Purification of a class C A-type β -lactamase from a derepressed strain of *Enterobacter cloacae*

Comparison of the wild-type and mutant enzyme with those from strains P99, 208 and GN7471

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Enterobacter cloacae strain 5822 expresses low levels of a class C β -lactamase which can be induced 100-fold by imipenem. Mutants that constitutively express high levels of β -lactamase can be selected on aztreonam or cefotaxime. The β -lactamase from one such mutant (5822M2) has been purified to homogeneity and compared on the basis of subunit M_r , pI, substrate specificity, inhibitor sensitivity and immunological cross-reactivity with the enzyme from strains P99, GN7471 and 208, which have been studied previously. The enzyme from strain 5822M2 is clearly related to these other forms and is of the A-type according to the criteria of Seeberg, Tolxdorff-Neutzling & Wiedemann [Antimicrob. Agents Chemother. (1983) 23, 918–925]. The enzyme from the wild-type strain (5822) is shown to be identical to that found in the depressed strain (5822M2), indicating that the mutation is in a regulatory gene. A detailed analysis of the kinetics of the enzyme from strain 5822M2 shows that all of the β -lactams studied are substrates and that a mechanism involving the formation of an acyl-enzyme is probably applicable in every case. The substrates however can clearly be grouped into two classes, i.e. ‘good’ substrates with k_{cat} values of 80–1200 s⁻¹ and ‘poor’ substrates/good inhibitors with k_{cat} values of 0.009–0.00007 s⁻¹. The permeability barrier to aztreonam is 4-fold less in the derepressed strain when compared with the wild-type strain. This is associated with significant changes in the expression of outer membrane porins. The observed resistance in the derepressed mutant appears to be linked to the elevated levels of β -lactamase (3000-fold) rather than to the modest changes in the permeability barrier.

INTRODUCTION

The introduction of the ‘third generation’ cephalosporins has provided a potent extension to earlier β -lactam antibiotics in that Enterobacteriaceae, gonococci, *Haemophilus influenzae* and *Pseudomonas aeruginosa* are now susceptible with a concomitant increase in clinical efficacy. Unfortunately, as with most advances in antibiotic design, a new group of resistant strains has emerged as the clinical usage of third generation cephalosporins, e.g. cefotaxime, has increased (see, e.g., Sanders & Sanders, 1983, 1985). These include *Enterobacter*, *Serratia* and *Aerobacter* species and in all cases which have been studied in sufficient detail resistance is associated with derepression of the chromosomal β -lactamase (Lampe *et al.*, 1982; Gootz *et al.*, 1982; Seeberg *et al.*, 1983). The increase in concentration of the β -lactamase in the periplasmic space can be as much as 1000-fold. Earlier studies suggested there was no requirement for a complete catalytic turnover as the level of free antibiotic was reduced simply by ‘trapping’ it in the form of an acyl-enzyme intermediate (Then & Angehrn, 1982). Such an explanation was based on early measurements which indicated there was no turnover with cefotaxime, ceftriaxone, cefmenoxime and moxalactam (Okonogi *et al.*, 1981; Fu & Neu, 1979; Minami *et al.*, 1980; Richmond, 1980; Matsuura *et al.*, 1980). More recent studies by Nikaido and co-workers have shown however that cefoxitin, ceftizoxime and cefotaxime are substrates, albeit extremely poor ones (Vu & Nikaido, 1985). There is also evidence that several mutant strains show a marked alteration in the expression

of outer membrane porin proteins (Bush *et al.*, 1985; Werner *et al.*, 1985) so that an altered permeability barrier may also function in the observed resistance. Unfortunately there have been few studies that have examined β -lactamase derepression and expression of porin proteins in both wild-type and phenotypically derived derepressed mutants. For example, the widely studied *Enterobacter cloacae* derepressed mutant P99 is a clinical isolate and the parent strain is not available (Fleming *et al.*, 1963). Indeed, there is no report, as far as we are aware, that has presented detailed evidence that the ‘mutant’ β -lactamase is identical with the wild-type enzyme, i.e. that the phenomenon is one of genuine derepression. Such a mechanism is likely however as the genomic structures of the *Enterobacter cloacae* and *Aerobacter aerogenes* β -lactamases are known to include a repressor gene 5′ upstream from the β -lactamase structural gene. In *Escherichia coli* the repressor gene is absent (the *Escherichia coli* enzyme is not inducible); however these cells contain another regulatory gene (*ampD*) that may act as a sensor with AmpR as a regulatory effector (see, e.g., Lindberg & Normark, 1987). Interestingly, Curtis *et al.* (1987) have recently described temperature-sensitive derepressed mutants of *Enterobacter cloacae* that express high levels of β -lactamase at 42 °C (in the absence of inducer) but not at 28 °C and have suggested that these contain a mutant repressor capable of adopting two temperature-dependent conformations.

In order to analyse the nature of the mechanism of resistance of *Enterobacter cloacae* to third-generation cephalosporins we have isolated wild-type, derepressed

and temperature-sensitive derepressed mutant strains and compared their β -lactamases with those derived from the well-studied P99, GN7471 and 208 strains using both a kinetic and structural approach. The results of this study are presented in the present paper together with the results of our studies on antibiotic permeability and outer membrane protein expression in these strains.

MATERIALS AND METHODS

Materials

The wild-type strain *Enterobacter cloacae* 5822 was isolated concurrently with a derepressed strain (R46) from a clinical source by Dr. D. Coleman. Derepressed mutants of strain 5822 were selected on agar plates containing either cefotaxime (20 μ g/ml) or aztreonam discs (30 μ g). To select temperature-sensitive mutants colonies were replica plated and then grown at 28 °C and 42 °C on agar plates containing cefotaxime (20 μ g/ml). Colonies that grew at 42 °C but not at 28 °C were provisionally identified as *ts*-mutants and this was confirmed by demonstrating a significant increase in β -lactamase activity when cells were grown at 42 °C as compared with cells grown at 28 °C (approx. 50-fold). Strains P99, GN7471 and 208 were obtained from Professor B. Wiedemann, Bonn, Germany.

CM-cellulose and DEAE-cellulose were obtained from Whatman, Sephadex G-100 and Ampholines were obtained from Pharmacia, and V8-protease was from Sigma. Cefotaxime, penicillin G, cephalixin, ceftazidime, and cephaloridine were gifts from Professor T. J. Foster, aztreonam was generously provided by Squibb, imipenem by Merck and nitrocefin by Glaxo, Greenford, Middlesex, U.K.

Purification of β -lactamases

All of the β -lactamases studied were purified using the same protocol. An overnight culture (2 litres) in L broth was harvested by centrifugation, washed once with 10 mM-Tris/HCl/30 mM-NaCl, pH 7.5, and then lysed using a French pressure cell. The suspension was centrifuged at 45 000 *g* for 45 min and the supernatant (approx. 20 ml) was gel-filtered on Sephadex G-25 into 10 mM-sodium phosphate, pH 7.5. This material was then passed through DEAE-cellulose equilibrated in 10 mM-sodium phosphate, pH 7.5. The flow-through material was pooled and the pH adjusted with 1 M-acetic acid to 6.5 and then applied to a column (2 cm $\times$ 10 cm) of CM-cellulose equilibrated in 10 mM-sodium phosphate, pH 6.5. The column was washed with the same buffer and the enzyme eluted with a 0–200 mM-KCl gradient. Active fractions were pooled and concentrated to 10 ml using an Amicon ultrafiltration cell. This material was gel filtered on Sephadex G-100 (2.5 cm $\times$ 90 cm) and the active fractions pooled, concentrated to 1 mg/ml by ultrafiltration and stored at –20 °C.

Purification of outer membranes

Cells (50 ml overnight culture at 37 °C in L broth) were harvested and then lysed by sonication. The suspension was clarified by centrifugation at 2000 *g* for 15 min at 4 °C followed by a further centrifugation at 16000 *g* for 20 min at 4 °C. Under these conditions the pellet consists mainly of outer membranes which were further purified by resuspension in 10 mM-Tris/HCl (pH 7.2) containing 10 mM-MgSO₄ and 2% (v/v) Triton

X-100 and centrifugation at 16000 *g* for 10 min at 4 °C. The pellets were finally resuspended in 10 mM-Tris/HCl, pH 7.2 (100 μ l).

Determination of permeability barriers

This was achieved using minor modifications of the method of Bush *et al.* (1985). Quantification of the permeability ratio (PR) was determined by using the methods of Zimmerman & Rosset (1977) and Kojo *et al.* (1980*a,b*). A ratio greater than 1 indicates the presence of a permeability barrier.

Enzyme assays

β -Lactamase activity was routinely measured at 30 °C using a spectrophotometric assay with cephalixin (100 μ M) and cephaloridine (100 μ M) as substrates and on occasion with aztreonam, cefotaxime and nitrocefin when activity was measured by monitoring the decrease in absorbance at 260 nm, 255 nm, 318 nm and 267 nm respectively, while with nitrocefin (100 μ M) the increase at 460 nm was used (Bush *et al.*, 1982). The buffer used in all assays was 0.1 M-sodium phosphate, pH 7, and the reaction was started by the addition of enzyme (10–100 μ l).

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out using the method of Laemmli (1970) with 15% or 20% acrylamide resolving gels.

Immunoblotting

Proteins resolved by SDS/polyacrylamide-gel electrophoresis were transferred electrophoretically to nitrocellulose paper and then blotted according to the procedure developed by Vaessen *et al.* (1981).

Isoelectric focusing

Isoelectric focusing was carried out using an LKB Multiphor apparatus with Ampholines in the pH ranges of 3–10, 6.5–9 and 8–10.5. Focusing was carried out for 90 min at 20 W. Proteins were then visualized by staining with Coomassie Brilliant Blue R or the β -lactamase activity was probed directly as described below.

Activity staining

Activity staining of focused gels was carried out by using an overlay method with substrate-saturated nitrocellulose. When the pH gradient was established (90 min) the gel was overlaid with nitrocellulose paper that had been soaked in 100 μ M-nitrocefin in 0.1 M-sodium phosphate, pH 7, and then blotted dry. This procedure allowed the red bands to develop and prevented the coloured product from diffusing.

Peptide mapping

Peptide maps were obtained by treating the purified β -lactamase with V8-protease as described by Cleveland *et al.* (1977).

RESULTS

The specific activity in lysate supernatants from the derepressed mutant strain 5822M2 is 4.6 μ mol/min per mg at 28 °C, which is a 3000-fold increase (see Table 1) when compared with the wild-type strain (5822). Table 1 also shows the specific activities of strains P99, GN7471,

208 and 5822ts when grown at 28 °C and 42 °C. It can be seen that only one strain (5822ts) is temperature-sensitive according to the criteria of Curtis *et al.* (1987), although both strains 5822wt and GN7471 show a modest increase (2-fold) in specific activity at 42 °C while conversely strain 208 shows a significant decrease. The purification of all the derepressed strains studied was straightforward and after passage through DEAE-cellulose to remove acidic proteins all of the β -lactamases were purified to homogeneity by chromatography on CM-cellulose and Sephadex G-100. Details of the purification for the enzyme from strain 5822M2 is shown in Table 2 together with a brief summary for the other strains, all of which exhibited very similar behaviour. By the criterion of SDS/polyacrylamide-gel electrophoresis all the preparations were homogeneous and all of the β -lactamases cross-reacted with rabbit anti-5822M2 on immunoblotting (results not shown). Seeberg *et al.* (1983) have characterized two classes of *Enterobacter cloacae* β -lactamases depending on their isoelectric point as A and B. These have pI values of 8.8 and 7.8 respectively. The enzyme from strain GN7471 does not fit into this scheme with a pI of 8.4. Fig. 1(a) shows an isoelectric focusing gel of P99, GN7471, 208 and 5822M2 stained with Coomassie Blue and Fig. 1(b) shows an activity stain of a similar gel blotted with nitrocellulose soaked in nitrocefin. It should be noted that the activity stain in Fig. 1(b) also includes the uninduced (lane 6) and induced (lane 5) wild-type enzyme from strain 5822. These results confirm that the β -lactamases from strains 208 and P99 are A and B type respectively and allows 5822M2 to be

classified as an A-type enzyme. A summary of the physical parameters of all of the purified forms from derepressed strains is shown in Table 3. The behaviour of the wild-type enzyme (5822) was also examined by SDS/polyacrylamide-gel electrophoresis of crude lysate supernatants and immunoblotting with anti-5822M2 (Fig. 1c). The wild-type enzyme has an identical subunit M_r (42000) to the derepressed 5822M2 enzyme and also an identical pI (8.8). To obtain further evidence that the wild type enzyme is identical with the derepressed enzyme from strain 5822M2 we partially purified the wild-type enzyme through the CM-cellulose chromatography step and then subjected this material to SDS/polyacrylamide-gel electrophoresis. A protein band with the same mobility as that of the β -lactamase from strain 5822M2 was excised from the gel and subjected to digestion by V8-protease using the method of Cleveland *et al.* (1977). The results of this experiment are shown in Fig. 2. The peptide map for the wild-type enzyme (lane 3) is identical with that of the derepressed enzyme (lane 1) and also with that of the wild-type enzyme induced using imipenem (1 μ g/ml) (see lane 2). This result is consistent with a mutation occurring in a regulatory protein and not in the β -lactamase structural gene.

The kinetics of all the forms were studied at 30 °C with cephaloridine, cephalexin and nitrocefin as substrates and cefotaxime, ceftazidime, benzylpenicillin and aztreonam as 'inhibitors'. The kinetic parameters K_m and V_{max} for the various substrates used are shown in Table 4 together with the inhibition constant K_i for aztreonam, ceftazidime, cefotaxime and imipenem. It was possible to show that this last group are also substrates for the β -lactamase from strain 5822M2, although the turnover numbers are very small (see Table 4). Indeed, for aztreonam the k_{cat} of 0.25 h⁻¹ is sufficiently slow to permit isolation of the acylated enzyme by isoelectric focusing (results not shown). It is possible to regain full activity and revert to the non-acylated form (free enzyme) by leaving the mixture overnight at room temperature (see below).

Outer membranes prepared from the wild-type strain grown in the presence and absence of inducer and from the derepressed strain 5822M2 show marked differences in the relative expression of porin proteins (Fig. 3). Both the mutant and the wild-type strains express four peptides of molecular masses 37, 39, 40 and 43 kDa respectively. All show the expected 'heat modifiability' (Lutgenberg *et al.*, 1977) on SDS/polyacrylamide-gel electrophoresis

Table 1. The effect of temperature on the specific activities of the various β -lactamases

Strain	Temperature ...	Specific activity (μ mol/min per mg)	
		28 °C	42 °C
5822wt		0.00135 $\pm$ 0.00052	0.00236 $\pm$ 0.000348
5822M2		4.6 $\pm$ 1.01	3.53 $\pm$ 0.35
5822ts		0.0134 $\pm$ 0.0082	0.499 $\pm$ 0.33
P99		3.16 $\pm$ 0.86	4.44 $\pm$ 1.6
208		4.2 $\pm$ 2.1	0.69 $\pm$ 0.35
GN7471		3.64 $\pm$ 0.55	7.7 $\pm$ 3

Table 2. Summary of the purification of the various β -lactamases

Step	<i>Enterobacter cloacae</i> strain	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)	Purification (fold)
French pressure cell	5822M2	43	1.2	—	1
G-25 gel filtration	5822M2	12	8.8	100	7
DEAE-cellulose	5822M2	0.71	90	60	48
CM-cellulose	5822M2	0.65	100	40	80
chromatography					
Sephadex G-100	5822M2	1.52	113	30	90
	R46	1.3	233	39	241
	208	0.83	240	31	258
	P99	0.9	148	38	114
	GN7471	1.2	280	58	288

Fig. 1. Isoelectric focusing, activity staining and immunoblotting of the various β -lactamases

Native isoelectric focusing was carried out on purified preparations of the β -lactamases from strains GN7471, P99, 5822M2, R46 and 208 and is shown (a) in lanes 1–5 respectively. The results of activity staining a similar gel are shown in (b) for purified preparations of GN7471, P99 and 5822M2 in lanes 1–3 respectively. Activity staining of cytosols from strain 5822M2, the wild-type strain (5822) induced with imipenem (1 μ g/ml) and uninduced wild-type are shown in lanes 4, 5 and 6 respectively. The results of immunoblotting 'cytosols' from the wild-type (5822) and derepressed strain (5822M2) are shown in (c). Lane 1 shows cytosol from the derepressed mutant 5822M2 immunoblotted with rabbit anti-5822M2 (1:1000) and lane 2 the result of a similar experiment except that the cells were incubated with imipenem (1 μ g/ml). Lanes 3 and 4 show the immunoblots of a similar experiment conducted with the wild-type strain (5822) in the presence (lane 3) and absence (lane 4) of imipenem (1 μ g/ml). To visualize the β -lactamase in the uninduced wild-type strain the experiment shown in lanes 3 and 4 was repeated and immunoblotted with a 1:50 dilution of rabbit anti-5822M2 for the control (lane 5) and imipenem-induced cells (lane 6).

(results not shown). The 39 kDa peptide is the major species in both strains; however, the wild-type (5822) expresses more of the peptides 37 and 43 kDa while the derepressed strain (5822M2) expresses more of the 40 kDa peptide. Interestingly, the pattern of expression in the cells treated with imipenem (a potent inducer of β -lactamase in the wild-type strain) is intermediate between that of the wild-type and derepressed mutant strains.

Table 3. Physical properties of the β -lactamases from the various *Enterobacter cloacae* strains

<i>Enterobacter cloacae</i> strain	Molecular mass (kDa) by:		pI	
	Gel filtration	SDS/PAGE	Native	Urea
R46	37.5	41.0	8.8	8.2
5822M2	37.5	41.0	8.8	8.2
208	37.5	40.5	8.8	8.2
GN7471	39.0	44.0	8.1	7.6
P99	38.5	43.0	7.8	7.4

Fig. 2. Peptide mapping of the β -lactamase from the wild-type and derepressed strains

Lanes 1, 2 and 3 show silver-stained SDS/polyacrylamide gels of the V8-protease-digested β -lactamases from the derepressed mutant (lane 1), the wild-type induced with imipenem (lane 2) and uninduced wild-type (lane 3).

There is a pronounced permeability barrier to aztreonam in the wild-type strain, as indicated by the permeability barrier of 85 (see Table 5). This value is increased 4-fold in the derepressed strain and may be associated with the altered expression of porin proteins.

DISCUSSION

Previous workers have purified 'cephalosporinases' from *Enterobacter cloacae* by a variety of techniques (Minami *et al.*, 1980; Lindstrom *et al.*, 1970; Charlier *et al.*, 1983; Ross, 1975; Cartwright & Waley, 1984; Vu & Nikaido, 1985), the most notable of which is the use of phenylboronic acid-agarose chromatography by Cartwright & Waley (1984). The procedure used in the present work is simple, rapid and affords good yields from several strains using commercially available ion-exchangers. The β -lactamase from strain 5822 and the derepressed mutant (5822M2) is a monomer of 41 kDa and this, coupled with the substrate specificity of this enzyme, is sufficient to classify it as a class C cephalosporinase (Jaurin & Grundström, 1981). It is possible to further classify the 5822 enzyme as of the A-type according to Seeberg *et al.* (1983) on the basis of its isoelectric point. Further work is necessary to determine the usefulness of the A- and B-type subclassification. From the present study it is clear that the two subtypes

Table 4. Kinetic parameters for the various forms of β -lactamase

Strain	Nitrocefin		Cephalexin		Cephaloridine	
	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}$ (s^{-1})	K_m (μM)
5822M2	305 $\pm$ 30	83 $\pm$ 13	307 $\pm$ 25	247 $\pm$ 8	1225 $\pm$ 111	249 $\pm$ 38
R46	228 $\pm$ 36	75 $\pm$ 18	316 $\pm$ 21	245 $\pm$ 19	1275 $\pm$ 115	200 $\pm$ 24
208	309 $\pm$ 23	81 $\pm$ 11	132 $\pm$ 20	240 $\pm$ 33	1197 $\pm$ 191	131 $\pm$ 40
P99	320 $\pm$ 25	78 $\pm$ 15	110 $\pm$ 22	69 $\pm$ 6	920 $\pm$ 165	305 $\pm$ 88
GN7471	80 $\pm$ 7	84 $\pm$ 15	80 $\pm$ 6	61 $\pm$ 8	800 $\pm$ 70	210 $\pm$ 24

$k_{cat.}$ (s^{-1})				
Imipenem	Ceftazidime	Ceftoxime	Aztreonam	
5822M2	0.0002	0.002	0.009	0.00007 s^{-1}

K_i (μM)				
Penicillin G	Aztreonam	Cefotaxime	Ceftazidime	
5822M2	2*	0.375* 0.6†	0.045* 0.045†	27*

With *cephalexin and †cephaloridine as substrates.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the outer membranes from the wild-type and mutant cells

Outer membranes from the wild-type strain (lane 1), the wild-type induced with imipenem (1 $\mu g/ml$) (lane 2) and the derepressed mutant (lane 3) were subjected to SDS/polyacrylamide-gel electrophoresis in 15% gels and stained with Coomassie Brilliant Blue R.

are immunologically related and exhibit very similar substrate specificities and inhibitor sensitivities. Sequence studies on A-type enzymes, e.g. from strains 5822M2 and 208, will allow a comparison with that of the B-type P99 enzyme (Galleni *et al.*, 1988). It is clear that the phenomenon observed in these strains is one of genuine derepression as we have shown that the enzyme in the mutant strain is clearly identical with the enzyme expressed in the wild-type strain.

Table 5. Permeability barrier to aztreonam entry into *E. cloacae*

Strain	[Aztreonam] (ng/ml) required to inhibit 50% of β -lactamase		Permeability ratio (O/T)
	Theoretical (T)	Observed (O)	
WT	0.07	6	85
5822M2	25	8000	320

It seems clear that all of the β -lactams that we have studied are substrates for these class C β -lactamases but that in the case of some of these antibiotics the acyl-enzyme is particularly stable and turns over with a half life of several minutes to hours depending on the β -lactam. A similar conclusion has been reached by Vu & Nikaido (1985) and Galleni & Frere (1988).

The mutant strain that we have elected to work with (5822M2) expresses 3000 times the level of β -lactamase seen in the parent strain. The derepressed strain also exhibits a marked change in the expression of outer membrane 'porin' proteins. It is unclear whether the altered expression of 'porins' is related to the modest (4-fold) increase in the permeability barrier or indeed how much the decreased permeability contributes to the observed resistance. Although the increase in permeability barrier is modest compared to the large increase in the level of β -lactamase, further experiments are required to delineate the contribution of these two factors to the observed resistance.

The regulation of expression of the porin proteins OmpF and OmpC in *Escherichia coli* is known to be regulated in a complex manner. It appears that at least two genes, *ompR* and *envZ*, regulate the expression of OmpF and OmpC in a highly co-ordinated manner. *ompR* is believed to activate transcription of *ompF* and

ompC in a reciprocal manner depending on the osmolality of the medium, with *envZ* acting as a sensor (Forst *et al.*, 1987). It is intriguing in this respect that imipenem, which presumably interacts with an AmpR analogue in *Enterobacter cloacae*, shifts the expression of the outer membrane proteins in strain 5822 to resemble that seen in the derepressed mutant and suggests that regulatory elements common to β -lactamase expression and to porin expression may exist. This hypothesis is strengthened by our recent observation that several osmoregulators affect β -lactamase levels as well as porin expression in strain 5822 (M. N. Graham & T. J. Mantle, unpublished work).

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