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1 **The *Pseudomonas aeruginosa* PilSR two-component system regulates both twitching and swimming**
2 **motilities**
3
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8 **Running Title:** PilS-PilR regulates FleSR expression
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ABSTRACT

Motility is an important virulence trait for many bacterial pathogens, allowing them to position themselves in appropriate locations at appropriate times. Motility structures - pili and flagella - are also involved in sensing surface contact, which modulates pathogenicity. In *Pseudomonas aeruginosa,* the PilS-PilR two-component system (TCS) regulates expression of the type IV pilus (T4P) major subunit PilA, while biosynthesis of the single polar flagellum is regulated by a hierarchical system that includes the FleSR TCS. Previous studies in *Geobacter sulfurreducens* and *Dichelobacter nodosus* implicated PilR in regulation of non-T4P-related genes, including some involved in flagellar biosynthesis. Here we used RNAseq analysis to identify genes in addition to *pilA* with changes in expression in the absence of *pilR*. Among these were 10 genes inversely dysregulated by loss of *pilA* versus *pilR,* even though both *pilA* and *pilR* mutants lack T4P and pilus-related phenotypes. The products of those genes - many of which were hypothetical - may be important for virulence and surface-associated behaviours, as mutants had altered swarming motility, biofilm formation, type VI secretion, and pathogenicity in a nematode model. Further, the PilSR TCS positively regulated transcription of *fleSR,* and thus many genes in the FleSR regulon. As a result, *pilSR* deletion mutants had defects in swimming motility that were independent of the loss of PilA. Together these data suggest that in addition to controlling T4P expression, PilSR have a broader role in the regulation of *P. aeruginosa* motility and surface sensing behaviours.

IMPORTANCE

Surface appendages like type IV pili and flagella are important for establishing surface attachment and infection in a host in response to appropriate cues. The PilSR regulatory system that controls type IV pilus expression in *Pseudomonas aeruginosa* has an established role in expression of the major pilin PilA. Here we provide evidence supporting a new role for PilSR in regulating flagellum-dependent swimming motility in addition to pilus-dependent twitching motility. Further, even though both *pilA* and *pilR* mutants lack PilA and pili, we identified sets of genes downregulated in the *pilR* mutant and upregulated in a *pilA* mutant as well as those downregulated only in a *pilR* mutant, independently of pilus expression. This finding suggests that change in the inner membrane levels of PilA is only one of the cues to which PilR responds to modulate gene expression. Identification of PilR as a regulator of multiple motility pathways may make it an interesting therapeutic target for anti-virulence compounds.

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55 **INTRODUCTION**

56 Prokaryotes rely on the use of two-component regulatory systems (TCS) to control many of their
57 cellular activities. Typically comprised of a membrane-bound histidine sensor kinase and a cytoplasmic
58 response regulator, TCSs allow bacteria to respond rapidly to chemical and physical changes in their
59 intra- or extracellular environments, altering expression of specific genes in response to a stimulus (1).
60 The opportunistic pathogen *Pseudomonas aeruginosa* encodes a higher-than-average number of TCSs
61 (2) that control diverse functions, including several motility phenotypes. Flagellum-dependent
62 swimming motility, for example, is controlled through a regulatory cascade that includes the
63 transcriptional regulator, FleQ (3) and the FleS-FleR TCS, which like many TCSs also requires the
64 alternate sigma factor RpoN (σ^{54})(4). FleQ controls transcription of *fleS-fleR* in addition to multiple other
65 flagellar, adhesion and biofilm-associated genes, in a c-di-GMP dependent manner (3, 5). FleSR has been
66 implicated in the expression of 20 or more flagellar biosynthetic genes in *P. aeruginosa*, as well as
67 additional genes not previously known to be involved in flagellar assembly or function (6).

68 The other major motility system in *P. aeruginosa* is the type IV pilus (T4P) system, which is used
69 for twitching across solid and semi solid surfaces (7, 8) among other important functions. In contrast to
70 the single polar flagellum that is used to propel the cell in low viscosity media, the cell extends multiple
71 pili that retract – independently or in a coordinated bundle – pulling it towards the point of attachment
72 (9-11). Pili can be extended from either pole, but typically a single pole is used at one time allowing for
73 directional movement (12). The majority of the pilus fibre is made of hundreds to thousands of subunits
74 of the major pilin protein, PilA (13), the expression of which could be energetically costly to the cell if
75 not tightly controlled.

76 *pilA* transcription is regulated by another TCS, PilS-PilR, in *P. aeruginosa* and many other T4P-
77 expressing bacteria (14-18). PilS is an atypical sensor histidine kinase (SK) with 6 transmembrane
78 segments (19-21) that allow PilS to interact directly with PilA for pilin autoregulation (22). PilR is the
79 cytoplasmic response regulator (RR) that binds in conjunction with σ^{54} to the *pilA* promoter to activate
80 transcription (23, 24). Neither *pilA* nor *pilR* mutants express PilA and therefore T4P, but they have
81 opposite PilR activation states. Activation of PilR upon transient decreases in PilA levels may be one way
82 in which pilus attachment events are detected by the cell.

83 In contrast to the response regulator FleR, which has a well-defined regulon in *P. aeruginosa* (6),
84 the suite of genes potentially controlled by PilR is poorly characterized. Genetic and *in silico* analyses of

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85 the PilR regulons of *Geobacter sulfurreducens* (16, 25) and *Dichelobacter nodosus* (17) have been
86 performed, but comparable studies are lacking in *P. aeruginosa*. Screening of the *G. sulfurreducens*
87 genome for putative PilR binding sites revealed 54 loci with hypothesized σ^{54}-dependent promoters,
88 many of which were upstream of genes for T4P and flagellar biosynthesis, or cell wall biogenesis (25).
89 Those data, in combination with work performed in *D. nodosus*, which identified several surface-
90 exposed proteins whose expression was controlled by PilR (17), suggest that *P. aeruginosa* PilR likely has
91 additional functions beyond control of *pilA* transcription. However, each of the cited studies focused
92 mainly on identification of genes and characterization of their pilus-related functions without examining
93 other phenotypic consequences of loss of *pilR*.

94 In this work, we used RNAseq analysis to identify genes that were dysregulated by loss of *pilR*.
95 Because *pilR* mutants lack pili, which are important for surface sensing (26) and control of downstream
96 events such as biofilm formation (27), we included a *pilA* mutant in our analysis to distinguish genes
97 whose expression is specifically controlled by PilR from those that are affected by the loss of PilA. In
98 addition to several genes that were co-regulated with *pilA*, which we have termed “pilin-responsive”
99 genes, we also identified multiple flagellar genes, including the FleSR TCS, as being downregulated only
100 in the absence of *pilR*, in a pilin unresponsive manner. We show that the consequence of this
101 downregulation is a previously unreported defect in swimming motility in both *pilS* and *pilR* mutants,
102 independent of the loss of PilA. This work defines the pilin-dependent and independent regulons of PilR
103 and provides evidence for a direct regulatory connection between the *P. aeruginosa* T4P and flagellar
104 motility systems.

105

106 **RESULTS**

107 ***The expression of multiple genes is similarly altered in pilA and pilR mutants***

108 We performed RNAseq analysis to identify genes in addition to *pilA* that might be controlled by
109 the PilSR TCS. However, in designing this experiment we considered that i) *pilR* mutants also lack
110 expression of PilA; ii) loss of PilA contributes to a decrease in intracellular levels of the messenger
111 molecule cyclic adenosine monophosphate (cAMP) (28); and iii) there are over 200 genes in *P.*
112 *aeruginosa* that are at least partially cAMP-dependent, including Vfr, a cAMP-binding virulence factor
113 regulator (28). To separate genes that are affected by the loss of PilA that occurs in both *pilA* and *pilR*
114 mutants from those that are truly regulated by PilR, we categorized genes as those whose expression

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115 was changed in only the *pilA* or *pilR* backgrounds, versus both backgrounds, compared to WT PAK
116 **(Figure 1)**. The former group may also include genes that are cAMP-dependent. We did not include a
117 *pilS* mutant in RNAseq analysis, because PilS potentially interacts with alternate response regulators,
118 making it more challenging to distinguish genes that are controlled by PilSR and those regulated by PilS
119 and other unidentified RRs (29). Genes that were dysregulated similarly by at least 2-fold in both the
120 *pilA* and *pilR* mutants are summarized in **Table S1**, and included several T4P-associated genes such as
121 *tsaP* (30), and minor pilins *fimU, pilV, pilW, pilY1* and *pilE* (31), previously identified as being Vfr
122 dependent (28). In total, 18 of 56 genes in this category (highlighted in gray in **Table S1**) are also Vfr and
123 cAMP-dependent (28). Since the expression of genes in this class was affected by loss of PilA, suggesting
124 PilR's role is indirect, they were not examined further. No genes whose expression was dysregulated by
125 loss of *pilA* but not *pilR* were identified.

126 ***Ten genes are inversely dysregulated by loss of pilA versus pilR***

127 The expression of a subset of ten genes was decreased in the *pilR* mutant but markedly
128 increased in the *pilA* mutant, even though *pilR* mutants also lack PilA (23) (**Figure 2, Table S2)**. We
129 categorized these genes as 'pilin-responsive', because similar to PilA, their expression was dependent on
130 PilR and increased when PilA levels were low. All but 3 of these genes encode hypothetical proteins or
131 are unannotated in the PAO1 genome. The co-regulation of these genes with *pilA* suggests that their
132 products could be previously unidentified contributors to T4P biogenesis and/or function, or to other
133 forms of motility.

134 To test this hypothesis, we extracted mutants with insertions in homologs of those PAK genes
135 from the ordered PA14 transposon (Tn) library (32). There were no transposon insertions in three of the
136 ten genes, and one additional mutant failed to grow in liquid culture. The PAO1 and PA14 designations
137 for the remaining 6 genes of interest for which mutants were available are listed in **Table 1.** We tested
138 these mutants for twitching, swimming, and swarming motilities. While all had wild-type twitching
139 motility, insertions in PA14_51940 (PA0952), PA14_11740 (PA4027) and PA14_69560 (PA5267, *hcpB)*
140 caused defects in swarming. Disruption of PA14_695060 (*hcpB)* also reduced swimming, alluding to a
141 role in flagellar function or biosynthesis, in addition to its established function in Type VI secretion
142 **(Figure 3)**. Together, these data indicate that genes co-regulated with *pilA* are not necessarily required
143 for T4P function, but a subset are involved in other forms of motility and in some cases, biofilm
144 formation (33) and pathogenicity in *C. elegans* (34) **(Figure S1).**

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145 ***A subset of genes is dysregulated only by loss of PilR***

146 In the *pilR* mutant, 89 genes were dysregulated ≥2-fold **(Table S3)**. To prioritize our follow-up
147 studies, we focused on a shorter list of genes with ≥3-fold changes in expression. Prior to this study, *pilA*
148 was the only known member of the PilS-PilR regulon in *P. aeruginosa* (19), though studies in *G.*
149 *sulfurreducens* and *D. nodosus* suggested its regulon was likely to be broader (16, 17, 25). Of particular
150 interest were 24 genes whose expression was ≥3-fold altered in *pilR* mutants but unaffected by loss of
151 *pilA.* These *pilR*-dependent but pilin unresponsive genes are highlighted in **Figure 2**. According to the
152 *Pseudomonas* genome database (35), these genes include five putative chemotactic transducers, two
153 biofilm-associated chemosensory proteins, six hypothetical proteins, and several metabolic enzymes.
154 However, motility-associated genes were the most common class identified. The genes encoding the
155 T4P assembly ATPase, PilB and prepilin peptidase, PilD, which share a divergently oriented promoter
156 with *pilA*, were downregulated in *pilR* but unaffected by loss of *pilA* **(Figure 2)**, even though previous
157 studies suggested they were controlled by σ^{70}, not PilSR and σ^{54} (36).

158 ***Multiple flagellum biosynthetic genes are downregulated in a pilR mutant***

159 In addition to the T4P-associated genes above, several flagellum biosynthetic genes had
160 decreased expression only in the *pilR* background **(Figure 2, Table S3 bolded text).** Among them were
161 *fleS-fleR* encoding the FleSR TCS, part of a regulatory cascade that controls the expression of genes
162 associated with flagellum biosynthesis and function (4, 6). Each had approximately 3-fold lower
163 expression in *pilR* compared to WT, while there was no difference in their expression in *pilA* versus WT.
164 This trend was verified using RT-PCR, though the magnitude was closer to 2-fold by this method
165 (Supplemental **Figure S2**). Of the flagellar genes in this category (**Figure 2)**, 10 of 12 (excluding *fleS* and
166 *fleR*) are *fleR* dependent (6). The remaining two, *fliE* and *fliF,* are FleQ dependent, but also had
167 decreased (≥2-fold) transcription in a *fleR* mutant in a previous study (6). These data suggest that PilSR
168 positively regulates *fleSR* expression, and when PilR is absent, expression of FleSR-dependent genes is
169 decreased accordingly.

170 ***Swimming motility is impaired by loss of pilS-pilR***

171 We next tested if downregulation of *fleSR* in the *pilS* and *pilR* backgrounds impacted swimming
172 motility, using a plate-based assay. A *fliC* mutant lacking the flagellin subunit was used as a negative
173 control. *pilA* mutants swam comparably to WT PAK, while *pilS* and *pilR* mutants – which also lack surface
174 pili – exhibited significant swimming defects ($p<0.005$), with uniform zones that reached about 40% of

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175 WT **(Figure 4, dashed line)**. Interestingly, both *pilS* and *pilR* mutants produced flares with increased
176 motility extending beyond these uniform swimming zones. These flares were hypothesized to be the
177 result of suppressor mutations that could overcome the effect of *pilS* or *pilR* deletion on swimming.

178 To test this idea, we isolated cells from the inner swimming zones of *pilS* and *pilR* plates (inside
179 the dashed line, **Figure 4**) and the putative suppressor mutants (flares outside the dashed line) and
180 reassessed their ability to swim after culturing them overnight. As controls, we took samples from the
181 WT zone close to the point of inoculation ('inner') and from the outer edge of the swimming zone
182 ('outer'). Repeating the swimming assays with these samples revealed no difference in swimming
183 between inner and outer samples from WT. However, *pilS* and *pilR* cells taken from the inner swimming
184 zones recapitulated the original swimming motility defects of the mutants – including the re-appearance
185 of highly motile suppressors – while cells taken from the outer flares had motility comparable to WT
186 **(Figure 4),** indicating that they likely acquired mutation(s) that allow for full motility in the absence of
187 *pilSR*.

188 To test if other flagellum-dependent phenotypes were affected by loss of *pilSR,* we measured
189 swarming motility, using the original mutants and the suppressors isolated from the swimming
190 experiments above. *pilSR* mutants in PAO1 were previously reported to be non-swarmers (37), but in
191 our hands the same mutants in the PAK background retain partial swarming motility, albeit with an
192 altered morphology compared to WT. The PAK *pilSR* mutants swarmed similarly to a *pilA* mutant, with
193 fewer and irregular tendrils **(Figure S2).** Interestingly, *pilSR* mutants isolated from the outer flares of the
194 swimming plates in **Figure 4** had swarming motility comparable to those isolated from the inner zones
195 and the parent *pilS* and *pilR* strains. While flagella are required for swarming, the suppressor mutations
196 that restored swimming motility in the *pilS* and *pilR* backgrounds did not restore swarming, suggesting
197 that expression of distinct swarming-related genes remains dysregulated.

198 ***FleSR impact twitching motility and pilA expression***

199 RNAseq analyses revealed that PilR was required for wild type expression of *fleSR*. We next
200 tested if this was a reciprocal regulatory pathway in which FleSR might contribute to regulation of *pilS*-
201 *pilR* and the PilSR regulon. We tested if loss of *fleSR* affected *pilA* expression and/or T4P function. A
202 double deletion of *fleSR* was made in the PAK background, and twitching motility measured. Loss of
203 *fleSR* reduced twitching motility to a modest but significant extent ($p<0.005$), with the double mutant
204 reproducibly twitching to approximately 80% of WT (**Figure 5A).** Interestingly, when *pilA* transcription

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205 was monitored using a *lux-pilA* reporter assay, *fleS-fleR* mutants had increased *pilA* transcription
206 compared to WT over a 5 h time course **(Figure 5B)**. Therefore, while FleS-FleR are involved in the
207 modulation of twitching motility and *pilA* transcription, it is not yet clear if this occurs directly through
208 regulation of *pilSR,* as increased levels of PilA can inhibit PilSR activation (22).

209

210 DISCUSSION

211 Two-component systems control a multitude of phenotypes, allowing for quick responses to
212 sudden changes in a bacterium's intra- and extracellular environments. These systems can be important
213 for survival, but also for coordinating virulence programs. Most TCSs explored to date control the
214 transcription of multiple genes, but prior to this work *P. aeruginosa* PilR had only a single known target,
215 *pilA* (23). Microarray and bioinformatics analyses of the *G. sulfurreducens* PilR regulon provided
216 evidence that PilR regulates multiple genes, including those required for soluble Fe(III) uptake (a pilin-
217 independent phenotype), flagellar assembly and function, and cell envelope biogenesis, though these
218 predictions were not confirmed with phenotypic assays (16, 25). Here, we showed that PilR controls the
219 expression of multiple genes, in pilin-responsive or unresponsive modes. Dysregulating expression of
220 select members of the *P. aeruginosa* PilR regulon resulted in changes in swimming, swarming, and/or
221 twitching motility, all phenotypes associated with virulence in specific hosts (27, 38-40).

222 The *G. sulfurreducens* and *D. nodosus* studies cited above failed to account for the confounding
223 variable that PilA is not expressed when *pilR* is deleted. This was an important consideration in designing
224 our RNAseq experiment **(Figure 1)**, as loss of PilA results in decreased cAMP levels and by extension,
225 downregulation of cAMP-dependent genes in the Vfr regulon, which includes a number of T4P-
226 associated genes (28). This design also enabled us to further classify genes in the PilR regulon based on
227 their responsiveness to pilin levels. As predicted, many of the genes that were similarly dysregulated by
228 loss of both *pilA* and *pilR* are Vfr-dependent (28) **(Table S1)**. Thus, we focused instead on those genes
229 that were dysregulated in a PilR-dependent manner and further categorized them as pilin responsive or
230 unresponsive.

231 We identified ten pilin-responsive genes with increased transcription in a *pilA* mutant but
232 significantly decreased transcription in the absence of *pilR*, even though *pilR* mutants also lack PilA
233 **(Figure 2)**. While this expression pattern initially seemed counterintuitive, we propose that these gene
234 products are regulated by PilS phosphorylation or dephosphorylation of PilR in response to fluctuating

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235 PilA levels. At high concentrations, PilA represses its own transcription by interacting directly with PilS in
236 the inner membrane, promoting its phosphatase activity on PilR (22). Conversely, when PilA is absent,
237 PilS phosphorylates PilR and *pilA* promoter activity is significantly increased, presumably in an attempt
238 to replenish intracellular PilA pools (41) but simultaneously increasing expression of other pilin
239 responsive genes **(Figure 2, Table S2)**. This signalling pathway may be one way in which adherence of a
240 pilus to a surface is detected, through transient depletion of pilin pools in the inner membrane when
241 attached pilus filaments fail to retract.

242 Many genes in this pilin-responsive category encoded hypothetical proteins or were
243 unannotated in the PAO1 and PAK genomes; the latter may encode regulatory RNAs. We used available
244 mutants from the PA14 Tn library to determine if the pilin-responsive genes were required for normal
245 T4P function. While all mutants tested had WT twitching motility, some had decreased swarming, and
246 one (PA14_69560) had decreased swimming motility. The only genes in this group that were
247 characterized previously are *hcpA* and *hcpB*, which encode proteins associated with the Type VI
248 secretion system. They are paralogs, possibly resulting from a gene duplication event (35). This finding
249 may represent a new link between T4P, flagellar function, and Type VI secretion, as the *hcpB* mutant had
250 defects in both swimming and swarming. This connection further explains the swimming defects of *pilS*
251 and *pilR* mutants (**Figure 4).**

252 We also identified genes that were affected only by loss of *pilR*, independent of PilA. These
253 genes might be modulated in response to cues detected by a different, pilin-insensitive sensor kinase
254 that can activate PilR. Alternatively, they may already be expressed in the WT at levels such that further
255 activation upon loss of *pilA* did not meet our 2-fold cutoff. A third possibility is that they are indirectly
256 upregulated as a result of PilR activity on adjacent promoters. For example, among these genes were
257 those encoding the T4P assembly ATPase PilB and the prepilin peptidase, PilD, which are contiguous
258 with *pilC* encoding the platform protein; however, there were insufficient reads in our RNAseq analysis
259 to accurately determine *pilC* expression levels **(Figure 2)**. Based on this and previous studies, *pilBCD* are
260 not co-transcribed (35, 36). *pilB* was reported to be σ^{70} dependent (36), but our data suggest that PilR
261 remodeling of the *pilA* promoter for transcription by the σ^{54} holoenzyme also facilitates transcription
262 from the divergent *pilB* promoter.

263 Of the pilin unresponsive genes identified, the most abundant class were involved in
264 biosynthesis, function, and regulation of the flagellum, including *fleSR* (**Figure 2, Table S3)** Most of the
265 others are members of the FleSR regulon (6) suggesting they are indirectly regulated by PilR. Swimming

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266 motility of *pilS* and *pilR* mutants was ~40% of WT, supporting the expression data **(Figure 4)**. By carefully
267 analyzing the swimming data, we hypothesized that suppressor mutations could overcome the defects
268 imposed by *pilS or pilR* deletion, allowing the mutants to swim normally. Preliminary sequence analyses
269 of these suppressors showed no mutations in *fleSR*, but it may be that mutations in *fleQ,* the promoter
270 regions of *fleSR*, or as yet unidentified genes could increase activity or expression of *fleS-fleR*. The as-yet
271 unidentified suppressors appear specific for flagellar function, as swarming motility (42) of the *pilS* and
272 *pilR* mutants and the highly motile suppressors, all of which lack PilA, was comparable to that of a *pilA*
273 mutant **(Figure S2)**,

274 Although *pilSR* were not considered members of the FleSR regulon (6), twitching motility was
275 modestly but reproducibly reduced to ~80% of WT in the absence of *fleSR* **(Figure 5A)**, while *pilA*
276 promoter activity was increased compared to WT **(Figure 5B)**. This phenotype is reminiscent of
277 mutations that inhibit pilus retraction, impairing twitching but increasing *pilA* transcription due to
278 depletion of PilA subunits from inner membrane pools (11, 41). During prior characterization of the
279 FleSR regulon, two new genes (PA3713 and PA1096/*fleP)* with motility phenotypes were identified.
280 Mutants were significantly impaired in swimming, and in the case of *fleP,* twitching motility (6). FleP was
281 proposed to control pilus length, as when it was deleted, surface pili were significantly longer than those
282 of WT, resulting in a form of hyperpiliation (6). Decreased *fleP* expression in our *fleSR* mutants could
283 impair twitching motility and alter *pilA* expression. Because of their FleSR dependence, expression of
284 *fleP* and PA3713 may be decreased in *pilSR* mutants; however, the reads for them in our RNAseq
285 experiment were too low to assess this idea.

286 Both the PilSR and FleSR TCSs are required for full virulence of *P. aeruginosa* –reviewed in (43) –
287 as each is involved in multiple virulence-associated phenotypes. PilSR and FleSR each contribute to
288 surface attachment and biofilm formation (27, 44), and are important for twitching and swimming
289 motilities. Both PilSR and FleSR are required for swarming motility due to their involvement in pilus and
290 flagellum function respectively (37, 42, 44), (**Figure S3)**. Given the overlap in phenotypes controlled by
291 PilSR and FleSR, it is perhaps not surprising that expression of the two systems may be linked. From our
292 RNAseq analysis and subsequent phenotypic assays, we propose a model in which PilSR positively
293 regulates *fleSR* transcription, independently of PilA depletion **(Figure 6).** The hierarchy for flagellar
294 biosynthesis proposed by Dasgupta *et al.* (6) suggests that transcription of *fleSR* is predominantly
295 dependent on FleQ. Since *fleQ* was not differentially expressed in *pilR*, we infer that PilSR promotes
296 *fleSR* transcription directly, rather than by modulating FleQ expression.

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297 Why, and under what conditions, might this regulatory circuit be active? Twitching motility is
298 normally deployed on solid or semi-solid surfaces (8) while flagella are typically used in liquid and low
299 viscosity conditions. One might predict that the systems are differentially activated in response to
300 relevant environmental conditions. Instead, the regulatory integration of these two systems may be an
301 adaptation to life as an opportunistic pathogen. T4P and flagella are typically expressed during the acute
302 phase of infection (4, 45) and during the transition to the chronic infection phase, motility systems are
303 downregulated in favour of those promoting Type VI secretion and biofilm formation (43, 46). Clinical
304 isolates of *P. aeruginosa* from chronically colonized patients are often both non-flagellated and non-
305 piliated (47). Lack of the immunogenic flagellum may help *P. aeruginosa* escape phagocytosis (47) and
306 aflagellate bacteria are better able to evade the inflammatory response of the host (48). Placing *fleSR*
307 under control of PilSR may facilitate a more rapid transition to the chronic disease state and more
308 efficient evasion of the host immune system. Similarly, both T4P and flagella are required for surface
309 sensing and surface-associated behaviours such as swarming motility and activation of virulence
310 cascades (26, 42, 49, 50). Co-regulation of their expression may allow *P. aeruginosa* and other motile
311 bacteria to amplify their responses to surface detection.

312 We identified 34 genes in addition to *pilA* whose expression was altered ≥3 fold by loss of *pilR*,
313 24 of which were dysregulated in a pilin unresponsive manner, supporting previous work in *G.*
314 *sulfurreducens* that identified putative PilR binding sites upstream of multiple genes (25). Importantly,
315 while *pilA* and *pilR* mutants look similar with respect to their T4P-related phenotypes, their transcription
316 profiles and other phenotypic outputs are different. For example, expression of genes encoding proteins
317 involved in flagellum biosynthesis, including *fleSR,* are downregulated in the absence of *pilR* but
318 unaffected by loss of *pilA*. This work reveals a previously unappreciated regulatory connection between
319 two diverse motility systems, with implications in detection of surface attachment and the transition
320 from acute to chronic disease states in a host.

321

322 **METHODS**

323 ***Bacterial strains and growth conditions***

324 Unless otherwise specified, *Pseudomonas aeruginosa* PAK strains were grown in Lennox Broth
325 (LB) (Bioshop) or on LB 1.5% agar plates at 37°C. Where the antibiotic kanamycin was used, it was
326 introduced at a final concentration of 150µg/mL. Mutants were generated by homologous

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327 recombination, using standard mating techniques described in (51). The strains and plasmids used in this
328 study are outlined in **Table 1.** Plasmids were prepared using standard cloning techniques and introduced
329 into *P. aeruginosa* using electroporation.

330 ***RNA isolation, library preparation, cDNA sequencing and analysis***

331 To isolate RNA, cells from strains of interest were streaked in triplicate onto half of an LB 1.5%
332 agar plate (100x15mm petri dishes) and grown overnight at 37°C. Cells were scraped from the plates
333 and resuspended in 1.5mL RNAprotect Bacteria Reagent (Qiagen) to maintain integrity of isolated RNA.
334 Cells were chemically lysed using 1mg/mL lysozyme in 10mM Tris-HCl and 1mM EDTA, pH 8.0 and RNA
335 isolated using the RNeasy mini kit (Qiagen) according to manufacturers' instructions. An on-column
336 DNase treatment was performed to minimize potential DNA contamination. Purified RNA was eluted
337 into 50μL nuclease free water and quantified.

338 The following steps were performed by the Farncombe Metagenomics Facility (McMaster
339 University, Hamilton, ON, Canada). For RNAseq analysis, ribosomal RNA was depleted from 9 RNA
340 samples (3x WT PAK, 3x *pilA* and 3x *pilR*) using the Ribo-zero rRNA depletion kit (Illumina) and cDNA
341 libraries prepared by the NEBnext Ultra Directional Library Kit. Libraries were sequenced using paired
342 end 75bp reads on the Illumina MiSeq platform. Reads were aligned to the PAO1 reference genome with
343 98% of reads mapped and normalization and differential gene expression were calculated using the
344 Rockhopper software (52). q-values for each identified gene are reported in **Tables S1-3.** The complete
345 RNAseq dataset has been deposited in NCBI GEO (Accession number: GSE112597).

346 ***Twitching motility assays***

347 Twitching motility assays were performed as described in (53). Briefly, strains of interest were stab
348 inoculated to the bottom of an LB 1% agar plate with a P10 pipette tip and plates were incubated upside
349 down at 37°C for 16-24h. Following incubation, agar was carefully removed and the plastic petri dish
350 was stained with 1% crystal violet for 20min. Excess dye was washed away with water and twitching
351 zone diameters were quantified using ImageJ ((http://imagej.nih.gov/ij/, NIH, Bethesda, MD). A one-way
352 ANOVA statistical test was used to determine significant differences in twitching compared to WT.

353 ***Swarming motility assays***

354 Swarming motility assays were performed as described in (54). Briefly, strains of interest were grown
355 overnight in 5mL LB cultures at 37°C. On the day of the assay, 0.5% agar plates with M8 buffer,

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356 supplemented with 2mM $MgSO_4$, 0.2% glucose, 0.05% L-glutamic acid and trace metals, were prepared
357 and allowed to solidify at room temperature for 1.5h. Then, 3.5µL of culture were spotted onto the
358 centre of a single plate and plates were incubated upright in a humidity-controlled 30°C incubator for
359 48h. Plates were imaged using a standard computer scanner. Figures shown are representative of 3
360 independent experiments.

361 ***Swimming motility assays***

362 Swimming motility plate assays were performed similarly to (55), with some modifications. Overnight
363 5mL cultures of strains of interest were grown at 37°C in LB with shaking. On the day of inoculation, LB
364 0.25% agar plates were prepared and allowed to solidify at room temperature for 1.5h. Cell cultures
365 were standardized to an OD_{600}=1.0 and 2µL were spotted onto the centre of each plate. Plates were
366 incubated upright for 16h at 37°C and swimming zone diameters were quantified using ImageJ
367 (http://imagej.nih.gov/ij/, NIH, Bethesda, MD). Where applicable, swimming zone diameters were
368 defined at the outer most part of the swimming zone that was still uniform in appearance. Images are
369 representative of 4 independent experiments. To determine statistical significance, a one-way ANOVA
370 analysis with Dunnett's post-test was performed, using WT as the control strain.

371 ***Biofilm assays***

372 Biofilm assays were performed similarly to the method described in (33), with some modifications.
373 Briefly, *P. aeruginosa* strains of interest were grown in 5mL liquid cultures of 50% LB/50% PBS (50/50
374 media) overnight at 37°C with shaking. The following day, strains were subcultured 1:25 into fresh 50/50
375 media and grown to a standardized OD_{600}=0.1. Standardized cultures were then diluted 1:500 and 150µL
376 of each strain of interest was plated in triplicate in a clear, 96 well plate (Nunc). The plate was closed
377 with a 96-peg lid, providing a surface on which biofilms can form, sealed with parafilm and incubated
378 with shaking for 18h at 37°C. To quantify planktonic growth, peg lids were removed and the 96-well
379 plate was scanned at a wavelength of 600nm. To quantify biofilms, peglids were washed in PBS and
380 stained with crystal violet for 15min. Following five 10min washes in water, crystal violet was solubilized
381 in 33% acetic acid in a fresh 96 well plate, which was scanned at 595nm. Biofilm data was graphed as %
382 WT, showing means and standard error of three independent experiments.

383 ***Caenorhabditis elegans slow killing pathogenicity assays***

384 Slow killing (SK) assays were performed as described previously (34). *Caenorhabditis elegans*
385 strain N2 populations were propagated and maintained on Nematode Growth Media (NGM) plates

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386 inoculated with *E. coli* OP50. Eggs were harvested to obtain a synchronized population by washing
387 worms and eggs from NGM plates with M9 buffer. Worms were degraded by adding buffered bleach,
388 leaving only eggs intact. Eggs were washed with M9 buffer and resuspended in M9 buffer with rocking
389 overnight to allow eggs to hatch into L1 larvae. Synchronized L1 worms were plated on NGM plates for
390 45h to develop into L4 worms. During this process, slow killing plates supplemented with 100µM 5-
391 Fluoro-2�-deoxyuridine (FUDR) were prepared and inoculated with 100µL of a 5mL LB overnight culture
392 of bacterial strains of interest and incubated at 37°C for 16-18h. Harvested and washed L4 worms (~30-
393 40) were dropped by Pasteur pipette onto each SK plate. Using a dissecting microscope, plates were
394 scored daily for dead worms, which were picked and removed. Survival curves were prepared using
395 Graphpad Prism 5.01 (La Jolla, CA) and statistically significant differences in pathogenicity between
396 strains were identified using Gehan-Breslow-Wilcoxon analysis.

397 ***pilA-lux reporter assay***

398 Luminescent reporter assays were performed as described previously (22). Strains of interest
399 were transformed by electroporation with the pMS402-p*pilA* plasmid, which contains the luciferase
400 genes under control of the *pilA* promoter. Strains were grown overnight in 5mL LB cultures
401 supplemented with 150 µg/mL kanamycin. The following day, a 1mL aliquot of a 1:20 dilution of cultures
402 was prepared and 100µL samples were plated in triplicate in a white walled, clear bottom 96-well plate
403 (3632 Costar, Corning Inc). Luminescence and OD_{600} were measured at 15min intervals over 5h using a
404 Synergy 4 microtitre plate reader (BioTek) programmed to shake continuously and incubate the plate at
405 37°C. Luminescence was normalized to OD_{600} and relative luminescence was plotted against time. Mean
406 and standard error of >4 biological replicates are shown.

407

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554

555

556 **FIGURE CAPTIONS**

557 **Figure 1. RNAseq experimental design**. Our RNAseq experiment was designed to distinguish between
558 genes dysregulated by loss of *pilR* versus loss of *pilA,* as *pilR* mutants also lack the pilin protein. Genes
559 coordinately dysregulated in both *pilA* and *pilR* compared to WT may be due to loss of PilA or pilus
560 expression. Genes that are inversely dysregulated in *pilA* and *pilR* mutants are upregulated in the
561 absence of PilA (pilin responsive), in a *pilR*-dependent manner. Genes dysregulated by loss of *pilR* but
562 not *pilA* are dependent only on PilR expression and referred to as pilin unresponsive in this study.

563 **Figure 2: Multiple T4P and non-T4P genes are dysregulated by loss of *pilR*.** The heat maps highlight
564 select genes of interest that are ≥3-fold dysregulated by the loss of *pilR,* or both *pilA* and *pilR*. In addition
565 to *pilA,* several other T4P associated genes have altered expression in *pilA* and/or *pilR* mutants.
566 Expression of ten genes, mostly hypothetical or unannotated, is increased in a *pilA* mutant but
567 decreased in *pilR*, which also lacks PilA. Of the genes dysregulated in a *pilR* mutant, many were
568 associated with flagellar biosynthesis and function, including those encoding the FleS-FleR TCS.

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569 **Figure 3: PA14 mutant homologs of inversely dysregulated genes affect motility phenotypes.** Available
570 PA14 transposon mutant homologs of inversely dysregulated genes identified in PAK were tested for **A)**
571 twitching, **B)** swarming and **C)** swimming motilities, **D)** biofilm formation, and **E)** pathogenicity towards
572 *Caenorhabditis elegans* to identify the functions of hypothetical proteins. All mutants displayed WT
573 twitching, but only a subset had defects in swimming and/or swarming motility. Several mutants
574 exhibited a hyperbiofilm phenotype, while two had defects in *C. elegans* killing (*** p<0.005).

575 **Figure 4: Swimming motility is impaired in *pilS* and *pilR* mutants.** Loss of *pilS* or *pilR* results in
576 decreased swimming motility (~40% of WT) in a plate-based assay. *pilA* mutants swim comparably to
577 WT, indicating that the swimming defect is not PilA dependent. *pilS* and *pilR* mutants appear to acquire
578 suppressors that overcome these defects resulting in asymmetrical flares. Reinoculation of swimming
579 plates with cells from the interior of swimming zones—inside the dotted white circles of *pilSR* (inner)
580 recapitulate the original phenotype, while cells taken from the flares (outer, from flares outside the
581 white circle, except for WT) swim to WT levels. Asterisks denote the location from which cells were
582 taken for the reinoculated swimming plates.

583 **Figure 5: Loss of *fleSR* reduces twitching motility but increases *pilA* transcription. A)** Loss of *fleSR*
584 reduces twitching motility by approximately 20%. Mean and standard error of 6 independent replicates
585 are shown. Significance determined by one-way ANOVA. **B)** A *lux-pilA* luminescent reporter assay
586 measuring *pilA* promoter activity indicated that *pilA* transcription is increased over 5h. Mean and
587 standard error of 4 biological replicates are shown.

588 **Figure 6: Model for *pilSR* dependent regulation of *fleSR* and the *fleSR* regulon.** Under conditions in
589 which *pilSR* expression is decreased (low cAMP) or perhaps when their activity is low (high intracellular
590 PilA), *fleSR* transcription is decreased. As a result, in *pilSR* mutants, as is expression of the the *fleSR*
591 regulon. Genes in red are those that had decreased expression in a *pilR* mutant in RNAseq. Grey genes
592 did not have sufficient reads assigned to them from RNAseq to accurately report differential expression.
593 FleQ (blue) was not differentially expressed between WT or *pilR* indicating that *pilSR* fits into the
594 flagellar regulatory hierarchy after FleQ but before *fleSR*, as *fleQ* itself, and most FleQ dependent genes
595 were unaffected by loss of *pilR*.

596

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597 **Figure S1: RT-PCR validation of *fleSR* transcription levels.** *fleS-fleR* were downregulated approximately
598 2-fold in *pilS* and *pilR*, but not *pilA.* Mean and standard error of 3 independent experiments are shown.

599 **Figure S2: Putative suppressors that restore swimming do not affect swarming motility in *pilSR***
600 ***mutants.*** Cells from *pilS* and *pilR* mutants with putative suppressor mutations that restore swimming
601 motility (outer) were inoculated in a swarming assay for comparison to *pilS* and *pilR* mutants that do not
602 have suppressors (inner). Strains with possible suppressors still exhibit *pilS* and *pilR*-like swarming
603 motility patterns indicating that the *pilSR* phenotype is dominant.

604

605

606 **TABLE LEGENDS**

607 **Table 1. PA and corresponding PA14 gene numbers of inversely dysregulated (pilin responsive) genes**

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609 **Table S1. Genes similarly dysregulated in *pilA* and *pilR***

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Table 1: PA and corresponding PA14 gene numbers of inversely dysregulated (pilin responsive) genes

PA Number (PAO1)	PA14 Number	Gene name	Product
PA0952	PA14_51940		Hypothetical protein
PA1512	PA14_44890	*hcpA*	Secreted protein (Type VI secretion)
PA4027	PA14_11740		Hypothetical protein: Putative bacterial extracellular solute binding protein
PA4683	PA14_61950		Hypothetical protein
PA5228	PA14_69040		Hypothetical protein: Putative 5-formyltetrahydrofolate cyclo-ligase
PA5267	PA14_69560	*hcpB*	Secreted protein (Type VI secretion)

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Table 2: Strains and plasmids used in this study

Strain	**Description**	**Source**
PAK WT	WT Group II strain of *P. aeruginosa*	(J. Boyd)
pilA	PAK with chromosomal deletion of *pilA*	(This study)
pilS	PAK with chromosomal deletion of *pilS*	(This study)
pilR	PAK with chromosomal deletion of *pilR*	(This study)
fliC	PAK wit FRT insertion in *fliC*	(This study)
fleSR	PAK with chromosomal of the full *fleS-fleR operon*	(This study)
PA14 WT	WT Group III strain of *P. aeruginosa*	(32)
PA14_51950	PA14 with a transposon insertion in gene PA14_51950	(32)
PA14_44890	PA14 with a transposon insertion in PA14_44890 (*hcpA)*	(32)
PA14_11740	PA14 with a transposon insertion in PA14_11740	(32)
PA14_61950	PA14 with a transposon insertion in PA14_61950	(32)
PA14_69040	PA14 with a transposon insertion in PA14_69040	(32)
PA14_69560	PA14 with a transposon insertion in PA14_69560 *(hcpB)*	(32)
Plasmid		
pMS402-p*pilA*	*pilA* promoter cloned into the BamHI site of pMS402, putting lux genes under control of *pilA* promoter	(22)

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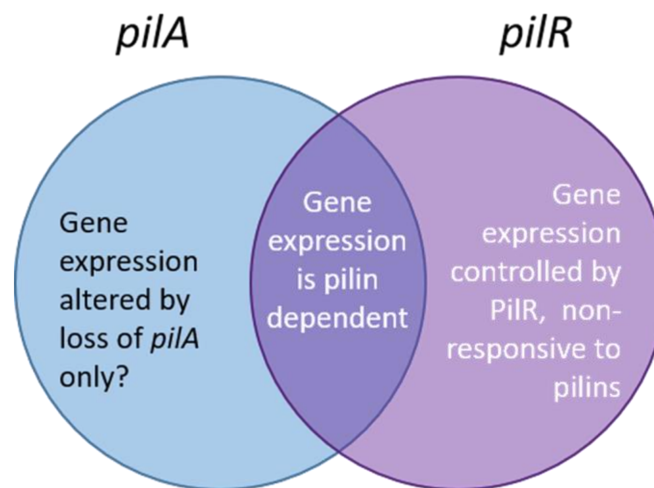

Figure 1. RNAseq experimental design. Our RNAseq experiment was designed to distinguish between genes dysregulated by loss of *pilR* versus loss of *pilA,* as *pilR* mutants also lack the pilin protein. Genes coordinately dysregulated in both *pilA* and *pilR* compared to WT may be due to loss of PilA or pilus expression. Genes that are inversely dysregulated in *pilA* and *pilR* mutants are upregulated in the absence of PilA (pilin responsive), in a *pilR*-dependent manner. Genes dysregulated by loss of *pilR* but not *pilA* are dependent only on PilR expression and referred to as pilin unresponsive in this study.

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Fold Change vs WT

<-5 0 >5

Inversely Regulated Genes

PA Number	Gene Name	Product	Fold Change (pilA vs WT)	Fold Change (pilR vs WT)
PA0507		acyl-CoA dehydrogenase		
PA0951a		unannotated		
PA0952		hypothetical protein		
PA0952a		unannotated		
PA1512	*hcpA*	secreted protein (T6S)		
PA4027		hypothetical protein		
PA4683		hypothetical protein		
PA5228		hypothetical protein		
PA5228a		unannotated		
PA5267	*hcpB*	secreted protein (T6S)		

PilR-dependent Genes

PA Number	Gene Name	Product	Fold Change (pilR vs WT)
PA0534	*pauB1*	FAD-Dependent oxidoreductase	
PA1077	***flgB***	**flagellar basal body rod protein**	
PA1078	***flgC***	**flagellar basal body rod protein**	
PA1079	***flgD***	**flagellar basal body rod modification protein**	
PA1081	***flgF***	**flagellar basal body rod protein**	
PA1082	***flgG***	**flagellar basal body rod protein**	
PA1083	***flgH***	**flagellar L-ring protein precursor**	
PA1092	***fliC***	**flagellin**	
PA1098	***fleS***	**Sensor histidine kinase (flagellar regulator)**	
PA1099	***fleR***	**Response regulator (flagellar regulator)**	
PA1422	*gbuR*	regulatory protein	
PA1423	*bdlA*	biofilm dispersion locus (chemosensor)	
PA1441	**fliK**	**flagellar hook length control**	
PA1697		hypothetical protein	
PA1967		**hypothetical protein**	
PA2274		hypothetical protein	
PA2654		chemotaxis transducer	
PA2867		chemotaxis transducer	
PA4310	*pctB*	chemotactic transducer	
PA4326		**hypothetical protein**	
PA4524	*nadC*	nicotinate-nucleotide pyrophosphorylase	
PA4526	*pilB*	T4P assembly ATPase	
PA4528	*pilD*	Type IV prepillin peptidase	
PA4547	***pilR***	**response regulator (T4P regulator)**	

Figure 2: Multiple T4P and non-T4P genes are dysregulated by loss of *pilR*. The heat maps highlight select genes of interest that are ≥3-fold dysregulated by the loss of *pilR,* or both *pilA* and *pilR*. In addition to *pilA,* several other T4P associated genes have altered expression in *pilA*

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and/or *pilR* mutants. Expression of ten genes, mostly hypothetical or unannotated, is increased in a *pilA* mutant but decreased in *pilR*, which also lacks PilA. Of the genes dysregulated in a *pilR* mutant, many were associated with flagellar biosynthesis and function, including those encoding the FleS-FleR TCS.

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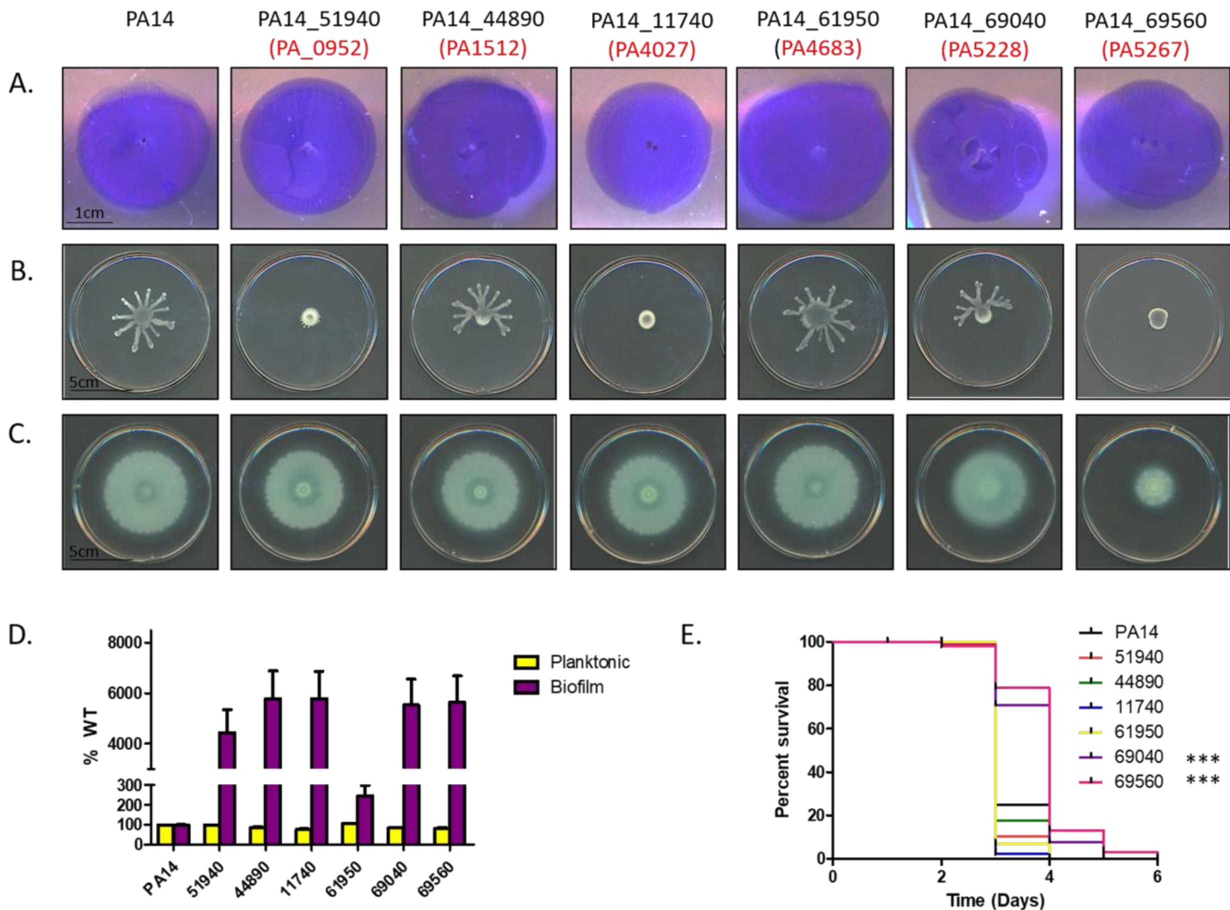

Figure 3: PA14 mutant homologs of inversely dysregulated genes affect motility phenotypes. Available PA14 transposon mutant homologs of inversely dysregulated genes identified in PAK were tested for **A)** twitching, **B)** swarming and **C)** swimming motilities, **D)** biofilm formation, and **E)** pathogenicity towards *Caenorhabditis elegans* to identify the functions of hypothetical proteins. All mutants displayed WT twitching, but some had varying defects in swimming and/or swarming motility. Several mutants also exhibited a hyperbiofilm phenotype, while two had defects in *C. elegans* killing (*** $p<0.005$).

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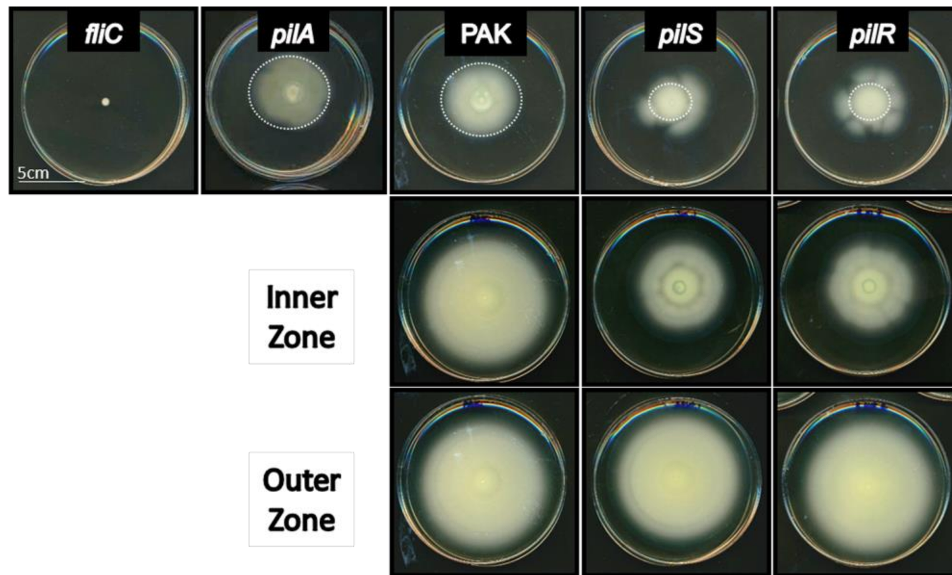

Figure 4: Swimming motility is impaired in *pilS* and *pilR* mutants. Loss of *pilS* or *pilR* results in decreased swimming motility (~40% of WT) in a plate-based assay. *pilA* mutants swim comparably to WT, indicating that the swimming defect is not PilA dependent. *pilS* and *pilR* mutants appear to acquire suppressors that overcome these defects resulting in asymmetrical flares. Reinoculation of swimming plates with cells from the interior of swimming zones—inside the dotted white circles of *pilSR* (inner) recapitulate the original phenotype, while cells taken from the flares (outer, from flares outside the white circle, except for WT) swim to WT levels. Asterisks denote the location from which cells were taken for the reinoculated swimming plates.

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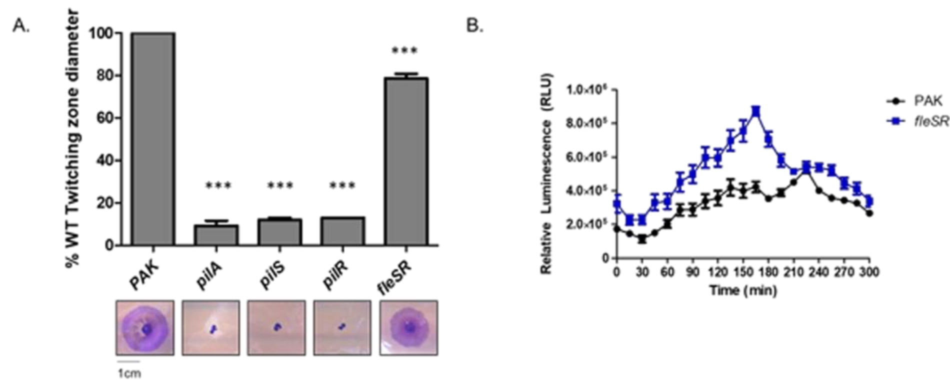

Figure 5: Loss of *fleSR* reduces twitching motility but increases *pilA* transcription. A) Loss of *fleSR* reduces twitching motility by approximately 20%. Mean and standard error of 6 independent replicates are shown. Significance determined by one-way ANOVA. **B)** A *lux-pilA* luminescent reporter assay measuring *pilA* promoter activity indicated that *pilA* transcription is increased over 5h. Mean and standard error of 4 biological replicates are shown.

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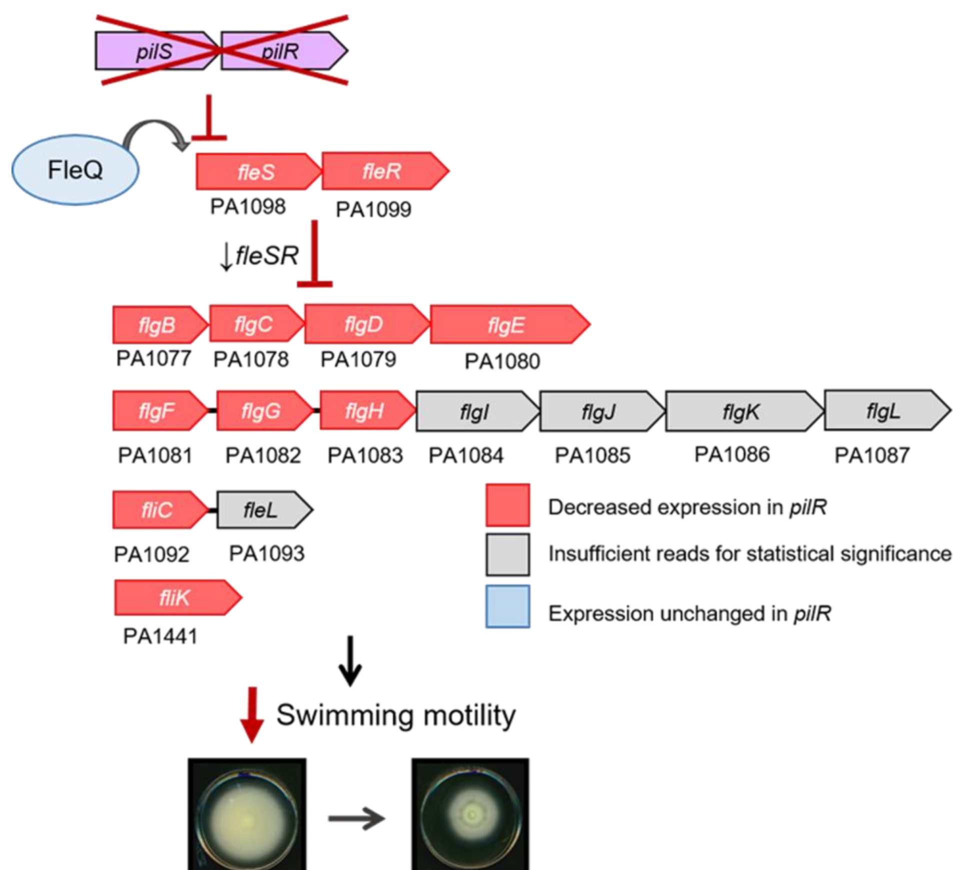

Figure 6: Model for *pilSR* dependent regulation of *fleSR* and the *fleSR* regulon. Under conditions in which *pilSR* expression is decreased (low cAMP) or perhaps when their activity is low (high intracellular PilA), *fleSR* transcription is decreased. As a result, in *pilSR* mutants, as is expression of the the *fleSR* regulon. Genes in red are those that had decreased expression in a *pilR* mutant in RNAseq. Grey genes did not have sufficient reads assigned to them from RNAseq to accurately report differential expression. FleQ (blue) was not differentially expressed between WT or *pilR* indicating that *pilSR* fits into the flagellar regulatory hierarchy after FleQ but before *fleSR*, as *fleQ* itself, and most FleQ dependent genes were unaffected by loss of *pilR*.