INTRODUCTION

Commensal and pathogenic bacteria frequently dwell within polymicrobial infections, and engage in interactions that impact both pathogenesis and the host response (Ramsey & Whiteley, 2009). Often, these consortia of bacteria work in concert to promote infection, cope with environmental stress, metabolize nutrients or resist clearing by the host immune response (Peters et al., 2012; Stacy et al., 2014). However, there are instances when bacteria living in these communities are in direct conflict. Within these polymicrobial environments several factors can influence the balance of power between microbes, including bacterial self-defence weapons such as bacteriocins, secondary metabolites or H₂O₂ (Kreth et al., 2008; Liu et al., 2011). Interestingly, host-derived nutrients can also play a role in promoting bacterial competition, as in the case of oral commensal streptococci and Pseudomonas aeruginosa.

Oral commensal streptococci are abundant in the oral cavity, and because they are early colonizers of the tooth surface, they play a role in facilitating the colonization of cariogenic bacteria such as Streptococcus mutans (Kolenbrander & London, 1993; Nobbs et al., 2009). Interestingly, viridans streptococci, including Streptococcus parasanguinis, Streptococcus gordonii and Streptococcus san- guinis, have been detected in the sputum of cystic fibrosis (CF) patients (Maeda et al., 2011). Moreover, some oral commensal bacteria have been associated with improved lung function in patients with CF (Filkins et al., 2012). Historically, P. aeruginosa has been a dominant pathogen during pulmonary CF infections, and has contributed to morbidity and mortality (Smith et al., 2006). P. aeruginosa is a major pathogen of multiple hosts, causes both acute and chronic infections, and has also been detected in some unique cases of periodontitis (Majorana et al., 1999). In addition to being multidrug-resistant, P. aerugi- nosa has the extraordinary ability to persist during an infection by altering the regulation of virulence and metabolic gene expression, with the most notable example being during CF pulmonary infections (Carmeli et al., 1999). The association between oral commensal streptococci in the CF lung and improved lung function suggested that some oral commensal streptococci could potentially interfere with the pathogenesis of P. aeruginosa. Previously, our laboratory reported that H₂O₂-producing oral commensal streptococci can inhibit P. aeruginosa in a nitrite-dependent manner through the production of reactive nitrogenous species.

Nitrite reductase is critical for Pseudomonas aeruginosa survival during co-infection with the oral commensal Streptococcus parasanguinis

Jessica A. Scoffield and Hui Wu

Department of Pediatric Dentistry, School of Dentistry, University of Alabama at Birmingham, Birmingham, AL, USA

Pseudomonas aeruginosa is the major aetiological agent of chronic pulmonary infections in cystic fibrosis (CF) patients. However, recent evidence suggests that the polymicrobial community of the CF lung may also harbour oral streptococci, and colonization by these micro-organisms may have a negative impact on P. aeruginosa within the CF lung. Our previous studies demonstrated that nitrite abundance plays an important role in P. aeruginosa survival during co-infection with oral streptococci. Nitrite reductase is a key enzyme involved in nitrite metabolism. Therefore, the objective of this study was to examine the role nitrite reductase (gene nirS) plays in P. aeruginosa survival during co-infection with an oral streptococcus, Streptococcus parasanguinis. Inactivation of nirS in both the chronic CF isolate FRD1 and acute wound isolate PAO1 reduced the survival rate of P. aeruginosa when co-cultured with S. parasanguinis. Growth of both mutants was restored when co-cultured with S. parasanguinis that was defective for H₂O₂ production. Furthermore, the nitrite reductase mutant was unable to kill Drosophila melanogaster during co-infection with S. parasanguinis. Taken together, these results suggest that nitrite reductase plays an important role for survival of P. aeruginosa during co-infection with S. parasanguinis.

Abbreviations: CF, cystic fibrosis; qRT, quantitative real-time; RNS, reactive nitrogenous species.
nitrogenous species (RNS) (Scofield & Wu, 2015). Nitrite is readily available within the oral cavity and CF lung, and is a byproduct of denitrifying bacteria (Grasemann et al., 1998; Hezel & Weitzberg, 2015). Nitrite reductase is one of several enzymes unique to the denitrification pathway of *P. aeruginosa* and is responsible for catalysing the reduction of nitrite to nitric oxide. As nitrite is crucial for the killing of *P. aeruginosa* by oral commensal streptococci, we questioned the role that the nitrite reductase of *P. aeruginosa* plays in facilitating its susceptibility to an oral commensal streptococcus, *S. parasanguinis*.

We report that *P. aeruginosa* isolates that are defective in nitrite reductase are susceptible to killing by *S. parasanguinis*. In addition, the inhibition of *P. aeruginosa* nirS mutants was mediated by an accumulation of nitrite in these mutants and by the production of H₂O₂ in *S. parasanguinis*. In addition, loss of nirS decreased the fitness of *P. aeruginosa* in a *Drosophila melanogaster* infection model and co-infection with *S. parasanguinis* further attenuated *P. aeruginosa* virulence in this model.

In summary, our study demonstrates the importance of nirS for *P. aeruginosa* pathogenesis and survival during polymicrobial infections with *S. parasanguinis*. Furthermore, it highlights the importance of understanding how polymicrobial interactions play a critical role in shaping the outcomes of disease.

**METHODS**

**Bacterial strains, culture conditions and reagents.** Bacterial strains and plasmids are listed in Table 1. *P. aeruginosa* was isolated on *Pseudomonas* Isolation Agar (PIA; Difco) and subsequently cultured in Luria broth (L-broth; Fisher) and incubated at 37 °C. *Escherichia coli* cells were also cultured in L-broth and incubated 37 °C. Oral streptococci were routinely cultured in Todd–Hewitt broth (THB; Difco) and incubated at 37 °C. *Escherichia coli* cells were also cultured in L-broth and incubated 37 °C. Oral streptococci were routinely cultured in Todd–Hewitt broth (THB; Difco) and incubated at 37 °C. *Escherichia coli* cells were also cultured in L-broth and incubated 37 °C.

**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. parasanguinis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FW213</td>
<td><em>S. parasanguinis</em> parent strain</td>
<td>Cole et al. (1976)</td>
</tr>
<tr>
<td>FW213 <em>poxl</em></td>
<td>Pyruvate oxidase/H₂O₂ mutant</td>
<td>Scofield &amp; Wu (2015)</td>
</tr>
<tr>
<td>FW213 <em>poxl</em> C</td>
<td>Pyruvate oxidase/H₂O₂ complemented</td>
<td>Scofield &amp; Wu (2015)</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wound isolate, non-mucoid</td>
<td>Holloway et al. (1979)</td>
</tr>
<tr>
<td>FRD1</td>
<td>CF isolate, mucoid</td>
<td>Ohman &amp; Chakrabarty (1981)</td>
</tr>
<tr>
<td>PAO1 <em>nirS</em></td>
<td>Nitrite reductase mutant</td>
<td>This study</td>
</tr>
<tr>
<td>FRD1 <em>nirS</em></td>
<td>Nitrite reductase mutant</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 <em>nirS</em> C</td>
<td>Nitrite reductase complemented</td>
<td>This study</td>
</tr>
<tr>
<td>FRD1 <em>nirS</em> C</td>
<td>Nitrite reductase complemented</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSS223</td>
<td>Transcriptional fusion</td>
<td>Suh et al. (2004)</td>
</tr>
<tr>
<td>pBluescript K(+)</td>
<td>Cloning vector</td>
<td>Addgene</td>
</tr>
</tbody>
</table>

**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 of each streptococcal species was inoculated onto a Todd–Hewitt agar (THA; Difco) plate as the early colonizer. After incubation overnight at 37 °C with 5 % CO₂, 10 μl of subcultured FRD1 (chronic *P. aeruginosa* CF isolate) or PAO1 (acute *P. aeruginosa* isolate) was inoculated next to the streptococci as the late colonizer. The plate was incubated overnight at 37 °C with 5 % CO₂. 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 medium, all streptococcal species were grown in THB overnight and 5 μl of cells were subcultured in a Costar 96-well microtitre plate (Corning) containing 200 μl fresh THB and grown to OD₄₇₀ 0.1, followed by the addition of 5 μl *P. aeruginosa* subculture (OD₄₇₀ 0.2–0.3). The cells were incubated overnight at 37 °C in the presence of 5 % CO₂. *P. aeruginosa* and oral streptococci cells were dispersed by vigorous pipetting, serially diluted and plated on PIA or THA in duplicate, and the c.f.u. counts were determined the next day.

**Construction of the *P. aeruginosa* nirS mutant and complemented strains.** To generate mutants of nirS, a DNA sequence containing ~500 bp upstream and 500 bp downstream of the nirS coding sequence was PCR amplified from FRD1 cells and cloned into the Smal site of pBluescript K(+) (*pBluescript K(+)*). An internal 800 bp fragment of the nirS coding sequence was removed using inverse PCR and replaced with the aacC1 gene encoding gentamicin resistance (Schweitzer, 1993) as a Smal fragment. This was followed by introduction of an origin of transfer (motII) of RP4 on a 230 bp HindIII fragment. The resulting plasmid was introduced into *P. aeruginosa* strains PAO1 and FRD1 by triparental mating, and potential nirS mutants were isolated as gentamicin-resistant, carbenicillin-sensitive colonies, indicating a double-crossover event. Replacement of the WT nirS gene with the nirS : : aacC1 allele was verified by PCR analysis. To complement the nirS mutant, the full-length nirS was PCR-purified from FRD1 cells and cloned into the Smal site of pBluescript K(+) (*pBluescript K(+)*) . The resulting plasmid was converted to a mobilizable plasmid.
via the addition of a mortT in the HindIII site and then introduced into P. aeruginosa through triparental mating.

**Construction of nirS transcriptional fusions.** The nirS:: lacZ transcriptional fusions were constructed using the nirS gene fragments isolated from PAO1 and FRD1. The fragments, which included 500 bp upstream of the coding sequence, were cloned into the Smal site of pSS223 (Suh et al., 2004). The plasmids containing the 5′ coding sequence for nirS, in the proper orientation, were verified by PCR and restriction digest. The plasmids containing the fusions were conjugated into FRD1 and PAO1 via triparental mating, and plasmid integration events were selected by carbenicillin resistance.

*S. parasanguinis* and *P. aeruginosa* oral infection of *D. melanogaster*. *D. melanogaster* were maintained on Jazz-mix *Drosophila* fly food (Fisher). To orally infect *Drosophila*, *P. aeruginosa* (PAO1) and *S. parasanguinis* were each grown to OD600 2.0, and 0.75 ml culture was centrifuged at 5000 g for 10 min to pellet cells. The bacterial pellet was resuspended in 100 μl of sterile 5% sucrose. The resuspended cells were spotted onto a sterile 21 mm filter (Whatman) that was placed on the surface of 5 ml 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 (1–3 days old) were starved for 3 h before being added to vials (10 flies per vial). Flies were anaesthetized using CO2 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.

**Biochemical assays.** β-Galactosidase assays were performed as described by Miller (1972). Nitrite concentrations were measured using the Griess reagent (Promega).

**Qualitative real-time (qRT)-PCR.** RNA was extracted from exponential-phase *P. aeruginosa* cultures using a Direct-zol kit (Zymo Research). Residual DNA was digested using RQ1 DNaase (Promega). RNA was purified with an RNeasy Mini kit (Qiagen), and converted into cDNA using an iScript cDNA Synthesis kit (Bio-Rad). cDNA was then used for qRT-PCR with iQ SYBR Green Supermix (Bio-Rad). The primers used to amplify 16S rRNA (reference) and the nirS gene were forward-GCTGGACTATCGCCGCTG/reverse-ATCTCGTAACCGGTGAAGGTG and forward-TGAAGTCTGGTTCTCGGTGTG/reverse-TCGTGCTGGTTGTAGAG, respectively.

**Statistical analysis.** Statistical significance was determined using Student’s t-test or the log-rank test. Data were considered statistically significant if P < 0.05.

**RESULTS**

*P. aeruginosa* isolates deficient for nitrite reductase are susceptible to *S. parasanguinis*-mediated killing

We previously reported that exogenous nitrite facilitates the inhibition of *P. aeruginosa* by H2O2-producing oral streptococci via the generation of a reactive nitrogenous intermediate (Scoffield & Wu, 2015). The denitrification pathway of *P. aeruginosa* contains a host of enzymes devoted to converting nitrate to nitric oxide and finally nitrogen. Nitrite reductase is the second enzyme in this pathway and is responsible for the conversion of nitrite to nitric oxide (Fig. 1a). Based on our previous study, we hypothesized that the activity of nitrite reductase could potentially be a major factor that contributed to the streptococcal and nitrite-mediated inhibition of *P. aeruginosa* due to an overproduction of RNS such as peroxy-nitrite or nitric oxide; hence, loss of nitrite reductase would result in increased *P. aeruginosa* survival due to a decreased production of RNS. Therefore, we wanted to explore the role that the nitrite reductase of *P. aeruginosa* plays during interactions with *S. parasanguinis* strain FW213. We constructed a mutation in the nirS gene that encodes nitrite reductase in both an acute (PAO1) and chronic (FRD1) isolate of *P. aeruginosa*. Next, we tested the fitness of this mutant in a plate competition assay with *S. parasanguinis*. As shown in Fig. 1(b), nirS mutations in both the acute and chronic *P. aeruginosa* isolates were sensitive to inhibition by *S. parasanguinis* compared with the WT and complemented strains. We previously reported that pyruvate oxidase, an enzyme required for the production of H2O2 in oral commensal streptococci, is required for *S. parasanguinis* and nitrite-mediated inhibition of *P. aeruginosa*. Therefore, we tested whether poxL, the gene that encodes pyruvate oxidase, facilitated inhibition of the *P. aeruginosa* nirS mutant during *P. aeruginosa* co-culture with *S. parasanguinis*. Loss of nirS resulted in a 2 and 4 log decrease in *P. aeruginosa* survival in the PAO1 and FRD1 isolates, respectively, when co-cultured with WT *S. parasanguinis* (Fig. 1c, d), even without the addition of exogenous nitrite. Growth of the nirS mutants was rescued by nirS complementation or during growth with the *S. parasanguinis* poxL mutant. Remarkably, co-culture of the *P. aeruginosa* nirS mutant with the *S. parasanguinis* poxL complemented strain resulted in complete inhibition of *P. aeruginosa* survival (Fig. 1c, d). Taken together, these results suggested that loss of nirS in *P. aeruginosa* did not abolish the production of potential nitrogenous byproducts that might promote inhibition by H2O2-producing streptococci.

**Loss of nitrite reductase increases NO2 levels in *P. aeruginosa***

Due to the increased sensitivity of the *P. aeruginosa* nirS mutant to *S. parasanguinis* without the addition of exogenous nitrite, we questioned whether the loss of nirS resulted in an accumulation of intracellular nitrite, which would explain why *P. aeruginosa* is sensitive to nitrite and streptococcal-mediated activity, as reported in our previous study (Scoffield & Wu, 2015). We measured intracellular and extracellular nitrite concentrations in mid-exponential-phase cultures of the PAO1 and FRD1 WT and nirS mutants. As shown in Fig. 2, the loss of nirS in PAO1 resulted in a significant increase in extracellular nitrite compared with WT PAO1. Interestingly, loss of nirS in FRD1 resulted in an increase in both intracellular and extracellular nitrite. A build-up of nitrite in the *P. aeruginosa* nirS mutant would contribute to the generation of reactive nitrogenous intermediates and increase the susceptibility of *P. aeruginosa* to killing by...
S. parasanguinis. These data were consistent with our previous study, which demonstrated that exogenous nitrite facilitated the generation of reactive nitrogenous intermediates when P. aeruginosa was co-cultured with H2O2-producing oral streptococci. Moreover, we previously observed that the chronic CF isolate FRD1 was more sensitive to oral streptococci and nitrite-mediated activity compared with the acute PAO1, as was the case with the FRD1 nirS mutant. Taken together, these observations implied that different P. aeruginosa isolates may have altered nitrite reductase activity.

Expression of nirS is reduced in the FRD1 isolate compared with the PAO1 isolate in response to nitrogenous intermediates and exposure to S. parasanguinis and nitrite

Previously, our laboratory reported that the FRD1 isolate displayed increased sensitivity to oral streptococci and nitrite-mediated activity compared with PAO1, which suggested that this isolate may be deficient in eliciting a response to nitrosative stress or was ineffective in reducing nitrite. Therefore, we constructed nirS::lacZ translational fusions in FRD1 and PAO1 to monitor promoter activity of nirS when grown on L-broth or L-broth that contained 2 mM H2O2, 1 mM NO2, 1 mM NO3 or 250 μM ONOO⁻ (peroxynitrite). We also measured nirS
expression in *P. aeruginosa* and *S. parasanguinis* co-cultures (±1 mM NO₂) using qRT-PCR analysis. As shown in Fig. 3(a), *nirS* expression was not induced in FRD1 compared with PAO1 when exposed to NO₂, NO₃ or ONOO⁻. In addition, *nirS* expression was not induced by the presence of H₂O₂ in either isolate. Furthermore, qRT-PCR analysis demonstrated that *nirS* expression was induced in PAO1, but not FRD1, when *P. aeruginosa* was co-cultured with *S. parasanguinis* in the presence of nitrite (Fig. 3b). It is important to note that catalase (*katA*) has been shown to be involved in *P. aeruginosa* resistance to nitric oxide (Su *et al.*, 2014); however, we measured *katA* expression in WT PAO1 and FRD1 and in the *nirS* mutants, and although the *nirS* mutants were marginally reduced in *katA* expression, we found no difference in *katA* expression when the WT isolates and *nirS* mutants were co-cultured with *S. parasanguinis* (data not shown). Altogether, these data suggested that *nirS* was largely induced by nitrogenous intermediates and not H₂O₂ produced by *S. parasanguinis*, and that *nirS* played a role in alleviating endogenous nitrogenous stress. Reduced activity of nitrite reductase by FRD1 during co-culture with *S. parasanguinis* and nitrite might have contributed to this isolate’s increased sensitivity to *S. parasanguinis* nitrite-mediated activity as previously reported, and signified the importance of this enzyme for the survival of *P. aeruginosa* during polymicrobial infections with oral streptococci.

**Loss of *nirS* in *P. aeruginosa* results in increased *D. melanogaster* survival during co-infection with *S. parasanguinis***

*P. aeruginosa* isolates defective for *nirS* display reduced survival in the presence of *S. parasanguinis*; thus, we wanted to test whether a mutation in *nirS* would render *P. aeruginosa* less fit in the *Drosophila in vivo* model of infection during co-infection with *S. parasanguinis*. *P. aeruginosa* strain

---

**Fig. 3.** Nitrite reductase expression is increased in response to nitrogenous intermediates in PAO1, but not FRD1. (a) *nirS* expression was measured in cultures of *P. aeruginosa* grown in L-broth that contained 2 mM H₂O₂, 1 mM NO₂, 1 mM NO₃ or 250 μM ONOO⁻ (peroxynitrite). (b) *nirS* expression was measured in *P. aeruginosa* and FW213 co-cultures (±1 mM nitrite). Data are means ± SD and are representative of three experiments.

**Fig. 4.** Nitrite reductase is required for the killing of *D. melanogaster* by PAO1 in the presence of FW213. (a) *Drosophila* were infected with WT PAO1 or the PAO1 *nirS* mutant, or co-infected with *S. parasanguinis* FW213. (b) *P. aeruginosa* c.f.u. counts during the single or co-infection. Data are mean ± SD and are representative of four biological replicates. *n = 40.*

*P < 0.05, **P < 0.005 (log-rank test).
PAO1, but not *S. parasanguinis*, readily kills *Drosophila*, and we previously established a *Drosophila* co-infection model to study *P. aeruginosa* and *S. parasanguinis* in vivo interactions (Scoffield & Wu, 2015). We infected *Drosophila* with WT PAO1 and the PAO1 nirS mutant, and also co-infected *Drosophila* with PAO1 and the PAO1 nirS mutant that contained equivalent numbers of *S. parasanguinis*. Compared with PAO1, the PAO1 nirS mutant displayed reduced virulence in the infection model. Following 8 days of infection, the survival rate for the PAO1 nirS mutant was 70%, compared with 0% in PAO1. However, when *Drosophila* were co-infected with the PAO1 nirS mutant and *S. parasanguinis*, the survival rate increased to 100% (Fig. 4a). Enumeration of *P. aeruginosa* in *Drosophila* revealed that PAO1 was able to better colonize the flies compared with the PAO1 nirS mutant. When we compared the number of *P. aeruginosa* bacteria remaining in the flies following co-infection, the PAO1 nirS mutant that was co-infected with *S. parasanguinis* had a severe reduction in c.f.u. compared with the PAO1 nirS mutant bacteria used for the single infection. Lastly, co-infection with *S. parasanguinis* did not affect the colonization of WT PAO1 in *Drosophila* (Fig. 4b). These data suggested that a functional nirS was critical for *P. aeruginosa* pathogenesis and was also important during polymicrobial infections with *S. parasanguinis*.

**DISCUSSION**

Oral commensal streptococci, including *S. parasanguinis*, have been shown to be prevalent in CF pulmonary infections (Maeda et al., 2011), which historically have been dominated by the presence of the major CF pathogen *P. aeruginosa*. Interestingly, the occurrence of oral commensal streptococci in the CF lung environment has been associated with improved lung function (Filkins et al., 2012); however, the mechanism by which *S. parasanguinis* benefits CF patients is unclear. We previously reported that exogenous nitrite facilitates the inhibition of *P. aeruginosa* by *S. parasanguinis* due to the production of reactive nitrogenous intermediates and the inhibition of *P. aeruginosa* in this manner could be beneficial for the host (Scoffield & Wu, 2015). In this study, we hypothesized that nitrite reductase, a *P. aeruginosa* enzyme involved in denitrification, could potentially be involved in the resistance or susceptibility of *P. aeruginosa* to reactive nitrogenous intermediates generated by *S. parasanguinis* activity. Therefore, we examined the role nitrite reductase plays in *P. aeruginosa* survival during co-infection with *S. parasanguinis*. Here, we report that loss of nitrite reductase promotes the accumulation of nitrite in *P. aeruginosa* cultures and thus renders *P. aeruginosa* nirS mutants sensitive to killing by H$_2$O$_2$-producing *S. parasanguinis*. These data are consistent with our previous report in which exogenous nitrite promoted the inhibition of *P. aeruginosa* by *S. parasanguinis* in an H$_2$O$_2$-dependent manner. Furthermore, the *P. aeruginosa* nirS mutant is less fit in the *Drosophila* melanogaster in vivo model of infection and, in addition, co-infection with *S. parasanguinis* in *Drosophila* further decreased the fitness of the nirS mutant. Taken together, our results demonstrate that nitrite reductase is critical for *P. aeruginosa* survival during polymicrobial infections with the oral commensal streptococcus *S. parasanguinis* and colonization of the host.

Nitrogenous intermediates generated within infection sites are often host-derived or are the result of nitrite or nitrate metabolism by denitrifying bacteria. Although little is known about how nitrogenous intermediates or denitrifying mechanisms influence competition between diverse micro-organisms within a polymicrobial environment, our study suggests denitrification processes may promote bacterial competition and could potentially impact disease outcomes in a manner that may be beneficial to the host. Nitrite is readily available in the human body for conversion to nitrogenous intermediates and has been linked to improved health, particularly in the oral cavity (Hyde et al., 2014). Elevated salivary nitrite concentrations have been associated with a reduction in dental caries, presumably due to the inhibition of the cariogenic pathogen *S. mutans* by nitric oxide-generating oral commensal bacteria (Doel et al., 2004). It is hypothesized that nitric oxide (or other RNS) generated by denitrifying oral commensals may modulate microbial homeostasis (Hyde et al., 2014), and thereby function as an infection control strategy. However, it is important to note that polymicrobial interactions involving *P. aeruginosa* and oral streptococci in the CF lung are complex, and are often controlled by the dynamics of specific streptococcal populations, colonization sequence and environmental conditions. For example, streptococcal species that belong to the *Streptococcus milleri* and *Streptococcus anginosus* groups have also been found to be co-colonized with *P. aeruginosa* in the CF airway and upregulate *P. aeruginosa* virulence, and as a result are considered pathogens in some cases of CF (Parkins et al., 2008; Sibley et al., 2008; Whiley et al., 2014). Furthermore, colonization sequence is also important for the ability of oral commensal streptococci to inhibit *P. aeruginosa*. *S. gordonii* and *S. sanguinis* inhibit the *P. aeruginosa* liverpool epidemic strain in an H$_2$O$_2$-dependent manner when these streptococci are inoculated as the primary colonizer in the presence of CO$_2$ (Whiley et al., 2015), which is consistent with our previous and current studies that demonstrate H$_2$O$_2$-producing streptococci inhibit a chronic and acute isolate of *P. aeruginosa* in the presence of nitrite when oral commensal streptococci are the primary colonizers (Scoffield & Wu, 2015). Overall, more studies are required to strengthen our understanding of how H$_2$O$_2$-producing commensal streptococci and nitrite can negatively impact *P. aeruginosa* virulence.

Micro-organisms are continuously exposed to nitrosative stress and are equipped with mechanisms that function to alleviate this stress encountered within the host. Moreover, the altered or reduced expression of denitrifying
enzymes, as reported in this study using the *P. aeruginosa* FRD1 clinical isolate, could render some bacterial isolates more susceptible to RNS stress mediated by the host or other bacteria. Functional denitrification enzymes have been previously reported to be crucial for the survival of other bacterial pathogens. For example, *Mycobacterium tuberculosis* survival in human macrophages is dependent upon a functional nitrite reductase (NirBD) (Akhtar et al., 2013). Moreover, HcpR, a regulator of hydroxylamine reductase, is required for *Porphyromonas gingivalis* growth in the presence of nitrite and nitric oxide, in addition to survival in host cells (Lewis et al., 2012). Furthermore, nitrite reductase has been shown to be required for the expression of the *P. aeruginosa* type III secretion system (Van Alst et al., 2009) and biofilm formation by *P. aeruginosa* (de la Fuente-Núñez et al., 2013). These studies suggest that mechanisms involved in nitrosative stress resistance or nitrite reduction are required for not only bacterial growth on nitrogenous substrates, but also bacterial virulence.

Few studies have examined the role that denitrification mechanisms have on bacterial competition within polymicrobial infections. Our findings demonstrate that nitrite reductase is required by *P. aeruginosa* in order to compete with the oral commensal *S. parasanguinis*. In addition, oral commensal bacteria may mediate antimicrobial mechanisms not only in the oral cavity, but also during systemic infections such as CF where *P. aeruginosa* and oral commensal streptococci are co-colonized. Furthermore, any alterations in the expression of enzymes that are important for the resistance or detoxification of nitrogenous intermediate could render micro-organisms susceptible to killing within a polymicrobial infection. Understanding the mechanisms that are required for pathogens to persist during polymicrobial infections could potentially lead to the development of novel therapeutics that promote bacterial competition and result in positive disease outcomes.

**ACKNOWLEDGEMENTS**

This work was supported by the National Institutes of Health grant R01 DE017954 (to H.W.). J. A. S. is supported by a National Institutes of Health (National Institute of Dental and Craniofacial Research) diversity supplement R01 DE022350-04-S1.

**REFERENCES**


Parkins, M. D., Sibley, C. D., Surette, M. G. & Rabin, H. R. (2008). The *Streptococcus milleri* group—an unrecognized cause of disease in...


Edited by: W. Crielaard