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Formation of Beta-Lactamase in *Bacteroides fragilis*: Cell-Bound and Extracellular Activity

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Nine strains of *Bacteroides fragilis* were cultivated in stirred fermentors and tested for their ability to produce β-lactamase. There was a correlation between formation of β-lactamase and high values of the minimal inhibitory concentration against β-lactam antibiotics. *B. fragilis* strain B70 was used for optimizing the production of β-lactamase. The highest bacterial yield was obtained in a proteose peptone-yeast extract medium. Optimal conditions for growth and β-lactamase production were obtained at 37 C and pH 7.0. The β-lactamase was released into the surrounding medium during the growth period to about 50%. Osmotic shock released about 20% of the total activity, and remaining activity was found in the cytoplasmic fraction. Substrate profile studies on four β-lactamase-producing strains showed that the enzymes were mainly cephalosporinases. They are inhibited by cloxacillin, *p*-chloromercuribenzoate, and iodine. Analytical isoelectric focusing in polyacrylamide gel gave an isoelectric point of 4.9 ± 0.2 for three of the strains and 5.6 ± 0.2 for one. Comparison with β-lactamases from aerobic gram-negative species with regard to isoelectric points showed no similarities. Also the molecular weight of the β-lactamase from strain B70 of 43,000 indicates that this is a new class of β-lactamase.

Strains of *Bacteroides fragilis* are frequently isolated from clinical specimens. Most of these strains are moderately or highly resistant to β-lactam antibiotics (5). This resistance seems to depend on the production of β-lactamases (EC 3.5.2.6), as reported by several authors. Garrod (8) found penicillinase-like activity in two strains of *B. fragilis*, and Pinkus et al. (26) demonstrated penicillin-hydrolyzing activity in 5 of 18 strains of *B. fragilis* and ampicillin-hydrolyzing activity in 2 of 18 strains, which was due to the production of a β-lactamase. Anderson and Sykes (1) described a β-lactamase from one highly resistant strain of *B. fragilis*, which was more active on cephaloridine than on penicillin, and Del Bene and Farrar (4) found only cephalosporinase activity in all of the 10 strains of *B. fragilis* tested. These findings are conflicting as to whether the enzymes are mainly penicillinases, cephalosporinases, or equally active against both types of β-lactam antibiotics.

The aim of the present study was to find optimal conditions for growth and β-lactamase production in strains of *B. fragilis* highly resistant to β-lactam antibiotics. We also wanted to make preliminary studies on some properties of the *B. fragilis* β-lactamases and compare them with β-lactamases from aerobic gram-negative organisms.

MATERIALS AND METHODS

Bacterial strains. *B. fragilis* subsp. *vulgatus* ATCC 8482, *B. fragilis* subsp. *ovatus* 8483, and *B. fragilis* subsp. *distasonis* ATCC 8503 were obtained from the American Type Culture Collection, Rockville, Md. *B. fragilis* subsp. *fragilis* NCTC 9343 and *B. fragilis* subsp. *thetaiotaomicron* NCTC 10582 were from the National Collection Type Cultures, London, England. These five strains were all fairly sensitive to β-lactam antibiotics (minimal inhibitory concentration [MIC] ≤1 μg/ml).

B. fragilis subsp. *ovatus* B4, *B. fragilis* subsp. *vulgatus* B70, *B. fragilis* subsp. *vulgatus* B72, and *B. fragilis* subsp. *fragilis* B153 were isolated from patients with septicemia and identified by the method of Holdeman and Moore (12). These four strains were all highly resistant to β-lactam antibiotics (MIC ≥ 250 μg/ml).

The anaerobic culture system, including the special equipment for subculturing (Bellco Glass Inc., Vineland, N.J.) developed by the Anaerobe Laboratory, Virginia Polytechnic Institute and State University (12), was used for subculturing the strains. After every batch culture reidentification of *B. fragilis* was made according to this system to confirm that no changes in biochemical activities of the strain had occurred during the experiment.

The following β-lactamase-producing, aerobic gram-negative rods were used in some experiments for comparative purposes. *Escherichia coli* TEM, *E. coli* 1573E, and *Klebsiella aerogenes* 1082E were kindly supplied by R. B. Sykes (Glaxo Research Ltd., Greenford, Middlesex, England). *Enterobacter cloacae* 265A was obtained from Eli Lilly & Company (Indianapolis, Ind.). *Pseudomonas aeruginosa* strains 18 S and 18 R were obtained from K. Nordström (Umeå University, Umeå, Sweden). *P. aeruginosa* P 13 and *Haemophilus influenzae* (Beecham 4482) were kindly given to us by B. Wretlind (Karolinska Sjukhuset, Stockholm, Sweden).

Prereduced media. Unless otherwise stated, the following basal medium was used: proteose peptone (Difco) (20 g)-yeast extract (Difco) (5 g)-cysteine-hydrochloride (0.55 g)-NaCl (5 g). The medium was prepared in a prereduced state by the method described by Nord et al. (21). The medium was dispensed into the fermentor, which was autoclaved for 15 min at 121 C. A solution of 10% (wt/vol) glucose was sterilized by filtration through a membrane filter (Millipore) (pore size, 0.22 μm) and was added to the fermentor to a final concentration of 10 g/liter. Four other prereduced complex media were also tested: (i) the basal medium in which cysteine-hydrochloride was substituted with thioglycollate, 0.5 g; (ii) a tryptone-yeast extract medium (33); (iii) a peptone-yeast extract medium (12); and (iv) a proteose peptone-whale extract medium (3). One defined amino acid medium, described by Wahren and Holme (34), was also used in the prereduced state. All media contained glucose, 10 g/liter. All manipulations of the media were performed in an anaerobic chamber (Coy, Ann Arbor, Mich.) by the method of Nord et al. (21).

Cultivation technique. Inocula were prepared by the method of Nord et al. (21). Stirred fermentors with a working volume of 1.0 liter were used for pilot studies (FL 101, Biotec, Stockholm, Sweden). Production of large quantities of β-lactamase was performed in fermentors of 3-liter working volume (FL 103, Biotec). Temperature was controlled with an accuracy of ±0.01 C, and pH was controlled with an accuracy of ±0.05 pH unit as previously described (21). The impeller speed was 200 rpm (Biotec LP 300).

A 10-ml amount was taken from the cultures at intervals and centrifuged at 4,000 × *g* for 15 min at 4 C. The supernatant fluid was tested for glucose concentration and enzyme activities. The pellet was used for determination of bacterial dry weight (21).

Assay of β-lactamases. Three methods for determination of β-lactamase activity were used. (i) A spectrophotometric method using the chromogenic cephalosporin analogue 87/312 as substrate was performed by the method of O'Callaghan et al. (24). Measurements were made at 386 nm in a Zeiss spectrophotometer PMQ 3. One unit of enzyme will hydrolyze 1 μmol of substrate per min at 37 C and pH 7.0.

(ii) An acidimetric method with phenol red as indicator (28) was used for measuring relative rates of hydrolysis of both penicillins and cephalosporins. Calculations were made on the basis of 1 mol of acid produced per mol of penicillin hydrolyzed and 2 mol of acid per mol of cephalosporin.

(iii) The spectrophotometric method based on absorption maxima in the ultraviolet region from 250 to 270 nm associated with the β-lactam ring (25) was used for substrate profile studies on cephalosporins only. Good agreement was obtained between the three methods.

Other enzyme assays. Alkaline phosphatase was determined by the method of Torriani (31) using *p*-nitrophenylphosphate as substrate. 3'-Nucleotidase and 5'-nucleotidase were determined by the method of Neu (17, 18) using bis-*p*-nitrophenylphosphate as substrate. Leucine aminopeptidase was determined by the method of Linder et al. (14) using L-leucyl-β-naphthylamide hydrochloride as substrate. Units of enzyme activities are expressed as micromoles of substrate hydrolyzed per minute at 37 C.

Studies on synthesis of β-lactamase. Induction of β-lactamase activity was attempted by the addition of 64 μg of benzylpenicillin (one-quarter the MIC value) per ml or 64 μg of cephalothin (one-quarter the MIC value) per ml to the culture medium initially. Samples were taken at intervals, and determinations of bacterial growth and β-lactamase activity were performed.

To stop protein synthesis, chloramphenicol at a final concentration of 50 μg/ml was added to a culture in the middle of the logarithmic phase. Samples taken at intervals were tested for bacterial growth and β-lactamase and leucine aminopeptidase activities.

Preparation of intracellular enzymes. Cultures of *B. fragilis* in the late logarithmic phase of growth were centrifuged, washed once in 0.01 M sodium phosphate buffer, pH 7.0, and resuspended in 20 ml of the same buffer. The cells were disrupted by the freeze-press procedure (6) (X-press, Biotec, Bromma, Sweden). After one passage of the frozen cell suspension through the X-press, about 90% of the cells were broken, as observed by phase-contrast microscopy. The supernatant after centrifugation (20,000 × *g*, 20 min, 4 C) constituted the crude β-lactamase preparation.

Intracellular enzymes from strains *E. coli* TEM, *E. coli* 1573E, *K. aerogenes* 1082E, and *E. cloacae* 265A were prepared by growing the organisms in 100 ml of brain heart infusion broth in 1-liter Erlenmeyer flasks with indentations for 6 h. Cells were harvested, washed once in 0.01 M sodium phosphate buffer, pH 7.0, resuspended in 10 ml of the same buffer, and disrupted by passing the cell suspension twice through the X-press. The supernatant after centrifugation constituted the crude β-lactamase preparation.

H. influenzae was cultivated in 100 ml of Todd-Hewitt broth supplemented with Iso-VitaleX and hemoglobin (1 g/liter) and was harvested after 18 h. Cells were treated as above.

Strains of *P. aeruginosa* were grown in 100 ml of brain heart infusion broth and were induced by the addition of benzylpenicillin to a final concentration of 1 mg/ml. Cells were harvested after 10 to 16 h, washed, and disrupted by freeze-pressing.

Isolation of cell-bound and intracellular en-

zymes. The isolation scheme described by Nord and Wadström (22) was followed. After treatment of disrupted cells by this scheme, the following fractions were obtained: cytoplasmic fraction, membrane-washed fraction, membrane protein fraction, washed cell wall fraction, LiCl-treated cell wall fraction, and lysozyme-treated cell wall fraction. All fractions were dialyzed and tested for enzymatic activities.

Inhibition of β-lactamase activity. Crude intracellular enzyme preparations were used in these experiments. Inhibition of the enzymatic hydrolysis of a 0.1 mM solution of cephaloridine by 0.1 mM solutions of cloxacillin, carbenicillin, methicillin, or cefoxitin was studied by the spectrophotometric method described by O'Callaghan and Morris (23). The inhibitory effects of 0.5 mM *p*-chloromercuribenzoate (PCMB) (dissolved in 0.1 M glycyl-glycine buffer, pH 8.5) and of 0.01 mM iodine on the enzymatic hydrolysis of a 0.1 mM solution of cephaloridine were also studied, after preincubation of the inhibitor with the enzyme preparation for 10 min at 37 C. Inhibition was calculated as the percentage decrease in the rate of hydrolysis as compared with the rate of hydrolysis of substrate without inhibitor.

Release of periplasmic enzymes. A culture in the late logarithmic phase of growth (10 to 12 h) was centrifuged (4,000 × *g*, 20 min, 4 C), and the pellet was divided into two equal parts and treated in two different ways.

(i) Osmotic shock was performed by a modification of the method described by Heppel (11). The pellet of cells was washed twice with 0.033 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.2) at 20 C. The cells were resuspended in the same buffer containing 20% (wt/vol) sucrose and 1 mM ethylenediaminetetraacetate at 20 C for 15 min and centrifuged again (4,000 × *g*, 20 min, 4 C). The cells were resuspended in 0.1 mM $MgCl_2$ at 4 C for release of periplasmic proteins and centrifuged immediately (4,000 × *g*, 20 min, 4 C). The remaining cell pellet was disintegrated in the X-press. The extracellular fluid, ethylenediaminetetraacetate wash, $MgCl_2$ wash (osmotic shock fluid), and cell lysate were all dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, prior to testing for enzymatic activities.

(ii) Release of periplasmic enzymes by treatment with polymyxin B was performed by the method of Cerny and Teuber (2) as modified by Evans et al. (7). Fluids and cell lysate were treated as above.

Isoelectric focusing and zymogram method. Isoelectric focusing in polyacrylamide gel was performed as previously described (30, 32) with the following modifications. Ampholine carrier ampholytes (pH range, 3 to 10) were used at a final concentration of 2% (wt/vol), 4.85% (wt/vol) γ-acrylamide and 0.15% (wt/vol) *N-N'*-methylene bisacrylamide (the data for the gels were T = 5% [wt/vol] and C = 3% [wt/wt]). Focusing was done on a cooling plate at 4 C on the LKB Multiphor apparatus (LKB-Produkter AB, Bromma, Sweden). Samples were applied onto the gel using absorbent pads (8 by 10 mm) (Paratex III/80 Lohmann KG, Fahl, Rein, German Federal Republic), and the amount of enzyme solution was 50 μl (approximately 0.05 U). Gels were run for 60 min with constant wattage (about 60 W). After focusing, the pH of the gel was measured with a flat membrane-combined microelectrode, and the carrier ampholytes were then removed by immersing the gel in 0.4 M sodium phosphate buffer, pH 7.0, at 20 C for 5 min. After equilibration, the gel was covered with a starch-agarose overlayer: a solution of 2% (wt/vol) soluble starch in 1.5% melted agarose (pH 7.0) containing benzylpenicillin or cephaloridine (5 mM) was poured onto the gel, which was then incubated at 37 C for 4 h. The zymogram was developed by soaking the gel in Lugol solution-distilled water (1:1) and photographed. The method described by Matthew et al. (16) using the chromogenic cephalosporin analogue 87/312 for detection of bands with β-lactamase activity was also used.

Molecular weight determination. (i) Gradient polyacrylamide electrophoresis. The gel electrophoresis apparatus (GE; Pharmacia, Uppsala, Sweden) was used according to the instructions from the manufacturer. Gradient polyacrylamide gels with T = 6.4 to 29.6 and C = 4.7 to 3.6 (batch LMW 740702) were generously supplied by the manufacturer. The gel was equilibrated with 0.09 M tris(hydroxymethyl)aminomethane-0.08 M borate buffer, pH 8.4. Samples containing approximately 0.01 U in 10 μl of crude enzyme preparation were applied in every second of the 12 application slits at the top of the gel. After running electrophoresis for 5 min (120 V, 6.5 mA), some of the slits were reloaded from two to five times. The experiment was run for 18 h at 5 C with an initial voltage of 120 V (35 mA) and a final voltage of 120 V (8 mA). The following reference substances were used: oligomers of cytochrome C and egg-white lysozyme polymerized by treatment with glutaraldehyde, human hemoglobin, bovine serum albumin, and lysostaphin. Detection of β-lactamase activity was performed by the starch-penicillin-agarose method used for isoelectric focusing in polyacrylamide gels.

(ii) Gel filtration on Bio-Gel P-60. Gel filtration on Bio-Gel P-60 was performed as an alternative method for molecular weight determination. A column (2.5 by 130 cm) containing P-60 was equilibrated with 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.0, containing 0.9% NaCl. A 2-ml amount of the crude enzyme preparation (approximately 2.0 U) was applied to the column. The effluent was fractionated in 5.2-ml samples with a flow rate of 10.4 ml/h. The experiments were always performed at 4 C.

Chemical analyses. Glucose was analyzed by the glucose oxidase method (Glox Kit, Kabi, Stockholm, Sweden). Protein was determined after extensive dialysis by the method of Lowry et al. (15) using bovine serum albumin as standard.

Chemicals. All chemicals were of analytical grade unless otherwise stated. Culture media were obtained from Difco (Detroit, Mich.). IsoVitaleX and hemoglobin were obtained from BBL, Division of BioQuest (Cockeysville, Md.). The salts and constituents for buffers were obtained from Merck AG (Darmstadt, Germany). The following chemicals

were obtained from Sigma Chemical Co. (St. Louis, Mo.): enzymes and enzyme substrates, unless otherwise stated, PCMB, glycyl-glycine, glutaraldehyde, bovine serum albumin, egg-white lysozyme, and cytochrome *c*. Lysostaphin, chymotrypsin, and ovalbumin were obtained from Schwarz/Mann (Orangeburg, N.Y.). Benzylpenicillin and human hemoglobin were obtained from AB Kabi (Stockholm, Sweden). Ampicillin, carbenicillin, cloxacillin, and methicillin were obtained from Astra (Södertälje, Sweden). Cephalothin and β-lactamase (lot no. 197-740B-287-1) were kindly supplied by Eli Lilly & Company (Indianapolis, Ind.); cephaloridine and chromogenic cephalosporin analogue (code no. 87/312) were kindly supplied by Glaxo Research Ltd. (Greenford, Middlesex, England). Cefoxitin was a gift from MSD (Merck, Sharp & Dohme International, Rahway, N.J.). Penicillinase from *Bacillus cereus* (Neutrapen, 400,000 U/ml) was obtained from Riker Laboratories (Loughborough, England). Polymyxin B was supplied by Novo (Novo Industri A/S, Bagsvaerd, Denmark). Chloramphenicol was obtained from Parke, Davis & Co. (Detroit, Mich.). Acrylamide and *N,N'*-methylene bisacrylamide were purchased from British Drug House (Poole, England), and ampholine was from LKB (Bromma, Sweden). Agarose was obtained from l'Industrie Biologique Francaise (Seine, France). Bio-Gel P-60 was obtained from Bio-Rad Laboratories (Richmond, Calif.).

RESULTS

Choice of strain. Nine strains of *B. fragilis* were cultivated in stirred fermentors under controlled conditions of pH, temperature, and agitation to determine their ability to produce β-lactamase. There were great variations among the ability of different strains to produce β-lactamase (Table 1). Strain B70 was the best producer of β-lactamase and was chosen for further studies of the influence of cultural conditions on the production of β-lactamase.

Medium composition. *B. fragilis* strain B70 was cultivated in six different prereduced media in stirred fermentors with a working volume of 1 liter. Both the highest yield of cells and extracellular β-lactamase activity were obtained in the proteose peptone-yeast extract medium (Table 2). No difference was found in enzymatic activity when using thioglycollate instead of cysteine as reducing agent in the medium, showing that cysteine was not an inhibitor of β-lactamase activity at the concentration used. The bacterial yield was lowest in an amino acid medium, and only low levels of β-lactamase activity were recorded.

Relationship between bacterial growth and formation of β-lactamase. The appearance of β-lactamase in the culture fluid during different phases of growth of *B. fragilis* strain B70 was studied in a fermentor of 1-liter volume with prereduced basal medium containing glucose (10 g/liter) at 37 C and pH 7.0 (Fig. 1). The period of active growth lasted for about 6 h, followed by a lytic phase. Glucose was the growth-limiting factor in the experiment. Release of β-lactamase from bacterial cells was parallel with the growth curve. After this period, a rapid decrease of β-lactamase activity was observed. Leucine aminopeptidase activity was followed as a marker for intracellular enzymes, but it did not increase at the beginning of the stationary phase, but rather showed a slow increase during the growth period. This indicates that cell lysis occurred in the culture only to a minor extent.

Addition to the medium of benzylpenicillin or cephalothin did not increase the level of β-lactamase activity of strain B70; on the contrary, cephalothin seemed to partially inhibit β-lactamase activity in this experiment.

Addition of chloramphenicol during the middle of the logarithmic phase stopped bacterial cell protein synthesis with an immediate rise in leucine aminopeptidase activity (Fig. 2), and extracellular β-lactamase appeared during the next 20 h without the rapid decrease in activity seen in the control experiment.

Influence of pH on growth and formation of

TABLE 1. *Extracellular β-lactamases of different strains of B. fragilis. Enzyme activities are expressed in units per milliliter of culture supernatant, and specific activities are expressed in units per milligram, dry weight, of bacteria*

Strain	Bacterial dry weight (mg/ml)	β-lactamase (U/ml)	Activity (U/mg)
B. fragilis subsp. *vulgatus* ATCC 8482	1.34	0	0
B. fragilis subsp. *ovatus* ATCC 8483	0.85	0.005	0.006
B. fragilis subsp. *distasonis* ATCC 8503	1.00	0	0
B. fragilis subsp. *fragilis* NCTC 9343	1.74	0.004	0.002
B. fragilis subsp. *thetaiotaomicron* NCTC 10582	3.46	0.008	0.002
B. fragilis subsp. *ovatus* B4	1.91	0.050	0.026
B. fragilis subsp. *vulgatus* B70	2.70	0.184	0.068
B. fragilis subsp. *vulgatus* B72	1.32	0.044	0.033
B. fragilis subsp. *fragilis* B153	1.39	0.087	0.063

TABLE 2. *Influence of the composition of prereduced medium on the yield of cells and the production of β-lactamase by B. fragilis B70 in a stirred fermentor at pH 7.0 and 37 C*

Medium	Bacterial dry weight (mg/ml)	β-lactamase (U/ml)	Activity (U/mg)
Proteose peptone-yeast extract medium (basal medium)	2.70	0.184	0.068
Basal medium with thioglycollate, 0.5 g	2.26	0.135	0.060
Tryptone-yeast extract medium (Wahren and Holme, [33])	2.38	0.036	0.015
Peptone-yeast extract medium (Holdeman and Moore [12])	1.67	0.041	0.025
Proteose peptone-whale extract medium (Dalland and Hofstad [3])	1.68	0.085	0.051
Defined amino acid medium (Wahren and Holme [34])	1.28	0.018	0.014

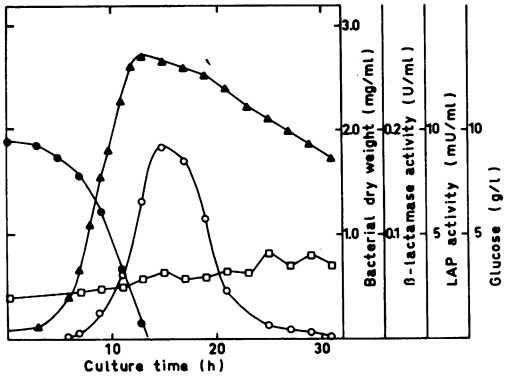

FIG. 1. *Relationship between bacterial growth and formation of extracellular β-lactamase and leucine amino-peptidase of B. fragilis B70. Cultivation was carried out in a stirred fermentor in prereduced proteose peptone medium. Symbols: ▲, bacterial dry weight; ○, β-lactamase activity; □, leucine aminopeptidase activity (LAP); ●, glucose.*

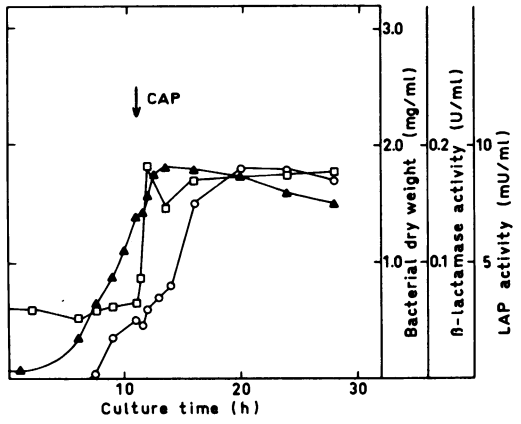

FIG. 2. *Relationship between protein synthesis and the appearance of extracellular β-lactamase and leucine aminopeptidase. Chloramphenicol (CAP) was added during the logarithmic phase of growth. For symbols, see legend to Fig. 1.*

β-lactamase. The influence of pH on the cell yield and the yield of β-lactamase was tested in the basal medium at 37 C (Fig. 3). The highest cell yield (2.7 mg, dry weight/ml) was observed at pH 7.0, which was also the pH optimum for production of extracellular β-lactamase (approximately 0.07 U/mg, dry weight of bacteria). At pH 5.5 and 8.0, a very low yield with low enzyme activity was obtained.

Influence of temperature on growth and formation of β-lactamase. The influence of temperature on the cell yield and the yield of β-lactamase was tested in the basal medium in the range 31 to 41 C at pH 7.0 (Fig. 4). The maximum cell yield (2.7 mg, dry weight/ml) was obtained at 37 C, but the difference between this and the yields at the other temperatures, 31 to 39 C, was only slight. The highest enzyme activity was obtained at 37 C (approximately 0.07 U/mg, dry weight).

Localization of enzyme activities. β-lactamases in aerobic gram-negative rods are considered by some investigators (11) to be located in the periplasmic space, as well as other enzymes (alkaline phosphatase, 3'-nucleotidase, and 5'-nucleotidase). These three enzymes were tested in parallel as reference enzymes for periplasmically located enzymes. Table 3 shows that the supernatant of the culture medium contained about 50% of the total β-lactamase activity and as much as 80 to 90% of the activity

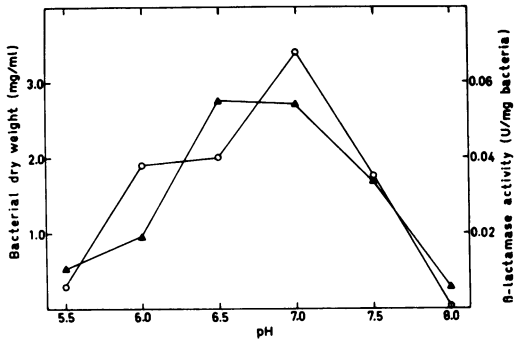

FIG. 3. *Influence of pH on growth and formation of extracellular β-lactamase of B. fragilis B70. Symbols: ▲, bacterial dry weight; ○, β-lactamase activity.*

of the other enzymes tested. Most of the remaining β-lactamase activity (~40%) was found in the cytoplasmic fraction, and ~9% was found in the washed cell wall fraction, whereas nucleotidase activity was found only in the cytoplasmic fraction, and alkaline phosphatase was found in equal amounts (~4%) in both fractions. The membrane protein fraction and lysozyme-treated cell wall fraction contained no enzymatic activities under the conditions used.

When the cells were subjected to osmotic shock, 20% of the total β-lactamase activity was released. Remaining cell-bound activity (~30%) was found in the cell lysate after disintegrating the osmotically shocked cells. Alkaline phosphatase, 3'-nucleotidase, and 5'-nucleotidase were not released by this method but were found either in the extracellular fluid (~70%) or in the cell lysate (~30%). No release of periplasmic enzymes by polymyxin B treatment occurred.

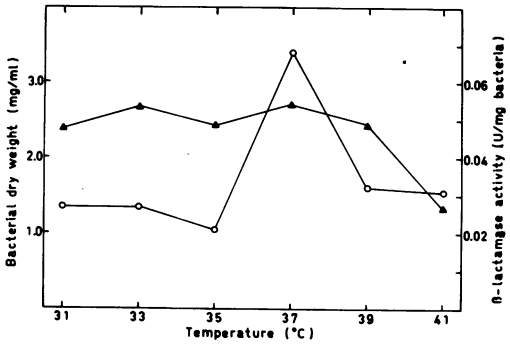

FIG. 4. *Influence of temperature on growth and formation of extracellular β-lactamase of B. fragilis B70. For symbols, see legend to Fig. 3.*

Substrate profile studies. These experiments were performed with crude enzyme preparations from the four β-lactamase-producing isolates of *B. fragilis*, B4, B70, B72, and B153.

Substrate profiles are expressed as the rate of hydrolysis of β-lactam antibiotics relative to the rate of hydrolysis of cephaloridine, which is given the value of 100. Table 4 shows the profiles for the four strains tested. Specific activities on cephaloridine expressed as units per milligram of bacterial protein are also given for the enzymes. Three of the enzymes (B4, B70, and B153) hydrolyze cephaloridine more rapidly than cephalothin, and cephalexin only to a minor extent, whereas the enzyme from B72 hydrolyzes both cephalothin and cephalexin more rapidly than cephaloridine. Hydrolysis of penicillins occurs at a very low rate, but B72 hydrolyzes both ampicillin and methicillin more rapidly than benzylpenicillin.

Inhibition studies. Inhibition of β-lactamase activity of the four preparations tested occurred with the β-lactam antibiotics used. Cefoxitin proved to be the strongest inhibitor; its stability to β-lactamase is attributed to the presence of a 7α-methoxy substituent. The other three inhibitors showed a varying degree of inhibition, but it was in no case less than 60% (Table 5).

With the sulfhydryl inhibitor PCMB, there was an inhibition of 100% for all four enzymes,

TABLE 3. *Localization of β-lactamase in B. fragilis B70 compared with alkaline phosphatase, 3'-nucleotidase, and 5'-nucleotidase. Enzyme activities are expressed as units per milliliter of culture fluid*

Sample	β-lactamase		Alkaline phosphatase		3'-Nucleotidase		5'-Nucleotidase		Protein (mg/ml)
	U/ml	%	U/ml	%	U/ml	%	U/ml	%	
Extracellular fluid	0.140	50	44.6	91	2.11	79	5.64	85	1.2
Cytoplasmic fraction	0.109	39	1.76	4	0.54	20	0.90	14	0.360
Membrane-washed fraction	0.003	1	0.44	1	0	0	0	0	0.004
Washed cell wall fraction	0.024	9	1.95	4	0.02	1	0.04	1	0.033
LiCl-treated cell wall fraction	0.002	1	0.10	<1	0	0	0	0	0.016

TABLE 4. *Specific activities and substrate profiles of β-lactamases from four strains of B. fragilis*

Strain	Hydrolysis rate[a]						
	Cephaloridine[b]	Cephalothin	Cephalexin	Penicillin G	Ampicillin	Methicillin	Cloxacillin
B4	100 (1.26)[c]	84	5	2	0.8	3	0
B70	100 (1.68)	70	6	1	0.6	2	0
B72	100 (0.11)	238	188	3	8	15	0
B153	100 (0.84)	80	12	1	0.6	3	0

[a] Rates of hydrolysis are relative to an arbitrary value of 100 for cephaloridine.
[b] Concentration of substrate in reaction mixture was 0.2 mM.
[c] Specific activities with cephaloridine as substrate expressed as units per milligram of bacterial protein.

which indicates that the enzymes have at least one cysteine residue essential for enzyme activity.

Iodine at a concentration of 0.01 mM inhibited the enzyme preparations from strains B4, B70, and B153 to 100%, but the enzyme from strain B72 was inhibited only to 65%.

Isoelectric focusing combined with zymogram. Gel electrofocusing of crude β-lactamase preparations from strains of *B. fragilis* revealed two patterns, as shown in Fig. 5. Strains B4, B70, and B153 showed identical patterns with one strong band (pI 4.9 ± 0.2). A weaker satellite band with pI of 5.3 ± 0.2 was observed for all three strains. Strain B72 showed a completely different pattern with at least three bands with pI 5.0 ± 0.2, 5.3 ± 0.2, and 5.6 ± 0.2, respectively.

Preparations from the aerobic gram-negative rods showed different and more complex patterns with one or two strong bands and several weaker satellite bands indicating microheterogeneity. No similarity between β-lactamase from *B. fragilis* and from aerobic gram-negative rods with respect to their isoelectric points could be found.

Application of samples at different points of the gel did not affect the appearance or the position of β-lactamase bands, but routinely samples were applied towards the anode.

Moleular weight. Preliminary results from the molecular weight determination by gradient polyacrylamide electrophoresis of β-lactamase from strain B70 indicated a molecular weight of 43,000. Gel filtration on Bio-Gel P-60 gave similar results, i.e., a molecular weight of 40,000 to 43,000. These values are unusually high compared with β-lactamases from aerobic gram-negative rods (20,000 to 30,000) (27).

TABLE 5. *Inhibition of β-lactamase activity on cephaloridine (0.1 mM) by different kinds of inhibitors*

Strain	Inhibition (%)					
	Cloxacillin (0.1 mM)	Carbenicillin (0.1 mM)	Methicillin (0.1 mM)	Cefoxitin (0.1 mM)	PCMB (0.5 mM)	Iodine (0.01 mM)
B4	85	72	65	100	100	100
B70	93	78	60	100	100	100
B72	100	73	60	100	100	65
B153	92	83	68	100	100	100

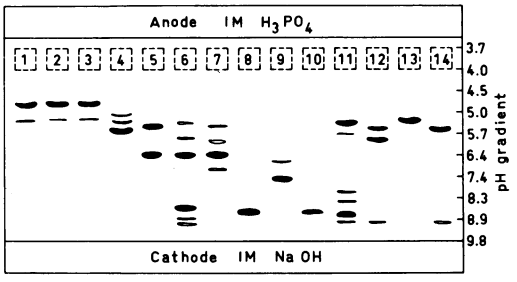

FIG. 5. *Isoelectric focusing on polyacrylamide gel of β-lactamases from different gram-negative species. Detection of enzyme activity with chromogenic cephalosporin 87/312. The following preparations were used: (1) B. fragilis B4; (2) B. fragilis B70; (3) B. fragilis B153; (4) B. fragilis B72; (5) E. coli TEM; (6) E. coli 1573E; (7) K. aerogenes 1082E; (8) E. cloacae 265A; (9) neutrapen, penicillinase from B. cereus; (10) β-lactamase from E. cloacae; (11) P. aeruginosa 18S; (12) P. aeruginosa 18R; (13) H. influenzae Beecham 4482; (14) P. aeruginosa P13. Main bands are marked as shaded areas. Open areas indicate bands with weak enzyme activity, probably due to microheterogeneity.*

DISCUSSION

B. fragilis is responsible for more than 60% of all anaerobic infections in humans (9). However, relatively little is known about the metabolism and enzymatic properties of these bacteria. Improvement in cultivation techniques for anaerobic bacteria during the last years (12) has created a new interest in these microorganisms, and reports on the growth condition of *B. fragilis* have appeared (3, 33). Other authors have studied β-lactamase production in *B. fragilis* (1, 4, 8, 26), but to our knowledge nobody has tried to optimize growth for production of β-lactamase or any other enzymes. Cultivations in stirred fermentors under strictly anaerobic conditions provide an excellent means of obtaining high yields of both cells and enzymes. Compared with cultivation in a prereduced medium without shaking and pH control, both the bacterial dry weight and the enzymatic activities increase about 10 times. This report shows that the composition of the medium, temperature, and pH are important factors for optimal growth and also that a strain with high specific activity of β-lactamase is needed for production of the enzyme in large amounts. Strain B70 seems to be such a strain, even though its specific activity of β-lactamase (~0.1 U/mg, dry weight) is relatively low when compared with many aerobic gram-negative rods. *Enterobacter* species, whose β-lactamases are inducible (10), as well as *E. coli* with R-factor-mediated β-lactamases (28), reach specific activities that are 10 to 100 times higher. Purification of the enzyme might, therefore, be difficult, and alternative ways to overcome this should be tried. Growth of the strain in continuous culture might provide a means of obtaining the enzyme in larger amounts (21).

The β-lactamase of strain B70 is neither inducible with benzylpenicillin nor with cephalothin, which is in agreement with the findings of Anderson and Sykes (1), whereas Del Bene and Farrar (4) observed increased activity in 8 of 10 strains when grown in the presence of benzylpenicillin. The enzyme is excreted into the surrounding medium during the period of active growth, a fact that cannot be explained by cell lysis when regarding leucine aminopeptidase activity which is constantly low. On the other hand, one would expect a significant rise in leucine aminopeptidase activity during the lytic phase if this enzyme is an intracellular one.

After the period of "active" release of β-lactamase, the enzyme is rapidly inactivated. This might be due to proteolytic activity, though we could detect only low activities. In the experiment with the addition of chloramphenicol, β-lactamase was not inhibited or inactivated but was found in the culture fluid after 20 h. This fact supports the theory of proteolytic activity, if the addition of chloramphenicol stopped the synthesis of proteolytic or other degradative enzymes that are normally found during the late exponential phase. Similar results with rapid decline in activity at the end of the exponential growth phase have been reported for phospholipase C in batch cultures of *Clostridium perfringens* type A (21).

Results from the studies of cellular location of β-lactamase in *B. fragilis* are not uniform as to whether the enzyme is cell-bound or periplasmically located. The release into the growth medium (~50%) suggests a rather loose association with the cell envelope, which is supported by the fact that ~20% of the activity is found in the osmotic shock fluid. One possibile explanation is that β-lactamase is synthesized in the cytoplasm and then transported to the periplasmic space, where it can easily attack all substrate molecules entering the cell through the outer membrane (27). However, the methods used in this study have previously been used only for aerobic bacteria, and, although the reference enzymes are well defined as periplasmically located in these bacteria, it is too early to draw conclusions for anaerobic bacteria. A recent report on the outer membrane complex in *B. fragilis* shows, however, that its cell envelope is typical of gram-negative bacteria (13).

It has been stated that R-factor-mediated β-lactamases in aerobic gram-negative rods are released by osmotic shock treatment (19), whereas chromosomally mediated ones are cell bound (20). One report proposes that the molecular weight of the β-lactamase should be a differentiating factor (29), so that molecules larger than 30,000 are retained within the cell, whereas smaller ones are released. Whether β-lactamase in *B. fragilis* is R-factor mediated is not yet known. Attempts have been made to show resistance transfer from *B. fragilis* to *E. coli* and vice versa and also from *B. fragilis* to other strains of this organism (1), but no detectable transfer occurred. According to the molecular weight determination of 43,000 for strain B70, the second theory does not seem applicable either.

Substrate profiles and inhibition studies were all performed with crude enzyme preparations and must, therefore, be reconfirmed with purified preparations, since inhibiting factors in the crude preparations might cause interference. The substrate profiles obtained for strains B4, B70, and B153, which seem almost identical, resemble those found by Del Bene and Farrar (4), and the profile for strain B72 could also be found among their strains. They found no penicillin-hydrolyzing activity in any of their strains, however, whereas we obtained low rates of hydrolysis for all four isolates. This might be explained by their shorter cultivation time and lower MIC values, producing hydrolyzing activity too low to be measurable. Also, the fact that iodine at very low concentrations inhibits enzyme activity should be considered, since this makes iodometric assays unsuitable for detection of β-lactamase from *B. fragilis*. Sensitivity to iodine is believed to be a common feature of β-lactamases from gram-negative species, although it has not been investigated thoroughly (27). However, the extreme sensitivity exhibited by *B. fragilis* β-lactamases has never been reported before. The inhibitory effect of PCMB is in agreement with the results of Del Bene and Farrar (4).

Isoelectric focusing of *B. fragilis* β-lactamases confirms that these enzymes are a kind of β-lactamases that does not fit in the classification scheme (27) worked out for aerobic gram-negative rods. Enzymes that are mainly cephalosporinases are referred to as class I enzymes according to this scheme. Class I enzymes are inhibited by cloxacillin but not by PCMB and have high pI values (~8.2) (16). *B. fragilis* β-lactamases are inhibited both by cloxacillin and PCMB and have a pI value of 4.9 (5.6 for strain B72). Thus, it seems that their properties are unique among β-lactamases described so far. Therefore, we would like to refer to them as a new class of β-lactamases. Further studies of their immunological properties and amino acid composition will reveal whether there are any similarities between these β-lactamases and those derived from aerobic strains.

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