

1 **The heme-responsive PrrH sRNA regulates *Pseudomonas aeruginosa* pyochelin gene ex-**
2 **pression**

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19 **Abstract**

20 *Pseudomonas aeruginosa* is an opportunistic pathogen that requires iron for growth and
21 virulence, yet this nutrient is sequestered by the innate immune system during infection. When
22 iron is limiting, *P. aeruginosa* expresses the PrrF1 and PrrF2 small regulatory RNAs (sRNAs),
23 which post-transcriptionally repress expression of non-essential iron-containing proteins thus
24 sparing this nutrient for more critical processes. The genes for the PrrF1 and PrrF2 sRNAs are
25 arranged in tandem on the chromosome, allowing for the transcription of a longer heme-respon-
26 sive sRNA, termed PrrH. While the functions of PrrF1 and PrrF2 have been studied extensively,
27 the role of PrrH in *P. aeruginosa* physiology and virulence is not well understood. In this study,
28 we performed transcriptomic and proteomic studies to identify the PrrH regulon. In shaking cul-
29 tures, the pyochelin synthesis proteins were increased in two distinct *prhH* mutants compared to
30 wild type, while the mRNAs for these proteins were not affected by *prhH* mutation. We identified
31 complementarity between the PrrH sRNA and sequence upstream of the *pchE* mRNA, suggesting
32 potential for PrrH to directly regulate expression of genes for pyochelin synthesis. We further
33 showed that *pchE* mRNA levels were increased in the *prhH* mutants when grown in static but not
34 shaking conditions. Moreover, we discovered controlling for the presence of light was critical for
35 examining the impact of PrrH on *pchE* expression. As such, our study reports on the first likely
36 target of the PrrH sRNA and highlights key environmental variables that will allow for future char-
37 acterization of PrrH function.

38

39 **Importance**

40 In the human host, iron is predominantly in the form of heme, which *Pseudomonas aeru-*
41 *ginosa* can acquire as an iron source during infection. We previously showed that the iron-respon-
42 sive PrrF sRNAs are critical for mediating iron homeostasis during *P. aeruginosa* infection; how-
43 ever the function of the heme-responsive PrrH sRNA remains unclear. In this study, we identified
44 genes for pyochelin siderophore biosynthesis, which mediate uptake of inorganic iron, as a novel
45 target of PrrH regulation. This study therefore highlights a novel relationship between heme avail-
46 ability and siderophore biosynthesis in *P. aeruginosa*.

47

48 Introduction

49 *P. aeruginosa* is a versatile environmental organism and opportunistic pathogen that can
50 survive in a wide range of environments. As a pathogen, *P. aeruginosa* causes acute lung and
51 blood infections in cancer patients and 10% of all hospital-acquired infections (1-4). *P. aeruginosa*
52 also causes life-long chronic lung infections in individuals with cystic fibrosis (CF) and is a signif-
53 icant contributor to chronic wound infections in diabetics and surgical patients (5-7). To evade the
54 immune system during infection, *P. aeruginosa* deploys numerous virulence factors, including
55 exotoxin A (8-10), type three secretion (11-13), and redox-active phenazine metabolites (14-16).
56 *P. aeruginosa* can also form biofilms, or adherent communities encased in a self-produced ex-
57 opolysaccharide (EPS) matrix, which protect the bacteria from immune assault during device-
58 mediated (e.g. ventilator associated pneumonia) and chronic infections (17-19). *P. aeruginosa* is
59 innately resistant to many therapeutic agents, and the emergence of multi-drug resistant (MDR)
60 strains of *P. aeruginosa* leads to persistent infections, longer hospital stays, and increased mor-
61 tality rates (20). Biofilm formation during chronic infection further complicates treatment due to
62 increased tolerance of these communities against antimicrobials (21). Timely expression of viru-
63 lence-related genes is essential for survival in the host, and *P. aeruginosa* regulates virulence-
64 associated processes in response to a variety of environmental cues, including nutrient availability
65 and quorum sensing factors (22). Understanding the regulatory pathways that mediate virulence
66 trait expression may therefore reveal novel strategies for therapeutic intervention.

67 As with many other pathogens, *P. aeruginosa* requires metallonutrients for growth and
68 virulence. *P. aeruginosa* has a particularly high requirement for iron, which plays a central role in
69 metabolism, oxygen and redox sensing, protection from oxidative stress, and nucleic acid syn-
70 thesis (23). To limit pathogen growth, the host restricts iron and other essential metals in a strat-
71 egy called “nutritional immunity” (24). *P. aeruginosa* overcomes nutritional immunity through a
72 variety of mechanisms, including the synthesis and uptake of two siderophores – pyoverdine and

73 pyochelin – which scavenge the oxidized (ferric) form of iron (Fe^{3+}) from the host iron sequestra-
74 tion proteins lactoferrin and transferrin (25-27). In reducing environments, *P. aeruginosa* acquires
75 the reduced (ferrous) form of iron (Fe^{2+}) via the Feo system (28). *P. aeruginosa* can also acquire
76 iron from heme, representing the most predominant source of iron in the human body (29). Heme
77 acquisition is mediated by the heme assimilation (Has) and *Pseudomonas* heme uptake (Phu)
78 systems, which transport heme into the cytosol (30), and a cytosolic heme oxygenase HemO that
79 cleaves the heme tetrapyrrole to yield biliverdin and inorganic iron for use in cellular processes
80 (31). Several studies have suggested that siderophore-mediated iron uptake is critical for acute
81 infections (26, 32), while ferrous and heme uptake become more prominent in chronic, biofilm
82 mediated infections that are characterized by biofilm communities, steep oxygen gradients, and
83 persistent inflammation (33-37).

84 Despite its essentiality, iron catalyzes the formation of reactive oxygen species via Fenton
85 chemistry, leading to damage of membranes, proteins, and DNA. In *P. aeruginosa* and many
86 other bacteria, the Ferric uptake regulator (Fur) when bound to cytosolic Fe^{2+} becomes an active
87 transcriptional repressor of genes involved in iron uptake (38, 39). *P. aeruginosa* Fur also re-
88 presses expression of two non-coding small RNAs (sRNAs) called PrrF1 and PrrF2 (40). The PrrF
89 sRNAs function by complementary base-pairing with, and destabilization of, mRNAs coding for
90 non-essential iron-containing proteins, resulting in what has been termed the “iron sparing re-
91 sponse” (41, 42). Owing to the central role of iron in *P. aeruginosa* physiology, deletion of both
92 the *prrF1* and *prrF2* genes results in a significant growth defect in low iron media, decreased
93 production of quorum sensing molecules, increased susceptibility to tobramycin during biofilm
94 growth, and attenuated virulence in an acute murine lung infection model (43-48).

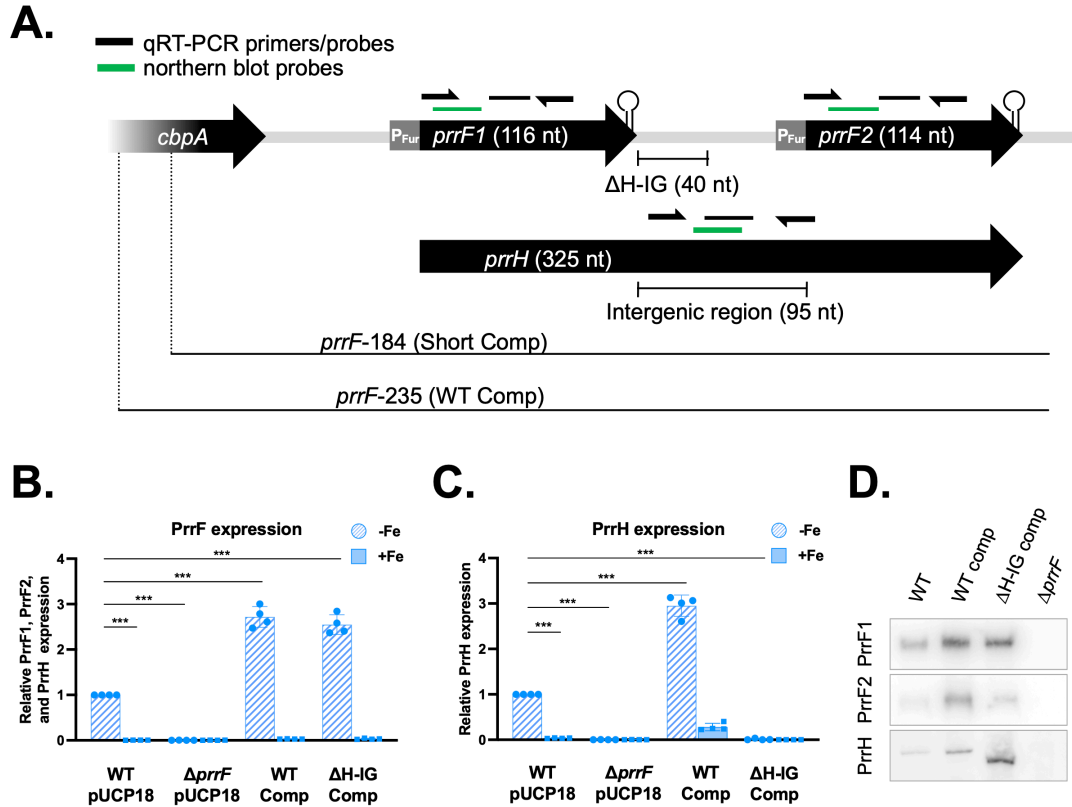
95 The *prrF1* and *prrF2* genes are located in tandem on the *P. aeruginosa* chromosome,
96 allowing for the transcription of a distinct third sRNA called PrrH (49). PrrH shares a promoter and
97 transcriptional start site with PrrF1, yet its expression is also dependent on read-through of the
98 *prrF1* Rho-independent terminator, *prrF1-prrF2* intergenic region, and *prrF2* sequence (**Fig. 1A**).

99 Due to sharing a promoter with *prrF1*, transcription of PrrH is similarly repressed by iron (49).
100 However, PrrH has also been shown to be regulated by heme (49, 50). Moreover, the PrrH sRNA
101 contains a unique sequence, derived from the *prrF1-prrF2* intergenic region (**PrrH-IG, Fig. 1A**),
102 that may be able to interact with and alter stability or translation of a distinct regulon of mRNAs.
103 The entire *prrH* sequence, including the PrrH-IG region, is broadly conserved in *P. aeruginosa*
104 clinical isolates, and the PrrH transcript is detected in clinical sputum samples, suggesting im-
105 portance of this sRNA during infection (34). An inherent challenge of studying PrrH is separating
106 its functions from those of the PrrF sRNAs, since it is not possible to transcribe PrrH without the
107 *prrF1* and *prrF2* genes (**Fig. 1A**). Given the model that the PrrH-IG region is required for PrrH
108 function, we previously generated a *prrF* locus allele with a deletion of the PrrH-IG region (Δ *prrH-*
109 *IG*) to distinguish PrrH and PrrF functions. We found that the PrrH-IG sequence is not responsible
110 for any of the previously identified phenotypes of the Δ *prrF* mutant (44). Thus, the role of PrrH in
111 mediating iron and heme homeostasis to date remains unclear.

112 In the current study, we continued our analysis of the Δ *prrH-IG* mutant, as well as a distinct
113 *prrH* mutant, to further investigate PrrH function. These strains were characterized by multiple
114 approaches, including simultaneous RNAseq and proteomics analyses. Our results revealed
115 genes for pyochelin biosynthesis as possible targets of PrrH regulation. We subsequently showed
116 that *prrH* mutation led to increased expression of *pchE*, and we identified static growth conditions
117 as more permissive of this regulation. We further found that controlling for light, which can be
118 sensed by *P. aeruginosa* through the photoreceptor BphP, led to more consistent and robust
119 repression of *pchE* by PrrH, suggesting this signal may have confounded previous PrrH regulation
120 studies. Lastly, we show that heme represses expression of the *pchE* gene, though the precise
121 role of PrrH in this regulation remains unclear. Overall, our data indicate that heme and PrrH affect
122 expression of pyochelin siderophore biosynthesis, either by distinct or overlapping pathways.

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125

126 **Fig 1. Characterization of transcripts produced by the $\Delta prrH$ -IG mutant.** Organization of the *prrF* locus

127 and design of complement plasmids (A). The WT comp includes the entire *prrF* locus plus 235 bp upstream

128 of the *prrF1* promoter to ensure regulation of locus is uninhibited. The Δ H-IG comp also includes the 235

129 bp region upstream of the *prrF1* promoter but has 40 bp of the *prrF1*-*prrF2* intergenic region removed. The

130 short comp contains the entire *prrF* locus but only includes 184 bp upstream of the *prrF1* promoter. Location

131 of qRT-PCR primers and probes are indicated in pink (PrrH) and red (PrrF). The PrrF primers and probes

132 cannot distinguish between PrrF1 and PrrF2. Northern blot probes are labeled in blue. For qRT-PCR (B,C),

133 PrrF and PrrH transcription are shown as an average of 3 biological replicates, relative to WT PAO1 in low

134 iron. Northern blot (D) is a representative from multiple experiments using radiolabeled DNA probes specific

135 to each transcript.

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138 **Results**

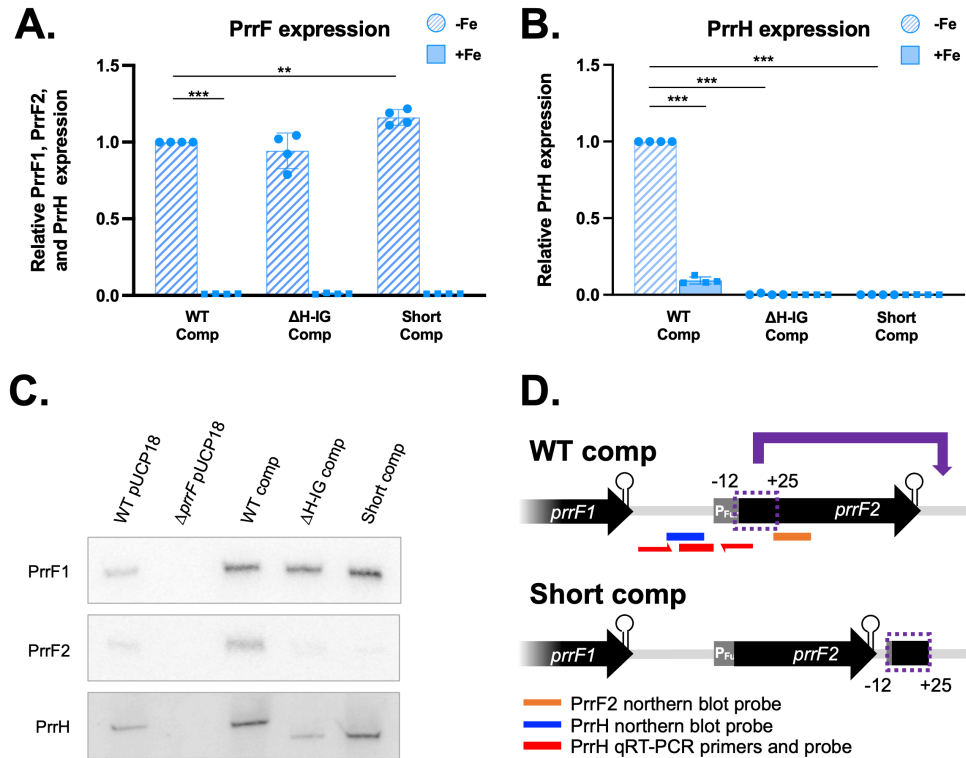
139 **Characterization of *prhH* mutants.** To investigate the functions of PrrH, we used the WT
140 and Δ H-IG complementation system previously developed by our laboratory (**Fig. 1A**) (44). In this
141 system, the Δ *prfF* mutant is complemented with either the entire *prfF* locus (WT-comp) or the *prfF*
142 locus lacking 40 bp of the *prfF1-prfF2* intergenic region (Δ H-IG-comp) (**Fig. 1A**) *in trans* using the
143 pUCP18 vector. Strains labeled as wild type (WT) and Δ *prfF* are the indicated PAO1 strains car-
144 rying the empty pUCP18 vector. As previously observed (44), quantitative real-time PCR (qRT-
145 PCR) using the primers in **Figure 1A** shows that WT-comp expresses PrrF and PrrH in low but
146 not in high iron M9 minimal medium (**Fig. 1B**). Furthermore, the PrrF, but not the PrrH, transcript
147 is detected in the Δ H-IG comp strain, indicating this strain is a *prhH* mutant that can still express
148 PrrF (**Fig. 1C**). Both PrrF and PrrH are expressed at about 3-fold higher levels in the comple-
149 mented strains compared to the WT vector control, likely due to ectopic expression from the
150 pUCP18 plasmid.

151 To confirm the qRT-PCR results, northern blot analysis was performed with probes spe-
152 cific for PrrF1, PrrF2, and PrrH as indicated in **Figure 1A**. As expected, the PrrF1 and PrrF2
153 transcripts were detected in RNA isolated from the WT-comp and the Δ H-IG-comp strains grown
154 in iron-depleted medium (**Fig. 1D**). In agreement with the qPCR results, both complemented
155 strains expressed higher levels of PrrF than the WT vector control (**Fig. 1D**). Because the PrrH
156 probe anneals to the intergenic region adjacent to the deleted 40 bp (**Fig. 1A**), we were also able
157 to determine that the Δ H-IG comp strain expresses a shorter PrrH transcript compared to that
158 expressed in the WT vector control and WT-comp strain, indicating that the Δ H-IG allele produces
159 a truncated PrrH sRNA (**Fig. 1D**).

160 We next characterized the transcripts of a distinct *prhH* mutant. This mutant was originally
161 constructed as a *prfF* complementation plasmid that contained less sequence upstream of the
162 *prfF1/prhH* transcriptional start site than the “WT comp” used in the above discussed studies. This
163 plasmid, which we refer to as the “short-comp”, was designed to contain the entire *prfF1-prfF2*

164 locus with only 184 bp upstream of the *prfF1/prfH* transcriptional start site, as compared to 235
165 bp upstream that is included in the WT-comp (**Fig 1A**) (44). Confirming our previous observation
166 (44), qRT-PCR analysis shows that the PrrF, but not the PrrH, transcript is detected in RNA iso-
167 lated from the short-comp strain (**Fig 2A, B**). We next performed northern blot analyses with
168 probes specific for PrrF1, PrrF2, and PrrH. The short-comp strain exhibited lower levels of the
169 PrrF2 sRNA and higher levels of the PrrF1 sRNA as compared to WT-comp (**Fig. 2C**). Addition-
170 ally, a PrrH transcript was detected from the short-comp strain, but it was slightly shorter than the
171 PrrH transcript produced by the WT strain (**Fig. 2C**). These observations were unexpected, as
172 the short complement was designed to only lack sequence well upstream of the *prfF1/prfH* tran-
173 scriptional start site. To investigate this further, we sequenced the short complement plasmid and
174 discovered a rearrangement in the *prfF2* region, as shown in the cartoon in **Figure 2D**. Specifi-
175 cally, 27 nucleotides flanking the *prfF2* start site (from -12 to +25) were translocated to down-
176 stream of the *prfF2* Rho-independent terminator (**Fig. 2D**). This would, therefore, account for both
177 the fainter PrrF2 transcript and truncated PrrH transcripts that were detected in the northern blots.
178 While we were still able to detect a PrrH transcript in the short comp, the re-arrangement led to a
179 PrrH transcript that was truncated in a distinct manner from the Δ H-IG mutant. Thus, we used
180 both the short-comp and Δ H-IG *prfH* mutants to investigate PrrH regulation in this study.

181



182

183 **Fig. 2. The short *prrF* complement has a rearrangement in *prrF2*.** qRT-PCR analyses of PrrF (A) and
 184 PrrH (B) transcription are shown as an average of 3 biological replicates, relative to WT PAO1 in low iron.
 185 Northern blot (C) is a representative from multiple experiments using radiolabeled DNA probes specific to
 186 each transcript (PrrF1, PrrF2, and PrrH). RNAs were isolated from samples collected after 8 hours of aer-
 187 obic growth in M9 media supplemented with 50 nM FeCl₃ (-Fe, low iron) or 100 μM FeCl₃ (+Fe, high iron)
 188 at 37°C. Panel D illustrates the re-arrangement that occurred in the short comp. Location of the northern
 189 blot probes used in 3C are shown in blue (PrrH) and orange (PrrF2). The qRT-PCR primers/probe for PrrH
 190 are shown in red. Sequencing was performed by Eurofins Genomics. Sequencing results were aligned and
 191 analyzed using MacVector software.

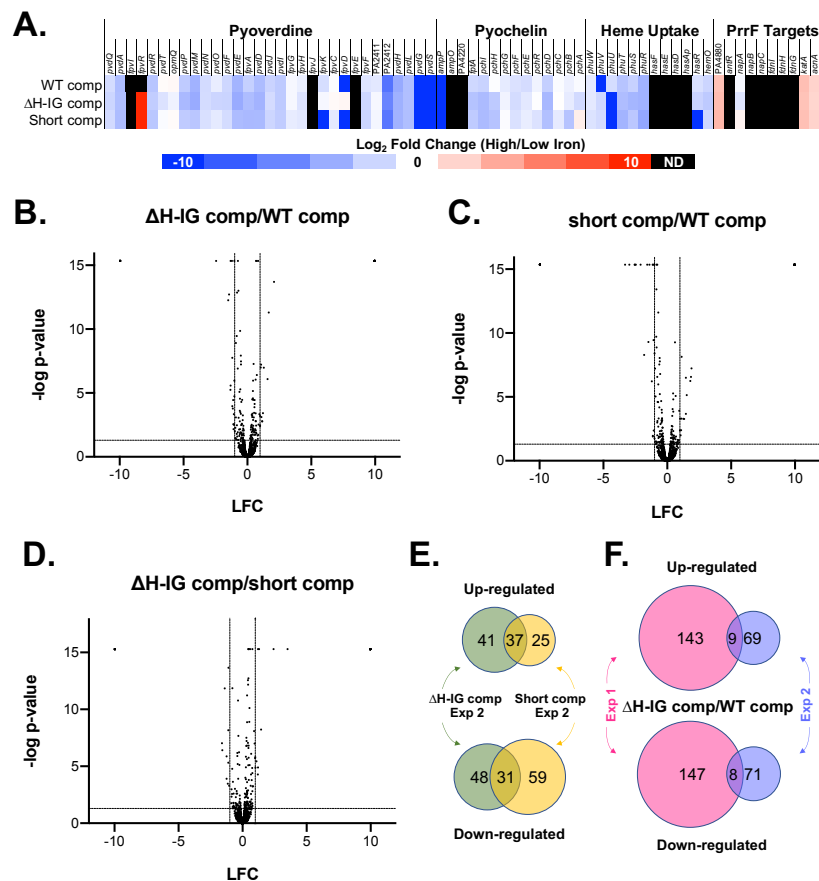
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193 **Transcriptomic and proteomic analysis of PrrF and PrrH regulation in PAO1 reveals**
 194 **pyochelin biosynthesis as a potential PrrH target.** Subsequent to validating the ΔH-IG-comp
 195 strain as a truncated *prrH* mutant, we performed RNAseq (51, 52) and label-free proteomics (53)
 196 on cultures of the WT vector control, Δ*prrF* vector control, WT-comp, and ΔH-IG-comp strains

197 grown in M9 minimal media, with or without FeCl₃ or 5μM heme supplementation. Five biological
198 replicates for each group were processed and the resulting data analyzed as described in the
199 Materials and Methods to generate a log fold change (LFC) for each RNA or protein in response
200 to: 1) iron or heme supplementation of each strain, 2) deletion of the *prrF* locus by comparing the
201 WT and Δ *prrF* vector controls, and 3) deletion of the H-IG sequence by comparing the WT-comp
202 and Δ H-IG-comp strains. Full datasets are provided in the supplementary materials as **Datasets**
203 **S1** (RNASeq) and **S2** (Proteomics). As expected, proteins involved in pyoverdine, pyochelin, and
204 heme uptake, as well as their corresponding mRNAs, were repressed by iron in each of the
205 strains, though the intensity of iron regulation varied somewhat amongst the individual strains,
206 particularly at the protein level (**Supplementary Materials, Fig. S1A**). Also as expected, most of
207 the known PrrF target mRNAs, and the proteins they encode, were activated by iron in the WT
208 vector control, WT-comp, and Δ H-IG strain, and this regulation was reduced or eliminated in the
209 Δ *prrF* vector control (**Supplementary Materials, Fig. S1A**), indicating that the plasmid-derived
210 PrrF sRNAs function similarly to those transcribed from the chromosome. Regulatory patterns for
211 these known iron-regulated genes and proteins varied slightly between the WT-comp and WT vec-
212 tor control, but they were comparable when comparing the WT-comp and Δ H-IG-comp strains
213 (**Supplementary Materials, Fig. S1A**).

214 We next determined how loss of the H-IG sequence affected PAO1 gene expression by
215 comparing the transcriptomes and proteomes of the Δ H-IG-comp strain, grown in low iron, to that
216 of the WT-comp strain, also grown in low iron. This analysis revealed no statistically significant
217 differences in the transcriptomes of these strains (**Supplementary Materials, Fig. S1B**), while
218 the proteome of the Δ H-IG comp was substantially altered compared to that of the WT comp
219 (**Supplementary Materials, Fig. S1C**). To validate observed changes in the PrrH-affected prote-
220 ome, we conducted a subsequent proteomics experiment with the WT-comp, Δ H-IG-comp, and
221 short-comp strains grown in M9 minimal medium with and without iron supplementation. As ob-
222 served in the first experiment, proteins involved in pyoverdine, pyochelin, and heme uptake were

223 similarly repressed by iron in all three strains, and PrrF-repressed targets showed similar effects
 224 in response to iron supplementation across all three strains (**Fig. 3A**), demonstrating that both
 225 *prhH* mutants exhibited iron and PrrF regulation similar to the WT strain. Also as observed for the
 226 first experiment, the proteomes of the Δ H-IG comp (**Fig. 3B**) and the short-comp (**Fig. 3C**) were
 227 significantly altered when compared to the WT-comp strain. Curiously, we also noted substantial
 228 differences in the proteomes of the short-comp and Δ H-IG strains (**Fig. 3D**). Moreover, proteins
 229 that were either upregulated or downregulated in each *prhH* mutant compared to the WT-comp in
 230 this dataset showed limited overlap with one another (**Fig 3E**, Δ H-IG-affected proteomes are rep-
 231 resented by green circles, short-comp-affected proteomes are represented by yellow circles).
 232 Likewise, proteins that were either upregulated or downregulated upon H-IG deletion in the first
 233 (**Fig. 3F**, represented by pink circles) and second (**Fig. 3F**, represented by purple circles) prote-
 234 omics experiments showed little overlap.



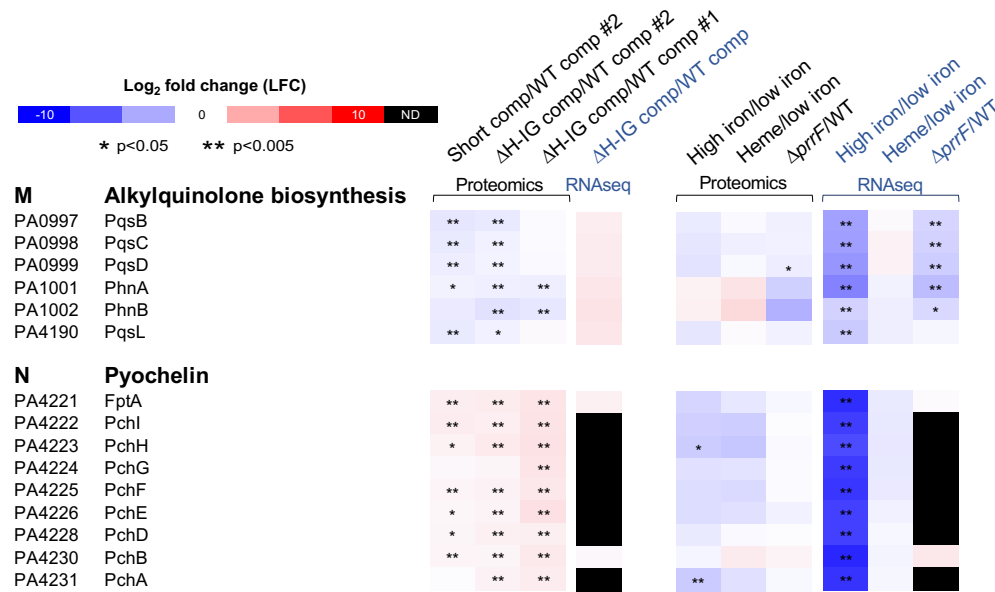
236 **Fig 3. Short complement and Δ H-IG complement substantially alter the proteome.** Proteomics results
237 when comparing protein samples collected after 8 hours of aerobic growth in M9 media supplemented with
238 50 nM FeCl₃ at 37°C. (A) Heatmap of a select group of iron-, heme-, and PrrF-regulated proteins shown as
239 the log₂ fold change of the abundance ratio between high iron and low iron. Undetected proteins are colored
240 in black (ND). (B-D) Volcano plots comparing the protein abundance of the Δ H-IG comp versus WT comp
241 (B), short comp versus WT comp (C), and Δ H-IG comp versus short comp (D). The log₂ fold change is
242 shown on the x-axes and the -log of the FDR p-value is on the y-axes. Horizontal dashed lines indicate
243 FDR p=0.05 and vertical dashed lines indicate LFC = \pm 1. (E-F) Comparison of the dysregulated proteins in
244 the Δ H-IG comp from experiments 1 and 2 are shown as Venn diagrams. (E) Panel E shows the overlap of
245 the dysregulated proteins in the Δ H-IG comp and short comp, each compared to WT comp, from experiment
246 2. (F) Panel F shows the overlap of the dysregulated proteins in the Δ H-IG comp compared to WT between
247 experiments 1 and 2. To be included in the Venn diagram analysis, changes in protein levels must have
248 demonstrated an FDR p-value <0.05 and -0.5 \leq LFC \geq 0.5.

249

250 Since the above datasets showed little consistency in robust PrrH effects, we sought to
251 identify smaller yet more consistent regulatory effects of *prhH* mutation. To investigate this, we
252 performed STRING network analysis on the proteins that were differentially regulated in any one
253 of the three comparisons (Δ H-IG comp/WT comp from experiment 1, Δ H-IG comp/WT comp from
254 experiment 2, and short comp/WT comp from experiment 2). To capture proteins that were weakly
255 but statistically significantly affected in each experiment, we lowered the LFC cutoff value for
256 proteins to be analyzed to 0.5 (-0.5 \leq LFC \leq 0.5). STRING network analysis revealed numerous
257 dysregulated functions and pathways amongst the proteins affected in all three experimental com-
258 parisons (**Supplementary Materials, Fig. S2-S3**). However, many of these clusters were not
259 consistently dysregulated. For example, the phenazine biosynthesis cluster was upregulated
260 upon *prhH* mutation in the first experiment yet downregulated by *prhH* mutation in the second
261 experiment. We also observed inconsistencies between comparisons when we considered how
262 iron, heme, and PrrF affected genes in each of these clusters. For example, proteins in cluster A

263 (sulfur metabolism) showed variable regulation by PrrH and were repressed in the *prfF* mutant
264 but induced by iron (**Supplementary Materials, Fig. S2-S3**).

265 Despite the variations in the effects of PrrH amongst the different experiments, we identi-
266 fied two clusters in which the effects of PrrH on proteins involved in specific cellular functions
267 were consistent across all three comparisons. Cluster M included proteins for 2-alkyl-4(1*H*)-quin-
268 olone biosynthesis which were reduced upon mutation of PrrH in all three comparisons (**Fig. 4**).
269 In contrast, Cluster N was comprised of pyochelin proteins that were largely induced by PrrH
270 mutations in all three comparisons (**Fig. 4**). Consistent with the analysis shown in **Figure S1**,
271 none of the RNAs encoding these proteins were significantly affected by PrrH mutation in the
272 RNASeq analysis of the first experiment (**Fig. 4**). Further analysis of Cluster M proteins showed
273 that these proteins were also affected by *prfF* deletion, which is consistent with previous studies
274 from our group (34, 48). Moreover, while iron repressed the levels of RNAs for the Cluster M
275 proteins, heme did not affect levels of the Cluster M proteins (**Fig. 4**), suggesting they are not
276 specifically regulated by PrrH. In contrast, proteomics and RNAseq showed no effect of PrrF on
277 proteins in Cluster N (**Fig. 4**). Moreover, heme repressed proteins in Cluster N, but had no effect
278 on the corresponding RNAs (**Fig. 4**), consistent with the lack of transcriptome effects observed
279 upon mutation of the PrrH sRNA (**Supplementary Materials, Figure S1**). Therefore, we focused
280 on the pyochelin biosynthesis genes and proteins in Cluster N as potential novel targets of the
281 PrrH sRNA.



282

283 **Fig 4. Proteins involved in alkylquinolone biosynthesis and pyochelin biosynthesis are dysregu-**

284 **lated in *prfH* mutants.** Expression data from proteomics and RNAseq are presented as heat maps where

285 up-regulated proteins are indicated in red and those down-regulated are in blue. Undetected proteins are

286 colored in black (ND). ** indicate p<0.005 and * indicate p<0.05.

287

288 **Static growth reveals potential for PrrH-mediated repression of the pyochelin bio-**

289 **synthesis *pchE* mRNA.** sRNA-mediated repression of gene expression is most often mediated

290 by pairing at or near the Shine Dalgarno or translational start site of an mRNA, precluding the

291 ribosome and in some cases resulting in destabilization of the mRNA. To determine the potential

292 for PrrH to directly regulate expression of proteins involved in pyochelin biosynthesis or uptake,

293 we analyzed the H-IG sequence to determine if pairing could occur with any of the PrrH affected

294 *pch* mRNAs using CopraRNA (54-56). This analysis identified PrrH complementarity sites within

295 the coding sequences of two distinct pyochelin genes: *pchI* and *pchE* (**Fig. 5A**), demonstrating

296 the capacity of the PrrH-IG sequence to directly pair with at least two mRNAs encoding proteins

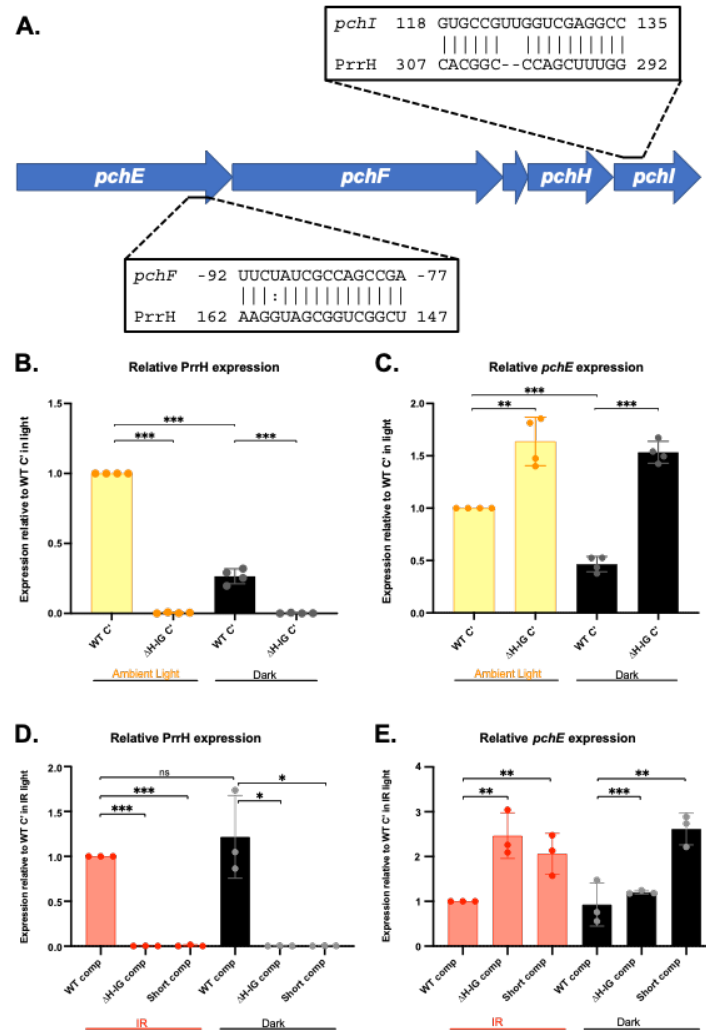
297 for pyochelin biosynthesis. Of note, the *pchI* and *pchE* are located within a single operon (**Fig.**

298 **5A**), suggesting PrrH may be able to bind at two distinct sites of the *pchEFGHI* mRNA.

299 We next sought to determine whether RNA levels for pyochelin biosynthesis are affected
300 by PrrH mutation under conditions where this system is more strongly expressed. Recently, our
301 lab observed that proteins involved in pyochelin biosynthesis are more robustly regulated by iron
302 in static compared to shaking conditions (57), suggesting static conditions may be more permis-
303 sive for expression of pyochelin mRNAs. Therefore, we performed qRT-PCR on samples col-
304 lected from both static and shaking cultures and, indeed, we observed an increase in *pchE* tran-
305 scription in each *prrH* mutant compared to the WT-comp when grown in static, but not shaking,
306 conditions (**Supplementary Materials Fig. S4A**). However, the increase in *pchE* expression, al-
307 beit statistically significant, was modest, and results of this experiment when repeated several
308 weeks apart gave inconsistent results (**Supplementary Materials Fig. S4B**). Thus, while these
309 data suggested that PrrH may affect *pchE* mRNA stability, they also indicated that confounding
310 variables were continuing to affect our ability to study PrrH regulation.

311
312 **Ambient and infrared light affect PrrH's impact on *pchE* mRNA levels.** Recent studies
313 from Mukherjee, *et al*, demonstrated that the photoreceptor BphP can mediate light-dependent
314 changes in biofilm-related gene expression via the response regulator AlgB (58). The BphP pho-
315 toreceptor activity is dependent on a biliverdin chromophore produced via the BphO heme oxy-
316 genase, which serves independent functions from the HemO heme oxygenase that mediates ac-
317 quisition of iron from heme (59, 60). Owing to its potential role in heme homeostasis, we wondered
318 whether BphP, and therefore light, may affect PrrH regulation of the potential *pchE* target. To
319 begin testing this, we first assessed the impact of ambient light on static cultures of the WT-comp
320 and Δ H-IG comp strains. Cultures were grown in a well-lit room (fluorescent lights on and near a
321 window) to provide an “ambient light” source, and parallel cultures were wrapped completely in
322 foil to produce a “dark” condition. Initial qPCR analysis revealed a significant decrease in PrrH
323 expression in dark compared to light conditions (**Fig. 5B**), though this trend was reversed in a
324 subsequent experiment (**Supplementary Materials Fig. S4C**). In contrast, we noted a significant

325 increase in *pchE* expression upon H-IG deletion in both light and dark conditions (Fig. 5C), a
 326 finding that was reproduced in a subsequent run of the experiment (Supplementary Materials
 327 Fig. S4D).



328

329 **Fig 5. Static growth and controlling for light promote consistent PrrH repression of the *pchEF***

330 **mRNA.** (A) CopraRNA identified sequences with complementarity to PrrH upstream of *pchF* and within

331 *pchI*. (B-E) qRT-PCR analysis of the PrrH sRNA (B,D) and *pchE* mRNA (C,E) levels relative to WT-comp

332 shown as an average of 3 or 4 biological replicates. RNA was isolated from cultures grown in M9 media

333 supplemented with 50 nM FeCl₃, grown in static conditions at 37°C for 8 hours, and exposed to either am-

334 biant or infrared (IR) light as described in the materials and methods. Expression is calculated as relative

335 to WT-comp in static, light conditions. Significance was calculated using a two-tailed Student's t test with
336 asterisks indicating the following P values: * indicates $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$.

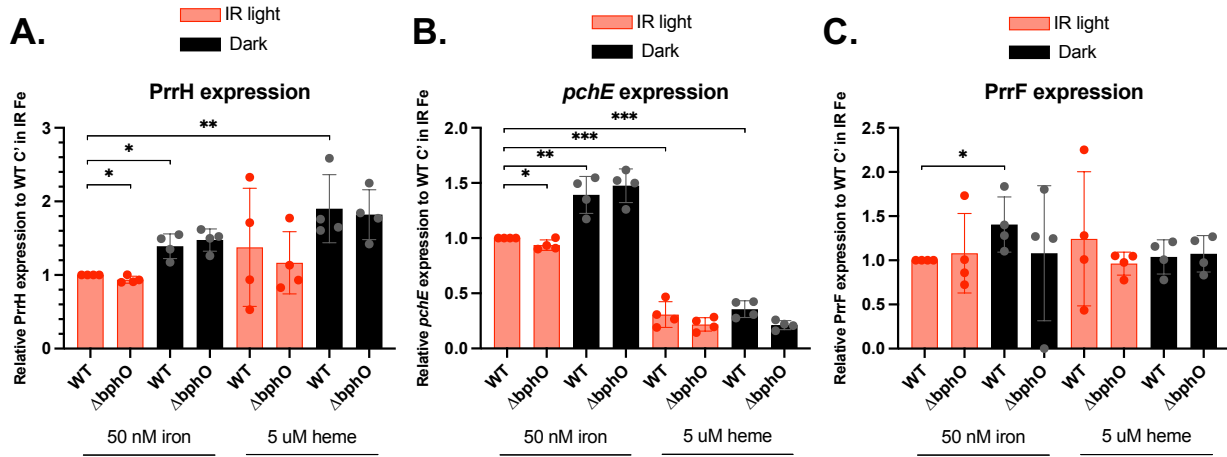
337

338 We next determined the impact of infrared (IR) light, which is specifically detected by the
339 BphP phytochrome, on static cultures of the WT comp, Δ H-IG comp, and short-comp strains. IR
340 light had no impact on expression of PrrH levels (**Fig. 5D**). However, IR light allowed for robust
341 induction of *pchE* expression upon *prhH* mutation (**Fig. 5E**). While the mechanistic rationale for
342 the effects of light on PrrH and *pchE* expression remain poorly understood, these experiments
343 demonstrate how controlling for light as an environmental variable may be critical to studying the
344 function of this unique sRNA.

345

346 **Heme negatively affects *pchE* expression.** Previous work demonstrates that heme posi-
347 tively affects PrrH sRNA levels via the PhuS heme binding protein (50). We therefore determined
348 if heme also affected expression of *pchE* in the WT PAO1 strain, lacking the pucP18 vector, in
349 static conditions either in the presence of IR light or wrapped in foil. At three hours post-inocula-
350 tion, one of each duplicate culture was supplemented with 5 μ M heme, and cultures were grown
351 for another 8 hours. Heme supplementation had a positive impact on PrrH levels, both in dark
352 and IR light conditions (**Fig. 6A**); however due to variability in PrrH expression, the increase was
353 only statistically significant when cultures were incubated in the dark (**Fig. 6A**). Notably, we ob-
354 served a robust and statistically significant decrease in *pchE* levels upon heme supplementation,
355 both in IR and dark conditions (**Fig. 6B**). We did not observe any repression of the PrrF sRNAs
356 (**Fig. 6C**), suggesting that heme repression of *pchE* is not due to Fur-mediated repression from
357 the iron that is enzymatically released upon heme degradation. Thus, our data suggest that heme
358 specifically represses expression of the *pchE* gene in WT PAO1.

359



360

361 **Figure 6. Heme negatively affects *pchE* expression.** Relative PrrH (A), *pchE* (B), and PrrF (C) transcript

362 levels relative to WT PAO1 when grown in the presence of absence of infrared (IR) light. Data are an

363 average of 4 biological replicates in M9 media supplemented with 50 nM FeCl₃ or 5 μ M heme grown stati-

364 cally at 37°C for 8 hours. Significance was calculated using an unpaired *t* test with two-tailed P values where

365 * indicates $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$.

366

367 We next determined if heme repression of *pchE* was dependent on the PrrH sRNAs using

368 the WT-comp, Δ H-IG, and short *prrF* complementation strains. The three strains were grown in

369 static conditions, with or without heme supplementation as above, and analyzed for PrrH, *pchE*,

370 and PrrF expression. We noted a similar positive but statistically insignificant effect of heme sup-

371 plementation on PrrH expression in this experiment (**Supplementary Materials, Fig. S5A**). Sur-

372 prisingly, we did not see any repression of *pchE* by heme in the WT comp strain, though heme

373 did appear to eliminate the induction of *pchE* in the *prrH* mutants (**Supplementary Materials,**

374 **Fig. S5B**). It is not clear why the heme regulation of *pchE* is not evident in the WT comp strain,

375 though we did note in our omics studies (above) that iron regulatory pathways are somewhat

376 altered in the complemented strains. Thus, we cannot assert, at this time, the role of PrrH in

377 heme-mediated repression of *pchE*. However, our data do indicate a role for both heme and PrrH

378 in affecting expression of *pchE*.

379

380 **BphO is not required for PrrH- or heme-mediated regulation of *pchE*.** Since our data
381 suggest that IR light may affect reproducibility of PrrH regulation on *pchE*, we next sought to
382 determine if the Bph phytochrome system affected expression of either PrrH or *pchE*. For this we
383 used a deletion mutant of the gene encoding the BphO heme oxygenase, which generates α -
384 biliverdin (α -BVIX) as the chromophore for the BphP phytochrome. The $\Delta bphO$ mutant showed
385 similar expression of PrrH, *pchE*, and PrrF under all conditions tested, regardless of IR light ex-
386 posure (**Fig. 6**). Thus, the impact of light on eliminating variability of this regulatory pathway does
387 not seem to be directly due to the BphP phytochrome. Instead, light may affect other metabolic
388 pathways that indirectly affect the ability of heme to affect expression of PrrH and *pchE*.

389

390 **Discussion**

391 This study aimed to determine the regulatory impact of the heme-responsive PrrH sRNA
392 in *P. aeruginosa*. Heme has emerged as a significant factor in chronic *P. aeruginosa* infections
393 over the past decade, with multiple studies demonstrating a reduced reliance on siderophore me-
394 diated iron uptake in the CF lung (33-37). Yet, only a few studies have rigorously addressed the
395 global impacts of heme on *P. aeruginosa* gene expression. Here, we characterized two distinct
396 *prhH* mutants, which express wild type levels of the PrrF sRNAs but produce truncated PrrH
397 sRNAs, and we determined the impact of these mutants on global gene expression using
398 RNASeq and proteomics. While experimental variations were observed in PrrH regulons from
399 separate experiments, we were able to identify pyochelin as a consistently dysregulated gene in
400 the *prhH* mutants and therefore a potential target of the PrrH sRNA. We further determined that
401 static growth promoted PrrH-dependent changes in *pchE* RNA levels, and we identified ambient
402 light as a likely confounding variable in our early studies. The impact of light builds on the recent
403 discovery that BphP, an α -biliverdin dependent photoreceptor (59), controls activity of the AlgB
404 response regulator in *P. aeruginosa* (58). This discovery led us to consider how light might affect

405 PrrH-dependent heme regulation, allowing us to control for this variable in the current study. The
406 result is the identification of *pchE* as the first verified PrrH-responsive gene in *P. aeruginosa*.

407 To our knowledge, a link between heme regulation and siderophore synthesis has only
408 been shown in one other bacterial species, *Staphylococcus aureus* (61). The *S. aureus* staphylo-
409 ferrin B (Sbn) biosynthetic locus contains a gene encoding the SbnI protein, which induces ex-
410 pression of the *sbn* operon in its apo form, and functions in Sbn synthesis when bound to heme
411 (61). Thus, SbnI plays a bifunctional role in siderophore biosynthesis and modulating heme-de-
412 pendent regulation of siderophore gene expression. The *P. aeruginosa* PhuS protein similarly
413 appears to have a bifunctional role, as it binds to the *prhH/prrF1* promoter in its apo form, and
414 functions as a shuttle to HemO when bound to heme (50, 60, 62, 63). Here, we show that PrrH
415 negatively impacts expression of *pchE*, and we show that heme similarly blocks *pchE* gene ex-
416 pression in *P. aeruginosa*. These results highlight heme as a preferred iron source under static
417 growth conditions, which promotes biofilm initiation and may more closely mimic chronic biofilm
418 infections where heme is a preferred iron source.

419 The impact of heme uptake and regulation on *P. aeruginosa* virulence has become in-
420 creasingly appreciated in the past decade. Recent work demonstrated a role for the unique β and
421 δ biliverdin isomers in post-transcriptional regulation of the Has heme uptake system (64), which
422 is required for *P. aeruginosa* virulence (51). Expression of the Has system is also subject to heme
423 binding to the secreted HasA hemophore, which binds to the HasR outer membrane receptor.
424 This, in turn, initiates a cell surface signaling (CSS) cascade through the HasI extracytoplasmic
425 function (ECF) sigma factor and cognate HasS anti-sigma factor (64). As indicated above, PhuS
426 functions as another heme-dependent regulatory protein, by binding in its apo form to the
427 *prrF1/prrH* promoter to affect PrrH expression (50). Heme has additionally been implicated in
428 virulence gene regulation via the BphP photoreceptor, which requires α -biliverdin produced by
429 the BphO heme oxygenase (58, 59). This recent study showed that AlgB acts as the response

430 regulatory to the BphP sensor kinase activity when sensing light, allowing light to negatively im-
431 pact biofilm formation (58). Notably, heme degradation by BphO does not contribute to *P. aeru-*
432 *ginosa*'s ability to use heme as an iron source, but instead seems to turn over intracellularly pro-
433 duced heme (60, 62). It is, therefore, intriguing that light was a confounding environmental varia-
434 ble in our studies of PrrH regulation, suggesting that these heme-dependent regulatory systems
435 are interlinked. Our studies suggest that the Bph system does not directly impact expression of
436 PrrH or *pchE* (**Fig. 6**). Studies of IR light regulation via BphP revealed a large metabolic regulon
437 (58), which may in turn indirectly influence many aspects of *P. aeruginosa* iron and heme home-
438 ostasis. Studies into how the PrrH heme regulatory system intersect with photosensing are con-
439 tinuing in our groups.

440 Understanding how heme regulation functions in *P. aeruginosa* has been complicated by
441 additional factors, including the transient presence of heme as an extracellular iron source. Once
442 extracellular heme is transported into the cell, it is shuttled to HemO to be degraded to biliverdin
443 which, as described above, exerts its own regulatory effects on gene expression. Heme oxygen-
444 ase also releases iron that can then function through the Fur protein to repress expression of
445 heme-responsive genes, including *hasR* and *prrF1/prrH*. Thus, time-dependent observations of
446 heme flux, promoter activity, RNA levels, and protein expression are likely all critical for under-
447 standing heme-dependent regulatory effects. Notably, supplementation of cultures with higher
448 concentrations of heme ($\geq 20 \mu\text{M}$) has been pursued in other works, yet these levels have the
449 potential to initiate toxicity toward cell membranes, nonspecific oxidative-cleavage prior to uptake,
450 and the formation of μ -oxo-dimers resulting in stable and biologically unavailable heme polymers.
451 In the current study, we began assessing the impact of heme on PrrH and *pchE* levels during
452 static growth, while all previous heme regulatory studies have been conducted in shaking growth.
453 Similar to observations by our group regarding the impacts of static growth on global iron regula-
454 tion (47), our work here suggests that heme and PrrH regulation is altered under static conditions.
455 Static culture conditions result in slower growth of *P. aeruginosa*, which will require us to reassess

456 the timing of heme uptake and metabolism to release iron under these conditions. Static cultures
457 also likely result in heterogenous communities with varying heme uptake and regulatory activities.
458 Continued work on heme uptake and regulation in complex *P. aeruginosa* communities, such as
459 within static cultures and biofilm communities, is required to understand more physiologically rel-
460 evant implications of these regulatory pathways.

461 An additional complexity for studies described here is the overlapping sequence of the
462 PrrF and PrrH sRNAs. Two recent studies ascribed a variety of physiological functions to PrrH,
463 yet both studies used knockouts and complementation constructs containing the entire *prrF-prrH*
464 sequence (65, 66). Therefore, any observations or phenotypes derived from these strains cannot
465 simply be designated as specific to PrrH. The genetic strategy previously developed by Reinhart,
466 *et al* (67) allowed us to overcome this issue by focusing on the sequence that is unique to PrrH.
467 We note that this system is not ideal because PrrF and PrrH are overexpressed in these strains,
468 leading to some changes in global gene expression (**Fig. S1, 3**), and indeed the WT comp showed
469 a different effect of heme on expression of *pchE* (**Fig. S4, 6**). We continue to work toward genetic
470 strategies that will allow us to specifically affect PrrH function while allowing the PrrF sRNAs to
471 remain unaffected.

472 Overall, this study identified the first regulatory target of the PrrH sRNA as *pchE* and pro-
473 vided evidence that this regulation could occur through direct post-transcriptional regulation of the
474 *pchE* mRNA. Additionally, we provide evidence that light may affect systems involved in heme
475 regulation, necessitating careful control of this environmental condition for our studies. Current
476 and future work will examine time-dependent effects of heme supplementation on these systems,
477 and work toward identifying more global impacts of the PhuS and PrrH regulatory molecules.

478

479

480 **Materials & Methods**

481 **Bacterial strains and growth conditions.** Strains used in this study are listed in **Table**
482 **S1.** *P. aeruginosa* strains were maintained on LB or BHI agar or broth. Strains carrying the com-
483 plementation plasmids were maintained with carbenicillin (250 µg/mL). Media was supplemented
484 with iron or heme as follows: 50 nM FeCl₃ (-Fe), 100 µM FeCl₃ (+Fe), and 1 µM or 5 µM Heme
485 (+He) prepared as previously described (44). Overnight cultures were grown in LB or BHI broth,
486 aerobically (250 RPM, 37°C) and washed in M9 media (Teknova, Hollister, CA) prior to inoculating
487 into M9 supplemented with iron or heme to a starting OD₆₀₀=0.05. Shaking cultures were grown
488 in 1:10 (media:flask) ratios, shaking aerobically at 250 RPM, 37°C and collected at 8 hours for
489 analyses. Static cultures were grown in 24-well cell culture plates (Greiner Bio-One, Kremsmun-
490 ster, Austria) at 37°C and collected at 8 hours for analyses. For ambient light conditions, cultures
491 were grown in a well-lit room (fluorescent overhead lights and near a window during the day). For
492 infrared (IR) light conditions, plates were placed under a 730 nM LED Lightbar (Forever Green
493 Indoors, Inc, Seattle, WA). Plates were wrapped in foil for dark conditions. For RNA isolation,
494 cultures were mixed with an equal volume of RNALater (Sigma-Aldrich, St. Louis, MO) and stored
495 at -80°C until processing.

496
497 **Quantitative real-time PCR (qRT-PCR).** RNA was isolated following manufacturer's sug-
498 gested protocol using the RNeasy Mini Kit (Qiagen, Hilden, Germany). An additional DNase I
499 (New England Biolabs, Ipswich, MA) treatment was performed at 37°C for 2 hours, ethanol pre-
500 cipitated, and eluted in RNase- free water. qRT-PCR was performed as previously described (43,
501 44). Primer and probe sequences are listed in **Table S2**.

502
503 **Northern blot analyses.** RNA was isolated following manufacturer's suggested protocol
504 using the RNeasy Mini Kit (Qiagen, Hilden, Germany). 5 µg (PrrF1, PrrF2) or 20 µg (PrrH) of total
505 RNA was electrophoresed on a 10% denaturing urea TBE gel (Bio-Rad, Hercules, CA). The RNA

506 was then transferred to a BrightStar-Plus Positively Charged Nylon Membrane (Invitrogen, Carls-
507 bad, CA) using a Trans-Blot Turbo Transfer System (Bio-Rad) and crosslinked for 2 minutes using
508 a UV Crosslinker (VWR, Radnor, PA). The blots were incubated with 32 P 5'-labelled probe at
509 42°C overnight and imaged using a phosphor screen on a Typhoon FLA 7000 Variable Mode
510 Imager System (GE Healthcare, Chicago, IL). Probe sequences are listed in **Table S2**.

511

512 **RNAseq.** Cultures were grown in shaking conditions as described above, without any ad-
513 ditional controls for light. Cultures were collected (at 8 hours of growth) directly into RNALater.
514 Iron was supplemented at a concentration of 50 nM (low iron) or 100 μ M (high iron), while heme
515 was used at 5 μ M. Sample preparation and subsequent RNA extraction and analyses were per-
516 formed as previously described (51). RNA integrity was validated using an Agilent 2100 Bioana-
517 lyzer. Libraries were prepared with samples with an RNA Integrity Number (RIN) greater or equal
518 to 8. Ribosomal RNA was depleted using the Ribo Zero kit and samples were converted into
519 Illumina sequencing libraries using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre,
520 Illumina). Libraries were sequenced using Illumina HiSeq (2 x 150 bp reads). Three biological
521 replicates were sequenced in each group and an average of 40 million reads were obtained for
522 each sample. Reads were mapped against the reference genome of *P. aeruginosa* PAO1
523 (NC_002516) with the following settings: mismatch cost = 2, insertion cost = 3, deletion cost = 3,
524 length fraction = 0.8, similarity fraction = 0.8. Fold changes in gene expression, and statistical
525 analyses were performed using the extraction of differential gene expression (EDGE) test as im-
526 plemented in CLC Genomics, which is based on the Exact Test. Differential gene expression was
527 calculated by comparing samples and setting a fold-change cut-off as described in the figure
528 legends. Genes were included in further analysis only if differences in expression yielded a FDR
529 p -value of $p \leq 0.05$.

530

531 **Quantitative label-free proteomics.** Cultures were grown in shaking conditions as de-
532 scribed above, without any additional controls for light. Cultures were collected at 8 hours of
533 growth. Iron was supplemented at a concentration of 50 nM (low iron) or 100 μ M (high iron), while
534 heme was used at 5 μ M. Sample preparation and subsequent proteomics analyses were per-
535 formed as previously described (47, 68, 69). In brief, cells were harvested by centrifugation at
536 2000 rpm for 30 s at 4 °C, and then subsequently lysed in 4% sodium deoxycholate, reduced,
537 alkylated, and trypsinolyzed on filter as previously described (70). Tryptic peptides were sepa-
538 rated on a nanoACQUITY UPLC analytical column (BEH130 C₁₈, 1.7 μ m, 75 μ m x 200 mm, Wa-
539 ters) over a 165 min linear acetonitrile gradient (3-40%) with 0.1 % formic acid using a Waters
540 nanoACQUITY UPLC system and analyzed on a coupled Thermo Scientific Orbitrap Fusion Lu-
541 mos Tribrid mass spectrometer. Full scans were acquired at a resolution of 120,000, and precur-
542 sors were selected for fragmentation by higher-energy collisional dissociation (normalized colli-
543 sion energy at 30%) for a maximum 3-second cycle. Tandem mass spectra were searched
544 against the *Pseudomonas* genome database PAO1 reference protein sequences (71) using the
545 Sequest-HT and MS Amanda algorithms with a maximum precursor mass error tolerance of
546 10 ppm (72, 73). Carbamidomethylation of cysteine and deamidation of asparagine and glutamine
547 were treated as static and dynamic modifications, respectively. Resulting hits were validated at a
548 maximum false-discovery rate (FDR) of 0.01 using the semi-supervised machine learning algo-
549 rithm Percolator (74). Protein abundance ratios were measured by comparing the MS1 peak vol-
550 umes of peptide ions, whose identities were confirmed by MS2 sequencing. Label-free quantifi-
551 cation was performed using an aligned AMRT (Accurate Mass and Retention Time) cluster quan-
552 tification algorithm (Minora; Thermo Fisher Scientific, 2017). Protein interactions were analyzed
553 using STRING 10.5 and visualized with Cytoscape 3.8.0 (75, 76)

554

555

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