Sessile growth reveals novel paradigms of *Pseudomonas aeruginosa* iron-regulated antimicrobial activity against *Staphylococcus aureus*

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ABSTRACT

Pseudomonas aeruginosa and *Staphylococcus aureus* are opportunistic pathogens that cause chronic, polymicrobial infections. Each pathogen possesses a complex regulatory network that modulates iron acquisition and virulence. However, our current knowledge of these networks is largely based on studies with shaking cultures, which are not likely representative of microbial communities *in vivo*. Here, we provide proteomic, metabolic, and genetic evidence that iron regulation is altered in sessile *P. aeruginosa* cultures. We further demonstrate that iron-regulated interactions between *P. aeruginosa* and *S. aureus* are mediated by distinct factors in shaking versus sessile bacterial cultures. Moreover, we identified type 6 secretion as a target of iron regulation in *P. aeruginosa* in static but not shaking conditions, and co-culture studies suggest this system may contribute to antimicrobial activity against *S. aureus* in static conditions. These results yield new bacterial iron regulation paradigms and highlight the need for re-defining iron homeostasis in sessile microbial communities.

INTRODUCTION

Antimicrobial-resistant pathogens represent a substantial health risk, raising concerns of a return to a “pre-antibiotic era”. The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* alone is responsible for over 10% of all hospital-acquired infections and is a leading cause of morbidity among nosocomial pathogens (1-6). *P. aeruginosa* also establishes chronic infections in surgical wounds, diabetic foot ulcers, and the lungs of individuals with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). Chronic infections in these populations are almost always polymicrobial in nature, and *P. aeruginosa* is often co-isolated with the Gram-positive pathogen *Staphylococcus aureus* (7, 8), which also exhibits significant antimicrobial resistance. Chronic co-infections with *S. aureus* and *P. aeruginosa* result in slower healing and worse prognoses due in part to antagonistic interactions between these two organisms (9-15). *S. aureus* is also the predominant pathogen in the lungs of young CF patients, but is eventually displaced by *P. aeruginosa* resulting in prolonged lung tissue inflammation and diminished lung function (16, 17). Interactions between these two species are thought to be driven by competition for limited resources in the host environment, which alters bacterial metabolic networks and virulence factor expression (11, 18, 19). However, despite the clear significance of polymicrobial interactions in infectious disease, the regulatory pathways that control these interactions remain largely undefined.

P. aeruginosa secretes multiple toxins and bio-active metabolites, which cause damage to host tissue and exhibit antimicrobial activity (AMA) against competing microorganisms. Amongst these is a collection of small secreted metabolites collectively referred to as 2-alkyl-4(1*H*)-quinolones (AQs), which mediate a range of toxic activities against *S. aureus* and are thought to drive interactions between these two species during infection (20-23). 2-alkyl-4-quinolone N-oxides (AQNOs) are potent cytochrome inhibitors that obstruct respiratory metabolism in *S. aureus* (24-26). 2-alkyl-3-hydroxy-quinolone, which is more commonly referred to as PQS, and 2-alkyl-4-hydroxyquinolones (AHQs) both function as quorum signaling

55 molecules and induce the expression of secreted factors that further inhibit *S. aureus* growth
56 (27-30). AQ synthesis is initiated by enzymes encoded by the *pqsABCDE* operon, with PqsA
57 catalyzing the first step with the conversion of anthranilate into anthraniloyl-CoA (31). Past
58 studies indicate AQ synthesis and anthranilate metabolism are subject to extensive regulation
59 by nutrient availability (21, 32, 33). However, the impact on these regulatory processes on
60 interactions of *P. aeruginosa* with other microorganisms remains unclear.

61 Iron is a required metallo-nutrient for most living organisms and as such has a significant
62 impact on the establishment and progression of *P. aeruginosa* and *S. aureus* infections (34, 35)
63 During infection, the host sequesters iron from invading pathogens through a process referred
64 to as nutritional immunity (36-38). In response to iron starvation, *P. aeruginosa* and *S. aureus*
65 express several virulence factors that cause host cell damage, presumably releasing iron and
66 other nutrients from host cells (33, 39-42). Iron starvation also induces the expression of
67 multiple systems that mediate high affinity uptake of iron and heme (43, 44). In *P. aeruginosa*,
68 iron starvation further induces expression of the PrrF small regulatory RNAs (sRNAs), which
69 post-transcriptionally reduce expression of non-essential iron-dependent metabolic enzymes,
70 including the anthranilate degradation enzyme complexes AntABC and CatBCA (33). As a result
71 of this regulation, the PrrF sRNAs spare anthranilate for AQ production and are therefore
72 required for optimal AQ production in iron-depleted conditions (32, 35).

73 In accordance with PrrF promoting AQ production in iron-depleted environments, we
74 recently discovered that iron starvation enhances AQ-dependent AMA against *S. aureus* on
75 agar plates as well as in a transwell co-culture system (21, 22). Surprisingly, the *prrF* locus was
76 not required for AMA in these assays. Several potential mechanisms were considered that could
77 explain this finding, including the possibility that co-culture with *S. aureus* may restore AQ
78 production through a PrrF-independent pathway. In support of this hypothesis, we found that co-
79 culture of the Δ*prrF* mutant with *S. aureus* in shaking liquid cultures restored PQS production to
80 wild type levels (21). However, technical issues prevented us from accurately quantifying AQs in

the agar plate and transwell co-culture assays, precluding a more thorough examination of the factors required for iron-regulated AMA.

In the current study, we sought to develop a co-culture assay that would allow us to examine the impact of PrrF and iron on gene expression, AQ production, and AMA under a variety of growth conditions. Analysis of metabolite levels, gene expression, and viability demonstrated that culture perturbation causes phenotypic changes in the *P. aeruginosa* Δ*prrF* mutant during co-culture with *S. aureus*. Proteomics further demonstrated that culture perturbation alters the activity of some iron homeostasis pathways in *P. aeruginosa*, including PrrF-regulated expression of anthranilate metabolism proteins. Analysis of microaerobic co-cultures indicated that changes in the Δ*prrF* mutant phenotypes during sessile culture were not solely due to decreased oxygen tensions. These studies indicate that sessile bacterial communities use distinct regulatory and metabolic networks to adapt to decreased nutrient availability, likely affecting the production of key virulence determinants and polymicrobial interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Supplementary Table 1. Lysogeny broth (LB) and agar (LA) were prepared using 10g/L NaCl (Sigma, St Louis, MO), 10g/L tryptone, and 5g/L yeast extract, 15g/L agar (when applicable) (Becton-Dickinson, Franklin Lakes, NJ). *P. aeruginosa* and *S. aureus* strains were both routinely grown on LA from freezer stock. Five isolated colonies of each strain were selected from overnight-incubated agar plates and inoculated into 5mL LB. For iron starvation studies, bacterial strains were grown in a Chelex-treated and dialyzed trypticase soy broth (DTSB) prepared as previously described (33). Media dialysis was carried out using Spectra/Por®2 dialysis membrane tubing (29mm diameter) with a molecular weight cutoff of 12-14kD (Repligen, Waltham, Ma). For co-culture assays, quantitative real time PCR (qRT-PCR)

analysis, and mass spectrometry-assisted metabolite and proteome studies, *S. aureus* and *P. aeruginosa* cultures were diluted into 1.5mL of DTSB with or without 100μM (high iron) $FeCl_3$ supplementation, to an absorbance (OD_{600}) of 0.08 and 0.05, respectively. Monocultures and co-cultures of *P. aeruginosa* and *S. aureus* were prepared in DTSB media and incubated at 37˚C for 18 hours in a shaking incubator. Shaking aerobic cultures were incubated in 1.5mL of DTSB media in 14 mL round bottom tubes and closed with a foam stopper to allow for sufficient aeration. Sessile cultures were incubated in 1.8mL DTSB media in 6-well polystyrene culture plates, which were covered in breathe-easy wrap to prevent evaporation. During incubation, cultures were grown at a shaking rate of either 250rpm (shaking conditions) or 0rpm (static conditions). Microaerobic co-cultures were grown in identical growth conditions as shaking cultures, except culture tubes were placed into an air-tight candle jar in the presence of a CampyPak (BD Diagnostics, NJ, USA). Sealed candle jars were secured in a shaking incubator and incubated for 18 hours at 37˚C and a shaking rate of 250rpm.

Colony forming unit determination. 400uL of *P. aeruginosa* and *S. aureus* mono- and co-cultures were harvested at 15000rcf for 5 minutes. Cell pellets were resuspended in 400uL of 0.1% Triton-X in phosphate-buffered saline and vortexed rigorously. From these resuspensions, serial dilutions were prepared in 0.1% Triton-X in PBS. 10uL of each dilution was spotted onto Baird-Parker agar plates and Pseudomonas Isolation Agar (PIA) plates to select for *S. aureus* and *P. aeruginosa* growth, respectively. Upon spotting 10uL of dilutions onto agar plates, the plates were tilted to facilitate even drips across the plate surface. PIA plates were incubated at 37˚C for 18 hours and colony forming units (CFU) were counted. Baird-Parker plates were incubated for 37˚C for 48 hours to allow for adequate growth of *S. aureus* small-colony variants observed in some assays prior to counting CFUs.

Quantitation of AQs using liquid chromatography-tandem mass spectrometry. AQ quantitation was carried out as previously reported (45). Briefly, 300uL of culture were harvested and spiked with a 25μM stock nalidixic acid internal standard to a final concentration of 500nM, followed by an extraction using ethyl acetate w/ 0.1% acetic acid. The organic phase containing extracted AQs was harvested from each sample, dried down, and resuspended in 300uL of 0.1% formic acid suspended in1:1:1 (v/v/v) mixture of methanol, water, and acetonitrile. Extracts were analyzed via quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) using multiple reaction monitoring performed on a Dionex Ultimate 3000 TSQ tandem quadrupole mass spectrometer in positive ion mode**.**

Real time PCR analysis. Quantitative real time PCR (qRT-PCR) analysis was conducted on three biological replicates of the PAO1 reference strain and isogenic Δ*prrF* mutant grown in DTSB supplemented with either 100μM or 0μM iron. *P. aeruginosa* cells were lysed after 18 hours using 2.5mg/mL lysozyme and incubated at 37˚C for 30 minutes. RNA extraction was performed using the RNeasy mini kit (QIAGEN, Germantown, MD). Real-time qualitative PCR analysis was performed as described previously (35) using an Applied Sciences StepOne Plus Real Time PCR System (Life Technologies, Carlsbad, CA). Primer and probe sequences used for gene expression analysis are listed in **Supplementary Table S2**. Quantitation of cDNA was carried out using standard curves compiled for individual gene targets, and *P. aeruginosa* expression data was normalized to *oprF* expression in aerobic conditions, or *omlA* in microaerobic and anaerobic conditions.

Quantitative label-free proteomics. Cell cultures were prepared for proteomics analysis as described previously (46). Briefly, cells were harvested by centrifugation and washed in phosphate-buffered saline prior to lysis in 4% sodium deoxycholate. Lysates were washed, reduced, alkylated and trypsinolyzed on filter (47, 48). Tryptic peptides were separated

158 using a nanoACQUITY UPLC analytical column (BEH130 C18, 1.7 µm, 75 µm x 200 mm,
159 Waters) over a 165-minute linear acetonitrile gradient (3 – 40%) with 0.1 % formic acid on a
160 Waters nano-ACQUITY UPLC system and analyzed on a coupled Thermo Scientific Orbitrap
161 Fusion Lumos Tribrid mass spectrometer. Full scans were acquired at a resolution of 120,000,
162 and precursors were selected for fragmentation by higher-energy collisional dissociation
163 (normalized collision energy at 32 %) for a maximum 3-second cycle. Tandem mass spectra
164 were searched against *Pseudomonas* genome database PAO1 reference protein sequences
165 (49) using Sequest HT algorithm and MS Amanda algorithm with a maximum precursor mass
166 error tolerance of 10 ppm (50, 51). Carbamidomethylation of cysteine and deamidation of
167 asparagine and glutamine were treated as static and dynamic modifications, respectively.
168 Resulting hits were validated at a maximum false discovery rate of 0.01 using a semi-
169 supervised machine learning algorithm Percolator (52). Label-free quantifications were
170 performed using Minora, an aligned AMRT (Accurate Mass and Retention Time) cluster
171 quantification algorithm (Thermo Scientific, 2017). Protein abundance ratios between the high
172 iron cultures and the low iron cultures were measured by comparing the MS1 peak volumes of
173 peptide ions, whose identities were confirmed by MS2 sequencing as described above. Gene
174 function and pathway analysis was completed using information from the *Pseudomonas*
175 genome database (49), KEGG database (53), *Pseudomonas* metabolome database (54), and
176 the STRING database (55).
177
178 **Statistics**. Statistically significant changes in cell viability, RNA gene expression, and
179 AQ concentrations between treatment groups were identified using a two-tailed students t-test
180 on Microsoft Excel 2013, with a significance threshold of $p \leq 0.05$. Protein expressions that
181 changed 2-fold or more with an FDR adjusted p-value < 0.05 were considered statistically
182 significant.
183

RESULTS

PrrF is required for AMA against *S. aureus* in shaking co-cultures. Previous co-culture studies performed in our lab examined the role of *P. aeruginosa* AMA against *S. aureus* in Chelex-treated and dialyzed tryptic soy broth (DTSB) supplemented with or without 100 μM $FeCl_3$ (21, 22). These previous assays were performed using either agar plate or liquid transwell co-culture systems. For agar co-cultures, *P. aeruginosa* strains were spotted onto confluent lawns of *S. aureus* on agar plates; for liquid transwell co-cultures, *P. aeruginosa* and *S. aureus* strains were co-incubated in transwell microtiter plates, with each species separated by a 0.2μm permeable transwell membrane (21). In each of these assays, *P. aeruginosa* demonstrated iron-regulated AMA against *S. aureus* that was dependent upon the *pqsA* gene, confirming the expected requirement for AQs. However, the *prrF* locus was not required for AMA in either of these assays (21). While analysis of shaking cultures showed that co-culture with *S. aureus* restored PQS production to the Δ*prrF* mutant (21), attempts to quantify AQs and analyze gene expression in the transwell plates and agar co-cultures were unsuccessful, preventing a more mechanistic analysis of how iron and PrrF affected AMA under these specific conditions.

We therefore grew mono- and co-cultures of *P. aeruginosa* strain PAO1 and *S. aureus* strain USA300 in a larger volume (1.5 mL) of DTSB, supplemented with or without 100μM $FeCl_3$, in a shaking incubator at 250 rpm. Viability of *P. aeruginosa* and *S. aureus* was then quantified by enumerating colony forming units on selective Pseudomonas isolation agar (PIA) and Baird Parker agar media, respectively. In agreement with earlier studies showing that AQs mediate AMA against *S. aureus*, USA300 viability was reduced approximately 50,000-fold during low iron co-culture with PAO1 but not during co-culture with the Δ*pqsA* mutant (**Fig. 1A**). Also in agreement with our earlier work, iron limitation significantly enhanced AMA of PAO1 against USA300 (**Fig. 1A**). Surprisingly, the Δ*prrF* mutant was defective for AMA relative to the isogenic parent strain PAO1 (**Fig. 1A**), a result that contrasted with our previously reported agar plate and transwell co-culture data (21). Viability of *P. aeruginosa* was not affected by iron

210 supplementation or deletion of either *pqsA* or *prrF* (**Fig. 1A**), demonstrating that increases in *S.*
211 *aureus* viability were not due to decreased viability of the *P. aeruginosa* Δ*pqsA* or Δ*prrF*
212 mutants.
213
214 **Co-culture with *S. aureus* does not uniformly restore AQ production to the Δ*prrF***
215 **mutant.** Previous studies in our lab demonstrated that production of the AQ congener, PQS,
216 could be restored to the Δ*prrF* mutant if grown in co-culture with the M2 methicillin-resistant
217 strain of *S. aureus* (21). The mechanism of this restoration was unknown, but it seemingly
218 explained why the Δ*prrF* mutant retained AMA in the transwell co-culture assay. However, PQS
219 itself was found to not be required for AMA in our earlier studies (21), and it was unknown
220 whether other AQs were similarly restored to the Δ*prrF* mutant during co-culture. We tested this
221 by quantifying various AQ congeners from mono- and co-cultures using liquid chromatography
222 coupled with tandem mass spectrometry (LC-MS/MS). In agreement with our previous studies,
223 LC-MS/MS analysis of Δ*prrF* culture extracts demonstrated increased levels of PQS, containing
224 either a C7 or C9 alkyl chain, in the Δ*prrF* mutant during co-culture with *S. aureus* strain M2
225 compared to mono-culture of the Δ*prrF* mutant (**Table S3**). However, no significant increase
226 upon co-culture was observed for any of the other AQs we quantified from the Δ*prrF* mutant co-
227 culture extracts (**Table S3**). In agreement with our previous findings, the Δ*pqsH* mutant, which
228 specifically lacks production of PQS, was not defective for AMA against *S. aureus* (**Fig. S1).**
229 These results indicate that restoration of PQS production by co-culture with *S. aureus* is unlikely
230 to influence AMA. Moreover, co-cultures of the Δ*prrF* mutant with *S. aureus* strain USA300
231 resulted in no significant increase in the concentrations of any AQs, including PQS (**Table S3).**
232 Combined with the data in **Fig. 1A**, these data disprove our previous conclusion that co-culture
233 with *S. aureus* promotes AQ production and AMA of the *P. aeruginosa* Δ*prrF* mutant.
234

PrrF is not required for AMA against *S. aureus* in sessile cultures. We next wondered if different results for the Δ*prrF* mutant obtained in the current (**Fig. 1A**) and previous studies (21) were due to differences in culture perturbation during incubation. Specifically, cultures using the agar and transwell systems were grown in static conditions, whereas the cultures for the studies described above were grown in shaking conditions. We hypothesized that culture agitation altered the impact of PrrF on AQ-mediated AMA. To test this, we grew mono-cultures and co-cultures of *P. aeruginosa* and *S. aureus* in DTSB supplemented with or without $FeCl_3$ under static conditions. Initially, these studies were performed with 15mL polystyrene culture tubes, but this eliminated AMA for all *P. aeruginosa* strains (**Fig. S2)**. This was presumably due to *S. aureus*, which is non-motile, settling at the bottom of the tubes and distal from the motile *P. aeruginosa* strains growing near the air-liquid interface. In order to facilitate interactions between *S. aureus* and *P. aeruginosa,* static co-cultures were instead prepared in six-well polystyrene cell culture plates, allowing for a shallower liquid depth and larger air-liquid interface, covered in breathe-easy wrap. While AMA was overall less robust in static conditions, we observed iron-regulated AMA against *S. aureus* by the wild-type PAO1 strain, as well as a complete loss of AMA in the Δ*pqsA* mutant (**Fig. 1B**). In contrast to the shaking assay, however, viability of *S. aureus* grown with the Δ*prrF* mutant in low iron conditions was not significantly different than when grown with the wild-type PAO1 strain (**Fig. 1B**), suggesting that PrrF-mediated AMA is altered during sessile growth. Importantly, changes in *S. aureus* CFU in this static assay were comparable to *S. aureus* culture densities previously obtained with transwell co-cultures (21). Thus, our data indicate that culture perturbation alters the factors that regulate *P. aeruginosa* AMA against *S. aureus* during co-culture.

AHQ concentrations are increased in static growth conditions. We next determined if PrrF was required for optimal AQ production during static growth. To test this, we used a recently validated LC-MS/MS method (45) to measure levels of various AQ species in shaking

and sessile cultures of wild-type *P. aeruginosa* and the Δ*prrF* mutant. As previously observed (21, 32), PrrF was required for optimal production of the C7 and C9 congeners of PQS, AQNO, and AHQ in shaking conditions (**Fig. 2A,B,C, white bars**). Surprisingly, PrrF was also required for optimal production of each of these AQs in static growth conditions (**Fig. 2A,B,C, gray bars**). However, levels of both the C7 and C9 congeners of PQS and AQNO were substantially decreased in static conditions relative to shaking conditions, and were in fact similar or lower than concentrations produced by the Δ*prrF* mutant in shaking cultures (**Fig. 2A,B, white versus gray bars**). This is likely due to the previously reported oxygen requirement for activity of the PqsH and PqsL enzymes. Moreover, these data suggest that the relative impact of these AQ congeners on AMA is lessened in sessile cultures. This idea is supported by data observed in static co-cultures of *S. aureus* and a *P. aeruginosa* Δ*pqsL* mutant, which demonstrate that the Δ*pqsL* still exhibits AMA in static conditions but is devoid of AMA in shaking conditions (**Fig. S3B**). Notably, levels of HHQ and NHQ were significantly increased in static cultures, with concentrations in the Δ*prrF* cultures equivalent or higher than that of the shaking wild type culture (**Fig. 2C, white versus gray bars**). Thus, while the Δ*prrF* mutant still exhibits an overall defect in AQ production during static growth, it may produce sufficient levels of AHQs to mediate AMA under these conditions.

AMA requirement for PrrF in shaking cultures is not impacted by oxygen availability. Previous reports have demonstrated that static growth can have dramatic impact on broad aspects of *P. aeruginosa* physiology, including expression of virulence traits. These reports have suggested that diminished oxygen availability in static conditions plays a defining role in mediating these effects (56-58). In agreement with this, LC-MS/MS analysis of static and shaking cultures revealed that oxygen-dependent PQS and AQNO molecules were produced at significantly lower concentrations in sessile cultures than in shaking (**Fig. 2A,B, white bars versus gray bars**). We therefore hypothesized that oxygen limitation may be responsible for

287 changes in the requirement for PrrF in *P. aeruginosa* AMA in sessile cultures. We tested this by
288 co-inoculating *S. aureus* strains with *P. aeruginosa* wild type PAO1, Δ*pqsA,* or Δ*prrF* strains in
289 DTSB media, and incubating these co-cultures with shaking in either microaerobic or aerobic
290 conditions for 18 hours (see **Materials and Methods**). This model system allowed us to directly
291 examine the impact of oxygen, while otherwise preserving additional variables such as culture
292 perturbation. Surprisingly, we observed that PrrF sRNAs were still necessary for AMA against *S.*
293 *aureus* in microaerobic conditions, similar to aerobic conditions (**Fig. 3A,B**), indicating that
294 oxygen availability alone does not alter the impact of PrrF on AMA.
295 We next measured AQ concentrations in microaerobic cultures to determine whether
296 oxygen limitation is responsible for the changes in AQ concentrations observed in sessile
297 cultures. Similar to what we observed in static cultures, AQNO and PQS molecules were
298 significantly lower in microaerobic cultures than in aerobic cultures; AQNOs, in particular, were
299 present in concentrations lower than even those of static Δ*prrF* cultures (**Fig. 2B, hashed bars**).
300 In addition, there was no statistically significant difference between concentrations of HQNO,
301 NQNO, or C9-PQS produced by the wild type and Δ*prrF* strains in microaerobic cultures (**Fig.**
302 **2A,B, hashed bars**), indicating that PrrF is not necessary for their production in microaerobic
303 conditions. In contrast to static cultures, AHQ concentrations in microaerobic shaking cultures
304 were comparable to those observed in aerobic shaking cultures (**Fig. 2C, white versus hashed**
305 **bars**). Moreover, AHQ concentrations were significantly reduced by *prrF* deletion in
306 microaerobic shaking conditions (**Fig. 2C, hashed bars**). Thus, while oxygen availability has
307 marked impact on the concentrations of different AQs, oxygen limitation alone does not fully
308 account for changes in AQ production and AMA of the Δ*prrF* mutant during static growth.
309 Combined, these results also suggest that the large increase in AHQ levels, which is observed
310 in sessile cultures but not microaerobic shaking cultures, allows the Δ*prrF* mutant to retain AMA
311 during sessile growth.
312

Iron regulation of anthranilate catabolism genes is retained during static growth. The above data demonstrate that the PrrF sRNAs promote production of AQs in both shaking and sessile cultures. In shaking conditions, PrrF contributes to AQ production via repression of anthranilate metabolism genes (32, 33). It is unclear, however, whether PrrF modulates AQ production in static conditions via the same metabolic pathways. To test this, first we performed targeted expression analysis of PrrF sRNAs and known PrrF-regulated genes in both static and shaking cultures of wild type *P. aeruginosa* using quantitative real time PCR (qRT-PCR). PrrF expression was markedly lower in sessile cultures as compared to shaking cultures, but it was still significantly repressed by iron supplementation **(Fig. 3A).** We next examined levels of the *antA* mRNA, encoding for a component of the anthranilate dioxygenase that degrades anthranilate, as well as *antR*, encoding a transcriptional activator of *antABC* that is directly repressed by PrrF (32). We observed iron-activated expression of both *antA* and *antR* when PAO1 was grown in either shaking or static conditions (**Fig. 3B-C**). As previously observed (32, 33), levels of *antA* and *antR* were significantly increased in the Δ*prrF* mutant compared to wild type PAO1 when grown in low iron shaking conditions, resulting in loss of iron regulation of *antR*. Interestingly, while expression of both *antR* and *antA* was derepressed in the Δ*prrF* mutant grown under static conditions, iron still activated each of these genes in sessile cultures of the Δ*prrF* mutant **(Fig. 3B-C)**. These results indicate that additional factors contribute to iron-regulated expression of the anthranilate degradation pathway under static conditions.

Proteomic analysis reveals PrrF-independent iron regulation of anthranilate metabolic proteins in static conditions. The studies above indicate that iron regulates AMA in a PrrF-independent manner under static growth conditions. However, our current knowledge of *P. aeruginosa* iron regulation is largely restricted to shaking cultures. To better understand changes in PrrF and iron regulation in sessile cultures *of P. aeruginosa*, we applied a label-free, LC-MS/MS-based proteomics methodology recently described by our laboratories (46). Using

this unbiased approach, we determined the proteomes of static and shaking cultures of PAO1 and the isogenic Δ*prrF* mutant grown in DTSB cultures supplemented with or without 100μM $FeCl_3$. Mass spectra were identified using a PAO1 reference proteome, which were in turn validated to a false discovery rate of 0.01 to ensure proper assignment of protein identities.

Iron regulation under shaking conditions was robust, with over 178 proteins significantly repressed by iron and 191 proteins induced by iron (**Supplementary Dataset 1**). As previously observed (40, 44, 59-63), iron supplementation significantly reduced proteins for the siderophore (pyoverdine and pyochelin) and heme uptake systems, as well as several iron-regulated virulence factors, during shaking growth (**Fig. 5A**). Well-known PrrF-repressed proteins involved in the tricarboxylic acid (TCA) cycle and oxidative metabolism, including aconitase A, succinate dehydrogenase B, and catalase, were induced by iron in a PrrF-dependent manner under shaking conditions (**Fig. 5B**) (33, 64). We also observed PrrF-dependent iron regulation of more recently identified PrrF targets in shaking conditions, such as genes involved in Fe-Sulfur cluster biogenesis (IscS, IscU) and amino acid metabolism (IlvD) (**Fig. 5B**) (46). Contrary to previous real-time PCR and microarray analyses showing iron induction of the *antABC* mRNAs (46), neither iron supplementation nor *prrF* deletion significantly affected levels of the AntABC proteins for anthranilate degradation under shaking conditions (**Fig. 5B**). Overall these studies replicated many of the global iron regulatory pathways observed in previous studies.

We next determined whether proteins that were regulated by iron and PrrF under shaking conditions were similarly regulated during sessile growth. Iron repression of siderophore and heme uptake proteins was largely retained during static growth, indicating that these iron regulatory pathways are not altered under these conditions (**Fig. 5A**). In contrast, iron and PrrF regulation of several proteins involved in the TCA cycle or oxidative stress protection was reduced or eliminated in static conditions (**Fig. 5B**). We also noted robust iron induction of the anthranilate degradation proteins AntABC and CatBCA, which occurred in a PrrF-

365 independent manner (**Fig. 5B**)**,** in agreement with trends we observed in our qRT-PCR analysis
366 of *antR* and *antA* **(Fig. 3B-C)**. Combined, these data indicate that several iron regulatory
367 pathways are altered in static conditions.
368
369 **Static growth reveals novel iron regulated proteins in *P. aeruginosa*.** To determine
370 how sessile growth might alter global regulatory effects of iron, we mined our proteomics
371 dataset for proteins whose levels were altered by iron depletion in either static or shaking
372 conditions, but not both, using a fold change ≥ 2, and an FDR adjusted *p* value ≤ 0.05 as our
373 cut-off. Approximately 410 proteins demonstrated significantly altered iron regulation in
374 response to growth conditions. Of these, 126 of these were specifically iron induced and 93
375 were iron repressed in shaking but not static conditions, while 78 were iron induced and 129
376 were iron repressed specifically in static conditions (**Supplementary Table S4 and Dataset**
377 **S1**). Approximately 50% of the proteins affected by iron supplementation in static conditions
378 were unaffected by iron in the Δ*prrF* mutant (**Supplementary Table S4 and Dataset S1**),
379 indicating PrrF still mediates iron homeostasis during these growth conditions, despite the loss
380 of PrrF-mediated iron regulation on previously identified targets as shown in **Fig. 5B**.
381 STRING network analysis was next used to identify relationships between the proteins
382 within each of these four groups. Several of the proteins that were induced by iron in shaking
383 conditions but not in static conditions were related to motility, including proteins for flagellar
384 assembly (FlgA, FlgL, FliC, and FliI), a chemotaxis-associated protein (ChpA), and proteins
385 involved in twitching pilus formation (PilV, PilY2, and FimU) **(Supplementary Dataset S1).**
386 Proteins that were significantly induced by iron depletion in static but not shaking cultures
387 included numerous enzymes required for synthesis of the redox cycling phenazine metabolites
388 and the pyochelin siderophore, as well as proteins encoded by the second type 6 secretion
389 system (T6SS) locus (HSI-II T6SS) (**Fig 6A)**. Iron regulation of the phenazine and pyochelin
390 synthesis proteins occurred in a PrrF-independent manner, while iron regulation of many of the

T6SS proteins was either reduced or lost in the Δ*prrF* mutant (**Fig. 6B**). Moreover, we found that *prrF* deletion had a negative effect on T6SS proteins in static but not shaking conditions **(Supplementary Dataset S1).** The identification of T6SS as iron- and PrrF-regulated during static but not shaking conditions was particularly intriguing, as this system is known to mediate cell-to-cell interactions that might not be possible in shaking cultures. No complementarity between the PrrF sRNAs and the mRNAs encoding these proteins, thus we hypothesize expression of these proteins is indirectly regulated by PrrF.

T6SS may play a role in AMA during static growth conditions. We next hypothesized that proteins that were induced by iron starvation in static conditions but not shaking conditions may contribute to iron-regulated AMA during static growth. We therefore tested the roles of phenazines, pyochelin, and T6SS on *P. aeruginosa* AMA in static conditions using static agar co-cultures of *S. aureus* strain USA300. *S. aureus* strain USA300 was swabbed onto agar plates containing low or high iron DTSB media, and wild-type *P. aeruginosa* strains or their respective mutant strains were spotted onto the monolayers. A mutant in the PA14 *P. aeruginosa* background, which is deleted for both operons encoding the core phenazine biosynthesis proteins (Δ*phz*), demonstrated wild type levels of AMA against *S. aureus* (**Fig. S4**). Likewise, a PAO1 deletion mutant defective for pyochelin siderophore synthesis (Δ*pchEF*) demonstrated wild type levels of AMA. These data suggest that neither phenazines nor pyochelin alone account for altered AMA in static conditions. Interestingly, a siderophore deficient double mutant containing deletions of *pchEF* and the pyoverdine synthesis gene *pvdA* was attenuated for AMA in these conditions (**Fig. S4**). Single deletion of the *pvdA* gene did not eliminate AMA, however, suggesting that the composite effect of these two siderophore is critical to AMA in static conditions.

Notably, a transposon mutant in *clpV2*, which encodes a key protein in the HSI-II T6SS, showed reduced AMA against *S. aureus* when compared to PAO1 (**Fig. S4**). However, it was

417 difficult to interpret the results obtained with the transposon mutants, as the parental mPAO1
418 strain exhibited very low baseline AMA (**Fig. S4**). To more reliably measure AMA by the HSI-II
419 T6SS mutants, we co-incubated wild type mPAO1 or the *clpV2* mutants in shaking and static
420 liquid co-cultures with *S. aureus.* While mPAO1 exhibited weak yet statistically significant AMA
421 against *S. aureus* in these conditions, one of the *clpV2* mutants exhibited no statistically
422 significant AMA against *S. aureus* (**Fig. 7B and Fig. S5A**). The same *clpV2* mutant showed no
423 defect in AMA during shaking growth (**Fig. 7A**). These results suggest a novel role for T6SS in
424 *P. aeruginosa* AMA that is specific to sessile growth conditions, where cell-to-cell contact is
425 more likely to occur.
426
427 **DISCUSSION**
428 The PrrF sRNAs regulate numerous biological functions in *P. aeruginosa* in response to
429 iron starvation and are thus critical for virulence (35). A Δ*prrF* mutant was also previously shown
430 to produce diminished quantities of AQs (21, 32), which are necessary for *P. aeruginosa* AMA
431 against *S. aureus* (20-22). Strikingly, previous reports indicated that the Δ*prrF* mutant still
432 exhibits AMA against *S. aureus* in static co-cultures, suggesting that PrrF is not necessary for
433 AMA (21). However, the current study shows that PrrF is required for AMA in shaking co-
434 cultures, while being dispensable for AMA in sessile cultures. We further showed that loss of the
435 Δ*prrF* mutant AMA phenotype during static conditions was not due to decreased oxygen, as the
436 Δ*prrF* mutant behaved similarly in microaerobic and aerobic shaking cultures. Moreover,
437 proteomics showed that iron and PrrF regulatory pathways are altered in static versus shaking
438 conditions, and revealed T6SS as a novel iron and PrrF responsive system in static conditions.
439 These results demonstrate that culture agitation has substantial impacts on *P. aeruginosa*
440 global iron homeostasis, which may further impact polymicrobial interactions and virulence
441 factor expression.

Previous reports showed that concentrations of PQS molecules were enhanced when *P. aeruginosa* is grown in the presence of *S. aureus*-derived N-acetylglucosamine (GlcNAc) (65). Our lab showed that the Δ*prrF* mutant exhibits a similar increases in PQS concentrations when co-cultured with the *S. aureus* strain, M2 (21), which we hypothesized had occurred via a similar mechanism. Importantly, analyses of a Δ*pqsH* mutant in the PA14 strain background by our lab and others showed that PQS is not required for AMA against *S. aureus*, either in static or biofilm growth (22, 23), and our current analysis shows that a PAO1 transposon mutant of *pqsH* similarly retains AMA in shaking and static conditions (**Fig. S1**). In the current report, we further examined whether PQS and other AQ metabolites were restored in the *P. aeruginosa* Δ*prrF* strain grown in the presence of *S. aureus*. Our results showed that *S. aureus* strain M2, but not USA300, restored PQS production to the Δ*prrF* mutant, and that neither strain of *S. aureus* restored levels of other AQs to the Δ*prrF* mutant (**Supplementary Table S3)**. Moreover, we observed no increase in AQ levels in the wild type PAO1 strain upon co-culture with either strain of *S. aureus* (**Supplementary Table S3)**. Combined, these results demonstrate that strain selection can alter the effects of co-culture on *P. aeruginosa* AQ production, and further demonstrate the importance of assessing quantities all AQs to define the mechanisms underlying polymicrobial interactions.

Since static growth is known to reduce oxygen availability, we next investigated the role of oxygen limitation in altering PrrF's effects on AQ production and AMA. In doing so, we observed that levels of the oxygen-dependent AQNO and PQS molecules (66, 67) were significantly reduced in both static and microaerobic shaking conditions as compared to aerobic shaking conditions. Strikingly, the quorum signaling AHQ molecules were substantially increased in both the wild type and Δ*prrF* mutant grown in static conditions, but not in microaerobic shaking conditions. Thus, despite a significant decrease in AHQs in the static Δ*prrF* mutant versus wild type cultures, the levels of AHQs in the static Δ*prrF* cultures remained relatively high in comparison to the aerobic or microaerobic shaking Δ*prrF* cultures (**Fig. 2**).

These results corresponded to the Δ*prrF* mutant retaining AMA in static conditions, while showing a defect in AMA in aerobic and microaerobic conditions (**Fig. 3**). These results suggest that increased AHQ production during static growth diminishes the impact of PrrF on AMA. Moreover, these results demonstrate that reduced oxygenation is not solely responsible for the altered phenotype of the Δ*prrF* mutant in static conditions.

In light of the distinct impacts of sessile growth on *P. aeruginosa* iron regulation, which are not mediated by oxygen limitation alone, this study establishes the need for additional research of iron regulatory mechanisms under sessile growth conditions. Indeed, a vast majority of iron regulation studies in *P. aeruginosa* have been conducted in planktonic cultures grown under shaking conditions, which bear little resemblance to the conditions observed during *P. aeruginosa* infections. Whereas aerobic shaking cultures are exposed to constant aeration and perturbation, *P. aeruginosa* infections are typically dynamic, and feature planktonic as well as sessile communities of *P. aeruginosa*, particularly during chronic infections. Contact-dependent interactions are likely highly variable under these conditions, as is quorum signaling activity. Furthermore, sessile cultures are capable of excreting a viscous matrix of extracellular DNA and exopolysaccharides during infection that form multicellular structures, known as biofilms (68). The formation of biofilms allows for steep concentration gradients of critical micronutrients, resulting in a high degree of physiological heterogeneity and variation in bacterial gene expression (58, 69-73). Our results suggest that the complex environmental cues that occur under sessile growth can influence iron regulatory networks, thus influencing the production of different virulence factors and metabolic pathways.

It is important to note that while *P. aeruginosa* has been shown to enhance production of virulence factors in response to iron starvation in shaking cultures, iron-regulation has not been well characterized during static growth. In this report we provide initial characterizations of these changes, revealing significant shifts in iron regulation of various virulence and AMA-associated proteins, including those for pyochelin and phenazine biosynthesis, as well as T6SS (**Fig. 6**).

494 Contrary to previous studies, in which iron starvation diminished phenazine biosynthesis gene
495 expression and phenazine production (38), we observed that iron limitation enhanced
496 expression of phenazine biosynthesis genes in our sessile cultures. It is unclear why iron
497 regulation of phenazines is altered in these different growth conditions, although it may be
498 related to the dynamic role of phenazines during cellular growth. For instance, it has been
499 shown that phenazines can induce auto-poisoning and extracellular DNA release in aerobic and
500 anaerobic cultures, which is further enhanced by nutrient depletion (74). However, phenazines
501 also contribute to iron acquisition and survival due to their inherent redox-cycling activity, by
502 which they are capable of catalyzing the formation of bioavailable ferrous iron from ferric iron
503 (75, 76). This may be especially pertinent in static conditions, where iron requirements for
504 biofilm formation are shown to be enhanced (77). Phenazines are also a contributing factor to *P.*
505 *aeruginosa* virulence during infection (78, 79), and induction of these biosynthetic pathways in
506 iron-depleted conditions may be part of an adaptive virulence response to low iron static
507 conditions. In support of this idea, we see that expression of other virulence-associated genes,
508 such as pyochelin synthesis genes, are more strongly upregulated in static as compared to
509 shaking conditions. Iron-bound pyochelin, in conjunction with the phenazine, pyocyanin, has
510 been shown to produce reactive oxygen species that can damage surrounding cells and tissue
511 during infection (80-83). In this case, phenazine and pyochelin production in sessile growth may
512 contribute to the extracellular release of iron by host cells and co-colonizing pathogens during
513 infection.

514 Expression of T6SS proteins were also increased by iron depletion in sessile cultures,
515 particularly proteins encoded by the HSI-II gene locus. T6SS in *P. aeruginosa* contributes to
516 virulence in several models of infection (84, 85), and facilitates inter-bacterial interactions in *P.*
517 *aeruginosa* and other bacterial species (86, 87). The cell-to-cell interactions mediated by T6SS
518 are contact dependent (86) and likely disrupted in shaking cultures. In turn, we hypothesized
519 that heightened iron regulation of HSI-II T6SS may correspond to increased contribution of

these systems to virulence and interbacterial interactions. Our analysis of a *clpV2* transposon mutant suggest that HSI-II T6SS contributes to AMA in sessile cultures but not in perturbed cultures (**Fig. 7**). Interestingly, an earlier study showed that T6SS is induced by the AQ-dependent regulator, PqsR (84), thus it is possible that T6SS provides a novel link between AQs and iron-regulated AMA during sessile cultures. Importantly, we note that interpretation of these results was complicated by weak AMA of the parental mPAO1 strain, thus further analyses are needed to carefully define the role of T6SS in iron-regulated interactions between *P. aeruginosa* and *S. aureus*.

Based on our co-culture studies, we were interested in the possible effects of static culture on PrrF-regulated expression of genes and proteins for anthranilate catabolism. PrrF sRNAs were previously shown to promote the production of PQS and other AQs through repression of anthranilate catabolism pathways in shaking cultures (32, 35). Surprisingly, our proteomics data did not reveal PrrF regulation of anthranilate metabolism proteins under the shaking conditions used in these studies, even though the mRNAs encoding these proteins were clearly regulated by iron and PrrF. Interestingly, we observed PrrF-independent iron regulation of both the anthranilate degradation mRNAs and proteins in sessile cultures. Nonetheless, the PrrF sRNAs were still required for optimal AQ production in most conditions that we tested, indicating a key role for these RNAs in modulating AQ metabolism. In contrast, a recent study in another strain of *P. aeruginosa* showed that PrrF is not required for AQ production in tobramycin-induced biofilms (88), although it is unclear whether this is due to a strain difference, or if PrrF simply does not impact AQ metabolism during biofilm growth.

Biofilms are an important adaptive feature of *P. aeruginosa* infection, and the appearance of robust biofilm-producing *P. aeruginosa* isolates during chronic lung infections is associated with increased mortality in CF patients (4, 89, 90). Iron availability plays a critical role in the ability of *P. aeruginosa* to form and maintain biofilms during infection, and virulence factors such as siderophores, phenazines, and antimicrobial exoproducts are thought to

546 contribute to iron homeostasis in these infections. *P. aeruginosa* mediates tight regulation of
547 these virulence factors in response to iron availability, although these regulatory networks have
548 not been fully characterized in sessile communities or biofilms. While previous work by our lab
549 and others have helped characterize some of these regulatory mechanisms in shaking cultures,
550 the results described herein highlight the need for further study of these iron regulatory
551 mechanisms in conditions that more closely reflect conditions in the host. Studies defining iron
552 regulation in biofilms and other complex communities are likely to reveal many more striking
553 physiological adaptations that are critical for chronic *P. aeruginosa* infections.
554

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561

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FIGURES AND FIGURE LEGENDS

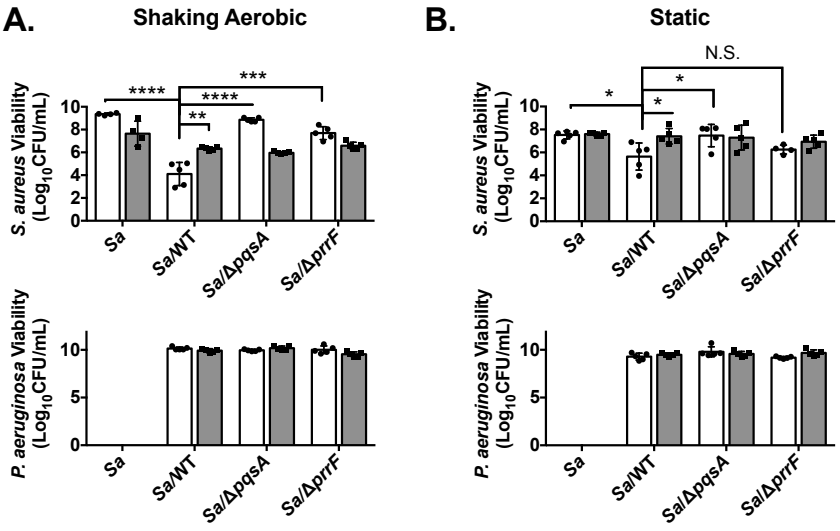


Figure 1. PrrF-mediated AMA is altered in shaking conditions. *P. aeruginosa* and *S. aureus* were co-inoculated in dialyzed trypticase soy broth (DTSB) media supplemented with (high iron) or without (low iron) 100μM $FeCl_3$. Co-cultures were incubated either in 14mL round bottom polystyrene cell culture tubes in shaking aerobic conditions (A) or in 6 well polystyrene cell culture plates in static conditions (B) for 18 hours. After incubation, colony forming units (CFUs) were enumerated as described in the **Materials and Methods**. Bars in each graph indicate the average value in low (white bars) or high (gray bars) iron conditions, and individual data points from biological replicates are indicated by circles (low iron) or squares (high iron). Error bars indicate the standard deviation of 5 independent experiments. Asterisks indicate a significant difference as determined by a two-tailed Student's *t* test as follows: *p≤0.05; **p≤0.005; ***p≤0.0005; ****p≤0.00005.

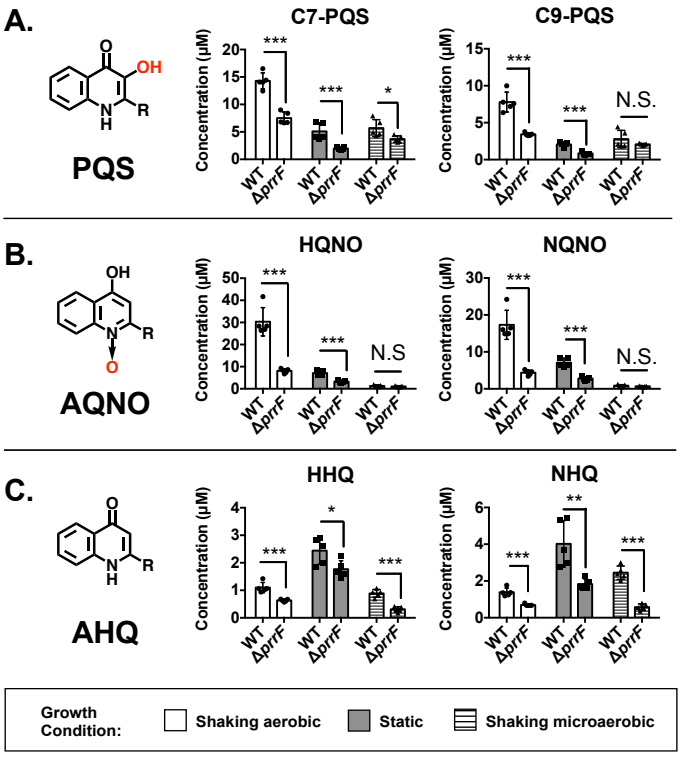


Figure 2. Concentrations of AQs in shaking, static, and microaerobic cultures of *P. aeruginosa.* Cultures were grown in 1.5 mL of iron-deplete DTSB media in 14mL polystyrene round bottom culture tubes and incubated for 18 hours at 37˚C. Microaerobic cultures were sealed in an air-tight GasPak™ system in the presence of an EZ Campy Container™ packet. Shaking and microaerobic cultures were incubated with perturbation (250rpm). Sessile cultures were incubated in shaking incubators with no perturbation (0rpm). Representative AQ core structures are depicted in black, with oxygen-dependent structural elements highlighted in red (R indicates alkyl chain). AQs were quantified by LC-MS/MS where bars in each graph indicate the average concentration of 5 independent experiments and individual data points from biological replicates are indicated by black circles (Error bars represent S.D.) Asterisks indicate a significant difference as determined by a two-tailed Student's *t* test as follows: *p<0.05; **p<0.005; ***p<0.0005.

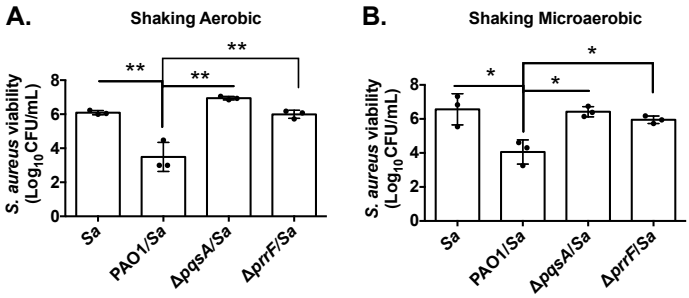


Figure 3. *P. aeruginosa* AMA requirement for PrrF is not influenced by oxygen availability in co-culture with *S. aureus*. *P. aeruginosa* and *S. aureus* were co-inoculated into 1.5 mL of iron-deplete dialyzed trypticase soy broth (DTSB) media in 14mL round-bottom polystyrene culture tubes. Cultures were incubated for 18hr at 37˚C in either (A) shaking aerobic or in (B) shaking microaerobic conditions. Microaerobic cultures were incubated in air-tight GasPak systems™ in the presence of a GasPak EZ Campy Container™ packet to ensure microaerobic conditions. After 18hr incubation, colony forming units (CFUs) were enumerated as described above. Bars in each graph indicate the average value, and individual data points from biological replicates are indicated with circles. Error bars indicate the standard deviation of 5 independent experiments. Asterisks indicate a significant difference as determined by a two-tailed Student's *t* test as follows: *p≤0.05; **p≤0.005.

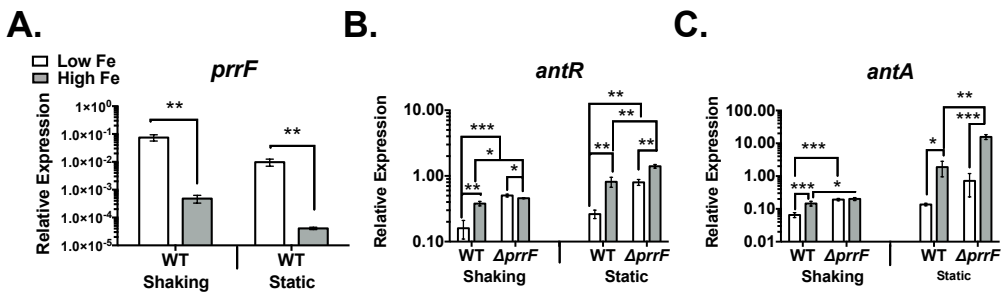


Figure 4. PrrF expression and regulation of anthranilate catabolism genes in shaking and static conditions. Wild type PAO1 and Δ*prrF* mutant strains were incubated in 1.5mL dialyzed trypticase soy broth (DTSB) supplemented with either low iron (white bars) or high iron (gray bars) in 14mL round bottom polystyrene culture tubes. Cultures were incubated for 18hr at 37˚C in either shaking aerobic conditions or static conditions prior to RNA extraction (**Methods and Materials).** Levels of the (A) *prrF*, (B) *antR*, and (C) *antA* RNAs were measured using qRT-PCR. Bars in each graph indicate the average value of 5 independent experiments (error bars represent standard deviation). Asterisks indicate a significant difference as determined by a two-tailed Student's *t* test as follows: *p<0.05; **p<0.005; ***p<0.0005.

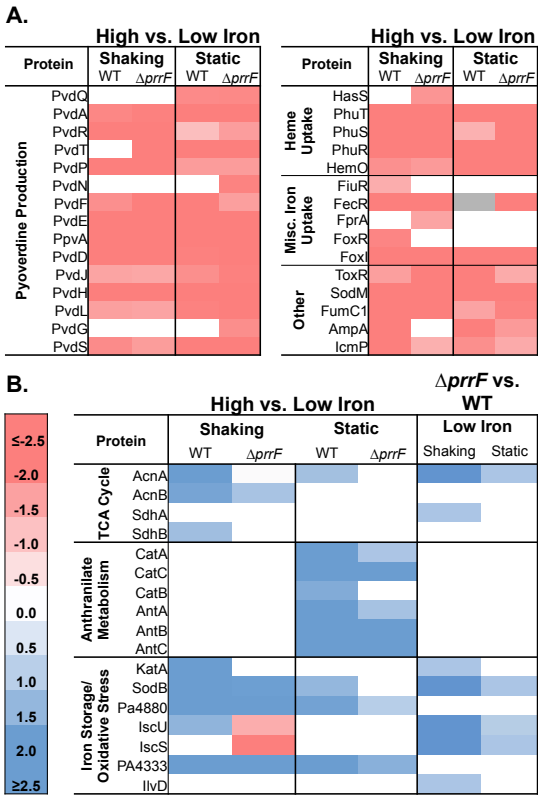
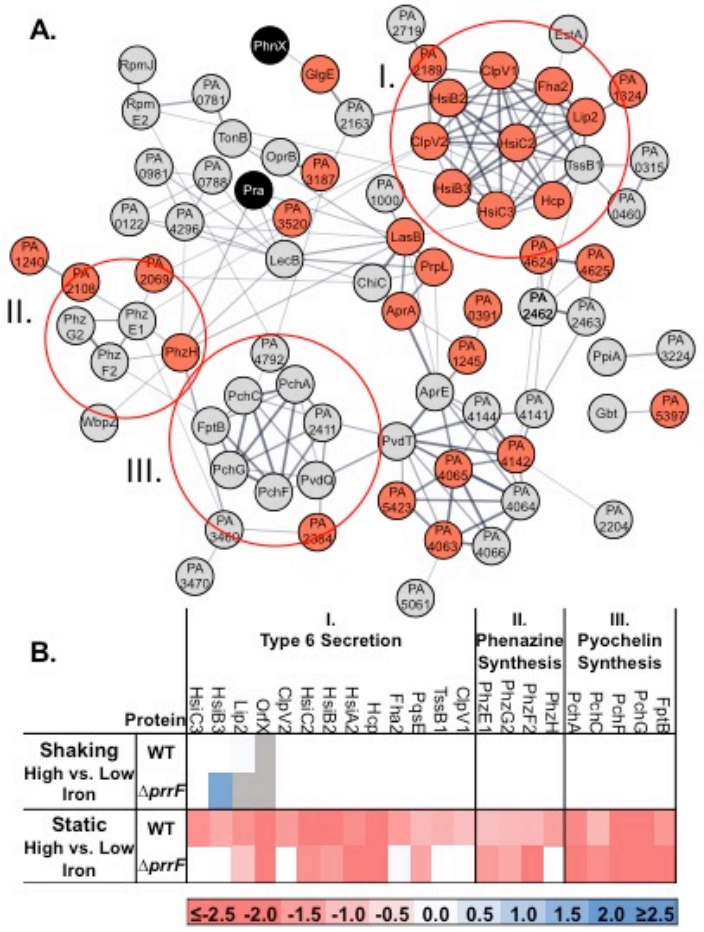


Figure 5. Static growth reduces the effects of classical PrrF regulation. Heatmaps showing the $\log_2$ fold change (LFC) of **(A)** known iron-repressed proteins and **(B)** known PrrF-regulated proteins, in the indicated strains grown in high versus low iron conditions or in the Δ*prrF* mutant versus wild type PAO1 grown in low iron conditions. Wild type PAO1 and Δ*prrF* strains were inoculated into 1.5mL DTSB supplemented with either iron (0μM) or high iron (100μM) and incubated in shaking or static conditions. LC-MS/MS-based proteomics analysis showed statistically significant protein regulation (FDR adjusted p-value < 0.05) of known iron and PrrF targets, as indicated by at least 2-fold induction or repression (i.e. 1 $\log_2$ fold change) in response to treatment. Gray boxes indicate proteins that were undetected in one or more condition. White boxes indicate no significant change in gene expression was observed.



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888 **Figure 6. Proteomics reveals novel targets of iron regulation in static conditions.** Wild
889 type PAO1 and the Δ*prrF* strain were inoculated into 1.5mL DTSB media and supplemented
890 with either low iron (0μM) or high iron (100μM) and incubated in either shaking or static
891 conditions. Statistically significant iron regulation (FDR adjusted p-value < 0.05) in static and
892 shaking conditions was identified by at least 2-fold (i.e.1 $\log_2$ fold change) induction in response
893 to iron starvation as described in **Materials and Methods**. (A) Network analysis of differentially
894 regulated genes was carried out using STRING database software, which revealed distinct
895 several virulence-associated genes that were similarly impacted by iron starvation under static
896 conditions. Line thickness indicates strength of data support for association between two
897 proteins, as calculated by STRING network analysis (55). Red nodes represent genes which
898 were significantly regulated by PrrF in low iron static conditions, gray nodes indicated genes that

899 do not exhibit regulation by PrrF. Black nodes represent genes that exhibited iron regulation in
900 static conditions but were not detected in one or more iron conditions in shaking cultures. (B)
901 Heatmaps of differentially regulated genes from type 6 secretion (T6SS), phenazine, and
902 pyochelin biosynthetic operons. Gray boxes indicate proteins that were not detected in one or
903 more condition. White boxes indicate that no significant change in gene expression was
904 observed.
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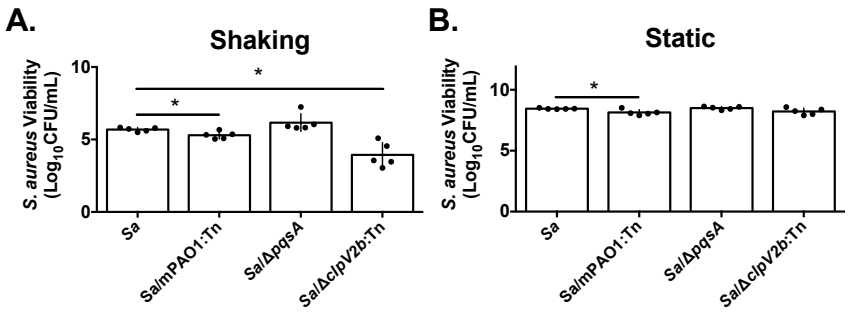

Figure 7. T6SS potentially impacts *P. aeruginosa* AMA. *P. aeruginosa* and *S. aureus* were co-inoculated in dialyzed trypticase soy broth (DTSB) media supplemented with (high iron) or without (low iron) 100μM $FeCl_3$. Co-cultures were incubated in 6 well polystyrene cell culture plates in static conditions for 18 hours. After incubation, colony forming units (CFUs) of *S. aureus* (A) and *P. aeruginosa* (B) were enumerated as described in the **Materials and Methods**. Bars in each graph indicate the average value, and individual data points from biological replicates are indicated by black circles. Error bars indicate the standard deviation of 5 independent experiments. Asterisks indicate a significant difference as determined by a two-tailed Student's *t* test with a significance threshold of *p≤0.05.