Purification and Properties of Chromosomally Mediated β -Lactamase from *Citrobacter freundii* GN7391

By M. TAJIMA,^{1*}† Y. TAKENOUCHI,² S. SUGAWARA,²
M. INOUE¹ AND S. MITSUHASHI¹

¹ Department of Microbiology, School of Medicine, Gunma University,
Maebashi, Gunma 371, Japan

² Central Research Laboratories, Sankyo Co. Ltd, Tokyo 140, Japan

(Received 26 February 1980; revised 19 May 1980)

Both a penicillinase and a cephalosporinase are present in a strain of *Citrobacter freundii* (GN7391) resistant to β -lactam antibiotics. The penicillinase was identical to the type Ia penicillinases (Type III by Richmond classification), mediated by Rms212 and R-TEM. A cephalosporinase, typical of enterobacteriaceae chromosomal β -lactamase (Type I by Richmond classification), was purified from the strain. It gave a single protein band on polyacrylamide gel electrophoresis and immunoelectrophoresis; the pI was 8.6 and its molecular weight was approximately 38000. Cysteine was not found among its amino acids. The specific activity was 388 units (mg protein)⁻¹ for the hydrolysis of cephaloridine, and the optimal pH was 8.0. Rabbit antiserum obtained against the purified enzyme showed cross-reaction with cephalosporinases produced by strains of *Enterobacter cloacae* in a neutralization test.

INTRODUCTION

β -Lactam antibiotics show relatively poor activity against *Citrobacter freundii* (Verbist, 1976; Bill & Washington, 1977). Many strains of *C. freundii* produce an inducible cephalosporinase, considered to be the 'species-specific' β -lactamase (Sawai *et al.*, 1968). Some properties of the cephalosporinase have been investigated and compared with β -lactamases of other bacteria, using crude enzyme preparations (Sawai *et al.*, 1970*b*; Newsom *et al.*, 1974).

In the present work, we have studied some of the physical, chemical and immunological properties of a β -lactamase produced by a strain of *Citrobacter freundii*. The neutralization reaction with a rabbit antiserum prepared against the purified β -lactamase is also reported.

METHODS

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

Bacterial strains and identification. All the strains of *Citrobacter freundii* studied were isolated from clinical sources and were maintained in cooked meat medium (Eiken Chemical Co., Tokyo, Japan). Species were identified according to the scheme of Cowan & Steel (1974).

Antibiotic resistance. Minimum inhibitory concentrations of various β -lactam antibiotics were determined by using twofold dilutions in heart infusion agar (Eiken Chemical Co.) with an inoculum of 10⁴ organisms.

Culture and harvesting of organisms. Trypto-soy broth (2 l; Eiken Chemical Co.) in a 5 l flask was inoculated with 100 ml of an overnight culture at 37°C and incubated on a rotary shaker at 37°C. After 4 h, the

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

bacteria were harvested by centrifuging and washed once with 0.15 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.5. The packed cells were resuspended in 200 ml 1 mM-phosphate buffer, pH 7.5 and the suspensions were treated for 4 min in an ultrasonic disintegrator (80 W, 25 kHz). The disrupted suspensions were centrifuged at 10000 g for 20 min at 5°C to remove unbroken cells and cell debris; the cell-free extract was retained as the crude enzyme preparation. Protein was determined by the Lowry method.

Purification of β -lactamase. (All buffer solutions were $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ unless otherwise stated.)

To the cell-free extract, streptomycin sulphate was added to give 1.5% (w/v). After removing the precipitate by centrifuging (10000 g for 20 min), the supernatant was dialysed overnight against distilled water and then against 2.15 M buffer, pH 7.2. The precipitate formed during dialysis was removed by centrifuging and the supernatant (step 2) was passed through a Sephadex CM-50 column (2.3 $\times$ 30 cm) equilibrated with 5 mM buffer, pH 7.2. The first peak contained a large amount of inactive protein and an R plasmid-mediated penicillinase. This penicillinase was purified by the methods of Yamanaka *et al.* (1973), with chromatography through DEAE-cellulose.

The chromosomal cephalosporinase appeared in the second peak after elution with 0.05 M buffer, pH 7.2 (step 3). This second peak was applied to another Sephadex CM-50 column (2.3 $\times$ 17 cm) and after washing with 300 ml 0.01 M buffer, pH 7.2, was eluted with 300 ml linear gradient of 0.01 to 0.05 M buffer, pH 7.2. The active fractions (eluted with 0.024 to 0.026 M buffer) were pooled and then dialysed against distilled water (step 4).

The dialysed solution (about 20 ml) was used for isoelectric focusing (see below; step 5). Fractions around the peak of enzyme activity (fractions 43 to 47, see Fig. 1) were pooled and applied to a Sephadex G-200 column (2.3 $\times$ 86 cm) equilibrated with 0.05 M-Tris/HCl/0.1 M-NaCl buffer, pH 8.0 and eluted with the same buffer (step 6). Active fractions were dialysed against distilled water and then lyophilized.

Isoelectric focusing. Isoelectric focusing was performed on a sucrose/ampholytes gradient (pH 3.5 to 10). Enzyme solution from step 4 (10 ml) mixed with 0.75 ml Ampholine in 60 ml total volume was used as the 'light solution'. The 'dense solution' contained the remaining enzyme solution, 2.25 ml Ampholine and 28 g sucrose in 60 ml total volume. The cathode (bottom) was filled with a solution consisting of 12 g sucrose, 0.2 ml H_3PO_4 and 14 ml distilled water. To prevent contact between the ampholytes and the anode (top), a solution containing 0.1 g NaOH in 10 ml distilled water was added at the top of the column. Experiments were carried out at 5°C in the LKB 8100 column over 72 h at 300 V. The column contents were then fractionated into 2 g per tube.

Polyacrylamide gel electrophoresis. The purity of the enzyme preparations was checked by discontinuous gel electrophoresis as described by Davis (1964), using 7.5% (w/v) polyacrylamide gel, pH 4.3, as the separation gel. Electrophoresis was carried out in duplicate gels: one was stained with Coomassie Brilliant Blue R250 and the other was cut into slices 4 mm thick. The slices were disrupted in 1 ml 0.01 M buffer, pH 7.5 and the β -lactamase activity was measured.

Determination of molecular weight. The molecular weight of the enzyme was determined by gel filtration through Sephadex G-200 with 0.05 M-Tris/HCl/0.1 M-NaCl buffer, pH 8.0, as described by Andrews (1964), and by sodium dodecyl sulphate (SDS)-polyacrylamide slab gel electrophoresis according to Weber & Osborn (1969), using bovine serum albumin (mol. wt 67000), ovalbumin (45000), chymotrypsinogen (25700) and lysozyme (14400) as molecular weight standards.

Assay of β -lactamase. Hydrolysis of β -lactam antibiotics was assayed spectrophotometrically (O'Callaghan *et al.*, 1969; Waley, 1974) by measuring the decrease in absorbance at the substrate specific wavelength at 30°C in 3 ml 0.01 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.5. The specific wavelengths of the substrates are given in Table 5. One unit of β -lactamase activity was defined as the amount catalysing the hydrolysis of 1 μmol substrate min^{-1} , and activities are expressed as units ($\text{mg enzyme solution}^{-1}$).

Estimation of kinetic parameters. Using the purified enzyme preparation, the K_m and $V_{\max}$ values for several substrates were estimated from Lineweaver-Burk plots. The K_m values are expressed as μM and $V_{\max}$ values as $\mu\text{mol substrate hydrolysed min}^{-1} (\text{mg protein})^{-1}$.

Immunological methods. Rabbit antisera were produced against the purified enzyme preparation from strain GN7391. The first injection, 1 mg protein in 1 ml 0.15 M-saline, was administered subcutaneously. The second subcutaneous injection, 1 mg protein in 0.5 ml saline, was emulsified with 0.5 ml complete Freund's adjuvant (Difco). After 3 weeks, a booster injection, 0.75 mg protein, was administered in the marginal ear vein. Antisera were collected about 2 weeks after the last injection.

From these antisera, gamma globulins were precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to give 50% saturation. After stirring overnight at 5°C, the precipitate was collected by centrifuging, then dissolved in and dialysed against 0.01 M-phosphate buffer, pH 7.0. The solution was passed through a column of DEAE-cellulose pre-equilibrated with the same buffer.

The effect of the antibodies on β -lactamase was determined by a neutralization test of the hydrolysing activity of the enzyme. Enzyme solution (4 units) was incubated with various amounts of antibody in 0.2 ml 0.01 M-phosphate buffer, pH 7.5 at 37°C for 20 min and then left at 5°C for 18 h.

Table 1. Minimum inhibitory concentrations of various antibiotics and β -lactamase activities of *Citrobacter freundii* strains

<i>C. freundii</i> strain	Minimum inhibitory concn ($\mu\text{g ml}^{-1}$)				Enzyme activity* (units mg^{-1})
	Cefmetazole	Cefoxitin	Cefazolin	Ampicillin	
GN299	100	200	1600	800	1.64
GN324	50	200	3200	800	3.63
GN346	100	200	1600	800	4.38
GN492	400	800	3200	800	0.45
GN517	50	100	1600	800	0.60
GN726	50	100	800	400	0.25
GN930	100	200	1600	800	0.57
GN1021	100	400	1600	400	0.18
GN1376	100	200	800	400	0.01
GN7084	200	400	> 3200	800	4.06
GN7085	50	100	1600	400	0.14
GN7090	200	400	3200	1600	0.01
GN7091	100	200	> 3200	3200	0.58
GN7092	200	400	> 3200	3200	0.45
GN7093	200	400	3200	3200	3.89
GN7099	50	200	3200	800	3.11
GN7390	> 400	> 400	3200	3200	0.22
GN7391	400	> 400	> 3200	> 3200	9.01
GN7539	50	50	1600	3200	1.14
GN7544	25	200	100	3200	0.35
GN7545	50	100	200	3200	0.43

* No inducing agent was added.

Immunoelectrophoresis was done in 1.2 % (w/v) agar containing veronal buffer, pH 8.6.

Amino acid analysis. The amino acid composition of the enzyme was determined as described by Spackman *et al.* (1958) and as previously reported (Fujii-Kuriyama *et al.*, 1977), with an amino acid analyser (Hitachi model KLA-2).

RESULTS

Resistance levels and β -lactamase activities

All the strains of *Citrobacter freundii* examined had a high degree of resistance to cefazolin and ampicillin (Table 1). Six strains (GN324, GN346, GN7084, GN7093, GN7099 and GN7391) had high activities of β -lactamase (Table 1). The β -lactamases produced by these strains hydrolysed cephaloridine, cefazolin and cephalothin more rapidly than penicillins (Table 2). Cefmetazole and cefoxitin, both cephamycin derivatives, were not hydrolysed.

Citrobacter freundii GN7391 had the highest enzyme activity and it hydrolysed not only cephalosporins but also penicillins such as penicillin G and ampicillin. This strain, which has an R-plasmid-mediated penicillinase (Tajima *et al.*, 1979) and a chromosomal cephalosporinase, was used as a source for purification of β -lactamase.

Purification of β -lactamase

Details of the purification procedure are given in Methods; a typical purification is summarized in Table 3. The penicillinase and cephalosporinase were separated in step 3. The penicillinase was purified (see Methods) and identified as a type Ia penicillinase (Sawai *et al.*, 1970a). The cephalosporinase was further purified as indicated in Table 3. After isoelectric focusing and subsequent fractionation, the pH, enzyme activity and protein concentration in each fraction were measured. The peak of enzyme activity coincided with

Table 2. *Substrate profile of β -lactamases from strains of Citrobacter freundii*

The rates of hydrolysis were determined at 30 °C with substrate concentrations of 100 μ M (cephalosporins and cephamycins) or 200 μ M (penicillins). The rates given are relative to an arbitrary value of 100 for cephaloridine.

Substrate	<i>C. freundii</i> strain					
	GN324	GN346	GN7084	GN7093	GN7099	GN7391
Cephaloridine	100	100	100	100	100	100
Cefazolin	388	518	408	418	494	436
Cephalothin	26	65	28	41	71	27
Cephalexin	23	23	22	34	41	26
Cefmetazole	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cefoxitin	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Penicillin G	2.5	4.7	1.7	42.9	3.3	26.5
Ampicillin	<0.1	<0.1	0.2	30.1	0.5	13.8
Carbenicillin	3.0	4.9	0.9	6.2	1.7	2.7

Table 3. *Summary of purification of β -lactamase from Citrobacter freundii strain GN7391*

These results were obtained for purification from a 20 l culture of strain GN7391. Activity was determined with 100 μ M-cephaloridine as the substrate.

Procedure	Total protein (mg)	Specific activity (units mg ⁻¹)	Recovery (%)
1. Ultrasonic disintegration	8430	9	100
2. Streptomycin treatment	3500	20	86.4
3. Chromatography on Sephadex CM-50	159.1	317	69.3
4. Re-chromatography on Sephadex CM-50	121.4	333	49.7
5. Isoelectric focusing	102.6	336	40.2
6. Gel filtration on Sephadex G-200	78.1	388	37.3

that of protein, at pI 8.6 (Fig. 1). Fractions around the peak (fractions 43 to 47) were pooled and applied to the Sephadex G-200 column. Elution of enzyme activity and protein coincided exactly and the specific activity in each fraction showed a constant value.

The purity of the lyophilized preparation was checked by polyacrylamide gel electrophoresis, SDS-polyacrylamide slab gel electrophoresis and immunoelectrophoresis. In polyacrylamide gel electrophoresis the enzyme activity and the stained band coincided, and SDS-polyacrylamide slab gel electrophoresis gave a single protein band. Agar gel immunoelectrophoresis using the anti-GN7391 antibody and the crude enzyme of *C. freundii* GN7391 gave a single precipitin arc, showing that the antibodies contained a single component reacting with crude enzyme; moreover, the precipitin arc for the antibody and the purified enzyme preparation was symmetrical with that of the crude enzyme.

These results indicated that the purified enzyme preparation was homogeneous.

Physicochemical properties of the purified enzyme

The molecular weight of the purified enzyme, determined by gel filtration through Sephadex G-200, was 38000. From the electrophoretic mobility in the SDS-polyacrylamide gel, the subunit molecular weight of the enzyme was also estimated to be 38000. Neutral sugar was not detectable in the purified enzyme by the phenol/sulphuric acid method (Dubois *et al.*, 1956). These results show that the purified enzyme is composed of a single polypeptide of molecular weight 38000.

The amino acid composition of the purified enzyme is given in Table 4. No cysteine was found. The pI was 8.6.

Fig. 1. Isoelectric focusing of β -lactamase from *Citrobacter freundii* strain GN7391. Step 4 fraction (6 mg) was focused on a sucrose/ampholytes gradient (pH 3.5 to 10) at 5 °C. Fractions (2 g) were collected and assayed for protein (○), β -lactamase activity (●) and pH (▲).

Table 4. Amino acid composition of β -lactamase from *Citrobacter freundii* strain GN7391

Amino acid	Content ($\mu\text{mol mg}^{-1}$)	Residues*
Aspartic acid	0.386	23
Threonine	0.317	19
Serine	0.309	18
Glutamic acid	0.769	46
Proline	0.412	25
Glycine	0.506	30
Alanine	0.633	38
Cysteine	0	0
Valine	0.404	24
Methionine	0.145	9
Isoleucine	0.299	18
Leucine	0.526	31
Tyrosine	0.243	14
Phenylalanine	0.114	7
Tryptophan	0.165	10
Lysine	0.410	24
Histidine	0.084	5
Arginine	0.227	14
Total	5.949	355
Ammonia	0.773	

* The calculated numbers of residues are based on an assumed molecular weight of 38000.

Enzymic properties of the purified enzyme

The enzyme was most active at pH 8.0 and its temperature optimum was 45 °C under the conditions of assay. Iodine at 0.01 mM inhibited the enzyme activity but 0.5 mM-*p*-chloromercuribenzoic acid did not.

The kinetic parameters of the enzyme activity toward cephalosporins, cephamycins and penicillins are given in Table 5. The relative V_{max} with cefazolin, cephaloridine, cephalothin, cephalexin and cephadrine was higher than with penicillins. Among the cephalosporin derivatives tested, cefamandole, cefuroxime and cefotaxime were resistant to hydrolysis by the β -lactamase. Cephamycins and semi-synthetic penicillins were hydrolysed to only a small extent by the enzyme and inhibited the enzyme activity competitively.

Table 5. *Kinetics of hydrolysis of β -lactam antibiotics by β -lactamase from *Citrobacter freundii* strain GN7391*

Antibiotic	Assay wavelength (nm)	$V_{\max}$ *	K_m (μM)	K_i † (μM)
Cephaloridine	260	100	72	
Cefazolin	263	288	254	
Cephalothin	262	27	9	
Cephalexin	262.5	24	10	
Cephadrine	263	20	15	
Cefsulodin	263	0.13		23
Cefamandole	273	<0.01		0.019
Cefoperazone	273	0.7		NT
Cefotiam	275	0.8		NT
Cefuroxime	262	<0.01		0.008
Cefotaxime	264	<0.01		0.008
Ceftizoxime	245	0.07		0.036
Cefmetazole	275	0.03		0.044
Cefoxitin	265	0.04		0.23
Penicillin G	232	2.8		NT
Ampicillin	235	0.07		0.11
Carbenicillin	235	<0.01		0.015
Cloxacillin	—	NT		0.005
Methicillin	—	NT		0.015

NT, Not tested.

* Rates are given relative to an arbitrary value of 100 for hydrolysis of cephaloridine.

† K_i values were determined from Lineweaver-Burk plots at various concentrations of inhibitors, using cephaloridine as the substrate.

Immunological properties of the purified enzyme

The effects of anti-GN7391 rabbit serum on the purified enzyme were studied as described by Jack & Richmond (1970). The standard curve obtained when increasing quantities of the antibody were added to a fixed quantity of the purified enzyme (constant antigen titration) is shown in Fig. 2. The maximum degree of neutralization of the purified enzyme was approximately 75%, even when an excess of the antibody was used. The reaction mixture was centrifuged to separate supernatants and immunoprecipitates. The supernatants had no β -lactamase activity with an excess of antibody. The remaining enzyme activity (some 25% of the initial activity) was not due to the presence of a different kind of β -lactamase unreactive with the antibody, but rather to precipitation of the antigen-antibody complex possessing some enzyme activity.

The neutralizing capacity of the anti-GN7391 β -lactamase antibody was checked with various β -lactamases obtained from clinical isolates: five *Citrobacter freundii*, one *Pseudomonas aeruginosa*, one *Pseudomonas cepacia*, three *Enterobacter cloacae*, one *Proteus morganii*, one *Proteus vulgaris*, one *Proteus rettgeri*, one *Serratia marcescens*, one *Bacteroides fragilis*, three R-plasmid-carrying *Escherichia coli* and one R-plasmid-carrying *Pseudomonas aeruginosa*. The antibody neutralized enzymes from *Citrobacter freundii* strains (96 to 100%) and from *Enterobacter cloacae* strains (92 to 95%); none of the other cephalosporinases from *Pseudomonas*, *Proteus*, *Serratia* and *Bacteroides* were affected at all. There was no reaction with R-plasmid-mediated penicillinases.

DISCUSSION

In clinical practice, a large number of strains of *Citrobacter freundii* show resistance to penicillins and cephalosporins. A previous study (Sawai *et al.*, 1970*b*) was devoted to precise characterization of various β -lactamases found in *C. freundii*. By combining several

Fig. 2. Neutralization curve obtained for titration of β -lactamase from *Citrobacter freundii* strain GN7391 with anti-GN7391 β -lactamase antibody (constant antigen titration). Purified enzyme (4 units) was incubated with various amounts of antibody at 37°C for 20 min and then left at 5°C for 18 h. Portions of the reaction mixture were assayed for β -lactamase activity (Δ). The remaining reaction mixture was centrifuged at 6000 g for 30 min to separate immunoprecipitates and supernatants. The precipitates were washed once with saline, and then the β -lactamase activities of the supernatant fractions ($\bullet$) and suspended precipitates ($\circ$) were assayed.

methods, including isoelectric focusing and neutralization with antisera, the authors were able to characterize differences in isoelectric point, molecular weight and immunological properties of β -lactamases in *C. freundii*.

The β -lactamase from *C. freundii* GN7391 presents a cephalosporinase profile as for class I β -lactamases described by Richmond & Sykes (1973) and as for typical cephalosporinases described by Sawai *et al.* (1968). As indicated by K_m and V_{max} values, this enzyme has a strong affinity and a high activity on cephalosporin derivatives and a low activity for ampicillin, novel cephalosporins and cephamycins. The GN7391 β -lactamase shares other properties of the class I β -lactamases: inhibition by cloxacillin and resistance to *p*-chloromercuribenzoic acid.

Isoelectric focusing gave a pI of 8.6 for the β -lactamase. This is consistent with a previous report (Matthew & Harris, 1976) of pI values of 8.3 and 7.5, respectively, for β -lactamase from *C. freundii* strains 1461E and 1088E, suggesting that cephalosporinases are basic proteins.

The β -lactamase protein of strain GN7391 has been detected among other strains of *C. freundii*, such as GN324, GN346, GN7084, GN7093 and GN7099, isolated from clinical materials, by the immunoelectrophoretic analysis and neutralization test. The protein also cross-reacted with chromosomal β -lactamases from strains of *Enterobacter cloacae*, although these β -lactamases have different molecular weight (approx. 42000; Minami *et al.*, 1979) and pI values (9.2 to 9.3). Cross-reaction between chromosomally mediated β -lactamases from different genera has been reported for *Enterobacter cloacae* and *Proteus morganii* (Letarte *et al.*, 1977) and *Alkalescens dispar*, *Escherichia coli* and *Shigella sonnei* (Sykes & Matthew, 1976).

REFERENCES

- ANDREWS, P. (1964). Estimation of the molecular weight of proteins by Sephadex gel filtration. *Biochemical Journal* **91**, 222-233.
- BILL, N. J. & WASHINGTON, J. A., II (1977). Comparison of in vitro activity of cephalixin, cephadrine, and cefaclor. *Antimicrobial Agents and Chemotherapy* **11**, 470-474.
- COWAN, S. T. & STEEL, K. J. (1974). *Manual for the Identification of Medical Bacteria*, 2nd edn. Cambridge: Cambridge University Press.
- DAVIS, B. J. (1964). Disc electrophoresis II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404-427.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.
- FUJII-KURIYAMA, Y., YAMAMOTO, M. & SUGAWARA, S. (1977). Purification and properties of beta-lactamase from *Proteus morganii*. *Journal of Bacteriology* **131**, 726-734.

- JACK, G. W. & RICHMOND, M. H. (1970). A comparative study of eight distinct β -lactamases synthesized by Gram-negative bacteria. *Journal of General Microbiology* **61**, 43–61.
- LETARTE, R., FELIX, M. D., PECHERE, J. C. & LEPROHON, D. A. (1977). Enzymatic and immunological characterization of a new cephalosporinase from *Enterobacter aerogenes*. *Antimicrobial Agents and Chemotherapy* **12**, 201–205.
- MATTHEW, M. & HARRIS, A. M. (1976). Identification of β -lactamases by analytical isoelectric focusing: correlation with bacterial taxonomy. *Journal of General Microbiology* **94**, 55–67.
- MINAMI, S., INOUE, M. & MITSUHASHI, S. (1979). Purification and properties of β -lactamase from *Enterobacter cloacae*. In *Proceedings of the 8th Gunma Symposium on Microbial Drug Resistance*, pp. 52–53. Edited by S. Mitsuhashi. Gunma: Gunma University Press.
- NEWSOM, S. W. B., MARSHALL, M. J. & HARRIS, A. M. (1974). Enterobacteria, β -lactam antibiotics and β -lactamases in clinical practice. *Journal of Medical Microbiology* **7**, 473–482.
- O'CALLAGHAN, C. H., MUGGLETON, P. W. & ROSS, G. W. (1969). Effects of β -lactamase from Gram-negative organisms on cephalosporins and penicillins. In *Antimicrobial Agents and Chemotherapy-1968*, pp. 55–63. Edited by G. L. Hobby. Maryland: American Society for Microbiology.
- RICHMOND, M. H. & SYKES, R. B. (1973). The β -lactamases of Gram-negative bacteria and their possible physiological role. *Advances in Microbial Physiology* **9**, 31–88.
- SAWAI, T., MITSUHASHI, S. & YAMAGISHI, S. (1968). Drug resistance of enteric bacteria. XIV. Comparison of β -lactamases in Gram-negative rod bacteria resistant to α -aminobenzylpenicillin. *Japanese Journal of Microbiology* **12**, 423–434.
- SAWAI, T., TAKAHASHI, K., YAMAGISHI, S. & MITSUHASHI, S. (1970a). Variant of penicillinase mediated by an R factor in *Escherichia coli*. *Journal of Bacteriology* **104**, 620–629.
- SAWAI, T., MITSUHASHI, S. & YAMAGISHI, S. (1970b). Stability of various derivatives of 7-aminocephalosporanic acid and 6-aminopenicillanic acid to cephalosporinases from Gram-negative bacteria. In *Progress in Antimicrobial and Anticancer Chemotherapy*, vol. 1, pp. 410–415. Edited by H. Umezawa. Tokyo: University of Tokyo Press.
- SPACKMAN, D. H., STEIN, W. H. & MOORE, S. (1958). Automatic recording apparatus for use in the chromatography of amino acid. *Analytical Chemistry* **30**, 1190–1206.
- SYKES, R. B. & MATTHEW, M. (1976). The β -lactamases of Gram-negative bacteria and their role in resistance to β -lactam antibiotics. *Journal of Antimicrobial Chemotherapy* **2**, 115–157.
- TAJIMA, M., TAKENOCHI, Y. & SUGAWARA, S. (1979). Purification and properties of β -lactamases from *Citrobacter freundii*. In *Proceedings of the 8th Gunma Symposium on Microbial Drug Resistance*, pp. 56–57. Edited by S. Mitsuhashi. Gunma: Gunma University Press.
- VERBIST, L. (1976). Comparison of the antibacterial activity of nine cephalosporins against *Enterobacteriaceae* and nonfermentative Gram-negative bacilli. *Antimicrobial Agents and Chemotherapy* **10**, 657–663.
- WALEY, S. G. (1974). A spectrophotometric assay of β -lactamase action on penicillins. *Biochemical Journal* **139**, 789–790.
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406–4412.
- YAMANAKA, Y., TAJIMA, M. & NAKAZAWA, S. (1973). Purification and characterization of β -lactamase from clinically isolated *E. coli* strain No. 24. *Chemotherapy, Tokyo* **21**, 1179–1183.