Oxidative stress tolerance is manganese (Mn\(^{2+}\)) regulated in *Streptococcus gordonii*

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The Sca permease in the oral bacterium *Streptococcus gordonii* is a member of a family of ATP-binding cassette (ABC)-type transporters for manganese (Mn\(^{2+}\)) and related cations that are associated with streptococcal virulence in a number of infection models. Since Mn\(^{2+}\) has a protective function against oxidative damage in a variety of bacteria, we have investigated the role of Sca permease in oxidative stress tolerance in *Streptococcus gordonii*. A single Mn\(^{2+}\)-dependent superoxide dismutase (SOD), encoded by *sodA*, is expressed by *S. gordonii* and was >10-fold up-regulated under oxidative stress conditions. Inactivation of *sodA* resulted in increased susceptibility of *S. gordonii* cells to growth inhibition by dioxygen (O\(_2\)), and to killing by paraquat (a superoxide anion generator) and by hydrogen peroxide (H\(_2\)O\(_2\)). Expression of thiol peroxidase, encoded by the *tpx* gene located immediately downstream of the *scaCBA* operon, was also up-regulated under oxidative conditions. Inactivation of *tpx* led to increased susceptibility of cells to H\(_2\)O\(_2\), but not to O\(_2\) or paraquat. In low-Mn\(^{2+}\) medium (0-01 µM Mn\(^{2+}\)) *sodA* and *tpx* genes were transcriptionally down-regulated, SOD activity was reduced and cells were more sensitive to growth inhibition by O\(_2\). A Sca permease-deficient (*scaC*) mutant showed further reduced SOD activity and hypersensitivity to O\(_2\) in medium containing <0-1 µM Mn\(^{2+}\). These results demonstrate that the Sca (Mn\(^{2+}\)) permease in *S. gordonii* is essential for protection against oxidative stress.

**Keywords**: manganese transport, oral streptococci, peroxide, reactive oxygen species

INTRODUCTION

The ability to tolerate oxidative stress is necessary for growth of bacteria in aerobic environments. Cellular macromolecules such as DNA, lipids and proteins must be protected from reactive oxygen species (ROS) produced both endogenously during metabolism of dioxygen (O\(_2\)) and exogenously, for example by polymorphonuclear leukocytes and macrophages of host immune responses (Miller & Britigan, 1997). In facultatively anaerobic streptococci, which lack catalase, the major mechanism for detoxification of ROS is the manganese form of superoxide dismutase (SOD). This is encoded by the *sodA* gene that is present in at least 28 species of streptococci (Poyart *et al*., 1998). MnSOD is found within the cytoplasm and upon the cell surface (Gerlach *et al*., 1998), where it may assist in protecting cells against exogenous superoxide (O\(_2\)\(^-\)).

It is suggested that O\(_2\)\(^-\) anions do not directly degrade DNA or polyunsaturated lipids to a significant extent, but that O\(_2\)\(^-\)-mediated cellular damage arises primarily by the release of Fe from proteins or chelates (Touati, 2000). This can facilitate Fenton-type reactions, in which the oxidation of metal ions, such as Fe\(^{2+}\) by hydrogen peroxide (H\(_2\)O\(_2\)), releases highly reactive hydroxyl radicals (HO\(^-\)). Many mitis-group streptococci produce relatively large amounts of H\(_2\)O\(_2\) during aerobic growth by the action of oxidase enzymes, e.g. NADH oxidase (Nox) and pyruvate oxidase (SpxB), the latter of which is up-regulated in response to O\(_2\) (Auzat *et al*., 1999; Pericone *et al*., 2000). The expression of *sodA* is also enhanced under oxidative stress in some streptococci (Gibson & Caparon, 1996; Yesilkaya *et al*., 2000). However, relatively little is known about the function or regulation of streptococcal peroxidase enzymes, which

Abbreviations:  NBT, nitro-blue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase.
in silico analysis of the Streptococcus pyogenes genome revealed genes encoding glutathione peroxidase (GpoA) and alkyl hydroperoxide reductase (AhpC) that were found to be involved in protection against O$_2^-$ and organic peroxides (King et al., 2000). In mitis-group streptococci (Whiteley & Beighton, 1998), including Streptococcus gordonii, Streptococcus parasanguis and Streptococcus pneumoniae, orthologous genes encoding proteins with about 50% amino acid sequence identities to Escherichia coli thiol peroxidase (Tpx) have been identified (Fenno et al., 1993; Dintilhac & Claverys, 1997; Kolenbrander et al., 1998). Purified Tpx from S. pneumoniae catalyses the degradation of H$_2$O$_2$ and protects glutamine synthetase from H$_2$O$_2$-mediated inactivation (Wan et al., 1997).

Manganese (Mn$^{2+}$) plays a major role in oxidative stress tolerance in a number of different bacteria. For example, SOD activity in Streptococcus suis is strongly influenced by Mn$^{2+}$ concentration in the growth medium (Niven et al., 1999). Also in Lactobacillus plantarum and Neisseria gonorrhoeae, which do not apparently produce MnSOD, Mn$^{2+}$ can directly protect cells against ROS (Archibald & Fridovich, 1981; Tseng et al., 2001). In S. gordonii, which colonizes the oral cavity and nasopharynx, and can cause endocarditis (Douglas et al., 1993), Mn$^{2+}$ uptake under low Mn$^{2+}$ (<0.5 µM) is mediated by an ATP-binding cassette (ABC)-type transporter, composed of ScaC (ATP-binding protein), ScaB (hydrophobic membrane protein) and ScaA (solute-binding lipoprotein) (Kolenbrander et al., 1998). Expression of the Sca permease is regulated in response to Mn$^{2+}$ by a metallorepressor protein ScaR (Jakubovics et al., 2000). In this study we demonstrate that the Sca (Mn$^{2+}$) permease is essential for oxidative stress tolerance in S. gordonii and that Mn$^{2+}$ regulates expression of superoxide dismutase and thiol peroxidase activities.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains used in this study are described in Table 1. E. coli cells were cultured at 37°C in Luria–Bertani (LB) medium (Sambrook et al., 1989) with shaking at 250 r.p.m. Streptococcal cells were routinely grown without shaking at 37°C in brain-heart infusion medium (Difco) containing 5% (w/v) yeast extract (BHY). Cells were propagated at 37°C in reduced oxygen atmosphere (candle jar) on BHYN agar medium consisting of BHY containing 5% (w/v) Neo-peptone (Difco) and 1.5% (w/v) Bacto-agar. Effects of O$_2$ on growth were determined on cells grown under atmospheric O$_2$ with shaking at 250 r.p.m. (aerated culture), or in capped tubes or bottles without shaking (aerobic). Alternatively, cells were cultured under strictly anaerobic conditions (gas phase 80% N$_2$, 10% CO$_2$, 10% H$_2$). Low-metal ion medium (TYH-C) for growth of streptococci contained 0.5% (w/v) yeast extract, 25 mM glucose, 25 mM HEPES, 10 mM (NH$_4$)$_2$SO$_4$, 2H$_2$O, 34 mM NaCl, 10 mM KCl and 10 mM Na$_2$HPO$_4$, pH 7.5. Low-metal ion medium (TYH-C) for growth of streptococci contained 0.5% (w/v) yeast extract, 25 mM glucose, 25 mM HEPES, 10 mM (NH$_4$)$_2$SO$_4$, 2H$_2$O, 34 mM NaCl, 10 mM KCl and 10 mM Na$_2$HPO$_4$, pH 7.5. Low-metal ion medium (TYH-C) for growth of streptococci contained 0.5% (w/v) yeast extract, 25 mM glucose, 25 mM HEPES, 10 mM (NH$_4$)$_2$SO$_4$, 2H$_2$O, 34 mM NaCl, 10 mM KCl and 10 mM Na$_2$HPO$_4$, pH 7.5. Low-metal ion medium (TYH-C) for growth of streptococci contained 0.5% (w/v) yeast extract, 25 mM glucose, 25 mM HEPES, 10 mM (NH$_4$)$_2$SO$_4$, 2H$_2$O, 34 mM NaCl, 10 mM KCl and 10 mM Na$_2$HPO$_4$, pH 7.5.

**Determination of SOD activity.** For visualization of SOD activity on non-denaturing polyacrylamide gels, S. gordonii cells were cultured to stationary phase in BHY medium and harvested by centrifugation at 5000 g for 10 min. Cells were washed once in 50 mM potassium phosphate buffer, pH 7.8, and suspended in 0.05 ml spheroplasting buffer (26%, w/v, (TYH-CM), MnCl$_2$ was added at a final concentration of 10 µM in TYH-C immediately before use. Standard inocula were prepared for TYH-C growth experiments as late-exponential-phase cells of S. gordonii cultured anaerobically in TYH-C, suspended in TYH-C containing 15% (v/v) glycerol and stored at $-70$ °C. Microwells containing TYH-C or MnCl$_2$-supplemented TYH-C were inoculated with 1% volume standard inoculum and incubated without shaking either aerobically or anaerobically at 37°C for 24 h. Cells were suspended homogeneously by pipetting and optical density (OD$_{590}$) values were determined. Mean OD$_{590}$ values for each strain in a single set of growth conditions were calculated from six independent cultures. When required, antibiotics were added to media at the following concentrations: ampicillin (Ap), 50 µg ml$^{-1}$; erythromycin (Em), 100 µg ml$^{-1}$ (E. coli) or 2 µg ml$^{-1}$ (S. gordonii); tetracycline (Tc), 15 µg ml$^{-1}$ (E. coli) or 5 µg ml$^{-1}$ (S. gordonii).

**Genetic manipulations.** Routine molecular biology procedures were performed according to Sambrook et al. (1989). Plasmids employed are listed in Table 1. These were purified from E. coli cells using the Concert miniprep kit (Gibco). Oligonucleotides were synthesized by MWG Biotech and chromosomal DNA was prepared from mutanolysin-treated streptococcal cells as described previously (Jenkinson, 1987). An isogenic tetracycline-resistant (Tc$^R$) sodA mutant was generated by insertional mutagenesis. An internal fragment of 418 bp from within the coding region of the sodA (GenBank accession no. Z95905) gene was PCR-amplified using primer pairs sodAf/sodAr (5’-TCTAGACTTGGCACCATTGACAA CACC-3’, 5’-CAAGCCCAAGATTGATCCTTACC-3’), and was cloned into pGEM-T (Promega). Recombinant plasmid was digested with XbaI and BamHI, and the streptococcal DNA fragment was ligated into XbaI/BamHI-digested streptococcal integrational plasmid pSF143 (Tc$^R$) (Tao et al., 1992). The resulting plasmid, pSF143-sodA (Table 1) was transformed into competent cells of S. gordonii DL1 (Haisman & Jenkinson, 1991), thus generating mutant strain UB1083 sodA (Table 1).

The tpx$^R$ gene was inactivated by allelic exchange with the erythromycin resistance determinant ermAM. PCR amplification with primers tpxf2/tpxr2 (5’-CGTCCGATGAAGACCCGGTTTC-3’, 5’-CCGCTGTAACCATCAATGCGG-3’) of S. gordonii DL1 DNA template generated a 1190 bp fragment comprising the entire tpx$^R$ gene (492 bp) and flanking sequences that was cloned into pGEM-T. A DNA fragment (1052 bp) containing the ermAM gene (GenBank accession no. AB057644) was PCR-amplified from plasmid pVA838 (Macrina et al., 1983) using primers ermR/ermF (5’-CCATATAAAAATCGATAACGC-3’, 5’-CCTTATICGATAC AATTCCCGC-3’) that contained ClaI restriction sites. The PCR product was digested with ClaI and ligated into a unique ClaI site within the cloned tpx$^R$ gene, thus generating plasmid pGEM-tpx$^R$.ermAM (Table 1). The insert DNA was excised with SalI, gel-purified and transformed into S. gordonii DL1 (to generate strain UB1313 tpx$^R$), and into S. gordonii UB1083 sodA (to generate double mutant UB1314 sodA tpx$^R$) (Table 1). Confirmation of predicted insertions was obtained by appropriate PCR-amplification and sequencing of products, or by DNA blot hybridization.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
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<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> JM109</td>
<td>e14 (McrA) recA1 endA1 gyrA96 thi-1 hsdR17(rK mK) supE44 relA1 Δ(lac-proAB) [F′ traD36 proAB lacI2ΔZAM15]</td>
<td>Promega</td>
</tr>
<tr>
<td>DH5α</td>
<td>deoR endA1 gyrA96 hsdR17(rK mK) recA1 supE thi-1 Δ(lacZYA-argF) 1800lacZAM15F′</td>
<td>Promega</td>
</tr>
<tr>
<td><strong>S. gordonii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL1 (Challis)</td>
<td>Wild-type</td>
<td>This laboratory</td>
</tr>
<tr>
<td>PK3041</td>
<td>sacC::ermAM</td>
<td>Kolenbrander <em>et al.</em> 1998</td>
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<td>UB1083</td>
<td>sodA::pSF143 tet</td>
<td>This study</td>
</tr>
<tr>
<td>UB1313</td>
<td>tpx::ermAM</td>
<td>This study</td>
</tr>
<tr>
<td>UB1314</td>
<td>tpx::ermAM sodA::pSF143 tet</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pGEM-T</td>
<td>3.0 kb; Ap™; ColElori</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-tpx.ermAM</td>
<td>pGEM-T-derived containing ermAM cassette within tpx gene sequence fragments</td>
<td>This study</td>
</tr>
<tr>
<td>pVA838</td>
<td>9.2 kb; Em™, Cm™; pACYCori E. coli–streptococcus shuttle vector</td>
<td>Macrina <em>et al.</em> 1983</td>
</tr>
<tr>
<td>pSF143</td>
<td>57 kb; Tc™; pACYCori</td>
<td>Tao <em>et al.</em> 1992</td>
</tr>
<tr>
<td>pSF143-sodA</td>
<td>pSF143-derived containing a fragment of internal sodA gene sequence</td>
<td>This study</td>
</tr>
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</table>

* All *S. gordonii* mutant strains were derived from DL1.

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**Fig. 1.** Non-denaturing PAGE stained for SOD activity (a) and Northern blots hybridized with radiolabelled internal fragments of sodA (b) or tpx (c) genes. Proteins or mRNAs were extracted from *S. gordonii* DL1 (lanes 1, 4 and 6), UB1313 tpx (lanes 2, 5 and 7) or UB1083 sodA (lane 3) cells cultured in BHY anaerobically (lanes 1–3), under vigorous aeration (lanes 4 and 5) or statically in the presence of 20 mM paraquat (lanes 6 and 7). The sizes of mRNAs were estimated to be: sodA, 0.6 kb; tpx::ermAM, 1.6 kb; tpx, 0.5 kb.

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Manganese and oxidative stress in *S. gordonii*

Raffinose, 10 mM MgCl₂, 20 mM Tris/HCl, pH 6.8 (Demuth *et al.*, 1996). Mutanolysin (25 U, Sigma) and PMSF (0.5 mM final concentration) were added and samples were incubated at 37 °C for 15 min. Cells were then broken by adding an equal volume of 0.10 mm glass beads (Sigma) and vortexing the suspensions vigorously for 2 min. Cell debris was pelleted by centrifugation at 12000 g for 2 min, and the cell-free supernatant was retained. Protein concentrations were determined by a modified Bradford assay (Bio-Rad), using bovine immunglobulin as standard. Non-denaturing PAGE of proteins was performed by the method of Davies (1964). Gels were stained with Coomassie brilliant blue, or SOD activity was detected as described elsewhere (Beauchamp & Fridovich, 1971). Briefly, gels were incubated at ambient temperature in 2.45 mM nitro-blue tetrazolium (NBT) solution for 20 min. This solution was replaced with riboflavin buffer (36 mM potassium phosphate, pH 7.8, containing 28 mM tetramethyl-p-phenylenediamine and 28 μM riboflavin) for 15 min. Gels were then transferred into distilled water and exposed to light from a 15 W fluorescent lamp in a foil-lined box for 5–15 min. Gel images were digitized using an Astra 1220U scanner and intensities of bands were quantified with Kodak digital science 1D image analysis software. SOD activities in *S. gordonii* cell extracts were quantified using the McCord–Fridovich assay (McCord & Fridovich, 1969). Early exponential-phase cells (OD₆₀₀ ~ 0.3) were harvested by centrifugation (5000 g for 10 min) and washed in PE buffer (26.5 mM KH₂PO₄, 0.33 M K₃HPO₄, 0.1 mM EDTA, pH 7.8). Bacteria were then suspended in spheroplasting buffer and cell-free extracts prepared as described above. Samples were stored on ice and SOD activities were determined from triplicate readings within 2 h of extract preparation. Specific SOD activities per mg protein were calculated as means from between five and seven independent cultures, where 1 U was defined as the amount of SOD required to reduce the initial rate of change of absorbance at 550 nm (ΔA₅₅₀) by 50 % (McCord & Fridovich, 1969).

**Determination of growth and kill rates.** To assess the effects of atmospheric conditions on the growth of *S. gordonii*, cultures were incubated at 37 °C anaerobically, aerobically (static incubation) or under vigorous aeration. Susceptibilities to paraquat and to H₂O₂ were determined as follows. Exponential-phase cells (OD₆₀₀ ~ 0.3) were harvested by centrifugation at 5000 g for 10 min and suspended at a density of 1×10⁶ cells ml⁻¹ in 1 % (w/v) Bacto-peptone (Difco) at 37 °C.
Paraquat (final concentration 10 mM) or H$_2$O$_2$ (final concentration 25 mM) was added and the suspensions were incubated at 37 °C for 1 h. Samples were removed at time zero and at intervals over 60 min, serially diluted, plated onto BHYN agar and numbers of c.f.u. were determined following 36 h incubation at 37 °C. S. gordonii strains formed only short chains of cells (usually 2–4 cells per chain) and so sonication, often employed to break chains of cells, was not applied.

**Northern analysis.** RNA was extracted from streptococcal cells as previously described (Jakubovics et al., 2000), separated by electrophoresis through 0.8% (w/v) agarose gels containing 3% (v/v) formaldehyde and transferred to Hybond-N+ membranes (Pharmacia). A probe comprising the internal coding region of sodA (418 bp) was generated by PCR amplification as described above. The primer pair tpxf1/tpxr1 (5'-CATCTAGAAGTAGCCGACACAGC-3', 5'-GCTATTGCCGGATCTAGTCAGG-3') was employed to amplify an internal fragment (431 bp) of tpx (GenBank accession no. L11577). PCR products were labelled with $^{32}$P using Prime-a-Gene (Promega) and purified on NICK columns (Pharmacia). Hybridizations were performed for 16 h at 68 °C in Church & Gilbert (1984) medium. Blots were washed twice in 2x SSC, 0.1% (v/v) SDS for 5 min at 20 °C and twice for 20 min in the same medium at 68 °C. Membranes were exposed to Fuji HR-E 30 X-ray film for 1–5 days and the intensities of the bands were quantified using Kodak digital science 1D image analysis software.

**Statistical analyses.** All statistical analyses of data were performed by Student’s t-test using Microsoft Excel software. Values of $P<0.05$ were considered to be statistically significant.

**RESULTS**

**SOD activity in S. gordonii**

A number of species of streptococci appear to express a single sod activity with Mn as preferred co-factor (Nakayama, 1992; Gibson & Caparon, 1996; Chang & Hassan, 1997; Pericone et al., 2000), while S. pneumoniae has been reported to produce MnSOD and FeSOD activities (Yesilkaya et al., 2000). To detect SOD activity in S. gordonii DL1, cell-free extracts of bacteria grown in BHY medium were subjected to non-denaturing PAGE and gels were activity-stained using NBT (Beauchamp & Fridovich, 1971). A single band of SOD activity was identified (Fig. 1a). This was insensitive to inhibition by ferricyanide or by H$_2$O$_2$, which inhibit Cu/Zn-SOD and FeSOD activities, respectively (data not shown). The band was absent from cell extracts of an isogenic mutant, UB1083 sodA::pSF143 tet, in which the sodA gene was insertionally inactivated (Fig. 1a, lane 3). This indicates that in wild-type S. gordonii cells, SOD activity is encoded by the sodA gene.

**O$_2$ and O$_2^-$ regulation of tpx**

Thiol peroxidase (Tpx) is an antioxidant enzyme present in a wide range of bacteria (Wan et al., 1997). In S. gordonii the tpx gene is located immediately downstream of the Sca (Mn$^{2+}$) permease operon (Kolenbrander et al., 1994). Inactivation of tpx in S. gordonii UB1313 tpx::ermAM had no apparent effect on SOD activity in anaerobic or aerated cultures (Fig. 1a). Similar to DL1 (wild-type), SOD activity and sodA mRNA levels in UB1313 tpx were increased under aeration and in cells grown with 20 mM paraquat (Figs 1a and 1b). Under anaerobic conditions, where SOD activities and sodA expression levels were low, tpx mRNA could barely be detected (Fig. 1c, lane 1). However tpx mRNA levels, like sodA mRNA levels, were increased dramatically by growth of cells in the presence of O$_2$ or paraquat (Fig. 1c, lanes 4 and 6). Inactivation of sodA did not lead to increased expression of tpx under anaerobic conditions (Fig. 1c, lane 3).

**O$_2$ and O$_2^-$ regulated expression of sodA**

In wild-type S. gordonii DL1, SOD activities were elevated in cells grown with aeration, and in cells grown with 20 mM paraquat, which generates intracellular O$_2^-$ (Fig. 1a). These increases were associated with significantly increased (>10-fold) levels of sodA mRNA (Fig. 1b). The SOD polypeptide could be easily identified as a major protein component in whole-cell protein extracts from wild-type cells grown under aeration (Fig. 2). Scanning densitometry of Coomassie-blue-stained proteins suggested that MnSOD constituted between 1.5 and 2% of total cell protein in aerated S. gordonii wild-type cells. The SOD band was barely visible in extracts from cells grown under anaerobic conditions and was absent from sodA mutant cell extracts (Fig. 2).

**Relative roles of SOD and Tpx in detoxification of ROS**

To determine more precisely the requirements for SOD and Tpx in protection against ROS, we generated an isogenic double mutant, UB1314 sodA tpx, as described in Methods. The effects of O$_2$ on growth, and of paraquat and H$_2$O$_2$ on survival of wild-type or mutant
strains were then tested. Wild-type cells entered stationary phase in batch culture at lower density when aerated as opposed to when grown anaerobically (Fig. 3). Inactivation of sodA or tpx, or of both genes in UB1314, did not affect growth of cells under anaerobic conditions (data not shown). However, the growth rate of UB1083 sodA (doubling time $t_d = 76.4 \pm 6.5$ min) in aerated culture was significantly ($P < 0.01$) less than that of the wild-type ($t_d = 44.8 \pm 3.3$ min) (Fig. 3). Although the growth rate of UB1313 tpx was not significantly different from that of DL1, the growth rate ($t_d = 81.3 \pm 9.5$ min) and growth yield of double mutant UB1314 were severely attenuated (Fig. 3). These data suggest that SOD is more important than Tpx for growth of S. gordonii in the presence of $O_2$. To determine the sensitivities of S. gordonii wild-type and mutant strains to intracellular $O_2^-$, exponential-phase cells were incubated in the presence of paraquat for 60 min and viable cell numbers were estimated at intervals. From preliminary experiments, it was found that >100 mM paraquat was required to kill wild-type cells. Sensitivity of UB1313 tpx mutant cells to paraquat was unaffected over a range of paraquat concentrations from 10 to 250 mM. Conversely, in 10 mM paraquat, viable cell numbers of UB1083 sodA or UB1314 sodA tpx were reduced by $>99\%$ within 15 min (Fig. 4a). Therefore SOD, but not Tpx, is necessary for protection against $O_2^-$-mediated toxicity. We also determined the sensitivity of wild-type and mutant strains to $25 \text{mM } H_2O_2$. Cells of UB1313 tpx were more sensitive to $H_2O_2$-mediated loss in viability than wild-type cells ($>95\%$ of UB1313 cells killed within 30 min, compared with $<75\%$ of wild-type cells), while UB1083 sodA mutant cells were more sensitive still ($>99\%$ of cells killed within 30 min) (Fig. 4b). The double mutant UB1314 was hypersensitive to killing by $H_2O_2$ (Fig. 4b), with a 5-log loss in viability within 30 min. Taken collectively, these data suggest that SOD and Tpx act in concert for the detoxification of ROS.

**SOD activity depends upon Mn$^{2+}$ availability**

To investigate the effect of Mn$^{2+}$ on SOD in S. gordonii, enzyme activities in cell-free extracts from wild-type or mutant cells were measured (McCord & Fridovich, 1969).
Table 2. Reduction of SOD enzyme activity in *S. gordonii* in response to Mn2⁺ limitation

<table>
<thead>
<tr>
<th>Strain*</th>
<th>SOD activity [U (mg protein)⁻¹] from cells cultured in†</th>
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<tbody>
<tr>
<td></td>
<td>BHY</td>
</tr>
<tr>
<td>DL1</td>
<td>5.22 (0.62)</td>
</tr>
<tr>
<td>UB1083 (sodA)</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PK3041 (scaC)</td>
<td>3.37 (1.79)</td>
</tr>
<tr>
<td>UB1313 (tpx)</td>
<td>4.89 (0.85)</td>
</tr>
</tbody>
</table>

* All strains were derivatives of strain DL1.
† Values in parentheses represent sd from five to seven independent cultures.

1969). SOD activities in wild-type DL1 or UB1313 tpx cells grown statically in BHY medium were similar (Table 2) and, as expected, SOD was undetectable in cell extracts of UB1083 sodA (Table 2). SOD activity was significantly reduced (P<0.01) in a Sca permease-deficient mutant, PK3041 scaC (Table 2) that is defective in uptake of Mn2⁺ (Kolenbrander *et al*., 1998). Addition of 10 µM Mn2⁺ to BHY medium improved the growth yields of all strains, and there were concomitant increases in SOD activities for DL1, UB1313 and PK3041 (Table 2). These data suggest that BHY medium does not satisfy fully the Mn2⁺ requirement for optimal growth of *S. gordonii* DL1.

Mn2⁺ availability regulates expression of sodA and tpx

For more detailed analyses of SOD regulation in response to Mn2⁺, a tryptone-yeast extract medium (TYH-C) was developed in which the Mn2⁺ concentration could be accurately defined (see Methods). To determine if SOD activity was modulated by Mn2⁺ availability at the level of transcription, mRNAs were extracted from cells cultured aerobically (static) in TYH-C (10 nM Mn2⁺) or TYH-CM (10 µM Mn2⁺) and analysed by Northern hybridization (Fig. 5). In wild-type cells, expression of sodA was reduced about 10-fold under low Mn2⁺ (TYH-C) conditions (Fig. 5b). tpx mRNA levels were also about 10-fold lower in cells cultured in low Mn2⁺ (Fig. 5c). Similar results were obtained from several independent sets of mRNA extractions. Expression levels of hppA encoding the binding protein component of the Hpp permease (oligopeptide transporter) (Jenkinson *et al*., 2000), confirming equal loadings of mRNA in the gel lanes.

Sca permease is essential for aerobic growth

To determine the effect of Mn2⁺ on O₂ tolerance, growth yields of wild-type and Sca permease mutant in TYH-C containing different concentrations of Mn2⁺. Growth is expressed as percentage of the maximum yield (OD₆₀₀). Data points represent mean values from six independent experiments. In each case the standard deviation was within 10% of the mean.

![Fig. 6. Effects of Mn²⁺ concentration on aerobic and anaerobic growth. *S. gordonii* DL1 (■, □) and PK3041 scaC (▲, △) cells were cultured anaerobically (■, ▲) or aerobically (□, △) for 24 h in TYH-C containing different concentrations of Mn²⁺. Growth is expressed as percentage of the maximum yield (OD₆₀₀). Data points represent mean values from six independent experiments. In each case the standard deviation was within 10% of the mean.](image)

**DISCUSSION**

In facultatively anaerobic bacteria, rapid and efficient oxidative stress response mechanisms are necessary to enable growth and survival under aerobic or reactive oxidant challenges. One of the most important enzymes in oxidative stress tolerance is SOD, and there is now substantial agreement that MnSOD acts as a major protectant against toxic O₂⁻ anions in streptococci.
In other Gram-positive bacteria more details are known about the regulatory networks that co-ordinate Mn⁡²⁺-sensing and ROS-sensing pathways. In *B. subtilis*, several peroxide stress genes are repressed by PerR, a metalloprotein that is activated by Mn⁡²⁺ or Fe⁡²⁺ cations and is susceptible to H₂O₂-inactivation, specifically in the Fe form (Bsat et al., 1998; Herbig & Helmann, 2001). *Staphylococcus aureus* PerR controls the expression of at least eight genes (Horsburgh et al., 2001). In *S. pyogenes*, PerR is essential for induced resistance to peroxide (King et al., 2000), although it does not apparently regulate expression of *ahpC*, encoding alkyl hydroperoxide reductase. It is possible therefore that a PerR orthologue may be involved in co-ordinating gene regulation in response to metal ions and ROS in *S. gordonii*. We are currently investigating this hypothesis with the aim of identifying PerR-regulated genes in *S. gordonii* and their respective regulatory promoter sequences.

For *S. gordonii* cells subjected to aerobic stress (in aerated culture) the Mn⁡²⁺ requirement must be substantially increased to provide for the large amounts of SOD enzyme being produced. Thus if Mn⁡²⁺ homeostasis is disrupted, then aerobic growth will be compromised. It is clearly evident that in low Mn⁡²⁺ (<0.1 μM) environments, such as are reported to be present in serum and peripheral tissues (Krachler et al., 1999), the Sca permease is required to provide for Mn⁡²⁺ homeostasis. In the absence of the permease there is no other mechanism in *S. gordonii* that will provide sufficient Mn⁡²⁺ for growth of the cells under aerobic or other oxidative stress conditions. These observations would also suitably account for the defects in virulence of Mn⁡²⁺ permease mutants of *Streptococcus mutans*, *S. parasanguis* and *S. pneumoniae* (Burnette-Curley et al., 1995; Berry & Paton, 1996; Kitten et al., 2000; Tseng et al., 2002). Identifying the critical genes and proteins involved in oxidative stress tolerance in these organisms may provide new targets for anti-streptococcal therapies.

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**REFERENCES**


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