R-PLASMID MEDIATED TRANSFER OF β-LACTAM RESISTANCE IN *BACTEROIDES FRAGILIS*

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We described plasmid mediated transfer of resistance to β-lactam antibiotics between *Bacteroides fragilis* strains. Ampicillin-resistance was transferred from *B. fragilis* strain GAI-10150 to a *B. fragilis* strain JC-101 with a frequency of 10^{-6}/input donor by a filter mating technique. A common plasmid band, named pBFKW1, was found in both the donor and the transconjugants. The plasmid was purified by an ethidium bromide-CsCl ultracentrifugation. The molecular size of the plasmid pBFKW1 which seemed to encode the β-lactam resistance and β-lactamase production was estimated *ca.* 40 kb by the analysis of endonuclease digest. Substrate profile of the enzymes derived from the donor and a transconjugant were of cephalosporinase character.

Most strains of *Bacteroides fragilis* constitutively produce a small amount of β-lactamase, and show moderate resistance to most β-lactam antibiotics except for the cephamycins such as cefoxitin and the carbapenems such as imipenem. Some strains of *B. fragilis* isolated from clinical materials produce large amounts of β-lactamase and therefore were resistant to β-lactam antibiotics.[1] It has been hypothesized that resistance of *B. fragilis* to β-lactam antibiotics is encoded by an extrachromosomal gene. Recently, several investigators have reported that resistance to β-lactam and production of β-lactamase in *B. fragilis* is transferable, which supports this hypothesis.[2~4] None of these reports provide biochemical evidence for a plasmid that encodes resistance to β-lactam antibiotics. In this paper, we describe the transfer of resistance to β-lactam antibitotics such as ampicillin and the production of β-lactamase, from donor to recipient cells, *via* an extrachromosomal gene.

Materials and Methods

Species Used

The β-lactam-resistant *B. fragilis* GAI-10150 strain was isolated from clinical materials. *B. fragilis* JC-101 was used as the recipient in the primary mating experiment and *B. fragilis* TM-4000 as the recipient in the secondary mating experiment (Table 1). These two strains were gifts from TALLY *et al.* All strains were preserved in 20% skim milk at −80°C.

Drug Used

The following drugs were used: Benzylpenicillin, cloxacillin, ampicillin, carbenicillin, piperacillin, cephaloridine, cefazolin, cefotaxime, cefmenoxime, ceftizoxime, latamoxef, cefoxitin, cefmetazole, imipenem, ampicillin plus clavulanic acid (2:1), tetracycline, and rifampicin.

Table 1. Bacterial strains and relevant characters.

Strain	Phenotype[a]	Comment
Bacteroides fragilis GAI-10150	Apc^R, Tc^R, Rif^S	Clinical isolate
B. fragilis JC-101	Apc^S, Tc^S, Rif^R, His^-, Arg^-[b]	Amino acid auxotroph of TM-4000 From F. P. TALLY
B. fragilis TM-4000	Apc^R, Tc^S, Rif^R	From F. P. TALLY
B. fragilis A-1～A-5	Apc^R, Tc^S, Rif^R, His^-, Arg^-	Transconjugants of GAI-10150 × JC-101 matings
B. fragilis A-2-1～3	Apc^R, Tc^S, Rif^R	Transconjugants of A-2 × TM-4000 matings

[a] Apc^R, Resistant to 200 μg/ml of ampicillin; Tc^R, resistant to 12.5 μg/ml of tetracyclin; Rif^R, resistant to 50 μg/ml of rifampicin; His^-, Arg^-, auxotrophs requiring histidine and arginine for growth.

[b] β-Lactamase activities of the strain was not detectable with 100 μM amplicillin as the substrate.

Drug Susceptibility Test

Testing was done in accordance with the standards established by the Japan Society of Chemotherapy. The MIC was determined by the agar-plate dilution method[5] using GAM agar (Nissui Seiyaku Co., Ltd., Tokyo). Plates were inoculated with 10^6 cfu/ml and incubated in an anaerobic chamber (CO_2 10%, H_2 10%, N_2 80%).

Table 2. Susceptibility of *Bacteroides fragilis* GAI-10150 against various β-lactam agents.

Antibiotics	MIC (μg/ml)
Benzylpenicillin	1,600
Cloxacillin	3,200
Ampicillin	1,600
Carbenicillin	1,600
Piperacillin	400
Cefazolin	400
Cefmenoxime	200
Latamoxef	400
Cefoxitin	25
Imipenem	1.56
Ampicillin/clavulanic acid	6.25

Resistance Transferability Test

Mating was done by the method of TALLY *et al.* using a membrane filter.[6]

Measurement of β-Lactamase Activity

Bacterial cells in the late logarithmic growth phase were collected, washed in a 0.1-M phosphate buffer at pH 7.0, and suspended in the same buffer. The bacteria were disrupted by sonication, and centrifuged at 10,000 × *g* for 20 minutes. The supernatant was used as a crude enzyme solution.

Spectrophotometry was used to assay β-lactamase activity with a substrate of 100 μM.[7] The decomposition of the drugs used in this study was monitored at the following wavelengths: 255 nm for cephaloridine, 267 nm for cefotaxime, 245 nm for ceftizoxime, 270 nm for latamoxef, 275 nm for cefmetazole, 236 nm for benzylpenicillin, and 235 nm for ampicillin.

Analysis of Plasmid

Cells were screened for plasmid DNA using the alkaline lysis technique.[8] DNA was isolated and refined by ethidium bromide-CsCl ultracenrifugation[9] and analyzed in 0.8% agarose gel by the method described by TALLY *et al.*[6]

Results

Table 2 shows the MICs of *B. fragilis* GAI-10150 against β-lactam antibiotics used in this study. The strains was resistant to 1,600 μg/ml of ampicillin and carbenicillin, to 3,200 μg/ml of cloxacillin, to 400 μg/ml of piperacillin, cefazolin, and latamoxef, and to 200 μg/ml of cefmenoxime. It was susceptible to 25 μg/ml of cefoxitin and to 1.56 μg/ml of imipenem. The MIC of mixture ampicillin and clavulanic acid (2 : 1) was 6.25 μg/ml, which supports a synergistic effects. This strain was resistant to tetracycline, while it was susceptible to clindamycin.

Transferable ampicillin-resistance was found at a frequency of 1.2×10^{-6}/input donor by filter mating with *B. fragilis* GAI-10150 as the donor, and JC-101 as the recipient. Both ampicillin and rifampicin were

Table 3. Transfer of ampicillin-resistance from *Bacteroides fragilis* donor cells to *B. fragilis* recipient cells by the filter mating.

Donor	Recipient	Selective medium	Transconjugant	
			Frequency	Phenotype
GAI-10150	JC-101	Rif, Apc	1.2×10^{-6}	Rif, Apc, His^-, Arg^-
		Rif, Tc	$<10^{-10}$	
A-2	TM-4000	Apc, His^+, Arg^+	4.0×10^{-7}	Rif, Apc

The selective media contained rifampicin (Rif, 50 μg/ml) plus appropriate antibiotics at the following concentration (μg/ml): Ampicillin (Apc, 200); tetracyclin (Tc, 10).

Table 4. Resistance levels of *Bacteroides fragilis* GAI-10150, JC-101, TM-4000 and transconjugants (A-2 and A-2-1).

Antibiotics	Strains				
	GAI-10150	JC-101	TM-4000	A-2	A-2-1
Ampicillin	1,600	12.5	6.25	800	400
Cloxacillin	3,200	25	12.5	3,200	800
Piperacillin	400	3.13	1.56	200	100
Cefazolin	400	12.5	6.25	200	200
Cefoxitin	25	3.13	1.56	12.5	12.5
Latamoxef	400	<0.39	<0.39	200	100

used as selective agents. In this mating, the transfer of tetracycline-resistance was not observed. Isolate A-2, one of the transconjugants obtained by the above primary transfer, was used as a donor and *B. fragilis* TM-4000 as the recipient in the secondary transfer experiment. Ampicillin-resistance was re-transferred at a frequency of 4.0×10^{-7}/input donor (Table 3).

The transconjugants obtained by the primary mating had a higher level of β-lactamase activity than the recipient. Table 4 shows β-lactam antibiotic susceptibility profile of the transconjugants and the parents. The MIC of strains A-2 and A-2-1, against ampicillin, cloxacillin, piperacillin, cefazolin, cefoxitin, and latamoxef was higher than that of the recipients *B. fragilis* JC-101 and TM-4000. The MIC of the recipients tended to be slightly lower than that of the donor *B. fragilis* GAI-10150.

Table 5. Substrate profile of β-lactamases from two strains *Bacteroides fragilis* GAI-10150 and A-2.

Substrate	Rate of hydrolysis	
	GAI-10150	A-2
Cephaloridine	100	100
Cefotaxime	33.1	40.9
Ceftizoxime	16.9	19.4
Latamoxef	0.4	0.6
Cefoxitin	0.3	0.4
Cefmetazole	0.1	0.3
Benzylpenicillin	6.8	11.6
Ampicillin	25.3	31.9

Rates of hydrolysis are relative to an arbitrary value of 100 for cephaloridine.
Crude preparations were used.

Table 5 shows the substrate specificity of crude enzymes from the donor GAI-10150 and the transconjugant A-2 obtained by the primary transfer between GAI-10150 and JC-101. The results suggest that β-lactamase produced by *B. fragilis* GAI-10150 and *B. fragilis* A-2 are of the cephalosporinase type.

Plasmid DNA was prepared by alkaline lysis technique from the following seven strains, *B. fragilis*; (1) GAI-10150, (2) JC-101, (3) A-2, a transconjugant obtained by the primary transfer, (4) TM-4000, a recipient used for the secondary transfer, (5) A-2-1, (6) A-2-2, and (7) A-2-3, transconjugants obtained by the secondary transfer. The DNA preparations were analyzed using electrophoresis in 0.8% agarose (Fig.

Fig. 1. Plasmid content of *Bacteroides fragilis* GAI-10150, JC-101, TM-4000 and transconjugants A-2, A-2-1～3.

Lanes 1: GAI-10150, 2: JC-101 (recipient), 3: A-2 (transconjugant from GAI-10150×JC-101 matings), 4: TM-4000 (recipient), 5～7: A-2-1～3 (transconjugant from A-2×TM-4000 matings), 8: *Hin*d III digests of λDNA.

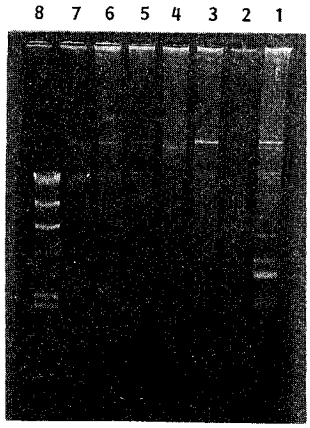

Fig. 2. Agarose gel electrophoresis of pBFKW1 cleaved with restriction endonucleases.

Lanes 1: *Eco*R I digest of pBFKW1, 2: *Hin*d III digest of pBFKW1, 3: *Hin*d III digests of λDNA.

1). A common plasmid band was observed in GAI-10150 and four transconjugants (lanes 3, 5, 6, and 7). A plasmid was isolated by ethidium bromide-CsCl ultracentrifugation from the transconjugant A-2, which correspond to one of several plasmids in *B. fragilis* GAI-10150.

The estimated size of this plasmid was around 39 kilobase pairs based on the sum of the four *Eco*R I restriction fragments or the ten *Hin*d III restriction fragments (Fig. 2).

Discussion

The production of β-lactamase in bacteria is governed by chromosomes or genes on the R plasmid. *B. fragilis* strains are known to be moderately or highly resistant to penicillins and cephalosporins. But until now, the location of the β-lactam resistance determinant in *B. fragilis* is unknown. The transfer of the production of β-lactamase between *B. fragilis* strains was unsuccessfully investigated by ANDERSON and SYKES, DEL BENE and FARRAR, and OLSSON-LILJEQUIST *et al.*[10～12] Recently, BUTLER *et al.*[2] were able to show transfer of ampicillin-resistance within *B. fragilis* from TMP 14, a highly ampicillin-resistant strain. The transfer of this determinant required the introduction of a tetracycline transfer element[13] or transfer factor, plasmid pBFTM10[14]. The recipient acquired the ampicillin-resistance and new β-lactamase activity. Although the donor strain contained two plasmids, the ampicillin-resistant transconjugant was plasmidless.

SATO *et al.*[3] also showed cotransfer of high level penicillin- and tetracycline-resistance between strains of *Bacteroides* but did not characterize the genetic element. RASCHTCHIAN *et al.*[4] reported that cefoxitin-resistance in *B. fragilis* is transferred, probably in the process of conjugation. But plasmid is not involved in this transfer, and the transcipient obtained could no longer retransfer the resistance. CUCHURAL *et al.*[15] also reported that cefoxitin-resistance is transferable. No plasmid was detected in this cefoxitin-resistant donor.

Our data document the intraspecies transfer of ampicillin-resistance within *B. fragilis* from *B. fragilis* GAI-10150, a highly ampicillin-resistant strain. In this strain, the transfer of ampicillin-resistance was clearly separate from a tetracycline-resistance transfer element and was shown to be associated with the transfer of one large conjugative plasmid. The ampicillin-resistant transconjugant acquiring the plasmid obtained the resistance to other β-lactam drugs as well and the ability to produce higher level of β-lactamase

than the recipient.

The plasmid which was related to β-lactamase-resistance transfer was refined by ethidium bromide-CsCl ultracentrifugation, and called pBFKW1. Based on the restriction endonuclease analysis, the estimated size of this plasmid was shown to be around 39 kilobase pairs. This plasmid had four *Eco*R I recognition sites, four *XBa* I recognition sites and one *Bgl* II recognition site. Further study is now going on to make the restriction map of this plasmid and to localize the ampicillin-resistance determinants on this plasmid.

B. fragilis GAI-10150 is more resistant to penicillins, such as ampicillin, cloxacillin and carbenicillin than to cephem drugs. This profile suggested that the enzyme responsible for β-lactam-resistance may be of penicillinase type. The substrate specificity however of crude enzyme collected from the donor and the transconjugant showed that this enzyme was cephalosporinase. The purification and characterization of the β-lactamase produced by *B. fragilis* GAI-10150 must be considered.

The ampicillin high resistant strains like *B. fragilis* GAI-10150 can be found frequently among the recent clinical isolates. The potential danger of a spread of this type of transferable plasmid carrying ampicillin-resistance determinants also remain to be evaluated.

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