β-LACTAMASE AND β-LACTAM ANTIBIOTICS RESISTANCE IN *ACINETOBACTER ANITRATUM* (SYN.: *A. CALCOACETICUS*)

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The cephalosporin β-lactamase (cephalosporinase) produced by *Acinetobacter* was studied. The enzyme was partially purified by means of column chromatography and its properties were investigated. The enzyme was induced by benzylpenicillin, 6-aminopenicillanic acid and cephaloridine. Its molecular weight is 30,000, its optimal temperature 40°C, and its optimal pH 7.25~7.50. Substrate specificity studies using various cephalosporins and penicillins, showed that the enzyme functioned as a cephalosporinase rather than penicillinase.

Gram-negative coccobacilli, such as *Acinetobacter anitratum* (syn.: *A. calcoaceticus*), have recently been isolated with increased frequency from clinical specimens[1]. Many clinical isolates of this species were found to be resistant to penicillins and cephalosporins, as well as to other antibiotics. We have attempted to examine the enzymological and physicochemical properties of the β-lactamase produced by *Acinetobacter* and its role in the bacterial resistance to penicillins and cephalosporins.

Materials and Methods

Bacterial strains: *Acinetobacter anitratum* NCTC 7844 and *Acinetobacter lwoffii* NCTC 5866 were obtained from The National Collection of Type Cultures; other *Acinetobacter* strains were isolated from clinical sources.

Bacterial cultivation and harvesting of cells: Bacteria were grown overnight in 500 ml of Heart Infusion Broth (Difco) at 37°C. The culture was diluted with 5 liters of fresh broth and grown at the same temperature until the absorbance at 610 nm (Shimadzu Bausch & Lomb Spectronic-20) reached 0.5~0.6. The cells were harvested by centrifugation and washed twice with 0.05 M sodium phosphate buffer (pH 6.9).

Drugs: Benzylpenicillin (PC-G), ampicillin (AB-PC), cephalexin (CEX), chloramphenicol (CP) and streptomycin (SM) were supplied by Toyo Jozo Co., Ltd. Cephaloridine (CER) was purchased from Torii Pharmaceuticals Co. Ltd., Tokyo, cefazolin (CEZ) from Fujisawa Pharmaceuticals Co., Ltd., Osaka, kanamycin (KM) from Meiji Seika Co., Ltd., Tokyo, and tetracycline (TC) from Takeda Co., Ltd., Osaka.

Drug resistance: Drug resistance was determined by the agar dilution method and level of resistance was expressed as the minimum inhibitory concentration (in μg/ml) of each drug. An overnight culture in Trypticase Soy Broth (B.B.L.) was diluted 100-fold with the same broth and one loopful of the diluted culture was spotted on agar plates containing serial two-fold dilutions of each drug. After 18 hours of incubation at 37°C, the spots were examined for the presence or absence of bacterial growth.

Preparation of column: Sephadex G-75 (Pharmacia, Uppsala, Sweden) was prepared and packed in column as described in the Pharmacia manual. Carboxymethyl (CM) Sephadex C-50 was washed before use and packed as above.

Determination of molecular weight: The molecular weight of the enzyme was determined by

gel filtration on a Sephadex G-75 column according to the method of ANDREUS[2]. Bovine serum albumin (M.W. 67,000), α-chymotrypsinogen (M.W. 25,000), and cytochrome C (M.W. 12,400) were used as reference markers. The void volume was estimated by the use of Blue Dextran 2000.

Enzyme assay: Cephalosporinase activity was determined iodometrically at pH 6.90 by a modification of PERRET's method[3]. One unit of enzyme activity was defined as the amount of enzyme which hydrolyzes 1 μmole of CER in 1 hour at 30°C in 0.1 M phosphate buffer (pH 6.90) with 8 mM substrate.

Determination of protein: Protein was determined by the method of LOWRY *et al*[4] using bovine serum albumin as the standard or by measuring the absorbance at 280 nm.

Results

Penicillin and Cephalosporin Resistance in *Acinetobacter* Species

Six strains of *A. anitratum* and *A. lwoffii* NCTC 5866, and one *lwoffii* type isolate (0926) were examined with respect to their levels of resistance to antibiotics and their ability to produce β-lactamase. The results are shown in Table 1.

Table 1. Levels of cephalosporinase production and resistance to β-lactam antibiotics in *A. anitratum* and *A. lwoffii* strains

Strain	CSase activity (units/mg of dry cells)[a]	Minimum inhibitory concentration (μg/ml)[b]								
		CER*	CEX	CEZ	AB-PC	PC-G	CP	TC	SM	KM
A. anitratum NCTC 7844	130	800	800	800	400	800	200	6.3	800	6.3
A. lwoffii NCTC 5866	—	1.6	3.1	6.3	3.1	1.6	1.6	3.1	1.6	3.1
A. anitratum 0917	31	200	800	400	100	200	100	6.3	6.3	6.3
A. anitratum 0933	16	100	400	400	50	100	200	50	800	200
A. anitratum 0919	30	100	400	400	50	200	200	3.1	50	3.1
A. anitratum 0932	17	200	800	400	100	200	100	3.1	6.3	6.3
A. anitratum 0922	16	200	800	400	50	100	200	3.1	50	3.1
A. anitratum 0926	—	3.1	12.5	12.5	1.6	3.1	6.3	3.1	100	1.6

a Specific activities of cephalosporinase in sonically disrupted cells (units/mg of dry weight of cells)
b The levels of drug resistance are expressed as described in Materials and Methods.
* CER: cephaloridine, CEX: cephalexin, CEZ: cefazolin, AB-PC: ampicillin, PC-G: benzylpenicillin, CP: chloramphenicol, TC: tetracycline, SM: streptomycin, KM: kanamycin.

Table 2. Hydrolysis (percent) of various cephalosporins and penicillins by cephalosporinase isolated from various *A. anitratum* strains

	Rate of hydrolysis[a]					
	CER	CEX	CEZ	CET	AB-PC	PC-G
A. anitratum NCTC 7844	100	59	108	63	1	3
A. anitratum 0917	100	86	193	125	—[b]	7
A. anitratum 0933	100	41	91	33	8	—
A. anitratum 0919	100	100	178	100	—	6
A. anitratum 0922	100	85	162	153	—	1
A. anitratum 7844 Km value (μM)	250	53			30	45

a The rate of hydrolysis was determined at 30°C in 0.1 M phosphate buffer (pH 6.9) with a substrate concentration of 8 mM. The rates given are expressed at the percentage of hydrolysis of cephaloridine.
b Not detected.

The MICs of penicillins and cephalosporins for all the *A. anitratum* strains were in excess of 100 μg/ml. On the other hand, both *A. lwoffii* strains were susceptible to penicillins and cephalosporins. All *A. anitratum* strains were found to produce β-lactamase, and the levels of β-lactamase present were roughly correlated with the levels of resistance to β-lactam antibiotics.

Substrate Profile: Five *A. anitratum* strains were selected for further study. Crude β-lactamases prepared from these strains were examined for their substrate specificities. The relative rates of hydrolysis of four cephalosporins and two penicillins, together with the MICHAELIS constant (Km) for some of the substrates, are shown in Table 2. The Km values were calculated from LINEWEAVER-BURK plots[5]. All the enzymes showed high cephalosporinase activities, but very low penicillinase activities. The enzyme activities of these strains increased about 5~10 fold after treatment with 100 μg of PC-G, CER or 6-aminopenicillanic acid for 1 hour at 37°C.

Partial Purification of β-Lactamase from *A. anitratum* NCTC 7844

Stage 1. The bacterial cells from the middle of the exponential growth phase were harvested by centrifugation, washed with 50 ml of 0.05 M sodium phosphate buffer (pH 6.90) and suspended in 50 ml of the same buffer. The cells (about 10 g, wet weight) in an ice-water bath were disrupted by a ultra sonic disintegrator (Kubota model 200 M, Tokyo) at 20 kHz for 5 minutes. The treated cell suspension was centrifuged at 15,000 $\times g$ for 30 minutes at 4°C. The supernatant fraction was used for the next stage of purification.

Stage 2. To the supernatant solution from Stage 1, streptomycin sulfate (2% of total volume) was added; the mixture was centrifuged and dialyzed against distilled water at 4°C for 18 hours. The dialyzed solution (60 ml) in the cellulose tube was concentrated to 3 ml by lyophilization.

Stage 3. The concentrated enzyme solution was applied to a CM-sephadex (C-50) column (2.5 × 50 cm) equilibrated with 0.05 M sodium phosphate buffer (pH 6.9), and the column was washed with 60 ml of the same buffer. A linear gradient of NaCl (0~0.3 M) was used to elute the enzyme. The enzyme was eluted at the concentration of 0.15 M NaCl. Active fractions (60 ml) were pooled.

Stage 4. The pooled fractions from Stage 3 were dialyzed against distilled water at 4°C for 18 hours and concentrated to 5 ml. The enzyme solution was applied to a Sephadex G-75 column (2.0 × 75 cm). The contents of the column were distributed into 5-ml fractions, and each fraction was assayed for protein concentration and cephalosporinase activity. The specific activity of the purified enzyme was 1,118.0 units per mg of protein. The partial purification of *Acinetobacter* β-lactamase is summarized in Table 3.

Table 3. Summary of purification of cephalosporinase from *A. anitratum* 7844

Stage	Purification	Total activity (units)	Specific activity[b] (units/mg protein)	Recovery (%)
1	Ultrasonic disintegration. Centrifugation.	34,400[a]	30.2	100
2	Streptomycin sulfate treatment. Dialysis.	33,600	32.8	97.6
3	CM-Sephadex column chromatography	17,800	492.0	51.7
4	Sephadex G-75 column chromatography	9,000	1,118.0	26.2

a Extracted from cells cultured in 5 liters of medium.
b units/mg protein.

Fig. 1. Effect of pH on the activity of cephalosporinase from *A. anitratum* 7844.
Incubations were carried out at 30°C. The cephalosporinase in 0.1 M phosphate buffer, pH 6.9, containing 8 mM of cephaloridine as substrate, was incubated for 15 minutes. Acetate buffer (0.1 M) was used at pH 5.8.
Phosphate buffers (0.1 M) were used for the range pH 6.8 to pH 7.6, 0.1 M tris-HCl buffers at pH 8.2 ~8.7.

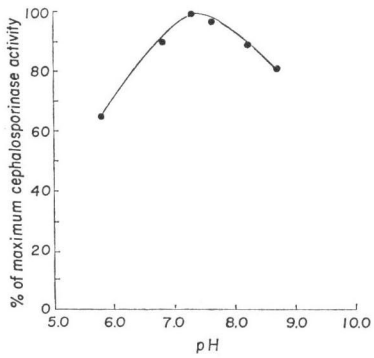

Fig. 2. Effect of temperature on the activity of cephalosporinase from *A. anitratum* 7844.
Incubations were carried out at the temperatures shown in the figure. Each incubation was for 15 minutes and the reaction was determined iodometrically.

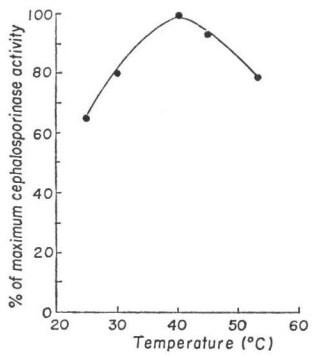

Fig. 3. Rate of heat inactivation of cephalosporinase of *A. anitratum* 7844 at 60°C.
Cephalosporinase preparation was incubated in 0.1 M phosphate buffer, pH 6.9, at 60°C for the periods shown in the figure. After incubation the samples were immediately frozen, and then the residual enzyme activity was estimated iodometrically.

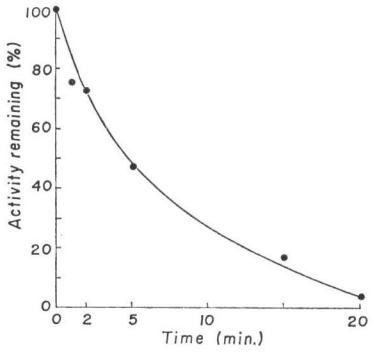

Table 4. Effects of various inhibitors on the cephalosporinase activity of *A. anitratum* 7844

Inhibitors or salts	Concentration (mM)	Inhibition (%)
Iodine	0.005	30
PCMB	1.0	—*
EDTA	1.0	—
$CuSO_4$	1.0	22
$NaNO_3$	1.0	—
NaCl	10	—

Each of the enzyme preparations was incubated in the presence or absence of each inhibitor or ion at the concentration indicated in the Table for 15 minutes at 30°C, and then assayed for enzyme activity which was determined on cephaloridine iodometrically.
* not detected.

Acinetobacter β-Lactamase

Optimal temperature and pH: The pH activity and temperature activity curves of *Acinetobactor* β-lactamase are shown in Fig. 1 and Fig. 2 respectively. Optimal pH of the enzyme was 7.25~7.50 and optimal temperature, 40°C.

Molecular weight: The molecular weight of the enzyme was estimated by gel filtration through Sephadex G-75 column. The molecular weight was estimated to be 30,000 and found to be similar to that of *Proteus vulgaris, Proteus rettgeri* and *Citrobacter freundii* cephalosporinases[6] and to be different from that of penicillinases belonging to type I and type II.

Heat stability: The rate of heat inactivation of the enzyme activity was determined at 60°C in

0.1 M sodium phosphate buffer, pH 6.90 (Fig. 3). The heat treatment of the enzyme for 2, 5 and 20 minutes resulted in retention of 72, 47 and 4% of the original activity, respectively.

Effects of inhibitors: The effects of some inorganic and organic compounds on the β-lactamase activity were determined. The results are summarized in Table 4: iodine showed a significantly inhibitory effect on the enzyme activity.

p-Chloromercuribenzoate and ethylendiaminetetraacetate showed no inhibition. $CuSO_4$ showed a slight inhibitory effect, while $NaNO_3$ did not inhibit.

Discussion

A. anitratum produces cephalosporin-inducible β-lactamase and the enzyme hydrolyzes cephalosporins more effectively than penicillins. The properties of the partially purified enzyme are as follows: molecular weight, 30,000; optimal temperature, 40°C; optimal pH, 7.25~7.50; its activity is inhibited by iodine and $CuSO_4$, but not by *p*-chloromercuribenzoate, ethylendiaminetetraacetate or $NaNO_3$.

β-Lactamases of other gram-negative bacteria, such as *Proteus morganii*, *P. vulgaris*, *Citrobacter freundii* and *Pseudomonas aeruginosa*, were characterized and their production is known to be inducible.[7,8] The *Acinetobacter* β-lactamase closely resembles that of those species in molecular weight, sensitivity to inhibitors and inducibility.

Our preliminary study on the location of the gene governing the production of cephalosporinase shows that the gene is not transferable and not cured by acridine dyes.*

The resistance to β-lactam antibiotics in gram-negative bacteria seems to be ascribable to the production of β-lactamase and also to intrinsic factors[9]. As *A. lwoffii* NCTC 5866 and one of our isolate are susceptible to β-lactam antibiotics and do not produce detectable β-lactamase, β-lactamase seems to be a major factor for the resistance to β-lactam antibiotics in *Acinetobacter*.

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* Data is not shown.