Comparison of genes required for \( \text{H}_2\text{O}_2 \) resistance in *Streptococcus gordonii* and *Streptococcus sanguinis*

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Hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) is produced by several members of the genus *Streptococcus* mainly through the pyruvate oxidase SpxB under aerobic growth conditions. The acute toxic nature of \( \text{H}_2\text{O}_2 \) raises the interesting question of how streptococci cope with intrinsically produced \( \text{H}_2\text{O}_2 \), which subsequently accumulates in the microenvironment and threatens the closely surrounding population. Here, we investigate the \( \text{H}_2\text{O}_2 \) susceptibility of oral *Streptococcus gordonii* and *Streptococcus sanguinis* and elucidate potential mechanisms of how they protect themselves from the deleterious effect of \( \text{H}_2\text{O}_2 \). Both organisms are considered primary colonizers and occupy the same intraoral niche making them potential targets for \( \text{H}_2\text{O}_2 \) produced by other species. We demonstrate that *S. gordonii* produces relatively more \( \text{H}_2\text{O}_2 \) and has a greater ability for resistance to \( \text{H}_2\text{O}_2 \) stress. Functional studies show that, unlike in *Streptococcus pneumoniae*, \( \text{H}_2\text{O}_2 \) resistance is not dependent on a functional SpxB and confirms the important role of the ferritin-like DNA-binding protein Dps. However, the observed increased \( \text{H}_2\text{O}_2 \) resistance of *S. gordonii* over *S. sanguinis* is likely to be caused by an oxidative stress protection machinery present even under anaerobic conditions, while *S. sanguinis* requires a longer period of time for adaptation. The ability to produce more \( \text{H}_2\text{O}_2 \) and be more resistant to \( \text{H}_2\text{O}_2 \) might aid *S. gordonii* in the competitive oral biofilm environment, since it is lower in abundance yet manages to survive quite efficiently in the oral biofilm.

INTRODUCTION

Oral commensals *Streptococcus gordonii* and *Streptococcus sanguinis* play an important role in the development of the oral biofilm. As initial colonizers of the saliva coated tooth-surface, they promote the successive integration of several bacterial species into the developing oral biofilm by providing specific surface receptors for later colonizers (Kolenbrander et al., 2006; Rosan & Lamont, 2000). Under healthy conditions, this would lead to a benign biofilm community able to provide protection against invading species and keep opportunistic pathogens like *Streptococcus mutans* in low abundance (Brook, 1999; He et al., 2014; Reid et al., 2001).

The mechanism of this clinically relevant microbial interference is not well understood, but several *in vitro* studies suggest that the ability of *S. gordonii* and *S. sanguinis* to produce copious amounts of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) plays an important role (Tong et al., 2007; Zhu & Kreth, 2012). The \( \text{H}_2\text{O}_2 \) dependent interference of *S. sanguinis* and *S. gordonii* with *S. mutans* has been the subject of several studies (Kreth et al., 2005, 2008, 2009; Zhu & Kreth, 2012; Zhu et al., 2014). Most importantly, \( \text{H}_2\text{O}_2 \) is the only agent inhibiting the growth of *S. mutans* in dual species biofilms with *S. sanguinis* (Kreth et al., 2005). Moreover, several important genetic and environmental factors modulating \( \text{H}_2\text{O}_2 \) production have been identified (Kreth et al., 2008, 2009; Zheng et al., 2011a, 2011b, 2012). \( \text{H}_2\text{O}_2 \) is produced by the oxido-reductase pyruvate oxidase (SpxB), catalysing the conversion of pyruvate to acetyl phosphate, \( \text{CO}_2 \) and \( \text{H}_2\text{O}_2 \) under aerobic conditions (Carlsson et al., 1987). Besides the inhibitory action by \( \text{H}_2\text{O}_2 \), two additional SpxB-dependent effects in increasing competitiveness of commensals are important: (i) ATP production from acetyl phosphate for energy generation...
and (ii) H₂O₂-induced release of extracellular DNA (eDNA) (Kreth et al., 2008). eDNA is a major component of the extracellular polymeric substance of biofilms, promoting cell-cell and cell-tooth contact (Das et al., 2011; Kreth et al., 2009). H₂O₂-induced release of eDNA can also serve in horizontal gene transfer promoting genetic diversity, as we presented recently (Itzek et al., 2011). Overall, SpxB and H₂O₂ seem to play a major role in oral streptococcal ecology, as demonstrated for the H₂O₂-dependent bacterial interference (Kuramitsu et al., 2007; Zhu & Kreth, 2012). In addition, SpxB confers a significant physiological advantage for S. gordonii during infection in a murine model of abscess formation (Ramsey et al., 2011). Considering the importance of H₂O₂ in microbial interference, it might not be surprising that streptococci do not encode enzymes for direct H₂O₂ detoxification, albeit encoding several proteins required to fight intrinsically generated reactive oxygen species (ROS). For example, the catalase used by several Gram-positive and Gram-negative bacteria to directly eliminate H₂O₂ is not encoded in the genome of any oral streptococci (Engelmann & Hecker, 1996; Kim & Holmes, 2012; Mishra & Imlay, 2012).

Alternative streptococcal mechanisms to avoid major damage from H₂O₂ and ROS to cellular components such as proteins, DNA and membranes have been described (Fujishima et al., 2013; Lei et al., 2011; Pericone et al., 2003). The mechanisms are best understood for Streptococcus pneumoniae, which also produces large amounts of H₂O₂ (Yesilkaya et al., 2013). S. pneumoniae seems to use a network of different H₂O₂ resistance mechanisms, but surprisingly lacks an inducible response to H₂O₂ (Pericone et al., 2003). Accordingly, S. pneumoniae does not encode the major transcriptional regulator PerR, known to coordinate the response to H₂O₂ in Streptococcus pyogenes, Streptococcus suis and Streptococcus oligofermentans (Brenot et al., 2005; King et al., 2000; Ricci et al., 2002; Wang et al., 2014; Zhang et al., 2012). Interestingly, SpxB itself seemed to play a role in protection against H₂O₂ in S. pneumoniae, since an SpxB null mutant showed increased H₂O₂ susceptibility (Pericone et al., 2003). Unlike S. pneumoniae, S. sanguinis and S. gordonii encode a PerR homologue, suggesting an orchestrated response toward oxidative stress. In this study, we found that SpxB plays no role in resistance against H₂O₂.

Therefore, to better understand the oxidative stress response in these important oral streptococci, two predicted PerR regulon members, Dps and TrxB, as well as SodA involved in peroxide resistance in other micro-organisms, were investigated and compared between S. sanguinis and S. gordonii.

**METHODS**

**Bacterial strains and media.** S. sanguinis SK36 (Xu et al., 2007) and 133-79 (Herzberg et al., 1990). S. gordonii DL1 (Pacula & Walczak, 1963) and V288 (Herzberg et al., 1997), and their isogenic mutants (Table 1) were routinely grown in an anaerobic chamber with catalyst (90% N₂ / 5% CO₂ / 5% H₂) or aerobically (5% CO₂) at 37 °C in Brain Heart Infusion Broth (BHI; Difco) or on BHI agar plates. When required, cultures were supplemented with 500 µg ml⁻¹ spectinomycin, 300 µg ml⁻¹ kanamycin or 5 µg ml⁻¹ erythromycin.

**DNA manipulations.** Standard recombinant DNA manipulations were used (Sambrook et al., 1989). PCR was performed with a G-Storm GS1 thermocycler (GeneTechnologies) according to the manufacturer’s protocol. Phusion DNA polymerase was obtained from New England Biolabs and Accuprime DNA polymerase from Life Technologies. Phusion and Accuprime DNA polymerase were used for overlapping PCR. Oligonucleotides (Table S1, available in the online Supplementary Material) were designed using sequence data obtained from the NCBI server (http://www.ncbi.nlm.nih.gov/genome/) and synthesized by Integrated DNA Technologies.

**Construction of isogenic Dps⁺, TrxB⁺ and SodA⁺ mutants.** Overlap extension PCR technique (Song et al., 2005) was used to construct mutants shown in Table 1. Oligonucleotide sequences for the construction of mutants are given in Table S1. Briefly, the upstream and downstream regions of the respective gene were PCR amplified with specific oligonucleotides, respectively. The spectinomycin resistance cassette (spc) encoded by ada9 was PCR amplified from plasmid pFW5 (Podbielski et al., 1996). The oligonucleotides for the up- and downstream regions of the respective genes were oligonucleotide hybrids with end sequences complementary to the spc cassette to allow for overlap extension PCR. PCR-generated fragments of the respective upstream region, the spc cassette, and the downstream region were purified with a Qiagen PCR purification kit and mixed at a 1:1:1 molar ratio and amplified with flanking oligonucleotides. This amplification led to the ‘ligation’ of the three fragments in the order of 5’-upstream-spc-downstream-3’. The resulting PCR product was used to transform S. sanguinis and S. gordonii to generate individual deletions in dps, trxB and sodA. Transformation was performed using a protocol described earlier (Zhu et al., 2011). Confirmation of gene deletions was performed by PCR.

**Complementation of mutant strains.** The TrxB⁻ and SodA⁻ mutations were complemented in trans using shuttle plasmid pDL276 (Dunny et al., 1991). Briefly, both genes were amplified from S. gordonii chromosomal DNA and placed under the control of the S. gordonii ldh promoter using the primers described in Table S1. The resulting plasmids carrying trxB and sodA from S. gordonii were transformed into S. gordonii and S. sanguinis. The SodA and TrxB proteins have an identity of 93% and 88%, respectively, and we reasoned that S. gordonii genes would be sufficient to complement the phenotypes of the SodA⁻ and TrxB⁻ mutations in S. sanguinis. For complementation of dps in S. sanguinis and S. gordonii, a knock-in strategy was used as described before (Zheng et al., 2011a). Briefly, to restore the Dps⁻ mutant with a functional dps, overlap extension PCR was performed. For the upstream fragment, approximately 1500 bp of the entire dps was amplified and about 600 bp downstream of the dps sequence was amplified from S. sanguinis and S. gordonii chromosomal DNA. The downstream and upstream fragments were homologous to the surrounding sequence of the dps region still present in the Dps⁻ mutant. Each of the oligonucleotides listed as Up R and Dn F incorporated about 25 bases complementary to the erythromycin (erm) resistance genes ermAM (Martin et al., 1987). The ermAM resistance genes were amplified by PCR using oligonucleotides erm F and erm R. All three PCR amplicons were purified with a Qiagen PCR purification kit and mixed in a 1:1:1 ratio. The mixture served as the template for a second round PCR with the appropriate Up F and Dn R oligonucleotides. The resulting
Table 1. Strains and plasmids used in this study

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<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tr>
<td>DL1</td>
<td>WT S. gordonii</td>
<td>Pakula &amp; Walczak (1963)</td>
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<tr>
<td>SK36</td>
<td>WT S. sanguinis</td>
<td>Xu et al. (2007)</td>
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<td>V288</td>
<td>WT S. gordonii</td>
<td>Herzberg et al. (1997)</td>
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<td>133-79</td>
<td>WT S. sanguinis</td>
<td>Herzberg et al. (1990)</td>
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<tr>
<td>DL1 Δdps</td>
<td>Δdps::aad9</td>
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<tr>
<td>DL1 ΔtrxB</td>
<td>ΔtrxB::aad9</td>
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<td>SK36 Δdps</td>
<td>Δdps::aad9</td>
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<td>SK36 ΔsodA</td>
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PCR products were transformed into the SK36 and DL1 Dps+ strains to restore the WT copy and verified by PCR.

**H₂O₂ sensitivity assays.** To test for H₂O₂ susceptibility of planktonic cells, a published assay for *S. pneumoniae* (Pericone et al., 2003) was modified by reducing the H₂O₂ concentration to 10 mM. Briefly, bacterial cultures were grown overnight in an anaerobic chamber in BHI at 37 °C to avoid any adaptive effects due to H₂O₂ produced during aerobic cultivation. After fresh inoculation, cells were grown until exponential phase in BHI and adjusted in PBS or BHI when indicated (A₆₀₀ nm 0.6). Five hundred microlitres of the bacterial suspension was subsequently treated with 10 mM H₂O₂ for 30 min at 37 °C. After 30 min, cells were washed with PBS to remove residual H₂O₂ and serially diluted on BHI agar plates. After overnight incubation in an anaerobic chamber at 37 °C, cfu were counted. Survival (%) was calculated as the ratio of cfu in the H₂O₂ treated sample versus the untreated control, as previously reported (Wang et al., 2014).

**RNA isolation, cDNA synthesis, and real-time reverse transcriptase PCR (RT-PCR).** Streptococcal cells were harvested by centrifugation (3000 g, 15 min, 4 °C). Cell pellets were resuspended in TRIzol (Life Technologies) and stored at −80 °C. To isolate RNA, cells were disrupted three times for 30 s each time using lysing matrix B (MP Biomedicals) in a FastPrep FP210 homogenizer (Thermo Scientific; speed setting of 6.0). Total RNA isolation was carried out according to the manufacturer’s instructions (isolation of total RNA using TRIzol, Life Technologies). RNA samples were treated with Turbo DNase (Ambion) to remove traces of chromosomal DNA. An RNeasy MiniElute cleanup kit (Qiagen) was used to purify RNA samples after DNase treatment. cDNA was synthesized using a qScript cDNA synthesis kit (Quanta Biosciences) according to the manufacturer’s protocol. Real-time RT-PCR was performed to determine specific cDNA copies with the comparative threshold cycle (Cₜ) method using a MyiQ single-colour real-time PCR detection system (Bio-Rad) and PerfeCta SYBR Green SuperMix for iTQ (Quanta Biosciences). Relative changes in cDNA copies representing differential gene expression were calculated using the ΔCₜ method described previously (Zheng et al., 2011a). The 16S rRNA gene was used as the housekeeping reference gene using the 16S rRNA oligonucleotides described in Table S1.

**Determination of H₂O₂ concentration.** The concentration of H₂O₂ in liquid cultures was determined as described earlier (Zheng et al., 2011a). Briefly, cell-free culture supernatants (40 μl) were mixed with 160 μl of freshly prepared 0.1 M sodium acetate (pH 4.5) containing 0.1 μg horseradish peroxidase (Thermo Scientific) and 10 μl of 1 mg ml⁻¹ o-dianisidine (Alfa Aesar) in methanol. The reaction mixture was incubated at room temperature (RT) for 10 min and protected from light before A₄₁₅ was determined using a microplate reader (model 680; Bio-Rad). The concentration was calculated from a standard curve prepared in the same medium or buffer using a serial dilution of a commercial 30% H₂O₂ solution in MilliQ water. The concentration of the initial dilution was determined spectrophotometrically ([ε₄₁₅] = 43.6/M cm) using a SmartSpec Plus UV–visible spectrophotometer (Bio-Rad) before each new experiment. The detectable range was 0.1–4.0 mM H₂O₂ in BHI.

The H₂O₂ accumulation of *S. gordonii* and *S. sanguinis* grown on BHI agar plates was evaluated using an H₂O₂ indicator mix with azido-bis-thiosulfonate (ABTS; Sigma; 30 mg ml⁻¹) and horseradish peroxidase (Sigma, 2 mg ml⁻¹). Two hundred and fifty microlitres of the indicator mix was spread on the BHI plates (approximately 20 ml volume) prior to inoculation of the plates with the respective bacteria. Indicator plates were incubated overnight in an anaerobic chamber, and colour development assessed following exposure to ambient air at RT and 37 °C, essentially as described previously (Brenot et al., 2005).

**Growth kinetics.** The growth of WT and mutant strains was monitored using a Bioscreen C analyser version 2.4 (Oy Growth Curves AB), which measures the turbidity in multiple cultures in parallel for static cultures. Growth kinetics were monitored at 37 °C in BHI in 20 min intervals with 15 s shaking prior to A₆₀₀ nm readings.

**Statistical analysis.** Statistical significance was calculated using a two-sided Student’s t-test and Quickcalcs online calculator (http://www.graphpad.com/quickcalcs). P-values of less than 0.05 were considered statistically significant.

**RESULTS**

**Differential susceptibility of *S. gordonii*, *S. sanguinis* and *S. mutans* to physiological H₂O₂ concentrations**

The inhibitory activity of H₂O₂ produced by *S. sanguinis* and *S. gordonii* against *S. mutans* has been demonstrated...
(Kreth et al., 2008). To assess a potential cross-inhibition of the H$_2$O$_2$ producers, a growth inhibition assay was performed. Aliquots of S. sanguinis and S. gordonii were inoculated onto a BHI agar plate and incubated aerobically overnight as initial colonizers. Subsequently, cultures of S. sanguinis and S. gordonii as well as H$_2$O$_2$ susceptible S. mutans as a control were inoculated close to the initial colonizers (Fig. 1a). Inhibition was determined after overnight incubation. Observable inhibition of S. mutans by both S. sanguinis and S. gordonii was consistent with earlier reports (Kreth et al., 2005). Conversely, S. gordonii showed growth at the interface to its own spotted colony as well as to the S. sanguinis colony, while S. sanguinis appeared to be slightly inhibited although not as severely as S. mutans. The inhibition was due to the production of H$_2$O$_2$, since addition of H$_2$O$_2$-degrading horseradish peroxidase and genetic inactivation of SpxB abolished the inhibition (Fig. 1b). These observations demonstrate a robust auto-protective response of S. sanguinis and S. gordonii against their own H$_2$O$_2$ production. Furthermore, which was evident from the inhibition zones at the interface to S. mutans, S. gordonii produces more H$_2$O$_2$ than S. sanguinis. In parallel, S. gordonii can cope with the H$_2$O$_2$ it produces itself, while S. sanguinis can survive with lower self-generated H$_2$O$_2$ amounts, but has difficulties with the S. gordonii-generated H$_2$O$_2$.

**S. gordonii shows increased H$_2$O$_2$ resistance when compared to S. sanguinis**

The slight inhibition of S. sanguinis to its self-produced H$_2$O$_2$ and the H$_2$O$_2$ produced by S. gordonii suggested differences in the ability to manage H$_2$O$_2$ stress when compared to S. gordonii. The sensitivity against H$_2$O$_2$ was therefore tested for both WT strains. In addition, SpxB mutants constructed earlier (Kreth et al., 2008) were included in the susceptibility test (Fig. 2a). SpxB is the main source for competitive H$_2$O$_2$ production in both species (Kreth et al., 2008) and has been linked to the ability to withstand H$_2$O$_2$ challenge in S. pneumoniae (Pericone et al., 2003). A 30 min challenge of both strains with 10 mM H$_2$O$_2$ resulted in a reduction of recoverable bacteria (Fig. 2a). About 65% of S. gordonii survived, while only about 10% survival was detected for S. sanguinis. This is consistent with the results observed in the growth inhibition assay. Surprisingly the reported involvement of SpxB in H$_2$O$_2$ resistance in S. pneumoniae (Pericone et al., 2003) did not occur in S. gordonii and S. sanguinis. Both mutant strains showed no difference when compared to the parent strains after H$_2$O$_2$ challenge. This is also supported by the growth inhibition assay, where the SpxB mutants did not show increased inhibition (Fig. 2b). To exclude a possible effect of the metabolic status of the cells during our initial survival assay, which was performed with cells resuspended in PBS buffer (Fig. 2a), we repeated the experiments with cells resuspended in BHI medium. No difference was observed between the WT strains and the SpxB mutants (Fig. S1).

To demonstrate that the difference in the survival rate between S. gordonii and S. sanguinis was strain independent, survival after H$_2$O$_2$ treatment was also compared between S. sanguinis strains SK 36 and 133-79 and S. gordonii strains DL1 and V288. The survival rate was comparable between S. gordonii strains. The survival of S. sanguinis showed an obvious difference, with SK36 being able to survive better than 133-79 (Fig. 2c). However, the two S. sanguinis strains used here were more susceptible to H$_2$O$_2$ than the two S. gordonii strains.

**Role of Dps, TrxB and SodA in resistance against H$_2$O$_2$ stress**

In contrast to the observation in S. pneumoniae (Pericone et al., 2003), SpxB did not play a role in resistance against H$_2$O$_2$ in both commensal streptococci. To understand the contribution of other oxidative stress-related enzymes in

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**Fig. 1.** Growth inhibition assay to test for the inhibiting ability of H$_2$O$_2$ producers S. sanguinis and S. gordonii against themselves, each other and S. mutans. (a) Initial colonizers (arrows) were inoculated first and grown for 16 h before the later colonizers were inoculated beside the formed colonies. After a further overnight incubation, pictures were taken to document inhibition. Pictures were adjusted for brightness and contrast over the entire image. (b) Initial colonizers (arrow) were inoculated first and grown for 16 h before the later colonizers were inoculated beside the formed colonies on plates containing horseradish peroxidase (HRP; 0.5 mg per plate). After a further overnight incubation, pictures were taken to document inhibition. Pictures were adjusted for brightness and contrast over the entire image.
the resistance against H$_2$O$_2$ stress, isogenic Dps, TrxB and SodA mutants were constructed. Dps is a DNA-binding ferritin-like protein involved in the sequestration of iron, thereby preventing the generation of reactive toxic radicals and functioning in oxidative damage protection (Yamamoto et al., 2002) (GenBank: ABN44083 (SK36); ABV10801 (DL1). TrxB is a thioredoxin reductase and part of the thioredoxin system. TrxB is required for the reduction of thioredoxin involved in the formation of reduced disulfide bonds in oxidized proteins, thus protecting cells against ROS damage (Marco et al., 2013) (GenBank: ABN45246 (SK36); ABV09235 (DL1), and SodA is the superoxide dismutase detoxifying superoxide (GenBank: ABN44155 (SK36); ABV09492 (DL1). As shown in Fig. 3, the Dps mutation had the most severe impact on the survival rate of S. sanguinis and S. gordonii, with a significant 10$^4$-fold reduction in recoverable bacteria after H$_2$O$_2$ treatment. Complementation restored WT resistance in both species (Fig. S2). Interestingly, the TrxB mutation caused also a significant reduction in the survival rate of S. sanguinis, but no effect was measured in S. gordonii when compared to the H$_2$O$_2$ treated WT. The SodA mutation had a significant impact on the survival of S. gordonii in the presence of hydrogen peroxide (P=0.001), while no significant difference was observed for S. sanguinis (Fig. 3). In summary, Dps seems to provide an important protection mechanism under the tested H$_2$O$_2$ stress condition in both commensal streptococci by preventing Fenton-mediated oxidative damage of DNA.

The TrxB mutation impairs growth under aerobic conditions

Growth curves of all strains showed that the TrxB mutants were impaired in growth, while the Dps and SodA mutants had a similar growth profile to the WT strains. Automatic growth measurements were taken in ambient air every 20 min with periodic shaking prior to absorption readings suggesting that the growth of the TrxB mutants might be influenced by oxygen (Fig. 4 and Fig. S3).

To test growth in cells exposed directly to oxygen, cultures were grown under anaerobic conditions until mid-exponential phase and serially diluted. Aliquots were inoculated onto duplicate BHI agar plates and incubated either without oxygen or with oxygen and 5% CO$_2$. As evident from Fig. 5, the TrxB mutant failed to grow under aerobic conditions in

![Fig. 2. Survival of S. sanguinis and S. gordonii after H$_2$O$_2$ challenge. (a) S. gordonii and S. sanguinis as well as their isogenic SpxB$^-$ mutants were treated with 10 mM H$_2$O$_2$ for 30 min. C.f.u. were determined after serial dilutions were plated onto BHI agar plates. Survival was calculated as the ratio of H$_2$O$_2$ treated cells to the untreated control. (b) Growth inhibition assay with WT strains and SpxB$^-$ mutants. Initial colonizers (arrows) were inoculated first and grown for 16 h before the later colonizers were inoculated beside the formed colonies. After a further overnight incubation, pictures were taken to document inhibition. Pictures were adjusted for brightness and contrast over the entire image. (c) Comparison of survival rates between different strains of S. sanguinis (SK36 versus 133-79) and S. gordonii (DL1 versus V288). Bars show mean ± SD of three independent experiments (a, c), or a representative picture of two independent experiments (b).]
both species while growth was not affected under anaerobic conditions. Complementation of TrxB using a plasmid-encoded copy restored WT growth (Fig. S4). No defect was observed for the other strains tested.

Gene expression response to oxygen and H$_2$O$_2$

To determine if exposure to oxygen or a physiologically relevant H$_2$O$_2$ concentration influences the expression of $dps$, $sodA$ or $trxB$, cells were grown under static conditions, under static conditions with added H$_2$O$_2$ (2 mM) or as shaken cultures, a growth condition known to induce maximal H$_2$O$_2$ production. In addition, expression of $recA$ was determined. Expression of $recA$ is induced under stress conditions in other streptococci (Giliberti et al., 2006; Len et al., 2004). As shown in Fig. 6, expression of $recA$ was only induced slightly under the tested conditions in $S$. $sanguinis$ or $S$. $gordonii$. Interestingly, despite the importance of Dps in the prevention of Fenton reactions caused by DNA damage, expression was reduced in both species. Expression of $sodA$ was induced under the tested conditions in $S$. $sanguinis$ and $S$. $gordonii$. A difference was also observed in the expression of $trxB$. $S$. $sanguinis$ responded with lower expression of $trxB$ compared to the static control, but $S$. $gordonii$ showed induced expression.

TrxB contributes to H$_2$O$_2$ balance in $S$. $gordonii$

To test if the investigated factors contributed to the H$_2$O$_2$ balance in $S$. $sanguinis$ and $S$. $gordonii$, H$_2$O$_2$ accumulation was investigated using specific H$_2$O$_2$ indicator medium, which develops a purple colour in the presence of H$_2$O$_2$ (Brenot et al., 2005). Cells were incubated for 16 h anaerobically to test for de novo produced H$_2$O$_2$ after the cells were shifted to an aerobic environment. Transfer to an aerobic environment resulted in colour development as early as 2 min after oxygen exposure for $S$. $gordonii$ at RT, while $S$. $sanguinis$ colonies remained white for up to 30 min when maintained at RT (Fig. 7a). Incubation at 37 °C also led to delayed development of the purple colour for $S$. $sanguinis$ colonies when compared to $S$. $gordonii$. The increased accumulation of H$_2$O$_2$ in the TrxB mutant of $S$. $gordonii$ was clear, with a darker colour development than the WT or any other mutant tested. The increased production of H$_2$O$_2$ by the $S$. $gordonii$ TrxB mutant was also confirmed, by growing cells in the same way as the on-plate assay, but subsequently resuspended from the plate and incubated for 1 h under de novo H$_2$O$_2$ producing conditions as planktonic cultures at 37 °C (Fig. 7b). The TrxB cells were able to produce about two to three times as much H$_2$O$_2$ when compared to the other $S$. $gordonii$ WT.

Manganese is not important for survival of H$_2$O$_2$ stress

The trace element manganese plays a pivotal role in the ability of several streptococci to cope with H$_2$O$_2$. A recent report demonstrated that the H$_2$O$_2$ producing $S$. $oligofermentans$ has an increased survival rate in the presence of manganese when challenged with H$_2$O$_2$ (Wang et al., 2014). To investigate whether the presence of elevated manganese concentrations in growth medium affects $S$. $gordonii$ and $S$. $sanguinis$ survival under conditions of H$_2$O$_2$ stress, BHI was supplemented with 0.25 mM MnCl$_2$, as reported for $S$. $oligofermentans$ (Wang et al., 2014). Cells were pre-grown overnight in the presence of MnCl$_2$. Overnight cultures were inoculated in BHI+MnCl$_2$, and grown either continuously as static cultures with no oxygen intake or placed on a rocker under aerobic conditions to promote endogenous H$_2$O$_2$ production for 1 h prior to challenge with 10 mM H$_2$O$_2$. Interestingly, no protective function of manganese was observed under these growth conditions. However, an increase in the survival rate of $S$. $sanguinis$ was observed when cells were exposed to oxygen when compared to the static culture condition (Fig. S5). This result suggests that $S$. $sanguinis$ is able to adapt to aerobic conditions with an increased resistance against ROS such as H$_2$O$_2$.

DISCUSSION

Bacteria generate and are exposed to ROS during growth under aerobic conditions, due to enzymic reactions in several biochemical pathways (Imlay, 2003). ROS include superoxide anion (O$_2^-_{}$), hydroxyl radicals (OH$^-$), and H$_2$O$_2$ (Imlay, 2013). Streptococci encoding the enzyme pyruvate oxidase or SpxB are known to produce considerable amounts of H$_2$O$_2$, exceeding 1 mM as reported for $S$. $pneumoniae$ and $S$. $gordonii$ (Itzek et al., 2011; Pericone et al., 2000). In the present study, the manganese concentration in the growth medium was increased to 0.25 mM to investigate whether manganese plays a role in $S$. $gordonii$ and $S$. $sanguinis$ survival in the presence of H$_2$O$_2$. Although the report by Wang et al. (2014) suggested that manganese is important for the survival of $S$. $oligofermentans$, no such protective function was observed in $S$. $gordonii$ and $S$. $sanguinis$. The $S$. $gordonii$ TrxB mutant displayed a higher resistance to H$_2$O$_2$, while the $S$. $sanguinis$ TrxB mutant showed a decreased resistance, compared to the respective WT strains. This suggests that manganese is not an essential factor for $S$. $gordonii$ and $S$. $sanguinis$ survival in the presence of H$_2$O$_2$. The observed differences in the survival rates of manganese-exposed cells compared to those grown without manganese might be explained by the presence of other factors that influence H$_2$O$_2$ production and resistance.
et al., 2003). Whether such high concentrations are actually achieved in the human host environment is debatable, since several scavenging mechanisms are in place, such as salivary peroxidase, which effectively removes H$_2$O$_2$ (Ashby et al., 2009). Furthermore, salvia or mucus flow would dilute bacterially generated H$_2$O$_2$. However, the H$_2$O$_2$ concentration at the biofilm–environment interface of S. gordonii biofilms demonstrates that local concentrations of H$_2$O$_2$ might be considerable and could affect bacterial and host cells in the immediate vicinity (Liu et al., 2011). The generation of ROS, and in particular H$_2$O$_2$, poses a risk to the cells since the presence of metal ions like Fe$^{2+}$ would promote the Fenton reaction causing DNA, protein, lipid and RNA damage (Imlay, 2003). Interestingly, H$_2$O$_2$ producing streptococci do not encode the catalase enzyme, which is considered the main enzyme for detoxification of high H$_2$O$_2$ concentrations (Mishra & Imlay, 2012), yet they are perfectly able to grow in the presence of their own produced H$_2$O$_2$. Our initial finding that S. sanguinis and S. gordonii showed minimal self-inhibitory effects against their intrinsically produced H$_2$O$_2$ prompted further investigation into the mechanism of H$_2$O$_2$ resistance. A difference in survival ability was determined, demonstrating that both commensals might rely on alternative mechanisms to detoxify and withstand intrinsic H$_2$O$_2$ production.

**Fig. 4.** Growth curves of S. sanguinis and S. gordonii WT strains and isogenic Dps$^{-}$, TrxB$^{-}$ and SodA$^{-}$ mutants. Strains were grown in BHI medium under aerobic conditions. Growth was monitored in a Bioscreen C system with shaking for 15 s every 20 min prior to absorption readings at 580 nm. The results are representative of two independent experiments with multiple technical replicates.

**Fig. 5.** Comparison of growth under aerobic and anaerobic conditions of S. sanguinis and isogenic Dps$^{-}$, TrxB$^{-}$ and SodA$^{-}$ mutants. Cells were grown to exponential phase, adjusted to the same cell density and serially diluted. Ten microlitres of every dilution step was spotted onto the plates, and the plates were incubated overnight under anaerobic or aerobic (in the presence of 5% CO$_2$) conditions. A representative picture of three independent experiments is shown.
Both *S. sanguinis* and *S. gordonii* encode a PerR homologue (*S. gordonii* SGO 0703 and *S. sanguinis* SSA 0686), suggesting an orchestrated response toward oxidative stress. The RegPrecise regulon database (Novichkov et al., 2010) lists four genes for *S. gordonii* and nine for *S. sanguinis*, regulated by PerR with predicted PerR binding sites (see Table 2). The expression of two genes predicted to be regulated by PerR in both species was examined in this study. Interestingly, expression of *dps* was reduced under aerobic growth conditions and in the presence of physiological concentrations of H2O2 in both species. This was a surprising result given the fact that a deletion of the *dps* gene had a strong effect on survival in the presence of H2O2. Expression of *dps* is growth-phase dependent as demonstrated for *S. suis* with a maximum expression during exponential growth and a decline in the stationary phase (Pulliainen et al., 2003). The protein abundance on the other hand is relatively constant, suggesting that functional Dps protein is available for the cell even during the stationary phase (Pulliainen et al., 2003). Whether a similar expression and protein abundance pattern exists in *S. sanguinis* and *S. gordonii* is yet to be determined. However, *dps* in *S. suis* is PerR regulated (Zhang et al., 2012) making it likely that the expression is comparable between all three species. Inactivation of PerR in *S. pyogenes* increased expression of *dps* about threefold, demonstrating that the repressive function of PerR is not very strong (Brenot et al., 2005). The second gene of the predicted PerR regulon examined was *trxB*. An obvious difference was observed between both species, with an increased expression of *trxB* in *S. gordonii* under oxidative stress, while the gene is not induced under oxidative stress in *S. sanguinis*. However, the role of PerR in the regulation of *dps* and *trxB* under the tested conditions is not clear, and further research is required to dissect the temporal regulatory role of PerR.

A similar induction under tested conditions was observed for *sodA* in both species, although again with a relatively low fold increase. Our data are consistent with the fold induction reported for the *sodA* gene in *S. pyogenes* after H2O2 challenge, although a lower H2O2 concentration was used and the challenge time was shorter (Grifantini et al., 2011). The overall low induction of these investigated genes raises the issue of why there is not a more pronounced response to oxygen or H2O2, especially considering that the inactivation of *dps*, *sodA* and *trxB* had a severe effect on the survival of the mutant strains during oxidative stress. However, the overall response to oxidative stress is likely to include more gene products than investigated here. For example, the global *S. pyogenes* H2O2 response includes 84 upregulated genes (Grifantini et al., 2011). Interestingly, the fold induction as a response to H2O2 stress was moderate as well, with most genes upregulated about two to threefold (Grifantini et al., 2011). A plausible explanation for this moderate increase in expression is that streptococci express oxidative stress-related genes more or less constitutively, and/or distribute the burden amongst several gene products. In addition, post-transcriptional regulation might be another regulatory mechanism for some streptococci to ensure an adequate response to oxidative stress. The aforementioned observed protein abundance of Dps in the early stationary phase despite a decrease of *dps* transcription exemplifies this.

In a recent report, the requirement for *trxB* during aerobic growth was demonstrated for *S. sanguinis* (Rhodes et al., 2014). We observed the same phenotype in our study for *S. sanguinis* and *S. gordonii*, highlighting the importance of thioredoxin reductase activity for both oral streptococci. *TrxB* is required for the reduction of thioredoxin and the reduction of disulfide bonds. This is especially important during the reduction of ribonucleotides to deoxyribonucleotides by aerobic class Ia (NrdEF) ribonucleotide reductases. The reduction to free thiols requires TrxB and thioredoxin for multiple turnovers essential for DNA replication and repair (Makhlynets et al., 2014; Rhodes et al., 2014). In addition, another important cytoplasmic protein required for the turnover of disulfide bonds is methionine sulfoxide reductase, MsrA (Ritz & Beckwith, 2001). Bacteria utilize MsrA to reverse oxidative damage on intra- and extracellular...
surface proteins and its function has been demonstrated in S. gordonii (Ezraty et al., 2004; Lei et al., 2011). A homologue of msrA is present in the genome of S. sanguinis (SSA_0374). However, deletion of the msrA gene in S. gordonii had a delayed growth phenotype, but was not lethal. The deletion of trxB has therefore several consequences on the ability of S. sanguinis and S. gordonii to cope with oxidative stress; interference with the NrdEF pathway being the most severe.

Manganese is an important co-factor for SodA, which has been demonstrated to be the only superoxide dismutase enzyme in S. gordonii and S. sanguinis (DiGuiseppi & Fridovich, 1982; Jakubovic et al., 2002). Furthermore, the importance of manganese in oxidative stress defence has recently been demonstrated for S. oligofermentans. Addition of Mn^{2+} to BHI medium increased the survival rate of S. oligofermentans up to 23-fold after H_{2}O_{2} stress (Wang et al.,

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**Fig. 7.** 
*De novo* production of H_{2}O_{2} in WT and isogenic Dps\(^{-}\), TrxB\(^{-}\) and SodA\(^{-}\) mutants. (a) *De novo* production of H_{2}O_{2} in S. gordonii, S. sanguinis and their respective mutants was evaluated following growth on H_{2}O_{2} indicator plates. Colonies producing H_{2}O_{2} appear purple, while non-producer colonies appear white. Cells were incubated overnight under anaerobic conditions and exposed to air. Colour development was followed over time with cells incubated at RT or at 37 °C and documented by photography. Shown is a representative composite figure of three independent experiments. (b) Determination of H_{2}O_{2} production with cells immediately removed from the plates and resuspended in BHI medium. The cells were incubated at 37 °C under shaking conditions to promote H_{2}O_{2} production for 1 h. H_{2}O_{2} concentration was measured with a colorimetric assay. The detection limit is 0.1 mM H_{2}O_{2}. Data presented are mean ± SD of two independent experiments.

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![Graph](http://mic.sgmjournals.org)
2014). Interestingly, we did not see an effect of Mn$^{2+}$ on the survival rate of $S.$ sanguinis or $S.$ gordonii. The concentration of Mn$^{2+}$ in BHI is approximately 0.2–0.4 μM (Crump et al., 2014; Wang et al., 2014), which is in the range of what was determined to be the salivary Mn$^{2+}$ concentration (Watanabe et al., 2009). It is possible that the Mn$^{2+}$ concentration in BHI medium is already saturated, and that the supplementation of Mn$^{2+}$ with 250 μM as reported for $S.$ oligofermentans and used in this study has no additional effect on the $S.$ sanguinis or $S.$ gordonii oxidative stress response. Our observation is in agreement with a previous report (Crump et al., 2014) arguing against a direct protective function of Mn$^{2+}$ against ROS by dissociating H$_2$O$_2$ as reported for other species (Archibald & Fridovich, 1981; Tseng et al., 2001). Alternatively, the failure to respond with an increase in ROS tolerance in the presence of Mn$^{2+}$ could be explained by the PerR dependent regulation of ROS responsive genes. PerR contains two metal-binding sites, a structural Zn$^{2+}$ site and a regulatory divalent metal ion site that preferentially binds Fe$^{2+}$ or Mn$^{2+}$ (Herbig & Helmann, 2001; Lee & Helmann, 2006). It has been shown that the presence of Mn$^{2+}$ in the regulatory site renders the repressor relatively insensitive to H$_2$O$_2$ (Fuangthong et al., 2002; Lee & Helmann, 2006) and the cells are therefore not able to respond with an increase in ROS resistance.

The original purpose of this study was to determine the reason for the observed difference in H$_2$O$_2$ susceptibility of $S.$ gordonii and $S.$ sanguinis. The answer to this question appears to be more complex and will take a more comprehensive analysis including an investigation of the global transcriptional profile in response to H$_2$O$_2$. However, this study might provide an initial explanation. One obvious difference between $S.$ gordonii and $S.$ sanguinis is the pattern of H$_2$O$_2$ production, which starts much earlier in $S.$ gordonii (see Fig. 7a, b). While after 20 min at 37 °C following a shift to ambient oxygen, $S.$ gordonii already produces H$_2$O$_2$, $S.$ sanguinis generates no measurable H$_2$O$_2$. This is in agreement with our earlier report showing increased H$_2$O$_2$ production of planktonic $S.$ gordonii during exponential growth when compared to $S.$ sanguinis (Kreth et al., 2008). This suggests that $S.$ gordonii is able to establish a response to H$_2$O$_2$ production much earlier. In addition, $S.$ gordonii has a similar survival rate after H$_2$O$_2$ treatment when non-aerated versus aerated cells are compared, while the survival rate increased for aerated $S.$ sanguinis cells when compared to non-aerated cells (see Fig. S5). It appears that $S.$ gordonii is ready to deal with oxidative stress much earlier during growth, with an oxidative stress protection machinery present even under anaerobic conditions, while $S.$ sanguinis requires a longer adaptation time. From an ecological perspective, this makes sense since $S.$ sanguinis is the numerically abundant initial colonizer occupying the same intraoral niche as $S.$ gordonii (Nobbs et al., 2007). However, $S.$ gordonii is lower in abundance, yet manages to survive in the oral biofilm. Its ability to produce more H$_2$O$_2$ much earlier and be more resistant to H$_2$O$_2$ might assist in this competition.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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**Table 2.** RegPrecise predicted PerR regulon members in *S. sanguinis* and *S. gordonii*

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<td>$S.$ sanguinis</td>
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<tr>
<td>$S.$ gordonii</td>
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<tr>
<td></td>
<td>mcrA Non-specific DNA-binding protein; Dps homologue</td>
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<tr>
<td></td>
<td>perR Peroxide stress response transcriptional regulator PerR</td>
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<td></td>
<td>trxB Thioredoxin reductase</td>
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<td>fhuA, fhuD Ferrichrome ABC transporter</td>
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<tr>
<td>mntC, mntB, mntA Manganese ABC transporter</td>
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Fuangthong, M., Herbig, A. F., Bsat, N. & Helmann, J. D. (2002). Regulation of the Bacillus subtilis fur and perR genes by PerR: not all members of the PerR regulon are peroxide inducible. J Bacteriol 184, 3276–3286.


