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The Road Less Traveled – Defining molecular commensalism with *Streptococcus sanguinis*

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Summary

The commensal oral microbial flora has evolved with the human host to support colonization of the various intraoral sites without triggering a significant immune response. In exchange, the commensal microbes provide critical protection against invading pathogens. The intrinsic ability of the oral flora to create a symbiotic microbial community with the host can be disturbed, selecting for the overgrowth of a dysbiotic community that can result in dental diseases, such as caries and periodontitis. While the mechanisms of molecular pathogenesis in oral diseases are well characterized, much less is known about the molecular mechanisms used by the commensal flora to maintain oral health. Here we focus on the commensal species *Streptococcus sanguinis*, which is found in abundance in the early oral biofilm and is strongly correlated with oral health. *S. sanguinis* exhibits a variety of features that make it ideally suited as a model organism to explore the molecular basis for commensalism. As such, this review will describe our current mechanistic understanding of *S. sanguinis* commensalism and speculate upon its molecular traits that may be exploitable to maintain or restore oral health under conditions that would otherwise lead to disease.

Introduction

Our view of the etiology of the main oral diseases, caries and periodontitis, has been refined in recent years, largely due to rapid improvements in high throughput sequencing technologies. Numerous recent studies have provided a detailed picture of the connection between oral disease status and microbial dysbiosis among the oral flora (Hajishengallis and Lamont, 2012; Simon-Soro and Mira, 2015). The severity of disease is heavily influenced by the synergistic interactions of the individual members of the polymicrobial consortium, including metabolic cross-feeding and interspecies signaling. Thus, the etiology of caries and periodontitis (and other mucosal polymicrobial diseases) is largely a consequence of

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microbial ecology and poorly defined by Koch's Postulates (Hajishengallis and Lamont, 2016; Magalhaes et al., 2016; Stacy et al., 2016).

The polymicrobial nature of oral diseases challenges current treatment approaches and a refocus might be required, since the overall incidence of caries and periodontal disease in the population has not significantly improved in multiple decades. In fact, they continue to be among the most common human diseases worldwide (Kassebaum et al., 2015). While there is a wealth of detailed knowledge regarding the pathogenic mechanisms triggered by dysbiosis, much less is known about the mechanisms used by the commensal flora to prevent pathology triggered by the abundance of microbes found at most mucosal surfaces. This is one area of research that is going to require a much greater emphasis in the future if we expect to effectively prevent and/or cure diseases caused by dysbiosis. Recent advances in the various -omics technologies have provided unparalleled insights into the actual outcomes of polymicrobial interactions. The remaining challenge is to decipher the regulatory and mechanistic events leading to these interactions. Such studies still require detailed knowledge of key processes from individual organisms in order to test specific hypotheses derived from big data studies (McLean, 2014). *S. sanguinis* exhibits a variety of features that make it ideally suited as a model organism to explore the molecular basis for commensalism among the flora. As such, this review will describe our current mechanistic understanding of the commensal aspects of *S. sanguinis* biology and discuss potential avenues to exploit commensalism therapeutically.

Historical excursion

S. sanguinis was originally named due to its role in infective endocarditis. In a note to the Journal of Bacteriology, Niven and White described a new species isolated from about 100 cases of subacute bacterial endocarditis (Niven and White, 1946). About one third of those isolates failed characterization and were simply referred to as *Streptococcus s.b.e.* (for subacute bacterial endocarditis) (Niven and White, 1946). Overall the group was quite homogenous in its physiological and biochemical characteristics (White and Niven, 1946). Initial attempts to isolate *Streptococcus s.b.e* had largely failed at the time, as samples were mainly focused on throat isolations. However, a confirmed isolate was later obtained from an extracted tooth, which we now know coincides with its preferred colonization site. Since the only source of *Streptococcus s.b.e.* in the original publication came from the blood of endocarditis patients, it was named *S. sanguis* from the latin word for blood (Niven et al., 1946; White and Niven, 1946). This was later amended to *S. sanguinis* for grammatical reasons (Truper and De' Clari, 1997). Further serological characterization confirmed the isolation of a new species, since no cross reactivity was observed between rabbit serum from *Streptococcus s.b.e.* and other identified streptococci belonging to the various Lancefield groups (Washburn et al., 1946).

During the 1960s and 70s several publications coauthored by Jan Carlsson first identified the primary habitat of *S. sanguinis*. Culture-dependent detection of *S. sanguinis* in infants was strongly correlated to tooth eruption providing evidence for the tooth surface as the preferred colonization site (Carlsson et al., 1970). Carlsson also performed some of the earliest mixed culture studies of *S. sanguinis* and *Streptococcus mutans* to examine the ecological aspects

of their interactions (Carlsson, 1971). In addition, he provided the first characterization of *S. sanguinis* pyruvate oxidase (SpxB, also PoxB), which is the main enzyme responsible for producing toxic quantities of hydrogen peroxide (H_2O_2) used to inhibit other species like *S. mutans* (Carlsson and Edlund, 1987; Carlsson et al., 1987; Kreth et al., 2008). The importance of H_2O_2 production for the promotion of homeostasis in the oral biofilm was first described in 1973 (Holmberg and Hallander, 1973) and is still the subject of ongoing research (see below).

Phenotypic characteristics

S. sanguinis is a Gram-positive, non-motile (although this has been challenged (Gurung et al., 2016); see below), non-spore forming, catalase-negative coccus. *S. sanguinis* is non-*beta*-hemolytic, but able to produce a green coloration on blood agar plates referred to as *alpha*-hemolysis (Facklam, 2002). *S. sanguinis* has been placed into the *Streptococcus mitis* group based upon 16S rRNA sequence analysis (Facklam, 2002). However, in a recent study based on the concatenated sequences of 50 ribosomal protein genes from 88 *Streptococcus* species, *S. sanguinis* is placed in it's own group together with *S. gordonii* (Teng et al., 2014). Thus far, *S. sanguinis* has only been isolated from humans.

Clinical evidence of *S. sanguinis* association with oral health

Several studies have demonstrated the importance of S*. sanguinis* during early colonization and its widespread distribution (Carlsson et al., 1970; Caufield et al., 2000; Aas et al., 2005). The cycle of early colonization by *S. sanguinis* likely occurs in every human after tooth eruption or extensive cleaning. Its contribution to oral biofilm homeostasis is inferred from its abundance at sites of oral health and its obvious decreased abundance at sites of active caries or periodontal disease (Becker et al., 2002; Colombo et al., 2009). Thus, *S. sanguinis* is not only one of the most ubiquitous and prolific inhabitants of the tooth surface, but it has evolved mechanisms to prevent obvious damage to the host as a consequence of its growth (i.e. a true commensal organism). Surprisingly little is known about the specific molecular mechanisms mediating this ability, with the exception of the few studies discussed later. Our limited knowledge in this area can be largely attributed to a historical emphasis upon mechanistic studies of pathogenesis, rather than commensalism.

In the seminal publication by Aas *et al.*, the authors were able to define the normal bacterial flora of the adult oral cavity using culture independent 16S rRNA sequencing of bacterial samples from 9 different intra-oral sites (Aas et al., 2005). In this study, the teeth of all five subjects that were free of oral health-related problems were colonized by *S. sanguinis*. This was true for only one other identified species, *S. mitis*, which in contrast was found in all tested intraoral locations (Aas et al., 2005). In more recent studies, *S. sanguinis* was found to be among the shared taxa strongly associated with oral health (Corby et al., 2005; Bik et al., 2010; Belda-Ferre et al., 2012), which is consistent with the inverse correlation observed between *S. sanguinis* and *S. mutans* in adults (de Stoppelaar *et al.* 1970)(Giacaman et al., 2015). The impact of *S. sanguinis* relative abundance in the oral biofilm is also reflected in the overall functional expression profile of the dental plaque microbiome. Transcripts derived from just 9 species represented 71% of the total dental plaque transcriptome, with *S.*

sanguinis providing the majority of detected transcripts (16%) (Peterson et al., 2014). In addition to adults, studies of children have also have reported a similar positive association between *S. sanguinis* abundance and oral health. In a defined study that compared 30 children with severe early childhood caries to healthy controls, *S. sanguinis* was only detected in healthy subjects or on teeth with sound enamel. In contrast, *S. sanguinis* was absent on white spot lesions, cavitated teeth, or on dentin (Becker et al., 2002). Later studies reported that *S. sanguinis* can still be detected in children with severe early childhood caries (Ge et al., 2008; Kanasi et al., 2010), but the relative levels of *S. sanguinis* and *S. mutans* in the oral cavity vary with oral health status (Ge et al., 2008). Similarly, the early colonization of *S. sanguinis* in infants has been shown to correlate with a significant delay in the colonization of *S. mutans* (Caufield et al., 2000). Overall, the vast majority of studies report an association of *S. sanguinis* with oral health. However, an over-representation of *S. sanguinis* in a caries active subject pool was recently reported, albeit with a very small cohort of 4 caries active subjects (Peterson et al., 2013). It is also worth noting that *S. sanguinis* seems to be among the species that are more prevalent in subjects with periodontal health as well (Stingu et al., 2008; Colombo et al., 2009; Mason et al., 2015). However, few specific details have been reported to explain why this is the case.

Two important questions remain: i) If *S. sanguinis* is associated with oral health and a delayed colonization of *S. mutans*, what causes its shift in abundance during disease development? Conventional wisdom posits this is simply a consequence of reduced viability due to acidification in dental plaque. However, it is also conceivable that initial impairments in *S. sanguinis* competitive abilities could precede and ultimately facilitate the sequence of events required for extensive plaque acidification. ii) Does *S. sanguinis* provide any protective function for periodontal health and perhaps more importantly, can this be exploited to prevent and/or mitigate periodontal disease (as well as caries)?

S. sanguinis presence in extra-oral diseases

Recent studies have suggested a potential mechanism for *S. sanguinis* to improve clinical outcomes in patients afflicted with cystic fibrosis, which is a systemic disease causing chronic respiratory infections due to defects in exocrine gland function (Elborn, 2016). *S. sanguinis* was demonstrated to inhibit the principal pathogen in cystic fibrosis lung infections, *P. aeruginosa*, due to its production of H_2O_2 (Whiley et al., 2015). The authors speculated that an early colonization of *S. sanguinis* and other H_2O_2-producing streptococcal species in the oral cavity and lung might influence the bacterial community structure of cystic fibrosis patients, potentially delaying disease onset and/or progression (Whiley et al., 2015). However, epidemiological evidence for such an effect is currently lacking. The authors also noted that synergistic effects were possible between other viridans group streptococci and *P. aeruginosa,* which actually led to enhanced pathogenicity (Whiley et al., 2015). While much is known about *P. aeruginosa* pathogenic mechanisms in cystic fibrosis, this is not the case for studies of commensal streptococci in this disease. Such studies may yield entirely new strategies to exploit bacterial antagonism as a therapeutic approach for cystic fibrosis. Although not the subject of this review, *S. sanguinis* is also known to be the etiological agent of several extra-oral diseases. The most prominent is its association with infective endocarditis, which is a relatively rare, but potentially fatal disease affecting the

heart valves or endocardium of patients with predisposing heart defects. For further details, the interested reader is directed to several excellent reviews on this topic (Cahill and Prendergast, 2015, 2016). In even rarer instances, *S. sanguinis* has also been reported to cause both meningitis and severe bacteremia, sometimes as a result of surgical procedures or cancer (Kampe et al., 1995; Macaluso et al., 1998; Moon et al., 2010; Liu et al., 2013; Bijlsma et al., 2016).

Molecular mechanisms of *S. sanguinis* commensalism in the oral cavity

Initial attachment and biofilm development

As a pioneer colonizer of the tooth surface, *S. sanguinis* facilitates the subsequent colonization of other species in the oral biofilm (Kolenbrander et al., 2006). This is not a unique ability of *S. sanguinis* per se, as there are a variety of other pioneer colonizers involved as well. However, the overall abundance of *S. sanguinis*, especially in early biofilms, suggests a dominant role in this process. Its success as a pioneer colonizer is also reflected in its relatively large abundance of salivary pellicle adhesins compared to most other oral bacterial species (Kolenbrander et al., 2006; Peterson et al., 2014). The abundance of *S. sanguinis* in newly formed oral biofilms also suggests that it likely plays a central role in shaping biofilm ecology as these communities develop, although detailed studies in this area are still lacking. For additional information regarding oral streptococcal coadhesion and community assembly, the interested reader is referred to several excellent recent reviews (Nobbs et al., 2009; Nobbs et al., 2011; Wright et al., 2013; Jakubovics et al., 2014; Nobbs et al., 2015).

Oral biofilm formation begins with the attachment of *S. sanguinis* and other pioneer colonizers to macromolecular complexes formed on saliva-coated tooth surfaces (Diaz et al., 2006; Kreth and Herzberg, 2015). Negatively charged residues and electrostatic interactions with hydrophilic regions in salivary proteins facilitate their attachment to the tooth surface (Lamkin and Oppenheim, 1993; Lindh, 2002) forming what is referred to as the acquired enamel pellicle (AEP). Although *S. sanguinis* is able to directly adhere to saliva-free hydroxyapatite (Tanaka et al., 1996), the main mineral found in tooth enamel, the initial attachment process is most-likely driven by an interaction of the streptococcal surface with salivary components. Binding to salivary proteins is mediated via protein-protein or protein-carbohydrate interactions with receptors exposed on the bacterial surface. Amylase is the most abundant salivary protein and is present both in the AEP and in dental plaque (Orstavik and Kraus, 1973; Aguirre et al., 1987). *S. sanguinis* specifically binds to amylase via long filamentous pili (Okahashi et al., 2011). The genes encoding these pili are organized in an operon encoding three putative structural pilin subunits as well as a sortase involved in the surface anchoring of the pili proteins. Disruption of the pilus locus results in decreased single species biofilm formation on saliva coated glass slides, although it does not abolish amylase binding completely (Okahashi et al., 2011). Thus, additional surface proteins are likely to be involved in amylase binding. Besides anchoring to the AEP, another major advantage of adherence to salivary amylase is that the enzyme retains about 50% of its enzymatic function (Scannapieco et al., 1990). Since amylase can efficiently hydrolyze the *alpha*-1,4-glucosidic linkages in starch to glucose, maltose, and maltodextrins (Ramasubbu

et al., 1996), they can provide a readily accessible source of easily metabolizable sugars that can be imported via high affinity carbohydrate transporters (Vadeboncoeur and Pelletier, 1997). This is presumably an important mechanism used to help newly attached cells of *S. sanguinis* rapidly spread over the tooth surface and develop into biofilm communities (Marsh et al., 1985).

SsaB is another surface exposed protein shown to mediate binding to saliva-coated hydroxyapatite, although the mechanism is unknown (Ganeshkumar et al., 1988). However, SsaB has been demonstrated to serve as the substrate-binding protein for an ATP-binding cassette (ABC) transporter for manganese (Mn^{2+}) (Crump et al., 2014). Therefore, it is unlikely to be a classical adhesin. Since salivary proteins are largely negative charged (Gibbins et al., 2014) and might interact with the divalent cation Mn^{2+}, it is possible that SsaB not only transports Mn^{2+}, but also functions as an adhesin when Mn^{2+} is bound to salivary proteins.

Another major component of saliva and AEP are the mucins, the gel-forming components of mucus. Mucins are a diverse group of >20 glycoproteins that primarily serve as a hydrating and lubricating layer for mucosal epithelial cells (Frenkel and Ribbeck, 2015) and *S. sanguinis* is known to adhere specifically to salivary MUC7 via the surface receptor SrpA (Plummer and Douglas, 2006). In addition, *S. sanguinis* has an unusually high number of uncharacterized lipoproteins (LP) and surface exposed cell-wall anchored proteins (CWA). The genome of the common lab strain SK36 contains 60 LPs and 30 CWAs (Xu et al., 2007), any of which could conceivably promote attachment to the AEP and/or facilitate coadherence to other species. The number of such proteins found in *S. sanguinis* is considerable higher than in *S. mutans* and *S. pneumoniae* (Xu et al., 2007) further highlighting its role as a central player in early oral biofilm development.

A feature that is rather unusual for streptococci has been recently investigated in greater detail and might also aid in *S. sanguinis* biofilm development. First described in the mid-1970s, *S. sanguinis* is capable of surface-associated twitching motility (Henriksen and Henrichsen, 1975). This ability is mediated by a type IV secretion system comprised of surface associated pili (Gurung et al., 2016). Motility is achieved by retracting pilus-like structures, thus pulling the cell in one direction (Jarrell and McBride, 2008). These pili are organized in an operon located within a 22 kb *pil* locus separate from the aforementioned amylase binding pili. Mutagenesis of the *pilT* component of the complex indicates that its ATPase activity is likely the principal mediator of *pilus* retraction during twitching motility (Gurung et al., 2016). It is perhaps even more striking that this pilus locus is thus far absent from all other streptococcal genomes. Currently, it is unknown whether twitching motility has any relevance *in vivo* or if it provides any colonization advantage, but it is tempting to speculate that this conserved function enhances *S. sanguinis* development of biofilms on the tooth surface. Since *S. sanguinis* lacks an obvious chemotaxis system, its twitching motility seems unlikely to be utilized for carbon source acquisition. One important function unrelated to motility could be as a surface adhesin via pili tethering. The forces generated through pilus retraction are surprisingly strong (Gurung et al., 2016) and could potentially help to anchor the bacteria when encountering excessive shear forces.

Biofilm maturation

The biofilm developmental program includes the formation of extracellular polymeric substances (EPS), generating a matrix component that has several functions for the biofilm. The biofilm EPS or matrix provides a diffusion barrier that limits the entry of antimicrobial components either through size exclusion or binding and immobilization, effectively reducing the local concentration of antimicrobials (Stewart, 2003; Davenport et al., 2014). The biofilm matrix contains carbohydrates, proteins, lipids and extracellular DNA (eDNA) and also provides a cohesive mesh-like structure supporting biofilm integrity (Flemming et al., 2007; Flemming and Wingender, 2010). Among the best-investigated matrix components of the oral biofilm are the glucans. Treatment of those polymeric carbohydrates with glucan degrading enzymes results in significantly less biofilm biomass (Klein et al., 2015). *S. sanguinis* reference strain SK36 encodes two glucan forming glucosyltranferases (GTF), GtfB and GtfP respectively (Nobbs et al., 2009). However, this might not be entirely representative, since GTF activity was characterized in 10 strains with considerable variability in GTF activities (Herzberg et al., 1990) as well as inefficient glucan production and biofilm formation (Hamada et al., 1981; Kopec et al., 2001). Other studies have indicated that *S. sanguinis* GTF activity is influenced by a variety of environmental factors, such as sodium and potassium concentration or external pH (Keevil et al., 1984; Vacca Smith et al., 2000). Glucans synthesized by *S. sanguinis* GTFs have been shown to promote the adherence of diverse oral bacteria to saliva-coated hydroxyapatite and to increase biofilm formation (Yoshida et al., 2014). However, there is still scant evidence among the literature to indicate the overall contribution of glucans and GTFs to *S. sanguinis* biofilm formation. It has been suggested that *S. sanguinis* might also adhere to the extracellular glucan produced by other streptococci via putative glucan-binding proteins, such as GbpB, SspC, and SspD (Moraes et al., 2014). However, this has yet to be confirmed experimentally.

Extracellular DNA (eDNA) is another important component of the biofilm matrix for *S. sanguinis* as well as many other bacteria (Okshevsky and Meyer, 2015). For example, DNase treatment effectively disrupts *S. sanguinis* biofilms (Moraes et al., 2014), while mutant strains impaired in eDNA production also exhibit defects in biofilm formation (Zheng et al., 2011b; Ge et al., 2016). *S. sanguinis* produces high molecular weight chromosomally derived eDNA during aerobic growth in response to the H_2O_2 produced by the pyruvate oxidase enzyme SpxB (Kreth et al., 2008; Kreth et al., 2009). Presumably, it is the reduced eDNA production of the *spxB* mutant that is responsible for its deficient biofilms, which appear more sparsely populated than the wild type (Zheng et al., 2011b). A similar result could also be observed if H_2O_2 production is hindered by growth in environments with low oxygen tensions (Zheng et al., 2011a; Zheng et al., 2011c). The connection between oxygen availability and eDNA production correlates well with *S. sanguinis* role as a pioneer colonizer and early biofilm producer. Newly colonized sites on the tooth surface would be expected to be highly aerobic and conducive to the production of eDNA. However, once a biofilm has been established, the oxygen tension within the biofilm declines sharply (de Beer et al., 1994; Xu et al., 1998), which should result in a concomitant decrease in H_2O_2 and eDNA production. At this point, it is currently unclear whether eDNA release continues to occur through an H_2O_2-independent mechanism or if further eDNA production is simply not required once a biofilm is established. It should also be noted that oxygen is a readily

available substrate to stimulate early supragingival biofilm formation, whereas the substrates for GTF activity (sucrose) are only available intermittently at best. In addition, since eDNA promotes *S. sanguinis* cell-cell aggregation (Kreth et al., 2009), this could be one of its key functions to stimulate biofilm formation, especially at newly colonized sites.

The social life of *S. sanguinis*

Both cell-cell and metabolic interactions between *S. sanguinis* and other members of the oral biofilm community are major determinants of oral ecology and ultimately oral health. Surface adhesin and/or lectin-mediated interactions with other species provide genetically encoded mechanisms to directly select the composition of organisms in developing mixed species biofilms. A variety of *S. sanguinis* coaggregation partners have been identified including species of *Actinomyces*, *Prevotella and Porphyromonas* (originally grouped in the genus *Bacteroides*), *Capnocytophaga*, *Fusobacterium*, and *Candida* (Jenkinson et al., 1990). *Porphyromonas gingivalis* was shown to adhere to *S. sanguinis* (Stinson et al., 1991; Lamont et al., 1992) via a surface exposed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Maeda et al., 2004). Given the pathogenic potential of *P. gingivalis*, *S. sanguinis* might have measures in place to limit the overgrowth of *P. gingivalis* and other potentially damaging coaggregation partners via its aforementioned H_2O_2 production ability and perhaps other uncharacterized mechanisms as well. *S. sanguinis* was shown to antagonize a variety of periodontal pathogens (Herrero et al., 2016) in addition to its role in inhibiting the principal cariogenic species *S. mutans* (Kreth et al., 2008). It is worth mentioning that certain species antagonized by *S. sanguinis* also have mechanisms to inhibit *S. sanguinis* or weaken the effect of H_2O_2. For example, *S. mutans* is able to produce antimicrobial peptides called mutacins, which target closely related streptococci, including *S. sanguinis* (Merritt and Qi, 2012). Curiously, *S. sanguinis* seems to produce an extracellular (challisin-like) protease that can interfere with mutacin production by degrading the competence stimulating peptide CSP regulating mutacin gene expression (Wang and Kuramitsu, 2005). Furthermore, several oral species produce catalase, which might decrease the efficiency of H_2O_2 antimicrobial activity (Jakubovics et al., 2008). As shown for *Aggregatibacter actinomycetemcomitans*, H_2O_2 can also serve as a signaling molecule to regulate gene expression (Ramsey and Whiteley, 2009; Stacy et al., 2014). Thus, *S. sanguinis* may be able to utilize H_2O_2 production for interspecies signaling as well. It is also worth noting that saliva contains several H_2O_2 detoxifying enzymes (Ihalin et al., 2006; Ashby, 2008) that likely degrade the free H_2O_2 in the oral cavity. Therefore, the utility of H_2O_2 for interspecies interactions in the biofilm is presumably confined to microenvironments proximal to producers like *S. sanguinis* (Zhu and Kreth, 2012; Jakubovics et al., 2014). It is also worth noting that *in* vitro studies suggest H_2O_2 produced from streptococci can trigger cytotoxicity in endothelial cells (Okahashi et al., 2014). Thus, saliva exposure could be important for protecting against collateral damage from streptococcal H_2O_2 production during early biofilm development. Given the variety of functions of H_2O_2 for biofilm development and ecology, there may be potential therapeutic opportunities to exploit these pathways in commensal species for the prevention of dysbiosis or even for reshaping the species composition of a dysbiotic microbial community (Zhu and Kreth, 2012; Jakubovics et al., 2014).

pH modulating abilities of *S. sanguinis* in the oral biofilm

Plaque acidification not only triggers a net demineralization of tooth enamel, it also selects for the overgrowth of aciduric species like the lactobacilli and the mutans streptococci. As a countermeasure to prevent this, *S. sanguinis* and other commensals have a variety of mechanisms to raise the local plaque pH. *S. sanguinis* has two principal approaches to do this. i) The aforementioned pyruvate oxidase enzyme SpxB generates both CO_2 and acetyl phosphate as end products in addition to H_2O_2 (Zhu and Kreth, 2012). ii) *S. sanguinis* catabolizes arginine via the arginine deiminase system (ADS) generating ornithine, ammonia, CO_2, and ATP (Burne and Marquis, 2000). Since ammonia is a base, it can directly increase the local pH and *S. sanguinis* was recently demonstrated to be a substantial contributor to the alkali generation capacity of dental plaque (Huang et al., 2015). Huang *et al.* speculated that deficiencies in alkali production by the ADS might be equally important for caries development as the production of acid itself (Huang et al., 2015). Similarly, a variety of recent clinical studies have demonstrated net increases in plaque pH and lower caries scores when given arginine-supplemented dentifrices (Kraivaphan et al., 2013; Nascimento et al., 2014). In future studies, it will be interesting to determine if/how arginine supplementation influences plaque species composition in these subjects.

Remaining under the radar – *S. sanguinis* interactions with the immune system

Commensal bacteria play crucial roles in the proper development of the immune system. For example, the development of intestinal immunity as well as the anatomical development of the gut-associated lymphoid tissue (GALT) is closely connected to the presence of the intestinal commensal microbiota (reviewed in (Palm et al., 2015). While those processes are well characterized for intestinal immunity, far less is known about analogous processes in the oral cavity (Lang et al., 2010). Current data suggests that commensal bacteria also influence immune system development during early colonization of the oral mucosa, while certain immune mechanisms are in place prior to colonization to provide initial protection (Lang et al., 2010). The gingival tissue must properly balance its response to bacterial colonization to benefit from the presence of commensal species, while still having the ability to mount a robust immune response when the ecology of the flora is disturbed. A comparative study of human immune cell responses to *S. sanguinis* and several other viridans streptococci illustrated the weak ability of *S. sanguinis* to activate CD45+, CD4+, and CD8+ cells, resulting in low levels of cytokine production and a poor anti–*S. sanguinis* humoral response (Salam et al., 2006). Likewise, incubating *S. sanguinis* with human oral keratinocytes results in no significant induction of antimicrobial peptide production, whereas both human beta defensin-3 and LL-37 are significantly induced by the closely related organism *S. gordonii* (Ji et al., 2007). While *S. gordonii* is also a pioneer colonizer of the human oral cavity, it is noteworthy that its abundance is typically maintained at much lower levels compared to *S. sanguinis* (Nyvad and Kilian, 1990; Tappuni and Challacombe, 1993; Mager et al., 2003; Li et al., 2004). Another study of gingival keratinocytes similarly observed no significant induction of matrix metalloproteinase 9 as well as a variety of beta defensins and cytokines in response to incubation with *S. sanguinis* cell wall extracts.

Furthermore, the authors also observed that coincubation of *F. nucleatum* and *S. sanguinis* was able to reduce the inflammatory potential of *F. nucleatum* via a TLR2-dependent mechanism (Peyret-Lacombe et al., 2009). Similarly, when the commensal species *S. sanguinis, S. mitis,* or *S. salivarius* were coincubated with the periodontopathogen *A. actinomycetemcomitans*, HOK-18A oral keratinocytes produced significantly less IL-8 in response to *A. actinomycetemcomitans* (Sliepen et al., 2009). In this study, a diffusible factor was most likely responsible for the reduction in IL-8 production, as cell-free supernatants of these commensals reproduced this same anti-inflammatory effect (Sliepen et al., 2009). Interestingly, *S. gordonii* was not found to exhibit this same ability (Sliepen et al., 2009). In addition to oral keratinocytes, *S. sanguinis* also fails to elicit IL-8 and tumor necrosis factor- α production from human whole blood cells (Tietze et al., 2006), while *S. sanguinis* peptidoglycan was shown to reduce cytokine production in THP-1 monocytes stimulated with the purified lipopolysaccharide (LPS) of several periodontopathogens (Lee, 2015). The latter result is of particular interest because Gram positive bacteria constantly release large amounts of peptidoglycan fragments called muropeptides as a consequence of cell wall remodeling during normal growth and cell division (Dworkin, 2014). The next logical step would be to determine whether diffusible muropeptides from commensals like *S. sanguinis* might serve as global anti-inflammatory agents of the mucosae. It would be particularly intriguing if this effect occurred through the previously described TLR2-dependent pathway (Peyret-Lacombe et al., 2009), since lipoteichoic acids in peptidoglycan normally serve as potent TLR2 agonists to trigger inflammation (Moreillon and Majcherczyk, 2003; Draing et al., 2008). It is conceivable that the anti-inflammatory ability of the commensal flora could be one of the key mechanisms required to reduce the inflammatory potential of other lower abundance members of the flora in order to maintain host symbiosis.

Genomic comparison of key genes involved in *S. sanguinis* commensalism

The universal success of *S. sanguinis* as an early colonizing commensal species in the oral cavity suggests that key genes and pathways crucial for its commensalism should be conserved among most or all isolates (Fig. 1). By comparing 25 publically available *S. sanguinis* genomes, we screened for the presence of key genes and operons previously described in this review. In all 25 strains, we could detect the arginine deiminase system, the glucan binding protein PcsB, the dual function adhesin/Mn^{2+} transporter SsaB, and the pyruvate oxidase SpxB. These genes are all highly conserved and likely to be part of the core *S. sanguinis* genome. The glucosyltransferase GtfP and the glucan binding proteins, SspC and SspD, can be found in 24 of the 25 strains, indicating they too have a similar level of conservation. Twitching motility is conserved in most strains, but is not as highly conserved overall (Fig. 2 and Table 1). Since adhesion is a critical trait for *S. sanguinis* ability to colonize and establish biofilms, we also examined these genomes for several uncharacterized putative adhesins identified in the genome of SK36 (SSA_0227, SSA_0453, SSA_0805, SSA_1019, and SSA_1666) (Kitten et al., 2011). In nearly all of the genomes, obvious orthologs of these proteins were detectable, with each sharing over 80% identity to SK36 (Table 2). The predicted adhesins encoded by SSA_0453 and SSA_1019 can be found in 22 out of the 25 genomes, while the putative adhesins SSA_0227, SSA_0805 and

SSA_1666 were found in 21 strains. It is worth noting that most publically available *S. sanguinis* genome sequences are draft genomes still in various stages of assembly. Adhesins in particular tend to be difficult to assemble due to the presence of repeat regions and are often found in the gaps between genome contigs. Therefore, it is possible that these aforementioned adhesins are actually present in all 25 strains, potentially even belonging to the core *S. sanguinis* genome. Overall, there seems to be a strong conservation for genes required for commensalism among *S. sanguinis* strains. In future studies, it will be of particular interest to determine whether the genetic pathways responsible for the anti-inflammatory qualities of *S. sanguinis* are also as highly conserved among strains. Such comparisons will have to wait until the genetic mechanisms of *S. sanguinis* immunomodulation are understood.

In the context of the oral biofilm, some of the traits described in this review are also conserved in other oral streptococcal species, such as the ability to produce competitive amounts of H_2O_2 via SpxB as well as alkali generation via the arginine deiminase system. The following oral streptococci encode *spxB* orthologs: *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus infantis*, *Streptococcus oralis*, *Streptococcus oligofermentans* and *Streptococcus cristatus* (Zhu et al., 2014) and a recent investigation identified arginine deiminase activity in *Streptococcus parasanguinis*, *Streptococcus intermedius*, *Streptococcus cristatus* and *Streptococcus gordonii* (Huang et al., 2015). This observation is consistent with the notion that certain metabolic functions of the oral biofilm determine health and disease status. Given the abundance of *S. sanguinis* in the oral cavity, it presumably makes a substantial contribution to the total metabolic output of these pathways.

Conclusion

There has been tremendous progress made in our understanding of the mechanisms of molecular pathogenesis in both caries and periodontal disease. However, much less is known about the other side of the equation, which is what we refer to as molecular commensalism. It is clear that shifts in oral bacterial ecology are harbingers of disease, especially when it occurs at the expense of the commensal flora, *S. sanguinis* among them. This raises the interesting question of whether it is possible to restore and/or bolster the commensal flora as an alternative approach to improve oral health. While such a strategy seems logical, if not obvious, there is a dearth of *in vivo* evidence to support this approach. As we develop a more thorough understanding of the mechanisms of molecular commensalism, we can expect to discover new strategies to promote the competitiveness of the commensal flora during ecologically challenging conditions that might otherwise lead to disease. This could be through the exogenous management of critical genetic responses in the flora or simply via probiotic supplementation. A case in favor of the former approach could be illustrated by the improvements observed in plaque pH due to arginine supplemented dentifrice usage (Nascimento et al., 2014), while the latter approach is supported by the recent successes with probiotic treatments for *Clostridium difficile* induced colitis (CDI) [a summary of successful application of probiotics to treat or prevent CDI can be found in (Spinler et al., 2016)]. For an oral probiotic approach, *S. sanguinis* could be regarded as an ideal candidate (Pamer, 2016), as it is an efficient colonizer, does not express obvious virulence factors, and it can modulate the inflammatory response. Given the relative simplicity of *S. sanguinis* isolation,

it may even be practical to create probiotic supplements using patient-specific isolates, thus providing *S. sanguinis* with its ideal ecological niche for colonization. Though, such an approach may not be appropriate for people with known heart valve defects, due to an elevated risk of infective endocarditis.

A surprisingly large number of polymicrobial diseases of the mucosae are the result of dysbiosis among the mucosal flora and most of these infections currently remain difficult to treat effectively. The oral system has proven to be an exceptional model system to characterize the key concepts driving pathogenesis in these types of diseases, since the organisms are easily accessible, well characterized, and many are genetically tractable. Indeed, the ecological basis for oral disease has been a driving force in the field for decades. For similar reasons, the oral system is ideally situated to serve as a preeminent model system of molecular commensalism as well. Presumably, the key mechanisms supporting symbiosis at the oral mucosa are also functioning analogously at other mucosal sites in the body, albeit with a different cast of characters. Thus, there is a prime opportunity for the oral microbiology and immunology community to further its leadership in our understanding of the interplay between the flora and host as well as potential strategies to exploit this knowledge for therapeutic benefit.

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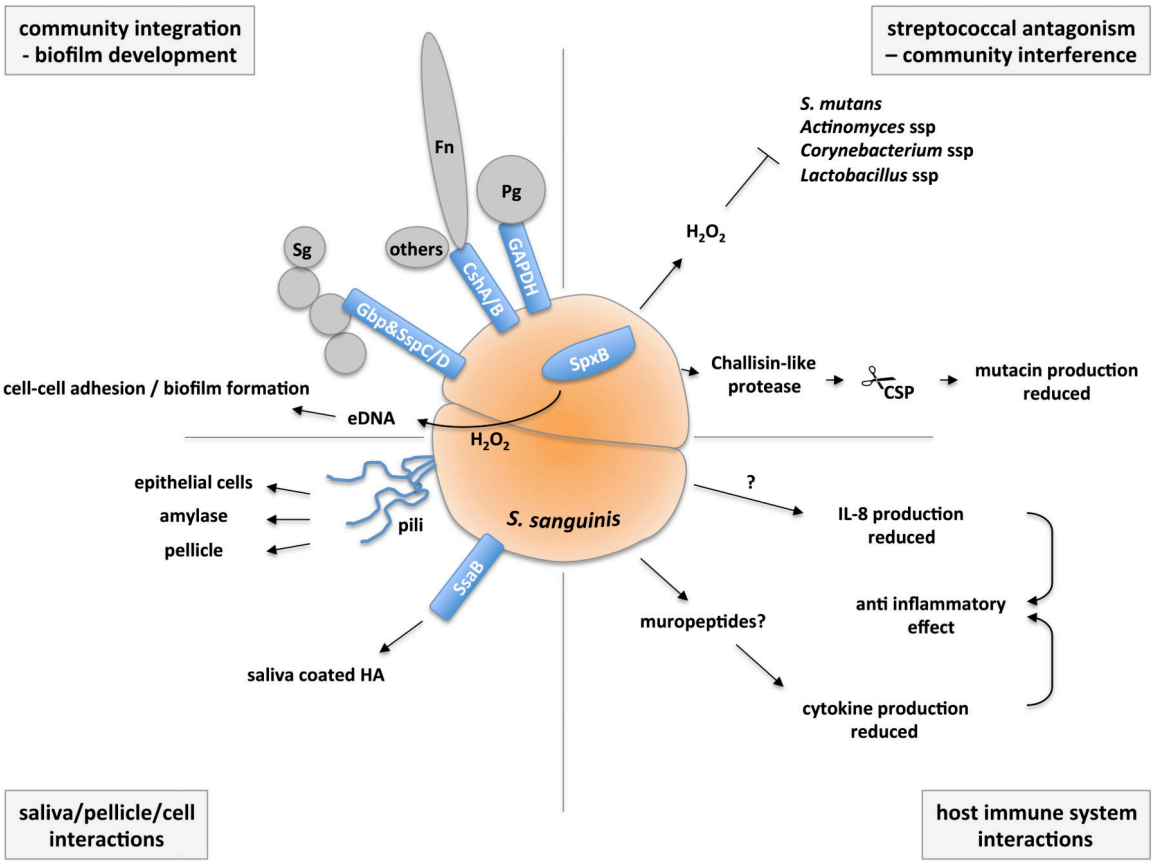

Fig. 1.

Summary of key *S. sanguinis* components important in commensalism. The schematic shows important components for *S. sanguinis* role as commensal organism, including community integration and biofilm development, community interference and streptococcal antagonism, and interactions with salivary proteins, host cells, and the immune system. Pg = *P. gingivalis*; Fn = *F. nucleatum*; Sg = *S. gordonii*; eDNA = extracellular DNA; CSP = competence stimulating peptide.

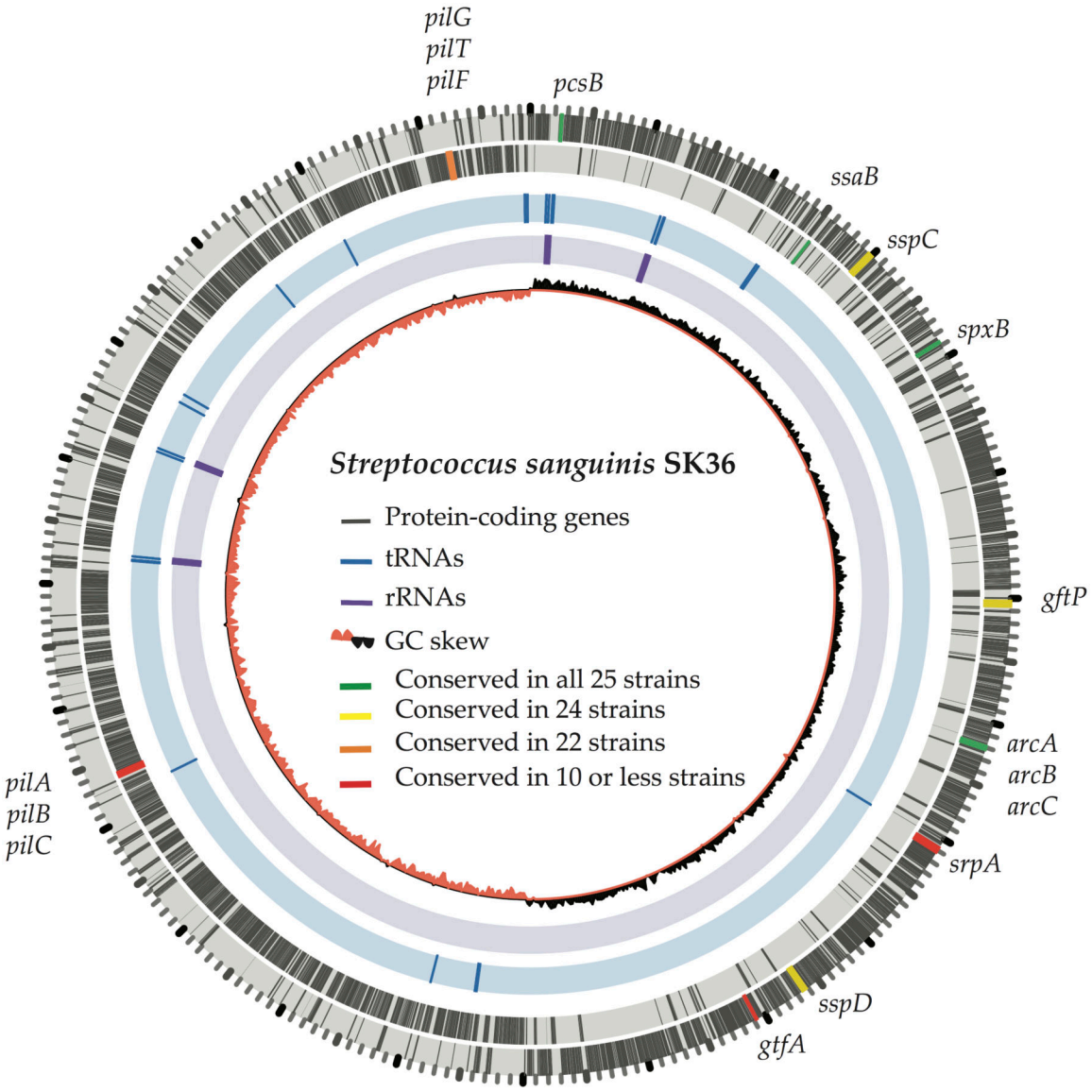

Fig. 2.

Genome of *Streptococcus sanguinis* SK36. The two outer rings illustrate protein-encoding genes (in grey) on forward and reverse strands, respectively. The position and conservation of several functional genes (Tab. 1) are also highlighted. The innermost ring shows the GC skew. Interestingly, most genes are located on the leading strand of each replichore. Additionally, most tRNAs (blue, ring 3) and rRNAs (purple, ring) are clustered around the origin of replication, possibly to optimize the growth rate.

Table 1

Conservation of selected genes implicated in commensalism in 25 sequenced strains of *S. sanguinis*

Gene	Function	No. of strains present
arcA	Arginine deiminase system	25
arcB	Arginine deiminase system	25
arcC	Arginine deiminase system	25
pcsB	Glucan-binding	25
spxB	Pyruvate oxidase	25
ssaB	Adhesion, Mn2+ transport	25
gtfP	Glucosyl transferase	24
sspC	Glucan-binding	24
sspD	Glucan-binding	24
pilF	Twitching motility	22
pilG	Twitching motility	22
pilT	Twitching motility	22
gtfA	Glucosyl transferase	10
pilA	Pilus, attachment to amylase	10
pilB	Pilus, attachment to amylase	10
pilC	Pilus, attachment to amylase	9
srpA	Mucin binding	1

Conservation of genes in 25 fully annotated genomes of *S. sanguinis* was determined by BLASTP. *S. sanguinis* SK36 proteins were used as queries, and an E-value of at least 1e-5, ≥70% coverage of the length of the query protein, and ≥70% amino acid identity was required to classify a protein as being conserved. The following strains were included in the analysis: SK36; VMC66; SK353; SK405; SK678; SK72; SK115; SK150; SK160; SK1; SK1057; SK330; SK408; SK1058; SK1087; SK1059; SK49; SK1056; SK355; SK340; ATCC 29667; CC94A; VT517; I141; 2908

Table 2

Conservation of predicted adhesins in 25 sequenced strains of *S. sanguinis*

SSA_designation	predicted function	No. of strains present
1019	collagen binding surface protein	22
453	pullanase/glycosidase	22
227	collagen-binding surface protein	21
805	collagen-binding surface protein	21
1666	collagen-binding surface protein	21

Conservation of open reading frames listed as SSA_designations from strain SK36 which served as template in the search of 25 fully annotated genomes of *S. sanguinis* using BLASTP. An E-value of at least 1e-5, ≥70% coverage of the length of the query protein, and ≥70% amino acid identity was required to classify a protein as being conserved.