

# Oral Streptococci and Nitrite-Mediated Interference of *Pseudomonas aeruginosa*

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The oral cavity harbors a diverse community of microbes that are physiologically unique. Oral microbes that exist in this polymicrobial environment can be pathogenic or beneficial to the host. Numerous oral microbes contribute to the formation of dental caries and periodontitis; however, there is little understanding of the role these microbes play in systemic infections. There is mounting evidence that suggests that oral commensal streptococci are cocolonized with *Pseudomonas aeruginosa* during cystic fibrosis pulmonary infections and that the presence of these oral streptococci contributes to improved lung function. The goal of this study was to examine the underlying mechanism by which *Streptococcus parasanguinis* antagonizes pathogenic *P. aeruginosa*. In this study, we discovered that oral commensal streptococci, including *Streptococcus parasanguinis*, *Streptococcus sanguinis*, and *Streptococcus gordonii*, inhibit the growth of *P. aeruginosa* and that this inhibition is mediated by the presence of nitrite and the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by oral streptococci. The requirement of both H<sub>2</sub>O<sub>2</sub> and nitrite for the inhibition of *P. aeruginosa* is due to the generation of reactive nitrogenous intermediates (RNI), including peroxynitrite. Transposon mutagenesis showed that a *P. aeruginosa* mutant defective in a putative ABC transporter permease is resistant to both streptococcus/nitrite- and peroxynitrite-mediated killing. Furthermore, *S. parasanguinis* protects *Drosophila melanogaster* from killing by *P. aeruginosa* in a nitrite-dependent manner. Our findings suggest that the combination of nitrite and H<sub>2</sub>O<sub>2</sub> may represent a unique anti-infection strategy by oral streptococci during polymicrobial infections.

The human oral cavity provides residence to more than 700 diverse bacterial species (1). A fraction of the microbial communities that colonize the oral cavity are responsible for localized pathogenic infections, such as dental caries and aggressive periodontitis (1, 2). In contrast, other oral microbes are classified as commensal bacteria (*Streptococcus parasanguinis*, *Streptococcus sanguinis*, *Streptococcus gordonii*, and *Streptococcus salivarius*) and are involved in facilitating the attachment of pathogenic bacteria to the tooth surface (3) and modulating bacterial dysbiosis (4). Microorganisms unique to the oral cavity have been considered to be mainly involved in localized oral infections; however, there is increasing evidence that suggests that oral microbes may play a more prominent role in systemic infections (5). Oral pathogens and commensals have the ability to disseminate through the bloodstream and cause infective endocarditis (6). Moreover, periodontal pathogens have been associated with cases of atherosclerosis and respiratory tract infections (5, 7).

Current evidence suggests that some oral streptococci reside in locations of the body that are distant to the oral cavity, including the respiratory tracts of cystic fibrosis (CF) patients (8). Historically, *Pseudomonas aeruginosa* was considered to be the most predominant and clinically important pathogen in CF patients (9, 10). The development of a chronic *P. aeruginosa* infection in the lungs of CF patients typically correlated with deteriorated lung function and mortality in these patients (11). There are several reasons why *P. aeruginosa* has become an incredibly successful pathogen in the lungs of CF patients. First, *P. aeruginosa* is equipped with an assortment of both cell-associated and extracellular virulence factors (12). Second, this bacterium has the ability to alter or redirect central metabolic activities or virulence mechanisms to adapt to stressful environments or acquire nutrients (10, 13–16) and is intrinsically resistant to many antimicrobials (17). All of these adaptation strategies enable *P. aeruginosa* to establish persistent infections in the lungs of CF patients and thus

render these infections difficult to treat. Interestingly, the oral commensal streptococci *S. parasanguinis* and *S. salivarius* have been found to be abundant in some CF patients and, remarkably, were associated with increased lung stability (8).

Previous reports have demonstrated that in the oral cavity, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production by oral commensal streptococci has an antagonistic effect on the pathogen *Streptococcus mutans* (18). Since the occurrence of oral streptococci in the lungs of CF patients has been linked to improved lung function, it is possible that the lungs of CF patients provide a unique environmental condition that allows oral streptococci to compete with *P. aeruginosa* in an H<sub>2</sub>O<sub>2</sub>-dependent manner. We aimed to investigate the underlying mechanism that enables oral streptococci to outcompete pathogenic *P. aeruginosa* under given circumstances, which may explain how the existence of oral streptococci impacts the progression of lung disease in CF patients.

We report that oral streptococci inhibit *P. aeruginosa* in an H<sub>2</sub>O<sub>2</sub>- and nitrite-dependent manner via the production of a reactive nitrogenous intermediate (RNI) such as peroxynitrite. In

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addition, *S. parasanguinis*/nitrite-mediated activity protects the host from killing by *P. aeruginosa* in the *Drosophila melanogaster* model of infection. Furthermore, transposon mutagenesis showed that a *P. aeruginosa* mutant defective in a putative ABC transporter permease is resistant to streptococcus/nitrite- and peroxy-nitrite-mediated killing. These data indicate that RNI production by the presence of oral streptococci and nitrite is the main antibacterial mechanism which could potentially represent an effective strategy utilized by oral commensal streptococci to inhibit the colonization or survival of *P. aeruginosa* in the lungs of CF patients.

## MATERIALS AND METHODS

**Bacterial strains, culture conditions, and reagents.** The bacteria used in this study were *S. parasanguinis* (FW213), *S. sanguinis* (SK36), *S. gordonii* (DL1), *S. mutans* (UA159), *S. salivarius* (K12), *P. aeruginosa* (PAO1 and FRD1), and *Escherichia coli* (TOP10). *P. aeruginosa* was isolated on *Pseudomonas* isolation agar (PIA) and subsequently cultured in Luria broth (LB) and incubated at 37°C. *E. coli* cells were also cultured in LB and incubated at 37°C. All oral streptococci were routinely cultured in Todd-Hewitt broth (THB) and incubated at 37°C with 5% CO<sub>2</sub>. Filtered-sterilized sodium nitrite was used at a concentration of 0.5 or 1.0 mM. Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and used at the following concentrations: 100 µg of ampicillin ml<sup>-1</sup> for *E. coli*; 125 µg of kanamycin ml<sup>-1</sup> for *S. parasanguinis*; 40 and 10 µg of erythromycin ml<sup>-1</sup> for *E. coli* and *S. parasanguinis*, respectively; and 180 µg of gentamicin ml<sup>-1</sup> and 100 µg of carbenicillin ml<sup>-1</sup> for *P. aeruginosa*. Peroxynitrite was purchased from Cayman Chemical Company (Ann Arbor, MI).

**Construction of the *S. parasanguinis* *poxL* mutant.** To generate *poxL* mutants of *S. parasanguinis*, a 2.564-kb fragment containing 500 bp upstream and 500 bp downstream of the *poxL* coding sequence was PCR amplified from *S. parasanguinis* cells and ligated into the pGEM-T Easy vector. An 879-bp internal fragment of the *poxL* coding region was removed using inverse PCR and replaced with the *aphA3* gene encoding kanamycin resistance as an XbaI fragment. The resulting plasmid was introduced into *S. parasanguinis* via electroporation, and potential *poxL* mutants were isolated as kanamycin-resistant transformants. Replacement of the wild-type *poxL* gene with the *poxL::aphA3* allele was verified by PCR analysis.

**Construction of the *poxL*-complemented strains.** To complement the *poxL* mutation, the wild-type gene was PCR amplified from *S. parasanguinis* cells. The resulting fragment was cloned into the Sall and KpnI sites of the streptococcus and *E. coli* shuttle vector pVPT-gfp. This plasmid was transformed into the *poxL* mutant. In *cis* complementation was verified by PCR analysis.

**Competition assays on solid medium and in liquid medium.** To examine the interactions between the oral streptococcal species and *P. aeruginosa*, a 10-µl subculture culture of each streptococcal species was inoculated onto a Todd-Hewitt agar (THA) plate (supplemented with 1 mM filtered-sterilized nitrite) as the early colonizer. After incubation overnight at 37°C with 5% CO<sub>2</sub>, 10 µl of subcultured FRD1 (chronic *P. aeruginosa* cystic fibrosis isolate) or PAO1 (acute-phase *P. aeruginosa* isolate) was inoculated next to the streptococci as the late colonizer. The plate was incubated overnight at 37°C with 5% CO<sub>2</sub>. Growth inhibition of *P. aeruginosa* was assessed by the presence of a proximal zone of inhibition at the intersection with the early colonizer.

For competition assays in liquid media, all streptococcal species were grown in THB overnight and 5 µl of cells was subcultured in a Costar 96-well microtiter plate (Corning, Inc., Corning, NY) containing 200 µl of fresh THB (with or without 1 mM nitrite) to an optical density at 470 nm (OD<sub>470</sub>) of 0.1, followed by the addition of 5 µl of a *P. aeruginosa* subculture (OD<sub>600</sub> = 0.2 to 0.3). The cells were incubated overnight at 37°C in the presence of 5% CO<sub>2</sub>. *P. aeruginosa* and oral streptococcal cells

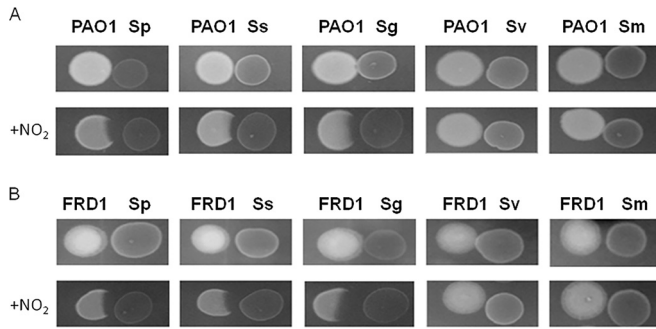
were dispersed by vigorous pipetting, serially diluted, and plated on PIA or THA in duplicate; CFU counts were determined the next day.

**Hydrogen peroxide and peroxy-nitrite quantification and peroxy-nitrite sensitivity assay.** The production of H<sub>2</sub>O<sub>2</sub> by *S. parasanguinis* was measured using the Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen, Carlsbad, CA). Briefly, exponential-phase cultures grown in THB (supplemented with sodium nitrite where indicated below) were pelleted and the supernatant was filtered sterilized prior to quantification of H<sub>2</sub>O<sub>2</sub>. To measure peroxy-nitrite, exponential-phase *S. parasanguinis* cells grown in THB were pelleted and resuspended in M9 medium. Five microliters of the cell suspension was added to 200 µl of M9 medium supplemented with 1 mM sucrose with or without 1 mM nitrite, followed by the addition of 100 µM 2',7'-dichlorodihydrofluorescein diacetate (Sigma-Aldrich) and incubation at 37°C. Fluorescence intensity exhibited by peroxy-nitrite was measured using a BioTek plate reader. To test the effect of peroxy-nitrite on cell viability, bacterial cultures were grown to an OD<sub>600</sub> of 0.4, exposed to 250 µM peroxy-nitrite for 20 min, and plated on PIA to calculate CFU.

**Transposon mutagenesis of *P. aeruginosa*.** A transposon insertion library was constructed in the *P. aeruginosa* chronic strain FRD1. FRD1 was mutagenized using a Tngfp transposon carried on plasmid pAG408 (19), which was introduced into *P. aeruginosa* via triparental mating. The conjugation mixtures were plated onto PIA containing gentamicin to select for *P. aeruginosa* containing transposon insertions. Transposon mutants were isolated as gentamicin-resistant, carbenicillin-sensitive colonies and subsequently pooled and screened for resistance to killing by oral streptococci.

**Construction of the *P. aeruginosa* PA3252 mutant and complemented strains.** To generate mutants of PA3252, a DNA sequence containing approximately 500 bp upstream and 500 bp downstream of the PA3252 coding sequence was PCR amplified from FRD1 cells and cloned into the SmaI site of pBluescript K(+). An internal 800-bp fragment of the PA3252 coding sequence was removed and replaced with the *aacC1* gene encoding gentamicin resistance as a SmaI fragment (20). This was followed by introduction of an origin of transfer (*moriT*) of RP4 on a 230-bp HindIII fragment (21). The resulting plasmid was introduced into *P. aeruginosa* strain FRD1 by triparental mating, and potential PA3252 mutants were isolated as gentamicin-resistant, carbenicillin-sensitive colonies, indicating a double-crossover event. Replacement of the wild-type PA3252 gene with the PA3252::*aacC1* allele was verified by PCR analysis. To complement the PA3252 mutant, full-length PA3252 was PCR purified from FRD1 cells and cloned into the SmaI site of pBluescript K(+). The resulting plasmid was converted to a mobilizable plasmid via the addition of a *moriT* in the HindIII site and then introduced into *P. aeruginosa* through triparental mating.

***S. parasanguinis* and *P. aeruginosa* oral infection of *Drosophila melanogaster*.** *Drosophila melanogaster* flies were maintained on Jazz-Mix *Drosophila* food (Fisher, Pittsburgh, PA). To orally infect *Drosophila*, wild-type *S. parasanguinis* and the *poxL* mutant were grown to an OD<sub>470</sub> of 2.0 and 1.5 ml of the culture was centrifuged at 5,000 × *g* for 10 min to pellet cells. The bacterial pellet was resuspended in 100 µl of sterile 5% sucrose (with or without 0.5 mM nitrite). The resuspended cells were spotted onto a sterile 21-mm filter (Whatman) that was placed on the surface of 5 ml of solidified 5% sucrose agar in a plastic vial (FlyBase). The filters were allowed to dry at room temperature for ~30 min before addition of *Drosophila*. To ensure maximum feeding on the discs containing bacteria, male Canton S flies (1 to 3 days old) were starved for 3 h before being added to vials (10 flies per vial). Flies were allowed to feed on wild-type *S. parasanguinis* and the *poxL* mutant 24 h prior to being infected with *P. aeruginosa* strain PAO1, which was prepared in the same manner as described above. Flies were anesthetized using CO<sub>2</sub> throughout the sorting and transferring process. The number of live flies to start the experiment was documented, and live flies were counted at 24-h intervals.



**FIG 1** Nitrite facilitates the inhibition of *P. aeruginosa* by some oral streptococci. Shown is a competition assay of the oral streptococci *S. parasanguinis* (Sp), *S. sanguinis* (Ss), *S. gordonii* (Sg), *S. salivarius* (Sv), and *S. mutans* (Sm) with *P. aeruginosa* on Todd-Hewitt agar (THA) with or without 1 mM nitrite. (A) *P. aeruginosa* acute strain PAO1; (B) *P. aeruginosa* chronic strain FRD1.

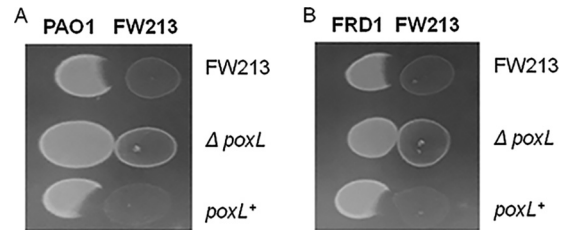
## RESULTS

**Nitrite is required for the killing of *P. aeruginosa* by oral streptococci.** To examine the competitive interactions that exist between oral streptococci and *P. aeruginosa*, we employed a variety of oral streptococci, including *S. parasanguinis* FW213, *S. sanguinis* SK36, *S. gordonii* DL1, *S. mutans* UA159, and *S. salivarius* K12, and two *P. aeruginosa* strains, an acute-phase (PAO1) and chronic (FRD1) isolate. We first evaluated the relationship between these two genera using a plate inhibition assay. As shown in Fig. 1, none of the oral streptococci had an effect on either PAO1 or FRD1 growth when plated on THA. Interestingly, when THA was supplemented with 1 mM nitrite, oral streptococci, with the exception of *S. salivarius* and *S. mutans*, displayed an inhibitory effect on bacterial growth of *P. aeruginosa* PAO1 and FRD1 (Fig. 1). This inhibition occurred when *S. parasanguinis* (or other streptococci) was placed as the early colonizer. THA supplemented with other chemical agents such as nitrate, arginine, or citrate did not support the inhibition of *P. aeruginosa* by *S. parasanguinis* (see Fig. SA1 in the supplemental material); therefore, the inhibition displayed in the plate assay was nitrite specific.

**H<sub>2</sub>O<sub>2</sub> mediates the killing of *P. aeruginosa* by *S. parasanguinis* in the presence of nitrite.** *S. salivarius* and *S. mutans* had no effect on the killing of *P. aeruginosa* and are also non-H<sub>2</sub>O<sub>2</sub>-producing streptococci; therefore, we hypothesized that production of H<sub>2</sub>O<sub>2</sub> is important for the killing of *P. aeruginosa*. To test this hypothesis, we constructed mutants that are defective in production of H<sub>2</sub>O<sub>2</sub> and tested the mutants' ability to kill *P. aeruginosa*. Pyruvate oxidase is required for the optimal production of H<sub>2</sub>O<sub>2</sub> and is a key virulence determinant in oral streptococci (22).

*S. parasanguinis* mutants defective in *poxL*, the gene that encodes pyruvate oxidase, lost the ability to produce H<sub>2</sub>O<sub>2</sub> and concurrently failed to inhibit PAO1 and FRD1 in the presence of nitrite (Fig. 2). Complementation of *poxL* restored the inhibitory activity (Fig. 2). These data demonstrate that H<sub>2</sub>O<sub>2</sub> production by oral streptococci is required for inhibition of *P. aeruginosa* in the presence of nitrite.

To obtain a quantitative measurement of the effects of oral streptococci on *P. aeruginosa*, we carried out coculture assays in the broth and then determined the CFU counts of both PAO1 and FRD1 obtained from coinfection with oral streptococci in the presence and absence of nitrite. There was an ~3-log decrease in PAO1 cells cocultured with *S. parasanguinis* in THB that con-



**FIG 2** Hydrogen peroxide production is required by *S. parasanguinis* strain FW213 to inhibit *P. aeruginosa* in the presence of 1 mM nitrite. (A) Competition assay between PAO1 and *S. parasanguinis* FW213 (wild-type,  $\Delta$ *poxL* mutant, and *poxL*-complemented strains). (B) Competition assay between FRD1 and *S. parasanguinis* FW213 (wild-type,  $\Delta$ *poxL* mutant, and *poxL*-complemented strains).

tained 1 mM nitrite (see Fig. SA2 in the supplemental material). Coculture of PAO1 with the *S. parasanguinis*  $\Delta$ *poxL* mutant resulted in no inhibition of PAO1 in the presence of nitrite. Interestingly, coculture of PAO1 with the complemented *S. parasanguinis*  $\Delta$ *poxL* mutant resulted in no detectable PAO1. In addition, there was an ~4-log decrease in PAO1 survival during coculture with *S. sanguinis* and *S. gordonii*. Furthermore, *S. mutans* and *S. salivarius* had no effect on PAO1 growth in cultures that contained nitrite (see Fig. SA2). There was a significant decrease in FRD1 survival in cocultures with wild-type *S. parasanguinis*, the *S. parasanguinis* *poxL*-complemented strain, *S. sanguinis*, or *S. gordonii* that contained 1 mM nitrite. However, there was an increase in survival of FRD1 during coculture with the *S. parasanguinis*  $\Delta$ *poxL* mutant (see Fig. SA3 in the supplemental material). These results are consistent with the data obtained from the plate inhibition assays, which further supports the conclusion that H<sub>2</sub>O<sub>2</sub>-producing streptococci have an inhibitory effect on *P. aeruginosa* in the presence of nitrite.

**Nitrite enhances peroxynitrite production but not H<sub>2</sub>O<sub>2</sub> in *S. parasanguinis* cultures.** H<sub>2</sub>O<sub>2</sub> has been shown to have antimicrobial activity at certain concentrations. We questioned whether nitrite enhanced the production of H<sub>2</sub>O<sub>2</sub> by oral streptococci, thereby promoting killing. To determine if 1 mM nitrite had an effect on H<sub>2</sub>O<sub>2</sub> production by oral streptococci, we measured H<sub>2</sub>O<sub>2</sub> production in exponential-phase cultures of *S. parasanguinis* and derivatives. The addition of nitrite had no significant effect on H<sub>2</sub>O<sub>2</sub> production by oral streptococci (Fig. 3A); as a result, we hypothesized that the combination of H<sub>2</sub>O<sub>2</sub> and nitrite generates a reactive nitrogenous intermediate (RNI), such as peroxynitrite. To test this theory, we measured peroxynitrite production by *S. parasanguinis* grown in the presence of nitrite. As shown in Fig. 3B, more peroxynitrite was detected by a fluorescent probe in *S. parasanguinis* cultures grown in nitrite than in cultures that contained no nitrite. In contrast, peroxynitrite generation was abolished in *S. parasanguinis* cultures defective for H<sub>2</sub>O<sub>2</sub> production. These data suggest that in some polymicrobial infections in which nitrite is present, oral streptococci promote the generation of an RNI, which has an antagonistic effect on neighboring bacteria.

**A PA3252 FRD1 mutant is more resistant to *S. parasanguinis*/nitrite and peroxynitrite inhibition than wild-type FRD1.** Given the fact that nitrite increased peroxynitrite production in *S. parasanguinis* cultures, we hypothesized that peroxynitrite may be an intermediate responsible for *P. aeruginosa* inhibition.



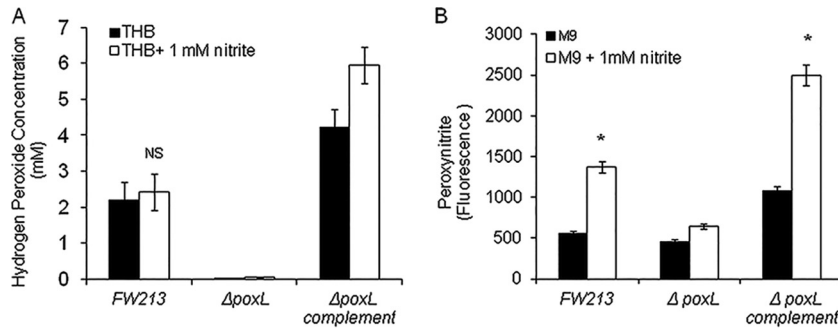


FIG 3 Nitrite does not increase hydrogen peroxide production by *S. parasanguinis* FW213 but induces the generation of peroxynitrite in FW213. (A) H<sub>2</sub>O<sub>2</sub> was measured in exponential-phase total cultures grown in THB with or without 1 mM nitrite. (B) Peroxynitrite was measured in exponential-phase cultures grown in M9 medium supplemented with 1 mM sucrose, with or without 1 mM nitrite. NS, not significant. \*, *P* < 0.05 (Student *t* test).

Therefore, we sought to determine the mechanism of action of peroxynitrite-mediated killing by screening the *P. aeruginosa* genome for mutants that confer resistance to killing by streptococcus/nitrite- and pure peroxynitrite-mediated activity. To test this, we mutagenized the FRD1 genome with a transposon and screened for mutants that were resistant to killing by *S. parasanguinis*. One resistant transposon mutant was isolated and mapped to PA3252, which encodes a probable permease of an ABC transporter. Next, we reconstructed a mutation in PA3252 in FRD1 to determine if a defect in PA3252 truly plays a role in resistance to both *S. parasanguinis*- and pure peroxynitrite-mediated killing. Indeed, the mutation in PA3252 in FRD1 background was more resistant to *S. parasanguinis*/nitrite-mediated (Fig. 4A) and pure peroxynitrite-mediated (Fig. 4B) inhibition than wild-type FRD1. In addition, complementation of PA3252 partially restored activity in FRD1 (Fig. 4).

**Primary infection with *S. parasanguinis* provides protection from *P. aeruginosa* in the *Drosophila melanogaster* model of infection.** *P. aeruginosa* displays significant inhibition when exposed to an established oral streptococcus culture in the presence of nitrite. To determine if this is the case *in vivo*, we utilized an *in vivo Drosophila* model, as it has been extensively used in bacterium-host interaction studies and proved to be relevant to human infection (23). We infected *Drosophila* with wild-type *S. parasanguinis* and the *poxL* mutant 24 h prior to coinfection with *P. aeruginosa* strain PAO1 (0.5 mM nitrite was added in some cases). As shown in Fig. 5A, neither wild-type *S. parasanguinis* nor the

*poxL* mutant had an effect on *P. aeruginosa* killing of *Drosophila* in the absence of nitrite. In contrast, when nitrite was administered, 80% of the *Drosophila* flies survived coinfection with wild-type *S. parasanguinis* and *P. aeruginosa*, compared to a 0% survival rate after 8 days of infection with *P. aeruginosa* alone (Fig. 5B). Furthermore, *Drosophila* coinfecting with PAO1 and *S. parasanguinis* defective for H<sub>2</sub>O<sub>2</sub> production had a reduction in survival rates compared to flies coinfecting with PAO1 and wild-type *S. parasanguinis*, again suggesting that both nitrite and H<sub>2</sub>O<sub>2</sub> are critical for the protection of *Drosophila* in this *in vivo* model of infection.

**DISCUSSION**

Historically, the manifestation of a chronic *P. aeruginosa* infection in the lungs of CF patients correlated with lung deterioration and increased mortality in these patients (11). Recent studies reveal that *P. aeruginosa* and oral streptococci are two types of organisms that cocolonize the polymicrobial environment constituted by the lungs of CF patients, and the presence of oral streptococci, including *S. parasanguinis*, offers beneficial outcomes to these patients (8). However, the underlying mechanism by which oral streptococci provide this health benefit is unknown. In this work, we report that oral streptococcal strains that produce H<sub>2</sub>O<sub>2</sub> inhibit *P. aeruginosa* in a nitrite-dependent manner. More importantly, the combination of hydrogen peroxide-producing oral streptococci and nitrite was required to protect *Drosophila melanogaster* from killing by *P. aeruginosa*. These studies suggest a unique anti-infective mechanism by oral streptococci. *P. aerugi-*

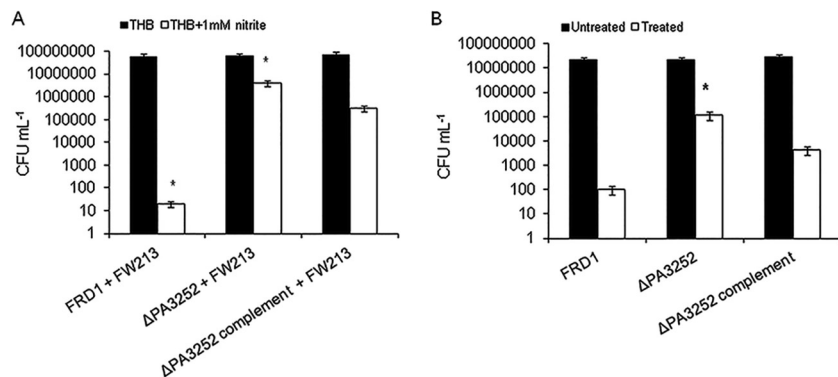
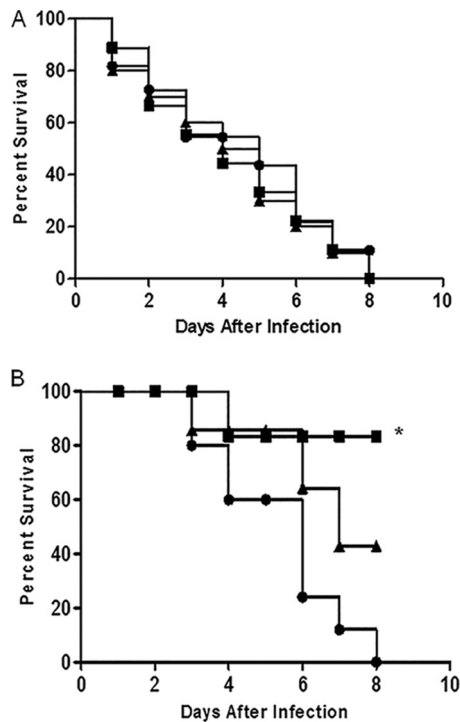


FIG 4 PA3252 is resistant to FW213 and peroxynitrite killing in the chronic isolate FRD1. (A) PA3252 mutant and FW213; (B) PA3252 resistance to peroxynitrite. Bacteria were exposed to 250 μM peroxynitrite for 20 min. \*, *P* < 0.05 (Student *t* test).



**FIG 5** *S. parasanguinis* and nitrite decrease *P. aeruginosa* virulence in a *Drosophila* model of infection. *Drosophila* flies were infected with *S. parasanguinis* FW213 wild-type and  $\Delta$ poxL mutant strains 24 h prior to *P. aeruginosa* PAO1 infection without (A) and with (B) supplementation with 0.5 mM nitrite. Curves represent 4 biological replicates.  $n = 40$ . \*,  $P < 0.05$  (log rank test). Circles, PAO1; squares, PAO1 plus wild-type FW213; triangles, PAO1 plus the  $\Delta$ poxL mutant.

*nosa* is well known for the ability to resist antibiotics and persist during chronic infections, and our findings suggest that there may be alternative methods to combat *P. aeruginosa* through the use of oral commensal bacteria.

Elevated levels of nitrite have been observed in the sputum of CF patients (24, 25); therefore, exploiting the abundance of nitrite during CF may be beneficial in clearing recalcitrant pathogens that colonize the lung. The average concentrations of nitrite in sputum have been reported to be between 400  $\mu$ M and 2 mM (24, 25). Although the prevalence of oral streptococci in CF patients is poorly understood, the ability of oral streptococci to exist in the lungs of these patients and utilize nitrite sources during chronic infection could potentially interfere with *P. aeruginosa* colonization or virulence. In support of our findings, *S. sanguinis* isolated from sputum has been shown to exhibit bactericidal activity against 19 nonmucoid and mucoid *P. aeruginosa* isolates recovered from patients with pulmonary disease, although the bactericidal agent was unknown (26). Other studies have shown that acidified nitrite inhibits *P. aeruginosa* cells grown under anaerobic conditions (27, 28). However, those particular studies used a very high concentration of nitrite (up to 15 mM; pH 6.5) to demonstrate killing of *P. aeruginosa*, which may not have physiological relevance in CF cases. In addition, *S. mutans* and *S. salivarius* produce copious amounts of lactic acid, which has been shown to permeate the outer membranes of Gram-negative cells (29, 30); however, neither of these acid-producing streptococci was able to significantly inhibit *P. aeruginosa*

in the presence of nitrite in our study, suggesting that acidified nitrite was not the underlying killing mechanism in our study. Furthermore, the benefits conferred by production of  $H_2O_2$  and the presence of nitrite may occur in the lung airways of CF patients (24). The requirement of nitrite and hydrogen peroxide for the killing suggests that combinatorial therapy may be effective in treating not only *P. aeruginosa* infections but also other recalcitrant infections.

The mechanism involved in *P. aeruginosa* inhibition by oral streptococci is not fully understood. In this study, a *P. aeruginosa* transposon mutant mapped to a permease of an ABC transporter (PA3252) and was resistant to the killing mediated by both *S. parasanguinis*/nitrite and pure peroxyntirite. Peroxyntirite has previously been shown to exhibit antibacterial activity by diffusing across bacterial cell membranes and inducing the oxidation or nitration of DNA, lipids, and proteins or promoting membrane damage (31, 32). Interestingly, Basic Local Alignment Search Tool (BLAST) analysis of the PA3252 amino acid sequence revealed that PA3252 shows significant homology to genes that encode transporters of polyamines. Polyamines have been demonstrated to decrease outer membrane permeability by inhibiting porin-mediated ion fluxes (33). Moreover, polyamines have also been shown to provide protection against nitrosative stress in *Escherichia coli* (34). Our working model proposes that mutation of the permease may alter membrane permeability or interfere directly with peroxyntirite transport, thereby rendering a PA3252 mutant more resistant to killing by peroxyntirite (Fig. 6). Alternatively, a mutation in PA3252 could possibly trap intracellular polyamines and enhance polyamine-mediated resistance to RNIs (Fig. 6). It is important to note that peroxyntirite may not be the only RNI produced by streptococcus/nitrite-mediated activity; therefore, we do not exclude the likelihood that other intermediates may play a role in *P. aeruginosa* inhibition. Furthermore, the precise function of PA3252 in the resistance to killing requires further investigation.

The antimicrobial effects of RNIs like peroxyntirite have been established for multiple pathogens, including *E. coli*, *Bacillus subtilis*, *Cryptosporidium parvum*, *Helicobacter pylori*, and *Rhodococcus equi* (35–39); however, our studies reveal a bacterium-mediated production of peroxyntirite, a new avenue that has not been explored before. The current study is not the first instance in which oral streptococci have protected against *P. aeruginosa*. In the oral cavity, streptococcal communities prevented the integration of *P. aeruginosa* into salivary biofilms and inhibited growth of *P. aeruginosa* using a different mechanism (40). It is evident that indigenous flora can employ diverse mechanisms to interfere with the colonization or pathogenesis of incoming pathogens under different environmental conditions, which safeguards healthy homeostasis of the human body and warrants further investigation.

In summary, our study highlights a new interaction between pathogenic *P. aeruginosa* and oral commensal streptococci and demonstrates how the infiltration of oral commensal bacteria into polymicrobial infection sites can favorably alter the outcomes of polymicrobial diseases that harbor pathogens such as *P. aeruginosa*. Our working model proposes that modulation of hydrogen peroxide and nitrite within a polymicrobial environment such as the lungs of CF patients might be a valuable defense strategy against the predominant CF pathogen *P. aeruginosa*. Understanding the strategies these oral communities employ to compete with pathogenic bacteria will provide new insight into the development

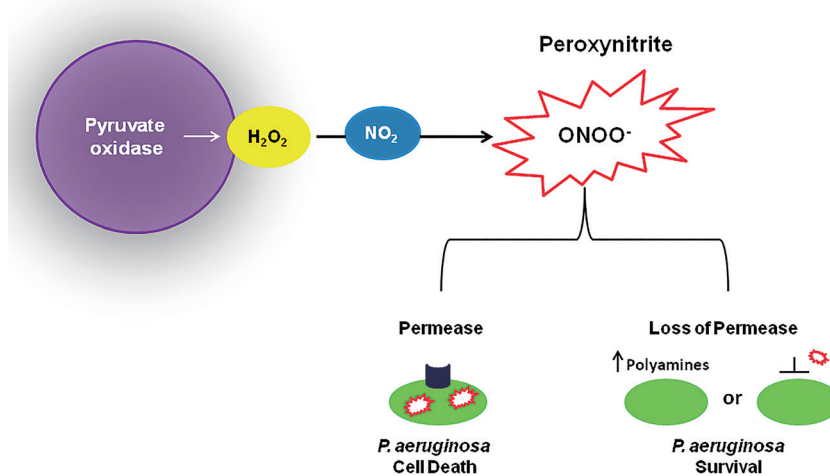
H<sub>2</sub>O<sub>2</sub>-producing Oral Streptococci

FIG 6 Model of oral streptococcus- and NO<sub>2</sub>-mediated inhibition of *P. aeruginosa*. During polymicrobial infections when nitrite is present, H<sub>2</sub>O<sub>2</sub>-producing oral streptococci can induce the production of the antimicrobial peroxynitrite, which may be transported by a *P. aeruginosa* ABC transporter permease. Loss of the permease results in polyamine-mediated resistance to peroxynitrite or decreased outer membrane permeability of peroxynitrite.

of therapeutic treatments that are effective in treating polymicrobial infections.

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