ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1990, p. 2169–2176
0066-4804/90/112169-08$02.00/0

Vol. 34, No. 11

Characterization of β-Lactamases from Non-*Bacteroides fragilis* Group *Bacteroides* spp. Belonging to Seven Species and Their Role in β-Lactam Resistance

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Received 24 May 1990/Accepted 7 September 1990

Twelve β-lactamase-positive non-*Bacteroides fragilis* group *Bacteroides* spp. belonging to seven different species were examined by MIC determination and enzyme characterization. MICs of most β-lactams except cefoxitin, cefotetan, imipenem, and meropenem were relatively high or very high. All enzymes hydrolyzed cephaloridine (V_{max}, 100%; K_m, 12 to 70 μM), cephalothin (V_{max}, 25 to 826%; K_m, 8 to 143 μM), cefamandole (V_{max}, 13 to 158%; K_m, 17 to 170 μM), and cefuroxime (hydrolysis rate, 19 to 98%), and 11 of 12 hydrolyzed cefotaxime (V_{max}, 26 to 145%; K_m, 13 to 127 μM); no hydrolysis of cefoxitin or moxalactam was observed. Penicillins were hydrolyzed at lower rates, with V_{max} values ≤20% of that obtained with cephaloridine. Addition of clavulanate, sulbactam, or tazobactam led to a 4- to 2,048-fold lowering of MICs of penicillins as well as cephalosporins. All enzymes were inhibited by clavulanate (50% inhibitory concentration [IC_{50}], 0.01 to 1.8 μM), sulbactam (IC_{50}, 0.02 to 1.9 μM), tazobactam (IC_{50}, 0.001 to 0.9 μM), cefoxitin (IC_{50}, 0.002 to 0.35 μM), and moxalactam (IC_{50}, 0.03 to 6.6 μM). No enzymes were inhibited by 100 μM EDTA or *p*-chloromercuribenzoic acid; an enzyme of one strain of *B. loescheii* was inhibited by 100 μM cloxacillin (IC_{50}, 2.35 μM). Ten enzymes had optimal activity at pH 5.0 to 6.0, and two had optimal activity at pH 8.0. Isoelectric focusing revealed pIs between 4.2 and 5.6. These enzymes seem to belong to a previously unclassified group of β-lactamases, related (but not identical) to β-lactamases of the *B. fragilis* group.

Anaerobic gram-negative rods are important hospital pathogens, especially among patients with weakened host defense mechanisms (2). β-Lactamases are almost always present in the *Bacteroides fragilis* group, the most common clinically encountered member of these organisms (3, 11, 30, 31, 47–49, 53). Many studies on the characteristics of β-lactamases in the *B. fragilis* group have been reported (1, 4, 10, 12–16, 23, 30–35, 38, 39, 41–43, 46–49, 54–56). A clear relationship between enzyme production and resistance to non-cephamycin β-lactam antimicrobial agents has been established, such that β-lactamase in the *B. fragilis* group seems to have clinical significance (12–15, 23, 32, 41, 46, 47, 49, 53).

Gram-negative anaerobic rods other than the *B. fragilis* group are increasingly encountered in clinical infections (2). β-Lactamase-production has been reported in some strains of *B. bivius*, *B. disiens*, the black-pigmented *Bacteroides* spp., *B. oris-B. buccae*, *B. oralis*, *B. splanchnicus*, *B. coagulans*, *B. ureolyticus*, *Mitsuokella multiacida*, and *Megamonas hypermegas* (3, 5, 18, 22, 26, 29–31, 37, 41, 42, 44, 46, 50, 51). Apart from a few studies with *B. bivius*, *B. disiens*, *B. oralis*, and black-pigmented *Bacteroides* spp., however, no systematic study of the characteristics of β-lactamases in these organisms has been undertaken (22, 26, 30, 31, 41, 42, 46, 50, 51).

This study describes the susceptibility patterns and enzyme characterization (including effects of β-lactamase inhibitors) of 12 β-lactamase-positive non-*B. fragilis* group *Bacteroides* strains from seven species, some of which have not previously been studied. An additional eight strains, belonging to five species, were examined by isoelectric focusing only.

MATERIALS AND METHODS

Bacterial strains and culture media. All bacteria tested were clinical isolates. Strains included three strains of *B. melaninogenicus*, four of *B. intermedius*, one of *B. loescheii*, one of *B. oralis*, five of *B. bivius*, four of *B. disiens*, and two of *B. capillosus*. Organisms were identified by standard methods (2, 44), and stored at −70°C in sterile defibrinated sheep blood until use. Vials were thawed, and organisms were plated onto enriched laked sheep blood agar containing kanamycin and vancomycin (44) to check for purity and viability. Purity was also checked by periodic Gram-staining.

Antibiotics and chemicals. Antibiotics were obtained as follows: amoxicillin, clavulanate, and ticarcillin, Beecham Laboratories, Bristol, Tenn.; cefoperazone and sulbactam, Pfizer Pharmaceuticals, New York, N.Y.; piperacillin and tazobactam, Lederle Laboratories, Pearl River, N.Y.; cephaloridine and cefuroxime, Glaxo Laboratories, Greenford, United Kingdom; cephalothin, cefamandole, and moxalactam, Lilly Laboratories, Indianapolis, Ind.; cefotaxime, Hoechst-Roussel Pharmaceuticals, Paris, France; imipenem and cefoxitin, Merck Sharp and Dohme Laboratories, West Point, Pa.; cefotetan and meropenem, Stuart Pharmaceuticals, Wilmington, Del.; mezlocillin, Bayer AG, Leverkusen, Federal Republic of Germany; metronidazole, Searle Labo-

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ratories, Chicago, Ill.; aztreonam, Squibb Pharmaceuticals, Princeton, N.J.; and cloxacillin, Sigma Laboratories, St. Louis, Mo. Nitrocefin was kindly provided by BBL Microbiology Systems (Cockeysville, Md.). Unless otherwise specified, all other reagents and chemicals were from Sigma.

Susceptibility testing. MICs were determined by agar dilution on Wilkins-Chalgren agar with 5% added sterile defibrinated sheep blood (3). Clavulanate, sulbactam, and tazobactam were added to β-lactams at a fixed concentration of 2 μg/ml (3). Synergy was determined as described previously (3). Plates were read after incubation at 37°C for 48 h in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.).

β-Lactamase extraction. Organisms were grown on 60 enriched sheep blood agar plates without antibiotics (44) for 48 h in GasPak jars (BBL). Growth was harvested from each plate with 5 ml of 10 mM phosphate buffer, pH 7.0, sedimented at 9,000 × *g* for 45 min, and washed with 50 ml of buffer. Cells (1.5 to 2.5 g [dry weight]) were suspended in 4.5 ml of 10 mM phosphate buffer (pH 7.0) to which 4.5 g of acid-washed glass beads (<150 μM) had been added, and were disrupted by shaking in a Mickle Laboratory Shaker (Mickle Laboratory Engineering Co., Gomshall, Surrey, United Kingdom) at 4°C for 90 min. Supernatants obtained after sedimenting at 25,000 × *g* for 60 min contained β-lactamase and were divided into aliquots and stored at −70°C until use. β-Lactamase of crude extracts was tested with 500 μg of nitrocefin solution (BBL) per ml as previously described, by using 75 μl of nitrocefin solution and 25 μl of extract (51). All extracts turned nitrocefin red within 5 min.

β-Lactamase assays. Hydrolysis of cephalosporins and penicillins was determined spectrophotometrically at 30°C with a double-beam spectrophotometer (model 550S) coupled to a 561 recorder (The Perkin-Elmer Corp.). Assays were carried out in 10 mM phosphate buffer (pH 7.0). The wavelengths of maximal absorption differences between hydrolyzed and nonhydrolyzed β-lactams were as previously described (24, 40). Substrate concentrations varied between 2.5 and 150 μM. For penicillins, cefoxitin, and moxalactam, substrate concentrations of up to 1 mM were used. For penicillinase detection, controls containing buffer and antibiotic only (instead of buffer and enzyme only, as was the case for other determinations) were also used. K_m and V_{max} values were calculated by computerized linear regression analysis of Lineweaver-Burk (1/v versus 1/S), Woolf-Augustinsson-Hofstee (v versus v/S), and S/v versus S plots. In all cases, values obtained from all three plots were very similar, and the mean of the three values was taken after linearity of the plots was checked.

Penicillin hydrolysis was also determined by two additional methods: (i) the microacidimetric method, using a pH-stat (20, 36), and (ii) the colorimetric method of Cohenford et al. (9).

The concentration of clavulanate, sulbactam, and tazobactam required to inhibit 50% of the β-lactamase activity (IC_{50}) was measured after 10 min of preincubation, with 20 μM nitrocefin as the substrate, whereas cefoxitin, moxalactam, and cloxacillin were preincubated for 3 min. Dilutions of inhibitors were between 0.001 and 25 μM. For EDTA, *p*-chloromercuribenzoic acid (*p*CMB), cloxacillin, aztreonam, sodium chloride, copper sulfate, zinc sulfate, manganese sulfate, and ferrous sulfate, one concentration of 100 μM was preincubated with the enzyme for 3 min and activity was assayed with 20 μM nitrocefin as the substrate. Apparent K_i values were calculated by using fixed concentrations of inhibitors and various concentrations of nitrocefin (2.5 to

TABLE 1. MICs of β-lactam antibiotics against β-lactamase-positive *Bacteroides* spp.

Antibiotic	MIC (μg/ml) for:											
	B. melaninogenicus		*B. loescheii*	*B. intermedius*		*B. oralis*	*B. bivius*		*B. disiens*		*B. capillosus*
	184	192	201	820	837	182	198	690	691	802	867	902
Amoxicillin[a]	64.0	64.0	256.0	32.0	32.0	128.0	8.0	128.0	128.0	8.0	64.0	8.0
Ticarcillin	64.0	32.0	8.0	2.0	16.0	32.0	32.0	8.0	8.0	4.0	16.0	16.0
Piperacillin	>64.0	16.0	>64.0	16.0	16.0	>64.0	64.0	16.0	16.0	64.0	8.0	16.0
Cefoperazone	0.5	2.0	>64.0	16.0	32.0	>64.0	32.0	32.0	1.0	1.0	8.0	32.0
Cephaloridine	>1,024.0	1,024.0	>1,024.0	>1,024.0	1,024.0	1,024.0	>1,024.0	>1,024.0	>1,024.0	1,024.0	1,024.0	512.0
Cephalothin	>1,024.0	1,024.0	>1,024.0	1,024.0	>1,024.0	1,024.0	1,024.0	1,024.0	1,024.0	1,024.0	1,024.0	128.0
Cefamandole	>1,024.0	1,024.0	>1,024.0	1,024.0	1,024.0	1,024.0	512.0	512.0	1,024.0	512.0	>1,024.0	256.0
Cefotaxime	1,024.0	512.0	1,024.0	512.0	256.0	512.0	1,024.0	512.0	512.0	256.0	512.0	64.0
Cefoxitin	16.0	16.0	<0.5	2.0	<0.5	16.0	<0.5	2.0	2.0	1.0	2.0	16.0
Cefotetan	8.0	8.0	2.0	2.0	2.0	32.0	2.0	4.0	1.0	2.0	2.0	8.0
Imipenem	1.0	0.5	1.0	1.0	1.0	1.0	0.5	1.0	0.5	0.25	0.125	0.5
Meropenem	0.25	0.25	0.25	0.25	0.25	1.0	0.25	0.5	0.25	0.125	0.25	0.125
Metronidazole	1.0	1.0	1.0	<0.5	2.0	<0.5	<0.5	2.0	2.0	1.0	<0.5	4.0

[a] Results similar to those of ampicillin.

75 μM) as the substrate, according to previously described methods.

One unit of β-lactamase activity was defined as the amount of enzyme which formed 1 μM nitrocefoic acid per min per mg of protein at pH 7.0 and at 30°C. Protein estimation was by the method of Lowry et al. (25), with bovine serum albumin as the standard.

The effect of various pH levels on enzyme activity was evaluated at pH 3.6, 4.0, 5.0, 5.7, 6.0, 7.0, 8.0, 9.2, 10.0, and 10.6, with a substrate of 20 μM nitrocefin.

Isoelectric focusing was performed as described by Matthew et al. (28), except that the acrylamide was polymerized by ammonium persulfate (0.50 mg/ml) and 0.05% *N, N, N', N'*-tetramethylethylenediamine (Bio-Rad Laboratories, Richmond, Calif.). Focusing was carried out at 4°C. Controls utilized with each run consisted of TEM-1 (pI, 5.4), TEM-2 (pI, 5.6), and a Bio-Rad control (pI, 4.65). Extracts were placed on the cathode or anode sections of gels, depending on the pI of the enzyme. After removal of electrode strips, β-lactamase bands were identified by flooding gels with nitrocefin (500 μg/ml). All extracts were tested three to five times.

RESULTS

Antibiotic susceptibilities. Antimicrobial susceptibilities of the 12 strains are presented in Table 1. As can be seen, cephalosporin MICs were higher than those of penicillins, and strains yielded relatively high or very high MICs of β-lactams, with the exception of cefoxitin, cefotetan, imipenem, and meropenem. All strains were highly resistant to cephaloridine, cephalothin, and cefamandole, with MICs ≥512 μg/ml for all strains but *B. capillosus* 902. Cefotaxime was almost always more active than the above-described cephalosporins. All strains were susceptible to cefoxitin, cefotetan (≤32 μg/ml), imipenem, meropenem (≤2 μg/ml), and metronidazole (≤4 μg/ml). Addition of clavulanate, sulbactam, or tazobactam in fixed concentration led to lowering of the β-lactam MIC (4- to >2,048-fold) with both penicillins and cephalosporins (Table 2).

Substrate profiles. Cephalosporin kinetic constants are presented in Table 3. As can be seen, cephaloridine, cephalothin, cefamandole, cefotaxime (for all strains except *B. capillosus* 902), and nitrocefin were hydrolyzed by all strains tested. No detectable hydrolysis of cefoxitin, moxalactam, amoxicillin, cloxacillin, or mezlocillin was detected with the spectrophotometric method, despite substrate concentrations as high as 1 mM. When hydrolysis rates of a single 100 μM substrate concentration of cephaloridine and cefuroxime were compared, all strains hydrolyzed cefuroxime, at rates between 19 and 98% of cehaloridine. K_m values varied between 12 and 70 μM for cephaloridine (relative V_{max} taken as 100%), 8 and 143 μM for cephalothin (relative V_{max}, 25 to 826%), 17 and 170 μM for cefamandole (relative V_{max}, 13 to 158%), 13 and 127 μM for cefotaxime (relative V_{max}, 26 to 145%) and 3 and 59 μM for nitrocefin (relative V_{max}, 85 to 668%). The *B. capillosus* enzyme which did not hydrolyze cefotaxime was not inhibited by cefotaxime (apparent K_i, >100 μM), with a cefotaxime MIC of 64 μg/ml (Tables 1 and 2). Differences in K_m and V_{max} were seen among organisms of the same species, with nitrocefin yielding the lowest K_m values. As a rule, cephaloridine and nitrocefin were hydrolyzed at higher rates than cephalothin, cefamandole, cefuroxime, or cefotaxime. Of note is the extremely high V_{max} for cephalothin (826%) observed in the one *B. loescheii* strain tested.

TABLE 2. MICs of β-lactam antibiotics alone and with inhibitors[a] against β-lactamase-positive *Bacteroides* spp.

Strain	MIC (μg/ml)																	
	Ampicillin			Piperacillin			Ticarcillin		Cephaloridine		Cephalothin		Cefamandole		Cefotaxime	
	Alone	+ CLA	+ SUL	+ TAZO	Alone	+ CLA	+ SUL	+ TAZO	Alone	+ CLA	Alone	+ CLA	Alone	+ CLA	Alone	+ CLA	Alone	+ CLA
B. melaninogenicus 184	64.0	2	2	2	>64.0	4	16	4	64.0	0.5	>1,024.0	2.0	>1,024.0	4.0	>1,024.0	4.0	1,024.0	<0.5
192	64.0	<0.125	<0.125	<0.125	16.0	<0.125	2	<0.125	32.0	1.0	1,024.0	<0.5	1,024.0	<0.5	1,024.0	<0.5	512.0	<0.5
B. loescheii 201	256.0	1	2	1	>64.0	4	16	16	8.0	0.5	>1,024.0	16.0	>1,024.0	32.0	>1,024.0	32.0	1,024.0	4.0
B. intermedius 820	32.0	<0.125	0.5	0.25	16.0	<0.125	1	0.25	2.0	0.5	>1,024.0	2.0	1,024.0	2.0	1,024.0	4.0	512.0	2.0
837	32.0	<0.125	<0.125	0.25	16.0	<0.125	0.25	<0.125	16.0	1.0	1,024.0	<0.5	>1,024.0	<0.5	1,024.0	<0.5	256.0	<0.5
B. oralis 182	128.0	2	4	4	>64.0	2	16	8	32.0	1.0	1,024.0	32.0	1,024.0	32.0	1,024.0	32.0	512.0	2.0
B. bivius 198	8.0	<0.125	<0.125	<0.125	64.0	<0.125	2	<0.125	32.0	0.5	>1,024.0	2.0	1,024.0	4.0	512.0	4.0	1,024.0	0.5
690	128.0	<0.125	<0.125	<0.125	16.0	0.25	1	<0.25	8.0	0.5	>1,024.0	<0.5	1,024.0	<0.5	512.0	<0.5	512.0	<0.5
691	128.0	0.5	0.5	0.5	16.0	2	4	2	8.0	0.5	>1,024.0	<0.5	1,024.0	<0.5	1,024.0	<0.5	512.0	<0.5
B. disiens 802	8.0	<0.125	<0.125	<0.125	64.0	<0.125	1	<0.125	4.0	0.5	1,024.0	<0.5	1,024.0	<0.5	512.0	<0.5	256.0	<0.5
867	64.0	1	2	1	8.0	1	4	1	16.0	1.0	1,024.0	8.0	1,024.0	32.0	>1,024.0	32.0	512.0	8.0
B. capillosus 902	8.0	1	1	1	16.0	4	4	4	16.0	1.0	512.0	<0.5	128.0	<0.5	256.0	<0.5	64.0	<0.5

[a] All inhibitors added at fixed concentrations of 2 μg/ml. CLA, Clavulanate; SUL, sulbactam; TAZO, tazobactam.

TABLE 3. Cephalosporin kinetic constants of β-lactamases from *Bacteroides* spp.[a]

Strain	Cephaloridine K_m[b]	Cephalothin		Cefamandole		Cefotaxime		Nitrocefin		Cefuroxime hydrolysis rate[c]
		V_{max}[d]	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	
B. melaninogenicus										
184	41	67	36	25	17	76	34	668	29	86
192	26	96	85	24	23	58	46	216	8	24
B. loescheii 201	62	826	143	158	53	110	41	208	10	98
B. intermedius										
820	12	65	8	66	47	145	84	112	4	56
837	62	116	66	41	170	58	13	352	39	69
B. oralis 182	26	55	8	33	41	26	48	216	6	50
B. bivius										
198	70	55	55	13	35	46	90	514	59	60
690	29	31	11	31	39	66	78	108	8	45
691	49	25	14	19	59	40	127	92	9	29
B. disiens										
802	40	36	32	20	66	35	84	85	11	19
867	70	33	138	28	46	28	98	415	12	62
B. capillosus 902	25	45	30	28	42	<0.1[e]		105	3	74

[a] No detectable hydrolysis of cefoxitin or moxalactam (relative V_{max}, <0.1).
[b] Micromolar.
[c] No K_m done. Hydrolysis rate of 100 μM cefuroxime, compared with that of 100 μM cephaloridine, taken as 100% hydrolysis.
[d] Relative to cephaloridine (100%).
[e] Cefotaxime MIC = 64 μg/ml; apparent K_i of cefotaxime >100 μM.

Results of assays for penicillin hydrolysis with the microacidimetric assay are listed in Table 4. As can be seen, hydrolysis rates for all penicillins were lower than that for cephaloridine, with V_{max} values of 2 to 12% (penicillin G), 0 to 4% (cloxacillin), 0.3 to 13% (amoxicillin), and 1 to 20% (mezlocillin). Although no hydrolysis could be demonstrated with the usual spectrophotometric method (control buffer plus enzyme, against enzyme plus substrate concentrations up to 1 mM), activity was observed when controls comprised buffer and antibiotic only. As an example, for *B. disiens* 867, the relative V_{max} for mezlocillin was found to be 1.6% compared with that of cephaloridine, at a substrate concentration of 100 μM; this value is similar to that observed with the microacidimetric method (Table 4). No hydrolysis of benzylpenicillin G could be demonstrated with the neocuproine-copper sulfate colorimetric assay (9), in the face of satisfactory concomitant hydrolysis of cephaloridine.

TABLE 4. Penicillinase activity of β-lactamases from *Bacteroides* spp.[a]

Strain	Relative V_{max}			
	Benzylpenicillin G	Cloxacillin	Amoxicillin	Mezlocillin
B. melaninogenicus				
184	4	4	2	4
192	2	1	ND[b]	1
B. loescheii 201	11	0	2	6
B. intermedius				
820	12	ND	13	20
837	10	ND	ND	ND
B. oralis 182	7	0	6	9
B. bivius				
198	3	1	1	2
690	4	4	4	3
691	2	2	1	2
B. disiens				
802	2	2	1	3
867	2	0.3	0.3	2
B. capillosus 902	11	ND	12	ND

[a] Microacidimetric method. Values expressed as a percentage of the value of cephaloridine (100%).
[b] ND, Not done.

Enzyme inhibition studies. IC_{50} and apparent K_i values are presented in Table 5. As can be seen, all enzymes were inhibited to various degrees by clavulanate, sulbactam, tazobactam, cefoxitin, and moxalactam. In most cases, tazobactam was more active than both sulbactam and clavulanate and clavulanate was more active than sulbactam, while cefoxitin was more active than moxalactam and yielded activity comparable to that of tazobactam. With 100 μM inhibitor and 20 μM nitrocefin substrate, only one enzyme (*B. loescheii* 201) was inhibited ≥90% by cloxacillin (93% inhibition; IC_{50}, 2.35 μM). No inhibition of any enzyme (≥90%) was observed by aztreonam, EDTA, *p*CMB, sodium chloride, zinc sulfate, manganese sulfate, or ferrous sulfate; only *B. disiens* 867 was inhibited by copper sulfate (96% inhibition).

Isoelectric focusing. Results of isoelectric focusing experiments are presented in Table 6 and Fig. 1. As can be seen, all pIs were in the acid range. For *B. melaninogenicus*, *B. intermedius*, *B. oralis*, *B. disiens*, and *B. capillosus*, pIs were usually 4.2 to 4.3. Exceptions were *B. melaninogenicus* 192, with a pI of 4.8, and *B. disiens* 867, with a pI of 4.9. The one *B. loescheii* strain yielded a pI of 5.0. All *B. bivius* strains yielded pIs of 5.6. The possibility of doublets in lanes 9 to 11 (Fig. 1B) cannot be excluded; however, the extremely acid nature of these pIs precluded accurate determination when samples were placed on the anode.

Results of enzyme activity as compared to amoxicillin MIC are presented in Table 7. As can be seen, there was a broad correlation between the two values when amoxicillin and ampicillin MICs (identical results for both drugs) were grouped as ≤32 μg/ml (mean activity [± standard deviation], 24.3 ± 15 mU) and ≥64 μg/ml (mean activity [± standard deviation], 102.9 ± 50 mU). Of the 12 β-lactamases, 8 yielded maximal activity at pH 5.0, 2 yielded maximal activity at pH 6.0, and 2 yielded maximal activity at pH 8.0 (Table 7).

TABLE 5. IC_{50} and apparent K_i values of five agents against β-lactamases of *Bacteroides* spp.

Strain	Clavulanate		Sulbactam		Tazobactam		Cefoxitin		Moxalactam	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
B. melaninogenicus										
184	0.17	0.07	0.90	0.13	0.04	0.015	0.002	0.003	0.26	0.013
192	0.02	0.05	0.10	0.15	0.01	0.003	0.003	0.016	0.03	0.046
B. loescheii 201	0.10	0.34	0.52	1.12	0.70	0.98	0.35	0.31	0.10	0.11
B. intermedius										
820	0.01	0.04	0.02	4.25	0.003	0.72	0.09	0.085	2.45	0.5
837	0.09	ND[a]	0.10	1.70	0.015	1.38	0.003	0.014	ND	0.04
B. oralis 182	0.04	0.003	0.03	0.011	0.01	0.002	0.03	0.005	0.11	0.022
B. bivius										
198	0.08	0.10	0.14	0.06	0.03	0.02	0.003	0.013	ND	0.10
690	0.01	0.01	0.035	0.03	0.001	0.005	0.07	0.016	2.7	0.69
691	0.08	0.17	0.10	0.55	0.001	0.002	0.008	0.005	1.9	0.79
B. disiens										
802	1.8	3.0	1.9	1.5	0.9	1.33	0.04	0.013	6.6	0.66
867	0.09	0.02	0.05	0.13	0.01	0.007	0.10	0.015	1.65	0.11
B. capillosus 902	0.08	0.25	0.10	0.40	0.001	0.002	0.04	0.002	2.9	0.025

[a] ND, Not done.

DISCUSSION

Results of MIC studies showed strains to be more resistant to cephalosporins than to penicillins (Table 1). However, all strains were much more susceptible to cefoxitin than to a broad-spectrum cephalosporin such as cefotaxime. Differences in piperacillin and ticarcillin activity compared with results of previous studies (3) may reflect different strains with increased β-lactamase production utilized in the current experiments. Hydrolysis studies are necessary to investigate this phenomenon. Because addition of clavulanate, sulbactam, and tazobactam led to a similar synergistic lowering of MICs of both penicillins and cephalosporins (Tables 1 and 2), β-lactamase production probably plays an important role in β-lactam resistance in these strains. Previous studies in the *B. fragilis* group as well as other β-lactamase-positive *Bacteroides* spp. have shown similar results for all three inhibitors when added to six different β-lactams (3). In view of the latter findings, it was decided to test clavulanate only in combination with the cephalosporins listed in Table 2.

With the exception of *B. capillosus* 902, which did not hydrolyze, had a very low affinity for cefotaxime, and had lower MICs than other strains, all organisms tested were resistant to cephaloridine, cephalothin, cefamandole, and cefotaxime with concomitant hydrolysis of all these agents by all β-lactamase extracts. In most cases, cephaloridine was hydrolyzed most rapidly, but in some cases (notably *B. loescheii* 201, which hydrolyzed cephalothin 8 times and cefamandole and cefotaxime 1 to 1.5 times more rapidly than cephaloridine), other cephalosporins were hydrolyzed at more rapid rates. K_m values were not always similar in different strains of the same species (Table 3). Timewell et al. (50) have described a β-lactamase from *B. oralis* with little activity on any cephalosporin other than cephaloridine. In the study presented here, the *B. oralis* strain hydrolyzed all cephalosporins other than cephaloridine at significant, albeit lower, rates than the latter compound, with relative V_{max} values ranging between 26 and 55%, compared with cephaloridine. The lower activity of extracts from *B. melaninogenicus* and *B. bivius* against cephalothin than against cephaloridine, reported by Timewell et al. (50), and the lower activity of *B. bivius* extracts against cephalothin, cefamandole, and cefotaxime than against cephaloridine, reported by Lacroix et al. (22), were observed for several extracts in the current study. Unlike Sherrill and McCarthy (41), we did encounter differences between kinetic patterns of β-lactamases from *B. melaninogenicus*, *B. intermedius*, and *B. oralis*. Similar to our findings, previous studies have documented lack of hydrolysis of cefoxitin and moxalactam by β-lactamases of non-*B. fragilis* group *Bacteroides* species (22, 41, 50). Malouin et al. (26), using a spectrophotometric method with a 24-h incubation period, have described slow hydrolysis rates of cefoxitin and moxalactam by *B. bivius*. This method was not used in the current study.

Using the microacidimetric method (20, 36), we detected low penicillin hydrolysis rates for all strains tested, as compared with cephaloridine hydrolysis rates. The colori-

TABLE 6. Isoelectric points of β-lactamases from 20 non-*B. fragilis Bacteroides* spp.[a]

Strain	pI
B. melaninogenicus	
184	4.3
192	4.8
637[b]	4.3
B. intermedius	
820	4.2
837	4.2
848[b]	4.2
850[b]	4.2
B. loescheii 201	5.0
B. oralis 182	4.3
B. bivius	
198	5.6
690	5.6
691	5.6
683[b]	5.6
687[b]	5.6
B. disiens	
802	4.2
867	4.9
868[b]	4.2
870[b]	4.2
B. capillosus	
902	4.2
935[b]	4.2

[a] Values are means of three to five tests; standard deviation ± 0.1.
[b] No kinetics performed.

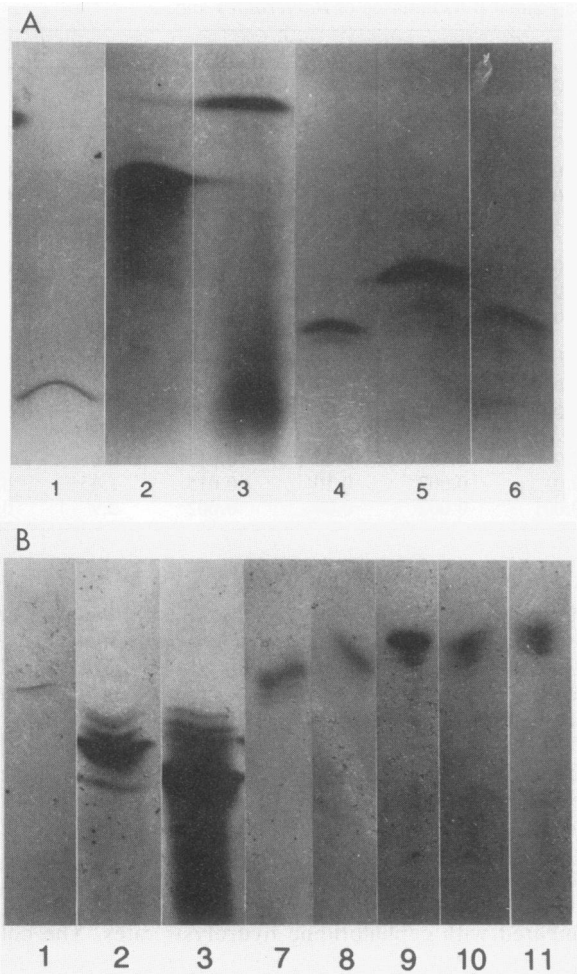

FIG. 1. Analytical isoelectric focusing pattern of enzyme extracts. Extracts placed on anode (panel A) or cathode (panel B). Lanes: 1, Bio-Rad control (pI 4.65); 2, TEM-1 (pI 5.4); 3, TEM-2 (pI 5.6); 4, *B. melaninogenicus* 192 (pI 4.8); 5, *B. loescheii* 201 (pI 5.0); 6, *B. disiens* 867 (pI 4.9); 7, *B. oralis* 182 (pI 4.3); 8, *B. melaninogenicus* 184 (pI 4.3); 9, *B. intermedius* 820 (pI 4.2); 10, *B. disiens* 802 (pI 4.2); 11, *B. capillosus* 902 (pI 4.2). *B. bivius* extracts yielded bands which, although reproducible, were too weak for satisfactory reproduction.

metric method of Cohenford et al. (9) did not appear to be sufficiently sensitive for detection of penicillin hydrolysis in our strains, probably because the low levels of enzyme present were insufficient to overcome spontaneous degradation of penicillin in the control without enzyme. Previous studies, mostly utilizing the microiodometric method of Sykes and Nordström (45), have described β-lactamases from *B. asaccharolyticus*, *B. melaninogenicus*, *B. disiens*, and *B. oralis* with predominantly penicillinase activity (29–31, 37). Lacroix et al. (22), also using the microiodometric method, have reported mixed penicillinase and cephalosporinase activity in *B. bivius*. By contrast, utilizing both microiodometric and microacidimetric methods, Sherrill and McCarthy (41) demonstrated significant cephalosporinase but low penicillinase activity in black-pigmented *Bacteroides* and *B. oralis*, with penicillin G and carbenicillin hydrolysis rates of 2 to 7% compared with that of cephaloridine (100%). Similarly, utilizing a modified spectrophotometric assay of β-lactamase action on penicillins (52), Tajima and coworkers (46) could only demonstrate 6 to 11% ampicillin hydrolysis

TABLE 7. Activity and pH optima of β-lactamases correlated with amoxicillin MIC

Strain	MIC (μg/ml)	Activity[a]	pH[b]
B. bivius 198	8.0	29.2	8.0
B. disiens 802	8.0	26.5	5.0
B. capillosus 902	8.0	12.5	5.0
B. intermedius			
820	32.0	45.5	5.0
837	32.0	7.6	8.0
B. bivius			
690	128.0	85.9	5.0
691	128.0	65.6	5.0
B. oralis 182	128.0	159.8	5.0
B. disiens 867	64.0	188.8	6.0
B. melaninogenicus			
192	64.0	65.7	6.0
184	64.0	83.2	5.0
B. loescheii 201	256.0	71.5	5.0

[a] Milliunits that formed 1 μM nitrocefoic acid per min/mg of protein at pH 7.0 and 30°C.
[b] pH at which the enzyme was most active.

compared with that of cephaloridine in two of three *B. bivius* strains, but no ampicillin hydrolysis in *B. oralis*, *B. intermedius*, *B. disiens*, or the remaining *B. bivius* strain; significant cephalosporinase activity was demonstrated in all strains tested. The reasons for these discrepant findings are unclear at present.

In the study presented here, we did not utilize the microiodometric method; had we done so, it is possible that more penicillinase activity could have been detected. However, the microacidimetric method is sensitive, while offering the least amount of interference of all penicillinase detection methods (9, 20, 36). Lack of penicillinase detection by the method of Cohenford et al. (9) and very low penicillinase activity (comparable to that observed with the microacidimetric technique) with the spectrophotometric method also argue against falsely low penicillinase detection levels in our study.

It is recognized that, because substrate profiles were performed with crude extracts in the current study, K_m and V_{max} values should be interpreted with caution. Future kinetic studies on purified enzymes are envisaged, to confirm existing date and eliminate spectral interference from crude extracts.

The high β-lactamase affinity of clavulanate, sulbactam, and tazobactam found in the study presented here (Table 5) reflects MIC results in which synergy with β-lactams was demonstrated with all three inhibitors (Table 2). The greater inhibitory activity of tazobactam relative to clavulanate and sulbactam and the greater activity of clavulanate relative to sulbactam generally encountered in the current study reflect previous findings in β-lactamases from aerobic gram-negative rods (21). This study confirms and extends previous studies which have documented the inhibitory activity of clavulanate and sulbactam on β-lactamases of non-*B. fragilis* group *Bacteroides* spp. (22, 30, 31, 41, 50). In contrast to the above findings, Tajima et al. (46) have described β-lactamases from *B. intermedius*, *B. bivius*, and *B. disiens* which were not inhibited by 10 μM clavulanate or sulbactam; MICs of β-lactams in combination with inhibitors were not provided. Little published information is currently available on the affinity of tazobactam to β-lactams of non-*B. fragilis* group *Bacteroides* spp. The clinical significance of differences in IC_{50} and apparent K_i values with different β-lacta-

mase inhibitors against enzymes from these and other β-lactamase-positive strains remains to be established. The difference in IC_{50} and apparent K_i values may reflect differences in inactivation mechanisms. It would be interesting to study whether the same differences occur without preincubation of enzyme with inhibitor. The latter experiments were not performed in the current study. Fu and Neu (16) have described greater moxalactam inhibitory activity relative to cefoxitin against *B. fragilis* β-lactamases. The opposite was encountered in the current study, in which excellent inhibitory activity was exhibited by cefoxitin against β-lactamases from non-*B. fragilis* group *Bacteroides* spp.

Lack of inhibitory activity by cloxacillin and *p*CMB was found in almost all β-lactamases of non-*B. fragilis* group *Bacteroides* spp. This phenomenon has been described before in some non-*B. fragilis Bacteroides* spp. and is an important distinguishing feature between these enzymes and β-lactamases of the *B. fragilis* group, which are inhibited by both compounds (30, 31, 50). An exception to the rule was cloxacillin inhibition of the one *B. loescheii* strain, which, however, yielded a relatively high cloxacillin IC_{50} (2.35 μM), compared to lower IC_{50} and apparent K_i values in strains susceptible to inhibition by the latter compound (6–8). The significance of inhibition by copper sulfate of *B. disiens* 867 is unclear at this time.

Results of isoelectric focusing studies are generally similar to those reported previously (22, 30, 51). However, three strains (*B. loescheii* 201, *B. disiens* 867, and *B. melaninogenicus* 192) had pI values higher than the levels of 4.2 to 4.3 usually encountered in non-*B. bivius Bacteroides* spp. Sherrill and McCarthy (41) have described a distinct band at pI 4.9 in *B. melaninogenicus*, which may correspond to our findings with *B. melaninogenicus* 192.

The specific place in the classification of β-lactamases of enzymes from the current strains is unclear. Susceptibility to clavulanate, resistance to cloxacillin and aztreonam, and acidic pIs are atypical features for group 1 cephalosporinases (6–8), and relatively low penicillin MICs, as well as inhibition by cefoxitin, are atypical for a cefuroximase (17, 19, 27). Although lack of inhibition by cloxacillin and *p*CMB may differentiate these enzymes from group 2e enzymes produced by *B. fragilis*, tentative assignment of these enzymes to group 2e could be justified, noting that cloxacillin and *p*CMB assay concentrations in the current study may be lower than the 100 μM used to define these as inhibitors: one 2e enzyme had a K_m for cloxacillin of 24 μM, while another was listed with an apparent K_i of <100 μM (8). Substrate profiles together with pI values differentiate them from other well-characterized β-lactamases (6–8). Further work is necessary to delineate the situation of these enzymes in the classification of β-lactamases.

ACKNOWLEDGMENTS

This study was supported by grants from Laboratoires Smith-Kline Beecham and Laboratories Pfizer, Paris, France, and C.N.A.M.T.S. grant no 17-3-22-03-E.

We thank J. Acar for use of facilities, E. Collatz and I. Podglajen for advice, and D. Shlaes for critical evaluation of the manuscript.

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