Induction of β -Lactamase in *Proteus vulgaris*

By KENJI OKONOGI,* MITSUZO KUNO AND EIJI HIGASHIDE

Central Research Division, Takeda Chemical Industries Ltd, Yodogawa-ku, Osaka 532, Japan

(Received 30 April 1985; revised 20 August 1985)

Various β -lactam antibiotics, including monocyclic β -lactams, induced the β -lactamase of *Proteus vulgaris*; when clinical isolates were induced by benzylpenicillin, each strain produced a single β -lactamase but the activity per milligram dry weight differed from strain to strain. The β -lactamases of the *P. vulgaris* strains were heterogeneous with respect to their isoelectric points, but had almost the same specific activities, substrate specificities and Michaelis constants. The kinetics of β -lactamase formation were investigated in three strains, each with a different β -lactamase activity. Differential rates of enzyme synthesis and peak activity depended on the concentration of inducer. The plots of the reciprocals of the differential rates versus the reciprocals of the inducer concentrations were linear, and the maximum rate of enzyme synthesis and the concentration of the inducer giving half-maximum induction were determined from this double reciprocal plot. The maximum rates of enzyme synthesis were different in the three strains. The kinetic analysis of β -lactamase formation revealed that the β -lactamase activities in a single bacterial species were determined by differences in the rate of enzyme synthesis and not by differences in the properties of the enzyme.

INTRODUCTION

Many β -lactamases, which are responsible for resistance to β -lactam antibiotics in pathogenic bacteria, are inducible enzymes (Richmond & Sykes, 1973), and the mechanisms of induction have been studied by many investigators (Nordström & Sykes, 1974; Sawai *et al.*, 1977; Gootz & Sanders, 1983). However, studies on the kinetics of induced β -lactamase formation in Gram-negative bacteria have been more difficult than those on β -galactosidase formation in *Escherichia coli* (Pardee & Prestidge, 1961; Kepes, 1963; Boezi & Cowie, 1961). One reason for this is a lack of appropriate gratuitous inducers (Jacob & Monod, 1961), such as isopropylthiogalactoside and methyl-1-thio- β -D-galactoside for β -galactosidase, which themselves are not substrates of the enzyme. Another difficulty is the growth inhibitory properties of the β -lactamase inducers; several milligrams of an inducer antibiotic per millilitre are needed for maximum induction of β -lactamase in Gram-negative bacteria such as *Pseudomonas aeruginosa* (Sabath *et al.*, 1965), *Enterobacter cloacae* (Minami *et al.*, 1980), and *Citrobacter freundii* (Sawai *et al.*, 1977), and growth is greatly impaired by these high concentrations of the antibiotic. The β -lactamase of *Proteus vulgaris*, however, can be induced by relatively low concentrations of β -lactam antibiotics (Okonogi *et al.*, 1981*a*) and hence *P. vulgaris* is a suitable organism for kinetic analysis of induced β -lactamase synthesis in Gram-negative bacteria.

This paper deals with the kinetics of β -lactamase formation by clinical isolates of *P. vulgaris*, each with different β -lactamase activity.

METHODS

Bacterial strains. Clinical isolates were identified using the API 20E System. The strains used in experiments on the kinetics of induction were suspended in Trypticase soy broth (TSB; obtained from BBL) containing 15% (v/v) glycerol and were stored at -80°C .

Antibiotics. Sulfazecin, nocardicin A, carumonam (AMA-1080), C-19393 S₂, clavulanic acid, sulbactam, 6-aminopenicillanic acid, and 7-aminocephalosporanic acid were prepared in our research division; aztreonam was from Hoffmann-La Roche and imipenem, nitrocefin and moenomycin were gifts from Merck, Glaxo and Hoechst, respectively. Other antibiotics were obtained from the following manufacturers: benzylpenicillin, ampicillin, sulbenicillin, mecillinam, cefsulodin, cefotiam and cefmenoxime from Takeda Chemical Industries, Japan; cloxacillin and cefazolin from Fujisawa Pharmaceuticals, Japan; cephaloridine and latamoxef from Shionogi, Japan; cefmetazole from Sankyo, Japan; cefoxitin from Daiichi Seiyaku, Japan; cefuroxime and ceftazidime from Glaxo, UK; cefoperazone and piperacillin from Toyama Chemical Industries, Japan; polymyxin B from Taito Pfizer, Japan; gramicidin S from Calbiochem, USA; bacitracin from Sigma and fosfomycin and cycloserine from Meiji Seika, Japan.

Susceptibility testings. The minimum inhibitory concentration (MIC) was determined by the standard agar dilution method using Trypticase soy agar (BBL). About 5 µl of bacterial suspension containing about 10⁶ colony forming units ml⁻¹ was inoculated with a multiple inoculator (Sakuma, Tokyo, Japan) onto agar plates containing twofold serial dilutions of each antibiotic. The plates were incubated at 37 °C for 18 h, and the MIC was defined as the lowest concentration of the drug on which there was either no visible growth or less than four colonies per spot.

Induction experiment. The β -lactamase activity of *P. vulgaris* GN4818 induced by 0.1 mg of various antibiotics ml⁻¹ for 1 h, and the activity of clinical isolates induced by 0.1 mg benzylpenicillin ml⁻¹ for 40 min, were determined as described previously (Okonogi *et al.*, 1981a). When induced with an antibiotic that might inhibit the enzyme, the enzyme preparation was dialysed extensively against 0.05 M-phosphate buffer (pH 6.9) before the enzyme was assayed. To analyse the kinetics of β -lactamase formation, 5 × 10⁸ cells stored at -80 °C were inoculated into 10 ml TSB in an L-tube, and incubated at 37 °C with shaking. An inducer was added when the OD₆₀₀ of the culture reached 1, and the incubation was continued. At 5 or 10 min intervals after induction, 0.75 ml of the culture was withdrawn and added to the same volume of 0.05 M-Na₂HPO₄/KH₂PO₄ buffer (pH 6.9) containing 50 µg chloramphenicol ml⁻¹ in an ice bath, and the cells were disrupted at 2 A for 1 min with an ultrasonic disintegrator (Kaijo Denki, Japan). The disrupted cell suspension was used to determine enzyme activity with 0.2 mM-cephaloridine as a substrate. Culture density was measured as OD₆₀₀ with a Gilford 250 spectrophotometer. One OD₆₀₀ unit corresponds to 0.45 mg dry weight cells ml⁻¹ (this value is the mean of ten representative clinical isolates which differed by less than 5%).

Enzyme assay. β -Lactamase activity was determined micro-iodometrically and spectrophotometrically as described previously (Okonogi *et al.*, 1981a). V_{max} and K_m were determined from Lineweaver-Burk plots. One unit (U) of β -lactamase was defined as the amount of enzyme that hydrolysed 1 µmol cephaloridine min⁻¹ at 30 °C.

Purification of β -lactamase. Overnight cultures (15 ml) of *P. vulgaris* GN4818, GN4413 or TN1945 were inoculated into 320 ml Brain Heart Infusion broth (Difco) in litre flasks, and the flasks were shaken for 2 h on a rotary shaker at 30 °C. Benzylpenicillin (0.5 mg ml⁻¹) was added to induce β -lactamase and incubation was continued for an additional 2 h. Cells harvested from 2 l of culture broth were suspended in 100 ml 0.01 M-sodium phosphate buffer (pH 6.0), and disrupted at 2 A for 3 min with an ultrasonic disintegrator in an ice bath. The crude enzyme solution obtained by removing cell debris by centrifugation at 30000 g for 30 min was dialysed against 0.02 M-sodium phosphate buffer (pH 6.0), and applied to a CM-Sephadex C-50 column (1.6 × 13 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0-0.5 M) in the phosphate buffer. Fractions with activity were lyophilized, dissolved in water, and subjected to Sephadex G-100 gel filtration (2 × 95 cm column) using 0.05 M-Na₂HPO₄/KH₂PO₄ buffer (pH 6.9).

Preparation of antiserum. Antiserum against the β -lactamase previously purified from *P. vulgaris* GN4413 (Okonogi *et al.*, 1981a) was prepared as follows. Mice (6-week-old female BALB/C) were immunized subcutaneously with 0.2 ml of an emulsion containing 100 µg enzyme and Freund's complete adjuvant (Difco) on day 0; they were then immunized (boosted) intraperitoneally with 0.2 ml of emulsion containing 10 µg enzyme and 2 mg aluminum hydroxide, and with 0.1 ml of emulsion containing 50 µg enzyme and Freund's incomplete adjuvant (Difco) on days 14 and 28, respectively. The mice were bled on day 42 and the serum was stored at -20 °C until used.

Analytical isoelectric focusing. This was done at 20 °C with a constant voltage of 200 V for 5.5 h on a 14 cm cylindrical polyacrylamide gel (Wrigley, 1971), consisting of 5% (w/v) acrylamide (Bio-Rad), 0.25% (w/v) *N,N'*-methylenebisacrylamide (Wako, Japan), 0.56 µl *N,N,N',N'*-tetramethylethylenediamine ml⁻¹ (Wako), 0.21 mg ammonium persulphate ml⁻¹ (Wako) and 2% (w/v) pH 3.5-10 ampholine (LKB). The anode solution was 20 mM-phosphoric acid and the cathode solution 1 M-sodium hydroxide. The enzyme was detected by laying a filter paper, which had been dipped in a solution of 0.5 mg nitrocefin ml⁻¹, over the surface of the gel. To measure pH, the gel was sliced into 14 pieces and each piece was incubated in 0.9 ml water at 30 °C for 2 h.

Determination of molecular weight and purity of β -lactamase. The purified β -lactamases were analysed by SDS-PAGE (Laemmli & Favre, 1973) and the gels were stained for proteins with 0.1% Coomassie brilliant blue. Cytochrome C, chymotrypsinogen A, ovalbumin and BSA (Boehringer-Mannheim) were used as molecular weight standards. The proteins were quantified by densitometry of the gels with a Shimadzu CS-910 dual-wavelength TLC scanner.

Protein determination. Protein was assayed by the Lowry method, using BSA as a standard.

Table 1. Induction of β -lactamase by β -lactam antibiotics in *P. vulgaris* GN4818

Inducer (100 μ g ml ⁻¹)	β -Lactamase activity after 1 h [U (mg dry weight) ⁻¹]	Inducer (100 μ g ml ⁻¹)	β -Lactamase activity after 1 h [U (mg dry weight) ⁻¹]
None	0.002	Monocyclic β -lactams	
Penicillins		Nocardicin A	0.264
6-Aminopenicillanic acid	0.192	Sulfazecin	0.343
Benzylpenicillin	0.724	Carumonam	0.122
Ampicillin	0.457	Aztreonam	0.269
Sulbenicillin	0.529	Carbapenems	
Cloxacillin	0.016	Imipenem	0.463
Mecillinam	0.169	C-19393 S ₂	<0.001
Piperacillin	0.141	Others	
Cephalosporins		Clavulanic acid	0.027
7-Aminocephalosporanic acid	0.153	Sulbactam	0.164
Cephaloridine	0.698		
Cefazolin	0.507		
Cefsulodin	0.650		
Cefotiam	0.819		
Cefuroxime	0.555		
Cefmenoxime	1.060		
Ceftazidime	0.993		
Cefoperazone	0.038		
Cefoxitin	0.627		
Cefmetazole	0.621		
Latamoxef	0.319		

RESULTS

Induction of β -lactamase by various antibiotics

The β -lactamase of *P. vulgaris* GN4818 was induced by various antibiotics and the activities were determined after dialysis (Table 1). When induced by antibiotics such as cefoxitin, clavulanic acid, sulbactam and C-19393 S₂ which inhibit the β -lactamase, the activities were negligible or quite low, but they were increased remarkably by dialysing the enzyme preparations except that induced by C-19393 S₂, which has a strong β -lactamase-inactivating activity (Okonogi *et al.*, 1981*b*). Dialysis had no effect on the activity of β -lactamase after induction with antibiotics having no β -lactamase-inhibitory activity. Benzylpenicillin, ampicillin, sulbenicillin and most cephalosporins were good inducers of the β -lactamase. Imipenem and monocyclic β -lactam antibiotics, such as sulfazecin and aztreonam, also had relatively high activity as inducers. Cloxacillin, cefoperazone, and clavulanic acid were poor inducers. Bicyclic amino acids, reported to induce the β -lactamase of *Enterobacter cloacae* strain 996/81 (Cullmann *et al.*, 1984), and non- β -lactam antibiotics, which inhibit cell wall synthesis or membrane functions, such as bacitracin, fosfomycin, meonomycin, cycloserine, polymyxin B and gramicidin S, did not induce the *P. vulgaris* GN4818 β -lactamase.

 β -Lactamase activity in clinical isolates

β -Lactamase activities were determined in clinical isolates of *P. vulgaris* having different susceptibilities to cephalosporins (Table 2). All strains produced only very small amounts of β -lactamase without an inducer, but produced much more enzyme after induction by 0.1 mg benzylpenicillin ml⁻¹. Each strain produced a single β -lactamase; only one activity band was detected on polyacrylamide gel after isoelectric focusing. The β -lactamases of *P. vulgaris* were heterogeneous with respect to their isoelectric points and the induced activity also varied from strain to strain, though the substrate specificities of the enzymes were almost the same. There was no correlation between the activities and the isoelectric points of the β -lactamases. Most strains were highly sensitive to cefmenoxime, though some with high β -lactamase activity were slightly less sensitive. Resistance to cefotiam was related to the β -lactamase activity. All strains were resistant to cefazolin.

Table 2. *β -Lactamase activity and cephalosporin susceptibility of clinical isolates of P. vulgaris*

β -Lactamase was induced by 0.1 mg benzylpenicillin ml⁻¹ for 40 min. The substrate concentration for the determination of enzyme activities was 0.2 mM. The β -lactamase activities for induced cultures are means $\pm$ SD of three independent experiments, and those for uninduced cultures were determined once. The substrate specificities, expressed as relative rate of hydrolysis taking the rate for cephaloridine as 100, are means $\pm$ SD of at least three assays for representative induced enzyme preparations. MICs are representative values of two to four assays.

Strain	pI of β -lactamase	β -lactamase activity [U (mg dry weight) ⁻¹]		Substrate specificity of β -lactamase (Cephaloridine = 100)			MIC (μ g ml ⁻¹)		
		Uninduced	Induced	Cefazolin	Cefotiam	Cefmenoxime	Cefazolin	Cefotiam	Cefmenoxime
GN5297	7.4	0.004	0.012 $\pm$ 0.005	400 $\pm$ 55	284 $\pm$ 76	48 $\pm$ 11	50	0.39	0.05
GN4420	8.9	0.002	0.085 $\pm$ 0.003	285 $\pm$ 24	196 $\pm$ 11	59 $\pm$ 8	100	25	0.10
TN1947	9.0	0.003	0.100 $\pm$ 0.018	314 $\pm$ 19	196 $\pm$ 3	63 $\pm$ 1	50	6.25	0.024
GN4414	8.9	0.002	0.108 $\pm$ 0.013	260 $\pm$ 31	184 $\pm$ 2	43 $\pm$ 4	50	12.5	0.10
TN225	8.5	0.002	0.313 $\pm$ 0.034	312 $\pm$ 9	202 $\pm$ 11	40 $\pm$ 4	200	6.25	0.20
GN4748	6.9	0.003	0.342 $\pm$ 0.061	399 $\pm$ 32	217 $\pm$ 7	41 $\pm$ 5	400	12.5	0.10
TN235	8.8	0.002	0.343 $\pm$ 0.021	463 $\pm$ 56	240 $\pm$ 31	51 $\pm$ 5	200	12.5	0.20
GN4418	7.0	0.004	0.348 $\pm$ 0.025	420 $\pm$ 22	234 $\pm$ 15	41 $\pm$ 3	800	100	0.20
GN4731	8.5	0.002	0.385 $\pm$ 0.050	379 $\pm$ 15	269 $\pm$ 9	44 $\pm$ 5	100	6.25	0.10
GN4422	8.5	0.001	0.429 $\pm$ 0.068	331 $\pm$ 13	239 $\pm$ 20	39 $\pm$ 4	200	12.5	0.05
TN224	7.6	0.004	0.461 $\pm$ 0.066	353 $\pm$ 36	202 $\pm$ 30	36 $\pm$ 3	400	50	0.20
GN4421	7.6	0.003	0.571 $\pm$ 0.084	473 $\pm$ 41	268 $\pm$ 24	42 $\pm$ 5	800	100	0.10
GN4712	8.2	0.002	0.571 $\pm$ 0.033	383 $\pm$ 29	249 $\pm$ 12	42 $\pm$ 3	200	25	0.10
GN4818	6.9	0.004	0.616 $\pm$ 0.016	401 $\pm$ 38	190 $\pm$ 7	42 $\pm$ 2	400	25	0.05
GN5293	8.1	0.001	0.619 $\pm$ 0.064	381 $\pm$ 19	219 $\pm$ 16	43 $\pm$ 4	200	50	0.39
GN4413	8.2	0.002	1.084 $\pm$ 0.135	437 $\pm$ 44	194 $\pm$ 30	52 $\pm$ 3	1600	800	0.78
TN1946	8.8	0.076	2.614 $\pm$ 0.291	379 $\pm$ 35	182 $\pm$ 9	38 $\pm$ 2	1600	400	3.13
TN1945	8.8	0.022	2.768 $\pm$ 0.234	363 $\pm$ 41	201 $\pm$ 12	42 $\pm$ 1	> 1600	400	6.25

Fig. 1. SDS-PAGE (a-c) and isoelectric focusing (d-f) of *P. vulgaris* β -lactamases. (a) and (d) GN4818; (b) and (e) GN4413; (c) and (f) TN1945.

Table 3. Properties of β -lactamases purified from *P. vulgaris* GN4818, GN4413 and TN1945

	GN4818	GN4413	TN1945
Molecular weight ($\pm$ the range)	27 000 $\pm$ 1000	27 500 $\pm$ 1000	28 000 $\pm$ 1000
Specific activity* [V_{max} ; $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$]			
Cephaloridine	522	556	559
Cephalothin	712	840	704
Cefazolin	2434	2641	1719
Cefotiam	1468	1485	1179
Cefmenoxime	293	339	229
Benzylpenicillin	51	57	49
K_m (μM)			
Cephaloridine	105	118	127
Cephalothin	30	31	39
Cefazolin	111	103	89
Cefotiam	161	228	143
Cefmenoxime	154	150	138
Benzylpenicillin	4.2	6.8	4.4

* The values were corrected by considering the purities of the enzyme preparations (90, 96 and 99% for the enzymes of *P. vulgaris* GN4818, GN4413 and TN1945, respectively).

Properties of β -lactamases

β -Lactamases were purified from *P. vulgaris* GN4818, GN4413 and TN1945, which have different β -lactamase activity and susceptibility to cephalosporins, and the properties of the enzymes were compared. The β -lactamases were purified more than 120-fold by the two-step purification procedure. The purities of the enzyme preparations from *P. vulgaris* GN4818, GN4413 and TN1945 were 90, 96 and 99%, respectively. The three enzymes had different isoelectric points (Fig. 1) and showed slightly different responses to inhibition by antiserum (Fig. 2). However, their specific activities, Michaelis constants for six substrates and molecular weights were all very similar (Table 3).

Fig. 2

Fig. 3

Fig. 2. Inhibition of β -lactamase of *P. vulgaris* by antiserum. The β -lactamases from *P. vulgaris* GN4818 ($\circ$), GN4413 ($\triangle$) and TN1945 ($\square$) were incubated with anti-*P. vulgaris* GN4413 β -lactamase serum for 1 h at 37°C, before enzyme assay. The results are the means of three determinations which differed by less than 10%.

Fig. 3. Induction of β -lactamase of *P. vulgaris* GN4818 (a), GN4413 (b) and TN1945 (c). The β -lactamase was induced by 1 ($\bullet$), 10 ($\square$), 100 ($\triangle$) and 1000 ($\circ$) μ g benzylpenicillin ml^{-1} . The results are the means of two determinations; bars indicate $\pm$ the range.

Fig. 4. Double reciprocal plot for β -lactamase induction. The differential rate of enzyme synthesis (increase in β -lactamase activity per increase in cell mass) in *P. vulgaris* GN4818 ($\circ$), GN4413 ($\triangle$) and TN1945 ($\square$) was determined by assaying the enzyme activity and the cell mass of the culture at 5 or 10 min intervals after induction as described in the text. Linear regression equations were estimated by the method of least squares; correlation coefficients were 0.97, 0.99 and 0.97 for *P. vulgaris* GN4818, GN4413 and TN1945, respectively.

Kinetics of β -lactamase production

The specific activity of β -lactamase after induction was determined in *P. vulgaris* GN4818, GN4413 and TN1945 (Fig. 3). When benzylpenicillin was added as an inducer to bacteria growing exponentially, β -lactamase synthesis began after a 5 to 10 min lag time, and continued at a constant rate for at least 20 min. As the inducer concentration was increased, the lag time decreased and the period of enzyme synthesis increased. The enzyme synthesis stopped 1 h after the addition of 1 mg benzylpenicillin ml^{-1} , probably because of depletion of the antibiotic by hydrolysis. The rate of enzyme synthesis during the early phase and the peak levels of the activity depended on the concentration of the inducer (Fig. 3). Differential rates of enzyme

synthesis (increase in enzyme activity divided by increase in cell mass) were determined after induction with various concentrations of benzylpenicillin. The relationship between the differential rate and the inducer concentration was similar to that between the reaction velocity and the substrate concentration in the enzyme reaction, and a plot of the reciprocal of the differential rates against the reciprocal of the concentrations of the inducer was found to be linear (Fig. 4). From this double reciprocal plot, the maximum rate of enzyme synthesis and the concentration of the inducer giving half-maximum induction were determined in the same way as are $V_{\max}$ and K_m by the Lineweaver-Burk method. The maximum rates of enzyme synthesis in *P. vulgaris* GN4818, GN4413 and TN1945 were estimated to be 2.1, 4.2 and 6.2 U (mg dry weight)⁻¹, respectively. The concentration of benzylpenicillin giving half-maximum induction was 12.7 $\mu\text{g ml}^{-1}$ in *P. vulgaris* GN4818 and TN1945, and 25.5 $\mu\text{g ml}^{-1}$ in *P. vulgaris* GN4413.

DISCUSSION

Induced β -lactamase formation in Gram-negative bacteria is considered to be controlled negatively by a repressor (Nordström & Sykes, 1974; Sawai *et al.*, 1977; Gootz & Sanders, 1983); the enzyme can be induced by various penicillins, cephalosporins (Minami *et al.*, 1980) and a carbapenem antibiotic (Grappel *et al.*, 1984). The present work has demonstrated that monocyclic β -lactam antibiotics are also inducers of β -lactamase. It has been reported that some bicyclic amino acids, and complex media such as TSB and Schaedler's broth, induced the β -lactamase of *Enterobacter cloacae* strain 996/81 (Cullmann *et al.*, 1984), and that bacitracin, a non- β -lactam antibiotic, induced the β -lactamase of *Bacillus cereus* (Citri, 1978), but such non-specific induction did not occur in *P. vulgaris*. These results suggest that the repressor of the β -lactamase of *P. vulgaris* recognizes the β -lactam structure only. A lack of enzyme activity after induction by C-19393 S₂ may be due to potent β -lactamase-inactivating activity (Okonogi *et al.*, 1981*b*) rather than to lack of inducer activity of the antibiotics; the fact that the enzyme activity after induction by C-19393 S₂ was lower than the basal level supports this assumption.

When induced with a given concentration of benzylpenicillin, the activity of β -lactamase in clinical isolates of *P. vulgaris* varied greatly from strain to strain. The different activities could result from a difference in either (i) the specific activities of the enzymes, (ii) the periods of β -lactamase synthesis after induction, (iii) the rate of enzyme synthesis or (iv) the concentration of inducer required for maximum induction. However, the enzymes purified from three strains with different total activity had almost the same specific activity. Also, the period during which enzyme synthesis continued depended only on the concentration of inducer, and was almost the same in the three strains. Therefore, both reasons (i) and (ii) can be disregarded.

Possibilities (iii) and (iv) were investigated by a kinetic analysis of β -lactamase induction. Boezi & Cowie (1961), and Müller-Hill *et al.* (1964) reported that the reciprocals of the specific β -galactosidase activities of *E. coli* induced by methyl-1-thio- β -D-galactoside were proportional to the reciprocals of the square of the inducer concentrations. The reciprocals of the differential rates of β -lactamase synthesis in *P. vulgaris* were proportional to the reciprocals of the inducer concentrations, and not to the reciprocals of their squares. The maximum rates of enzyme synthesis obtained from the double reciprocal plots were different in the three strains, and the concentrations of the inducer giving half-maximum induction were independent of enzyme activity, indicating that the strains with high activity synthesize the enzyme protein at a higher rate than those with low activity. Differences in the rate of enzyme synthesis would result from differences in either the efficiency of the β -lactamase promoter or the number of β -lactamase genes.

The β -lactamases of the *P. vulgaris* strains differed in their isoelectric points, indicating that there are some variations in their amino acid composition, which might have resulted either from mutations in the β -lactamase gene or from different post-translational processing in the different strains. The changes in amino acid composition need only be small; plasmid-mediated TEM-1 and TEM-2 β -lactamases, which have different isoelectric points, differ by only a single amino acid (Ambler, 1978; Sutcliffe, 1978). Alternatively, some amino acids may be lacking in some β -lactamases; the β -lactamases of *P. vulgaris* GN4818 and GN4413 migrated slightly more

quickly than that of TN1945 on SDS-polyacrylamide gel and Sephadex G-100 gel. Since the β -lactamase is a periplasmic protein and has a leader peptide, the latter possibility could result from different processing of the leader peptide as well as by mutational deletion within the β -lactamase gene. In any case, the structure of the active site must be conserved because the three enzymes had almost the same specific activities, substrate specificities and Michaelis constants. Slight differences in responses to antiserum may have resulted from differences in structure in the vicinity of the active site which altered binding affinity to antibody. Matthew *et al.* (1975) introduced analytical isoelectric focusing to identify β -lactamases, and suggested that the technique could be used in bacterial taxonomy (Matthew & Harris, 1976). However, observations that the β -lactamases of clinically isolated *P. vulgaris* were heterogeneous with respect to their isoelectric point, indicate that the use of the isoelectric focusing of β -lactamases in taxonomy may be misleading.

We thank Dr S. Shiho for preparing the antiserum and Mrs E. Saga for her technical assistance. We also thank Drs Y. Nakao and M. Yoneda for their encouragement throughout this work.

REFERENCES

- AMBLER, R. P. (1978). Partial amino acid sequence of penicillinase coded by *Escherichia coli* plasmid R6K. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 3732-3736.
- BOEZI, J. A. & COWIE, D. B. (1961). Kinetic studies of β -galactosidase induction. *Biophysical Journal* **1**, 639-647.
- CITRI, Y. (1978). Induction of penicillinase by bacitracin. *Biochemical and Biophysical Research Communications* **80**, 573-579.
- CULLMANN, W., DALHOFF, A. & DICK, W. (1984). Nonspecific induction of β -lactamase in *Enterobacter cloacae*. *Journal of General Microbiology* **130**, 1781-1786.
- GOOTZ, T. D. & SANDERS, C. C. (1983). Characterization of β -lactamase induction in *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy* **23**, 91-97.
- GRAPPEL, S. F., GIOVENELLA, A. J., NEWMANN, D. J. & NISBET, L. J. (1984). β -Lactamase induction by *N*-formidoylthienamycin. *Journal of Antibiotics* **37**, 1101-1102.
- JACOB, F. & MONOD, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology* **3**, 318-356.
- KEPES, A. (1963). Kinetics of induced enzyme synthesis. Determination of the mean life of galactosidase-specific messenger RNA. *Biochimica et biophysica acta* **76**, 293-309.
- LAEMMLI, U. K. & FAVRE, M. (1973). Maturation of the head of bacteriophage T4. I. DNA packaging events. *Journal of Molecular Biology* **80**, 575-599.
- 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.
- MATTHEW, M., HARRIS, A. M., MARSHALL, M. J. & ROSS, G. W. (1975). The use of analytical isoelectric focusing for detection and identification of β -lactamases. *Journal of General Microbiology* **88**, 169-178.
- MINAMI, S., YOTSUJI, A., INOUE, M. & MITSUHASHI, S. (1980). Induction of β -lactamase by various β -lactam antibiotics in *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy* **18**, 382-385.
- MÜLLER-HILL, B., RICKENBERG, H. V. & WALLENFELS, K. (1964). Specificity of the induction of the enzymes of the lac operon in *Escherichia coli*. *Journal of Molecular Biology* **10**, 303-318.
- NORDSTRÖM, K. & SYKES, R. B. (1974). Induction kinetics of β -lactamase biosynthesis in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **6**, 734-740.
- OKONOGI, K., KUNO, M., KIDA, M. & MITSUHASHI, S. (1981a). β -Lactamase stability and antibacterial activity of cefmenoxime (SCE-1365), a novel cephalosporin. *Antimicrobial Agents and Chemotherapy* **20**, 171-175.
- OKONOGI, K., NOZAKI, Y., IMADA, A. & KUNO, M. (1981b). C-19393 S₂ and H₂, new carbapenem antibiotics. IV. Inhibitory activity against β -lactamases. *Journal of Antibiotics* **34**, 212-217.
- PARDEE, A. B. & PRESTIDGE, L. S. (1961). The initial kinetics of enzyme induction. *Biochimica et biophysica acta* **49**, 77-88.
- RICHMOND, M. H. & SYKES, R. B. (1973). The β -lactamase of Gram-negative bacteria and their possible physiological role. *Advances in Microbial Physiology* **9**, 31-88.
- SABATH, L. D., JAGO, M. & ABRAHAM, E. P. (1965). Cephalosporinase and penicillinase activities of a β -lactamase from *Pseudomonas pyocyanea*. *Biochemical Journal* **96**, 739-752.
- SAWAI, T., NAKAJIMA, S., MOROHOSHI, T. & YAMAGISHI, S. (1977). Thermolabile repression of cephalosporinase synthesis in *Citrobacter freundii*. *Microbiology and Immunology* **21**, 631-638.
- SUTCLIFF, J. G. (1978). Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 3737-3741.
- WRIGLEY, C. W. (1971). Gel electrofocusing. *Methods in Enzymology* **22**, 559-564.