ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1986, p. 645–648
0066-4804/86/110645-04$02.00/0

Vol. 30, No. 5

β-Lactamase-Mediated Imipenem Resistance in *Bacteroides fragilis*

GEORGE J. CUCHURAL, JR.,[1]* MICHAEL H. MALAMY,[2] AND FRANCIS P. TALLY[1,3]

Infectious Disease Service, Department of Medicine,[1] and Clinical Microbiology Laboratory, Department of Pathology,[3] Tufts-New England Medical Center Hospital, and the Department of Molecular Biology, Tufts University School of Medicine,[2] Boston, Massachusetts 02111

Received 20 February 1986/Accepted 6 August 1986

Imipenem has excellent antimicrobial activity owing in part to β-lactamase stability. We found that only 2 of over 350 *Bacteroides fragilis* group clinical isolates were resistant to imipenem, with an MIC of more than 16 μg/ml. These two isolates from the Tufts Anaerobe Laboratory (TAL) were resistant to all other β-lactam agents tested. The organisms were able to inactivate imipenem in broth cultures and contained similar β-lactamases that were able to hydrolyze carbapenems, cephamycins, cephalosporins, and penicillins. The molecular sizes of the β-lactamases in TAL2480 and TAL3636 were estimated to be 44,000 daltons. The novel β-lactamase contained Zn^{2+} as a cofactor. An additional factor contributing to resistance was determined. The outer membranes of these two organisms were found to limit free diffusion of the drugs into the periplasm. This novel β-lactamase, associated with a barrier to drug permeation, resulted in high-grade β-lactam drug resistance.

Bacteroides fragilis is the most important anaerobic bacterial pathogen of humans. Clinical isolates generally contain β-lactamase and as a result are frequently resistant to the cephalosporins, including the broad-spectrum cephalosporins, as well as penicillins (2, 19). Only cephamycins, such as cefoxitin, and carbapenems, such as imipenem, are resistant to hydrolysis by the frequently described *B. fragilis* β-lactamase (2, 5, 14). Resistance to imipenem is rare in *B. fragilis*. We found that only 2 of over 350 isolates tested were resistant to imipenem. We investigated the β-lactamase activity in these two strains.

MATERIALS AND METHODS

Antimicrobial drugs. Standard powders were obtained from various manufacturers (19).

Isolates. Bacterial identification was performed by the established method (9). Tufts Anaerobe Laboratory (TAL) isolate 2480 was isolated from the blood of a patient at the New England Deaconess Hospital, Boston, Mass. The patient died of uncontrolled sepsis while receiving cefoxitin and clindamycin. TAL3636 was isolated from a patient at the New England Medical Center Hospitals, Boston, Mass. This diabetic patient failed to respond to cefoxitin therapy and required an amputation for a foot infection.

Media and incubation. The isolates were transported, stored, and incubated as previously described (10, 19). Brain heart infusion broth (Scott Laboratories, Inc., Fiskeville, R.I.) supplemented with 0.0005% hemin and 0.5% yeast extract (BHIS) was used for the liquid growth medium. BHIS was solidified with 1.5% agar. Blood agar plates consisted of BHIS plates with 5% defibrinated sheep blood.

Susceptibility testing. The MICs were determined by an agar dilution method using a Steers replicator and anaerobic chamber techniques previously described (4, 19, 20). The MICs and MBCs were also determined by microtiter methods (20). The inoculum effect on the MIC and MBC was investigated by increasing the initial inoculum from 10^6 to 10^8 CFU/ml. This represents 5×10^4 and 5×10^6 CFU per well for microtiter testing and 3×10^3 and 3×10^5 CFU per spot for agar dilution testing. Induction of resistance was assayed by growing the organisms in broth medium with and without imipenem (5 μg/ml), cefoxitin (5 μg/ml), or ampicillin (20 μg/ml). The organisms were exposed to the drugs in both overnight and 4-h incubations.

β-Lactamase induction. β-Lactamase induction was tested by incubating isolates in BHIS with and without cefoxitin (5 μg/ml) or ampicillin (20 μg/ml). Serial samples were obtained hourly for 8 h after the addition of the drug. Whole and toluene-treated cells were assayed for β-lactamase-specific activity by using nitrocefin as described below.

Drug assay. The cefoxitin concentration in BHIS was measured by both high-pressure liquid chromatography and bioassays (5). The concentrations of SCH34343, imipenem, and aztreonam in broth cultures were determined by a bioassay identical to the method used for cefoxitin.

β-Lactamase assay. The β-lactamase activity of the crude enzyme extracts and purified fractions was determined by a spectrophotometric technique (11, 13). Drug destruction was monitored at the following wavelengths: cefoxitin, 261 nm; nitrocefin, 482 nm; cephaloridine, 255 nm; moxalactam, 270 nm; imipenem, 297 nm; cefotaxime, 267 nm; cephalothin, 265 nm (3, 11, 13, 16, 22). Penicillin hydrolysis was assayed by spectrophotometry at 236 nm (11). The reactions were monitored in a dual-beam spectrophotometer (Coleman 124; The Perkin-Elmer Corp., Maywood, Ill.) maintained at a temperature of 30°C. One unit of β-lactamase activity was defined as that amount of enzyme that can destroy 1 μmol of drug per min in the initial linear phase of the reaction. β-Lactamase-specific activity of a sample was calculated by dividing the β-lactamase activity by the amount of protein (units per milligram) in the reaction mixture. Michaelis-Menten constants were calculated from initial reaction velocities by the method of least-squares using at least eight concentrations of substrate (Lineweaver-Burk plots). Concentrations (1 mM) of aztreonam, iodine, *p*-chloromercuribenzoate, and clavulanic acid and sulbactam and various concentrations of EDTA and 1,10-phenanthroline were incubated with the purified enzyme for 5 min at 22°C and tested as inhibitors of the β-lactamase reaction with nitrocefin (100 μM) as the substrate.

* Corresponding author.

TABLE 1. Susceptibility of imipenem-resistant *B. fragilis* isolates[a]

Isolate	MIC (μg/ml)[b]				
	Imipenem	SCH34343	Cefoxitin	Moxalactam	Piperacillin
2480	128	16	128	>128	>128
3636	128	16	128	>128	>128
Control[c]	0.5	<0.12	16	4	8

[a] MICs of cefazolin, cefamandole, cefotaxime, cefoperazone, cefotetan, aztreonam, penicillin, ampicillin, ticarcillin, and carbenicillin for both isolates were >128 μg/ml.
[b] MICs were determined by the agar dilution method.
[c] Control MICs are the median values for 350 *B. fragilis* clinical isolates.

β-Lactamase preparation. Cell suspensions were obtained by growing the isolates in 20 ml of BHIS overnight and washing the cells three times with 0.1 M potassium phosphate buffer (pH 7). Intact cells, cells treated with 1% (vol/vol) toluene for 30 min at 22°C, and cells sonicated with a Branson sonicator (Branson Sonic Power Co., Danbury, Conn.) for 2 min at a power setting of 7 with 50% duty cycle in an ice slush bath were used in this study. Clarified crude enzyme solutions were obtained from cell sonic extracts after centrifugation to remove cell debris. Freezing or prolonged exposure to room temperature resulted in the loss of enzyme activity. Therefore, fresh enzyme samples were obtained for each series of experiments and were maintained in an ice slush bath before use.

β-Lactamase crypticity measurement. β-Lactamase crypticity was defined as the ratio of the activity of the disrupted cells to that of the intact cells. The cells were disrupted by sonication for 3 min by using a Branson sonicator or by treatment with 1% toluene for at least 30 min.

β-Lactamase purification. A clarified crude enzyme solution was obtained after sonication of cells obtained from 1- or 2-liter cultures of TAL240 harvested in late-exponential-growth phase and was used as the starting material for purification. The crude enzyme solution was subjected to ammonium sulfate precipitation, and the fraction that was precipitated between 50 and 70% saturation was retained. This sample was applied to a Sephadex G-75 gel-filtration column equilibrated with 0.01 M potassium phosphate buffer (pH 8) and eluted with the same buffer. Active fractions were applied to a DEAE-DE52-cellulose-anion-exchange column (Whatman Ltd., Kent, England) equilibrated with 0.01 M potassium phosphate buffer (pH 8). Elution of active fractions from this column was performed by maintaining a constant potassium phosphate concentration of 0.01 M (pH 8) and by increasing NaCl concentrations. The starting NaCl concentration in the elution buffer was adjusted to 0.05 M NaCl. A linear gradient from 0.05 to 0.1 M NaCl was used to elute the active fractions. β-Lactamase activity of the fractions was assayed by using nitrocefin and cefoxitin as substrates. All purification steps were performed at 4°C.

Protein concentrations. Protein concentrations were determined by the Lowry method or by measuring optical density at 280 nm when appropriate. The protein content of the cell samples was determined by a modified Lowry technique that allowed the solubilization of protein from the whole cells (7).

Isoelectric focusing. Isoelectric focusing of β-lactamase-containing samples (clarified crude sonic extracts) was performed with prepared polyacrylamide gel plates (Ampholine Pagplate; pH gradient 3.5 to 9.5; LKB Stockholm, Bromma, Sweden). Electrofocusing was done at 4°C for 4 h at an average power of 5 W. TEM-2 β-lactamase, sonic extracts from other β-lactamase-containing *B. fragilis* isolates, and the pigmented proteins myoglobin and cytochrome *c* were used as controls. The pH gradient was determined by a surface electrode with measurements taken at 1-cm intervals. β-Lactamase-containing bands were detected by overlaying the gel with a solution of 500 μg of nitrocefin per ml. The gel was observed for the development of red bands. The coordinates of the active bands were recorded.

Molecular sizes. The molecular sizes of β-lactamases of TAL2480 and TAL3636 were estimated by using a Sephadex G-75 gel filtration column calibrated with five proteins of known molecular sizes ranging from 12,300 to 97,400 daltons (7).

RESULTS

Susceptibility. The two imipenem-resistant isolates, TAL2480 and TAL3636, were found to be highly resistant to all other β-lactam drugs tested. The most active drugs for the control isolates are shown in Table 1. This resistance included cephalosporins, including broad-spectrum cephalosporins, carbapenems, penicillins, and monobactams. No induction to higher levels of resistance was found when the two isolates were preincubated in the presence of cefoxitin, ampicillin, or imipenem. Increasing the inoculum from 10^6 to 10^8 resulted in twofold higher MICs and MBCs when tested with imipenem, ampicillin, and cefoxitin (data not shown).

Broth inactivation. Both TAL2480 and TAL3636 were able to rapidly inactivate imipenem and cefoxitin in broth culture. Less than 1% of the initial 50 μg of imipenem per ml remained 8 h after incubation with either TAL2480 or TAL3636. At 8 h, 66% of the drug remained in the control tube. The controls consisted of the drug in broth with a susceptible *B. fragilis* isolate or drug in broth alone. Likewise, at 24 h, less than 1% of the initial 100 μg of cefoxitin per ml remained after incubation with TAL2480 or TAL3636 compared with 81% in the controls. SCH34343 was also destroyed but aztreonam was not destroyed in broth cultures under the same conditions.

β-Lactamase content. No induction to higher levels of cellular β-lactamase content was detected when broth cultures of TAL2480 or TAL3636 were exposed to either 20 μg of ampicillin or 5 μg of cefoxitin per ml during an 8-h period.

β-Lactamase physical characteristics. The β-lactamases of TAL2480 and TAL3636 had the same estimated molecular size of 44,000 daltons. The β-lactamases failed to focus well enough to establish a reliable pI, in contrast to control samples containing TEM-1 or the β-lactamase from other *B. fragilis* isolates. The optimal pH of β-lactamase activity of the purified TAL2480 enzyme was 7.0. The enzyme maintained 50% of maximal activity between pH 6.0 and 7.7. The optimum temperature for the β-lactamase reaction was 36°C. Freezing of the enzyme samples resulted in >99% loss of activity.

β-Lactamase purification and substrate profile. The β-

TABLE 2. Partial purification of β-lactamase of TAL2480

Step	Purification (fold)	% β-Lactamase activity recovered[a]
Sonicated cells		100
50–70% Ammonium sulfate fraction	4	95
Sephadex G-75 gel filtration	49	89
DEAE DE52 cellulose-anion exchange	162	37

[a] β-Lactamase activity was assayed with either nitrocefin or cefoxitin.

lactamase of TAL2480 was partially purified (Table 2). The purest sample had four contaminating bands seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fractions were assayed for both cefoxitin- and nitrocefin-degrading activity throughout the purification procedure. Both activities copurified. The kinetic parameters of the partially purified TAL2480 enzyme were determined (Table 3). The enzyme hydrolyzes most β-lactams. The substrates that are usually considered more stable to *B. fragilis* β-lactamases, cefoxitin and moxalactam, had higher K_ms and modestly lower relative V_{max}s compared with the older cephalosporins. Imipenem was hydrolyzed more rapidly than moxalactam or cefoxitin were. These results suggest a broad substrate profile for this enzyme.

There was no significant difference in the substrate profiles between the purified and crude enzymes of TAL2480. The substrate profiles of the clarified crude lysates of TAL2480 and TAL3636 were the same.

Inhibitors. The partially purified β-lactamase of TAL2480 was not inhibited by 1 mM clavulanic acid or sulbactam. It was inhibited by 1 mM iodide, dithiothreitol, *p*-chloromercuribenzoate, EDTA, and 1,10-phenanthroline (less than 1% activity remained). After a 10-fold dilution of the EDTA-treated enzyme, inhibition was not reversed by Mn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, or Ni^{2+}; however, 1 mM Zn^{2+} or Co^{2+} completely reversed the inhibition. Aztreonam at a concentration of 1 mM did not inhibit nitrocefin hydrolysis.

β-Lactamase crypticity. The crypticity values for imipenem, cefoxitin, and nitrocefin varied from 14 to 40 (Table 4).

DISCUSSION

The resistance of *B. fragilis* to penicillins and cephalosporins is mediated primarily by the chromosomal β-lactamases present in most isolates (14, 15, 23). The activity of cefoxitin and imipenem against these organisms is largely due to resistance of the drug to the frequently described β-lactamases (2). Our data indicated that *B. fragilis* acquired a new class of β-lactamase that inactivates a wide range of β-lactam substrates usually considered stable to hydrolysis, including cephamycins and carbapenems. The β-lactamase inhibition studies suggested that this β-lactamase contains Zn^{2+} as a cofactor essential for catalysis and that Co^{2+} may substitute for Zn^{2+}. Other β-lactamases containing Zn^{2+} have been described, and β-lactamase II of *Bacillus cereus* has been extensively studied (1, 8). This β-lactamase does have the ability to hydrolyze imipenem (Harold Neu, personal communication). *B. cereus* β-lactamase II differs from non-zinc-containing enzymes in many respects, including enzymatic mechanism, molecular size, and lack of amino acid sequence homology (1, 8). β-Lactamases produced by *Pseudomonas maltophilia* and *Flavobacterium odoratum* have also been shown to inactive imipenem and to be zinc containing (16, 17). It is of great interest to define the relationship of the TAL2480 enzyme to other zinc-containing β-lactamases. The inability of aztreonam to inhibit nitrocefin hydrolysis and the lack of inactivation in broth incubations suggest that aztreonam does not have great affinity for the β-lactamase. *B. fragilis* is intrinsically resistant to the monobactams owing to the poor affinity of penicillin-binding proteins for aztreonam (6). The β-lactamases of TAL2480 and TAL3636 are similar to the frequently described *B. fragilis* β-lactamases in that they also are cell bound and expressed constitutively (15).

TABLE 3. Kinetic parameters of purified β-lactamase from TAL2480

Drug	K_m (μM)	V_{rel}[a]
Cefoxitin	102	0.23
Moxalactam	160	0.75
Cephaloridine	30	1.0
Cefotaxime	15	0.43
Imipenem	190	2.1
Cephalothin	13	0.5
Nitrocefin	19	3.6
Mezlocillin	ND[b]	1.32
Ampicillin	ND	2.6
Carbenicillin	ND	3.0
Penicillin G	ND	3.3
Azlocillin	ND	5.6

[a] V_{rel} represents relative the V_{max} of drug compared with the V_{max} of cephaloridine for cephalosporins. Penicillin hydrolysis rates were determined at a concentration of 1,000 μM only, and V_{rel} represents the velocity of the penicillin hydrolysis relative to the V_{max} of cephaloridine.

[b] ND, Not determined.

TABLE 4. β-Lactamase crypticity

Drug (concn, 100 μM)	Ratio of velocity of drug inactivation in broken/intact cells in isolate	
	TAL2480	TAL3636
Imipenem	18	14
Cefoxitin	22	30
Nitrocefin	40	35

Other laboratories have recently reported novel β-lactamase activity in *B. fragilis*. Sato et al. reported an enzyme which primarily acts as a penicillinase (18). Yotsuji et al. reported a broad-spectrum β-lactamase that has a substrate profile similar to those of the TAL2480 and TAL3636 enzymes (23). However, their isolate is probably different in that it is susceptible to piperacillin and imipenem. EDTA inhibition was not mentioned in their report.

Outer membrane permeability to antibiotics can be an integral component of β-lactam drug resistance in gram-negative bacteria. β-Lactam drug hydrolysis in the periplasmic space coupled with limited permeability and, thus, limited drug influx can interact synergistically to maintain low levels of drug near the penicillin-binding proteins (12, 21, 24). Crypticity values are inversely related to degree of permeability and are greater than 1 when the drug permeation is the rate-limiting step for β-lactam hydrolysis. The values of 14 to 40 do suggest a degree of inhibition of drug penetration through the outer membrane for imipenem, cefoxitin, and nitrocefin in these isolates. Olsson et al. showed previously that limited outer membrane permeability in *B. fragilis* may contribute to β-lactam drug resistance (14).

The discovery of this new resistance to a broad range of β-lactam drugs in *B. fragilis* is clinically relevant. These isolates were fully pathogenic and were recovered from human infections. β-Lactam drug resistance in *B. fragilis* is a dynamic process and may respond to selection pressure, as shown by outbreaks and clustering of cefoxitin resistance in various clinical centers (4). Increased use of β-lactamase-stable compounds will result in new strategies by organisms to overcome the drug challenge. β-Lactam drug resistance in

B. fragilis will probably continue to be a problem in the foreseeable future.

ACKNOWLEDGMENTS

These studies were supported in part by Public Health Service grant 5 KO4-AI00393-05 from the National Institute for Allergy and Infectious Diseases and research career development award 2 RO1-AI19497-04 A1, both to F.P.T.

We thank Nilda V. Jacobus for her excellent technical assistance.

LITERATURE CITED

1. **Baldwin, G. S., A. Goldes, H. A. Hill, S. G. Waley, and E. P. Abraham.** 1980. A spectroscopic study of metal ion and ligand binding to beta-lactamase II. J. Inorg. Biochem. **13**:189–204.
2. **Brown, J. E., V. E. Del Bene, and C. D. Collins.** 1981. In vitro activity of *N*-formimidoyl thienamycin, moxalactam, and other new beta-lactam agents against *Bacteroides fragilis*: contribution of beta-lactamase to resistance. Antimicrob. Agents Chemother. **19**:248–252.
3. **Bush, K., J. S. Freudenberger, and R. B. Sykes.** 1982. Interaction of azthreonam and related monobactams with β-lactamases from gram-negative bacteria. Antimicrob. Agents Chemother. **22**:414–420.
4. **Cuchural, G. J., Jr., F. P. Tally, N. V. Jacobus, S. L. Gorbach, K. Aldridge, T. Cleary, S. M. Finegold, G. Hill, P. Iannini, J. P. O'Keefe, and C. Pierson.** 1984. Antimicrobial susceptibilities of 1,292 isolates of the *Bacteroides fragilis* group in the United States: comparison of 1981 with 1982. Antimicrob. Agents Chemother. **26**:145–148.
5. **Cuchural, G. J., Jr., F. P. Tally, N. V. Jacobus, P. K. Marsh, and J. W. Mayhew.** 1983. Cefoxitin inactivation by *Bacteroides fragilis*. Antimicrob. Agents Chemother. **24**:936–940.
6. **Georgopapadakou, N. H., S. A. Smith, and R. B. Sykes.** 1983. Penicillin binding proteins in *Bacteroides fragilis*. J. Antibiot. **36**:907–910.
7. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
8. **Hill, H. A., P. G. Sammes, and S. G. Waley.** 1980. Active-sites of beta-lactamases from *Bacillus cereus*. Philos. Trans. R. Soc. London Ser. B1 **289**:333–344.
9. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
10. **Marsh, P. K., M. H. Malamy, M. J. Schimell, and F. P. Tally.** 1983. Sequence homology of clindamycin resistance determinants in clinical isolates of *Bacteroides* spp. Antimicrob. Agents Chemother. **23**:726–730.
11. **Neu, H.** 1986. Antibiotic inactivating enzymes and bacterial resistance, p. 757–789. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 2nd ed. The Williams & Wilkins Co., Baltimore.
12. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1–32.
13. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
14. **Olsson, B., K. Dornbusch, and C. E. Nord.** 1979. Factors contributing to resistance to beta-lactam antibiotics in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **15**:263–268.
15. **Olsson, B., C.-E. Nord, and J. Wadstrom.** 1976. Formation of β-lactamase in *Bacteroides fragilis* cell bound and extracellular activity. Antimicrob. Agents Chemother. **9**:727–735.
16. **Saino, Y., F. Kobayashi, M. Inoue, and S. Mitsuhashi.** 1982. Purification and properties of inducible penicillin β-lactamase isolated from *Pseudomonas maltophilia*. Antimicrob. Agents Chemother. **22**:564–570.
17. **Sato, K., T. Fujii, R. Okamoto, M. Inoue, and S. Mitsuhashi.** 1985. Biochemical properties of β-lactamase produced by *Flavobacterium odoratum*. Antimicrob. Agents Chemother. **27**:612–614.
18. **Sato, K., Y. Matsuura, M. Inoue, and S. Mitsuhashi.** 1982. Properties of a new penicillinase type produced by *Bacteroides fragilis*. Antimicrob. Agents Chemother. **22**:579–584.
19. **Tally, F. P., G. J. Cuchural, N. V. Jacobus, S. L. Gorbach, K. E. Aldridge, T. J. Cleary, S. M. Finegold, G. B. Hill, P. B. Iannini, R. V. McCloskey, J. P. O'Keefe, and C. L. Pierson.** 1983. Susceptibility of the *Bacteroides fragilis* group in the United States in 1981. Antimicrob. Agents Chemother. **23**:536–540.
20. **Tally, F. P., N. V. Jacobus, J. G. Bartlett, and S. L. Gorbach.** 1975. Susceptibility of anaerobes to cefoxitin and cephalosporins. Antimicrob. Agents Chemother. **7**:128–132.
21. **Vu, H., and H. Nikaido.** 1985. Role of β-lactam hydrolysis in the mechanism of resistance of a β-lactamase-constitutive *Enterobacter cloacae* strain to expanded-spectrum β-lactams. Antimicrob. Agents Chemother. **27**:393–398.
22. **Waley, S.** 1974. A spectrophotometric assay of β-lactamase action on penicillins. Biochem. J. **139**:789–790.
23. **Yotsuji, A., S. Minami, M. Inoue, and S. Mitsuhashi.** 1983. Properties of novel β-lactamase produced by *Bacteroides fragilis*. Antimicrob. Agents Chemother. **24**:925–929.
24. **Zimmermann, W., and A. Rosselet.** 1977. Function of the outer membrane of *Escherichia coli* as a permeability barrier to β-lactam antibiotics. Antimicrob. Agents Chemother. **12**: 368–372.