BIOCHEMICAL PROPERTIES OF β-LACTAMASE PRODUCED BY *BACTEROIDES DISTASONIS*

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Three of 29 clinical isolates of *Bacteroides distasonis* strains, GAI2095, GAI2361 and GAI6270 were found to produce high levels of β-lactamase relative to the remaining 26 strains. The enzymes from these strains showed a broad substrate profile, hydrolyzing cephaloridine, cephalothin, cefazolin, oxyimino-cephalosporins such as cefuroxime, cefotaxime and cefmenoxime, and piperacillin at high rates. Examination of the substrate profiles indicated that the enzymes were mainly oxyimino-cephalosporinases. Inactivation of latamoxef over a long time by crude enzyme extracts of GAI2095 and GAI2361 was detected by a microbiological assay. The enzyme activities were inhibited by imipenem, cefoxitin, clavulanic acid and sulbactam but not by EDTA. The majority of β-lactamase activity was found in the cell extract prepared by ultrasonic treatment, especially in the precipitate by ultracentrifugation including membrane fraction. When the cells of *B. distasonis* were subjected to osmotic shock, negligible levels of β-lactamase activity were found in the supernatant fluid. The enzymes appeared to be tightly associated with the cell envelope since detergents were required to elute these activities.

Members of the *Bacteroides fragilis* group have been recognized as important opportunistic pathogens and are known to be moderately or highly resistant to β-lactam antibiotics[1].

β-Lactamases produced by Gram-positive and Gram-negative bacteria are considered to be the most important biochemical mechanism of resistance to β-lactam antibiotics. Several investigations have already described β-lactamases from *Bacteroides* species[2~7]. However, only limited work has been done on the β-lactamase from *Bacteroides distasonis*.

In this paper, we deal with the biochemical properties of β-lactamase produced by *B. distasonis*, and also investigate the cellular location of the enzyme.

Materials and Methods

Bacterial Strains

Twenty-nine strains of *B. distasonis* isolated from clinical specimens were used. All of the strains were identified by the method of the Virginia Polytechnic Institute and were given our laboratory strain numbers. *B. fragilis* GAI558 (a clinical isolate) was chosen as representative control strain produced the typical oxyimino-cephalosporinase. These strains were stored in skim milk (10%) at −70°C.

Antibiotics

Benzylpenicillin, ampicillin, cloxacillin, piperacillin, sulbenicillin, cephaloridine, cephalothin, cefazolin, cefuroxime, cefotaxime, cefmenoxime, cefoxitin and latamoxef were commercially available materials. The following compounds were gifts: Imipenem from Banyu Pharmaceutical Co., Ltd.; sulbactam from Pfizer Taito Co., Ltd.; and clavulanic acid from Beecham Research Laboratories.

Susceptibility Testing

Drug resistance was determined by an agar dilution method with GAM agar (Nissui Seiyaku

Co., Ltd., Tokyo). Of a diluted culture (*ca.* 10^6 cells per ml), one loopful (*ca.* 5 μl) was inoculated onto assay media containing serial 2-fold dilutions of drug. MICs of each drug were scored after incubation at 37°C for 24 hours in an anaerobic chamber.

Culture Condition

Cells were grown in GAM broth (Nissui Seiyaku Co., Ltd., Tokyo). Usually, a 10% inoculum from an overnight starter culture was added to bottles of broth and the cultures were grown statically at 37°C in an anaerobic chamber.

Preparation of Crude β-Lactamase

Cultures in the late exponential phase of growth were harvested by centrifugation at $5,000 \times g$ for 15 minutes at 4°C and washed twice in 0.1 M phosphate buffer (pH 7.0). The cells were resuspended at about 0.05 of the volume of the original culture, in 0.1 M phosphate buffer (pH 7.0) and sonicated in an ultrasonic disruptor (TOMY SEIKO) for three periods of 1 minute each at 0°C. The supernatants after centrifugation (10,000 or $20,000 \times g$, 20 minutes at 4°C) constituted the crude enzyme.

Enzyme Assay

β-Lactamase activity was assayed by a modification of the micro-iodometric method of NOVICK[8] with penicillins as substrates or by a direct spectrophotometric method of WALEY[9] with cephalosporins as substrates. One U of β-lactamase activity was expressed as 1 μmol of substrate hydrolyzed per minute at 30°C in 50 mM phosphate buffer. The *Km* and the maximum rate of hydrolysis (relative Vmax) values were determined from Lineweaver-Burk plots.

Alkaline phosphatase activity was measured by using *p*-nitrophenylphosphate as the substrate; μmol of end product (*p*-nitrophenol) were estimated.

Antibiotic Assay

Concentrations of cephamycins and imipenem were measured by a microbiological assay with *Escherichia coli* NIHJ as the test strain.

Inhibition Study

The inhibition of the enzyme activity in the crude extract by various inhibitors was estimated by the micro-iodometric method using 200 μM piperacillin or cephaloridine. The inhibition activities of 0.1, 1.0 and 10.0 μM solutions of imipenem, cefoxitin, clavulanic acid and sulbactam, and 10 mM EDTA were determined after preincubation for 5 minutes at 30°C.

Isoelectric Focusing

Isoelectric focusing of crude sonicates was performed on a sucrose/ampholytes gradient (pH 3~10). The experiment was carried out at 4°C in the LKB8100 column over 24 hours at 900 V.

Solubilization of β-Lactamase Activity

To elute β-lactamase activity from the centrifugal precipitates, the precipitates were resuspended in each of the following: 1 M NaCl, 2% Triton X-100, 2% sodium dodecyl sulfate, 2% sodium deoxycholate. After 20 minutes at room temperature, the mixture was centrifuged at $20,000 \times g$ for 30 minutes at 4°C. The supernatant and resuspended pellet were assayed for β-lactamase activity.

Purification of β-Lactamase

Preliminary attempts to purify the crude β-lactamase preparations involved treatment of the enzyme with 2% sodium deoxycholate and elution through a Toyopearl HW-55F column (2.0×85 cm) which was equilibrated at 4°C in glycine - EDTA buffer (pH 9.0), containing 0.5% deoxycholate and eluted with the same buffer.

The β-lactamase was partially purified from the crude enzyme preparations described above by gel filtration on Toyopearl HW-65F and HW-55F columns. Toyopearl HW-65F was equilibrated at 4°C in 10 mM phosphate buffer (pH 7.0), containing 0.1 M KCl in a column (2.5×90 cm) and eluted with the same buffer. The concentrated elute from the Toyopearl HW-65F column was applied to the Toyopearl HW-55F column described above and eluted with the same buffer. Fractions showing β-lactamase activity were pooled and dialyzed against 10 mM phosphate buffer (pH 7.0). The

enzyme preparation was used for kinetics.

Cellular Location of the Enzyme

The cellular location of β-lactamase in *B. distasonis* was explored in two ways.

(i) Osmotic shock was performed by the method described by NEU and CHOU[10]. The washed late exponential-phase cells were suspended in 20% sucrose - 30 mM Tris buffer (pH 7.3). EDTA was added to a concentration of 0.5 mM, and after 5 to 10 minutes of mixing, the cells were removed by centrifugation. The pellet of cells was resuspended in water at 0°C and mixed for 5 to 10 minutes. The cells were removed by centrifugation. To elute the enzyme from the centrifugal precipitate, the precipitate was resuspended in 2% Triton X-100.

Viability was determined on serial dilutions, made in GAM broth, which were plated on GAM agar.

(ii) The supernatants after centrifugation of the sonicated cell suspensions at $10,000 \times g$ for 20 minutes at 4°C were ultracentrifuged at $140,000 \times g$ for 2 hours at 4°C. To elute β-lactamase from ultracentrifugal precipitates, the precipitates were resuspended in 2% Triton X-100. After 30 minutes at room temperature, the mixture was ultracentrifuged at $140,000 \times g$ for 2 hours at 4°C.

All supernatant fluids and cells were reserved for enzyme assay.

Results

Susceptibility to β-Lactam Antibiotics

Twenty-nine strains of *B. distasonis* were screened with a nitrocefin assay[11] to determine their ability to produce β-lactamase. Strains GAI2095, GAI2361 and GAI6270 were the best producers of β-lactamase and were chosen for the biochemical studies. Resistance levels of these strains to β-lactam antibiotics are shown in Table 1. *B. distasonis* GAI2095, GAI2361 and GAI6270 were highly resistant to penicillins and cephalosporins except cefoxitin and imipenem. *B. fragilis* GAI558, which was chosen for comparison, was moderately resistant to penicillins and cephalosporins, except cefoxitin, latamoxef and imipenem.

β-Lactamase Formation during the Growth

The appearance of β-lactamase activity in the surrounding medium and crude extract prepara-

Table 1. *In vitro* activity of β-lactam antibiotics against *Bacteroides distasonis* strains.

Compound	MIC (μg/ml)			
	B. distasonis			*B. fragilis* GAI558
	GAI2095	GAI2361	GAI6270	
Cephaloridine	1,600	1,600	800	400
Cephalothin	1,600	1,600	800	400
Cefazolin	1,600	1,600	400	400
Cefuroxime	1,600	1,600	1,600	200
Cefoxitin	50	50	50	6.25
Latamoxef	1,600	1,600	100	12.5
Cefotaxime	400	400	400	100
Cefmenoxime	800	400	400	100
Benzylpenicillin	3,200	3,200	1,600	400
Ampicillin	3,200	3,200	3,200	200
Carbenicillin	>3,200	>3,200	3,200	400
Cloxacillin	>3,200	>3,200	1,600	400
Sulbenicillin	3,200	3,200	1,600	200
Piperacillin	400	200	400	50
Imipenem	1.56	1.56	1.56	0.39

tions during different phases of growth of *B. distasonis* GAI2095 was studied. The maximal activity in the crude cell extracts was obtained in the late exponential phase after 7-hour incubation. Enzyme activity was not detected in the surrounding medium during cell growth.

Solubilization of β-Lactamase Activity

The β-lactamase activities of *B. distasonis* GAI2095, GAI2361 and GAI6270 could be extracted from the centrifugal precipitate at almost the same level with 2% Triton X-100 and 2% deoxycholate, but not with 1 M NaCl. The enzyme activities were lost when 2% sodium dodecyl sulfate was added to the pellet. Approximately 5% of β-lactamase activity still remained in the Triton X-100-treated precipitates. Triton X-100 and deoxycholate in these concentrations had no effect on the *B. distasonis* β-lactamase activity.

Preliminary Purification of β-Lactamase

Fig. 1 (A) and (B) represent the UV absorption and β-lactamase activity profile during gel filtration of crude sonicates from *B. fragilis* GAI558 (A) and *B. distasonis* GAI2095 (B) on Toyopearl HW-55F. Peak enzyme activity for the extract of strain GAI558 was eluted in fraction 71.

However, the enzyme activity from strain GAI2095 was eluted in the void volume of the column. The range of the molecular weight calibration of Toyopearl HW-55F is 1×10^3 to 7×10^5. Therefore, the apparent molecular weight of the enzyme was $>7\times10^5$.

Fig. 1 (C) shows the UV absorption and β-lactamase activity profile of the same crude sonicated extracts after treatment with deoxycholate as Fig. 1 (B). The β-lactamase activity peak shifted from the void volume of the column to fractions 58.

Calibration of the Toyopearl HW-55F column in the presence of deoxycholate with the protein standards indicated that the molecular weight of the deoxycholate-treated β-lactamase of *B. distasonis* GAI2095 was approximately 43,000.

Enzymatic Properties of the Enzyme

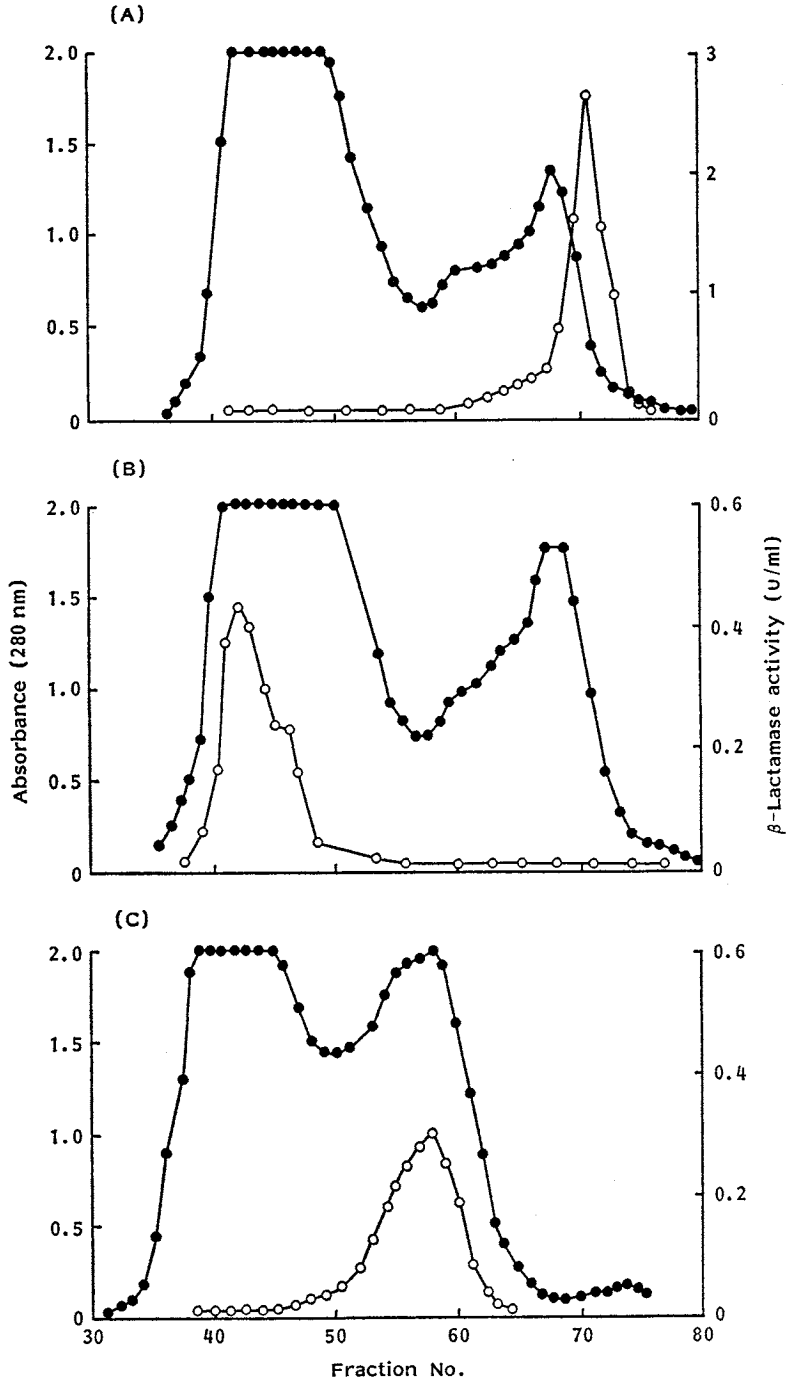
The kinetic parameters (*Km* and relative Vmax) of partially purified enzymes from *B. distasonis* and *B. fragilis* are shown in Table 2. The enzymes from *B. distasonis* GAI2095 and GAI2361 showed high activity against cephaloridine, cefazolin, cefuroxime, cefotaxime and piperacillin. Cephalothin, cefmenoxime and penicillins such as benzylpenicillin, ampicillin, carbenicillin, cloxacillin and sulbenicillin were also hydrolyzed, although the rates of hydrolysis were lower than that of cephaloridine. The enzymes from *B. distasonis* GAI6270 and *B. fragilis* GAI558 showed high activity against cephalosporins, including oxyimino-cephalosporins. By the spectrophotometric method, detectable hydrolysis of cefoxitin, latamoxef and imipenem by the enzymes was not observed.

Hydrolysis of cefoxitin, latamoxef and imipenem was studied with crude enzyme extracts from *B. distasonis* strains. Each antibiotic (at a final concentration of 100 μM) was added to the crude enzyme extract. After 6 hours of incubation at 30°C, the residual amounts of the antibiotics were determined by a microbiological assay. Latamoxef was hydrolyzed by crude enzyme extracts from *B. distasonis* GAI2095 and GAI2361 (Table 3).

A number of enzyme inhibitors were tested for their inhibitory effect on the activity of the enzymes. The enzyme activities were almost completely inhibited by 0.1 μM clavulanic acid, sulbactam, cefoxitin and imipenem, but not by 10 mM EDTA.

Fig. 1. Toyopearl HW-55F column chromatography of crude sonicates from *Bacteroides fragilis* GAI558 (A) and *B. distasonis* GAI2095 (B), and the deoxycholate-treated enzyme of strain GAI2095 (C).

● UV absorbance (280 nm), ○ β-lactamase activity.



Isoelectric Focusing

Crude sonicates of strains GAI2095 and GAI2361 showed two peaks of β-lactamase, with isoelectric points of 4.0 and 3.5, while strain GAI6270 displayed one peak at 3.7.

Table 2. Kinetic parameters of crude β-lactamase[a] from *Bacteroides distasonis* strains.

Compound	*B. distasonis* GAI2095		*B. distasonis* GAI2361		*B. distasonis* GAI6270		*B. fragilis* GAI558	
	Vmax[b]	*Km* (μM)	Vmax	*Km* (μM)	Vmax	*Km* (μM)	Vmax	*Km* (μM)
Cephaloridine	100	17.4	100	17.0	100	112	100	112
Cephalothin	34	4.8	40	8.4	21	18.1	78	72.5
Cefazolin	270	45.9	225	44.6	20	40.3	57	196
Cefuroxime	201	90.9	252	192	63	75.2	56	109
Cefotaxime	104	84.0	93	65.8	28	28.3	8	69.4
Cefmenoxime	37	27.9	46	35.5	34	27.2	10	76.3
Benzylpenicillin	17	18.3	21	8.3	6.7	31.6	1.7	10.8
Ampicillin	22	12.2	38	17.6	6.3	10.0	0.77	10.8
Carbenicillin	11	109	12	86.2	2.2	43.9	0.44	13.3
Cloxacillin	7.8	45.9	9.4	47.2	2.2	24.2	—	ND
Sulbenicillin	5.6	63.3	5.4	67.1	1.0	41.8	0.56	38.2
Piperacillin	238	194	220	333	17	46.5	—	ND

[a] β-Lactamase activities for cephaloridine of *B. distasonis* GAI2095, GAI2361 and GAI6270, and *B. fragilis* GAI558 were 0.36, 0.26, 0.78 and 1.71 U/mg of protein, respectively.

[b] Hydrolysis is expressed as the relative rate of hydrolysis, taking the hydrolysis of cephaloridine as 100.

ND: Not detectable.

Table 3. Hydrolysis of antibiotics by crude enzymes from *Bacteroides distasonis* strains.

Organism	Residual amounts (%)[a]		
	Latamoxef	Cefoxitin	Imipenem
B. distasonis GAI2095	4 (1,600)[b]	78 (50)	75 (1.56)
B. distasonis GAI2361	6 (1,600)	82 (50)	72 (1.56)
B. distasonis GAI6270	89 (100)	98 (50)	71 (1.65)
B. fragilis GAI558	95 (12.5)	95 (6.25)	77 (0.39)
Control[c]	99	99	72

[a] Residual amounts of cephamycins and imipenem were determined by a microbiological assay.

[b] MIC (μg/ml).

[c] 50 mM phosphate buffer (pH 7).

Cellular Location of the Enzyme

β-Lactamases in aerobic Gram-negative rods are considered to be located in the periplasmic space, as well as other enzyme (alkaline phosphatase, 3′-nucleotidase and 5′-nucleotidase). Alkaline phosphatase was measured in parallel as a reference enzyme for periplasmically located enzymes.

Cells of *B. distasonis* GAI2095, GAI2361 and GAI6270, and *B. fragilis* GAI558 were subjected to osmotic shock. Fig. 2 shows that a high level of alkaline phosphatase activity of strains GAI2095, GAI2361, GAI6270 and GAI558 was found in the water shocking fluid (70~95% of the total activity), when the total enzyme was determined as a total of the activities in the water shocking fluid and Triton X-100-treated extracts from the sedimented materials. Similarly, strain GAI558 released a high level of β-lactamase in the water shocking fluid (60%), whereas strains GAI2095, GAI2361 and GAI6270 released only 3~12% of total activity. Remaining cell-bound activity (88~97%) was found in the cell lysate. More than 99% of *B. distasonis* and *B. fragilis* cells were irrevocably damaged by the EDTA treatment and the cold shock.

When sonicated cell suspensions were centrifuged for 20 minutes at 10,000×*g* and the supernatants were ultracentrifuged for 2 hours at 140,000×*g*, 98% of β-lactamase activity of strain GAI558 ap-

Fig. 2. Release of enzymes from *Bacteroides distasonis* cells by osmotic shock.

Open bar: β-Lactamase, shadow bar: alkaline phosphatase.

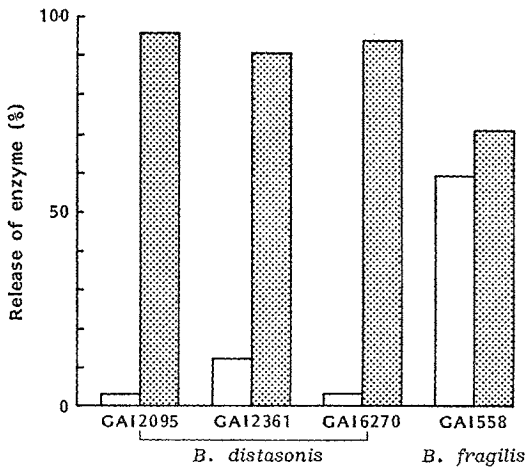
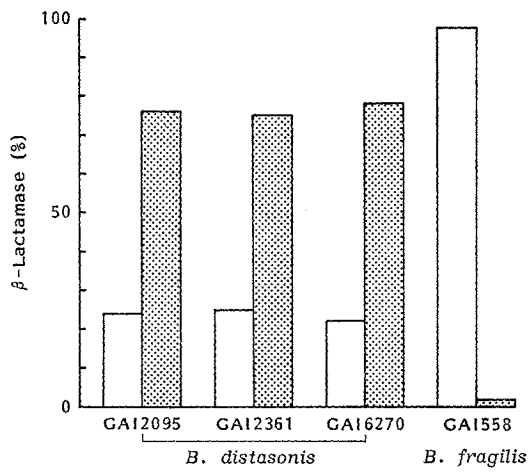


Fig. 3. Cellular location of β-lactamase in *Bacteroides distasonis*.

Open bar: Supernatant (140,000×*g*, 2 hours), shadow bar: precipitate.



peared in the supernatant, but in the case of strains GAI2095, GAI2361 and GAI6270, over 75% of the activity was found in the pellet (Fig. 3).

Discussion

Several authors have suggested that many strains of *B. fragilis* group produce β-lactamase that contributed to their resistance to β-lactam antibiotics, and there is a general correlation between the β-lactamase activity and the strain's antibiotic MIC[3~5]. Our results confirmed *B. distasonis* GAI2095, GAI2361 and GAI6270 were highly resistant to penicillins and cephalosporins, including oxyimino-cephalosporins and latamoxef, and also β-lactamase high producer. The β-lactamases of GAI2095, GAI2361 and GAI6270 showed relatively high activity against cephalosporins, especially oxyimino-cephalosporins. The substrate profiles and isoelectric points obtained for strains GAI2095 and GAI2361 seem almost identical, and are different from that of strain GAI6270. By the spectrophotometric method, detectable hydrolysis of latamoxef was not observed.

However, inactivation of latamoxef over a long time by crude enzyme extracts of GAI2095 and GAI2361 was detected by the microbiological assay. MITSUHASHI *et al.*[12] suggested that inactivation due to enzymatic hydrolysis over a long time may play an important role in resistance to some cephamycins in strains of *B. fragilis*. We conclude that the enzymatic hydrolysis of latamoxef over a long time may contribute to the resistance of these strains.

The sensitivity to different β-lactamase inhibitors was similar to that of the enzymes from the other *B. fragilis* group. They were inhibited by cefoxitin, clavulanic acid and sulbactam at low concentrations but not by EDTA.

MITSUHASHI *et al.* reported that oxyimino-cephalosporins were hydrolyzed at high rates by β-lactamase from *B. fragilis*[3], *Proteus vulgaris*[13], *Pseudomonas cepacia*[14], *Pseudomonas maltophilia*[15], *Flavobacterium odoratum*[16], *Flavobacterium meningosepticum*[17] and *Legionella gormanii*[18]. These enzymes can be divided into two subgroups, type I (*B. fragilis*, *P. vulgaris*, *P. cepacia*, L-2 from *P. maltophilia* and *F. meningosepticum*) and type II (*F. odoratum*, L-1 from *P. maltophilia* and *L. gormanii*), by substrate and inhibitor profiles. According to this classification, the *B. distasonis* β-lactamase belongs to type I.

Results from the studies of cellular location of the enzyme suggest that the enzyme was tightly associated with the cell envelope, possibly membrane-bound. The enzyme was not released into the

surrounding medium during growth. Only levels of the enzyme were released by osmotic shock. The enzymatic activity remains associated with the sedimented material after ultracentrifugation and the enzyme could be solubilized with Triton X-100 from this precipitate. The cellular location of the *B. distasonis* enzyme seems to be distinct from that of the *B. fragilis* enzyme which was periplasmically located or in loose association with the cell envelope[2].

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