The *Bth_i0249* Regulates *Burkholderia thailandensis* Biofilm Formation

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(Received: *May 9, 2022*; Revised: *May 25, 2022*; Accepted: *May 25, 2022*)

ABSTRACT

Biofilm forming bacteria are a structured community of microorganisms enclosed in a self-produced polymatrix. Biofilm forming bacteria are responsible for a variety of chronic infections. *Burkholderia pseudomallei* is a causative agent of a broad spectrum of clinical symptoms collectively known as melioidosis. Biofilm formation and maturation in *Burkholderia* subspecies is poorly defined. *Burkholderia thailandensis* is a closely related subspecies to *B. pseudomallei*. Because of the broad similarities between these two bacteria, *B. thailandensis* is sometimes used as a model for study of *B. pseudomallei*. The aim of this study was to investigate the *B. thailandensis* biofilms phenotype in static and shear conditions using confocal laser scanning microscopy (CLSM). It was demonstrated that *B. thailandensis* was able to form mature biofilms over 96 hours that expressed multiple three-dimensional biofilm structures under both static and laminar-shear conditions. Using the microarray data from other Gram-negative bacteria that exhibited differential expression in biofilms as compared with free-living cells it was possible identify a candidate *bth_i0249* gene of the YcgR protein superfamily of proteins that contains the *PilZ* conserved domain of *B. thailandensis* that plays a role in biofilm formation. Both the *Δbth_i0249* and its conserved domain, *Δbth_pilZ* mutants, significantly reduced biofilm production both in static and laminar-shear conditions. Taken together, the *bth_i0249* and its conserved domain, *pilZ*, are involved in biofilm formation.

Keywords: Biofilm formation, *B. thailandensis*, Mutation

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Introduction

Bacterial colonization is initiated with freely suspended or planktonic cells that subsequently develop into a complex group of organisms encased in a polymeric matrix [1-2]. Biofilm formation is a response to environmental factors, such as a low nutritional source, oxidative stress, or exposure to sub-inhibitory concentrations of antimicrobial agents. Previous studies have shown that biofilms are dynamic with a biological cycle that includes; initiation, maturation, maintenance and dissolution with cells in the biofilms returning to a planktonic lifestyle [3]. Additionally, biofilms possess several characteristics that are considered to be involved in many chronic infections. Importantly, more than 80% of chronic bacterial infections are associated with biofilms [4]. For example, biofilms have significantly higher resistance to antimicrobial agents and the host immune systems than their planktonic counterparts [4-5].

Cyclic-dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger known to regulate a variety of cellular processes controlling biofilm formation, motility, production of extracellular polysaccharides and multicellular behavior in diverse bacteria. It has emerged as a conserved bacterial intracellular second messenger. It is synthesized by proteins with diguanylate cyclase activity with a characteristic GGDEF motif. Degradation of cyclic di-GMP is affected by phosphodiesterase activity. The PilZ (‘pills’) domain (Pfam domain PF07238), enterobacterial YcgR, firmicute YpfA families, and other proteins in the bacterial genomes are part of the c-di-GMP-binding proteins. Association of these domains could provide clues to multiple functions of the c-di-GMP in bacterial development. YcgR bound to c-di-GMP stimulates YcgR to interact with the flagella and thus promotes the motile-to-sessile transition. The role of c-di-GMP, PilZ domain and YcgR in biofilm formation has been reported in several bacteria [6].

Burkholderia thailandensis is a Gram-negative bacillus that is found naturally in soil and water. It rarely causes disease in humans or animals. It has been described by Brett *et al.* [7]. It is subspecies a closely related to *B. pseudomallei*, a bacterial pathogen responsible for melioidosis, a fatal infectious disease. Due to the broad similarities between these two bacteria, *B. thailandensis* is sometimes used as a model for study of *B. pseudomallei* biofilms growing as it does not require biosafety category 3 facilities which makes it easier to work. The biofilm production ability was found varied but not related to virulence [8]. Interestingly, biofilms are one of the important candidate mechanisms for antibiotic resistance in various bacteria including *B. pseudomallei*. Several reports demonstrated the *B. pseudomallei* biofilm, although the gene(s) that control the biofilm formation are still unknown.

Objectives of the study

The aims were to study *B. thailandensis* as a model for investigate biofilm formation, development, and maintenance over time under static and laminar-shear conditions. In addition, using the microarray data from other Gram-negative bacteria that exhibited differential expressions of biofilms, as compared to free-living cells, to identify a candidate *bth_i0249* gene of the YcgR protein superfamily proteins containing the PilZ conserved domain of *B. thailandensis* that plays a role in biofilm formation.

The flow cell system in conjunction with a confocal laser scanning microscope (CLSM), enabled the detailed study of the growth of biofilms over a number of days, with the ability to control parameters such as growth, medium composition and flow rate. The CLSM combined with an appropriate staining method observed biofilm maturation. The understanding of biofilm formation process can describe an association between biofilm formation and antimicrobial resistance.

Methodology

1. Bacterial isolates

B. thailandensis strains E264 and UE5 [7] were isolated from the environment of the northeast of Thailand. The *Escherichia coli* mobilizer strain RHO3 was used as the donor host cells for *B. thailandensis* conjugation. *E. coli* DH5α was used for general DNA cloning and mutant constructions.

2. Validation of gene expression by End-point RT-PCR

To confirm the expression of *B. thailandensis bth_i0249* in biofilm and planktonic forms, RT-PCR was used to amplify *bth_i0249* mRNA extracted when *B. thailandensis* E264 was grown in either planktonic or biofilm conditions. 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-h biofilm bacteria. Total RNA was extracted from planktonic and biofilm samples using TRIZOL reagent (Invitrogen, USA). To remove contaminated genomic DNA from RNA samples, 2 U of RNase-Free DNase (Promega, Madison, WI) in a final volume of 20 µL was used. 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 RTBT0249-F and RTBT0249-R primers (Table 1). 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 second, and extension at 72°C for 1 minute. The 16S rRNA gene was used as an internal control.

To measure gene expression levels, 10 µL of each PCR product was electrophoresed on 1% agarose gel, stained with ethidium bromide, and observed under a gel documentation (Syngene, USA). The density of the PCR product bands was analyzed using ImageJ, a Java-based image analysis program.

3. Construction of *Δbth_i0249* and its conserved domain, the *PilZ* mutants

Clean deletions were generated in these genes using the I-SceI- and *sacB*-based allele replacement strategies in the combination of *E. coli* mobilizer strains RHO3 and pEXKm5. The fragment mutagenesis method described by Lopez *et al*. [9] was employed to generate *Δbth_i0249* and its conserved domain, the *pilZ* defective mutants in *B. thailandensis* strain E264. The *B. thailandensis* strain E264 *bth_i0249* and its *pilZ* sequences were obtained from the GenBank. PCR primers (Table 1 and Figure 1A) corresponding to 5′ (BTH_I0249-F1 and BTH_I0249-R1) and 3′ fragments (BTH_I0249-F2 and BTH_I0249-R2) of *bth_i0249* and 5′ (PilZ_BT0249-F1 and PilZ_BT0249-R1) and 3′ fragments (PilZ_BT0249-F2 and PilZ_BT0249-R2) of *pilZ* were

designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/ tools/ primer-blast). The primer sequences and product sizes are shown in Table 1. Forward primers amplifying the 3′ fragments of these genes (*bth_i0249* and *pilZ*) were designed to have an oligonucleotide tail homologous to the 3′ ends of 5′ fragments. The 5′ and 3′ fragments of each gene were joined by PCR using BTH_I0249-F1 and BTH_I0249-R2 or PilZ_BT0249-F1and PilZ_BT0249-R2 primers, which was facilitated by a tail on the 3′ forward primer to give a new PCR product with a deletion region between BTH_I0249-R1 and BTH_I0249-F2 or between PilZ_BT0249-R1 and PilZ_BT0249-F2. These mutant constructs were cloned into pGEM®T-Easy of *E. coli* DH5α. White colonies were selected using β- galactosidase indicator medium containing 50 µg/ ml 5- bromo- 4- chloro- 3- indolyl- β- D-galactopyranoside (X-Gal) (Promega, WI, USA) plates containing 100 µg/ml ampicillin. Colonies containing the desired plasmids were analyzed by PCR using primers flanking the mutant alleles, BTH_I0249- F1 and BTH_I0249- R2 or PilZ_BT0249- F1 and PilZ_BT0249- R2. The products were confirmed by agarose gel electrophoresis and verified by DNA sequencing. The unmarked knockout cassettes assembled by PCR that contained deletions in the *bth_i0249* and *PilZ* domains were cloned into the non-replicative plasmid, pEXKm5 [9]. The pEXKm5-mutant allele constructs were transformed into *E. coli* DH5α. Plasmids were extracted and checked by PCR with primers BTH_I0249-F1 and BTH_I0249-R2 or PilZ_BT0249-F1and PilZ_BT0249-R2 for the correct product sizes of target gene constructs. The pEXKm5-mutant plasmids were then transformed into *E. coli* RHO3 and delivered to the host *B. thailandensis* strain E264 by conjugation as previously described [9], resulting in integration of the allelic replacement constructs into the *B. thailandensis* chromosome by homologous recombination between cloned and chromosomal sequences. The merodiploid clones visualized as blue colonies on LB agar containing 1000 µg/ml kanamycin and 50 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) were selected for PCR with primers flanking the mutant alleles, BTH_I0249-F1 and BTH_I0249-R2 or PilZ_BT0249-F1and PilZ_BT0249-R2 (Figure 1 and Table 1). For merodiploid resolution, clones were streaked onto yeast extract tryptone (YT) agar (Yeast extract and tryptone, BD; agar, Oxoid) containing 15% sucrose and 50 µg/ml X-Gluc and incubated at 25℃ for 72 h. White colonies growing on X-Gluc-containing medium (YT-sucrose-X-Gluc plate) were selected and purified by streaking on the same medium and incubated as described above. Merodiploid resolution leads to formation of either a wild type or a mutant strain, which were distinguished by using PCR primer sets flanking the mutant deletion alleles primers (BTH_I0249-F1 and BTH_I0249-R2 or PilZ_BT0249-F1and PilZ_BT0249-R2) and the *oriT* pEXKm5 plasmid backbone sequences. In contrast to *B. thailandensis* E264, *Δbth_i0249* and *Δbth_pilZ* mutants yielded smaller DNA fragments of the deleted region and did not yield *oriT* associated PCR products. Neither the mutants nor the wild type strains grew on LB agar supplemented with 1000 µg/ml kanamycin.

Table 1 Oligonucleotides used in this study

Primers	Nucleotide sequences (5' to 3')	corresponding gene	Source of reference
RTBT0249-F	TTGCGCAACCTCGTCA	*bth_i0249*	this study
RTBT0249-R	ACGAGTTGCAGGTCGA	*bth_i0249*	this study
BTH_I0249-F1	TCGAAGATGTACACGTCGATG	Upstream of	this study
BTH_I0249-R1	GCGCCGCGCGTTTCGGGCTTGCCGGTATTTTATC	*bth_i0249*	this study
BTH_I0249-F2	GATAAAATACCGGCAAGCCCGAAACGCGCGGCGC	Downstream of	this study
BTH_I0249-R2	TGAGCTGCGAATTCACGCTC	*bth_i0249*	this study
PilZ_BT0249-F1	GAACAGTCGACGAGTCCGCCCGC	Upstream of *PilZ*	this study
PilZ_BT0249-R1	GCCCGTGCGCAGCCCCAGCTGCACGCAGTACAGCAC	domain	this study
PilZ_BT0249-F2	GTGCTGTACTGCGTGCAGCTGGGGCTGCGCACGGGC	Downstream of	this study
PilZ_BT0249-R2	CGCGAGTTGCCGGCGCTTCATCT	*PilZ* domain	this study

4. Quantification of Biofilm Formation

A modified microtiter plate test was used to determine the biofilm-forming capacity of all isolates as previously described [8]. A single colony of *B. thailandesis* E264 or UE5, grown on a nutrient agar (NA) plate, was inoculated into a sterile tube containing 1 ml of modified Vogel and Bonner's medium (MVBM) and grown overnight at 37°C in a 200-rpm shaker incubator (Innova™, New Brunswick Scientific, NJ, USA) and used as an inoculum. Thereafter, an MVBM tube was inoculated with 2% inoculum (v/v) for 18 h. The 18 h-broth cultures of each bacterial strain were adjusted to give an optical density (OD) at 540 nm of 0.8-0.9 in fresh medium. Two hundred microliters of each bacterial suspension were added into 8 wells of a sterile 96-well flat-bottomed plastic tissue culture plate (Nunclon™, Roskilde, Denmark). Wells containing only the medium served as the negative controls. The plates were incubated aerobically at 37°C for 3 h to allow adhesion. Thereafter, the supernatant fluid of each well

was aspirated gently to remove non-adherent bacteria and replaced with 200 µl of fresh MVBM. After incubation at 37°C for an additional 21 h, the non-adherent bacteria were again removed and the wells containing adherent bacteria were washed with 200 µl of sterilized deionized water and fresh medium was added once more. After incubation for an additional 24 h, the supernatant was again removed, and the wells were finally washed three times with 200 µl of sterile deionized water. The attached bacteria, representing a 2-day biofilm culture were fixed with 200 µl of 99% methanol for 15 min and allowed to dry at room temperature. The plates were stained for 5 min with 200 µl of 2% crystal violet. The unbound stain was removed with running tap water. The plates were air dried and the crystal violet in each well was solubilized by adding 200 µl of 33% (v/v) glacial acetic acid. The plates were read at 630 nm using a microplate reader. The ability of each isolate to produce biofilm was determined twice and the results reported are the average from three independent experiments.

5. Biofilm formation in static conditions

For characterization of static biofilm phenotypes in *B. thailandensis,* the method previously described by Taweechaisupapong *et al*. [8] was employed with some modification. The 18 h-broth cultures of each bacterial strain were adjusted to give an OD at 540 nm of 0.8-0.9 in fresh medium and 2 ml of cultures were transferred onto glass cover slips (22 by 22 mm) and submerged horizontally in a six-well plate (Greiner bio-one, Frickenhausen, Germany). The plates were incubated aerobically at 37°C for 3 h to allow adhesion. Thereafter, the supernatant fluid of each well was aspirated gently to remove non-adherent bacteria and replaced with 2 ml of fresh MVBM. After incubation at 37°C for an additional 21 h, the non-adherent bacteria were again removed and the wells containing adherent bacteria were washed with 2 ml of sterilized deionized water and fresh medium was added once more. After incubation for an additional 24 h, the medium was removed by aspiration and the wells were washed three times with 2 ml of sterile deionized water to remove non-adherent cells. The attached bacteria, representing 2-day biofilm cultures were maintained under static conditions.

6. Biofilm formation in dynamic flow cell conditions

A single *B. thailandensis* colony was inoculated in a sterile tube containing 1 ml of MVBM and incubated overnight at 37°C in a 220-rpm shaker incubator and used as inoculum. Six ml of 2% (v/v) inoculum from the overnight culture were incubated at 37°C with 220 rpm shaking for 18 h. The cultures of each bacterial strain were adjusted to give an OD at 540 nm of 0.8-0.9 in fresh medium. Five ml of each bacterial culture were aseptically injected into each flow channel. The flow system was assembled and prepared as described previously [10-12]; biofilms were grown in individual 1 mm square glass tubing (0.15 mm wall), supplied with a flow of 3 ml/h with 10% MVBM. After inoculation, bacterial cells were first allowed to initiate attachment to the cell surface for 30 min. Subsequently, flow was then applied and maintained at a constant rate of 3 ml/h using a MasterFlex® pump set (Cole-Parmer, USA). *B. thailandensis* biofilm formation was monitored at 24, 48, 72 and 96 h under laminar shear conditions.

7. Microscopy and image acquisition

The images of static biofilms were carried out as previously described [13]. *B. thailandensis* biofilms were statically grown on glass cover slips on the bottom of six-well microtiter plates, then the cover slips were transferred and fixed in glutaraldehyde. 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; plates were then covered with aluminum foil and incubated for 30 min at room temperature.

For the flow cell studies, the Live/Dead BacLight Bacterial Viability Kits (Invitrogen, USA) were used to stain biofilms in the flow cells (3 µl SYTO 9 and 3 µl Propidium iodide (PI) in 1ml 1x PBS buffer). One ml SYTO9/PI was applied to each channel using sterile 1 ml syringes with 26½ gauge needles. Biofilms were stained for 30 min while covered with aluminum foil at room temperature. The stained biofilms were observed under a Zeiss LSM 510 Meta confocal microscope and the 3D structure images were analyzed with LSM Image Browser software (Carl Zeiss, Jena, Germany).

8. 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.

Results

1. Identification and mutagenesis of the *Δbth_i0249* gene and the *pilZ* conserved domains of *B. thailandensis*

Candidate genes suspected to play a role in biofilm formation in *B. thailandensis* genomes were identified based on the microarray data from other Gram-negative bacteria that exhibited differential expressions in biofilms, as compared with free-living cells. To identify genes of *B. thailandensis*, bioinformatics analysis was targeted on hypothetical genes or operons of suspected biofilm related genes. Using these criteria, *bth_i0249* genes of *B. thailandensis* showed the highest homology scores. This might provide significant evidence of this gene being involved in biofilm formation. The *bth_i0249* is annotated as a YcgR protein superfamily protein similar to the YcgR protein of *E. coli*, which acts as a flagellar brake, regulating swimming and swarming motility. Analysis of the *B. pseudomallei* and *B. thailandensis* genomes identified an ortholog of *ycgR* in both *B. thailandensis* (*bth_i0249*) and *B. pseudomallei* (*bpsl0279*) that shared 100% homology. They were 100% homologous on the protein level. A confirmation of expression of the gene during growth and during biofilm formation in *B. thailandensis* was performed using end-point RT-PCR. The results found that *bth_i0249* expression was expressed higher in biofilms compared to planktonic cells (Figure 1D). To determine if *bth_i0249* played a role in biofilm formation, deletion constructs of the *bth_i0249* and *pilZ* domain were created. The presence of deleted *bth_i0249* gene and *pilZ* domains were verified by PCR, as shown in Figure 1 and sequence analysis (Supporting Information S1-S2).

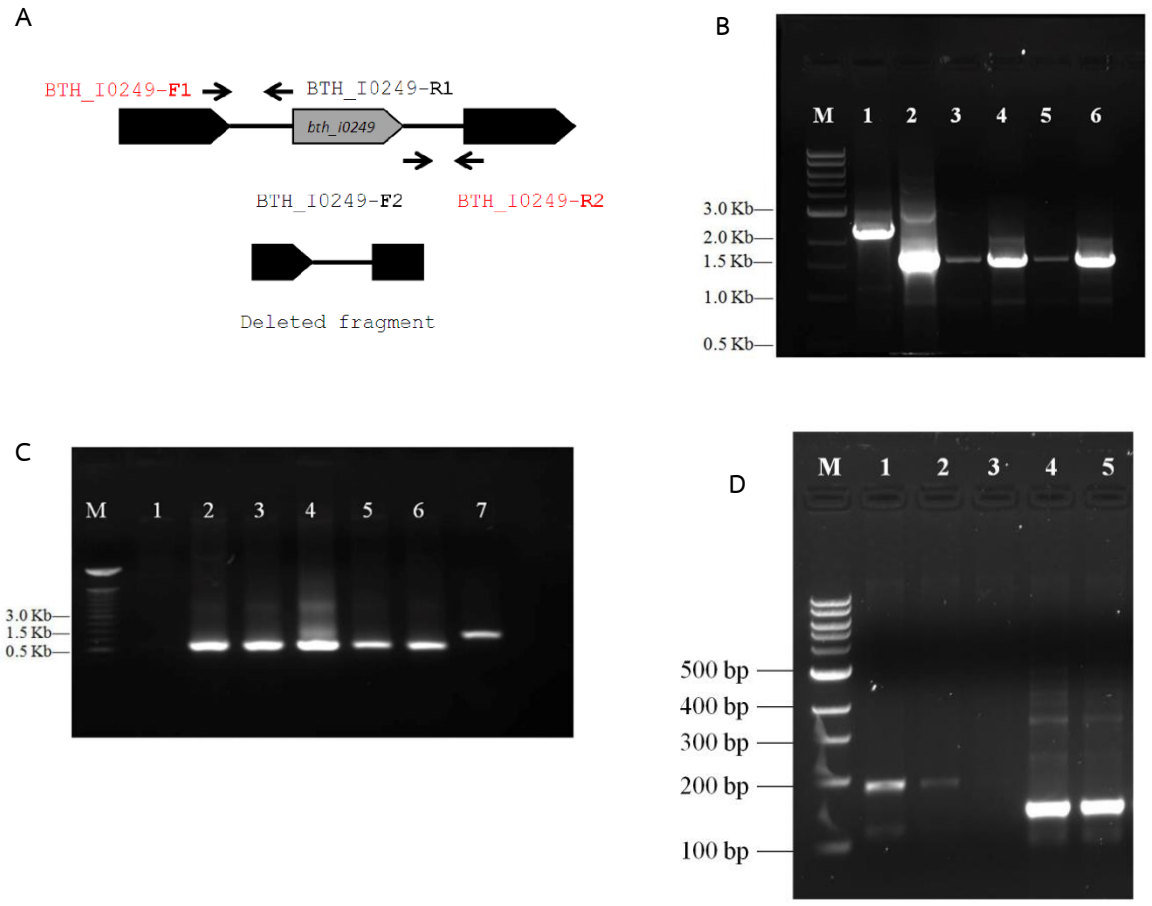
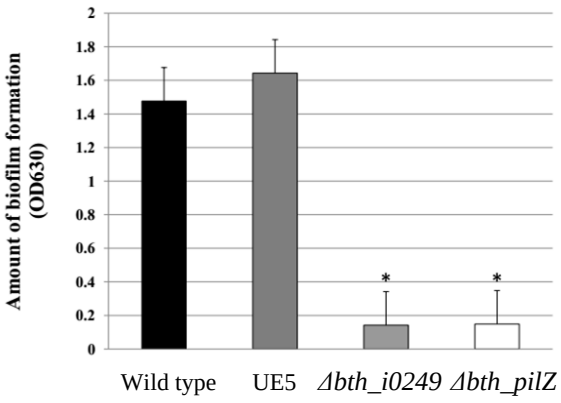


Figure 1 Gene organization and PCR analysis of the *bth_i0249* of *B. thailandensis* E264 and its mutants. **(A)** Primer binding sites are indicated by arrows. Highlights in gray color indicate the extent of DNA deleted in the *Δbth_i0249* mutants (see Supporting Information S1). PCR analysis of selected *B. thailandensis Δbth_i0249* mutant strains. PCR was performed on boiled colony preparations with primers flanking the mutant alleles BTH_I0249-F1 and BTH_I0249-R2 (Supporting Information S1). **(B)** PCR products from *B. thailandensis* wild type genomic DNA (lane 1) or *Δbth_i0249 B. thailandensis* mutants (lanes 2-6) were separated by agarose gel and stained with ethidium bromide. **(C)** For PCR analysis of selected *ΔbthpilZ* mutant strains, PCR was done using primers flanking the mutant alleles (PilZ_BT0249-F1 and PilZ_BT0249-R2) (Supporting Information S2). PCR products from *B. thailandensis* wild type genomic DNA (lane 7) or *ΔbthpilZ* mutants (lanes 2-6) were separated by agarose gel. **(D)** End-point RT-PCR of cDNA from biofilm (lane 1, 4) and planktonic (lane 2, 5) cells of *B. thailandensis* E264. Lanes 3 is negative control. The primers designed for amplification of 16s rRNA were used as an internal control for gene expression control (lane 4, 5). Lane M is 100 bp DNA Ladder.

2. *B. thailandensis* Δbth_i0249 and Δbth_pilZ mutants were reduced in biofilm formation under static conditions

The *Δbth_i0249* and its conserved domains, *Δbth_pilZ* mutants, were constructed and these two deletion strains grew similarly compared with their wild type counterparts (Supporting Information S3). Using the *Δbth_i0249* and its conserved domain, the *Δbth_pilZ* mutants, their ability to form biofilms under static growth conditions was assayed. Static biofilms were grown using microplates. Both the *Δbth_i0249* and its conserved domain, *Δbth_pilZ* mutants, showed an approximately 90% ($P > 0.05$) (OD 630 nm, 0.142 ±0.03) reduction in biofilm production, as compared to the wild type (OD 630 nm, 1.423 ±0.56) (Figure 2A). As shown in Figure 2B, neither mutant was able to initiate attachment to the glass cover slip when grown under static conditions.

A



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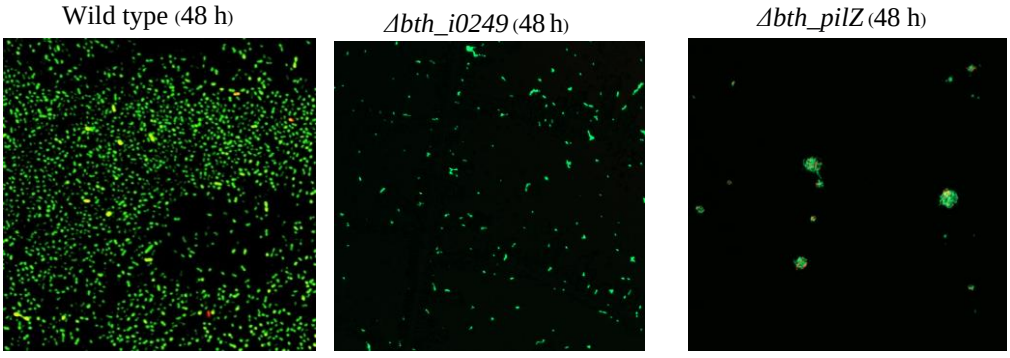


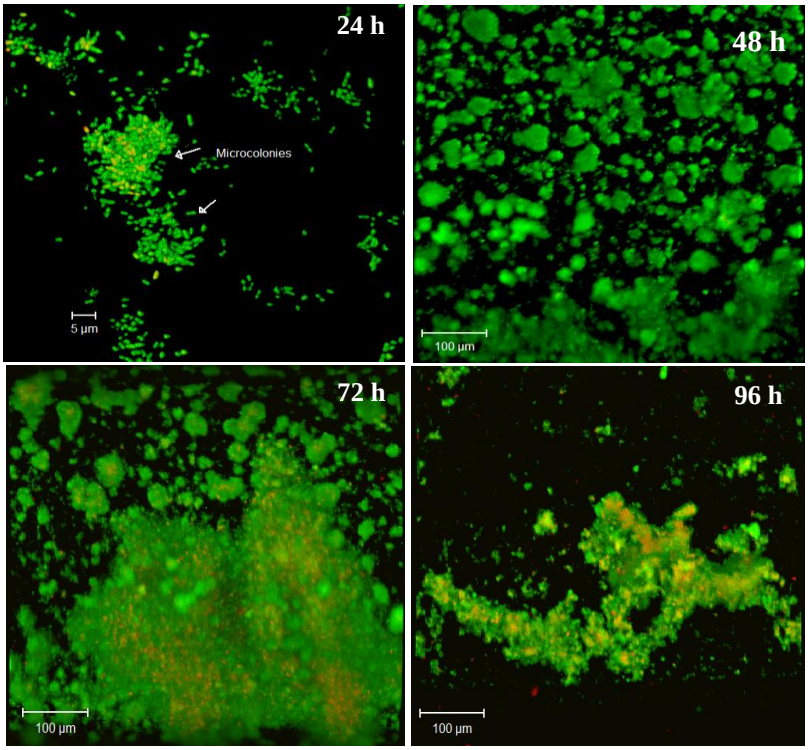
Figure 2 Biofilm-forming capacity of *B. thailandensis* wild type, *Δbth_i0249* and *Δbth_pilZ* mutant strains. **(A)** The ability of each isolate to produce biofilms was determined twice and the results reported are the average. Data represents mean±SD of 3 independent experiments. The asterisks (*) indicate significantly different ($P<0.05$) compared to the wild type control. **(B)** Biofilm formation of *B. thailandensis* wild type and *Δbth_i0249* and *Δbth_pilZ* mutant strains under static conditions by confocal microscopy. The 18 hour-broth cultures were diluted to give an OD at 540 nm of 0.8 - 0.9 in fresh medium and 2 ml of cultures were transferred to glass cover slips submerged in six-well plates. The plates were incubated at 37°C, under static conditions for 48 hours. Modified Vogel and Bonner's medium (MVBM) was replaced on each plate every 24 h. The attached bacteria, representing a 2-day biofilm culture under static conditions were analyzed by confocal microscopy (Zeiss, Jena, Germany).

3. *B. thailandensis Δbth_i0249* and *Δbth_pilZ* mutants were reduced in biofilm formation under laminar shear conditions

Using laminar shear flow cell conditions, the ability of *B. thailandensis* to form biofilms was determined. *B. thailandensis* was grown in a flow cell and biofilm formation was characterized over time (12-96 hours) using confocal microscopy. By 24 hours, microcolony formation was observed throughout the flow cell surface, with colonies sizes 10-20 μm (Figure 3A). No attached cells were observed at 12 hours (data not shown). After 48 hours of biofilm formation, macrocolony formation was observed, as shown in Figure 3A. A phenotypic switch was clearly observed at 72 hours with the larger macrocolonies being replaced with a mix of small and large cell clusters indicating biofilm maturation. Following mature biofilm formation, biofilm detachment was observed after 96 hours of incubation. In contrast to static conditions, biofilms grown in flow-thought medium conditions exhibited rough curved structures and covered all of the surfaces.

For the *Δbth_i0249* and its conserved domain, the *Δbth_pilZ* mutants, biofilm formations, both micro- and macrocolonies, were delayed (Figure 3B). After 48 hours, microcolonies (10 μm) and small macrocolonies (20 μm) were observed, however, cell clusters around the macrocolonies were not observed at 72 hours, as compared to wild type controls. Taken together, inactivation of *bth_i0249* and its conserved domain *pilZ*, affects biofilm formation and maturation under both static and dynamic conditions.

A



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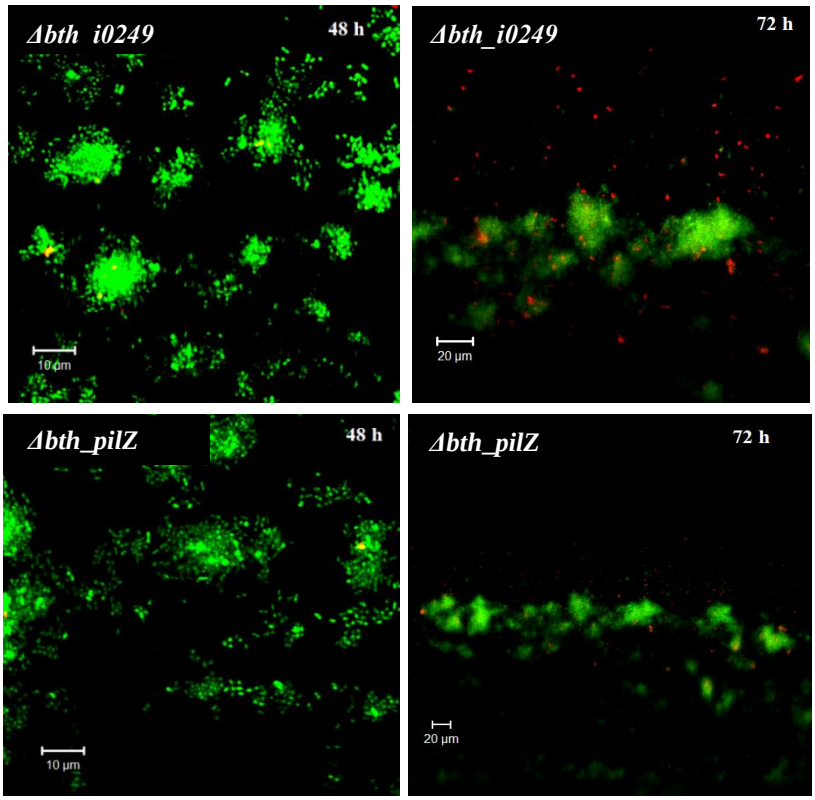


Figure 3 Phenotypic analysis of *B. thailandensis* wild type biofilm formation under laminar shear in flow cells **(A).** Phenotypic analysis of *B. thailandensis* **Δ***bth_i0249* and **Δ***bth_pilZ* mutant biofilm formation at 48 and 72 h under confocal microscopy (B).

Discussion and Conclusion

Biofilm formation is a biological process that bacteria enter in response to environmental conditions, such as a low nutritional source, oxidative stress, or exposure to sub-inhibitory concentrations of antibiotics. When bacteria switch from planktonic to biofilm mode of growth, they undergo a phenotypic shift. There are several steps involved in this phenotypic shift commencing with reversible attachment, followed by irreversible attachment, maturation, and dispersion. In the present study, two different biofilm growth conditions, static and shear force (flow cell), were used to characterize and identify genes involved in biofilm formation of *B. thailandensis.* These data provided the first steps in unraveling the functional consequences of the biofilm formation process in *Burkholderia* subspecies.

The phenotypic characterization of *B. thailandensis* biofilm formation and maturation demonstrated that this organism is capable of forming biofilms under static conditions, as previously described [8, 14-16]. Under static conditions, large macrocolonies were observed exhibiting a uniformly flat structure when seen under CLSM analysis after 48 h of incubation.

Model biofilms grown in the laboratory likely differ from the biofilms developed in clinical and environmental settings, which are more dynamic. To more closely mimic these dynamic environments, biofilms were grown under laminar shear force in flow cells. When *B. thailandensis* biofilms were grown in the presence of laminar shear in flow cells, the biofilm phenotypes were different from those being grown under static growth conditions. In the flow cell experiments, initial cell attachment and microcolony formations were observed earlier at 24 h. The flow cell phenotypes showed larger and taller clusters of cells that had a haze surrounding individual bacterial cell clusters at 48 h of incubation. These results were similar to those seen with *Pseudomonas aeruginosa* biofilms, in which extracellular DNA has been identified as a part of exopolysaccharide matrices [17-18]. A mature biofilm formation was seen at 72 h of incubation by achieved with thicker and more organized mushroom-shape masses with the maximal cell clusters surrounded by water channel voids. This phenotype has been reported frequently for developmentally mature biofilms in flowing conditions with the complex architecture known to facilitate efficient nutrient uptake [19], thereby delivering nutrients and other essential components to deeply embedded regions of a biofilm community. Biofilms entered into the detachment process of the biofilm life cycle at 96 h of the incubation. The detachment process in biofilm maturation is poorly understood but it is assumed that physical forces, such as hydrodynamics or shear forces are known to cause biofilm detachment either via erosion of single cells or sloughing of large aggregates of biomass [20]. Collectively, wild-type *B. thailandensis* readily formed biofilms under static and shear conditions and exhibited multiple phenotypes during biofilm formation and maturation.

The broad similarities between *B. pseudomallei* and *B. thailandensis* also raise the possibility that *B. thailandensis* could be useful as a potential model organism to study certain aspects of *B. pseudomallei* biology [7]. Herein, the *Δbth_i0249* and its conserved domain of *Δbth_pilZ* mutants were generated. The PilZ domain (Pfam domain PF07238) is identified in the sequences of bacterial

cellulose synthases, alginate biosynthesis protein Alg44, and proteins of enterobacterial YcgR. The PilZ domain can bind with the central regulator of the prokaryote, 3',5'-cyclic diguanylic acid (c-di-GMP) to control the biofilm lifestyle, indicating that this domain might play an important role in biofilm formation [21]. In *B. thailandensis*, *bht_i0249* encoded a 252 amino acid that is similar to the YcgR protein of *E. coli*, which functions as a motility regulatory protein. Interestingly, *bht_i0249* itself possesses the conserved PilZ domain (gi|83718394:282218-282331). The *bht_i0249* and its *PilZ* mutants biofilms were characterized under static and laminar shear conditions and that *bht_i0249* and its *PilZ* mutants were unable to produce flat biofilm structures on glass cover slips after 48 hours of growing under static conditions when compared with their parental strains. For the flow cell studies, formation of microcolonies and macrocolonies of *bht_i0249* mutants was delayed, with small microcolonies (10-20 μm) after 72 hours of incubation under flow-cell growth suggesting that the PilZ domain is required for biofilm formation.

The genes involve in biofilm formation of *B. thailandensis* were identified using differential microarray data from other Gram-negative bacteria that exhibited differential expressions in biofilms, as compared with free-living cells. The *bht_i0249* and its conserve *PilZ* domain with central regulator of the prokaryote, 3',5'-cyclic diguanylic acid (c-di-GMP) were found to regulate the biofilm formation both in static and flow cell conditions. Although *B. thailandensis* is not an important bacterial pathogen for human, its close relation with *B. pseudomallei*, a causative agent of fetal melioidosis, make this biofilm study is useful. Because in the biofilm induced conditions, *B. pseudomallei* will resist to ceftazidime, a drug of choice for melioidosis treatment [15]. Continued studies will elucidate more about genetic mechanisms of biofilm production, as well as the role of biofilms in infections of melioidosis.

Acknowledgements

The authors would like to thank Professor Robert Kenneth Ernst and Assoc. Prof. Mark E. Shirtliff, Department of Microbial Pathogenesis, School of Dentistry, University of Maryland, Baltimore, Maryland, USA for providing laboratory facilities. We also acknowledge Prof. James A. Will for editing the MS via Publication Clinic KKU, Thailand.

Funding information

This work was supported by the Royal Golden Jubilee Ph.D. (RGJ-PHD) program under Grant Number PHD/0179/2549 and Melioidosis Research Center, Khon Kaen University.

References

1. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, *et al.* Bacterial biofilms in nature and disease. Annu Rev Microbiol. 1987;41:435-464.
2. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbiol. 1995;49: 711-745.
3. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol. 2000;54:49-79.
4. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999 May 21;284(5418):1318-1322.
5. Donlan RM. Role of biofilms in antimicrobial resistance. ASAIO J. 2000 Nov-Dec;46(6):S47-52.
6. Hengge R. Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol. 2009 Apr;7(4):263-273.
7. Brett PJ, DeShazer D, Woods DE. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int J Syst Bacteriol. 1998 Jan;48 Pt 1:317-320.
8. Taweechaisupapong S, Kaewpa C, Arunyanart C, Kanla P, Homchampa P, Sirisinha S, *et al.* Virulence of *Burkholderia pseudomallei* does not correlate with biofilm formation. Microb Pathog. 2005 Sep;39(3):77-85.
9. Lopez CM, Rholl DA, Trunck LA, Schweizer HP. Versatile dual-technology system for markerless allele replacement in *Burkholderia pseudomallei*. Appl Environ Microbiol. 2009 Oct;75(20):6496-6503.
10. Bjarnsholt T, Jensen PO, Burmolle M, Hentzer M, Haagensen JA, Hougen HP, *et al. Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology. 2005 Feb;151 (Pt 2):373-383.
11. Leid JG, Shirtliff ME, Costerton JW, Stoodley P. Human leukocytes adhere to, penetrate, and respond to *Staphylococcus* aureus biofilms. Infect Immun. 2002 Nov;70(11):6339-6345.
12. Stapper AP, Narasimhan G, Ohman DE, Barakat J, Hentzer M, Molin S, *et al.* Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation. J Med Microbiol. 2004 Jul;53(Pt 7):679-690.
13. Takenaka S, Iwaku M, Hoshino E. Artificial *Pseudomonas aeruginosa* biofilms and confocal laser scanning microscopic analysis. J Infect Chemother. 2001 Jun;7(2):87-93.
14. Korbsrisate S, Vanaporn M, Kerdsuk P, Kespichayawattana W, Vattanaviboon P, Kiatpapan P, *et al.* The *Burkholderia pseudomallei* RpoE (AlgU) operon is involved in environmental stress tolerance and biofilm formation. FEMS Microbiol Lett. 2005 Nov 15;252(2):243-249.
15. Sawasdidoln C, Taweechaisupapong S, Sermswan RW, Tattawasart U, Tungpradabkul S, Wongratanacheewin S. Growing *Burkholderia pseudomallei* in biofilm stimulating conditions significantly induces antimicrobial resistance. PLoS One. 2010;5(2):e9196.

16. Tunpiboonsak S, Mongkolrob R, Kitudomsub K, Thanwatanaying P, Kiettipirodom W, Tungboontina Y, et al. Role of a *Burkholderia pseudomallei* polyphosphate kinase in an oxidative stress response, motilities, and biofilm formation. J Microbiol. 2010 Feb;48(1):63-70.
17. Steinberger RE, Holden PA. Extracellular DNA in single- and multiple-species unsaturated biofilms. Appl Environ Microbiol. 2005 Sep;71(9):5404-5410.
18. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science. 2002 Feb 22;295(5559):1487.
19. Stoodley P, Debeer D, Lewandowski Z. Liquid flow in biofilm systems. Appl Environ Microbiol. 1994 Aug;60(8):2711-2716.
20. Stoodley P, Cargo R, Rupp CJ, Wilson S, Klapper I. Biofilm material properties as related to shear-induced deformation and detachment phenomena. J Ind Microbiol Biotechnol. 2002 Dec;29(6):361-367.
21. Amikam D, Galperin MY. PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics. 2006 Jan 1;22(1):3-6.

Supporting Information

Supporting Information S1: Show sequence of deleted fragment (*bth_i0249*) and position of primer binding sites

>gi|83718394:280837-283595 Burkholderia thailandensis E264 chromosome I, complete sequence

BTH_I0249 – F1

AGATGATGTTGAAGAGCATGCGCGATGCGACGCCGTCGGACGGCCTGCTCGATTCGAACTCGTCGAAGATGTACA

CGTCGATGCTCGATCAGCAGCTCGCGCAGCAGATGTCGTCGAAGGGCATCGGCGTTGCCGACGCGCTGACGAAGC

AGTTGCTGCGCAACGCGAACGTGGCGCCCGACGCGCAGAGCGAAGGCGGGCTCGCGGCGATGAACGCGCTCGCGA

AGGCGTACGCGAATTCGAATGCGTCGTCCGGCAACGGCGCGCTCGCGGGCACGCACGGCTATTCGGCGGCGAGCG

CGCTCACGCCGCCGCTCAAGGGCAACGGCTCCCCGCAGGCGGAGGCGTTCGTCGAGAAGATGGCGGGCGCCGCGC

AGGCGGCGAGCGCGGCCACCGGCATTCCGGCGCGCTTCATCGTCGGCCAGGCGGCGCTCGAATCCGGCTGGGGCA

AGCGCGAGATTCGCGGCGCGAACGGCGAATCGAGCTACAACGTATTCGGCATCAAGGCGACGAAGGGCTGGACCG

GCCGCACGGTGTCGGCCGTCACGACCGAGTACGTGAACGGCAAGCCGCACCGCGTCGTCGCGCGGTTCCGCGCGT

ACGATTCGTACGAGCACGCGATGACCGATTACGCGAGCCTCCTGAGGAACAACCCGCGCTACGCGAGCGTGCTGA

ACGCGGGCCACAGCGCGGAGGGCTTCGCCAACGGGATGCAGAAGGCCGGCTACGCGACCGATCCGCACTACGCGA

AGAAGCTGATTTCGATCATGCAGCAGATCGGCTGATGTAAGCCGAGCAACCGTTCGGTACATTTCGGACCGCGCC

GGGCTTCGCCTCGCGCGGTTTTAACGTTTGCTGAAAAAATAAACTCCGGTTTCGATCTAAACTTTGCGCAGCCGT

TGCCGCTAAACGTGAGAGACCAGCGATCGGGCGGATCACGCCCGGTCATAACGCGCCCCCGAAATCCGCCAGGCG

BTH_I0249 – R1

CATAGAGATAAAATACCGGCAAGCCATGAATACCGAACAGTCGACGAGTCCGCCCGCGAACGCCGCCGCGCATTC

CGGCCACGATTACGGCCGCCGCAATCCGCTCGAGATCGGCGTGCAGTTGCGCAACCTCGTCAATCGCGGCGATTT

CCTGACCGTCCAGTATCAGGGCGGCCAACTCGTCACCCGCATCCTCGACGTCGACGTCGGCGCGCGGACCTTCGT

ATTCGACTGGGGCGCGCTTGCCGACCAGAACGCGGGCATCCTCGCCGCGCAGCGCTGCGTGTTCCATGCGTCGCC

GGACGGCGTGCGCGTCGAATTCTCGACCGCGACCCCGCGCGAAACCCGTTACGAGAACCTGCCGGCGTTCGAGGC

CGACTTTCCCGATGTGCTGTACTGCGTGCAGCGCCGCGAATATTTCCGGGTCGACGCGCCGATCCTCGATCCGTA

CGTGTGCCGAGGCAAGCTGCCCGACGGCGAGAGCTTCCTGTTCGAGGTGCACAACCTGTCGCTCGGCGGCCTGGG

GCTGCGCACGGGCGACGATCGCGTGGCGTCGTTCGAGCCGGGGATGACGCTGCCCGACGTGGAGCTGAACCTGAA

CGGCCACGGGATGCTGTCGCTCGACCTGCAGCTCGTGTCGCACCGCGCAACCGAGACGCCGAGCGGTGCGCGCCG

CTACCAGTTGGGCTTTCGCTTCCTGTCGCTGCCGGGCAATGCCGAGAATACGTTGCAGCGCATCATCACGCAGCT

CGAGATGAAGCGCCGGCAACTCGCGCGCGCCTGACGAAACGCGCGGCGCGCGGCCGGCGAACCCGCTCGGGGCGG

BTH_I0249 – F2

CAGAAACGCATGCGGCGGCGCTCGCGCGCAGGCCGCACGCCGCGGCACGCATTTTTGCGCGTGCGATCCCTCAAT

TTTCTCGACGCGCGGCCGTTATACCGGGAGTCCACGCAACCCGGCGGCCGCGGCGCGGCCGGCTCATAGGATCGC

GCATGTCCAATACCCTCATGAACCTCGGGGTCAGCGGCCTGAACGCCGCACTCTGGGGGCTCACCACGACCGGCC

AGAACATCAGCAACGCGGCGACGCCCGGCTATTCCGTCGAGCGCCCCGTCTACGCGGAGGCGAGCGGCCAGTACA

CGAGCAGCGGCTATCTGCCGCAAGGCGTGTCGACCGTCACCGTCGAGCGCCAGTACAACCAGTATCTGTCGAATC

AGCTGAACGCCGCGCAGACGCAGGGCAGTTCGCTGTCGACCTACTACACGCTCGTCGCGCAACTGAACAACTACG

TCGGCAGCCCGACGGCCGGCATCGCGACCGCGATCACGAACTACTTCACCGGCCTGCAGACGGTCGCGAACAACG

CGGCTGACCCGTCCGCGCGGCAGACCGCGATGAGCAACGCGCAGACGCTCGCGAGCCAGCTCGTCGCCGCGGGCC

AGCAATACTCGCAACTGCGCCAGAGCGTGAATTCGCAGCTCACCGATACCGTCACGCAGATCAACAGCTACACCT

BTH_I0249 – R2

CGCAGATCGCGCAGCTCAACGAGCAGATCGCGTCGGCGAGCTCGCAGGGGCAGCCGCCGAACCAGCTTCTCGATC

**Gray color is the region that was deleted.

Supporting Information S2: Show sequence of deleted fragment (*pilZ*) and position of primer binding sites

>gi|83718394:281837-282595 Burkholderia thailandensis E264 chromosome I, complete sequence

PilZ_BT0249 – F1

ATGAATACCGAACAGTCGACGAGTCCGCCCGCGAACGCCGCCGCGCATTCCGGCCACGATTACGGCCGCC

GCAATCCGCTCGAGATCGGCGTGCAGTTGCGCAACCTCGTCAATCGCGGCGATTTCCTGACCGTCCAGTA

TCAGGGCGGCCAACTCGTCACCCGCATCCTCGACGTCGACGTCGGCGCGCGGACCTTCGTATTCGACTGG

GGCGCGCTTGCCGACCAGAACGCGGGCATCCTCGCCGCGCAGCGCTGCGTGTTCCATGCGTCGCCGGACG

GCGTGCGCGTCGAATTCTCGACCGCGACCCCGCGCGAAACCCGTTACGAGAACCTGCCGGCGTTCGAGGC

PilZ_BT0249 – R1

CGACTTTCCCGATGTGCTGTACTGCGTGCAGCGCCGCGAATATTTCCGGGTCGACGCGCCGATCCTCGAT

CCGTACGTGTGCCGAGGCAAGCTGCCCGACGGCGAGAGCTTCCTGTTCGAGGTGCACAACCTGTCGCTCG

GCGGCCTGGGGCTGCGCACGGGCGACGATCGCGTGGCGTCGTTCGAGCCGGGGATGACGCTGCCCGACGT

PilZ_BT0249 – F2

GGAGCTGAACCTGAACGGCCACGGGATGCTGTCGCTCGACCTGCAGCTCGTGTCGCACCGCGCAACCGAG

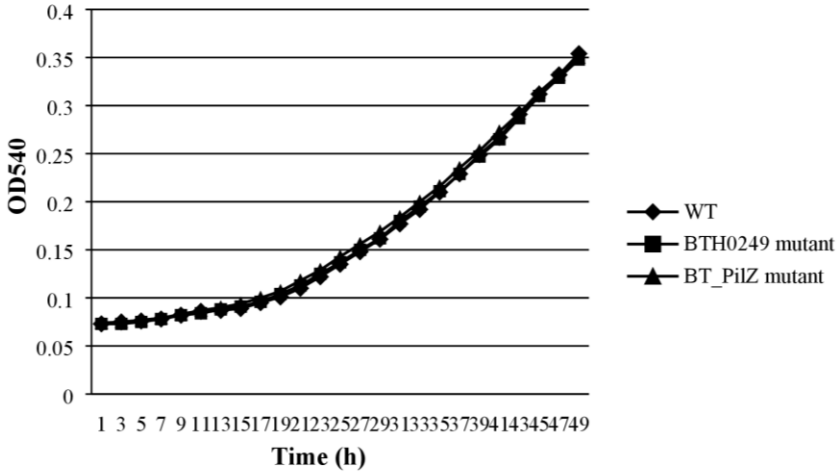
ACGCCGAGCGGTGCGCGCCGCTACCAGTTGGGCTTTCGCTTCCTGTCGCTGCCGGGCAATGCCGAGAATA

CGTTGCAGCGCATCATCACGCAGCTCGAGATGAAGCGCCGGCAACTCGCGCGCGCCTGA

PilZ_BT0249 – R2

**Gray color is the region that was deleted.

Supporting Information S3: Comparison of the growth between *B. thailandensis* wild type, *Δbth_i0249* and *Δbth_pilZ* mutants in MVBM medium



Comparison of the growth between *B. thailandensis* wild type, *bth_i0249* and *pilZ* mutants in MVBM. The bacteria were cultured in MVBM medium and measured for turbidity (wave length 540 nm) at various time points.