Availability of Zinc Impacts Interactions Between *Streptococcus sanguinis* and *Pseudomonas aeruginosa* in Co-culture

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ABSTRACT

Airway infections associated with cystic fibrosis (CF) are polymicrobial. We reported previously that clinical isolates of *P. aeruginosa* promote the growth of a variety of streptococcal species. To explore the mechanistic basis of this interaction, we performed a genetic screen to identify mutants of *Streptococcus sanginuis* SK36 whose growth was no longer enhanced by *P. aeruginosa* PAO1. Mutations in zinc uptake systems of *S. sanginuis* SK36 reduced growth of these strains by 1-3 log compared to wild-type *S. sanginuis* SK36 when grown in coculture with *P. aeruginosa* PA01, while exogenous zinc (0.1-10 μm) rescued the coculture defect of zinc uptake mutants of *S. sanginuis* SK36. Zinc uptake mutants of *S. sanginuis* SK36 had no obvious growth defect in monoculture. Consistent with a competition for zinc driving coculture dynamics, *S. sanginuis* SK36 grown in coculture with *P. aeruginosa* showed increased expression of zinc uptake genes compared to *S. sanginuis* grown alone. Strains of *P. aeruginosa* PAO1 defective in zinc transport also supported more robust growth by *S. sanginuis* compared to coculture with wild-type *P. aeruginosa* PAO1. An analysis of 118 CF sputum samples revealed that total zinc levels varied from ~5-145 μM. At relatively low zinc levels, *Pseudomonas* and *Streptococcus* were found in approximately equal abundance; at higher zinc levels, we observed an increasing relative abundance of *Pseudomonas* and decline of *Streptococcus*, perhaps as a result of increasing zinc toxicity. Together, our data indicate that the relative abundance of these microbes in the CF airway may be impacted by zinc levels.

IMPORTANCE. Polymicrobial infections in CF likely impact patient health, but the mechanism(s) underlying such interactions are poorly understood. Here we show that interactions between *Pseudomonas* and *Streptococcus* are modulated by zinc availability using an *in vitro* model system, and clinical data are consistent with this model. Together with previous studies, our work supports a role for metal homeostasis as a key factor driving microbial interactions.

INTRODUCTION

Cystic fibrosis (CF) is a monogenic autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1). It is estimated that ~70,000 individuals in the world are affected by CF and the most common mutation, caused by a deletion of phenylalanine at the 508th amino acid within the CFTR protein (ΔF508), is found in approximately 70% of this population (2, 3). CFTR dysfunction affects several body systems, and progressive lung disease due to chronic and recurrent microbial infections is the leading cause of morbidity and mortality in individuals with CF (4, 5). It has been shown that CF airway infections are polymicrobial (6, 7), and the composition and interspecies interactions within the polymicrobial communities can have profound and diverse consequences, including on bacterial growth (8-10), as well as disease progression and therapeutic outcomes (6, 11).

An example of such microbial interactions includes the CF-associated streptococcal species, the presence of which may influence the growth and/or virulence of other CF pathogens, including the important pathogen *Pseudomonas aeruginosa* (12, 13). In turn, *P. aeruginosa* can impact the growth and/or persistence of streptococci (8-10, 14), with the net impact of these interactions resulting in exacerbation (4, 15-17) or less server loss of lung function (11, 16, 18-21). Therefore, understanding how these pathogens interact with each other and their multicellular host to impact disease progression, as well as how these interactions are modified by the CF airway environment, is of high significance.

Zinc is an essential micronutrient for all organisms and serves as a structural or catalytic cofactor in 5–6% of proteins in bacterial proteome (22-24). However, high concentrations of zinc are toxic, possibly due to competition with other relevant metal ions (24, 25), inhibition of key enzymes and essential metabolic reactions in the cell (26-28) and inducing membrane stress (29). For most pathogens, there is intense competition for zinc in the face of the host's defense mechanisms, called nutritional immunity (30), and conversely elevating zinc level is

75 also a strategy used by macrophages to kill pathogens encapsulated in their phagosomes
76 (31, 32).

77 To maintain zinc homeostasis, bacterial species have evolved multiple systems to import and
78 export zinc in environments of zinc limitation or excess, respectively (33, 34). In *P.*
79 *aeruginosa*, the two best-studied zinc acquisition systems are the high-affinity ZnuABC
80 system (35-37) and the pseudopaline system (38, 39). ZnuABC is a member of the
81 ATP-binding cassette (ABC) transporters, consisting of a zinc-specific binding periplasmic
82 protein (ZnuA), an inner membrane permease (ZnuB), and an ATPase (ZnuC) (35, 37, 40).
83 Loss of ZnuA in *P. aeruginosa* PAO1 results in an ~60% reduction in cellular zinc
84 accumulation (35). The pseudopaline system is primarily involved in zinc uptake in zinc poor
85 environments and zinc transport into the cell is achieved via the action of a four-gene operon
86 (*cntOLMI*, also termed *zrmABCD*) (38, 39). This operon includes the genes for a
87 TonB-dependent outer membrane pseudopaline receptor (CntO), two biosynthetic enzymes
88 (CntL and CntM) responsible for synthesizing pseudopaline and an inner membrane
89 transporter (CntI) involved in pseudopaline secretion (36, 38, 39). Both the ZnuABC system
90 and the pseudopaline operon are negatively regulated by zinc level through the Fur-like zinc
91 uptake regulator Zur (35, 37, 39), which can sense and respond to femtomolar changes of
92 cytosolic zinc concentrations (41). When zinc is bound, Zur represses transcription of the zinc
93 uptake systems and loss of Zur results in higher cytoplasmic zinc in *P. aeruginosa* (36, 37).

94 Similarly, several zinc transporters have been identified to contribute to zinc homeostasis and
95 virulence in pathogenic streptococci (42, 43). In most streptococci, zinc acquisition involves a
96 high-affinity zinc-ABC transporter, which is comprised of an integral membrane component
97 (AdcB), an ATPase (AdcC) and one or several zinc-binding proteins (AdcA, AdcAII, Lbp, or
98 Lmb) (42, 44-46). The streptococcal AdcR repressor, a MarR family regulator, is involved in
99 regulation of this transporter (47).

Recent studies have suggested that total zinc levels are much higher in sputum from CF patients compared with healthy controls (48), but zinc availability is limited in the lung mucosa, leading to zinc starvation for *P. aeruginosa* during CF lung infection (49). Moreover, the host also employs zinc starvation or intoxication to retard streptococcal growth during colonization and infection (42, 43, 50). Nevertheless, the potential significance of zinc in the interplay between *P. aeruginosa* and *Streptococcus*, especially in the context of a polymicrobial infection in the CF airway, remains to be explored.

We recently showed that *P. aeruginosa* can enhance the growth of multiple oral *Streptococcus* spp. in coculture conditions (8, 10), but the molecular mechanism underlying such interactions is still poorly understood. In the present work, we used a comprehensive *Streptococcus sanguinis* SK36 mutant library to determine the genetic requirements for *S. sanguinis* SK36 to benefit from interaction with *P. aeruginosa*. Our results show that efficient zinc acquisition in *S. sanguinis* SK36 plays a critical role in *P. aeruginosa*-induced growth enhancement. We observed increased transcription of zinc uptake genes in *S. sanguinis* SK36 and demonstrate that *P. aeruginosa* and *S. sanguinis* SK36 are competing for zinc during co-cultivation. Furthermore, by coupling analysis of microbial communities and zinc content within CF sputum samples, we discovered a relationship between the relative abundance of *Streptococcus* and *Pseudomonas* with the concentrations of total zinc in CF sputum. These results suggest that zinc availability may play a role in *Pseudomonas*-*Streptococcus* interactions in vivo, and furthermore, changes in zinc levels may also be related to the development of the respiratory microflora in patients with CF.

RESULTS

***P. aeruginosa* and its conditioned medium stimulate *S. sanguinis* growth on both plastic and CF-derived airway cells.** We have previously shown that *P. aeruginosa* can

enhance *S. sanguinis* SK36 growth either as planktonic or biofilm cells in coculture compared to that of *S. sanguinis* SK36 monoculture, while the *P. aeruginosa* population was not significantly impacted by the presence of *S. sanguinis* (8, 10), a finding confirmed here Fig. S1A. Furthermore, an assay monitoring biofilm formation over time shows that the higher viable count of *S. sanguinis* SK36 in coculture with *P. aeruginosa* is due to enhanced growth of *S. sanguinis* SK36 after a modest reduction in viability (Fig. 1A). Interestingly, *S. sanguinis* SK36 did show enhanced growth at later time points in monoculture, indicating that *P. aeruginosa* may stimulate transition out of lag phase as one possible mechanism of the observed increased viable count of *S. sanguinis* SK36 observed in the coculture assay system. The viable counts of *P. aeruginosa* were similar in monoculture and coculture at all timepoints (Fig. 1B).

In an attempt to understand the basis of this observed growth enhancement of *S. sanguinis* SK36, we tested whether a cell free supernatant of *P. aeruginosa* PAO1 could stimulate the growth of *S. sanguinis* SK36. We found that *P. aeruginosa* cell-free supernatant was indeed capable of promoting *S. sanguinis* biofilm growth on plastic (Fig. 1C). We noted a significant increase in the number of *S. sanguinis* cells grown in the presence of a 1/2 or 1/4 dilution of the original *P. aeruginosa* PAO1 supernatant (Fig. S1B), with a dose-dependent decrease in growth enhancement with increasing dilution of the supernatant. Undiluted *P. aeruginosa* PAO1 supernatant showed a less robust effect on *S. sanguinis* SK36 growth than the 1/2 or 1/4 dilution, perhaps due to the known antimicrobial factors in *P. aeruginosa* supernatant, including siderophores, phenazines, cyanide and elastase (51-53). Taken together, these data suggest that one or more secreted factors in the *P. aeruginosa* PAO1 supernatant can enhance growth of *S. sanguinis* SK36.

To determine whether the interactions between *Streptococcus* and *P. aeruginosa* also occur when these microbes are grown in the presence of airway cells derived from patients with CF, we extended our observation to CF-derived bronchial epithelial cells homozygous for the

ΔF508 allele of CFTR (referred to as "CFBE monolayers" hereafter) (54). We evaluated *S. sanguinis* SK36 growth on CFBE monolayers in the absence or presence of *P. aeruginosa* cells or supernatants. As a control, *S. sanguinis* growth on tissue culture plates without host cells was also included (Fig. 1D, left-most column, plastic). Interestingly, *S. sanguinis* growth enhancement was observed with monocultures biofilms on host cells, and *P. aeruginosa* cells and supernatants further enhanced the growth of *S. sanguinis* SK36 (Fig. 1D). Collectively, our results demonstrate that the ability of *P. aeruginosa* PAO1 cells and supernatant to promote the growth of *S. sanguinis* SK36 under a variety of conditions.

Identification of *S. sanguinis* mutants defective in growth enhancement mediated by *P. aeruginosa*. To identify *S. sanguinis* genes that are potentially required for the increase in *S. sanguinis* SK36 growth in the *P. aeruginosa* PAO1-*S. sanguinis* SK36 coculture, we performed a genome-wide screen of an available comprehensive *S. sanguinis* SK36 mutant library (55), which covers ~90% of the predicted 2270 protein coding genes in the *S. sanguinis* SK36 genome. In this screen we sought to identify mutants with reduced enhancement phenotypes in the presence of *P. aeruginosa* PAO1 (Fig. 2A). Of 2048 mutants screened, a total of 80 mutants showed a measurable and repeatable reduction in the enhancement of *S. sanguinis* growth (Table S1).

Among those 80 candidates, mutations were found in genes belonging to a wide variety of functional classes (Fig. 2B), including: (i) carbohydrate transport and metabolism (*SSA_0222*, *SSA_0773*, *SSA_1261*, *SSA_1521*, *SSA_1749*, etc.), (ii) amino acid transport and metabolism (*SSA_0564*, *SSA_1043*, *SSA_1044*, *SSA_1341-1343*, etc.), (iii) cell division and cell envelope biogenesis (*SSA_0015*, *SSA_0655*, etc.), (iv) coenzyme metabolism (*SSA_1201*, *SSA_1202*, *SSA_1536*, etc.), (v) DNA replication (*SSA_1626*), (vi) nucleotide transport and metabolism (*SSA_0568*, *SSA_1163*, etc.), and (vii) translation, ribosomal structure and protein biosynthesis (*SSA_0820*, *SSA_1272*, *SSA_1611*, *SSA_1613*, *SSA_1895*, *SSA_1896*, *SSA_2033*, *SSA_2058*, etc.).

We also found genes involved in metal ion (zinc, iron and manganese) transport and metabolism (*SSA_0136*, *SSA_0137*, *SSA_0260*, *SSA_0261*, *SSA_1955*, *SSA_1956*, *SSA_2365-2367*, etc.; Table S1 and Fig. 2B,C), which are involved in many crucial biological processes and are essential for bacterial survival in the environment or in the infected host (28). In particular, zinc has recently been shown to be essential for pathogenic streptococci in their growth, morphology and virulence during infection (42, 43).

Zinc is required for the promotion of *S. sanguinis* growth by *P. aeruginosa.* To further probe the basis for the enhanced *S. sanguinis* growth mediated by *P. aeruginosa*, we focused on the role of zinc importers identified in our screen (SSA_0136, SSA_0137, SSA_0260 and SSA_0261; Fig. 2C). In *S. sanguinis* SK36, the *SSA_0136* and *SSA_0137* genes encode components of an Adc zinc ATP-binding cassette (ABC) transporter, which is involved in zinc uptake in several pathogenic streptococci. The *SSA_0136* and *SSA_0137* genes are called *adcC* and *adcB*, respectively (42, 45). The *SSA_0260* gene, also termed SsaB (*S. sanguinis* adhesin B) (56), is an LraI family lipoprotein and serves as the substrate-binding protein for a Mn/Zn ABC import system (57). The *SSA_0261* gene, also named *ssaC*, is located upstream of *SSA_0260* and encodes a putative Mn/Zn ABC transporter permease (UniProt: A3CKL5). The *SSA_0260* and *SSA_0261* genes are predicted to be co-transcribed.

To validate our screen results, the four zinc transporter mutants were further tested in the coculture assay with *P. aeruginosa* PAO1. We found that the *SSA_0136* and *SSA _0137* mutants showed a ~2-log reduction in growth enhancement relative to that of the wild-type *S. sanguinis* SK36, while the *SSA_0260* and *SSA_0261* mutants displayed a 1-log reduction compared the wild-type *S. sanguinis* SK36 (Fig. 3A). In addition, the four mutants were also tested in monoculture to determine their growth behavior; the results revealed that these mutants grew like *S. sanguinis* WT (Fig. S2), suggesting that the growth enhancement deficiency observed for these mutants was not due to a general growth defect.

To confirm that the observed growth promotion defect was indeed caused by inactivation of the individual genes, we complemented each of the mutants with its corresponding gene under the control of an IPTG-inducible promoter, as reported (58). As shown in Fig. 3B, the complemented mutants showed growth enhancement phenotypes similar to that of wild-type *S. sanguinis* SK36, indicating that the mutation in these genes is solely responsible for the observed growth enhancement deficiency. Together, our data indicate that the functions of these genes are required for *S. sanguinis* SK36 to show enhanced growth in coculture with *P. aeruginosa*.

As zinc-ABC transporters are required for zinc acquisition (42), we reasoned that the defects in growth enhancement of these mutants during coculture might be due to the lack of zinc uptake. Consequently, the effect of supplementing additional zinc to the coculture medium was tested. We first measured the zinc levels of the medium used in our coculture conditions (MEM+L-Gln) and found the concentration of zinc is 0.024 ± 0.007 μM. Supplementation of the medium with 0.1 μM, 1 μM and 10 μM zinc chloride restored the growth enhancement phenotype of each of the mutants to wild-type levels (Fig. 3A), and growth of the wild-type *S. sanguinis* SK36 was slightly promoted upon addition of 0.1 μM and 1 μM zinc (Fig. 3A). There was no significant difference in *P. aeruginosa* growth in monoculture or coculture in the zinc-amended and unamended media (Fig. S3). These results indicate that zinc is required for efficient growth enhancement of *S. sanguinis* during coculture with *P. aeruginosa*.

Notably, zinc added to 10 μM slightly inhibited the growth of wild-type *S. sanguinis* SK36 and the SSA_0136 and SSA _0137 mutants, but not the SSA_0260 and SSA_0261 mutants (Fig. 3A), suggesting that high concentrations of zinc are toxic to *S. sanguinis*, that these zinc transporters play an important role in zinc acquisition, and may have distinct affinities for zinc.

As mentioned above, *P. aeruginosa* can also promote *S. sanguinis* SK36 growth on airway cells (Fig. 1D). To further investigate the role of zinc in the *P. aeruginosa*-induced growth enhancement, we extended our observation to cocultures on CFBE monolayers. The

wild-type *S. sanguinis* SK36 strain and zinc transporter mutants were cocultured with *P. aeruginosa* with 1 μM zinc chloride added to the medium. Similar to the observations in our coculture model on plastic, the zinc transporter mutants exhibited reduced growth enhancement, and supplementing the medium with 1 μM zinc rescued the growth enhancement defect of these mutants (Fig. 3C). It is worth noting that there was no significant difference in growth of the wild-type *S. sanguinis* SK36 and the zinc transporter mutants on CFBE monolayers with or without addition of zinc (Fig. 3D). The growth of *S. sanguinis* was enhanced when cultured with CFBE cells even in the absence of zinc (Fig. 1D), indicating that CFBE monolayers produce factor(s) other than zinc that can enhance the growth of *S. sanguinis*. Taken together, these data demonstrate that enhanced *S. sanguinis* growth mediated by *P. aeruginosa* requires the zinc importers of *S. sanguinis* SK36.

Coculture with *P. aeruginosa* upregulates zinc transporter gene expression in *S. sanguinis*. We next examined the expression patterns of zinc transporter genes in both *S. sanguinis* and *P. aeruginosa* using qRT-PCR. As shown above in Fig. 1A, in the first 2 hours of coculture with *P. aeruginosa* PAO1, *S. sanguinis* did not show a significant enhancement in growth. The mixed cultures showed a significant trend toward enhanced *S. sanguinis* growth and exhibited a robust increase in growth at 6 h. We saw no differences between the pure and the mixed cultures when we examined the growth kinetics of *P. aeruginosa* PAO1 over the course of 24 h. As a consequence, qRT-PCR studies were performed on samples of 2 h and 6 h for both monocultures and cocultures.

At 2 h, *S. sanguinis* zinc transporter genes *SSA_0136* and *SSA_0137* were upregulated 2.2- and 2.3-fold in coculture compared to *S. sanguinis* monoculture (Fig. 4A), respectively, while the expression of *SSA_0260* and *SSA_0261* genes were not significantly changed at this time point (Fig. 4A). At 6 h, *S. sanguinis* expression of all of the four zinc transporter genes was upregulated (4.4-fold for *SSA_0136*; 4.7-fold for *SSA_0137*; 8.2-fold for *SSA_0260*; 4.3-fold for *SSA_0261*) when co-cultured with *P. aeruginosa* (Fig. 4A). In contrast, the addition of

exogenous zinc (1 μM) to the coculture medium reduced the expression of *S. sanguinis* zinc transporter genes to the same level as monocultures with zinc supplementation (Fig. 4B). The expression of *P. aeruginosa* PAO1 zinc uptake and regulator genes (*zur*, *znuA*, *cntI*, *cntO*) was minimally impacted by the presence of *S. sanguinis* compared to *P. aeruginosa* PAO1 alone at either 2 h or 6 h (Fig. S4), although the zinc transporters of *P. aeruginosa* PAO1 were significantly upregulated in monoculture and coculture at 6 h compared to 2 h (Fig. S4). Notably, the robust increase in zinc transporter gene expression by *S. sanguinis* at 6 h corresponds to the induction of *P. aeruginosa* uptake systems at this same time point. Taken together, our studies are consistent with a model that *S. sanguinis* zinc transporter genes are induced by zinc deficiency when *S. sanguinis* is grown in coculture with *P. aeruginosa*, presumably due to zinc competition between these organisms.

Mutations in zinc-related genes of *P. aeruginosa* promote growth of *S. sanguinis* in coculture. Our data suggest that in coculture, *P. aeruginosa* and *Streptococcus* compete for zinc. As shown above, *S. sanguinis* SK36 mutants defective in zinc uptake show less robust viability when grown in coculture with *P. aeruginosa*. Thus, we would predict that mutations in zinc uptake in *P. aeruginosa* would result in *S. sanguinis* SK36 more effectively competing for zinc, likely reflected by an increase in viable counts of *S. sanguinis*.

We first assessed the impact of mutating zinc transport systems of *P. aeruginosa* PAO1 by testing strains with mutations in the *cntI*, *znuA* and *cntI-O* genes (Fig. 5A-C). In all cases, coculture with the *P. aeruginosa* zinc transport mutants resulted in a modest enhancement (~2-3-fold) of growth of *S. sanguinis* SK36 compared to coculture with the wild-type *P. aeruginosa* PAO1. One confounding factor in the interpretation of these results is that all of the *P. aeruginosa* zinc transport mutants show a small but consistent ~50% reduction in growth in coculture, which may be due to the lack of effective zinc transport or other anticipated defects, as reported (35, 37).

In *P. aeruginosa*, the expression of zinc importer genes is controlled by the Zur protein, a zinc responsive repressor (36), and loss of Zur results in a higher intracellular zinc concentration in *P. aeruginosa* (37). We generated a *P. aeruginosa* PAO1 Δ*zur* mutant and cocultured this mutant with *S. sanguinis* SK36 with or without zinc addition. Coculture of the wild-type *P. aeruginosa* PAO1 with *S. sanguinis* served as the control. In the absence of added zinc, there was no difference in the growth of *S. sanguinis* SK36 in the presence of the wild-type versus *P. aeruginosa* PAO1 Δ*zur* mutant (Fig. 5D-E). As described above, in mixed cultures of *S. sanguinis* and wild-type *P. aeruginosa* PAO1, we found excessive zinc concentration (10 μM) led to a 9-fold decline in *S. sanguinis* growth compared to cells grown without zinc supplementation (Fig. 3A and Fig. 5D). In contrast, mixed cultures of *S. sanguinis* and *P. aeruginosa* PAO1 Δ*zur* mutant in the presence of 10 μM zinc resulted in a 3.5-fold increase in *S. sanguinis* growth compared to that observed for coculture with WT *P. aeruginosa* PAO1 (Fig. 5D). Given the protective action of the zinc hyper-accumulating *P. aeruginosa* PAO1 Δ*zur* mutant towards *S. sanguinis* SK36, these results further support the idea that *P. aeruginosa* and *S. sanguinis* compete for zinc when grown in coculture.

The relationship between sputum zinc concentration and the relative abundance of *Streptococcus* and *Pseudomonas*. In previous studies we had collected sputum samples from patients with CF and assessed the relative abundance of *Streptococcus* and *Pseudomonas* in these samples (59). These same samples were analyzed by ICP-MS, as reported (59, 60), to determine the concentration of zinc in the sputum. We observed that the concentration of zinc in these samples ranged from 4.8-145 μM with a median of 36.4 μM (n = 118 sputum samples; Fig. S5).

We examined the relationship between zinc concentration and the relative abundance of *Streptococcus* and *Pseudomonas* in these samples and observed that Streptococcus dominated the sputum samples when the zinc concentration was low (Fig. 6, orange dots), but

304 that the relative abundance of *Pseudomonas* was higher in samples with high zinc levels (Fig.
305 6, blue dots). Although measures like Pearson or Spearman correlation have been used to
306 quantify this type of relationship and attach statistical significance, the compositional nature of
307 bacterial community abundance suggests that these statistical approaches are inappropriate
308 (61, 62). Therefore, we interpret *in vivo* associations observed between *Streptococcus*
309 abundance*, Pseudomonas* abundance and zinc concentration as broadly consistent with
310 the hypothesis that they are related without attaching a specific measure of statistical
311 significance.

312

313 **Physiologically relevant levels of zinc impact the competition between *Streptococcus***
314 **and *Pseudomonas.*** Given the observations described above, we decided to assess how
315 varying zinc concentrations might impact the competition between various *Streptococcus*
316 species found in the CF airway and *P. aeruginosa* (Fig. 7). For clinical isolates of *S.*
317 *intermedius* (two isolates), S*. constellatus*, *S. parasanguinis* and *S. salivarius*, all of which
318 have been found in the CF airway (11), increasing zinc across a range of concentrations
319 measured in sputum resulted in progressively lower viability of many of these streptococci
320 when grown in monoculture (Fig. 7A) or in coculture with *P. aeruginos*a (Fig. 7B). Thus, many
321 of these strains appear to be sensitive to clinically-relevant concentrations of zinc.
322 Interestingly, two isolates (*S. anginosus* and *S. oralis*) showed robust growth even at the
323 highest level of zinc tested, indicating that these strains have increased tolerance to zinc for
324 reasons we do not understand. Interestingly, the data in Figure 6 show that some patients
325 with high levels of zinc in their sputum also have high relative abundance of *Streptococcus*;
326 perhaps these patients harbor zinc-resistant streptococci.

327

DISCUSSION

In this study, we characterized the interaction between *P. aeruginosa* and *S. sanguinis* SK36 in a dual species coculture model system. We demonstrated that zinc uptake by *S. sanguinis* SK36 was necessary for the *P. aeruginosa*-mediated promotion of *S. sanguinis* SK36 growth, and that *P. aeruginosa* competed with *S. sanguinis* SK36 for zinc during cocultivation. Additionally, we described a new association between zinc levels and the abundance of *Pseudomonas* and *Streptococcus* in CF sputum, thus highlighting the potential role of zinc in the interaction between these CF pathogens, as well as a potential role for this metal in shaping microbiome dynamics in the context of polymicrobial CF airway infections.

We report that co-cultivation with *P. aeruginosa* results in enhanced growth of *S. sanguinis* SK36 on either plastic or CF-derived airway cells, while *P. aeruginosa* growth is relatively unaffected during coculture. These data are consistent with previous reports from our group and others that coculture of streptococci with *P. aeruginosa* promotes the growth of streptococci, but with no obvious benefit to *P. aeruginosa* growth (8-10). Interestingly, in a study examining the interactions between *P. aeruginosa* and oral streptococci, including *S. sanguinis* (12, 63), *P. aeruginosa* growth was inhibited when streptococci were grown as a pioneer colonizer; streptococci can produce hydrogen peroxide (H_2O_2) to react with excess nitrite in the medium to generate reactive nitrogenous intermediates (RNI) for the inhibition of *P. aeruginosa* growth (12, 13, 63). We note that *P. aeruginosa* was inoculated in combination with *S. sanguinis* in our study under conditions that differ from these previous reports, and we did not observe streptococcus-mediated inhibition of *P. aeruginosa*. These observations suggest that the relationships between *P. aeruginosa* and streptococci are complex and likely are influenced by metabolic/environmental factors and colonization sequence. Understanding how various *in vitro* models reflect dynamic *in vivo* environments will contribute to efficiently synthesizing and better understanding the data obtained from various laboratories.

By screening a genome-wide non-essential gene mutant library of *S. sanguinis* SK36, we discovered 80 mutants with attenuation in *P. aeruginosa*-mediated growth enhancement (summarized in Table S1). The large number of genes (3.5% of the *S. sanguinis* SK36 genome) found to be involved in this interspecies interaction and the variety of functions performed by these gene products provide new insights into the mechanism(s) of interaction between these two pathogens. Among the candidate genes we identified in our screen, we focused on a set of genes involved in the import of zinc. We demonstrate that the ability to obtain zinc as one factor contributing to the *P. aeruginosa*-induced enhancement of *S. sanguinis* SK36 growth, both in the presence and absence of human airway cells. Future studies will focus on the other genes identified in our screen.

Our observations here raise the question of whether zinc is a factor that drives growth enhancement of streptococci in the presence of *P. aeruginosa*, or whether efficient uptake of zinc is required to allow the growth-promoting factors produced by *P. aeruginosa* to exert their effect. We favor the latter model, in large part because we show that adding additional zinc to monocultures of *S. sanguinis* SK36 or other streptococci has, at best, a very modest effect on growth of the streptococci. That is, it does not appear that the streptococci are obtaining zinc from *P. aeruginosa* to enhance streptococcal growth. Instead, we observed that zinc starvation might be triggered by competition for this metal between *P. aeruginosa* and *S. sanguinis* SK36. Indeed, the elevated expression of the zinc transporters of wild-type *S. sanguinis* SK36 in mixed cultures, and the lack of such induction with zinc supplementation in the coculture medium, indicates that *S. sanguinis* SK36 becomes zinc-starved during its interaction with *P. aeruginosa*. In contrast, there was no significant change in the expression of *P. aeruginosa* zinc transporters under coculture conditions, indicating that *P. aeruginosa* is not lacking for this metal under these conditions; thus it is unlikely that *S. sanguinis* is “stealing” the zinc in the culture from *P. aeruginosa*. Taken together, we argue that zinc provided by *P. aeruginosa* is not a key factor enhancing streptococcal growth in coculture.

379 An intriguing observation here is the relationship between the sputum zinc levels and the
380 relative fraction of *Pseudomonas* and *Streptococcus* in the CF sputum. In the human body,
381 the zinc concentration varies among different tissues and total zinc concentration in induced
382 sputum from control patients is around 1 μM (50 μg/l) (42, 64), although higher levels of total
383 sputum zinc have been reported in patients with CF (48), a finding consistent with our sputum
384 measurements here. Furthermore, the zinc-sequestering protein calprotectin is present in
385 CF sputum in high concentrations (65, 66), potentially resulting in limited bioavailability of zinc
386 for CF pathogens like *P. aeruginosa* (49). Thus, at present it is difficult to conclude how much
387 of the increased zinc measured in CF sputum is actually available to the microbes; knowing
388 the answer to this question is key to understanding disease progression. For example, based
389 on *in vitro* studies, efficient zinc uptake is critical for *P. aeruginosa* to express several
390 virulence traits associated with lung colonization, including swarming, swimming motility and
391 the ability to form biofilms (49). It has been shown that high concentrations of total zinc are
392 correlated with airway inflammation (48), indicating that perhaps *P. aeruginosa* can access
393 some of the large pool of zinc in some circumstances. Furthermore, sputum zinc levels were
394 found to decrease following antibiotic treatment of CF exacerbation (48, 49), again
395 suggestive that loss of access to zinc reduces virulence. Interestingly, we found a possible
396 relationship between the concentrations of zinc and relative abundance of *Pseudomonas* and
397 *Streptococcus*, however, because of the relative abundance data available for this analysis,
398 determining a statistical correlation is difficult. Nevertheless, we do note that at higher zinc
399 measured concentrations, the relative abundance of *Streptococcus* appears to be lower than
400 at low concentrations of this metal, which may be related to the toxicity observed when
401 *Streptococcus* is grown in high levels of zinc, resulting in impaired growth or death (67).
402 These data do suggest that increased total zinc may be associated with increased
403 bioavailable zinc. A more detailed analysis of the physiology of zinc metabolism in CF sputum
404 and measurements of the bioavailabilty of this metal will be required to definitely address the
405 questions raised here, and these questions should be addressed in future studies.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table S2. *S. sanguinis* SK36 and other clinical streptococcal isolates were grown statically in Bacto™ Todd-Hewitt (TH) broth supplemented with 0.5% (w/v) yeast extract (THY), or on Trypticase™ soy agar plates supplemented with 5% (v/v) defibrinated sheep blood (blood agar) at 37ºC with 5% CO_2. *P. aeruginosa* and *Escherichia coli* strains were grown in lysogeny broth (LB) medium (68) with shaking or on LB agar at 37ºC unless otherwise noted. As indicated, the following antibiotics and concentrations were used: 500 µg/ml kanamycin and 200 µg/ml spectinomycin for *S. sanguinis*; 50 µg/ml gentamycin and 150 µg/ml carbenicillin for *P. aeruginosa*; 10 µg/ml gentamicin, 50 µg/ml carbenicillin and 100 µg/ml spectinomycin for *E. coli*. For IPTG-inducible plasmids, IPTG was added to cultures to a 100 µM final concentration.

Coculture assays. Coculture assays were performed as previously described with minor modifications (8). Briefly, overnight cultures of *P. aeruginosa* and *Streptococcus* spp. were centrifuged at 13,000 × *g* for 3 min, washed twice with phosphate buffered saline (PBS), and resuspended in minimal essential medium (MEM) supplemented with 2 mM L-glutamine (MEM+L-Gln). For coculture samples, *P. aeruginosa* inoculum was prepared to an OD_{600} of 0.05 and *Streptococcus* spp. inoculum was prepared to an OD_{600} of 0.001 in MEM+L-Gln. For monoculture controls, the inocula for *P. aeruginosa* or *Streptococcus* spp. were prepared to the same OD_{600} as for the coculture samples. Three wells of a 96-well deep well plate were inoculated per monoculture and coculture condition with 400 µl per well. Culture plates were then incubated statically at 37°C with 5% CO_2 for 2 h, at which point the unattached planktonic cells were removed by aspiration and 400 µl of fresh MEM+L-Gln was once again added to each well. The cultures were then incubated for an additional 20 h, and both planktonic and biofilm cells were harvested together using a 96 pin replicator. Bacterial growth was determined by 10-fold serial dilutions in PBS and plated in 3 µl aliquots on

Pseudomonas isolation agar (PIA) or blood agar supplemented with 10 µg/ml neomycin and 10 µg/ml polymixin B (SBA) for *P. aeruginosa* and *Streptococcus* spp. selective growth, respectively. After overnight incubation, bacterial colonies were counted and the colony forming units (CFU) per ml of culture were determined.

Genetic screen of the SK36 library for mutants defective in growth enhancement. To investigate *S. sanguinis* SK36 genes involved in *P. aeruginosa*-mediated enhancement of growth, the *S. sanguinis* SK36 non-essential gene mutant library (55) was screened for growth enhancement defects as previously described for *P. aeruginosa* with some modifications (8). Briefly, a 96 pin replicator was used to transfer inocula from the frozen library to a 96-well plate containing 150 ml of THY broth per well. The plate was then incubated statically for 24 h at 37ºC with 5% CO_2. The *P. aeruginosa* PAO1 culture was grown overnight in LB broth, adjusted to an OD_{600} of 0.05 in MEM+L-Gln as described above, and 400 µl of this adjusted inoculum suspension was added to each well of a 96-well deep well plate. The 96 pin replicator was then used to transfer 2-3 µl of the 24 h culture from the mutant library plate into the 96-well deep well plate containing *P. aeruginosa* PAO1. Unattached bacteria were removed after 2 h, and 400 µl of fresh MEM+L-Gln was added to each well and the plates were grown for an additional 20 h at 37°C with 5% CO_2. The 96 pin replicator was again used to disrupt the biofilms into the planktonic fraction, and large petri dish plates containing either PIA or SBA media were spot inoculated with each culture and grown as described above. Candidates that showed low or undetectable growth based on differences compared to the wild-type *S. sanguinis* SK36 (which formed small lawns when inoculated onto an agar plate by the 96 pin replicator) were stored at -80ºC in 30% glycerol in a sterile 96-well plate. To confirm the phenotype, a second and third round of mutant screening was performed as described above.

Kinetic growth assay. The relative ability of *S. sanguinis* SK36 and *P. aeruginosa* PAO1 to grow in the coculture model system was determined by kinetic growth assays. Briefly, *S.*

458 *sanguinis* SK36 was grown in coculture with *P. aeruginosa* PAO1 as described above in a
459 96-well deep well plate. The cultures were grown at 37°C with 5% CO_2 for 24 h, and were
460 assessed for viable cell counts (CFU/ml) at seven time points: 0, 2, 4, 6, 8, 10, and 24 h. The
461 0 h time point corresponds to the initial inoculum. Cells were collected at the 2 h time point
462 prior to the 2 h medium replacement. At each time point, a combination of the planktonic and
463 biofilm cells from triplicate wells were serially diluted and plated on PIA and SBA agar media,
464 and CFU counts determined after overnight incubation as described above.

465 ***P. aeruginosa* supernatant assays.** To prepare *P. aeruginosa* conditioned medium,
466 bacteria were grown in 0.5 ml per well of MEM+L-Gln in 24-well plates with media changes as
467 described above. At the 22 h time point, the planktonic fractions were collected, centrifuged
468 and supernatants were filter-sterilized through a 0.22 µm syringe filter. The effect of the
469 sterile *P. aeruginosa* supernatants on the growth of *S. sanguinis* was tested on both plastic
470 and CFBE monolayers. For experiments on plastic, supernatant with different levels of
471 dilution in fresh MEM+L-Gln was added to *S. sanguinis* monocultures when the medium was
472 replaced at the 2 h time point. For assays on CF airway cells, a 1/2 dilution of *P. aeruginosa*
473 supernatant in MEM+L-Gln was supplemented with 0.4% L-arginine and gently added to
474 each well of CF airway cells that had been cocultured with *S. sanguinis* for 1 h or 5.5 h when
475 the medium was exchanged. *S. sanguinis* growth was evaluated after 22 h of incubation at
476 37°C with 5% CO_2 as described above.

477 **Tissue culture cells and coculture on CFBE monolayers.** The cystic fibrosis bronchial
478 epithelial (CFBE) monolayers used in the coculture model (54, 69) are immortalized cells that
479 overexpress ΔF508-cystic fibrosis transmembrane conductance regulator (10, 70). CFBE
480 monolayers were grown as previously described (10, 69). In brief, the CFBE monolayers

were seeded at a concentration of 100,000 cells/well in a 24-well tissue culture plate and fed every other day with MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 µg/ml puromycin and 5 µg/ml Plasmocin. Cells were grown at 37°C with 5% CO_2 for 5-7 days to form a confluent monolayer and tight junctions before inoculation with bacteria. For coculture assays with mono- and dual-bacterial species, liquid cultures of *P. aeruginosa* and *Streptococcus* spp. were prepared as described above and 500 µl of bacterial inocula were gently added to triplicate wells of CFBE monolayers that had been washed twice with MEM. The cocultures were incubated at 37°C with 5% CO_2 for 1 h, at which point unattached bacteria were removed by aspiration, 500 µl of MEM+L-Gln+0.4% L-arginine was added to each well and incubated for an additional 4.5 h. At this point, planktonic cells were removed by aspiration, and 500 µl of fresh MEM+L-Gln+0.4% L-arginine was once again added into each well. The established coculture was incubated for an additional 16.5 h. At 22 h post-inoculation, both planktonic and biofilm-grown bacteria were collected together by scraping with a pipette tip, and bacteria were serially diluted and plated on PIA and SBA plates, as described above, to identify *P. aeruginosa* and *Streptococcus* spp., respectively. Following overnight incubation, the resulting colonies were counted and the CFU/ml of the culture was determined.

Zinc supplementation. For zinc supplementation assays, wild-type *Streptococcus* SK36 and individual mutants with growth enhancement defects, wild-type *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 zinc homeostasis associated mutants were grown as described above for monoculture and coculture assays. After 2 h, unattached bacteria were removed by aspiration, MEM+L-Gln with or without additional zinc (at the indicated concentrations diluted from 1 mM zinc chloride stock solution in MEM+L-Gln) was added to each well and cocultures were then treated as described above. Nutritional complementation on CF airway cells were performed as described for biofilms on plastic, except that MEM+L-Gln supplemented with 0.4% arginine (to enhance biofilm formation) with or without additional zinc chloride at the indicated concentration was used for medium exchange when indicated.

Construction of mutants and complementation. In-frame deletions of *P. aeruginosa* genes were constructed by allelic exchange employing the sucrose counter-selection system with the gene replacement vector pEX18Ap (71). Mutant strains were confirmed by PCR analysis of genomic DNA. *S. sanguinis* mutants were derived from a defined mutant library described previously (55). For complementation of each targeted *S. sanguinis* gene, a suicide vector pJFP126 was used to allow for the insertion of complementing genes into an ectopic chromosomal site (*SSA_0169*) via homologous recombination and expression of each gene is under the control of an IPTG inducible promoter *hyper-spank* (8, 58). Transformation was performed essentially as described previously (72) with the competence stimulating peptide (CSP sequence: DLRGVPNPWGWIFGR) custom-synthesized by GenScript Inc. (Piscataway, NJ). Primers used for PCR amplification of selected genes are listed in Table S3.

Expression studies. For qRT-PCR studies, the overnight culture used as inoculum was prepared as described above from which three replicates cocultures of *P. aeruginosa* and *S. sanguinis,* or the *S. sanguinis* monoculture, were prepared in 100 ml of warm MEM+L-Gln in a 250 ml flask. The inoculum of *P. aeruginosa* and *S. sanguinis* was prepared and brought to an OD_{600} of 0.05 and 0.02, respectively, as described above. Cultures were incubated at 37ºC with 5% CO_2. After 2 h and 6 h of incubation, samples were pelleted, and bacterial cells were mechanically lysed with 10 cycles of 30 s bead beating, 30 s on ice with a 1:1 mixture of 0.1 mm and 0.5 mm glass beads. Total RNA was isolated using TRIzol and the Direct-zol™ RNA MiniPrep Kit with two times of in-column DNase I treatment according to the manufacturer's instructions (Zymo Research, R2053). RNA purity and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). For each sample, 1 µg of RNA was converted to cDNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) and then diluted 1:50. qRT-PCR were carried out in triplicate in a StepOnePlus Real-Time PCR System (Applied Biosystems) using iTaq Universal SYBR Green Supermix (Bio-Rad). The qRT-PCR primers are listed in Table S3. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method with DNA gyrase subunit gene *gyrA* as a

535 normalization control for *S. sanguinis* (73, 74) and *PA2875* as the reference gene for *P.*
536 *aeruginosa* (75).

537 **Measurement of zinc in sputum samples.** Sputum samples for zinc analysis were stored at
538 −80°C until processed. Sputum zinc was quantified by inductively coupled plasma-mass
539 spectrometry (ICP-MS) following nitric acid digestion of organic material according to the
540 method of Heck et al. and is expressed as micromolar zinc (60).

541 **Statistical Analysis.** Statistical analysis was performed using GraphPad Prism 7 program
542 and results were expressed as the mean values ± standard deviations. Unless otherwise
543 noted, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test
544 or Student's *t*-test analysis was performed to determine statistical significance of the data. *,
545 $P < 0.05$; **, $P < 0.01$. See figure legends or text for other specific statistical tests used.

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554 **Literature Cited.**

555 1. Elborn J. 2016. Cystic fibrosis. Lancet 388:2519-2531.

556 2. Cutting GR. 2015. Cystic fibrosis genetics: from molecular understanding to clinical
557 application. Nat Rev Genet 16:45-56.

558 3. Rowe SM, Miller S, Sorscher EJ. 2005. Cystic fibrosis. N Engl J Med 352:1992-2001.

559 4. Sibley CD, Parkins MD, Rabin HR, Duan K, Norgaard JC, Surette MG. 2008. A
560 polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in
561 cystic fibrosis patients. Proc Natl Acad Sci U S A 105:15070-5.

562 5. Sherrard LJ, Tunney MM, Elborn JS. 2014. Antimicrobial resistance in the respiratory
563 microbiota of people with cystic fibrosis. Lancet 384:703-13.

564 6. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoli JD,
565 VanDevanter DR, Murray S, Li JZ, Young VB, LiPuma JJ. 2012. Decade-long bacterial
566 community dynamics in cystic fibrosis airways. Proc Natl Acad Sci U S A 109:5809-14.

567 7. Filkins LM, O'Toole GA. 2015. Cystic fibrosis lung infections: Polymicrobial, complex,
568 and hard to treat. PLoS Pathog 11:e1005258.

569 8. Scott J, Li K, Filkins L, Zhu B, Kuchma S, Schwartzman J, O'Toole G. 2019.
570 *Pseudomonas aeruginosa* can inhibit growth of streptococcal species via siderophore
571 production. J Bacteriol 201:e00014-19.

572 9. Scoffield JA, Duan D, Zhu F, Wu H. 2017. A commensal streptococcus hijacks a
573 *Pseudomonas aeruginosa* exopolysaccharide to promote biofilm formation. PLoS
574 Pathog 13:e1006300.

10. Price KE, Naimie AA, Griffin EF, Bay C, O'Toole GA. 2016. Tobramycin-treated *Pseudomonas aeruginosa* PA14 enhances *Streptococcus constellatus* 7155 biofilm formation in a cystic fibrosis model system. J Bacteriol 198:237-47.

11. Filkins LM, Hampton TH, Gifford AH, Gross MJ, Hogan DA, Sogin ML, Morrison HG, Paster BJ, O'Toole GA. 2012. Prevalence of streptococci and increased polymicrobial diversity associated with cystic fibrosis patient stability. J Bacteriol 194:4709-17.

12. Scoffield JA, Wu H. 2015. Oral streptococci and nitrite-mediated interference of *Pseudomonas aeruginosa*. Infect Immun 83:101-7.

13. Scoffield JA, Wu H. 2016. Nitrite reductase is critical for *Pseudomonas aeruginosa* survival during co-infection with the oral commensal *Streptococcus parasanguinis*. Microbiology-Sgm 162:376-383.

14. Bastaert F, Kheir S, Saint-Criq V, Villeret B, Dang PM, El-Benna J, Sirard JC, Voulhoux R, Sallenave JM. 2018. *Pseudomonas aeruginosa* LasB subverts alveolar macrophage activity by interfering with bacterial killing through downregulation of innate immune defense, reactive oxygen species generation, and complement activation. Front Immunol 9:1675.

15. Sibley CD, Grinwis ME, Field TR, Parkins MD, Norgaard JC, Gregson DB, Rabin HR, Surette MG. 2010. McKay agar enables routine quantification of the '*Streptococcus milleri*' group in cystic fibrosis patients. J Med Microbiol 59:534-40.

16. Scott JE, O'Toole GA. 2019. The yin and yang of *Streptococcus* lung infections in cystic fibrosis: a model for studying polymicrobial interactions. J Bacteriol 201:e00115-19.

17. Dennis EA, Coats MT, Griffin S, Pang B, Briles DE, Crain MJ, Swords WE. 2018. Hyperencapsulated mucoid pneumococcal isolates from patients with cystic fibrosis have increased biofilm density and persistence in vivo. Pathog Dis 76: doi: 10.1093/femspd/fty073.

18. Acosta N, Whelan FJ, Somayaji R, Poonja A, Surette MG, Rabin HR, Parkins MD. 2017. The evolving cystic fibrosis microbiome: A comparative cohort study spanning 16 years. Ann Am Thorac Soc 14:1288-1297.

19. Flight WG, Smith A, Paisey C, Marchesi JR, Bull MJ, Norville PJ, Mutton KJ, Webb AK, Bright-Thomas RJ, Jones AM, Mahenthiralingam E. 2015. Rapid detection of emerging pathogens and loss of microbial diversity associated with severe lung disease in cystic fibrosis. J Clin Microbiol 53:2022-9.

20. Acosta N, Heirali A, Somayaji R, Surette MG, Workentine ML, Sibley CD, Rabin HR, Parkins MD. 2018. Sputum microbiota is predictive of long-term clinical outcomes in young adults with cystic fibrosis. Thorax 73:1016-1025.

21. Coburn B, Wang PW, Diaz Caballero J, Clark ST, Brahma V, Donaldson S, Zhang Y, Surendra A, Gong Y, Elizabeth Tullis D, Yau YC, Waters VJ, Hwang DM, Guttman DS. 2015. Lung microbiota across age and disease stage in cystic fibrosis. Sci Rep 5:10241.

615 22. Andreini C, Banci L, Bertini I, Rosato A. 2006. Zinc through the three domains of life.
616 Journal of Proteome Research 5:3173-3178.
617 23. Finney LA, O'Halloran TV. 2003. Transition metal speciation in the cell: Insights from
618 the chemistry of metal ion receptors. Science 300:931-936.
619 24. Mikhaylina A, Ksibe AZ, Scanlan DJ, Blindauer CA. 2018. Bacterial zinc uptake
620 regulator proteins and their regulons. Biochem Soc Trans 46:983-1001.
621 25. McDevitt CA, Ogunniyi AD, Valkov E, Lawrence MC, Kobe B, McEwan AG, Paton JC.
622 2011. A molecular mechanism for bacterial susceptibility to zinc. PLoS Pathog
623 7:e1002357.
624 26. Yamamoto K, Ishihama A. 2005. Transcriptional response of *Escherichia coli* to
625 external zinc. J Bacteriol 187:6333-6340.
626 27. Hantke K. 2005. Bacterial zinc uptake and regulators. Curr Opin Microbiol 8:196-202.
627 28. Porcheron G, Garenaux A, Proulx J, Sabri M, Dozois CM. 2013. Iron, copper, zinc, and
628 manganese transport and regulation in pathogenic Enterobacteria: correlations
629 between strains, site of infection and the relative importance of the different metal
630 transport systems for virulence. Front Cell Infect Microbiol 3:e00090.
631 29. Poole K, Hay T, Gilmour C, Fruci M. 2019. The aminoglycoside resistance-promoting
632 AmgRS envelope stress-responsive two-component system in *Pseudomonas*
633 *aeruginosa* is zinc-activated and protects cells from zinc-promoted membrane
634 damage. Microbiology 165:563-571.

635 30. Kehl-Fie TE, Skaar EP. 2010. Nutritional immunity beyond iron: a role for manganese
636 and zinc. Curr Opin Chem Biol 14:218-24.
637 31. Ong CL, Gillen CM, Barnett TC, Walker MJ, McEwan AG. 2014. An antimicrobial role
638 for zinc in innate immune defense against group A streptococcus. J Infect Dis
639 209:1500-8.
640 32. Botella H, Peyron P, Levillain F, Poincloux R, Poquet Y, Brandli I, Wang C, Tailleux L,
641 Tilleul S, Charriere GM, Waddell SJ, Foti M, Lugo-Villarino G, Gao Q,
642 Maridonneau-Parini I, Butcher PD, Castagnoli PR, Gicquel B, de Chastellier C,
643 Neyrolles O. 2011. Mycobacterial p(1)-type ATPases mediate resistance to zinc
644 poisoning in human macrophages. Cell Host Microbe 10:248-59.
645 33. Blencowe DK, Morby AP. 2003. Zn(II) metabolism in prokaryotes. FEMS Microbiol Rev
646 27:291-311.
647 34. Capdevila DA, Wang J, Giedroc DP. 2016. Bacterial strategies to maintain zinc
648 metallostasis at the host-pathogen interface. J Biol Chem 291:20858-20868.
649 35. Pederick VG, Eijkelkamp BA, Begg SL, Ween MP, McAllister LJ, Paton JC, McDevitt
650 CA. 2015. ZnuA and zinc homeostasis in *Pseudomonas aeruginosa*. Sci Rep 5:13139.
651 36. Gonzalez MR, Ducret V, Leoni S, Perron K. 2019. *Pseudomonas aeruginosa* zinc
652 homeostasis: Key issues for an opportunistic pathogen. Biochim Biophys Acta Gene
653 Regul Mech 1862:722-733.

654 37. Ellison ML, Farrow JM, 3rd, Parrish W, Danell AS, Pesci EC. 2013. The transcriptional
655 regulator Np20 is the zinc uptake regulator in *Pseudomonas aeruginosa*. PLoS ONE
656 8:e75389.
657 38. Lhospice S, Gomez NO, Ouerdane L, Brutesco C, Ghssein G, Hajjar C, Liratni A,
658 Wang SL, Richaud P, Bleves S, Ball G, Borezee-Durant E, Lobinski R, Pignol D,
659 Arnoux P, Voulhoux R. 2017. *Pseudomonas aeruginosa* zinc uptake in chelating
660 environment is primarily mediated by the metallophore pseudopaline. Scientific
661 Reports 7:17132.
662 39. Mastropasqua MC, D'Orazio M, Cerasi M, Pacello F, Gismondi A, Canini A, Canuti L,
663 Consalvo A, Ciavardelli D, Chirullo B, Pasquali P, Battistoni A. 2017. Growth of
664 *Pseudomonas aeruginosa* in zinc poor environments is promoted by a
665 nicotianamine-related metallophore. Mol Microbiol 106:543-561.
666 40. Patzer SI, Hantke K. 1998. The ZnuABC high-affinity zinc uptake system and its
667 regulator Zur in *Escherichia coli*. Mol Microbiol 28:1199-210.
668 41. Outten CE, O'Halloran TV. 2001. Femtomolar sensitivity of metalloregulatory proteins
669 controlling zinc homeostasis. Science 292:2488-92.
670 42. Shafeeq S, Kuipers OP, Kloosterman TG. 2013. The role of zinc in the interplay
671 between pathogenic streptococci and their hosts. Mol Microbiol 88:1047-1057.
672 43. Makthal N, Kumaraswami M. 2017. Zinc'ing it out: zinc homeostasis mechanisms and
673 their impact on the pathogenesis of human pathogen group A streptococcus.
674 Metallomics 9:1693-1702.

675 44. Plumptre CD, Eijkelkamp BA, Morey JR, Behr F, Counago RM, Ogunniyi AD, Kobe B,
676 O'Mara ML, Paton JC, McDevitt CA. 2014. AdcA and AdcAII employ distinct zinc
677 acquisition mechanisms and contribute additively to zinc homeostasis in
678 *Streptococcus pneumoniae*. Mol Microbiol 91:834-51.
679 45. Moulin P, Patron K, Cano C, Zorgani MA, Camiade E, Borezee-Durant E, Rosenau A,
680 Mereghetti L, Hiron A. 2016. The Adc/Lmb system mediates zinc acquisition in
681 *Streptococcus agalactiae* and contributes to bacterial growth and survival. J Bacteriol
682 198:3265-3277.
683 46. Linke C, Caradoc-Davies TT, Young PG, Proft T, Baker EN. 2009. The laminin-binding
684 protein Lbp from *Streptococcus pyogenes* is a zinc receptor. J Bacteriol 191:5814-23.
685 47. Panina EM, Mironov AA, Gelfand MS. 2003. Comparative genomics of bacterial zinc
686 regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal
687 proteins. Proc Natl Acad Sci U S A 100:9912-7.
688 48. Gray RD, Duncan A, Noble D, Imrie M, O'Reilly DS, Innes JA, Porteous DJ, Greening
689 AP, Boyd AC. 2010. Sputum trace metals are biomarkers of inflammatory and
690 suppurative lung disease. Chest 137:635-41.
691 49. Mastropasqua MC, Lamont I, Martin LW, Reid DW, D'Orazio M, Battistoni A. 2018.
692 Efficient zinc uptake is critical for the ability of *Pseudomonas aeruginosa* to express
693 virulence traits and colonize the human lung. Journal of Trace Elements in Medicine
694 and Biology 48:74-80.

695 50. Makthal N, Nguyen K, Do H, Gavagan M, Chandrangsu P, Helmann JD, Olsen RJ,
696 Kumaraswami M. 2017. A critical role of zinc importer AdcABC in Group A
697 Streptococcus-host interactions during infection and its implications for vaccine
698 development. Ebiomedicine 21:131-141.
699 51. Britigan BE, Railsback MA, Cox CD. 1999. The *Pseudomonas aeruginosa* secretory
700 product pyocyanin inactivates alpha1 protease inhibitor: implications for the
701 pathogenesis of cystic fibrosis lung disease. Infect Immun 67:1207-12.
702 52. Lyczak JB, Cannon CL, Pier GB. 2000. Establishment of *Pseudomonas aeruginosa*
703 infection: lessons from a versatile opportunist. Microbes Infect 2:1051-60.
704 53. Gallagher LA, Manoil C. 2001. *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis*
705 *elegans* by cyanide poisoning. J Bacteriol 183:6207-6214.
706 54. Anderson GG, Moreau-Marquis S, Stanton BA, O'Toole GA. 2008. In vitro analysis of
707 tobramycin-treated *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived
708 airway epithelial cells. Infect Immun 76:1423-33.
709 55. Xu P, Ge X, Chen L, Wang X, Dou Y, Xu JZ, Patel JR, Stone V, Trinh M, Evans K,
710 Kitten T, Bonchev D, Buck GA. 2011. Genome-wide essential gene identification in
711 *Streptococcus sanguinis*. Sci Rep 1:125.
712 56. Das S, Kanamoto T, Ge X, Xu P, Unoki T, Munro CL, Kitten T. 2009. Contribution of
713 lipoproteins and lipoprotein processing to endocarditis virulence in *Streptococcus*
714 *sanguinis*. J Bacteriol 191:4166-79.

715 57. Crump KE, Bainbridge B, Brusko S, Turner LS, Ge X, Stone V, Xu P, Kitten T. 2014.
716 The relationship of the lipoprotein SsaB, manganese and superoxide dismutase in
717 *Streptococcus sanguinis* virulence for endocarditis. Mol Microbiol 92:1243-59.
718 58. Rhodes DV, Crump KE, Makhlynets O, Snyder M, Ge X, Xu P, Stubbe J, Kitten T.
719 2014. Genetic characterization and role in virulence of the ribonucleotide reductases of
720 *Streptococcus sanguinis*. J Biol Chem 289:6273-87.
721 59. Gifford AH, Alexandru DM, Li Z, Dorman DB, Moulton LA, Price KE, Hampton TH,
722 Sogin ML, Zuckerman JB, Parker HW, Stanton BA, O'Toole GA. 2014. Iron
723 supplementation does not worsen respiratory health or alter the sputum microbiome in
724 cystic fibrosis. J Cyst Fibros 13:311-8.
725 60. Heck JE, Andrew AS, Onega T, Rigas JR, Jackson BP, Karagas MR, Duell EJ. 2009.
726 Lung cancer in a U.S. population with low to moderate arsenic exposure. Environ
727 Health Perspect 117:1718-23.
728 61. Carr A, Diener C, Baliga NS, Gibbons SM. 2019. Use and abuse of correlation
729 analyses in microbial ecology. ISME J doi:10.1038/s41396-019-0459-z.
730 62. Knight R, Vrbanac A, Taylor BC, Aksenov A, Callewaert C, Debelius J, Gonzalez A,
731 Kosciolek T, McCall LI, McDonald D, Melnik AV, Morton JT, Navas J, Quinn RA,
732 Sanders JG, Swafford AD, Thompson LR, Tripathi A, Xu ZZ, Zaneveld JR, Zhu Q,
733 Caporaso JG, Dorrestein PC. 2018. Best practices for analysing microbiomes. Nat
734 Rev Microbiol 16:410-422.

735 63. Whiley RA, Fleming EV, Makhija R, Waite RD. 2015. Environment and colonisation
736 sequence are key parameters driving cooperation and competition between
737 *Pseudomonas aeruginosa* cystic fibrosis strains and oral commensal streptococci.
738 PLoS One 10:e0115513.
739 64. Jayaram L, Chunilal S, Pickering S, Ruffin RE, Zalewski PD. 2011. Sputum zinc
740 concentration and clinical outcome in older asthmatics. Respirology 16:459-66.
741 65. Gray RD, Imrie M, Boyd AC, Porteous D, Innes JA, Greening AP. 2010. Sputum and
742 serum calprotectin are useful biomarkers during CF exacerbation. J Cyst Fibros
743 9:193-8.
744 66. Gray RD, MacGregor G, Noble D, Imrie M, Dewar M, Boyd AC, Innes JA, Porteous DJ,
745 Greening AP. 2008. Sputum proteomics in inflammatory and suppurative respiratory
746 diseases. Am J Respir Crit Care Med 178:444-52.
747 67. Ong CL, Walker MJ, McEwan AG. 2015. Zinc disrupts central carbon metabolism and
748 capsule biosynthesis in *Streptococcus pyogenes*. Sci Rep 5:10799.
749 68. Bertani G. 2004. Lysogeny at mid-twentieth century: P1, P2, and other experimental
750 systems. J Bacteriol 186:595-600.
751 69. Moreau-Marquis S, Redelman CV, Stanton BA, Anderson GG. 2010. Co-culture
752 models of *Pseudomonas aeruginosa* biofilms grown on live human airway cells. J Vis
753 Exp doi:10.3791/2186.

754 70. Hentchel-Franks K, Lozano D, Eubanks-Tarn V, Cobb B, Fan L, Oster R, Sorscher E,
755 Clancy JP. 2004. Activation of airway Cl- secretion in human subjects by adenosine.
756 Am J Respir Cell Mol Biol 31:140-6.
757 71. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A
758 broad-host-range Flp-FRT recombination system for site-specific excision of
759 chromosomally-located DNA sequences: application for isolation of unmarked
760 *Pseudomonas aeruginosa* mutants. Gene 212:77-86.
761 72. Chen L, Ge X, Xu P. 2015. Identifying essential *Streptococcus sanguinis* genes using
762 genome-wide deletion mutation. Methods Mol Biol 1279:15–23.
763 73. Zhu B, Ge X, Stone V, Kong X, El-Rami F, Liu Y, Kitten T, Xu P. 2017. *ciaR* impacts
764 biofilm formation by regulating an arginine biosynthesis pathway in *Streptococcus*
765 *sanguinis* SK36. Sci Rep 7:17183.
766 74. Zhu L, Zhang Y, Fan J, Herzberg MC, Kreth J. 2011. Characterization of competence
767 and biofilm development of a Str*eptococcus sanguinis* endocarditis isolate. Mol Oral
768 Microbiol 26:117-26.
769 75. Costaglioli P, Barthe C, Fayon M, Christoflour N, Bui S, Derlich L, Domblides P,
770 Crouzet M, Vilain S, Garbay B. 2014. Selection of *Pseudomonas aeruginosa* reference
771 genes for RT-qPCR analysis from sputum of cystic fibrosis patients. Mol Cell Probes
772 28:10-2.
773
774

FIGURE LEGENDS

Figure 1. ***P. aeruginosa*** **can stimulate the growth of** ***S. sanguinis*** **when grown in co-cultures**. (A and B) Growth kinetics of *S. sanguinis* SK36 (labeled SK36) and *P. aeruginosa* PAO1 (labeled PAO1) in a 96-well deep well plate either in coculture or as a monoculture. (C-D) *P. aeruginosa* cells or a 1:2 dilution of *P. aeruginosa* supernatant (super) enhances *S. sanguinis* growth in coculture on a plastic substratum (C) or CFBE monolayers (D). Error bars indicate standard deviation of the means from a representative triplicate assay. (**, $P < 0.01$, Student's *t*-test).

Figure 2. **Identification of** ***S. sanguinis*** **SK36 mutants that exhibits reduced growth enhancement mediated by** ***P. aeruginosa***. (A) Schematic diagram of the genome-wide screen of *S. sanguinis* SK36 mutant library. (B) Functional classification of screen hits using KEGG Orthology (KO) database shown here as the number of candidate mutants identified in each of the indicated pathways. (C) Genes required for zinc uptake identified in the screen and corresponding functions (https://www.genome.jp/dbget-bin/get_linkdb?-t+genes+gn:T00473).

Figure 3. Zinc is required for the ***P. aeruginosa*****-induced enhancement of** ***S. sanguinis*** **growth**. (A) Growth of wild-type *S. sanguinis* and zinc transporter mutants with *P. aeruginosa* (expressed as CFU/ml) in media with or without addition of zinc at the indicated concentration. Statistical significance was assessed by one-way ANOVA with a Turkey's multiple comparison, and different letters indicates statistically significant differences ($P < 0.05$). Identical letters indicate no significant difference. In this and all subsequent panels, the *SSA* designation indicates the wild-type gene, while the Ssx designation indicates a mutation in that gene, using the convention reported in the original description of these mutant strains (55). (B) Complementation assays with the zinc transporter mutant strains. Significant differences in growth compared to the uncomplemented vector control are indicated (*, $P < 0.05$; **, $P < 0.01$, Student's *t*-test). (C) Growth of indicated *S. sanguinis* strains with *P. aeruginosa* on CF airway cells in the presence or absence of 1 μM zinc. Statistical significance was determined

by one-way ANOVA with a Turkey's multiple comparison. Different letters indicates statistically significant differences ($P < 0.05$). Identical letters indicate no significant difference. (D) Growth of indicated *S. sanguinis* strains in monoculture on CF airway cells in the presence or absence of 1 μM zinc. Data are representative of three experiments performed in triplicate, and none of the differences are significant.

Figure 4. *S. sanguinis* zinc transporter genes are upregulated in the presence of *P. aeruginosa*. (A) Relative mRNA expression of *S. sanguinis* zinc transporter genes in monoculture and coculture with *P. aeruginosa* at 2 h and 6 h. The relative mRNA expression was measured using qRT-PCR, normalized to the expression of the *gyrA* control, and calculated using the $2^{-\Delta\Delta CT}$ method setting the value of SK36 (2 h) as one. (B) Relative expression of *S. sanguinis* zinc transporter genes at 6 h in monoculture and coculture with *P. aeruginosa* with or without zinc (1 μM) supplementation. Error bars represent deviations of the means. ANOVA with Turkey's multiple comparison test was used for statistical analysis (**, $P < 0.01$).

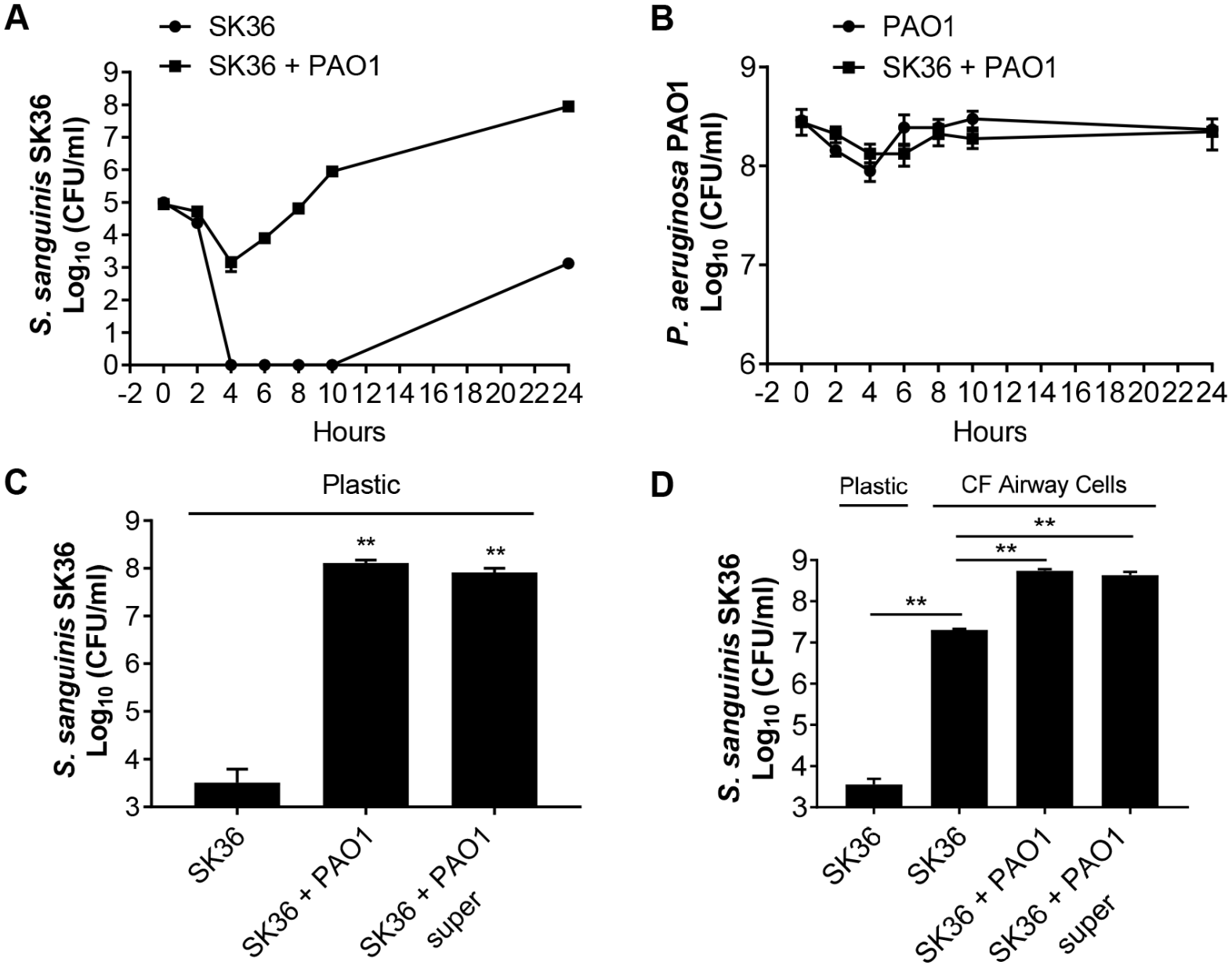
Figure 5. Analysis of zinc transporter mutants. (A-C) Growth of *S. sanguinis* SK36 with *P. aeruginosa* PAO1 wild type and indicated mutant strains in coculture. The strains tested carry deletion mutations in the *cntI* (A), *znuA* (B) and *cntI-O* (C) genes of *P. aeruginosa* PAO1. (D-E) Growth of *S. sanguinis* SK36 (D) and *P. aeruginosa* wild-type and ΔzurA mutants (E) in coculture assays in MEM (no added zinc) or with 10 μM of added zinc. ANOVA with Turkey's multiple comparison test was used for statistical analysis (**, $P < 0.01$) in panel D. There were no significant differences in panel E. SK36, *S. sanguinis* SK36 and PAO1, *P. aeruginosa* PAO1.

Figure 6. The relationship between sputum zinc concentration and the relative abundance of *Streptococcus* and *Pseudomonas*. The relative abundance of *Streptococcus* (orange dots, left Y axis) and *Pseudomonas* (blue dots, right Y axis) in each sputum sample (indicated as a fraction of 100%) is plotted versus the concentration of zinc in

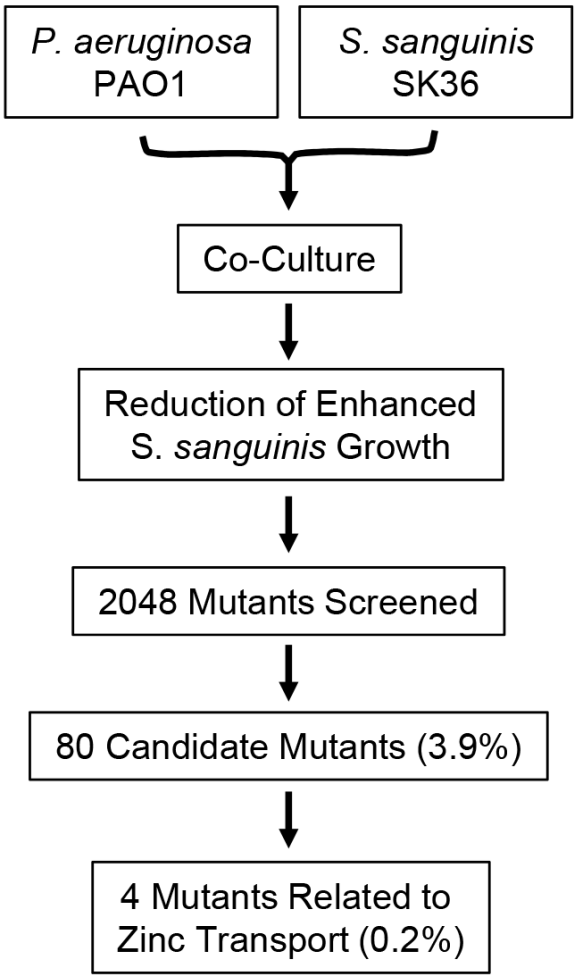
827 the corresponding sample (expressed as μM zinc on the X-axis). Total zinc was measured
828 by ICP-MS analysis of nitrate acid-dissolved samples and normalized to the volume of the
829 sample.

830 **Figure 7. Impact of zinc level on viability of *Streptococcus* in monoculture and**
831 **coculture with *Pseudomonas*.** Growth of the indicated streptococci (expressed as CFU/ml)
832 when grown in monoculture (A) or coculture with *P. aeruginosa* PAO1 (B) at the indicated
833 concentration of supplemented zinc chloride. The 0 addition has only the zinc present in the
834 medium (0.024 ± 0.007 μM). The other zinc additions were based on the data shown in Fig. S5
835 and indicate the lowest (4.7 μM), median (36.4 μM) and highest concentration (145 μM) of zinc
836 found in the 118 sputum samples analysed. Statistical significance was determined by
837 one-way ANOVA with a Turkey's multiple comparison. Different letters indicates statistically
838 significant differences ($P < 0.05$). Identical letters indicate no significant difference.

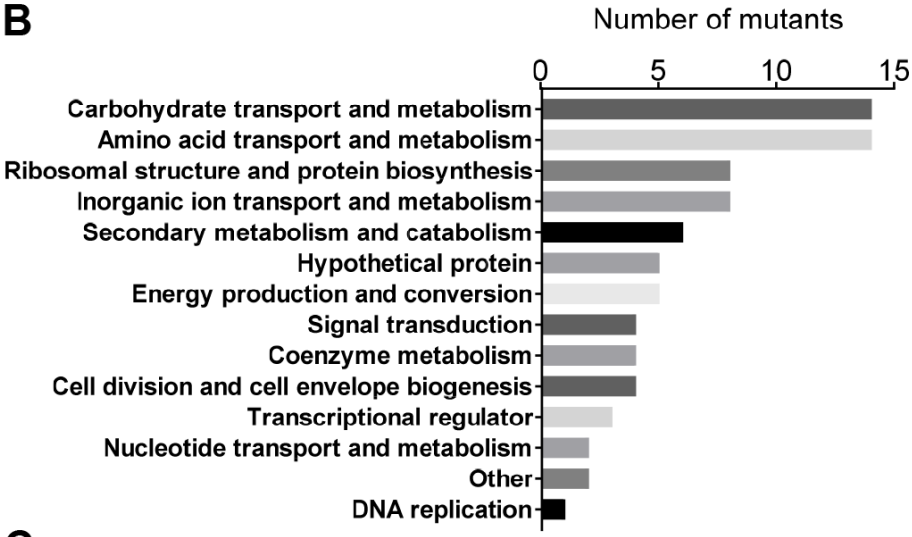
839


A
SK36
SK36 + PAO1
S. sanguinis SK36
Log10 (CFU/ml)
Hours
B
PAO1
SK36 + PAO1
P. aeruginosa PAO1
Log10 (CFU/ml)
Hours
C
Plastic
S. sanguinis SK36
Log10 (CFU/ml)
SK36
SK36 + PAO1
SK36 + PAO1 super
D
Plastic
CF Airway Cells
S. sanguinis SK36
Log10 (CFU/ml)
SK36
SK36
SK36 + PAO1
SK36 + PAO1 super

A

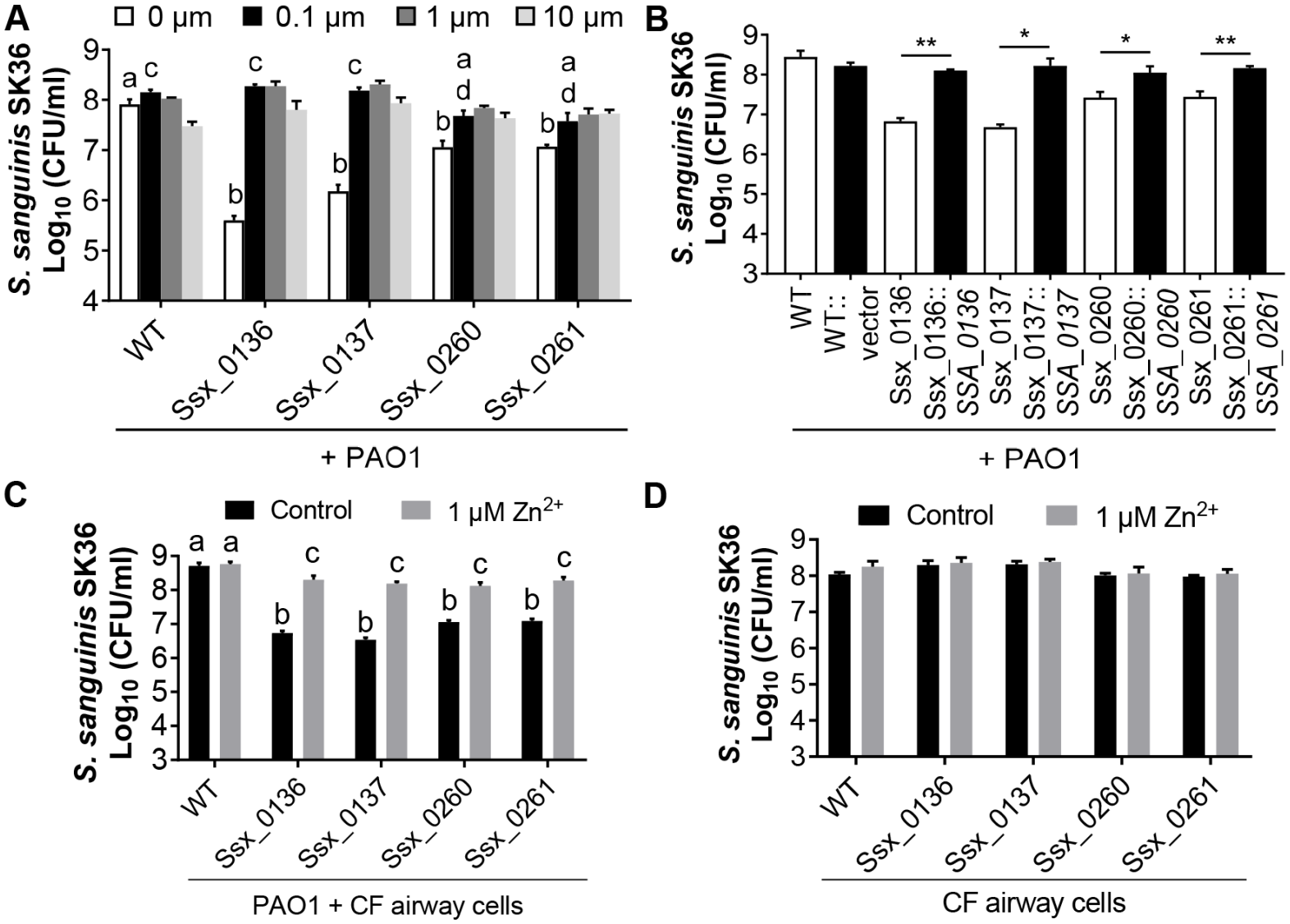


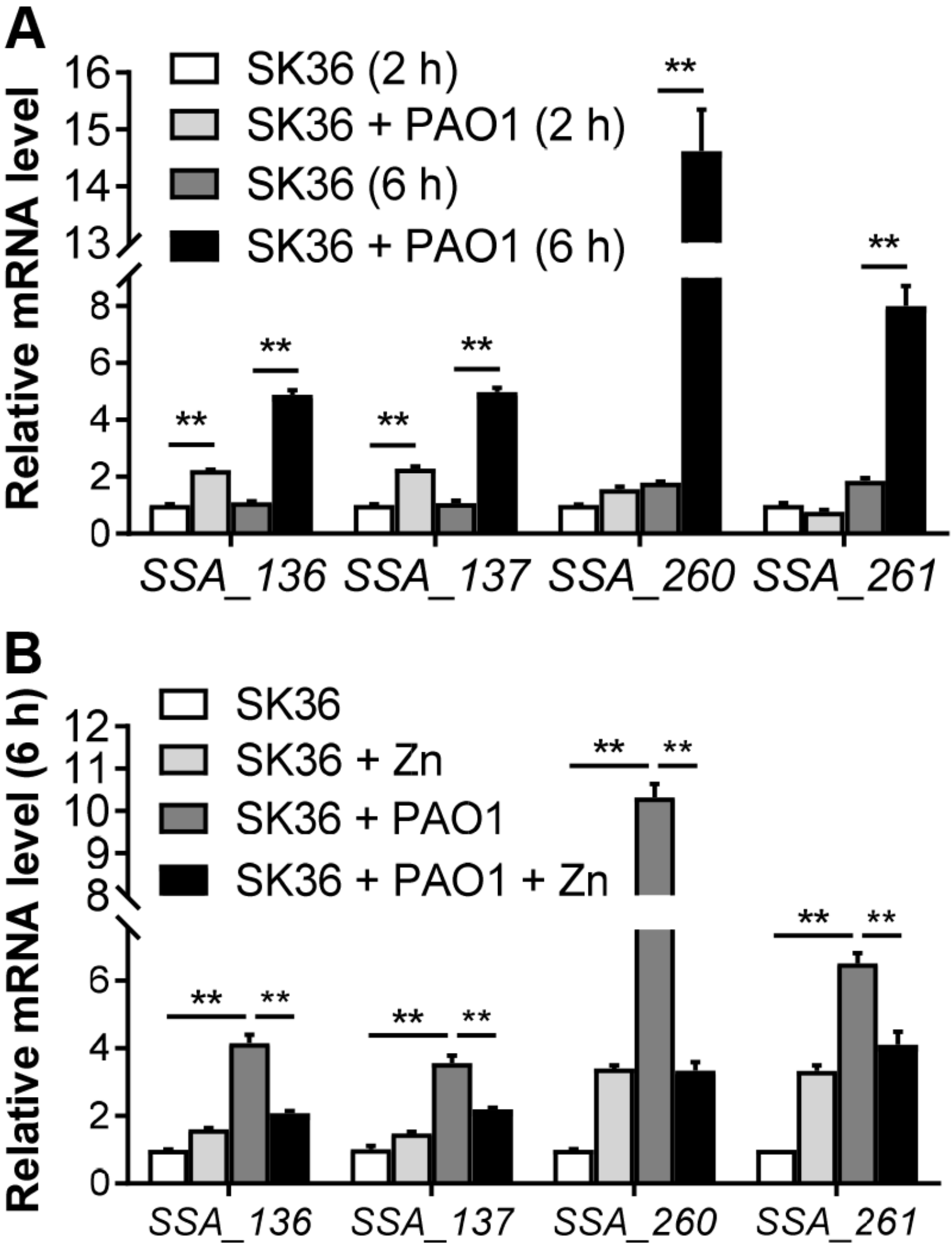
B

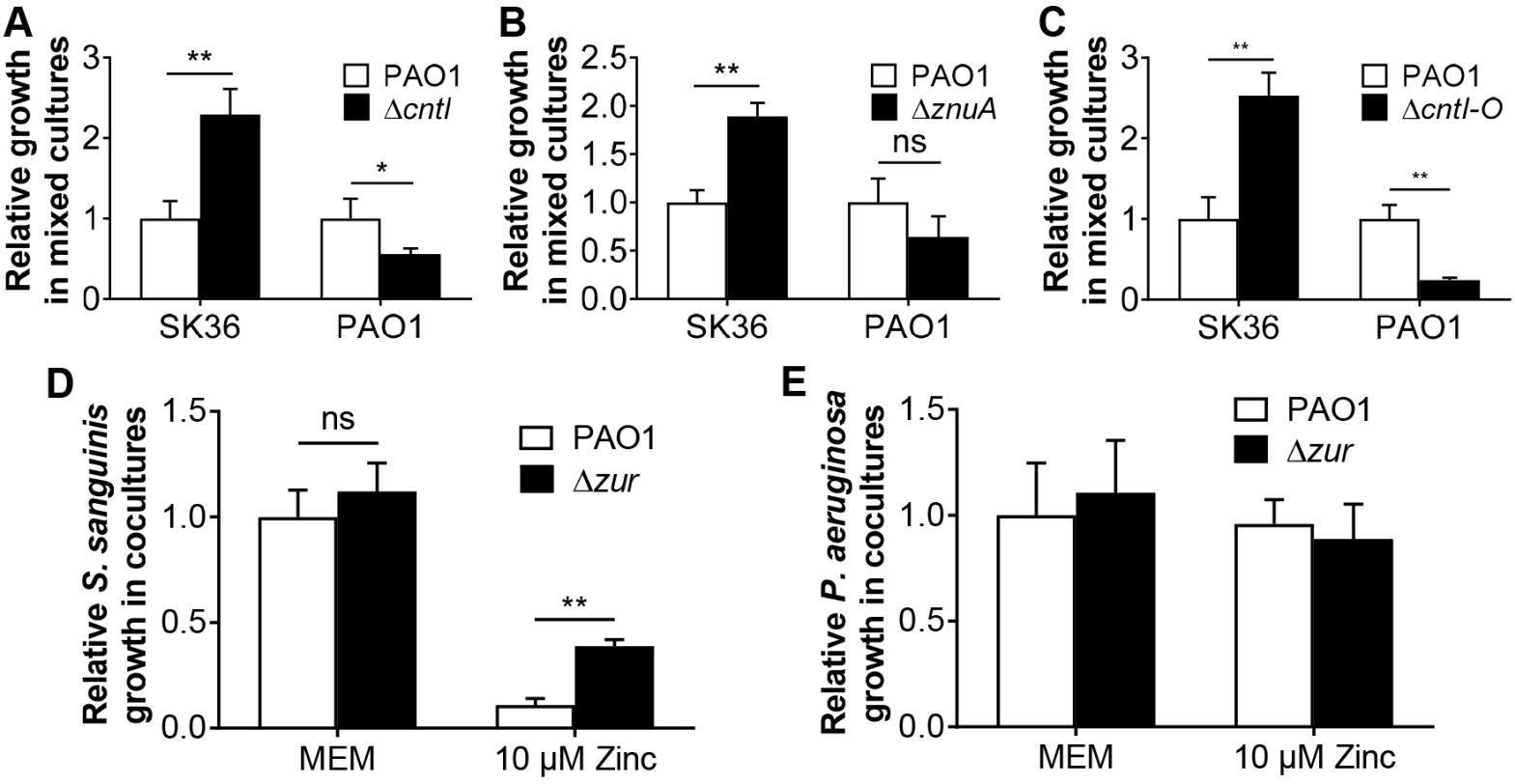


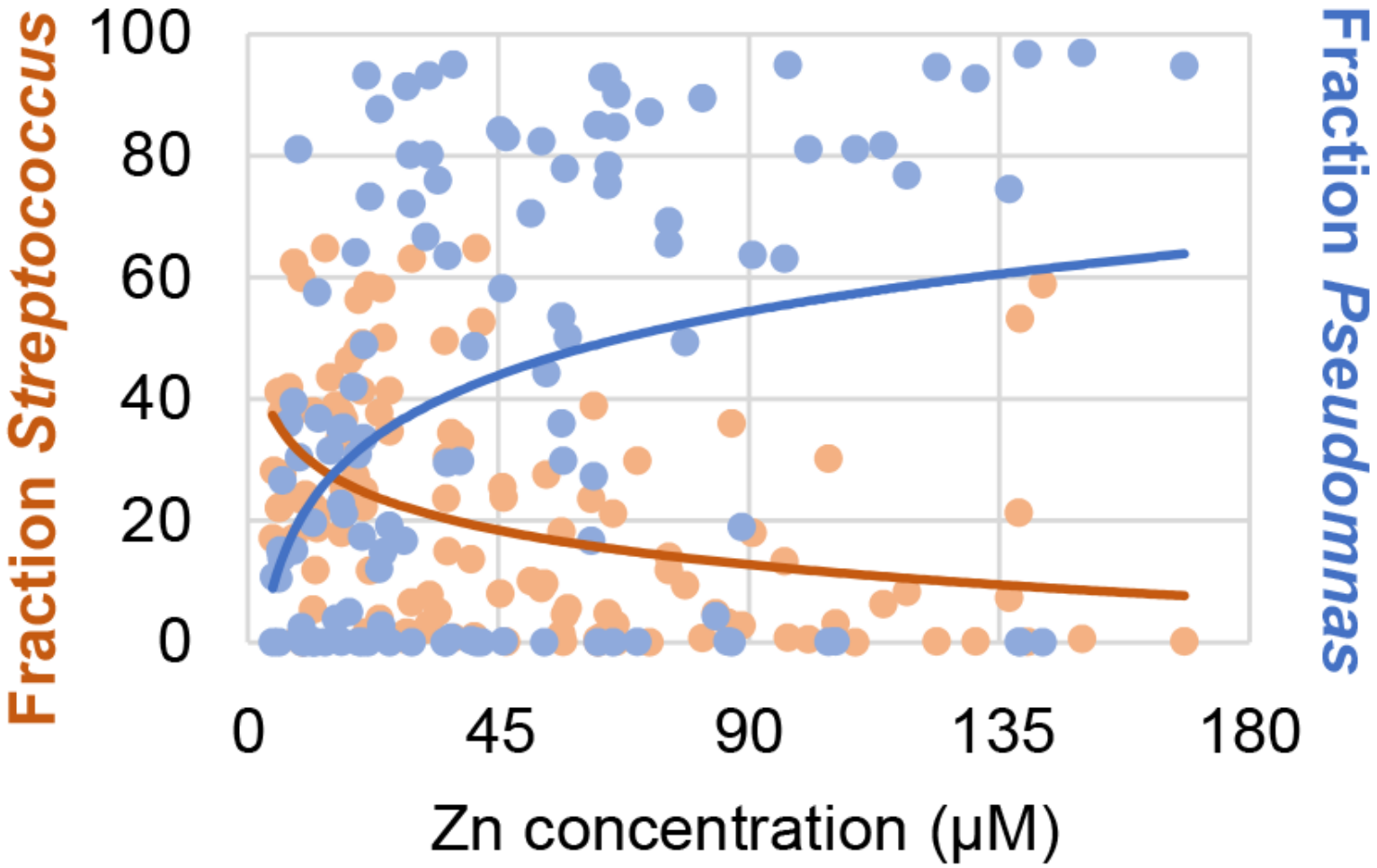
C

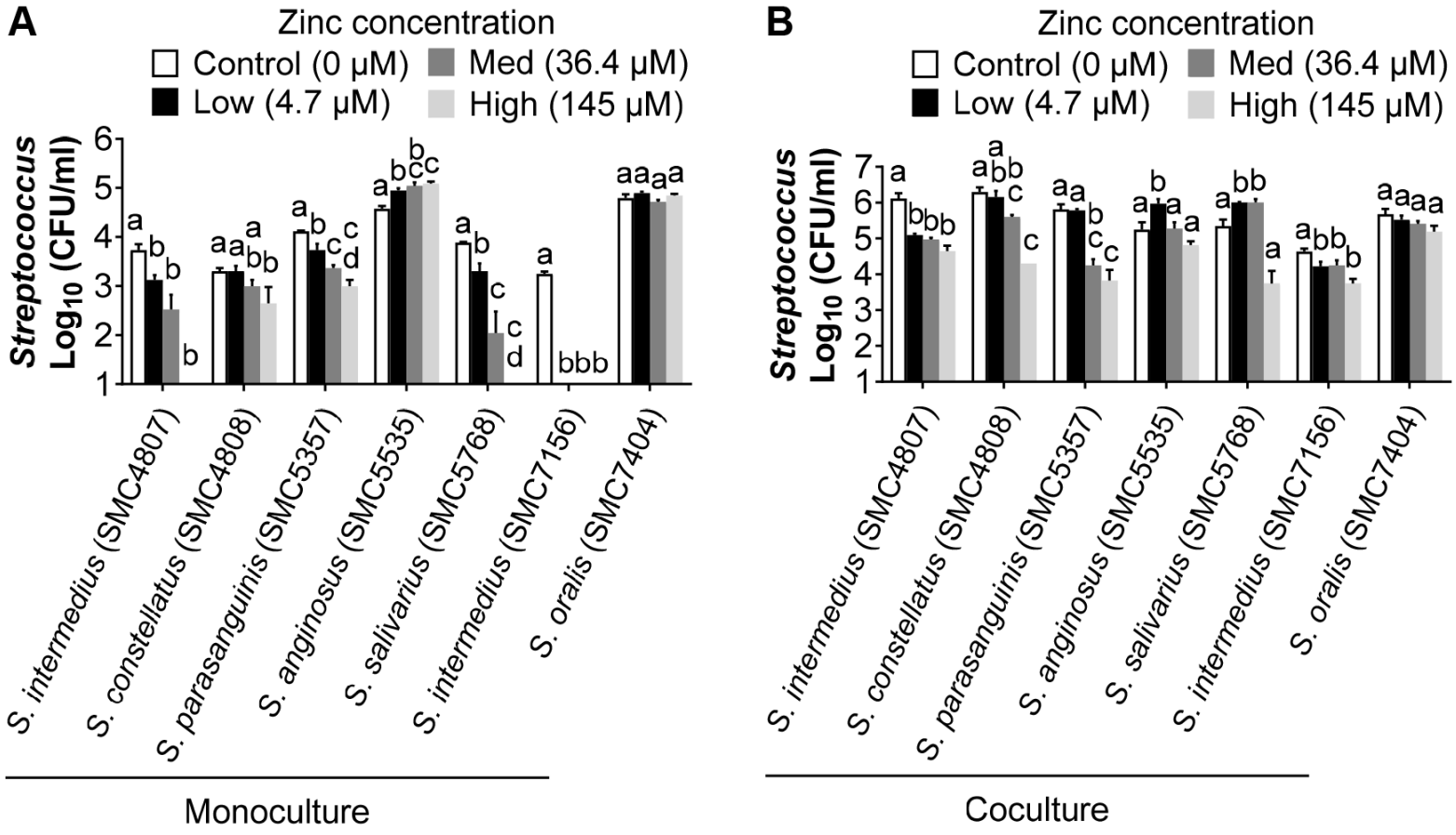
Gene #	Predicted Function
SSA_0136	ABC transporter, ATP-binding protein, Zn porter
SSA_0137	ABC transporter (permease), Zn porter
SSA_0260	manganese/Zinc ABC transporter substrate-binding protein
SSA_0261	ABC-type Mn^{2+}/Zn^{2+} transport systems, permease component


A
0 µm
0.1 µm
1 µm
10 µm
S. sanguinis SK36
Log10 (CFU/ml)
WT
Ssx_0136
Ssx_0137
Ssx_0260
Ssx_0261
+ PAO1
B
S. sanguinis SK36
Log10 (CFU/ml)
**
*
*
**
WT
WT::
vector
Ssx_0136
Ssx_0136::
SSA_0136
Ssx_0137
Ssx_0137::
SSA_0137
Ssx_0260
Ssx_0260::
SSA_0260
Ssx_0261
Ssx_0261::
SSA_0261
+ PAO1
C
Control
1 µM Zn2+
S. sanguinis SK36
Log10 (CFU/ml)
WT
Ssx_0136
Ssx_0137
Ssx_0260
Ssx_0261
PAO1 + CF airway cells
D
Control
1 µM Zn2+
S. sanguinis SK36
Log10 (CFU/ml)
WT
Ssx_0136
Ssx_0137
Ssx_0260
Ssx_0261
CF airway cells


A
Relative mRNA level
SK36 (2 h)
SK36 + PAO1 (2 h)
SK36 (6 h)
SK36 + PAO1 (6 h)
16
15
14
13
8
6
4
2
0
**
SSA_136
SSA_137
SSA_260
SSA_261
B
Relative mRNA level (6 h)
SK36
SK36 + Zn
SK36 + PAO1
SK36 + PAO1 + Zn
12
11
10
9
8
6
4
2
0
**
SSA_136
SSA_137
SSA_260
SSA_261


A
Relative growth in mixed cultures
PAO1
Δcntl
**
*
SK36
PAO1
B
Relative growth in mixed cultures
PAO1
ΔznuA
**
ns
SK36
PAO1
C
Relative growth in mixed cultures
PAO1
Δcntl-O
**
**
SK36
PAO1
D
Relative S. sanguinis growth in cocultures
PAO1
Δzur
ns
**
MEM
10 μM Zinc
E
Relative P. aeruginosa growth in cocultures
PAO1
Δzur
MEM
10 μM Zinc


Fraction Streptococcus
100
80
60
40
20
0
0
45
90
135
180
Zn concentration (μM)
Fraction Pseudomnas


A
Zinc concentration
Control (0 μM)
Med (36.4 μM)
Low (4.7 μM)
High (145 μM)
Streptococcus
Log10 (CFU/ml)
S. intermedius (SMC4807)
S. constellatus (SMC4808)
S. parasanguinis (SMC5357)
S. anginosus (SMC5535)
S. salivarius (SMC5768)
S. intermedius (SMC7156)
S. oralis (SMC7404)
Monoculture
B
Zinc concentration
Control (0 μM)
Med (36.4 μM)
Low (4.7 μM)
High (145 μM)
Streptococcus
Log10 (CFU/ml)
S. intermedius (SMC4807)
S. constellatus (SMC4808)
S. parasanguinis (SMC5357)
S. anginosus (SMC5535)
S. salivarius (SMC5768)
S. intermedius (SMC7156)
S. oralis (SMC7404)
Coculture