Identification of hydrogen peroxide production-related genes in *Streptococcus sanguinis* and their functional relationship with pyruvate oxidase

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Hydrogen peroxide (H₂O₂), an important substance produced by many members of the genus *Streptococcus*, plays important roles in virulence and antagonism within a microbial community such as oral biofilms. The *spxB* gene, which encodes pyruvate oxidase, is involved in H₂O₂ production in many streptococcal species. However, knowledge about its regulation and relation with other genes putatively involved in the same pathway is limited. In this study, three genes – *ackA*, *spxR* and *tpk* – were identified as contributing to H₂O₂ production in *Streptococcus sanguinis* by screening mutants for opaque colony appearance. Mutations in all three genes resulted in significant decreases in H₂O₂ production, with 16–31 % of that of the wild-type. H₂O₂ production was restored in the complemented strains. Antagonism against *Streptococcus mutans* by these three *S. sanguinis* mutants was reduced, both on plates and in liquid cultures, indicating the critical roles of these three genes for conferring the competitive advantage of *S. sanguinis*. Analysis by qPCR indicated that the expression of *spxB* was decreased in the *ackA* and *spxR* mutants and significantly increased in the *tpk* mutant.

INTRODUCTION

Hydrogen peroxide (H₂O₂) is produced by many members of the genus *Streptococcus* (García-Mendoza et al., 1993; Kreth et al., 2005; Ramos-Montañez et al., 2008) and is important in three aspects. First, H₂O₂ is reported to correlate with virulence in *Streptococcus pneumoniae* (Auzat et al., 1999; Ramos-Montañez et al., 2008; Weiser et al., 1994). *S. pneumoniae* undergoes spontaneous phase variation resulting in opaque and transparent colony forms, and the differences in colony opacity correlate with virulence (Weiser et al., 1994). Recent research indicates that transparent variants are more proficient in colonization, with more production of teichoic acid and H₂O₂, but with less production of capsule than opaque variants (Ramos-Montañez et al., 2008). Studies also suggest that the H₂O₂ produced by *Streptococcus pyogenes* acts as a potential virulence factor by exerting direct damage to host tissues (Ginsburg & Sadovnic, 1998; Ginsburg & Varani, 1993). Second, H₂O₂ production is related to competition and co-existence within microbial communities such as oral biofilms. Many streptococci are able to produce inhibitory substances such as H₂O₂ to reduce the growth of co-resident micro-organisms. For example, *S. sanguinis* can produce H₂O₂ that will inhibit growth of *Staphylococcus aureus* (Uehara et al., 2006). *S. sanguinis* and *Streptococcus gordonii* demonstrate antagonistic activity against *Streptococcus mutans* via H₂O₂ production (Kreth et al., 2005). Other studies also investigated the inhibitory capacity of H₂O₂ produced by various species of oral streptococci (García-Mendoza et al., 1993; Kreth et al., 2005, 2008). Recently, H₂O₂ was also shown to contribute to the release of DNA from *S. sanguinis* and *S. gordonii*, which appears to support oral biofilm formation and facilitate exchange of genetic material among competent strains (Kreth et al., 2009). Third, H₂O₂ is a by-product of aerobic metabolism (Jakubovics et al., 2002). Many streptococci produce relatively large amounts of H₂O₂ during aerobic growth by the action of oxidase enzymes such as pyruvate oxidase and NADH oxidase (Auzat et al., 1999; Tittmann et al., 2005).

*S. sanguinis* is a member of the human indigenous oral microflora and one of the major microbes colonizing teeth (Kuramitsu et al., 2007; Rosan & Lamont, 2000). It is also one of the most common causative agents of infective endocarditis (Douglas et al., 1993; Mylonakis & Calderwood, 2001; Tleyjeh et al., 2005). On the other hand, *S. sanguinis* is considered an antagonistic bacterium against *S. mutans* (Becker et al., 2002; Caufield et al., 2000). Relatively high proportions of *S. sanguinis* are generally found in dental plaque with lower levels of *S. mutans*. High levels of *S. mutans* in the oral cavity correlate with low levels of *S. sanguinis* (Caufield et al., 2000; Ge et al., 2008b).

In *S. pneumoniae*, the function of pyruvate oxidase (SpxB) in H₂O₂ production has been well characterized (Weiser et al.,...
Although H₂O₂ production is involved in important metabolic pathways, potential pathogenic virulence, and interspecies competition, knowledge of its metabolic basis and regulation remains limited in S. sanguinis. The spxB gene is involved in H₂O₂ production (Weiser et al., 2014 Microbiology). To identify SpxR, a regulator of spxB required for spxB transcription and for full virulence in a murine model of infection (Ramos-Montañez et al., 2008).

When needed, medium was supplemented with kanamycin (500 μg ml⁻¹), chloramphenicol (5 μg ml⁻¹) or erythromycin (10 μg ml⁻¹). The mutants were named by using “ssx” to refer the corresponding ssa gene in the NCBI database.

**Methods**

**Bacterial strains and growth media.** The strains used are described in Table 1. S. sanguinis strain SK36 (obtained from Dr Mogens Kilian, Århus University, Denmark) was isolated from human dental plaque (Kilian & Holmgren, 1981). This strain and its derivatives were routinely grown in brain heart infusion broth (BHI; Difco) supplemented with 1.5 % (w/v) agar under microaerobic conditions (7.2 % H₂, 7.2 % CO₂, 79.6 % N₂ and 6 % O₂) in an Anoxomat jar at 37 °C as described previously (Ge et al., 2008a; Paik et al., 2005). S. mutans UA159 or its derivative was also routinely grown in BHI under microaerobic conditions as for S. sanguinis. When needed, medium was supplemented with kanamycin (500 μg ml⁻¹), chloramphenicol (5 μg ml⁻¹) or erythromycin (10 μg ml⁻¹). The mutants were named by using “ssx” to refer the corresponding ssa gene in the NCBI database.

**Table 1. Bacterial strains used in this study**

| Strain | Phenotype or description | Source |
|--------|--------------------------|--------|
| **S. sanguinis** | | |
| SK36 | Human plaque isolate | Kilian & Holmgren (1981) |
| sxx_0169 | Km<sup>+</sup> ΔiddA::aphA-3 | This study |
| sxx_0192 | Km<sup>+</sup> ΔackA::aphA-3 | This study |
| sxx_0192C | Em<sup>+</sup> ΔackR::pcerm | This study |
| sxx_0190 | Km<sup>+</sup> Δssa_0190::aphA-3 | This study |
| sxx_0191 | Km<sup>+</sup> Δssa_0191::aphA-3 | This study |
| sxx_0193 | Km<sup>+</sup> Δssa_0193::aphA-3 | This study |
| sxx_0195 | Km<sup>+</sup> Δssa_0195::aphA-3 | This study |
| sxx_0391 | Km<sup>+</sup> ΔspxB::aphA-3 | This study |
| sxx_0391C | Cm<sup>+</sup> spxB<sup>+</sup> Δmagellan2 | This study |
| sxx_1492 | Km<sup>+</sup> ΔspxB::aphA-3 | This study |
| sxx_1492C | Cm<sup>+</sup> spxB<sup>+</sup> Δmagellan2 | This study |
| sxx_1494 | Km<sup>+</sup> Δssa_1494::aphA-3 | This study |
| sxx_1493 | Km<sup>+</sup> Δssa_1493::aphA-3 | This study |
| sxx_1490 | Km<sup>+</sup> Δssa_1490::aphA-3 | This study |
| sxx_1489 | Km<sup>+</sup> Δssa_1489::aphA-3 | This study |
| sxx_2118 | Km<sup>+</sup> Δtpk::aphA-3 | This study |
| sxx_2118C | Km<sup>+</sup> Δtpk::aphA-3 | This study |
| sxx_2120 | Km<sup>+</sup> Δssa_2120::aphA-3 | This study |
| sxx_2119 | Km<sup>+</sup> Δssa_2119::aphA-3 | This study |
| sxx_2117 | Km<sup>+</sup> Δssa_2117::aphA-3 | This study |
| sxx_2116 | Km<sup>+</sup> Δssa_2116::aphA-3 | This study |
| **S. mutans** | | |
| UA159 | Wild-type, serotype c | ATCC 700610 |
| smx_42 | Cm<sup>+</sup> Δsmx.42::magellan2 | This study |
Mutant construction and complementation. For the construction of precise single gene deletion mutants in *S. sanguinis* SK36, we developed a PCR-based recombinant method employing linear DNA for deletion construction *in vitro* (P. Xu and others, unpublished data). Briefly, for each targeted gene, three sets of primers were designed to amplify a linear DNA fragment containing the kanamycin resistance cassette (*aphA-3*) (Turner *et al.*, 2009) with two flanking arms of DNA upstream and downstream of the targeted gene. The linear recombinant PCR amplicon was directly transformed into *S. sanguinis* competent cells as described previously (Ge *et al.*, 2008a). A 96-well high-throughput format was used to generate a genome-wide mutant library. The mutants were confirmed by PCR and RT-PCR analyses.

To construct the complemented strain, a DNA fragment containing the targeted gene followed by a selectable marker (either chloramphenicol or erythromycin resistance cassette) (Turner *et al.*, 2009) was integrated via double homologous recombination into the corresponding mutant (Table 1) to replace the kanamycin resistance cassette. Chloramphenicol- or erythromycin-resistant and kanamycin-sensitive transformants were selected and confirmed by PCR analysis.

Screening for H$_2$O$_2$ production-defective mutants by opaque colony morphology. For opaque colony observation, 5 μl overnight culture of *S. sanguinis* SK36 or different mutants was spotted on the surface of BHI plates and incubated at 37 °C under microaerobic conditions. For catalase-containing plates, 100 μl catalase from bovine liver (Sigma) was spread on the BHI plate surface (~880 U H$_2$O$_2$). The plates were air-dried for 10 min in a hood before bacterial inoculation. Bacterial suspension (5 μl) was spotted on the catalase-containing plate and incubated at 37 °C under microaerobic conditions for 2 days. The opaque colonies on the plate were recorded and photographed using a BioDoc-It imaging system. After obtaining the opaque mutants in the assays, the integrity of the mutations was confirmed by using PCR amplification and sequencing.

H$_2$O$_2$ release assays. H$_2$O$_2$ production was quantified using the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen) as described by the manufacturer, with minor modifications (Ramos-Montañez *et al.*, 2008). Briefly, 100 μl reaction mixture (50 mM Amplex Red reagent, 0.1 U horseradish peroxidase ml$^{-1}$ in 0.05 M sodium phosphate buffer, pH 7.4) was dispensed into wells of a 96-well microtitre plate in triplicate. The cells were incubated overnight in static culture under microaerobic conditions. Cells were dispersed by vigorous pipetting and serial dilutions were plated on BHI agar plates supplemented with chloramphenicol in triplicate and the c.f.u. was determined.

RNA extraction and qPCR analyses. Total RNA was prepared from the cells growing in liquid exponential phase in BHI medium under microaerobic conditions to OD$_{660}$ 0.6–1.0. Cells were lysed after lysozyme treatment and mechanical disruption using FastPrep lysing matrix B (Qiogene). RNA was isolated by using the RNeasy mini kit (Qiagen). DNA was removed from the RNeasy mini kit column by DNase I treatment. Total RNA was quantified using a NanoDrop ND 1000 spectrophotometer. First-strand cDNA synthesis was performed in a 20 μl reaction mixture containing 100 ng RNA, 0.5 μl random primers (3 μg μl$^{-1}$), 1.0 μl dNTP mix (10 mM each dNTP), 1.0 μl 100 mM DTT, 1.0 μl RNAout (40 U; Invitrogen) and 0.5 μl SuperScript III reverse transcriptase (200 U μl$^{-1}$) in first-strand buffer (Invitrogen). Reactions lacking reverse transcriptase were prepared in parallel as controls for possible DNA contamination. First strand cDNA from each reaction was subjected to 90-fold dilutions, and 2 μl of each dilution was used as template for each PCR. Quantitative real-time PCR was performed in reactions containing 5 μl SYBR Green PCR master mix (Applied Biosystems), 1 μl each PCR primer (2 mM) using the ABI 7500 fast real-time PCR system. The housekeeping gene *gyrA* was used as a normalization control. The data were collected and statistically analysed from triplicates. Serial dilutions of chromosomal DNA from wild-type strain SK36 were used for standard curves.

**RESULTS**

H$_2$O$_2$ production determines colony morphology in *S. sanguinis* SK36

During the process of creating a genome-wide single gene deletion mutant library of *S. sanguinis* SK36, it was found that some mutants showed different colony morphologies. In comparison with the semi-transparent colony of the wild-type strain SK36, certain mutants presented an opaque colony when grown on BHI agar plates under microaerobic conditions. It was reported previously that the colony morphology variation between transparent and opaque colonies in *S. pneumoniae* is related to H$_2$O$_2$ production and can be detected on tryptic soy agar plates by the addition of catalase (Weiser *et al.*, 1994). Such an opaque colony marker has been used to identify genes involved in H$_2$O$_2$ production in *S. pneumoniae* (Ramos-Montañez *et al.*, 2008). To investigate the existence of similar morphological variation in *S. sanguinis* and to establish a condition to screen genes involved in H$_2$O$_2$ production, *S. sanguinis* SK36 was cultured on BHI plates with and without the addition of excess catalase to hydrolyse peroxidases. The colony opacity was compared to that of the wild-type strain, SK36. Paired t-test was used for statistical analysis.
for 2 days when catalase was added (data not shown). This showed that in *S. sanguinis*, the opaque colony variations were related to H$_2$O$_2$ production. The result also suggested that H$_2$O$_2$ production-defective mutants of *S. sanguinis* SK36 might be identified by colony morphology in our system. We hypothesized that the mutants with opaque colonies had lower H$_2$O$_2$ production.

Next, to identify the potential genes involved in H$_2$O$_2$ production, we screened over 1000 available single gene deletion mutants for variation in colony morphology to identify potential H$_2$O$_2$-production-defective mutants, as described above. Four mutants showing obvious opaque colonies were identified. The morphological variations of the four mutants were further confirmed by comparison with the wild-type strain SK36 on BHI plates, including a control strain ssx_0169 with kanamycin resistance (data not shown) to determine that the kanamycin resistance gene did not interfere with the phenotypes being investigated. This control strain was selected because it was demonstrated that the *ssx_0169* gene did not affect important cellular phenotypes (Turner et al., 2009). The deletion locus of each mutant was confirmed to have the expected structure by PCR analysis and DNA sequencing. One of the mutants (*ssx_0391*) had a deletion in the *spxB* gene, whose product, pyruvate oxidase, is known to catalyse the production of H$_2$O$_2$ (Ramos-Montañez et al., 2008). The other three genes identified were *ackA*, encoding acetate kinase, *spxR*, encoding a conserved hypothetical protein, and *tpk*, encoding thiamine pyrophosphokinase (Table 1). The four opaque mutants (including *ssx_0391*) were further characterized.

**Opaque mutants have reduced rates of H$_2$O$_2$ production**

Since H$_2$O$_2$ production is proposed to relate to the opaque morphology, we next quantified H$_2$O$_2$ production in the four mutants identified above, and compared it with that of the wild-type strain SK36. The control strain *ssx_0169* was also included in this analysis. All four opaque mutants displayed significantly reduced rates of H$_2$O$_2$ production compared with the semi-transparent parent strain SK36 (Fig. 1). H$_2$O$_2$ production rates of the mutants were only 16–31% of that of SK36. Similar H$_2$O$_2$ production to the wild-type strain SK36 was found in the kanamycin-resistant control strain (*ssx_0169*). Though each of the four opaque mutants displayed decreased rates of H$_2$O$_2$ production, none of them lost the capacity of H$_2$O$_2$ production completely, including the *spxB* mutant. It should be noted that the intact ORF of each mutant was precisely deleted in each of our mutants, so it was impossible that any partial gene function remained. This suggested that the pyruvate oxidase activity might not be the only oxidase activity responsible for H$_2$O$_2$ production.

Next, to ensure that these identified genes function in H$_2$O$_2$ production, we checked the H$_2$O$_2$ production in the relative single gene mutants of their upstream and downstream genes (i.e. *ssx_0190, ssx_0191, ssx_0193* and *ssx_0195; ssx_1494, ssx_1493, ssx_1490* and *ssx_1489; ssx_2120, ssx_2119, ssx_2117* and *ssx_2116*). The mutant for *ssx_1491* is not available because the gene was found to be essential). There was no statistically significant defect in H$_2$O$_2$ production by any of these mutants compared with that of the wild-type. This result supports the hypothesis that the defects in H$_2$O$_2$ production in the identified mutants are not related to the neighbouring genes. To ensure this, we introduced the genes back to the mutants. A chloramphenicol resistance cassette was placed downstream of each gene for selection. After obtaining the complemented strains, their morphology and H$_2$O$_2$ production were examined. The results showed that the morphology of three strains, *ssx_0391C, ssx_1492C* and *ssx_2118C* (Table 1), was restored to semi-transparent and the rates of H$_2$O$_2$ production were restored to the wild-type level (Fig. 1). In the first attempt to complement *ssx_0192* gene function, we failed to fully restore the phenotype. We then examined the mRNA level of downstream genes *ssa_0193, ssa_0195* and *ssa_0197* in the mutant *ssx_0192*, which did not show significant changes compared with that of the wild-type. Given these data, we deduced that there might be some errors with the complemented strain. We therefore performed this complementation again employing an erythromycin resistance cassette (pSerm). The resulting strain *ssx_0192C* was successfully restored for H$_2$O$_2$ production (Fig. 1). All of the data indicated that the identified genes are involved in H$_2$O$_2$ production.

**Opaque mutants demonstrate reduced antagonistic activity against *S. mutans* UA159 both on plates and in liquid media**

Because the formation of H$_2$O$_2$ in *S. sanguinis* plays an important role in interspecies interactions within the oral microflora, we performed competition assays to examine whether the four H$_2$O$_2$-defective mutants showed any difference from the parent strain, SK36, in their capacity for antagonism against a primary dental cariogen, *S. mutans*.

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**Fig. 1.** H$_2$O$_2$ production in *S. sanguinis* strains. H$_2$O$_2$ production normalized to culture densities was determined relative to that produced by the wild-type strain SK36. Data indicate mean±SD from three biological repeats. Statistical significance is indicated (**P<0.01**). Black bars, SK36 or a mutant; grey bars, complemented strain.
We first examined the antagonistic activity on agar plates. *S. sanguinis* and *S. mutans* cells were spotted next to one another on agar plates. The inhibition zones of *S. sanguinis* and the mutants against *S. mutans* were determined (Fig. 2a). The results showed that all four mutants lost the ability to inhibit *S. mutans* UA159 on BHI plates under microaerobic conditions (Fig. 2a).

To further quantify the antagonism of the H2O2-defective mutants against *S. mutans*, we performed competition assays in liquid culture using mixed species. We first constructed a chloramphenicol-resistant control strain of *S. mutans* by integrating the chloramphenicol resistance gene magellan2 (Turner et al., 2009) into the *S. mutans* UA159 chromosome. The *smu.42* gene encoding a hypothetical protein (SMU.42) which did not affect its sensitivity to antagonism by *S. sanguinis* (data not shown) was selected as the target location for integration in the *S. mutans* genome. Because the *S. mutans* derivative can be distinguished from *S. sanguinis* on chloramphenicol selection agar plates, the inhibition effect of *S. sanguinis* on *S. mutans* could be determined by bacterial colony numbers on agar plates supplemented with chloramphenicol. We mixed the same amount of each *S. sanguinis* mutant, as assessed by OD660, with *S. mutans* and co-cultured the two species mixture. *S. mutans* cells were counted on BHI plates supplemented with chloramphenicol after 48 h. This indicated that all four mutants were less able to inhibit *S. mutans* in liquid culture compared with the wild-type strain, SK36, and the control strain *ssx_0169* (Fig. 2b).

**Transcriptional level of spxB in H2O2 production-defective mutants**

To examine whether *spxB* expression changes in the mutants with decreased H2O2 production, we determined the transcriptional level of *spxB* by real-time qPCR (Fig. 3). The results showed that the expression of *spxB* in *ssx_0192* and *ssx_1492* decreased significantly compared with SK36. The significant decrease in *spxB* transcription suggested that the effects of the deleted gene products in *ssx_0192* and *ssx_1492* on H2O2 production might occur via SpxB. In contrast, the *ssx_2118* mutant demonstrated increased expression of *spxB*, indicating that SSA_2118 affects H2O2 production by a mechanism other than affecting *spxB* expression (Fig. 3).

**DISCUSSION**

In this study, we describe three genes involved in the production of H2O2 and the preliminary study of their...
effects on \textit{spxB} expression in \textit{S. sanguinis}. We also show that all the genes involved in the production of \textit{H}_2\textit{O}_2
defined here were also critical for the antagonism of \textit{S. sanguinis} against \textit{S. mutans}.

The three non-\textit{spxB} mutants identified in this study demonstrated defects in \textit{H}_2\textit{O}_2 production similar to that in the \textit{spxB} mutant. \textit{SSA}_0192 is annotated as acetate kinase (Xi \textit{et al}., 2007), which converts acetetyl phosphate, the other product derived from the decarboxylation of pyruvate besides \textit{CO}_2 and \textit{H}_2\textit{O}_2, to acetate. Our results indicated that \textit{spxB} expression was reduced in the deletion mutant \textit{ssx}_0192. A possible mechanism of this regulation might be that the gene deletion in \textit{ssx}_0192 caused acetetyl phosphate accumulation, which caused feedback suppression of \textit{spxB} expression (Wang \textit{et al}., 1999). We tried to determine the acetetyl phosphate concentration using the hydroxamate assay (Gorrell \textit{et al}., 2005) in \textit{ssx}_0192 and the wild-type strain to examine this hypothesis. However, the acetetyl phosphate concentrations in both strains were too low to give a reliable result. The gene product in \textit{SSX}_1492 (\textit{SSA}_1492) showed high identity (76\% identity in amino acid sequence) to SpxR in \textit{S. pneumoniae}, which was identified as a regulator of \textit{spxB} (Ramos-Montañez \textit{et al}., 2008). The conserved domain analysis of \textit{SSA}_1492 showed that the protein contains a putative helix–turn–helix domain (Ramos-Montañez \textit{et al}., 2008) located at the amino terminus, followed by a DRTGG-CBS domain, which are hypothesized to bind to DNA and adenosyl compounds (such as AMP and ATP), respectively. Combined with the significantly decreased expression of \textit{spxB} observed in the \textit{SSX}_1492 mutant, our data suggest that \textit{SSA}_1492 might act as a positive regulator of \textit{spxB} (Kemp, 2004; Ramos-Montañez \textit{et al}., 2008; Rigali \textit{et al}., 2002; Scott \textit{et al}., 2004). It was hypothesized that SpxR in \textit{S. pneumoniae} regulates \textit{spxB} transcription in response to energy and metabolic state, and the SpxR regulon includes comparatively few genes (Ramos-Montañez \textit{et al}., 2008).

As some other species of streptococci, including \textit{S. mutans}, \textit{S. pyogenes} and \textit{Streptococcus agalactiae} (group B Streptococcus), lack \textit{spxB} but contain homologues of SpxR (Ramos-Montañez \textit{et al}., 2008), presumably the regulatory targets of the SpxR homologues are species-specific. Our study suggests that the role of SpxR in regulating \textit{spxB} is not confined to \textit{S. pneumoniae}, because it seems to have the same function in \textit{S. sanguinis}. The thiamine pyrophosphokinase encoded by \textit{ssa}_2118 catalyses the transfer of a pyrophosphate moiety from ATP to thiamine and produces thiamine pyrophosphate. Thiamine pyrophosphate has been reported to be an important cofactor for pyruvate oxidase activity (Carlsson & Kujala, 1984; Muller \textit{et al}., 1994; Tittmann \textit{et al}., 1998, 2005). The deletion of \textit{ssa}_2118, therefore, presumably decreases the rate of \textit{H}_2\textit{O}_2 formation by decreasing the activity of pyruvate oxidase (Fig. 1). Our results indicated that \textit{SSA}_2118 was required for \textit{H}_2\textit{O}_2 production. This is also consistent with the finding that a site-specific mutation of an amino acid in SpxB that is required for thiamine pyrophosphate binding reduces \textit{H}_2\textit{O}_2 production significantly in \textit{S. pneumoniae} (Ramos-Montañez \textit{et al}., 2008). At the same time, it is possible that \textit{SSA}_2118 involves \textit{H}_2\textit{O}_2 production by affecting not only SpxB but also other enzymes that require thiamine pyrophosphate. It was interesting that \textit{spxB} expression in mutant \textit{ssx}_2118 showed a significant increase. All three non-\textit{spxB} mutants exhibited decreased \textit{H}_2\textit{O}_2 production but the expression levels of \textit{spxB} were distinct. We hypothesize that \textit{SSA}_0192 and \textit{SSA}_1492 were required for the normal expression of \textit{spxB}, while \textit{SSA}_2118 was required for the thiamine pyrophosphate, which is the cofactor for SpxB. Both SpxB and \textit{SSA}_2118 were necessary for \textit{H}_2\textit{O}_2 production.

Our study suggests that it is practical to identify \textit{H}_2\textit{O}_2 production-defective mutants in \textit{S. sanguinis} by their colony morphology variation. In \textit{S. pneumoniae}, the proposed roles of \textit{spxB} function and regulation in pneumococcal phase variation have been somewhat contradictory (Ramos-Montañez \textit{et al}., 2008). Some research suggested that \textit{spxB} expression level was unlikely to directly determine colony morphology (Overweg \textit{et al}., 2000) since the \textit{spxB} mutant still varied in colony morphology, while another study indicated that some opaque variants were later found to be defective in SpxB function (Pericone \textit{et al}., 2002). It has been suggested recently that SpxB does determine colony morphology and might play a role in phase variation (Belanger \textit{et al}., 2004). The contradiction may be related to other components contributing to the phenotype, such as a capsule. In \textit{S. sanguinis}, our results indicated that it was not the expression of \textit{spxB} that is responsible for the morphological variation, but the \textit{H}_2\textit{O}_2 the strain produces, because all of the four \textit{H}_2\textit{O}_2 production-defective mutants had an opaque appearance, and this appearance was not dependent on the expression of \textit{spxB}. For example, the expression of \textit{spxB} in mutant \textit{ssx}_2118 was significantly increased compared with that in the wild-type strain SK36 (Fig. 3), but the mutant \textit{ssx}_2118 still presented an opaque morphology, which is presumably due to the \textit{H}_2\textit{O}_2 production deficiency of the mutant.

In \textit{S. pneumoniae}, a similar screening study was performed by Ramos-Montañez \textit{et al}., 2008. From screening ~232 000 colonies, seven spontaneous mutants were identified that showed opaque appearance; six of them were found to produce less \textit{H}_2\textit{O}_2 than the wild-type strain. All were related to \textit{spxB} and one of the genes, \textit{spxR}, was found to regulate \textit{spxB} expression. In our study, in addition to \textit{spxB} and \textit{spxR}, we identified two other mutants, \textit{ssx}_0192 and \textit{ssx}_2118, that had an opaque appearance and produced less \textit{H}_2\textit{O}_2. It is interesting that these genes were not identified in the \textit{S. pneumoniae} study, even though we would expect that identical mutants in \textit{S. pneumoniae} would have the same phenotype. This could be because their spontaneous screen carried out by Ramos-Montañez \textit{et al}., 2008 was not saturating. It would be interesting to determine whether mutations in the \textit{ssa}_0192 and \textit{ssa}_2118 orthologues in \textit{S. pneumoniae} (\textit{spd}_1853 and \textit{spd}_1779) would have also demonstrated this phenotype. If so, they will be potential virulence factors in \textit{S. pneumoniae}. 

\textit{L. Chen and others}
The competition between pioneer colonizing oral streptococci in the oral community is of continued interest. Our studies show that the four genes identified are critical for conferring a competition advantage to S. sanguinis. This might contribute to a better understanding of interspecies interactions within oral microbial communities and serve as a foundation on which the molecular mechanisms of \( \text{H}_2\text{O}_2 \) production and its regulation by oral streptococci could be elucidated.

ACKNOWLEDGEMENTS

This work was supported by a National Institutes of Health grant (R01DE018138) (P.X). We thank Todd Kitten (VCU School of Dentistry) for critical discussions and suggestions about experiments.

REFERENCES

Auzat, I., Chapuy-Regaud, S., Le Bras, G., Dos, S. D., Oggunniyi, A. D., Le, T. I., Garei, J. R., Paton, J. C. & Trombe, M. C. (1999). The NADH oxidase of Streptococcus pneumoniae: its involvement in competence and virulence. Mol Microbiol 34, 1018–1028.

Becker, M. R., Paster, B. J., Leys, E. J., Moeschberger, M. L., Kenyon, S. G., Galvin, J. L., Boches, S. K., Dewhirst, F. E. & Griffen, A. L. (2002). Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 40, 1001–1009.

Belanger, A. E., Clague, M. J., Glass, J. I. & LeBlanc, D. J. (2004). Pyruvate oxidase is a determinant of Avery's rough morphology. J Bacteriol 186, 8164–8171.

Carlsson, J. & Kujala, U. (1984). Pyruvate oxidase activity dependent on thiamine pyrophosphate, flavin adenine dinucleotide and orthophosphate in Streptococcus sanguis. FEMS Microbiol Lett 25, 53–56.

Caufield, P. W., Dasanayake, A. P., Li, Y., Pan, Y., Hsu, J. & Hardin, J. M. (2000). Natural history of Streptococcus sanguinis in the oral cavity of infants: evidence for a discrete window of infectivity. Infect Immun 68, 4018–4023.

Douglas, C. W., Heath, J., Hampton, K. K. & Preston, F. E. (1993). Identity of viridans streptococci isolated from cases of infective endocarditis. J Med Microbiol 39, 179–182.

Garcia-Mendoza, A., Liebana, J., Castillo, A. M., de la Higuera, A. & Piedrola, G. (1993). Evaluation of the capacity of oral streptococci to produce hydrogen peroxide. J Med Microbiol 39, 434–439.

Ge, X., Kitten, T., Chen, Z., Lee, S. P., Munro, C. L. & Xu, P. (2008a). Identification of Streptococcus sanguinis genes required for biofilm formation and examination of their role in endocarditis virulence. Infect Immun 76, 2551–2559.

Ge, Y., Caufield, P. W., Fisch, G. S. & Li, Y. (2008b). Streptococcus mutans and Streptococcus sanguinis colonization correlated with caries experience in children. Caries Res 42, 444–448.

Ginsburg, I. & Sadovnic, M. (1998). Gamma globulin, Evan’s blue, aprotinin A PL21 inhibitor, tetracycline and antibiotics protect epithelial cells against damage induced by synergism among streptococcal hemolysins, oxidants and proteinases: relation to the prevention of post-streptococcal sequelae and septic shock. FEMS Immunol Med Microbiol 22, 247–256.

Ginsburg, I. & Varani, J. (1992). Interaction of viable group A streptococci and hydrogen peroxide in killing of vascular endothelial cells. Free Radic Biol Med 14, 495–500.

Gorrell, A., Lawrence, S. H. & Ferry, J. G. (2005). Structural and kinetic analyses of arginine residues in the active site of the acetate kinase from Methanosarcina thermophila. J Biol Chem 280, 10731–10742.

Jakubovics, N. S., Smith, A. W. & Jenkinson, H. F. (2002). Oxidative stress tolerance is manganese (Mn2+) regulated in Streptococcus gordonii. Microbiology 148, 3255–3263.

Kemp, B. E. (2004). Bateman domains and adenosine derivatives form a binding contract. J Clin Invest 113, 182–184.

Kilian, M. & Holmgren, K. (1981). Ecology and nature of immunoglobulin A1 protease-producing streptococci in the human oral cavity and pharynx. Infect Immun 31, 868–873.

Kreth, J., Merritt, J., Shi, W. & Qi, F. (2005). Competition and coexistence between Streptococcus mutans and Streptococcus sanguinis in the dental biofilm. J Bacteriol 187, 7193–7203.

Kreth, J., Zhang, Y. & Herzberg, M. C. (2008). Streptococcal antagonism in oral biofilms: Streptococcus sanguinis and Streptococcus gordonii interference with Streptococcus mutans. J Bacteriol 190, 4632–4640.

Kreth, J., Vu, H., Zhang, Y. & Herzberg, M. C. (2009). Characterization of hydrogen peroxide-induced DNA release by Streptococcus sanguinis and Streptococcus gordonii. J Bacteriol 191, 6281–6291.

Kuramitsu, H. K., He, X., Lux, R., Anderson, M. H. & Shi, W. (2007). Interspecies interactions within oral microbial communities. Microbiol Mol Biol Rev 71, 653–670.

Muller, Y. A., Schumacher, G., Rudolph, R. & Schulz, G. E. (1994). The refined structures of a stabilized mutant and of wild-type pyruvate oxidase from Lactobacillus plantarum. J Mol Biol 237, 315–335.

Mylonakis, E. & Calderwood, S. B. (2001). Infective endocarditis in adults. N Engl J Med 345, 1318–1330.

Overweg, K., Pericone, C. D., Verhoef, G. G. C., Weiser, J. N., Meiring, H. D., De Jong, A. P. J. M., De Groot, R. & Hermans, P. W. M. (2000). Differential protein expression in phenotypically variant streptococci of Streptococcus pneumoniae. Infect Immun 68, 4604–4610.

Paik, S., Senty, L., Das, S., Noe, J. C., Munro, C. L. & Kitten, T. (2005). Identification of virulence determinants for endocarditis in Streptococcus sanguinis by signature-tagged mutagenesis. Infect Immun 73, 6064–6074.

Pericone, C. D., Overweg, K., Herrmans, P. W. M. & Weiser, J. N. (2000). Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract. Infect Immun 68, 3990–3997.

Pericone, C. D., Bae, D., Shchepetov, M., McCool, T. & Weiser, J. N. (2002). Short-sequence tandem and nontandem DNA repeats and endogenous hydrogen peroxide production contribute to genetic instability of Streptococcus pneumoniae. J Bacteriol 184, 4392–4399.

Ramos-Montañez, S., Tsui, H. C., Wayne, K. J., Morris, J. L., Peters, L. E., Zhang, F., Kazmierczak, K. M., Sham, L. T. & Winkler, M. E. (2008). Polymorphism and regulation of the spxB (pyruvate oxidase) virulence factor gene by a CBS-HotDog domain protein (SpxR) in serotype 2 Streptococcus pneumoniae. Mol Microbiol 67, 729–746.

Rigali, S., Derouaux, A., Giannotta, F. & Dusart, J. (2002). Subdivision of the helix–turn–helix GntR family of bacterial regulators in the FadR, HutC, MocR, and YtrA subfamilies. J Biol Chem 277, 12507–12515.

Rosan, B. & Lamont, R. J. (2000). Dental plaque formation. Microbes Infect 2, 1599–1607.

Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G. & Hardie, D. G. (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. J Clin Invest 113, 274–284.
Tittmann, K., Proske, D., Spinka, M., Ghisla, S., Rudolph, R., Hubner, G. & Kern, G. (1998). Activation of thiamin diphosphate and FAD in the phosphate-dependent pyruvate oxidase from Lactobacillus plantarum. J Biol Chem 273, 12929–12934.

Tittmann, K., Wille, G., Golbik, R., Weidner, A., Ghisla, S. & Hubner, G. (2005). Radical phosphate transfer mechanism for the thiamin diphosphate- and FAD-dependent pyruvate oxidase from Lactobacillus plantarum. Kinetic coupling of intercofactor electron transfer with phosphate transfer to acetyl-thiamin diphosphate via a transient FAD semiquinone/hydroxyethyl-ThDP radical pair. Biochemistry 44, 13291–13303.

Tleyjeh, I. M., Steckelberg, J. M., Murad, H. S., Anavekar, N. S., Ghomrawi, H. M., Mirzoyev, Z., Moustafa, S. E., Hoskin, T. L., Mandrekar, J. N. & other authors (2005). Temporal trends in infective endocarditis: a population-based study in Olmsted County, Minnesota. JAMA 293, 3022–3028.

Turner, L. S., Das, S., Kanamoto, T., Munro, C. L. & Kitten, T. (2009). Development of genetic tools for in vivo virulence analysis of Streptococcus sanguinis. Microbiology 155, 2573–2582.

Uehara, Y., Agematsu, K., Kikuchi, K., Matsuzaki, S., Imai, S., Takamoto, M., Sugane, K., Sugiura, T., Konishi, Y. & other authors (2006). Secretory IgA, salivary peroxidase, and catalase-mediated microbicidal activity during hydrogen peroxide catabolism in viridans streptococci: pathogen coaggregation. J Infect Dis 194, 98–107.

Wang, H., Tseng, C. P. & Gunsalus, R. P. (1999). The napF and narG nitrate reductase operons in Escherichia coli are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite. J Bacteriol 181, 5303–5308.

Weiser, J. N., Austrian, R., Sreenivasan, P. K. & Masure, H. R. (1994). Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect Immun 62, 2582–2589.

Xu, P., Alves, J. M., Kitten, T., Brown, A., Chen, Z., Ozaki, L. S., Manque, P., Ge, X., Serrano, M. G. & other authors (2007). Genome of the opportunistic pathogen Streptococcus sanguinis. J Bacteriol 189, 3166–3175.

Edited by: M. Kilian