Pseudomonas aeruginosa Can Inhibit Growth of Streptococcal Species via Iron Sequestration

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Abstract

Cystic Fibrosis (CF) is a genetic disease that causes patients to accumulate thick, dehydrated mucus in the lung and develop chronic, polymicrobial infections due to reduced mucociliary clearance. These chronic polymicrobial infections and subsequent decline in lung function are significant factors in the morbidity and mortality of CF. *Pseudomonas aeruginosa* and *Streptococcus* spp. are among the most prevalent pathogens in the CF lung; the presence of *P. aeruginosa* correlates with lung function decline and the *Streptococcus milleri* group (SMG) is associated with exacerbations in CF patients. Here we characterize the interspecies interactions that occur between these two genera. We demonstrated that multiple *P. aeruginosa* laboratory strains and clinical CF isolates promote the growth of multiple SMG strains and oral *Streptococci* in an *in vitro* coculture system. We investigated the mechanism by which *P. aeruginosa* enhances growth of streptococci by screening for mutants of *P. aeruginosa* PA14 unable to enhance *Streptococcus* growth, and we identified the *P. aeruginosa* *pqsL*::*TnM* mutant, which failed to promote growth of *S. constellatus* and *S. sanguinis*. Characterization of the *P. aeruginosa* Δ*pqsL* mutant revealed that this strain cannot promote *Streptococcus* growth. Our genetic data and growth studies support a model whereby the *P. aeruginosa* Δ*pqsL* mutant outcompetes *Streptococcus sanguinis* for limited iron. We propose a model whereby competition for iron represents one important means of interaction between *P. aeruginosa* and *Streptococcus* spp.

Importance

Cystic fibrosis (CF) lung infections are increasingly recognized for their polymicrobial nature. These polymicrobial infections could alter the biology of the organisms involved in CF-related infections, leading to changes in growth or virulence and could affect patient health. In this study, we demonstrate interactions between *P. aeruginosa* and streptococci using a coculture model, and demonstrate that one interaction between these microbes is likely competition for iron. Thus, these data indicate that one CF pathogen may influence the growth of another. This study adds to our limited knowledge of polymicrobial interactions.

Introduction

Cystic fibrosis (CF) is a genetic disease caused by a defect in the cystic fibrosis transmembrane conductance regulator (1), which leads to reduced mucociliary clearance in the lungs of these patients (2). Due to the reduced mucociliary clearance, bacteria colonize the lungs of patients with CF and establish chronic, polymicrobial infections that cause increased inflammation and respiratory function decline (2). Recent studies have demonstrated that the microbiota in the lungs form polymicrobial biofilms, and that mixed bacterial biofilm populations can affect antibiotic tolerance and bacterial virulence (3, 4).

The *Streptococcus milleri* group (SMG), which is composed of three species (*S. anginosus, S. constellatus,* and *S. intermedius*) has been isolated from sputum samples of patients with CF. When these microbes are the numerically dominant species in the lung, these organisms correlate with exacerbation in patients with CF (5–8). In contrast, previous research from our laboratory (9) and a second group (10) demonstrated that increased relative abundance of *Streptococcus* spp. within the CF lung microbiome correlates with better lung function and clinical stability. Together these data indicate a possible complex relationship between *Streptococcus* spp. and the host, and *Streptococcus* spp. and the other microbes in the CF airway.

Pseudomonas aeruginosa becomes the dominant microorganism in the lungs of ~45% of adults patients with CF (11), is cultured from >80% of these patients (12), and is the dominant microbe in the lung at end stage disease (13). Both *P. aeruginosa* and streptococci have been found to co-colonize CF patients (5–8, 14), but the polymicrobial interactions that occur between these organisms are not well studied. Previous studies

77 investigating interactions between *P. aeruginosa* and *Streptococcus* spp. demonstrated
78 that *Streptococcus* spp. can influence production of *P. aeruginosa* virulence factors
79 such as rhamnolipids, elastase, and phenazines (15–19) and can suppress *P.*
80 *aeruginosa* growth through hydrogen peroxide production (15) and subsequent reactive
81 nitrogenous intermediates (20, 21). Conversely, *P. aeruginosa* was found to influence
82 the growth (15, 17, 19, 22) and biofilm formation (22, 23) of *Streptococcus* spp. Work
83 from our lab demonstrated that *P. aeruginosa* PA14 produces the surfactants β-
84 hydroxyalkanoyl-β-hydroxyalkanoic acids (HAAs) and monorhamnolipids which caused
85 a 6-fold reduction in *S. constellatus* 7155 biofilm formation in coculture (22). The
86 surfactant-induced biofilm suppression was relieved when *P. aeruginosa* and *S.*
87 *constellatus* 7155 were cocultured in the presence of tobramycin, an antibiotic used for
88 maintenance therapy by patients with CF. We determined that tobramycin suppressed
89 *P. aeruginosa* production of HAAs and monorhamnolipids, and that in the presence of
90 tobramycin, *P. aeruginosa* can enhance *S. constellatus* 7155 growth on a CF-derived
91 bronchial epithelial cell (CFBE) monolayer (22). These data indicate that *P. aeruginosa*
92 can both positively and negatively impact cocultured microbes, including *Streptococcus*
93 spp., and the interaction between the microbes can be influenced by environmental
94 context.

95 In this study, we investigate the ability of *P. aeruginosa* to influence
96 *Streptococcus* growth in our *in vitro* coculture system. We demonstrate that multiple *P.*
97 *aeruginosa* strains and clinical isolates can enhance the growth of multiple
98 *Streptococcus* spp. We used a candidate genetic approach and a genetic screen to
99 identify *P. aeruginosa* mutants that were unable to support *Streptococcus* growth, and

found a single mutant of *P. aeruginosa* that no longer enhances growth of streptococci. We found that the *P. aeruginosa* Δ*pqsL* mutant suppressed *S. sanguinis* growth, likely via a mechanism that involves iron sequestration. These data indicate that competition for iron can impact this polymicrobial interaction.

Results

***P. aeruginosa* promotes streptococcal growth in a coculture system.** We reported previously that *P. aeruginosa* can enhance viable *S. constellatus* 7155 cell number when grown as a coculture on CF-derived bronchial epithelial (CFBE) cells (22). We first sought to recapitulate the finding that *P. aeruginosa* promotes the *Streptococcus* biofilm population by using the model organism *S. sanguinis* SK36 in coculture conditions in the absence of CFBE cells. We used *P. aeruginosa* PAO1 and *S. sanguinis* SK36 for these experiments because both are sequenced strains (24–26) with available genetic mutant libraries (27–29). This simplified coculture system allowed us to test the interaction between *P. aeruginosa* and *Streptococci* without confounding factors contributed by the CFBE cells.

To test the hypothesis that *S. sanguinis* SK36 viable cell number increases in coculture with *P. aeruginosa* PAO1 in absence of CFBE cells, we grew *P. aeruginosa* PAO1 and *S. sanguinis* SK36 in coculture in the wells of a plastic culture dish. We observed that the number of viable *S. sanguinis* SK36 in a biofilm was enhanced 100-1055-fold by coculture with *P. aeruginosa* PAO1 compared to *S. sanguinis* SK36 grown as a monoculture (Fig. 1A, see also Fig. S1A). *P. aeruginosa* PAO1 biofilm growth was not significantly affected by coculture with *S. sanguinis* SK36 (Fig. S1B). These data indicate that the enhancement of *S. sanguinis* SK36 populations in a biofilm by *P. aeruginosa* PAO1 does not require the CFBE cells.

126 ***P. aeruginosa* enhances growth of *S. sanguinis* SK36**. We considered two models of
127 polymicrobial interaction that may be enhancing viable, *S. sanguinis* SK36 cells in the
128 biofilm when grown in co-culture with *P. aeruginosa*. *P. aeruginosa* might promote
129 *Streptococcus* adhesion and biofilm formation, or *P. aeruginosa* may promote
130 streptococcal growth. To distinguish between these models, we conducted a time
131 course experiment with *P. aeruginosa* PA14, *P. aeruginosa* PAO1, and *S. sanguinis*
132 SK36. If *P. aeruginosa* was promoting adhesion of *S. sanguinis* SK36 cells rather than
133 growth, we predict that we would detect more *S. sanguinis* SK36 in the biofilm and
134 fewer planktonic cells, but total c ell number is not increased compared to *S. sanguinis*
135 SK36 monoculture. In contrast, if *P. aeruginosa* were enhancing *S. sanguinis* SK36
136 growth, then both total biofilm and planktonic *S. sanguinis* SK36 populations should
137 increase in coculture compared to *S. sanguinis* SK36 monoculture. As demonstrated by
138 the increased *S. sanguinis* SK36 biofilm and planktonic cells recovered from coculture
139 compared to monoculture, *P. aeruginosa* appears to promote the growth of *S. sanguinis*
140 SK36 (Fig. 1B and Fig. S2A and S2B), thus accounting for the increased population of
141 *S. sanguinis* SK36 biofilm cells.
142
143 **Multiple *P. aeruginosa* strains enhance the growth of multiple streptococci.** Based
144 on current evidence that multiple *Streptococcus* species inhabit the CF lung (30, 31)
145 and influence patient health (5–10, 14), we sought to determine whether the observed
146 enhancement of *Streptococcus* viable counts in coculture with *P. aeruginosa* may be
147 more broadly generalized to other streptococci, including the *Streptococcus milleri*
148 group (SMG), which has been implicated in CF-related exacerbations (5–8). To assess

149 the ability of *P. aeruginosa* to promote multiple *Streptococcus* spp., we cocultured *P.*
150 *aeruginosa* PAO1 with 6 SMG isolates and 8 oral *Streptococcus* spp. Figure 1C depicts
151 a representative strain of each streptococcal species assayed, and shows the biofilm
152 population obtained from monoculture and coculture with *P. aeruginosa* PAO1,
153 respectively (see Fig. S3A for all 14 strains tested). *P. aeruginosa* PAO1 growth was not
154 significantly affected by coculture with any of the *Streptococcus* spp. tested (Fig. S3B).
155 We found that *P. aeruginosa* PAO1 significantly enhanced the growth of one of
156 the two *S. anginosus*, two of two *S. intermedius*, and neither of the two *S. constellatus*
157 strains tested. Additionally, of the oral *Streptococcus* spp. tested, *P. aeruginosa* PAO1
158 significantly promoted the growth of one of the two *S. oralis*, the one *S. parasanguinis,*
159 and one of the three *S. salivarius* isolates, but not the *S. pneumoniae* or *S. peroris*
160 isolates tested (Fig. 1C and Fig. S3A). While not every *Streptococcus* isolate tested
161 demonstrated significant increase in viable population recovered from the coculture,
162 most species tested exhibited a trend toward increased growth when cocultured with *P.*
163 *aeruginosa* PAO1. These coculture data suggest that *P. aeruginosa* may be promoting
164 *Streptococcus* growth through a pathway that affects many *Streptococcus* species.
165 Next we assessed whether multiple *P. aeruginosa* clinical and laboratory strains
166 could promote the growth of *S. sanguinis*. Additionally, given that *S. parasanguinis* was
167 found to bind extracellular alginate produced by mucoid *P. aeruginosa* strains (23), we
168 tested whether mucoid or nonmucoid *P. aeruginosa* could better promote growth in our
169 coculture system. We cocultured *S. sanguinis* SK36 with seven nonmucoid *P.*
170 *aeruginosa* and four mucoid *P. aeruginosa* laboratory and clinical strains, and observed
171 a significant growth enhancement of *S. sanguinis* SK36 by ten out of eleven *P.*

aeruginosa strains tested in coculture biofilms (Fig. 1D) and planktonic growth (Fig. S4A). The growth of all tested *P. aeruginosa* strains was not affected by coculture with *S. sanguinis* SK36 (Fig. S4B, S4C).

Additionally, *P. aeruginosa* PAO1 (parental) and *P. aeruginosa* PDO300 *mucA22* are isogenic nonmucoid and mucoid strains, respectively. We found a significant enhancement in viable *S. sanguinis* SK36 biofilm cells recovered from coculture with *P. aeruginosa* PDO300 *mucA22* compared to *P. aeruginosa* PAO1, suggesting that mucoid *P. aeruginosa* strains may better enhance *Streptococcus* growth. Additionally, these mucoid *P. aeruginosa* strains showed among the most robust promotion of viable counts when cocultured with *Streptococcus*.

In summary, we have demonstrated that our minimal medium coculture assay using a plastic substratum can recapitulate our prior observation that *P. aeruginosa* promotes streptococcal growth. We were able to determine that *P. aeruginosa* is likely promoting *Streptococcus* growth rather than increasing the biofilm population via enhanced adherence. The *Streptococcus* growth-enhancement phenotype occurred among most oral *Streptococci* tested, and the majority of *P. aeruginosa* clinical and laboratory strains are capable of promoting *Streptococcus* growth, which lends support to the idea that these interactions are common among these two genera.

Known *P. aeruginosa* virulence pathways are not involved in the *Streptococcus* growth-promoting phenotype. *P. aeruginosa* has many well characterized virulence factors that have been demonstrated to impact polymicrobial interactions, including pathways for quorum sensing (32), biofilm formation, and the production of secreted

195 molecules such as phenazines (33, 34), siderophores (3, 35), alginate (36), and
196 rhamnolipids (22). We hypothesized that one or more of these virulence factors might
197 be altering *Streptococcus* growth in our system. To test this idea, we utilized a
198 candidate genetic approach to assess whether any of these virulence pathways may be
199 involved in the observed growth-enhancing phenotype. We cocultured *P. aeruginosa*
200 PA14 mutants in each of the above pathways with *S. constellatus* 7155 as a model
201 streptococcal strain know to positively respond to *P. aeruginosa* growth enhancement
202 (22), and assessed whether any of these mutants lost the ability to enhance *S.*
203 *constellatus* 7155 growth. We found that none of the pathways tested were involved in
204 enhancement of *S. constellatus* 7155 growth (Table S1).
205 We also constructed *P. aeruginosa* PA14 Δ*katA*, Δ*katB*, and Δ*katAkatB* mutant
206 strains in order to test the hypothesis that extracellular *P. aeruginosa* catalase is
207 enhancing *S. sanguinis* SK36 growth by breaking down hydrogen peroxide produced by
208 *S. sanguinis* SK36 given that KatA has been found in the supernatant of *P. aeruginosa*
209 cultures (37–39). It has previously been reported that *S. sanguinis* and other oral
210 streptococci can inhibit *P. aeruginosa* growth through hydrogen peroxide production
211 (15, 20, 21), and that the hydrogen peroxide produced by oral streptococci plays an
212 important role in growth inhibition, eDNA release, and biofilm formation within the oral
213 microbiome (reviewed in 40 and 41). We chose to mutate the *katA and katB* genes and
214 not the *katE* gene because previous reports indicate that KatA is the major catalase
215 utilized by *P. aeruginosa*, and that KatB can partially recover hydrogen peroxide
216 resistance in the absence of KatA (37, 42). Meanwhile, KatE was not demonstrated to
217 play a role in alleviating hydrogen peroxide stress (42). *S. sanguinis* SK36 did not

demonstrate reduced growth in coculture with the *P. aeruginosa* PA14 Δ*katA*, Δ*katB*, or Δ*katA*Δ*katB* mutant strains compared to wild-type *P. aeruginosa* PA14, indicating that catalase is not playing a role in the *Streptococcus* growth enhancement phenotype (Fig. S5A). The *P. aeruginosa* Δ*katA* mutant displays a slight, significant growth defect in the coculture compared to *P. aeruginosa* PA14 in coculture, and the Δ*katB* mutant displays a modest, but significant growth defect in monoculture compared to *P. aeruginosa* PA14 in coculture (Fig. S5B). Taken together, these data suggest known virulence factors, on their own, do not contribute to *P. aeruginosa*-mediated growth enhancement of *Streptococcus* spp.

Screening the *P. aeruginosa* PA14NR Set for *P. aeruginosa* PA14 transposon insertion mutant strains that do not support *S. constellatus* growth. The Ausubel lab reported a nonredundant library of *P. aeruginosa* PA14 transposon insertion mutants (PA14NR Set) (43). The PA14NR Set contains 5,459 transposon insertion mutant strains with mutations in 4,596 genes. Each of these *P. aeruginosa* PA14 transposon mutant strains was tested in coculture with *S. constellatus* 7155 (Fig. 2A). Of the 5,459 mutant strains in the library, 48 strains were unable to promote *S. constellatus* 7155 growth in two replicate experiments (Table S2). Two of these 48 mutants were eliminated when we tested available deletion mutants as they did not recapitulate the phenotype of the transposon mutation (not shown). The remaining 46 transposon mutants (Table S2) were tested in our standard coculture with *S. sanguinis* SK36 to determine which *P. aeruginosa* PA14 transposon mutants are unable to enhance *Streptococcus* growth in a second strain. 44 of the 46 *P. aeruginosa* PA14

241 transposon mutants were capable of enhancing growth of *S. sanguinis* SK36, and thus
242 were unlikely involved in a general pathway for enhancing growth of *Streptococcus*. We
243 found that two transposon mutants were unable to promote either *S. constellatus* 7155
244 or *S. sanguinis* SK36 growth: *P. aeruginosa pqsL*::*TnM* and *P. aeruginosa dbpA*::*TnM*.
245 The *dbpA* gene codes for the RNA helicase DbpA, which has been demonstrated
246 to play a role in the formation of the 50S ribosomal subunit in *Escherichia coli* (reviewed
247 in 44). *E. coli* is able to compensate for Δ*dbpA* deletionsin forming the 50S ribosomal
248 subunit, as described previously (45). An inability to form the 50S ribosomal subunit
249 would otherwise cause a lethal protein synthesis defect, and a dominant negative *dbpA*
250 mutant strain is necessary to observe a defect in DbpA function. We built and assayed
251 the *P. aeruginosa* PA14 Δ*dbpA* mutant strain and found no significant defect in *S.*
252 *sanguinis* SK36 growth enhancement (Fig. S6A) or in *P. aeruginosa* growth (Fig. S6B),
253 and thus did not pursue further study of this mutant.
254 We previously studied the effects of the *Pseudomonas* quinolone signal pathway
255 (*pqs*) on interactions between *P. aeruginosa* and *Staphylococcus aureus,* including the
256 utilization of 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), a respiratory chain inhibitor,
257 to drive *S. aureus* to fermentative metabolism (3, 35, 36). As was observed for *S.*
258 *constellatus* 7155, there was a significant reduction in the ability of *P. aeruginosa*
259 *pqsL*::*TnM* mutant to support *S. sanguinis* SK36 growth compared to the wild-type *P.*
260 *aeruginosa* PA14 (Fig. 2B). There was no detectable growth defect of the *pqsL*::*TnM*
261 mutant strain compared to wild-type *P. aeruginosa* PA14 in our assay condition (Fig.
262 S7). We chose to focus on the *P. aerugionsa pqsL*::*TnM* mutant for the remainder of our
263 study.

The *P. aeruginosa* Δ*pqsL* mutant has a defect in *Streptococcus* growth enhancement. The PQS pathway involves the production of multiple 4-hydroxy-2-alkylquinolones (HAQs) and begins with anthranilic acid, which is converted to intermediates of unknown structure by the enzymes PqsA and PqsD (Fig. 3A). These unknown intermediates can then be converted into HQNO by PqsL, our gene of interest, or 4-hydroxy-2-heptylquinoline (HHQ) by PqsB and PqsC. HHQ can then be converted into 3,4-dihydroxy-2-heptylquinoline (PQS) by PqsH (46–48). MvfR (also known as PqsR) is the transcriptional regulator that is activated by HHQ and PQS and positively regulates the transcription of operons involved in PQS, LasR, and RhlR quorum sensing pathways, as well as the operons required for production of the siderophores pyoverdine and pyochelin (49, 50). MvfR/PqsR and PQS have also been demonstrated to indirectly increase expression of the phenazine pyocyanin (49).

We considered two different mechanisms that could explain why the growth of *S. sanguinis* SK36 is no longer promoted by coculture with the *P. aeruginosa* PA14 *pqsL*::*TnM*. We hypothesized that either the *pqsL*::*TnM* strain may no longer be able to promote *S. sanguinis* SK36 growth, or the loss of PqsL function resulted in a *P. aeruginosa* strain that reduced *S. sanguinis* SK36 viability. To distinguish between these hypotheses, we assessed the *Streptococcus* growth enhancement capabilities of *P. aeruginosa* PA14 deletion mutants in the *pqs* pathway when grown in coculture with *S. sanguinis* SK36. We found that the Δ*pqsL* mutant was the only mutant in the *pqs* pathway that was unable to promote *S. sanguinis* SK36 growth (Fig. 3B and Fig. S8A

for *S. sanguinis* SK36 planktonic growth, Fig. S8B-C for *P. aeruginosa* biofilm and planktonic growth).

We complemented the Δ*pqsL* strain with an arabinose inducible pMQ72-*pqsL* construct and demonstrated a significant increase in viable *S. sanguinis* SK36 biofilm cells recovered when the complemented strain was induced with 0.2% arabinose (Fig. 3C and Fig. S9A for planktonic growth); there was no significant difference between wild-type *P. aeruginosa* PA14 and the complemented Δ*pqsL*/pMQ72-*pqsL* strain. Additionally, there was no significant difference in *P. aeruginosa* biofilm and planktonic growth in medium amended with 0.2% arabinose, the inducer of the expression for the P_{BAD} promoter on the pMQ72 plasmid (Fig. S9B-C).

Additionally, we assayed the *P. aeruginosa* Δ*pqsL* mutant strain in coculture with a few representative *Streptococcus* spp. from Fig. 1B to determine if the Δ*pqsL* mutant strain has a broad defect in *Streptococcus* growth enhancement (Fig. 3D). We found that for *S. intermedius* 4807, there was a slight but nonsignificant growth decrease during coculture with *P. aeruginosa* Δ*pqsL*, indicating that the mutant strain is unable to enhance *Streptococcus* growth. Similarly, we saw a nonsignificant decrease in *S. parasanguinis* 5357 growth in coculture with the Δ*pqsL* mutant compared to wild-type *P. aeruginosa* PA14. We did observe a significant decrease in *S. anginosus* 5535 cells recovered from the coculture with the Δ*pqsL* mutant strain compared to monoculture and coculture conditions with *P. aeruginosa* PA14, indicating that the Δ*pqsL* mutation is contributing to the repression of the growth of *S. anginosus* 5535. We found that both wild-type *P. aeruginosa* PA14 and the Δ*pqsL* mutant strain caused a nonsignificant reduction in *S. intermedius* 7155 cells recovered from coculture, indicating that both of

309 these *P. aeruginosa* strains may be able to outcompete *S. intermedius* 7155. We saw
310 no significant changes to *P. aeruginosa* growth while in coculture with these
311 representative *Streptococcus* spp. (Fig. S10).
312
313 **The *P. aeruginosa* Δ*pqsL* mutant likely suppresses *S. sanguinis* SK36 growth via**
314 **iron sequestration.** It has been demonstrated previously that a *pqsL* mutant is deficient
315 in HQNO production and overproduces PQS (47). Exogenous PQS has been
316 demonstrated to chelate iron and to increase the expression of the genes coding for
317 siderophore and phenazine biosynthesis enzymes in *P. aeruginosa* (51–53). Thus, we
318 hypothesized that it was the increased production of PQS and/or increased expression
319 of one or more PQS-regulated genes that caused the observed loss in growth
320 promotion of *S. sanguinis* SK36.
321 To test our hypothesis, we assayed *S. sanguinis* SK36 in coculture with the
322 Δ*pqsL* mutant strains deficient in production of the virulence factors regulated by PQS:
323 siderophores (Δ*pqsL*Δ*pvdA*Δ*pchE*) and phenazines (Δ*pqsL*Δ*phzA-G1/2*) (49, 51–53).
324 We found that the *P. aeruginosa* Δ*pqsL*Δ*pvdA*Δ*pchE* deletion mutant strain restored *S.*
325 *sanguinis* SK36 growth enhancement to levels similar to wild-type *P. aeruginosa* PA14
326 (Fig. 4A) without affecting *P. aeruginosa* growth (Fig. S11). In contrast, the *P.*
327 *aeruginosa* Δ*pqsL*Δ*phzA-G1/2* mutant did not restore *S. sanguinis* SK36 growth (Fig.
328 4A, Fig. S11).
329 One prediction of this iron sequestration model is that iron supplementation
330 should restore the *Streptococcus* enhancing activity of the *P. aeruginosa* Δ*pqsL* mutant.
331 We added 50μM $FeCl_3$ to our minimal medium coculture conditions and saw restoration

of *S. sanguinis* SK36 growth enhancement 6 out of 18 times. We explored this phenotype using different $FeCl_3$ concentrations, making a fresh $FeCl_3$ solution daily, using buffered media in our assay and when making the $FeCl_3$ stock solution, but the phenotype was still variable. We do not fully understand why the iron rescue phenotype was so variable. We measured the iron levels of the medium used in our coculture conditions (MEM) using ICP/MS and showed that the concentration of iron is below the limit of detection (<5 ppb), so it is plausible that the streptococci are iron limited in our coculture conditions.

Next, we tested the idea that coculture in anaerobic conditions would also lead to recovery of the *Streptococcus* growth promotion phenotype in the Δ*pqsL* mutant, because *P. aeruginosa* has been demonstrated to reduce pyoverdine and pyochelin production in anoxic conditions (54). Upon anaerobic coculture in an AnaeroPak-Anaero container with a GasPak satchet, the *P. aeruginosa* Δ*pqsL* mutant significantly enhanced *S. sanguinis* SK36 viability compared to coculture under aerobic conditions (Fig. 4B and Fig. S12A). The level of *S. sanguinis* growth enhancement promoted by the *P. aeruginosa* Δ*pqsL* mutant in anaerobic conditions was equivalent to that observed for the WT *P. aeruginosa*. Additionally, if *P. aeruginosa* were enhancing *S. sanguinis* SK36 growth solely through oxygen consumption, we would expect to see enhanced *S. sanguinis* growth in monoculture under anaerobic conditions, which we do not observe here.

We note that *P. aeruginosa* biofilm and planktonic growth were decreased under the anaerobic growth conditions used in these experiments compared to what we typically observe under aerobic conditions (Fig. S12B-C), which is not surprising given

that aerobic respiration is the main means of energy generation for this microbe. Together, these data indicate that wild-type *P. aeruginosa* is contributing to the growth *S. sanguinis* SK36 via a mechanism independent of oxygen consumption in our coculture system, and are consistent with our hypothesis that reduced siderophore production under anaerobic conditions mitigates the phenotype of the Δ*pqsL* mutant.

An iron ABC transporter of *S. sanguinis* SK36 participates in competition with *P. aeruginosa*. Our data suggest that one component of the interaction between *P. aeruginosa* and *Streptococcus* spp. is the competition for iron. The genome of *S. sanguinis* SK36 has been sequenced and annotated, and using this information we identified several gene products that, based on their annotation, might be involved in iron uptake. We predicted that if *S. sanguinis* SK36 is indeed competing with *P. aeruginosa* for iron, loss of one or more of these iron uptake system would compromise the ability of *S. sanguinis* SK36 to grow in coculture with *P. aeruginosa*. Given that the Δ*pqsL* mutant likely has an enhanced capacity to scavenge iron as indicated by the restoration of *S. sanguinis* SK36 growth enhancement by the Δ*pqsL*Δ*pvdA*Δ*pchE* mutant, any compromise observed for *S. sanguinis* SK36 iron acquisition mutants should be exacerbated in coculture with *P. aeruginosa* Δ*pqsL*.

Xu and colleagues reported a mutant library of *S. sanguinis* SK36 wherein non-essential genes are deleted and replaced with a kanamycin resistance cassette (27, 28). Using this library, we examined whether selected *S. sanguinis* SK36 mutant strains lacking genes involved in iron uptake (Table 1) have reduced growth in the presence of wild-type *P. aeruginosa* PA14 or the *P. aeruginosa* Δ*pqsL* strain. We tested *S. sanguinis*

SK36 strains carrying mutations in genes coding for iron regulatory proteins, an iron-binding lipoprotein, a ferrichrome-binding protein, and predicted iron-uptake ABC transporters using the coculture assay. We found that all of the mutant strains tested behaved like wild-type *S. sanguinis* SK36 in coculture, except for the *S. sanguinis* SK36 Ssx_1742 and Ssx_1744 mutant strains (Fig. 4C and S13A). The SSA_1742 gene codes for a predicted ferrichrome-binding protein and the SSA_1744 gene codes for a predicted permease protein of an iron compound ABC transporter.

The *S. sanguinis* SK36 Ssx_1742 and Ssx_1744 mutant strains demonstrated reduced growth in monoculture conditions, 6.5-fold and 6.9-fold respectively, indicating that they may be iron starved in our minimal medium growth conditions (Fig. 4C). This iron starvation phenotype is exacerbated in coculture with wild-type *P. aeruginosa* PA14 with a 44.9-fold reduction in Ssx_1742 cells and a 5.5-fold reduction in Ssx_1744 cells obtained from coculture compared to wild-type *S. sanguinis* SK36 coculture.

In coculture with the *P. aeruginosa* Δ*pqsL* mutant strain, there is a significant, 4.9-fold reduction in Ssx_1742 cells compared to wild-type *S. sanguinis* SK36 in coculture with the Δ*pqsL* mutant. Importantly, coculture of the *S. sanguinis* SK36 Ssx_1744 mutant strain showed no additional, significant growth defect when grown in coculture with the *P. aeruginosa* Δ*pqsL* mutant compared to the WT *P. aeruginosa* strain. We take this result to mean that the increased iron sequestration by the Δ*pqsL* mutant is competing for the iron typically transported by the *S. sanguinis* SK36 Ssx_1744-encoded iron ABC transporter; thus loss of Ssx_1744 confers no additional phenotype when cocultured with the *P. aeruginosa* PA14 Δ*pqsL* mutant. Finally, we

400 observed no significant difference in *P. aeruginosa* PA14 and the Δ*pqsL* mutant strain

401 growing in coculture with *S. sanguinis* SK36 mutant strains (Fig. S13B and S13C).

Discussion

In this study, we sought to characterize a polymicrobial interaction that occurs between *P. aeruginosa* and *Streptococcus* spp. We previously demonstrated that *P. aeruginosa* can suppress *S. constellatus* 7155 biofilms through surfactant production, and that this suppression can be alleviated through treatment with the CF maintenance antibiotic, tobramycin (22). Our current work adds to our understanding of *P. aeruginosa* – *Streptococcus* interactions by demonstrating the widespread ability of multiple *P. aeruginosa* clinical isolates from CF patients and laboratory strains to enhance the growth of multiple species of *Streptococcus.* To better understand the basis of the ability of *P. aeruginosa* to promote the growth of *Streptococcus* spp., we screened *P. aeruginosa* transposon insertion mutants to identify factors that contribute to the ability of *P. aeruginosa* to enhance growth of *S. constellatus* - we identified 46 candidate mutants. Following up on these mutants, we identified only one strain carrying a mutation in the *pqsL* gene that has a consistent, reduced *Streptococcus* spp. growth enhancement phenotype versus multiple species of *Streptococcus*. Upon further investigation we revealed that this mutant no longer promotes *Streptococcus* growth because the *P. aeruginosa* Δ*pqsL* mutant strain likely actively competes with *Streptococcus* for iron. Loss of PqsL function has been reported to enhance PQS production (47), excess PQS has been demonstrated to enhance siderophore biosynthesis gene transcription (51–53), and PQS-mediated iron sequestration by *P. aeruginosa* has been demonstrated to reduce growth of both gram positive and gram negative soil bacteria (32). This PQS-mediated growth inhibition of soil bacterial growth can be restored upon addition of 50μM $FeCl_3$ (32). Similarly, our data show the ability to

restore *Streptococcus* growth by introducing mutations in the siderophore genes to the Δ*pqsL* mutant or by growing the cocultures anaerobically, a growth condition where *P. aeruginosa* is known to reduce pyoverdine and pyochelin production (54). We cannot rule out that the increased siderophore production in the Δ*pqsL* mutant impacts production of reactive oxygen species that may be toxic to *Streptococcus* spp.; the fact that growth of the Δ*pqsL* mutant under anaerobic conditions reverses its phenotype is consistent with this idea.

An interesting observation from this study was the demonstration of a significant increase in *S. sanguinis* SK36 biofilm growth between an isogenic nonmucoid and mucoid *P. aeruginosa* PAO1 strain (Fig. 1D). Previous work demonstrated that *S. parasanguinis* is able to use the streptococcal surface adhesin BapA1 to bind alginate produced by mucoid *P. aeruginosa* and enhance *S. parasanguinis* biofilm formation *in vitro*, however *S. gordonii* and *S. sanguinis* SK36 did not demonstrate enhanced biofilm formation (23). Here we demonstrate a significant growth increase of *S. sanguinis* SK36 when in coculture with *P. aeruginosa* PDO300 *mucA22* compared to the isogenic nonmucoid strain. It is possible that *S. sanguinis* SK36 can also bind to alginate. Alternatively, we hypothesize that the growth enhancement induced by the mucoid *P. aeruginosa* strain may be due to decreased rhamnolipid production that has been described in mucoid strains (36) and the corresponding relief of rhamnolipid-induced *Streptococcus* killing (22). Furthermore, mucoid strains were shown to produce lower levels of products of the PQS pathway and reduced levels of siderophores (36). Thus, *Streptococcus* spp. may more readily co-exist, and perhaps grow to larger numbers, in patients with mucoid *P. aeruginosa*, a question that could be answered by performing a

clinical study assessing relative levels of *Streptococcus* spp. as a function of mucoid *P. aeruginosa*. Furthermore, these data indicate that the interactions between *P. aeruginosa* and *Streptococcus* may change over the lifetime of patients with CF as the colonizing *P. aeruginosa* converts to mucoidy.

Oral streptococci have been demonstrated to utilize hydrogen peroxide to inhibit the growth and colonization of competing microorganisms (15, 20, 21, 40), and we hypothesized that *P. aeruginosa* catalase might play a role in enhancing *Streptococcus* growth as catalase has been found in the supernatant of *P. aeruginosa* cultures (38, 39, 42). However, we found no significant defect in *S. sanguinis* SK36 growth enhancement by our *P. aeruginosa* Δ*katA*, Δ*katB*, and Δ*katA*Δ*katB* compared to wild-type *P. aeruginosa* PA14 indicating that catalase is not the factor produced by *P. aeruginosa* that is enhancing *Streptococcus* growth. It has been demonstrated that *P. aeruginosa* does not secrete catalase and that it is found in the supernatant due *P. aeruginosa* cell lysis (38) – it may be that catalase found in the supernatant is too dilute to have a positive influence on *Streptococcus* growth in coculture, or that the hydrogen peroxide is not growth limiting to the streptococci in our coculture conditions. Thus, we conclude that *P. aeruginosa* catalase is not influencing *Streptococcus* growth in our model system. It is also worth noting that anaerobic coculture was not sufficient to enhance *S. sanguinis* SK36 monoculture growth to the same levels achieved during coculture with *P. aeruginosa* PA14 in aerobic conditions (Fig. 4B). These data indicate that *P. aeruginosa*-mediated growth enhancement of streptococci cannot be explained by oxygen consumption via *P. aeruginosa*.

To better understand how *S. sanguinis* SK36 might compete with *P. aeruginosa* for iron in iron deplete conditions, we examined a set of *S. sanguinis* SK36 mutants lacking putative iron uptake systems or regulatory genes. Of the nine mutants tested, only two showed reduced growth of *S. sanguinis* SK36 when in coculture with *P. aeruginosa* PA14, Ssx_1742 lacking a ferrichrome-binding protein and Ssx_1744 lacking the permease protein of an iron-compound ABC transporter (Fig. 4C and S12). The Ssx_1742 mutant demonstrated a significant growth defect in monoculture, and during coculture with *P. aeruginosa* PA14. The growth defect of the Ssx_1742 mutant was worsened when cocultured with the Δ*pqsL* mutant. Together, these data indicate that the Ssx_1742 mutant strain is unable to compete with *P. aeruginosa* for the limited iron in our co-culture conditions, and that the ferrichrome-binding protein encoded by Ssx_1742 is not involved in the competition for this metal with *P. aeruginosa*. In contrast, the Ssx_1744 mutant showed no additional defect when cocultured with the Δ*pqsL* mutant versus the WT *P. aeruginosa*. We take this result to mean that the increased production of the siderophores in the Δ*pqsL* mutant is competing for the iron typically transported by the *S. sanguinis* SK36 Ssx_1744-encoded iron ABC transporter; thus loss of Ssx_1744 confers no additional phenotype when cocultured with the *P. aeruginosa* PA14 Δ*pqsL* mutant. These data indicate that the Ssx_1744-encoded iron ABC transporter of *S. sanguinis* SK36 plays a key role in the competition with *P. aeruginosa*.

Our data support a second mechanism whereby *P. aeruginosa* can limit the growth of *Streptococcus* spp. (Figure 5), including SMG, via iron sequestration. We previously reported that *P. aeruginosa* rhamnolipid surfactants could reduce the viability

493 of *S. constellatus*. *P. aeruginosa* can also influence the biofilm formation of *S.*
494 *parasanguinis* through alginate production (23) and the growth of *Streptococcus* spp.
495 via a currently undescribed mechanisms (15, 17, 22). Conversely, previous studies
496 investigating interactions between *P. aeruginosa* and *Streptococcus* spp. also showed
497 that *Streptococcus* spp. influences transcription of *P. aeruginosa* virulence genes,
498 including rhamnolipids, elastase, and phenazine biosynthesis genes through AI-2
499 signaling (16) and an undescribed mechanism (15, 17, 18), and can suppress *P.*
500 *aeruginosa* growth when they are a primary colonizer through production of H_2O_2(15,
501 20) and reactive nitrogenous intermediates (20, 21). Thus, this polymicrobial interaction
502 is complex.

503 Our data also indicate that *P. aeruginosa* can promote the growth of various
504 *Streptococcus* spp., but we do not understand the basis of this growth promotion. We
505 anticipated that the genetic screen described here would likely identify components of
506 such a growth-promoting pathway in *P. aeruginosa*; instead, our screen only identified a
507 single locus apparently involved in a competitive interaction. We suggest two possible
508 explanations for our findings. First, perhaps *P. aeruginosa* determinants that promote
509 *Streptococcus* growth are essential; we think this explanation unlikely, but a formal
510 possibility. More likely is that *P. aeruginosa* has multiple, redundant pathways to boost
511 *Streptococcus* growth. Thus, our genetic approach would be expected to fail to identify
512 such redundant pathways, and alternative strategies to explore *P. aeruginosa*-
513 *Streptococcus* interactions must be employed in future studies.

514 Finally, the observations we present here may be of relevance in the CF lung, as
515 many patients are co-colonized by *P. aeruginosa* and *Streptococcus* spp. (9, 55).

Analysis of the average available iron in the airway varies markedly between ~0.02 μM in healthy individuals and ~8 μM in patients with CF, and there is a great deal of variability within patients with CF (56, 57). The increased iron in the CF airway is likely due to the reported enhanced levels of extracellular iron in the apical surface liquid of airway cells with a mutation in CFTR (58) and the bleeding into the airway (hemoptysis) associated with this patient population (59). Thus, in CF patients, iron levels in the airway can range from concentrations wherein we might expect direct competition between *P. aeruginosa* and *Streptococcus* for this limited resource, to levels wherein abundant iron would mitigate such competition. Additional studies are necessary to determine if *Streptococcus* spp. are iron limited (or not) in the CF airway, or in sufficiently close proximity to *P. aeruginosa* in the airway (i.e., in mixed microcolonies) to expect direct competition for iron in a local niche.

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Materials and Methods

Bacterial strains and growth conditions. Strains used in this study are listed in Supplemental Table S3. *P. aeruginosa* strains were grown on lysogeny broth (LB) agar or in LB liquid with shaking at 37ºC, and where indicated, in the presence of antibiotics at the following concentrations: 25μg/ml gentamicin, 250μg/ml kanamycin, 75μg/ml tetracycline. *Streptococcus* spp. were grown as previously described (22) on tryptic soy agar supplemented with 5% defibrinated sheep's blood (blood agar) or statically in Todd Hewitt broth supplemented with 0.5% yeast extract (THY) and 20μl/ml oxyrase (Oxyrase, Inc.) at 37ºC with 5% CO_2. *S. sanguinis* SK36 gene replacement mutant strains were grown on blood agar or THY with 500μg/ml kanamycin (27). At the end of each coculture assay, *P. aeruginosa* was grown overnight on *Pseudomonas* Isolation agar (PIA) at 37ºC, and *Streptococcus* spp. were grown overnight on blood agar at 37ºC anaerobically in AnaeroPak-Anaero containers (Thermo Fisher) or on blood agar supplemented with 10μg/ml neomycin and 10μg/ml polymixin B (*Streptococcus* selection agar) when specified. *Saccharomyces cerevisiae* strain InvSc1 (Invitrogen), was used for homologous recombination to build the pMQ30-*katA*, pMQ-30-*katB*, and pMQ30-*dbpA* deletion vector and pMQ72-*pqsL* complementation vector. InvSc1 was grown as previously described in 1% Bacto yeast extract, 2% Bacto peptone, and 2% dextrose (60). Synthetic defined agar-uracil (4813-065;Qbiogene) was used for InvSc1 selections.

Species identification of *Streptococci.* *Streptococci* were isolated at the Dartmouth Hitchcock Medical Center in Lebanon, NH. *Streptococcus* clinical isolates were speciated using 16S rRNA gene sequencing. Genomic DNA (gDNA) was extracted from each strain from overnight cultures using the Gentra Puregene Yeast/Bact. Kit (QIAGEN) followed with 16S-ITS PCR as previously described (61) using the Strep16S-1471F and 6R-IGS primers (listed in Table S4). *Streptococcus oralis, S. mitis,* and *S. pneumoniae* were further differentiated by PCR of a region of the *gdh* gene and sequencing as previously described using the Strep-gdhF and Strep-gdhR primers (listed in Table S4) (61). The Phusion Polymerase PCR protocol (New England Biolabs) was followed for preparing 50µl reactions, and the PCR conditions for the 16S-ITS region were: 98ºC for 30s followed by 25 cycles of 98ºC for 10s, 61ºC for 15s, 72ºC for 30s, a final extension at 72ºC for 7 minutes. The PCR conditions for amplifying *gdh* were as follows: 98ºC for 30s, followed by 30 cycles of 98ºC for 10s, 57.9ºC for 15s, 72ºC for 30s followed by a final extension at 72ºC for 7 minutes. The resulting PCR products were imaged on a 1% agarose gel with Sybr Safe (Thermo Fisher Scientific Inc.). The remaining PCR reaction was purified using the QIAquick PCR Purification kit (QIAGEN), and the purified DNA product was sequenced at the Dartmouth Molecular Biology Core Facility using the Applied Biosystems 3730 DNA Analyzer. Sequence results were analyzed using NCBI BLAST for species identification.

Mixed microbial coculture system. Cocultures were conducted as previously described in the CFBE model system (3, 22, 35, 36) with some modifications. Overnight cultures of *P. aeruginosa* and *Streptococcus* spp. were individually centrifuged at

10,000 x *g* for 3 minutes, the cell pellet was washed with 1.5 ml minimal essential medium (MEM) supplemented with 2 mM L-glutamine (MEM+L-Gln), centrifuged again, and the cell pellet was resuspended in 1.5 ml MEM+L-Gln. The optical density at 600nm (OD_{600}) of each culture was determined and the *P. aeruginosa* cultures were adjusted in MEM+L-Gln to an OD_{600} of 0.05. The *Streptococcus* spp. cultures were adjusted to an OD_{600} of 0.1. *S. sanguinis* SK36 overnight cultures were adjusted to an OD_{600} of 0.1, then further diluted 1:100 in MEM+L-Gln due to the robust growth *S. sanguinis* SK36 exhibits in monoculture. A 1:1 mixture of *P. aeruginosa* and *Streptococcus* spp. was prepared from the adjusted cultures. Three wells of a 96-well plate were inoculated per monoculture and coculture condition with 100uL per well. The culture plates were then incubated statically for 1 hour at 37ºC with 5% CO_2, at which point the unattached planktonic cells were aspirated with a multichannel pipette and replaced with 100µl MEM supplemented with 2mM L-glutamine and 0.4% L-arginine (MEM+L-Gln+L-Arg). The culture plates were incubated statically for an additional 5.5 hours, at which point the supernatant was removed and replaced with 100µl MEM+L-Gln+L-Arg. At 21 hours post-inoculation, planktonic cells were removed to be plated and biofilms were disrupted using a 96 pin replicator in 100µl of MEM+L-Gln. Both planktonic and biofilm cells were 10-fold serially diluted and plated on selective media. PIA plates were grown overnight aerobically, and blood agar plates were grown overnight in AnaeroPak-Anaero containers (Thermo Scientific) with GasPak satchets (BD) to selectively grow *P. aeruginosa* and *Streptococcus* spp., respectively. Following overnight incubation, colonies were counted and the colony forming units (CFU) per milliliter of culture were determined.

Growth kinetics in mixed microbial coculture system. *P. aeruginosa* PA14 and PAO1 were grown in coculture with *S. sanguinis* SK36 as described above, with one 96-well plate per time point. Six time points were assessed: 0, 3, 5.5, 7.5, 10, and 24 hours. The 0-hour time point corresponds to the initial inoculum. At each time point, the planktonic and biofilm cells from the same wells were serially diluted and plated on PIA and blood agar. Cells were harvested from the 5.5 hour time point plate prior to the 5.5 hour medium exchange.

Construction of *P. aeruginosa* PA14 deletion mutant strains. The pMQ30 vector (Table S3) was used to generate the *P. aeruginosa* PA14 Δ*katA*, Δ*katB*, Δ*katA*Δ*katB*, and Δ*dbpA* mutant strains. The pMQ30-*katA*, pMQ30-*katB*, and pMQ30-*dbpA* deletion constructs were built using homologous recombination of the PCR products made with the respective "KO" primers (listed in Table S4) with the Xba1 restriction enzyme-digested pMQ30 in yeast as previously reported (60). Plasmid integrants were isolated on LB agar supplemented with gentimicin and nalidixic acid followed by counterselection on sucrose medium. Deletion mutants were confirmed by PCR with respective "conf." primers (Table S4), followed by sequencing. Coculture was conducted as described above with the confirmed *P. aeruginosa* PA14 deletion mutant strains.

Genetic screen. The Ausubel lab created a nonredundant *P. aeruginosa* PA14 transposon library (PA14NR set) in 96-well plate format (43). Initially, a 96 pin replicator was used to transfer inocula from the frozen library to a sterile 96-well plate containing

627 150µl of LB per well. The plate was then incubated statically for 24 hours at 37ºC. *S.*
628 *constellatus* 7155 frozen aliquots were made from 750µl of overnight culture mixed with
629 750µl of 40% glycerol. The day of the coculture experiment, frozen *S. constellatus* 7155
630 aliquots were thawed and 500µl of aliquot were added to 4.5ml THY cultures with 100µl
631 Oxyrase, and were grown for 6-8h at 37ºC with 5% CO_2. The *S. constellatus* 7155
632 culture was then adjusted to an OD_{600} of 0.05 in MEM+L-Gln, and 100µl of adjusted
633 culture were added to each well of a sterile 96-well plate. The PA14NR set was grown
634 in LB for 24 hr in a 96 well plate format - each well contained a transposon mutant from
635 the *P. aeruginosa* PA14NR set. A 96 pin replicator was then used to transfer 2-3µl of
636 culture from the transposon library plate into the plate containing *S. constellatus* 7155.
637 The coculture plates were then incubated statically for 2 hours at 37ºC with 5% CO_2.
638 After 2 hours, the supernatant and unattached bacteria were aspirated using a
639 multichannel pipette and 100µl MEM+L-Gln with 5µg/ml tobramycin to suppress *P.*
640 *aeruginosa* PA14 rhamnolipid production were added to each well. The plates were
641 then incubated statically for an additional 20 hours at 37ºC with 5% CO_2. At 22 hours
642 post-inoculation, the 96 pin replicator was used to disrupt the biofilms into the
643 supernatant fraction. The 96 pin replicator was then used to spot culture onto large petri
644 plates containing either PIA or *Streptococcus* selection agar. PIA plates were incubated
645 overnight at 37ºC, and *Streptococcus* selection agar plates were incubated for 24 hours
646 at 37ºC with 5% CO_2. In the initial screen, we identified P. aeruginosa mutants that
647 showed low or undetectable *S. constellatus* 7155 growth. To confirm the phenotype, the
648 candidate *P. aeruginosa* PA14 transposon mutant strain was picked from the PIA plate
649 and grown statically overnight at 37ºC in a sterile 96-well plate in 125µl LB. The next

650 morning, 125µl of 40% glycerol was added to each well containing *P. aeruginosa* PA14
651 candidate mutants, and these "candidate mutant" plates were stored at -80ºC for the
652 next round of screening. For the second round of the screen, the coculture process
653 described above was repeated with the plates containing candidate mutants.
654 If we had clean deletions of the candidate mutants, they were also tested in the
655 assay above. If the clean deletion did not recapitulate the original transposon mutant,
656 that transposon mutant was eliminated from the list of candidate mutants. Table S2
657 shows the final list of *P. aeruginosa* PA14 transposon insertion mutant strains that
658 yielded low or undetectable *S. constellatus* 7155 growth after rescreening.
659 We then tested each individual *P. aeruginosa* PA14 transposon mutant in Table
660 S2 in our standard 96-well coculture assay as described above with *S. sanguinis* SK36.
661 The two mutants that yielded consistently low *S. sanguinis* SK36 growth in our standard
662 coculture are in bold in Table S2.
663
664 ***P. aeruginosa* Δ*pqsL* complementation.** The pMQ72 vector (Table S3) with an
665 arabinose-inducible promoter was used to complement the *P. aeruginosa* PA14 Δ*pqsL*
666 deletion mutant. The pMQ72-*pqsL* complementation plasmid was built using
667 homologous recombination of the PCR product made with the pqsL comp 3' and pqsL
668 comp 5' primers (listed in Table S4) with SacI restriction enzyme-digested pMQ72 in
669 yeast as previously reported (60). *P. aeruginosa* PA14 Δ*pqsL*/pMQ72-*pqsL* and *P.*
670 *aeruginosa* PA14 Δ*pqsL*/pMQ72-empty vector control strains were cocultured with *S.*
671 *sanguinis* SK36 as described above with the following changes: at 1 and 5.5h post-

672 inoculation, MEM+L-Gln+L-Arg supplemented with L-arabinose at 0% and 0.2% final
673 concentration was added to the medium to induce pMQ72-*pqsL* gene expression.
674
675 **Coculture with 50μM ferric chloride.** Coculture was conducted as described above,
676 but with the following changes: at 1 and 5.5 hours post-inoculation, supernatants were
677 aspirated with a multichannel pipette, and replaced with MEM+L-Gln+L-Arg, with or
678 without freshly prepared, filter sterilized 50μM ferric chloride hexahydrate.
679
680 **Anaerobic coculture.** Coculture was conducted as described above, but with the
681 following alterations: once the plates were inoculated, they were incubated in
682 AnaeroPak-Anaero containers with a GasPak satchet. At each medium change (1 hour,
683 5.5 hours), a new satchet was added to the container to ensure anaerobic coculture
684 conditions. The AnaeroPak container was incubated in the same incubator as the
685 aerobic plate to control for any environmental effects.

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889

890 **Figure Legends**

891

892 **Figure 1. *P. aeruginosa* enhanced growth of *Streptococcus* spp. in coculture.** (A to
893 D) Coculture assays were conducted to investigate streptococcal growth when exposed
894 to *P. aeruginosa*. (A) *S. sanguinis* SK36 (Ss) cocultured with *P. aeruginosa* PAO1. (B)
895 Coculture growth kinetics of *S. sanguinis* with *P. aeruginosa* PA14 were investigated.
896 The time points each represent the average of three biological replicates with three
897 technical replicates. The error bars indicate SD. (C) A representative group of clinical
898 and reference *Streptococcus* strains were tested in coculture with *P. aeruginosa* PAO1
899 (see Fig. S3A for all *Streptococcus* spp. strains tested). In this panel, each strain is
900 denoted by their strain number, *S. anginosus* 5535, *S. intermedius* 4807, *S. constellatus*
901 7155, *S. parasanguinis* ATCC15912, *S. pneumoniae* D39, *S. peroris* ATCC700780, *S.*
902 *oralis* 7404, and *S. salivarius* JIM8780. (D) *S. sanguinis* SK36 was tested in coculture
903 with multiple *P. aeruginosa* clinical and laboratory strains. (A, C, D) Each column
904 represents the average of three biological replicates with three technical replicates. The
905 error bars indicate the standard deviation. ns, not significant, *, $P < 0.05$, **, $P < 0.01$,
906 and ***, $P < 0.001$ by paired two-tailed student's *t*-test (A and C), repeated measures
907 one-way analysis of variance (ANOVA) with Tukey's multiple comparisons posttest (D),
908 and paired two-tailed student's *t*-test between Ss + PAO1 DHL08 and Ss + PAO1
909 mucA22 (D). The corresponding graphs depicting *P. aeruginosa* growth in these assays
910 can be found in Figures S1-4.

911

912 **Figure 2. Screening for *P. aeruginosa* PA14 mutants altered in interaction with**

***Streptococcus*.** (A) An overview of the *P. aeruginosa* PA14NR Set transposon mutant screen used to identify *P. aeruginosa* transposon insertion mutants that can no longer enhance *S. constellatus* 7155 growth, and the number of *P. aeruginosa* PA14 transposon insertion mutants identified in each step. (B) Coculture between a mutant strain identified in the screen, *P. aeruginosa pqsL*::*TnM*, and S*. sanguinis* SK36. Each bar represents the average of three biological replicates, each with three technical replicates. Error bars represent SD. **, P < 0.01 and ***, P < 0.001 by repeated measures ANOVA with Tukey's multiple comparison posttest. *P. aeruginosa* growth data from this assay can be found in Figure S7.

Figure 3. The *P. aeruginosa* Δ*pqsL* mutant inhibits *Streptococcus* growth. (A) The PQS biosynthetic pathway and the enzymes that catalyze each step are shown. (B) Coculture of *S. sanguinis* SK36 with wild-type *P. aeruginosa* PA14 and *P. aeruginosa* PA14 mutant strains lacking each enzyme in the PQS biosynthetic pathway. (C) Coculture of *S. sanguinis* SK36 with *P. aeruginosa* PA14, the Δ*pqsL* mutant, its complement pMQ72-*pqsL* and the vector control pMQ72 in the presence of 0.2% arabinose. (D) Coculture of representative *Streptococci* from Figure 1 in coculture with the WT *P. aeruginosa* PA14 and the Δ*pqsL* mutant strain. In each panel, bars represent the average of three biological replicates, each with at least three technical replicates. Error bars indicate SD. ns, not significant, *, P < 0.05, **, P < 0.01, and ***, P < 0.001 by repeated measures ANOVA with Dunnett's multiple comparisons posttest with *Streptococcus*-only as the control condition (B) and repeated measures ANOVA with Tukey's multiple comparisons posttest (C and D). The corresponding *P. aeruginosa*

936 growth data for these experiments can be found in Figures S8-10.

937

938 **Figure 4. *P. aeruginosa* Δ*pqsL* mutant likely inhibits *Streptococcus* growth by**
939 **sequestering iron.** (A) Coculture of *S. sanguinis* SK36 with *P. aeruginosa* PA14 mutant
940 strains lacking *pqsL,* and siderophore (Δ*pqsLpvdApchE*) or phenazine genes
941 (Δ*pqsLphzA-G1/2*). (B) Coculture of *S. sanguinis* SK36 with *P. aeruginosa* PA14 mutant
942 strains in 5% CO_2 (aerobic) or in anaerobic conditions. (C) Coculture of *S. sanguinis*
943 SK36 gene replacement mutants lacking putative iron acquisition genes with *P.*
944 *aeruginosa* PA14 and the Δ*pqsL* mutant. Each bar represents the average of three
945 biological replicates, each with at least three technical replicates. Error bars indicate
946 SD. ns, not significant, *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ by repeated
947 measures ANOVA with Tukey's multiple comparisons posttest (A), paired two-tailed
948 student's *t*-test (B), and repeated measures ANOVA with Dunnett's multiple
949 comparisons posttest with *S. sanguinis* (Ss) as the control condition (C). The
950 corresponding *P. aeruginosa* growth data for these experiments can be found in Supp.
951 Figure S11-13.

952

953 **Figure 5. A model for *P. aeruginosa-Streptococcus* interactions**. (A-B) *P.*
954 *aeruginosa* has both positive and negative interactions with *Streptococcus* spp. (A) It
955 has been demonstrated previously that *P. aeruginosa* can enhance *S. parasanguinis*
956 biofilm formation through alginate secretion (23) or inhibit *Streptococcus* growth with
957 rhamonolipid secretion (22). Here we describe a negative interaction wherein iron
958 sequestration by *P. aeruginosa* limits *Streptococcus* spp. growth. *Streptococcus*
959 promoting factors produced by *P. aeruginosa* have not yet been identified (indicated by

960 the question mark). (B) Previous evidence also demonstrates that *Streptococci* can
961 influence *P. aeruginosa* through AI-2 signaling (16), leading to enhanced *lasB*, *rhlA*,
962 *exoT*, and *phzA1/*2 gene expression, or inhibit *P. aeruginosa* viability through H_2O_2
963 production (15, 20, 21) and subsequent generation of reactive nitrogenous
964 intermediates (20, 21).

965 **Table 1: *S. sanguinis* SK36 iron-related gene products.**

Gene Number	Predicted Gene Product
Ssx_0256	ScaR metalloregulator
Ssx_0686	Fe^{2+}/Zn^{2+} uptake regulation protein
Ssx_1129	periplasmic iron transport lipoprotein
Ssx_1578	ABC-type Fe^{3+}-siderophore transport system, permease component
Ssx_1581	FatB, metal-binding ABC transporter
Ssx_1741	ABC-type Fe3+-siderophores transporter, ATPase component
Ssx_1742	ferrichrome-binding protein
Ssx_1743	ABC-type Fe3+-siderophore transport system, permease component
Ssx_1744	iron compound ABC transporter, permease protein

966

Figure 1

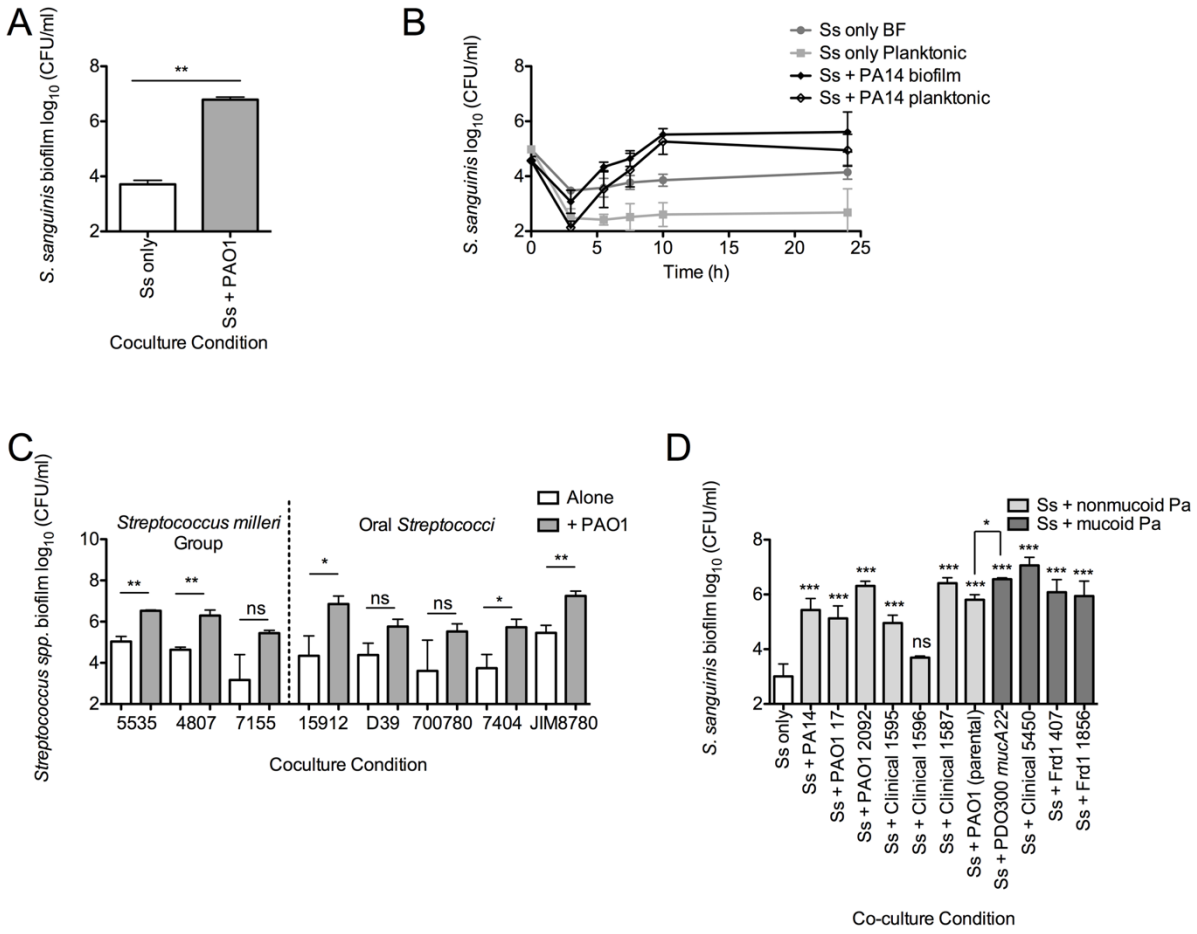
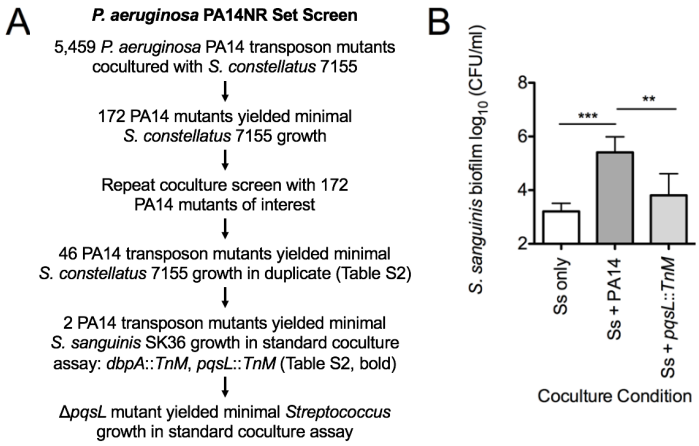


Figure 2



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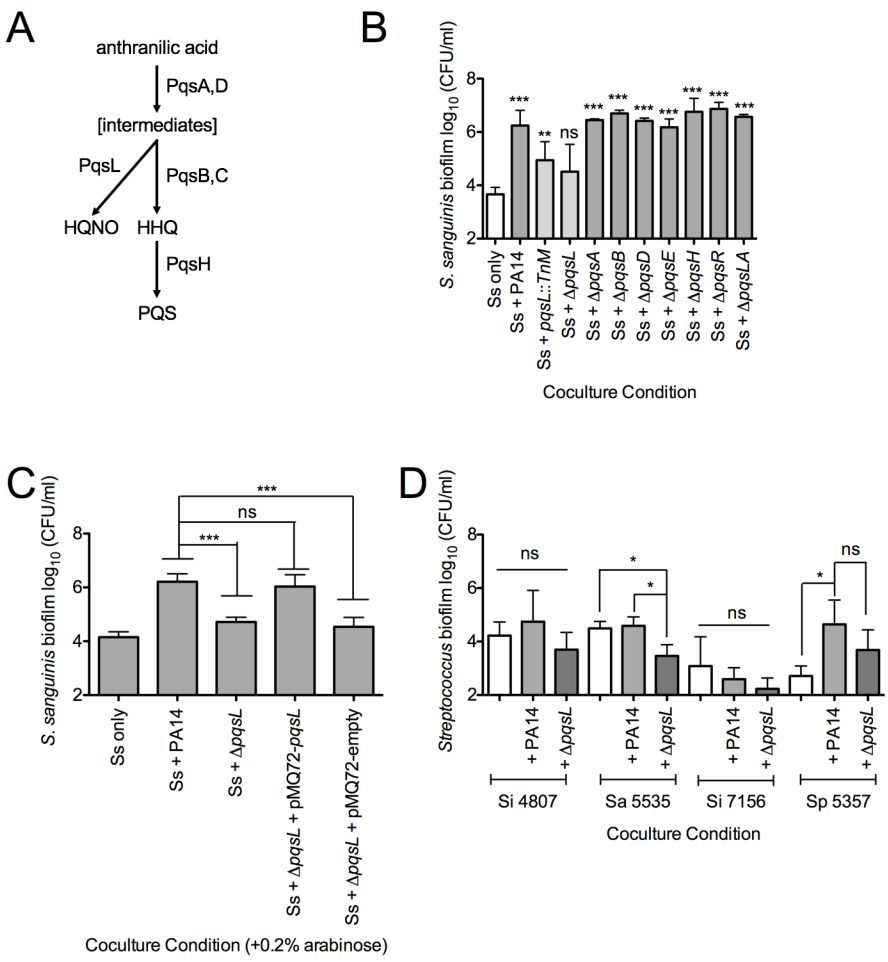

Figure 4

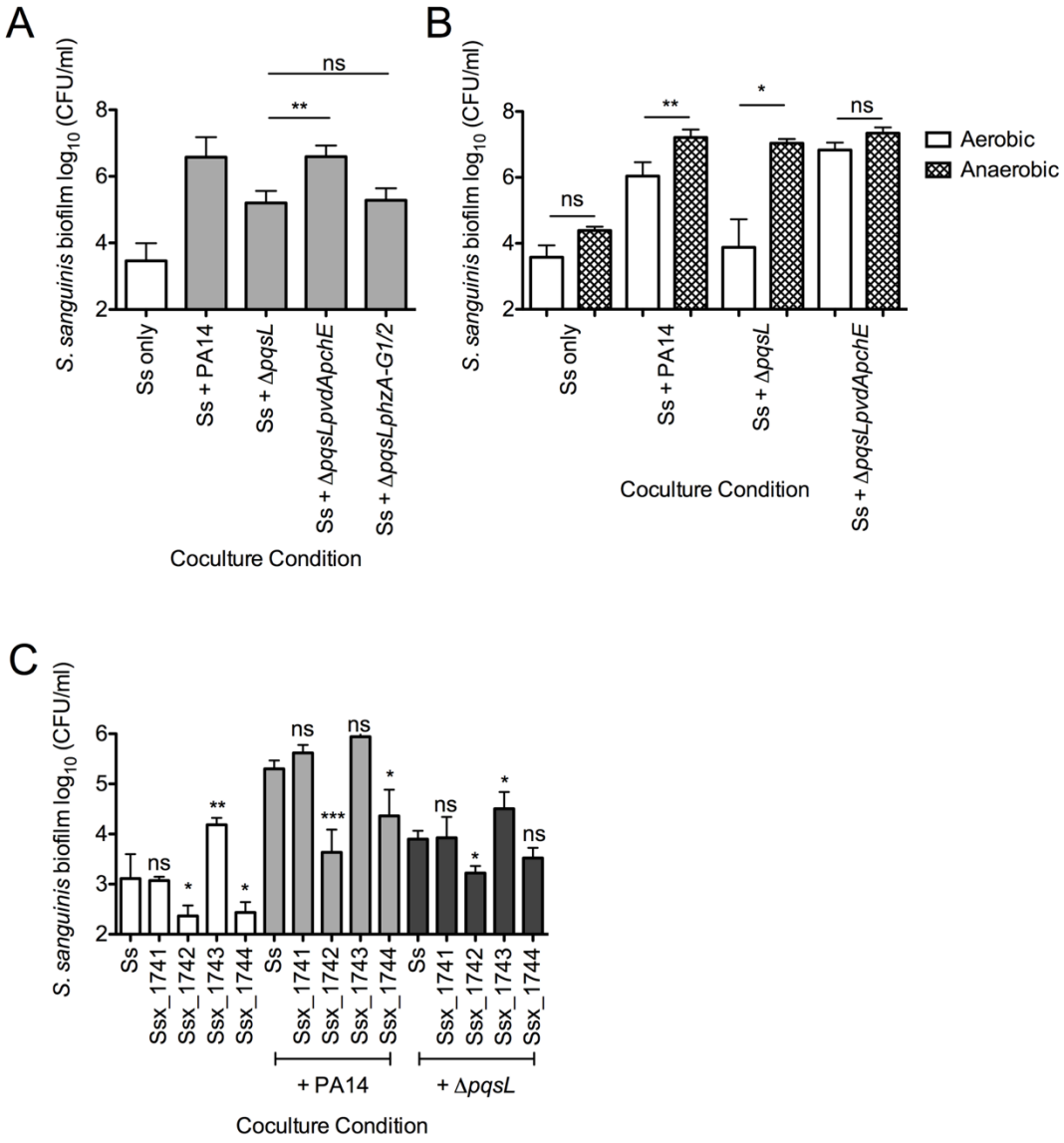

Figure 5