Communication

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Pallav Joshi [1,2,†], Stefan Krco [1,2,†], Samuel J. Davis [1,2], Lachlan Asser [1], Thomas Brück [3], Rochelle M. Soo [1,2], Mikael Boden [1], Philip Hugenholtz [1,2], Liam A. Wilson [4], Gerhard Schenk [1,5,*] and Marc T. Morris [1,2,*]

[1] School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia.
[2] Australian Centre for Ecogenomics, The University of Queensland, Brisbane, Australia.
[3] Werner Siemens-Chair of Synthetic Biotechnology, Technical University of Munich (TUM), TUM School of Natural Sciences, Lichtenbergstr. 4, 85748 Garching, Germany.
[4] Chemistry Research Laboratory, Department of Chemistry and the Ineos Oxford Institute for Antimicrobial Research, Oxford University, Oxford, OX1 3TA United Kingdom.
[5] Australian Institute of Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Australia.
* Correspondence: marc.morris@uq.edu.au; G.S. schenk@uq.edu.au
† These authors contributed equally to this work and share first authorship.

Abstract: Metallo-β-lactamases (MBLs) are members of the structurally conserved but functionally diverse MBL-fold superfamily of metallohydrolases. MBLs are a major concern for global health care as they efficiently inactivate β-lactam antibiotics, including the "last-resort" carbapenems, and no clinically suitable inhibitors are currently available. Increasingly, promiscuous β-lactamase activity is also observed in other members of the superfamily, including from viruses, which represents an underexplored reservoir for future pathways to antibiotic resistance. Here, two such MBL-fold enzymes from *Bacillus* phages, the cyclic mononucleotide-degrading proteins Apyc$_{Goe3}$ and Apyc$_{Grass}$, are shown to efficiently degrade β-lactam substrates *in vitro*. In particular, Apyc$_{Grass}$ displays a distinct preference for carbapenem substrates with a catalytic efficiency that is within one order of magnitude of that of the clinically relevant MBL NDM-1. Mutagenesis experiments also demonstrate that the loss of a metal-bridging aspartate residue reduces nuclease activity up to 35-fold, but improves carbapenemase activity. In addition, we propose that the oligomeric state significantly influences β-lactamase activity by modifying access to the active site pocket. Together, these observations hint at a possible new avenue of resistance via the spread of phage-borne MBL-fold enzymes with β-lactamase activity.

Keywords: virus; metallo-beta-lactamase (MBL); MBL-fold superfamily; antimicrobial resistance; anti-pycsar; carbapanemase

Introduction

Antibiotic resistance has long been recognized as a critical global health concern [1]. The excessive use of antibiotics as preventive and curative measures against infection not only for human health but also in intensive animal husbandry has resulted in a widespread rise in antimicrobial resistance [2]. Amongst the varied mechanisms of antibiotic resistance, the production of β-lactamases, enzymes that break down β-lactam antibiotics, is of particular concern as they are the most widely used (~65% globally) and include 'last-resort' antibiotics such as the carbapenems (Figure 1A) [1,3]. These β-lactamases are classified based on their structure and mechanism as serine-β-lactamases (SBLs - Ambler classes A, C, and D; see Bush [4]), which utilise a conserved serine residue to hydrolyse the four-membered β-lactam ring, and metallo-β-lactamases (MBLs – Ambler class B), which catalyse the same reaction using a catalytic hydroxide coordinated by one or two zinc ions [3,5]. MBLs are of particular interest as currently no clinically useful inhibitors are available [3,5–7]. In contrast, SBLs are targeted by several potent and widely used drugs (e.g., clavulanic acid) [3,8,9].

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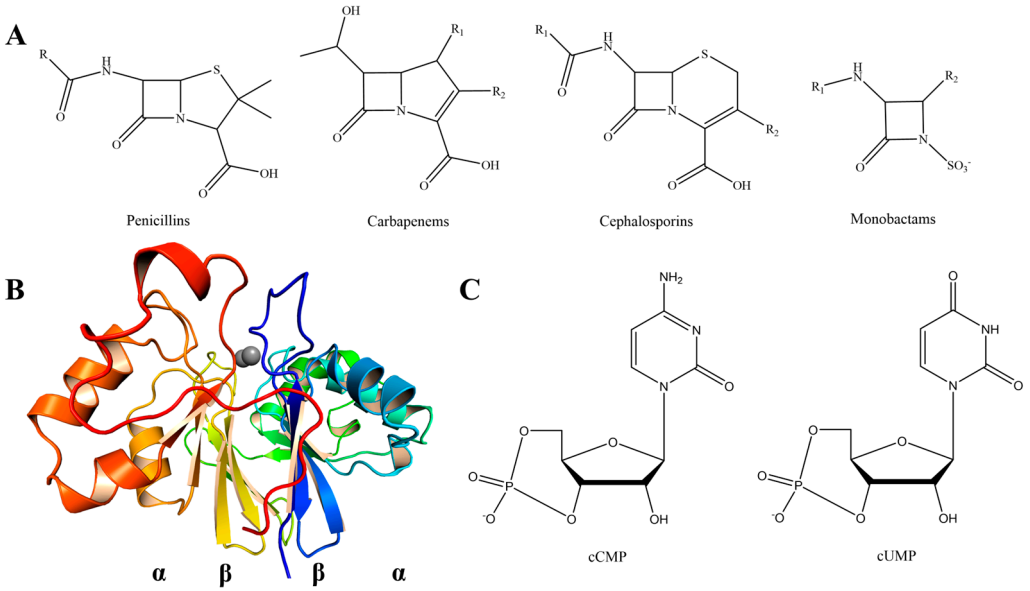

Figure 1. (**A**) Chemical structure of the backbones of the four major β-lactam antibiotic classes. (**B**) Overall structure of Apyc$_{Goe3}$. Structural model was predicted using AlphaFold3 [10] (**C**) Chemical structures of two cyclic nucleotides: cyclic cytidine monophosphate (cCMP) and cyclic uridine monophosphate (cUMP).

MBLs are divided into the B1, B2, and B3 subgroups according to their phylogeny, active site residues, metal content, and substrate preferences [3,11,12]. The B1 and B2 subgroups contain the majority of the MBLs of current clinical concern [13], whereas B3 MBLs are primarily associated with environmental microorganisms [11,12,14,15], although some notable B3 members of concern (*e.g.*, AIM-1) have been encountered in clinical environments [16,17]. The three subgroups belong to the MBL-fold protein superfamily [3,11], named after the first enzyme that was shown to contain the characteristic αββα or 'MBL'-fold (Figure 1B), the MBL from *Bacillus cereus* (BcII) [18]. The MBL-fold superfamily is present in all domains of life, and while functionally diverse they predominantly function as hydrolases. Members include, but are not limited to, endo- and exoribonucleases [19–21], phosphatases [22–24], lactonases [25,26], glyoxalases [27–29], dehalogenases [30], sulfatases [31,32], oxidases [33], and β-lactamases [11,12,34–38]. Recent studies have shown that numerous members of this superfamily exhibit catalytic promiscuity [39–43]. Promiscuous β-lactamase activity is of concern as the corresponding enzymes may represent a cryptic reservoir of antibiotic resistance. For example, two MBL-fold enzymes recovered from deep sea viral metagenomes, *i.e.*, TupBlac and PNGM-1, are nucleases with promiscuous β-lactamase activity [41,44,45]. While these native nucleases were likely acquired from a bacterial host, and their β-lactamase activity is currently marginal, their presence in a virus raises the question about the possible evolution and dissemination of antimicrobial resistance through horizontal gene transfer back into bacterial hosts.

During a sequence-based survey of B3 MBLs [12], we discovered two distantly related viral MBL-fold enzymes in the genomes of the *Bacillus* phages vB_Bsum_Goe3 (KY368640) and Grass virus (KF669652), which both belong to the "Bastille-like" viruses within the *Spounavirinae* subfamily of the *Myoviridae* [46,47]. These virally-encoded MBL-fold enzymes appear to have been horizontally-acquired from their bacterial hosts, but no closely-related, characterised MBL-fold homologs were reported at that time. The recent characterisation of anti-Pycsar enzymes [48], sheds light on the likely *in vivo* function of these enzymes. The Pyrimidine Cyclase System for Antiphage Resistance (Pycsar) is a recently identified but seemingly widespread bacterial defence mechanism against phages, which is activated by cyclic nucleotide messengers (Figure 1C) [49,50]. In response, *Bacillus* phages have evolved an anti-Pycsar system, by expressing MBL-fold proteins which specifically cleave and thus inactivate cyclic nucleotide messengers [48]. One such anti-Pycsar protein, Apyc1 from the *Bacillus* phage Bsp38, shares 78.9% and 88.2% sequence identity with the vB_Bsum_Goe3 and Grass virus MBL-fold enzymes [48]. Therefore, we predicted that these MBL proteins should possess nuclease

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activity (*i.e.* Apyc$_{Goe3}$ and Apyc$_{Grass}$, respectively). However, since virally encoded β-lactamase activity has been demonstrated in TupBlac and PNGM-1, we evaluated the potential of both enzymatic properties in Apyc$_{Goe3}$ and Apyc$_{Grass}$. We recombinantly-expressed the enzymes in an *Escherichia coli* host, followed by affinity chromatography purification *via* either an N-terminal maltose binding protein (MBP) or hexahistidine tag[1]. To assess their likely nucleolytic function, the RNase activity of these enzymes was quantitatively assessed using a fluorescence-based activity assay kit (RNase QC Alert kit, Thermo Fisher) [44]. To test their β-lactamase activity, we performed continuous *in vitro* UV-Vis assays following the hydrolysis of representative β-lactam antibiotics.

Materials and Methods

The genes for Apyc$_{Goe3}$ (A0A217ER65) and Apyc$_{Grass}$ (U5PU04) as well as Apyc$_{Goe3}$ (D178S) and Apyc$_{Grass}$ (D161S) were cloned into both pMAL-c5x and pET-24a(+) vectors for subsequent tag-based affinity purification using either Maltose Binding Protein (MBP) or polyhistidine (6xHis) tags, respectively. The expression vectors containing the relevant genes were synthesized commercially (pMAL-c5x : Gene Universal Inc.; pET-24a(+) : Twist Biosciences).

The vectors were transferred into chemically-competent *E. coli* Rosetta (DE3) cells via heat shock at 42°C. Single, isolated colonies were picked and grown in LB medium supplemented with 100 μg/ml of either ampicillin (pMAL-c5x vectors) or kanamycin (pET-24a(+) vectors) for selection. These cultures were used to inoculate larger expression cultures of LB medium with appropriate antibiotics and were grown shaking (200 rpm) at 37 °C until they reached an $OD_{600} \approx 0.6$. At this point, protein expression was induced by addition of 500 μM IPTG, the temperature reduced to 18 °C, and the culture left to grow for a further 12 h. The cells were harvested by centrifugation (20 min, 5,000 xg), resuspended in lysis buffer (20 mM Tris buffer, pH 8.0, containing 0.15 M NaCl, 150 μM $ZnCl_2$) supplemented with 1 mg/ml lysozyme, 1 mg/ml DNase I, and 1.5 mg/ml EDTA-free protease inhibitor cocktail, and lysed on ice by sonication. Cell debris was removed by centrifugation (40 min, 14,000xg), and the supernatant loaded onto 5 mL MBPTrap HP or HisTrap FF column for MBP- and polyhistidine-tagged enzymes, respectively, pre-equilibrated with purification buffer (20 mM Tris buffer, pH 8.0, containing 0.15 M NaCl, 150 μM ZnCl2). The proteins were eluted against 10 mM maltose (MBPTrap HP) or 500 mM imidazole (HisTrap FF). Fractions containing the enzyme (determined by SDS-PAGE analysis) were pooled and excess NaCl was removed via buffer exchange using EconoPac 10DG desalting columns (Bio-Rad). The purified proteins were stored at 4°C in 20 mM Tris buffer (pH 8.0).

To quantify hydrolytic activity towards nucleic acids, activity of the two viral enzymes and two mutant enzymes was assessed against a mixed RNA substrate supplied by a commercially available nuclease activity kit (RNase QC Alert, Thermo Fisher), which had been previously used to assess the nuclease activity of TupBlac [44]. The substrate is composed of ribonucleic acids with both a fluorescent probe and quencher. Upon hydrolysis of the substrate, the fluorescent probe is no longer quenched, and allows for quantitative analysis of the amount of substrate hydrolysed. The assays were conducted in Costar 96-well flat bottom plates with final volumes of 200 μL at 37 °C for 1 hour using a MBG Clariostar TRF plate reader instrument at 490/520 nm excitation/emission via the time resolved fluorescence function and analysed using Reader Control and MARS Data Analysis Software. Enzyme concentrations of 50 nM were used for all four tested enzymes. Nucleolytic activity of each enzyme was determined by comparison to a standard curve generated with RNase A. The standard curve was produced with 5 pg, 10 pg, 25 pg, and 50 pg of RNase A.

The β-lactamase activity of the wild-type and serine mutant enzymes was tested by continuous *in vitro* UV-Vis assays against representative substrates from each major class of β-lactam antibiotics:

[1] Both systems resulted in the production of soluble, pure enzyme. However, preliminary assays indicated that only the MBP-tagged enzymes displayed significant β-lactamase activity and were hence used for more detailed catalytic characterisation. Any attempts to remove the MBP tag by proteolytic cleavage resulted in precipitation of the viral MBL proteins. Unless otherwise specified, the terms Apyc$_{Goe3}$ and Apyc$_{Grass}$ correspond to the MBP-tagged constructs.

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ampicillin (λ = 235 nm; ε = 900 M^{-1} cm^{-1}), carbenicillin (λ = 235 nm; ε= 1,190 M^{-1} cm^{-1}), penicillin G (λ = 235 nm; ε = 936 M^{-1} cm^{-1}), biapenem (λ = 293 nm; ε = 8,630 M^{-1} cm^{-1}), imipenem (λ = 295 nm; ε = 9,000 M^{-1} cm^{-1}), meropenem (λ = 297 nm; ε = 6,500 M^{-1} cm^{-1}), cefaclor (λ = 280 nm; ε = 6,410 M^{-1} cm^{-1}), cefuroxime (λ = 260 nm; ε = 9,320 M^{-1} cm^{-1}), cephalothin (λ = 265 nm; ε = 8,790 M^{-1} cm^{-1}), and aztreonam (λ = 318 nm; ε = 660 M^{-1} cm^{-1}). The assays were run for 1 min at 25 °C in 50 mM Tris (pH 8.0) using enzyme concentrations of 0.5 μM for $Apyc_{Goe3}$, $Apyc_{Goe3}$ (D178S), and $Apyc_{Grass}$, and 0.3-0.5 μM for $Apyc_{Grass}$ (D161S). All β-lactamase assays were run on an Agilent Cary 60 UV-Vis Spectrophotometer.

Results and Discussion

As predicted, $Apyc_{Goe3}$ and $Apyc_{Grass}$ possess significant RNAse activity (see Figure 2 and details below), supporting the designation of these enzymes as orthologs of Apyc1. Concerningly, $Apyc_{Goe3}$ and $Apyc_{Grass}$ also display significant levels of β-lactamase activity (Table 1). For $Apyc_{Grass}$, the catalytic efficiencies (k_{cat}/K_M) are greatest for carbapenem substrates (10-15 s^{-1} mM^{-1}), followed by penicillins (0.5-3 s^{-1} mM^{-1}), while cephalosporins are less readily hydrolysed (≥0.1 s^{-1} mM^{-1}). The preference for carbapenems is due to both higher catalytic rates and lower K_M values when compared to penicillins. Interestingly, cephalosporins bind more strongly to $Apyc_{Grass}$ than the other substrates but are turned over extremely slowly. For $Apyc_{Goe3}$, the catalytic efficiencies are similar for carbapenems (~1 s^{-1} mM^{-1}) and penicillins (1-3 s^{-1} mM^{-1}), and are approximately two- to ten-fold higher than for cephalosporins (0.1-0.5 s^{-1} mM^{-1}). Similar to $Apyc_{Grass}$, the cephalosporins bind significantly tighter than the other substrates but are turned over very slowly. This suggests that while cephalosporins can bind tightly, they are likely oriented in a catalytically non-competent conformation. Consistent with this interpretation is the observation that cephalosporins, but not the other major classes of β-lactams, are competitive inhibitors of other MBL-fold enzymes, namely SNM1A and SNM1B, two human MBL-fold enzymes involved in DNA repair, with IC50 values in the low μM range [51]. These results suggest that non-competent binding of cephalosporins or inhibitory effects of these β-lactams may be a more common characteristic across the MBL-fold superfamily than previously appreciated and hence warrants further investigation.

Table 1. Catalytic parameters of the MBP-tagged wild-type and mutant forms of the virally encoded $Apyc_{Goe3}$ and $Apyc_{Grass}$ recorded with representative substrates from all major classes of β-lactam antibiotics. Units of k_{cat}, K_M, and k_{cat}/K_M are s^{-1}, μM, and s^{-1} mM^{-1} | N.H. - no hydrolytic activity detected. [a]Lee *et al.* [53].

	$Apyc_{Goe3}$			$Apyc_{Goe3}$ (D178S)			$Apyc_{Grass}$			$Apyc_{Grass}$ (D161S)			PNGM-1[a]		
Substrate	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M
Penicillins															
Penicillin G	0.24	230	1.04	0.45	751	0.60	0.67	231	2.90	0.48	180	2.70	$7.5x10^{-2}$	16	4.7
Ampicillin	1.8	663	2.67	0.72	526	1.37	0.54	418	1.29	0.39	891	0.44	$2.7x10^{-2}$	15	1.8
Carbenicillin	0.32	205	1.56	0.13	157	0.83	0.15	330	0.45	0.24	462	0.52	-	-	-
Carbapenems															
Meropenem	0.22	215	1.02	0.43	160	2.69	1.1	98	11.2	4.4	287	15.3	$8.0x10^{-4}$	2	0.42
Imipenem	0.27	436	0.62	-	-	-	3.1	200	15.5	4.2	253	16.6	$1.1x10^{-3}$	2	0.55
Cephalosporins															
Cefuroxime	$8.6x10^{-3}$	18	0.48	$8.3x10^{-3}$	3.5	2.37	$5.3x10^{-3}$	44	0.12	$6.0x10^{-3}$	142	0.042	-	-	-
Cephalothin	$3.0x10^{-3}$	19	0.16	$7.0x10^{-3}$	23	0.30	$5.6x10^{-3}$	51	0.11	$1.3x10^{-3}$	54	0.024	0.13	62	2.1
Monobactams															
Aztreonam	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	N.H.	-	-	-

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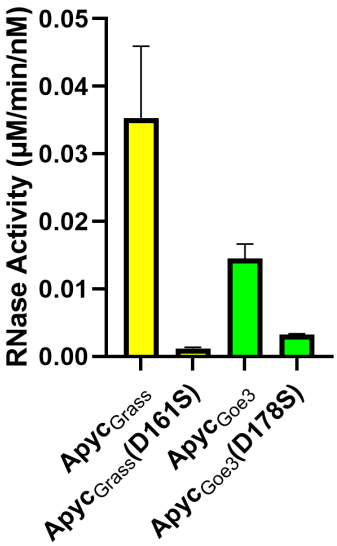

Figure 2. Nuclease activity of Apyc$_{Grass}$, Apyc$_{Grass}$(D161S), Apyc$_{Goe3}$, and Apyc$_{Goe3}$(D178S) using a mixed ribonucleic acid substrate tagged with a fluorescent probe from the RNase QC Alert kit (Thermo Fisher). Activity is obtained by comparison to a standard curve generated with an RNase A positive control.

It should be noted that Apyc$_{Goe3}$ and Apyc$_{Grass}$ are significantly more efficient as β-lactamases than other virally encoded enzymes from the MBL-fold superfamily. For comparison, PNGM-1 has k_{cat}/K_M ratios comparable to those of Apyc$_{Goe3}$ and Apyc$_{Grass}$ for penicillins and carbapenems (~0.5 – 5 s^{-1} mM^{-1}); however, they all appear to bind in a catalytically non-competent conformations (leading to k_{cat} values of 10^{-2} - 10^{-4} s^{-1}; Table 1). In particular, the carbapenemase activity of Apyc$_{Grass}$ (k_{cat} ~ 1 – 3 s^{-1}; k_{cat}/K_M ~ 10 – 15 s^{-1} mM^{-1}) is remarkable, even when compared to "true" MBLs such as the B1 MBL NDM-1, an enzyme that is recognised as a globally-distributed clinical concern [1,52] (Table S1). The k_{cat} and k_{cat}/K_M values of the NDM-1-catalysed hydrolysis of the carbapenems meropenem (12 s^{-1}, 250 s^{-1} mM^{-1}) and imipenem (20 s^{-1}, 210 s^{-1} mM^{-1}) are only one order of magnitude greater than those of Apyc$_{Grass}$. Furthermore, we also note that the turnover rates of both Apyc$_{Goe3}$ and Apyc$_{Grass}$ are comparable, and in some cases superior, to those of some SBLs such as the clinically relevant AmpC and OXA-48 (Table S1). This may indicate that these enzymes are predisposed to further evolve into efficient carbapenemases, which could constitute a threat to current treatment options for infections.

Sequence alignments and AlphaFold3 models suggest that both Apyc$_{Goe3}$ and Apyc$_{Grass}$ contain the canonical HHH/DHH metal-binding motif observed in B3 MBLs and across the broader MBL-fold superfamily [3,11] (Figures 3 and S1). Notably, they possess the aspartate residue found in the majority of non-β-lactamase MBL-fold hydrolases which bridges the two metal ions in the active site. This aspartate residue has been suggested to be critical to catalysis in several MBL-fold enzymes [39,54], but is notably substituted by non-metal-coordinating residues in all true β-lactamase lineages of the superfamily (*i.e.*, in the B1, B2, and B3 MBLs), potentially implying a key role for this residue in the evolution of β-lactamase activity. The replacement of this metal-bridging ligand in class B β-lactamases may facilitate greater structural flexibility (*i.e.*, reduced rigidity), possibly a feature that is important to accommodate a large number of diverse β-lactam substrates. To test this hypothesis, mutant variants of Apyc$_{Goe3}$ and Apyc$_{Grass}$ were generated where this aspartate residue was replaced by a serine residue, as is found in B3 MBLs [11].

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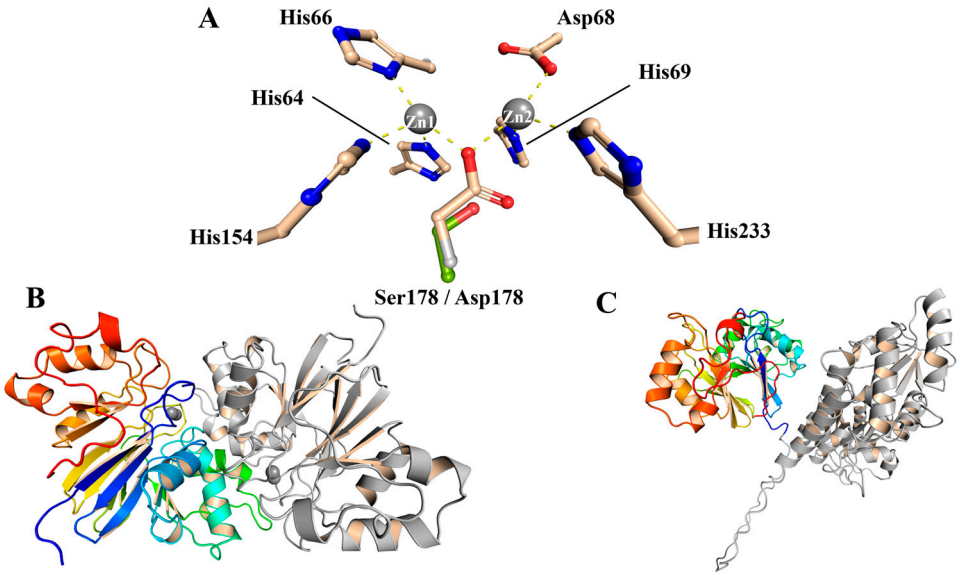

Figure 3. (**A**) Active site of Apyc$_{Goe3}$ showing the aspartate to serine mutation observed in Apyc$_{Goe3}$ (D178S), which removes a coordination point for each metal (Zn^{2+}) ion. (**B**) Overall structure of the polyhistidine-tagged Apyc$_{Goe3}$ dimer. One monomer is coloured in a gradient from blue (N-terminus) to red (C-terminus), and the other in grey. (**C**) Overall structure of the MBP-tagged Apyc$_{Goe3}$ monomer. The Apyc$_{Goe3}$ portion of the fusion protein is coloured in a gradient from blue (N-terminus) to red (C-terminus), whereas the MBP tag is coloured grey. All structural models were predicted using AlphaFold3 [10].

The mutation has, however, minimal impact on the catalytic properties using carbapenems and penicillins as substrates. The preference for carbapenems is retained with k_{cat}/K_M values of ~2 s^{-1} mM^{-1} and 15 s^{-1} mM^{-1} for Apyc$_{Goe3}$ and Apyc$_{Grass}$, respectively (Table 1). For the penicillin substrates, the corresponding values are 0.5-1.5 s^{-1} mM^{-1} and 0.5-3 s^{-1} mM^{-1}. The cephalosporins are again turned over very slowly, but due to the very low K_M values in Apyc$_{Goe3}$ they reach k_{cat}/K_M values similar to that of the other substrates. While the introduction of the aspartate to serine mutation had modest impact on the β-lactamase activity of Apyc$_{Goe3}$ and Apyc$_{Grass}$, it reduced their nuclease activity by 5- and 35-fold, respectively. The RNase activity of the native forms of Apyc$_{Goe3}$ and Apyc$_{Grass}$ displayed nuclease activities of 4,054 mU/min and 10,115 mU/min (equivalent to ~0.015 µM/min/nM and ~0.035 µM/min/nM; Figure 2), while the mutant enzymes displayed activities of 810 mU/min and 289 mU/min, respectively. In comparison, TupBlac, a dual-activity MBL-fold enzyme from a giant mimivirus, was reported to possess a nuclease activity of 0.451 mU/min [44]. It thus appears that both the native and mutant forms of Apyc$_{Goe3}$ and Apyc$_{Grass}$ readily degrade β-lactam antibiotics as well as ribonucleic acids and do so more efficiently than comparable viral MBL-fold nucleases, although nuclease activity is greatly diminished in the serine mutant enzymes. Hence, our study demonstrates that while the aspartate residue that is present in the active site of many MBL-fold superfamily members is important for nuclease activity, its contribution to β-lactamase activity is less significant. The loss of this aspartate residue may thus have been a mechanism to enable ancestral MBL-fold hydrolases to accelerate their evolution towards β-lactamase, but other as of yet unknown factors also contribute towards this functional transition.

Interestingly, the hexahistidine-tagged variants of both enzymes display nuclease but no β-lactamase activity (data not shown). A possible explanation for the observed discrepancy in the activity profiles of the different variants may be the impact of the tags on the oligomeric state of these enzymes. We hypothesise that due to its size, the MBP tag prevents the oligomerisation of Apyc$_{Goe3}$ and Apyc$_{Grass}$, while the histidine-tagged variants may form dimers or even higher oligomers. Indeed, structural prediction using AlphaFold3 suggested that the native and polyhistidine-tagged variants form dimers, whereas the enzymes tagged with the larger MBP tag were monomeric (Figure 3). It is noteworthy that most of the highly active "true" MBLs (e.g., NDM-1, AIM-1) are monomeric, while

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many nucleases such as the RNase Zs are dimeric [19]. In addition, the crystal structure of PNGM-1 (PDB code: 6J4N) suggests that the protein may be tetrameric [45], while structural modelling of TupBlac reveals considerable structural similarity to the dimeric RNase Zs [44]. Indeed, Size Exclusion Chromatography with Multi Angle Light Scattering (SEC-MALS) experiments with Apyc$_{Goe3}$ confirm that the polyhistidine-tagged, but not the MBP-tagged, variant formed dimers (data not shown). These results thus raise the possibility that both TupBlac and PNGM-1, as well as multimeric MBL-fold hydrolases more broadly, may be functionalised into more efficient β-lactamases by altering their oligomeric states to monomers, which may enhance the accessibility of the active site for β-lactam substrates.

Conclusions

The present study provides evidence that anti-Pycsar proteins from the MBL-fold superfamily are capable of efficient β-lactam hydrolysis, and notably display a preference for the 'last-resort' carbapenems. However, their β-lactamase activity may be dependent on their oligomeric state. The metal-bridging aspartate residue present in the large majority of MBL-fold enzymes, but absent in "true" MBLs, appears to have minimal effect on β-lactamase activity (Table 1), but its removal greatly impairs nuclease activity (Figure 2). The evolution of "true" MBLs from an MBL-fold hydrolase precursor could thus have arisen from a concerted effect of structural changes that alters the oligomeric state and the removal of a metal ligand that leads to a change of the preferred substrate towards β-lactams. Given that phages can be vectors of horizontal transfer of genes between bacterial hosts, the possible evolution of an efficient β-lactamase from a phage-encoded MBL-fold nuclease, in particular anti-Pycsar enzymes, could represent a new avenue for rapid dissemination of antimicrobial resistance, and thus pose a significant threat to human health.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Materials & Methods; Figure S1: Alignment of metal-binding residues of Apyc orthologs and representatives of the MBL-fold superfamily; Table S1: Catalytic parameters of representative members of each Ambler class of β-lactamases.

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