THE β-LACTAMASES OF GENUS BACTEROIDES

MASAZO TAJIMA,* KAKUYO SAWA, KUNITOMO WATANABE and KAZUE UENO

Institute of Anaerobic Bacteriology, Gifu University School of Medicine, 40 Tsukasa-Machi, Gifu-Shi, Gifu 500, Japan

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Two hundred anaerobic isolates from clinical specimens were examined for β-lactamase production by Nitrocefin methodology. Altogether, 77 strains were β-lactamase producers. Organisms belonging to *Bacteroides melaninogenicus* group, *i.e.*, *B. intermedius* and *B. bivius*, were found to have a significant frequency of β-lactamase production. Substrate profile and sensitivity to the β-lactamase inhibitors, sulbactam, clavulanic acid, cloxacillin, and cefmetazole, a cephamycin derivative, were determined for these enzymes. All enzymes hydrolyzed cephalosporins more rapidly than penicillins. Cefmetazole was not hydrolyzed at all. Strains of *B. fragilis*, *B. thetaiotaomicron*, *B. uniformis*, *B. ovatus* and *B. oralis* produced β-lactamases sensitive to all four inhibitors. Strains of *B. intermedius*, *B. bivius* and *B. disiens* produced enzymes of different nature which were inhibited only by cefmetazole. *B. vulgatus* enzyme was inhibited by three of the four inhibitors. These results suggest that the β-lactamases of the genus Bacteroides may be classified by substrate profile and inhibitor pattern.

The β-lactamase activity in Bacteroides species was first reported by GARROD in 1955.[1] He suspected penicillinase production in two strains of *Bacteroides fragilis*. PINKUS *et al.*[2] also reported on penicillinase activity in these strains. KANAZAWA *et al.*[3] reported both penicillinase and cephalosporinase activities in *B. thetaiotaomicron*. The β-lactamse of *B. fragilis* was characterized devotedly by DEL BENE and FARRAR[4] and ANDERSON and SYKES[5] in 1973. Thereafter, the enzymes from Bacteroides species have been characterized,[6,7,8] using the following parameters: molecular weight, isoelectric point, substrate profile, and sensitivity to β-lactamase-inhibitors, such as pCMB, clavulanic acid and cefoxitin. The purpose of the present study was to determine the incidence of β-lactamase-producing strains among Bacteroides species from clinical specimens and to characterize these enzymes by a study of their substrate profile and sensitivity to β-lactamase-inhibitors.

Materials and Methods

Bacterial Strains and Identification

A total of 200 clinical anaerobic isolates tested are listed in Table 1. Isolates were identified by the criteria of the VPI manual[9] in the Institute of Anaerobic Bacteriology, Gifu University School of Medicine.

Antibiotics

The β-lactam antibiotics used (see Results) were gifts from the respective manufacturers.

Detection of β-Lactamase

The method used routinely for detection of β-lactamase activity was the cell suspension method with Nitrocefin,[10] a chromogenic cephalosporin as the substrate. The cell suspensions were prepared from cells grown on GAM (Gifu anaerobic medium; Nissui Seiyaku Co., Ltd., Tokyo) agar plates supple-

* Present address: Biological Research Laboratories, Sankyo Co., Ltd., 1–2–58, Hiromachi, Shinagawa-ku, Tokyo 140, Japan

mented with vitamin K. An aliquot (50 μl) of cell suspensions was placed in a well of a spot plate containing 50 μl of Nitrocefin solution (500 μg/ml). The plate was incubated at room temperature (25~28°C). Readings for color change were performed at 10 minutes and 30 minutes. The rate of β-lactamase activity was expressed semiquantitatively as following: − no color change at 30 minutes, + orange at 30 minutes, ++ red at 30 minutes and +++ red at 10 minutes.

Preparation of Crude β-Lactamase

Organisms were grown in Trypto-Soy broth (250 ml; Eiken Chemical Co., Ltd., Tokyo) supplemented with vitamin K in 500 ml flask. Broths were inoculated with several colonies grown on the GAM agar plate and incubated in an anaerobic chamber (Model 1024, Forma Scientific; Ohio, U. S. A.) at 35°C. After 24 hours, the bacteria were harvested by centrifugation and washed once with 0.1 M phosphate buffer, pH 7.5. Packed cells were resuspended in 10 ml of the same buffer, and disrupted by ultrasound as previously described[11] and the cell-free extract was retained as the crude enzyme preparation. Protein was determined by the LOWRY method[12].

Assay of β-Lactamase

Hydrolysis of β-lactam antibiotics was assayed spectrophotometrically[11,13,14] by measuring the decrease in absorbance at the substrate specific wavelength at 30°C in 3 ml of 0.01 M phosphate buffer, pH 7.5. One unit of β-lactamase activity was defined as the amount catalyzing the hydrolysis of 1 μmole substrate per minute at 30°C. Activities are expressed as units per mg enzyme protein.

Substrate Profiles

Substrate profiles were determined with cephaloridine, cefazolin, cephalothin, cefamandole, cefuroxime, cefmenoxime, ceftizoxime, cefmetazole and ampicillin. The rate of hydrolysis of each antibiotic was calculated relative to the rate of hydrolysis of cephaloridine which was given an arbitrary value of 100.

Sensitivity to β-Lactamase-inhibitors

Cephaloridine (100 μM) was used as the substrate and cloxacillin, clavulanic acid, sulbactam and cefmetazole were tested as inhibitors (10 μM). The substrate and an inhibitor were incubated simultaneously. The rates of cephaloridine hydrolysis with and without the inhibitor were compared to determine the sensitivity of the enzymes to the β-lactamase-inhibitors.

Results

β-Lactamase Producers in Genus Bacteroides

The species of genus Bacteroides examined and their β-lactamase activity assayed with Nitrocefin are listed in Table 1. Of the total, 77 (38.5%) of 200 Bacteroides isolates produced β-lactamase. These included 32 of 107 *B. fragilis*, 5 of 21 *B. thetaiotaomicron*, 21 of 27 *B. bivius*, and 19 of 45 other Bacteroides species.

As shown in Table 1 and Fig. 1, the *B. melaninogenicus* group showed a higher frequency of the β-lactamase production than the *B. fragilis* group, and in addition produced larger amount of β-lactamase. *B. distasonis* and *B. asaccharolyticus* produced no detectable β-lactamase activity.

Substrate Profile

The rates of hydrolysis of the cephalosporins, a cephamycin and a penicillin are shown in Table 2 and are presented as histograms for each group in Fig. 2. All β-lactamases tested in the Bacteroides species have a cephalosporinase character. They hydrolyzed cephalosporins, including cefamandole, cefuroxime and cefmenoxime more rapidly than ampicillin. The β-lactamases from two strains each of *B. fragilis* and *B. ovatus* were not significantly different and so were included in one group (profile 1 in Fig. 2). The β-lactamases from *B. thetaiotaomicron* and *B. vulgatus* were different from each other and also from other groups (profile 2, 3). *B. uniformis* had a unique substrate profile (profile 4). The

Fig. 1. Frequency of β-lactamase-producing isolates among Bacteroides species.

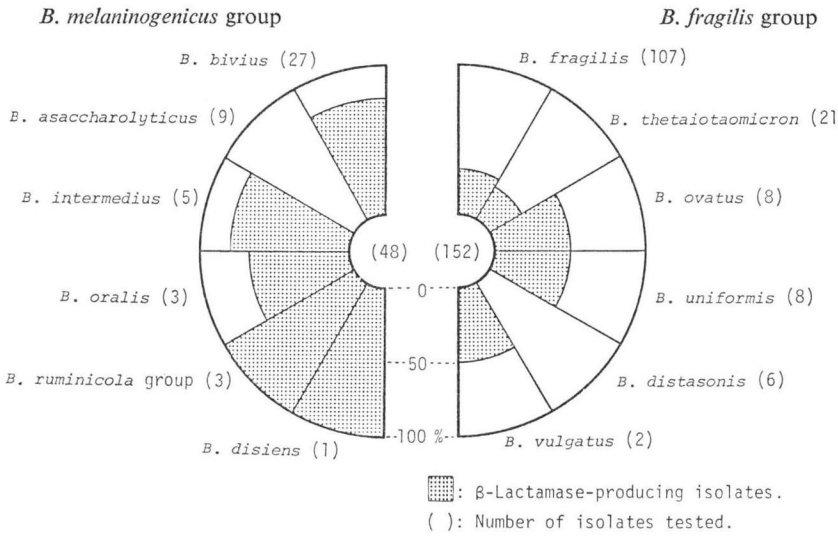

Table 1. β-Lactamase activity in Bacteroides strains isolated from clinical specimens.

Species	No. of tested	No. of positive	β-Lactamase activity*				% of positive
			+++	++	+	−	
B. fragilis	107	32	1	2	29	75	29.9
B. distasonis	6	0				6	0
B. ovatus	8	4			4	4	50
B. thetaiotaomicron	21	5	1	1	3	16	23.8
B. vulgatus	2	1	1			1	50
B. uniformis	8	4		1	3	4	50
B. oralis	3	2			2	1	66.7
B. asaccharolyticus	9	0				9	0
B. intermedius	5	4	4			1	80
B. disiens	1	1		1			100
B. ruminicola	3	3	1	1	1		100
B. bivius	27	21	8	6	7	6	77.8
Total	200	77	16	12	49	123	38.5

* −, No color change at 30 minutes; +, orange at 30 minutes; ++, red at 30 minutes; +++, red at 10 minutes.

β-lactamase from *B. oralis* was different from *B. fragilis* and *B. ovatus* β-lactamases (profile 5). The β-lactamases from two strains of *B. intermedius* (profile 6) were different from two *B. bivius* β-lactamases (profile 8). The profiles of β-lactamase from *B. disiens* and a strain of *B. bivius* (ATCC 29303) were not significantly different and were grouped together (profile 7). The inability to hydrolyze cefmetazole and low or no hydrolysis of ampicillin was common to all groups.

Sensitivity to β-Lactamase-inhibitors

The sensitivities to inhibitors of β-lactamases from eight species of Bacteroides are shown in Table 3. The enzymes from *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron* and *B. oralis* were sensitive to all four in-

Fig. 2. Substrate profiles of β-lactamases from nine species of Bacteroides.
CER Cephaloridine, CEZ cefazolin, CET cephalothin, CMD cefamandole, CXM cefuroxime, CMX cefmenoxime, CZX ceftizoxime, CMZ cefmetazole, ABPC ampicillin.

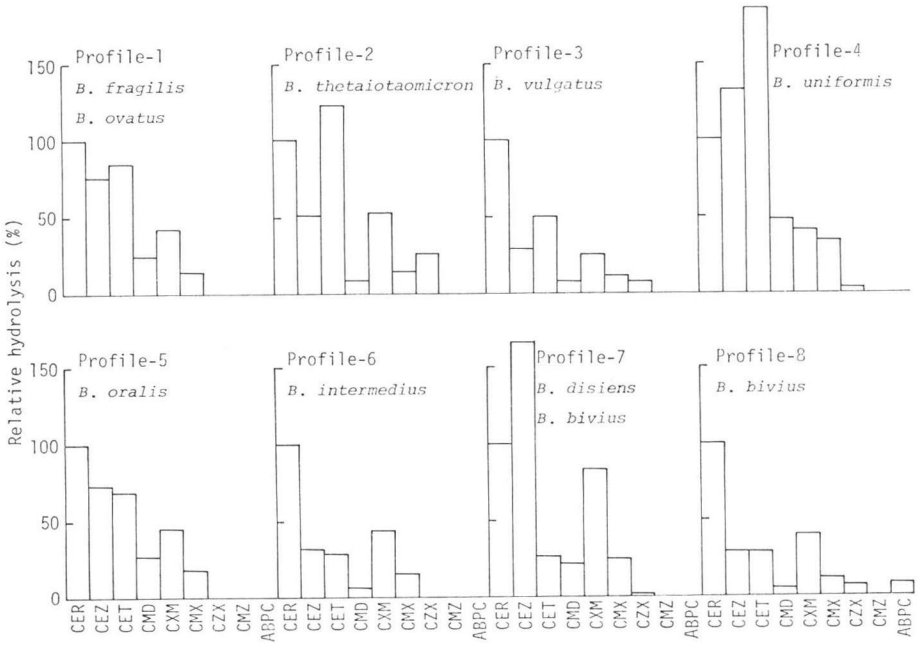

Table 2. Relative hydrolysis by Bacteroides β-lactamases of β-lactam antibiotics.

Organism	Specific activity*	Relative hydrolysis (%)									Profile
		CER	CEZ	CET	CMD	CXM	CMX	CZX	CMZ	ABPC	
B. fragilis GAI3025	1.085	100	59	63	24	37	15	0	0	0	1
B. ovatus GAI3513	0.095	100	90	102	25	47	15	0	0	0	1
B. thetaiotaomicron GAI2140	0.062	100	51	122	8	53	15	26	0	0	2
B. vulgatus GAI2303	0.340	100	28	49	4	25	11	4	0	0	3
B. uniformis GAI3510	0.007	100	132	186	48	41	34	3	0	0	4
B. oralis GAI3674	0.173	100	74	68	26	44	18	0	0	0	5
B. intermedius GAI4874	0.251	100	34	30	6	45	18	0	0	0	6
B. intermedius GAI4913	0.154	100	27	27	6	43	12	0	0	0	6
B. disiens GAI2900	0.071	100	204	28	27	96	26	0	0	0	7
B. bivius ATCC29303	0.581	100	137	23	15	69	21	5	0	0	7
B. bivius GAI1834	0.462	100	29	29	6	46	13	6	0	11	8
B. bivius GAI4100	0.519	100	23	22	4	33	11	8	0	6	8

* Specific activity was determined with 100 μM cephaloridine as the substrate. Abbreviations: CER cephaloridine, CEZ cefazolin, CET cephalothin, CMD cefamandole, CXM cefuroxime, CMX cefmenoxime, CZX ceftizoxime, CMZ, cefmetazole, ABPC ampicillin.

Table 3. Sensitivity of Bacteroides β-lactamases to inhibitors.

Species	Inhibitor (10 μM)			
	Cefmetazole	Sulbactam	Clavulanic acid	Cloxacillin
B. fragilis	S*	S	S	S
B. ovatus	S	S	S	S
B. thetaiotaomicron	S	S	S	S
B. oralis	S	S	S	S
B. vulgatus	S	R	S	S
B. intermedius	S	R	R	R
B. disiens	S	R	R	R
B. bivius	S	R	R	R

* S: sensitive (75% inhibition); R: resistant (no significant inhibition).

hibitors, cefmetazole, sulbactam, clavulanic acid and cloxacillin. The β-lactamase from *B. vulgatus* was inhibited by all inhibitors with the exception of sulbactam. The β-lactamases from *B. intermedius*, *B. disiens* and *B. bivius* were inhibited only by cefmetazole. Thus, the β-lactamases from genus Bacteroides were classified into three groups on the basis of inhibition profile.

Discussion

There have been many studies on β-lactamases from the *Bacteroides fragilis* and *B. melaninogenicus* groups. However, the incidence of β-lactamase production among Bacteroides species has not been clarified. OKUBADEJO *et al.*[15] found no β-lactamase activity in approximately 60 strains of *B. fragilis*. On the other hand, GARROD[1] suspected penicillinase activity in 2 (25%) of 8 isolates of *B. fragilis*. Furthermore, PINCUS *et al.*[2] reported 5 (28%) of 18 isolates of *B. fragilis* destroyed penicillin. Other reports[16,17] on β-lactamase producing *B. fragilis* gave frequencies between 40 and 88%. The present study demonstrated 32 (30%) of 107 isolates of *B. fragilis* produced β-lactamase.

MURRAY and ROSENBLATT[18] reported that most clinical isolates (60%) of *B. melaninogenicus* group produced β-lactamase, with no difference in the qualitative production of β-lactamase in the three *B. melaninogenicus* subspecies. BOURGAULT and ROSENBLATT[17] reported 25 (54%) of 46 of *B. melaninogenicus* gave positive results. BROOK *et al.*[19] also reported the incidence of β-lactamase producing Bacteroides species including the *B. melaninogenicus* group, isolated from children, and its correlation between the source of isolates and ability to produce the enzyme. They reported that 53 (82%) of 65 of *B. fragilis* group and 28 (38%) of 73 isolates of *B. melaninogenicus* group were β-lactamase producers. Our results did not confirm the findings of MURRAY and ROSENBLATT[18] and TIMEWELL *et al.*[20] that almost all of isolates of *B. asaccharolyticus* have β-lactamase activity. We found no β-lactamase activity in 9 strains of *B. asaccharolyticus*. BROOK *et al.*[19] reported that *B. distasonis* has no β-lactamase and we confirmed these results in our studies.

We have classified β-lactamases from genus Bacteroides into three groups according to sensitivity to β-lactamase-inhibitors and into eight profiles according to hydrolysis of β-lactam antibiotics. TIMEWELL *et al.*[20] grouped together the β-lactamases from *B. melaninogenicus* and *B. bivius*, however, we observed the β-lactamase from *B. bivius* to have a broader substrate profile than that of *B. intermedius*. β-Lactamase activity was detected in three isolates of *B. ruminicola* but no β-lactamase activity could be detected in the crude enzyme preparation from these strains. The isolates of *B. intermedius* and *B. bivius* produced a large amount of β-lactamase with a high frequency (approximately 80%). Vaginal isolates of *B. bivius* produced β-lactamase which hydrolyzed various cephalosporins. These observations are practically important for β-lactam chemotherapy in the fields of obstetrics and gynecology.

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