Full title: Non-hierarchical, RhlR-regulated acyl-homoserine lactone quorum sensing in a cystic fibrosis isolate of *Pseudomonas aeruginosa*

Short title: RhlR quorum sensing in a *Pseudomonas aeruginosa* clinical isolate

Renae L. Cruz[1], Kyle L. Asfahl[2], Sara Van den Bossche[3], Tom Coenye[3], Aurélie Crabbé[3], Ajai A. Dandekar[1,2]

Departments of [1]Microbiology and [3]Medicine, University of Washington, Seattle, WA 98117 USA; [3]Laboratory of Pharmaceutical Microbiology, Ghent University, Ghent, Belgium

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Correspondence author:

Ajai A. Dandekar

K-359A HSB, Box 356522

1705 NE Pacific St

Seattle, WA 98195

dandekar@u.washington.edu

1 **ABSTRACT**

2 The opportunistic pathogen *Pseudomonas aeruginosa* is a leading cause of airway infection in

3 cystic fibrosis (CF) patients. *P. aeruginosa* employs several hierarchically arranged and

4 interconnected quorum sensing (QS) regulatory circuits to produce a battery of virulence factors

5 such as elastase, phenazines, and rhamnolipids. The QS transcription factor LasR sits atop this

6 hierarchy, and activates the transcription of dozens of genes, including that encoding the QS

7 regulator RhlR. Paradoxically, inactivating *lasR* mutations are frequently observed in isolates from

8 CF patients with chronic *P. aeruginosa* infections. In contrast, mutations in *rhlR* are rare. We have

9 recently shown that in CF isolates, the QS circuitry is often "rewired" such that RhlR acts in a

10 LasR-independent manner. To begin understanding how QS activity differs in this "rewired"

11 background, we characterized QS activation and RhlR-regulated gene expression in *P.*

12 *aeruginosa* E90, a LasR-null, RhlR-active chronic infection isolate. In this isolate, RhlR activates

13 the expression of 53 genes in response to increasing cell density. The genes regulated by RhlR

14 include several that encode virulence factors. Some, but not all, of these genes are present in the

15 QS regulon described in the well-studied laboratory strain PAO1. We also demonstrate that E90

16 produces virulence factors at similar concentrations to that of PAO1. Unlike PAO1, cytotoxicity by

17 E90 in a three-dimensional lung epithelium cell model is also RhlR-regulated. These data

18 illuminate a "rewired" LasR-independent RhlR regulon in chronic infection isolates and suggest

19 that RhlR may be a target for therapeutic development in chronic infections.

20 **AUTHOR SUMMARY**

21 *Pseudomonas aeruginosa* is a prominent cystic fibrosis (CF) pathogen that uses quorum sensing

22 (QS) to regulate virulence. In laboratory strains, the key QS regulator is LasR. Some isolates from

23 patients with chronic CF infections appear to use an alternate QS circuitry in which another

24 transcriptional regulator, RhlR, mediates QS. We show that a LasR-null CF clinical isolate

25 engages in QS through RhlR and remains capable of inducing cell death in an *in vivo*-like lung

26 epithelium cell model. Our findings support the notion that LasR-null clinical isolates can engage

27 in RhlR QS and highlight the centrality of RhlR gene regulation in chronic *P. aeruginosa* infections.

28 **INTRODUCTION**

29 Many species of bacteria are able to sense and communicate with each other via quorum sensing

30 (QS), a cell-density dependent gene regulation mechanism[1]. In *Proteobacteria*, acyl-

31 homoserine lactones (HSL) are used as QS signals. Commonly, signals are produced by acyl-

32 HSL synthases of the *luxI* family and are recognized by their cognate receptors, transcription

33 factors of the *luxR* family[2].

34

35 *Pseudomonas aeruginosa*, a leading cause of airway infection in cystic fibrosis (CF) patients,

36 uses QS to regulate the production of a wide array of virulence factors including phenazines,

37 rhamnolipids, and hydrogen cyanide[3]. *P. aeruginosa* possesses two complete LuxI/LuxR QS

38 regulatory circuits: LasI/LasR and RhlI/RhlR[4,5]. The signal synthase LasI produces the signal

39 *N*-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL). Above a certain concentration, 3OC12-

40 HSL binds to and facilitates the dimerization of LasR[6]. The LasR homodimer functions as a

41 transcriptional activator promoting the expression of hundreds of genes including *rhlR* and *rhlI,*

42 thereby linking the two acyl-homoserine lactone (AHL) QS regulatory circuits[4,5]. Similarly, RhlI

43 produces the signal *N*-butanoyl-homoserine lactone (C4-HSL), which binds to RhlR, initiating

44 transcription of an additional set of target genes that overlap somewhat with the LasR

45 regulon[1,7]. There is a third, non-AHL QS circuit in *P. aeruginosa* that involves a quinolone signal

46 (*Pseudomonas* quinolone signal; PQS), which activates the transcription factor PqsR[8]. PqsR

47 and RhlR co-regulate the production of some extracellular products[9].

48

49 In laboratory strains of *P. aeruginosa*, deletion or deleterious mutation of *lasR* results in

50 attenuated virulence in various animal models of infection[10,11]. Despite the importance of LasR

51 in regulating virulence, several studies have shown that *lasR* mutations are commonly observed

52 in isolates collected from the lungs of chronically infected CF patients[12–14]. In some patients,

53 the frequency of isolates with a mutant *lasR* has been reported to be greater than 50%[13,15].

3

54   These findings led to the notion that QS is not essential during chronic stages of infection,

55   dampening enthusiasm for QS inhibitors as potential therapeutics. Contrary to this idea, we and

56   others have shown that many LasR-null *P. aeruginosa* chronic infection isolates remain capable

57   of engaging in QS activity through the RhlI/RhlR circuit[16–18]. CF strains appear to "rewire" their

58   QS circuitry so that RhlR is the key transcription factor.

59

60   We are interested in the regulatory remodeling of QS that occurs in isolates of *P. aeruginosa* from

61   chronic infections, including those in CF. To begin to understand how RhlR-mediated QS in

62   clinical isolates might be different from that of laboratory strains[15,16,18,19], we studied a CF

63   isolate called E90[20], which contains a single base-pair deletion in *lasR* at base 170, and uses

64   RhlR to mediate QS. E90 produces QS-regulated virulence factors at levels comparable to that

65   of PAO1. We used RNA-seq to analyze the RhlR regulon of this isolate by comparing its

66   transcriptome with that of an isogenic RhlR deletion mutant. We determined that the E90 RhlR

67   regulon consists of over 83 genes including those that encode virulence factors. Using a three-

68   dimensional tissue culture model, we also observed that E90 induces cell death in a RhlR-

69   dependent manner. Together our data provide a more complete picture of the "rewiring" of QS

70   that can take place in CF-adapted *P. aeruginosa,* while also providing a basis for understanding

71   the gene targets of RhlR without the confounding effects of the QS hierarchy.

72   **RESULTS**

73   **RhlR and C4-HSL-dependent QS activity is conserved in LasR-null isolate E90**

74   We identified isolate E90 from a phenotypic survey of chronic infection isolates collected in the

75   Early *Pseudomonas* Infection Control (EPIC) Observational Study [16]. This isolate, an apparent

76   LasR mutant, still engaged in activities that are putatively QS-regulated such as rhamnolipid,

77   exoprotease, and phenazine production. The *lasR* gene of E90 features a 1 bp deletion at

78   nucleotide position 170, a frameshift mutation which results in a premature stop codon (at residue

79   114). To confirm that this single nucleotide polymorphism encodes a nonfunctional LasR

80   polypeptide, we transformed the strain with a LasR-specific reporter plasmid consisting of *gfp*

81   fused to the promoter region of *lasI,* which encodes the signal synthase and is strongly activated

82   by LasR [21]. GFP fluorescence in E90 transformed with this reporter plasmid was nil and mirrored

83   that of a PAO1Δ*lasR* mutant (Fig. 1A). As a complementary approach, we measured the

84   concentration of 3OC12-HSL produced by E90 using a bioassay. We found that E90 after

85   overnight growth produced very low amounts of 3OC12-HSL (40 nM) compared to PAO1 (1.5

86   µM) and in contrast to PAO1Δ*lasR* for which no 3OC12-HSL was detected (Fig. 1B)*.* The apparent

87   differences in E90 LasR activity reported by the transcriptional reporter assay and the bioassay

88   is likely explained by the greater sensitivity of the latter method. E90 produced approximately 8.3

89   µM C4-HSL after overnight growth, comparable to what we measured for PAO1 (9.8 µM).

90   Altogether, these data confirmed that E90 encodes a non-functional LasR, and suggested that

91   QS in this isolate is regulated by either RhlR, PqsR, or both transcription factors.

92

93   To determine if RhlR QS activity in E90 was AHL-dependent, we examined the expression of

94   several well-studied quorum-regulated genes in the presence or absence of AiiA lactonase, an

95   enzyme that degrades AHL signals[22]. Using qRT-PCR, we observed that expression of *lasB*

96   and *rhlA* were increased in the presence of AHLs (Fig. 2). These genes, which encode the

97   exoprotease elastase and a rhamnosyltransferase involved in rhamnolipid production, were

5

98   identified as QS-regulated in PAO1 [3,23]. *rhlI*, which encodes the C4-HSL synthase, was also

99   AHL-regulated (Fig. 2). Because we had determined that little 3OC12-HSL is produced by E90

100   (Fig. 1), we reasoned that expression of QS-regulated genes likely was dependent on C4-HSL.

101

102   Previous work has shown that RhlR activity can be uncoupled from LasR regulation in LasR-null

103   backgrounds [24–26]. Given that C4-HSL production is robust in E90 (Fig. 1B), we queried if this

104   strain similarly engaged in RhlR-dependent QS activity. To address this question, we engineered

105   a *rhlR* deletion in the E90 background to observe its effect on quorum-regulated phenotypes. We

106   found that the E90Δ*rhlR* deletion mutant displayed nil *rhlA* promoter activity and produced little to

107   no exoprotease and pyocyanin, consistent with the idea that RhlR regulates QS activity in E90

108   (Fig. 3). As a whole, our results showed that the LasR-null isolate E90 retains QS activity in a

109   RhlR- and C4-HSL-dependent manner, and suggested that regulation by RhlR in this strain

110   parallels that of LasR in PAO1. Because RhlR-dependent QS regulation appears to be common

111   in CF isolates[16–18], we reasoned that a study of the genes regulated by RhlR in this background

112   would give insight into which QS-regulated gene products might be important in chronic CF

113   infections. Furthermore, because *rhlR* is not regulated by LasR in E90 (and other clinical isolates),

114   a study of the E90 QS transcriptome has the potential to disentangle genes that are regulated

115   solely by RhlR from those that require both LasR and RhlR.

116

Identification of the RhlR regulon of E90

118   To determine which genes are regulated by RhlR, we performed an RNA-seq-based differential

119   gene (DE) expression analysis comparing RNA collected from cultures of the parent strain E90

120   to the isogenic RhlR deletion mutant. First, we sought to generate a *de novo*-assembled genome

121   for E90 to use as an RNA-seq mapping reference which would account for the potential genomic

122   differences between E90 and reference strains of *P. aeruginosa*. Using a hybrid approach

123   combining both short- and long-read high-throughput sequencing, we were able to assemble the

124   genome of E90 into a single circular contig of approximately 6.8 Mb that harbors 6650 annotated

125   features (Figure 4; 6650 features total, 6503 protein coding sequences). In addition to being

126   roughly 550 kb larger than the published sequence of laboratory strain PAO1 [27], the genome of

127   E90 includes 862 features with no homology to PAO1. Also present is a 4.4 Mb inversion relative

128   to PAO1, which includes an internal reorder of roughly 250 kb. The inversion appears to be the

129   result of a recombination event between two roughly 5 kb repeat regions that do not have

130   homology to PAO1, but flank the *rrnA/rrnB* region previously implicated in restructuring of the *P.*

131   *aeruginosa* genome [27]. A brief search of the E90 genome for *P. aeruginosa* genes previously

132   reported to be under purifying selection in CF isolates revealed a nonsynonymous mutation in the

133   gene coding for the probable oxidoreductase MexS (locus PAE90_2949/PA2491;

134   nonsynonymous SNP), as well as the resistance-nodulation-division multidrug efflux membrane

135   fusion protein precursor MexA (locus PAE90_0464/PA0425; 33bp deletion) [13].

136

137   Next, to facilitate a comparison to previously published QS regulons in our transcriptome analysis

138   [3,28], we grew strains in LB-MOPS to an OD_{600} of 2.0. The growth of E90 and E90Δ*rhlR* are

139   indistinguishable in this medium (S1 Fig.). Our DE analysis identified 53 genes that were

140   upregulated in the E90 vs. E90Δ*rhlR* comparison (Table 1 and S1 Table). Forty-four (83%) of

141   these genes were identified as QS-regulated in a previous microarray study of the PAO1 [3] and

142   21 belong to the core quorum-controlled genome characterized in the laboratory strain PAO1 [28].

Table 1. The 20 most highly RhlR-activated genes in isolate E90.

De novo ID[a]	Gene name[b]	Product description[c]	Fold change
PAE90_1621	*rhlB*	rhamnosyltransferase chain B	5688.8
PAE90_1620	*rhlA*	rhamnosyltransferase chain A	1956.4
PAE90_0833	*phzC1*	phenazine biosynthesis protein PhzC	129.4
PAE90_1777		probable FAD-dependent monooxygenase	64.5
PAE90_1773		conserved hypothetical protein	60.9
PAE90_1775		probable short chain dehydrogenase	52.0
PAE90_3307	*hcnA*	hydrogen cyanide synthase HcnA	35.0

7

PAE90_1771		probable acyl carrier protein	32.6
PAE90_3647		probable acyl carrier protein	32.1
PAE90_1364	*lasB*	elastase LasB	32.1
PAE90_0834	*phzB1*	probable phenazine biosynthesis protein	30.9
PAE90_0835	*phzA1*	probable phenazine biosynthesis protein	29.0
PAE90_1778		probable non-ribosomal peptide synthetase	28.3
PAE90_1772	*fabH2*	3-oxoacyl-[acyl-carrier-protein] synthase III	23.5
PAE90_1776		hypothetical protein	20.4
PAE90_2705		**hypothetical protein**	**15.5**
PAE90_0837		**hypothetical protein**	**14.7**
PAE90_2723	***vqsR***	**VqsR**	**14.4**
PAE90_1770		hypothetical protein	13.2
PAE90_0133		**hypothetical protein**	**12.2**

a. "PAE90" identification numbers correspond to locus tags in the E90 *de novo* genome.

b. Gene names from PAO1-UW reference annotation (PAO1_107; see Materials and Methods) available on the Pseudomonas Genome Database (https://www.pseudomonas.com).

c. Product descriptions from PAO1-UW reference annotation, with the exception of those genes not present in the PAO1 genome (see Materials and Methods) which are described as annotated in the *de novo* genome.

Bold denotes genes not previously identified as QS-regulated (Schuster et al., 2003).

143

144 We also identified several well-known virulence genes including those that encode biosynthetic

145 machinery required for rhamnolipid (*rhlAB*), hydrogen cyanide (HCN; *hcnABC*), elastase (*lasB*),

146 and pyocyanin synthesis (*phzABC1*). Elastase is an exoprotease known to degrade various

147 components of the innate and adaptive immune system including surfactant proteins A and D

148 [29,30]. Rhamnolipid and pyocyanin have also been previously appreciated for their roles in

149 airway epithelium infiltration and damage [31,32]. In addition, our RNA-seq analysis revealed

150 *hsiA2,* the first gene in the cluster encoding the Second Type VI Secretion System, which

151 facilitates the uptake of *P. aeruginosa* by lung epithelial cells [33].

152

153 While QS control of the phenazine biosynthesis pathway has been reported previously, only one

154 of the two "redundant" operons ("*phz1*"; *phzA1-G1*) was indicated [3]. Interestingly, our

155 transcriptome analysis found that RhlR also regulates the first two genes of the second phenazine

156 operon ("*phz2*"; *phzA2-G2*) in E90, albeit at a slightly lower level than *phz1*. Both operons encode

157 nearly identical sets of proteins, each with the capacity to synthesize the precursor (phenazine-

158 1-carboxylic acid) of many downstream phenazine derivatives, including the virulence factor

159 pyocyanin [34]. Despite their seemingly redundant function, *phz1* and *phz2* do not appear to be

160 regulated in concert. In strain PA14, although *phz1* is more highly expressed than *phz2* in liquid

161 culture, similar to what we observed in the E90 RhlR regulon, *phz2* actually contributes more to

162 overall phenazine production in liquid culture [35]. Furthermore, *phz2* is the only active *phz* operon

163 in colony biofilms, and was the only *phz* operon implicated in lung colonization in a murine model

164 of infection [35].

165

166 Moreover, we observed that the RhlR regulon included genes that likely confer a growth

167 advantage in the CF lung. For example, *cbpD* encodes a chitin-binding protein shown to

168 contribute to the thickness of biofilms, the development of which is important for nutrient

169 acquisition and stress resistance [36]. The gene encoding the monodechloroaminopyrrolnitrin 3-

170 halogenase PrnC was also present in the E90 RhlR regulon, which has not been reported in

171 previous *P. aeruginosa* transcriptomes and is not present in the PAO1 reference genome.

172 Halogenase PrnC has only previously been described in *P. protegens* (formerly *P. fluorescens*),

173 where it is involved in the synthesis of pyrrolnitrin, an antifungal antibiotic [37].

174

175 Among the most highly regulated genes [3,28] were those belonging to a conserved nonribosomal

176 peptide synthetase (NRPS) pathway (PaE90_1770-1779; PA3327-3336). The products of this

177 NRPS pathway have been identified as azetidine-containing alkaloids referred to as

178 azetidomonamides[38]. The biological significance of this widely conserved NRPS pathway in

9

179   *Pseudomonas* species or what roles azetidomonamides may play in virulence or interspecies

180   interaction is not well understood, but regulation by QS appears to be a common feature.

181

182   Our interrogation of the E90 RhlR regulon also revealed 30 genes that were RhlR-repressed (S2

183   Table); none of these genes were reported in previous reports of QS-repressed genes [3] and 19

184   are not present in the PAO1 genome. We found two genes of the *alpBCDE* lysis cassette, *alpB*

185   and *alpC*, were repressed by RhlR in E90 under the conditions of our experiments. While induction

186   of *alpBCDE*, via de-repression of the *alpA* gene, has been shown to be lethal to individual cells,

187   it may benefit infecting cells at the population level [39]. We also observed down-regulation of the

188   gene encoding the posttranscriptional regulatory protein RsmA by RhlR in E90. RsmA is nested

189   in a host of regulatory machinery important in infection, and mutation of RsmA has been observed

190   to favor chronic persistence and increased inflammation in a murine model of lung infection [40].

191   Lastly, we identified RhlR regulation of phage loci not found in the PAO1 genome. The RhlR-

192   repressed phage loci correspond to E90 genes PaE90_2433 through PaE90_2442.

193

194   **RhlR is the primary driver of cytotoxicity in a lung epithelium model**

195   LasR-null laboratory strains are less virulent than the WT in acute infection settings [10,11,41].

196   However, as the RhlR-dependent QS regulon of E90 includes several factors implicated in

197   virulence (Table 1), we queried if E90 might be capable of inducing host cell death. To address

198   this question, we incubated an *in vivo*-like three-dimensional (3D) lung epithelial cell culture model

199   (A549 cell line) [42] with either PAO1, E90, or engineered QS transcription-factor mutants. The

200   3D lung cell model possesses several advantages over the standard A549 monolayer as an

201   infection model, including increased production of mucins, formation of tight junctions and polarity,

202   decreased expression of carcinoma markers, and physiologically relevant cytokine expression

203   and association of *P. aeruginosa* with the epithelial cells [42,43]. Following an incubation period

204   of 24 hours, we measured cell death of the 3-D cell cultures via cytosolic lactate dehydrogenase

10

205   (LDH) release. Consistent with prior studies, WT PAO1 cytotoxicity is abrogated in a LasR

206   deletion mutant; however, cytotoxicity of a PAO1 RhlR-null mutant is similar to that of the wild-

207   type, because in this assay the secreted products responsible for cytotoxicity are LasR-regulated

208   in PAO1, with little or no contribution from RhlR. Strikingly, the opposite was true for E90: deletion

209   of RhlR significantly reduces cytotoxicity (Fig. 5). This RhlR-dependent cytotoxicity might be

210   related to the different timing of RhlR activation in E90, the specific set of genes regulated by

211   RhlR in this strain, or both. Together, these results highlight the restructuring of QS gene

212   regulation in this clinical isolate and underscore implications for virulence during chronic infection.

213 **DISCUSSION**

214 A substantial body of literature now suggests that the QS hierarchy of *P. aeruginosa* is adaptable

215 and that LasR mutants can be "rewired" to be AHL QS proficient [16,25,26,44]. These "rewired"

216 LasR-null clinical isolates retain the QS regulation of several exoproducts through the RhlI/RhlR

217 circuit [16,17]. Prior studies examining a RhlR-dependent variant of PAO1 [24], and another LasR-

218 null and RhlR-active clinical isolate[17], show that the parent strain outcompetes RhlR-null

219 derivatives when grown in co-culture [17,24]. These findings support the notion that there is

220 something inherently disadvantageous about mutation of RhlR and point to RhlR as a key QS

221 transcription factor in chronic infections like CF.

222

223 We do not know the mechanism or genetic modifications that resulted in Las- independent RhlR

224 activity in isolate E90. In strain PAO1, in which the hierarchy of QS was initially described, LasR

225 mutants can readily evolve an independent RhlR QS system through inactivating mutations of

226 *mexT*, which encodes a non-QS transcriptional regulator [24,26]. However, this is not the case in

227 isolate E90, which possesses a functional *mexT* allele. We did observe that *rhlI* expression is

228 upregulated by RhlR in E90 unlike in PAO1, where *rhlI* expression is predominately LasR-

229 regulated [45]. These data suggest that in E90 RhlR and RhlI may constitute a positive

230 autoregulatory loop that may facilitate Las-independent RhlR activity. We are interested in

231 investigating alternate mechanisms, other than inactivation of *mexT,* through which RhlR escapes

232 LasR regulation in these "rewired" backgrounds.

233

234 In the present study, we aimed to identify which genes comprise the RhlR regulon in a clinical

235 isolate, which may shed light on factors important for establishment or continuation of a chronic

236 infection. Our RNA-seq analysis revealed that the E90 RhlR regulon bears a substantial amount

237 of overlap with the suite of AHL-regulated genes previously identified in PAO1[3] and consists of

238 virulence factors that are likely advantageous in the context of the CF lung.

12

239

240   A portion of the genes found to be RhlR-regulated in our transcriptomic analysis of E90 were not

241   previously reported to be QS-regulated. These genes include *vqsR* (PAE90_2723/PA2591) and

242   two genes of the *phz2* operon (PAE90_3614-3615/PA1899-1900). VqsR is itself a LuxR-homolog

243   that serves to augment QS gene regulation, possibly through activation of the orphan QS receptor

244   QscR, although the precise mechanism and biological outcomes of this interaction are still

245   mysterious [46,47]. Our finding that the *phz2* operon, in addition to *phz1*, is activated by RhlR

246   may reflect ongoing QS adaptation in our selected CF isolate. While E90 appears to produce

247   slightly less bulk pyocyanin in broth culture than PAO1, pyocyanin production by E90 may be

248   comparatively greater in the biofilm lifestyle of the CF lung. The *phz2* locus, while showing roughly

249   98% nucleotide identity with *phz1*, has been shown to be responsible for nearly all the pyocyanin

250   produced in biofilms by PAO1 and is the dominant contributor to murine lung colonization between

251   the two loci [35]. It is possible that some of these previously unreported QS-regulated genes were

252   excluded from earlier transcriptome analyses[3,28] due to different analysis approaches or

253   methodology. Of particular note, we compared a RhlR-deletion mutant to the parent strain to

254   derive our transcriptome, while some of these previous studies used signal-synthase mutants with

255   and without signal, which has been demonstrated, in the case of RhlR QS, to yield a different

256   phenotype [48].

257

258   We also discovered RhlR-QS-regulation of many genes that are not present in the PAO1 genome.

259   This list includes several hypothetical proteins activated as much as 15-fold in E90 compared to

260   the RhlR mutant. The list also includes the gene encoding the halogenase PrnC, a protein

261   involved in production of the antifungal antibiotic pyrrolnitrin which may be important in

262   interspecific interactions in the CF lung [49]. Our finding that RhlR-QS in E90 also appears to

263   repress genes in the programmed cell death cassette *alpBCDE*, points to additional potential for

264   QS regulation of population level interactions in CF-adapted strain E90.

13

265

266 Although we do not yet fully understand the biological significance of the RhlR-mediated

267 suppression of the phage identified in this study, we are interested in exploring its role, if any, in

268 fitness and inter- and intra-species competition in the near future. We note that had we used the

269 PAO1 genome, as opposed to the E90 *de novo* genome, for read alignment, we would have failed

270 to identify the phage loci and a handful of other genes. These findings therefore argue in favor of

271 using *de novo* genomes to improve comprehensive transcriptome analyses of clinical and

272 environmental isolates moving forward.

273

274 Strikingly, we found that in E90, RhlR but not LasR is the critical determinant of cytotoxicity in a

275 three-dimensional lung epithelium cell aggregate model. Though our study did not reveal exactly

276 which virulence factors are important for cell death in this model, our results nevertheless

277 challenge the idea that LasR-null isolates are avirulent. Instead, our data argue that some

278 virulence activity is conserved in "rewired" isolates, but that RhlR is the primary regulator of

279 several such functions.

280

281 The scope of our analysis is limited by our examination of a single clinical isolate and laboratory

282 growth conditions were used for RNA-seq analysis, but our data provide a basis for understanding

283 regulatory remodeling of QS activity and provide avenues for future investigation. Several

284 important questions remain about QS in clinical isolates, including whether or not there is a "core"

285 regulon that is common to isolates that use either LasR or RhlR as the primary QS transcription

286 factor. Our work also serves as a starting point to test hypotheses regarding the role of RhlR-

287 regulated genes during chronic infection, the possible fitness advantage associated with LasR-

288 independent RhlR activity, and mediators of sustainable chronic infections. Our work reveals the

289 potential breadth of QS activity and virulence functions retained in LasR-null, CF-adapted isolates

290   and suggests that the development of anti-QS therapeutics for chronic *P. aeruginosa* infections

291   should be focused on RhlR, not LasR.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in S3 Table in Supporting Information. E90 is part of a collection of clinical isolates obtained via the Early *Pseudomonas* Infection Control Observational (EPIC Obs) Study[20]. The isolates are from oropharyngeal and sputum samples from 5-12 year-old patients. Further details regarding the EPIC Obs study design and results have been described previously [20,50].

For the transcriptional reporter assays as well as pyocyanin and AHL measurements, overnight cultures were started from single colonies grown in 3 mL of Luria-Bertani (LB) broth buffered with 50 mM morpholinopropanesulfonic acid (MOPS) in an 18 mm culture tube. For the cytotoxicity experiments, overnight cultures were started from single colonies in 5 mL of unbuffered LB broth. When appropriate, antibiotics were added at the following concentrations: 10 µg/mL gentamicin or 100 µg/mL ampicillin for *Escherichia coli*, and 100 µg/mL gentamicin for *P. aeruginosa*. Cells were grown at 37˚C with shaking at 250 RPM unless stated otherwise.

LasR and RhlR activity. LasR and RhlR-specific promoter fusions constructed in pPROBE-GT have been described previously [16] and are listed in S3 Table. Electrocompetent *P. aeruginosa* cells were prepared through repeated washing and resuspension of cell pellets in 300 mM sucrose[51]. Transformants were obtained by plating on LB agar supplemented with gentamicin and verified by PCR.

Experimental cultures were prepared as follows: first, overnight cultures were grown with the addition of 100 µg/mL gentamicin and 100 µg/mL AiiA lactonase, the latter inhibiting AHL-mediated QS[22]. The addition of AiiA lactonase eliminates residual GFP fluorescence that would otherwise arise from previously induced reporter gene expression during overnight growth. Overnight cultures were then diluted to an optical density (OD$_{600}$, 1 cm pathlength) of 0.001

16

318   (approximately 1-5 x10^6 CFU/mL) in 3 mL MOPS-buffered LB supplemented with AiiA lactonase

319   in 18 mm culture tubes. After these cultures grew to an approximate OD_{600} of 0.2, they were

320   diluted to OD_{600} 0.001 in 400 µL of MOPS-buffered LB alone in a 48-well plate with a clear bottom

321   (Greiner Bio-One). To prevent evaporation, strains were only grown in wells that did not line the

322   edges of the plate and all empty wells were filled with 400 µL water. We monitored GFP

323   fluorescence and OD_{600} at 30-minute intervals for 15 hours using a BioTek Synergy HI microplate

324   reader (excitation: 489 nm, emission: 520 nm, gain: 80). All strains were grown at 37˚C with

325   shaking for the duration of the assay. To account for differences in growth, results were

326   normalized to OD_{600}. As a negative control, each strain was electroporated with an empty vector,

327   which was used to establish a baseline level of background fluorescence. The fluorescence

328   intensity was calculated by subtracting the background fluorescence from the total fluorescence

329   measured at every time point. All experiments were performed in biological triplicate.

330

Construction of the E90Δ*rhlR* mutant

331

332   A homologous recombination approach was used to generate an in-frame deletion mutant[52,53].

333   Fragments flanking *rhlR* were PCR-amplified from E90 genomic DNA and cloned into pEXG2 to

334   yield pEXG2.E90Δ*rhlR*, which was then transformed into *E. coli* S17-1 in order to facilitate

335   conjugal transfer of pEXG2.E90Δ*rhlR* into E90. Transconjugants were selected by plating on

336   *Pseudomonas* isolation agar supplemented with gentamicin, and deletion mutants were counter-

337   selected by plating onto LB agar with 10% (wt/vol) sucrose. Deletion of *rhlR* was confirmed by

338   PCR and targeted sequencing.

339

AHL signal extraction and measurement

340

341   Experimental cultures were prepared from overnight cultures diluted to OD_{600} 0.001 in 3 mL of

342   MOPS-buffered LB in an 18 mm culture tube. Experimental cultures were grown with shaking until

343   they reached OD_{600} 2.0. Then, AHL signals were extracted from experimental cultures using

17

344   acidified ethyl acetate as described elsewhere[54]. We used an *E. coli* DH5α strain containing

345   either pJN105L and pSC11 in conjunction with the Tropix® Galacto-Light™ chemiluminescent

346   assay (Applied Biosystems) to measure 3OC12-HSL, or containing pECP65.1 to measure C4-

347   HSL[23,55,56]. The bioassay strains and plasmids are listed in S3 Table in the Supporting

348   Information.

349

350   **Protease and pyocyanin measurements**

351   Experimental cultures were prepared from overnight cultures by diluting to OD_{600} 0.001 in 3 mL

352   MOPS-buffered LB in 18 mm culture tubes. For secreted protease measurements, experimental

353   cultures were grown with shaking to OD_{600} 2.0. Then, cells were pelleted and 100 μL of the

354   supernatant was collected to measure protease production using the FITC-Casein for Pierce

355   Fluorescent Protease Assay Kit (ThermoFisher Scientific). For pyocyanin measurements,

356   experimental cultures were grown with shaking for 18 h and pyocyanin was extracted from

357   cultures as described previously[16]. We grew strains in MOPS-buffered LB to remain consistent

358   with the growth conditions used for RNA-seq analysis.

359

360   **Cytotoxicity of three-dimensional A549 cell cultures**

361   A three-dimensional lung epithelial cell culture model was generated by culturing A549 cells

362   (ATCC CCL-185) on porous microcarrier beads in a rotating well vessel (RWV) bioreactor system,

363   as described previously [42]. A549 cells were grown in GTSF-2 medium (GE Healthcare)

364   supplemented with 2.5 mg/L insulin transferrin selenite (ITS) (Sigma-Aldrich), 1.5 g/L sodium

365   bicarbonate, and 10% heat-inactivated FBS (Invitrogen), and incubated at 37°C under 5% CO_2,

366   >80% humidity conditions. Infection studies were performed on cultures grown for 11 to 14 days

367   in the RWV. Thereafter, 3-D cell cultures were equally distributed in a 48-well plate at a

368   concentration of 2.5×10^5 cells/well (250 μL volume), and infected with the different strains at a

369   targeted multiplicity of infection of 30:1 as described previously[43]. All infection studies were

370 performed in the above-described cell culture medium, with the exception that no FBS was added

371 given the interference of serum compounds with QS signaling [57]. After 24 h infection, the release

372 of cytosolic lactate dehydrogenase (LDH) from 3-D lung epithelial cell cultures was determined

373 using a LDH activity assay kit (Sigma-Aldrich) according to the manufacturer's instructions. A

374 standard curve using NADH was included. The positive control (theoretical 100% LDH release)

375 was obtained by lysing 2.5×10^5 cells with 1% Triton-X100. All LDH release values were

376 expressed as a percentage of the positive control.

377

378 **RNA isolation and qRT-PCR**

379 Overnight cultures were started from single colonies grown in 3 mL of MOPS-buffered LB in 18

380 mm culture tubes. Experimental cultures were prepared by diluting overnight cultures to OD_{600}

381 0.01 in 25 mL of MOPS-buffered LB in 125-mL baffled flasks. Experimental cultures were grown

382 at 37˚C with shaking at 250 RPM. Approximately 1×10^9 cells were pelleted at OD_{600} 2.0 and

383 mixed with RNA Protect Bacteria reagent (Qiagen) before being stored at -80˚C. Thawed cell

384 pellets were resuspended in QIAzol reagent and mechanically lysed by bead beating. To extract

385 RNA, we used the RNeasy kit (Qiagen) according to manufacturer's instructions. Isolated RNA

386 was then treated with Turbo DNAse (Ambion) and purified using the MinElute cleanup kit

387 (Qiagen). Three biological replicates were processed for each strain (E90 and E90Δ*rhlR*). Next,

388 cDNA was prepared using the iScript™ cDNA Synthesis Kit (BioRad). Then, expression of target

389 genes was analyzed by following the protocol for the iQ™ SYBR® Green SuperMix (BioRad) on

390 a CFX96 Real-Time PCR cycler. We analyzed expression of the following genes: *lasI, lasB, rhlI,*

391 *rhlR, rhlA, pqsA, chiC,* and *aprA.* We used *rplU* as a reference gene. Primers used for qRT-PCR

392 are listed in S3 Table in Supporting Information.

393

394 **Whole-genome sequencing, RNA-seq, and differential gene expression analysis**

395   We generated the complete circular sequence of E90 using a *de novo* whole-genome sequencing

396   approach. High-molecular weight (HMW) genomic DNA was isolated from overnight E90 liquid

397   culture using the Genomic-tip 20/G kit (Qiagen). Genomic DNA was sequenced separately using

398   the following two approaches. For short reads, genomic DNA was subjected to 300 bp PE

399   sequencing on the Illumina MiSeq platform using TruSeq v3 reagents to yield approximately 20

400   M raw reads which were then groomed using Trimmomatic (v0.36; adapter trimming, paired reads

401   only, Phred score cutoff=15) [58]. For long reads, genomic DNA was prepared into two ligation-

402   mediated (SQK-LSK109, Oxford Nanopore) libraries: one barcoded via PCR (EXP-PBC001) and

403   the other via native barcoding (EXP-NBD114). Libraries were then subjected to sequencing on

404   the Nanopore MinION platform using R9.4.1 pores. Nanopore reads were base-called and de-

405   multiplexed using Guppy (v3.1.5, Oxford Nanopore), further groomed to remove adapters and for

406   quality using Porechop (v0.2.4) [59], and final statistics determined in NanoPack (NanoPlot

407   v1.27.0; NanoQC v0.9.1) [60] (read length N50=12kb; median read quality=Q12.6). All reads were

408   then combined in a hybrid *de novo* assembly approach using the Unicycler pipeline [61], including

409   short-read assembly via SPAdes(v3.13.0)[62], long-read assembly via Racon (v1.4.3), and

410   polishing via Pilon [63], to yield the complete E90 genome. The E90 genome was then annotated

411   using the RAST pipeline [64]. The E90 genome is available via the National Center for

412   Biotechnological Information (NCBI) under BioProject accession PRJNA559863.

413

414   For RNA-seq experiments, cultures were prepared, and RNA was extracted and purified as

415   described above for qRT-PCR with 2 biological replicates per treatment. Genewiz, LLC performed

416   rRNA depletion, library generation, and sequencing for all samples. RNA reads were obtained

417   using the Illumina HiSeq platform with an average of 15.3M 150-bp paired-end raw reads per

418   sample which were then groomed using Trim Galore (v0.4.3;

419   https://github.com/FelixKrueger/TrimGalore). Reads were then aligned against the E90 genome

420   and counted using the Subread/featureCounts suite of command line tools to produce a final

421   count matrix of 4 by 6478 which was then loaded into the R statistical environment [65,66].

422   Differential expression (DE) analysis was performed using DESeq2 using a fold-change cut-off of

423   2 and an adjusted p=0.05 [67]. The raw reads and count matrix associated with this transcriptome

424   analysis have been deposited in the of the NCBI Sequence Read Archive under BioProject

425   accession PRJNA559863.

426

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REFERENCES

436

437   1.   Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev

438   Cell Dev Biol. 2005;21: 319–346. doi:10.1146/annurev.cellbio.21.012704.131001

439   2.   Case RJ, Labbate M, Kjelleberg S. AHL-driven quorum-sensing circuits: their frequency

440   and function among the Proteobacteria. ISME J. 2008;2: 345–349.

441   doi:10.1038/ismej.2008.13

442   3.   Schuster M, Lostroh CP, Ogi T, Greenberg EP. Identification, timing, and signal specificity

443   of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J

444   Bacteriol. 2003;185: 2066–2079. doi:10.1128/JB.185.7.2066

445   4.   Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A. A hierarchical quorum-sensing

446   cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR

447   (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol Microbiol. 1996;21:

448   1137–46.

449   5.   Pesci EC, Pearson JP, Seed PC, Iglewski BH. Regulation of *las* and *rhl* quorum sensing in

450   *Pseudomonas aeruginosa*. J Bacteriol. 1997;179: 3127–32.

451   6.   Kiratisin P, Tucker KD, Passador L. LasR, a transcriptional activator of *Pseudomonas*

452   *aeruginosa* virulence genes, functions as a multimer. J Bacteriol. 2002;184: 4912–9.

453   7.   Schuster M, Greenberg EP. A network of networks: quorum-sensing gene regulation in

454   *Pseudomonas aeruginosa*. Int J Med Microbiol. 2006;296: 73–81.

455   doi:10.1016/j.ijmm.2006.01.036

456   8.   Wade DS, Calfee MW, Rocha ER, Ling EA, Engstrom E, Coleman JP, et al. Regulation of

457   *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. J Bacteriol.

458   2005;187: 4372–80. doi:10.1128/JB.187.13.4372-4380.2005

459   9.   Farrow JM, Sund ZM, Ellison ML, Wade DS, Coleman JP, Pesci EC. PqsE functions

460   independently of PqsR-*Pseudomonas* quinolone signal and enhances the *rhl* quorum-

461   sensing system. J Bacteriol. 2008;190: 7043–7051. doi:10.1128/JB.00753-08

462   10.   Rumbaugh KP, Diggle SP, Watters CM, Ross-Gillespie A, Griffin AS, West SA. Quorum

463   sensing and the social evolution of bacterial virulence. Curr Biol. 2009;19: 341–345.

464   doi:10.1016/J.CUB.2009.01.050

465   11.   Tang HB, DiMango E, Bryan R, Gambello M, Iglewski BH, Goldberg JB, et al. Contribution

466   of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a

467   neonatal mouse model of infection. Infect Immun. 1996;64: 37–43.

468   12.   D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Déziel E, Smith EE, et al. Growth

469   phenotypes of *Pseudomonas aeruginosa lasR* mutants adapted to the airways of cystic

470   fibrosis patients. Mol Microbiol. 2007;64: 512–533. doi:10.1111/j.1365-2958.2007.05678.x

471   13.   Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, et al.

472   Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients.

473   Proc Natl Acad Sci U S A. 2006;103: 8487–92. doi:10.1073/pnas.0602138103

474   14.   Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, et al.

475   *Pseudomonas aeruginosa lasR* mutants are associated with cystic fibrosis lung disease

476   progression. J Cyst Fibros. 2009;8: 66–70. doi:10.1016/J.JCF.2008.09.006

477   15.   Wilder CN, Allada G, Schuster M. Instantaneous within-patient diversity of *Pseudomonas*

478   *aeruginosa* quorum-sensing populations from cystic fibrosis lung infections. Infect Immun.

479   2009;77: 5631–9. doi:10.1128/IAI.00755-09

480   16.   Feltner JB, Wolter DJ, Pope CE, Groleau MC, Smalley NE, Greenberg EP, et al. LasR

481   variant cystic fibrosis isolates reveal an adaptable quorum-sensing hierarchy in

482   *Pseudomonas aeruginosa*. MBio. 2016;7. doi:10.1128/mBio.01513-16

483   17.   Chen R, Déziel E, Groleau M-C, Schaefer AL, Greenberg EP. Social cheating in a

484   *Pseudomonas aeruginosa* quorum-sensing variant. Proc Natl Acad Sci U S A. 2019;116:

485   7021–7026. doi:10.1073/pnas.1819801116

486   18.   Bjarnsholt T, Jensen PØ, Jakobsen TH, Phipps R, Nielsen AK, Rybtke MT, et al. Quorum

487   sensing and virulence of *Pseudomonas aeruginosa* during lung infection of cystic fibrosis

488   patients. PLoS One. 2010;5: e10115. doi:10.1371/journal.pone.0010115

489   19.   Wang Y, Gao L, Rao X, Wang J, Yu H, Jiang J, et al. Characterization of *lasR*-deficient

490   clinical isolates of *Pseudomonas aeruginosa*. Sci Rep. 2018;8: 13344.

491   doi:10.1038/s41598-018-30813-y

492   20.   Treggiari MM, Rosenfeld M, Mayer-Hamblett N, Retsch-Bogart G, Gibson RL, Williams J,

493   et al. Early anti-pseudomonal acquisition in young patients with cystic fibrosis: rationale

494   and design of the EPIC clinical trial and observational study. Contemp Clin Trials. 2009;30:

495   256–268. doi:10.1016/j.cct.2009.01.003

496   21.   Seed PC, Passador L, Iglewski BH. Activation of the *Pseudomonas aeruginosa lasI* gene

497   by LasR and the *Pseudomonas* autoinducer PAI: an autoinduction regulatory hierarchy. J

498   Bacteriol. 1995;177: 654–9.

499   22.   Dong Y-H, Xu J-L, Li X-Z, Zhang L-H. AiiA, an enzyme that inactivates the acylhomoserine

500   lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc

501   Natl Acad Sci. 2000;97: 3526–3531. doi:10.1073/pnas.97.7.3526

502   23.   Pearson JP, Pesci EC, Iglewski BH. Roles of *Pseudomonas aeruginosa las* and *rhl*

503   quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J

504   Bacteriol. 1997;179: 5756–67. doi:10.1128/JB.179.18.5756-5767.1997

505   24.   Kostylev M, Kim DY, Smalley NE, Salukhe I, Greenberg EP, Dandekar AA. Evolution of

506   the *Pseudomonas aeruginosa* quorum-sensing hierarchy. Proc Natl Acad Sci U S A.

507   2019;116: 7027–7032. doi:10.1073/pnas.1819796116

508   25.   Dekimpe V, Deziel E. Revisiting the quorum-sensing hierarchy in *Pseudomonas*

509   *aeruginosa*: the transcriptional regulator RhlR regulates LasR-specific factors.

510   Microbiology. 2009;155: 712–723. doi:10.1099/mic.0.022764-0

511   26.   Oshri RD, Zrihen KS, Shner I, Omer Bendori S, Eldar A. Selection for increased quorum-

512   sensing cooperation in *Pseudomonas aeruginosa* through the shut-down of a drug

513   resistance pump. ISME J. 2018;12: 2458–2469. doi:10.1038/s41396-018-0205-y

514   27.   Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete

515   genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature.

516   2000;406: 959–964. doi:10.1038/35023079

517   28.   Chugani S, Kim BS, Phattarasukol S, Brittnacher MJ, Choi SH, Harwood CS, et al. Strain-

518   dependent diversity in the *Pseudomonas aeruginosa* quorum-sensing regulon. Proc Natl

519   Acad Sci U S A. 2012;109: E2823–E2831. doi:10.1073/pnas.1214128109

520   29.   Kuang Z, Hao Y, Walling BE, Jeffries JL, Ohman DE, Lau GW. *Pseudomonas aeruginosa*

521   elastase provides an escape from phagocytosis by degrading the pulmonary surfactant

522   protein-A. PLoS One. 2011;6: e27091. doi:10.1371/journal.pone.0027091

523   30.   Alcorn JF, Wright JR. Degradation of pulmonary surfactant protein D by *Pseudomonas*

524   *aeruginosa* elastase abrogates innate immune function. J Biol Chem. 2004;279: 30871–9.

525   doi:10.1074/jbc.M400796200

526   31.   Zulianello L, Canard C, Kohler T, Caille D, Lacroix J-S, Meda P. Rhamnolipids are virulence

527   factors that promote early infiltration of primary human airway epithelia by *Pseudomonas*

528   *aeruginosa*. Infect Immun. 2006;74: 3134–3147. doi:10.1128/IAI.01772-05

529   32.   Caldwell CC, Chen Y, Goetzmann HS, Hao Y, Borchers MT, Hassett DJ, et al.

530   *Pseudomonas aeruginosa* exotoxin pyocyanin causes cystic fibrosis airway pathogenesis.

531   Am J Pathol. 2009;175: 2473–88. doi:10.2353/ajpath.2009.090166

532   33.   Sana TG, Hachani A, Bucior I, Soscia C, Garvis S, Termine E, et al. The second type VI

533   secretion system of *Pseudomonas aeruginosa* strain PAO1 is regulated by quorum sensing

534   and Fur and modulates internalization in epithelial cells. J Biol Chem. 2012;287: 27095–

535   105. doi:10.1074/jbc.M112.376368

536   34.   Mavrodi D V., Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS. Functional

537   analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from

538   *Pseudomonas   aeruginosa*   PAO1.   J   Bacteriol.   2001;183:   6454–6465.

539   doi:10.1128/JB.183.21.6454-6465.2001

25

35. Recinos DA, Sekedat MD, Hernandez A, Cohen TS, Sakhtah H, Prince AS, et al. Redundant phenazine operons in *Pseudomonas aeruginosa* exhibit environment-dependent expression and differential roles in pathogenicity. Proc Natl Acad Sci U S A. 2012; doi:10.1073/pnas.1213901109

36. Zhang W, Sun J, Ding W, Lin J, Tian R, Lu L, et al. Extracellular matrix-associated proteins form an integral and dynamic system during *Pseudomonas aeruginosa* biofilm development. Front Cell Infect Microbiol. 2015;5: 40. doi:10.3389/fcimb.2015.00040

37. Hammer PE, Hill DS, Lam ST, Van Pée KH, Ligon JM. Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. Appl Environ Microbiol. 1997;63: 2147–54.

38. Hong Z, Bolard A, Giraud C, Prévost S, Genta-Jouve G, Deregnaucourt C, et al. Azetidine-containing alkaloids produced by a quorum-sensing regulated nonribosomal peptide synthetase pathway in *Pseudomonas aeruginosa*. Angew Chemie Int Ed. 2019;58: 3178–3182. doi:10.1002/anie.201809981

39. McFarland KA, Dolben EL, LeRoux M, Kambara TK, Ramsey KM, Kirkpatrick RL, et al. A self-lysis pathway that enhances the virulence of a pathogenic bacterium. Proc Natl Acad Sci U S A. 2015;112: 8433–8438. doi:10.1073/pnas.1506299112

40. Mulcahy H, O'Callaghan J, O'Grady EP, Macia MD, Borrell N, Gomez C, et al. *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation. Infect Immun. 2008;76: 632–638. doi:10.1128/IAI.01132-07

41. Rumbaugh KP, Griswold JA, Iglewski BH, Hamood AN. Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. Infect Immun. 1999;67: 5854–62.

42. Carterson AJ, Höner zu Bentrup K, Ott CM, Clarke MS, Pierson DL, Vanderburg CR, et al. A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture

566   model for *Pseudomonas aeruginosa* pathogenesis. Infect Immun. 2005;73: 1129–40.

567   doi:10.1128/IAI.73.2.1129-1140.2005

568   43. Crabbé A, Liu Y, Matthijs N, Rigole P, De La Fuente-Nùñez C, Davis R, et al. Antimicrobial

569   efficacy against *Pseudomonas aeruginosa* biofilm formation in a three-dimensional lung

570   epithelial model and the influence of fetal bovine serum. Sci Rep. 2017;7: 43321.

571   doi:10.1038/srep43321

572   44. Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J, et al. A cell-cell communication signal

573   integrates quorum sensing and stress response. Nat Chem Biol. 2013;9: 339–343.

574   doi:10.1038/nchembio.1225

575   45. de Kievit TR, Kakai Y, Register JK, Pesci EC, Iglewski BH. Role of the *Pseudomonas*

576   *aeruginosa las* and *rhl* quorum-sensing systems in *rhlI* regulation. FEMS Microbiol Lett.

577   2002;212: 101–6.

578   46. Liang H, Deng X, Ji Q, Sun F, Shen T, He C. The *Pseudomonas aeruginosa* global

579   regulator VqsR directly inhibits QscR to control quorum-sensing and virulence gene

580   expression. J Bacteriol. 2012;194: 3098–108. doi:10.1128/JB.06679-11

581   47. Juhas M, Wiehlmann L, Huber B, Jordan D, Lauber J, Salunkhe P, et al. Global regulation

582   of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa.* Microbiology.

583   2004;150: 831–841. doi:10.1099/mic.0.26906-0

584   48. Mukherjee S, Moustafa D, Smith CD, Goldberg JB, Bassler BL. The RhlR quorum-sensing

585   receptor controls *Pseudomonas aeruginosa* pathogenesis and biofilm development

586   independently of its canonical homoserine lactone autoinducer. PLOS Pathog. 2017;13:

587   e1006504. doi:10.1371/journal.ppat.1006504

588   49. Wynands I, van Pée K-H. A novel halogenase gene from the pentachloropseudilin producer

589   *Actinoplanes* sp. ATCC 33002 and detection of in vitro halogenase activity. FEMS Microbiol

590   Lett. 2004;237: 363–367. doi:10.1111/j.1574-6968.2004.tb09718.x

591   50. Mayer-Hamblett N, Rosenfeld M, Gibson RL, Ramsey BW, Kulasekara HD, Retsch-Bogart

592 GZ, et al. *Pseudomonas aeruginosa in vitro* phenotypes distinguish cystic fibrosis infection

593 stages and outcomes. Am J Respir Crit Care Med. 2014;190: 140617081504001.

594 doi:10.1164/rccm.201404-0681OC

595 51. Choi K-H, Kumar A, Schweizer HP. A 10-min method for preparation of highly

596 electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer

597 between chromosomes and plasmid transformation. J Microbiol Methods. 2006;64: 391–

598 397. doi:10.1016/J.MIMET.2005.06.001

599 52. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. A broad-host-range Flp-

600 FRT recombination system for site-specific excision of chromosomally-located DNA

601 sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants.

602 Gene. 1998;212: 77–86. doi:10.1016/s0378-1119(98)00130-9

603 53. Kostylev M, Otwell AE, Richardson RE, Suzuki Y. Cloning should be simple: *Escherichia*

604 *coli* DH5α-mediated assembly of multiple DNA fragments with short end homologies. PLoS

605 One. 2015;10: e0137466. doi:10.1371/journal.pone.0137466

606 54. Shaw PD, Ping G, Daly SL, Cha C, Cronan JE, Rinehart KL, et al. Detecting and

607 characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography.

608 Proc Natl Acad Sci U S A. 1997;94: 6036–41. doi:10.1073/PNAS.94.12.6036

609 55. Chugani SA, Whiteley M, Lee KM, D'Argenio D, Manoil C, Greenberg EP. QscR, a

610 modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*.

611 Proc Natl Acad Sci U S A. 2001;98: 2752–7. doi:10.1073/pnas.051624298

612 56. Lee J-H, Lequette Y, Greenberg EP. Activity of purified QscR, a *Pseudomonas aeruginosa*

613 orphan quorum-sensing transcription factor. Mol Microbiol. 2006;59: 602–609.

614 doi:10.1111/j.1365-2958.2005.04960.x

615 57. Smith AC, Rice A, Sutton B, Gabrilska R, Wessel AK, Whiteley M, et al. Albumin Inhibits

616 *Pseudomonas aeruginosa* quorum sensing and alters polymicrobial interactions. Infect

617 Immun. 2017;85. doi:10.1128/IAI.00116-17

618   58.   Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data.

619   Bioinformatics. 2014;30: 2114–2120. doi:10.1093/bioinformatics/btu170

620   59.   Wick RR, Judd LM, Gorrie CL, Holt KE. Completing bacterial genome assemblies with

621   multiplex   MinION   sequencing.   Microb   Genomics.   2017;3:   e000132.

622   doi:10.1099/mgen.0.000132

623   60.   De Coster W, D'Hert S, Schultz DT, Cruts M, Van Broeckhoven C. NanoPack: visualizing

624   and   processing   long-read   sequencing   data.   Bioinformatics.   2018;34:   2666–2669.

625   doi:10.1093/bioinformatics/bty149

626   61.   Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies

627   from   short   and   long   sequencing   reads.   PLOS   Comput   Biol.   2017;13:   e1005595.

628   doi:10.1371/journal.pcbi.1005595

629   62.   Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: A

630   new genome assembly algorithm and its applications to single-cell sequencing. J Comput

631   Biol. 2012;19: 455–477. doi:10.1089/cmb.2012.0021

632   63.   Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: An

633   integrated   tool   for   comprehensive   microbial   variant   detection   and   genome   assembly

634   improvement. PLoS One. 2014;9: e112963. doi:10.1371/journal.pone.0112963

635   64.   Antipov D, Hartwick N, Shen M, Raiko M, Lapidus A, Pevzner PA. plasmidSPAdes:

636   assembling   plasmids   from   whole   genome   sequencing   data.   Bioinformatics.   2016;32:

637   btw493. doi:10.1093/bioinformatics/btw493

638   65.   Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server:

639   rapid   annotations   using   subsystems   technology.   BMC   Genomics.   2008;9:   75.

640   doi:10.1186/1471-2164-9-75

641   66.   Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for

642   assigning   sequence   reads   to   genomic   features.   Bioinformatics.   2014;30:   923–930.

643   doi:10.1093/bioinformatics/btt656

644 67. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping

645 by seed-and-vote. Nucleic Acids Res. 2013;41: e108–e108. doi:10.1093/nar/gkt214

646 68. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-

647 seq data with DESeq2. Genome Biol. 2014;15: 550. doi:10.1186/s13059-014-0550-8

648

649 **FIGURE LEGENDS**

650 **Figure 1. LasR activity is absent in E90, a cystic fibrosis-adapted chronic infection isolate.**

651 A) p*lasI-gfp* reporter activity over time (Fluorescence/OD$_{600}$). PAO1, open circles; PAO1Δ*lasR*,

652 blue circles; E90, red triangles. Data from the first three hours are excluded from analysis because

653 cell density measurements were below the limit of detection. B) AHL signal concentrations. Black

654 bars, 3OC12-HSL; grey bars, C4-HSL. The dashed line indicates the limit of detection for the

655 3OC12 and C4-HSL bioassay (10 nM in each case). Both the PAO1Δ*lasR* mutant and E90

656 produce concentrations of 3OC12-HSL and C4-HSL that are significant from PAO1 (p-value <

657 0.05 by t-test). For (A) and (B), means and standard deviation of biological replicates are shown

658 (*n*=3). In some cases, error bars are too small to be seen.

659

660 **Figure 2. In isolate E90, expression of several canonical QS-regulated genes is AHL-**

661 **dependent.** The following target genes were measured in the presence or absence of AiiA

662 lactonase using qRT-PCR: *lasI,* 3OC12-HSL signal synthase; *lasB,* elastase; *rhlI,* C4-HSL signal

663 synthase; *rhlR,* RhlR, *pqsA,* coenzyme A ligase involved in *Pseudomonas* Quinolone Signal

664 synthesis; *chiC,* chitinase; *aprA;* alkaline metalloprotease. The differences in threshold cycle (Δ

665 Ct) are measured relative to the housekeeping gene *rplU*. Fold changes in gene expression (table

666 on right) are reported relative to cultures incubated with AiiA lactonase. Asterisks (*) indicate

667 statistical significance (p < 0.05 by *t* test). Error bars represent the standard deviation for results

668 of three independent experiments.

30

669

670   **Figure 3. RhlR regulates QS in E90.** Production of (A) pyocyanin or (B) protease in either PAO1,

671   E90, PAO1Δ*lasR* or E90Δ*rhlR*. The dashed lines indicate the detection limits for the pyocyanin

672   and protease assays, which are 0.2 µg/mL and 0.008 µg/mL, respectively. C) *rhlA-gfp* reporter

673   activity over time. Data from the first five hours are excluded because cell density measurements

674   were below the limit of detection of the plate reader. Error bars represent the standard deviation

675   for results of three independent experiments. In some cases, error bars are too small to be seen.

676   Both the PAO1Δ*lasR* mutant and E90 produce concentrations of pyocyanin or exoprotease that

677   are significantly different from PAO1 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by t-test).

678

679   **Figure 4. General features of the complete E90 genome.** This circular representation of the

680   E90 genome includes rings indicating the following features described from the outer-most to

681   inner-most rings: annotated features of CDS (blue) or rDNA (grey) on the forward (outer) or

682   reverse (inner) strands; all-by-all blastn hits (red) in a comparison against PAO1_107 (nucleotide

683   identity >40%); GC content deviation (black); GC skew (+, green; -, purple). Additional outer,

684   partial rings indicate the 4.4 Mb inversion (bright red) and the 250 kb reorder (light blue).

685

686   **Figure 5. RhlR regulates cytotoxicity in E90, but not PAO1**. We measured cell lysis (as a

687   percentage of the total lactate dehydrogenase release caused by incubation with a lysis agent) of

688   A549 cells incubated with PAO1, E90, or QS transcription factor mutants of PAO1 or E90.

689   Asterisks (*) indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by t-test). Error

690   bars represent the standard deviation for results of at least three independent experiments.
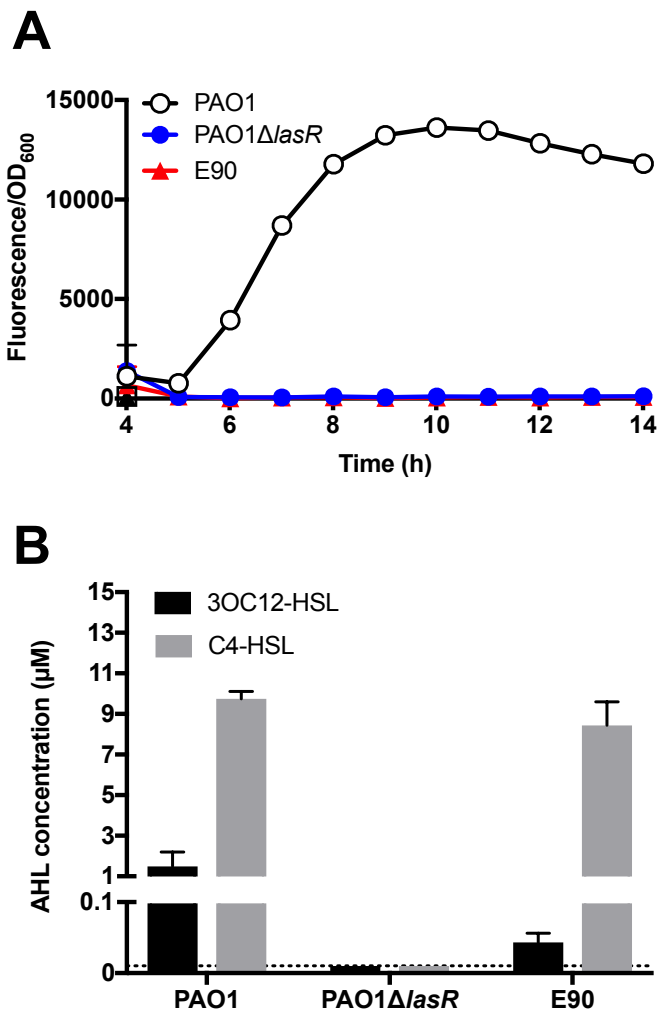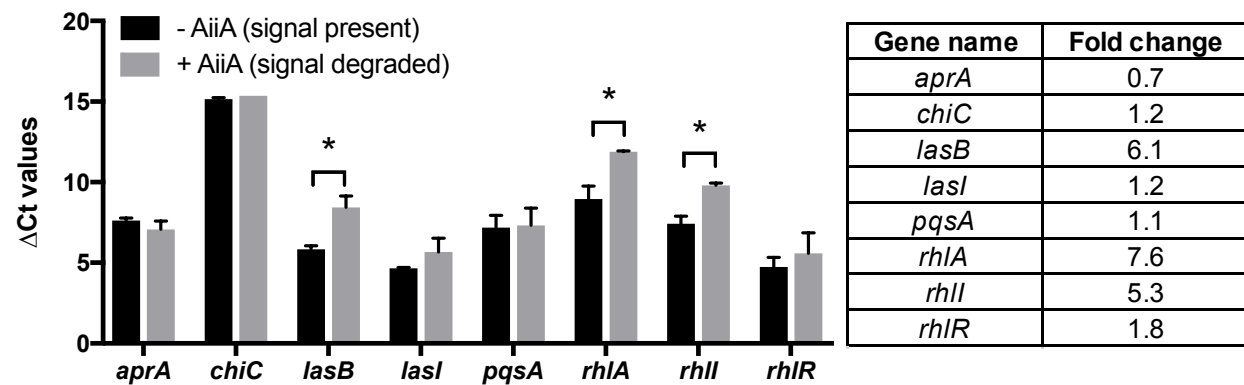
Figure 1

A

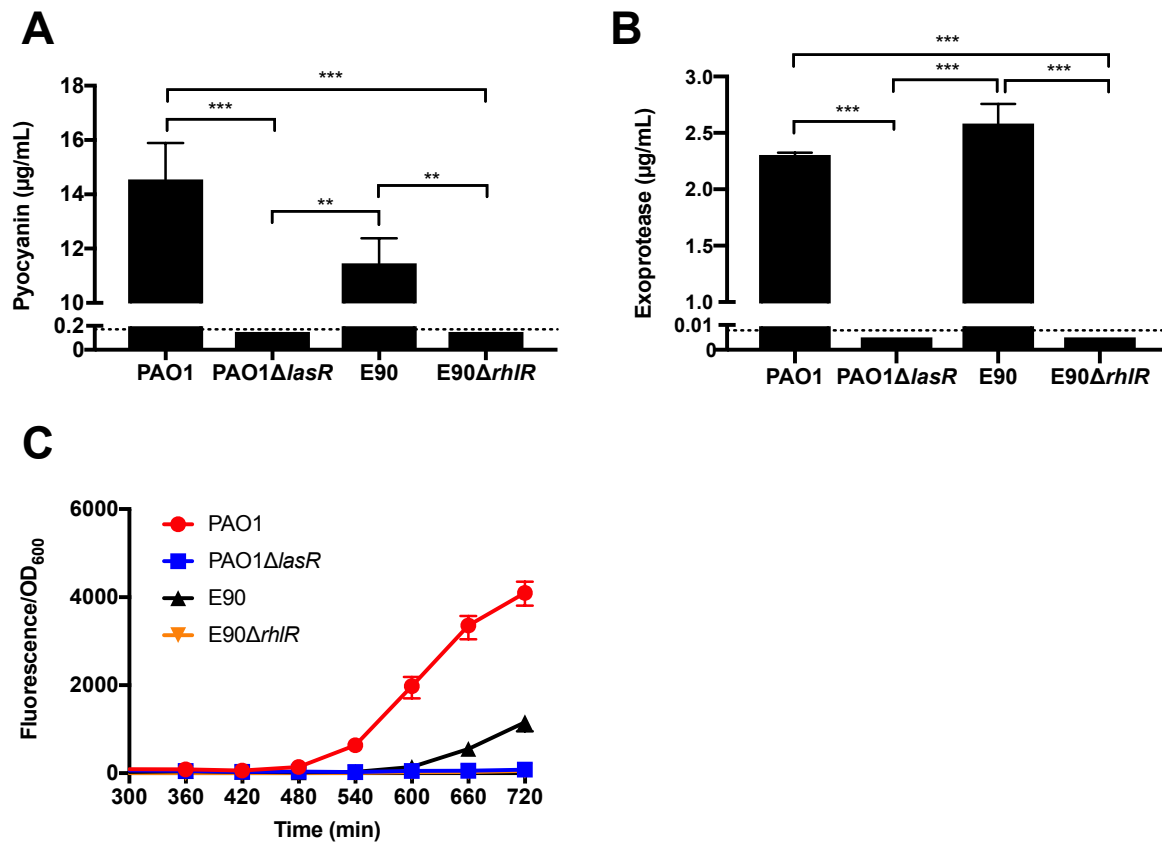**B**

Figure 2

Gene name	Fold change
aprA	0.7
chiC	1.2
lasB	6.1
lasI	1.2
pqsA	1.1
rhlA	7.6
rhlI	5.3
rhlR	1.8

33

Figure 3

Figure 4
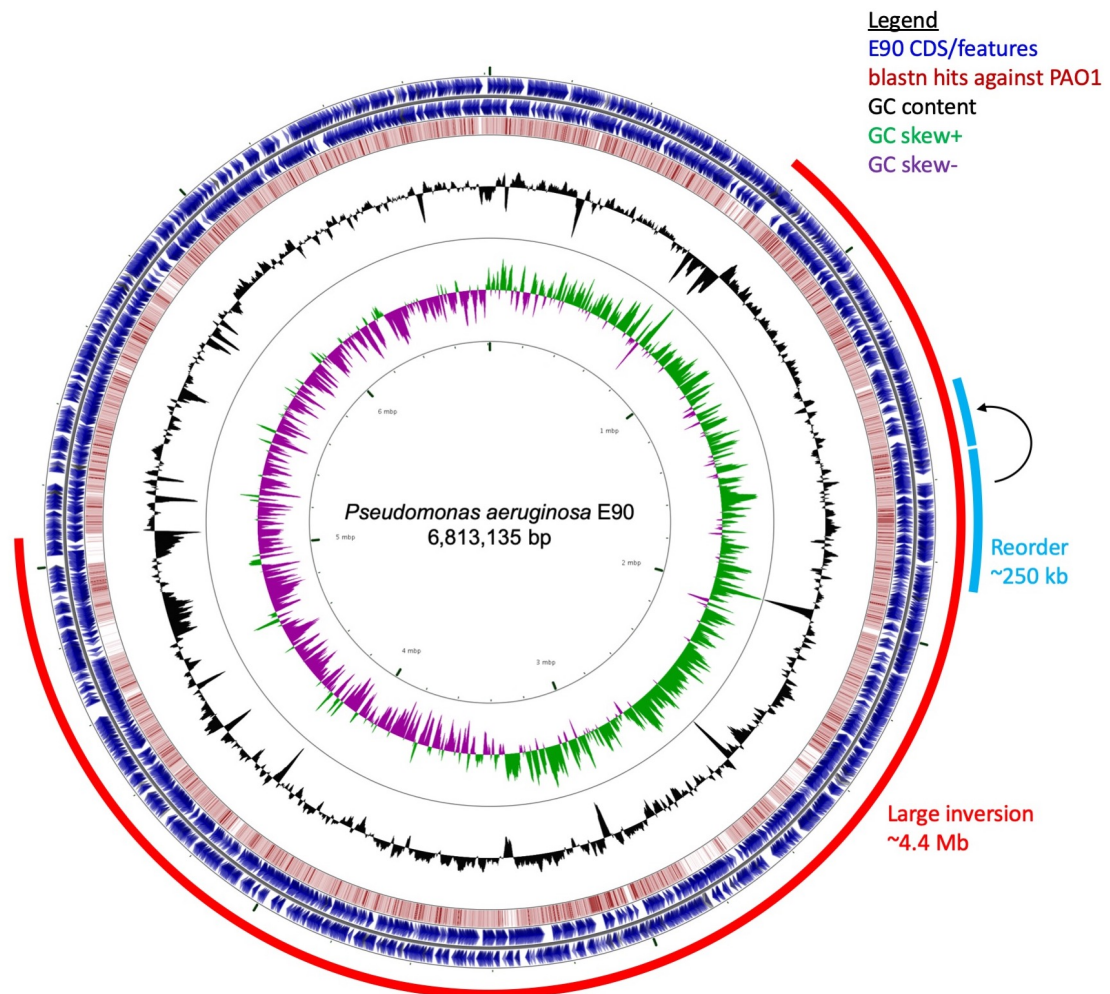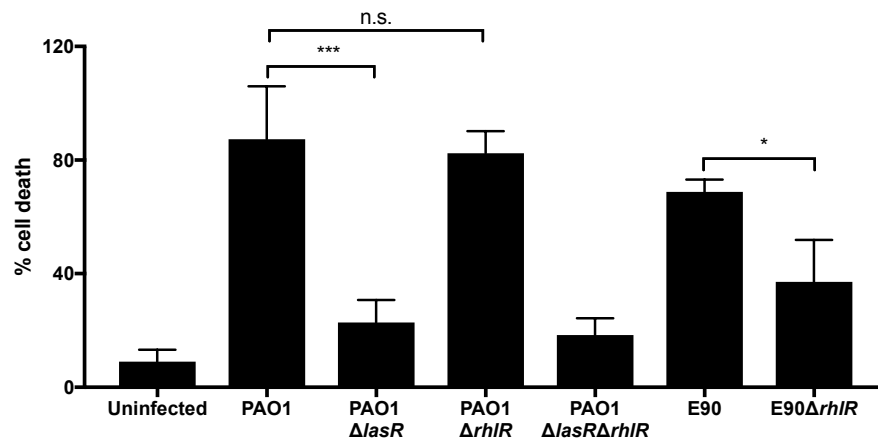
Figure 5

SUPPORTING INFORMATION

S1 Fig. Growth curves of E90 and E90Δ*rhlR* in buffered Luria-Bertani Broth.
Means and standard deviation of biological replicates are shown (n=3). In some cases, error bars are too small to be seen.

S1 Table. Complete list of RhlR-activated genes in strain E90.

S2 Table. Complete list of RhlR-repressed genes in strain E90.

S3 Table. Bacterial strains, plasmids, and primers used in this study.