Superoxide dismutase C is required for intracellular survival and virulence of *Burkholderia pseudomallei*

Muthita Vanaporn,^{1,2} Matthew Wand,³ Stephen L. Michell,¹
Mitali Sarkar-Tyson,⁴ Philip Ireland,⁴ Stan Goldman,⁵
Chidchamai Kewcharoenwong,⁶ Darawan Rinchai,⁶
Ganjana Lertmemongkolchai⁶ and Richard W. Titball¹

Correspondence

Richard W. Titball
r.w.titball@exeter.ac.uk
Muthita Vanaporn
tmuthita@mahidol.ac.th

¹College of Life and Environmental Sciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK

²Department of Microbiology and Immunology, Mahidol University, Rajvithi Road, Bangkok 10400, Thailand

³Health Protection Agency, Porton Down, Salisbury SP4 0JG, UK

⁴Department of Biomedical Sciences, Defence Science and Technology Laboratory, Porton Down, Salisbury SP4 0JQ, UK

⁵Genetic Chemistry Inc., 200 Page Mill Road, Palo Alto, CA 94306, USA

⁶The Center for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand

Burkholderia pseudomallei is an intracellular pathogen and the causative agent of melioidosis, a life-threatening disease of humans. Within host cells, superoxide is an important mediator of pathogen killing. In this study, we have identified the *B. pseudomallei* K96243 *sodC* gene, shown that it has superoxide dismutase activity, and constructed an allelic deletion mutant of this gene. Compared with the wild-type, the mutant was more sensitive to killing by extracellular superoxide, but not to superoxide generated intracellularly. The *sodC* mutant showed a markedly decreased survival in J774A.1 mouse macrophages, and reduced numbers of bacteria were recovered from human polymorphonuclear neutrophils (PMNs) when compared with the wild-type. The numbers of wild-type or mutant bacteria recovered from human diabetic neutrophils were significantly lower than from normal human neutrophils. The *sodC* mutant was attenuated in BALB/c mice. Our results indicate that SodC plays a key role in the virulence of *B. pseudomallei*, but that diabetics are not more susceptible to infection because of a reduced ability of PMNs to kill by superoxide.

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INTRODUCTION

Burkholderia pseudomallei is a Gram-negative facultative intracellular bacterium and the causative agent of melioidosis, an often fatal disease of humans. The disease is endemic in South-East Asia and Northern Australia. Although melioidosis can occur in apparently healthy individuals, it is known that conditions such as diabetes predispose individuals to disease (White, 2003). The manifestations of melioidosis range from an asymptomatic infection to sepsis with a high mortality rate (White, 2003). The bacterium has the ability to establish persistent but asymptomatic infections in humans that can last for over 60 years (Ngauy *et al.*, 2005). Treatment is not always successful

because the bacterium is inherently resistant to many antibiotics. Consequently, a protracted course of treatment with a combination of antibiotics is required, and relapse following treatment is common (White, 2003). There is currently no vaccine available for the prevention of disease.

It has been shown that *B. pseudomallei* has the ability to survive and multiply inside host cells such as epithelial cells and macrophages (Harley *et al.*, 1998). However, the mechanisms by which the bacterium invades cells and resists host cell killing mechanisms are poorly understood. *B. pseudomallei* has been found in mature phagosomes (Stevens *et al.*, 2002), and therefore should possess mechanisms that provide resistance to killing by reactive oxygen intermediates, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Bogdan *et al.*, 2000). The bactericidal action of these molecules normally results in damage to bacterial proteins, membranes

Abbreviations: GST, glutathione S-transferase; i.p., intraperitoneal; PMNs, polymorphonuclear neutrophils; RE, restriction endonuclease; SOD, superoxide dismutase.

and nucleic acids (Imlay, 2003). The most potent reactive oxygen intermediate is superoxide (O_2^-), and a key strategy by which bacteria avoid killing by O_2^- involves a group of enzymes called superoxide dismutases (SODs). These enzymes catalyse the conversion of superoxide to hydrogen peroxide (H_2O_2), which can then be detoxified by other enzymes (Fridovich, 1995). Three types of SOD have been reported in bacteria (SodA, SodB and SodC), classified according to their corresponding metal co-factor [Mn^{2+} , Fe^{3+} or Cu^{2+}/Zn^{2+} , respectively (Grace, 1990)]. SodC enzymes have been reported to act on exogenous O_2^- and have therefore been linked to virulence. For example, *sodC* mutants of *Salmonella typhimurium*, *Salmonella choleraesuis* and *Salmonella dublin* are attenuated in mice (Farrant *et al.*, 1997), and *sodC* has been shown to play a role in the virulence of *Mycobacterium tuberculosis*, *Neisseria meningitidis*, *Vibrio vulnificus* and *Burkholderia cenocepacia* (Dunn *et al.*, 2003; Kang *et al.*, 2007; Keith & Valvano, 2007; Piddington *et al.*, 2001). A defect in the virulence of a *Francisella tularensis* *sodC* mutant is not apparent in interferon-gamma ($-/-$), inducible nitric oxide synthase ($-/-$) or NADPH oxidase disorder phox ($-/-$) mice, confirming the role of SodC in resisting host-generated reactive oxygen species (Melillo *et al.*, 2009).

In this study, we have determined the role of *B. pseudomallei* *sodC* in virulence, and investigated whether phagocytes from diabetics might have an impaired ability to kill *B. pseudomallei* by superoxide generation.

METHODS

Bacterial stains, macrophage cell line, culture conditions and reagents. *Escherichia coli* strains DH5 α and S17-1 λ pir, and *B. pseudomallei* K96243, were grown at 37 °C in Luria–Bertani (LB) broth. Chloramphenicol (30 μ g ml $^{-1}$ for *E. coli* and 50 μ g ml $^{-1}$ for *B. pseudomallei*) and kanamycin (50 μ g ml $^{-1}$ for *E. coli* and 400 μ g ml $^{-1}$ for *B. pseudomallei*) were added as required. LB agar without sodium chloride but containing 10% (w/v) sucrose was used in the final step of mutant selection. Bacterial growth was determined by measuring OD $_{600}$ in triplicate. The mouse macrophage cell line J774A.1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Hyclone), 1% L-glutamine (250 mM) (Hyclone) and 1% penicillin/streptomycin (Hyclone) at 37 °C with 5% CO $_2$. For infection with *B. pseudomallei*, macrophages were cultured in Gibco L-15 medium (Invitrogen) at 37 °C without CO $_2$. Reagents used in this study were obtained from Sigma-Aldrich, unless stated otherwise.

***B. pseudomallei* SodC overexpression and purification.** The *sodC* gene (BPSL1001) is located on chromosome 1 between nucleotides 1 164 723 and 1 165 262. The gene was PCR-amplified without the region encoding the signal peptide and cloned into pSTBlue-1 (Novagen). After sequencing to confirm the authenticity of the cloned DNA, the fragment was excised using *Eco*RI and *Sal*I, cloned into suitably digested pGEX4T-2 (GE Healthcare) and transformed into *E. coli* strain BL21 Star (Invitrogen). For expression of glutathione S-transferase (GST)–SodC, *E. coli* strain BL21 Star harbouring pGEX-SodC was cultured overnight at 37 °C in LB broth supplemented with 100 μ g ampicillin ml $^{-1}$, and 0.1 ml of this culture was used to inoculate 10 ml LB broth containing 100 μ g ampicillin

ml $^{-1}$. The culture was incubated at 37 °C with shaking at 250 r.p.m. until the OD $_{600}$ reached 0.5, when expression was induced by adding IPTG (Sigma) to a final concentration of 0.1 mM with continued incubation for 4 h. The culture was then centrifuged to harvest the cell pellet and a cell lysate prepared using BugBuster (Novagen). To purify the GST–SodC fusion protein, the soluble fraction was loaded onto a glutathione affinity column (Novagen), and after washing, the bound protein was eluted using reduced glutathione according to the manufacturer's protocol. Purified SodC was analysed by SDS-PAGE using NuPAGE Bis-Tris 4–12% gels (Invitrogen).

Measurement of SodC activity. SodC activity was measured using a commercial assay (Fluka) according to the manufacturer's instructions. The microtitre plate was incubated at 37 °C for 20 min, then the A $_{450}$ was read by a Model 680 microtitre plate reader (Bio-Rad) and the SOD activity calculated.

PCR amplification. Hot Start *Taq* (Invitrogen) or the FailSafe PCR system (EpiBio) was used to amplify DNA. Primers SodC F (5'-CCAGGAATTCAGGCCATCGGCCTGTTGGCC-3') and SodC R (5'-TCGAGTCGACTCATTGCCGCACGACGCCGC-3') were used to amplify *sodC* with flanking *Eco*RI and *Sal*I restriction endonuclease (RE) sites (shown underlined). Primers for mutant verification were UpC fw (5'-AGGACAACCCGATGGTGTG-3') and DnC rev (5'-CGGCGTCGTGTTTCGAGAATTC-3'). Primers for complementation were Ccom fw (5'-CGTAAACTCGAGCGCCG-3') and Ccom rev (5'-GCACCGACTAGTGCATTGC-3'), and included *Xho*I or *Spe*I RE sites (shown underlined).

***sodC* mutant construction.** A DNA fragment that included 500 bp regions upstream and downstream of the *B. pseudomallei* *sodC* coding region and flanked by *Sac*I and *Sal*I RE sites was synthesized by GENEART. The DNA fragment was ligated to *Sac*I- and *Sal*I-digested plasmid pDM4. The ligation product was electroporated into *E. coli* DH5 α , and the recombinant pDM4 was selected on LB agar containing 30 μ g chloramphenicol ml $^{-1}$. The recombinant plasmid was isolated and electroporated into *E. coli* S17-1 λ pir, then conjugated with *B. pseudomallei* K96243. The conjugation and *sacB* counter selection were carried out as described by Logue *et al.* (2009). Transconjugants were selected on LB agar containing 50 μ g chloramphenicol ml $^{-1}$. Colonies were selected and subcultured into LB broth, and an overnight culture was diluted 10 4 times and plated onto LB agar without salt containing 10% sucrose. After incubation at 24 °C for 2–3 days, colonies were picked and the *sodC* deletion verified by PCR using primers UpC fw and DnC rev.

Complementation. The *sodC* gene was amplified to include 150 bp of DNA upstream of the ATG start codon using primers Ccom fw and Ccom rev, cloned into pSTBlue-1 (Novagen) and verified by sequencing. After digestion of the recombinant plasmid with *Xho*I and *Spe*I, the DNA fragment was gel-purified and ligated into *Xho*I/*Spe*I-digested plasmid pBBR (Kovach *et al.*, 1994). The recombinant pBBRsodC plasmid was electroporated into *E. coli* DH5 α . The *E. coli* harbouring pBBRsodC were selected by plating on LB agar plates containing 50 μ g kanamycin ml $^{-1}$. Plasmid pBBRsodC was isolated from the recombinant *E. coli* DH5 α and electroporated into *E. coli* S17-1 λ pir. The plasmid was then mobilized into *B. pseudomallei* by conjugation with *E. coli* S17-1 λ pir containing pBBRsodC, and transformants were selected on LB agar plates containing 400 μ g kanamycin ml $^{-1}$. The authenticity of the transformants was verified by PCR.

In vitro sensitivity to superoxide. To determine sensitivity to intracellular superoxide, a disc-diffusion assay was used. Overnight cultures were diluted to OD $_{600}$ 1.0 and spread onto LB agar. Sterile filter discs containing 10 μ l of 0.25 or 0.5 mM paraquat were placed onto each plate. The zone of growth inhibition around the disc was

measured after overnight incubation. To generate extracellular superoxide, xanthine/xanthine oxidase (250 µM/0.14 U final concentrations, respectively) was used. Overnight cultures were diluted to 1×10^8 c.f.u. ml⁻¹, and to 1 ml of culture, catalase (100 U ml⁻¹ final concentration) was added to protect cells from H₂O₂ generated as a superoxide degradation product. At 30, 60 and 120 min, samples were serially diluted in $1 \times$ PBS, pH 7.2, and plated on LB agar plates. After 1–2 days incubation at 37 °C, colonies were counted.

Macrophage infection. Prior to infection, macrophages were cultured in L-15 medium. Overnight cultures of *B. pseudomallei* were diluted to 1×10^6 c.f.u. ml⁻¹ in L-15 medium, then added to wells seeded with 1×10^5 macrophages (m.o.i. of 10). After incubation at 37 °C for 2 h, extracellular bacteria were killed by replacing the growth medium with L-15 medium containing 50 µg ceftazidime ml⁻¹ for 2 h, followed by maintenance in L-15 medium containing 10 µg ceftazidime ml⁻¹. At 2, 4, 6 or 8 h post-infection, cells were washed with pre-warmed PBS to remove the antibiotic. Viable intracellular bacteria were released from the infected cells by adding 0.1% (v/v) Triton X-100. The cell lysates were serially diluted with sterile distilled water and appropriate dilutions plated on LB agar. The numbers of c.f.u. were counted after incubation at 37 °C for 24–36 h. All experiments were performed in triplicate with three technical replicates each.

Phagocytosis and oxidative burst in human polymorphonuclear neutrophils (PMNs). *B. pseudomallei* was labelled with FITC (Sigma), as previously described (Chanchamroen *et al.*, 2009), and suspended in PBS, pH 7.4, at a concentration of 1×10^8 c.f.u. ml⁻¹. FITC-labelled *B. pseudomallei* was analysed by flow cytometry. Whole blood samples from five healthy subjects were stimulated *in vitro* with FITC-labelled bacteria at an m.o.i. of 10 for 60 min, or with 3200 ng ml⁻¹ phorbol 12-myristate 13-acetate (PMA) (Sigma) for 15 min at 37 °C; then 25 µl 2800 ng hydroethidine ml⁻¹ (Sigma) was added and the preparation was incubated for 5 min at 37 °C. Erythrocytes were then lysed with Lysing Buffer (BD Biosciences), washed twice, and fixed with 10% (v/v) paraformaldehyde for decontamination prior to analysis by flow cytometry (FACSCalibur, BD Biosciences).

Enumeration of bacteria in human PMNs. Human PMNs were isolated from heparinized venous blood using 3.0% (w/v) Dextran T-500 sedimentation and Ficoll-Paque PLUS centrifugation (Amersham Biosciences). In all experiments, the PMN purity was 95%, as determined by Giemsa staining and microscopy, while the cell viability was 98%, as determined by trypan blue exclusion. Purified PMNs in RPMI 1640 were infected with *B. pseudomallei* at an m.o.i. of 0.3 at 37 °C for 30 min. The intracellular survival of *B. pseudomallei* in PMNs was determined after the extracellular bacteria were killed with 250 µg kanamycin ml⁻¹ at 37 °C for 30 min, and culture supernatants were checked for sterility by plating on LB agar plates.

Virulence studies. Female BALB/c age-matched mice, approximately 6 weeks old, were used in this study. The mice were grouped together in cages of five with free access to food and water and subjected to a 12 h light/dark cycle. For challenge, the animals were handled under bio-safety level III containment conditions. All investigations involving animals were carried out according to the requirements of the Animal (Scientific Procedures) Act 1986. In two separate experiments, groups of six mice were challenged with wild-type *B. pseudomallei* strain K96243 or Δ sodC mutant by the intraperitoneal (i.p.) route, and the infection was monitored for 5 weeks. In the first and second experiments, 3.7×10^4 and 4.98×10^4 c.f.u. of wild-type *B. pseudomallei* strain K96243, and 9×10^4 and 1.17×10^5 c.f.u. of the Δ sodC mutant, respectively, were given. Data from both experiments were combined into a meta-analysis, and survival was compared using logrank tests. Humane end points were

strictly observed and animals deemed incapable of survival were humanely killed by cervical dislocation.

RESULTS

The *B. pseudomallei* genome encodes a functional SodC

We identified a putative SodC-encoding gene (BPSL1001) from the genome sequence of *B. pseudomallei* K96243 (Holden *et al.*, 2004). The gene, which is located on the large chromosome, was found in all *B. pseudomallei* and *Burkholderia mallei* isolates for which complete genome sequence data are available (*B. pseudomallei* K96243, 1106a, 668, 1710b, MSHR346; *B. mallei* ATCC23344, NCTC10229, NCTC10247, SAVP1). The gene would encode a 179 aa protein with 80% sequence identity to the SodC of *B. cenocepacia* strain J2315 (Keith *et al.*, 2005). The protein was predicted by SignalP-HMM to possess a signal peptide (probability 0.81) that included a lipobox [Leu-Ala-Gly-Cys (Babu *et al.*, 2006)] between amino acids 26 and 29.

To investigate whether this gene encoded an SOD enzyme, BPSL1001 was expressed in *E. coli* strain BL21 Star as a GST fusion protein and purified using glutathione affinity chromatography (Fig. 1a). The purified *B. pseudomallei* protein showed SOD activity [$5.39 \text{ U } (\mu\text{g protein})^{-1}$] when 1 mM Cu²⁺ and 1 mM Zn²⁺ were added to the reaction. In the absence of either of these metal ions, enzyme activity was not detected. These findings confirmed that BPSL1001 encodes a Cu²⁺- and Zn²⁺-dependent SOD, consistent with it being a SodC enzyme.

Fig. 1. (a) SDS-PAGE of purified *B. pseudomallei* SodC protein. M, protein size markers. (b) PCR amplification from *B. pseudomallei* Δ sodC (lane 1) or wild-type (lane 2) DNA using primers flanking the *sodC* gene. M, molecular size markers.

Construction of a *B. pseudomallei* *sodC* deletion mutant

To assess the role of *sodC* in virulence we constructed a BPSL1001 allelic deletion mutant in *B. pseudomallei* K96243. DNA encoding the upstream and downstream regions of BPSL1001 was cloned into the pDM4 suicide vector and conjugated into *B. pseudomallei*. Mutants were selected on LB agar containing 10% (w/v) sucrose and verified by PCR using specific primers upstream and downstream of the gene (Fig. 1b). The PCR product generated from the mutant (predicted 1245 bp) was approximately 500 bp smaller than the amplified fragment from the wild-type (predicted 1769 bp). Growth (measured as OD₆₀₀) in LB broth of the wild-type, Δ *sodC* mutant and the complemented mutant was determined at 4, 6, 8, 12 and 24 h. There was no significant difference in the growth rates of the strains (Fig. 2), although the mutant entered the stationary phase at a lower density ($P=0.032$, comparing the wild-type and mutant at 12 h).

B. pseudomallei Δ *sodC* shows increased sensitivity to killing by extracellular superoxide

Compared with wild-type *B. pseudomallei*, the Δ *sodC* mutant did not show increased sensitivity to superoxide generated intracellularly using paraquat (Fig. 3a). However, after 30 min exposure to extracellular superoxide, generated using xanthine and xanthine oxidase, the Δ *sodC* mutant showed decreased survival compared with the wild-type. This trend continued over the course of the experiment (Fig. 3b). A wild-type phenotype was restored

Fig. 2. Growth of *B. pseudomallei* K96243 (●), Δ *sodC* mutant (▲) or Δ *sodC* mutant complemented with a plasmid-borne *sodC* gene (Δ) in LB broth aerobically at 37 °C, determined by OD₆₀₀. Error bars, SD from triplicate experiments.

Fig. 3. Susceptibility of *B. pseudomallei* to intracellularly (a) or extracellularly (b) generated superoxide. (a) Zones of inhibition of growth of *B. pseudomallei* K96243 (white bars), Δ *sodC* mutant (grey bars) or Δ *sodC* mutant complemented with a plasmid-borne *sodC* gene (black bars) around filter paper discs soaked with different concentrations of paraquat. Error bars, SD from three independent experiments. (b) Survival of 1×10^8 c.f.u. of *B. pseudomallei* K96243 (●), Δ *sodC* mutant (▲) or Δ *sodC* mutant complemented with a plasmid-borne *sodC* gene (Δ) after incubation with xanthine, xanthine oxidase and catalase. Error bars, SD from triplicate experiments.

by complementation of the Δ *sodC* mutant with a plasmid-borne *sodC* gene and associated upstream sequence.

B. pseudomallei Δ *sodC* has a reduced ability to survive in murine macrophages

To determine the role of *sodC* in intracellular survival, J774A.1 macrophages were infected with *B. pseudomallei* K96243 or the Δ *sodC* mutant. The numbers of intracellular bacteria at 2, 4, 6 and 8 h post-infection were determined. Whereas *B. pseudomallei* K96243 grew in macrophages over the course of the experiment (Fig. 4), the numbers of Δ *sodC* bacteria progressively decreased over the course of the study ($P=0.00003$ at 8 h post-infection). This defect in

Fig. 4. Number of intracellular *B. pseudomallei* K96243 (white bars), Δ sodC mutant (grey bars) or Δ sodC mutant complemented with a plasmid-borne sodC gene (black bars) recovered at 2, 4, 6 or 8 h post-infection of J774A.1 macrophages (initial m.o.i. 10). Error bars, SD from triplicate experiments. Asterisks indicate significant differences ($P < 0.01$).

macrophage survival was reversed in the complemented mutant.

SodC does not modulate uptake of *B. pseudomallei* or oxidative burst activity by human PMNs

Next, human PMNs were isolated and infected with either the wild-type or the Δ sodC mutant. Initially, bacteria were labelled with FITC, and FACS analysis was used to measure the uptake of bacteria. At 30 min post-infection there was no significant difference in the rate of phagocytosis shown by PMNs infected with wild-type, Δ sodC mutant or complemented Δ sodC cells (Fig. 5a) ($P > 0.05$). In addition, the oxidative burst in PMNs infected with wild-type, Δ sodC mutant or complemented Δ sodC cells was not significantly different (Fig. 5b) ($P > 0.05$).

Neutrophils from diabetics are not defective in superoxide killing of *B. pseudomallei*

Altered neutrophil function is believed to play an important role in the increased susceptibility of diabetics to infection with *B. pseudomallei* (Chanchamroen *et al.*, 2009), and studies have indicated that PMNs from diabetics have a reduced ability to generate superoxide (Shah *et al.*, 1983; Wykretowicz *et al.*, 1993). Therefore we investigated whether SodC played a different role in the survival of *B. pseudomallei* in PMNs from healthy and diabetic individuals. We purified PMNs from healthy ($n=3$) or diabetic individuals ($n=3$), and incubated the PMNs with wild-type, Δ sodC mutant or complemented Δ sodC *B. pseudomallei*.

At 1 h post-infection we recovered significantly fewer wild-type ($P < 0.001$), Δ sodC mutant ($P < 0.01$) or complemented Δ sodC bacteria ($P < 0.05$) from PMNs from diabetic donors than from PMNs from healthy donors (Fig. 5c). However, compared with wild-type bacteria, the recovery of the Δ sodC mutant was reduced by a similar proportion in healthy and in diabetic PMNs (Fig. 5c).

A *B. pseudomallei* sodC mutant is attenuated in a mouse model of infection

To further investigate the role of SodC in virulence we infected BALB/c mice with strains of *B. pseudomallei*. In two separate experiments, mice were challenged with 3.7×10^4 [141 minimum lethal dose (MLD)] or 4.98×10^4 (190 MLD) c.f.u. of *B. pseudomallei* K96243, or with 9×10^4 or 1.17×10^5 c.f.u. of the Δ sodC mutant (Fig. 6). By day 35 post-challenge, only one of 12 mice challenged with the wild-type survived, whereas eight of 12 of the mice challenged with the Δ sodC mutant were alive ($P < 0.05$). At day 35, all of the surviving mice which had been challenged with the Δ sodC mutant were culled and *B. pseudomallei* was readily isolated from the spleen, lung and liver of these mice (data not shown).

DISCUSSION

B. pseudomallei is an intracellular pathogen able to survive in a range of host cells, including professional phagocytes (Harley *et al.*, 1998; Jones *et al.*, 1996). The ability of the bacterium to survive within host cells is central to its ability to cause disease and to the evasion of the host responses to infection. *B. pseudomallei* is able to survive within phagosomes, and its type III secretion system then mediates bacterial escape into the cytoplasm, which is the primary site of replication in the host cell (Stevens *et al.*, 2002; Wiersinga *et al.*, 2006). Phagosome escape is reported to occur at 1–3 h post-infection (Burtnick *et al.*, 2008; Stevens *et al.*, 2002), with some bacteria found in late endosomes (Stevens *et al.*, 2002). Subsequently, autophagosomes may play a role in the control of infection, though *B. pseudomallei* appears to have evolved mechanisms to avoid extensive uptake and killing by this pathway (Cullinane *et al.*, 2008; Gong *et al.*, 2011).

Much of the recent work on mechanisms of survival and the growth of *B. pseudomallei* in host cells has focused on its ability to escape from the phagosome (Puthucheary & Nathan, 2006; Stevens *et al.*, 2002, 2005). The strategies

Fig. 5. Infection of human PMNs by *B. pseudomallei*. (a) Phagocytosis of *B. pseudomallei* by PMNs measured by FACS analysis of cells which had been incubated with FITC-labelled bacteria. (b) Oxidative burst in infected PMNs again measured by FACS analysis. (c) Ability of *B. pseudomallei* to infect PMNs from healthy (H) or diabetes mellitus (DM) individuals. Bacteria within PMNs were enumerated at 60 min post-infection. Data shown are the means of two determinations each from three healthy and three diabetic individuals (* P <0.05, ** P <0.01, *** P <0.001).

Fig. 6. Survival of BALB/c mice challenged via the i.p. route with *B. pseudomallei* K96243 (●, dashed line) or the Δ SodC mutant (■, unbroken line). Data from two experiments were combined into a meta-analysis, survival was compared using logrank tests, and the percentage survival plotted up to 35 days after infection, after which surviving animals were culled.

which allow bacterial survival in endosomes have received much less attention. A previous study by Miyagi and co-workers reported that reactive oxygen intermediates play only a minor role in killing *B. pseudomallei* within phagocytes (Miyagi *et al.*, 1997). The limited role of reactive oxygen intermediates in killing was also supported by Breitbach *et al.* (2006), who showed that compared with wild-type mice, bone marrow macrophages from C57BL/6gp91phox^{-/-} mice show only a slightly impaired capacity to kill internalized *B. pseudomallei*. Conversely, these workers showed that C57BL/6gp91phox^{-/-} mice are markedly more susceptible to infection, suggesting a role for reactive oxygen intermediates in resistance to infection. This could indicate a role for superoxide generated in neutrophils rather than in macrophages for the control of infection (Breitbach *et al.*, 2006).

The role of reactive nitrogen intermediates in the control of infection is equally unclear, with Miyagi *et al.* (1997) showing that they play a major role in the control of infection in J774 cells, but Breitbach *et al.* (2006) reporting that compared with the wild-type, macrophages from C57BL/6iNOS^{-/-} mice are equally resistant to infection and C57BL/6iNOS^{-/-} mice are no more susceptible to infection than wild-type mice.

In this study we show that *sodC* encodes an SOD. *B. pseudomallei* SodC would be able to catalyse the conversion of superoxide to hydrogen peroxide, which is less harmful to the bacterial cell and could potentially be degraded by other *B. pseudomallei* enzymes, such as KatG (a catalase-peroxidase) and AhpC (an alkyl hydroperoxide reductase) (Loprasert *et al.*, 2003, 2004). The ability of this enzyme to protect against exogenous rather than endogenous superoxide is consistent with the presence of a predicted signal

peptide and the consequent export of the protein across the cytoplasmic membrane. We also identified a lipobox within the SodC signal sequence, although it is not clear what role this plays in membrane anchoring. The *B. cenocepacia* and *M. tuberculosis* SodC proteins both include a lipobox, but whereas the former protein is found predominantly as an unanchored protein in the periplasmic space (Keith & Valvano, 2007), the latter is membrane-bound (D'Orazio *et al.*, 2001).

The roles of other SODs in the lifestyle of *B. pseudomallei* are not known. BPSL0880 in *B. pseudomallei* K96243 would encode a protein with 96% sequence identity to the *B. cenocepacia* SodB. Like *sodC*, the gene encoding the putative SodB is found in all of the *B. pseudomallei* and *B. mallei* strains for which complete genome sequences are available. SodB is not predicted to possess a signal sequence and therefore may be located in the cytoplasm. It is possible that this enzyme is responsible for the resistance to paraquat that we have demonstrated.

Chanchamroen and co-workers have previously reported that *B. pseudomallei* is phagocytosed and killed by human PMNs (Chanchamroen *et al.*, 2009). Our results indicate that there is no significant difference in the ability of human PMNs to phagocytose or mount an oxidative burst towards the wild-type or the *sodC* mutant. However, our finding that at 1 h post-infection the numbers of the *sodC* mutant were lower than the number of wild-type bacteria indicates that the mutant is defective in survival and/or growth in these cells.

We also investigated the possibility that the *sodC* mutant would show a further reduced ability to survive in PMNs from diabetics. The possibility that PMNs from diabetics show altered abilities to ingest and kill different micro-organisms has received considerable attention in the past. However, these studies have drawn different and inconsistent conclusions. Some studies have shown that compared with normal PMNs, cells from diabetics have a reduced ability to phagocytose micro-organisms (Delamaire *et al.*, 1997; Marhoffer *et al.*, 1992), but other studies have found no difference (Balasoiu *et al.*, 1997; Tater *et al.*, 1987). Similarly, several reports show a reduced ability of PMNs from diabetics to kill ingested micro-organisms (Gin *et al.*, 1984; Tan *et al.*, 1975; Tater *et al.*, 1987), whilst others report that killing capacity is unaffected (Balasoiu *et al.*, 1997). Experiments on PMNs have shown that a reduced oxidative burst can be linked to reduced microbicidal activity (Delamaire *et al.*, 1997; Marhoffer *et al.*, 1992; Shah *et al.*, 1983; Wykretowicz *et al.*, 1993), and indeed superoxide generation has been shown to be reduced (Shah *et al.*, 1983; Wykretowicz *et al.*, 1993). Conversely, other studies have suggested that the fact that PMNs are from diabetics has no impact on their ability to kill bacteria (Balasoiu *et al.*, 1997).

In studies with *B. pseudomallei*, it has previously been reported that PMNs from diabetics show a reduced ability to phagocytose this bacterium compared with PMNs from

healthy individuals (Chanchamroen *et al.*, 2009). Similarly, there is reduced uptake of *B. pseudomallei* by bone marrow dendritic cells from chronic diabetic mice compared with normal mice (Williams *et al.*, 2011). The reduced phagocytic ability of these cells gives an explanation for the reduced numbers of bacteria isolated from the diabetic PMNs tested in our study. Importantly, this study shows that the numbers of wild-type, Δ *sodC* mutant or complemented mutant cells recovered from diabetic PMNs are similarly reduced in diabetic PMNs. This finding indicates that superoxide-mediated killing, at least during the early stages of infection, is not impaired in PMNs from diabetics.

In conclusion, our findings have shown that BPSL1001, when expressed in *E. coli*, encodes a protein with SOD activity. A *B. pseudomallei* deletion mutant in this gene showed increased sensitivity to killing by extracellular superoxide. When complemented with a plasmid-borne *sodC* gene, wild-type resistance levels were restored. We have also shown the importance of *sodC* in virulence and survival within host cells by the reduced numbers of the Δ *sodC* mutant isolated from murine macrophages and the reduced virulence of the Δ *sodC* mutant in BALB/c mice when compared with the wild-type.

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