Multiple Mobile Promoter Regions for the Rare Carbapenem Resistance Gene of Bacteroides fragilis

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Received 30 October 2000/Accepted 20 March 2001

Bacterial insertion sequences (IS), integral parts of the chromosome of many bacterial species, are mobile DNA elements which typically code only for the transposase which promotes their mobility. These elements are capable of generating mutations and genome rearrangements as a result of translocation, of promoting gene acquisition, and of mobilizing DNA fragments via the formation of compound transposons (13, 14). By various mechanisms, the presence or mobility of these elements may affect degradative pathways (9, 10), bacterial pathogenicity or virulence (8, 35), and resistance to antibiotics (15, 17) and may condition gene expression. Genes may be silenced by insertional gene disruption and reactivated by precise excision (8, 11, 35). Gene activation also may result from the provision of efficient promoters, carried either entirely by the element (10, 17) or generated as hybrid structures between IS elements and genome rearrangements as a result of translocation, of promoting gene acquisition, and of mobilizing DNA fragments via the formation of compound transposons (13, 14). By various mechanisms, the presence or mobility of these elements may affect degradative pathways (9, 10), bacterial pathogenicity or virulence (8, 35), and resistance to antibiotics (15, 17) and may condition gene expression. Genes may be silenced by insertional gene disruption and reactivated by precise excision (8, 11, 35). Gene activation also may result from the provision of efficient promoters, carried either entirely by the element (10, 17) or generated as hybrid structures between IS, upon insertion, and target sequences (5, 9, 12, 15).

Like most bacterial genera in which insertion sequences have been searched for, Bacteroides harbors several such elements. They are IS4351, IS942, IS1186, and IS224 (or their isofoms), and they belong to the IS30, IS4, IS5, and IS21 families, respectively (13, 27). Only IS4351 has also been observed as part of a compound transposon (20). The four elements have been found preferentially in Bacteroides fragilis, the anaerobe species most frequently isolated from human infections (24).

No involvement other than that in the expression of antibiotic resistance genes has, as yet, been observed for the Bacteroides IS elements. While it was speculated that IS1224 may activate the endogenous B. fragilis cephalosporinase gene, cepA, either via inactivation of a repressor function or generation of a hybrid promoter (22, 27), it has been shown that the carbapenemase gene cfa (cfrA) is activated by an IS1186-borne promoter (17). It seemed likely that there was a similar contribution to transcription of cfrA by IS942 (19) and IS4351 (I. Podglajen, unpublished data), of the macrolide resistance gene ermF by IS4351 (20), and of the metronidazole resistance genes nimA and nimB by IS1168 (7), nimD by IS1169 and nimC by IS1170 (32).

The carbapenemase CfaA confers resistance to practically all β-lactams, the most widely used class of antibiotics. Surprisingly, this conceivably advantageous resistance trait seems to have remained confined to a small, genotypically identified subgroup (or subspecies) of B. fragilis (6, 16, 23) despite the presence of a variety of genetic elements with the potential for gene mobilization and transfer within the genus Bacteroides (25–27). In the present study we describe two novel insertion sequences and their capacity to efficiently promote the expression of cfrA.

Identification and characterization of two novel IS elements in B. fragilis. During the systematic analysis of carbapenem resistance in clinical isolates of B. fragilis we observed five imipenem-resistant strains, by using dot blot hybridization (data not shown), which carried cfrA but none of the promoter-bearing insertion sequences IS1186 (17), IS942 (19), or IS4351 (20 and I. Podglajen, unpublished) or closely related IS isoforms. Since cfrA has generally been found to be silent unless at least one of these IS is present, we explored the mode of its activation in the five clinical strains which are part of our collection of 65 cfrA-positive strains.

Species identification was carried out using API 20A and API 32A strips (bioMérieux, Marcy-l’Etoile, France). Identification of all strains given in Fig. 1 was confirmed by nucleotide sequence analysis of 16S ribosomal DNA segments amplified with the primers BF16SF (TAACTCCGTGCCAGCAGC) and BF16SR (GTGGACTACCAGGGTATC) and Extra-pol II DNA polymerase (Eurobio, Les Ulis, France) under standard conditions (1). The regions upstream of cfrA were amplified with primers E (CTTCGAATTCGGCGAGGGATACATA) and G (CGCCAAGCTTTGCCTGCCATTAT), specific for sequences upstream of the known IS insertion sites and cfrA, respectively (17). The nucleotide sequences of the PCR-generated fragments were determined by a commercial provider (Génome Express, Montreuil, France) or by using the Thermo
Sequenase [\textsuperscript{33}P] Terminator Cycle Sequencing kit from Amer- sham. New primers were synthesized as sequence determination proceeded. All sequences were determined in duplicate on both strands.

Two novel IS elements were identified, IS\textsuperscript{1187} in four of the imipenem-resistant strains and IS\textsuperscript{1188} in one strain (Fig. 1). IS\textsuperscript{1187} was 1,026 bp in length, had 21-bp inverted repeats (with one mismatch), and contained one major open reading frame, spanning most of the element, with a protein-coding capacity of 326 amino acids (aa), a deduced molecular size of 37.5 kDa, and a theoretical pl of 9.3. The stop codon of this reading frame was located within the right inverted repeat. The inverted repeats of this element were flanked by direct repeats of 9 bp in BFr1757 and BFr1761 and 8 bp (with one mismatch) in BFr1758 and BFr1760 (Fig. 2). The second element, IS\textsuperscript{1188}, was 1,691 bp in length, had 17-bp inverted repeats (with three mismatches), and contained two major open reading frames, one with a protein-coding capacity of 448 aa, a deduced molecular size of 52.5 kDa, and a theoretical pl of 9.5; the other, on the opposite strand, with a protein-coding capacity of 140 aa, a molecular size of 16.3 kDa, and a theoretical pl of 9.7. The inverted repeats of this element were flanked by direct repeats of 4 bp (Fig. 2).

Insertion of both elements occurred within ca. 90 bp upstream from the initiation codon of cfiA and in all but one case occurred very close to the sites at which insertion of all IS elements known or suspected to carry cfiA-activating promoters has been observed previously (Fig. 2).

| Strain   | IS   | cfiA | Hybridization | Relative intensity | MIC IMI (\mu g/ml) |
|----------|------|------|---------------|-------------------|-------------------|
| BFr1765  | 1188 | -d   |               |                   | 0.25              |
| BFr902   | 1188 | +    |               |                   | 64                |
| BFr1757  | 1187 | +    |               |                   | 32                |
| BFr1758  | 1187 | +    |               |                   | 64                |
| BFr1760  | 1187 | +    |               |                   | 64                |
| BFr1761  | 1187 | +    |               |                   | 32                |
| TAL2480  | 942  | +    |               |                   | 128               |
| BFr81R   | 1186 | +    |               |                   | 128               |
| BFr81    | 1186 | +    |               |                   | 1                 |

**FIG. 1.** Relative efficiency of cfiA transcription mediated by various IS elements as related to susceptibility to imipenem. a, autoradiograph of RNA-DNA slot blot hybridization with total RNA and a \textsuperscript{32}P-labeled cfiA probe; b, relative intensity determined after densitometry of the autoradiograph; c, MIC IMI, minimal inhibitory concentration of imipenem; d, absence (−) or presence (+) of cfiA (in BFr81 there is no IS upstream of cfiA).

**FIG. 2.** Insertion sites and direct repeats of insertion sequences activating cfiA transcription. Direct repeats are underlined and mismatches are shown below the line. a, sequence upstream from cfiA in strain BFr81 with the ribosomal binding site (RBS) and translation initiation codon shown in bold; b, sequence in BFR81R (17); c, e, and f, sequences in BFr908, BFr271R, and BFr930, respectively (I. Podglajen, unpublished); d, sequence in strains TAL2480 (31) and TAL3636 (18, 19); g, h, i, and k, sequences in strains BFr902, BFr1757, BFr1758 and BFr1760, and BFr1761, respectively. Nucleotide variations with respect to BFr81 are shown in lower case; −, no nucleotide at that position.
The copy numbers of IS1187 and IS1188 were estimated in cfIA-positive and cfIA-negative strains (see below) using IS-specific probes after digestion of chromosomal DNA with AvaI and Southern hybridization (17). Probes specific for the respective transposase-coding regions were generated with primers BF1187F (CGTATTGCAGAATGGATAAGTG) and BF1187R (GTTCCACGTCTGGTGTCCTGTTC) amplifying a 725-bp fragment of IS1187 and primers BF1188F (GGCCTGTGCTCACCAGAC) and BF1188R (CGGATCCGCTAGGCTCATATGC) amplifying a 778-bp fragment of IS1188. The probes were randomly labeled with the MegaPrime kit and [32P]dCTP from Amersham. The copy numbers of the two elements varied between one in the two cfIA-negative strains, as for IS1187 in BFr1763 and IS1188 in BFr1765, and three to at least five in the cfIA-positive strains (Fig. 3). Since neither IS contained an AvaI site, each band should represent at least one element. The differences in hybridization intensity might reflect the existence of incomplete copies or, perhaps more likely, of copies with some sequence divergence (IS isoforms). Such an element, with 74% homology to IS1187, has been observed in a clinical B. fragilis isolate in the United Kingdom while this work was under way (3).

When criteria for grouping IS (13, 14) were applied, IS1187 and IS1188 appeared to be members of the IS5 family. There was agreement with the overall size of the elements and the size of the inverted and direct repeats. Furthermore, identities in the vicinity of the amino acids of the DDE triad, a conserved motif of the active site of IS transposases (13, 14), indicated a relationship of the putative transposase of IS1187 with that of IS5 and of the transposase of IS1188 with that of IS1031, representative of a subgroup of the IS5 family (Fig. 4) (13).

When the amino acid sequences of the putative transposases were compared with sequences in the databases, that of IS1187 was most closely related to a plasmid-coded transposase of ISR1 from Riemerella anatipestifer (33), with 45% identities and 66% equivalencies over a stretch of 300 aa, to the transposase from Clostridium cellulovorans (30) (34% identities, 50% equivalencies, over a stretch of 251 aa) and to the transposase of IS982 from lactococci (34) (29% identities, 45% equivalencies, over a stretch of 189 aa). The putative transposase of IS1188 was most closely related to similar proteins coded by an IS element from a Sphingomonas sp. (34) (32% identities, 51% equivalencies, over a stretch of 418 aa), by IS1380 from Acetobacter pasteurianus (29) (31% identities, 47% equivalencies, over a stretch of 447 aa), and by the chromosome of Bacillus halodurans (28).

The G+C contents of IS1187 (42.9 mol%) and IS1188 (46.1 mol%) were quite close to the values of 40.1, 42, and 46.6 mol% for IS942 (19), IS4351 (20), and IS1186 (17), respectively, and 42 mol% for the B. fragilis chromosome overall (27). These values neither support nor preclude the possibility of a foreign origin of the IS elements.

Incidence of IS1187 and IS1188 among B. fragilis isolates.

The IS elements reported previously in Bacteroides appear to exist primarily in the small cfIA-positive subgroup of B. fragilis, where they are chromosome borne (16). To test the possibility that this was true also for IS1187 and IS1188, we carried out dot blot hybridization experiments using the IS-specific probes. Seventy-five randomly collected cfIA-negative B. fragilis strains, mostly from our laboratory collection or kindly provided by I. Casin and L. Dubreuil, were assayed for the presence of the two elements. Hybridization was positive with each element in one strain but did not occur with any of the several plasmids in the size range of ca. 3 to 8 kb that were present in both strains (data not shown). This suggested a chromosomal location of the elements in these strains. Except for IS1224 (27), no IS has been reported as being chromosome borne outside of the cfIA-positive group. In the cases where IS942 or IS1186 or isolomers thereof have been described outside of this group (7, 32), they were carried on small plasmids. There was no coresidence in the IS1187- or IS1188-bearing strains with one of the three known IS elements IS942, IS1186, or IS4351, combinations of which, by contrast, were observed in about one-third of the 50 cfIA-positive strains analyzed previously (16). Whether the absence of such a coresidence reflects a recent introduction of

| IS5  | TiVDTA (76) | v-aDaG (45) | iE (5) | 1R | v |
|------|------------|-------------|-------|----|---|
| IS1186 | V-D (58) | DaG (54) | -E (7) | vR |
| IS1187 | V-D (85) | D-G (42) | -E (5) | 1- |
| IS1031 | iDSQ (71) | i-Ds-Y (41) | LprRvV (7) | R |
| IS1188 | i-D (80) | D- (64) | L-prV (5) | R |

FIG. 4. DDE motifs in the putative transposases of IS1187 and IS1188. Data for IS5 and IS1031 were taken from Mahillon and Chandler (13), who also assigned IS1186 (17) to the IS5 family.
IS1187 or IS1188 or both into B. fragilis remains a matter of speculation.

Expression of cfiA in IS1187- or IS1188-bearing strains. Expression of cfiA was studied by Northern hybridization using a slot blot procedure with cesium chloride gradient-purified RNA (10 μg per slot) and a 32P-labeled cfiA probe. The relative efficiency of cfiA transcription was estimated after densitometry of the autoradiograph (Fig. 1). There was an apparent correlation between the degree of cfiA transcription and the level of resistance to imipenem, with a somewhat lower relative transcription in strain BFr902. Considering that IS1187 level of resistance to imipenem, with a somewhat lower relative transcription and the cfia promoter activity in cepA (2). There were two consecutive –7 motifs in IS1187. In IS1186 and IS1188, the octamer motifs overlapped with a putative consensus E. coli σ70 promoter at the –10 hexamer, and the TTG triplet of the –33 motif overlapped with the –35 hexamer. There was no such overlap in IS942 and IS1187, but putative E. coli σ70 promoter sequences were observed at some distance downstream or upstream, respectively (Fig. 6). Whether the –10 and –35 hexamers may be functional in σ70 homologue-producing species more closely related to the Enterobacteriaceae remains to be tested experimentally. This possibility leads to the further speculation that the multiplicity of consensus sequences might endow Bacteroides IS elements with the potential to provide mobile promoters to distantly related bacterial species.

FIG. 5. Mapping of cfiA transcripts by 5’ RACE. Lane 1, strain BFr1757 (IS1187); lane 2, BFr902 (IS1188); lane 3, TAL2480 (IS942); lane 4, BFr81R (IS1186); lane M, molecular size standards (123-bp DNA ladder; Gibco BRL-Life Sciences).

FIG. 6. Start sites of cfiA transcription downstream from putative promoter sequences in B. fragilis IS elements. Start sites determined by 5’ RACE are indicated by an arrow, and the distance to the translation initiation codon is indicated in base pairs. Consensus sequences of the B. fragilis promoter sequences (TTTG [−33]), TAnnTTTG [−7]) (2) are shown in bold. Putative –35 and –10 hexamers of the E. coli Eσ70 promoter (21) are underlined.
With respect to cfiA, the number of known mobile promoters, currently five, that this rare and normally promoterless gene is able to recruit in order to ensure its transcription is quite remarkable. It is conceivable that the use of carbapenemases has somehow favored the accumulation of IS elements in the cfiA-positive *B. fragilis* population, or alternatively, that this population might possess particular factors enhancing their acquisition or transposition.

**Nucleotide sequence accession numbers.** The nucleotide sequences of IS1187 and IS1188 have been deposited in the GenBank and EMBL databases under the accession numbers Y18979 and AJ277413, respectively.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (CRI 95-06) and the MENRT-sponsored Programme de Recherche Fondamentale en Microbiologie et Maladies Infectieuses et Parasitaires.

We gratefully acknowledge I. Casin and L. Dubreuil for the gift of strains and P. Bertin for critically reading the manuscript.

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