

Review Article

The Role of Hydrogen Peroxide in Environmental Adaptation of Oral Microbial Communities

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Oral streptococci are able to produce growth-inhibiting amounts of hydrogen peroxide (H_2O_2) as byproduct of aerobic metabolism. Several recent studies showed that the produced H_2O_2 is not a simple byproduct of metabolism but functions in several aspects of oral bacterial biofilm ecology. First, the release of DNA from cells is closely associated to the production of H_2O_2 in *Streptococcus sanguinis* and *Streptococcus gordonii*. Extracellular DNA is crucial for biofilm development and stabilization and can also serve as source for horizontal gene transfer between oral streptococci. Second, due to the growth inhibiting nature of H_2O_2 , H_2O_2 compatible species associate with the producers. H_2O_2 production therefore might help in structuring the initial biofilm development. On the other hand, the oral environment harbors salivary peroxidases that are potent in H_2O_2 scavenging. Therefore, the effects of biofilm intrinsic H_2O_2 production might be locally confined. However, taking into account that 80% of initial oral biofilm constituents are streptococci, the influence of H_2O_2 on biofilm development and environmental adaptation might be under appreciated in current research.

1. The Oral Biofilm: A Highly Adapted Microbial Consortium

Oral bacteria residing in the supragingival biofilm have a remarkable degree of structural organization [1, 2]. This organization is the result of a successive buildup and continuous integration of new species into the developing biofilm. Starting with a cleaned or recently emerged tooth, initial oral streptococcal colonizers adhere via specific surface proteins to salivary proteins covering the tooth surface [1]. Oral streptococci by themselves provide surface proteins for the attachment and integration of other oral bacteria [3]. Initial binding of oral streptococci therefore sets the stage for the development of a mature biofilm community. Beside the physical contact, biofilm development involves several layers of interactions among the biofilm community members. This includes efficient nutrient usage by metabolic cooperativity, communication by small signal molecules, and genetic exchange [4, 5].

The crucial steps in initial attachment and biofilm development have been well documented in the past years.

Using specific removable appliances harboring dental enamel chips, Diaz et al. were able to trace the spatiotemporal pattern of oral biofilm formation in the human host [6]. Oral streptococci were the predominant species in the initial colonization stage after 4 and 8 hours. Up to 80% of the detected initial colonizers belonged to the genus *Streptococcus* with some species discussed as constant members presenting a core group of initial biofilm formation [6, 7]. The biofilm developmental process starts with small microcolonies consisting mainly of streptococci and few non-streptococci [6]. This developmental process has implications on other species efforts to join the biofilm community or attach in close proximity. Oral streptococci are known for their production and secretion of antimicrobial substances, one of them is hydrogen peroxide (H_2O_2) [8, 9]. The production of antimicrobial substances like H_2O_2 could therefore be regarded as an important protection mechanism of the initial colonizers of the resident biofilm community against invading and competing species. More importantly, it might also be a mechanism to shape the colonization process toward a specific

species composition. Only species coevolved with oral streptococci and therefore adapted to withstand H_2O_2 can integrate or colonize in close proximity to the initial colonizers and extend the developing biofilm community.

After initial attachment of streptococci, the biofilm builds up and several other species join the biofilm community [1, 6]. This also leads to an increase in biofilm thickness and subsequent anaerobic conditions [10–12], which in turn can attract anaerobic bacteria. H_2O_2 production inside the oral biofilm most likely declines under these conditions due to insufficient oxygen availability. The role of H_2O_2 becomes less important and other factors might influence biofilm maturation. From the perspective of the oral streptococci, H_2O_2 fulfills its purpose exactly when it is needed, during initial biofilm formation, when oxygen for H_2O_2 production is readily available [13]. The ecological niche of oral streptococci is freely accessible for competing species during initial biofilm formation, and this competition is counteracted either by the direct bactericidal effect of H_2O_2 or the preferred integration of compatible species into the growing community. Once the streptococci are established and have built up an association of compatible neighboring biofilm inhabitants, they already occupy their favorite ecological niche and the antimicrobial activity of H_2O_2 is no longer required.

The multispecies oral biofilm community provides a protective function to prevent invasion of foreign (pathogenic) bacteria [14]. Unfortunately, some of the bacterial species commonly found in the human oral biofilm consortium have the ability to cause diseases like tooth decay (caries). Under healthy conditions, these species would not cause any harm. Disease development is the result of a disturbed biofilm homeostasis leading to an overgrowth of conditional pathogenic bacteria and a general reduction of the species composition normally found in healthy supragingival plaque [15, 16]. Interestingly, clinical evidence emerges that some of the H_2O_2 producing oral streptococci seem to be reduced in their abundance in subjects having oral diseases like caries or periodontal disease [17–19].

The available *in vivo* and *in vitro* studies point to H_2O_2 as an important metabolic product generated in the early cycles of oral biofilm formation. In the following sections, specific examples important in biofilm development and in the adaptation to the oral biofilm environment are discussed.

2. Sources of H_2O_2

H_2O_2 in the oral cavity originates from bacteria and from the host [20]. At the present time, it is not clear how both sources influence each other and if at all the production of H_2O_2 by the host directly impacts the biofilm and *vice versa*. H_2O_2 has not been detected directly in saliva [21, 22]. The transient concentration has been calculated to be around $10\ \mu\text{M}$ based on known concentrations of thiocyanate and hypothiocyanite in saliva [22]. One potential reason is the presence of a salivary scavenging system for H_2O_2 to protect the host from H_2O_2 toxicity [23, 24]. Two host-derived peroxidases are present in the human oral cavity, salivary peroxidase, and myeloperoxidase [23]. Both are able to

use H_2O_2 as an oxidant and thiocyanate as a substrate to produce hypothiocyanite [23]. Interestingly, hypothiocyanite is not only a detoxification product, but also a general antimicrobial substance, and the combination of H_2O_2 , hypothiocyanite, and salivary peroxidase seems to be most potent in inhibiting bacterial metabolism [25, 26]. Salivary peroxidase, a noninducible component of saliva originates in the parotid and submandibular glands [27]. Myeloperoxidase is an offensive component of polymorphonuclear leukocytes [28], which are present in saliva with elevated levels during inflammatory diseases like periodontal disease [29].

2.1. Sources of H_2O_2 in the Oral Biofilm. Oral streptococci have long been known to produce H_2O_2 , mainly due to their ability to inhibit various other species in *in vitro* tests. Early reports already indicate that H_2O_2 production might be widely distributed among oral streptococci. Thompson and Shibuya tested 55 *alpha*-hemolytic oral streptococci and found that 48 were able to inhibit the growth of *Corynebacterium diphtheria* [30]. Tests with identified streptococcal species showed that *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus sanguinis*, and *Streptococcus sobrinus* all were able to produce significant amounts of H_2O_2 during growth *in vitro*, which can be detected in the supernatants of the growth medium [31]. These oral streptococci are commonly isolated and present in a relatively high abundance in the human oral biofilm [32]. Variations in H_2O_2 production among streptococci were shown to be growth medium and carbohydrate dependent [31], indicating environmental influences on regulatory mechanisms of H_2O_2 production.

The enzyme responsible for the production of H_2O_2 in *S. sanguinis* and *S. gordonii* was identified as pyruvate oxidase, encoded by gene *spxB* (also referred to as *pox*) [33–35]. The pyruvate oxidase is an oxidoreductase that catalyzes the conversion of pyruvate, inorganic phosphate (P_i), and molecular oxygen (O_2) to H_2O_2 , carbon dioxide (CO_2), and the high-energy phosphoryl group donor acetyl phosphate in an aerobic environment. Genetic inactivation of the respective open reading frames encoding for putative pyruvate oxidase orthologs in *S. sanguinis* and *S. gordonii* confirmed the pyruvate oxidase as the enzyme responsible for significant H_2O_2 production [35]. The production of growth inhibiting amounts of H_2O_2 is not exclusive to the pyruvate oxidase in oral streptococci. Detailed genetic inactivation studies in *Streptococcus oligofermentans* showed that at least two other enzymes in addition to the pyruvate oxidase are able of producing growth-inhibiting amounts of H_2O_2 [36, 37]. The lactate oxidase, gene *lctO* (also referred to as *lox*), catalyzes the formation of pyruvate and H_2O_2 from L-lactate and oxygen and an L-amino acid oxidase generates H_2O_2 from amino acids and peptones. Dual species biofilm antagonism assays with *S. oligofermentans* and *S. mutans* demonstrated that the H_2O_2 produced by LctO activity is still able to antagonize *S. mutans* in an *spxB* background. The role of the L-amino acid oxidase in interspecies competition is not clear since its H_2O_2 producing activity is low, and only visible in a *lctO/spxB* double knockout mutant [36, 38]. Nonetheless, the L-amino acid oxidase seems to be important as suggested

TABLE 1: Distribution and nucleotide identity of *spxB* and *lctO* among sequenced oral streptococcal isolates.

Species	Strain	<i>spxB</i> identity (%)	<i>lctO</i> identity (%)
<i>S. mitis</i>	B6	100	100
	NCTC 12261	97	95
	SK564	97	95
	SK321	97	94
	SK597	96	94
	F0392	96	93
	SK95	96	—
	ATCC 6249	96	90
<i>S. sanguinis</i>	SK36	94	—
	SK49	95	—
	AATCC 49296	96	91
<i>S. gordonii</i>	CH1	96	—
<i>S. oralis</i>	Uo5	96	91
	ATCC 35037	96	91
<i>S. parasanguinis</i>	SK236	95	—
<i>S. vestibularis</i>	FO396	95	—
	ATCC 49124	95	—
<i>S. peroris</i>	ATCC 700780	95	90
<i>S. cristatus</i>	ATCC 51100	—	87
<i>S. oligofermentans</i>	AS 1.3089	95	88

by a recent study, Boggs et al. showed that the L-amino acid oxidase gene *aao* from *S. oligofermentans* was probably acquired via horizontal gene transfer from a source closely related to *S. sanguinis* and *S. gordonii*, while evolutionary *S. oligofermentans* seems to be more closely related to *S. oralis*, *S. mitis*, and *S. pneumoniae* [39]. The authors speculate that the *aao* gene is important for *S. oligofermentans* to occupy a specific ecological niche in the oral biofilm [39]. The regulation of *aao* gene expression is not known, and the gene might be induced under specific conditions *in vivo*.

Using the available genome sequence data from the Human Oral Microbiome Database (<http://www.homd.org/>), the distribution of *spxB* and *lctO* among oral streptococci was determined using *spxB* and *lctO* from *S. mitis* B6 as a template. As shown in Table 1, several important oral streptococci encode open reading frames with a high homology to *spxB* and *lctO*. All species listed in Table 1 are commonly isolated from subjects suggesting a wide distribution of *spxB* and *lctO* in oral streptococci. Interestingly, *spxB* seemed to be more conserved among species when compared to *lctO*. The relatively wide distribution of *spxB* and *lctO* and the high degree of conservation suggest that both genes play an important role in the H₂O₂ production capabilities of the oral biofilm and might be considered as oral streptococcal community genes. Interestingly, inactivation of *spxB* in *S. sanguinis* diminishes competitive H₂O₂ production, suggesting that *lctO* plays no role in interspecies competition under the tested conditions in *S. sanguinis* [35].

2.2. Sources of H₂O₂ from the Host. H₂O₂ originates from several sources in the human body. Mitochondria are well-known producers of reactive oxygen species (ROS) as a byproduct of respiration [40]. Effective intracellular scavenging systems are in place to avoid ROS inflicted damage [41] and the H₂O₂ might not leave the oral mucosa in sufficient amounts to play a role in oral microbial biofilm ecology. A regulated production of ROS is observed as part of the oxidative burst from phagocytic cells [42]. The ROS production is directed towards the outsides of the phagocytic cell to defend the host from microbial pathogens and might therefore freely diffuse to nearby locations. Polymorphonuclear leukocytes seem to be the predominant phagocytic cells in saliva originating from the gingival crevice fluid and are constantly replenished [29]. However, one study with healthy individuals observed a high intraindividual day-to-day variability of salivary polymorphonuclear leukocyte content [43], making it difficult to judge how much H₂O₂ is being released as a consequence of phagocytic cell activity.

A more constant source of H₂O₂ supplied into saliva could originate from salivary gland cells expressing the dual oxidase 2 gene (*Duox2*) as shown by Geiszt et al. [44]. The same study also suggests that that ROS production occurs in the last step of saliva formation for direct delivery of ROS into the oral cavity [42, 44] and could therefore be the major source for salivary H₂O₂ originating from the host.

3. Hydrogen Peroxide in Oral Bacterial Ecology

3.1. Where Does It Matter: The Importance of Bacterial Proximity. The fact that H₂O₂ was never detected in saliva so far and the existence of a major scavenging system comprised of salivary peroxidases raise an important question: how likely does H₂O₂ affect oral bacterial ecology or aid in biofilm community adaptation? This question might be addressed by the fact that a bacterial biofilm comprises its own microcosm with intrinsic biofilm H₂O₂ production and most likely has a localized effect due to diffusion restrictions. By measuring the H₂O₂ concentration produced by single species, *S. gordonii* biofilms, Liu et al. were able to show that a steady state level of 1.4 mM H₂O₂ was produced at a distance of 100 μ m above the biofilm surface [45]. Only 0.4 mM H₂O₂ is produced when measured 200 μ m above the biofilm. This localized production of 1.4 mM is a concentration able to inhibit H₂O₂ susceptible bacteria, which have to be in close proximity. Remarkably, the same study also measured higher concentrations of H₂O₂ close to the surface of the biofilm as compared to planktonic grown cells [45]. This is in contrast to an earlier study by Nguyen et al. showing that *S. sanguinis* and *S. gordonii* had lower H₂O₂ production rates in biofilms when compared to planktonic cells [46]. This discrepancy might be partially explainable by the advanced method used in the study by Liu et al., allowing realtime detection with an H₂O₂ specific probe measuring directly above the biofilm surface [45]. Also, the study by Nguyen et al. used a higher concentration of glucose in the growth media, which might have repressed the H₂O₂ production rate [46] (see below: regulatory studies on H₂O₂ production). The difference in H₂O₂ concentration as a function of biofilm surface distance

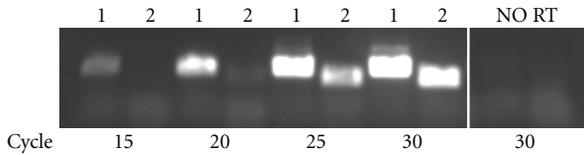


FIGURE 1: Expression of *spxB* in freshly isolated human plaque. To detect the expression of *spxB* among streptococcal species in the oral biofilm, plaque samples were collected from a healthy subject without active caries. Bacterial RNA was isolated and cDNA synthesized after standard protocols [59]. The *spxB* gene was PCR amplified from the synthesized cDNA with primers described by us earlier specific for *spxB* and 16S rRNA [59]. Samples were removed during the PCR run after 15, 20, 25, and 30 PCR cycles and loaded on an agarose gel for visualization. 1 = 16S rRNA; 2 = *spxB*; no RT = control for chromosomal DNA contamination.

supports the suggestion that H_2O_2 producing species most likely have an effect on close neighboring species. When the cells dislodge and enter a planktonic state, H_2O_2 production becomes irrelevant. Taking into account that the oral biofilm is a diffusion barrier for larger proteins and molecules [47], the intrinsic H_2O_2 production of biofilm would also be more protected against the action of salivary lactoperoxidases, which might not penetrate preformed biofilms [48].

Detection of actual *spxB* expression in the human oral biofilm would support the importance of *spxB*-dependent H_2O_2 production. If *spxB* plays a vital role in oral biofilm ecology, one would expect that cells residing in the human oral biofilm express the *spxB* gene. Using freshly isolated plaque samples from a subject with no active caries, *spxB* specific cDNA was synthesized from RNA isolated from human oral biofilm bacteria and *spxB* expression confirmed (Figure 1; unpublished results). This observation not only shows for the first time the expression of an oral biofilm relevant gene *in vivo* but also strongly supports *spxB* relevance in the human dental plaque and suggests that *spxB* plays a role in biofilm specific processes.

3.2. Adaptation to a Competitive Environment-Genetic Exchange. Adaptation to the constantly changing oral environment requires some kind of genetic flexibility. This can be achieved by specific gene expression regulation and the adjustment of the transcriptome to sudden perturbations in the environment or by the acquisition of new genetic traits to cope with long-term environmental changes. Oral streptococci are known for their natural ability to take up extracellular DNA, a physiological state called competence [49]. Bacterial competence has long been recognized as the ability to take up DNA, but recent studies show that competence is part of a larger stress response, which enables competent bacteria to cope with a stressful environment [50]. Competent oral streptococci are able to take up homologous and heterologous DNA [51–53]. This increases the available DNA pool and allows for acquisition of new genetic traits from other species. Expression of newly acquired genetic traits depends on the homologous recombination of the incorporated DNA into the host chromosome [54, 55].

The mechanisms and genetic regulation of natural competence leading to the uptake and integration of DNA via homologous recombination are documented in numerous studies, and the basic blueprint of competence seems to be similar among oral streptococci [49, 56]. What is less known is how the biofilm community generates the extracellular DNA for DNA uptake by competent bacteria. A general mechanism of bacteria to produce extracellular DNA is an autolytic event leading to bacterial disintegration. Recent studies show that autolysis is a regulated process.

The release of DNA into the environment by *S. gordonii* and *S. sanguinis* is closely associated with the production of H_2O_2 [35]. The wild type organisms release high molecular weight DNA during aerobic growth, which was shown to be of chromosomal origin [57]. A deletion of the pyruvate oxidase gene affected this release process dramatically [57]. In addition, a significant reduced concentration of extracellular DNA was detected under oxygen limited growth conditions [58], correlating with a reduced expression of *spxB* and a lower amount of SpxB [59, 60]. Further studies showed that H_2O_2 is the only requirement to induce the DNA release process. Addition of H_2O_2 to anaerobically grown cells does induces DNA release. Although mechanistic studies are still in progress and the release process is not fully understood, our group has demonstrated a correlation between H_2O_2 induced DNA damage and extracellular DNA generation. Treatment with DNA damaging agents like UV light and mitomycin C also triggered the release of DNA under anaerobic conditions [58].

Initial evidence of an autolytic activity involved in the DNA release process comes from Robert A. Burne's group, showing that the major autolysin AtlS is involved in DNA release [61]. A deletion of AtlS in *S. gordonii* prevented autolysis under aerobic conditions, and as a consequence, a decreased production of extracellular DNA was observed [61]. Their observation, however, is in contrast to an observation by our group, showing that under anaerobic conditions, extracellular DNA release can be induced by H_2O_2 addition without any obvious bacterial cell lysis [58]. A possible explanation for these observations is that streptococci may have several mechanisms to trigger lysis responding to different internal and/or external stimuli. Autolysis may also not necessarily mean complete lysis of the bacterial cell or might only affect a small portion of the population. A recent report showed that *S. gordonii* expresses a murein hydrolase, *LytF*, involved in competence dependent bacterial lysis [62]. In fact, *lytF* is only expressed during competence because its expression is under the control of the competence stimulating peptide CSP, a small secreted peptide which accumulates in the environment after reaching a critical threshold concentration initiating the competence signaling cascade (see [50] for a detailed overview of competence in bacteria). DNA transfers experiments relying on *LytF* dependent cell lysis, and subsequent DNA uptake by *S. gordonii* showed that most cells are protected from the muralytic activity of *LytF* [62]. This is in agreement with our observation of a lysis resistant population [57, 58]. A close association, however, of H_2O_2 induced release of DNA and competence development is evident since cells

grown under H_2O_2 producing conditions are also induced for competence development [58]. Interestingly, competence development in *S. pneumoniae* can be initiated by mitomycin C induced DNA damage, which also leads to the release of DNA [63]. This is reminiscent of our observation that DNA damaging agents induce DNA release [58], which is associated with the ecological advantage of H_2O_2 induced DNA release and the adaptation of oral streptococci to stress. *S. gordonii* and probably other H_2O_2 -producing oral streptococci release DNA into the environment as a consequence of DNA damage. This pool of released DNA likely contains mutations in various genes because of the DNA damage. If such mutated DNA is taken up and integrated into the chromosome, the transformation event would lead to a bacterium able to grow and outcompete bacteria without the respective mutation under selective conditions. Even nonmutated extracellular DNA or genes would be useful as a template for the repair of stress-induced DNA damage [58]. The extracellular DNA is precisely produced at a time when it is biologically meaningful, under aerobic conditions during initial biofilm formation with its fierce interspecies competition and environmental stress, hence, when the cells are most competent for DNA transformation. Finally, H_2O_2 can also cause the release of DNA from streptococci not producing H_2O_2 , but the mechanism for this is not known (unpublished results).

3.3. The Other Role of Extracellular DNA. Besides providing genetic information for transformation of competent oral streptococci, the DNA released as a consequence of H_2O_2 -production might aid in initial biofilm development [64]. Although not directly shown for H_2O_2 producing oral streptococci, studies with *S. mutans* demonstrate the importance of extracellular DNA in initial adhesion. Das et al. showed that adhesion kinetics in the presence and absence of naturally occurring extracellular DNA were different. *S. mutans* cells adhered better and in greater numbers to the provided test surface when extracellular DNA was present [65, 66].

Initial biofilm formation involves the adhesion of pioneer colonizers to the tooth surface [3]. Another important event in early biofilm formation is bacteria-bacteria aggregation: (1) aggregation of bacteria before the actual attachment event in saliva increases the cluster size of bacteria able to adhere; (2) bacterial aggregation will also aid in the recruitment of other bacteria into the developing biofilm. Although aggregation of bacteria is well described with the identification of several surface proteins involved in the process [3], the role of extracellular DNA in oral bacterial aggregation is not well investigated. Studies with fresh water bacteria show that the released DNA functions in a netlike manner able to trap bacteria [67]. Initial evidence shows that extracellular DNA plays a role in the intraspecies aggregation of *S. sanguinis*. When grown as a planktonic culture, addition of extracellular DNA degrading DNase inhibits partially the aggregation [57]. Further studies are required to fully understand the role of extracellular DNA in multispecies biofilm formation and bacterial aggregation.

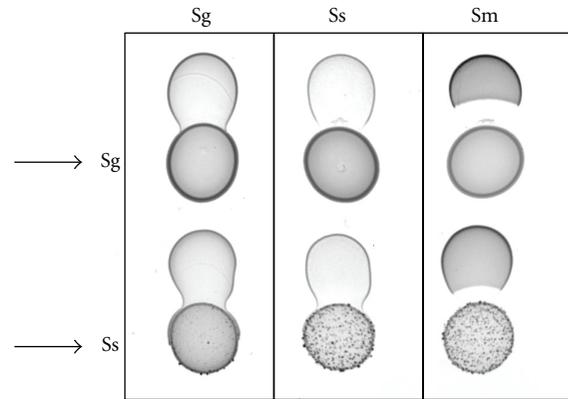


FIGURE 2: Oral streptococcal antagonism assay with *S. sanguinis*, *S. gordonii*, and *S. mutans*. The lower row in the plate dual-species antagonism assay were inoculated first (indicated by an arrow) and allowed to grow for 16 h. Subsequently, the to be tested species was inoculated in close proximity. Diffusible H_2O_2 produced by *S. sanguinis* (Ss) and *S. gordonii* (Sg) during growth caused inhibition of *S. mutans* (Sm), while no obvious growth inhibition was observed when *S. sanguinis* or *S. gordonii* was tested against themselves or against each other.

3.4. Biofilm Community Development. Earlier clinical studies have demonstrated the inverse relationship between *S. sanguinis* and cariogenic *S. mutans* [17, 19]. A recent study showed that *S. oligofermentans* is also frequently isolated from healthy human subjects [68]. The clinical evidence suggests that the initial colonization by H_2O_2 -producing bacteria has a beneficial aspect for the human host with regard to caries development, possibly through the influential role of H_2O_2 on biofilm community development. Detailed *in vitro* experiments and relevant biofilm studies confirmed that *S. sanguinis*, *S. gordonii*, and *S. oligofermentans* produce H_2O_2 to inhibit *S. mutans* [8, 35, 37]. Although, the produced H_2O_2 has a slight self-inhibitory effect on the producing species in batch cultures, no obvious inhibition occurs when H_2O_2 producers are tested against each other in an antagonistic plate diffusion assay (Figure 2). As a consequence, community development favors integration of species that are compatible with the production of H_2O_2 . Jakubovics et al. showed an interesting relationship between *S. gordonii* and *Actinomyces naeslundii*. Although *A. naeslundii* is severely inhibited in the aforementioned antagonistic plate diffusion assay, coaggregation cultures showed that both species could grow together in close proximity [69, 70]. *S. gordonii* is, however, the dominant species in this consortium, leading to a ratio of about 9 to 1. *S. gordonii* might benefit from this relationship by the fact that the H_2O_2 degrading catalase produced by *A. naeslundii* can reduce oxidative damage to *S. gordonii* proteins inflicted by its own H_2O_2 [70]. The low ratio of *A. naeslundii* to *S. gordonii* would still allow for sufficient inhibition of H_2O_2 susceptible species, but a clear ecological niche is necessary to support growth of both species, which could lead to the formation of more stable plaque communities. Another common oral isolate found in close association with *S. gordonii* is *Veillonella* ssp. [71].

Both species interact at the physiologic and metabolic level as shown by several studies [72–74]. Some strains of *Veillonella* also produce catalase, indicating that a similar effect as described for *A. naeslundii* might exist in the relationship between *S. gordonii* and *Veillonella* *ssp.* The biological relevance of the interactions between *Streptococci*, *Veillonella*, and *Actinomyces* has recently been demonstrated *in vivo* by confirming the spatial association of the three species in human plaque samples [2]. Further studies are required to determine the exact role of catalase production in the dual species relationship between H_2O_2 -producing streptococci and catalase-expressing species.

The production of H_2O_2 seems to select for a close association with compatible bacteria during biofilm community development. Therefore, H_2O_2 might shape the colonization pattern during initial biofilm formation and provide an ecological advantage for the producer and the accompanying H_2O_2 resistant species.

4. Regulatory Studies on H_2O_2 Production

S. gordonii and *S. sanguinis*. A detailed analysis of environmental influences on *S. gordonii*'s H_2O_2 production showed two important behaviors. (1) During growth under limited glucose and sucrose availability, *S. gordonii* produces only H_2O_2 , while H_2O_2 and L-lactic acid are produced in equal amounts when concentrations of carbohydrates were higher than 0.1 mM. Since lower carbohydrate availability means increased competition among the biofilm microflora, a switch to only H_2O_2 production might increase the ecological competitiveness. (2) High glucose and sucrose concentrations inhibit the production of H_2O_2 [75]. This observation prompted us to further investigate the mechanism of H_2O_2 production control by determining *spxB* expression and SpxB abundance in *S. sanguinis* and *S. gordonii* under different environmental conditions. We could confirm the influence of carbohydrate concentration on *spxB* expression and abundance showing glucose repression in *S. gordonii* [59]. The carbohydrate dependent repression of *spxB* expression was also confirmed for galactose, maltose, and lactose, while sucrose and fructose seemed to have no effect in our strain [59]. This indicates that strain variability among *S. gordonii* might exist in the regulation of *spxB* expression. A detailed analysis of the promoter region of *spxB* from *S. gordonii* showed the existence of two putative binding sites for the catabolite control protein A (CcpA). CcpA is the main regulator of carbon catabolite repression in Gram-positive bacteria [76]. Mutational analysis of the promoter sequence confirmed the role of the CcpA binding sites and purified CcpA was able to bind to the respective regions in *in vitro* electromobility shift assays [59]. Surprisingly, the *spxB* expression in *S. sanguinis* is not influenced by carbohydrate availability, despite a high degree of promoter homology between both species and the presence of respective CcpA binding sites. However, a deletion of CcpA in *S. sanguinis* increased expression of *spxB* several folds [77]. This suggests that *S. sanguinis* constantly represses the expression of *spxB* or only lifts the repression due to a yet unknown environmental signal. One reason for this alternative *spxB* expression

control could be *S. sanguinis* increased susceptibility to H_2O_2 when compared to *S. gordonii* (unpublished results). By keeping the production of H_2O_2 low, *S. sanguinis* might prevent self-damage of cellular components like surface adhesins, making it less competitive in the oral environment. The observation that monospecies biofilms of a *S. sanguinis* CcpA mutant had a higher proportion of dead cells when compared to the wild type further supports this hypothesis [77].

Both species do not produce competitive H_2O_2 under anaerobic growth conditions. Accordingly, *spxB* expression and SpxB abundance is greatly reduced under anaerobic growth conditions, but the protein is still detectable [59, 60]. This finding suggests that both streptococci keep a low level of SpxB present to remain competitive once they encounter aerobic conditions. The mechanisms of oxygen-dependent *spxB* expression control are not known at this time.

The *spxB* expression control involves additional regulators and proteins. Most notable is the identification of an SpxR homolog in *S. sanguinis* [78]. SpxR was originally identified in *S. pneumoniae* and it was hypothesized that SpxR in *S. pneumoniae* regulates *spxB* transcription in response to the energy and metabolic state of the cell [79]. Although not confirmed experimentally, this regulatory function might well be active in *S. sanguinis*, since no carbohydrate-dependent regulation was detected. Future research might address this question and identify what actual signal is involved in *spxB* regulation in *S. sanguinis*.

S. oligofermentans. *S. oligofermentans* developed an interesting mechanism to produce antagonistic H_2O_2 and maximize its competitiveness. SpxB produces the majority of H_2O_2 during active growth [36] leading to the generation of an extra ATP through the *spxB* pathway. This ATP provides a metabolic growth advantage in addition to the ecological advantage of H_2O_2 production. The *lctO*-dependent H_2O_2 generation on the other hand is more prominent in the early stationary phase, due to an increased availability of lactate [36]. Several other oral streptococci encode genes for both H_2O_2 forming enzymes suggesting a similar role in H_2O_2 production. This dual SpxB/LctO presence indicates that even under starving conditions, oral streptococci might still produce competitive amounts of H_2O_2 to shape biofilm development towards a health compatible composition.

5. H_2O_2 in Oral Bacterial-Host Interactions

Oral streptococcal interactions occur in the mouth and therefore in close proximity to human host cells and the mucosal surface. Interactions with human innate immunity components are inevitable. Marvin Whiteley's group showed that the production of H_2O_2 has an unexpected effect on the recognition of pathogenic species by the immune response [80]. Using the recognized periodontal pathogen *Aggregatibacter actinomycetemcomitans* and *S. gordonii* as model organisms to study a combined effect on the host innate immune response, they described an interesting relationship between both species. Not only is *A. actinomycetemcomitans* able to effectively use the lactic acid produced by *S. gordonii*

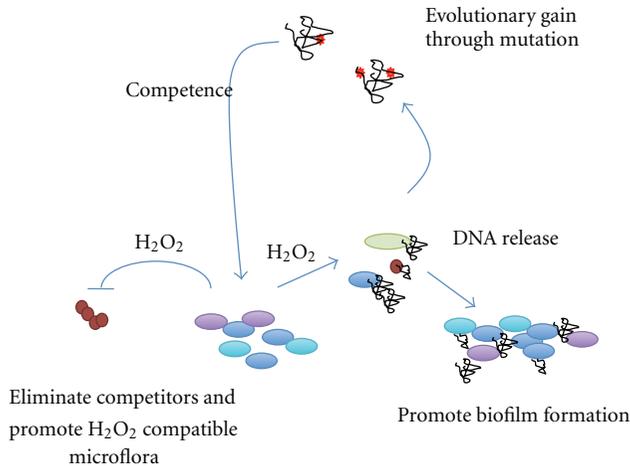


FIGURE 3: Overview of the effects of H₂O₂ production on oral biofilm development. Initially, the antagonistic effect of streptococcal H₂O₂ production was described. As a consequence, competitors are eliminated, and the integration of H₂O₂ compatible species into the developing biofilm is promoted. H₂O₂ production also causes the release of DNA into the environment. The extracellular DNA promotes biofilm formation and cell-cell aggregation. In addition, H₂O₂ causes DNA damage, which in turn could lead to beneficial mutations in competent oral streptococci uptake of extracellular DNA. Extracellular DNA could therefore support adaptational processes to changing environmental conditions and promote evolution of oral biofilm development.

for growth [81] but it also responded to H₂O₂ as a signal to induce the expression of an immune evasion gene, *apiA*. This gene encodes an outer membrane protein able to bind factor H, conferring protection against killing by the alternative complement component of the innate immunity. In addition, the *katA* gene encoding cytoplasmic catalase is also induced, conferring resistance to the destructive action of H₂O₂ on *A. actinomycetemcomitans* cellular components [80].

This observation demonstrates that biofilm community development is capable of remarkable evolutionary adaptations and that H₂O₂ plays a prominent role in the process of oral biofilm development (Figure 3).

6. Concluding Remarks

One of the most important problems in current oral microbial research is to confirm biological relevance of *in vitro* experimental results. Accepted animal models to simulate oral biofilm ecology are generally rodent models. Although these models increase complexity, the transplanted human oral flora faces a rodent oral microbial consortium and a distinct oral environment. It therefore competes with species and conditions not encountered under normal conditions. It is not known if this complexity affects competition studies. A recent rodent study actually questions the validity of the importance of H₂O₂ production in *S. gordonii* competitiveness. Performing coinoculation studies in rats, Tanzer et al. showed that *S. mutans* is always able to outcompete

S. gordonii under all experimental conditions [82]. Unfortunately, it was not determined whether the *S. gordonii* strain in their study produced competitive amounts of H₂O₂ or if the *spxB* gene was expressed in the rat oral biofilm. It is also unclear if the respective *S. mutans* strain was H₂O₂ susceptible. It is therefore important that animal studies about ecological questions actually demonstrate that the respective competitive gene set(s) are expressed under animal test conditions. It is also important to verify the expression of the gene(s) of interest in the human oral biofilm. Our initial data for *spxB* gene expression in the human oral biofilm are promising and warrant further research regarding the ecological role of H₂O₂ production in human oral biofilm.

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