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Nucleotide Sequence Analysis of Two 5-Nitroimidazole Resistance Determinants from *Bacteroides* Strains and of a New Insertion Sequence Upstream of the Two Genes

ABDELLATIF HAGGOUD, GILLES REYSSET,* HOUSSINE AZEDDOUG, AND MADELEINE SEBALD

Unité des Anaérobies, Institut Pasteur, 75724 Paris Cedex 15, France

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DNA sequence analysis of regions from plasmid pIP417 and chromosome BF8 which encode 5-nitroimidazole resistance in *Bacteroides* strains allowed the identification of two open reading frames corresponding to new genes, *nimA* (528 bp) and *nimB* (492 bp). Either gene may confer 5-nitroimidazole resistance to susceptible strains of *Bacteroides*. The encoded polypeptides have deduced molecular masses of 20.1 and 18.6 kDa, respectively, and share about 73% identity and 85% similarity. A new insertion sequence (IS) element named IS*1168* lies 14 bases upstream of the *nimA* gene. The complete sequence of IS*1168* was determined. A similar IS exists 12 bp upstream of the *nimB* gene. About 60% of the BF8 IS element was also sequenced and shown to be almost identical to IS*1168*.

The 5-nitroimidazoles (5-Ni) are antimicrobial agents used extensively for the treatment of infections due to anaerobic eucaryotes and procaryotes. The broad spectrum of activity against anaerobic organisms is probably related to the mechanism of action of the compounds. According to published data (for a review, see reference 5), the 5-Ni molecule is a prodrug whose activation depends on reduction of the nitro group in the absence of oxygen.

Despite the widespread use of 5-Ni, resistance to 5-Ni (Ni^r) in anaerobes has been reported infrequently (for a review, see references 6 and 20). The presence of specific transferable Ni^r determinants in *Bacteroides* strains was first reported in 1989 (2), and at least three Ni^r determinants have been identified. Two of them were shown to be located on small mobilizable plasmids named pIP417 and pIP419 (19). The third one was shown to be located on the chromosome of *Bacteroides fragilis* BF8 (8). The three determinants are transferable to *Bacteroides* 5-Ni-susceptible strains through a conjugation-like process (2, 22). We report here the nucleotide sequences of the two Ni^r genes (*nimA* and *nimB*) that have been cloned previously (8, 19), together with the characterization of a new putative insertion element (IS*1168*) located upstream of both genes.

MATERIALS AND METHODS

Bacterial strains and growth. The *Bacteroides* strains used in this study were *Bacteroides vulgatus* BV17 harboring plasmid pIP417 and *B. fragilis* BF8 from J. Breuil and A. Dublanchet (2, 18). The plasmid-free *B. fragilis* BF638R was used as a recipient for transformation and conjugation experiments (13). Strain BF638R-XB2 is an Ni^r transconjugant obtained after the mating of strain BF8 with BF638R (8). TGY medium was used for routine growth (13), and Wilkins Chalgren medium (Oxoid) was used for testing antibiotic susceptibility and plating of transformants. Growth conditions have already been described (13). The following antibiotic concentrations were used: clindamycin, 5 μg ml^{-1}; rifampin, 20 μg ml^{-1}; metronidazole or tinidazole, 4 μg ml^{-1}. *Escherichia coli* HB101 (1) and *E. coli* JM109 (28) were used as cloning hosts and were cultivated aerobically in L broth supplemented with ampicillin (50 μg ml^{-1}) when appropriate.

Plasmid constructions and DNA techniques. All the recombinant plasmids used for cloning or phenotypic expression in *Bacteroides* strains were constructed with the cloning vector pBI191 (23) or pFK707ΔHI (8), whereas recombinant plasmids used for sequencing purposes were constructed with pUC19 (28). Large-scale plasmid and chromosomal DNA preparations, restriction endonuclease analysis, and transformation were performed as described previously (19). The presence and locations of IS*1168* and the *nim* genes were checked by PCR. Two sets of oligonucleotide primers were synthesized. The primers 5′-TTACGGCTACAAGCAGC-3′ and 5′-GGGTCTTGGACTTGCGC-3′ are homologous, respectively, to nucleotides (nt) 166 to 182 and nt 424 to 440 of the IS*1168* copy of BF8. The primers 5′-ATGTTTAGA GAAATGCG-3′ and 5′-GTCATTCCACAGTTCGT-3′ are homologous, respectively, to nt 794 to 810 and nt 921 to 936 of the *nimB* gene. Reaction conditions for PCR were as described previously (21). Amplified fragments were purified from agarose gel (1%) with the Geneclean II kit (Gibco BRL). They were radiolabelled with [α-^{32}P]dCTP (110 Bq/mmol; Amersham) by using the Boehringer-Mannheim nick translation kit. The Southern blotting techniques used were described previously (3). Hybridizations and washes were done at 60°C.

DNA sequencing and sequence analysis. DNA sequencing was done with Sanger dideoxy sequencing reactions using modified T7 polymerase (Sequenase 2.0 kit; U.S. Biochemicals Corp.). Reaction mixtures were analyzed on 6 or 8% polyacrylamide gels (21). Computer analysis was performed with Genetics Computer Group (GCG) programs (University of Wisconsin, Madison).

Nucleotide sequence accession numbers. The nucleotide sequences will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession numbers X71443 for the fragment sequenced from *B. fragilis* BF8 and X71444 for the fragment of plasmid pIP417.

* Corresponding author. Mailing address: Unité des Anaérobies, Institut Pasteur, 75724 Paris Cédex 15, France. Phone: (1) 45 68 83 11. Fax: (1) 40 61 31 23.

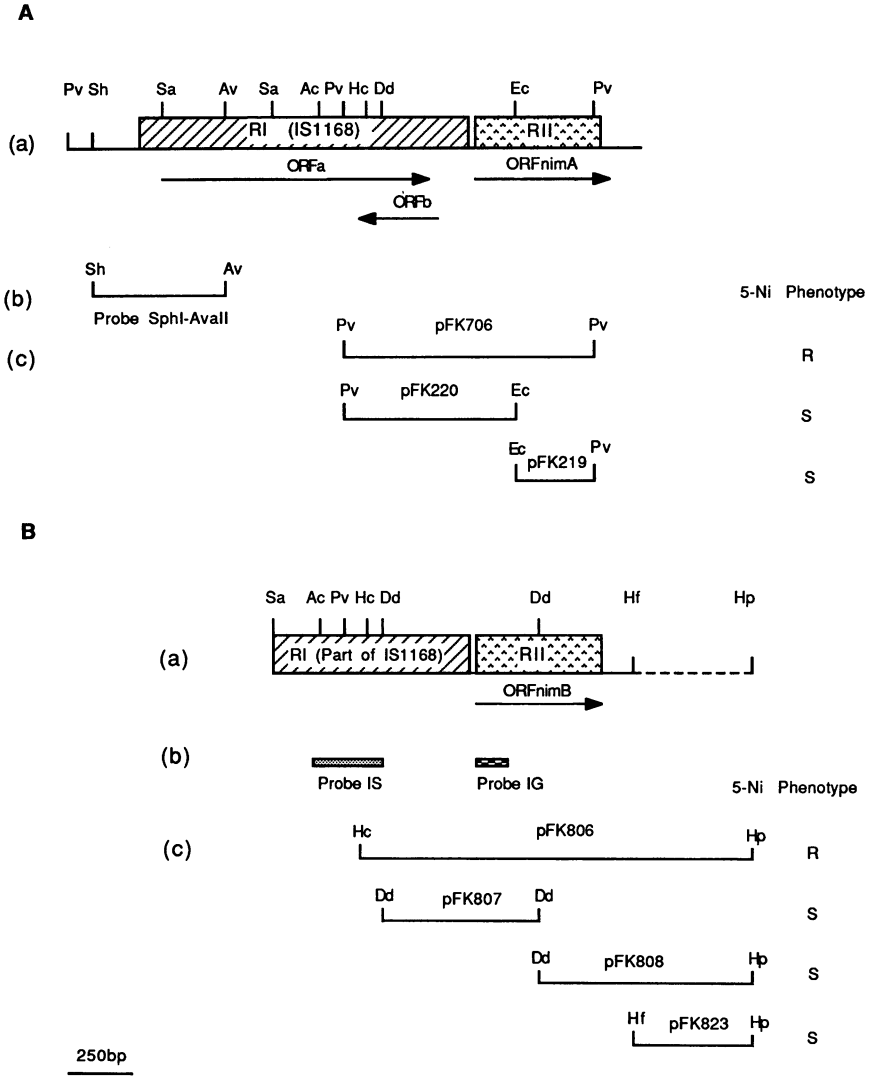

FIG. 1. Partial physical maps for the *nimA* gene from plasmid pIP417 (A) and the chromosomal *nimB* gene of strain BF8 (B). Restriction enzyme sites are as follows: Ac, *Acc*I; Av, *Ava*II; Dd, *Dde*I; Ec, *Eco*RV; Hc, *Hin*cII; Hf, *Hin*fI; Hp, *Hpa*II; Pv, *Pvu*II; Sa, *Sau*3A; Sh, *Sph*I; Xb, *Xba*I. (A) Part a, physical map. Arrows indicate the hypothetical length and direction of translation of the different ORFs. Part b, location of the probe *Sph*I-*Ava*II used for the determination of the right end of IS*1168*. Part c, subclones of pIP417 analyzed for expression of Ni^r in *B. fragilis* BF638R. Phenotypes are given on the right. R, resistance; S, sensitivity. (B) Part a, partial restriction map of plasmid pFK801. The dashed line indicates the nonsequenced region. Part b, location of probes IS and IG. Part c, subclones of pFK806 and the corresponding Ni^r phenotypes in *B. fragilis* BF638R.

RESULTS AND DISCUSSION

Nucleotide sequence of the Ni^r determinants. The Ni^r determinant encoded by plasmid pIP417 previously located on a *Pvu*II fragment (19) was sequenced from both strands, as well as adjacent regions, after subcloning into the pUC19 vector. To locate the chromosomal determinant cloned on plasmid pFK707ΔHI (8), deletion analysis was performed prior to sequencing. Figure 1 presents partial restriction maps for the two sequences, the various probes used in Southern hybridization experiments, and the results of the deletion studies. Both pFK706 and pFK806 confer 5-Ni resistance to susceptible *Bacteroides* strains. Southern blot analysis showed that the *Dde*I restriction fragment of plasmid pFK807 hybridized with the *Pvu*II fragment of plasmid pIP417, in contrast to the insert from pFK823 (data not shown). These results indicated that the chromosomal Ni^r determinant was located on pFK806 between the *Hin*cII and *Hin*fI restriction sites. This restriction fragment and the adjacent *Sau*3A-*Hin*cII fragment located just upstream were sequenced in both directions. The complete nucleotide sequences of the pIP417 and BF8 restriction fragments are given in Fig. 2.

The plasmidic RII region, extending from nt 1614 to nt 2144, and the corresponding chromosomal region from nt 794 to nt 1286 exhibit 70% identity. The deletion analysis showed that these RII regions are necessary and sufficient to confer resistance to 5-Ni (Fig. 1A, part c, and B, part c). These regions correspond to two open reading frames (ORFnimA and ORFnimB) that have three possible translational start codons: ATG codons at positions 1614, 1626, and 1683 in the pIP417 sequence and at positions 794, 806, and 863 in the BF8

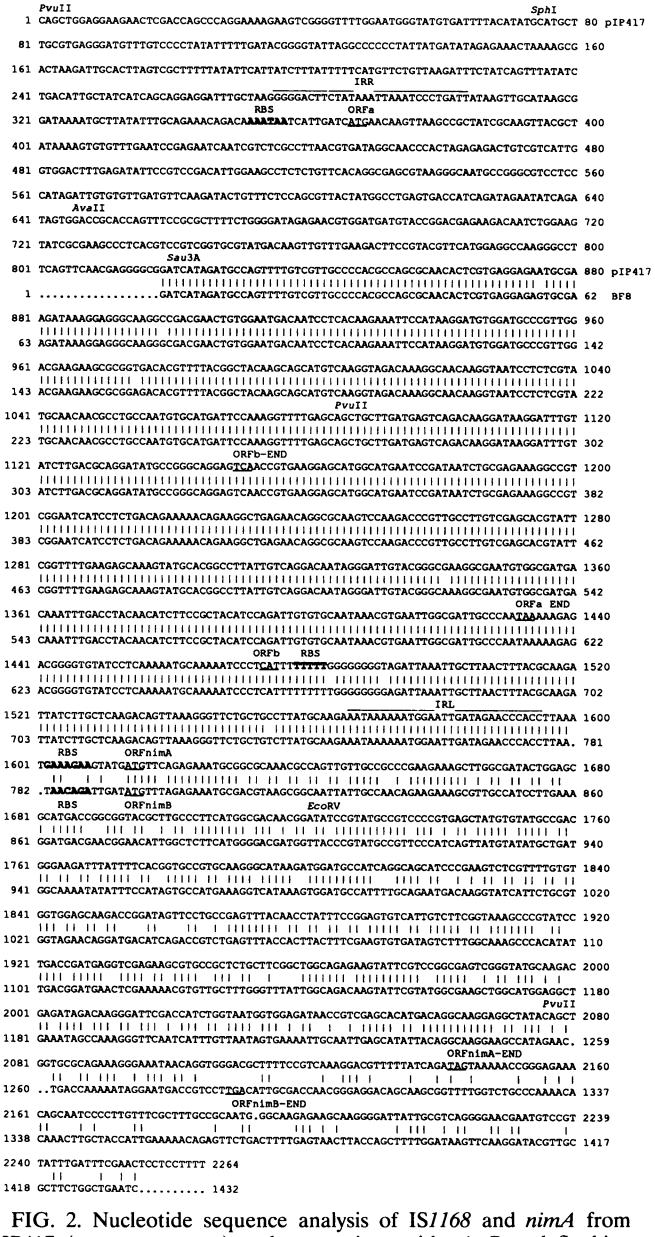

FIG. 2. Nucleotide sequence analysis of IS*1168* and *nimA* from pIP417 (upper sequence) and comparison with *nimB* and flanking regions from *B. fragilis* BF8 (lower sequence). The comparison was done with the Gap program of the GCG package. Nucleotide identities between the two sequences are indicated by vertical lines and gaps are indicated by periods. Relevant restriction sites (identified in the legend to Fig. 1) are shown on the sequence of pIP417. The two inverted repeat sequences of IS*1168* are indicated by lines above the corresponding nucleotides and named IRL (left) and IRR (right). The positions of the different ORFs of the two sequences are indicated; the start and stop codons of the ORFs are underlined. The sequences of the putative ribosome-binding sites (RBS) are indicated by bold letters.

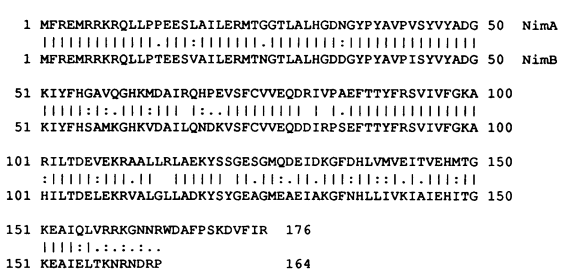

FIG. 3. Comparison of the deduced amino acid sequences of the *nimA* and *nimB* genes. The Gap program of the GCG package was used. Matching amino acids are indicated by vertical lines. Amino acids that are positively related are shown with a colon on the line between the amino acids, those with a zero value relationship are shown with a period, and those that are negatively related are shown as a blank space.

sequence. The only stop codons in phase with these start sites are at position 2142 (TAG) in pIP417 and position 1286 (TGA) in BF8. Sequences complementary to parts of the 3′ end of *B. fragilis* 16S rRNA, 5′-GAACACCUCCUUUCU-3′ (26), are present upstream of the three possible start sites.

***nim* gene products.** The two ORFs just described may encode NimA and NimB polypeptides of 153 to 176 (molecular mass, ≤20.1 kDa) and 141 to 164 (molecular mass, ≤18.6 kDa) amino acid residues for the plasmidic and chromosomal genes, respectively. As shown in Fig. 3, the two largest polypeptides share about 73% identity and 85% similarity, considering conservative substitutions. The percentage for each amino acid is within the range usually found for procaryotic proteins (4). It is noteworthy that the stop codon of the *nimA* gene of pIP417 is located downstream of the *Pvu*II site (position 2081), although plasmid pFK706 still confers Ni^r. The last 21 amino acids of the protein can thus be deleted without affecting the activity of *nimA*. The nucleotide and amino acid sequences of the *nimA* and *nimB* genes were compared with sequences available in the GenBank (release 76.0), EMBL (release 34.0), and SWISS-PROT (release 25.0) data bases by using the FASTA and TFASTA programs of the GCG. No significant homology with any published sequences was found. In particular, the *nim* genes share no relationship with the gene isolated from another strain of *B. fragilis* which would encode a product involved in metronidazole resistance in *E. coli* (25).

The hydrophobicity profiles of the *nim* gene products do not suggest a membrane location for the proteins (data not shown). Moreover, a similar uptake of metronidazole was observed with strain BF638R, whether or not it carried the recombinant Ni^r plasmids pFK706 and pFK806 (data not shown). The mechanism of resistance conferred by the *nimA* and *nimB* genes thus does not involve a modification of the penetration of the drug into the cells.

Characterization of a new IS element upstream of the *nimA* and *nimB* genes. The two sequences upstream of the genes are more identical than the *nimA* and *nimB* coding sequences. The two RI regions, positions 819 to 1599 in pIP417 and positions 1 to 781 in BF8, share 99.1% identity. Regions RI and RII are separated by short nonhomologous sequences of 14 bp for pIP417 and 12 bp for BF8. The similarity between the two RI regions, directly visible on the restriction maps, argues for the presence of an insertion sequence (IS) upstream of the two genes. Southern analysis performed under high-stringency conditions (<30% mismatching allowed) demonstrates that the homology between the RI region of BF8 and that of pIP417 extends upstream of the *Sau*3A site but stops before the *Sph*I site (Fig. 1). The presence and copy numbers of the IS and *nim* genes on the chromosomes of strains BF8 and BF638R-XB2 and on plasmid pIP417 have been determined with two internal specific probes, IS and IG, respectively (Fig. 1B, part b). With strain BF8, about 10 *Sau*3A restriction fragments hybridized with the IS probe but only 2 of them, of 5.4 and 4.3 kb, also

hybridized with the IG probe (data not shown). In the transconjugant BF638R-XB2, two restriction fragments of 5.4 and 1.3 kb hybridized with the IS probe; the 5.4-kb restriction fragment also gave a positive signal with the IG probe. In control experiments performed with the *Dde*I restriction fragment of plasmid pFK807, which covers both RI and RII regions, no hybridization with the DNA of the susceptible strain BF638R was observed (8), demonstrating that neither the IS nor the *nim* genes were present in the recipient strain. Our conclusion is that strain BF8 carries two copies of the *nim* genes, each one associated with an IS, and eight copies of the IS. Through conjugation, BF638R has acquired a copy of *nimA* with its associated IS, plus an additional copy of the IS. At present, every *nim* gene has been shown to be associated with an IS element. In a recent survey of 5-Ni resistant strains of *Bacteroides*, 88.8% were shown to harbor 1 to 10 copies of IS*1168*, and among 30 sensitive strains tested, the presence of an IS element was detected in only one strain (reference 18 and data not shown).

With plasmid pIP417, only one copy of the repeated sequence was found located near the *nimA* gene, showing that it is not part of a transposon (data not shown). The IS upstream of the *nimA* gene on plasmid pIP417 was entirely sequenced (Fig. 2). This element, named IS*1168*, contained 1,320 bp with imperfect inverted repeats at its ends for which 19 of 29 bp match (Fig. 2). The moles percent of G+C of IS*1168* (46.7) is within the range (39 to 46) determined for genomic DNAs of *Bacteroides* spp. (9). Two ORFs were found within IS*1168* (Fig. 1A, part a). The largest ORF (ORFa) extends from positions 367 to 1432 and could code for a basic polypeptide of 41.2 kDa with a calculated pI value of 9.93. The other ORF (ORFb) is located on the opposite strand and could code for a polypeptide of 12.5 kDa. A search in the GenBank and EMBL data bases for sequences related to ORFa was performed with the TFASTA program of the GCG package. Homologies were found for polypeptides encoded by other IS elements. The highest score was obtained with a large ORF in a repeated sequence harbored by plasmid pWWO of *Pseudomonas putida* (17; accession number L11583). The two putative polypeptides shared 59% similarity and 42% identity. ORFa also displayed significant but lower identities with putative transposases of different ISs: 29.1% identity in a 227-amino-acid overlap with ORF1 of IS*1106* from *Neisseria meningitidis* (10), 22.3% identity in a 179-amino-acid overlap with ORF5A of IS*5* from phage λKH100 (11), and 26.5% identity in a 155-amino-acid overlap with ORF1 of IS*52* from *Pseudomonas syringae* (27). These ISs are ca. 1,200 bp in length (1,274 bp for the IS from pWWO, 1,137 bp for IS*1106*, 1,195 bp for IS*5*, 1,209 bp for IS*52*, and 1,320 bp for IS*1168* as described here). In contrast, IS*1168* shares no significant homology with the IS elements already described for *Bacteroides* strains IS*4351* (16) and IS*942* (14).

The location of the IS elements less than 20 bp upstream of the predicted initiation codons of the two *nim* genes reinforces the hypothesis of the IS-activated expression of *nim* genes as has been previously shown for the macrolide-lincosamide-streptogramin B resistance genes of *Bacteroides* strains (15, 16, 24). In parallel with this study, I. Podglajen and E. Collatz (12) have characterized an IS, named IS*1186*, able to activate a silent carbapenemase gene (*cfiA*) in *B. fragilis* BFr81 (also called BF8). Nucleotide sequence comparison reveals that IS*1168* and IS*1186* are almost identical, and this also suggests potential gene activation by IS*1168*. Activation of downstream genes by ISs is well documented for several IS elements, including IS*1*, IS*3*, IS*4*, and IS*10*, as well as IS*5*, and can be due to either the presence of outwardly directed promoters within the element or the formation of new promoters after insertion (7).

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