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Genes Expression in Biosynthesis Lipopolysaccharide of *Burkholderia pseudomallei* the Causative Agent of Melioidosis

[1, 2]Nagi A. Al-Haj, [1]Lai L. Suang, [1, 2]Mariana N. Shamsudin, [2]Rasedee Abdullah,
[3]Rahmah Mohamed and [1]Zamberi Sekawi
[1]Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences,
[2]Laboratory of Immunotherapeutic and Vaccine, Institute of Bioscience,
[3]Department of Marine Science and Aquaculture, Institute of Bioscience,
University Putra Malaysia, 43400 Serdang, Malaysia

Abstract: *Burkholderia pseudomallei* is the causative agent of melioidosis, a serious disease of man and animals. The high mortality of *B. pseudomallei* infections may cause by Lipopolysaccharides (LPS), an endotoxin. The biosynthesis of LPS is complex comprising three components, lipid A, core oligosaccharide and O-specific antigen. In the current study was designed to further elucidate genes involved in the biosynthesis pathway of LPS in melioidosis agent followed with selected gene product expression with essential function for survival and virulence melioidosis agent. Expression of *Bplps0013*/*lpxA* and *Bplps0007*/*rfaF* successful expressed the entire proteins in 2 h with sizes of approximately 29 kDa and 43.7 kDa, respectively. The baseline information provided through the present research can be a preliminary approach towards the development of effective therapeutics against melioidosis.

Key words: Lipopolysaccharides, melioidosis, B*urkholderia pseudomallei,* expression, restriction enzyme digestion, Western blot

INTRODUCTION

Lipopolysaccharide (LPS) is a major structure of *B. pseudomallei*, which contributes greatly to the structural integrity of the bacteria and protects them from the host immune defenses. The formation of LPS is a complex process involving the synthesis of activated precursors by enzymatic activities, followed by the assembly of the lipid A core (Raetz and Chris, 2002). Therefore, disruption of the vital biosynthetic enzymes that cannot be compensated by other genes will lead to *B. pseudomallei* death. The role of exotoxins in the pathogenesis of melioidosis is unresolved. The high mortality of *B. pseudomallei* infections is related to an increased propensity to develop high bacteraemias (>1 cfu mL^{-1}). But the relation between bacterial counts in blood and mortality is similar to that of other gram-negative pathogens. This finding suggests that exotoxins do not contribute directly to outcome (Walsh *et al*., 1995). The endotoxin, or Lipopoly-saccharide (LPS), the immunodominant antigens is highly conserved (Charuchaimontri *et al*., 1999). LPS can evade killing by serum bactericidal system. The ability to evade complement mediated killing is considered to be an important virulence determinant (David *et al*., 1998). LPS is a good vaccine candidate due to it elicits the immune response, antibodies production from host. Desirably, vaccines should cause the patients to produce an antibody that binds to an epitope in LPS vaccines. IgM antibodies that produce can provide suitable and specific protection against endotoxemia. Thus, the administered of vaccine have to be sufficient in advance to permit IgM antibodies to be produced prior to potential endotoxin expose. Intervention to generate active vaccine comprise of LPS therefore are interested for many researchers. Matthias *et al.* (1997) desire to develop a composite *Klebsiella* vaccine containing other surface determinants whose seroepidemiology is less complex than that of the capsular antigens. They found that LPS O-antigen of *Klebsiella* is an attractive candidate for such a second generation *Klebsiella* vaccine. One of the reasons is because a Monoclonal Antibody (MAb) directed against the O1 serogroup antigen was protective in animal models of septicemia and peritonitis caused by a highly virulent *Klebsiella* strain. Therefore, it may provide superior coverage against clinical*Klebsiella* strains. Joyce *et al.* (1999) suggested that inner core lipopolysaccharide epitope may serve as vaccine

Corresponding Author: Nagi AL-Haj, Laboratory of Immunotherapeutic and Vaccine, Institute of Bioscience, University Putra Malaysia, 43400, Serdang, Malaysia

candidate against *Neisseria meningitides* due to it capable to elicit functional antibodies specific to inner core structure of *N. meningitides*. While, Bennett-Guerrero *et al*. (2004) have patented their invention regarding the vaccination with complete core rough LPS antigen provides both strain-specific protection and cross-core protection without unacceptable toxicity or other side effects against *E. coli*.

Upon the vaccine administration to laboratory animal, the compositions stimulate the synthesis of antibodies, which recognize an epitope in the core region of LPS molecule and which are protective against endotoxemia cause by at least 2 different gram-negative bacterial strains having different core structure. O-PS region of LPS has been found to be a major antigenic determinant (Shin-ichi *et al*., 1998). Previous studies demonstrate that both polyclonal antiserum and MAb's raised against *B. pseudomallei* flagellin protein, LPS and a tetanus toxin-PS glycoconjugate are capable of passively immunizing diabetic infant rats against challenge with the organism (Brett *et al*., 1994). Based on the preliminary success of these studies, Brett and Woods (2000) again synthesized a glycoconjugate molecule that incorporated both flagellin protein and O-PS antigen as a reasonable vaccine candidate and they have demonstrated that immune serum rose against the conjugate. It was proved that LPS may serve as a protective immunogen against *B. pseudomallei*.

The whole genome of *B. pseudomallei* has been successfully sequenced by Institute of Wellcome Trust Sanger on 4th July 2002. The total size of the genome is 7,247,547 bp and consists of two chromosomes with the size 4,074,543 and 3,173,005 bp, respectively. The C + G content of *B. pseudomallei* is 68.06% and the sequence for strain K96243 can be accessed from http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_pseudolammei. Expression of recombinant proteins can be approached in general by starting with a plasmid that encodes the desired protein, introducing the plasmid into the required host cell, growing the host cells and inducing expression with IPTG and end with cell lysis and SDS-PAGE or Western blot analysis to verify the presence of the protein. The expression of recombinant protein with pQE plasmid encodes 6×His tag. The 6×His tagged protein can be purified using Ni-NTA Spin Kit and Columns (QIAGEN), which contains NTA (Nitrilotriacetic Acid) matrices. NTA occupied four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sited free to interact with 6×His tag.

Therefore, it can bind 6×His tagged proteins more tightly and allowing the purification of proteins more pure and high yield. The buffer pH for purification is important in order to obtained high yield and high purity of recombinant proteins.

MATERIALS AND METHODS

***B. pseudomallei* stock:** All *B. pseudomallei* culture researches were done in the Pathogen Laboratory, Department of Biochemical, University Kebangsaan Malaysia (UKM) laboratory. *B. pseudomallei* strain D286 isolate was cultured on Ashdown medium, a selected medium for *B. pseudomallei*. The *B. pseudomallei* picture was provided by research assistant Mr. Lim Boon San from Pathogen Laboratory (UKM). The biochemical tests of *B. pseudomallei* were done by using Microbact™ 24E gram negative bacteria confirmation kit, which was also carried out by the research assistant Mr. Lim Boon San.

Expression system: Protein expression system began with constructing expression clones, followed by the expression of 6X His-tagged proteins and purification on Ni-NTA matrices (QIA expressionist™, QIAGEN).

Ligation: The vector used in expression system was pQE-30 UA (QIAexpressionist™, QIAGEN), which is designed for direct cloning of PCR product. Ligation of PCR insert was performed using 2XLigation Master Mix supplied with pQE-30 UA vector in the QIAexpress UA Cloning Kit (QIAexpressionist™, QIAGEN). The master mix contains all reagents and cofactors required for efficient ligation. The reaction mixture was as below with total up 10 μL: pQE-30 UA vector (50 ng μL^{-1}), 1 μL PCR product, 4 μL 2×Ligation Master Mix (added last) 5 μL. The mixture was mix gently and incubated at 16°C overnight to have better ligation.

Transformation: The *E. coli* host strain that used in transformation is SG13009 (pREP4) (QIAGEN). This strain derived from *E. coli* K12 and has the phenotype Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+ and Lon^+. The transformation was carried out using TransformAid™ bacterial transformation system (Fermentas®, USA). The transformants was plated out on LB agar plates containing 25 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin. The plates were incubated at 37°C overnight. A control transformation without insert was performed to ensure that the antibiotic ampicillin is working effectively and a control of transformation efficiency with a know amount of intact plasmid DNA also been included. The tranformants were screened for correct insertion and frame of the coding fragment by restriction analysis of the pQE

plasmid DNA, PCR the clone using forward vector primer and gene specific reverse primer and commercially sequencing of the cloning plasmid.

Restriction enzyme digestion: Restriction enzyme digestion was performed by the mixing of 1 μL plasmid, 0.5 μL of enzyme *EcoR1* (Fermentas), 0.5 μL enzyme *HindIII* (Fermentas) and 2.0 μL of buffer y+/tango™ (Fermentas) to determine the presence of the insert. The mixture was incubated at 37°C for overnight then 65°C for 10 min to stop the reaction. An aliquot of 10 μL mixture was subjected to electrophoresis. Host cells containing the recombinant plasmid should be maintained in the presence of kanamycin at 25 μg mL^{-1} and ampicillin at 100 μg mL^{-1}.

Time-course analysis of protein expression: Plasmid pQE-40, which expresses 6×His-tagged DHFR was also cultured as control. The cultures were incubated overnight and then transferred 1.0 mL overnight cultures into 50 mL LB medium (including antibiotic) and grown at 37°C for 1 min with vigorous shaking. When the cultures OD_{600} is 0.5-0.7, 5 mL of culture was transferred in a new microcentrifuge tube and stored in -20°C. The cultures were then induced expression by adding IPTG to a final concentration of 1 mM. A total of 5 mL sample cultures were transferred to new microcentrifuge tube in every 2 h for 8 h and stored in -20°C to prevent the further expression. The cultures in microcentrifuge tube were then harvested by centrifuged for 1 min at 130,000 rpm and the supernantants were discarded. The cells were then resuspended in 400 μL of buffer B and be gently vortexed. The lysates were pelleted down for 20 min at 130,000 rpm to remove cellular debris. An aliquot of 15 μL samples were mixed with 5 μL of 3×SDS-PAGE sample buffer and boiled for 5 min at 96°C. Lastly, the samples were analyzed by SDS-PAGE. The optimal induction period of protein expression was determined by observed the thickness of 6×His-tagged protein band on SDS-PAGE.

Protein expression and purification: The positive expressed clone was inoculated in 10 mL LB broth media containing ampicillin (100 μg mL^{-1}) and kanamycin (25 μg mL^{-1}) and incubated overnight at 37°C. Then 10 mL overnight culture was transferred into 100 mL antibiotics LB broth media. For the protein expression, 1 mM final concentration of IPTG was added after incubated 1 h. The culture was then incubated for several hours depend on time course where protein was optimum expressed. The culture was then harvested by centrifuged for 1 min at 130,000 rpm and the supernantant was discarded. The cells were then resuspended in 400 μL of buffer B and be gently vortexed. The lysate was pelleted down for 20 min at 130,000 rpm to remove cellular debris. The supernatant was then transfers to a fresh tube. The 600 μL of buffer B was equilibrated in a Ni-NTA spin column and centrifuged for 2 min at 2000 rpm. The cleared lysate supernatant containing the 6×His-tagged protein was loaded onto an equilibrated Ni-NTA spin column. The Ni-NTA spin column was centrifuged for 2 min at 2000 rpm and washed twice with 600 μL buffer C. The protein was then eluted with 2×200 μL buffer E and centrifuged for 2 min at 2000 rpm to collect the eluate. An aliquot of 15 μL samples were mixed with 5 μL of 3×SDS-PAGE sample buffer and boiled for 5 min at 96°C. Lastly the samples were analyzed by SDS-PAGE.

SDS-PAGE: The expressed proteins were analysed using SDS-PAGE essentially as described by Laemmli. The separating gel with 12% acrylamide, 0.1% SDS and stacking gel with 5% acrylamide, 0.1% SDS were prepared using minigel system apparatus (Mini Protean II Vertical Slab Gel) by Bio-Rad. The gel solution for resolving gel was prepared and poured onto glass by using 1 mL pipettor up to 75% of height of the glass plate. It then was layered with 1 mL of distilled water using pipette and waited for 20 min to solidify. Once the gel solidified, the water was tip off. The stacking gel was prepared and layered on the solidified resolving gel and inserted the comb immediately. After 15 min the comb was removed, the well was washed with water to remove unpolymerized acrylamide. The gel was then set into the buffer tank and added with running buffer up to the top and bottom reservoirs. An aliquot of 10 μL proteins samples were suspended and mixed well with 5 μL 2× loading buffer in 0.5 mL eppendorf tube and were heated in thermal cycler at 98% for 5 min to denature the protein before loading into the gel. The gel was then subjected to electrophoresis at 100 V for about 1 h until the bromophenol blue reaches the bottom of the resolving gel. After the power supply turned off, the glass plate was removed and the plate was pried apart using extra part of the gel spacer. The gel was detached slowly from the glass plates and subjected to staining. The staining was done by place the gel in container contains coomasive blue staining solution (about 5× volume of the gel) and agitated on a belly dance for 1 h the gel then washed with distilled water several times and destained in destaining solution with gently agitated overnight. The protein bands were clearly seen and the image was captured using imager white light (Alpha Imager™ 2200, Alpha Innotech Corporation).

Western blotting: Before the blotting, the PVDF membrane was soaked in 100% methanol for 15 sec and be let in distilled water for 2 min and finally in Towbin buffer for 1 h. The material that used in assemble sandwich cassette western blot were presoaked in Townbin buffer for 1 h as well. The blocking stack was assembled on top of the stainless grid cathode in the rough of the frame stand.

Detection of fusion protein by anti-His antibody: After the transferring of protein to the membrane, the membrane was blocked with blocking buffer for overnight at 4°C with gentle agitation. The blocking buffer was then discarded. Immediately, the 10 mL of dilution primary antibody (5 μL of anti-His antibody in 10 mL blocking buffer), was added to cover the membrane and incubated for 1 h. After 1 h, the primary antibody was aspirated out and stored at -20°C for reused purpose. The membrane was washed 3 times with washing buffer and incubated for another 1 h in 10 mL of diluted secondary antibody (5 μL of concentrated alkaline phosphatase conjugated goat anti-IgG mouse antibody in 10 mL blocking buffer). Again, after the secondary antibody solution was removed, the membrane was washed 3 times with agitation in washing buffer. When the washing was completed, the bound alkaline phosphatase conjugated secondary antibody on the membrane was detected using BCIP/NBT substrate (provided in Western MAX Alkaline Phosphatase (AP) kit) and incubated at room temperature until dark blue-gray color developed. The reaction was stopped with washed in distilled water for 5 min and air-dried. Immediately the membrane was captured using imager white light (Alpha Imager™ 2200, Alpha Innotech Corporation).

RESULTS

The restriction enzyme digestion showed appropriate bands formation. Two bands of the appropriate sizes were observed in the electrophoresis gel. The two bands in the restriction enzyme analysis results comprised of the pZT57R/T vector of 2886 bp and another ligation band with a size similar to each ORF fragment, respectively (Fig. 1). The clone containing Bplps0022/waaC1, however did not show any band formation in restriction enzyme digestion.

Protein detection-SDS-PAGE: Figure 2 showed the expression of Bplps0007/rfaF protein band analysis, which depend on the time course of 0 (before IPTG was added), 2, 4, 6 and 8 h after added with IPTG. Before inducing with IPTG at the 0 time course, no band was observed in the gel region with the molecular weight between 42.7 and 55.6 kDa. After 2 h, a thick band can be observed at the location between 42.7 and 55.6 kDa, which is approximately 43.7 kDa indicating that putative Bplps0007/rfaF protein was successfully expressed. At the time course of 4, 6 and 8 h, the bands were thicker then the 2nd h. The bands located below 42.7 kDa are the protein from *E. coli* host strain SG13009 (pREP4) (QIAGEN). Positive control used in this expression is the expressed pQE-40 control plasmid provided together in the QIAexpress UA cloning kit (QIAGEN, Research Biolabs). It showed a correct band at the molecular weight of 26 kDa on SDS-PAGE gel, indicating the expression methods was successful. The negative control is also using pQE-40 control plasmid, which does not show the band at the location around 42.7 kDa indicating no expression. Figure 3 showed the expression of putative Bplps0013/lpxA protein depending on time course of 0 (before IPTG was added), 2, 4 and 6 h after adding IPTG. Before inducing with IPTG at the time course of 0, no band was observed at the molecular weight region between 25 and 32.5 kDa. After 2 h, a thick band can be observed at the location between 25 and 32.5 kDa, which is approximately 29 kDa indicating that putative Bplps0013/lpxA protein was successfully expressed. At the time course of 4 and 6 h, the bands were also observed but not as thick as the 2nd h. The expression is

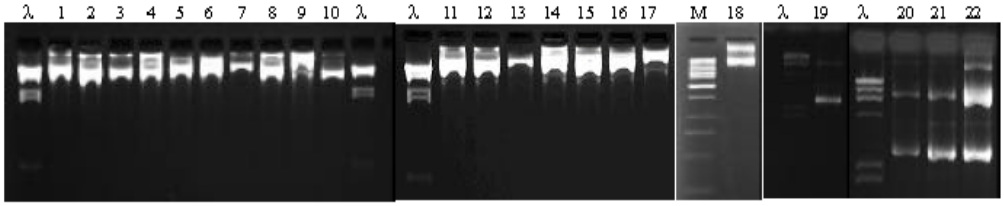

Fig. 1: Recombinant plasmids extraction. Lane 1: fabG, Lane 2: adk, Lane 3: kdsB, Lane 4: rfaF, Lane 5: wzyC, Lane 6: Dpm1, Lane 7: lpxB, Lane 8: lpxD, Lane 9: fabZ, Lane 10: fabH, Lane 11: waaE, Lane 12, udg, Lane 13: waaB, Lane 14: wbyC, Lane 15: wbiA, Lane 16: wzt, Lane 17: lpxC, Lane 18: wbiI, Lane 19: wzm, Lane 20: lpxL, Lane 21: rfaQ, Lane 22: lpxA, λ, λ: Hind III DNA ladder (Fermentas), M: 1 kb DNA Ladder (Fermentas)

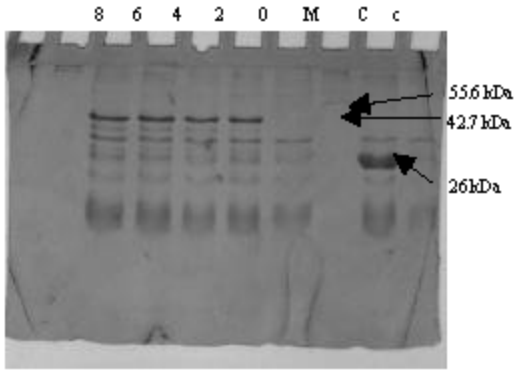

Fig. 2: Time course analysis of Bplps0007/rfaF (~43.7 kDa) protein expression from pQE-30 UA cloned by SDS-PAGE technique. M, protein marker (New England Biolabs); C, positive control with expression; c, control without expression; 0, *RfaF* clone before induce expression; 2, 4, 6 and 8 h after induce expression

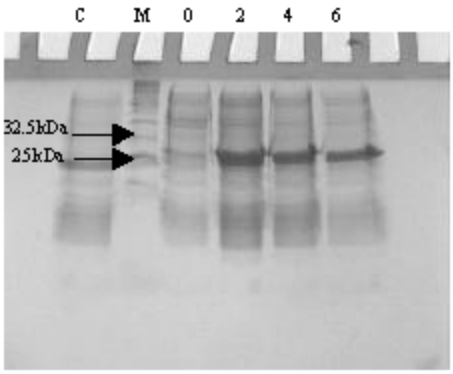

Fig. 3: Time course analysis of Bplps0013/LpxA (~29 kDa) protein from pQE-30 UA cloned by SDS-PAGE technique. M: Prestained protein marker (New England Biolabs); C: positive control with expression; 0: LpxA clone before induce expression; 2, 4 and 6 are the hours after induce expression

high at the 2nd h. The multiple unclear bands are the protein from *E. coli* host strain SG13009 (pREP4) (QIAGEN).

Protein detection Western blot: The protein expression of the 2 putative ORFs confirmed through western blot showed the reciprocal signal bands on the membrane. The rabbit polyclonal anti-His protein and the secondary antibody detected the reciprocal proteins on the membrane by producing the dark band signal as shown in Fig. 4. The signals were seen at the membrane region

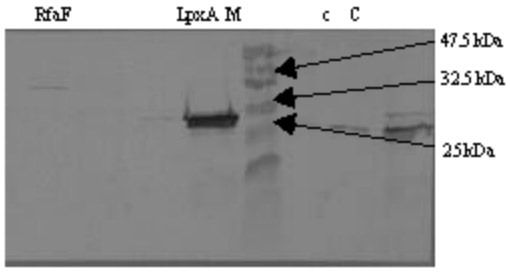

Fig. 4: Analysis of RfaF (~43.7 kDa) and LpxA (~29 kDa) protein from pQE-30 UA cloned by Western blot technique. M: Prestained protein marker (New England Biolabs); c: Negative control; C: Positive control

corresponding to the molecular weight of 43.7 and 29 kDa, respectively. The prestained protein marker confirmed the size of the signals.

DISCUSSION

Lipopolysaccharide (LPS) or endotoxin expressed by *B. pseudomallei* is both a virulence determinant and a protective antigen (Charuchaimontri *et al*., 1999). Therefore, several current programs of research aim at neutralizing this endotoxin or eliminating from the circulation (Woods *et al*., 1999). LPS biosynthesis pathway involves a series of genes may represent an ideal target for the attack of disease-producing bacteria by antibodies and other immunological or pharmacological agents (Rietschel *et al*., 1994). Additional, the study of molecular properties of core oligosaccharide (Bennett-Guerrero *et al*., 2004; Raetz and Chris, 2002) and O-antigen (Brett and Woods, 1996) may also contribute to the development pharmaceutical product for the prevention or treatment of the infection of particular pathogen. This is the significant of the current study of molecular level of LPS biosynthesis genes since LPS genes for *B. pseudomallei* has not been fully annotated and the comparison of local *B. pseudomallei* strain to the sequence for strain K96243 has not been studied, which could contribute to the epidemiological study of the pathogen. Gene expression step through vector pQE-30 UA was used. It contains 6×His tag at the N-terminus of the protein of interest. This vector is the most commonly used and is generally the easiest to prepare since it allows the direct insertion of PCR product into the prelinearized pQE-30 UA vector, which has a U overhang on each 3'end. Therefore, it can eliminate the need for restriction digestion of the vector and insert. Only 5'end of the orf must be ligated in frame.

Therefore, all primers at 5'end were designed in frame to provide correct expression. However, because bacteria cannot process introns, the cloned sequences must be stripped of introns. The cloned gene is inserted next to appropriate bacterial transcription and translation start signals. Some expression vectors have been designed with restriction sites located just next to a *lac* regulatory region. These restriction sites permit foreign DNA to be spliced into the vector for expression under the control of the *lac* regulatory system. Very small proteins and peptides are sometimes difficult to express stably in *E. coli* because they cannot fold correctly and are often subject to proteolytic degradation. While, very long recombinant proteins, may be subject to premature termination. The proteins, which are desired to be expressed are BPlps0007/rfaF, BPlps0013/lpxA and BPlps0036/wzm, which contain approximately 41.4, 27.9 and 30.6 kDa, respectively. Protein of BPlps0013/lpxA, UDP-GlcNAc acyltransferase, was favorable in the study of the expression because it is the enzyme catalazes the 1st step of lipid A biosynthesis. The biosynthesis pathway of lpxA may serve as drug target site (Raetz, 1998). BPlps0007/rfaF is a structural gene for heptosyltransferase, which aid in the adding of Hep II to Hep I to complete lipopolysaccharide inner-core backbone. *RfaF* is reported as an essential gene in *Pseudomonas aeruginosa*, which may be a drug target (Kishore *et al*., 2004). The 3rd expressed protein is BPlps0036/Wzm, which encodes protein homologous to O-antigen and capsular transport proteins. It is identified as components of ABC transport system, which Wzm protein is an integral membrane protein (Rocchetta *et al*., 1999). A DNA vaccine can be developed to target this membrane protein. BPlps0007/RfaF and BPlps0013/lpxA were successfully cloned and the proteins were induced to be expressed. BPlps0036/Wzm gene was first successfully ligated and transformed into the host. However, due to the incompatible condition for BPlps0036/wzm sequence with the host cell, the ligant jumping out. Although, the cloning was carried out several times for BPlps0036/wzm gene, the ligation still failed. The vector and the host used in this study may not be suitable for *wzm* gene cloning. Examination of the SDS-PAGE and Western Blot revealed that BPlps0007/rfaF and BPlps0013/lpxA expressed a protein with a molecular mass in agreement with the predicted mass (41.4 and 27.9 kDa, respectively). All plasmids expressed the vector encoded His tag sequence protein (~2 kDa). Therefore, proteins with apparent molecular masses of 43.4 and 29.9 kDa were visualized in SDS-PAGE and Western blot membrane. The expression of fusion protein for BPlps0007/rfaF and BPlps0013/lpxA can be observed in the 2nd h of IPTG induction. This expression system takes only 2 h enough for the gene expression and it was suggested that big amount of LPS fusion proteins were expressed in <2 h. Strong protein band was showed through out 6-8 h of induction indicated that promoter on plasmid pQE-30 expression vector is a strong promoter to express foreign genes in *E. coli* system. High level production of this protein in the current research using this system expression plasmid could lead to use it as immunostimulator by direct injection. Ease in purification of protein study in details is the other advantage of the high expression of ORFs in this study. In near future, a suitable vaccine or antimicrobial may be developed by targeting the genes encoding the various components essential in LPS biosynthesis and survival of the pathogen.

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