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

Short title: Minor pilins regulate virulence by modulating FimS-AlgR activity

Authors: Victoria A. Marko, Sara L.N. Kilmury, Lesley T. MacNeil, and Lori L. Burrows*.

Affiliations: Department of Biochemistry and Biomedical Sciences and the Michael G. DeGroote Institute for Infectious Diseases Research, McMaster University, Hamilton, ON, Canada.

*For correspondence: burrowl@mcmaster.ca

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 and 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 and activation. 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 deployment 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 to explain the frequent co-occurrence of the
48 non-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 intrinsic antibiotic resistance and recalcitrance to therapy [1]. Among its virulence factors
54 are filamentous surface appendages called 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. The operon is positively regulated by the
62 virulence factor regulator Vfr, and the two-component system (TCS) FimS (AlgZ)-AlgR. FimS is
63 a predicted histidine sensor kinase and AlgR is a response regulator that promotes expression of
64 genes important for biofilms and chronic cystic fibrosis (CF) lung infections [17-21]. The N-
65 termini of immature pilins are cleaved and methylated at the cytoplasmic face of the inner
66 membrane (IM) by the prepilin peptidase, PilD, while PilY1 may be processed by signal
67 peptidase 1 [22-25]. Mature pilins are polymerized into a T4P fibre via an envelope-spanning
68 assembly machinery, where individual PilA subunits are added or removed at the platform
69 protein, PilC, via action of the ATPases PilB and PilT, respectively [2, 26].

The MPs and PilY1 are required for T4P function in several bacterial species, including *P. aeruginosa*, *Escherichia coli*, *Neisseria meningitidis*, *N. gonorrhoeae*, and *Myxococcus xanthus* [12-15, 27-30]. PilY1 and the MPs were originally proposed to oppose pilus retraction, as some surface pili remain in *pilY1* and MP mutants when retraction is blocked via deletion of *pilT* [23, 28, 29, 31, 32]. We recently showed that deletion of the minor pseudopilins of the Xcp type II secretion system in a *pilT* background lacking the T4P MPs abolished pilus assembly, suggesting that when MPs are missing, the minor pseudopilins can prime extension, but cannot counteract retraction [24]. We also demonstrated that PilY1 and the MPs are present in sheared pili, and that the loss of PilV, PilW, PilX, or PilY1 excludes the other three components from the pilus [24]. Thus, PilVWXY1 are proposed to form a core assembly-initiation subcomplex, while FimU and PilE are thought to connect this complex to PilA. Initiation of assembly with subsequent addition of multiple PilA subunits would place the MPs at the pilus tip, with PilY1 – the largest component – at the distal position, supporting the hypothesis that PilY1 is a T4P-associated adhesin [31].

PilY1 and the MPs (and their regulators FimS-AlgR) are required for T4P biogenesis, and therefore T4P-mediated function [12-15, 17, 19]. However, recent studies hinted at more enigmatic roles of PilWXY1 in virulence. Bohn et al. [33] showed that in a non-piliated *P. aeruginosa* background, subsequent loss of *pilY1* reduced virulence in a *Caenorhabditis elegans* fast killing assay and in a mouse airway infection model, and increased resistance to killing by neutrophils. Thus, PilY1 has a role in virulence that does not require functional pili. Other studies using *C. elegans* infection models suggested that MP and *pilY1* mutants had attenuated virulence relative to WT, and in one case, to a non-piliated mutant [34-37]. Recently, Siryaporn et al. [38] showed that PilWXY1 were required for surface-activated virulence towards amoebae,

while other non-piliated mutants had WT virulence. The N-terminal region of PilY1 has limited sequence similarity to the eukaryotic von Willebrand factor A (VWFa) domain, which can be deformed by shear forces [39]. In-frame deletion of this domain from PilY1 allowed normally avirulent planktonic cells to kill amoebae [38]. PilY1 was therefore proposed to be a mechanosensor, where deformation of its VWFa domain upon surface interaction led – by an as-yet unknown mechanism – to increased expression of virulence factors. One important caveat of that study was that an *algR* mutant (which also lacks PilY1 and the MPs) had WT virulence towards amoebae [38].

Deformation of PilA subunits by tensile forces acting upon surface-attached pili was also proposed as a possible way to signal attachment. Detection of partly unfolded pilins by the Pil-Chp chemotaxis system could lead to increased cyclic adenosine monophosphate (cAMP) synthesis via the CyaB adenylate cyclase [40, 41]. cAMP is bound by Vfr, a key transcription factor that promotes expression of virulence factors involved in motility, attachment, and secretion [20, 40, 41]. *fimS-algR* transcription is activated by Vfr, leading to increased transcription of *fimU-pilVWXY1E* [40]. PilVWXY1 were proposed to repress their own expression in an AlgR-dependent manner, as the loss of *pilV*, *pilW*, *pilX*, or *pilY1* led to elevated expression of the MP operon and *fimS-algR* [23, 33, 38, 40]. The mechanism of this putative feedback inhibition is largely uncharacterized, but was speculated to involve FimS [40].

Once expression of the MP operon is activated, extracellular PilY1 may sense surface association via its VWFa domain and transduce this information through the T4P assembly machinery [38, 40]. This signal is thought to activate an IM-localized diguanylate cyclase, SadC, to increase levels of c-di-GMP, promoting expression of genes associated with a biofilm lifestyle, while repressing early-phase virulence traits such as swarming motility [40, 42]. This

model was supported by studies demonstrating that loss of *pilW*, *pilX*, or *pilY1* in a high-c-di-GMP background resulted in hyper-swarming and reduced c-di-GMP levels, as measured by liquid chromatography-mass spectrometry of extracts from surface-grown cells [39, 43]. Rodesney et al. [44] showed that c-di-GMP levels increased in response to shear forces, and that functional T4P were required for this phenomenon, further supporting this hypothesis. However, unlike *pilW*, *pilX*, and *pilY1* mutants, a *sadC* mutant had WT virulence towards amoebae, suggesting the PilWXY1-SadC pathway may be important for surface sensing, but not necessarily for surface-activated virulence [38].

Although PilY1 and the MPs clearly influence virulence, the underlying mechanism remains to be established [33-36, 38, 45]. We hypothesized that a subset of these components represses FimS activity, such that loss of *pilW*, *pilX*, or *pilY1* activates FimS-AlgR, shifting the bacteria to a less pathogenic phenotype typically associated with chronic infection. We found that *pilW*, *pilX*, and *pilY1* mutants had attenuated virulence in *C. elegans* slow killing (SK) assays compared to WT or a *pilA* mutant, and this was dependent on FimS-AlgR, because double mutants had WT virulence. Hyperactivation (via phospho-mimetic point mutation) or overexpression of AlgR alone was sufficient to attenuate virulence. Together, these data are consistent with a model where loss of PilWXY1 relieves feedback inhibition on expression of the AlgR regulon, resulting in dysregulation of virulence factors that are important for *C. elegans* pathogenesis.

Results

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

Specific genes in the MP operon were reported to be important for virulence in amoebae, nematodes, and mouse models, but those studies were done using different strains of *P. aeruginosa* [33-36, 38, 45]. We first sought to confirm these results in the *C. elegans* SK model, using two well-studied strains. SK assays were performed using PA14 with deletions of *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, or *pilE* (Fig 1A). An *E. coli* OP50 plate was included as a negative control for pathogenicity; worms began to senesce on these plates around day 7-8, consistent with published data regarding temperature-dependent effects on lifespan [46]. Given that worms at later time points were at increased risk of death due to ageing in addition to *P. aeruginosa* infection, statistical significance was assessed using the Gehan-Breslow-Wilcoxon test, which places greater weight on earlier time points [47]. A *pilA* (major pilin) mutant was slightly less virulent than WT; subsequent comparisons were made relative to *pilA,* since all mutants lack pili. *fimU* and *pilE* mutants had increased virulence relative to the *pilA* mutant, with similar virulence to WT. In contrast, *pilW*, *pilX*, and *pilY1* mutants had reduced virulence relative to the *pilA* mutant, suggesting their reduced virulence was not due to loss of functional T4P. Virulence of the *pilV* mutant was similar to the *pilA* mutant. The twitching and virulence defects of *pilW*, *pilX*, and *pilY1* mutants could be partially complemented by expression of the relevant gene *in trans* (Supplementary Fig S1). The stoichiometry of PilY1 and the MPs is important for optimal T4P function [23], which may explain the lack of full complementation. To verify that these phenotypes were not strain-specific, we tested PAO1 transposon-insertion mutants of *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* in the SK assay (Fig 1B). Similar to the results in PA14, PilWXY1 were important for T4P-independent virulence. However, the *fimU* and *pilV* mutants were also less pathogenic than *pilA*; the PA14 and PAO1 MPs are divergent (61-75% amino acid similarity), so it is possible that FimU and PilV function slightly differently in PAO1 versus

PA14 [48]. To focus on genes that were generally important for virulence of *P. aeruginosa*, we undertook studies of the mechanism responsible for loss of virulence in the *pilW*, *pilX*, and *pilY1* mutants.

PilWXY1 promote virulence in a SadC-independent manner

PilWXY1 were previously proposed to increase c-di-GMP production by SadC, such that loss of *pilW*, *pilX*, or *pilY1* resulted in a biofilm-deficient phenotype, indicative of low intracellular c-di-GMP [39, 40, 43]. Therefore, we hypothesized that biofilm defects of *pilW*, *pilX*, and *pilY1* might impede their ability to colonize the *C. elegans* gut, leading to reduced virulence. The PA14 and PAO1 parent strains and their cognate *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants formed negligible levels of biofilm in liquid SK medium, chosen to approximate the growth conditions used for the SK assay (Supplementary Fig S2). To assess the levels of cyclic-di-GMP in these strains, we constructed a luminescence-based *cdrA* promoter reporter based on an extensively-characterized green fluorescent protein-based reporter system [44, 49-54]. *cdrA* promoter activity has been positively correlated with c-di-GMP levels, as measured by liquid chromatography-mass spectrometry [49, 51, 53, 54]. We verified that overexpression of SadC led to a ~60-fold increase in *cdrA* promoter activity, while overexpression of AlgR, which positively regulates genes that promote c-di-GMP production [55, 56], led to a ~2-fold increase in promoter activity that was enhanced to ~4-fold when *algR* expression was increased with 0.05% L-arabinose (Fig 2A). Deletion of *sadC* or *algR* led to a ~2-fold decrease in *cdrA* promoter activity relative to WT. *cdrA* promoter activity in WT is expected to be relatively low in liquid media because c-di-GMP levels increase upon surface attachment [43]. Compared to WT, *pilW*, *pilX*, and *pilY1* had ~3-fold lower *cdrA* promoter

activity, indicative of reduced c-di-GMP (Fig 2B). These results are consistent with reports that PilWXY1 promote c-di-GMP production via SadC [39, 40, 43]. We next investigated whether SadC was required for virulence towards *C. elegans*, as would be predicted if decreased virulence in *pilW*, *pilX*, and *pilY1* mutants was due to dysregulation of SadC activity. A small decrease in virulence towards *C. elegans* was previously reported for a PA14 *sadC* mutant [57]; however, we saw no difference in virulence between WT and a *sadC* mutant in either the PA14 or PAO1 backgrounds (Supplementary Fig S3). Further, overexpression of SadC led to a hyper-biofilm phenotype *in vitro* in SK medium, but a slight reduction in virulence, demonstrating that the amount of biofilm formed *in vitro* does not correlate with virulence in *C. elegans* (Fig 3). Although the exact mechanisms of *P. aeruginosa* pathogenesis in *C. elegans* are not fully understood, biofilms were suggested to be important for establishment of infection [57-59]. Our *in vitro* biofilm data suggests that biofilms may not be a major contributor to *P. aeruginosa* pathogenesis in this model, but direct visualization and quantification of biofilms within the nematode gut will be required to support this conclusion.

PilVWXY1 repress expression of the MP operon

After ruling out involvement of the SadC pathway, we next explored the potential role of FimS-AlgR in PilWXY1-mediated virulence. Informed by previous work in our laboratory showing that the sensor kinase PilS of the PilSR TCS interacts directly with PilA in the inner membrane to decrease PilR-dependent major pilin expression [60], we hypothesized that FimS interacts with one or more MPs, and that loss of that interaction could lead to activation of AlgR and subsequent upregulation of the MP operon. Bacterial two-hybrid (BACTH) assays were used to identify potential interactions between FimS and PilA, FimU, PilV, PilW, PilX, or PilE (Fig

4A). We also screened for interaction of FimS and AlgR, which has been inferred but never demonstrated [19]. Interactions between FimS and each pilin were identified; however, based on our experience with PilS [60], binding of pilins is necessary but not sufficient for inhibition. We also demonstrated interaction of FimS and AlgR (Fig 4A), providing further support for the hypothesis that FimS is the sensor kinase for AlgR.

To decipher which MPs might modulate expression of the operon, we monitored expression from the *fimU* promoter using a *luxCDABE* reporter. Compared to WT PA14, there was a ~25-fold increase in luminescence in *pilV*, *pilW*, *pilX*, and *pilY1* mutants, which could be restored to WT levels by expressing the corresponding pilin *in trans* (Fig 4B, Supplementary Fig S4). *fimU* and *pilA* mutants had ~5-fold increased promoter activity, while a *pilE* mutant was comparable to WT. *fimS* and *algR* mutants had low baseline luminescence, ~10-fold lower than WT. To determine whether the increased promoter activity in *pilV*, *pilW*, *pilX*, and *pilY1* mutants depended on FimS-AlgR, either *fimS* or *algR* was deleted in the *pilY1* mutant background. The *pilY1 algR* double mutant had low luminescence (~10-fold lower than WT), consistent with AlgR acting as a positive regulator of the MP operon [40]. Loss of *fimS* in the *pilY1* mutant background also abolished *fimU* promoter activity (~10-fold lower than WT), supporting the idea that FimS may monitor PilVWXY1 levels and activate AlgR when levels are low. Based on these data, PilA, FimU, and PilE are unlikely to modulate FimS-AlgR activity even though they can interact with FimS.

PilVWXY1 were previously proposed to form a complex in the inner membrane, such that loss of any one component destabilizes the others [24]. Since PilY1 is thought to be cleaved on the periplasmic side of the inner membrane, it is unlikely to interact directly with the transmembrane domains of FimS [24]. Thus, we suspected that high *fimU* promoter activity in

the *pilY1* mutant was due to reduced levels of one or more of the other pilins. To address this, we overexpressed FimU, PilV, PilW, PilX, or PilE in the *pilY1* mutant and measured *fimU* promoter activity. All these strains had luminescence comparable to the *pilY1* mutant (Supplementary Fig S4). Conversely, distinct effects have been observed in other studies upon overexpression of PilY1 [39, 40, 43]. Therefore, we overexpressed PilY1 in the *pilW* and *pilX* (high-luminescence) backgrounds; but PilY1 alone was insufficient to alter *fimU* promoter activity. Together, the data suggest that no individual component of the PilVWXY1 subcomplex is capable of modulating FimS activity when others are absent.

We also tested whether PilD processing of PilVWX was required for modulation of FimS activity. We constructed a *pilD* mutant, which lacks twitching motility since unprocessed pilins remain in the inner membrane [23, 61]. The absence of *pilD* had no impact on *fimU* promoter activity (Supplementary Fig S5), and a *pilD* mutant had virulence equivalent to a *pilA* mutant, likely attributable to its lack of T4P. Thus, PilVWX can modulate FimS activity in their unprocessed form.

Hyperactivation of AlgR attenuates virulence

Because the results suggested that loss of PilWXY1 relieves feedback inhibition on FimS-AlgR, resulting in AlgR activation, we tested whether hyperactivation of AlgR alone could decrease virulence. We made chromosomal $algR_{D54E}$ phospho-mimetic point mutants [62] in both PA14 and PAO1 backgrounds. We also made $algR_{D54A}$ point mutants, as AlgR phosphorylation is required for transcription of a subset of genes in its regulon, including the MP operon [17, 62, 63]. We verified that the $algR_{D54A}$ mutant was defective for twitching motility, while the $algR_{D54E}$ mutant had WT twitching (Supplementary Fig S6). Unexpectedly, a *fimS*

253 mutant retained ~50% twitching motility, in contrast to previous reports [18, 62]. In the absence
254 of FimS, AlgR might be phosphorylated by small phosphate donors [64]. Based on the *fimS* data,
255 we also questioned the assumption that AlgR phosphorylation was necessary for expression from
256 the *fimU* promoter. When we overexpressed WT AlgR or $AlgR_{D54A}$ in the *algR* mutant
257 (Supplementary Fig S6), its twitching defect was fully complemented by AlgR, and partially
258 complemented (25%) by $AlgR_{D54A}$. Thus, although it increases binding to the *fimU* promoter [17,
259 62], phosphorylation of AlgR is not essential for transcription of the MP operon.
260 SK assays were then performed for PA14 and PAO1 $algR_{D54A}$ and $algR_{D54E}$ mutants, plus
261 PA14 and PAO1 *fimS* and *algR* deletion mutants. PA14 and PAO1 $algR_{D54E}$ mutants were less
262 pathogenic than the corresponding WT strains, while *fimS*, *algR* and $algR_{D54A}$ mutants had WT
263 virulence (Fig 5AB). Loss of FimS-AlgR decreases expression of the MPs and PilY1 and
264 prevents pilus assembly [17, 40]. Because our data show that loss of FimS-AlgR (and thus MP
265 expression) had no impact on virulence, we conclude that reduced virulence of *pilW*, *pilX*, and
266 *pilY1* mutants is due to the resulting activation of FimS-AlgR.
267
268 **Overexpression of AlgR attenuates virulence**
269 Increased transcription of *fimS-algR* in a *pilY1* mutant relative to WT has been reported
270 [38], suggesting that reduced virulence could arise through expression of increased amounts of
271 the FimS-AlgR TCS, as well as its activation. Therefore, we asked whether increased AlgR
272 levels would attenuate virulence, as previously demonstrated in a mouse infection model [65].
273 When *algR* was expressed *in trans* from a multicopy plasmid in PA14 *algR*, virulence was
274 reduced compared to the vector control (Fig 6A). Because un-phosphorylated AlgR can also
275 affect transcription of a subset of genes [66, 67], we tested the same mutant complemented with

276 $AlgR_{D54A}$. Complementation of the *algR* mutant with $AlgR_{D54A}$ resulted in a severe virulence
277 defect relative to the vector-only control. Thus, AlgR hyperactivation and overexpression
278 independently diminish *P. aeruginosa* virulence towards *C. elegans*. Lastly, as AlgR is a positive
279 regulator of biofilm formation [17, 55, 56], we performed biofilm assays for PA14 *algR*
280 complemented with AlgR or $AlgR_{D54A}$. Expression of either variant led to hyper-biofilm
281 formation (Fig 6B), further emphasizing that the ability of a strain to form biofilms in SK
282 medium does not correlate with virulence in worms. Instead, we suggest that virulence factors
283 repressed by FimS-AlgR are important for *C. elegans* SK, and an increase in AlgR levels and/or
284 activity attenuates virulence.
285
286 **The virulence defects of *pilW*, *pilX* and *pilY1* mutants are dependent on FimS-AlgR**
287 To provide further support for this model, we asked whether the virulence defects of
288 PA14 *pilW*, *pilX*, and *pilY1* mutants required FimS-AlgR. We deleted *fimS* or *algR* in the *pilW*,
289 *pilX*, and *pilY1* backgrounds, and tested virulence of the double mutants (Fig 7). We also deleted
290 *pilW*, *pilX*, and *pilY1* in the $algR_{D54A}$ background, to test if AlgR activation was necessary for the
291 loss of virulence in *pilW*, *pilX*, and *pilY1* mutants. In all cases, the double mutants had WT
292 virulence, equivalent to that of the *fimS*, *algR*, or $algR_{D54A}$ single mutants. These results
293 demonstrate that decreased virulence resulting from loss of PilWXY1 requires both FimS and
294 AlgR. Although overexpression of $AlgR_{D54A}$ *in trans* repressed virulence (Fig 6A), the
295 chromosomal mutation was sufficient to alleviate the virulence defect of *pilW*, *pilX*, and *pilY1*
296 mutants, suggesting that AlgR phosphorylation is important for PilWXY1-modulated virulence.
297 The sigma factor AlgU ($AlgT/\sigma^{22}/\sigma^{E}$) acts upstream of FimS-AlgR to promote *algR*
298 transcription [68-70], thus we tested its potential involvement in modulation of virulence by

299 PilWXY1. An *algU* mutant was more virulent than WT (Fig 8), as previously demonstrated in
300 mouse models [71], while *pilW algU*, *pilX algU*, and *pilY1 algU* double mutants had near-WT
301 virulence (less than an *algU* mutant, but more than *pilW*, *pilX*, and *pilY1* single mutants).
302 Although AlgU promotes *algR* transcription [69], loss of AlgU alone does not prevent expression
303 of AlgR [68]. Given the reduced virulence of the *pilW algU*, *pilX algU*, and *pilY1 algU* double
304 mutants relative to *algU*, PilWXY1 modulation of FimS-AlgR signalling appears to be intact in
305 the *algU* mutant. These data are consistent with studies showing that *mucA* and *mucD* mutants,
306 in which *algR* and *algU* are highly transcribed [69, 72-74], are less virulent towards *C. elegans*
307 [75-77].
308

309 ## Discussion

310 *P. aeruginosa* uses T4P to attach to surfaces and host cells, for biofilm maturation, and to
311 move across surfaces via twitching motility [2]. The MPs and PilY1 are important players in T4P
312 biogenesis and function, but also in regulation of swarming motility, surface attachment,
313 mechanosensation, and virulence [38-40, 43]. The MP operon is positively regulated by FimS-
314 AlgR, a TCS implicated in regulation of chronic *P. aeruginosa* lung infections [17-19]. Here, we
315 explored the connection between loss of PilWXY1 (and thus, loss of T4P) and AlgR activation in
316 virulence towards *C. elegans*, as summarized in Fig 9. We showed that *pilW*, *pilX*, and *pilY1*
317 mutants were less virulent than WT or a *pilA* mutant, supporting the idea that PilWXY1
318 modulate virulence independently of their role in T4P assembly. We confirmed previous reports
319 [23, 33, 40] that in the absence of *pilV*, *pilW*, *pilX*, or *pilY1*, expression of the MP operon is
320 significantly increased, and that this requires FimS-AlgR. Either hyperactivation or

overexpression of AlgR reduced virulence, while loss of *fimS* or *algR* in *pilW*, *pilX*, or *pilY1* reverted virulence to WT levels.

These data – coupled with BACTH data showing that the minor pilins interact directly with FimS in the membrane (Fig 4) – suggest that FimS may act as a molecular thermostat to monitor MP levels, and in their absence, activates AlgR to upregulate expression of the MP operon. A similar inventory control mechanism was recently described for the PilSR TCS, where PilS phosphorylates PilR when PilA levels are low, and dephosphorylates PilR when PilA levels are high [60]. It is not yet clear if FimS responds to changes in levels of the PilVWXY1 subcomplex, thought to prime assembly of T4P [24, 78, 79]. When overexpressed individually *in trans*, each of the MPs inhibited twitching motility in PAO1 [23], but since the others were still expressed from the chromosome, the exact nature of the signal detected by FimS remains to be determined. When expressed *in trans*, no single component of the PilVWXY1 subcomplex reduced *fimU* promoter activity if others were absent (Supplementary Fig S4). The specific signal that inhibits FimS activity remains to be deciphered. Whether the FimS-inhibitory signal is the same in PA14 and PAO1 also remains unknown. Though PilWXY1 were required for virulence in PA14 and PAO1, FimU and PilV influenced virulence only in PAO1 (Fig 1A-B). Given the MPs are divergent, FimU and PilV may play different roles in PAO1 versus PA14 [48]. It is possible that FimU and PilV are more important for stability of the PilWXY1 subcomplex in PAO1 than in PA14, and/or that PAO1 FimU and PilV can directly modulate FimS activity.

Kuchma et al. [39, 43] reported that loss of *pilW*, *pilX*, or *pilY1* increased swarming motility and decreased biofilm formation, both indicative of low c-di-GMP levels. As biofilms were proposed to contribute to *P. aeruginosa* pathogenesis in *C. elegans*, we investigated

344 whether the reduction in virulence in the absence of PilWXY1 was linked to decreased biofilm
345 via loss of SadC activation [57-59, 80]. In our hands, levels of *sadC* had no impact on virulence
346 even though they clearly modulated the amount of biofilm produced in SK media (Fig 3A-B,
347 Supplementary Fig S3). Irazoqui et al. [59] examined the *C. elegans* gut during *P. aeruginosa*
348 infection and described extracellular material that they suggested might indicate presence of a
349 biofilm. Anti-biofilm compounds reduced *P. aeruginosa* virulence towards *C. elegans*, but a
350 mechanism of action for those compounds has not been described [58]. Recently, the small RNA
351 SrbA was shown to modulate both biofilm and virulence towards *C. elegans*; however, deletion
352 of *srbA* led to altered transcription of at least 26 other genes that may also affect virulence [81].
353 Rather than using standard biofilm media, we performed these assays in liquid SK media
354 to more closely mimic the conditions to which bacteria are exposed in the SK assay. To our
355 knowledge, this is the first report to use SK media for biofilm assays. As we found no correlation
356 between biofilm formation and virulence, we suggest that acute-phase virulence factors may be
357 more important for *C. elegans* pathogenesis in the SK model. However, we recognize that *in*
358 *vitro* biofilm assays may not replicate the conditions within the *C. elegans* gut; direct
359 visualization of bacteria in worms will be needed to clarify the role of biofilm formation.
360 PilY1 and the MPs have been implicated in surface detection and activation of virulence,
361 via signalling through SadC [38, 40]. Because loss of PilY1 or the MPs prevents T4P assembly
362 and function, it is crucial to distinguish phenotypes resulting from lack of specific proteins
363 versus loss of piliation [24]. Luo et al. [40] suggested that association of PilY1 with surfaces
364 transduces a signal through the T4P machinery to stimulate c-di-GMP production by SadC, while
365 Rodesney et al. [44] showed that loss of *pilA*, *pilY1*, or *pilT* prevents surface-activated c-di-GMP
366 production. Rodesney et al. [44] proposed that both PilY1 and functional T4P are required for

367 mechanosensation; however, it is not possible to delete *pilY1* without ablating T4P assembly.
368 Our *cdrA* promoter reporter data support the idea that PilWXY1 promote cyclic-di-GMP
369 production by SadC, as loss of *pilW*, *pilX*, or *pilY1* decreased *cdrA* promoter activity (Fig 2B).
370 However, we argue that the PilWXY1-SadC pathway – though important for c-di-GMP
371 signalling – is not critical for virulence towards *C. elegans*. Instead, our data show that
372 PilWXY1-FimS-AlgR signalling axis is responsible for T4P-independent changes in virulence of
373 *pilW*, *pilX*, and *pilY1* mutants. Thus, surface attachment may induce c-di-GMP production via
374 PilWXY1-SadC [40, 43], while the brief trapping of T4P outside the cell upon contact with a
375 surface might transiently deplete PilVWXY1 levels in the IM, resulting in increased FimS-AlgR
376 activity and transition towards a sessile, biofilm lifestyle.

377 Whether the loss of *pilW*, *pilX*, or *pilY1* leads to increased amounts of AlgR, its increased
378 phosphorylation via FimS, or both, remains to be clarified. Okkotsu et al. [62] showed that AlgR
379 and $AlgR_{D54E}$ levels are comparable, suggesting that the loss of virulence we observed for PA14
380 $algR_{D54E}$ is attributable to the D54E phospho-mimetic mutation alone. Overexpression of
381 $AlgR_{D54A}$ *in trans* reduced virulence (Fig 6A), but the same mutation on the chromosome
382 reverted virulence of *pilW*, *pilX*, and *pilY1* mutants to WT levels (Fig 7). Therefore, we suspect
383 that it is primarily AlgR phosphorylation (or lack of AlgR dephosphorylation) that leads to
384 decreased virulence. However, it is possible that both increased AlgR protein levels and
385 phosphorylation contribute to the reduced pathogenicity of *pilW*, *pilX*, and *pilY1* mutants. Kong
386 et al. [55] showed that AlgR binds *fimS-algR*, suggesting that the TCS could positively regulate
387 its own transcription in response to reduced PilWXY1 levels.

388 In addition to being essential for T4P function, FimS and AlgR control alginate
389 production in the context of chronic CF infections, where *algR* transcription is high [18, 82].

Phosphorylation of AlgR increases binding affinity at some – but not all – of its target sequences [17, 62, 63, 67]. For example, $AlgR_{D54N}$ failed to support twitching motility, but did not affect alginate production [17, 63]. Our twitching motility data suggests that $AlgR_{D54A}$ is capable of binding to the *fimU* promoter, albeit less efficiently than WT AlgR (Supplementary Fig S6). FimS is an unorthodox histidine kinase, with four transmembrane domains instead of the typical two, and lacks both a periplasmic sensing domain and the canonical motif involved in ATP coordination that mediates auto-phosphorylation [19, 83]. Direct interaction and/or phospho-transfer between FimS and AlgR have not been reported. Rather, the idea that FimS acts as a kinase for AlgR comes from this and other studies demonstrating similar phenotypes for *fimS,* *algR,* and $algR_{D54N}$ mutants [17, 18, 84]. Here, we demonstrated that FimS and AlgR interact in the BACTH assay (Fig 4) lending further support to this model.

FimS and AlgR promote expression of genes important for production of alginate, biofilms, and c-di-GMP, and inhibit expression of virulence factors such as the T3SS, pyocyanin, and quorum sensing [55, 56, 74, 85, 86]. The observation that the loss of *algR* had no impact on virulence towards amoebae [38] or nematodes (Fig 5AB) suggests that the AlgR-activated genes may not contribute to virulence, although the mechanisms of killing could differ. In mouse models, *fimS* and *algR* deletion mutants are attenuated, though overexpression of AlgR also markedly reduces virulence [55, 65, 87]. Further, Little et al. demonstrated that PAO1 $algR_{D54E}$ had WT virulence in *Drosophila melanogaster* and mouse infection models, while an $algR_{D54A}$ mutant had highly attenuated virulence [87]. The outcomes that result from interaction of *P. aeruginosa* with different hosts will depend on a combination of factors including host defenses, site of infection, available nutrients, and virulence repertoire of a particular strain. However, our

412 results suggest that changes in the specific repertoire of bacterial virulence factors, or the timing
413 of their production, can tip the balance in the host's favour.

414 The subset of AlgR-regulated virulence genes important for *C. elegans* pathogenesis is
415 not defined. Screening of a PA14 transposon library for loss of virulence implicated several
416 genes encoding regulators rather than individual virulence factors, suggesting that *C. elegans*
417 pathogenesis is multifactorial [35]. Consistent with this hypothesis, a study of 18 WT *P.*
418 *aeruginosa* strains revealed no correlation between pathogenicity and any specific virulence
419 factors [88]. We saw WT or greater levels of virulence for *algR* and *algU* mutants, respectively,
420 consistent with a role for AlgRU in repression of acute phase virulence factors (Figs 5, 8).
421 Factors under positive control of AlgRU may be important during later stages of infection in
422 more complex mammalian infection models, but not crucial for pathogenesis in nematodes [89,
423 90]. In support of this hypothesis, past studies have demonstrated that increased mucoidy, via
424 mutation of *mucA* or *mucD*, reduced nematode killing [75-77].

425 While important for the initial stages of infection, T4P are less critical in chronic CF lung
426 infections and are often lost over time [5, 91, 92]. *P. aeruginosa* CF isolates frequently become
427 mucoid via activation of AlgR, and production of many virulence factors is reduced [82, 93, 94].
428 Although the two outcomes are not necessarily temporally or mechanistically linked, mutations
429 that achieve both may be advantageous during chronic CF lung infections. Specifically, loss of
430 PilWXY1 may be adaptive in the context of CF, leading to AlgR activation and loss of T4P
431 function. To test this idea, it will be interesting to examine the genotypes of mucoid CF isolates
432 for these types of mutations. In conclusion, our results suggest that PilWXY1 promote virulence
433 towards *C. elegans* by inhibiting FimS-AlgR activation. These data demonstrate how loss of one
434 virulence factor (T4P) may activate others (via AlgR). Because the interplay between virulence

435 factors in *P. aeruginosa* is complex and dynamic, careful consideration will be required when
436 designing potential anti-virulence therapeutic strategies.
437

438 # Materials and methods

439 ## Bacterial strains and plasmids

440 Strains and plasmids used in this work are listed in Supplementary Table S1. Bacteria
441 were grown at 37°C for 16 h in 5 ml lysogeny broth (LB) Lennox, or on 1.5% agar LB plates,
442 unless otherwise specified. Plasmids were transformed into chemically-competent *E. coli* by
443 heat-shock, and into *P. aeruginosa* by electroporation [95]. Where appropriate, gentamicin (Gm)
444 was added at 15 µg/ml for *E. coli*, and 30 µg/ml for *P. aeruginosa*. Kanamycin (Kan) was added
445 at 50 µg/ml for *E. coli*, and 150 µg/ml for *P. aeruginosa*. Ampicillin (Amp) was added at 100
446 µg/ml for *E. coli*. L-arabinose was added at 0.05% where indicated to induce expression from the
447 pBADGr promoter [96].
448

449 ## Cloning procedures

450 Vectors were constructed using standard cloning procedures, using the primers listed in
451 Supplementary Table S2. Deletion constructs were designed to contain 500-1000 bp homology
452 upstream and downstream the gene to be deleted. Deletion constructs for PA14 *fimU*, *pilV*, *pilW*,
453 *pilX*, *pilY1*, and *pilE* were synthesized by Genscript in the pUC57Kan vector. pEX18Gm-*sadC*
454 was created by amplifying the *sadC* deletion region from PA14 *sadC roeA* [42], followed by
455 digestion and ligation into pEX18Gm. pEX18Gm-*fimS*, pEX18Gm-$algR_{D54A}$, and pEX18Gm-
456 $algR_{D54E}$ were made by overlap extension PCR [97]. Restriction digestion followed by ligation of
457 the upstream and downstream fragments was used to create the deletion constructs pEX18Gm-

458 *algR*, pEX18Gm-*algU*, and pEX18Gm-*pilD*. pMS402-P*fimU* and pMS402-P*cdrA* were created
459 by amplifying and digesting the promoter regions of the PA14 MP operon and *cdrA* gene,
460 respectively. Digested pBADGr was treated with alkaline phosphatase prior to ligation to avoid
461 re-circularization of the vector. Constructs were verified by Sanger sequencing (MOBIX lab,
462 McMaster, Hamilton, ON).
463
464 **Mutant generation by allelic exchange**
465 Allelic exchange was used to remove or alter specific genes [98]. pEX18Gm suicide
466 plasmid derivatives (see Cloning procedures and Table 1) were used to create all mutants in this
467 work. After heat-shock transformation into *E. coli* SM10 cells, pEX18Gm constructs were
468 conjugated into corresponding PA14 or PAO1 parent strains. Cells were then transferred to
469 *Pseudomonas* isolation agar (PIA) Gm100 plates and incubated for 18 h at 37°C, to select for
470 integration of pEX18Gm derivatives into the chromosome. Colonies were streaked onto
471 LB/sucrose and incubated at 30°C for 18 h to select against merodiploids. Resultant colonies
472 were patched onto LB and LB Gm30 to identify gentamicin-sensitive colonies. Regions flanking
473 the desired mutations were amplified and sequenced to confirm success.
474
475 **Twitching motility assays**
476 Twitching motility assays were performed as previously described [99], with the
477 following modifications. Individual colonies were stab-inoculated in triplicate into 1% agar LB
478 solidified in plasma-treated tissue culture-grade plates (Thermo Fisher) and incubated at 30°C for
479 48 h. Agar was carefully removed and plates were stained with 1% crystal violet for 5 min.

480 Unbound dye was removed by rinsing with water, then stained twitching areas were measured
481 using ImageJ. Twitching zones were normalized to WT (100%).
482
483 **Biofilm assays**
484 Biofilm assays were performed as previously described, with modifications [100]. *P.*
485 *aeruginosa* cultures were grown for 16 h at 37°C, diluted 1:200 in fresh LB, and grown to OD_{600}
486 ~0.1. Cultures were then diluted 1:500 in liquid SK media (50 mM NaCl, 0.35% peptone, 1 mM
487 $CaCl_2$, 1 mM $MgSO_4$, 5 µg/ml cholesterol in EtOH, 20 mM KH_2PO_4, and 5 mM K_2HPO_4), then
488 96-well plates were inoculated with 150 µl each strain, in triplicate. Sterility controls (liquid SK
489 media) were included throughout the plate to check for contamination. Plates were covered with
490 peg lids (Nunc) then wrapped in parafilm and incubated at 37°C for 24 h, shaken at 200 rpm.
491 After incubation, the OD_{600} of the plate was measured to check for uniform growth and lack of
492 contamination. Peg lids were washed for 10 min in 200 µl/well 1X phosphate-buffered saline
493 (PBS), then stained with 200 µl/well 0.1% (w/v) crystal violet for 15 min. Unbound crystal violet
494 was removed by washing lids in 70 ml distilled water 5 times at 10 min intervals. Crystal violet
495 was solubilized from lids in 200 µl/well 33.3% acetic acid, then the absorbance at 600 nm was
496 measured. Optical density and absorbance at 600 nm were plotted for growth and biofilm
497 formation, respectively, then analyzed by one-way ANOVA followed by Dunnett post-test to
498 compare each mutant to the WT control, $p = 0.05$. Error bars indicate standard error of the mean.
499 Representative wells of acetic acid-solubilized crystal violet were imaged.
500
501 ***Caenorhabditis elegans* slow killing assay**

SK assays were performed as described previously [101]. SK plates (0.35% peptone, 50 mM NaCl, 2% agar, 1 mM $CaCl_2$, 5 µg/ml cholesterol, 1 mM $MgSO_4$, 20 mM KH_2PO_4, 5 mM K_2HPO_4, 100 µM FUDR) were seeded with 100 µl of an overnight culture and incubated overnight at 37°C. The following day, plates were enriched with 1 ml of an overnight culture concentrated to 100 µl. Synchronized L4 worms were collected from *E. coli* OP50 plates, washed twice in M9 buffer, and then >50 worms were seeded onto each bacterial lawn on individual SK plates. SK plates were incubated at 25°C and scored for dead worms every 24 h. Worms were considered dead when they did not respond to touch, and were removed from the plate. OP50 was included as a negative control for virulence. Percent survival was plotted as a function of time. Survival curves were plotted on GraphPad Prism 5.00 for Windows, then compared using the Gehan-Breslow-Wilcoxon test, $p = 0.05$. Given that larvae were synchronized at 20°C then transferred at L4 to 25°C for the duration of the assay, worms were at risk of death due to senescence, rather than direct killing by *P. aeruginosa*, before day 10 [46]. Therefore, the Gehan-Breslow-Wilcoxon test, which gives weight to earlier timepoints, was used in favour of the standard log-rank test (notably, all reported differences were also significant by the standard log-rank test). To correct for multiple analyses, the critical p-value of 0.05 was divided by the number of pairwise comparisons made within an individual trial, as per the Bonferroni method [102]. Each assay was performed at least 3 times, and differences were only considered significant if they were reproducible in the majority of trials. Representative trials are shown; all replicates can be viewed in the Supplemental Material (Supplementary File S1).

Luminescent reporter assay

524 Luminescent reporter assays were performed as previously described, with minor
525 modifications [60]. Various strains harbouring the pMS402-P*fimU* or pMS402-P*cdrA* plasmids,
526 encoding the luciferase genes under control of the *fimU* or *cdrA* promoters, respectively, were
527 grown for 16 h at 37°C in LB Kan150, then diluted 1:50 in fresh liquid SK media with Kan150,
528 in addition to Gm30 and 0.05% L-arabinose where appropriate. Subsequently, 100 µl of each
529 culture was added to white-walled, clear-bottom 96-well plates (Corning) in triplicate, and
530 incubated with shaking at 37°C in a Synergy 4 microtiter plate reader (BioTek). Luminescence
531 readings were taken every 15 min for 5 h, and normalized to growth (OD_{600}) at each time point.
532 Readings that exceeded the limit of detection (>4 000 000 luminescence units) were discarded.
533 At least 3 individual trials were performed. Error bars indicate standard error of the mean.
534
535 **Bacterial two-hybrid β-galactosidase activity assay**
536 To test for interactions between FimS and AlgR or individual pilins, bacterial two-hybrid
537 (BACTH) assays were performed as previously described [103]. pUT18C and pKT25
538 derivatives, encoding the T18 and T25 domains of the *Bordetella pertussis* CyaA adenylate
539 cyclase fused to the N-terminus of FimS, AlgR, PilA, FimU, PilV, PilW, PilX, or PilE [24, 60,
540 104], were co-transformed into *E. coli* BTH 101 to screen for pairwise interactions. Single
541 colonies were inoculated in 5 ml LB Amp100 Kan50 and grown overnight. The following day,
542 100 µl was inoculated into 5 ml fresh media and grown to OD_{600} = 0.6, then 5 µl was spotted
543 onto MacConkey plates (1.5% agar, 100µg/ml ampicillin, 50µg/ml kanamycin, 1% (w/v)
544 maltose, 0.5mM isopropyl b-D-thiogalactopyranoside) (Difco) or LB Amp100 Kan50 plates
545 supplemented with 100 µl of 20 mg/ml X-gal. Plates were incubated at 30°C for 24 h. An
546 interaction was considered positive when colonies appeared pink or blue on MacConkey and LB

547 + X-gal plates, respectively. BTH 101 expressing pUT18C and pKT25 empty vectors was used
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549 positive control [49].
550

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900

901 **Figure captions**

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

903 (A) SK assays for PA14 *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. Synchronized L4
904 worms were seeded onto SK plates and scored for death every 24 h, then plotted as "percent

survival" over the course of the assay. "Day" represents the number of days after L4 on which the plates were scored. PA14 *fimU* and *pilE* mutants had similar virulence to WT, *pilA* and *pilV* mutants were slightly less virulent than WT, and *pilW, pilX,* and *pilY1* mutants were less virulent than all other strains tested. (B) SK assays for PAO1 *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. The PAO1 *pilE* mutant had similar virulence to WT, the *pilA* mutant was slightly less virulent, and *fimU*, *pilV*, *pilW*, *pilX*, and *pilY1* mutants were much less virulent. In (A) and (B), asterisks indicate strains that were significantly different from a *pilA* mutant by Gehan-Breslow-Wilcoxon test at p = 0.05 (p = 0.00625 with a Bonferroni correction), n = 3 trials.

Fig 2. *pilW*, *pilX*, and *pilY1* mutants have reduced *cdrA* promoter activity.

(A) *cdrA* promoter activity in PA14 *sadC* and *algR* deletion and overexpression strains. pMS402-P*cdrA*, containing the *lux* genes under expression of the *cdrA* promoter, was introduced into strains of interest, along with pBADGr (vector-only control), pBADGr-*sadC*, or pBADGr-*algR*. Assays were set up in technical triplicate in SK media, with or without 0.05% L-arabinose to induce expression of the pBADGr promoter, and measurements were taken every 15 min over 5 h. Loss of *sadC* or *algR* led to a subtle decrease in *cdrA* promoter activity, while SadC overexpression led to a dramatic increase in *cdrA* promoter activity. Overexpression of AlgR also led to a subtle increase in *cdrA* promoter activity that was enhanced upon addition of L-arabinose. n = 3 trials. (B) *cdrA* promoter activity in PA14 *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. Loss of *pilW*, *pilX*, or *pilY1* led to a decrease in *cdrA* promoter activity. n = 3 trials.

Figure 3. SadC promotes biofilm formation but is not required for virulence.

928 (A) Biofilm assays for *sadC* deletion and overexpression strains. PA14 *sadC* biofilm levels were
929 similar to WT. Expression of SadC *in trans* from a multicopy plasmid led to increased biofilm
930 formation relative to WT at 0% (due to leaky promoter) and 0.05% L-arabinose, $p < 0.001$.
931 Significance was determined by one-way ANOVA followed by Dunnett post-test relative to
932 PA14 + pBADGr, n = 3. (B) SK assays for *sadC* deletion and overexpression strains.
933 Overexpression of SadC led to a subtle but reproducible loss of virulence relative to WT at 0%
934 L-arabinose. A *sadC* mutant had WT virulence. Asterisks indicate strains that were significantly
935 different from PA14 + pBADGr by Gehan-Breslow-Wilcoxon test at $p = 0.05$ ($p = 0.0125$ with a
936 Bonferroni correction), n = 3.
937
938 **Fig 4. PilVWXY1 repress their expression via FimS-AlgR.**
939 (A) BACTH assays for FimS, AlgR, PilA, and MPs. Protein fusions with T18 and T25 fragments
940 of the CyaA adenylate cyclase were screened for interactions on MacConkey and LB + X-gal
941 plates. FimS interacted with AlgR, PilA, FimU, PilV, PilW, PilX, and PilE. Positive (+) or
942 negative (-) interactions are indicated below each image, n = 3. (B) *fimU* promoter activity in
943 PA14 *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilE*, *fimS*, *algR*, *pilY1 fimS*, or *pilY1 algR* mutants.
944 pMS402-P*fimU*, containing the *fimU* promoter upstream of the *lux* genes, was introduced into
945 strains of interest. Loss of *pilV*, *pilW*, *pilX*, or *pilY1* led to highly elevated *fimU* promoter
946 activity. *pilA* and *fimU* mutants had moderately increased *fimU* promoter activity relative to WT.
947 *fimS* and *algR* mutants had negligible luminescence, and loss of *fimS* or *algR* also reverted *fimU*
948 promoter activity in the *pilY1* mutant to baseline. n = 3 trials.
949
950 **Fig 5**. **AlgR hyperactivation reduces virulence.**

951 SK assays for (A) PA14 and (B) PAO1 *fimS*, *algR*, *algR*$_{D54A}$, and *algR*$_{D54E}$ mutants. The *fimS*,
952 *algR,* and *algR*$_{D54A}$ mutants had WT virulence, while the *algR*$_{D54E}$ mutants were less virulent
953 than WT. For (A) and (B), asterisks indicate strains that were significantly different from WT by
954 Gehan-Breslow-Wilcoxon test at p = 0.05 (p = 0.01 with a Bonferroni correction), n = 3 trials.
955
956 **Fig 6. AlgR promotes biofilm formation and represses virulence.**
957 (A) SK assays for *algR* deletion and overexpression strains. Loss of *algR* led to a small increase
958 in virulence, while overexpression of pBADGr-*algR* or pBADGr-*algR*$_{D54A}$ reduced virulence at
959 0.05% L-arabinose. Asterisks indicate strains that were significantly different from PA14 +
960 pBADGr by Gehan-Breslow-Wilcoxon test at p = 0.05 (p = 0.00833 with a Bonferroni
961 correction), n = 3 trials. (B) Biofilm assays for *algR* deletion and overexpression strains.
962 Microtiter plate biofilm assays were performed in liquid SK media over 24 h, in triplicate.
963 Biofilms were stained with 1% crystal violet then solubilized in acetic acid. Loss of *algR* had no
964 effect on biofilm formation. When grown at 0.05% L-arabinose, overexpression of pBADGr-
965 *algR* or pBADGr-*algR*$_{D54A}$ increased biofilm formation, p < 0.001. Significance was determined
966 by one-way ANOVA followed by Dunnett post-test relative to WT, n = 3 trials.
967
968 **Fig 7. The virulence defect of *pilW*, *pilX*, and *pilY1* mutants is dependent on FimS-AlgR.**
969 SK assays for *pilW*, *pilX*, *pilY1*, *fimS*, *algR*, and *algR*$_{D54A}$ single and double mutants. *fimS*, *algR*,
970 and *algR*$_{D54A}$ mutants have WT virulence. *pilW*, *pilX*, and *pilY1* have reduced virulence relative
971 to WT, *fimS*, *algR*, and *algR*$_{D54A}$ mutants. Combination of *pilW*, *pilX*, or *pilY1* mutations with
972 *fimS*, *algR*, or *algR*$_{D54A}$ mutations results in virulence equivalent to *fimS*, *algR*, and *algR*$_{D54A}$
973 single mutants, respectively. All graphs represent 1 trial, separated into 3 graphs where strains

974 relevant to (A) *pilW*, (B) *pilX*, and (C) *pilY1* mutants are included. Asterisks indicate strains that
975 were less virulent than PA14 by Gehan-Breslow-Wilcoxon test at p = 0.05 (p = 0.003125 with a
976 Bonferroni correction), n = 3.
977
978 **Fig 8. PilWXY1-mediated virulence is not dependent on AlgU.**
979 SK assays for PA14 *pilW*, *pilX*, *pilY1*, *algU*, *pilW algU*, *pilX algU*, and *pilY1 algU* mutants. Loss
980 of *algU* led to increased pathogenicity relative to WT, while *pilW algU*, *pilX algU*, and *pilY1*
981 *algU* mutants had near-WT virulence. Asterisks indicate strains that were significantly different
982 from PA14 by Gehan-Breslow-Wilcoxon test at p = 0.05 (p = 0.00625 with a Bonferroni
983 correction), n = 3.
984
985 **Fig 9. Hypothesized model for regulation of the MP operon via FimS-AlgR.**
986 When PilVWXY1 are absent, FimS may directly or indirectly promote increased protein levels
987 and/or phosphorylation of AlgR (phosphate indicated by yellow star). Phospho-AlgR binds the
988 *fimU* promoter to promote expression of *pilY1* and the MP genes. Phospho-AlgR also promotes
989 expression of genes associated with chronic infections, and represses those associated with acute
990 infections. As PilVWXY1 accumulate in the IM, they are likely detected by FimS, potentially
991 leading to reduced AlgR protein levels and/or phosphorylation. Abbreviations: PilV, V (orange);
992 PilW, W (cyan); PilX, X (pink); PilY1, Y1 (dark purple); IM, inner membrane.
993
994 **Supporting information**
995 **Fig S1. Twitching motility and virulence of *pilW*, *pilX*, and *pilY1* mutants can be**
996 **complemented *in trans*.**

997 (A) Twitching motility assays for complemented PA14 *pilW*, *pilX*, and *pilY1* mutants. Colonies
998 were stab-inoculated into 1% agar LB plates, in triplicate. Plates were stained with crystal violet
999 after 48 h at 30°C. Complementation of PA14 *pilW*, *pilX*, and *pilY1* mutants with pBADGr-*pilW*,
1000 pBADGr-*pilX*, or pBADGr-*pilY1*, respectively, led to increased TM relative to complementation
1001 with pBADGr alone. Numbers indicate percent twitching area relative to WT, n = 3. (B) SK
1002 assays for complemented PA14 *pilW*, *pilX*, and *pilY1* mutants. Complementation of *pilW*, *pilX*,
1003 and *pilY1* mutants with pBADGr-*pilW*, pBADGr-*pilX*, or pBADGr-*pilY1*, respectively, restored
1004 virulence to near-WT levels. Asterisks indicate strains that were less virulent than PA14 +
1005 pBADGr by Gehan-Breslow-Wilcoxon test at p = 0.05 (p = 0.00833 with a Bonferroni
1006 correction), n = 3. Individual graphs represent separate trials.
1007
1008 **Fig S2. PA14 and PAO1 produce low levels of biofilm in liquid slow killing media.**
1009 Biofilm assays for (A) PA14 and (B) PAO1 *pilA, fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants.
1010 Very little biofilm formation was detectable in liquid SK media for any strains. There were no
1011 differences in biofilm formation as determined by one-way ANOVA followed by Dunnett post-
1012 test relative to WT at p = 0.05, n = 3.
1013
1014 **Fig S3. SadC is not required for virulence in PA14 or PAO1.**
1015 SK assays for (A) PA14 and (B) PAO1 *sadC* mutants. Loss of *sadC* had no impact on
1016 pathogenicity relative to each respective WT strain, as measured by Gehan-Breslow-Wilcoxon
1017 test at p = 0.05 (p = 0.025 with a Bonferroni correction), n = 3.
1018

Fig S4. *pilV*, *pilW*, *pilX*, and *pilY1* mutants cannot be cross-complemented for *fimU* promoter activity.

(A) *fimU* promoter activity of *pilV*, *pilW*, *pilX*, and *pilY1* mutants complemented with the respective gene *in trans*. The high luminescence of each mutant was restored to WT level when *pilV*, *pilW*, *pilX*, and *pilY1* were complemented with PilV, PilW, PilX, and PilY1, respectively. (B) *fimU* promoter activity of a *pilY1* mutant expressing each MP *in trans*. Expression of FimU, PilV, PilW, PilX, or PilE in the *pilY1* background had no impact on *fimU* promoter activity relative to the *pilY1* + empty vector control. (C) *fimU* promoter activity of *pilW* and *pilX* mutants overexpressing PilY1. Overexpression of PilY1 had no impact on *fimU* promoter activity in *pilW* and *pilX* backgrounds relative to the respective vector-only controls. Assays in (A), (B), and (C) were carried out in the presence of 0.05% L-arabinose to induce expression of the pBADGr promoter, n = 3.

Fig S5. PilD is not required for PilWXY1-mediated modulation of FimS-AlgR activity.

(A) Twitching motility assays for PA14 *pilA* and *pilD* mutants. Loss of *pilD* resulted in loss of twitching motility. Numbers indicate percent twitching area relative to WT, n = 3. (B) *fimU* promoter activity of a *pilD* mutant compared to PA14, *pilA*, and *pilY1*. Loss of *pilD* had no impact on *fimU* promoter activity relative to WT, n = 3. (C) SK assays for PA14, *pilA*, *pilY1*, and *pilD* mutants. A *pilD* mutant had equivalent virulence to a *pilA* mutant; less pathogenic than WT but more pathogenic than a *pilY1* mutants. Asterisks represent strains that were significantly different from the *pilA* mutant by Gehan-Breslow-Wilcoxon test at p = 0.05 (p = 0.0125 with a Bonferroni correction), n = 3.

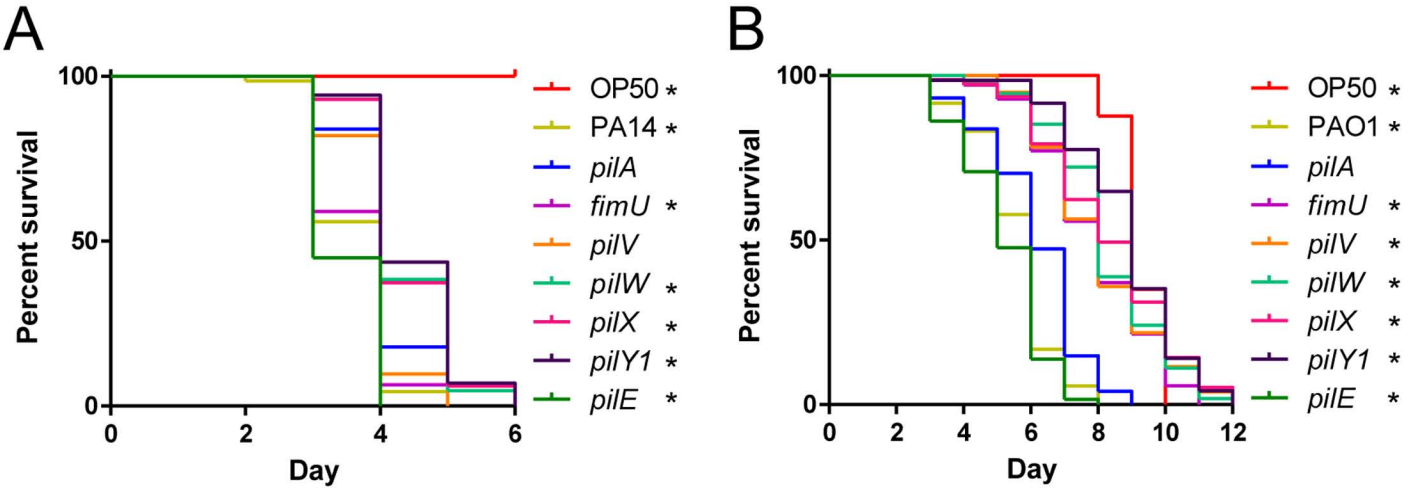
Fig S6. Phosphorylation of AlgR is required for optimal twitching motility.

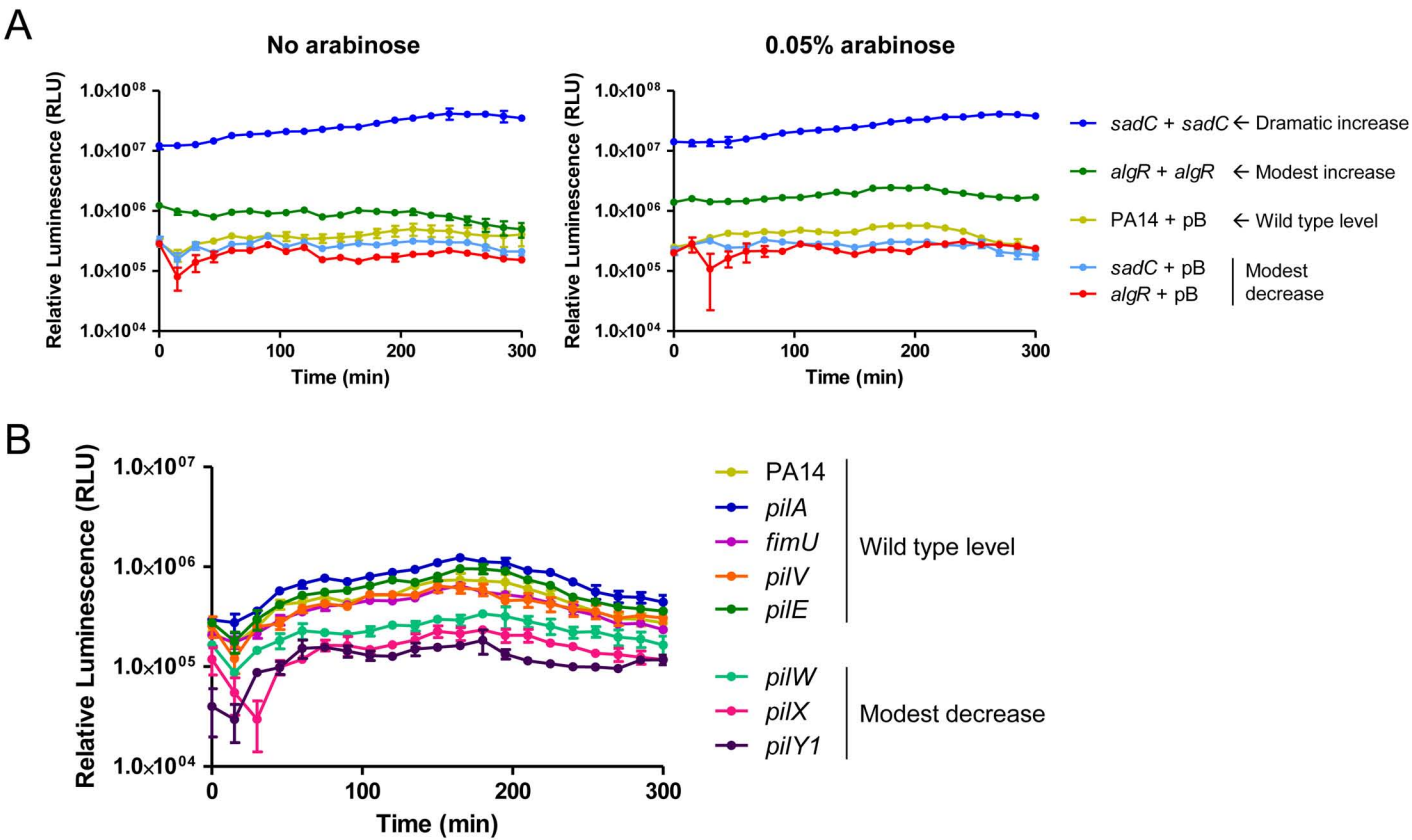
(A) Twitching motility assays for PA14 *pilA*, *fimS*, *algR*, *algR*$_{D54A}$, and *algR*$_{D54E}$ mutants. Twitching motility was abolished in *pilA*, *algR*, and *algR*$_{D54A}$ mutants, and fully retained in the *algR*$_{D54E}$ mutant. A *fimS* mutant twitched to ~50% WT levels. (B) Twitching motility assays for PA14 *algR* complemented with AlgR or AlgR$_{D54A}$. An *algR* mutant was fully complemented by AlgR with and without induction by 0.05% L-arabinose. The AlgR$_{D54A}$ variant supported twitching motility in the *algR* mutant background in the presence of 0.05% L-arabinose, to ~25% WT levels. In (A) and (B), numbers indicate percent twitching area relative to WT, n = 3.

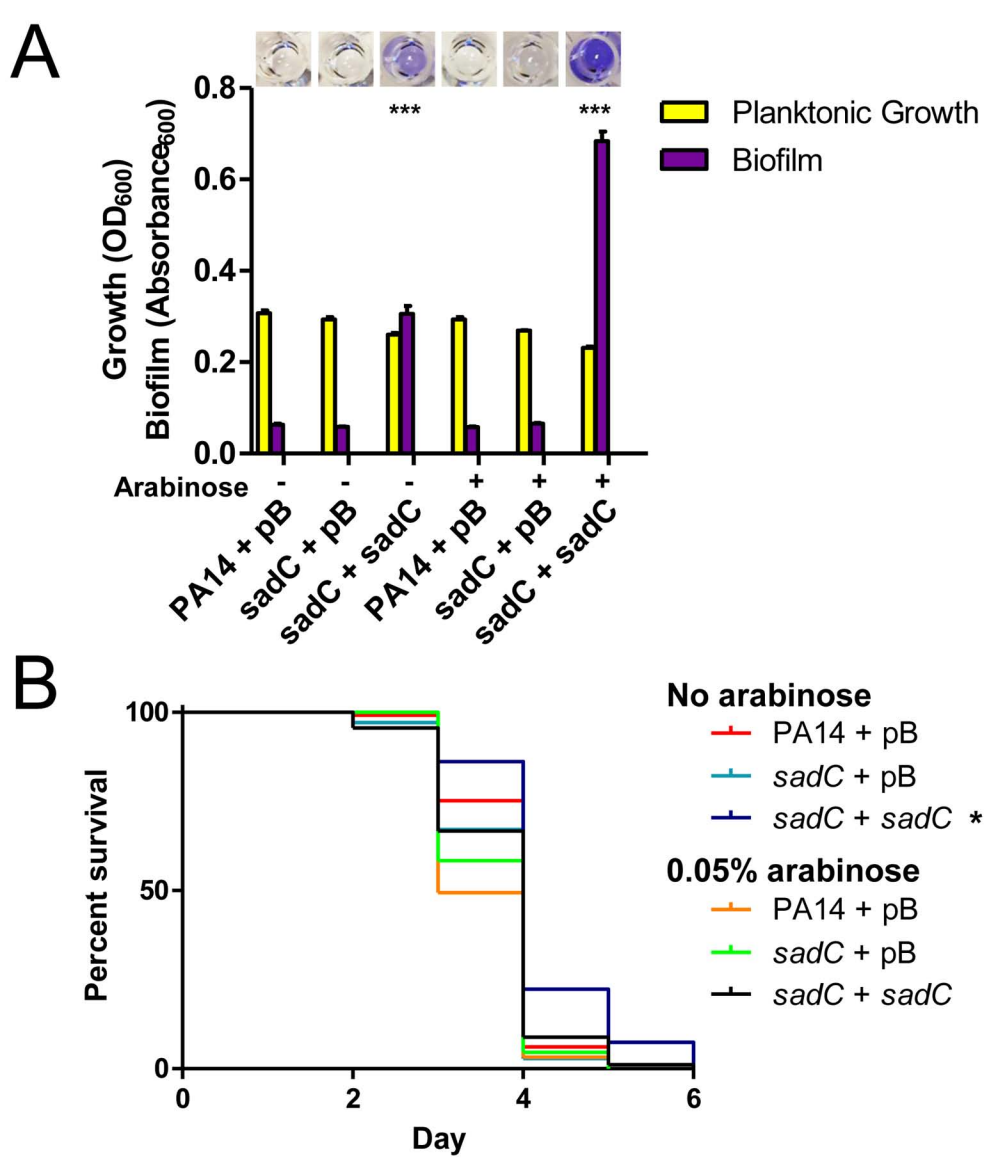
Table S1. Bacterial strains and plasmids used in this study.

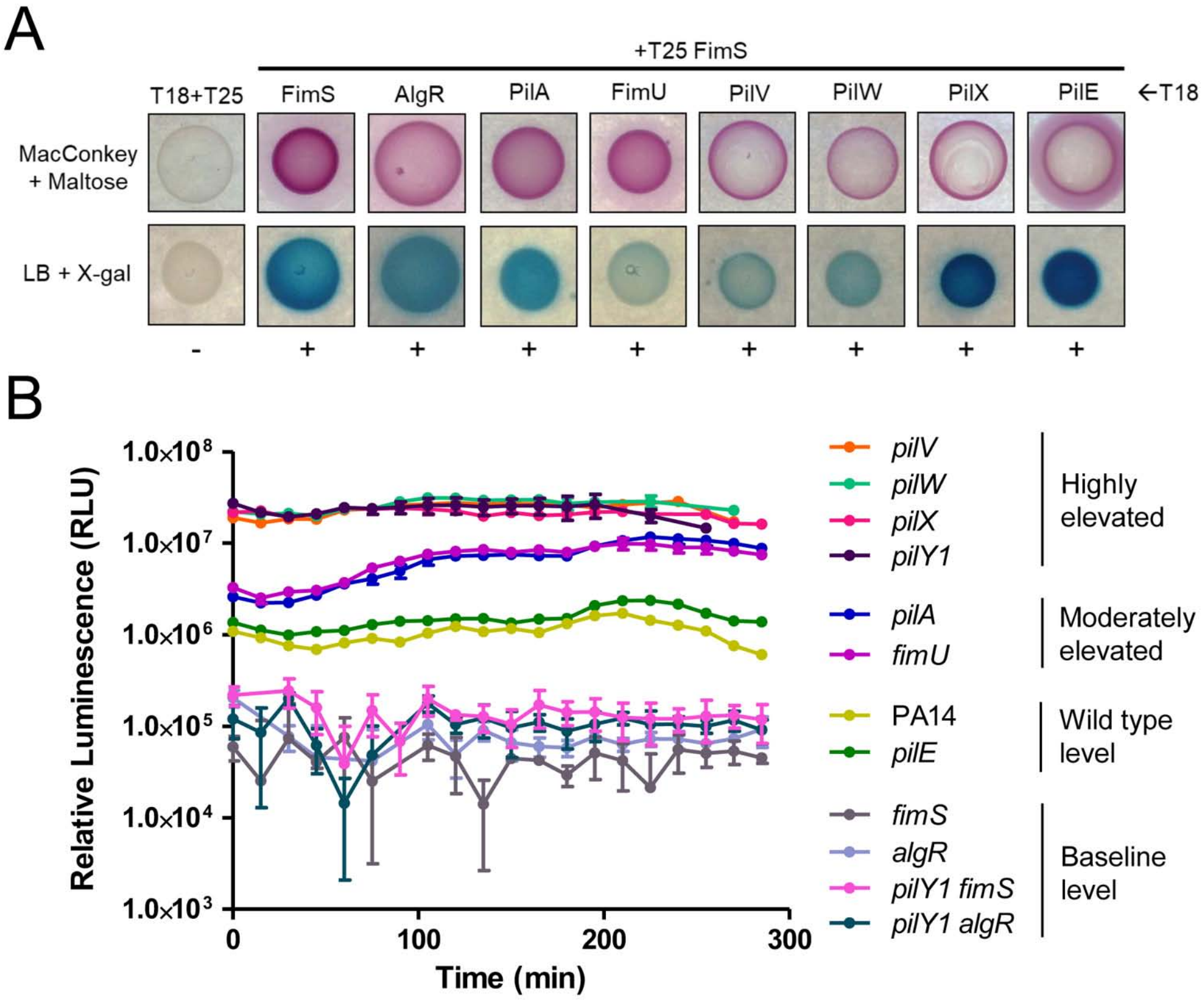
Table S2. Primers used in this study. Restriction sites are underlined.

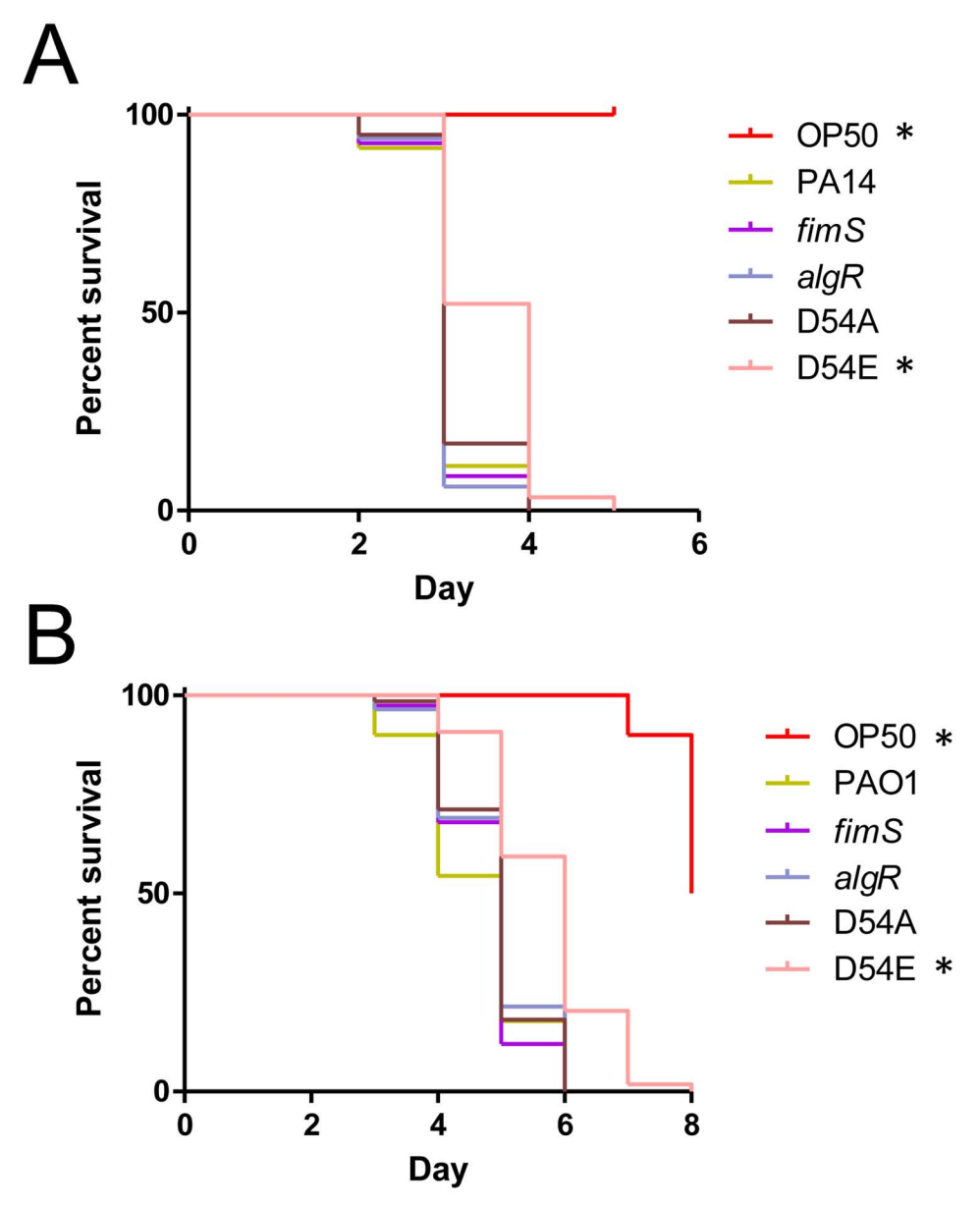
File S1. Replicates for slow killing assays. Three independent experiments for Figs 1A-B, 3B, 5A-B, 6A, 7A-C, 8, and Supplementary Figs S1B, S3A-B, S5C.

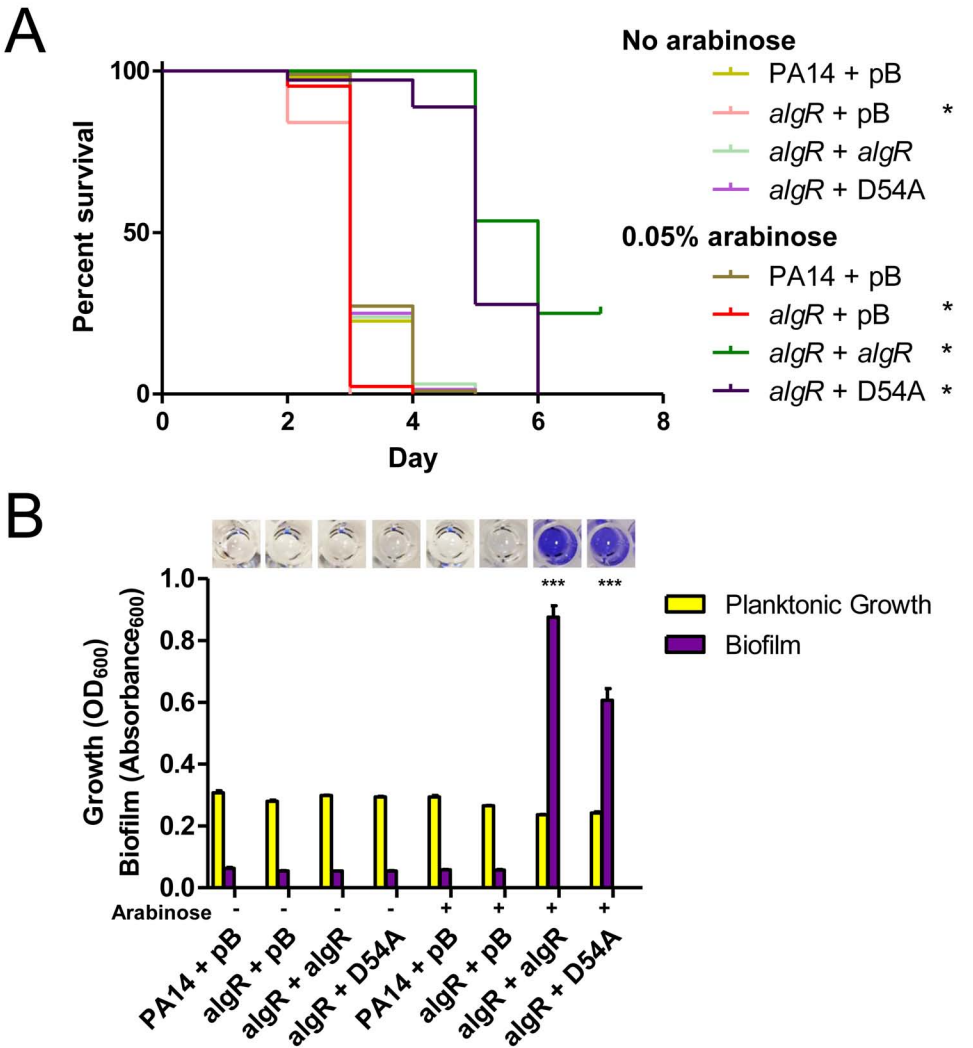

A
Percent survival
100
50
0
0
2
4
6
Day
OP50 *
PA14 *
pilA
fimU *
pilV
pilW *
pilX *
pilY1 *
pilE *
B
Percent survival
100
50
0
0
2
4
6
8
10
12
Day
OP50 *
PAO1 *
pilA
fimU *
pilV *
pilW *
pilX *
pilY1 *
pilE *

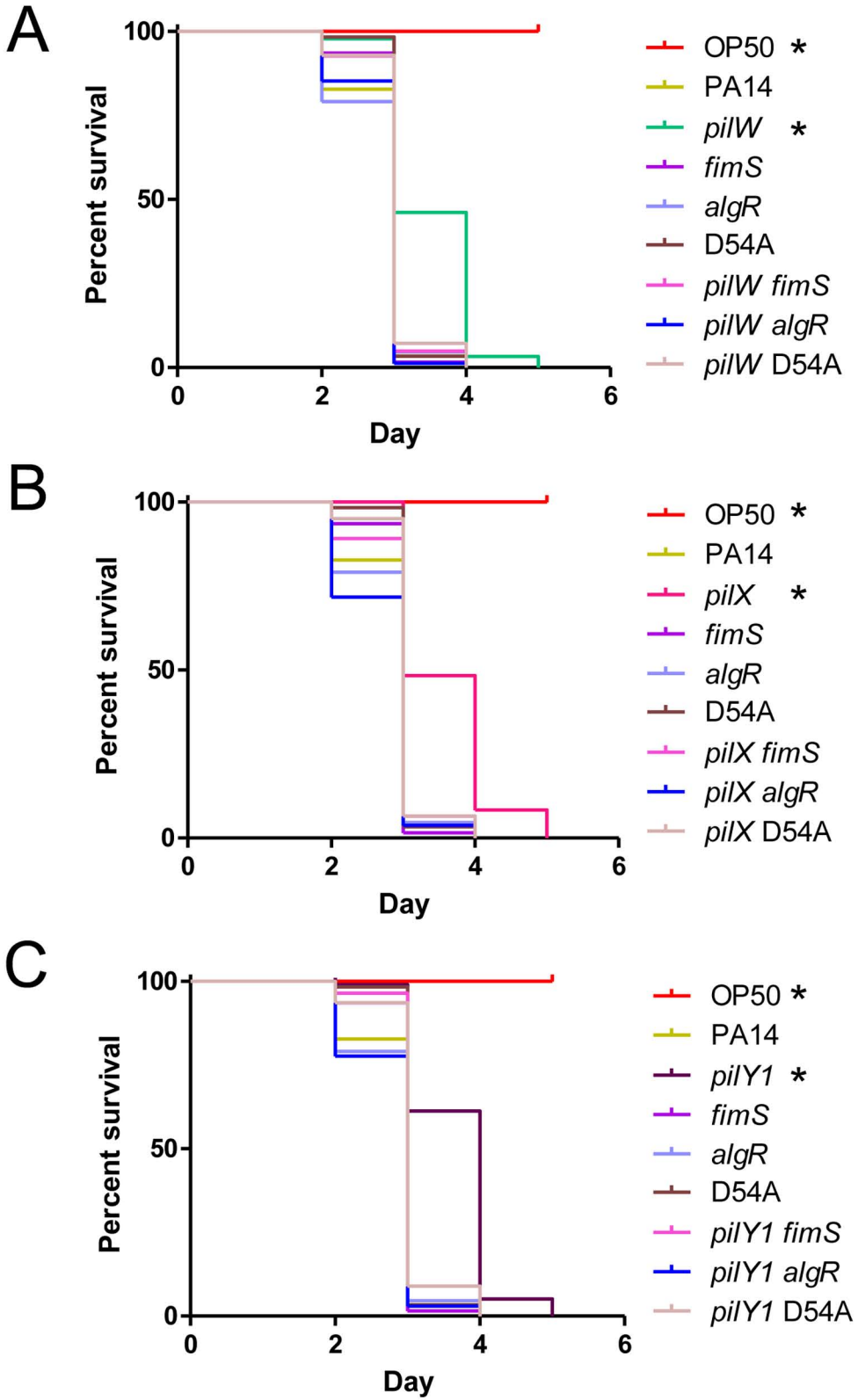

A
No arabinose
0.05% arabinose
Relative Luminescence (RLU)
1.0x10^08
1.0x10^07
1.0x10^06
1.0x10^05
1.0x10^04
0
100
200
300
Time (min)
sadC + sadC ← Dramatic increase
algR + algR ← Modest increase
PA14 + pB ← Wild type level
sadC + pB
algR + pB
Modest decrease
B
Relative Luminescence (RLU)
1.0x10^07
1.0x10^06
1.0x10^05
1.0x10^04
0
100
200
300
Time (min)
PA14
pilA
fimU
pilV
pilE
Wild type level
pilW
pilX
pilY1
Modest decrease

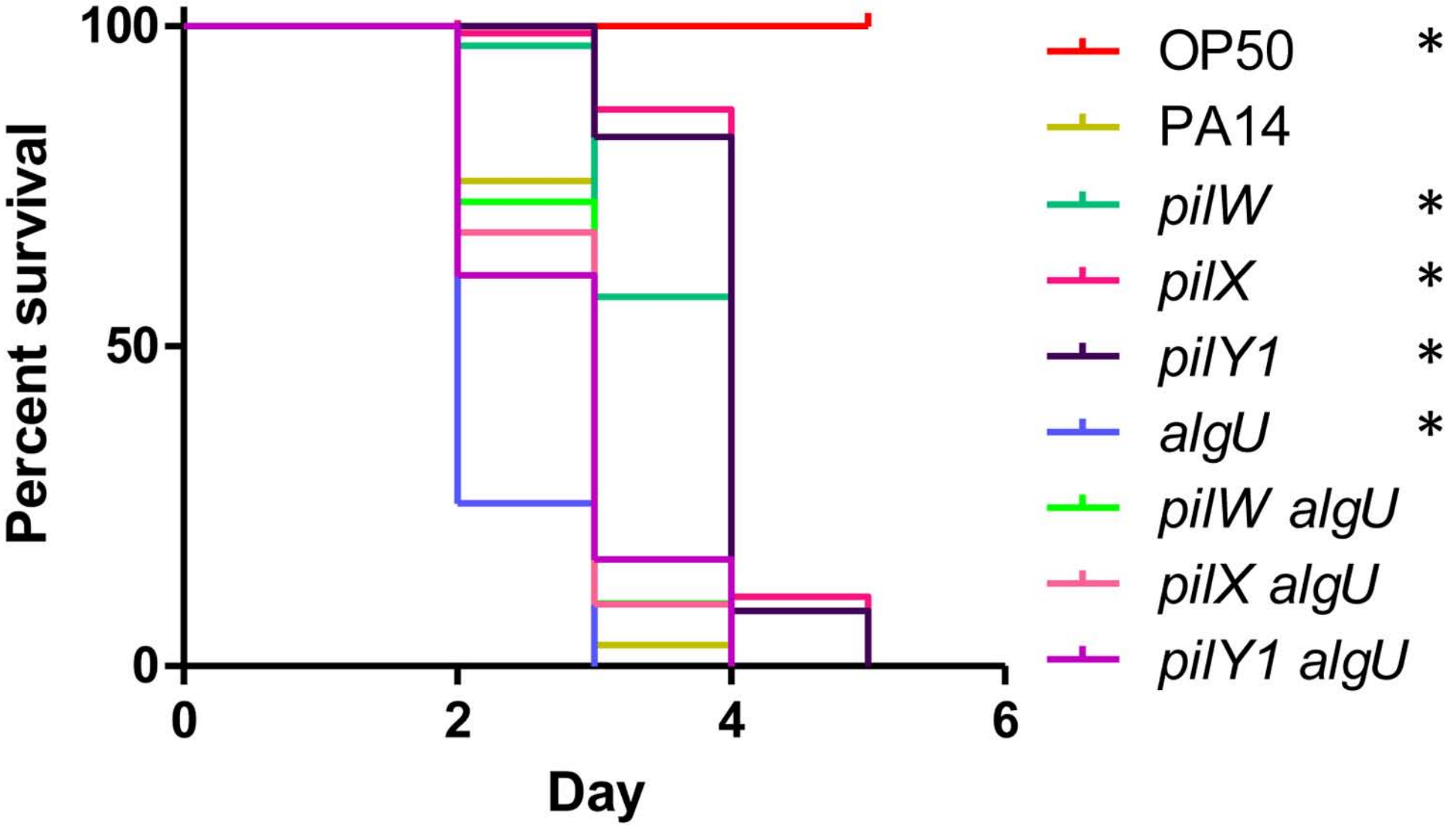

A
Growth (OD600)
Biofilm (Absorbance600)
0.8
0.6
0.4
0.2
0.0
***
***
Planktonic Growth
Biofilm
Arabinose
-
-
-
+
+
+
PA14 + pB
sadC + pB
sadC + sadC
PA14 + pB
sadC + pB
sadC + sadC
B
Percent survival
100
50
0
0
2
4
6
Day
No arabinose
PA14 + pB
sadC + pB
sadC + sadC *
0.05% arabinose
PA14 + pB
sadC +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


A
+T25 FimS
T18+T25
FimS
AlgR
PilA
FimU
PilV
PilW
PilX
PilE
←T18
MacConkey
+ Maltose
LB + X-gal
-
+
+
+
+
+
+
+
+
B
Relative Luminescence (RLU)
1.0×10^8
1.0×10^7
1.0×10^6
1.0×10^5
1.0×10^4
1.0×10^3
0
100
200
300
Time (min)
pilV
pilW
pilX
pilY1
pilA
fimU
PA14
pilE
fimS
algR
pilY1 fimS
pilY1 algR
Highly elevated
Moderately elevated
Wild type level
Baseline level


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
Percent survival
100
50
0
0
2
4
6
8
Day
No arabinose
PA14 + pB
algR + pB *
algR + algR
algR + D54A
0.05% arabinose
PA14 + pB
algR + pB *
algR + algR *
algR + D54A *
B
Growth (OD600)
Biofilm (Absorbance600)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
Planktonic Growth
Biofilm
Arabinose - - - - + + + +
PA14 + pB
algR + pB
algR + algR
algR + D54A
PA14 + pB
algR + pB
algR + algR
algR + D54A


A
Percent survival
100
50
0
0
2
4
6
Day
OP50 *
PA14
pilW *
fimS
algR
D54A
pilW fimS
pilW algR
pilW D54A
B
Percent survival
100
50
0
0
2
4
6
Day
OP50 *
PA14
pilX *
fimS
algR
D54A
pilX fimS
pilX algR
pilX D54A
C
Percent survival
100
50
0
0
2
4
6
Day
OP50 *
PA14
pilY1 *
fimS
algR
D54A
pilY1 fimS
pilY1 algR
pilY1 D54A


100
50
0
Percent survival
0
2
4
6
Day
OP50 *
PA14
pilW *
pilX *
pilY1 *
algU *
pilW algU
pilX algU
pilY1 algU

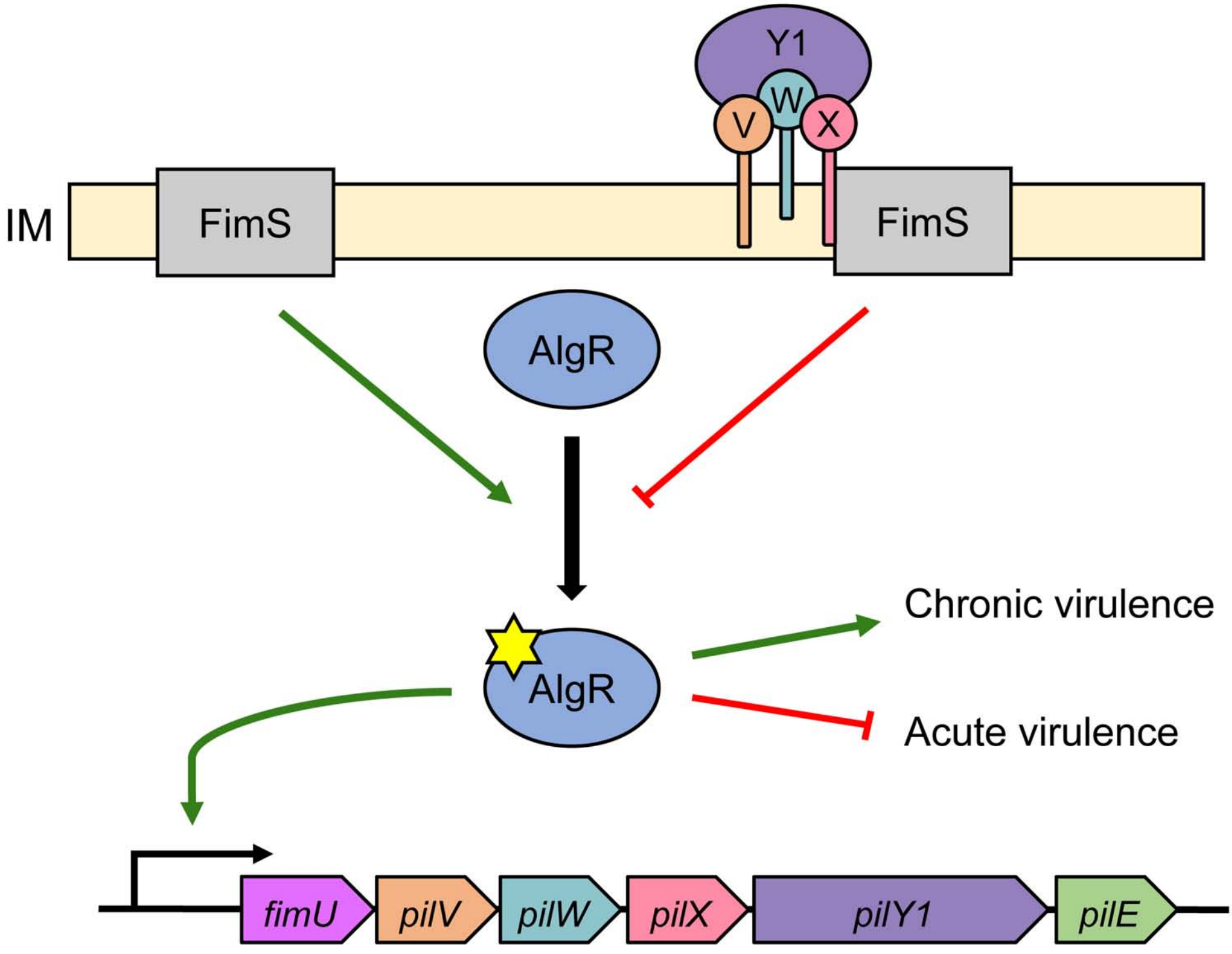

Y1
W
V
X
IM
FimS
FimS
AlgR
AlgR
Chronic virulence
Acute virulence
fimU
pilV
pilW
pilX
pilY1
pilE

Table S1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Characteristics	Source
Plasmids		
pEX18Gm	Suicide vector for gene replacement	[1]
pEX18Gm-*pilA*	Deletion construct for PA14 *pilA*	This work
pEX18Gm-*fimU*	Deletion construct for PA14 *fimU*	This work
pEX18Gm-*pilV*	Deletion construct for PA14 *pilV*	This work
pEX18Gm-*pilW*	Deletion construct for PA14 *pilW*	This work
pEX18Gm-*pilX*	Deletion construct for PA14 *pilX*	This work
pEX18Gm-*pilY1*	Deletion construct for PA14 *pilY1*	This work
pEX18Gm-*pilE*	Deletion construct for PA14 *pilE*	This work
pEX18Gm-*sadC*	Deletion construct for *sadC*	This work
pEX18Gm-*fimS*	Deletion construct for *fimS*	This work
pEX18Gm-*algR*	Deletion construct for *algR*	This work
pEX18Gm-*algR*$_{D54A}$	Mating construct for *algR* D54A substitution	This work
pEX18Gm-*algR*$_{D54E}$	Mating construct for *algR* D54E substitution	This work
pEX18Gm-*algU*	Deletion construct for *algU*	This work
pEX18Gm-*pilD*	Deletion construct for *pilD*	This work
pBADGr	Arabinose-inducible complementation vector	[2]
pBADGr-*pilW*	Complementation construct for *pilW*	[3]
pBADGr-*pilX*	Complementation construct for *pilX*	This work
pBADGr-*pilY1*	Complementation construct for *pilY1*	This work
pBADGr-*sadC*	Complementation construct for *sadC*	This work
pBADGr-*algR*	Complementation construct for *algR*	This work
pBADGr-*algR*$_{D54A}$	Complementation construct for *algR*$_{D54A}$	This work
pMS402	Transcriptional reporter vector carrying the promoterless *luxCDABE* genes	[4]
pMS402-P*fimU*	Transcriptional reporter for *fimU* promoter	This work
pMS402-P*cdrA*	Transcriptional reporter for *cdrA* promoter	This work
pKT25	Vector encoding T25 fragment of *B. pertussis* CyaA	[5]
pKT25-*fimS*	Vector encoding *fimS* fused to T25	This work
pUT18C	Vector encoding T18 fragment of *B. pertussis* CyaA	[6]
pUT18C-*fimS*	Vector encoding *fimS* fused to T18	[7]
pUT18C-*algR*	Vector encoding *algR* fused to T18	This work
pUT18C-*pilA*	Vector encoding *pilA* fused to T18	This work
pUT18C-*fimU*	Vector encoding *fimU* fused to T18	[8]
pUT18C-*pilV*	Vector encoding *pilV* fused to T18	This work
pUT18C-*pilW*	Vector encoding *pilW* fused to T18	This work
pUT18C-*pilX*	Vector encoding *pilX* fused to T18	This work
pUT18C-*pilE*	Vector encoding *pilE* fused to T18	[9]
***E. coli* strains**		
DH5α	*F$^-$φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk$^-$, mk$^+$) phoA supE44 thi-1 gyrA96 relA1 λ−*	Invitrogen

SM10	*thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu* (Km^R)	Invitrogen
OP50	*Uracil auxotroph, C. elegans food source*	[10]
BTH 101	*Bacterial two-hybrid reporter strain*	Euromedex
***P. aeruginosa* strains**		
PAO1	WT	[11]
PAO1 *pilA*	ISphoA/hah transposon insertion at position 163	[11]
PAO1 *fimU*	ISlacZ/hah transposon insertion at position 237	[11]
PAO1 *pilV*	ISphoA/hah transposon insertion at position 122	[11]
PAO1 *pilW*	ISlacZ/hah transposon insertion at position 381	[11]
PAO1 *pilX*	ISphoA/hah transposon insertion at position 182	[11]
PAO1 *pilY1*	ISlacZ/hah transposon insertion at position 1407	[11]
PAO1 *pilE*	ISphoA/hah transposon insertion at position 183	[11]
PAO1 *sadC*	Deletion of *sadC*	This work
PAO1 *fimS*	Deletion of *fimS*	This work
PAO1 *algR*	Deletion of *algR*	This work
PAO1 $algR_{D54A}$	PAO1 expressing the phospho-inactive form of *algR*	This work
PAO1 $algR_{D54E}$	PAO1 expressing the phospho-mimetic form of *algR*	This work
PA14	WT	[12]
PA14 + pBADGr	WT with pBADGr	This work
PA14 + pMS402-P*fimU*	WT with pMS402 containing *fimU* promoter	This work
PA14 + pMS402-P*fimU* + pBADGr	WT with pMS402 containing *fimU* promoter and pBADGr	This work
PA14 + pMS402-P*cdrA*	WT with pMS402 containing *cdrA* promoter	This work
PA14 + pMS402-P*cdrA* + pBADGr	WT with pMS402 containing *cdrA* promoter and pBADGr	This work
PA14 *pilA*	Deletion of *pilA*	This work
PA14 *pilA* + pMS402-P*fimU*	Deletion of *pilA* with pMS402 containing *fimU* promoter	This work
PA14 *pilA* + pMS402-P*cdrA*	Deletion of *pilA* with pMS402 containing *cdrA* promoter	This work
PA14 *fimU*	Deletion of *fimU*	This work
PA14 *fimU* + pMS402-P*fimU*	Deletion of *fimU* with pMS402 containing *fimU* promoter	This work
PA14 *fimU* + pMS402-P*cdrA*	Deletion of *fimU* with pMS402 containing *cdrA* promoter	This work
PA14 *pilV*	Deletion of *pilV*	This work
PA14 *pilV* + pMS402-P*fimU*	Deletion of *pilV* with pMS402 containing *fimU* promoter	This work
PA14 *pilV* + pMS402-P*fimU* + pBADGr	Deletion of *pilV* with pMS402 containing *fimU* promoter and pBADGr	This work
PA14 *pilV* + pMS402-P*fimU* + pBADGr-	Deletion of *pilV* with pMS402 containing *fimU* promoter and complemented with *pilV*	This work

pilV		
PA14 *pilV* + pMS402-P*cdrA*	Deletion of *pilV* with pMS402 containing *cdrA* promoter	This work
PA14 *pilW*	Deletion of *pilW*	This work
PA14 *pilW* + pBADGr	Deletion of *pilW* containing pBADGr	This work
PA14 *pilW* + pBADGr-*pilW*	Deletion of *pilW* complemented with *pilW*	This work
PA14 *pilW* + pMS402-P*fimU*	Deletion of *pilW* with pMS402 containing *fimU* promoter	This work
PA14 *pilW* + pMS402-P*fimU* + pBADGr	Deletion of *pilW* with pMS402 containing *fimU* promoter and pBADGr	This work
PA14 *pilW* + pMS402-P*fimU* + pBADGr-*pilW*	Deletion of *pilW* with pMS402 containing *fimU* promoter and complemented with *pilW*	This work
PA14 *pilW* + pMS402-P*fimU* + pBADGr-*pilY1*	Deletion of *pilW* with pMS402 containing *fimU* promoter and complemented with *pilY1*	This work
PA14 *pilW* + pMS402-P*cdrA*	Deletion of *pilW* with pMS402 containing *cdrA* promoter	This work
PA14 *pilX*	Deletion of *pilX*	This work
PA14 *pilX* + pBADGr	Deletion of *pilX* containing pBADGr	This work
PA14 *pilX* + pBADGr-*pilX*	Deletion of *pilX* complemented with *pilX*	This work
PA14 *pilX* + pMS402-P*fimU*	Deletion of *pilX* with pMS402 containing *fimU* promoter	This work
PA14 *pilX* + pMS402-P*fimU* + pBADGr	Deletion of *pilX* with pMS402 containing *fimU* promoter and pBADGr	This work
PA14 *pilX* + pMS402-P*fimU* + pBADGr-*pilX*	Deletion of *pilX* with pMS402 containing *fimU* promoter and complemented with *pilX*	This work
PA14 *pilX* + pMS402-P*fimU* + pBADGr-*pilY1*	Deletion of *pilX* with pMS402 containing *fimU* promoter and complemented with *pilY1*	This work
PA14 *pilX* + pMS402-P*cdrA*	Deletion of *pilX* with pMS402 containing *cdrA* promoter	This work
PA14 *pilY1*	Deletion of *pilY1*	This work
PA14 *pilY1* + pBADGr	Deletion of *pilY1* containing pBADGr	This work
PA14 *pilY1* + pBADGr-*pilY1*	Deletion of *pilY1* complemented with *pilY1*	This work
PA14 *pilY1* + pMS402-P*fimU*	Deletion of *pilY1* with pMS402 containing *fimU* promoter	This work
PA14 *pilY1* + pMS402-P*fimU* +	Deletion of *pilY1* with pMS402 containing *fimU* promoter and pBADGr	This work

pBADGr		
PA14 *pilY1* + pMS402-P*fimU* + pBADGr-*pilY1*	Deletion of *pilY1* with pMS402 containing *fimU* promoter and complemented with *pilY1*	This work
PA14 *pilY1* + pMS402-P*fimU* + pBADGr-*fimU*	Deletion of *pilY1* with pMS402 containing *fimU* promoter and complemented with *fimU*	This work
PA14 *pilY1* + pMS402-P*fimU* + pBADGr-*pilV*	Deletion of *pilY1* with pMS402 containing *fimU* promoter and complemented with *pilV*	This work
PA14 *pilY1* + pMS402-P*fimU* + pBADGr-*pilW*	Deletion of *pilY1* with pMS402 containing *fimU* promoter and complemented with *pilW*	This work
PA14 *pilY1* + pMS402-P*fimU* + pBADGr-*pilX*	Deletion of *pilY1* with pMS402 containing *fimU* promoter and complemented with *pilX*	This work
PA14 *pilY1* + pMS402-P*fimU* + pBADGr-*pilE*	Deletion of *pilY1* with pMS402 containing *fimU* promoter and complemented with *pilE*	This work
PA14 *pilY1* + pMS402-P*cdrA*	Deletion of *pilY1* with pMS402 containing *cdrA* promoter	This work
PA14 *pilE*	Deletion of *pilE*	This work
PA14 *pilE* + pMS402-P*fimU*	Deletion of *pilE* with pMS402 containing *fimU* promoter	This work
PA14 *pilE* + pMS402-P*cdrA*	Deletion of *pilE* with pMS402 containing *cdrA* promoter	This work
PA14 *sadC roeA*	Deletion of *sadC* and *roeA*	[13]
PA14 *sadC*	Deletion of *sadC*	This work
PA14 *sadC* + pBADGr	Deletion of *sadC* with pBADGr	This work
PA14 *sadC* + pMS402-P*cdrA* + pBADGr	Deletion of *sadC* with pMS402 containing *cdrA* promoter and pBADGr	This work
PA14 *sadC* + pBADGr-*sadC*	Deletion of *sadC* complemented with *sadC*	This work
PA14 *sadC* + pMS402-P*cdrA* + pBADGr-*sadC*	Deletion of *sadC* with pMS402 containing *cdrA* promoter and complemented with *sadC*	This work
PA14 *fimS*	Deletion of *fimS*	This work
PA14 *fimS* + pMS402-P*fimU*	Deletion of *fimS* with pMS402 containing *fimU* promoter	This work
PA14 *algR*	Deletion of *algR*	This work
PA14 *algR* + pBADGr	Deletion of *algR* with pBADGr	This work
PA14 *algR* + pMS402-P*cdrA* +	Deletion of *algR* with pMS402 containing *cdrA* promoter and PA14	This work

pBADGr		
PA14 *algR* + pBADGr-*algR*	Deletion of *algR* complemented with WT *algR*	This work
PA14 *algR* + pMS402-P*cdrA* + pBADGr-*algR*	Deletion of *algR* with pMS402 containing *cdrA* promoter and complemented with *algR*	This work
PA14 *algR* + pBADGr-*algR*$_{D54A}$	Deletion of *algR* complemented with phospho-inactive *algR*	This work
PA14 *algR* + pMS402-P*fimU*	Deletion of *algR* with pMS402 containing *fimU* promoter	This work
PA14 *algR*$_{D54A}$	PA14 expressing the phospho-inactive form of *algR*	This work
PA14 *algR*$_{D54E}$	PA14 expressing the phospho-mimetic form of *algR*	This work
PA14 *algU*	Deletion of *algU*	This work
PA14 *pilD*	Deletion of *pilD*	This work
PA14 *pilW fimS*	Deletion of *fimS* in *pilW* background	This work
PA14 *pilW algR*	Deletion of *algR* in *pilW* background	This work
PA14 *pilW algR*$_{D54A}$	Deletion of *pilW* in phospho-inactive *algR* background	This work
PA14 *pilW algU*	Deletion of *algU* in *pilW* background	This work
PA14 *pilX fimS*	Deletion of *fimS* in *pilX* background	This work
PA14 *pilX algR*	Deletion of *algR* in *pilX* background	This work
PA14 *pilX algR*$_{D54A}$	Deletion of *pilX* deletion in phospho-inactive *algR* background	This work
PA14 *pilX algU*	Deletion of *algU* in *pilX* background	This work
PA14 *pilY1 fimS*	Deletion of *fimS* in *pilY1* background	This work
PA14 *pilY1 fimS* + pMS402-P*fimU*	Deletion of *pilY1*/*fimS* with pMS402 containing *fimU* promoter	This work
PA14 *pilY1 algR*	Deletion of *algR* in *pilY1* background	This work
PA14 *pilY1 algR* + pMS402-P*fimU*	Deletion of *pilY1*/*algR* with pMS402 containing *fimU* promoter	This work
PA14 *pilY1 algR*$_{D54A}$	Deletion of *pilY1* in phospho-inactive *algR* background	This work
PA14 *pilY1 algU*	Deletion of *algU* in *pilY1* background	This work

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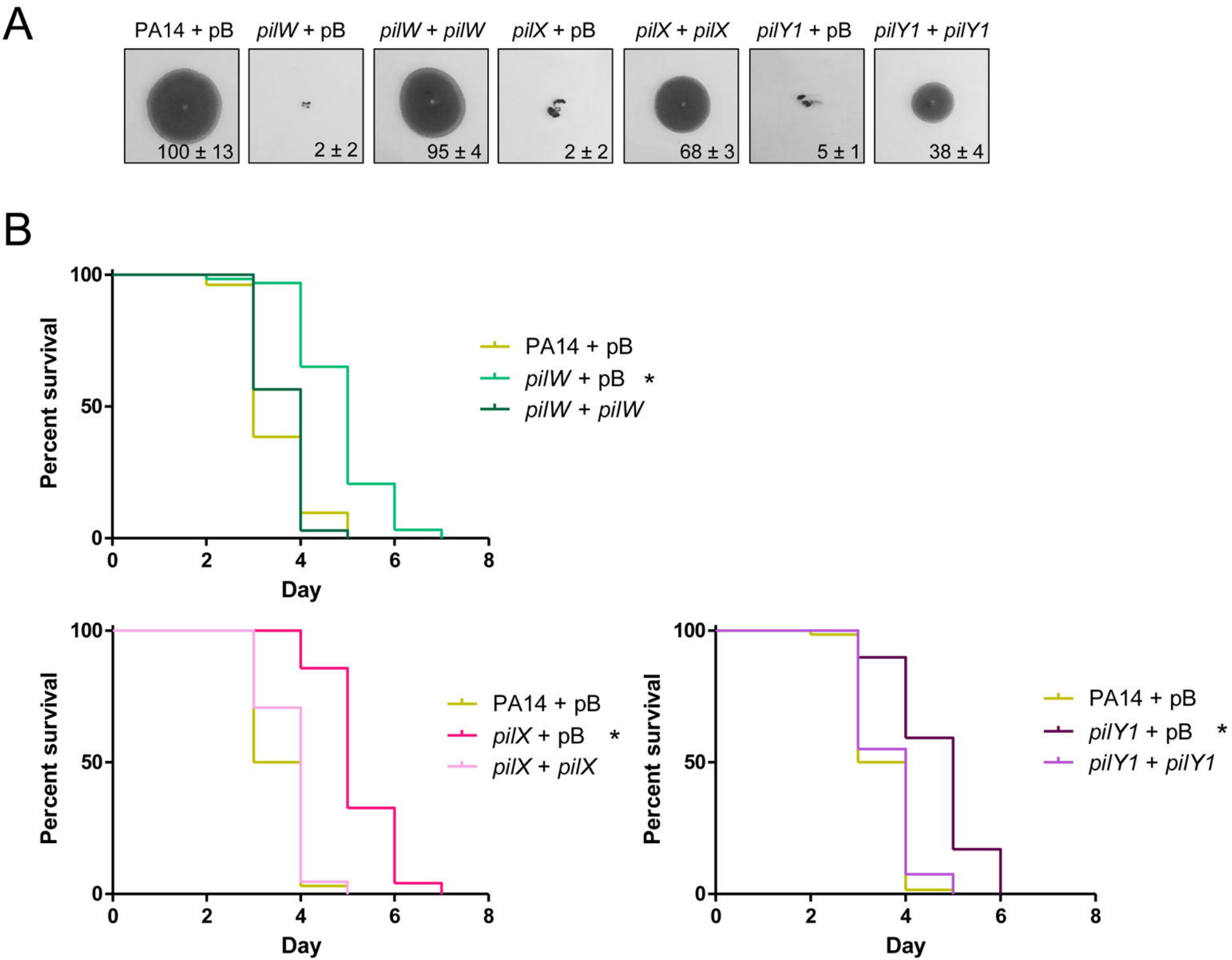
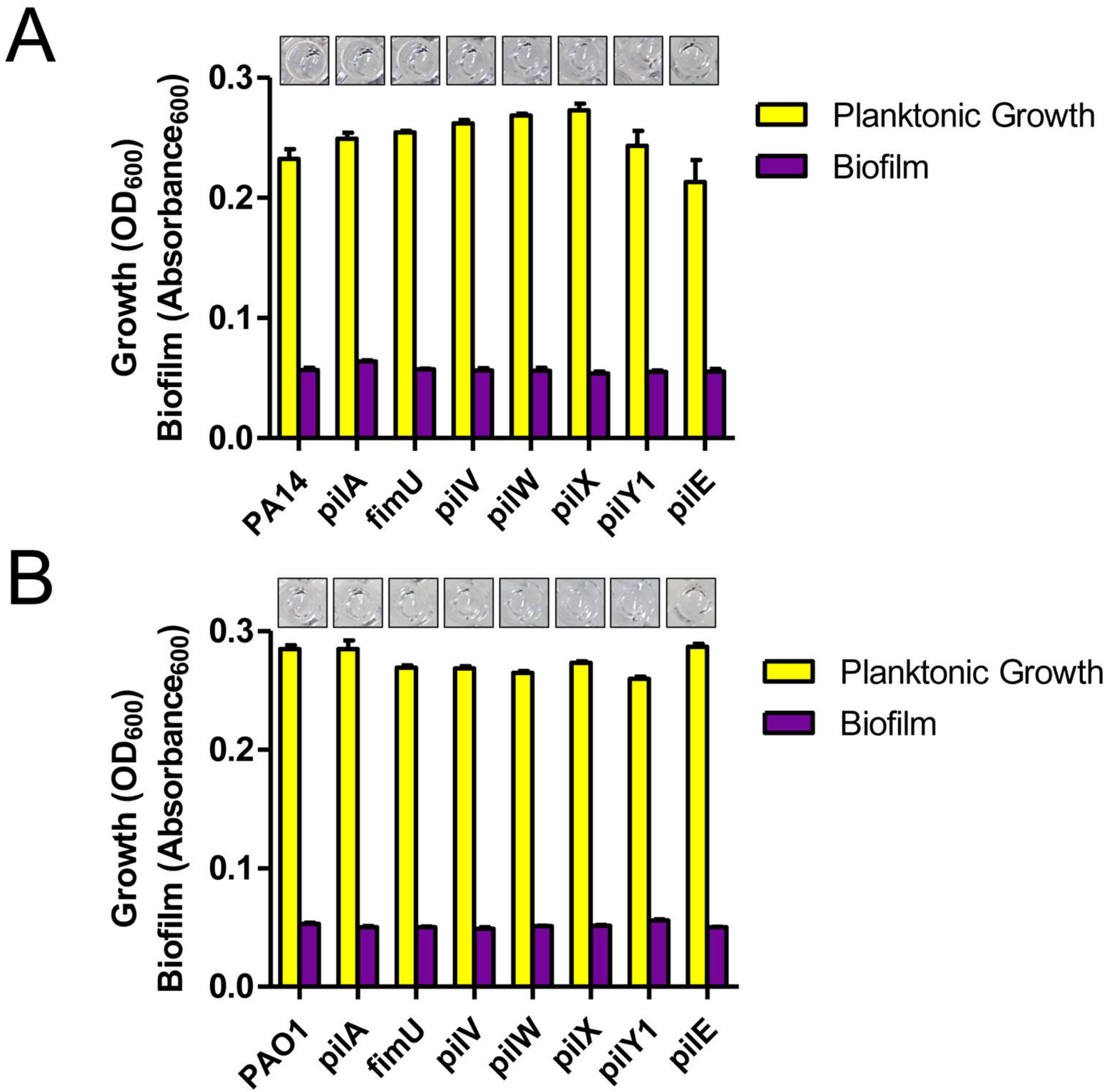
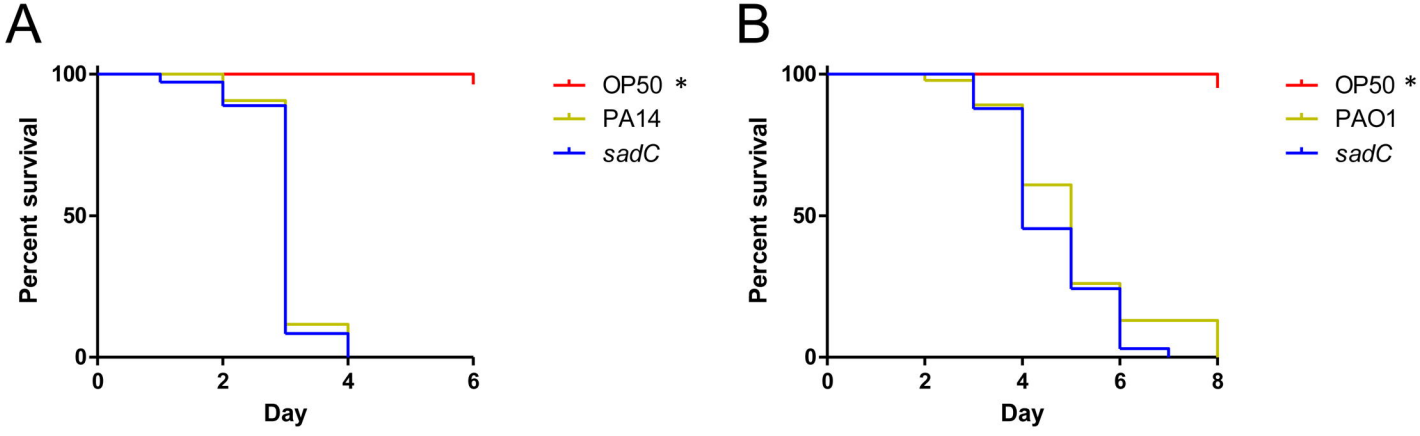
Table S2. Primers used in this study. Restriction sites are underlined.

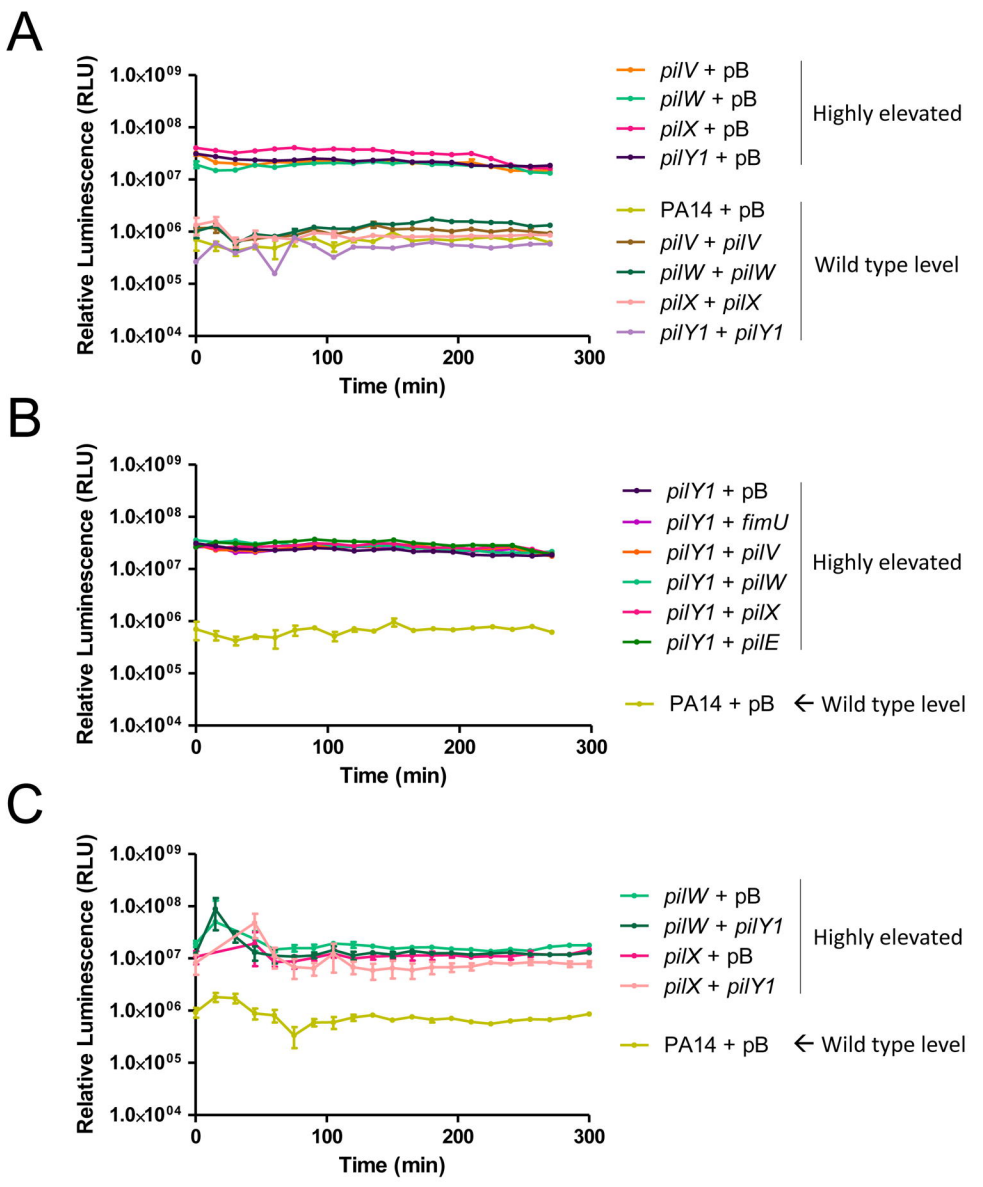
Primer name	Sequence
pilA(-1100)F	TCGAGGATCCGCATCACGATCTTCTC
pilA(52)R	TACCTGCAGTCGCAACCACGATCATCAG
pilA(62)F	ACCTGCAGATATGCCTGCCCTGACTGCA
pilA(+1056)R	CTGGAAGCTTCCGGCGGAATCAACG
pilX CF	GTCGAATTCATGACCCTGCGCCATACCTCTC
pilX CR	GACAAGCTTTCAGTTGGTATACAGGCGTGCA
pilY1 CF	GTCAGAATTCTGGAGCCAGCGCATGATC
pilY1 CR	CTATCCCGGGTCATTTCTCCTCGACGAC
sadC(-500)F	GATTGAATTCGAGCTCGAACACGGTGACGATCCCG
sadC(+558)R	CTAATCTAGAGGATCCCAGTCCGGCTCGTAGCGC
algR F1	GCAGAATTCGGCCGAGCATGCGGTG
algR R1	GCAGGATCCGAGGTTCGTCATCGA
algR F2	GCAGGATCCGCCGGAGTCAGGCG
algR R2	GCAAAGCTTCGCAGGCTGGAGGTG
fimS(-500)F	GACTGGTACCGTTCATGTGCACGTCTTCCAG
fimS(+500)R	GCCGAAGCTTTGTGGTCGGCAATGAAGAAG
fimS(18)F	GTACAACCATGGTAAGTTCCTTGAATCGGATAGGC
fimS(15)R	GAACTTACCATGGTTGTACATGCAGGAAGCCTGA
algRD54-500F	CGGCTCTAGATGAGCAGTATCGTCTTGGCGATCG
algRD54+500R	GATTAAGCTTGCACGAAGCGCTCGCCGAAC
algR(D54A)F	ATCGTCCTGCTGGCTATCCGCATGCC
algR(D54A)R	GGCATGCGGATAGCCAGCAGGACGAT
algR(D54E)F	ATCGTCCTGCTGGAAATCCGCATGCCC
algR(D54E)R	GGGCATGCGGATTTCCAGCAGGACGAT
algU 74R	GACTAGATCTAGACATGTCTGAGCAGATCGAAAGC
algU 51F	CAGACATGTCTAGATCTAGTCGCTCGTGAAGCAATC
algU -478F	GTGAGCTCTCAAGGCCAGACTCAG
algU +499R	GAAAGCTTGGTATCGCTGGACGAGGAG

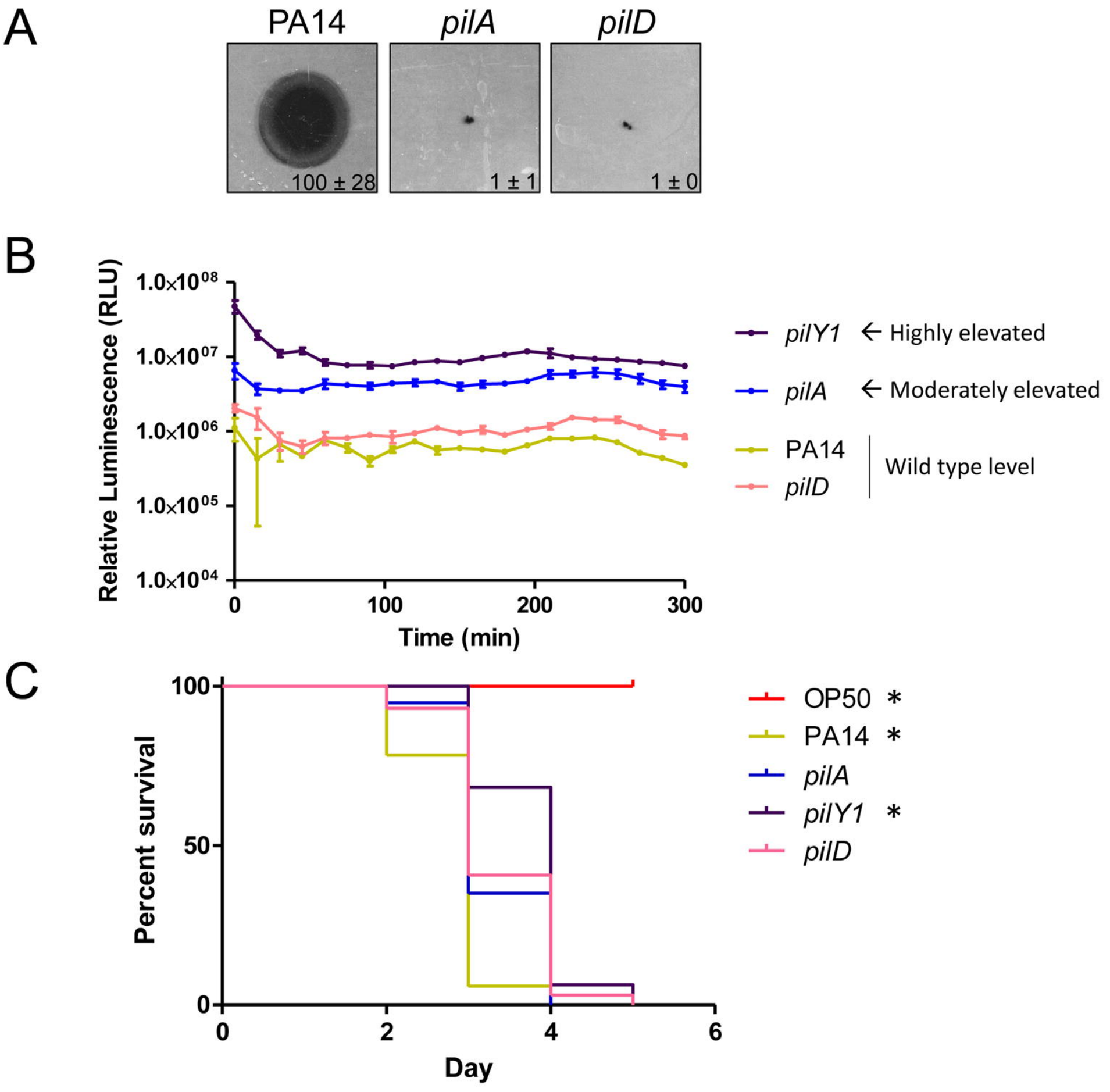
pilD 90R	GACTACTCTAGACGATGGTTGAGGAAGCTGCC
pilD 85F	CATCGTCTAGAGTAGTCCTATCTGGCGATTGC
pilD -638F	CGGAGCTCCAGTTCCAGTCCGTATTTG
pilD +456R	GTAAGCTTCCTGGAGGATCGAGCGC
algR CF	GTAACCATGGCTCATGCAGGAAGCCTGAGCTTATG
algR CR	CAGTAAGCTTTCAGAGCTGATGCATCAGACGCCTG
PfimU F	GTTAGGATCCGCTCTCTTACCTGTGCTCCA
PfimU R	GCATGGATCCGCAGTACTCCACAAGGAAAAG
PcdrA-500F	GAGGATCCGATCGGCGCCTTGTTGCTG
PcdrA-1R	GCGGATCCGAAAATCTCCCTATCTGCGTGGC
FimS Bac-F	CATTCTAGACATGCCTATCCGATTCAAG
FimS Bac-R	CCTGAATTCTCAGGCTTCCTGCATGAGTCG
AlgR BACTH F	GCAGGATCCCATGAATGTCCTGATTGTCG
AlgR BACTH R	GCAGGTACCGAGAGCTGATGCATCAGACG
pilAB2HFor	GCATCTAGACTTTACCTTGATCGAACTGATGATCGTGGTTG
pilA2B2HRev	CATGAATTCTTAGTTATCACAACCTTTCGGAGTGAACATCGG
pilVB2HFor	GCATCTAGACTTCAGCATGATCGAAGTGCTGGTCG
pilVB2HRev	CATGGTACCTCATGGCTCGACCCTGAGG
pilWB2HFor	GCATCTAGACCTGTCCATGATCGAACTACTGGTGGCC
pilWMCS2Rev	AAGGTACCTCATGGCACGAGATTCCTGAGTGTCTGG
pilXB2HFor	GTATCTAGACGCCACGCTGGTCATCGCC
pilXMCS2Rev	AAGGTACCTCAGTTGGTATAGAGACGGGCGAGAA

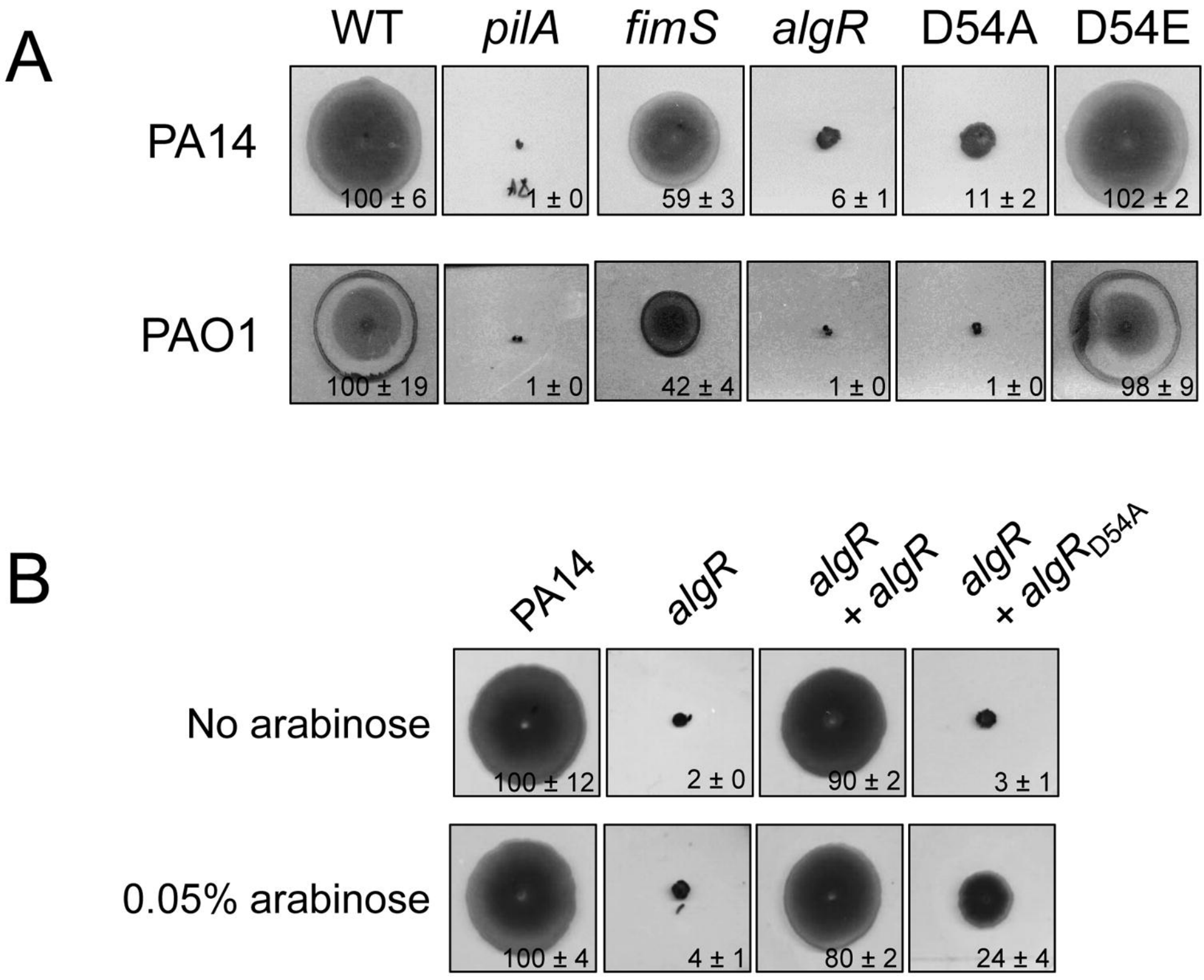

A
PA14 + pB
pilW + pB
pilW + pilW
pilX + pB
pilX + pilX
pilY1 + pB
pilY1 + pilY1
100 ± 13
2 ± 2
95 ± 4
2 ± 2
68 ± 3
5 ± 1
38 ± 4
B
Percent survival
Day
PA14 + pB
pilW + pB *
pilW + pilW
pilX + pB *
pilX + pilX
pilY1 + pB *
pilY1 + pilY1


A
Growth (OD600)
Biofilm (Absorbance600)
0.3
0.2
0.1
0.0
PA14
pilA
fimU
pilV
pilW
pilX
pilY1
pilE
Planktonic Growth
Biofilm
B
Growth (OD600)
Biofilm (Absorbance600)
0.3
0.2
0.1
0.0
PAO1
pilA
fimU
pilV
pilW
pilX
pilY1
pilE
Planktonic Growth
Biofilm


A
100
50
0
Percent survival
0
2
4
6
Day
OP50 *
PA14
sadC
B
100
50
0
Percent survival
0
2
4
6
8
Day
OP50 *
PAO1
sadC


A
Relative Luminescence (RLU)
1.0x10^09
1.0x10^08
1.0x10^07
1.0x10^06
1.0x10^05
1.0x10^04
0
100
200
300
Time (min)
pilV + pB
pilW + pB
pilX + pB
pilY1 + pB
Highly elevated
PA14 + pB
pilV + pilV
pilW + pilW
pilX + pilX
pilY1 + pilY1
Wild type level
B
Relative Luminescence (RLU)
Time (min)
pilY1 + pB
pilY1 + fimU
pilY1 + pilV
pilY1 + pilW
pilY1 + pilX
pilY1 + pilE
Highly elevated
PA14 + pB ← Wild type level
C
Relative Luminescence (RLU)
Time (min)
pilW + pB
pilW + pilY1
pilX + pB
pilX + pilY1
Highly elevated
PA14 + pB ← Wild type level


A
PA14
pilA
pilD
100 ± 28
1 ± 1
1 ± 0
B
Relative Luminescence (RLU)
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
0
100
200
300
Time (min)
pilY1 ← Highly elevated
pilA ← Moderately elevated
PA14
pilD
Wild type level
C
Percent survival
100
50
0
0
2
4
6
Day
OP50 *
PA14 *
pilA
pilY1 *
pilD


A
WT
pilA
fimS
algR
D54A
D54E
PA14
100 ± 6
1 ± 0
59 ± 3
6 ± 1
11 ± 2
102 ± 2
PAO1
100 ± 19
1 ± 0
42 ± 4
1 ± 0
1 ± 0
98 ± 9
B
PA14
algR
algR + algR
algR + algR D54A
No arabinose
100 ± 12
2 ± 0
90 ± 2
3 ± 1
0.05% arabinose
100 ± 4
4 ± 1
80 ± 2
24 ± 4