A novel metallo-β-lactamase, Mbl1b, produced by the environmental bacterium *Caulobacter crescentus*1

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Abstract *Caulobacter crescentus* 101123 possesses a gene (Mbl1b) encoding a metallo-β-lactamase with 32% amino acid identity to the L1 metallo-β-lactamase from *Stenotrophomonas maltophilia*. The gene was cloned into an expression vector and the enzyme, Mbl1b, was expressed in *Escherichia coli*. Mbl1b was purified. Catalytic properties for several antibiotics were determined. The enzyme exhibits Michaelis–Menten kinetics for imipenem, meropenem and nitrocefin but substrate inhibition kinetics with cefoxitin, cefaloridine, penicillin G and ampicillin. A homology model predicts Mbl1b has the same structural fold as other metallo-β-lactamasess with a detailed structure very similar to L1 but whereas L1 is a homotetramer, Mbl1b is monomeric. The main differences between Mbl1 and L1 are in the N-terminal region. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Antibiotic; Metallo-β-lactamase; *Caulobacter crescentus*; *Stenotrophomonas maltophilia*

1. Introduction

The metallo-β-lactamasess are an important class of Zn2+-dependent hydrodases. They share the zinc-binding HAHD active site motif with a variety of other hydrolytic enzymes: the arylsulphatases, glyoxalase II enzymes [1] and polyketide cyclases [2]. A general picture concerning catalytic activity is emerging of zinc acting as a Lewis acid to polarise a water molecule or hydroxyl ion to facilitate a hydrolytic nucleophilic attack on the carbonyl group of the substrate [3,4]. Metallo-β-lactamasess readily hydrolyse carbapenems, a relatively new and important class of β-lactam antibiotics, to confer resistance and thus are assuming increasing clinical significance. Metallo-β-lactamasess, for example IMP1 and VIM, can be mobilised on mobile genetic elements, particularly class 1 integrons [5]. Unfortunately there are no clinically useful inhibitors of the metallo-β-lactamasess. Bacteria, which are widely distributed in the environment, and are opportunist pathogens, for example *Stenotrophomonas maltophilia* which causes several diseases in immunocompromised patients, are of growing clinical concern [6]. The L1 metallo-β-lactamase from *S. maltophilia* has β-lactamase activity against a wide range of clinically useful antibiotics, including the carbapenems, conferring a broad spectrum of β-lactam resistance. L1 is unique amongst metallo-β-lactamasess in that it is tetrameric [7] and recent pre-steady-state kinetic analysis suggests that L1 uses a mechanism that differs in important details from those of other metallo-β-lactamasess [8]. Homologues to L1 are therefore of great interest since the sequence and kinetic differences can facilitate understanding of this interesting enzyme. An L1 homologue from *Janthinobacterium lividum* has previously been described [9].

*Caulobacter crescentus* is a widespread Gram-negative bacterium found in soil and aquatic habitats [10]. *Caulobacter* spp. have two distinct life-cycle stages: a motile, chemotactically active swarm cell and a sessile stalked cell [11], and *C. crescentus* is an important model system in cell differentiation studies.

This study reports the discovery of a metallo-β-lactamase gene in the chromosome of *C. crescentus* (Mbl1b related to the L1 gene of *S. maltophilia*, and the cloning and sequencing of one variant, Mbl1b, and the purification and characterisation of its β-lactamase (Mbl1b).

2. Materials and methods

2.1. Organisms and culture conditions

*C. crescentus* strain 101123 (Pasteur Institute Culture Collection, Paris, France) was used in this study and was cultured aerobically at 30°C on Medium 28 broth or agar, as appropriate (Oxoid, Basingstoke, UK). *Escherichia coli* strains were grown aerobically at 37°C on Nutrient Broth or Agar (Oxoid).

2.2. Identification, TA-cloning and expression of the Mbl1 gene

The *C. crescentus* genome, strain CB15 [12], was used searching the Wu-BLAST 2.0 algorithm (tBLASTn option) [13], with the known amino acid sequence for the L1 metallo-β-lactamase from *S. maltophilia* [14] as the query sequence. The Blastn62 comparison matrix was used with gap opening and extension penalties of 10 and 0.1, respectively.

PCR primers were selected to isolate the entire Mbl1 gene sequence. The forward primer sequence (5’-3’) was ATGAGCGGCTTGAGCTGTCGGC and the reverse primer (5’-3’) was GATCGGGTACCGTCTGCCC. The PCR products were performed using the methods of Avison et al. ([15]. TA cloning of the resulting amplicon into a TOPO-pTrcHis expression vector (Invitrogen, Carlsbad, CA, USA) was carried out as previously reported by Avison et al. [16].
2.3. Purification of Mbl1b

Mbl1b was purified from a periplasmic preparation of an *E. coli* strain transformed with *Mbl1* as described for the L1 metallo-β-lactamase [16]. The purity of the purified protein was assessed by electrospray mass spectrometry using a VG Quattro mass spectrometer. The purity of the purified protein was determined from its absorbance at 280 nm using a molar extinction coefficient of 30 680 AU M⁻¹ cm⁻¹ calculated from the primary protein sequence using the method of Parson and Fitzgerald [17]. N-terminal sequencing was performed on the same preparation used for mass spectrometry using an ABI 477/210A protein sequencer.

2.4. Steady-state kinetics of antibiotic hydrolysis

The kinetic parameters *k_cat* and *K_m* were determined in 50 mM cacodylate buffer at pH 7.0 in the presence of 100 μM *MZ* from initial rate measurements at different substrate concentrations using the Hanes–Woolf linearisation of the Michaelis–Menten equation [19]. Initial rates were determined by following product accumulation, at 482 nm for nitrocefin (Becton-Dickinson, Cockeysville, MD, USA), or substrate depletion, at 299 nm for ampicillin and penicillin G, 265 nm for cefoxitin, cefaloridine, cefmetazole and ceftazidime, and 260 nm for ceftazidime, in an UltroSpec III spectrophotometer (Amersham Pharmacia Biotec, Amersham, UK). Enzyme activity was also determined in the absence of 1 mM BRL42715, a competitive inhibitor of serine β-lactamases (Smith Kline Beecham, Harlow, UK), or 50 mM EDTA which inhibits metallo-β-lactamases. For those antibiotics for which substrate inhibition was seen, the initial rate data were fitted to a minimal kinetic mechanism of the form:

\[
\frac{v = \frac{E_0 K_{cat}}{1 + [S]}}{K_{app} + [S]}
\]

where *v* is the enzyme rate in μM s⁻¹, *E_0* is the enzyme concentration (in μM) and [S] is the substrate concentration (μM). Fitting was achieved using the non-linear least squares algorithm with the program GRAFIT [20].

2.5. Zinc assay

The zinc content per monomer of Mbl1 was determined by dialysing a 5 mM solution of protein against 100 volumes of zinc-free 50 mM cacodylate buffer, pH 6.0, for 2 days with daily buffer changes. The protein was then concentrated to approximately 20 μM using a Centricon column (molecular weight cutoff > 10,000, Millipore Corporation, Watford, UK) following the manufacturer's instructions. The concentrated protein was acid-hydrolysed with an equal volume of HNO₃ (BDH, Aristar grade) at 20°C for a minimum of 24 h and analysed with a Unicam 919 atomic absorption spectrometer at 213.9 nm.

2.6. Homology modeling of the Mbl1b enzyme

A comparative homology model of Mbl1b was created. The primary sequence of Mbl1b was threaded into the L1 single chain crystal structure (chain A, pdb id: 1sml) using the program SWISS MODEL [21]. The resultant molecule was energy-minimised using the program Gromos96 [22] using parameter set 43B1 until the average absolute derivative of coordinates with respect to energy fell below 0.01 kcal A⁻¹⁻¹.

3. Results

The initial Blast search of the *C. crescentus* CB15 genome (EMBL accession number AE005673) identified a 867 bp open reading frame (ORF) encoding a protein with significant similarity to the L1 metallo-β-lactamase from *S. maltophilia*. The translation products of *Mbl1* from *C. crescentus* CB15 (Mbl1a) and *C. crescentus* 10123 (Mbl1b). The arrow indicates the cleavage site for the signal peptide of precursor Mbl protein. The N-terminal sequence of mature L1 is AEVPL.
reading frame, Mbl1, the translated product of which is 32% identical to the L1 L-lactamase monomer from S. maltophilia in a ClustalW [23] alignment. A PCR reaction using genomic DNA from C. crescentus strains 101123 produced an approximate 850 bp amplification product with a nucleotide sequence almost identical to that of strain CB15. The gene product, Mbl1b, differs from that of CB15 by six amino acids. Fig. 1 shows the alignment of these two C. crescentus protein sequences with L1.

The PCR product was TA-cloned into TOPO-pTrcHis and the gene resequenced to check that the desired clone had been recovered. The cloned gene was 100% homologous to that of C. crescentus 101123.

Purification of the protein expressed from the cloned ampliﬁcoun of strain 101123 yielded a gel ﬁltration peak corresponding to a protein of molecular weight 29 kDa. Mass spectrometry of this protein showed a single peak of molecular mass 28790 ± 50 with an N-terminal sequence of DDMPAN, consistent with the mature protein starting at residue 22 (see arrow in Fig. 1). The predicted mass of the mature product (assuming the mature protein starts at D22) is 28744.

The puriﬁed protein contained 1.87 ± 0.3 mol of zinc per mol of protein. This protein showed good β-lactamase activity against nitrocefin that was completely abolished by incubation with 50 mM EDTA, but was insensitive to BRL47215. The steady-state kinetic parameters for the enzyme against several different types of β-lactam are shown in Table 1. The enzyme shows conventional Michaelis–Menten kinetics with imipenem, meropenem and nitrocefin as substrate (Fig. 2a). However, with cefoxitin, cefaloridine, penicillin G and ampicillin substrate inhibition was seen (Fig. 2b).

During the creation of the homology model of Mbl1b, the preliminary threading produced a remarkable ﬁt. With the exception of residues E56, R57 and N58 the RMS deviation of the K carbon chain of Mbl1b from that of L1 was 0.76 Å, even prior to energy minimisation. The ﬁnal model clearly demonstrated the KLLK fold typical of other metallo-β-lactamases and all the putative active site residues of Mbl1b are predicted to be in identical orientations to those in the L1 reference structure (Fig. 3). A copy of the pdb ﬁle of the model is available by e-mail from the corresponding author.

### 4. Discussion

The activity of puriﬁed Mbl1b was tested against all β-lactams and the strong similarity of the homology model to the L1 crystal structure clearly indicates that Mbl1b is a metallo-β-lactamase. The presence of a signal peptide in the primary translation product indicates that the enzyme is normally periplasmic, a prediction subsequently conﬁrmed in E. coli. The principal difference between the L1 sequence and that of Mbl1 lies in the N-terminal region. In L1 the ﬁrst 16 N-terminal amino acids form an extension relative to other metallo-β-lactamases. Extensions of the A and C (and B/D) L1 subunits interact via residues L5, L8 and Y11. Mbl1 lacks this region.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>$K_a$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (µM⁻¹ s⁻¹)</th>
<th>$K_i$ (µM)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>200 (90)</td>
<td>940 (65)</td>
<td>4.7 (0.73)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Meropenem</td>
<td>330 (250)</td>
<td>650 (44)</td>
<td>1.97 (2.8)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>130 (2)</td>
<td>1300 (1.1)</td>
<td>10 (0.55)</td>
<td>260</td>
<td>7</td>
</tr>
<tr>
<td>Cefaloridine</td>
<td>580 (300)</td>
<td>950 (28)</td>
<td>1.64 (0.09)</td>
<td>260</td>
<td>8</td>
</tr>
<tr>
<td>Ceftazidine</td>
<td>5000 (145)</td>
<td>260 (27)</td>
<td>0.05 (0.2)</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Nitrocefin</td>
<td>96 (7)</td>
<td>1800 (20)</td>
<td>18.8 (2.9)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>20 (50)</td>
<td>770 (100)</td>
<td>38.5 (22)</td>
<td>880</td>
<td>4</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>84 (40)</td>
<td>57 (175)</td>
<td>0.68 (4.4)</td>
<td>910</td>
<td>3</td>
</tr>
</tbody>
</table>

Comparable ﬁgures for L1 (taken from [25] or [26]) are in parentheses. $n$ is the value required to ﬁt the observed kinetics to Eq. 1. Kinetic estimates were the result of three replicates and standard errors were less than 10% of mean values. The enzyme concentration used in the assay was 0.01 µM except for nitrocefin and imipenem where 0.005 µM was used and ceftazidine for which the concentration was 0.05 µM.

*For those substrates for which substrate inhibition was observed, $K_{app}$ is reported rather than $K_i$.
In addition to the crystal structure, Ullah et al. [7] presented models of ampicillin, ceftazidime and imipenem bound to the active site of the L1 metallo-β-lactamase. For each β-lactam the N-terminal extensions were important for binding the antibiotic. In particular, hydrophobic interactions between Y11 and aromatic groups on the various β-lactams were identified as factors that may stabilise transition states. The kinetic data for Mbl1b are broadly consistent with this model of metallo-β-lactamase. For each β-lactam the N-terminal extensions were important for binding the antibiotic. In particular, hydrophobic interactions between Y11 and aromatic groups on the various β-lactams were identified as factors that may stabilise transition states. The kinetic data for Mbl1b are broadly consistent with this model of metallo-β-lactamase, in that for all of the β-lactams tested (with the exception of penicillin G) the $K_m$ (or $K_{cat}$) was significantly greater than that for L1. Interestingly, $k_{cat}/K_m$ values are also greater than those for L1 in five out of eight cases, suggesting that once the β-lactam substrate molecule is productively bound to the active site, hydrolysis in these cases is more efficient than with L1.

Substrate inhibition of Mbl1b by some but not other β-lactams is a new finding for metallo-β-lactamases. The exact mechanism of substrate inhibition is unknown but was seen with all penicillins and most cephalosporins tested (the exception being nitrocefin). The carbapenems tested showed conventional Michaelis–Menten kinetics. These findings imply that the different kinetics have a fundamental structural basis and that different antibiotics may have different mechanisms of hydrolysis. The principal difference between the carbapenems and other β-lactams is that both penicillins and cephalosporins have bulky aromatic ring substitutions which the first lack. The active sites of metallo-β-lactamases are shallow open grooves lined with hydrophobic groups and, from the homology model, Mbl1b is similar. We speculate that the value of $n$ in Table 1 may be a function of the number of unproductive conformations of these bulky substitutions.

In the L1 crystal structure, tryptophan 17 helps stabilise the zinc ligand histidine 225 via edge–face contacts. In Mbl1 this tryptophan (W7) is conserved in Mbl1b. All the other active site ligands identified in L1 are present and their orientations in the homology model are almost identical to those in L1 (Fig. 3). Given this, as expected, the protein binds two zinc ions per subunit.

L1 is unique amongst metallo-β-lactamases in that it is tetrameric; the others are monomeric [3]. One of the principal residues involved in L1 inter-chain stabilisation is Met140, which interacts with a hydrophobic pocket on the adjacent subunit (A/D, and B/C). An equivalent methionine is absent from the Mbl1b sequence and, as expected, Mbl1 is monomeric. These differences between Mbl1 and L1 suggest that Mbl1 is likely to be of considerable use as a model system for elucidating the detailed mechanism of the metallo-β-lactamases, serving as a link between the monomeric enzyme and the tetramer, L1.

The biological role of the Mbl1 β-lactamase in *C. crescentus* is uncertain. Strain 101123 was isolated before antibiotics were administered to farm animals [24] and comes from an environment in which significant exposure to β-lactams is unlikely. However, it is possible that the organism has experienced sufficiently persistent selective pressure due to natural environmental β-lactams, for example from fungi. Stalked bacteria have been observed attached to trichomycetous fungi in the hindgut of soil-inhabiting millipedes. *Caulobacter* spp. can be isolated readily both from soil samples and from such hindgut fungal samples [10]. It is quite possible that this protein represents an ancestral hydrolase that has been selected to counteract the effect of naturally occurring fungal β-lactams in the normal environment of the bacteria.

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**References**

