Characterization of a β -lactamase produced by *Pseudomonas paucimobilis*

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A novel β -lactamase enzyme produced by a strain of *Pseudomonas paucimobilis* is described. The enzyme differs from other recorded β -lactamases from Gram-negative aerobic bacteria. It was constitutive, and had the characteristics of a penicillinase. One single band of β -lactamase activity at pI 4.6 was seen on iso-electric focusing. The enzyme had a molecular mass of 30 kDa. The β -lactamase was strongly inhibited by tazobactam, sulbactam and clavulanic acid but not by the thiol residue inhibitors *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulphonic acid, or by metallo-enzyme inhibitors. Plasmid DNA was not demonstrable, suggesting that the enzyme was chromosomally encoded.

Introduction

β -Lactamases (EC 3.5.2.6) have been detected in bacteria isolated long before the introduction of penicillin into therapeutic use in 1942, and the debate regarding the primary physiological role of these specialized peptidases still continues. The development of new β -lactam antibiotics is only just keeping ahead of the ability of bacteria to produce β -lactamases that inactivate them. The original β -lactamase (penicillin-amido- β -lactam hydrolase; Abraham & Chain, 1940) was particularly efficient at hydrolysing penicillin, but as newer β -lactam compounds were introduced into clinical practice so the hydrolytic spectrum of β -lactamases widened.

Pseudomonas paucimobilis is a yellow-pigmented, motile pseudomonad which has been isolated from aquatic environmental sites (Holmes *et al.*, 1977), hospital hot water supplies and respiratory therapy equipment (Crane, *et al.*, 1981). *P. paucimobilis* is an opportunist pathogen in patients with underlying serious medical problems, particularly in those undergoing continuous ambulatory peritoneal dialysis (Glupczynski *et al.*, 1984; Calubiran *et al.*, 1990).

Recently we isolated an environmental strain of *P. paucimobilis* from an episode of 'pseudobacteraemia' associated with contaminated blood culture vials. Pre-

vious workers have described susceptibility profiles of *P. paucimobilis* to various β -lactams without comment regarding the mechanism of resistance (Peel *et al.*, 1979; Southern & Kutscher, 1981; Smalley *et al.*, 1983). The type strain NCTC 11030 is reported to be resistant to penicillin but sensitive to broad-spectrum β -lactams. However, our isolate appeared unusual with regard to its antibiotic susceptibility profile in that it resembled bacteria expressing the newly described 'extended broad-spectrum' β -lactamases (for a review, see Philippon *et al.*, 1989). It was resistant to the recently introduced monobactam aztreonam, partially susceptible to the third-generation aminothiazoyl cephalosporin ceftazidime, yet fully susceptible to second-generation cephalosporins such as cefuroxime and to cephamycins, such as cefoxitin. Therefore, we have undertaken a study of the β -lactamase produced by this environmental isolate of *P. paucimobilis* to determine its spectrum of activity, inhibition profile, physical properties and the location of the gene(s) responsible for its production.

Methods

Chemicals. All chemicals were of analytical grade. Nitrocef [3-(2,4-dinitrostyryl)-(6*R*,7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid], a chromogenic cephalosporin, was supplied by Glaxo (Greenford, UK). Assay-grade antibiotics and inhibitors of known potency were received as gifts as follows: penicillin, ampicillin, cloxacillin, carbenicillin and clavulanic acid (Smith Kline Beecham, UK), piperacillin and tazobactam (Lederle, UK), cephaloridine and ceftazidime (Glaxo, UK), aztreonam (Squibb, UK), sulbactam (Pfizer, UK)

Abbreviations: Nitrocef, 3-(2,4-dinitrostyryl)-(6*R*,7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid; p-CMB, *p*-chloromercuribenzoate; p-CMS, *p*-chloromercuriphenylsulphonic acid; PEN-Y, penicillin-hydrolysing β -lactamases inhibited by clavulanic acid.

and imipenem (Merck Sharp Dohme, UK). Powders were reconstituted in appropriate buffers and filter-sterilized. Stock solutions not used immediately were stored at -70°C . Paper disks incorporating known amounts of antimicrobials were purchased from Oxoid. Proteins of known molecular mass (range 14.2–66 kDa), ethidium bromide. Tris/HCl buffer, 2-mercaptoethanol, lysozyme and agarose were from Sigma. Proteins of known iso-electric point (range 4.7–10.6) were from LKB Bromma, Sweden. *p*-Chloromercuribenzoic acid (p-CMB), *p*-chloromercuriphenylsulphonic acid (p-CMS), *N*-ethylmaleimide and *o*-phenanthroline (Sigma), and EDTA and potassium iodide (BDH) were used for enzyme-inhibition kinetic studies.

Organism and cultural conditions. The isolate was identified as *Pseudomonas paucimobilis* at 30°C using a commercial identification kit (API). Crude enzyme sonicates were prepared from overnight cultures harvested on 10% (v/v) horse blood agar (Lab M). Resistance phenotypes were determined by controlled disk diffusion using diagnostic sensitivity testing agar (DSTA, Lab M) with the addition of 5% (v/v) lysed horse blood (Lab M) at 37°C . Minimum inhibitory concentrations (MICs) were determined in Iso-Sensitest broth (Oxoid) at 10^5 c.f.u. ml $^{-1}$ using a commercial microtitre plate containing lyophilized antimicrobials (Sensititre, Gibco Life Technologies). Plasmid curing with ethidium bromide was performed in tryptic soya broth (TSB, Lab M).

β -Lactamase extraction. Overnight bacterial growth on horse blood agar was suspended in 5 ml 0.1 M-potassium phosphate buffer, pH 7.0. Sonication was performed using an Ultrasonics Disruptor, model Rapidis. The suspension was sonicated for up to five 30 s pulses. Samples were cooled during and between sonication by immersion in an ice-water slurry bath. Cell-free extracts were prepared by centrifugation at 4°C for 15 min at 13 000 r.p.m. Protein concentration (mg l $^{-1}$) was determined at 650 nm in a Unicam Sp1800 UV spectrophotometer by the Lowry method. Extracts were stored at -70°C .

Purification and PAGE molecular mass determination. Purification of the β -lactamase was performed in two stages. Crude sonicate was loaded onto a 1.6×80 cm column of Ultrogel AcA44 (LKB) and run in 50 mM-potassium phosphate pH 7.5, 10% (v/v) glycerol, 1 mM-EDTA and 5 mM-2-mercaptoethanol (flow rate 8 ml h $^{-1}$). Fractions of 2 ml were collected and the presence of β -lactamase assayed by addition of nitrocefin. Active fractions were pooled, and applied to a 1 ml MonoQ HR 5/5 column (Pharmacia LKB Biotechnology) equilibrated in 20 mM-Tris/HCl pH 8.0, 10% (v/v) glycerol and 1 mM-2-mercaptoethanol (flow rate 1 ml min $^{-1}$). After elution of the unbound protein, the column was developed with a 20 ml gradient of 0–0.35 M-NaCl in 20 mM-Tris/HCl pH 8.0, 10% (v/v) glycerol, 1 mM-2-mercaptoethanol (flow rate 1 ml min $^{-1}$). Fractions of 0.5 ml were collected and assayed for β -lactamase activity. Samples (10 μ l) of the active fractions were analysed by SDS-PAGE in an 8×10 cm 10% (w/v) gel according to Laemmli (1970). Protein bands were visualized by staining with Coomassie Blue.

Iso-electric point determination. Analytical iso-electric focusing was performed in a Multiphor apparatus (LKB) with the cell-free sonicate on polyacrylamide gels (5%, w/v) containing ampholines in the range pH 3.5–9.5 (LKB). Controls included β -lactamases TEM-1 and TEM-2 and a range of proteins of known mobility (LKB). Electrophoresis was performed at 1500 V for 90 min at 10°C . After electrophoresis, β -lactamase bands were identified by placing a filter paper soaked in a 100 μ M solution of nitrocefin on the gel.

Estimation of β -lactamase kinetic constants. The method used was adapted from that described by O'Callaghan *et al.* (1972). β -Lactamase

was assayed in a Unicam Sp1800 UV spectrophotometer at 37°C . Decreasing concentrations of nitrocefin from 100 μ M in 0.1 M-phosphate buffer, pH 7.0, were mixed with sonicate. A typical reaction mixture in a quartz cell (1 cm pathlength) contained 3.0 ml 100 μ M-nitrocefin solution and 0.1 ml sonicate. Hydrolysis rates measured by change in absorbance at 482 nm were obtained on a Unicam AR25 linear recorder. Activity of the enzyme was defined as μ mol nitrocefin destroyed min $^{-1}$ (ml enzyme) $^{-1}$ at pH 7.0. Double-reciprocal plots of reaction rate and concentration were constructed and mean Michaelis constants (K_m) calculated from two replicate experiments.

Inhibition profile. The previous procedure was followed but using sonicate pre-incubated (room temperature for 30 min) with an equal volume of the following compounds: p-CMB, 10^{-4} M; p-CMS, 10^{-4} M; clavulanic acid, 10 mg l $^{-1}$; sulbactam, 10 mg l $^{-1}$; tazobactam, 10 mg l $^{-1}$; cloxacillin, 10^{-2} M; aztreonam, 10 and 100 mg l $^{-1}$; potassium iodide, 20 mg l $^{-1}$; EDTA, 10^{-3} M; *o*-phenanthroline, 10^{-3} M; and *N*-ethylmaleimide, 10^{-3} M. Percentage decrease in hydrolysis of 100 μ M-nitrocefin = $[(A - B)/A] \times 100$, where A was the hydrolysis rate in the uninhibited reaction and B was the hydrolysis rate in the inhibited reaction. The I_{50} value (the inhibitor concentration inhibiting 50% of the enzyme activity) was calculated as $I_{50} = I(V_i/V_0 - V_i)$, where I is the concentration of inhibitor which gives a rate V_i and V_0 is the rate of the control lacking the inhibitor (Neu, 1985).

Hydrolysis rates as measured by HPLC. HPLC was used to determine hydrolysis by measuring residual concentrations of test β -lactams when incubated with cell-free sonicate. The HPLC equipment consisted of a solvent delivery system and injection valve (Waters Associates, model U6K), a variable-wavelength photodiode-array detector (Hitachi, model L-3000), and an integrator (Hitachi, model D-2000). Separation was performed using a reversed-phase guard column and a C $_{18}$ analytical column (Waters Associates, Radial Pak μ -Bondapak C $_{18}$). Details of respective mobile phases and wavelength of detection are summarized in Table 1. Individual assay procedures were based on the following: penicillin (Ghebre-Sellassie *et al.*, 1982), ampicillin (Smith-Kline Beecham, 1981), cloxacillin (Lauriault *et al.*, 1982), carbenicillin (Twoomey, 1981), piperacillin (Sorgel *et al.*, 1986), cephaloridine and ceftazidime (de Neeling *et al.*, 1988), aztreonam (Squibb Pharmaceuticals, 1985), and imipenem (Gravallese *et al.*, 1984).

Comparative β -lactamase activity was determined by the method of de Neeling *et al.* (1988). Briefly, 0.1 ml of antibiotic solution (100 μ g ml $^{-1}$) in 0.1 M-potassium phosphate buffer, pH 7.0, was mixed with an equal volume of crude sonicate, previously diluted such that a 50% decrease in penicillin concentration was obtained after approximately 3 min incubation at room temperature. At 1 min intervals, 40 μ l samples were injected into the HPLC. Reduction in β -lactam concentration was quantified by integration of peak area under curve by a computing integrator. Spontaneous degradation of β -lactam was determined by mixing antibiotic and heat-inactivated (100 $^{\circ}\text{C}$, 1 h) sonicate. The logarithm of the residual substrate concentration was plotted against time on semi-logarithmic paper. Other β -lactams were incubated with this pre-diluted sonicate and examined at 1 min injection intervals until a 50% reduction in concentration was achieved and new peaks of degradation products found (see retention times, Table 1). Therefore, for these other β -lactams initial reaction rates were not determined but an arbitrary hydrolysis rate was considered to be the residual concentration at a single time point (1 min) compared to penicillin (100%).

Plasmid analysis. Plasmid DNA analysis was attempted by the rapid screening method of Birnboim & Doly (1979). Electrophoresis was run in Tris/borate buffer, pH 8.0, with horizontal agarose gels (0.7%) for approximately 3.5 h at 120 mV. Gels were stained with 10% (w/v) ethidium bromide for 15 min and plasmid bands viewed with a UV transilluminator (302 nm).

Table 1. HPLC parameters for β -lactam antibiotic hydrolysis studies

β -Lactam	Organic solvent* (%, v/v)	Phosphate buffer:			Detection wavelength (nm)	Flow rate (ml min ⁻¹)	Retention time (min)	Retention time of major hydrolysis product (min)
		mmol l ⁻¹	Salt†	pH				
Penicillin	25 (A)	6	K ₂ H ^a	7.5	236	1.5	4.8	3.5
Ampicillin	6 (M)	100	KH ₂	3.2	227	2.5	5.4	3.6
Cloxacillin	20 (A)	50	KH ₂	4.5	254	4.0	8.6	3.2
Carbenicillin	35 (M)	50	KH ₂ ^b	3.4	220	4.0	2.8	2.3
Piperacillin	40 (M)	40	KH ₂ + K ₂ H	6.2	220	2.5	4.9	2.6
Cephaloridine	20 (M)	50	NH ₄ H ₂	4.4	240	4.0	4.2	2.6
Ceftazidime	10 (M)	50	NH ₄ H ₂	4.4	257	4.0	4.0	ND
Aztreonam	20 (M)	100	KH ₂ ^c	2.0	313	2.0	4.4	3.2
Imipenem	0	200	H ₃ BO ₃	7.2	313	4.0	4.5	ND

ND, Not detected.

* A, acetonitrile; M, methanol.

† a, + ion-pair reagent 0.008 M-tetrabutyl ammonium chloride; b, + ion-pair reagent 0.1% tetrabutyl ammonium bromide; c, + 1% KCl.

Results and Discussion

This isolate of *P. paucimobilis* was fully resistant to penicillin (MIC > 8.0 mg l⁻¹), ampicillin (MIC > 128 mg l⁻¹), cephaloridine (MIC 32 mg l⁻¹), carbenicillin, ticarcillin, piperacillin (all MICs > 256 mg l⁻¹), and aztreonam (MIC 64 mg l⁻¹), but only partially resistant to ceftazidime (MIC 8.0 mg l⁻¹). It was fully sensitive to cefuroxime, cefoxitin, cefotaxime, ceftizoxime, ceftriaxone and imipenem (all MICs < 1.0 mg l⁻¹). Using disk sensitivity testing, the isolate was also shown to be sensitive to amoxycillin + clavulanic acid, ticarcillin + clavulanic acid and piperacillin + tazobactam.

The above resistance profile suggests that the β -lactamase produced by *P. paucimobilis* is a penicillinase. Quantitative hydrolytic activity of the β -lactamase towards five penicillins, two cephalosporins, one carbapenem and one monobactam substrate was determined by HPLC (Table 2). Many β -lactams exhibit substrate inhibition (Bush, 1983) above 1.0 mM; HPLC allows the use of lower substrate concentrations (50 μ g ml⁻¹ or less). This is particularly useful for penicillins, which lack the high UV absorption of the cephalosporins at low assay values (Waley, 1974). An added advantage of HPLC over spectrophotometry is its ability to detect the formation and increase, with time, of the open-ring degradation products following β -lactam hydrolysis. Using HPLC, the rate of penicillin hydrolysis was estimated to be 230 μ mol min⁻¹ (mg protein)⁻¹ and this was assigned a nominal value of 100% hydrolysis. Enzyme activity was relatively penicillin specific, with minimal hydrolysis of cephaloridine (3.9%) and aztreonam (1.6%), and no demonstrable activity (< 0.1%) towards ceftazidime (aminothiazoyl cephalosporin) or imipenem (carbapenem).

Table 2. Characteristics of the β -lactamase from *P. paucimobilis*

The enzyme had a pI of 4.6 and a molecular mass of 30 kDa.

(a) Substrate profile		(b) Inhibition profile	
Substrate (50 μ g ml ⁻¹)	Relative hydrolysis rate	Inhibitor†	Inhibition (%)‡
Penicillin	100*	<i>N</i> -Ethylmaleimide	19
Cloxacillin	15.4	Cloxacillin	31
Ampicillin	61.5	Clavulanic acid	69
Carbenicillin	46.2	Sulbactam	78
Piperacillin	23	Tazobactam	89
Cephaloridine	3.9		
Ceftazidime	< 0.1		
Aztreonam	1.6		
Imipenem	< 0.1		

* The observed rate was 230 μ mol penicillin hydrolysed min⁻¹ (mg protein)⁻¹ (by HPLC).

† See Methods for inhibitor concentrations.

‡ Expressed as the percentage reduction in the rate of hydrolysis of nitrocefin (100 μ M).

Compared to penicillin, nitrocefin was hydrolysed slowly, with a mean K_m value of 18 μ M (obtained from 1/*v* versus 1/*S* plots using four different concentrations). Specific activity of the enzyme was 0.1 μ mol nitrocefin destroyed min⁻¹ (ml enzyme)⁻¹ (O'Callaghan *et al.*, 1972). Enzyme inhibitors can be used both to characterize β -lactamases and to infer the nature of the catalytically important site(s). Using 100 μ M-nitrocefin as substrate, β -lactamase activity was inhibited following pre-incubation with the β -lactamase inhibitors clavulanic acid, sulbactam and tazobactam. Tazobactam was the most potent inhibitor: 89% reduction of enzyme activity and I_{50} of 1.2 μ g ml⁻¹. Clavulanic acid is normally more

or equipotent towards the broad-spectrum plasmid-mediated β -lactamases as compared with the two penicillanic acid sulphones tazobactam and sulbactam (Jacobs *et al.*, 1986). In contrast, tazobactam demonstrates greater inhibition of chromosomally-encoded β -lactamases (Gutmann *et al.*, 1986). The metal ion chelators EDTA and *o*-phenanthroline at a concentration of 1.0 mM had no inhibitory effect on the β -lactamase, indicating that the enzyme does not belong to the group 3 metalloenzymes as described by Bush (1989*b*). Resistance to the amino acid modifiers p-CMB and p-CMS indicates the absence of a catalytically important cysteine residue. However, *N*-ethylmaleimide, another thiol group directed inhibitor, had marginal activity (19%) inhibition. Despite the finding that the β -lactamase showed some hydrolysis (15.4%) of cloxacillin from an initial concentration of 50 $\mu\text{g ml}^{-1}$, at higher values substrate inhibition did occur: 31% and 86% at 1 mM and 10 mM, respectively, with an I_{50} of 1000 $\mu\text{g ml}^{-1}$. The combination of greater penicillin hydrolysis and inhibition by clavulanate (I_{50} 4.6 $\mu\text{g ml}^{-1}$) suggests that the enzyme be allocated to group 2a (PEN-Y) β -lactamases of Bush (1989*a*). Group 2a penicillinases (inhibited by clavulanic acid) include many of the enzymes from Gram-positive organisms and represent many of the original members of molecular class A (Ambler, 1980). Hydrolysis rates for penicillins are much higher than those for cephalosporins and cloxacillin is not an effective inhibitor of group 2a β -lactamases.

Aztreonam can form stable covalent acyl enzymes with some cephalosporinases, such that the enzyme remains effectively inactivated (trapped) for several generations of bacterial growth (Bush *et al.*, 1985), but not with penicillinases (Bush, 1988). Pre-incubation of the *P. paucimobilis* β -lactamase with aztreonam at 10 and 100 mg l^{-1} did not inhibit its activity towards nitrocefin. Addition of clavulanate solution (10 mg l^{-1}) to an aztreonam disk (30 μg) did render the *P. paucimobilis* isolate sensitive. Therefore, the observed resistance to aztreonam in this isolate is due to a different mechanism than that for 'extended broad spectrum' β -lactamases. It cannot be wholly attributed to β -lactamase hydrolysis (1.6%) but could be compounded by permeability and/or target site modification.

It was not possible to abolish β -lactamase expression by prolonged exposure to sub-inhibitory concentrations of ethidium bromide. Also, our inability to demonstrate plasmid DNA by agarose gel electrophoresis is evidence that the β -lactamase produced by *P. paucimobilis* is chromosomally encoded.

On iso-electric focussing a single band of activity was detectable at pI 4.6. Iso-electric points below 5.0 are rare for cephalosporin-hydrolysing β -lactamases (*Bacteroides fragilis* pI 4.7; Yotsuji *et al.*, 1983) and relatively

uncommon for penicillin-hydrolysing β -lactamases (Bush, 1989*a, b*). Three of the four recorded penicillinases with a pI below 5.0 were found in anaerobic bacteria and the fourth in *Streptomyces cacaoi* (Ogarawa *et al.*, 1981). Therefore, the β -lactamase of pI 4.6 expressed by *P. paucimobilis* appears to be an unusual penicillinase within the Gram-negative aerobic bacteria. Calibration of the Ultrogel Ac44 column used in the purification scheme with molecular mass standards showed this enzyme to have a native molecular mass of 30 kDa. Since the purified enzyme displayed a major single band of the same size upon SDS-PAGE, it can be concluded that it consists of a single 30 kDa polypeptide.

Concluding remarks

The data presented here demonstrate that the β -lactamase expressed by *P. paucimobilis* is a constitutive protein of 30 kDa, with an iso-electric point of 4.6 and exhibiting preferential hydrolysis of penicillin substrates compared to cephalosporins. The enzyme was strongly inhibited by tazobactam, sulbactam and clavulanic acid but not by thiol group or metalloenzyme inhibitors or by the monobactam aztreonam. The properties of this β -lactamase are in accordance with the group 2a penicillin-hydrolysing β -lactamases (Bush, 1989*a*). Attempts to abolish enzyme activity by exposure to ethidium bromide were unsuccessful. Furthermore, it was not possible to detect extrachromosomal plasmid DNA, indicating that the enzyme seems to be encoded by a chromosomal gene.

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