Loss of LasR function leads to decreased repression of *Pseudomonas aeruginosa* PhoB activity at physiological phosphate concentrations

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Abstract

While *Pseudomonas aeruginosa* LasR plays a role in quorum sensing (QS) across all phylogenetically-distinct lineages, isolates with loss-of-function mutations in *lasR* (LasR– strains) are commonly found in diverse settings including infections where they are associated with worse clinical outcomes. In LasR– strains, another QS transcription factor RhlR can be reactivated in low inorganic phosphate (Pi) concentrations via the two-component system PhoR-PhoB, restoring expression of some of the genes in the QS network. Here, we demonstrate a new link between LasR and PhoB in which the absence of LasR increases PhoB activity at physiological Pi concentrations and raises the Pi concentration necessary for PhoB inhibition. PhoB activity was also less repressed by Pi in mutants lacking different QS regulators (RhlR, and PqsR) and in mutants lacking genes required for the production of QS-regulated phenazines suggesting that decreased phenazine production was one reason for decreased PhoB repression by Pi in LasR–. In addition, the CbrA-CbrB two-component system, which is elevated in LasR– strains, was necessary for reduced PhoB repression by Pi and a Δ*crc* mutant, which lacks the CbrA-CbrB-controlled translational repressor, activated PhoB at higher Pi concentrations than the in the wild type. The Δ*lasR* mutant had a PhoB-dependent growth advantage in a medium with no added Pi and increased virulence-determinant gene expression in a medium with 0.7 mM Pi. Reanalysis of published RNA-seq data found evidence for PhoB activity in *P. aeruginosa* in cystic fibrosis sputum indicating that potential strain differences in PhoB activation may impact disease state.

23 **Introduction**

24 *Pseudomonas aeruginosa* is a pernicious pathogen that infects burn wounds,
25 nonhealing wounds, eyes, and the airways of people with cystic fibrosis (pwCF) or chronic
26 obstructive pulmonary disease (COPD). One key *P. aeruginosa* attribute that contributes
27 to pathogenicity is its ability to acquire nutrients, such as phosphorus, that are restricted
28 in the host environment. Phosphate is required for cell membranes, nucleic acids,
29 metabolic intermediates, and is utilized in signal transduction. The most accessible form
30 of phosphorus is free inorganic phosphate (Pi), which is found in human serum at
31 concentrations near 1.3 mM Pi in healthy adults [1, 2]. However, during infection, Pi is
32 highly restricted by the host as part of nutritional immunity through mechanisms including
33 the secretion of phosphate-binding proteins [3-5]. Additionally, the majority of phosphorus
34 in the body is present in organic forms which require degradation prior to uptake and
35 utilization by microbes [6].

36 In response to low Pi levels, *P. aeruginosa* induces the production of enzymes
37 such as phosphatases, phospholipases, and DNases to access phosphate from organic
38 sources and high-affinity phosphate transporters via the transcription factor PhoB and its
39 sensor kinase PhoR [7-10]. PhoB activity can also induce the production of specific
40 phenazine small molecules [11-15] which solubilize phosphate from minerals [16]. PhoR
41 activity is regulated by interactions with the high-affinity Pi transporter PstABC via PhoU
42 [9, 17-23] such that PhoR is inhibited by Pi transport into the cell [23, 24]. PhoB positively
43 regulates its own expression [25]. However, published evidence suggests a negative
44 feedback loop also exists in *P. aeruginosa* [26] as has been described in *Escherichia coli*
45 [27], presumably to control intracellular Pi. Homologous PhoR-PhoB two-component

46 systems play significant roles in Pi acquisition across gram-negative bacteria including *E.*
47 *coli* [23, 28] *Salmonella enterica* [29], and *Vibrio cholerae* [30].

48 In addition to nutrient acquisition, PhoB also regulates virulence in multiple
49 pathogens [9]. In *E. coli*, type III secretion system (T3SS) components and effectors are
50 upregulated during Pi-limitation in a PhoB-dependent manner [31]. A different relationship
51 has been found in *V. cholerae* where PhoB directly binds and inhibits transcription of
52 genes encoding virulence regulators TcpPH [30]. In *P. aeruginosa,* cross-regulation
53 between PhoR-PhoB and quorum sensing (QS) has been described [12-15, 32, 33]. *P.*
54 *aeruginosa* QS is largely controlled by three transcription factors, LasR, RhlR, and PqsR,
55 that are active when QS inducers are at sufficient concentrations i.e. high cell densities
56 or environments with decreased diffusion. While LasR positively regulates both RhlR and
57 PqsR, many groups have shown that RhlR and PqsR activity can occur in the absence of
58 LasR [34-37]. It has also been established that activation of PhoB by Pi limitation induces
59 QS [13-15, 38, 39] and this can occur in a LasR-independent manner [12, 32, 33]. QS regulation
60 contributes to virulence and biofilm formation in many contexts [40-42]. One group of
61 virulence factors induced by both PhoB and QS are phenazines which inhibit immune cell
62 function [43, 44] and kill other microbes [45-49] through oxidative stress. Though PhoB-
63 binding sites have been identified upstream of some phenazine biosynthesis genes [15],
64 PhoB induction of phenazine production is largely attributed to increased expression of
65 *rhlR* [12, 15, 38]. Furthermore, *phoR, phoB, rhlR,* and *pqsR* are all required for *P. aeruginosa*
66 phenazine production in co-culture with *Candida albicans* [26], a fungus that frequently co-
67 infects the airways of pwCF [50]. PhoB regulation of QS suggests low Pi could be a signal
68 for QS remodeling to circumvent reliance on LasR.

69 LasR– *P. aeruginosa* are present in the environment [51-53] and also often evolve
70 during chronic infections like those observed in pwCF [34, 54] where they make up
71 approximately one-third of clinical isolates [55]. As QS positively correlates with virulence,
72 it is of significant interest that these LasR– isolates are associated with worse disease
73 progression in pwCF [55] and worse lesions during acute ocular infections [52]. LasR– *P.*
74 *aeruginosa* competitive fitness in laboratory evolution experiments depends on increased
75 CbrA-CbrB activity [56, 57], which provides growth advantages in complex media [35, 56, 58].
76 CbrA-CbrB is a two-component system that relieves carbon catabolite repression (CCR)
77 in *P. aeruginosa* by inducing the production of the small RNA *crcZ* which sequesters Crc
78 [59-62]. Crc interacts with Hfq to bind to the ribosome binding site of multiple mRNA targets,
79 inhibiting their translation. Crc-Hfq target mRNAs encode transporters, enzymes, pili, and
80 more [63]. CbrA is required for full *P. aeruginosa* virulence [64] and both CbrA and CbrB
81 are rarely mutated in clinical isolates [65].

82 Investigations of *P. aeruginosa* virulence regulation are often conducted in
83 laboratory media with millimolar Pi concentrations that repress PhoR activation of PhoB
84 (e.g. synthetic CF medium (SCFM), 5.1 mM [66]; RPMI medium, 5.6 mM [67, 68]; Luria Broth,
85 6 mM [69]; or M9 medium, 64 mM [69]). Further, PhoR-PhoB activity is most often studied
86 in media with less than 0.5 mM Pi [12, 19, 23, 33]. These two extremes either strongly repress
87 or strongly induce PhoB activity, making it difficult to investigate the contributions of PhoB
88 to *P. aeruginosa* virulence inside the host at physiological Pi concentrations (1.3 mM Pi
89 in serum [1, 4]) or when Pi is transiently available. Thus, we aimed to elucidate the
90 relationship between LasR and PhoB at physiologically relevant Pi concentrations. In
91 these studies, we show that LasR– isolates and a Δ*lasR* mutant had elevated PhoR-

92 dependent PhoB activity relative to comparator strains with functional LasR at Pi
93 concentrations in the 0.7 – 1.1 mM range. In contrast, LasR+ and LasR– strains had
94 similar PhoB activity in both low (0.2 mM) and high (10 mM) Pi conditions. Our data
95 demonstrate that a lack of phenazines or reduced Crc activity via CbrA-CbrB led to higher
96 PhoB activity and decreased repression of PhoR-PhoB by Pi. We show that PhoB is
97 required for the Δ*lasR* mutant growth advantages in a medium with no added Pi and the
98 increased expression of multiple virulence factors including phenazine biosynthetic
99 enzymes and phospholipases. This work establishes a novel connection between QS and
100 PhoB wherein LasR represses PhoB activity and PhoB is required for increased virulence
101 gene expression in the Δ*lasR* mutant in part through reactivation of QS. This model for
102 virulence regulation may aid in understanding why *P. aeruginosa* LasR– strains are
103 associated with poor clinical outcomes.
104

105 **Results**

106 **PhoB activity is repressed at lower Pi concentrations in *P. aeruginosa* LasR+**
107 **strains than in their *P. aerugiosa* LasR– counterparts.**

108 Though PhoB regulates virulence factor production across species [31, 70], there are
109 only a limited number of *in vitro* studies on PhoB activity at Pi concentrations similar to
110 those found *in vivo*. Thus, we sought to determine the Pi concentrations necessary to
111 repress PhoB activity in *P. aeruginosa* strain PA14. PhoB activity was monitored by
112 assessing the activity of alkaline phosphatase (AP), which is encoded by the PhoB-
113 regulated gene *phoA* [7, 71, 72], on agar containing the colorimetric AP substrate bromo-4-
114 chloro-3-indolyl phosphate (BCIP) [8, 73] (Fig. 1A). Using gradient plates with range of Pi

115 concentrations (0.1 mM - 1 mM) [26] in a MOPS-glucose medium base [74], we found 0.7
116 mM Pi repressed PhoB activity in the wild-type *P. aeruginosa*. Interestingly, a Δ*lasR*
117 mutant showed AP activity across the entire gradient though AP activity showed some
118 reduction at higher Pi concentrations (Fig. 1A). Consistent with PhoB regulation of AP
119 activity, the Δ*phoB* mutant had no detectable AP activity at any concentration, and the
120 Δ*pstB* mutant, which has constitutive PhoB activity due to de-repression of PhoR [26, 75],
121 had strong AP production at all Pi concentrations tested (Fig. 1A). At 0.7 mM Pi, which
122 repressed PhoB activity in the wild type but not the Δ*lasR* mutant, the Δ*lasR* mutant
123 complemented with *lasR* at the native locus phenocopied the wild type (Fig. 1B).

124 Consistent with PhoR activation of PhoB, we found neither the Δ*lasR*Δ*phoB* or
125 Δ*lasR*Δ*phoR* mutants had AP activity (Fig. 1B). Furthermore, a clinical isolate from a
126 corneal infection, DH2590 (262K in Hammond, *et al.* [52]), which has a loss-of-function
127 mutation in *lasR,* also expressed AP at 0.7 mM Pi and this high level was reduced by
128 complementing a functional copy of *lasR* at the native locus (Fig. 1B). In addition, we
129 compared AP activity in two clinical isolate pairs (Fig. 1C) from CF sputum samples
130 wherein one isolate has a LOF mutation in *lasR* and the other does not [76]. In both cases,
131 the LasR– isolates had more AP activity than their LasR+ counterparts at 0.7 mM Pi.

132 Both the Δ*lasR* mutant and the wild type showed PhoB activity at lower Pi
133 concentrations (< 0.7 mM). We found that at low Pi (0.2 mM), the concentration frequently
134 used to study PhoB activity [12, 13, 19, 23, 77], the wild type and Δ*lasR* strains had similar AP
135 activity (Fig. 1D). Additionally, neither strain showed AP production at 10 mM Pi.
136 Additionally, no AP activity was observed in either the wild-type or the Δ*lasR* strain on LB
137 agar which is reported to have 6 mM Pi [69] (Fig. S1A). The Δ*phoB* mutant did not have

138 AP activity even at 0.2 mM Pi and the Δ*pstB* mutant had AP production at both 0.2 mM
139 Pi, 10 mM Pi, and on LB. AP activity was quantified using the soluble colorimetric
140 substrate p-nitrophenyl phosphate (PNPP). The Δ*lasR* mutant had significantly more AP
141 activity than the wild type at 0.7 mM Pi (Fig. 1E) and this difference was not attributable
142 to differences in growth (Fig. S1B). Similarly, qRT-PCR analysis of *phoA*, which encodes
143 AP, normalized to the reference transcript *ppiD* found significantly lower levels in the wild
144 type when compared to the Δ*lasR* mutant (Fig. 1F).

145 To directly assess the levels of transcripts from other genes within the PhoB
146 regulon, we utilized NanoString multiplex technology as previously published [26]. At 0.7
147 mM Pi, the Δ*lasR* mutant had significantly higher levels of PhoB-regulated transcripts
148 including *phoB* itself and genes encoding phosphatases (*phoA* and *lapA*), a phosphate
149 transporter (*oprO*), a type two secretion system protein required for secretion of LapA
150 (*hxcT*) [78], a putative TonB transporter (*exbD2*) [79], a phosphodiesterase (*glpQ*), a low-
151 phosphate ornithine lipid biosynthetic protein (*olsA*) [80], and a Pi chemotaxis protein (*ctpL*)
152 [81]. None of these transcripts were significantly different when the Δ*phoB* and
153 Δ*lasR*Δ*phoB* mutants were compared (Fig. 1G and Supplemental File 1).

154 **Other quorum-sensing mutants have active PhoB at higher Pi concentrations than**
155 **the wild type which correlates with decreased phenazine production.**

156 LasR positively regulates other QS transcription factors including RhlR and PqsR
157 and some Δ*lasR* mutant phenotypes are due to their decreased activity. At the same time,
158 RhlR and PqsR are capable of inducing QS-controlled genes in the absence of LasR in
159 specific strains and conditions [34, 35, 57, 82]. Thus, we assessed the role of RhlR and PqsR

160 in the repression of PhoB. As shown in Fig. 2A, AP activity was similarly significantly
161 elevated in the Δ*lasR*, Δ*rhlR,* and Δ*pqsR* mutants from the wild type at 0.7 mM Pi. Across
162 a gradient plate of 0.5 – 1.5 mM Pi, PhoB activity in all QS mutants was inhibited at a
163 significantly higher Pi concentration than the wild type (Fig. 2B). However, the absence
164 of LasR (Δ*lasR,* Δ*lasR*Δ*rhlR,* and Δ*lasR*Δ*pqsR*) led to decreased repression by Pi at
165 concentrations >1 mM, while the Δ*rhlR* and Δ*pqsR* were inhibited by 0.9 mM Pi.

166 As each of the QS transcription factor mutants, Δ*lasR*, Δ*rhlR*, and Δ*pqsR*, produce
167 fewer phenazines than the wild type in late exponential and early stationary phase
168 cultures [83, 84], we tested whether the absence of phenazines was sufficient to increase
169 PhoB activity at moderate Pi concentrations. *P. aeruginosa* produces multiple phenazines
170 including phenazine-1-carboxylic acid (PCA) as well as the PCA derivatives 5-methyl-
171 PCA (5MPCA), pyocyanin (PYO), and phenazine-1-carboxamide. PCA is synthesized by
172 proteins encoded by two highly similar operons, *phzA1-G1* and *phzA2-G2.* The mutant
173 lacking both *phz* operons (Δ*phzA1-G1*Δ*phzA2-G2*), referred to as Δ*phz*, had significantly
174 elevated AP activity compared to the wild type at 0.7 mM Pi (Fig. 3A). The Δ*phz1* mutant
175 (Δ*phzA1-G1*) was not different from the wild type, and deletion of either of the adjacent
176 phenazine-modifying genes required for 5MPCA and PYO biosynthesis, *phzM* or *phzS*,
177 did not lead to changes in AP production at 0.7 mM (Fig. S2A). In contrast, the Δ*phz2*
178 mutant (Δ*phzA2-G2*) phenocopied Δ*phz* and had significantly more AP production than
179 the Δ*phz1* mutant or the wild type (Fig. 3A). We and others have previously published
180 data identifying *phzA2-G2* as the predominant contributor of PCA [26, 84-86].

181 We found no difference in AP activity at 0.7 mM Pi between the Δ*lasR* and Δ*phz*
182 mutants (Fig. 3B). However, a Δ*lasR*Δ*phz* mutant had significantly elevated AP activity

183 compared to both single mutants (Fig. 3B). Both the Δ*lasR* and Δ*lasR*Δ*phz* mutants had
184 detectable AP activity at a significantly higher Pi concentration than Δ*phz* (Fig. 3C)
185 suggesting that PhoB activity was derepressed by an additional phenazine-independent
186 mechanism in the Δ*lasR* mutant. Consistent with this model, the Δ*phz* mutant was
187 repressed by a similar Pi concentration as the Δ*rhlR* and Δ*pqsR* mutants, but not their
188 LasR– counterparts (Fig. 2B).

189 **CbrA-CbrB-Crc impacts PhoB activity in the Δ*lasR* mutant.**

190 Several studies suggest that *P. aeruginosa* strains lacking LasR activity have
191 elevated CbrA-CbrB activity in many conditions [56, 58]. CbrA-CbrB activity leads to relief
192 of Crc-mediated translation repression of its targets including some that encode catabolic
193 enzymes and transporters [62, 63, 87] (Fig. 4A). In both BCIP and PNPP assays, we found
194 that deletion of *cbrB* eliminated AP activity at 0.7 mM Pi in a Δ*lasR* mutant and that the
195 Δ*lasR* phenotype was restored by complementation with the restoration of *cbrB* at the
196 native locus (Fig 4B). PhoB activity in both the Δ*cbrB* and Δ*lasR*Δ*cbrB* mutants was
197 similarly repressed below 0.5 mM Pi (Fig. S2B). Additionally, AP activity at 0.7 mM Pi on
198 BCIP is restored to a Δ*lasR*Δ*cbrB* mutant by deletion of *crc* (Fig. 4B) suggesting that the
199 increase in Pi repression of PhoB in the Δ*lasR*Δ*cbrB* is due to elevated Crc activity (Fig.
200 4A for pathway). A Δ*crc* single mutant also had significantly elevated AP activity
201 compared to the wild type and *crc* complemented strain (Fig. 4C). There was no difference
202 in AP activity at 0.7 mM Pi between the Δ*lasR* and Δ*lasR*Δ*crc* mutants, though both were
203 significantly elevated compared to the Δ*crc* mutant (Fig. 4D). Additionally, the Δ*lasR* and
204 Δ*lasR*Δ*crc* mutants both showed significantly decreased PhoB sensitivity to Pi than the
205 Δ*crc* mutant (Fig. 4E).

206 **PhoB activity in the *P. aeruginosa* Δ*lasR* strain provides fitness advantages when**
207 **Pi is limited.**

208 To determine if the increased PhoB activity in *P. aeruginosa* Δ*lasR* cells increased
209 fitness upon depletion of Pi, we grew the wild-type, the Δ*lasR* mutant, and their respective
210 *phoB* mutants overnight in LB, then sub-cultured into MOPS-glucose medium with no
211 added Pi (Fig. 5A). The wild type and the Δ*lasR* mutant showed similar initial growth rates.
212 However, in the post-exponential phase, the Δ*lasR* mutant reached significantly higher
213 densities than the wild type. Both the Δ*phoB* and Δ*lasR*Δ*phoB* mutants showed minimal
214 growth in the no added Pi media and were not significantly different from each other.

215 **PhoB activity mediates the expression of virulence determinants in LasR– *P.***
216 ***aeruginosa,* in part through QS*.***

217 Multiple studies have shown that there are alternative mechanisms for the
218 activation of RhlR in LasR– strains [12, 32-36] and that phosphate limitation promotes RhlR
219 activity [12-15, 32, 33]. We have previously published that both PhoB and RhlR are required
220 for antagonistic 5MPCA production by *P. aeruginosa* in co-culture with *Candida albicans*
221 [26]. Here we show that the Δ*lasR* mutant produces more of the antifungal phenazine
222 5MPCA in co-culture with *Candida albicans* and that 5MPCA production by both LasR+
223 and LasR– *P. aeruginosa* required PhoB and CbrB (Fig. 5B). Using NanoString multiplex
224 technology and a previously published code set [26], we compared virulence determinant
225 transcripts from the wild type and Δ*phoB,* Δ*lasR,* Δ*lasR*Δ*phoB* mutants grown as colony
226 biofilms on MOPS agar with 0.7 mM Pi. Transcripts encoding multiple virulence
227 associated proteins, including phenazine biosynthetic enzymes (*phzM* and *phzS*),

228 phospholipases (*plcH* and *plcN*), and an exopolysaccharide (EPS) matrix regulator
229 (*phdA*) [88], were significantly higher in the Δ*lasR* mutant compared to the wild type (Fig.
230 5C and Supplemental File 1). These differences were not detected when the Δ*phoB* and
231 Δ*lasR*Δ*phoB* mutants were compared, though all transcripts were significantly lower in
232 the Δ*lasR*Δ*phoB* mutant compared to the Δ*lasR* strain. As expected, expression of *plcN*
233 [11, 89] and *phdA* [79, 90], which are impacted by PhoB activity, were significantly lower in the
234 Δ*phoB* mutant compared to the wild type. However, it is interesting that *phzM, phzS,* and
235 *plcH* expression were only PhoB-dependent in the Δ*lasR* mutant under these conditions.

236 As the production of phenazines by LasR– *P. aeruginosa* requires RhlR [12, 32, 33, 35]
237 and Pi limitation promotes RhlR activity [12-14, 32, 33], we sought to determine if PhoB
238 contributes to QS at 0.7 mM Pi. We found significantly higher abundance of transcripts
239 encoding QS proteins (*lasI, rhlR, pqsR,* and *pqsH)* and those regulated by QS
240 transcription factors (*lasB* and *rhlA*) in the Δ*lasR* mutant compared to the Δ*lasR*Δ*phoB*
241 mutant at 0.7 mM Pi (Fig. 5D and Supplemental File 1). The transcripts *lasB, rhlR, pqsR*,
242 and *pqsH* were significantly lower in the Δ*lasR* mutant compared to the wild type, though
243 there were no differences in *lasI* or *rhlA* abundance. We found no significant differences
244 in QS transcript abundance between the Δ*phoB* mutant and the wild type at 0.7 mM Pi.

245 **Cystic fibrosis sputum isolates have active PhoB.**

246 CF sputum is frequently colonized by *P. aeruginosa* but is difficult to assess free
247 Pi levels in sputum as there are many organic forms also present. Thus, we sought to
248 determine if conditions in sputum, including Pi levels, suppressed PhoB activity by
249 analyzing high-quality RNA-seq data of *P. aeruginosa* from sputum published by Lewin,

250 *et al.* [91]. In this study, RNA-seq analysis of RNA from *in vivo* CF sputum samples was
251 compared to that of the laboratory strain PAO1 grown in synthetic cystic fibrosis medium
252 (SCFM2) and other *in vitro* conditions. SCFM2 contains 5.1 mM Pi [92]. We used the *in*
253 *vivo* and SCFM2 samples from this published dataset to compare expression of 85 genes
254 in the PhoB regulon, as defined by a gene ADAGE signature [93] (Supplemental File 2).
255 Overall, we found higher transcripts of PhoB-regulated genes including those encoding
256 phosphatases, phospholipases, and transporters *in vivo* than *in vitro* (Fig. 6). In fact, 42%
257 of the Adage-defined PhoB regulon was significantly higher *in vivo* (Supplemental File 2).
258 Across the sputum samples, there was heterogeneity suggesting that sputum conditions
259 and/or infecting strains led to varied levels of PhoB activity.

260 **Discussion**

261 In this manuscript, we showed that LasR– *P. aeruginosa* laboratory strains and
262 clinical isolates had elevated PhoB-dependent AP activity and increased expression of
263 the PhoB regulon at 0.7 mM Pi. We found that the range of PhoB-permissive Pi
264 concentrations was broader for LasR– *P. aeruginosa*, though PhoB was still repressed at
265 high Pi concentrations. Importantly, these intermediate Pi concentrations where LasR–
266 *P. aeruginosa* showed more PhoB activity are similar to those found in the serum of
267 healthy adults [1, 2]. We also found that elevated PhoB activity was common across QS
268 mutants and that this was possibly due to reduced phenazine production. However,
269 LasR– *P. aeruginosa* had active PhoB at even higher Pi concentrations than the other
270 QS mutants, suggesting differences in PhoB regulation.

271 LasR– *P. aeruginosa* are more fit than their LasR+ counterparts in many contexts.
272 LasR– *P. aeruginosa* are known to have elevated CbrA-CbrB activity, conferring growth

273 advantages in complex media through decreased Crc activity [56, 58]. Other studies have
274 shown that LasR– *P. aeruginosa* are more resistant to cell lysis at high pH [94] and more
275 fit in microoxic conditions and normoxically grown colony biofilms [95]. Future studies are
276 necessary to determine if these phenotypes are interrelated. We showed that CbrB was
277 required for PhoB activation in LasR– *P. aeruginosa* and a *crc* mutation was sufficient to
278 induce PhoB activity, even in a LasR+ strain. Importantly, the Δ*crc* mutant had less AP
279 activity and was inhibited by lower Pi concentrations than the Δ*lasR* mutant. These data
280 suggest Crc- *P. aeruginosa* do not phenocopy LasR– strains.

281 The elevated PhoB activity in the Δ*lasR* mutant contributed to fitness upon a shift
282 from LB to a medium without added Pi. As phosphate accessibility can be transient inside
283 the host, increased PhoB activity may be beneficial in building up stores of polyphosphate
284 for utilization when cells experience low Pi stress. In addition to regulating phosphate
285 acquisition, PhoB activity has also been shown to promote RhlR expression and
286 subsequent PYO production, swarming motility, and cytotoxicity [12, 14, 32, 33, 89]. Here we
287 showed that the Δ*lasR* mutant had PhoB-dependent increased expression of virulence
288 genes including phenazine biosynthetic enzymes and phospholipases like PlcH. PlcH is
289 known to degrade airway surfactant and we have previously shown that its expression
290 leads to a decline in lung function [96]. We also showed PhoB and CbrB are both required
291 for increased production of the antifungal phenazine 5MPCA by the Δ*lasR* mutant in co-
292 culture with *C. albicans*.

293 The roles for phenazines in PhoB regulation are complex. As all QS mutants
294 produce fewer phenazines in late exponential phase and early stationary phase [83, 84], we
295 proposed phenazines may repress PhoB activity. We demonstrated that phenazine-

296 deficient *P. aeruginosa* had similarly elevated PhoB activity to the single QS mutants.
297 Unlike the Δ*rhlR* and Δ*pqsR* mutants, LasR– *P. aeruginosa* are capable of producing
298 phenazines in certain contexts [12, 34-36]. We observed that the Δ*lasR* mutant appeared
299 greener on BCIP agar at 0.7 mM Pi which we speculate is due to the production of the
300 blue-green phenazine PYO as this was not observed in the phenazine-deficient
301 Δ*lasR*Δ*phz* mutant (Fig. 3B). Thus, the higher AP activity in the Δ*lasR*Δ*phz* compared to
302 the Δ*lasR* may be due to the absence of phenazines that reduce PhoB activation at
303 moderate Pi concentrations. It is also of note that PhoB activity promotes phenazine
304 production, potentially through direct binding upstream of the *phz* operons [15], but more
305 likely through RhlR activity [12, 32, 33]. This increased phenazine production could then
306 inhibit further PhoB activity, providing negative feedback. However, our data suggests
307 PCA, not PYO or 5MPCA, inhibits PhoB as loss of the biosynthetic enzymes PhzM or
308 PhzS did not induce PhoB activity. It cannot be assumed that cells producing visible PYO
309 also have high PCA and reduced PhoB activity. An example of this is the Δ*crc* mutant
310 which we showed had elevated AP activity and is known to produce more PYO through
311 de-repression of PhzM [97]. In some instances, increased PCA conversion to PYO may
312 even result in PCA depletion and thus aid in the activation of PhoB. Further work is
313 needed to fully understand the interplay between PhoB activity and phenazines.

314 It is well established that in permissive Pi conditions, PhoB activity can directly
315 promote the expression of QS-regulated genes through RhlR [14, 15, 32, 89], allowing cells to
316 circumvent reliance on LasR as a QS regulator [12, 33]. Our data support this existing model
317 by showing QS gene expression is PhoB-dependent in a Δ*lasR* mutant but not the wild
318 type at 0.7 mM Pi. Importantly, we have shown increased PhoB activity in LasR– *P.*

319 *aeruginosa* compared to LasR+ strains and this has not been identified in previous
320 publications. The relatively low (0.4 – 0.5 mM) and high (4 – 4.5 mM) Pi concentrations
321 used in the past likely hindered the observation of increased PhoB activity in LasR– *P.*
322 *aeruginosa* and demonstrates the utility of gradient agar plates. As we found PhoB is
323 active in the Δ*lasR* mutant at higher Pi concentrations than the wild type, the previously
324 reported PhoB-driven reorganization of QS may occur in LasR– *P. aeruginosa* under
325 conditions that otherwise repress PhoB activity in LasR+ strains.

326 It is challenging to determine extracellular Pi concentrations in the infection
327 environment due to the abundance of organic phosphate in membrane lipids, DNA, and
328 metabolites. To address this, we reanalyzed an existing RNAseq data set of CF sputum
329 samples and PAO1 grown in SCFM2 [91]. We found the majority of the PhoB regulon was
330 elevated in the sputum samples, suggesting they may contain less Pi than what is
331 provided in SCFM2 (5.1 mM) [92]. Our data indicate that PhoB activity in LasR– *P.*
332 *aeruginosa* differs from the wild type at intermediate Pi concentrations (0.7 – 1.1 mM).
333 increased levels of the PhoB regulon in sputum was variable across samples from
334 different donors. This variability could be due to differences in the genotype of infecting
335 *P. aeruginosa,* differences in inflammation, or the presence of other microbes that may
336 compete for Pi.

337 Further work is needed to elucidate the mechanisms by which LasR activity
338 represses PhoB activity at Pi concentrations in the intermediate Pi range. There is
339 evidence that PhoB can be spontaneously phosphorylated by acetyl phosphate *in vitro*
340 [98] but the concentrations required for this reaction suggest it is unlikely to occur inside
341 cells [99]. Therefore, we expect changes in PhoB activity are mediated by its sensor kinase

342 PhoR. PhoR activity is suppressed by interactions with the Pi transporter PstABC and
343 PhoU [17, 20, 22]. Thus, changes in expression of these proteins could alter PhoR-PhoB
344 activity. We observed no differences in transcript abundance of *phoU* or *pstA* between
345 the wild type and Δ*lasR* mutant (Supplemental File 1) and so believe this model is unlikely.
346 However, other changes in Pi transport into the cell could still contribute to differences in
347 PhoB activity. While the mechanisms of PhoR-PhoU-PstABC interactions are not well
348 understood, PhoR has been shown to interact with PhoU through its Per-Arnt-Sim (PAS)
349 domain [22]. PAS domains in other organisms bind a broad range of ligands [100] and can
350 modify kinase activity [101, 102]. Thus, we propose that changes to the intracellular
351 environment in phenazine-deficient cells or cells with low Crc activity may stimulate PhoR
352 activity through its PAS domain. Future work into the mechanisms of the PhoR PAS
353 domain would also be beneficial for understanding similar kinases in *P. aeruginosa* and
354 other organisms as PAS domains are abundant but remain poorly understood [103].
355 Understanding LasR– *P. aeruginosa* virulence in physiologically relevant conditions is
356 critical as LasR– isolates are frequently found in both acute and chronic infections and
357 these findings may be relevant to *P. aeruginosa* when in settings that do not induce QS
358 regulation. We propose that increased PhoB-mediated QS and virulence gene expression
359 in LasR– *P. aeruginosa* may contribute to their association with worse outcomes [52, 55].
360 Our data support a novel model where LasR represses PhoB activity and thus
361 LasR– *P. aeruginosa* have elevated PhoB activity at physiological Pi concentrations.
362 Others have shown that PhoB activity can induce RhlR activity in the absence of LasR,
363 leading to increased production of virulence factors like PYO [12, 33]. Here we have shown

364 QS and virulence-related gene expression in the Δ*lasR* mutant is highly PhoB dependent
365 at a physiologically relevant Pi concentration.
366
367 **Materials and Methods**

368 **Strains and growth conditions**
369 Bacterial strains and plasmids used in this study are listed in **Table S1**. Bacteria were
370 streaked from frozen stocks onto LB (lysogeny broth) with 1.5% agar [104]. Planktonic
371 cultures were grown in 5 mL LB medium in 18 mm Borosilicate glass tubes on a roller
372 drum at 37 °C in the indicated medium.
373
374 **Construction of in-frame deletion, complementation, and expression plasmids**
375 Construction of in-frame deletion and complementation constructs was performed using
376 yeast cloning techniques in *Saccharomyces cerevisiae* as previously described [104] or
377 Gibson assembly [105, 106]. In-frame deletion and chromosomal complementation
378 constructs were made using the allelic replacement vector pMQ30 [107]. Plasmids were
379 purified from yeast using Zymoprep Yeast Plasmid Miniprep II according to the
380 manufacturer's protocol and transformed into *E. coli* strain S17/λpir by electroporation.
381 Plasmids were introduced into *P. aeruginosa* by conjugation and recombinants were
382 obtained using sucrose counter-selection. Genotypes were screened by PCR and
383 plasmid constructs were confirmed by sequencing.
384
385 **Alkaline phosphatase activity assessment**

386 Agar plates were made as previously described [26] with MOPS minimal medium [74] with
387 0.7 mM K_2HPO_4/KH_2PO_4, 60 µg/mL BCIP (Sigma Aldrich # 11383221001), 20 mM
388 glucose and 15 g/L agar (referred to here as MOPS agar). Overnight cultures of *P.*
389 *aeruginosa* were grown in LB, normalized to an OD_{600} of 1, then 5 µl were spotted on
390 MOPS agar and incubated at 37 °C for 24 h. Plates with a gradient of phosphate were
391 made based on the methodology described for pH gradient plates [108]. First 35 mL of
392 molten MOPS agar at one end of the gradient was pipetted into a 10 cm square petri dish
393 (Corning, BP124-05) that rested in a custom 3D-printed prop that held the plate slanted
394 at a 30° angle. Once the bottom layer had solidified, the plate was laid flat and 35 mL of
395 molten medium agar containing the second desired concentration on the gradient was
396 poured atop. BCIP was added as described above.
397
398 **Calculating alkaline phosphatase enzymatic activity**
399 A colorimetric assay using the substrate p-Nitrophenyl phosphate (PNPP) (New England
400 Biolabs) was used to quantify alkaline phosphatase activity in colonies grown on MOPS
401 agar plates with no added BCIP using the inoculation regime described above. After 12-
402 24 h incubation, the colonies were scraped from the agar with a pipette tip and
403 resuspended in 1 mL 10 mM Tris-HCL buffer at pH 8. Fifty microliters of the cell
404 suspension was mixed with 50 µl of room temperature 1-step PNPP solution or 50 µl of
405 Tris-HCL buffer at pH 8 and incubated for 1 h. A_{405} and A_{600} were recorded at t = 0 min
406 and t = 60 min. AP activity was calculated using the equation 1000 x (ΔA_{405}/(time (min) x
407 A_{600})) where $\Delta A_{405} = (A_{405}PNPP - A_{405}NoPNPP)_{t60} - (A_{405}PNPP - A_{405}NoPNPP)_{t0}$.
408

409 **NanoString analysis**

410 Total RNA was collected from *P. aeruginosa* colony biofilms grown on MOPS agar with
411 0.7 mM K_2HPO_4/KH_2PO_4. Bacteria were grown overnight in LB then sub-cultured in LB
412 until OD_{600} = 0.5 before 5 µl were spotted on MOPS agar and incubated at 37 °C for 12
413 h. Cells were then scraped from the agar and resuspended in 200 µl Tris-EDTA buffer.
414 RNA was extracted using the Qiagen RNeasy kit (Qiagen 74004) using the protocol
415 provided by the manufacturer. Samples were not treated with DNAse. NanoString
416 analysis of 80 ng of isolated RNA using codeset PaV5 [26] was performed as previously
417 reported [109]. Counts were normalized to the geometric mean of spiked-in technical
418 controls and five housekeeping genes (*ppiD*, *rpoD*, *soj*, *dnaN*, *pepP*, *dapF*). Normalized
419 counts were used for heatmap construction.

420

421 **Growth curves**

422 *P. aeruginosa* was grown in LB medium for 16 h and then sub-cultured to a starting OD_{600}
423 = 0.05 in 5 mL of MOPS minimal medium + 16.7 mM glucose in glass culture tubes. OD_{600}
424 was recorded using a Spectronic 20D+.

425

426 **Transcriptomic analysis**

427 Publicly available transcriptomics data was accessed from Lewin *et al.* [91]. Transcripts
428 were Log_2 transformed, z-scored, and represented in a heatmap generated in R [110]. Full
429 details of this analysis and the code used to produce the heatmap are accessible through
430 the Github repository associated with this publication (https://github.com/hoganlab-
431 dartmouth/PhoB-regulon-in-sputum).

432

433 **Statistical analysis**

434 Statistical analyses were performed using GraphPad Prism version 10.1.1 for macOS.

435

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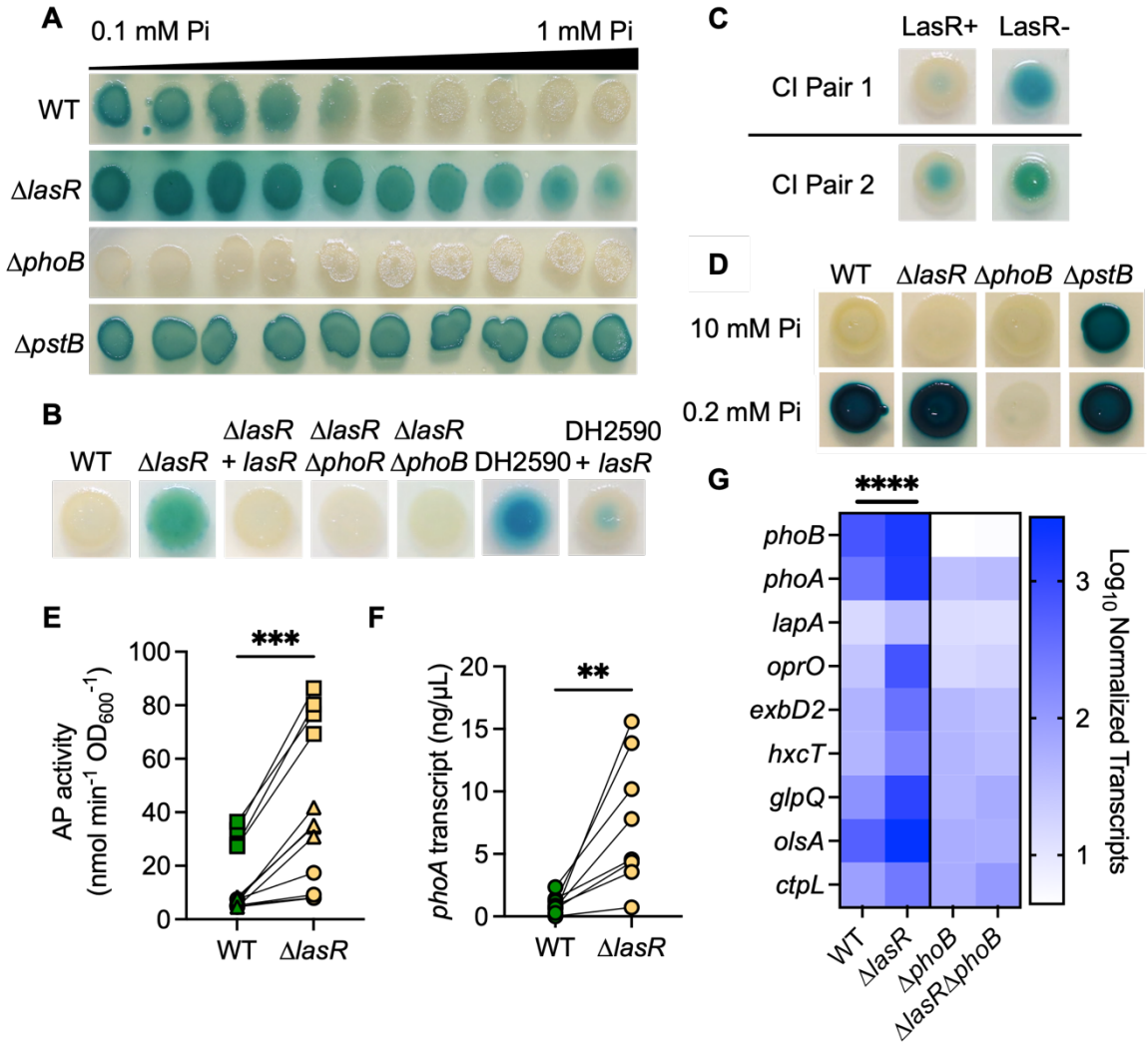

Fig. 1 *P. aeruginosa* LasR- strains require lower Pi to repress PhoB activity. A) *P. aeruginosa* WT, Δ*lasR*, Δ*phoB* and Δ*pstB* were spotted on gradient plates of MOPS agar with a range of Pi concentrations (0.1 - 1 mM) and 60 µg/mL BCIP to indicate alkaline phosphatase (AP) activity. **B)** *P. aeruginosa* strains, including DH2590 (a LasR- clinical isolate (CI) and a derivative in which the *lasR* gene was replaced with the gene from strain PA14) on MOPS agar with BCIP and 0.7 mM Pi C. **C)** *P. aeruginosa* CI LasR+/LasR- pairs from the sputum of two pwCF. **D)** Colony biofilms on MOPS agar with 0.2% glucose and either 0.2 or 10 mM Pi. For **A-D**, similar results were obtained in three replicate experiments; a representative experiment is shown. For panels **E-G**, *P. aeruginosa* was grown as colony biofilms on MOPS agar with 0.7 mM Pi. **E)** AP activity in WT and Δ*lasR* after 12 h at 37 °C. Data from replicates collected on the same day have the same shape. Data analyzed using a paired, two-tailed t-test (n = 12). **F)** *phoA* transcripts in WT and Δ*lasR* were measured by qRT-PCR on different days and normalized to the housekeeping gene transcript *ppiD*. Data analyzed using a paired, two-tailed t-test (n = 8). **G)** Levels of PhoB-controlled transcripts and *phoB* itself were assessed using NanoString multiplex technology. Nine PhoB-regulated transcripts are shown as normalized counts. Data were analyzed using a two-way ANOVA; significant differences between wild type-Δ*lasR,* wild type-Δ*phoB*, and Δ*lasR*-Δ*lasR*Δ*phoB* ($p < 0.0001$, n = 2-3). There were no significant differences between the Δ*phoB* and Δ*lasR*Δ*phoB* mutants ($p = 0.46$, n = 3). Statistical differences for each transcript are available in Supplemental File 1. For all panels, asterisks denote significance ($p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****).

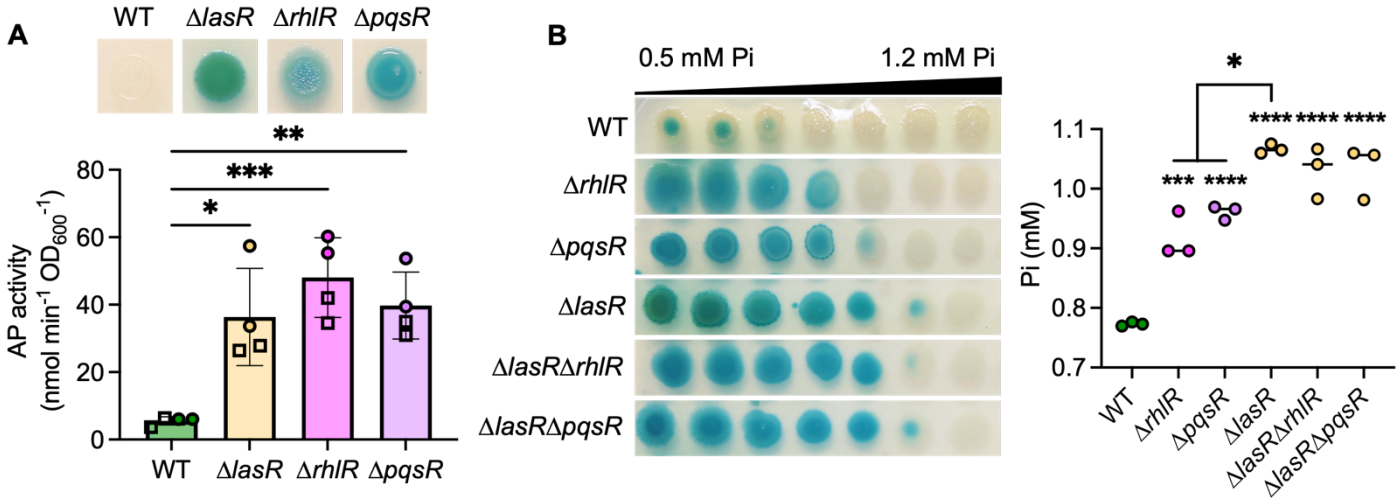

Fig. 2 *P. aeruginosa* quorum qensing (QS) mutants have active PhoB at higher Pi concentrations than the wild type. A) Colony biofilms of WT and the indicated QS mutants (Δ*lasR*, Δ*rhlR*, Δ*pqsR*) were grown on MOPS agar with 0.7 mM Pi and BCIP (top) or on medium without BCIP for analysis of AP activity using the colorimetric PNPP substrate. Data from replicates collected on the same day have the same shape. Data analyzed by an ordinary one-way ANOVA with Tukey's multiple comparisons tests (n = 4). **B)** *P. aeruginosa* was grown on a plate with a gradient of Pi concentrations with 0. 5 to 1.2 mM shown. Quantitative data analyzed using a one-way ANOVA (n = 3) (asterisks denote significance from the wild type). Asterisks denote significance ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****).

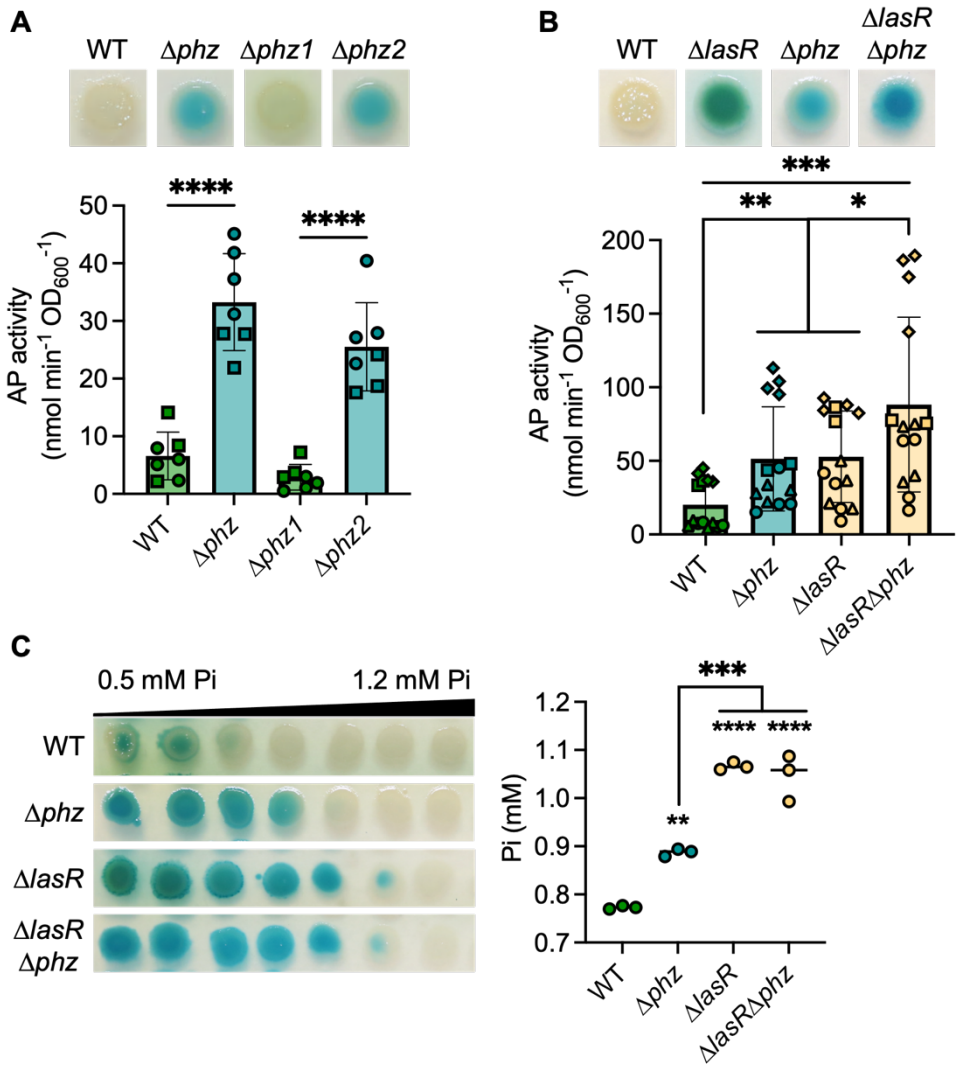

Fig. 3 The loss of phenazines promotes PhoB activity. A) AP activity in WT and mutants Δ*phz1*, Δ*phz2*, and Δ*phz* (lacking *phz1* and *phz2* operons) grown on MOPS agar with 0.7 mM Pi and BCIP (top) or on medium without BCIP for analysis of AP activity using the colorimetric PNPP substrate. Data from replicates collected on the same day have the same shape. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test (n = 7). **B)** Analysis of wild type, and the Δ*lasR*, Δ*phz*, and Δ*lasR*Δ*phz* mutants. The Δ*lasR*Δ*phz* mutant had significantly more AP activity than the Δ*lasR* and Δ*phz* strains. There are no significant differences between Δ*lasR* and Δ*phz* (p=0.99) (n = 12). **C)** *P. aeruginosa* was grown on plates with a gradient of Pi; the average concentration of Pi that inhibits AP activity is graphed to the right (n =3). Data analyzed using a one-way ANOVA; asterisks denote significance from the wild type. Asterisks denote significance ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****).

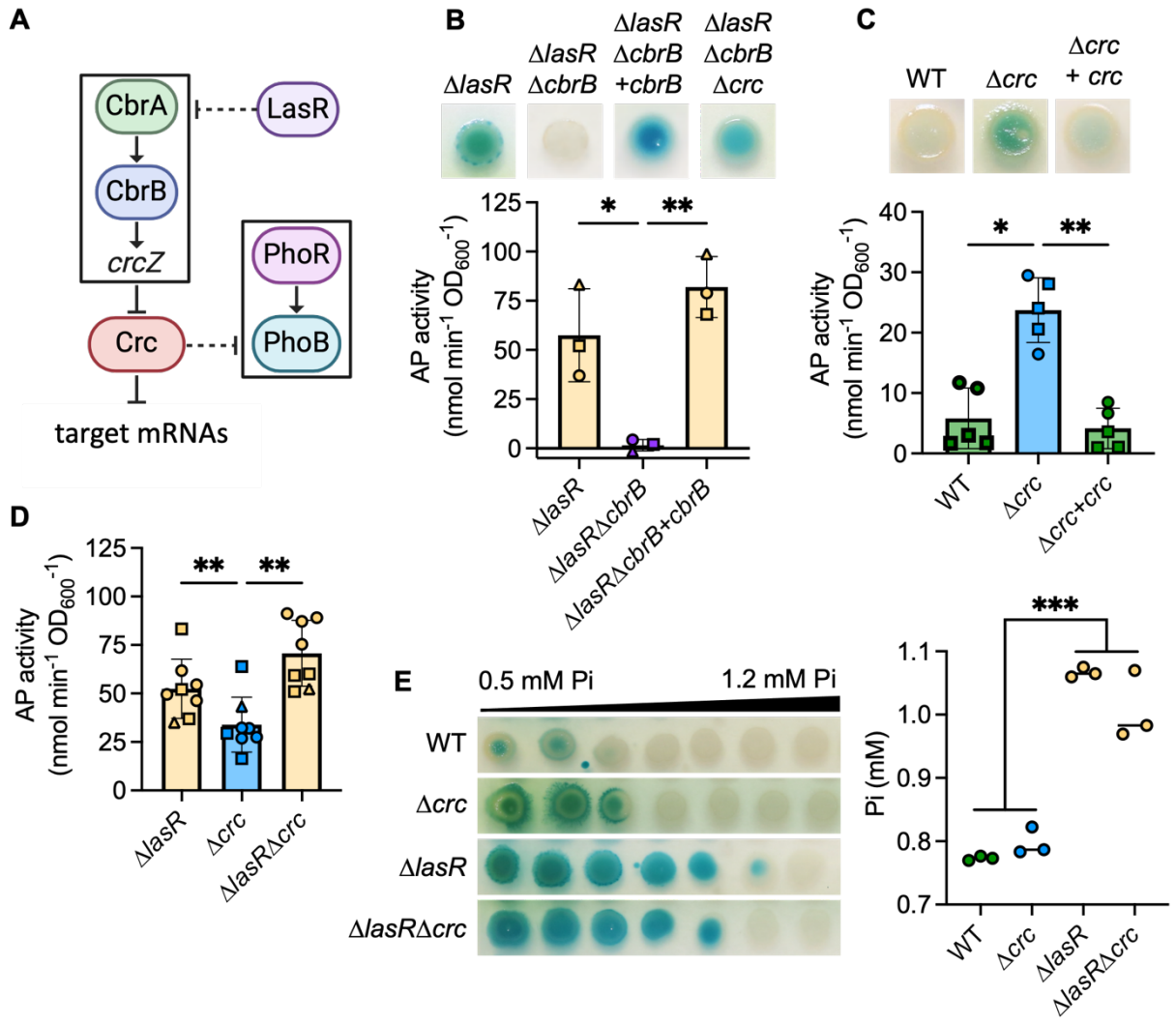

Fig. 4. The CbrA-CbrB-Crc pathway promotes PhoR-PhoB activity in LasR- strains. A) A proposed model of the relationship between the CbrA-CbrB and PhoR-PhoB two component systems; *crcZ*, a small RNA; Crc, which acts as a translational repressor in complex with Hfq. For figures **B** and **C**, AP activity in indicated strains grown on MOPS agar with 0.7 mM Pi and BCIP (top) or on medium without BCIP for analysis of AP activity using the colorimetric PNPP substrate. Data from replicates collected on the same day have the same shape. Data were analyzed using a one-way ANOVA and Tukey's multiple comparisons test. **B)** AP activity in Δ*lasR*, Δ*lasR*ΔcbrB, Δ*lasR*Δ*cbrB*+cbrB, and Δ*lasR*Δ*cbrB* +*crc* mutants (n = 3). **C)** AP activity in the wild type (WT), Δ*crc* mutant and its complemented derivative is shown (n = 5). **D)** AP activity in Δ*lasR*, Δ*crc*, and Δ*lasR*Δ*crc* (n = 8). **E)** *P. aeruginosa* was grown on plates with a gradient of Pi (0.5 – 1.5 mM Pi) and BCIP. The average concentration of Pi which inhibited AP activity is graphed to the right (n = 3). Asterisks denote significance ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***).

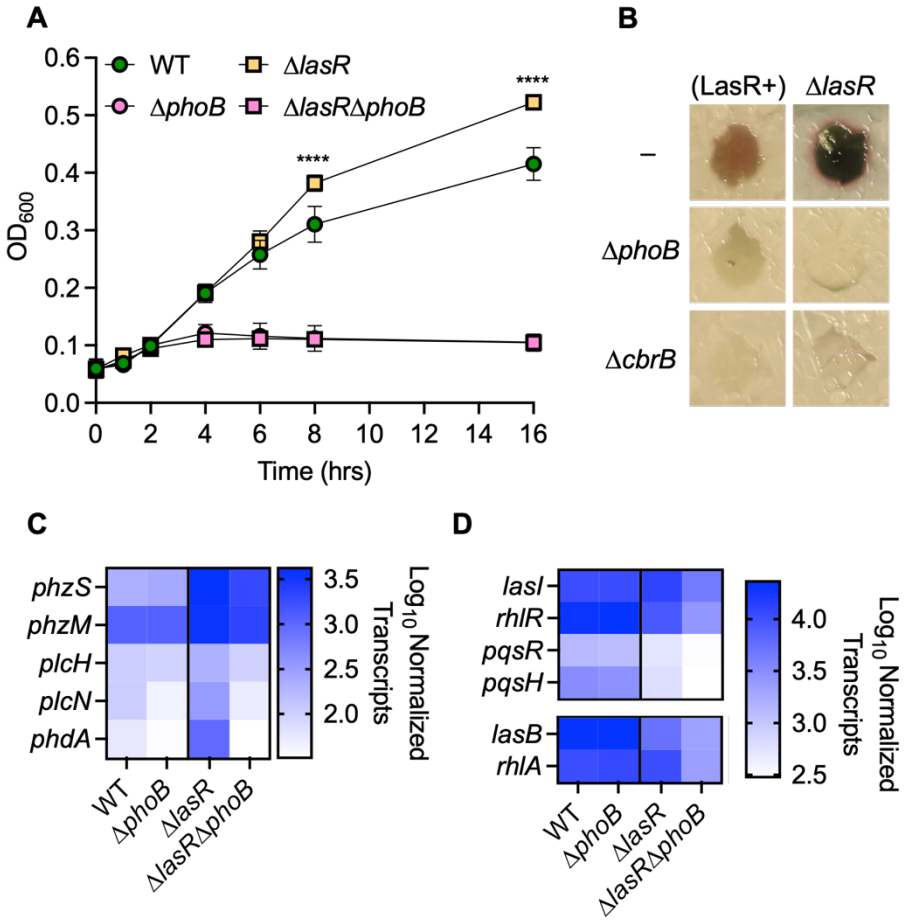

Fig. 5 PhoB activity mediates fitness advantages and antagonism in Δ*lasR P. aeruginosa*
A) *P. aeruginosa* wild type (WT), Δ*lasR*, Δ*phoB*, and Δ*lasR*Δ*phoB* was grown in MOPS liquid medium with no added Pi at 37 °C. Data analyzed using a two-way ANOVA with Tukey's multiple comparisons test (n = 3). Asterisks denote significance (p ≤ 0.0001 = ****). **B)** *P. aeruginosa* colonies were grown on a lawn of *Candida albicans*. Wild-type (LasR+) *P. aeruginosa* produces 5MPCA which is converted to red pigment inside *C. albicans*. The Δ*lasR* mutant produced more 5MPCA than the wild type. 5MPCA production required PhoB and CbrB in both strains (n = 3). **C)** NanoString analysis of PhoB-regulated transcripts shown as normalized counts (n = 2 - 3). All transcripts shown were significantly higher in the Δ*lasR* mutant compared to both the wild type (p ≤ 0.0001) and the Δ*lasR*Δ*phoB* mutant (p ≤ 0.0016). **D)** PhoB contribution to regulation of QS genes by NanoString analysis of QS-regulated transcripts between the wild type (WT) or Δ*phoB* mutant (p>0.30) and the Δ*lasR* and Δ*lasR*Δ*phoB*. All transcripts were significantly lower in the Δ*lasR*Δ*phoB* mutant than the Δ*lasR* mutant (p ≤ 0.01). *lasB, rhlR, pqsR,* and *pqsH* were significantly lower in the Δ*lasR* mutant than the wild type (p < 0.0001). Complete NanoString dataset available in Supplemental File 1.

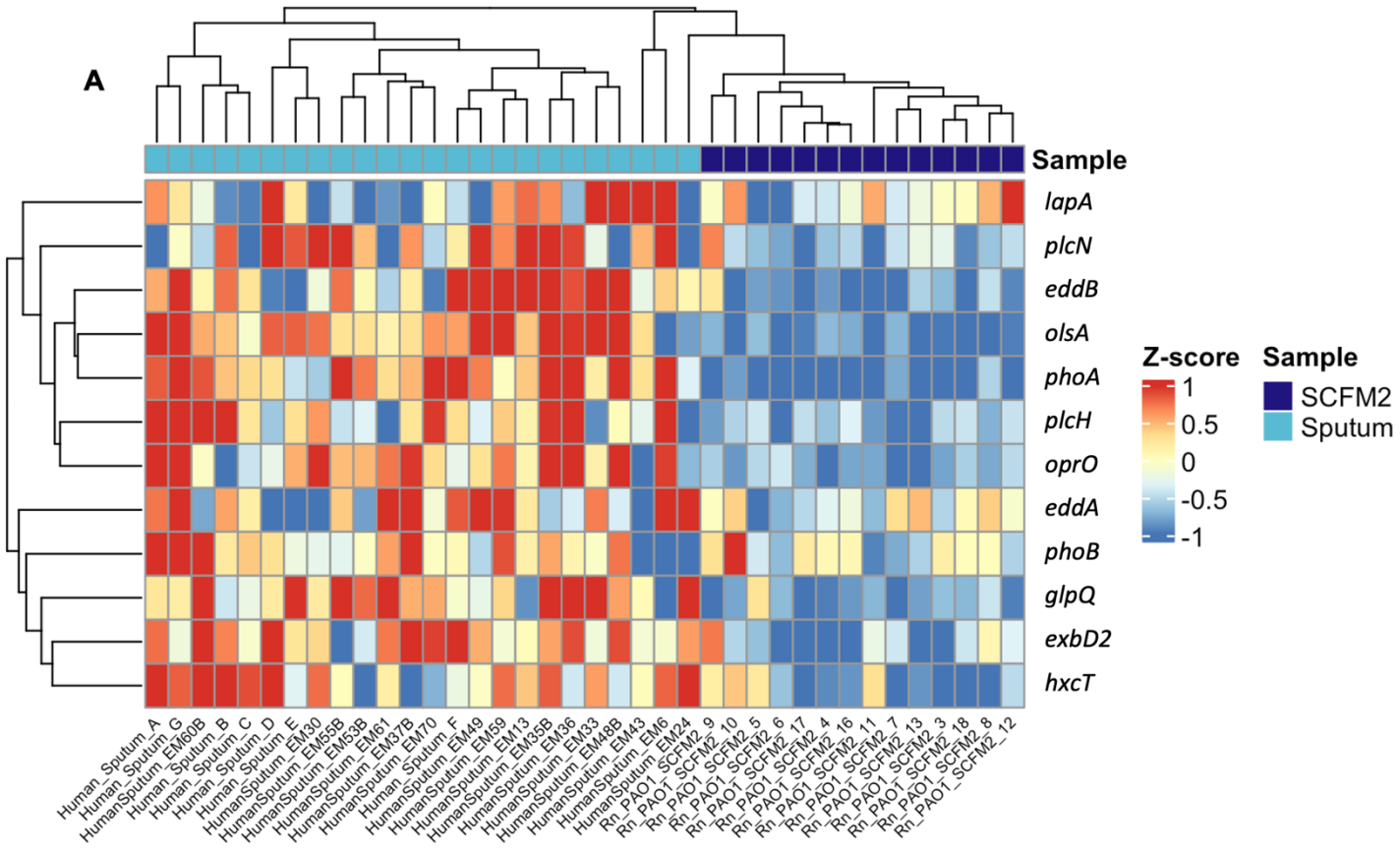

Fig. 6 PhoB-regulated transcripts are higher *in vivo*. RNA-seq data from Lewin, *et al.* (2023) were arranged by sample type (Human Sputum or PAO1 grown in SCFM) and PhoB-regulated transcripts were Z-scored and sorted by unsupervised hierarchical clustering via the R pheatmap function. RNA-seq data is available in Supplemental File 2.