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β-Lactamases from *Yersinia enterocolitica*

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SUMMARY

Two β-lactamases, A and B, have been shown to be present in a strain of *Yersinia enterocolitica* (W222). β-Lactamase A hydrolyses a variety of penicillins and cephalosporins. This enzyme is sensitive to thiol reagents, is only partially inhibited by 0·1 mM-cloxacillin and has a molecular weight of approximately 20000. β-Lactamase B shows strong cephalosporinase activity but does not hydrolyse some of the penicillins. It is more resistant than β-lactamase A to thiol reagents, is completely inhibited by 0·1 mM-cloxacillin and has a molecular weight of about 34000. With cephaloridine as a substrate, which is readily hydrolysed by both enzymes, about 85% of the total activity of a cell extract is due to β-lactamase A and 15% to B. Addition of 6-aminopenicillanic acid to the culture during growth results in a 2- to 4-fold selective increase in the amount of β-lactamase B.

Two β-lactamases similar to enzymes A and B have been found in five other strains of *Y. enterocolitica*. In contrast, only one β-lactamase, similar to enzyme B, has been detected in a different strain of *Y. enterocolitica* (H66), which is abnormal in that it is sensitive to ampicillin. Addition of 6-aminopenicillanic acid to cultures of this strain results in an 8- to 10-fold increase in β-lactamase production.

INTRODUCTION

Antibiotics of the β-lactam group show relatively poor activity against *Yersinia enterocolitica* (Niléhn, 1967, 1969; Wauters, 1970). Many strains of these bacteria are β-lactamase producers (Cornelis, Wauters & Vanderhaeghe, 1973*b*). No β-lactamase activity was detected by the same techniques in *Y. pseudotuberculosis*. Mishankin, Ryzhko & Grigorian (1973) also concluded that β-lactamase was present in *Y. enterocolitica* ('Pasteurella X'), but not in strains of *Y. pseudotuberculosis*.

In the present work, an attempt has been made to study some of the properties of β-lactamases produced by strains of *Y. enterocolitica* belonging to the serological groups 3 and 9 (Winblad, 1967, 1968; Wauters, Le Minor & Chalon, 1971; Wauters, Le Minor, Chalon & Lassen, 1972). Strains W222, W239 and W267 were chosen at random among strains of group 3 recently isolated from patients with enteritis. Strain IP134 of group 3 from the collection of l'Institut Pasteur, Paris, France (also called the Winblad strain, see Winblad, Niléhn & Sternby, 1966, and isolated from a patient with acute terminal ileitis and mesenteric lymphadenitis), was chosen because it is now widely known. In strain H66, isolated from a patient with abdominal pain and diarrhoea, the pattern of resistance to β-lactam antibiotics was different from that of other strains of the same group (Cornelis *et al.* 1973*b*). Although strain H66 is resistant to cephalothin and is a β-lactamase producer it is sensitive to ampicillin and carbenicillin. Strains W285 (isolated from a normal pig) and W227 (from a patient with symptoms simulating acute appendicitis) were strains of serological group 9.

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All these strains are sensitive to the broad spectrum antibiotics other than those of the β-lactam family and there is no reason to suspect that their resistance to the latter is mediated by a plasmid. Although more than 3000 strains of *Y. enterocolitica* have now been classified at l'Institut Pasteur (H. H. Mollaret), naturally occurring R factor-containing strains have been described in only three cases (Cornelis, Wauters & Bruynoghe, 1973*a*; Cornelis *et al.* 1973*b*).

METHODS

Bacteria. All the strains of *Y. enterocolitica* studied came from Professor G. Wauters, Department of Microbiology, University of Louvain. Strain IP134 ('Winblad') is a member of the *Y. enterocolitica* collection of Professor H. H. Mollaret, l'Institut Pasteur.

Growth of cultures. Brain–heart infusion broth (Oxoid; 200 ml) in a 500 ml conical flask with four baffles (Smith, Warren, Newton & Abraham, 1967) was inoculated with a 24 h culture grown at 27 °C on a brain–heart agar slope (10 ml) in a 25 ml bottle. The flask was placed on a rotary shaker (160 rev./min) at 27 °C (Wauters, 1970). After overnight incubation, 10 ml of the culture was added to each of a variable number of similar flasks containing 190 ml brain–heart infusion. These flasks were incubated at 27 °C, 160 rev./min. The bacteria were harvested after 7 h, at the end of the exponential phase, when the extinction was about 6. Extinctions of the cultures were measured with a Spekker absorptiometer, using a neutral grey filter H 508.

Preparation of crude extracts. Cultures were centrifuged at 4 °C for 90 min at 2000 *g*. The supernatants were discarded and the bacteria kept frozen at −20 °C. Before use the thawed bacteria were mixed with phosphate buffer (0·025 M, pH 7·3) to give a suspension of 17 ml for 1 l culture. Samples of this suspension (7 ml) were disintegrated ultrasonically (in a jacketed glass vessel cooled with ethanol at 0 °C) by treatment for 4 min in an MSE ultrasonic disintegrator (60 W, 20 kHz). Centrifugation at 100000 *g* for 3 h at 4 °C yielded a clear yellow supernatant which is referred to as crude extract.

Column chromatography. The eluates from columns were monitored by use of a Uvicord II detector, at 280 ± 5 nm. The concentration of NaCl in fractions obtained during gradient elution was calculated from conductivity measurements of the fractions at room temperature, made with a Radiometer conductimeter type CDM 2E.

Before chromatography the crude extract of strain W222 was treated with DNase (final concentration 10 μg/ml), kept for 3 h at 20 °C and used immediately. After 2 to 3 days at 4 °C, precipitation occurred and most of the β-lactamase activity was associated with the precipitate. The treated extract was dialysed against phosphate buffer (0·025 M, pH 7·3) and applied to a column of Sephadex CM 50.

One active component, B, passed straight through the column (Fig. 2, below) to give a cloudy solution. This solution was centrifuged at 100000 *g* for 2 h. The clear supernatant (40 ml) was dialysed against diethanolamine–HCl buffer (0·05 M with respect to diethanolamine, pH 8·6) and applied to a column (24 × 2·6 cm) of DEAE-Sephadex. Component B was eluted at 0·05 M-NaCl by a NaCl gradient (0 → 0·2 M) in the same buffer.

A second active component, A, was retained on the column of Sephadex CM 50 and eluted at 0·15 M-NaCl by a NaCl gradient (0 → 0·5 M-NaCl) in phosphate buffer (Fig. 2).

Assay of β-lactamases. β-Lactamase activity is expressed as μmol substrate hydrolysed/min/ml enzyme solution, at 30 °C and pH 6·5. Three methods of assay were used.

(i) Rates of hydrolysis were usually determined titrimetrically in a pH stat (Sabath, Jago & Abraham, 1965; Hou & Poole, 1972). The apparatus (Radiometer, Copenhagen, Denmark) comprised a TTT 2 titrator, an ABU 12 autoburette and an SBR 3 titrigraph recorder. The reaction was carried out in a jacketed vessel at 30 °C.

The solutions to be assayed (5 to 200 μl) were added to 1·6 to 1·8 ml water in the reaction vessel. After equilibration at the chosen pH, 200 μl of a concentrated substrate solution (at a lower or approximately the same pH) were added to give a final substrate concentration (unless otherwise stated) of 1 mg/ml. Hydrolysis of the substrate was followed by the addition of 40 mM-NaOH to the reaction mixture, except in measurements for the determination of K_m for cephaloridine when 10 mM-NaOH was used.

Rates of hydrolysis (μmol/min) were calculated on the assumption that 1 equivalent of acid is liberated per mole of substrate hydrolysed for penicillins, cephalexin and cefoxitin, while two equivalents of acid are liberated on hydrolysis of the other cephalosporins used (Sabath *et al.* 1965).

(ii) For the determination of the rate of hydrolysis of 6-aminopenicillanic acid (6-APA) a spectrophotometric assay was used (Waley, 1974). The change in absorption at 250 nm was followed, at 30 °C and pH 6·8, in a Cecil spectrophotometer (Cecil Instruments, Cambridge) with a Servoscribe recorder.

(iii) For the determination of Michaelis constants for benzylpenicillin, rates of hydrolysis were measured by a microiodometric method as described by Novick (1962) except that a phosphate buffer (0·05 M, pH 6·5) was used. The data were plotted as described by Eadie (1942).

Detection of β-lactamases. The presence of β-lactamase in fractions from chromatographic columns was detected by using cephalosporin 87/312 (O'Callaghan, Morris, Kirby & Shingler, 1972), which undergoes a colour change from yellow to red on hydrolysis of its β-lactam ring. A sample (10 μl) of the solution to be tested was added to 0·5 ml of a solution of the cephalosporin in water (0·1 mM, 51·6 μg/ml).

Substrates and inhibitors. Benzylpenicillin, cephalosporin C, cephaloridine and cephalosporin 87/312 were from Glaxo Research Laboratories, Greenford, Middlesex; cephalothin, cephalexin, cefamandole and *O*-formylcefamandole (Eykyn, Jenkins, King & Phillips, 1973) were from Eli Lilly & Co., Indianapolis, U.S.A. 6-APA, cloxacillin, methicillin, carbenicillin and ampicillin were from the Beecham Research Laboratories, Brockham Park, Surrey. Oxacillin was from Bristol Laboratories, Syracuse, New York, U.S.A., and cefoxitin (a 7-methoxycephalosporin) was from Merck, Sharp & Dohme Research Laboratories, Rahway, New Jersey, U.S.A.

Isoelectric focusing. A Multiphor isoelectric focusing apparatus and Ampholines from LKB were used. Gels were made according to the maker's instructions for the pH range 3·5 to 9·5, except that 0·6 ml instead of 0·4 ml of riboflavin solution was used. The runs were performed lengthways. After prefocusing for 1·5 to 2 h, each channel received 5 or 10 μl of the enzyme solutions and the run was continued for a further 4 h.

For pH measurements, the two outside channels were cut into sections 5 mm wide and the sections soaked in 0·6 ml of degassed water in stoppered tubes. For colorimetric location of the enzyme, channels were cut into sections in the same way and each section dropped into 0·25 ml phosphate buffer (0·025 M) pH 7·3. After a few hours, samples of the solutions (100 or 50 μl, according to the activity present) were added to 0·5 ml of a solution of cephalosporin 87/312 (0·1 mM).

Para-chloromercuribenzoate (PCMB), Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) (Habeeb, 1972), ε-2,4-dinitrophenyl-L-lysine (ε-DNP-lysine) and DNase from bovine pancreas (about 350 units/mg) were from BDH. *N*-ethylmaleimide (NEM), bovine serum albumin, soybean trypsin inhibitor, ovalbumin and cytochrome *c* were from Sigma. Preparations of Sephadex and Dextran Blue were from Pharmacia.

Table 1. *Substrate profiles of β-lactamase from Y. enterocolitica, strain* W222

The rates of hydrolysis were determined at 30 °C with substrate concentrations of 2 mg/ml (about 5 mM). With 6-APA the rate was measured spectrophotometrically (Waley, 1974) at pH 6·8. With other substrates measurements were made in the pH stat at pH 6·5. The rates given are relative to an arbitrary value of 100 for benzylpenicillin.

		β-Lactamase A		β-Lactamase B	
Substrate	Crude extract	Rate of hydrolysis	K_m (μM)	Rate of hydrolysis	K_m (μM)
Penicillin G	100	100	39	100	17
Ampicillin	43	77	—	<1	—
Carbenicillin	21	25	—	<1	—
Cephaloridine	248	300	175	277	—
Cephalosporin C	88	35	—	605	—
Cephalothin	—	134	—	214	—
Oxacillin	—	16	—	<1	—
Cloxacillin	—	2	—	<1	—
Methicillin	—	8	—	<1	—
Cephalexin	—	5	—	8	—
Cefoxitin	—	<1	—	<1	—
Formylcefamandole	—	317	—	40	—
Cefamandole	—	127	—	<1	—
6-APA	—	13	—	—	—

RESULTS

β-Lactamase of strain W222

Amounts of enzyme produced. All the β-lactamase is cell-bound. The specific enzyme activity of the crude bacterial extract without any potential inducer (in units/mg dry wt organisms, with benzylpenicillin as substrate) is about 7×10^{-1}. This corresponds to an activity of about 7 units/ml of crude extract. The substrate profile of the crude extract is shown in Table 1.

Effect of 6-APA on β-lactamase production. In a preliminary attempt to determine whether the organism produced an inducible β-lactamase, 6-APA was added to the culture 4 h after inoculation (to give a final concentration of 200 μg/ml) and the bacteria were harvested after 6 h. The quotient (β-lactamase activity of cultures grown with 6-APA)/(β-lactamase activity of cultures grown without 6-APA) was 1·9 when cephalosporin C was used as a substrate but 1·4 when cephaloridine was used. No increase in the latter value was observed in cultures grown in higher concentrations (400 and 800 μg/ml) of 6-APA.

The addition of 6-APA to cultures had a much greater effect on the activity of the resulting extracts against cephalosporin C than against cephaloridine. Figure 1 shows the increase in the quotient (rate of hydrolysis of cephalosporin C)/(rate of hydrolysis of cephaloridine) obtained with bacterial extracts from cultures grown in the presence of increasing amounts of 6-APA. The quotient varied from 0·33 when no 6-APA was added, to 0·99 when its concentration was 800 μg/ml. This indicated that two β-lactamases were produced by the organism but that only one (which hydrolysed cephalosporin C more rapidly than cephaloridine) was inducible.

Separation of two β-lactamases. Preliminary experiments (in which β-lactamase activity with cephalosporin 87/312 was revealed colorimetrically) showed that the crude extract yielded two peaks of activity when chromatographed on DEAE-Sephadex at pH 8·6 or on Sephadex CM 50 at pH 7·3.

One component, B, was not retarded on Sephadex CM 50 but a second component, A,

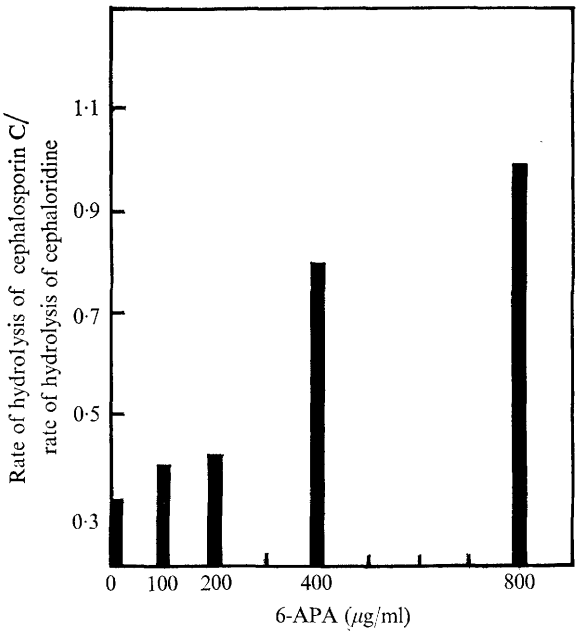

Fig. 1. Effect of induction by 6-APA on the relative rates of hydrolysis of cephalosporin C and cephaloridine by an extract of *Y. enterocolitica* strain W222. Rates of hydrolysis were measured with suspensions subjected to ultrasonic treatment. Different amounts of 6-APA were added to cultures 4 h after inoculation. The cultures were harvested after 7 h.

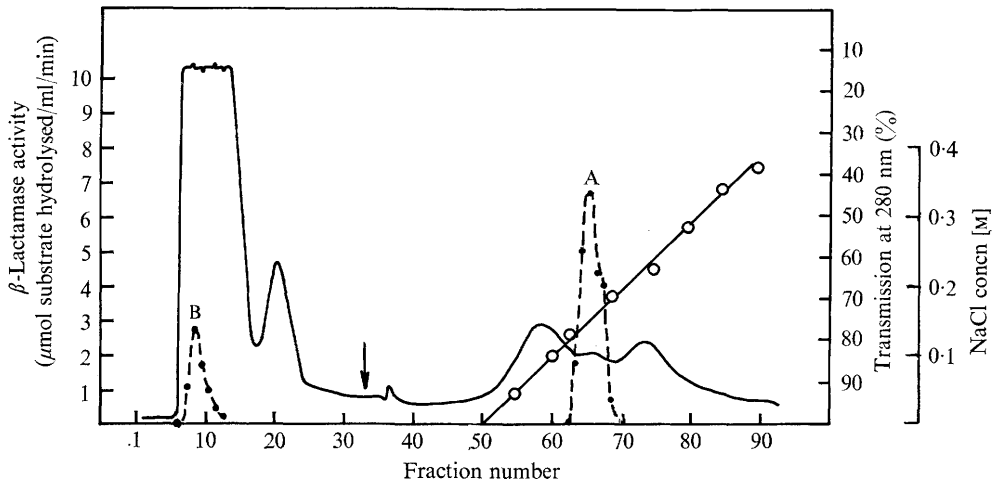

Fig. 2. Chromatographic separation of *β*-lactamases A and B from *Y. enterocolitica*, strain W222. Crude extract (14 ml) was chromatographed on a column (2·6 × 24 cm) of Sephadex CM 50 in sodium phosphate buffer (0·025 M) pH 7·3 at 4 °C. The flow rate was 26 ml/h and fractions were collected every 15 min. NaCl gradient (0 → 0·5 M) was applied after the collection of 33 fractions. Cephalosporin C was used as a substrate in the assay of enzyme B, and cephaloridine in the assay of enzyme A. ●--●, *β*-Lactamase activity; ○—○, NaCl concentration; ——, light transmission at 280 nm.

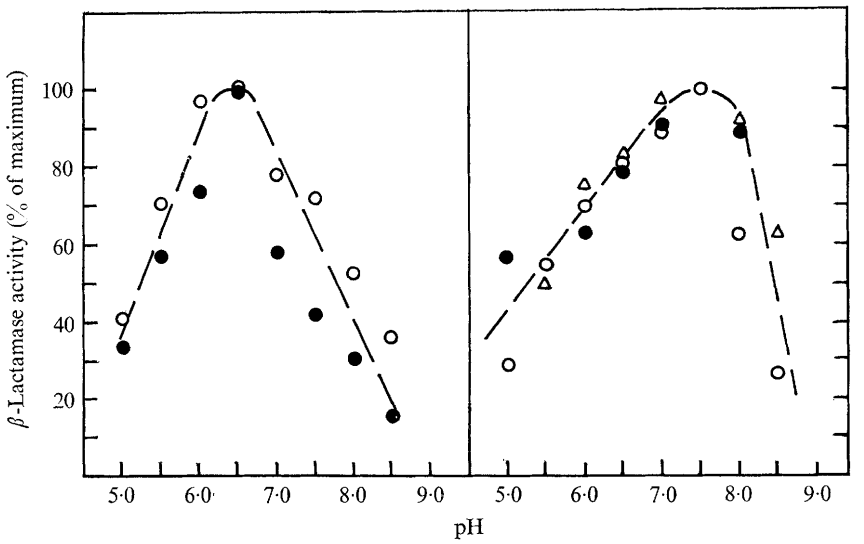

Fig. 3. pH–activity curves for β-lactamases A (left) and B (right) from *Y. entercolitica*, strain W222. The symbols ●, ○, and △ show the results obtained in different experiments. The pH was adjusted with 2 N-HCl or 2 N-NaOH in the pH stat.

was adsorbed and could subsequently be eluted in a NaCl gradient (Fig. 2). In contrast, component B was adsorbed on a column of DEAE-Sephadex (see Methods).

Component A contained about 85 % of the β-lactamase activity, when assayed against cephaloridine, if no potential inducer had been added to the culture during growth (Fig. 2). To obtain larger amounts of component B, 6-APA (1 mg/ml) was added to the cultures. The proportion of B then increased from 15 to 33 % of the total, as determined from β-lactamase activity with cephaloridine as substrate.

Enzymic properties of β-lactamases A and B. Thin-layer chromatography showed that both enzymes hydrolysed benzylpenicillin to benzylpenicilloic acid. Both enzymes also rapidly changed the colour of cephalosporin 87/312 from yellow to red, which is characteristic of the opening of the β-lactam ring of this compound (O'Callaghan *et al.* 1972).

Cephaloridine is hydrolysed more rapidly than benzylpenicillin by both enzymes (Table 1). On the other hand, the relative rate of hydrolysis of cephalosporin C is much greater with β-lactamase B than with β-lactamase A. Ampicillin, carbenicillin and cefamandole were substrates for A but resistant to hydrolysis by B. Cefoxitin was resistant to both enzymes and cloxacillin and cephalexin were resistant or relatively poor substrates. The K_m values for enzymes A and B with benzylpenicillin as a substrate were similar.

The pH–activity curves of β-lactamase A (with cephaloridine as substrate) and β-lactamase B (with cephalosporin C as substrate) are shown in Fig. 3. The curve for enzyme A is relatively sharp and has a maximum near pH 6·5. The curve for enzyme B is less sharp and has a maximum at about pH 7·5.

Under the conditions used, treatment with three thiol reagents caused some loss of activity with both enzymes but this was much greater with enzyme A than enzyme B (Table 2). On the other hand, 0·1 mM-cloxacillin completely inhibited the activity of enzyme B (with cephalosporin C as a substrate) but only partially inhibited the activity of enzyme A.

Molecular weights and isoelectric points. An estimate of the molecular weights of the two β-lactamases was made by gel-filtration on Sephadex (Andrews, 1964).

Table 2. *Inhibition of β-lactamase from Y. enterocolitica by thiol reagents and by cloxacillin*

The activities of enzymes A and B were determined with cephaloridine and cephalosporin C, respectively, as substrates. The thiol reagents were allowed to react with the enzymes at 37 °C in 0·025 M-sodium phosphate buffer at pH 7·3 (PCMB and NEM) or pH 8·0 (Ellman's reagent), and for 20 min (PCMB) or 90 min (NEM and Ellman's reagent). Cloxacillin was added to the enzyme in the pH-stat 5 min before the addition of substrate.

Inhibitors	Concentration (mM)	Percentage inhibition of β-lactamase A	Percentage inhibition of β-lactamase B
PCMB	0·5	100	14
NEM	2·0	39	19
Ellman's reagent	1·0	57	29
Cloxacillin	0·1	28	100

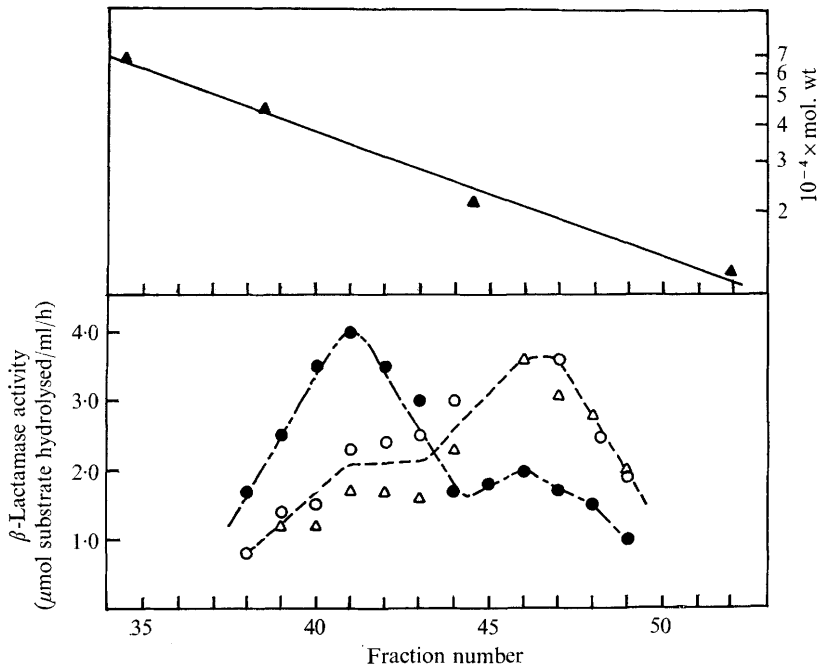

Fig. 4. Estimation of the molecular weights of β-lactamases A and B. Gel filtration of the crude extract was carried out on a column (2·7 × 100 cm) of Sephadex G 100 at 4 °C in sodium phosphate buffer, 0·025 M, pH 7·3; the flow rate was 35 ml/h and 8·6 ml fractions were collected. The reference substances (▲) were: bovine serum albumin (mol. wt 67000); ovalbumin (mol. wt 45000); soybean trypsin inhibitor (mol. wt 21500); cytochrome *c* (mol. wt 12500). The Dextran Blue peak appeared in fraction 27 and the DNP-lysine peak in fraction 71. ●, Rate of hydrolysis with cephalosporin C as substrate; ○, rate with cephaloridine as substrate; △, rate with formylcefamandole as substrate.

Solutions of β-lactamase A (100 units, assayed with cephaloridine as substrate) and β-lactamase B (100 units, assayed with cephalosporin C as substrate) were mixed with Dextran Blue, ε-DNP-lysine and four proteins of known molecular weight as standards, and applied to a column of Sephadex G 100. The filtrate was assayed for β-lactamase activity with three substrates: cephalosporin C (rapidly hydrolysed by B), cephaloridine (rapidly hydrolysed by both enzymes) and *O*-formylcefamandole (rapidly hydrolysed by A).

Table 3. *Substrate profile of β-lactamase of Y. enterocolitica, strain* H66

Assays were done at pH 6·5 with substrate concentrations of 2 mg/ml.

	Relative rates of hydrolysis with β-lactamase from					
	Strain H66 (non-induced)*		Strain H66 (non-induced)†		Strain H66 (induced)‡	Strain W222 (β-lactamase B)§
	Expt 1	Expt 2	Expt 1	Expt 2		
Penicillin G	100	100	100	100	100	100
Cephaloridine	230	196	96	278	286	277
Cephalosporin C	738	493	802	721	656	605
Cephalothin	—	—	—	—	222	214
Ampicillin	—	—	—	—	<1	<1
Carbenicillin	—	—	—	—	<1	<1
Oxacillin	—	—	—	—	<1	<1
Cloxacillin	—	—	—	—	<1	<1
Methicillin	—	—	—	—	<1	<1
Cephalexin	—	—	—	—	19	8
Cefoxitin	—	—	—	—	<1	<1
Formylcefamandole	—	—	—	—	24	40
Cefamandole	—	—	—	—	<1	<1

—, Not tested.

* Values refer to the crude extract of strain H66 grown without inducer.

† Values refer to samples obtained by chromatography on Sephadex CM 50 (see text) and concentrated by dialysis against Carbowax.

‡ Values refer to the crude extract of cultures grown with 6-APA.

§ These values are given for comparison, and refer to β-lactamase B from strain W222.

The distribution of activity as determined with the different substrates is shown in Fig. 4. The two enzymes were clearly separated from each other. Comparison of the positions of the peak fractions with those of the reference proteins indicated that the molecular weight of β-lactamase A was 20000 and that of β-lactamase B 34000. The isoelectric point of enzyme A, determined by isoelectric focusing, was at pH 8·1 ± 0·1, while that for B was at 5·4 ± 0·1.

The β-lactamase of strain H66

Production and nature. Crude extracts of *Y. enterocolitica*, strain H66 (grown without any potential inducer), showed activities ranging from 1·3 to 2·3 units/ml with cephalosporin C as substrate. The corresponding activity of the crude extract of strain W222 grown under similar conditions was about 6 units/ml with cephalosporin C as substrate and about 18 units/ml with cephaloridine as substrate.

Chromatography of the crude extract of strain H66 on Sephadex CM 50 at pH 7·3, under the conditions used with the extract of strain W222, revealed only one β-lactamase. This enzyme emerged from the column near the solvent front, like β-lactamase B from strain W222, and was detected colorimetrically with cephalosporin 87/312. It accounted for more than two-thirds of the small total activity of the crude extract. No enzyme corresponding to β-lactamase A from strain W222 was eluted with the NaCl gradient.

The substrate profile of the β-lactamase in the crude extract from strain H66 resembled that of enzyme B from strain W222, and differed from that of enzyme A (Table 3). Apparent differences in some of the values obtained in two different experiments can probably be attributed to the very low activities of these extracts.

Induction of β-lactamase from strain H66. In view of its similarity to β-lactamase B from

Table 4. *Induction of β-lactamase production in Y. enterocolitica strain* H66 *by 6-APA*

6-APA was added 4·5 h after inoculation and the cultures harvested after 7 h. Enzyme activities are related to an arbitrary value of 100 for the culture grown without 6-APA.

6-APA (μg/ml)	β-Lactamase activity (relative)
0	100
25	260
50	407
100	584
250	840
500	815

Table 5. *β-Lactamase production by different strains of Y. enterocolitica*

Rates of hydrolysis were measured at 30 °C and pH 6·5. Fraction B contains β-lactamase not adsorbed on Sephadex CM 50 at pH 7·3. Fraction A contains β-lactamase eluted from Sephadex CM 50 after adsorption at pH 7·3. The low total β-lactamase activities of fractions B and A, relative to those of the crude extract, can be attributed partly to dilution of the enzymes during adsorption and elution (for details see text).

	Rate of hydrolysis (μmol/min/ml of solution) by					
	Crude extract of:		Fraction B of:		Fraction A of:	
Strain	Cephlor	CephC	Cephlor	CephC	Cephlor	CephC
W222	15·4	11·0	0·21	0·66	1·7	0·1
W267	18·1	13·8	0·33	0·89	2·0	<0·1
W239	17·7	9·2	0·27	0·57	1·2	<0·1
IP134	22·0	10·0	0·25	0·46	0·8	<0·1
W285	15·0	12·0	0·35	0·91	1·6	0·1
W227	29·5	28·0	0·97	2·50	1·0	<0·1

Cephlor, cephaloridine; cephC, cephalosporin C.

strain W222, it was of interest to determine whether the enzyme from strain H66 was inducible. 6-APA was added to the culture 4·5 h after inoculation. Because of the relatively low resistance of strain H66 to a number of β-lactam antibiotics (Cornelis *et al.* 1973*b*), the amounts of 6-APA used were smaller than with strain W222.

The results of one experiment are given in Table 4. The quotient (β-lactamase activity produced in the presence of 6-APA)/(β-lactamase activity produced in the absence of 6-APA) increased with the concentration of 6-APA and reached a maximum of 8·4 when the latter was 250 μg/ml. In two other experiments, with 200 μg 6-APA/ml, the corresponding values were 8·1 and 10·0.

The substrate profile of the crude β-lactamase obtained from strain H66 after induction with 6-APA (Table 3) is similar to that of enzyme B from strain W222. The pH activity curve of the crude induced enzyme from strain H66 showed a maximum at pH 7·5 to 8·0 and closely resembled the curve obtained with β-lactamase B from strain W222.

β-Lactamases of other strains

Production and nature of the enzymes. Five strains of *Y. enterocolitica* were grown in parallel with the strain W222. After 4 h growth, 6-APA was added to the cultures to give a final concentration of 1000 μg/ml. The β-lactamase activity of crude extracts of all the

strains was of the same order when determined with either cephaloridine or cephalosporin C as the substrate (Table 5). The variation found in the ratio of activity against cephaloridine to activity against cephalosporin C may be attributed to variation in the response to the inducer.

Evidence that all the extracts contained two β-lactamases similar to enzymes A and B from strain W222 was obtained as follows. The extracts were dialysed against phosphate buffer (pH 7·3, 0·025 M) for 16 h, and 0·5 ml samples of each were then added to 13 ml of wet Sephadex CM 50, equilibrated with the same buffer at the same pH, in 15 ml centrifuge tubes. After the tubes had been kept for 2 h at 4 °C, with regular gentle shaking, the resin was spun down (1500 *g*, 30 min) and the supernatant assayed against cephaloridine and cephalosporin C.

The quotient (rate of hydrolysis of cephaloridine)/(rate of hydrolysis of cephalosporin C) was smaller with the β-lactamases unadsorbed on Sephadex CM 50 (Table 5) than with those of the crude extracts. The average value of this quotient for the unadsorbed enzymes of the group was 0·43. The corresponding value for β-lactamase B of strain W222 was 0·45, and that for the B-like enzyme from strain H66 was 0·44. These results were consistent with the presence of a B-like enzyme in all the five strains.

In a second experiment, 1 ml of each crude extract was added to 2 ml Sephadex CM 50 and the mixtures kept for 1 h with gentle shaking. The absorbent was then washed ten times with 2 ml of buffer, and 0·5 ml of 1 M-NaCl in the same buffer was added to elute the adsorbed β-lactamase. The results of assays of the supernatants obtained on centrifugation, with cephaloridine and cephalosporin C as substrates, are given in Table 5. The rates of hydrolysis of cephalosporin C were too low for precise determination of the relative activities against the two substrates to be made, but the values obtained were consistent with the presence in all the eluates of an enzyme resembling β-lactamase A from strain W222.

DISCUSSION

The specific β-lactamase activities of the strains of *Y. enterocolitica* studied fall within the range of values given for 46 strains of β-lactamase-producing Gram-negative bacteria by Jack & Richmond (1970), who defined the unit of activity as μmol benzylpenicillin hydrolysed/h. Although the strains of *Y. enterocolitica* may be regarded as relatively weak producers, they still show higher β-lactamase activities than many of the bacteria studied by these authors.

The finding that *Y. enterocolitica*, strain W222, produced two different β-lactamases, and that the same or two similar enzymes were produced by five other strains chosen at random from the serological groups 3 and 9, suggests that this is a common property of strains belonging to these groups. In general, these strains resemble each other closely in the pattern of their resistance to β-lactam antibiotics. Strain H66, which is abnormal among the strains of group 3 in that it is sensitive to ampicillin although still relatively resistant to cephalothin (Cornelis *et al.* 1973*b*), produced a β-lactamase of only one type, similar to enzyme B of strain W222. Ampicillin is not hydrolysed by this enzyme, whereas cephalothin is a good substrate.

Although the existence of two 'species specific' β-lactamases in the same organism is apparently uncommon, an analogous situation may exist within the Pseudomonas group and in *Bacillus cereus*. In addition to the inducible enzyme in *Pseudomonas aeruginosa* (Sabath *et al.* 1965) which seems to be generally present (Richmond, Jack & Sykes, 1971), another β-lactamase is constitutive and is thought not to be R factor-mediated (Newsom,

Sykes & Richmond, 1970; Richmond *et al.* 1971). Two different β-lactamases are produced by *B. cereus* (Sabath & Abraham, 1966; Crompton *et al.* 1962), and neither has been reported to be mediated by an R factor.

The tenfold increase in the production of the β-lactamase B-like enzyme by strain H66 in the presence of 6-APA indicates that this enzyme is inducible. Although the increase in the enzyme produced on induction was relatively small, it is no smaller than that observed with some of the β-lactamases in Enterobacter (Hennessey, 1967). In its relatively high cephalosporinase activity the enzyme from strain H66 resembles the inducible β-lactamases in Enterobacteriaceae (Ayliffe, 1964, 1965; Hennessey, 1967) and *P. aeruginosa* (Sabath *et al.* 1965; Zyk, Kalkstein & Citri, 1972; Yaginuma *et al.* 1973; McPhail & Furth, 1973).

The activity of the β-lactamase B of strain W222 was increased only 2- to 4-fold by the addition of 6-APA to the growing culture. Since this increase was observed with preparations which had been subjected to ultrasonic treatment but from which particulate matter had not been removed by centrifugation, it was probably due to enzyme induction, and not to cell damage by 6-APA allowing the subsequent liberation of the β-lactamase or increased accessibility to substrates. The relatively small increase in activity on induction resembles the observations of Richmond *et al.* (1971), who had difficulty in demonstrating the presence of an inducible β-lactamase in strains of *P. aeruginosa* which also produced a constitutive β-lactamase; they suggested that the constitutive enzyme destroyed the inducer before it reached its site of action.

β-Lactamase A of *Y. enterocolitica* resembles some other PCMB-sensitive β-lactamases from Gram-negative bacteria in its ability to hydrolyse a variety of penicillins and cephalosporins and in its relative resistance to inhibition by cloxacillin (Jack & Richmond, 1970). Like the enzyme described by Smith (1963) from *Aerobacter cloacae*, β-lactamase A appears to be less sensitive to NEM than to PCMB.

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