



CLONING, SEQUENCE ANALYSIS, AND CHARACTERIZATION OF PUTATIVE BETA-LACTAMASE OF *STENOTROPHOMONAS MALTOPHILIA*

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ABSTRACT

The main objective of current study was to explore the function of chromosomal putative beta-lactamase gene (smlt 0115) in clinical *Stenotrophomonas maltophilia*. Antibiotic susceptibility test (AST) screening for current antimicrobial drugs was done and Minimum Inhibitory Concentration (MIC) level towards beta-lactams was determined by E-test. Putative beta-lactamase gene of *S. maltophilia* was amplified via PCR, with specific primers, then cloned into pET-15 expression plasmid and transformed into *Escherichia coli* BL21. The gene was sequenced and analyzed. The expressed protein was purified by affinity chromatography and the kinetic assay was performed. *S. maltophilia* ATCC 13637 was included in this experiment. Besides, a hospital strain which exhibited resistant to a series of beta-lactams including cefepime was identified via AST and MIC, hence it was named as S2 strain and was considered in this study. Sequencing result showed that putative beta-lactamase gene obtained from ATCC 13637 and S2 strains were predicted to have cephalosporinase activity by National Center for Biotechnology Information (NCBI) blast program. Differences in the sequences of both ATCC 13637 and S2 strains were found via ClustalW alignment software. Kinetic assay proved a cephalosporinase characteristic produced by *E. coli* BL21 clone that overexpressed the putative beta-lactamase gene cloned under the control of an external promoter. Yet, expressed protein purified from S2 strain had high catalytic activity

against beta-lactam antibiotics which was 14-fold higher than expressed protein purified from ATCC 13637 strain. This study represents the characterization analysis of putative beta-lactamase gene (smlt 0115) of *S. maltophilia*. The presence of the respective gene in the chromosome of *S. maltophilia* suggested that putative beta-lactamase gene (smlt 0115) of *S. maltophilia* plays a role in beta-lactamase resistance.

Keywords: *S. maltophilia*, putative beta-lactamase gene, beta-lactamase resistant

INTRODUCTION

Stenotrophomonas maltophilia is recognized as opportunistic nosocomial pathogen especially for immunocompromised patients since it is frequently isolated in Intensive Care Unit (ICU). Though they are omnipresent in environment particularly rhizosphere of plants root, they have been associated in the act of colonization and infection to hospitalized patients (Ryan et al., 2009). It has been reported causing wide range of disease, such as bacteremia, meningitis, pneumonia, and skin infections (Rojas et al., 2008; Ryan et al., 2009). *S. maltophilia* is intrinsically resistant to most of the current antimicrobial agents. So far, studies done by several researchers were focused on the L1 and L2 beta-lactamases, and efflux system (Zhang et al., 2000). L1 and L2 beta-lactamases were found to be responsible for most of the beta-lactams resistance (Avison et al., 2002). However, a putative beta-lactamase encoded by smlt 0115 was found in *S. maltophilia* genome as shown in National Center for Biotechnology Information (NCBI) database and it was predicted to have beta-lactam resistance activity. Thus, we decided to explore the function of putative beta-lactamase (smlt 0115) in *S. maltophilia*. In this study, the biological properties of the putative beta-lactamase in *S. maltophilia* were predicted using bioinformatics tools. We also report here the cloning and expression of the putative beta-lactamase gene in *Escherichia coli* BL21 and further characterization of the purified beta-lactamase.

MATERIAL AND METHODS

Bacterial strain, susceptibility test and MIC

The AST testing of isolates used in this study was performed by Oxoid, USA antibiotics disc (including penicillin, amoxicillin clavulanate, gentamicin, netilmicin,

piperacillin tazobactam, cefoperazone sulbactam, cefoxitin, ceftazidime, cefepime, ciprofloxacin, imipenem, meropenem, co-trimoxazole, and polymyxin-B). Besides, Minimum Inhibitory Concentration (MIC) of beta-lactams was done by E-test (AB Biodisk, Sweden). All the procedures were according to Clinical Laboratory Standards document (CLSI, 2009).

Bacterial DNA preparation, polymerase chain reaction (PCR) and putative beta-lactamase gene sequencing

Sequence of putative beta-lactamase gene of the *S. maltophilia* K279a in NCBI was used as the guideline. This search yielded the presence of a putative beta-lactamase gene with location sequence: NC_010943.1. To clone this gene, chromosomal DNA of ATCC 13637 strain and S2 strain were extracted using chromosomal DNA extraction kit (Promega, USA). Followed by PCR with the oligonucleotides forward primer (5' GGC AGC **CAT ATG** TCC CGC CCA ATG CTC 3') and reverse primer (5' CCA **CCG CTG AGC** CTA TGG CCC TGC GAT GAC 3'), amplified by Phusion High-Fidelity PCR Mastermix (Finnzymes). The PCR was performed under standard condition. After that, the PCR products were sequenced using Applied Biosystems 3130 genetic analyzer and the sequences were analysed by ClustalW software.

Cloning and expression of putative beta-lactamase gene in *E. coli* BL21

His-tag pET-15 plasmid (Novagen) and PCR products were subjected to *Nde*I and *Bln*I digestion, bold and underlined sequence in oligonucleotides are the cutting side for these restriction enzymes. Then, both cutted pET-15 plasmid and the PCR product were ligated by T4 DNA Ligase (New England Biolabs), followed by heat-shock transformation into *E. coli* BL21. Transformants selected on Luria-Bertani (LB) agar containing 100µg/ml ampicillin. One of the constructed plasmid named as pET-AT^S (carrying putative beta-lactamase gene from ATCC 13637), another named as pET-S2^R (carrying putative beta-lactamase gene from S2 strain). Isopropylthio-beta-galactoside (IPTG) was used to induce the putative beta-lactamase gene expression of the plasmid in *E. coli* BL21.

Protein extraction and purification

Bacterial proteins were extracted via freeze-thaw method. Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acylamide was run at 200V for 40 minutes to examine the efficiency of protein extraction. Western blot was done to confirm the expressed proteins were His-tagged. Primary antibody was mouse-anti-Histidine antibody; secondary antibody was rabbit-anti-mouse antibody. Substrate used in western blot was 4-chloro-1-naphthol (4CN). Then, proteins were purified via His-trap FF affinity column (GE Healthcare). Purified proteins were examined again under SDS-PAGE for purity assurance.

Kinetic assay

Concentration of purified protein was determined by Bradford assay (Bio-rad). Beta-lactams (100 μ M) was prepared in 1ml of 50mM phosphate buffer (pH7.0). 50 μ L of purified enzyme was added to initiate the reaction. Beta-lactams hydrolysis was measured as the change in absorbance at 240nm for penicillin; 260nm for cefoxitin; 257nm for ceftazidime; 265nm for cefepime in a spectrophotometer (Beckman Coulter DU730) at room temperature. The rate of enzyme hydrolysis was determined from the initial velocity over a 1-min reaction period. Hydrolysis of the substrate was estimated from the absorbancy decrease at the corresponding wavelength and was assumed to be complete after a reaction for 1-minute. The kinetic assay was repeated three times to obtain precise result. One enzyme unit was defined as the amount of beta-lactamase necessary to hydrolyse 1 μ mol substrate per minute under the assay condition. Specific activity was expressed as enzyme units per milligram protein. The data represents the average of three independent experiments. Kinetic parameters V_{max} and K_m were determined by initial velocity kinetic analysis by fitting to the Michaelis-Menton equation (Labia et al., 1973).

RESULTS

Disc diffusion method showed that *S. maltophilia* ATCC 13637 was susceptible to most of the common antibiotics used in hospital excluding imipenem and meropenem as aspected. MIC value of ceftazidime and cefepime for ATCC 13637 were quite low, which had the same reading at 0.75mg/L. Meanwhile, S2 strain was resistant to a series of beta-lactams including penicillin, amoxicillin clavulanic acid, cefoxitin, ceftazidime, and cefepime. MIC

value of ceftazidime and cefepime for S2 strain were 64mg/L and 48mg/L, respectively, which was far higher than ATCC 13637. Co-trimoxazole was used to treat the patient. The recombinant strain (pET-S2^R) also exhibited high level resistance to ceftazidime and cefepime. The control *E. coli* BL21 carrying pET-15 was susceptible to all antibiotics except ampicillin.

PCR products showed a single band with approximately 1.2kbp after the amplification of putative beta-lactamase gene using forward and reverse primers, which was in accord with the theoretical size of putative beta-lactamase gene and this amplicon was confirmed to have cephalosporins activity. From NCBI gene bank database, putative beta-lactamase gene sequence of ATCC 13637 has 99% similarity with putative beta-lactamase gene of *S. maltophilia* K279a, with 1144 matched in 1152 bp. Meanwhile, putative beta-lactamase gene sequence of S2 strain also has 99% similarity with putative beta-lactamase gene of *S. maltophilia* K279a in NCBI data, with 1141 matched in 1152 bp. Differences in the sequence are at different sites. Interestingly, blasted result between putative beta-lactamase gene of ATCC 13637 and S2 strain also has 99% similarity, with 1141 matched in 1152. The translated amino acids sequence is the main factor which directly contributes to the resistance characteristic. Amino acids sequence prediction showed existence of sequence changes between ATCC 13637 and S2 (Figure 1). Amino acids predicted from putative beta-lactamase gene of *S. maltophilia* S2 strain differs by four amino acid substitutions from the ATCC 13637 strain. In addition, a stop codon at position of 376 was determined in amino acids sequence of ATCC 13637. Amino acids sequence of S2 strain had a full length sequence as K279a strain in NCBI database. Hence, it could be tested via kinetic assay.

Recombinant plasmid pET-15 that contains putative beta-lactamase gene was constructed and transformed into *E. coli* BL21. Colonies containing recombinant plasmid were confirmed by colony-PCR. A thick band with molecular weight at approximately 43kDa was observed from the crude protein sample by SDS-PAGE after expression of putative beta-lactamase gene by *E. coli* BL21, which was tally to the predicted molecular weight. The predicted peptide of 383 amino acids has a calculated molecular weight of 42kDa. Western blot with anti-His antibody was used to confirm the expression of the protein. A single band was determined in SDS-PAGE after the protein purification by His-trap FF affinity column (Figure 2).

Kinetic assay with homogeneous preparation of the *E. coli* BL21 pET-AT^S and pET-S2^R was done. Kinetic parameters (K_m and K_{cat}) and catalytic efficiency (V_{max}/K_m) of putative beta-lactamase were determined for a representative set of beta-lactam antibiotics (Table 1).

E. coli BL21 pET-AT^S has low activity towards the beta-lactams series. Meanwhile, *E. coli* BL21 pET-S2^R exhibited higher hydrolysis rate to the series of beta-lactams.

Table 1 Kinetic parameters of putative beta-lactamase

Substrate	pET-AT ^S			pET-S2 ^R		
	K _m (mM)	K _{cat} (s ⁻¹)	K _{cat} /K _m (M ⁻¹ s ⁻¹)	K _m (mM)	K _{cat} (s ⁻¹)	K _{cat} /K _m (M ⁻¹ s ⁻¹)
Penicillin	0.50	0.10	200	0.16	0.80	5000
Cefoxitin	0.28	0.11	410	0.08	0.59	7374
Ceftazidime	0.25	0.105	420	0.10	0.88	8800
Cefepime	0.12	0.60	5000	0.05	3.60	72000

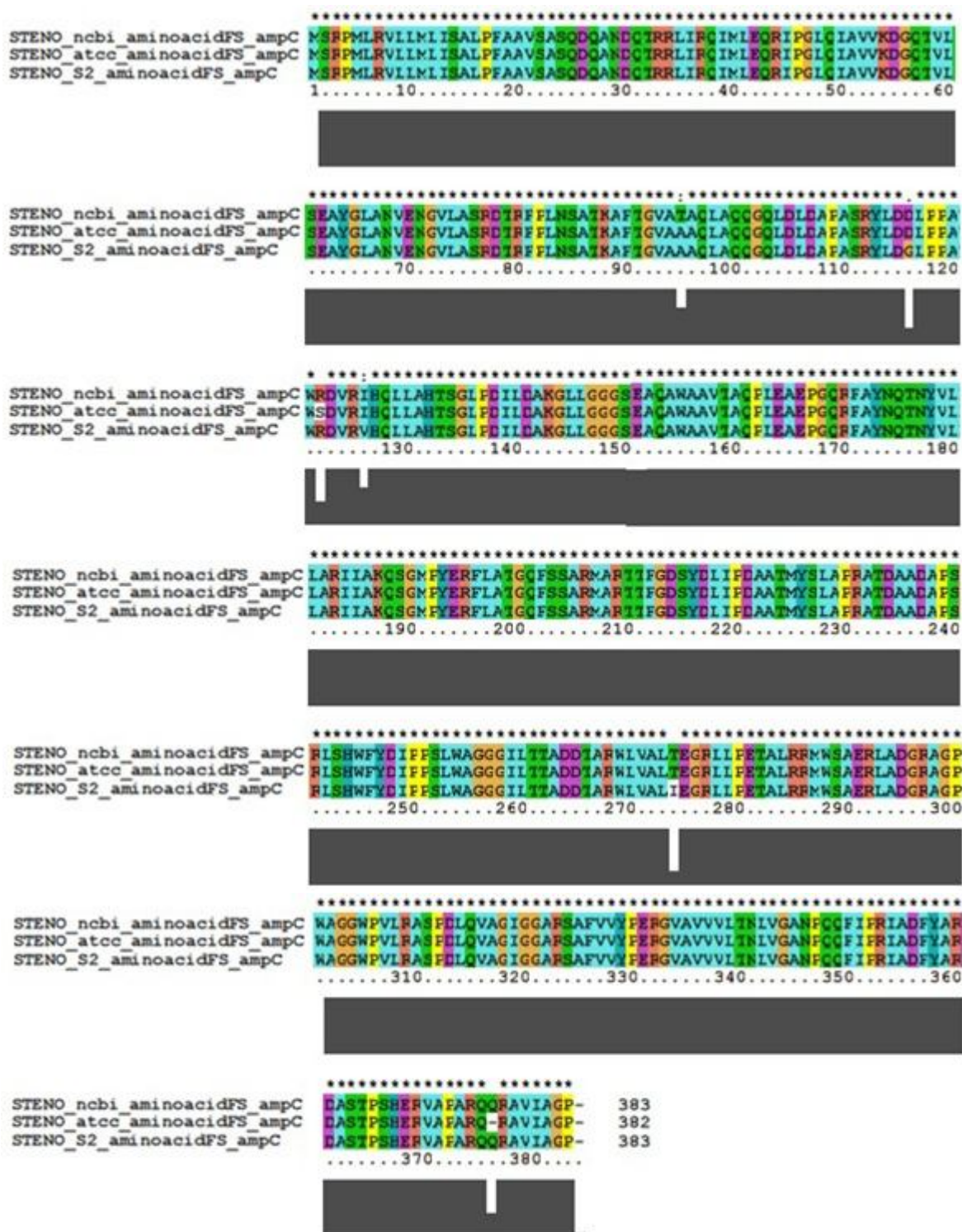


Figure 1 Homology of predicted chromosomally encoded putative beta-lactamase. The figure shows multiple amino acid sequence alignment of putative beta-lactamases from *S. maltophilia* K279a, ATCC 13637, and S2 strain. Asterisks indicate identical amino acids. Dashes indicate stop codon. Few amino acid substitutions could be observed via the alignment.

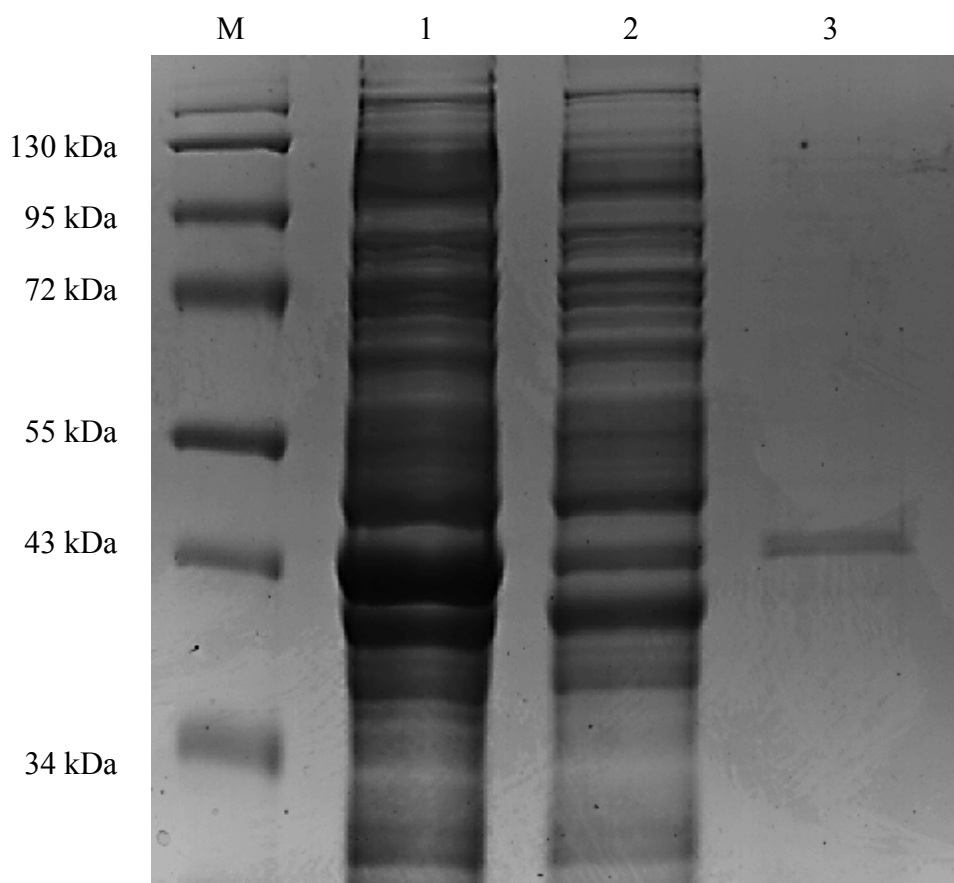


Figure 2 Expression of putative beta-lactamase via *E. coli* BL21. M, molecular mass standards; lane 1, crude protein of *E. coli* BL21 carrying recombinant plasmid; lane 2, flow through of unbound protein; lane 3, purified putative beta-lactamase. The purified protein matched with the predicted size of expressed putative beta-lactamase which is 43 kDa.

DISCUSSION

The clinical *S. maltophilia* S2 strain was isolated from the blood of a patient intubated in ICU of a teaching hospital in Malaysia. Before the isolation of *S. maltophilia* S2 strain, patient had been empirically treated with beta-lactams and carbapenems to against other potential pathogens that causing infection. Prior antibiotics treatment with broad spectrum antibiotics could be a factor causing *S. maltophilia* isolation (**VanCouwenberghe et al., 1997**). From the AST result, *S. maltophilia* S2 strain showed resistant to a series of beta-lactams antibiotics, and carbapenems as usual. Besides, high MIC value towards ceftazidime and cefepime was determined. This result might be interfered by intrinsically production of L1 and L2 beta-lactamase (**Okazaki and Avison, 2008**). In order to study the putative beta-

lactamase gene (smlt0115), cloning and expression of that gene was performed. The expressed putative beta-lactamase was not AmpC beta-lactamase since the absence of SXSK in the amino acids sequence, which is a characteristic motif of AmpC beta-lactamases with the conserved serine active site and also YXN motif as shown in the sequencing result **(Rajnish et al., 2011)**.

In the kinetic assay, the expressed putative beta-lactamase gene by *E. coli* harboring pET-AT^S plasmid had very weak activity towards the tested beta-lactams. Presence of a stop codon at position 376 of ATCC 13637 strain's amino acids could be the factor since it was a truncated enzyme. Truncated amino acids will interfere the folding of the protein hence causing low activity towards tested antibiotics **(Kingsley et al., 2004)**. On the other hand, *E. coli* harboring pET-S2^R plasmid exhibited detectable hydrolytic activity towards the tested beta-lactams. Lower K_m value for hydrolysis of cefepime was found in putative beta-lactamase produced from recombinant pET-S2^R strain, which was 0.05mM, whereas K_m for penicillin was 0.16mM. Low in K_m value of an enzyme showed the enzyme had higher affinity towards its substrates, cefepime. Besides, catalytic efficiency of pET-S2^R putative beta-lactamase towards cefepime was $72000 \text{ M}^{-1}\text{s}^{-1}$ which was 14-fold higher than towards penicillin, which was just $5000 \text{ M}^{-1}\text{s}^{-1}$. This means our pET-S2^R putative beta-lactamase had high affinity and high catalytic activity towards cefepime. From the alignment of amino acids sequences shown in Figure 1, besides the presence of stop codon at position 376 of ATCC 13637 strain's amino acids, that four amino acids substitution was found between ATCC 13637 and S2 strain. Substitution of amino acids might be the factor that enhanced hydrolyzing activity towards cefepime **(Cantu et al., 1996; Corkill et al., 2000)**. Yet, mutations in chromosomally encoded beta-lactamases might be happened under certain circumstance in order to enhance survival rate of bacteria when they were exposed to extended spectrum cephalosporins. Thus give rise to the production of strong hydrolyzing beta-lactamase which may lead to therapeutic failure.

However, catalytic efficiency of pET-S2^R putative beta-lactamase towards cefepime was $72000\text{M}^{-1}\text{s}^{-1}$, which was considered low as compared to other cefepime strong hydrolyzing beta-lactamase, especially AmpC beta-lactamases. But, high mutation rate and rapid resistant gene acquisition characteristic of *S. maltophilia* could not be neglected since this microorganism may generate stronger beta-lactamase in future **(Turrientes et al., 2010)**.

CONCLUSION

Herein, we reported that a putative beta-lactamase of *S. maltophilia* has been cloned and expressed in *E. coli* BL21, and was proved to have higher activity towards cefepime than penicillin. This finding could be correlated to the high level of resistance to cefepime in *S. maltophilia*.

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