recipient plated. Although NBU1 transconjugants that did not contain Tc^r Em^r DOT were isolated, it was still possible that functions provided by Tc^r Em^r DOT were required for NBU1 insertion. The lack of the Tc^r Em^r DOT in the NBU1 transconjugants could have been due to aborted integration of the Tc^r element or to segregation of the two inserted elements following recipient cell division.

Since the transfer frequency for NBU1 was comparable to that for the Tc^r elements and not considerably higher, independent transfer of the two elements could not have resulted in the relatively high cotransfer frequencies actually observed (Table 2). From the high frequency of cotransfer of the Tc^r elements and the NBUs, it is also clear that Tc^r elements and NBUs do not exclude each other. The *B. uniformis* recipient (BU1001) received both Tc^r ERL and NBU2 from BF-ERL Thy, although it already contained both NBU1 and XBU4422 (Table 1). When the resulting BU1001 transconjugant (BU1004) was the donor, Tc^r ERL cotransferred with NBU1 and NBU2. Southern analysis of 10 transconjugants showed that Tc^r ERL cotransferred with NBU1 and NBU2 together to *B. thetaiotaomicron* BT4100 at about the same frequency as NBU1 or NBU2 alone (Table 2). NBU2 integrates preferentially into two sites in the chromosome of *B. thetaiotaomicron* at approximately equal frequency (1). NBU1 could also insert into two different sites in the *B. thetaiotaomicron* chromosome, although one was preferred at a ratio of about 4:1 or higher (see below).

Mobilization of NBU1 by Tc^r elements and IncP plasmids. Previously, we had shown that functions provided by the Tc^r elements were needed for the excision and circularization of the NBUs (12, 14, 15). One model that followed from these observations was that the excised circular forms were transfer intermediates. The Tc^r element genes required for the excision-circularization step are regulatory genes that also control many Tc^r element functions (14, 15). Apparently, an excision gene(s) on the NBUs is activated in *trans* by the Tc^r element regulatory proteins. Evidence that the circular form of NBU1 was the transfer intermediate and that Tc^r element functions were required for the mobilization of this circular form came from experiments using fortuitously isolated cointegrates of NBU1 and a mobilization-defective *E. coli-Bacteroides* shuttle vector, pEG920. pEG920 contains the *Bacteroides* plasmid pB8-51, which can be mobilized by IncP plasmids and the Tc^r elements. In pEG920, pB8-51 is defective for mobilization

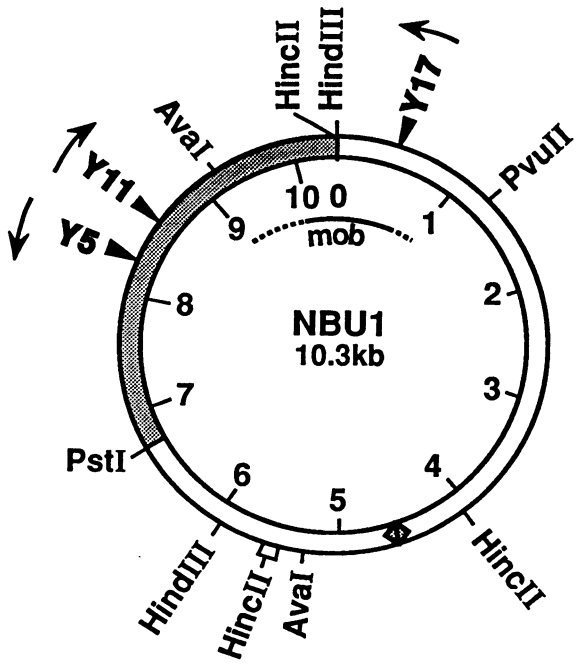

FIG. 1. Map of NBU1 with locations of the pEG920 insertions that produce Y5, Y11, and Y17. The arrows indicate the orientations of pEG920 in the cointegrates, and the arrowheads point upstream of *tetX* (*Tc^r) on pEG920 (see Fig. 2). The shaded area is the region on NBU1 which hybridizes to other NBU elements, especially NBU2 and NBU3. The mobilization region (mob) recognized by IncP plasmids and conjugal Tc^r elements is indicated (6). The shaded diamond at the bottom is the region of NBU1 where insertion into the chromosomal target sites occurs.

(1,000-fold decrease) by both groups of elements (9). The pEG920-NBU1 cointegrates were isolated in experiments involving the transfer of pEG920 from BU1004 (which contains Tc^r ERL and NBU1) to *E. coli* recipients and were the result of pEG920 integrating into NBU1 in three different locations. The cointegrates were designated Y5, Y11, and Y17. Sequence analysis of the cointegrates showed that the site on pEG920 where the integrations occurred was the same site where XBU4422 inserted (1, 10) and may be a recombinational or insertion hot spot in *Bacteroides* hosts. The location and the orientation of the pEG920 insertions in NBU1 are shown in Fig. 1. The region on NBU1 involved in integration into chromosomal sites, indicated as a shaded diamond within the 1.5-kbp *Hin*cII fragment (Fig. 1), was not involved in the pEG920-NBU1 cointegrate formation. Two of these cointegrates, Y5, and Y11, were mobilized from *E. coli* donors by the IncP plasmid R751 to *E. coli* or to *Bacteroides* recipients at 100- to 1,000-fold-higher frequencies than the frequency of mobilization of pEG920 alone (Tables 3 and 4). Y17 was not mobilized at a detectable frequency by R751 and was not used in the following experiments.

Y5 and Y11 were tested for self-transfer from *B. uniformis* and *B. thetaiotaomicron* donors. Y5 and Y11 were transferred from BU1001 to *E. coli* at a low frequencies (Table 3). However, there was no detectable transfer ($<10^{-10}$) of Y11 or Y5 (data not shown) from *B. thetaiotaomicron* 5482 BT4100 to *E. coli* (Table 4). The transfer from BU1001 was probably due to XBU4422, a cryptic member of the conjugal Tc^r element family which is in this strain and has been shown to have low-level transfer and plasmid mobilization properties (13). Transfer of Y5 and Y11 was increased 100- to 1,000-fold by the presence of an exogenous Tc^r element in *B. uniformis* donors. Transfer of Y5 and Y11 (data are shown for Y11) from *B. thetaiotaomicron* donors required a Tc^r element and occurred at relatively high frequencies. Thus, genes on the Tc^r elements appeared to be required for transfer of Y5 and Y11. The fact that both Y5 and Y11 transferred at much higher frequencies than pEG920 alone suggested that NBU1 contained a mobilization region recognized by IncP plasmids and by Tc^r elements but did not rule the possibility that the defective pB8-51 mobilization region was contributing to the transfer. Deletion of pB8-51 from Y11 and Y5 (Fig. 2) did not affect the mobilization of either of the resultant vectors, Y11D and Y5D, by R751. (Results for Y11D are given in Table 4). Therefore,

TABLE 3. Mobilization of pEG920::NBU1 cointegrates by IncP R751 or Tc^r ERL

Donors		Frequency of transfer to[a]:	
Strain	Vector	*E. coli*	*B. uniformis*
HB101(R751)	Y5	2×10^{-3}	1.5×10^{-5}–6.4×10^{-4}
	Y11	1.5×10^{-5}–1×10^{-4}	1.8×10^{-5}–5×10^{-4}
	Y17	$<10^{-9}$	$<3 \times 10^{-9}$
	pEG920	1.1×10^{-7}	1.8×10^{-7}
BU1001	Y5	4×10^{-8}	$<10^{-9}$
	Y11	3.5×10^{-8}	1.1×10^{-8}
	pEG920	$<10^{-9}$	$<10^{-9}$
BU1004 (Tc^r ERL)	Y5		
	−Tc[b]	8×10^{-5}	8×10^{-5}
	+Tc	1.1×10^{-4}	4×10^{-4}
	Y11		
	−Tc	5×10^{-4}	10^{-4}
	+Tc	8.5×10^{-5}	2.8×10^{-4}
	pEG920		
	−Tc	7×10^{-10}	$<10^{-9}$
	+Tc	1.3×10^{-7}	4.5×10^{-7}

[a] Frequencies are the numbers of transconjugants per recipient at the end of the mating. The frequencies are the averages of three or more matings. Ranges are given for values which varied more than fourfold. EM24NR and BU1001 were the recipients for HB101(R751) donors, and HB101 and BU1100 were the recipients for *B. uniformis* donors.

[b] Tetracycline (1 μg/ml) was added (+) or omitted (−) to induce functions on Tc^r ERL.

TABLE 4. Use of pEG920::NBU1 Y11 derivatives[a] to monitor excision, transfer, and integration of NBU1 in *Bacteroides* hosts

Donors		Frequency of transfer to[b]:	
Strain	Vector	*E. coli*	*B. thetaiotaomicron*
DH5αMCR(R751)[c]	Y11	2.3×10^{-4}	10^{-4}
	Y11D	3.2×10^{-4}	2×10^{-7}–1×10^{-6}
BT4100	Y11	$<10^{-10}$	$<10^{-9}$
BT4104 (Tc^r ERL)[c]	Y11		
	−Tc[d]	$<3 \times 10^{-9}$	$<10^{-9}$
	+Tc	8×10^{-5}	3×10^{-5}
	::Y11D[e]		
	−Tc	$<10^{-9}$	$<10^{-9}$
	+Tc	1.3×10^{-7}–1.4×10^{-6}	3×10^{-9}–1×10^{-8}

[a] The data presented are for the Y11 derivatives. Results obtained for Y5 derivatives were comparable (data not shown). All of the Y11 derivatives replicate in *E. coli*. Y11D is a suicide vector and integrates in *Bacteroides* recipients.

[b] The frequencies are the averages of at least three experiments. Ranges are given if the values varied more than fourfold. EM24NR and either BT4104 or BT4001 (with similar results) were the recipients from the DH5αMCR(R751) donors. HB101 or DH5αMCR and BT4001 were the recipients from the BT4104 and BT4100 donors.

[c] R751 transferred at a frequency of 0.2 to 0.5 to *E. coli* recipients. Tc^r ERL transferred at a frequency of 10^{-7} to 10^{-6} to BT4001.

[d] Tetracycline was added (+) or omitted (−) to induce functions on Tc^r ERL in the BU1104 donors.

[e] Integrated Y11D.

NBU1 was providing all the necessary sequences for mobilization of the cointegrates by the IncP plasmids. The results of a detailed study localizing and sequencing the mobilization region on NBU1 are presented in an accompanying paper (6). This study shows that the mobilization region is internal to the NBU1, as shown in Fig. 1, and not at the ends, supporting the model that the circular form of NBU1 is the transfer intermediate. The results given in Tables 3 and 4 suggest that the frequency of mobilization of the NBU1 circular forms approaches 10^{-4} per recipient.

Previous studies showed that the excision and circularization of NBUs requires both the presence of a Tc^r element and the induction of the Tc^r element functions by preexposure of the cells to tetracycline (12, 14, 15). Similarly, mobilization of coresident plasmids by Tc^r elements is stimulated 10- to 1,000-fold by preincubation of *Bacteroides* donors with low levels of tetracycline (9, 16). In contrast, mobilization of Y5 and Y11 from *B. uniformis* (BU1004) was not enhanced by preexposure to tetracycline (Table 3). However, tetracycline stimulation of Y5 and Y11 mobilization was seen if *B. thetaio-*

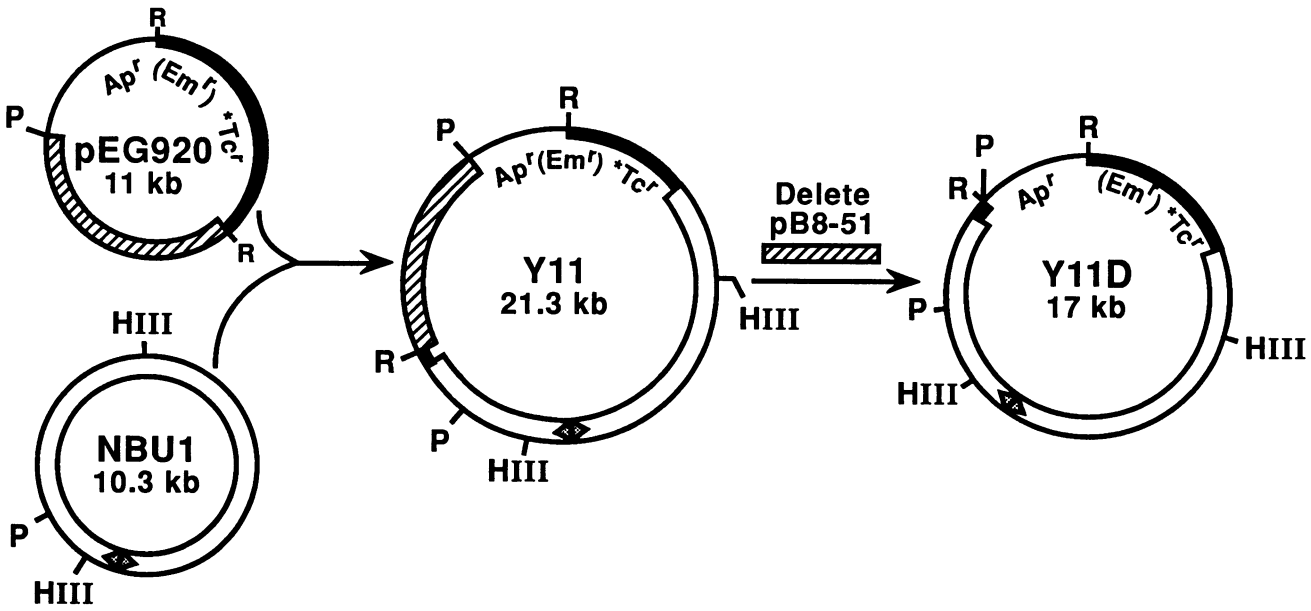

FIG. 2. Formation of pEG920::NBU1 Y11 and construction of Y11D. Y11 was formed when the plasmidlike form of NBU1 was excised from the *B. uniformis* 0061 chromosome and was recombined with mobilization-defective pEG920. The shaded diamonds indicate the region of NBU1 found at the ends of the integrated form. The thin black line indicates pUC19 sequences, the thick black line represents the 3.9-kbp *Eco*RI fragment of Tn*4400* containing *ermF* (Em^r) and *tetX* ($*Tc^r$), and the hatched line indicates the *Bacteroides* plasmid pB8-51. The Y11 cointegrate is replication and mobilization proficient in both *Bacteroides* and *E. coli* hosts. Deletion of pB8-51 (hatched region) from Y11 formed Y11D. Y11D was no longer capable of replication in *Bacteroides* hosts and integrated into the chromosome. Abbreviations: HIII, *Hin*dIII; R, *Eco*RI; P, *Pst*I; Ap^r and $*Tc^r$, ampicillin and tetracycline resistances, respectively, that are expressed in *E. coli*; (Em^r), erythromycin resistance that functions in *Bacteroides* spp.

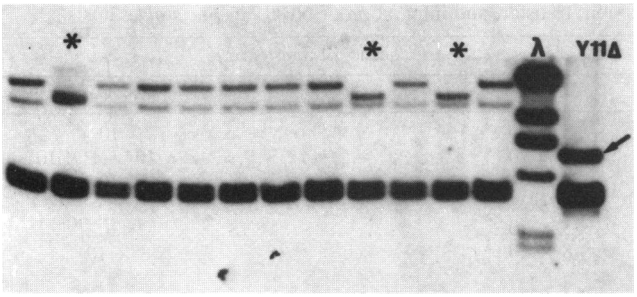

FIG. 3. Southern blot of Y11D insertions into the chromosome of *B. thetaiotaomicron* (BT4001). Twelve independent Y11D, erythromycin-resistant transconjugants in BT4001 were selected. The Southern blot of *Hin*dIII digests of DNA from the transconjugants was probed with ^{32}P-labelled Y11D and lambda DNA. The sizes of the *Hin*dIII lambda standard fragments are 23, 9.5, 6.7, 4.4, 2.3, and 2.0 kbp. The arrow points to the 5-kbp *Hin*dIII fragment of NBU1 in Y11D (last lane on the right), which contains the insertion region of NBU1 and separates into two large *Hin*dIII Y11D-chromosomal junction fragments in the transconjugants. Nine of the 12 BT4001::NBU1 transconjugants have Y11D inserted in the same chromosomal site, and three of the isolates (lanes indicated by the asterisks) appear to have Y11D integrated into a second site.

taomicron BT4104 was the donor. The results for Y11 are given in Table 4. Since the constitutive transfer from *B. uniformis* could have been due to the interaction of Tc^r ERL and other elements, such as XBU4422, NBU1, and NBU2, known to be present in this *B. uniformis* strain, *B. thetaiotaomicron* 5482 derivatives were used in the remainder of the experiments.

Calculation of integration and excision frequencies for NBU1. To determine if NBU1 could replicate in *Bacteroides* strains, we used Y11D (Fig. 2) and Y5D (not shown). If NBU1 carries a replication region, Y5D and/or Y11D ought to be able to replicate in *Bacteroides* recipients. Y5D and Y11D were mobilized by R751 to BT4001 and to BT4104, which contains Tc^r ERL. Both Y5D and Y11D integrated into the *B. thetaiotaomicron* chromosome at about the same frequency (10^{-7} to 10^{-6}), and the results for Y11D are shown in Table 4. Southern blot analysis of the transconjugants indicated that Y11D integrated within the same *Hin*dIII fragment containing the insertion region of NBU1 as seen in the natural transfers of NBU1 (12, 15) (Fig. 1) and in the same primary and secondary sites (ratio, 4:1). The Southern blot of 12 Y11D transconjugants is shown in Fig. 3. Failure of Y5D and Y11D to replicate in *B. thetaiotaomicron* could be due to the interruption of a replication function by the pEG920 insertion. However, the failure of both Y5D and Y11D, each with pEG920 inserted into a different location, to replicate in *B. thetaiotaomicron*

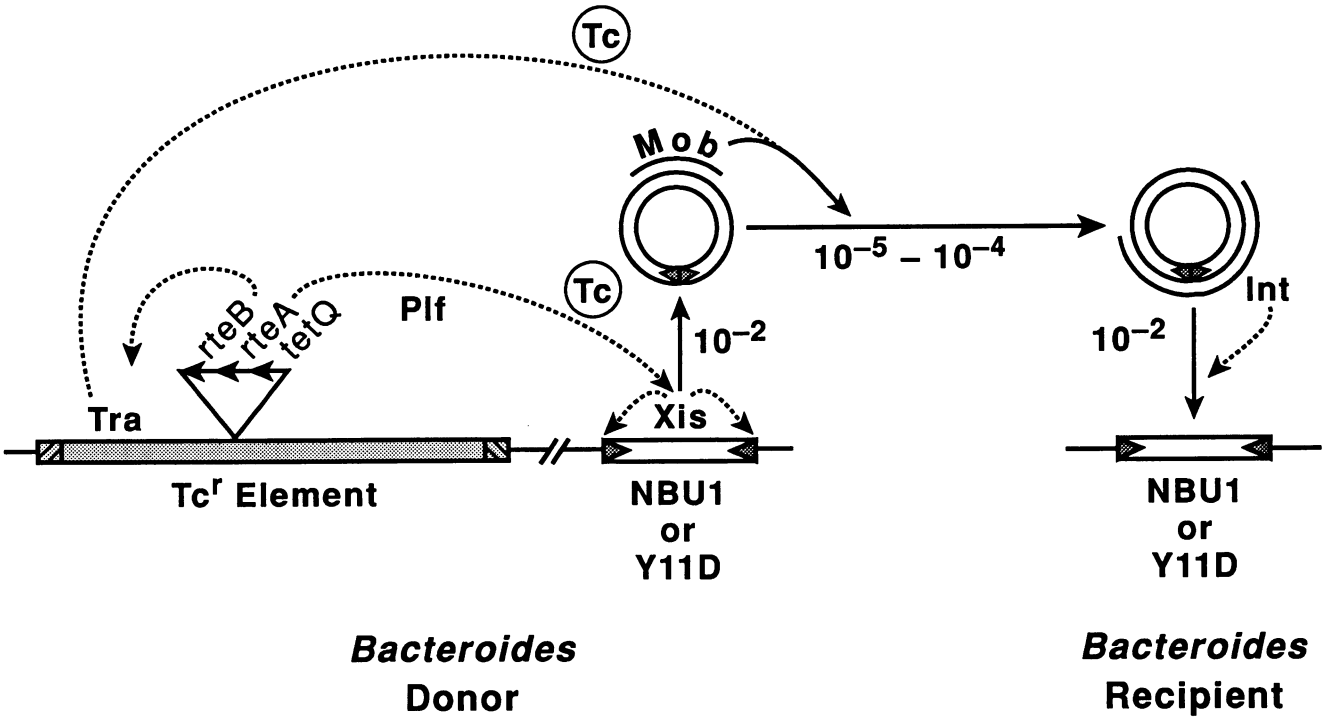

FIG. 4. Model for the dissemination of NBU1 among *Bacteroides* spp. Several tetracycline-inducible (Tc) functions carried on a coresident conjugal integrated Tc^r element are required for the excision and transfer of the integrated NBU1 from a donor chromosome to a recipient (14, 15). The production of the circular form of NBU1 requires regulatory genes (*tetQ*, *rteA*, and *rteB*) provided by the Tc^r element and excision (Xis) functions provided by NBU1 (14). The excision and mobilization frequencies of 10^{-2} and 10^{-5} and 10^{-4}, respectively, were calculated from experiments using Y11D and Y11. Mobilization required additional genes on the Tc^r element (e.g., transfer genes [Tra]) as well as a region (Mob) on NBU1 which is interrupted in Y17 (Fig. 1) (6). Since no Tc^r element functions were required for the integration of NBU1 (Y11D), the integration (Int) functions are presumably provided by NBU1. The location of the integration functions must be between the Mob region and the pEG920-Y5 insertion site indicated by the outer line. The NBU1 frequency of integration was estimated from the experiments with Y11 and Y11D to be about 10^{-2} per recipient receiving a circular copy of the element.

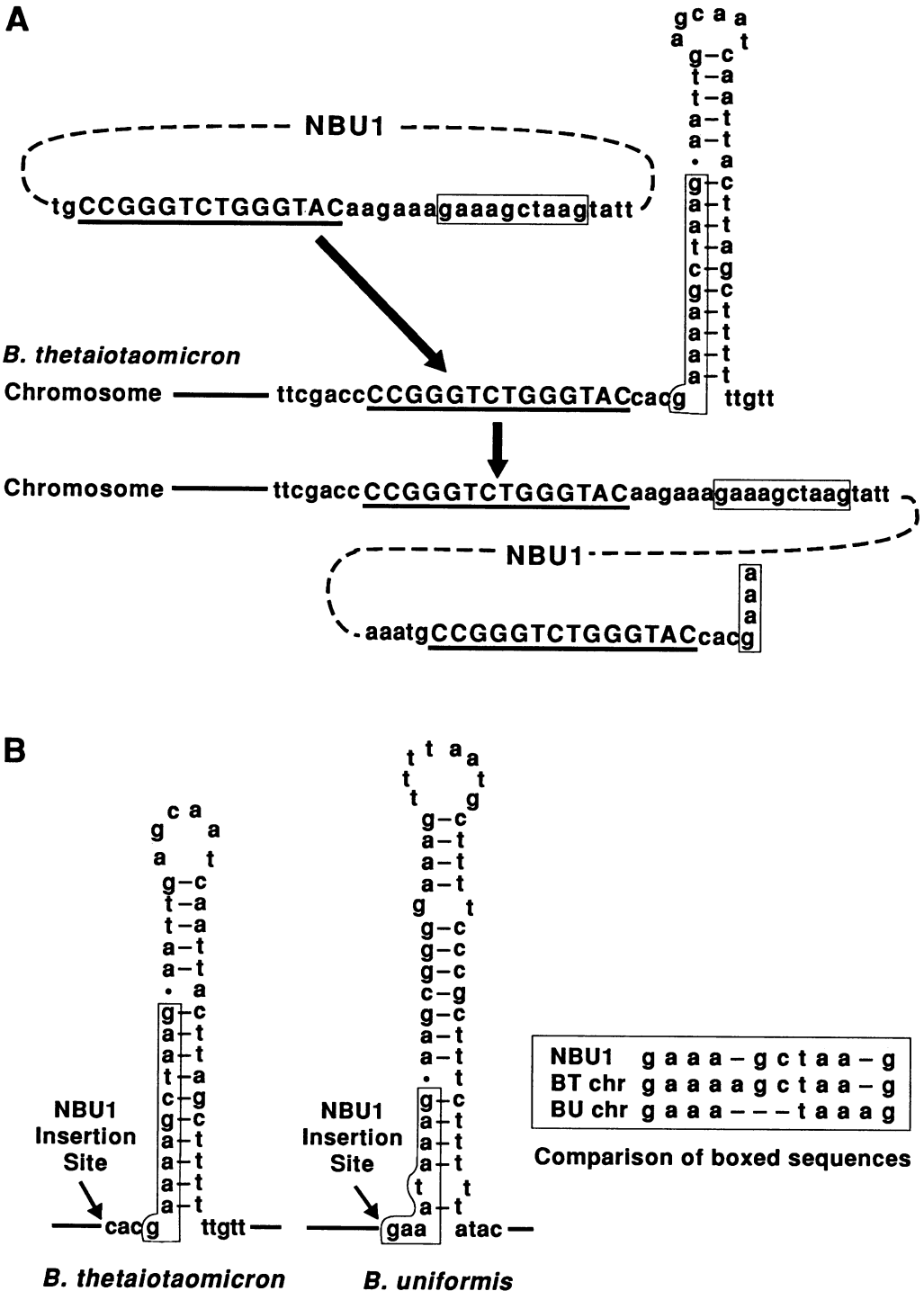

FIG. 5. DNA sequences of NBU1 and its chromosomal insertion regions in *B. uniformis* 0061 and *B. thetaiotaomicron* 5482. The sequence of the region of the circular NBU1 which inserts into the chromosomal sites (shaded diamonds in Fig. 1, 2, and 4) is shown at the top of panel A. The 14 bases in capital letters and underlined were also found in the primary NBU1 insertion site in *B. thetaiotaomicron* 5482. This exact 14-bp sequence was found at both the right junction, J_R, and the left junction, J_L, of *B. thetaiotaomicron*::NBU1 and at the J_R of *B. uniformis*::NBU1 (not shown). The 10-base sequence in the boxed region of NBU1, 3′ to the junction sequence, was found with some changes in both of the chromosomal targets. A sequence comparison of the boxed 10-base region of NBU1 and the two chromosomal sites is shown at the right of panel B. The possible stem-and-loop structures containing the boxed sequences in the target sites of both *B. uniformis* and *B. thetaiotaomicron* are shown at the left in panel B. The 10-base sequence in NBU1 itself is not part of a stem-and-loop structure. Positions of the NBU1 insertion sites relative to the stem-and-loop structures are indicated by the arrows.

supports the hypothesis that NBU1 does not contain functions for replication in *Bacteroides* spp.

Y5D and Y11D integration occurred at the same frequency regardless of whether there was Tc^r element in the recipient (Table 4). This showed that NBU1 could mediate its own insertion into the recipient chromosome without the aid of a Tc^r element and that the presence of a Tc^r element did not stimulate integration. The frequency of NBU1 insertion could be estimated from the data in Table 4. Since transfer of Y11D to BT4001 or BT4104 (mobilization plus integration) occurred at a 100- to 1,000-fold-lower frequency than transfer of Y11 (mobilization only), the integration frequency for Y11D approaches 10^{-2} per recipient receiving a copy of Y11D. Both the integrated Y11D and Y5D (data for Y5D are similar but not shown) could be transferred to *E. coli* recipients by the coresident Tc^r ERL, indicating that neither the integration nor the excision genes of NBU1 were interrupted by the pEG920 insertions (Fig. 1). The frequency of transfer of the integrated Y11D from the chromosome of BT4104 to *E. coli*, where Y11D replicates as a plasmid, was used to estimate the frequency of Y11D excision. Transfer of Y11D to *E. coli* (excision plus mobilization) occurred 10- to 100-fold less frequently than the transfer of Y11 (mobilization only). Thus, the frequency of Y11D excision from the *B. thetaiotaomicron* chromosome was about 10^{-2} to 10^{-1} (Fig. 4).

Transfer of Y11D from the chromosome of *B. thetaiotaomicron* 5482 to the chromosome of a derivative of 5481 (excision plus transfer plus integration) occurred at a frequency of 10^{-9} to 10^{-8} (Table 4). This is the frequency expected from multiplying the frequencies estimated for the separate steps (Fig. 4). However, this frequency was 100-fold lower than the frequency (10^{-6}) estimated for the natural transfer of the native form of NBU1 (see above). Since it is difficult to accurately measure the natural transfer of NBU1 (see Materials and Methods), it is possible that the estimated frequency is too high. If trimethoprim does not kill cells rapidly, transfer on the heavily seeded selection plates could have occurred. The natural transfer was also done with Tc^r Em^r DOT from BU1006, which gave the highest and most consistent transfer of NBU1 (Table 1), especially compared with Tc^r ERL. We had to use Tc^r ERL in the experiments with Y11D because Y11D already contained the Em^r marker and we wanted to avoid interactions due to DNA homologies between the two elements during the transfer process, which could influence the frequencies observed. Another factor is that although the transfer frequencies are probably not influenced, the excision and insertion events could be adversely affected by the size (11 kbp) of the pEG920 insertion. Comparisons of Southern blot analysis of the excised form of NBU1 versus that of Y11D by tetracycline-induced Tc^r ERL in *B. thetaiotaomicron* showed the concentration of the Y11D circular form to be much weaker than that of the native NBU1 (not shown). Despite the fact that our estimated values for the excision and possibly the insertion of NBU1, determined by using Y11D, are probably too low, it is still clear that the frequencies of the excision and the integration steps are at least 10- to 100-fold higher than that of the mobilization step.

DNA sequences of NBU1 insertion region, preferred chromosomal target site, and chromosomal junctions. Southern blot analysis of the inserted NBU1 and the circular form was used to localize the NBU1 insertion region to near one end of a 1.5-kbp *Hin*cII fragment (shaded diamonds in Fig. 1 and 2). The 1.5-kbp *Hin*cII NBU1 fragment was isolated from Y11, cloned into pUC19, and sequenced as described in Materials and Methods. The NBU1-*B. uniformis* right junction, J_R, was also cloned and sequenced as described in Materials and Methods. The integration point on NBU1 was determined by comparison of the two sequences. Both J_R and the left junction, J_L, of NBU1-*B. thetaiotaomicron* in the preferred insertion site were cloned as described in Materials and Methods, using the Y11 suicide derivative, Y11DP (Table 1). The chromosomal target site was then amplified by the polymerase chain reaction, cloned, and sequenced. The sequence of the *B. uniformis* 0061 target could not be obtained because we have no strains which have lost NBU1. A summary of the results of the sequencing is shown in Fig. 5.

The 14 bases on NBU1 that are capitalized and underlined in Fig. 5A were also found in the *B. thetaiotaomicron* primary insertion site for NBU1 and ultimately at both NBU1-*B. thetaiotaomicron* junctions. Integration appeared to have occurred by recombination within or adjacent to these 14 bases. These 14 bases were also found at the right junction of NBU1-*B. uniformis* (not shown). A potential stem-and-loop structure was found 3′ to the NBU1 J_R in both *B. thetaiotaomicron* and *B. uniformis* (Fig. 5B). The sequences of the stem and loop in these two species were not identical, but both contained most of a 10-base sequence that was also found near the integration region of NBU1 shown boxed in Fig. 5. A comparison of these sequences is shown in Fig. 5B. The 14 bases of identity and the stem-and-loop structure with 10 bases of partial identity probably contribute to the site specificity of the NBU1 insertion. It remains to be determined exactly what sequences on NBU1 and its target sites are required for insertion and how the NBU1 mechanism of insertion compares with those of other site-specific insertion elements.

There was no sequence similarity between the ends of NBU1 (Fig. 5) and either the ends of XBU4422 (1) or the ends of the mobilizable *Bacteroides* transposon, Tn*4399*, described by Hecht et al. (3, 4). Also, the target site selections and the deduced mechanisms of integration of the three elements were quite dissimilar. Taken together, the differences between NBU1, XBU4422, and Tn*4399* suggest that NBU1 represents a family of mobilizable integrating elements that are not related to the mobilizable Tn*4399* and are not minature versions of the conjugal Tc^r elements.

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