CHARACTERIZATION OF A NOVEL TWO-COMPONENT SYSTEM RESPONSE REGULATOR INVOLVED IN BIOFILM FORMATION AND A LOW-IRON RESPONSE OF *BURKHOLDERIA PSEUDOMALLEI*

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Abstract. Two-component systems (TCSs) regulate an adaptive response to environmental conditions, leading to changes in bacterial cellular processes. In this study, we identified a novel TCS response regulator gene, designated as *bfmR* (biofilm formation-associated regulator) that regulates biofilm formation by *Burkholderia pseudomallei* (Bp). An insertion mutant of the Bp *bfmR* gene resulted in a significant decrease in expression of fimbriae chaperone-usher assembly genes (*BPSL2028* and *BPSL2027*), leading to suppression of assembly of fimbriae on the cell surface and reduced biofilm formation. The defective phenotypes of the mutant strain were restored by introducing a complementing plasmid having an intact *bfmR* gene. Using RT-PCR analyses, we found that *bfmR* gene expression was upregulated under low-iron growth conditions. In addition, the *bfmR* mutant strain showed retarded growth in low-iron medium and in phagocytic cells compared to the wild-type strain. These results indicate that *bfmR* is a novel positive regulator for controlling assembly of fimbriae and biofilm formation, and is upregulated under low-iron conditions.

Keywords: *Burkholderia pseudomallei*, *bfmR* gene, two-component system, biofilm formation, fimbriae assembly

INTRODUCTION

Burkholderia pseudomallei (Bp) is a gram-negative bacillus and is a facultative intracellular pathogen (Currie *et al*, 2000) opportunistically causing melioidosis, a life-threatening infectious disease of humans and animals. Bp can persist under a wide range of conditions and can survive as a saprophyte in the environment (Thomas and Forbes-Faulkner, 1981; Inglis and Sagripanti, 2006). Like other bacteria, Bp has two-component systems (TCSs), the signal transduction pathways which regulate a wide variety of cellular processes involved in adaptation and responses to the environment (Alm *et al*, 2006). A TCS consists of a sensor histidine kinase that catalyzes its autophosphorylation after exposure to environmental

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stimuli and then transfers the phosphoryl group to a response regulator (RR). The activated RR then regulates downstream gene expression which alters the cellular physiology (Laub and Goulian, 2007). There is increasing interest in developing TCS inhibitors for the prevention and treatment of diseases caused by pathogenic bacteria (Chauhan and Calderone, 2008; Gotoh *et al*, 2010).

Growing as a biofilm form, a population of bacterial cells growing on a surface and surround themselves within an exopolysaccharide matrix, is a strategy by Bp for adaptation and survival in their environment leading to increased resistance to conventional antibiotics such as doxycycline, ceptazidime, imipenem and trimethoprim (Sawasdidoln *et al*, 2010). Many pathogenic bacteria use TCSs to regulate biofilm formation by controlling the production of extracellular appendages. For example, the BfmR/BfmS regulatory system of *Acinetobacter baumannii* controls the Csu (A/B) ABCDE chaperone–usher assembly system, which is involved in the assembly of pili on the cell surface, and affects biofilm formation (Tomaras *et al*, 2008). *Escherichia coli* RcsB response regulator is important for controlling the expression of cell surface fimbriae which are involved in biofilm formation (Schwan *et al*, 2007; Lehti *et al*, 2012b). It is possible that Bp may use a similar regulatory mechanism to control the cell surface appendages that are involved in biofilm formation.

A novel TCS response regulator gene, designated as *bfmR* (named after biofilm formation associated regulator, corresponding to the locus tag *BPSL2024*, the accession number NC_006350.1) was upregulated when Bp was grown under low-iron conditions (Tuanyok *et al*, 2005), suggesting the possible involvement of this *bfmR* gene in adaptation and survival mechanisms. Previous study showed that this gene is involved in adaptation of Bp to thermal- and pH-stress conditions (Neamnak *et al*, 2014). In this study, we demonstrated the role of *bfmR* in production of cell surface fimbriae, biofilm formation and bacterial growth under low-iron conditions.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids

Bp K96243 and *Burkholderia thailandensis* UE5 (Bt UE5) were used as representatives of the clinical (Holden *et al*, 2004) and environmental (Kespichayawattana *et al*, 2004) isolates, respectively. Bp and *E. coli* were routinely maintained on Luria-Bertani (LB) plates or in LB broth. For cloning experiments, 100 μg/ml ampicillin (Ap), 60 μg/ml tetracycline (Tc), and 30 μg/ml chloramphenicol (Cm) were added to the culture medium when required. LB medium supplemented with 5 μg/ml gentamicin (Gm) and Tc was used as the selective medium for the *bfmR* mutant strain, and LB plus Tc, Gm, and Cm was used as the selective medium for the complemented strain. Low-iron LB medium was prepared by the addition of a chelator, 200 μM 2,2′-dipyridyl (DIP; Sigma Chemical, St Louis, MO) (Jarosik *et al*, 1998). LB medium containing 10 μM ferric chloride ($FeCl_3$) was used as a high-iron medium. To minimize the iron contamination, all glassware was washed with 5% HCl and rinsed with deionized water before use. For biofilm assays, cultures were grown in modified Vogel and Bonner's (MVBM) minimal medium (Lam *et al*, 1980). All cultures were incubated at 37°C. The plasmids used in this study are listed in Table 1.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
B. pseudomallei		
K96243	Wild-type, clinical isolate, Gm^r	(Holden *et al*, 2004)
bfmR mutant	Mutant, K96243 derivative; *bfmR* insertion mutant (*ΔbfmR*), Tc^r	This study
ΔbfmR complement	Complemented strain; carrying pBBR1MCS-2024, Cm^r, Tc^r	This study
B. thailandensis		
UE5	Environmental isolate	(Rainbow *et al*, 2002; Kespichayawattana *et al*, 2004)
E. coli		
S17-1*λpir*	S17 with a λ prophage carrying the π protein	(de Lorenzo and Timmis, 1994)
DH5α	Cloning strain	Invitrogen
Plasmids		
pKNOCK-Tc	Mobilizable suicide vector, Tet^r	(Alexeyev, 1999)
pKNL2024	pKNOCK-Tc containing a 212 bp internal fragment of *bfmR* gene, Tet^r	This study
pGEM-T easy vector	TA cloning vector, Ap^r	Promega
pGEM-2024	pGEM-T easy vector containing a 212 bp internal fragment of *bfmR* gene, Ap^r	This study
pGEM-F2024	pGEM-T easy vector containing full length of *bfmR* gene, Ap^r	This study
pBBR1MCS	Broad-host-range expression vector, Cm^r	(Kovach *et al*, 1995)
pBBR1MCS-2024	pBBR1MCS containing full length of *bfmR* gene, Cm^r	This study

Cell lines and media

RAW 264.7 cells (a murine macrophage cell line) were grown in RPMI 1640 (Gibco BRL, Grand Island, NY) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco BRL) at 37°C with a 5% CO_2 atmosphere. Kanamycin (Km, 250 μg/ml) was added for routine culture.

Construction of the *bfmR* mutant

To create the *bfmR* insertion mutant strain, a 212-base pair (bp) internal fragment [nucleotide (nt) 58 to 270] of the *bfmR* gene was amplified by PCR from Bp K96243 genomic DNA using primers BPSL2024_F and BPSL2024_R flanked by *SalI* and *PstI* restriction sites, respectively (Table 2). The PCR amplification was performed as follows: denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute; and final extension at 72°C for 7 minutes. The amplified product was cloned into pGEM-T easy (Promega, Madison, WI) to create pGEM-2024 and was confirmed by DNA sequencing (data not

Table 2
Oligonucleotides used in this study.

Primer	Sequence (5'→3')
BPSL2024_F	ATGTCGACGGCATGGGCGACGTATC
BPSL2024_R	CTCTGCAGATCAGCACCGGATTGTC
C2024-2_F	ACTTGGGTACCAACCATCTGCTGCGCTGCGTTG
C2024-2_R	CTTACTCTAGAGTGCGACGCGCATCGCACGGC
RT2024F	CGTGCGACATCGTCATCA
RT2024R	GCATCGTGTGCATCAGCA
RT25-24F	CTCGACGAATTCCACCATCT
RT25-24R	ATCGCGAAATCGGTGATG
RT2027F	ATCGCGACGGCATCTACTAC
RT2027R	ATTGAACAGGAGCCCGAAG
RT2028F	GCGAGGATGTCTACGACGAG
RT2028R	CACCGAGTAGTGCATCTGGA

Underlines indicate restriction endonuclease cleavage sites.

shown). The 212 bp *bfmR* fragment was then subcloned into *SalI* and *PstI*-digested pKNOCK-Tc plasmid (Alexeyev, 1999) to produce pKNL2024. This recombinant plasmid was transformed into *E. coli* S17-1*λpir* by electroporation and was then mobilized into Bp K96243 by conjugation using the filter-mating method (Clarke *et al*, 2005). The transconjugants were selected on LB plates supplemented with Tc and Gm. The single crossover-mediated allelic exchange mutant was confirmed by Southern blotting (Southern, 1992; Sambrook and Russell, 2006).

Construction of a Δ*bfmR* complemented strain

A complemented strain was constructed by introducing a plasmid containing an intact *bfmR* gene into the mutant strain. To construct the complementing plasmid, a 748 bp fragment containing the full-length *bfmR* gene was amplified by PCR using primers C2024-2_F and C2024-2_R, which contain *KpnI* and *XbaI* restriction sites, respectively (Table 2). The PCR product was cloned into the pGEM-T easy vector to create pGEM-F2024. The plasmid insert was confirmed by sequencing (data not shown) and subsequently subcloned into the *KpnI-XbaI* site of the expression plasmid pBBR1MCS-Cm^r (Kovach *et al*, 1994) to create plasmid pBBR1MCS-2024. The recombinant plasmid was transformed into *E. coli* S17-1*λpir* by electroporation and then mobilized into the *bfmR* 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 plasmid was extracted and digested with restriction endonucleases, and it was also sequenced (data not shown).

Determination of bacterial growth rate

The *bfmR* mutant and wild-type strains were grown in 100 ml of LB medium and low-iron LB medium. Each flask of medium was inoculated with 1 ml (1% inoculation) of an overnight culture, and then the cells were cultured at 37°C with shaking at 200 rpm for 24 hours. The bacterial growth rate was determined

using optical density (OD_{550}) and viable bacterial cell counts of each culture every 4 hours.

Transcriptional analysis of the *bfmR* gene in low- and high-iron conditions

To evaluate the expression level of *bfmR* under high- and low-iron conditions, Bp was cultured in high- and low-iron LB medium. Total RNA was extracted from cells harvested during the exponential or stationary phases using TRIZOL reagent (Invitrogen, Carlsbad, CA). To remove contaminating genomic DNA from the RNA samples, DNase I (Fermentas, Ontario, Canada) was used. Gene expression was determined by semi-quantitative one-step RT-PCR using the SuperScript® III One-Step RT-PCR System with Platinum®*Taq* DNA Polymerase (Invitrogen) and with primers RT2024F and RT2024R. The 16S rRNA gene was used as an internal control for the transcription. To measure gene expression levels, 10 μl of each PCR product was electrophoresed on 1% agarose gel, stained with ethidium bromide, and observed using UV transillumination. The density of the PCR product bands was analyzed using ImageJ, a Java-based image analysis program (Abramoff, 2004). The expression analysis was performed in three independent experiments.

Transcriptional and regulatory analysis of the *bfmR* gene

Total RNA was extracted from wild-type K96243, the *bfmR* mutant, and from complemented strains according to the TRIZOL reagent protocol (Invitrogen). Expression analysis of the target genes was performed using the SuperScript® III One-Step RT-PCR System with Platinum®*Taq*DNA Polymerase (Invitrogen). For transcriptional analysis of the *bfmR*/*BPSL2025* locus, the primers RT25-24F and RT25-24R were used to amplify the intergenic region of the *bfmR*/*BPSL2025* genes in an RNA sample from the wild-type strain. The analysis of *bfmR* regulation of fimbriae-associated genes *BPSL2027* and *BPSL2028* was performed on RNA samples from wild-type, *bfmR* mutant, and complemented strains using the primers RT2027F and RT2027R for the *BPSL2027* gene and the primers RT2028F and RT2028R for the *BPSL2028* gene (Table 2). Ten μl of each amplicon was analyzed by agarose gel electrophoresis as described above. Promoter prediction was conducted using Microbes Online Operon Predictions (www.microbesonline.org/about_operon.html).

Transmission electron microscopy (TEM) assay

To evaluate production of fimbriae on the bacterial cell surface, bacterial strains were cultured in LB broth and observed under TEM. Briefly, 3 ml of LB broth was inoculated with a single colony of the wild-type, the *bfmR* mutant, or the complemented strain and incubated statically at 37°C for 24 hours. A drop of each bacterial suspension was placed onto the surface of a Formvar-coated grid, air dried for 30 minutes and observed under TEM (Zeiss EM902, Oberkochen, Germany).

Biofilm formation assay

Bacterial biofilm formation was quantitatively estimated according to the method of Stepanovic *et al* (2000). Briefly, overnight cultures of wild-type, *bfmR* mutant, and complemented strains were suspended in MVBM medium and adjusted to an OD_{540} of 0.8-0.9. Bt UE5 was used as a reference for biofilm production. Suspensions (200 μl) from each bacterial culture were added into eight wells of a sterile 96-well plate (Nunclon™, Roskilde, Denmark). Another eight wells containing

only medium served as negative controls. Plates were statically incubated at 37°C for 3 hours to allow bacterial adhesion. After incubation, non-adherent bacteria were removed, the culture medium was replaced with 200 μl of fresh MVBM medium, and the plates were incubated for an additional 21 hours. Culture medium containing non-adherent bacteria was then aspirated, the plates were washed with 200 μl of sterile distilled water and fresh medium was added. After 24 hours of further incubation, the supernatant was removed, and the plate was washed three times with 200 μl of sterile distilled water. Plates containing attached bacteria, representing 2-day biofilm formation, were fixed with 200 μl of 99% (v/v) methanol for 15 minutes at room temperature. The methanol was then removed, and the plate was dried at room temperature and stained with 200 μl of 2% (w/v) Hucker crystal violet for 5 minutes. Excess stain was washed away with running tap water. The stained plates were air dried at room temperature, and the stained biofilm was solubilized in 200 μl of 33% (v/v) glacial acetic acid by gentle mixing for 5 minutes. The quantity of biofilm produced by each culture was measured at OD_{630} using a microtiter plate reader. The OD values were adjusted to the biofilm values produced by Bt UE5. The relative biofilm formation capacity of each strain was then compared to the others by Student's *t*-test.

Bacterial motility assay

The swimming motility of the bacteria was evaluated by the standard method (Deziel *et al*, 2001). Briefly, the wild-type, *bfmR* mutant and complemented strains were grown overnight on LB agar plates. Cells of each strain were picked with a sterile toothpick and inoculated onto a swimming agar plate containing 0.3% (w/v) agar, 1% (w/v) tryptone, and 0.5% (w/v) NaCl. Plates were incubated at 30°C for 24 to 48 hours. Bacterial motility was then evaluated by measuring the size of the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation from three replicated experiments.

Multiplication of the *bfmR* mutant in a murine macrophage cell line RAW 264.7

To assess the internalization and survival ability of the *bfmR* mutant in phagocytic cells, RAW 264.7 cells were seeded into 6-well plates ($2.5x10^6$ cells/well) and incubated overnight at 37°C in a 5% CO_2 atmosphere. A 1% inoculum of the wild-type or the mutant strain was incubated at 37°C with shaking at 200 rpm until it reached mid-log phase (~7 hours). Cells were then collected by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in RPMI medium. On the day of infection, the medium in the cell culture plates was replaced with fresh medium. Bacterial cells were added into each well at a multiplicity of infection (MOI) of 2. After 2 hours of incubation, the cells were washed with PBS, and culture medium containing 250 μg/ml Km was added to kill the residual extracellular bacteria. After a further 2 hours of incubation, the culture medium was replaced with the medium containing 20 μg/ml Km. The infected cells were lysed with 0.1% Triton X-100 at 4, 6, 12, 18 and 24 hours post-infection. The number of intracellular bacteria was quantified by serial dilution, dropping on LB agar plates and counting the colonies.

RESULTS

Analysis of *bfmR* and its operon

According to the *B. pseudomallei* K96243 complete genome database, accession number NC_006350.1, the *bfmR* gene

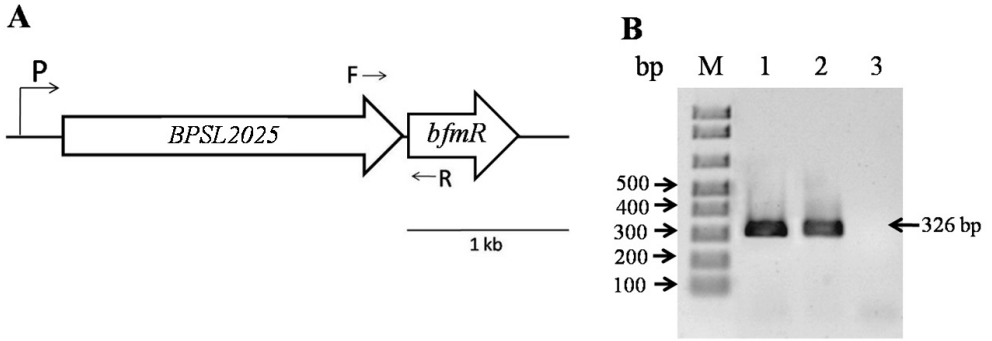

Fig 1–(A) Gene organization of *bfmR* (a response regulator, RR) and *BPSL2025* (a sensor kinase, SK) of *B. pseudomallei* K96243. **P** indicates the promoter region and the direction of transcription. **F** and **R** indicate the primer binding sites for RT-PCR. (B) Gel electrophoresis of the RT-PCR products amplified using specific primers spanning the intergenic region between the *bfmR* and *BPSL2025* genes. Lane M, DNA molecular marker (bp); lane 1, a product from genomic DNA (positive control); lane 2, a product from RNA sample (test); lane 3, a negative control.

is 621 nucleotides (nt) in length encoding a TCS response regulator consisted of 206 amino acids with an estimated molecular weight of 21.8 kDa. The *bfmR* gene is positioned immediately downstream of its predicted cognate sensor kinase gene, *BPSL2025*, separated by an intergenic region of 13 nt (Fig 1A). The promoter for the *BPSL2025* gene was predicted to be upstream of the gene, not in the intergenic region. They were assumed to be co-transcribed, forming a bicistronic operon. This possibility was confirmed by RT-PCR using the intergenic primer set across the *bfmR* and *BPSL2025* locus. A 326 bp amplicon was produced by these intergenic primers (Fig 1B), suggesting that *bfmR* and *BPSL2025* are co-transcribed as an operon.

Determination of the bacterial growth rate and *bfmR* expression under low-iron conditions

According to a previous report (Tuanyok *et al*, 2005), *bfmR* was upregulated when Bp was grown in iron-limited conditions. In this study, we compared the growth rates of a *bfmR* mutant strain and the wild-type strain in low iron medium and in normal LB medium. The results show that the growth rate of the wild-type bacteria in LB medium was not different from that of *bfmR* mutant strain. On the other hand, the growth rate of the *bfmR* mutant under iron-limiting conditions was significantly lower than that of the wild-type strain, p=0.015 at 12 hours and p=0.028 at 16 hours (Fig 2A). Also, the growth rate of the mutant and wild-type strains are similar in high-iron medium (supplemented with 10 μM $FeCl_3$), and both strains grew faster in the high-iron medium than in LB or low-iron medium (data not shown).

The relative expression of the *bfmR* gene was measured semi-quantitatively by one-step RT-PCR using RNA samples extracted from the wild-type strain cultured in low- and high-iron medium during the exponential and stationary growth phases. The results showed that the expression of *bfmR* in low-iron conditions was significantly higher than in high-iron conditions at both of the bacte-

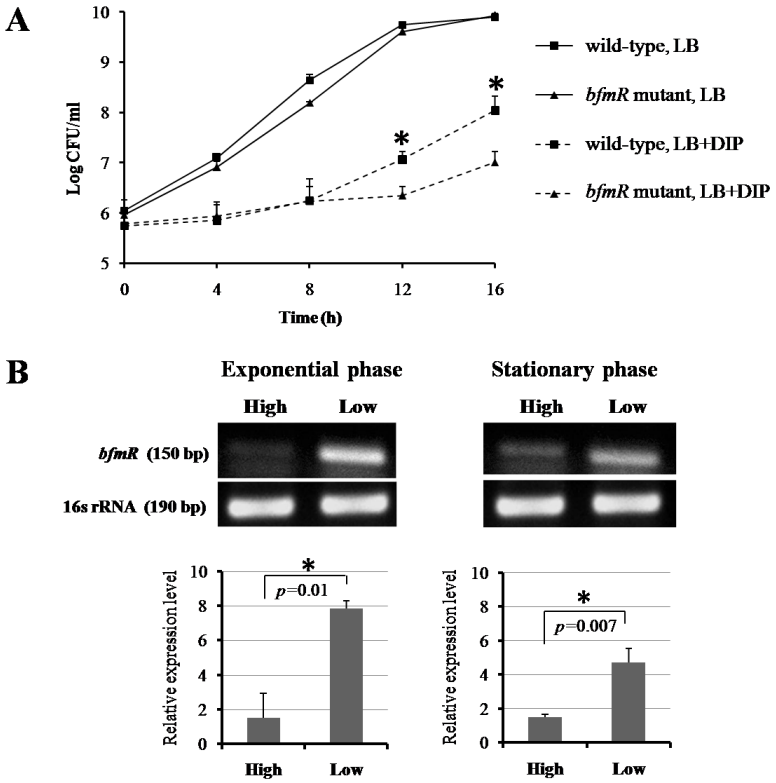

Fig 2–Comparison of bacterial growth rates and *bfmR* gene expression in low- and high-iron conditions. (A) Growth rate of the wild-type K96243 and the *bfmR* mutant in LB medium (solid line) and LB medium supplemented with iron chelator 200 μM DIP (dotted line). The viable bacterial cell number was counted in CFU/ml (colony forming unit/ml). (B) The relative expression levels of the *bfmR* gene under low- and high-iron conditions in exponential (left) and stationary phase (right) after analysis with RT-PCR and gel electrophoresis using 16S rRNA as an internal control for gene expression. Asterisks denote statistical significance using a paired-samples Student's *t*-test ($p<0.05$). Mean values ±SDs are presented and the data show an average from two independent experiments

rial growth phases (exponential phase, $p=0.01$; stationary phase, $p=0.007$) (Fig 2B).

The effect of *bfmR* mutation on fimbrial assembly gene expression

Based on the sequence analysis, *bfmR* coding sequence is located downstream of fimbriae chaperone-usher assembly genes, *BPSL2027* (encoding for a putative fimbriae-related protein) and *BPSL2028* (encoding for a putative fimbriae-assembly chaperone). We therefore, hypothesized that BfmR may regulate the expression of its upstream fimbriae-associated genes, and we measured the expression of fimbrial assembly genes of the mutant and wild-type strain of Bp by RT-PCR analysis. The results showed that expression of *BPSL2028* in the mutant strain was significantly reduced ($p=0.005$) compared to that of the wild-type strain, whereas expression of *BPSL2027* in the mutant strain was slightly reduced but not to the level of statistical significance

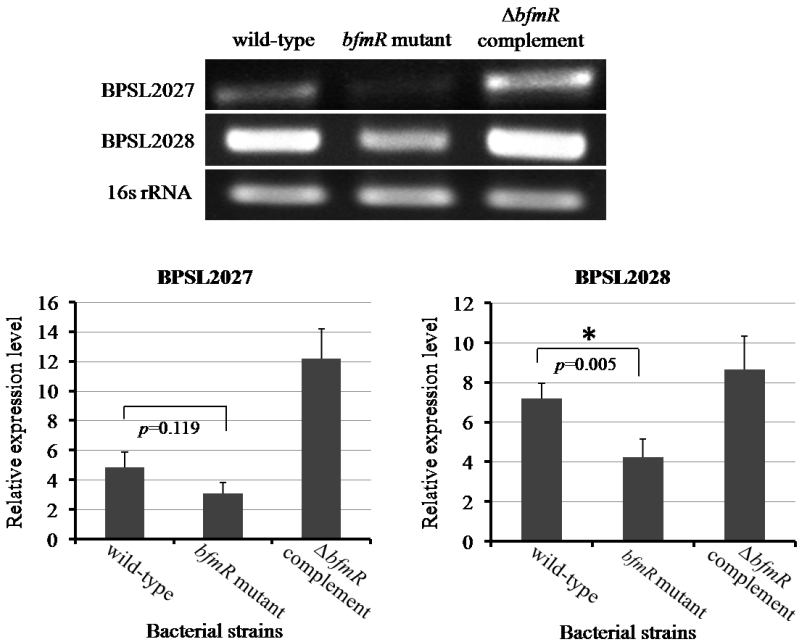

Fig 3–The effect of *bfmR* mutant on expression of the fimbriae chaperone-usher assembly genes. The relative expression levels of *BPSL2027* (fimbriae-related protein) and *BPSL2028* (fimbriae assembly chaperone) were analyzed by RT-PCR and gel electrophoresis from RNA sampled isolated from the wild-type K96243, the *bfmR* mutant, and the complemented strains. Asterisks denote statistical significance using a paired-samples Student's *t*-test ($p<0.05$), and the mean values ±SDs are presented. The data show an average from two independent experiments.

(p=0.119). Expression of these two genes in the complemented strain carrying the constitutive expression pBBR1MCS-2024 plasmid were higher than in the wild-type (Fig 3).

The *bfmR* mutation impaired assembly of the cell surface fimbriae and biofilm formation

Since *bfmR* mutation caused a reduction of expression of the fimbrial assembly genes, we investigated further the extent of fimbriation on the cell surface of the *bfmR* mutant using a transmission electron microscope (TEM). Most of the observed cells of the mutant strain displayed notable reductions in fimbrial density compared to the wild-type in randomly selected fields. Complementation of the mutant by pBBR1MCS-2024 plasmid fully restored the production of fimbriae, sometimes to densities exceeding those in the wild-type (Fig 4A).

Because fimbriae and pili are important for bacterial adhesion to solid surfaces during the initial step of biofilm formation (Tomaras *et al*, 2003; Vallet *et al*, 2004), we investigated the ability of the Bp *bfmR* mutant strain to form biofilms in 96-well plates. The results showed that the biofilm formation by the *bfmR* mutant was significantly lower than that by the wild-type ($p< 0.001$), with a reduction of approximately 70%. In contrast, biofilm formation of the complemented strain was

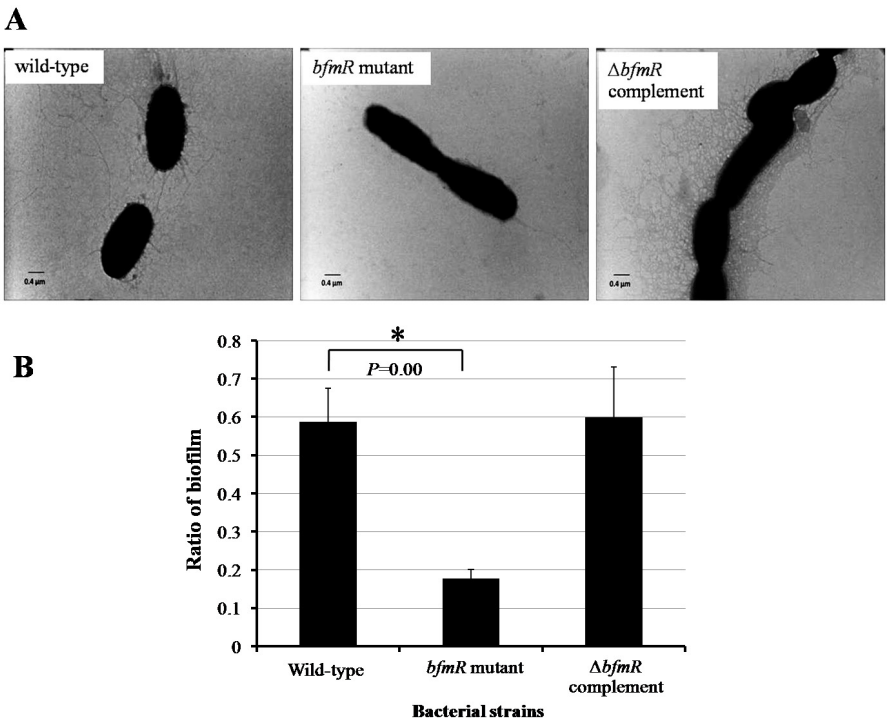

Fig 4–(A) Electron microscopic images of bacterial cell surface fimbriae and biofilm formation of the wild-type, the *bfmR* mutant, and the complemented strains. The fimbriae produced by each strain were visualized using TEM. (B) Bar graphs indicate the biofilm-forming ability of each Bp strain after 48 hours incubation. The turbidity of biofilm samples stained with crystal violet was measured at OD_{630}. Asterisks denote statistical significance using a paired-samples Student's *t*-test ($p \leq 0.05$); each bar represents the mean value ±SD. The results are given as the average of three independent experiments.

restored to the level of wild-type (Fig 4B).

The role of *bfmR* on bacterial migration ability

Because *bfmR* was found to be involved in the assembly of fimbriae on the cell surface as shown above, we examined whether fimbriae are involved in bacterial swimming motility, which was measured using semisolid agar plates. We found that the motility of the *bfmR* mutant was comparable ($p>0.05$) with that of the wild-type strain. In contrast, the motility of the complemented strain harboring pBBR1MCS-2024 plasmid and constitutively expressing BfmR protein, was significantly lower compared to that of the wild-type ($p=0.003$) and with *bfmR* mutant ($p=0.016$) strains (Fig 5).

Survival of the *bfmR* mutant in phagocytic cells

Bp can effectively survive in professional phagocytic cells (Jones *et al*, 1996; Allwood *et al*, 2011). However, iron deficiency within macrophages could limit intracellular growth of bacterial pathogens (Weinberg, 2000). Because the growth ability of the *bfmR* mutant strain was reduced in the low-iron medium,

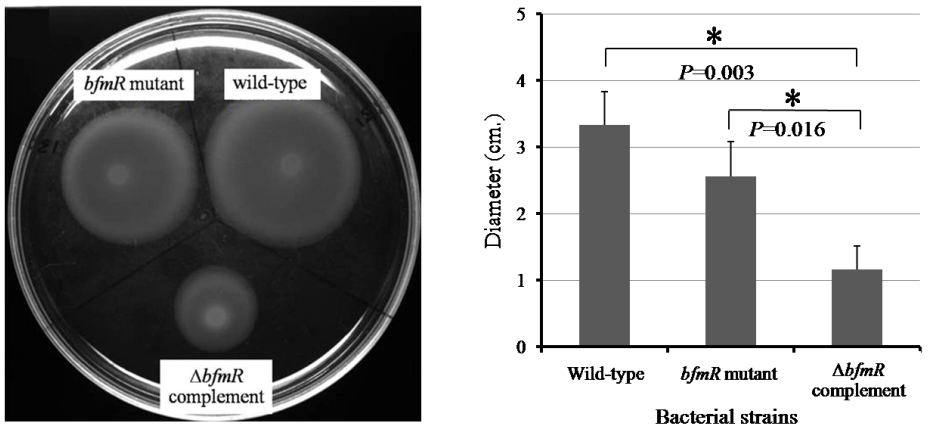

Fig 5–The swimming motility of the wild-type, the *bfmR* mutant and the complemented strains on semi-solid agar plates. The migration zone was measured in centimeters (cm). Asterisks denote statistical significance using a paired-samples Student's *t*-test ($p \leq 0.05$); each bar represents the mean value ±SD. The experiment was performed in triplicate.

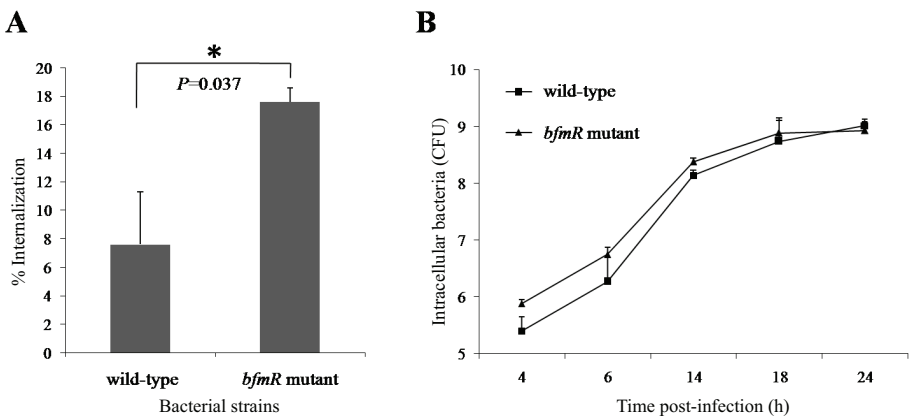

Fig 6–Bacterial internalization and intracellular survival in the RAW 264.7 macrophage cell line. (A) The percentage of internalized bacteria at 2 hours post-infection is represented in the bar graph for the wild-type and the *bfmR* mutant strains. Asterisks denote statistical significance using a paired-samples Student's *t*-test ($p \leq 0.05$) and the data were obtained from two independent experiments. (B) Intracellular survival of the bacteria was measured at 4, 6, 14, 18, 24 hours post-infection. The number of bacteria is presented as CFU.

its intracellular replication potential in murine macrophage RAW 264.7 cells was determined in 6-well plates. The results showed that, after 2 hours of infection, the number of *bfmR* mutant strain cells taken up by phagocytic cells was significantly (p=0.037) higher than that of the wild-type strain (Fig 6A). In addition, the intracellular replication of the *bfmR* mutant was slightly (but not significantly) higher than that of the wild-type at 4 (p=0.285), 6 (p=0.260), and 14 hours (p=0.283) post-infection. By 24 hours post-infection, the numbers of intracellular bacteria of the

mutant and wild-type strains were almost equal (Fig 6B).

DISCUSSION

Two-component systems (TCSs) occur widely in prokaryotes and play regulatory roles in adapting cellular functions in response to environmental stimuli. Although many putative TCS coding sequences have been found in the Bp genome (Holden *et al*, 2004), only a few TCSs have been identified empirically. In this study, therefore, we aimed to investigate the function of a novel TCS response regulator gene, *bfmR* in Bp.

In this study, the mutant showed reduced expression of the fimbriae-associated genes, which was confirmed by actual reduction of the fimbriae as observed using TEM. In association with the reduction of the fimbriae, biofilm formation was defective in the mutant strain, but was restored by complementation with the intact gene. Our findings agreed with the case of the *csu* chaperone-usher assembly operon (*csuAB-ABCDE*) in *A. baumannii*, homologous to the fimbriae chaperone-usher assembly operon (*BPSL2031-BPSL2026*) of Bp (Nuccio and Baumler, 2007). The *A. baumannii* Csu pilus chaperone-usher assembly system is positively regulated by a response regulator protein (BfmR). In this species, a *bfmR* mutant was defective in *csu* expression and lost the capability for assembly of pili and biofilm formation (Tomaras *et al*, 2003; 2008). Similarly, in *E. coli*, RcsB is known to activate the expression of the Mat fimbrial protein, which is important to promote biofilm formation (Lehti *et al*, 2012b). Our data strongly suggest that BfmR is a transcriptional regulator for the expression of fimbriae assembly genes, which are required in Bp for fimbriae assembly, cell attachment to solid surfaces and biofilm production. The precise regulatory mechanisms should be explored in the future.

The swimming motility of the *bfmR*-complemented strain, carrying a plasmid constitutively expressing the BfmR protein, was significantly lower compared with the wild-type or *bfmR* mutant (Fig 5). The reduced swimming capability of the complemented strain might be due to the overproduction of cell surface fimbriae. Similar to our results, the overproduction of CupD fimbriae assembled on the cell surface of *Pseudomonas aeruginosa* resulted in reduced swimming motility (Mikkelsen *et al*, 2009). Also, constitutive expression of type I fimbriae decreased the swimming motility of *E. coli* (Bryan *et al*, 2006; Lane *et al*, 2007; Lehti *et al*, 2012a). Another types of motility including swarming, and twitching were also tested but we found no significant difference between all three strains.

In general, iron concentrations influence bacterial cell metabolism and enzyme activity that are associated with pathogenicity. The iron-associated function of the *bfmR* gene was shown (Fig 2). It can therefore be inferred that iron deficiency induces the *bfmR* gene and that the expression of this gene is associated with adaptation to this stress condition such as might be experienced during intracellular growth in host macrophages.

In the present study, the intracellular replication rate of the mutant was lower than that of the wild-type strain (Fig 6). This would be explained by the limited availability of free iron inside of the macrophages (Weinberg, 2000; Olakanmi *et al*, 2002; Paradkar *et al*, 2008). This possibility is supported by the decreased growth rate of the mutant in low-iron medium (Fig 2B). Similarly, intracellular survival

of *S. typhimurium* is limited in RAW264.7 macrophages with decreased iron levels (Nairz *et al*, 2007). Taken all these findings together, *bfmR* has roles in the bacterial resistance mechanism against phagocytosis and in intra-macrophage survival.

In conclusion, our results show the important role of a novel *bfmR* response regulator gene in Bp, including promotion of biofilm formation by regulating expression of fimbriae assembly genes. *bfmR* also appears to mediate the adaptive response to low-iron conditions. This is the first study to demonstrate the molecular mechanism of a TCS involved in biofilm formation and adaptation in Bp. In this study, we investigated only the *bfmR* response regulator gene but not its cognate *BPSL2025* sensor kinase gene, which is expected to play a role in environmental signal recognition. The interaction of regulatory cascades between BfmR and BPSL2025 are currently under investigation. Our finding may be useful in developing of a TCS inhibitor for prevention and treatment of melioidosis in the future.

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