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NBU1, a Mobilizable Site-Specific Integrated Element from *Bacteroides* spp., Can Integrate Nonspecifically in *Escherichia coli*

NADJA B. SHOEMAKER,* GUI-RONG WANG, AND ABIGAIL A. SALYERS

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

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NBU1 is an integrated *Bacteroides* element that can be mobilized from *Bacteroides* donors to *Bacteroides* recipients. Previous studies have shown that a plasmid carrying the internal mobilization region of NBU1 could be transferred by conjugation from *Bacteroides thetaiotaomicron* to *Escherichia coli*. In this report, we show that NBU1 can integrate in *E. coli*. Whereas integration of NBU1 in *B. thetaiotaomocron* is site specific, integration of NBU1 in *E. coli* was relatively random, and the insertion frequency of NBU1 into the *E. coli* chromosome was 100 to 1,000 times lower than the frequency of integration in *B. thetaiotaomicron*. The frequency of NBU1 integration in *E. coli* could be increased about 10- to 70-fold, to a value close to that seen with *B. thetaiotaomicron*, if the primary integration site from *B. thetaiotaomicron*, BT1-1, was provided on a plasmid in the *E. coli* recipient or the NBU1 integrase gene, *intN1*, was provided on a high-copy-number plasmid to increase the amount of integrase available in the recipient. When the primary integration site was available in the recipient, NBU1 integrated site specifically in *E. coli*. Our results show that NBUs have a very broad host range and are capable of moving from *Bacteroides* spp. to distantly related species such as *E. coli*. Moreover, sequence analysis of NBU1 integration sites provided by integration events in *E. coli* has helped to identified some regions of the NBU1 attachment site that may play a role in the integration process.

Human colonic *Bacteroides* spp. harbor at least two types of integrated elements that can be transferred by conjugation: large (>65-kbp) self-transmissible conjugative transposons (CTs) and smaller 10- to 12-kbp elements that are mobilized in *trans* by the CTs. We have called these smaller elements NBUs (nonreplicating *Bacteroides* units) (19, 22). Another group has given a recently discovered NBU-type element a transposon designation, Tn*4555* (24). NBUs require *trans*-acting factors provided by the CTs to carry out the first two steps in transfer. A regulatory protein encoded on the CT, RteB, is necessary to stimulate the integrated NBU to excise and form a covalently closed circle (25, 26). This circular form does not replicate, but it has an internal transfer origin (*oriT*) that allows it to be mobilized by the CT (9, 10). In this second step of transfer, the CT provides proteins that form the mating apparatus, through which a copy of the NBU circle form is presumably transferred to the recipient. The NBU provides its own nickase, which is encoded by the *mob* gene (9, 10). Once the NBU has entered the recipient cell, it appears to be able to integrate on its own without the intervention of CT-encoded functions (22). Despite the fact that the NBUs rely on CTs for transfer functions, the NBUs appear to be unrelated to the CTs at the DNA sequence level (2, 22). So far, no sequence similarities between the NBUs and CTs have been reported.

CTs are found in many clinical isolates of *Bacteroides* spp. (2, 20) and appear to be responsible for much of the transfer of antibiotic resistance genes that is occurring within *Bacteroides* spp. NBUs are also found in many *Bacteroides* clinical isolates, as well as in natural isolates, indicating that NBUs are also being spread among *Bacteroides* strains in nature (2, 19). The first NBUs to be described were cryptic, but an NBU-like element that carries a cefoxitin resistance gene, Tn*4555*, has now been identified (24). Thus, NBUs may also be contributing to the spread of antibiotic resistance genes within *Bacteroides* spp.

Although *Bacteroides* spp. have a gram-negative cell wall, they are not closely related to *Escherichia coli* and other facultative gram-negative bacteria (13, 15). In general, *Bacteroides* promoters do not work in *E. coli* and vice versa, and the broad-host-range IncP plasmids that can replicate widely in bacterial genera related to *E. coli* do not replicate in *Bacteroides* spp. (17, 28). Nonetheless, previous work had suggested that NBUs might be capable of transferring from *Bacteroides* spp. to *E. coli* and related genera (22). This possibility was raised by our finding that when the mobilization region of NBU1 or NBU2 was cloned into a specially constructed shuttle vector, the chimeric plasmid could be mobilized by coresident CTs from *Bacteroides* donors to *E. coli* recipients (9, 10, 22). Plasmids carrying the NBU1 mobilization region were also mobilized by IncP plasmids from *E. coli* donors to *E. coli* or *Bacteroides* recipients (9, 10, 22). For productive transfer of wild-type NBUs from *Bacteroides* spp. to *E. coli* to occur, however, the NBU would have to be able to integrate in *E. coli*. To move from *E. coli* to another host, it would have to be able to excise in *E. coli*. In this report, we present evidence that NBU1 can integrate in *E. coli.*

In an accompanying paper (21), we report the sequence of the primary integration site of NBU1, BT1-1. A comparison of the sequence of BT1-1 with that of the joined ends of NBU1 (*attN1*) revealed two short regions of sequence similarity that might be important for integration. Region I consisted of a 14-bp sequence, within which the integration crossover occurred. Region II, which was within a few base pairs of region I, consisted of a 10-bp sequence that was part of an inverted-repeat region (Fig. 1). We report here that integration events in *E. coli* provided us with the sequences of several other NBU1 integration sites, which were much poorer targets than the primary site was. Analysis of these sites shows that inte-

* Corresponding author. Mailing address: Department of Microbiology, 407 S. Goodwin Ave., University of Illinois, Urbana, IL 61801. Phone: (217) 333-2061. Fax: (217) 244-6697.

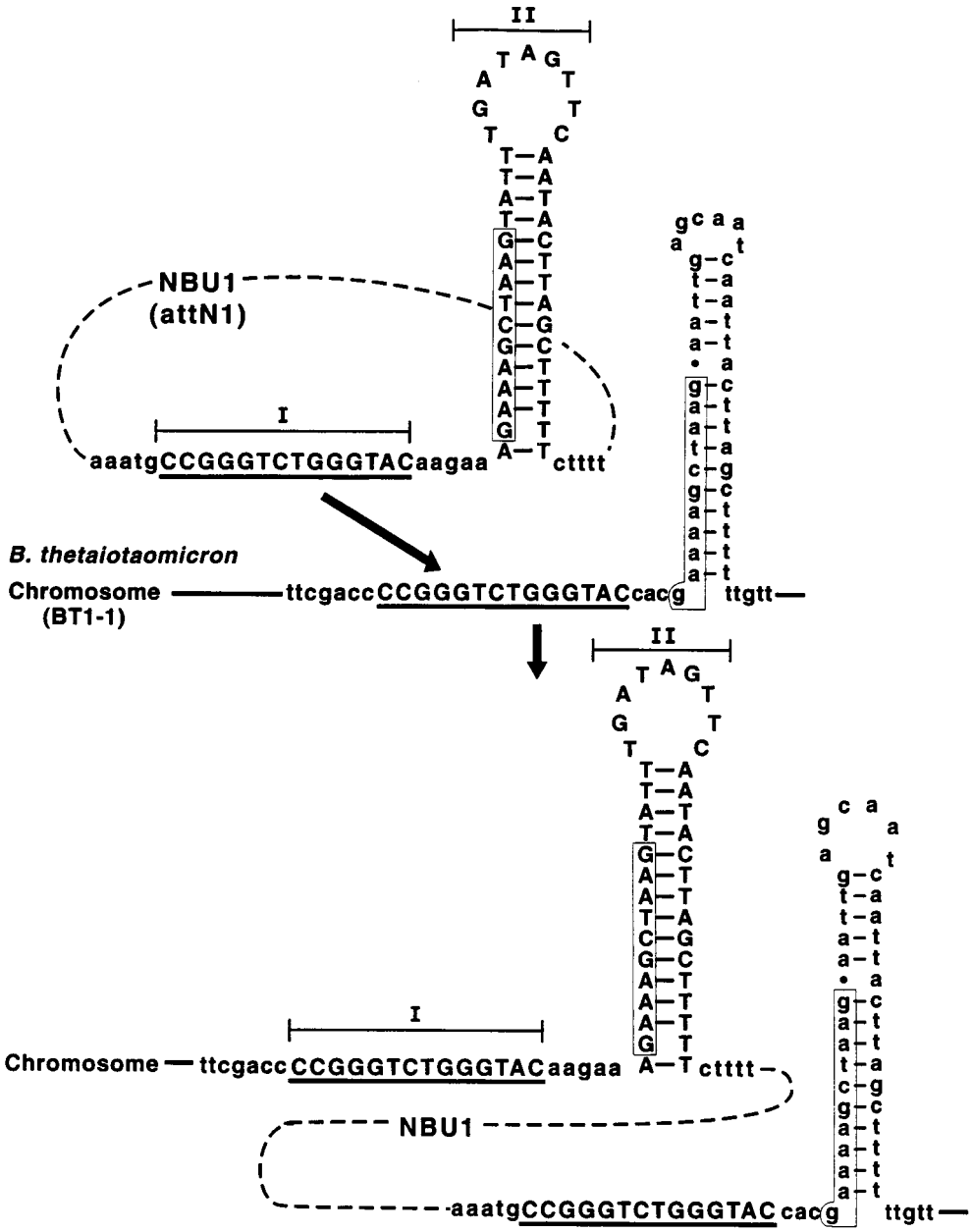

FIG. 1. Diagram of NBU1 integration into its primary site in *B. thetaiotaomicron*, showing region I and region II of *attN1* in relation to the primary integration site, BT1-1. Region I comprises the 14-bp region on *attN1* (underlined) that is identical to a corresponding 14-bp region in the primary *attB* site, BT1-1. The crossover event takes place within or at an end of region I. Region II is a 10-bp sequence (boxed) that lies within a region of dyad symmetry in both *attN1* and *attB*. The positions of region I and region II of integrated NBU1 are shown in the lower portion of the figure. Note that *attN1* is reconstructed at the left junction, *attL*, and the *attB* site, BT1-1, is reconstructed at the right junction, *attR*.

gration of NBU1 can occur within an imperfect region I, with no region II homology nearby.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown in Luria broth (LB) or on LB agar plates (16).

DNA extractions and Southern blot analysis. Plasmids were isolated by the Ish-Horowitz modification of the alkaline lysis procedure (16). Total DNA was isolated by using a modification of the method of Saito and Miura (14). Restriction enzyme digests of the DNA and cloning procedures were done according to the manufacturers' suggestions (GIBCO-BRL, New England Biolabs, or Promega) or as described by Sambrook et al. (16). Southern blot analysis was done as previously described (16, 20). The probes were gel-purified DNA fragments which were labelled with [^{32}P]dCTP by using the Random Prime-It II kit (Stratagene, La Jolla, Calif.).

Filter matings. The conditions for filter matings have been described previously (17, 28). Briefly, the mating mixture was incubated aerobically at 37°C on LB agar plates; the donor-to-recipient ratio was either 1:1 or 1:3. The antibiotic concentrations for the selection of the *E. coli* transconjugants were as follows: streptomycin, 200 μg/ml; nalidixic acid, 100 μg/ml; trimethoprim, 100 μg/ml; ampicillin, 100 μg/ml; and chloramphenicol, 20 μg/ml.

Studies of NBU1 integration into plasmid R388 in *E. coli*. Initially, we used conjugation experiments to determine if NBU1 could integrate into the IncW plasmid, R388 (29). In these experiments, the *E. coli* donor strain (DH5αMCR) contained R388 (trimethoprim resistant) (Tp^r) and an NBU1-containing plasmid, Y11DP (tetracycline resistant and ampicillin resistant) (Tc^r Ap^r) (18, 22). Y11DP is derived from Y11, a chimera that was produced when the *E. coli-Bacteroides* shuttle vector pEG920 inserted into the circular form of NBU1 in

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Description and/or source (reference)
E. coli strains		
DH5αMCR	RecA	GIBCO-BRL
HB101	Str^r RecA	3
EM24	Str^r RecA	RecA derivative of LE392 (17)
SF8	Str^r RecB RecC	W. Resnikoff
SY327	Nal^r Rif^r λ*pir*	12
BW19851	RecA Tra^+ Tp^r Sm^r Pir^+	*E. coli* S-17 (23) with R6K *pir* in *uidA* (11)
Plasmids		
R388	Tp^r	IncW plasmid used to trap NBU1 cointegrate in *E. coli* (29)
pUC19	Ap^r	*E. coli* cloning vector (30)
pUC::BT1-1	Ap^r	NBU1 primary *B. thetaiotaomicron* integration site, BT1-1, cloned in pUC19 (22)
pUC::BT1-2	Ap^r	NBU1 secondary *B. thetaiotaomicron* integration site, BT1-2, cloned in pUC19 (this study)
pIA_{18}	Ap^r Mob^+ (Int^+ Exc^-)	2.7-kbp region of NBU1 cloned in pNV19, containing *attN1* and *intN1* (21); same as NBU1 region cloned in pNPR-IA except for 60 bp of additional DNA upstream of *attN1*
pEP185.2	Cm^r, R6K *oriV*	*pir*-requiring R6K *oriV* replicon containing RP4 transfer origin and Cm^r from pACYC184 (12)
pNPR*attN1*	Cm^r, R6K *oriV*	NBU1 *attN1* cloned in *Sma*I site of pEP185.2 (this study)
pNPR-IA	Cm^r, R6K *oriV* Int^+	2.6-kbp *Pvu*II fragment of pIA_{46}, containing NBU1 *attN1* and *intN1*, cloned in *Sma*I site of pEP185.2 (this study)
Y5D, Y11D	Ap^r Tc^r Mob^+ (Em^r Mob^+ Rep^- Int^+ Exc^+)	pEG920::NBU1 cointegrates, Y5 and Y11, with pB8-51 replicon deleted (18, 22)
Y11DP	Same as Y11D except Exc^-	3-kbp *Pst*I deletion of NBU1 sequences in Y11DP (22)

[a] Phenotypes in parentheses are expressed only in *Bacteroides* spp.; phenotypes not in parentheses are expressed in *E. coli*. Abbreviations: Em^r, erythromycin resistant; Rif^r, rifampin resistant; Sm^r, streptomycin resistant due to Tn7; Mob, mobilization by Tra function of IncP plasmids or (if in parentheses) by *Bacteroides* conjugative transposons; Rep, replication; Int, integration; Exc, excision; *oriV*, origin of replication.

such a way as to leave the joined ends and integrase gene of NBU1 intact (18, 21, 22). The *Bacteroides* replication region of pEG920 and part of NBU1 were deleted from Y11 to produce Y11DP. Y11DP still contains enough of NBU1 to integrate into the *Bacteroides thetaiotaomicron* chromosome but is excision defective. The donor strain containing Y11DP and R388 was filter mated with *E. coli* EM24, and streptomycin-resistant (Str^r) Tp^r Tc^r transconjugants were selected. Cotransfer of these resistances should occur only if Y11DP had formed a cointegrate with R388. Plasmid preparations were made from Str^r Tp^r Tc^r transconjugants to confirm that a cointegrate had formed. Southern blot analysis was done to determine whether the NBU1 in Y11DP had integrated into R388 via its ends. In these experiments, the probe consisted of a 1.5-kbp *Hinc*II fragment of NBU1 that contained *attN1*. To determine the locations of NBU1 insertions in R388, plasmid preparations of the R388::Y11DP cointegrates were digested with *Bam*HI and then ligated and transformed into DH5αMCR. Tc^r Tp^s transformants were isolated. Since there are no *Bam*HI sites on Y11DP, the resulting transconjugants yielded plasmids containing Y11DP plus both of the NBU1-R388 junctions. Some of the insertion events had occurred within a 0.4-kbp *Bam*HI fragment of R388, whose sequence was known (27). The left and right junction primers of NBU1 (22) were used to obtain enough DNA sequence from the junctions to determine the precise insertion site of NBU1 in the R388 *Bam*HI fragment. Although these experiments showed that NBU1 could integrate into R388, the cointegrates containing Y11DP and R388 were quite unstable, probably because of the presence of two *E. coli* plasmid replication origins. Accordingly, a different approach to detecting integration of NBU1 was taken in subsequent experiments.

Construction of new NBU1 insertion vectors for use in *E. coli*. To develop a more defined and stable system for investigating NBU1 integration in *E. coli*, insertion vectors based on the R6K replication system were constructed (7). Plasmids carrying the R6K *oriV* can replicate only in hosts that also carry the RGK *pir* gene. In strains that do not contain *pir*, such plasmids do not replicate and can integrate into the genome. A 530-bp fragment of NBU1, which included the joined ends of NBU1 but lacked the NBU1 integrase, was cloned into the *pir*-dependent vector pEP185.2 (6, 12) to form pNPR*attN1*. The joined ends of NBU1 that were cloned into pEP185.2 included region I and region II (Fig. 1), which are identified in an accompanying paper as being potentially important for NBU1 integration (21). Another plasmid was constructed (pNPR-IA) that contained the joined ends of NBU1 plus the NBU1 integrase gene (*intN1*). This was done by cloning of a 2.6-kbp *Pvu*II fragment from pIA_{46}, a plasmid that contained the minimal region of NBU1 required for integration in *B. thetaiotaomicron* (21), into the *pir*-requiring plasmid pEP185.2. pEP185.2 carries a chloramphenicol resistance (Cm^r) gene that confers Cm^r on *E. coli* regardless of whether it is present on the multicopy plasmid or integrated into the *E. coli* chromosome. *E. coli* BW19851 (11) was used as the *pir*-containing host, and *E. coli* EM24 and HB101 were used as hosts that did not contain *pir*.

To test for integration of NBU1 into the *E. coli* chromosome, pNPR-IA or pNPR*attN1* was mobilized by the transfer (Tra) functions of the IncP plasmid RP4 integrated in BW19851 (*pir* Tp^r Cm^r) to *E. coli* HB101 or EM24. In some cases, HB101 or EM24 recipients contained plasmids which provided *attN1*, *attB*, and/or *intN1*. Transconjugants containing insertions of pNPR-IA or pNPR*attN1* in the chromosome or in the resident plasmid were selected as Str^r Cm^r Tp^s transconjugants from the BW19851 (Tp^r Cm^r) donors. The streptomycin concentration necessary for selecting the transconjugants had to be 200 μg/ml or higher because BW19851, a *pir*-containing derivative of S17-1, contains Tn7 (11, 23). In some experiments, the plasmids were transferred to a *pir*-containing recipient, SY327 (12). In this case, selection was for nalidixic acid-resistant (Nal^r) Cm^r Tp^s transconjugants.

The *E. coli* EM24 chromosomal insertion sites for NBU1 were cloned by digesting the DNA of the EM24::NPR-IA transconjugants with *Pst*I; ligating the mixture and transforming the *pir*-containing recipient strain, SY327; and selecting for Cm^r transformants. This method for cloning the junction region of the integrated NBU1 was possible because the single *Pst*I site of pNPR-IA (which occurred in the multiple cloning site) had been eliminated during the cloning process. The NBU1::chromosome junctions cloned in these transformants were sequenced by using the left and right junction primers of NBU1, and the sequences obtained were analyzed by computer searches with the BLAST and Genetics Computer Group sequence analysis programs (1, 5) to determine if the insertion had occurred within a reported *E. coli* gene or sequence. To test for integration of NBU1-containing plasmids into a plasmid that carried the primary or secondary NBU1, integration sites from *B. thetaiotaomicron* (BT1-1 or BT1-2, respectively) were cloned into pUC19 (30) and provided in *E. coli* HB101 or EM24 recipients. In some cases, pIA_{18} (21), which carried the NBU1 *intN1* and *attN1* genes, was also present in the donor or recipient to provide the NBU1 integrase in *trans*. The main difference between pIA_{18} and pNPR-IA was that pIA_{18} replication was *pir* independent whereas replication of pNPR-IA was *pir* dependent.

RESULTS AND DISCUSSION

NBU1 integration into R388. To determine if NBU1 could integrate in *E. coli*, we took advantage of the fact that the IncW plasmid R388 does not mobilize the NBU1-containing plasmid Y11DP in *trans* (22). Since the joined ends of NBU1 in Y11DP

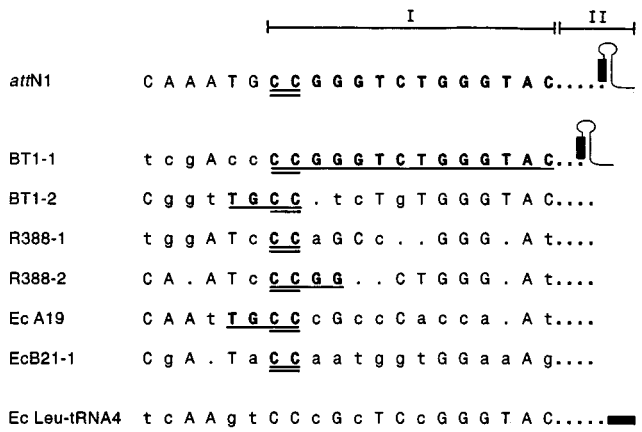

FIG. 2. Comparison of NBU1 *attB* sites in *B. thetaiotaomicron* and *E. coli*. The NBU1 *attN1* is shown at the top with the 14-bp sequence of region I in bold capital letters and a representation of the 10-bp region (filled rectangles) included in an inverted-repeat region at the 3′ end (shown as a stem-loop structure). The primary *B. thetaiotaomicron attB* site, BT1-1, contains both region I and region II, but the secondary *attB* site, BT1-2, contains only an imperfect region I and lacks all of region II. The *E. coli attB* sites in the IncW plasmid R388 (R388-1 and R388-2) and the *E. coli* chromosomal *attB* sites (Ec A19 and EcB21-1) are also shown. EcB21-1 was deduced from *attL* and *attR* sequences of integrated NBU1, whereas the other *attB* sites were determined directly. All four of the *E. coli attB* sites had partial identity to region I and no region II. The identical nucleotides in the target sequences are shown as capital letters. The bold, underlined capital letters were found at both of the NBU1-*attB* junctions and are where the crossovers must have occurred. For the NBU1 insertions into any of the *attB* sites, the *attL* was the *attN1* sequence and the *attR* was the *attB* sequence. This could occur if the crossover between *attN1* and the *attB*s was adjacent to or within the doubly underlined C's or if the crossover occurred at the first mismatch between the region I sequences of *attN1* and *attB*. The sequence of the *E. coli* (Ec) Leu-tRNA4 (8), as a possible *attB* site, is shown at the bottom. The nucleotides with identity to region I are shown as capitals, and the location of the 10-bp sequence of region II with 90% identity to *attN1* but which is not included in an inverted-repeat region is indicated by the filled rectangle. No NBU1 insertions in this sequence were isolated.

are free to integrate, Y11DP should be able to invade R388 if NBU1 is capable of integrating in *E. coli* and if R388 carries a suitable integration site. Cointegrates containing both R388 and Y11DP were isolated at frequencies of 5.6×10^{-7} to 6.4×10^{-6}, relative to the transfer of R388 alone. Restriction and Southern blot analyses of the cointegrates indicated that integration had occurred via the ends of NBU1 and that insertions had occurred in more than one site on R388.

The sequences of the two R388 insertion sites, R388-1 and R388-2, are shown in Fig. 2 and compared with the *B. thetaiotaomicron* primary and secondary insertion sites (BT1-1 and BT1-2, respectively). The bold, underlined bases shown in Fig. 2 were repeated at both ends of the inserted Y11DP. These results suggest that the crossover points on R388 lay within or at either end of these sequences. The sequence data also confirmed that the integration event had occurred within region I of *attN1*, the region where the integration occurs in *B. thetaiotaomicron*. In contrast to the situation for the *B. thetaiotaomicron* primary site, there were several mismatches in region I and all of region II was missing from the target sites on R388. Previously, we had found that an NBU1 secondary site in *B. thetaiotaomicron*, BT2-1 (Fig. 2), also had an imperfect region I and no region II, but integration in this site had only been seen to occur in one isolate of the many screened. Thus, we could not rule out the possibility that integration into this site had occurred by some abnormal route. The finding that integration events that occurred readily in *E. coli* also occurred at target sites without an intact region I and with no region II provided further evidence that a perfect region I and region II are not essential for integration.

Integration of *pir*-dependent vectors, pNPR-IA and pNPR*attN1*, in *E. coli*. There were two possible explanations for the fact that NBU1 integrated site specifically in *B. thetaiotaomicron* but relatively randomly in *E. coli* when R388 was the target. First, R388 may not have contained a good primary-site sequence. Consistent with this hypothesis, the frequency of integration into R388 was orders of magnitude lower than that estimated for integration into the *B. thetaiotaomicron* chromosomal site (10^{-3} to 10^{-2} transconjugants per recipient). Alternatively, *Bacteroides* spp. might have a special host factor that was responsible for site specificity in *B. thetaiotaomicron* but was missing in *E. coli*. If the first explanation is correct, it should be possible to increase the frequency of NBU1 integration in *E. coli* and make integration site specific by providing a good primary site in *E. coli*. Previously, we had found that in two different *Bacteroides* spp., *B. thetaiotaomicron* and *B. uniformis*, the primary NBU1 insertion sites were located at the 3′ end of a Leu-tRNA gene (21). Since the *E. coli* Leu-tRNA4 sequence had significant sequence similarity to the NBU1 *attN1* and to the *B. thetaiotaomicron* and *B. uniformis* primary attachment sites (8, 21), we wanted to determine whether NBU1 would integrate site specifically in this *E. coli* site.

To monitor integration of NBU1 into the *E. coli* chromosome, we constructed insertion vectors based on the R6K *pir* system (7, 12) (Fig. 3). One of these (pNPR-IA) contained both the NBU1 *attN1* region and the NBU1 integrase gene. The region of NBU1 cloned in pNPR-IA had proved to be sufficient for site-specific integration into the *B. thetaiotaomicron* primary site, BT1-1, at frequencies similar to those for integration of wild-type NBU1 into this site (21, 22). This plasmid was used for the initial experiments to test for integration of NBU1 into the *E. coli* chromosome. A second vector that contained only a 530-bp *attN1* region segment of NBU1 (pNPR*attN1*) was used as a control to determine whether integration of pNPR-IA into the *E. coli* chromosome, if it occurred, was dependent on the NBU1 integrase gene. Both of these vectors replicated in the *pir*-containing hosts, such as BW19851, and could be mobilized out of BW19851 donors to a *pir*-containing recipient, SY327, at frequencies of 0.4 to 0.8 per recipient (Table 2). pNPR-IA integrated in recipients that lacked *pir* at frequencies of over 10^{-5} per recipient, regardless of whether the recipient was $RecA^+$ or $RecA^-$ (Table 2). The fact that pNPR*attN1* did not integrate into the *E. coli* chromosome ($<10^{-8}$ transconjugants per recipient) shows that the NBU1 integrase gene was required for integration in *E. coli.*

To determine if the insertions into the *E. coli* chromosome were occurring site specifically in the 3′ end of the Leu-tRNA gene, chromosomal DNA from several independently isolated *E. coli* transconjugants was probed with the 1.5-kbp *Hin*cII fragment of NBU1, which included *attN1* (Fig. 2). If pNPR-IA integrated site specifically, all of the transconjugants would have hybridizing bands of the same size. This was not the case (Fig. 4), although several integration events appeared to have occurred in the same site. The junction regions of three of the insertions, which appeared to be in the same site, were cloned and sequenced. None of the three insertions had occurred in the 3′ end of Leu-tRNA4. One had occurred downstream of the 3′ end of the *relA* gene of *E. coli* (Ec A19, Fig. 2). The other two occurred in the same site, EcB21-1, but no gene in this region was identified in the computer searches. These *E. coli attB* sites are shown in Fig. 2. Neither of these sites had the 10-bp sequence of region II or a region of dyad symmetry similar to that of region II near the crossover region. They also lacked a complete region I sequence.

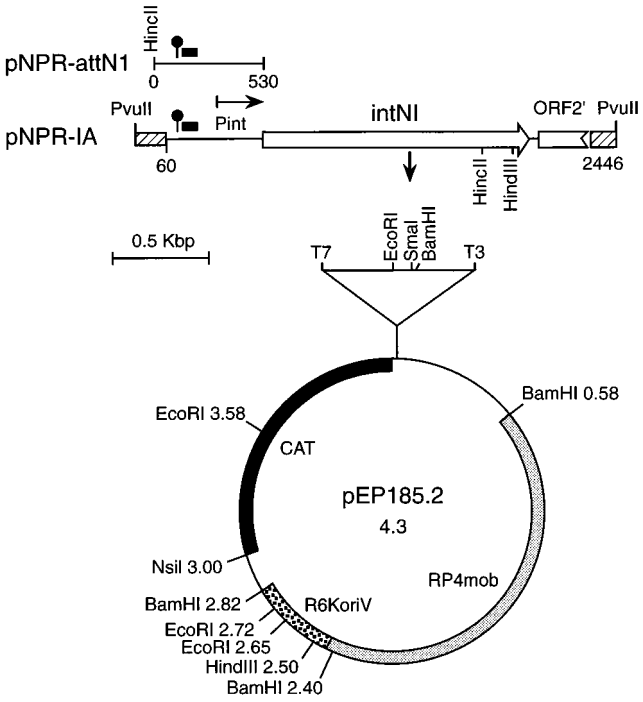

FIG. 3. Construction of the R6K *oriV pir*-requiring NBU1 integration vectors, pNPR*attN1* and pNPR-IA. A 530-bp stretch of NBU1 containing the *attN1* region was cloned into the *Sma*I site of pEP185.2 (12), in the direction indicated, to form pNPR*attN1*. The filled-lollipop structure and the filled rectangle represent, respectively, the region of dyad symmetry (which contains region II) and the 14-bp crossover sequence (region I) of *attN1*. Both the NBU1 *attN1* and the NBU1 integrase gene (*intN1*) were cloned from pIA_{46} (21) on a 2.6-kbp *Pvu*II fragment into the *Sma*I site of pEP185.2, to form pNPR-IA. The residual pUC19 sequences are shown as filled regions at the end of the *Pvu*II fragment. The direction and extent of NBU1 *intN* and the location of the truncated ORF2, ORF2′, are indicated. pEP185.2 contains the origin of transfer for RP4 (RP4mob), the origin of replication of R6K (R6KoriV), the chloramphenicol acetyltransferase (CAT) gene from pACYC184, and the multiple cloning region from the Bluescript plasmid (6, 12).

TABLE 2. Frequency of transfer and insertion of pNPR*attN1* and pNPR-IA from BW19851 donors to *E. coli* recipients

Plasmid(s) in donor	Plasmid(s) in recipient[a]	Frequency[b]	Relative frequency[c]
pNPR*attN1*	pUC19	$<4 \times 10^{-8}$	1
	pUC::BT1-1	$<6 \times 10^{-8}$	
	pUC::BT1-2	$<6 \times 10^{-8}$	
	pIA_{18}	0.1×10^{-3}–1×10^{-3}	>2,000
	pUC::*attN1*	$<6 \times 10^{-8}$	
pNPR*attN1*, pIA_{18}	pUC19	$<1.7 \times 10^{-8}$	1
	pUC::BT1-1	$<1.8 \times 10^{-8}$	
	pUC::BT1-2	$<7.0 \times 10^{-8}$	
	pIA_{18}	0.1×10^{-3}–1.0×10^{-3}	>5,000
pNPR-IA	pUC19	1.0×10^{-5}–7.0×10^{-5}	1
	pUC::BT1-1	7.0×10^{-4}	10–70
	pUC::BT1-2	1.1×10^{-5}	0.6–1
	pIA_{18}	0.1×10^{-3}–4.0×10^{-3}	10–400
	pUC::*attN1*	7.0×10^{-4}	10–70

[a] HB101 or EM24 containing the indicated pUC19-based vector.
[b] Frequency of Cm^r transconjugants per recipient, averaged over four or more experiments. Ranges are given when greater-than-fivefold differences were observed. The transfer frequency to SY327 was 0.4 to 0.8 for both pNPR*attN1* and pNPR-IA.
[c] Frequency relative to that seen when pUC19 was in the recipient.

There was a high degree of sequence similarity between region I of *attN1* and region I in the *E. coli* Leu-tRNA4 3′ end and between the 10-bp sequence of region II of *attN1* and a similarly positioned 10-bp region downstream of the *E. coli* Leu-tRNA4 gene, although this 10-bp *E. coli* sequence did not occur within a region of dyad symmetry. One explanation for the fact that no events of NBU1 integration into this site were detected, despite the fact that it was more similar to the sequence of the primary site in *B. thetaiotaomicron* than were the sites into which NBU1 actually integrated in *E. coli*, is that insertion of NBU1 in this site was a lethal event. We noted that recipient viability was 10-fold lower than expected (10^8 rather than 10^9 CFU/ml) for both $RecA^+$ and $RecA^-$ recipients, and this could be the result of deleterious effects of integrating NBU1 into the *E. coli* Leu-tRNA gene.

To test the hypothesis that NBU1 would integrate site specifically and at higher frequency in *E. coli* if the primary *B. thetaiotaomicron attB* site, BT1-1, was provided in the *E. coli* recipients, the primary *B. thetaiotaomicron attB* (BT1-1) was cloned into pUC19 to form pUC::BT1-1. The frequency of pNPR-IA integration in recipients containing pUC::BT1-1 was 10 to 70 times higher than it was if the recipient contained no plasmid (Table 2). Plasmid preparations obtained from the transconjugants revealed cointegrates containing both pNPR-IA and pUC::BT1-1, but the cointegrates were only a minor component of the plasmid population. Accordingly, cointegrates had to be isolated for further analysis. Restriction mapping and Southern blot analysis showed that in all cases, integration had occurred between the *attN1* of pNPR-IA and the *attB* of pUC::BT1-1 (data not shown). Thus, the presence of BT1-1 in the recipient not only increased the frequency of the insertions but also increased site specificity. This result suggests that the site specificity in *B. thetaiotaomicron* is due to the availability of a good primary *attB* site and not to a special host factor that is present only in *B. thetaiotaomicron*. It is important to keep in mind, however, that BT1-1 in *E. coli* was supplied on a high-copy-number plasmid, rather than as a single chromosomal copy as in *Bacteroides* spp. The high copy number of the *attB*

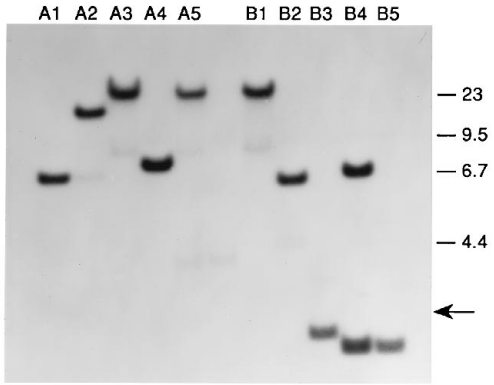

FIG. 4. Location of NBU1 (pNPR-IA) insertions in EM24 transconjugants. The DNAs from five chloramphenicol-resistant transconjugants from each of two matings (A and B lanes, respectively) between BW19851 (pNPR-IA) and EM24 were digested with *Bam*HI and *Eco*RI and run on an agarose gel. The Southern blot of the gel was probed with the ^{32}P-labelled 1.5-kbp *Hin*cII fragment of NBU1 containing *attN1* (Fig. 3). Each insertion should result in two junction bands; however, one of the junction bands is very faint because of the proximity of the *attN1* region to the end of the probe. The isolate in lane B4 had two insertions, but this was the only such double insertion isolated. The sizes in kilobase pairs and locations of the *Hin*dIII fragments of lambda are indicated at the right, and the arrow indicates where the 2.7-kbp *Bam*HI-*Eco*RI *attN1* fragment of pNPR-IA would run if the circular form were present. The failure to see a band at this point indicates that the integrated pNPR-IA is not excising in *E. coli.*

site could have influenced the frequency of integration into this site. The secondary site, cloned into pUC19 (pBT1-2), was also tested as a target. In this case, there was no increase in integration frequency (Table 2), and all of the integration events occurred in the *E. coli* chromosome rather than in the plasmid.

A comparison of the NBU1 secondary *attB* sites found in *E. coli* and *Bacteroides* hosts (Fig. 2) suggests that the crossovers could be occurring adjacent to or within the first two CC's of region I. This would explain why the sequence of the *attL* at the left junction of an NBU1 insertion was always the *attN1* sequence and the *attR* sequence at the right junction was always the *attB* sequence. Alternatively, the same results would be obtained if the crossovers occurred at the first mismatched base pair within or immediately following region I. When either of these possible mechanisms is taken into consideration, a closer examination of the possible *attB* region I of the *E. coli* Leu-tRNA4 suggests that an insertion in this 14-bp region with the crossover occurring either adjacent to or within the first two C's or at the first mismatch would result in two major changes in the tRNA sequence and structure. First, it would create disruption of the stem portion of the T-psi-C loop of the tRNA because of two mismatches (C to G in positions 3 and 5), and secondly, it would change the conserved CCA-3′ of *E. coli* tRNA sequences (8) to CAA-3′. Either of these disruptions could be lethal in *E. coli*, and this lethality might be partially responsible for the low integration frequency observed and the lower-than-expected viability of recipients.

Integrase is required in the recipient. Bringel et al. (4) reported that Tn*916* integrase produced in the donor was transferred to the recipient with the transposon and was sufficient for integration in the recipient, although the frequency of integration was over 1,000-fold lower than it was if the integrase was provided in the recipient. In this case, an integrase-defective derivative of Tn*916*Kan was inserted in the chromosome and the Tn*916* integrase was provided in *trans* on a plasmid or on a wild-type Tn*916*(Tc). Transfer of the defective Tn*916*Kan was monitored. In our experiments, NBU1 was already in the excised circular form in pNPR*attN1* and thus would not require integrase for the excision step. To determine whether IntN1 could be provided in the donor or if IntN1 had to be provided in the recipient, we did the following experiments. The NBU1 *attN1* was on one plasmid (pNPR*attN1*) in the donor, and the NBU1 integrase was provided in *trans* on a second compatible plasmid (pIA_{18}) in either the donor (BW19851) or the recipient strain (EM24 or HB101). No transconjugants were observed from matings in which the donor carried pIA_{18}, but transconjugants were obtained when pIA_{18} was provided in the recipient (Table 2). Therefore, the NBU1 integrase must be present in the recipient.

pIA_{18} contained not only the *intN1* gene but also the *attN1* site adjacent to the *intN1* gene plus 60 bp of additional DNA upstream of the *attN1* region compared with the DNA cloned in pNPR-IA. The fact that *attN1* was on this plasmid raised the question of whether NBU1 was integrating into its own *attN1* on pIA_{18} rather than into the *E. coli* chromosome. We analyzed 20 transconjugants of matings in which pNPR*attN1* was in the donor and pIA_{18} was in the recipient. In all 20 cases, pNPR*attN1* had integrated into the *attN1* site of pIA_{18}. Thus, NBU1 can integrate into its own *att* site. The frequency of integration into pIA_{18} was nearly 10-fold higher than that into pBT1-1 (Table 2). If the *attN1* alone was provided on a plasmid without the *intN1* gene (pUC::*attN1*), pNPR-IA could still integrate into *attN1* but the integration frequency was the same as when pUC::BT1-1 was the target (Table 2). The fact that integration into pIA_{18} occurred at a higher frequency than did integration into pUC::*attN1* is probably due to the increased amount of IntN1 in the recipients carrying pIA_{18}.

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