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 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.

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$_2^-$). Both spontaneous and enzymatically catalysed reactions lead to the production of other reactive oxygen species (ROS), including hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH$^-$) (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.
(Edwards et al., 2001; Fang et al., 1999; Kanafani & Martin, 1985). SODs catalyse the dismutation of $O_2^{-}$ 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 $O_2^{-}$ can be detoxified by catalase and this enzyme is an important line of defence against peroxide stress. The response 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 (Bsat et al., 1998; Mongolosuk & 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 Δ*perR*, ΔkatB and ΔsodA1 mutant strains, created by replacement of their coding sequences with an omega kanamycin resistance cassette (Saile & Koehler, 2002). An isogenic ΔsodA1 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 μg ml$^{-1}$), erythromycin (400 μg ml$^{-1}$ for *E. coli*; 5 μg ml$^{-1}$ for *B. anthracis*), kanamycin (20 μg ml$^{-1}$), chloramphenicol (6 μg ml$^{-1}$) and spectinomycin (100 μg ml$^{-1}$ for *B. subtilis*).

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Trait or relevant genotype</th>
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<tr>
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</tr>
<tr>
<td>UM23C1-2</td>
<td>pXO1$^{-}$ pXO2$^{-}$ Ura$^{-}$ Rif$^{R}$</td>
<td>Hoffmaster &amp; Koehler (1997)</td>
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<td>Δ<em>perR</em></td>
<td>UM23C1-2Δ<em>perR</em>: Km$^{R}$</td>
<td>Tu et al. (2012a)</td>
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<tr>
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<td>UM23C1-2ΔkatB*: Km$^{R}$</td>
<td>This work</td>
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<tr>
<td>ΔsodA1</td>
<td>UM23C1-2ΔsodA1*: Km$^{R}$</td>
<td>Tu et al. (2012b)</td>
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<td><strong>B. subtilis</strong></td>
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<tr>
<td>168</td>
<td>trpC2</td>
<td>Anagnostopoulos &amp; Spizizen (1961)</td>
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<tr>
<td>AH1490</td>
<td>trpC2 metC3 sodA::pAH402, Sp$^{R}$ derivatives of <em>B. subtilis</em> MB24, trpC2 metC3 Spo$^{R}$</td>
<td>Henriques et al. (1998)</td>
</tr>
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<td>This work</td>
</tr>
<tr>
<td>ΔkatA</td>
<td>trpC2 katA::pHTKA1, Erm$^{R}$</td>
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</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pUTE583</td>
<td>Cm$^{R}$ in <em>E. coli</em>; Erm$^{R}$ in <em>B. anthracis</em></td>
<td>Chen et al. (2004)</td>
</tr>
<tr>
<td>pUTE618</td>
<td>Cm$^{R}$ Km$^{R}$ in <em>E. coli</em></td>
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<td>pGEM-T</td>
<td>Amp$^{R}$ in <em>E. coli</em></td>
<td>Promega</td>
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**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 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.

**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.

**Allelic exchange** 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). 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* (AnthraList) 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.

<table>
<thead>
<tr>
<th>Primer name (function)</th>
<th>Sequence (5’–3’)</th>
<th>PCR amplification product (bp)</th>
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<tr>
<td><strong>Real-time RT-PCR</strong></td>
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<td>katB-F</td>
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<td>katB-R</td>
<td>GGTAAGCTGGGTCTTCACCA</td>
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<tr>
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<td>sodA1-R</td>
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<tr>
<td>Ban gyrb-F*</td>
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<tr>
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<td>katA-R</td>
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<td>sodA-R</td>
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<td>Bsu gyrb-F†</td>
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<td>Bsu gyrb-R†</td>
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<td><strong>Northern blot</strong></td>
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<td>katB-R2</td>
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<td><strong>Mutant construction</strong></td>
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<tr>
<td>katB up-F</td>
<td>gctgctctgaATGAATCCAAATAATCGCTTAAACA</td>
<td><em>katB</em> upstream region (500)</td>
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<td>gtcattcgaTCTGAAATTCCCGATGCAAAGCTT</td>
<td><em>katB</em> downstream region (500)</td>
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<tr>
<td>katB down-R</td>
<td>gtaccagctTTGAACCATCTGAAGATAATTTAT</td>
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*Primers amplify the specific region of the gyrb gene in *B. anthracis*.  
†Primers amplify the specific region of the gyrb gene in *B. subtilis*.  

The process was repeated three or four times with at least 8 min cooling on ice in between treatments. The lysates were centrifuged (12000 g, 15 min, 4 °C) to remove cell debris and the resulting supernatant was cleared by a second centrifugation step (20000 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.
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-Toq 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 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 ΔsodA1 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 ΔsodA1 mutant is likely to be contributed by SodA2 (Passalacqua et al., 2006; Tu et al., 2012b) since SodC (BA5139) is likely to be secreted (Bendsen 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 ΔkatA 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
catalase genes peaked 10 min after the addition of H$_2$O$_2$, 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 H$_2$O$_2$, 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 H$_2$O$_2$ (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* ΔkatA 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 H$_2$O$_2$ (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 ΔkatB 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* ΔsodA 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 ΔsodA mutant was fourfold higher in the absence of stress, and 1.4-fold and 6.1-fold higher, respectively, in the presence of H$_2$O$_2$ 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 H$_2$O$_2$ and 8.7-fold with paraquat; Fig. 3b). When this analysis was repeated with the *B. anthracis* ΔsodA1 mutant, the measured catalase activities and katB gene expression profiles were indistinguishable from those of the wild-type (Fig. 3c, d).
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 ferrochelatase, the last enzyme in the haem biosynthesis pathway (Taketani, 1993). The *B. anthracis* genome encodes two ferrochelatases, 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 (DG value of ~11.6 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...
ΔperR mutant to oxidative stress, and this was analysed in comparison with the wild-type (Fig. 5). The ΔperR 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 ΔperR mutant to 1.0 mM H$_2$O$_2$, 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$_2$O$_2$ was increased by fivefold, to

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**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 ΔperR mutant. Total RNA was extracted from the cells during mid-exponential growth in LB broth after 10 min exposure to H$_2$O$_2$ (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 predicted 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 ΔG = −11.8 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.
5.0 mM, the growth of the wild-type was severely inhibited, while that of the ΔperR mutant was comparable to that of the untreated culture (Fig. 5b).

The catalase activity of the ΔperR 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 ΔperR mutant may, at least in part, account for its increased resistance to peroxide (Fig. 5b). The increased catalase activity of the ΔperR 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 ΔperR mutant, implies that PerR regulates both promoters.

Putative canonical B. subtilis-like α^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 ΔkatB mutant was identical to that of the wild-type (Fig. 6c, d) whereas the growth rate of the B. subtilis ΔkatA mutant reproducibly showed a shorter lag and mean generation time than the wild-type (Fig. 6a, b). The B. subtilis ΔkatA mutant was more sensitive to H2O2 stress (Fig. 6a), as observed by Bol & Yasbin (1991), but recovered to reinitiate growth. The B. anthracis ΔkatB mutant was extremely sensitive to H2O2, and as a result the cells underwent lysis (Fig. 6c). Neither the B. subtilis ΔkatA mutant nor the B. anthracis ΔkatB mutants showed increased sensitivity to paraquat (Fig. 6b, d).

The growth of the B. subtilis ΔsodA 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 ΔsodA mutant was reproducibly less sensitive to H2O2 than the wild-type (Fig. 7b), presumably a reflection of the
increased catalase activity of this mutant (Fig. 3b). The _B. anthracis_ Δ sodA1 mutant had no observable growth defect in the absence of oxidative stress but, like the _B. subtilis_ Δ sodA mutant, showed a marked increase in its susceptibility to paraquat-induced superoxide stress (Fig. 7c). In contrast with the _B. subtilis_ Δ sodA mutant, _B. anthracis_ Δ 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 conditions.
conditions. Since superoxide radicals are converted to \( \text{H}_2\text{O}_2 \) by enzymic and spontaneous dismutation (Beyer et al., 1991; Imlay, 2008), the induction of the PerR regulon in \( B. \text{subtilis} \) presumably reflects an intracellular accumulation of \( \text{H}_2\text{O}_2 \). If the same mechanism operates in \( B. \text{anthracis} \), it is not clear how this bacterium avoids the accumulation of \( \text{H}_2\text{O}_2 \) to levels that lead to the induction of its PerR-regulated \( \text{katB} \) gene.

\( B. \text{subtilis} \) 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 \( \text{H}_2\text{O}_2 \) to the extent that the manganese-bound form is \( 10^6 \)-fold less sensitive to peroxide than the iron-bound form. Transcriptional reporter experiments with \( \text{lacZ} \) show that the extent of \( \text{katA} \) induction in \( B. \text{subtilis} \) by \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) (Fuangthong & Helmann, 2003; Herbig & Helmann, 2001). Hence, it is tempting to speculate that the elevated intracellular iron in superoxide-stressed \( B. \text{subtilis} \) cells might displace the coordinated manganese in PerR and ultimately derepress \( \text{katA} \) expression more effectively.

In \( B. \text{anthracis} \), increases in intracellular \( \text{H}_2\text{O}_2 \) 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 \( \text{hemH-2} \) and \( \text{katB} \) promoter regions revealed the presence of putative Fur boxes in addition to the expected PerR boxes, indicating that Fur might be involved in repressing \( \text{katB} \) expression in an iron-dependent manner. Fur and PerR might compete with each other to regulate \( \text{katB} \) expression and indeed \( \text{katB} \) is repressed in response to iron starvation (Carlson et al., 2009). In contrast, \( B. \text{subtilis} \) \( \text{katA} \) 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 \( \text{Campylobacter jejuni} \) catalase in an iron-dependent manner, and catalase expression was limited by Fur under high iron conditions (van Vliet et al., 1999). In \( \text{Staphylococcus aureus} \), while PerR represses catalase expression, Fur positively regulates catalase expression in an iron-dependent manner, despite the observation that the \( \text{katA} \) 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 H₂O₂; the increase in mRNA levels in H₂O₂-induced cells is comparable to that in a ΔperR mutant (Fuangthong et al., 2002). In contrast, the increase in B. anthracis katB mRNA levels in the ΔperR mutant is significantly greater than that in the H₂O₂-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, aroA/EF, hisC and tyrA), oligopeptide transport and acetoin utilization (Hoffmaster & Koehler, 1997; Bourgogne et al., 2003). Moreover, the Sox 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 O₂⁻ in particular has been implicated in anthrax proliferation, via a lethal toxin-mediated killing of the host cell (Hanna et al., 1994). Moreover, O₂⁻ actually stimulates spore germination, an essential step in the pathogenesis of B. anthracis (Baillie et al., 2005).

ACKNOWLEDGEMENTS

This study was supported by the EU-funded BaSysBio project LSHG-CT-2006-037469. W.Y.T. was supported by the Overseas Research Student Award Scheme (ORSAS) from UK higher education funding bodies, and the Sarawak Higher Education Scheme, Sarawak Foundation, Malaysia. B. subtilis ΔsoxA was a gift from A. O. Henriques, Universidade Nova de Lisboa, Lisbon, Portugal, and B. subtilis ΔkatA was from Ulrike Mader, Ernst Moritz Arndt University, Greifswald, Germany.

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Edited by: T. Msadek

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