COMPARISON OF STRESS ADAPTATION AND SURVIVAL RATE BETWEEN *BURKHOLDERIA PSEUDOMALLEI* WITH MUTANT AND WILD TYPE *BFMR*

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Abstract. *Burkholderia pseudomallei* (Bp) is highly adaptable to a wide range of environmental changes for survival and pathogenesis. However, the underlying mechanisms of such adaptability are still unclear. Two-component system (TCS) is a common signal transduction used by bacteria in response to environmental changes. A gene designated as *bfmR* (locus tag of BPSL2024) has been proposed to encode a response regulator, a member of the TCS, and was studied by mutagenesis and comparison of its phenotypic changes compared with those of the wild type. The growth rates of the mutant Bp at temperatures of 37°-39°C and pH 5-8 were significantly lower than the wild type strain ($p < 0.05$), especially at 39°C ($p = 0.01$) and pH 7 ($p = 0.01$). The survival rate of the mice infected with the mutant strain is not significantly different from mice infected with wild type strain. The defective phenotypes were recovered in the complemented strain. These results indicated that *bfmR* is involved in adaptation of Bp to thermal- and pH-induced stress conditions.

Keywords: *Burkholderia pseudomallei*, *bfmR*, melioidosis, two component system

INTRODUCTION

Burkholderia pseudomallei (Bp), the causative agent of melioidosis, is most commonly found in Southeast Asia and northern Australia (Yabuuchi and Arakawa, 1993). This pathogen lives in soils and is highly adaptive to a wide range of environmental changes in its natural habitat and host.

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Similar to most of bacterial pathogens, Bp has two-component systems (TCS), which comprise the majority of signal transduction pathways and regulate a wide variety of cellular processes (Cock and Whitworth, 2007). TCS is a common signal transduction mechanism used in response to environmental changes. TCS enables bacteria to sense, and respond and adapt to a wide range of environments stress and growth conditions. The system consists of a histidine kinase (HK) and a response regulator (RR). HK receives environmental signals from outer membrane and RR functions in response to signals received from HK. Generally, HK possesses an N-terminal domain, which

perceives an environmental stimulus and a C-terminal transmitter domain, which is autophosphorylated upon HK activation. Input domains are often transmembrane (TM) perceiving extracellular signals. A typical RR has a C-terminal effector domain and an N-terminal receiver domain, which is phosphorylated upon interaction with the transmitter domain of HK. This alters the activity of the effector domain, often regulating transcription (Parkinson and Kofoid, 1992).

bfmR (a locus tag of BPSL2024, according to NCBI annotation) is a gene of unknown function. It is located downstream of *BPSL2025* on Bp large chromosome, which encodes a protein containing a putative histidine kinase and histidine kinase-like ATPase conserved domains with three possible transmembrane helices. Whereas *BPSL2024* encodes a protein containing a putative phosphoacceptor site and a helix-turn-helix DNA-binding motif, *BPSL2025* and *bfmR* was proposed to encode a putative sensor kinase and a putative response regulator protein of two-component signaling system, respectively. DNA microarray analysis showed upregulation of *bfmR* in iron-limited condition, whereas disruption of *BPSL2025* dramatically decreases virulence of the mutant pathogen in mice (Tuanyok *et al*, 2006). Several TCSs are found in pathogenic bacteria, suggesting a role in bacterial pathogenesis in a network manner.

In this study, the role of *bfmR* in Bp virulence was examined by determination survival rate of BALB/c mice infected with wild type and mutant *bfmR* Bp strains. An adaptation-associated role of *bfmR* was elucidated by monitoring bacterial growth rate during cultivation in different environmental factors.

MATERIALS AND METHODS

Bacterial strains and culture condition

K96243 wild type (WT) strain of Bp was obtained from the Melioidosis Research Center, Faculty of Medicine, Khon Kaen University, Thailand. Mutant *bfmR* (MT) strain and *bfmR* complemented (COM) Bp strains were generated from a previous study (unpublished). In brief, a 212 bp PCR amplicon of *bfmR* was inserted into pKNOCK (Tn) and the recombinant plasmid transfected into *E. coli* and transformant conjugated with Bp. MT strain was generated through the insertion of the recombinant plasmid into the chromosomal targeted gene and was verified by Southern blot analysis. COM strain was constructed by transferring PBMC plasmid containing *bfmR* into MT strain.

The gram-negative bacteria used in this study were maintained in glycerol stocks. Bacterial inoculum was prepared by growing the strains on Luria–Bertani (LB) agar. A single colony was randomly selected, suspended in 3 ml of LB broth and incubated at 37°C overnight. Bacterial cells were subcultured at a 1:100 dilution in fresh LB broth and then incubated at 37°C with shaking until an $OD_{550\ nm}$ reached to 0.1.

Effect of temperature and pH on bacteria growth

The effect of temperature on growth was studied in WT, MT and COM strains using 200 l of LB containing 1% bacteria inoculum cultured at 37°, 38° and 39°C in 96-well plates with shaking for 24 hours. Similarly, bacterial cell suspensions were grown at pH values of 5, 6, 7, and 8. $OD_{550\,nm}$ of the cultures were measured at the start and every 2 hours for 24 hours using Varioskan Flash Multimode Reader (Thermo Scientific, Rockford, IL). Experiments

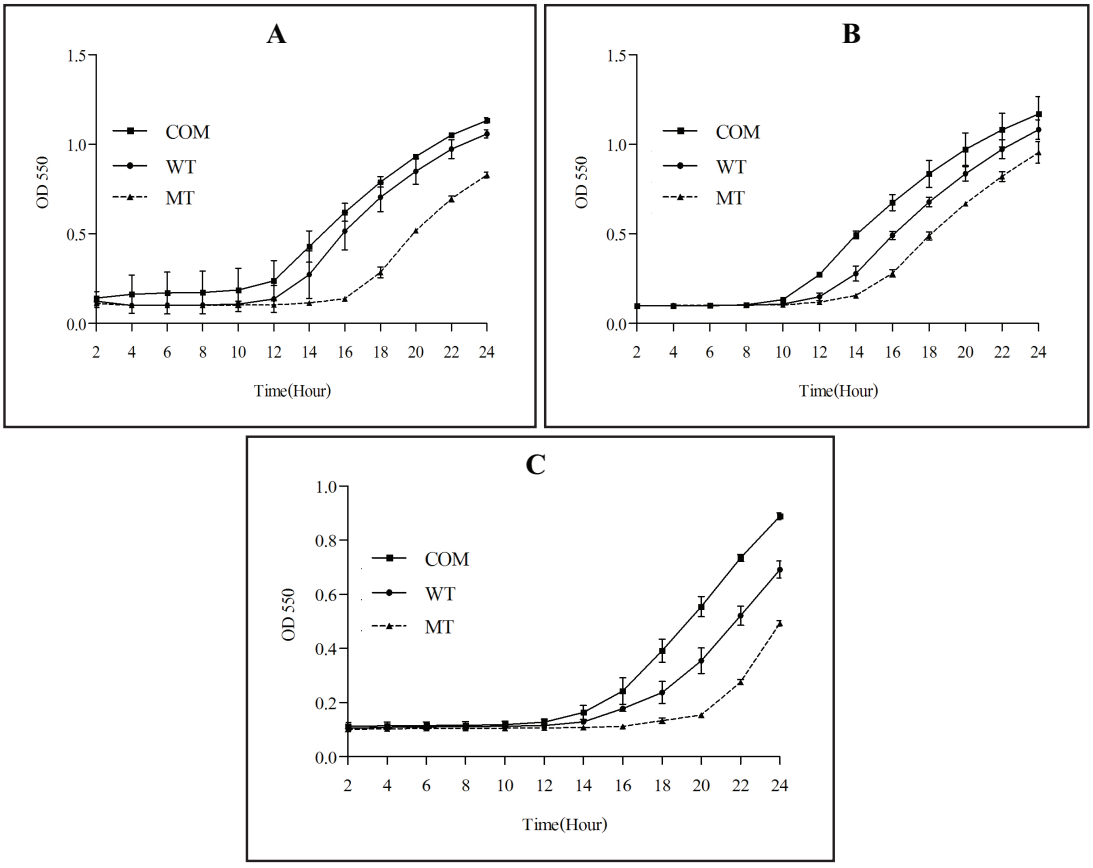

Fig 1–Growth curves of mutant (MT), wild type (WT) and complemented (COM) *bfmR B. pseudomallei* strains at 37ºC (A), 38ºC (B) and 39ºC (C). Cell amounts were measured by optical density at 550 nm.

were performed in triplicate, and the average results were plotted against time. Differences among groups were tested using one-way ANOVA.

Survival analysis of Bp-infected BALB/c mice

Twenty-six 4-weeks old male BALB/c mice, housed under biosafety level 3 conditions, were divided into 3 groups of 8 mice with free access to food and water. Each of these 3 groups of mice was subjected to challenge with either WT, MT or COM strain. BALB/c mice were injected through intraperitoneal (i.p.) route with 100 l containing 1x10^6 CFU bacteria. The remaining 2 mice were used as a control group and were injected with 100 l of phosphate-buffered saline. Mice were observed daily. Comparisons of the survival rates among the three infected groups of the mice were preformed using Log rank test.

Histopathological analysis of Bp-infected mice

On day 3 after infection, 3 mice from each group were anesthetized with an overdose of diethyl ether. The spleen, lung and liver of each mouse were collected and embedded in methyl methacrylate resin. A total of three cross-sections (3 to

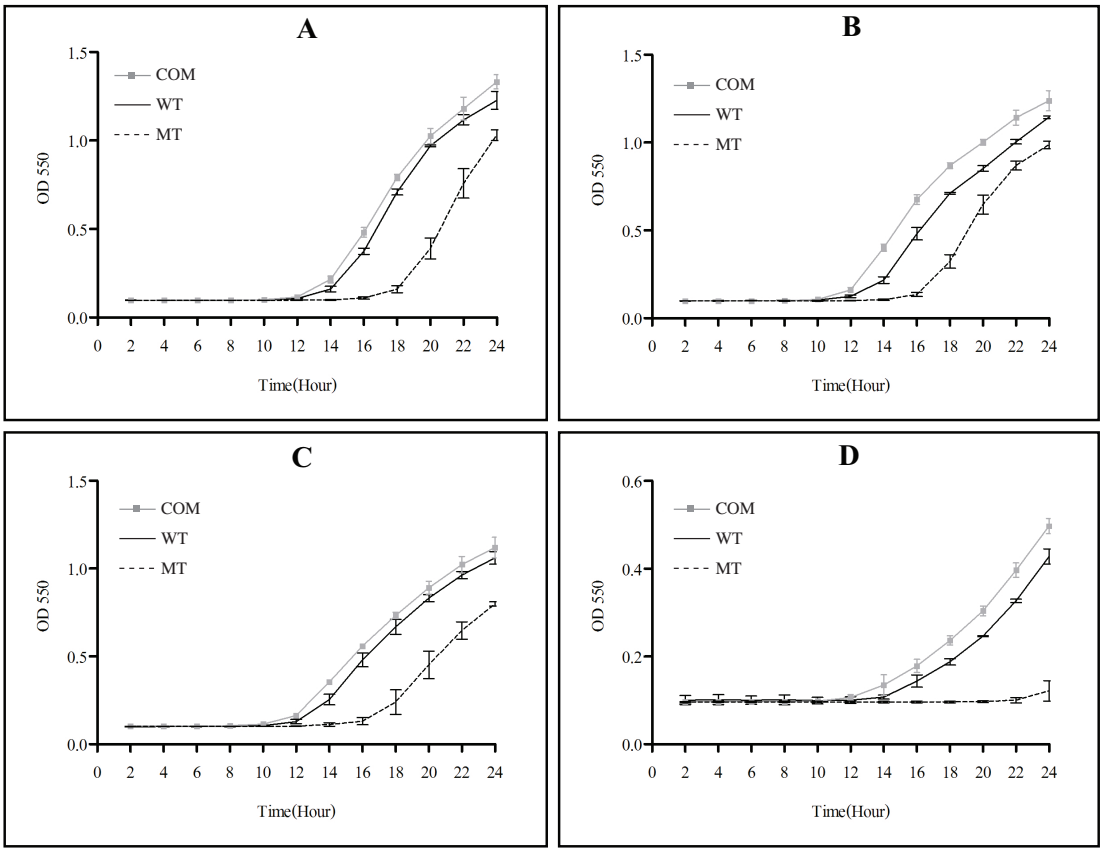

Fig 2–Growth curves of mutant (MT), wild type (WT) and complemented (COM) *bfmR B. pseudomallei* strains at various pH conditions. MT, WT and COM *B. pseudomallei* strains were cultured in LB broth at pH of 5 (A), 6 (B), 7 (C) and 8 (D) for 24 hours. Cell amounts were measured by optical density at 550 nm.

6 m in thickness) were obtained from the organs of each mouse using a rotary microtome and stained with hematoxylin and eosin. Granuloma and necrosis lesions were microscopically analyzed.

The protocols of animal experiments was approved by the Animal Ethics Committee of Khon Kaen University, Thailand (no. AEKKU51/2554).

RESULTS

Effect of temperature and pH on growth of WT, MT and COM Bp strains

Growth curves of WT, MT and COM Bp strains at temperatures ranging from 37°-39°C revealed growth retardation of MT over WT strain at all tested temperatures (Fig 1). The lag phase of growth of MT strain was dramatically longer than that of WT, whereas the curves in the log phase showed similar growth rates. Statistically significant difference between the growth rate of WT and MT strains are found at all three temperatures, 37°, 38° and 39°C (p = 0.008, 0.006 and 0.019, respectively). However the COM strain showed recovery of the delayed lag phase in all three thermal conditions.

Similarly, with changes of pH the lag phase of MT growth was dramatically longer than that of WT strain, especially

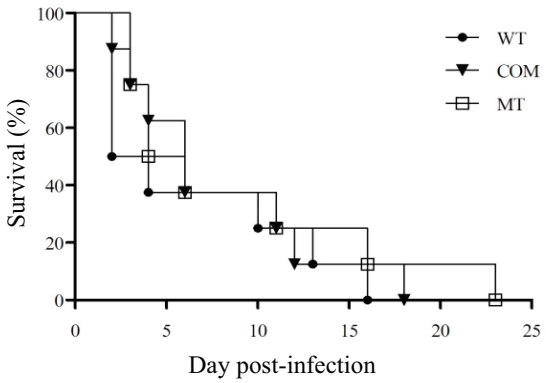

Fig 3–Survival analysis of BALB/c mice infected with mutant (MT), wild type (WT) and complemented (COM) *bfmR B. pseudomallei* strains.

at pH 8 where the lag period of MT (23 hours) was almost twice as long as the optimal growth condition of WT strain (Fig 2). Statistically significant differences between the growth rates of WT and MT strains are found at all four pH conditions, pH value of 5, 6, 7 and 8 (p = 0.023, 0.016, 0.009 and 0.035, respectively). Furthermore, COM strain showed the shortest lag phase of growth at all pH conditions compared with other two strains.

Survival analysis of BALB/c infected with wild type and mutant *bfmR* Bp strains

In order to investigate virulence-association of *bfmR*, MT, WT and COM strains were inoculated into BALB/c mice and observed daily. Fifty percent mortality (4/8 mice) were observed with those infected with WT strain by day 2 post-infection, whereas that of MT-infected mice was day 4 (Fig 3). All WT- and MT-infected mice were dead by day 16 and 23, respectively, but this difference is not statistically significant. Interestingly, survival curve of COM-infected mice was intermediate between that of WT- and MT-infected groups.

Histopathological analysis of mice infected with wild type and mutant *bfmR* Bp strains

In order to investigate the pathology associated with *bfmR*, histological analysis of the spleen, lung and liver of 3 WT-, MT- and COM-infected mice were performed on day 3 post-infection. Spleen tissues from MT-infected mice showed relatively lower levels of granuloma than those of WT-infected animals, but spleens of COM-infected mice had the lowest level of granuloma formation (Table 1). In the liver, necrosis was found at the same pathologic level between WT and MT infection, but COM infection caused dramatically higher necrosis level. Furthermore, granuloma in the liver was found only in COM-infected mice. No pathologic lesions were found in the lung of all infected mice.

DISCUSSION

The mechanisms involved in the high adaptability of Bp are still needed to be elucidated. Bp *bfmR*, a response regulator of the two component regulatory system, has been found to be upregulated in iron-limited condition by DNA microarray analysis (Tuanyok *et al*, 2006). In this study *bfmR* was found to be associated with adaptation to thermal- and pH-induced stress, and also demonstrated as a possible virulence-associated gene. As control, Bp carrying the mutant *bfmR* was complemented with the wild type gene.

Although growth of Mt Bp strain was slower than WT strain under thermal- and pH- induced stress conditions, the growth lag period was the shortest with COM strain. This may be due to the COM being constructed to express *bfmR* constitutively whereas in WT strain this gene is inducible under stress conditions. Moreover, *bfmR* copy numbers in the COM strain

Table 1
Histopathologic analysis of the spleen, and liver from mice infected with mutant (MT), wild type (WT) and complemented (COM) *bfmR B. pseudomallei* strains.

B. pseudomallei strain	Spleen		Liver	
	Granuloma	Necrosis	Granuloma	Necrosis
MT	++	-	-	+
WT	+++	-	-	+
COM	+	-	+	+++

Level of abnormality: +1, few ; +2, moderate; +3, many. The histopathological examinations were examined independently by two histopathologists.

might be higher than WT strain, giving greater support the suggestion that *bfmR* is involved in Bp adaptation to thermal- or pH- induced stress conditions.

MT-infected mice had the lowest pathology of internal organs than WT- and COM-infection, but this was not reflected in the survival curve. As the two component system coordinately works in a signaling network, the mutated function of *bfmR* might be compensated by other virulence determinants.

In summary, using a gene knock-out system, we have elucidated the role of *bfmR* of Bp in adaptation to thermal- and pH-induced stress, but its role in virulence was only suggestive due to statistical constraints.

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