Identification and lead-in characterization of novel B3 metallo-*β*-lactamases

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Abstract: Metallo-*β*-lactamases (MBLs) are zinc ion dependent enzymes that are responsible for the emergence and spread of *β*-lactam resistance among bacterial pathogens. There are uncharacterized putative MBLs in the environment and their emergence is major interference in the generation of universal MBL inhibitors so it is important to identify and characterize novel MBLs. In this study two novel MBLs from *Luteimonas sp. J29* and *Pseudoxanthomonas mexicana* were identified using B3 MBLs as query in BLAST database search. 3D models of putative MBLs generated by SWISS-MODEL server taking AIM-1 as a structural template were verified using web based structure assessment and validation programs. Multiple sequence alignment revealed that residues important for substrate binding were conserved and loop region residues (156-162 and 223-230) important for catalysis are variable in these novel MBLs. Homology models showed typical MBL *α*/*β*/*β*/*α* sandwich fold containing six *α* helices, twelve *β* strands and metal interacting residues are conserved in similar way as with other B3 MBLs. We report promising putative B3 MBLs with some variations and substrate docking studies revealed that novel MBLs have attributes close to acquired B3 MBLs.

Keywords: *β*-lactam antibiotics; *Luteimonas sp. J29*; *Pseudoxanthomonas mexicana*; homology modeling.

INTRODUCTION

Accession of *β*-lactamases is the most common mechanism of resistance against *β*-lactam antibiotics in both gram positive and gram negative bacteria to hydrolyze *β*-lactams by cleavage of the amide bond of *β*-lactam ring (Babic *et al*., 2006). *β*-lactamases are molecularly classified on the basis of amino acid sequence and divided into class A, C and D enzymes which employ serine for substrate hydrolysis and class B enzymes, metallo-*β*-lactamases (MBLs), which need divalent zinc ions for activity whereas functional classification categorize these enzymes on the basis of selective resistance or substrate profile (Bush and Jacoby, 2010). Sequence characteristics have resulted in establishment of MBL subclasses B1, B2, B3 and B4. B1 and B3 are dizinc enzymes and possess broad substrate specificity while B2 is monozinc with narrow substrate spectrum and B4 mononuclear in the absence of substrates. The group is structurally identical and characterized by *α*/*β*/*β*/*α* sandwich fold where upto six residues can coordinate zinc ions at active sites (Phelan *et al*., 2014).

Members of B1, among others, include IMP-1 from *Pseudomonas aeruginosa* and NDM-1 from *Klebsiella pneumonia* (Concha *et al*., 2000; Yong *et al*., 2009). Examples of B2 subgroup include CphA from *Aeromonas hydrophila* and Sfh-I from *Serratia fonticola* (Segatore *et al*., 1993; Fonseca *et al*., 2011). SMB-1 from *Serratia marcesens*, THIN-B from *Janthinobacterium lividum* and L1 from *Stenotrophomonas maltophilia* are some representatives of B3 subgroup (Wachino *et al*., 2013; Docquier *et al*., 2004; Costello *et al*., 2006). B4 subgroup is represented by SPR-1 from *Serratia proteamaculans* (Phelan *et al*., 2014). AIM-1 (Adelaide IMipenemase-1) from *Pseudomonas aeruginosa* was the first mobile enzyme from B3 subgroup that was found in the chromosome and flanked by two copies of IS*CR* elements that are implicated in the mobilisation of three MBL genes. AIM-1 was the first mobile enzyme from B3 subgroup which might accelerate the spread of these resistance genes among clinically opportunistic pathogens (Yong *et al*., 2009; Toleman *et al*., 2002; Yong *et al*., 2012).

As there is prompt exposure of antibiotic resistant factors and the emergence of novel MBLs is the obstacle in the generation of clinically useful MBL inhibitors so it is important to identify and characterize them before emergence. In the present study we focus on B3 subgroup as they are less well studied and have a broad substrate spectrum. Putative MBLs were identified by protein database search and their 3D structural models were built. Further, enzyme ligand interactions were identified by docking Imipenem to putative MBL receptors. In depth structural insights of these novel MBLs might facilitate design of future universal MBL inhibitors.

MATERIALS AND METHODS

Protein database search using BLAST

The entire sequences of AIM-1 (accession: 4AWY) and SMB-1 (accession: BAL14456.1) were taken from NCBI

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protein database as query sequences. The protein Basic Local Alignment Search Tool (BLAST) was used to recognize potential MBLs (data not showed).

Multiple sequence alignments

The sequence alignment between novel MBLs and other important B3 MBLs (AIM-1, L1 and SMB-1) was performed by CLUSTAL OMEGA program version 1.2.1 (available from http://www.ebi.ac.uk/Tools/msa/clustalo) with default settings and the alignment was edited using ESPript3.0(http://espript.ibcp.fr/ESPript/ESPript/index.php) (Sievers *et al*., 2011). The phylogenetic tree was built using the MEGA6 software by Maximum Likelihood model (1000 iterations were used to test analysis) (Tamura *et al*., 2013).

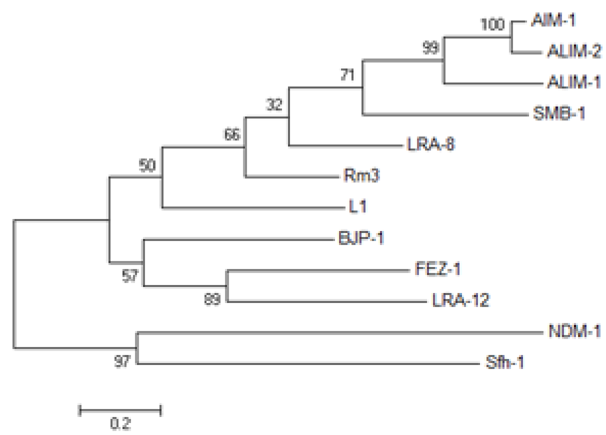

Fig. 1: Phylogenetic Tree of Selected Metallo-*β*-Lactamases. The analysis was conducted using the Neighbor-joining tree model, based on the amino acid sequences, using the MEGA6 software (1000 bootstrap replicates).

Structural homology modeling and docking studies

AIM-1 was selected as a structural template to generate 3D models of novel MBLs by SWISS-MODEL server (http://swissmodel.expasy.org) using automated mode. 3D models were endorsed by PROCHECK, ERRAT and VERIFY 3D programs available from the Structural Analysis and Verification Server (SAVES) (http://nihserver.mbi.ucla.edu/SAVES) and SWISS-MODEL structure assessment tool (http://swissmodel.expasy.org) (Laskowski *et al*., 1993; Colovos and Yeates, 1993; Bowie *et al*., 1991). Molecular docking studies were carried out to get the binding interaction mode with Imipenem using Autodock 4.2. Lamarckian Genetic Algorithm (LGA) was implemented for docking simulation and conformational search (Rizvi *et al*., 2013).

RESULTS

Protein database search and identification of novel MBLs

By using BLAST two novel proteins, *Luteimonas sp. J29* (accession code: WP_051322755) and *Pseudoxanthomonas mexicana* (accession code: WP_062355367), were identified as putative MBLs. Sequence identity of MBL from *Luteimonas sp. J29* with B3 subgroup is high *i.e.* with AIM-1, L1 and SMB1 is 63%, 48% and 49% respectively. Sequence comparison with NDM-1 (B1- MBL) and CphA (B2-MBL) is 29% and 25% respectively. Sequence identity of MBL from *Pseudoxanthomonas mexicana* with AIM-1, L1, SMB1, NDM-1 and CphA is 85%, 38%, 44%, 28% and 24% respectively. Sequence based alignment and phylogenetic analysis showed that these novel MBLs closely related to B3 MBLs (fig. 1).

Table 1: Substrate interacting residues (according to BBL numbering)

	Substrate interacting residues
ALIM-1	Trp38,His116,His118,Asp120,His121,Gln157, His196,Thr223,Ser265,His263,Ala266
ALIM-2	Trp38,His118,Asp120,Gln157,His196,Ser221, Leu222,Thr223,His263,lle225,Ala266,Tyr293

These results strongly suggest to classify novel MBL like proteins to B3 subgroup. For convenience, MBLs from *Luteimonas sp. J29* and *Pseudoxanthomonas mexicana* were named AIM-1 Like Imipenemase-1 (ALIM-1) and AIM-1 Like Imipenemase-2 (ALIM-2) respectively because of higher sequence similarity with AIM-1. Both MBLs shows significant resemblance (59% for ALIM-1 and 83% for ALIM-2) with putative B3 MBL from uncultured bacteria (accession code: AMP57086.1) from wastewater metagenome environmental sample. They also shows resemblance to protein (accession code: OHE90088.1) identified from environment metagenome by Xanthomonadales bacterium (63% for ALIM-1 and 82% for ALIM-2). Interestingly phylogenetic analysis revealed that novel MBLs, in addition to AIM-1 and SMB-1, also closely related to Rm3 MBL isolated from metagenomic environmental sampling and belongs to the clade of B3 MBLs differs from BJP-1 and FEZ-1 group (fig. 1) (Salimraj *et al*., 2016).

Attributes of novel MBLs

BBL numbering is used to specify important amino acids in this paper (Garau*et al*., 2004). Primary metal coordinating residues for B3 MBLs His116, His118, Asp120, His121, His196, and His263 are conserved in ALIM-1 and ALIM-2 (fig. 2). Other residues important for substrate binding, orientation and inhibitor (mercaptoacetate) interaction (Trp38, Ser221 and Thr223) are also conserved in both MBLs (Wachino *et al*., 2013). The loop region important for substrate interaction spanning residues 156-166 is less variable and more like AIM-1 in case of ALIM-2 whereas in ALIM-1 there is high variability. Active site might be more defined and influence substrate orientation in case of ALIM-2 (Ile at position 225) as compared to ALIM-1 (Val at position 225) (Leiros *et al*., 2012). In ALIM-1 Val160 is

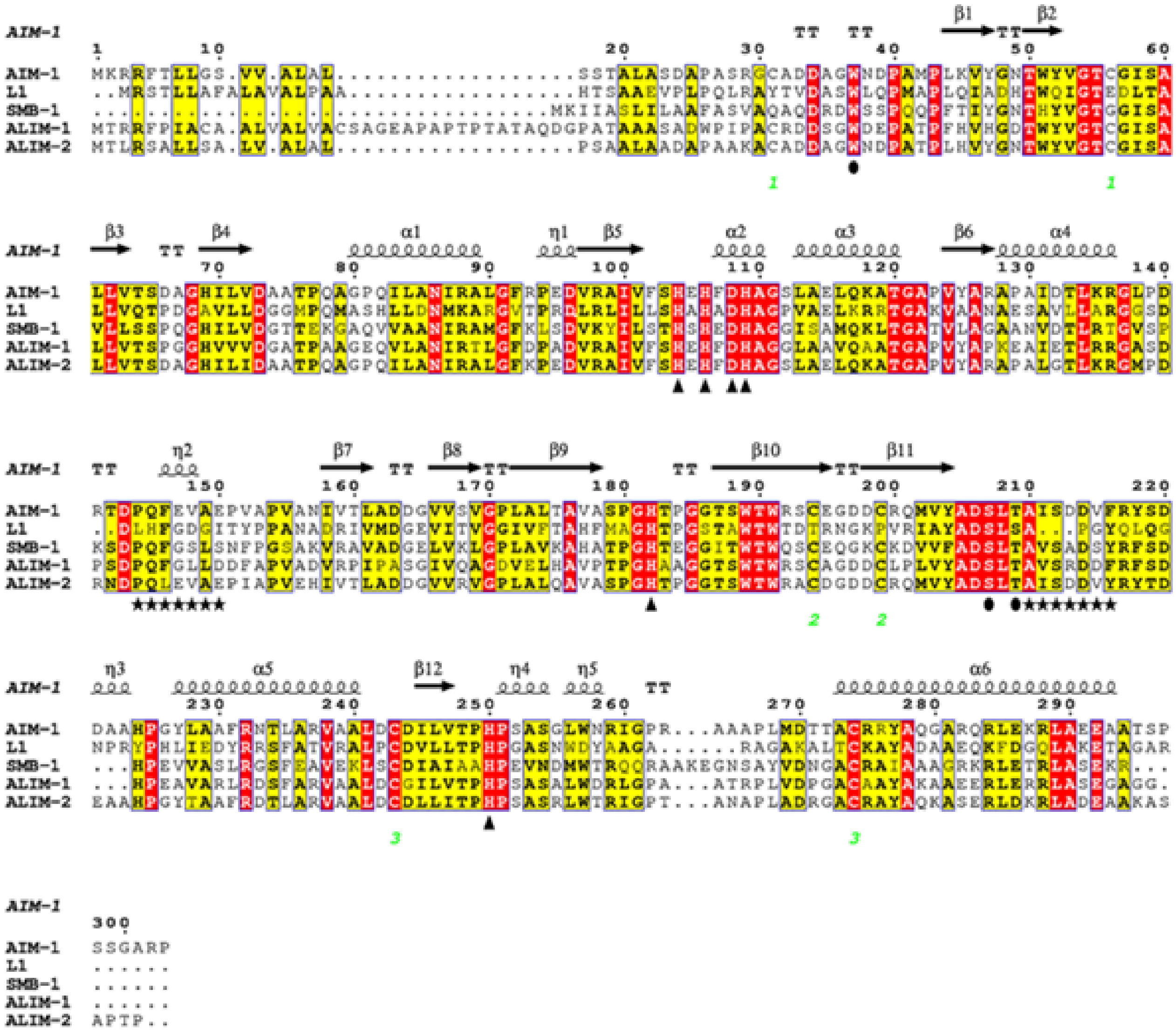

Fig. 2: Multiple Sequence Alignment of ALIM-1 and ALIM-2 with B3 MBLs (AIM-1, L1 and SMB-1). Secondary structure elements of AIM-1 also included. Primary zinc binding ligands (His116, His118, Asp120, His121, His196, and His263) are indicated by triangles. Residues important for substrate binding (Trp38, Ser221 and T223) are marked by circle. Important loop regions (156-162 and 224-230) are represented by star. The figure was made with ESPript.

substituted with charged residue Lys whereas Glu is replaced by Asp at position 162 and there is Val at position 225 (fig. 2). In case of ALIM-2 the bulky hydrophobic residue Phe at position 158 which is conserved in AIM-1, L1, SMB-1 and ALIM-1 is replaced by Lys.

Homology-modeled 3D structures of ALIM-1 and ALIM-2

The sequences of ALIM-1 and ALIM-2 were taken from NCBI Protein database and the sequence alignment between novel MBLs and AIM-1 (based on higher sequence similarity) was performed by SWISS-MODEL server. The resulted overall structure of both ALIM-1 and ALIM-2 is dimer, each chain consist of six α helices, twelve β strands and ~ five 3_{10} helices. The quality of the 3D model was evaluated via the Ramachandran plot using PROCHECK software where 88.4% of residues were in the most favorable region for ALIM-1 and 86.9% for ALIM-2, while 11.2% and 12.2% were in the allowed region for ALIM-1 and ALIM-2 respectively, validating predicted models are of good quality. For homology models, the overall quality factor predicted by the ERRAT server was 88.6 for ALIM-1 and 94.5 for ALIM-2. As predicted by Verify 3D 99.24% and 92.96% of the residues in model had an average 3D-1D score >0.2 for ALIM-1 and ALIM-2 respectively, thus verifying the models. For model reliability prediction the tool of

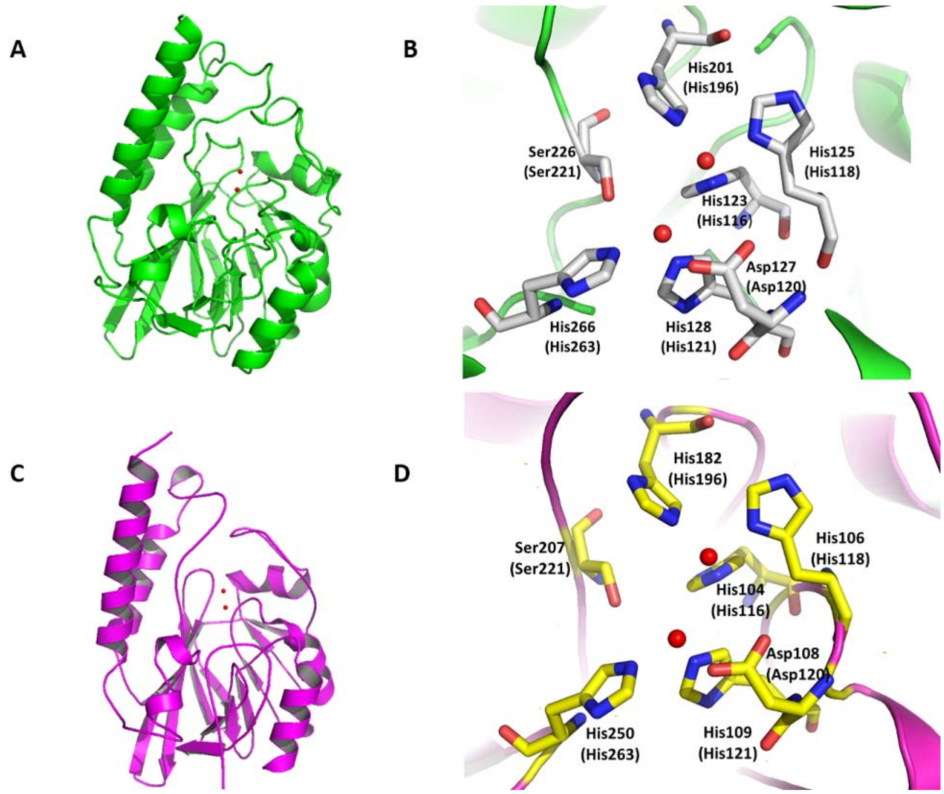

Fig. 3: Illustration to show (A) homology modeled 3D structure of ALIM-1 its (B) zinc coordinates, and (C) homology modeled 3D structure of ALIM-2 its (D) zinc coordinates at metal center. (Spheres indicate Zinc and residues in brackets numbered according to BBL numbering).

QMEAN (Qualitative Model Energy ANalysis) was used, the QMEAN score for the ALIM-1 and ALIM-2 computational models is 0.78 and 0.84 respectively and the density plots of QMEAN score are also computed. Z-score was also computed in comparison with the average X-ray structures to estimate the absolute quality of the models; Z score for ALIM-1 and ALIM-2 is 0.29 and 0.98 respectively. Based on these analyses, per-residue error plots further were examined to determine if potentially unreliable regions present. Both homology models confirmed the presence of typical MBL *α/β/β/α* fold and primary metal coordinates similar to subgroup B3 metal center (fig. 3).

Docking of imipenem at active site

After docking conformations showing best down binding energy were selected for analysis. The binding site pocket of ALIM-1 and ALIM-2 (fig. 4) was formed by eleven and twelve residues respectively as shown in table 1. Residues Thr223 and Ser221were found to be involved in carbapenem interaction at active site in other acquired B3 MBLs whereas involvement of Ser265 in case of ALIM-1 is unusual (Wachino *et al*., 2016).

DISCUSSION

We have identified putative B3 MBLs from *Luteimonas sp. J29* and *Pseudoxanthomonas mexicana.* Both organisms are Gram-negative, little is known about *Luteimonas sp. J29* as DNA genome sequence of *Luteimonas sp. J29* has been determined recently (NCBI Accession: PRJNA184586) whereas *Pseudoxanthomonas mexicana* has been isolated from diverse environments (human urine, riverside urban soil and anaerobic digester) (Thierry *et al*., 2004). In context of environment decontamination applications of *Pseudoxanthomonas mexicana* described by Singh *et al*., 2015, presence of potential MBL can pose future problem because of rapid spread of antibiotic resistance. After homology modeling, docking studies revealed substrate interacting residues. One important thing is at binding pocket unique amino acid residue Gln157 is present, which is found only in horizontally acquired B3 MBLs (AIM-1 and SMB-1) and thought to play an important role in substrate recognition in AIM-1 and SMB-1 as described by Leiros *et al*., 2012 and Wachino *et al*., 2013; in this context the possibility of these novel proteins as acquired B3 MBLs cannot be excluded. It was found that mode of interaction with substrate is quite similar to acquired B3 MBLs where ALIM-2 is more close to AIM-1 with the involvement of bulky residue Ile225 at active site. In order to get insights into the catalytic mechanisms next step will be their recombinant expression and kinetic studies. It is expected that information gained from this study in combination with other findings will be useful in the design of

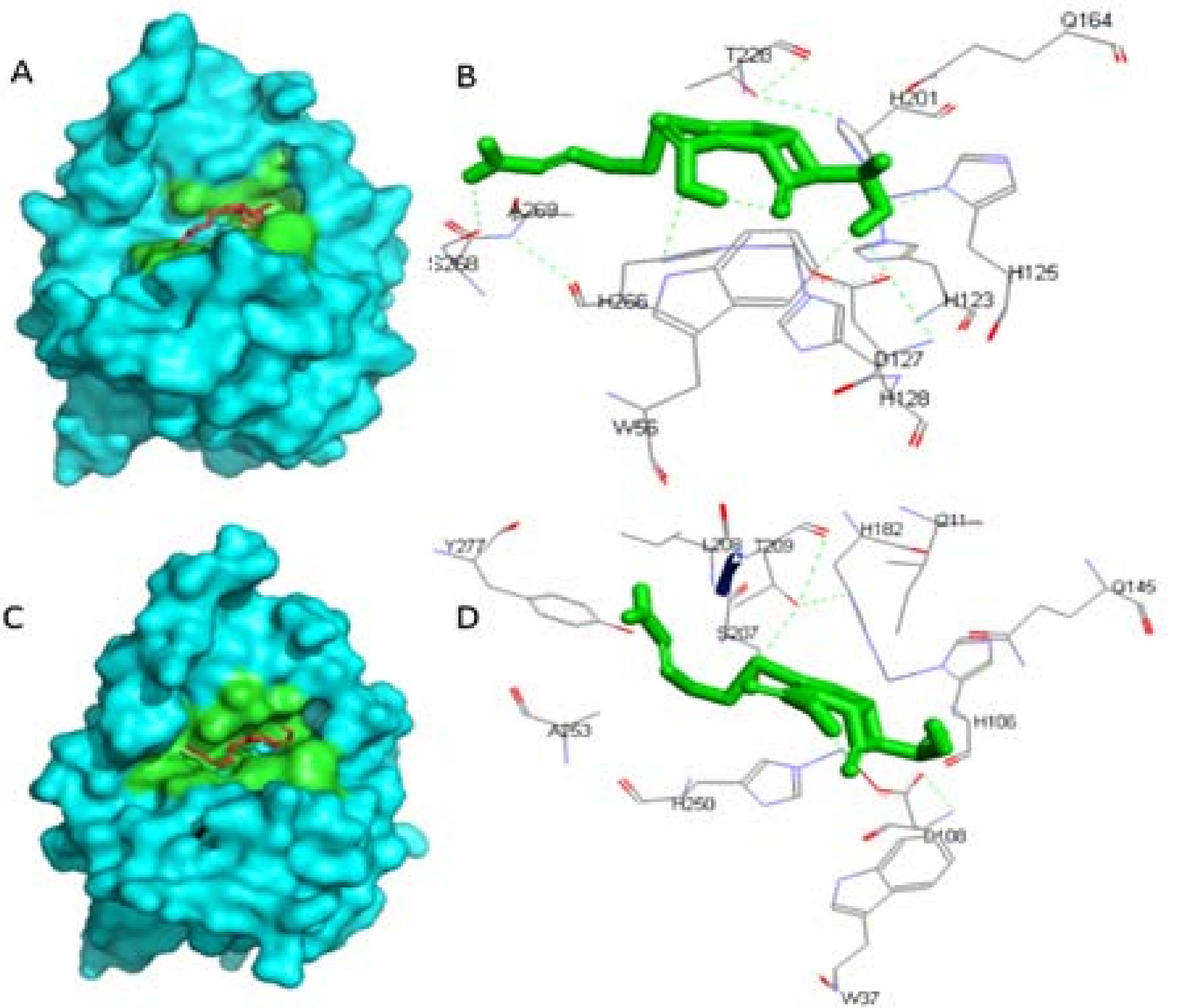

Fig. 4: Substrate Docking with ALIM-1 and ALIM-2. Views of Imipenem (red) docked at active site of ALIM-1 (A) and ALIM-2 (C) where green surface showed substrate interacting residues. Substrate interaction explained for ALIM-1(B) and ALIM-2 (D).

inhibitors that could be used as anti-bacterial agents in future.

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