Role of RelA and SpoT in *Burkholderia pseudomallei* survival, biofilm formation and ceftazidime tolerance during nutritional stress

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Abstract

Burkholderia pseudomallei is a saprophyte found in soil and stagnant water and is the causative agent of human melioidosis, an often fatal disease. *B. pseudomallei* is intrinsically resistant to many antibiotics. The stringent response is a global bacterial adaptation process in response to nutritional limitation and is mediated by the alarmone (p)ppGpp, which is produced by two proteins, RelA and SpoT. In order to test whether the stringent response is involved in ceftazidime tolerance, biofilm formation, and bacterial survival in the soil microcosm, *B. pseudomallei* strain K96243 and its isogenic Δ*relA* and Δ*relA*Δ*spoT* mutants were grown in rich and nutrient-limited media. In nutrient-limiting conditions, both the wild type and mutants were up to up to 64-times more tolerant to ceftazidime than when grown in rich culture conditions. Moreover, the biofilm formation of all bacterial isolates tested was significantly higher under nutrient-limiting conditions than under nutrient-rich conditions. The Δ*relA*Δ*spoT* mutant produced less biofilm than its wild type or Δ*relA* mutant under nutrient-limiting conditions. The survival of the Δ*relA*Δ*spoT* double mutant cultured in 1% moisture content soil was significantly decreased compared to the wild type and the Δ*relA* mutant. Therefore, the Rel/SpoT protein family might represent a promising target for the development of novel antimicrobial agents to combat *B. pseudomallei*.

INTRODUCTION

Burkholderia pseudomallei is classified by the Centers for Disease Control and Prevention as a category B warfare agent (Rotz *et al.*, 2002) and is the causative agent of melioidosis, a severe infectious disease that is endemic in areas of Southeast Asia and Northern Australia (Wiersinga *et al.*, 2012; Foong *et al.*, 2014; Stone *et al.*, 2014; Limmathurotsakul *et al.*, 2016). *B. pseudomallei* is an environmental saprophyte that can be isolated from soil and water in these endemic areas. High rainfall, surface water, and soil moisture appear to be common factors associated with disease distribution in the wet tropics (Palasatien *et al.*, 2008). A soil water content of 20% was reported to support *B. pseudomallei* survival for more than 1 year and *B. pseudomallei* can persist for 2 years in soil with a water content of greater than 40%, while soil with a water content of less than 10% led to the death of *B. pseudomallei* within 70 days (Tong *et al.*, 1996). However, several studies reported that *B. pseudomallei* can remain residual and viable in dry soil (Chen *et al.*, 2003; Larsen *et al.*, 2013). This may be associated with the high incidence of disease in dry tropical area. Infection is thought to be acquired either through a wound in the skin, or through the inhalation of aerosolized bacteria. Overall, the mortality rate is ranging from 14% in northern Australia to 61.5% in Cambodia (Currie *et al.*, 2010; Rammaert *et al.*, 2014; Stone *et al.*, 2014). *B. pseudomallei* exhibits resistance to diverse groups of antibiotics, including third-generation cephalosporins, penicillins, rifamycins, and aminoglycosides (Schweizer, 2012) and is relatively resistant to quinolones and macrolides. This limits the therapeutic options for the treatment of melioidosis (Wiersinga *et al.*, 2012; Stone *et al.*, 2014). In addition, *B. pseudomallei* is known to form biofilm and microcolonies (Kanthawong *et al.*, 2012; Anutrakunchai *et al.*, 2015; Mongkolrob *et al.*, 2015; Puknun *et al.*, 2016). Recently, we found that *B. pseudomallei* growing in a biofilm became tolerant to several antibiotics that planktonic bacteria were susceptible to (Sawasdidoln *et al.*,

2010; Anutrakunchai *et al.*, 2015). In addition, nutrient-limited condition could induce biofilm formation and drug tolerance of *B. pseudomallei* (Anutrakunchai *et al.*, 2015). However, the mechanisms leading to drug tolerance remain largely unknown. Therefore, the identification of tolerance mechanisms is important when aiming to devise new therapeutic strategies.

Bacteria have evolved various general response mechanisms towards extracellular stresses, and these may also promote antibiotic tolerance (Poole, 2012). Marked antibiotic tolerance can be produced by starving bacteria for nutrients in the laboratory (Eng *et al.*, 1991). Recently, the starvation-induced so-called stringent response has been shown to mediate antibiotic tolerance in nutrient-limited and in biofilm cells of *Pseudomonas aeruginosa* (Nguyen *et al.*, 2011). The molecular hallmark of this response is the synthesis of the small molecules guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) – together denoted (p)ppGpp – by Rel/SpoT homolog (RSH) and small alarmone synthetase proteins (Magnusson *et al.*, 2005; Potrykus & Cashel, 2008; Tozawa & Nomura, 2011). (p)ppGpp regulates many aspects of the microbial cell biology that are sensitive to changing nutrient availability, including growth, adaptation, secondary metabolism, survival, persistence, cell division, motility, biofilm formation, development, competence, and virulence (Potrykus & Cashel, 2008). However, the many effects of (p)ppGpp on metabolism and physiology are complex and seem to differ greatly among different organisms. Several studies report that deleting the *relA* and *spoT* genes of *Salmonella typhimurium*, but not *relA* alone, gave a complete deficiency of (p)ppGpp, resulting in strong attenuation in mice and non-invasiveness *in vitro* (Pizarro-Cerda & Tedin, 2004; Song *et al.*, 2004; Thompson *et al.*, 2006). Recently, a *B. pseudomallei* Δ*relA*Δ*spoT* mutant was found to display a defect in stationary phase survival and intracellular replication in murine macrophages. Moreover, the mutant was attenuated in the *Galleria mellonella* insect model and in both acute

and chronic mouse models of melioidosis (Muller *et al.*, 2012). In this study, we further investigated the role of RelA and SpoT in *B. pseudomallei* survival, biofilm formation and drug tolerance during nutritional stress. Since the soil water content is another important factor affecting the survival of *B. pseudomallei,* the role of RelA and SpoT in survival kinetics of *B. pseudomallei* in soils of various water content levels was also evaluated. Our results suggest that the Rel/SpoT protein family might be considered when trying to develop new strategies to combat *B. pseudomallei*.

MATERIALS AND METHODS

Bacteria and growth conditions

The wild-type *B. pseudomallei* K96243 and its isogenic Δ*relA* and Δ*relA*Δ*spoT* mutants (Muller *et al.*, 2012) were used. The media used in this study were Luria-Bertani broth (LB, CRITERION™) for nutrient-rich conditions, and modified Vogel and Bonner's medium (Lam *et al.*, 1980) containing 2 g/L glucose (0.2G MVBM) for nutrient-limiting conditions. A single colony of bacteria initially grown on nutrient agar (NA, CRITERION™) was inoculated into 2 ml of LB and 0.2G MVBM, incubated overnight at 37^{o}C in a 200 rpm shaker-incubator and used as inoculum in all experiments.

Growth rate measurement

The growth rates were determined using a computerized spectrophotometric incubator (Varioskan Flash, Thermo Fisher Scientific, USA). *B. pseudomallei* K96243 wild type, Δ*relA*, and Δ*relA*Δ*spoT* mutants grown in either LB or 0.2G MVBM from an overnight culture were adjusted to give an optical density (OD) at 540 nm of 0.1 (~1×10^{8} CFU/ml). Two percent inocula (v/v) from

each medium were inoculated into fresh media. Then, 200 µl of each bacterial suspension were added into a sterile 96-well round-bottomed plate, with 6 replicates each. Wells containing only medium served as a negative control. The microtiter plates were placed in a computerized spectrophotometric incubator and incubated at 37°C. Growth of bacterial cells was automatically monitored by the computerized instrument in terms of the change in turbidity (absorbance at 540 nm), at 60-min intervals, for a period of 72 hours. To determine the number of surviving cells, each bacterial suspension was taken at 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 22, 24 and 48 hours, serially diluted and plated in triplicate on NA plates. Colonies were counted after 24 hours of incubation at 37°C. The growth rate measurements were repeated on three separate occasions.

Drug susceptibility test

Ceftazidime (CAZ), the drug of choice for the treatment of melioidosis, was used in this study. The drug susceptibility of each bacterial strain was determined using a broth microdilution method and the interpretation of the results was conducted according to the criteria established by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2009). Two percent inocula (v/v) of *B. pseudomallei* K96243 wild type, Δ*relA*, and Δ*relA*Δ*spoT* mutant grown either in LB or 0.2G MVBM overnight were inoculated into fresh medium and incubated at 37°C in a 200 rpm shaker-incubator to yield stationary phase bacteria. The bacterial cells were further diluted to provide a final inoculum density of ~1×10^6 CFU/ml in each medium. The CAZ was 2-fold serially diluted in LB and 0.2G MVBM with a final volume of 50 µl in each well of the 96-well microtiter plates. Then the final inoculum (50 µl) was added to each well of a 96-well plate. The final concentrations of CAZ were ranging from 0.5 to 2048 µg/ml. Wells containing only media and culture-free CAZ were included as negative controls. The

plates were incubated at 37°C for 24 hours. Then, the bacterial growth was examined and the lowest concentration of CAZ which inhibited visible growth of the bacteria was recorded as the minimum inhibitory concentration (MIC). Aliquots of the mixture of CAZ and bacteria which showed negative visible growth after the first 24 hours of incubation were inoculated onto the surface of NA plates. The lowest concentration of CAZ giving negative growth of the bacteria was recorded as the minimum bactericidal concentration (MBC). All experiments were repeated on three separate occasions, with triplicate determinations on each occasion.

Quantification of biofilm formation

A modified microtiter plate test was used to determine the 2-day biofilm-forming capacities of all isolates as previously described (Taweechaisupapong *et al.*, 2005). Briefly, a single bacterial colony was inoculated into 2 ml LB and 0.2G MVBM and incubated overnight at 37°C in a 200 rpm shaker-incubator. Then, 2% inocula (v/v) were prepared in 10 ml of each fresh medium and incubated overnight at 37°C in a 200 rpm shaker-incubator. After incubation, bacteria were adjusted to OD at 540 nm of 0.8-0.9 in fresh medium. Each bacterial suspension (200 µl) in two different media were added into 96-well flat-bottomed plates, with 8 replicates each. Wells containing only the medium served as negative control. The plates were incubated aerobically at 37°C for 3 hours to allow adhesion. Thereafter, the supernatant fluid of each well was gently aspirated to remove non-adherent bacteria, and replaced with 200 µl of each fresh medium. After incubation at 37°C for an additional 21 hours, the non-adherent bacteria were again removed and the wells containing adherent bacteria were washed with 200 µl of sterilized deionized water and fresh media were added once more. After incubation for an additional 24 hours, the supernatants were again removed and the wells were finally washed three more times with 200 µl of sterilized

deionized water. The attached bacteria, representing a 2-day biofilm culture, were fixed with 200 µl of 99% methanol for 15 minutes and the plates were dried at room temperature. The plates were stained with 200 µl of 2% Hucker crystal violet for 5 minutes. Excess stain was removed with running tap water. The plates were air dried and the dye bound to the adherent cells was solubilized with 200 µl of 33% (v/v) glacial acetic acid/well. The OD of each well was measured at 620 nm using a microplate reader. The ability of each isolate to produce biofilm was determined in four independent experiments and the results reported are the average from these four independent experiments. To compare the relative capacity of different isolates to produce biofilm, their OD values were adjusted against that produced by a strain of *B. thailandensis* (UE5) which had been randomly selected and used as reference in all experiments as previously reported (Taweechaisupapong *et al.*, 2005). The corrected OD_{620} was calculated by the following formula: Corrected OD_{620} = OD of tested strain x (mean OD of UE5 on all plates / OD of UE5 on test plate)

Microcosms preparation and determination of *B. pseudomallei* survival

Sandy loam soil obtained from a *B. pseudomallei*-positive site (site 39, Ban Kai Na) in the Nam Phong District, Khon Kaen Province, Thailand (average moisture content 5.86%) was used throughout this study (Kamjumphol *et al.*, 2015). The soil samples were sieved through a 2 mm sieve and dried at ambient temperature for 24 hours. Soil microcosms were prepared in flasks (250 ml) using 100 grams of carefully mixed soil taken from the initial stock and autoclaved at 121°C for 15 minutes. Following sterilization, the soil was adjusted to a moisture content (MC) of 1% (~1 ml/100 grams soil) and 5% (~5 ml/100 grams soil) with sterile distilled water and mixed well. The final moisture content was determined using moisture analyser (HB43-S Halogen, Mettler Toledo, USA). Sterile soil, as checked by plate counting, was inoculated with 10^8 CFU/ml of *B. pseudomallei* K96243 wild type, Δ*relA*, or Δ*relA*Δ*spoT* mutants by applying a drop of 200 µl of

each bacterial suspension over the soil in each flasks in 5 different locations (one drop per quarter-section of the soil surface, and one drop around the centre area) and incubated statically at 30°C. At time 0, 1, 2, 3, 4, 5, 10, 15 and 20 weeks, soil samples were taken from individual microcosm and 200 ml of polyethylene glycol (PEG) - sodium deoxycholate (DOC) solution which contained 2.5% (w/v) PEG and 0.1% (w/v) DOC (Trung *et al.*, 2011) was added to 100 g of soil. The suspension was shaken (200 rpm) for 2 hours at 30°C and then allowed to settle for 5 minutes. Then the supernatant was taken, serially diluted, plated in triplicate on Ashdown's agar and incubated for 48 hours to allow colony counting. Each assay was performed on two separate occasions.

Statistical analysis

The differences in biofilm formation and bacterial survival in soil microcosm among the tested groups were compared using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). The level required for statistical significance was $P < 0.05$.

RESULTS

Growth rate under nutrient-rich and nutrient-limiting conditions

The growth curves of *B. pseudomallei* K96243 wild type and the isogenic Δ*relA* and Δ*relA*Δ*spoT* mutants grown in either LB or 0.2G MVBM are shown in Fig. 1. The measurement of turbidity revealed that the wild type and the Δ*relA* mutant had similar growth rates in the two different media, while the Δ*relA*Δ*spoT* mutant had a longer lag phase (Fig. 1A). In nutrient-limiting media (0.2G MVBM), *B. pseudomallei* K96243 wild type and the Δ*relA* mutant both grew exponentially and growth abruptly stopped at 14-16 hours, but afterwards, growth resumed slowly

and the cultures entered stationary phase. In contrast, the *B. pseudomallei* Δ*relA*Δ*spoT* double mutant grow exponentially and then growth gradually slowed down as cultures entered stationary phase. Higher OD levels with the double mutant were apparent after 22 hours when compared to the wild type and the Δ*relA* single mutant. However, the number of surviving *B. pseudomallei* Δ*relA*Δ*spoT* mutant cells in nutrient-limiting media determined by colony counting assays was lower than in the wild type and Δ*relA* mutant (Fig. 1B).

Drug susceptibility and biofilm formation

The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ceftazidime (CAZ) against the panel of strains grown in different media are shown in Table 1. The MBC values of CAZ against all tested *B. pseudomallei* strains in LB medium were lower than those in 0.2G MVBM. Interestingly, the wild type K96243 was less susceptible to CAZ in 0.2G MVBM than its isogenic Δ*relA* and Δ*relA*Δ*spoT* mutants, whereas the MBC values in LB against all tested bacteria were not much different. Moreover, the *B. pseudomallei* Δ*relA* single mutant appeared to be less susceptible to CAZ than the Δ*relA*Δ*spoT* double mutant in 0.2G MVBM medium, but more susceptible in LB.

The results of the quantitative microtiter-plate test showed that all tested strains of *B. pseudomallei* in LB produced statistically lower levels of biofilm (fold-change levels between 9- and 32-times; $P < 0.001$ in all cases) than those in 0.2G MVBM (Fig. 2). In addition, *B. pseudomallei* K96243 wild type produced statistically significant 1.5-fold higher levels of biofilm ($P < 0.01$) than the *ΔrelAΔspoT* double mutant in 0.2G MVBM medium. Although *B. pseudomallei* K96243 also produced 1.2-fold higher levels of biofilm than the *ΔrelA* mutant in nutrient-limiting conditions, the difference was not statistically significant.

Effect of soil moisture content on *B. pseudomallei* survival

B. pseudomallei K96243 wild type and isogenic Δ*relA* and Δ*relA*Δ*spoT* mutants were compared in terms of their ability to survive in soil with 1% and 5% moisture content over 20 weeks (Fig. 3). The survival of *B. pseudomallei* wild type and mutants in soil with 1% MC was significantly lower than that in 5% MC soil ($P < 0.01$). In addition, the survival of the *B. pseudomallei* Δ*relA*Δ*spoT* double mutant cultured in 1% MC soil was significantly lower ($P < 0.01$) than that of the wild type and Δ*relA* mutant from one week of incubation onwards.

DISCUSSION

The results from this study indicate that the RelA and SpoT proteins are important for survival and persistence of *B. pseudomallei* in harsh environments such as nutrient-limitation and desiccation. RelA/SpoT Homologue (RSH) proteins represent a superfamily of enzymes that synthesize and/or hydrolyze the nucleotide alarmone ppGpp, which in turn activates the stringent response (Magnusson *et al.*, 2005; Potrykus & Cashel, 2008; Tozawa & Nomura, 2011). This response is a conserved regulatory system for mediating the growth response to various environmental conditions. In the present study, we compared the growth kinetics and survival of wild-type *B. pseudomallei* strain K96243 and its isogenic Δ*relA*, and Δ*relA*Δ*spoT* mutants in nutrient-rich and nutrient limiting conditions. As shown in Fig. 1A, the Δ*relA*Δ*spoT* double mutant exhibited a longer lag phase in both conditions. Our results are consistent with previous studies of *E. coli* Δ*relA*Δ*spoT* double mutant ($ppGpp^0$) strains, which showed a prolong lag phase in isoleucine starved cultures (Traxler *et al.*, 2008). The lag phase represents the earliest period in the life of a bacterial population before the start of exponential growth. It is the most poorly understood

stage of the bacterial growth cycle. However, it has been assumed that the lag phase allows the adaptation required for bacterial cells to begin to exploit new environmental conditions. Therefore, the extended lag phase found in the *B. pseudomallei* Δ*relA*Δ*spoT* double mutant may be due to a defect in the regulatory system for mediating this growth response.

The stringent response molecule, ppGpp, triggered by nutritional deprivation, also causes an arrest of cell cycle in bacteria (Ferullo & Lovett, 2008). It should be noted that under nutrient-limiting conditions, growth arrest was observed in the wild-type *B. pseudomallei* K96243 and the Δ*relA* single mutant when turbidity was measured, while the Δ*relA*Δ*spoT* double mutant was noticeably impaired for arrest and a higher OD level was apparent after 22 hours when compared to the wild type and the Δ*relA* mutant (Fig. 1A). The higher OD values of the Δ*relA*Δ*spoT* double mutant after 22 hours observed in this study may be due to the defect in the stringent response, which leads to a prolonged period of growth arrest. This is in agreement with the suggestion that ppGpp mutants in general are unable to modulate their growth rates and prepare for stationary phase (Magnusson *et al.*, 2005). Our results also indicate that SpoT contributes to ppGpp synthesis in the Δ*relA* single mutant, thereby enabling a similar growth pattern as observed in the wild type.

In this study, although the *B. pseudomallei* Δ*relA*Δ*spoT* mutant had a longer lag phase in rich media, the number of viable cells in both the lag and the exponential phase was not different from that of the wild type and Δ*relA* mutant, suggesting that ppGpp-synthesizing enzymes may not play a role during the lag and exponential growth phases in nutrient-rich conditions. In contrast, the Δ*relA*Δ*spoT* double mutant showed a rapid loss in viable, colony-forming units (CFU) in nutrient limiting conditions (Fig. 1B), indicating that both growth as well as survival mechanisms were affected. Our results are consistent with previous studies in *B. pseudomallei* and other bacteria, which showed that lack of ppGpp-synthesizing enzymes leads to a defect in survival

(Primm *et al.*, 2000; Gaynor *et al.*, 2005; Mouery *et al.*, 2006; Muller *et al.*, 2012). In addition, several studies reported that bacteria alter their morphology from rod-like to coccoid or reduce their cell size upon starvation (Baker *et al.*, 1983; Kjelleberg *et al.*, 1993; Chen *et al.*, 2009), which represents one of the starvation survival patterns of bacteria. However, our previous study demonstrated that the morphology of Δ*relA*Δ*spoT* double mutant cells in stationary phase in LB cultures appeared slightly bigger on average or had formed filaments of various lengths, which differed from that of wild-type cells (Muller *et al.*, 2012). These results indicate that the Δ*relA*Δ*spoT* double mutant is unable to decrease its cell size in response to nutrient deprivation upon entry into stationary phase. The filamentous morphology of *B. pseudomallei* the Δ*relA*Δ*spoT* double mutant demonstrated previously is consistent with several studies using *E. coli* Δ*relA*Δ*spoT* double mutants (Xiao et al., 1991; Magnusson et al., 2007; Traxler et al., 2008), and might contribute to the reduced survival observed in nutrient-limiting conditions of this study (Fig. 1B).

Biofilm formation is an important strategy for bacterial survival in hostile environments or *in vivo* during infections. Several studies indicate that the stringent response promotes biofilm formation in many bacteria (Balzer & McLean, 2002; He *et al.*, 2012; Sugisaki *et al.*, 2013). In this study, the role of the stringent response in *B. pseudomallei* biofilm formation was similar to those reported in other bacteria. The *B. pseudomallei* Δ*relA*Δ*spoT* mutant had a significantly reduced ability to form biofilms under nutrient-limiting conditions compared to the wild type ($P < 0.01$), although the mutant was not completely deficient in biofilm formation. Moreover, a lower level of antibiotic tolerance was observed with both *B. pseudomallei* mutants under nutrient-limiting conditions compared to the wild type, which is in accordance with previous report in *Pseudomonas aeruginosa* (Nguyen *et al.*, 2011). Nguyen *et al.* showed that nutrient-limited planktonic cells and biofilm cells of *P.aeruginosa* that are defective in the *relA* and *spoT* genes

were markedly less antimicrobial-tolerant than their wild-type counterparts. Their study indicated that the increased antimicrobial susceptibility of a Δ*relA*Δ*spoT* mutant in *P. aeruginosa* is related to increased oxidative stress. The increase in hydroxyl radical formation and oxidative stress in Δ*relA*Δ*spoT* cells was linked to increased production of pro-oxidant 4-hydroxy-2-alkylquinoline molecules and reduced levels of catalase and superoxide dismutase activity (Nguyen *et al.*, 2011). However, quantification of biofilm formation of wild type and Δ*relA*Δ*spoT* mutant was not conducted in the study of Nguyen *et al*.. In the present study, lower level of biofilm formation observed in *B. pseudomallei* mutants under nutrient-limiting conditions was related to decreased antimicrobial tolerance compared to the wild type. These results highlight the importance of biofilm recalcitrance toward antibiotics.

B. pseudomallei is a difficult organism to kill. For example, the bacteria can survive in triple distilled water for years (Wuthiekanun *et al.*, 1995; Pumpuang *et al.*, 2011). The unusual ability of *B. pseudomallei* to survive from months to years in distilled water, as well as the unusually long environmental persistence in soil, makes treatment and remediation of environments contaminated with *B. pseudomallei* extremely challenging. Several studies reported that *B. pseudomallei* can remain residual and viable in dry soil (Chen *et al.*, 2003; Larsen *et al.*, 2013). However, some studies showed that *B. pseudomallei* strains isolated from soil and water in China and Taiwan survived in desiccated soil only up to 30 days, and some clinical strains did not survive for longer than ca. 25 days at a soil MC of 5% (Tong *et al.*, 1996; Chen *et al.*, 2003). Here, the role of RelA and SpoT in survival kinetics of *B. pseudomallei* in soils with MC of 1% and 5% was evaluated. The results revealed that *B. pseudomallei* exposed in this study remained viable in 1% and 5% MC soil for at least 20 weeks, although the survival of the *ΔrelA spoT* double mutant cultured in 1% MC soil was significantly less than that of the wild type and the *ΔrelA* mutant after

the first week of incubation (Fig. 3). Our results are similar to those reported by Larsen *et al*. (Larsen *et al.*, 2013). However, this is in contrast to results for *B. pseudomallei* strains studied in China and Taiwan (Tong *et al.*, 1996; Chen *et al.*, 2003). Tong *et al*. (Tong *et al.*, 1996) reported that soil with a water content of less than 10% led to the death of *B. pseudomallei* within 70 days, while soil with a water content of more than 40% maintained bacterial survival for 726 days. Chen *et al*. (Chen *et al.*, 2003) concluded that *B. pseudomallei* could survive extended periods in soil only at a minimum 15% water content. This apparent discrepancy may be the result of methodological differences as well as differences in the strain background of the bacteria.

Taken as a whole, this study provides new insights into the role of the stringent response of *B. pseudomallei* in survival, biofilm formation and drug tolerance during nutritional stress, as well as the survival kinetics in soils of various moisture contents. Recently, Relacin, a novel ppGpp analogue, was shown to inhibit RelA and Rel/Spo proteins (Wexselblatt et al., 2012). Relacin affects entry into stationary phase in Gram positive bacteria and leads to cell death, inhibits sporulation and biofilm formation, placing it as an attractive new antibacterial agent. The findings of this study supported the role of Rel/SpoT in *B. pseudomallei* survival strategies and suggest that the Rel/SpoT may represent a promising target for the development of agents to combat *B. pseudomallei*.

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Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ceftazidime against *B. pseudomallei* K96243 wild type and Δ*relA*, and Δ*relA*Δ*spoT* mutants in nutrient-rich (LB) and nutrient-limited conditions (0.2G MVBM).

Isolate	LB		0.2G MVBM	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
K96243	4	8	2	512
Δ*relA*	4	4	2	256
Δ*relA*Δ*spoT*	4	8	2	128

LB : Luria-Bertani broth , MVBM : modified Vogel and Bonner's medium,

0.2G MVBM : modified Vogel and Bonner's medium containing 2g/L glucose.

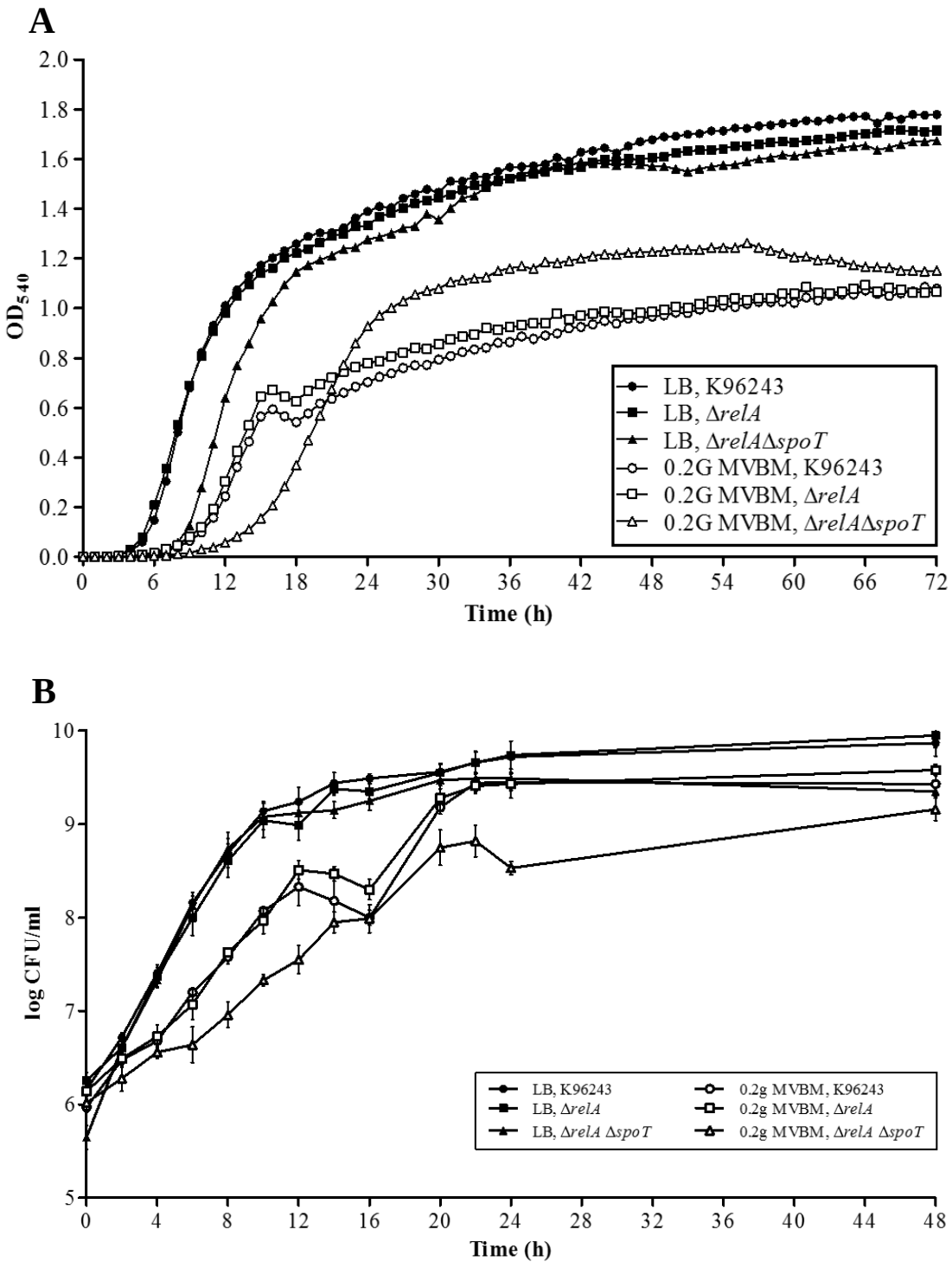
A
OD540
Time (h)
LB, K96243
LB, ΔrelA
LB, ΔrelAΔspoT
0.2G MVBM, K96243
0.2G MVBM, ΔrelA
0.2G MVBM, ΔrelAΔspoT
B
log CFU/ml
Time (h)
LB, K96243
LB, ΔrelA
LB, ΔrelA ΔspoT
0.2g MVBM, K96243
0.2g MVBM, ΔrelA
0.2g MVBM, ΔrelA ΔspoT

Figure 1

Figure 1. (A) Growth curves of *B. pseudomallei* K96243 wild type and isogenic Δ*relA* and Δ*relA*Δ*spoT* mutants in Luria-Bertani broth (LB; nutrient-rich) and modified Vogel and Bonner's medium containing 2g/L glucose (0.2G MVBM; nutrient-limited) monitored by a computerized spectrophotometric incubator in terms of changes in turbidity at the indicated time points. Data are the mean value of three independent experiments carried out in six replicates. (B) Survival of the strains determined by colony culturing assay at the indicated time points. Data are the mean value of three independent experiments carried out in triplicate.

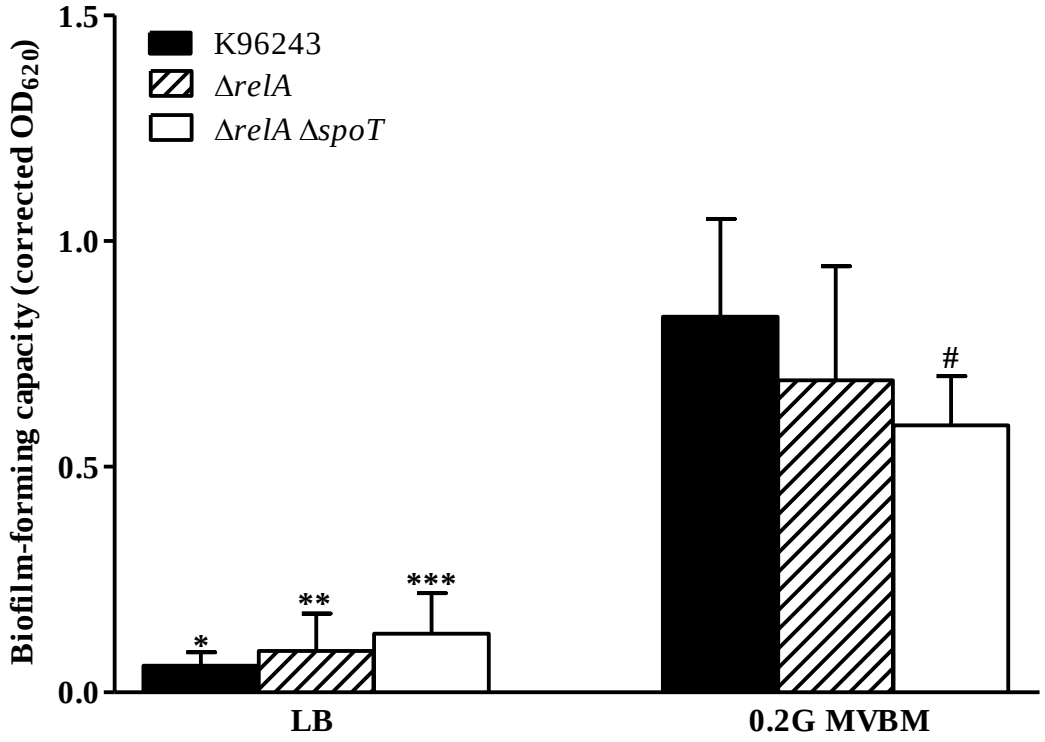

Figure 2. Biofilm-forming capacity of *B. pseudomallei* K96243 wild type and isogenic Δ*relA* and Δ*relA*Δ*spoT* mutants in Luria-Bertani broth (LB; nutrient-rich) and modified Vogel and Bonner's medium containing 2g/L glucose (0.2G MVBM; nutrient-limited) relative to a reference strain for standardisation. Data are the mean value of four independent experiments carried out in eight replicates.

$^{*}P < 0.001$ compared to *B. pseudomallei* K96243 wild type cultured in 0.2G MVBM

$^{**}P < 0.001$ compared to the *B. pseudomallei* Δ*relA* mutant cultured in 0.2G MVBM

$^{***}P < 0.001$ compared to the *B. pseudomallei* Δ*relA*Δ*spoT* mutant cultured in 0.2G MVBM

$^{\#}P < 0.01$ compared to *B. pseudomallei* K96243 wild type cultured in 0.2G MVBM

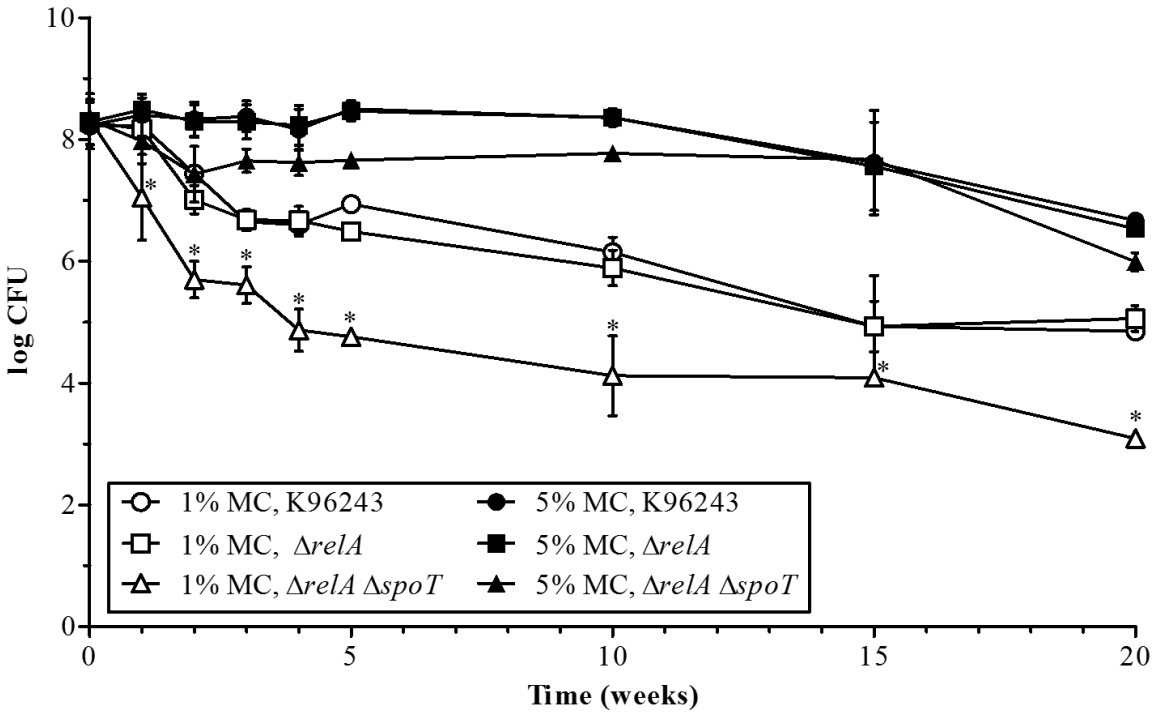

Figure 3. Survival of *B. pseudomallei* K96243 wild type and isogenic Δ*relA* and Δ*relA*Δ*spoT* mutants in 1% and 5% moisture content (MC) soil over 20 weeks. Data are the mean value of two independent experiments.

*$P < 0.01$ compared to *B. pseudomallei* K96243 wild type in 1 % MC