Comparative analysis of the responses of related pathogenic and environmental bacteria to oxidative stress

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Bacillus anthracis, the causative agent of anthrax, is exposed to host-mediated antibacterial activities, such as reactive oxygen species (ROS), during the early stages of its disease process. The ability to resist these host-mediated stresses is an essential characteristic of a successful pathogen while it is generally assumed that non-pathogenic environmental bacteria succumb to these antimicrobial activities. In order to gain insights into the underlying mechanisms that pathogens use to resist host-mediated oxidative stress, we have compared the oxidative stress responses of *B. anthracis* and *Bacillus subtilis*, a well-studied environmental bacterium. Among the four putative catalases encoded by *B. anthracis* we identified KatB as the main vegetative catalase. Comparative analysis of catalase production in *B. anthracis* and *B. subtilis* in response to superoxide and peroxide stress reveals different expression profiles, even though both are regulated by the PerR repressor, which senses and responds to peroxide stress. A *B. anthracis* *perR* deletion mutant exhibits enhanced KatB activity and is hyper-resistant to peroxide stress. Superoxide dismutase A1 (SodA1) is the main contributor to the intracellular superoxide dismutase activity in vegetative cells and the gene encoding this enzyme is constitutively expressed. Although aspects of the ROS detoxifying systems of *B. anthracis* and *B. subtilis* are similar, their responses to superoxide stress are different. The observed differences are likely to reflect adaptations to specific environmental niches.

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INTRODUCTION

Bacillus anthracis and *Bacillus subtilis* are closely related bacteria that perform similar basic cellular processes (Read *et al.*, 2003). *In vivo*, *B. anthracis* is a pathogen while *B. subtilis* is a free-living soil bacterium. In the case of *B. anthracis*, spores are the main agent for initiating infection via a respiratory, gastrointestinal or cutaneous route of entry into the host. Following infection, spores are engulfed by macrophages where they germinate to form metabolically active vegetative cells. The spores and vegetative cells must be able to evade the antibacterial activities of the innate immune system and, ultimately, to establish the disease anthrax. Consequently, *B. anthracis* needs to adapt rapidly to the hostile environment encountered within the macrophage, including a potentially bactericidal oxidative burst catalysed by the NADPH oxidase complex located in the phagosomal membrane. This transfers a single electron to molecular oxygen, generating superoxide radicals ($O_2^{\cdot-}$). Both spontaneous and enzymically catalysed reactions lead to the production of other reactive oxygen species (ROS),

including hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) (Raupach & Kaufmann, 2001). In contrast, the levels of oxidative stress to which an environmental bacterium such as *B. subtilis* is exposed to are generally low and are mainly associated with toxic forms of oxygen generated as a consequence of aerobic growth (Messner & Imlay, 1999).

In response to oxidative stress, bacteria upregulate the expression of genes whose products function to either protect cells from the effects of ROS or repair any resulting damage. These products include key detoxifying enzymes such as catalases, peroxidases, superoxide dismutases (SODs), alkyl hydroperoxide reductase and the organic hydroperoxide resistance proteins (Imlay, 2008; Seaver & Imlay, 2001). Mechanisms regulating the oxidative stress response have been extensively studied in *B. subtilis* (Zuber, 2009), but less so in *B. anthracis*. *B. subtilis* is therefore an attractive model for studying the regulation and functional activity of proteins required for oxidative stress resistance and for comparative analyses with related pathogenic species that routinely encounter exogenous ROS as a consequence of their life style.

The antioxidant enzymes SOD and catalase have long been implicated as virulence determinants of pathogenic bacteria

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase.

(Edwards *et al.*, 2001; Fang *et al.*, 1999; Kanafani & Martin, 1985). SODs catalyse the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2 , while catalase breaks down H_2O_2 to water and O_2 . In *B. subtilis*, SodA is the main intracellular SOD and is required for resistance to superoxide stress (Herbig & Helmann, 2001; Inaoka *et al.*, 1999). Two other putative SODs in *B. subtilis*, SodF and YojM, are not as well characterized although the latter is likely to reside in a non-cytoplasmic location. Four SODs have been identified in *B. anthracis* (Passalacqua *et al.*, 2006). *B. anthracis* SodA1 (BA4499) and SodA2 (BA5696) are structurally related cytoplasmic proteins that are annotated as iron/manganese SODs. Recently, however, we have shown that SodA1 is cambialistic for iron and magnesium while SodA2 is exclusively coordinated with iron, and therefore more correctly annotated as SodB (Tu *et al.*, 2012b). The biochemistry of the remaining SODs, Sod15 (BA1489) and SodC (BA5139), has not been analysed in detail, but they are putatively active with iron/manganese and copper/zinc, respectively (Passalacqua *et al.*, 2006; Read *et al.*, 2003). The Sod15 preprotein has a putative type II signal peptide at its N terminus and is therefore likely to be anchored to the outer surface of the cytoplasmic membrane. Although SodA1 is the main SOD present in exponentially growing cells, phenotypic and mutagenesis studies indicate that all four SODs contribute to the virulence of *B. anthracis* (Cybulski *et al.*, 2009; Passalacqua *et al.*, 2006).

Hydrogen peroxide generated from the dismutation of $O_2^{\cdot-}$ can be detoxified by catalase and this enzyme is an important line of defence against peroxide stress. Comparison of the oxidative stress responses of *B. anthracis* and *B. subtilis* under comparable growth conditions and stress levels shows that KatB, a homologue of *B. subtilis* KatA, is the main vegetative catalase protecting *B.*

anthracis against peroxide stress. The response of *B. subtilis* to peroxide stress is regulated by the metalloregulatory protein PerR (peroxide regulator), a homologue of bacterial Fur-like (ferric-uptake regulator) proteins (Bsar *et al.*, 1998; Mongkolsuk & Helmann, 2002). *B. subtilis* PerR is a repressor, and derepression of the PerR regulon is dependent on the presence of bound Mn^{2+} and Fe^{2+} ions (Herbig & Helmann, 2001; Storz & Imlay, 1999). Genes regulated by *B. subtilis* PerR include *kata* (catalase), *mrgA* (a Dps homologue), *ahpCF* (alkyl hydroperoxide reductase) and *hemAXCDBL* (haem biosynthesis enzymes) (Chen & Helmann, 1995; Haikarainen & Papageorgiou, 2010; Zuber, 2009). We show that the deletion of *B. anthracis* PerR leads to the derepression of *katB* and to an increase in KatB activity. However, the expression profile of *B. anthracis* *katB* expression in response to superoxide stress was significantly different from that of *B. subtilis* *kata*, and the physiological significance of this is discussed in relation to the different lifestyles of these bacteria.

METHODS

Bacterial strains and culture conditions. The bacterial strains are listed in Table 1. *B. anthracis* strain UM23C1-2 (Hoffmaster & Koehler, 1997) was used for the generation of $\Delta perR$, $\Delta katB$ and $\Delta sodA1$ mutant strains, created by replacement of their coding sequences with an omega kanamycin resistance cassette (Saile & Koehler, 2002). An isogenic $\Delta sodA$ mutant of *B. subtilis* 168 was generated by transformation of chromosomal DNA from *B. subtilis* AH1490 (Table 1). Bacterial cultures were routinely grown at 37 °C, in Luria–Bertani (LB) broth or on LB agar. The culture medium was supplemented as necessary with antibiotics: ampicillin (50 $\mu g\ ml^{-1}$), erythromycin (400 $\mu g\ ml^{-1}$ for *E. coli*; 5 $\mu g\ ml^{-1}$ for *B. anthracis*), kanamycin (20 $\mu g\ ml^{-1}$), chloramphenicol (6 $\mu g\ ml^{-1}$) and spectinomycin (100 $\mu g\ ml^{-1}$ for *B. subtilis*).

Table 1. Strains and plasmids used in this study

Rif^r, Rifampimycin resistance; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance; Erm^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance.

Bacterial strain or plasmid	Trait or relevant genotype	Source or reference
<i>B. anthracis</i>		
UM23C1-2	pXO1 ⁻ pXO2 ⁻ Ura ⁻ Rif ^r	Hoffmaster & Koehler (1997)
$\Delta perR$	UM23C1-2 $\Delta perR$::Km ^r	Tu <i>et al.</i> (2012a)
$\Delta katB$	UM23C1-2 $\Delta katB$::Km ^r	This work
$\Delta sodA1$	UM23C1-2 $\Delta sodA1$::Km ^r	Tu <i>et al.</i> (2012b)
<i>B. subtilis</i>		
168	<i>trpC2</i>	Anagnostopoulos & Spizizen (1961)
AH1490	<i>trpC2 metC3 sodA</i> ::pAH402, Sp ^r ; derivatives of <i>B. subtilis</i> MB24, <i>trpC2 metC3 Spo</i> ⁺	Henriques <i>et al.</i> (1998)
$\Delta sodA$	<i>trpC2 sodA</i> ::pAH402	This work
$\Delta katA$	<i>trpC2 katA</i> ::pHTKA1, Erm ^r	Engelmann & Hecker (1996)
Plasmids		
pUTE583	Cm ^r in <i>E. coli</i> ; Erm ^r in <i>B. anthracis</i>	Chen <i>et al.</i> (2004)
pUTE618	Cm ^r Km ^r in <i>E. coli</i>	Chen <i>et al.</i> (2004)
pGEM-T	Amp ^r in <i>E. coli</i>	Promega

DNA manipulations and general techniques. DNA was extracted with the DNeasy Blood and Tissue kit (Qiagen). Transformation of *B. subtilis* was performed according to the method of Anagnostopoulos & Spizizen (1961). All PCRs were carried out with Platinum *Pfx* DNA polymerase (Invitrogen) with primers (Table 2) purchased from Invitrogen. PCR products were purified by using the QIAquick PCR purification kit (Qiagen).

Growth curve assays. Overnight pre-cultures were grown at 37 °C with agitation (220 r.p.m.) in LB broth. To monitor growth, overnight cultures were diluted into fresh pre-warmed LB broth to OD₅₄₀ 0.05 and growth was continued under the same conditions. Oxidative compounds (H₂O₂ or paraquat) were added at mid-exponential phase (OD₅₄₀ 0.3) at the indicated concentrations. The sensitivity of the cells to the compounds was determined by comparison with an untreated control. Growth curves were determined on at least two separate occasions using independent cultures.

Enzyme assays for SOD and catalase. Oxidative stress was induced by the addition of either H₂O₂ or paraquat at the indicated final concentrations to cultures at mid-exponential phase (OD₅₄₀ 0.3) and the cells were harvested 60 min later by centrifugation (3700 g, 10 min, 4 °C). The cell pellets were washed once in cold 50 mM phosphate buffer, pH 8.0, and resuspended in 750 µl 50 mM phosphate buffer, pH 8.0, in a 2.0 ml Teflon disruption vessel containing approximately equal volumes of glass beads (diameter 0.10–0.11 mm). Cells were homogenized by vigorous shaking for 2 min in a Mikro-Dismembrator (Braun) operated at 2600 r.p.m.

The process was repeated three or four times with at least 8 min cooling on ice in between treatments. The lysates were centrifuged (12 000 g, 15 min, 4 °C) to remove cell debris and the resulting supernatant was cleared by a second centrifugation step (20 000 g, 30 min, 4 °C). Glycerol was added to a final concentration of 12–15 % (v/v) prior to storage at –20 °C. The protein content was quantified using the 2-D Quant kit (GE Healthcare) and 1–2.5 µg was used for the determination of SOD or catalase activities. SOD activity was measured with a SOD assay kit-WST (Fluka Analytical, Sigma) and standardized using recombinant *Escherichia coli* MnSOD (Sigma-Aldrich). The Amplex Red Catalase Assay kit (Molecular Probes) was used to determine catalase activity.

RNA extraction. Cultures (~10⁸ cells) were resuspended in an equal volume of RNA stabilization solution (RNAlater, Ambion) and harvested by centrifugation (5000 g, 10 min, 4 °C). The RiboPure Bacterial kit (Ambion) was used for the extraction of total cell RNA according to the manufacturer's protocol with modifications to optimize RNA yield. The cells were disrupted with 450 µl RNAwiz (Ambion) and 250 µl ice-cold zirconia beads for 15 min using a Mikro-Dismembrator (Braun). Chloroform (0.2 vols) was added to the harvested bacterial lysate for phase separation. After centrifugation at 4 °C (5000 g, 10 min), the aqueous phase containing partially purified RNA (200–250 µl) was recovered and purified on a glass-fibre cartridge. Bound RNA was eluted with 70 µl elution solution and RNase-free DNase was added to remove trace amounts of genomic DNA. The RNA integrity was determined by ensuring an A₂₆₀:A₂₈₀ ratio of between 1.8 and 2 and by agarose gel electrophoresis on a formaldehyde denaturing gel.

Table 2. Oligonucleotide primers used in this study

Primer sequences were derived from the genome sequences of *B. anthracis* (AnthrList) or *B. subtilis* (SubtiList). Lower-case letters indicate a 5' extension with T7 promoter sequence for the creation of an antisense RNA probe. Restriction sites are underlined and additional clamp bases are in bold.

Primer name (function)	Sequence (5'–3')	PCR amplification product (bp)
Real-time RT-PCR		
katB-F	ATCCAGATACACAGCGTTAC	180
katB-R	GGTAAGCTGGGTCTTCAAC	
sodA1-F	CTAACCACACATTCTTCTGG	170
sodA1-R	TACTACTAACCAAGCCCAAC	
Ban gyrB-F*	ACCGTTCTTCATGCTGGTG	170
Ban gyrB-R*	GCAACCGGAATACCTCTTTC	
katA-F	ACTCAATGACTGCCGGTTC	158
katA-R	GTTACGTCGTTTGTCACCTC	
sodA-F	ATGGCTTACGAACCTCCAG	184
sodA-R	CAAGATCTGCAACAAGCTC	
Bsu gyrB-F†	ACGGCATTACGGTTGAAGTG	199
Bsu gyrB-R†	TCATCTCCGCTTAGGTTTGG	
Northern blot		
katB-F2	ATGAATCCAAATAATCGCTT	624
katB-R2	ctaatacgactcactatagggagaGAATGAATGTACACCGAAAC	
Mutant construction		
katB up-F	gcctgctctcga ATGAATCCAAATAATCGCTTAACA	<i>katB</i> upstream region (500)
katB up-R	gtaccggaattc GTCATGAAATCCCAGTAACGATCT	
katB down-F	gtaccgaagctt TTATTTCGGAGCATTTCTACATA	<i>katB</i> downstream region (500)
katB down-R	gtaccggaattc TTGAACCATCTGAAGATAAATTAT	

*Primers amplify the specific region of the *gyrB* gene in *B. anthracis*.

†Primers amplify the specific region of the *gyrB* gene in *B. subtilis*.

Real-time quantitative RT-PCR. HPLC-purified RT-PCR primers (Table 2) were purchased from Invitrogen. First strand cDNA was generated from total RNA with a reverse transcription (RT) reaction using the Moloney murine leukaemia virus (MMLV)-RT kit (Promega). Briefly, 1 µg total RNA was denatured at 65 °C for 5 min in the presence of random hexamer primers pd(N)₆ (GE Healthcare) followed by 2 h incubation at 42 °C for primer extension. Relative quantitative real-time RT-PCR amplification and analysis were performed using a LightCycler 480 and software system (Roche Diagnostics) and SYBR Green fluorescence chemistry (SYBR Green I Master kit, Roche Diagnostics). The RT-PCRs (20 µl) with cDNA template were prepared according to the manufacturer's instructions. Real-time PCR amplification was accomplished with an initial 10 min denaturation and anti-*Taq* DNA polymerase antibody-inactivation step at 95 °C, followed by 40–45 cycles of amplification: 10 s at 95 °C, 20 s at 55 °C and 5 s at 72 °C. Measurement of the SYBR Green fluorescence signal at 72 °C was performed at the end of each amplification cycle. Melting-curve analysis from 65 to 97 °C, followed by the final cooling step to 40 °C was carried out to confirm that only a single specific product was amplified. The housekeeping/reference genes (*gyrB* of both *B. anthracis* and *B. subtilis*) were used to compensate for variations in the cDNA input and sample-to-sample differences. All experiments were carried out in duplicate. Target gene expression was calculated relative to the non-regulated reference gene (*gyrB*) and the results were expressed as a normalized ratio in arbitrary units.

Northern blot analysis. For Northern blot analysis, 3 µg total RNA was incubated (65 °C for 10 min) in 60 % v/v formamide, 3 mM NaH₂PO₄, 0.2 mM EDTA, 3.6 mM Tris/HCl (pH 7.8), and resolved on a 1.2 % agarose gel containing 6 % (w/v) formaldehyde in 20 mM MOPS buffer (pH 7.0) (Invitrogen). RNA was transferred to a positively charged nylon membrane (Hybond-N, Amersham Pharmacia Biotech) and fixed by treatment with UV light (UV Stratalinker, Stratagene). The *B. anthracis* *katB*-specific DIG-labelled RNA probe was synthesized by *in vitro* transcription with T7 RNA polymerase using a DIG RNA labelling kit (Roche) and specific PCR products as templates. Synthesis of the template by PCR was performed using the oligonucleotide primers listed in Table 2. Northern hybridization was performed as described by Eymann *et al.* (2002).

RESULTS

Following infection and engulfment by macrophages, *B. anthracis* is exposed to superoxide anions generated by the host NADPH oxidase. Cellular damage is restricted by a combination of spontaneous non-enzymic dismutation and superoxide dismutases (Beyer *et al.*, 1991; Imlay, 2008). Paraquat is a redox cycling agent that reacts with components of the respiratory chain in bacterial cells, leading to the univalent reduction of oxygen, and the generation of endogenous superoxide stress (Hassan, & Fridovich, 1979). The addition of paraquat to respiring bacterial cells results in the generation of intracellular O₂^{•−} and, by dismutation, to H₂O₂. Both endogenously generated and exogenous H₂O₂ are detoxified by catalase, the efficiency of which is essential for avoiding the generation of Fe²⁺-mediated hydroxyl radicals. *B. anthracis* is twice as resistant to superoxide stress and ten times more resistant to peroxide stress than *B. subtilis*. We therefore carried out a comparative analysis of the production of SOD and catalase by *B. subtilis* and *B.*

anthracis under comparable growth and stress conditions to account for their differing responses to superoxide and peroxide stress (Mostertz *et al.*, 2004; Pohl *et al.*, 2011).

The main SODs of *B. anthracis* and *B. subtilis* are constitutively expressed

B. subtilis SodA is the only SOD detected in the cytoplasm of *B. subtilis* vegetative cells and this enzyme has been shown to play an important role in protection against superoxide stress (Henriques *et al.*, 1998; Inaoka *et al.*, 1999). We found that, irrespective of the application of oxidative stress – paraquat (0.4 mM) or H₂O₂ (0.1 mM) – *sodA* gene expression was constitutive in *B. subtilis* (Fig. 1a, insert). The lack of change in *sodA* gene expression in response to oxidative stress was reflected in the measured cytoplasmic SOD enzyme activity, albeit with a small reduction (0.73-fold) in SOD activity in the H₂O₂-treated samples (Fig. 1a). Deletion of *B. subtilis* *sodA* resulted in ~98 % loss of SOD activity under all conditions (Fig. 1a).

SodA1 and SodA2 are the only SODs detectable in cytoplasmic extracts of *B. anthracis* (Passalacqua *et al.*, 2006; Tu *et al.*, 2012b). Our quantitative analysis of the SOD activity in the wild-type and Δ *sodA1* mutant showed that SodA1 was responsible for ~90 % of the intracellular SOD activity in this bacterium (Fig. 1b) in untreated control samples and in cells exposed to paraquat (0.8 mM) or H₂O₂ (1.0 mM). The SOD activity remaining in the cytoplasm of the Δ *sodA1* mutant is likely to be contributed by SodA2 (Passalacqua *et al.*, 2006; Tu *et al.*, 2012b) since SodC (BA5139) is likely to be secreted (Bendtsen *et al.*, 2004; Passalacqua *et al.*, 2006) and Sod15 (BA1489) to be spore-specific (Cybulski *et al.*, 2009; Liu *et al.*, 2004; Passalacqua *et al.*, 2006). Quantification of its expression revealed that *sodA1*, like *B. subtilis* *sodA*, is constitutively expressed in the wild-type (Fig. 1b, insert). The *sodA1* expression profile therefore correlates well with the largely unaltered cytoplasmic SOD activity (Fig. 1b).

The SOD activity of the *B. subtilis* Δ *katA* mutant was similar to that in the wild-type strain, except for a small reduction in the wild-type under peroxide stress that was not observed in the mutant (Fig. 1a). The SOD activity in *B. anthracis* was also not affected by inactivation of *katB* under any of the tested conditions (Fig. 1b).

The catalase genes of *B. anthracis* and *B. subtilis* are differentially expressed

Catalase is a highly efficient enzyme and therefore likely to be transiently induced. We analysed the induction kinetics of the main catalases in *B. subtilis* (KatA) and *B. anthracis* (KatB) in response to peroxide stress, measuring, respectively, *katA*- and *katB*-specific mRNA and catalase activity. Samples were taken at various times following the addition of H₂O₂ (0.1 mM for *B. subtilis*, 1.0 mM for *B. anthracis*) at concentrations that perturb growth but do not cause cell lysis. In both organisms, the expression of their respective

Fig. 1. Total SOD activities in response to peroxide and superoxide stress. (a) *B. subtilis* 168 and Δ sodA and Δ katA mutants (mid, dark and light grey, respectively), (b) *B. anthracis* UM23C1-2 and Δ sodA1 and Δ katB mutants (mid, dark and light grey, respectively). Enzyme activities were determined from cytoplasmic extract prepared 60 min after exposure to the oxidative compounds (0.1/1.0 mM H₂O₂ or 0.4/0.8 mM paraquat, *B. subtilis*/*B. anthracis*). The inset panels indicate the relative levels of expression of the *sodA* (*B. subtilis*) and *sodA1* (*B. anthracis*) genes in the respective wild-type strains. Error bars indicate SD from three independent experiments.

catalase genes peaked 10 min after the addition of H₂O₂, declining rapidly to a non-induced level within 25 min (Fig. 2). This pattern of gene expression results in a time-dependent increase in catalase activity that reaches a maximum ~30 min after exposure to H₂O₂, followed by a gradual reduction in specific activity. The absolute levels of catalase activity were similar in both organisms.

Previous transcriptional profiling reported that *B. subtilis* *katA* was induced in response to both peroxide and superoxide stress (Mostertz *et al.*, 2004), while our data show that *B. anthracis* *katB* is only induced in response to peroxide stress (Pohl *et al.*, 2011). We therefore compared the responses of *B. anthracis* and *B. subtilis* to oxidative stress under similar growth conditions and levels of stress (Fig. 3) to ensure the comparability of the data. *B. subtilis* *katA* was induced in response to both H₂O₂ (58-fold increase) and paraquat (19-fold increase) (Fig. 3a), and catalase activity was similarly increased (40- and 11-fold, respectively; Fig. 3b). *B. subtilis* *katA* is a member of the PerR regulon (Fuangthong *et al.*, 2002) and these results indicate that paraquat, directly or indirectly, activates the PerR regulon in this bacterium. The lack of significant levels of catalase activity in the *B. subtilis* Δ katA mutant confirmed that KatA is the main vegetative catalase in this bacterium (Fig. 3b).

B. anthracis encodes four putative catalases [BA0843, KatB (BA1159), BA3030, BA3164]. *B. anthracis* KatB is most closely related to *B. subtilis* KatA (53 % identity, 70 % similarity), and therefore we investigated the role of KatB in oxidative stress resistance. In contrast with *B. subtilis*

katA, the transcription of the *B. anthracis* *katB* gene was induced in response to H₂O₂ (11-fold) but not paraquat. This was confirmed by qRT-PCR analysis of the *katB* (Fig. 3a, c) and measurement of the catalase activity (Fig. 3d). To confirm that the lack of *katB* induction in response to superoxide stress was independent of the paraquat concentration, additional experiments were carried out with a growth-limiting concentration of paraquat (1.6 and 3.2 mM). In both cases, *katB* expression was similar to that at 0.8 mM paraquat (Fig. 3c, insert). Analysis of the Δ katB mutant indicated that KatB is responsible for between 70 and 90 % of the observed wild-type catalase activity (Fig. 3d).

Since treatment with paraquat induces catalase activity in *B. subtilis* but not *B. anthracis*, we determined the effect of increasing the superoxide stress by inactivating the main SODs in both of these strains. When catalase was assayed in the *B. subtilis* Δ sodA mutant, we found a higher level of activity than in the wild-type under all tested conditions. Compared with the wild-type strain, the catalase activity in the Δ sodA mutant was fourfold higher in the absence of stress, and 1.4-fold and 6.1-fold higher, respectively, in the presence of H₂O₂ and paraquat (Fig. 3b). The increased levels of catalase activity were reflected in the over-expression of the *katA* gene (2.3-fold untreated, 2.1-fold with H₂O₂ and 8.7-fold with paraquat; Fig. 3b). When this analysis was repeated with the *B. anthracis* Δ sodA1 mutant, the measured catalase activities and *katB* gene expression profiles were indistinguishable from those of the wild-type (Fig. 3c, d).

Fig. 2. Time-course of catalase gene expression (closed symbols) and enzyme activity (open symbols) in response to exposure to H₂O₂. (a) *B. subtilis* (0.1 mM H₂O₂) and (b) *B. anthracis* (1.0 mM H₂O₂). The catalase gene transcript levels (*katA* in *B. subtilis*, *katB* in *B. anthracis*) were determined by SYBR Green real-time qRT-PCR, normalized to the *gyrB* genes of their respective organisms which do not show any significant changes in expression under the experimental conditions. Data are the mean $\pm$ SD of three independent experiments. Soluble protein extracts were obtained from cells broken by glass bead lysis.

A major difference between the expression profile of *B. subtilis katA* and *B. anthracis katB* (Fig. 3) was the presence of two transcripts hybridizing to the *katB* probe of *B. anthracis*, one ~1.5 kb in length, the other ~2.5 kb in length (Fig. 4a), while *B. subtilis katA* is encoded by a monocistronic transcript of ~1.5 kb (data not shown). The size of the shorter transcript was consistent with a monocistronic transcript encoding *katB* while that of the less abundant larger transcript was consistent with a bicistronic transcript encoding the upstream gene *hemH-2* as well as *katB* (Fig. 4a, b). This was confirmed by Northern blotting using a *hemH-2* probe (data not shown). HemH-2 is predicted to be a ferroxidase, the last enzyme in the haem biosynthesis pathway (Taketani, 1993). The *B. anthracis* genome encodes two ferroxidases, HemH-1 and HemH-2, both of which show a high degree of sequence similarity with *B. subtilis* HemH (Olsson *et al.*, 2002). Treatment of *B. anthracis* with H₂O₂ upregulated the less abundant 2.5 kb *hemH-2-katB* bicistronic transcript to the same extent as the 1.5 kb monocistronic transcript, indicating that they are similarly regulated from independent promoters (Fig. 4b). A putative transcription termination element, comprising a 32 bp palindromic sequence (ΔG value of -11.8 kcal mol⁻¹), was identified 5 bp downstream of the *katB* translational stop codon (Fig. 4c).

B. anthracis katB is a member of the PerR regulon

B. anthracis BA0537 is a homologue of *B. subtilis* PerR, encoding the regulator of the peroxide response. To confirm the identity of BA0537 as PerR and to determine its role in *B. anthracis*, we determined the response of a

Fig. 3. Total catalase activity and catalase gene expression (*katA* and *katB*, respectively) in *B. subtilis* and *B. anthracis* in response to superoxide and peroxide stress. Catalase gene expression (a, c) and total catalase activity (b, d) were determined in *B. subtilis* strain 168 (a, b) and *B. anthracis* strain UM23C1-2 (c, d) and their respective Δ *sodA*/ Δ *sodA*1 and Δ *katA*/ Δ *katB* mutants. Relative gene expression was determined from total RNA extracted from the cells 10 min after exposure to the stressor and enzyme activities 60 min after exposure. Error bars indicate SD from three independent experiments. Bars: white, Δ *katA* (b) or Δ *katB* (d); grey, 168 (a, b) or UM23C1-2 (c, d); black, Δ *sodA*.

Fig. 4. *B. anthracis* *katB* is regulated by PerR and expressed as a monocistronic transcript or as a bicistronic transcript with *hemH-2*. (a) Northern hybridization analysis of *katB* transcription in *B. anthracis* UM23C1-2 and $\Delta perR$ mutant. Total RNA was extracted from the cells during mid-exponential growth in LB broth after 10 min exposure to H_2O_2 (H) and paraquat (P) and compared with that of the untreated control (C). The probe was an internal *katB* gene fragment. The approximate sizes of the transcripts are indicated (kb). (b) Schematic representation of the *hemH-2* and *katB* genetic organization and structure. The large arrows indicate the ORFs and their direction of transcription. The arrows below the map indicate the lengths of the transcripts and their orientation. The bent arrows indicate the putative promoters. The predicted hairpin loop structure immediately downstream of *katB* is shown. (c) Nucleotide sequence of the promoter 5' of *katB* and *hemH-2*. *hemH-2* is located immediately upstream of *katB*. Putative -35 and -10 regions, and ribosome-binding sites (RBS) are indicated in bold lower case type. Predicted PerR and Fur-binding sites are boxed. The start codon (ATG) and stop codon (TAA) for *katA* (single underline) and *hemH-2* (double underline) are as indicated. The 32 bp palindromic sequence predicted to be the transcriptional terminator (calculated $\Delta G = -11.8 \text{ kcal mol}^{-1}$) for *katB* is indicated by a dotted line. (d) Nucleotide sequence of the promoter 5' of *B. subtilis* *katA*. The putative PerR-binding site is boxed.

$\Delta perR$ mutant to oxidative stress, and this was analysed in comparison with the wild-type (Fig. 5). The $\Delta perR$ mutant exhibited the same growth kinetics as the wild-type in both untreated and paraquat-treated cultures (Fig. 5a), indicating that *B. anthracis* PerR does not play a role in

resistance to superoxide stress. In contrast, exposure of the $\Delta perR$ mutant to 1.0 mM H_2O_2 , the concentration of stressor normally used to perturb but not inhibit growth, had no visible effect on the growth (Fig. 5b). When the concentration of H_2O_2 was increased by fivefold, to

Fig. 5. Growth kinetics of wild-type *B. anthracis* UM23C1-2 and Δ perR mutant in LB medium in response to oxidative stress: (a) paraquat and (b) H₂O₂. Similar growth kinetics were obtained from two independent experiments. Arrows indicate the times at which the oxidative compounds were added. (c) Total enzyme catalase activity in *B. anthracis* UM23C1-2 and Δ perR mutant. Levels of total catalase activity of *B. anthracis* UM23C1-2 (dark grey bars) and Δ perR mutant (light grey bars) were determined from whole-cell extracts after 60 min induction with H₂O₂ or paraquat. Error bars indicate SD from two independent experiments.

5.0 mM, the growth of the wild-type was severely inhibited, while that of the Δ perR mutant was comparable to that of the untreated culture (Fig. 5b).

The catalase activity of the Δ perR mutant was constitutive and more than 30 times higher than the untreated wild-type and four times higher than the peroxide-treated

wild-type (Fig. 5c). The increased catalase activity of the Δ perR mutant may, at least in part, account for its increased resistance to peroxide (Fig. 5b). The increased catalase activity of the Δ perR mutant was reflected in the level of expression of the *katB* gene and expression was no longer responsive to oxidative stress (Fig. 4a). The constitutive expression of both the monocistronic *katB*, and the bicistronic *hemH-2-katB* transcripts in the Δ perR mutant, implies that PerR regulates both promoters.

Putative canonical *B. subtilis*-like σ^A -dependent promoter sequences (TTGACA-16/18 bp-TATAAT) (Helmann & Moran, 2002) were identified upstream of *hemH-2* and *katB* (Fig. 4c). A PerR consensus recognition sequence has not been defined for *B. anthracis*; however, overlapping the putative -10 elements of both of these promoters are sequences that conform to the *B. subtilis* PerR recognition site (TTATAATAATTATAA) (Fuangthong & Helmann, 2003), with nucleotide identities of 11/15 (*hemH-2*) and 14/15 (*katB*) nucleotides (Fig. 4c). Taken together, the data in Figs 4 and 5 indicate that *B. anthracis* BA0537 is a functional PerR that responds to peroxide stress and regulates *katB* and *hemH-2* expression. Additionally, putative Fur boxes, similar to the *B. subtilis* Fur recognition site (TGATAATnATTATCA) (Fuangthong & Helmann, 2003), were found at the promoter regions of both *katB* and *hemH-2* (11/15 and 12/15 nt, respectively) (Fig. 4c). This suggests a possible involvement of iron homeostasis in the regulation of *katB* and *hemH-2* and this has been confirmed in the case of *katB* which is downregulated in response to iron starvation (Carlson *et al.* 2009).

The roles of the main catalases and SODs in oxidative stress resistance

The importance of the main vegetative catalases and superoxide dismutases of *B. subtilis* (KatA/SodA) and *B. anthracis* (KatB/SodA1) in oxidative stress resistance was determined by monitoring their growth in identical media and at similar stress levels (Figs 6 and 7). In the absence of stress, the growth rate of the *B. anthracis* Δ katB mutant was identical to that of the wild-type (Fig. 6c, d) whereas the growth rate of the *B. subtilis* Δ katA mutant reproducibly showed a shorter lag and mean generation time than the wild-type (Fig. 6a, b). The *B. subtilis* Δ katA mutant was more sensitive to H₂O₂ stress (Fig. 6a), as observed by Bol & Yasbin (1991), but recovered to reinitiate growth. The *B. anthracis* Δ katB mutant was extremely sensitive to H₂O₂, and as a result the cells underwent lysis (Fig. 6c). Neither the *B. subtilis* Δ katA mutant nor the *B. anthracis* Δ katB mutants showed increased sensitivity to paraquat (Fig. 6b, d).

The growth of the *B. subtilis* Δ sodA mutant exhibited a reduced growth rate compared with the wild-type in the absence of oxidative stress, and addition of paraquat further impaired its growth (Fig. 7a). Interestingly, the Δ sodA mutant was reproducibly less sensitive to H₂O₂ than the wild-type (Fig. 7b), presumably a reflection of the

Fig. 6. Growth of *B. subtilis* and its $\Delta sodA$ mutant (a, b) and *B. anthracis* and its $\Delta sodA1$ mutant (c, d) in LB medium with and without the indicated oxidative stress compounds. Arrows indicate the times at which the oxidative compounds were added to the culture. Similar growth kinetics were obtained from two independent experiments.

increased catalase activity of this mutant (Fig. 3b). The *B. anthracis* $\Delta sodA1$ mutant had no observable growth defect in the absence of oxidative stress but, like the *B. subtilis* $\Delta sodA$ mutant, showed a marked increase in its susceptibility to paraquat-induced superoxide stress (Fig. 7c). In contrast with the *B. subtilis* $\Delta sodA$ mutant, *B. anthracis* $\Delta sodA1$ showed the same level of sensitivity to H₂O₂ as the wild-type (Fig. 7d), reflecting the lack of change in the catalase activity of this mutant (Fig. 3d).

DISCUSSION

Oxidative stress results from the generation of ROS, such as superoxide (O₂^{•−}), peroxide (H₂O₂) and hydroxyl radicals (OH[•]) that damage essential macromolecular components (Halliwell & Gutteridge, 2007). One of the mechanisms by which ROS exerts its effect is by increasing the intracellular free iron pools through the oxidation and subsequent release of iron from iron-containing proteins. SOD-proficient *E. coli* cells grown in the presence of paraquat, or SOD-deficient mutants, have excess levels of intracellular superoxide and free iron, leading to accelerated rates of DNA damage and mutagenesis. This occurs because both O₂^{•−} and H₂O₂ leach iron from [Fe-S] cluster proteins (Jang & Imlay, 2007; Keyer & Imlay, 1996; Nunoshiba *et al.*, 1999). Elevated

levels of intracellular free iron can potentially increase oxidative stress by reacting with H₂O₂ to generate hydroxyl radicals (Taketani, 1993). While interactions between the oxidative stress response and iron homeostasis in *B. anthracis* are beginning to be understood (Passalacqua *et al.*, 2007; Pohl *et al.* 2011), the regulatory proteins that control the oxidative stress responses have not previously been identified.

We show, using a combination of enzyme activity assay, mutagenesis and growth analyses, that *katB* encodes the main vegetative catalase of *B. anthracis*, and plays a predominant role in protection against peroxide stress. *katB* expression is derepressed in response to peroxide stress, and deletion of PerR abolishes *katB* repression, resulting in hyper-resistance to H₂O₂. These results indicate that PerR coordinates the peroxide response of *B. anthracis* in a manner similar to that of *B. subtilis*.

The expression of the main vegetative catalase genes of *B. anthracis* and *B. subtilis* differs in their response to superoxide stress (Mostertz *et al.*, 2004; Pohl *et al.*, 2011). In *B. subtilis*, endogenous superoxide stress, or the accumulation of superoxide radicals in the absence of the main cytoplasmic SOD (SodA), leads to the induction of *katA*. In contrast, only relatively modest changes in *katB* expression are observed in *B. anthracis* under these

Fig. 7. Growth of *B. subtilis* and its Δ sodA mutant (a, b), and *B. anthracis* and its Δ sodA1 mutant (c, d) in LB medium with and without the indicated oxidative stress compounds. Arrows indicate the times at which the oxidative compounds were added to the culture. Similar growth kinetics were obtained from two independent experiments.

conditions. Since superoxide radicals are converted to H₂O₂ by enzymic and spontaneous dismutation (Beyer *et al.*, 1991; Imlay, 2008), the induction of the PerR regulon in *B. subtilis* presumably reflects an intracellular accumulation of H₂O₂. If the same mechanism operates in *B. anthracis*, it is not clear how this bacterium avoids the accumulation of H₂O₂ to levels that lead to the induction of its PerR-regulated *katB* gene.

B. subtilis PerR requires either iron or manganese as a co-repressor and these metal ions compete for binding to PerR. The specific metallated form of PerR influences its reactivity with H₂O₂ to the extent that the manganese-bound form is 10⁶-fold less sensitive to peroxide than the iron-bound form. Transcriptional reporter experiments with *lacZ* show that the extent of *katA* induction in *B. subtilis* by H₂O₂ is enhanced in growth medium supplemented with iron rather than with manganese. This is because the metal components in the medium affect the metal bound to PerR and, as a consequence, the reactivity of PerR to H₂O₂ (Fuangthong & Helmann, 2003; Herbig & Helmann, 2001). Hence, it is tempting to speculate that the elevated intracellular iron in superoxide-stressed *B. subtilis* cells might displace the coordinated manganese in PerR and ultimately derepress *katA* expression more effectively.

In *B. anthracis*, increases in intracellular H₂O₂ and/or the free iron pools in the presence of superoxide stress might be expected to similarly affect PerR. However, despite observing that the iron concentration of paraquat-treated cells doubles during the ensuing 60 min (Tu *et al.*, 2012b), the resulting superoxide stress failed to induce catalase gene expression. Analysis of the *hemH-2* and *katB* promoter regions revealed the presence of putative Fur boxes in addition to the expected PerR boxes, indicating that Fur might be involved in repressing *katB* expression in an iron-dependent manner. Fur and PerR might compete with each other to regulate *katB* expression and indeed *katB* is repressed in response to iron starvation (Carlson *et al.*, 2009). In contrast, *B. subtilis* *katA* is repressed solely by PerR and is Fur-independent (Fuangthong *et al.*, 2002; Fig. 4d).

Dual regulation of catalase synthesis by PerR and Fur has been widely described in other pathogens. For example, PerR and Fur regulate the expression of *Campylobacter jejuni* catalase in an iron-dependent manner, and catalase expression was limited by Fur under high iron conditions (van Vliet *et al.*, 1999). In *Staphylococcus aureus*, while PerR represses catalase expression, Fur positively regulates catalase expression in an iron-dependent manner, despite the observation that the *katA* promoter region does not

have an obvious Fur box (Horsburgh *et al.*, 2001). The generation of a Δfur mutant would allow us to address the question of whether *B. anthracis* Fur interacts with PerR to regulate *katB* expression; however, despite extensive efforts, we have not succeeded in isolating a Δfur mutant. A similar failure has been reported previously, suggesting that this gene is essential for survival (Gat *et al.*, 2008).

Transcriptional profiling indicates that *B. subtilis* *katA* is fully derepressed in the presence of H_2O_2 ; the increase in mRNA levels in H_2O_2 -induced cells is comparable to that in a $\Delta perR$ mutant (Fuangthong *et al.*, 2002). In contrast, the increase in *B. anthracis* *katB* mRNA levels in the $\Delta perR$ mutant is significantly greater than that in the H_2O_2 -treated cells, implying that there may be an additional level of regulation of *katB* expression during oxidative stress.

A potential criticism of the current study is the absence, in *B. anthracis* strain UM23C1-2, of the virulence plasmids pXO1 and pXO2 and, in particular, the pXO1-encoded AtxA regulator. The AtxA regulon has been defined in a fully virulent strain of *B. anthracis*, and deletion of *atxA* results in decreased transcription of the structural genes for the pXO1-encoded anthrax toxin (*pagA*, *lef* and *cya*), the pXO2-encoded capsule biosynthesis operon (*capBCADE*) and chromosome-encoded biosynthetic enzymes for branched-chain and aromatic amino acids (*ilvCD*, *aroAEF*, *hisC* and *tyrA*), oligopeptide transport and acetoin utilization (Hoffmaster & Koehler, 1997; Bourgogne *et al.*, 2003). However, the Sod and catalase genes discussed in this paper were not identified as members of the AtxA regulon.

The results of our comparative studies reveal similarities and differences in the mechanisms by which *B. anthracis* and *B. subtilis* respond to superoxide stress. The resulting differential gene regulation might provide some clues to the *B. anthracis* pathway of pathogenesis. Intriguingly, previous studies indicate that *B. anthracis* manipulates the host's innate immunity for its own benefit. The release of macrophage O_2^- in particular has been implicated in anthrax proliferation, via a lethal toxin-mediated killing of the host cell (Hanna *et al.*, 1994). Moreover, O_2^- actually stimulates spore germination, an essential step in the pathogenesis of *B. anthracis* (Baillie *et al.*, 2005).

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