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Characterization of Three Different β-Lactamases from the *Bacteroides fragilis* Group

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β-Lactamases from five strains of *Bacteroides fragilis* and two strains of *Bacteroides uniformis*, all resistant to β-lactam antibiotics, were compared by means of isoelectric focusing and enzyme kinetic measurements. β-Lactamases from the five *B. fragilis* strains were identical, whereas those from the two *B. uniformis* strains were distinguished from each other and also from the *B. fragilis* enzymes. The two *B. uniformis* strains were relatively resistant to cefoxitin (minimal inhibitory concentration, 32 μg/ml), but only one of the strains, *B. uniformis* 2986, was found to rapidly inactivate cefoxitin. This apparently enzymatic inactivation of cefoxitin seemed to be of minor importance, and the main factor for cefoxitin resistance was considered to be a decreased permeability of the drug. Transfer of resistance to β-lactam antibiotics from these β-lactamase-producing strains of *B. fragilis* and *B. uniformis* to a non-β-lactamase-producing strain of *Bacteroides distasonis* was attempted, but with these isolates no transfer of resistance to β-lactam antibiotics was demonstrated.

The *Bacteroides fragilis* group of organisms comprises the following species: *B. fragilis*, *B. thetaiotaomicron*, *B. distasonis*, *B. ovatus*, *B. vulgatus*, and *B. uniformis* (9). These were formerly regarded as subspecies of *B. fragilis*, except *B. uniformis*, which is a new species now included in this group (9).

Bacteria belonging to this group are the anaerobes most frequently isolated from clinical specimens (7), and among them *B. fragilis* is the single most common pathogen (12). They are known to be resistant to many antimicrobial agents, including some β-lactam antibiotics, tetracyclines, and aminoglycosides, whereas clindamycin, chloramphenicol, and metronidazole in most cases are useful in treating infections caused by organisms from the *B. fragilis* group. Cefoxitin, belonging to a new kind of β-lactam antibiotics called cephamycins, exhibits properties which make it a useful alternative in treatment of these infections, since it is resistant to the hydrolytic action of β-lactamases from the *B. fragilis* group (4).

However, cefoxitin-resistant strains of the *B. fragilis* group have already been found (K. Dornbusch, B. Olsson-Liljequist, and C. E. Nord, J. Antimicrob. Chemother., in press). Their resistance was considered to be of intrinsic character rather than caused by an enzymatic degradation of cefoxitin, since only 1 of 11 strains tested could inactivate cefoxitin and 2 of the cefoxitin-resistant strains were β-lactamase negative. This paper describes some characteristics of the β-lactamase produced by the above-mentioned cefoxitin-inactivating strain, *B. uniformis* 2986, and compares its properties with those of previously described β-lactamases from the *B. fragilis* group.

Strains of the *B. fragilis* group have been shown to contain plasmids of various sizes (8, 19, 22). However, the presence of plasmids could in no case be correlated to resistance to β-lactam antibiotics. Anderson and Sykes (1) attempted transfer of resistance to β-lactam antibiotics between *B. fragilis* and *Escherichia coli* and also between two strains of *B. fragilis*, but with negative results. In contrast to this, Mancini and Behme (11) demonstrated one case of transfer of multiple antibiotic resistance from *B. fragilis* to *E. coli*. Recently, plasmid-mediated transfer of resistance to clindamycin and erythromycin between strains of the *B. fragilis* group has been demonstrated by several groups, all using the filter mating technique in their transfer experiments (17, 21, 23). The mechanism of transfer of tetracycline resistance has also been clarified (18). In view of these results, the filter mating technique was applied to transfer experiments between β-lactamase-producing donor strains and a rifampin-resistant recipient strain, all belonging to the *B. fragilis* group.

MATERIALS AND METHODS

Bacterial strains. Strains of *B. fragilis*, *B. uniformis*, and *B. distasonis* used in this study are listed in Table 1. *B. fragilis* strains B8, B34, B105, B122, B147, and B70 and *B. uniformis* strains B72 and 2986 produce β-lactamase. Strain B98 is a cefoxitin- and rif-

TABLE 1. *Characterization of strains of B. fragilis, B. uniformis, and B. distasonis*

Strain no.	Species	β-Lactamase pI	Minimal inhibitory concn (μg/ml)			
			Pc[a]	Tc	Rif	Cfx
B34	*B. fragilis*	4.9	>128	<1	<1	4
B105	*B. fragilis*	4.9	>128	>16	<1[b]	4
B122	*B. fragilis*	4.9	>128	>16	<1[b]	2
B147	*B. fragilis*	4.9	>128	>16	<1[b]	8
B70	*B. fragilis*	4.9	128	16	<1	4
B72	*B. uniformis*	5.6	64	16	<1	32
2986	*B. uniformis*	5.3	>128	<1	<1[b]	32
B98	*B. distasonis*	—[c]	16	<1	>100	32

[a] Pc, Benzylpenicillin; Tc, tetracycline; Rif, rifampin; Cfx, cefoxitin.
[b] Mutation to rifampin resistance occurred at a frequency of one in 10^9 colony-forming units.
[c] Strain produces no β-lactamase.

ampin-resistant isolate of *B. distasonis*. All strains are Swedish clinical isolates except *B. uniformis* 2986, which is a cefoxitin-resistant strain kindly supplied by Vera Sutter (Wadsworth Veterans Administration Hospital Center, Los Angeles, Calif.). *B. fragilis* strains TMP10 and TM2000 (21) were kindly supplied by Francis P. Tally (Tufts New England Medical Center, Boston, Mass.).

The anaerobic culture system (Bellco Glass, Inc., Vineland, N.J.) developed by the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va., was used for subculturing the strains. Identification of the strains was made by the method of Holdeman et al. (9).

Media. The strains were maintained in rubber-stoppered tubes containing prereduced chopped-meat broth with glucose. For liquid culture, prereduced proteose peptone broth with 1% (wt/vol) glucose was used (14). Antibiotic susceptibility tests and selection of transcipients were performed on PDM-ASM agar (AB Biodisk, Solna, Sweden) with 5% (vol/vol) defibrinated horse blood (5).

Susceptibility tests. Antibiotic susceptibility tests were performed by using the agar dilution technique as described previously (12).

Purification of β-lactamase. Crude β-lactamase preparations were prepared by ultrasonication as described previously (13). These preparations were purified by affinity chromatography in a single step, using the following method: AH-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) with an amino-terminal six-atom spacer arm (1,6-diaminohexane) was used to couple cloxacillin with a reactive carboxyl group. Coupling was performed through a carbodiimide condensation as recommended by the manufacturer. One milliliter of swollen gel contained 6 to 10 μmol of spacer groups, and the concentrations of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and cloxacillin used were 200 and 100 μmol/ml of gel, respectively. A K9/15 column (Pharmacia Fine Chemicals) was filled with 3.5 ml of coupled gel, which gave, upon settling, a column of bed dimensions 0.9 by 5.5 cm. The gel was equilibrated with 24 mM phosphate buffer with an ionic strength of 0.05 and a pH of 7.0, and the primary elution of the sample was performed with the same buffer. To this buffer was added 1.0 M NaCl for the secondary elution. Protein determinations were performed with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.), using bovine serum albumin as the standard.

Isoelectric focusing. Analytical isoelectric focusing of crude and purified β-lactamase preparations from strains B34, B70, B72 and 2986 was performed as described previously (15). An LKB Ampholine PAG plate, pH 3.5 to 9.5 or pH 4 to 6.5, was used (LKB, Bromma, Sweden). Zymogram staining for β-lactamase activity was performed by using an overlayer technique, in which a 2% agarose solution containing chromogenic cephalosporin analog 87/312 at a concentration of 1 mM was poured onto the polyacrylamide gel to give a layer of 1-mm thickness. Protein staining of the gel was performed according to Söderholm et al. (20).

Assay of β-lactamase. The method used routinely for assay of β-lactamase was the spectrophotometric method, with chromogenic cephalosporin analog 87/312 (Glaxo Research Ltd., Greenford, Middlesex, England) as the substrate (14). Change in optical density was measured at 386 nm. One unit of enzyme will hydrolyze 1 μmol of substrate per min at 37°C and pH 7.0.

For estimation of kinetic parameters, the spectrophotometric method based on absorption maxima in the ultraviolet region from 250 to 270 nm and the acidmetric method with phenol red as indicator were used as described previously (14). The kinetic parameters K_m and V_{max} were estimated from a least-squares fit to Lineweaver-Burk plots, with substrate concentrations of 0.05 to 0.2 mM.

Inactivation of cefoxitin. Crude β-lactamase preparations from strains B34, B70, B72, and 2986 were incubated with cefoxitin at a final concentration of 50 μg/ml. Samples were removed for antibiotic assays after 30 min, 3 h, 6 h, and 24 h as described by Dornbusch et al. (in press). Inhibition experiments in which strain 2986 was grown in the presence of 8 μg of cefoxitin per ml (one-fourth of the minimal inhibitory concentration) and with or without the β-lactamase inhibitor clavulanic acid (1 μg/ml) were performed as described previously (13). In control experiments cephaloridine (64 μg/ml; one-fourth of the minimal inhibitory concentration) was substituted for cefoxitin.

Transfer experiments. *B. fragilis* strains B34, B105, B122, B147, and B70 and *B. uniformis* strains

B72 and 2986 were used as donors. *B. distasonis* B98 was used as the recipient strain in all transfer experiments. Donor and recipient strains were grown separately in cooked meat-glucose medium for 6 h to a bacterial density of approximately 10^9 colony-forming units/ml. One milliliter of each was mixed and filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) The filter was placed on a PDM-ASM agar plate and incubated anaerobically overnight at 37°C. Bacteria were harvested from the filter by suspension in 1 ml of phosphate-buffered saline and immediately plated on medium containing either benzylpenicillin (50 μg/ml) and rifampin (25 μg/ml) or tetracycline (2.5 μg/ml) and rifampin (25 μg/ml). Identity of transcipients could be confirmed by their resistance to cefoxitin and by their ability to ferment arabinose and rhamnose. Donor and recipient strains were treated identically as controls.

Chemicals. All chemicals were of analytical grade unless otherwise stated. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was obtained from Fluka AG (Buchs SG, Basle, Switzerland). Benzylpenicillin was obtained from KABI (Stockholm, Sweden), and cloxacillin was from Astra (Södertälje, Sweden). Cephaloridine, cefuroxime, and chromogenic cephalosporin (87/312) were from Glaxo Research Ltd. Cephalothin, cephalexin, cefamandole, and cefazolin were obtained from Lilly Research Centre Ltd. (Windlesham, Surrey, England). Cefoxitin was supplied by Merck, Sharp & Dohme (Rahway, N.J.). Tetracycline was obtained from Novo Industri A/S (Bagsvaerd, Denmark), and rifampin was from Ciba-Geigy AG (Basel, Switzerland). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, Mo.).

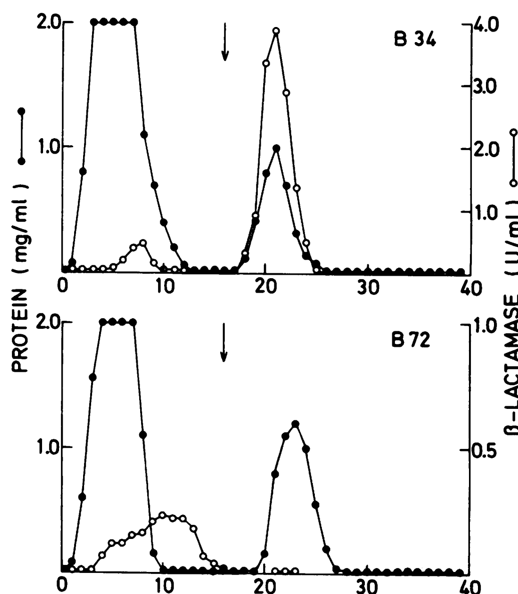

FIG. 1. *Affinity chromatography elution profiles of β-lactamase preparations from B. fragilis B34 and B. uniformis B72. Fractions of 1.0 ml were collected at a flow rate of 0.5 ml/min and tested for protein (●) and β-lactamase activity (○). Primary elution was carried out using 24 mM phosphate buffer, pH 7.0, and secondary elution was done using the same buffer with the addition of 1.0 M NaCl. Arrows indicate the buffer change.*

RESULTS

Purification of β-lactamase. Chromatography of crude enzyme preparations from *B. fragilis* strains B34 and B70 on the AH-Sepharose 4B-cloxacillin column resulted in purified preparations, with approximately 80% recovery of enzyme activity (Fig. 1). The β-lactamase focused as one distinct band on zymogram staining of the focused gel, whereas protein staining of the gel revealed four or five additional protein bands. Chromatography of crude preparations from *B. uniformis* strains B72 and 2986 on the same column gave a different result, as exemplified by strain B72 in Fig. 1. β-Lactamase activity partly coincided with the first protein peak, but fractions with β-lactamase activity were also eluted immediately after this protein peak. These fractions were pooled and contained 50% of total enzyme activity.

Isoelectric focusing. Results of isoelectric focusing of crude β-lactamase preparations are listed in Table 1. A β-lactamase with an isoelectric point (pI) of 4.9 was found in all five *B. fragilis* strains. *B. uniformis* strains B72 and 2986 produced β-lactamases with pI's of 5.6 and 5.3, respectively. All preparations gave rise to one strong band (the pI of the enzyme) and one or several weak adjacent bands. Purified β-lactamase preparations from strains B34, B70, B72, and 2986 all focused as one distinct band at their respective pI's (Table 1).

Kinetic parameters. The kinetic parameters K_m and V_{max} were determined with crude and purified β-lactamase preparations from strains B34, B70, B72, and 2986 (Table 2). Six cephalosporins, cefoxitin, and benzylpenicillin were used as substrates. Two strains, both producing the pI 4.9 β-lactamase, were included to demonstrate the usefulness of determining K_m and V_{max} in combination with pI for identification of β-lactamases. These two *B. fragilis* strains, B34 and B70, showed almost identical patterns, with cephaloridine and cefazolin being the substrates most readily hydrolyzed and cephalexin, cefamandole, and cefuroxime being those least well hydrolyzed. The affinities of the enzymes were greatest for cefamandole. Crude enzyme preparations and purified preparations gave similar results.

The β-lactamase produced by *B. uniformis* strain B72 hydrolyzed all cephalosporins, but it hydrolyzed cefuroxime more readily than cephaloridine and its affinity was also highest for

TABLE 2. *Kinetic parameters of crude β-lactamases from B. fragilis and B. uniformis*

Substrate	Strain (pI)							
	B34 (4.9)		B70 (4.9)		B72 (5.6)		2986 (5.3)	
	V_{max}[a]	K_m (μM)	V_{max}	K_m (μM)	V_{max}	K_m (μM)	V_{max}	K_m (μM)
Cephaloridine	100	98	100	139	100	108	100	32
Cephalothin	77	67	76	117	134	222	52	43
Cephalexin	6	213	3	96	126	261	28	345
Cefamandole	20	48	17	28	156	214	76	73
Cefuroxime	45	79	36	107	38	64	165	101
Cefazolin	90	247	126	536	139	204	370	48
Cefoxitin	—[b]		—		—		—[c]	
Benzylpenicillin	3[d]	ND[e]	3	ND	6	ND	3	ND

[a] V_{max} relative to cephaloridine = 100.
[b] No hydrolysis of substrate detected with any β-lactamase assay method.
[c] Inactivation of cefoxitin shown in a microbiological assay.
[d] Determined by the acidimetric method with 0.2 mM substrate concentration and related to hydrolysis of cephaloridine measured identically.
[e] ND, Not determined.

cefuroxime. The β-lactamase from *B. uniformis* strain 2986, on the other hand, was able to hydrolyze both cefuroxime and cefazolin more readily than cephaloridine and, to a lesser extent, cephalothin, cephalexin, and cefamandole. Also, with these two strains crude and purified enzyme preparations gave comparable results. All four enzymes had very low hydrolytic activity on benzylpenicillin. No hydrolysis of cefoxitin could be detected by any of the four enzymes using either the ultraviolet method (262 nm) or the acidimetric method under standardized conditions.

Inactivation of cefoxitin. Cefoxitin was inactivated by the crude β-lactamase preparation from *B. uniformis* strain 2986 within 30 min, whereas it was unaffected by crude β-lactamase preparations from the other three strains tested. Growth of strain 2986 was not affected by the presence of 8 μg of cefoxitin per ml or by cefoxitin in combination with clavulanic acid when compared with a growth control with no antibiotic added (Fig. 2). However, inactivation of cefoxitin was delayed when clavulanic acid was present, indicating an inhibitory effect of clavulanic acid on this particular β-lactamase. The combination of cephaloridine and clavulanic acid inhibited growth but was not bactericidal to strain 2986 (Fig. 2). Hydrolysis of cephaloridine was much slower in the presence of clavulanic acid than without this β-lactamase inhibitor.

Transfer experiments. Several transfer experiments using the *B. fragilis* or *B. uniformis* strains as donors and *B. distasonis* strain B98 as recipient were performed. Primarily, transfer of β-lactam antibiotic resistance was investigated (i.e., presence of β-lactamase in a *B. distasonis* transcipient) and, secondarily, transfer of tetracycline resistance in those cases where the donor was resistant to tetracycline was studied. No transcipients were found in any of the mating experiments, whereas spontaneous rifampin-resistant mutants were found in donor strains B105, B122, B147, and 2986 in a frequency of approximately 10^{-9}. In a control experiment for checking the reliability of the filter mating technique used, the transfer of clindamycin and erythromycin resistance from *B. fragilis* TMP10 to *B. fragilis* TM2000 was successfully repeated.

DISCUSSION

A multistep procedure has been described for the purification of β-lactamase from *B. fragilis* (2). Purification of several other types of β-lactamases, originating from both gram-positive and gram-negative organisms, has been performed in a single step by means of affinity chromatography, using various gel supports in combination with a range of β-lactams as ligands (3, 6, 10). This technique was applied to the purification of β-lactamases from strains of *B. fragilis* and *B. uniformis* for comparison of enzyme characteristics. Cloxacillin, with inhibitory effects on the *B. fragilis* β-lactamase (14), was used herein as the ligand coupled to AH-Sepharose 4B. This gel absorbed the β-lactamases from the two *Bacteroides* species differently (Fig. 1), indicating a clear distinction between these β-lactamases.

Determination of the pI of a β-lactamase is essential for its identification. In this study it was shown that the β-lactamase from *B. unifor-*

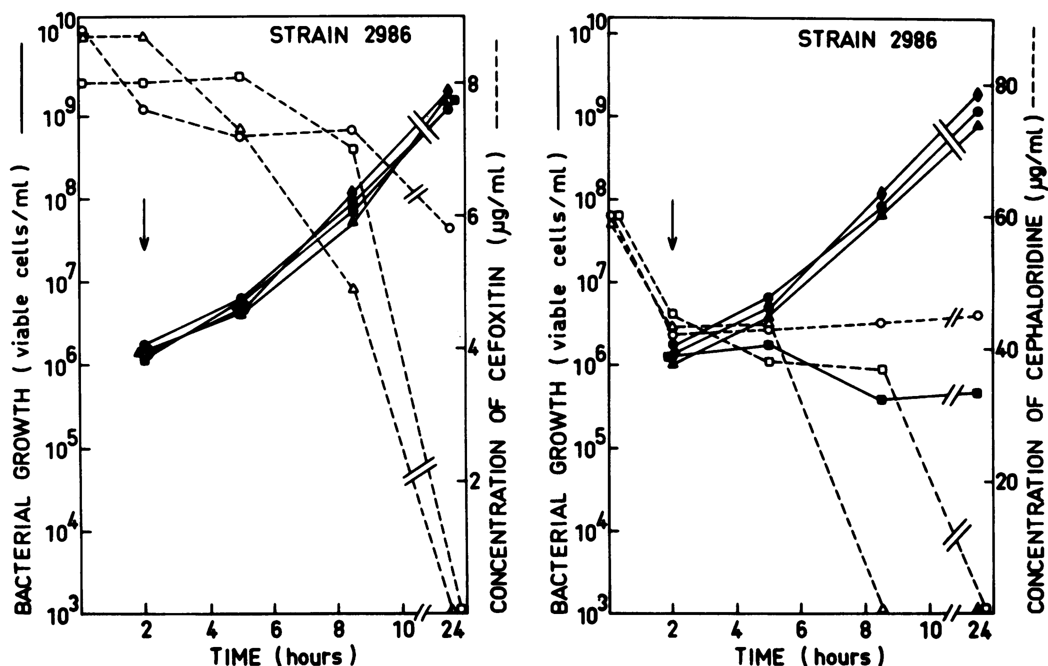

FIG. 2. *Effect of clavulanic acid in combination with cefoxitin and cephaloridine, respectively, on growth of B. uniformis strain 2986. Arrows indicate addition of clavulanic acid. Solid lines indicate bacterial growth and the symbols represent: ●, controls for bacterial growth; ▲, bacterial growth in the presence of cefoxitin and cephaloridine, respectively; ■, bacterial growth in the presence of β-lactam antibiotic and clavulanic acid; ◆, growth in the presence of clavulanic acid. Dashed lines indicate concentrations of cefoxitin and cephaloridine, respectively, and the symbols represent: ○, controls for spontaneous inactivation of β-lactam antibiotic; △, concentration of β-lactam antibiotic in bacterial culture; □, concentration of β-lactam antibiotic in bacterial culture with clavulanic acid added.*

mis strain 2986 differed from two other β-lactamases, one produced by *B. fragilis* strains and the other produced by *B. uniformis* strain B72 (Table 1). The enzyme with pI 4.9 has been found in most strains of *B. fragilis* that possess β-lactamase activity (unpublished data), and it is probably species specific. Neither of the two β-lactamases from *B. uniformis* has been found in any other strain tested so far.

By determining the enzyme kinetics of the three β-lactamases, using a range of cephalosporins and benzylpenicillin as substrates, the differences between the enzymes were further evidenced. When looking at the kinetic parameters for two substrates with relative stability to hydrolytic action by β-lactamases, cefamandole and cefuroxime, these differences were clearly demonstrated (Table 2). The pI 4.9 and pI 5.3 enzymes showed high affinity for cefamandole but rather poor hydrolytic activity. The pI 5.6 enzyme, on the other hand, showed low affinity but high hydrolytic activity. For cefuroxime the situation was different, with the pI 5.3 enzyme showing higher hydrolytic activity than the other two β-lactamases.

B. uniformis strain 2986 is unique in that it is relatively resistant to cefoxitin (minimal inhibitory concentration, 32 μg/ml) and is also able to inactivate cefoxitin as well as other cephamycins and cephalosporins (Dornbusch et al., in press). So far, no cefoxitin-resistant Swedish isolates from the *B. fragilis* group have shown the ability to inactivate cefoxitin. Also, among members of the family *Enterobacteriaceae* very few clinical isolates were able to inactivate cefoxitin (16).

Inactivation of cefoxitin could only be detected by a microbiological assay and not by two spectrophotometric methods. The effect of clavulanic acid on the growth of strain 2986 in the presence of a subinhibitory concentration of cefoxitin was investigated to find out whether the β-lactamase caused the inactivation of cefoxitin. Growth of the strain was not inhibited by clavulanic acid in combination with cefoxitin, but inactivation of cefoxitin was delayed in the presence of clavulanic acid (Fig. 2). A subinhibitory concentration of cephaloridine, on the other hand, was able to inhibit growth of the strain when combined with clavulanic acid. Thus, it is suggested that the inactivation of cefoxitin (as

well as of cephaloridine) by strain 2986 is caused by the β-lactamase. However, the main factor for resistance to cefoxitin must be of another nature, since inhibition of the β-lactamase did not render strain 2986 susceptible to cefoxitin. Thus, a permeability barrier specific for cefoxitin (and possibly other cephamycins) in *B. uniformis* strain 2986 is proposed (13; Dornbusch et al., in press).

In this report several mating experiments, with the β-lactamase-producing strains used as presumptive donors and strain B98 used as the recipient, gave negative results. Despite the more efficient filter mating technique used here, no evidence of transferable β-lactam antibiotic resistance could be obtained. The function of the plasmids present in these donor strains thus remains unsolved, and until data are presented to the contrary, it must be assumed that in strains of the *B. fragilis* group of organisms the genes coding for β-lactamase production are located on the chromosome.

In conclusion, it has been clearly shown that enzyme kinetics and pI's are characteristics of β-lactamases well suited to differentiate between β-lactamases from the *B. fragilis* group. A β-lactamase from a *B. uniformis* strain with the unique property of apparently inactivating cefoxitin was characterized and found to differ from the more frequently found *B. fragilis* β-lactamases. None of the β-lactamases were located on transferable plasmids as judged from the present results, but more investigations are needed to clarify the genetic control of antibiotic resistance in *B. fragilis*.

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