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

Subject Area: Microbiology and Infectious Disease

Luke K. Brewer[1], Weiliang Huang[1], Maureen A. Kane[1], Amanda G. Oglesby-Sherrouse[1,2]*

University of Maryland, Baltimore, [1]School of Pharmacy, Department of Pharmaceutical Sciences and [2]School of Medicine, Department of Microbiology and Immunology, Baltimore, Maryland, 21201

*To whom correspondence should be addressed: aoglesby@rx.umaryland.edu

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.

29 **INTRODUCTION**

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

47 *P. aeruginosa* secretes multiple toxins and bio-active metabolites, which cause damage
48 to host tissue and exhibit antimicrobial activity (AMA) against competing microorganisms.
49 Amongst these is a collection of small secreted metabolites collectively referred to as 2-alkyl-
50 4(1*H*)-quinolones (AQs), which mediate a range of toxic activities against *S. aureus* and are
51 thought to drive interactions between these two species during infection (20-23). *2*-alkyl-4-
52 quinolone N-oxides (AQNOs) are potent cytochrome inhibitors that obstruct respiratory
53 metabolism in *S. aureus* (24-26). 2-alkyl-3-hydroxy-quinolone, which is more commonly referred
54 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

81 the agar plate and transwell co-culture assays, precluding a more thorough examination of the
82 factors required for iron-regulated AMA.
83 In the current study, we sought to develop a co-culture assay that would allow us to
84 examine the impact of PrrF and iron on gene expression, AQ production, and AMA under a
85 variety of growth conditions. Analysis of metabolite levels, gene expression, and viability
86 demonstrated that culture perturbation causes phenotypic changes in the *P. aeruginosa* Δ*prrF*
87 mutant during co-culture with *S. aureus*. Proteomics further demonstrated that culture
88 perturbation alters the activity of some iron homeostasis pathways in *P. aeruginosa*, including
89 PrrF-regulated expression of anthranilate metabolism proteins. Analysis of microaerobic co-
90 cultures indicated that changes in the Δ*prrF* mutant phenotypes during sessile culture were not
91 solely due to decreased oxygen tensions. These studies indicate that sessile bacterial
92 communities use distinct regulatory and metabolic networks to adapt to decreased nutrient
93 availability, likely affecting the production of key virulence determinants and polymicrobial
94 interactions.
95
96 **MATERIALS AND METHODS**
97 **Bacterial strains and growth conditions.** Bacterial strains used in this study are listed
98 in Supplementary Table 1. Lysogeny broth (LB) and agar (LA) were prepared using 10g/L NaCl
99 (Sigma, St Louis, MO), 10g/L tryptone, and 5g/L yeast extract, 15g/L agar (when applicable)
100 (Becton-Dickinson, Franklin Lakes, NJ). *P. aeruginosa* and *S. aureus* strains were both routinely
101 grown on LA from freezer stock. Five isolated colonies of each strain were selected from
102 overnight-incubated agar plates and inoculated into 5mL LB. For iron starvation studies,
103 bacterial strains were grown in a Chelex-treated and dialyzed trypticase soy broth (DTSB)
104 prepared as previously described (33). Media dialysis was carried out using Spectra/Por®2
105 dialysis membrane tubing (29mm diameter) with a molecular weight cutoff of 12-14kD
106 (Repligen, Waltham, Ma). For co-culture assays, quantitative real time PCR (qRT-PCR)

107 analysis, and mass spectrometry-assisted metabolite and proteome studies, *S. aureus* and *P.*
108 *aeruginosa* cultures were diluted into 1.5mL of DTSB with or without 100μM (high iron) $FeCl_3$
109 supplementation, to an absorbance (OD_{600}) of 0.08 and 0.05, respectively. Monocultures and
110 co-cultures of *P. aeruginosa* and *S. aureus* were prepared in DTSB media and incubated at
111 37˚C for 18 hours in a shaking incubator. Shaking aerobic cultures were incubated in 1.5mL of
112 DTSB media in 14 mL round bottom tubes and closed with a foam stopper to allow for sufficient
113 aeration. Sessile cultures were incubated in 1.8mL DTSB media in 6-well polystyrene culture
114 plates, which were covered in breathe-easy wrap to prevent evaporation. During incubation,
115 cultures were grown at a shaking rate of either 250rpm (shaking conditions) or 0rpm (static
116 conditions). Microaerobic co-cultures were grown in identical growth conditions as shaking
117 cultures, except culture tubes were placed into an air-tight candle jar in the presence of a
118 CampyPak (BD Diagnostics, NJ, USA). Sealed candle jars were secured in a shaking incubator
119 and incubated for 18 hours at 37˚C and a shaking rate of 250rpm.
120
121 **Colony forming unit determination.** 400uL of *P. aeruginosa* and *S. aureus* mono- and
122 co-cultures were harvested at 15000rcf for 5 minutes. Cell pellets were resuspended in 400uL of
123 0.1% Triton-X in phosphate-buffered saline and vortexed rigorously. From these resuspensions,
124 serial dilutions were prepared in 0.1% Triton-X in PBS. 10uL of each dilution was spotted onto
125 Baird-Parker agar plates and Pseudomonas Isolation Agar (PIA) plates to select for *S. aureus*
126 and *P. aeruginosa* growth, respectively. Upon spotting 10uL of dilutions onto agar plates, the
127 plates were tilted to facilitate even drips across the plate surface. PIA plates were incubated at
128 37˚C for 18 hours and colony forming units (CFU) were counted. Baird-Parker plates were
129 incubated for 37˚C for 48 hours to allow for adequate growth of *S. aureus* small-colony variants
130 observed in some assays prior to counting CFUs.
131

132 **Quantitation of AQs using liquid chromatography-tandem mass spectrometry.** AQ
133 quantitation was carried out as previously reported (45). Briefly, 300uL of culture were
134 harvested and spiked with a 25μM stock nalidixic acid internal standard to a final concentration
135 of 500nM, followed by an extraction using ethyl acetate w/ 0.1% acetic acid. The organic phase
136 containing extracted AQs was harvested from each sample, dried down, and resuspended in
137 300uL of 0.1% formic acid suspended in1:1:1 (v/v/v) mixture of methanol, water, and
138 acetonitrile. Extracts were analyzed via quantitative liquid chromatography-tandem mass
139 spectrometry (LC-MS/MS) using multiple reaction monitoring performed on a Dionex Ultimate
140 3000 TSQ tandem quadrupole mass spectrometer in positive ion mode**.**
141
142 **Real time PCR analysis.** Quantitative real time PCR (qRT-PCR) analysis was
143 conducted on three biological replicates of the PAO1 reference strain and isogenic Δ*prrF* mutant
144 grown in DTSB supplemented with either 100μM or 0μM iron. *P. aeruginosa* cells were lysed
145 after 18 hours using 2.5mg/mL lysozyme and incubated at 37˚C for 30 minutes. RNA extraction
146 was performed using the RNeasy mini kit (QIAGEN, Germantown, MD). Real-time qualitative
147 PCR analysis was performed as described previously (35) using an Applied Sciences StepOne
148 Plus Real Time PCR System (Life Technologies, Carlsbad, CA). Primer and probe sequences
149 used for gene expression analysis are listed in **Supplementary Table S2**. Quantitation of cDNA
150 was carried out using standard curves compiled for individual gene targets, and *P. aeruginosa*
151 expression data was normalized to *oprF* expression in aerobic conditions, or *omlA* in
152 microaerobic and anaerobic conditions.
153
154 **Quantitative label-free proteomics.** Cell cultures were prepared for proteomics
155 analysis as described previously (46). Briefly, cells were harvested by centrifugation and
156 washed in phosphate-buffered saline prior to lysis in 4% sodium deoxycholate. Lysates were
157 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

184 **RESULTS**

185 **PrrF is required for AMA against *S. aureus* in shaking co-cultures.** Previous co-
186 culture studies performed in our lab examined the role of *P. aeruginosa* AMA against *S. aureus*
187 in Chelex-treated and dialyzed tryptic soy broth (DTSB) supplemented with or without 100 μM
188 $FeCl_3$ (21, 22). These previous assays were performed using either agar plate or liquid transwell
189 co-culture systems. For agar co-cultures, *P. aeruginosa* strains were spotted onto confluent
190 lawns of *S. aureus* on agar plates; for liquid transwell co-cultures, *P. aeruginosa* and *S. aureus*
191 strains were co-incubated in transwell microtiter plates, with each species separated by a 0.2μm
192 permeable transwell membrane (21). In each of these assays, *P. aeruginosa* demonstrated iron-
193 regulated AMA against *S. aureus* that was dependent upon the *pqsA* gene, confirming the
194 expected requirement for AQs. However, the *prrF* locus was not required for AMA in either of
195 these assays (21). While analysis of shaking cultures showed that co-culture with *S. aureus*
196 restored PQS production to the Δ*prrF* mutant (21), attempts to quantify AQs and analyze gene
197 expression in the transwell plates and agar co-cultures were unsuccessful, preventing a more
198 mechanistic analysis of how iron and PrrF affected AMA under these specific conditions.

199 We therefore grew mono- and co-cultures of *P. aeruginosa* strain PAO1 and *S. aureus*
200 strain USA300 in a larger volume (1.5 mL) of DTSB, supplemented with or without 100μM
201 $FeCl_3$, in a shaking incubator at 250 rpm. Viability of *P. aeruginosa* and *S. aureus* was then
202 quantified by enumerating colony forming units on selective Pseudomonas isolation agar (PIA)
203 and Baird Parker agar media, respectively. In agreement with earlier studies showing that AQs
204 mediate AMA against *S. aureus*, USA300 viability was reduced approximately 50,000-fold
205 during low iron co-culture with PAO1 but not during co-culture with the Δ*pqsA* mutant (**Fig. 1A**).
206 Also in agreement with our earlier work, iron limitation significantly enhanced AMA of PAO1
207 against USA300 (**Fig. 1A**). Surprisingly, the Δ*prrF* mutant was defective for AMA relative to the
208 isogenic parent strain PAO1 (**Fig. 1A**), a result that contrasted with our previously reported agar
209 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

235 **PrrF is not required for AMA against *S. aureus* in sessile cultures**. We next
236 wondered if different results for the Δ*prrF* mutant obtained in the current (**Fig. 1A**) and previous
237 studies (21) were due to differences in culture perturbation during incubation. Specifically,
238 cultures using the agar and transwell systems were grown in static conditions, whereas the
239 cultures for the studies described above were grown in shaking conditions. We hypothesized
240 that culture agitation altered the impact of PrrF on AQ-mediated AMA. To test this, we grew
241 mono-cultures and co-cultures of *P. aeruginosa* and *S. aureus* in DTSB supplemented with or
242 without $FeCl_3$ under static conditions. Initially, these studies were performed with 15mL
243 polystyrene culture tubes, but this eliminated AMA for all *P. aeruginosa* strains (**Fig. S2)**. This
244 was presumably due to *S. aureus*, which is non-motile, settling at the bottom of the tubes and
245 distal from the motile *P. aeruginosa* strains growing near the air-liquid interface. In order to
246 facilitate interactions between *S. aureus* and *P. aeruginosa,* static co-cultures were instead
247 prepared in six-well polystyrene cell culture plates, allowing for a shallower liquid depth and
248 larger air-liquid interface, covered in breathe-easy wrap. While AMA was overall less robust in
249 static conditions, we observed iron-regulated AMA against *S. aureus* by the wild-type PAO1
250 strain, as well as a complete loss of AMA in the Δ*pqsA* mutant (**Fig. 1B**). In contrast to the
251 shaking assay, however, viability of *S. aureus* grown with the Δ*prrF* mutant in low iron conditions
252 was not significantly different than when grown with the wild-type PAO1 strain (**Fig. 1B**),
253 suggesting that PrrF-mediated AMA is altered during sessile growth. Importantly, changes in *S.*
254 *aureus* CFU in this static assay were comparable to *S. aureus* culture densities previously
255 obtained with transwell co-cultures (21). Thus, our data indicate that culture perturbation alters
256 the factors that regulate *P. aeruginosa* AMA against *S. aureus* during co-culture.
257
258 **AHQ concentrations are increased in static growth conditions.** We next determined
259 if PrrF was required for optimal AQ production during static growth. To test this, we used a
260 recently validated LC-MS/MS method (45) to measure levels of various AQ species in shaking

261 and sessile cultures of wild-type *P. aeruginosa* and the Δ*prrF* mutant. As previously observed
262 (21, 32), PrrF was required for optimal production of the C7 and C9 congeners of PQS, AQNO,
263 and AHQ in shaking conditions (**Fig. 2A,B,C, white bars**). Surprisingly, PrrF was also required
264 for optimal production of each of these AQs in static growth conditions (**Fig. 2A,B,C, gray**
265 **bars**). However, levels of both the C7 and C9 congeners of PQS and AQNO were substantially
266 decreased in static conditions relative to shaking conditions, and were in fact similar or lower
267 than concentrations produced by the Δ*prrF* mutant in shaking cultures (**Fig. 2A,B, white versus**
268 **gray bars**). This is likely due to the previously reported oxygen requirement for activity of the
269 PqsH and PqsL enzymes. Moreover, these data suggest that the relative impact of these AQ
270 congeners on AMA is lessened in sessile cultures. This idea is supported by data observed in
271 static co-cultures of *S. aureus* and a *P. aeruginosa* Δ*pqsL* mutant, which demonstrate that the
272 Δ*pqsL* still exhibits AMA in static conditions but is devoid of AMA in shaking conditions (**Fig.**
273 **S3B**). Notably, levels of HHQ and NHQ were significantly increased in static cultures, with
274 concentrations in the Δ*prrF* cultures equivalent or higher than that of the shaking wild type
275 culture (**Fig. 2C, white versus gray bars**). Thus, while the Δ*prrF* mutant still exhibits an overall
276 defect in AQ production during static growth, it may produce sufficient levels of AHQs to
277 mediate AMA under these conditions.
278
279 **AMA requirement for PrrF in shaking cultures is not impacted by oxygen**
280 **availability.** Previous reports have demonstrated that static growth can have dramatic impact
281 on broad aspects of *P. aeruginosa* physiology, including expression of virulence traits. These
282 reports have suggested that diminished oxygen availability in static conditions plays a defining
283 role in mediating these effects (56-58). In agreement with this, LC-MS/MS analysis of static and
284 shaking cultures revealed that oxygen-dependent PQS and AQNO molecules were produced at
285 significantly lower concentrations in sessile cultures than in shaking (**Fig. 2A,B, white bars**
286 **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

313 **Iron regulation of anthranilate catabolism genes is retained during static growth.**
314 The above data demonstrate that the PrrF sRNAs promote production of AQs in both shaking
315 and sessile cultures. In shaking conditions, PrrF contributes to AQ production via repression of
316 anthranilate metabolism genes (32, 33). It is unclear, however, whether PrrF modulates AQ
317 production in static conditions via the same metabolic pathways. To test this, first we performed
318 targeted expression analysis of PrrF sRNAs and known PrrF-regulated genes in both static and
319 shaking cultures of wild type *P. aeruginosa* using quantitative real time PCR (qRT-PCR). PrrF
320 expression was markedly lower in sessile cultures as compared to shaking cultures, but it was
321 still significantly repressed by iron supplementation **(Fig. 3A).** We next examined levels of the
322 *antA* mRNA, encoding for a component of the anthranilate dioxygenase that degrades
323 anthranilate, as well as *antR*, encoding a transcriptional activator of *antABC* that is directly
324 repressed by PrrF (32). We observed iron-activated expression of both *antA* and *antR* when
325 PAO1 was grown in either shaking or static conditions (**Fig. 3B-C**). As previously observed (32,
326 33), levels of *antA* and *antR* were significantly increased in the Δ*prrF* mutant compared to wild
327 type PAO1 when grown in low iron shaking conditions, resulting in loss of iron regulation of
328 *antR*. Interestingly, while expression of both *antR* and *antA* was derepressed in the Δ*prrF*
329 mutant grown under static conditions, iron still activated each of these genes in sessile cultures
330 of the Δ*prrF* mutant **(Fig. 3B-C)**. These results indicate that additional factors contribute to iron-
331 regulated expression of the anthranilate degradation pathway under static conditions.
332
333 **Proteomic analysis reveals PrrF-independent iron regulation of anthranilate**
334 **metabolic proteins in static conditions.** The studies above indicate that iron regulates AMA in
335 a PrrF-independent manner under static growth conditions. However, our current knowledge of
336 *P. aeruginosa* iron regulation is largely restricted to shaking cultures. To better understand
337 changes in PrrF and iron regulation in sessile cultures *of P. aeruginosa*, we applied a label-free,
338 LC-MS/MS-based proteomics methodology recently described by our laboratories (46). Using

339 this unbiased approach, we determined the proteomes of static and shaking cultures of PAO1
340 and the isogenic Δ*prrF* mutant grown in DTSB cultures supplemented with or without 100μM
341 $FeCl_3$. Mass spectra were identified using a PAO1 reference proteome, which were in turn
342 validated to a false discovery rate of 0.01 to ensure proper assignment of protein identities.
343 Iron regulation under shaking conditions was robust, with over 178 proteins significantly
344 repressed by iron and 191 proteins induced by iron (**Supplementary Dataset 1**). As previously
345 observed (40, 44, 59-63), iron supplementation significantly reduced proteins for the
346 siderophore (pyoverdine and pyochelin) and heme uptake systems, as well as several iron-
347 regulated virulence factors, during shaking growth (**Fig. 5A**). Well-known PrrF-repressed
348 proteins involved in the tricarboxylic acid (TCA) cycle and oxidative metabolism, including
349 aconitase A, succinate dehydrogenase B, and catalase, were induced by iron in a PrrF-
350 dependent manner under shaking conditions (**Fig. 5B**) (33, 64). We also observed PrrF-
351 dependent iron regulation of more recently identified PrrF targets in shaking conditions, such as
352 genes involved in Fe-Sulfur cluster biogenesis (IscS, IscU) and amino acid metabolism (IlvD)
353 (**Fig. 5B**) (46). Contrary to previous real-time PCR and microarray analyses showing iron
354 induction of the *antABC* mRNAs (46), neither iron supplementation nor *prrF* deletion significantly
355 affected levels of the AntABC proteins for anthranilate degradation under shaking conditions
356 (**Fig. 5B**). Overall these studies replicated many of the global iron regulatory pathways observed
357 in previous studies.
358 We next determined whether proteins that were regulated by iron and PrrF under
359 shaking conditions were similarly regulated during sessile growth. Iron repression of
360 siderophore and heme uptake proteins was largely retained during static growth, indicating that
361 these iron regulatory pathways are not altered under these conditions (**Fig. 5A**). In contrast, iron
362 and PrrF regulation of several proteins involved in the TCA cycle or oxidative stress protection
363 was reduced or eliminated in static conditions (**Fig. 5B**). We also noted robust iron induction of
364 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

391 T6SS proteins was either reduced or lost in the Δ*prrF* mutant (**Fig. 6B**). Moreover, we found that
392 *prrF* deletion had a negative effect on T6SS proteins in static but not shaking conditions
393 **(Supplementary Dataset S1).** The identification of T6SS as iron- and PrrF-regulated during
394 static but not shaking conditions was particularly intriguing, as this system is known to mediate
395 cell-to-cell interactions that might not be possible in shaking cultures. No complementarity
396 between the PrrF sRNAs and the mRNAs encoding these proteins, thus we hypothesize
397 expression of these proteins is indirectly regulated by PrrF.
398
399 **T6SS may play a role in AMA during static growth conditions.** We next
400 hypothesized that proteins that were induced by iron starvation in static conditions but not
401 shaking conditions may contribute to iron-regulated AMA during static growth. We therefore
402 tested the roles of phenazines, pyochelin, and T6SS on *P. aeruginosa* AMA in static conditions
403 using static agar co-cultures of *S. aureus* strain USA300. *S. aureus* strain USA300 was
404 swabbed onto agar plates containing low or high iron DTSB media, and wild-type *P. aeruginosa*
405 strains or their respective mutant strains were spotted onto the monolayers. A mutant in the
406 PA14 *P. aeruginosa* background, which is deleted for both operons encoding the core
407 phenazine biosynthesis proteins (Δ*phz*), demonstrated wild type levels of AMA against *S.*
408 *aureus* (**Fig. S4**). Likewise, a PAO1 deletion mutant defective for pyochelin siderophore
409 synthesis (Δ*pchEF*) demonstrated wild type levels of AMA. These data suggest that neither
410 phenazines nor pyochelin alone account for altered AMA in static conditions. Interestingly, a
411 siderophore deficient double mutant containing deletions of *pchEF* and the pyoverdine synthesis
412 gene *pvdA* was attenuated for AMA in these conditions (**Fig. S4**). Single deletion of the *pvdA*
413 gene did not eliminate AMA, however, suggesting that the composite effect of these two
414 siderophore is critical to AMA in static conditions.
415 Notably, a transposon mutant in *clpV2*, which encodes a key protein in the HSI-II T6SS,
416 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.

442 Previous reports showed that concentrations of PQS molecules were enhanced when *P.*
443 *aeruginosa* is grown in the presence of *S. aureus*-derived N-acetylglucosamine (GlcNAc) (65).
444 Our lab showed that the Δ*prrF* mutant exhibits a similar increases in PQS concentrations when
445 co-cultured with the *S. aureus* strain, M2 (21), which we hypothesized had occurred via a similar
446 mechanism. Importantly, analyses of a Δ*pqsH* mutant in the PA14 strain background by our lab
447 and others showed that PQS is not required for AMA against *S. aureus*, either in static or biofilm
448 growth (22, 23), and our current analysis shows that a PAO1 transposon mutant of *pqsH*
449 similarly retains AMA in shaking and static conditions (**Fig. S1**). In the current report, we further
450 examined whether PQS and other AQ metabolites were restored in the *P. aeruginosa* Δ*prrF*
451 strain grown in the presence of *S. aureus*. Our results showed that *S. aureus* strain M2, but not
452 USA300, restored PQS production to the Δ*prrF* mutant, and that neither strain of *S. aureus*
453 restored levels of other AQs to the Δ*prrF* mutant (**Supplementary Table S3)**. Moreover, we
454 observed no increase in AQ levels in the wild type PAO1 strain upon co-culture with either strain
455 of *S. aureus* (**Supplementary Table S3)**. Combined, these results demonstrate that strain
456 selection can alter the effects of co-culture on *P. aeruginosa* AQ production, and further
457 demonstrate the importance of assessing quantities all AQs to define the mechanisms
458 underlying polymicrobial interactions.
459 Since static growth is known to reduce oxygen availability, we next investigated the role
460 of oxygen limitation in altering PrrF's effects on AQ production and AMA. In doing so, we
461 observed that levels of the oxygen-dependent AQNO and PQS molecules (66, 67) were
462 significantly reduced in both static and microaerobic shaking conditions as compared to aerobic
463 shaking conditions. Strikingly, the quorum signaling AHQ molecules were substantially
464 increased in both the wild type and Δ*prrF* mutant grown in static conditions, but not in
465 microaerobic shaking conditions. Thus, despite a significant decrease in AHQs in the static
466 Δ*prrF* mutant versus wild type cultures, the levels of AHQs in the static Δ*prrF* cultures remained
467 relatively high in comparison to the aerobic or microaerobic shaking Δ*prrF* cultures (**Fig. 2**).

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

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

489 It is important to note that while *P. aeruginosa* has been shown to enhance production of
490 virulence factors in response to iron starvation in shaking cultures, iron-regulation has not been
491 well characterized during static growth. In this report we provide initial characterizations of these
492 changes, revealing significant shifts in iron regulation of various virulence and AMA-associated
493 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

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

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

541 Biofilms are an important adaptive feature of *P. aeruginosa* infection, and the
542 appearance of robust biofilm-producing *P. aeruginosa* isolates during chronic lung infections is
543 associated with increased mortality in CF patients (4, 89, 90). Iron availability plays a critical role
544 in the ability of *P. aeruginosa* to form and maintain biofilms during infection, and virulence
545 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

555 **ACKNOWLEDGMENTS**

556 This work was funded in part by the National Institutes of Health (NIH) grants R01 AI123320 (to
557 AGO-S) and T32 GM066706 (to LKB), and the University of Maryland, School of Pharmacy
558 Mass Spectrometry Center (SOP1841-IQB2014). We thank members of the Oglesby-Sherrouse
559 laboratory for editing of the manuscript, and Dr. Angela Wilks for thoughtful discussions and
560 feedback as the study progressed.
561

562 **REFERENCES**

563 1. Koch C (2002) Early infection and progression of cystic fibrosis lung disease. *Pediatr*
564 *Pulmonol* 34(3):232-236.10.1002/ppul.10135
565 2. Li Z*, et al.* (2005) Longitudinal development of mucoid Pseudomonas aeruginosa
566 infection and lung disease progression in children with cystic fibrosis. *JAMA* 293(5):581-
567 588.10.1001/jama.293.5.581
568 3. Paganin P*, et al.* (2015) Changes in cystic fibrosis airway microbial community
569 associated with a severe decline in lung function. *PLoS One*
570 10(4):e0124348.10.1371/journal.pone.0124348

571 4. Kerem E, Corey M, Gold R, & Levison H (1990) Pulmonary function and clinical course
572 in patients with cystic fibrosis after pulmonary colonization with *Pseudomonas*
573 *aeruginosa*. *J Pediatr* 116(5):714-719
574 5. Scheetz MH*, et al.* (2009) Morbidity associated with *Pseudomonas aeruginosa*
575 bloodstream infections. *Diagn Microbiol Infect Dis* 64(3):311-
576 319.10.1016/j.diagmicrobio.2009.02.006
577 6. Wisplinghoff H*, et al.* (2004) Nosocomial bloodstream infections in US hospitals: analysis
578 of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis*
579 39(3):309-317.10.1086/421946
580 7. Baldan R*, et al.* (2014) Adaptation of *Pseudomonas aeruginosa* in Cystic Fibrosis
581 airways influences virulence of *Staphylococcus aureus* in vitro and murine models of co-
582 infection. *PLoS One* 9(3):e89614.10.1371/journal.pone.0089614
583 8. Hoffman LR*, et al.* (2006) Selection for *Staphylococcus aureus* small-colony variants due
584 to growth in the presence of *Pseudomonas aeruginosa*. *Proceedings of the National*
585 *Academy of Sciences of the United States of America* 103(52):19890-
586 19895.10.1073/pnas.0606756104
587 9. Aliaga L, Mediavilla JD, Llosa J, Miranda C, & Rosa-Fraile M (2000) Clinical significance
588 of polymicrobial versus monomicrobial bacteremia involving *Pseudomonas aeruginosa*.
589 *Eur J Clin Microbiol Infect Dis* 19(11):871-874
590 10. Serra R*, et al.* (2015) Chronic wound infections: the role of *Pseudomonas aeruginosa*
591 and *Staphylococcus aureus*. *Expert Rev Anti Infect Ther* 13(5):605-
592 613.10.1586/14787210.2015.1023291
593 11. Barnabie PM & Whiteley M (2015) Iron-Mediated Control of Pseudomonas aeruginosa-
594 Staphylococcus aureus Interactions in the Cystic Fibrosis Lung. *Journal of bacteriology*
595 197(14):2250-2251.10.1128/JB.00303-15

596 12. DeLeon S*, et al.* (2014) Synergistic interactions of *Pseudomonas aeruginosa* and
597 *Staphylococcus aureus* in an in vitro wound model. *Infect Immun* 82(11):4718-
598 4728.10.1128/IAI.02198-14
599 13. Pastar I*, et al.* (2013) Interactions of methicillin resistant *Staphylococcus aureus* USA300
600 and *Pseudomonas aeruginosa* in polymicrobial wound infection. *PLoS One*
601 8(2):e56846.10.1371/journal.pone.0056846
602 14. Rosenbluth DB, Wilson K, Ferkol T, & Schuster DP (2004) Lung function decline in
603 cystic fibrosis patients and timing for lung transplantation referral. *Chest* 126(2):412-
604 419.10.1378/chest.126.2.412
605 15. Hendricks KJ*, et al.* (2001) Synergy between *Staphylococcus aureus* and *Pseudomonas*
606 *aeruginosa* in a rat model of complex orthopaedic wounds. *J Bone Joint Surg Am*
607 83(6):855-861.10.2106/00004623-200106000-00006
608 16. Foundation CF (2016) 2016 Patient Registry Annual Data Report. (Cystic Fibrosis
609 Foundation; Bethesda, Maryland).
610 17. Ballmann M, Rabsch P, & von der Hardt H (1998) Long-term follow up of changes in
611 FEV1 and treatment intensity during *Pseudomonas aeruginosa* colonisation in patients
612 with cystic fibrosis. *Thorax* 53(9):732-737.10.1136/thx.53.9.732
613 18. Skaar EP (2010) The battle for iron between bacterial pathogens and their vertebrate
614 hosts. *PLoS Pathog* 6(8):e1000949.10.1371/journal.ppat.1000949
615 19. Bouvier NM (2016) Cystic fibrosis and the war for iron at the host-pathogen battlefront.
616 *Proc Natl Acad Sci U S A* 113(6):1480-1482.10.1073/pnas.1525101113
617 20. Mashburn LM, Jett AM, Akins DR, & Whiteley M (2005) *Staphylococcus aureus* serves
618 as an iron source for *Pseudomonas aeruginosa* during *in vivo* coculture. *Journal of*
619 *bacteriology* 187(2):554-566

620 21. Nguyen AT, Jones JW, Ruge MA, Kane MA, & Oglesby-Sherrouse AG (2015) Iron
621 Depletion Enhances Production of Antimicrobials by *Pseudomonas aeruginosa*. *J*
622 *Bacteriol* 197(14):2265-2275.10.1128/JB.00072-15
623 22. Nguyen AT*, et al.* (2016) Cystic Fibrosis Isolates of *Pseudomonas aeruginosa* Retain
624 Iron-Regulated Antimicrobial Activity against *Staphylococcus aureus* through the Action
625 of Multiple Alkylquinolones. *Front Microbiol* 7:1171.10.3389/fmicb.2016.01171
626 23. Filkins LM*, et al.* (2015) Coculture of *Staphylococcus aureus* with *Pseudomonas*
627 *aeruginosa* Drives *S. aureus* towards Fermentative Metabolism and Reduced Viability in
628 a Cystic Fibrosis Model. *J Bacteriol* 197(14):2252-2264.10.1128/JB.00059-15
629 24. Szamosvari D & Bottcher T (2017) An Unsaturated Quinolone N-Oxide of *Pseudomonas*
630 *aeruginosa* Modulates Growth and Virulence of *Staphylococcus aureus*. *Angew Chem*
631 *Int Ed Engl* 56(25):7271-7275.10.1002/anie.201702944
632 25. Lightbown JW & Jackson FL (1956) Inhibition of cytochrome systems of heart muscle
633 and certain bacteria by the antagonists of dihydrostreptomycin: 2-alkyl-4-
634 hydroxyquinoline N-oxides. *Biochem J* 63(1):130-137
635 26. Reil E, Hofle G, Draber W, & Oettmeier W (1997) Quinolones and their N-oxides as
636 inhibitors of mitochondrial complexes I and III. *Biochim Biophys Acta* 1318(1-2):291-
637 298.10.1016/s0005-2728(96)00150-8
638 27. Collier DN*, et al.* (2002) A bacterial cell to cell signal in the lungs of cystic fibrosis
639 patients. *FEMS Microbiol Lett* 215(1):41-46
640 28. Bredenbruch F, Geffers R, Nimtz M, Buer J, & Haussler S (2006) The *Pseudomonas*
641 *aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environ Microbiol*
642 8(8):1318-1329.10.1111/j.1462-2920.2006.01025.x
643 29. Pesci EC*, et al.* (1999) Quinolone signaling in the cell-to-cell communication system of
644 *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the*
645 *United States of America* 96(20):11229-11234

646 30. Florez C, Raab JE, Cooke AC, & Schertzer JW (2017) Membrane Distribution of the
647 Pseudomonas Quinolone Signal Modulates Outer Membrane Vesicle Production in
648 *Pseudomonas aeruginosa*. *MBio* 8(4).10.1128/mBio.01034-17
649 31. Coleman JP*, et al.* (2008) *Pseudomonas aeruginosa* PqsA is an anthranilate-coenzyme
650 A ligase. *Journal of bacteriology* 190(4):1247-1255.10.1128/JB.01140-07
651 32. Djapgne L*, et al.* (2018) The Pseudomonas aeruginosa PrrF1 and PrrF2 small regulatory
652 RNAs (sRNAs) promote 2-alkyl-4-quinolone production through redundant regulation of
653 the antR mRNA. *Journal of bacteriology*.10.1128/JB.00704-17
654 33. Oglesby AG*, et al.* (2008) The influence of iron on *Pseudomonas aeruginosa* physiology:
655 a regulatory link between iron and quorum sensing. *J Biol Chem* 283(23):15558-
656 15567.10.1074/jbc.M707840200
657 34. Reid DW, Withers NJ, Francis L, Wilson JW, & Kotsimbos TC (2002) Iron deficiency in
658 cystic fibrosis: relationship to lung disease severity and chronic *Pseudomonas*
659 *aeruginosa* infection. *Chest* 121(1):48-54
660 35. Reinhart AA*, et al.* (2017) The *Pseudomonas aeruginosa* PrrF small RNAs regulate iron
661 homeostasis during acute murine lung infection. *Infection and immunity*
662 85(5).10.1128/IAI.00764-16
663 36. Hood MI & Skaar EP (2012) Nutritional immunity: transition metals at the pathogen-host
664 interface. *Nat Rev Microbiol* 10(8):525-537.10.1038/nrmicro2836
665 37. Wakeman CA*, et al.* (2016) The innate immune protein calprotectin promotes
666 *Pseudomonas aeruginosa* and *Staphylococcus aureus* interaction. *Nat Commun*
667 7:11951.10.1038/ncomms11951
668 38. Zygiel EM, Nelson CE, Brewer LK, Oglesby-Sherrouse AG, & Nolan EM (2019) The
669 human innate immune protein calprotectin induces iron starvation responses in
670 *Pseudomonas aeruginosa*. *J Biol Chem* 294(10):3549-3562.10.1074/jbc.RA118.006819

671 39. Kim EJ, Sabra W, & Zeng AP (2003) Iron deficiency leads to inhibition of oxygen transfer
672 and enhanced formation of virulence factors in cultures of *Pseudomonas aeruginosa*
673 PAO1. *Microbiology* 149(Pt 9):2627-2634.10.1099/mic.0.26276-0
674 40. Vasil ML & Ochsner UA (1999) The response of *Pseudomonas aeruginosa* to iron:
675 genetics, biochemistry and virulence. *Molecular microbiology* 34(3):399-413
676 41. Wiens JR, Vasil AI, Schurr MJ, & Vasil ML (2014) Iron-regulated expression of alginate
677 production, mucoid phenotype, and biofilm formation by *Pseudomonas aeruginosa*.
678 *MBio* 5(1):e01010-01013.10.1128/mBio.01010-13
679 42. Torres VJ*, et al.* (2010) *Staphylococcus aureus fur* regulates the expression of virulence
680 factors that contribute to the pathogenesis of pneumonia. *Infection and immunity*
681 78(4):1618-1628.10.1128/IAI.01423-09
682 43. Friedman DB*, et al.* (2006) *Staphylococcus aureus* redirects central metabolism to
683 increase iron availability. *PLoS pathogens* 2(8):e87.10.1371/journal.ppat.0020087
684 44. Ochsner UA & Vasil ML (1996) Gene repression by the ferric uptake regulator in
685 *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. *Proceedings of the*
686 *National Academy of Sciences of the United States of America* 93(9):4409-4414
687 45. Brewer LK*, et al.* (2019) Development and Bioanalytical Method Validation of an LC-
688 MS/MS Assay for Simultaneous Quantitation of 2-Alkyl-4(1H)-Quinolones for Application
689 in Bacterial Cell Culture and Lung Tissue. *Submitted*
690 46. Nelson CE*, et al.* (2019) Proteomic analysis of the *Pseudomonas aeruginosa* iron
691 starvation response reveals PrrF sRNA-dependent iron regulation of twitching motility,
692 amino acid metabolism, and zinc homeostasis proteins. *J Bacteriol*.10.1128/JB.00754-
693 18
694 47. Erde J, Loo RR, & Loo JA (2014) Enhanced FASP (eFASP) to increase proteome
695 coverage and sample recovery for quantitative proteomic experiments. *J Proteome Res*
696 13(4):1885-1895.10.1021/pr4010019

697 48. Wisniewski JR, Zougman A, Nagaraj N, & Mann M (2009) Universal sample preparation
698 method for proteome analysis. *Nat Methods* 6(5):359-362.10.1038/nmeth.1322
699 49. Winsor GL*, et al.* (2016) Enhanced annotations and features for comparing thousands of
700 Pseudomonas genomes in the Pseudomonas genome database. *Nucleic Acids Res*
701 44(D1):D646-653.10.1093/nar/gkv1227
702 50. Eng JK, Fischer B, Grossmann J, & Maccoss MJ (2008) A fast SEQUEST cross
703 correlation algorithm. *J Proteome Res* 7(10):4598-4602.10.1021/pr800420s
704 51. Dorfer V*, et al.* (2014) MS Amanda, a universal identification algorithm optimized for high
705 accuracy tandem mass spectra. *J Proteome Res* 13(8):3679-3684.10.1021/pr500202e
706 52. Kall L, Canterbury JD, Weston J, Noble WS, & MacCoss MJ (2007) Semi-supervised
707 learning for peptide identification from shotgun proteomics datasets. *Nat Methods*
708 4(11):923-925.10.1038/nmeth1113
709 53. Kanehisa M, Sato Y, Kawashima M, Furumichi M, & Tanabe M (2016) KEGG as a
710 reference resource for gene and protein annotation. *Nucleic Acids Res* 44(D1):D457-
711 462.10.1093/nar/gkv1070
712 54. Huang W*, et al.* (2018) PAMDB: a comprehensive Pseudomonas aeruginosa
713 metabolome database. *Nucleic Acids Res* 46(D1):D575-D580.10.1093/nar/gkx1061
714 55. Szklarczyk D*, et al.* (2017) The STRING database in 2017: quality-controlled protein-
715 protein association networks, made broadly accessible. *Nucleic Acids Res* 45(D1):D362-
716 D368.10.1093/nar/gkw937
717 56. Gaines JM, Carty NL, Colmer-Hamood JA, & Hamood AN (2005) Effect of static growth
718 and different levels of environmental oxygen on toxA and ptxR expression in the
719 *Pseudomonas aeruginosa* strain PAO1. *Microbiology* 151(Pt 7):2263-
720 2275.10.1099/mic.0.27754-0

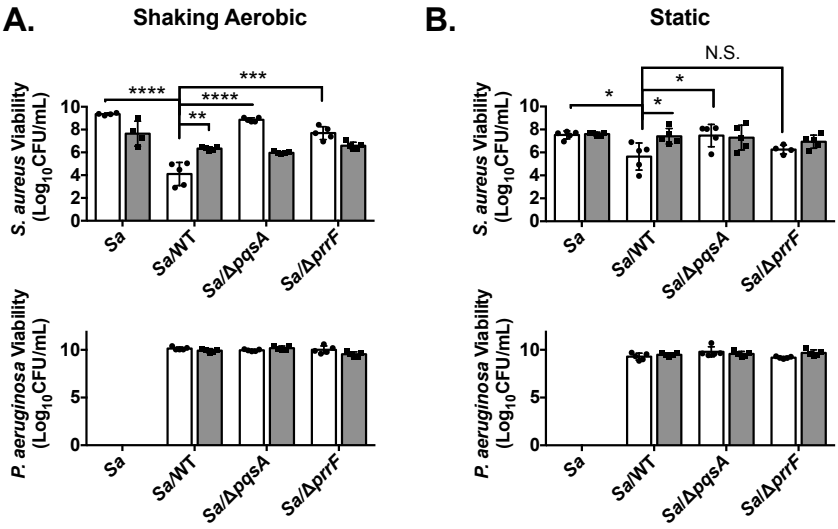
721 57. Wyckoff TJ, Thomas B, Hassett DJ, & Wozniak DJ (2002) Static growth of mucoid
722 *Pseudomonas aeruginosa* selects for non-mucoid variants that have acquired flagellum-
723 dependent motility. *Microbiology* 148(Pt 11):3423-3430.10.1099/00221287-148-11-3423
724 58. Dotsch A*, et al.* (2012) The *Pseudomonas aeruginosa* transcriptome in planktonic
725 cultures and static biofilms using RNA sequencing. *PLoS One*
726 7(2):e31092.10.1371/journal.pone.0031092
727 59. Poole K, Heinrichs DE, & Neshat S (1993) Cloning and sequence analysis of an EnvCD
728 homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in
729 the secretion of the siderophore pyoverdine. *Molecular microbiology* 10(3):529-544
730 60. Ankenbauer RG & Quan HN (1994) FptA, the Fe(III)-pyochelin receptor of
731 *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to
732 hydroxamate siderophore receptors. *J Bacteriol* 176(2):307-319
733 61. Dean CR & Poole K (1993) *Cloning and characterizati*on of the ferric enterobactin
734 receptor gene (pfeA) of Pseudomonas aeruginosa. *Journal of bacteriology* 175(2):317-
735 324
736 62. Serino L*, et al.* (1997) Biosynthesis of pyochelin and dihydroaeruginoic acid requires the
737 iron-regulated pchDCBA operon in *Pseudomonas aeruginosa*. *J Bacteriol* 179(1):248-
738 257
739 63. Hassett DJ*, et al.* (1997) An operon containing fumC and sodA encoding fumarase C
740 and manganese superoxide dismutase is controlled by the ferric uptake regulator in
741 *Pseudomonas aeruginosa*: fur mutants produce elevated alginate levels. *J Bacteriol*
742 179(5):1452-1459
743 64. Wilderman PJ*, et al.* (2004) Identification of tandem duplicate regulatory small RNAs in
744 *Pseudomonas aeruginosa* involved in iron homeostasis. *Proceedings of the National*
745 *Academy of Sciences of the United States of America* 101(26):9792-
746 9797.10.1073/pnas.0403423101

747 65. Korgaonkar AK & Whiteley M (2011) *Pseudomonas aeruginosa* enhances production of
748 an antimicrobial in response to N-acetylglucosamine and peptidoglycan. *Journal of*
749 *bacteriology* 193(4):909-917.10.1128/JB.01175-10
750 66. Drees SL*, et al.* (2018) PqsL uses reduced flavin to produce 2-
751 hydroxylaminobenzoylacetate, a preferred PqsBC substrate in alkyl quinolone
752 biosynthesis in *Pseudomonas aeruginosa*. *J Biol Chem* 293(24):9345-
753 9357.10.1074/jbc.RA117.000789
754 67. Schertzer JW, Brown SA, & Whiteley M (2010) Oxygen levels rapidly modulate
755 *Pseudomonas aeruginosa* social behaviours via substrate limitation of PqsH. *Mol*
756 *Microbiol* 77(6):1527-1538.10.1111/j.1365-2958.2010.07303.x
757 68. Kovach K*, et al.* (2017) Evolutionary adaptations of biofilms infecting cystic fibrosis lungs
758 promote mechanical toughness by adjusting polysaccharide production. *NPJ Biofilms*
759 *Microbiomes* 3:1.10.1038/s41522-016-0007-9
760 69. Stewart PS & Franklin MJ (2008) Physiological heterogeneity in biofilms. *Nat Rev*
761 *Microbiol* 6(3):199-210.10.1038/nrmicro1838
762 70. Werner E*, et al.* (2004) Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl*
763 *Environ Microbiol* 70(10):6188-6196.10.1128/AEM.70.10.6188-6196.2004
764 71. Wessel AK*, et al.* (2014) Oxygen limitation within a bacterial aggregate. *MBio*
765 5(2):e00992.10.1128/mBio.00992-14
766 72. de Beer D, Stoodley P, Roe F, & Lewandowski Z (1994) Effects of biofilm structures on
767 oxygen distribution and mass transport. *Biotechnol Bioeng* 43(11):1131-
768 1138.10.1002/bit.260431118
769 73. Xu KD, Stewart PS, Xia F, Huang CT, & McFeters GA (1998) Spatial physiological
770 heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability.
771 *Appl Environ Microbiol* 64(10):4035-4039

772 74. Meirelles LA & Newman DK (2018) Both toxic and beneficial effects of pyocyanin
773 contribute to the lifecycle of *Pseudomonas aeruginosa*. *Mol Microbiol* 110(6):995-
774 1010.10.1111/mmi.14132
775 75. Glasser NR, Kern SE, & Newman DK (2014) Phenazine redox cycling enhances
776 anaerobic survival in *Pseudomonas aeruginosa* by facilitating generation of ATP and a
777 proton-motive force. *Mol Microbiol* 92(2):399-412.10.1111/mmi.12566
778 76. Wang Y*, et al.* (2011) Phenazine-1-carboxylic acid promotes bacterial biofilm
779 development via ferrous iron acquisition. *J Bacteriol* 193(14):3606-
780 3617.10.1128/JB.00396-11
781 77. O'May CY, Sanderson K, Roddam LF, Kirov SM, & Reid DW (2009) Iron-binding
782 compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under
783 anaerobic conditions. *J Med Microbiol* 58(Pt 6):765-773.10.1099/jmm.0.004416-0
784 78. Hall S*, et al.* (2016) Cellular Effects of Pyocyanin, a Secreted Virulence Factor of
785 *Pseudomonas aeruginosa*. *Toxins (Basel)* 8(8).10.3390/toxins8080236
786 79. Ho Sui SJ*, et al.* (2012) Raloxifene attenuates *Pseudomonas aeruginosa* pyocyanin
787 production and virulence. *Int J Antimicrob Agents* 40(3):246-
788 251.10.1016/j.ijantimicag.2012.05.009
789 80. Wang Y & Newman DK (2008) Redox reactions of phenazine antibiotics with ferric
790 (hydr)oxides and molecular oxygen. *Environ Sci Technol* 42(7):2380-
791 2386.10.1021/es702290a
792 81. Britigan BE*, et al.* (1992) Interaction of the *Pseudomonas aeruginosa* secretory products
793 pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to
794 endothelial cells. Implications for *Pseudomonas*-associated tissue injury. *J Clin Invest*
795 90(6):2187-2196.10.1172/JCI116104

796 82. Britigan BE, Rasmussen GT, & Cox CD (1997) Augmentation of oxidant injury to human
797 pulmonary epithelial cells by the *Pseudomonas aeruginosa* siderophore pyochelin. *Infect*
798 *Immun* 65(3):1071-1076
799 83. Coffman TJ, Cox CD, Edeker BL, & Britigan BE (1990) Possible role of bacterial
800 siderophores in inflammation. Iron bound to the *Pseudomonas* siderophore pyochelin
801 can function as a hydroxyl radical catalyst. *J Clin Invest* 86(4):1030-
802 1037.10.1172/JCI114805
803 84. Lesic B, Starkey M, He J, Hazan R, & Rahme LG (2009) Quorum sensing differentially
804 regulates *Pseudomonas aeruginosa* type VI secretion locus I and homologous loci II and
805 III, which are required for pathogenesis. *Microbiology* 155(Pt 9):2845-
806 2855.10.1099/mic.0.029082-0
807 85. Sana TG*, et al.* (2012) The second type VI secretion system of *Pseudomonas*
808 *aeruginosa* strain PAO1 is regulated by quorum sensing and Fur and modulates
809 internalization in epithelial cells. *J Biol Chem* 287(32):27095-
810 27105.10.1074/jbc.M112.376368
811 86. Russell AB*, et al.* (2013) Diverse type VI secretion phospholipases are functionally
812 plastic antibacterial effectors. *Nature* 496(7446):508-512.10.1038/nature12074
813 87. Hood RD*, et al.* (2010) A type VI secretion system of *Pseudomonas aeruginosa* targets
814 a toxin to bacteria. *Cell Host Microbe* 7(1):25-37.10.1016/j.chom.2009.12.007
815 88. Tahrioui A*, et al.* (2019) Extracellular DNA release, quorum sensing, and PrrF1/F2 small
816 RNAs are key players in *Pseudomonas aeruginosa* tobramycin-enhanced biofilm
817 formation. *NPJ Biofilms Microbiomes* 5:15.10.1038/s41522-019-0088-3
818 89. Henry RL, Mellis CM, & Petrovic L (1992) Mucoid *Pseudomonas aeruginosa* is a marker
819 of poor survival in cystic fibrosis. *Pediatr Pulmonol* 12(3):158-161
820 90. Pedersen SS, Hoiby N, Espersen F, & Koch C (1992) Role of alginate in infection with
821 mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* 47(1):6-13
822

823 **FIGURES AND FIGURE LEGENDS**



824

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

836

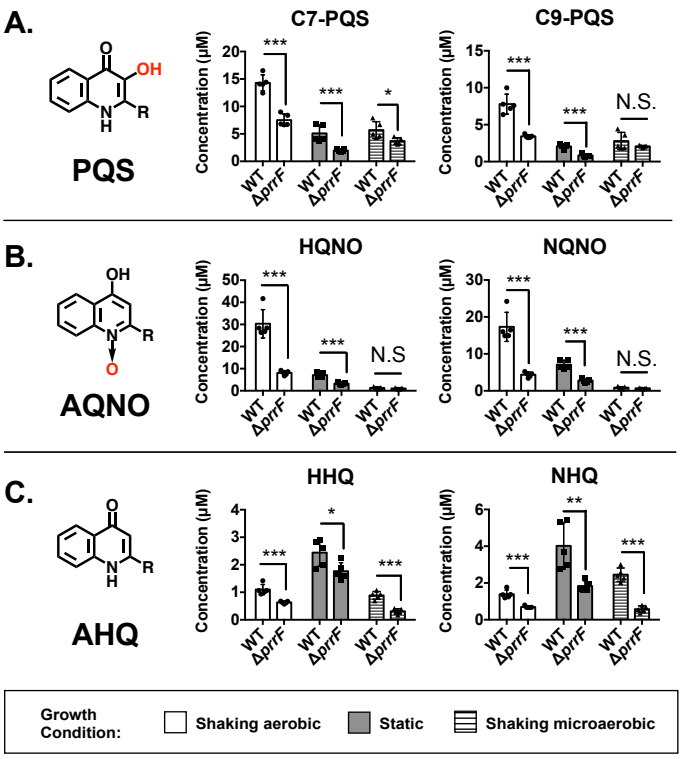


Figure 2. Concentrations of AQs in shaking, static, and microaerobic cultures of *P. aeruginosa.* Cultures were grown in 1.5 mL of iron-deplete DTSB media in 14mL polystyrene round bottom culture tubes and incubated for 18 hours at 37˚C. Microaerobic cultures were sealed in an air-tight GasPak™ system 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.

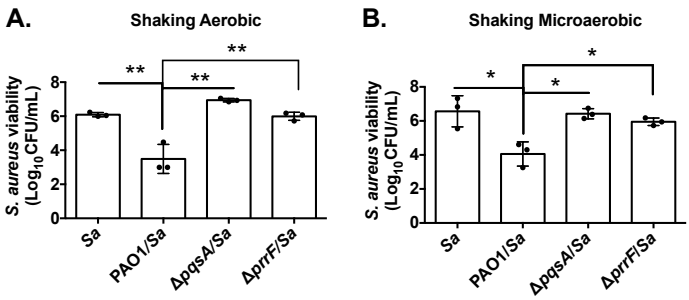


Figure 3. *P. aeruginosa* AMA requirement for PrrF is not influenced by oxygen availability in co-culture with *S. aureus.* *P. aeruginosa* and *S. aureus* were co-inoculated into 1.5 mL of iron-deplete dialyzed trypticase soy broth (DTSB) media in 14mL round-bottom polystyrene culture tubes. Cultures 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.

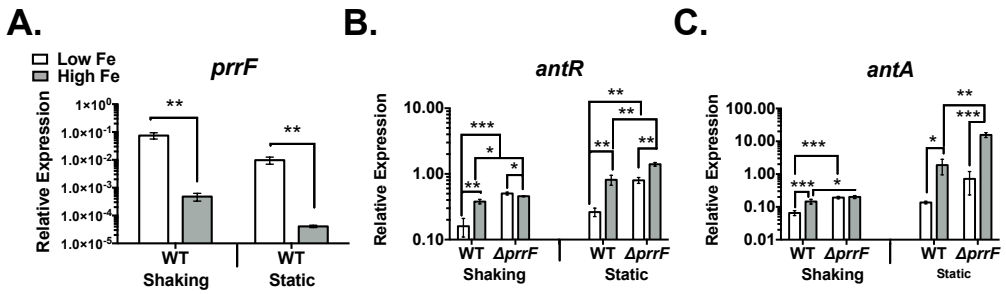


Figure 4. PrrF expression and regulation of anthranilate catabolism genes in shaking and static conditions. Wild type PAO1 and Δ*prrF* mutant strains were incubated in 1.5mL dialyzed trypticase soy broth (DTSB) supplemented with either low iron (white bars) or high iron (gray bars) in 14mL round bottom 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.

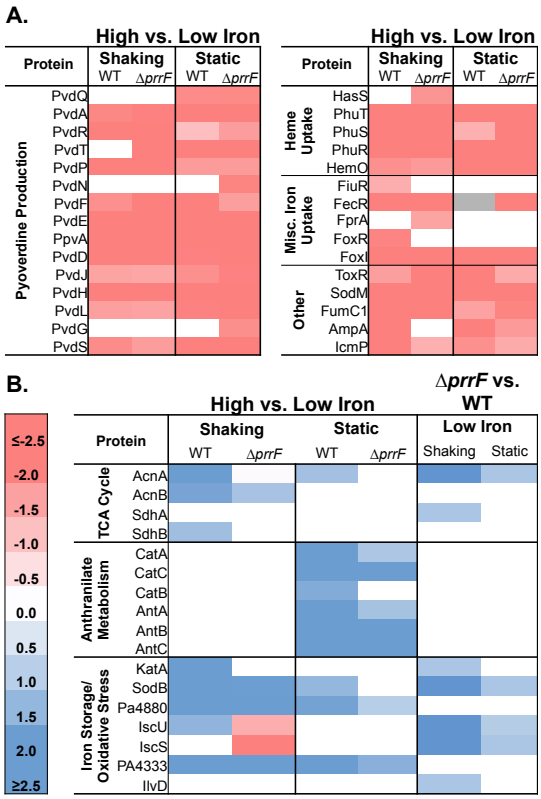


Figure 5. Static growth reduces the effects of classical PrrF regulation. Heatmaps showing the $\log_2$ fold change (LFC) of **(A)** known iron-repressed proteins and **(B)** known PrrF-regulated proteins, in the indicated strains grown in high versus low iron conditions or in the Δ*prrF* mutant versus 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.

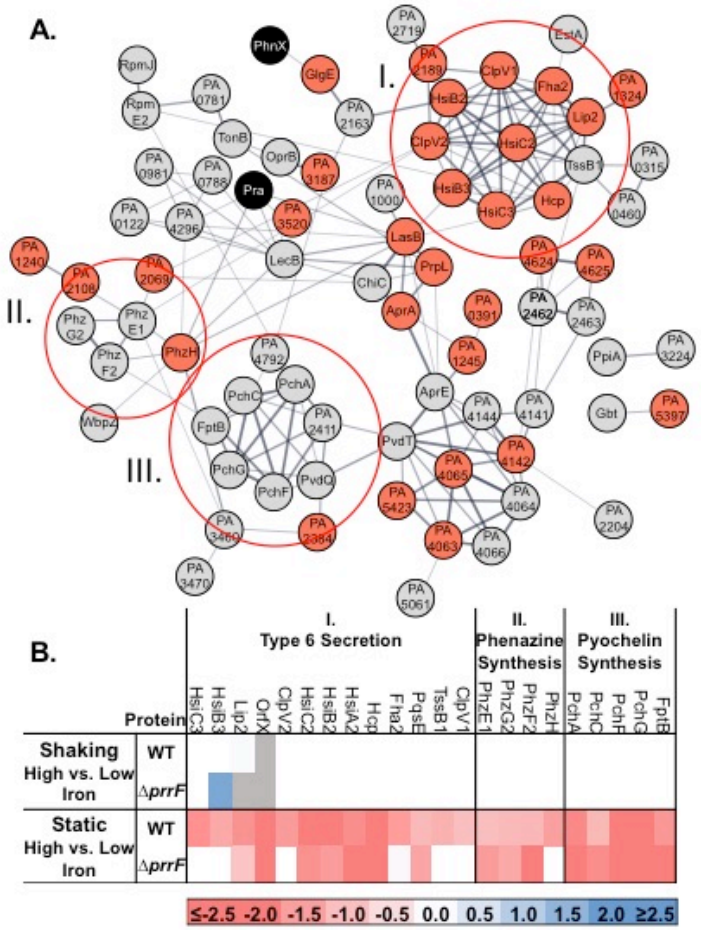


Figure 6. Proteomics reveals novel targets of iron regulation in static conditions. Wild type PAO1 and the Δ*prrF* strain were inoculated into 1.5mL DTSB media and supplemented with either low iron (0μM) or high iron (100μM) and incubated in either shaking or static conditions. Statistically significant iron regulation (FDR adjusted p-value < 0.05) in static and shaking conditions was identified by at least 2-fold (i.e.1 $\log_2$ fold change) induction in response to iron starvation as described in **Materials and Methods**. (A) Network analysis of differentially regulated genes was carried out using STRING database software, which revealed distinct several virulence-associated genes that were similarly impacted by iron starvation under static conditions. Line thickness indicates strength of data support for association between two proteins, as calculated by STRING network analysis (55). Red nodes represent genes which 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.
905

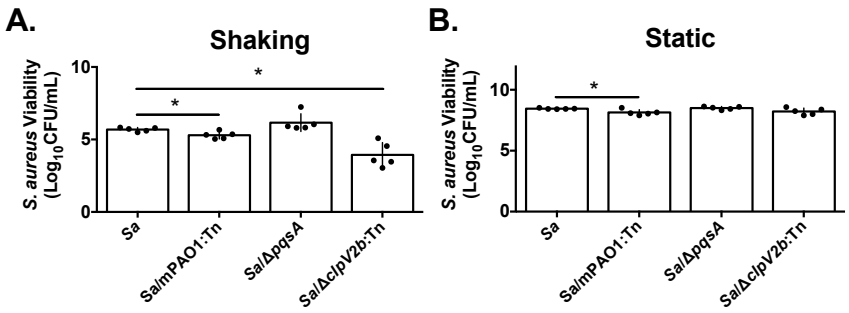


Figure 7. T6SS potentially impacts *P. aeruginosa* AMA. *P. aeruginosa* and *S. aureus* were co-inoculated in dialyzed trypticase soy broth (DTSB) media supplemented with (high iron) or without (low iron) 100μM $FeCl_3$. Co-cultures were incubated in 6 well polystyrene cell culture plates in static conditions 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 *$p \leq 0.05$.