

Role of RelA and SpoT in *Burkholderia pseudomallei* Virulence and Immunity

Claudia M. Müller,^a Laura Conejero,^b Natasha Spink,^b Matthew E. Wand,^{a*} Gregory J. Bancroft,^b and Richard W. Titball^a

College of Life and Environmental Sciences, Biosciences, University of Exeter, Exeter, Devon, United Kingdom,^a and London School of Hygiene and Tropical Medicine, London, United Kingdom^b

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 U.S. Centers for Disease Control and Prevention, and there is currently no melioidosis vaccine available. Small modified nucleotides such as the hyperphosphorylated guanosine molecules ppGpp and pppGpp play an important role as signaling molecules in prokaryotes. They mediate a global stress response under starvation conditions and have been implicated in the regulation of virulence and survival factors in many bacterial species. In this study, we 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* $\Delta relA \Delta spoT$ 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 $\Delta relA \Delta spoT$ 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 $\Delta relA \Delta spoT$ mutant may be a promising live-attenuated vaccine candidate.

Burkholderia pseudomallei, a Gram-negative, motile, aerobic bacterium of the class betaproteobacteria, 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, which last for several decades (9). Despite antibiotic treatment, mortality rates for acute melioidosis can be 40 to 50% in areas where the disease is endemic, and relapse rates in surviving individuals are also high [for a review, see the work of Wiersinga et al. (48)]. Currently, there is no vaccine, and therefore, *B. pseudomallei* is listed as a category B select agent by the U.S. Centers 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 the works of Cashel et al. (6), Magnusson et al. (27), Potrykus and 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 downregulation of ribosomal proteins and DNA replication and an upregulation 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 gammaproteobacteria, 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 *spoT* genes has a dramatic impact on gene ex-

pression profiles: microarray analyses of strains lacking ppGpp-synthesizing enzymes have revealed deregulation of several hundred 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 involved in survival and adaptation to stress conditions but also others, 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* spp., *Campylobacter jejuni*, *Legionella pneumophila*, *Helicobacter pylori*, and *Mycobacterium tuberculosis*, the 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

Received 17 February 2012 Returned for modification 15 March 2012

Accepted 28 June 2012

Published ahead of print 9 July 2012

Editor: A. Camilli

Address correspondence to Claudia M. Müller, C.Mueller@exeter.ac.uk.

* Present address: Matthew E. Wand, Health Protection Agency, Porton Down, Salisbury, United Kingdom.

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doi:10.1128/IAI.00178-12

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>B. pseudomallei</i> strains		
K96243	Clinical isolate from Thailand	23
K96243 Δ <i>relA</i>	Inactivation of BPSL1946 (<i>relA</i>) by complete deletion	This study
K96243 Δ <i>relA</i> Δ <i>spoT</i>	Inactivation of BPSL1946 (<i>relA</i>) and BPSL2561 (<i>spoT</i>) by complete deletion	This study
576	Clinical isolate from Thailand	2
2D2	Auxotroph transposon mutant of <i>B. pseudomallei</i> 576	3
<i>E. coli</i> strains		
DH5 Δ <i>pir</i>	<i>recA1 gyrA</i> (Nal) Δ (<i>lacIZYA-argF</i>) (ϕ 80 <i>dlac</i> Δ [<i>lacZ</i>]M15) <i>pirRK6</i>	43
S17-1 Δ <i>pir</i>	RPA-2 <i>tra</i> regulon; <i>pirRK6</i> Sm ^r Tp ^r	43
Plasmids		
pDM4	Suicide vector with R6K origin; Cm ^r	29
pDM4- Δ <i>relA</i>	600 bp up- and downstream of <i>relA</i> cloned into pDM4 via XhoI/SphI sites	This study
pDM4- Δ <i>spoT</i>	600 bp up- and downstream of <i>spoT</i> cloned into pDM4 via Sall/XmaI sites	This study

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, 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 gamma interferon (IFN- γ) and transforming growth factor (TGF)- β 4 production in spleens (37). This may render ppGpp-deficient *Salmonella* strains promising candidates as live-attenuated vaccines against typhoid in chicken.

Here, we evaluated a Δ *relA* Δ *spoT* double mutant of *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 long-lasting immunity. Several live-attenuated vaccine strains of *B. pseudomallei* have been tested to date [reviewed by Sarkar-Tyson and Titball (42) and Patel et al. (38)]. Most of them are mutants with defects in the intracellular life stages, such as mutations 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 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, the antibiotics chloramphenicol (Sigma-Aldrich, Gillingham, United Kingdom), kanamycin (Sigma-Aldrich), and gentamicin (Sigma-Aldrich) were used at concentrations of 25 μ g/ml, 50 μ g/ml, and 50 μ g/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 the primer combinations *relA*-1 and *relA*-2 (upstream), *relA*-3 and *relA*-4 (downstream), *spoT*-1 and *spoT*-2 (upstream), and *spoT*-3 and *spoT*-4 (downstream), respectively, and K96243 genomic DNA as a template. A second, recombinant PCR was performed using the outside primers (*relA*-1 plus *relA*-4 and *spoT*-1 plus *spoT*-4) and the respective up- and downstream PCR fragments from the first PCR as templates. The resulting 1.2-kb recombinant PCR products were cloned into the suicide vector pDM4 via its XhoI/SphI and Sall/XmaI 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 plus *relA*-down and *spoT*-up plus *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 overnight in LB medium at 37°C with aeration. One milliliter of the cultures was pelleted by centrifugation, and the cells were resuspended in 0.5 ml fresh LB medium. Ten microliters of these suspensions was transferred onto 1-cm by 1-cm squares of nitrocellulose membrane (Hybond-ECL; GE Healthcare, Little Chalfont, United Kingdom) placed on brain heart infusion (BHI; Fluka Sigma-Aldrich) agar plates, either individually for control plates or with donor and recipient together for the conjugations, and the plates were incubated overnight at 37°C. The cells were scraped off the membranes using sterile inoculation loops and transferred to tubes containing 1 ml sterile PBS. One hundred microliters of these suspensions was 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.

TABLE 2 Oligonucleotides used in this study

Name	Sequence (5'–3') ^a	Purpose
relA-1-fw	CCCCTCGAGGAAGGCGCGGACAAGCGG	Mutagenesis
relA-2-rv	CGGCGCTCACTGCAGCATCGTCGGGTATGGCCAGTT	Mutagenesis
relA-3-fw	CCGACGATGCTGCAGTGAGCGCCCGGTGGGCCCGC	Mutagenesis
relA-4-rv	CCCGCATGCCGTATCGACGAGTTCGCCGTC	Mutagenesis
spoT-1-fw	GCGGTGCGACCTGCCCGCTCGCTCGCGGC	Mutagenesis
spoT-2-rv	GCGGCGCTAACTAGTCAITTTTCGCTCCTGGGTTCG	Mutagenesis
spoT-3-fw	GCGAAAATGACTAGTTAGCGCCGCGCGGCCCGAC	Mutagenesis
spoT-4-rv	GCGCCCGGGCCTCGCGCAAATCGAGGTTCG	Mutagenesis
relA-up-fw	ACAACGGCACCGTCTATCTC	Confirmation of plasmids
relA-down-rv	GCGGTGTCATAACCGTATTC	Confirmation of plasmids
spoT-up-fw	CTCGATTACCGTTGAAGAC	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	CGCCCGAAAACAATGGCGG	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

^a Restriction sites are underlined. Complementary regions are in italics. Translational start and stop codons are in bold.

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 counterselection. Overnight cultures of *sacB*⁺ transconjugants were serially diluted in sterile PBS and plated onto salt-free LB agar plates containing 10% (wt/vol) sucrose, which were incubated at 24°C for 2 to 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 either with a reverse primer that binds within the coding region of the target gene (primer series 5), resulting in a PCR product only with wild-type cells, or with a reverse primer that binds within the downstream region used for mutagenesis (primer series 4), resulting in different-sized fragments with the wild type and deletion mutants. Mutants were also confirmed by performing RT-PCR using the primer combinations relA-RT-1 plus relA-RT-2 and spoT-RT-1 plus spoT-RT-2, which specifically amplify the *relA* and *spoT* transcripts, respectively, as described below.

The *relA spoT* double mutant was obtained by first deleting the *relA* gene, followed by a second round of mutagenesis deleting the *spoT* gene. A $\Delta spoT$ single 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, United Kingdom) according to the manufacturer's recommendations. In brief, cells were harvested by centrifugation at 12,000 rpm for 5 min. 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 the addition of chloroform and precipitated in isopropanol overnight at –20°C. The RNA was pelleted by centrifugation, washed with 70% ethanol, air dried for 5 min at room temperature, and

resuspended in nuclease-free water. Contaminating DNA was removed by DNase I (Ambion, through Life Technologies, Paisley, United Kingdom) digestion for 45 min at 37°C, followed by phenol-chloroform extractions, isopropanol precipitation, and resuspension of the total RNA in nuclease-free water as described above.

Semiquantitative 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 deoxynucleoside triphosphate (dNTP) mixture at 10 mM each (Promega, Southampton, United Kingdom). After primer annealing at 65°C for 5 min, a mix of first-strand buffer, dithiothreitol (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 min, followed by heat inactivation at 70°C for 15 min. cDNA samples were 10× 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 and 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 previously (47). In summary, J774A.1 murine macrophages were infected at a multiplicity of infection (MOI) of 10 and incubated at 37°C for 2 h 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 and lysed in 0.1% (vol/vol) Triton X-100, and serial dilutions of the lysis mixture were plated on LB agar plates for the determination of intracellular bacterial numbers.

Galleria mellonella killing assays. Bacterial survival and virulence for *G. mellonella* larvae were assessed by killing assays as described previously (47). Briefly, 1 × 10⁴ CFU of bacteria in a volume of 10 µl were injected into the hemocoels 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 h postinfection by draining the hemocoels of three larvae per strain and plating serial dilutions onto LB agar plates.

Animal infection studies. Female C57BL/6 mice (6 to 8 weeks old; Harlan Laboratories, Bicester, Oxon, United Kingdom) 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 Health, IA) and xylazine (10 mg/kg; Rompum; Bayer, Leverkusen, Germany) diluted in PFS. Infection was performed by intranasally (i.n.) administering a total volume of 50 μ l containing 2,500 CFU (acute model) or 100 CFU (chronic model) of wild-type *B. pseudomallei* K96243 or the isogenic Δ relA Δ spoT mutant. The infection dose was confirmed as described elsewhere (22). Control uninfected mice received 50 μ l of PFS i.n.

Immunization studies. Female C57BL/6 mice were immunized i.n. with 1×10^5 CFU of *B. pseudomallei* 2D2 (3) or the Δ relA Δ spoT mutant in a volume of 50 μ l. Sham-immunized mice received 50 μ l PFS. Five weeks later they were challenged i.n. with 1×10^3 CFU of *B. pseudomallei* strain 576 in a volume of 50 μ l, and survival was monitored. On day 55 postinfection, survivors were culled and lungs and spleens aseptically removed into cold PBS. Organs were homogenized through a 100- μ m cell strainer (BD Falcon, CA), 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 to 48 h at 37°C. The limit of detection was 50 CFU/organ.

Bright-field microscopy. Ten microliters of bacterial cultures was applied to coverslips placed inside the wells of a 24-well plate, and cells were fixed using paraformaldehyde (4% in PBS) for 30 min. The coverslips 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 slides were fumigated overnight before they could be removed from the BSL3 suite. Samples were imaged at a magnification of $\times 100$ using a Zeiss Axiophot microscope equipped with VisiV-view 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 (Softberry).

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, CA). *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 molecular mass = 82.3 kDa) had 40% overall identity to RelA_{*E.coli*}, whereas BPSL2561 (predicted molecular mass = 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 of *B. pseudomallei* (RelA_{*Bps*}), whereas the start codon of SpoT_{*Bps*} seemed to be misannotated 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 (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 Δ relA single mutant and a Δ relA Δ spoT double mutant grew at rates similar to that of the wild type during exponential growth in LB medium (Fig. 2A). Cultures of the Δ relA Δ spoT mutant reached higher optical densities than cultures of the wild type and the Δ relA mutant at the transition into stationary phase between 12 and 24 h postinoculation, but the optical density decreased steadily during the remainder of the experiment. The optical densities of wild-type and Δ relA mutant cultures remained at a constant level throughout stationary growth phase and were found to be twice as high as the values observed with the Δ relA Δ spoT mutant on day 13. Despite the elevated optical density after 24 h incubation, the number of CFU in cultures of the Δ relA Δ spoT mutant was lower than in cultures of the wild type and the Δ relA single mutant (Fig. 2B), and numbers further decreased over time until no viable bacteria could be detected at day 3 after inoculation (Fig. 2B). In contrast, the number of viable cells remained constant in cultures of the wild type for up to 1 week postinoculation and decreased only marginally during the second week. The survival of the Δ relA single mutant was more variable, with two of four cultures exhibiting a decrease in the number of CFU over time. However, on average, there was no significant difference relative 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 for other organisms (21, 31, 41).

In keeping with this finding, early-stationary-growth-phase cells of the Δ relA single mutant did not appear to differ in size and shape from wild-type cells (Fig. 1C and D). In contrast, cells of the Δ relA Δ spoT mutant appeared slightly bigger on average (Fig. 1E, left) or had formed filaments of various lengths (Fig. 1E, right), 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 h postinfection. 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 h postinfection [p.i.]) (Fig. 3). The extent of bacterial uptake into phagocytic cells, represented by the number of bacteria at 2 h after initial infection,

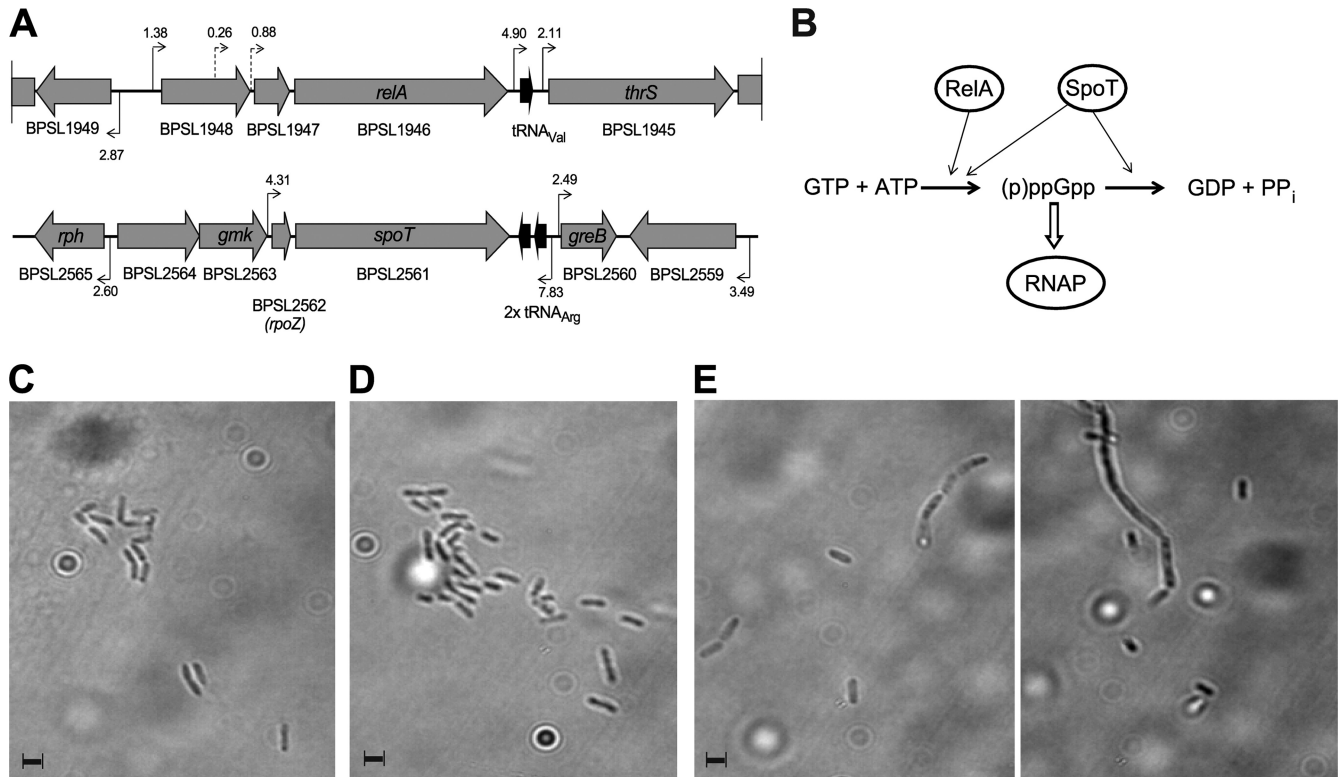


FIG 1 Genetic organization and enzymatic activities of RelA-SpoT family proteins in *B. pseudomallei* and cell morphology of RelA-SpoT family mutants. (A) Schematic overview of the chromosomal regions surrounding the *relA* gene (BPSL1946 [top]) and the *spoT* gene (BPSL2561 [bottom]). Predicted promoters and their linear discriminant function (LDF) score are indicated. (B) Schematic of the classical synthesis pathway of (p)ppGpp. ppGpp 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 to E) Cell morphology of *B. pseudomallei* wild-type strain K96243 (C), a K96243 $\Delta relA$ single mutant (D), and a K96243 $\Delta relA \Delta spoT$ double mutant (E). All strains were grown to early stationary growth phase (24-h incubation at 37°C) in LB broth, and bacterial cells were visualized by bright-field microscopy at a magnification of $\times 100$. The scale bars represent the average length of K96243 wild-type cells. In panel E, two images of K96243 $\Delta relA \Delta spoT$ mutant cells are presented, one focused on single cells (left) and one focused on a filament (right).

was not altered between the mutant and the wild type. The $\Delta relA$ 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 $\Delta relA \Delta spoT$ mutant of *B. pseudomallei* is attenuated in an insect model of infection. Previously, we 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 time of larvae infected with the wild type was 24 h, whereas infection with the $\Delta relA$ mutant resulted in a median survival time of 30 h (log rank test, $P = 0.004$). No larvae infected with the $\Delta relA \Delta spoT$ double mutant had died by the end of 35 h ($P = 0.002$). When bacteria inside the larvae were enumerated prior to the onset of paralysis at 20 h postinfection, a $>2,000$ -fold increase in bacterial numbers compared to the input was observed with the wild type (Fig. 4B). The growth rates of the $\Delta relA$ and $\Delta relA \Delta spoT$ mutants were 2 times and 11 times lower than that of 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 $\Delta relA \Delta spoT$ 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 $\Delta relA \Delta spoT$ 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 postinfection, whereas a lower dose of $<1,000$ CFU results in a chronic infection (8). The $\Delta relA \Delta spoT$ 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 postinfection (Fig. 5A and B). None of the mice infected with the *B. pseudomallei* $\Delta relA \Delta spoT$ mutant exhibited any outward signs of disease or weight loss.

To test whether the $\Delta relA \Delta spoT$ mutant is able to protect mice from subsequent infection, C57BL/6 mice were immunized i.n. with 1×10^5 CFU of K96243 $\Delta relA \Delta spoT$ 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 postinfection. Immunization of mice with K96243 $\Delta relA \Delta spoT$ resulted in significantly increased survival of mice compared to PFS control mice, with 100% survivors up to 30 days

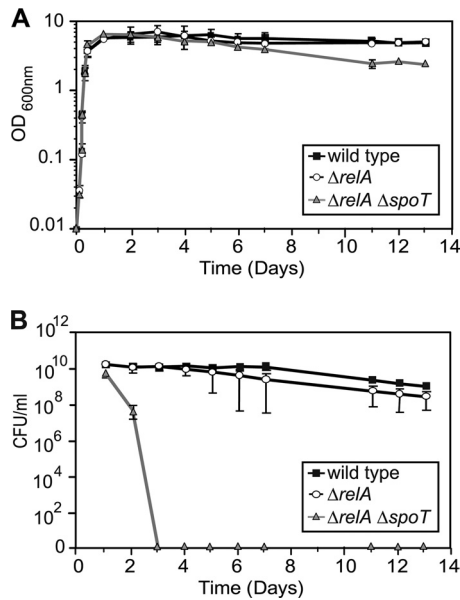


FIG 2 Growth and stationary-phase survival of wild-type K96243 and isogenic mutants. All strains were inoculated into LB broth at an optical density of 0.01, and cultures were incubated at 37°C with aeration. (A) Growth curve representing the optical densities of the cultures at the indicated time points. (B) Stationary-phase survival of the strains determined by plating samples onto LB agar plates at the 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 deviations (A) or standard errors of the means (B) for those experiments.

postchallenge ($P = 0.0061$) (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 $\Delta relA \Delta spoT$ mutant showed undetectable CFU levels in three of five mice at day 55 after infection with the wild type (Fig. 5D and E). However, bacteria could be detected in the remaining mice, and one spleen sample exhibited extremely large abscesses. This indicates that vaccination with a $\Delta relA \Delta spoT$ mutant of *B. pseudomallei* induces high levels of protective immunity against *B. pseudomallei* infection, but as with all other live-

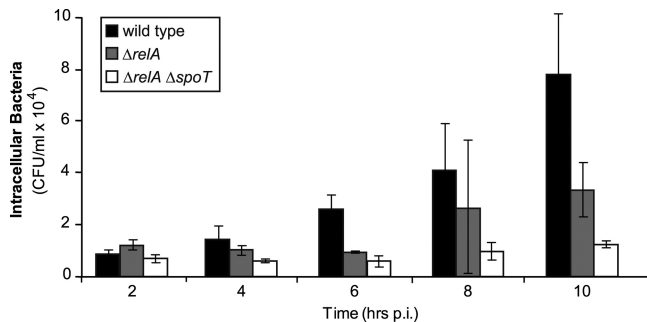


FIG 3 Intracellular survival of wild-type K96243 and isogenic mutants. J774A.1 murine macrophages were infected with wild-type K96243 and isogenic mutants at an MOI of 10, and intracellular bacterial numbers were determined at the indicated time points after initial infection by plating onto LB agar plates. Bars and error bars represent the means and standard deviations of a representative experiment performed in three technical replicates for each time point.

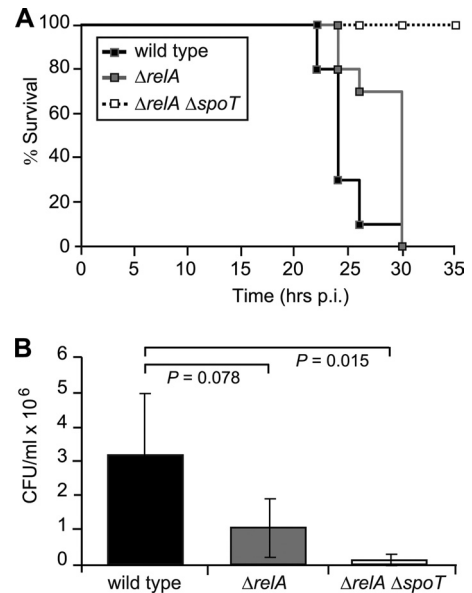


FIG 4 Killing of *G. mellonella* larvae by wild-type K96243 and isogenic mutants. (A) Groups of 10 larvae per strain were injected with 1×10^4 CFU of bacteria and maintained at 37°C. Dead and live larvae were scored at indicated time points and plotted as Kaplan-Meier survival curves. All curves were 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 h postinfection was determined by aseptically removing the bottom 2 mm of five larvae per strain, draining the hemocoel, and plating serial dilutions onto LB agar plates. Results are means and standard deviations from two independent experiments.

attenuated vaccines tested to date, sterile immunity was not achieved.

DISCUSSION

In this study, we characterized the phenotype of a $\Delta relA \Delta spoT$ 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 $\Delta relA \Delta spoT$ mutant may be a promising live-attenuated vaccine candidate.

The ppGpp-mediated stringent response is not well characterized in members of the betaproteobacteria. In phylogenetic analyses, it has been shown that RelA and SpoT proteins of betaproteobacteria such as *Bordetella* spp. and *Neisseria* spp. cluster next to the gammaproteobacterial branch (30). ppGpp production has been described for *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*. First, 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

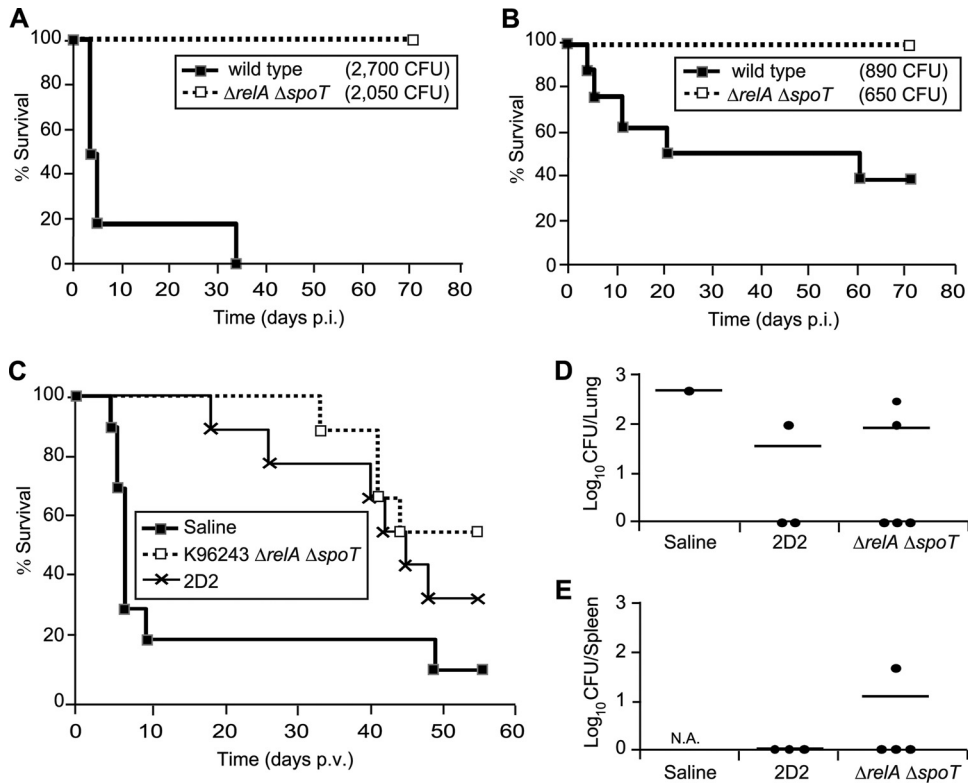


FIG 5 Survival and protection of mice infected with wild-type K96243 and its isogenic $\Delta relA \Delta spoT$ mutant. (A and B) C57BL/6 mice were challenged intranasally with an intended dose of 2,500 CFU (acute challenge [A]) or 500 CFU (chronic challenge [B]). Actual infection doses for each experiment are given in parentheses. Log rank tests confirmed a significant difference in survival between the wild type and the $\Delta relA \Delta spoT$ mutant in both cases ($P < 0.001$ and $P < 0.05$, respectively). (C to E) C57BL/6 mice were immunized intranasally with 1×10^5 CFU of strain 2D2 or K96243 $\Delta relA \Delta spoT$. After 5 weeks of 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 (C). Log rank tests confirmed a significant difference in survival of the K96243 $\Delta relA \Delta spoT$ and the 2D2 cohorts compared to the saline control ($P < 0.01$ and $P < 0.05$, respectively). At day 55 after infection with the wild type, bacterial burdens were assessed in lungs (D) and spleens (E) of survivors. Note that only one mouse had survived up to this time point in the saline-treated sample group. The spleen of this mouse and that of one survivor in the K96243 $\Delta relA \Delta spoT$ sample group had extremely large abscesses and could not be harvested. N.A., not available.

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 and SpoT enzymes, which are 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 $\Delta relA \Delta spoT$ mutant had lost all viability after 3 days of incubation (Fig. 2B). The viability of the $\Delta relA$ single mutant seemed to be variable, with some cultures having lost viability after 1 week, some exhibiting an initial decrease in CFU followed by a plateau at intermediate levels, and others showing no difference relative 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 $\Delta relA \Delta spoT$ mutant cells differed from that of wild-type cells, as the mutant cells appeared slightly bigger on average (compare Fig. 1C and E). This suggests that the $\Delta relA \Delta spoT$ 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 assessed the virulence of *relA* and *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 $\Delta relA \Delta spoT$ double mutant was found to be severely attenuated in all of the models, whereas the $\Delta relA$ single mutant exhibited intermediate levels of attenuation in the two systems. Previously, we demonstrated the usefulness of the macrophage model and the *Galleria mellonella* insect model for comparing the virulence of different *Burkholderia* isolates (47). The 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 nonmammalian 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 attenuated not because of reduced bacterial uptake or due to enhanced killing and clearance by macrophages and insect hemocytes but rather because of a defect in intracellular replication (see bacterial numbers in Fig. 3 and 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, in both an acute and a chronic model of disease, the K96243 $\Delta relA \Delta spoT$ double mutant also partially protected immunized animals from intranasal challenge with virulent wild-type bacteria (Fig. 5C). Pioneering studies by Dannenberg and Scott (13–15) had 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 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 (four 50% lethal doses), but deaths of challenged mice were recorded from day 15 postchallenge 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 were carried out in BALB/c mice, and we 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, as in 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 medium plates (6, 32, 50). Therefore, more studies on the proneness 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.

ACKNOWLEDGMENTS

This work was supported by Wellcome Trust award WT085162AIA.

We thank Muthita Vanaporn for her help in constructing the mutants and Sok Lau for her help with the microscopy. We thank Robert Gilbert and all of the members of the London School of Hygiene and Tropical Medicine Biological Services Facility for animal husbandry. We also thank Carlos Balsalobre for critically reading the manuscript and his helpful comments.

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