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Role of a *Burkholderia pseudomallei* Polyphosphate Kinase in an Oxidative Stress Response, Motilities, and Biofilm Formation

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***Burkholderia pseudomallei*, a motile and rod Gram-negative bacterium, is the causative agent of melioidosis. The bacterium is an intracellular pathogen and that motility is generally crucial for their survival in a natural environment and for systemic infection inside a host. We report here a role of *B. pseudomallei* polyphosphate kinase in virulence, such as an oxidative stress response, motilities and biofilm formation. The polyphosphate kinase (*ppk*) mutant is susceptible to hydrogen peroxide in an oxidative stress condition, unable to perform swimming, swarming motilities, and has lower density biofilm forming capacity than the wild-type strain. We also demonstrated that both polyphosphate kinase and motile flagella are essential and independently involved in biofilm formation. The *B. pseudomallei* flagellin (*fliC*) mutant and *B. mallei*, a non-motile species, are shown to produce higher density biofilm formation than the *ppk* mutant, but less than wild type *B. pseudomallei*.**

***Keywords*:** biofilm, *B. mallei*, *B. pseudomallei*, flagellin, oxidative stress, polyphosphate kinase

Burkholderia pseudomallei, a motile and Gram-negative rod, is the causative agent of a life-threatening disease known as melioidosis (White, 2003). The bacterium is an intracellular pathogen due to its ability to penetrate and to infect by colonization, invasion, survival and growth inside the host cell. The pathogenic bacteria have special properties, virulence factors, which enhance the ability to cause disease. Bacterial motility is one virulence factor that is needed for the colonization and the invasive capacities in several pathogenic bacteria (Chua, 2003). Potential benefits of motility include increased efficiency of nutrient acquisition, avoidance of toxic substances, ability to translocate into preferred hosts and access to optimal colonization sites within them, and dispersal in the environment during the course of transmission. The cost of motility is significant considering the metabolic burden of synthesizing and assembling various flagella and pili components and the energetic expense of fueling flagella and presumably the pili motor. It is often crucial to bacteria survival in a natural environment and for systemic infection inside a host. The dependence of motility on the presence of polyphosphate kinase reveals important roles for polyphosphate in diverse processes such as virulence, biofilm formation, and symbiosis in *Pseudomonas aeruginosa* (Rashid *et al.*, 2000).

Burkholderia mallei, a non-motile and Gram-negative bacillus, is a zoonotic pathogen that causes a disease known as glanders. The organism has been phylogenetically classified as a clone of *B. pseudomallei*. As compared *B. pseudomallei*, *B. mallei* had limited surviving capabilities in the environment (Godoy *et al.*, 2003) and it is host-restricted (Schell *et al.*, 2008). Flagella are used to differentiate *B. mallei* from *B. pseudomallei*. *B. mallei* is non-motile as a result of the lack of flagella, although flagellin genes are present (Nierman *et al.*, 2004). The physiological lack of flagella marks its importance to this study.

The polyphosphate kinase genes encoded polyphosphate kinase enzymes of which two families, PPK1 and PPK2, have been identified in many bacteria (Brown and Kornberg, 2008). The enzymes are responsible for the synthesis of inorganic polyphosphate from ATP. The recent availability of genome sequences of *B. pseudomallei* and *B. mallei*, has shown that a *ppk* gene occurs in both species with 99% nucleotide identity and has 78% similarity to the *ppk*1 of *Ralstonia solanacearum* but not to the *ppk*2. To study the role of *ppk* in *B. pseudomallei*, a *ppk* mutant was constructed and analyzed with regard to oxidative stress response to hydrogen peroxide, motilities and biofilm formation in comparison with the parent strain. In order to find the relationship between bacterial flagellum and the polyphosphate kinase in the ability to form biofilm, we compared the biofilm production with the *B. pseudomallei* flagellin mutant (*fliC*) as well as *B. mallei*. Our report here demonstrates that both polyphosphate kinase and motile flagella are essential and independently involved in the biofilm formation process.

Materials and Methods

Bacterial strain and growth conditions

The bacterial strains used are listed in Table 1. MM35 is a *B. pseudomallei* flagellin (*fli*C) mutant from a clinical isolated 1026b wild type. *B. pseudomallei* and *B. mallei* were routinely maintained in Luria-Bertani (LB) medium. Pseudomonas agar base supplemented with SR103E (Cetrimide, Fucidin, and Cephaloridine) from Oxoid

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Table 1. Bacterial strains

Strain or plasmid	Genotype or relevant characteristic	Source of references
B. pseudomallei		
NF10/38 (BpWT)	Wild type, clinical isolate from blood	This study
PPKM	NF10/38: :pKPPK	This study
PPKM/pBSDPPK	NF10/38: :pKPPK containing pBSDPPK	This study
MM35	1026b: :Tn5-OT182*fliC*	DeShazer *et al.* (1997)
B. mallei EY2233 (BmWT)	Wild type, infected human	Tanpiboonsak *et al.* (2004)
Plasmids		
pKNOCK-Tc	Mobilizable suicide vector, Tet^r	Alexyev (1999)
pKPPK	pKNOCK-Tc containing a 500-bp internal segment of *B. Pseudomallei ppk* gene	This study
pBR1MCS	Broad-host-range cloning vector, Cm^r	Kovach *et al.* (1994)
pBSDPPK	pBR1MCS containing full-length *ppk* gene	This study

was used after conjugation as selective medium to inhibit growth of *E. coli*. All cultures were grown at 37°C in an aerobic condition with 250 rpm shaking. Tetracycline (60 μg/ml) and chloramphenicol (40 μg/ml) were added to media when required.

Construction of a *B. pseudomallei ppk* mutant and its complemented strain

A *ppk* knockout mutant, PPKM (Table 1), was created with pKPPK according to a previously described procedure (Low, 1991). The pKPPK was constructed by transferring the 500-bp *Kpm*I-*Xba*I fragment from pUCPPK into the mobilizable suicide vector pKNOCK-Tc (Alexyev, 1999). The constructed *B. pseudomallei ppk* mutant was analyzed by Southern blot analysis and PCR as described elsewhere (Sambrook and Russell, 2001). To confirm that all changes in phenotypes were caused by the disruption of *ppk* and were not due to polar effects on downstream genes, a plasmid (pBSDPPK) containing the complete *ppk* coding sequence under control of the *lac*Z and *cat* promoters was constructed and transferred into *B. pseudomallei* wild-type and mutant strains for complementation analysis.

Growth inhibition zone assay under hydrogen peroxide treatment

Bacterial cultures grown overnight in LB-medium were adjusted to OD 600 nm of 1.0 and added to 3 ml warm top LB agar. The mixtures were overlaid onto LB agar plates. Paper discs containing 10 μl of a 5, 50, 500, and 5,000 mM hydrogen peroxide solution were put on the cell lawn. The diameters of growth inhibition zones were measured after 24 h incubation as described by Loprasert *et al*. (2004).

Motility assay in swimming, swarming, and twitching

Motility assays in swimming, swarming and twitching were performed as described by Rashid and Kornberg (2000). The medium used for the swimming motility assay was tryptone broth [10 g/L trypton (Difco); 5 g/L NaCl] that contained 0.3% (w/v) bacto agarose. Swim plates were inoculated with bacteria from activated cultures which were prepared in LB agar (1.5%, w/v) plates at 37°C for overnight. The swim plates were then incubated at 30°C for 12 h.

Medium used for swarming motility assay consisted of 0.5% (w/v) bacto-agar with 8 g/L Difco-nutrient broth, to which 5 g/L glucose was added. Swarm plates are typically allowed to dry at room temperature overnight before being used. Swarming efficiency was improved when cells from swim agar (0.3%, w/v) plates were inoculated onto swarm plates and were incubated overnight at 37°C. An inoculation from an overnight LB agar (1.5%, w/v) plate also supported swarming.

Medium used for twitching motility assay consisted of LB broth (10 g/L trypton; 5 g/L yeast extract; 10 g/L NaCl) solidified with 1% (w/v) bacto-agar. Twitch plates were briefly dried and strains were stab inoculated with a sharp toothpick to the bottom of the Petri dish from an overnight-grown LB agar (1.5%, w/v) plate. After incubation at 37°C for 24 h, the zone of motility at the agar/Petri dish interface was measured.

Colorimetric measurement of biofilm formation density

Aseptically transferred 10^9 cell/ml of bacterial culture was directly used for general assay. In case of pool down or driven force condition, the bacterial culture was centrifuged at 2,000 rpm for 5 min in a sterile 96-well polysterene plate before assay. Then they were incubated at 37°C for 12, 24, 36, 48, and 60 h. At the end of the incubation period the wells were drained and immediately washed three times with distilled water. Crystal violet dye (1%) was added to cover the depth of the culture in each well. After staining for 15 min, the crystal violet was suctioned out and washed three times with distilled water. The soluble crystal violet was removed from the complex in 200 ml of 95% ethanol and the absorbance was measured by spectrophotometer at OD 540 nm as described elsewhere (O'Toole *et al.*, 1999).

Imaging of biofilm formation by Confocal Laser Scanning Microscope (CLSM)

The image of biofilm forming bacteria by a CLSM method was carried out as previously described (Takenaka, 2001). Briefly, bacteria were grown in the same condition as used for the colorimetric method until 24 h and then transferred and fixed on the cover slips by glutaraldehyde. Then the fixed-died bacterium was stained with green- fluorescein of isothiocyanate-concanavalinA or FITC-ConA which reacts to exopolysaccharide matrixes of the biofilm. After washing out the green fluorescein, the bacterial DNA was stained by the red-fluorescein of Topro3. The cover slip with 100 μl of 80% glycerol in 20% 1× PBS was imaged by FluoView FV1000 confocal microscope (OLYMPUS) at wavelengths of 488 nm for FITC-ConA and 633 nm for Topro3, respectively.

Results

Defect in oxidative stress response in the *ppk* mutant treated with hydrogen peroxide

To test whether the *ppk* gene is involved in oxidative stress response in *B. pseudomallei*, an inhibition zone assay was performed as shown in Fig. 1A. Approximately 55% up to 60% of the *ppk* mutant (PPKM) are susceptible to hydrogen peroxide in a dose dependent manner from 5 to 500 mM compared to its wild type (BpWT) as represented in Fig. 1B.

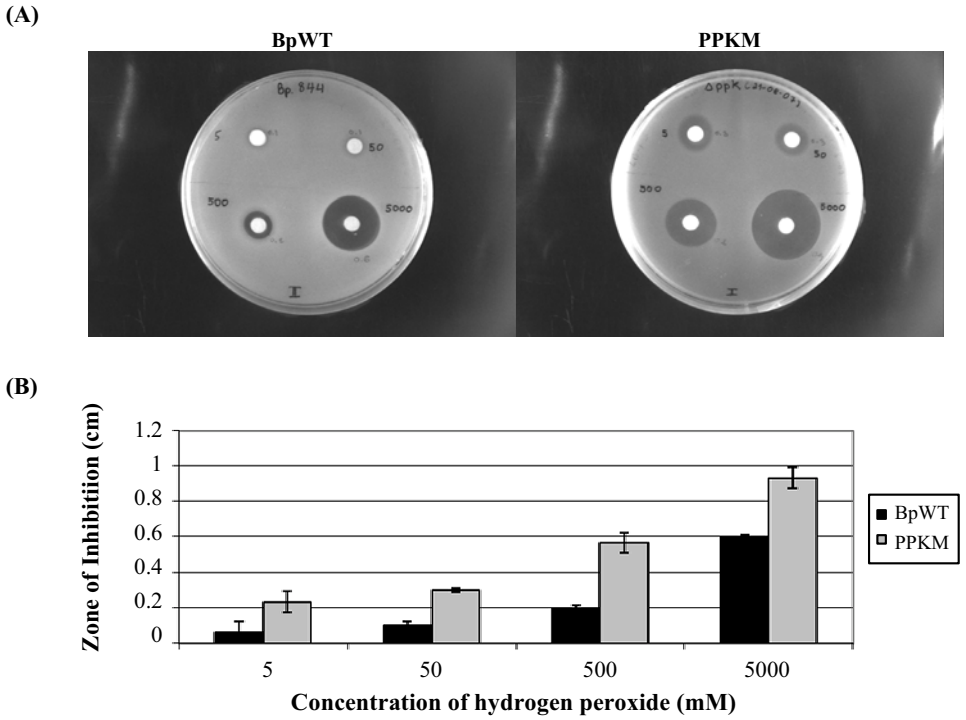

Fig. 1. Determination of sensitivity to hydrogen peroxide, an oxidative stress condition. The BpWT and PPKM were exposed to hydrogen peroxide in various concentrations from 5 mM, 50 mM, 500 mM, and 5000 mM. The zone of inhibition (A) were determined and presented in bar graph (B). Data are the means for three independent experiments and are presented as Mean±SEM.

Characterization of Motility in a *B. pseudomallei ppk* mutant

To examine whether the *ppk* mutant has any defects in the swimming, swarming and twitching motilities, the motility assays of the *ppk* mutant and its complement strain were compared with that of the corresponding wild type strain. On swimming and swarming plates as shown in Figs. 2A and B, respectively, the *ppk* mutant was severely impaired in both motilities. The motilities defects were complemented by introducing the corresponding gene on a medium-copy-number plasmid, pBSDPPK, into the mutant and both the swimming and swarming motilities were completely restored. Migration of the swimming and the swarming cells from the points of inoculation (turbid zone) could be observed distinguishable around the growth in both of wild type strain and complement strain.

Twitching motility was not significantly different in all strains (Fig. 2C). The bacterial motility appeared when cells were stabbed through an agar layer to the bottom of the petri dish, and after 24 h a slightly spreading colony expansion at the interstitial surface between the agar and the plastic was detected in all strains.

Defects in biofilm formation of the *ppk* mutant, the *fliC* mutant and *B. mallei*

The biofilm formation as a function of time in various bacteria

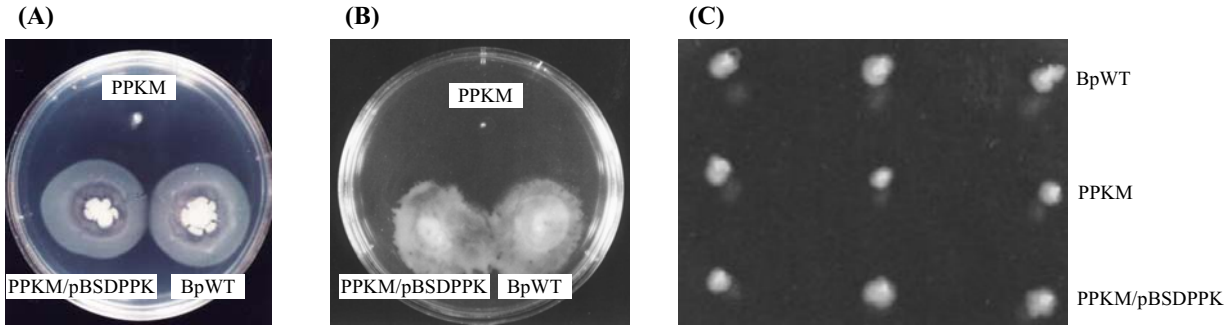

Fig. 2. Motility assay for swimming, swarming, and twitching. Motility of *B. pseudomallei* wild type (BpWT), the *ppk* mutant (PPKM), and its complementation strain (PPKM/pBSDPPK) were assessed on the swimming (A), swarming (B), and twitching (C) plates. Data are the means for three independent experiments and are presented as Mean±SEM.

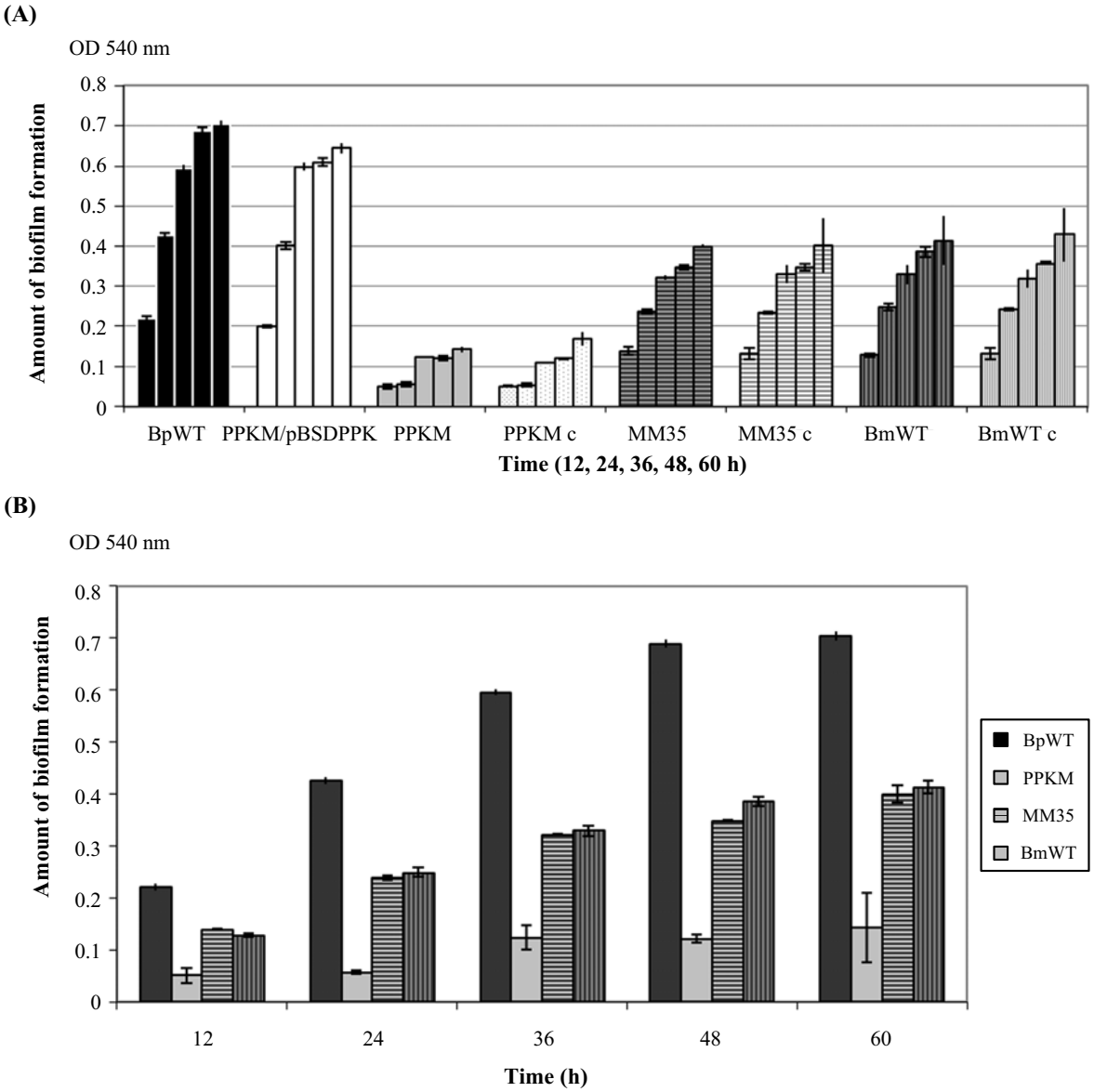

Fig. 3. Determination of biofilm production by colorimetric method. (A) Colorimetric measurement of the amount of biofilm formed by various strains with and without centrifugation steps at each time growth. Error bars indicated SEM. The strains are BpWT, PPKM/pBSDPPK, PPKM, PPKMc, MM35, MM35 c, BmWT, BmWT c, respectively. (B) Colorimetric measurement of relative biofilm forming capacity of BpWT, PPKM, MM35, and BmWT, respectively. Data are the means for three independent experiments and are presented as Mean±SEM.

was compared to the *B. pseudomallei* wild type strain by colorimetric assay as shown in Fig. 3A. Biofilm density of the wild type (BpWT) at 12 up to 24 h (initial attachment) of bacterial growth produced approximately 60% of the final phase and then the biofilm formation was increased up to the maximum at the late stationary phase of growth (60 h). In order to illustrate a defect of the *ppk* in biofilm production, the mutant with its complement strain (PPKM/pBSDPPK) was tested and its yields were comparable with that of the corresponding wild type strain, whereas the *ppk* mutant (PPKM) was defect in biofilm production at 12 up to 24 h. The biofilm density was only 20% that of the wild type whereas it was 15% less than the maximum production of the wild type at the late stationary phase (60 h). In an attempt to demonstrate that the non-motile flagella of the *ppk* mutant could play an important role as an adhesin in the initial attachment for biofilm formation, a driven force in the initial attachment of the *ppk* mutant under centrifugation condition (PPKMc) was performed and a similar pattern of the biofilm production without centrifugation was detected. This result of the centrifuged *ppk* mutant indicated that the energy driven force from centrifugation could not increase the initial attachment step. Therefore, a flagellum alone without internal energy of the bacterium could not act as an adhesion to support the biofilm production.

To demonstrate that the flagellum plays an important role in biofilm formation, the two aflagellated strains, *B. pseudomallei* flagellin (*fliC)* mutant (MM35) and *B. mallei* (BmWT) was

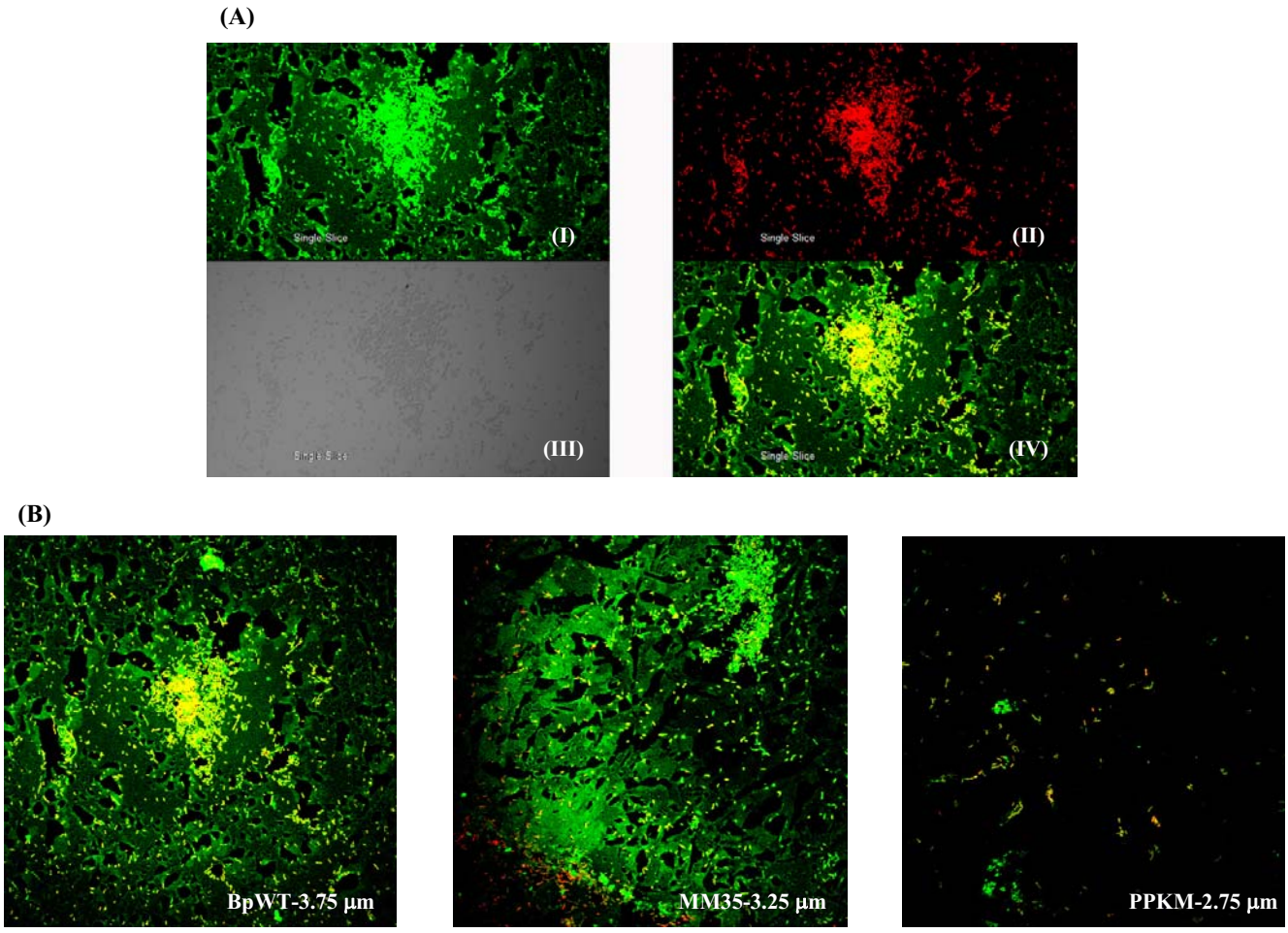

Fig. 4. Imaging of biofilm production by Confocal Laser Scanning Microscope. (A) The images of BpWT were observed under Confocal Laser Scanning Microscope (CLSM). Exopolysaccharide matrixes stained with FITC-ConA (I), the bacterial DNA stained with Topro3 (II), the bacterial cells from light microscope (III) and merged pictures between (I) and (II) with the mixed color between green and red showing yellow color that is the bacteria cell having an exopolysaccharide matrix (IV). (B) The CLSM images of relative biofilm formation capacity among the BpWT, MM35, and PPKM, respectively. The thickness of the exopolysaccharide is indicated in µm.

carried out and both yielded similar biofilm production. The results showed that at each time of bacterial growth from 12 up to 60 h, the biofilm densities were produced only 60% of the wild type. The similar results were obtained when the two aflagellated strains (MM35c and BmWTc) were pooled down to driven the initial attachment by centrifugation. Our results indicated that a *ppk* and a motile flagellum rather than aflagellated or non-motile flagellum, need for initial attachment and movement before bacterial growth into the late stationary phase to form biofilm.

Comparative biofilm production among the *B. pseudomallei ppk* mutant, *B. pseudomallei fliC* mutant with its wild type and its clone species, *B. mallei*, as studied by a colorimetric method as shown in Fig. 3B. The results illustrated that the *ppk* mutant produced the lowest biofilm production in all stages of growth and yielded about 15-20% of the wild type, while the two aflagellated strains produced approximately 40% less biofilm than that of the wild type and its clone species. The image of biofilm forming bacteria by Confocal Laser Scanning Microscope (CLSM) method was illustrated in Fig. 4 and a similar result as with the colorimetric method was obtained (Fig. 4B). These images indicated that both *ppk* and *fliC* mutants have defects in micro-colony formation, showing wide distribution of the bacteria in contrast to the wild type. Furthermore, the *fliC* mutant strain revealed higher production of exopolysaccharide than the *ppk* mutant. Our results suggested that the polyphosphate kinase is involved in all three steps of bacterial movement, micro-colonies formation and exopolysaccharide production, whereas the flagellum is involved in the first two steps of bioflim formation.

Discussion

The potential roles of polyphosphate kinase in *B. pseudomallei* were studied with respect to oxidative stress, motilities and biofilm formation and also compared to the two aflagellated strains, a *B. pseudomallei fli*C mutant and *B. mallei*. Many functions of polyphosphate kinases have been reported (Korn-

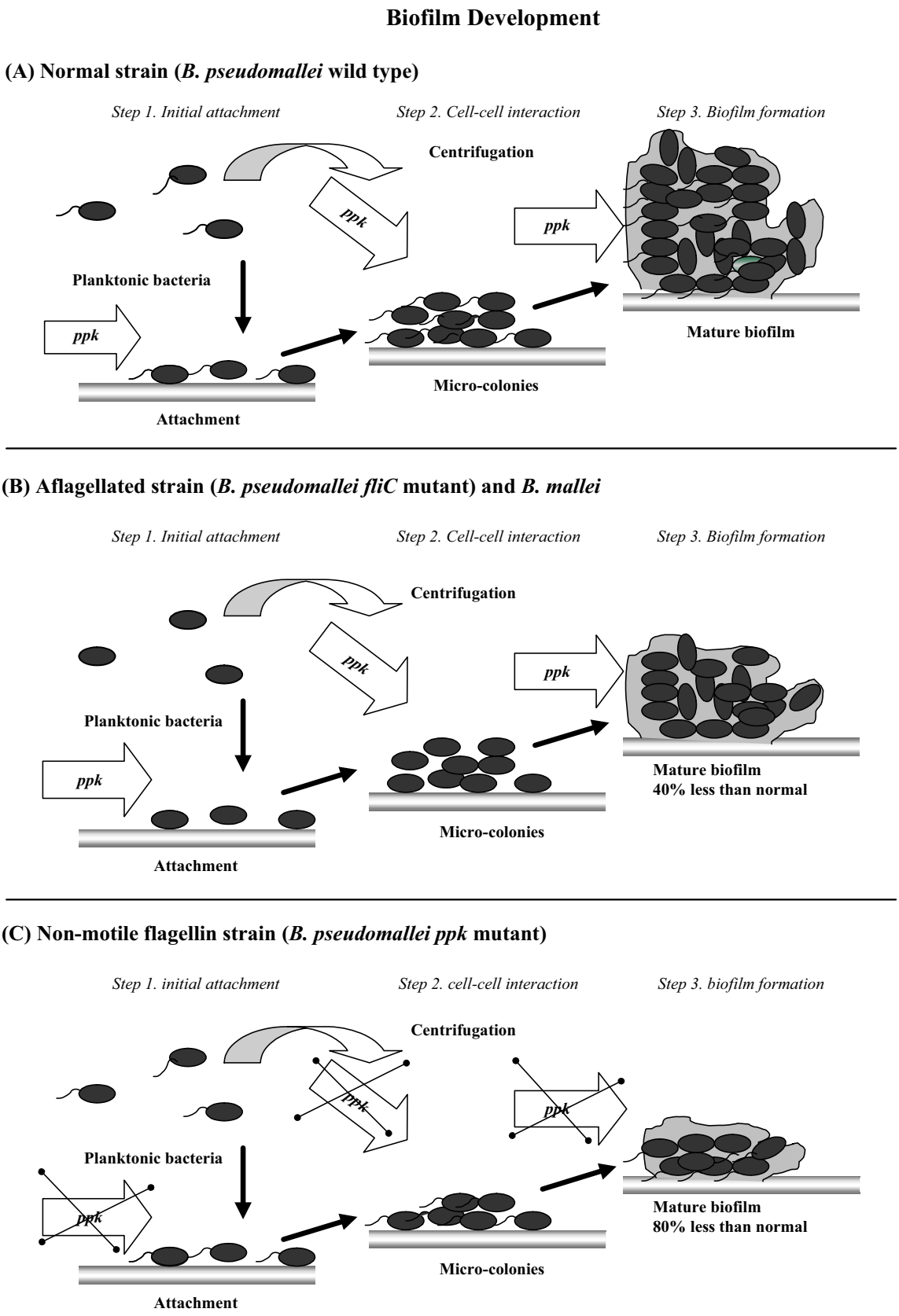

Fig. 5. Diagram showing steps of biofilm development. (A) the normal strain (*B. pseudomallei* wild type), (B) the aflagellated strains (*B. pseudomallei fliC* mutant), the non-motile strain (*B. mallei* wild type), and (C) the non-motile flagellin strain (*B. pseudomallei ppk* mutant).

berg *et al.*, 1999; Rashid *et al.*, 2000; Rashid and Kornberg, 2000; Candon *et al.*, 2007; Fraley *et al.*, 2007; Richards *et al.*, 2008), however the present study is the first demonstration of an insight relationship among aflagellated bacteria, non-motile-flagella and motile-flagella via polyphosphate kinase function.

The *ppk* mutant was shown to be more susceptible to hydrogen peroxide than the wild type. A defect in an oxidative stress response could be explained by the lack of ability in movement to avoid chemical damage. It is not related with retardation in growth, since we had started to demonstrate a growth rate of all the mutants performed in this study and found that all growth curves had no significant difference (data not shown). In the motility assays, both swimming and swarming defects were detected in the *ppk* mutant, whereas twitching motility was not significantly different from the wild type. In general, defects in both swimming and swarming are due to flagella functioning (Arkhipov *et al.*, 2006), whereas twitching motility is involved with pili functioning (Fraley *et al.*, 2007). In order to illustrate whether both defects on swimming and swarming were due to flagella formation or motile flagella, we therefore detected a flagella structure of the *ppk* mutant comparing with its parental strain by electron-microscope. The result indicated that the *ppk* mutant produces the same flagella structure as its parental strain (data not shown). Thus, we are able to conclude that the *ppk* gene involves in motile flagella which supports to the bacterial movement for their survival in a toxic chemical or an oxidative condition whereas pili are not significantly played in this role. A similar result has been reported in *Pseudomonas aerugenosa* and it has been concluded that flagella in a *ppk* mutant are not able to function which due to inadequate supply of energy to flagella (Rashid and Kornberg, 2000; Fraley *et al.*, 2007).

Many recent studies have been performed in order to find the relationship between bacteria flagella and the ability to form biofilms. Biofilm formation by many bacteria involves three major steps (McLean *et al.*, 2005; Ma *et al.*, 2006; Ryder *et al.*, 2007). The first step is the movement of the free floating bacteria to an abiotic site and permanent attachment to the surface by using flagella or pili (Kornberg *et al.*, 1999). The second step is micro-colonies formation or cell-cell interaction. The last step is secretion of exo-polysaccharide to form biofilm. It has been reported that flagella mediated motility has often been associated with the initial step of biofilm development (Prigent-Combaret *et al.*, 2000). However, whether flagellum is required in the transport of microbes to a surface or plays a role in initial attachment (acts as an adhesin) is not yet classified. A study in *P. aeruginosa* has shown the relationship between flagella and/or flagella-mediated motility to the ability to form biofilm (Klausen *et al.*, 2003). Furthermore a study by Watnick and Kolter showed that flagella, along with the type IV pilus, accelerate attachment to the abiotic surface and flagella alone mediates spread along the abiotic surface (Pratt and Kolter, 1998; Watnick and Kolter, 1999). In our studies, we performed an additional centrifugation step to accelerate or mimic a motile flagellum for the initial attachment of the bacteria to the abiotic surface. The results in all bacterial types showed no significant difference between with and without centrifugation step (Fig. 3A). We therefore are able to conclude that transportation of microbes to an abiotic surface is not essential in the initial attachment of biofilm formation, since centrifugation of the bacteria to attach on abiotic surface did not increase biofilm production. Furthermore, the flagellum seemed to have a limited role in initial attachment for the bacterial biofilm formation, since the two aflagellated strain, a *B. pseudomallei fli*C mutant and *B. mallei*, produced higher biofilm formation than a *B. pseudomallei ppk* mutant which contains flagellum but is non-motile. Thus our finding indicated that the motile-bacteria with either motile flagella or motile pili or both are important for the initial attachment to a form biofilm. Moreover, motile bacteria are required to increase the biofilm structure formation that is involved in the third step of biofilm development. In addition to our CLSM results, secretion of exo-polysaccharide in the third step might require energy production via polyphosphate kinase (*ppk*) as demonstrated in Fig. 4B.

Overall, the role of *B. pseudomallei* motile-flagella and polyphosphate kinase in biofilm formation is depicted in Fig. 5. The polyphosphate kinase is critical as it is involved in all three steps of the biofilm formation.

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