Full title: Promoter selectivity of the RhlR quorum-sensing transcription factor receptor in *Pseudomonas aeruginosa* is coordinated by distinct and overlapping dependencies on C_4-homoserine lactone and PqsE

Short title: C_4HSL and PqsE coordinate RhlR promoter binding

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ABSTRACT

Quorum sensing is a mechanism of bacterial cell-cell communication that relies on the production and detection of small molecule autoinducers, which facilitate the synchronous expression of genes involved in group behaviors, such as virulence factor production and biofilm formation. The *Pseudomonas aeruginosa* quorum sensing network consists of multiple interconnected transcriptional regulators with the transcription factor, RhlR, acting as one of the main drivers of quorum sensing behaviors. RhlR is a LuxR-type transcription factor that regulates its target genes when bound to its cognate autoinducer, C_4-homoserine lactone, which is synthesized by RhlI. RhlR function is also regulated by the metallo-β-hydrolase enzyme, PqsE. We recently showed that PqsE binds RhlR to alter its affinity for promoter DNA, a new mechanism of quorum-sensing receptor activation. Here, we perform ChIP-seq analyses of RhlR to map C_4-homoserine lactone-dependent and PqsE-dependent RhlR binding sites across the *P. aeruginosa* genome. We identify 40 RhlR binding sites, all but three of which are associated with genes known to be regulated by RhlR. While PqsE is generally required for full activation of RhlR transcriptional activity, it is also responsible for preferentially driving RhlR to a distinct subset of binding sites. RhlR binds to PqsE-dependent sites and regulates transcription from these sites independent of

35 the presence of autoinducer. Relatedly, autoinducer directly affects RhlR binding to all promoters,
36 but its effect on binding does not correlate with its role in regulating RhlR-dependent gene
37 expression. Thus, our work reveals three distinct modes of activation by RhlR: i) when RhlR is
38 unbound by autoinducer but bound by PqsE, ii) when RhlR is bound by autoinducer but not bound
39 by PqsE, and iii) when RhlR is bound by both autoinducer and PqsE, establishing a stepwise
40 mechanism for the progression of the RhlR-RhlI-PqsE quorum sensing pathway in *P. aeruginosa*.
41

42 **AUTHOR SUMMARY**

43 *Pseudomonas aeruginosa* represents a serious threat to public health in the United
44 States because of its intrinsic mechanisms of antimicrobial resistance. One of the primary drivers
45 of its antimicrobial resistance profile is biofilm formation. Biofilms are multicellular communities
46 that are formed in an ordered mechanism by the collective. In the case of *P. aeruginosa*, biofilm
47 formation is controlled by a cell-cell communication process called quorum sensing. Quorum
48 sensing underpins transcriptional changes in a bacterium that allows for the transition from
49 individual, planktonic behaviors to group, sessile behaviors. Group behaviors are behaviors that
50 are only beneficial to engage in once a critical threshold of kin population is reached. The
51 transition to group behaviors is mediated by quorum sensing through an interconnected regulatory
52 system, with the transcription factor receptor RhlR regulating the expression of hundreds of
53 genes. RhlR-dependent transcription relies on the detection of a ligand produced by its partner
54 synthase, RhlI, and an accessory binding protein that alters its affinity for promoter DNA, PqsE,
55 resulting in a dual regulatory mechanism that was not previously observed in quorum sensing.
56 The main goal of this study was to determine the molecular basis for promoter selection by RhlR
57 using bacterial genetics and DNA-based sequencing methods for measuring site-specific protein
58 binding. Our approach established the entirety of the RhlR direct regulon and the contributions of
59 each of the known regulators to RhlR-dependent promoter binding and gene regulation.
60

61 **INTRODUCTION**

62 *Pseudomonas aeruginosa* is a Gram-negative bacterium that can grow in nutrient-limited
63 environments such as the reservoirs of water around sinks and in ventilators found in hospital
64 settings. As a result, it is one of the most common nosocomial pathogens and is the primary cause
65 of ventilator-associated pneumonia in the United States [1,2]. Quorum sensing (QS) is a process
66 of bacterial cell-cell communication that controls pathogenesis in many bacterial species,
67 including *P. aeruginosa*. QS relies on the production, accumulation, detection, and population-
68 wide response to extracellular signal molecules called autoinducers (AI) [3]. QS allows bacteria
69 to synchronously alter gene expression patterns that underpin collective behaviors, such as
70 virulence factor production and biofilm formation [4,5]. QS signaling is relayed through a series of
71 AI synthase/receptor pairs [6–12]. At high cell density, the synthase produces a secreted AI that
72 diffuses across the membrane and binds its cognate cytosolic receptor, ultimately resulting in
73 changes in gene expression [13–17].

74 *P. aeruginosa* has two LuxI/R-type synthase/receptor pairs that act in concert to
75 regulate virulence factor expression: LasI/R and RhlI/R [8,18–20]. QS in *P. aeruginosa* is best
76 described as a hierarchical system of interconnected regulatory networks, starting with the LasI/R
77 circuit. LasI synthesizes the AI *N*-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}HSL$), which
78 binds the transcription factor receptor LasR. LasR upregulates the expression of *rhlI/R*, thereby
79 initiating a second wave of QS gene regulation [20–22]. Homologous to LasI/R, RhlI synthesizes
80 the AI *N*-butyryl-L-homoserine lactone (C_4HSL), which binds the transcription factor receptor
81 RhlR. Both systems function in a positive feedback manner with the receptor upregulating the
82 transcription of its partner synthase, thus maximizing the transcriptional response of the system
83 [8,11,21–27]. LasR and RhlR regulate the transcription of hundreds of genes, both directly and
84 indirectly [13,19,21,28–32]. Each transcription factor has its own regulon, with a subset of genes
85 co-regulated by both systems. LasR and RhlR co-regulate a third QS pathway, the Pseudomonas
86 quinolone signaling (PQS) system [33–35]. LasR upregulates the transcription of *pqsR*. PqsR is

87 a LysR transcription factor receptor that binds to the AI 2-heptyl-3-hydroxy-4(1*H*)-quinolone
88 (PQS), which is produced by the biosynthetic pathway enzymes encoded by the *pqsABCDE*
89 operon and *pqsH* [36–40]. Similar to the Las and Rhl system, PqsR upregulates the transcription
90 of genes involved in the synthesis of its AI, thereby establishing another positive feedback loop,
91 resulting in a fully activated QS network [35,36]. RhlR transcriptionally represses the *pqsABCDE*
92 operon, highlighting the interconnected regulatory mechanisms that govern the progression of QS
93 in *P. aeruginosa* [35]. PqsE is a metallo-β-hydrolase enzyme encoded in the *pqsABCDE* operon
94 and is involved in PQS synthesis. Recent work from our group and others has shown that PqsE
95 plays a more nuanced role in QS signaling, outside of its role as an enzyme in the PQS
96 biosynthetic pathway [29,41–47]. Moreover, PqsE is dispensable for the production of PQS, as
97 the deletion of *pqsE* results in wild-type (WT) levels of PQS [45,46].

98 Distinct from its role as an enzyme, PqsE plays an important role in the production of
99 virulence factors; deletion of *pqsE* results in a failure of the strain to produce pyocyanin, an
100 important virulence factor that is produced in a QS-dependent manner via the regulation of the
101 paralogous *phzABCDEFG1* and *phzABCDEFG2* operons as well as *phzH*, *phzM*, and *phzS*
102 [29,41,45,48,49]. Furthermore, pyocyanin production could not be restored by the addition of
103 PQS, indicating a non-enzymatic role for PqsE in the regulation of pyocyanin production [50]. We
104 recently discovered that PqsE physically interacts with RhlR to regulate the ability of RhlR to bind
105 DNA and, thus, contributes to the regulation of pyocyanin production [29,44,47]. More globally,
106 RNA-seq experiments revealed that the PqsE interaction with RhlR and C_4HSL-bound RhlR
107 contribute to RhlR-dependent gene regulation in an overlapping manner, with a few notable
108 exceptions [28,29]. For example, the rhamnolipid synthase, *rhlA*, is regulated by RhlR in a C_4HSL-
109 dependent manner, with PqsE exerting only a minor influence on its transcription. Conversely,
110 the hydrogen cyanide synthase, *hcnA*, is regulated by RhlR in a PqsE-dependent manner, with
111 C_4HSL exerting only a minor influence on its transcription. RNA-seq analyses revealed that these
112 two examples occur on the far ends of a wide spectrum of RhlR-dependent regulation. To date,

113 the RhlR regulon has been inferred from gene expression analyses [42,50]. Thus, it is unknown
114 which genes are directly regulated by RhlR promoter binding. Moreover, the dependence of RhlR
115 on PqsE and C_4HSL for binding different DNA sites has not been determined. Here, we perform
116 ChIP-seq analyses of RhlR in different genetic backgrounds to comprehensively map the direct
117 RhlR regulon in *P. aeruginosa*. We also assess the different and overlapping contributions of
118 C_4HSL and PqsE to RhlR-dependent regulation.
119
120 **RESULTS**
121
122 **Identification of 40 RhlR binding sites across the *P. aeruginosa* PA14 genome**
123 Previous studies identified RhlR-regulated genes but could not determine the direct
124 RhlR-mediated regulation due to the lack of binding data [19,27–29,42,51]. To distinguish
125 between direct and indirect regulation, we mapped RhlR binding to the *P. aeruginosa* PA14
126 genome using ChIP-seq in WT PA14 with a polyclonal antibody raised against RhlR [41]. To
127 account for the possibility that the antibody binds proteins other than RhlR, we performed a
128 parallel ChIP-seq experiment in a Δ*rhlR* strain. Of the 168 genomic regions enriched in ChIP-seq
129 with the WT strain (Table S1), 40 had decreased enrichment in the Δ*rhlR* strain (Figure S1). We
130 consider these 40 regions to be true RhlR-bound regions (Table 1). We speculate that the other
131 128 regions are not RhlR-bound. Indeed, many of these 128 regions are known MvaT/U binding
132 sites, suggesting that their enrichment in the RhlR ChIP-seq was due to cross-reactivity of the
133 RhlR antibody with MvaT and/or MvaU [52,53].
134 The 40 RhlR-bound regions we identified include many with well-established RhlR
135 regulatory elements, such as *rhlA*, *hcnA*, and *phzA1* (Figure 1A-C) [19,20,29,54,55]. We identified
136 a strongly enriched DNA sequence motif associated with 26 of the 40 RhlR-bound regions (Figure
137 1D; Table 1). This sequence motif matches the known RhlR binding consensus, which was
138 previously determined using promoter sequences from the core regulon. Interestingly, we did not

139 find a match to the RhlR motif in the regions upstream of genes *phzH* and *rahU* (encodes
140 hemolysin) that are well-established RhlR-regulated genes. These findings suggest that the
141 presence of a *lux*-box sequence alone is insufficient to explain RhlR binding to all DNA targets,
142 or that some *lux*-boxes deviate considerably from the consensus motif.

143 We next determined the position of RhlR-bound regions relative to genes (Table 1). 32
144 RhlR binding sites are in intergenic regions, <500 bp upstream of the start of a gene. This is an
145 8-fold enrichment over the number of intergenic regions expected by chance (~10% of genome
146 is intergenic). Eight RhlR binding sites are within genes; these include sites within *phzB1* and
147 *phzB2* that also have stronger upstream RhlR binding sites (Figure 1C). Five of the eight
148 intragenic RhlR-bound regions are <500 bp upstream of a neighboring gene, suggesting that RhlR
149 bound to these regions may regulate transcription of the neighboring gene. Overall, RhlR binds
150 predominantly to regions upstream of genes, consistent with its role as a transcription regulator.
151

152 **Identification of directly RhlR-regulated genes**

153 Intergenic RhlR binding sites are associated with 17 transcripts (defined as single-gene
154 or multi-gene operons) that showed significant (q<0.01) differential expression +/- *rhlR* in our
155 recent RNA-seq study (Figure 1A-C; Figure 2; Table 1). An additional 26 transcripts with
156 associated intergenic RhlR binding sites (e.g., *lasB*) did not have significant differential expression
157 in our data, but 14 of these transcripts were shown to be regulated by RhlR in other RNA-seq
158 studies, bringing the total number of transcripts with an upstream RhlR binding site and evidence
159 of RhlR-dependent regulation to 31. We consider these 31 transcripts to be direct regulatory
160 targets of RhlR (Table 1). RhlR-dependent regulation of some genes is likely to be condition-
161 specific, which would explain apparent disparities in RNA-seq studies. All but one of the directly
162 RhlR-regulated transcripts is positively regulated by RhlR, consistent with the known function of
163 RhlR as a transcription activator. Some genes (e.g., *chiC*, *opmD*, *pelG,* and *mucA*) have been
164 shown to be *rhlR*-dependent by RNA-seq in multiple studies, but were not associated with RhlR

165 binding [19,29,50]. These genes are likely indirectly regulated by RhlR. Consistent with this idea,
166 multiple genes encoding known or predicted transcription regulators, including *vqsR*, have
167 associated RhlR binding sites.
168
169 **Characterization of the role of RhlI-synthesized C_4HSL in RhlR promoter selection**
170 Previous work in our lab and others suggested that RhlI-synthesized C_4HSL has an
171 unequal impact on regulation of different genes by RhlR [19,29,56,57]. RNA-seq analyses of a
172 Δ*rhlR* strain and a Δ*rhlI* strain revealed that RhlR-controlled genes are regulated by C_4HSL to
173 varying degrees [19,29], suggesting that RhlR may bind and regulate transcription from some
174 DNA sites in the absence of its ligand and/or that there are additional factors that assist RhlR
175 binding DNA. To understand the impact of C_4HSL on RhlR binding to its DNA sites, we repeated
176 ChIP-seq of RhlR in a Δ*rhlI* strain and compared the occupancy of RhlR at each of the 40 RhlR-
177 bound regions. The Δ*rhlI* strain served to assess the role of C_4HSL in regulating RhlR binding;
178 analysis of a strain lacking *rhlI* is viewed as a strain lacking C_4HSL. The effect of C_4HSL on RhlR
179 binding to DNA varied considerably between RhlR-bound regions (Figure 3A; Table 1). For
180 example, binding of RhlR upstream of *rhlA* decreased 4-fold in the Δ*rhlI* strain compared to WT
181 PA14 (Figure 1A). Conversely, binding of RhlR upstream of *hcnA* increased 1.47-fold in the Δ*rhlI*
182 strain relative to WT PA14 (Figure 1B, Table 1). Thus, our data support a variable role of C_4HSL
183 in RhlR binding to DNA sites, with some sites requiring C_4HSL for maximal RhlR binding and other
184 sites being unaffected by the loss of C_4HSL.
185 The identification of binding sites upstream of *rhlA* and *hcnA* are examples of two broad
186 classes of RhlR-dependent binding sites that are more or less C_4HSL-dependent, respectively.
187 However, these dependencies are not binary, and occur on a spectrum with the least C_4HSL-
188 dependent binding site upstream of *hcnA*, and the most C_4HSL-dependent binding site upstream
189 of *arcD* (16-fold decrease in RhlR binding when *rhlI* was deleted) (Table 1). Known QS-regulated
190 genes associated with RhlR binding sites that are dependent on C_4HSL for RhlR binding include,

191 in order from less to more C_4HSL-dependent: *phzA1*, *phzH*, *lasA*, *lasB*, and *phzA2* (Table 1).
192 Known QS-regulated genes associated with RhlR binding sites that are not dependent on C_4HSL
193 for RhlR binding include *lecB*, *phzB1*, and *phzB2* (Table 1). We note that few RhlR-bound regions
194 are completely dependent upon C_4HSL for RhlR binding, indicating that RhlR can bind most of its
195 DNA sites in the absence of C_4HSL, albeit with reduced affinity in many cases.

196 To determine the role of C_4HSL in the regulation of RhlR target genes, we compared
197 expression of direct RhlR target genes +/- C_4HSL from our recent RNA-seq study (Figure 3B;
198 Table 1). We focused on genes associated with strong RhlR binding sites (see Methods for a
199 description of strong sites) and at least a 2-fold difference in expression +/- *rhlR*. C_4HSL-
200 dependent changes in expression of RhlR target genes correlated positively with C_4HSL-
201 dependent changes in RhlR binding (Figure 3C). The effect of deleting *rhlI* on expression of *rhlA*
202 was similar to the effect of deleting *rhlR*, consistent with strong C_4HSL-dependent binding of RhlR
203 upstream of *rhlA*. However, even for genes such as *hcnA*, where RhlR binding is unaffected by
204 C_4HSL, we still observed a substantial requirement for C_4HSL for regulation by RhlR (Figure 3C).
205 These data strongly suggest that even at sites where C_4HSL is not required for RhlR binding to
206 DNA, C_4HSL is required for RhlR to maximally activate transcription.

207

208 **Characterization of the role of PqsE in RhlR promoter selection**

209 Previous studies showed that PqsE forms a complex with RhlR and increases the affinity
210 of RhlR for some DNA sites [29,44]. PqsE also promotes transcription activation by RhlR
211 [28,29,43]. We speculated that PqsE can assist RhlR binding to some sites in the absence of
212 C_4HSL, which would explain the variable dependence of different RhlR binding sites for C_4HSL.
213 To investigate the effect of PqsE on RhlR binding to its DNA sites, we repeated ChIP-seq of RhlR
214 in a Δ*pqsE* strain and compared the binding of RhlR in the WT strain to each of the 40 RhlR-
215 bound regions +/- *pqsE* (Figure 4A). The effect of *pqsE* deletion on RhlR binding varied
216 dramatically for different RhlR-bound regions. For example, binding of RhlR upstream of *hcnA*

217 decreased 10-fold in the Δ*pqsE* strain compared to WT PA14 (Figure 1B). Conversely, binding of
218 RhlR upstream of *rhlA* decreased only 1.5-fold in the Δ*pqsE* strain (Figure 1A). Thus, our data
219 support a variable role of PqsE in RhlR binding to its DNA sites, with some sites requiring PqsE
220 for maximal RhlR binding and other sites being unaffected by loss of PqsE, similar to the role of
221 C_4HSL.

222 The identification of binding sites upstream of *hcnA* and *rhlA* are examples of two broad
223 classes of RhlR-dependent binding sites that are more or less PqsE-dependent, respectively.
224 However, these dependencies are not binary, and occur on a spectrum with the least PqsE-
225 dependent binding site upstream of *PA14_48530* (no impact of PqsE on RhlR binding), and the
226 most PqsE-dependent binding site upstream of *PA_30840* (189-fold decrease in RhlR binding
227 when *pqsE* was deleted). Known QS-regulated genes associated with RhlR binding sites that are
228 dependent on PqsE for RhlR binding include, in order from less PqsE-dependent to more PqsE-
229 dependent include *lecB* and *hcnA*. Known QS-regulated genes associated with RhlR binding sites
230 that are not dependent on PqsE for RhlR binding include *azeB*, *azeA*, and *rhlI* [50]. We note that
231 17 of the 31 regulated sites had less than a 2-fold change in RhlR occupancy in the Δ*pqsE* strain
232 relative to that in WT PA14, indicating that RhlR binding to many sites is stimulated by PqsE.

233 We previously showed that a D73A substitution in PqsE abolishes catalytic activity but has
234 no impact on the interaction with RhlR [50]. We also showed that PqsE with the triple substitution
235 R243A/R246A/R247A (the "Non-Interacting" [NI] mutant) was impaired for interaction with RhlR
236 [29,44,58]. To test the effect of these mutations on the genome-wide binding profile of RhlR, we
237 performed ChIP-seq of RhlR in strains expressing *pqsE* D73A or *pqsE*-NI. Consistent with our
238 prior study, binding of RhlR in cells expressing *pqsE* D73A was very similar to that in WT cells
239 (Figure S2A), and binding of RhlR in cells expressing *pqsE*-NI was very similar to that of Δ*pqsE*
240 cells (Figure S2B), consistent with the overlapping expression profiles of both *pqsE* D73A with
241 WT (Figure S2C) and *pqsE*-NI with Δ*pqsE* (Figure S2D) [29,47].

242 To determine the role of PqsE in the regulation of RhlR target genes, we compared
243 expression of direct RhlR target genes +/- *pqsE* from our recent RNA-seq study (Figure 4B; Table
244 1). We focused on genes associated with strong RhlR binding sites and at least a two-fold
245 difference in expression +/- *rhlR*. PqsE-dependent changes in expression of RhlR target genes
246 correlated positively with PqsE-dependent changes in RhlR binding (Figure 4C). For example, the
247 effect of deleting *pqsE* on the expression of *hcnA* was similar to the effect of deleting *rhlR*,
248 consistent with strong PqsE-dependent binding of RhlR upstream of *hcnA*. By contrast, deleting
249 *pqsE* had no impact on expression of *rhlA*, consistent with a modest impact of PqsE on RhlR
250 binding upstream of *rhlA*. Strikingly, expression of some genes was largely independent of PqsE
251 despite a strong dependence of RhlR binding on PqsE. For example, binding of RhlR upstream
252 of *PA14_01490* was 3.7-fold lower in the Δ*pqsE* strain than in WT PA14, but the effect of deleting
253 *pqsE* on expression of *PA14_01490* was only 30% the effect of deleting *rhlR*. This suggests that
254 RhlR bound to DNA in the absence of PqsE is a stronger transcription activator than RhlR bound
255 in complex with PqsE.
256
257 **Comparison of the roles of C_4HSL and PqsE in RhlR promoter selection**
258 ChIP-seq and RNA-seq data showed that RhlR binding and RhlR-dependent
259 transcription activation of *rhlA* and *lasB* are more dependent on C_4HSL than PqsE, whereas the
260 opposite is true for *hcnA* and *lecB*. As an independent test of the impact of C_4HSL and PqsE on
261 RhlR-dependent transcription activation of *rhlA*, *lasB*, *hcnA*, and *lecB*, we used quantitative RT-
262 PCR to measure RNA levels for these genes in WT PA14, Δ*rhlR*, Δ*rhlI*, Δ*pqsE*, and Δ*rhlI*Δ*pqsE*
263 strains (Figure 5A). Expression of all five genes was reduced at least 2-fold in Δ*rhlR* relative to
264 WT PA14. These data are largely consistent with RNA-seq data. Expression of *rhlA* and *lasB* was
265 substantially more dependent upon *rhlI* than *pqsE*. By contrast, expression of *hcnA* was
266 substantially more dependent upon *pqsE* than *rhlI*. Expression of *lecB* was roughly equally
267 dependent upon *pqsE* and *rhlI*. For all five genes, simultaneous deletion of both *rhlI* and *pqsE*

268 mirrored the effect of deleting *rhlR*, suggesting that RhlR requires one or both of C_4HSL and PqsE
269 to bind DNA and activate transcription from any DNA site. The effect of deleting both *rhlI* and
270 *pqsE* on expression of RhlR target genes was consistent with our ChIP-seq analyses of RhlR
271 performed in a Δ*rhlI*Δ*pqsE* strain, as deletion of both regulators resulted in nearly the same
272 decrease in binding occupancy at almost all sites compared to the deletion of *rhlR* (Figure 5B).
273 To further assess the role of C_4HSL and PqsE on gene expression, we performed quantitative
274 RT-PCR on *rhlA*, *hcnA*, and *lecB* in Δ*rhlI* and Δ*rhlI*Δ*pqsE* strain backgrounds with increasing
275 concentrations of C_4HSL. Expression of *rhlA* was sensitive to the addition of C_4HSL in both the
276 Δ*rhlI* and Δ*rhlI*Δ*pqsE* backgrounds, indicating that C_4HSL is the main driver of its expression
277 (Figure 5C). Conversely, *hcnA* and *lecB* expression levels were largely independent of C_4HSL
278 across all concentration ranges tested in the Δ*rhlI* strain, and maximal expression was dependent
279 on the presence of PqsE, as the Δ*rhlI* strain had higher expression levels for both genes than the
280 Δ*rhlI*Δ*pqsE* strain (Figure 5C).

281 To directly assess the role of each of the regulators in gene expression, we compared the
282 effect of C_4HSL and PqsE on RhlR-dependent transcription activation of *rhlA*, *lasB*, *hcnA*, and
283 *lecB* using luciferase reporter fusions in *Escherichia coli* expressing either *rhlR* or *rhlR* and *pqsE*,
284 +/- C_4HSL (Figure 6A) [29,47,59]. These data are largely consistent with RNA-seq data and the
285 regulator dependencies described above. Expression of *rhlA* and *lasB* was strongly dependent
286 upon C_4HSL and only modestly stimulated by PqsE. By contrast, expression of *hcnA* and *lecB*
287 was largely independent of C_4HSL but was strongly stimulated by PqsE (Figure 6A). We view the
288 *E. coli* reporter system as being equivalent to an *in vitro* assessment of RhlR function. To
289 determine the effect of PqsE on RhlR binding to DNA sites *in vitro*, we performed electrophoretic
290 mobility shift assays (EMSA) using 200 bp fragments of the *rhlA*, *hcnA*, and *lecB* promoters
291 centered around the determined RhlR DNA-binding motif sequence (Figure 1D) with purified RhlR
292 +/- PqsE added at equimolar concentrations (Figure 6B; Table 1). We note that we, nor others in
293 the field, have purified WT RhlR bound to its native AI. Instead, we solubilized and purified RhlR

294 in the presence of the synthetic agonist meta-bromothiolactone (mBTL), as previously described
295 [41]. mBTL behaves identically to C_4HSL in cell-based reporter assays [60,61]. To the best of our
296 knowledge, native WT RhlR cannot be purified from *E. coli* without a ligand present. Thus, the
297 reciprocal experiment with RhlR +/- C_4HSL could not be performed. Consistent with our previous
298 work and with the work shown above, RhlR bound the *rhlA* promoter in the absence of PqsE, but
299 its binding was enhanced when in complex with PqsE [29]. Conversely, at the concentrations
300 tested, RhlR alone could not bind the *hcnA* or *lecB* promoter and required PqsE to bind DNA
301 (Figure 6B). These data are consistent with our ChIP-seq, *E. coli* reporter, and *P. aeruginosa*
302 transcription analyses, and indicate a potential hierarchy of promoter specificity that results from
303 the mixture of C_4HSL and PqsE dependencies at RhlR-regulated promoters.
304
305 **DISCUSSION**
306 This is the first comprehensive assessment of the direct RhlR regulon. Our findings are
307 largely consistent with prior studies that focused on RhlR-dependent changes in RNA levels
308 [27,29,42,50], but we clarify what regulation is direct versus indirect. Moreover, apparent
309 inconsistencies in RNA-seq data between studies, and the presence of RhlR binding sites
310 upstream of genes without apparent RhlR-dependent changes in RNA levels, suggest that direct
311 regulation of some genes by RhlR is condition-specific, requiring additional factors. Most direct
312 RhlR target genes have established roles in QS. Direct RhlR regulation of several uncharacterized
313 genes, such as *PA14_59180*, *PA14_22090,* and *PA14_10360*, strongly suggests a role for these
314 genes in QS. Regarding the role of PqsE, our data are also largely consistent with an RNA-seq
315 study that assessed the effect of RhlR and PqsE on transcript levels genome-wide [42]. However,
316 the majority of RhlR-regulated genes described in the earlier study are likely to be indirectly
317 regulated, highlighting the value of combining RNA-seq and ChIP-seq data.
318 Both C_4HSL and PqsE contribute to RhlR-dependent transcription activation, but to
319 varying degrees depending on the RhlR site. In the absence of both C_4HSL and PqsE, RhlR

320 binding to DNA was abolished for almost all sites (Figure 5B), resulting in no RhlR-dependent
321 gene activation (Figure 5A). We conclude that in most cases, RhlR needs to bind at least one of
322 C_4HSL or PqsE to be functional. We speculated that there is an inverse relationship between
323 dependence on C_4HSL and dependence on PqsE, as appears to be the case for sites associated
324 with *rhlA*, *lasB*, *hcnA*, and *lecB* (Figures 5 and 6). However, this is not generally the case (Figure
325 7). Moreover, C_4HSL is required for binding at some places more than others but is essential for
326 maximal expression everywhere. We conclude that C_4HSL is required for binding only at certain
327 sites but is required for efficient transcription activation at all sites, suggesting that C_4HSL binding
328 to RhlR imparts substantial conformational changes in RhlR. These findings indicate that RhlR
329 could be stable and functional without a ligand in *P. aeruginosa*, which is not the case in
330 heterologous expression systems. The prevailing dogma was that this class of LuxR-type
331 receptors folded around their native ligand during complex assembly. However, previous Western
332 blot analyses on RhlR in a Δ*rhlI* strain revealed that RhlR remained in the soluble fraction and
333 was not targeted for degradation; it was unclear if the protein was functional [19]. Our results point
334 to a scenario where RhlR is stable without C_4HSL because of the presence of PqsE. PqsE is
335 required for RhlR binding to some sites more than others, but the ability of RhlR to activate
336 transcription is only weakly affected by loss of *pqsE* at sites that lose a significant amount of RhlR
337 binding when *pqsE* is absent. We speculate that C_4HSL:RhlR bound to PqsE, while having a
338 higher affinity for promoter DNA than C_4HSL:RhlR alone, is an overall weaker activator of
339 transcription.

340 The dependence on C_4HSL and/or PqsE for RhlR binding is likely due to differences in
341 the DNA sequences of the RhlR binding sites, or the surrounding sequence. Initially, we
342 hypothesized that PqsE could drive RhlR to promoters with a more degenerate binding motif, or
343 promoters without a detectable binding motif. While that appears to be the case with *hcnA*, it is
344 not true for *lecB* (Table 1). We were unable to infer a PqsE-dependent motif because so few
345 binding sites are strongly associated with PqsE for binding. Future experiments involving chimeric

346 binding sites may reveal the molecular basis for PqsE promoter dependency. Additionally, regions
347 surrounding the RhlR-dependent motif could have an effect on binding to the motif sequence in a
348 PqsE-dependent or -independent manner. For example, integration host factor (IHF) in *Vibrio*
349 *harveyi* regulates LuxR binding and transcription at the *luxC* promoter by binding to regions
350 upstream of the *lux*-box binding motif, bending the DNA, and physically interacting with LuxR [62].
351 Thus, additional regulatory sequences or regulatory elements could alter PqsE-dependency by
352 specifically interacting with PqsE only when it is in complex with RhlR.

353 The activation of a transcription factor, in this case, RhlR, via an accessory protein, such
354 as PqsE, is unique in QS. We speculate that the relative abundance of C_4HSL and PqsE might
355 differ according to different growth conditions, particularly with respect to cell density, and these
356 relative differences affect the timing of RhlR-dependent gene expression. For example, conditions
357 might exist where PqsE concentrations are low, which would result in a higher dependence on
358 C_4HSL binding and activation, leading to a subset of RhlR-dependent genes being expressed.
359 Given that RhlR represses *pqsE* expression, we speculate that expression of the PqsE-dependent
360 RhlR regulon decreases as C_4HSL accumulates. Thus, there is likely a window at which the
361 C_4HSL and PqsE concentrations are "optimal" that maximal gene expression of both regulons
362 can occur. Further studies are needed to determine whether the timing of RhlR activation for
363 different genes is impacted by the dependence on PqsE.

364

365 **FIGURE LEGENDS**

366 **Figure 1.** *RhlR-, C_4HSL-, and PqsE-dependent binding occupancy and gene expression of known*
367 *QS regulated genes.* Genome browser depictions of ChIP-seq and RNA-seq profiles for WT PA14
368 (JPS0222) (black), Δ*rhlR* (JPS0151) (red), Δ*rhlI* (JPS0154) (blue), and Δ*pqsE* (JPS0561) (purple)
369 strains at the (A) *rhlA* operon, (B) *hcnA* operon, and (C) *phzA1* operon. ChIP-seq read counts per
370 base were normalized to reads/100,000,000 reads. RNA-seq data were normalized as a part of

371 genome alignment using Rockhopper. D) MEME analysis was conducted on all 40 RhlR-bound
372 sequences using default parameters, optionally looking for any number of repetitions [63].
373
374 **Figure 2.** *RhlR directly regulates 40 genes in the PA14 genome.* Global RNA-seq analysis
375 comparing normalized RNA levels from the WT PA14 strain to the normalized RNA levels from
376 the Δ*rhlR* strain for all genes in the *P. aeruginosa* genome. Red dots correspond to genes
377 containing a RhlR binding site <500bp upstream of the start site as determined by ChIP-seq
378 analyses. Characterized QS genes of interest are highlighted: *lecB* (magenta), *lasB* (dark green),
379 *hcnA* (light green), *rhlA* (cyan), *phzA1* (olive), and *phzA2* (brown).
380
381 **Figure 3.** *RhlR dependence on C_4HSL for gene activation correlates with its dependence on*
382 *C_4HSL for binding to target sites.* (A) Comparison of ChIP enrichment from the WT PA14 and
383 Δ*rhlI* strains, highlighting the 40 RhlR DNA binding sites from Figure 2. (B) Global RNA-seq
384 analysis comparing normalized RNA levels from the WT PA14 strain to the normalized RNA levels
385 from the Δ*rhlI* strain for all genes in the *P. aeruginosa* genome. Blue dots correspond to genes
386 containing a RhlR binding site <500bp upstream of the start site as determined by ChIP-seq
387 analyses. For (A) and (B) characterized QS genes of interest are highlighted: *lecB* (magenta),
388 *lasB* (dark green), *hcnA* (light green), *rhlA* (cyan), *phzA1* (olive), and *phzA2* (brown). (C)
389 Correlation of RhlR DNA binding dependence and gene activation dependence of RhlI. RhlR
390 dependence on C_4HSL for both gene expression and binding at a given binding site were
391 calculated by subtracting the expression (RNA-seq) or binding (ChIP-seq) values of the Δ*rhlR*
392 strain from the WT control strain and dividing that value by the resulting value of the subtraction
393 of the expression or binding values of the Δ*rhlI* from the WT control strain (i.e., ($WT_{expression}$ -
394 Δ$rhlR_{expression}$)/($WT_{expression}$ - Δ$rhlI_{expression}$)). A value approaching 1 indicates a strong dependency
395 on C_4HSL for expression or binding. A value approaching 0 or lower indicates a low or lack of
396 dependency on C_4HSL for expression or binding.

397

398 **Figure 4.** *RhlR dependence on PqsE for gene activation correlates with its dependence on PqsE*
399 *for binding to target sites.* (A) Comparison of ChIP enrichment from the WT PA14 and Δ*pqsE*
400 strains, highlighting the 40 RhlR DNA binding sites from Figure 2. (B) Global RNA-seq analysis
401 comparing normalized RNA levels from the WT PA14 strain to the normalized RNA levels from
402 the Δ*pqsE* strain for all genes in the *P. aeruginosa* genome. Purple dots correspond to genes
403 containing a RhlR binding site <500bp upstream of the start site as determined by ChIP-seq
404 analyses. For (A) and (B) characterized QS genes of interest are highlighted: *lecB* (magenta),
405 *lasB* (dark green), *hcnA* (light green), *rhlA* (cyan), *phzA1* (olive), and *phzA2* (brown). (C)
406 Correlation of RhlR DNA binding dependence and gene activation dependence of PqsE. RhlR
407 dependence on PqsE for both gene expression and binding were calculated by subtracting the
408 expression (RNA-seq) or binding (ChIP-seq) values for each in the Δ*rhlR* strain from the WT
409 control strain and dividing that value by the resulting value of the subtraction of the expression or
410 binding values of the Δ*pqsE* from the WT control strain, (i.e., ($WT_{expression}$ -
411 Δ$rhlR_{expression}$)/($WT_{expression}$ - Δ$pqsE_{expression}$)). A value approaching 1 indicates a strong dependency
412 on PqsE for expression or binding. A value approaching 0 or lower indicates a low or lack of
413 dependency on PqsE for expression or binding.

414

415 **Figure 5.** *PqsE and C_4HSL coordinate full RhlR-dependent DNA binding and gene expression.*
416 (A) Quantitative RT-PCR analysis of *rhlA*, *lasB*, *hcnA*, and *lecB* in Δ*rhlR* (red), Δ*rhlI* (blue), Δ*pqsE*
417 (purple), and Δ*rhlI*Δ*pqsE* (JPS0278) (orange) strains compared to WT expression levels (dotted
418 line). *gyrA* was used as an internal housekeeping control. Bars represent the mean of three
419 biological replicates. Two technical replicates were performed and averaged for each gene in
420 each biological replicate. Error bars represent standard deviations of the means of biological
421 replicates. (B) Comparison of ChIP enrichment from the WT PA14 and Δ*rhlI*Δ*pqsE* strains,
422 highlighting the 40 RhlR DNA binding sites from Figure 2. Characterized QS genes of interest are

423 highlighted: *lecB* (magenta), *lasB* (dark green), *hcnA* (light green), *rhlA* (cyan), *phzA1* (olive), and
424 *phzA2* (brown). (C) Quantitative RT-PCR analysis of *rhlA* (cyan), *hcnA* (light green), and *lecB*
425 (magenta) in Δ*rhlI* and Δ*rhlI*Δ*pqsE* strains without (-) or with increasing concentrations of C_4HSL.
426 *gyrA* was used as an internal housekeeping control. Transcript levels were normalized to the
427 Δ*rhlI*Δ*pqsE* (-) C_4HSL control. Bars represent the mean of three biological replicates. Two
428 technical replicates were performed and averaged for each gene in each biological replicate. Error
429 bars represent standard deviations of the means of biological replicates.
430
431 **Figure 6.** *PqsE is required for RhlR binding to certain promoters and maximal gene expression.*
432 (A) *E. coli* reporter system expressing RhlR alone (blue) or RhlR and PqsE (purple) in the
433 presence of *rhlA* (JPS0477 and JPS0476, respectively), *lasB* (JPS0924 and JPS0925,
434 respectively), *hcnA* (JPS0924 and JPS0925, respectively), and *lecB* (JPS0948 and JPS0949,
435 respectively) promoters fused to the *luxCDABE* operon (luciferase). All samples received 0.1%
436 arabinose to induce RhlR expression from the pBAD promoter. PqsE was constitutively expressed
437 from the *lac* promoter. The strains expressing RhlR alone contained an empty vector plasmid
438 equivalent to that of the plasmid containing *pqsE*. All samples received 1 μM of C_4HSL or the
439 equivalent volume of DMSO as a carrier control. Light values were normalized to the specific gene
440 reporter control that received only arabinose. Bars represent the mean of three biological
441 replicates. Two technical replicates were performed and averaged for each gene in each
442 biological replicate. Error bars represent standard deviations of the means of biological replicates.
443 (B) Representative images of electrophoretic mobility shift assays using 200 bp fragments of the
444 *rhlA*, *hcnA*, and *lecB* promoters with increasing concentrations of RhlR (left) or RhlR complexed
445 with PqsE (right). A representative SDS-PAGE loading control depicting the concentrations of
446 RhlR and RhlR-PqsE (bottom). RhlR was purified using the synthetic agonist mBTL.
447

448 **Figure 7.** *PqsE and C_4HSL binding dependencies for RhlR occur on a spectrum.* Correlation of
449 relative RhlR binding dependencies on both C_4HSL and PqsE. Correlation values for binding
450 dependence from Figures 3C and 4C were plotted against each other. A value approaching 1
451 indicates a strong dependency on C_4HSL or PqsE for binding. A value approaching 0 or lower
452 indicates a low or lack of dependency on C_4HSL or PqsE for binding.
453
454 **Table 1.** *List of RhlR binding sites in WT PA14 and their associated expression and binding*
455 *dependencies in different genetic backgrounds.* Expression values were determined using
456 Rockhopper [66] with data from Simanek *et al*. 2022 [29]. Q-values represent the probability of a
457 false discovery of differentially expressed genes. Expression values used to calculate relative
458 expression all increased by 1 to prevent div/0.
459
460 **MATERIALS & METHODS**
461
462 **Bacterial strains and growth conditions**. Specific growth conditions for the different strains of
463 *P. aeruginosa* and *E. coli* are described below for their corresponding experiments. Standard
464 molecular biology techniques were used to amplify and clone promoter fragments for the creation
465 of *luxCDABE* fusions. The primers used in this study are listed in Table S3. The plasmids and
466 strains used in this study are listed in Table S4.
467
468 **Chromatin Immunoprecipitation Sequencing (ChIP-seq).** *P. aeruginosa* strains were grown
469 overnight, back diluted 1:100 in fresh LB media and grown to $OD_{600\ nm}$ = 2.0. Crosslinking, lysis,
470 and immunoprecipitation were performed as previously described [64] with the following changes:
471 a polyclonal antibody raised against WT RhlR from PA14 from our previous work was used [41],
472 and the DNA library preparation was performed using Q5 high-fidelity DNA polymerase (New
473 England Biolabs). Peak-calling was performed as previously described [65].

474

475 **Data analysis.**

476 Initial analysis of RNA-seq data [29] was conducted using Rockhopper [66] without strand
477 specificity to normalize, align, and conduct a pairwise comparison of all WT and mutant strains
478 combining two independent sequencing runs [66–68]. The total reads for each called peak in each
479 replicate of the ChIP-seq data were calculated by summing the read count within 100 bases of
480 the center of each peak and normalizing by the total reads of the replicate multiplied by 1,000,000.
481 The read counts for each peak were then averaged with their duplicate. The value for each peak
482 in the WT average was then divided by the scores for the *rhlR* deletion average, and peaks with
483 a ratio > 1 were identified and referred to as "real" RhlR binding sites. The original list of ChIP-
484 sites was used to determine the genes with internal ChIP sites or a ChIP site within 500 bases
485 upstream of the gene or its operon start (Table S1). Genes were then further filtered by association
486 with a "real" RhlR binding site as identified using R (Table 1). Plots of relative ChIP-enrichment
487 and expression were generated using R. Genome browser images were generated using signal
488 map. ChIP-seq data used in the genome browser were normalized to reads/100,000,000 reads.
489 RNA seq data were normalized as part of Rockhopper alignment. To identify MvaT binding
490 detected by RhlR-ChIP, the sequences of sites without a RhlR deletion-associated change in
491 binding were entered into multiblast for PAO1. Previously detected MvaT binding sites in PAO1
492 were then compared to the artifactual sites [52,53]. The probability of a site being intergenic was
493 calculated using a binomial test, predicting the likelihood of 32 sites being intergenic out of 40
494 total and testing with the proportion of the genome that is intergenic (non-coding bases/total
495 bases). The code for data processing and analysis was deposited in the Wade Lab Github at:
496 https://github.com/wade-lab

497

498 ***E. coli* luciferase reporter assay.** *E. coli* TOP10 strains were constructed to express *rhlR* and
499 *pqsE* with the following promoters fused to *luxCDABE* (luciferase): p*rhlA,* p*hcnA,* p*lecB,* and

500 p*lasB*. The *E. coli* strains contained a pBAD vector harboring *rhlR* coupled with one of two pACYC
501 vectors, either containing WT *pqsE* or an empty plasmid, as well as the pCS26 vector harboring
502 promoters of interest fused to *luxCDABE* at the 3'-end of the promoter to assess transcriptional
503 activity. The strains were inoculated from glycerol stocks and grown overnight at 37 °C with
504 shaking in 5 mL LB supplemented with ampicillin (100 µg/mL), kanamycin (50 µg/mL), and
505 tetracycline (15 µg/mL). Overnight cultures were diluted 1:100 in 20 mL LB antibiotic media and
506 incubated at 37 °C with shaking for 3 h. Once the optimal cell density was reached ($OD_{600\ nm}$ =
507 0.6), each culture was supplied with 0.1% arabinose. A black-bottom 96-well plate was prepared
508 with 1 µM C_4HSL or DMSO. To each well containing 1 µL of AI or DMSO, 99 µL of culture was
509 added. The plates were incubated for 4 h at 37 °C with shaking. The Veritas Microplate
510 Luminometer (Promega) was used to measure and record luminescence.
511
512 **RNA extraction, cDNA synthesis, and qPCR.** WT PA14, Δ*rhlI*, and Δ*rhlI*Δ*pqsE* strains were
513 inoculated from glycerol stocks and grown in 5 mL LB overnight at 37 °C with shaking. Overnight
514 cultures were allocated to make 4 subcultures per strain, with each containing a 1:100 dilution in
515 20 mL LB. Two 5-fold serial dilutions of C_4HSL starting at 25 mM were dispensed into its
516 corresponding flask to make a 1 µM, 5 µM, and 25 µM dilution for each strain. 20 µL of DMSO
517 was added to the last flask for the equivalent volume of a carrier control. Cultures were grown for
518 5 h until the optimal cell density was achieved and pyocyanin production was visualized. The
519 cultures were pelleted via centrifugation and saved at -80 °C. Cells were thawed on ice and then
520 resuspended with 700 µL TRIzol reagent (ThermoFisher). Samples were transferred to screw-
521 cap tubes with homogenization beads and homogenized for two 50 sec cycles. 100 µL chloroform
522 was added, hand mixed for 15 sec, and left on ice for 2 min. Samples were pelleted via
523 centrifugation at 12,000 x *g* for 20 min at 4 °C. The aqueous, RNA-containing layer was extracted
524 without disturbing the phenol layer and dispensed into a new microcentrifuge tube, where an
525 additional 500 µL isopropanol was added. Samples were inverted to mix and left at RT for 10 min.

526 Samples were pelleted by centrifugation at 12,000 x *g* for 10 min at 4 °C, and the supernatant
527 was discarded. Pellets were resuspended in 1 mL 70% ethanol and were pelleted by
528 centrifugation at 7,500 x g for 5 min at 4 °C. Supernatant was discarded and the pellets were
529 resuspended in 100 µL sterile, nuclease-free water and solubilized in a 37 °C water bath. RNA
530 concentration and purity were measured on a Nanodrop (ThermoFisher). In new microcentrifuge
531 tubes, DNase reactions were set up using 3 µg RNA, 3 µL 10X DNase Buffer, 1 µL TURBO
532 DNase, and 1 µL SUPERase-IN RNase Inhibitor (ThermoFisher) in a total reaction volume of 30
533 µL. Samples were incubated in a 37 °C water bath for 90 min, with an additional 1 µL DNase
534 added at the 1 h mark. A volume of 3 µL DNase Inactivation Slurry (ThermoFisher) was added to
535 each tube and samples were incubated with mixing at RT for 5 min. Samples were spun via
536 centrifugation at 10,000 x *g* for 2 min at 4 °C. Supernatant was transferred to new microcentrifuge
537 tubes and stored at -80ºC. Once thawed on ice, RNA concentration and purity was measured on
538 the NanoDrop. cDNA reactions were set up in PCR tubes using 500 ng DNA-free RNA, 1 µL 10
539 mM random hexamer, and 1 µL 10 mM dNTPs. Sterile, nuclease-free water was added to each
540 tube to bring the samples to a final volume of 13 µL. The reactions were incubated at 65 °C for 5
541 min in a thermocycler (Bio-Rad) and chilled on ice for 5 min. From the SuperScript III Kit, reactions
542 received 4 µL 5X First Strand Buffer, 1 µL 0.1 M DTT, 1 µL SUPERase-IN RNase Inhibitor, and
543 1 µL SuperScript III Reverse Transcriptase (ThermoFisher). Samples were incubated at 50 °C for
544 45 min, 70 °C for 15 min, and held at 4 °C in a thermocycler (Bio-Rad). The newly synthesized
545 20 µL cDNA reactions were diluted 1:5 with 80 µL sterile, nuclease-free water. A master mix for
546 each gene (*hcnA*, *lasB*, *rhlA*, *lecB*, *gyrA*, and *sigX*) was made using 10 µL SYBR Select Master
547 Mix (ThermoFisher), 0.6 µL forward and reverse primers, as well as 6.8 µL sterile, nuclease-free
548 water. Master mix volumes were multiplied by the number of samples for each gene. A 96-well
549 reaction plate (ThermoFisher) was prepared by supplying each well with 2 µL diluted cDNA and
550 18 µL master mix. A clear plate sealer was firmly adhered to the plate and was quickly spun down

551 via centrifugation at 4 °C. A 7500 Fast real-time PCR system (Applied Biosystems) and software
552 (v2.3) were utilized for relative gene expression analysis and cycle threshold quantification.
553
554 **Protein expression and purification.** 1 L cultures of BL21 strains containing pET28b-*pqsE* or
555 pETDuet-1-*rhlR* were grown in LB and protein production was induced as previously described
556 [47]. RhlR was solubilized using 50 μM mBTL during expression. After growth, cells were pelleted
557 via centrifugation at 12,000 x *g* at 4 °C for 30 min. Cell pellet was frozen at -80 °C until protein
558 purification. Lysis was achieved via sonication by resuspending pellet in lysis buffer (50 mM Tris
559 [pH 8.0], 150 mM NaCl, 20 mM imidazole), followed by centrifugation at 4 °C for 30 min at 15,000
560 x g. Supernatants for each protein were saved for affinity purification pulldown. Different protocols
561 were utilized for affinity purification pulldown for supernatants containing PqsE, and RhlR:mBTL.
562 Supernatant containing PqsE was incubated with 1.5 mL of previously washed Ni-NTA resin
563 (Qiagen) and mixed at 4 °C for 1 h. After incubation, sample was washed 3 times with 25 mL of
564 lysis buffer and then eluted 10x with 1 mL of elution buffer (50 mM Tris [pH 8.0], 150 mM NaCL,
565 500 mM imidazole). For supernatant containing RhlR:mBTL, Buffer A (20 mM Tris [pH 8.0], 1 mM
566 dithiothreitol) was mixed with the sample for a total of 50 mL and loaded onto a column for affinity
567 purification pulldown utilizing a HiTrap™ Heparin HP (Cytiva) column. Protein samples were then
568 subjected to separation on a Superdex-200 column (Cytiva) equilibrated with 50 mM Tris HCl [pH
569 8.0] and 150 mM NaCl. Gels were then stained with Coomassie brilliant blue and imaged on a
570 Bio-Rad EZ-Doc gel imager. SDS-Page was used to assess the purity of proteins after separation.
571
572 **Electrophoretic mobility shift assays.** Concentrations of PqsE, RhlR:mBTL, and PqsE-
573 RhlR:mBTL were standardized to be equal per reaction. EMSA reactions consisted of 17 μL of
574 EMSA Buffer (200 mM KCl, 50 mM Tris-HCl [pH 7.5], 250 μg/mL bovine serum albumin, 50 mM
575 NaCl, 5 nM EDTA, 5 μM $MgCl_2$, 5 μM dithiothreitol), 2 μL of protein dilution, and 1 μL of 10 ng/μL
576 of the DNA fragment to be tested. The reactions were initiated, vortexed briefly and incubated at

577 RT for 15 min. 2 µL of Novex™ Hi-Density TBE 5X Sample Buffer (Invitrogen) was mixed with 8
578 µL of the completed EMSA reaction and loaded on an 8% acrylamide gel. Electrophoresis was
579 performed in 1X TBE buffer at 100 V for 60 minutes followed by washing gel with 0.5X TB buffer
580 at RT for 15 min. Gels were stained with 50 mL of 1X SYBR-Gold in 0.5X TB buffer for 30 minutes
581 at RT and blocked from light. After staining, gel was washed with 50 mL of 0.5X TB buffer with
582 shaking for 15 min. The washing step was repeated an additional two times, followed by
583 visualization on a Bio-Rad EZ-Doc gel imager.
584

585 **DATA AVAILABILITY**

586 EBI ArrayExpress accession numbers for all RhlR ChIP-seq data were deposited under E-MTAB-
587 12966.
588

589 **ACKNOWLEDGMENTS**

590 This work was supported by National Institutes of Health grant R01GM14436101, New York
591 Community Trust Foundation grant P19-000454, and Cystic Fibrosis Foundation grant
592 PACZKO21G0 to J.E.P, NIH Research Supplements to Promote Diversity in Health-Related
593 Research grant 1R01GM14436101-S1 to N.J.C.T and J.E.P., NIH training grant T32GM132066
594 to L.I.P and M.W.B, and NIH grant R35GM144328 to J.T.W. The authors thank the research
595 laboratories in the Division of Genetics at the Wadsworth Center, New York State Department of
596 Health for helpful discussions on the research and for resource sharing. We thank the dedicated
597 staff scientists at the Advanced Genomics Technologies Center and Media & Tissue Core
598 facilities at the Wadsworth Center, New York State Department of Health.
599

600 **SUPPORTING INFORMATION CAPTIONS**

601 **Figure S1.** *RhlR binds to 40 sites in the PA14 genome.* Comparison of ChIP enrichment from the
602 WT PA14 and Δ*rhlR* strains. The 40 sites bound specifically by RhlR are highlighted in red. All

603 non-specific sites are shown in black. See Table 1 for details on all RhlR specific binding sites.
604 See Table S1 for a list of all RhlR specific and non-specific sites.
605
606 **Figure S2.** *The PqsE-RhlR interaction interface is required for PqsE-dependent DNA*
607 *binding.* Comparison of ChIP enrichment between the (A) WT PA14 and *pqsE*-D73A strains and
608 (B) Δ*pqsE* and *pqsE*-NI strains. Comparison of gene expression between the (C) WT PA14 and
609 *pqsE*-D73A strains and (D) Δ*pqsE* and *pqsE*-NI strains
610
611 **Table S1.** *List of all RhlR ChIP-seq binding sites from WT PA14.* The average peak values are
612 the average of two biological replicates and two independent ChIP experiments. Peak values
613 were calculated as the sum of all enrichment within 100 bases upstream and downstream of the
614 center of the ChIP peak.
615
616 **Table S2.** *Gene expression levels for all genes in WT PA14 for the indicated genotype and their*
617 *associated RhlR binding dependency.* Expression values were determined using Rockhopper [66]
618 with data from Simanek *et al*. 2022 [29]. Q-values represent the probability of a false discovery of
619 differentially expressed genes.
620
621 **Table S3.** *List of all primers used in this study.*
622
623 **Table S4.** *List of all plasmids and strains used in this study.*
624
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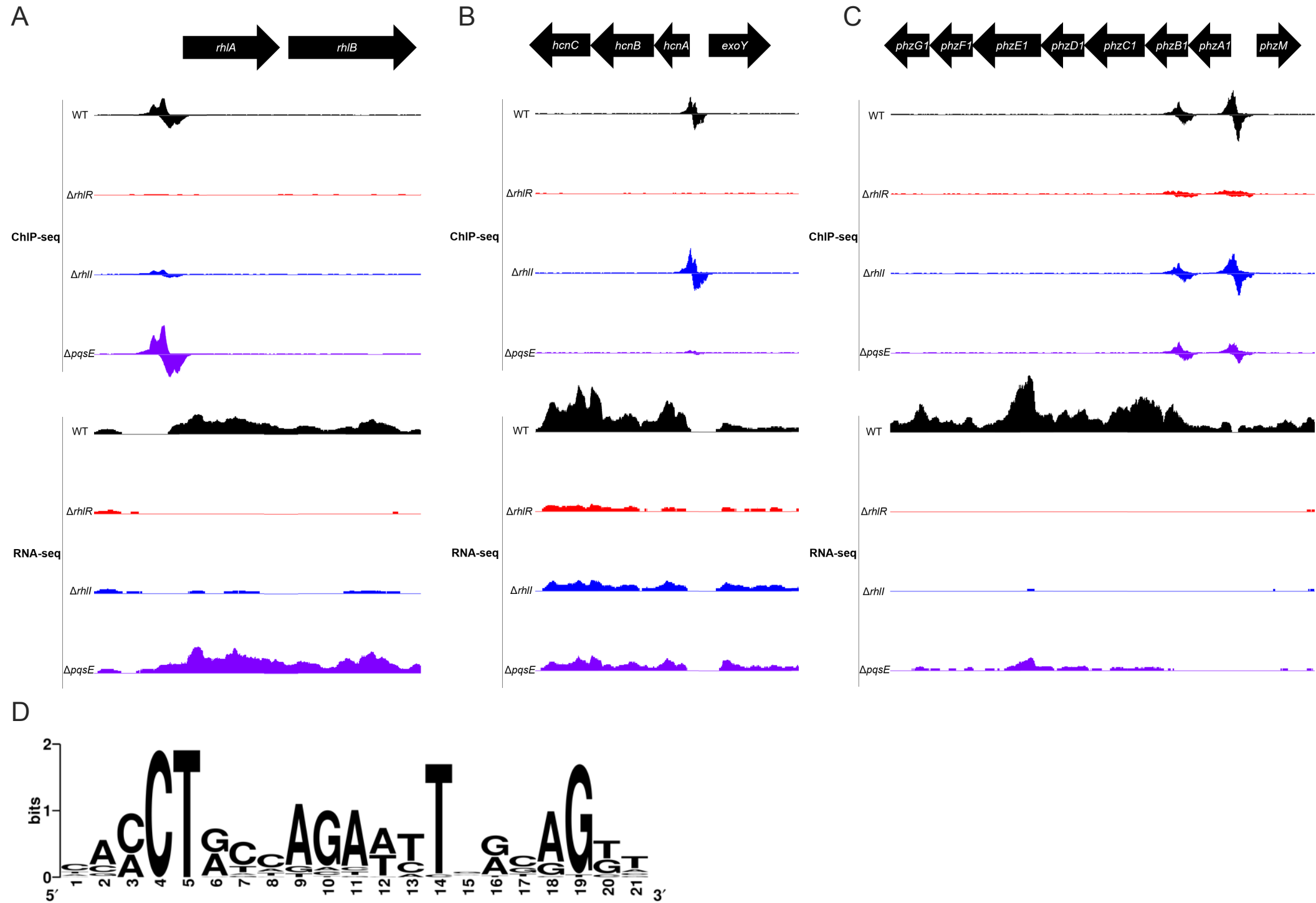
Figure 1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rhlA
rhlB
ChIP-seq
WT
ΔrhlR
ΔrhlI
ΔpqsE
RNA-seq
WT
ΔrhlR
ΔrhlI
ΔpqsE
B
hcnC
hcnB
hcnA
exoY
ChIP-seq
WT
ΔrhlR
ΔrhlI
ΔpqsE
RNA-seq
WT
ΔrhlR
ΔrhlI
ΔpqsE
C
phzG1
phzF1
phzE1
phzD1
phzC1
phzB1
phzA1
phzM
ChIP-seq
WT
ΔrhlR
ΔrhlI
ΔpqsE
RNA-seq
WT
ΔrhlR
ΔrhlI
ΔpqsE
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bits
2
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0
5′
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
3′

Figure 2

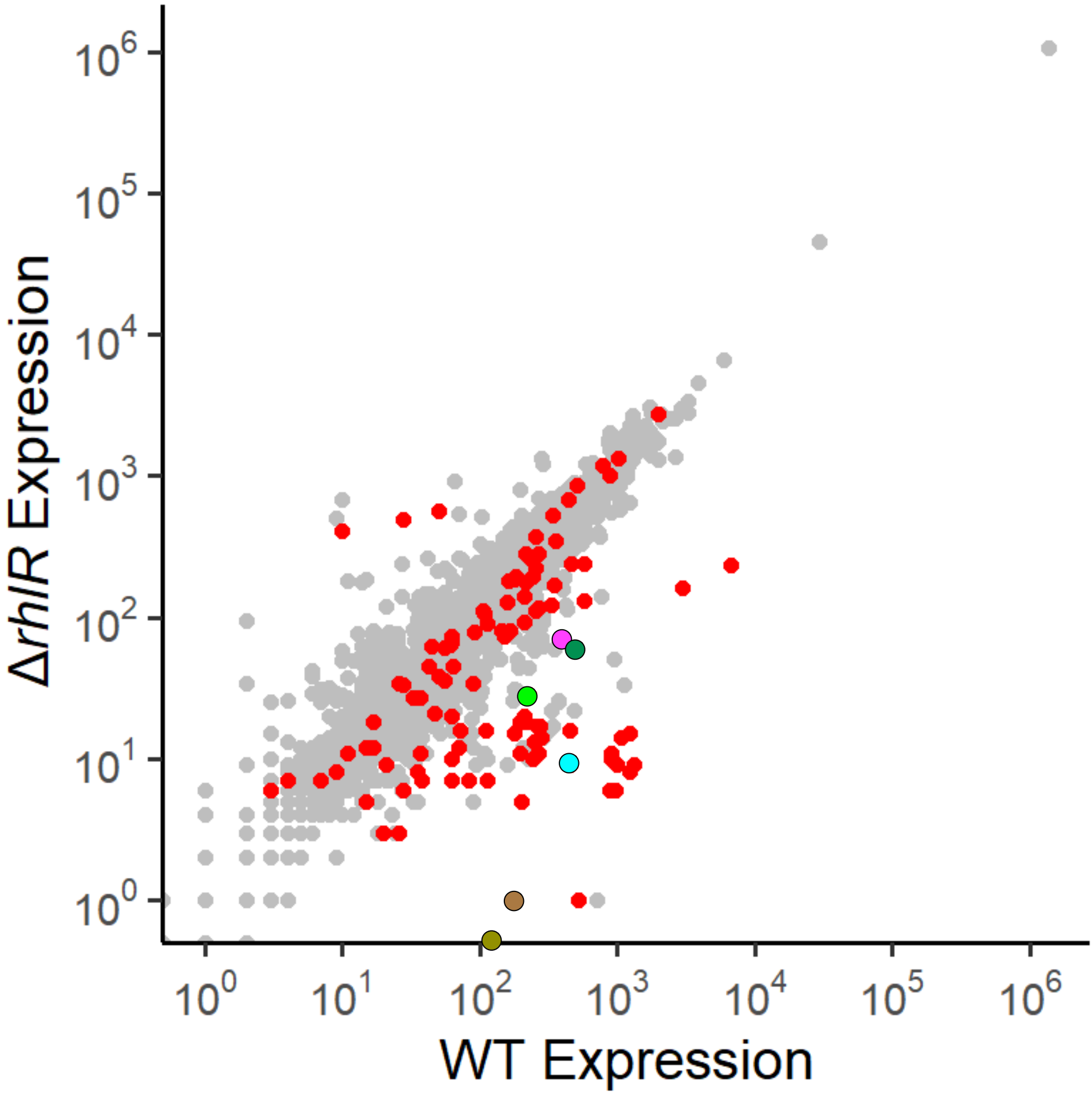

Figure 3

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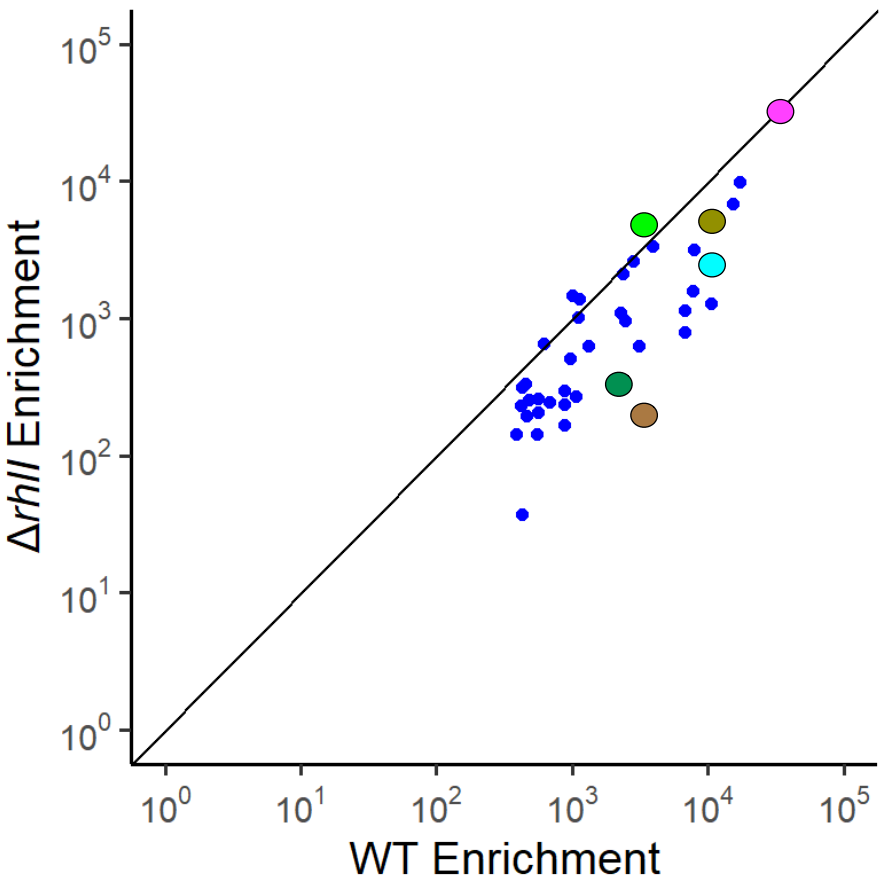
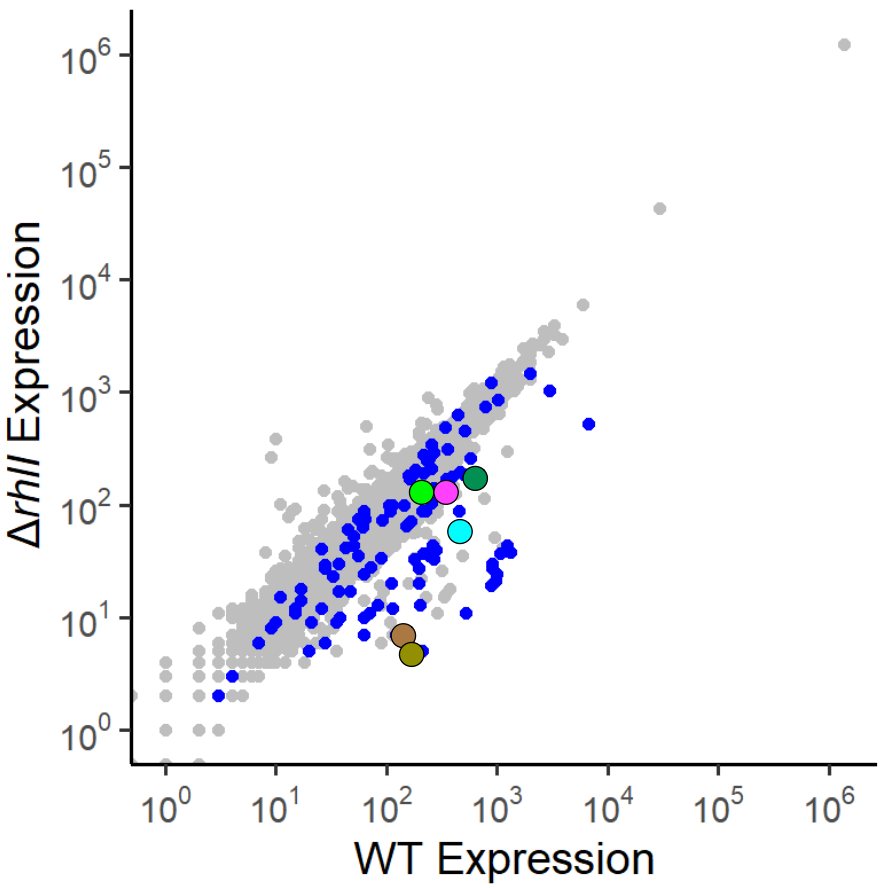

Δrhll Enrichment
WT Enrichment
10^0
10^1
10^2
10^3
10^4
10^5

B


Δrhll Expression
WT Expression
10^0
10^1
10^2
10^3
10^4
10^5
10^6

C

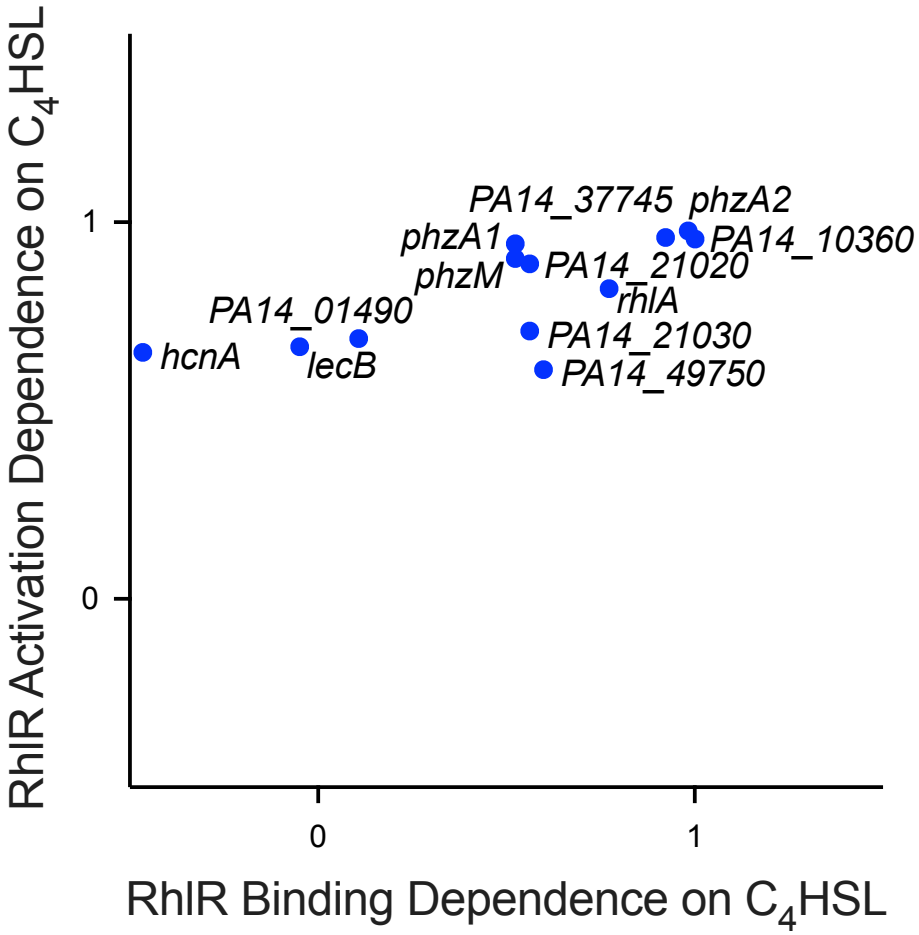
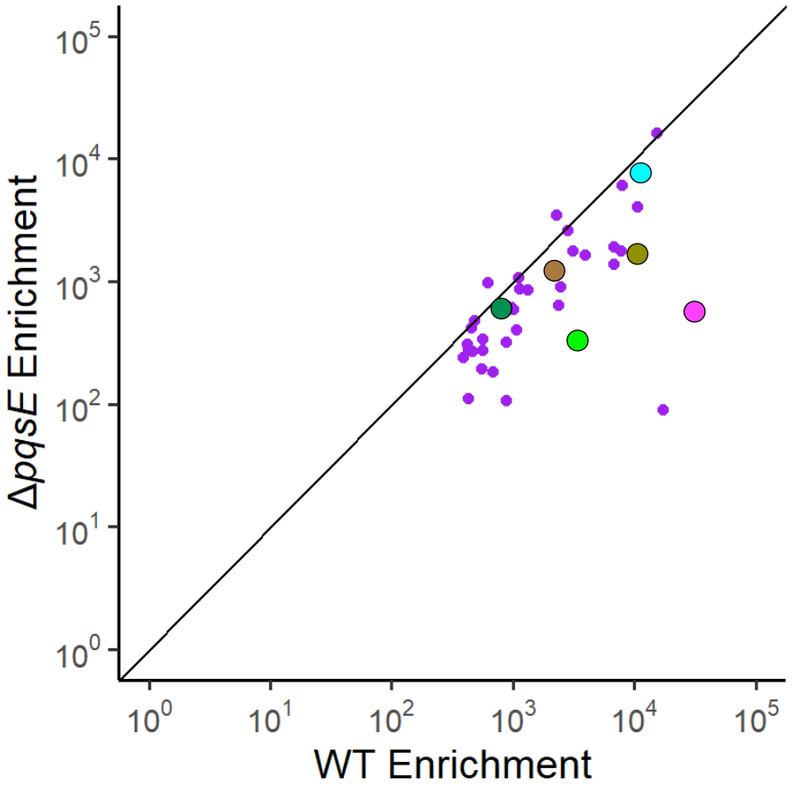
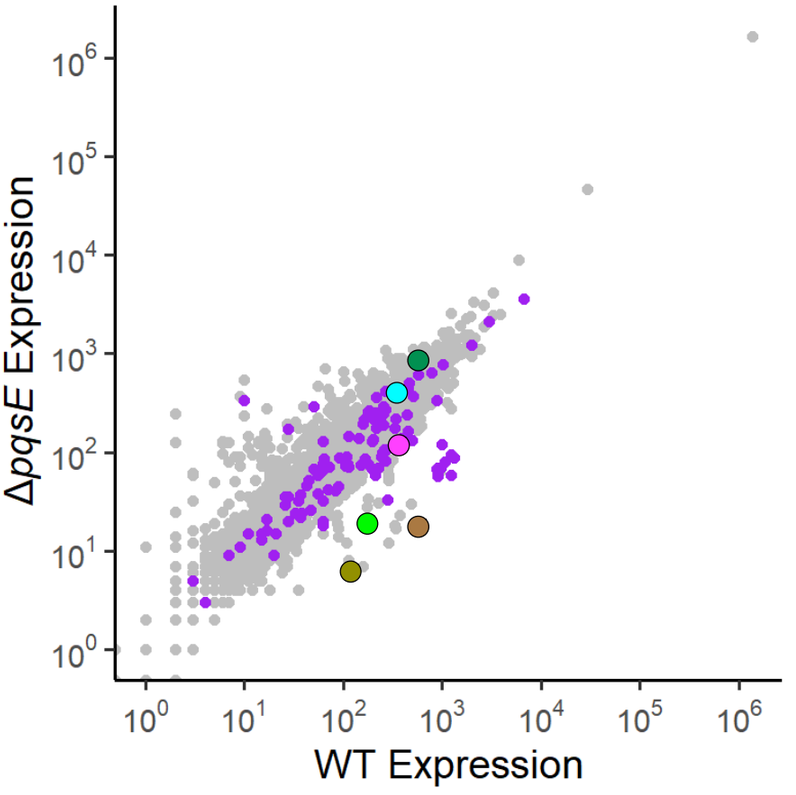

RhlR Activation Dependence on C_4HSL
RhlR Binding Dependence on C_4HSL
0
1
PA14_37745
phzA2
PA14_10360
phzA1
phzM
PA14_21020
rhlA
PA14_01490
PA14_21030
hcnA
lecB
PA14_49750

Figure 4

A


ΔpqsE Enrichment
WT Enrichment
10^0
10^1
10^2
10^3
10^4
10^5

B


ΔpqsE Expression
WT Expression
10^0
10^1
10^2
10^3
10^4
10^5
10^6

C

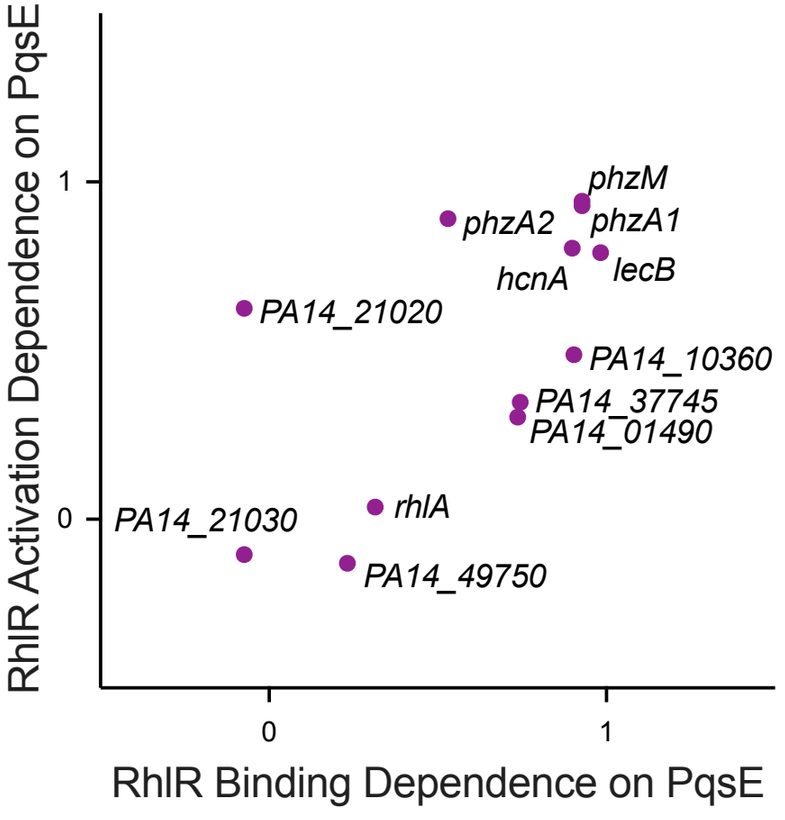
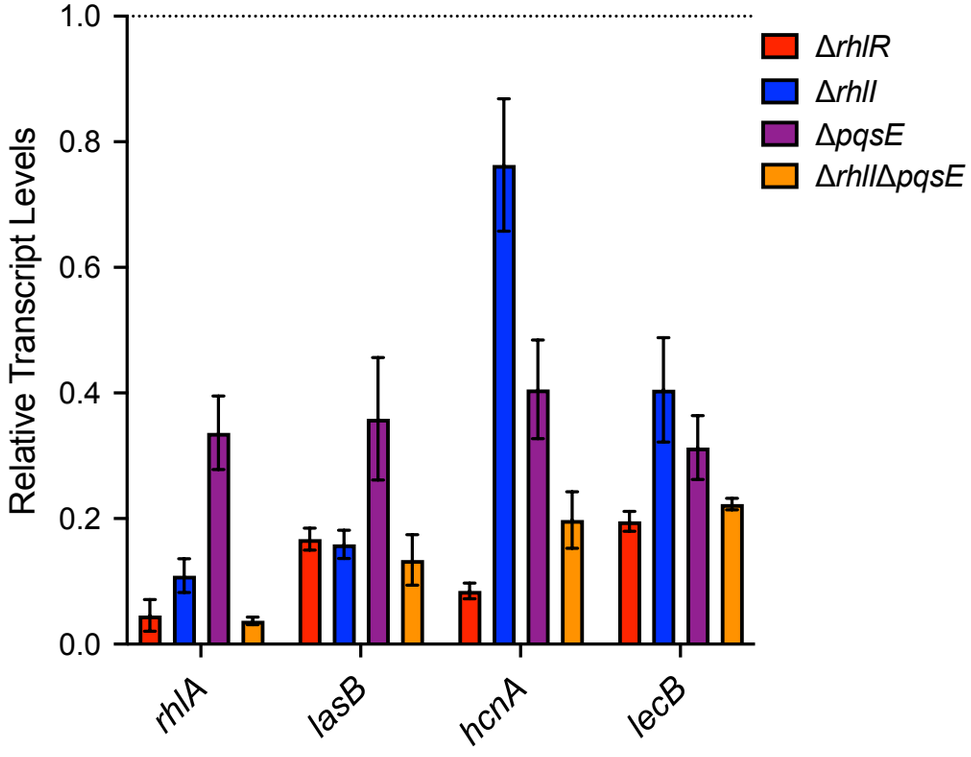

RhlR Activation Dependence on PqsE
RhlR Binding Dependence on PqsE
1
0
0
1
phzM
phzA2
phzA1
hcnA
lecB
PA14_21020
PA14_10360
PA14_37745
PA14_01490
rhlA
PA14_21030
PA14_49750

Figure 5

A


Relative Transcript Levels
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0.8
0.6
0.4
0.2
0.0
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ΔrhlI
ΔpqsE
ΔrhlIΔpqsE
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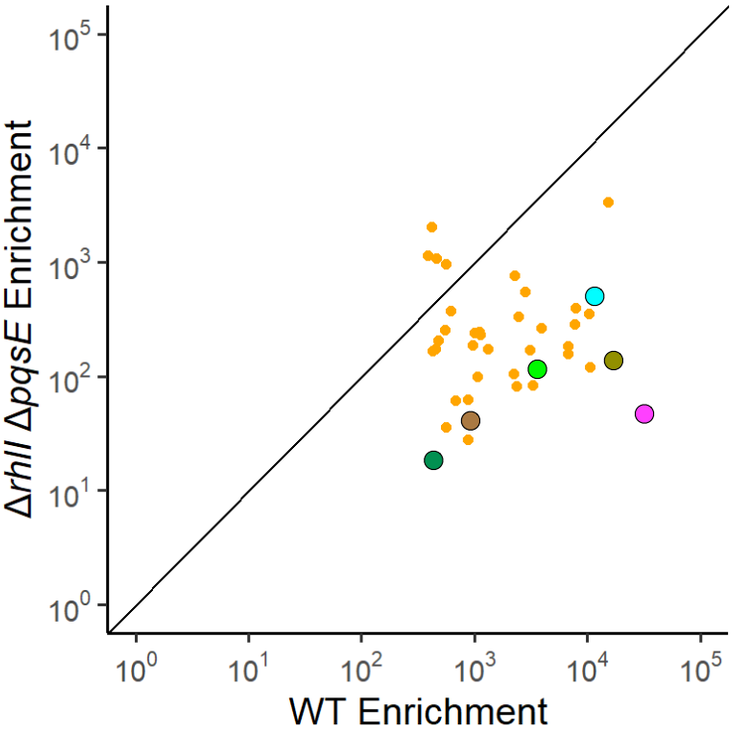
lasB
hcnA
lecB

B


ΔrhlI ΔpqsE Enrichment
WT Enrichment
10^0
10^1
10^2
10^3
10^4
10^5

C

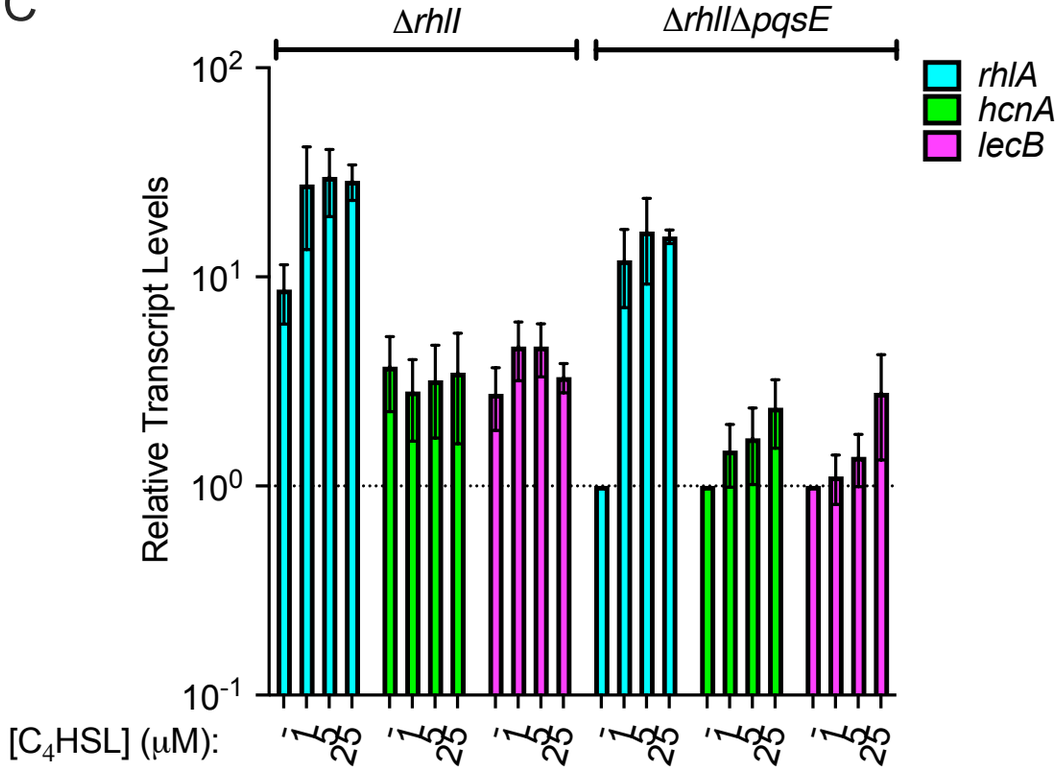
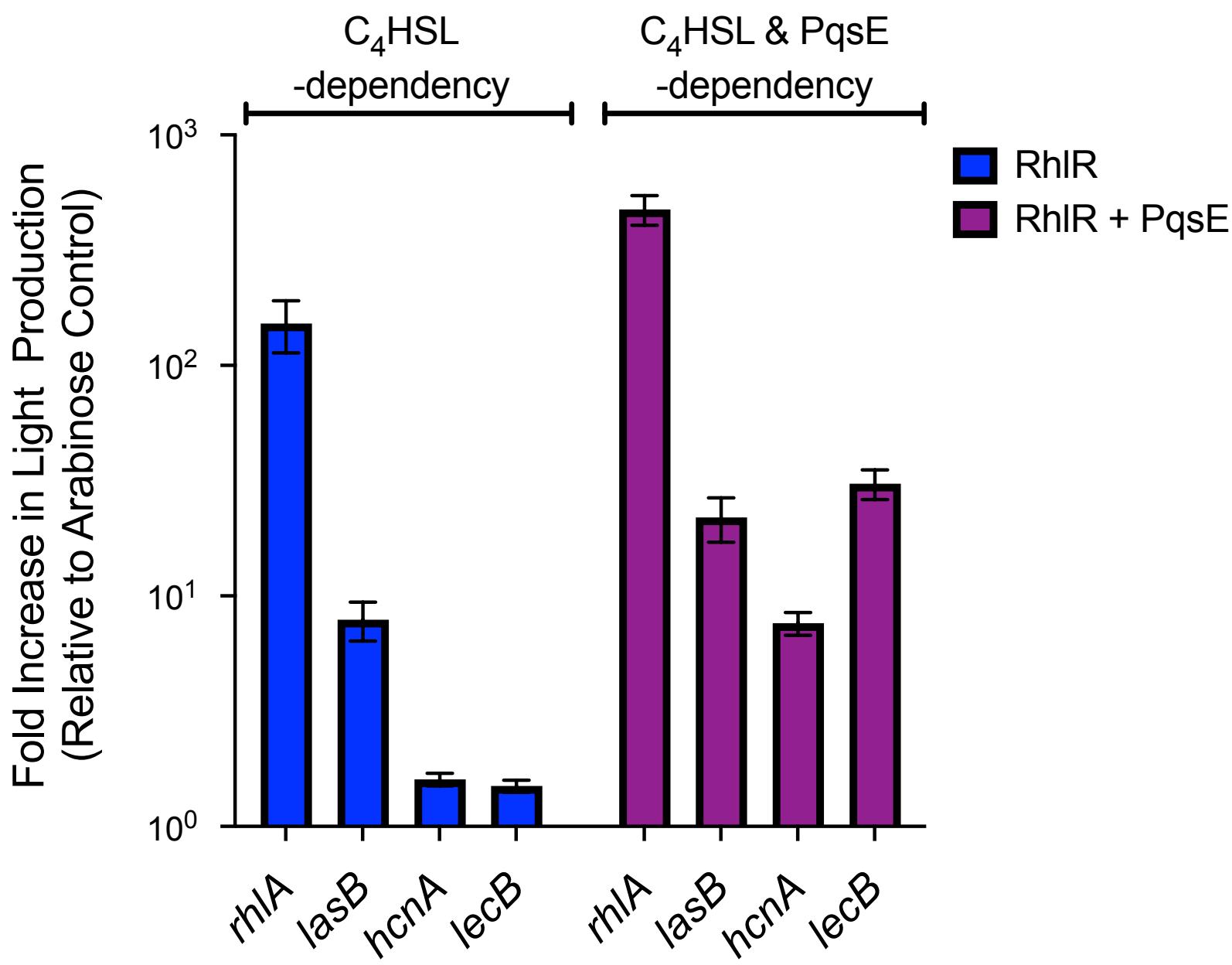

ΔrhlI
ΔrhlIΔpqsE
rhlA
hcnA
lecB
Relative Transcript Levels
10^2
10^1
10^0
10^{-1}
[C_4HSL] (μM):
0
1
5
25

Figure 6

A


C_4HSL
-dependency
C_4HSL & PqsE
-dependency
RhlR
RhlR + PqsE
Fold Increase in Light Production
(Relative to Arabinose Control)
10^3
10^2
10^1
10^0
rhlA
lasB
hcnA
lecB
rhlA
lasB
hcnA
lecB

B

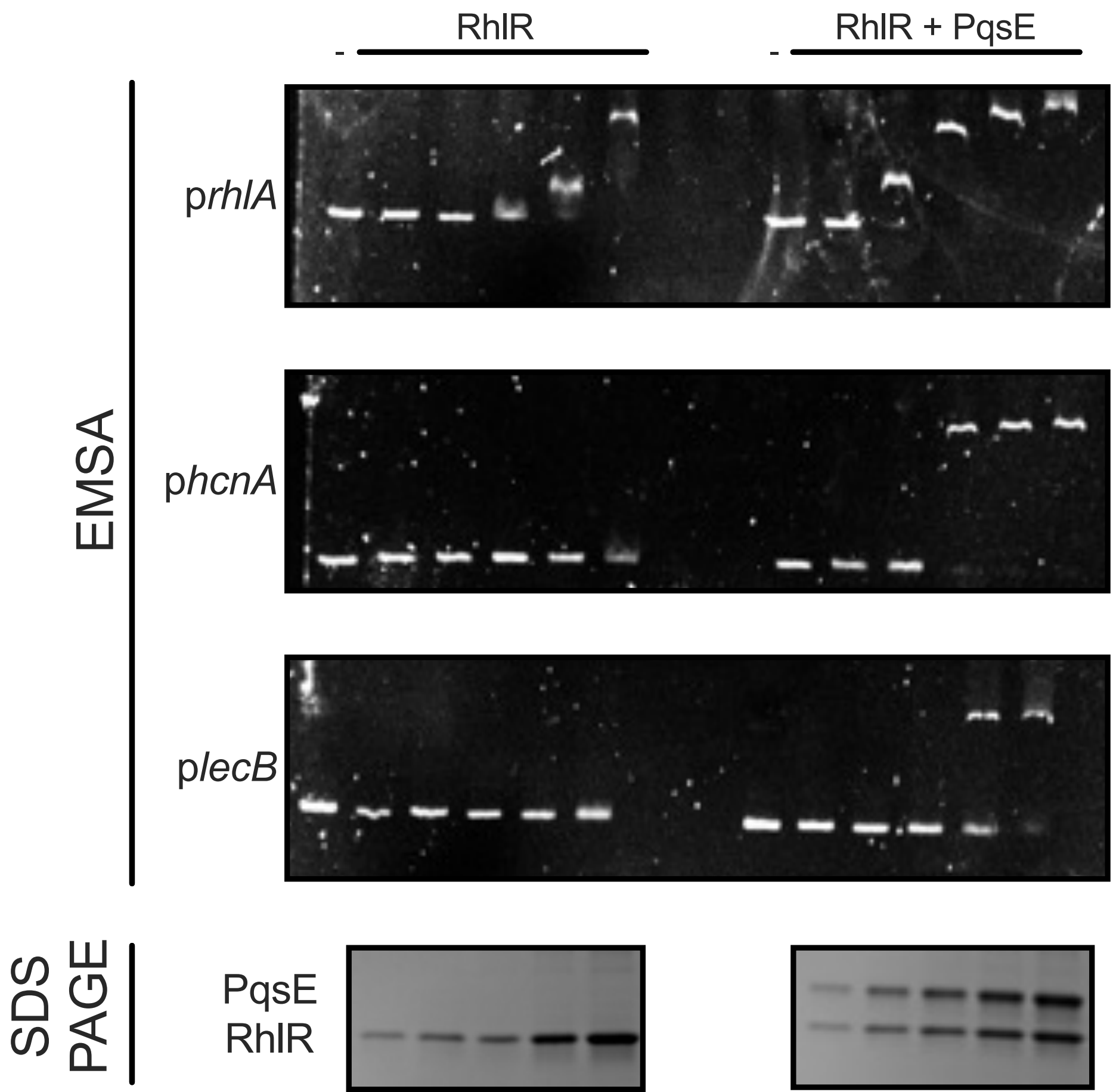
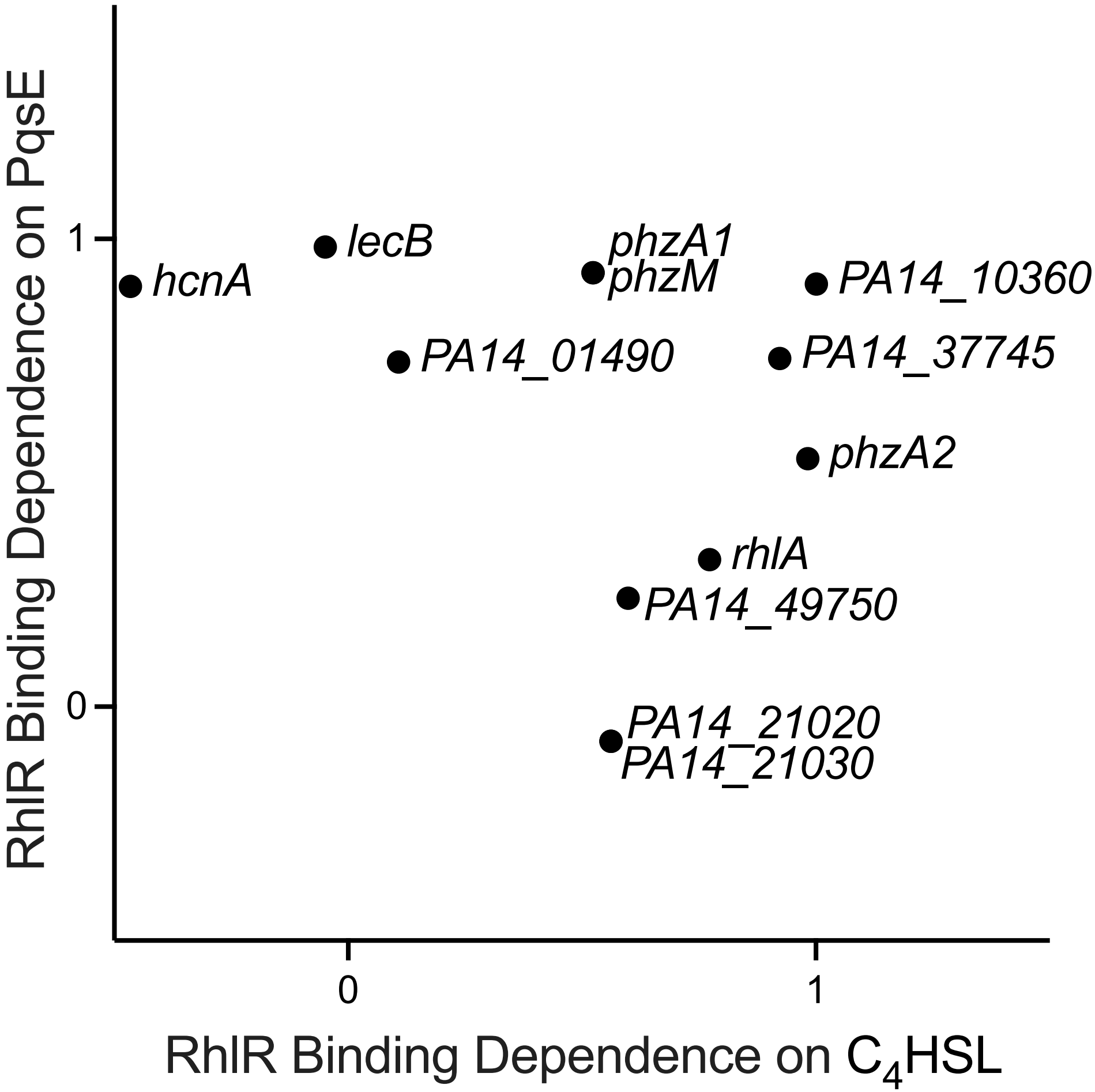

RhlR
RhlR + PqsE
-
-
EMSA
prhlA
phcnA
plecB
SDS
PAGE
PqsE
RhlR

Figure 7


RhlR Binding Dependence on PqsE
1
0
hcnA
lecB
phzA1
phzM
PA14_10360
PA14_01490
PA14_37745
phzA2
rhlA
PA14_49750
PA14_21020
PA14_21030
0
1
RhlR Binding Dependence on C_4HSL