# HHS Public Access

Author manuscript

*Mol Oral Microbiol*. Author manuscript; available in PMC 2019 October 01.

Published in final edited form as:

*Mol Oral Microbiol*. 2018 October ; 33(5): 337–352. doi:10.1111/omi.12231.

# Live and let die: hydrogen peroxide production by the commensal flora and its role in maintaining a symbiotic microbiome

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## Summary

The majority of commensal oral streptococci are able to generate hydrogen peroxide ($H_2O_2$) during aerobic growth, which can diffuse through the cell membrane and inhibit competing species in close proximity. Competing $H_2O_2$ production is mainly dependent upon the pyruvate oxidase, SpxB and to a lesser extent the lactate oxidase LctO, both of which are important for energy generation in aerobic environments. Several studies point to a broad impact of $H_2O_2$ production in the oral environment, including a potential role in biofilm homeostasis, signaling, and interspecies interactions. Here, we summarize the current research regarding oral streptococcal $H_2O_2$ generation, resistance mechanisms, and the ecological impact of $H_2O_2$ production. We also discuss the potential therapeutic utility of $H_2O_2$ for the prevention/treatment of dysbiotic diseases as well as its potential role as a biomarker of oral health.

## Introduction

The human host is closely associated with complex microbial communities consisting primarily of bacteria, but also of viruses, fungi, archaea, and certain protozoa[1]. The colonization of oral mucosal and hard tissue surfaces by microorganisms is accompanied by the vigorous intra- and interspecies exchange of information among diverse microbial communities as well as cross-kingdom communication between the microbes and host [2,3]. The ideal outcome of microbial colonization is to create a host-protective environment characterized by a consortium of commensal and mutualistic flora species. The protection

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CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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afforded by the resident commensal flora can be viewed both temporally as well as spatially: temporally, since the initial colonization after birth can contribute to a lifetime of proper host immune system function [4] and spatially, since community interactions amongst the flora as well as flora-host interactions both yield mostly local effects [5]. To receive these protective benefits from the flora, it is critical to maintain microbial symbiosis [6]. There are various recognized mechanisms that promote a symbiotic relationship with the flora, including the production of hydrogen peroxide ($H_2O_2$) by the commensal oral streptococci.

The oral cavity has several surfaces covered with microbes dwelling in biofilms and the composition of the biofilm community is determined by the respective environment [7–9]. For example, the process of dental biofilm formation has been the center of several *in vivo* oral microbial ecology studies [10–13]. Distinctive patterns of colonization sequences have been described, which can develop into a mature biofilm community with enormous complexity and structure. Initial colonization of the saliva bathed tooth surface in the first 4 to 8 hours is dominated by oral streptococci and *Corynebacterium* ssp. as recently shown [12,14]. This dominance extended up to 16 hours into biofilm development and *Streptococcus* accounted for about 20% of the taxa [14]. Incidentally, oral streptococci are also the main source for ecologically relevant $H_2O_2$ production [15]. The growth of initial colonizers during biofilm development in the first 16 hours seemed to be exponential, suggesting ideal conditions for facultative anaerobic and aerotolerant species. Further biofilm development favors the integration of Gram-negative anaerobic species including *Fusobacterium*, *Prevotella* and *Porphyromonas*, but growth of the biofilm population seemed to slow down after 16 hours [14].

Initial biofilm development requires the formation of saliva-derived macromolecular complexes on the tooth surface leading to the formation of the acquired enamel pellicle. This process takes place within seconds after a clean enamel surface is exposed to saliva [16]. Mechanistically, the abundance of streptococci as initial colonizers can be explained by surface adhesins specifically recognizing salivary proteins that are part of the acquired enamel pellicle. Integration of other species, including later colonizers, occurs via specific cell-surface receptors also displayed by streptococci [17], thus streptococci together with a few other species are able to determine the spatial and temporal development of oral biofilms and are important for biofilm homeostasis. Several synergistic and antagonistic mechanisms have been described that contribute to oral biofilm dynamics [18], and $H_2O_2$ production seems to play a significant role [15].

In the time since our previous review about the role of $H_2O_2$ in oral biofilm ecology [15], there have been important advances in our understanding of oral microbial ecology and species composition in health and disease. One of the key outcomes of those studies is that polymicrobial diseases such as caries and periodontal disease are the ecological consequences of dysbiosis. For example, caries is triggered by overgrowth of a subset of the oral flora that are inherently aciduric and acidogenic [19]. We have previously suggested that the conserved ability of commensal oral streptococci to produce $H_2O_2$ is important for oral health [20]. Here we review the latest evidence detailing our current knowledge of the biology of streptococcal $H_2O_2$ production as well as the role of $H_2O_2$ as key component of oral ecology.

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## Relevant Chemistry of hydrogen peroxide and reactive oxygen species (ROS) production

### i) Oxygen redox pathways generate $H_2O_2$ as an intermediate

The generation of $H_2O_2$ during cellular redox reactions occurs due to the sequential univalent reduction of molecular oxygen [21]. $H_2O_2$ is the second intermediate formed by the addition of one electron and two protons to the highly unstable superoxide anion. $H_2O_2$ is relatively stable and unreactive in an abiotic environment [22,23]. Pure solutions of macromolecules like nucleic acids, proteins (metal-free), lipids, and polysaccharides are typically resistant to $H_2O_2$ oxidation. However, this is not the case in a biological environment, which is replete with transition metals such as Fe(ii) or $Cu^{2+}$ that act as reducing agents for $H_2O_2$ [22,24]. The further reduction of $H_2O_2$ results in the addition of an electron and a proton to generate $H_2O$ and the extremely reactive and short-lived hydroxyl radical, which is the major reactive oxygen species (ROS) reacting with biomolecules [21]. The half-life of the hydroxyl radical in a biological system is only about 1 ns. Its reaction with biomolecules is non-selective and is only limited by diffusion [25].

### ii) $H_2O_2$ involvement in the production of hydroxyl radicals via Fenton chemistry

In a cellular context, the generation of hydroxyl radicals is primarily catalyzed by Fenton chemistry via the transition metal ion pool inside the cell, especially soluble Fe(II) [26]. Fe(II) donates one electron to one $H_2O_2$ molecule to generate a hydroxyl radical, a hydroxide ion, and oxidized Fe(III). The hydroxyl radical will subsequently react rapidly with any organic cellular compound in the immediate vicinity [27]

### iii) Cytotoxicity of $H_2O_2$

Much of the cytotoxicity of $H_2O_2$ is the result of oxidative DNA damage induced by hydroxyl radicals generated through Fenton chemistry [27,28]. Hydroxyl radicals can trigger direct strand breakage through the oxidation of the ribose moieties of the DNA backbone [27,29,30]. In addition, the nucleobases can be oxidized, with guanine being one of the major targets of oxidation [31]. Interestingly, two kinetically distinguishable modes of killing by $H_2O_2$ seem to exist as shown with the model organism *Escherichia coli* [32,33]. Cells treated with $H_2O_2$ concentrations lower than 3 mM were more susceptible compared to $H_2O_2$ concentrations between 5 to 20 mM. Above 20 mM $H_2O_2$, the survival rate is inversely proportional to the $H_2O_2$ concentration, as would be expected. The dual range of $H_2O_2$ susceptibility is attributed to separate killing mechanisms. Lower concentrations of $H_2O_2$ will mostly damage DNA due to Fenton reaction hydroxyl radicals inducing a lethal mutation rate [27,34]. The ability of Fe to bind to DNA is a key feature of this mechanism [35]. The second mode of killing at higher $H_2O_2$ concentrations seems to be the result of damage to other target(s) [33,34], including proteins and lipids. However, the biologically relevant $H_2O_2$ concentration bacteria normally encounter is usually in the nM to lower mM range [36–39]. Hydrogen peroxide concentrations up to 7 mM have been reported for streptococci [37], although those measurements were mostly performed in batch cultures that may not be reflective of the normal $H_2O_2$ production capacity of streptococcal biofilms. Regardless, it seems unlikely that *E. coli* would encounter >20 mM $H_2O_2$ during growth in bacterial

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communities. A similar dual susceptibility to $H_2O_2$ has also been reported for *Streptococcus thermophilus* [40], which is intriguing since both organisms utilize distinct mechanisms to detoxify $H_2O_2$. *E. coli* directly degrades $H_2O_2$ via the catalase enzyme [41], whereas streptococci typically lack catalase and rely on other mechanisms [42,43]. It is worth noting that *S. mutans* is equally susceptible to killing when treated with 1 to 5 mM $H_2O_2$ [44]. Thus, the dual susceptibility to $H_2O_2$ killing might not be generalizable for all species and/or growth conditions.

$H_2O_2$ mediated damage can also occur to enzymes with iron-sulfur clusters abolishing enzyme activity, as well as enzymes that require a single $Fe^{2+}$ ion as cofactor for enzymatic activity [31]. Other potential targets are lipids in the cell membrane of bacterial cells. However, this has not been studied with oral biofilm bacteria and the lack of the main target for lipid peroxidation, polyunsaturated fatty acid chains in most bacterial membranes, makes it unlikely that this mode of killing action occurs [27,38].

## Sources of microbial hydrogen peroxide production in the oral biofilm

In the oral biofilm, naturally produced $H_2O_2$ crosses the bacterial membrane of producer cells and is released into the environment where it can influence neighboring cells and ultimately oral biofilm ecology. Our group and others have demonstrated that abundant $H_2O_2$ production in the pioneer colonizer *S. sanguinis* is critically dependent upon the activity of the pyruvate oxidase, SpxB. SpxB knock-out mutants do not produce sufficient extracellular $H_2O_2$ to inhibit competing $H_2O_2$-susceptible species like *Streptococcus mutans* [45]. SpxB-dependent $H_2O_2$ production has also been shown experimentally for *S. gordonii* [45] and *Streptococcus parasanguinis* [46] as well as *Streptococcus infantis*, *Streptococcus oralis*, *Streptococcus cristatus* and *Streptococcus mitis* as shown on Prussian Blue $H_2O_2$ indicator plates [47,48] (Fig. 1, unpublished).

### i) Pyruvate oxidase, SpxB

The initial characterization of the pyruvate oxidase from *S. sanguinis* was reported by Jan Carlsson and colleagues. Permeabilized cells exhibited pyruvate oxidase activity dependent upon thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), $Mg^{2+}$, and orthophosphate [49]. Catalytic activity was also significantly increased in cells grown aerobically [49], while the reaction itself is oxygen-dependent [50]. SpxB catalyzes the oxidative decarboxylation of pyruvate to the high energy metabolite acetyl-phosphate in addition to $CO_2$, $H_2O_2$, and ATP. Thus, the reaction provides an energetic growth advantage for the producer [15]. Our group confirmed the presence of SpxB under anaerobic conditions, suggesting that the enzyme is present in cells even when $H_2O_2$ production is not detectable [51]. Consistent with the results from Carlsson, we observed *S. sanguinis spxB* expression to increase about 25-fold in aerobically grown cultures with the highest *spxB* expression occurring in late log phase [51]. Even so, *S. sanguinis* still produces small amounts of SpxB under anaerobic conditions when pyruvate is primarily metabolized via the pyruvate formate lyase [49]. The reason for this is not entirely clear, but it could potentially provide a growth advantage once oxygen becomes available. Alternatively, SpxB might have additional moonlighting functions that have yet to be discovered.

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The biochemical mechanism of SpxB catalysis has not been elucidated in oral streptococci, but this has been investigated using the *Lactobacillus plantarum* pyruvate oxidase (*Lp*Pox) ortholog. Several characteristics between *Lp*Pox and *S. sanguinis* SpxB are conserved and they share 49% identity (Table 1) [52]. Molecular modeling using the automated protein structure homology-modelling server SWISS-MODEL (https://swissmodel.expasy.org) [53] predicted a high conservation of the *S. sanguinis* SpxB protein structure compared to the known structures of the pyruvate oxidases from *L. plantarum* [54] and *Aerococcus viridans* [55] (Fig. 2).

From the *Lp*Pox crystal structure, several aspects of the catalytic process have been revealed, including the roles of the enzyme-bound co-factors TPP and FAD [56,57]. The reaction occurs in several steps beginning with a nucleophilic attack on the bound pyruvate molecule by TPP leading to acetyl-TPP and $FADH_2$. In a subsequent oxygen-dependent mechanism, $FADH_2$ is oxidized to FAD generating $H_2O_2$, the acetyl-TPP reacts with inorganic phosphate to create acetyl-phosphate, and TPP is regenerated [57,58] (Fig. 3). A similar catalytic mechanism would be expected for *S. sanguinis* SpxB. However, *Lp*Pox can substitute any of the divalent metal ions $Mg^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ to fix the di-phosphate moiety of TPP, while the *S. sanguinis* SpxB seems to favor $Mg^{2+}$ [52].

#### i.i) Distribution and chromosomal location of spxB in oral streptococci—

SpxB-dependent $H_2O_2$ generation is conserved among most of the oral streptococci. Using the amino acid sequence of SpxB from *S. sanguinis* SK36 as a reference, we performed a BlastP search of the entire Human Oral Microbiome Database (http://www.homd.org/) [59]. A surprisingly high conservation of the amino acid composition ( 95 identity/ 98% similarity) and protein length (591 amino acids) was observed among oral streptococci (Table 1).

Among streptococci, *spxB* is typically expressed as a single gene controlled by its own promoter (Fig. 4). In several species, the immediate upstream open reading frame encodes a P-type ATPase copper transporter. Copper is a trace metal that can participate in the Fenton reaction and is also able to disrupt the iron sulfur clusters of various proteins, several of which participate in a variety of cellular processes influenced by $H_2O_2$. Accordingly, exposure of *Streptococcus pyogenes* to $H_2O_2$ leads to the induction of genes with predicted roles in iron sulfur cluster assembly [60]. Therefore, a coordinated regulation of copper homeostasis and hydrogen peroxide production could be one mechanism employed as a strategy to mitigate the potential toxicity triggered by iron-sulfur cluster protein oxidation.

**i.ii) Evidence of in vivo expression—**In an effort to demonstrate the *in vivo* relevance of pyruvate oxidase, *spxB* gene expression was quantified in clinical plaque samples from different individuals [61]. Universal *spxB* primers were developed for streptococci and could readily amplify *spxB* from isolates identified as $H_2O_2$ producers on Prussian Blue agar plates [47,48]. Conversely, isolates exhibiting no $H_2O_2$ production also failed to yield *spxB* PCR amplicons [61]. The expression characteristics of *spxB* was also determined in these clinical plaque samples. While *spxB* transcript levels were quite variable between the 9 subjects, the day to day variability was quite low and seemed relatively constant over time for one selected subject [61]. The ability to detect *spxB* expression in oral plaque samples points to its active role in oral streptococcal physiology. Moreover, *spxB* is expected to play

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a particularly important role in early biofilm development, since SpxB-positive streptococci comprise significant fractions of newly formed oral biofilms [62] and these biofilms also contain significantly higher oxygen concentrations compared to older, mature biofilms [63].

### ii) Other sources of streptococcal hydrogen peroxide production

Another commonly encoded $H_2O_2$-producing enzyme of oral streptococci is the FMN (Flavin mononucleotide)-dependent lactate oxidase (LctO; sometimes referred to as Lox) [6415]. The metabolic function of LctO is the aerobic oxidation of L-lactate to generate pyruvate. Since pyruvate is also the substrate for SpxB, both LctO and SpxB are linked in the central metabolism of oral streptococci during aerobic growth. The catalytic mechanisms of SpxB and LctO are different, since no TPP is involved in LctO oxidation of L-lactate. However, for both enzymes the cofactor FMN is initially reduced to $FMNH_2$ and then subsequently oxidized to regenerate FMN and produce $H_2O_2$ [65].

The LctO-dependent inhibitory ability of *Streptococcus oligofermentans* toward *S. mutans* has been studied in detail [66–68]. (Note: *Streptococcus oligofermentans* has been recently re-classified as *Streptococcus cristatus* [69], but for the reminder of the review we adhere to its old designation). Overall, the $H_2O_2$-dependent inhibition of *S. mutans* by *S. oligofermentans* seems to be a coordinated event between SpxB and LctO, with SpxB producing the majority of $H_2O_2$ during early and logarithmic growth and LctO dominating during stationary phase. This might be partially due to the fact that the lactate concentration is initially limited, thus precluding LctO from producing much $H_2O_2$. However, lactate will accumulate during growth and can later be used by LctO to produce pyruvate and $H_2O_2$. Interestingly, inactivation of SpxB in *S. oligofermentans* has a negative effect on the activity of LctO, further suggesting these two enzymes are coordinately controlled. Furthermore, in the context of a multispecies biofilm, LctO activity would presumably play an important ecological role in the presence of copious lactate producers like *S. mutans*. It has been suggested that this lactate-dependent oxidase activity ensures successful competition with *S. mutans* and may partially explain the high prevalence of *S. oligofermentans* in certain caries-free individuals [68,70]. Other than *S. oligofermentans*, the role of LctO has only been studied in *S. pyogenes* [71] and *S. pneumoniae* [72]. Therefore, it remains to be determined whether other oral streptococci can exploit LctO activity to inhibit cariogenic species.

In addition to SpxB and LctO, *S. oligofermentans* may utilize another oxidase to produce competitive, *S. mutans*-inhibiting quantities of $H_2O_2$ [73–75]. This enzyme was initially classified as an L-amino acid oxidase (LAAO) because of its ability to use peptone and amino acids such as L-aspartic acid, L-tryptophan, L-lysine, L-isoleucine, L-arginine, L-asparagine and L-glutamine to produce significant amounts of $H_2O_2$. Tong and colleagues suggested LAAO activity could be important under specific growth conditions [74]. For example, when saliva is used as sole nutrient source, polypeptides might be catabolized into free amino acids that could serve as LAAO substrates to yield $\alpha$-keto acids, ammonia, and $H_2O_2$. However, the activity of the enzyme was later revised and aminoacetone was reported to be the preferred substrate for purified LAAO [75]. This was independently confirmed and the structure of the enzyme solved. Thus, the enzyme was renamed to aminoacetone oxidase or AAO. Its activity previously measured using amino acids substrates is therefore likely the

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result of either promiscuous catalysis on non-preferred substrates or an artifact of the *in vitro* reaction conditions [73]. Nonetheless, AAO seems to be a unique enzyme in oral streptococci and there is strong evidence to suggest that *S. oligofermentans* acquired this gene via horizontal gene transfer [76]. Although LctO-mediated inhibition of *S. mutans* is ten-fold stronger as compared to AAO [74], it might still provide a selective advantage for *S. oligofermentans* under certain ecological conditions. Unlike SpxB and LctO, AAO also has the added advantage of producing ammonia as a byproduct [73], which, like the arginine deiminases of other commensal oral streptococci, might similarly provide protection against plaque acidification [77].

Other fundamental redox reactions in the cell can also yield modest amounts of $H_2O_2$. For example, the regeneration of $NAD^+$ via NADH oxidase can yield $H_2O_2$ [78]. In general, NADH oxidases reduce molecular oxygen to either $H_2O_2$ (via Nox-1) or $H_2O$ (via Nox-2) using NADH as a substrate [78]. Among streptococci, differences exist on the distribution of the *nox-1/2* genes. *S. mutans* encodes *nox-1* and *nox-2* [79], while *S. sanguinis* only encodes *nox-2* [78]. The $H_2O_2$ production capacity of *nox-1* is apparently limited, since *S. mutans* does not produce inhibiting amounts of $H_2O_2$ [45]. In a recent study, Ge *et al.* demonstrated that a *nox-2* knock-out mutant in *S. sanguinis* loses its ability to compete with *S. mutans* due to a decrease in extracellular $H_2O_2$ production [78]. Interestingly, this effect on $H_2O_2$ production was suggested to be indirect since the intracellular concentration of $H_2O_2$ was actually slightly increased and no deleterious impacts were observed upon SpxB activity. The authors also reported that the *nox-2* deletion affects membrane fluidity, which has led to the speculation that the observed lack of *S. mutans* inhibition might result from a decrease in $H_2O_2$ diffusion across the membrane [78].

## Self-compatibility and the response to hydrogen peroxide stress

When comparing the aerobic growth of $H_2O_2$-producing streptococci in the presence and absence of externally added catalase, it is evident that $H_2O_2$ production yields self-toxicity [80]. Typically, streptococci do not encode catalase, suggesting that the benefit from excreting relatively large amounts of $H_2O_2$ outweighs the potential negative effects its production might pose on the producer itself. The absence of catalase or other commonly employed $H_2O_2$-detoxifying enzymes in oral streptococci also suggests that other important, perhaps uncharacterized mechanisms are involved in protection from $H_2O_2$ toxicity. Certainly, it is also possible that in the oral cavity $H_2O_2$ toxicity might not be as pronounced as under laboratory conditions. Saliva has an inherent capacity to deplete $H_2O_2$ via the salivary lactoperoxidase system, which catalyzes the oxidation of thiocyanate by $H_2O_2$ to produce the antimicrobial compound hypothiocyanite [81–83]. It is currently unclear to what extent, if any, that lactoperoxidase might influence the local $H_2O_2$ concentrations in oral biofilms. Furthermore, saliva flow aides in the dilution of $H_2O_2$ [84]. However, saliva flow can be limited, for example, in biofilms or interproximal spaces. The actual concentration of $H_2O_2$ in saliva or at the biofilm-tooth interface has not been determined *in vivo*. The detection is hindered by several problems, including the presence of the salivary lactoperoxidase system which can eliminate $H_2O_2$ before its reliable detection. The lactoperoxidase substrates (thiocyanate) and products (hypothiocyanite) can be measured in saliva however, and this allows salivary $H_2O_2$ concentrations to be extrapolated to approximately 10 μM [85]. We

currently lack the sensor technologies required to directly measure $H_2O_2$ at the biofilm interface of patients, but one would expect this value to be significantly higher than the 10 μM value estimated for saliva. Recent real-time measurements of the $H_2O_2$ concentration 100 μm above an *in vitro* polymicrobial biofilm found up to 1.4 mM of $H_2O_2$ can be generated [86]. This suggests that during early biofilm development, in which there is an abundance of SpxB positive streptococci, considerable amounts of $H_2O_2$ can be produced that presumably requires efficient cellular defense mechanisms to avoid $H_2O_2$-dependent toxicity.

A key bacterial mechanism to prevent $H_2O_2$ toxicity is through the avoidance of the Fenton reaction. We have previously demonstrated in *S. sanguinis* and *S. gordonii* that mutants lacking Dps, a ferritin-like protein involved in iron sequestration from DNA, are about $10^4$-fold more susceptible to exogenous $H_2O_2$ compared to their respective wild types [43]. A similar hypersensitivity was also reported for *S. mutans* as well as other streptococci, suggesting that Dps is a broadly conserved and critical protein for the defense against $H_2O_2$ [87,88]. In *S. mutans*, superoxide dismutase (Sod) mutants exhibit a similar hypersensitivity as Dps mutants [89], while the same Sod mutations in *S. sanguinis* and *S. gordonii* only result in about two to three-fold increases in $H_2O_2$ sensitivity [43]. TrxB mutants of *S. sanguinis* and *S. gordonii* also exhibit dramatically different $H_2O_2$ sensitivity phenotypes. TrxB is a thioredoxin reductase that catalyzes the reduction of thioredoxin and plays important role in the formation of disulfide bonds in oxidized proteins. An *S. sanguinis* TrxB mutant is severely impaired in its $H_2O_2$ resistance, whereas the *S. gordonii* TrxB mutant exhibits no obvious survival defects after $H_2O_2$ challenge [43].

Studies of the *S. pneumoniae* pyruvate oxidase SpxB demonstrated an unexpected direct role for SpxB in the protection against $H_2O_2$ toxicity. *S. pneumoniae* SpxB deletion mutants are $10^2$ to $10^3$-fold more susceptible to exogenously added $H_2O_2$ [90]. The source of this change in $H_2O_2$ susceptibility was determined to be dependent upon post translational modifications of SpxB itself, in which sulfenylation of SpxB occurs during endogenous $H_2O_2$ production as part of a reactive oxygen species adaptation mechanism [91]. Although not entirely understood, the sulfenylation of SpxB is postulated to serve as an "$H_2O_2$ sink" that scavenges intracellular $H_2O_2$ [91]. Thus, *S. pneumoniae* has apparently evolved an intriguing mechanism to use SpxB to mitigate the oxidative stress that is created through its own catalytic activity. However, this mechanism does not appear to be a conserved characteristic of SpxB orthologs, as *spxB* mutations do not impact $H_2O_2$ sensitivity for either *S. sanguinis* or *S. gordonii* [43]. Based upon our current knowledge of oxidative stress tolerance, it would appear that the basic machinery employed by oral streptococci are part of a common toolbox. Yet, the observed differences among streptococci for proteins like SpxB, Sod, and TrxB suggest that there are significant aspects of $H_2O_2$ resistance that have yet to be discovered in these species. This is particularly evident from a recent publication illustrating a wide variety of oxidative stress tolerance phenotypes among different strains of *S. mutans* that all exhibit similar expression patterns for known components of the ROS response machinery [92]. For further information about streptococcal ROS response mechanisms, see [93].



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## Hydrogen peroxide production and biofilm ecology

Our view of oral disease development has evolved in the last two decades due to advances in our understanding of the ecology within oral biofilms [94]. It is now widely accepted that caries and periodontal disease are byproducts of oral dysbiosis [95]. However, due to a historical focus upon pathogenic bacteria, a major aspect of oral health has been relatively understudied: the protective abilities of the commensal flora. Currently, there is a paucity of studies revealing the biological mechanisms that support symbiosis, a research area that we refer to as molecular commensalism [20]. A better understanding of such mechanisms could provide valuable new strategies to prevent dysbiotic diseases. In fact, there is already evidence that such an approach is feasible. By promoting the growth of alkali-producing oral streptococci via arginine supplementation, it is possible to achieve a net increase in dental plaque pH and lower caries scores [77,96]. $H_2O_2$ production is another aspect that could be exploited, as $H_2O_2$ is specifically associated with the commensal flora. Cariogenic species like *S. mutans* and most periodontopathogens are all exquisitely sensitive to $H_2O_2$ toxicity [15,97].

### i) $H_2O_2$ and interspecies competition in the oral biofilm

To better understand how $H_2O_2$ production is influenced by the immediate biofilm environment, scanning electrochemical microscopy (SECM) has been used to monitor $H_2O_2$ production in real-time at the surface of an *S. gordonii*-*S. mutans* dual species biofilm [98]. In combination with a pH probe, Joshi and colleagues identified the Achilles heel of *S. gordonii* $H_2O_2$ production. In an *in vitro* dual species biofilm assay grown in artificial saliva, *S. gordonii* is able to produce a considerable amount of $H_2O_2$ at a neutral pH. In fact, *S. gordonii* can slow the process of biofilm acidification by increasing *spxB* expression and $H_2O_2$ production once the environmental pH starts to fall due to sugar fermentation. This adjustment in $H_2O_2$ production provides a temporary check on *S. mutans* growth. However, once the environmental pH falls below the physiological buffering capacity of saliva, both *spxB* expression and $H_2O_2$ production diminish, which allows *S. mutans* to eventually dominate [98]. This clearly shows that a prolonged reduction of the salivary pH not only promotes the growth of aciduric species, but also impairs the ability of some commensal species to effectively antagonize $H_2O_2$-sensitive pathobionts like *S. mutans* [98].

The effect of the environmental pH on the $H_2O_2$ production capability of oral streptococci seems to be a key issue in their competitive abilities. Contrary to the observation with *S. gordonii*, *S. sanguinis* exhibited no change in the production of $H_2O_2$ when growing at pH6, demonstrating that the response to a lower pH is not a universal decrease in $H_2O_2$ production [99]. Although the observed phenotypic response showed consistency among different strains, the authors would like to emphasize that the ability of streptococci to adjust to environmental perturbations needs to be assessed on the species and sometimes strain level and should not be generalized [92,99]. Interestingly, the observed reduction of *spxB* expression and $H_2O_2$ production in *S. gordonii* leads to a redirection of the metabolic flux at the pyruvate node [99]. Since less pyruvate is metabolized through SpxB catalytic activity, an increase in lactic acid production was observed, which is also reflected in an increased expression of the lactate dehydrogenase gene *ldh*. The increase in lactic acid production

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itself is an adaptation mechanism improving the survival of *S. gordonii* at lower pH [99]. *S. sanguinis* in general produces less $H_2O_2$ than *S. gordonii* and is more susceptible towards lower pH. Furthermore, the reduced $H_2O_2$ production at lower pH observed in *S. gordonii* still exceeds *S. sanguinis* production ability [99]. Perhaps, the production of $H_2O_2$ serves a slightly different ecological purpose since *S. sanguinis* is one of the most-abundant early colonizers [20] and biofilm mediated protection mechanisms may protect the *S. sanguinis* population against low pH [100]. At the same time, higher abundance means less energy needs to be channeled towards $H_2O_2$ production in general, while *S. gordonii* as a species with lower abundance needs to be more aggressive to ensure competitiveness.

The impact of environmental pH upon competitive $H_2O_2$ production has also been examined in other species [99,101,102]. *S. oligofermentans*, a potent $H_2O_2$ producer, is able to inhibit the growth of *S. mutans* at pH6 in deferred antagonism assays, which is quite surprising, since *S. oligofermentans* growth at pH6 is compromised to a much greater degree compared to *S. mutans*. Even with its impaired growth at pH6, *S. oligofermentans* still produces up to 1.5-fold more $H_2O_2$ compared to pH7 [101]. At a pH of 5.5, *S. oligofermentans* growth defects are too severe to remain competitive with *S. mutans*, despite its aggressive $H_2O_2$ production. The inhibitory spectrum of commensal oral streptococcal $H_2O_2$-production was also confirmed for periodontopathogens like *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia,* and *Porphyromonas gingivalis* [97,103], suggesting that $H_2O_2$ production might similarly influence the composition of subgingival biofilms.

### ii) Tipping the balance – influencing the production of $H_2O_2$

A key outcome for studies of molecular commensalism would be to develop new approaches to sustain a symbiotic biofilm community, even under conditions that otherwise would lead to dysbiosis. Conceivably, this could be achieved either by increasing the number of health-associated bacteria, such as SpxB-encoding streptococci, and/or by increasing the $H_2O_2$ output of producer species. Clinically, it has been shown that *S. sanguinis* isolates from caries-free individuals have increased $H_2O_2$ production relative to high caries individuals, which further supports the health-protective role of $H_2O_2$ [104]. In addition, our previous studies of an *S. gordonii* CcpA mutant have demonstrated the conceptual utility of exogenously manipulating $H_2O_2$ production to achieve a desired ecological outcome. CcpA is a transcriptional regulator of *spxB* and its deletion increases *spxB* expression and $H_2O_2$ production [105]. Consequently, CcpA mutants also exhibit an enhanced ability to antagonize the growth of *S. mutans*. Similarly, *in vitro* studies of a 3-species *S. gordonii, S. mutans,* and *A. naeslundii* biofilm model have demonstrated that L-arginine supplementation can prevent *S. mutans* dominance and favor *S. gordonii* growth even in the presence of sucrose [106], a carbohydrate that strongly promotes the dysbiotic overgrowth of *S. mutans* [107]. In this system, L-arginine was able to increase the pH of the biofilm, as expected, but it had the added benefit of increasing the production of $H_2O_2$ by *S. gordonii* as well. Conversely, in a 14-species biofilm model containing both $H_2O_2$-producing streptococci and periodontopathogens, the addition of clinically relevant concentrations of the $H_2O_2$-inactivating enzymes myeloperoxidase, lactoperoxidase, and erythrocyte catalase strongly favored the growth of the periodontopathogens at the expense of the commensals [108]. Overall, there is ample clinical and experimental evidence establishing a clear inverse

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correlation between the abundance of $H_2O_2$ produced in an oral biofilm community and the prevalence of pathobionts.

When comparing the oral biofilms of different people, it is evident that considerable variability exists not only at the species level, but even among different strains of the same species [109]. This has important ecological implications for one's individual likelihood of developing oral dysbiosis. For example, differences in the $H_2O_2$ susceptibility of an individual's pathobionts can influence how much $H_2O_2$ production is required to sustain symbiosis. This is evident from a recent study of *S. mutans* clinical isolates, which demonstrated how variability among the oxidative stress tolerance phenotypes of these strains has a dramatic influence upon their competitiveness in a multispecies biofilm setting [92]. *S. mutans* clinical isolates were mixed with *S. oralis* and *A. naeslundii* and grown as biofilms for up to 91 hours. One particular *S. mutans* strain (Smu81) that was isolated from subgingival plaque [110] exhibited poor competitive abilities after 67 and 91 hours of incubation in the presence of sucrose, while all other *S. mutans* strains eventually became the dominant biofilm species under the same growth conditions [92]. In a deferred antagonism assay, *S. oralis* was able to severely inhibit the growth of Smu81, but this effect was completely abolished in the presence of catalase [92]. It is still a mystery why different strains of *S. mutans* can exhibit such a wide range of oxidative stress tolerance.

Similarly, clinical isolates of $H_2O_2$-producing streptococci also exhibit a range of $H_2O_2$-production phenotypes. Thus far, we have identified three distinct groups of $H_2O_2$-producers: group I – high-level $H_2O_2$ production, carbon catabolite repression (CCR) insensitive; group II – low-level $H_2O_2$ production, CCR insensitive; and group III – CCR-sensitive $H_2O_2$ production (Fig. 5). Importantly, like *S. mutans* oxidative stress tolerance, the $H_2O_2$-production phenotypes of these isolates were largely determined at the strain level [121]. Further details regarding the role of CCR-dependent regulation of $H_2O_2$-production will be described later.

As mentioned previously, environmental pH is another factor that can have a profound impact upon $H_2O_2$ production. In a recent study by Huang and colleagues, alkali-generating arginolytic species were isolated from subjects with and without caries [111]. Overall, 6 different *Streptococcus* species were detected with *S. sanguinis* being predominant (67.9%) followed by *S. gordonii* (8.9%), *S. intermedius* (8.9%), *Streptococcus cristatus* (8.9%), *S. australis* (3.6%), and *S. parasanguinis* (1.8%) [111]. Each of these species also encodes *spxB* (see Table 1) and further analysis determined $H_2O_2$ production to be their principal mechanism of *S. mutans* antagonism [112]. While $H_2O_2$ production is negatively impacted by acidic growth conditions for some streptococci [99], the arginine deiminase system is highly acid tolerant [113,114] and could serve to bolster SpxB activity to prolong competitive $H_2O_2$ production.

### iii) Beyond competition – $H_2O_2$ signaling, biofilm formation, and community development

Another emerging aspect of $H_2O_2$ production is its influence on bacterial synergistic interactions, potentially functioning as a signal molecule. The role of streptococcal $H_2O_2$ in interspecies interactions with *A. actinomycetemcomitans* has been the subject of several excellent publications [115–117]. Initial investigations with *S. gordonii* and *A.*

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*actinomycetemcomitans* revealed that the latter is able to utilize the lactic acid produced by *S. gordonii* as a carbon source, thus providing metabolic complementation and potentially promoting virulence [116]. The dilemma for *A. actinomycetemcomitans* is its susceptibility to the $H_2O_2$ produced by *S. gordonii*. To minimize its $H_2O_2$ exposure, *A. actinomycetemcomitans* increases the production of its catalase gene *katA* as well as a novel gene *dspB* [115]. DspB is an extracellular enzyme that promotes the dispersal of *A. actinomycetemcomitans* biofilms by hydrolyzing the major component of its extracellular matrix, poly-N-acetylglucosamine. This allows *A. actinomycetemcomitans* to keep a "safe" distance from *S. gordonii* in mixed species murine abscesses. Normally, *A. actinomycetemcomitans* and *S. gordonii* both form small aggregates of about 250–1000 cells in experimental abscesses, while those aggregates increased about two-fold in an *A. actinomycetemcomitans* DspB mutant [115]. Moreover, the DspB mutant aggregates were either contiguous to or within 4 μm of those of *S. gordonii*, while the wild type maintained a distance between 4 and 13 μm from the *S. gordonii* aggregates. Presumably, this distance provides the optimal balance between the benefits provided by carbon source acquisition and the limitations of its catalase activity [115]. In a separate mixed species study of *A. actinomycetemcomitans* and *S. parasanguinis*, it was demonstrated that a close proximity between both species can be maintained by triggering a decrease in *spxB* expression [118]. In fact, dual species biofilms exhibited a significant increase in bio-volume and biofilm thickness as compared to mono-species biofilms, while both species remained intimately associated. Interestingly, *A. actinomycetemcomitans* catalase did not play an important role in this assay, presumably because the down regulation of *spxB* limited $H_2O_2$ production to a level at which *katA* expression is unnecessary.

A similar phenomenon has been reported for mixed species biofilms of *S. gordonii* and *A. naeslundii* [119]. Like *A. actinomycetemcomitans, A. naeslundii* is very susceptible to inhibition by $H_2O_2$ and encodes catalase. Interestingly, in co-cultures with *A. naeslundii*, *S. gordonii* exhibits reduced oxidative damage to its surface proteins, which is likely a benefit provided *in trans* from *A. naeslundii* catalase [119]. Thus, certain combinations of species might prove beneficial to $H_2O_2$ producers by minimizing the self-toxicity associated with their $H_2O_2$ production. Taken together, multiple lines of evidence support the role of $H_2O_2$ in shaping the ecology within the polymicrobial dental biofilm through both pathobiont inhibition and interspecies interactions. Further knowledge of the biogeographical landscape of the numerous polymicrobial physical [12] and chemical interactions [120] will be required to fully understand the dynamics of these interactions and how they may influence the balance between symbiosis and dysbiosis.

## Genetic regulation of streptococcal *spxB* expression

The expression of *spxB* is under the control of several transcriptional regulators that are conserved in oral streptococci and respond to either metabolic or environmental stimuli. Some species- and strain-specific variations exist in their mode of control and it is currently unknown how these different control elements are coordinated in a hierarchy of transcriptional regulation. In addition, a specific regulatory mechanism has yet to be established for the decreased *spxB* expression observed during the switch from aerobic to anaerobic growth conditions. Likewise, it is currently unclear whether the previously

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described pH dependence of $H_2O_2$ production is the result of transcriptional regulation, post-transcriptional regulation, or an indirect consequence of a metabolic adjustment.

### i) CcpA

Oral streptococci have access to and metabolize a large variety of carbohydrates. Glucose is the most important and preferred carbon source, but its concentration in the oral cavity fluctuates as a consequence of the host diet. In the presence of glucose, most streptococci will suppress the expression of genes involved in the utilization of other carbon sources, a process referred to as carbon catabolite repression (CCR) [121]. We previously identified distinct *spxB* transcriptional responses to glucose from *S. sanguinis* and *S. gordonii*. *S. gordonii* follows the classic carbon catabolite repression response and decreases *spxB* expression in the presence of glucose, while *spxB* expression remains unchanged in *S. sanguinis*, although its expression is consistently lower than in *S. gordonii* overall [105,122,123]. Given this difference in regulation, it was surprising to find a consensus sequence for the catabolite control protein A (CcpA) (*cre*) in both species. CcpA is the main global regulator for carbohydrate metabolism in Gram-positives [121]. Interestingly, *S. pneumoniae spxB* expression is also controlled by CcpA, but is not affected by glucose similar to *S. sanguinis* [124]. The regulatory role of CcpA has been confirmed by mutagenesis, and as expected, *spxB* expression is increased in the CcpA mutants of both *S. sanguinis* and *S. gordonii*, suggesting that *S. sanguinis* CcpA-dependent control of *spxB* expression is glucose independent [123]. To further explore the regulatory role of CcpA in both species, promoter binding studies were performed and confirmed to depend upon the two *cre* sites for both species [123]. Despite this, it is still not clear why the two species differ in their CCR response for *spxB*, but we have some evidence indicating that a regulatory mechanism upstream of CcpA is most likely responsible. Furthermore, we observed similar carbohydrate-dependent and carbohydrate-independent *spxB* control mechanisms from a variety of clinical isolates of different oral streptococci, which indicates that the distinctions between *S. gordonii* and *S. sanguinis* are commonly found in other species as well [123].The ecological implications of this differential regulation of *spxB* are still unclear and this is a subject of active investigation.

### ii) SpxR

One of the first *spxB* transcriptional regulators identified in *S. pneumoniae* is SpxR. Mutational studies confirmed the role of SpxR as a transcription activator of *spxB* gene expression. SpxR mutants exhibit decreased $H_2O_2$ production and are severely impaired for virulence in a murine infection model [124]. The regulatory role of SpxR was also demonstrated in *S. sanguinis* [125]. The domain architecture of SpxR contains a helix-turn-helix motif for DNA binding as well as CBS and HotDog domains. CBS and HotDog domains seem to be ancient and ubiquitous motifs with potential sensory functions monitoring the cellular energy status by binding to AMP, ATP, or S-adenosyl methionine [126]. Thus, it has been suggested that SpxR connects *spxB* expression to the energy and metabolic status of the cell [124,125], but this prediction has yet to be experimentally confirmed and the signal sensed by SpxR remains unknown.

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#### iii) SpxA1 and SpxA2

SpxA1 and SpxA2 are both transcriptional regulators implicated in the control of *spxB* expression. Both regulators appear conserved among streptococci and fulfill several important functions in oxidative stress tolerance as well as biofilm and competence development. A detailed analysis in *S. sanguinis* demonstrated that a deletion of *spxA1* decreased the production of $H_2O_2$ threefold along with a similar reduction in *spxB* expression. In the *spxA2* deletion background, $H_2O_2$ production was not found to be significantly affected [127], but this result has been recently challenged [128]. Interestingly, SpxA1/2 exhibit significant homology, including a Cys-X-X-Cys motif that has previously been shown to sense the intracellular redox state [129]. Further studies are required to better understand the mechanistic role of both transcriptional regulators for *spxB* regulation.

#### iv) Two-Component Systems (TCS) VicRK and SptRS

Environmental information is commonly transmitted via TCS signaling in bacteria [130,131]. Two *S. sanguinis* TCS have been previously demonstrated to influence *spxB* gene expression [132,133]. The initial discovery that the TCS VicRK positively regulates *spxB* expression as well as biofilm formation and the release of extracellular DNA demonstrates that $H_2O_2$ production is involved in important cellular developmental processes [133]. This is consistent with our previous studies of the connection between *spxB* expression, $H_2O_2$ production, and the induction of extracellular DNA release [134]. More recently, the *S. sanguinis* SptSR TCS has also been demonstrated to control *spxB* expression [132]. In contrast to the VicRK TCS, a deletion of either *sptS* or *sptR* increases the production of eDNA and $H_2O_2$. While VicR was shown to bind the *spxB* promoter [133], this was not possible for SptR, although a putative consensus binding site was identified in the *spxB* promoter [132]. Therefore, it is currently unknown whether the regulatory effect of the SptRS TCS is direct or indirect. The latter is a distinct possibility, since the *sptR* and *sptS* mutations trigger significant transcriptional changes in the regulatory genes *vicR* and *spxR* [132].

## Streptococcal $H_2O_2$ production at extra-oral locations

The reservoir of oral streptococci is not confined to the oral cavity, as S. dentisani, S. tigurinus, S. oralis, S. oligofermentans, S. mitis, S. infantis, and S. gordonii have all been isolated from a various areas of nasopharynx from healthy adults [135]. In addition, it has been suggested that streptococci are generally protective for paranasal sinuses to certain diseases such as chronic rhinosinusitis [136]. Although there is currently no experimental evidence of $H_2O_2$ production as a key facet of this protection, the availability of oxygen in the nasopharynx would certainly allow for its production.

Oral streptococci are also commonly associated with the lung microbiome, and their presence in patients with either chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF) has been shown [137,138]. For COPD patients, the streptococcal community in the lungs was shown to originate primarily from the oral cavity through aspiration based upon comparisons of the overall similarity of the bronchial, peripheral lung, nasal, and oral microbiomes [138]. This suggests that aspiration and colonization by oral streptococci is likely a common occurrence. A recent clinical study found that a pediatric control cohort had a

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higher percentage of *Streptococcus* in their broncho-alveolar lavage fluid compared to an age-matched cohort of pediatric CF patients, while the study failed to detect any *Streptococcus* in adult CF patients [137]. Over time, the non-traditional CF taxa like the streptococci seem to be replaced by the traditional CF taxa including *Pseudomonas*, *Staphylococcus,* and *Stenotrophomonas* [137]. This observation may indicate that the non-traditional taxa including *Streptococcus* can colonize better and initially provide some level of protection. It has also been hypothesized that oral streptococci may play an important role in increasing the diversity of the cystic fibrosis microbiome, thus promoting patient stability [139]. Mechanistically, this is consistent with the findings by Scoffield and Wu, which demonstrated that oral streptococci successfully interfere with *Pseudomonas aeruginosa* through $H_2O_2$ production [46]. Surprisingly, multiple streptococci including *S. parasanguinis*, *S. sanguinis* and *S. gordonii* all failed to inhibit the growth of *P. aeruginosa* in antagonism assays when using regular growth medium, but exhibited inhibition when nitrite was supplemented. This effect was also SpxB-dependent. In a *Drosophila melanogaster* infection model, the authors demonstrated that $H_2O_2$-producing oral streptococci are similarly protective against *P. aeruginosa* in the presence of nitrite [46]. Nitrite concentrations seem to be elevated in the sputum of CF patients [140] and can be converted to peroxynitrite in the presence of $H_2O_2$. It has been suggested that peroxynitrite is potentially internalized through a permease to exert its antimicrobial effect upon *P. aeruginosa* [46]. It is also worth noting that multiple CF studies have detected oral streptococci from the *Streptococcus anginosus* group (formerly Milleri group) as being strongly associated with CF pathogenesis, namely *S. intermedius* and *S. constellatus* [141,142]. Coincidently, after searching the annotated genomes available in the Human Oral microbiome Database (HOMD), both *S. intermedius* and *S. constellatus* were not found to encode *spxB* and are thus not expected to be significant $H_2O_2$ producers.

In conclusion, oral streptococci are present in multiple sites of the human host. Most likely, their dissemination originates from the oral cavity. In future studies, it would be interesting to determine whether commensal oral streptococci exert similar health protective benefits outside of the oral cavity via their $H_2O_2$ production.

## Outlook

Hydrogen peroxide production plays an important role in the metabolism of oral streptococci and effects the ecology of dental biofilms. Experimental evidence over the last decade supports a critical role for $H_2O_2$ in maintaining oral symbiotic homeostasis. With our deeper understanding of the ecological aspects of oral diseases, it has been suggested that future treatments are likely to remodel dysbiotic communities back to a state of symbiosis with the host [19,20,143]. This concept is a promising alternative to conventional therapies, but its translation is only likely to occur with a more thorough understanding of molecular commensalism [20]. The manipulation of $H_2O_2$ production among the commensal flora is certainly one conceivable strategy to prevent and/or reverse dysbiosis.

Furthermore, a recent systematic review about the diagnostic accuracy of current Caries Risk Assessment (CRA) methods concluded “the analyzed methods may lead to patients with increased risk not being identified” [144]. Clinical CRA guidelines approved by the American

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Dental Association fail to consider the polymicrobial nature of caries and lack any molecular markers sampled directly at the tooth surface where caries occurs [145]. The identification of such microbial biomarkers to monitor changes at the tooth surface before the clinical manifestation of caries is detectable would greatly improve early interventions. Currently, the caries disease process cannot be accurately determined in the absence of observable demineralization. Therefore, predictive preclinical biomarkers are clearly desirable and much needed. $H_2O_2$ production might prove to be a useful biomarker of the underlying ecology within the oral cavity.

## Acknowledgements

This project was supported by NIH grants DE018893, DE022083, and DE023850 to JM and an NIH grant DE021726 to JK.

## References

1. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. Genome Med. 2016;8(1):51. [PubMed: 27122046]
2. Marsh PD, Devine DA. How is the development of dental biofilms influenced by the host? J Clin Periodontol. 2011;38 Suppl 11:28–35. [PubMed: 21323701]
3. Jakubovics NS. Talk of the town: interspecies communication in oral biofilms. Mol Oral Microbiol. 2010;25(1):4–14. [PubMed: 20331790]
4. Lang ML, Zhu L, Kreth J. Keeping the bad bacteria in check: interactions of the host immune system with oral cavity biofilms. Endodontic Topics. 2012;22(1):17–32.
5. Chiu L, Bazin T, Truchetet ME, Schaeverbeke T, Delhaes L, Pradeu T. Protective Microbiota: From Localized to Long-Reaching Co-Immunity. Front Immunol. 2017;8:1678. [PubMed: 29270167]
6. Zaura E, ten Cate JM. Towards understanding oral health. Caries Res. 2015;49 Suppl 1:55–61. [PubMed: 25871419]
7. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol. 2005;43(11):5721–5732. [PubMed: 16272510]
8. Simon-Soro A, Belda-Ferre P, Cabrera-Rubio R, Alcaraz LD, Mira A. A tissue-dependent hypothesis of dental caries. Caries Res. 2013;47(6):591–600. [PubMed: 24080530]
9. Sanz M, Beighton D, Curtis MA, et al. Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases. Consensus report of group 1 of the Joint EFP/ORCA workshop on the boundaries between caries and periodontal disease. J Clin Periodontol. 2017;44 Suppl 18:S5–S11. [PubMed: 28266109]
10. Valm AM, Mark Welch JL, Rieken CW, et al. Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. Proc Natl Acad Sci U S A. 2011;108(10):4152–4157. [PubMed: 21325608]
11. Zijnge V, van Leeuwen MB, Degener JE, et al. Oral biofilm architecture on natural teeth. PLoS One. 2010;5(2):e9321. [PubMed: 20195365]
12. Mark Welch JL, Rossetti BJ, Rieken CW, Dewhirst FE, Borisy GG. Biogeography of a human oral microbiome at the micron scale. Proc Natl Acad Sci U S A. 2016;113(6):E791–800. [PubMed: 26811460]
13. Diaz PI, Hoare A, Hong BY. Subgingival Microbiome Shifts and Community Dynamics in Periodontal Diseases. J Calif Dent Assoc. 2016;44(7):421–435. [PubMed: 27514154]
14. Wake N, Asahi Y, Noiri Y, et al. Temporal dynamics of bacterial microbiota in the human oral cavity determined using an in situ model of dental biofilms. NPJ Biofilms Microbiomes. 2016;2:16018. [PubMed: 28721251]
15. Zhu L, Kreth J. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. Oxidative medicine and cellular longevity. 2012;2012:717843. [PubMed: 22848782]

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16. Hannig M, Joiner A. The structure, function and properties of the acquired pellicle. Monogr Oral Sci. 2006;19:29–64. [PubMed: 16374028]
17. Wright CJ, Burns LH, Jack AA, et al. Microbial interactions in building of communities. Mol Oral Microbiol. 2013;28(2):83–101. [PubMed: 23253299]
18. Kreth J, Merritt J, Qi F. Bacterial and host interactions of oral streptococci. DNA Cell Biol. 2009;28(8):397–403. [PubMed: 19435424]
19. Hoare A, Marsh PD, Diaz PI. Ecological Therapeutic Opportunities for Oral Diseases. Microbiol Spectr. 2017;5(4).
20. Kreth J, Giacaman RA, Raghavan R, Merritt J. The road less traveled - defining molecular commensalism with *Streptococcus sanguinis*. Mol Oral Microbiol. 2017;32(3):181–196. [PubMed: 27476770]
21. Wood PM. The potential diagram for oxygen at pH 7. Biochem J. 1988;253(1):287–289. [PubMed: 2844170]
22. Mahaseth T, Kuzminov A. Potentiation of hydrogen peroxide toxicity: From catalase inhibition to stable DNA-iron complexes. Mutat Res. 2017;773:274–281. [PubMed: 28927535]
23. Pryor WA. Oxy-radicals and related species: their formation, lifetimes, and reactions. Annu Rev Physiol. 1986;48:657–667. [PubMed: 3010829]
24. Goldstein S, Meyerstein D, Czapski G. The Fenton reagents. Free Radic Biol Med. 1993;15(4): 435–445. [PubMed: 8225025]
25. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 5th Edition ed: Oxford University Press; 2015.
26. Winterbourn CC. Toxicity of iron and hydrogen peroxide: the Fenton reaction. Toxicol Lett. 1995;82–83:969–974.
27. Imlay JA. Pathways of oxidative damage. Annu Rev Microbiol. 2003;57:395–418. [PubMed: 14527285]
28. Mello Filho AC, Hoffmann ME, Meneghini R. Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. Biochem J. 1984;218(1):273–275. [PubMed: 6712613]
29. Iqbal M, Sharma SD, Mizote A, Fujisawa M, Okada S. Differential role of hydrogen peroxide and organic hydroperoxides in augmenting ferric nitrilotriacetate (Fe-NTA)-mediated DNA damage: implications for carcinogenesis. Teratog Carcinog Mutagen. 2003;Suppl 1:13–21.
30. Storz G, Imlay JA. Oxidative stress. Curr Opin Microbiol. 1999;2(2):188–194. [PubMed: 10322176]
31. Imlay JA. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat Rev Microbiol. 2013;11(7):443–454. [PubMed: 23712352]
32. Brandi G, Sestili P, Pedrini MA, Salvaggio L, Cattabeni F, Cantoni O. The effect of temperature or anoxia on *Escherichia coli* killing induced by hydrogen peroxide. Mutat Res. 1987;190(4):237–240. [PubMed: 3550449]
33. Imlay JA, Linn S. Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. J Bacteriol. 1986;166(2):519–527. [PubMed: 3516975]
34. Linley E, Denyer SP, McDonnell G, Simons C, Maillard JY. Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. J Antimicrob Chemother. 2012;67(7):1589–1596. [PubMed: 22532463]
35. Rai P, Cole TD, Wemmer DE, Linn S. Localization of Fe(2+) at an RTGR sequence within a DNA duplex explains preferential cleavage by Fe(2+) and H2O2. J Mol Biol. 2001;312(5):1089–1101. [PubMed: 11580252]
36. Gonzalez-Flecha B, Demple B. Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing *Escherichia coli*. J Bacteriol. 1997;179(2):382–388. [PubMed: 8990289]
37. Barnard JP, Stinson MW. The alpha-hemolysin of Streptococcus gordonii is hydrogen peroxide. Infect Immun. 1996;64(9):3853–3857. [PubMed: 8751938]
38. Pesakhov S, Benisty R, Sikron N, et al. Effect of hydrogen peroxide production and the Fenton reaction on membrane composition of *Streptococcus pneumoniae*. Biochim Biophys Acta. 2007;1768(3):590–597. [PubMed: 17292324]

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39. Seaver LC, Imlay JA. Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. J Bacteriol. 2001;183(24):7182–7189. [PubMed: 11717277]
40. Thibessard A, Fernandez A, Gintz B, Leblond-Bourget N, Decaris B. Hydrogen peroxide effects on *Streptococcus thermophilus* CNRZ368 cell viability. Res Microbiol. 2001;152(6):593–596. [PubMed: 11501678]
41. Clark JB. Catalase activity in *Escherichia coli*. J Bacteriol. 1952;64(4):527–530. [PubMed: 12999680]
42. Henningham A, Dohrmann S, Nizet V, Cole JN. Mechanisms of group A Streptococcus resistance to reactive oxygen species. FEMS Microbiol Rev. 2015;39(4):488–508. [PubMed: 25670736]
43. Xu Y, Itzek A, Kreth J. Comparison of genes required for $H_2O_2$ resistance in *Streptococcus gordonii* and *Streptococcus sanguinis*. Microbiology. 2014;160(Pt 12):2627–2638.
44. Thomas EL, Milligan TW, Joyner RE, Jefferson MM. Antibacterial activity of hydrogen peroxide and the lactoperoxidase-hydrogen peroxide-thiocyanate system against oral streptococci. Infect Immun. 1994;62(2):529–535. [PubMed: 8300211]
45. Kreth J, Merritt J, Shi W, Qi F. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. J Bacteriol. 2005;187(21):7193–7203. [PubMed: 16237003]
46. Scoffield JA, Wu H. Oral streptococci and nitrite-mediated interference of *Pseudomonas aeruginosa*. Infect Immun. 2015;83(1):101–107. [PubMed: 25312949]
47. Saito M, Seki M, Iida K, Nakayama H, Yoshida S. A novel agar medium to detect hydrogen peroxide-producing bacteria based on the prussian blue-forming reaction. Microbiol Immunol. 2007;51(9):889–892. [PubMed: 17895606]
48. Sumioka R, Nakata M, Okahashi N, et al. *Streptococcus sanguinis* induces neutrophil cell death by production of hydrogen peroxide. PLoS One. 2017;12(2):e0172223. [PubMed: 28222125]
49. Carlsson J, Edlund MB. Pyruvate oxidase in *Streptococcus sanguis* under various growth conditions. Oral Microbiol Immunol. 1987;2(1):10–14. [PubMed: 3295684]
50. Carlsson J, Kujala U. Pyruvate oxidase activity dependent on thiamine pyrophosphate, flavin adenine dinucleotide and orthophosphate in *Streptococcus sanguis*. FEMS Microbiology Letters. 1984;25(1):53–56.
51. Zheng LY, Itzek A, Chen ZY, Kreth J. Oxygen dependent pyruvate oxidase expression and production in *Streptococcus sanguinis*. Int J Oral Sci. 2011;3(2):82–89. [PubMed: 21485312]
52. Carlsson J, Edlund MB, Lundmark SK. Characteristics of a hydrogen peroxide-forming pyruvate oxidase from *Streptococcus sanguis*. Oral Microbiol Immunol. 1987;2(1):15–20. [PubMed: 3473418]
53. Biasini M, Bienert S, Waterhouse A, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. 2014;42(Web Server issue):W252–258. [PubMed: 24782522]
54. Muller YA, Schumacher G, Rudolph R, Schulz GE. The refined structures of a stabilized mutant and of wild-type pyruvate oxidase from *Lactobacillus plantarum*. J Mol Biol. 1994;237(3):315–335. [PubMed: 8145244]
55. Juan EC, Hoque MM, Hossain MT, et al. The structures of pyruvate oxidase from *Aerococcus viridans* with cofactors and with a reaction intermediate reveal the flexibility of the active-site tunnel for catalysis. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2007;63(Pt 11):900–907.
56. Meyer D, Neumann P, Koers E, et al. Unexpected tautomeric equilibria of the carbanion-enamine intermediate in pyruvate oxidase highlight unrecognized chemical versatility of thiamin. Proc Natl Acad Sci U S A. 2012;109(27):10867–10872. [PubMed: 22730460]
57. Wille G, Meyer D, Steinmetz A, Hinze E, Golbik R, Tittmann K. The catalytic cycle of a thiamin diphosphate enzyme examined by cryocrystallography. Nat Chem Biol. 2006;2(6):324–328. [PubMed: 16680160]
58. Tittmann K, Golbik R, Ghisla S, Hubner G. Mechanism of elementary catalytic steps of pyruvate oxidase from *Lactobacillus plantarum*. Biochemistry. 2000;39(35):10747–10754. [PubMed: 10978159]

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59. Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. Database (Oxford). 2010;2010:baq013. [PubMed: 20624719]
60. Grifantini R, Toukoki C, Colaprico A, Gryllos I. Peroxide stimulon and role of PerR in group A Streptococcus. J Bacteriol. 2011;193(23):6539–6551. [PubMed: 21949080]
61. Zhu L, Xu Y, Ferretti JJ, Kreth J. Probing oral microbial functionality--expression of *spxB* in plaque samples. PLoS One. 2014;9(1):e86685. [PubMed: 24489768]
62. Belstrom D, Constancias F, Liu Y, et al. Metagenomic and metatranscriptomic analysis of saliva reveals disease-associated microbiota in patients with periodontitis and dental caries. NPJ Biofilms Microbiomes. 2017;3:23. [PubMed: 28979798]
63. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. Nat Rev Microbiol. 2008;6(3): 199–210. [PubMed: 18264116]
64. Lockridge O, Massey V, Sullivan PA. Mechanism of action of the flavoenzyme lactate oxidase. J Biol Chem. 1972;247(24):8097–8106. [PubMed: 4640938]
65. Maeda-Yorita K, Aki K, Sagai H, Misaki H, Massey V. L-lactate oxidase and L-lactate monooxygenase: mechanistic variations on a common structural theme. Biochimie. 1995;77(7–8): 631–642. [PubMed: 8589073]
66. Bao X, de Soet JJ, Tong H, et al. *Streptococcus oligofermentans* Inhibits *Streptococcus mutans* in Biofilms at Both Neutral pH and Cariogenic Conditions. PLoS One. 2015;10(6):e0130962. [PubMed: 26114758]
67. Liu L, Tong H, Dong X. Function of the pyruvate oxidase-lactate oxidase cascade in interspecies competition between *Streptococcus oligofermentans* and *Streptococcus mutans*. Appl Environ Microbiol. 2012;78(7):2120–2127. [PubMed: 22287002]
68. Tong H, Chen W, Merritt J, Qi F, Shi W, Dong X. *Streptococcus oligofermentans* inhibits *Streptococcus mutans* through conversion of lactic acid into inhibitory $H_2O_2$: a possible counteroffensive strategy for interspecies competition. Mol Microbiol. 2007;63(3):872–880. [PubMed: 17302806]
69. Jensen A, Scholz CF, Kilian M. Re-evaluation of the taxonomy of the Mitis group of the genus Streptococcus based on whole genome phylogenetic analyses, and proposed reclassification of *Streptococcus dentisani* as *Streptococcus oralis* subsp. *dentisani* comb. nov., *Streptococcus tigurinus* as *Streptococcus oralis* subsp. *tigurinus* comb. nov., and *Streptococcus oligofermentans* as a later synonym of *Streptococcus cristatus*. Int J Syst Evol Microbiol. 2016;66(11):4803–4820. [PubMed: 27534397]
70. Tong H, Gao X, Dong X. *Streptococcus oligofermentans* sp. nov., a novel oral isolate from caries-free humans. Int J Syst Evol Microbiol. 2003;53(Pt 4):1101–1104. [PubMed: 12656152]
71. Seki M, Iida K, Saito M, Nakayama H, Yoshida S. Hydrogen peroxide production in *Streptococcus pyogenes*: involvement of lactate oxidase and coupling with aerobic utilization of lactate. J Bacteriol. 2004;186(7):2046–2051. [PubMed: 15028688]
72. Taniai H, Iida K, Seki M, et al. Concerted action of lactate oxidase and pyruvate oxidase in aerobic growth of Streptococcus pneumoniae: role of lactate as an energy source. J Bacteriol. 2008;190(10):3572–3579. [PubMed: 18344365]
73. Molla G, Nardini M, Motta P, D'Arrigo P, Panzeri W, Pollegioni L. Aminoacetone oxidase from *Streptococcus oligofermentans* belongs to a new three-domain family of bacterial flavoproteins. Biochem J. 2014;464(3):387–399. [PubMed: 25269103]
74. Tong H, Chen W, Shi W, Qi F, Dong X. SO-LAAO, a novel L-amino acid oxidase that enables *Streptococcus oligofermentans* to outcompete *Streptococcus mutans* by generating $H_2O_2$ from peptone. J Bacteriol. 2008;190(13):4716–4721. [PubMed: 18469105]
75. Zhou P, Liu L, Tong H, Dong X. Role of operon *aaoSo-mutT* in antioxidant defense in *Streptococcus oligofermentans*. PLoS One. 2012;7(5):e38133. [PubMed: 22666463]
76. Boggs JM, South AH, Hughes AL. Phylogenetic analysis supports horizontal gene transfer of L-amino acid oxidase gene in *Streptococcus oligofermentans*. Infect Genet Evol. 2012;12(5):1005–1009. [PubMed: 22414918]

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77. Nascimento MM, Browngardt C, Xiaohui X, Klepac-Ceraj V, Paster BJ, Burne RA. The effect of arginine on oral biofilm communities. Mol Oral Microbiol. 2014;29(1):45–54. [PubMed: 24289808]

78. Ge X, Yu Y, Zhang M, et al. Involvement of NADH Oxidase in Competition and Endocarditis Virulence in *Streptococcus sanguinis*. Infect Immun. 2016;84(5):1470–1477. [PubMed: 26930704]

79. Higuchi M, Yamamoto Y, Poole LB, et al. Functions of two types of NADH oxidases in energy metabolism and oxidative stress of *Streptococcus mutans*. J Bacteriol. 1999;181(19):5940–5947. [PubMed: 10498705]

80. Gualdi L, Hayre JK, Gerlini A, et al. Regulation of neuraminidase expression in *Streptococcus pneumoniae*. BMC Microbiol. 2012;12:200. [PubMed: 22963456]

81. Ashby MT. Inorganic chemistry of defensive peroxidases in the human oral cavity. J Dent Res. 2008;87(10):900–914. [PubMed: 18809743]

82. Ashby MT, Kreth J, Soundarajan M, Sivuilu LS. Influence of a model human defensive peroxidase system on oral streptococcal antagonism. Microbiology. 2009;155(Pt 11):3691–3700. [PubMed: 19684069]

83. Welk A, Rudolph P, Kreth J, Schwahn C, Kramer A, Below H. Microbicidal efficacy of thiocyanate hydrogen peroxide after adding lactoperoxidase under saliva loading in the quantitative suspension test. Arch Oral Biol. 2011;56(12):1576–1582. [PubMed: 21621190]

84. Britse A, Lagerlof F. The diluting effect of saliva on the sucrose concentration in different parts of the human mouth after a mouth-rinse with sucrose. Arch Oral Biol. 1987;32(10):755–756. [PubMed: 3482155]

85. Pruitt KM, Tenovuo J, Mansson-Rahemtulla B, Harrington P, Baldone DC. Is thiocyanate peroxidation at equilibrium in vivo? Biochim Biophys Acta. 1986;870(3):385–391. [PubMed: 3697358]

86. Liu X, Ramsey MM, Chen X, Koley D, Whiteley M, Bard AJ. Real-time mapping of a hydrogen peroxide concentration profile across a polymicrobial bacterial biofilm using scanning electrochemical microscopy. Proc Natl Acad Sci U S A. 2011;108(7):2668–2673. [PubMed: 21282623]

87. Tsou CC, Chiang-Ni C, Lin YS, et al. An iron-binding protein, Dpr, decreases hydrogen peroxide stress and protects *Streptococcus pyogenes* against multiple stresses. Infect Immun. 2008;76(9): 4038–4045. [PubMed: 18541662]

88. Yamamoto Y, Higuchi M, Poole LB, Kamio Y. Role of the *dpr* product in oxygen tolerance in *Streptococcus mutans*. J Bacteriol. 2000;182(13):3740–3747. [PubMed: 10850989]

89. Fujishima K, Kawada-Matsuo M, Oogai Y, Tokuda M, Torii M, Komatsuzawa H. dpr and sod in *Streptococcus mutans* are involved in coexistence with *S. sanguinis*, and PerR is associated with resistance to $H_2O_2$. Appl Environ Microbiol. 2013;79(5):1436–1443. [PubMed: 23263955]

90. Pericone CD, Park S, Imlay JA, Weiser JN. Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. J Bacteriol. 2003;185(23):6815–6825. [PubMed: 14617646]

91. Lisher JP, Tsui HT, Ramos-Montanez S, et al. Biological and Chemical Adaptation to Endogenous Hydrogen Peroxide Production in *Streptococcus pneumoniae* D39. mSphere. 2017;2(1).

92. Liu Y, Palmer SR, Chang H, Combs AN, Burne RA, Koo H. Differential oxidative stress tolerance of *Streptococcus mutans* isolates affects competition in an ecological mixed-species biofilm model. Environ Microbiol Rep. 2018;10(1):12–22. [PubMed: 29124888]

93. Yesilkaya H, Andisi VF, Andrew PW, Bijlsma JJ. *Streptococcus pneumoniae* and reactive oxygen species: an unusual approach to living with radicals. Trends Microbiol. 2013;21(4):187–195. [PubMed: 23415028]

94. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol. 2012;27(6):409–419. [PubMed: 23134607]

95. Marsh PD. In Sickness and in Health-What Does the Oral Microbiome Mean to Us? An Ecological Perspective. Adv Dent Res. 2018;29(1):60–65. [PubMed: 29355410]

96. Zheng X, He J, Wang L, et al. Ecological Effect of Arginine on Oral Microbiota. Sci Rep. 2017;7(1):7206. [PubMed: 28775282]

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97. Herrero ER, Slomka V, Bernaerts K, et al. Antimicrobial effects of commensal oral species are regulated by environmental factors. J Dent. 2016;47:23–33. [PubMed: 26875613]
98. Joshi VS, Sheet PS, Cullin N, Kreth J, Koley D. Real-Time Metabolic Interactions between Two Bacterial Species Using a Carbon-Based pH Microsensor as a Scanning Electrochemical Microscopy Probe. Anal Chem. 2017;89(20):11044–11052. [PubMed: 28920437]
99. Cheng X, Redanz S, Cullin N, et al. Plasticity of the Pyruvate Node Modulates Hydrogen Peroxide Production and Acid Tolerance in Multiple Oral Streptococci. Appl Environ Microbiol. 2018;84(2).
100. Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. Trends Microbiol. 2005;13(1):34–40. [PubMed: 15639630]
101. Liu Y, Chu L, Wu F, et al. Influence of pH on inhibition of *Streptococcus mutans* by *Streptococcus oligofermentans*. Eur J Oral Sci. 2014;122(1):57–61. [PubMed: 24267570]
102. Bao X, Yang J, de Soet JJ, et al. Factors Influencing the Competition between S*treptococcus oligofermentans* and *Streptococcus mutans* in Dual-Species Biofilms. Caries Res. 2017;51(5): 507–514. [PubMed: 28965113]
103. Kreth J, Zhang Y, Herzberg MC. Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*. J Bacteriol. 2008;190(13):4632–4640. [PubMed: 18441055]
104. Giacaman RA, Torres S, Gomez Y, Munoz-Sandoval C, Kreth J. Correlation of *Streptococcus mutans* and *Streptococcus sanguinis* colonization and *ex vivo* hydrogen peroxide production in carious lesion-free and high caries adults. Arch Oral Biol. 2015;60(1):154–159. [PubMed: 25455129]
105. Zheng L, Chen Z, Itzek A, Ashby M, Kreth J. Catabolite control protein A controls hydrogen peroxide production and cell death in *Streptococcus sanguinis*. J Bacteriol. 2011;193(2):516–526. [PubMed: 21036992]
106. He J, Hwang G, Liu Y, et al. l-Arginine Modifies the Exopolysaccharide Matrix and Thwarts *Streptococcus mutans* Outgrowth within Mixed-Species Oral Biofilms. J Bacteriol. 2016;198(19):2651–2661. [PubMed: 27161116]
107. Kreth J, Zhu L, Merritt J, Shi W, Qi F. Role of sucrose in the fitness of *Streptococcus mutans*. Oral Microbiol Immunol. 2008;23(3):213–219. [PubMed: 18402607]
108. Herrero ER, Boon N, Bernaerts K, et al. Clinical concentrations of peroxidases cause dysbiosis in in vitro oral biofilms. J Periodontal Res. 2018.
109. Sato Y, Yamagishi J, Yamashita R, et al. Inter-Individual Differences in the Oral Bacteriome Are Greater than Intra-Day Fluctuations in Individuals. PLoS One. 2015;10(6):e0131607. [PubMed: 26121551]
110. Palmer SR, Miller JH, Abranches J, et al. Phenotypic heterogeneity of genomically-diverse isolates of *Streptococcus mutans*. PLoS One. 2013;8(4):e61358. [PubMed: 23613838]
111. Huang X, Schulte RM, Burne RA, Nascimento MM. Characterization of the arginolytic microflora provides insights into pH homeostasis in human oral biofilms. Caries Res. 2015;49(2): 165–176. [PubMed: 25634570]
112. Huang X, Browngardt CM, Jiang M, Ahn SJ, Burne RA, Nascimento MM. Diversity in Antagonistic Interactions between Commensal Oral Streptococci and *Streptococcus mutans*. Caries Res. 2018;52(1–2):88–101. [PubMed: 29258070]
113. Curran TM, Lieou J, Marquis RE. Arginine deiminase system and acid adaptation of oral streptococci. Appl Environ Microbiol. 1995;61(12):4494–4496. [PubMed: 8534118]
114. Marquis RE, Bender GR, Murray DR, Wong A. Arginine deiminase system and bacterial adaptation to acid environments. Appl Environ Microbiol. 1987;53(1):198–200. [PubMed: 3103530]
115. Stacy A, Everett J, Jorth P, Trivedi U, Rumbaugh KP, Whiteley M. Bacterial fight-and-flight responses enhance virulence in a polymicrobial infection. Proc Natl Acad Sci U S A. 2014;111(21):7819–7824. [PubMed: 24825893]
116. Ramsey MM, Rumbaugh KP, Whiteley M. Metabolite cross-feeding enhances virulence in a model polymicrobial infection. PLoS pathogens. 2011;7(3):e1002012. [PubMed: 21483753]

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117. Ramsey MM, Whiteley M. Polymicrobial interactions stimulate resistance to host innate immunity through metabolite perception. Proc Natl Acad Sci U S A. 2009;106(5):1578–1583. [PubMed: 19164580]
118. Duan D, Scoffield JA, Zhou X, Wu H. Fine-tuned production of hydrogen peroxide promotes biofilm formation of *Streptococcus parasanguinis* by a pathogenic cohabitant *Aggregatibacter actinomycetemcomitans*. Environ Microbiol. 2016;18(11):4023–4036. [PubMed: 27348605]
119. Jakubovics NS, Gill SR, Vickerman MM, Kolenbrander PE. Role of hydrogen peroxide in competition and cooperation between *Streptococcus gordonii* and *Actinomyces naeslundii*. FEMS microbiology ecology. 2008;66(3):637–644. [PubMed: 18785881]
120. Edlund A, Garg N, Mohimani H, et al. Metabolic Fingerprints from the Human Oral Microbiome Reveal a Vast Knowledge Gap of Secreted Small Peptidic Molecules. mSystems. 2017;2(4).
121. Deutscher J The mechanisms of carbon catabolite repression in bacteria. Curr Opin Microbiol. 2008;11(2):87–93. [PubMed: 18359269]
122. Zheng L, Itzek A, Chen Z, Kreth J. Environmental influences on competitive hydrogen peroxide production in *Streptococcus gordonii*. Appl Environ Microbiol. 2011;77(13):4318–4328. [PubMed: 21571883]
123. Redanz S, Masilamani R, Cullin N, Giacaman RA, Merritt J, Kreth J. Distinct Regulatory Role of Carbon Catabolite Protein A (CcpA) in Oral Streptococcal *spxB* Expression. J Bacteriol. 2018;200(8).
124. Ramos-Montanez S, Tsui HC, Wayne KJ, et al. 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. 2008;67(4):729–746. [PubMed: 18179423]
125. Chen L, Ge X, Dou Y, Wang X, Patel JR, Xu P. Identification of hydrogen peroxide production-related genes in *Streptococcus sanguinis* and their functional relationship with pyruvate oxidase. Microbiology. 2011;157(Pt 1):13–20. [PubMed: 21718486]
126. Dillon SC, Bateman A. The Hotdog fold: wrapping up a superfamily of thioesterases and dehydratases. BMC Bioinformatics. 2004;5:109. [PubMed: 15307895]
127. Chen L, Ge X, Wang X, Patel JR, Xu P. SpxA1 involved in hydrogen peroxide production, stress tolerance and endocarditis virulence in *Streptococcus sanguinis*. PLoS One. 2012;7(6):e40034. [PubMed: 22768210]
128. Li T, Xu M, Zheng L. Is SpxA2 involved in hydrogen peroxide production and competence development in *Streptococcus sanguinis*? J Med Microbiol. 2017;66(7):981–989. [PubMed: 28693663]
129. Nakano S, Erwin KN, Ralle M, Zuber P. Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. *Mol Microbiol*. 2005;55(2):498–510. [PubMed: 15659166]
130. Mattos-Graner RO, Duncan MJ. Two-component signal transduction systems in oral bacteria. J Oral Microbiol. 2017;9(1):1400858. [PubMed: 29209465]
131. Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. Annu Rev Biochem. 2000;69:183–215. [PubMed: 10966457]
132. Camargo TM, Stipp RN, Alves LA, Harth-Chu EN, Hofling JF, Mattos-Graner RO. Novel Two-Component System of *Streptococcus sanguinis* Affecting Functions Associated with Viability in Saliva and Biofilm Formation. Infect Immun. 2018;86(4).
133. Moraes JJ, Stipp RN, Harth-Chu EN, Camargo TM, Hofling JF, Mattos-Graner RO. Two-component system VicRK regulates functions associated with establishment of *Streptococcus sanguinis* in biofilms. Infect Immun. 2014;82(12):4941–4951. [PubMed: 25183732]
134. Itzek A, Zheng L, Chen Z, Merritt J, Kreth J. Hydrogen peroxide-dependent DNA release and transfer of antibiotic resistance genes in *Streptococcus gordonii*. J Bacteriol. 2011;193(24):6912–6922. [PubMed: 21984796]
135. De Boeck I, Wittouck S, Wuyts S, et al. Comparing the Healthy Nose and Nasopharynx Microbiota Reveals Continuity As Well As Niche-Specificity. Front Microbiol. 2017;8:2372. [PubMed: 29238339]
136. Wilson MT, Hamilos DL. The nasal and sinus microbiome in health and disease. Curr Allergy Asthma Rep. 2014;14(12):485. [PubMed: 25342392]

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137. Zemanick ET, Wagner BD, Robertson CE, et al. Airway microbiota across age and disease spectrum in cystic fibrosis. Eur Respir J. 2017;50(5).
138. Pragman AA, Lyu T, Baller JA, et al. The lung tissue microbiota of mild and moderate chronic obstructive pulmonary disease. Microbiome. 2018;6(1):7. [PubMed: 29316977]
139. Filkins LM, Hampton TH, Gifford AH, et al. Prevalence of streptococci and increased polymicrobial diversity associated with cystic fibrosis patient stability. J Bacteriol. 2012;194(17): 4709–4717. [PubMed: 22753064]
140. Linnane SJ, Keatings VM, Costello CM, et al. Total sputum nitrate plus nitrite is raised during acute pulmonary infection in cystic fibrosis. Am J Respir Crit Care Med. 1998;158(1):207–212. [PubMed: 9655731]
141. Sibley CD, Parkins MD, Rabin HR, Duan K, Norgaard JC, Surette MG. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. Proc Natl Acad Sci U S A. 2008;105(39):15070–15075. [PubMed: 18812504]
142. Whelan FJ, Heirali AA, Rossi L, Rabin HR, Parkins MD, Surette MG. Longitudinal sampling of the lung microbiota in individuals with cystic fibrosis. PLoS One. 2017;12(3):e0172811. [PubMed: 28253277]
143. Nascimento MM. Oral microbiota transplant: a potential new therapy for oral diseases. J Calif Dent Assoc. 2017;45(10):565–568. [PubMed: 29497269]
144. Tellez M, Gomez J, Pretty I, Ellwood R, Ismail AI. Evidence on existing caries risk assessment systems: are they predictive of future caries? Community Dent Oral Epidemiol. 2013;41(1):67–78. [PubMed: 22978796]
145. American Dental Association. 2017; https://www.ada.org/en/member-center/oral-health-topics/caries-risk-assessment-and-management.
146. Tittmann K, Wille G, Golbik R, Weidner A, Ghisla S, Hubner G. 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. 2005;44(40):13291–13303. [PubMed: 16201755]

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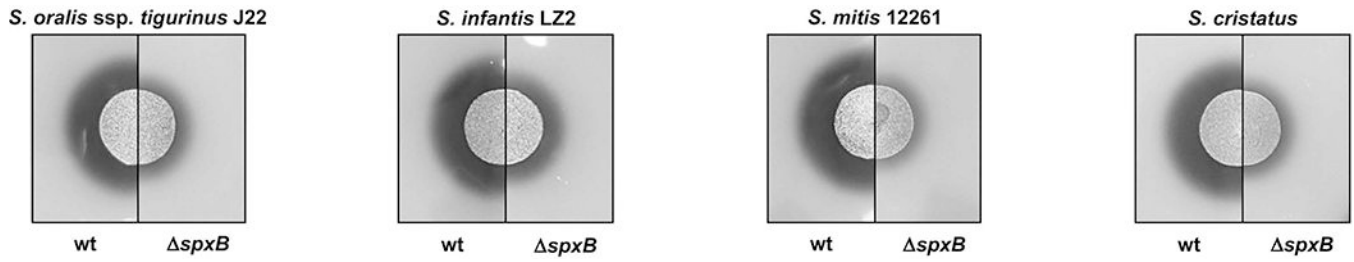


**Fig. 1:**
Comparison of $H_2O_2$ production by *S. oralis* ssp. *tigurinus* J22, *S. infantis* LZ2, *S. mitis* 12261, and *S. cristatus* wild type strains and their respective isogenic *spxB* mutants. $H_2O_2$ production from all strains was assessed on $H_2O_2$ indicator plates after overnight incubation at 37°C aerobically. $H_2O_2$ production leads to the precipitation of Prussian blue during aerobic growth, which forms a blue halo around the producer [47,48].

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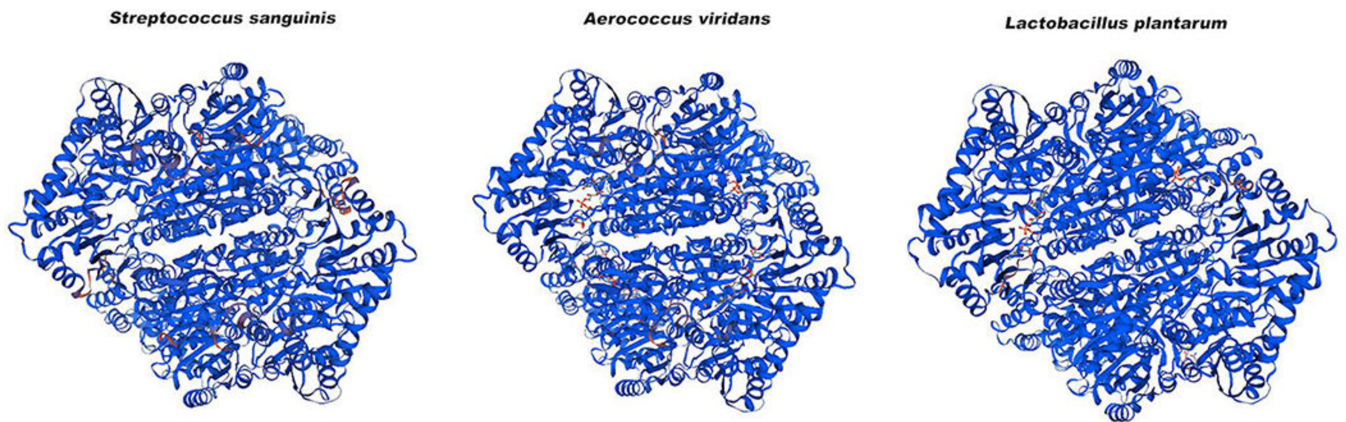


**Fig. 2:**

Protein structure homology modeling of SpxB from *S. sanguinis* using the automated protein structure homology-modelling server SWISS-MODEL. The reference proteins used were from *Aerococcus viridans* and *Lactobacillus plantarum*. The quaternary structure quality estimate (QSQE) based upon the modeling with *Aerococcus viridans* SpxB is 0.78. This protein has 69% sequence identity to the *S. sanguinis* SpxB. The QSQE score ranges from 0 to 1 with higher numbers indicating higher reliability.

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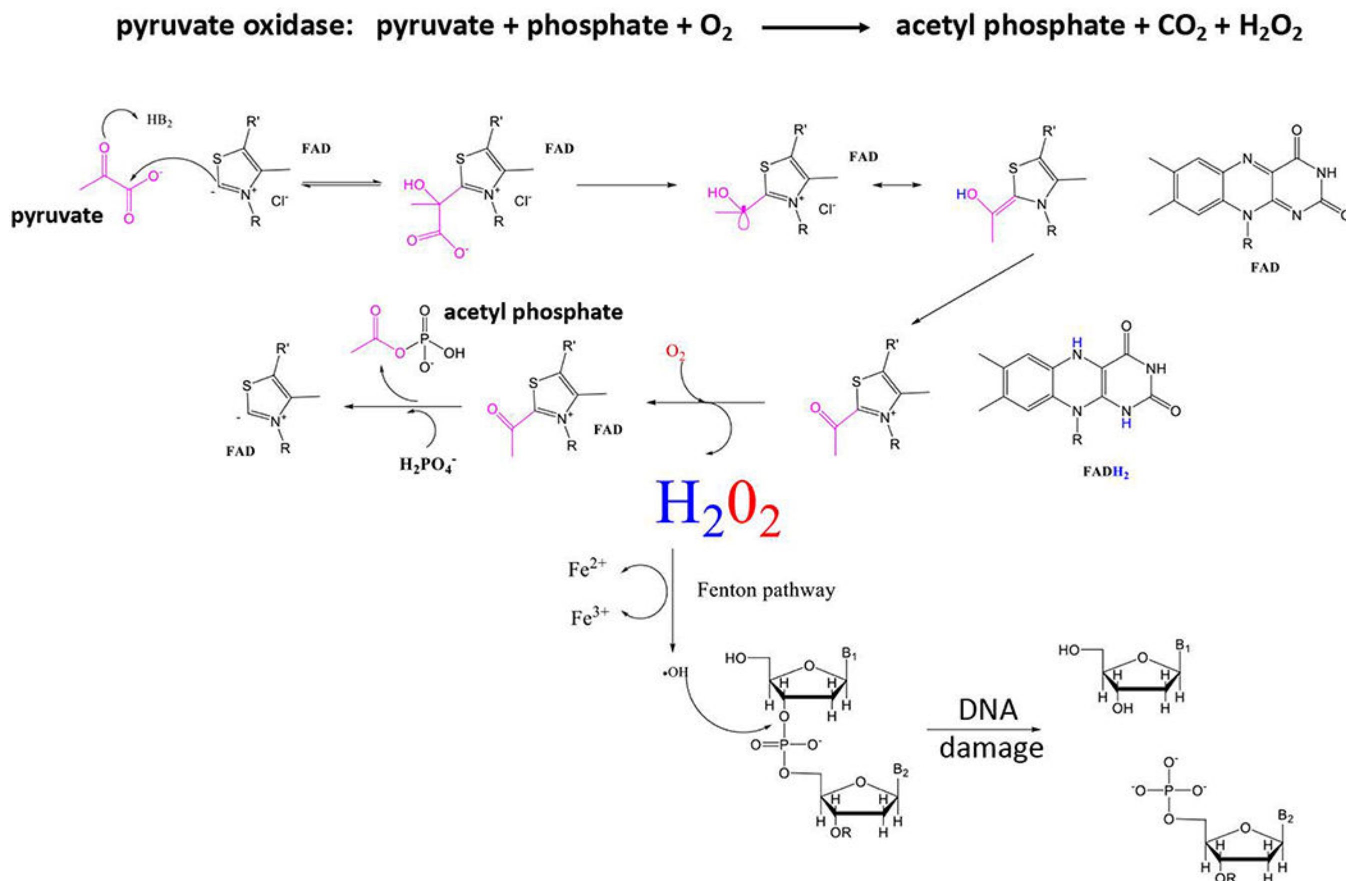


**Fig. 3:**

Generalized overview of pyruvate oxidase mechanism, Fenton reaction, and DNA damage. The pyruvate oxidase catalyzes the oxidative decarboxylation of pyruvate and requires the cofactors TPP and FAD. During the multistep reaction, two reducing equivalents are transferred from TPP to FAD, yielding 2-acetyl-thiamine pyrophosphate and $FADH_2$. $FADH_2$ is re-oxidized by $O_2$ which generates $H_2O_2$ and FAD. Ac-TPP is then cleaved phosphorolytically to yield acetyl phosphate and TPP [146]. $Fe^{2+}$ is able to non-specifically bind to DNA and react with the generated $H_2O_2$ in the Fenton reaction to cause DNA damage in the form of DNA strand breakage (as shown here) or base alterations in the DNA.

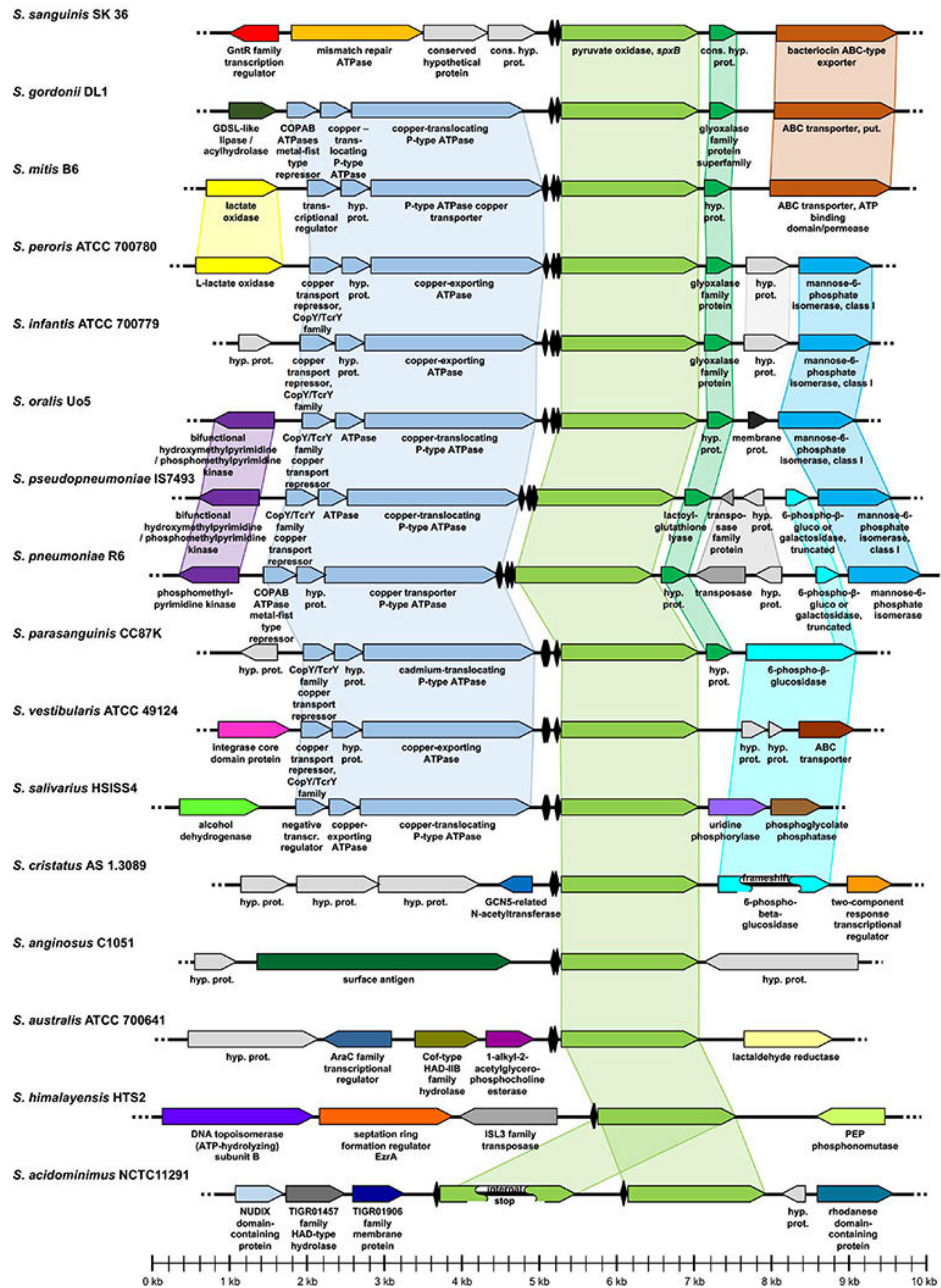


**Fig. 4:**

Chromosomal context of *spxB* in multiple oral streptococci. The sequences of the given species and strains as well as the displayed gene names and functions were obtained from NCBI. Species were sorted with respect to their analogy in gene assortment. Related or functionally connected genes are shown in matching colors. In case the gene function was not already given in the NCBI database ("conserved hypothetical protein" or "hypothetical protein"), related or functionally connected genes were identified by BLAST sequence comparison. Figures were manually generated using Microsoft Powerpoint. Catabolite



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responsive elements (*cre* boxes) are indicated (♦) and were predicted as previously described [121]. The respective *cre* boxes of *S. sanguinis* and *S. gordonii* were experimental validated [123].

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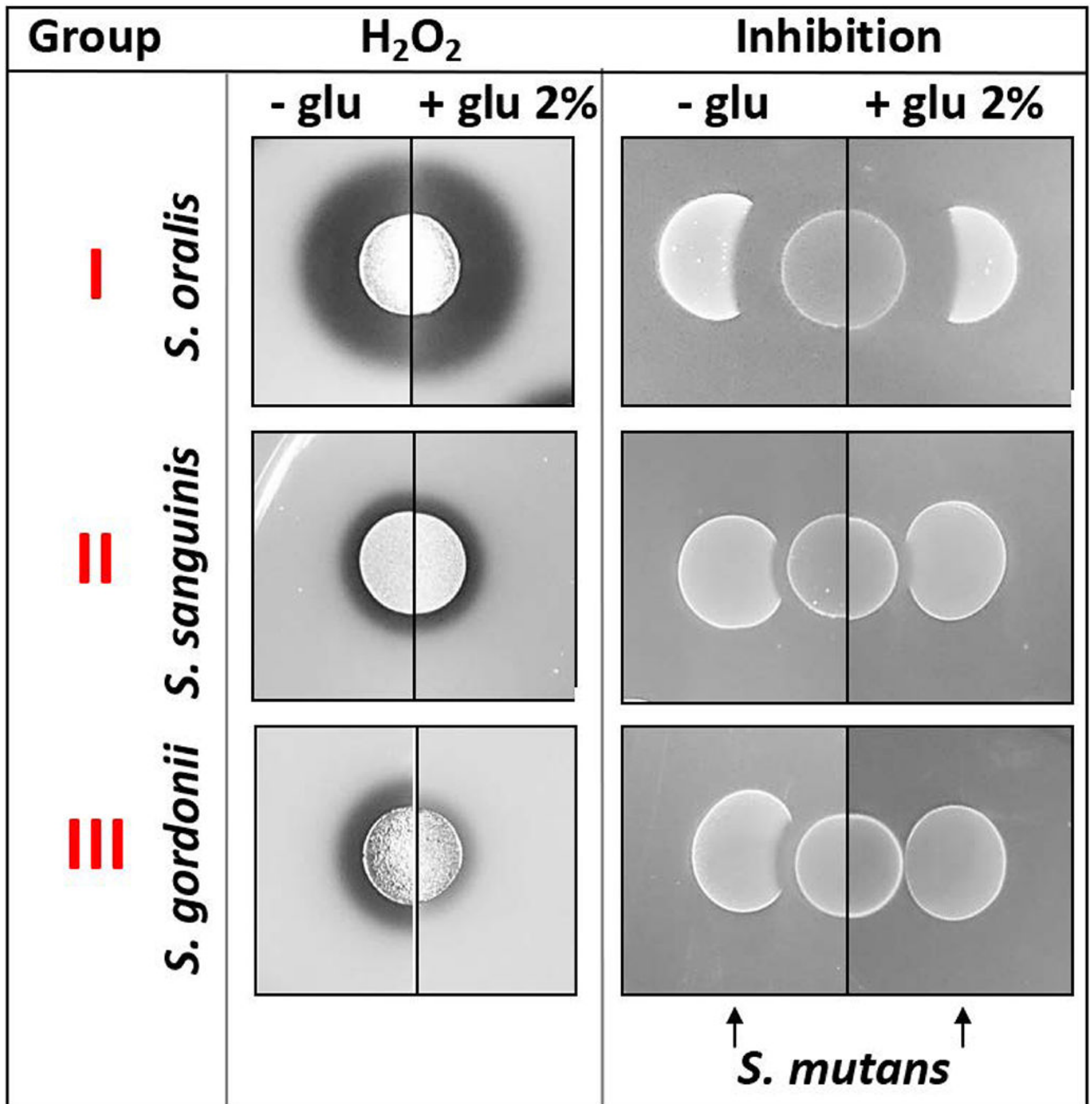


**Fig. 5:**

Representative species for the three identified $H_2O_2$-production groups and their ability to inhibit *S. mutans* ± glucose.

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**Table 1:**

Comparison of SpxB among different species

| Species | Strain | number of amino acids | % identity | % positives |
|---|---|---|---|---|
| *Streptococcus sanguinis* | SK36 | 591 | 100 | 100 |
| *Streptococcus gordonii* | CH1 | 591 | 98 | 99 |
| *Streptococcus mitis* | B6 | 591 | 98 | 98 |
| *Streptococcus peroris* | ATCC 700780 | 591 | 98 | 98 |
| *Streptococcus infantis* | ATCC 700779 | 591 | 98 | 98 |
| *Streptococcus oralis* | Uo5 | 591 | 98 | 98 |
| *Streptococcus pseudopneumoniae* | IS7493 | 591 | 98 | 98 |
| *Streptococcus pneumoniae* | R6 | 591 | 98 | 98 |
| *Streptococcus parasanguinis* | CC87K | 591 | 98 | 98 |
| *Streptococcus vestibularis* | ATCC 49124 | 591 | 98 | 99 |
| *Streptococcus salivarius* | HSISS4 | 591 | 97 | 98 |
| *Streptococcus cristatus* | AS1.3089 | 591 | 98 | 99 |
| *Streptococcus anginosus* | C1051 | 591 | 98 | 99 |
| *Streptococcus australis* | ATCC 700641 | 591 | 96 | 98 |
| *Streptococcus himalayensis* | HTS2 | 593 | 89 | 94 |
| *Streptococcus acidominimus* | NCTC11291 | 593 | 85 | 92 |
| | | 583 (pseudogene) * | 70 | 83 |
| *Aerococcus viridans* | CCUG4311 | 592 | 69 | 82 |
| *Lactobacillus plantarum* | ZJ316 | 603 (PoxB) ** | 49 | 66 |

% identity: identity on the amino acid level to the query sequence of *spxB* from *S. sanguinis*.

% positives: amino acids with a conservative exchange were physiochemical properties are preserved when compared to the query sequence of *spxB* from *S. sanguinis*.

*The second pyruvate oxidase gene in *S. acidominimus* possesses an internal stop codon.

** L. plantarum encodes several pyruvate oxidases, shown here is the relevant pyruvate oxidase used for molecular modeling.