ORIGINAL ARTICLE

Oxygen dependent pyruvate oxidase expression and production in *Streptococcus sanguinis*

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The objective of this study was to characterize the oxygen dependent regulation of pyruvate oxidase (SpxB) gene expression and protein production in *Streptococcus sanguinis* (*S. sanguinis*). SpxB is responsible for the generation of growth-inhibiting amounts of hydrogen peroxide (H_2O_2) able to antagonize cariogenic *Streptococcus mutans* (*S. mutans*). Furthermore, the ecological consequence of H_2O_2 production was investigated in its self-inhibiting ability towards the producing strain. Expression of *spxB* was determined with quantitative Real-Time RT-PCR and a fluorescent expression reporter strain. Protein abundance was investigated with FLAG epitope engineered in frame on the C-terminal end of SpxB. Self inhibition was tested with an antagonism plate assay. The expression and protein abundance decreased in cells grown under anaerobic conditions. *S. sanguinis* was resistant against its own produced H_2O_2, while cariogenic *S. mutans* was inhibited in its growth. The results suggest that *S. sanguinis* produces H_2O_2 as antimicrobial substance to inhibit susceptible niche competing species like *S. mutans* during initial biofilm formation, when oxygen availability allows for *spxB* expression and Spx production.

Keywords: *Streptococcus sanguinis*; pyruvate oxidase; oxygen dependent

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Introduction

Oral streptococci are important members of the oral biofilm. Their ability to initially colonize saliva-bathed tooth surfaces enables subsequent colonization by other oral bacteria [1-2]. Clinical studies suggest that successful colonization and high abundance of oral streptococci might be beneficial to oral health. For example, *Streptococcus sanguinis* (*S. sanguinis*) and the cariogenic *Streptococcus mutans* (*S. mutans*) are mutually antago-

nistic. Caries-free individuals have significantly higher numbers of *S. sanguinis* associated with almost non-detectable levels of *S. mutans*. This ratio changes in individuals with caries in an opposite manner [3]. Furthermore, initial colonization with abundant *S. sanguinis* delays acquisition of *S. mutans* in human subjects [4]. This information suggests an intrinsic capability of initial biofilm colonizers, comprised of up to 80% of oral streptococci [2], to promote biofilm homeostasis.

The expression of specific oral streptococcal surface proteins able to bind to salivary components is important for initial colonization and might explain the successful exclusion of pathogenic species [1, 5]. Once a specific niche is occupied, invasion by other species might be prevented. Another important factor in the interference

with potential pathogens is the production of anti-microbial components [6-7]. Oral streptococci, including *S. sanguinis*, are known to produce growth inhibiting amounts of hydrogen peroxide (H_2O_2) [8]. Several *in vitro* studies demonstrate that H_2O_2 is the only agent produced by *S. sanguinis* able to inhibit the growth of *S. mutans* [6-7]. Although *S. sanguinis* also produces anti-microbial peptides [9-10], these peptides do not appear to play a role in interspecies competition with *S. mutans*.

H_2O_2 is produced by pyruvate oxidase (SpxB) in a reaction converting pyruvate to acetyl phosphate. The reaction requires oxygen, with H_2O_2 and CO_2 generated during the catalytic process [11-12]. H_2O_2 most likely diffuses out of the cell and exerts its antimicrobial effect on neighboring H_2O_2 susceptible species. Two recent studies have shown that *spxB* gene expression is controlled by at least two transcriptional regulators. Chen *et al.* identified several genes affecting the production of H_2O_2, including an *spxR* homolog [13]. SpxR was previously identified as a transcriptional activator of *spxB* expression in *Streptococcus pneumonia* (*S. pneumoniae*). SpxR potential binding abilities to adenosyl compounds and CoA containing compounds suggested an expression control of *spxB* in response to the energy and metabolic state of *S. pneumoniae* [14]. In addition, the *spxB* gene in *S. sanguinis* is under the transcriptional control of catabolite control protein A, CcpA [15]. CcpA is the main transcriptional regulator of carbon catabolite repression in Gram-positives [16].

An important environmental factor influencing the interference capability of *S. sanguinis* against *S. mutans* is oxygen. Under anaerobic conditions, *S. sanguinis* is no longer able to inhibit the growth of *S. mutans* in an *in vitro* antagonism assay [6]. In the present study, the effect of oxygen on *spxB* expression and SpxB abundance was further characterized. Moreover, the effect of H_2O_2 produced by *S. sanguinis* was investigated with respect to its self-inhibiting potential.

Materials and Methods

Bacterial strains and media

Bacterial strains used in this study are listed in Table 1. Strains were routinely grown aerobically as static cultures (5% CO_2) at 37 ℃ in brain heart infusion (BHI; Difco, Sparks, MD) or on BHI agar plates. Cells were grown anaerobically in an anaerobic chamber (90% N_2, 5% CO_2, 5% H_2). Shaking cultures were grown in ambient air. *Escherichia coli* (*E. coli*) DH5α was grown at 37 ℃ in Luria-Bertani medium (LB, Difco) with agitation at 250 $r \cdot min^{-1}$. When required for selection, cultures were supplemented with the following antibiotics: spectinomycin at 500 $\mu g \cdot mL^{-1}$ for *S. sanguinis*; spectinomycin at 150 $\mu g \cdot mL^{-1}$ and ampicillin 100 $\mu g \cdot mL^{-1}$ for *E. coli*.

Table1 Strains and primers used in this study

Strain	Relevant Characteristics	Reference
SK36	*S. sanguinis* wild type	[17]
DL1	*S. gordonii* wild type	[18]
UA140	*S. mutans* wild type	[19]
SK36 *spxB-luc*	SK36::pFW5Φ (*spxB*$_p$-*luc*)	[15]
SK36 *spxB-gfp*	SK36::pFW5Φ (*spxB*$_p$-*gfp*)	This study
SK36 *spxB*-FLAG	SK36::pFW5 *spxB*-FLAG epitope	[15]
DH5α	*E. coli* cloning strain	[20]

Primer	Sequence (5' →3')
spxB –flag tag F	AGCGCTCGAGCGTGATTACATGAACAAACTCG
spxB –flag tag R	CGCTAGATCTTTACTTGTCATCATCGTCTTTGTAATCTTTAATTGCGCGTGATTGCA
Ss spxB F	AGCCGTCGACCGCAGATCCAATTGCTGT
Ss spxB R	AGCGGGATCCTGCTGCAGATGCAGTAAT
16S rRNA F	AAGCAACGCGAAGAACCTTA
16S rRNA R	GTCTCGCTAGAGTGCCCAAC
spxB-RT-F	AATTCGGCGGCTCAATCG
spxB-RT-R	AAGGATAGCAAGGAATGGAGTG

DNA manipulations

Standard recombinant DNA manipulations were used [21]. Restriction enzymes and DNA ligase were obtained from New England Biolabs (Beverly, MA) or Promega (Madison, WI) and used as specified by the manufacturer. PCR products were cloned into the pGEM®-T vector from Promega. All plasmids were extracted and purified from *E. coli* with a QIAGEN Miniprep kit (Valencia, CA). DNA extracted from agarose gels (1%) was purified with a QIAGEN QIAquick gel extraction kit. PCR was performed with a G-Storm GS1 thermocycler (GeneTechnologies; Essex, UK) according to the manufacturer's protocol. GoTaq-DNA polymerase was obtained from Promega, and Phusion high-fidelity DNA polymerase was obtained from New England Biolabs. Primer sequences (Table 1) were designed using sequence data obtained from the Los Alamos National Laboratory Oral Pathogens Sequence Database (http://www.oralgen.lanl.gov) and synthesized by Integrated DNA Technologies (Coralville, IA).

Construction of a pyruvate oxidase carrying a C-terminal FLAG epitope

The FLAG epitope construction was described earlier [15]. Briefly, PCR amplification of a region about 700 bp from the 3′ end of the pyruvate oxidase gene (*spxB*) was accomplished with specific primers incorporating a 6 FLAG epitope sequence (GATTACAAAGACGATGA TGACAAG) before the stop codon using primer pairs spxB-flag tag F/ spxB-flag tag R. The PCR product was inserted into the pGEM®-T Easy vector (Promega) to create pGEM®-T-spxB-flag. The construct was sequenced to confirm the in-frame presence of the FLAG epitope sequence. The cloned fragment was released with *Xho* Ⅰ / *Bgl* Ⅱ, and inserted into pFW5 [22] digested with the same enzymes. The recombinant plasmid was transformed into SK36 wild type. Transformants were selected on BHI plates containing appropriate antibiotics and were further analyzed by PCR for integration of the plasmid at the correct locus. Overnight cultures of strains carrying the Spx-FLAG construct were grown in the presence of spectinomycin.

Construction of a spxB-gfp reporter strain

A 0.7-kb fragment containing the *spxB* promoter region was amplified by PCR from chromosomal DNA of *S. sanguinis* using primers Ss *spxB* F, including a *Sal* Ⅰ linker, and Ss *spxB* R, including a *Bam*H Ⅰ linker. The PCR product was cloned in front of the *gfp* gene of plasmid pFW5-*gfp* [23] to generate plasmid pFW5-*spxB*-GFP. The correct construct was confirmed by DNA sequencing and subsequently transformed into SK36.

Western immunoblots

Western immunoblotting was essentially performed as described earlier [15]. Briefly, after cell disruption, cytoplasmatic extracts were obtained by centrifugation and the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with a solution of 5% skim milk dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h. Subsequently, the membrane was incubated in primary antibody solution (anti-FLAG M2 antibody; Stratagene) overnight at 4 ℃, followed by three wash steps with TBST, and then incubated with horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific) for 1 h at room temperature. After washing with TBST, the blots were developed using the enhanced chemiluminescence (ECL) detection system (Thermo Scientific).

RNA isolation, cDNA synthesis, and real-time PCR

RNA was isolated using QIAGEN RNeasy kit, cDNA was synthesized using Stratascript reverse transcriptase (Stratagene) according to the manufacturer's protocol. Quantitative real-time-PCR (qRT-PCR) was performed to detect specific transcripts with the comparative threshold cycle (CT) method using the Bio-Rad MyiQ Cycler (Hercules, CA). Relative changes in gene expression were calculated using the ΔCT method described previously. The 16S rRNA gene was used as the housekeeping reference gene. All primers used for qRT-PCR are listed in Table 1.

Competition assay on solid medium

To compare competitive growth between different oral streptococci, a protocol described previously was used with modifications [6]. Briefly, 8 μL of an overnight culture of the respective species in BHI medium adjusted to an absorption at 600 nm of 0.8 was inoculated onto a BHI agar plate as the pioneer colonizer. After incubation overnight (16 h) at 37 ℃ in 5% CO_2, 8 μL of the competing species adjusted to an absorption at 600 nm of 0.8 was inoculated next to the pioneer colonizer such that the colonies almost touched each other. The plate was incubated overnight and subsequently photographed.

Fluorescence microscopy

Cells were grown in BHI on a rocking platform for maximal aeration as described earlier [6] or as static anaerobic cultures at 37 ℃ until late logarithmic phase ($A_{600\,nm}$ ~ 0.6 to 0.7). An aliquot was removed from both cultures, centrifuged at 13 200 r·min^{-1} in a table-top centrifuge and the liquid was replaced by fresh medium. Cells were resuspended and aerated by vigorous pipetting.

This was performed to ensure oxygen availability to the anaerobic culture, since Gfp maturation requires oxygen presence. The cells were incubated at room temperature for 15 min, which was shown before to be sufficient for Gfp maturation [24]. Fluorescent pictures were taken with an Olympus BX51 fluorescent microscope using appropriate fluorescent filter settings for Gfp detection and an UPlan FL N 40× or 100× oil immersion lens. Pictures were taken with Olympus DP72 digital camera and CellSens 1.3 software. Exposure times were kept constant for all images. Pictures were adjusted for brightness and contrast with photo processing software GIMP 2.6.10. (http://www.gimp.org/) applied to all parts of the image.

Results

S. sanguinis is resistant to its own H_2O_2 during competitive growth

The production of H_2O_2 by *S. sanguinis* is considered to be an antagonistic measure towards competing species during oral biofilm development. Several studies employing an *in vitro* antagonism assay have shown that the initial colonizer *S. sanguinis* is able to inhibit the later colonizer *S. mutans* [7, 13]. *S. mutans* is also able to inhibit other species by the production of bacteriocins, named mutacins [7]. *S. mutans* is not inhibited by its own mutacins [25], most likely due to the expression of its own resistance genes. To further confirm H_2O_2 as an antimicrobial compound produced against competing species, we tested the self-inhibiting ability against *S. sanguinis* in an antagonism plate assay. When spotted first as an initial colonizer, *S. sanguinis* was able to inhibit the growth of proximally spotted *S. mutans*. However, the growth of *S. sanguinis* and H_2O_2 producing *Streptococcus gordonii* (*S. gordonii*) was not affected. When *S. gordonii* was used as an initial colonizer, a similar result was observed, with a weak growth inhibition of *S. sanguinis* at the border of *S. gordonii* (Figure 1). The ability of *S. gordonii* to produce relatively more H_2O_2 compared to *S. sanguinis* might explain this observation [6]. The respective SpxB⁻ mutants of *S. sanguinis* and *S. gordonii* did not inhibit the growth of any tested species, supporting the observation that the inhibiting substance is H_2O_2 (data not shown). In conclusion, *S. sanguinis* is able to tolerate the H_2O_2 produced both by itself and the commensal *S. gordonii,* and this observation supports the idea that H_2O_2 is produced as a competitive measure against susceptible bacteria in order to maintain biofilm homeostasis.
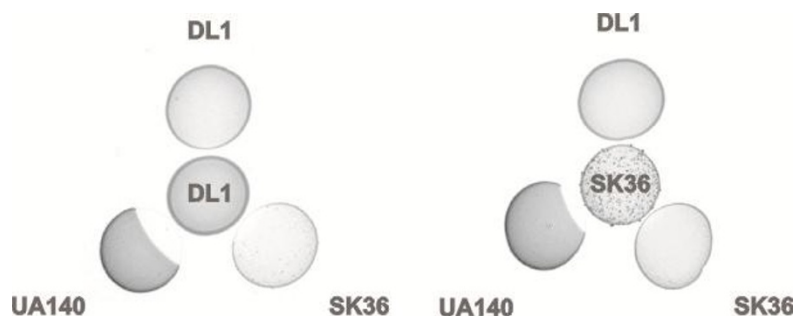

Figure 1 Antagonism assay. The strain spotted in the middle was incubated for 16h before subsequent inoculation of the surrounding strains. After additional overnight incubation, growth was documented photographically.

Oxygen influences spxB transcription

The enzymatic conversion of pyruvate to acetyl phosphate requires oxygen [12]. As a consequence, anaerobic growth conditions diminish the ability of *S. sanguinis* to produce antagonistic amounts of H_2O_2 [6]. To test if oxygen availability influences *spxB* expression, the expression profile of *spxB* was first determined using a *spxB*-luciferase reporter. As shown in Figure 2A, the relative luciferase activity of the reporter strain was similar over the measured time period, indicating that *spxB* is constitutively expressed in *S. sanguinis* under the tested growth conditions. Oxygen dependence of *spxB* expression was therefore measured with cells grown to mid-logarithmic phase as anaerobic static and aerobic shaken cultures. Using qRT-PCR, expression of *spxB* was increased about 25-fold under aerobic conditions when compared to anaerobic grown cells (Figure 2B), suggesting that *spxB* expression is regulated depending on oxygen availability.

SpxB abundance is decreased but not absent under anaerobic conditions

SpxB abundance was determined to test for protein production correlation with differential *spxB* expression

under aerobic *vs.* anaerobic conditions. An isogenic *S. sanguinis* strain was constructed carrying a SpxB C-terminal fusion of the FLAG epitope detectable with FLAG specific antibody. The FLAG epitope did not interfere with the H_2O_2 production. Cells were grown on BHI agar plates with and without oxygen and RNA isolated as described earlier [15]. The SpxB abundance during aerobic growth was increased noticeably when compared to anaerobic grown cells (Figure 3). Interestingly, a faint band was detected for anaerobically grown cells, although no H_2O_2 was produced under these conditions [6]. A similar result was obtained with planktonic grown cells (data not shown). These results indicate that *S. sanguinis* couples *spxB* expression and protein production.
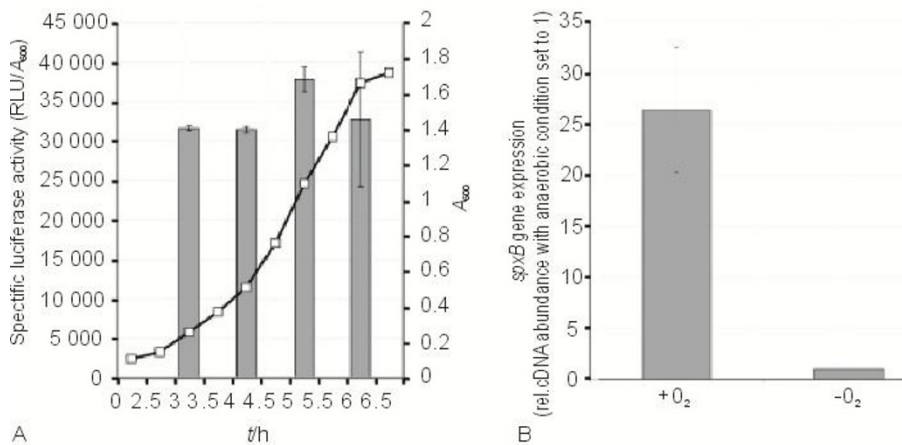

Figure 2 Growth phase and oxygen dependent regulation of *spxB* expression. (**A**) Specific luciferase activity monitored during growth of *S. sanguinis*. (**B**) qRT-PCR with cDNA prepared from aerobically and anaerobically grown cells.
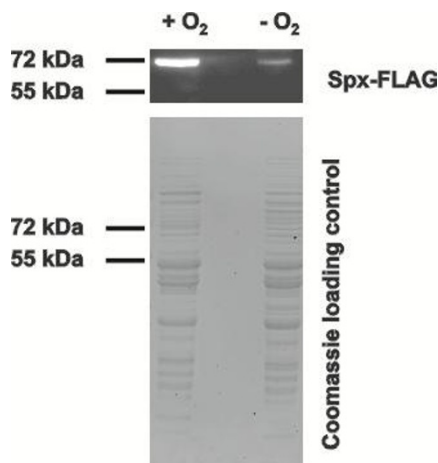

Figure 3 Effect of oxygen on SpxB abundance. Western blot analysis of SpxB protein abundance. Production of the pyruvate oxidase was detected using an antibody against the Spx-FLAG epitope. A replicate SDS gel was prepared and stained with Coomassie blue as a control. The PageRuler™ prestained protein ladder (Fermentas) was used as a size reference. Black bars depict the relative protein sizes.

Detection of spxB expression on the cellular level

Common techniques of specific gene expression analysis like qRT-PCR rely on averaging gene expression of the whole population. To determine *spxB* expression on the cellular level, a *spxB-gfp* fluorescent reporter was constructed. Planktonic cultures were grown with and without oxygen until mid-logarithmic growth phase. After aeration of the anaerobically grown culture to promote Gfp maturation, cells were analyzed with fluorescent microscopy. A weak fluorescence was detected for cells grown without oxygen, while cells grown under shaking conditions to promote aeration had a bright fluorescence (Figure 4A). The fluorescent reporter data confirm the increased expression of *spxB* under aerobic conditions.

After careful inspection of individual chains of aerobically grown *S. sanguinis*, differences in fluorescence intensity were observed in neighboring cells. As shown in Figure 4B, some neighboring cells showed bright fluorescence compared to others with a decreased fluorescence. Occasionally, single cells were observed with no fluorescence (Figure 4B, white arrow). These results suggest that at the individual cell level, differences in *spxB* expression exist among the investigated population.
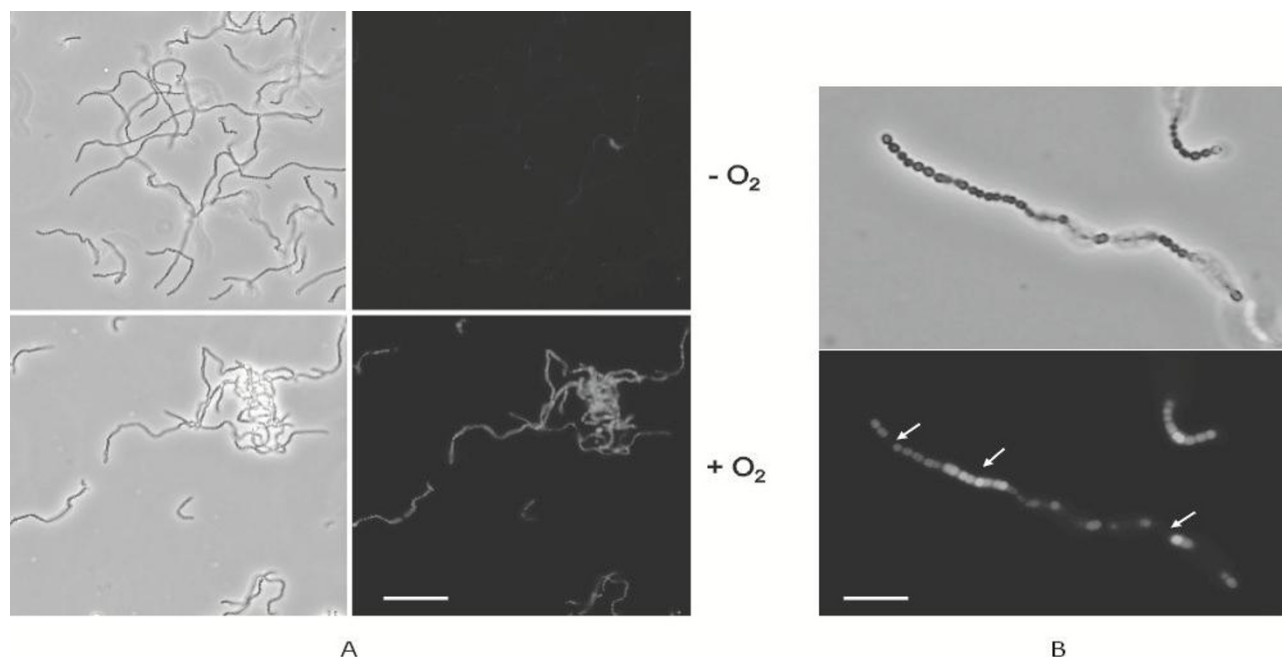
Figure 4 Fluorescent microscopy of spx-gfp reporter strain grown with and without oxygen. (**A**) Bright field and fluorescent image of cells grown with and without oxygen. Magnification is 400 fold. White bar = 25 μm. (**B**) Bright field and fluorescent image of cells grown with oxygen. Magnification is 1 000 fold. White bar = 10 μm.

Discussion

In this report, the expression and protein production of the H_2O_2 generating enzyme pyruvate oxidase was further characterized in *S. sanguinis* and the consequence of H_2O_2 production on neighboring cells of its own kind was investigated. We now show that *S. sanguinis* regulates the expression of the pyruvate oxidase gene *spxB* according to the availability of oxygen. Under anaerobic conditions, *spxB* expression is decreased and this is also reflected in a lower abundance of SpxB. Oxygen is an important environmental factor able to influence bacterial processes in the oral biofilm [6, 26]. It was shown previously that aerobic conditions enable *S. sanguinis* and *S. gordonii* to antagonize *S. mutans* [6]. Interestingly, the ability of *S. sanguinis* and *S. gordonii* to produce H_2O_2 under aerobic conditions correlates with a decreased fitness of *S. mutans* when grown in the presence of oxygen. Ahn *et al.* demonstrated that several phenotypic traits related to virulence properties of *S. mutans*, like biofilm formation and acid tolerance, are affected during aerobic growth. Furthermore, the ability to grow in the presence of H_2O_2 was also decreased. Although aerobically grown *S. mutans* cells were able to cope better with superoxide ions produced during treatment with paraquat, no adaptation was measured for H_2O_2 [22].

Therefore aerobically produced H_2O_2 by commensals would be the ideal antimicrobial component against susceptible species like *S. mutans* especially considering that H_2O_2 does not affect the producing cell as demonstrated in the antagonism assay (Figure 1). However, considering that oxygen tension could decrease during biofilm formation due to diffusion limitation and rapid consumption [27], H_2O_2 as an antimicrobial component would seem to be counterproductive when cells grow under anaerobic conditions. *S. mutans,* for example, is able to produce growth inhibiting mutacins regardless of oxygen availability [6-7]. Nonetheless, H_2O_2 production under aerobic conditions might be sufficient to function in interspecies competition. For example, an environment of high oxygen availability is most likely to occur in conditions of low biofilm abundance during initial biofilm development. As the commensals, including *S. sanguinis*, start to produce H_2O_2 they would naturally favor the growth of the H_2O_2 tolerant species at the expense of sensitive species, such as *S. mutans*. Another consequence of H_2O_2 production is the release of extracellular DNA (eDNA) [28], which is known to support initial biofilm formation. eDNA is an important structural component of biofilms [29] and H_2O_2 seems to be the only agent required to induce eDNA release in *S. sanguinis* and *S. gordonii* [24]. Likewise, it was shown for several species

that eDNA is required for biofilm formation [30-32] by promoting cell-cell aggregation in *S. sanguinis* [28]. The H_2O_2 dependent production of eDNA might therefore promote the initial adhesion to the tooth surface and subsequent biofilm formation. Since *S. sanguinis* is an initial biofilm colonizer, its ecological fitness might be adapted to this initial stage of biofilm formation. Once the initial biofilm is established, initial colonizers might not need to produce antimicrobial components to maintain biofilm homeostasis, since the ecological niche is already occupied by up to 80% of oral streptococci [2].

The expression of *spxB* is not evenly distributed in a culture grown under optimal H_2O_2 producing conditions, although all cells should have equal access to oxygen during agitation. Non-*spxB* expressing cells were also detected beside cells expressing *spxB* in a relatively high abundance as visualized by Gfp fluorescence. Several recent studies have shown that bacterial populations exhibit heterogeneity in the expression of various characteristics [33-34]. For example, competence development in *Bacillus subtilis* occurs only in about 10% of the population [35]. This phenomenon is also referred to as bi-stability, caused by a dual pattern of gene expression in an otherwise clonal population. Another example is the occurrence of persister cells in bacterial populations. Persister cells are dormant cells in a metabolically active population and therefore highly tolerant to antibiotic treatment [33]. The sparse occurrence of *spxB* non-expressing cells resembles metabolically inactive cells detected for *S. sanguinis* when grown under similar conditions [28]. Additional studies are required to confirm the existence of persister cells in *S. sanguinis* populations.

In conclusion, *S. sanguinis* produces H_2O_2 to antagonize cariogenic *S. mutans*, but is resistant to its own and closely related *S. gordonii* H_2O_2 *in vitro*. *spxB* expression and production of SpxB is dependent on oxygen availability and decreases during anaerobic growth. Ongoing studies aim to identify the transcriptional regulator responsible for the aerobic-anaerobic regulation in *S. sanguinis* to further broaden our understanding of how oral streptococci adapt to growth in the oral biofilm.

Acknowledgement

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