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

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Abstract

While the *Pseudomonas aeruginosa* LasR transcription factor plays a role in quorum sensing (QS) across 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, the transcription factor RhlR, which is controlled by LasR, can be alternately activated in low inorganic phosphate (Pi) concentrations via the two-component system PhoR-PhoB. 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– strains. 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 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 physiological Pi, in part through reactivation of QS. This work suggests PhoB activity may contribute to the virulence of LasR– *P. aeruginosa* and subsequent clinical outcomes.

22 **Importance**

23 Loss-of-function mutations in the gene encoding the *Pseudomonas aeruginosa* quorum
24 sensing (QS) regulator LasR occur frequently and are associated with worse clinical
25 outcomes. We have found that LasR– *P. aeruginosa* have elevated PhoB activity at
26 physiological concentrations of inorganic phosphate (Pi). PhoB activity promotes Pi
27 acquisition as well as the expression of QS and virulence-associated genes. Previous
28 work has shown that PhoB induce RhlR, another QS regulator, in a LasR- mutant in low
29 Pi conditions. Here, we demonstrate a novel relationship wherein LasR represses PhoB
30 activity, in part through the production of phenazines and Crc-mediated translational
31 repression. This work suggests PhoB activity may contribute to the increased virulence
32 of LasR– *P. aeruginosa.*

33

34 **Introduction**

35 *Pseudomonas aeruginosa* is a pernicious pathogen that infects burns, nonhealing
36 wounds, eyes, and the airways of people with cystic fibrosis (pwCF) or chronic obstructive
37 pulmonary disease (COPD). *P. aeruginosa* fitness in vivo is due to its ability to establish
38 persistent biofilms and acquire critical nutrients, such as phosphorus, in the host
39 environment. Phosphate is required for cell membranes, nucleic acids, metabolic
40 intermediates, and is used in some signal transduction pathways. The most accessible
41 form of phosphorus is free inorganic phosphate (Pi) which is at ~1.3 mM in the serum of
42 healthy adults.[1, 2] During infection, however, Pi is restricted by the host as part of
43 nutritional immunity through different mechanisms including the secretion of phosphate-
44 binding proteins.[3-5]

45 Most host phosphorus is in organic forms that require degradation prior to uptake
46 and utilization by microbes.[6, 7] *P. aeruginosa* induces the production of phosphatases,
47 phospholipases, and DNases to access organic phosphate along with high-affinity
48 phosphate transporters in response to low Pi levels via the transcription factor PhoB and
49 its sensor kinase PhoR.[8-11] In *P. aeruginosa*, PhoB also induces the production of
50 specific phenazine small molecules [12-16] which solubilize phosphate from minerals.[17]
51 PhoR, which activates PhoB through phosphorylation, is regulated by interactions with
52 the high-affinity Pi transporter PstABC via PhoU such that Pi transport inhibits PhoR
53 activation.[10, 18-25] While PhoB is known to positively regulate its own expression [26], data
54 suggest that PhoB also participates in a negative feedback loop [27], presumably to limit
55 intracellular Pi concentrations, as has been described in *Escherichia coli*.[28]

56 In addition to the role of the PhoR-PhoB two-component system in Pi acquisition
57 in *P. aeruginosa* and other species such as *E. coli* [24, 29], *Salmonella enterica* [7], and
58 *Vibrio cholerae* [30], these regulators also play diverse roles in the regulation of virulence
59 in multiple pathogens.[10] In *E. coli*, type III secretion system components and effectors
60 are upregulated during Pi limitation in a PhoB-dependent manner.[31] In *V. cholerae*, PhoB
61 inhibits transcription of the genes encoding TcpPH, which positively regulate expression
62 of virulence factors including toxin co-regulated pilus.[30, 32] In *P. aeruginosa,* cross-
63 regulation between PhoR-PhoB and quorum sensing (QS), which contributes to virulence
64 and biofilm formation [33-35], has been described. *P. aeruginosa* QS is largely controlled
65 by three transcription factors, LasR, RhlR, and PqsR, that are active when QS inducers
66 are at sufficient concentrations, i.e. high cell densities or environments with decreased
67 diffusion. While LasR positively regulates both RhlR and PqsR, many groups have shown
68 that RhlR and PqsR can be activated in the absence of LasR.[36-39] Furthermore, in low Pi
69 conditions, PhoB induces QS [14-16, 40, 41] and this can occur in a LasR-independent
70 manner.[13, 42, 43] One group of virulence factors induced by both PhoB and QS are
71 phenazines which inhibit immune cell function [44, 45] and kill other microbes [46-50] through
72 oxidative stress. Though PhoB binding sites have been identified upstream of some
73 phenazine biosynthesis genes [16], PhoB induction of phenazine production is largely
74 attributed to increased expression of *rhlR*.[13, 16, 40] Furthermore, *phoR, phoB, rhlR,* and
75 *pqsR* are all required for *P. aeruginosa* phenazine production in co-culture with *Candida*
76 *albicans* [27], a fungus that frequently co-infects the airways of pwCF.[51] PhoB regulation
77 of QS suggests low Pi could be a signal for QS remodeling to circumvent reliance on
78 LasR.

79 *P. aeruginosa* LasR– strains are found in the environment and isolates from both
80 acute and chronic infections like those in the lungs of pwCF where they make up
81 approximately one-third of clinical isolates.[36, 52-57] LasR– isolates are associated with
82 worse disease progression in pwCF [57] and worse lesions during acute ocular
83 infections.[54] In laboratory evolution experiments, the increased fitness of *P. aeruginosa*
84 LasR– lineages depends on increased CbrA-CbrB activity [58, 59] which leads to growth
85 advantages in complex media.[37, 58, 60] *P. aeruginosa* CbrA-CbrB is a two-component
86 system that induces expression of the small RNA *crcZ* which sequesters Crc.[61-64] Crc,
87 with Hfq, binds to multiple mRNA targets, many of which encode transporters and
88 catabolic enzymes [65], and inhibits their translation. CbrA is required for full *P. aeruginosa*
89 virulence [66].

90 *P. aeruginosa* virulence regulation is often studied in laboratory media with Pi
91 concentrations that repress PhoR activation of PhoB (e.g. synthetic CF medium (SCFM),
92 5.1 mM [67]; RPMI medium, 5.6 mM [68, 69]; Luria Broth, 6 mM [70]; or M9 medium, 64 mM
93 [70]). PhoR-PhoB activity is generally studied in media with less than 0.5 mM Pi.[13, 20, 24,
94 42] These two extremes in Pi concentrations either strongly repress or strongly induce
95 PhoB activity and thus may not be relevant to our understanding of any contributions of
96 PhoB to *P. aeruginosa* virulence at physiological Pi concentrations (1.3 mM Pi in serum
97 [1, 4]). Thus, we aimed to elucidate the relationship between LasR and PhoB at
98 physiologically relevant Pi concentrations. In these studies, we show that LasR– isolates
99 and a Δ*lasR* mutant had elevated PhoR-dependent PhoB activity relative to comparator
100 strains with functional LasR at Pi concentrations in the 0.7 – 1.1 mM range. In contrast,
101 LasR+ and LasR– strains had similar PhoB activity in both low (0.2 mM) and high (10

102 mM) Pi conditions. Our data demonstrate that a lack of phenazines or reduced Crc activity
103 via CbrA-CbrB led to higher PhoB activity and decreased repression of PhoR-PhoB by
104 Pi. We show that PhoB is required for the Δ*lasR* mutant growth advantages in a medium
105 with no added Pi and the increased expression of multiple virulence factors, including
106 phenazine biosynthetic enzymes and phospholipases, at physiological Pi. This work
107 establishes a novel connection between QS and PhoB wherein LasR represses PhoB
108 activity and PhoB is required for increased virulence gene expression in the Δ*lasR* mutant,
109 in part through reactivation of QS. This model for virulence regulation may aid in
110 understanding why *P. aeruginosa* LasR– strains are associated with poor clinical
111 outcomes.
112

113 **Results**

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

116 Though PhoB regulates virulence factor production across species [10, 30, 31], there
117 are only a limited number of in vitro studies on PhoB activity at Pi concentrations similar
118 to those found in human hosts. Thus, we sought to determine the Pi concentrations
119 necessary to repress PhoB activity. In *P. aeruginosa* strain PA14, PhoB activity was
120 monitored by assessing the activity of alkaline phosphatase (AP), which is encoded by
121 the PhoB-regulated gene *phoA* [8, 71, 72] in colonies on agar containing the colorimetric AP
122 substrate bromo-4-chloro-3-indolyl phosphate (BCIP) [9, 73] (Fig. 1A). Using gradient
123 plates with range of Pi concentrations (0.1 mM - 1 mM) [27] in MOPS-glucose medium [74],
124 we found that 0.7 mM Pi repressed AP activity in the wild type. Interestingly, a Δ*lasR*

125 mutant had AP activity across the entire gradient with a reduction in activity at higher Pi
126 concentrations (Fig. 1A). Consistent with PhoB regulation of AP activity, the Δ*phoB*
127 mutant had no detectable AP activity at any concentration, and the Δ*pstB* mutant, which
128 has constitutive PhoB activity due to de-repression of PhoR [27, 75], had strong AP
129 production at all Pi concentrations tested (Fig. 1A). At 0.7 mM Pi, these was AP activity
130 in colonies of the Δ*lasR* mutant, but not the wild type colonies or those formed by the
131 Δ*lasR* mutant complemented with *lasR* at the native locus (Fig. 1B). As expected, neither
132 the Δ*lasR*Δ*phoB* or Δ*lasR*Δ*phoR* mutants had AP activity (Fig. 1B). Furthermore, a clinical
133 isolate from a corneal infection, DH2590 (262K in Hammond, *et al.* [54]), which has a loss-
134 of-function (LOF) mutation in *lasR,* encoding the non-functional $LasR^{I215S}$ variant, also
135 expressed AP at 0.7 mM Pi and AP activity was reduced by replacing the endogenous
136 *lasR* allele with a functional PA14 *lasR* allele at the native locus (Fig. 1B). In addition, we
137 compared AP activity in two clinical isolate pairs (Fig. 1C) from CF sputum samples
138 wherein one isolate has a LOF mutation in *lasR* and the other does not.[76] In both cases,
139 the LasR– isolates had more AP activity than their LasR+ counterparts at 0.7 mM Pi.

140 Both the Δ*lasR* mutant and the wild type showed PhoB activity at lower Pi
141 concentrations (< 0.7 mM). We found that at 0.2 mM Pi, the concentration frequently used
142 to study PhoB activity [13, 14, 20, 24, 77], the wild type and Δ*lasR* strains had similar AP activity
143 (Fig. 1D). Additionally, neither strain showed AP production at 10 mM Pi (Fig. 1D) and no
144 AP activity was observed in either the wild-type or the Δ*lasR* strain on LB agar, which is
145 reported to have 6 mM Pi [70] (Fig. S1A). The Δ*phoB* mutant did not have AP activity even
146 at 0.2 mM Pi and the Δ*pstB* mutant had AP production at 0.2 mM Pi, 10 mM Pi, and on
147 LB. To quantify differences AP activity, we used the soluble colorimetric substrate p-

148 nitrophenyl phosphate (PNPP). The Δ*lasR* mutant had significantly more AP activity than
149 the wild type at 0.7 mM Pi (Fig. 1E) while growth was similar (Fig. S1B). Similarly, qRT-
150 PCR analysis of *phoA*, which encodes AP, found significantly higher transcript levels in
151 the Δ*lasR* mutant compared to the wild type (Fig. 1F).

152 To directly assess the levels of transcripts of other genes within the PhoB regulon,
153 we utilized NanoString multiplex technology as previously published [27]. At 0.7 mM Pi,
154 the Δ*lasR* mutant had significantly higher levels of PhoB-regulated transcripts including
155 *phoB* itself and genes encoding phosphatases (*phoA* and *lapA*), a phosphate transporter
156 (*oprO*), a type two secretion system protein required for secretion of LapA (*hxcT*) [78], a
157 putative TonB transporter (*exbD2*) [79], a phosphodiesterase (*glpQ*), a low-phosphate
158 ornithine lipid biosynthetic protein (*olsA*) [80], and a Pi chemotaxis protein (*ctpL*).[81] None
159 of these transcripts were significantly different when the Δ*phoB* and Δ*lasR*Δ*phoB* mutants
160 were compared (Fig. 1G and Supplemental File 1).

161 **Other QS mutants and a mutant lacking QS-controlled phenazines have active**
162 **PhoB at higher Pi concentrations than the wild type**

163 LasR positively regulates other QS transcription factors including RhlR and PqsR
164 and some Δ*lasR* mutant phenotypes are due to their decreased activity. However, RhlR
165 and PqsR are capable of inducing QS-controlled genes in the absence of LasR in specific
166 strains and conditions.[36, 37, 59, 82] Thus, we assessed the role of RhlR and PqsR in the
167 repression of PhoB. As shown in Fig. 2A, AP activity was significantly elevated in the
168 Δ*rhlR* and Δ*pqsR* mutants, as in the Δ*lasR* mutant, relative to the wild type at 0.7 mM Pi.
169 Across a gradient plate of 0.5 – 1.5 mM Pi, AP activity in all QS mutants was inhibited at

170 a significantly higher Pi concentration than the wild type (Fig. 2B). While AP activity in the
171 Δ*rhlR* and Δ*pqsR* strains was inhibited by Pi > 0.9 mM, activity in the LasR— strains
172 (Δ*lasR,* Δ*lasR*Δ*rhlR,* and Δ*lasR*Δ*pqsR*) was inhibited by Pi > 1 mM (Fig. 2B).

173 As each of the QS transcription factor mutants, Δ*lasR*, Δ*rhlR*, and Δ*pqsR*, produce
174 fewer phenazines than the wild type in late exponential and early stationary phase
175 cultures [83, 84], we tested whether the absence of phenazines was sufficient to increase
176 PhoB activity at moderate Pi concentrations. *P. aeruginosa* produces multiple phenazines
177 including phenazine-1-carboxylic acid (PCA and the PCA derivatives 5-methyl-PCA
178 (5MPCA), pyocyanin (PYO), and phenazine-1-carboxamide. PCA is synthesized by
179 proteins encoded by two highly similar operons, *phzA1-G1* and *phzA2-G2.* The mutant
180 lacking both *phz* operons (Δ*phzA1-G1*Δ*phzA2-G2*), referred to as Δ*phz*, had significantly
181 elevated AP activity compared to the wild type at 0.7 mM Pi (Fig. 3A). The Δ*phz1* mutant
182 (Δ*phzA1-G1*) was not different from the wild type and deletion of either of the adjacent
183 phenazine-modifying genes required for 5MPCA and PYO biosynthesis, *phzM* or *phzS*,
184 did not lead to changes in AP production at 0.7 mM (Fig. S2A). In contrast, the Δ*phz2*
185 mutant (Δ*phzA2-G2*) phenocopied Δ*phz* and had significantly more AP production than
186 the Δ*phz1* mutant or the wild type (Fig. 3A). We and others have previously published
187 data identifying *phzA2-G2* as the predominant contributor of PCA.[27, 84-86]

188 We found no difference in AP activity at 0.7 mM Pi between the Δ*lasR* and Δ*phz*
189 mutants (Fig. 3B). However, a Δ*lasR*Δ*phz* mutant had significantly elevated AP activity
190 compared to both single mutants (Fig. 3B). Both the Δ*lasR* and Δ*lasR*Δ*phz* mutants had
191 detectable AP activity at significantly higher Pi concentrations than Δ*phz* (Fig. 3C)
192 suggesting that PhoB activity was derepressed by an additional phenazine-independent

193 mechanism in the Δ*lasR* mutant. Consistent with this model, the Δ*phz* mutant was
194 repressed by a similar Pi concentration as the Δ*rhlR* and Δ*pqsR* mutants, but not their
195 LasR– counterparts (Fig. 2B).

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

197 Several studies suggest that *P. aeruginosa* strains lacking LasR activity have
198 elevated CbrA-CbrB activity in many conditions.[58, 60] CbrA-CbrB activity leads to relief of
199 Crc-mediated translational repression of diverse mRNA targets including many that
200 encode catabolic enzymes and transporters [64, 65, 87] (Fig. 4A). In both BCIP and PNPP
201 assays, we found that in a Δ*lasR*Δ*cbrB* mutant did not have AP activity at 0.7 mM Pi and
202 that AP production was restored by complementation of *cbrB* at the native locus (Fig 4B).
203 PhoB activity in both the Δ*cbrB* and Δ*lasR*Δ*cbrB* mutants was similarly repressed when
204 Pi > 3 mM (Fig. S2B). Additionally, AP activity in colonies grown on medium with 0.7 mM
205 Pi was restored to a Δ*lasR*Δ*cbrB* mutant by deletion of *crc* (Fig. 4B) suggesting that the
206 increase in Pi repression of PhoB in the Δ*lasR*Δ*cbrB* is due to elevated Crc activity (Fig.
207 4A for pathway). A Δ*crc* single mutant also had significantly elevated AP activity
208 compared to the wild type and *crc*-complemented strain (Fig. 4C). There was no
209 difference in AP activity between the Δ*lasR* and Δ*lasR*Δ*crc* mutants at 0.7 mM Pi, though
210 both were significantly elevated compared to the Δ*crc* mutant (Fig. 4D). Additionally, the
211 Δ*lasR* and Δ*lasR*Δ*crc* mutants both showed significantly decreased PhoB sensitivity to Pi
212 than the Δ*crc* mutant (Fig. 4E).

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

215 To determine if the increased PhoB activity in Δ*lasR* cells increased fitness upon
216 depletion of Pi, we grew the wild type, the Δ*lasR* mutant, and their respective Δ*phoB*
217 derivatives overnight in LB followed by sub-culture into MOPS-glucose medium with no
218 added Pi (Fig. 5A). The wild type and the Δ*lasR* mutant showed similar initial growth rates.
219 However, in the post-exponential phase, the Δ*lasR* mutant reached significantly higher
220 densities than the wild type. Both the Δ*phoB* and Δ*lasR*Δ*phoB* mutants showed minimal
221 growth in the no added Pi media and were not significantly different from each other.

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

224 Multiple studies have shown that there are alternative mechanisms for the
225 activation of RhlR in LasR– strains [13, 36-38, 42, 43] and that phosphate limitation promotes
226 RhlR activity.[13-16, 42, 43] We have previously published that both PhoB and RhlR are
227 required for 5MPCA production by *P. aeruginosa* resulting in antagonism of *C. albicans* in
228 co-culture.[27] A derivative of 5MPCA accumulates inside *C. albicans* cells as a red
229 pigment. Here we show evidence of increased 5MCPA production by the Δ*lasR* mutant in
230 co-culture with *C. albicans.* 5MPCA production by both LasR+ and LasR– *P. aeruginosa*
231 required PhoB and CbrB (Fig. 5B).

232 The NanoString codeset used in Fig. 1G included genes that encode proteins
233 associated with phosphate acquisition, virulence, and QS.[27] We analyzed the abundance
234 of multiple transcripts encoding virulence-associated proteins, including phenazine
235 biosynthetic enzymes (*phzM* and *phzS*), phospholipases (*plcH* and *plcN*), and an
236 exopolysaccharide matrix regulator (*phdA*) [88] in the wild type and Δ*lasR,* Δ*phoB,* and

237 Δ*lasR*Δ*phoB* mutants. These virulence-associated transcripts were significantly higher in
238 the Δ*lasR* mutant compared to the wild type (Fig. 5C and Supplemental File 1). Three of
239 these transcripts have also been identified as part of the PhoB regulon (*plcH* [89], *plcN* [12,
240 90, 91] and *phdA* [79, 92]) while others have not (*phzM* and *phzS*). However, all five transcripts
241 were significantly lower in the Δ*lasR*Δ*phoB* mutant compared to the Δ*lasR* strain and no
242 differences were detected when the Δ*phoB* and Δ*lasR*Δ*phoB* mutants were compared.
243 While *plcN* and *phdA* were significantly lower in Δ*phoB* compared to the wild type, *phzM,*
244 *phzS,* and *plcH*, were only PhoB-dependent in the Δ*lasR* mutant. We then sought to
245 determine if PhoB contributes to QS. We found significantly higher abundance of
246 transcripts encoding proteins involved in QS regulation (*rhlR* and *pqsR*) and autoinducer
247 synthesis (*lasI* and *pqsH*) as well as transcripts that can be induced by RhlR (*rhlA* and
248 *lasB*) in the Δ*lasR* mutant compared to the Δ*lasR*Δ*phoB* mutant (Fig. 5D and
249 Supplemental File 1). As expected, the *lasB, rhlR, pqsR*, and *pqsH* transcripts were also
250 significantly lower in the Δ*lasR* mutant compared to the wild type. Consistent with the
251 model that PhoB is not active in the wild type at 0.7 mM Pi, there were no significant
252 differences in QS-related transcript abundance between the Δ*phoB* mutant and the wild
253 type.

254 **Discussion**

255 In this manuscript, we showed that LasR– *P. aeruginosa* laboratory strains and
256 clinical isolates had elevated PhoB-dependent AP activity and increased expression of
257 the PhoB regulon at 0.7 mM Pi. We found that the range of PhoB-permissive Pi
258 concentrations was broader for LasR– *P. aeruginosa*, though PhoB was still repressed at
259 high Pi concentrations. Importantly, these intermediate Pi concentrations where LasR–

260 *P. aeruginosa* showed more PhoB activity are similar to those found in the serum of
261 healthy adults.[1, 2] We also found that elevated PhoB activity was common across QS
262 mutants and this was possibly due to reduced phenazine production. However, LasR– *P.*
263 *aeruginosa* had active PhoB at even higher Pi concentrations than the other QS mutants,
264 suggesting differences in PhoB regulation.

265 LasR– *P. aeruginosa* are more fit than their LasR+ counterparts in many contexts.
266 LasR– *P. aeruginosa* are known to have elevated CbrA-CbrB activity, conferring growth
267 advantages in complex media through decreased Crc activity.[58, 60] Other studies have
268 shown that LasR– *P. aeruginosa* are more resistant to cell lysis at high pH [93] and more
269 fit in microoxic conditions and normoxically grown colony biofilms.[94] Future studies are
270 necessary to determine if these phenotypes are interrelated. We showed that CbrB was
271 required for increased PhoB activation in LasR– *P. aeruginosa* and a *crc* mutation was
272 sufficient to induce PhoB activity, even in a LasR+ strain. Importantly, the Δ*crc* mutant
273 had less AP activity and was inhibited by lower Pi concentrations than the Δ*lasR* mutant.
274 These data suggest Crc– *P. aeruginosa* do not phenocopy LasR– strains.

275 The elevated PhoB activity in the Δ*lasR* mutant contributed to fitness upon a shift
276 from LB to a medium without added Pi. As phosphate accessibility can be transient inside
277 the host, increased PhoB activity may be beneficial in building up stores of polyphosphate
278 for utilization when cells experience low Pi stress. In addition to regulating phosphate
279 acquisition, PhoB activity has also been shown to promote RhlR expression and
280 subsequent PYO production, swarming motility, and cytotoxicity.[13, 15, 42, 43, 90] Here we
281 showed that the Δ*lasR* mutant had PhoB-dependent increased expression of virulence-
282 associated genes including phenazine biosynthetic enzymes and phospholipases like

283 PlcH. PlcH is known to degrade airway surfactant and we have previously shown that its
284 expression leads to a decline in lung function.[95] We also showed PhoB and CbrB are
285 both required for increased production of the antifungal phenazine 5MPCA by the Δ*lasR*
286 mutant in co-culture with *C. albicans*.

287 The roles for phenazines in PhoB regulation are complex. As all QS mutants
288 produce fewer phenazines in late exponential phase and early stationary phase [83, 84], we
289 proposed phenazines may repress PhoB activity. We demonstrated that phenazine-
290 deficient *P. aeruginosa* had similarly elevated PhoB activity to the single QS mutants.
291 Unlike the Δ*rhlR* and Δ*pqsR* mutants, LasR– *P. aeruginosa* can still produce phenazines
292 in certain contexts.[13, 36-38] We observed that the Δ*lasR* mutant appeared greener on BCIP
293 agar at 0.7 mM Pi which we speculate is due to the production of the blue-green
294 phenazine PYO as this was not observed in the phenazine-deficient Δ*lasR*Δ*phz* mutant
295 (Fig. 3B). Thus, the higher AP activity in the Δ*lasR*Δ*phz* compared to the Δ*lasR* may be
296 due to the absence of phenazines that reduce PhoB activation at moderate Pi
297 concentrations. It is also of note that PhoB activity promotes phenazine production,
298 potentially through direct binding upstream of the *phz* operons [16], but more likely through
299 RhlR activity.[13, 42, 43] This increased phenazine production could then inhibit further PhoB
300 activity, providing negative feedback. However, our data suggests PCA, not PYO or
301 5MPCA, inhibits PhoB as loss of the biosynthetic enzymes PhzM or PhzS did not induce
302 PhoB activity. It cannot be assumed that cells producing visible PYO also have high PCA
303 and reduced PhoB activity. An example of this is the Δ*crc* mutant which we showed had
304 elevated AP activity and is known to produce more PYO through de-repression of
305 PhzM.[96] In some instances, increased PCA conversion to PYO could result in PCA

306 depletion and thus aid in the activation of PhoB. Further work is needed to fully
307 understand the interplay between PhoB activity and phenazines.

308 It is well established that in permissive Pi conditions, PhoB can promote the
309 expression QS genes directly [40] or through RhlR [15, 16, 43, 90], allowing cells to circumvent
310 reliance on LasR as a QS regulator.[13, 42] Our data support this existing model by showing
311 QS gene expression is PhoB-dependent in a Δ*lasR* mutant but not the wild type at 0.7
312 mM Pi. Importantly, we have shown increased PhoB activity in LasR– *P. aeruginosa*
313 compared to LasR+ strains and this has not been identified in previous publications. The
314 relatively low (0.4 – 0.5 mM) and high (4 – 4.5 mM) Pi concentrations used in the past
315 likely hindered the observation of increased PhoB activity in LasR– *P. aeruginosa* and
316 demonstrates the utility of gradient agar plates. As we found PhoB is active in the Δ*lasR*
317 mutant at higher Pi concentrations than the wild type, the previously reported PhoB-driven
318 reorganization of QS may occur in LasR– *P. aeruginosa* under conditions that otherwise
319 repress PhoB activity in LasR+ strains.

320 Further work is needed to elucidate the mechanisms by which LasR activity
321 represses PhoB activity at intermediate Pi concentrations. There is evidence that PhoB
322 can be spontaneously phosphorylated by acetyl phosphate in vitro [97] but the
323 concentrations required for this reaction suggest it is unlikely to occur inside cells.[98]
324 Therefore, we expect changes in PhoB activity are mediated by its sensor kinase PhoR.
325 PhoR activity is suppressed by interactions with the Pi transporter PstABC and PhoU.[18,
326 21, 23] Thus, changes in expression of these proteins could alter PhoR-PhoB activity. We
327 observed no differences in transcript abundance of *phoU* or *pstA* between the wild type
328 and Δ*lasR* mutant (Supplemental File 1) and so believe this model is unlikely. However,

329 other changes in Pi transport into the cell could still contribute to differences in PhoB
330 activity. While the mechanisms of PhoR-PhoU-PstABC interactions are not well
331 understood, PhoR has been shown to interact with PhoU through its Per-Arnt-Sim (PAS)
332 domain.[23] PAS domains in other organisms bind a broad range of ligands [99] and can
333 modify kinase activity.[100, 101] Thus, we propose that changes to the intracellular
334 environment in phenazine-deficient cells or cells with low Crc activity may stimulate PhoR
335 activity through its PAS domain. Future work into the mechanisms of the PhoR PAS
336 domain would also be beneficial for understanding similar kinases in *P. aeruginosa* and
337 other organisms as PAS domains are abundant but remain poorly understood.[102]
338 Understanding LasR– *P. aeruginosa* virulence in physiologically relevant conditions is
339 critical as LasR– isolates are frequently found in both acute and chronic infections and
340 these findings may be relevant to *P. aeruginosa* when in settings that do not induce QS
341 regulation. We propose that increased PhoB-mediated QS and virulence gene expression
342 in LasR– *P. aeruginosa* may contribute to their association with worse outcomes.[54, 57]
343 In conclusion, our data support a novel model where LasR represses PhoB activity
344 and thus LasR– *P. aeruginosa* have elevated PhoB activity at physiological Pi
345 concentrations. Others have shown that PhoB activity can induce RhlR activity in the
346 absence of LasR, leading to increased production of virulence factors like PYO.[13, 42] Here
347 we have shown QS and virulence-related gene expression in the Δ*lasR* mutant is highly
348 PhoB dependent at a physiologically relevant Pi concentrations.
349

350 **Materials and Methods**

351 **Strains and growth conditions**

352 Bacterial strains and plasmids used in this study are listed in **Table S1**. Bacteria were
353 streaked from frozen stocks onto LB (lysogeny broth) with 1.5% agar.[103] Planktonic
354 cultures were grown in 5 mL LB medium in 18 mm Borosilicate glass tubes on a roller
355 drum at 37 °C in the indicated medium.
356

357 **Construction of in-frame deletion, complementation, and expression plasmids**

358 Construction of in-frame deletion and complementation constructs was performed using
359 yeast cloning techniques in *Saccharomyces cerevisiae* as previously described [103] or
360 Gibson assembly.[104, 105] In-frame deletion and chromosomal complementation
361 constructs were made using the allelic replacement vector pMQ30.[106] Plasmids were
362 purified from yeast using Zymoprep Yeast Plasmid Miniprep II according to the
363 manufacturer's protocol and transformed into *E. coli* strain S17/λpir by electroporation.
364 Plasmids were introduced into *P. aeruginosa* by conjugation and recombinants were
365 obtained using sucrose counter-selection. Genotypes were screened by PCR and
366 plasmid constructs were confirmed by sequencing.
367

368 **Alkaline phosphatase activity assessment**

369 Agar plates were made as previously described [27] with MOPS minimal medium [74] with
370 0.7 mM K_2HPO_4/KH_2PO_4, 60 µg/mL BCIP (Sigma Aldrich # 11383221001), 20 mM
371 glucose and 15 g/L agar (referred to here as MOPS agar). Overnight cultures of *P.*
372 *aeruginosa* were grown in LB, normalized to an OD_{600} of 1, then 5 µL were spotted on
373 MOPS agar and incubated at 37 °C for 24 h. Plates with a gradient of phosphate were
374 made based on the methodology described for pH gradient plates [107]. First 35 mL of

375 molten MOPS agar at one end of the gradient was pipetted into a 10 cm square petri dish
376 (Corning, BP124-05) that rested in a custom 3D-printed prop that held the plate slanted
377 at a 30° angle. Once the bottom layer had solidified, the plate was laid flat and 35 mL of
378 molten medium agar containing the second desired concentration on the gradient was
379 poured atop. BCIP was added as described above.
380

381 **Calculating alkaline phosphatase enzymatic activity**

382 A colorimetric assay using the substrate p-Nitrophenyl phosphate (PNPP) (New England
383 Biolabs) was used to quantify alkaline phosphatase activity in colonies grown on MOPS
384 agar plates with no added BCIP using the inoculation regime described above. After 12-
385 24 h incubation, the colonies were scraped from the agar with a pipette tip and
386 resuspended in 1 mL 10 mM Tris-HCL buffer at pH 8. Fifty µL of the cell suspension was
387 mixed with 50 µL of room temperature 1-step PNPP solution or 50 µL of Tris-HCL buffer
388 at pH 8 and incubated for 1 h. A_{405} and A_{600} were recorded at t = 0 min and t = 60 min.
389 AP activity was calculated using the equation 1000 x (ΔA_{405}/(time (min) x A_{600})) where
390 $\Delta A_{405} = (A_{405}PNPP - A_{405}NoPNPP)_{t60} - (A_{405}PNPP - A_{405}NoPNPP)_{t0}$.
391

392 **NanoString analysis**

393 Total RNA was collected from *P. aeruginosa* colony biofilms grown on MOPS agar with
394 0.7 mM K_2HPO_4/KH_2PO_4. Bacteria were grown overnight in LB then sub-cultured in LB
395 until OD_{600} = 0.5 before 5 µL were spotted on MOPS agar and incubated at 37 °C for 12
396 h. Cells were then scraped from the agar and resuspended in 200 µL Tris-EDTA buffer.
397 RNA was extracted using the Qiagen RNeasy kit (Qiagen 74004) using the protocol

398 provided by the manufacturer. Samples were not treated with DNAse. NanoString
399 analysis of 80 ng of isolated RNA using codeset PaV5 [27] was performed as previously
400 reported [108]. Counts were normalized to the geometric mean of five housekeeping genes
401 (*ppiD*, *rpoD*, *soj*, *dnaN*, *pepP*, *dapF*). Normalized counts were used for heatmap
402 construction.
403
404 **Growth curves**
405 *P. aeruginosa* was grown in LB medium for 16 h and then sub-cultured to a starting OD_{600}
406 = 0.05 in 5 mL of MOPS minimal medium + 16.7 mM glucose in glass culture tubes. OD_{600}
407 was measured using a Spectronic 20D+.
408
409 **Statistical analysis**
410 Statistical analyses were performed using GraphPad Prism version 10.1.1 for macOS.
411
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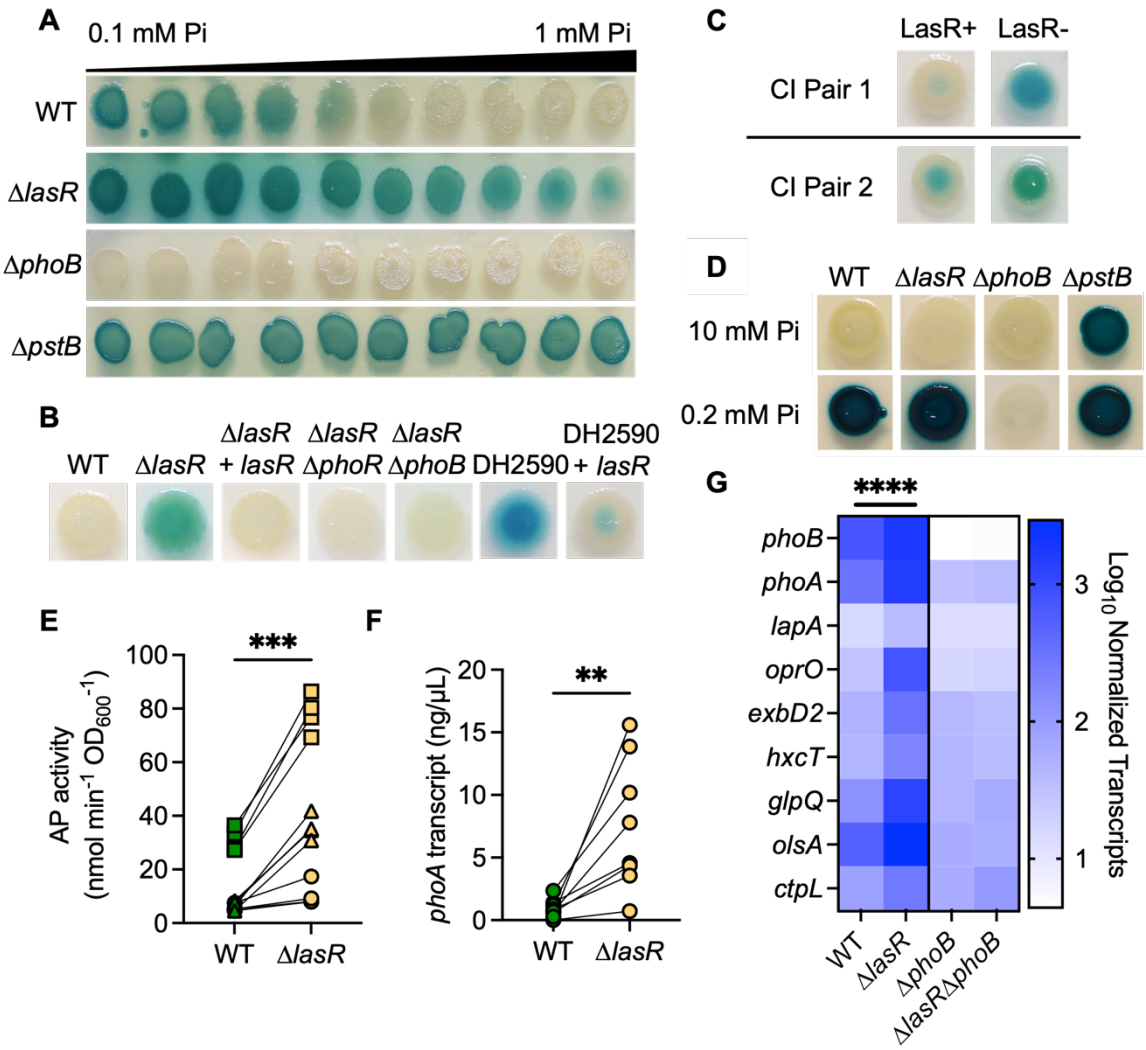

Fig. 1 *P. aeruginosa* LasR– strains require higher Pi to repress PhoB activity. A) *P. aeruginosa* wild type (WT), Δ*lasR*, Δ*phoB* and Δ*pstB* were spotted on gradient plates of MOPS agar with a range of Pi concentrations (0.1 - 1 mM) and 60 µg/mL BCIP to indicate alkaline phosphatase (AP) activity. **B)** *P. aeruginosa* strains, including DH2590 (a LasR– clinical isolate (CI) and a derivative in which the *lasR* allele was replaced with PA14 *lasR*) on MOPS agar with BCIP and 0.7 mM Pi. **C)** *P. aeruginosa* CI LasR+/LasR– pairs from the sputum of two pwCF on MOPS agar with BCIP and 0.7 mM Pi. **D)** Colony biofilms on MOPS agar with 0.2% glucose and either 0.2 or 10 mM Pi and BCIP. 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 WT-Δ*lasR,* WT-Δ*phoB*, and Δ*lasR*-Δ*lasR*Δ*phoB* ($p < 0.0001$, n = 2-3). There were no significant differences between the Δ*phoB*-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 ($p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****).

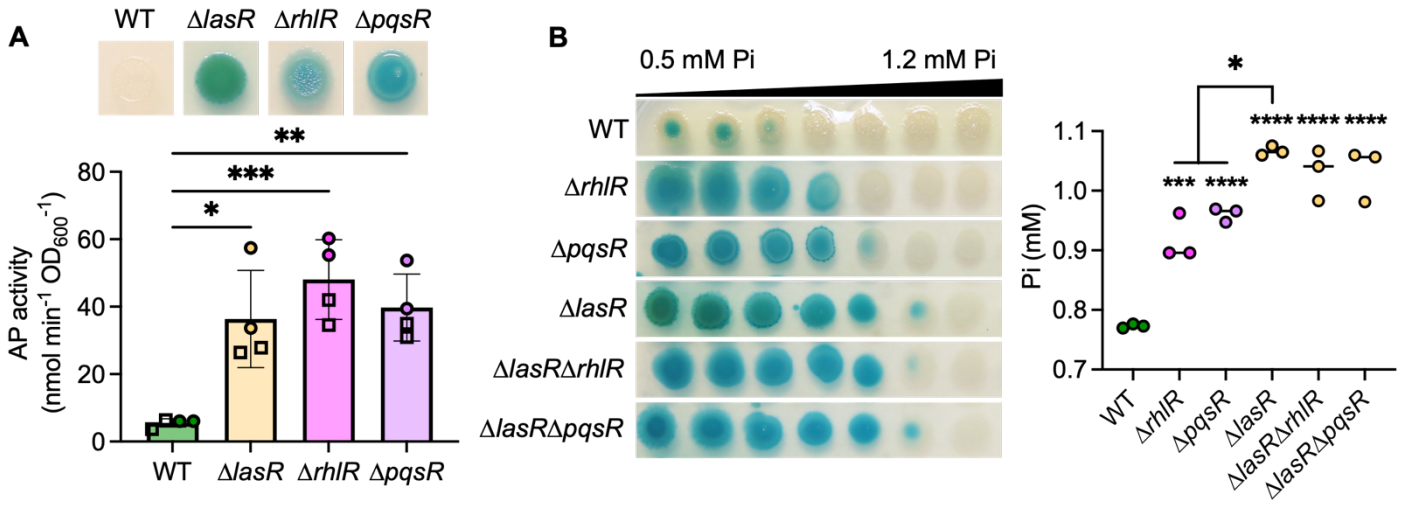

Fig. 2 *P. aeruginosa* quorum sensing (QS) mutants have active PhoB at higher Pi than the wild type. A) Colony biofilms of wild type (WT) and the indicated QS mutants (Δ*lasR*, Δ*rhlR*, Δ*pqsR*) were grown on MOPS agar with 0.7 mM Pi and BCIP (top) or on medium without BCIP for analysis of AP activity 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). For all panels, asterisks denote significance ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****).

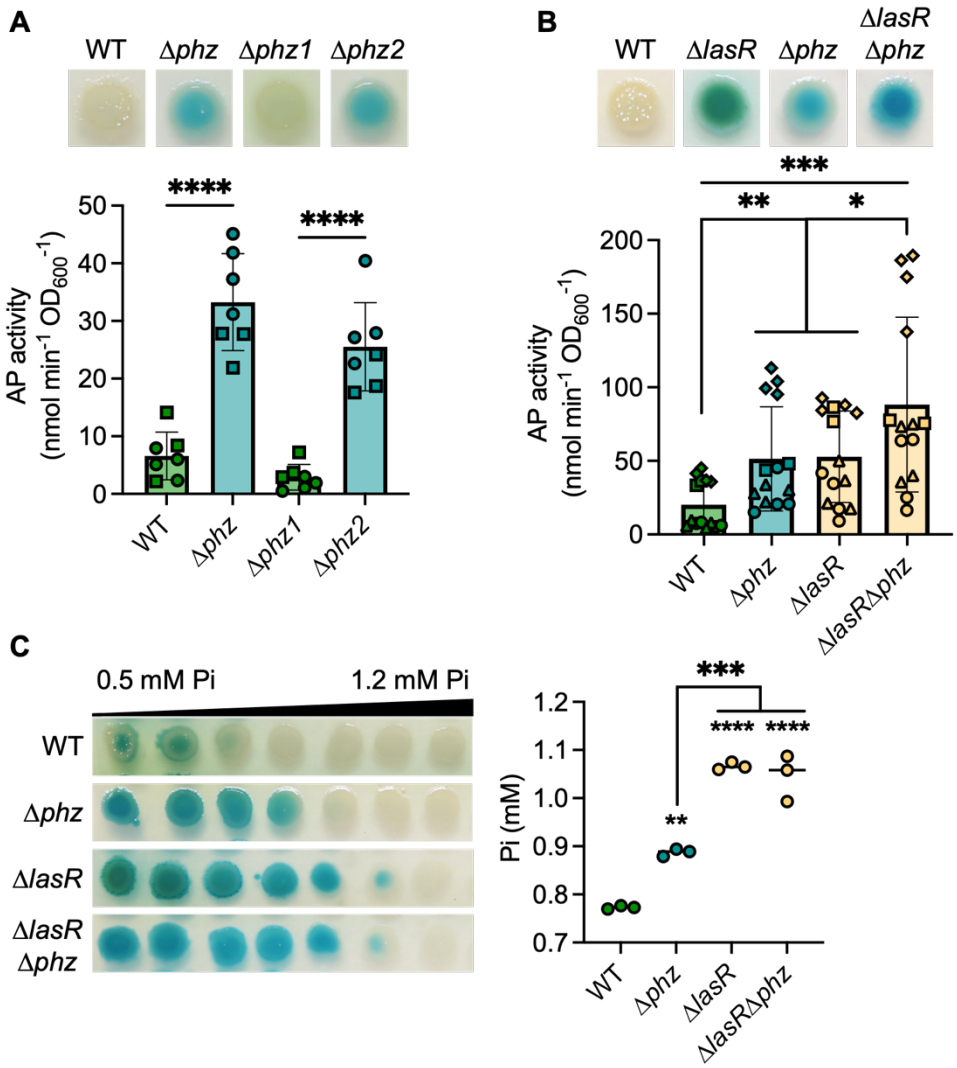

Fig. 3 The loss of phenazines promotes PhoB activity. A) AP activity in wild type (WT) and mutants Δ*phz1*, Δ*phz2*, and Δ*phz* (lacking *phz1* and *phz2* operons) grown on MOPS agar with 0.7 mM Pi and BCIP (top) or on medium without BCIP for analysis of AP activity using the colorimetric PNPP substrate. 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 WT 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 ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****).

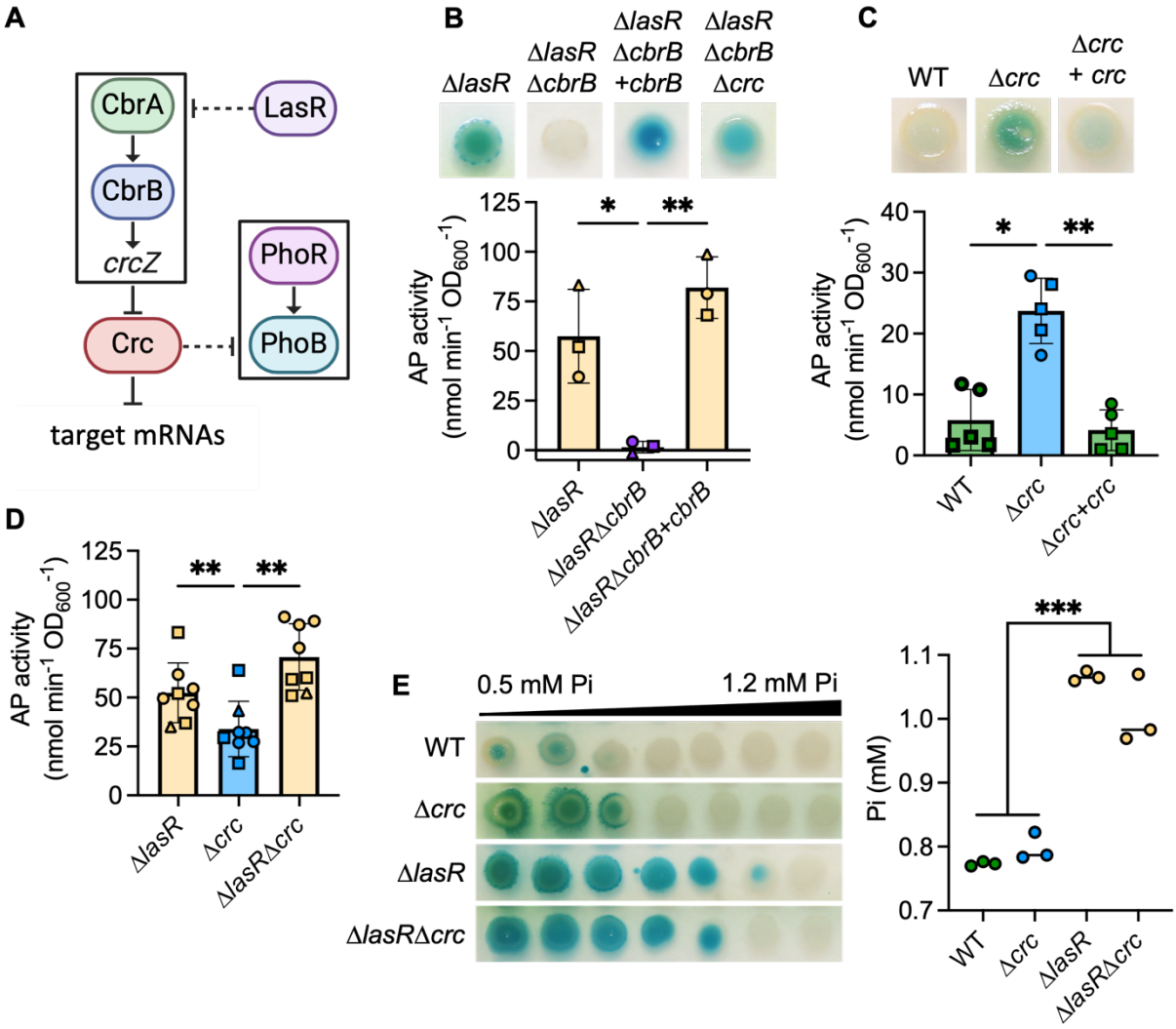

Fig. 4. The CbrA-CbrB-Crc pathway promotes PhoR-PhoB activity in LasR– strains. A) A proposed model of the relationship between the CbrA-CbrB and PhoR-PhoB two component systems; *crcZ*, a small RNA; Crc, which acts as a translational repressor in complex with Hfq. For figures **B** and **C**, AP activity 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 (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 ($p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***).

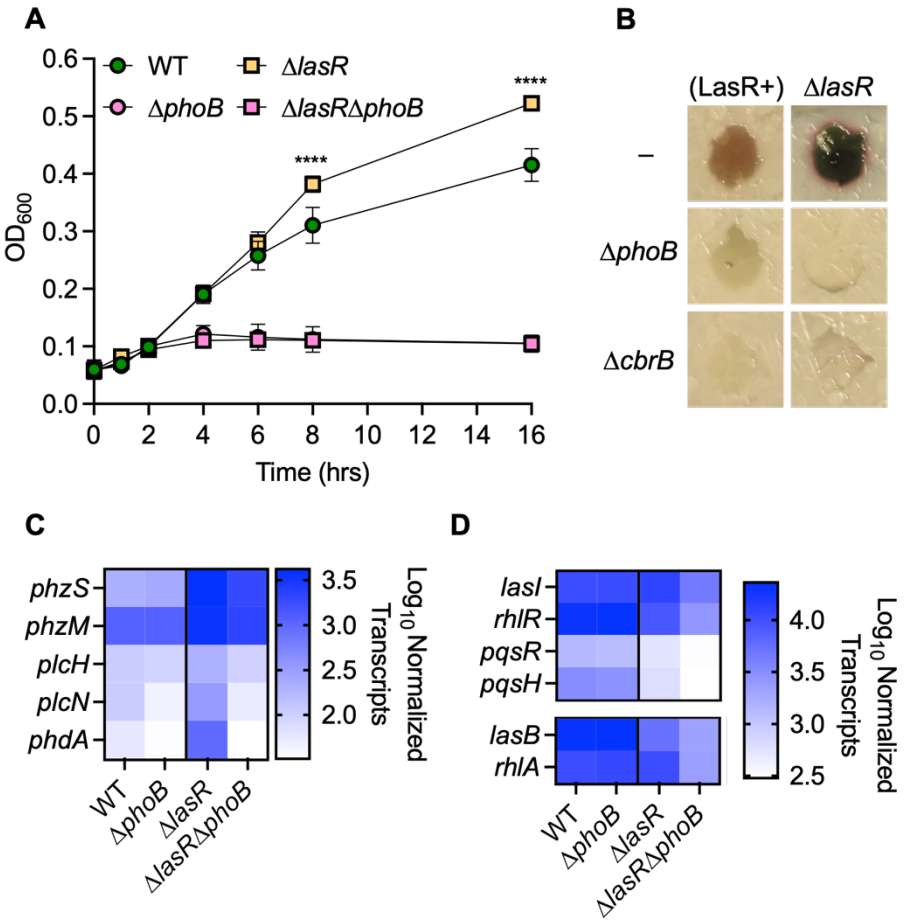

Fig. 5 PhoB activity mediates fitness advantages and antagonism in Δ*lasR P. aeruginosa*. **A)** *P. aeruginosa* wild type (WT), Δ*lasR*, Δ*phoB*, and Δ*lasR*Δ*phoB* were grown in MOPS liquid medium with no added Pi at 37 °C. Data analyzed using a two-way ANOVA with Tukey's multiple comparisons test (n = 3). Asterisks denote significance ($p \leq 0.0001$ = ****). **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 Log_{10} normalized counts (n = 2 - 3). All transcripts shown were significantly higher in the Δ*lasR* mutant compared to both the wild type ($p \leq 0.0001$) and the Δ*lasR*Δ*phoB* mutant ($p \leq 0.0016$). **D)** PhoB contribution to regulation of QS genes by NanoString analysis of QS-regulated transcripts between the 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 ($p \leq 0.01$). *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.