

## Gene Context and DNA Rearrangements in the Carbapenemase Locus of Division II Strains of *Bacteroides fragilis*<sup>∇</sup>

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**The *cfiA* gene is clustered in a bicistronic operon encoding an *N*-acetyltransferase and an *O*-acetyltransferase related to resistance markers. This genetic context, exclusively found in strains of *Bacteroides fragilis* division II, has been highly rearranged by the successive integration of two new mobile sequences, a miniature element and ISB $\beta$ 9. Besides that, among the DNA polymorphisms detected in the *cfiA* locus, only the integration of IS942 at its promoter was a determinant for expression of carbapenemase activity.**

The *cfiA* gene, encoding the unique carbapenemase enzyme found in *Bacteroides*, is restricted to the division II group of *Bacteroides fragilis* strains (5). Bacteria belonging to this group were isolated in previous studies of human (3) or animal (unpublished data) infections, where identification of the *cfiA* genes was performed by PCR with primers P1 and P4 (Table 1) and confirmed by Southern hybridization on genomic DNA digestions (not shown).

**Imipenemase activity.** Division II strains were cultivated under anaerobic conditions (3), and enzyme activity was determined at 37°C with 0.1 mM imipenem and 50 mM phosphate buffer (pH 7.0) by using an  $\epsilon_{299}$  (extinction coefficient at 299 nm) value of 9,670 M<sup>-1</sup> cm<sup>-1</sup>. Only strains MN11 and MN32 expressed imipenemase activity and were resistant to imipenem (Fig. 1).

**Allelic polymorphism.** The sequences of *cfiA* genes revealed that they encode previously described protein variants (4). The allele from strain MN5 (*cfiA11*) corresponds to protein polymorphisms in residues 79 (T), 85 (T), 113 (K), 188 (T), and 225 (N), referred to as TTKTN; alleles from strains MN11 (*cfiA12*), MN32 (*cfiA13*), and 4a (*cfiA14*) encode the protein variant MTRAD; and those from strains MN36 (*cfiA15*) and

MN41 (*cfiA16*) presented the polymorphisms TAKAD and TTKAD, respectively. Only MTRAD alleles were presented in the two strains, MN11 and MN32, expressing imipenemase activity (Fig. 1). However, the occurrence of a particular protein variant does not correlate with the metallolactamase phenotype, since the 4a strain (allele *cfiA14*; protein variant MTRAD) is sensitive to carbapenems. Accordingly, a previous work reported TAKTN and TAKAD alleles in strains sensitive to carbapenem, while TAKAD, TTKTN, and TAKTD alleles were found in resistant strains, where the expression of imipenemase activity was determined by the integration of IS elements near the promoter of the gene (4).

**Upstream sequences.** Inverse PCR (iPCR) was performed to isolate the DNA regions upstream from the *cfiA* genes. Genomic DNAs were digested by HaeIII, and after ligation (3), the R1 and R2 primers (Table 1) were used in a PCR of 35 cycles with annealing/extension at 68°C. A fragment of 0.7 kb was expected from the HaeIII restriction site placed 157 bp upstream of the *cfiA* start codon (Fig. 2C), but the display of iPCR fragments revealed the polymorphism of that region (Fig. 2A). Thus, strains MN5, MN35, MN41, and 4a presented

TABLE 1. Primers used in this work

Primer	Sequence (5'–3')	Position <sup>b</sup>	Usage of primer pair
P1 <sup>a</sup>	AAAGAATAAAATGAAAACAGT	1855	PCR of <i>cfiA</i> , probe for Southern hybridization, detection of protein polymorphisms
P4 <sup>a</sup>	GATAAAAGTTTTTCGCCCTCTTC	2260	
P5	AAGCAGGGATCTGCTTAC	164	PCR of <i>orf1-orf3</i> intergenic sequence: detection of sequence polymorphisms
P6	ATGACCGTATTCAGATAG	1109	
R1	TGCGACAGGGAAAAGCATGGAGA	1867	iPCR of <i>cfiA</i> upstream region: detection of sequence polymorphisms.
R2	CCCTCGCCGAAATCGAAGGATG	1990	

<sup>a</sup> Described in reference 2.

<sup>b</sup> The 3' ends of primers are positioned in relation to the sequence under accession no. FM200792.

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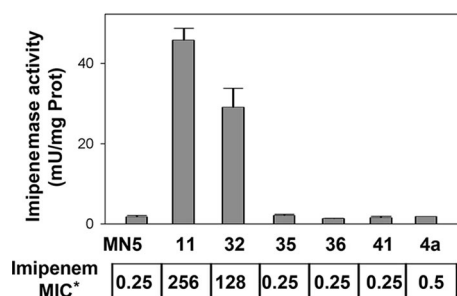


FIG. 1. Imipenemase activities and imipenem resistance of the *B. fragilis* strains. MICs ( $\mu\text{g/ml}$ ) for some strains were reported previously (3).

fragments of the predictable size, while PCR products of 2.4 kb were detected in strains MN11, MN32, and MN36. Sequencing of iPCR fragments showed that the *cfiA* upstream sequences from strains MN11 and MN32 share the insertion, 7 bp upstream of the gene, of an IS942-like mobile element. As described previously (4), this element might determinate the imipenem resistance phenotype (Fig. 1). In contrast, the DNA fragment from strain MN36 is produced accidentally, since a sequence polymorphism abolished the proximal restriction site. This situation makes available for research the DNA region upstream of the *cfiA* gene.

**Gene context.** Three coding sequences were detected upstream of the *cfiA* gene from strain MN36 (Fig. 2C). The first sequence, *orf1*, is closely related to genes Bf3550 and Bf3356 from the genomes of *B. fragilis* YCH46 and NCTC9343, respectively, where they are annotated as hypothetical membrane proteins. The genes *orf2* and *orf3*, which are minimally overlapped (1 bp), encode nonrelated proteins belonging to the Gcn5-related *N*-acetyltransferase (8) and streptogramin A acetyltransferase (7) families, respectively.

All DNA fragments spanning further than 151 bp upstream of the *cfiA* gene, including sequences available in public databases, share the sequence of *orf3*. The primers P5 and P6 were used in a PCR of 30 cycles, with an annealing temperature at 54°C, which showed the exclusive detection of the *orf1-orf2-orf3* gene cluster in division II strains (5) and that the intergenic sequence between *orf1* and *orf2* is highly polymorphic (Fig. 2B). Sequencing of the amplified fragments revealed that the *orf2* upstream sequence is the target for integration of two new mobile sequences (Fig. 2C), a putative miniature element (MITE*Bf1*) and a member of the ISAs1 family, named IS*Bf9*, following the standard nomenclature (6).

Two *cfiA* gene sequences that exist in public databases (GenBank), under accession numbers M34831 and AY372696, extend downstream from the *cfiA* gene and span a sequence closely related to Bf3360 from *B. fragilis* NCTC9343, encoding a member of the small multidrug resistance protein family (1). Thus, the DNA region between Bf3356 and Bf3360 in *B. fragilis* NCTC9343 could have been exchanged in the strains carrying the metallo-lactamase gene, a hypothesis supported by the GC content bias (roughly 5%) existing between the *orf2-orf3-cfiA* gene cluster and its genomic environment (Fig. 2C). The presence of *cfiA* in a region of genomic plasticity, where sequences

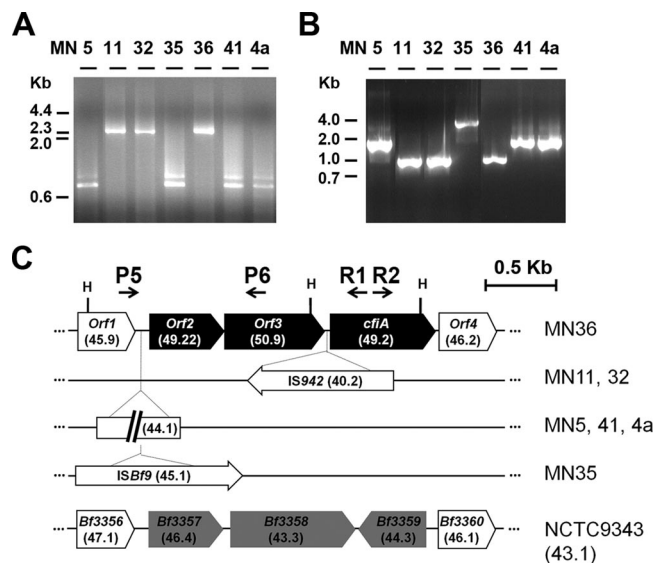


FIG. 2. Structures of *cfiA* upstream sequences. (A) iPCR fragments amplified with the R1/R2 primer pair and HaeIII (H) digestions. (B) *orf1-orf3* intergenic polymorphisms displayed by PCR with the P5/P6 primer pair. (C) Genomic map of the *cfiA* gene cluster and surrounding regions. Numbers in parentheses indicate %GC of sequence elements. See the text for accession numbers.

related to resistance markers or mobilization functions are detected, might condition the potential for spreading carbapenem resistance among *Bacteroides* and related bacteria, a clinically relevant gene context that paradoxically remained unknown.

**Nucleotide sequence accession numbers.** Accession numbers for *cfiA* coding and upstream sequences determined in this work are FM200784 to FM200794.

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