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Factors Contributing to Resistance to Beta-Lactam Antibiotics in *Bacteroides fragilis*

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Fifteen strains of *Bacteroides fragilis*, five highly resistant, five moderately resistant, and five susceptible to benzylpenicillin and cephaloridine, were tested for β-lactamase production. In the highly resistant and the moderately resistant groups of strains, a correlation between formation of β-lactamase and minimal inhibitory concentrations was demonstrated. In the presence of the β-lactamase inhibitors clavulanic acid and CP-45,899 at concentrations of 1 μg/ml, the susceptibility to cephaloridine increased fourfold or more in the β-lactamase-producing strains. Crypticity measurements (β-lactamase activity broken/intact cells) with cephaloridine as substrate could indicate a diffusion barrier in the cell wall.

The role of beta-lactamase production in resistance of aerobic gram-negative bacteria to beta-lactam antibiotics has been extensively investigated and was recently reviewed (16). The beta-lactamases produced by aerobic gram-negative bacteria represent a wide variety of enzymes with regard to both physiology and enzyme characteristics. They are either chromosomally mediated or R plasmid mediated, and they differ in substrate profiles and in physicochemical characteristics such as isoelectric points and molecular weights.

Several methods have been used to determine the relative importance of a beta-lactamase versus a permeability barrier in resistance of gram-negative bacteria to beta-lactam antibiotics. Such information is lacking from studies of beta-lactamase from *Bacteroides fragilis*, the most common anaerobe in clinical infections (4).

The aim of the present investigation was therefore to study both the significance of beta-lactamase production and of a permeability barrier in *B. fragilis* in relation to resistance to beta-lactam antibiotics.

MATERIALS AND METHODS

Strains. From a recent study of the susceptibility of 231 clinical isolates of *B. fragilis* to beta-lactam antibiotics (10), 15 strains were selected for further studies. They had been identified as *B. fragilis* following the criteria outlined by Holdeman et al. (5). They were chosen so that five strains were relatively susceptible (minimal inhibitory concentration [MIC] < 4 μg/ml), five strains were relatively resistant (MIC = 4 to 64 μg/ml), and five strains were highly resistant (MIC > 128 μg/ml) to benzylpenicillin when determined by the standard agar dilution method (3).

The anaerobic culture system (Bellco Glass, Inc., Vineland N.J.) developed by the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, was used for subculturing the strains. Reidentification of *B. fragilis* was made after every cultivation by the method of Holdeman et al. (5).

Cultivation technique. Prereduced proteose peptone-yeast extract medium was prepared as described earlier and was used for all cultures (6). The bacteria were either cultured in 1.0-liter flasks under static conditions or in stirred fermentors (FL 101, Biotec, Stockholm, Sweden) with 1.0-liter working volumes (6). Preparation of inocula and determination of bacterial cell dry weight were performed as previously described (11).

Preparation of intracellular enzymes. The cultures were harvested after 16 h (in the late logarithmic phase of growth) and centrifuged at 4,000 × *g* for 20 min at 4°C. The pellets were washed once in 0.05 M sodium phosphate buffer, pH 7.0, centrifuged, and finally resuspended in 1/20 of the culture volume in the same buffer. The suspension was then disrupted for 5 min at 100 W in an ultrasonic disintegrator (Labsonic 1510, B. Braun Melsungen AG, Germany) while kept in an ice bath. The supernatant after centrifugation (20,000 × *g* for 20 min at 4°C) constituted the crude β-lactamase preparation.

Beta-lactamase assay. The spectrophotometric assay with the chromogenic cephalosporin analog 87/312 (Glaxo) was performed by the method of O'Callaghan et al. (8). One unit of enzyme hydrolyzed 1 μmol of substrate per min at 37°C and pH 7.0. The spectrophotometric method based on the absorption maximum at 255 nm of cephaloridine, which is associated with the β-lactam ring (9), was used for crypticity measurements.

Effect of beta-lactamase inhibitors on MICs of cephaloridine. A 1-μg amount of clavulanic acid (Beecham Pharmaceuticals, Betchworth, Surrey, England) or CP-45,899 (Pfizer Inc., Sandwich, England) per ml was incorporated into agar plates containing cephaloridine in doubling concentrations in the range

of 0.5 to 512 μg/ml. In parallel, agar plates were prepared containing either of the β-lactamase inhibitors only in doubling concentrations for MIC determinations. Bacterial inocula comprised approximately 10^4 colony-forming units and were applied by means of a Steers replicator (10). The plates were read after 48 h of incubation at 37°C in anaerobic jars (GasPak, Baltimore Biological Laboratory, Cockeysville, Md.). The MIC was defined as the lowest concentration of antibiotic inhibiting growth completely.

Growth of strains in broths containing cephaloridine and β-lactamase inhibitors. The strain to be tested was grown in four flasks, each containing 100 ml of prereduced proteose peptone medium. The inoculum comprised approximately 10^4 bacteria per ml. One flash served as the control for bacterial growth, the second flask received cephaloridine to a concentration of one quarter of the MIC of the actual strain, the third flask received the same amount of cephaloridine plus the β-lactamase inhibitor at a concentration of 1 μg/ml, and the fourth flask received the β-lactamase inhibitor only (1 μg/ml). Addition of cephaloridine took place when the experiment was started, and the β-lactamase inhibitor was added in the early logarithmic growth phase. Samples were taken every 2 h during the logarithmic phase of growth, and a final sample was taken after overnight incubation. Viable counts were performed on every sample. The concentration of cephaloridine in the samples was followed by microbiological assay with *Staphylococcus aureus* Oxford strain 209 as indicator, and that of CP-45,899 was followed with *Commononas terrigena* as the indicator strain. The spontaneous inactivation of cephaloridine was followed by simultaneous incubation of an additional flask containing broth and antibiotic.

Crypticity measurements. Strains were grown in 100 ml of prereduced proteose peptone medium to a density of approximately 10^9 bacteria per ml, harvested by centrifugation (4,000 × *g* for 20 min at 4°C), washed once in 0.05 M sodium phosphate buffer, pH 7.0, and then resuspended in 5 ml of the same buffer. A sample of this bacterial suspension was assayed for β-lactamase with cephaloridine as substrate (activity of intact bacteria), while the remainder was disrupted for 5 min in an ultrasonic disintegrator before β-lactamase assay. The crypticity is defined as the enzymatic activity, expressed in units per milligram (dry weight), of broken bacteria divided by enzymatic activity of intact bacteria (14).

Chemicals. All chemicals were of analytical grade, unless otherwise stated. Culture media were obtained from Difco Laboratories, Detroit, Mich. The salts and constituents for buffers were obtained from Merck AG, Darmstadt, Germany. Cephaloridine and chromogenic cephalosporin 87/312 were kindly supplied by Glaxo Research Ltd., Greenford, Middlesex, England. Clavulanic acid was kindly provided by Beecham Pharmaceuticals, Betchworth, Surrey, England, and CP-45,899 was a gift from Pfizer Inc., Sandwich, England.

RESULTS

Correlation between MIC and β-lactamase activity. MICs for benzylpenicillin and cephaloridine were compared to β-lactamase activity for 15 strains of *B. fragilis* when the strains were cultivated in 1.0-liter flasks under static conditions (Table 1). Strains that were highly resistant to benzylpenicillin (MIC ≥ 128 μg/ml) produced about 10 times as much β-lactamase (units per milligram [dry weight] of bacteria) as produced by the relatively resistant strains (MIC, 4 to 64 μg/ml). Strains that were relatively sensitive to benzylpenicillin (MIC < 4 μg/ml) produced only minute amounts of β-lactamase or no β-lactamase at all. Cultivation of strains in stirred fermentors under controlled conditions with regard to pH and temperature resulted in higher yields of β-lactamase.

Influence of β-lactamase inhibitors on MICs of cephaloridine for *B. fragilis*. The presence of 1 μg of clavulanic acid or CP-45,899 per ml of agar considerably reduced the MICs for β-lactamase-producing strains of *B. fragilis* but did not influence the MICs for strains showing low or no enzyme production (Table 2). MICs for clavulanic acid were 8 to 16 μg/ml and those for CP-45,899 were 16 to 32 μg/ml.

Growth of *B. fragilis* in broth containing cephaloridine and β-lactamase inhibitors. The effects of clavulanic acid or CP-45,899 (1 μg/ml) on growth of strains B 105, B 53, and B 157 are presented in Fig. 1 and 2. The highly resistant strain B 105 and the relatively resistant strain B 53 were both inhibited by cephaloridine at a concentration four times less than their respective MICs when grown also in the pres-

TABLE 1. *Correlation between MICs and β-lactamase activity for 15 strains of B. fragilis*

Strain	MIC (μg/ml) of:		β-lactamase[a] activity (U/mg)
	Benzylpenicillin	Cephaloridine	
B 8	128	256	0.109
B 34	512	512	0.553
B 105	512	512	0.106
B 122	256	256	0.572
B 147	512	512	0.356
B 1	8	16	0.022
B 53	16	32	0.047
B 62	8	8	0.014
B 80	8	16	0.015
B 85	16	32	0.025
B 65	0.125	0.5	0
B 117	2	16	0.003
B 157	2	8	0.006
B 174	0.125	0.5	0
B 305	0.125	0.5	0

[a] Intracellular β-lactamase activities as obtained after sonic oscillation were expressed in units per milligram (dry weight) of bacteria.

TABLE 2. *Effect of the β-lactamase inhibitors clavulanic acid and CP-45,899 on the MICs for cephaloridine against strains of B. fragilis determined by the agar dilution method*

Strain	MIC (μg/ml) of:				
	CER[a]	CA[b]	CER + CA (1 μg/ml)	CP[c]	CER + CP (1 μg/ml)
B 8	256	8	8	32	16
B 34	512	16	16	32	32
B 105	512	16	4	32	16
B 122	256	16	4	32	16
B 147	512	8	4	32	32
B 1	16	8	2	32	0.5
B 53	32	16	2	32	1
B 62	8	8	2	32	1
B 80	16	8	2	32	1
B 85	32	8	4	32	1
B 65	0.5	8	0.5	16	0.5
B 117	16	16	16	32	16
B 157	8	16	8	32	8
B 174	0.5	8	0.5	32	0.5
B 305	0.5	8	0.5	16	0.5

[a] CER, Cephaloridine.
[b] CA, Clavulanic acid.
[c] CP, CP-45,899.

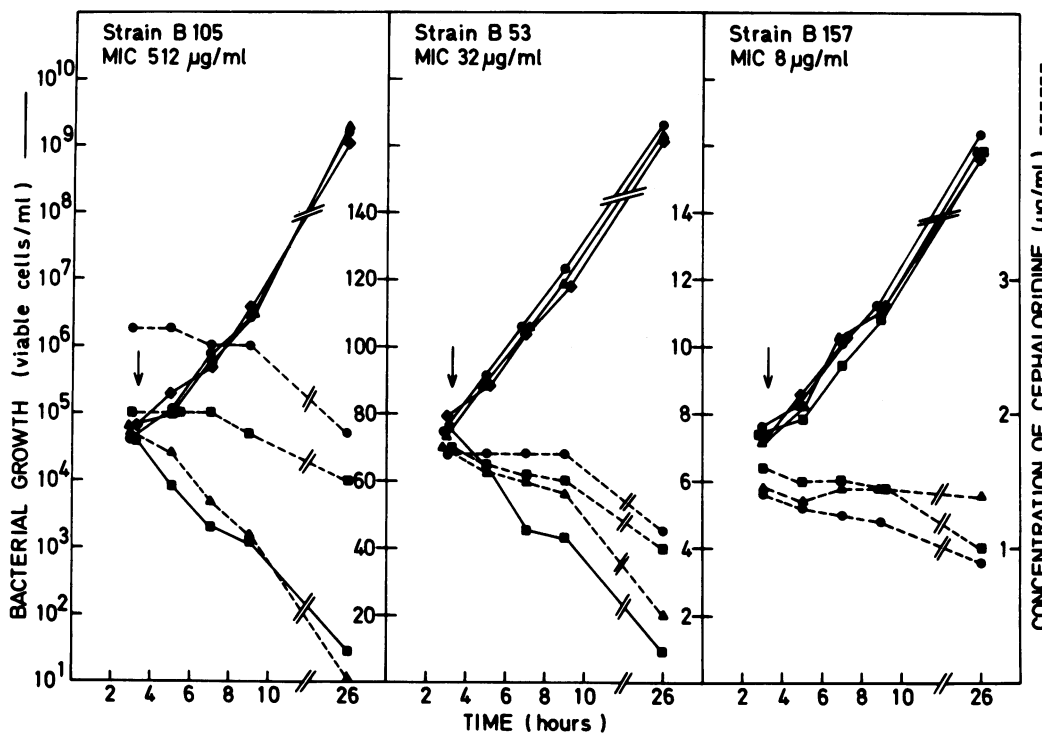

FIG. 1. *Effect of cephaloridine and clavulanic acid on growth of B. fragilis strains B 105, B 53, and B 157. Arrows indicate the addition of clavulanic acid. Symbols: ●, controls for bacterial growth and spontaneous inactivation of cephaloridine; ▲, bacterial growth in the presence of cephaloridine and corresponding concentrations of cephaloridine; ■, growth in the presence of cephaloridine and clavulanic acid and corresponding concentrations of cephaloridine; ◆, growth in the presence of clavulanic acid. Solid line: Bacterial growth in the presence of drugs. Dashed lines: Concentrations of cephaloridine.*

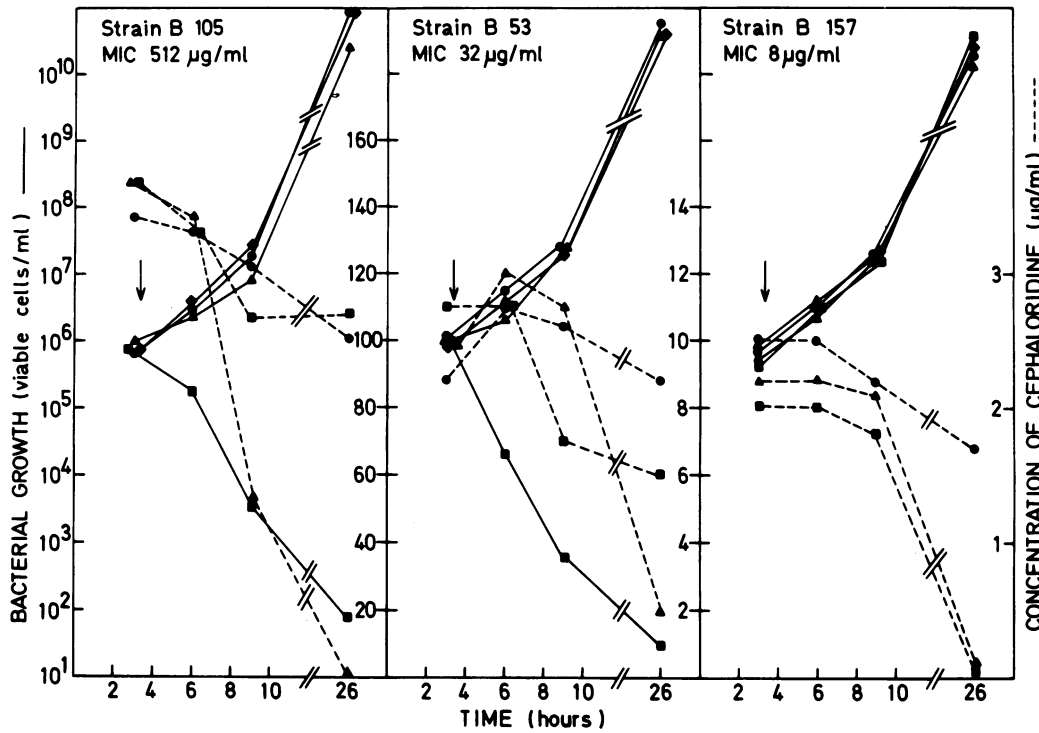

FIG. 2. *Effect of cephaloridine and CP-45.899 on growth of B. fragilis strains B 105, B 53, and B 157. For symbols, see legend to Fig. 1.*

ence of either β-lactamase inhibitor, whereas strain B 157 was not affected. It can be seen that the inhibitors themselves had no inhibitory effects on the bacterial growth at 1 µg/ml. Cephaloridine was inactivated in the presence of bacteria but to a much lesser extent in the presence of bacteria plus β-lactamase inhibitor. The concentration of CP-45,899 remained constant in the broth culture during the duration of these experiments.

Crypticity measurements. Crypticity values could only be obtained for the five highly resistant strains, because no β-lactamase activity could be detected in unbroken cells of the other strains. The values for strains B 8, B 34, B 105, B 122, and B 147 with cephaloridine as substrate are shown in Table 3.

TABLE 3. *Crypticity measurements of the five highly resistant strains of B. fragilis with cephaloridine as substrate*

Strain	β-Lactamase activity[a]		Crypticity value (broken/intact cells)
	broken	Intact	
B 8	0.11	0.015	7.3
B 34	0.50	0.24	2.1
B 105	0.56	0.25	2.2
B 122	0.23	0.12	1.9
B 147	0.50	0.09	5.5

[a] β-Lactamase activity expressed in units per milligram (dry weight) of bacteria.

DISCUSSION

The presence of a β-lactamase in clinical isolates of *B. fragilis* has been reported by several authors (7). The percentage of strains with β-lactamase activity varied between these investigations. These differences could perhaps be accounted for by different screening techniques. Darland and Birnbaum (2) employed a modified microiodometric method and found that approximately 50% of 79 isolates possessed at least small amounts of β-lactamase. Weinrich and Del Bene (17) studied only 10 strains of *B. fragilis* and found β-lactamase in 8 of these by using an alkalimetric method.

In a previous report from our laboratory (10), it was found that approximately 90% of 231 clinical isolates of *B. fragilis* were β-lactamase producers by using the chromogenic cephalosporin 87/312 in a simple screening test. Strains of *B. fragilis* could be divided into three resistance categories, one highly resistant group, one relatively resistant group, and one relatively suscep-

tible group, and these groups correlated well with the amount of β-lactamase produced by each strain. The significance of these findings needed to be studied further, and it was therefore decided to concentrate on a limited number of strains from different resistance categories. The exact amount of β-lactamase in 15 strains of *B. fragilis*, 5 from each of the above-mentioned groups, showed a positive correlation to the level of resistance in each strain (Table 1). Similar results have been reported by Darland and Birnbaum (2), who made a statistical analysis for association between resistance and presence of β-lactamase.

The possibility of inhibiting β-lactamase activity in *B. fragilis* by certain β-lactam compounds like cefoxitin or compounds that resemble the β-lactam nucleus like clavulanic acid and CP-45,899 have opened new ways of assessing the role which the β-lactamase plays in resistance to β-lactam antibiotics. Cefoxitin was the first substance reported to be an effective inhibitor of β-lactamase activity in *B. fragilis* (2).

Clavulanic acid, which is a naturally occurring substance produced by a strain of *Streptomyces clavuligerus*, was first shown to be a potent inhibitor of β-lactamases from many aerobic bacteria such as *S. aureus, Escherichia coli, Klebsiella, Proteus, Shigella, Pseudomonas,* and *Haemophilus influenzae* (13). The cephalosporinases produced by strains of *Pseudomonas aeruginosa* and *Enterobacter cloacae* were, however, less well inhibited. Two reports have appeared recently on the ability of clavulanic acid to act synergistically with penicillin and thereby lower MICs in strains of *B. fragilis* (18, 19). The present results show that clavulanic acid is a very effective inhibitor of β-lactamase from *B. fragilis* at a concentration as low as 1 μg/ml, thus allowing cephaloridine to act at readily attainable concentrations (Table 2). Only two strains of *B. fragilis* that produced β-lactamase were unaffected by the action of clavulanic acid. Wüst and Wilkins (19) reported that strains of *B. fragilis* belonging to homology group II were not susceptible to clavulanic acid. They supposed that these strains lacked β-lactamase and thus possessed only intrinsic resistance, although they never actually performed assays to confirm the absence of β-lactamase.

The second inhibitor tested, CP-45,899, had an inhibitory effect on *B. fragilis* β-lactamase similar to that of clavulanic acid.

The function of the outer membrane in gram-negative bacteria as a permeability barrier to β-lactam antibiotics has attracted the attention of many authors (1, 12, 14, 15, 20). The synergistic effect of the β-lactamase and the barrier function of the outer membrane has been determined by measuring the crypticity for a given strain and substrate. Such measurements are useful under certain conditions, but they also have some limitations, because they can only be used for substrates against which the β-lactamase is active (14).

In our investigation, neither cell wall-defective mutants of *B. fragilis* nor mutants which lacked the ability to produce β-lactamase could be obtained. Accordingly, determination of the crypticity of strains with cephaloridine as enzyme substrate was carried out. The results for three of the strains are in good agreement with those obtained by Darland and Birnbaum (2), but higher crypticity values were found in two of the strains.

In conclusion, our findings demonstrate that resistant *B. fragilis* strains are resistant because of beta-lactamase production and a permeability barrier, but the other *B. fragilis* strains are relatively resistant because of an unknown mechanism.

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