Full title: *Pseudomonas aeruginosa* type IV minor pilins and PilY1 regulate virulence by modulating FimS-AlgR activity

Short title: Minor pilins regulate virulence via modulation of FimS-AlgR activity

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Abstract

Type IV pili are expressed by a wide range of prokaryotes, including the opportunistic pathogen *Pseudomonas aeruginosa*. These flexible fibres mediate twitching motility, biofilm maturation, surface adhesion, and virulence. The pilus is composed mainly of major pilin subunits while the low abundance minor pilins FimU-PilVWXE, plus the putative adhesin PilY1, prime pilus assembly and are proposed to form the pilus tip. The minor pilins and PilY1 are encoded in an operon that is positively regulated by the FimS-AlgR two-component system. Independent of pilus assembly, PilY1 is proposed to be a mechanosensory component that - in conjunction with minor pilins - triggers up-regulation of acute virulence phenotypes upon surface attachment. Here, we investigated the link between the minor pilins and virulence. *pilW, pilX*, and *pilY1* mutants had reduced virulence towards *Caenorhabditis elegans* relative to wild type or a major pilin mutant, implying a role in pathogenicity that is independent of pilus assembly. We hypothesized that loss of specific minor pilins relieves feedback inhibition on FimS-AlgR, increasing transcription of the minor pilin operon and other members of the AlgR regulon.

Reporter assays confirmed that FimS-AlgR were required for the increased expression from the minor pilin operon promoter upon loss of select minor pilins. Overexpression of AlgR or its hyperactivation via point mutation reduced virulence, and the virulence defects of *pilW*, *pilX*, and *pilY1* mutants were dependent on FimS-AlgR expression. We propose that PilY1 and the minor pilins inhibit their own expression, and that loss of these proteins leads to FimS-mediated activation of AlgR, and reduced expression of acute-phase virulence factors. This mechanism could contribute to adaptation of *P. aeruginosa* in chronic lung infections, as mutations in the minor pilin operon result in the loss of piliation and increased expression of AlgR-dependent virulence factors – such as alginate – that are characteristic of such infections.

Author summary

Pseudomonas aeruginosa causes dangerous infections, including chronic lung infections in cystic fibrosis patients. It uses many strategies to infect its hosts, including the use of grappling hook-like fibres called type IV pili. Among the components involved in assembly and function of the pilus are five proteins called minor pilins that - along with a larger protein called PilY1 - may help the pilus attach to surfaces. In a roundworm infection model, loss of PilY1 and specific minor pilins delayed killing, while loss of other pilus proteins did not. We traced this effect to increased activation of the FimS-AlgR regulatory system that inhibits expression of virulence factors used to initiate infections, while positively regulating chronic infection traits such as alginate production, a phenotype called mucoidy. A disruption in the appropriate timing of FimS-AlgR-dependent virulence factor expression when select minor pilins or PilY1 are missing may explain why those pilus-deficient mutants have reduced virulence compared with others whose products are not under FimS-AlgR control. Increased FimS-AlgR activity upon

47 loss of PilY1 and specific minor pilins could help explain the frequent co-occurrence of the non-
48 piliated and mucoid phenotypes that are hallmarks of chronic *P. aeruginosa* lung infections.
49

50 ## Introduction

51 *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen, recently listed as
52 one of the highest priority antimicrobial-resistant threats by the World Health Organization, due
53 to its high intrinsic antibiotic resistance and recalcitrance to therapy [1]. Among its virulence
54 factors are filamentous surface appendages called the type IV pili (T4P), sophisticated biological
55 nanomachines that are broadly distributed among bacteria and archaea [2, 3]. In *P. aeruginosa*,
56 T4P facilitate surface and host cell adhesion, colonization, biofilm maturation, virulence, and
57 twitching, a form of surface-associated motility facilitated by cycles of extension, adhesion, and
58 retraction of T4P fibres [3-11]. T4P are composed of hundreds to thousands of copies of small
59 proteins called major pilins (PilA in *P. aeruginosa*) along with the low abundance minor pilins
60 (MPs), FimU-PilVWXE [12-16]. The MPs are encoded in a polycistronic operon with the *pilY1*
61 gene that codes for a large ~125 kDa non-pilin protein. This operon is positively regulated by the
62 two-component system (TCS) FimS (AlgZ)-AlgR, where FimS is a predicted histidine sensor
63 kinase and AlgR is a response regulator that promotes expression of genes important for biofilms
64 and chronic cystic fibrosis (CF) lung infections [17-19]. The N-termini of immature pilins are
65 cleaved and methylated at the cytoplasmic face of the inner membrane (IM) by the prepilin
66 peptidase, PilD, while PilY1 may be processed by signal peptidase 1 [20-23]. Mature pilins are
67 polymerized into a T4P fibre via an envelope-spanning assembly machinery, where individual
68 PilA subunits are added or removed at the platform protein, PilC, via action of the ATPases PilB
69 and PilT, respectively [2, 24].

70 The MPs and PilY1 are required for T4P function in several bacterial species, including
71 *P. aeruginosa*, *Escherichia coli*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Myxococcus*
72 *xanthus* [12-15, 25-28]. PilY1 and the MPs were originally proposed to oppose pilus retraction,
73 as some surface pili remain in *pilY1* and MP mutants when retraction is blocked via deletion of
74 *pilT* [21, 26, 27, 29, 30]. We recently showed that deletion of the minor pseudopilins of the Xcp
75 type II secretion system in a *pilT* background lacking the T4P MPs abolished pilus assembly,
76 suggesting that when MPs are missing, the minor pseudopilins can prime extension, but cannot
77 counteract retraction [22]. We also demonstrated that PilY1 and the MPs are present in sheared
78 pili, and that the loss of PilV, PilW, PilX, or PilY1 excludes the other three components from the
79 pilus [22]. Thus, PilVWXY1 form a core assembly-initiation subcomplex, while FimU and PilE
80 connect this complex to PilA. Initiation of assembly with subsequent addition of PilA subunits
81 beneath would place the MPs at the pilus tip, with PilY1 – the largest component – at the distal
82 position, supporting the hypothesis that PilY1 is a T4P-associated adhesin [29].
83 PilY1 and the MPs (and their regulators FimS-AlgR) are required for T4P biogenesis, and
84 therefore T4P-mediated function [12-15, 17, 19]. However, recent studies hinted at more
85 enigmatic roles of PilWXY1 in virulence. Bohn et al. [31] showed that in a non-piliated *P.*
86 *aeruginosa* background, subsequent loss of *pilY1* reduced virulence in a *Caenorhabditis elegans*
87 fast killing assay and in a murine airway infection model, and increased resistance to killing by
88 neutrophils. Thus, PilY1 has a role in virulence that does not require functional pili. Other
89 studies using *C. elegans* infection models suggested that MP and *pilY1* mutants had attenuated
90 virulence relative to WT, and in one case, to a non-piliated mutant [32-35]. Recently, Siryaporn
91 et al. [36] showed that PilWXY1 were required for surface-activated virulence towards amoebae,
92 while other non-piliated mutants had WT virulence. The N-terminal region of PilY1 has limited

93 sequence similarity to the eukaryotic von Willebrand factor A (VWFa) domain, which can be
94 deformed by shear forces [37]. In-frame deletion of this domain from PilY1 allowed normally
95 avirulent planktonic cells to kill amoebae [36]. PilY1 was therefore proposed to be a
96 mechanosensor, where deformation of its VWFa domain upon surface interaction induced
97 expression of virulence factors. One important caveat of that study was that an *algR* mutant
98 (which also lacks PilY1 and the MPs) had WT virulence towards amoebae [36].

99 Deformation of PilA subunits by tensile forces acting upon surface attached pili was also
100 proposed as a possible way to signal surface attachment. Detection of partly unfolded pilins by
101 the Pil-Chp chemotactic system could lead to increased cyclic adenosine monophosphate
102 (cAMP) synthesis via the CyaB adenylate cyclase [38, 39]. cAMP is bound by Vfr (virulence
103 factor regulator), a key transcription factor that promotes expression of virulence factors
104 involved in motility, attachment, and secretion [38-40]. *fimS-algR* transcription is activated by
105 Vfr, leading to increased transcription of *fimU-pilVWXY1E* [38]. PilVWXY1 were proposed to
106 repress their own expression in an AlgR-dependent manner, as the loss of *pilV*, *pilW*, *pilX*, or
107 *pilY1* led to elevated expression of the MP operon and *fimS-algR* [21, 31, 36, 38]. The
108 mechanism of this putative feedback inhibition is largely uncharacterized, but has been
109 speculated to involve FimS [38].

110 Once expression of the MP operon is activated, extracellular PilY1 may sense surface
111 association via its VWFa domain and transduce this information through the T4P assembly
112 machinery [36, 38]. This signal is thought to activate an IM-localized diguanylate cyclase, SadC,
113 and increase levels of c-di-GMP, a second messenger that promotes expression of genes
114 associated with a biofilm lifestyle while repressing early-phase virulence traits such as swarming
115 motility [38, 41]. This model was supported by studies demonstrating that loss of *pilW*, *pilX*, or

116 *pilY1* in a high-c-di-GMP background resulted in hyper-swarming, indicative of reduced c-di-
117 GMP [37, 42]. Rodesney et al. [43] showed that c-di-GMP levels increased in response to shear
118 forces, and that functional T4P were required for this phenomenon, further supporting this
119 hypothesis. However, unlike *pilW*, *pilX*, and *pilY1* mutants, a *sadC* mutant had WT virulence
120 towards amoebae, suggesting the PilWXY1-SadC pathway may be important for surface-
121 sensing, but not necessarily for surface-activated virulence [36].

122 Although PilY1 and the MPs influence virulence, the underlying mechanism remains
123 unclear [31-34, 36]. We hypothesized that a subset of these components represses FimS activity,
124 such that loss of *pilW*, *pilX*, or *pilY1* activates FimS-AlgR, shifting the bacteria to a less
125 pathogenic phenotype typically associated with chronic infection. We found that *pilW*, *pilX*, and
126 *pilY1* mutants had attenuated virulence in *C. elegans* slow killing (SK) assays compared to WT
127 or a *pilA* mutant, and this was was dependent on FimS-AlgR, as double mutants had wild type
128 (WT) virulence. Hyperactivation (via phospho-mimetic point mutation) or overexpression of
129 AlgR alone was sufficient to attenuate virulence. Together, these data are consistent with a
130 model where loss of PilWXY1 relieves feedback inhibition on expression of the AlgR regulon,
131 resulting in dysregulation of virulence factors that are important for *C. elegans* pathogenesis.
132

133 ## Results

134 **PilWXY1 are important for T4P-independent virulence in PA14 and PAO1**

135 Specific genes in the MP operon were reported to be important for virulence in amoebae,
136 nematodes, and mouse models, but those studies were done using various strain backgrounds
137 [31-34, 36]. We first sought to confirm these results in the *C. elegans* SK model, using two well-
138 studied strains. SK assays were performed using PA14 with deletions of *pilA*, *fimU*, *pilV*, *pilW*,

139 *pilX*, *pilY1*, or *pilE* (Fig 1A). *fimU* and *pilE* mutants had WT virulence, while *pilW*, *pilX*, and
140 *pilY1* mutants had reduced virulence compared to WT. *pilW*, *pilX*, and *pilY1* mutants were also
141 less virulent than a *pilA* mutant, suggesting their reduced virulence was not due to loss of
142 functional T4P. *pilA* and *pilV* mutants had intermediate virulence, where they were significantly
143 less pathogenic than WT only in some trials. The twitching and virulence defects of *pilW*, *pilX*,
144 and *pilY1* mutants could be partially complemented by expression of the relevant gene *in trans*
145 (Supplementary Fig S1 and S2). The stoichiometry of PilY1 and the MPs is important for
146 optimal T4P function, which may explain the lack of full complementation [21]. To verify that
147 these phenotypes were not strain-specific, we tested PAO1 transposon-insertion mutants of *pilA*,
148 *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* in the SK assay (Fig 1B). Similar to the results in PA14,
149 PilWXY1 were important for T4P-independent virulence. However, the *fimU* and *pilV* mutants
150 were also less pathogenic than *pilA*; the PA14 and PAO1 MPs are divergent (61-75% amino acid
151 similarity), so it is possible that FimU and PilV function slightly differently in PAO1 versus
152 PA14 [44]. To focus on genes that were generally important for virulence of *P. aeruginosa*, we
153 undertook studies of the mechanism responsible for loss of virulence in the *pilW*, *pilX*, and *pilY1*
154 mutants.
155

156 **PilWXY1 promote virulence in a SadC-independent manner**

157 PilWXY1 were previously implicated in promoting c-di-GMP production via SadC, such
158 that loss of *pilW*, *pilX*, or *pilY1* resulted in a hyper-swarming, biofilm-deficient phenotype,
159 indicative of low intracellular c-di-GMP [37, 38, 42]. Therefore, we hypothesized that biofilm
160 defects of *pilW*, *pilX*, and *pilY1* might impede their ability to colonize the *C. elegans* gut, leading
161 to reduced virulence, and thus tested their ability to form biofilms in liquid SK medium. The

162 PA14 and PAO1 parent strains and their cognate *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE*
163 mutants formed negligible levels of biofilm in that medium (Supplementary Fig S3). Next, we
164 performed swarming motility assays using 0.5% agar SK plates, as swarming is inversely
165 correlated with biofilm formation [41, 45]. PA14 *pilW*, *pilX*, and *pilY1* mutants had swarming
166 defects (Fig 2), consistent with their reduced virulence in *C. elegans*, while all PAO1 strains had
167 low swarming on this media (S4 Fig). These data are inconsistent with reports that PilWXY1
168 promote c-di-GMP production, and instead suggest that the absence of *pilW*, *pilX*, or *pilY1*
169 promotes c-di-GMP production, resulting in repression of swarming. We next investigated
170 whether SadC-mediated biofilm formation affected virulence towards *C. elegans*, as would be
171 expected if the loss of virulence in *pilW*, *pilX*, and *pilY1* mutants was due to dysregulation of
172 SadC activity. However, there was no difference in virulence between PA14 and a *sadC* mutant
173 (Supplementary Fig S5). Further, overexpression of SadC led to a hyper-biofilm phenotype *in*
174 *vitro* but a slight reduction in virulence, demonstrating that the amount of biofilm formed does
175 not correlate with virulence in *C. elegans* (Supplementary Fig S5). Although the exact
176 mechanisms of *P. aeruginosa* pathogenesis in *C. elegans* are not fully understood, biofilms were
177 suggested to be important for establishment of infection [46-48]. However, in these assay
178 conditions, biofilms do not appear to be a major contributor to *P. aeruginosa* pathogenesis.
179

180 **PilVWXY1 repress expression of the minor pilin operon**

181 After ruling out involvement of the SadC pathway, we next explored the potential role of
182 FimS-AlgR in PilWXY1-mediated virulence. Informed by previous work in our laboratory,
183 showing that the sensor kinase PilS of the PilSR TCS interacts directly with PilA in the inner
184 membrane to regulate major pilin levels [49], we hypothesized that FimS interacts with one or

185 more MPs, and that loss of that interaction could lead to phosphorylation of AlgR and
186 subsequent upregulation of the MP operon. Bacterial two-hybrid (BACTH) assays were used to
187 identify potential interactions between FimS and PilA, FimU, PilV, PilW, PilX, or PilE (Fig 3A).
188 Positive interactions were identified between FimS and each pilin; however, based on our
189 experience with PilS, binding of pilins is necessary but not sufficient for regulation.

190 To decipher which MPs might modulate expression of the operon, we monitored
191 expression from the *fimU* promoter using a *luxCDABE* reporter. Compared to WT PA14, there
192 was a ~25-fold increase in luminescence in *pilV*, *pilW*, *pilX*, and *pilY1* mutants (Fig 3B). *fimU*
193 and *pilA* mutants had ~5-fold increased promoter activity, while a *pilE* mutant was comparable
194 to WT. *fimS* and *algR* mutants had low baseline luminescence, ~10-fold lower than WT. To
195 determine whether the increased promoter activity in *pilV*, *pilW*, *pilX*, and *pilY1* mutants was
196 dependent on FimS-AlgR, either *fimS* or *algR* was deleted in the *pilY1* mutant background. As
197 previously reported, the *pilY1 algR* double mutant had low luminescence (~10-fold lower than
198 WT), consistent with AlgR acting as a positive regulator of the MP operon [38]. Loss of *fimS* in
199 the *pilY1* mutant background also abolished *fimU* promoter activity (~10-fold lower than WT),
200 supporting the idea that FimS may monitor PilVWXY1 levels and activate AlgR when levels are
201 low. Based on these data, PilA, FimU, and PilE are unlikely to modulate FimS-AlgR activity
202 even though they can interact with FimS.

203

204 **Hyperactivation of AlgR results in attenuated virulence**

205 Thus far, our results supported the hypothesis that loss of PilWXY1 relieves feedback
206 inhibition on FimS-AlgR, resulting in AlgR activation. Next, we aimed to test whether
207 hyperactivation of AlgR alone could decrease virulence. We constructed chromosomal $algR_{D54E}$

208 phospho-mimetic point mutants [50] in both the PA14 and PAO1 backgrounds. We also
209 constructed *algR*$_{D54A}$ point mutants, as AlgR phosphorylation is required for transcription of a
210 subset of genes in its regulon, including the MP operon [17, 50, 51]. SK assays were performed
211 for PA14 and PAO1 *algR*$_{D54A}$ and *algR*$_{D54E}$ mutants, along with PA14 and PAO1 *fimS* and *algR*
212 deletion mutants. *algR*$_{D54E}$ mutants of both PA14 and PAO1 were less pathogenic than the
213 corresponding WT strains, while *algR* deletion and *algR*$_{D54A}$ mutants had WT virulence (Fig 4).
214 *fimS* mutants also had WT virulence. Loss of FimS-AlgR was shown previously to decrease
215 expression of the minor pilins and PilY1, and prevent pilus assembly [17, 38]. Because our data
216 show that loss of FimS-AlgR (and thus loss of minor pilin expression) had no impact on
217 virulence, we conclude that reduced virulence of *pilW*, *pilX*, and *pilY1* mutants is not due to loss
218 of those gene products directly, but rather to the resulting activation of FimS-AlgR.
219

220 **Overexpression of AlgR results in attenuated virulence**

221 Increased transcription of *fimS-algR* in a *pilY1* mutant relative to WT has been reported
222 [36], suggesting that reduced virulence could arise through increased levels of FimS-AlgR
223 expression as well as activation of the TCS. Therefore, we asked whether increased AlgR levels
224 would attenuate virulence. When *algR* was expressed *in trans* from a multicopy plasmid in PA14
225 *algR*, virulence was reduced compared to the vector-only control (Fig 5A). Because un-
226 phosphorylated AlgR can also affect transcription of a subset of genes [52, 53], we also tested
227 the same mutant complemented with AlgR$_{D54A}$. Complementation of the *algR* mutant with
228 AlgR$_{D54A}$ resulted in a severe virulence defect relative to the vector-only control. Thus, AlgR
229 hyperactivation and overexpression independently diminish *P. aeruginosa* virulence towards *C.*
230 *elegans*. Lastly, as AlgR was reported to be a positive regulator of biofilm formation [54], we

231 performed biofilm assays for PA14 *algR* complemented with AlgR or $AlgR_{D54A}$. Expression of
232 both AlgR and $AlgR_{D54A}$ led to hyper-biofilm formation (Fig 5B), further emphasizing that the
233 ability of a strain to form biofilms in SK medium does not correlate with virulence. Instead, we
234 suggest that virulence factors that are repressed by FimS-AlgR are important for *C. elegans* slow
235 killing, and thus an increase in AlgR levels and/or activity attenuates virulence.
236

237 **The virulence defects of *pilW*, *pilX* and *pilY1* mutants are dependent on FimS-AlgR**

238 To provide further support for this model, we asked whether the virulence defects of
239 PA14 *pilW*, *pilX*, and *pilY1* mutants were dependent on FimS-AlgR. We deleted *fimS* or *algR* in
240 the *pilW*, *pilX*, and *pilY1* backgrounds, and tested virulence of the double mutants (Fig 6). We
241 also deleted *pilW*, *pilX*, and *pilY1* in the $algR_{D54A}$ background, to test if AlgR activation was
242 required for the loss of virulence in *pilW*, *pilX*, and *pilY1* mutants. In all cases, the double
243 mutants had WT virulence, equivalent to that of the *fimS*, *algR*, or $algR_{D54A}$ single mutants.
244 These results demonstrate that both FimS and AlgR are required for the reduction in virulence
245 resulting from loss of PilWXY1. Although overexpression of $AlgR_{D54A}$ *in trans* repressed
246 virulence (Fig 5A), the chromosomal point mutation was sufficient to alleviate the virulence
247 defect of *pilW*, *pilX*, and *pilY1* mutants, suggesting that AlgR phosphorylation at residue D54 is
248 important for PilWXY1-mediated virulence.
249

250 **Loss of FimS-AlgR rescues swarming motility defects of *pilW*, *pilX*, and *pilY1* mutants**

251 After confirming that reduced virulence in PA14 *pilW*, *pilX*, and *pilY1* mutants was
252 dependent on FimS-AlgR, we compared swarming motility of PA14 *pilW*, *pilX*, *pilY1*, *fimS*,
253 *algR*, and $algR_{D54A}$ single and double mutants (Fig 7). *pilW*, *pilX*, and *pilY1* mutants had

254 swarming defects, while swarming of *fimS*, *algR*, and *algR*$_{D54A}$ mutants was similar to WT
255 PA14. Deletion of *fimS* or *algR* in the *pilW*, *pilX*, and *pilY1* backgrounds restored swarming to
256 the level of *fimS* and *algR* single mutants. Swarming of the *pilW algR*$_{D54A}$, *pilX algR*$_{D54A}$, and
257 *pilY1 algR*$_{D54A}$ mutants resembled that of the *algR*$_{D54A}$ single mutant. These data mirror the SK
258 assay results, in that the swarming defects of the *pilW*, *pilX*, and *pilY1* mutants are dependent on
259 FimS and AlgR. Taken together, our data support a model where PilWXY1 repress their own
260 expression via FimS-AlgR, and loss of PilWXY1 relieves this inhibition, activating AlgR in a
261 FimS-dependent manner. Activated AlgR then represses genes that are important for swarming
262 and virulence towards *C. elegans* under SK conditions.
263

264 **Discussion**

265 *P. aeruginosa* uses T4P to attach to surfaces and host cells, during biofilm maturation,
266 and to move across surfaces via twitching motility [2]. The MPs and PilY1 are important players
267 in T4P biogenesis and function, but also in regulation of swarming motility, surface attachment,
268 mechanosensation, and virulence [36-38, 42]. The MP operon is positively regulated by FimS-
269 AlgR, a TCS implicated in regulation of chronic *P. aeruginosa* lung infections [17-19]. Here, we
270 explored the potential connection between loss of PilWXY1 (and thus, loss of T4P) and AlgR
271 activation in virulence towards *C. elegans*. We showed that *pilW*, *pilX*, and *pilY1* mutants were
272 less virulent than WT or a *pilA* mutant – similar to the reduced virulence of these mutants
273 towards amoebae [36]. These data support the idea that PilWXY1 modulate virulence
274 independently of their role in T4P assembly. We confirmed previous reports [21, 31, 38] that in
275 the absence of *pilV*, *pilW*, *pilX*, or *pilY1*, expression of the MP operon is significantly increased,

276 and that this requires FimS-AlgR. Either hyperactivation or overexpression of AlgR reduced
277 virulence, while loss of *fimS* or *algR* in *pilW*, *pilX*, or *pilY1* backgrounds reverted it to WT levels.
278 These data suggest that FimS acts as a molecular thermostat to monitor MP levels, and in
279 their absence, activates AlgR to upregulate expression of the MP operon (Fig 8). A similar
280 regulatory mechanism was recently described for the PilSR TCS, where PilS phosphorylates
281 PilR when PilA levels are low, and dephosphorylates PilR when PilA levels are high [49]. It is
282 not yet clear whether FimS responds to PilVWXY1 individually or as a group, as these four
283 proteins are thought to form an inner membrane subcomplex that primes assembly of T4P [22,
284 55, 56]. When overexpressed individually *in trans*, each of the MPs inhibited twitching motility
285 in PAO1 [21], but since the others were still expressed from the chromosome, the exact nature of
286 the signal detected by FimS remains to be determined.
287 Kuchma et al. [37, 42] reported that loss of *pilW*, *pilX*, or *pilY1* increased swarming
288 motility and decreased biofilm formation, both indicative of low c-di-GMP levels. As biofilms
289 were proposed to contribute to *P. aeruginosa* pathogenesis in *C. elegans*, we investigated
290 whether the reduction in virulence in the absence of PilWXY1 was linked to decreased biofilm
291 via loss of SadC activation [46-48, 57]. In our hands, levels of *sadC* had no impact on virulence
292 even though they clearly modulated the amount of biofilm produced in SK media
293 (Supplementary Fig S5). Irazoqui et al. [48] examined the *C. elegans* gut during *P. aeruginosa*
294 infection, and described extracellular material that they suggested might indicate presence of a
295 biofilm. Anti-biofilm compounds reduced *P. aeruginosa* virulence towards *C. elegans*, but a
296 mechanism of action for those compounds has not been described [47]. Recently, the small RNA
297 SrbA was shown to modulate both biofilm and virulence towards *C. elegans*; however, deletion
298 of *srbA* led to altered transcription of at least 26 other genes that may also affect virulence [58].

299 Rather than using standard biofilm and swarming motility media, we performed these assays in
300 SK media to more closely mimic the conditions that the bacteria are exposed to in the SK assay.
301 To our knowledge, using SK media for swarming motility assays has only been attempted once
302 [33], and it has not been used for biofilm assays. As we found no correlation between biofilm
303 formation and virulence, we suggest that acute-phase virulence factors may be more important
304 for *C. elegans* pathogenesis in the SK model.

305 PilY1 and the MPs have been implicated in surface detection and activation of virulence
306 [36, 38]. Because loss of PilY1 or any of the MPs prevents T4P assembly and function, it is
307 important to distinguish phenotypes resulting from lack of those components specifically, rather
308 than loss of piliation [22]. Luo et al. [38] suggested that association of PilY1 with surfaces
309 transduces a signal through the T4P system to stimulate c-di-GMP production by SadC, while
310 Rodesney et al. [43] showed that loss of *pilA*, *pilY1*, or *pilT* prevents surface-activated c-di-GMP
311 production. Rodesney et al. [43] proposed that both PilY1 and functional T4P are required for
312 mechanosensation; however, it is not possible to delete *pilY1* without ablating T4P assembly.
313 Rather than PilY1 signalling through the T4P assembly machinery and SadC [38, 42], T4P-
314 mediated surface attachment may transiently deplete PilVWXY1 levels in the IM, resulting in
315 increased FimS-AlgR activity and transition towards a sessile, biofilm lifestyle. Surface-
316 activated increases in c-di-GMP levels are thought to occur within hours of attachment [38, 43];
317 this idea is consistent with our swarming data, where activation of FimS-AlgR in response to
318 PilVWXY1 depletion reduces swarming motility (Fig 2).

319 Whether the loss of *pilW*, *pilX*, or *pilY1* leads to increased amounts of AlgR, its increased
320 phosphorylation via FimS, or both remains to be clarified. Okkotsu et al. [50] showed that AlgR
321 and $AlgR_{D54E}$ levels are comparable, suggesting that the loss of virulence we observed for PA14

322 *algR*$_{D54E}$ is attributable to the D54E phospho-mimetic mutation alone. While overexpression of
323 AlgR$_{D54A}$ *in trans* reduced virulence (Fig 5A), the same mutation on the chromosome reverted
324 virulence of *pilW*, *pilX*, and *pilY1* mutants to WT levels (Fig 6). Therefore, we suspect that it is
325 primarily AlgR phosphorylation (or lack of AlgR dephosphorylation) that is responsible for loss
326 of virulence. However, it is possible that increased AlgR protein levels and phosphorylation both
327 contribute. Kong et al. [54] showed that AlgR binds *fimS-algR*, suggesting that the TCS could
328 positively regulate its own transcription in response to reduced PilWXY1 levels.

329 In addition to being essential for T4P function, FimS and AlgR control alginate
330 production in the context of chronic CF infections, where *algR* transcription is high [18, 59].
331 Phosphorylation of AlgR increases binding affinity at some – but not all – of its target sequences
332 [17, 50, 51, 53]. For example, AlgR$_{D54N}$ failed to support twitching motility, but did not affect
333 alginate production [17, 51]. FimS is an unorthodox histidine kinase, with 4 transmembrane
334 domains instead of the more typical 2, and lacks both a periplasmic sensing domain and the
335 canonical motif involved in ATP coordination, which mediates auto-phosphorylation [19, 60].
336 Direct interaction and/or phospho-transfer between FimS and AlgR have not been reported.
337 Rather, the idea that FimS acts as a kinase for AlgR comes from this and other studies
338 demonstrating similar phenotypes for *fimS, algR,* and *algR*$_{D54N}$ mutants [17, 18, 61]. FimS and
339 AlgR promote expression of genes important for production of alginate, biofilms, and c-di-GMP,
340 and inhibit expression of virulence factors such as the T3SS, pyocyanin, and quorum sensing
341 [54, 62-65]. Dynamic regulation of AlgR levels are important for pathogenesis, as either deletion
342 or overexpression of *algR* attenuated virulence in a murine model of acute septicemia [66].
343 However, an *algR* mutant was also reported to have WT virulence towards amoebae [36]. The
344 outcomes that result from interaction of *P. aeruginosa* with different hosts will depend on a

345 variety of factors including host defenses, site of infection, available nutrients, and virulence
346 repertoire of a particular strain. However, our results suggest that changes in the specific
347 repertoire of bacterial virulence factors, or the timing of their production, can tip the balance in
348 the host's favour.

349 The subset of AlgR-regulated virulence genes important for *C. elegans* pathogenesis is
350 not clearly defined. Screening of a PA14 transposon library for loss of virulence implicated
351 several genes encoding regulators rather than individual virulence factors, suggesting that *C.*
352 *elegans* pathogenesis is multifactorial [33]. In support of this hypothesis, a study of 18 WT *P.*
353 *aeruginosa* strains revealed no correlation between virulence and the presence of any specific
354 virulence gene [67]. We saw WT virulence for *algR* mutants, consistent with a role for AlgR in
355 negative regulation of acute phase virulence factors. Factors under positive control of AlgR may
356 be important during later stages of infection in more complex mammalian infection models [68,
357 69].

358 While important for the initial stages of infection, T4P are less critical in chronic CF lung
359 infections and are often lost when the bacteria grow for extended periods as biofilms [5, 70, 71].
360 *P. aeruginosa* CF isolates frequently become mucoid via activation of AlgR, leading to reduced
361 production of other virulence factors [59, 72, 73]. Although the two outcomes are not necessarily
362 temporally or mechanistically linked, mutations that achieve both may be advantageous during
363 chronic CF lung infections. Specifically, loss of PilWXY1 may be adaptive in the context of CF,
364 leading to AlgR activation and loss of T4P function. It will be interesting to examine the
365 genotypes of mucoid CF isolates for these types of mutations.

366

367

368 # Materials and methods

369 **Bacterial strains and plasmids**

370 Strains and plasmids used in this work are listed in Supplementary Table S1. Bacteria
371 were grown at 37°C for 16 h in 5 ml Luria Bertani Lennox (LB) broth, or on 1.5% agar LB
372 plates, unless otherwise specified. Plasmids were transformed into chemically-competent *E. coli*
373 by heat-shock, and into *P. aeruginosa* by electroporation [74]. Where appropriate, gentamicin
374 (Gm) was added at 15 µg/ml for *E. coli*, and 30 µg/ml for *P. aeruginosa*. Kanamycin (Kan) was
375 added at 50 µg/ml for *E. coli*, and 150 µg/ml for *P. aeruginosa*. Ampicillin (Amp) was added at
376 100 µg/ml for *E. coli*. L-Arabinose was added at 0.05% where indicated to induce expression
377 from the pBADGr promoter [75].

378

379 **Cloning procedures**

380 Vectors were constructed using standard cloning procedures, using the primers listed in
381 Supplementary Table S2. Deletion constructs were designed to contain 500-1000 bp homology
382 upstream and downstream the gene to be deleted. Deletion constructs for PA14 *fimU*, *pilV*, *pilW*,
383 *pilX*, *pilY1*, and *pilE* were synthesized by Genscript in the pUC57Kan vector. pEX18Gm-*sadC*
384 was created by amplifying the *sadC* deletion region from PA14 *sadC roeA* [41], followed by
385 digestion and ligation into pEX18Gm. pEX18Gm-$algR_{D54A}$, pEX18Gm-$algR_{D54E}$, and
386 pEX18Gm-*fimS* were made by overlap extension PCR [76]. pMS402-P*fimU* was created by
387 amplifying and digesting the promoter region of the PA14 MP operon. Digested pBADGr was
388 treated with alkaline phosphatase prior to ligation to avoid re-circularization of the vector.
389 Constructs were verified by Sanger sequencing (MOBIX lab, McMaster, Hamilton, ON).

390

391 **Mutant generation by allelic exchange**

392 Allelic exchange was used to remove or alter specific genes [77]. pEX18Gm suicide
393 plasmid derivatives (see Cloning procedures and Table 1) were used to create all mutants in this
394 work. After heat-shock transformation into *E. coli* SM10 cells, pEX18Gm constructs were
395 conjugated into corresponding PA14 or PAO1 parent strains. Cells were then transferred to
396 *Pseudomonas* isolation agar (PIA) Gm100 plates and incubated for 18 h at 37°C, to select for
397 integration of pEX18Gm derivatives into the chromosome. Colonies were streaked onto
398 LB/sucrose and incubated at 30°C for 18 h to select against merodiploids. Resultant colonies
399 were patched onto LB and LB Gm30 to identify gentamicin-sensitive colonies. Regions flanking
400 the desired mutations were amplified and sequenced to confirm success.

401

402 **Twitching motility assays**

403 Twitching motility assays were performed as previously described [78], with the
404 following modifications. Individual colonies were stab-inoculated in triplicate into 1% agar LB
405 solidified in plasma-treated tissue culture-grade plates (Thermo Fisher) and incubated at 30°C for
406 48 h. Agar was carefully removed and plates were stained with 1% crystal violet for 5 min.
407 Unbound dye was removed by rinsing with water, then stained twitching areas were measured
408 using ImageJ. Twitching zones were normalized to WT (100%).

409

410 **Swarming motility assays**

411 SM assays were performed as previously described [79], with the following
412 modifications. The concentration of agar in SK plates (50 mM NaCl, 0.5% agar, 0.35% peptone,
413 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 µg/ml cholesterol in 100% ethanol, 20 mM KH_2PO_4, and 5 mM

414 K_2HPO_4) was reduced to 0.5% (w/v) to promote swarming. Plates were dried individually for 1.5
415 h at room temperature then 3.5 µl of an overnight culture was spotted onto the center of the
416 duplicate plates. Plates were individually incubated upright at 37°C for 24 h, followed by
417 incubation at 22°C for 24 h. Empty swarming motility plates were placed around the perimeter
418 of arrayed experimental plates to reduce edge effects and promote formation of reproducible
419 swarming zones. Experiments were repeated three times, 2 technical replicates per trial.
420

421 **Biofilm assays**

422 Biofilm assays were performed as previously described, with modifications [80]. *P.*
423 *aeruginosa* cultures were grown for 16 h at 37°C, diluted 1:200 in fresh LB, and grown to OD_{600}
424 ~ 0.1. Cultures were then diluted 1:500 in liquid SK media (50 mM NaCl, 0.35% peptone, 1 mM
425 $CaCl_2$, 1 mM $MgSO_4$, 5 µg/ml cholesterol in EtOH, 20 mM KH_2PO_4, and 5 mM K_2HPO_4), then
426 96-well plates were inoculated with 150 µl each strain, in triplicate. Sterility controls (liquid SK
427 media) were included throughout the plate to check for contamination. Plates were covered with
428 peg lids (Nunc) then wrapped in parafilm and incubated at 37°C for 24 h, shaken at 200 rpm.
429 After incubation, the OD_{600} of the plate was measured to check for uniform growth and lack of
430 contamination. Peg lids were washed for 10 min in 200 µl/well 1X phosphate-buffered saline
431 (PBS), then stained with 200 µl/well 0.1% (w/v) crystal violet for 15 min. Unbound crystal violet
432 was removed by washing lids in 70 ml distilled water 5 times at 10 min intervals. Crystal violet
433 was solubilized from lids in 200 µl/well 33.3% acetic acid, then the absorbance at 600 nm was
434 measured. Optical density and absorbance at 600 nm were plotted for growth and biofilm
435 formation, respectively, then analyzed by one-way ANOVA followed by Dunnett post-test to

436 compare each mutant to the WT control, p = 0.05. Error bars indicate standard error of the mean.
437 Representative wells of acetic acid-solubilized crystal violet were imaged.
438
439 ***Caenorhabditis elegans* slow killing assay**
440 SK assays were performed as described previously [81]. SK plates (0.35% peptone, 50
441 mM NaCl, 2% agar, 1 mM $CaCl_2$, 5 µg/ml cholesterol, 1 mM $MgSO_4$, 20 mM KH_2PO_4, 5 mM
442 K_2HPO_4, 100 µM FUDR) were seeded with 100 µl of an overnight culture and incubated
443 overnight at 37°C. The following day, plates were enriched with 1 ml of an overnight culture
444 concentrated to 100 µl. Synchronized L4 worms were collected from *E. coli* OP50 plates, washed
445 twice in M9 buffer, and then >50 worms were seeded onto each bacterial lawn on individual SK
446 plates. SK plates were incubated at 25°C and scored for dead worms every 24 h. Worms were
447 considered dead when they did not respond to touch, and were removed from the plate. OP50
448 was included as a negative control for virulence. Percent survival was plotted as a function of
449 time. Survival curves were plotted on GraphPad Prism 5.00 for Windows, then compared using
450 the Gehan-Breslow-Wilcoxon test, p = 0.05. Each assay was performed at least 3 times, and
451 differences were only considered significant if they were reproducible in all trials.
452 Representative trials are shown.
453
454 **Luminescent reporter assay**
455 Luminescent reporter assays were performed as previously described, with minor
456 modifications [49]. Various strains harbouring the pMS402-P*fimU* plasmid, encoding the
457 luciferase genes under control of the *fimU* promoter, were grown for 16 h at 37°C in LB Kan150,
458 then diluted 1:50 in fresh LB Kan150. Subsequently, 100 µl of each culture was added to white-

459 walled, clear-bottom 96-well plates (Corning) in triplicate, and incubated with shaking at 37°C in
460 a Synergy 4 microtiter plate reader (BioTek). Luminescence readings were taken every 15 min
461 for 5 h, and normalized to growth (OD_{600}) at each time point. Readings that exceeded the limit of
462 detection (>4 000 000 luminescence units) were discarded. At least 3 individual trials were
463 performed. Error bars indicate standard error of the mean.
464
465 **Bacterial two-hybrid β-galactosidase activity assay**
466 To test for interactions between FimS and individual pilins, bacterial two-hybrid
467 (BACTH) assays were performed as previously described [82]. pUT18C and pKT25 derivatives,
468 encoding the T18 and T25 domains of the *Bordetella pertussis* CyaA adenylate cyclase fused to
469 the N-terminus of FimS, PilA, FimU, PilV, PilW, PilX, or PilE [22, 49, 83], were co-transformed
470 into *E. coli* BTH 101 to screen for pairwise interactions. Each interaction was tested in both
471 orientations, with FimS in pUT18C and each pilin in pKT25, or FimS in pKT25 and each pilin in
472 pUT18C. Single colonies were inoculated in 5 ml LB Amp100 Kan50 and grown overnight. The
473 following day, 100 µl was inoculated into 5 ml fresh media and grown toOD_{600} = 0.6, then 5 µl
474 was spotted onto MacConkey plates (1.5% agar, 100µg/ml ampicillin, 50µg/ml kanamycin, 1%
475 (w/v) maltose, 0.5mM isopropyl b-D-thiogalactopyranoside) (Difco). Plates were incubated at
476 30°C for 24 h. An interaction was considered positive when colonies appeared pink, as a result
477 of fermentation of maltose. BTH 101 expressing pUT18C and pKT25 empty vectors was used as
478 a negative control, and BTH 101 expressing pUT18C-*fimS* and pKT25-*fimS* was used as a
479 positive control [49].
480
481

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486

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717

718 **Figure captions**

719 **Fig 1**. **PilWXY1 contribute to T4P-independent virulence.**

720 (A) SK assays for PA14 *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. Synchronized L4
721 worms were seeded onto SK plates and scored for death every 24 h, then plotted as "percent
722 survival" over the course of the assay. "Day" represents the number of days after L4 on which
723 the plates were scored. PA14 *fimU* and *pilE* mutants had similar virulence to WT, *pilA* and *pilV*

724 mutants were slightly less virulent than WT in some trials, and *pilW, pilX,* and *pilY1* mutants
725 were less virulent than all other strains tested. (B) (A) SK assays for PAO1 *pilA*, *fimU*, *pilV*,
726 *pilW*, *pilX*, *pilY1*, and *pilE* mutants. The PAO1 *pilE* mutant had similar virulence to WT, the *pilA*
727 mutant was slightly less virulent in some trials, and *fimU*, *pilV*, *pilW*, *pilX*, and *pilY1* mutants
728 were much less virulent. In (A) and (B), asterisks indicate strains that were consistently less
729 virulent than a *pilA* mutant in all trials by Gehan-Breslow-Wilcoxon test at p = 0.05, n = 3 trials.
730

731 **Fig 2. PA14 *pilW*, *pilX*, and *pilY1* mutants have swarming motility defects.**
732 SM assays for PA14 *pilA, fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. SM assays were
733 performed in duplicate on 0.5% agar SK plates, and imaged after 24 h incubation at 37°C and 24
734 h at 22°C. PA14 *pilW*, *pilX*, and *pilY1* mutants had reduced SM relative to WT. Representative
735 images are shown, n = 3 trials.
736

737 **Fig 3. PilVWXY1 repress their expression via FimS-AlgR.**
738 (A) BACTH assays for FimS, PilA, and MPs. Protein fusions with T18 and T25 fragments of the
739 CyaA adenylate cyclase were screened for interactions on MacConkey plates. FimS interacted
740 with PilA, FimU, PilV, PilW, PilX, and PilE in at least one orientation. Positive (+) or negative
741 (-) interactions are indicated below each image, n = 3. (B) *fimU* promoter activity in PA14 *pilA*,
742 *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilE*, *fimS*, *algR*, *pilY1 fimS*, or *pilY1 algR* mutants. pMS402-
743 P*fimU*, containing the *fimU* promoter upstream of the *lux* genes, was introduced into strains of
744 interest. Assays were set up in technical triplicate, and measurements were taken every 15 min
745 over 5 h. Loss of *pilV*, *pilW*, *pilX*, or *pilY1* led to highly elevated *fimU* promoter activity. *pilA*

746 and *fimU* mutants had slightly increased promoter activity relative to WT. Loss of *fimS* or *algR*
747 reverted *fimU* promoter activity in the *pilY1* mutant to baseline. n = 3 trials.
748
749 **Fig 4**. **AlgR hyperactivation reduces virulence.**
750 (A) SK assays for PA14 *fimS*, *algR*, $algR_{D54A}$, and $algR_{D54E}$ mutants. The *fimS*, *algR,* and
751 $algR_{D54A}$ mutants had WT virulence, while the $algR_{D54E}$ mutant was less virulent than WT. (B)
752 SK assays for PAO1 *algR*, $algR_{D54A}$, and $algR_{D54E}$ mutants. The *algR* and $algR_{D54A}$ mutants had
753 WT virulence, while the $algR_{D54E}$ mutant was less virulent than WT. For (A) and (B), asterisks
754 indicate strains that were less virulent than WT in all trials, by Gehan-Breslow-Wilcoxon test at
755 $p = 0.05$, n = 3 trials.
756
757 **Fig 5. AlgR promotes biofilm formation and represses virulence.**
758 (A) SK assays for *algR* deletion and overexpression strains. Loss of *algR* had no effect on SK,
759 while overexpression of pBADGr-*algR* or pBADGr-$algR_{D54A}$ reduced virulence at 0.05%
760 arabinose. Asterisks indicate strains that were consistently less virulent than PA14 + pBADGr in
761 all trials by Gehan-Breslow-Wilcoxon test at $p = 0.05$, n = 3 trials. (B) Biofilm assays for *algR*
762 deletion and overexpression strains. Microtiter plate biofilm assays were performed in liquid SK
763 media over 24 h, in triplicate. Biofilms were stained with 1% crystal violet then solubilized in
764 acetic acid. Loss of *algR* had no effect on biofilm formation. When grown at 0.05% arabinose,
765 overexpression of pBADGr-*algR* or pBADGr-$algR_{D54A}$ increased biofilm formation, $p < 0.001$.
766 Significance was determined by one-way ANOVA followed by Dunnett post-test relative to WT,
767 n = 3 trials.
768

769 **Fig 6. The virulence defect of *pilW*, *pilX*, and *pilY1* mutants is dependent on FimS-AlgR.**

770 SK assays for *pilW*, *pilX*, *pilY1*, *fimS*, *algR*, and $algR_{D54A}$ single and double mutants. *fimS*, *algR*,

771 and $algR_{D54A}$ mutants have WT virulence. *pilW*, *pilX*, and *pilY1* have reduced virulence relative

772 to WT, *fimS*, *algR*, and $algR_{D54A}$ mutants. Combination of *pilW*, *pilX*, or *pilY1* mutations with

773 *fimS*, *algR*, or $algR_{D54A}$ mutations results in virulence equivalent to *fimS*, *algR*, and $algR_{D54A}$

774 single mutants, respectively. All graphs represent 1 trial, separated into 3 graphs for clarity.

775 Asterisks indicate strains that were consistently less virulent than PA14 by Gehan-Breslow-

776 Wilcoxon test at $p = 0.05$, $n = 3$.

777

778 **Fig 7. The swarming motility defect of *pilW*, *pilX*, and *pilY1* mutants is dependent on FimS-**

779 **AlgR.**

780 SM assays for *pilW*, *pilX*, *pilY1*, *fimS*, *algR*, and $algR_{D54A}$ single and double mutants. The

781 reduction in SM of *pilW*, *pilX*, and *pilY1* mutants was dependent on FimS-AlgR. *pilW*, *pilX*, and

782 *pilY1* had reduced SM relative to WT. *fimS*, *algR*, and $algR_{D54A}$ mutants had WT swarming.

783 Double mutants swarmed similar to *fimS*, *algR*, and $algR_{D54A}$ single mutants. Representative

784 images are shown, $n = 3$.

785

786 **Fig 8. Model for regulation of the MP operon via FimS-AlgR.**

787 When PilVWXY1 are absent, FimS promotes phosphorylation of AlgR (phosphate indicated by

788 yellow star). Phospho-AlgR binds the *fimU* promoter to promote expression of *pilY1* and the MP.

789 Phospho-AlgR promotes expression of genes associated with chronic infections, and represses

790 those associated with acute infections. As PilVWXY1 accumulate in the IM, they are detected by

791 FimS, leading to reduced AlgR phosphorylation. Abbreviations: PilV, V (orange); PilW, W
792 (cyan); PilX, X (pink); PilY1, Y1 (dark purple); IM, inner membrane.
793
794 **Supporting information**
795 **S1 Fig. Twitching motility of *pilW*, *pilX*, and *pilY1* mutants can be complemented *in trans*.**
796 Colonies were stab-inoculated into 1% agar LB plates, in triplicate. Plates were stained with
797 crystal violet after 48 h at 30°C. Complementation of PA14 *pilW*, *pilX*, and *pilY1* mutants with
798 pBADGr-*pilW*, pBADGr-*pilX*, or pBADGr-*pilY1*, respectively, led to increased TM relative to
799 complementation with pBADGr alone. Numbers indicate percent twitching area relative to WT,
800 n = 3.
801
802 **S2 Fig. Virulence of *pilW*, *pilX*, and *pilY1* mutants can be complemented *in trans*.**
803 Complementation of PA14 *pilW*, *pilX*, and *pilY1* mutants with pBADGr-*pilW*, pBADGr-*pilX*, or
804 pBADGr-*pilY1*, respectively, restored virulence to near-WT levels. Asterisks indicate strains that
805 were consistently less virulent than WT by Gehan-Breslow-Wilcoxon test at p = 0.05, n = 3.
806 Individual graphs represent separate trials.
807
808 **S3 Fig. PA14 and PAO1 produce low levels of biofilm in liquid slow killing media.**
809 Biofilm assays for (A) PA14 and (B) PAO1 *pilA, fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants.
810 Very little biofilm formation was detectable in liquid SK media for any strains. There were no
811 differences in biofilm formation as determined by one-way ANOVA followed by Dunnett post-
812 test relative to WT at p = 0.05, n = 3.
813

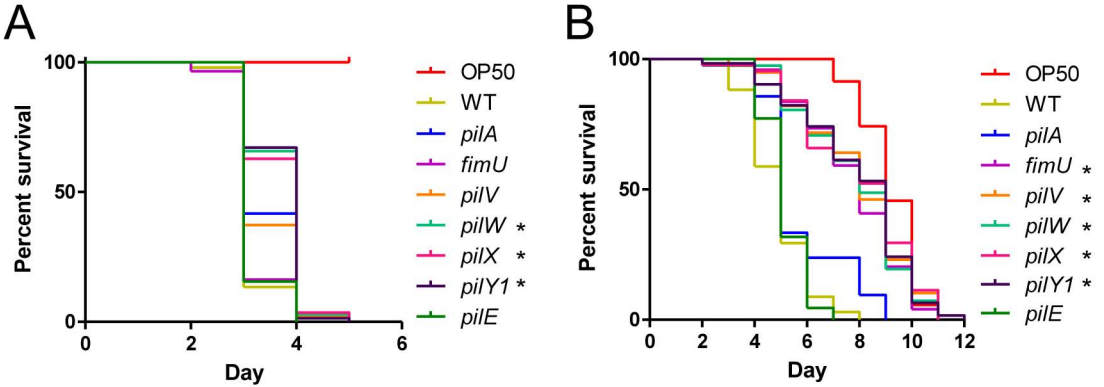
814 **S4 Fig. PAO1 does not swarm optimally on 0.5% agar slow killing plates.**

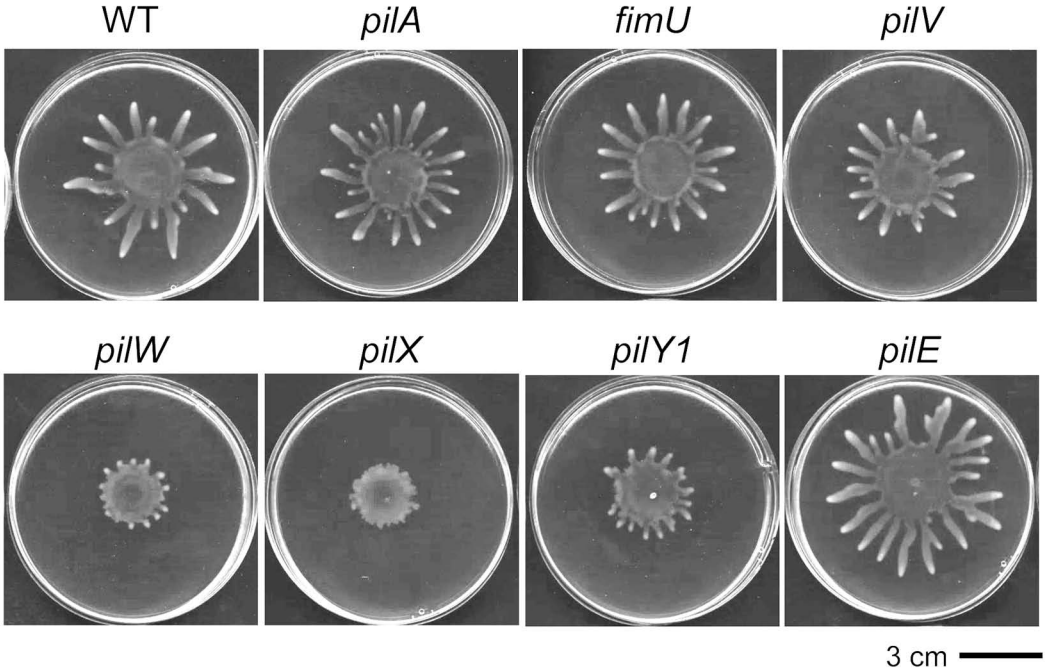
815 SM assays for PAO1 *pilA, fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. SM assays were
816 performed in duplicate on 0.5% agar SK plates, and imaged after 24 h incubation at 37°C and 24
817 h at 22°C. All strains formed very small smarming zones, and the patterns were not reproducible
818 between strains. Representative images are shown, n = 3.

819

820 **S5 Fig. SadC promotes biofilm formation but is not required for virulence.**

821 (A) Biofilm assays for *sadC* deletion and overexpression strains. PA14 *sadC* biofilm levels were
822 similar to WT. Expression of SadC *in trans* from a multicopy plasmid led to increased biofilm
823 formation relative to WT at 0% (due to leaky promoter) and 0.05% arabinose, $p < 0.001$.
824 Significance was determined by one-way ANOVA followed by Dunnett post-test relative to
825 PA14 + pBADGr, n = 3. (B) SK assays for *sadC* deletion and overexpression strains.
826 Overexpression of SadC led to a subtle but reproducible loss of virulence relative to WT at 0%
827 and 0.05% arabinose. A *sadC* mutant had WT virulence. Asterisks indicate strains that were
828 consistently less virulent than PA14 + pBADGr by Gehan-Breslow-Wilcoxon test at $p = 0.05$, n
829 = 3.


A
Percent survival
100
50
0
0
2
4
6
Day
OP50
WT
pilA
fimU
pilV
pilW *
pilX *
pilY1 *
pilE
B
Percent survival
100
50
0
0
2
4
6
8
10
12
Day
OP50
WT
pilA
fimU *
pilV *
pilW *
pilX *
pilY1 *
pilE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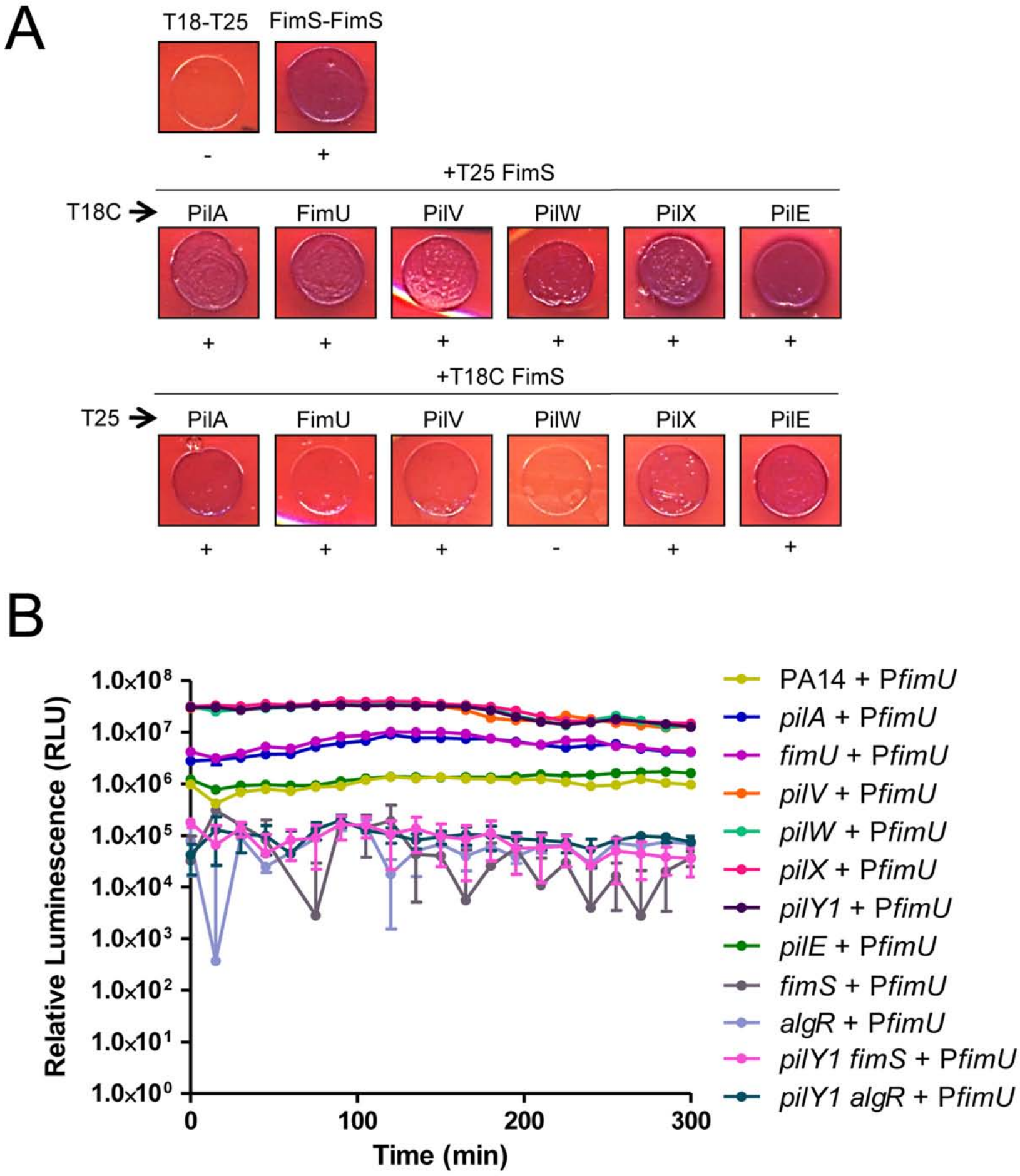
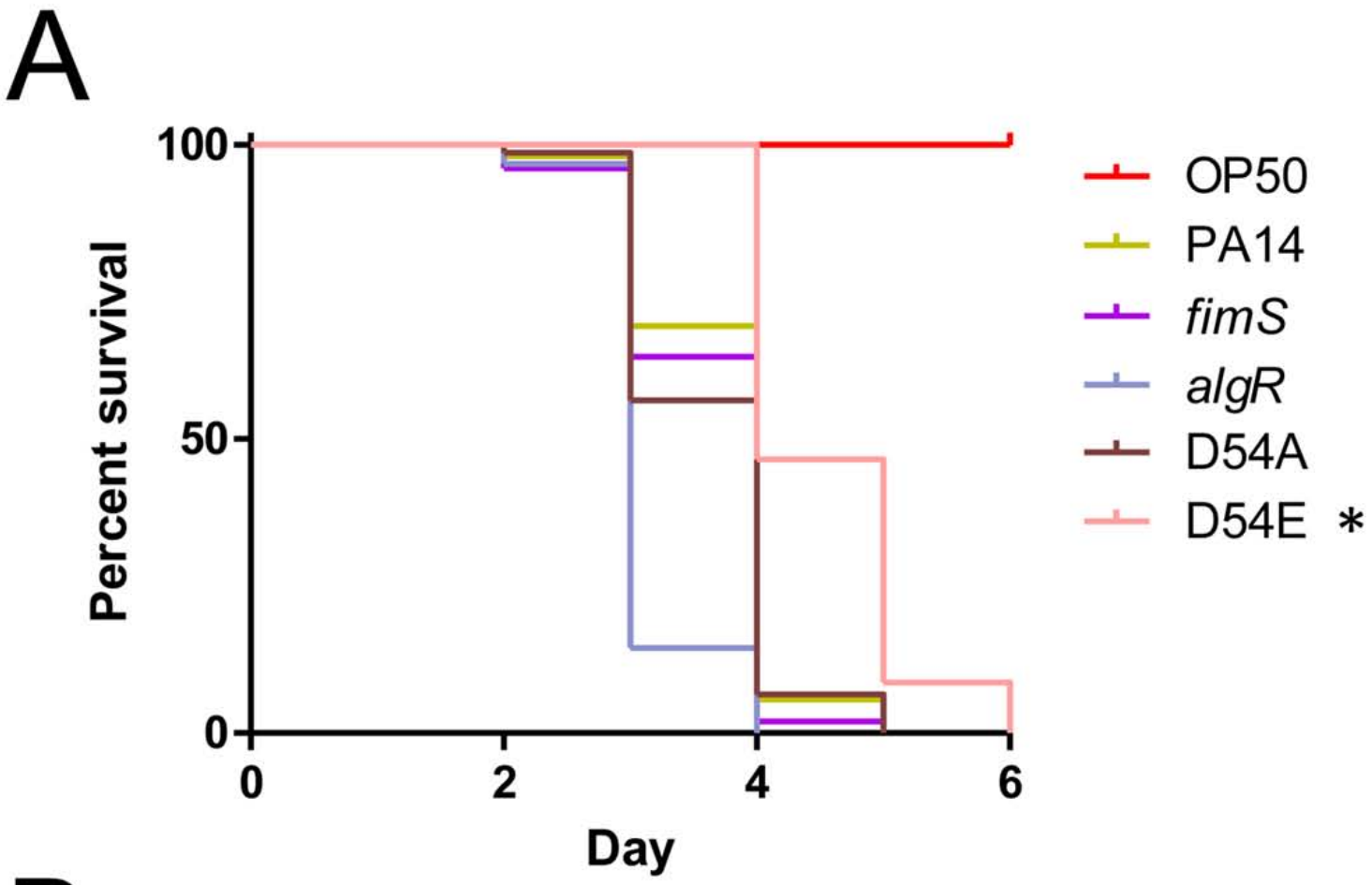
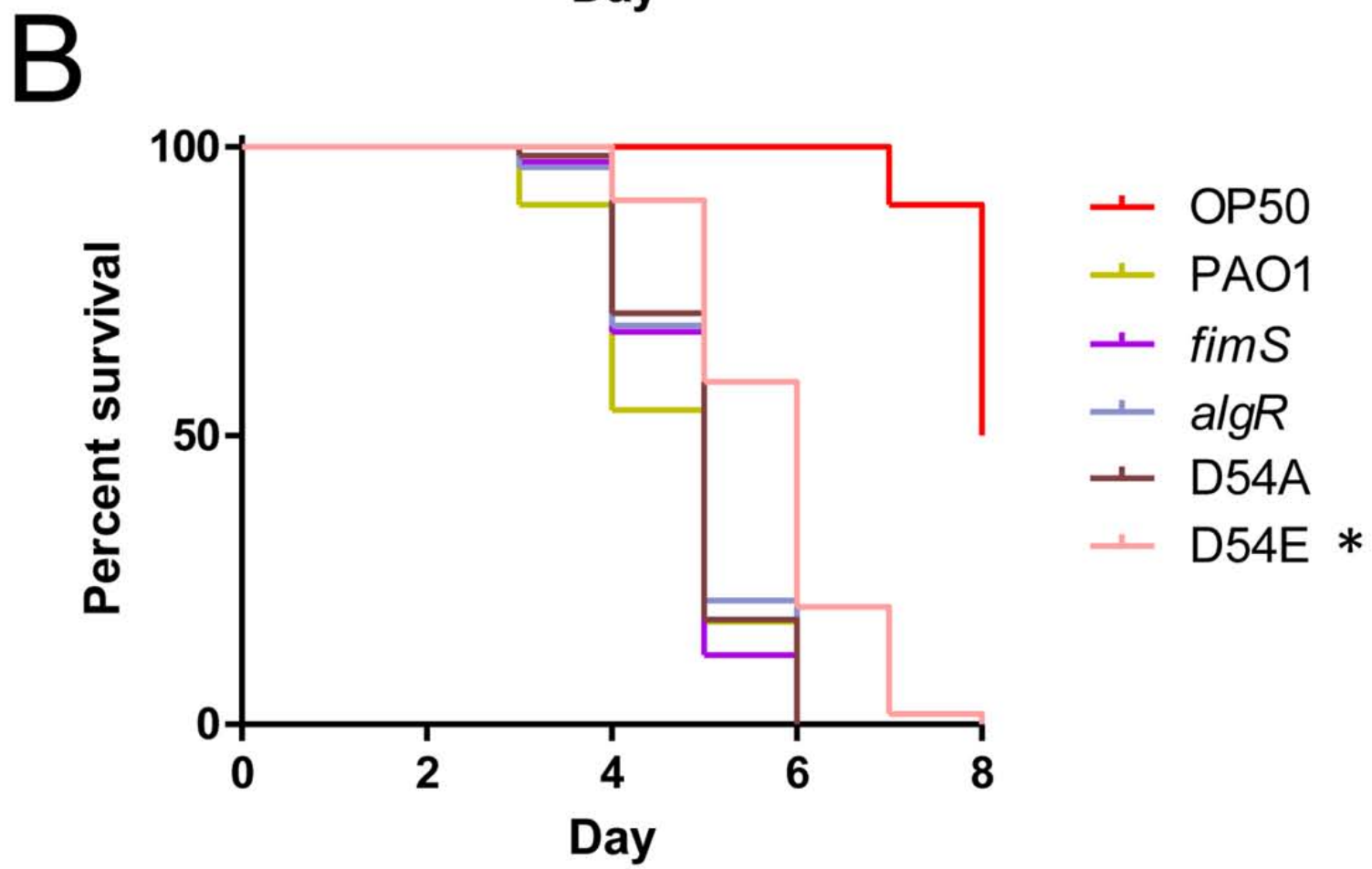
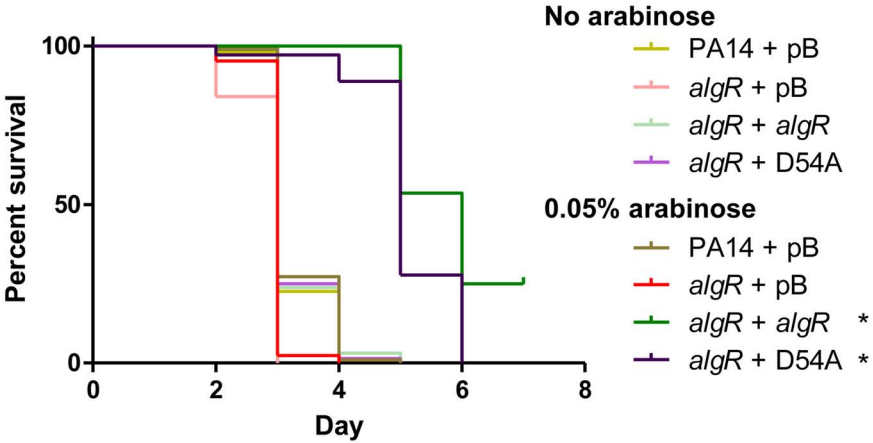
pilA
fimU
pilV
pilW
pilX
pilY1
pilE
3 cm


A
T18-T25
FimS-FimS
-
+
+T25 FimS
T18C
PilA
FimU
PilV
PilW
PilX
PilE
+
+
+
+
+
+
+T18C FimS
T25
PilA
FimU
PilV
PilW
PilX
PilE
+
+
+
-
+
+
B
Relative Luminescence (RLU)
1.0x10^8
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
1.0x10^0
0
100
200
300
Time (min)
PA14 + PfimU
pilA + PfimU
fimU + PfimU
pilV + PfimU
pilW + PfimU
pilX + PfimU
pilY1 + PfimU
pilE + PfimU
fimS + PfimU
algR + PfimU
pilY1 fimS + PfimU
pilY1 algR + PfimU

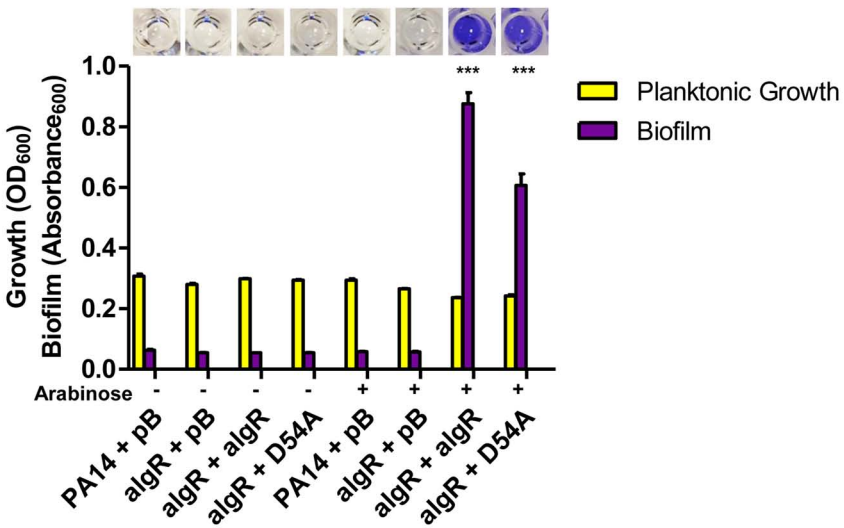

A
100
50
0
Percent survival
0
2
4
6
Day
OP50
PA14
fimS
algR
D54A
D54E *

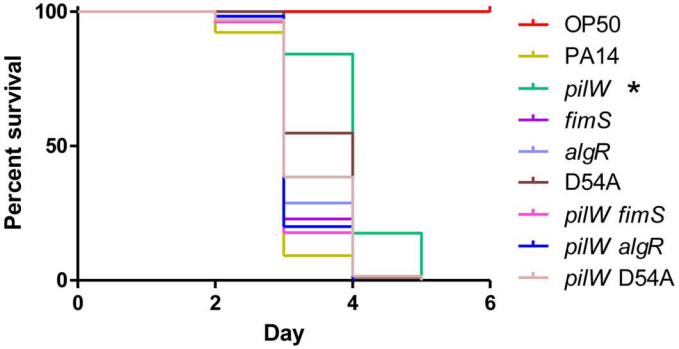

B
100
50
0
Percent survival
0
2
4
6
8
Day
OP50
PAO1
fimS
algR
D54A
D54E *

A


No arabinose
PA14 + pB
algR + pB
algR + algR
algR + D54A
0.05% arabinose
PA14 + pB
algR + pB
algR + algR *
algR + D54A *
Percent survival
100
50
0
0
2
4
6
8
Day

B


Planktonic Growth
Biofilm
Growth (OD600)
Biofilm (Absorbance600)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
Arabinose
- - - - + + + +
PA14 + pB
algR + pB
algR + algR
algR + D54A
PA14 + pB
algR + pB
algR + algR
algR + D54A


100
50
0
Percent survival
0
2
4
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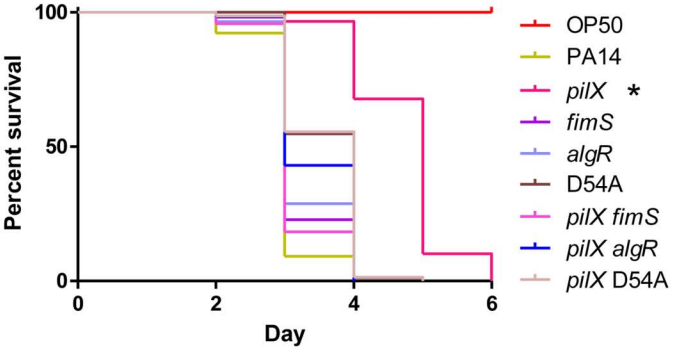
Day
OP50
PA14
pilW *
fimS
algR
D54A
pilW fimS
pilW algR
pilW D54A


100
50
0
Percent survival
0
2
4
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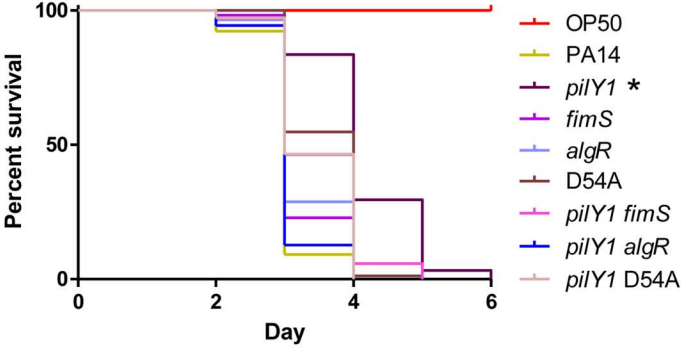
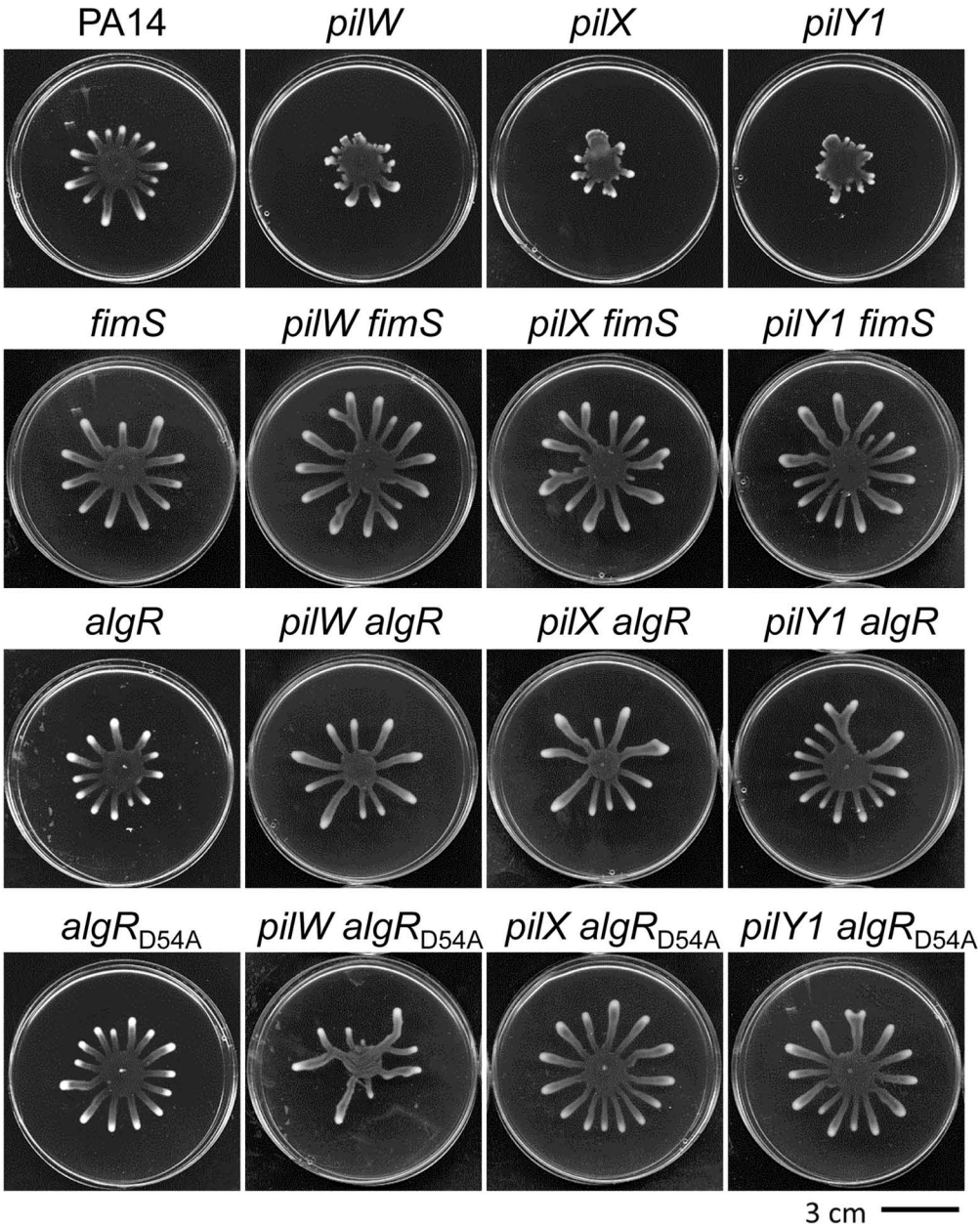
Day
OP50
PA14
pilX *
fimS
algR
D54A
pilX fimS
pilX algR
pilX D54A


100
50
0
Percent survival
0
2
4
6
Day
OP50
PA14
pilY1 *
fimS
algR
D54A
pilY1 fimS
pilY1 algR
pilY1 D54A


PA14
pilW
pilX
pilY1
fimS
pilW fimS
pilX fimS
pilY1 fimS
algR
pilW algR
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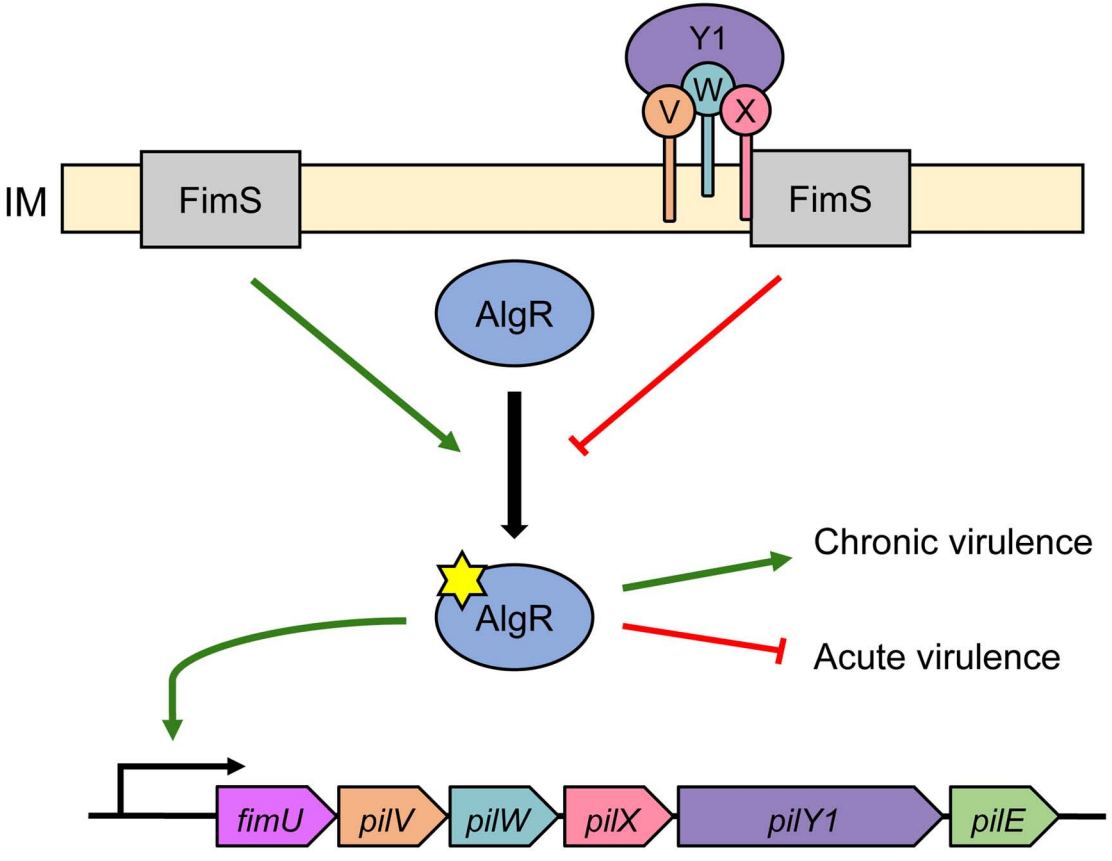
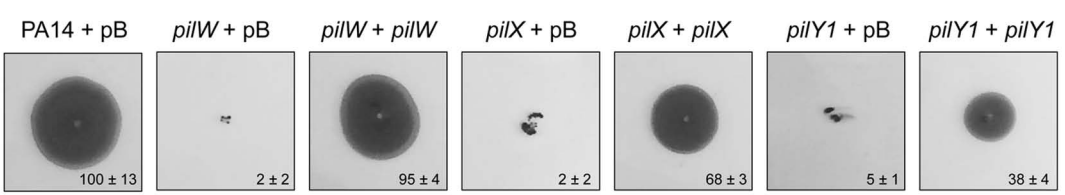
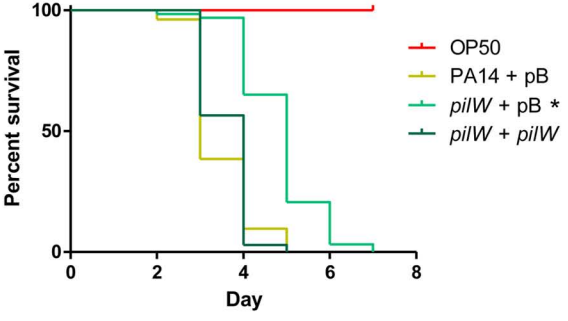
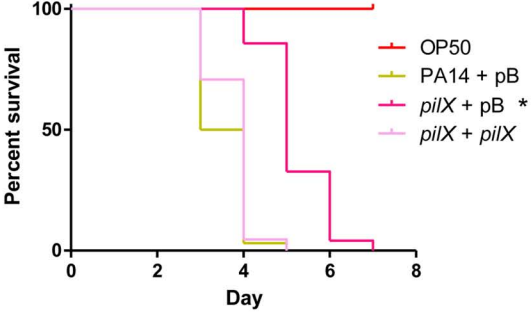
pilY1 algR D54A
3 cm


Y1
W
V
X
IM
FimS
FimS
AlgR
AlgR
Chronic virulence
Acute virulence
fimU
pilV
pilW
pilX
pilY1
pilE


PA14 + pB
pilW + pB
pilW + *pilW*
pilX + pB
pilX + *pilX*
pilY1 + pB
pilY1 + *pilY1*
100 ± 13
2 ± 2
95 ± 4
2 ± 2
68 ± 3
5 ± 1
38 ± 4


100
50
0
Percent survival
0
2
4
6
8
Day
OP50
PA14 + pB
pilW + pB *
pilW + *pilW*


100
50
0
Percent survival
0
2
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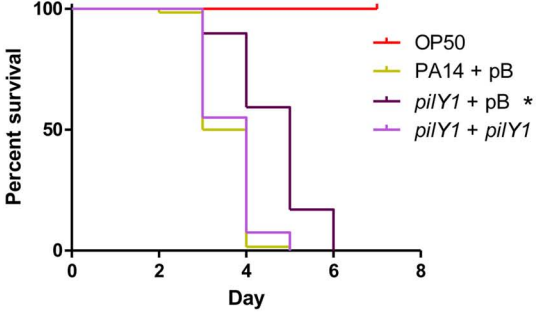
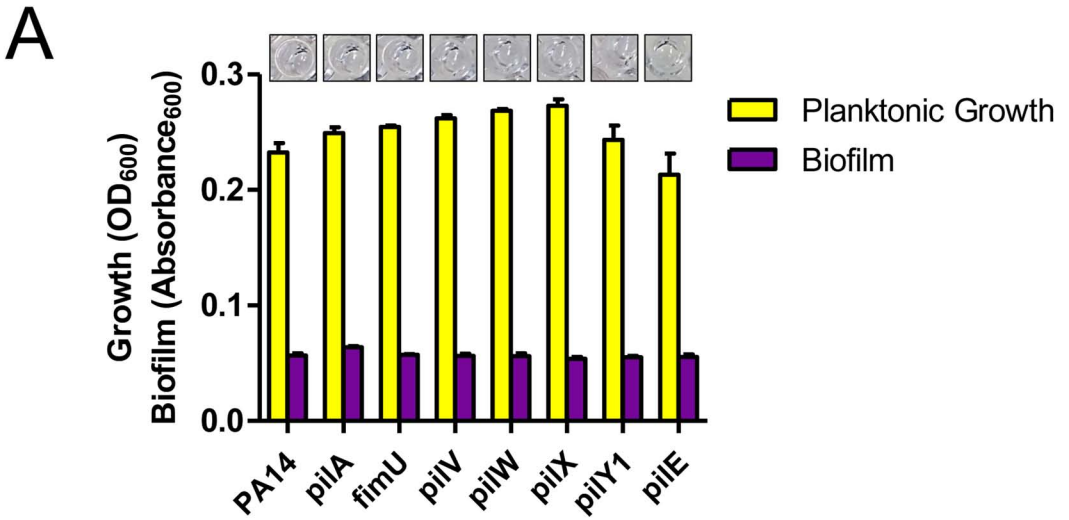
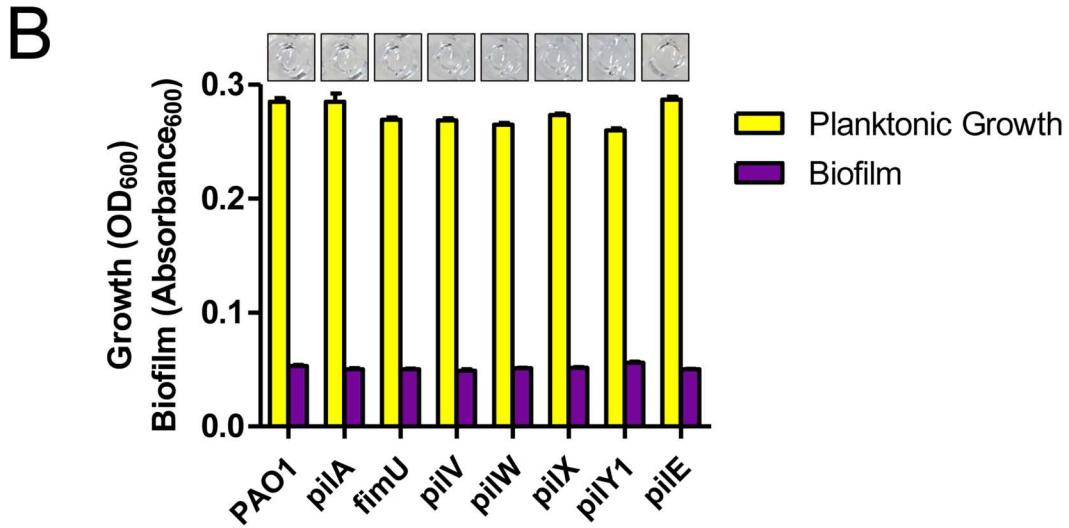
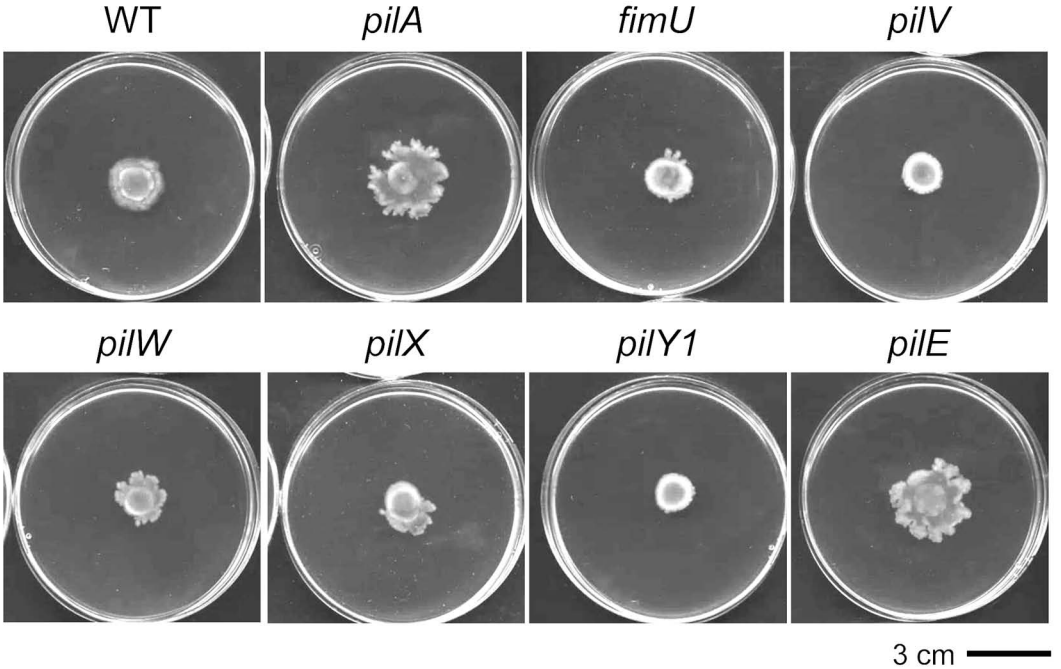
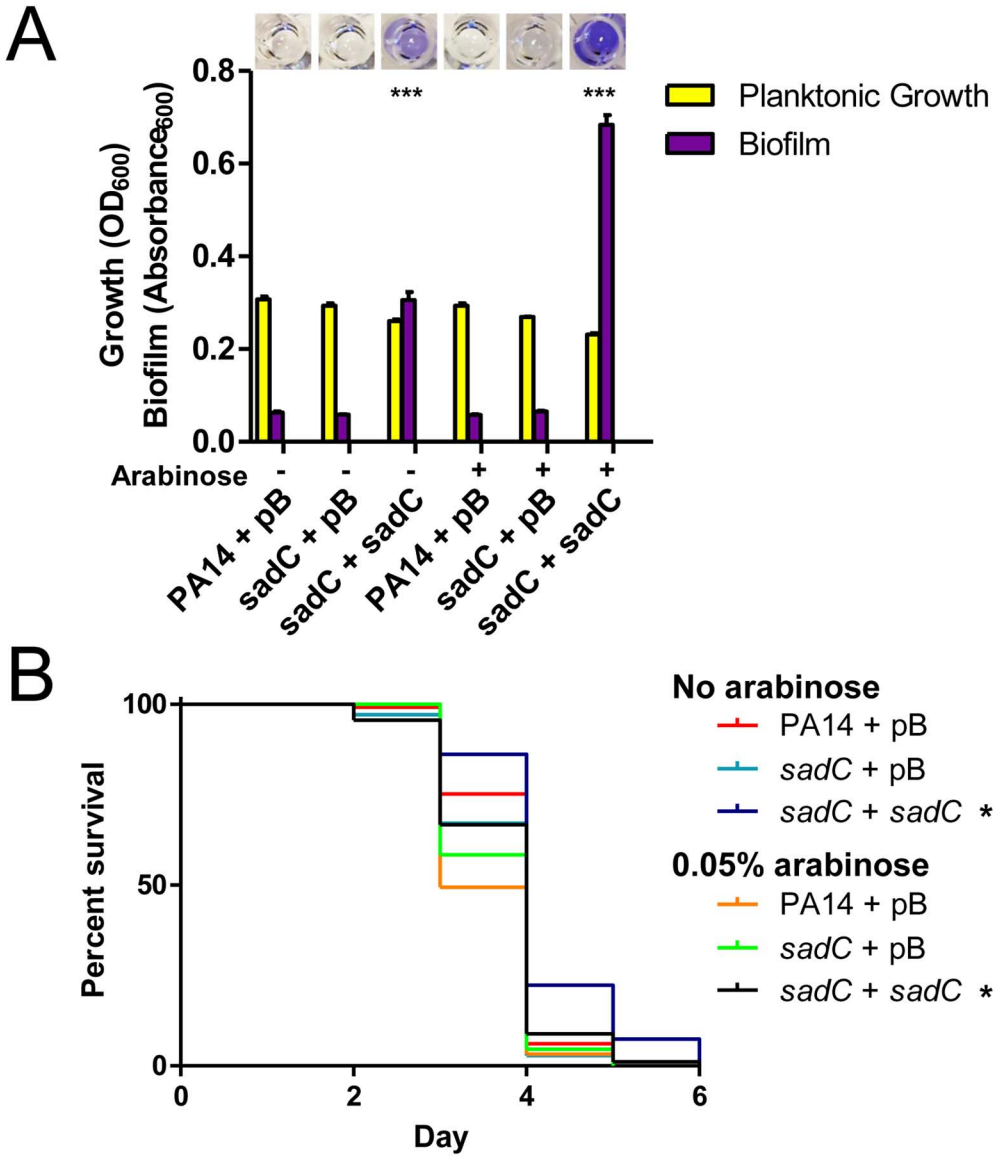
8
Day
OP50
PA14 + pB
pilX + pB *
pilX + *pilX*


100
50
0
Percent survival
0
2
4
6
8
Day
OP50
PA14 + pB
pilY1 + pB *
pilY1 + *pilY1*


A
Growth (OD600)
Biofilm (Absorbance600)
0.3
0.2
0.1
0.0
Planktonic Growth
Biofilm
PA14
pilA
fimU
pilV
pilW
pilX
pilY1
pilE


B
Growth (OD600)
Biofilm (Absorbance600)
0.3
0.2
0.1
0.0
Planktonic Growth
Biofilm
PAO1
pilA
fimU
pilV
pilW
pilX
pilY1
pilE


WT
pilA
fimU
pilV
pilW
pilX
pilY1
pilE
3 cm


A
Growth (OD600)
Biofilm (Absorbance600)
0.8
0.6
0.4
0.2
0.0
***
***
Planktonic Growth
Biofilm
Arabinose
-
-
-
+
+
+
PA14 + pB
sadC + pB
sadC + sadC
PA14 + pB
sadC + pB
sadC + sadC
B
Percent survival
100
50
0
0
2
4
6
Day
No arabinose
PA14 + pB
sadC + pB
sadC + sadC *
0.05% arabinose
PA14 + pB
sadC + pB
sadC + sadC *