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Research Letter

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Burkholderia pseudomallei rpoS mediates iNOS suppression in human hepatocyte (HC04) cells

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One sentence summary: This study investigated the induction of nitric oxide synthase (iNOS) in human hepatocyte cells (HC-04) infected with wild-type *Burkholderia pseudomallei* or an *rpoS* mutant. RpoS was implicated in the ability to suppress iNOS induction and with the induction of autophagy, indicating that it plays an important role in *B. pseudomallei* interactions with liver cells.
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ABSTRACT

Burkholderia pseudomallei is an intracellular Gram-negative bacterial pathogen and the causative agent of melioidosis, a widespread disease in Southeast Asia. Reactive nitrogen, in an intermediate form of nitric oxide (NO), is one of the first lines of defense used by host cells to eliminate intracellular pathogens, through the stimulation of inducible nitric oxide synthase (iNOS). Studies in phagocytotic cells have shown that the iNOS response is muted in *B. pseudomallei* infection, and implicated the *rpoS* sigma factor as a key regulatory factor mediating suppression. The liver is a main visceral organ affected by *B. pseudomallei*, and there is little knowledge about the interaction of liver cells and *B. pseudomallei*. This study investigated the induction of iNOS, as well as autophagic flux and light-chain 3 (LC3) localization in human liver (HC04) cells in response to infection with *B. pseudomallei* and its *rpoS* deficient mutant. Results showed that the *rpoS* mutant was unable to suppress iNOS induction and that the mutant showed less induction of autophagy and lower co-localization with LC3, and this was coupled with a lower intracellular growth rate. Combining these results suggest that *B. pseudomallei rpoS* is an important factor in establishing infection in liver cells.

Keywords: autophagy; *Burkholderia pseudomallei*; hepatocyte; inducible nitric oxide synthase (iNOS); melioidosis; RpoS

INTRODUCTION

The intracellular Gram-negative bacterium *Burkholderia pseudomallei* is the causative agent of melioidosis (Woods *et al.* 1999; White 2003; Cheng and Currie 2005), a tropical disease that is present throughout Southeast Asia (Woods *et al.* 1999; White 2003; Cheng and Currie 2005). Clinical symptoms of infection are commonly characterized by pneumonia and multiple abscesses, which are mostly found at bacteria dissemination sites such as the lungs, liver and spleen (Cheng and Currie 2005), with the liver being implicated as a primary target for *B. pseudomallei* (Ben *et al.* 2004; Lee *et al.* 2006; Pal *et al.* 2014). The liver is a critical organ that plays an important role in maintaining homeostasis (Parker and Picut 2005) and is comprised of several cell types

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including hepatocytes, which can generate and secrete inflammatory cytokines and chemokines in response to bacterial infections (Parker and Picut 2005).

The *rpoS* gene of *B. pseudomallei* encodes for the important transcription factor, sigma S, which plays a role in transcriptional regulation and which responds during stress conditions (Lange and Hengge-Aronis 1991; Hengge-Aronis *et al.* 1993; Hengge-Aronis 2000; Osiriphun *et al.* 2009). Notably, it has been reported that the RpoS of *B. pseudomallei* regulates many factors involved in pathogenesis such as multinucleated giant cell formation and the induction of apoptosis in the RAW264.7 murine macrophage cell line (Utaisincharoen *et al.* 2006; Lengwehasatit *et al.* 2008).

As previously reported, *B. pseudomallei* can interfere with the induction of nitric oxide synthase (iNOS) expression, which is involved in the elimination of intracellular bacteria via the reactive nitrogen intermediate nitric oxide (NO) in the RAW264.7 murine macrophage cell line (Utaisincharoen *et al.* 2001, 2003). However, a recent publication by Bast *et al.* (2011) showed that iNOS-derived NO might not be the main mechanism that eliminates the bacteria in human hepatocytes. Besides iNOS-derived NO production, the induction of autophagy has been associated with the elimination of intracellular pathogens (Campoy and Colombo 2009; Gomes and Dikic 2014).

Autophagy is the catabolic pathway which cells use to degrade unnecessary or dysfunctional cellular components through the action of lysosomes (Mizushima 2007; Glick, Barth and Macleod 2010). Moreover, it also acts as an antimicrobial response found in many cell types (Campoy and Colombo 2009; Gomes and Dikic 2014). Autophagy has been reported as a mechanism that is used to eliminate several bacteria such as *Mycobacterium tuberculosis* and Group A Streptococcus (Campoy and Colombo 2009; Mostowy and Cossart 2012). Recently, several studies have reported that the LC3 (light-chain 3)-associated phagosome helps in the elimination of *B. pseudomallei* from infected mouse macrophages (Gong *et al.* 2011; Randow, MacMicking and James 2013; Romao and Münz 2014). However, any relationship between iNOS expression and the stimulation of autophagy in host cells infected with *B. pseudomallei* has not been shown.

In this study, HC04 cells were used to determine any relationship between iNOS expression and the induction of autophagy as a consequence of infection with *B. pseudomallei* PP844, a wild-type strain, and an *rpoS*-deficient mutant strain. Evidence was obtained at both the transcriptional and the translational levels, and showed an inverse correlation between expression of iNOS and the induction of autophagy in *B. pseudomallei*-infected HC04 cells.

MATERIALS AND METHODS

Cells and culture conditions

The human hepatocyte cell line HC04, which was established and characterized by Sattabongkot *et al.* (2006), was maintained in equal volumes of DMEM and Ham's F-12 media (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and incubated in a 37°C-humidified incubator in a 5% CO_2 atmosphere.

Bacterial strains

Burkholderia pseudomallei PP844 was originally isolated from a melioidosis patient at Srinagarind Hospital, Khon Kaen, Thailand (Wuthiekanun *et al.* 1996; Anuntagool *et al.* 1998). The *B. pseudomallei rpoS* mutant strain used in this study was originally constructed and verified by Subsin *et al.* (2003). *Escherichia coli* B was purchased from Coli Genetic Stock Center (CGSC, Yale University, CT, USA).

Infection

The HC04 cell line was infected at an interested multiplicity of infection (MOI) following a previously established standard methodology (Utaisincharoen *et al.* 2001). Briefly, a total of 5 $\times$ 10^5 cells were seeded into wells of a 6-well plate and incubated overnight under standard conditions, after which cells were counted before co-culture with bacteria at MOI 10 or 100 and incubation for the times specified. Mock infections (no bacteria) were undertaken in parallel.

Evaluation of intracellular survival of *Burkholderia pseudomallei*

Burkholderia pseudomallei-infected HC04 cells were pelleted by centrifugation at 130 $\times$ *g* and lysed with 0.1% TritonX-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS pH 7.4. Ten-fold of serial dilution were undertaken and the number of bacteria was determined as colony forming units at each time point post infection using the drop plate method. The colony forming unit (CFU) calculations were determined using the standard method (Herigstad, Hamilton and Heersink 2001).

Immunofluorescence analysis

Burkholderia pseudomallei-infected and mock-infected HC04 cells were incubated with 4% paraformaldehyde for 10 min and permeabilized with 0.3% TritonX-100 in 1$\times$ PBS pH 7.4. After washing with 1$\times$ PBS pH 7.4, the cells were incubated with a 1:1000 dilution of a rabbit anti-NOS2 polyclonal antibody (sc651; Santa Cruz Biotechnology, Inc. Dallas, TX, USA) or a 1:500 dilution of a rabbit anti-LC3 polyclonal antibody (2775S; Cell Signaling Technology, Danvers, MA, USA) as appropriate for 1 h. Slides were subsequently incubated with a 1:500 dilution of a goat anti-rabbit IgG, conjugated with Alexa fluor®488 (A11008; Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) and were additionally stained with DAPI (4′,6-diamidino-2-phenylindole) (D1306; Invitrogen, ThermoFisher Scientific) to detect bacteria under an Olympus FV10i confocal microscope.

RNA extraction and gene expression

Total RNA was extracted from *B. pseudomallei*-infected and mock-infected HC04 cells using TRIzol® reagent (Invitrogen, ThermoFisher Scientific) and was subsequently treated with DNaseI (Promega, Madison, WI, USA). The cDNA was synthesized using gene-specific reverse primers and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega). The sequences of iNOS primers are (forward) 5′-ATGCCAGATGGCAGCATCAGA-3′ and (reverse) 5′-ATCTCCTCCAGGATGTTGTA-3′. The sequences of β-actin primer are (forward) 5′-CTCTTCCAGCCTTCCTTCCT-3′ and (reverse) 5′-AGCACTGTGTTGGCGTACAG-3′. The total cDNA of each sample were used to quantitate the levels of gene expression using KAPA SYBR FAST qPCR Kit (Kapabiosystems Co.). The expression of iNOS and β-actin was analyzed using the qPCR_Mx3000P software (Agilent Technologies, CA, USA).

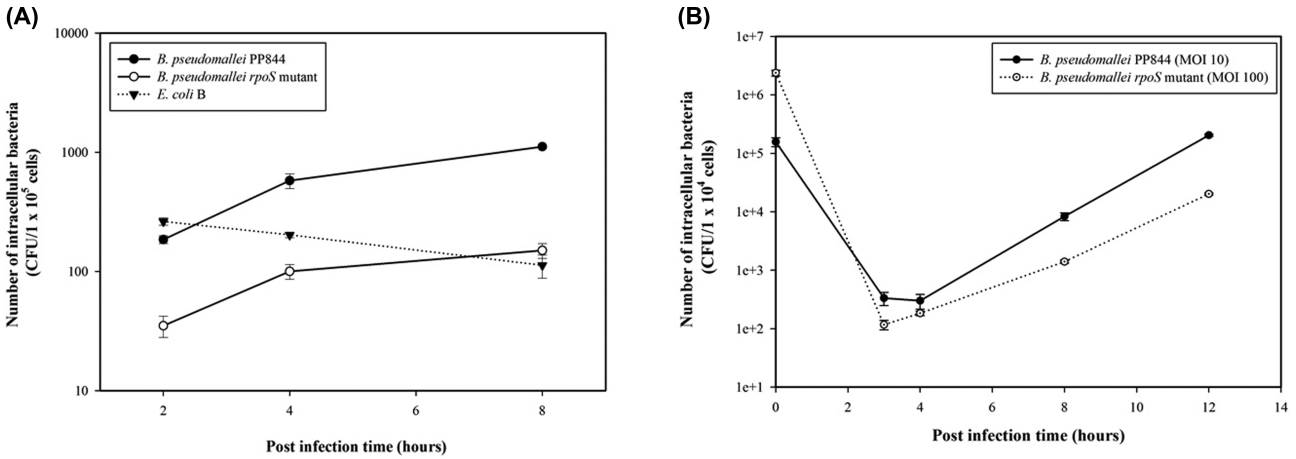

Figure 1. The survivability of *B. pseudomallei* PP844 and its *rpoS* mutant in HC04 cell. (**A**) The intracellular replication of *B. pseudomallei* PP844, *B. pseudomallei rpoS* mutant and *E. coli* B in HC04 cells starting with the same MOI 10 at 2, 4 and 8 h.p.i. The number of intracellular bacteria was determined using the drop plate technique and calculated by using a CFU method. Data are Means ± SEMs from experiments carried out in duplicate. (**B**) The intracellular replication of *B. pseudomallei* PP844 (MOI 10) and *B. pseudomallei rpoS* mutant (MOI100) in HC04 cells. The number of intracellular bacteria was determined using the drop plate technique and calculated by using a CFU method. Data are Means ± SEMs from experiments carried out in duplicate.

Table 1. The representative of data from Fig. 1B.

B. pseudomallei strains / Post infection time (hours)	PP844 (wild type) (CFU)	*rpoS* mutant (CFU)	P-value
0	156 666 ± 26 870	2366 667 ± 268 701	ND
3	333 ± 85	117 ± 21	*
4	300 ± 85	183 ± 17	*
8	8333 ± 1273	1400 ± 85	0.019
12	203 333 ± 8485	20 167 ± 778	<0.001

ND means not determined.
Asterisk (*) means there is no significant different.

Autophagic flux analysis

HC04 cells were either not treated or treated with 125 nM bafilomycin A1 for 2 h before mock infection or infection with *B. pseudomallei* at MOI 10. Cells and bacteria were co-cultured under standard conditions for the times indicated after which cells were washed with 1× PBS pH 7.4, and total proteins extracted. The expression levels of LC3-II and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) were determined by western blotting using a rabbit anti-LC3 polyclonal antibody (2775S; Cell Signaling Technology) and a mouse anti-GAPDH monoclonal antibody (611463; BD Pharmacia, San Jose, CA, USA) followed by a Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (sc2004; Santa Cruz Biotechnology, Inc.) and a HRP-conjugated goat anti-mouse IgG antibody (sc2005; Santa Cruz Biotechnology, Inc.). Signal was observed using Luminata Forte Western HRP substrate (WBLUF0100; Merck Millipore, Darmstadt, Germany). Band intensities were quantitated using the ImageJ software.

Statistical analysis

Graphs, means and standard deviations were calculated using SigmaPlot 11.0 (Informer Technologies, Inc.). Statistical analyses were determined by one-way Analysis of Variance (ANOVA) using SigmaPlot 11.0 (Informer Technologies, Inc.). A *P*-value ≤0.05 was considered statistically significant.

RESULTS

The intracellular survival of *Burkholderia pseudomallei* in HC04 cells

While it has been established that *Burkholderia pseudomallei* PP844 and its *rpoS* mutant do not show differences in growth rate when grown in Luria-Bertani (LB) medium (Subsin *et al.* 2003), we initially sought to determine whether there were differences in intracellular survival in liver cells. HC04 cells were therefore infected with either *B. pseudomallei* PP844, its *rpoS* mutant or *Escherichia coli* B and at selected times post infection the intracellular bacterial numbers determined by the drop plate method with results expressed in terms of CFUs of the intracellular bacteria (Herigstad, Hamilton and Heersink 2001). Results showed that *B. pseudomallei* PP844 produced significantly higher levels of intracellular bacteria than the *rpoS* mutant strain at all time points examined (Fig. 1A), suggesting that RpoS plays a role in the invasion and/or intracellular survival of *B. pseudomallei*.

To confidently conclude whether the *rpoS* gene influence on the intracellular survival of *B. pseudomallei* inside the HC04 cells, the increasing MOI of the *rpoS*-deficient *B. pseudomallei* was performed at MOI 100 to properly assess the ability of bacteria replication. Results showed that *B. pseudomallei rpoS* mutant still exhibited lower level of intracellular replication than its parental strain (Fig. 1B) (Table 1), which indicated that *rpoS* influence on

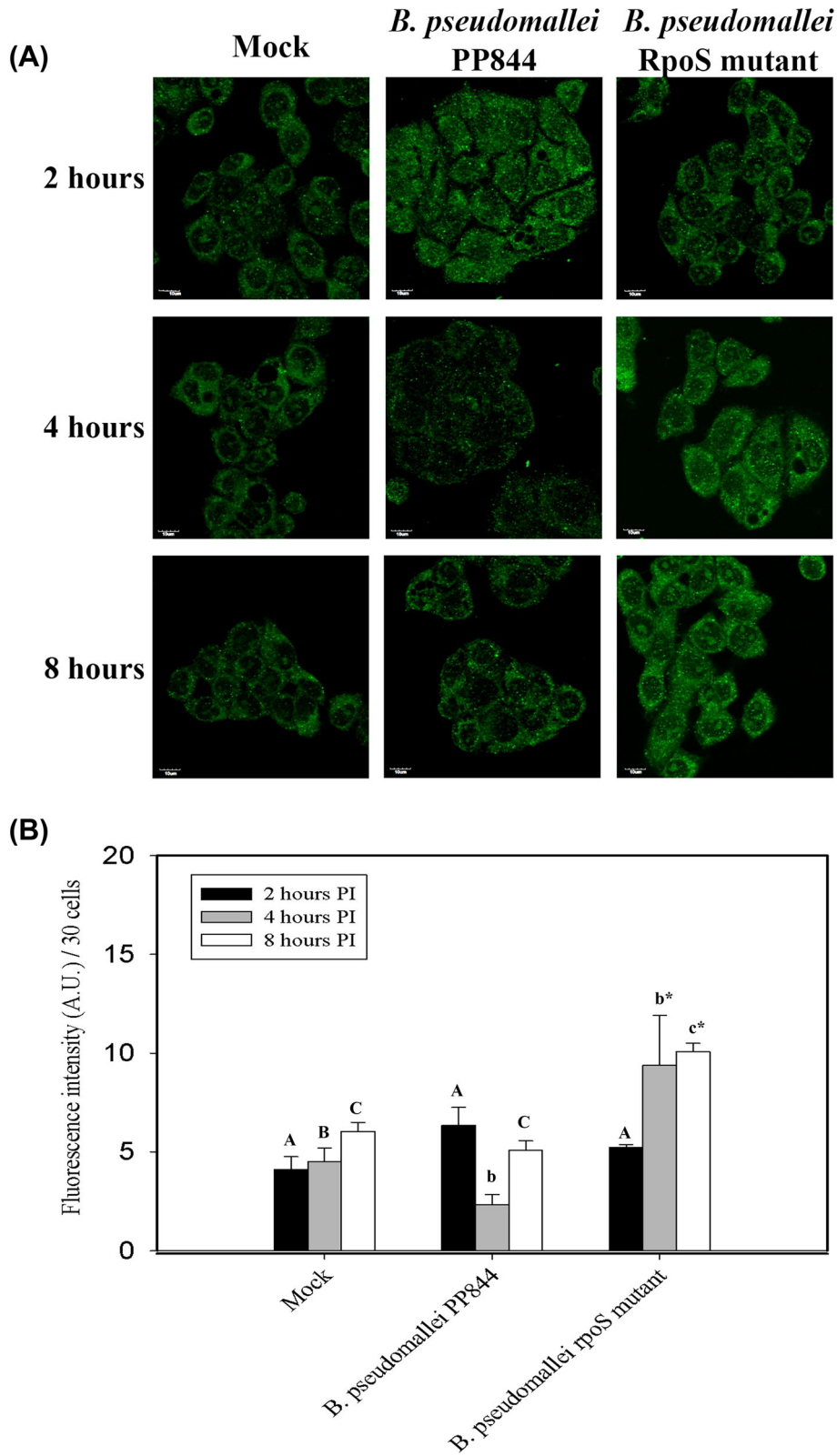

Figure 2. Expression of iNOS in response to *B. pseudomallei* infection of HC04 cells. (**A**) Representative fluorescence microscope images of human liver HC04 cells mock infected or infected with *B. pseudomallei* or its *rpoS* mutant at MOI 10. Images were captured at 2, 4 and 8 h.p.i. The scale bar in each panel is 10 μm. (**B**) Measurement of fluorescence intensity was undertaken using ImageJ software. Statistical analyses were determined using SigmaPlot 11.0 one-way ANOVA. Lower case letters indicate a significant difference from the corresponding upper case letters (*P*-value <0.05). Lower case letters with a star indicate that *rpoS* mutant was statistically different from wild type.

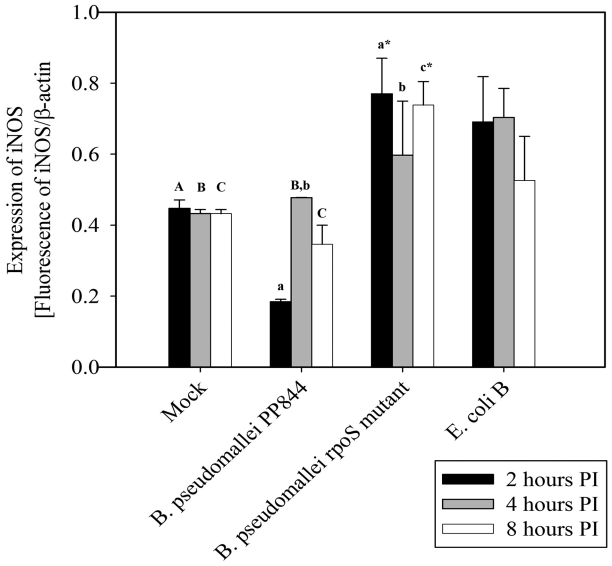

Figure 3. Quantification of iNOS expression. Human liver HC04 cells were mock infected or infected with *B. pseudomallei* or its *rpoS* mutant or *E. coli* B at MOI 10 and at 2, 4 and 8 h.p.i. RNA was extracted and expression of iNOS determined by qRT-PCR. The level of iNOS expression was normalized against the expression of β-actin. Statistical analyses were undertaken using SigmaPlot 11.0 one-way ANOVA. Lower case letters indicate a significant difference from the corresponding upper case letters (P-value <0.05). Lower case letters with a star indicate that the *rpoS* mutant was statistically different from wild type.

the intracellular survivability of *B. pseudomallei* both invasion and replication inside the cell.

Expression of iNOS in HC04 cells after infection with *Burkholderia pseudomallei*

To investigate the expression of iNOS in response to infection, HC04 cells were mock infected or infected with *B. pseudomallei* PP844 and its *rpoS* mutant under standard conditions and at appropriate time points, cells were examined under a confocal microscope after staining with appropriate antibodies. Results showed the clear induction of iNOS starting from 4 h post infection (h.p.i.) in response to *B. pseudomallei* infection of the *rpoS* mutant, but not by the parental PP844 strain (Fig. 2A and B).

To confirm this result, levels of iNOS mRNA expression were investigated using quantitative RT-PCR (qRT-PCR). HC04 cells were therefore mock infected or infected with *B. pseudomallei* PP844 and its *rpoS* mutant under standard conditions, in parallel with infection with *E. coli* B which is known to induce iNOS expression (Titheradge 1997) as a positive control. Results showed that iNOS mRNA expression was significantly higher in cells infected with the *rpoS* mutant at all time points examined compared to mock, and higher at 2 and 8 h.p.i compared to the parental PP844 strain. At 4 h.p.i, expression of iNOS in response to *rpoS* mutant infection was increased as compared to the parental strain, but this did not reach statistical significance. Levels of iNOS expression in the *rpoS* mutant infection were not statistically different from the levels of iNOS expression seen in response to infection with *E. coli* B (Fig. 3). Interestingly, a significant inhibition of iNOS expression was seen at 2 h.p.i after infection by the parental *B. pseudomallei* PP844 strain (Fig. 3). Importantly, the results were consistent with the results of the immunofluorescence assay, although increased expression of the iNOS message was seen somewhat earlier than the concomitant increase in protein expression in *B. pseudomallei rpoS* mutant infection. However, these results are consistent with previous reports that RpoS is involved in inhibition of iNOS expression in response to infection by *B. pseudomallei* (Utaisincharoen *et al.* 2006).

Autophagic flux analysis

To determine whether the level of iNOS expression might be related to the induction of autophagy, the expression of the microtubule-associated protein light-chain 3 (LC3), a key autophagy-related protein (Tanida, Ueno and Kominami 2004), was determined and used to calculate the autophagic flux. After infecting HC04 cells in the presence or absence of bafilomycin A1 to inhibit fusion between autophagosomes and lysosomes (Yamamoto *et al.* 1998), total proteins were collected at 2, 4 and 8 h.p.i and the levels of LC3-II determined by western blotting. The expression of GAPDH was additionally determined as an internal control to normalize the LC3 expression.

Results (Fig. 4) indicated that at 2 h.p.i. *B. pseudomallei* PP844 showed increased autophagic flux as compared to the *rpoS* mutant, while at 4 and 8 h.p.i. the autophagic flux in *B. pseudomallei* PP844-infected cells was slightly higher than in cells infected by the *rpoS* mutant. A comparison with the results for iNOS expression suggests that the suppression of iNOS might be associated with the induction of autophagy in the host cell.

Co-localization of LC3 and *Burkholderia pseudomallei*

The previous results showed decreased autophagic flux in the *rpoS* mutant as compared to *B. pseudomallei* PP844. To further investigate this, the expression of LC3 was examined by confocal microscopy, and cells were counterstained with DAPI to allow visualization of the nucleus and the bacterial genome. Results (Fig. 5) showed a markedly high level of expression of LC3 in *B. pseudomallei* PP844 as compared to the *rpoS* mutant, and moreover analysis of co-localization between LC3 and the bacterial genome at 4 h.p.i. showed a significantly higher co-localization between *B. pseudomallei* PP844 and LC3 than between the *rpoS* mutant and LC3 (Fig. 5). By 8 h.p.i., the co-localization between the bacterial genome and LC3 was still higher for *B. pseudomallei* PP844 than for the *rpoS* mutant, although this was no longer statistically significant.

DISCUSSION

Previous studies have implicated the liver as one of the visceral organs infected by *Burkholderia pseudomallei*, resulting in the formation of abscesses and contributing to the pathology of melioidosis (Ben *et al.* 2004; Lee *et al.* 2006; Pal *et al.* 2014). However, the interaction between *B. pseudomallei* and liver cells remains poorly characterized. To better understand the pathophysiology of liver cells under *B. pseudomallei* infection, this study used the human hepatocyte HC04 cell line which has previously been shown to be an appropriate model cell line for liver cell pathogens (Sattabongkot *et al.* 2006).

The *rpoS* gene encodes the alternative RNA polymerase sigma factor, and previous studies have implicated RpoS as a global regulatory factor induced in response to a variety of cellular stresses conditions (Lange and Hengge-Aronis 1991; Hengge-Aronis *et al.* 1993; Hengge-Aronis 2000; Osiriphun *et al.* 2009). In *B. pseudomallei*, it has been shown that lack of *rpoS* does not affect bacterial growth in LB medium (Subsin *et al.* 2003). As shown in the result, *B. pseudomallei* parental strain exhibited higher invasive ability into HC04 than the *rpoS*-deficient

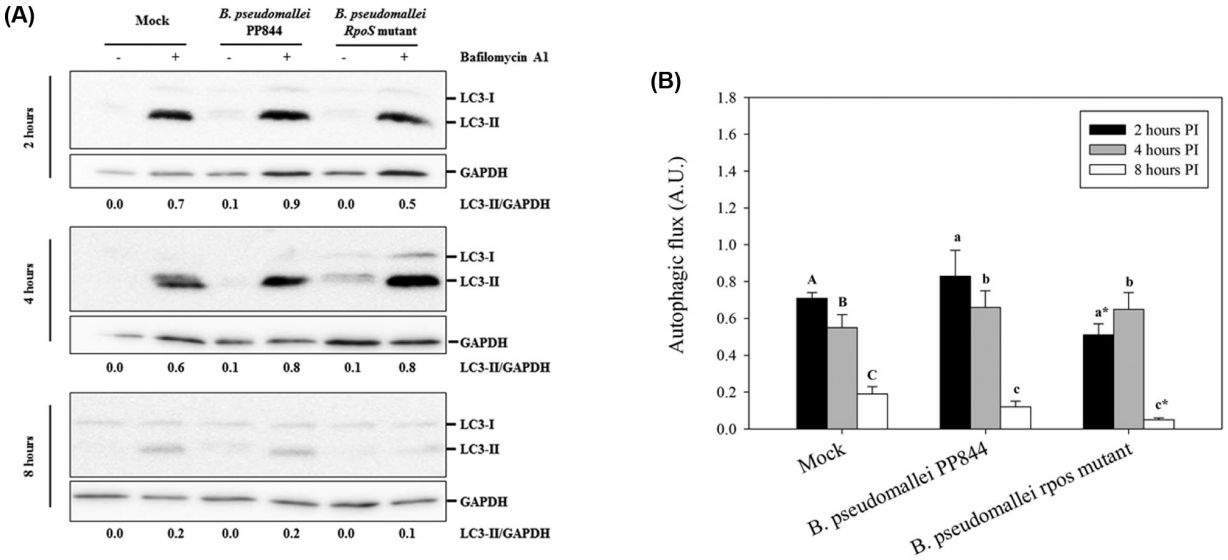

Figure 4. Assessment of autophagic flux in HC04 cells. (**A**) Human liver HC04 cells were mock infected or infected with *B. pseudomallei* or its *rpoS* mutant at MOI 10 in the presence or absence of bafilomycin A1 and at 2, 4 and 8 h.p.i. expression of LC3-I (14kDa) and II (16kDa) determined by western blotting. GAPDH were used as a loading control for each condition. The intensity of LC3-II and GAPDH were determined using ImageJ software and the numbers below the blots show the ratio between LC3-II and GAPDH. (**B**) The plot of autophagic flux in HC04 cells as determined from A. Statistical analyses were undertaken using SigmaPlot 11.0 one-way ANOVA. Lower case letters indicate a significant difference from the corresponding upper case letters (*P*-value <0.05). Lower case letters with a star indicate that the *rpoS* mutant was statistically different from wild type.

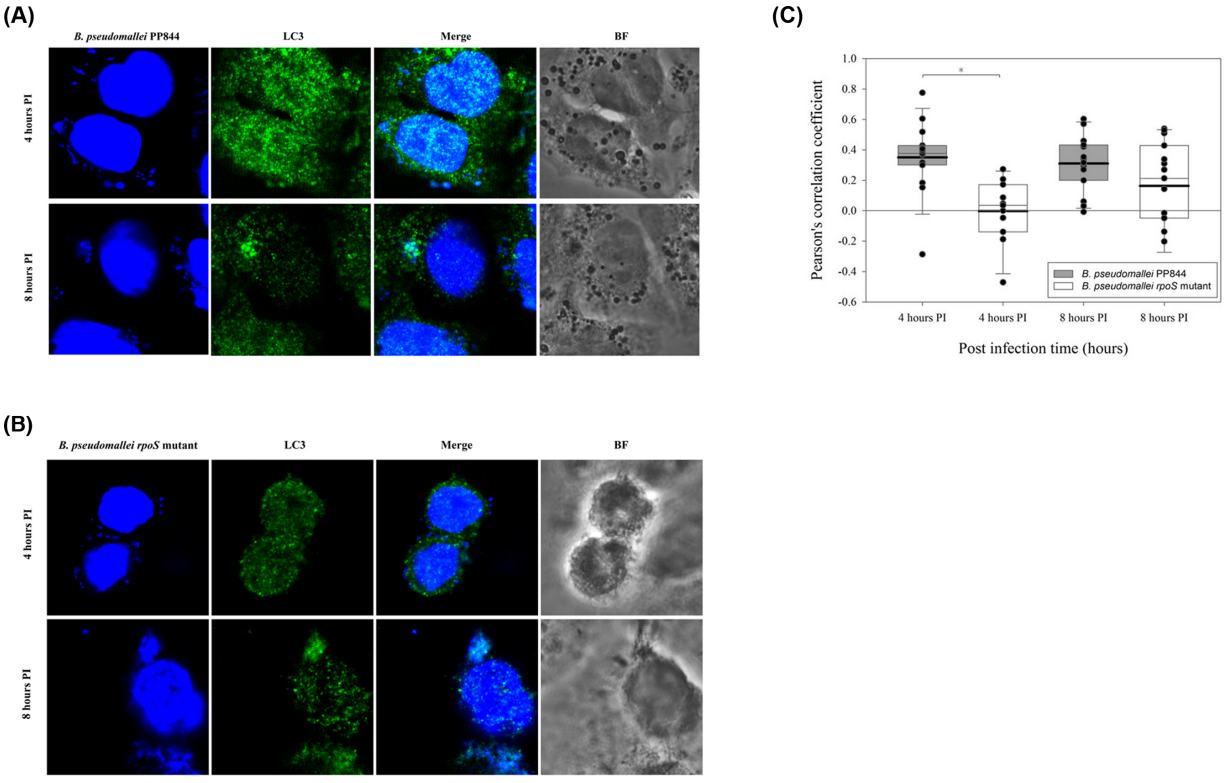

Figure 5. Co-localization of *B. pseudomallei* and LC3 in HC04 cells. Human liver HC04 cells were mock infected or infected with (**A**) *B. pseudomallei* or (**B**) its *rpoS* mutant at MOI 10 and at 4 and 8 h.p.i. examined for LC3 expression using an Alexa Fluor 488 antibody (green) and the presence of bacteria through DAPI (blue) staining and examination under a fluorescent microscope. (**C**) Pearson's correlation coefficients (PCC) for LC3 and bacteria (extra nuclei DAPI signal) were determined using the ImageJ software with the Pearson-spearman correlation (PSC) co-localization plug-in. PCC values for each condition are shown in a box chart which shows the mean (black line) and median (gray line) values. The lower and the upper borders of the box are 5th and 95th percentiles, respectively.

strain (Fig. 1A). Moreover, the results in Fig. 1B and Table 1 show that *B. pseudomallei* deficient *rpoS* gene markedly reduced numbers of bacteria in liver cells, suggesting that *rpoS* plays a role in the intracellular survival of *B. pseudomallei* PP844 (Fig. 1B and Table 1).

The exact mechanism by which the pathogen survives the intracellular environment is still unclear. However, *Escherichia coli* DpsA has been studied, and it has been suggested that DpsA acts as a regulator of the cell-cycle check point during oxidative stress to reduce cell growth, providing an opportunity for repairing oxidative DNA damage (Chodavarapu *et al.* 2008). Consistent with the role of *B. pseudomallei* DpsA in enhancing intracellular survival, a decreased level of *dpsA* expression in the *rpoS*-mutant strain under oxidative stress results in a reduced intracellular survival (Jangiam *et al.* 2010; Al-Maleki *et al.* 2014).

Host cells have a number of innate immune responses to suppress or eliminate invading pathogens, and the pathogens adapt to suppress or subvert these responses, allowing their survival and replication. Inducible nitric oxide synthase (iNOS) is one such system (Bogdan 2015) and previous studies in the mouse macrophage RAW264.7 cell line showed early suppression of iNOS by *B. pseudomallei* (Utaisincharoen *et al.* 2001), a result consistent with our present study in liver cells. Markedly however, suppression of iNOS expression was limited to *B. pseudomallei* PP844 and the *rpoS* mutant showed no inhibition of iNOS expression. Indeed, robust induction of iNOS expression was observed in the *rpoS* mutant, consistent with the levels of induction of iNOS expression seen with the control *E. coli* B infection.

Autophagy is a cellular degradation pathway that delivers sequestered cytoplasmic constituents such as damaged cell organelles to lysosomes for degradation and the subsequent recycling of cellular constituents (Klionsky and Emr 2000). However, autophagy has additionally been shown to be an essential component of the host response to a number of different pathogens (Levine 2005). Previous studies in RAW264.7 cells have shown that activation of autophagy could decrease the intracellular survival of *B. pseudomallei*, and the secreted protein BopA was suggested to be the factor mediating *B. pseudomallei* escape from autophagy (Cullinane *et al.* 2008). As shown here, there was an increase in autophagic flux in response to infection of liver cells with *B. pseudomallei* PP844, but not with the *rpoS* mutant (Fig. 4A and B).

The link between iNOS and autophagy is somewhat contradictory. In macrophages, stimulation of iNOS-derived NO by Lipopolysaccharide (LPS) leads to a triggering of autophagy via MAPK/NF-κB/iNOS signaling (Han *et al.* 2013), whereas in Hela cells it was shown that inhibition of iNOS increased autophagic flux (Sarkar *et al.* 2011). Our results suggest that the *rpoS* mutant which induces a strong induction of iNOS blocks increased autophagic flux seen with *B. pseudomallei* PP844.

Several studies have reported the co-localization of LC3 and *B. pseudomallei* containing phagosomes and it is suggested that the autophagic machinery is recruited to *B. pseudomallei* containing phagosomes through the action of the *B. pseudomallei* Bsa type III secretion system (D'Cruze *et al.* 2011; Gong *et al.* 2011; Rinchai *et al.* 2015). However, as observed here, co-localization of LC3 and *B. pseudomallei* was significantly reduced in early infection in the *rpoS* mutant as compared to *B. pseudomallei* PP844. Interestingly, the hydrophobic affinity chromatography technique which was used to investigate the interaction between LC3 and bacterial protein found that there is some bacterial protein that interacts with LC3 and it is under the RpoS regulation (Joompa, pers. comm.). This supports our finding in Figs 4 and 5. Collectively our results show an inverse relationship between iNOS expression and autophagy, and show that *rpoS* is pivotal in suppressing the host cell induction of iNOS in response to *B. pseudomallei* in liver cells.

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Conflict of interest. None declared.

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