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An overview of the kinetic parameters of class B β-lactamases

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The catalytic properties of three class B β-lactamases (from *Pseudomonas maltophilia*, *Aeromonas hydrophila* and *Bacillus cereus*) were studied and compared with those of the *Bacteroides fragilis* enzyme. The *A. hydrophila* β-lactamase exhibited a unique specificity profile and could be considered as a rather specific 'carbapenemase'. No relationships were found between sequence similarities and catalytic properties. The problem of the repartition of class B β-lactamases into sub-classes is discussed. Improved purification methods were devised for the *P. maltophilia* and *A. hydrophila* β-lactamases including, for the latter enzyme, a very efficient affinity chromatography step on a Zn^{2+}-chelate column.

INTRODUCTION

β-Lactamases are extracellular or periplasmic enzymes produced by bacteria which confer resistance to β-lactam antibiotics (Sykes and Matthew, 1976; Medeiros, 1984; Waley, 1992). The enzymes have been divided into four classes on the basis of their primary structures and catalytic mechanisms. Enzymes of classes A, C and D are active-site-serine enzymes, but can be distinguished on the basis of their primary structures (Jaurin and Gundström, 1981; Dale et al., 1985; Ambler et al., 1991; Joris et al., 1991).

Class B β-lactamases are metalloproteins which require a bivalent transition-metal ion for their activity, most often Zn^{2+}. These enzymes were found to be produced by *Bacillus cereus* (β-lactamase II) (Kubawara and Abraham, 1967), which also produces two distinct class A enzymes (Pollock, 1956; Davies et al., 1974; Thatcher, 1975; Connolly and Waley, 1983; Nielsen and Lampen, 1983), *Pseudomonas maltophilia* (L-1) (Saino et al., 1982; Bicknell et al., 1985), which also produces a class C enzyme, *Aeromonas hydrophila* (A2) (Iaconis and Sanders, 1990) and *Bacteroides fragilis* (Cuchural et al., 1986; Bandoh et al., 1991). The sequences of these proteins have also been established (Lim et al., 1988; Rasmussen et al., 1990; Thompson and Malamy, 1990; Massidda et al., 1991; R. Levesque, personal communication).

The production of metallo-β-lactamases by pathogenic strains is likely to result in clinical problems. The mechanism-based inactivators which have been utilized to fight the active-site-serine enzymes will probably be useless against Zn^{2+}-dependent enzymes, and the acquisition of the genes coding for these proteins by other strains is a distinct and frightening possibility.

It is thus becoming urgent to understand the catalytic mechanism of these enzymes. In this paper we report the kinetic parameters of the *A. hydrophila*, *P. maltophilia* and *Bacillus cereus* β-lactamases, and compare them with those of the *Bacteroides fragilis* enzyme (Yang et al., 1992).

MATERIALS AND METHODS

Bacterial strains

Bacillus cereus and *P. maltophilia* enzymes were respectively produced by strains 5/B/6 and ULA-511 (collection strain of University of L'Aquila), a clinical isolate. The *A. hydrophila* enzyme was produced in *Escherichia coli* strain DH5α, containing plasmid pAA20R, which was resistant to tetracyclin (50 μg/ml). This strain was a gift from Professor G. Satta (Catholic University of Rome, Italy) and Dr. O. Massidda (University of Siena, Italy).

Antibiotics

Benzylpenicillin was from Rhône-Poulenc (Paris, France). Ampicillin and oxacillin were from Bristol Benelux (Brussels, Belgium), and carbenicillin was from Beecham Research Laboratories (Brentford, Middx., U.K.). Cephaloridine, cephalexin, cephaloglycin and moxalactam were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.), cefuroxime was from Glaxo Group Research (Greenford, Middx., U.K.) and cefotaxime was from Hoechst-Roussel (Romainville, France). Aztreonam SQ 81804 was from the Squibb Institute for Medical Research (Princeton, NJ, U.S.A.), cefoxitin and imipenem were from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.), and sulbactam and β-iodopenicillanic acid were from Pfizer Central Research (Sandwich, Kent, U.K.). All of these compounds were kindly given by the respective companies. In addition, nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.), and tetracycline was from Sigma (Deisenhofen, Germany).

Production and purification of class B β-lactamases

Bacillus cereus β-lactamase II was produced and purified as described by Davies et al. (1974).

The *P. maltophilia* ULA-511 L-1 β-lactamase was obtained as follows. An overnight culture in Brain/Heart Infusion Broth (BioMérieux, Charbonnières-les-Bains, France) was diluted 10-fold with the same medium and grown for 1 h at 30 °C under orbital shaking. Imipenem was added as an inducer to a final concentration of 50 μg/ml and incubation was continued under the same conditions. After 3 h, the production of L-1 β-lactamase reached a maximum and the bacteria were harvested by centrifugation at 10000 *g* for 30 min at 4 °C. The periplasmic extract, containing the zinc β-lactamase, was obtained by converting the cells into protoplasts in 30 mM Tris/HCl buffer

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(pH 8.0), containing 27% sucrose, 0.5 mM EDTA and 2 mg/ml lysozyme, according to the procedure described by Lindström et al. (1970). The suspension was centrifuged at 23000 *g* for 15 min at 4 °C and the supernatant dialysed overnight against 50 mM Tris/HCl buffer (pH 8.0), containing 0.1 mM $ZnCl_2$. The extract was then concentrated by ultrafiltration and adsorbed on to a Q-Sepharose fast-flow column (2.0 cm × 30 cm) (Pharmacia-LKB Biotechnology, Uppsala, Sweden), previously equilibrated with the same buffer. The column was washed and the elution carried out with a linear gradient of NaCl (0–1 M) in the same buffer. The fractions containing the L-1 enzyme were pooled, concentrated by ultrafiltration and dialysed overnight against 50 mM sodium acetate buffer, pH 5.0, containing 0.1 mM $ZnCl_2$. The dialysed sample was applied on a Mono S HR 5/5 prepacked column (Pharmacia-LKB Biotechnology) previously equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 0.1 mM $ZnCl_2$. After washing, elution of the β-lactamase was performed using a linear gradient of NaCl (0–1 M) in the same buffer. Active fractions were pooled, concentrated and dialysed against 50 mM Tris/HCl buffer, pH 8.0, containing 0.1 mM $ZnCl_2$ and 0.1 M NaCl. This buffer was also used to store the enzyme at 4 °C. No activity was lost after a 5-month storage period under these conditions.

The *A. hydrophila* A2 β-lactamase was produced and purified as follows. An overnight culture of *E. coli* DH5α in Luria–Bertani broth containing 50 μg/ml tetracycline was diluted 10-fold with the same medium and grown for 6 h at 37 °C under orbital agitation. The preparation of the periplasmic content was carried out as described above. The extract was dialysed overnight against 30 mM sodium cacodylate buffer, pH 6.5. The first purification step was carried out by affinity chromatography as described by Porath and co-workers (Porath et al., 1975; Porath and Olèn, 1983) for enzymes requiring metal cofactors. A column (1.5 cm × 10 cm) of iminodiacetic acid conjugated with 4% cross-linked agarose (Affiland, Liège, Belgium) was modified by treatment with 50 mM $ZnCl_2$ in distilled water, extensive washing with distilled water to remove the unbound Zn^{2+} and equilibration with 30 mM sodium cacodylate buffer, pH 6.5. The periplasmic preparation was concentrated by ultrafiltration and loaded on to the column. Elution of the A2 enzyme was carried out by adding 0.5 M NaCl and 20 mM $ZnCl_2$ to the same buffer. The fractions containing the β-lactamase activity were pooled, concentrated, dialysed overnight against 30 mM sodium cacodylate buffer (pH 6.5) containing 0.1 mM $ZnCl_2$, centrifuged at 10000 *g* for 10 min at 4 °C and filtered through a Superdex 200 column (bed vol. 160 ml; Pharmacia-LKB Biotechnology). The fractions exhibiting β-lactamase activity were pooled, concentrated and dialysed overnight against 50 mM sodium cacodylate buffer (pH 7.0) containing 0.1 mM $ZnCl_2$ and 0.5 M NaCl. The enzyme was stored at 4 °C, and no activity was lost after 4 months under these conditions.

Determination of β-lactamase activity

The activities of the *P. maltophilia* ULA-511 and *A. hydrophila* enzymes were routinely determined by monitoring the hydrolysis of 100 μM imipenem at 300 nm ($\Delta\epsilon = -9000\ M^{-1}\cdot cm^{-1}$). One unit of β-lactamase activity is defined as that amount that hydrolyses 1 μmol of imipenem/min at 30 °C in 50 mM sodium cacodylate buffer, pH 6.5, containing 0.1 mM $ZnCl_2$.

Protein determination

Protein concentrations were determined by the method of Lowry et al. (1951), with BSA as standard.

PAGE and M_r determinations

M_r values were determined by heating the proteins (unknown and standards) at 100 °C for a few minutes in the presence of 1% SDS, running the samples (at 150 V for 2 h at 4 °C) on a 12% (w/v) polyacrylamide gel in the presence of 0.1% SDS and measuring their relative mobilities as described by See and Jackowski (1990). The gels were stained with Coomassie Brilliant Blue R-250 (E. Merck, Darmstadt, Germany).

Isoelectric focusing analysis

Gel isoelectric focusing was performed in 5% polyacrylamide gels containing ampholytes (pH range 3.5–9.5) using a Multiphor II Apparatus (Pharmacia-LKB Biotechnology). Gels were focused at 10 °C and 25 W for 90 min.

Assays

The kinetic experiments with the β-lactamases from *P. maltophilia* ULA-511 and *A. hydrophila* were performed in 50 mM sodium cacodylate buffer, pH 7.0, containing 0.1 mM $ZnCl_2$, at 35 °C and 30 °C, respectively. For *Bacillus cereus* β-lactamase II, all the experiments were performed at 30 °C in 10 mM sodium cacodylate buffer, pH 6.0, containing 0.5 M NaCl and 0.3 mM $ZnSO_4$.

Determination of K_m and $k_{cat.}$ values

The hydrolysis of the antibiotics was monitored by following the absorbance variation resulting from the opening of the β-lactam ring, using an Uvikon 860 spectrophotometer equipped with thermostatically controlled cells and connected to a Copam PC88C microcomputer via a RS232C serial interface. The wavelengths and absorbance variations were those described by Matagne et al. (1990). Cells with 0.2–1.0 cm pathlengths were used, depending on the solution concentration. K_m and $k_{cat.}$ values for all the compounds were measured either under initial-rate conditions, using the Hanes' linearization of the Michaelis–Menten equation, or by analysing the complete hydrolysis time courses, as described by De Meester et al. (1987). K_m values lower than 10 μM were determined in competition experiments using nitrocefin as reporter substrate. The total reaction volume was 0.5 ml in all cases.

Table 1 Summary of purification of the zinc-dependent β-lactamase from *P. maltophilia* ULA-511

The preparation obtained after the Mono S column was considered as at least 95% pure on the basis of the SDS/PAGE results. For more details, see the Materials and methods section. Activity was assayed spectrophotometrically using 100 μM imipenem as substrate. Errors are within ±5%.

Purification step	Total volume (ml)	Total protein content (mg)	Specific activity (units/mg)	Purity (%)
Periplasm	350	1700	3.4	5.2
Q-Sepharose (fast flow)	6.5	40	50.0	77.0
Mono S	4.5	12	62.5	> 95.0

RESULTS

Purification of the β-lactamases

P. maltophilia ULA-511 L-1 enzyme

The purification of the L-1 enzyme is summarized in Table 1. As shown by Saino et al. (1982), *P. maltophilia* produces a second, inducible, class C β-lactamase (L-2). This was efficiently separated from the L-1 β-lactamase in the first step of purification, since it did not adsorb on the ion exchanger; its activity was found during the column washing. The purification yield of L-1 was 13%.

The purified enzyme yielded a single protein band on SDS/PAGE (Figure 1). From the same data, the subunit M_r could be estimated as 28500 (the enzyme contains four identical subunits; Bicknell et al., 1985). The pI was 5.91 (results not shown). These results are in good agreement with those previously reported.

A. hydrophila β-lactamase

Table 2 summarizes the purification of the A2 β-lactamase to protein homogeneity, and Figures 2(a) and 2(b) show the chromatographic profiles obtained. Note that after the first affinity chromatographic step, the enzyme was already more than 80% pure.

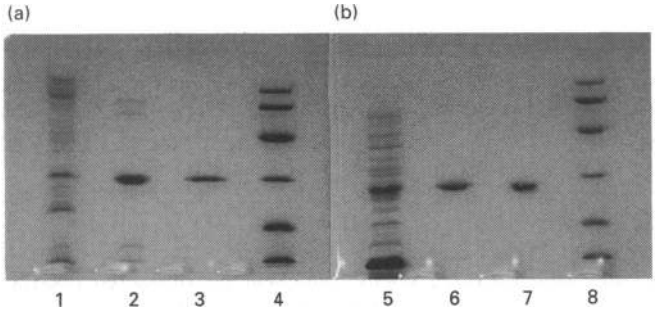

Figure 1 SDS/PAGE of the purified *P. maltophilia* ULA-511 (a) and *A. hydrophila* (b) β-lactamases

(**a**) Lane 1, periplasm extract; lane 2, Q-Sepharose fast-flow eluate; lane 3, purified enzyme; lane 4, M_r markers (Bio-Rad): rabbit muscle phosphorylase *b* (97400), BSA (66200), hen egg white ovalbumin (45000), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (21500), hen egg white lysozyme (14400). (**b**) Lane 5, periplasm extract; lane 6, enzyme from affinity chromatography; lane 7, purified enzyme; lane 8, M_r markers (see lane 4).

Table 2 Purification of the *A. hydrophila* zinc-dependent β-lactamase produced by *E. coli* DH5α

The preparation obtained after the Superdex column was considered as at least 95% pure on the basis of the SDS/PAGE results. For more details, see the Materials and methods section. Activity was assayed spectrophotometrically using 100 μM imipenem as substrate. Errors are ±5%.

Purification step	Total volume (ml)	Total protein content (mg)	Specific activity (units/mg)	Purity (%)
Periplasm	800	14500	0.2	0.12
Affinity chromatography	4.0	5.6	145.0	86.0
Superdex	3.0	3.6	163.0	> 95.0

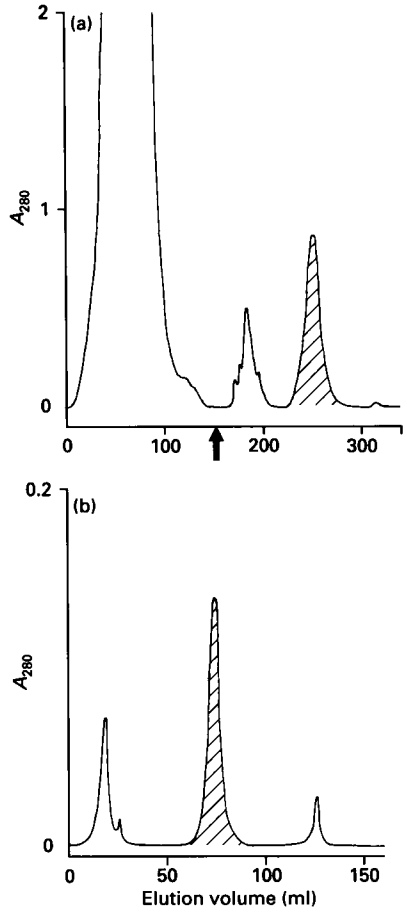

Figure 2 Elution profiles of the *A. hydrophila* A2 β-lactamase

(**a**) Affinity chromatography. The hatched fractions exhibited β-lactamase activity and were pooled. The arrow indicates the change of buffer. For conditions, see the Materials and methods section. (**b**) Filtration on a Superdex 200 column. The activity was found in the hatched fractions.

The purified zinc β-lactamase gave a single protein band on SDS/PAGE (Figure 1) and the calculated M_r was 27500. The purification yield was 20%. The pI, determined by isoelectric focusing analysis, was 8.10 (results not shown).

Kinetic parameters

Table 3 lists the kinetic parameters (K_m, $k_{cat.}$ and $k_{cat.}/K_m$) determined with the three purified class B β-lactamases and compares them with those reported by Yang et al. (1992) for the *Bacteroides fragilis* enzyme. Two classical inhibitors of active-site-serine β-lactamases were also tested; 6-β-iodopenicillanate and sulbactam were hydrolysed by all three class B β-lactamases. Among the compounds usually considered as 'β-lactamase stable', cefoxitin and moxalactam were hydrolysed by β-lactamase L-1 from *P. maltophilia* ULA-511 and by β-lactamase II from *Bacillus cereus*, but behaved as inactivators of the A2 enzyme from *A. hydrophila*. Aztreonam did not seem to interact with any of the enzymes. Since aztreonam had been previously reported to be a substrate for the *A. hydrophila* and *P. maltophilia* enzymes (Iaconis and Sanders, 1990), we carefully checked its influence on the hydrolysis of a reporter substrate (imipenem). At a concentration of 0.5 mM, i.e. about 10 times the published

Table 3 Kinetic parameters determined for class B β-lactamases

For more details, see the Materials and methods section. The *Bacteroides fragilis* data are from Yang et al. (1992). For all data, the S.D. values are below 10% of the mean. Abbreviations: Inact., inactivation; N.I., no interaction; N.H., no hydrolysis detected.

	Aeromonas hydrophila A2 enzyme			*Pseudomonas maltophilia* ULA-511 L-1 enzyme			*Bacillus cereus* β-lactamase II			*Bacteroides fragilis* β-lactamase ccrA		
Antibiotic	K_m (μM	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/k_{cat.}$ ($M^{-1} \cdot s^{-1}$)	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
Ampicillin	> 2000	> 500	2.70×10^{5}*	40±3	175	4.40×10^{6}	1530±115	1105	7.20×10^{5}	120	190	1.60×10^{6}
Carbenicillin	450±30	16.00	3.60×10^{4}	194±15	277	1.40×10^{6}	4200±300	755	1.80×10^{5}	240	190	8.00×10^{5}
Penicillin G	700±50	5.21	7.40×10^{3}	50±6	1100	2.20×10^{7}	1500±200	678	4.50×10^{5}	40	193	4.80×10^{6}
Oxacillin	25±3	0.75	3.00×10^{4}	27±3	285	1.10×10^{7}	1750±20	325	1.90×10^{5}	–	–	–
Cephaloridin	190±8	0.12	6.30×10^{2}	300±50	28	9.30×10^{4}	1350±140	25	1.90×10^{4}	6	42	7.00×10^{6}
Cephalexin	230±20	0.65	2.90×10^{2}	100±8	37	3.70×10^{5}	300±26	1.7	5.70×10^{3}	49	95	1.90×10^{6}
Cephaloglycin	280±30	0.20	7.10×10^{2}	36±2	19	5.30×10^{5}	190±5	61	3.20×10^{5}	–	–	–
Nitrocefin	100±8	0.31	3.10×10^{2}	7±1	20	2.90×10^{6}	70±5	45	6.40×10^{5}	16	200	1.25×10^{7}
Cefuroxime	20±3	0.07	3.50×10^{3}	30±3	80	2.70×10^{6}	45±5	35	7.80×10^{5}	4	29	7.30×10^{6}
Cefotaxime	26±3	0.07	2.70×10^{3}	26±3	66	2.60×10^{6}	90±10	60	6.70×10^{5}	27	98	3.60×10^{6}
Imipenem	80±8	168.00	2.10×10^{6}	90±8	65	7.30×10^{5}	> 1000	> 100	1.20×10^{5}*	270	200	7.40×10^{5}
6-β-IP	530±50	0.64	1.20×10^{3}	640±30	648	1.00×10^{6}	7460±590	450	6.00×10^{4}	–	–	–
Sulbactam	37±4	0.12	3.24×10^{3}	76±4	210	2.80×10^{6}	5200±325	10	1.90×10^{3}	–	–	–
Cefoxitin	Inact.	Inact.	Inact.	2+0.05	1.10	5.50×10^{5}	2100±465	0.2	9.50×10^{1}	110	10	9.00×10^{4}
Moxalactam	Inact.	Inact.	Inact.	1±0.05	0.29	2.90×10^{5}	644±90	1.2	1.90×10^{3}	160	(30)	(1.90×10^{5})‡
Aztreonam	N.I.†	N.I.†	N.I.†	N.I.†	N.I.†	N.I.†	N.I.†	N.I.†	N.I.†	N.H.	N.H.	N.H.

* Only the $k_{cat.}/K_m$ ratio was determined because v_0 remained proportional to S_0 up to the highest substrate concentration which could be used.

† Up to 0.5 mM aztreonam.

‡ Cuchural et al. (1986). Values in parentheses were computed by us from their data.

K_m values, we did not observe any effect of aztreonam on the rate of hydrolysis of imipenem by either enzyme.

DISCUSSION

In the present study, we devised new methods for the purification of the *A. hydrophila* and *P. maltophilia* ULA-511 β-lactamases. For the former enzyme, affinity chromatography on a Zn^{2+} chelate column was found to be extremely efficient, yielding a 700-fold purification in one step. The method was much simpler than that already described (Iaconis and Sanders, 1990), and the yield was 20-fold higher. The same affinity method was unsuccessful with the *P. maltophilia* enzyme, even when buffer pH was varied from 5.5 to 8.5, i.e. from below to above the enzyme's pI value. In all cases, the enzyme failed to adsorb on to the support. Nevertheless, the method described here for purifying this protein is simpler and faster than the previously reported techniques, although the yield was only marginally better than that reported by Bicknell et al. (1985).

The kinetic parameters which were determined in the present study are generally in good agreement with those found by other workers, except that aztreonam was previously reported to be a substrate of the *A. hydrophila* and *P. maltophilia* enzymes (Iaconis and Sanders, 1990). This discrepancy might be explained by the difficulty in measuring the hydrolysis of this substrate, which is accompanied by a small variation in absorbance at low substrate concentrations.

It was interesting to include the *Bacteroides fragilis* enzyme in the comparison of the kinetic parameters. The genes coding for the β-lactamases produced by two different *Bacteroides fragilis* strains have been sequenced and were found to encode proteins of identical primary structures (Rasmussen et al., 1990; Thompson and Malamy, 1990) and thus it was concluded that *Bacteroides fragilis* TAL 3636 and TAL 2480 strains produce the same enzyme. Furthermore a β-lactamase purified from another strain of *Bacteroides fragilis*, isolated in a different country, did not seem to exhibit a very different substrate profile (Hedberg et al., 1992), although their study was much less extensive than that of Yang et al. (1992).

Table 3 gives an overview of the catalytic properties of the well-characterized class B β-lactamases. The *A. hydrophila* enzyme clearly and strikingly exhibits the most specific substrate profile, while the three other enzymes can be considered as rather broad-spectrum. The specificity of the *A. hydrophila* enzyme is extremely narrow. Imipenem, and to a lesser degree ampicillin, are its only good substrates. It can thus be considered as a rather specific 'carbapenemase'.

Among the three other enzymes, the *Bacteroides fragilis* β-lactamase can probably be considered as the most 'dangerous' one, since its $k_{cat.}$ and $k_{cat.}/K_m$ values are rather large with all tested substrates, including cefoxitin and moxalactam (which behave as inactivators of the A2 enzyme) and third-generation cephalosporins, such as cefotaxime, which are usually considered to be β-lactamase-stable. Do the known sequences supply a clue to these different specificities? Surprisingly, a sequence comparison (Table 4), performed as in Joris et al. (1991), indicated that the *P. maltophilia* enzyme was only remotely related to the three other ones, which appeared to constitute a more homogenous group. Indeed, the four enzymes exhibited only 14 strictly conserved residues, but this number increased to 37 if the *P. maltophilia* protein was excluded. Surprisingly, His-86, one of the histidine residues which seem to be involved as Zn^{2+}-binding ligands in the *Bacillus cereus* 5/B/6 enzyme (Lim et al., 1988), was replaced by an Asn residue in the *A. hydrophila* A2 β-lactamase. It will be interesting to compare the dissociation constants of the metal ions in the four enzymes. It is possible that this could be related to the peculiar substrate profile of the *A. hydrophila* enzyme.

The renal dehydropeptidase I, which is also a Zn^{2+}-dependent metalloprotein, is another enzyme known for its ability to

Table 4 Comparison of the four class B β-lactamases

The numbers represent the percentages of very similar or identical residues computed on the basis of the 'Distances' algorithm (Devereux et al., 1984), as done by Joris et al. (1991). For normalization purposes, short portions of the proteins were eliminated at the C- and N-termini of the mature proteins. The comparison thus encompassed residues 42–256 for the *Bacillus cereus* 5/B/6 enzyme (Lim et al., 1988), 25–240 for *Bacteroides fragilis* (Rasmussen et al., 1990), 27–250 for *A. hydrophila* (Massidda et al., 1991) and 33–280 for *P. maltophilia* (R. Levesque, personal communication) enzymes respectively. Note that the Zn^{2+}-dependent β-lactamase from *Bacillus cereus* 569/H (Hussain et al., 1985) only differs by 24 positions from *B. cereus* 5/B/6, and its inclusion in the comparison would not modify the conclusions.

	Identity (%)		
	B. fragilis	*B. cereus* 5/B/6	*A. hydrophila*
B. cereus 5/B/6	41.4	–	–
A. hydrophila	34.3	34.9	–
P. maltophilia	22.7	21.4	21.0

hydrolyse carbapenems (Kropp et al., 1982). However, a comparison of its sequence (Adachi et al., 1990) with that of the *A. hydrophila* β-lactamase failed to reveal any sequence similarity.

In consequence, it seems that class B β-lactamases comprise two sub-classes, one containing (at the present time) only the *P. maltophilia* enzyme, and the second the three other β-lactamases. This distinction, based on the enzyme sequences, does not directly reflect the substrate profiles of the various enzymes, a situation similar to that observed with class A β-lactamases (Matagne et al., 1990).

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REFERENCES

Adachi, H., Tawaragi, Y., Inuzuka, C., Kubota, I., Tsujimoto, M., Nishihara, T. and Nakazato, H. (1990) J. Biol. Chem. **265**, 3992–3995

Ambler, R. P., Coulson, A. F. W., Frère, J.-M., Ghuysen, J.-M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G. and Waley, S. G. (1991) Biochem. J. **276**, 269–272

Bandoh, K., Muto, Y., Watanabe, K., Katoh, N. and Ueno, K. (1991) Antimicrob. Agents Chemother. **35**, 371–372

Bicknell, R., Emanuel, E. L., Gagnon, J. and Waley, S. G. (1985) Biochem. J. **229**, 791–797

Connolly, A. K. and Waley, S. G. (1983) Biochemistry **22**, 4647–4651

Cuchural, G. J., Jr., Malamy, M. H. and Tally, F. (1986) Antimicrob. Agents Chemother. **30**, 645–648

Dale, J. W., Godwin, D., Mossakowska, D., Stephenson, P. and Wall, S. (1985) FEBS Lett. **191**, 39–44

Davies, R. B., Abraham, E. P. and Melling, J. (1974) Biochem. J. **143**, 115–127

De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourgignon, C., Frère, J.-M. and Waley, S. G. (1987) Biochem. Pharmacol. **36**, 2393–2403

Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. **12**, 387–395

Hedberg, M., Edlund, C., Lindqvist, L., Rylander, M. and Nord, C. E. (1992) J. Antimicrob. Chemother. **29**, 105–113

Hussain, M., Carlino, A., Madonna, M. J. and Lampen, O. (1985) J. Bacteriol. **164**, 223–229

Iaconis, J. P. and Sanders, C. C. (1990) Antimicrob. Agents Chemother. **34**, 44–51

Jaurin, B. and Gundström, T. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 4897–4901

Joris, B., Ledent, P., Dideberg, O., Fonzè, E., Lamotte-Brasseur, J., Kelly, A., Ghuysen, J.-M. and Frère, J.-M. (1991) Antimicrob. Agents Chemother. **35**, 2294–2301

Kropp, H., Sundelof, J. G., Hajdu, R. and Kahan, F. M. (1982) Antimicrob. Agents Chemother. **22**, 62–70

Kubawara, S. and Abraham, E. P. (1967) Biochem. J. **103**, 23C–30C

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lim, H. M., Pène, J. J. and Shaw, R. (1988) J. Bacteriol. **170**, 2873–2878

Lindström, E. B., Boman, H. G. and Steele, B. B. (1970) J. Bacteriol. **101**, 218–231

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

Massidda, O., Rossolini, G. M. and Satta, G. (1991) J. Bacteriol. **173**, 4611–4617

Matagne, A., Misselyn-Baudin, A.-M., Joris, B., Epircum, T., Granier, B. and Frère, J.-M. (1990) Biochem. J. **265**, 131–146

Medeires, A. A. (1984) Br. Med. Bull. **40**, 18–27

Nielsen, J. B. K. and Lampen, J. O. (1983) Biochemistry **22**, 4652–4656

Pollock, M. R. (1956) J. Gen. Microbiol. **15**, 154–169

Porath, J. and Olèn, B. (1983) Biochemistry **22**, 1621–1630

Porath, J., Carlsson, J., Olssin, I. and Belfrage, G. (1975) Nature (London) **258**, 598–599

Rasmussen, B. A., Gluzman, Y. and Tally, F. P. (1990) Antimicrob. Agents Chemother. **34**, 1590–1592

Saino, Y., Kobayashi, F., Inoue, M. and Mitsuhashi, S. (1982) Antimicrob. Agents Chemother. **22**, 564–570

See, Y. P. and Jackowski, G. (1990) in Protein Structure: A Practical Approach (Creighton, E. T., ed.), pp. 1–21, IRL Press, Oxford

Sykes, R. B. and Matthew, M. (1976) J. Antimicrob. Chemother. **2**, 115–157

Thatcher, D. R. (1975) Methods Enzymol. **43**, 640–652

Thompson, J. S. and Malamy, M. H. (1990) J. Bacteriol. **172**, 2584–2593

Waley, S. G. (1992) in The Chemistry of Beta-Lactams (Page, M. I., ed.), pp. 198–228, Chapman and Hall, London

Yang, Y., Rasmussen, B. A. and Bush, K. (1992) Antimicrob. Agents Chemother. **36**, 1155–1157

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