Role and regulation of the superoxide dismutases of *Staphylococcus aureus*

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*Staphylococcus aureus* has two superoxide dismutases (SODs), encoded by the sodA and sodM genes, which inactivate harmful superoxide radicals (O2−) encountered during host infection or generated from aerobic metabolism. The transcriptional start sites have been mapped and expression analysis on reporter fusions in both genes has been carried out. Under standard growth conditions, manganese (Mn), a mineral superoxide scavenger, elevated total SOD activity but had no effect on the transcription of either gene. Transcription of sodA and sodM was most strongly induced by either internally or externally generated O2−, respectively. Sensitivity to internally generated O2− was linked with SodA deficiency. Mn supplementation completely rescued a sodA mutant when challenged by internally generated O2−, and this was growth-phase-dependent. Sensitivity to externally generated O2− stress was only observed in a sodA sodM mutant and was Mn-independent. In a mouse abscess model of infection, isogenic sodA, sodM and sodA sodM mutants had reduced virulence compared to the parental strain, showing the importance of the enzymic O2− scavenging system for the survival of the pathogen.

INTRODUCTION

Reactive oxygen species (ROS) such as the superoxide anion (O2−), hydrogen peroxide (H2O2) and hydroxyl radical (‘OH) are natural products of metabolism formed when oxygen becomes partially reduced and are crucial to host defence for the optimal antimicrobial activity of neutrophils and other phagocytes (Hurst & Barrette, 1989; Miller & Britigan, 1995). Internal generation of these reactive species can result in damage to DNA, proteins and lipids (Imlay, J. A. & Linn, 1988).

Superoxide dismutases (SODs) are metalloproteins that catalyse dismutation of O2−, the first ROS produced upon reduction of oxygen. By converting O2− to H2O2 and O2, not only do they protect against direct damage caused by O2−, but also against indirect O2− toxicity by preventing a Fe3+-dependent catalytic reaction leading to the production of ‘OH via the Haber–Weiss reaction (Haber & Weiss, 1934). There is also evidence that O2− can reduce hypochlorous acid (HOCI), a potent oxidant derived from the interaction of H2O2 with phagocyte-derived peroxidases, to form ‘OH (Candeias et al., 1993; Ramos et al., 1992).

Many Gram-negative bacteria, including *Escherichia coli* and *Salmonella typhimurium*, have three SODs that are classified according to their metal cofactor and cellular localization. Iron (Fe) and manganese (Mn) SODs are present in the cytoplasm, where they protect cellular macromolecules from endogenous superoxide, whereas copper–zinc (Cu–Zn) SODs reside in the periplasm, protecting from superoxide generated externally by host phagocytes during infection (Benov & Fridovich, 1994; Imlay, K. R. & Imlay, J. A., 1996). Other metal cofactors have been found in SODs, including a cytoplasmic nickel (Ni) SOD in *Streptomyces coelicolor* (Kim et al., 1998).

*Staphylococcus aureus* has two SOD-encoding genes, sodA and sodM (Clements et al., 1999; Valderas & Hart, 2001). The products of these genes combine to form three SOD activity zones as observed by zymogram analysis (Clements et al., 1999; Valderas & Hart, 2001), two homodimers and a heterodimer. *In vitro* data so far indicate that the SodA homodimer is responsible for the majority of the *S. aureus* SOD activity. SodA is a Mn cofactor enzyme (Clements et al., 1999) and the metal requirement of SodM has been proposed to be Mn also (Valderas & Hart, 2001).

The role of SODs in virulence has been investigated in several bacterial species. *Mycobacterium tuberculosis* survival in macrophages was not affected by loss of its Cu–ZnSOD (Dussurget et al., 2001). However, increased expression of the periplasmic Cu–ZnSOD in invasive *E. coli* enhanced survival within non-phagocytic cells (Battistoni et al., 2000).
Streptococcal MnSOD also contributes to virulence (Poyart et al., 2001; Yesilkaya et al., 2000). In S. aureus, there is contradicting evidence as to the roles of the SODs in virulence. A study of S. aureus clinical isolates concluded that SOD activity did not correlate with lethality in a mouse model of infection (Mandell, 1975). Furthermore, 8325-4 isogenic sodA (Clements et al., 1999) or sodM (Schneider et al., 2002) mutants were not attenuated in animal models of infection (Chan et al., 1998; Schneider et al., 2002). However, SOD activity was significantly higher in S. aureus strains isolated from patients with staphylococcal disease compared to those isolated from non-staphylococcal disease patients (Kanafani & Martin, 1985).

As well as enzymic SOD activity, it is now becoming apparent that, in many organisms, several Mn2+ complexes can catalyse the elimination of O2− (Archibald & Fridovich, 1982; Beyer & Fridovich, 1989; Darr et al., 1987; Faulkner et al., 1994; Kono et al., 1976). In Bacillus subtilis, both sodA and Mn are essential for resisting superoxide stress (Iinoka et al., 1999). In Neisseria gonorrhoeae, accumulation of Mn correlates with resistance to oxidative killing by O2− and is independent of SOD activity (Tseng et al., 2001). Lactoc acid bacteria lack enzymic SOD but accumulate Mn to levels that can protect them from O2−, by a mechanism that is proposed to involve oxidation of Mn2+ to Mn3+ by O2− and then subsequent reduction of Mn3+ to Mn2+ by the H2O2 generated or an intracellular reductant (Archibald & Fridovich, 1981). Furthermore, in E. coli, Mn supplementation relieves the phenotypic defects seen in SOD-deficient strains (Al-Maghrebi et al., 2002).

We have recently characterized Mn2+ homeostasis in S. aureus and have proposed a similar non-enzymic Mn2+-associated protection mechanism. S. aureus has two Mn uptake systems that consist of an ABC-type permease, MntABC and an Nramp homologue, MntH (Horsburgh et al., 2002b). The expression of these transporters is regulated by MntR, a DtxR-like metal regulatory protein (O’Halloran, 1993; Que & Helmann, 2000), and is required for Mn2+-dependent mediation of oxidative stress resistance in response to internal O2− (Horsburgh et al., 2002b). Inactivation of MntA results in methyl viologen sensitivity (Gaballa & Helmann, 1998; Lindsay & Foster, 2001). Fur, PerR and MntR form an integrated network controlling peroxide stress resistance as well as Fe2+ and Mn2+ homeostasis (Horsburgh et al., 2002c).

Most physiological studies on S. aureus have used strains of the 8325 lineage (including RN6390 and 8325-4). However, these strains have an 11 bp deletion in rsbU, which is involved in the positive control of SigB activity (Giachino et al., 2001). SigB is an alternative sigma factor with a role in stress resistance. To circumvent possible problems associated with the rsbU mutation, we have recently constructed an RsbU+ derivative of 8325-4 called SH1000 (Horsburgh et al., 2002a).

To further understand the mechanisms by which S. aureus resists oxidative stress and evades host defences, we have characterized the two staphylococcal SODs and describe the superoxide resistance in S. aureus SH1000.

**METHODS**

**Strains and growth conditions.** Strains and plasmids used in this study are listed in Table 1. E. coli was grown in Luria–Bertani (LB) medium at 37 °C. S. aureus was grown in brain–heart infusion (BHI; Oxoid) or Chelex-treated BHI (CT-BHI) containing 400 μM MgSO4 at 37 °C with shaking at 250 r.p.m. CT-BHI was prepared by adding 25 g Chelex-100 1−1 and stirring at room temperature for 6 h prior to removal by filtration (Horsburgh et al., 2001a). Experimental 25 ml cultures in acid-washed (0.1 M HCl, 24 h) 250 ml flasks were inoculated at a starting OD600 value of 0.005 prior to growth at 37 °C. When required, antibiotics were added at the following concentrations: 100 mg ampicillin 1−1; 50 mg kanamycin 1−1; 50 mg neomycin 1−1; 5 mg tetracycline 1−1; 5 mg erythromycin 1−1; 25 mg lincomycin 1−1.

**Construction of reporter and mutant strains.** To construct a sodM mutant, primers (5′-AAAGATTCCTGAACACATTTT-3′ and 5′-AAGAATTCGATATCTCATC-3′; restriction sites underline) were designed using sequences from the S. aureus NCTC 8325 Genome Sequencing Database maintained by the University of Oklahoma’s Advanced Center for Genome Technology (http://www.genome.ou.edu/staph.html) and used to PCR-amplify a 2345 bp region containing sodM. The PCR product was cut with BamHI and EcoRI and cloned into BamHI/EcoRI-digested suicide vector pOB (Horsburgh et al., 2002b) to create pMKM. A Km-digested tet cassette amplified from pDG1513 (Guerout-Fleury et al., 1995) using primers OL32 and OL33 (Horsburgh et al., 2001b) was ligated into Km (unique within sodM) digested pMKM to generate pMKMET.

The suicide plasmid pMKMET was used to transform electro-competent S. aureus RN4220 (Schenk & Ladaga, 1992), resulting in erythromycin- and tetracycline-resistant strains harbouring one disrupted and one functional copy of sodM through single-crossover homologous recombination. SH1000 was transduced (Novick, 1991) with a ph1 lysate from one of these integrants to resolve the sodM locus by transductional outcross. Tetracycline-resistant transductants were screened for loss of erythromycin resistance and thus loss of the plasmid. One such clone was isolated, producing strain MHKM (sodM::tet). To obtain a sodA mutant, the sodA::::Tn917-LTV1 locus was transduced from strain SPW1 (Clements et al., 1999) into SH1000 using 411, giving rise to strain MHKA (sodA::Tn917). Southern blotting was used in each case to verify the location and structural integrity of the DNA at the integration site.

Transcriptional sodA::lacZ and sodM::lacZ reporter fusions were made by PCR amplification (primers 5′-AACAGATCTCAGC- TTTCACATG-3′ and 5′-AAGAATTCCTGAACACATTTT-3′; for sodA; primers 5′-ATGGATCCAGAATCAGAA-3′ and 5′-AAGAATTCGATATCTCATC-3′; for sodM; restriction sites underline) of suitable DNA fragments incorporating 950 and 847 bp of the promoter regions of the above genes, respectively. The purified

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Superoxide dismutases in Staphylococcus aureus

DNA fragments were digested with appropriate restriction enzymes and cloned into similarly digested pAZ106 (Kemp et al., 1991), to generate pMK1A and pMK1M, respectively. The plasmids were electroporated into RN4220 and the resulting erythromycin-resistant clones were transduced into recipient SH1000 using φ11, producing lacZ fusions strains MHK1A (sodA::pAZ106 SodA+), and MHK1M (sodM::pAZ106 SodM+). Southern blotting was used in each case to verify the location and structural integrity of the DNA at the integration site.

Transcriptional start-site mapping. RNA was isolated from late-exponentially growing (BHI) cells of S. aureus (OD600 8.0) using the Fast-prep blue kit (Bio-101). Primer extension reactions (Horsburgh & Moir, 1999) were performed using 20 μg total RNA and 10 pmol primer (5'-AGTTTCTTGTGAAAAATGGG-3', sodA; 5'-ATTGTTCTTGTGCTATATG-3', sodM) and labelling with [α-32P]ATP (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹). Sequencing (Amersham Sequenase 2.0 kit) was performed on purified pMK1A and pMK1M (Qiagen Mini-Prep kit) using the above oligonucleotides and labelling with 35S-labelled ATP (1000 Ci mmol⁻¹, 37 TBq mmol⁻¹).

SOD and β-galactosidase assays. Levels of β-galactosidase activity were measured, using 4-methylumbelliferyl β-d-galactoside (Sigma) as substrate, as described previously (Horsburgh et al., 2002b). Twenty-millilitre CT-BHI agar (1 %, w/v) plates (size/20 mM MnSO₄) that had just been inoculated with 5 μl of stationary-phase CT-BHI-grown S. aureus (OD₆₀₀ 14). The overlay was allowed to set and an assay disc (5 mm diameter) was placed in the middle of the plate followed by 5 μl of the test compound (Sigma) (methyl viologen, 0-2 M). Plates were incubated overnight at 37 °C. Assays were performed in duplicate.

For determination of SOD activity, samples were collected at the late-exponential phase of growth (OD₆₀₀ 10), washed in PBS and lysed using osmotic stress and lysostaphin (Clements et al., 1999). Total protein concentration was determined by Bradford assay (Bio-Rad) and samples were stored at −70 °C until needed. Spectrophotometric determination of total SOD activity was determined by the inhibition of autoxidation of pyrogallol according to the method of Marklund & Marklund (1974) using 1 μg of total protein per assay. Samples were analysed twice. For enzymic activity determination, 1 μg of total protein was applied onto a native PAGE gel. Bands were visualized by negative staining using the nitro blue tetrazolium method of Beauchamp & Fridovich (1971).

Disc-diffusion assay. Sensitivity to methyl viologen was assessed by disc diffusion (Horsburgh et al., 2002b). Twenty-millilitre CT-BHI agar (1 %, w/v) plates (+/-20 μM MnSO₄) were poured. Plates were overlaid with 5 ml of CT-BHI agar (0-7 %, w/v; +/-20 μM MnSO₄) that had just been inoculated with 5 μl of stationary-phase CT-BHI-grown S. aureus (OD₆₀₀ 14). The overlay was allowed to set and an assay disc (5 mm diameter) was placed in the middle of the plate followed by 5 μl of the test compound (Sigma) (methyl viologen, 0-2 M). Plates were incubated overnight at 37 °C. Assays were performed in duplicate.

Internal superoxide stress assay. Experimental cultures in CT-BHI with or without 20 μM Mn²⁺ were inoculated from an overnight CT-BHI culture to an OD₆₀₀ value of ~0.005. Cells were challenged with methyl viologen (10 mM) at the early-exponential (T = 2 h), post-exponential (T = 6 h) and stationary (T = 12 h) phases of growth. Samples were collected at appropriate intervals post-challenge and viable bacteria determined in duplicate by plating onto BHI agar. The experiment was performed twice and a representative dataset is shown.

External superoxide stress assay. Experimental cultures in CT-BHI with or without 20 μM Mn²⁺ were inoculated from an

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/description</th>
<th>Reference/source</th>
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</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli DH5x</td>
<td>∅80 Δ(lacZ)M15 Δ(argF–lac)U169 endA1 recA1 hsdR17</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>S. aureus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8325-4</td>
<td>Wild-type strain cured of prophages</td>
<td>Lab. stock</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction-deficient transformation recipient</td>
<td>Lab. stock</td>
</tr>
<tr>
<td>SH1000</td>
<td>8325-4 RsbU⁺</td>
<td>Horsburgh et al. (2002a)</td>
</tr>
<tr>
<td>MJH502</td>
<td>SH1000 RsbU⁺ sigB::tet</td>
<td>Horsburgh et al. (2002a)</td>
</tr>
<tr>
<td>MHKA</td>
<td>SH1000 sodA::pAZ106 SodA⁺</td>
<td>This study</td>
</tr>
<tr>
<td>MHKM</td>
<td>SH1000 sodM::pAZ106 SodM⁺</td>
<td>This study</td>
</tr>
<tr>
<td>SPW1</td>
<td>8325-4 sodA::Tn917</td>
<td>Clements et al. (1999)</td>
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<tr>
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<td>This study</td>
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<tr>
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<td>This study</td>
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<tr>
<td><strong>Plasmid</strong></td>
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<tr>
<td>pAZ106</td>
<td>Promoterless lacZ crm insertion vector</td>
<td>Kemp et al. (1991)</td>
</tr>
<tr>
<td>pDG1513</td>
<td>tet-cassette-containing vector</td>
<td>Guerout-Fleury et al. (1995)</td>
</tr>
<tr>
<td>pOB</td>
<td>pGEM3Zf(+) crm-containing cloning vector</td>
<td>Horsburgh et al. (2002b)</td>
</tr>
<tr>
<td>pMK1A</td>
<td>1 kb fragment of the sodA promoter region in pAZ106</td>
<td>This study</td>
</tr>
<tr>
<td>pMK1M</td>
<td>1-2 kb fragment of the sodM promoter region in pAZ106</td>
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</tr>
<tr>
<td>pMKM</td>
<td>2-2 kb PCR fragment containing sodM gene in pOB</td>
<td>This study</td>
</tr>
<tr>
<td>pMKM Tet</td>
<td>pMKM containing a tetracycline cassette in sodM</td>
<td>This study</td>
</tr>
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</table>
overnight CT-BHI culture to an OD$_{600}$ value of ~0.005. Cells from the early-exponential (T = 2 h), post-exponential (T = 6 h) and stationary (T = 12 h) phases of growth were diluted in PBS to an OD$_{600}$ value of 0.1 and the test compounds (X/XO, 1 mM/1.5 U ml$^{-1}$) were added. Cells were incubated stationary at 37°C for 1 h. Suitable dilutions were plated in duplicate on BHI plates to determine viable counts. The experiment was repeated twice and a representative set of values is shown.

Pathogenicity study. A mouse abscess model of infection was used, as described in Chan et al. (1998). Results from 10 mice were recorded, and their significance was determined by the Student’s t-test.

**RESULTS**

**SOD expression and activity**

RNA extracted from late-exponential phase cells of (BHI) SH1000 or MJH502 (sigB) was subjected to primer extension analysis and transcription start sites were visualized on a 6% (w/v) sequencing gel (Fig. 1). Under the conditions tested, transcription of sodA initiated from two σ$^{A}$-type promoters (Fig. 1a). These are designated as σ$^{A1}$ and σ$^{A2}$. More transcription from σ$^{A1}$ was observed in a sigB mutant background. Expression of sodM occurs from a single σ$^{A}$-type promoter that is significantly more active in MJH502 (sigB) and almost undetectable in the wild-type SH1000 (Fig. 1b).

To measure gene expression during growth, reporter fusion strains were made. Expression of sodA and sodM was maximal at the mid- (Fig. 2a, 60 000 β-galactoside units) and late- (Fig. 2b, 12 500 β-galactoside units) exponential phases of growth, respectively. In MJH502 (sigB), expression levels of sodA were unaffected (Fig. 2a), while sodM levels were elevated (Fig. 2b, 30 000 β-galactoside units). Similar expression levels were obtained in the presence or absence of added Mn (results not shown).

In metal-depleted cultures, SodM homodimer was the major SOD activity on an activity gel, while SodA homodimer activity was barely visible (Fig. 2c). Addition of Mn caused a dramatic increase in SodA homodimer.

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**Fig. 1.** Transcriptional analysis of the SODs in *S. aureus* SH1000 and MJH502 (sigB). (a) Primer extension analysis of the sodA promoter region showing the two (P$_{A1}$, P$_{A2}$) start sites. (b) The sodM promoter region has a single start site (P$_{M}$). Promoter −10 and −35 regions of (c) sodA and (d) sodM are underlined. Transcriptional start sites are enlarged. The ribosome-binding site and translational start of each gene are shown in bold.
activity and the disappearance of the SodM homodimer activity (Fig. 2c). Conversely, in MJH502 (\textit{sigB}), SodM homodimer activity was independent of Mn levels and less SodA homodimer activity was apparent in the presence of Mn when compared to SH1000 (Fig. 2d).

Spectrophotometrically measured SOD activity in metal-depleted culture extracts of \textit{S. aureus} was approximately 300 U mg$^{-1}$ (Fig. 3). The addition of Mn increased specific activity to $\sim$2500 U mg$^{-1}$ (Fig. 3). Strain MHKM (\textit{sodM}) grown in the absence of metals had no detectable SOD activity and upon addition of Mn its activity levels reached $\sim$1900 U mg$^{-1}$ (Fig. 3). Conversely, strain MHKA (\textit{sodA}) grown in the absence of metals had an activity of $\sim$500 U mg$^{-1}$ which was reduced to $\sim$200 U mg$^{-1}$ upon addition of Mn (Fig. 3). Strain MHKAM (\textit{sodA sodM}) growing in the absence or presence of Mn had residual activity of $\sim$100 U mg$^{-1}$ (Fig. 3). Thus, regulation of \textit{sodA} and \textit{sodM} is complex and occurs at the transcriptional and post-transcriptional levels.

**Effect of oxidative stress on SOD expression and activity**

To determine if the expression of the SODs was induced by stress, the \textit{lacZ} fusion strains were sampled after the addition of either methyl viologen (100 \textmu M) or X/XO (0\textendash 5 mM/60 mU ml$^{-1}$) at early-exponential phase (OD$_{600}$ 0\textendash 1). Under the conditions tested here, the addition of the superoxide-generators had no effect on the cell growth rate.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Expression of SODs. Growth (closed symbols) and \textbeta-galactosidase activity (open symbols) in CT-BHI of \textit{sodA}::\textit{lacZ} (a) and \textit{sodM}::\textit{lacZ} (b) in SH1000 (\square, \blacksquare) and MJH502 (\textit{sigB}) (\textcircled{C}, \textcircled{M}). Protein-associated SOD activity of \textit{S. aureus} SH1000 (c) and MJH502 (\textit{sigB}) (d) during growth in CT-BHI with no added metals (\textendash) and 20 \textmu M Mn$^{2+}$ (+Mn). The top and bottom band represent a SodM homodimer and SodA homodimer, respectively. The middle band is thought to represent a heterodimer. The experiment was repeated twice and a representative is shown here.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{SOD activity in \textit{S. aureus}. Comparison of SOD-specific activity of \textit{S. aureus} SH1000 and strains MHKA (\textit{sodA}), MHKM (\textit{sodM}) and MHKAM (\textit{sodA sodM}) in CT-BHI under metal limitation and upon addition of 20 \textmu M Mn. Error bars are shown.}
\end{figure}
In metal-depleted cultures, addition of either methyl viologen or X/XO did not significantly alter expression of sodA (results not shown). In Mn-containing cultures (20 μM), the expression of sodA increases threefold (180,000 β-galactoside units, T = 3 h) after addition of methyl viologen, while addition of X/XO had no effect compared to the control (60,000 β-galactoside units, T = 3 h; results not shown).

In the absence of metals, expression of sodM was not increased by addition of either methyl viologen or X/XO. In the presence of Mn, sodM transcription was induced by addition of X/XO (26,000 β-galactoside units, T = 5 h) compared to the control (18,000 β-galactoside units, T = 5 h; results not shown).

**Mn affects resistance to oxidative-stress-generating compounds**

We have previously shown that Mn uptake contributes to superoxide stress resistance (Horsburgh et al., 2002b) and have proposed that elemental dismutation of superoxide by Mn acts in concert with the SOD enzymes. The role of SODs and Mn availability in stress resistance was tested by disc-diffusion assay. Under the metal-depleted conditions used, strains SH1000 and MHKM (sodM) were not sensitive to methyl viologen stress (Fig. 4). However, strains MHKA (sodA) and MHKAM (sodA sodM) were sensitive (Fig. 4, 8–8.5 mm inhibition zones). Upon addition of Mn, MHKA (sodA) and MHKAM (sodA sodM) became fully resistant to methyl viologen. Thus, superoxide sensitivity due to the lack of both SOD enzymes can be relieved by the presence of Mn.

**Role of Mn and SODs on the survival of S. aureus during oxidative stress throughout growth**

Temporal expression of sodA and sodM was shown earlier (Fig. 2). Superoxide stress was applied at different points during growth to determine if there was any growth-phase-associated role for the SODs. In the absence of metals, only strain MHKAM (sodA sodM) showed a growth defect which was restored to near wild-type levels upon incorporation of Mn in the growth medium (results not shown).

Exposure to methyl viologen during the early-exponential phase of growth (2 h) and in the absence of Mn resulted in a 10^3- to 10^4-fold loss of viability for strains MHKA (sodA) and MHKAM (sodA sodM) within 24 h post-addition of the compound (Fig. 5a I). Viability levels were not affected by the addition of Mn (Fig. 5a I). Viability of SH1000 and MHKM (sodM) was largely unaffected in either Mn-depleted or replete media (Fig. 5a I).

During the post-exponential phase of growth (6 h) and in the absence of Mn, exposure to methyl viologen resulted in a 10^3- to 10^4-fold drop in the viability of strains MHKA (sodA) and MHKAM (sodA sodM) within 24 h post-addition of the compound (Fig. 5a II). Growth of strains MHKA (sodA) and MHKAM (sodA sodM) in Mn-replete cultures resulted in a 100-fold increase in cell survival after 24 h (Fig. 5a II). Viability of strains SH1000 and MHKM (sodM) was unaffected in either metal-depleted or Mn-containing media (Fig. 5a II).

During stationary phase (12 h) and in the absence of Mn, viability of strains MHKA (sodA) and MHKAM (sodA sodM) was reduced by 10^3-fold after 24 h (Fig. 5a III). Mn improved MHKA (sodA) and MHKAM (sodA sodM) viability by 10-fold (Fig. 5a III). Viability of MHKAM (sodM) reduced twofold and was unaffected by the presence or absence of Mn in the culture. The viability of SH1000 was unchanged in either metal-depleted or Mn-containing media (Fig. 5a III).

Strains MHKA (sodA), MHK (sodM) and MHKAM (sodA sodM) all showed less survival than SH1000 after exposure to X/XO stress only during the early- (2 h) and post- (6 h) exponential phases of growth (Fig. 5b). Resistance could only be restored for MHKA (sodA) and MHKAM (sodM) during the post-exponential phase by the addition of Mn to the medium (Fig. 5b, 6 h). In the stationary phase, only MHKAM (sodA sodM) was more sensitive than SH1000, this being irrespective of Mn level (Fig. 5b, 12 h).

**Role of SODs in virulence**

The role of the SODs in virulence was tested in an established mouse subcutaneous model of infection (Chan et al., 1998). Strains MHKA (sodA), MHK (sodM) and MHKAM (sodA sodM) had significantly reduced virulence compared to the wild-type SH1000, using the Student’s t-test (MHKA, P < 0.01; MHKM, P < 0.02; MHKAM, P < 0.02) (Fig. 6). Thus, both SodA and SodM contribute to the virulence of S. aureus SH1000 in this model.

**Fig. 4.** The effect of Mn supplementation (20 μM) on the sensitivity of wild-type S. aureus SH1000 and isogenic mutants to methyl viologen using disc-diffusion assays. Cells were grown on CT-BHI plates with discs containing 0.2 M methyl viologen (5 μl). Error bars are shown. Solid bars, SH1000; hatched bars, MHKA (sodA); open bars, MHK (sodM); horizontal-striped bars, MHKAM (sodA sodM).
**DISCUSSION**

We have characterized important aspects of the role and regulation of *S. aureus* SODs in response to superoxide stress and with respect to Mn availability.

Both *S. aureus* SODs are transcribed from $\sigma^A$-type promoters. However, unlike *E. coli* (Dubrac & Touati, 2000; Hassan & Schrum, 1994; Imlay, K. R. & Imlay, J. A., 1996) or *B. subtilis* (Iinoaka et al., 1998), where expression of SODs is carried out from single $\sigma^A$-type promoters, in *S. aureus sodA* is transcribed from two $\sigma^A$-type promoters ($P_{A1}$ and $P_{A2}$). So far, the significance of this finding remains unclear. Furthermore, transcriptional data show an indirect repression of $sodM$ $P_M$ and $sodA$ $P_{A1}$ promoters by $\sigma^B$, which is in agreement with the recent finding that $\sigma^B$ indirectly represses a number of staphylococcal genes (Giachino *et al.*, 2001; Horsburgh *et al.*, 2002a). Gel activity evidence also suggests that $\sigma^B$ is also involved in the post-transcriptional regulation of SodM homodimer activity. $\sigma^B$ is a pleiotropic regulator in *S. aureus* with roles in stress resistance and virulence-determinant production (Horsburgh *et al.*, 2002a). The indirect control of gene expression by $\sigma^B$ alludes to the presence of further $\sigma^B$-controlled regulatory components.

In *E. coli*, Mn and Fe play an important role in MnSOD and FeSOD biosynthesis, respectively, and MnSOD or FeSOD activity increases in the presence of Mn or Fe, respectively (Pugh *et al.*, 1984). Our work with *S. aureus* has shown that the presence of Mn increases SodA homodimer activity and concomitantly decreases SodM homodimer activity. This occurs primarily due to post-transcriptional...
effects, as the expression of the respective genes is independent of Mn availability, in the absence of superoxide-generating compounds. Synthesis of SodA in the presence of Mn (its co-factor) may enhance enzyme stability and perhaps heterodimer formation. In turn, this would deplete the level of SodM available for homodimer formation.

Superoxide induction of sodA and sodM transcription is Mn-dependent. sodM is most strongly induced in response to externally generated superoxide stress, whereas sodA is induced by internal stress. In Gram-negative bacteria, SODs are compartmentalized in order to deal with different sources of superoxide stress. MnSODs are present in the cytoplasm, protecting cell components from internal superoxide, whereas Cu–ZnSODs are periplasmic, protecting from external superoxide encountered during infection (Benov & Fridovich, 1994; Imlay, K. R. & Imlay, J. A., 1996). The importance of SodA in resisting internal superoxide stress in S. aureus has been highlighted previously (Clements et al., 1999) and is a reflection of its preferential induction by internal superoxide stress.

Regulation of SODs in response to stress has been studied extensively in E. coli (Compan & Touati, 1993; Dubrac & Touati, 2000, 2002; Fee, 1991; Greenberg et al., 1990), where sodA expression is controlled by SoxRS in response to oxidative stress (Compan & Touati, 1993; Greenberg et al., 1990). There is no apparent SoxRS in S. aureus and how superoxide influences sodA expression is unknown. 

Superoxide induction of the expression of the Mn transporter mntA by an unknown regulator has been noted previously (Horsburgh et al., 2002b). Interestingly, in E. coli, a small RNA molecule, RyhB, has recently been shown to be involved in the positive regulation of sodB (Masse & Gottesman, 2002).

Mn can completely rescue the sensitivity of S. aureus MHKA (sodA) and MHKAM (sodA sodM) to internal superoxide (methyl viologen) stress and the growth defect of MHKAM. Mn homeostasis is important for several micro-organisms in surviving oxidative stress (Archibald & Fridovich, 1981, 1982; Inaoka et al., 1998; Horsburgh et al., 2002b; Tseng et al., 2001). This has been proposed to occur by a direct elemental superoxide scavenging by complexes of Mn with cellular ligands (Archibald & Fridovich, 1981, 1982; Al-Maghrebi et al., 2002; Horsburgh et al., 2002c). The SOD- and Mn-dependent superoxide-scavenging systems act synergistically to relieve the burden of superoxide stress on the cell.

The importance of Mn in the response of S. aureus to internal (methyl viologen) superoxide stress is growth-phase-dependent. S. aureus uses Mn to combat internal superoxide stress encountered mostly during the post-exponential and stationary phases of growth. Previously, Valderas & Hart (2001) showed that the double mutant (sodA sodM) had a growth defect in the absence of methyl viologen. We were able to minimize this defect by supplementing the medium with Mn (results not shown).

Also, in the Valderas & Hart study (2001), SodM had a role in resisting internal superoxide stress encountered during the stationary phase of growth. Here, we find a similar slight SodM-dependent effect. Furthermore, they found the sodA mutant was insensitive to methyl viologen at either the post-exponential or stationary phase of growth. Throughout our experiments, it has always been the case that methyl viologen stress induces a virtually identical phenotype for the sodA and sodA sodM mutants that can be attributed to the absence of SodA. The differences between these studies may be related to the strain backgrounds used (RN6390 vs SH1000). RN6390 is of the 8325 lineage and has an rsbU defect which results in greatly reduced SigB activity (Giachino et al., 2001; Horsburgh et al., 2002a).

So far, there have been no reports on the sensitivity of S. aureus to externally generated superoxide stress. Here, we show that Mn has a growth-phase- as well as a SOD-dependent effect on external superoxide stress resistance. Very interestingly, both SodA and SodM have a role in resisting external superoxide stress. Absence of both SODs leads to significantly reduced viability and Mn supplementation is incapable of restoring resistance. It is also apparent that the Mn system contributes to survival in the post-exponential phase of growth, where it is complementary to the SODs in overcoming external superoxide stress.

The different effect of external compared to internal superoxide on the cells is probably a reflection of the targets that might be involved in bringing about its toxicity. It is
likely that proteins or lipids in the staphylococcal cell wall or membrane are damaged by external superoxide leading to loss of viability. Also, the fact that SodA, the major internal superoxide-scavenging dismutase, is sufficient to resist external stress supports the idea that external superoxide radicals may enter the cell and disrupt the internal superoxide equilibrium. External superoxide enters the cell via anion channels (Miller & Britigan, 1997). We have so far been unable to find SOD activity in the staphylococcal cell wall, despite testing under many different growth conditions (results not shown).

Contrary to previous studies (Clements et al., 1999; Schneider et al., 2002), we have found that both SodA and SodM contribute to the survival of the organism in an animal model of infection. Clements et al. (1999) used the S. aureus 8325-4 background to show that SodA was not important in virulence. This may be a reflection of the constitutively high levels of SodM homodimer activity observed in 8325-4, a SigB under-producing strain (M. H. Karavolos & S. J. Foster, unpublished data). In a different study by Schneider et al. (2002) conducted in S. aureus 8325-4, lack of SodM did not affect survival of the organism in either a systemic or a kidney model of infection.

These results accentuate the significance of SOD activity for the survival of staphylococci either within the host or in the external environment. It is becoming apparent in a wide range of organisms that this activity can be achieved by complementary mechanisms involving enzyme activity and elemental dismutation. These dual systems allow efficient resistance mechanisms to be maintained throughout growth. Oxidative stress resistance is a key component of the organism’s survival machinery and further elucidation of its intricate mechanisms will allow further understanding of S. aureus physiology and pathogenesis.

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