Research Journal of Medical Sciences 3 (3): 104-110, 2009
ISSN: 1815-9346

Polymerase Chain Reaction and Cloning of *Burkholderia pseudomallei* Putative Genes

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Abstract: Total of 23 putative Open Read Farms from *B. pseudomallei* strain D286 was successfully cloned and the nucleotide sequence analysis of the putative genes showed the homologue (98-100%) to strain K96243. The high similarity in gene sequences between these strains is confirmed for presence of the necessary ORF for LPS biosynthesis through PCR amplification the application of the ORFs in the PCR amplification and expression method. The findings of this study have contributed to some information on the molecular bases of the LPS biosynthesis genes in *B. pseudomallei* specifically for strain D286. PCR amplification, a specific pair of primer for each ORFs was proving specific for amplification of genes in *B. pseudomallei* strain D286. The PCR mixture with addition of DMSO, formamide and glycerol could ease the PCR optimization where different pairs of primers were involved. The specific primer pairs with the PCR mixture could be used in developing a PCR diagnosis of melioidosis.

Key words: Polymerase chain reaction, cloning, melioidosis, burkholderia pseudomallei, open reading frames, putative genes, primers

INTRODUCTION

Melioidosis is one of the most severe community-acquired sepsis caused by bacteria and now is known to be a major cause of human morbidity and mortality in tropical or endemic areas (Chaowagul *et al*., 1989). This life threatening melioidosis is caused by a gram-negative bacterium namely *Burkholderia pseudomallei*. It appears on the category B list of critical agents published by the US Centers for Disease Control and Prevention (CDC). Previous studies have demonstrated that the 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 may represent an ideal target for the attack of disease-producing bacteria by antibodies and other immunological or pharmacological agents (Rietschel *et al*., 1994). In order these beneficial effects of LPS could be exploited in clinical medicine against melioidosis, study was performed for further applications in reduction of LPS sepsis by harvesting the beneficial property of LPS through the bioinformatics in conjunction with molecular approach. Molecular-based characterization selected LPS genes carried out in the present study is fundamental to future studies such as in developing vaccine for prevention or drug for treatment of melioidosis.

PCR is a powerful molecular biology technique introduced in 1980 by Kary Mullis and is widely use (Mullis and Faloona, 1987). The technique allows amplification of specific fragments of DNA by using a pair of primer.

The amplified DNA fragment is then enough in quantity to be used for further study, for example, cloning and expression analysis. The PCR produces large amounts of a specific DNA fragment by the enzymatic amplification *in vitro* which the phenomenon is same as the DNA replication *in vivo* (Saiki *et al*., 1985). The basic components that are needed in the PCR reaction include a pair of specific primers, DNA template, dNTPs and *Taq* polymerase (*Thermus aquaticus* DNA polymerase I). The enzyme works optimally at 72°C to make copy strands of DNA and allows only specific regions of the genome to be replicated *in vitro*.

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The technique rapidly gained acceptance as an indispensable tool in molecular biology, population genetics and medical diagnosis. Cloning is the insertion of DNA with information of interest into a specific vector that allows replication and transfer of DNA that inserted into the vector from one host to another and allows production of large quantities of the DNA fragment for physical or biological analysis. The cloning is important in biology study because it can provide a pure sample of an individual gene, separated from all the other genes that it normally shares in the cells (Brown, 1995). In this study, cloning was carried out to store the gene information for further study and for expression purposes.

MATERIALS AND METHODS

***B. pseudomallei* stock:** All *B. pseudomallei* culture works were done in the Pathogen Laboratory, Department of Biochemical, Universiti 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.

Primers design and synthesis: Primers sets based on annotated ORFs used for PCR were designed specifically based on the sequences of annotated LPS putative genes manually and confirm by using PrimerPrimier 3 (CGAT, UKM). The optimum CG content and optimum melting point for each set of primer were considered. The primers were then synthesized by Research Biolabs (Malaysia). The oligonucleotide sequences of primers are shown in Table 1.

Amplification of putative ORFs by PCR using designed set primers: A total of 25 μL amplification reaction mixture was prepared in 0.5 mL centrifuge tube. The cocktail components of the reaction mixture are presented in appendix C. The PCR mixtures were subjected to thermal cycling using the thermal cycler (Biometra-Trio Themoblock, Biometra Co., Ltd, Gottingen, Germany). The DNA amplification steps performed with initial denaturation at 96°C for 5 min, followed by 30 cycles steps consisting of denaturation at 96°C for 1 min, annealing at 60°C, elongation at 72°C for 2 min. The amplification ended with a single final extension at 72°C for 20 min for cloning purposes. After the amplification process, an aliquot of 10 μL of each reaction mixture was loaded into the well of a 1.8% agorose gel (1.2 g agarose gel powder in 100 mL 1×TBE buffer) and electrophoresed. The gel was stained with ethidium bromide and the image captured using imager (Alpha Imager™ 2200, Alpha Innotech Corporation). For negative control, several Gram negative bacteria were used to test the specificity of the primer which includes isolate *Pseudomonas aeruginosa*, *Vibrio cholera*, *Vibrio alginolyticus*, *Klebsiella pneumoniae* and *Escherichia coli*.

Table 1: The oligonucleotide sequences of primers used in PCR

Gene putative/ Gene name		5'-3' sequence	Product length (bp)
Bplps0001/*lpxL*	F	5' AGGATTCGCATGCTAGGCC 3'	943
	R	5' GAGTTTCATGGGCGCTATTG 3'	
Bplps0003/*fabG*	F	5' GCAAACATGGAGATTCGCG 3'	780
	R	5' CGCCTCGTCATTTCGTCACTT 3'	
Bplps0004/*Adk*	F	5' ATGCGTTTGATCCTGTTGGGC 3'	672
	R	5' CGCCGCGATTCACTTGAG 3'	
Bplps0005/*kdsB*	F	5' ATGACCTCCCCGCTCCCTT 3'	861
	R	5' GGGCTTGGGGCGAATCTC 3'	
Bplps0006/*lpxK*	F	5' GCGCATCGCGTCATGA 3'	1072
	R	5' CGGACCCTTGCAAATCGG 3'	
Bplps0007/*rfaF*	F	5' CGAATGAGCAGACTCCACGG 3'	1195
	R	5' GGACAGGCGATCCAAAGC 3'	
Bplps0008/*wzyC*	F	5' GCCGTCAAGTTCAAGCCG 3'	1315
	R	5' ATCGTGCGTCCGCGTTC 3'	
Bplps0009/*waaB*	F	5' CGCGGACGCACGATGACG 3'	1293
	R	5' CGTGCGAAGCGAGCGAGC 3'	
Bplps0010/*rfaQ*	F	5' GACATGATCGTGTTTCGCGG 3'	1108
	R	5' ATCGCCGAATCCGCTGC 3'	
Bplps0011/*dpm1*	F	5' GCGGACATGACTCACCCTG 3'	1043
	R	5' GTGATACGCGAAGACGACGG 3'	

F = Forward; R = Reverse

Gel purification: The gel purification was carried out according to the manufacturer's instruction (QIAquick Gel extraction kit, QIAGEN Hilden, Germany). Each target DNA fragments from electrophoresed agarose gel was excised with a clean, sharp scalpel and placed in a microfuge tube. Three volumes of buffer QG (QIAquick Gel extraction kit, QIAGEN Hilden, Germany) was added to one volume of the gel and incubated at 50°C for 10 min. The samples were mixed by vortexing every 2-3 min during the incubation to help dissolve the gel. After the gel slice was completely dissolved, one gel volume of isopropanol (Sigma Chemical Co., USA) was added to the samples and mixed. The content of tubes were then transferred to a spin column and centrifuged at 8,000× g for 1 min at room temperature. The flow through was discarded. The 500 μL of buffer QG was added to the spin column and centrifuged from 1 min to remove all trace of agarose. The flow though was again discarded and the column was washed with 750 μL of buffer PE by centrifugation at 8,000× g for 1 min. After discarding the flow through, the column was allowed to spin for an additional 1 min at maximum speed for the complete removal of ethanol in the washing buffer. Finally, the purified PCR DNA was eluted in a clean microfuge tube with 30 μL of elution buffer.

Cloning of putative ORFs into pTZ57R/T vector

Ligation: Cloning of PCR product was carried out using InsT/Aclone™ PCR Product Cloning Kit (Fermentas®, USA). The vector that used in ligation was plasmid vector pTZ57R/T DNA and the components as below were mixed well in 1.5 mL microcentrifuge tube for ligation purpose: 3.0 μL plasmid vector pTZ57R/T DNA, 4.0 μL purified PCR fragment, 3.0 μL 10× ligation buffer, 3.0 μL PEG 4000 solution, 16.0 μL Distilled water, 1.0 μL T4 DNA Ligase, 5U and the mixture was then incubated at 22°C overnight to yield maximum recombinant before transformed into competent cell.

Transformation: The transformation was carried out using TransformAid™ Bacterial Transformation System (Fermentas®, USA). The competent *Escherichia coli* XL1-Blue which obtained from CGAT, UKM, was cultured on tetracycline agar plate overnight. A single colony of overnight culture was transferred into 2.0 mL microcentrifuge tube contained 1.5 mL of pre-warmed C-Medium (TransformAid™) and incubated for 30 min at 37°C with gentle agitation. Meanwhile, 400 μL T-Solution was prepared by mixing 200 μL of T-Solution (A) TransformAid™ and 200 μL of T-Solution (B) TransformAid™ and kept on ice. The fresh culture was centrifuged at 130,000 rpm for 1 min at 4°C to pellet down the cells. The supernatant was discarded and the pellet cells was resuspended in 300 μL of T-Solution and incubated on ice for 5 min. The mixture was spin down for 1 min at 4°C and the supernatant was removed. About 100 μL of T-Solution was added to dilute the cells and incubated on ice for 5 min. Meanwhile, DNA for transformation was prepared by dispensing 2.5 μL of ligation mixture into a new 0.5 mL centrifuge tube and incubated on ice for 2 min. An aliquot of 50 μL resuspended competent cells was added to the ligation mixture and incubated on ice for 5 min. The cells lastly was plated on a pre-warm LB-Ampicillin (50 μg mL^{-1}) agar plated, which spreaded with 40 μL of 0.1 M IPTG and 40 μL of 20 mg mL^{-1} X-gal and incubated overnight at 37°C.

Analysis of positive clones: The recombinant clones of *E. coli* XL1-Blue were identified by blue-white colony selection on LB-Ampicillin + IPTG + X-gal agar plated. Seven to 10 single colonies with white color were selected as positive recombinant clones transferred each colony to 10 mL LB/ampicillin (50 μg mL^{-1}) broth medium. Same colonies were also subjected to PCR analysis for confirmation.

Extraction of plasmid: The overnight broth culture was harvested by centrifuged at 130,000 rpm for 2 min and supernatant was discarded. Plasmid extraction was performed using QIAprep Plasmid Kit (QIAGEN. Germany). The pelleted bacteria cells were resuspended in 250 μL buffer P1 followed by the addition of 250 μL buffer P2 and buffer N3. The tube was inverted gently 4-6 times to mix for each buffer addition. The mixture was then centrifuged at 130,000 rpm for 10 min. The supernatant obtained was transferred into QIAprep spin column and centrifuged at 130,000 rpm for 1 min. The flow through was discarded and the column was washed by adding 750 μL of buffer P3. After centrifuged for 1 min, the flow through was again discarded and the column was centrifuged again to remove residual wash buffer. The column was then placed in a sterilled 1.5 mL centrifuge tube. The DNA was eluted by adding 50 μL of TE buffer to the center of the column. The plasmid was stored at -20°C.

Restriction enzyme digestion: Restriction enzyme digestion was performed to determine the presence of the insert. An aliquot of 1 μL plasmid was cut with 1 μL of *EcoR1* (Fermentas) in 2 μL buffer *EcoR1* and distilled water was added up to 20 μL. The tube was mixed by tapping and the mixture was incubated at 37°C for overnight. The reaction was stopped by increasing temperature to 65°C for 10 min. An aliquot of 10 μL mixture was subjected to electrophoresis.

DNA sequencing analysis: Confirmation of DNA target sequence was done commercially using automated sequencing (Reseach Biolabs, Malaysia). The orientation and frame of the insert was confirmed using Biology Workbench available access online.

RESULTS

Cloning of putative ORFs into pTZ57R/T vector: After overnight incubation on the LB agar containing 50 μg mL^{-1} ampicillin, the white and blue colonies were seen growing on IPTG/X-gal agar plate (Fig. 1). Blue colonies were the clones with insert which do not undergo ligation while white colonies contained insert with ligatant. As a control in transformation process, competent *Escherichia coli* XL1-Blue without insert was spread on 50 μg mL^{-1} ampicillin agar plate and showed no colony growth after overnight incubation. For each transformation reaction, 3-10 white colonies were randomly chosen from the LB agar plate and subjected to PCR analysis.

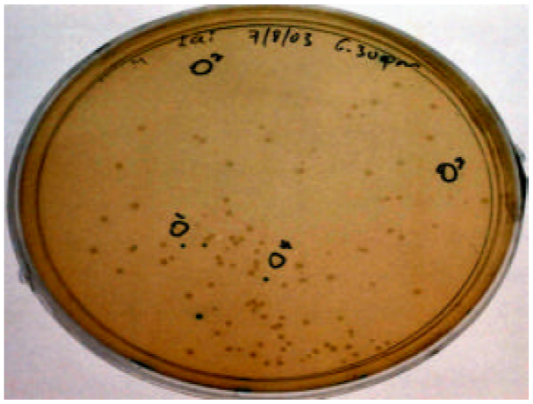

Fig. 1: The cloned ORFs on the LB agar/amp. Overnight growth of *Escherichia coli* XL1-Blue colonies after transformation with pTZ57R/T plasmid vector at 37°C. Two types of colonies were observed as white and blue colonies on medium containing IPTG and X-gal

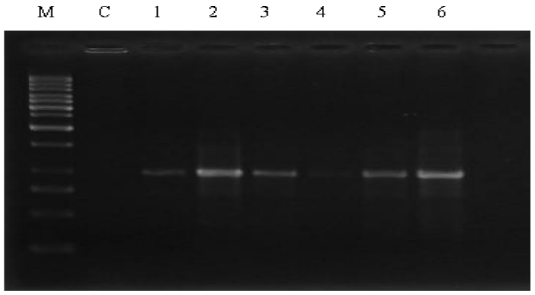

Fig. 2: Colony PCR for Bplps0001/*lpxL*. Clone screening analysis of Bplps0001/*lpxL* gene by PCR using specific primer gives the band size ~943 bp. Clone in lane 4 that does not contain cloned gene will be eliminated. M, 1 Kb DNA Ladder (Fermentas); C, Negative control

Colony PCR. Screening of ORFs genes from the cloned *Escherichia coli* XL1-Blue by the PCR: Colony PCR was performed using respected specific set primers to screen and select for the colonies that carried the ORFs. The primer sets that use in colony PCR consist of a forward primer from vector and reverse primer from respective specific ORFs. Blue colony was used as negative control in this colony PCR step. The conditions of PCR were same as described for PCR but the colonies was used as templates. The colonies that produced the correct size band of PCR represent the amplified ORFs gene. Thus, it was confirmed that these colonies are the positive clones that carried the ORFs and were in a correct orientation.

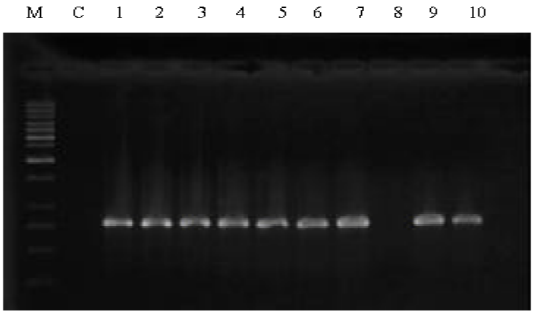

Fig. 3: Colony PCR for Bplps0003/*fabG*. Clone screening analysis of Bplps0003/*fabG* gene by PCR using specific primer gives the band size ~780 bp. Clone in lane 7 that does not contain cloned gene will be eliminated. M, 1 Kb DNA Ladder (Fermentas); C, Negative control

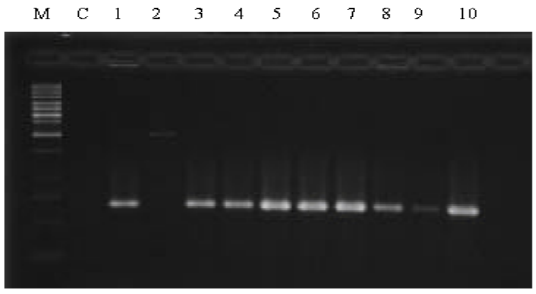

Fig. 4: Colony PCR for Bplps0004/*adk*. Clone screening analysis of Bplps0004/*adk* gene by PCR using specific primer gives the band size ~672 bp. Clone in lane 2 that does not contain cloned gene will be eliminated. M, 1 Kb DNA Ladder (Fermentas); C, Negative control

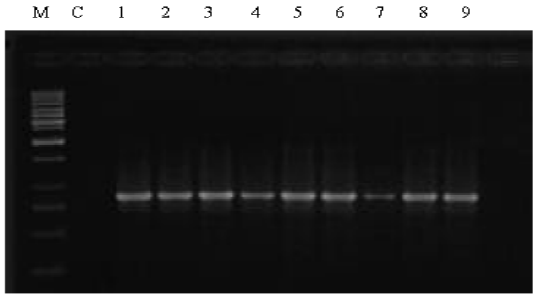

Fig. 5: Colony PCR for Bplps0005/*kdsB*. Clone screening analysis of Bplps0005/*kdsB* gene by PCR using specific primer gives the band size ~861 bp. M, 1 Kb DNA Ladder (Fermentas); C, Negative control

While no band was produced indicate that no ligatant or incorrect orientation. Total of 23 successfully amplified

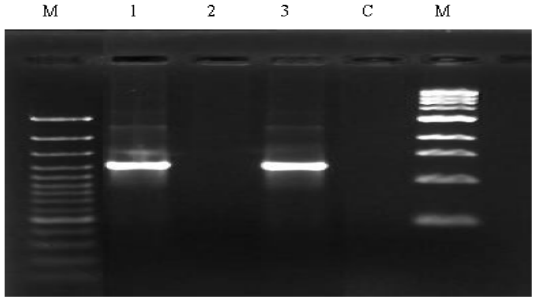

Fig. 6: Colony PCR for Bplps0007/*rfaF*. Clone screening analysis of Bplps0007/*rfaF* gene by PCR using specific primer gives the band size ~1195 bp. Clone in lane 2 that does not contain cloned gene will be eliminated. m, 100 bp DNA Ladder (Fermentas) M, 1 Kb DNA Ladder (New England Biolabs); C, Negative control

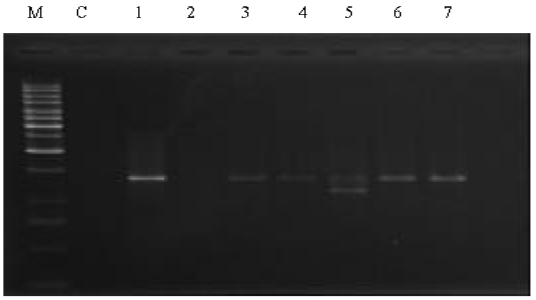

Fig. 7: Colony PCR for Bplps0008/*wzyC*. Clone screening analysis of Bplps0008/*wzyC* gene by PCR using specific primer gives the band size ~1315 bp. Clone in lane 2 that does not contain cloned gene will be eliminated. M, 100 bp DNA Ladder (Fermentas) M, 1 Kb DNA Ladder (Fermentas); C, Negative control

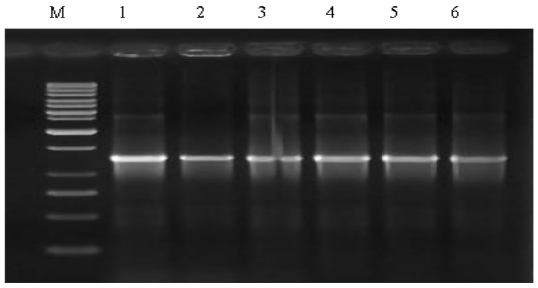

Fig. 8: Colony PCR for Bplps0009/*waaB*. Clone screening analysis of Bplps0009/*waaB* gene by PCR using specific primer gives the band size ~1293 bp. M, 1 Kb DNA Ladder (Fermentas)

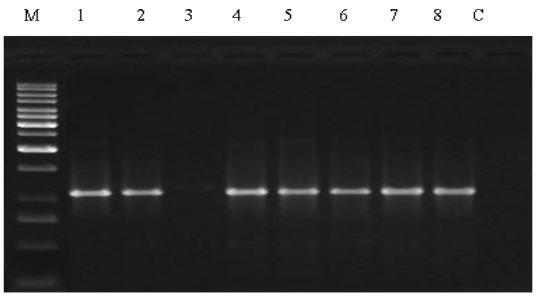

Fig. 9: Colony PCR for Bplps0010/*rfaQ*. Clone screening analysis of Bplps0010/*rfaQ* gene by PCR using specific primer gives the band size ~1108 bp. Clone in lane 3 that does not contain cloned gene will be eliminated. M, 1 Kb DNA Ladder (Fermentas); C, Negative control

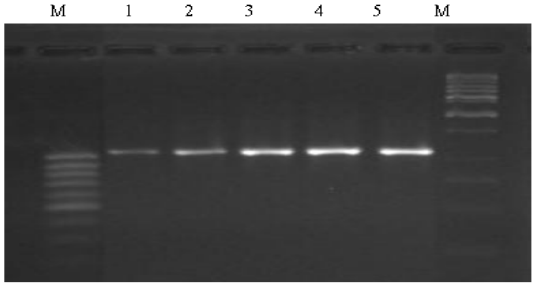

Fig. 10: Colony PCR for Bplps0011/*Dpm1*. Clone screening analysis of Bplps0011/*Dpm1* gene by PCR using specific primer gives the band size ~1043 bp. m, 100 bp DNA Ladder (Fermentas); M, 1 Kb DNA Ladder (Fermentas)

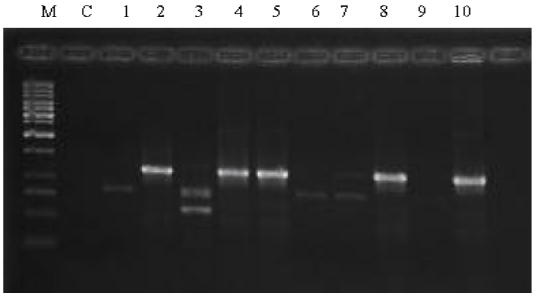

Fig. 11: Colony PCR for Bplps0012/*lpxB*. Clone screening analysis of Bplps0012/*lpxB* gene by PCR using specific primer gives the band size ~1182 bp. Clone in lane 1, 3, 6, 7 and 9 that do not contain cloned gene will be eliminated. M, 1 Kb DNA Ladder (Fermentas); C, Negative control

ORFs, only 19 ORFs successfully cloned into pTZ57R/T plasmid (Fig. 2-11). Putative genes Bplps0012 (*lpxD*), Bplps0018 (*waaE*), Bplps0019 (*udg*) and Bplps0020 (*waaC2*) were not successful cloned.

DISCUSSION

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. According to Wyckoff *et al.* (1998) inhibition of lipid A biosynthesis may kill most gram-negative bacteria, increases bacterial permeability to antibiotics and decreases endotoxin production. As a conclusion, interference of the lipid A biosynthesis mechanisms could be exploited for therapeutic intervention in melioidosis. Additional, the study of molecular properties of core oligosaccharide (Bennett-Guerrero *et al*., 2000; 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. A total of 21 ORFs were annotated and found located in a cluster which may be involved in biosynthesis of O-antigen in *B. pseudomallei*. All these ORFs were identified 100% similar as genes identified by David *et al.* (1998). According to the authors, this moiety is considered as type II O-antigen which is essential for *B. pseudomallei* serum resistance and virulence.

All the ORFs which provided the new information about *B. pseudomallei* biology are those that could be assigned as a probable function on the basis of similarity to established sequence motifs, but could not be assigned a definite name. Therefore, the ORFs' name can be given based on the similar protein and the sequence arrangement on *B. pseudomallei* genome. Due to the results of data mining in this study are based on *in silico*, all the annotated ORFs are considered putative or probable. Most of the genes involve in LPS biosynthesis are essential. For example, *lpxK*, *waaA*, *kdsB*, *waaF*, *lpxD*, *fabA*, *fabZ* and *fabH* were found as essential genes in DEG (Database of Essential Gene). Essential genes are genes that are indispensable to support cellular life. The functions encoded by this gene set are essential and could be considered as a foundation of life itself (Ren *et al*., 2004). It is even belief that some basic functions and principles are common to all cellular life on this planet. The importance in supporting cellular line emphasize the necessity to characterize and study the genes since most antibiotics target essential cellular processes and thus essential gene products of microbial cells represent promising new targets for antibiotics. In addition, it is well established that bacterial LPS is a permeability barrier that confers resistance to a variety of antimicrobial agents. Alteration of this barrier often leads to increased sensitivity to hydrophobic and cationic compounds (Nikaido, 1989).

The characterization of LPS could have a significant impact on the focus of antimicrobial and vaccine research. All of the ORFs which provided the latest new information about *B. pseudomallei* biology are those that could be assigned a probable function on the basis of similarity to established sequence motifs, but could not be assigned a definite name. Therefore, the ORFs' name was given base on the similar protein and the sequence arrangement on *B. pseudomallei* genome: for example, *BPlps0001/lpxL* Present study cannot predict exactly all gene components due to the limitation of our knowledge of complex biological processes and signals regulating gene expression. To confirm that the amplified PCR fragments are the correct sequence instead of an artifact PCR, all amplified PCR fragment were inserted into a plasmid vector pTZ57 followed by automated DNA sequencing. This method also allows the ORFs to be maintained in *E. coli* cell for future research. Plasmid vector pTZ57 contains 2 main genes, *ampR* and *lacZ*, which allows ampicillin selection and β-galactosidase activity, respectively.

Therefore the recombinant clones can be identified by blue and white selection. Ligation stage was achieved by adding T4 DNA ligase to solution containing PCR product and pTZ57R vector in 22°C overnight. The procedure was modified to overnight ligation since overnight ligation can produce more white colonies compared to 2 h ligation time. The vector is also an efficient means of amplifying cloned DNA because there are many copies per cell, as many as several hundred for some plasmids. Due to the *E. coli* XL1-Blue competent cell is sensitive to ampicilin, the cell that do not undergo transformation may not grow on the plate. The vector and the competent cell are suitable to be used in the current study. Verification for positive clones was performed by

RE digestion, colony PCR and DNA sequencing. In colony PCR, the colonies that grew on plate could be amplified for the respective gene using T7 forward primer and reverse specific primer. Only the right orientation of the insert can be amplified using such primers and showed approximately correct band size on agaros gel. It is because T7 forward primer prime on vector sequence could polymerize through out the insert and stop till reverse specific primer. No band was seen for those insert without ligation or with incorrect orientation.

Through this step, the orientation of the insert was verified. Restriction enzyme digestion was used to confirm the insertion of the ORFs by cutting the plasmid and observing the present of band after electrophoresis on agarose gel. This step reconfirmed the existence of the insert before proceeding to commercial sequencing. *EcoR1* and *HindIII* which were used in this double digestion of plasmid yielded 2 separate bands with the size approximately similar to the desired DNA size. The result showed that the cloning procedure was successful. DNA sequencing analysis was the last step and accurately provided the desire of information.

CONCLUSION

The results showed 98-99% matching of *B. pseudomallei* strain D286 ORFs with *B. pseudomallei* K96243. The results were not only verifying the successful of cloning of research DNA fragments, it also provide the inside view of the genetically relation or different between *B. pseudomallei* strain D286 and *B. pseudomallei* K96243. Furthermore, the comparison of these both strains are not yet been reported. Comparison of these 2 strains may contribute to future epidemiology study of these strains.

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