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Identification and DNA Sequence of the Mobilization Region of the 5-Nitroimidazole Resistance Plasmid pIP421 from *Bacteroides fragilis*

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The nucleotide sequence of the DNA mobilization region of the 5-nitroimidazole resistance plasmid pIP421, from strain BF-F239 of *Bacteroides fragilis*, was determined. It contains a putative origin of transfer (*oriT*) including three sets of inverted repeats and two sequences reminiscent of specific integration host factor binding sites. The product of the mobilization gene mob_{421} (42.2 kDa) is a member of the *Bacteroides* mobilization protein family, which includes the MobA of pBI143, NBUs, and Tn*4555*. Sequence similarity suggests that it has both *oriT* binding and nicking activities. The transfer frequency of pIP421 in a *B. fragilis* donor strain possessing a Tc^r or Tc^r Em^r-like conjugative transposon was significantly enhanced by tetracycline. Moreover, the mobilization region of pIP421 confers the ability to be mobilized from *Escherichia coli* by an IncP plasmid.

Moderate resistance of *Bacteroides* spp. to 5-nitroimidazole (5-Ni) has only recently appeared in some clinical isolates (2). The resistance of these strains is due to the presence of specific 5-Ni resistance *nim* genes (18). Epidemiological data have shown that in France the frequency of 5-Ni-resistant *Bacteroides* strains is increasing. The spread of the resistance among these significant opportunistic pathogens, which are responsible for various infectious diseases, could be explained by the fact that *nim* genes are usually found on mobilizable genetic elements, either in non-self-transferable plasmids or on the chromosome. Three such 5-Ni^r plasmids (pIP417, pIP419, and pIP421) have already been characterized, and the mobilization properties of one of them, pIP417, has been fully analyzed (25). The mobilization region of plasmid pIP417 is closely related to that of both the erythromycin resistance plasmid pBFTM10 from *Bacteroides fragilis* (8) and pIP419 but not to that of pIP421. Conjugal transfer of pIP417 is stimulated by tetracycline (TET) in a strain carrying a conjugative transposon of the Tc^r Em^r DOT family on the chromosome. These elements possess regulatory genes involved in control of their self-transfer activities and can also interfere with the regulation of coresident mobilizable plasmids (22). They could thus be responsible for the rapid spread of plasmid-borne resistance genes. We therefore examined the transfer properties of pIP421 and compared them with those of mobilizable genetic elements previously identified in *Bacteroides* species. The experimental procedures used in the present study have been previously described (25).

The conjugal transfer properties of plasmid pIP421 in *B. fragilis*. The conjugal transfer properties of pIP421, native to the strain BF-F239 (5-Ni^r Tc^r Em^r) of *B. fragilis*, were first investigated through mating experiments in which the 5-Ni^s strain BF-2 of *B. fragilis* was used as the recipient (Table 1). When the donor strain was pregrown in the absence of inducer, pIP421 was transferred at a low frequency (3.5×10^{-8} per recipient). The frequency was increased up to 100-fold if TET or chlortetracycline (ClTET) was added to the growth medium of the donor strain. Thus, strain BF-F239 may contain a chromosomal conjugative transposon required for efficient pIP421 transfer, probably regulated by the products of the *rteB* and/or *rteC* genes (21). To test this hypothesis, the conjugal transfer of the plasmid pIP421 was studied through mating experiments with strain BF-2, containing a Tc^r Em^r DOT conjugative-type transposon (25), as the donor and strain BF-638R (5-Ni^s Rf^r) of *B. fragilis* as the recipient. The transfer of pIP421 was effectively enhanced up to 500-fold in the presence of TET, and the transfer frequency of the plasmid was in the same range as that of the chromosomal Tc^r determinant of strain BF-2 (1×10^{-5} versus 1.3×10^{-5}). In these matings the cotransfer of both determinants was approximately 10%. Reciprocal matings were also performed. Plasmid pIP421 was transferred back from the donor strain BF-638R to strain BF-2 at a frequency of 1.4×10^{-7} transconjugants per recipient. Thus, pIP421 was transferable from all donor strains tested, but the presence of coresident conjugative transposons providing a transfer function in *trans* was required for efficient mobilization of the plasmid.

Localization and DNA analysis of the mobilization region of pIP421. Based on previous studies (7, 26), the mobilization region was suspected to be located within the 2.9-kb *Dra*I restriction fragment of plasmid pIP421 or within the 1.2-kb fragment between the *nimD* and *repA* genes (Fig. 1). To identify the genetic locus more precisely, three deleted derivatives of pIP421 (pFK326, pFK329, and pFK331) were constructed and tested for their mobilization properties (Fig. 1). The results suggest that the mobilization region of pIP421 maps to the 2.75-kb *Dra*I-*Ava*II restriction fragment. The complete DNA sequence of this restriction fragment was determined (Fig. 2). Based upon an ATG start codon, a large open reading frame of 426 amino acids was identified. The open reading frame, 1,278 bp long, possesses 52.2 mol% G + C, which is out of the range (39 to 46 mol%) of those of genomic DNAs of *Bacteroides* spp. (9). The putative corresponding gene, located within the 1.1-kb *Eco*RV restriction fragment, was not entirely present in either of the mobilization-deficient plasmids pFK326 and pFK329 but was not disrupted in pFK331. It was thus concluded that the product encoded by the gene was

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TABLE 1. Mobilization of plasmid pIP421[a]

Donor		Recipient		Induction[d]	Transfer frequency
Strain[b]	Relevant phenotype[c]	Strain[b]	Relevant phenotype[c]		
BF-F239	5-Ni^r Tc^r Em^r Cc^r	BF-2	Cc^r Tc^r Im^r	None	3.5×10^{-8}
				+ TET	5.6×10^{-6}
				+ CITET	6.2×10^{-6}
BF-2(pIP421)	5-Ni^r Tc^r Cc^r Im^r	BF-638R	Rf^r	None	8.6×10^{-7}
				+ TET	1.0×10^{-5}
BF-638R(pIP421)	5-Ni^r Rf^r	BF-2	Cc^r Tc^r Im^r	None	1.4×10^{-7}

[a] Frequencies are expressed as the number of transconjugants per recipient at the end of the mating. In mating of strains BF-F239 and BF-2, the 5-Ni^r transconjugants were selected on Wilkins Chalgren solid medium containing tinidazole (4 μg/ml) and imipenem (1 μg/ml). In mating of strains BF-2(pIP421) and BF-638R, the 5-Ni^r transconjugants were selected on the same medium containing tinidazole (4 μg/ml) and rifampin (25 μg/ml), and in mating of strains BF-638R(pIP421) and BF-2, transconjugants were selected on the same medium containing tinidazole (4 μg/ml) and clindamycin (5 μg/ml).

[b] References: BF-F239, 19; BF-2, 20; BF-638R, 17.

[c] Abbreviations used for resistance phenotypes are as follows: 5-Ni, 5-nitroimidazole; Tc, tetracycline; Em, erythromycin; Cc, clindamycin; Im, imipenem; Rf, rifampin.

[d] The donor was grown in medium containing a low concentration (1 μg/ml) of TET or CITET before mating.

required for the mobilization of pIP421. The deduced amino acid sequence shares similarities with those of proteins encoded by other mobilization genes from *Bacteroides* spp. found in GenBank, EMBL, and SWISS-PROT databases. The percentages of identity were 40.9% with the MobA protein of the mobilizable plasmid pBI143 (24), 37.6% with that of the chromosomal NBU1 element (10), 36.3% with that of the NBU2 element (11), and 33.8% with that of the transposon Tn*4555* (23). The polypeptide was also very similar in size (425 amino acids) and in molecular mass (42.2 kDa) to these proteins and was therefore designated Mob_{421}. This polypeptide thus belongs to the family of *Bacteroides* mobilization proteins, defined by Smith and Parker (23), which appear to be bifunctional. They bind to the *oriT* region and catalyze the nicking reaction that initiates the transfer process. The amino acid sequences of these proteins present four conserved blocks, some of which are cognates of the characteristic motifs I to IV of the TraI and TraJ proteins from various conjugative plasmids of the RP4 family (16). The amino acid sequence of Mob_{421} was searched for these motifs (Fig. 2). There is a strong homology with motifs I and II, although no significant similarity was detected by pairwise comparisons between amino acid sequences of Mob_{421} and TraI or TraJ of the IncP plasmids performed by the GAP and BESTFIT programs of the Genetics Computer Group package. In contrast, there was no significant similarity with motifs III and IV, despite motif III having been defined as containing the active site of TraI, which is directly involved in the nicking at the *oriT* region.

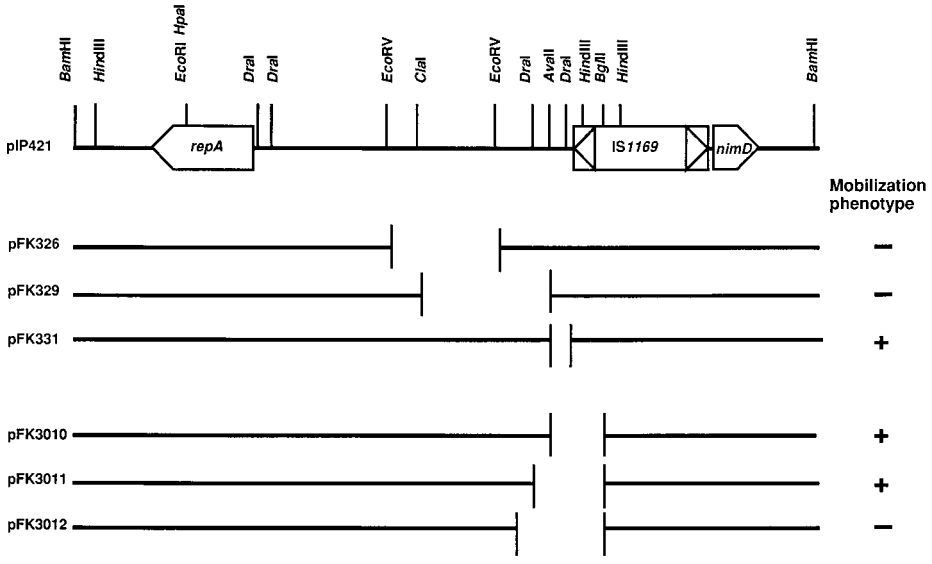

FIG. 1. Partial physical map of plasmid pIP421 and its subclones. The *repA* and *nimD* genes and IS*1169* are shown as open boxes. pFK326 corresponds to a deletion of the 1.1-kb *Eco*RV restriction fragment, pFK329 corresponds to a deletion of the 1.4-kb *Cla*I-*Ava*II restriction fragment, and pFK331 corresponds to a deletion of the 0.45-kb *Ava*II-*Bgl*II restriction fragment. Plasmids pFK3010, pFK3011, and pFK3012 were constructed by deletion of 570, 745, and 920 bp, respectively, from the *Bgl*II site. The mobilization phenotype of the derivatives is indicated on the right of the figure: (+) indicates efficient mobilization and (−) indicates the absence of mobilization from *B. fragilis* BF-2 to BF-638R. The mating experiments were performed by using TET to stimulate the transfer, and the 5-Ni^r transconjugants were selected on Wilkins Chalgren solid medium containing tinidazole (4 μg/ml) and rifampin (25 μg/ml).

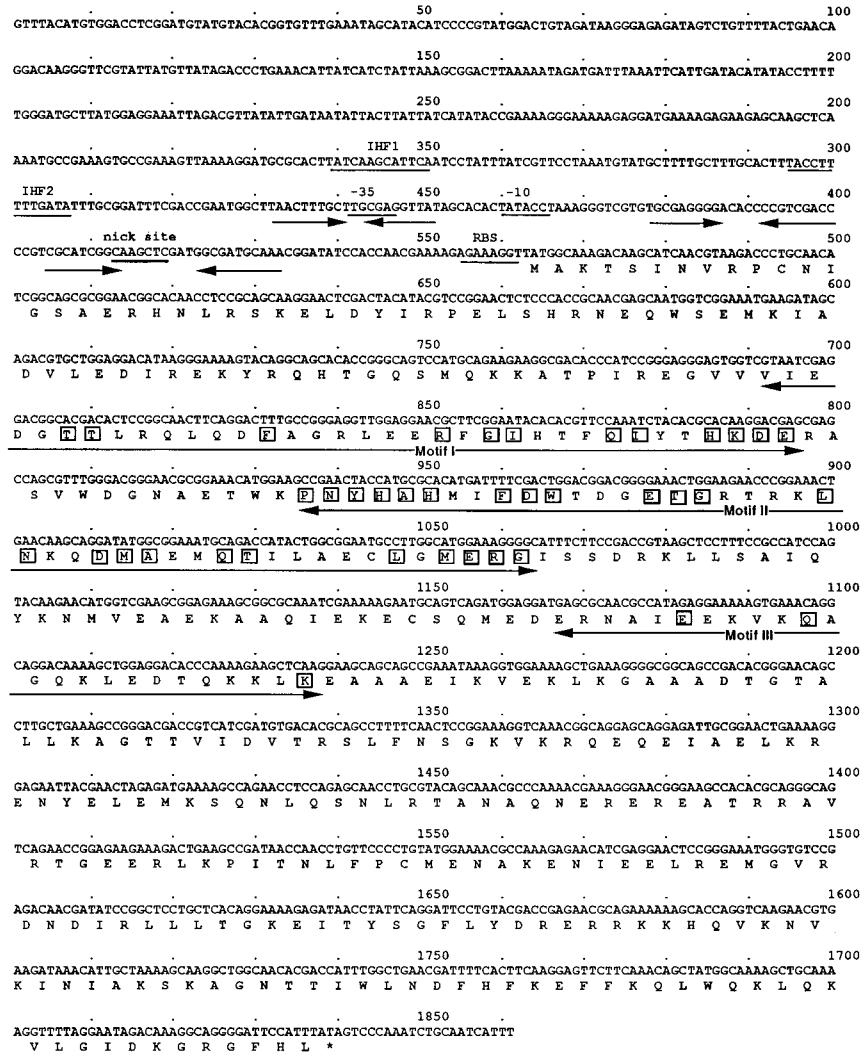

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the mob_{421} gene of plasmid pIP421. The nucleotide sequence of the mob_{421} gene was determined by the Sanger dideoxy sequencing reactions with modified T7 polymerase (Sequenase 2.0 kit; U.S. Biochemical Corp., Cleveland, Ohio). Relevant restriction sites are underlined. The proposed nick site and the putative −35 and −10 promoter sequences of the *mob* gene are underlined and labeled. The possible *Bacteroides* ribosome binding site (RBS) (AGAAAGG) defined by Weisburg et al. (27) is also indicated. The three IR sequences are indicated by arrows. Left and right arms of IR1, IR2, and IR3 present one or two mismatches (IR1L, TAACtTtGC; IR1R, GCgAgGTTA; IR2L, TGCGAgGGG; IR2R, CCCgTCGCA; IR3L, TcGCATCGgCA; and IR3R, GgCGATGCaA). The nucleotide sequences of the binding sites IHF_1 (TAGTTCGTAAgT) and IHF_2 (TAcCTTTTTGATA) present one mismatch with the consensus sequence (YAAnnnnTTGATW/WATCAAnnnnTTR) defined by Friedman (5). Motifs I, II, and III of MobA from *Bacteroides* spp., defined by Smith and Parker (23), are indicated, and the residues conserved in pBI143, NBU1, NBU2, and Tn*4555* are in boxes.

The Mob_{421} protein was also compared to the product of the other two *Bacteroides* mobilization gene families. Pairwise comparisons revealed that the unique mobilization polypeptide of pIP421 is definitively unrelated to the mobilization proteins of both plasmids pIP417 (25) and pBFTM10 (8) and also to those of plasmid pLV22a (15) and the transposon Tn*4399* (13, 14).

An internal region of Mob_{421}, between amino acids 170 and 360 (data not shown), displays limited similarity with recombinases, including those encoded by plasmids pGI2 from *Bacillus thuringiensis* (12), R46 from *Escherichia coli* (3), and TOLpww53 and TOLpDK1 from *Pseudomonas putida* (1). A search for specific sequence motifs of the Mob_{421} protein with the PROSITE dictionary protein sites and patterns was also performed. The sequence between amino acids 239 and 250 was identical to the consensus sequence (AAADTGTALLKA) of the active site of eukaryotic and viral aspartyl proteases. In particular, these proteases are involved in the degradation or processing of the mating pheromones during the sexual fission of *Saccharomyces cerevisiae* (4). No such site was found in the other *Bacteroides* mobilization proteins, and therefore this motif may not be functional or essential for the transfer process.

The *oriT* region of plasmid pIP421. The location of the *cis*-acting transfer origin (*oriT*) required for plasmid mobilization was determined by testing transfer properties of subclones and deletion derivatives of pIP421. Plasmids pFK3010, pFK3011, and pFK3012, devoid of 570, 745, and 920 bp, respectively, were obtained by making unidirectional exonuclease III deletions in the region upstream from the single *Bgl*II restriction site of pIP421 (Fig. 1). Mobilization assays were performed with TET-induced strain BF-2 as the donor and strain BF-638R as the recipient. The transfer frequencies of pFK3010 and pFK3011 (7.2×10^{-5} and 4.7×10^{-5} transfor-

mants per recipient, respectively) were similar to those of pIP421 (1.0×10^{-5}). In contrast, no significant mobilization of pFK3012 was observed (transfer frequency $\leq 2.8 \times 10^{-9}$), although the mob_{421} gene was not truncated in this deleted plasmid. This suggested that the *oriT* maps within the 217-bp region upstream from the ATG of mob_{421}.

This region contained several features commonly found in the *oriT* of conjugative or mobilizable plasmids. A putative nick site was identified (Fig. 2) presenting two mismatches (gAgCTTG) with the consensus sequence established by Pansegrau et al. (16). Surprisingly, in view of the similarity of the corresponding mobilization proteins, there was no sequence similarity with the proposed nick region of the NBU elements. In contrast, the putative nick site of pIP421 shared sequence similarity with those of other *Bacteroides* mobilizable elements, such as pIP417, pBFTM10, and Tn*4399*. Surrounding the nick site, three sets of inverted repeats, IR1 and IR2 (9 bp) and IR3 (11 bp), and two sequences reminiscent of specific integration host factor (IHF) binding sites were also found (Fig. 2). These structures are recognition sites for specific DNA binding proteins (6). In particular the binding of IHF proteins locally alters the *oriT* structure, thus facilitating the access of the *mob* gene products to the nick region (5). The genetic organization of the *oriT* of pIP421 was very similar to those of pIP417, pBFTM10, Tn*4399*, and NBU elements.

Mobilization properties of plasmid pIP421 in *E. coli*. Most *Bacteroides* elements are mobilizable in *E. coli* by IncP plasmids such as RP4 or R751 (22). To verify that this was also the case for pIP421, the 2.9-kb *Dra*I restriction fragment was inserted into pUC19 to give the recombinant plasmid pFK323. The vector pUC19 is a nonconjugal plasmid with no self-transfer sites or functions and is not mobilized by IncP plasmids. Strains ECJ62(R751) and DH5α were electrotransformed with both pUC19 and pFK323 plasmids and used as donor strains in matings with strain RR1 of *E. coli*. The frequency of mobilization of pFK323 from the ECJ62 donor harboring R751 was very high (2.0×10^{-2} transformants per recipient), whereas no transfer of pUC19 from the same strain was observed, and neither plasmid was mobilized from the DH5α donor strain. The IncP plasmids were thus able to direct the transfer of a coresident plasmid containing the mobilization region of pIP421.

Nucleotide sequence accession number. The DNA sequence of the mob_{421} gene will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y10480.

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