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Genetics of *sanguinis*-group streptococci in health and disease

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INTRODUCTION

Our view of oral streptococci has largely been influenced by the approach taken in the last century to identify etiologic agents of disease. As a consequence, beneficial aspects of streptococcal colonization of the oral cavity were initially overlooked. The first comprehensive analysis of the resident oral microbiota was accomplished in 2005 (1) and with this, a new picture began to emerge. With the availability of high throughput sequencing techniques and an increased sensitivity in analysis methods, the presence of a defined microbiome associated with oral health has been shown (2). Alongside this, 'omics' techniques have revealed that prevalent oral diseases such as caries and periodontal disease are polymicrobial in nature and the result of microbial dysbiosis (3, 4). Even more striking, the metabolic output of these mixed microbial communities seems to be more relevant than their precise microbial composition (4). This is also reflected by the fact that the severity of caries and periodontal disease is heavily influenced by the synergistic interactions of the individual members of the polymicrobial consortium, including metabolic cross-feeding and interspecies signaling with transcriptional adjustment to the metabolic output. Thus, the ecological context of the microbial community seems to be of importance to understand oral health and disease development. As a consequence, polymicrobial diseases cannot be explained by the behavior of one bacterial species and certainly cannot be treated like diseases that follow Koch's postulates (5-7). Novel approaches to combat oral polymicrobial diseases should therefore focus on the bacterial community that is present in the healthy oral cavity. Since oral streptococci are abundant during initial colonization of the tooth (8, 9), their function is to provide a favorable environment for incorporation of later species and to support

24 accretion of the mature oral biofilm, which in general has a health-protecting function (10,
25 11).

26 One of the oral *Streptococcus* species that is repeatedly isolated in great
27 abundance as part of the health-associated microbiome is the initial colonizer
28 *Streptococcus sanguinis* (12-14). Because of the strong association of *S. sanguinis* with
29 oral health, this commensal can serve as a model to understand how an individual species
30 is able to interact with other members of the bacterial community to shape the composition
31 of a benign oral biofilm. *S. sanguinis* is in general classified as a non-spore-forming,
32 catalase-negative, chain-forming coccus. *S. sanguinis* is non-beta-hemolytic, but is able
33 to produce a green coloration on blood agar plates referred to as *alpha*-hemolysis, which
34 is a consequence of hydrogen peroxide (H_2O_2) production (15). *S. sanguinis* has been
35 placed into the *mitis* group of streptococci based on 16S rRNA sequence analysis;
36 however, it has also been classified in its own group together with *S. gordonii* and *S.*
37 *parasanguinis* (15). A more recent analysis using the housekeeping genes *rpoB*, *sodA*,
38 *ddl*, and *gdh* showed a more distant relationship with *S. parasanguinis*, but confirmed the
39 phylogenetic relationship with *S. gordonii* (16). Here we present the *sanguinis*-group
40 streptococci in the context of molecular commensalism, highlighting those aspects of their
41 biology that are important for health-associated biofilm development, including
42 polymicrobial interactions, regulatory and mechanistic events (Figure 1).

43

44 **MOLECULAR DETERMINANTS OF *S. SANGUINIS* AS A COMMENSAL PIONEER**
45 **COLONIZER**

46 Initial colonization is intimately linked to the adhesion capabilities of oral
47 streptococci. *S. sanguinis*, together with *S. gordonii*, *S. oralis* and *S. mitis*, are well

48 adapted for initial colonization. These streptococci express a multitude of bacterial surface
49 proteins, named adhesins, which are able to interact with salivary proteins covering the
50 tooth surface (17-19). *S. sanguinis* seems to be particularly well equipped with adhesins
51 that recognize this salivary pellicle (20). Overall, initial colonization has two important
52 consequences. First, the ecological niche is occupied, which plays a significant role in the
53 process of colonization resistance and the exclusion of potential incoming pathogens.
54 Second, initial colonization shapes the overall composition of the biofilm, since the pioneer
55 colonizer can produce metabolic products or provide a substratum for compatible partner
56 species. Initial biofilm development requires the formation of macromolecular complexes.
57 Complex formation is facilitated through electrostatic interactions of salivary proteins with
58 the tooth surface to form the acquired enamel pellicle, a process that occurs within
59 seconds after a clean enamel surface is exposed to saliva (21). Microbial attachment to
60 the acquired enamel pellicle is then mediated via protein-protein and lectin-like
61 interactions. The protein content of the acquired enamel pellicle is thus a major
62 determinant of colonization sequence, dictating strength of microbial adhesion as well as
63 localization, since pellicle proteins seem to differ according to anatomical site (22).

64 A prominent protein in saliva and the acquired enamel pellicle is α-amylase,
65 responsible for the catalytic hydrolysis of starch (23, 24). Amylase binding proteins have
66 been identified in several oral streptococcal species (24). Best studied is the amylase-
67 binding protein A (AbpA) in *S. gordonii* (25). Mutation of AbpA results in deficient biofilm
68 formation and bacterial adhesion *in vitro* (26). Although the sequenced reference strain *S.*
69 *sanguinis* SK36 seems to encode an *abpA* homolog in a similar chromosomal context with
70 its accessory sortase, *srtB* (27), its function is currently unknown. Interestingly, *S.*

71 *sanguinis* is able to bind directly to surface-bound amylase and *vice versa* (24). This
72 function has been shown to be mediated by long filamentous pili, organized in a four-
73 gene operon (ssa1631-1634) that encodes three pilin subunits (PilA-C) and a dedicated
74 sortase, SrtC, for cell-surface anchoring of the pilin structural proteins (28). Pili are
75 relatively long and thin appendages and the pili of *S. sanguinis* can be as long as 1 μm,
76 as shown by immune-gold staining of PilA (28). This poses a potentially interesting
77 dynamic with AbpA, which is strictly confined to the outer cell surface, as shown for *S.*
78 *parasanguinis,* also with immune-gold labeling (27). Taking into account that pili are
79 flexible, one could hypothesize that binding to amylase in acquired enamel pellicle is
80 possible even when the molecule is scarce, since the pili could serve as a flexible "arm",
81 latching onto free amylase within saliva. A recent publication demonstrating that
82 amylase in acquired enamel pellicle is actually less abundant when compared to saliva
83 (29) is in agreement with this hypothesis, suggesting an advantage of a flexible "arm" over
84 a rigid arrangement on the bacterial surface. The pili also showed binding to other salivary
85 proteins and their deletion diminished biofilm formation on saliva-coated surfaces (28).
86 However, the mutant was still able to bind amylase, albeit with lower efficiency (28),
87 suggesting that other surface proteins are also able to bind amylase, possibly the
88 aforementioned AbpA homolog (27). A pilus-bound amylase also offers the advantage of
89 retaining about 50% of its enzymatic function (30). Gaining access to the amylase
90 substrate starch through a flexible pilus would increase the chance for hydrolysis of the
91 alpha-1,4-glycosidic linkage into glucose, maltose and maltodextrins, promoting sugar
92 uptake and subsequent metabolism by simply increasing the accessible radius of the cell.
93 Two major mucins are found in saliva, MUC7 (low molecular weight) and MUC5B
94 (high molecular weight) (31, 32). The majority of mucins are synthesized and secreted

95 by the submandibular and sublingual glands, as well as minor glands located in the
96 palatal, buccal and labial mucosae. Mucins are heavily glycosylated glycoproteins and
97 form a lubricating, viscoelastic coating on all oral surfaces. They are abundant proteins
98 in saliva and the acquired enamel pellicle (31, 32). Both MUC7 and MUC5B contain
99 sialic acid as a glycoconjugate and this can be targeted by *S. sanguinis* SK36 sialic-
100 acid-binding adhesin SrpA (33). SrpA contains a subdomain in its binding region that is
101 similar to the V-set Ig-like fold adopted by mammalian Siglecs (sialic acid-binding
102 immunoglobulin-like lectins) (34, 35). Indeed, Siglec-like domains have been identified
103 in potential adhesins of several *S. sanguinis* isolates and other oral streptococci (34).
104 Glycoarray dot blots with human salivary samples and naturally occurring
105 glycoconjugates have demonstrated a high specificity of *S. sanguinis* SK36 SrpA for
106 MUC7, but no binding to MUC5B. This is in contrast to *S. gordonii*, which showed in
107 general better binding to several glycoconjugates, including MUC5B and amylase (34),
108 which are known to form a heterotypic complex (36). As mentioned above, amylase and
109 MUC5B seem to be depleted in the acquired enamel pellicle compared to saliva (29),
110 but this was not seen for MUC7. Thus specificity of binding to MUC7 within the acquired
111 enamel pellicle may go some way to explain why *S. sanguinis* seems to be one of the
112 first oral colonizers and found in greater abundance compared to *S. gordonii*. The
113 glycoarray also revealed an interesting role for divalent cations Ca^{2+} and Mg^{2+} in the
114 binding of *S. sanguinis* SK36 to MUC7, as well as to other glycoconjugates (34).
115 Chelation of Ca^{2+} and Mg^{2+} decreased binding to several of the tested components.
116 This was also observed with two *S. gordonii* strains (34), suggesting a dominant role
117 for divalent cations in the binding process of not only *S. sanguinis*, but of other oral
118 streptococci to salivary components and the acquired enamel pellicle. This is further

119 supported by the increased abundance of proteins known to bind divalent cations like
120 Ca^{2+} in the acquired enamel pellicle compared to saliva (29). Interestingly, *S. sanguinis*
121 encodes a surface-associated, dual-function protein that bridges substratum
122 attachment and interactions with divalent cations. SsaB is able to bind to saliva-coated
123 hydroxyapatite through an unknown mechanism (37), but its principal function seems
124 to be the transport of divalent cations (38). While this transport was demonstrated to be
125 specific for Mn^{2+} and Fe^{2+}, playing a pivotal role in oxidative stress defense (38), other
126 divalent cations like Ca^{2+} and Mg^{2+} might still be able to interact with SsaB to facilitate
127 binding to the acquired enamel pellicle.

128 Recently the ability of *S. sanguinis* to be motile on surfaces like solidified agar has
129 been reported for several strains. Spreading zones after prolonged incubation can be
130 observed around colonies, indicating active movement (39). Whether or not this so-called
131 'twitching motility' has any function or is used as a means to disseminate within the oral
132 biofilm is not yet clear. However, the *pil* locus that encodes the type IV pilus involved in
133 twitching motility is conserved in most of the sequenced *S. sanguinis* genomes (39),
134 suggesting biological importance.

135 Overall, *S. sanguinis* does not rely on a single mechanism to bind and establish
136 itself within the acquired enamel pellicle. Rather the process is elaborate and ensures
137 the role of *S. sanguinis* as a pioneer colonizer. This correlates with the observation that
138 signal peptidase I is required for biofilm development (40). In general, signal peptidases
139 are membrane-bound endo-proteases that cleave the signal peptide portion from the
140 majority of secreted proteins (41). *S. sanguinis* encodes two signal peptidases,
141 SSA_0849 and SSA_0351, which are crucial for biofilm formation. Deletion of
142 SSA_0351 abolishes biofilm formation but does not affect planktonic growth. Although

143 the substrates for signal peptidase processing are not experimentally verified, *in silico*
144 prediction identified 168 potential candidates, including several adhesins (40). Further
145 characterization of signal peptidase processed surface proteins will most likely identify
146 new proteins involved in the adhesion process.
147
148 ***S. SANGUINIS* IN BIOFILM FORMATION**
149 The initial step in biofilm development is attachment, followed by micro-colony
150 formation of newly attached cells through growth expansion. This process then leads into
151 a series of ordered and temporal events, in which coadhesion predominates, ultimately
152 resulting in formation of a mature biofilm. The production of extracellular matrix material
153 is a defining step in biofilm development and maturation. Matrix materials include
154 polymers such as carbohydrates, proteins, extracellular DNA (eDNA) and lipids, and are
155 collectively referred to as extracellular polymeric substances (EPS) (42). Since EPS is
156 produced by the biofilm inhabitants themselves, specific enzymatic functions and cellular
157 processes are associated with its formation.
158 Exploiting the intake of dietary sugars, many oral *Streptococcus* species have
159 evolved glucosyltransferases (Gtfs) that hydrolyze sucrose and polymerize the glucose
160 into glucans. These, in turn, promote biofilm development. A single Gtf, GtfP, is carried
161 on the *S. sanguinis* genome, which synthesizes mainly water-soluble α-1,6-linked glucans
162 that branch at α-3,6-linked glucose residues (43). TetR family regulator BrpT was recently
163 identified as a repressor of *gtfP* expression. While a *gtfP* mutant formed only a fragile
164 biofilm, the biofilm formed by the *brpT* mutant was thicker, more robust, and with a higher
165 glucan content (44). By modulating levels of glucan synthesis, BrpT may therefore control

166 switching of *S. sanguinis* biofilms from an adherent to a dissemination state. *S. gordonii*
167 also carries a single *gtf* gene, *gtfG*, encoding an enzyme that synthesizes both α-1,3- and
168 α-1,6-linked glucans. This activity was found to promote mixed biofilm formation with
169 *Candida albicans* (45), and a *gtfG* mutant was unable to persist on the tooth surface in
170 rats (46). Expression of GtfG is under the positive regulation of Rgg (47), a homolog of
171 which is present in *S. sanguinis*. As for other oral streptococci, it is likely that expression
172 of Gtfs and thus glucan content of biofilms is a tightly controlled process, affected by
173 multiple factors and transcriptional regulators.

174 Compared to carbohydrates, the presence of eDNA within EPS is a relatively
175 recent discovery. Nonetheless, growing evidence suggests that eDNA is a critical
176 contributor to cell-to-cell adherence and overall biofilm stability (48). Courtesy of its
177 negative charge, eDNA may facilitate association with the acquired salivary pellicle (49),
178 and cell aggregation was shown to be promoted by eDNA for *S. sanguinis*, although not
179 for *S. gordonii* (50). Visualization of 'yarn' and 'sweater' structures of eDNA that wrap
180 around cells within *Enterococcus faecalis* biofilms (51) provides compelling evidence for
181 how eDNA may contribute to the structural integrity of biofilms, and similar structures have
182 been seen for biofilms of *S. gordonii* (A.H. Nobbs, unpublished data). Moreover, eDNA
183 regulates the viscoelastic properties of biofilms that allows them to withstand mechanical
184 stress (52), a property that is particularly pertinent to the oral cavity environment. In line
185 with this, the presence of DNABII proteins within EPS of *S. gordonii* and other oral
186 bacterial biofilms was recently found to be essential for eDNA integrity and biofilm
187 structure (53). The regulation of eDNA release is not fully understood, and both lytic and
188 active mechanisms have been reported. For *S. sanguinis* and *S. gordonii*, autolysins LytF

189 and AtlS play significant roles via mechanisms closely linked to H_2O_2 production and
190 competence development. These are discussed in more detail later.

191 Multicellular entities such as biofilms require a high level of coordination, and
192 quorum sensing (QS) circuits and two component signaling systems (TCS) are intimately
193 involved in these processes. One QS system found across several bacteria is LuxS/AI-2.
194 LuxS is an integral component of the activated methyl cycle (AMC) for correct methylation
195 of nucleic acids and proteins, but as a by-product of this cycle generates autoinducer 2
196 (AI-2), an interspecies chemical signal. LuxS mutants in *S. gordonii* and *S. sanguinis* were
197 altered in their ability to form biofilms, and lack of AI-2 resulted in altered *S. gordonii*
198 microcolony architecture (54). However, a definitive role for AI-2 has not been identified
199 and for *S. sanguinis*, a disrupted AMC rather than absence of AI-2 was found to underpin
200 the *luxS* mutant biofilm phenotype (55). A TCS associated with regulation of biofilm
201 formation for both *S. sanguinis* and *S. gordonii* is BfrAB (56). This modulates expression
202 of two ABC transporters (BfrCD, BfrEFG) and a putative membrane-bound
203 metalloprotease (BfrH). Given such functions, it is postulated that this system transports
204 and processes proteins or peptides across the cell membrane that promote biofilm
205 development, although the precise targets have yet to be identified. More recently, defects
206 in TCS SptRS have been found to promote biofilm formation by *S. sanguinis*, associated
207 with elevated levels of H_2O_2 and eDNA (57), while standalone ArcR was identified as a
208 key regulator of *S. gordonii* biofilm development, perhaps via modulation of the
209 phosphotransferase system (58).

210 A final aspect of streptococcal biology that is closely associated with biofilm
211 formation is competence development. This is a QS system that controls transformation,
212 i.e. the capacity for bacteria to actively take up exogenous eDNA, and in species such as

213 *S. sanguinis* and *S. gordonii*, is regulated via the *comCDE* operon (59, 60). Gene *comC*
214 encodes a precursor molecule that is cleaved and exported via ABC transporter ComAB,
215 releasing the mature competence stimulating peptide (CSP) into the local environment.
216 For *S. gordonii* this is a 19-amino acid peptide, while the CSP of *S. sanguinis* is 17-amino
217 acid residues, thereby minimizing interspecies cross-talk. The CSP is sensed by TCS
218 ComDE. Once the CSP pheromone exceeds a threshold concentration, ComD
219 phosphorylates ComE, which upregulates expression of early competence genes
220 including *comCDE* and *comAB*, establishing a positive feedback loop, and *comX*. ComX
221 then drives expression of the late competence genes required for DNA binding, uptake
222 and recombination. The coordination of competence and biofilm development contributes
223 to the adaptability of bacteria such as *S. sanguinis* to changing environmental conditions
224 via horizontal gene transfer (HGT). This will be explored in more detail later.
225

226 **_S. SANGUINIS_ IN COMMUNITY DEVELOPMENT**

227 *S. sanguinis* and *S. gordonii* can form monospecies biofilms, but within the host the
228 biofilm communities are typically polymicrobial in nature. As pioneer colonizers, these
229 streptococci can have profound consequences for niche occupation and subsequent
230 colonization by incoming species and thus significantly influence whether a community is
231 predisposed to health or disease. Such interactions do not occur at random, but rather
232 are directed in an ordered and temporal manner as a consequence of direct physical
233 engagement (coadhesion), metabolic relationships and interspecies communication.

234 Alongside streptococci, *Actinomyces* species constitute the predominant, health-
235 associated early colonizers of the oral cavity, and both *S. sanguinis* and *S. gordonii* are
236 able to coadhere with *Actinomyces oris*. This is mediated by recognition of streptococcal

237 receptor polysaccharide (RPS) containing linkages GalNAcβ1-3Gal or Galβ1-3GalNAc by
238 the FimA subunit of *A. oris* type 2 fimbriae (61, 62), with variations in the genetic loci for
239 synthesis of RPS (*rps*) and RPS precursors (*rml*, *galE1*, *galE2*) subtly altering the
240 coadhesion profile with different streptococcal species (63). In addition, *S. gordonii*
241 antigen I/II family protein SspB targets an extracellular polysaccharide produced by *A.*
242 *oris*, although the precise composition and structure of this polysaccharide has yet to be
243 determined (64). Once bound, *A. oris* may then promote *S. gordonii* survival under low
244 arginine conditions by stabilization of arginine biosynthesis (65). Similarly, *S. gordonii*
245 adhesin Hsa binds surface receptor Hag1 of early colonizer *Veillonella* species (66), but
246 this interaction is also underpinned by a strong metabolic dependency. Lacking a fully
247 functional glycolytic pathway, veillonellae must utilize hydroxyl acids for growth. These
248 are provided by streptococci as excreted metabolic waste product lactate and utilization
249 of lactate, in turn, protects streptococci from low pH (67). Once established, this
250 community of pioneer colonizers then supports the incorporation of secondary or late
251 colonizers, with *Fusobacterium nucleatum* serving as an important 'bridging' organism
252 due to its promiscuous coadhesion capabilities. *S. sanguinis* supports this engagement
253 via interaction with the arginine-inhibitable adhesin RadD of *F. nucleatum* (68), while a
254 second fusobacterial outer membrane protein, coaggregation mediating protein A
255 (CmpA), has recently been shown to promote biofilm formation with *S. gordonii* (69).

256 These community interactions with compatible species illustrate how *sanguinis*-
257 group bacteria are able to promote development of a health-associated microbiota.
258 Nonetheless, the social life of these bacteria is not exclusively beneficial and other
259 microbial partnerships may facilitate a more disease-prone state. Such examples have
260 largely been described for *S. gordonii*, rather than for *S. sanguinis*, leading to the

261 designation of *S. gordonii* as an 'accessory pathogen'. Some of the best characterized
262 interactions are with periodontopathogens *Porphyromonas gingivalis* (70, 71) and
263 *Aggregatibacter actinomycetemcomitans* (72), and with fungal pathogen *C. albicans* (73).
264 Evidence from in vitro studies and animal models shows that these relationships can
265 enhance both the persistence and virulence potential of the microbes involved (74).

266 Chemical communication in modulation of the oral microbial community is
267 exemplified by the complex effects of interspecies signaling molecule AI-2. This molecule
268 promotes dual species biofilm formation between *S. gordonii* and *S. oralis* but can also
269 modulate the relative proportions of these species in a concentration-dependent manner
270 (75). Likewise, while AI-2 from *F. nucleatum* promotes biofilm development with *S.*
271 *gordonii*, it has the opposing effect on *S. oralis* (76). AI-2 from *S. gordonii* is essential for
272 mutualistic biofilm growth with *A. oris* (77), but may also promote biofilm formation with *P.*
273 *gingivalis* (78) and *C. albicans* (79). Ultimately, QS molecules and peptide pheromones
274 work together with the molecular mechanisms described above to exquisitely coordinate
275 biofilm development. The result is a community optimized to survive and persist under the
276 prevailing environmental conditions in a manner that exceeds the capabilities of the
277 individual component species.

278

279 **COMPETITIVE BEHAVIOR - INHIBITION BY *S. SANGUINIS* H_2O_2 AND BACTERIOCIN**
280 **PRODUCTION**

281 Addressed above are examples of synergistic interactions with *sanguinis*-group
282 streptococci that promote incorporation and retention of the partner microbes within the
283 biofilm community. Nonetheless, within a defined ecological niche, resources are limited
284 and thus, competitive forces also work to shape the developing biofilm. One of the best

285 investigated competitive measures of *S. sanguinis* (and the vast majority of oral
286 streptococci) is the production of H_2O_2 (80). The enzyme responsible for the production of
287 H_2O_2 is pyruvate oxidase, SpxB (81, 82). SpxB is encoded by the majority of commensal
288 oral streptococci, with an unusually high degree of conservation of over 96% amino acid
289 identity when compared to SpxB of *S. sanguinis* strain SK36. SpxB catalyzes the
290 conversion of pyruvate to acetyl phosphate, CO_2, H_2O_2, and ATP. Therefore, besides
291 generating inhibitory amounts of H_2O_2, SpxB confers a growth advantage on the producer
292 via ATP generation during biofilm development (81, 82).

293 The overall importance of H_2O_2 production is further indicated by the fact that
294 neither *S. sanguinis* nor *spxB*-encoding oral streptococci seem to encode for the H_2O_2
295 detoxifying enzyme catalase. Consequently, these bacteria are able to produce
296 considerable amounts of H_2O_2 that can influence the surrounding environment and inhibit
297 susceptible species. H_2O_2-dependent competitive behavior faces two challenges. First,
298 SpxB requires oxygen for its activity (83, 84). Its production therefore declines once biofilm
299 formation reaches a certain density resulting in an anaerobic environment. Second,
300 released H_2O_2 is a substrate for detoxifying enzymes like salivary lacto-peroxidase (85).
301 Therefore, the effect of H_2O_2 production on biofilm development is confined to the
302 immediate vicinity of the producer and most likely does not result in active killing of
303 competitors. Rather, H_2O_2 will affect susceptible species just enough to gain a growth
304 advantage. For *S. sanguinis*, the oxygen dependent production of H_2O_2 makes perfect
305 sense from an ecological point of view. As initial colonizer, *S. sanguinis* finds a sparse
306 inhabited environment with enough salivary oxygen tension to promote H_2O_2 production
307 (86). However, once biofilm growth through proliferation and integration of compatible
308 species is sufficient to result in a decline in oxygen tension, *S. sanguinis* will already be

309 an established member of the community and so H_2O_2 production is no longer required
310 (80). The inhibitory spectrum of H_2O_2 as evaluated *in vitro* with deferred antagonism plate
311 assays includes cariogenic *S. mutans*, as well as periodontopathogens *A.*
312 *actinomycetemcomitans*, *Prevotella intermedia* and *P. gingivalis* (83, 87).

313 The genetic regulation of *spxB* expression holds a mystery. A clear repressive
314 function of the carbon catabolite repressor CcpA has been shown since deletion of CcpA
315 in *S. sanguinis* strain SK36 lifts *spxB* repression (88). Further, two *cre* sites (carbon
316 responsive element) for the binding of CcpA have been verified experimentally (89), yet
317 no glucose effect on *spxB* expression and H_2O_2 production can be observed. This is in
318 contrast to other species like *S. gordonii*, which follows classic carbon catabolite
319 repression in the presence of glucose (90). Why CcpA in *S. sanguinis* does not respond
320 to the presence of glucose, and if any environmental factor influences *spxB* expression,
321 has yet to be determined. Compared to *S. gordonii* and other oral streptococci, *S.*
322 *sanguinis* H_2O_2 production in general seems to be less (89, 91) and a clue to the
323 consequences of low H_2O_2 production and tight control by CcpA might be explained by
324 the increased susceptibility of a *S. sanguinis* CcpA knockout mutant towards its own H_2O_2
325 production (88). However, whether or not this is a direct effect of H_2O_2 or the result of
326 altered gene expression from other CcpA-controlled genes is not currently known. From
327 the ecological standpoint, the numerical abundance of *S. sanguinis* over other species
328 might not require high production of H_2O_2 while other species like *S. gordonii,* known to
329 be a less prominent member of the oral biofilm, might require more aggressive H_2O_2
330 production to establish itself within the oral community. Taking into account that oral
331 streptococci intermingle during biofilm formation and can be found at the same location
332 (92), the sum of H_2O_2 production could be more important than the quantity produced by

333 individual species. Nevertheless, competitive H_2O_2 production and its role in community
334 development is important and can be used to study biofilm dynamics.

335 Interestingly, *S. sanguinis* is also able to produce antimicrobial activity via
336 bacteriocins. Several reports describe the antimicrobial activity of sanguicins (93, 94).
337 Initial characterization showed bacteriostatic activity against a number of oral species,
338 including *A. naeslundii,* but not periodontopathogens *P. gingivalis*, *P. intermedia* and *F.*
339 *nucleatum* (53). However, a more recent study using purified sanguicin demonstrated
340 antimicrobial activity against such pathogens (93). Since both studies used a different
341 strain of *S. sanguinis* and reported different molecular weights (65 kDa *vs* 280 kDa) for
342 the purified peptide, it is most likely that different sanguicins were characterized. It is also
343 worth mentioning that none of the studies determining the effect of H_2O_2 on
344 periodontopathogens or *S. mutans* reported an inhibitory effect when catalase was added
345 to the deferred antagonism assay (87, 95). Therefore, either strain-specific antagonistic
346 activity exists, or the culture conditions used in the studies were selective for specific
347 production of bacteriocins or H_2O_2.

348 *S. sanguinis* bacteriocins are also reported to exhibit anti-fungal activity (96, 97).
349 As part of the normal microbiota, *C. albicans* is commonly isolated from subjects, but can
350 also cause problems like oral candidiasis (98). *S. sanguinis* produces a bacteriocin that
351 can cause changes in cell surface hydrophobicity of several *Candida spp.*, a factor that
352 influences the initial adhesion of *Candida* to oral epithelium. Furthermore, the bacteriocin
353 can impair fungal cell membrane permeability and general cell structure (96, 97). How this
354 ultimately affects survival of the fungal cell *in vivo* is not known but, analogous to the effect
355 of H_2O_2 production, it might provide a competitive advantage to *S. sanguinis* by impairing
356 growth of competing oral fungi.

357

358 **HORIZONTAL GENE TRANSFER AND ANTIBIOTIC RESISTANCE RESERVOIR**

359 An important feature of oral streptococci is the ability to take up eDNA when
360 developing competence, as discussed earlier. While the process of competence
361 development is well characterized at the molecular level (99), the origin of DNA in the
362 environment and the molecular mechanisms of DNA release from bacterial cells are less
363 well understood. As a vital part of the oral biofilm matrix, eDNA available for uptake by
364 competent bacteria is quite abundant (100, 101). In general, cell lysis can contribute to
365 the release of DNA from bacterial cells. This process can be an active autolysis process
366 involving murein hydrolases that weaken or completely lyse the cell wall, consequently
367 expelling DNA into the environment (102). Interestingly, both *S. sanguinis* and *S. gordonii*
368 release DNA in a H_2O_2-dependent manner under aerobic conditions (83). The eDNA is
369 largely intact, high molecular weight DNA of chromosomal origin. Release of eDNA peaks
370 when *S. sanguinis* grows aerobically under optimal H_2O_2-producing conditions and release
371 declines when cells are grown under oxygen-limiting conditions, when no H_2O_2 is
372 produced. Furthermore, deletion of *spxB* severely impacts the release (83). However,
373 eDNA release can be induced even under anaerobic conditions with the addition of H_2O_2,
374 as shown for *S. gordonii*, but only when cells are metabolically active (103). Addition of
375 H_2O_2 to cells suspended in buffer will not release DNA, indicating the requirement for
376 active metabolism, gene expression or protein synthesis. This is also consistent with the
377 observation of a time delay between the production of H_2O_2 and the appearance of the
378 released DNA, and the evidence that addition of chloramphenicol, known to block
379 synthesis of new proteins, also blocks release of DNA (103). This is relevant in the context
380 of the oral biofilm, where SpxB-positive streptococci that have access to oxygen can

381 produce H_2O_2 that might then diffuse through the oral biofilm with limited or no oxygen.
382 Remarkably, the H_2O_2-dependent release of DNA is not the result of obvious cell lysis
383 (50), as reported for other streptococci like *S. pneumoniae* (104). Although the exact
384 molecular mechanism involved in the release is not known at this moment, *S. sanguinis*
385 might encode a dedicated system for eDNA release. This could involve partial lysis and/or
386 active transport across the membrane. In a recent study, the transport of eDNA in *H.*
387 *influenzae* was found to be dependent upon competence-related protein ComE (105), the
388 transporter involved in the uptake of DNA. *S. sanguinis* encodes for ComE and intriguingly
389 expression of competence genes seems to be increased under aerobic conditions ideal
390 for H_2O_2 production in streptococci (83, 103). However, experimental evidence that this
391 mechanism is conserved in *S. sanguinis* is not available. When grown under anaerobic
392 conditions, eDNA release is not completely absent suggesting another mechanism
393 involved in the release (50). One autolytic enzyme involved in this process in *S. sanguinis*
394 is LytF, also under the control of the competence system as one of the early competence
395 genes (106). LytF is a secreted autolysin and can be recovered from the environment in
396 its active form. LytF is a fratricin, but also acts on neighboring cells causing release of
397 eDNA not only from *S. sanguinis* but also other streptococci (106). In general, streptococci
398 seem to control or connect competence development with the release of eDNA (107),
399 exemplifying their role as masters of efficacy. Utilizing this approach increases the chance
400 that DNA is available for uptake and transformation.

401 Uptake of eDNA has two pronounced functions; first, it serves in horizontal gene
402 transfer during bacterial transformation and therefore plays a pivotal role in the promotion
403 of diversity among oral bacterial species, and is an important mechanism of evolution
404 allowing the acquisition of new genetic traits stored in the genomic information contained

405 in the multispecies biofilm (108). Second, eDNA in the oral cavity can serve as a reservoir
406 for diverse antibiotic resistance mechanisms (109). A recent comparison of *S. sanguinis*
407 and *S. gordonii* genomes revealed the presence of several genes potentially associated
408 with antibiotic resistance determinants, including GNAT acetyltransferases, *parE*, and
409 TetR family regulators (110). Combined they potentially provide resistance to diverse
410 antibiotics like aminoglycosides, fluoroquinolones and many more. Additionally, several
411 genes were identified encoding anion channels in *S. sanguinis* that confer resistance to
412 fluoride (111), particularly important since fluoride is successfully used to prevent caries.
413 Although the direct transfer of antibiotic resistance determinants via eDNA from other
414 species to *S. sanguinis* has not been confirmed, evidence exists that in general the
415 acquisition of new genetic traits occurs. For example, the pathway for vitamin B_{12}
416 biosynthesis as well as the degradation of ethanolamine and propanediol, which are
417 encoded on a large genomic region, has been acquired through horizontal gene transfer
418 (112), although it is unknown if this was mediated through conjugation, transduction or
419 transformation. The best evidence for the potential of H_2O_2-induced transfer of antibiotic
420 resistance was shown with *S. gordonii*, using engineered strains carrying antibiotic
421 resistance cassettes. Co-incubation of strains encoding distinct antibiotic cassettes under
422 ideal H_2O_2-producing conditions increased the occurrence of intraspecies genetic
423 exchange by 300-fold when compared to non-permissive conditions (103). Overall,
424 antibiotic resistance in the oral biofilm seems to be mainly acquired through horizontal
425 gene transfer as suggested by a recent review (113). The dental biofilm is an ideal
426 environment for the development and transfer of antibiotic resistance, even under
427 conditions where no external pressure through antibiotic administration is present. This is
428 supported by the finding that biofilm evolution and selective pressure through competitive

429 bacterial interactions mediated by bacteriocins alone gave rise to antibiotic resistance
430 (114). The oral microbiome is able to elicit this evolutionary pressure due to the
431 abundance of bacteriocins (115).
432
433 ## ***S. SANGUINIS*** **AS A MODULATOR OF THE HOST**
434 While the predominant ecological niche of *S. sanguinis* is the tooth surface, dental
435 plaque formed at the gingival margin brings the oral biofilm into contact with the oral
436 epithelium and associated immune defenses. The host-microbe interplay that occurs at
437 these sites represents a critical step in determining progression of the biofilm community
438 below the gum line, and potential transition from oral health to onset of gingivitis,
439 periodontitis or other disease manifestations. Another facet of the *S. sanguinis* persona
440 as coordinator of the microbial community is therefore its interactions with host tissues.
441 Under conditions of oral health, an equilibrium exists between microbiota and host,
442 representing a delicate balance of antimicrobial factors from immune cells, together with
443 pro- and anti-inflammatory molecules released from the host in response to the sustained
444 microbial challenge. By contrast, disruption of this homeostasis is the hallmark of chronic
445 inflammatory periodontal disease and resultant tissue damage. In keeping with this, *S.*
446 *sanguinis* biofilms were found to be a poor stimulant of proinflammatory cytokines IL-1α,
447 IL-6 and IL-8 from OKF4 oral epithelial cells compared to biofilms of *F. nucleatum* (116).
448 Likewise, in contrast to cell wall extracts of *F. nucleatum* or *P. gingivalis*, those of *S.*
449 *sanguinis* failed to induce significant upregulation by gingival keratinocytes of genes
450 encoding human β defensin peptides, proinflammatory cytokines (e.g. IL-8) or matrix
451 metalloproteinase-9. These keratinocyte responses were mediated by TLR2, with

452 differences in acylation patterns of bacterial lipopeptides purported to underpin the
453 differential outcomes of TLR2 stimulation by the bacterial species (117). Moreover, while
454 only a weak stimulant of host responses itself, *S. sanguinis* is also able to suppress the
455 effects of other microbes. In mixed culture, *S. sanguinis* impaired induction of IL-8 release
456 from gingival HOK-18A epithelial cells by *A. actinomycetemcomitans*. Such effects were
457 also seen with *S. sanguinis* spent culture medium alone, implying a secreted molecule as
458 the mediator (118). Similarly, through blocking LPS engagement with monocyte receptors
459 LPS-binding protein (LBP) and CD14, peptidoglycan from *S. sanguinis* was able to inhibit
460 induction of genes encoding TNFα, IL-6 and IL-8 by periodontopathogens *P. gingivalis*,
461 *A. actinomycetemcomitans* and *Tannerella forsythia* (119). This mechanism correlates
462 well with the observation that Gram-positive bacteria release large quantities of
463 peptidoglycan fragments, muropeptides, during cell division (120). Such coordination of
464 both host responses and of the oral microbiota is likely critical to the role of *S. sanguinis*
465 as a health-associated member of the oral biofilm community.

466

467 ## ***S. SANGUINIS*** **AS AN ETIOLOGICAL AGENT OF EXTRAORAL DISEASES**

468 Despite its main role as a benign oral commensal, the name *S. sanguinis* derives
469 from its role in cardiovascular disease infective endocarditis (IE). In a note to the Journal
470 of Bacteriology, Niven and White described a new species isolated from approximately
471 100 cases of subacute bacterial endocarditis (121). About one third of the isolates failed
472 characterization as previously described streptococci and were referred to as
473 *Streptococcus s.b.e.* (for subacute bacterial endocarditis) (121). Overall the group was
474 quite homogeneous in its physiological characteristics (122). Further serological

475 characterization confirmed the isolation of a new species since no cross reactivity was
476 observed between rabbit serum from *Streptococcus s.b.e.* and other identified
477 streptococci belonging to various Lancefield groups (123). Interestingly, isolation of
478 *Streptococcus s.b.e.* from the human throat was unsuccessful at that time, despite testing
479 over 800 streptococcal isolates. The only other positive culture came from an extracted
480 tooth, which we now know coincides with the preferred colonization site. However, in the
481 original publication, the natural habitat was not identified and the only source was from
482 the blood of endocarditis patients, hence the species name *sanguis*; Latin for blood (122,
483 124). This has been changed fairly recently to the grammatically correct version, *S.*
484 *sanguinis* (125).

485 IE is a relatively rare, but potentially fatal disease and can affect the heart valves
486 or endocardium. The annual incidence ranges from 3-7 cases per 100,000 people per
487 year and has been relatively constant, whereas the etiology of IE has changed over time
488 (126, 127). A major concern with this disease is that mortality rates remain high; the in-
489 hospital mortality rate ranges from 15-22%, with a 5-year mortality rate around 40%.
490 Currently, *Staphylococcus aureus* is the most common pathogen associated with IE.
491 However, oral streptococci are responsible for an estimated 35-40% of cases, with
492 *sanguinis*-group species the most common isolates (128).

493 Understanding the molecular mechanisms that might contribute to the capacity for
494 *S. sanguinis* to cause IE has benefited from the availability of an excellent animal model
495 for IE. New Zealand white rabbits can be catheterized at specific sides of the heart causing
496 injury-induced IE after bacterial blood inoculation (129). The injury provides exposed ECM
497 components, fibrin and platelets for attachment of the bacterial cells and subsequent
498 aberrant clot formation, which leads to infective vegetations forming on the heart valves.

499 Todd Kitten's group, Virginia Commonwealth University, have used this model to decipher
500 molecular determinants expressed by *S. sanguinis* during the infection process. Using a
501 random signature-tagged mutagenesis approach, six chromosomal loci were identified
502 from an initial screen of 800 mutants. The mutants carried transposons in an intergenic
503 region and in genes encoding undecaprenol kinase, homoserine kinase, anaerobic
504 ribonucleotide reductase, adenylosuccinate lyase, and a hypothetical protein (130). The
505 screening method is certainly elegant but has some technical limitations, since important
506 determinants like surface-exposed proteins, which have been shown to contribute to the
507 infection process (131), were missed. Nonetheless, the identification of determinants
508 regarded as house-keeping genes involved in cell wall (undecaprenol kinase), amino acid
509 (homoserine kinase) and nucleic acid (ribonucleotide reductase) synthesis suggested a
510 potential role for these genes in pathogenesis that had not previously been appreciated.
511 Furthermore, since these genes are not found in humans, they may represent potential
512 targets for drug development. However, a functional analysis of the clonal structure of *S.*
513 *sanguinis* strains isolated from the oral cavity and from subjects with IE demonstrated that
514 house-keeping and virulence genes are subject to considerable intra-species
515 recombination events. Thus potential drug targets in such genes may correlate with a high
516 potential for the selection of resistant mutants (132). Interestingly, the same study also
517 concluded that the endocarditis strains did not form a distinct sub-cluster. This supports
518 the notion that *S. sanguinis* strains are human pathobionts, and thus all strains have the
519 potential to cause IE (132).

520 Following on from this study, a targeted signature-tagged mutagenesis approach
521 was employed to identify surface proteins important in IE. Thirty three proteins were
522 classified as cell wall-associated and of these, mutants in 31 were tested in the

523 aforementioned rabbit model of IE, alongside mutants in 3 sortase genes (133).
524 Interestingly, no single cell wall-associated protein was found to be essential for the
525 development of IE (133). It may be concluded from this that no specific virulence
526 determinants are required by *S. sanguinis* to cause IE. Rather, what is expressed and
527 present during colonization of the oral cavity may be sufficient to colonize the
528 endocardium. In support of this, surface adhesins Hsa, PadA and SspA/B from *S. gordonii*
529 have been implicated in IE through their engagement with platelets, yet also contribute to
530 the capacity for *S. gordonii* to bind salivary pellicle and other oral microbes (134-137).
531 Such highly optimized genomes might explain the success of these bacteria as early
532 colonizers, with their ability to cause IE on rare occasions coincident in their ubiquitous
533 presence and abundance within the oral cavity.

534 Mutations in the genes encoding lipoprotein SsaB (a putative manganese transport
535 protein) (38), *spxA1* (encoding a global regulator involved in H_2O_2 production) (138) and
536 *nox* (encoding an NADH oxidase that also influences H_2O_2 production) (139), together
537 with the anaerobic ribonucleotide reductase already mentioned, have all been shown to
538 impair the capacity of *S. sanguinis* to cause IE. This suggests that the ability of *S.*
539 *sanguinis* to adapt to differences in oxygen tension and the production of and resistance
540 to reactive oxygen species are important in the development of IE, although molecular
541 details have yet to be explained. A surface-bound nuclease, designated SWAN
542 (streptococcal wall-anchored nuclease), has also been proposed to enable *S. sanguinis*
543 to evade killing by neutrophil extracellular traps (NETs), and so promote survival both in
544 the bloodstream and within infective vegetations (140).

545 Survival within the bloodstream to access extraoral sites and induction of
546 thrombosis are key steps in IE, but these capabilities also associate *S. sanguinis* with

547 other systemic diseases. These include meningitis, following infection of the lining of the
548 spinal cord or brain, and disseminated intravascular coagulation, in which aberrant
549 activation of the coagulation cascade leads to the formation of small clots that can occlude
550 blood flow to major organs and tissues (141, 142). It is also widely recognized that oral
551 streptococci such as *S. sanguinis* exist in biofilm communities with *Pseudomonas*
552 *aeruginosa* in the lungs of cystic fibrosis (CF) patients. However, with evidence that H_2O_2
553 production by these streptococci can impair *P. aeruginosa* growth (143), the impact of *S.*
554 *sanguinis* on CF disease progression remains to be fully understood.
555
556 **CONCLUSION/OUTLOOK**
557 With the impact upon the individual, it is easy to understand why disease outcomes
558 have historically dominated microbiological research. Nonetheless, technological
559 advances are providing a growing appreciation for the importance of our resident
560 microbiota. Within the oral cavity, bacteria such as *S. sanguinis* are ubiquitous and
561 abundant, reflecting their evolution to be exquisitely adapted to colonization of their
562 ecological niche. As a consequence, *S. sanguinis* is able to orchestrate accretion of the
563 dental plaque biofilm, promoting the acquisition of beneficial microbes while serving as an
564 imposing competitor to others, and ultimately serving as the foundation of a health-
565 associated biofilm community. Complex molecular mechanisms regulating physical
566 interactions and communication networks underpin these capabilities. If these can be
567 understood at both the molecular and ecological level, they offer immense potential for
568 exploitation in the development of novel strategies to combat infections from a point of
569 health as opposed to disease.
570

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977 **FIGURE LEGENDS**

978 Figure 1. Summary of key *Streptococcus sanguinis* components important in
979 commensalism. The schematic shows important components for the role of *S. sanguinis*
980 as a commensal organism, including community integration and biofilm development,
981 community interference and streptococcal antagonism, and interactions with salivary
982 proteins, host cells, and the immune system. Pg, *Porphyromonas gingivalis*; Fn,
983 *Fusobacterium nucleatum*; Sg, *Streptococcus gordonii*; eDNA, extracellular DNA; CSP,
984 competence stimulating peptide. Reprinted with permission from Kreth et al. (2017). The
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