ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1999, p. 2808–2810 Vol. 43, No. 11
0066-4804/99/$04.00+0

Distribution of the *cfiA* Gene among *Bacteroides fragilis* Strains in Japan and Relatedness of *cfiA* to Imipenem Resistance

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Received 15 March 1999/Returned for modification 23 June 1999/Accepted 4 September 1999

The *cfiA* gene, encoding an imipenem-hydrolyzing metallo-β-lactamase produced by *Bacteroides fragilis*, and insertion-like elements were detected by PCR amplification with *B. fragilis* strains isolated in Japan. The *cfiA* gene was found in 1.9 and 4.1% of the imipenem-susceptible *B. fragilis* isolates collected from 1987 to 1988 and from 1992 to 1994, respectively. Insertion-like elements adjacent to the *cfiA* gene were found in all nine metallo-β-lactamase-producing imipenem-resistant strains tested but not in nine *cfiA*-positive strains with no detectable metallo-β-lactamase activity.

Bacteroides fragilis is an anaerobic bacterium most frequently isolated from suppurative anaerobic infections and exhibits a broad spectrum of resistance to antimicrobial agents (17). Nationwide surveys in Japan and the United States showed imipenem to be very active against *B. fragilis* (2, 4). However, the emergence of resistance to imipenem among *B. fragilis* strains has been reported (1, 3, 5). It has been suggested that the production of an imipenem-hydrolyzing metallo-β-lactamase contributes to imipenem resistance among *B. fragilis* strains (1, 10).

The metallo-β-lactamase produced by *B. fragilis* is encoded by the *cfiA* gene (22), which has also been called the *ccrA* gene (15). A recent study demonstrated that an insertion element (IS), IS*1186*, located immediately upstream of the *cfiA* gene promoted the expression of this carbapenemase gene (13) as well as other insertion elements (14). Podglajen et al. suggested that a one-step mutation can allow the silent *cfiA* gene to be expressed (12). If so, *B. fragilis* strains carrying the silent *cfiA* gene would be expected to be eradicated in clinical settings before mutation occurs.

The aim of this study was to investigate the distribution of the *cfiA* gene among *B. fragilis* strains in Japan and to analyze the relationships between susceptibility to imipenem, metallo-β-lactamase production, and the presence of the *cfiA* gene adjacent to IS-like elements. A one-step mutation of *cfiA*-positive, imipenem-susceptible *B. fragilis* strains was also tested.

B. fragilis clinical strains used were placed into one of three groups. (i) The first group consisted of 21 stock strains, including 7 imipenem-resistant strains (MIC, ≥256 μg/ml, 4 strains; 32 μg/ml, 1 strain; and 16 μg/ml, 2 strains) from our laboratory, which were collected between 1986 and 1994 from various hospitals in Japan, and 13 imipenem-susceptible strains (MIC, 4 μg/ml, 1 strain; 1 μg/ml, 5 strains; and 0.5 μg/ml, 7 strains), and 1 imipenem-intermediate strain (MIC, 8 μg/ml), which were collected before 1987. (ii) The second group included 162 isolates, collected between 1987 and 1988, from a central clinical laboratory in Tokyo, Japan. (iii) The third group consisted of 124 isolates collected at Gifu University Hospital, Gifu, Japan, between 1992 and 1994.

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Susceptibility was tested by an agar dilution method (8). Imipenem of known potency was obtained from Banyu Pharmaceutical, Tokyo, Japan.

Metallo-β-lactamase activity was assayed by both a spectrophotometric technique (1) and a biological method. For the biological assay, a 2-day culture of *B. fragilis* on modified Gifu anaerobe medium (GAM) agar (Nissui Pharmaceutical, Tokyo, Japan) was suspended in Anaerobe Broth MIC medium (Difco Laboratories, Detroit, Mich.). The cell suspension of 10^6 CFU/ml was mixed with the same volume of 200 mM 3-(*N*-morpholino)propanesulfonic acid–potassium hydroxide buffer (pH 7.2) containing imipenem at a final concentration of 6.3 μM or with imipenem solution supplemented with 2 mM EDTA. The mixture was incubated anaerobically for 18 h at 37°C. Imipenem alone and a mixture of imipenem and EDTA were incubated in parallel as controls.

To measure the remaining imipenem bioactivity, blank paper disks (Toyo-roshi, Tokyo, Japan) were impregnated with 30 μl of the mixture and placed on Antibiotic Medium 3 (Difco) plus 1.5% agar which was seeded with *Bacillus subtilis* MB-32 as an indicator strain. Plates were read for the presence of inhibition zones after overnight aerobic incubation at 37°C.

Bacterial DNA was obtained by heating cells for 10 min at 95°C. The primers for detection of the *cfiA* gene and IS-like elements and the predicted size of PCR products with primer sets are listed in Table 1. PCR amplification was run for 35 cycles consisting of 20 s at 95°C and 2 min at 64°C as described elsewhere (9). Southern hybridization was performed as described previously (7). Oligonucleotide probe GBI-3 was used for a PCR product with GBI-1 and GBI-2 primers, and oligonucleotide probe GBI-2 was used for an amplicon with GBI-3 and GBI-4 primers (Table 1).

Four *cfiA*-positive and four *cfiA*-negative imipenem-susceptible strains were tested for a one-step mutation resulting in imipenem resistance. A 48-h culture of each of these strains was suspended in Anaerobe Broth MIC medium at a concentration of 10^9 CFU/ml. A 100-μl aliquot of cell suspension was spread on modified GAM agar containing 16 μg of imipenem per ml and incubated anaerobically for 72 h at 37°C. Ten colonies on each agar plate, if available, were subcultured on modified GAM agar and subjected to imipenem susceptibility testing as described above.

To detect the *cfiA* gene, PCR amplification with three

TABLE 1. Sequences of oligonucleotide primers and probes

Genetic element and oligonucleotide	Sequence (5′–3′)	Position[a]	Usage	Amplicon (predicted size)
cfiA gene				
GBI-1	CCCAACTCTCGGACAAAGTG	624–643	Forward primer	GBI-1–GBI-2 (340 bp)
GBI-2	AGTGAATCGGTGAATCCATG	944–963	Reverse primer and probe for a GBI-3 and GBI-4 primer set	
GBI-3	CGAACCAGATGACGATAGAC	891–910	Forward primer and probe for a GBI-1 and GBI-2 primer set	GBI-3–GBI-4 (358 bp)
GBI-4	ACGATCTGCTTGGTATGCTC	1229–1248	Reverse primer	GBI-1–GBI-4 (625 bp)
IS				
G[b]	CGCCAAGCTTTGCCTGCCATTAT	Upstream of *cfiA*	Forward primer	G-E (approx. 2 kbp)
E[b]	CTTCGAATTCGGCGAGGGATACATAA	Inside of *cfiA*	Reverse primer	

[a] Thompson and Malamy (22).
[b] Podglajen et al. (13).

primer sets (GBI-1 and GBI-2, GBI-3 and GBI-4, and GBI-1 and GBI-4) was carried out. A positive PCR test was detected in seven imipenem-resistant laboratory stock strains of *B. fragilis* which produced detectable levels of metallo-β-lactamase by spectrophotometric assay or bioassay; in one imipenem-susceptible strain, which produced no detectable metallo-β-lactamase; and in one imipenem-intermediate strains, which generated no detectable metallo-β-lactamase. Twelve other imipenem-susceptible strains, which had no detectable metallo-β-lactamase, had a negative PCR test. Representative PCR results are shown in Fig. 1A to C. The results of the Southern hybridization agreed with those of the PCR assay (data not shown). All seven imipenem-resistant strains were PCR positive for IS-like elements; a PCR product of approximately 2 kbp in size was generated (Fig. 1D, lanes 2 and 5). One imipenem-susceptible strain, which gave a positive PCR test for *cfiA*, was PCR negative for IS-like elements with an amplicon of approximately 400 bp (Fig. 1D, lane 6), a DNA size which indicates that there is no IS-like element immediately upstream of *cfiA*.

Prevalence of *cfiA*, susceptibility to imipenem, metallo-β-lactamase production, and carriage of IS-like elements were studied in two cohorts of *B. fragilis* strains (Table 2). Based on the results from the stock strains mentioned above, a primer set of GBI-1 and GBI-4 was used to detect *cfiA*. All *cfiA*-positive strains were subjected to a test for metallo-β-lactamase production by both spectrophotometric assay and bioassay.

Imipenem resistance was found in 2 (1.2%) of 162 strains recovered between 1987 and 1988 and 1 (0.8%) of 124 strains isolated between 1992 and 1994. Two resistant isolates collected between 1987 and 1988 had the *cfiA* gene and IS-like elements and produced metallo-β-lactamase, whereas one resistant strain (MIC of imipenem, 32 μg/ml) isolated between 1992 and 1994 was *cfiA*- and IS-negative and showed no detectable metallo-β-lactamase activity. The *cfiA* gene was detected in 1.9% of the 159 imipenem-susceptible strains isolated between 1987 and 1988 and in 4.1% of the 122 imipenem-susceptible strains recovered between 1992 and 1994. Regardless of the susceptibility to imipenem, the *cfiA* gene was found in 6 (3.7%) of the 162 strains isolated between 1987 and 1988 and 5 (4.0%) of the 124 strains isolated between 1992 and 1994.

Although tiny colonies were found after eight imipenem-susceptible strains were cultured on imipenem-supplemented agar plates, recovered colonies (irrespective of *cfiA* carriage) developed no resistance to imipenem by susceptibility testing and produced no detectable metallo-β-lactamase.

In this study of two cohorts of *B. fragilis* strains, the prevalence of the *cfiA* gene was 3.7 and 4.0%, respectively. Of imipenem-susceptible *B. fragilis* strains, 1.9% of the first cohort

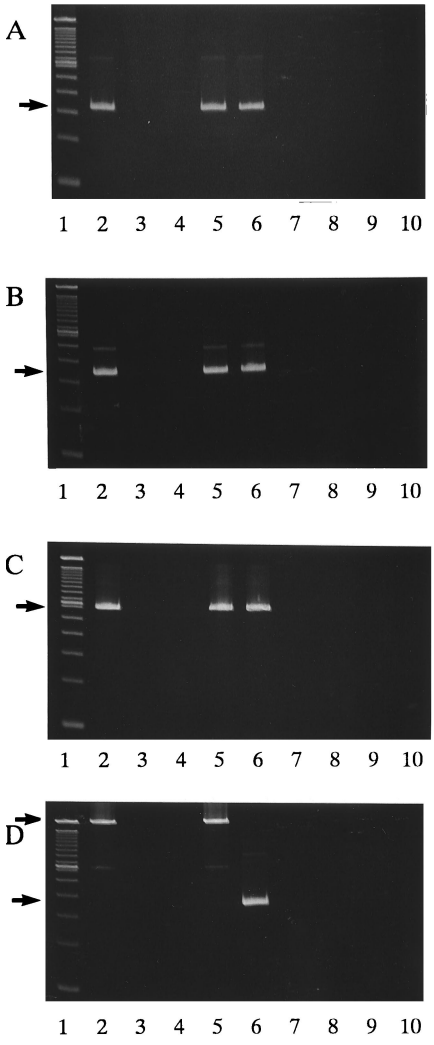

FIG. 1. PCR amplification for detection of the *cfiA* gene with primers GBI-1 and GBI-2 (A), GBI-3 and GBI-4 (B), and GBI-1 and GBI-4 (C) and for detection of IS-like element with primers G and E (D). Lane 1, 100-bp DNA ladder (Gibco BRL); lanes 2 and 5, metallo-β-lactamase-producing, imipenem-resistant *B. fragilis* strains; lanes 3, 4, and 6 to 9, detectable metallo-β-lactamase-negative, imipenem-susceptible strains; lane 10, negative control without DNA sample. Arrows indicate 340-bp (A), 358-bp (B), 625-bp (C), ca. 2-kbp (D), and ca. 400-bp (D) amplicons. Lanes 2, 5, and 6 were PCR positive for the *cfiA* gene. Lanes 2 and 5 were positive for IS-like elements immediately upstream of the *cfiA* gene.

TABLE 2. Distribution of the *cfiA* gene and IS-like element and metallo-β-lactamase production among clinical isolates of *B. fragilis*[a]

No. of strains tested (yr isolated)	Susceptibility to imipenem	*cfiA* gene	IS-like element	Metallo-β-lactamase production	No. of productive strains
162 (1987–1988)	R	+	+	+	2
	I	+	−	−	1
	S	+	−	−[b]	3
	S	−	−[c]	ND	156
124 (1992–1994)	R	−	−	−	1
	I	−	−	ND	1
	S	+	−	−[b]	5
	S	−	−[c]	ND	117

[a] R, resistant with MICs of ≥16 μg/ml; I, intermediate with a MIC of 8 μg/ml; S, susceptible with MICs of ≤4 μg/ml; +, positive; −, negative; ND, not done.
[b] One strain was tested.
[c] Ten strains were tested.

and 4.1% of the second cohort carried the *cfiA* gene. These results are relatively similar to those obtained in previous studies from France showing that approximately 3% of 500 randomly selected strains of *B. fragilis* were *cfiA* positive (14) and that a silent *cfiA* gene was found in 1.6% of the isolates (12, 13). The similarities derived from geographically distinct surveys suggest that the prevalence of *cfiA*-positive strains among *B. fragilis* may be relatively constant in each country.

Our study suggests that metallo-β-lactamase production is clearly related to the presence of the *cfiA* gene and IS-like elements immediately upstream of the metallo-β-lactamase gene. Gene activation by IS elements in *B. fragilis* is being identified: for example, IS*21* (21) activation of the *cepA* gene (20); IS*4351*, IS*942*, and IS*1186* (13, 16) activation of the *ccrA* or *cfiA* gene; IS*4351* activation of the *ermF* gene (18, 19); and IS*1170* and IS*1169* activation of *nimC* and *nimD* (23).

In this study, one strain of *B. fragilis* (MIC of imipenem, 32 μg/ml) lacked production of metallo-β-lactamase. By contrast, *Bacteroides distasonis* (6) and *Enterobacter cloacae* (11) have been shown to have other imipenem resistance mechanisms, including reduced outer membrane permeability and the production of other types of β-lactamase, such as serine β-lactamase. Further studies are needed to determine the other resistance mechanism(s) of *B. fragilis* strains against imipenem.

The intraspecific transfer of imipenem resistance in a *B. fragilis* strain associated with the production of an imipenem-hydrolyzing metallo-β-lactamase has been previously reported (2). However, this earlier study has been the sole report of plasmid-mediated transmission of metallo-β-lactamase. Activation of the silent *cfiA* gene by one-step mutation was not confirmed in this study. Taken together, our data suggest that neither transfer of imipenem resistance by a plasmid nor spontaneous mutation leading to resistance seems to be a common way for *B. fragilis* to acquire resistance to imipenem.

Our study did not prove the conversion of *cfiA* gene-harboring imipenem-susceptible strains to imipenem resistance by a single mutation. This failure may be due to the lack of the IS element necessary for imipenem resistance within the strains tested.

The PCR assay described here, in combining detection of the *cfiA* gene and of IS-like elements immediately upstream of the *cfiA* gene, may be a useful tool to monitor the prevalence of metallo-β-lactamase-mediated imipenem-resistant *B. fragilis* strains.

This study was supported in part by a grant for the "Study of Drug-Resistant Bacteria," founded by the Ministry of Health and Welfare, Japan, in 1996.

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