β -Lactamase of *Lysobacter enzymogenes*: induction, purification and characterization

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Lysobacter enzymogenes produces an inducible β -lactamase and induction with 100 μg ampicillin ml^{-1} resulted in an increase of more than 100-fold in enzyme activity. Various other β -lactam antibiotics also served as effective inducers. The enzyme was obtained from cells by osmotic shocking to release periplasmic components and it was purified primarily by ion-exchange chromatography and PAGE. The β -lactamase consists of one polypeptide with a molecular mass of about 28 kDa and an isoelectric point greater than 9.6. It is strongly inhibited by *p*-chloromercuribenzoate and clavulanic acid but not by EDTA. The enzyme readily hydrolyses several penicillins and cephalosporins, but not oxacillin or cloxacillin. The enzyme therefore belongs to group 2b of the bacterial β -lactamases.

Introduction

Lysobacter enzymogenes and related species are Gram-negative gliding bacteria noted for the production of extracellular enzymes which have a role in biodegradation (Christensen & Cook, 1978; Reichenbach & Dworkin, 1981; Rosenberg & Varon, 1984). A number of these enzymes have been studied in detail. The proteases (Jackson & Matsueda, 1970; Whitaker, 1970), the chitinase (Hedges & Wolfe, 1974), the nucleic acid hydrolases (von Tigerstrom, 1980, 1981), a phosphatase (von Tigerstrom, 1984), an amylase and an esterase (von Tigerstrom & Stelmaschuk, 1987, 1989) were found to be secreted into the exterior milieu, mainly after the exponential growth phase. Another phosphatase (von Tigerstrom & Stelmaschuk, 1986) and another esterase (von Tigerstrom & Stelmaschuk, 1989) are associated with the outer membrane.

Little is known about the periplasm of these organisms or about the effect of the production of secreted hydrolases on the integrity of the outer membrane and the periplasmic components. For instance, it is possible that some of the hydrolases found in the exterior milieu could have come from the periplasm. Therefore, in order to study the secretion and localization of enzymes in

L. enzymogenes, it will be important to have a periplasmic marker enzyme.

Christensen & Cook (1978) reported that most species of *Lysobacter* are resistant to 10 units of penicillin G. To our knowledge, there have not been any reports of β -lactamases from *Lysobacter* species or related gliding bacteria (Bush, 1989*a, b, c*). It was not known, therefore, whether the resistance to penicillin was due to a β -lactamase, to structural constraints imposed by the outer membrane or to a low affinity of the penicillin-binding proteins for penicillin.

Recently we found that *L. enzymogenes* produces a β -lactamase which appears to be located in the periplasm. This paper describes the induction of the β -lactamase, as well as the purification and some properties of the enzyme.

Methods

Chemicals. The β -lactamase substrate PADAC [7-(thienyl-2-acetamido)-3-[2-(4-*N,N*-dimethyl-aminophenylazo)-pyridinium methyl]-3-cephem-4-carboxylic acid] was obtained from Calbiochem and all other β -lactams were purchased from Sigma. The protein standards phosphorylase *b*, ovalbumin, α -chymotrypsin and cytochrome *c* from horse heart also were from Sigma, whereas the bovine serum albumin (BSA) and chymotrypsinogen A were from Pierce. Bovine pancreatic ribonuclease was the product of the Worthington Biochemical Corporation, *p*-chloromercuribenzenesulphonic acid (*p*CMBS) and *p*-chloromercuribenzoate (*p*CMB) were obtained from Sigma and clavulanic acid was a gift from Dr S. E. Jensen of the Department of Microbiology, University of Alberta.

Abbreviations: PADAC, 7-(thienyl-2-acetamido)-3-[2-(4-*N,N*-dimethyl-aminophenylazo)-pyridinium methyl]-3-cephem-4-carboxylic acid; *p*CMB, *p*-chloromercuribenzoate; *p*CMBS, *p*-chloromercuribenzenesulphonic acid.

Organism and culture conditions. *Lysobacter enzymogenes* UASM 495 (ATCC 29487) was obtained from Dr F. D. Cook, Department of Soil Microbiology, University of Alberta. It was grown at 22 °C in 1% (w/v) yeast extract on an orbital shaker at 250 r.p.m. To obtain a growing culture with induced levels of β -lactamase, ampicillin (100 μ g ml⁻¹) was added immediately after the medium was inoculated with stationary-phase cells to a cell density of 0.8 OD₆₀₀. The culture was then grown to an OD₆₀₀ of about 3.5. Other β -lactam antibiotics were also added to the medium to a concentration of 100 μ g ml⁻¹ and non-specific inducers (Sanders, 1987) such as tryptophan, glycine, haemin and thiamin were used at 500 μ g ml⁻¹. The cell-free extract was prepared as described previously (von Tigerstrom & Stelmashuk, 1989).

Enzyme assays. β -Lactamase was assayed in a Perkin Elmer λ 3 spectrophotometer with recorder at a constant temperature of approximately 22 °C. A typical reaction mixture contained 850 μ l 50 mM-Tris/HCl, pH 7.5, and 100 μ l 0.1 mM-PADAC. To initiate the reaction, 50 μ l enzyme source, diluted with 10 mM-potassium phosphate, 1 mg BSA ml⁻¹ pH 7.5, was added. The decrease in the A_{570} was recorded over a 2 to 3 min period. The β -lactamase activities were routinely determined with PADAC as the substrate using the molar absorption coefficient of 52700 M⁻¹ cm⁻¹. Activities with other β -lactam substrates also were determined spectrophotometrically at the wavelengths reported by Hirai *et al.* (1980), using the methods of Waley (1974) and Samuni (1975) for penicillins and of O'Callaghan *et al.* (1968) and Ross & O'Callaghan (1975) for cephalosporins. One unit of β -lactamase is the amount that hydrolyses 1 μ mol of substrate min⁻¹.

The activities of glucose-6-phosphate dehydrogenase (DeMoss, 1955), alkaline phosphatase (von Tigerstrom, 1984) and ribonuclease (von Tigerstrom, 1980) were assayed by the published procedures.

Purification of β -lactamase. *L. enzymogenes* was grown in 1% (w/v) yeast extract, 100 μ g ampicillin ml⁻¹ as indicated above. The cells from 355 ml culture were obtained by centrifugation at 22 °C for 15 min and the cell pellets were rinsed with 71 ml of 10 mM-Tris/HCl, 50 mM-NaCl, 1.5 mM-MgCl₂, pH 7.5, and centrifuged for 5 min at 22 °C. The following steps were carried out at between 0 and 4 °C and centrifugations were at 15300 g, except where indicated otherwise.

(a) **Preparation of shock fluid.** The cells were resuspended in 71 ml 10 mM-Tris/HCl, 200 mM-MgCl₂, pH 8.4, and mixed gently at 37 °C for 15 min. They were then centrifuged at 22 °C for 5 min, resuspended in 5 ml 10 mM-Tris/HCl, 200 mM-MgCl₂, pH 8.4, and quickly added to 350 ml 10 mM-Tris, 2 mM-EDTA, 1 mM-dithiothreitol, pH 11.0, kept at 0 °C. The suspension was mixed gently for 20 min to release the periplasmic components. The shocked cells were sedimented by centrifugation at 0 to 4 °C for 15 min. The supernatant was retained and the pH was adjusted to pH 7.5 with 1 M-HCl to yield 355 ml of shock fluid. For some experiments the shocking procedure was done using 10 mM-Tris/HCl, pH 8.4 as the shocking buffer.

(b) **Concentration with CM-Sephadex.** CM-Sephadex (0.7 g) was added to 353 ml shock fluid. The suspension was stirred for 30 min and filtered using a 2.5 cm diameter column. The packed CM-Sephadex was then eluted with 50 ml 10 mM-Tris/HCl, 2 mM-EDTA, 1 mM-dithiothreitol, pH 7.5, and then with the same buffer at pH 11.0. Fractions of 5.9 ml were collected and fractions 39 to 42 (23.5 ml), containing 78% of the β -lactamase activity applied to the column, were pooled for further purification. The pH of the pooled fractions was adjusted from 9.8 to 7.5 before dialysis against 10 mM-potassium phosphate, pH 7.5.

(c) **CM52-cellulose chromatography.** The dialysed preparation from step (b) was applied to a 2.5 cm diameter $\times$ 11 cm column of CM52-cellulose equilibrated with 10 mM-potassium phosphate, pH 7.5 (starting buffer). It was then eluted with 50 ml starting buffer followed by a

linear gradient formed from 150 ml starting buffer and 150 ml 10 mM-potassium phosphate, 0.4 M-KCl, pH 7.5. The β -lactamase was eluted at approximately 0.13 M-KCl and fractions 32 to 34 (15.5 ml) were pooled. They contained 59% of the activity applied to the CM52 column.

(d) **Gel filtration.** Purified preparation (5 ml) from step (c) was applied to a 2.5 cm diameter $\times$ 42.5 cm column of Sephadex G-75 superfine equilibrated with 10 mM-potassium phosphate, 0.1 M-KCl, pH 7.4, and eluted with the same buffer, collecting 2.13 ml fractions. The β -lactamase was eluted after 125.7 ml. About 50% of the β -lactamase activity applied to the column was recovered and there was no increase in specific activity.

PAGE molecular mass determination and estimation of isoelectric point. SDS-PAGE was carried out according to the method of Maizel (1971) as described previously (von Tigerstrom & Stelmashuk, 1985). Phosphorylase b (97 kDa), BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome c (12.5 kDa) were used as molecular mass markers.

Electrophoresis of the native β -lactamase was carried out in 7% (w/v) polyacrylamide gel as described by Maizel (1971) for basic proteins, except that sodium thioglycollate (0.1 mM) was added to the electrophoresis buffer. After electrophoresis, the gel was covered for 3 min with 0.2 M-Tris/HCl, pH 7.5, and then for about 15 min with 60 μ g PADAC ml⁻¹. Excess PADAC was then removed and the gel was photographed before staining the gel for protein by the method of Fairbanks *et al.* (1971). A duplicate gel was stained for protein only.

The molecular mass of the native β -lactamase was estimated by gel filtration using a 2.5 cm diameter $\times$ 42.5 cm column of Sephadex G-75 superfine. Blue dextran, *Escherichia coli* C₄F₁ alkaline phosphatase (84 kDa), ovalbumin (45 kDa), secreted *L. enzymogenes* phosphatase (25 kDa) (von Tigerstrom, 1984) and cytochrome c (12.5 kDa) were used as the standards. The column was eluted with 10 mM-potassium phosphate, 0.1 M-KCl, 0.1 mM-sodium thioglycollate, pH 7.5. Fractions of 2.3 ml were collected. The K_{av} , as defined by the supplier of Sephadex, was determined for each standard protein and used to determine graphically for each protein.

To estimate the isoelectric point of the β -lactamase, the elution of the enzyme from CM-Sephadex by increasing the pH while maintaining a constant, low ionic strength (conductivity of 8 mS) was compared to the elution of pancreatic ribonuclease and cytochrome c. CM-Sephadex (0.1 g) was added to 50 ml of 10 mM-KCl, 5 mM-Tris/HCl, 10 mM-glycine, pH 7.5, containing 2.1 units β -lactamase, 2 mg cytochrome c and 0.5 mg ribonuclease. The suspension was stirred for 30 min at 0 °C and poured into a 1.2 cm diameter column to obtain 5 ml of packed CM-Sephadex below 6 ml buffer (as a mixing chamber). The column was eluted with 16 ml of 10 mM-KCl, 5 mM-Tris/HCl, 10 mM-glycine, pH 7.5, and then with 5 mM-Tris, 10 mM-glycine adjusted to pH 10.8 with KOH, and 1.35 ml fractions were collected. The β -lactamase and ribonuclease activities and cytochrome c concentrations were determined in each fraction and plotted against the pH. The pH value corresponding to the midpoint of the increasing slope of the peak was determined graphically for each protein.

Inhibition of β -lactamase. The enzyme (0.015 units ml⁻¹) was incubated at 22 °C in 10 mM-potassium phosphate, pH 7.5, in the absence and in the presence of BSA (1 mg ml⁻¹), with different concentrations of pCMBS and pCMB ranging from 0 to 100 μ M. Clavulanic acid, in the presence of BSA, was used from 0 to 33 μ M. The β -lactamase activities remaining after 10 min were determined and the results were used to estimate the inhibitor concentrations that would cause 50% inhibition of the enzyme.

Amino acid sequence. β -Lactamase was prepared as outlined above. After purification step (b) the preparation was dialysed against 10 mM-

Tris/HCl, pH 8.0, and freeze-dried. The Sequencing Laboratory of the Department of Biochemistry and Microbiology at the University of Victoria, Victoria, British Columbia, Canada, performed the SDS-PAGE, blotting of the 28 kDa protein band and the N-terminal sequence analysis.

Analytical procedures. Protein determinations were carried out by the Lowry method or, where suitable, by the direct spectrophotometric method (Warburg & Christian, 1941). Cytochrome *c* was determined by measuring A_{530} .

Results and Discussion

Production of β -lactamase

Preliminary results indicated that *L. enzymogenes* produced only a small amount of β -lactamase when grown in 1% yeast extract medium and that the enzyme was inducible. The rate of production of the β -lactamase was greatest during active growth of the organism in medium containing 100 to 200 μg of inducer (ampicillin) (ml culture^{-1}). No significant induction was observed during the stationary growth phase and little β -lactamase was produced if the medium containing the inducer was inoculated with a small inoculum (to give an OD_{600} of 0.05) and then allowed to grow for 22 h to the stationary phase. This was observed with both ampicillin and oxacillin and was probably due to the inducer being inactivated during the early period of growth. For these

Table 1. Induction of β -lactamase with different β -lactam antibiotics

L. enzymogenes was grown with 100 $\mu\text{g ml}^{-1}$ of the inducers as described in Methods. The β -lactamase activities were determined in cell-free extracts prepared from samples taken at the times indicated. PADAC was used as the substrate.

Inducer	$10^2 \times \beta$ -Lactamase activity (units per OD_{600} unit)	
	5 h	7.5 h
None	0.097	0.085
Ampicillin	8.7	9.1
Oxacillin	5.3	7.0
Methicillin	4.4	6.8
Penicillin G	6.6	7.4
Nafcillin	7.6	12.8
Cephaloridine	7.3	6.6
Cephalothin	6.9	6.2

reasons and because the enzyme could best be obtained by osmotic shocking of cells that were actively growing, the organism was grown routinely for 5 to 7 h (approximately 2 to 3 generations) with 100 μg ampicillin ml^{-1} using a large inoculum. Fig. 1 shows the growth of *L. enzymogenes* and the production of β -lactamase with different inducer concentrations. The rate of growth was not affected by ampicillin, even at 300 $\mu\text{g ml}^{-1}$ (not shown). The highest yield of β -lactamase per unit of cell mass was usually obtained after 5 to 7 h and the enzyme activity was more than 100-fold higher in induced cells than in uninduced cells. Nearly identical amounts of β -lactamase activity were obtained with ampicillin concentrations of 100 $\mu\text{g ml}^{-1}$ and 200 $\mu\text{g ml}^{-1}$. Using lower concentrations of ampicillin we found that 50% of maximal induction was obtained with 35 μg ampicillin ml^{-1} .

Although ampicillin was used routinely as the inducer, other β -lactam antibiotics also induced the *Lysobacter* β -lactamase (Table 1). The growth of *L. enzymogenes* was not inhibited by these antibiotics and most were less effective as inducers of β -lactamase than ampicillin. An exception was nafcillin, which produced the highest β -lactamase activity at 7.5 h.

In a separate experiment, the induction of β -lactamase by ampicillin, which is hydrolysed rapidly, at 2 to 160 $\mu\text{g ml}^{-1}$ was compared to the induction achieved by oxacillin, which is hydrolysed very slowly by the β -lactamase. Interestingly, the levels of β -lactamase produced at different concentrations of the two inducers were nearly identical. Therefore, the nature of the mechanism of induction and of the actual inducing molecule are not clear.

The addition of tryptophan, glycine, haemin or thiamin to growing cultures of *L. enzymogenes* had no

Fig. 1. Growth of *L. enzymogenes* and production of β -lactamase in the presence and absence of ampicillin. The organism was grown as described in Methods. The OD_{600} of the cultures (○) and the β -lactamase activities in cell-free extracts of cultures grown with 0 μg (●), 100 μg (■) and 200 μg (▲) ampicillin ml^{-1} were determined.

Table 2. Purification of β -lactamase

The purification was very reproducible. Similar results were obtained twice at this scale and several times with smaller cultures.

Step	Fraction	Volume (ml)	Protein (mg)	β -Lactamase activity	
				(units)	[units (mg protein) ⁻¹]
	Cell extract	355	309	103	0.33
(a)	Shock fluid	355	100	84	0.84
(b)	CM-Sephadex	23.5	6.4	65	10
(c)	CM52	15.5	1.0	38	38

effect on the growth of the organism or the β -lactamase activities. Thus, non-specific induction as seen with *Aerobacter cloacae* (Sanders, 1987) did not occur.

Purification and stability

The β -lactamase was purified 115-fold with a 37% yield by using three simple steps. The purity of the final preparation was estimated from the results of SDS-PAGE to be 80% to 90%. A typical purification is summarized in Table 2.

In addition to full induction of the enzyme, an effective shocking procedure was important for a successful purification. During early experiments, a comparison of the shocking procedures of Neu & Heppel (1965) and of Cheng *et al.* (1970) was made. These procedures released 31% and 66%, respectively, of the β -lactamase present in the cell-free extract. The Mg²⁺-shocking procedure (Cheng *et al.*, 1970) was modified slightly so that the shock fluid was obtained in 10 mM-Tris/HCl, pH 8.4, at 0 °C without the 10 mM-MgCl₂. Under these conditions we found that few, if any, cytoplasmic components and only 3% of the outer-membrane components were released into the shock fluid. This was determined by assaying glucose-6-phosphate dehydrogenase as the cytoplasmic marker enzyme and the membrane-bound alkaline phosphatase (von Tigerstrom & Stelmaschuk, 1985) as the outer-membrane marker enzyme. Therefore, it is clear that the inducible β -lactamase of *L. enzymogenes* is a periplasmic enzyme.

The shocking procedure for the large-scale purification was carried out at pH 11, as described in Methods. This increased the yield of the β -lactamase from that found using pH 8.4, to about 84% (large-scale purification) or nearly 100% (small-scale purification). However, the specific activity was not increased, indicating that the additional release of the enzyme was non-specific.

The enzyme activity was not affected by short exposure to pH 5.0 or to high pH values such as those

used during the shocking procedure and the elution from CM-Sephadex. A considerable loss of enzyme activity was, however, noticed during storage in 10 mM-potassium phosphate, pH 7.5. The addition of 1 mg bovine serum albumin ml⁻¹ or the use of siliconized glassware allowed storage for several days at 0 °C or several weeks at -22 °C without loss of activity. The addition of 1 mM-dithiothreitol also stabilized the enzyme at 0 °C but in its presence there was almost complete loss of enzyme activity upon freezing and thawing. Exposure of the β -lactamase to 0.1% SDS at room temperature also resulted in complete inactivation.

Molecular mass and isoelectric point

Fig. 2 shows SDS-PAGE of shock fluids from cells grown with and without ampicillin and of the β -lactamase preparations after steps (b) and (c) of the purification. Activity staining was not possible due to the instability of the enzyme in the presence of SDS, but it seemed clear that the band positioned just above the chymotrypsinogen A (25 kDa) was the β -lactamase. A corresponding band was not detectable in preparations from uninduced cells. A molecular mass for β -lactamase of 28.2 kDa was calculated from each of two gels obtained on different occasions.

Non-denaturing PAGE of native β -lactamase from concentrated shock fluid [step (b) of the purification] was also done, and the gel was stained first for β -lactamase activity and then for protein. β -Lactamase activity was only seen at the position coinciding with the major protein band (not shown). These results support the conclusion reached from the SDS-PAGE that the major inducible protein is the β -lactamase of *L. enzymogenes*.

The native enzyme was eluted from the Sephadex G-75 column with a K_{av} of 0.37. The K_{av} values of the standard proteins were 0.14 (*E. coli* alkaline phosphatase), 0.28 (ovalbumin), 0.45 (*L. enzymogenes* phosphatase) and 0.57 (cytochrome *c*). From these data an apparent molecular mass of approximately 30 kDa was estimated for the β -lactamase. A comparison of the molecular mass values from the SDS-PAGE and Sephadex G-75 chromatography indicated that the native enzyme is composed of one polypeptide chain.

During step (b) of the purification the β -lactamase was eluted from CM-Sephadex at pH 9.8. This suggested that the enzyme has a high isoelectric point. Since isoelectric points of very basic proteins are difficult to determine in isoelectric focusing gels, we compared the elution of the β -lactamase from CM-Sephadex to that of two other basic proteins. The β -lactamase, pancreatic ribonuclease and cytochrome *c* were eluted from a small column of CM-Sephadex using a pH gradient as described in Methods. Pancreatic ribonuclease, with an isoionic point

Fig. 2. SDS-PAGE of *L. enzymogenes* β -lactamase. The electrophoresis was carried out as described in Methods. The shock fluids were prepared in 10 mM-Tris/HCl, pH 8.4. They were concentrated 50-fold by freeze-drying and samples were dialysed and prepared for SDS-PAGE. The lanes contained: 1, 15 μ l (~ 60 μ g protein) shock fluid from cells grown without ampicillin; 2, 15 μ l (~ 90 μ g protein) shock fluid from cells grown with ampicillin; 3, 10 μ l (~ 3 μ g protein) after purification step (b); 4, 20 μ l (~ 1.3 μ g protein) after purification step (c); and 5, 20 μ l standard protein mixture (phosphorylase b, BSA, ovalbumin, chymotrypsinogen A, cytochrome c).

of 9.6 and an isoelectric point of 9.45 (Richards & Wyckoff, 1971), was eluted at pH 9.40. The β -lactamase was eluted together with cytochrome c at pH 9.60. Mammalian cytochrome c has an isoionic point and isoelectric point of 10.04 and this value is unaffected by the presence of various positive ions according to Barlow & Margoliash (1966). However, an isoelectric point of 9.6 is reported by Bio-Rad (1988). In the absence of any ionic effect, a basic protein should be eluted from a cation exchanger at its isoelectric pH and if the ionic strength of the solution is increased it should be eluted slightly below the isoelectric pH. Since the proteins in this study were eluted at low ionic strength with the hydroxyl and glycine

ions as the only major negative ions, we estimate that the isoelectric point of the β -lactamase is similar to that of cytochrome c, i.e. approximately 9.6 or higher.

Inhibitors of β -lactamase

EDTA at concentrations of up to 5 mM had no inhibitory effect on the β -lactamase activities in cell-free extracts and in shock fluids of the induced cells or, as expected, in more highly purified enzyme preparations. This indicated that the organism does not also produce a β -lactamase which is dependent on metal ions for activity and is in agreement with the PAGE results, which indicated that the organism produces only one major β -lactamase. The concentrations of the other inhibitors tested were chosen to give between 0% and greater than 90% inhibition under the conditions described in Methods. The inhibitor concentrations causing approximately 50% inhibition were determined to be 25 μ M for pCMBS and 35 μ M for pCMB in the presence of BSA and 0.08 μ M for pCMBS and 0.04 μ M for pCMB in the absence of BSA. Clavulanic acid at 0.28 μ M inhibited the β -lactamase by 50%. The sensitivity to pCMBS and pCMB indicates that the enzyme requires thiol groups for activity, and this is in agreement with the stabilizing effect of dithiothreitol.

Substrate specificity of β -lactamase

The activities of the β -lactamase with nine different substrates and the K_m values for the substrates that were readily hydrolysed were determined in order to further characterize the enzyme (Table 3). The K_m values and maximum velocity (V) values were obtained from $1/v$ versus $1/[S]$ plots using seven different concentrations for each substrate, except where indicated otherwise in Table 3.

The enzyme was relatively non-specific, degrading both penicillins and cephalosporins, but it was considerably more active with ampicillin and penicillin G than with cephaloridine and cephalothin. Oxacillin and cloxacillin were hydrolysed very slowly, if at all. Although the induction pattern of β -lactamases in different organisms is complex (Sanders, 1987), it is interesting to note that oxacillin was a poor substrate but a relatively good inducer of the *L. enzymogenes* enzyme.

Both cefotaxime and PADAC were hydrolysed slowly. However, because of the high molar absorption coefficient of PADAC, its use as the substrate for the routine assay nevertheless resulted in a very sensitive assay method.

According to this substrate profile, the *L. enzymogenes* β -lactamase could belong to either the group 2b, group

Table 3. Rates of hydrolysis by shock fluid-derived β -lactamase and K_m values for some β -lactam substrates

Substrate	V		K_m (μM)
	(units ml ⁻¹)	(%) [*]	
Penicillin G	5.3	100	116
Ampicillin	10.9	206	438
Carbenicillin	2.0	37	141
Oxacillin	<0.06†	<1	ND
Cloxacillin	<0.06†	<1	ND
Cephaloridine	0.87	16	91
Cephalothin	1.88	35	57
Cefotaxime	0.41†	8	ND
PADAC	0.12	2	ND

ND, Not determined.

^{*} The activity obtained with penicillin G was assigned a value of 100%.[†] The activities were obtained with 120 mM-oxacillin or cloxacillin and 100 mM-cefotaxime. Therefore, they may not represent maximum velocities.

2b' or, possibly, group 2c of β -lactamases (Bush, 1989a, b).

N-terminal amino acid sequence

The β -lactamase of *L. enzymogenes* was induced and purified for amino acid sequencing as described in Methods. As shown in Fig. 3, a complete sequence was obtained up to amino acid 20 and a partial sequence for amino acids 21 to 41. Although the sequence contains many amino acids with degenerate codons, the results have been used to construct an oligonucleotide probe which is being used to characterize the gene for the β -lactamase.

Concluding remarks

The results of our experiments show that the β -lactamase of *L. enzymogenes* is an inducible, periplasmic enzyme of about 28 kDa which has a very high isoelectric point. It is relatively non-specific with respect to the substrate it hydrolyses and is strongly inhibited by pCMBS, pCMB and clavulanic acid but not by EDTA. According to these properties the enzyme is similar to the group 2b β -lactamase produced by *Pseudomonas cepacia* (Hirai *et al.*, 1980; Bush, 1989b). Furthermore, preliminary data suggest that the enzyme seems to be coded for by a chromosomal gene. We were unable to isolate β -lactamase-negative strains after growth with 50 μg and 100 μg ethidium bromide ml⁻¹ or after extended cultivation at the organism's maximum growth temperature (36 °C) (results not shown).

1	Leu	Ser	Pro	Lys	Pro	Ala	Ala	Ser	Ala	Asp	10
11	Ala	Ala	Leu	Arg	Ala	Arg	Leu	Ala	Glu	Leu	20
21	Asp?	Lys	Arg	Ser	Gly	Gly?	-	Leu	Gly	Val	30
31	Ser?	Ala	Leu	Asp	Thr	Ala	Phe?	-	Gly	-	40
41	Phe										

Fig. 3. N-terminal amino acid sequence of *L. enzymogenes* β -lactamase. -, Undetermined amino acid residue.

Electrophoresis of the enzyme under denaturing and non-denaturing conditions, in combination with the activity staining, indicates that the purified protein is the β -lactamase of *L. enzymogenes*. Furthermore, the response of the enzyme to inhibitors and the good recoveries of enzyme activity during the purification steps indicate that the enzyme is the only major β -lactamase of the organism.

Our main interest is to determine the mechanism of secretion and the localization of extracellular proteins by *L. enzymogenes*. The β -lactamase will be a readily detectable periplasmic marker and, thus, should be very useful for these studies.

The physiological function of this enzyme in *L. enzymogenes* is also of interest. The β -lactamases have been reviewed by Richmond & Sykes (1973) and more recently by Sanders (1987) and Bush (1989a, b, c). The interest in these enzymes stems mainly from their possible role in the resistance of pathogenic bacteria to β -lactam antibiotics. However, non-pathogenic organisms also produce these enzymes and *L. enzymogenes* seems to be a typical example of such a Gram-negative bacterium. The function of β -lactamases in these organisms was discussed by Richmond & Sykes (1973) but they did not arrive at a definite conclusion and it is not clear why *L. enzymogenes* and other non-pathogenic bacteria produce this enzyme. It may be a protective mechanism for some other organisms, but *L. enzymogenes* seems to have a natural resistance to β -lactam antibiotics without the β -lactamase. The rate of growth of *L. enzymogenes* was not affected by 300 μg ampicillin ml⁻¹ or 100 μg ml⁻¹ of the other β -lactam antibiotics listed in Table 1. This lack of inhibition was particularly surprising in the case of antibiotics like oxacillin which were not degraded by the enzyme. Impermeability of the outer membrane does not seem to be a factor in the resistance since the antibiotics function as inducers. Perhaps the target structure for the antibiotic is insensitive. Furthermore, it is unlikely that *L. enzymogenes* encounters high enough inducer concentrations in its natural habitat to significantly increase the

β -lactamase levels. It also is unlikely that the enzyme has a nutritional function as do many of the other hydrolases that this organism produces since synthesis of the β -lactamase does not seem to be affected by catabolite repression. Thus, the metabolic function of the β -lactamase in *L. enzymogenes* is as yet unknown and the induction of the enzyme is a poorly understood and, perhaps, fortuitous event.

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