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The effect of *Bpsl0279* mutation on biofilm formation in *Burkholderia pseudomallei*

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Abstract

Burkholderia pseudomallei is a causative agent of a fatal disease, melioidosis, which needs prolonged antibiotic treatment. It can produce biofilms that play some roles in either antibiotic resistance or relapse. Knowledge related to gene(s) that controlling biofilm formation in *B. pseudomallei* is still limited. From bioinformatics analysis, *bpsl0279* and *bpsl1080*, the hypothetical genes in *B. pseudomallei* K96243, were found to be homologous with some of 80 biofilm related genes in other bacteria. Reverse transcription polymerase chain reaction (RT-PCR) showed their expression to be higher when growing in biofilm conditions compared to planktonic. Mutagenesis of *bpsl0279* gene led to significantly lower biofilm productions. Approximately 75% of biofilm formation was reduced in *Δbpsl0279* in static and easily observed in dynamic laminar shear conditions that can be restored by its complementation. The *Δbpsl0279* formed only small microcolonies of 10-20 µm in diameter while the wild type established the roughness macrocolonies (> 50 µm) and reached 100 µm after 48 h. In addition, *gfp*-tagged wild type attached to the glass surface (264 ± 32 cells/field) significantly better than the mutants (120 ± 30 cells/field). The *bpsl0279* was later reported as a putative flagella brake protein YcgR1 and was homolog with *bth_i0249* in *Burkholderia thailandensis*. This gene contains PilZ domain, of which is a c-di-GMP binding and involved in many aspects of biofilm formation. Our study concluded *bpsl0279* to be involved in the early stage of biofilm formation that may be a good target to interrupt for the benefit of treatment.

Keywords: YcgR1, Biofilm, *Burkholderia pseudomallei*, Mutagenesis, Flow cell, *Bpsl0279*

1. Introduction

Bacterial colonization is initiated by the attachment of freely suspended planktonic cells. They subsequently develop into a complex group of organisms encased in a polymeric matrix known as biofilms [1] in response to environmental changes such as a low nutritional source, oxidative stress, or exposure to sub-inhibitory concentrations of antimicrobial agents. Biofilms are a dynamic biological cycle includes initiation, maturation, maintenance, and dissolution of cells in the biofilms returning to a planktonic lifestyle [2]. Importantly, more than 80% of chronic bacterial infections are associated with biofilms [3]. Furthermore, bacteria in biofilms exhibited significantly higher resistance to antimicrobial agents and the host immune systems than their planktonic counterparts [3].

B. pseudomallei is a causative agent of a fatal disease, melioidosis, and recognized as the Tier 1 select agent by CDC, USA [4]. The hot spots of endemic areas were reported in Southeast Asia and northern Australia. In northeast Thailand, melioidosis was reported as the third most common cause of death among infectious diseases [5]. *B. pseudomallei* was reported to produce biofilms and microcolonies [6], however, there was no correlation between the capacity to produce biofilm and their virulence [7]. Despite these findings, the high mortality and relapse rates of *B. pseudomallei* infection compared to other bacterial infections are still of concern [8,9]. Growing

B. pseudomallei in biofilm stimulating conditions could induce the bacteria to be more resistant to some antimicrobial agents tested when compared to the planktonic counterpart [10]. Moreover, the later study provided the first evidence that relapse patients were associated with biofilm formation of the primary infecting isolate [11]. Nevertheless, knowledge about gene(s) that control the biofilm formation is still limited.

In this study, the microarray data obtained from other Gram-negative bacteria that differentially expressed in biofilms form when compared with the planktonic cells were analyzed. We could identify a candidate *bpsl0279* gene of the YcgR superfamily proteins containing the PilZ conserved domain in *B. pseudomallei* K96243 that plays a role in biofilm formation. The knock-out mutant, *B. pseudomallei* K96243 *Δbpsl0279*, was constructed and some phenotypes was evaluated in both static and the flow cell system that may provide us some clues to alter the biofilm formation in the future.

2. Materials and methods

2.1 Bacterial strains, growth conditions, and plasmids

B. pseudomallei K96243 is a sequenced strain obtained from a septicemic patient admitted in Khon Kaen hospital, Khon Kaen province, Thailand that used to construct a *Δbpsl0279* mutant. The Luria-Bertani (LB) medium was used to culture *B. pseudomallei* K96243 and *Escherichia coli*. The 100 µg/mL of ampicillin (Ap) was used in *gfp-B. pseudomallei* selection. MM35 is a flagellum-lacking *fliC* mutant of *B. pseudomallei* 1026b that used as a negative control in motility study [12]. Antibiotics were used to maintain some strains which are 5 µg/mL gentamicin (Gm) and 60 µg/mL tetracycline (Tc) for the *bpsl0279* mutant strain, Tc, Gm, and Cm for the complemented strain. The modified Vogel and Bonner's (MVBM) minimal medium was used as biofilm-induced condition [7]. The details of plasmids in this study are listed in Table 1.

2.2 Bioinformatics analysis

Criteria for candidate genes of interest in this study are those that showed higher expression in biofilm culture and ultimately be involved in biofilm function in Gram-negative bacteria. Forty-five candidate genes were selected from microarray data of four pathogens, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Vibrio cholera*, that differentially expressed in biofilms compared with planktonic cells [13-16]. In addition, 35 candidate genes from other pathogens published in literature that had been identified to involve in biofilm functions were also included. Amino acid sequences of the 80 (45+35) candidate genes (Appendix 1) were used to compare with the sequence of *B. pseudomallei* K96243 by using Blastp program. In this study, the novel genes involved in biofilm formation in *B. pseudomallei* were identified. The hypothetical genes were selected based on these criteria as described in Appendix 1. Based on these criteria, the *bpsl0279* and *bpsl080* showed the highest homology scores and may indicate their roles to be involved in biofilm formation. The *bpsl0279* encoded a 252 amino acids hypothetical protein that similar to *E. coli* YcgR protein, of which functioned as motility regulatory protein. The *bpsl1080* encoded 786 amino acids hypothetical protein that similar to *P. aeruginosa* signaling protein that has been reported to function as regulatory protein during biofilm formation.

Table 1 Plasmids used in this study.

Plasmids	Characteristics	References
pKNOCK-Tc	Mobilizable suicide vector, Tc^r	[17]
pGEM-T easy vector	TA cloning vector, Ap^r	Promega
pGEM-*bpsl0279*	pGEM-T easy vector containing a 515-bp internal fragment of *bpsl0279* gene, Ap^r	this study
pGEM-F*bpls0279*	pGEM-T easy vector containing full length of *bpsl0279* gene, Ap^r	this study
pBBR1MCS	Broad-host-range expression vector, Cm^r	[18]
pBBR1MCS-*bpsl0279*	pBBR1MCS containing full length of *bpsl0279* gene, Cm^r	this study

Table 2 Oligonucleotides used in this study.

Primers	Nucleotide sequences (5' to 3')	Corresponding genes	References
RT0279-F	TTGCGCAACCTCGTCA	*bpsl0279*	this study
RT0279-R	ACGAGTTGCAGGTCGA		
RT1080-F	ACGACCAGGGCTTCGA	*bpsl1080*	this study
RT1080-R	GCGCCTGCTTGAGGTA		
SL0279-F	AAACCCGTTACGAGAACCTG	*bpsl0279*	this study
SL0279-R	GCACTTCGAACAGGAAGGTC		
C0279_F	ACTTGGGTAC*CAACCATCTGCTGCGCTGCGTTG	*bpsl0279*	this study
C0279_R	CTTACTCTAGA*GTGCGACGCGCATCGCACGGC		
pKNOCK-Tc specific primer	CACTTAACGGCTGACATGG	pKNOCK-Tc vector	this study

*Underlines indicate restriction endonuclease cleavage sites.

2.3 Validation of gene expression

The expression of the selected genes in biofilm and planktonic forms was confirmed by using Reverse transcription polymerase chain reaction (RT-PCR), to amplify *bpsl0279* and *bpsl1080* mRNA extracted when *B. pseudomallei* K96243 was grown in either planktonic or biofilm conditions [7]. For planktonic growth condition, the bacteria were cultured in LB broth at 37°C with 200 rpm agitation using Shaker Incubator (New Brunswick, Eppendorf, Germany) for 18 h to reach the mid-log state. For biofilm growth conditions, the bacteria were grown in 24-well plates at 37°C under static conditions using modified Vogel and Bonner's medium (MVBM), representing 48-hour biofilm bacteria. Total RNA was extracted from planktonic and biofilm samples using TRIZOL reagent (Invitrogen, USA). The contaminated genomic DNA was removed by adding 2 U of RNase-Free DNase (Promega, Madison, WI) in a final volume of 20 µL. Total RNA samples were reverse transcribed into cDNA first strand using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (RT) (Invitrogen, USA) with random primers. An aliquot of the first strand cDNA was then amplified with RT0279-F and RT0279-R primers for *bpsl0279* or RT1080-F and RT1080-R primers for *bpsl1080* (Table 2). The amplification reactions were performed for 35 cycles with the PCR profiles of 1-minute denaturation at 95°C, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. The 16S rRNA gene was used as an internal control. The PCR product was separated on 1% agarose gel, stained, and analyzed by a gel documentation (Syngene, USA). The expression level was analyzed based on the density of PCR products bands by using ImageJ analysis program.

2.4 Construction of bpsl0279 mutant (Δbpsl0279)

The SL0279-F and SL0279-R primers (Table 1) were used to obtain a 515-base pair (bp) internal fragment of the *bpsl0279* gene from *B. pseudomallei* K96243 genomic DNA and cloned into pGEM-T Easy plasmid vector (Promega, Wisconsin, USA). The fragment was then sub-cloned into *Not*I and *Sal*I-digested pKNOCK-Tc plasmid [17] to generate pKNOCK-SL0279 and electroporated into *E. coli* S17-1λpir for further mobilized into *B. pseudomallei* K96243 by conjugation [19]. LB supplemented with Tc and Gm was used to select the transconjugants and confirmed by PCR, Southern blotting, and sequencing.

2.5 Construction of a Δbpsl0279 complemented strain

Primers C0279-F and C0279-R containing *Kpn*I and *Xba*I restriction sites were used to obtain a 759 bp of the full-length *bpsl0279* gene (Table 1) and cloned into pGEM-T easy vector to create a pGEM-FULL0279. The correct sequence of the plasmid was confirmed before sub-cloned into *Kpn*I-*Xba*I sites of the expression plasmid PBBR1MCS-Cmr [18] to create pBBR1MCS-bpsl0279. The recombinant plasmid was sequenced and the transconjugant was obtained as previously described (Appendix 2).

2.6 Construction of gfp-tagged B. pseudomallei

The *gfp*-tagged *B. pseudomallei* wild type, *Δbpsl0279* and *Δbpsl0279* complementation strains were constructed by using pAL778 plasmid and the helper plasmid, pTNS3 (kindly provided by Prof. Ben Adler, Monash University, Clayton, VIC, Australia). To create the *gfp*-tagged bacteria, the triparental conjugation method described by Choi et al. [20] was performed for transferring the *gfp* carrying plasmid (pAL778) into *B.*

pseudomallei genome. The *gfp*-tagged strains were used in attachment and Air-Liquid Interface (ALI) assay and observed under fluorescence microscopy.

2.7 Determination of bacterial growth rates

One percent overnight culture of *Δbpsl0279*, wild type and the *Δbpsl0279* complemented strains were inoculated into 100 mL of LB medium in 250 mL Erlenmeyer flask and cultured at 37°C with shaking at 200 rpm for 24 h. In time course of 2 h, 1 mL of each culture was taken to measure the OD540nm to obtain the bacterial growth rate.

2.8 Quantification of biofilm formation

To quantify the relative amount of bacterial biofilm formation, the approach following the method of Stepanovic *et at.* [21] was performed. Two hundred microliters of the 0.8-0.9 OD540nm culture of wild type, *Δbpsl0279*, and *Δbpsl0279* complemented strains in MVBM medium were applied into eight wells of a sterile 96-well flat-bottomed plate (Nunclon™, Roskilde, Denmark) and medium alone was served as a negative control. The plates were incubated at 37°C for 3 h in static condition for bacterial adhesion and then replaced the medium with 200 µL MVBM before continuing cultured for 21 h, washed with 200 µL of sterile distilled water, refilled with 200 µL MVBM and incubated for another 24 h. Lastly, the plates were washed for three times to obtain 2-day biofilms. Subsequently, the biofilms were stained and measured at 630nm using a microtiter plate reader. *B. thailandensis* UE5 was used as a biofilm reference strain to normalize across the plates. The relative capacity of biofilm formation was compared by Student's *t*-test.

To evaluate the static biofilm phenotypes, the 18-hour broth cultures of wild type and *Δbpsl0279* strains were grown on glass cover slips (22 by 22 mm) and submerged horizontally in a six-well plate (Greiner bio-one, Frickenhausen, Germany). The attached bacteria after 48 h of incubation represented a 48-hour biofilm culture under static conditions.

2.9 Biofilm formation in dynamic flow cell conditions

An overnight culture of *B. pseudomallei* wild type and the mutant in MVBM were adjusted to 0.8-0.9 OD540nm and 5 mL were used aseptically to inject into each flow channel of flow cell (BioSurface Technologies, Montana, USA). The flow system was set up as described previously [22] in 1 mm individual square glass tubing (0.15 mm wall). Briefly, the bacterial cells were first allowed to initiate attachment for 30 min before a flow of 10% MVBM was applied at the rate of 3 mL/h using a MasterFlex® pump set (Cole-Parmer, USA). *B. pseudomallei* biofilm formation was monitored at 24 and 48 h under laminar shear conditions.

2.10 Microscopy and image acquisition

B. pseudomallei biofilms that grown on glass cover slips were fixed by glutaraldehyde in 6-wells plates. In preparation for microscopy, 1 mL SYTO9/PI (Live/Dead BacLight Bacterial Viability Kits; Invitrogen, USA) was added to the 24-h and 48-h biofilms on the cover slips, covered the plates with aluminum foil and incubated for 30 minutes at room temperature.

For the flow cell studies, the biofilms were stained with the Live/Dead BacLight Bacterial Viability Kits (Invitrogen, USA) by using the mixture of 3 µL SYTO9 and 3 µL Propidium iodide (PI) in 1mL 1x PBS buffer. One milliliter of the mixture was injected to each channel using sterile 1 mL syringe with 26½ gauge needle. Biofilms were shielded with aluminum foil to prevent light interference while staining for 30 minutes at room temperature and then observed under a Zeiss LSM 510 Meta confocal microscope and the 3D structure images were analyzed with LSM Image Browser software (Carl Zeiss, Germany).

2.11 Attachment and air-liquid interface (ALI) assay

An attachment assay was used to determine the attachment capacity of wild type, *Δbpsl0279* and *Δbpsl0279* complemented strains at the early phase as previously described with some modifications [23]. All strains that were tagged with *gfp* were cultured from 2% inoculum (v/v) in MVBM for 18 h and then adjusted to 0.8-0.9 OD540nm and 2 mL were added onto glass cover slips (22 by 22 mm) and then submerged (horizontally) in MVBM medium in 24-well plates (Greiner bio-one, Frickenhausen, Germany). The plates were incubated for 30 minutes at 37°C, washed twice with sterile distilled water. The *gfp*-tagged bacterial cells were counted under a fluorescence microscope (Nikon, Japan) and presented as the average number of bacteria per field (magnification, x40) for 14 fields.

To study the later stage of microcolony formation at the area between medium and air, the air-liquid interface (ALI) [24] was used. A 24-well plate was placed horizontally at an angle of 30° to 50°. The *gfp*-tagged bacterial cultures for inoculation were prepared as in attachment assay. Three hundred microliters of diluted culture were transferred into a separate well in the angled 24-well plates, covered with a lid and incubated at 37°C for 24 h. Each well was gently aspirated and washed twice with 400 µL sterile medium and then 200 µL of fresh medium was added into each well before observed by an inverted microscope or fluorescence microscopy (Nikon, Japan).

2.12 Motility assay

To assess the swimming motility, the experiment was conducted on tryptone swim plates (1% tryptone, 0.5% NaCl, 0.3% agar) that were inoculated with a sterile toothpick and followed by incubation for an overnight at 37°C. The motility under either aerobic or anaerobic growth conditions was examined by incubating the plates in an incubator or anaerobic jars including GasPak (Oxoid, UK). The motility was qualitatively assessed by measuring the circular turbid zone formed by the bacterial cells moving away from the inoculation point [25]. The assay was performed in three independent experiments.

2.13 Statistical analysis

The statistical significance of the biofilm mass thickness data, the thickness of biofilm masses of wild-type and mutant strains were compared by the student's *t* test. The data were compared at each time point and $p < 0.05$ was considered to be significant.

3. Results

3.1 Bpsl0279 as a candidate gene related to biofilm formation in B. pseudomallei

By comparing the amino acids sequences of 80 biofilm-related genes from other organisms to those unknown function genes in *B. pseudomallei* K96243 by Blasp program, *bpsl0279* and *bpsl1080* were selected. The relative expression of both genes was measured by semi-quantitative one-step RT-PCR using RNA samples extracted from the *B. pseudomallei* K96243 wild type strains cultured in planktonic and biofilm growth conditions. The *bpsl0279* and *bpsl1080* were significantly expressed higher in biofilm growing condition when compared to planktonic, of which prominently observed in *bpsl0279* (Figure 1). To confirm such finding, the *bpsl0279* was therefore selected for knock out using pKNOCK-Tc suicide vector and performed the phenotypic studies.

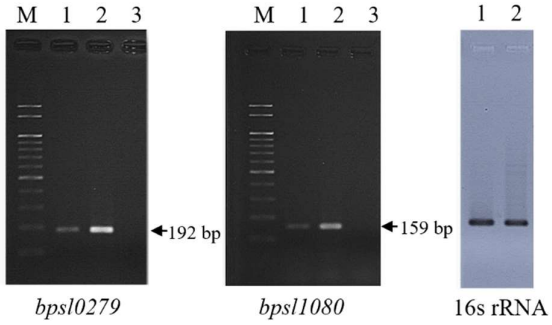

Figure 1 Gel electrophoresis of RT-PCR products from *bpsl0279* and *bpsl1080* genes in *B. pseudomallei* K96243 growing in planktonic and biofilm conditions. Total RNA obtained from B. pseudomallei K96243 when growth in planktonic (lane 1), or biofilm conditions (lane 2) were used to perform RT-PCR using primers designed to detect *bpsl0279* or *bpsl1080*. The primers designed for amplification of 16S rRNA were used as an internal control for gene expression. M: 100 bp DNA marker.

3.2 Mutagenesis of the bpsl0279 in B. pseudomallei K96243 (Δbpsl0279)

The *bpsl0279* was successfully knock out in *B. pseudomallei* K96243 as verified by PCR amplification using primers designed to detect the presence of the 650 bp insertion. (Figure 2A, 2B). The gene interruption by pKNOCK was also confirmed by Southern blot hybridization using *Δbpsl0279* and wild type genomic DNA digested with *EcoR*I/*Not*I and hybridized with DIG-labeled 600-bp tetracycline resistant gene probe. The 2.7-kb band of digested product could only be detected in *Δbpsl0279* but not in the wild type (Figure 2B).

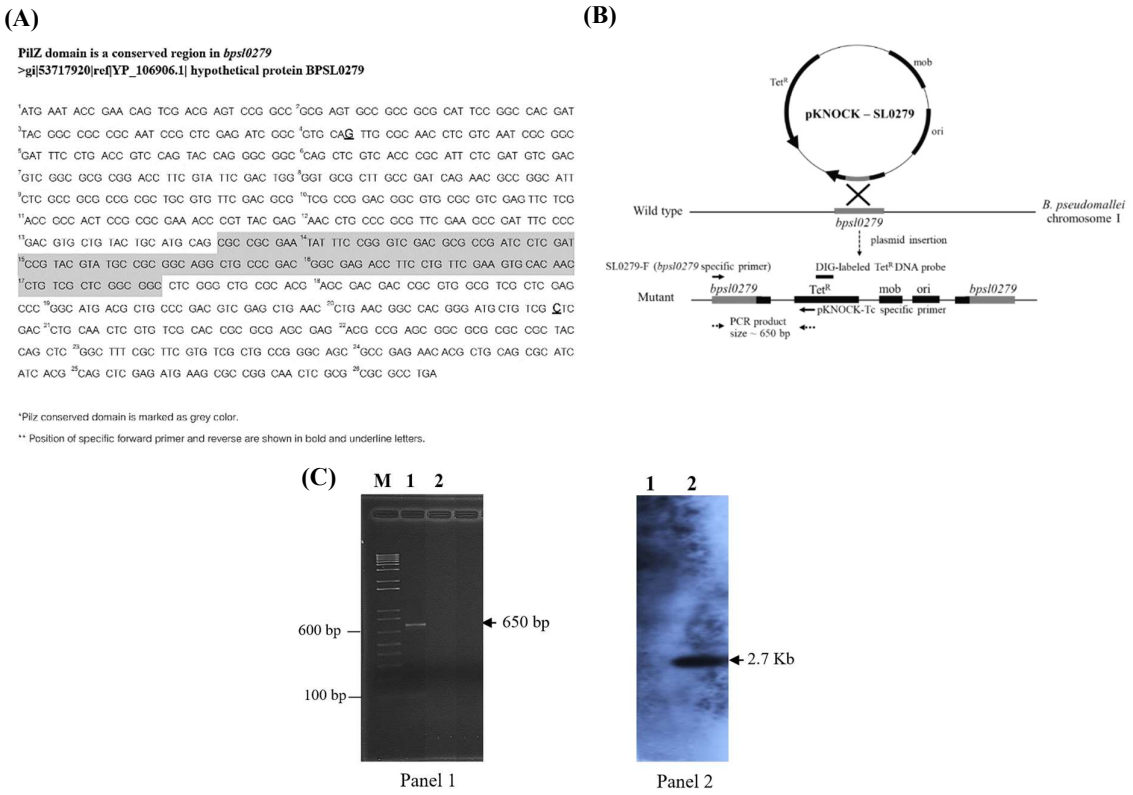

Figure 2 Mutagenesis of *bpsl0279* gene on the *B. pseudomallei* chromosome. (A) Map of *bpsl0279* and its pilZ conserved domain was shown with the position of specific forward and reverse primers for the internal fragment of *bpsl0279*. (B) Schematic diagram demonstrates the construction of *Δbpsl0279* strain. Chromosomal integration of suicide vector on wild type chromosome occurred by the allelic exchange between *bpsl0279* sequences presented on the constructed plasmid and the homologous allele on the chromosome sequence. (C) Agarose gel electrophoresis of the 650 bp PCR products (Panel 1) from *Δbpsl0279* (lane 1) and wild type (lane 2) using SL0279-F and pKNOCK-Tc specific primers and Southern blot hybridization (Panel 2). For Southern blot, *B. pseudomallei* genomic DNA from wild type (lane 1) and *Δbpsl0279* (lane 2) were digested with *Eco*RI/*Not*I and hybridized with DIG-labeled 600-bp tetracycline resistant gene probe. The 2.7 kb band of digested product only can be detected in lane 2 but not in lane 1 of the wild type. Lane M: 100 bp DNA marker.

3.3 The impairment of biofilm formation in Δbpsl0279 strain

The *Δbpsl0279* mutant strain produced biofilms approximately 75% lower than the wild type ($p < 0.05$) (Figure 3A). Moreover, the biofilm formation was restored in the complementation strain to have similar level as measured in the wild type. We further investigated the structure of the biofilms produced by the mutant under static and laminar shear growth conditions. Under static growth condition, wild type biofilms exhibited a uniformly flat structure, homogeneously covered the glass surface and produced the biofilm mass with thickness of 6.5 µm. On the contrary, *Δbpsl0279* formed its biofilms as small microcolonies scattering throughout the glass surface with thickness of 2.33 µm as shown in Figure 3C.

When the biofilms formation of the wild type and *Δbpsl0279* was investigated under dynamic laminar shear condition under a flow cell reactor, the wild type biofilms exhibited roughness and form large macrocolonies (size > 50 µm) with some of them reached a maximum size of 100 µm after 48 h after incubation (Figure 3C). In contrast, the *Δbpsl0279* was able to form only microcolonies (size 10 - 20 µm) (Figure 3B). These results indicated that inactivation of *bpsl0279* could alter the biofilm formation under both static and dynamic growth conditions.

3.4 The impairment of biofilm formation in Δbpsl0279 was observed at the early stage

As the attachment of bacterial cells to the surface and microcolony formation are the important steps to initiate the biofilm formation, we therefore investigated the ability of *Δbpsl0279* to form the biofilms at the early state by labeled it with *gfp*. The results showed that wild type cells attached to the glass surface significantly better (264 ± 32 cells per field) than *Δbpsl0279* (120 ± 30 cells per field; $p < 0.05$). Again, the attachment of the *Δbpsl0279*

complemented strain was restored to be similar to the wild type (Figure 3B). The later stage of the biofilm formation was observed at the ALI area. The result demonstrated that *Δbpsl0279* failed to form microcolony at the ALI area (Appendix 3). These results suggested that inactivation of *bpsl0279* affected the attachment of cells to the surface or microcolony formation, of which is the early stage of the biofilm process and resulting in decreasing of biofilm formation.

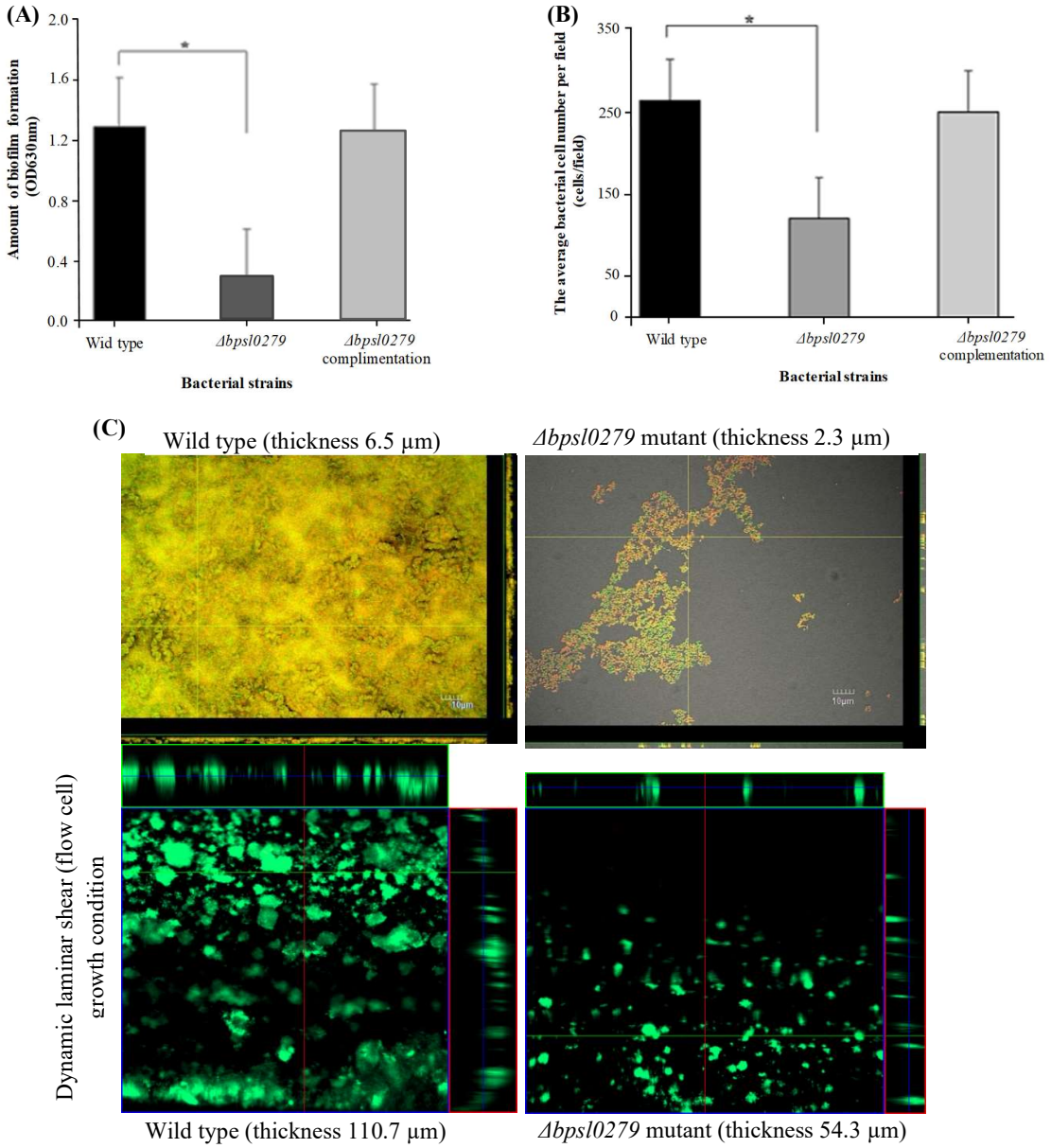

Figure 3 Biofilm-forming capacity and attachment of wild type and *Δbpsl0279*. (A) The amounts of biofilm quantitation in 96-well plates were in Y-axis and X-axis showed the results from *B. pseudomallei* K96243 wild type, *Δbpsl0279* and *Δbpsl0279* complementation. The asterisks (*) indicates significantly different ($p < 0.05$), (B) The *gfp*-tagged wild type, *gfp*-tagged *Δbpsl0279* strains and *gfp*-tagged *Δbpsl0279* complementation were grown on glass cover slips (22 by 22 mm) submerged (horizontally) in 6-well plates. The number of attached bacteria is presented as the average number of bacteria ± SD per field (cells/field), and (C) Biofilm characteristics of wild-type *B. pseudomallei* and *Δbpsl0279* strains grown on glass cover slip (static condition) and in square glass tubes supplied with dynamic flow (dynamic lamina shear conditions) for 48 h. The exopolysaccharide matrix was stained with FITC-ConA (green), and the bacterial DNA was stained with propidium iodide (red) and observed under confocal laser scanning microscope (CLSM). The thickness was represented in µm.

3.5 The mutation of bpsl0279 gene enhanced the motility under anaerobic growth condition

Motility is an important key for bacterial attachment, to assess swimming motility of the bacteria, swim plates were inoculated with either wild type, *Δbpsl0279*, or *Δbpsl0279* complemented strains for overnight at 37°C. There was no significant difference of the turbid zone at 24 h in aerobic condition when compared between wild type and *Δbpsl0279* strain (Figure 4A). Additionally, the motility assay was also observed at 24 h under anaerobic growth condition, but similar result was obtained. Interestingly at 48 h of incubation, the turbid zone of *Δbpsl0279* was larger than the wild type and the complement strains (Figure 4B, 4C). When the exopolysaccharide from the biofilms were stained under anaerobic growth condition, the results showed that *Δbpsl0279* produced significantly lower exopolysaccharide than the wild type (Appendix 3).

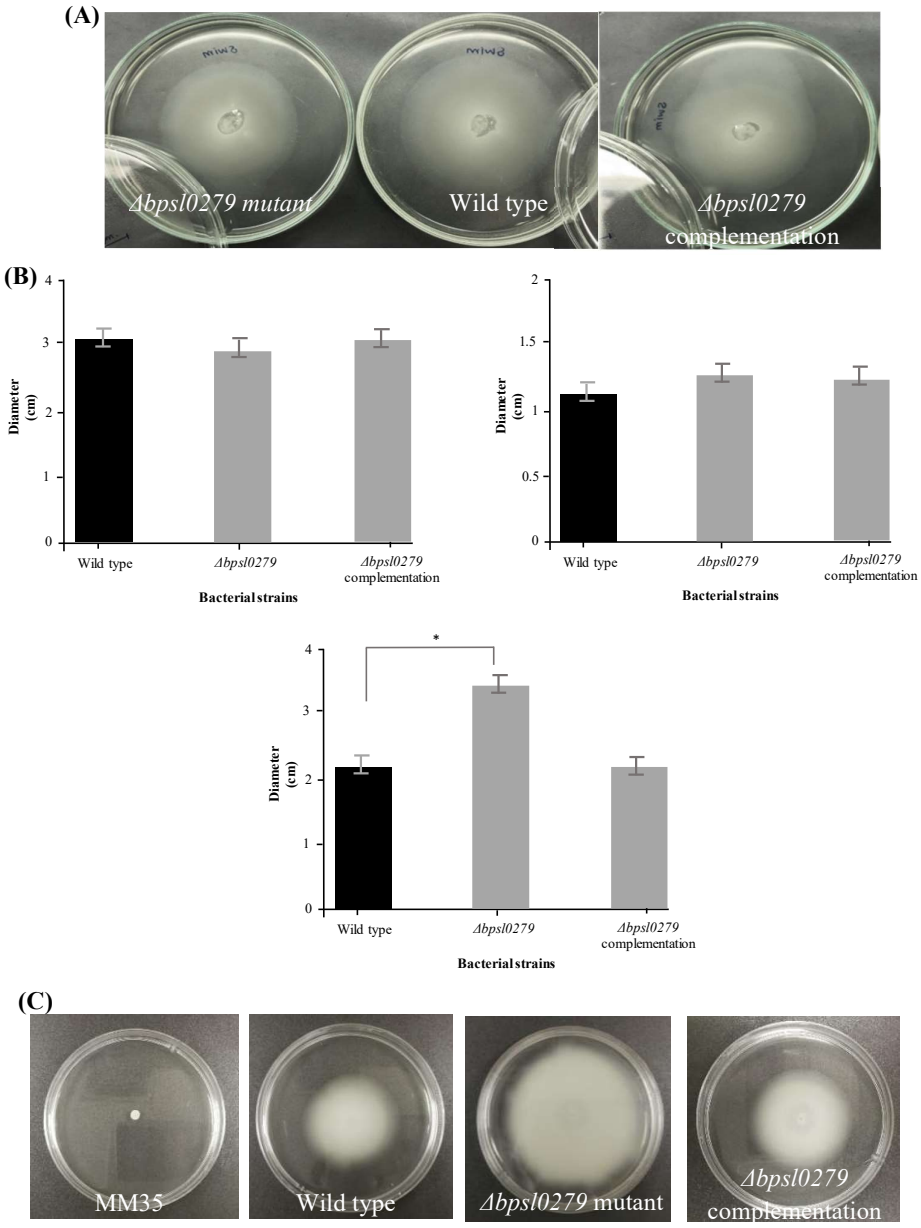

Figure 4 The swimming motility of the wild type, *Δbpsl0279,* and the complemented strains on semi-solid agar plates under aerobic and anaerobic growth conditions. The bacteria were incubated in aerobic (A) or anaerobic jars (B). The assessment of the circular turbid zone formed by the bacterial cells was determined in centimeters (cm), each bar represents the mean ± SD and (C) the experiment was performed in triplicate. *B. pseudomallei* flagella mutant (MM35) was served as a negative control. Asterisks are statistical significance ($p<0.05$).

4. Discussion

Biofilm formation is a biological process that bacteria produced in response to drastic environmental conditions for living such as a low nutritional source, oxidative stress, or exposure to sub-inhibitory concentrations of antibiotics. Our previous data indicated that *B. pseudomallei* relapse strains produced more biofilm than their original isolates [11].

In the present study, the *bpsl0279*, a hypothetical gene that is homologous to biofilms-related genes in other bacteria, was selected and explored. *B. pseudomallei Δbpsl0279* was successfully constructed. We investigated *bpsl0279* further as its ability to produce biofilm of *Δbpsl0279* were significantly reduced (75%) when compared to *Δbpsl1080* (50%). Although *Δbpsl0279* showed significantly lower biofilm production but not absent. As biofilm production is a complex process associated with various system, *bpsl0279* is therefore not the key important gene to abolish the biofilm formation. Several proteins or genes associated with biofilm formation have been reported in *B. pseudomallei* such as amylo-alpha-1, 6-glucosidase [26], BbeR-BbeS system (BPSL1036-BPSL1037) [27], capsule I polysaccharide biosynthesis gene cluster [28], surface-associated motility, surface composition and cell wall biogenesis [29]. In addition, the cyclic or c-di-GMP was demonstrated to regulate bacterial behaviors, including biofilm formation [30]. It enhances biosynthesis of capsular and fimbrial components required for biofilm formation while inhibiting flagella and pili that allow bacterial movement [31-34]. All of them showed significant reduction in biofilm formation as observed in *Δbpsl0279*. Transcriptome analysis of high and low biofilm producers found that approximately 9.5% of the total *B. pseudomallei* genes were associated with biofilm formation [29]. Therefore, interrupting a few genes related to biofilm formation could not inhibit the whole process.

Two different biofilm growth conditions, static and shear force (flow cell) were used to investigate the biofilm formation in *Δbpsl0279*. The in vitro flow cell method gives more advantages over static conditions as it evaluates bacterial biofilms under hydrodynamic and nutrient conditions coupled with continuous and non-destructive ability of growing biofilms which similar to what happened in vivo. We found in our study that both methods gave similar result of biofilm production in *Δbpsl0279*. In addition, this gene might relate with biofilm formation at the initial cell attachment as significantly lower cell attached and exopolysaccharide were noted in mutant compared to wild type. (Figure 3C and Appendix 3). Lower cell attachment led to small microcolonies formation documented in this mutant compared to the macrocolonies in wild type (Figure 3).

The *bpsl0279* has been recently reported as *B. pseudomallei* flagellar brake protein YcgR1 (GenBank Accession No. VUD41981). This protein functions as a flagellar brake in Gram-negative bacteria such as *E. coli* and *S. typhimurium* [35]. It regulates swimming and swarming in c-di-GMP-dependent mechanism that led to a decrease motility. Our study found that knockout of *bpsl0279* gene led to the increase in cell mobility (Figure 4). The *E.coli* YcgR attaches to MotA via its PilZ domain, of which is a c-di-GMP binding, so disturbs the MotA-FliG interaction and other motor proteins via its YcgR-N domain to inhibit flagellar motility. However, it is not clear why *Δbpsl0279* showed differences in motility only in anaerobic conditions after growing for 48 h. It may partly be due to the time of biofilm formation and the anaerobic environment as can happen inside the biofilms complex. The PilZ domain can bind with the central regulator of the prokaryote, c-di-GMP, to control the biofilm lifestyle, indicating that this domain might play an important role in biofilm formation. Most bacteria can switch between a planktonic motile mode and a biofilm mode, in which bacterial cells exhibit social behaviors by aggregating and attaching to a surface. It is assumed that motility is one of the contributing factors towards biofilm formation in early stage and the inhibition of motility promotes biofilm production in later stage. We showed for the first time that *bpsl0279* gene control motility and its mutant leads to impairment in attachment thus affecting the early stage of the biofilm process, resulting in decreasing of biofilm formation.

B. thailandensis, a non-pathogenic bacterium, also found in soil where *B. pseudomallei* resides has its genome and phenotypes similar to each other that could prove useful as a potential model organism to study certain aspects of *B. pseudomallei* biology [36]. We found that the *bth_i0249* gene, and their conserved pilZ domain were homolog to *bpsl0279* gene in *B. pseudomallei*. When they were mutated using non-replicative vector, pEXKm5 [37], *Δbth_i0249* and *Δbth_pilZ* were unable to produce flat biofilm structure on glass cover slips after 48 h of biofilm growing in both static and flow cell conditions (unpublished data). The result also correlated with the attachment assay, that inactivation of *Δbth_i0249* and *Δbth_pilZ* affect the biofilm production at the early stage of the process. All this information indicated that protein with PilZ domain might be relevant to this event. The PilZ domain proteins were found in many bacteria with highly conserved residues include the motif RRxxxR [38]. Blastp analysis showed the RRxxxR motif was also found in *bpsl0279* and *bth_i0249* genes with the motif RREYFR. Interestingly, many studies have revealed the role of PilZ domain in affecting the biofilm production in many aspects; initial attachment and twitching motility, EPS synthesis [39]. Therefore, it is possible that inactivation of the conserved domain, PilZ in *bpsl0279* and *bth_i0249* gene reduced the initial attachment capacity at the early stage of biofilm formation that caused diminished biofilm production under static and shear force conditions. Nevertheless, the biofilm formation by microbes is possibly revealed by numerous redundancies and overlaps of pathways involved in biofilm formation, as suggested by gene knockout in *bpsl0279* does not totally

prevent biofilm formation but either retarded or reduced. It indicated that biofilm mode of growth is an integral component of bacterial life cycle, in addition, it is a key factor for survival in environment of diverse bacteria.

5. Conclusion

In conclusion, the gene involved in biofilm formation of *B. pseudomallei* was identified using differential microarray data from other Gram-negative bacteria that exhibited differential expressions in biofilms, as compared with planktonic cells. The *bpsl0279* was found to regulate the biofilm formation observed in both static and flow cell conditions. As *B. pseudomallei* showed tolerance to ceftazidime, the drug of choice, in the biofilm induced conditions [40], continue studies of the candidate gene(s) involving in the biofilm process will elucidate more about genetic mechanisms of biofilm production as well as the role of biofilms in melioidosis.

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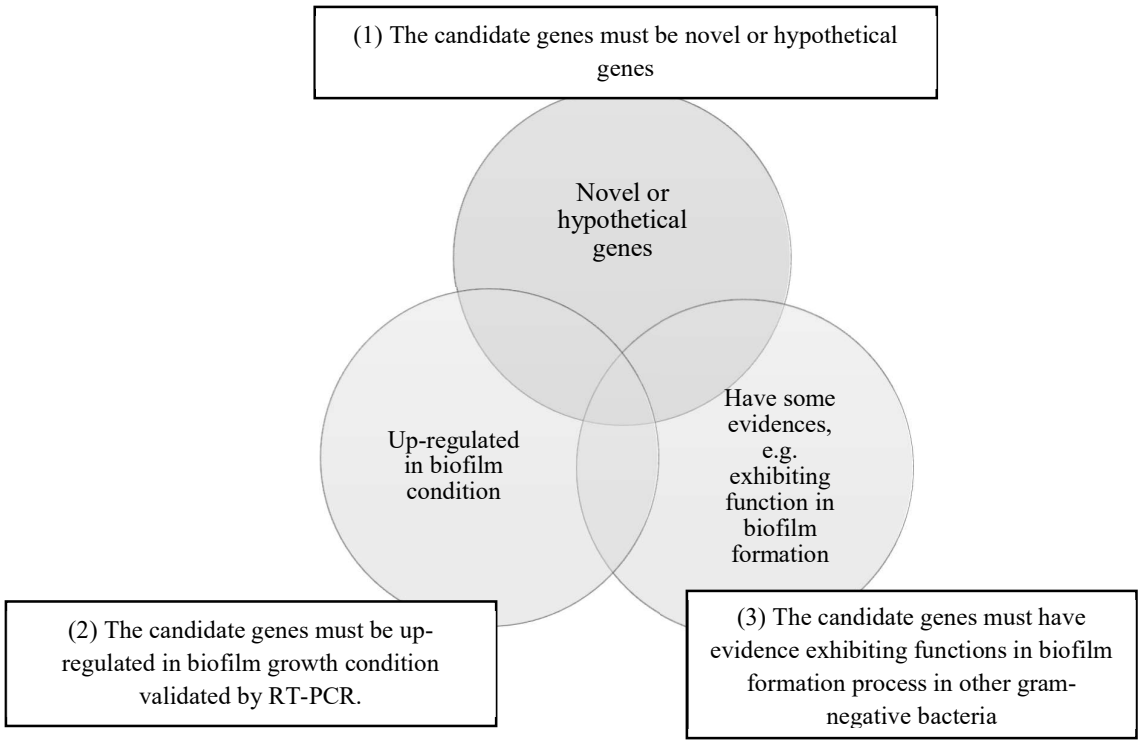
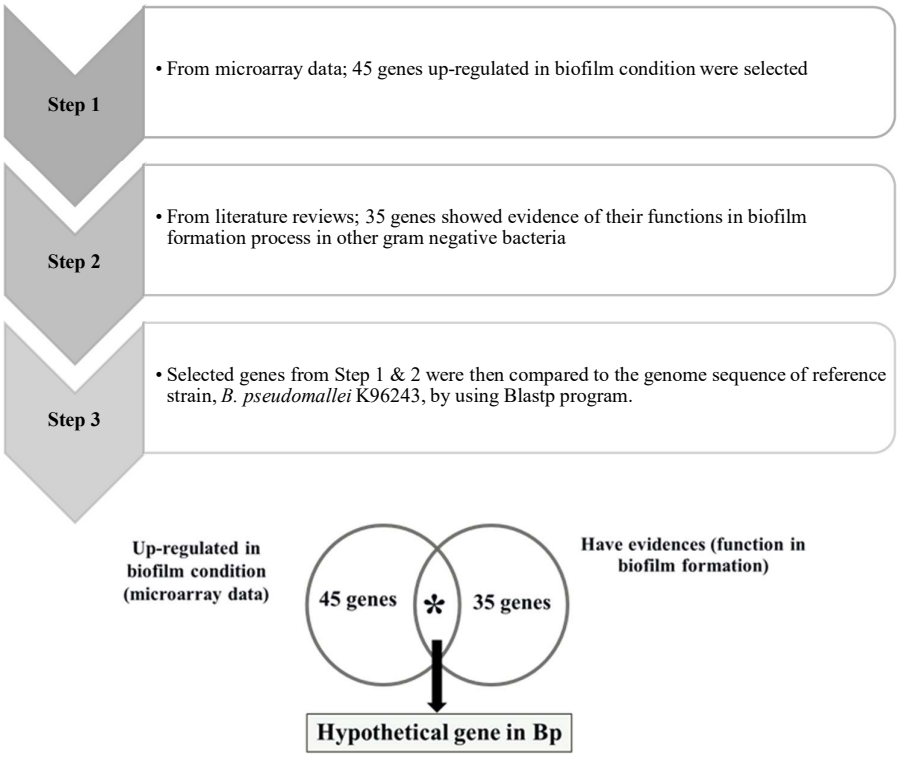
Appendix 1 Candidate genes selection approach based on bioinformatic study and microarray data.

1.1 Criteria for gene selection



1.2 Candidate genes selection procedure



The *bpsl0279 and bpsl1080* were then validated RT-PCR

1.3 List of candidate genes

1.3.1 45 genes up-regulated in biofilm condition

Gene/Gene ID	Description	*Bacterial species*
hslS	Heat shock protein	*E. coli*
soxS	Regulation of superoxide response regulon, global regulator	*E. coli*
hha	Haemolysin expression modulating protein, regulator	*E. coli*
glnA	Glutamine synthetase	*E. coli*
ybaJ	Unknown	*E. coli*
b2377	Unknown	*E. coli*
b1112	Unknown, possible stress response	*E. coli*
hslT	Heat shock protein	*E. coli*
PA5553 – PA5561	ATP synthase genes (up-regulated in developing biofilms, 8 h)	*Pseudomonas. aeruginosa*
mvaT	global regulator of gene expression (expression peaked in 8 h-biofilms)	*P. aeruginosa*
PA0718- PA0727	The functional class 'phage, transposon, or plasmid	*P. aeruginosa*
VC1332-VC1334	Conserved hypothetical protein	*Vibrio cholerae*
VC1335	Transcriptional regulator, GntR family	*V. cholerae*
VC1336	Carboxyphosphonoenolpyruvate phosphonomutase	*V. cholerae*
VC2705	Sodium/solute symporter, putative	*V. cholerae*
VCA0682	Transcriptional regulator UhpA	*V. cholerae*
csgA, csgB	Encoding the curlin fimbrial subunits (Up-regulated in mature biofilm)	*Salmonella enterica serovar Typhimurium*
cheA, cheR, motA, motB	Required for motility and chemotaxis	*E. coli*
csrA	Global gene regulator	*S. Typhimurium*
ompX	Encoding the major outer membrane protein OmpX	*S. Typhimurium*
ycgR	Flagellar brake protein YcgR	*E. coli*
cpxR	Periplasmic Stress Response Protein CpxP	*E. coli*

1.3.2 35 genes showed evidence of their functions in biofilm formation process

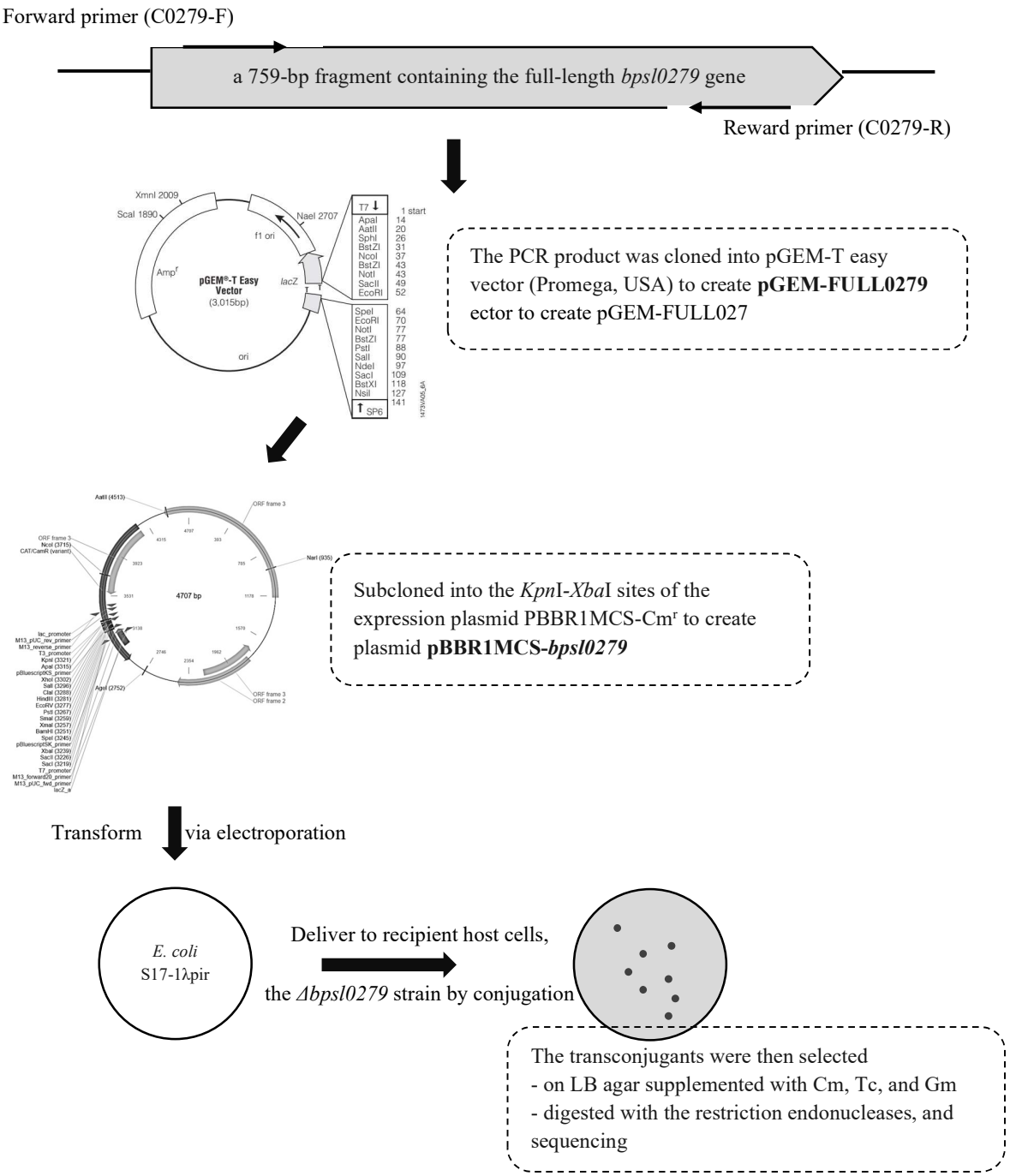
Gene/Gene ID	Description	*Bacterial species*
barA, uvrY	Activates biofilm formation	*E. coli*
cpxR, cpxA	Senses surface perturbation and required for optimal cell-to-cell interactions	*E. coli*
ompR, envZ	Increases attachment via curli and cellulose gene activation	*E. coli*
rcsB, yojN, rcsC	Activates biofilm formation via remodeling of cell surface composition	*E. coli*
rpoS	Reduces or increases depth of the biofilm	*E. coli, P. aeruginosa*
crc	Required for normal biofilm development (activation of type IV motility)	*P. aeruginosa*
gacAS	Required for microcolonies formation	*P. aeruginosa*
rpoN	Role in initial adhesion and biofilm architecture	*P. aeruginosa*
	Role in biofilm architecture	*Vibrio fisheri*
sodC	Superoxide dismutase	*E. coli*
tpx	Thiol peroxidase	*E. coli*
dsbA	Disulfide oxidoreductase	*E. coli*
soxS	Regulatory protein SoxS	*E. coli*
sadB (PA5346)	Required for the transition from reversible to irreversible attachment	*P. aeruginosa*
bifA (PA4367)	A cyclic-di-GMP phosphodiesterase	*P. aeruginosa*
alg44	Alginate biosynthesis	*P. aeruginosa*
motA, motB	bacterial flagellar motor proteins	*E. coli*
cupA	chaperone-usher pathway (required for biofilm formation on abiotic surfaces)	*P. aeruginosa*
vfr	Regulates the las quorum sensing system and twitching motility	*P. aeruginosa*
cheY-3	Chemotaxis protein CheY	*V. cholerae*
leuO	LysR family transcription factor	*V. cholerae*
bap1	Encoding haemolysin-related proteins (biofilm-associated protein 1)	*V. cholerae*
pela	Encoding a protein with a predicted polysaccharide deacetylase domain	*P. aeruginosa*
vps	Vibrio polysaccharide exopolysaccharide	*V. cholerae*
rpoS	An RNA polymerase subunit	*P. aeruginosa*
pslA (PA2231)	Required for polysaccharide synthesis	*P. aeruginosa*
aglC	Encodeing phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core	*P. aeruginosa*
qseC	The quorum-sensing E. coli regulator C	*E. coli*
cyaA, crp	required for the expression of flagella genes	*E. coli*

1.4 Candidate genes selection approach based on bioinformatic study and microarray data

To begin with the criteria for candidate genes of interest in this study are those that showed higher expression in biofilm culture and ultimately be involved in biofilm function in Gram- negative bacteria. The first 45 candidate genes were selected from microarray data of four other Gram-negative pathogens, that differentially expressed in biofilms form comparing with free-living cells. In addition, 35 candidate genes from other pathogens published in literature that had been identified to involve in biofilm functions were also included. Amino acid sequences of

80 candidate genes were used to compare with the sequence of *B. pseudomallei* K96243 reference strain by using Blastp program. The hypothetical genes under these criteria were selected. From such criteria, the *bpsl0279* and *bpsl080* showed the highest homology scores when comparing with 80 candidate genes and might indicate their roles to be involved in biofilm formation.

Appendix 2 Construction of a *Δbpsl0279* complemented strain.



A complementation strain of *bpsl0279* was constructed by introducing a plasmid containing an intact *bpsl0279* gene into the *Δbpsl0279* mutant strain. Firstly, a 759 bp nucleotide sequence of the full-length *bpsl0279* gene was amplified by PCR using C0279-F and C0279-R primers containing *Kpn*I and *Xba*I restriction sites. Secondly, the PCR product was cloned into pGEM-T easy vector to create pGEM-FULL0279. The recombinant plasmid was confirmed by sequencing and subsequently sub-cloned into *Kpn*I-*Xba*I sites of the expression plasmid

pBBR1MCS-Cmr [19] to create pBBR1MCS-*bpsl0279*. The recombinant plasmid was transformed into *E. coli* S17-1λpir and then mobilized into the *Δbpsl0279* mutant strain by conjugation. The transconjugants were then selected on LB agar supplemented with Cm, Tc, and Gm. To ensure that the complementation clone was correct, the recombinant plasmid was digested with the restriction endonucleases, and sequencing.

Appendix 3 The Air-Liquid Interface (ALI) assay and the exopolysaccharide production of the wild type, *Δbpsl0279*, and the complemented strains.

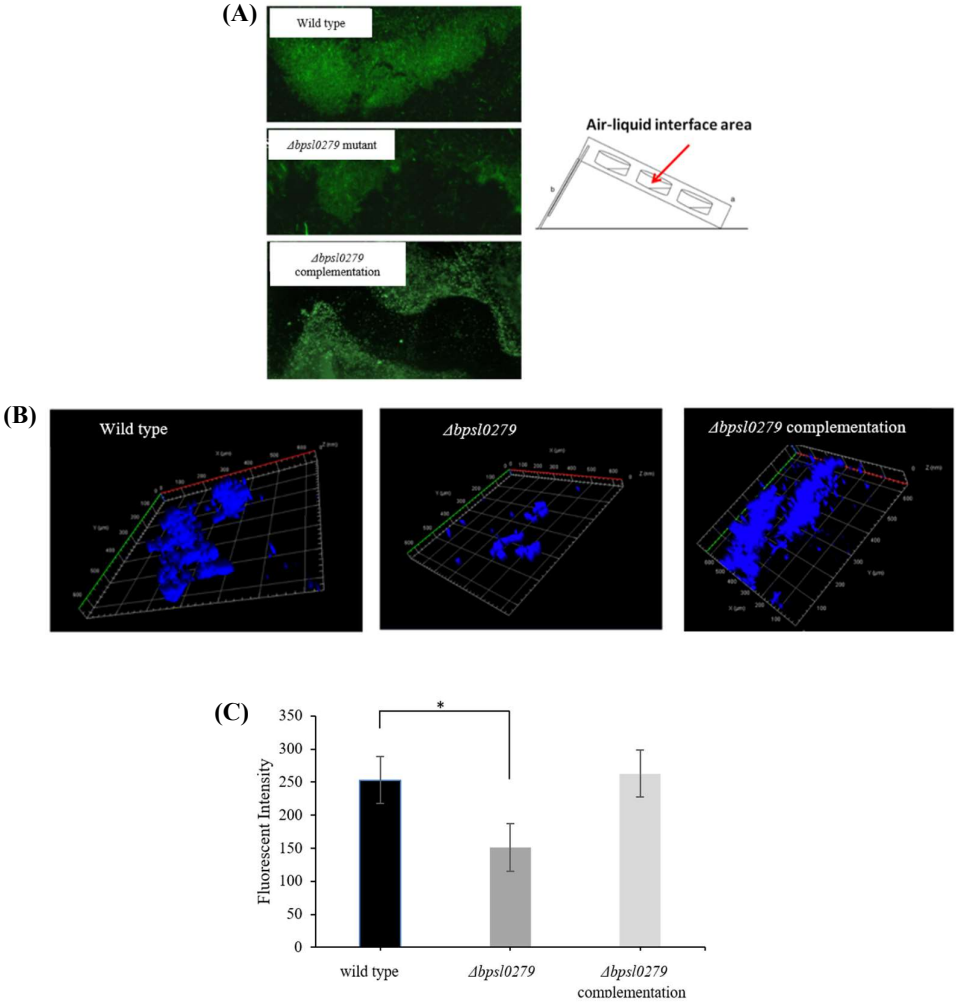


Figure 3A (A) Microcolony formation at the air-liquid interface area of the *gfp*-tagged wild type, *gfp*-tagged *Δbpsl0279* mutant, and *gfp*-tagged *Δbpsl0279* complemented strains. The *gfp*-tagged bacterial cultures were inoculated in a 24-well plate placed at an angle of 30° to 50° relative to horizontal for 24 h, was then visualized by fluorescence microscopy at 20x magnification (Nikon, Japan). (B) The exopolysaccharide production of the bacteria was observed under static anaerobic growth conditions, where the bacteria were grown on the glass cover slip and incubated in anaerobic jars using Gas Pak (Oxoid, UK). The exopolysaccharide matrixes of the bacteria were stained with FITC-ConA, then observed under CLSM (represented as blue color). The relative amount of exopolysaccharide matrixes was represented as the fluorescent intensity. (C) Fluorescent intensity was analyzed by Zen Blue edition software (Carl Zeiss Microscopy, GmbH). Each bar represents the fluorescent intensity as mean ± SD per field for 10 fields. Asterisks denote statistical significance using a paired-samples Student's *t*-test ($p<0.05$).