Plasmids pIP419 and pIP421 from *Bacteroides*: 5-nitroimidazole resistance genes and their upstream insertion sequence elements

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The genetic organization of two different 5-nitroimidazole (5-Ni) resistance genes was investigated: *nimC* and *nimD* from *Bacteroides* plasmids pIP419 and pIP421, respectively. The *nimC* gene (492 bp) and the *nimD* gene (495 bp) directed the synthesis of polypeptides with deduced molecular masses of 18.37 kDa and 18.48 kDa, respectively. The predicted proteins showed 67–83% identity and 78–91% similarity with the products of two other *nimA* and *nimB* genes previously described and could be derived from a common ancestral gene. An insertion sequence element (IS1170) was identified upstream of the *nimC* gene. IS1170 is 1604 bp in length and is flanked by imperfect inverted repeats (15 bp). IS1170 is similar to the *Bacteroides* insertion sequence element IS942 with an identity of 70% at the nucleotide level. The single copy of IS1170 present on plasmid pIP419 is integrated 24 bp upstream of the initiation codon of *nimC*. Similar genetic organization was found on plasmid pIP421. One copy of another insertion sequence (IS1169) was found 4 bp upstream of the first ATG codon of the *nimD* gene. This element (1325 bp) shows a strong homology at the nucleotide level (70% identity) with IS1186 and IS1168 found to be associated with the *Bacteroides* carbapenem resistance gene *cfiA*, and the 5-Ni genes *nimA* and *nimB*, respectively. There is strong evidence that, as in the case of the *cfiA* gene, the transcription of the four *nim* genes so far studied is directed by outward-oriented promoters, carried on the right ends of the different insertion sequence elements.

Keywords: *Bacteroides*, plasmids pIP419 and pIP421, 5-nitroimidazole resistance genes, insertion sequence elements

INTRODUCTION

Among the anaerobic bacteria involved in human infections, *Bacteroides* species are the most frequently isolated (Salyers, 1984). These bacteria are commonly resistant to antibiotics, particularly to many β -lactam antibiotics, tetracycline and macrolides, but remain sensitive to the 5-nitroimidazoles. Despite the clinical use of these antimicrobial agents for more than 30 years, the incidence of

5-nitroimidazole (5-Ni) resistance in *Bacteroides* is still very low.

The demonstration that specific transmissible genetic determinants conferring a low level of resistance occur in *Bacteroides* was reported in 1989 (Breuil *et al.*, 1989). Two resistance determinants (*nimA* and *nimC*) were shown to be located on small mobilizable plasmids, designated pIP417 (7.7 kb) and pIP419 (10 kb) derived from *Bacteroides vulgatus* BV17 and *Bacteroides thetaiotaomicron* BT13, respectively (Reyssset *et al.*, 1992). Although differing in their restriction maps, the two plasmids showed homology in a region involved in their mobilization (Reyssset *et al.*, 1992). Another resistance determinant (*nimB*) was found to be located on the chromosome of *Bacteroides fragilis* strain BF8 (Sebald *et al.*, 1990; Haggoud *et al.*, 1994). This determinant was also

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Abbreviation: 5-Ni, 5-nitroimidazole.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of the fragments from pIP419 and pIP421 are X76948 and X76949, respectively.

shown to be transferable homo- or heterospecifically through a conjugation-like process. Subsequently, another 5-Ni^r plasmid was found in three clinical isolates of *Bacteroides* (Reysset *et al.*, 1993).

The 5-Ni^r genes of strain BF8 (*nimB*) and of plasmid pIP417 (*nimA*) have been sequenced (Haggoud *et al.*, 1994). The nucleotide sequences and the deduced amino acid sequences show about 70% identity suggesting that, although different, the two genes are closely related. Moreover, an identical insertion sequence element (IS1168, 1320 bp) was found a few base pairs upstream of the start codons of both genes (Haggoud *et al.*, 1994). An identical insertion sequence element (IS1186) was shown to activate the carbapenemase gene *cfiA* of *B. fragilis* when present immediately upstream of the gene (Podglajen *et al.*, 1994). The transcription of *cfiA* was found to be driven from a promoter identified on the right end of the insertion sequence element (Podglajen *et al.*, 1994). The same promoter should be efficient in the transcription of the *nimA* and *nimB* genes.

There is therefore strong evidence that these newly described insertion sequences contain the regulatory signals for the expression of the resistance genes. However, our studies have indicated that IS1168 fails to hybridize with pIP419 and therefore transcription of *nim* genes is not always directed by IS1168. The aim of this work was to compare the four *nim* genes and their upstream regions.

METHODS

Bacterial strains and plasmids. The *Escherichia coli* strains used in this study were HB101 (Boyer & Roulland-Dussoix, 1969), JM109 (Yanisch-Perron *et al.*, 1985) and GT869 (Parsot, 1986). The *B. fragilis* strains were the plasmid-free strain BF-638R (Privitera *et al.*, 1979) used as a recipient for transformation, and BF-F238, BF-F239 and BF-M6 (Reysset *et al.*, 1993). The following plasmids were also used: pIP419 from *B. thetaiotaomicron* BT13 and the recombinant plasmid pFK100 (Reysset *et al.*, 1992), pIP421 (7.3 kb) isolated from strain BF-F239, pUC19 (Yanisch-Perron *et al.*, 1985) and pBI191 (Smith, 1985).

Media and culture conditions. *Bacteroides* strains were cultured in an anaerobic chamber (La Calhène, 78140 Velizy, France) in 5% H₂, 5% CO₂ and 90% N₂. TGY medium was used as basal medium (Privitera *et al.*, 1979). Wilkins-Chalgren medium (Oxoid) was used for antibiotic susceptibility testing and plating of transformants. *E. coli* strains used as cloning hosts were cultivated aerobically in L-broth supplemented with ampicillin (100 µg ml⁻¹) where appropriate.

Preparation and analysis of plasmid DNA. Small scale preparations of plasmid DNA were obtained from exponential phase cells (OD₆₆₀ 0.6) by the alkaline lysis procedure (Birnboim & Doly, 1979). The DNA was purified by isopycnic ethidium bromide-CsCl ultracentrifugation. DNA was analysed by horizontal electrophoresis on 0.7% agarose gels in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM Na₂EDTA, pH 8.25). Restriction endonuclease analysis was performed under the conditions recommended by the suppliers (Amersham and Boehringer Mannheim).

Plasmid constructions and electrotransformation. All the recombinant plasmids used for cloning or phenotypic ex-

pression in *Bacteroides* were constructed using the cloning vector pBI191 (Smith, 1985), whereas recombinant plasmids needed for DNA sequencing or *in vitro* transcription-translation assays were constructed using the cloning vector pUC19 (Yanisch-Perron *et al.*, 1985). Plasmids pFK110 and pFK111 are recombinant plasmids from pUC19 containing a 2 kb *Sau3A* restriction fragment from the *nimC* gene of pIP419 cloned in opposite orientations. Similarly, plasmids pFK310 and pFK311 contained the 1.8 kb *HindIII* restriction fragment from the *nimD* gene of pIP421 cloned in opposite orientations. In recombinant plasmids pFK110 and pFK310, the *nim* genes are located in the opposite orientation to the *plac* promoter. Electrotransformations of *E. coli* and *Bacteroides* were performed according to Dower *et al.* (1988) and Reysset *et al.* (1992) respectively.

DNA sequencing, sequence analysis, Southern blotting and DNA-DNA hybridization. DNA sequencing was performed by dideoxy sequencing reactions (Sanger *et al.*, 1977) using modified T7 polymerase (Sequenase 2.0 kit, US Biochemical). Reaction mixtures were analysed by electrophoresis on 6 or 8% (w/v) polyacrylamide gels (Sambrook *et al.*, 1989). Computer analysis was performed by using the Genetic Computer Group (GCG) programs (University of Wisconsin, Madison). The presence and copy number of IS1169 and IS1170 were determined by Southern blot analysis performed according to Bryant *et al.* (1988) on nylon N⁺ membranes (Gene Screen⁺, Dupont de Nemours). Probes were purified restriction fragments labelled with [³²P]dATP by nick translation (Boehringer Mannheim). The location of the Ni^r determinant of plasmid pIP421 was checked using an internal probe of the *nimB* gene (IG probe) synthesized by PCR using the following oligonucleotide primers: 5'-ATGTTTAGAGAAATGCG-3' and 5'-GTCATTCCACAGTTCGT-3' (Haggoud *et al.*, 1994). The amplified fragment was purified from agarose gel (1%) with the GeneClean II kit (Gibco BRL) and radiolabelled as described previously.

***In vitro* transcription-translation.** Recombinant plasmids pFK110, pFK111, pFK310 and pFK311, containing the pIP419 and pIP421 5-Ni^r inserts cloned in opposite orientations, were subjected to transcription-translation in an *in vitro* prokaryotic DNA-directed system (Amersham). The proteins were separated by SDS-PAGE, and the ³⁵S-labelled synthesized polypeptides detected by autoradiography.

RESULTS

Location and nucleotide sequences of the 5-Ni^r determinants of plasmids pIP419 and pIP421

The 5-Ni^r determinant encoded by plasmid pIP419 was previously cloned on vector pBI191 as a 2 kb *Sau3A* restriction fragment (Reysset *et al.*, 1992) to give the plasmid pFK100. Deletion analysis of pFK100 was performed in order to locate precisely the 5-Ni^r gene, and the subclones were analysed for expression of 5-Ni resistance in *B. fragilis* BF-638R. It was demonstrated that a recombinant plasmid containing the 1.25 kb *EcoRI*-*Sau3A* restriction fragment (Fig. 1) confers 5-Ni resistance to susceptible *Bacteroides* strains, whereas the *DraI*-*Sau3A* and the *Sau3A*-*PvuII* restriction fragments (approx. 1 and 1.1 kb, respectively) did not.

Plasmids (7.3 kb) detected in three 5-Ni^r clinical isolates of *B. fragilis* (strains BF-F238, BF-F239 and BF-M6), were

Fig. 1. Physical map and subclones of plasmid pIP419 and the *nimC* region. (a) Physical map, with unique restriction sites indicated by asterisks. An expanded map of the sequenced region is also shown. The location and the putative direction of transcription of the ORFs (a and b) within the insertion sequence element IS1170, and the single ORF of the *nimC* gene are indicated by arrows. The location of IS1170 used as an internal probe in hybridization experiments is indicated by a hatched box. (b) Subclones of pFK100 and the corresponding 5-Ni phenotypes in *B. fragilis* BF-638R. R, resistance; S, sensitivity.

Fig. 2. Physical map of plasmid pIP421 and the *nimD* region. The unique restriction sites are indicated in bold type. An expanded map of the sequenced region is also shown. The location and putative direction of transcription of the three major ORFs (a to c) within the insertion sequence element IS1169, and the single ORF of the *nimD* gene are indicated by arrows. The location of IS1169 used as an internal probe in hybridization experiments is indicated by a hatched box.

shown to confer resistance when transferred by conjugation or transformation to *B. fragilis* strain BF-638R (Reysset *et al.*, 1993). Preliminary restriction analysis of

the three plasmids with *Hind*III and *Pvu*II enzymes has shown similar restriction patterns. However, these are distinct from those of the previously isolated 5-Ni^r

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1 CTACAAATAT CTCATTTTCG GCACACTCTT TATCCTCTCA TGACACGACA GTTTTTTTCG GATTTTCATG AAAAGTCTGA AGACTTCTGT GTGTCATGAG
   IRL
101 AGCTTGGGTC GTACCTTTTCA GGCATAACTT CCCCTTACCC CACAGGCAA CTGAGGCAA TATGCCATAA ATCCACAAA GCATAAGGCT AATCGGCCCC
   ORFb end
201 GCGCTTAAAG ACATCTCTAT AGCCCTGTTT TCCGGTGTAG ATATTGACA TGTCATTCTT GGAAGTCTTT ATCAITTAG CAACACAGA AACAATCTTG
   HindIII
301 AATACGAAAG CTTGATGCG GCTTGTCTGT TGAGTCGAA TGCTTTTACA TTCAATCTGC GGATATGGT TTTGTAGAG TTCCTTATCA GTGGGGTCAT
   EcoRI
401 CAGCAGTAG ACCGTATTTT TCAGCCGAGA AGGATTGGG CAGAGCTTC CATCCGATC CTTGTTCATG TCTGTCAAG ATTCTTTCTC TTCCCTCAGC
   EcoRI
501 CAGGTTGTAG AATCTACGA TATCTCTGAC ATCGGATTGC AATCATTTGG TGAGAATGTA CCGTAGGTA TATTCACCTT CCCATAGTTC CTGAGTTTGG
   EcoRI
601 TCAGCTCTTC TCTGTCTCG AACAACTAGG CGATAAGGCT TTCCCTTCCA CTTTTCACG ATAATGGAGT TCAGTTCCGA TTCCTATCCG TTGATTTCCT
   Sau3A
701 CAGTCTCCCA TCCTCGGAGA ATGAACATGT CATCTAAAA CGCGAGCAT CTGTGGCGG GAGTGTAGAA ATGGTTACAA TGATCTTTTA CCACATCCAC
   Sau3A
801 AATCTCTTCG GAACATGACC CGCAGTCCAT ACGTACCTCG TCTATATGGA TACCTGTGTC TTCAAGCCTT GTAAGATCC TTCAAGGCT ATCCTGTTGG
   ORFb start
901 CGGAACCGA CATTGGTATT CCGCTCGCGG TTTCTATCG CTACGATAG GTCCCGGATG ACAGCTACTC CGGGGCTGTA TCCGGTAAAC TTCTGTAGG
1001 TGGGCTTGGC GTCACTACTG CCGTGTCTGA TAAACTGATG GTCGAAATCG AAATCAATAT TCTGTCCCTC GTTTAATCT CCGGTAGATA TGAATGCCCT
   ORFb end
1101 GACGAAGCAG TTGCTTCATG CCGTCTGCGA TATTGAATC ATAGGATTTT CCCGAGACGG AAGACGTATA GGTATGTTG GGTACGGTCA GTTCTTGAT
   PstI
1201 GGCACGAGA ATCGTATCTG CGCTGCACT CTTTGACACA GGATGAAGG AAGATGAGA CATGAGTGG GTGGATATGT CTTCAACACA GGAGCCGCCA
   AvalI
1301 CAGAAATACA CGCAGATGAG GAACAGGAGG ATTTCACTGT ATTGATAACC GAATGCTCTG GTACGCTGGT CCAAAGTGG ATCAATTATG TTAGACAAAA
   EcoRI
1401 GAGCATCGAA TCTTCCATT ACACAAAAA TTCACCAAA AGGAGTGATT TGTTCAAGT TTATTGTAT TTTTGGATG CCGTAGAAG ATTTGTTGA
   SspI
1501 TTTTATTGCG AACGTAAGT TAAGTGAATC TTCTGACATG GCAAAATACT GGGTAATATA TTATTACTCA GTATTTTAAA TTATTGTA ACAATATGTG
   ORFb start
   -35 DraI -10
1601 TTCGGGAATT AAGGATGATT ATGGAACAGC AAACAACAT GTTCAGAGCG ATCGCTCGA AGCGGCACGA GTTGCCTACC GATGAGAGCG TAGGCATATT
   IRR RBS M F R A M R P K R H E L P T D E S V G I L
   ORFnimC start
1701 GAAGCGAATG ACCAAGCGCA CGCTGGCCTT GCATGGCGAT GGCATTATTC CGTATCGCGT GCGCTGACG TATGTGTACA CGGACGGGCG AATCATTTTC
   K R M T N G T L A L H G D G Y P Y A V P V S Y V Y S D G R I Y F
1801 CACACGGCCA CGCAAGGCGA TAAGGTAGAT GCGCTGATCG GGAACGACAA GGTATCTGTC TCGGTGGTGG AGCAAGATGA CCGTAATCTT CCGCAGTTCA
   H T A T Q G H K V D A L M R N D K V S F C V V E Q D D V K S A E F T
1901 CCACTTACTT CCGGAGCGTA ATACCGCTCG CGAGGCGCAG CATCTGTGAG CAGCAGACGG AGAAGCGTGC CGCATTCGAG CTGCTTGGC ACATATATTC
   PvuII
   T Y F R S V I P F G R A R I L D E T E N G A A L Q L L A D K Y S
2001 GTCCGGTATG CCGGCTGTGG AGGCGGTGAT AGCCAAGGCG TTCCGTACCC TGCTGATGTT GGAGATAGAT ATTGAGCACC TGACGGGCGA GGAATCTATC
   S G M P G L E A V I A K G F R H L L M V E I D I E H L T G K E S I
2101 GAGCTGTGCA GGGAAAGAA TCACATGTAA GTTATCTCTT ACAGGACTAT TTCAACAAGC GAAGTGTGTC CAGCATGTTT TTAATGCGGA TGGCTGTGGC
   E L V R E K N D M *
   ORFnimC end EcoRV
2201 GGGCATAGT TCGGCTACGG TCACGCCGAC AATATCTTTT CCGCGCGAT ATCGTTTATC ACACGCACCA CTTCGCGAT TTTCAITCCA TCCGGGACCA
   EcoRV
2301 CACCGACCGC CGCGATGATT TCCCGCGGTC GAGTACATCC ATACCAAGT GTATGACACAC CTTGGATGTT CCGCAGGATT GTACCATGTG CGAAGCGATC
   NcoI
2401 ACTGTCTCCG GGACATCTTC CCGTGTGAGA TGTGGATGTC CATATCGTTC CTGCTTGGCC TTGATTCTCG CACGCTCCCA ATCGCGCAAT CCGACGAAAA
2501 AGGATTTTGG AAGGGGCTAT CTTCACTCGG AGTTCAGAAA CAAGCTGTTT TGCCGCCAAC CCATGTCAGG CTGTATCCGC CATGGCGATGA TAGCCCGCAT
   BglII Sau3A
2601 AGGATTCGCC TGCCAAGGTC ATGTCGGGAT GTGCTCGAAA TCCAGATCTG GAGCGTATGA AGAAGCGGCC GGAAGTCAG CCGTTTTCGA ATAACCTCTG
2701 TGACTACATA AGCCGACAGG AAGTAACCTT CAAATAGAC AACAAACAGG CAAAATTAAG CCGGAACGAT GCGAAGTTCT ATGTGA

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Fig. 3. Nucleotide sequence of the *nimC* region of plasmid pIP419. Relevant restriction sites are indicated below the sequence. The two inverted repeat sequences of IS1170 [IRL (left) and IRR (right)] are indicated by lines below the corresponding nucleotides. The position of the start and stop codons of ORFa and ORFb within the IS element is also underlined. The three possible ATG start codons at nt positions 1639, 1651 and 1708 (underlined) and the stop codon TAA at nt 2130 (indicated by an asterisk) of ORFnimC (labelled in bold type) are shown. The predicted amino acid sequence for *nimC* is shown under the sequence, starting at nt 1639. The putative ribosome-binding site (RBS) and promoter sequences of ORFnimC are double-underlined.

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1 CAGCTGTACA TACATCCGAG GTTCACATGT AACCATCGTT GGTTCATAT TCTTCAGGTA AAGTCGCTAC CTTAGTGATT TGTGTGTTT CACCGACAGG
   AvalI
101 TATTCACCGG CTTGTGCTTT CTTGTGTAAA GAATCAGGCC ATTACTATCA AATGAATAG AATCCTTTTC ATTATCATAT CTTTIAAAG TTCATTTACA
   SspI
201 TCTACGGCAA AAGTATTGAT TTTAGACAGT TCTATAAATT AAATATATGT AGTACAGTTG TTTATATGGT TCCAAGAGCG TAAATTTGCA GAAATAAGA
   IRL
301 ATTITATAT GAACAAGTA TCCCGATACC GTAGGCTTCG TTATAATCAA CTATTGAGT CAGAGAATCG TGAATTCGCT TTGAATGAAA TGGGCAATCC
   ORFb start
   BglII MboI
401 TCTTGAATG TTGCTCAGT ATGTTGATT TGAGATCTTT COTCCTACTC TTGAATCAGC CTTTITACC GGAGGCGCA AAAGTAATGC CGTGTGCTCG
   ORFb end
501 CCGTAGACTG GTGTGCTGAT GTTCAAGGTC TTGTTCTTC ACGGTTATTA TGGTTTGAGT GACCATCAGA TAGAGTATCA GATAGTTGAC CGTACGAGTT
601 TCCGCAAGTT TCTTGTGATT GAATGTGTTG ACGATGTTCC TGACGAGAGG ACGGTGTGGA AGTATCGCGA ACTCCTGACA AATACAGGCG TTTATGACAA
   HindIII
701 GCTTTTTTCG GAATTCATA GTTTCATGGA AAGTAAGGCT CTGCAATTCA ATGAGGCTCG CATCATGATT GCGAGTTTG TTATTGCCCC TTGCCAACGT
801 AACACCGGTG ATGAATGTA CGAGATAAAA CAGGAGCGGG GTGATAAGTT GTGAATGAC AATCCGCCCA AGAAGCTCCA CAAGGATGTA GATGCCCGCT
   HindIII
901 GGACAAGAAA CGGTGATGAG ACTTTTACG GCTACAAGCA GCAIACATAA GCCTTCGAGA AGCAATAAG ATCATCTTT CTTATGATAC CAGCTGGGCA
   ORFb start
1001 GAAAGCATG ATTCCAAAGG CTTTGAGGGA CTGCTGGATG AAAAGACGA AGGCAAGGAC TTGTATTGG AGCGCGGTA TGTGCGACAA GAGGAGATTG
1101 TAAACAGCA TAAGATGAAT CGATAAATT GCGAAAGGG CTACCGTAAC CGTCCGCTTA CCAAGAGACA GAAATCAGAC AATAGGAAAA AATCCAAGAC
   ORFb end
1201 ACGTTGCTTT GTCCAGCATG TATTCGGGTT TGAGAACAAA ACCATGCTTG GACTTGTGTT GCGTACAGTA GCGCTTATTC GTGCTAAAGC CAATGTAGCA
1301 CTCACGATC TTGTGTATAA CATCAGTCTG TACACGCAAA TAATCAGGCT GAAACCTGAG TTGTTGGGTT GAATTATGCC TGACATCCA ATTTTACAAA
   -35 SspI -10
1401 CAAACACAAG TTAATTATTG TAAAAATTGG GGGGGGTAGA AAATATTCGC TAAATTTGAA AGACTAAAC TCACAATTG GGCAGTAAA GAGTTCTGCT
1501 GTCTGATACA AGAGGATAAA TGCAATGAT AGACTACCC AAAGCAATAT GTTTAGAGAA ATCGCGGTA AGCGGCAATT GTTGCCAACA GAAGAAAGCG
   IRR RBS M F R E M P R K R L L P T E E S V
   ORFnimD start
1601 TTGCACTTCT TGAACGGAAT ACAACGGGGA COTTGCTCTC TCATGGGGAT GACGCTATC CGTATGCGCT CCGTGTGATG TATGTATATG CCGATGCCAA
   A I L E R M T N G T L A L H G D D G Y P Y A V P V S Y V Y A D G K
1701 AATTACTTCT CACAGTGCCA TGCAAGGGCC AAAAGTGGAT GCCATCTGCG GGAATGACAA GGTCTGCTTC TCGGTATGAG AGCAGTACGA GGTCAAGGCG
   I Y F H S A M Q G P K V D A I L R N D K V S F C V V E Q D E V K P
1801 GCGGAGTTTA CCACCTATTT TCGGAGCGTG ATAGCTTTTG GCAMGGCCCG CATACTGACC GAGGAGAACG AAAAGCGAAA TGCTTTAAAC CTGCTGGCGG
   A E F T T Y F R S V I V F G K A R I L T D E N E K R N A L N L L A D
1901 ACAGTATTCG GCATGGCGGA CGGGGATGAG AGGCTGAATG GCGCAAGGG TTAATCATTT TCGTATGATG AGAATATACA GAAATGACGA TGACCGGAAA
   K Y S H G E A G M E A E M A K G F N H L L M I E I T V E Q M T G K
2001 AGAAGCATCT GAATGACAAA GGGGAGAAA CCGATGTTCT TCACAAGAAA TGCGTACTG ACGGAGAAA AAAGCTGTTT CCGCTCGCGG
   E A I E L T R G R N G C S *
   ORFnimD end

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Fig. 4. Nucleotide sequence of the *nimD* region of plasmid pIP421. Relevant restriction sites (see Fig. 2) are indicated below the sequence. The two inverted repeat sequences of IS1169 [IRL (left) and IRR (right)] are indicated by lines below the corresponding nucleotides. The position of the start and stop codons of ORFa, b and c within the IS element is also underlined. The three possible ATG start codons at nt positions 1549, 1561 and 1618 (underlined) and the stop codon TGA at nt 2043 (indicated by an asterisk) of ORFnimD (labelled in bold type) are shown. The predicted amino acid sequence for *nimD* is shown under the sequence. The putative ribosome-binding site (RBS) and promoter sequences of ORFnimD are double-underlined.

plasmids. This result was confirmed by examining the physical map of plasmid pIP421 of strain BF-F239. Restriction enzyme sites were identified and localized by analysis of DNA cleaved by various enzymes (unique and double digestions). The linear map of pIP421 is given in Fig. 2. Plasmid pIP421 had unique restriction sites for *Ava*II, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hpa*I and *Sca*I, two sites for *Eco*RV, and three sites for *Dra*I. Plasmid pIP421 was not digested by the following restriction enzymes: *Ava*I, *Hae*II, *Kpn*I, *Nco*I, *Nsi*I, *Pvu*II, *Sal*I, *Sma*I and *Xba*I. The location of the 5-Ni^r determinant of plasmid pIP421 was then determined by Southern blot analysis, using the probe IG defined in Methods. Strong hybridization was only observed with a 1.8 kb *Hind*III restriction fragment (data not shown).

Both 5-Ni^r determinants, and the adjacent regions, were then sequenced from both strands after subcloning into pUC19. The complete nucleotide sequences of the pIP419 and pIP421 5-Ni resistance regions are given in Figs 3 and 4, respectively. The location of the ORFs (ORFnimC and ORFnimD) corresponding to the 5-Ni^r determinants of plasmids pIP419 and pIP421, respectively, was determined and compared with the nucleotide sequences of the *nimA* and *nimB* genes (Haggoud et al., 1994). As already observed in the case of the 5-Ni^r determinants cloned from plasmid pIP417 and from the chromosome of strain BF8, the two ORFs (492 and 495 bp, respectively) have three possible ATG start codons (Figs 3 and 4).

The nim gene products

The largest ORF's, ORFnimC and ORFnimD, may encode polypeptides of 163 and 164 amino acid residues (molecular mass 18.37 and 18.48 kDa), respectively. Pairwise comparisons of the deduced amino acid sequences of the polypeptides encoded by the four *nim* genes so far sequenced were done with the Gap program of the GCG package. The polypeptides show 67–83% identity, and 78–91% similarity. The higher values were obtained with the polypeptides encoded by the chromosomal *nimB* gene of strain BF8 and those of the *nimD* gene of plasmid pIP421. No significant homology was found between the nucleotide and predicted amino acid sequences for the *nim* genes and those available in the GenBank, EMBL or SWISS-PROT databases.

An *E. coli* *in vitro* transcription–translation system was used to investigate the polypeptide synthesis directed by the 2 kb *Sau*3A and the 1.8 kb *Hind*III 5-Ni^r inserts of plasmids pIP419 and pIP421, respectively. The two restriction fragments were cloned in both orientations into pUC19. The results are shown in Fig. 5. In all cases, polypeptides of the expected molecular mass were synthesized from the genes cloned in both orientations, suggesting that the promoters of both genes were functional in *E. coli*. The molecular masses of NimC and NimD determined on polyacrylamide gels were 19 and 17 kDa, respectively, in agreement with the predicted

Fig. 5. Autoradiograph of plasmid-encoded proteins detected in an *in vitro* *E. coli* transcription–translation assay. The [³⁵S] methionine-labelled proteins were separated on an SDS polyacrylamide gel (15%, w/v, acrylamide). Lanes: 1, pUC19; 2, pFK110; 3, pFK111; 4, pFK310; 5, pFK311. Standard proteins are indicated on the left. Products of the *nimC* and *nimD* genes are indicated by arrows.

theoretical values of 18.37 and 18.48 kDa. Similar results were obtained using the *nimA* and *nimB* cloned genes (data not shown).

Characterization of an insertion sequence element, IS1170, present upstream of the *nimC* gene

Analysis of the nucleotide sequence upstream of the *nimC* gene of plasmid pIP419 revealed the presence of an insertion sequence element (IS1170). IS1170 (1604 bp) extends from nucleotide 10 to 1614 (Fig. 3) and is located 24 bp upstream of the first ATG codon of *nimC*. IS1170 is flanked by 15 bp imperfect inverted repeats (IRL and IRR) 12 bp of them matching. The G+C content of IS1170 (42.8 mol%) is within the range (39–46 mol%) determined for genomic DNAs of *Bacteroides* spp. (Johnson, 1978). Two ORFs were present on the same strand within IS1170. They extend from nucleotide 830 to 189 and from nucleotide 1479 to 1054 and could code for polypeptides of 141 and 213 amino acids, respectively. A search in the databases for amino acid sequences related to the two ORFs revealed an identity of 70 and 76%, respectively, with both ends of the unique ORF of *Bacteroides* IS942 described by Rasmussen & Kovacs (1991), suggesting that mutations occurred in the ancestral sequence of the gene encoding the putative transposase. Comparison of the nucleotide sequences of IS942 and IS1170 revealed that they show 70% identity, the two inverted repeats of IS1170 and IS942 being more conserved. The left and right IR of IS942 and IS1170 show 80

Fig. 6. Comparison of the nucleotide sequences of the inverted repeat left (IRL) and right (IRR), and the outward-oriented promoter of the two insertion sequence families. (a) Comparison of IS1186 (Podglajen *et al.*, 1994; GenBank accession number X72301); IS1168 (Haggoud *et al.*, 1994; GenBank accession number X71444) and IS1169 (this work). (b) Comparison of IS942 (Rasmussen *et al.*, 1991; GenBank accession number J03326) and IS1170 (this work). The figures in parentheses indicate the distances between the regions. Nucleotide identity is indicated by asterisks and mismatches by dashes.

CAGCATAT (CCT---IS942---AGG) **CAGCATAT**----- (ATG of *ccrA*)
CAGCATAT (CCT---IS942---AGG) **CATCATAT**----- (ATG of *ccrA*)
 -----**AT** (CCT---IS1170---AGG) **AT**----- (ATG of *nimC*)

-----**TACA** (AGA---IS1186 ---ACC) **TACA**----- (ATG of *cfiA*)
 -**AAAAAGAA** (AGA---IS1186 ---ACC) **AGAATAAA**-- (ATG of *cfiA*)
TAAAAAGAA (AGA---IS1186 ---ACC) **TAAAAAGAA**-- (ATG of *cfiA*)
 -----**TAAG** (AGA---IS1168 ---ACC) **TTAA**----- (ATG of *nimA*)
 -----IS1168 ---ACC) **TTAA**----- (ATG of *nimB*)
 ---**GA** (TTTGTAGA---IS1169 ---ACCAAAA) **GA**----- (ATG of *nimD*)

Fig. 7. Comparison of the insertion site duplication sequences of different *Bacteroides* IS elements upstream of the *ccrA*, *cfiA* and *nim* genes. The insertion site duplication sequences are written in bold type. Data are from Rasmussen & Kovacs (1991) for IS942, Podglajen *et al.* (1994) for IS1168 and Haggoud *et al.* (1994) for IS1169. Other data are from this work.

and 100% identity respectively (Fig. 6). A putative duplication of the target site (Galas & Chandler, 1989), created by the insertion of IS1170 upstream of the *nimC* gene, was only 2 bp in length (AT) instead of the 8 bp target duplication found by Rasmussen & Kovacs (1991) for two integration events of IS942 upstream of the *ccrA* gene (Fig. 7).

Characterization of an insertion sequence element, IS1169, present upstream of the *nimD* gene

Sequence analysis revealed the presence of another insertion sequence element (IS1169) 4 bp upstream of the first ATG codon of the *nimD* gene. IS1169 (1325 bp)

(G + C 41 mol%) contains imperfect inverted repeat ends of 29 bp and shows strong homology with IS1168 adjacent to the *nimA* and *nimB* genes, both in the IR and ORF regions. The IRL and IRR of IS1169 show 62 and 76% identity, respectively, to the corresponding regions of IS1168 (Fig. 6). The three ORFs (ORFa, b and c) found within IS1169 potentially code for basic polypeptides of molecular mass 31.57, 8.17 and 18.34 kDa, respectively. The largest ORF (ORFa) shows 78.5% identity at the amino acid level with the corresponding ORF of IS1168. The results of a search in the GenBank and EMBL databases for sequences related to IS1169 were identical to those found for IS1168 (Haggoud *et al.*, 1994), i.e. IS1246 harboured by plasmid pWVO of *Pseudomonas putida* (Reddy *et al.*, 1994, accession number L11583), IS1106 from *Neisseria meningitidis* (Knight *et al.*, 1992), IS5 from IKH100 (Kröger & Hobom, 1982) and IS52 from *Pseudomonas syringae* (Yamada *et al.*, 1986).

The duplication of the target site expected for the insertion sequence of the IS5 family is 4 bp in length (Galas & Chandler, 1989). Some examples of the target duplication of IS1186, IS1168 and IS1169 are illustrated in Fig. 7. For IS1186, Podglajen *et al.* (1994) found in all cases but one a 4 bp target duplication. In one strain the target duplication was 9 bp in length. The situation for IS1168 present upstream of the *nim* genes is less clear. In the copy of IS1168 present upstream of the *nimA* gene a 3 bp target duplication (TAA) was found, separated by one nucleotide from both ends of the inverted repeats. A similar situation was observed by Knight *et al.* (1992) for IS1106

Fig. 8. Southern blot analysis of digested DNA of pIP419 and pIP421 using IS1170 and IS1169 as internal probes. (a) Plasmid DNA restriction profiles on stained agarose gel; (b) Southern analysis of the gel using the IS1170 probe; (c) Southern analysis of the gel using the IS1169 probe. Lanes: 1, λ DNA digested by *Hind*III and *Eco*RV (molecular mass standard); 2, pIP419 digested by *Dra*I; 3, pIP421 digested by *Dra*I-*Sca*I; 4, pIP421 digested by *Eco*RV-*Sca*I (note that a 6.15 kb restriction fragment which also hybridizes with the IS1169 probe is from a partial digestion fragment of the plasmid consisting of the 3.25 plus 2.9 kb restriction fragments).

in *N. meningitidis*. For the copy of IS1169 present upstream of the *nimD* gene, a 2 bp sequence (GA) was found as a potential target duplication site.

Absence of a composite transposon-like structure on plasmids pIP419 and pIP421

It has been previously shown that only one copy of IS1168 is present on plasmid pIP417, demonstrating that the *nimA* gene is not part of a transposon (Haggoud *et al.*, 1994). The copy number of IS1170 and IS1169 from plasmids pIP419 and pIP421, respectively, was determined by Southern blot analysis. The probes used in this study were the internal *Sau*3A-*Eco*RI restriction fragment of length 626 bp (nt 782-1408, Fig. 3) for IS1170, and the internal *Hind*III restriction fragment of length 366 bp (nt 333-699, Fig. 4) for IS1169. The location of the two probes are shown in Figs 1 and 2, respectively. Plasmid pIP419 was digested by *Dra*I to give restriction fragments of lengths 3.1, 5.5 and 1.5 kb. Plasmid pIP421 was doubly digested by *Sca*I-*Dra*I and *Sca*I-*Eco*RV to give restriction fragments of lengths 2.24, 2.1, 2.65 and 0.26 kb, and 2.9, 3.25 and 1.1 kb, respectively. Results of hybridization experiments are shown in Fig. 8. For plasmid pIP419, only the expected 2.95 kb *Dra*I restriction fragment hybridized with the IS1170 probe (lane 2) but not with the IS1169 probe. For plasmid pIP421, the 2.24 kb *Sca*I-*Dra*I (lane 3) and the 2.9 kb *Eco*RV-*Sca*I (lane 4) restriction fragments hybridized with the IS1169 probe. From the physical maps we concluded that in both cases only one copy of the insertion sequence element is present upstream of each *nim* gene. Thus, the insertion sequence elements of plasmids pIP419 and pIP421 are not parts of a composite transposon.

DISCUSSION

The two 5-Ni^r genes *nimC* and *nimD*, present on plasmids pIP419 and pIP421, have been cloned and sequenced. Nucleotide sequence analysis revealed the presence of two ORFs, ORF_{nimC} and ORF_{nimD}, which may encode polypeptides of 163 and 164 amino acid residues (mo-

lecular mass 18.37 and 18.48 kDa), respectively. The two predicted proteins show 72.5% identity and 84.7% similarity.

The four *nim* genes so far studied (this work, and Haggoud *et al.*, 1994) share some common features. The nucleotide sequence length is from 492 to 534 bp with a G+C content varying from 42.8 to 55.9 mol%. Pairwise comparisons of the nucleotide sequences reveal an identity of about 70%, which suggests that the *nim* genes are closely related. Nevertheless, the multiple alignment of the four sequences reveals that they show approximately 49% identity, indicating a high degree of variability at the nucleotide level. The genes could direct the synthesis of polypeptides of molecular mass 18-20 kDa showing 67-83% identity and 78-91% similarity when the conservative amino acid substitutions are taken into account. The multiple alignment of the four amino acid sequences disclosed a greater homology of the *nim* gene products, with an identity of 57% and a similarity of 78%. We suggest that the four genes, whether chromosomally determined or plasmid-borne, are derived from a common ancestral gene. No significant homology was found with either the nucleotide or amino acid sequences in the GenBank and EMBL databases.

Another important feature is that each *nim* gene is preceded by an insertion sequence element located a few base pairs upstream of the putative start codon. The same insertion sequence element, IS1168, was found upstream of the *nimA* and *nimB* genes, whereas other insertion sequences, IS1169 and IS1170, were found upstream of the *nimD* and *nimC* genes, respectively. These insertion sequence elements belong to two families as indicated in Fig. 6.

The first family consists of the three *Bacteroides* insertion sequence elements IS1168, IS1169 and IS1186. The latter element was detected upstream of the carbapenemase gene *cfiA* in a clinical isolate of *B. fragilis* (Podglajen *et al.*, 1994). In this carbapenem-resistant strain, the transcription of *cfiA* was found to be driven from a promoter identified on the right end of IS1186. The -35 region

sequence (TTGGGG) is separated by the optimal spacing distance of 17 bp from the -10 region (TAACCTT). Comparison of the nucleotide sequence of IS1168 and IS1186 revealed that the two insertion sequence elements were almost identical (99.7% identity), suggesting that the transcription of the *nimA* gene is also directed by the same promoter. Furthermore, analysis of the nucleotide sequence of IS1169, which shows 71% identity to IS1168, reveals that there is a nearly perfect match (five out of six base pairs) between both the -35 and -10 sequences of IS1186 (see Fig. 6). We thus conclude that, as for the *cfiA* gene, the *nimA*, *B* and *D* genes are transcribed from an internal insertion sequence promoter. In a survey of 36 5-Ni^r clinical isolates of *Bacteroides* strains, the insertion sequence elements of the first family were found to be associated with a *nim* gene in 94% of the strains (Reysset *et al.*, 1993, and data not shown), which suggests a wide distribution amongst resistant strains.

IS942 and IS1170 belong to a second *Bacteroides* insertion sequence family. One copy of IS942 was found integrated 19 bp upstream of the predicted initiation codon of the metallo- β -lactamase gene *ccrA* cloned from *B. fragilis* TAL3636 (Rasmussen & Kovacs, 1991). The authors have also suggested that the transcription of the *ccrA* gene was driven from a promoter within the IS element, and have defined the -35 and -10 promoter-like sequences (Rasmussen *et al.*, 1990). As shown in Fig. 7, two related -35 and -10 boxes were also found in the corresponding regions of IS1170. Moreover, although the 1 kb *DraI*-*Sau3A* restriction fragment of plasmid pIP419 contained the entire ORF_{nimC}, it did not confer the 5-Ni^r phenotype, the *DraI* site being located between the proposed -35 and -10 sequences (see Fig. 3).

An identical tetranucleotide AGAA sequence, which appeared to be an efficient ribosome-binding site in *Bacteroides* (Podglajen *et al.*, 1994), was found between the end of the IRR of each insertion sequence element and the ATG start codon of the *cfiA*, *ccrA* or *nim* genes. We have also shown by *in vitro* transcription-translation assay that the promoters of IS1168, IS1169 and IS1170 are functional in *E. coli*, suggesting that the outward-oriented promoters of the two *Bacteroides* IS families could be functional in this species.

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