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Inactivation of Cephalosporins by *Bacteroides*

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We investigated the relationship between β-lactamases of *Bacteroides fragilis* organisms and their resistance to cephalosporins. Timed killing curves were used to study the in vitro activity of three cephalosporins, cephalothin, cefazolin, and cefamandole, and a semisynthetic cephamycin, cefoxitin. Measurements of residual antibiotic concentrations in culture supernatants were made, and they were compared with the β-lactamase activity of the microorganism. A cephalosporin-susceptible strain was rapidly killed by cephalothin, cefazolin, cefamandole, and cefoxitin. Four cephalosporin-resistant strains were not killed by cephalothin, cefazolin, or cefamandole but were killed by cefoxitin. An inoculum effect was noted with cefazolin and not with cefoxitin. The resistant strains of *Bacteroides* inactivated the three cephalosporins, but there was no inactivation of cefoxitin. A constitutive β-lactamase was detected in all the isolates of the *B. fragilis* group that were resistant to the cephalosporins. There was no distinction of the species based on isoelectric focusing of the enzyme. These data suggest that inactivation by β-lactamase may be the mechanism for resistance of *B. fragilis* to the cephalosporins and would explain the enhanced in vitro activity of cefoxitin.

The *Bacteroides fragilis* group, which includes *B. fragilis*, *B. vulgatus*, *B. ovatus*, *B. distasonis*, and *B. thetaiotaomicron*, is known to be relatively resistant to penicillins and cephalosporins (9, 17). The minimal inhibitory concentration (MIC) of penicillin against most organisms is in the range of 8 to 32 μg/ml, and approximately 10% of the strains are highly resistant, with an MIC greater than 128 μg/ml (18). This resistance has been ascribed to the production of membrane-associated β-lactamases which appear to have their greatest activity against cephalosporin antibiotics (1, 4, 13, 15, 20).

Recently, there have been efforts to develop β-lactam antibiotics which are poor substrates for this enzyme. Cefoxitin, a new cephamycin antibiotic, is such an agent, and it provides an excellent tool to study the relationship between the β-lactamases and antibiotic resistance within the *B. fragilis* group (4). The purpose of this study was to determine the interaction between various cephalosporin antibiotics and cefoxitin against selected strains of *B. fragilis* and to correlate these findings with their β-lactamase activity.

MATERIALS AND METHODS

Antibiotics. Antibiotic standard powders were obtained from the following sources: cephalothin, cefazolin, and cefamandole from Eli Lilly & Co., Indianapolis, Ind.; cefoxitin from Merck Sharpe & Dohme, West Point, Pa.; and chloramphenicol from Parke, Davis & Co., Detroit, Mich.

Organisms. Five strains of organisms within the *B. fragilis* group with a wide range of susceptibility to β-lactam antibiotics were selected for kill curve studies. Sixteen strains representing all the species of the *B. fragilis* group were used to characterize the β-lactamases. These organisms were obtained from the culture collection of the Tufts Anaerobic Laboratory (TAL), and the strains were identified by the criteria in the Virginia Polytechnic Institute and State University manual (5).

Susceptibility studies. MICs and minimal bactericidal concentrations were determined by broth dilution, using microtiter techniques in an anaerobic chamber, as previously described (18). The medium (BHIS) was brain heart infusion (BHI) broth supplemented with yeast extract (0.5%), hemin (0.1%), and vitamin K (1 μg/ml), but without cystine. The inoculum was adjusted at 10^5 to 10^6 colony-forming units (CFU) per ml, using a late-log-phase culture.

Kill curves. Five colonies of the test strain were picked from an anaerobic blood agar plate which contained BHI agar with sheep blood (5%) and hemin (0.1%) and were inoculated into 5 ml of BHIS. The broth was incubated overnight in the anaerobic chamber, and this culture was diluted into 1,000 ml of reduced BHIS to give approximately 10^7 to 10^8 CFU/ml. This inoculated broth was then divided into 200-ml portions, one as a control and four to which the antibiotics were added. The final concentrations of the drugs were 128 μg/ml for the highly resistant isolates and 32 μg/ml for the intermediate and susceptible organisms. Samples were obtained at 0, 1, 2, 4, and 24 h for colony counts, determination of antibiotic levels, and assay of β-lactamase activity.

Effect of inoculum size. Strain TAL 153 (ATCC 23745) was grown overnight in 5 ml of BHIS and was diluted to give 10^8 CFU/ml. Two further 10^{-2} dilutions were also done to obtain inocula of approximately 10^4, 10^6, and 10^8. The cultures were divided, one as a control and others for the addition of antimicrobial agents to achieve a final concentration of 128 μg/ml. Samples were taken at 0, 1, 2, 4, and 24 h for assay of antibiotic levels and total colony counts.

The antibacterial activity of cefoxitin was further tested in chloramphenicol-inhibited cells. *B. fragilis* 153 was grown to approximately 10^7 CFU/ml, and the culture was divided into two portions, one as a control and the other for the addition of 8 μg of chloramphenicol per ml. After 90 min, the two samples were again divided, one as control and the second for treatment with cefoxitin. Bacterial counts were performed when the chloramphenicol was added and at 0, 1, 2, 4, and 24 h after adding cefoxitin.

Antibiotic assays. Cephalothin, cefazolin, and cefamandole concentrations were determined by bioassay, utilizing *Bacillus subtilis* as previously described (F. P. Tally, N. V. Jacobus, and M. Barza, J. Antimicrob. Chemother., in press). Cefoxitin levels were determined by bioassay with highly sensitive *Escherichia coli* A-658, kindly supplied by Arnold Demain, Massachusetts Institute of Technology, Boston, Mass. The organism was grown overnight in nutrient broth supplemented with thiamine (2 μg/ml) and 10% Casamino Acids (1 ml per liter of broth) at 37°C in a shaker water bath. A 1-ml amount of the seed culture was added to 100 ml of 1.5% nutrient agar (supplemented as above) which had been brought to 45°C, and assay plates were poured.

β-lactamase studies. The isolates were grown in 5 ml of BHIS and were harvested by centrifugation at 12,000 × *g* for 10 min. The pellet was washed with 2 ml of 0.01 M $NaPO_4$, pH 7.0, and was centrifuged. The cells were suspended in 1 ml of $NaPO_4$ buffer and were sonicated on ice by four 30-s bursts with a Branson sonicator utilizing a small probe (Bronwill Scientific Inc., Rochester, N.Y.). The sonic extract was centrifuged at 20,000 × *g* for 20 min, and the supernatant was assayed for β-lactamase.

Qualitative analysis was performed by adding 0.1 ml of the supernatant to 0.1 ml of chromatogenic cephalosporin 87/312 at a concentration of 51.6 μg/ml in 0.05 M $NaPO_4$, pH 7.0 (11). The development of a red color was observed after 30 min of incubation at 37°C. The chromatogenic cephalosporin was kindly supplied by C. O'Callaghan, Glaxo Research Ltd., Middlesex, England.

Quantitative assay of β-lactamase activity was performed on selected strains by using a spectrophotometric assay of the reduction of chromatogenic cephalosporin 87/312. Induction of enzyme product was attempted by growth of the organisms in subinhibitory concentrations of cefazolin. The quantitative studies were performed at 0, 4, and 24 h after treatment with cefazolin and cefoxitin. The results were reported as units of protein per milligram; protein concentration was determined by the method of Lowry et al. (8). The *B. fragilis* cells were harvested from 35 ml of BHIS, washed, and sonicated as described above. A 2-ml amount of test solution which contained 1 ml of 87/312 (51.6 μg/ml) and 1 ml of supernatant from the sonic extract was mixed in a 3-ml cuvette. (A 1:10 dilution of the supernatant from highly resistant organisms was employed.) The mixture was immediately placed into a Perkin-Elmer spectrophotometer with a regulated temperature of 37°C by a constant water flow; the change in optical density at 386 nm (pH 7.0) was measured. The activity was calculated by the equation of O'Callaghan et al. (11).

Isoelectric focusing of β-lactamase. The isoelectric focusing points of the β-lactamases were determined in preparations from the various members of the *B. fragilis* group. The enzyme was harvested by sonication as described above. A broad-range polyacrylamide gel plate, with ampholite ranging from pH 3.5 to 10, was used in a flat-bed electrophoresis system (LKB Instruments Inc., Stockholm, Sweden). Whatman filter strips (10 by 12 mm) were dipped into the supernatant of the sonic extract from each organism, and they were positioned on the plate at 10-mm intervals at the anode. Two filter strips were saturated with either 1 M H_3PO_4 for the anode or 1 M NaOH for the cathode. The initial electrophoresis conditions were 200 V, 48 mA, 10 W for 10 min; then it was increased to 20 W. The samples were focused for 2 h. Horse spleen ferritin was used as a control to indicate focusing completion; the pH of the polyacrylamide gel plate was measured at 10-ml intervals, and a standard line was plotted as pH versus distance. The polyacrylamide gel plate was removed from the electrophoresis unit, and the plate was sprayed with a solution of the chromatogenic cephalosporin 87/312 (51.6 μg/ml) to visualize the enzyme. The plate was incubated at 37°C until the red color developed, indicating the location of the β-lactamase. The enzyme bands were measured, and the isoelectric points were determined from the standard line.

RESULTS

Inoculum effect. Five strains of *Bacteroides* were used in the studies of timed bactericidal activity (kill curves). The in vitro activity of cephalothin, cefazolin, cefamandole, and cefoxitin against these organisms is shown in Table 1. One strain was inhibited at low concentrations, three isolates had intermediate susceptibility, and one organism was highly resistant.

Utilizing kill curve techniques, it was found that a high inoculum had a pronounced effect on the susceptibility of *B. fragilis* 153 to cefazolin; indeed, at the 10^8 inoculum, no killing could be observed (Fig. 1). On the other hand, inoculum size had no effect on the bactericidal activity of cefoxitin (Fig. 1). The bactericidal activity of cefoxitin required actively growing cells, since antimicrobial killing was abolished when growth of the organism was inhibited by preincubating with chloramphenicol (Fig. 2).

Timed bactericidal activity and drug concentrations. The timed killings of the five organisms, with simultaneous measurements of antibiotic concentrations in the broth, are shown

TABLE 1. *Susceptibility of Bacteroides to cephalosporins*

Organism	MIC (μg/ml)[a]			
	CEPH	CEZ	CEF	CEX
B. vulgatus 10	1	2	8	2
B. thetaiotamicron 11	64	16	32	16
B. fragilis 118	64	16	32	16
B. fragilis 153	32	64	32	16
B. thetaiotamicron 790	>256	>256	>256	128

[a] CEPH, Cephalothin; CEZ, cefazolin; CEF, cefamandole; CEX, cefoxitin.

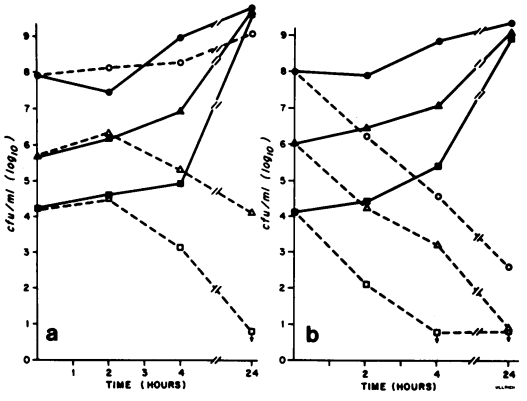

FIG. 1. *Effect of increased inoculum size on the antimicrobial activity of cefazolin (a) and cefoxitin (b) against B. fragilis, tested by kill curve techniques. Control cultures (——) and antibiotic-treated cultures (-----). The inocula were 10^4 (□, ■), 10^6 (△, ▲), and 10^8 (○, ●) cells. Viable colony counts were done at the indicated times.*

in Fig. 3 to 6. (The controls for each organism, which are not shown, grew up to approximately 5×10^9 CFU/ml within 4 to 6 h.) Cephalothin failed to inhibit the growh of the four resistant strains of *Bacteroides*, and at the same time there was a rapid and total reduction in the concentration of antibiotic in the broth culture (Fig. 3). With the susceptible strain 10 of *B. vulgatus*, there was complete bacterial killing; the concentration of cephalothin in the broth did not decrease in the initial 4 h, but the drug was completely destroyed at 24 h. With cefazolin, there was no inhibition of growth of the four resistant organisms, and there was rapid inactivation of antibiotic in the broth (Fig. 4). However, the susceptible strain 10 showed a prompt onset of bactericidal activity with a decrease in the CFU below detectable levels, and the level of antibiotic declined only 30% in the broth. Cefamandole slightly inhibited the growth of the four resistant organisms, but it was not bactericidal (Fig. 5). Concomitantly, there were substantial decreases in the broth concentrations of cefamandole with these isolates. With the susceptible organism 10, cefamandole was bactericidal, with a decrease in CFU of 10^6, and no significant change was observed in the concentration of drug.

Cefoxitin was bactericidal against all of the strains of *Bacteroides* tested, including the highly resistant *B. thetaiotaomicron* 790 (Fig. 6). In contrast to the other agents, there was no significant inactivation of cefoxitin in the broth cultures.

β-Lactamase activity. Measurement of β-lactamase activity with the chromatogenic cephalosporin 82/312 revealed that four bacterial strains, 790, 153, 11, and 118, possessed constitutive β-lactamases, whereas the susceptible strain 10 did not have any enzyme activity.

The quantitative activity of the β-lactamase of four *Bacteroides* strains was determined from late-log-phase cells harvested from control cultures and from cells stimulated by subinhibitory levels of cefazolin (Table 2). There was no enzyme activity detected in strain 10. With the two intermediately resistant strains 153 and 511, there were low levels of enzyme and there was no induction. The highly resistant strain 790

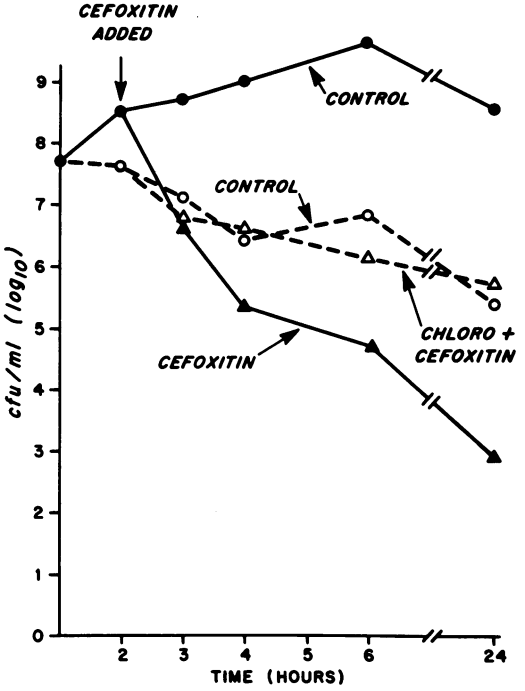

FIG. 2. *Effect of cefoxitin on the viability of rapidly growing (●) and chloramphenicol-inhibited (○) cells of B. fragilis. Cefoxitin was added 2 h after chloramphenicol to chloramphenicol-inhibited cells (△) and to control cells (▲).*

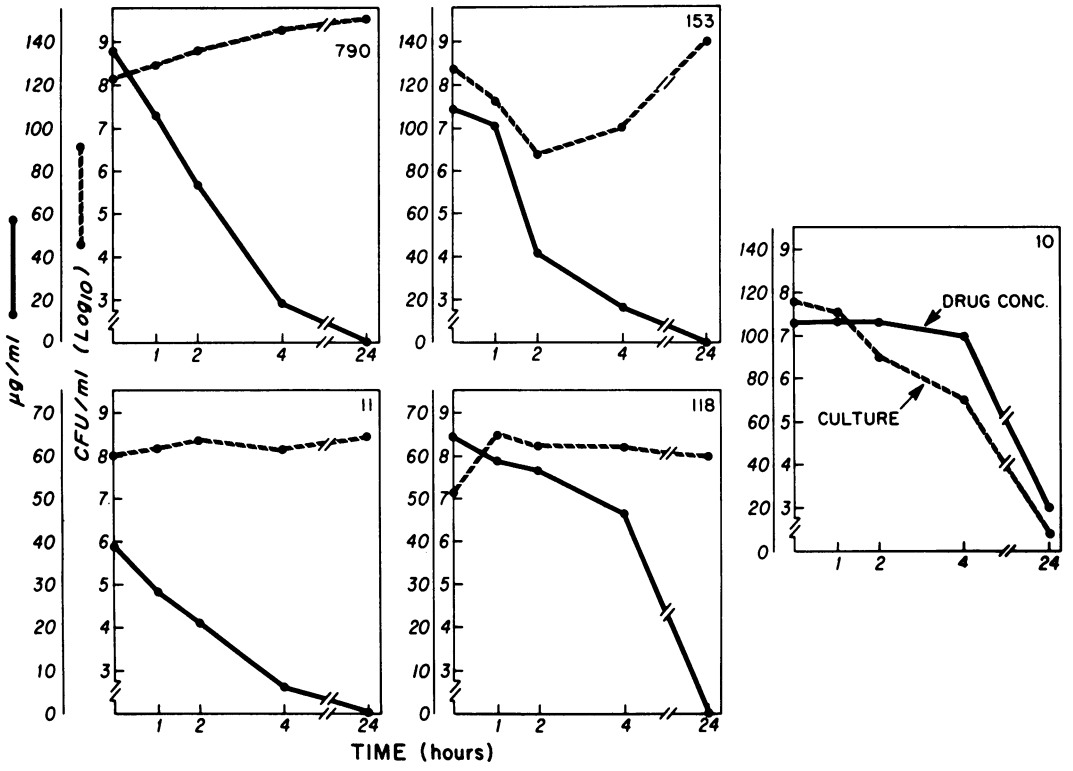

FIG. 3. *In vitro activity of cephalothin versus the B. fragilis group by kill curve. Antibiotic concentration* (——); *viable cell count* (----).

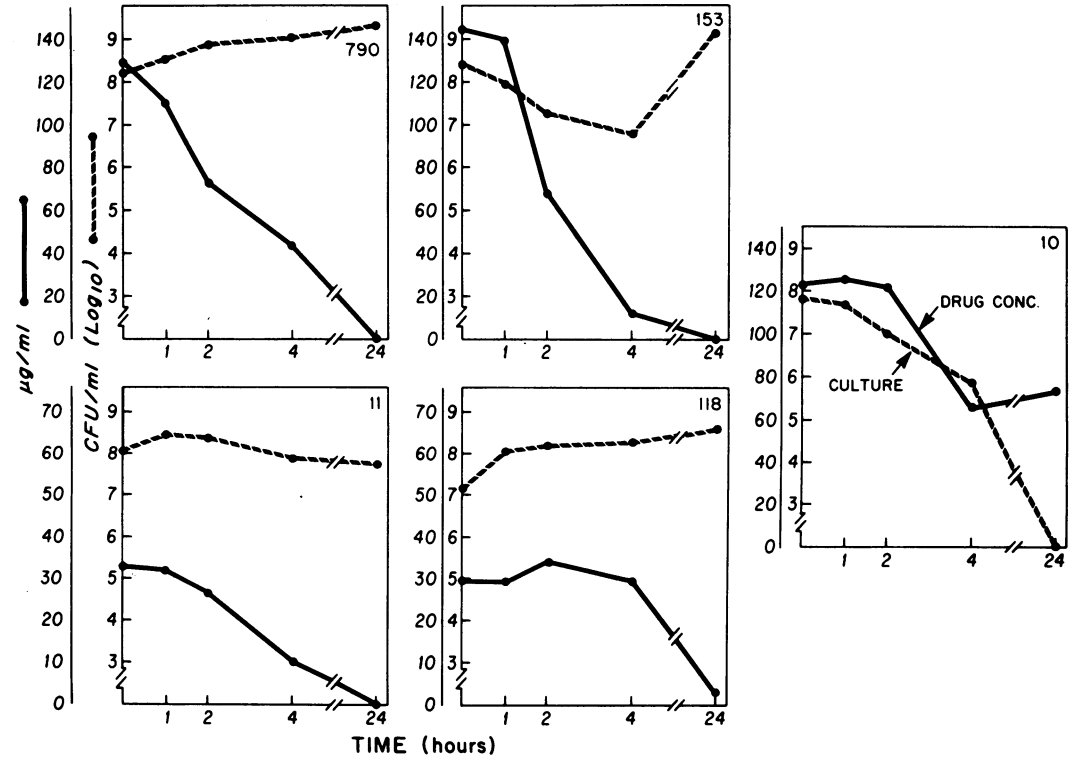

FIG. 4. *In vitro activity of cefazolin against the B. fragilis group by kill curve. Antibiotic concentration* (——); *viable cell count* (----).

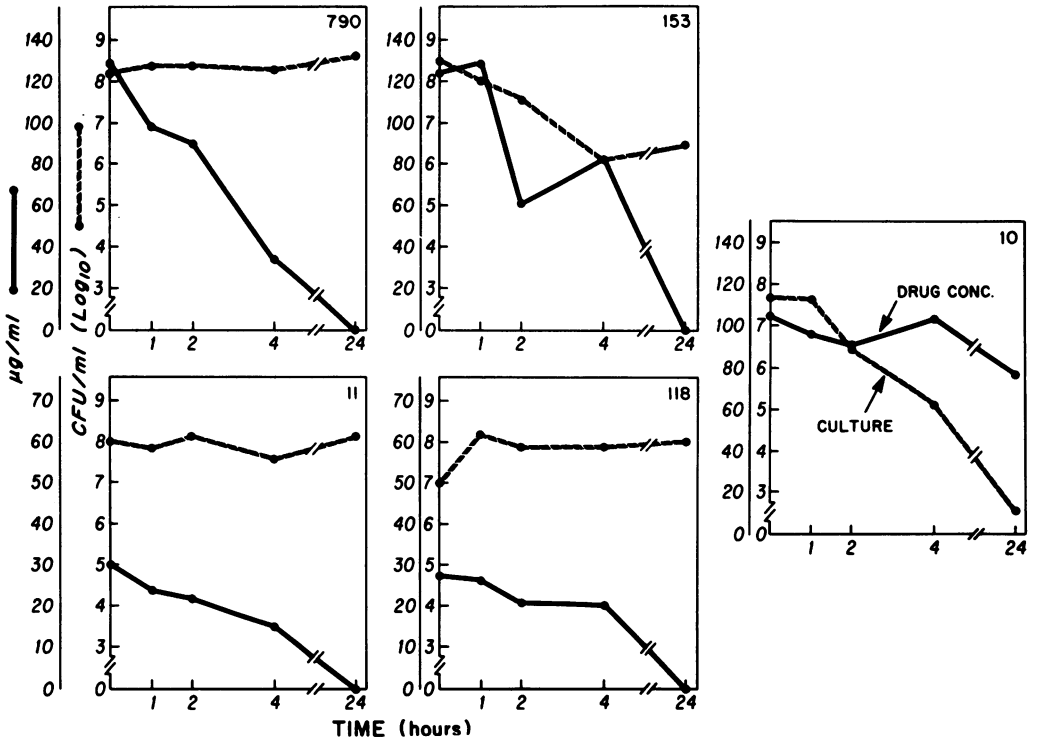

FIG. 5. *In vitro activity of cefamandole against the B. fragilis group by kill curve. Antibiotic concentration* (——); *viable cell count* (----).

produced 10 times the concentrations of enzyme, but again there was no induction. Cefoxitin competitively inhibited the β-lactamase activity in these strains of *Bacteroides*.

The β-lactamases of 16 strains of *Bacteroides*, representing all of the species of the "*Bacteroides fragilis* group," were further analyzed by isoelectric focusing. There were 3 highly cephalosporin-resistant organisms and 13 isolates with intermediate resistance. There was β-lactamase activity detectable in all of the strains (Table 3). The isoelectric focusing characteristics of the enzyme revealed four distinct focusing points. Most of the strains had an enzyme which focused at approximately pH 4.6. Two highly resistant strains had enzymes that focused at 4.8 and 5.1 pH units. There were five isolates with two distinct bands which may represent different enzymes or isoenzymes. It was impossible to make any correlation between specific enzymes and the various species of *Bacteroides*, based on the isoelectric focusing points.

DISCUSSION

The results of our study indicate that the β-lactamases of *B. fragilis* are important determinants of in vitro resistance to the cephalosporins. The resistance appears to be mediated by inactivation of susceptible antibiotic compounds. Cephalothin, which is the most resistant cephalosporin to the β-lactamases of *Staphylococcus aureus*, was rapidly inactivated by the enzymes of the *B. fragilis* group (16). The inactivation of cefazolin was similar to that of cephalothin, whereas cefamandole was slightly more resistant. Cefoxitin, on the other hand, was completely resistant to degradation by these enzymes and exerted excellent antimicrobial activity. These findings correlate with those from an in vivo study of the efficacy of these drugs in a model of anaerobic infection in rats (7).

A high inoculum was found to reduce the bactericidal activity of cefazolin but had no effect on cefoxitin, again suggesting a role for β-lactamases. Inoculum size has also been shown to determine the in vivo inactivation of penicillin in tissue chambers monoinfected with *B. fragilis* (12). High bacterial counts of 10^7 to 10^9/ml are also found in anaerobic abscesses in humans (2). These data suggest that the in vitro susceptibility testing of anaerobic bacteria, particularly the *B. fragilis* group, with current techniques which employ a low inoculum (~10^4 to 10^5) may not be adequate to detect β-lactamase-producing organisms.

Characterization of the β-lactamase reveals

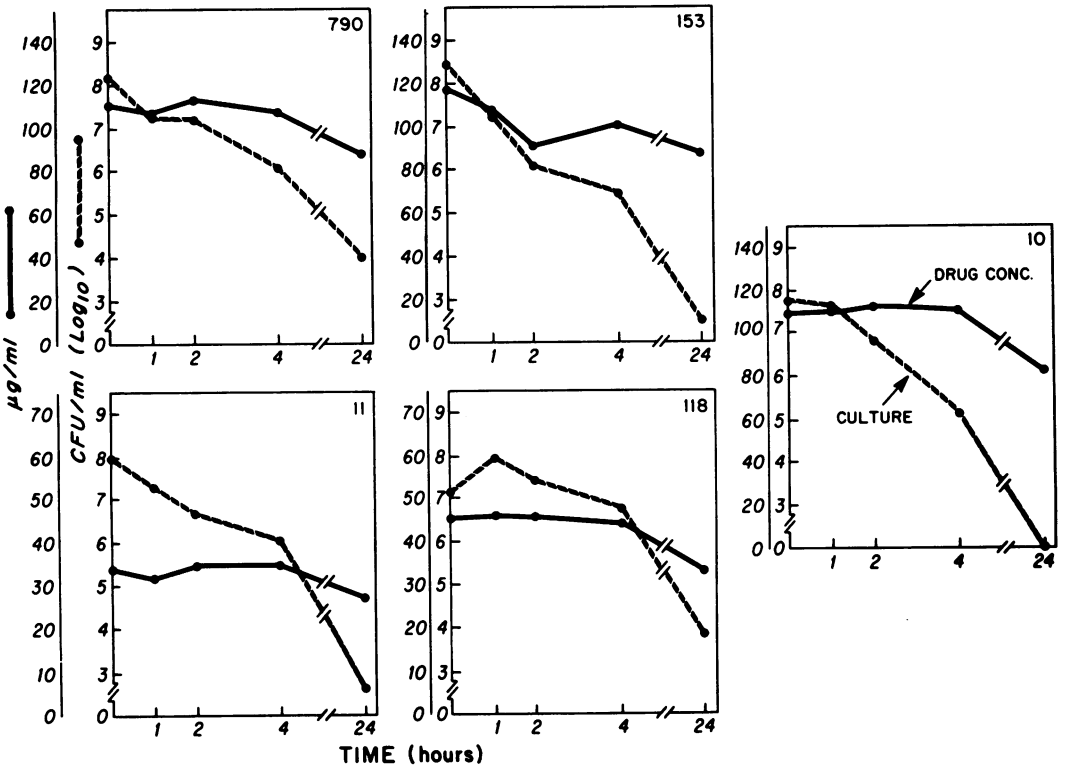

FIG. 6. *In vitro activity of cefoxitin against the B. fragilis group. Antibiotic concentration* (——); *viable cell count* (-----).

TABLE 2. *β-Lactamase activity in the B. fragilis group*

Organism	MIC of cefazolin (μg/ml)	β-Lactamase activity (U)[a]	
		Control	Cefazolin
B. vulgatus 10	2	0	0
B. fragilis 153	16	0.060	0.044
Bacteroides spp. 511	32	0.041	0.076
B. thetaiotamicron 790	>256	0.582	0.428

[a] One unit = hydrolysis of 1 μm of 87/312 per min at 37°C per mg of protein.

that it is membrane associated, is constitutively produced, and is present in all of the isolates of *Bacteroides* which are considered intermediately or fully resistant to penicillins and cephalosporins. These findings are in agreement with previously published reports, and they extend the observations of Darland and Birnbaum to include all strains of the *B. fragilis* group which show resistance to cephalosporin and penicillin antibiotics (4). The highly resistant organisms also possessed a constituitive enzyme, but they produced 10 times more. None of our isolates produced an extracellular β-lactamase such as described by Olsson et al. (14).

Isoelectric focusing of the β-lactamase of the *B. fragilis* group revealed a pattern of focusing points between 4.0 and 5.0 pH units. This pattern is different from that of the β-lactamases found in the *Enterobacteriaceae*, and in this regard our findings agree with previous studies (6, 14). However, we could not detect differences based on the focusing points between the various species of *Bacteroides*, as were suggested by the studies of Leung and Williams and Olsson et al. (6, 14). Several strains had two distinct bands which may have been either different enzymes or isoenzymes. To further define the β-lactamase, physiochemical analysis, as described by Britz and Wilkinson, and immunological characterization of the enzymes are necessary (3).

The unique isoelectric focusing points of the *B. fragilis* group could provide a useful tool for genetic studies of β-lactam resistance in anaerobic bacteria. However, it is likely that the genes coding for β-lactamase production in the *B. fragilis* group are located on the chromosome. Evidence for this is derived from the fact that the enzyme is constitutively produced by all strains thus far tested. In addition, we have analyzed several β-lactamase-producing strains which did not possess plasmids (19; F. P. Tally, unpub-

TABLE 3. *Isoelectric focusing of β-lactamase of the B. fragilis group*

Organism	Strain no.	Isoelectric points at pH:			
		4.0 ± 0.1	4.6 ± 0.1	4.8	5.1
Intermediately resistant[a]					
B. fragilis	44		+		
	153		+		
	202		+		
B. vulgatus	47		++		
	57		+		
	112		+		
B. ovatus	154	+			
	257		+		
B. thetaiotamicron	11		+		
	104		+		
	123		+		
	240	+	+		
B. distasonis	109	+		+	
Highly resistant[b]					
B. fragilis	1,002		+		+
B. thetaiotamicron	790		+	+	
Bacteroides spp.	511		+		

[a] MIC < 16 μg/ml to 128 μg/ml.
[b] MIC < 256 μg/ml.

lished data). Plasmid-mediated β-lactam resistance is usually associated with inducibility, loss of resistance on storage or passage in the laboratory, and extracellular enzyme production. None of the features has been observed with the *Bacteroides* strains described in this study and in other reports. More research is needed to detect differences in β-lactam resistance and to define the genetic system in *Bacteroides* which determines antimicrobial resistance.

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