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

Nicholas S. Jakubovics,¹ Anthony W. Smith² and Howard F. Jenkinson¹

Author for correspondence: Howard F. Jenkinson. Tel: +44 117 928 4358. Fax: +44 117 928 4313.
e-mail: howard.jenkinson@bris.ac.uk

¹ Oral Microbiology Unit, Department of Oral and Dental Science, University of Bristol Dental School, Lower Maudlin Street, Bristol BS1 2LY, UK

² Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK

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_2O_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_2O_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_2O_2), releases highly reactive hydroxyl radicals ($HO^\bullet$). Many mitis-group streptococci produce relatively large amounts of H_2O_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.

inactivate H_2O_2 directly. *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 (Whiley & 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.*, 1995; Dintilhac & Claverys, 1997; Kolenbrander *et al.*, 1998). Purified Tpx from *S. pneumoniae* catalyses the degradation of H_2O_2 and protects glutamine synthetase from H_2O_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+} ($<0.5 \mu\text{M}$) 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 metalloregressor 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 (anaerobic). 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) Tryptone (Difco), 0.5% (w/v) yeast extract, 25 mM glucose, 25 mM HEPES, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 H_2O , 34 mM NaCl, 10 mM KCl and 10 mM Na_2HPO_4 , pH 7.5. Divalent metal ions were depleted from this medium by treatment with Chelex-100 resin (Niven *et al.*, 1999) and, after autoclaving, the medium was adjusted to 1 mM MgCl_2 and 0.1 mM CaCl_2 . For Mn^{2+} -defined medium

(TYH-CM), MnCl_2 was added at a final concentration of $10 \mu\text{M}$ 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_{600}) values were determined. Mean OD_{600} 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), $50 \mu\text{g ml}^{-1}$; erythromycin (Em), $100 \mu\text{g ml}^{-1}$ (*E. coli*) or $2 \mu\text{g ml}^{-1}$ (*S. gordonii*); tetracycline (Tc), $15 \mu\text{g ml}^{-1}$ (*E. coli*) or $5 \mu\text{g ml}^{-1}$ (*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'-TCTAGACTTGACCATGACAAA CACC-3', 5'-CAAGCCCAAGATTGGATCCTTACC-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* gene was inactivated by allelic exchange with the erythromycin resistance determinant *ermAM*. PCR amplification with primers *tpxf2/tpxr2* (5'-CGTCCGATGAAGACCGTTTC-3', 5'-CGCTGTAACCATCAATGCGG-3') of *S. gordonii* DL1 DNA template generated a 1190 bp fragment comprising the entire *tpx* 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 *ermf/ermr* (5'-CCATATCATAAAATCGATACAGC-3', 5'-CCTTATCGATACA AATCCCCCG-3') that contained *Clal* restriction sites. The PCR product was digested with *Clal* and ligated into a unique *Clal* site within the cloned *tpx* gene, thus generating plasmid pGEM-*tpx.ermAM* (Table 1). The insert DNA was excised with *SalI*, gel-purified and transformed into *S. gordonii* DL1 (to generate strain UB1313 *tpx*), and into *S. gordonii* UB1083 *sodA* (to generate double mutant UB1314 *sodA tpx*) (Table 1). Confirmation of predicted insertions was obtained by appropriate PCR-amplification and sequencing of products, or by DNA blot hybridization.

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,

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/reference
<i>E. coli</i>		
JM109	<i>e14⁻</i> (McrA ⁻) <i>recA1endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1</i> Δ (<i>lac-proAB</i>) [F' <i>traD36 proAB lacI^q</i> Z Δ M15]	Promega
DH5 α	<i>deoR endA1 gyrA96 hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1 supE thi-1</i> Δ (<i>lacZYA-argFV169</i>) Φ 80 Δ <i>lacZ</i> Δ M15F ⁻ λ ⁻	Promega
<i>S. gordonii</i> *		
DL1 (Challis)	Wild-type	This laboratory
PK3041	<i>scaC::ermAM</i>	Kolenbrander <i>et al.</i> (1998)
UB1083	<i>sodA::pSF143 tet</i>	This study
UB1313	<i>tpx::ermAM</i>	This study
UB1314	<i>tpx::ermAM sodA::pSF143 tet</i>	This study
Plasmids		
pGEM-T	3.0 kb; Ap ^R ; ColE1ori	Promega
pGEM- <i>tpx.ermAM</i>	pGEM-T-derived containing <i>ermAM</i> cassette within <i>tpx</i> gene sequence fragments	This study
pVA838	9.2 kb; Em ^R , Cm ^R ; pACYC <i>ori</i> <i>E. coli</i> -streptococcus shuttle vector	Macrina <i>et al.</i> (1983)
pSF143	5.7 kb; Tc ^R ; pACYC <i>ori</i>	Tao <i>et al.</i> (1992)
pSF143- <i>sodA</i>	pSF143-derived containing a fragment of internal <i>sodA</i> gene sequence	This study

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

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.

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 12 000 *g* for 2 min, and the cell-free supernatant was retained. Protein concentrations were determined by a modified Bradford assay (Bio-Rad), using bovine immunoglobulin 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 tetramethylethylenediamine and 28 μ M 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 K₂HPO₄, 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 (ΔA_{550}) 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 $\times$ 10⁹ cells ml⁻¹ in 1% (w/v) Bacto-peptone (Difco) at 37 °C.

Paraquat (final concentration 10 mM) or H_2O_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'-CATCTAGAAGTAGGCGACACAGC-3', 5'-GCTATTGCCGATCCTAGTCAGG-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 $2 \times \text{SSC}$, 0.1% (w/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_2O_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^- -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

Fig. 2. Non-denaturing PAGE stained with Coomassie blue. Proteins were extracted from mid-exponential phase *S. gordonii* cells. Lanes: 1, DL1 anaerobic culture; 2, aerated DL1 cells; 3, UB1083 *sodA* anaerobic culture.

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).

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).

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_2O_2 on survival of wild-type or mutant

Fig. 3. Retardation of growth by O_2 . *S. gordonii* DL1 (■, □), UB1083 *sodA* (▽), UB1313 *tpx* (○) or UB1314 *tpx sodA* (△) cells were cultured in BHY medium anaerobically (■) or with aeration by shaking at 250 r.p.m. (□, ▽, ○, △). Growth profiles of strains UB1313, UB1083 and UB1314 under anaerobic conditions are not shown, but were essentially identical to DL1.

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 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,

Fig. 4. Lethal effects of ROS on *S. gordonii* cells. Rates of killing were determined for strains DL1 (□), UB1083 *sodA* (▽), UB1313 *tpx* (○) and UB1314 *tpx sodA* (△) in the presence of 10 mM paraquat (a) or in 25 mM H_2O_2 (b). No viable UB1314 cells were detected after incubation in 25 mM H_2O_2 for > 30 min. Control samples of DL1 cells were included (■) that were not exposed to either reagent. Standard deviations from triplicate samples are indicated.

Table 2. Reduction of SOD enzyme activity in *S. gordonii* in response to Mn^{2+} limitation

Strain*	SOD activity [U (mg protein) ⁻¹] from cells cultured in†	
	BHY	BHY + 10 μ M $MnCl_2$
DL1	5.22 (0.62)	8.05 (1.25)
UB1083 (<i>sodA</i>)	<0.5	<0.5
PK3041 (<i>scaC</i>)	3.37 (1.79)	8.74 (1.26)
UB1313 (<i>tpx</i>)	4.89 (0.85)	9.69 (1.28)

* All strains were derivatives of strain DL1.

† Values in parentheses represent SD from five to seven independent cultures.

Fig. 5. Influences of Mn^{2+} on the transcription of oxidative stress response genes. mRNAs were extracted from *S. gordonii* DL1 (wild-type) cells cultured in TYH-C (no added Mn^{2+}) or TYH-CM (10 μ M Mn^{2+}) and analysed by Northern hybridization. Blots were probed with ³²P-labelled internal fragments of *hppA* (a), *sodA* (b) or *tpx* (c).

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 Mn^{2+} (Kolenbrander *et al.*, 1998). Addition of 10 μ M Mn^{2+} 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 Mn^{2+} requirement for optimal growth of *S. gordonii* DL1.

Mn^{2+} availability regulates expression of *sodA* and *tpx*

For more detailed analyses of SOD regulation in response to Mn^{2+} , a tryptone-yeast extract medium (TYH-C) was developed in which the Mn^{2+} concentration could be accurately defined (see Methods). To

Fig. 6. Effects of Mn^{2+} 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 Mn^{2+} . Growth is expressed as percentage of the maximum yield (OD_{600}). Data points represent mean values from six independent experiments. In each case the standard deviation was within 10% of the mean.

determine if SOD activity was modulated by Mn^{2+} availability at the level of transcription, mRNAs were extracted from cells cultured aerobically (static) in TYH-C (10 nM Mn^{2+}) or TYH-CM (10 μ M Mn^{2+}) and analysed by Northern hybridization (Fig. 5). In wild-type cells, expression of *sodA* was reduced about 10-fold under low Mn^{2+} (TYH-C) conditions (Fig. 5b). *tpx* mRNA levels were also about 10-fold lower in cells cultured in low Mn^{2+} (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 (oligo-peptide transporter) (Jenkinson *et al.*, 1996) were identical under the different Mn^{2+} concentrations (Fig. 5a) (see also Jakubovics *et al.*, 2000), confirming equal loadings of mRNA in the gel lanes.

Sca permease is essential for aerobic growth

To determine the effect of Mn^{2+} on O_2 tolerance, growth yields of wild-type and Sca permease mutant in TYH-C medium were measured following anaerobic or aerobic (static) growth across a range of Mn^{2+} concentrations. Maximal growth yields of wild-type or PK3041 *scaC* mutant strains could be obtained with 0.1 μ M Mn^{2+} under anaerobic or aerobic growth conditions (Fig. 6). However, specifically under aerobic conditions, the Sca permease-deficient mutant failed to grow at <0.1 μ M Mn^{2+} (Fig. 6). Thus the Sca transporter is critical to support aerobic growth of *S. gordonii* under limiting Mn^{2+} .

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 O_2^- anions in streptococci

(Jakubovics & Jenkinson, 2001). In this article we demonstrate that *S. gordonii* produces a single SOD, which is Mn^{2+} -dependent, based upon sequence comparisons with other MnSOD proteins and our enzyme inhibition profile. Although it has recently been reported that the related bacterium *S. pneumoniae* produces FeSOD in addition to MnSOD (Yesilkaya *et al.*, 2000), we could not demonstrate production of FeSOD by *S. gordonii*. Expression of *sodA* in *S. gordonii* is up-regulated under aerobic stress and SOD protein constitutes between 1.5 and 2% of total cell protein in aerated cultures, as reported also for *S. sanguis* (Di-Giuseppi & Fridovich, 1982). In support of a major role for MnSOD in oxidative stress tolerance, *S. gordonii* *sodA* mutants were exquisitely sensitive to paraquat and to molecular oxygen, both of which result in the generation of O_2^- within cells. The increased sensitivity of *sodA* mutants to H_2O_2 is most likely due to synergy between O_2^- and H_2O_2 for causing cellular damage (Touati, 2000).

In streptococci, which do not produce catalase, protection against H_2O_2 may be afforded by peroxidase enzymes. In *S. gordonii*, the *tpx* gene, located immediately downstream of the *sca* Mn^{2+} -permease operon (Kolenbrander *et al.*, 1998), encodes a protein with significant sequence identity to *E. coli* Tpx. It appears that SOD and Tpx may form part of a co-ordinated response to oxidative stress, since the expression of both genes was up-regulated > 10-fold under vigorous aeration of cultures. Tpx expression is also up-regulated by O_2 in *S. parasanguis* (Spatafora *et al.*, 2002). A major role for *S. gordonii* Tpx appears to be in protection against H_2O_2 . In aerated cultures the *tpx* mutant (and *tpx sodA* mutant) entered stationary phase at a lower cell density than the wild-type. It has been shown that inhibition of *S. gordonii* growth under these conditions is partly the result of accumulation of H_2O_2 (Barnard & Stinson, 1996).

Our data suggest that a complex Mn^{2+} -dependent regulatory system operates in the control of the oxidative stress response in *S. gordonii*. Under low Mn^{2+} there is reduced expression of *sodA* and of *tpx*, but the enzymes are sufficiently active for normal growth, even under conditions of mild oxidative stress. The mechanism by which Mn^{2+} influences transcription of these genes is unknown, but it is unlikely to involve the metallo-repressor protein ScaR (Jakubovics *et al.*, 2000) since transcription of *sodA* and *tpx* in *scaR* mutants is still Mn^{2+} -responsive (data not shown). It is possible that decreased *sodA* expression under low Mn^{2+} may benefit cells by co-ordinating SOD production with the amount of Mn^{2+} available for enzyme activation. Alternatively, SOD may act as an intracellular store for Mn, in which case up-regulation of *sodA* expression under high Mn^{2+} might regulate the concentration of free intracellular Mn^{2+} cations. In contrast, there is no evidence that Tpx binds Mn^{2+} and peroxidase activity is dependent upon oxidation and reduction of one or more active site cysteine residues (Zhou *et al.*, 1997).

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 H_2O_2 -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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