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ORIGINAL PAPER

Function of *Burkholderia pseudomallei* RpoS and RpoN2 in bacterial invasion, intracellular survival, and multinucleated giant cell formation in mouse macrophage cell line

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Abstract Melioidosis, a human infectious disease with a high mortality rate in many tropical countries, is caused by the pathogen *Burkholderia pseudomallei (B. pseudomallei)*. The function of the *B. pseudomallei* sigma S (RpoS) transcription factor in survival during the stationary growth phase and conditions of oxidative stress is well documented. Besides the *rpoS*, bioinformatics analysis of *B. pseudomallei* genome showed the existence of two *rpoN* genes, named *rpoN1* and *rpoN2*. In this study, by using the mouse macrophage cell line RAW264.7 as a model of infection, the involvement of *B. pseudomallei* RpoS and RpoN2 in the invasion, intracellular survival leading to the reduction in multinucleated giant cell (MNGC) formation of RAW264.7 cell line were illustrated. We have demonstrated that the MNGC formation of RAW264.7 cell was dependent on a certain number of intracellular bacteria (at least 5×10^4). In addition, the same MNGC formation (15%) observed in RAW264.7 cells infected with either *B. pseudomallei* wild type with multiplicity of infection (MOI) 2 or RpoN2 mutant (Δ*rpoN2*) with MOI 10 or RpoS mutant (Δ*rpoS*) with MOI 100. The role of *B. pseudomallei* RpoS and RpoN2 in the regulation of type III secretion system on *bipB-bipC* gene expression was also illustrated in this study.

Keywords *Burkholderia pseudomallei* · Multinucleated giant cell formation · RpoS · RpoN2 · Type III secretion system

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Introduction

According to a systemic analysis in 2019, infectious diseases are leading cause of gobal morbidity and mortality with 13.9 million of death worldwide (Gray and Sharara, 2022; GBD 2019 Antimicrobial Resistance Collaborators 2022). Identification and characterization of diverse strategies used by pathogenic

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bacteria during their pathogenesis is a key components in fighting against bacterial diseases. Melioidosis is an endemic infectious disease in tropical areas caused by *Burkholderia pseudomallei* (*B. pseudomallei*) (Wiersinga et al. 2012), a saprophytic Gram-negative pathogenic rod-shaped bacterium belongs to beta-Proteobacteria (Lazar Adler et al. 2009). *B. pseudomallei* genome analysis and several other studies have demonstrated the availability of many virulence genes including type III secretion system cluster 3 (T3SS3) genes which are coding for *B. pseudomallei* secretion machinery (Cornelis 2006; Costa et al. 2015). During *B. pseudomallei* pathogenesis the T3SS3 genes are expressed to promote internalization of bacteria into host cells, the escape of infecting bacteria from the phagocytic endosome into the cytoplasm, the actin-based motility, the cell spreading and the induction of multinucleated giant cell (MNGC) formation in host cells (Jones et al. 1996; Kespichayawattana et al. 2000; Pruksachartvuthi et al. 1990). Previously, it has been demonstrated that *B. pseudomallei* could invade and survive within both phagocytic and non-phagocytic cells and that T3SS3 *bipB* gene was involved in MNGC formation of infected host cells (Suparak et al. 2005).

As a facultative intracellular pathogen, *B. pseudomallei* is exposed to conditions of nutrient limitation and oxidative stress in the host environment. Under these conditions, alternative transcription factors (RpoS) play an important role in bacterial survival and in preventing cellular and genetic damages. The *B. pseudomallei* RpoS is capable of repressing iNOS expression and is important for apoptosis induction in host cells (Lengwehasatit et al. 2008; Utaisincharoen et al. 2001). In addition, *B. pseudomallei* RpoS has been proposed to promote MNGC formation, a mediator of cell to cell spreading of bacteria (Utaisincharoen et al. 2006). However, the role of RpoS in controlling these processes in infected host cells remains to be elucidated.

Besides bacterial RpoS, RpoN was previously known to have a function in the assimilation of nitrogen. Due to the additional RpoN-dependent genes that are not necessary part of the nitrogen metabolic pathways, other functions of RpoN are considered (Gussin et al. 1986). In *Pseudomonas aeruginosa*, RpoN has been shown to be involved in virulence and pathogenesis as well as bacterial motility, fimbriae formation, nutrient transport, and intracellular signaling (Boucher et al. 2000; Dasgupta et al. 2003; Heurlier et al. 2003; Ishimoto & Lory 1989; Mattick et al. 1996; Strom & Lory 1993; Thompson et al. 2003; Totten et al. 1990). Recently, *B. pseudomallei rpoN2* was mutated and characterized (Diep et al. 2015). Our previous study presented a discovery revealing that *B. pseudomallei* harbors two copies of the RpoN gene (RpoN1 and RpoN2), with RpoN2 situated on chromosome 2 (Diep et al. 2015). Contrary to direct involvement in amino acid utilization within *B. pseudomallei*, RpoN2 exhibits the capability to reinstate this function when introduced into *Escherichia coli*. Utilizing a *B. pseudomallei* rpoN2 mutant strain lacking KatE activity, we demonstrated that RpoN2, but not RpoN1, plays a specific role in regulating catalase E expression at both the transcriptional and translational levels (Diep et al. 2015). To extend the knowledge of RpoS and RpoN2 functions, this study focused on the involvement of *B. pseudomallei* RpoS and RpoN2 in the bacterial invasion, intracellular survival, and MNGC formation in bacterially infected mouse macrophage cells. BipB and BipC, proteins within *B. pseudomallei*, are integral components of the type III secretion system (T3SS), a critical virulence mechanism employed by the bacterium in the development of melioidosis. While BipB facilitates the translocation of effector proteins across the host cell membrane, BipC acts as a chaperone protein, promoting the secretion and stability of T3SS effectors. These proteins collectively enhance the pathogenicity of *B. pseudomallei* by actively manipulating host cell functions, enabling the bacterium to evade immune responses and successfully establish infection (Suparak et al. 2005; Wiersinga et al. 2012).

The involvement of RpoS and RpoN2 in *B. pseudomallei* virulence was investigated using a mouse macrophage cell line (RAW264.7) model. We demonstrated that *B. pseudomallei* RpoS and RpoN2 are required for efficient invasion of bacteria into host cells and subsequent intracellular survival. In addition, our results also provided evidence that MNGC formation is activated by a certain number of at least 5×10^4 intracellular survival bacteria either the wild type or both *rpoS* and *rpoN2* mutants. Furthermore, previously unknown roles for *B. pseudomallei* RpoS and RpoN2 in the regulation of TTSS/bipB and bipC expression were identified.

Materials and methods

Cell line and culture conditions

The mouse macrophage, Abelson murine leukemia virus-transformed cell line RAW264.7 (ATCC® TIB-71™), was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium-High Glucose (DMEM) (Thermo Scientific HyClone Laboratory, Logan, UT) supplemented with 2 mM L-glutamine (Biochrome, Germany) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) in a 37 °C humidified incubator with a 5% CO_2 atmosphere. Subcultures were prepared by scraping cells at passage number six for use in infection experiments.

Bacterial strains and growth conditions

B. pseudomallei strain PP844, used as a wild type strain in this study, was originally isolated from a patient admitted to Srinagarind Hospital in Khon Kaen province, a melioidosis endemic region of Thailand (Utaisincharoen et al. 2001). The *rpoS* and *rpoN2* deletion mutants (Δ*rpoS* and Δ*rpoN2*) used for comparison throughout this study were constructed as previously described (Diep et al. 2015; Subsin et al. 2003).

Prior to the experiment, PP844 WT, Δ*rpoS,* and Δ*rpoN2* strains were cultured in 10 ml Tryptic Soy Broth (TSB), supplemented with tetracycline at a final concentration 60 µg/ml and incubated at 37 °C with shaking at 200 rpm. Overnight bacterial cultures were inoculated at 0.1% (v/v) in 5 ml fresh TSB without antibiotics and grown with shaking for an additional 6 h. The bacterial cells were collected by centrifugation at 7000 × *g* at 4 °C for 5 min and washed twice with phosphate buffer saline (PBS). The optical density of cultures was measured at 600 nm (OD_{600}) and bacterial content was adjusted to the desired number.

Infection assays

Infection assays were performed using the antibiotic (kanamycin) protection method as described previously (Jones et al. 1996; Kespichayawattana et al. 2000) with some modifications. Briefly, 5.8×10^4 RAW264.7 cells were seeded in 12-well sterile non-pyrogenic cultured plates (SPL life sciences) for overnight in 37 °C humidified incubator with 5% CO_2 atmosphere. Bacterial cells were collected from the 0.1% inoculated bacterial cultures in 5 ml TSB by centrifugation 7000×g. Bacterial cell pellets were washed twice by PBS and re-suspended in DMEM media with the density of 10^7 bacterial CFUs/ml. The infection assay was initiated by addition of prepared cultures of either *B. pseudomallei* WT or Δ*rpoS* or Δ*rpoN2* cells to the monolayer of RAW264.7 cells in 12-well plates. Multiplicity of infection (MOI) of 2, 10 or 20 bacteria per one RAW264.7 cell were used and plates were briefly shaken to equally distribute the bacteria in each well (Utaisincharoen et al. 2006). Infected cultures were incubated for 2 h at 37 °C in 5% CO_2 to allow bacteria to enter the host cells. The monolayer of RAW264.7 cells was then washed twice with pre-warmed PBS and further incubated for an additional 2 h in 2 ml fresh complete DMEM medium containing 250 µg/ml kanamycin (Gibco Labs) to eliminate extra cellular bacteria. At each indicated time point post-infection (PI), the media was removed, the monolayer was washed twice with PBS and the infected RAW264.7 cells were lysed to liberate the intracellular bacteria by addition of 200 µl 0.1% Triton X-100 (Sigma Chemicals, Co.).

To evaluate the invasiveness of *B. pseudomallei* WT, Δ*rpoS,* and Δ*rpoN2* strains, RAW264.7 cell lysates at 0 h PI with MOI 2, MOI 10, and MOI 20 were subjected to serial dilution and plated onto the TSA (tryptic soy agar) for determination of bacterial CFUs after 48 h incubation at 37 °C.

Intracellular survival assays

Infection assays with MOI2 (two bacteria per one RAW264.7 cell) as described above were used for intracellular survival experiments. The infected RAW264.7 cells were collected at the indicated times (4 h, 6 h, 8 h, 12 h, 16 h, and 20 h) PI and lysed. Serial dilutions of RAW264.7 cell lysates were plated onto the TSA. After 48 h incubation at 37 °C, the number of bacterial colonies was determined and the value was used as the intracellular bacteria in each sample.

Giemsa staining and %MNGC counting

RAW264.7 cells were seeded and cultured overnight on glass coverslips in 12-well plates. The viability

of the cells before and after infection was assayed by staining with trypan blue. Following infection at MOI 2 with *B. pseudomallei* WT or Δ*rpoS* or Δ*rpoN2*, the infected RAW264.7 cells were washed twice with PBS pH7.4 and fixed with cold methanol for 15 min. Coverslips were stained with Giemsa (BDH medicals) and air-dried. Images were captured using an inverted microscope (Nikon, TE2000U). To evaluate the %MNGC formation of the infected RAW264.7 cells, approximately 5000 nuclei per coverslip were counted and analyzed using ImageJ software. The percent MNGC formation was calculated as:

$$\%\text{MNGC} = (\text{number of nuclei within multinucleated giant cells} / \text{total number counted}) \times 100$$

Total *B. pseudomallei* RNA isolation

Total *B. pseudomallei* RNA isolation was performed using the phenol–chloroform method containing the TRIzol reagent following the manufacturer's protocol (Invitrogen, Life technologies, USA). Total RNA was re-suspended in RNase-free water and stored at − 80 °C prior to use.

qRT-PCR for comparative quantitation of bipB-bipC gene expression in B. pseudomallei WT, ΔrpoS and ΔrpoN2 strains

RNA samples were treated with RQ1 RNase-free DNase (Promega, USA) at 37 °C for 60 min to remove genomic DNA contamination and double-tested by running PCR with specific primers. The specific bands for small ribosomal RNAs were monitored and the RNA concentrations, as well as the purity, were measured by using a Nanodrop TM 2000 (Thermo Fisher Scientific, USA).

To confirm whether *bipB* and *bipC* genes are co-transcribed in a single transcript, reverse transcription PCR (RT-PCR) was performed using the Improm–II Reverse Transcriptase kit (Promega, USA). The newly synthesized first strand of cDNAs with an expected size of about 286 bp was checked by conventional PCR with bipB73 and bipC72 specific primers and the results were observed using 1.5% agarose gel electrophoresis.

The specific primer sequences were designed as shown in Fig. 5A, and were obtained from Suparak and co-workers (Suparak et al. 2005) and anneal pair-wise between *bipB* and *bipC* (bipB73 forward: 5′-CTG CTC GGC GAT CTG CTC AA-3′ and bipC72 reverse: 5′-ACC GCC TTG TCG CCC TG-3′). The RT-PCR mixture contained 200 ng *B. pseudomallei* total RNA, 20 pmol of each either *23S* rRNA primers as an internal control (forward: 5′-CGA ATG GGG AAA CCC GGC CC-3′, reverse: 5′-GGC CGC ACT TTC CAG AGC GT-3′).

The expression of *bipB-bipC* genes along different growth phases in TSB was studied by using Kapa SYBR FAST Universal kit (Kapa Biosystems) for real-time qPCR using a Stratagene MX3000P QPCR system (Agilent technologies). To investigate the *bipB-bipC* gene expression under RpoS or RpoN2 regulation, the relative quantitation of gene expression in *B. pseudomallei* WT, Δ*rpoS,* and Δ*rpoN2* samples using real-time quantitative PCR was performed as described above. The results were analyzed using the comparative Ct method or ΔΔCt method (Applied Biosystems).

Statistical analysis

All results in this study were from at least 3 times independent experiments, each carried out in duplicate or triplicate. Values were presented as means ± standard error. Statistical significance of differences between the two means was calculated using SigmaStat 3.5 software and evaluated by the Student's t-test and P value < 0.01 was considered significant.

Results

Correlation between multinucleated giant cell (MNGC) formation in infected mouse macrophage cells and intracellular survival of *B. pseudomallei* wild type, ΔrpoS and ΔrpoN2

The results in Fig. 1A illustrate that uninfected RAW264.7 cells were still in inactivated stage with a spherical shape. On the contrary, all the infected cells clearly exhibited MNGC formation (Fig. 1B–D). It should be noted that the RAW264.7 activated by lipopolysaccharide (LPS), which is well-known to

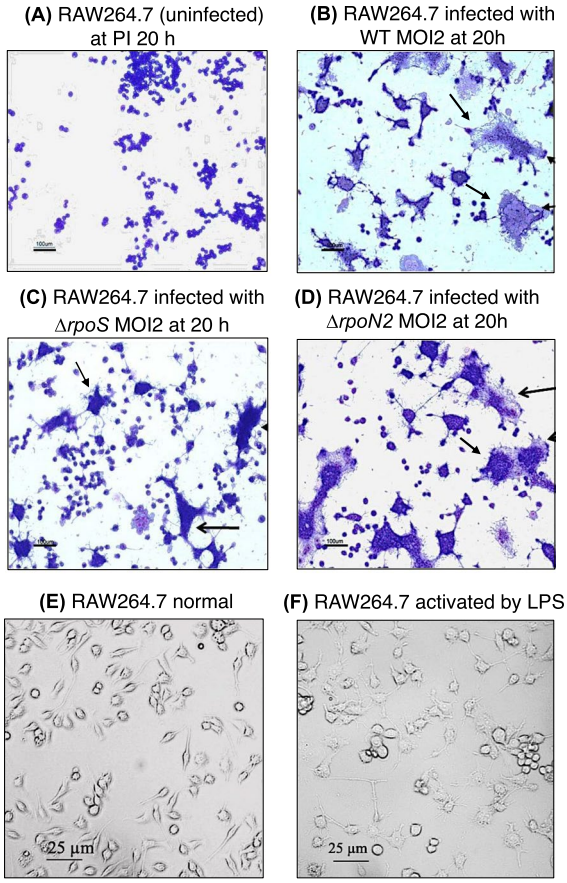

Fig. 1 Giemsa staining for observation MNGC formation of the 10^5 RAW264.7 cells infected with (**B**) *B. pseudomallei* WT, (**C**) Δ*rpoS* and (**D**) Δ*rpoN2* with MOI2 at 20 h compared to (**A**) the uninfected RAW264.7 cell at 20 h PI. The cells were fixed, stained with Giemsa, then the pictures were taken under Inverted Microscope (Nikon, TE2000U) and counted by ImageJ program. Arrows indicate MNGC formation of RAW264.7 cells. **E** and **F** RAW264.7 cell at 24 h treated without and with lipopolysaccharide (LPS, 1 µg/ml)

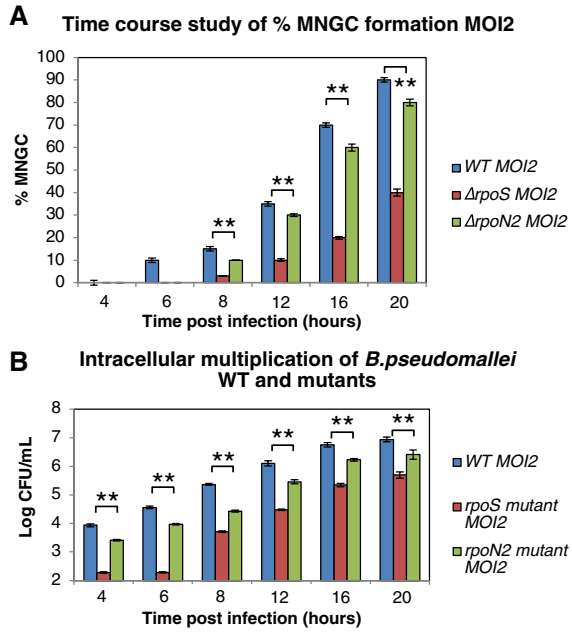

Fig. 2 Time course studies on multinucleated giant cell (MNGC) formation in mouse macrophage cell line and intracellular survival of *B. pseudomallei* wild type, Δ*rpoS* and Δ*rpoN2*. **A** The percent MNGC formation in RAW264.7 cells infected with MOI2 of *B. pseudomallei* wild type, Δ*rpoS* and Δ*rpoN2* at 4 h, 6 h, 8 h, 12 h, 16 h and 20 h PI. **B** The intracellular survival of *B. pseudomallei* wild type, Δ*rpoS* and Δ*rpoN2* after infected in RAW264.7 cell at 4 h, 6 h, 8 h, 12 h, 16 h and 20 h PI using drop plate technique and colony forming units (CFUs) by plotted based on the logarithm of means and standard errors from three independent experiments. The graphs plotted based on the mean of three independent experiments, each was performed in duplicate, $^{**}P < 0.01$

stimulate the macrophage activation, did not display the formation of MNGC although the morphology of cells changed, including production of filopodia and lamellipodia (Fig. 1E, F). At 20 h PI, RAW264.7 cells infected with either *B. pseudomallei* WT or Δ*rpoS* or Δ*rpoN2* display 90%, 40%, and 80% MNGC formation, respectively. The percentages of MNGC in RAW264.7 cells were calculated during the time of infection and plotted as shown in Fig. 2A. For the first 6 h PI, MNGCs were not observed in RAW264.7 cells infected with either Δ*rpoS* or Δ*rpoN2*; however, at 8 h PI the formation of MNGCs was detected and the number of MNGCs increased until the termination of the experiment at 20 h PI. Although the level of MNGC formation in RAW264.7 infected with Δ*rpoS* and Δ*rpoN2* was lower than those infected with WT at each time point of PI, the similar increasing pattern suggested a delay in MNGC formation in host cells infected with Δ*rpoS* and Δ*rpoN2*.

To determine the intracellular survival of *B. pseudomallei* WT, Δ*rpoS,* and Δ*rpoN2* in RAW264.7 cells after invasion, the doubling time for each strain was calculated. At 4 h and 6 h PI, the doubling time of WT was calculated at 44 min and 48 min, respectively. After that at 8 h PI, the replication rate of Δ*rpoS* and Δ*rpoN2* was calculated to be 129 min and 71 min, respectively, and then cell numbers increased at a similar rate as WT (Fig. 2B). The number %MNCG formation

increased propotional to the number of bacteria and the formation of MNCG was observed when the number of bacteria was higher than 10^4 (Fig. 2A, B). These results lead us to the hypothesis that the MNGC formation in the infected host cells depends on the number of bacteria inside the host (at least 10^4 bacterial cells). Intracellular survival of Δ*rpoN2* at each PI time point during infection experiment is lower than WT but higher than Δ*rpoS*. These findings lead us to look insight into the invasion capability of each employed strain.

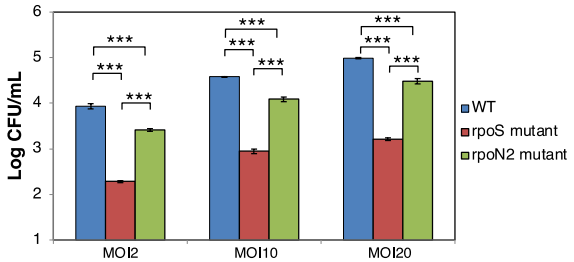

Fig. 3 The invasiveness of Δ*rpoS* and Δ*rpoN2* into RAW264.7 cell line in comparison with *B. pseudomallei* wild type. The 1×10^5 RAW264.7 cells/well in 12 well-plates were infected with either bacteria WT (wild type), *rpoS* mutant (Δ*rpoS*) or *rpoN2* mutant (Δ*rpoN2*) with MOI 2, MOI 10 and MOI 20. Data are expressed as the mean and standard error of the mean (SEM) for three independent experiments, each carried out triplicate. *** $P<0.001$

Involvement of RpoS and RpoN2 in the invasion of *B. pseudomallei* into mouse macrophage cell line (RAW264.7)

In this experiment, the assigned number of infected bacteria of *B. pseudomallei* WT or Δ*rpoS* or Δ*rpoN2* strain was performed with MOI of 2, 10, and 20 bacteria per RAW264.7 cell. Figure 3 and data in Table 1 show that the invasiveness of *B. pseudomallei* WT was 50 times higher than Δ*rpoS* and about 5 times higher than Δ*rpoN2* after 4 h PI. Thus to force the numbers of 10^4 bacterial cells to enter the host cell, the infection assay with MOI 2 for wild type, MOI 100 for Δ*rpoS,* and MOI 10 for Δ*rpoN2* was designed and carried out as shown in Fig. 4 and Table 2. At 8 h PI, the same intracellular number of WT or Δ*rpoS* or Δ*rpoN2* was found at about 10^5 bacteria by CFU counting (Table 2). It is correlated with the same percentage of MNGC formation (15%) in the RAW264.7 infected with either WT or Δ*rpoS* or Δ*rpoN2* (Fig. 4), suggesting the certain number of intracellular bacteria at least 5×10^4 is required to cause the morphological changes of RAW264.7 cells and that *B. pseudomallei* RpoS and RpoN2 may not be directly involved in the MNGC formation of the host.

Table 1 Invasion of either *B. pseudomallei* wild type (WT), Δ*rpoS* or Δ*rpoN2* strain into RAW 264.7 cell line, as measured by the kanamycin protection assay

Bacterial strain	Innoculum size [a] (CFU)	Mean no. of intracellular bacteria after 4 h incubation [b] (CFU)	Internalization (%)
WT MOI 2	1.89×10^5	$8.63\times10^3\pm6.01\times10^2$	4.57
WT MOI 10	8.20×10^5	$3.86\times10^4\pm2.05\times10^2$	4.71
WT MOI 20	2.00×10^6	$9.69\times10^4\pm1.80\times10^3$	4.84
Δ*rpoS* MOI 2	1.97×10^5	$1.91\times10^2\pm6.25\times10^0$	0.10
Δ*rpoS* MOI 10	9.30×10^5	$8.83\times10^2\pm6.47\times10^1$	0.09
Δ*rpoS*MOI 20	1.89×10^6	$1.64\times10^3\pm6.95\times10^1$	0.09
Δ*rpoN2* MOI 2	2.10×10^5	$2.58\times10^3\pm8.47\times10^1$	1.23
Δ*rpoN2* MOI 10	9.71×10^5	$1.23\times10^4\pm8.26\times10^2$	1.27
Δ*rpoN2* MOI 20	2.21×10^6	$3.05\times10^4\pm2.19\times10^3$	1.38

[a]Innoculum size = number of bacteria added into 10^5 RAW 264.7 host cells. The mixtures were incubated for 4 h

[b]Number CFU of liberated intracellular bacteria after 4 h incubation

Internalization (%) is percentage of number of intracellular bacteria over innoculum size. Data are means ± standard error of the mean (SEM) from three independent experiments, each carry out duplicate

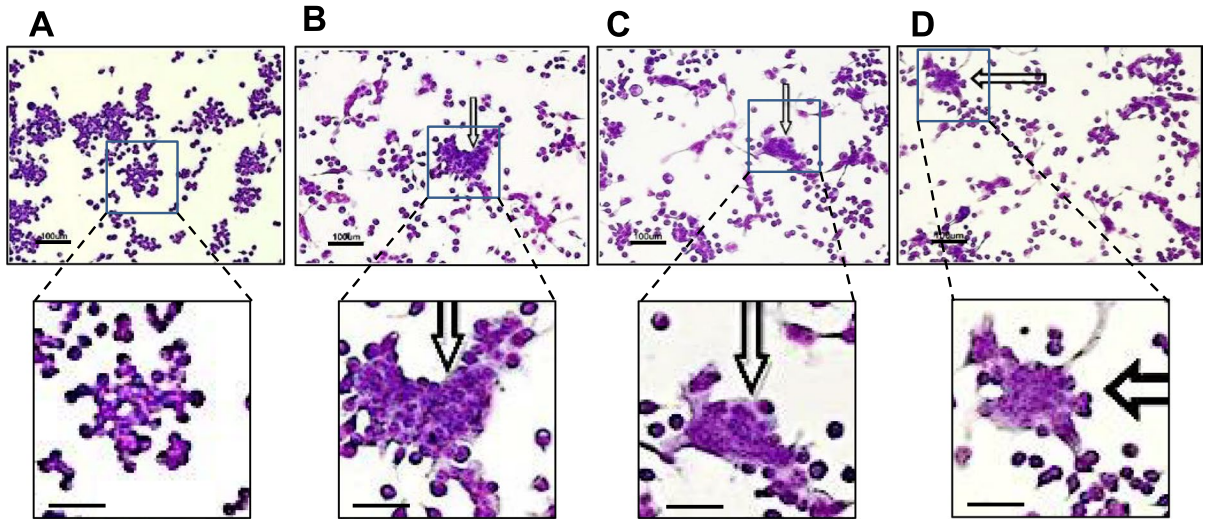

Fig. 4 The MNGC formation in RAW 264.7 cells infected either with *B. pseudomallei* WT MOI 2 or Δ*rpoS* MOI 100 or Δ*rpoN2* MOI 10 at PI 8 h. Giemsa staining of (**a**) uninfected RAW264.7 at 8 h PI, **b** infected RAW264.7 cells with MOI 2 of *B. pseudomallei* wild type (WT), **c** infected RAW264.7 cells with MOI 100 of Δ*rpoS*, **d** infected RAW264.7 cells with MOI 10 of Δ*rpoN2*. The arrows indicated the MNGC formation in an infected RAW264.7 cells. Scale bars are 100 µm and 50 µm

Table 2 MNGC formation in RAW 264.7 cells infected with either *B. pseudomallei* WT MOI2 or Δ*rpoS* MOI 100 or Δ*rpoN2* MOI 10 at PI 8 h

Bacterial strain	Innoculum size [a] (CFU)	No. of intracellular bacteria at PI 8 h[b] (CFU)	MNGC formation[c] in RAW 264.7 cells infected with bacteria at PI 8 h (%)
WT MOI2	1.93×10^5	$1.42\times10^5\pm4.73\times10^3$	15.32 ± 0.73[d]
Δ*rpoS* MOI100	1.27×10^7	$1.58\times10^5\pm1.04\times10^4$	15.87 ± 0.22[d]
Δ*rpoN2* MOI 10	9.72×10^5	$1.68\times10^5\pm1.04\times10^4$	16.12 ± 0.13[d]

[a]Innoculum size = number of indicated *B. pseudomallei* added into 10^5 RAW 264.7 host cells

[b]Number CFU (Mean) of intracellular bacteria at post infection 8 h (PI 8 h) ± standard error of the mean (SEM) from three independent experiments, each carried out in duplicate

[c]%MNGC = (number of nuclei within multinucleated giant cells/total number counted) × 100

[d]Percentages were determined at 8 h after RAW 264.7 cells infected with indicated strains of *B. pseudomallei*

Data are Means ± SEMs from at least three independent experiments, each carried out in duplicate

Involvement of *B. pseudomallei* RpoS and RpoN2 in regulation of the TTSS cluster 3 bipB-bipC gene expression

We next investigated the potential mechanism by which *B. pseudomallei* could modulate the MNGC formation of infected RAW264.7 cells. It has been demonstrated previously that BipB may play an important role in the induction of MNGC formation of the host cells due to the reduction in MNGC formation of host cells infected with *bipB* mutant strain (Suparak et al. 2005). In addition, computational analysis of *B. pseudomallei* genome by using PROMSCAN program predicts that the consensus sequence of RpoN promoter is located upstream of 179 genes, which include T3SS cluster 3 on the chromosome II with a very high confidentiality of the score. Therefore, we hypothesize that RpoN and RpoS may be involved in *bipB* expression regulation at transcriptional level. By using DOOR program (Mao et al. 2014) and information of gene organization in NCBI, *B. pseudomallei bipB* and *bipC* were predicted to

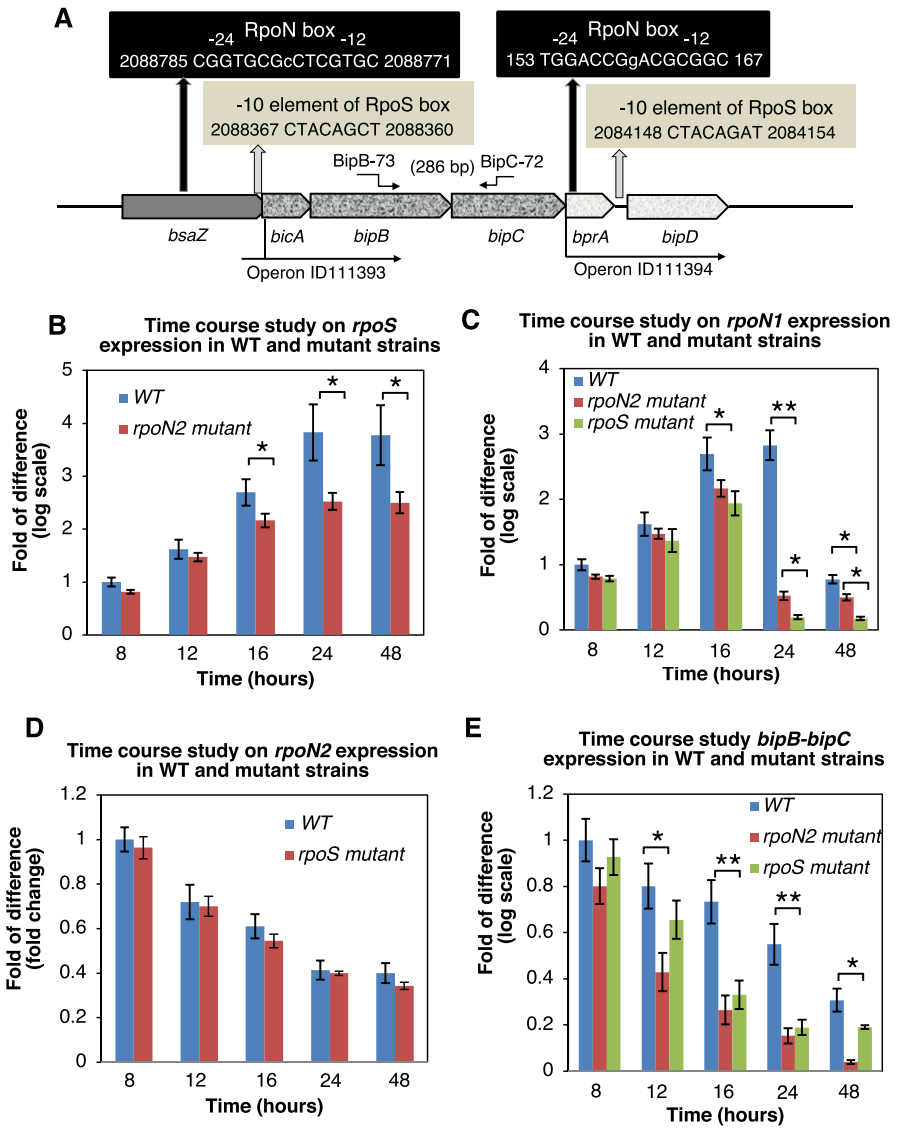

Fig. 5 Analysis of *bipB-bipC* gene expression at transcriptional level in *B. pseudomallei* WT, Δ*rpoS* and Δ*rpoN2* strains. **A** Physical gene map of *bsaZ-bicA-bipB-bipC-bprA-bipD* region with potential RpoS and RpoN boxes. Putative RpoN boxes predicted by HMMer search with -24/-12 promoter training set in 300 bp UPS (upstream) region of *bipB* operon and 300 bp UPS of *bipD*. (**B**–**E**). Time course study and relative quantitation of (**B**) *rpoS*, **C** *rpoN1*, **D** *rpoN2* and **E** *bipB-bipC* gene expressions in *B. pseudomallei* WT and mutant strains. Data are expressed as the mean and standard error of the mean (SEM) of three independent experiments, each carried out triplicate. * $P < 0.05$, ** $P < 0.01$

belong to operon ID 111393 (Fig. 5A) and *bipD* to belonged to operon ID 111394, which is a single gene operon. Base on this prediction, the potential RpoS and RpoN boxes in 300 bp UPS (upstream region) of these two operons were then predicted by HMMer software (Osiriphun et al. 2009). The *bipB-bipC* expression in *B. pseudomallei* WT, Δ*rpoS,* and Δ*rpoN2* were examined firstly in traditional RT-PCR and PCR in agarose gel stained with ethidium bromide (Supplementary Fig. 1). The qRT-PCR data were further analyzed by using ΔΔCt method to compare the level expression of *bipB-bipC* genes between WT and the two mutants (Fig. 5B–E).

Consistent with previous studies, *rpoS* was not expressed or below detection in Δ*rpoS* strain, but it was constitutively expressed in WT and the Δ*rpoN2* mutant, showing the pattern of increased expression during the period of exponential growth and reached a maximum level during the steady phase of growth, indicating that RpoS is essential for bacteria growth and RpoN2 may regulate the expression of *rpoS* at stationary phase (Fig. 5B). *B. pseudomallei rpoN1* is expressed constitutively in WT, Δ*rpoS,* and Δ*rpoN2* strains; however, after 16 h of growth, it seems to be regulated by RpoS and RpoN2 (Fig. 5C). The mechanism of how RpoS and RpoN2 involve in regulation

of *rpoN1* expression needs to be studied further. On the other hand, *rpoN2* expression decreased gradually over time and no difference was observed in WT and Δ*rpoS* (Fig. 5D), suggesting that *rpoN2* does not belong to a group of RpoS-dependent genes. The *rpoN2* promoter region in the RpoN box was founded, suggesting that it is an auto-regulated gene. The level of *rpoN2* expression is lower than that of *rpoN1* judging by the RT-qPCR Ct value and the thinner band in agarose gel (Supplementary Fig. 2).

The expression of *bipB-bipC* in Fig. 5E confirms the bioinformatics prediction that *bipB* and *bipC* are co-transcribed together as an operon as well as the appearance of a clearer single band detected in WT sample by agarose gel (Supplementary Fig. 1). At each indicated time point during bacterial growth in TSB, the expression level of *bipB-bipC* genes in Δ*rpoS* and Δ*rpoN2* mutant strains was substantially lower than in WT at all time-points examined. The result indicates that both RpoS and RpoN2 were involved in regulation the multi-steps of the bacterial invasion and intracellular survival genes and operons, but not directly involved in MNGC formation.

Discussion and conclusions

In this study, we observed that *B. pseudomallei* RpoS and RpoN2 are indeed involved in the entry and intracellular survival. The number of Δ*rpoS* and Δ*rpoN2* bacteria that successfully infect RAW264.7 cells are, however, lower than the wild type, leading to a reduction of MNGC formation in macrophage cells infected with the mutant strains at every time point PI monitored (Fig. 2A, B). In addition, the results in Tables 1 and 2 extend our finding, confirming that the %MNGC formation in RAW264.7 cells was dependent on the bacterial number inside the host cells (at least 5×10^4 bacteria). Our analysis indicates a strong correlation between intracellular survival of bacteria and MNGC formation in infected host cells which was not observed in the previous studies that monitored a single time point PI (Burtnick el al. 2011). Utainsincharoen and co-workers were the first to demonstrate that *B. pseudomallei* null mutant *rpoS* (Δ*rpoS*) strain failed to induce MNGC formation in the infected host cells at 8 h PI (Utaisincharoen et al. 2006). In our work, we expanded on the previous analysis with modified invasion assays to allow for investigation of MNGC formation in infected host RAW264.7 cells up to 20 h PI (Figs. 1 and 2).

The result shown in Fig. 3 demonstrated that the invasion capability of the Δ*rpoS* and Δ*rpoN2* strains was significantly lower than WT in all cases with the same MOI. Similarly, the different invasion efficiency of Δ*rpoS* (about 0.1%), Δ*rpoN2* (1.2%), and its WT (about 4.8%) at various MOIs was investigated (Table 1). These data are consistent with previous reports (Utaisincharoen et al. 2006), especially for the analysis of *bopE*, *bop*A, *bipB*, *bipC*, *bipD* null mutants that exhibited reduced or impaired entry into eukaryotic host cells (Kang et al. 2015; Stevens et al. 2004; Suparak et al. 2005). Taken together, these results suggest that *B. pseudomallei* RpoS and RpoN2 are involved in bacterial invasion.

The exact mechanism of cell invasion is not clearly understood but the utilization of a sophisticated array of bacterial effector proteins that are injected into the host cell cytoplasm through a TTSS apparatus has been reported (Hii et al. 2008; Stevens et al. 2002, 2003). The mechanism by which bacteria can subvert the host signaling and cytoskeletal machinery for their own purposes has not been clearly established. The impaired entry into the host cells of *B. pseudomallei bipB* or *bipD* (coding for translocator proteins) inactivated strains was found to be due to the impaired delivery into the host cells cytoplasm of several effector proteins, for example BopE, which contributes to invasion (Stevens et al. 2003; Suparak et al. 2005). Hence it is believed that several Bsa proteins act in concert with BopE to facilitate bacterial invasion and it is interesting to know how these genes are regulated (Stevens et al. 2003, 2004). The reduction in invasion efficiency of the Δ*rpoS* and Δ*rpoN2* strains into RAW264.7 cells led us to hypothesize that the *B. pseudomallei* RpoS and RpoN2 may be involved in the transcriptional regulation of one or more genes in TTSS/bsa cluster, particularly *bip*s and *bop*s coding for the invasion and outer membrane proteins.

The lower rate of invasion and intracellular replication of the Δ*rpoS* and Δ*rpoN2* strains lead to a reduction in MNGC formation of infected RAW264.7 cells. Our results demonstrated a correlation between %MNGC formation of RAW264.7 and intracellular *B. pseudomallei* number. At 4 h PI, when the number of WT *B. pseudomallei* was below 10^4 CFU, no MNGC formation was observed in RAW264.7 cells infected

the WT. At 6 h PI, bacterial level was over 10^4 CFU and 10% MNGC formation of the host was observed. This analysis suggests that at least 5×10^4 CFU of bacteria is required for induction of MNGC formation in host cells. Moreover, at 12 h PI, the number of intracellular Δ*rpoS* cells reached a similar value as that seen for the WT at 6 h PI. In each case, approximately 10%MNGC formation was observed in RAW264.7 cells infected with Δ*rpoS* and Δ*rpoN2* compared to its WT (Fig. 4). The correlation between intracellular *B. pseudomallei* and MNGC formation was confirmed by producing conditions in which both Δ*rpoS* and Δ*rpoN2* strains with their parental strain were present at similar intracellular levels (*B. pseudomallei* WT MOI 2 or Δ*rpoS* MOI 100 or Δ*rpoN2* MOI 10) as shown in Table 2.

The involvement of RpoS and RpoN2 in *bipB-bipC* gene expression during stationary and late-stationary phase (Fig. 5B-E) is confirmed in agreement with the reduction in invasion efficiency of the Δ*rpoS* and Δ*rpoN2* strains into RAW264.7 cells (Fig. 4 and Table 1). These results indicate that *B. pseudomallei* RpoS and RpoN2 are involved in the transcriptional regulation of genes in TTSS/*bsa* cluster, particularly *bipB-bipC* genes encode translocator proteins that may inject bacterial proteins responsible for invasion into the host. Both RpoS and RpoN2 are able to regulate the same level of *bipB-bipC* gene expression (Fig. 5E). Moreover, the expression of *B. pseudomallei katE* gene has been recently illustrated to be regulated by both RpoS and RpoN2 (Diep et al. 2015; Jangiam et al. 2010). We hypothesized the crosstalk between these two sigma families might involve via an enhancing binding protein (EBP) which is necessary for the RpoN function and that is currently under our investigation.

In Table 1 and 2, the significant reduction of invasiveness was observed in Δ*rpoN2* and Δ*rpoS* strains, suggesting the functions of RpoN2 and RpoS in the cellular internalization and MNGC formation. The expression of *rpoN1* was significantly suppressed in the *rpoS and rpoN2*-inactivated strains as compared to wild type (Fig. 5C). Moreover, the Δ*rpoS* cells require more time to adapt to the intracellular environment compared to Δ*rpoN2* and its wild type. The exact mechanism by which this pathogen survives inside macrophages is unknown; however, an important role for antioxidant enzymes in the inhibition of macrophage bactericidal activity is known (Miyagi et al. 1997). *B. pseudomallei* oxidative stress responses genes have been partially characterized, among them *oxyR* and the *katG-dpsA* operon are under RpoS control (Jangiam et al. 2010; Loprasert et al. 2002, 2003). Although the expression of *katG* and *dpsA* is tightly regulated by RpoS, expression of this operon in the Δ*rpoS* strain in hostile conditions has been documented (Jangiam et al. 2010). Since the RpoS also independently control the activation of *katG* and *dpsA*, the Δ*rpoS* strain exhibited a delaying survival inside RAW264.7 cells. When activation of KatG induces the production of peroxides and hydroxyl radicals by the host, DpsA binds to the chromosome to prevent DNA oxidative damage (Loprasert et al. 2002). In *E. coli* DpsA interacts with DnaA to impede initiation by interfering with strand opening at the origin of replication. This suggests that DpsA acts as a regulator of the cell cycle checkpoint during oxidative stress to reduce initiation, providing an opportunity for the repair of oxidative DNA damage (Chodavarapu et al. 2008). Consistent with its proposed role, DpsA protects the intracellular *Salmonella* Typhimurium from killing by H_2O_2, facilitates *Salmonella* survival in murine macrophages, and enhances *Salmonella* virulence (Halsey et al. 2004). The role of DpsA protein in *B. pseudomallei* has not been established however, *dpsA* expression is elevated at all stages of growth in Δ*rpoS* strain in comparison with its wild type. In addition, increasing level of the DpsA protein results in a reduced growth rate but enhanced intracellular survival (Al-Maleki et al. 2014; Jangiam et al. 2010). These findings may help to explain our results that the Δ*rpoS* strain can survive and replicate inside RAW264.7, however at the lower rate than the Δ*rpoN2* and its wild type.

Taken together, our results demonstrate important roles for *B. pseudomallei* RpoS and RpoN2 in invasion and intracellular survival via regulation of TTSS/bsa genes. We also illustrate that MNGC formation of RAW264.7 cells requires a certain number of bacterial intracellular survival and replication and that bacterial *rpoS* and *rpoN2* contribute to all these processes in the host cell. Overall, this study revealed that MNGC formation in host infected cells is linked to bacterial survival and proliferation PI and not directly to genes regulated by either RpoS or RpoN2.

Author's contribution D.T.H. Diep: concept, acquisition, analysis, interpretation of data. L.B.Vong: acquisition, analysis,

or interpretation of data. S. Tungpradabkul: concept, supervision. All authors prepared the manuscript and approved the version to be submitted.

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Data availability Data available on request from the authors.

Declarations

Conflict of interest No authors have any conflicts of interest to declare.

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