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DNA AMPLIFICATION OPTIMIZATION AND CLONING OF SEVERAL TARGET GENES FROM *BURKHOLDERIA PSEUDOMALLEI*

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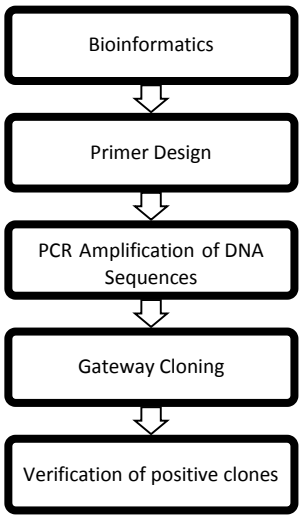
Graphical abstract



Abstract

Burkholderia pseudomallei is a saprophytic Gram-negative bacillus that is found in soil and surface water. It causes the disease melioidosis, which infect humans and animals. Melioidosis can be fatal in human, where it causes fever and commonly present with pneumonia, with or without septicaemia. Melioidosis is primarily endemic in Southeast Asia and Northern Australia, but has also been found in the Middle East, China and South America. It is challenging to treat melioidosis, as it is intrinsically resistant to many antibiotics, and can cause latent infection. By characterizing identified protein targets from *B. pseudomallei*, we can gain fundamental knowledge on how this bacterium behaves, and thus provide us strategies to combat them. We report here the recent progress of DNA amplification and cloning of four target genes from *B. pseudomallei* strain D286 performed in Kulliyyah of Science, IIUM. Genomic DNA of *B. pseudomallei* strain D286 is obtained from School of Biosciences & Biotechnology, UKM. The four target genes; *BPSL1612, BPSL1618, BPSL1691* and *BPSL2054* were chosen from 52 hypothetical proteins predicted to be essential in *B. pseudomallei* by transposon-directed insertion site sequencing (TraDIS) technique (Moule *et al.*, 2014). All four target genes were subjected to nested PCR amplification for subsequent Gateway™ cloning protocols, expression and purification studies.

Keywords: Melioidosis, *B. pseudomallei*, hypothetical proteins, Gateway™ cloning

1.0 INTRODUCTION

Burkholderia pseudomallei (also known as *Pseudomonas pseudomallei*) are a Gram-negative, bipolar, aerobic, motile rod-shaped bacterium. These bacteria derived from the genus Burkholderia. It can infect humans and animals and causes melioidosis disease. It is also capable of infecting plants. There is evidence that this bacterium does not cause disease in all individuals, instead the bacterium is persisting at

unknown sites in the body to reactivate later in life (1).

Melioidosis disease can be active as lethal acute infection or persist as a chronic infection before reactivating itself after several decades. According to Holden *et al.,* (1), it can cause death within the first 48 hours due to septic shock, even with optimal antimicrobial chemotherapy given. Melioidosis often affects individuals with pre-existing conditions associated with an altered immune response, the most common being is diabetes mellitus (50% of cases) (2, 3, 4).

This bacterium is normally found in soil or on water surface (1, 5). Infection can occur through inhalation, ingestion or through skin abrasions exposed to this bacterium. The most common examples of clinical manifestation are metastatic pneumonia, hepatic and splenic abscesses, demonstrating bacterial dissemination to distant sites (1, 2). At least 20 weeks of initial treatment is required with different intravenous and oral phases. Initial intravenous therapy is given for 10–14 days with ceftazidime or a carbapenem as drugs of choice (2).

B. pseudomallei is intrinsically resistant to many antibiotics, i.e. penicillin, ampicillin, first-generation and second- generation cephalosporins, gentamicin, tobramycin, streptomycin, and polymyxin (1). It is susceptible to amoxicillin-clavulanate, chloramphenicol, doxycycline, trimethoprim-sulphamethoxazole, ureidopenicillins, ceftazidime and carbapenems, imipenem, ciprofloxacin, and augmentin (2, 6).

In Malaysia, melioidosis is indicated as endemic in Pahang (incidence of 6.1 per 100,000 population per year) (3, 7), and has been recorded in Johor Bahru (8) and Kedah (incidence of 16.35 per 100,000 population per year) (4). In July 2010, a co-infection of melioidosis and leptospirosis occurs in an outbreak resulting in eight fatalities (9). In terms of mortality, the overall mortality from primary disease is from 50% to 51% in northeast Thailand (35% in children) and ~20% overall in the Northern Australia (1, 2). In Kedah, Malaysia, the overall mortality rate is 33.8%, and it increase to 48% among bacteremics (4).

The genome of *B. pseudomallei* (strain K96243 from Thailand was first to be fully sequenced) is known to be one of the largest and most complex genome (6). It comprises of two circular chromosomes of 4.07 megabase pairs and 3.17 megabase pairs that contains approximately 6,332 predicted coding sequences within 7.25 Mb of the two chromosomal DNA. The large chromosome encodes many of the core functions related with central metabolism and cell growth and the small chromosome carries more accessory functions associated with adaptation and survival in different niches (1, 2, 6). In addition, the *B. pseudomallei* genome demonstrates a high degree of plasticity, with frequent acquisition of genomic islands by horizontal transfer. This important feature of genetic evolution has resulted in a genetically diverse pathogenic species (1, 5).

Moule *et al.* (6) in 2014 has published a compilation of predicted essential genes library using transposon-directed insertion site sequencing (TraDIS) technique (6). The list includes hypothetical proteins or conserved hypothetical proteins with no functional data available. These hypothetical proteins may possess unique biological function that is important for the pathogenicity and viability of *B. pseudomallei.*

By characterizing identified protein targets from *B. pseudomallei,* we can gain fundamental knowledge on how this bacterium behaves, and thus provide us strategies to combat them. To better understand this complex bacterium and to identify new drug targets, we seek to investigate and characterize several identified essential target genes from *B. pseudomallei*.

2.0 EXPERIMENTAL

2.1 Bioinformatics

Target genes were selected from the TraDIS library list of 52 hypothetical proteins predicted to be essential, published by Moule *et al.,* 2014 (6). Bioinformatic analysis was performed to choose four suitable target genes. The gene and protein sequences for target open reading frames were retrieved from the geneDB database (http://www.genedb.org/Homepage). Protein sequence data was analysed using various ExPasy proteomics tools (http://www.expasy.org/proteomics). Searches for related protein sequences were carried out using the National Center for Biotechnology (NCBI) website with BLAST (Basic Local Alignment Search Tool against the non-redundant (nr) database, or the Protein Data Bank (PDB) sequence database to identify structures with similar amino acid sequences.

2.2 Primer Design and PCR Amplification

The open reading frame encoding the selected hypothetical proteins (*BPSL1612, BPSL1618, BPSL1691, BPSL2054*) was amplified from *B. pseudomallei* strain D286 genomic DNA using the oligonucleotide sequences of primers outlined in Table 1. Primers were purchased from Integrated DNA Technologies (IDT). The purity grade was 'desalted'. They were re-dissolved in sterile Milli-Q water to a concentration of 100 µM and stored at -20°C. The genomic DNA was sourced from School of Biosciences & Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

Primers were designed for directional cloning of inserts into the Gateway cloning system (10). A two-stage nested PCR strategy was used to flank the target sequences with the *att*B recombination sequences for Gateway cloning (11).

An rTEV protease-cleavage site was included in between the *att*B sequence and the original ORF translational start. The first round of PCR used the

gene-specific primers (Table 1) to amplify the gene of interest. The second PCR utilized the product from the first round of PCR as template and used generic primers (Table 2) to incorporate the *att*B sites required for the Gateway BP recombination reaction. PCR product sample from each run was purified and quantified before being used.

Table 1 Gene-specific primers used for the amplification of selected hypothetical protein gene constructs

Constructs	Primer Sequences (5'-3')		T_m	%GC
BPSL1612	Forward	GGC AGC GGC GCG ATG AAA ACG AAC GGC AAG	69.7	63.3
	Reverse	GAA AGC TGG GTG CTA CGC GCG GGA TGC GTC	70.4	66.7
BPSL1618	Forward	GGC AGC GGC GCG ATG AGG GAA ATT CTC GAA	68.4	60.0
	Reverse	GAA AGC TGG GTG TCA GTG GGC GAG CGC CAG	70.3	66.7
BPSL1618_2nd construct	Forward	CTG TAT TTT CAG GGC AGC GGC GCG ATG AGG GAA ATT CTC GAA ACA	69.4	51.1
	Reverse	GAA AGC TGG GTG TCA GTG GGC GAG CGC CAG TTT	70.3	60.6
BPSL1691	Forward	GGC AGC GGC GCG ATG AGT GAT TCT GTC AGT	68.2	60.0
	Reverse	GAA AGC TGG GTG TCA GAG CGC ATG ATC GGC	67.3	60.0
BPSL2054	Forward	GGC AGC GGC GCG ATG CGA CGC GAG ACT CGC	74.8	76.7
	Reverse	GAA AGC TGG GTG TCA TGC CGG ATG CCA GGT	68.6	60.0
BPSL2054_2nd construct	Forward	TAT TTT CAG GGC AGC GGC GCG ATG CGA CGC GAG ACT CGC	73.3	64.1
	Reverse	GAA AGC TGG GTG TCA TGC CGG ATG CCA GGT TGG ATT CGC CGC	72.9	61.9

Table 2 Generic primers used for the amplification of Gateway recombination sites

Constructs	Primer Sequences (5'-3')	
Generic_Forward_attB1	Forward	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC GAA AAC CTG TAT TTT CAG GGC AGC GGC GCG
Generic_Reverse_attB2	Reverse	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTG

Gradient PCR was performed at the initial stage to optimize the annealing temperature for specific primers used, among others. The T_m values for the forward primer and reverse primer were noted to test a range of temperature points for the gradient PCR. Two Taq polymerase kits were tested; (1) MyTaq™ Polymerase (Bioline), and (2) Primestar® HS DNA Polymerase (Takara). The typical PCR steps for each MyTaq™ Polymerase and Primestar® HS DNA Polymerase are outlined in Table 3 and Table 4 respectively. The presence of DNA was visualized with 1.0 % (w/v) agarose gel electrophoresis under UV light. The second round of PCR utilized nested PCR steps (Table 5) to incorporate the *att*B sites.

Table 3 Typical PCR cycle for MyTaq™ Polymerase (Bioline) kit (based on published PCR protocol (12)). Optimal annealing temperature (xx°C) is pre-determined using gradient PCR

Step	Temperature	Time	Cycle
Initial denaturation	96°C	5 min	1 cycle
Denaturation	96°C	1 min	30 cycles
Annealing	xx°C	2 min	
Extension	72°C	2 min	
Final Extension	72°C	20 min	1 cycle
End/Hold	4°C	To hold	-

After presence of DNA band at approximately the correct size was confirmed, the PCR product sample was purified using PCR cleanup kit (Axygen) or gel-purified using PCR Gel Cleanup Kit (Axygen). DNA was quantified by using Nanodrop 2000.

Table 4 Typical PCR cycle for Primestar® HS DNA Polymerase (Takara) kit. Optimal annealing temperature (xx°C) is pre-determined using gradient PCR

Step	Temperature	Time	Cycle
Initial denaturation	98°C	5 min	1 cycle
Denaturation	98°C	10 sec	30 cycles
Annealing	xx°C	5 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	1 cycle
End/Hold	4°C	To hold	-

Table 5 Nested PCR cycle for *att*B sites recombination

Step	Temperature	Time	Cycle
Initial denaturation	96°C	5 min	1 cycle
Denaturation	96°C	30 sec	5 cycles
Annealing	45°C	30 sec	
Extension	68°C	1 min	
Denaturation	96°C	30 sec	25 cycles
Annealing	55°C	30 sec	
Extension	68°C	1 min	
Final Extension	68°C	10 min	1 cycle
End/Hold	4°C	To hold	-

PCR products were recombined (BP reaction) with pDONR221 to generate Entry clones. Approximately

40 femtomoles of nested PCR product were used in a BP reaction. For a 5 µL BP reaction, the components consisted of 40 fmol PCR product, 150 ng/µL pDONR221 vector, 1X BP clonase buffer, 1X TE buffer, and 1 µL BP clonase. The reaction was incubated at room temperature for 18 hours. To terminate the reaction, 0.5 µL Proteinase K was added and the reaction was incubated at 37°C for 10 minutes. The recombination product was transformed into *E. coli* DH5α cells using heat-shock method, and plated onto LB-agar plate supplemented with 50µg/mL Kanamycin. Positive *att*L-flanked entry clones containing the gene of interest were screened by *Bsr*GI restriction digest and colony PCR.

3.0 RESULTS AND DISCUSSION

3.1 PCR Amplification

Three out of four genes were successfully amplified and purified (Figure 1 and Figure 2). One gene, *BPSL1612* was readily amplified by MyTaq™ Polymerase (Bioline). *BPSL 1691* could only be amplified using Primestar® HS DNA Polymerase (Takara). New primer constructs were designed for three other genes, *BPSL1618, BPSL1691* and *BPSL2054*, to increase the probability it can be amplified. *BPSL1618* and *BPSL1691* gene constructs were then successfully amplified by using Primestar® HS DNA Polymerase (Takara). *BPSL2054* gene constructs however, could not be amplified using both primer sets and taq polymerases. The optimization of PCR amplification of *BPSL2054* has not been exhausted however, and will be pursued in the near future. The PCR amplification results are summarized in Table 6.

3.2 Cloning Experiments

The cloning experiments are still in progress. We attempted transformation of *BPSL1612, BPSL1618* and *BPSL1691* nested PCR products using heat-shock method, but no colony growth was observed for both *BPSL1612* and *BPSL1618* recombination products. A false positive result was obtained for *BPSL1691*. Using *Bsr*GI restriction digest and colony PCR, we confirmed that the colonies growing on LB-agar plate supplemented with 50µg/mL Kanamycin do not contain the gene of interest (data not shown). Future transformation experiments will be performed using fresh competent *E. coli* cells and adopting electroporation method to improve transformation efficiency. From this ongoing study, we currently possess three purified PCR products for three hypothetical genes from *B. pseudomallei*, namely *BPSL1612, BPSL1618* and *BPSL1691*, at an acceptable concentration and purity ready for subsequent cloning, expression and purification experiments.

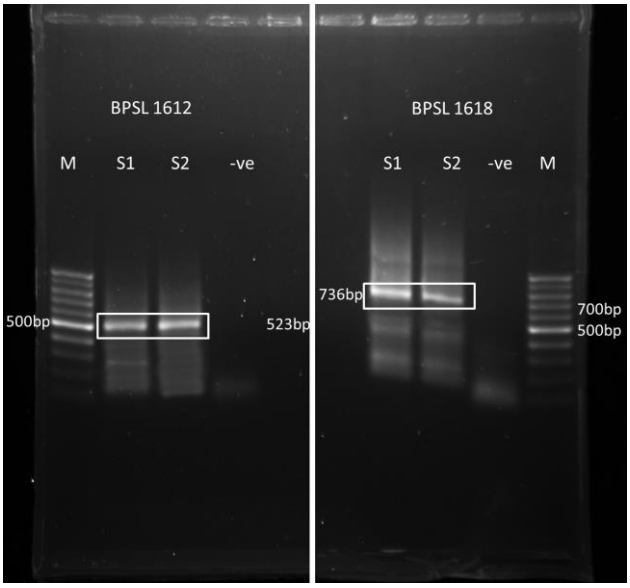


Figure 1 PCR products for *BPSL1612* and *BPSL1618* gene constructs. S1: Sample replicate 1, S2: Sample replicate 2, -ve: negative control, M: 100bp molecular marker (Vivantis)

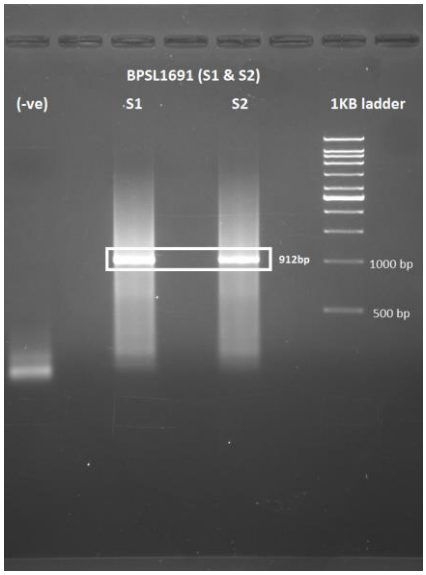


Figure 2 PCR products for *BPSL1691* gene construct. S1: Sample replicate 1, S2: Sample replicate 2, -ve: negative control, 1KB ladder: 1kb molecular marker (Vivantis)

.

Table 6 A summary of results

Target Gene	Nucleo-tide length	pI	Amino acid/ mass (kDa)	PDB BLASTp	Gateway PCR		DNA Purity (A_{260}/A_{280})	Cloning
					MyTaq™ Polymerase (Bioline)	Primestar® HS DNA Polymerase (Takara)		
BPSL1612	523bp	pH 10.3	161/ 17.3	Crystal structure of Dextranase from Streptococcus mutans (30%)	✓	✓	1.85	In progress
BPSL1618	736bp	pH 5.5	232/ 26.6	No similarity	✗	✓	1.88	In progress
BPSL1691	912bp	pH 5.0	303/ 32.9	Crystal structure of a Duf692 Family Protein from Haemophilus Somnus (31%)	✗	✓	1.68	In progress
BPSL2054	477bp	pH 11.9	158/ 18.0	Crystal structure of a Mammalian Reductase (35%)	✗	✗	-	Repeating PCR

4.0 CONCLUSION

The amplification of hypothetical genes from *B. pseudomallei* proves to be a challenging process. This is mainly due to the high G-C content and subsequently, a high annealing temperature. More strategies need to be adopted to improve this. We first attempted PCR amplification of four hypothetical genes using MyTaq™ polymerase (Bioline). Only one out of four genes, *BPSL1612* was successfully amplified at first trial. After re-design of primers and adopting high fidelity Primestar® HS DNA polymerase (Takara), two out of four genes, *BPSL1618* and *BPSL1691* were successfully amplified. This study has provided the optimized PCR amplification procedures for three target genes predicted to be essential in *B. pseudomallei*. This important data can be utilized to prepare sufficient DNA material for future Gateway cloning experiments on *B. pseudomallei,* and subsequently for its protein expression and purification assays. At the time of writing this article, one out of the four targeted genes, *BPSL2054* has not been successfully amplified.

Meanwhile, we have yet to obtain positive results in cloning the three successfully amplified genes of interests, *BPSL1612, BPSL1618* and *BPSL1691*. The results are very preliminary, and we anticipate to successfully clone all three target genes in the near future.

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References

[1] Holden, M. T. G., Titball, R. W., Peacock, S. J., Cerdeño-Tárraga, A. M., Atkins, T., Crossman, L. C., ... & Parkhill, J. 2004. Genomic Plasticity of the Causative Agent of melioidosis, Burkholderia pseudomallei. *Proceedings of the National Academy of Sciences of the United States of America*. 101(39): 14240-14245.

[2] Wiersinga, W. J., Van der Poll, T., White, N. J., Day, N. P., & Peacock, S. J. 2006. Melioidosis: Insights Into the Pathogenicity of Burkholderia pseudomallei. *Nature Reviews Microbiology*. 4(4): 272-282.

[3] Jabatan Kesihatan Negeri Pahang. 2012. Guidelines for Clinical and Public Health Management of Melioidosis in Pahang. *Jabatan Kesihatan Negeri Pahang*. 1-55. Accessed through http://jknpahang.moh.gov.my/v3/uploaddir/muatturun/204PAHANG%20MELIOIDOSIS%20GUIDELINE%20170311.pdf.

[4] Hassan, M. R., Pani, S. P., Peng, N. P., Voralu, K., Vijayalakshmi, N., Mehanderkar, R., ... & Michael, E. 2010. Incidence, Risk Factors and Clinical Epidemiology of Melioidosis: A Complex Socio-Ecological Emerging Infectious Disease in the Alor Setar Region of Kedah, Malaysia. *BMC Infectious Diseases*. 10(1): 302.

[5] Wiersinga, W. J., Currie, B.J., Peacock, S. J. 2012. Melioidosis. *The New England Journal of Medicine*. 367: 1035-1044.

[6] Moule, M. G., Hemsley, C. M., Seet, Q., Guerra-Assunção, J. A., Lim, J., Sarkar-Tyson, M., & Wren, B. W. 2014. Genome-Wide Saturation Mutagenesis of Burkholderia pseudomallei K96243 Predicts Essential Genes and Novel Targets for Antimicrobial Development. *MBio*. 5(1): e00926-13.

[7] How, S. H., Ng, K. H., Jamalludin, A. R., Shah, A., & Rathor, Y. 2005. Melioidosis in Pahang. *Med J Malaysia*. *60*(5): 606-613.

[8] Pagalavan L. 2005. Melioidosis: the Johor Bahru Experience**.** *Med J Malaysia.* 60(5): 599-605.

[9] Sapian, M., Khair, M. T., How, S. H., Rajalingam, R., Sahhir, K., Norazah, A., ... & Jamalludin, A. R. 2012. Outbreak of

Melioidosis and Leptospirosis Co-Infection Following A Rescue Operation. *Med J Malaysia*. 67(3): 293-7.

[10] Moreland, N., Ashton, R., Baker, H. M., Ivanovic, I., Patterson, S., Arcus, V. L., ... & Lott, J. S. 2005. A Flexible and Economical Medium-Throughput Strategy for Protein Production and Crystallization. *Acta Crystallographica Section D: Biological Crystallography*. 61(10): 1378-1385.

[11] Landy, A. 1989. Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. *Ann. Rev. Biochem.* 58: 913-949.

[12] Suang, L. L., Sekawi, Z., Al-Haj, N. A., Shamsudin, M. N., Abdullah, R., & Mohamed, R. 2009. Molecular Procedure for Detection of *Burkholderia pseudomallei*. *Research Journal of Applied Sciences*. 4(2): 73-7.