

CONJUGAL TRANSFER OF IMIPENEM RESISTANCE IN *Bacteroides fragilis*

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Transfer of imipenem resistance in *Bacteroides fragilis* was studied. Clinical isolate *B. fragilis* 10-73 was highly resistant to imipenem. Imipenem resistance was transferred from 10-73 to *B. fragilis* strain TM4000 at a frequency of 10^{-6} /input recipient by a filter mating technique. The resistance could also be retransferred. *B. fragilis* 10-73 and both primary and secondary transciipients produced an imipenem-hydrolyzing metallo- β -lactamase. Acquisition of imipenem resistance correlated with the appearance of plasmid DNA with a size (ca. 13.6 kb) similar to that of the donor strain. TM4000 transformed by electroporation with purified DNA of the 13.6-kb plasmid pBFUK1 produced the metallo- β -lactamase and was resistant to imipenem. Transfer was resistant to DNase treatment and no transfer was seen with a sterile filtrate of the donor culture. It is suggested that gene transfer in *B. fragilis* has the properties of a conjugation system rather than those of transformation or transduction.

Bacteroides fragilis is an important pathogen in anaerobic infections. Clinically isolated *B. fragilis* generally produce β -lactamase and are moderately or highly resistant to penicillins and cephalosporins. Imipenem has excellent antimicrobial activity against these organisms. Resistance to imipenem is rare in *B. fragilis* but a few strains have been reported to be resistant^{1~3)}. We previously reported two *B. fragilis* clinical isolates, GAI-30079 and 30144 that are highly resistant to imipenem and to most other β -lactam antibiotics³⁾. Both strains had an imipenem-hydrolyzing β -lactamase. The enzyme activity was inhibited by EDTA. It was suggested that this β -lactamase was a metalloenzyme. Recently we found imipenem-resistant *B. fragilis* 10-73 in which resistance was transferrable by a conjugation system. This study investigates the transferrability of imipenem resistance and plasmid in matings involving *B. fragilis* 10-73.

Materials and Methods

Bacterial Strains

The strains of *B. fragilis* used in this work are described in Table 1. *B. fragilis* 10-73, isolated from materials of a wounded infection, was resistant to imipenem, tetracycline, penicillins, cephalosporins, cephamycins, and latamoxef (Table 2). Rifampicin-resistant mutant strains, *B. fragilis* TM4000 and JC101 were used as recipients in the matings.

Media Used

For the liquid culture, brain heart-infusion broth (BHIB) (Becton Dickinson & Co., Maryland, U.S.A.) supplemented with hemin (5 μ g/ml) and yeast extract (5 μ g/ml), and GAM broth (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with hemin (5 μ g/ml) and menadione (0.1 μ g/ml) were used. Anaerobic minimum media were prepared using a slightly modified version of the method of VAREL and BRYANT⁴⁾.

Susceptibility Tests

The MICs were determined by an agar dilution method using modified GAM agar (Nissui Seiyaku

Table 1. *Bacteroides fragilis* strains and relevant characteristics.

Strain	Phenotype ^{a,b}	Comment
<i>B. fragilis</i> 10-73	Carb ^r , Rif ^s , Tet ^r , His ⁻ Arg ⁻	Clinical isolate
TM4000	Carb ^s , Rif ^r	Plasmid free
JC101	Carb ^s , Rif ^r , His ⁻ Arg ⁻	Amino acid auxotroph of TM4000, plasmid free
Tc 1	Carb ^r , Rif ^r , His ⁻ Arg ⁻ , Tet ^s	Transcipient of 10-73 × JC101 matings
Tc 12, Tc 30	Carb ^r , Rif ^r , Tet ^s	Transcipient of 10-73 × TM4000 matings
Tf3	Carb ^r , Rif ^r	TM4000 transformant containing pBFUK1 from Tc 30

^a Carb; carbapenem, Rif; rifampicin, Tet; tetracycline.^b His⁻Arg⁻; auxotrophs requiring histidine and arginine for growth.Table 2. Susceptibility of *Bacteroides fragilis* 10-73 and reference strains to various β -lactams.

Antibiotic	MIC (μ g/ml)			Antibiotic	MIC (μ g/ml)		
	10-73	GAI-30144	ATCC 25285		10-73	GAI-30144	ATCC 25285
Ampicillin	100	200	12.5	Cefotetan	100	100	3.13
Piperacillin	50	50	3.13	Cefbuperazone	200	200	1.56
Cefazolin	200	>200	25	Clavulanic acid/ampicillin	200	50	0.78
Cefoperazone	>200	>200	12.5	Sulbactam/cefoperazone	>200	400	3.13
Ceftizoxime	>200	100	3.13	Imipenem	200	100	0.2
Cefoxitin	100	25	6.25	Clindamycin	6.25	0.2	0.78
Cefmetazole	100	100	6.25	Tetracycline	100	12.5	ND
Latamoxef	200	100	0.78				

ND: Not determined.

Co., Ltd., Tokyo, Japan). A final inoculum of 10^6 cfu/ml was applied with a replicating device. Anaerobic cultures were incubated for 48 hours at 37°C in an anaerobic chamber (Model 1204; Forma Scientific, Ohio, U.S.A.).

Transfer Experiment

Matings were done by the filter mating procedure⁵. The parental strains were grown separately in BHIB until the mid-logarithmic phase. Donor and recipient strains were mixed in 1:10 ratio, respectively and approximately 5 ml of the mixture was filtered onto the surface of a 0.45 μ m Nalgene filter (Nalgene Co., New York, U.S.A.). For dummy mating, donor culture filtrate passed through a 0.45 μ m filter was used as the donor. The filter was placed on a modified GAM agar and incubated overnight at 37°C in the anaerobic chamber. The cells were harvested from the filter by suspension in BHIB. Aliquots were placed on selective media. Rifampicin, tetracycline and meropenem were used for selection.

β -Lactamase Assay

Cells from an overnight culture in GAM broth were suspended in 50 mM MOPS-KOH (pH 7.2) buffer and disrupted by sonication. Cell extracts centrifuged at 10,000 \times g for 15 minutes at 4°C were used as a crude enzyme preparation. The enzyme activity was assayed using a spectrophotometric technique⁶. The reaction mixture contained a 100 μ M β -lactam compound in a 200 mM MOPS-KOH (pH 7.2) buffer and was maintained at 30°C. Reaction was initiated by adding an aliquot of crude enzyme. Drug inactivation was monitored at the following wavelengths: benzylpenicillin 233 nm, ampicillin 235 nm, cephaloridine 260 nm, cefoxitin 270 nm, cefotaxime 264 nm, ceftizoxime 257 nm, cefoperazone 273 nm, latamoxef 275 nm, imipenem 299 nm, and aztreonam 318 nm.

DNA Preparation and Analysis

Routine screening for plasmid content was performed using the alkaline lysis technique⁷. Purified plasmid DNA was prepared using cesium chloride-ethidium bromide ultracentrifugation⁸. Restriction

endonuclease digestions were performed at 37°C for 2 hours in the buffers recommended by the manufacturer (Takara Shuzo Co., Ltd., Kyoto, Japan). DNA preparations were analyzed in 0.8% agarose gel by the method described by TALLY *et al.*⁵⁾.

Transformation Experiment

For the transformation experiment, purified plasmid DNA from *B. fragilis* transciplient Tc 30 was used. Transformation of *B. fragilis* TM4000 was performed using the electrotransformation technique (Gene pulsar™ apparatus Bio-Rad Laboratories, Richmond, CA, U.S.A.) as described by SEBALD *et al.*⁹⁾.

Results

The susceptibility of *B. fragilis* 10-73 is shown in Table 2. Compared to reference strain ATCC 25285, strain 10-73 was highly resistant to cephamycins, ceftizoxime, latamoxef, and imipenem. It was also resistant to ampicillin/clavulanic acid and cefoperazone/sulbactam. Its susceptibility pattern is quite similar to that of highly imipenem-resistant strain GAI-30144 which produces metallo- β -lactamase³⁾. Besides β -lactam antibiotics, 10-73 was also resistant to 50 μ g/ml of tetracycline.

To investigate the ability of 10-73 to transfer antibiotic resistance, we mated this strain with *B. fragilis* TM4000. As shown in Table 3, imipenem resistance was transferred at a frequency of 10⁻⁶/input recipient. However, transfer of tetracycline resistance was not observed (Table 3). The transciplients (Tc) obtained by primary mating were resistant to β -lactams at levels comparable to those of the donor strain 10-73, but were sensitive to tetracycline (data not shown). When plasmid DNA from twenty strains of transciplient was analyzed by electrophoresis in 0.8% agarose, it was found that the migration patterns of plasmid DNA fell into three classes (Fig. 1: Lanes 1 and 7; Type 1, Lanes 2~5; Type 2, Lane 6; Type 3) but all transciplients contained plasmid DNA which was homologous with the plasmid DNA of a similar size in the donor strain (Fig. 1; indicated by arrows).

Secondary mating was done between Tc 1, a transciplient with JC101 background and TM4000. This was because we didn't have a suitable selective marker in secondary mating between a transciplient with TM4000 background and JC101. Imipenem resistance was transferred at a frequency of 10⁻⁸/input recipient in a primary mating between 10-73 and JC101, and retransferred at a frequency of 10⁻⁵/input recipient (Table 3). As with the primary mating with TM4000, common plasmid DNA was also observed in 10-73, Tc 1 and transciplients of secondary mating (data not shown). Thus, acquisition of imipenem resistance was accompanied by the appearance of plasmid DNA and was separated from a tetracycline-resistance transfer element.

Table 3. Transfer of resistance between *Bacteroides fragilis* by filter mating.

Donor	Recipient	Selective medium ^a	Transciplient	
			Frequency (per/recipient)	Phenotype
10-73	TM4000	Rif, Mepm	10 ⁻⁶	Mepm ^r , Rif ^r
10-73	TM4000	Rif, Tet	< 10 ⁻¹⁰	Tet ^s
10-73	JC101	Rif, Mepm	10 ⁻⁸	Mepm ^r , Rif ^r , Tet ^s , His ⁻ Arg ⁻
Tc 1	TM4000	Rif, Mepm/AMM ^b	10 ⁻⁵	Mepm ^r , Rif ^r , Tet ^s , His ⁺ Arg ⁺

^a The selective media contained rifampicin (Rif, 25 μ g/ml) plus appropriate antibiotics at the following concentrations (μ g/ml): Meropenem (Mepm, 50); tetracycline (Tet, 5).

^b Colonies, which grew on selective media containing rifampicin and meropenem, were replica plated on anaerobic minimum media (AMM) and confirmed their ability to grow on AMM plate.

Cell extracts from 10-73 had β -lactamase activity as shown in Table 4. The enzyme hydrolyzed penicillins, oxyiminocephalosporins, cefoxitin, latamoxef, and carbapenems, but not aztreonam. Comparison of susceptibility and substrate profile suggests that resistance to a broad range of β -lactams, including carbapenems, was due to in β -lactamase activity. Cell extracts from some transciipients were also subjected to β -lactamase assay. As shown in Table 4, β -lactamases from transciipients (Tc) showed a very similar substrate hydrolysis pattern as that of 10-73, but different from that of typical cephalosporinase from TM4000. It is reasonable to think that imipenem-hydrolyzing β -lactamases of transciipients are derived from the donor strain, 10-73. Substrate profiles of the enzyme from 10-73 and transciipients were almost identical to those of metallo- β -lactamase from GAI-30144 (Table 4). In addition, as with GAI-30144 enzyme, their activity was strongly inhibited by EDTA (100 μ M), but not by clavulanic acid (500 μ M). There is a possibility that the enzymes from 10-73 and transciipients are identical to those from GAI-30144. However, in GAI-30144 and another metallo- β -lactamase-producing *B. fragilis*, GAI-30079, imipenem resistance was never transferred to *B. fragilis* JC101 (data not shown).

Plasmid DNA was purified using cesium chloride-ethidium bromide ultracentrifugation from Tc 30, one of the transciipients which contained a minimal number of DNA bands in electrophoresis (example Fig. 1: Lanes 1 and 7). Analysis of the products of restriction enzyme cleavage of plasmid DNA confirmed the presence of a single plasmid of

Fig. 1. Agarose gel analysis of DNA in parents and transciipients of *Bacteroides fragilis*.

Lanes 1~7: transciipients from 10-73 \times TM4000 matings, 8: TM4000 (recipient), 9: 10-73 (donor), 10: *Hind* III digests of lambda-DNA.

Plasmid bands which were observed in all transciipients and the donor strain, 10-73, are indicated by arrows at the left.

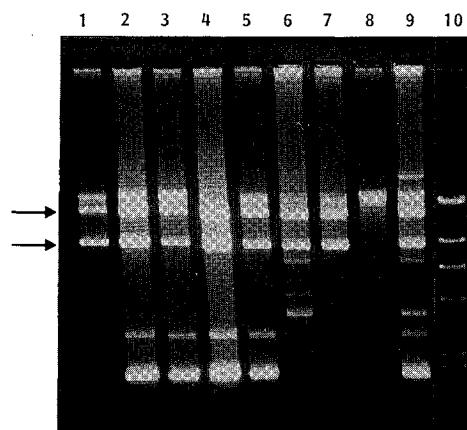


Table 4. β -Lactamase activities of cell extracts from *Bacteroides fragilis* 10-73, transciipients, transformant and a reference strain.

Substrate	Rate of hydrolysis ^a (%)				
	10-73	Tc ^b 12	Tc 30	Tf ^c 3	30144
Benzylpenicillin	252	161	285	354	498
Ampicillin	178	104	159	236	90
Cephaloridine	100	100	100	100	100
Cefoperazon	334	228	181	247	206
Cefotaxime	90	84	62	89	94
Ceftizoxime	147	162	136	147	265
Cefoxitin	8	7	6	11	11
Latamoxef	164	146	78	71	108
Imipenem	127	120	102	99	182
Meropenem	184	146	111	147	—
Aztreonam	ND ^d	ND	ND	ND	ND

^a The rate of hydrolysis was expressed using cephaloridine as 100%.

^b Tc: transciipient of 10-73 \times TM4000.

^c Tf: transformant of TM4000 with pBFUK1 from Tc 30.

^d ND: Not detectable.

approximately 13.6 kilobase pairs (kb), designated pBFUK1. Plasmid pBFUK1 was subjected to electrotransformation into TM4000. Imipenem-resistant transformants (Tf) were obtained at a frequency of 10^{-5} transformant/survivor. Eight strains selected randomly from those transformants were analyzed for their plasmid content and it was found that all strains contained a plasmid band which seemed to be the plasmid pBFUK1. Crude enzyme prepared from a transformant (Tf) had a similar substrate hydrolysis pattern as that of Tc 30 (Table 4). The enzyme was also very sensitive to EDTA, but not to clavulanic acid. These results suggest that plasmid pBFUK1, which was present in Tc 30 and consequently in 10-73, bore imipenem resistance gene coding for metallo- β -lactamase.

To investigate the nature of transfer, we performed mating on a GAM plate containing 1 mg/ml of DNase. Dummy mating, using donor (10-73), was performed using a culture sterile filtrate. Transfer of imipenem resistance was not prevented by the presence of DNase and no transfer was observed in mating between a sterile filtrate of the donor culture and TM4000. Therefore, gene transfer by *B. fragilis* 10-73 was suggested to have the properties of a conjugation system rather than those of transformation or transduction.

Discussion

Imipenem has excellent antimicrobial activity against a broad range of bacterial species. An increasing number of research groups has recently been involved with imipenem resistance. Species studied include *Xanthomonas maltophilia*¹⁰⁾, *Flavobacterium odoratum*¹¹⁾, *Legionella gormanii*¹²⁾, *Serratia marcescens*¹³⁾, mutant strains of *Aeromonas hydrophilia*¹⁴⁾, and *B. fragilis*^{1~3)}. Resistance to imipenem is associated with imipenem-hydrolyzing metallo- β -lactamase in those species. In *Pseudomonas aeruginosa*, imipenem resistance is known to be associated with a specific decrease in imipenem permeability¹⁵⁾, but recently it has also been shown to be associated with an imipenem-hydrolyzing metallo- β -lactamase¹⁶⁾. Imipenem-hydrolyzing metallo- β -lactamases thus play an important role in many imipenem-resistant organisms. In this study, we demonstrated the intraspecific transfer of imipenem-resistance within *B. fragilis* from a highly imipenem-resistant *B. fragilis* 10-73, resistance being due to the production of an imipenem-hydrolyzing metallo- β -lactamase. Electrotransformation studies indicated that the transfer of imipenem resistance was associated with that of the conjugative plasmid pBFUK1. Others, such as, WATANABE *et al.*, have recently demonstrated that the metallo- β -lactamase from *P. aeruginosa* is plasmid-mediated and that the plasmid is transferrable¹⁶⁾. The enzyme encoded by pMS350 from *P. aeruginosa* has a similar substrate and inhibitor profile as that of the *B. fragilis* 10-73 enzyme. It would be of great interest to define the relationship between the β -lactamase gene on pMS350 and pBFUK1, as well as their products.

In *B. fragilis*, imipenem-hydrolyzing β -lactamases have been reported by YOTSUJI *et al.* (G237)¹⁾, CUCHURAL *et al.* (TAL2480)²⁾, and BANDOH *et al.* (GAI-30144)³⁾. On the basis of the properties of their substrate and inhibitor profiles, these three enzymes and the 10-73 enzyme can be classified as type II oxyiminocephalosporinase (CXase) by MITSUHASHI *et al.*¹¹⁾, as they are quite similar to one another. It is tempting to speculate that these imipenem-hydrolyzing enzymes found in *B. fragilis* were derived from the same ancestor. In GAI-30144, however, the transfer of imipenem resistance was not observed as described in this paper. A gene coding for the metallo- β -lactamase from TAL2480 has been shown to be present in the chromosome¹⁷⁾, this is in contrast to the plasmid of our strain 10-73. These observations suggest a variety of genes coding for imipenem-hydrolyzing β -lactamase in *B. fragilis*.

The precise mechanism of the resistance transfer process in *B. fragilis* 10-73 remains unknown, but our data suggest that imipenem resistance transfer is independent of the tetracycline-resistance transfer element. Plasmid pBFUK1 seems to be self-transferrable. However, the size of the plasmid is rather small compared to most of the self-transferrable plasmids and there is a possibility that the plasmid is mobilized.

In Japan, imipenem resistance in *B. fragilis* is currently rare. According to research at our institute, 6 of 433 (about 1.4%) strains of *B. fragilis*, which were isolated from clinical specimens over the 1986 to 1989 period, were resistant to 8 μ g/ml of imipenem. However, the present observation that the transfer of

a plasmid-bearing metallo- β -lactamase can naturally occur is of great significance. This is because transferable plasmids play an important role in the rapid dissemination of resistance to antibiotics.

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