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STRUCTURE AND FUNCTIONS OF A METALLO-BETA-LACTAMASE LIKE HYPOTHETICAL PROTEIN Bleg1_2437 FROM ALKALI-TOLERANT SOIL BACTERIUM Bacillus lehensis G1

TAN SOO HUEI

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfilment of the requirement for the degree of Master of Science

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β-lactam antibiotics are the most useful chemotherapeutic agents in the treatment of diseases of bacterial origin. However, the emergence of antibiotic resistance mechanism among pathogenic bacteria has downsized the efficacy of antibiotics via the production metallo-β-lactamase (MBL) which enables pathogens to destroy the β-lactam ring of β-lactam antibiotics. Although MBL is not ubiquitously found in all pathogenic bacteria, its presence is a public health concern. In addition, the presence of unknown and uncharacterised MBL in the environment signal the possible emergence of ―sidntò superbug. Therefore, the study aimed to identify a functional cluster of Hypothetical proteins (HPs) and screen the HP pool for the presence of MBL domain from the locally isolated *Bacillus lehensis* G1 via *in silico* prediction. Furthermore, predicts the structure and function of the selected HP and characterise the antibiotic-degrading ability of the selected HP. In the present study, there are 1202 hypothetical proteins (HPs) have been discovered from newly sequenced genome of alkali-tolerant soil bacterium *B. lehensis* G1, HP Bleg1_2437 may likely be an MBL. Domain and sequence analysis of HP Bleg1_2437 using InterProScan and DELTA-BLAST revealed that this 23 kDa protein contains highly conserved metal-binding residues such as H54, H56, D58, H59, H131 and H191 that are similar with the those in subclass B3 of MBL that are involved in the coordination of two Zn^{2+} ions. The three-dimensional protein structure of Bleg1_2437 built using Modeller 9v10 exhibited an αββα sandwich layer similar to the well conserved global topology of MBL superfamily. Docking of several β-lactam antibiotics to the predicted structure of Bleg1_2437 using Maestro v9.3 revealed that the antibiotics interact with residues in the binding pocket of Bleg1_2437 such as the Zn^{2+} binding residues mentioned above, hydrophobic residues such as I10, Y15, F153, I157 and G158, as well as polar residues such as Q11, T12, N13, D150 and S156 with significant binding energy. The ORF of Bleg1_2437 with approximately 633 nucleotides was amplified by PCR and cloned into pET-32(b) to form pET-32(b)::*Bleg1_2437* recombinant plasmid. This recombinant plasmid was transformed into *Escherichia coli* Rosetta-gami (DE3) for Bleg1_2437 protein expression and purification. The optimum condition for intracellular expression of Bleg1_2437 was achieved at 20 ºC, with Isopropyl-β-D-Thiogalactopyranoside (IPTG) concentration of 0.1 mM and incubated for 18 hours. The crude extract of Bleg1_2437 was purified through Hi-Trap Sepharose affinity chromatography.The yield of purified recombinant

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Bleg1_2437 with protein tags (Trx-tag + S-tag + His-tag) was about 22 %. It displayed hydrolysis activity towards several β-lactam antibiotics with preference towards ampicillin. It exhibited the highest catalytic efficiency k_{cat}/K_m (86.4 $\mu M^{-1}s^{-1}$) towards ampicillin compared with meropenem (16.9 $\mu M^{-1}s^{-1}$) and nitrocefin (5.0 $\mu M^{-1}s^{-1}$). Furthermore, with the addition of 100 μM of zinc sulfate, the k_{cat}/K_m values of Bleg1_2437 towards all tested β-lactam antibiotics were increased approximately 2 to 5 fold. The findings of this study reveal on the presence of the clinically important and dangerous antibiotics-degrading enzyme, MBL, within the pool of HPs which are generally regarded as proteins of unknown functions and of no particular importance.

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KAJIAN STRUKTUR DAN FUNGSI PROTEIN HIPOTETIKAL Bleg1_2437 SEAKAN METALLO-BETA-LACTAMASE DARI BAKTERIA TANAH ALKALI-TOLERAN *Bacillus lehensis* G1

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Antibiotik β-laktam merupakan salah satu agen kemoterapi yang paling berguna dalam rawatan jangkitan bakteria. Walaubagaimanapun, kemunculan mekanisme kerintangan antibiotik di kalangan bakteria patogenik telah mengurangkan keupayaan antibiotik melalui penghasilan ―metallo-β-lactamaseò (MBL) yang berupaya menguraikan antibiotik β-laktam. Walaupun MBL tidak kerap dijumpai di dalam semua bakteria patogenik, kehadirannya merupakan suatu kebimbangan untuk kesihatan awam sejagat. Selain itu, kewujudan MBL yang tanpa diketahui dalam alam sekitar telah mengakibatkan kemunculan ―superbugò. Oleh itu, objektif untuk penyelidikan ini adalah mengidentifikasikan kluster protein yang fungsinya tidak diketahui (HPs) mengikut fungsi-fungsinya dan menyaringkan HP yang berciri MBL di kalangan kluster HP daripada genom *B. lehensis* G1. Seterusnya, struktur dan fungsi HP yang dipilih akan diramalkan dan keupayaan penguraian antibiotik HP tersebut akan diuji. Dalam penyelidikan ini, sebanyak 1202 HP telah ditemui daripada genom bakteria yang baru dijujuk, iaitu bakteria tanah alkali-toleran ―*Bacillus lehensis* G1ò, HP bersiri Bleg1_2437 dijangka merupakan suatu MBL. Analisis ―domainò dan jujukan HP Bleg1_2437 dengan menggunakan INTERPROSCAN dan DELTA-BLAST, mendedahkan bahawa protein 23 kDa ini mengandungi residu-residu pengikatan logam yang sangat terpelihara seperti H54, H56, D58, H59, H131 dan H191 sama seperti sub-klas B3 MBL yang terlibat dalam koordinasi dua ion Zn^{2+}. Struktur protein tiga dimensi Bleg1_2437 yang dibina dengan Modeller 9v10 menunjukkan lapisan ―sandwichò αββα sama seperti topologi global terpelihara kepunyaan superfamily MBL. Penetapan beberapa antibiotik β-laktam pada struktur ramalan Bleg_2437 menggunakan Maestro v9.3 mendedahkan bahawa antibiotik tersebut berinteraksi dengan residu-residu di dalam poket pengikatan Bleg1_2437 seperti residu-residu pengikat ion Zn^{2+} yang tersebut di atas, residu-residu hidrofobik seperti I10, Y15, F153, I157 dan G158, residu-residu polar seperti Q11, T12, N13, D150 dan S156 dengan menghasilkan tenaga pengikatan yang keertian. Rangka bacaan terbuka (ORF) bagi Bleg1_2437 sepanjang 633 nukleotide telah diamplifikasi dengan PCR dan diklonkan ke dalam vektor pET-32(b) untuk membentuk pET-32(b)::*Bleg1_2437* plasmid rekombinan. Plasmid ini dieskpres dalam *Escherichia coli* Rosetta-gami (DE3) secara intrasel. Keadaan ekspresi optimum untuk Bleg1_2437 dicapai pada suhu 20 ºC, kepekatan IPTG 0.1 mM dan jangkamasa pengkulturan selama 18 jam. Enzim kasar

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yang didapati daripada eskpresi intrasel ditulenkan melalui kromatografi afiniti –Hi-Trap Sepharoseò. Proses penulenan dapat mengahsilkan Bleg1_2437 sebanyak 22.0 % yang mengandungi peptide isyarat (Trx-tag + S-tag + His-tag). Bleg1_2437 yang telah ditulenkan terbukti menunjukkan aktiviti hidrolase terhadap beberapa β-lactam dengan keutamaan terhadap ampisilin. Ia menunjukkan keupayaan katalitik yang paling tinggi k_{cat}/K_m (86.4 $\mu M^{-1}s^{-1}$) berbanding dengan meropenem (16.9 $\mu M^{-1}s^{-1}$) dan nitrocefin (5.0 $\mu M^{-1}s^{-1}$). Selain itu, dengan penambahan 100 μM zink sulfate, nilai k_{cat}/K_m Bleg1_2437 terhadap semua β-laktam yang diuji telah meningkat sebanyak 2 hingga 5 kali ganda. Penemuan penyelidikan ini mendedahkan bahawa kehadiran enzim pengurai-antibiotik, MBL yang penting secara klinikal dan merbahaya di kalangan kumpulan HP yang selalu dianggap sebagai protein yang tidak diketahui fungsinya dan tidak menpunyai kepentingan tertentu.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

Appr1p	ADP-ribose 1''-2'' cyclic phosphate
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
B-CASP	Beta C-terminal Artemis SNM1 and PSO2
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CA-MRSA	Community-Acquired Methicillin Resistant *Staphylococcus aureus*
CDC	Center for Disease Control and Prevention
CRE	Carbapenem Resistant Enterobacteriacae
DAP	Diamino Pimelic Acid
DOPE	Discrete Optimized Protein Energy
DSF	Differential Scanning Fluorimetry
DSLS	Differential Static Light Scattering
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Center for Disease Control and Prevention
EDTA	Ethylene Diamine Tetra Acetic acid
ESBL	Extended Spectrum Beta-Lactamase
E-value	Expectation value
FASTA	FAST-All
GRAVY	Grand Average of Hydrophobicity
HA-MRSA	Hospital-Acquired Methicillin Resistant *Staphylococcus aureus*
HMM	Hidden Markov Model
HP	Hypothetical Protein
IMP	Imipenemase
IPTG	Isopropyl-β-D-Thiogalactopyranoside
I-TASSER	Iterative Threading Assembly Refinement
ITC	Isothermal Titration Calorimetry
kDa	Kilodalton
LB	Lysogeny Broth
MBL	Metallo-β-lactamase
MBLED	Metallo-β-lactamase Engineered Database
MDR	Multidrug Resistance

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MSA	Multiple Sequence Alignment
MWCO	Molecular Weight Cut Off
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
NBT	Nitro Blue Tetrazolium
NCBI CDD	National Center of Biotechnology Information Conserved Domain Database
NDM-1	New Delhi Metallo-β-lactamase-1
Ni-NTA	Nickel Nitrilotriacetic acid
NMR	Nuclear Magnetic Resonance
NSAR	National Surveillance of Antibiotic Resistance
OD	Optical Density
PFAM	Protein Families
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PHYLIP	Phylogeny Inference Package
PNPP	Paranitrophenylphosphate
PSI-BLAST	Position Specific Interative-BLAST
PSSMs	Position Specific Score Matrices
RMSD	Root Mean Square Deviation
SBL	Serine-β-lactamase
SDM	Site Directed Mutagenesis
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TAE	Tris Acetate EDTA
TBS	Transfer Buffer Saline
TBSTT	Transfer Buffer Saline with Triton X-100 and Tween-20
TEMED	Tetramethylethylenediamine
v/v	Volume per volume
VIM	Verona Integron-encoded Metallo-β-lactamase

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VRE	Vancomycin Resistant *Enterococci*
w/v	Weight per volume
Wat1	Water molecule 1
Wat2	Water molecule 2
WHO	World Health Organization
ZN1	Zinc 1
ZN2	Zinc 2

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CHAPTER 1

INTRODUCTION

β-lactam antibiotics are the first line of antimicrobial chemotherapeutic agents for treatment of diseases of bacterial origin due to their ability to inhibit the synthesis of specific transpeptidases involved in peptidoglycan biosynthesis (Fabiane et al., 1998; Wilke et al., 2005). They do not have functional and structural counterpart in human and hence have minimal side effects to the patients. Their low cost of production enables them to be widely used in the health care industry (Wilke et al., 2005). However, the emergence of antibiotic resistance mechanism among pathogenic bacteria has downsized the efficiency of antibiotics, thus requiring the usage of an arsenal of antibiotics whilst patients are besieged by infections by pathogens (Wang et al., 1999). Such situation is made graver with the ability of pathogens acquiring resistance within a few years of new antibiotic usage in a hospital setting (Schmieder and Edwards, 2012) .

One of the most powerful weapons used by pathogens to counter the efficiency of β-lactam antibiotics is through the production of β-lactamases. β-lactamases act by degrading the amide bond of β-lactam ring in the antibiotics. This enzyme can be categorised into four groups: classes A, B, C and D (Ambler et al., 1991). Classes A, C and D β-lactamases, also known as serine-β-lactamases (SBLs), employ an active serine residue as a nucleophile to attack the carbonyl carbon of the β-lactam ring covalently. While class B β-lactamases, also known as metallo-beta-lactamases (MBLs), use one or two zinc ions for the inactivation of β-lactam antibiotics (Bebrone, 2007; Crowder et al., 2006). Based on the number of zinc ions utilized by these MBLs, they can be further categorised into three subclasses, B1, B2 and B3. B1 and B3 MBLs need two zinc ions to stabilise the hydroxide ion which acts as nucleophile to attack the β-lactam ring (Palzkill, 2013). B2 MBLs only require a single zinc ion for their activity (Bebrone, 2007). Compared to SBLs, MBLs are more dangerous because they can hydrolyse almost all types of β-lactams, with the exception of monobactam antibiotic. In addition to this, MBLs cannot be inhibited with clinical inhibitors such as clavulanate and tazobactam which are effective against SBLs.

Therefore MBLs have a wider antibiotic degradation spectrum and this impose a serious threat to public health (Bebrone et al., 2009; Palzkill, 2013).

To add to the gravity of this situation, the knowledge on the distribution and diversity of resistance genes from environmental bacteria is somewhat limited (Schmieder and Edwards, 2012). Most of the known chromosomal MBLs are produced by soil microorganisms outside clinical settings. Examples include BcII, CphA, and L1 resident MBLs originating from *Bacillus cereus*, *Aeromonas* spp and *Stenotrophomonas maltophilia* soil bacteria respectively (Crowder et al., 1998; Fabiane et al., 1998; Garau et al., 2005). This indicates that soil environment can act as a reservoir in mobilising MBLs among the soil consortium (Aminov, 2009). In addition to soil, MBL-producing strains have been found in drinking water and sewage as well. One example is the most recently discovered *Klebsiella pneumoniae* strain, which produces the wide-spectrum New Delhi MBL-1 (NDM-1) in India (Walsh et al., 2011) . Findings such as these suggest that the

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antibiotic resistance threat is circulating in environmental microorganisms that normally are non-pathogenic.

More in-depth knowledge on currently existing MBLs is crucial in the fight against the persistence of bacterial pathogens which are able to withstand a wide spectrum of antibiotics. Equally crucial is the hunt for unknown, uncharacterised MBLs to predict and prepare the possible emergence of –silent‖ superbugs. It is important to note that 30% of genes in any sequenced genome code for –orphan‖ proteins with unknown functions, due to their low sequence and structural similarity to well-characterised proteins (Galperin and Koonin, 2004). These proteins, generally known as hypothetical proteins (HPs) are often omitted due to their dissimilarity to well-characterised proteins. In the pursuit of finding possible unknown and uncharacterised MBLs, HPs provide the best pool of proteins for such a quest. Some significant examples include CAU-1 and BJP-1 HPs from *Caulobacter crescentus* and *Bradyrhizobium japonicum* which have been reported to share sequence identity to B3 MBLs and exhibited β-lactam hydrolysis activity (Docquier et al., 2002; Stoczko et al., 2006).

As the threat of uncharacterised MBLs proved to be an important but constantly ignored determinant in rising antibiotic-resistance worldwide, the quest to search and characterise MBL-like proteins within the pool of HPs from local isolates become crucial. Hence, the present study aimed to:

1. identify a functional cluster of HPs of the locally isolated *Bacillus lehensis* G1 based on *in silico* prediction
2. screen the HP pool for the presence of MBL domain
3. predict the structure, function and possible mechanism of the selected HP
4. biochemically characterise the antibiotic-degrading ability of the selected HP

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