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Identification and preliminary characterization of novel B3-type metallo-*β*-lactamases

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ABSTRACT

Antibiotic resistance has emerged as a major global threat to human health. Among the strategies employed by pathogens to acquire resistance the use of metallo-*β*-lactamases (MBLs), a family of dinuclear metalloenzymes, is among the most potent. MBLs are subdivided into three groups (*i.e.* B1, B2 and B3) with most of the virulence factors belonging to the B1 group. The recent discovery of AIM-1, a B3-type MBL, however, has illustrated the potential health threat of this group of MBLs. Here, we employed a bioinformatics approach to identify and characterize novel B3-type MBLs from *Novosphingobium pentaromativorans* and *Simiduia agarivorans*. These enzymes may not yet pose a direct risk to human health, but their structures and function may provide important insight into the design and synthesis of a still elusive universal MBL inhibitor.

Keywords: Antibiotic Resistance; *β*-Lactam Antibiotics; Metallo-*β*-Lactamases; Sequence Homology; Novosphingobium Pentaromativorans; Simiduia Agarivorans

1. INTRODUCTION

The introduction of *β*-lactam antibiotics (**Figure 1**) in the 1940s has been considered as a breakthrough, if not the most significant breakthrough in the history of medicine. However, only a few years after introducing penicillin resistance was observed in *Staphylococcus aureus* and meanwhile a large and increasing number of pathogens have acquired resistance to the most commonly used antibiotics [1,2], triggering some experts to liken antibiotic resistance to terrorism in terms of its global impact. http://www.bbc.co.uk/news/health-21737844

One of the most frightening forms of antibiotic resistance occurs through the action of metallo-*β*-lactamases (MBLs), and enzymes are capable of breaking down most widely used *β*-lactam antibiotics [1-5]. These Zn^{2+}-dependent enzymes are not susceptible to any known drugs [5]. MBLs have been divided into three subgroups (*i.e.* B1, B2 and B3) based on their sequences and metal requirements [1,4,5]. Despite only low levels of sequence similarity these subgroups show homology at the level of structure; they all exhibit *α*/*β*/*β*/*α* folded with two metal binding sites located between the two central *β* sheets (**Figure 2**). The key active site residues responsible for binding the metal ions in the three subgroups show variations that result in differences in metal requirements and catalytic mechanisms [5]. Most of the known MBL virulence factors belong to subgroup B1 and include BCII from *Bacillus cereus* [6], CcrA from *Bacteroides fragilis* [7], as well as IMP-1 and SPM-1, both initially identified in *Pseudomonas aeruginosa* [8,9]. The recently identified NDM-1 ("New Delhi Imipenemase-1") has acquired particular notoriety as it induces resistance to virtually all known *β*-lactam antibiotics [10]. Subgroup B2 enzymes share only ~11% sequence ho-

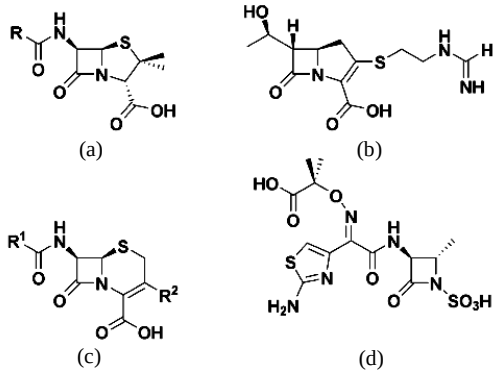

Figure 1. Representatives for the most common *β*-lactam antibiotic families. (a) Penicillin; (b) carbapenem; (c) cephalosporin; and (d) monobactam.

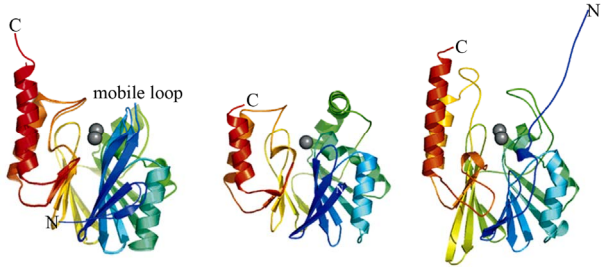

Figure 2. Representative MBL structures. Left: B1-type CcrA from *B. fragilis*; center: B2-type CphA from *A. hydrophila*; right: B3-type L1 from *S. maltophilia* (only one subunit shown). The protein main chains are color-ramped from the N-(blue) to the C-terminus (red). Zinc ions are rendered as gray spheres.

mology with B1-type MBLs, hydrolyze exclusively carbapenems (e.g. meropenem and imipenem) and require only one metal ion for catalysis [5,11]. Representative B2-type MBLs are CphA from *Aeromonas hydrophila* [12], ImiS from *A. veronii* [13] and Sfh-1 from *Serratia fonticola* [14]. Subgroup B3 is closer related to B1-type MBLs rather than B2-type MBLs, requiring two bound metal ions for catalysis (**Figure 2**). The most studied representative is the tetrameric L1 from *Stenotrophomonas maltophilia* [15]. Other members include FEZ-1 from *Legionella gormanii* [16], GOB-1 from *Elizabethkingia meningoseptica* (of which to date 18 variants have been reported) [17] and SMB-1 from *Serratia marcesens* [18], the most recently identified MBL, which has a higher hydrolytic activity against a wide range of *β*-lactams than other B3-type MBLs [18]. Of clinical relevance is in particular the enzyme AIM-1 from *Pseudomonas aeruginosa*, which has been identified recently in multi-drug resistant isolating in a hospital in Adelaide, Australia (hence, the nomenclature of "Adelaide Imipenemase-1"—AIM-1) [19].

Despite rather modest homology across their full length amino acid sequences MBLs share considerable similarities in their active site structures [5]. All MBLs provide binding sites for two closely spaced metal ions that are invariably Zn^{2+} *in vivo* (*i.e.* Zn1 and Zn2). Some variations are observed among the ligands that bind to the zinc ions in the active site. B1- and B3-type MBLs have three histidines (His) on the Zn1 site. In the B2-type MBLs one of these histidines (His116) is replaced by an asparagine (Asn). For B1- and B2-type MBLs the second metal binding site is conserved with an aspartate (Asp), a histidine and a cysteine (Cys) coordinating the metal ion. In the B3 subclass the cysteine is replaced by another histidine (**Figure 3**). A standard numbering scheme for residues in MBLs has been developed based on sequence alignments for B1, B2 and B3 MBLs, and is used throughout this work to simplify comparisons between different MBLs [4,20,21].

In light of the rapid spread of antibiotic resistance and

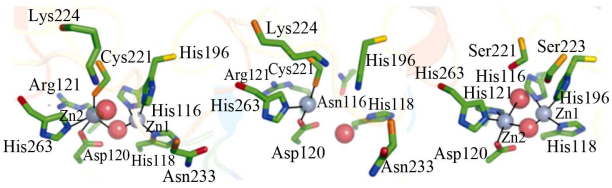

Figure 3. Active site structures of representative MBLs. Left: B1-type BcII from *B. cereus*; center: B2-type CphA from *A. hydrophila*; right: B3-type L1 from *S. maltophilia*. Zinc ions are rendered as grey spheres, and water molecules are shown as red spheres. Coordination bonds are shown as solid lines.

the increasing emergence of novel virulence factors (exemplified by NDM-1 and AIM-1). It is essential to identify novel putative MBLs, ideally before they become a threat to health care. Furthermore, novel MBLs may also provide essential insight into the structure and/or functional aspects relevant to the design and synthesis of universal inhibitors that may be clinically useful to combat antibiotic resistance. Here, our focus was on the B3 subgroup because they appear to be less known than their counterparts in the other two subgroups, but they are a danger to be reckoned as the recent emergence of AIM-1 and SMB-1 illustrated.

2. METHODS

2.1. Selection of the Query Sequence and Protein Database Search Using BLAST

The B3-type AIM-1 from *P. aeruginosa* was used as query sequence for the protein database search. The protein sequence of AIM-1 was obtained from the Protein Data Bank (PDB; accession code: 4AWY), and the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify homologues. The two most promising candidates, from *Novosphingobium pentaromativorans* and *Simiduia agarivorans* were selected for multiple sequence comparisons including known B3-type MBLs.

2.2. Multiple Sequence Alignments

Multiple sequence alignments including the novel B3-type MBLs and well known members of this group of enzymes (*i.e.* AIM-1 [19], L1 [15] and SMB-1 [18]) were carried out using ClustalW2, a multiple sequence alignment program, available via The European Bioinformatics Institute website. http://www.ebi.ac.uk /Tools/msa/clustalw2/

3. RESULTS AND DISCUSSION

3.1. Protein Database Search, Nomenclature and Classification of Novel MBLs

Using the BLAST search engine with AIM-1 as the

query two promising candidate sequences were retrieved, *i.e.* MBL-like sequences from *N. pentaromativorans* (accession code: ZP_09194167.1) and *S. agarivorans* (accession code: YP_006917856.1). These microorganisms are both Gram-negative. *N. pentaromativorans* is a polycyclic aromatic hydrocarbon-degrading bacterium [22], while *S. agarivorans* is a heterotrophic marine bacterium [23]. None of these organisms poses a direct current threat to human health, however, the observation that they harbor a potential MBL may not only foreshadow a future problem, they also could represent a genetic pool from which horizontal genetic transfer can occur and they could lead to the design and development of universally applicable inhibitors against MBLs. It is thus essential to investigate the properties of these novel MBL-like proteins and compare them with those of well-known MBLs (e.g. AIM-1, L1 or SMB-1).

As a step towards their characterization, the sequences of the *N. pentaromativorans* and *S. agarivorans* MBL-like proteins were compared with those of well-characterized MBLs from the B3 subgroup, *i.e.* AIM-1 [19], L1 [15] and SMB-1 [18]. The results from pairwise sequence comparisons are summarized in Table 1. Not surprisingly, the two sequences are most closely related to AIM-1. The MBL-like protein from *N. pentaromativorans* shares 53% sequence identity and 65% homology (including conserved amino acid substitutions) with AIM-1. The sequence identity/homology to the other two well characterized B3-type MBLs, L1 and SMB-1, is smaller (38%/54% and 41%/58%, respectively) but still strongly indicative that the *N. pentaromativorans* protein is indeed a B3-type MBL. In comparison, pairwise sequence comparisons with the B1-type NDM-1 and B2-type CphA indicate only 26%/39% and 23%/42%, respectively. A similar conclusion can be drawn for the MBL-like sequence from *S. agarivorans*. Its similarity/homology with AIM-1 (47%/64%) is less than that of the *N. pentaromativorans* MBL, but it appears to be closer related to SMB-1 instead (**Table 1**). The two MBL-like proteins share 47%/63% identity/homology in a direct pairwise sequence comparison. In summary, these pairwise comparisons strongly support the classification of these novel proteins sequences from *N. pentaromativorans* and *S. agarivorans* as MBLs from the B3 subgroup. In accordance with frequently applied nomenclature procedures (e.g. “Adelaide Imipenemase-1” or AIM-1) the MBL-like sequences from *N. pentaromativorans* and *S. agarivorans* are labeled here “Maynooth Imipenemase-1” (MIM-1) and “Maynooth Imipenemase-2” (MIM-2), respectively.

3.2. Important Amino Acid Residues

The above discussion demonstrated that the MBL-like sequences in the genomes of *N. pentaromativorans* and *S. agarivorans* are likely members of the B3 subgroup in the MBL family. However, in order to substantiate this interpretation it is essential to ascertain that amino acid residues that are essential for MBL function are conserved in the amino acid sequences of MIM-1 and MIM-2. The most relevant amino acid residues with respect to MBL function are those that form the metal binding site. Other residues in the proximity of the metal ion binding sites may be important for substrate or inhibitor binding or both. In order to evaluate the functionality of MIM-1 and MIM-2 a multiple sequence alignment between these sequences and the structurally well-characterized AIM-1 [19], L1 [15] and SMB-1 [18] was carried out (**Figure 4**).

Importantly, the six amino acids that form the metal ion binding site are also invariant in both MIMs (*i.e.* His116, His118 and His196 in the Zn1 site and Asp120, His121 and His263 in the Zn2 site; see also **Figure 3**). Other amino acid side chains that were identified as important in MBL function are well conserved, including those in positions 221 (Ser) and 223 (Ser/Thr) that line the pocket where the β-lactam substrate may bind [5]. Tyr228, a residue that aids the polarization of the β-lactam carbonyl oxygen as a means to increase the susceptibility of the carbonyl bond for a nucleophilic attack by the “bridging” hydroxide is conserved in all MBLs compared here except AIM-1 (**Figure 4**). Conserved is also Trp39, another residue that has been shown to play an important role in substrate binding [24]. Furthermore, in AIM-1 and to a large extent SMB-1 (but not L1) the structure of the enzyme is stabilized by the presence of three disulfide bridges (*i.e.* the pairs Cys32-Cys66, Cys208-Cys213 and Cys256-Cys290 [18,19]). These six cysteine residues are conserved in both MIM-1 and

Table 1. Pairwise sequence comparisons between MBL-like sequences from *N. pentaromativorans* (MIM-1; see text for details) and *S. agarivorans* (MIM-2) and selected MBLs from the B1 (NDM-1), B2 (CphA) and B3 (AIM-1, L1, SMB-1) subgroups.

	MBL	Identity (%)	Homology (%)
MIM-1	AIM-1	53	65
	L1	38	54
	SMB-1	41	58
	NDM1	26	39
	CphA	23	42
MIM-2	MIM-1	47	63
	AIM-1	47	64
	L1	33	51
	SMB-1	43	63
	NDM-1	37	59
	CphA	24	44

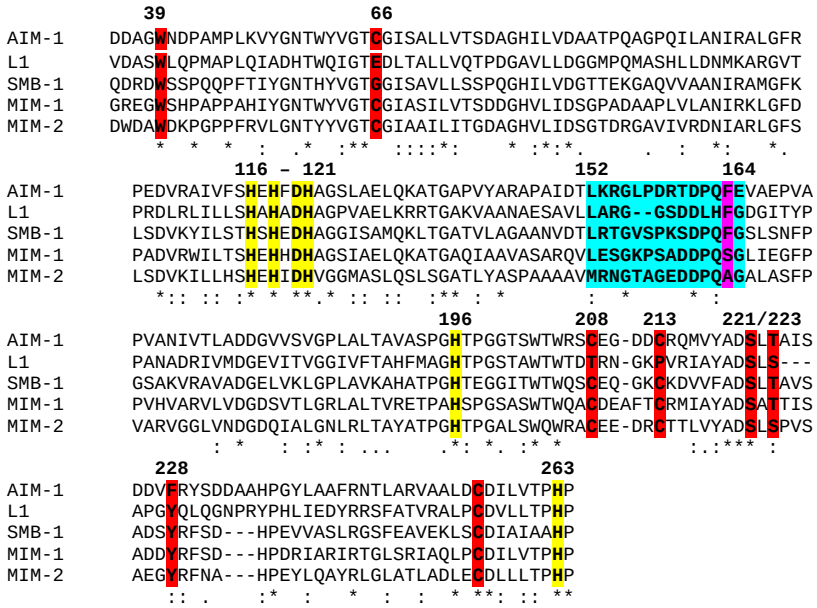

Figure 4. Multiple sequence alignment between known (AIM-1, L1 and SMB-1) and putative (MIM-1 and MIM-2) B3-type MBLs. Amino acid side chains involved in Zn^{2+} binding are shown in yellow. Other relevant residues are also indicated in color and described in the text.

MIM-2, suggesting that these enzymes possess a similar overall fold as AIM-1 and SMB-1.

Some observed sequence variations may, however, deserve mentioning here as they may be significant for differences in substrate preference, inhibitor binding and/or catalysis. The region between residues 152 and 164 forms a flexible loop that may clamp down on the bound substrate and thus assist catalysis [5]. In this loop, the degree of sequence conservation is low (**Figure 4**) which may indicate variations in substrate selection, catalytic efficiency and possibly also interactions with potential enzyme inhibitors.

While there is currently no clinically useful MBL inhibitor available, mercaptoacetates (MCRs) are known to be potent *in vitro* inhibitors of some MBLs [25]. A recent crystallographic study with SMB-1 has shown that MCR interacts with active site residues Ser221 and Thr223 and has a K_i = 9.4 ± 0.4 μM (**Figure 4**) [18]. MIM-1 and AIM-1 have a sequence identical to that of SMB-1 in this so-called "MCR binding" region, whereas L1 and MIM-2 have a Ser instead of Thr. Although this represents a conserved substitution, it may nonetheless be of significance as the different sizes of these side chains may affect the modes of substrate/inhibitor binding. Of particular interest may also be the residue in position 162, occupied by the bulky and nonpolar Phe residue in AIM-1, L1 and SMB-1, but by the small and polar Ser in MIM-1 and the small and nonpolar Ala in MIM-2. This residue lies within the flexible loop mentioned in the previous paragraph and may thus play an essential role in substrate and inhibitor binding.

It is now essential to characterize the properties of these novel B3-type MBLs to determine which of these variations are functionally relevant and in particular which of those may affect the mode and magnitude of inhibition by known inhibitors (such as MCR). Ultimately, it is anticipated that the functional and structural comparison of a multitude of related MBLs will identify residues that are suitable targets to develop universally applicable inhibitors that may be resistant to frequent mutational changes characteristic of this family of enzymes.

3.3. Conclusion

The main finding of this study is the identification of two novel MBLs from *N. pentaromativorans* (MIM-1) and *S. agarivorans* (MIM-2) that belong to the B3 sub-group of this family of enzymes. Both proteins containing the amino acid ligands necessary to bind two zinc ions in their active sites and various residues in the vicinity of the catalytic center are invariant or highly conserved, indicating that MIM-1 and MIM-2 should be efficient catalysts for the hydrolysis of *β*-lactam antibiotics. While MIM-1 and MIM-2 are not expected to represent an immediate threat to human health, they may harbor information that is crucial for 1) our understanding of the reaction mechanism(s) MBLs may employ; and 2) the development of universal MBL inhibitors that are resistant to frequent mutational variations observed among members of this family of enzymes. This study is an initial step towards the characterization of these novel MBLs. Steps toward

their recombinant expression and purification, as well as their catalytic and structural characterization are currently in progress. Importantly, we and others have developed an arsenal of *in vitro* inhibitors, mainly against B1-type MBLs [26], and these compounds will be tested for their effects against MIM-1 and MIM-2. It is hoped that in due course a universal inhibitor may emerge from these and related studies to combat successfully the threat of antibiotic resistance which poses an immediate threat to global health.

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