Static growth alters PrrF- and 2-alkyl-4(1*H*)-quinolone regulation of virulence trait expression in *Pseudomonas aeruginosa*

Running title: Static growth alters *P. aeruginosa* iron regulation

Subject Area: Microbiology and Infectious Disease

Luke K. Brewer[1], Weiliang Huang[1], Brandy Hackert[1], Maureen A. Kane[1], Amanda G. Oglesby[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 is an opportunistic pathogen that is frequently associated with both acute and chronic infections, the latter of which are often polymicrobial. *P. aeruginosa* possesses a complex regulatory network that modulates nutrient acquisition and virulence, but our knowledge of these networks is largely based on studies with shaking cultures, which are not likely representative of conditions during infection. Here, we provide proteomic, metabolic, and genetic evidence that regulation by iron, a critical metallo-nutrient, is altered in static *P. aeruginosa* cultures. We identified type VI secretion as a target of iron regulation in *P. aeruginosa* in static but not shaking conditions, and we present evidence that this regulation occurs via PrrF sRNA-dependent production of 2-alkyl-4(1*H*)-quinolone metabolites. We further discovered that iron-regulated interactions between *P. aeruginosa* and a Gram-positive opportunistic pathogen, *Staphylococcus aureus*, are mediated by distinct factors in shaking versus static bacterial cultures. These results yield new bacterial iron regulation paradigms and highlight the need to re-define iron homeostasis in static microbial communities.

31 **INTRODUCTION**

32 Antimicrobial-resistant bacterial pathogens represent a substantial health risk, raising
33 concerns of a return to a "pre-antibiotic era." One prominent example is multi-drug resistant
34 (MDR) *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen that is capable of
35 establishing acute infections of the blood and lung in immunocompromised individuals. *P.*
36 *aeruginosa* also causes chronic infections in surgical wounds, diabetic foot ulcers, and in the
37 lungs of individuals with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD).
38 *P. aeruginosa* is the leading cause of morbidity among nosocomial pathogens, and is
39 responsible for over 10% of all hospital-acquired infections (1-7). Therapeutic options for *P.*
40 *aeruginosa* are rapidly dwindling due to the spread of antimicrobial resistance, underscoring the
41 need for novel antimicrobials that can be used to treat MDR infections. To that end, a more
42 complete understanding of the metabolic processes that contribute to *P. aeruginosa* survival
43 and pathogenesis during infection is necessary for the discovery and development of effective
44 therapeutics.

45 Iron is a required metallo-nutrient for most living organisms and has a significant impact
46 on the establishment and progression of *P. aeruginosa* infections (8, 9). During infection, the
47 host sequesters iron from invading pathogens through a process referred to as nutritional
48 immunity (10-12). In response to iron starvation, *P. aeruginosa* expresses several virulence
49 factors that cause host cell damage, presumably releasing iron and other nutrients from host
50 cells (13-17). Iron starvation also induces the expression of multiple systems that mediate high
51 affinity uptake of iron and heme (18, 19). Additionally, iron starvation induces expression of the
52 PrrF small regulatory RNAs (sRNAs), which post-transcriptionally reduce expression of non-
53 essential iron-dependent metabolic enzymes. The PrrF sRNAs are required for virulence in an
54 acute murine lung infection model and are also highly expressed in the lungs of CF patients (9,
55 20, 21). However, the specific mechanisms by which the PrrF sRNAs contribute to mammalian
56 infection remain unclear.

57 One prominent function of the PrrF sRNAs is their ability to indirectly promote the
58 production of several small secreted metabolites collectively referred to as 2-alkyl-4(1*H*)-
59 quinolones (AQs), which mediate a range of toxic activities against the host, as well as microbial
60 pathogens that co-colonize *P. aeruginosa*-infected hosts. (20, 22-24). 2-alkyl-4-quinolone N-
61 oxides (AQNOs) are potent cytochrome inhibitors that obstruct respiratory metabolism in
62 mammalian and microbial cells (25-27). 2-alkyl-3-hydroxy-quinolone, which is more commonly
63 referred to as PQS, and 2-alkyl-4-hydroxyquinolones (AHQs) both function as quorum signaling
64 molecules and induce the expression of secreted factors that further inhibit growth of
65 mammalian and microbial cells (28-31). AQ synthesis is initiated by enzymes encoded by the
66 *pqsABCDE* operon, with PqsA catalyzing the first step with the conversion of anthranilate into
67 anthraniloyl-CoA (32). The PrrF sRNAs promote AQ production by repressing the expression of
68 the anthranilate degradation enzyme complexes AntABC and CatBCA (16), which degrade
69 anthranilate to metabolites that enter the tricarboxylic acid (TCA) cycle. Thus, the PrrF sRNAs
70 spare anthranilate for AQ production and are therefore required for optimal AQ production in
71 iron-depleted conditions (9, 20, 23, 33).
72 In accordance with PrrF promoting AQ production in iron-depleted environments, we
73 previously discovered that iron starvation enhances AQ-dependent antimicrobial activity against
74 the Gram-positive pathogen *Staphylococcus aureus*, which can co-colonize with *P. aeruginosa*
75 in chronic wounds and in CF lung infections (20, 23). Surprisingly, these previous studies
76 showed that the *prrF* locus was not required for iron-regulated antimicrobial activity, and several
77 possible explanations for this have been considered. One explanation that has not been
78 investigated is that earlier studies used either agar plates or static liquid cultures, which are
79 likely distinct from the shaking liquid cultures that have historically been used to study iron
80 regulation. Whereas aerobic shaking cultures are exposed to constant aeration and perturbation
81 to promote homogenous cultures, *P. aeruginosa* infections are typically more heterogeneous
82 and feature planktonic as well as adherent biofilm communities of *P. aeruginosa*, particularly

83 during chronic infections. Changes in the iron metabolism and iron regulatory factors in
84 response to aeration and perturbation have been similarly documented in other bacterial
85 species, suggesting a regulatory link between iron homeostasis mechanisms and static growth
86 that may be common among other bacterial species (34, 35). Because of the broad impact of
87 iron on *P. aeruginosa* physiology, we hypothesized that static growth would likewise alter the
88 iron-regulated metabolism in *P. aeruginosa*, potentially revealing novel iron regulatory pathways
89 that are more relevant during infection.

90 In the current study, we sought to identify targets of iron and PrrF regulation during static
91 growth of *P. aeruginosa* using an unbiased proteomics-based approach. Our results revealed a
92 number of proteins that were differentially affected by iron and PrrF in static versus shaking
93 cultures. Amongst these were proteins for phenazine biosynthesis, which were repressed by
94 iron in static conditions, a result that is in contrast to our own shaking cultures and recent
95 studies showing that phenazine synthesis genes are iron-induced in shaking conditions (12, 36).
96 We also discovered PrrF positively affects the expression of the Hcp1 secretion island II type VI
97 secretion system (HSI-II T6SS) in *P. aeruginosa*, and we provide evidence that this regulation is
98 due to PrrF-regulated AQ production. Moreover, while PrrF is not required for antimicrobial
99 activity against *S. aureus* in static conditions, we found that it still promotes the production of
100 AQs in static conditions. However, the relative abundances and roles of individual AQ species
101 for interspecies interactions varied between shaking versus static cultures, thereby altering the
102 requirement of PrrF for antimicrobial activity. Lastly, we show that reduced oxygen availability in
103 static cultures is not solely responsible for the changes in iron regulation observed in this study.
104 Combined, these results indicate that static bacterial communities use unique regulatory and
105 metabolic networks to adapt to decreased nutrient availability, likely affecting the production of
106 key virulence determinants.
107
108

109 **MATERIALS AND METHODS**

110 **Bacterial strains and growth conditions.** Bacterial strains used in this study are listed
111 in **Supplementary Table S1**. Lysogeny broth (LB) and agar (LA) were prepared using 10g/L
112 NaCl (Sigma, St Louis, MO), 10g/L tryptone, and 5g/L yeast extract, 15g/L agar when applicable
113 (Becton-Dickinson, Franklin Lakes, NJ). *P. aeruginosa* and *S. aureus* strains were both routinely
114 grown on LA from freezer stock. Five isolated colonies of each strain were selected from
115 overnight-incubated agar plates and inoculated into 5mL LB. For iron starvation studies,
116 bacterial strains were subcultured into Chelex-treated and dialyzed trypticase soy broth (DTSB)
117 prepared as previously described (16). Media dialysis was carried out using Spectra/Por®2
118 dialysis membrane tubing (29mm diameter) with a molecular weight cutoff of 12-14kD
119 (Repligen, Waltham, Ma).

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

134

135 **Quantitative label-free proteomics.** Cell cultures were prepared for proteomics
136 analysis as described previously (36). Briefly, cells were harvested by centrifugation and
137 washed in phosphate-buffered saline prior to lysis in 4% sodium deoxycholate. Lysates were
138 washed, reduced, alkylated and trypsinolyzed on filter (37, 38). Tryptic peptides were separated
139 using a nanoACQUITY UPLC analytical column (BEH130 C18, 1.7 µm, 75 µm x 200 mm,
140 Waters) over a 165-minute linear acetonitrile gradient (3 – 40%) with 0.1 % formic acid on a
141 Waters nano-ACQUITY UPLC system and analyzed on a coupled Thermo Scientific Orbitrap
142 Fusion Lumos Tribrid mass spectrometer. Full scans were acquired at a resolution of 120,000,
143 and precursors were selected for fragmentation by higher-energy collisional dissociation
144 (normalized collision energy at 32 %) for a maximum 3-second cycle. Tandem mass spectra
145 were searched against *Pseudomonas* genome database PAO1 reference protein sequences
146 (39) using Sequest HT algorithm and MS Amanda algorithm with a maximum precursor mass
147 error tolerance of 10 ppm (40, 41). Carbamidomethylation of cysteine and deamidation of
148 asparagine and glutamine were treated as static and dynamic modifications, respectively.
149 Resulting hits were validated at a maximum false discovery rate (FDR) of 0.01 using a semi-
150 supervised machine learning algorithm Percolator (42). Label-free quantifications were
151 performed using Minora, an aligned AMRT (Accurate Mass and Retention Time) cluster
152 quantification algorithm (Thermo Scientific, 2017). Protein abundance ratios between the high
153 iron cultures and the low iron cultures were measured by comparing the MS1 peak volumes of
154 peptide ions, whose identities were confirmed by MS2 sequencing as described above. Gene
155 function and pathway analysis was completed using information from the *Pseudomonas*
156 genome database (39), KEGG database (43), *Pseudomonas* metabolome database (44), and
157 the STRING database (45).
158
159 **Real time PCR analysis.** Quantitative real time PCR (qRT-PCR) analysis was
160 conducted on at least three biological replicates of the PAO1 reference strain, the isogenic

161 Δ*prrF* mutant, or Δ*pqsA* mutant grown in DTSB supplemented with (high iron) or without (low
162 iron) 100 μM $FeCl_3$. *P. aeruginosa* cells were lysed after 18 hours using 2.5mg/mL lysozyme
163 and incubated at 37˚C for 30 minutes. RNA extraction was performed using the RNeasy mini kit
164 (QIAGEN, Germantown, MD). Real-time qualitative PCR analysis was performed as described
165 previously (9) using an Applied Sciences StepOne Plus Real Time PCR System (Life
166 Technologies, Carlsbad, CA). Primer and probe sequences used for gene expression analysis
167 are listed in **Supplementary Table S2**. Quantitation of cDNA was carried out using standard
168 curves generated by performing qPCR of individual gene targets using cDNA reverse-
169 transcribed from serial dilutions of RNA. *P. aeruginosa* expression data was normalized to *oprF*
170 expression in aerobic shaking conditions, or *omlA* in either microaerobic shaking conditions or
171 static growth condition, as expression of these genes is not impacted by iron in each respective
172 growth condition (9).
173
174 **Quantitation of AQs using liquid chromatography-tandem mass spectrometry.** AQ
175 quantitation was carried out as previously reported (46). Briefly, 300 L of culture were harvested
176 and spiked with a 25 μM stock nalidixic acid internal standard to a final concentration of 500 nM,
177 followed by an extraction using ethyl acetate with 0.1% acetic acid. The organic phase
178 containing extracted AQs was harvested from each sample, dried down, and resuspended in
179 300 μL of 0.1% formic acid suspended in 1:1:1 (v/v/v) mixture of methanol, water, and
180 acetonitrile. Extracts were analyzed via quantitative liquid chromatography-tandem mass
181 spectrometry (LC-MS/MS) using multiple reaction monitoring (MRM) performed on a Waters
182 ACQUITY UHPLC coupled to a Xevo TQ-XS tandem quadrupole mass spectrometer in positive
183 ion mode**.**
184
185 **Quantification of viable cells in co-culture studies.** 400 μL of *P. aeruginosa* and *S.*
186 *aureus* mono- and co-cultures were harvested at 15000 rcf for 5 minutes. Cell pellets were

187 resuspended in 400 μL of 0.1% Triton-X in phosphate-buffered saline (PBS) and vortexed
188 rigorously. From these resuspensions, serial dilutions were prepared in 0.1% Triton-X in PBS.
189 10 μL of each dilution was spotted onto Baird-Parker agar plates and Pseudomonas Isolation
190 Agar (PIA) plates to select for *S. aureus* and *P. aeruginosa* growth, respectively. Upon spotting
191 10 μL of dilutions onto agar plates, the plates were tilted to facilitate even drips across the plate
192 surface. PIA plates were incubated at 37˚C for 18 hours and colony forming units (CFU) were
193 counted. Baird-Parker plates were incubated for 37˚C for 48 hours to allow for adequate growth
194 of *S. aureus* small-colony variants observed in some assays prior to counting CFUs.
195
196 **Statistics**. Statistically significant changes in cell viability, RNA gene expression, and
197 AQ concentrations between treatment groups were identified using one-way ANOVA two-tailed
198 on Prism 6, using either Tukey's, Sidak's, or Dunnett's post test for multiple comparisons, with a
199 significance threshold of p value < 0.05. Protein expressions that changed 2-fold or more with
200 an FDR adjusted p-value < 0.05 were considered statistically significant.
201
202 **RESULTS**
203 **Proteomics reveals altered iron-regulation in static *P. aeruginosa* cultures.** Our
204 current knowledge of *P. aeruginosa* iron regulation is largely restricted to shaking cultures (14,
205 16, 19, 23, 36, 47). To determine whether global iron regulatory pathways are altered in static
206 cultures *of P. aeruginosa*, we applied a label-free, LC-MS/MS-based proteomics methodology
207 recently described by our laboratories (36). Using this unbiased approach, we determined the
208 proteomes of static and shaking cultures of PAO1 as well as the isogenic Δ*prrF* mutant grown in
209 Chelexed and dialyzed TSB medium (DTSB), supplemented with or without 100 μM $FeCl_3$.
210 Mass spectra were identified using a PAO1 reference proteome, which were in turn validated to
211 a false discovery rate (FDR) of 0.01 to ensure proper assignment of protein identities.

212 Initially, we sought to confirm whether iron regulatory effects previously identified in
213 shaking conditions were similar to shaking cultures analyzed in the current study. For this, we
214 identified proteins whose levels showed a statistically significant (FDR adjusted *p*-value <0.05)
215 log fold change (LFC) > 1 upon iron starvation. This analysis revealed 178 and 191 proteins that
216 were significantly induced and repressed, respectively, by iron starvation (**Supplementary**
217 **Dataset 1**). As previously observed (14, 19, 48-52), iron starvation significantly increased levels
218 of proteins for the siderophore (pyoverdine and pyochelin) and heme uptake systems, as well as
219 several iron-regulated virulence factors, during shaking growth (**Fig. 1A**). Also as previously
220 observed, iron-containing proteins involved in the tricarboxylic acid (TCA) cycle and oxidative
221 metabolism, including aconitase A (AcnA), succinate dehydrogenase B (SdhB), and catalase
222 (KatA), were induced by iron in a PrrF-dependent manner under shaking conditions (**Fig. 1B**)
223 (16, 53). We also observed PrrF-dependent iron regulation of more recently identified PrrF
224 targets in shaking conditions, including proteins involved in Fe-Sulfur cluster biogenesis (IscS,
225 IscU) and amino acid metabolism (IlvD) (**Fig. 1B**) (36). Contrary to previous real-time PCR and
226 microarray analyses showing iron induction of the *antABC* mRNAs (36), neither iron
227 supplementation nor *prrF* deletion significantly affected levels of the AntABC proteins for
228 anthranilate degradation under shaking conditions (**Fig. 1B**). Overall however, this analysis
229 replicated many of the global iron regulatory pathways observed in previous studies of shaking
230 *P. aeruginosa* cultures.
231 We next determined whether known targets of PrrF and iron regulation under shaking
232 conditions were similarly regulated under static conditions. Iron repression of siderophore and
233 heme uptake proteins was largely retained during static growth, indicating that these iron
234 regulatory pathways are not altered under static conditions (**Fig. 1A**). In contrast, iron and PrrF
235 regulation of several proteins involved in the TCA cycle or oxidative stress protection was
236 reduced or eliminated in static conditions (**Fig. 1B**). We also noted robust iron induction of the
237 anthranilate degradation proteins AntABC and CatBCA, which occurred in a PrrF-independent

manner (**Fig. 1B**), in agreement with trends we observed in subsequent qRT-PCR analysis of *antR* and *antA* under identical growth conditions **(Fig. S1B,C)**. Combined, these data indicate that PrrF-dependent iron regulatory pathways are altered in static conditions.

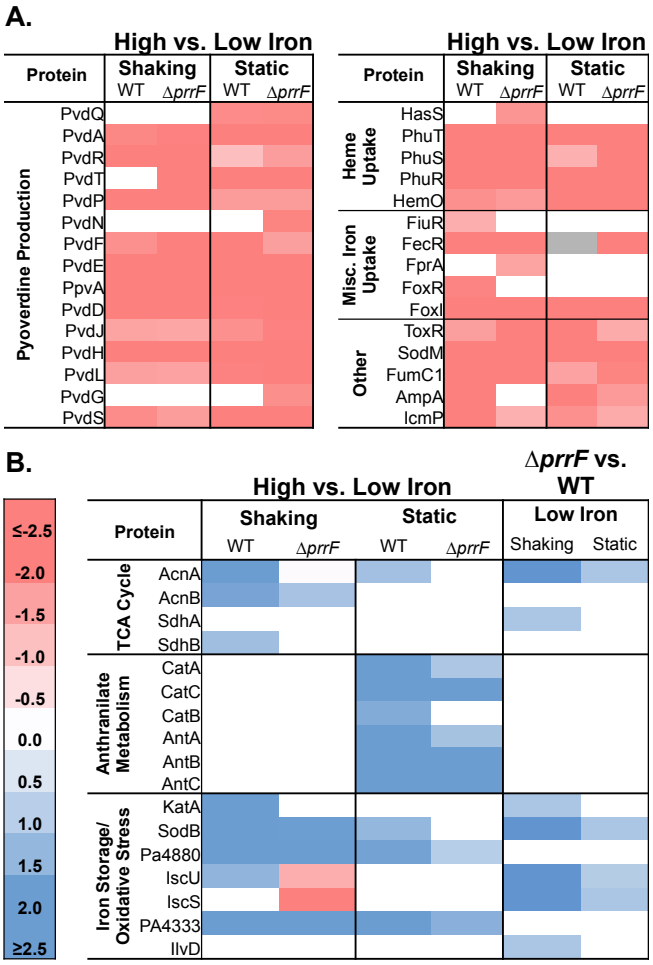


Figure 1. Static growth reduces the effects of classical PrrF regulation. Heatmaps showing the $\log_2$ fold change (LFC) of **(A)** known iron-repressed proteins and **(B)** known PrrF-regulated proteins, in the indicated strains grown in high versus low iron conditions or in the Δ*prrF* mutant versus wild type PAO1 grown in low iron conditions. Wild type PAO1 and Δ*prrF* strains were inoculated into 1.5mL DTSB supplemented with $FeCl_3$ and incubated in shaking or static conditions as described in the Materials and Methods. 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 LFC) 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.

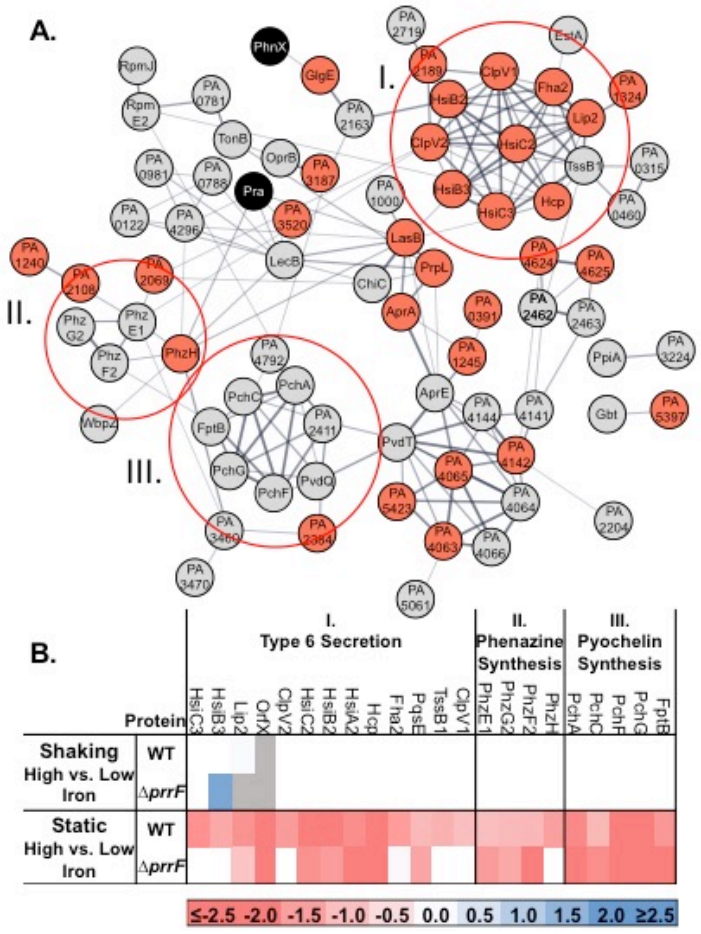
To determine if there were additional iron or PrrF regulatory effects that were unique to either static or shaking growth, we mined our proteomics dataset for proteins whose levels were altered by iron depletion in either static or shaking conditions, but not in both, by an LFC ≥ 1 and

255 an FDR adjusted *p* value ≤ 0.05. Approximately 410 proteins demonstrated significantly altered
256 iron regulation in response to growth conditions. Of these, 126 of these were specifically iron
257 induced and 93 were iron repressed in shaking but not static conditions, while 78 were iron
258 induced and 129 were iron repressed only in static conditions (**Supplementary Table S3-7 and**
259 **Dataset S1**). Approximately 50% of the proteins affected by iron supplementation in static
260 conditions were unaffected by iron in the Δ*prrF* mutant (**Supplementary Table S3-7 and**
261 **Dataset S1**), indicating PrrF still contributes to iron regulation during these growth conditions,
262 despite the loss of PrrF-mediated iron regulation on previously identified targets as shown in
263 **Fig. 1B**.
264 STRING network analysis was next used to identify relationships between the proteins
265 within each of these four groups (45). Several of the proteins that were induced by iron in
266 shaking conditions but not in static conditions were related to motility, including proteins for
267 flagellar assembly (FlgA, FlgL, FliC, and FliI), a chemotaxis-associated protein (ChpA), and
268 proteins involved in twitching pilus formation (PilV, PilY2, and FimU) **(Supplementary Dataset**
269 **S1).** Proteins that were significantly induced by iron depletion in static but not shaking cultures
270 included numerous enzymes required for synthesis of the redox cycling phenazine metabolites
271 and the pyochelin siderophore, as well as proteins encoded by the HSI-II T6SS locus (**Fig 2A,**
272 **Supplementary Fig S4-5)**. Iron regulation of the phenazine and pyochelin synthesis proteins
273 occurred in a PrrF-independent manner, while iron regulation of many of the T6SS proteins was
274 either reduced or lost in the Δ*prrF* mutant (**Fig. 2B**). Moreover, we found that *prrF* deletion had a
275 negative effect on T6SS proteins in static but not shaking conditions **(Supplementary Dataset**
276 **S1**). The identification of T6SS as iron- and PrrF-regulated during static but not shaking
277 conditions was particularly intriguing, as this contact-dependent secretion system is known to
278 mediate virulence and interbacterial interactions that may be more relevant in static conditions
279 than in highly perturbed, shaking conditions. No complementarity was identified between the

280 PrrF sRNAs and the mRNAs encoding these proteins, thus we hypothesized that PrrF-regulated

281 expression of these proteins occurs via an indirect mechanism.

282

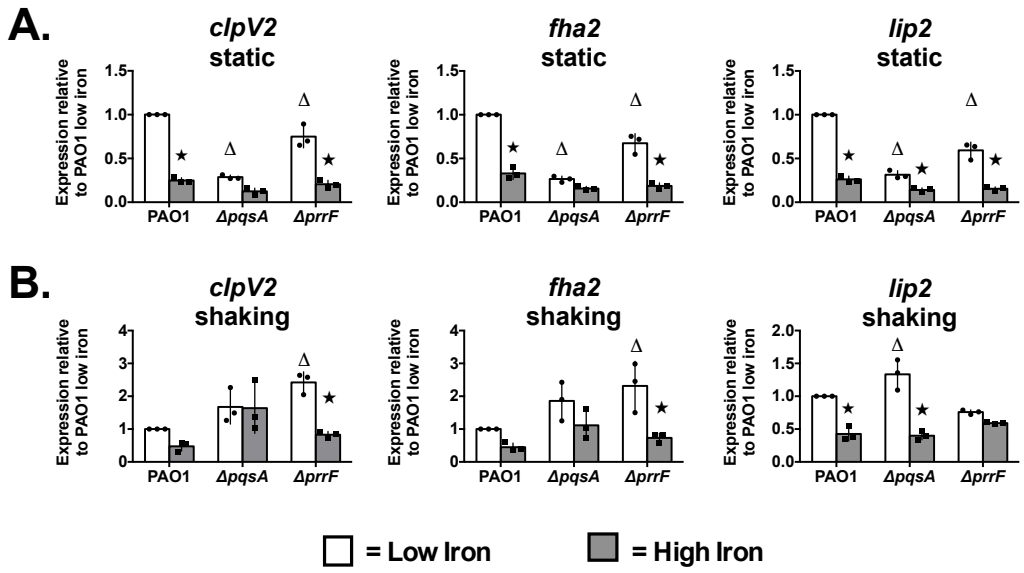


283

284 **Figure 2. Proteomics reveals novel targets of iron regulation in static conditions.** Wild type PAO1
285 and the Δ*prrF* strain were inoculated into 1.5mL DTSB media and supplemented with either low iron (0
286 μM) or high iron (100 μM) and incubated in either shaking or static conditions. Statistically significant iron
287 regulation (FDR adjusted p-value < 0.05) in static and shaking conditions was identified by at least 2-fold
288 (i.e.1 LFC) induction in response to iron starvation as described in Materials and Methods. **(A)** Network
289 analysis of differentially regulated genes was carried out using STRING database software, which
290 revealed distinct several virulence-associated proteins that were similarly impacted by iron starvation
291 under static conditions. Line thickness indicates strength of data support for association between two
292 proteins, as calculated by STRING network analysis (45). Red nodes represent proteins that were
293 significantly regulated by PrrF in low iron static conditions, gray nodes indicated proteins that do not
294 exhibit regulation by PrrF. Black nodes represent proteins that exhibited iron regulation in static conditions
295 but were not detected in one or more iron conditions in shaking cultures. **(B)** Heatmaps of differentially
296 regulated proteins encoded by the HSI-II T6SS, phenazine, and pyochelin biosynthetic operons. Gray
297 boxes indicate proteins that were not detected in one or more condition. White boxes indicate that no
298 significant change in protein levels was observed.
299

300 **HSI-II T6SS genes are differentially regulated by AQs in shaking versus static**
301 **conditions.** No previous reports have demonstrated a regulatory link between the PrrF sRNAs
302 and T6SS, nor were we able to identify complementarity between PrrF and T6SS secretion
303 mRNA sequences. Previous reports, however, have demonstrated that PrrF sRNAs are
304 necessary for full AQ production via repression of the anthranilate-degrading enzymes (9, 33).
305 AQs, in turn, induce expression of several virulence-associated genes, including genes involved
306 in T6SS (54, 55). We therefore sought to determine whether reduced levels of T6SS proteins in
307 the Δ*prrF* mutant during our proteomics analysis were due to a partial loss of AQ production.
308 To investigate this idea, we first performed targeted expression analysis of T6SS genes
309 in our wild type, Δ*prrF,* and Δ*pqsA* strains grown in static conditions using quantitative real time
310 PCR (qRT-PCR). Specifically, we analyzed the expression of genes encoding proteins that
311 demonstrated PrrF dependent iron regulation in our proteomics analysis, including *clpV2*, *fha2*,
312 and *lip2*. In agreement with our proteomics analysis, the Δ*prrF* mutant exhibited statistically
313 significant decreases in expression of each of these genes when grown in static conditions (**Fig.**
314 **3A**). The Δ*pqsA* mutant demonstrated even more dramatic decreases in the expression of
315 *clpV2, fha2,* and *lip2* relative to wild type PAO1 (**Fig. 3A**), supporting the notion that AQs are
316 necessary for the expression of T6SS genes in static cultures. Similar AQ-dependent
317 expression of other T6SS genes was also observed (**Fig. S2).** In contrast, neither *prrF* nor *pqsA*
318 were required for T6SS expression in shaking conditions (**Fig. 3B and Fig. S2**), supporting the
319 hypothesis that AQ regulation of T6SS is enhanced by static growth. In agreement with our
320 proteomics, T6SS expression was iron regulated in static cultures of wild type PAO1, but not in
321 shaking cultures (**Fig. 3A,B),** with expression of *clpV2, fha2,* and *lip2* repressed by an average
322 of 4.07-, 3.12-, and 3.86-fold in high iron static cultures, respectively. Iron repression of these
323 genes was diminished or was not statistically significant in static Δ*pqsA* cultures (*clpv2*
324 repressed 2.40-fold; *fha2* repressed 1.72-fold; *lip2* repressed 2.25-fold). Interestingly,
325 expression of T6SS synthesis proteins was still repressed by iron in static Δ*prrF* cultures, with

326 expression of *clpV2, fha2, and lip2* repressed by an average of 3.78-fold, 3.63-fold, and 3.83-
327 fold in high iron static conditions, respectively, suggesting that PrrF is not the sole mediator of
328 iron-regulated T6SS gene expression in PAO1. We noted that expression of the T6SS genes
329 was greatly reduced in low iron shaking cultures of PAO1, with C_t values 2-3 higher than what
330 was observed in low iron static cultures of PAO1 (data not shown). Thus, shaking growth
331 appears to reduce T6SS expression by PAO1 when grown in DTSB low iron medium, masking
332 iron, PrrF, and AQ regulation to be observed.
333



334

335 **Figure 3. PrrF and AQs differentially regulate expression of T6SS genes in static and shaking**
336 **conditions.** Cultures were grown in 1.5 mL of DTSB media supplemented with either 0 μM $FeCl_3$ or 100
337 μM $FeCl_3$ in 14mL polystyrene round bottom culture tubes for 18 hours at 37˚C. Cultures were incubated
338 **(A)** without perturbation (0rpm) or (**B**) with perturbation (250rpm) prior to RNA extraction (**Methods and**
339 **Materials)**. Bars in each graph indicate the average value of 3 independent experiments (error bars
340 represent standard deviation), and individual data points from biological replicates are indicated by black
341 circles. Triangles represent a significant difference of each mutant grown in low iron conditions relative to
342 the low iron cultures PAO1, whereas black stars indicate a significant difference in expression in high iron
343 versus low iron conditions within a strain, as determined by one-way ANOVA with Tukey's post-test for
344 multiple comparisons, with a significance cutoff of $p \leq 0.05$.
345

346 **PrrF-mediated AQ production is retained in static growth conditions.** Our
347 proteomics and qRT-PCR analysis indicated that AQ regulation of T6SS genes is specific to
348 static conditions, although the underlying mechanism of this shift was not clear. Previous reports

349 have demonstrated that static growth can dramatically impact *P. aeruginosa* physiology,
350 including regulated expression of virulence traits. These reports have suggested that diminished
351 oxygen availability in static conditions plays a defining role in mediating these effects (56-58).
352 Additional studies showed that oxygen limitation decreases PQS production while increasing
353 production of the PQS precursor molecules, AHQs (59). As such, we hypothesized that oxygen
354 limitation resulting from static growth conditions would favor production of AHQs, potentially
355 influencing AQ regulation of T6SS.

356 To test this, we used a validated LC-MS/MS method (46) to measure levels of various
357 AQ species in shaking and static cultures of wild-type PAO1 and the isogenic Δ*prrF* mutant. As
358 previously observed (23, 33), PrrF was required for optimal production of the C7 and C9
359 congeners of PQS, AQNO, and AHQ in shaking conditions (**Fig. 4A,B,C, white bars**). Similarly,
360 PrrF was required for optimal production of all AQ congeners except HQNO in static cultures
361 (**Fig. 4A,B,C, gray bars**), indicating that PrrF still promotes production of most AQs in static
362 growth conditions. Production of C7-PQS, C9-PQS, and NQNO was diminished in static
363 conditions, likely due to the previously reported oxygen requirement for activity of the PqsH and
364 PqsL enzymes (59, 60). In contrast, production of AHQ congeners was more robust in static
365 than in shaking conditions, both in the wild type and Δ*prrF* strains (**Fig. 4C, gray bars versus**
366 **white bars**). However, when we performed analysis of cultures grown in shaking microaerobic
367 conditions, AHQ levels were not significantly enhanced relative to shaking aerobic cultures,
368 despite decreased production of PQS and AQNO congeners (**Fig. 4A,B,C striped bars versus**
369 **white bars**). This indicates that increased AHQ levels in static cultures is not solely due to
370 reduced oxygen availability, but that static growth causes distinct changes in *P. aeruginosa* AQ
371 metabolite profiles.

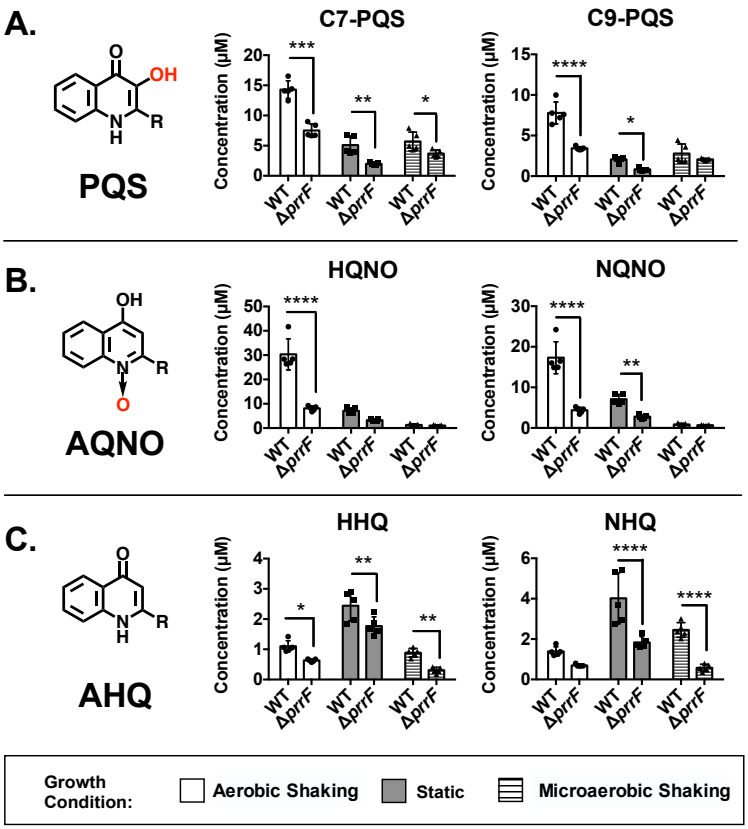


Figure 4. Concentrations of AQs in shaking, static, and microaerobic cultures of *P. aeruginosa.* Cultures were grown in 1.5 mL of DTSB media without iron supplementation in 14mL polystyrene round bottom culture tubes and incubated for 18 hours at 37˚C. Microaerobic cultures were sealed in an air-tight GasPak™ system in the presence of an EZ Campy Container™ packet. Shaking and microaerobic cultures were incubated with perturbation (250rpm). Static cultures were incubated in shaking incubators with no perturbation (0rpm). The core structures of (**A**) PQS, (**B**) AQNO, and (**C**) AHQ molecules are depicted in black, with oxygen-dependent structural elements highlighted in red (R indicates alkyl chain). AQs were quantified by LC-MS/MS. Bars in each graph indicate the average concentration of 5 independent experiments, and individual data points from each biological replicate are indicated by black circles (error bars represent standard deviation). Asterisks indicate a significant difference compared to PAO1 within each growth condition as determined by one-way ANOVA with Sidak's post test for multiple comparisons, with significance thresholds as follows: *p≤0.05; **p≤0.005; ***p≤0.0005; ****p≤0.00005.

PrrF-mediated antimicrobial activity is altered by static growth conditions. We next probed the impact of altered AQ profiles in static growth on iron-regulated interactions with *S. aureus*. As discussed above, we previously discovered that iron starvation enhances AQ-dependent antimicrobial activity against *S. aureus* on agar plates as well as in a transwell co-culture system (20, 23). Surprisingly, the Δ*prrF* mutant demonstrated similar antimicrobial activity to wild type PAO1 in this previous study, suggesting that PrrF's effects on AQ-mediated

392 antimicrobial activity may be impacted by static growth (23). This idea was further supported by
393 our proteomic, qPCR, and metabolite analysis discussed above, showing that both PrrF-
394 mediated iron regulatory pathways and AQ profiles are altered in static growth (**Fig. 2 and Fig.**
395 **3)**. To investigate how culture perturbation would affect antimicrobial activity, we co-inoculated
396 mono- and co-cultures of *P. aeruginosa* strain PAO1 and *S. aureus* strain USA300 in DTSB,
397 supplemented with or without 100 μM $FeCl_3$, and grew them either statically or in a shaking
398 incubator at 250rpm. After 18 hours, viability of *P. aeruginosa* and *S. aureus* was quantified by
399 enumerating CFUs on selective PIA and Baird Parker agar, respectively. In agreement with
400 previous studies showing that AQs mediate antimicrobial activity against *S. aureus*, USA300
401 viability was reduced significantly during low iron co-culture with PAO1 under static conditions,
402 whereas the Δ*pqsA* mutant did not exhibit antimicrobial activity against USA300 (**Fig. 5B, white**
403 **bars**). Also in agreement with our earlier work, iron limitation significantly enhanced
404 antimicrobial activity of PAO1 against USA300 in static co-cultures (**Fig. 5B, white versus gray**
405 **bars**). Iron limitation similarly enhanced the antimicrobial activity of the Δ*prrF* mutant, although
406 this trend was not statistically significant, perhaps due to overall weak growth of *S. aureus* in
407 static conditions.
408 In shaking cultures, we observed similar AQ-dependent, iron-regulated antimicrobial
409 activity. However, *S. aureus* viability was reduced approximately 50,000-fold in low iron co-
410 cultures with PAO1, signifying more robust killing than in static co-cultures (**Fig. 5A, white**
411 **bars).** Notably, the Δ*prrF* mutant was defective for antimicrobial activity relative to the isogenic
412 PAO1 parent strain in low iron shaking co-cultures (**Fig. 5A, white bars**), a result that
413 contrasted with our previous and current reports on static co-cultures (23). *P. aeruginosa*
414 viability was not affected by iron supplementation or deletion of either *pqsA* or *prrF* in any of the
415 conditions tested (**Fig. 5**), verifying that changes in antimicrobial activity were not the result of
416 changes in the viability of the *P. aeruginosa* Δ*pqsA* or Δ*prrF* mutants.
417

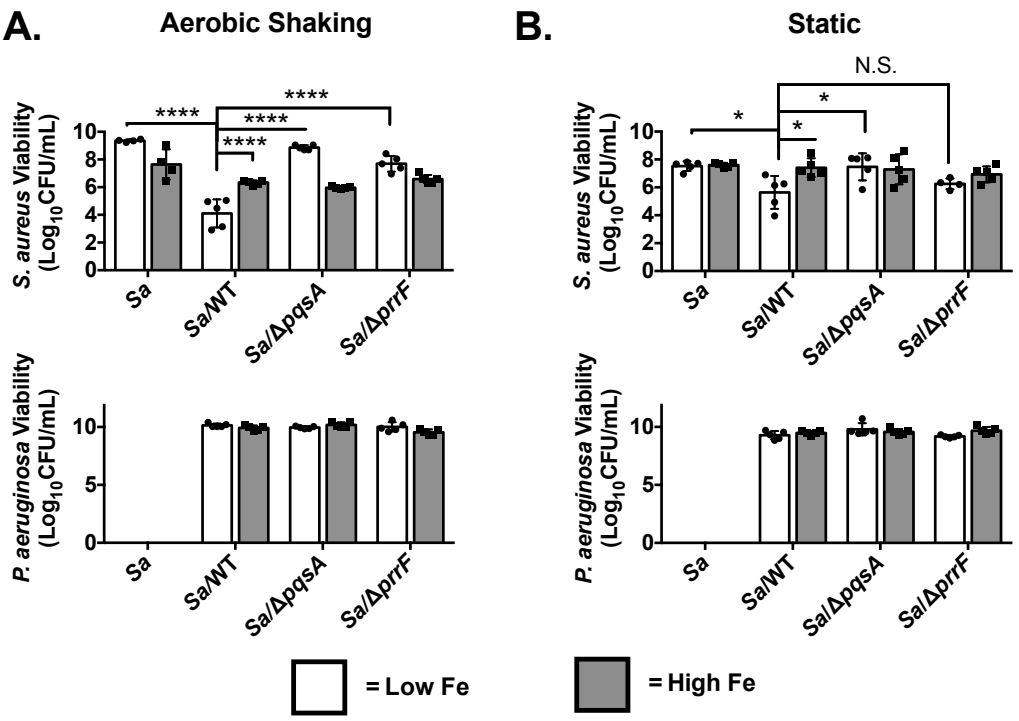


Figure 5. PrrF-mediated antimicrobial activity is altered in shaking conditions. *P. aeruginosa* and *S. aureus* were co-inoculated in DTSB media supplemented with (high iron) or without (low iron) 100 μM $FeCl_3$. Co-cultures were incubated either in 14mL round bottom polystyrene cell culture tubes in shaking aerobic conditions **(A)** or in 6 well polystyrene cell culture plates in static conditions **(B)** for 18 hours. After incubation, 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 compared to *S. aureus* monoculture as determined by one-way ANOVA with Tukey's post test for multiple comparisons, with significance thresholds as follows: *p≤0.05; **p≤0.005; ***p≤0.0005; ****p≤0.00005.

Static growth alters the impact of specific AQs on antimicrobial activity. We observed that the impact of PrrF on antimicrobial activity is reduced during static growth, which was surprising since PrrF is still required for optimal production of most AQs during static growth (**Fig. 4, white and gray bars)**. Because antimicrobial activity was AQ-dependent in both shaking and static co-cultures, we next questioned whether individual AQ species differentially impacted antimicrobial activity in shaking versus static cultures. LC-MS/MS analysis showed that levels of both HQNO and NQNO are dramatically reduced in static conditions, and HQNO, which was the most abundant AQNO congener observed in our analysis of shaking cultures,

was not dependent on PrrF in these conditions (**Fig. 4B**). This led us to hypothesize that AQNO-mediated antimicrobial activity would be greatly reduced, and likely unaffected by PrrF, in static conditions. To test the relative contributions of AQNOs in static versus shaking conditions, we co-incubated *S. aureus* with an AQNO-deficient PAO1 Δ*pqsL* strain in liquid co-cultures. Similar to previous analyses reported by our lab and others (20, 24), Δ*pqsL* was completely defective for antimicrobial activity against *S. aureus* in shaking cultures (**Fig. 6A).** In contrast, the Δ*pqsL* retained modest antimicrobial activity in static co-cultures (**Fig. 6B**). Considering the dramatic decrease in AQNOs during static growth, these data suggest the relative contribution of AQNOs towards antimicrobial activity is reduced in static co-cultures.

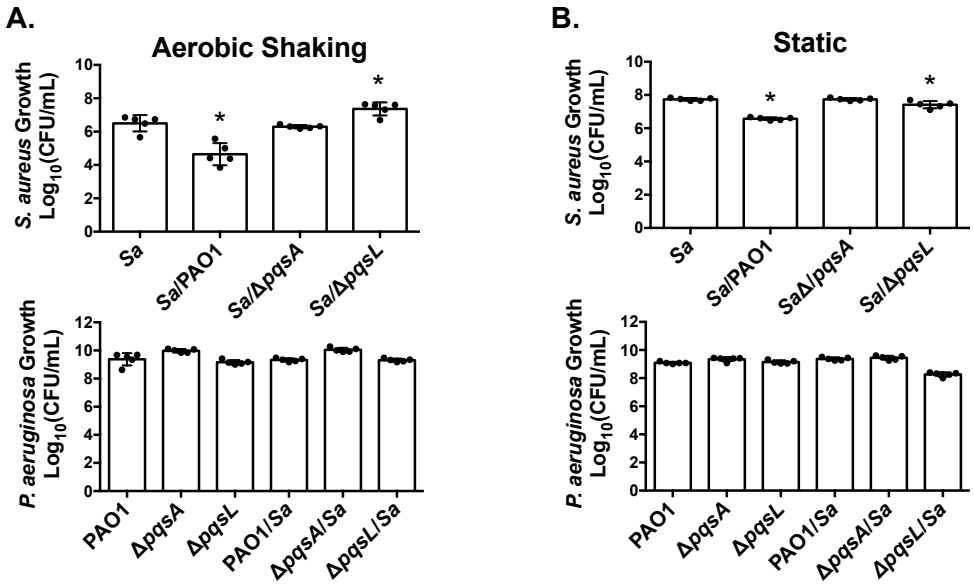
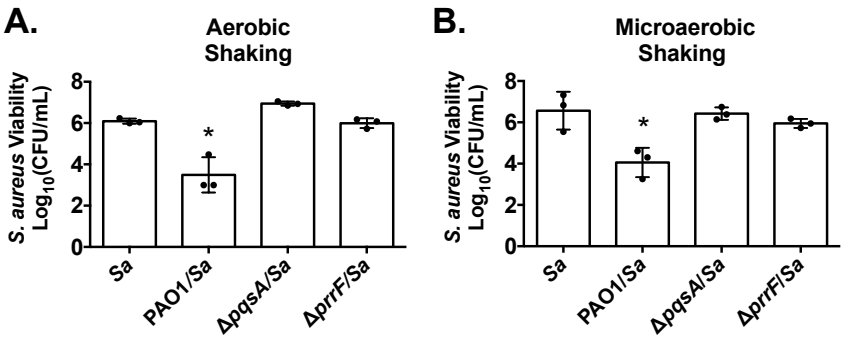


Figure 6. AQNO-mediated antimicrobial activity is reduced in static conditions.
P. aeruginosa and *S. aureus* were co-inoculated in DTSB media supplemented with (high iron) or without (low iron) 100 μM $FeCl_3$. Co-cultures were incubated either in 14mL round bottom polystyrene cell culture tubes in shaking aerobic conditions **(A)** or in 6 well polystyrene cell culture plates in static conditions **(B)** for 18 hours. After incubation, CFUs 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 circles. Error bars indicate the standard deviation of 5 independent experiments. Asterisks indicate a significant difference compared to *S. aureus* monoculture as determined by one-way ANOVA with Dunnett's post test for multiple comparison, with a significance threshold of *p≤0.05.

Our LC-MS/MS analysis also showed that AHQs were much more abundant in static cultures compared to shaking cultures of both the wild type and Δ*prrF* mutant (**Fig. 4C, gray**

460 **bars**). Thus, AHQ production by the Δ*prrF* mutant in static culture, even if it is reduced
461 compared to wild type, may still be robust enough to promote antimicrobial activity against *S.*
462 *aureus* in a seemingly PrrF-independent manner. Conversely, our LC-MS/MS analysis showed
463 that shaking microaerobic cultures did not allow for a similar increase in AHQ levels (**Fig. 4C,**
464 **striped bars**). To determine if the ability of the Δ*prrF* mutant to maintain antimicrobial activity in
465 static growth was due solely to oxygen limitation or due to the increase in AHQs, we co-cultured
466 *S. aureus* with *P. aeruginosa* wild type PAO1, Δ*pqsA,* or Δ*prrF* strains with shaking in either
467 microaerobic or aerobic conditions. Similar to what was observed in shaking aerobic cultures,
468 the Δ*prrF* mutant was defective for antimicrobial activity in shaking microaerobic conditions (**Fig.**
469 **7A,B**), indicating that changes in PrrF-dependent antimicrobial activity during static growth are
470 not solely due to changes in oxygen availability, but may instead correlate with an overall
471 increase in AHQ levels. Combined, these results demonstrate that different classes of AQ
472 metabolites have distinct impacts on polymicrobial interactions in static versus shaking cultures,
473 with AHQs potentially playing a more prominent role than AQNOs during static growth.



474

475 **Figure 7. *P. aeruginosa* antimicrobial activity requirement for PrrF is not influenced by oxygen**
476 **availability in co-culture with *S. aureus.*** *P. aeruginosa* and *S. aureus* were co-inoculated into 1.5 mL of
477 iron-deplete DTSB media in 14mL round-bottom polystyrene culture tubes. Cultures were incubated for
478 18hr at 37˚C in either **(A)** shaking aerobic or in **(B)** shaking microaerobic conditions. Microaerobic cultures
479 were incubated in air-tight GasPak systems™ in the presence of a GasPak EZ Campy Container™
480 packet to achieve microaerobic conditions. After 18hr incubation, CFUs were enumerated as described
481 above. Bars in each graph indicate the average value, and individual data points from biological replicates
482 are indicated with circles. Error bars indicate the standard deviation of 5 independent experiments.
483 Asterisks indicate a significant difference compared to *S. aureus* monoculture as determined by one-way
484 ANOVA with Dunnett's post test for multiple comparisons, with a significance threshold of *p≤0.05.
485

486 **DISCUSSION**

487 The PrrF sRNAs are critical for *P. aeruginosa* pathogenesis due to their ability to
488 mediate numerous biological functions upon iron starvation (9). Amongst these is the Δ*prrF*
489 mutant's well-documented defect in the production of AQs (23, 33), which are necessary for
490 virulence in several infection models (61-63). In the current study, we identified the HSI-II T6SS
491 loci as a novel PrrF-responsive system, and we provide evidence that this iron regulatory effect
492 is promoted by static growth and mediated by AQs. T6SS contributes to *P. aeruginosa* virulence
493 in several models of infection (54, 55) and also facilitates inter-bacterial interactions in *P.*
494 *aeruginosa* and other bacterial species (64, 65). T6SS mediates cell-to-cell interactions that are
495 contact dependent and likely disrupted in shaking cultures (64), making this newly elucidated
496 iron regulatory pathway more relevant to static growth. This study therefore provides yet another
497 potential mechanism for how the PrrF sRNAs may contribute to pathogenesis. More broadly,
498 our results highlight how iron regulatory pathways are highly dynamic and subject to changing
499 environmental conditions, which may have broad implications for the heterogeneous conditions
500 within the host.

501 Iron depletion was previously reported to enhance expression of T6SS systems in *P.*
502 *aeruginosa*, though this was presumed to be the result of ferric uptake regulator (Fur) regulation
503 due to the presence of a Fur consensus site in one of the promoters of the HSI-II locus (54). In
504 the current study, we show that expression of T6SS genes is dependent on PrrF in static
505 conditions, which themselves are regulated by iron via the Fur protein. The PrrF sRNAs appear
506 to regulate the HSI-II locus island through AQs, likely the PQS or AHQ molecules that function
507 as co-inducers of the PqsR/MvfR regulatory protein. Previous studies suggest that MvfR
508 activates expression of the HSI-II T6SS loci via another quorum sensing regulator, RhlR, which
509 directly activates HSI-II T6SS promoter activity in *P. aeruginosa* strain PAO1. Interestingly,
510 these previous studies showed that AQs induced HSI-II T6SS in shaking cultures grown in
511 nutrient-rich LB medium (55), whereas we found that AQs were not necessary for HSI-II T6SS

512 expression in shaking cultures grown in a nutrient depleted medium (DTSB). There are several
513 reasons for this apparent discrepancy, including media selection and the well-documented
514 variability of *P. aeruginosa* strains between laboratories (66). As such, additional studies will be
515 needed to determine the mechanism by which static growth conditions alter AQ-mediated
516 regulation T6SS. However, it is clear that iron substantially affects T6SS gene expression, and
517 the use of static conditions in our current study allowed us to elucidate how the PrrF sRNAs
518 contribute to this regulatory effect.

519 Static growth also revealed changes in how iron affects the expression of phenazine
520 biosynthetic proteins (**Fig. 2**). Recent studies of iron regulation in shaking conditions showed
521 that iron starvation diminished phenazine biosynthesis gene expression and phenazine
522 production (12, 36), while the static growth conditions in the current studies showed that iron
523 depletion enhanced expression of phenazine biosynthesis proteins. This difference may be
524 related to the varying role of phenazines in shaking versus static growth conditions. For
525 instance, phenazines can induce auto-poisoning and extracellular DNA release, and these toxic
526 effects are enhanced by nutrient depletion during aerobic shaking growth (67). However,
527 phenazines also contribute to iron acquisition and survival due to their inherent redox-cycling
528 activity, by which they are capable of catalyzing the formation of bioavailable ferrous iron from
529 ferric iron (68, 69). This may be especially pertinent in static conditions, where iron requirements
530 for biofilm formation are enhanced (70). The inherent redox-cycling activity of phenazines
531 promotes biofilm formation by promoting the reduction of ferric iron to more bioavailable ferrous
532 iron (68-70).

533 Our study also showed that pyochelin siderophore biosynthetic proteins were more
534 strongly induced by iron depletion in static cultures as compared to shaking cultures (**Fig. 2**).
535 While pyochelin is primarily appreciated for its role in the acquisition of ferric iron, iron-bound
536 pyochelin can also promote the production of reactive oxygen species, particularly in
537 conjunction to phenazines (71-74). As such, coordinated production of phenazines and

538 pyochelin in static cultures may function to promote *P. aeruginosa* biofilm formation. Biofilms are
539 an important adaptive feature of *P. aeruginosa* infection, and the appearance of robust biofilm-
540 producing *P. aeruginosa* isolates during chronic lung infections is associated with increased
541 mortality in CF patients (4, 75, 76). While the current study did not specifically address the
542 impact of iron depletion within biofilm communities, the distinct effects of iron during static
543 growth may be relevant to *P. aeruginosa's* ability to form biofilms during chronic infections.
544 In addition to promoting virulence in mammalian hosts, many of the iron-regulated
545 systems discussed in this report have been implicated in polymicrobial interactions. Previous
546 work from our groups showed that iron depletion enhanced the production of AQs, thereby
547 enhancing antimicrobial activity during co-culture with *S. aureus* (23). While this earlier work
548 suggested that PrrF was not required for antimicrobial activity, the current study demonstrates
549 that the role of PrrF in antimicrobial activity is dependent on culture agitation. Specifically, we
550 show that PrrF is required for antimicrobial activity in shaking cultures, but dispensable in static
551 cultures. Our work further highlighted distinct roles of different AQ species in mediating
552 antimicrobial activity in shaking versus static conditions. Notably, we observed greatly
553 diminished levels of AQNOs in static cultures, while AHQ levels were substantially increased.
554 Thus the mechanism of antimicrobial activity against *S. aureus* is likely quite different between
555 shaking and static cultures. Interestingly, Filkins *et al* previously showed that AQNOs were
556 critical for antimicrobial activity against *S. aureus* in biofilms grown on CF bronchial epithelial
557 cells (24), which might be expected to more closely mimic our static versus shaking conditions.
558 This apparent inconsistency could be due to any number of differences between the culture
559 conditions used here and in the Filkins study, including the time to form a mature biofilm, media
560 selection, and the presence of mammalian cell lines. The potential role of AHQs in mediating
561 antimicrobial activity in static cultures has yet to be elucidated but may be linked to enhanced
562 QS-based regulation of antimicrobial factors. As such, further studies are needed to identify the
563 extent and mechanism of AHQ activity in static cultures.

564 Iron availability plays a critical role in the ability of *P. aeruginosa* to express a variety of
565 virulence traits, including the production of secondary metabolites, toxin secretion, and biofilm
566 formation. The Fur protein and PrrF sRNAs maintain tight control over the expression of many
567 of these activities in response to iron availability. However, these regulatory networks have not
568 been fully characterized in static communities, which are likely to more closely reflect conditions
569 in the host. The current study demonstrates that static growth allows for distinct iron regulatory
570 pathways in *P. aeruginosa*, and highlights to need to further investigate iron regulation in static
571 microbial communities such as biofilms. Such studies are likely to reveal many more striking
572 physiological adaptations that are critical for chronic *P. aeruginosa* infections, yielding novel
573 targets for the development of effective antimicrobial therapies.
574

575 **ACKNOWLEDGMENTS**

576 This work was funded in part by the National Institutes of Health (NIH) grants R01
577 AI123320 (to AGO), T32 GM066706 (to LKB), the Cystic Fibrosis Foundation grant
578 CFFOGLESB17G0 (to AGO), and the University of Maryland, School of Pharmacy Mass
579 Spectrometry Center (SOP1841-IQB2014). We thank members of the Oglesby laboratory for
580 editing of the manuscript, and Dr. Angela Wilks for thoughtful discussions and feedback as the
581 study progressed.
582
583
584
585

586 **REFERENCES**

587 1. Koch C (2002) Early infection and progression of cystic fibrosis lung disease. *Pediatr*
588 *Pulmonol* 34(3):232-236.10.1002/ppul.10135
589 2. Li Z*, et al.* (2005) Longitudinal development of mucoid Pseudomonas aeruginosa
590 infection and lung disease progression in children with cystic fibrosis. *JAMA* 293(5):581-
591 588.10.1001/jama.293.5.581
592 3. Paganin P*, et al.* (2015) Changes in cystic fibrosis airway microbial community
593 associated with a severe decline in lung function. *PLoS One*
594 10(4):e0124348.10.1371/journal.pone.0124348
595 4. Kerem E, Corey M, Gold R, & Levison H (1990) Pulmonary function and clinical course
596 in patients with cystic fibrosis after pulmonary colonization with *Pseudomonas*
597 *aeruginosa*. *J Pediatr* 116(5):714-719
598 5. Scheetz MH*, et al.* (2009) Morbidity associated with *Pseudomonas aeruginosa*
599 bloodstream infections. *Diagn Microbiol Infect Dis* 64(3):311-
600 319.10.1016/j.diagmicrobio.2009.02.006
601 6. Wisplinghoff H*, et al.* (2004) Nosocomial bloodstream infections in US hospitals: analysis
602 of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis*
603 39(3):309-317.10.1086/421946
604 7. CDC (2019) *Antibiotic Resistance Threats in the United States, 2019* (Atlanta, GA.),
605 U.S. Dept of Health and Human Services.
606 8. Reid DW, Withers NJ, Francis L, Wilson JW, & Kotsimbos TC (2002) Iron deficiency in
607 cystic fibrosis: relationship to lung disease severity and chronic *Pseudomonas*
608 *aeruginosa* infection. *Chest* 121(1):48-54
609 9. Reinhart AA*, et al.* (2017) The *Pseudomonas aeruginosa* PrrF small RNAs regulate iron
610 homeostasis during acute murine lung infection. *Infection and immunity*
611 85(5).10.1128/IAI.00764-16

612 10. Hood MI & Skaar EP (2012) Nutritional immunity: transition metals at the pathogen-host
613 interface. *Nat Rev Microbiol* 10(8):525-537.10.1038/nrmicro2836
614 11. Wakeman CA*, et al.* (2016) The innate immune protein calprotectin promotes
615 *Pseudomonas aeruginosa* and *Staphylococcus aureus* interaction. *Nat Commun*
616 7:11951.10.1038/ncomms11951
617 12. Zygiel EM, Nelson CE, Brewer LK, Oglesby-Sherrouse AG, & Nolan EM (2019) The
618 human innate immune protein calprotectin induces iron starvation responses in
619 Pseudomonas aeruginosa. *J Biol Chem* 294(10):3549-3562.10.1074/jbc.RA118.006819
620 13. Kim EJ, Sabra W, & Zeng AP (2003) Iron deficiency leads to inhibition of oxygen transfer
621 and enhanced formation of virulence factors in cultures of *Pseudomonas aeruginosa*
622 PAO1. *Microbiology* 149(Pt 9):2627-2634.10.1099/mic.0.26276-0
623 14. Vasil ML & Ochsner UA (1999) The response of *Pseudomonas aeruginosa* to iron:
624 genetics, biochemistry and virulence. *Molecular microbiology* 34(3):399-413
625 15. Wiens JR, Vasil AI, Schurr MJ, & Vasil ML (2014) Iron-regulated expression of alginate
626 production, mucoid phenotype, and biofilm formation by *Pseudomonas aeruginosa*.
627 *MBio* 5(1):e01010-01013.10.1128/mBio.01010-13
628 16. Oglesby AG*, et al.* (2008) The influence of iron on *Pseudomonas aeruginosa* physiology:
629 a regulatory link between iron and quorum sensing. *J Biol Chem* 283(23):15558-
630 15567.10.1074/jbc.M707840200
631 17. Torres VJ*, et al.* (2010) *Staphylococcus aureus fur* regulates the expression of virulence
632 factors that contribute to the pathogenesis of pneumonia. *Infection and immunity*
633 78(4):1618-1628.10.1128/IAI.01423-09
634 18. Friedman DB*, et al.* (2006) *Staphylococcus aureus* redirects central metabolism to
635 increase iron availability. *PLoS pathogens* 2(8):e87.10.1371/journal.ppat.0020087

636 19. Ochsner UA & Vasil ML (1996) Gene repression by the ferric uptake regulator in
637 *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. *Proceedings of the*
638 *National Academy of Sciences of the United States of America* 93(9):4409-4414
639 20. Nguyen AT*, et al.* (2016) Cystic Fibrosis Isolates of *Pseudomonas aeruginosa* Retain
640 Iron-Regulated Antimicrobial Activity against *Staphylococcus aureus* through the Action
641 of Multiple Alkylquinolones. *Front Microbiol* 7:1171.10.3389/fmicb.2016.01171
642 21. Reinhart AA*, et al.* (2015) The prrF-encoded small regulatory RNAs are required for iron
643 homeostasis and virulence of Pseudomonas aeruginosa. *Infect Immun* 83(3):863-
644 875.10.1128/IAI.02707-14
645 22. Mashburn LM, Jett AM, Akins DR, & Whiteley M (2005) *Staphylococcus aureus* serves
646 as an iron source for *Pseudomonas aeruginosa* during *in vivo* coculture. *Journal of*
647 *bacteriology* 187(2):554-566
648 23. Nguyen AT, Jones JW, Ruge MA, Kane MA, & Oglesby-Sherrouse AG (2015) Iron
649 Depletion Enhances Production of Antimicrobials by *Pseudomonas aeruginosa*. *J*
650 *Bacteriol* 197(14):2265-2275.10.1128/JB.00072-15
651 24. Filkins LM*, et al.* (2015) Coculture of *Staphylococcus aureus* with *Pseudomonas*
652 *aeruginosa* Drives *S. aureus* towards Fermentative Metabolism and Reduced Viability in
653 a Cystic Fibrosis Model. *J Bacteriol* 197(14):2252-2264.10.1128/JB.00059-15
654 25. Szamosvari D & Bottcher T (2017) An Unsaturated Quinolone N-Oxide of *Pseudomonas*
655 *aeruginosa* Modulates Growth and Virulence of *Staphylococcus aureus*. *Angew Chem*
656 *Int Ed Engl* 56(25):7271-7275.10.1002/anie.201702944
657 26. Lightbown JW & Jackson FL (1956) Inhibition of cytochrome systems of heart muscle
658 and certain bacteria by the antagonists of dihydrostreptomycin: 2-alkyl-4-
659 hydroxyquinoline N-oxides. *Biochem J* 63(1):130-137

660 27. Reil E, Hofle G, Draber W, & Oettmeier W (1997) Quinolones and their N-oxides as
661 inhibitors of mitochondrial complexes I and III. *Biochim Biophys Acta* 1318(1-2):291-
662 298.10.1016/s0005-2728(96)00150-8
663 28. Collier DN*, et al.* (2002) A bacterial cell to cell signal in the lungs of cystic fibrosis
664 patients. *FEMS Microbiol Lett* 215(1):41-46
665 29. Bredenbruch F, Geffers R, Nimtz M, Buer J, & Haussler S (2006) The *Pseudomonas*
666 *aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environ Microbiol*
667 8(8):1318-1329.10.1111/j.1462-2920.2006.01025.x
668 30. Pesci EC*, et al.* (1999) Quinolone signaling in the cell-to-cell communication system of
669 *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the*
670 *United States of America* 96(20):11229-11234
671 31. Florez C, Raab JE, Cooke AC, & Schertzer JW (2017) Membrane Distribution of the
672 Pseudomonas Quinolone Signal Modulates Outer Membrane Vesicle Production in
673 *Pseudomonas aeruginosa*. *MBio* 8(4).10.1128/mBio.01034-17
674 32. Coleman JP*, et al.* (2008) *Pseudomonas aeruginosa* PqsA is an anthranilate-coenzyme
675 A ligase. *Journal of bacteriology* 190(4):1247-1255.10.1128/JB.01140-07
676 33. Djapgne L*, et al.* (2018) The Pseudomonas aeruginosa PrrF1 and PrrF2 small regulatory
677 RNAs (sRNAs) promote 2-alkyl-4-quinolone production through redundant regulation of
678 the antR mRNA. *Journal of bacteriology*.10.1128/JB.00704-17
679 34. Beauchene NA*, et al.* (2015) Impact of Anaerobiosis on Expression of the Iron-
680 Responsive Fur and RyhB Regulons. *mBio* 6(6):e01947-01915.10.1128/mBio.01947-15
681 35. Sampathkumar B*, et al.* (2006) Transcriptional and translational expression patterns
682 associated with immobilized growth of Campylobacter jejuni. *Microbiology* 152(Pt 2):567-
683 577.10.1099/mic.0.28405-0
684 36. Nelson CE*, et al.* (2019) Proteomic analysis of the *Pseudomonas aeruginosa* iron
685 starvation response reveals PrrF sRNA-dependent iron regulation of twitching motility,

686 amino acid metabolism, and zinc homeostasis proteins. *J Bacteriol*.10.1128/JB.00754-
687 18
688 37. Erde J, Loo RR, & Loo JA (2014) Enhanced FASP (eFASP) to increase proteome
689 coverage and sample recovery for quantitative proteomic experiments. *J Proteome Res*
690 13(4):1885-1895.10.1021/pr4010019
691 38. Wisniewski JR, Zougman A, Nagaraj N, & Mann M (2009) Universal sample preparation
692 method for proteome analysis. *Nat Methods* 6(5):359-362.10.1038/nmeth.1322
693 39. Winsor GL*, et al.* (2016) Enhanced annotations and features for comparing thousands of
694 Pseudomonas genomes in the Pseudomonas genome database. *Nucleic Acids Res*
695 44(D1):D646-653.10.1093/nar/gkv1227
696 40. Eng JK, Fischer B, Grossmann J, & Maccoss MJ (2008) A fast SEQUEST cross
697 correlation algorithm. *J Proteome Res* 7(10):4598-4602.10.1021/pr800420s
698 41. Dorfer V*, et al.* (2014) MS Amanda, a universal identification algorithm optimized for high
699 accuracy tandem mass spectra. *J Proteome Res* 13(8):3679-3684.10.1021/pr500202e
700 42. Kall L, Canterbury JD, Weston J, Noble WS, & MacCoss MJ (2007) Semi-supervised
701 learning for peptide identification from shotgun proteomics datasets. *Nat Methods*
702 4(11):923-925.10.1038/nmeth1113
703 43. Kanehisa M, Sato Y, Kawashima M, Furumichi M, & Tanabe M (2016) KEGG as a
704 reference resource for gene and protein annotation. *Nucleic Acids Res* 44(D1):D457-
705 462.10.1093/nar/gkv1070
706 44. Huang W*, et al.* (2018) PAMDB: a comprehensive Pseudomonas aeruginosa
707 metabolome database. *Nucleic Acids Res* 46(D1):D575-D580.10.1093/nar/gkx1061
708 45. Szklarczyk D*, et al.* (2017) The STRING database in 2017: quality-controlled protein-
709 protein association networks, made broadly accessible. *Nucleic Acids Res* 45(D1):D362-
710 D368.10.1093/nar/gkw937

711 46. Brewer LK*, et al.* (2019) Development and Bioanalytical Method Validation of an LC-
712 MS/MS Assay for Simultaneous Quantitation of 2-Alkyl-4(1H)-Quinolones for Application
713 in Bacterial Cell Culture and Lung Tissue. *Submitted*
714 47. Ochsner UA, Wilderman PJ, Vasil AI, & Vasil ML (2002) GeneChip expression analysis
715 of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel
716 pyoverdine biosynthesis genes. *Mol Microbiol* 45(5):1277-1287.10.1046/j.1365-
717 2958.2002.03084.x
718 48. Poole K, Heinrichs DE, & Neshat S (1993) Cloning and sequence analysis of an EnvCD
719 homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in
720 the secretion of the siderophore pyoverdine. *Molecular microbiology* 10(3):529-544
721 49. Ankenbauer RG & Quan HN (1994) FptA, the Fe(III)-pyochelin receptor of
722 *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to
723 hydroxamate siderophore receptors. *J Bacteriol* 176(2):307-319
724 50. Dean CR & Poole K (1993) *Cloning and characterizati*on of the ferric enterobactin
725 receptor gene (pfeA) of Pseudomonas aeruginosa. *Journal of bacteriology* 175(2):317-
726 324
727 51. Serino L*, et al.* (1997) Biosynthesis of pyochelin and dihydroaeruginoic acid requires the
728 iron-regulated pchDCBA operon in *Pseudomonas aeruginosa*. *J Bacteriol* 179(1):248-
729 257
730 52. Hassett DJ*, et al.* (1997) An operon containing fumC and sodA encoding fumarase C
731 and manganese superoxide dismutase is controlled by the ferric uptake regulator in
732 *Pseudomonas aeruginosa*: fur mutants produce elevated alginate levels. *J Bacteriol*
733 179(5):1452-1459
734 53. Wilderman PJ*, et al.* (2004) Identification of tandem duplicate regulatory small RNAs in
735 *Pseudomonas aeruginosa* involved in iron homeostasis. *Proceedings of the National*

736 *Academy of Sciences of the United States of America* 101(26):9792-
737 9797.10.1073/pnas.0403423101
738 54. Sana TG*, et al.* (2012) The second type VI secretion system of *Pseudomonas*
739 *aeruginosa* strain PAO1 is regulated by quorum sensing and Fur and modulates
740 internalization in epithelial cells. *J Biol Chem* 287(32):27095-
741 27105.10.1074/jbc.M112.376368
742 55. Lesic B, Starkey M, He J, Hazan R, & Rahme LG (2009) Quorum sensing differentially
743 regulates *Pseudomonas aeruginosa* type VI secretion locus I and homologous loci II and
744 III, which are required for pathogenesis. *Microbiology* 155(Pt 9):2845-
745 2855.10.1099/mic.0.029082-0
746 56. Gaines JM, Carty NL, Colmer-Hamood JA, & Hamood AN (2005) Effect of static growth
747 and different levels of environmental oxygen on toxA and ptxR expression in the
748 *Pseudomonas aeruginosa* strain PAO1. *Microbiology* 151(Pt 7):2263-
749 2275.10.1099/mic.0.27754-0
750 57. Wyckoff TJ, Thomas B, Hassett DJ, & Wozniak DJ (2002) Static growth of mucoid
751 *Pseudomonas aeruginosa* selects for non-mucoid variants that have acquired flagellum-
752 dependent motility. *Microbiology* 148(Pt 11):3423-3430.10.1099/00221287-148-11-3423
753 58. Dotsch A*, et al.* (2012) The *Pseudomonas aeruginosa* transcriptome in planktonic
754 cultures and static biofilms using RNA sequencing. *PLoS One*
755 7(2):e31092.10.1371/journal.pone.0031092
756 59. Schertzer JW, Brown SA, & Whiteley M (2010) Oxygen levels rapidly modulate
757 *Pseudomonas aeruginosa* social behaviours via substrate limitation of PqsH. *Mol*
758 *Microbiol* 77(6):1527-1538.10.1111/j.1365-2958.2010.07303.x
759 60. Drees SL*, et al.* (2018) PqsL uses reduced flavin to produce 2-
760 hydroxylaminobenzoylacetate, a preferred PqsBC substrate in alkyl quinolone

761 biosynthesis in *Pseudomonas aeruginosa*. *J Biol Chem* 293(24):9345-
762 9357.10.1074/jbc.RA117.000789
763 61. Deziel E*, et al.* (2005) The contribution of MvfR to Pseudomonas aeruginosa
764 pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-
765 regulated genes are modulated without affecting lasRI, rhlRI or the production of N-acyl-
766 L-homoserine lactones. *Mol Microbiol* 55(4):998-1014.10.1111/j.1365-
767 2958.2004.04448.x
768 62. Xiao G*, et al.* (2006) MvfR, a key Pseudomonas aeruginosa pathogenicity LTTR-class
769 regulatory protein, has dual ligands. *Mol Microbiol* 62(6):1689-1699.10.1111/j.1365-
770 2958.2006.05462.x
771 63. Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC, & Manoil C (2002) Functions
772 required for extracellular quinolone signaling by Pseudomonas aeruginosa. *J Bacteriol*
773 184(23):6472-6480
774 64. Russell AB*, et al.* (2013) Diverse type VI secretion phospholipases are functionally
775 plastic antibacterial effectors. *Nature* 496(7446):508-512.10.1038/nature12074
776 65. Hood RD*, et al.* (2010) A type VI secretion system of *Pseudomonas aeruginosa* targets
777 a toxin to bacteria. *Cell Host Microbe* 7(1):25-37.10.1016/j.chom.2009.12.007
778 66. Chandler CE*, et al.* (2019) Genomic and Phenotypic Diversity among Ten Laboratory
779 Isolates of Pseudomonas aeruginosa PAO1. *J Bacteriol* 201(5).10.1128/JB.00595-18
780 67. Meirelles LA & Newman DK (2018) Both toxic and beneficial effects of pyocyanin
781 contribute to the lifecycle of *Pseudomonas aeruginosa*. *Mol Microbiol* 110(6):995-
782 1010.10.1111/mmi.14132
783 68. Glasser NR, Kern SE, & Newman DK (2014) Phenazine redox cycling enhances
784 anaerobic survival in *Pseudomonas aeruginosa* by facilitating generation of ATP and a
785 proton-motive force. *Mol Microbiol* 92(2):399-412.10.1111/mmi.12566

786 69. Wang Y*, et al.* (2011) Phenazine-1-carboxylic acid promotes bacterial biofilm
787 development via ferrous iron acquisition. *J Bacteriol* 193(14):3606-
788 3617.10.1128/JB.00396-11
789 70. O'May CY, Sanderson K, Roddam LF, Kirov SM, & Reid DW (2009) Iron-binding
790 compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under
791 anaerobic conditions. *J Med Microbiol* 58(Pt 6):765-773.10.1099/jmm.0.004416-0
792 71. Wang Y & Newman DK (2008) Redox reactions of phenazine antibiotics with ferric
793 (hydr)oxides and molecular oxygen. *Environ Sci Technol* 42(7):2380-
794 2386.10.1021/es702290a
795 72. Britigan BE*, et al.* (1992) Interaction of the *Pseudomonas aeruginosa* secretory products
796 pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to
797 endothelial cells. Implications for *Pseudomonas*-associated tissue injury. *J Clin Invest*
798 90(6):2187-2196.10.1172/JCI116104
799 73. Britigan BE, Rasmussen GT, & Cox CD (1997) Augmentation of oxidant injury to human
800 pulmonary epithelial cells by the *Pseudomonas aeruginosa* siderophore pyochelin. *Infect*
801 *Immun* 65(3):1071-1076
802 74. Coffman TJ, Cox CD, Edeker BL, & Britigan BE (1990) Possible role of bacterial
803 siderophores in inflammation. Iron bound to the *Pseudomonas* siderophore pyochelin
804 can function as a hydroxyl radical catalyst. *J Clin Invest* 86(4):1030-
805 1037.10.1172/JCI114805
806 75. Henry RL, Mellis CM, & Petrovic L (1992) Mucoid *Pseudomonas aeruginosa* is a marker
807 of poor survival in cystic fibrosis. *Pediatr Pulmonol* 12(3):158-161
808 76. Pedersen SS, Hoiby N, Espersen F, & Koch C (1992) Role of alginate in infection with
809 mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* 47(1):6-13
810