The role of RelA and SpoT in *Burkholderia pseudomallei* virulence and immunity

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Abbreviations: i.n. = intranasal; i.p. = intraperitoneal

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ABSTRACT

Burkholderia pseudomallei is a gram-negative soil bacterium and the causative agent of melioidosis, a disease of humans and animals. It is also listed as a category B bioterrorism threat agent by the US Center for Disease Control and Prevention, and there is currently no melioidosis vaccine available. Small modified nucleotides such as the hyper-phosphorylated guanosine molecules ppGpp and pppGpp play an important role as signaling molecules in prokaryotes. They mediate a global stress response during starvation conditions and have been implicated in the regulation of virulence and survival factors in many bacterial species. In this study, we have created a *relA spoT* double mutant in *B. pseudomallei* strain K96243, which lacks (p)ppGpp-synthesizing enzymes, and investigated its phenotype *in vitro* and *in vivo*. The *B. pseudomallei* ΔrelAΔspoT mutant displayed 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. Vaccination of mice with the ΔrelAΔspoT mutant resulted in partial protection against infection with wild type *B. pseudomallei*. In summary, (p)ppGpp signaling appears to represent an essential component of the regulatory network governing virulence gene expression and stress adaptation in *B. pseudomallei* and the ΔrelAΔspoT mutant may be a promising live-attenuated vaccine candidate.

INTRODUCTION

Burkholderia pseudomallei, a Gram-negative, motile, aerobic bacterium of the class β-proteobacteria, is widely distributed throughout the biosphere and is the causative agent of the life threatening disease melioidosis (7). Infection with *B. pseudomallei* presents with a variety of nonspecific symptoms and can range from acute disease, which is rapidly fatal, to chronic or latent (i.e. asymptomatic) infections that last for several decades (9). Despite antibiotic treatment, mortality rates for acute melioidosis can be 40- 50% in endemic areas, and relapse rates in surviving individuals are also high [for a review see Wiersinga *et al.* (48)]. Currently, there is no vaccine available and therefore, *B. pseudomallei* is listed as a category B select agent by the U.S. Center for Disease Control and Prevention.

Guanosine tetra- and pentaphosphates, collectively referred to as ppGpp, are small signaling molecules that are produced in response to various stress conditions such as amino acid starvation [for reviews see Cashel *et al.* (6), Magnusson *et al.* (27), Potrykus & Cashel (39), and Dalebroux *et al.* (12)]. ppGpp signaling is widespread across various genera of the bacterial kingdom, but can also be found in plant chloroplasts (4). The ppGpp response has been extensively studied in *Escherichia coli*. In this organism, stress-induced ppGpp interacts with the RNA polymerase core enzyme and elicits a down-regulation of ribosomal proteins and DNA replication and an up-regulation of amino acid synthesis operons and genes involved in adaptation to stasis. This so-called "stringent response" mechanism causes the bacterial cell to stall growth and to redirect its resources to promote survival mechanisms until nutrient conditions improve.

In many γ-proteobacteria, ppGpp is synthesized from GTP and ATP by two enzymes, RelA and SpoT (see Fig. 1B). The latter is a bifunctional enzyme that has both synthetase and hydrolase

activities. Deletion of the *spoT* gene is lethal due to toxic accumulation of ppGpp in the cell. On the other hand, deletion of both the *relA* and the *spoT* gene has a dramatic impact on gene expression profiles: microarray analyses of strains lacking ppGpp-synthesizing enzymes have revealed de-regulation of several hundreds of genes (18, 34, 46), thereby highlighting the role of ppGpp as a global signaling molecule.

The diverse phenotypes of ppGpp-deficient strains indicate that this molecule affects various cellular processes, many of them involved in survival and adaptation to stress conditions, but also other cellular processes such as sporulation, competence, quorum sensing and virulence [reviewed by Magnusson *et al.* (27), Braeken *et al.* (5), and Dalebroux *et al.* (12)]. For example, in pathogenic bacteria such as *Francisella tularensis*, *Brucella* sp., *Campylobacter jejuni, Legionella pneumophila*, *Helicobacter pylori*, and *Mycobacterium tuberculosis*, absence of ppGpp-synthesizing enzymes results in defects in stationary phase survival, defects in intracellular replication and attenuation in animal models of disease (10, 11, 16, 17, 21, 24, 41, 49).

In addition to their greatly reduced virulence, strains defective in ppGpp synthesis have been shown to confer protective immunity against challenge with wild type bacteria. This is the case in *Salmonella enterica* serovar Typhimurium (33), *Salmonella enterica* serovar Gallinarum (37), *Francisella tularensis* (16) and *Yersinia pestis* (44). In *Y. pestis*, protection was conferred via a mixture of Th1 and Th2 immune responses, as measured by IgG1 and IgG2a levels (44). In *S.* Typhimurium, the ppGpp mutant elicited serum and mucosal antibody responses, measured by IgA and IgG titers, as well as cellular immune responses as measured by a Delayed Type Hypersensitivity (DTH) response (33). Similarly, in *S. enterica*, vaccination induced a B-cell response in blood measured by IgM, IgA and IgG levels, and induced CD4+ and CD8+ T-cell proliferation and IFN-γ and TGF-β4 cytokine production in spleens (37). This may render

ppGpp-deficient *Salmonella* strains a promising live-attenuated vaccine against typhoid in chicken.

Here, we have evaluated a Δ*relA* Δ*spoT* double mutant in *B. pseudomallei* strain K96243 as a vaccine candidate. Live-attenuated vaccines offer the advantage of containing complex epitopes, thus stimulating both the antibody and cell-mediated arms of the immune system. This is of particular importance with facultative intracellular pathogens such as *B. pseudomallei*. Moreover, live-attenuated vaccines usually confer strong and longer lasting immunity. Several live-attenuated vaccine strains of *B. pseudomallei* have been tested to date [reviewed by Sarkar-Tyson & Titball (42) and Patel *et al.* (38)]. Most of them represent mutants with defects in the intracellular life stages such as mutants in biosynthetic pathways or type III secretion systems. For example, the most successful *B. pseudomallei* vaccine strain characterized to date, strain 2D2, carries a mutation in the *ilvI* gene, which renders the strain auxotrophic for the synthesis of branched chain amino acids (3). However, none of the live-attenuated vaccine candidates tested to date are able to confer sterilizing immunity or are able to protect against chronic disease (38). This demonstrates the need to assess whether different classes of mutants exhibit improved protective efficacy.

Here, we have targeted an important signaling system, namely synthesis of the alarmone ppGpp, for mutagenesis in *B. pseudomallei*. We found that the Δ*relA*Δ*spoT* derivative was severely attenuated in both acute and chronic models of disease and subsequently, we tested the ability of the mutant to confer protective immunity. Our results from protection studies in mice indicated that this mutant might be a medically relevant addition to the list of live-attenuated vaccines against melioidosis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains and plasmids used in this study are listed in Table 1. Unless stated otherwise, bacteria were grown with aeration in Luria broth (LB) at 37°C. When required, antibiotics chloramphenicol (Sigma-Aldrich, Gillingham, UK), kanamycin (Sigma-Aldrich) and gentamicin (Sigma-Aldrich) were used at concentrations of 25 µg/ml, 50 µg/ml, and 50 µg/ml, respectively.

Mutant construction. In-frame deletion mutants were constructed using a suicide plasmid containing regions homologous to up- and downstream regions of the target genes (26). Amplified DNA fragments used for constructing the suicide plasmid derivatives were generated by recombinant PCR. Briefly, 600 bp up- and downstream fragments of the *relA* (BPSL1946) and *spoT* (BPSL2561) genes of *B. pseudomallei* strain K96243 were PCR amplified using primer combinations relA-1&relA-2 (upstream), relA-3&relA-4 (downstream), spoT-1&spoT-2 (upstream), and spoT-3&spoT-4 (downstream), respectively, and K96243 genomic DNA as a template. A second, recombinant PCR was performed using the outside primers (relA-1&relA-4 and spoT-1&spoT-4) and the respective up- and downstream PCR fragments from the first PCR as a template. The resulting 1.2 kb recombinant PCR products designated Δ*relA* and Δ*spoT* were cloned into the suicide vector pDM4 via its *Xho*I/*Sph*I and *Sal*I/*Xma*I-sites, respectively. The presence of the recombinant PCR fragments in the resulting plasmids pDM4-Δ*relA* and pDM4-Δ*spoT* was confirmed by PCR using primer combinations relA-up&relA-down and spoT-up&spoT-down, respectively. All plasmids were maintained in *E. coli* DH5 λ *pir* cells. The sequences of all primers are listed in Table 2.

Biparental mating. The pDM4-derivatives were introduced into *E. coli* strain S17 λ *pir* by electroporation. The resulting donor strains and the *B. pseudomallei* recipient strains were grown over-night in LB medium at 37°C with aeration. 1 ml of the cultures was pelleted by centrifugation and the cells were resuspended in 0.5 ml fresh LB medium. 10 µl of these suspensions were transferred onto 1 cm x 1 cm sized squares of nitrocellulose membrane (Hybond-ECL; GE Healthcare, Little Chalfont, UK) placed on brain heart infusion (BHI; Fluka_Sigma-Aldrich) agar plates, either singularly for the control plates or donor and recipient together for the conjugations, and the plates were incubated over-night at 37°C. The cells were scraped off the membranes using sterile inoculation loops and transferred to tubes containing 1 ml sterile PBS. 100 µl of these suspensions were plated onto LB agar plates supplemented with gentamicin to select against the donor strain and chloramphenicol to select for *B. pseudomallei* transconjugants carrying the pDM4 constructs integrated into the chromosome.

Allelic exchange. The generation of clean in-frame deletion mutants relied on a second recombination event, where the integrated pDM4 plasmid constructs were deleted from the chromosome by using *sacB*-mediated counter-selection. Overnight cultures *sacB*$^+$ transconjugants were serially diluted in sterile PBS and plated onto salt-free LB agar plates containing 10% (w/v) sucrose, which were incubated at 24°C for 2-3 days. Colonies obtained on these plates were tested for sensitivity to chloramphenicol by patching them onto LB agar plates with and without chloramphenicol. Chloramphenicol-sensitive colonies were analyzed for carrying a deletion of the target gene. Confirmation of the mutants was performed by PCR using a primer that binds upstream of the fragment used for mutagenesis (primer series 3) combined with a reverse primer that binds within the coding region of the target gene (primer series 5), thus only resulting in a PCR product with wild-type cells, or a reverse primer that binds within

the downstream region used for mutagenesis (primer series 4), thus resulting in different sized fragments with wild-type and deletion mutants. Mutants were also confirmed by performing RT-PCR using primer combinations relA-RT-1& relA-RT-2 and spoT-RT-1& spoT-RT-2, which specifically amplify the *relA* and *spoT* transcripts, respectively, as described below.

The *relA spoT* double mutant was obtained by firstly deleting the *relA* gene, followed by a second round of mutagenesis deleting the *spoT* gene. A single *spoT* deletion mutant could not be obtained, despite several attempts.

RNA extractions. For transcriptional studies, bacteria were grown aerobically at 37°C in LB broth overnight. Total RNA was extracted from 4.5 ml of these stationary phase cultures using Trizol reagent (Invitrogen, through Life Technologies, Paisley, UK) according to the manufacturer's recommendations. In brief, cells were harvested by centrifugation at 12,000 rpm for 5 mins. Each cell pellet from 1.5 ml culture was resuspended in 1 ml Trizol reagent. Cells were lysed by pipetting and samples were stored at -80°C until they had passed a sterility check. Total RNA was extracted after adding chloroform and precipitated in isopropanol over-night at -20°C . The RNA was pelleted by centrifugation, washed with 70% ethanol, air dried for 5 mins at room temperature and resuspended in nuclease-free water. Contaminating DNA was removed by DNase I (Ambion, through Life Technologies, Paisley, UK) digestion for 45 mins at 37°C, followed by phenol / chloroform extractions, isopropanol precipitation and re-suspension of the total RNA in nuclease-free water as described above.

Semi-quantitative RT-PCR. For cDNA synthesis, 4 µg of total RNA was mixed with 3 µl of random primers at 3 µg/µl (Invitrogen) and 1 µl of a dNTP mixture at 10 mM each (Promega, Southampton, UK). After primer annealing at 65°C for 5 mins, a mix of first-strand buffer, DTT, 40 U RNase OUT recombinant RNase inhibitor (Invitrogen), and 200 U Superscript III reverse transcriptase (Invitrogen) was added according to the manufacturer's recommendations. cDNA

synthesis was performed at 50°C for 60 mins, followed by heat inactivation at 70°C for 15 mins. cDNA samples were 10x diluted in water and directly used for PCR amplification. For the adjustment of cDNA amounts, 16S rRNA was used as an internal standard, using primers 16S-RT-1 & 16S-RT-2. The sequences of all primers are listed in Table 2.

Macrophage uptake and intracellular survival assays. Bacterial uptake and survival inside macrophages were quantified using a modified kanamycin protection assay as described (47). In summary, J774A.1 murine macrophages were infected at an MOI of 10 and incubated at 37°C for 2 hrs to allow bacterial internalization to occur. The medium was then replaced with fresh medium containing 250 μg/ml kanamycin in order to suppress growth of extracellular bacteria. At appropriate time points, cells were washed, lysed in 0.1% (v/v) Triton X-100 and serial dilution of the lysis mixture were plated out on LB agar plates for the determination of intracellular bacterial numbers.

Galleria mellonella **killing assays**. Bacterial survival and virulence towards *G. mellonella* larvae was assessed by killing assays as described (47). Briefly, $1x10^4$ CFU of bacteria in a volume of 10 μl were injected into the hemocoel of 10 larvae per bacterial strain. Larvae were incubated statically at 37°C inside petri dishes and the number of dead larvae was scored periodically. Intracellular bacterial numbers were determined 20 hrs post infection by draining the hemocoel of three larvae per strain and plating out serial dilutions onto LB agar plates.

Animal infection studies. Female C57BL/6 mice (6-8 week-old; Harlan Laboratories, Bicester, Oxon, UK) were used throughout the studies. All animal experiments were performed in accordance with the guidelines of the Animals (Scientific Procedures) Act of 1986 and were approved by the local ethical review committee at the London School of Hygiene and Tropical Medicine. For each infection, aliquots were thawed from frozen bacteria stocks and diluted in

pyrogen-free saline (PFS). Prior to intranasal infection, mice were anesthetized intraperitoneally with ketamine (50 mg/kg; Ketaset; Fort Dodge Animal, Iowa, USA) and xylazine (10 mg/kg; Rompum; Bayer, Leverkusen, Germany) diluted in PFS. Infection was performed administering a total volume of 50 µl i.n. containing 2,500 colony forming units (CFU; acute model) or 100 CFU (chronic model) of *B. pseudomallei* K96243 wild type or isogenic Δ*relA*Δ*spoT* mutant. Infection dose was confirmed as described elsewhere (22). Control uninfected mice received 50 µl i.n. of PFS.

Immunisation studies. Female C57BL/6 mice were immunized i.n. with $1x10^5$ CFU of *B. pseudomallei* 2D2 (3) or the Δ*relA*Δ*spoT* mutant in a volume of 50 µl. Sham immunized mice received 50 µl PFS. Five weeks later they were challenged i.n. with $1x10^3$ CFU of *B. pseudomallei* strain 576 in a volume of 50 µl and survival was monitored. On day 55 post infection, survivors were culled and lungs and spleens aseptically removed into cold PBS. Organs were homogenized through a 100 µm cell strainer (BD Falcon, California, USA), serial 10-fold dilutions of tissue homogenates were plated onto tryptic soy agar plates (Sigma-Aldrich), and bacterial colonies were counted after incubation for 24-48 hours at 37^oC. The limits of detection were 50 CFU/organ.

Bright field microscopy. 10 µl of bacterial cultures were applied onto cover slips placed inside the wells of a 24-well plate and cells were fixed using paraformaldehyde (4% in PBS) for 30 mins. The cover slips were washed three times in phosphate-buffered saline and inverted onto a drop of Vectashield mounting medium (Vector Laboratories) on top of a glass slide. The microscope slide were fumigated over night before they could be removed from the BSL3 suite. Samples were imaged at 100x magnification using a Zeiss Axiophot microscope equipped with VisiView software (Visitron Systems GmbH).

***In silico* analyses**. Homologies between amino acid sequences were determined using NCBI's BLASTP algorithm. Conserved domains were analyzed using NCBI's conserved domain database (CDD). Promoter predictions were performed using the Bprom software (www.softberry.com).

Statistical analyses. Differences between average values were tested for significance by performing an unpaired, two-sided Student's t-test. Log-Rank tests of survival data were performed using the GraphPad Prism software version 5.01 (GraphPad Software, San Diego California USA). *p* values of ≤ 0.05 were considered significant.

RESULTS

RelA and SpoT proteins in *B. pseudomallei*.

RelA and SpoT play key roles in the generation of ppGpp in bacteria. The genome of *B. pseudomallei* strain K96243 was searched for homologues of the RelA and SpoT proteins of *Escherichia coli*, which produced two matches. BPSL1946 (predicted MW = 82.3 kDa) had 40% overall identity to RelA$_{E.coli}$, whereas BPSL2561 (predicted MW = 88 kDa) had 45% identity to SpoT$_{E.coli}$. Interestingly, no significant similarity was detected in the first 40 amino acid residues of the N-terminus of RelA$_{Bps,}$ whereas the start codon of SpoT$_{Bps}$ seemed to be miss-annotated by 59 amino acid residues. The identity between the two paralogs was 34%, and both proteins were conserved in the closely related species *B. mallei* and *B. thailandensis*. The *B. pseudomallei* proteins possess typical conserved domains for RelA-SpoT-family proteins (28). No other RelA SpoT family proteins were found in the genome by BLAST and PFAM searches. The organization of the chromosomal regions surrounding the *relA* and *spoT* genes, respectively, is depicted in Fig. 1A.

To further characterize the two proteins and the contribution of ppGpp synthesis in *B. pseudomallei*, a Δ*relA* single mutant and a Δ*relA*Δ*spoT* double mutant were created in *B. pseudomallei* strain K96243. The correct genotypes of the mutants were confirmed by PCR, and the absence of *relA* and / or *spoT* transcripts was confirmed by reverse-transcription PCR (data not shown). Since both genes are located at the end of putative transcriptional units, no polar effects on downstream genes is expected (see Fig. 1A). A Δ*spoT* single mutant could not be obtained despite several attempts.

Stationary phase survival is decreased in a Δ*relA*Δ*spoT* double mutant of *B. pseudomallei*.

As a first phenotypic test, the growth of the mutants in axenic culture was analyzed. Both the ΔrelA single mutant and a ΔrelAΔspoT double mutant grew at similar rates as the wild type during exponential growth in LB medium (Fig. 2A). Cultures of the ΔrelAΔspoT mutant reached higher optical densities than cultures of the wild type and the ΔrelA single mutant at the transition into stationary phase between 12 and 24 hrs post inoculation, but the optical density decreased steadily during the remainder of the experiment. The optical densities of wild type and ΔrelA mutant cultures remained at a constant level throughout stationary growth phase and were found to be 2-times higher than the values observed with the ΔrelAΔspoT mutant on day 13. Despite the elevated optical density after 24 hrs incubation, the number of colony forming units (CFU) in cultures of the ΔrelAΔspoT mutant was lower than in cultures of the wild type and the ΔrelA single mutant (Fig. 2B), and numbers further decreased over time until no viable bacteria could be detected at day three after inoculation (Fig. 2B). In contrast, the number of viable cells remained constant in cultures of the wild type for up to one week post inoculation, and only decreased marginally during the second week. The survival of the ΔrelA single mutant was more variable, with two out of four cultures exhibiting a decrease in the number of CFUs over time. However, on average, there was no significant difference to the wild type (Fig. 2B). This indicates a severe defect in stationary phase survival only in the absence of both ppGpp-synthesizing enzymes, similar to what has been described in other organisms (21, 31, 41).

In keeping with this finding, early stationary growth phase cells of the ΔrelA single mutant did not appear to differ in size and shape from wild type cells (Fig. 1C&D). In contrast, cells of the ΔrelAΔspoT mutant appeared slightly bigger on average (Fig. 1E, left panel), or had formed filaments of various lengths (Fig. 1E, right panel), thereby indicating stress-related changes in cell morphology in the absence of both ppGpp-synthesizing enzymes.

A Δ*relA*Δ*spoT* mutant of *B. pseudomallei* is unable to replicate inside macrophages.

B. pseudomallei is a facultative intracellular organism that can survive inside macrophages. In order to assess the intracellular survival ability of the Δ*relA* and Δ*relA*Δ*spoT* mutants, murine macrophages were infected with *B. pseudomallei* K96243 or isogenic mutants and the numbers of intracellular bacteria were determined up to 10 hrs post infection. While the wild type exhibited intracellular replication over the course of the experiment, the numbers of the Δ*relA*Δ*spoT* mutant did not increase compared to the input ($p = 0.024$ at 10 hrs p.i.; see Fig. 3). The extent of bacterial uptake into phagocytic cells, represented by the number of bacteria at 2 hrs post initial infection, was not altered between the mutant and the wild type. The Δ*relA* single mutant exhibited an intermediate replication rate and no significant difference in initial bacterial numbers. This suggests that the presence of RelA/SpoT family proteins is crucial for intracellular survival in phagocytic cells.

A Δ*relA*Δ*spoT* mutant of *B. pseudomallei* is attenuated in an insect model of infection.

Previously, we have demonstrated the value of wax moth (*G. mellonella*) larvae as a model system to assess virulence of *B. pseudomallei* isolates (47). Here, *G. mellonella* larvae were infected with 1×10^4 CFU of *B. pseudomallei* K96243 or isogenic mutants and survival of the larvae was monitored (Fig. 4A). The median survival of larvae infected with the wild type was 24 hrs, whereas infection with the Δ*relA* mutant resulted in a median survival of 30 hrs (Log-rank test $p = 0.004$). No larvae infected with the Δ*relA*Δ*spoT* double mutant had died by the end of 35 hrs ($p = 0.002$). When enumerating bacteria inside the larvae prior to the onset of paralysis at 20 hrs post infection, a >2,000-fold increase in bacterial numbers compared to the input was

observed with the wild type (Fig. 4B). The growth of the Δ*relA* and Δ*relA*Δ*spoT* mutants was 2-times and 11-times lower compared to wild type, respectively. This indicates a severe intracellular growth defect and a significant attenuation in insect virulence in the absence of RelA/SpoT family proteins.

K96243 Δ*relA*Δ*spoT* is attenuated in murine models of infection and is able to induce a protective immune response.

To further investigate the role of RelA and SpoT in virulence, C57BL/6 mice were infected i.n. with *B. pseudomallei* K96243 or the Δ*relA*Δ*spoT* double mutant at two different doses. A high dose of 2,500 CFU of *B. pseudomallei* K96243 has been shown to result in acute disease with death of the mice occurring within 10 days post infection, whereas a lower dose of <1,000 CFU results in a chronic infection (8). The Δ*relA*Δ*spoT* double mutant was attenuated in both the acute and the chronic model of infection and all mice survived for at least up to 70 days post infection (Fig. 5A&B). None of the mice infected with the *B. pseudomallei* Δ*relA*Δ*spoT* mutant exhibited any outward signs of disease or weight loss.

To test whether the Δ*relA*Δ*spoT* mutant is able to protect mice from subsequent infection, C57BL/6 mice were immunized i.n. with 1×10^5 CFU of K96243 Δ*relA*Δ*spoT* or *B. pseudomallei* strain 2D2 as a positive control and challenged 5 weeks later with 1×10^3 CFU of *B. pseudomallei* strain 576 given i.n., which usually results in an acute infection in naïve mice with a time to death of <10 days post infection. Immunization of mice with K96243 Δ*relA*Δ*spoT* resulted in significantly increased survival of mice compared to PFS control mice with 100% survivors up to 30 days post challenge ($p = 0.0061$; Fig. 5C). The pattern of survival of the immunized animals was comparable to the pattern of survival of mice that had been immunized with strain

2D2. Colonization data of lungs and spleens of surviving animals that had been vaccinated with the K96243 Δ*relA*Δ*spoT* mutant resulted in undetectable CFU levels in three out of five mice at day 55 p.i. with the wild type (Fig. 5D&E). However, bacteria could be detected in the remaining mice and one spleen sample exhibited extremely large abscesses. This indicates that vaccination with a Δ*relA*Δ*spoT* mutant of *B. pseudomallei* induces high levels of protective immunity against *B. pseudomallei* infection but, as with all other live-attenuated vaccines tested to date, sterile immunity could not be achieved.

DISCUSSION

In this study we have characterized the phenotype of a *ΔrelAΔspoT* derivative of *B. pseudomallei* and have assessed its potential to confer protective immunity on mice. Our results demonstrate that RelA/SpoT family proteins play an important role in survival and virulence of *B. pseudomallei* and that a *ΔrelAΔspoT* mutant may be a promising live-attenuated vaccine candidate.

The ppGpp-mediated stringent response is not well characterized in members of the β-proteobacteria. In phylogenetic analyses, it has been shown that RelA and SpoT proteins of β-proteobacteria such as *Bordetella* sp. and *Neisseria* sp. cluster next to the γ-proteobacterial branch (30). ppGpp production has been described in *Neisseria gonorrhoeae* (20). In contrast to *E. coli*, the gonococcal SpoT protein does not seem to contribute to ppGpp production and a *relA* single mutant had a growth defect on rich solid medium. The *B. pseudomallei* proteins share 45% and 44% identity with the RelA and SpoT proteins of *N. gonorrhoeae*, respectively.

From our data, we propose that the stringent response in *B. pseudomallei* may be similar to the one observed in *E. coli*. Firstly, we were unable to create a *spoT* single mutant, which indicates that SpoT is the only enzyme that can hydrolyze ppGpp in *B. pseudomallei* and thus prevent toxic accumulation. Moreover, the intermediate phenotype of the *relA* single mutant and its normal growth rate indicate that SpoT$_{Bps}$ also contributes to ppGpp synthesis. Interestingly, as seen in Fig. 1A, SpoT$_{Bps}$ is encoded upstream of the transcription elongation factor GreB, which also interacts with the RNA-polymerase through the secondary channel (25), similarly to ppGpp but with different effects (1, 40). More detailed studies on the stringent response in *B. pseudomallei* could provide a better understanding of survival and adaptability capacities of this organism.

Here, we characterized the phenotype of a *B. pseudomallei* mutant that is defective in RelA/SpoT enzymes usually involved in ppGpp synthesis and degradation. In other bacteria, it has been shown that lack of ppGpp-synthesizing enzymes results in defects in stationary phase survival (21, 31, 41). Our results show that this is also the case in *B. pseudomallei*. The Δ*relA*Δ*spoT* mutant had lost all viability after three days incubation (Fig. 2B). The viability of the Δ*relA* single mutant seemed to be variable, with some cultures having lost viability after one week, some exhibiting an initial decrease in CFU followed by a plateau at intermediate levels, and others showing no difference to the wild type (data not shown), which might indicate the involvement of suppressor mutants or other stochastic effects, which remain to be elucidated.

The assumed role of the ppGpp-mediated stringent response is to reduce growth when cells encounter unfavorable growth conditions, including nutrient deprivation upon entry into stationary phase, and to redirect resources into adaptation and survival mechanisms (27). In general, ppGpp mutants seem to be unable to modulate their growth rates and prepare for stationary phase survival. Stationary phase adaptation includes morphological changes such as a reduced cell size and rounding of cells (36). The morphology of Δ*relA*Δ*spoT* mutant cells differed from wild type cells as the mutant cells appeared slightly bigger on average (compare Fig. 1C and E). This suggests that the Δ*relA*Δ*spoT* mutant is unable to adopt a different morphology in response to stationary phase growth, whereas the filamentation indicates that the bacteria experience stress, and we speculate that these two effects might contribute to the reduced survival.

In this study, we have assessed the virulence of the *relA spoT* mutants in three different infection models: a macrophage *in vitro* model, an insect model, and mouse models of acute and chronic melioidosis. The Δ*relA*Δ*spoT* double mutant was found to be severely attenuated in all of the

models, whereas the Δ*relA* single mutant exhibited intermediate levels of attenuation in the two model systems tested. Previously, we have demonstrated the usefulness of the macrophage model and the *Galleria mellonella* insect model for comparing the virulence of different *Burkholderia* isolates (47). Our results presented here demonstrate that either model can also be used to assess the virulence of mutants, and in our case, the virulence in the non-mammalian models reflected the virulence in mice. Both model systems also provided further insights into the possible mode of attenuation: mutants lacking ppGpp-synthesizing enzymes are not attenuated due to reduced bacterial uptake or due to enhanced killing and clearance by macrophages and insect hemocytes, but rather due to a defect in intracellular replication (see bacterial numbers in Fig. 3 and Fig. 4B). The macrophage model may also prove useful to elucidate the role of ppGpp in the intracellular lifestyle of *B. pseudomallei* in future studies.

In addition to being severely attenuated in mice, both in an acute and a chronic model of disease, the K96243 Δ*relA*Δ*spoT* double mutant also partially protected immunized animals from intranasal challenge with virulent wild type bacteria (Fig. 5C). Pioneering studies of Dannenberg and colleagues (13-15) already demonstrated the feasibility of immunization against experimental melioidosis using attenuated *B. pseudomallei*. However, the genetic basis of attenuation of these strains is not known. More recently, other workers have assessed the feasibility of immunization against experimental melioidosis using rationally attenuated *B. pseudomallei* mutants. Immunization by the i.p. route, with a range of mutants, has been shown to protect against acute disease following a subsequent i.p. challenge with wild type *B. pseudomallei* (35, 42). However, disease following an i.n. or inhalational challenge is more severe than that seen following i.p. challenge (45) and there are few studies which have demonstrated protection against an i.n. or inhalational challenge. In part, this might reflect the

finding that protection against an i.n. challenge appears to be dependent on immunization by the same route (19). Immunization of BALB/c mice i.n. with two doses of an Δasd mutant has been shown to provide protection against acute disease following a low dose challenge (4 LD_{50} doses), but deaths of challenged mice were recorded from day 15 post challenge onwards (35). A similar pattern of protection was observed after i.n. immunization of BALB/c mice with an *ilvI* mutant (2D2), after i.n. challenge with 1×10^2 CFU of wild type *B. pseudomallei* (19). However, these studies have been carried out in BALB/c mice and we have recently reported that the C57BL/6 infection model reflects the spectrum of melioidosis seen in humans (8). Therefore, we considered that the C57BL/6 infection model would allow a more meaningful assessment of the potential for live-attenuated mutants to induce protective immunity in humans. In this study, we report that C57BL/6 mice immunized i.n. with either 2D2 or the $\Delta relA\Delta spoT$ mutant of *B. pseudomallei* were protected against acute disease following an i.n. challenge (1×10^3 CFU) of *B. pseudomallei* strain 576, which has proven to provide a robust measure of protection in many other vaccination studies. However, like previous studies, mice eventually succumbed to disease. In addition to incomplete protection by *B. pseudomallei* live-attenuated vaccines, concerns exist over their safety. The vaccine strain, even though unable to cause overt disease, might persist in the host and cause disease later in life. All our data indicate a defect of the *B. pseudomallei* $\Delta relA\Delta spoT$ mutant in long-term survival in broth culture and in macrophages, but the strain has not been tested for latency *in vivo*. Moreover, suppressor mutations of ppGpp-deficient strains have been reported in *E. coli,* which abolish their auxotrophy on minimal media plates (6, 32, 50). Therefore, more studies on the liability to acquire suppressor mutations and on the molecular mechanism of the intracellular replication defect, including subcellular localization experiments, have to be performed on the *B. pseudomallei* $\Delta relA\Delta spoT$ mutant before it can be

considered safe. Nevertheless, identification of different classes of protective vaccine strains may be useful for the creation of a live-attenuated vaccine strain carrying a combination of mutations, which could increase both protection and safety.

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TABLES

Table 1 – Strains and plasmids used in this study.

	Description	Reference
Strains		
B. pseudomallei K96243	Clinical isolate from Thailand	(23)
B. pseudomallei K96243 Δ*relA*	Inactivation of BPSL1946 (*relA*) by complete deletion	This study
B. pseudomallei K96243 Δ*relA*Δ*spoT*	Inactivation of BPSL1946 (*relA*) and BPSL2561 (*spoT*) by complete deletion	This study
B. pseudomallei 576	Clinical isolate from Thailand	(2)
B. pseudomallei 2D2	Auxotroph transposon mutant of *B. pseudomallei* 576	(3)
E. coli DH5 λ *pir*	*recA1 gyrA* (Nal) Δ(*lacIZYA-argF*) (φ80d*lac*Δ [*lacZ*]M15) *pirRK6*	(43)
E. coli S17-1 λ *pir*	RPA-2 *tra* regulon, *pirRK6,* Sm^R, Tp^R	(43)
Plasmids		
pDM4	Suicide vector with R6K origin; Cm^R	(29)
pDM4-Δ*relA*	600 bp up-and downstream of *relA* cloned into pDM4 via *Xho*I/*Sph*I sites	This study
pDM4-Δ*spoT*	600 bp up-and downstream of *spoT* cloned into pDM4 via *Sal*I/*Xma*I sites	This study

Table 2 – Oligonucleotides used in this study. Restriction sites are underlined. Complementary region are indicated in italics. Translational start and stop codons are highlighted in bold.

| Name	Sequence (5' – 3')	Purpose
relA-1-fw	CCC<u>CTCGAG</u>GAAGGCGCGCGGACAAGCGG	Mutagenesis
relA-2-rv	*CGGCGC**TCA**<u>CTGCAG</u>CATCGTCGG*GTATGGCCAGTT	Mutagenesis
relA-3-fw	*CCGACGATG<u>CTGCAG</u>**TGA**GCGCCG*CCGGTGGGCCGC	Mutagenesis
relA-4-rv	CCC<u>GCATGC</u>CGTATCGACGAGTTCGCCGTC	Mutagenesis
spoT-1-fw	GCG<u>GTCGAC</u>CTGCCGCCGTCGCTCGCGGC	Mutagenesis
spoT-2-rv	*GCGGCG**CTA**<u>ACTAGT</u>CATTTTCGC*CTCCTGGGTTCG	Mutagenesis
spoT-3-fw	*GCGAAAA**TG**<u>ACTAGT</u>**TAG**CGCCGC*GCGGCGCCCGAC	Mutagenesis
spoT-4-rv	GCG*<u>CCCGGG</u>*GCCTGCGCGAAATCGAGGTCG	Mutagenesis
relA-up-fw	ACAACGGCACCGTCTATCTC	Confirmation of plasmids
relA-down-rv	GCGGTGTCATAACCGTATTC	Confirmation of plasmids
spoT-up-fw	CTCGCATTACCGTTGAAGAC	Confirmation of plasmids
spoT-down-rv	GCCCTTTCAGTTCGAAGTTG	Confirmation of plasmids
relA-3-fw	GCGTGCAGTCGGACCTGCTG	Confirmation of mutation
relA-4-rv	GTTCGGGAAAATTCGCAAGCC	Confirmation of mutation
relA-5-wt	GTGGAAGCGGAATCGGCTG	Confirmation of mutation
spoT-3-fw	GGTGAAGAAGCAGTTCCGCA	Confirmation of mutation
spoT-4-rv	CGCCCGAAACAAATGGCGG	Confirmation of mutation
spoT-5-wt	GCGGACGATGGTGTAGTGC	Confirmation of mutation
relA-RT-1	AGATCCGCACGCAGGAAATG	RT-PCR analysis
relA-RT-2	TTCGCTGTGCAGGTGGTAAG	RT-PCR analysis
spoT-RT-1	GCGCATCAATGGCTCAAGTC	RT-PCR analysis
spoT-RT-2	TTCAGGCGCATCGTCTTCAG	RT-PCR analysis
16S-RT-1	GCCAGTCACCAATGCAGTTC	RT-PCR normalization
16S-RT-2	ACCAAGGCGACGATCAGTAG	RT-PCR normalization

FIGURES
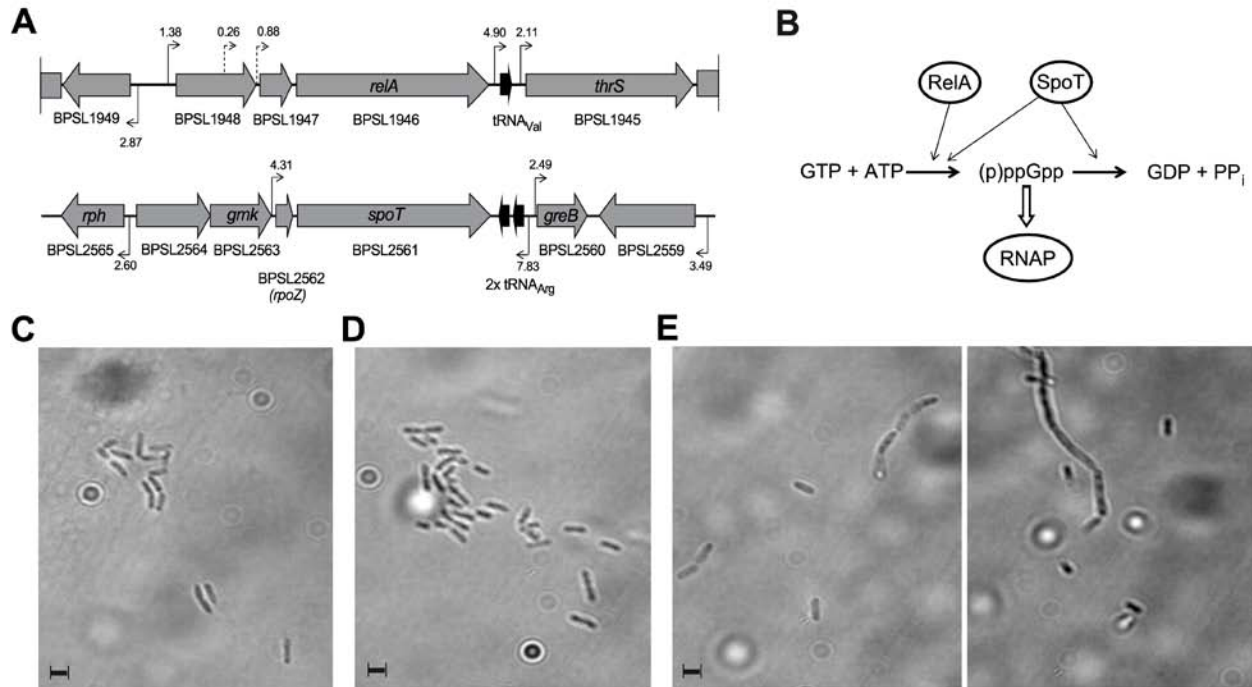
Fig. 1: Genetic organization and enzymatic activities of RelA-SpoT family proteins in *B. pseudomallei* and cell morphology of RelA-SpoT family mutants. A) Schematic overview of the chromosomal regions surrounding the *relA* gene (BPSL1946; upper panel) and the *spoT* gene (BPSL2561; lower panel). Predicted promoters and their linear discriminant function (LDF) score are indicated in the figure. B) Schematic of the classical synthesis pathway of (p)ppGpp. pppGpp is produced from GTP and ATP by either RelA- or SpoT-dependent mechanisms and is subsequently converted to ppGpp. ppGpp interacts directly with the RNA polymerase enzyme (RNAP) and thus affects gene expression. The effects of ppGpp induction are relieved by SpoT-mediated hydrolysis of ppGpp. C-E) Cell morphology of *B. pseudomallei* strain K96243 wild type (C), a K96243 Δ*relA* single mutant (D), and a K96243 Δ*relA*Δ*spoT* double mutant (E). All strains were grown to early stationary growth phase (24 hrs incubation at 37°C) in LB broth and bacterial cells were visualized by bright field microscopy at 100x magnification. The scale bars represent the average length of K96243 wild type cells. Two images of K96243 Δ*relA*Δ*spoT* mutant cells are presented, one focused on single cells (left panel), and one focused on a filament (right panel).
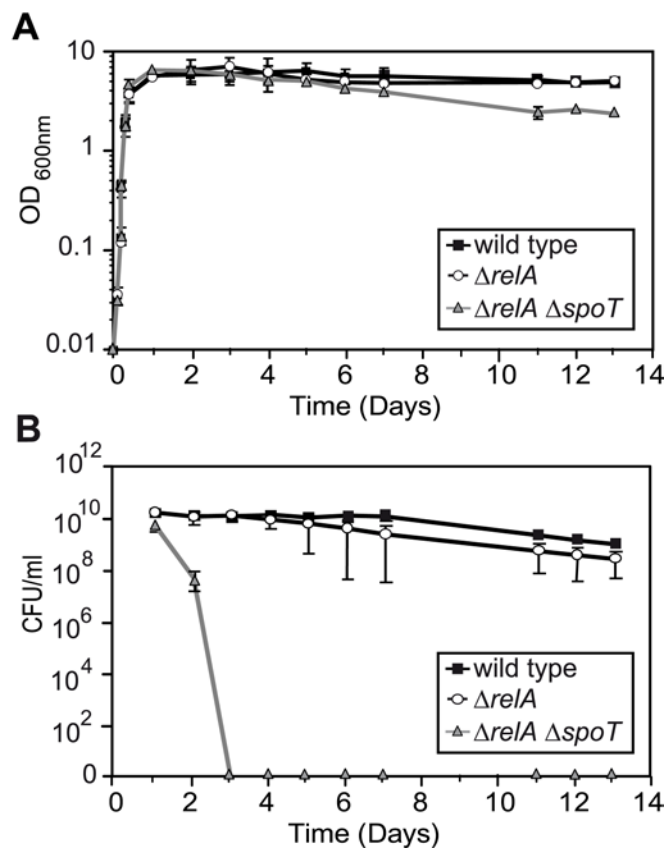
Fig. 2: Growth and stationary phase survival of K96243 wild type and isogenic mutants. All strains were inoculated into LB broth at an optical density of 0.01 and cultures were incubated at 37°C with aeration. A) Growth curve representing the optical densities of the cultures at indicated time points. B) Stationary phase survival of the strains determined by plating out samples onto LB agar plates at indicated time points. All experiments were performed in at least three independent experiments and data are plotted on a logarithmic scale as means with standard deviation (A) or standard error of the mean (B) between those experiments.
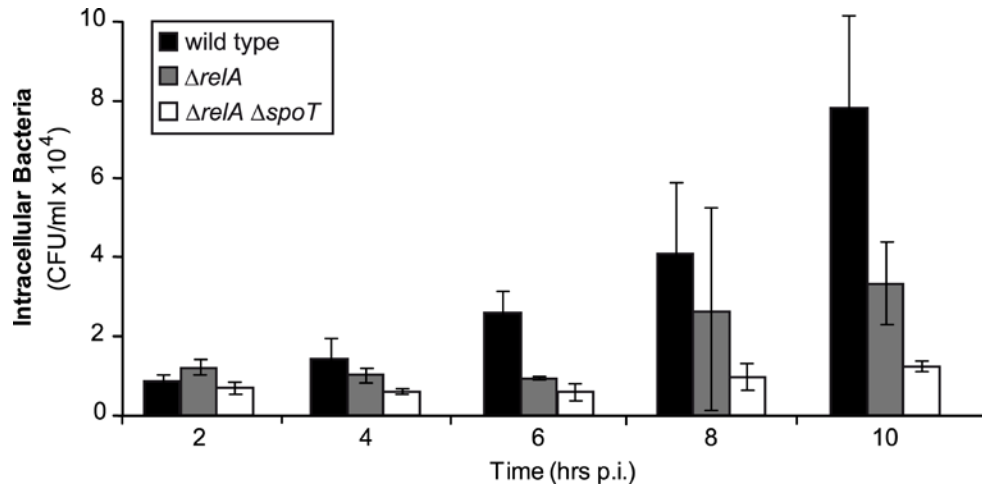
Fig. 3: Intracellular survival of K96243 wild type and isogenic mutants. J774A.1 murine macrophages were infected with K96243 wild type and isogenic mutants at an MOI of 10 and intracellular bacterial numbers were enumerated at indicated time points after initial infection by plating onto LB agar plates. Bars and error bars represent the means and standard deviation of a representative experiment performed in three technical replicates for each time point.
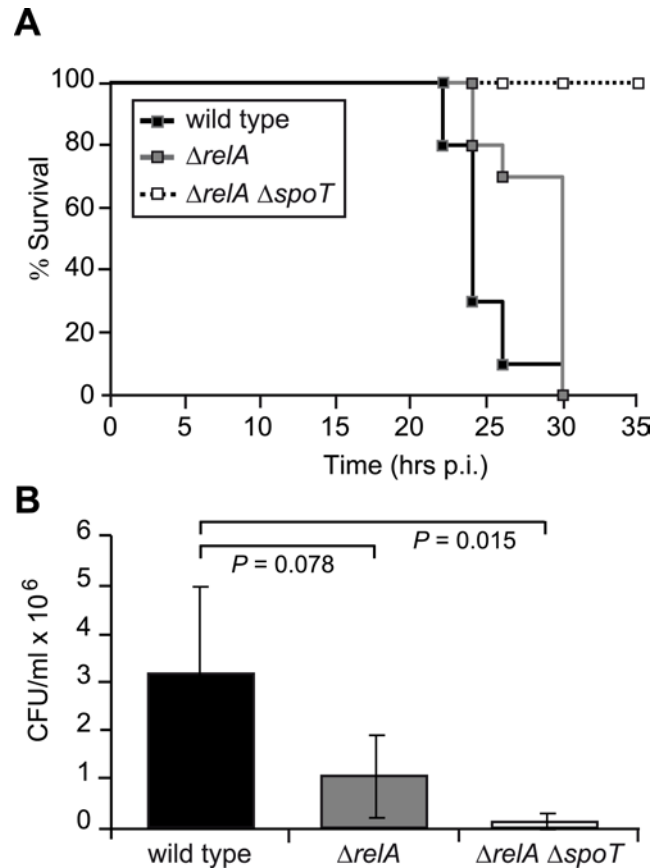
Fig. 4. Killing of *G. mellonella* larvae by K96243 wild type and isogenic mutants. A) Groups of ten larvae per strain were injected with 1×10^4 CFU of bacteria and maintained at 37°C. Dead and live larvae were scored at indicated time points and plotted as Kaplan-Meier survival curves. All curves were significantly different from each other (Log-rank test; $p < 0.01$ in all cases). B) The number of bacteria present inside the larvae at 20 hrs post infection was determined by aseptically removing the bottom 2 mm of five larvae per strain, draining the hemocoel and plating out serial dilutions onto LB agar plates. Results are shown as means and standard deviation of two independent experiments.
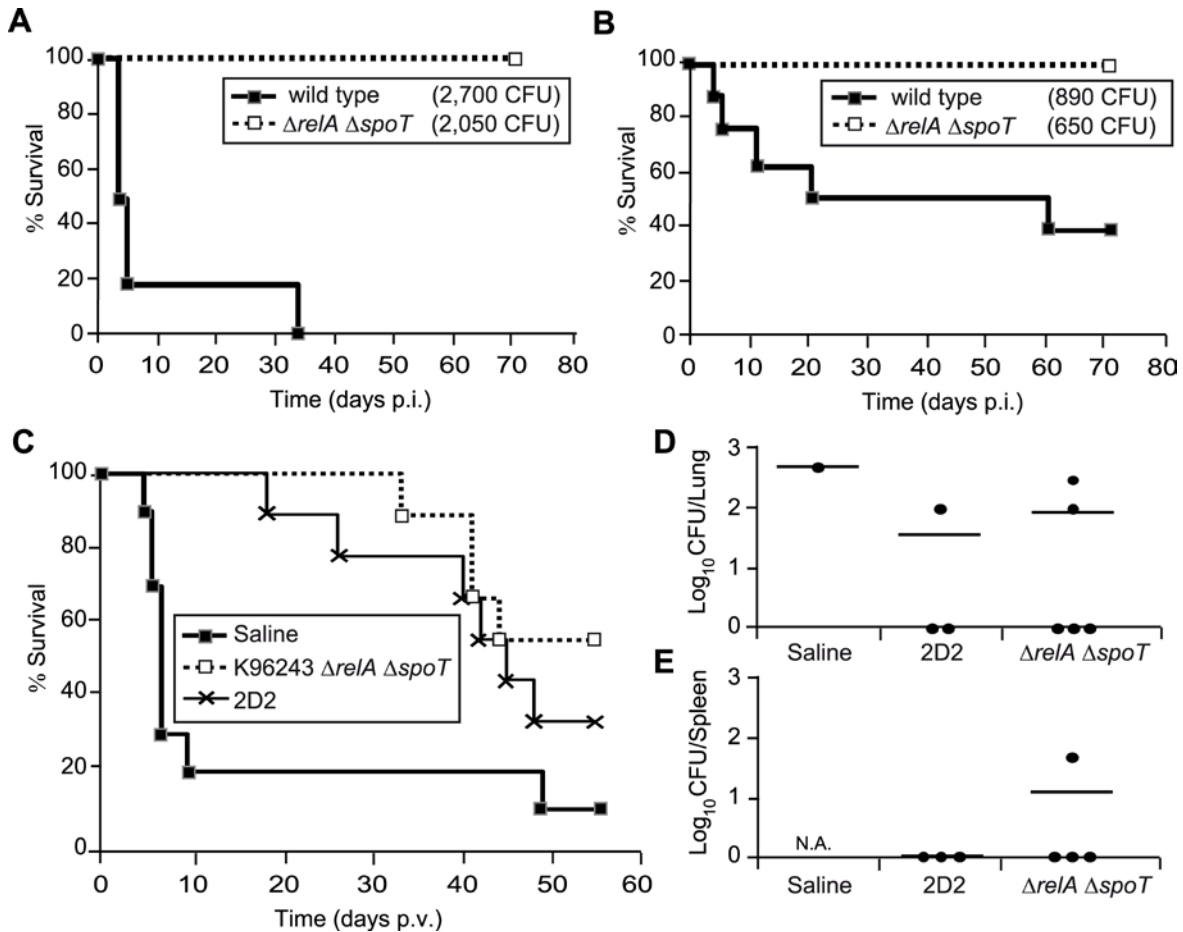
Fig. 5. Survival and protection of mice infected with K96243 wild type and isogenic Δ*relA*Δ*spoT* mutant. A-B) C57BL/6 mice were challenged intranasally with an intended dose of 2,500 CFU (acute challenge; panel A) or 500 CFU (chronic challenge; panel B). Actual infection doses for each experiment are given in brackets in the figure. Log-rank tests confirmed a significant difference in survival between the wild type and the Δ*relA*Δ*spoT* mutant in both cases ($p < 0.001$ and $p < 0.05$, respectively). C-E) C57BL/6 mice were immunized intranasally with 1×10^5 CFU of strains 2D2 or K96243 Δ*relA*Δ*spoT*. After 5 weeks incubation, mice were challenged intranasally with 1,000 CFU of *B. pseudomallei* strain 576. Mice were monitored for survival and numbers are plotted as Kaplan-Meier survival curves (panel C). Log-rank tests confirmed a significant difference in survival between the K96243 Δ*relA*Δ*spoT* and the 2D2 cohorts compared to the saline control ($p < 0.01$ and $p < 0.05$, respectively). At day 55 post infection with the wild type, bacterial burdens were assessed in lungs (panel D) and spleens (panel E) of survivors. Note: Only one mouse had survived up to this time point in the saline-treated sample group. The spleen of this mouse and one survivor of the K96243 Δ*relA*Δ*spoT* sample group had extremely large abscesses and could not be harvested. N.A. = not available.