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Inactivation of Cefoxitin and Moxalactam by *Bacteroides bivius* β-Lactamase

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Moxalactam and cefoxitin are known for their high stability against *Bacteroides* β-lactamases. We investigated the β-lactamase activity of crude extracts obtained from three strains of *Bacteroides bivius* and two strains of *Bacteroides fragilis* against cefoxitin and moxalactam. In a spectrophotometric antibiotic assay with a 24-h incubation period, *B. bivius* extracts decreased the initial concentration (10 μg/ml) of moxalactam and cefoxitin by 60%, whereas *B. fragilis* extracts had no effect. In a microbiological assay, when *B. bivius* or *B. fragilis* extracts were added to cephalothin (10 μg/ml) or cefamandole (4 μg/ml), we observed complete disappearance of the inhibitory zones against the indicator strain (*Clostridium perfringens* ATCC 13124). Only the *B. bivius* extracts were able to decrease the inhibitory activity (from 10 to 100%) of cefoxitin and moxalactam (each at 10 μg/ml). Prior addition of clavulanic acid to crude extracts prevented the losses of antibacterial activity. Furthermore, the inhibition of the β-lactamase hydrolysis of nitrocefin by cefoxitin or moxalactam was prevented by a 12-h preincubation of the β-lactam with the *B. bivius* extracts but not with the *B. fragilis* extracts. Finally, with the *B. bivius* strain producing the most β-lactamase, we showed an effect of inoculum size on the MICs of cefoperazone, cefoxitin, and moxalactam with a broth dilution technique. Increasing the inoculum size with the *B. fragilis* strains had no effect on the MICs of cefoxitin and moxalactam. These results indicate a slow and clavulanate-sensitive β-lactamase activity of *B. bivius* extracts against cefoxitin and moxalactam.

Bacteroides fragilis and *Bacteroides bivius* are anaerobes frequently found in specimens from human infections. *B. fragilis* is usually recovered from intraabdominal infections (2, 19, 22), whereas *B. bivius* is frequently isolated from obstetric and gynecologic infections (1, 12, 33). Both species produce a β-lactamase which may play an important role in their resistance to β-lactams (15, 16, 25, 32, 37). Moxalactam and cefoxitin are known to be resistant to the β-lactamases generally found in *B. fragilis* (10, 11, 27, 30, 37), and there is little or no effect for clavulanic acid, a β-lactamase inhibitor, on the susceptibility of *B. fragilis* to cefoxitin (17, 39, 41), although some effect on the inhibitory activity of moxalactam in some resistant strains was reported recently (41). We reported previously that for β-lactamase-positive strains of *B. bivius*, there is a potentiating effect of clavulanic acid on their susceptibilities to cefoxitin and moxalactam which correlates with the amount of β-lactamase produced (16). The purpose of this study was to further evaluate the activity of *B. bivius* β-lactamase against moxalactam, cefoxitin, and some other cephalosporins and compare it with that of the *B. fragilis* β-lactamase.

Because of the relative stability of cefoxitin and moxalactam compared with that of other β-lactams, it has been difficult to measure the slow hydrolysis of these agents with a standard spectrophotometric assay. Therefore, three other methods were used to determine the specific enzymatic activities of the crude β-lactamase extracts against several β-lactams, including cefoxitin and moxalactam: (i) a spectrophotometric antibiotic assay measuring decreases in antibiotic concentration during a prolonged incubation with crude extracts; (ii) a microbiological assay showing the effect of crude extracts on antibiotic inhibitory activities, with or without the addition of clavulanic acid; and (iii) a spectrophotometric method showing the depletion of the competitive inhibitory activity of cefoxitin and moxalactam against β-lactamase in the crude extracts by using nitrocefin to detect resulting free β-lactamase. Finally, we also looked for an effect of inoculum size on the MICs of cefoperazone, cefoxitin, and moxalactam for the strains used.

This study confirmed in several ways the slow β-lactamase activity of *B. bivius* against cefoxitin and moxalactam detected previously (16). In addition, this report introduces simple techniques for the evaluation of slow β-lactamase activity. The possible clinical relevance of slow β-lactamase activity in *B. bivius* is also discussed.

MATERIALS AND METHODS

Antimicrobial agents. Six β-lactams were used in this work: cephaloridine (Glaxo Ltd., Toronto, Ontario, Canada), cefoperazone (Pfizer Canada Inc., Montreal, Quebec, Canada), cefoxitin (C. E. Frosst and Co., Pointe-Claire, Quebec, Canada), and cephalothin, cefamandole, and moxalactam (Eli Lilly and Co., Scarborough, Ontario, Canada). The β-lactamase inhibitor clavulanic acid was kindly furnished by Beecham Laboratories, Pointe-Claire, Quebec, Canada.

β-Lactamase extraction. Three strains of *B. bivius* (strains 396, 537, and 1127) and two strains of *B. fragilis* (strains 434 and 907) isolated from clinical specimens in the microbiology laboratory of Hôpital Saint-Luc, Montreal, Quebec, Canada, were used in this study. The *B. bivius* strains were chosen because of their increased β-lactamase production (16). The

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B. fragilis strains both produce a β-lactamase typical for that species. Their identification was confirmed by the methods described in the *Anaerobe Laboratory Manual* (14). All strains were β-lactamase positive within 2 min in a nitrocefin (Glaxo Pharmaceuticals, Ltd., Greenford, England) microwell test (17). Crude β-lactamase extracts were obtained by ultrasonic treatment and differential ultracentrifugation, as previously described (16).

Standard spectrophotometric β-lactamase assay. The standard spectrophotometric method of O'Callaghan et al. (24) was used to measure the β-lactamase activity of crude extracts against cefamandole, cefoxitin, cephaloridine, cephalothin, and moxalactam (each at 10^{-4} M); wavelengths used were 274 (13), 265 (20), 255 (24), 262 (13), and 275 (20) nm, respectively. Assays were performed in 0.05 M phosphate buffer (pH 7.0) at 37°C with a Gilford spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). One unit of enzyme was able to hydrolyze 1 nmol of substrate per min at 37°C and pH 7.0. To calculate the rate of hydrolysis of cefoxitin and moxalactam, we used the millimolar absorbancy difference ($\Delta\varepsilon$) of the molecules. The $\Delta\varepsilon$ values were 7.38 mM^{-1} cm^{-1} at 265 nm and 7.96 mM^{-1} cm^{-1} at 275 nm for cefoxitin and moxalactam, respectively (20). In addition, the decrease in the specific UV absorption of cefoxitin and moxalactam at 10 μg/ml brought about by crude extracts was measured spectrophotometrically over a 24-h period. The concentration of cefoxitin and moxalactam in the presence of an extract was calculated, and corrected for spontaneous decay, by comparison with a standard curve derived from the UV absorptions of a duplicate set of standard antibiotic concentrations (0, 5, 10, 20, and 40 μg/ml) incubated under identical conditions. The standard curves were constructed at 0, 3, 6, 9, 13, and 24 h of incubation, and each of the curves was tested for linearity of regression.

Microbiological assay. Leveled glass slides (83 by 102 mm; Eastman Kodak Co., Rochester, N.Y.) were flooded with 15 ml of Wilkins-Chalgren agar (Difco Laboratories, Detroit, Mich.) preinoculated with ~10^6 bacteria per ml. The indicator bacterium was *Clostridium perfringens* ATCC 13124 (American Type Culture Collection, Rockville, Md.). Wells (diameter, 7 mm) were cut in the agar and filled with 25 μl of the reaction mixture. The reaction mixture consisted of the crude enzyme extract and a defined concentration of the test antibiotic. The slides were incubated anaerobically at 37°C for 18 h. The resulting concentrations of active antibiotic were estimated by comparison of the zone sizes produced around the test wells with those produced around control wells containing standard antibiotic concentrations in the absence of extracts. The precision and accuracy of the microbiological assay were evaluated by the repeatability of 10 measurements of standard antibiotic concentrations.

Standardized crude extracts (500, 1,000, 2,000, and 3,000 U/ml measured against cephaloridine by spectrophotometry) were used in assays for cefamandole (4 μg/ml) and for cefoxitin, cephalothin, and moxalactam (each at 10 μg/ml). For each extract, duplicate assays were run and the results were averaged. In some experiments, clavulanic acid at 0.75, 8, or 32 μg/ml was preincubated (for 30 min) with the crude extract before the addition of the antibiotic to be tested.

Coupling of spectrophotometric method and microbiological assay. The concentration of cefoxitin incubated in the presence of the *B. bivius* 1127 β-lactamase extract was determined by both the spectrophotometric method and the microbiological assay. Simultaneously for a duplicate set of six tubes, 0.5 ml of cefoxitin at 40 μg/ml was added to 0.5 ml of the β-lactamase extract at 400 U/ml and the reaction mixtures were incubated at 37°C for 24 h. The hydrolysis was stopped at 0, 3, 6, 9, 13, and 24 h by the addition of 1 ml of cold methanol. The UV absorption of the cefoxitin in the reaction mixtures was measured at 275 nm. The concentration of cefoxitin in the mixtures was calculated, and corrected for spontaneous decay, by comparison with standard antibiotic concentrations (0, 5, 10, 20, and 40 μg/ml) incubated under identical conditions. The standard curves were constructed at 0, 3, 6, 9, 13, and 24 h of incubation, and each of the curves was tested for linearity of regression.

In parallel, the cefoxitin concentration in the reaction mixtures was determined with 25-μl samples by using the microbiological assay described above. The concentration was calculated, and corrected for spontaneous decay, by comparison with a standard curve derived from the inhibitory zone sizes for the duplicate set of standard antibiotic concentrations used above. A statistical test of significance for the correlation coefficient found between the two methods was also performed.

Prevention of competitive inhibitory activity of cefoxitin and moxalactam against β-lactamases. The β-lactamase activity of crude extracts was measured spectrophotometrically at 482 nm with 10^{-4} M nitrocefin (23). Inhibition of the β-lactamase was observed when cefoxitin or moxalactam (each at 15 μg/ml) was added as a competitive substrate before the addition of the nitrocefin.

Hydrolysis of cefoxitin or moxalactam by the crude extracts was detected by measuring spectrophotometrically against nitrocefin the free β-lactamase activity resulting from a 12-h preincubation of the crude extracts wtih each β-lactam at 37°C. The absence of competitive inhibition was interpreted as indicating the hydrolysis of cefoxitin or moxalactam by the extract during the preincubation period. Controls with crude extracts and cefoxitin or moxalactam incubated separately were included to make sure that spontaneous degradation of the β-lactam was not responsible for the absence of competitive inhibition.

Effect of inoculum size. The effect of the inoculum size on the MICs of cefoperazone, cefoxitin, and moxalactam for the *B. bivius* and *B. fragilis* strains was determined by a broth dilution technique with Wilkins-Chalgren medium. Cefoperazone was compared with the other two β-lactams because of the well-known effect of *B. fragilis* inoculum size on its MIC (4, 42). The inoculum was prepared from an 18-h broth culture; inocula (1.0 ml) containing 10^4, 10^5, and 10^7 CFU of *B. bivius* per ml and 10^5, 10^6, and 10^8 CFU of *B. fragilis* per ml were added to tubes containing 1.0 ml of antibiotic in broth. The MIC represented the lowest concentration of the antibiotic yielding no visible growth after 48 h of incubation in an anaerobic chamber at 37°C.

RESULTS

Standard spectrophotometric method. The β-lactamase activity of crude extracts expressed in units per milligram of cells (dry weight) against five cephalosporins is shown in Table 1. The mean β-lactamase activity for the *B. fragilis* strains was 1.2, 4.4, and 8.0 times that observed for the *B. bivius* strains against cephaloridine, cephalothin, and cefamandole, respectively. Hydrolysis of the molecules was performed in short incubations (5 to 15 min, depending upon the β-lactamase dilution and the cephalosporin used). In contrast, cefoxitin and moxalactam seemed to be slowly hydrolyzed by *B. bivius* extracts only. This β-lactamase activity was apparent spectrophotometrically by a slight

TABLE 1. β-Lactamase activity of crude extracts against five cephalosporins, determined with standard spectrophotometric method

Cephalosporin	λ[a]	β-Lactamase activity[b] of crude extract of indicated strain						
		B. bivius				*B. fragilis*		
		1127	396	537	Mean	434	907	Mean
Cephaloridine	255	666	312	60	346.0	346	452	399.0
Cephalothin	262	143	85	22	83.3	317	422	369.5
Cefamandole	274	23	23	6	17.3	126	152	139.0
Moxalactam	275	S[c]	S	S	S	0	0	0
Cefoxitin	265	S	S	0	S	0	0	0

[a] Wavelengths used in nanometers.
[b] Expressed in units per milligram of cells (dry weight).
[c] S, Slow activity observed after a 3-h incubation.

decrease in the UV absorption of the antibiotic molecules. However, the slow hydrolysis was not calculable after a 3-h period of incubation by using the conventional method of calculation, which requires the variation in UV absorption observed before and after complete hydrolysis of the substrate (24). Instead it was possible to use the $\Delta\varepsilon$ value (20) for these molecules to calculate the rate of hydrolysis observed in this period of incubation. As previously reported, by using the $\Delta\varepsilon$ values (16), we found some activity against these antibiotics: 0.1 U/mg of cells (dry weight) against cefoxitin and moxalactam for the *B. bivius* 1127 extract and 0.03 and 0.05 U/mg of cells against cefoxitin and moxalactam, respectively, for the *B. bivius* 537 extract. However, the significance of the small variations in UV absorption measured by this method remains disputable. A modification of the conventional spectrophotometric method was used to determine the effect of slow hydrolysis in an extended period of incubation. The hydrolysis rate of moxalactam and cefoxitin by *B. bivius* extracts, over a 24-h period, led eventually to a decrease of the antibiotic concentration ranging from 50 to 65% for moxalactam and 34 to 77% for cefoxitin (Fig. 1). The curves indicated slow hydrolysis rates, and the hydrolysis was noticeable within 3 h of incubation for the extracts from strains 396 and 1127. The moxalactam and cefoxitin concentrations were not decreased by the two *B. fragilis* extracts. In Fig. 1, each calculated antibiotic concentration came from a standard curve derived from a set of standard antibiotic concentrations. The standard curves constructed at 0, 3, 6, 9, 13, and 24 h of incubation each showed linear regression with significant correlation coefficients (r) ($P < 0.001$).

Microbiological assay. The inhibitory activity of cefamandole and cephalothin against *C. perfringens* ATCC 13124 disappeared completely in the presence of *B. bivius* or *B. fragilis* crude extracts previously adjusted to 500 U/ml against cephaloridine (Table 2). However, only the *B. bivius* extracts decreased the initial concentration of moxalactam and cefoxitin. This ranged from a 10 to 100% decrease, depending upon the antibiotic and the amount of β-lactamase present in the assay. The preaddition of clavulanic acid to the crude extracts prevented the loss of antibacterial activities. The concentration of clavulanic acid needed to preserve the initial antibiotic concentration was between 0.75 and 32 μg/ml. The *B. fragilis* extracts had no effect on cefoxitin or moxalactam even in the absence of clavulanic acid.

Control tests demonstrated that 32 μg of clavulanic acid per ml had no inhibitory effect on the indicator strain. Also, comparison of the inhibition zones caused by any of the antibiotics alone with zones obtained when the cephalosporins were combined with clavulanic acid showed no synergistic effect for the combinations (data not shown). The precision of the microbiological assay was evaluated, and it was determined that the measured concentration represented ±5% of the actual concentration in the wells for cefamandole, ±4% for cefoxitin, and ±3% for cephalothin and moxalactam.

Coupling of spectrophotometric method and microbiological assay. For cefoxitin, the concentration of the β-lactam incubated with the *B. bivius* 1127 β-lactamase extract was measured by both the spectrophotometric method and the microbiological assay over a 24-h period (Table 3). A significant correlation between the two methods was shown ($P < 0.01$). Consequently, a correlation between the decrease in the microbiological inhibitory activity of cefoxitin and the specific decrease in the UV absorption of the β-lactam ring

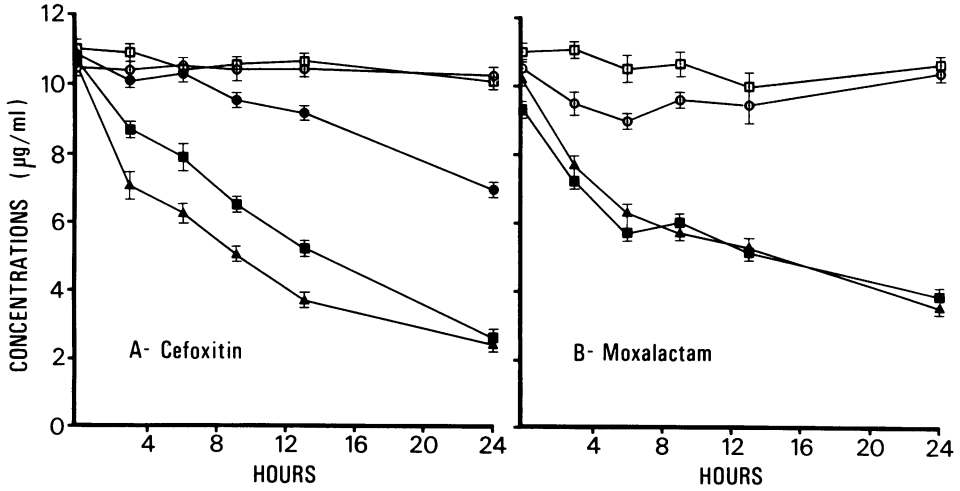

FIG. 1. Cefoxitin (A) and moxalactam (B) concentrations in the presence of crude β-lactamase extracts, measured in the spectrophotometric antibiotic assay over a 24-h period. Symbols (crude extract activities previously calculated spectrophotometrically against cephaloridine are shown in parentheses): ⊡, *B. fragilis* 907 (119 U/ml); ⊙, *B. fragilis* 434 (105 U/ml); ●, *B. bivius* 537 (34 U/ml; in panel A only); ■, *B. bivius* 396 (81 U/ml); ▲, *B. bivius* 1127 (362 U/ml in panel A and 145 U/ml in panel B). The bars indicate the standard deviation of the mean of duplicate assays.

TABLE 2. Percentage of initial antibiotic concentration recovered in the presence of crude β-lactamase extracts with or without clavulanic acid[a]

Cephalosporin	Source of extract		Extract activity[b] (U/ml)	% of initial antibiotic concn recovered[c] with clavulanic acid concn (μg/ml):			
				0	0.75	8	32
Cephalothin (±3%)[d]	*B. bivius*	396	500	0	50	84	96
		537	500	0	50	86	95
		1127	500	0	100	ND[e]	ND
	B. fragilis	434	500	0	79	95	92
		907	500	0	80	94	98
Cefamandole (±5%)	*B. bivius*	396	500	0	98	100	100
		537	500	0	97	99	100
		1127	500	0	100	ND	ND
	B. fragilis	434	500	0	97	100	100
		907	500	0	99	100	100
Moxalactam (±3%)	*B. bivius*	396	500	82	95	96	100
		537	500	79	96	95	99
		1127	1,000	0	95	98	100
	B. fragilis	434	3,000	98	98	100	100
		907	3,000	99	100	100	100
Cefoxitin (±4%)	*B. bivius*	396	500	70	75	99	100
		537	500	52	70	98	99
		1127	500	85	95	99	100
		1127	1,000	81	96	99	100
		1127	2,000	0	95	99	100
	B. fragilis	434	3,000	99	100	100	100
		907	3,000	99	99	100	99

[a] Determined in the microbiological assay.
[b] β-Lactamase activity previously calculated spectrophotometrically against cephaloridine.
[c] Mean of duplicate assays.
[d] The numbers in parentheses represent the precision of the measurements.
[e] ND, Not done.

was observed, suggesting that hydrolysis, and not a nonspecific binding of cefoxitin, occurred in the microbiological assay.

Prevention of competitive inhibitory activity of cefoxitin against β-lactamases. All crude extracts preincubated without a β-lactam showed β-lactamase activity against nitrocefin (100% hydrolysis rate) (Table 4). When the crude extracts and cefoxitin or moxalactam were preincubated separately and then mixed together before the addition of nitrocefin, little or no β-lactamase activity was observed against the chromogenic cephalosporin due to the competitive inhibition by the two β-lactams (5). When crude extracts and cefoxitin or moxalactam were preincubated together before nitrocefin addition, the chromogenic substrate could still be hydrolyzed by the *B. bivius* extracts. In contrast, the *B. fragilis* extracts remained inhibited to the same level by cefoxitin or moxalactam.

TABLE 3. Cefoxitin concentration recovered in the presence of *B. bivius* 1127 β-lactamase extract[a]

Incubation time (h)	Cefoxitin concn (μg/ml; mean ± SD[b]) recovered as determined by:	
	Spectrophotometric method	Microbiological assay
0	9.6 ± 0.2	10.0 ± 1.0
3	7.0 ± 0.3	8.6 ± 0.5
6	4.7 ± 0.6	6.8 ± 0.3
9	3.3 ± 0.3	5.7 ± 0.6
13	1.2 ± 0.5	4.5 ± 0.2
24	0.9 ± 0.5	3.1 ± 0.3

[a] The activity of the *B. bivius* 1127 β-lactamase extract was 200 U/ml.
[b] Standard deviation of the mean of duplicate assays.

Effect of inoculum size. An important effect of inoculum size on the MICs of cefoperazone was observed with *B. fragilis* 907 and *B. bivius* 1127 (Table 5). However, only *B. bivius* 1127, which has the most β-lactamase activity against moxalactam and cefoxitin, generated an inoculum effect with more than a fourfold increase in the MICs of these agents.

DISCUSSION

Cefoxitin and moxalactam are extremely resistant to β-lactamase hydrolysis (7, 28, 29), and their therapeutic efficacy in anaerobic infections involving *B. fragilis* has been demonstrated (6, 21, 35). Many in vitro studies have shown the inefficiency of *B. fragilis* β-lactamase in hydrolyzing β-lactams bearing a 7-α-methoxy group (10, 11, 27, 30, 37), although investigators (8, 40) have isolated rare cefoxitin- and moxalactam-resistant *B. fragilis* strains. It was shown

TABLE 4. Relative hydrolysis rates of nitrocefin (10^{-4} M) by crude extracts preincubated with or without cefoxitin or moxalactam (each at 15 μg/ml)

Treatment[a]	Relative hydrolysis rate (%)[b] with indicated antibiotic treatment by crude extracts of strain:							
	B. bivius 396		*B. bivius* 1127		*B. fragilis* 434		*B. fragilis* 907	
	Cefoxitin (135 U/ml[c])	Moxalactam (125 U/ml)	Cefoxitin (850 U/ml)	Moxalactam (1,000 U/ml)	Cefoxitin (790 U/ml)	Moxalactam (720 U/ml)	Cefoxitin (893 U/ml)	Moxalactam (2,100 U/ml)
Extract and β-lactam preincubated separately	1.9 ± 0.9[d]	8.1	1.9 ± 0.7	29.6	0.9	ND[e]	0.6 ± 0.2	0.6 ± 0.3
Extract and β-lactam preincubated together	15.2 ± 1.7	60.3	83.6 ± 3.4	92	0.9	ND	2.8 ± 2.0	0.4 ± 0.2

[a] Extract and β-lactam were preincubated separately or together for 12 h at 37°C before the addition of nitrocefin. When incubated separately, they were mixed before nitrocefin addition.
[b] Relative hydrolysis rate measured spectrophotometrically at 482 nm and expressed as a percentage of the hydrolysis rate by the extract alone (100%).
[c] β-lactamase activity measured spectrophotometrically against cephaloridine.
[d] Standard deviation of the mean of duplicate assays.
[e] ND, Not done.

by Yotsuji et al. (40) that some of these strains produce a new β-lactamase able to hydrolyze many β-lactams, including cefoxitin, moxalactam, and imipenem. More recently, the same group found slow β-lactamase inactivation of moxalactam and other cephamycins, but not of cefoxitin, in some strains of *B. fragilis* by using a microbiological assay and high-pressure liquid chromatography (41). In our study, we used *B. fragilis* strains showing typical β-lactamase substrate profiles as compared with profiles of other strains previously used (3, 18, 26, 27, 31, 32, 37). Despite the relatively high β-lactamase activity of these strains (>300 U/mg of cells [dry weight] against cephaloridine and cephalothin), we were unable to demonstrate enzymatic activity against cefoxitin and moxalactam in our different assays. Also, the moxalactam MICs for our *B. fragilis* strains were lower than the ones reported by Yotsuji et al. (41) for their strains with the most activity against the agent (12.5 μg/ml), and clavulanic acid did not potentiate the susceptibilities of our strains to moxalactam. Finally, high-pressure liquid chromatography techniques for β-lactamase assays may be more sensitive than the assays used in this study.

TABLE 5. Effect of inoculum size on cefoperazone, cefoxitin, and moxalactam MICs for *B. bivius* and *B. fragilis*

Strain and inoculum size (CFU/ml)	MIC (μg/ml)		
	Cefoperazone	Cefoxitin	Moxalactam
B. bivius 396			
3×10^4	1	2	8
4×10^5	1	2	8
3×10^7	1	2	16
B. bivius 537			
3×10^4	1	1	2
3×10^5	2	2	8
4×10^7	2	2	8
B. bivius 1127			
2.5×10^4	8	8	8
2.5×10^5	8	8	16
2.5×10^7	256	64	64
B. fragilis 434			
2×10^5	256	4	4
2×10^6	256	8	4
2×10^8	512	8	4
B. fragilis 907			
1.5×10^5	128	8	8
1.5×10^6	256	8	8
1.5×10^8	4,096	16	8

In contrast, the three *B. bivius* strains used in this study and showing different levels of β-lactamase production were all able to hydrolyze cefoxitin and moxalactam to some extent. The specific hydrolysis of the β-lactam ring of these molecules was demonstrated by the use of specific UV absorption as in a conventional method (24). In addition, the modified spectrophotometric assay described in this paper allowed the detection of slow hydrolysis.

The use of a microbiological assay permitted observation of the biological inactivation of cefoxitin and moxalactam by *B. bivius* crude extracts. The addition of clavulanic acid in such assays prevented the inactivation; this suggested a role for β-lactamase in this phenomenon since clavulanic acid is a potent inhibitor of *B. fragilis* (31, 32, 37) and *B. bivius* (16, 37) β-lactamases.

Finally, the observations that cefoxitin and moxalactam inhibited the *B. bivius* β-lactamase activity on nitrocefin and that this inhibitory activity disappeared with a preincubation of the antibiotic with the β-lactamase extract suggested the hydrolysis of both β-lactams during the preincubation (12 h at 37°C). The primary inhibition step can be easily explained since cefoxitin and moxalactam have been described as competitive inhibitors (5, 28). Both cefoxitin and moxalactam can be slowly hydrolyzed by *B. bivius* β-lactamase. Other specific characteristics of *B. bivius* β-lactamase (inhibition profile and pI) have been reported previously (16).

To the best of our knowledge, no therapeutic failures have been observed with cefoxitin or moxalactam in the treatment of *B. bivius* infections. In clinical use, moxalactam has been found to be effective in obstetric and gynecologic infections in which *B. bivius* was implicated (9, 34). Moreover, in the present study cefoxitin and moxalactam still showed in vitro activity against the *B. bivius* strains below the breakpoints for resistance to these agents (36).

The clinical relevance of the slow hydrolysis of cefoxitin and moxalactam by the *B. bivius* enzyme must be considered since 75 to 85% of the clinical isolates produce a β-lactamase (16, 33). The observed inoculum effect of *B. bivius* on the MICs of cefoxitin and moxalactam in the present study is intriguing, and individuals should be aware of such an effect when treating high-inoculum infections in humans.

Vu and Nikaido (38) recently suggested a role for an apparently slow β-lactam hydrolysis in the mechanism of resistance of a β-lactamase-constitutive *Enterobacter cloacae* strain to most of the broad-spectrum β-lactams. They suggested that hydrolysis may be more important for the

expression of the resistance phenotype if low extracellular concentrations of the antibiotics were used for the evaluation of the phenomenon. In turn, we speculate that even slow *B. bivius* β-lactamase activity could be sufficient to maintain cefoxitin or moxalactam periplasmic concentrations below their effective inhibitory levels if at the infection site a diffusion barrier regulates extracellular concentration of these β-lactams. Inoculum size, along with slow β-lactamase activity, may thus contribute to cefoxitin or moxalactam therapeutic failure in *B. bivius* infections, although there is a lack of clinical evidence supporting this idea.

In summary, this report provides further evidence regarding the different properties of *B. fragilis* and *B. bivius* β-lactamases. We confirmed the *B. bivius* β-lactamase activity against cefoxitin and moxalactam suggested in a previous study (16). This β-lactamase activity was real but slow when compared with the hydrolysis observed for cephaloridine, cephalothin, and cefamandole. Clavulanic acid could inhibit this β-lactamase activity, as demonstrated in a microbiological assay. Finally, this report introduces simple techniques that may be useful for the detection of slow β-lactamase activity when high-pressure liquid chromatography is not available.

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