



Pseudomonas aeruginosa Can Inhibit Growth of Streptococcal Species via Siderophore Production

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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 organisms in the CF lung; the presence of *P. aeruginosa* correlates with lung function decline, and the *Streptococcus milleri* group (SMG), a subgroup of the viridans streptococci, is associated with exacerbations in patients with CF. Here we characterized 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 that are unable to enhance *Streptococcus* growth, and we identified the *P. aeruginosa* *pqsL::TnM* mutant, which failed to promote growth of *Streptococcus constellatus* and *S. sanguinis*. Characterization of the *P. aeruginosa* $\Delta pqsL$ mutant revealed that this strain cannot promote *Streptococcus* growth. Our genetic data and growth studies support a model whereby the *P. aeruginosa* $\Delta pqsL$ mutant overproduces siderophores and thus likely 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 may alter the biology of the organisms involved in CF-related infections, leading to changes in growth, virulence, and/or antibiotic tolerance, and could thereby affect patient health and response to treatment. In this study, we demonstrate interactions between *P. aeruginosa* and streptococci using a coculture model and show 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, and they add to our limited knowledge of polymicrobial interactions in the CF airway.

KEYWORDS *Pseudomonas aeruginosa*, *Streptococcus*, biofilm, cystic fibrosis, polymicrobial

Cystic fibrosis (CF) is a genetic disease caused by a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) (1), which leads to reduced mucociliary clearance in the lungs of these patients (2). Due to this reduced mucociliary clearance, bacteria colonize the lungs of patients with CF and establish chronic, polymicrobial infections that cause increased inflammation and respiratory function

Citation Scott JE, Li K, Filkins LM, Zhu B, Kuchma SL, Schwartzman JD, O'Toole GA. 2019. *Pseudomonas aeruginosa* can inhibit growth of streptococcal species via siderophore production. J Bacteriol 201:e00014-19. <https://doi.org/10.1128/JB.00014-19>.

Editor Thomas J. Silhavy, Princeton University

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Received 7 January 2019

Accepted 29 January 2019

Accepted manuscript posted online 4 February 2019

Published 26 March 2019

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, 65).

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 (4, 5, 7, 66). In contrast, previous research from our laboratory (8) and two other groups (9, 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 between *Streptococcus* spp. and the other microbes in the CF airway.

Pseudomonas aeruginosa is the dominant microorganism (>50% relative abundance) in the lungs of ~45% of adults patients with CF (11), is cultured from >80% of these patients (12), and is the predominant microbe in the lung at end-stage disease (13). *P. aeruginosa* and streptococci have been found to cocolonize CF patients (4, 5, 7, 14, 15), but the polymicrobial interactions that occur between these organisms are not well studied. Previous studies investigating interactions between *P. aeruginosa* and *Streptococcus* spp. demonstrated that *Streptococcus* spp. can influence production of *P. aeruginosa* virulence factors such as rhamnolipids, elastase, and phenazines (6, 16–19) and can suppress *P. aeruginosa* growth through hydrogen peroxide production (16) and production of reactive nitrogenous intermediates (20, 21). Conversely, *P. aeruginosa* was found to influence the growth (16–19, 22, 23) and biofilm formation (23, 24) of *Streptococcus* spp. Work from our lab demonstrated that *P. aeruginosa* PA14 produces the surfactants β -hydroxyalkanoyl- β -hydroxyalkanoic acids (HAAs) and monorhamnolipids, which caused a 6-fold reduction in *S. constellatus* 7155 biofilm formation in coculture (23). The surfactant-induced biofilm suppression was relieved when *P. aeruginosa* and *S. constellatus* 7155 were cocultured in the presence of tobramycin, an antibiotic used for maintenance therapy by patients with CF. We determined that tobramycin suppressed *P. aeruginosa* production of HAAs and monorhamnolipids and that in the presence of tobramycin, *P. aeruginosa* can enhance *S. constellatus* 7155 growth on a CF-derived bronchial epithelial cell (CFBE) monolayer (23). These data indicate that *P. aeruginosa* can both positively and negatively impact cocultured microbes, including *Streptococcus* spp., and that the interaction between the microbes can be influenced by environmental and/or clinical context.

In this study, we investigated the ability of *P. aeruginosa* to influence *Streptococcus* growth in our *in vitro* coculture system. We demonstrated that multiple *P. aeruginosa* strains and clinical isolates can enhance the growth of multiple *Streptococcus* spp. We used a candidate gene approach and a genetic screen to identify *P. aeruginosa* mutants that were unable to support *Streptococcus* growth, and we found a single mutant of *P. aeruginosa* that no longer enhances growth of streptococci. We found that the *P. aeruginosa* $\Delta pq s L$ mutant suppressed *S. sanguinis* growth, likely via a mechanism that involves siderophore overproduction and thus 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 numbers when grown as a coculture on CF-derived bronchial epithelial (CFBE) cells (23). We first sought to recapitulate the finding that *P. aeruginosa* promotes the *Streptococcus* biofilm population by using the model organism *S. sanguinis* SK36 under 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 (25–27) with available genetic mutant libraries (28–30). This simplified coculture system allowed us to test the interaction between *P. aeruginosa* and streptococci without confounding factors contributed by the CFBE cells.

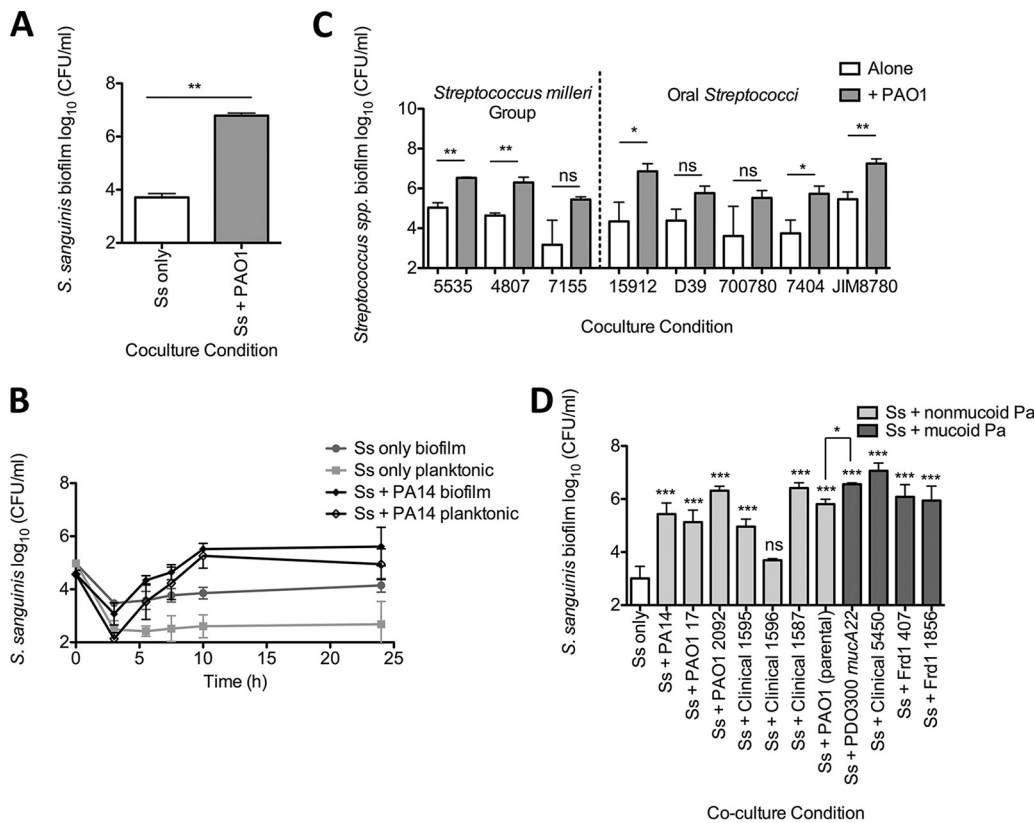


FIG 1 *P. aeruginosa* enhances growth of *Streptococcus* spp. in coculture. Coculture assays were conducted to investigate streptococcal growth when cocultured with *P. aeruginosa*. (A) *S. sanguinis* SK36 (Ss) cocultured with *P. aeruginosa* PAO1. (B) The coculture growth kinetics of *S. sanguinis* with *P. aeruginosa* PA14 were investigated. Each time point represents the average of three biological replicates with three technical replicates. The error bars indicate standard deviation (SD). (C) A representative group of clinical and reference *Streptococcus* strains were tested in coculture with *P. aeruginosa* PAO1 (see Fig. S3A in the supplemental material for all *Streptococcus* species strains tested). Each strain is denoted by its strain number: *S. anginosus* 5535, *S. intermedius* 4807, *S. constellatus* 7155, *S. parasanguinis* ATCC 15912, *S. pneumoniae* D39, *S. peroris* ATCC 700780, *S. oralis* 7404, and *S. salivarius* JIM8780. (D) *S. sanguinis* SK36 was tested in coculture with multiple *P. aeruginosa* clinical and laboratory strains. (A, C, and D) Each bar represents the average of three biological replicates with three technical replicates. The error bars indicate the standard deviation. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by the paired two-tailed Student *t* test [A and C], repeated-measures one-way analysis of variance [ANOVA] with Tukey's multiple-comparison posttest [D], and the paired two-tailed Student *t* test between Ss + PAO1 DHL08 and Ss + PAO1 mucA22 [D]). The corresponding graphs depicting *P. aeruginosa* growth in these assays can be found in Fig. S1 to S4 in the supplemental material.

To test the hypothesis that the *S. sanguinis* SK36 viable cell number increases in coculture with *P. aeruginosa* PAO1 in the absence of CFBE cells, we grew *P. aeruginosa* PAO1 and *S. sanguinis* SK36 in coculture in the wells of a plastic culture dish in minimal essential medium (MEM) tissue culture medium containing glucose. We observed that the number of viable *S. sanguinis* SK36 cells in a biofilm was enhanced 100- to 1,055-fold by coculture with *P. aeruginosa* PAO1 compared to *S. sanguinis* SK36 grown as a monoculture in MEM (Fig. 1A; see Fig. S1A in the supplemental material). *P. aeruginosa* PAO1 biofilm growth was not significantly affected by coculture with *S. sanguinis* SK36 (Fig. S1B). These data also indicate that the enhancement of the *S. sanguinis* SK36 population in a biofilm by *P. aeruginosa* PAO1 does not require the CFBE cells.

***P. aeruginosa* enhances growth of *S. sanguinis* SK36.** We considered two models of polymicrobial interaction that may be enhancing viable *S. sanguinis* SK36 cells in the biofilm when grown in coculture with *P. aeruginosa*. *P. aeruginosa* might promote *Streptococcus* adhesion and biofilm formation, or *P. aeruginosa* might promote streptococcal growth. To distinguish between these models, we conducted a time course experiment with *P. aeruginosa* PA14, *P. aeruginosa* PAO1, and *S. sanguinis* SK36. If *P.*

aeruginosa was promoting adhesion of *S. sanguinis* SK36 cells rather than growth, we predict that we would detect more *S. sanguinis* SK36 in the biofilm and fewer planktonic cells but that the total cell number would not increase compared to that of *S. sanguinis* SK36 monoculture. In contrast, if *P. aeruginosa* was enhancing *S. sanguinis* SK36 growth, then both total biofilm and planktonic *S. sanguinis* SK36 populations should increase in coculture compared to that of *S. sanguinis* SK36 monoculture. As demonstrated by the increased *S. sanguinis* SK36 biofilm and planktonic cells recovered from coculture compared to monoculture, *P. aeruginosa* appears to promote the growth of *S. sanguinis* SK36 (Fig. 1B; see Fig. S2A and B in the supplemental material), thus accounting for the increased population of *S. sanguinis* SK36 biofilm cells.

Multiple *P. aeruginosa* strains enhance the growth of multiple streptococci.

Based on current evidence that multiple *Streptococcus* species inhabit the CF lung (7, 31) and influence patient health (4, 5, 7–10, 14, 15), we sought to determine whether the observed enhancement of *Streptococcus* viable counts in coculture with *P. aeruginosa* may be more broadly generalized to other streptococci, including the *Streptococcus milleri* group (SMG), which has been implicated in CF-related exacerbations (4, 5, 7, 14). To assess the ability of *P. aeruginosa* to promote multiple *Streptococcus* spp., we cocultured *P. aeruginosa* PAO1 with 6 SMG isolates and 8 oral *Streptococcus* spp. Figure 1C depicts a representative strain of each streptococcal species assayed and shows the biofilm populations obtained from monoculture and coculture with *P. aeruginosa* PAO1 (see Fig. S3A in the supplemental material for all 14 strains tested). *P. aeruginosa* PAO1 growth was not significantly affected by coculture with any of the *Streptococcus* spp. tested (Fig. S3B).

We found that *P. aeruginosa* PAO1 significantly enhanced the growth of one of the two *S. anginosus*, two of two *S. intermedius*, and neither of the two *S. constellatus* strains tested. Additionally, of the oral *Streptococcus* spp. tested, *P. aeruginosa* PAO1 significantly promoted the growth of one of the two *S. oralis* isolates, the one *S. parasanguinis* isolate, and one of the three *S. salivarius* isolates tested but not that of the *S. pneumoniae* or *S. peroris* isolates tested (Fig. 1C and S3A). While not every *Streptococcus* isolate tested demonstrated a significant increase in viable population recovered from the coculture, most species tested exhibited a trend toward increased growth when cocultured with *P. aeruginosa* PAO1. These data suggest that *P. aeruginosa* may be promoting *Streptococcus* growth through a pathway that affects many *Streptococcus* species.

Next, we assessed whether multiple *P. aeruginosa* clinical and laboratory strains could promote the growth of *S. sanguinis*. Additionally, given that *S. parasanguinis* was found to bind extracellular alginate produced by mucoid *P. aeruginosa* strains (24), we tested whether mucoid or nonmucoid *P. aeruginosa* could better promote growth in our coculture system. We cocultured *S. sanguinis* SK36 with seven nonmucoid *P. aeruginosa* and four mucoid *P. aeruginosa* laboratory and clinical strains and observed a significant growth enhancement of *S. sanguinis* SK36 by 10 out of 11 *P. aeruginosa* strains tested in coculture biofilms (Fig. 1D) and planktonic growth (see Fig. S4A in the supplemental material). The growth of all tested *P. aeruginosa* strains was not affected by coculture with *S. sanguinis* SK36 (Fig. S4B and C).

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 (Fig. 1D), 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*.

To extend our findings here, we next tested the growth-enhancing capability of *P. aeruginosa* PA14 in rich medium, using both lysogeny broth (LB) and Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). Notably, *S. sanguinis* SK36 growth in rich medium monoculture reached a level comparable to that for coculture in minimal medium (Fig. S4D). However, there was still a significant increase in *S. sanguinis* SK36

growth when in coculture with *P. aeruginosa* PA14 in rich medium compared to *S. sanguinis* SK36 monoculture under the same conditions.

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 on airway cells. This observation extends to coculture assays in rich medium. Furthermore, 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 molecules such as phenazines (33, 34), siderophores (35, 65), alginate (67), and rhamnolipids (23). We hypothesized that one or more of these virulence factors might be altering *Streptococcus* growth in our system. To test this idea, we utilized a candidate genetic approach to assess whether any of these virulence pathways may be involved in the observed growth-enhancing phenotype. We cocultured *P. aeruginosa* PA14 mutants with mutations in each of the above-mentioned pathways with *S. constellatus* 7155 as a model streptococcal strain known to positively respond to *P. aeruginosa* growth enhancement (23) and assessed whether any of these mutants lost the ability to enhance *S. constellatus* 7155 growth. We found that none of the pathways tested were involved in enhancement of *S. constellatus* 7155 growth (see Table S1 in the supplemental material).

Given that KatA has been found in the supernatants of *P. aeruginosa* cultures (36–38), we also constructed *P. aeruginosa* PA14 $\Delta katA$, $\Delta katB$, and $\Delta katA \Delta katB$ mutant strains in order to test the hypothesis that extracellular *P. aeruginosa* catalase is enhancing *S. sanguinis* SK36 growth by degrading hydrogen peroxide produced by *S. sanguinis* SK36. It has previously been reported that *S. sanguinis* and other oral streptococci can inhibit *P. aeruginosa* growth through hydrogen peroxide production (16, 20, 21) and that the hydrogen peroxide produced by oral streptococci plays an important role in growth inhibition, extracellular DNA (eDNA) release, and biofilm formation within the oral microbiome (39, 40). We chose to mutate the *katA* and *katB* genes and not the *katE* gene because previous reports indicate that KatA is the major catalase utilized by *P. aeruginosa* and that KatB can partially recover hydrogen peroxide resistance in the absence of KatA (36, 41). KatE was not demonstrated to play a role in alleviating hydrogen peroxide stress (41). *S. sanguinis* SK36 did not demonstrate reduced growth in coculture with the *P. aeruginosa* PA14 $\Delta katA$, $\Delta katB$, or $\Delta katA \Delta katB$ mutant strains compared to wild-type *P. aeruginosa* PA14, indicating that catalase is not playing a role in the *Streptococcus* growth enhancement phenotype (see Table S1 and Fig. S5A in the supplemental material). The *P. aeruginosa* $\Delta katA$ mutant displayed a slight but significant, growth defect in the coculture compared to wild-type *P. aeruginosa* PA14 in coculture, and the $\Delta katB$ mutant displayed a modest but significant, growth defect in monoculture compared to *P. aeruginosa* PA14 in coculture (Fig. S5B), but these strains still stimulated *S. sanguinis* SK36 growth to the level observed for wild-type *P. aeruginosa*. Taken together, these data suggest that 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), which contains 5,459 transposon insertion mutant strains with mutations in 4,596 genes (42). Each of these *P. aeruginosa* PA14 transposon mutant strains was tested in coculture with *S. constellatus* 7155 (Fig. 2A). Of the 5,459 mutant

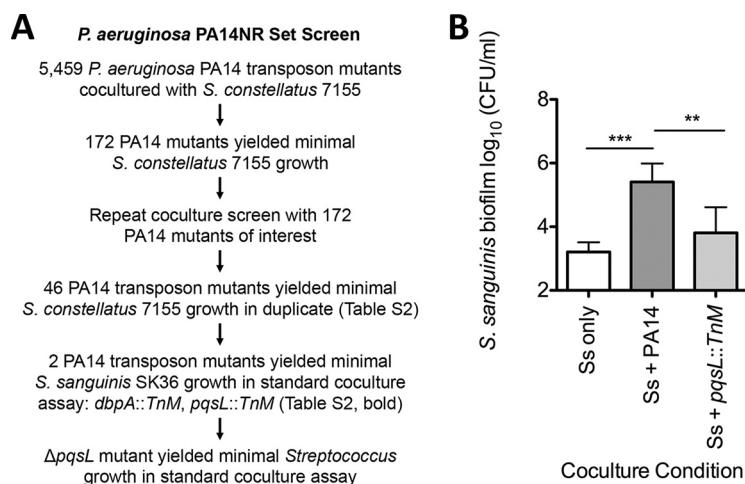


FIG 2 Screening for *P. aeruginosa* PA14 mutants altered in interaction with *Streptococcus*. (A) An overview of the *P. aeruginosa* PA14NR set transposon mutant screen used to identify *P. aeruginosa* transposon insertion mutants that can no longer enhance *S. constellatus* 7155 growth and the number 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. **, $P < 0.01$; ***, $P < 0.001$ (by repeated-measures ANOVA with Tukey's multiple-comparison posttest). *P. aeruginosa* growth data from this assay can be found in Fig. S7 in the supplemental material.

strains in the library, 48 strains were unable to promote *S. constellatus* 7155 growth in two replicate experiments (see Table S2 in the supplemental material). Two of these 48 mutants were eliminated when we tested available deletion mutants, as the deletion mutant strains did not recapitulate the phenotype of the transposon mutation (not shown). The remaining 46 transposon mutants (Table S2) were tested in our standard coculture assay with *S. sanguinis* SK36 to determine which *P. aeruginosa* PA14 transposon mutants are unable to enhance *Streptococcus* growth in a second strain. A total of 44 of the 46 *P. aeruginosa* PA14 transposon mutants were capable of enhancing growth of *S. sanguinis* SK36, and thus these genes were unlikely to be involved in a general pathway for enhancing growth of *Streptococcus*. We found that two transposon mutants were unable to promote either *S. constellatus* 7155 or *S. sanguinis* SK36 growth: *P. aeruginosa pqsL::TnM* and *P. aeruginosa dbpA::TnM*.

The *dbpA* gene codes for the RNA helicase DbpA, which has been demonstrated to play a role in the formation of the 50S ribosomal subunit in *Escherichia coli* (43). *E. coli* is able to compensate for a *dbpA* deletion in forming the 50S ribosomal subunit, as described previously (44); an inability to form the 50S ribosomal subunit would otherwise cause a lethal protein synthesis defect, and a dominant negative *dbpA* mutation is necessary to observe a defect in DbpA function in *E. coli*. We built and assayed the *P. aeruginosa* PA14 Δ *dbpA* mutant strain and found no significant defect in *S. sanguinis* SK36 growth enhancement (see Fig. S6A in the supplemental material) or in *P. aeruginosa* growth (Fig. S6B), and thus we did not pursue further study of this mutant.

We previously studied the effects of the *Pseudomonas* quinolone signal (PQS) pathway on interactions between *P. aeruginosa* and *Staphylococcus aureus*, including the utilization of 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), a respiratory chain inhibitor, to drive *S. aureus* to fermentative metabolism (45, 65, 67). As was observed for *S. constellatus* 7155, there was a significant reduction in the ability of the *P. aeruginosa pqsL::TnM* mutant to support *S. sanguinis* SK36 growth compared to the wild-type *P. aeruginosa* PA14 (Fig. 2B). There was no detectable growth defect of the *pqsL::TnM* mutant strain compared to wild-type *P. aeruginosa* PA14 under our assay conditions (see Fig. S7 in the supplemental material). We chose to focus on the *P. aeruginosa pqsL::TnM* mutant for the remainder of our study.

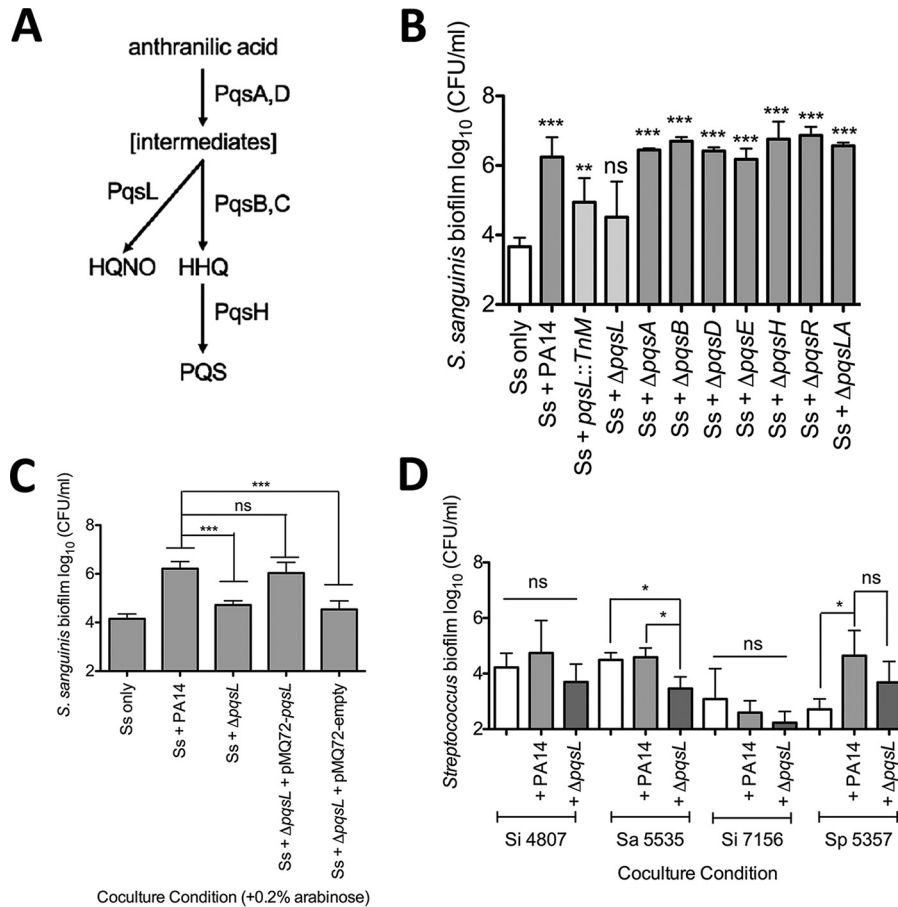


FIG 3 The *P. aeruginosa* $\Delta pqsL$ mutant inhibits *Streptococcus* growth. (A) The PQS biosynthetic pathway and the enzymes that catalyze each step. (B) Coculture of *S. sanguinis* SK36 with wild-type *P. aeruginosa* PA14 and *P. aeruginosa* PA14 mutant strains lacking each enzyme in the PQS biosynthetic pathway. (C) Coculture of *S. sanguinis* SK36 with *P. aeruginosa* PA14, the $\Delta pqsL$ mutant, its complement pMQ72-*pqsL*, and the vector control pMQ72 in the presence of 0.2% arabinose. (D) Coculture of representative streptococci from Fig. 1 in coculture with the wild-type *P. aeruginosa* PA14 and the $\Delta pqsL$ 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; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by repeated-measures ANOVA with Dunnett's multiple-comparison posttest with *Streptococcus* only as the control condition [B] and repeated-measures ANOVA with Tukey's multiple-comparison posttest [C and D]). The corresponding *P. aeruginosa* growth data for these experiments can be found in Fig. S8 to S10 in the supplemental material.

The *P. aeruginosa* $\Delta pqsL$ mutant has a defect in *Streptococcus* growth enhancement.

The PQS pathway involves the production of multiple 4-hydroxy-2-alkyl-quinolones (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, the product of 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, PQS, and MvfR (PqsR) positively regulates the transcription of operons involved in PQS production and the LasR and RhIR 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 mutants with deletions in the *pqs* pathway when grown in coculture with *S. sanguinis* SK36. We found that the $\Delta pqsL$ mutant was the only *pqs* pathway mutant that was unable to promote *S. sanguinis* SK36 growth (Fig. 3B; see Fig. S8A in the supplemental material for *S. sanguinis* SK36 planktonic growth and Fig. S8B and C for *P. aeruginosa* biofilm and planktonic growth).

We complemented the $\Delta pqsL$ 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; see Fig. S9A in the supplemental material for planktonic growth); there was no significant difference between wild-type *P. aeruginosa* PA14 and the complemented $\Delta pqsL$ /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 and C).

Additionally, we assayed the *P. aeruginosa* $\Delta pqsL$ mutant strain in coculture with a few representative *Streptococcus* spp. from Fig. 1B to determine if the $\Delta pqsL$ 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* $\Delta pqsL$, 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 $\Delta pqsL$ 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 $\Delta pqsL$ mutant strain compared to monoculture and coculture conditions with *P. aeruginosa* PA14, indicating that the $\Delta pqsL$ mutation is contributing to the repression of the growth of *S. anginosus* 5535. We found that both wild-type *P. aeruginosa* PA14 and the $\Delta pqsL$ mutant strain caused a nonsignificant reduction in *S. intermedius* 7155 cells recovered from coculture, indicating that both of these *P. aeruginosa* strains may be able to outcompete *S. intermedius* 7155. We saw no significant changes to *P. aeruginosa* growth while in coculture with these representative *Streptococcus* spp. (see Fig. S10 in the supplemental material). Taking the data together, we can extend our finding to at least one other strain of *Streptococcus*.

The *P. aeruginosa* $\Delta pqsL$ mutant likely suppresses *S. sanguinis* SK36 growth by siderophore production via iron sequestration. It has been demonstrated previously that a *pqsL* mutant is deficient in HQNO production and overproduces PQS (47). Exogenous PQS has been demonstrated to chelate iron and to increase the expression of the genes coding for siderophore and phenazine biosynthesis enzymes in *P. aeruginosa* (51–53). Thus, we hypothesized that it was the increased production of PQS and/or increased expression of one or more PQS-regulated genes that caused the observed loss in growth promotion of *S. sanguinis* SK36.

To test our hypothesis, we assayed *S. sanguinis* SK36 in coculture with the $\Delta pqsL$ mutant strains deficient in production of the virulence factors regulated by PQS: siderophores ($\Delta pqsL \Delta pvdA \Delta pchE$) and phenazines ($\Delta pqsL \Delta phzA-G1/2$) (49, 51–53). We found that the *P. aeruginosa* $\Delta pqsL \Delta pvdA \Delta pchE$ deletion mutant strain restored *S. sanguinis* SK36 growth enhancement to levels similar to those with wild-type *P. aeruginosa* PA14 (Fig. 4A) without affecting *P. aeruginosa* growth (see Fig. S11 in the supplemental material). In contrast, the *P. aeruginosa* $\Delta pqsL \Delta phzA-G1/2$ mutant did not restore *S. sanguinis* SK36 growth (Fig. 4A and S11).

We performed a complementation experiment to further confirm a role for siderophores in the ability of the $\Delta pqsL$ mutant to reduce the viability of *Streptococcus*. Specifically, we asked whether complementing the mutation in one of the genes required for siderophore production (*pchE*) would restore the growth defect of the $\Delta pqsL \Delta pvdA \Delta pchE$ mutant to a phenotype similar to that observed for the $\Delta pqsL$ mutant. We cloned *pchE* into the vector pMQ72 and compared it to the vector control strain ($\Delta pqsL \Delta pvdA \Delta pchE$ /pMQ72). The complemented $\Delta pqsL \Delta pvdA \Delta pchE$ /pMQ72-

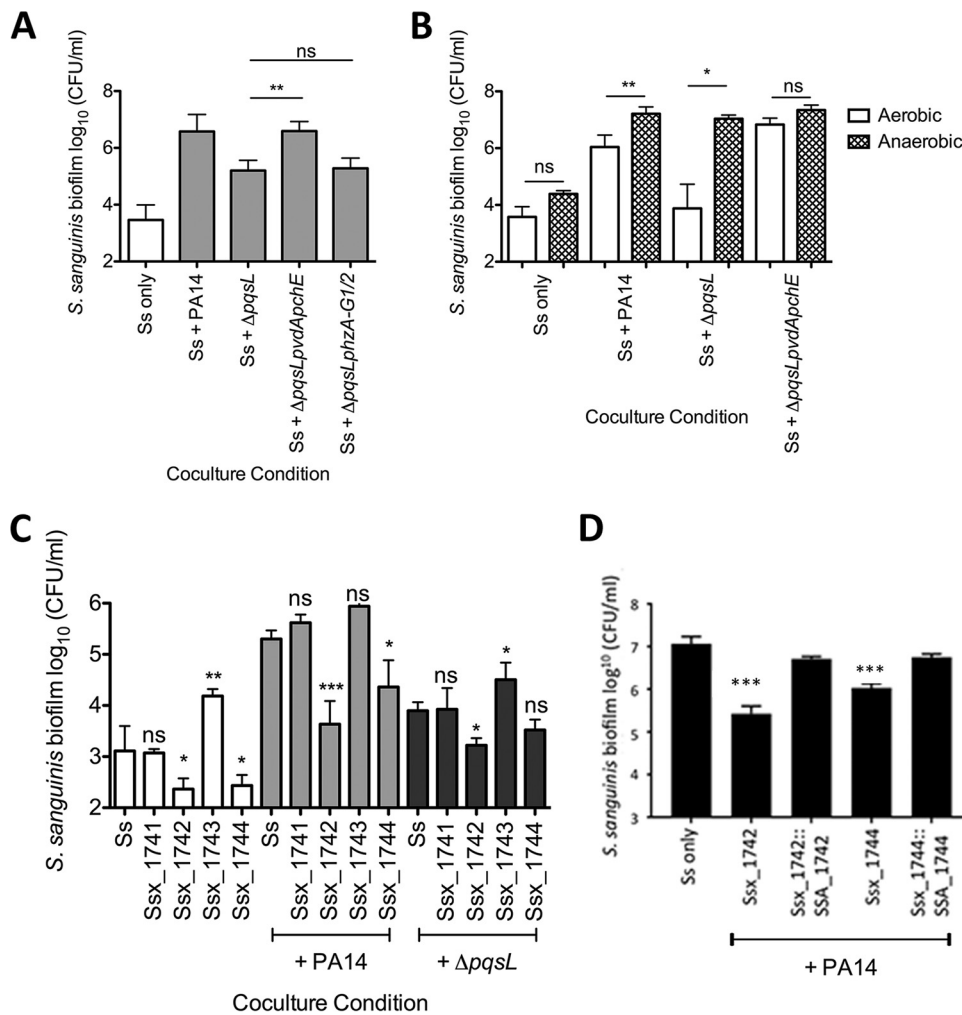


FIG 4 The *P. aeruginosa* Δ*pqsL* mutant likely inhibits *Streptococcus* growth by sequestering iron via siderophore production. (A) Coculture of *S. sanguinis* SK36 with *P. aeruginosa* PA14 mutant strains lacking *pqsL* and siderophore (Δ*pqsL* Δ*pvdA* Δ*pchE*) or phenazine (Δ*pqsL* Δ*phzA-G1/2*) genes. (B) Coculture of *S. sanguinis* SK36 with *P. aeruginosa* PA14 mutant strains in 5% CO₂ (aerobic) or under anaerobic growth conditions. (C) Coculture of *S. sanguinis* SK36 gene replacement mutants lacking putative iron acquisition genes with *P. aeruginosa* PA14 and the Δ*pqsL* mutant. (D) Complementation assays with the rescued Ssx_1742 and Ssx1744 strains. Each bar represents the average of three biological replicates, each with at least three technical replicates. There was no significant difference between the wild-type *S. sanguinis* SK36 and the two complemented strains. Error bars indicate SD. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by repeated-measures ANOVA with Tukey's multiple-comparison posttest [A], the paired two-tailed Student *t* test [B], repeated-measures ANOVA with Dunnett's multiple-comparisons posttest with *S. sanguinis* [Ss] as the control condition [C], and the paired two-tailed Student *t* test with a Bonferroni correction with the wild type compared to both complemented and vector control strain and the paired two-tailed Student *t* test with a Bonferroni correction with the wild type compared to both complemented and vector control strain [D]). The corresponding *P. aeruginosa* growth data for these experiments can be found in Fig. S11, S13, and S14 in the supplemental material.

pchE strain showed a phenotype not significantly different from that of the Δ*pqsL* mutant (see Fig. S12 in the supplemental material). These data confirmed that it was indeed loss of siderophore production in the Δ*pqsL* mutant background that allowed the Δ*pqsL* Δ*pvdA* Δ*pchE* strain to enhance the growth of *S. sanguinis* SK36.

A prediction of this iron sequestration model is that iron supplementation should restore the *Streptococcus*-enhancing activity of the *P. aeruginosa* Δ*pqsL* mutant. We added 50 μM FeCl₃ to our minimal medium coculture conditions and saw restoration of *S. sanguinis* SK36 growth enhancement 6 out of the 18 times we performed the experiment. We explored this phenotype using a range of FeCl₃ concentrations from 5 μM to 50 μM, making a fresh FeCl₃ solution daily and using buffered media in our assay and when making the FeCl₃ stock solution, but the phenotype was still variable.

TABLE 1 *S. sanguinis* SK36 iron-related gene products

Gene	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 Fe ³⁺ -siderophore transporter, ATPase component
Ssx_1742	Ferrichrome-binding protein
Ssx_1743	ABC-type Fe ³⁺ -siderophore transport system, permease component
Ssx_1744	Iron compound ABC transporter, permease protein
Ssx_1745	General stress response protein CsbD

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 inductively coupled plasma mass spectrometry (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 under our coculture conditions.

Next, we tested the idea that coculture under anaerobic conditions would also lead to recovery of the *Streptococcus* growth promotion phenotype in the $\Delta pq s L$ mutant, because *P. aeruginosa* has been demonstrated to reduce pyoverdine and pyochelin production under anoxic conditions (54). Upon anaerobic coculture in an AnaeroPak-Anaero container with a GasPak sachet, the *P. aeruginosa* $\Delta pq s L$ mutant significantly enhanced *S. sanguinis* SK36 viability compared to that in coculture under aerobic conditions (Fig. 4B; see Fig. S13A in the supplemental material). The level of *S. sanguinis* growth enhancement promoted by the *P. aeruginosa* $\Delta pq s L$ mutant under anaerobic conditions was equivalent to that observed for the wild-type *P. aeruginosa*. Additionally, if *P. aeruginosa* were enhancing *S. sanguinis* SK36 growth solely through oxygen consumption, we would expect to see enhanced *S. sanguinis* SK36 growth in monoculture under anaerobic conditions, which we did 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. S13B and 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* contributes to the growth of *S. sanguinis* SK36 via a mechanism independent of oxygen consumption in our coculture system, and they are consistent with our hypothesis that reduced siderophore production under anaerobic conditions mitigates the phenotype of the $\Delta pq s L$ 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 $\Delta pq s L$ mutant likely has an enhanced capacity to scavenge iron as indicated by the restoration of *S. sanguinis* SK36 growth enhancement by the $\Delta pq s L \Delta pvdA \Delta pchE$ mutant, any compromise observed for *S. sanguinis* SK36 iron acquisition mutants should be exacerbated in coculture with the *P. aeruginosa* $\Delta pq s L$ mutant.

Xu and colleagues reported a mutant library of *S. sanguinis* SK36 wherein nonessential genes are deleted and replaced with a kanamycin resistance cassette (28, 29). 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* $\Delta pq s L$ 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; see Fig. S14A in the supplemental material). 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 under monoculture conditions (6.5-fold and 6.9-fold, respectively), indicating that they may be iron starved under 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.

To confirm that the observed competition defect was indeed due to the Ssx_1742 and Ssx_1744 mutations, we complemented each of the mutants as described in Materials and Methods. As shown in Fig. 4D, the complemented mutants showed competition phenotypes similar to that observed for wild-type *S. sanguinis* SK36.

We next explored the phenotypes of the Ssx_1742 and Ssx_1744 mutants in coculture with the *P. aeruginosa* $\Delta pqsL$ mutant strain. In coculture with the *P. aeruginosa* $\Delta pqsL$ mutant strain there was a significant, 4.9-fold reduction in the Ssx_1742 mutant compared to wild-type *S. sanguinis* SK36 in coculture with the $\Delta pqsL$ 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* $\Delta pqsL$ mutant compared to the wild-type *P. aeruginosa* strain. We take this result to mean that the increased iron sequestration by the $\Delta pqsL$ 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 $\Delta pqsL$ mutant. Finally, we observed no significant difference in *P. aeruginosa* PA14 and the $\Delta pqsL$ mutant strain growing in coculture with *S. sanguinis* SK36 mutant strains (Fig. S14B and C).

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 (23). 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*, and 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* 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* $\Delta pqsL$ 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 inhibition of soil bacterial growth can be restored upon addition of 50 μM FeCl_3 (32). Similarly, our data show the ability to restore *Streptococcus* growth by introducing mutations in the siderophore genes to the $\Delta pqsL$ mutant or by growing the cocultures anaerobically, a growth condition where *P. aeruginosa* is known

to reduce pyoverdine and pyochelin production (54). Consistent with these data, we observed that *S. sanguinis* SK36 grew slightly more in the presence of a *P. aeruginosa* PA14 $\Delta pq s L \Delta p v d A \Delta p c h E$ mutant than in the presence of wild-type *P. aeruginosa* PA14. The increased *S. sanguinis* SK36 growth indicates that these microbes are competing for iron during coculture and that changes to iron uptake can alter the competition between these two microbes. In addition, PQS has been demonstrated to act as both an anti- and pro-oxidant under different conditions (55), and we cannot rule out that the increased PQS production in the $\Delta pq s L$ mutant impacts production of reactive oxygen species that may be toxic to *Streptococcus* spp.; the fact that growth of the $\Delta pq s L$ mutant under anaerobic conditions reverses the growth 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. 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 (24). 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, which has been described in mucoid strains (67), and the corresponding relief of rhamnolipid-induced *Streptococcus* killing (23). Furthermore, mucoid strains were shown to produce lower levels of products of the PQS pathway and reduced levels of siderophores (67). Thus, *Streptococcus* spp. may more readily coexist, 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 (16, 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 supernatants of *P. aeruginosa* cultures (37, 38, 41). However, we found no significant defect in *S. sanguinis* SK36 growth enhancement by the *P. aeruginosa* $\Delta katA$, $\Delta katB$, and $\Delta katA \Delta katB$ mutants compared to wild-type *P. aeruginosa* PA14, indicating that catalase is not the factor produced by *P. aeruginosa* that enhances *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 (37); 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 under 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 under aerobic conditions. 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 under iron-limiting 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: the *Ssx_1742* mutant, lacking a ferrichrome-binding protein, and the *Ssx_1744* mutant, lacking the permease protein of an iron compound ABC transporter. The *Ssx_1742* mutant demonstrated a significant growth defect in monoculture and

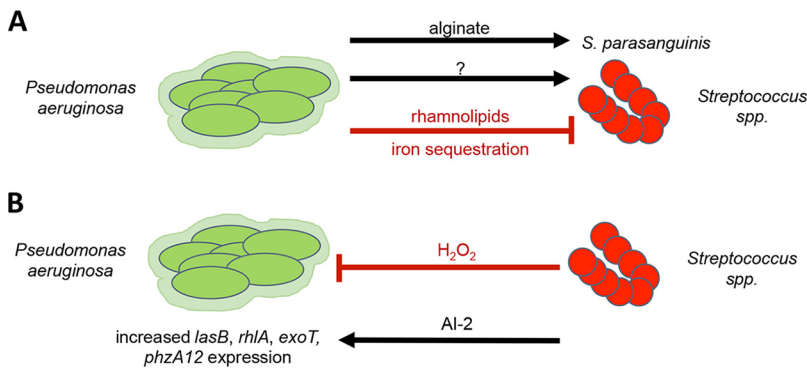


FIG 5 A model for *P. aeruginosa*-*Streptococcus* interactions. *P. aeruginosa* has both positive and negative interactions with *Streptococcus* spp. (A) It has been demonstrated previously that *P. aeruginosa* can enhance *S. parasanguinis* biofilm formation through alginate secretion (24) or inhibit *Streptococcus* growth via rhamnolipid secretion (23). Here we propose a negative interaction wherein iron sequestration by *P. aeruginosa* limits *Streptococcus* sp. growth. *Streptococcus*-promoting factors produced by *P. aeruginosa* have not yet been identified (indicated by the question mark). (B) Previous evidence also demonstrates that streptococci can influence *P. aeruginosa* through AI-2 signaling (18), leading to enhanced *lasB*, *rhlA*, *exoT*, and *phzA12* gene expression, or can inhibit *P. aeruginosa* viability through H₂O₂ production (16, 20, 21) and subsequent generation of reactive nitrogenous intermediates (20, 21).

during coculture with *P. aeruginosa* PA14. The growth defect of the Ssx_1742 mutant was worsened when it was cocultured with the $\Delta pqsL$ mutant. Together, these data indicate that the Ssx_1742 mutant strain is unable to compete with *P. aeruginosa* for the limited iron under our coculture 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 $\Delta pqsL$ mutant versus the wild-type *P. aeruginosa*. We take this result to mean that the increased production of the siderophores in the $\Delta pqsL$ 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 $\Delta pqsL$ 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. (Fig. 5), including the SMG, via iron sequestration. We previously reported that *P. aeruginosa* rhamnolipid surfactants could reduce the viability of *S. constellatus*. *P. aeruginosa* can also influence the biofilm formation of *S. parasanguinis* through alginate production (24) and the growth of *Streptococcus* spp. via a currently undescribed mechanisms (16, 17, 23). Conversely, previous studies investigating interactions between *P. aeruginosa* and *Streptococcus* spp. also showed that *Streptococcus* spp. influence transcription of *P. aeruginosa* virulence genes, including rhamnolipid, elastase, and phenazine biosynthesis genes, through AI-2 signaling (18) and an undescribed mechanism (6, 16, 17) and can suppress *P. aeruginosa* growth when they are a primary colonizer through production of H₂O₂ (16, 21) and reactive nitrogenous intermediates (20, 21). Thus, this polymicrobial interaction is complex.

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

Finally, the observations we present here may be of relevance in the CF lung, as many patients are cocolonized by *P. aeruginosa* and *Streptococcus* spp. (8, 56). Analysis of the average available iron in the airway varies markedly, between $\sim 0.02 \mu\text{M}$ in healthy individuals and $\sim 8 \mu\text{M}$ in patients with CF, and there is a great deal of variability among patients with CF (57, 58). 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 (59) and the bleeding into the airway (hemoptysis) associated with this patient population (60). Thus, in CF patients, iron levels in the airway can range from concentrations where we might expect direct competition between *P. aeruginosa* and *Streptococcus* for this limited resource to levels where 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.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table S3 in the supplemental material. *P. aeruginosa* strains were grown on lysogeny broth (LB) agar or in LB liquid with shaking at 37°C and, where indicated, in the presence of antibiotics at the following concentrations: 25 $\mu\text{g}/\text{ml}$ gentamicin, 250 $\mu\text{g}/\text{ml}$ kanamycin, and 75 $\mu\text{g}/\text{ml}$ tetracycline. *Streptococcus* spp. were grown as previously described (23) 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 20 $\mu\text{l}/\text{ml}$ Oxyrase (Oxyrase, Inc.) at 37°C with 5% CO_2 . *S. sanguinis* SK36 gene replacement mutant strains were grown on blood agar or THY with 500 $\mu\text{g}/\text{ml}$ kanamycin (29). For antibiotic selection during construction of the *S. sanguinis* SK36 complementation strains, spectinomycin (Spc) was used at a concentration of 100 $\mu\text{g}/\text{ml}$ in *E. coli* and 200 $\mu\text{g}/\text{ml}$ in *S. sanguinis* SK36.

At the end of each coculture assay, *P. aeruginosa* was grown overnight on *Pseudomonas* isolation agar (PIA) at 37°C, and *Streptococcus* spp. were grown overnight on blood agar at 37°C anaerobically in AnaeroPak-Anaero containers (Thermo Fisher) or on blood agar supplemented with 10 $\mu\text{g}/\text{ml}$ neomycin and 10 $\mu\text{g}/\text{ml}$ polymyxin 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 vectors and pMQ72-*pqsL* complementation vector. InvSc1 was grown as previously described in 1% Bacto yeast extract, 2% Bacto peptone, and 2% dextrose (61). Synthetic defined agar-uracil (4813-065; Qbiogene) was used for InvSc1 selections.

Species identification of streptococci. *Streptococcus* spp. were isolated at the Dartmouth Hitchcock Medical Center in Lebanon, NH. The species of *Streptococcus* clinical isolates were determined 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 by 16S-internal transcribed spacer (ITS) PCR as previously described (62) using the Strep16S-1471F and 6R-IGS primers (listed in Table S4 in the supplemental material). *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) (62). The Phusion polymerase PCR protocol (New England Biolabs) was followed for preparing 5- μl reaction mixtures, and the PCR conditions for the 16S-ITS region were as follows: 98°C for 30 s followed by 25 cycles of 98°C for 10 s, 61°C for 15 s, and 72°C for 30 s and a final extension at 72°C for 7 min. The PCR conditions for amplifying *gdh* were as follows: 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 57.9°C for 15 s, and 72°C for 30 s, followed by a final extension at 72°C for 7 min. The resulting PCR products were imaged on a 1% agarose gel with Sybr Safe (Thermo Fisher Scientific Inc.). The remaining PCR product 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 in the CFBE model system as previously described (8, 23, 65, 67) with some modifications. Overnight cultures of *P. aeruginosa* and *Streptococcus* spp. were individually centrifuged at 10,000 $\times g$ for 3 min, the cell pellet was washed with 1.5 ml minimal essential medium (MEM) supplemented with 2 mM L-glutamine (MEM+L-Gln) and centrifuged again, and the cell pellet was resuspended in 1.5 ml MEM+L-Gln. The optical density at 600 nm (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* sp. cultures were adjusted to an OD_{600} of 0.1. *S. sanguinis* SK36 overnight cultures were adjusted to an OD_{600} of 0.1 and then further diluted 1:100 in MEM+L-Gln due to the robust growth that *S. sanguinis* SK36 exhibits in monoculture. A 1:1 mixture by volume of *P. aeruginosa* and *Streptococcus* spp. was prepared from the adjusted cultures. Three wells of a 96-well plate per monoculture and coculture condition were inoculated with 100 μl per well. The culture plates were then incubated statically for 1 h at 37°C with 5% CO_2 , at which point the unattached planktonic cells were aspirated with a multichannel pipette and replaced with 100 μl MEM supplemented with 2 mM L-glutamine and 0.4% L-arginine (MEM+L-Gln+L-Arg). The culture plates were incubated statically for an additional 5.5 h, at which point the supernatant was removed and replaced

with 100 μ l MEM+L-Gln+L-Arg. L-Arginine (0.4%) was added to the minimal medium at the 1- and 5.5-h medium changes to promote *P. aeruginosa* biofilm formation (63). At 21 h postinoculation, planktonic cells were removed to be plated, and biofilms were disrupted using a 96-pin replicator in 100 μ l 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 sachets (BD) to selectively grow *P. aeruginosa* and *Streptococcus* spp., respectively. Following overnight incubation, colonies were counted and the 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 h. The 0-h 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 at the 5.5-h time point plate prior to the 5.5-h medium exchange.

Coculture in rich media. *P. aeruginosa* PA14 was grown in coculture with *S. sanguinis* SK36 as described above but with the following changes: at the 1-h medium change, the MEM+L-Gln was removed and replaced with 100 μ l LB or THY. Fresh LB or THY (100 μ l) was used at the 5.5-h medium change as well. This allows all of the culture conditions to originate from the same inocula.

Construction of *P. aeruginosa* PA14 deletion mutant strains. The pMQ30 vector (Table S3) was used to generate the *P. aeruginosa* PA14 $\Delta katA$, $\Delta katB$, $\Delta katA \Delta katB$, and $\Delta dbpA$ 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 XbaI restriction enzyme-digested pMQ30 in yeast as previously reported (61). Plasmid integrants were isolated on LB agar supplemented with gentamicin 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 (42). Initially, a 96-pin replicator was used to transfer inocula from the frozen library to a sterile 96-well plate containing 150 μ l of LB per well. The plate was then incubated statically for 24 h at 37°C. *S. constellatus* 7155 frozen aliquots were made from 750 μ l of overnight culture mixed with 750 μ l of 40% glycerol. The day of the coculture experiment, frozen *S. constellatus* 7155 aliquots were thawed, 500 μ l of aliquot was added to 4.5-ml THY cultures with 100 μ l Oxyrase, and the cultures were grown for 6 to 8 h at 37°C with 5% CO₂. The *S. constellatus* 7155 culture was then adjusted to an OD₆₀₀ of 0.05 in MEM+L-Gln, and 100 μ l of adjusted culture was added to each well of a sterile 96-well plate. The PA14NR set was grown in LB for 24 h in a 96-well plate format; each well contained a transposon mutant from the *P. aeruginosa* PA14NR set. A 96-pin replicator was then used to transfer 2 to 3 μ l of culture from the transposon library plate into the plate containing *S. constellatus* 7155. The coculture plates were then incubated statically for 2 h at 37°C with 5% CO₂. After 2 h, the supernatant and unattached bacteria were aspirated using a multichannel pipette, and 100 μ l MEM+L-Gln with 5 μ g/ml tobramycin to suppress *P. aeruginosa* PA14 rhamnolipid production was added to each well. The plates were then incubated statically for an additional 20 h at 37°C with 5% CO₂. At 22 h postinoculation, the 96-pin replicator was used to disrupt the biofilms into the supernatant fraction. The 96-pin replicator was then used to spot culture onto large petri plates containing either PIA or *Streptococcus* selection agar. PIA plates were incubated overnight at 37°C, and *Streptococcus* selection agar plates were incubated for 24 h at 37°C with 5% CO₂. In the initial screen, we identified *P. aeruginosa* mutants that showed low or undetectable *S. constellatus* 7155 growth. To confirm the phenotype, the candidate *P. aeruginosa* PA14 transposon mutant strain was picked from the PIA plate and grown statically overnight at 37°C in a sterile 96-well plate in 125 μ l LB. The next morning, 125 μ l of 40% glycerol was added to each well containing *P. aeruginosa* PA14 candidate mutants, and these “candidate mutant” plates were stored at -80°C for the next round of screening. For the second round of the screen, the coculture process described above was repeated with the plates containing candidate mutants.

If we had clean deletions of the candidate mutants, they were also tested in the assay described above. If the clean deletion did not recapitulate the original transposon mutant, that transposon mutant was eliminated from the list of candidate mutants. Table S2 shows the final list of *P. aeruginosa* PA14 transposon insertion mutant strains that yielded low or undetectable *S. constellatus* 7155 growth after rescreening.

We then tested each individual *P. aeruginosa* PA14 transposon mutant listed in Table S2 in our standard 96-well coculture assay as described above with *S. sanguinis* SK36. The two mutants that yielded consistently low *S. sanguinis* SK36 growth in our standard coculture are in bold in Table S2.

***P. aeruginosa* $\Delta pqsL$ complementation.** The pMQ72 vector (Table S3) with an arabinose-inducible promoter was used to complement the *P. aeruginosa* PA14 $\Delta pqsL$ deletion mutant. The pMQ72-*pqsL* complementation plasmid was built using homologous recombination of the PCR product made with the *pqsL* comp 3' and *pqsL* comp 5' primers (listed in Table S4) with SacI restriction enzyme-digested pMQ72 in yeast as previously reported (61). *P. aeruginosa* PA14 $\Delta pqsL$ /pMQ72-*pqsL* and *P. aeruginosa* PA14 $\Delta pqsL$ /pMQ72-empty vector control strains were cocultured with *S. sanguinis* SK36 as described above with the following change: at 1 and 5.5 h postinoculation, MEM+L-Gln+L-Arg supplemented with L-arabinose at 0% and 0.2% final concentrations was added to the medium to induce pMQ72-*pqsL* gene expression.

***P. aeruginosa* Δ pchE complementation construct.** Due to the gene length (4.3 kb) and content of repetitive DNA, the *pchE* gene was amplified in two overlapping PCR fragments using Phusion polymerase (NEB). Fragment 1 was amplified using the primers *pchE* 5'0.2 and *pchE* int R, and fragment 2 was amplified with primers *pchE* int 1B F (see Table S4 for primer sequences). The resulting PCR fragments were cloned into pMQ72 by homologous recombination in yeast as described above.

Coculture with ferric chloride. Coculture was conducted as described above but with the following change: at 1 and 5.5 h postinoculation, supernatants were aspirated with a multichannel pipette and replaced with MEM+L-Gln+L-Arg with or without freshly prepared, filter-sterilized ferric chloride hexahydrate (ranging from 5 to 50 μ M).

Anaerobic coculture. Coculture was conducted as described above but with the following alterations. Once the plates were inoculated, they were incubated in AnaeroPak-Anaero containers with a GasPak sachet. At each medium change (1 h and 5.5 h), a new sachet was added to the container to ensure anaerobic coculture conditions. The AnaeroPak container was incubated in the same incubator as the aerobic plate to control for any environmental effects.

Complementing mutations of *S. sanguinis* SK36. The open reading frames (ORFs) of SSA_1742 and SSA_1744 were PCR amplified with PfuUltra II fusion HS DNA polymerase (Agilent Technologies) using primers F-1742-oe/R-1742-oe and F-1744-oe/R-1744-oe, respectively (Table S4). The PCR products and the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible plasmid pJFP126 were digested with SphI and/or HindIII, ligated, and electroporated into *E. coli* DH5 α (64). Plasmid DNA was purified from DH5 α cells using a Qiagen Miniprep kit (Qiagen). Transformation of *S. sanguinis* strains with each plasmid was carried out as previously described (28). The DNA fragment containing gene expression elements and the *aad9* gene, encoding resistance to Spc, was transferred from the plasmid to the genomes of the resulting *S. sanguinis* strains.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00014-19>.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Ping Xu for providing his *S. sanguinis* SK36 mutant library and the pVA838 vector and Deborah Hogan, Nicholas Jacobs, and Dominique Limoli for providing bacterial strains. We thank Brian Jackson for quantifying the iron concentration in our media with ICP-MS.

This work was supported by the Cystic Fibrosis Foundation (OTOOLE16GO), a Molecular and Cellular Biology at Dartmouth training grant (T32GM008704), the Munk-Pfefferkorn Fund, an NIH grant (R37 AI83256-06) to G.A.O., and a China Scholarship Council (CSC) grant (201708330005) to K.L.

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