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1 **cAMP-independent control of twitching motility in *Pseudomonas aeruginosa***

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19 **ABSTRACT**

20 FimV is a *Pseudomonas aeruginosa* inner membrane hub protein that modulates levels
21 of the second messenger, cyclic AMP (cAMP), through activation of the adenylate cyclase, CyaB.
22 Although type IVa pilus (T4aP)-dependent twitching motility is modulated by cAMP levels,
23 mutants lacking FimV are twitching impaired,even when exogenous cAMP is provided. Here we
24 further define FimV's cAMP-dependent and -independent regulation of twitching. We
25 confirmed that the response regulator of the T4aP-associated Chp chemotaxis system, PilG,
26 required both FimV and the CyaB regulator, FimL, to activate CyaB. However, in cAMP-replete
27 backgrounds - lacking the cAMP phosphodiesterase CpdA or the CheY-like protein PilH, or
28 expressing constitutively-active CyaB - *pilG* and *fimV* mutants failed to twitch. Both cytoplasmic
29 and periplasmic domains of FimV were important for its cAMP-dependent and -independent
30 roles, while its septal peptidoglycan-targeting LysM motif was required only for twitching
31 motility. Polar localization of the sensor kinase PilS, a key regulator of transcription of the major
32 pilin, was FimV-dependent. However, unlike its homologues in other species that localize
33 flagellar system components, FimV was not required for swimming motility. These data provide
34 further evidence to support FimV's role as a key hub protein that coordinates the polar
35 localization and function of multiple structural and regulatory proteins involved in *P. aeruginosa*
36 twitching motility.

37

38 **IMPORTANCE**

39 *Pseudomonas aeruginosa* is a serious opportunistic pathogen. Type IVa pili (T4aP) are important
40 for its virulence, because they mediate dissemination and invasion via twitching motility, and

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41 are involved in surface sensing which modulates pathogenicity via changes in cAMP levels. Here
42 we show that the hub protein FimV and the response regulator of the Chp system, PilG, regulate
43 twitching independently of their roles in modulation of cAMP synthesis. These functions do not
44 require the putative scaffold protein FimL, proposed to link PilG with FimV. PilG may regulate
45 asymmetric functioning of the T4aP system to allow for directional movement, while FimV
46 appears to localize both structural and regulatory elements – including the PilSR two-
47 component system – to cell poles for optimal function.

48

49 **INTRODUCTION**

50 Type IV pili (T4P) are polar filamentous surface appendages made by a broad range of
51 bacteria and archaea (1, 2). They can be divided into two sub-families, type IVa (T4aP) and type
52 IVb (T4bP), which – though clearly related – differ in their pilin subunits and assembly system
53 architectures (2). T4aP are involved in several processes including DNA uptake, surface
54 attachment, and twitching motility (3-5). During twitching motility, T4aP undergo repeated
55 cycles of assembly and disassembly, acting as molecular grappling hooks to pull the cells along
56 surfaces. Well-studied T4aP model species include *Neisseria spp.*, *Myxococcus xanthus*, and
57 *Pseudomonas aeruginosa* (6, 7). Although core structural components of the T4aP assembly
58 machinery and pilus fibre are shared, each species has unique regulatory elements that control
59 the function of the T4aP machinery in response to their specific environmental requirements.
60 Without these regulatory proteins, the bacteria make non-functional T4aP systems (8-11).

61 The *P. aeruginosa* Chp system is a putative chemosensory system that controls both
62 twitching motility and intracellular levels of the second messenger, cyclic adenosine

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63 monophosphate (cAMP) (12-14). It resembles the well-studied Che system of *E. coli,* but lacks a
64 CheZ-like phosphatase. Rather, similar to *Sinorhizobium meliloti* (15), it has two CheY-like
65 response regulators, PilG and PilH (14, 16). PilG is proposed to regulate activation of CyaB and
66 pilus extension (17), while PilH has been proposed to be either a phosphate sink that limits
67 downstream signalling through PilG in lieu of a phosphatase (17, 18), or a separate response
68 regulator controlling function of the T4aP retraction ATPase, PilT (12).
69 The Chp system positively regulates intracellular levels of cAMP by activating the major
70 adenylate cyclase, CyaB (17). Deletion of *pilG* results in decreased cAMP, surface piliation, and
71 twitching motility, while *pilH* mutants have increased cAMP and surface piliation but decreased
72 twitching relative to wild type (17). Supplementation of a *pilG* mutant with exogenous cAMP
73 restored surface piliation but not twitching motility (17), suggesting that PilG regulates pilus
74 biogenesis and function by at least two pathways. A recent study (18) showed that of the two
75 proteins, PilH is the preferred target of ChpA phosphorylation, consistent with its proposed role
76 as a phosphate sink. Decreased twitching motility in the *pilH* background may reflect
77 hyperphosphorylation of PilG, perturbation of the chemotactic response, and uncoordinated
78 movement.
79 Important for *P. aeruginosa* virulence is its ability to switch from a planktonic to sessile
80 state when cells contact surfaces (19, 20). T4aP-mediated surface interaction is proposed to
81 lead to signalling through the Chp system, upregulating surface-associated virulence
82 phenotypes by increasing intracellular levels of cAMP (20). Vfr (virulence factor regulator) binds
83 cAMP and modulates the expression of >200 genes, including the type II secretion system (T2SS)
84 and its effectors, and T4aP assembly components including the motor ATPases PilBTU, the

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85 alignment subcomplex PilMNOP, the secretin PilQ, and the PilSR two-component system that
86 regulates PilA levels (21). This regulatory circuitry allows for just-in-time expression of
87 components required for a surface-associated lifestyle in response to surface contact.
88 FimV is also required for T4aP function and CyaB activation (17), and is proposed to link
89 into the Chp system via the cytoplasmic protein, FimL (22). FimV is a 97 kDa inner-membrane
90 protein with one transmembrane segment. Its periplasmic domain contains a lysin (LysM) motif
91 that binds peptidoglycan (PG) (23), and its cytoplasmic domain contains three discontinuous
92 tetratricopeptide repeat (TPR) motifs involved in protein-protein interactions (24). FimV
93 homologs have been identified in other T4P-producing bacteria (10) although their overall
94 sequence identity is low, with the most conserved features being the LysM motif (COG3170),
95 the single transmembrane segment, and a highly conserved cytoplasmic "FimV C-terminal
96 domain" – TIGR03504 – encompassing a single TPR repeat and capping helix (25).
97 FimV homologs have been characterized in several species (8-11, 26-28), but their
98 functions are not necessarily conserved. Deletion of FimV in *Legionella pneumophila* resulted in
99 loss of twitching motility and cell elongation, while deletion of the *Neisseria meningitidis* FimV
100 homolog TspA led to decreased host cell adhesion but no effect on twitching motility or surface
101 piliation. The *Vibrio cholerae* homolog, HubP, functions as a protein interaction hub, although its
102 role is not limited to T4P localization. Deletion of HubP altered the cellular distribution of the
103 chemotactic and flagellar machinery, and the chromosomal origin, *oriCI* (28). HubP from
104 *Shewenella putrifaciens* is responsible for localization of the chemotactic machinery, but not the
105 flagellar system (27). Yamaichi et al. (28) showed that polar localization of *V. cholerae* HubP was
106 dependent on the conserved LysM motif (25). Wehbi et al. (29) showed that *P. aeruginosa fimV*

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107 mutants have decreased levels of the T4aP alignment subcomplex proteins, PilMNOP, while in-
108 frame deletion of FimV's LysM motif resulted in fewer PilQ multimers, suggesting that PG
109 binding is important for optimal secretin formation. A recent study (30) confirmed that FimV
110 participates in localization of PilMNOPQ to sites of future cell division, ultimately placing T4aP
111 assembly systems at both poles of newly divided cells.
112 T4aP-mediated twitching motility requires both cAMP-dependent and independent
113 inputs (17). For example, provision of exogenous cAMP to mutants lacking PilG restored
114 piliation but not motility, and a mutant expressing a constitutively active form of CyaB but
115 lacking FimV failed to twitch (25). FimL was proposed to be a scaffold protein linking PilG to the
116 C-terminal TPR motif of FimV, leading to CyaB activation, and FimV localized both FimL and PilG
117 to cell poles (22, 25). However, of these three proteins, only FimL is dispensable for twitching
118 motility in cAMP-replete conditions. Thus, the FimV-FimL-PilG model fails to explain the cAMP-
119 independent roles of FimV and PilG in twitching.
120 Here we provide evidence supporting different cAMP-independent roles for FimV and
121 PilG in regulation of twitching motility. We show that in addition to polar localization of FimL,
122 PilG, and PilMNOPQ (22, 30), FimV is responsible for polar localization of PilS, the membrane-
123 bound sensor kinase that controls *pilA* transcription. These data show that FimV plays a central
124 role in control of twitching motility that overlaps with – but is distinct from – that of the Chp
125 system.
126
127 **RESULTS**
128 **FimV is required for Chp activation of CyaB**

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129 FimL, FimV, and PilG are all required for activation of CyaB, with FimL proposed to link
130 FimV to the Chp system through PilG (17, 22, 31, 32). However, while phenotypes associated
131 with *fimL* deletion could be rescued by deletion of *cpdA* or by increasing intracellular cAMP
132 levels in other ways (22, 32, 33), provision of exogenous cAMP failed to restore motility in a *pilG*
133 mutant (17). We investigated whether the cAMP-independent function of PilG also required
134 FimV by comparing PilU levels – a proxy for intracellular cAMP levels (21, 25) – twitching, and
135 piliation in *fimL*, *fimV*, and *pilG* single mutants or in double mutants also lacking *cpdA* to prevent
136 degradation of endogenous cAMP (32) (**Figure 1**). To confirm that FimV and FimL were epistatic
137 to PilG, we also examined *pilH fimL* and *pilH fimV* double mutants. In the absence of PilH, cells
138 are predicted to have hyper-phosphorylated PilG, consistent with the high levels of cAMP
139 observed in a *pilH* background (17, 18).

140 PilU levels were decreased in *fimL*, *fimV*, and *pilG*, to 28%, 10% and 22% of wild type,
141 respectively, consistent with roles in regulating cAMP synthesis (10, 12, 17, 32), (**Figure 1**). Both
142 *pilG* and *fimV* were twitching deficient, while *fimL* twitching resembled that of wild type, as
143 reported previously (32, 34). The *cpdA* mutant had high levels of PilU, surface piliation, and wild
144 type twitching, consistent with high cAMP levels (17, 32). Deletion of *cpdA* in *fimL*, *fimV*, or *pilG*
145 increased PilU levels relative to the corresponding single mutants, to at least wild-type levels
146 (**Figure 1**), showing that CyaB retains residual activity in those backgrounds. However, only the
147 *cpdA fimL* mutant was motile, confirming that both PilG and FimV have cAMP-independent roles
148 in T4aP function. Also consistent with previous reports (17), a *pilH* mutant assembled surface
149 pili but was twitching impaired (~39% of wild type). The *pilH fimV* and *pilH fimL* double mutants
150 had PilU levels similar to those of *fimV* and *fimL* single mutants, suggesting that despite its

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151 hyper-phosphorylation in the absence of *pilH* (18), PilG was unable to activate CyaB without
152 FimV or FimL, confirming that all three are required for the Chp system to stimulate cAMP
153 synthesis.
154

155 **Decreased levels of PilMNOPQ in *fimV* are due to decreased cAMP**

156 Wehbi et al. (29) showed previously that *fimV* mutants had reduced levels of PilMNOP,
157 and that a $fimV_{\Delta LysM}$ mutant with an in-frame deletion of the LysM motif had fewer PilQ
158 secretins. However, transcription of the *pilMNOPQ* operon is Vfr- and thus cAMP-dependent
159 (21). To determine if any of these phenotypes were independent of cAMP, we examined levels
160 of PilMNOPQ and PilU, and twitching motility in *fimV*, $fimV_{\Delta LysM}$, a mutant encoding only the
161 cytoplasmic domain of FimV ($fimV_{1194}$), and in a *fimV cyaB-R456L* double mutant that expresses
162 constitutively active CyaB (25, 35).

163 The *fimV* mutant had low levels of PilU (~23% of wild type), reflecting low cAMP levels,
164 while the *fimV cyaB-R456L* double mutant had wild type levels of PilU (**Figure 2**). The $fimV_{1194}$
165 mutant had ~29% of wild-type PilU, suggesting that even though its protein partners PilG and
166 FimL are cytoplasmic, expression of FimV's cytoplasmic domain alone was insufficient to
167 activate CyaB. Complementation of *fimV* and $fimV_{1194}$ *in trans* with a construct expressing full-
168 length FimV increased PilU levels to ~58% and ~65% of wild type, respectively. Surprisingly, the
169 $fimV_{\text{LysM}}$ mutant had ~89% of wild type PilU, suggesting that the LysM motif and thus PG binding
170 was dispensable for CyaB activation.

171 The *fimV* mutant had decreased levels of PilMNOP, and few detectable PilQ multimers,
172 and all were restored to wild type with full-length FimV. Supporting the hypothesis that their

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173 levels were dependent on Vfr and cAMP, the *fimV cyaB-R456L* double mutant had wild type
174 levels of PilMNOPQ. Despite this, the *fimV cyaB-R456L* double mutant had no recoverable
175 surface pili (**Figure 2**) and could not twitch, confirming a cAMP-independent role(s) for FimV in
176 pilus assembly and twitching motility. The *fimV*$_{1194}$ mutant had low levels of PilMNOP and no
177 detectable PilQ multimers, but these could be rescued by complementation with full length
178 FimV. *fimV*$_{\Delta LysM}$ had essentially wild type PilMNOPQ levels, and could assemble surface pili
179 (**Figure 2**); however, twitching was ~37% of wild type. The motility defect in *fimV*$_{\Delta LysM}$ suggests
180 that PG binding is important for FimV's cAMP-independent function(s).

181 Wehbi et al. (29) showed that the *fimV*$_{1194}$ mutant (which expresses the cytoplasmic
182 domain of FimV) was unable to twitch, but motility could be rescued by complementation with
183 a plasmid expressing only the periplasmic domain of FimV (residues 1-507, pFimV$_{507}$), suggesting
184 that together, the two FimV fragments could restore function without being physically
185 connected. A *fimV* deletion mutant complemented with empty vector or pFimV$_{507}$ had similar
186 PilU levels, suggesting that the periplasmic domain alone is not sufficient to activate CyaB
187 (**Figure 3A**). Unexpectedly, despite its ability to restore motility in the *fimV*$_{1194}$ background
188 (**Figure 3B**), pFimV$_{507}$ did not significantly increase PilU levels in that background, suggesting
189 that the cytoplasmic and periplasmic domains cannot activate CyaB efficiently when they are
190 not covalently linked. However, CyaB activation is not strictly required for twitching, as both
191 *fimL* (**Figure 1**) and *cyaAB* mutants have low cAMP levels and piliation, but near wild-type
192 motility (17, 32). We next tested if FimV's periplasmic domain played a cAMP-independent role
193 in twitching by complementing the *fimV cyaB-R456L* mutant with pFimV$_{507}$. pFimV$_{507}$ failed to
194 restore twitching in *fimV cyaB-R456L* (**Figure 3B**), suggesting that the cytoplasmic region of FimV

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195 plays a cAMP-independent role in motility. Taken together, the data show that the cAMP-
196 independent role(s) of FimV requires both domains.
197
198 **FimV is required for PilS localization**
199 The *V. cholerae* homolog of FimV, HubP, interacts with multiple proteins and has broad
200 regulatory function (28). FimV is required for bipolar localization of PilG and FimL and the T4aP
201 structural proteins PilMNOPQ, but not the Chp methyl-accepting chemotaxis protein (MCP) PilJ
202 (22, 30). To determine if FimV was required for localization of other T4aP regulators, we
203 examined its effects on localization of PilS (36, 37), the histidine sensor kinase component of the
204 PilRS two-component system that regulates *pilA* transcription in response to changes in PilA
205 levels in the inner membrane (38).
206 In wild-type cells, PilS-YFP was localized to both poles (**Figure 4**) as reported previously
207 (36), while in the absence of FimV, PilS-YFP was diffuse in the inner membrane. Interestingly,
208 the localization pattern of PilS-YFP in $fimV_{\Delta LysM}$ was similar to wild type, suggesting that PG-
209 binding was not critical for PilS localization. However, PilS-YFP was mislocalized in $fimV_{1194,}$
210 suggesting that the cytoplasmic domain is insufficient for PilS localization. Finally, because PilG
211 also has cAMP-independent effects on motility (17) (**Figure 1)**, we examined PilS-YFP localization
212 in a *pilG* mutant. Localization was similar to wild type, suggesting that PilG and FimV have
213 distinct cAMP-independent roles in motility (**Figure 4**). These data also suggest that PilS
214 localization is independent of intracellular cAMP concentration.
215
216 **FimV deletion does not affect swimming motility**

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217 As the *V. cholerae* and *S. putrefaciens* homologs of FimV modulate swimming motility
218 (27, 28), we tested whether loss of *fimV* impaired swimming in *P. aeruginosa*. We saw no effect
219 of FimV deletion on swimming motility (**Figure 5**), suggesting that it is not essential for flagellar
220 function in *P. aeruginosa*. Consistent with reports that swimming is negatively regulated by high
221 cAMP (21), the *cpdA* mutant was swimming impaired (~53% relative to wild type). Deletion of
222 *cpdA* in the *fimV* (~77%), *fimL* (~90%), and *pilG* (~85%) backgrounds reduced swimming relative
223 to the single mutants, potentially due to increased cAMP levels (**Figure 1**). Suprisingly, despite
224 having very high cAMP levels (17), and levels of piliation similar to the *cpdA* mutant (**Figure 1**),
225 *pilH* had ~93% swimming motility relative to wild type (**Figure 5**). This finding suggests that high
226 levels of cAMP and piliation do not necessarily inhibit swimming motility.
227
228 **DISCUSSION**

229 PilG, FimL, and FimV were recently proposed to be components of a surface-sensing
230 pathway that activates CyaB (22), with FimL acting as a scaffold protein connecting PilG to FimV.
231 Consistent with this model, we saw that increasing the level of PilG phosphorylation through
232 deletion of *pilH* (18) failed to increase levels of PilU if either FimV or FimL was missing (**Figure 1**).
233 However, only the *fimL* mutant twitched (**Figure 1**) following introduction of compensatory
234 mutations that increased cAMP levels (17, 32), confirming that both PilG and FimV have cAMP-
235 independent roles in twitching (17, 25). Thus, FimL's role in twitching is limited to its ability to
236 connect PilG and FimV, leading to CyaB activation via an as-yet unknown mechanism.
237 Interestingly, Nolan et al. (33) identified other suppressors of *fimL* that mapped outside the

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238 *cyaA, cyaB*, *pilG, pilH*, *vfr*, and *cpdA* loci. How those uncharacterized loci fit into the FimV-FimL-
239 PilG signalling axis remains to be determined.
240 Since FimL's role is limited to the cAMP-dependent pathway (22, 32) and twitching
241 motility in the *fimL* background is essentially wild type (**Figure 1**), PilG and FimV both function –
242 together or independently of one another – in its absence. PilG polar localization is dependent
243 on FimV, but PilG remains localized to the poles when FimL is absent (22). These data imply that
244 PilG interacts with FimV directly, or indirectly via another, as-yet unidentified adaptor protein.
245 That component is unlikely to be part of the Chp system; the MCP PilJ localizes to the poles
246 independently of FimV, and PilG retains bipolar localization in the absence of both PilJ (**Figure**
247 **S1**) and the Chp system kinase, ChpA (22). Identifying this interaction partner – potentially
248 among the list of proteins recovered in a recent PilG pulldown/mass spectrometry study (22)
249 could help to clarify how PilG contributes to cAMP-independent regulation of twitching.
250 Because restoration of cAMP levels in a *pilG* mutant by supplying exogenenous cAMP
251 (17), constitutively activating CyaB (35), or deleting *cpdA* (**Figure 1A**) restores piliation but not
252 twitching motility, the cAMP-independent role of PilG may be the coordination of pilus
253 retraction to permit directional movement. In *M. xanthus*, the Chp-like Frz system controls the
254 asymmetric subcellular distribution of the PilB and PilT motor ATPases in cells undergoing T4aP-
255 mediated S-motility, to coordinate movement (39). It is likely that asymmetric T4aP retraction
256 similarly occurs in *P. aeruginosa*, as pilus retraction at both poles simultaneously would result in
257 zero net movement.
258 How PilG might regulate pilus retraction remains unclear. CheY interacts with FliM at the
259 *E. coli* flagellar switch complex to control the direction of flagellum rotation (40), but the T4aP

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260 system lacks an obvious FliM equivalent. However, the T4aP system was recently discovered to
261 have a rotary motor (41-43). A hexameric PilB or PilT ATPase docks into the PilM ring at the base
262 of the apparatus and encircles the cytoplasmic domains of the PilC platform protein (7), rotating
263 it clockwise or counterclockwise, respectively, to insert or extract pilin subunits from the pilus in
264 a stepwise manner (43). Transient interactions of phospho-PilG with PilM, PilC, or the motor
265 ATPases might dictate which ATPase is docked at the leading versus lagging pole. In *pilH*
266 mutants, hyperactivation of PilG may dysregulate asymmetric pilus retraction, leading to
267 hyperpiliation and impaired motility (**Figure 1A**).

268 Interestingly, the cAMP-independent role of PilG appears dependent on, but distinct
269 from, that of FimV. In addition to FimL and PilG (22), FimV is required for polar localization of
270 the structural components PilMNOPQ (30) and the PilSR two-component system (**Figure 4**).
271 However, unlike its homologues in *V. cholerae* and *S. putrefaciens* (27, 28), its deletion does not
272 affect swimming (**Figure 5**). FimV and its homologs are emerging as protein interaction hubs
273 that bind to septal PG via their LysM motif to target their partners to the septum during division,
274 ensuring the correct placement of polar and partitioning systems during and after separation of
275 daughter cells (28, 30). Although studies of *L. pneumophila* FimV and *N. meningitidis* TspA (9,
276 11) did not address the role of the LysM motif or localization in function, the phenotypes of
277 mutants lacking these proteins could reflect consequent mislocalization of motility or adhesion
278 systems.

279 Septal PG binding by FimV was dispensable for CyaB activation (**Figure 2**) even though
280 FimL, PilG, and CyaB are located at the cell poles (22, 32). It is possible that deletion of the LysM
281 motif alone does not completely mislocalize FimV, as it likely has other interaction partners that

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282 help to confine it to the cell poles; attempts to test this hypothesis using a $\text{FimV}_{\Delta\text{LysM}}$-YFP fusion
283 have not been successful to date. Alternatively, $\text{FimV}_{\Delta\text{LysM}}$ may be present at the cell pole due to
284 diffusion in the inner membrane in sufficient quantities to promote CyaB activity. Consistent
285 with only partial mislocalization of $\text{FimV}_{\Delta\text{LysM}}$, PilS-YFP remained mostly localized to the poles in
286 that background, while deletion of FimV's entire periplasmic region led to PilS delocalization
287 (**Figure 4**). Transmembrane domains 5 and 6 of PilS (44), and the membrane-embedded MASE2
288 domain of CyaB (35) are sufficient for their polar localization. It is possible that they interact
289 with FimV via its transmembrane segment, or are integrated into the FimV hub through as-yet
290 unidentified intermediaries.

291 In summary, this work helps to resolve the cAMP-dependent and independent
292 regulation of *P. aeruginosa* twitching motility by FimV and PilG. The cAMP-independent role of
293 FimV is likely coordinate localization of multiple T4aP structural and regulatory components to
294 the cell poles, while that of PilG may be to control pilus retraction in a way that allows for
295 directional movement; experiments to test this idea are underway. Characterization of the FimV
296 protein-interaction network will identify its full repertoire of direct and indirect interaction
297 partners, and clarify the links between polar localization and function.
298

299 **MATERIALS AND METHODS**

300 **Bacterial growth and culture conditions**

301 Bacterial strains and plasmids are listed in **Table 1**. Unless otherwise stated,
302 untransformed *P. aeruginosa* strains and all *E. coli* strains were grown on LB agar at 37 °C.
303 Antibiotic selection was as follows unless stated otherwise: gentamicin, 15µg/ml for *E. coli* and

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304 30µg/ml for *P. aeruginosa*; kanamycin, 50µg/ml for *E. coli*; ampicillin, 100mg/ml for *E. coli*. All *P.*
305 *aeruginosa* strains containing a FimV complementation construct were grown on media
306 supplemented with 0.1% (w/v) arabinose.
307
308 **Mutant generation**
309 Mutants were made as previously described (17). Deletion constructs for the generation
310 of *fimL*, *cpdA*, and *fimV* mutants were designed to include 100 nucleotides upstream and
311 downstream of the gene to be deleted. The *pilH* construct was designed to include the first 12
312 nucleotides and last 30 nucleotides of the gene. The upstream and downstream fragments were
313 amplified from the PAK chromosome using the primer sets described in Table 2. Inserts were
314 cloned into the pEX18Gm suicide vector at the SacI and HinDIII sites (pEX18Gm::*fimL*), KpnI and
315 EcoRI sites (pEX18Gm::*cpdA*), and HinDIII and KpnI sites (pEX18Gm::*pilH*). Suicide vectors were
316 verified by DNA sequencing.
317 After verification, plasmids (pEX18GM::*fimL*, pEX18Gm::*cpdA*, pEX18Gm::*pilH*, and
318 pEX18Gm::$fimV_{LysM}$) were transformed into *E. coli* SM10 cells. Plasmids were transferred to *P.*
319 *aeruginosa* by conjugation at a ratio of 6:1 (*E. coli* to *P. aeruginosa*). 100 µl of the 6:1 mixed
320 culture were spotted onto LB 1.5% agar (w/v) and incubated overnight at 37°C. The mating
321 mixture was resuspended in 5 ml of LB and 100 µl was plated onto *Pseudomonas* isolation agar
322 supplemented with Gm 100 µg/ml and grown overnight at 37°C. Single colonies were
323 resuspended in 1 ml LB and plated onto LB 1.5% agar lacking sodium chloride and supplemented
324 with 8% (w/v) sucrose, and grown overnight at 30°C. Resulting single colonies were replica
325 plated onto LB and LB supplemented with Gm 30 µg/ml. Mutants were verified by PCR, and the

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326 *pilH* mutant was screened by western blotting with anti-PilH antiserum. Double mutants were
327 generated in the same manner.
328
329 **Plasmid construction**
330 The coding region of the first 507 residues of FimV was amplified by PCR using
331 pBADGr::*fimV* as a template, and cloned into pBADGr. The PCR amplified DNA was digested with,
332 purified, and ligated into pBADGr at the KpnI and XbaI sites with T4 DNA ligase according to
333 manufacturer's instructions (Thermo Scientific).
334 A version of *pilS* lacking its stop codon was amplified from the PAK chromosome and
335 cloned into pBADGr::*yfp* in-frame with *yfp* at the EcoRI and SmaI sites. In-frame ligation was
336 confirmed by DNA sequencing.
337 A version of PilG lacking its stop codon was amplified from the PAO1 chromosome and
338 cloned into the pMarkiC vector, which encodes (Gly_3-$Ser)_3$-YFP at the HinDIII site of pUCP20Gm.
339 PilG was ligated into the EcoRI and XbaI sites. In-frame ligation was confirmed by DNA
340 sequencing.
341
342 **Immunoblotting**
343 Western blotting of whole cell lysates was performed as previously described (45). In
344 brief, whole cell lysates were prepared from strains were grown overnight on LB 1.5% agar, or in
345 the case of plasmid transformed strains, LB 1.5% agar supplemented with 0.1% (w/v) arabinose.
346 Cell growth was then resuspended in 1X PBS and normalized to an OD_{600} of 0.6. Cells were
347 pelleted by centrifugation at 2,300 $\times g$ for 5 min. Pellets were then resuspended in 175 µl of 1X

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348 SDS-PAGE loading dye. Cell lysates were resolved on 15% SDS-PAGE gels and transferred to
349 nitrocellulose membranes. Membranes were blocked in 5% skim milk dissolved in PBS (pH 7.4)
350 for 1 h, washed in PBS, and incubated with PBS-diluted antisera raised against the FimV
351 periplasmic domain (1:1000), PilU (1:5000), PilM (1:1000), PilN (1:1000), PilO (1:1000), PilP
352 (1:1000), or PilQ (1:1000), or polyclonal anti-GFP antibody (Novus Biologicals; 1:5000) for 1 h,
353 washed, incubated with alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody
354 (1:3000, Bio-Rad) for 1 h, and washed. Blots were developed using 5-bromo-4-chloro-3-
355 indolylphosphate (BCIP) and nitro blue tetrazolium (NBT). Data are representative of n = 3
356 independent experiments.
357

358 **Sheared surface protein preparation**

359 Surface pili were analyzed as previously described (46). In brief, strains of interest were
360 streaked in a grid-like pattern onto LB 1.5% agar, or in the case of plasmid-transformed strains,
361 LB 1.5% agar supplemented with 0.1% (w/v) arabinose and grown overnight at 37 °C. Cells were
362 gently scraped from the plates using a sterile coverslip and resuspended in 4.5 ml PBS (pH 7.4).
363 Surface appendages were sheared by vortexing the cells for 30 s. The OD_{600} for each strain was
364 measured, and an amount of cells equivalent to 4.5 ml of the sample with the lowest OD_{600} was
365 pelleted by centrifugation at 16,100 *x g* for 5 min. When necessary, PBS was added to samples
366 to a final volume of 4.5 ml prior to centrifugation. Supernatants were removed and centrifuged
367 again at 16,100 *x g* for 20 min to remove remaining cells. Supernatants were collected and
368 mixed with 5 *M* NaCl and 30% (w/v) polyethylene glycol (Sigma; molecular weight range ~8000)
369 to a final concentration of 0.5 *M* NaCl and 3% (w/v) polyethylene glycol, and incubated on ice

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370 for 30 min. Precipitated surface proteins were collected by centrifugation at 16,100 x g for 30
371 min. Supernatants were discarded and samples were centrifuged again at 16,100 x g for 2 min.
372 Pellets were resuspended in 150 μl of 1X SDS-PAGE sample buffer (80 m*M* Tris, pH 6.8, 5.3%
373 (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 2% (w/v) SDS).
374 Samples were boiled for 10 min and resolved by 15% SDS-PAGE. Bands were visualized by
375 staining with Coomassie brilliant blue (Sigma). Data are representative of n = 3 independent
376 experiments.
377
378 **Twitching assay**
379 Twitching motility was tested as previously described (46). In brief, cells from an overnight
380 culture were stab inoculated to the interface between LB 1% agar, or in the case of plasmid-
381 transformed strains, LB 1% agar supplemented with 0.1% (w/v) arabinose and the underlying
382 tissue culture-treated polystyrene petri dish, and incubated at 37 °C for 16 h (Thermo Fisher).
383 Twitching zones were visualized by removing the agar and staining cells on the petri dish with
384 1% (w/v) crystal violet and washing with water to remove unbound dye. Twitching zones were
385 measured by analyzing the diameter of each twitching zone in pixels using ImageJ software
386 (NIH). Twitching zones were normalized to the twitching diameter of wild type PAK in each
387 individual experiment. Data are representative of n = 3 independent experiments.
388
389 **Fluorescence microscopy**
390 *P. aeruginosa* strains transformed with pBADGr::FimV-eYFP were grown overnight.
391 Microscopy was performed using 8-well 1.0 borosilicate chambered coverglass (LabTek).

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392 Chamber slides were prepared by adding LB 1% agar supplemented with 0.1% (w/v) arabinose
393 to create an agar layer ~3mm in thickness and covering the bottom of the chamber. Agar was
394 allowed to solidify with the lid off to prevent condensation. Bacteria were stab inoculated to the
395 interface between the agar and coverglass. Slides were wrapped in foil to prevent
396 photobleaching, and incubated at 37°C for 1h in the dark. Cells were then imaged using an EVOS
397 FL Auto microscope, with a monochrome camera for brightfield imaging and a YFP LED light
398 cube for fluorescence imaging, through a 60X oil immersion objective at room temperature.
399 Representative fields were cropped from larger images and enlarged using ImageJ software
400 (NIH) (47).

401 Fluorescence images were quantified using the MicrobeJ plugin for ImageJ (48).
402 Brightfield and fluorescence images were arranged into a stack on ImageJ. Regions of interest
403 corresponding to the bacteria were selected based on the brightfield image, and thresholding
404 particles based on length (0.5μm-5μm), width (0.2μm-1.5μm), and area (0.75μm^2-max), and fit
405 to rod-shaped bacteria. Pixel intensity profiles were generated by MicrobeJ using the profile
406 option on the fluorescence image, using 1μm width and 0.5μm extensions. Intensity profiles
407 were plotted along a Y-axis of range 0-140, and the X-axis was partitioned into 50 bins. Pixel
408 intensity profiles were generated for the YFP channel. Data are representative of at least 3
409 independent trials.

410

411 **Swimming assay**

412 Cells from overnight cultures were resuspended in sterile PBS and standardized to OD_{600}
413 0.6. Two μl of cell suspension were spotted onto LB 0.3% agar and allowed to dry onto the

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414 surface of the agar. Plates were incubated at 30°C for 16h with the agar side down. Data are

415 representative of n=3 independent experiments.

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565 **Table 1 – Strains and plasmids used in this study.**

Strains	Genotype/Description	Source
P. aeruginosa		
PAK	Wild type *P. aeruginosa* strain K	(17)
NP	PAK with a deletion of *pilA*	(49)
pilU	PAK with a deletion of *pilU*	(25)
fimV	PAK with a deletion of *fimV*	(25)
fimV$_{\text{LysM}}$	PAK with an in-frame deletion of the LysM motif, nucleotides 519-690	This study
fimV cyaB-R456L	PAK with a deletion of *fimV* and an arginine to lysine substitution in *cyaB* at position 456	(25)
fimV1194	PAK with an FRT insertion at nucleotide position 1194 in *fimV*	(25)
fimL	PAK with a deletion of *fimL*	This study
pilG	PAK with a deletion of *pilG*	(17)
cpdA	PAK with a deletion of *cpdA*	This study
cpdA fimV	PAK with deletions of *cpdA* and *fimV*	This study
cpdA fimL	PAK with deletions of *cpdA* and *fimL*	This study
cpdA pilG	PAK with deletions of *cpdA* and *pilG*	This study
pilH	PAK with a deletion of *pilH*	This study
pilH fimV	PAK with deletions of *pilH* and *fimV*	This study

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pilH fimL	PAK with deletions of *pilH* and *fimL*	This study
E. coli		
DH5a	F-, *Φ80lacZΔM15, Δ(lacZYA-argF), U169, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, thi-1, gyrA96, relA1, λ-;* General cloning strain	Invitrogen
SM10	*thi-1 thr leu tonA lacy supE recARP42`-2* Tcr::Mu Kmr; mating donor strain	(50)
Plasmids		
pBADGr	Arabinose-inducuble expression construct	(51)
pBADGr::*fimV*	Arabinose-inducible expression construct encoding full length FimV	(25)
pBADGr::*fimV507*	Arabinose-inducible expression construct encoding the periplasmic domain of FimV, residues 1-507	This study
pEX18Gm::*fimL*	Suicide vector containing 1kb upstream and downstream from the *fimL* locus	This study
pEX18Gm::*cpdA*	Suicide vector containing 1kb upstream and downstream from the *cpdA* locus	This study
pEX18Gm::*pilH*	Suicide vector containing 1kb upstream and downstream from the *pilH* locus	This study

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pEX18Ap-*fimV*-GmFRT	Suicide vector containing *fimV* amplified from PAO1 and disrupted at nucleotide position 1194 with an FRT-flanked gentamicin resistance cassette	(29)
pEX18Gm-*fimV*-ΔLysM	Suicide vector containing residues 1-1521 of *fimV* with a deletion of nucleotides 519-690	(29)
pBADGr::eYFP(HinDIII)	Arabinose-inducible expression construct encoding eYFP cloned into the HinDIII site	(30)
pBADGr::PilS-YFP	Arabinose-inducible expression construct with pilS cloned upstream of eYFP	This study
pMarkiC	puCP20Gm-based vector with $(Gly_3\text{-}Ser)_3$-YFP cloned in at the HinDIII site	This study
pMarkiC::pilG	pMarkiC with PilG cloned into the EcoRI and XbaI sites	This study

566

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568 **Table 2 – List of oligonucleotides used in this study**

Primers	Sequence
FimV-F	5' - GCGGGTACCATGGTTCGGCTTCGTACACTGGTTCGGG - 3'
$FimV_{507}$-R	5' - GCGTCTAGACTAGTGGTGATGGTGATGATGCTGTTCCTCGCCGGTATCCGCGGC - 3'
PilS YFP-F	5'-GACGAATTCATGCGCGCTGAACGGCTA-3'
PilS YFP-R	5'-GCACCCGGGTGCTGAGTTTGCGTGGGTGGGC-3'
ko FimL F1	5’ CGCGAGCTCAATGGGCGTGCCGTGCATCA -3’
ko FimL R1	5’- CGCGGATCCCGGTCTAGTGCGCCTCCC -3’
ko FimL F2	5’- CGCGGATCCTGGCCGGCGAGTTCCGCT -3’
ko FimL R2	5’- CGCAAGCTTGGACCGTCAGCTCGCTGCTC -3’
ko cpdA F1	5' - TCAAGCTTGGATCAGCTCGACGCCCGGCA - 3'
ko cpdA R1	5' - TCGGTACCTCTTCGAAGTGGACTACGACA - 3'
ko cpdA F2	5' - TACGGTACCAGGCGTCGGTGGCGGGAGT - 3'
ko cpdA R2	5' - TCGAATTCACGACCCGCAGCGCGATTG C - 3'
ko pilH F1	5' -GCGAAGCTTCGTTATCGAAGGGCGGGTCC - 3'
ko pilH R1	5' - GGGTCTAGAATCAACAATCAAAATACGAGCCATGGGG - 3'
ko pilH F2	5' - GGGTCTAGAAATGCGGTGCTGGCGGGCTGA - 3'
ko pilH R2	5' - GCGGGTACCCGTTTCTCGAAGTCGTTGCG - 3'
G_MarkiC_F	5’-ATTTAAGAATTCAGGAGGATATATATGGAACAGCAATCCGACG - 3'
G_MarkiC_R	5’-AATCATTCTAGAGGAAACGGCGTCCACCGGG - 3'

569

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570 **Figure Captions**

571 **Figure 1. FimV and PilG have separate cAMP-independent roles in T4aP function. A.** Loss of
572 *fimL* reduces the levels of PilU (a proxy for intracellular cAMP levels), surface piliation, and
573 twitching motility, and all phenotypes are restored to wild type levels in a *cdpA fimL* double
574 mutant. In contrast, *fimV* and *pilG* mutants lack surface piliation and motility even in a *cpdA*
575 background, while PilU levels are restored to wild type or greater. Scale bar = 1 cm. **B.**
576 Quantification of relative PilU levels and twitching zone diameters compared to wild type PAK
577 set to 100%; measurements are the average of n=3. Matching letters indicate statistically
578 significant differences at $p<0.05$.
579

580 **Figure 2. FimV, and its ability to bind PG, are essential for motility.**
581 **A.** Schematic of FimV fragments used in this work. FimV's periplasmic and cytoplasmic domains
582 are connected by a single transmembrane segement. The N-terminal periplasmic domain
583 contains a PG-binding LysM motif (green square), which is deleted in-frame in the *fimV*$_{\Delta LysM}$
584 mutant (white square)**. B.** The levels of PilU (a proxy for intracellular cAMP levels; (25)), and
585 assembly machinery components PilMNOPQ were assessed by Western blot in various *fimV*
586 backgrounds, complemented with empty vector or with full-length *fimV;* a representative blot is
587 shown. Surface piliation and twitching motility are shown in the bottom two rows. Scale bar = 1
588 cm. **C.** Quantification of protein levels and motility, n = 3. Note that PilQ multimer levels cannot
589 be accurately quantified by this method. In the absence of *fimV*, levels of PilU, PilN, and PilO and
590 PilQ multimers were reduced, and surface piliation and motility were lost. These phenotypes
591 were restored by complementation with *fimV* in trans. *fimV*$_{1194}$ expressing only the cytoplasmic

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592 domain phenocopied the *fimV* deletion mutant. When FimV's LysM motif was deleted, levels of
593 the proteins of interest and piliation were similar to wild type, but motility remained severely
594 impaired. CyaB requires FimV for its activity, but in the absence of *fimV* can be constitutively
595 activated by the R456L mutation (35). However, the cells have very few surface pili and are non-
596 motile.
597
598 **Figure 3. CyaB activation - but not twitching motility - requires that FimV's cytoplasmic and**
599 **periplasmic domains be connected. A.** Representative Western blot of whole cell lysates with
600 anti-PilU antiserum, and quantification of PilU levels (a intreacellular for cAMP levels) by
601 densitometry, n = 3. The reduced levels of PilU in *fimV* and $fimV_{1194}$ mutants are not restored to
602 wild type by *in trans* expression of the N-terminal domain ($FimV_{507}$). **B.** Quantification of
603 twitching motility. As reported previously (29), expression of $pFimV_{507}$ *in trans* complements
604 motility in the *fimV1194* mutant that expresses the cytoplasmic domain of FimV. It cannot
605 complement motility of a *fimV* mutant, even when cAMP levels are restored in a background
606 expressing constitutively-active CyaB-R456L.
607
608 **Figure 4. PilS localization is dependent on FimV.** Brightfield and fluorescence microscopy was
609 used to image PilS-YFP localization in the wild type, *fimV* mutants, or a *pilG* mutant and the
610 average pixel intensity of the YFP signal along the long axis of the cell was quantified using
611 MicrobeJ (48). The number of cells averaged for each population is shown on the graph. PilS-YFP
612 is localized to the poles in wild type cells but fluorescence becomes circumferential in the *fimV*
613 and $fimV_{1194}$ backgrounds. In a mutant expressing FimV with an in-frame deletion of its LysM

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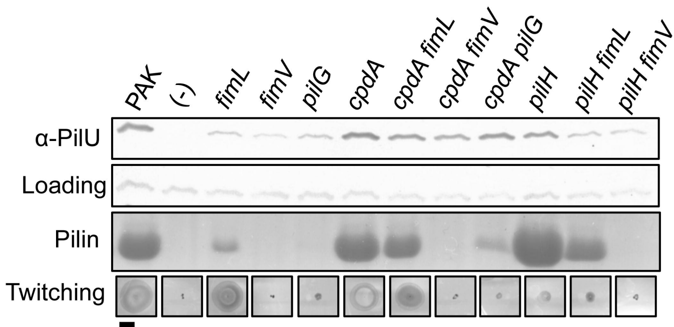
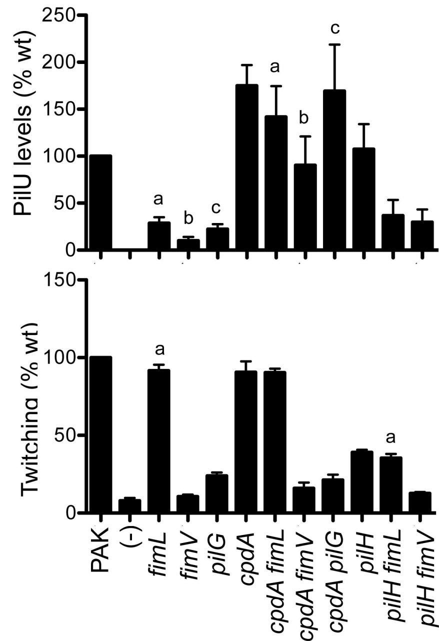
614 peptidoglycan-binding motif, fluorescence is polar but patchy circumferential fluorescence is
615 also visible. PilS-YFP localization remains polar in a *pilG* mutant. Scale bar = 5 µm.
616
617 **Figure 5. Swimming motility of PAK wild type and mutant strains. A.** Representative swimming
618 motility assays in 0.3% LB agar. Scale bar = 1 cm. **B.** Quantification of swimming zones, average
619 of n = 3. Lowercase letters indicate paired samples that are statistically different from one
620 another. Loss of *fimV* does not impact swimming motility. PAK is the parent strain; the *fliC*
621 mutant that lacks flagellin was included for comparison.


A.
PAK
(-)
fimL
fimV
pilG
cpdA
cpdA fimL
cpdA fimV
cpdA pilG
pilH
pilH fimL
pilH fimV
α-PilU
Loading
Pilin
Twitching


B.
PilU levels (% wt)
250
200
150
100
50
0
a
b
c
a
b
c
Twitching (% wt)
150
100
50
0
a
a
PAK
(-)
fimL
fimV
pilG
cpdA
cpdA fimL
cpdA fimV
cpdA pilG
pilH
pilH fimL
pilH fimV

A.

periplasmic

transmembrane

cytoplasmic

LysM motif

FimV

fimV$_{\Delta LysM}$ *fimV*$_{1194}$ pFimV$_{507}$

B.

PAK (-) *fimV* *fimV*$_{1194}$ *fimV*$_{\Delta LysM}$ *fimV* *cyaB-R456L*

+p*fimV* - - - + - + - + - +

PilU

PilM

PilN

PilO

PilP

Loading

PilQ$_{multimer}$

PilQ$_{monomer}$

Pilin

Motility

C.

PilU PilM PilN PilO PilP Twitching motility

% control

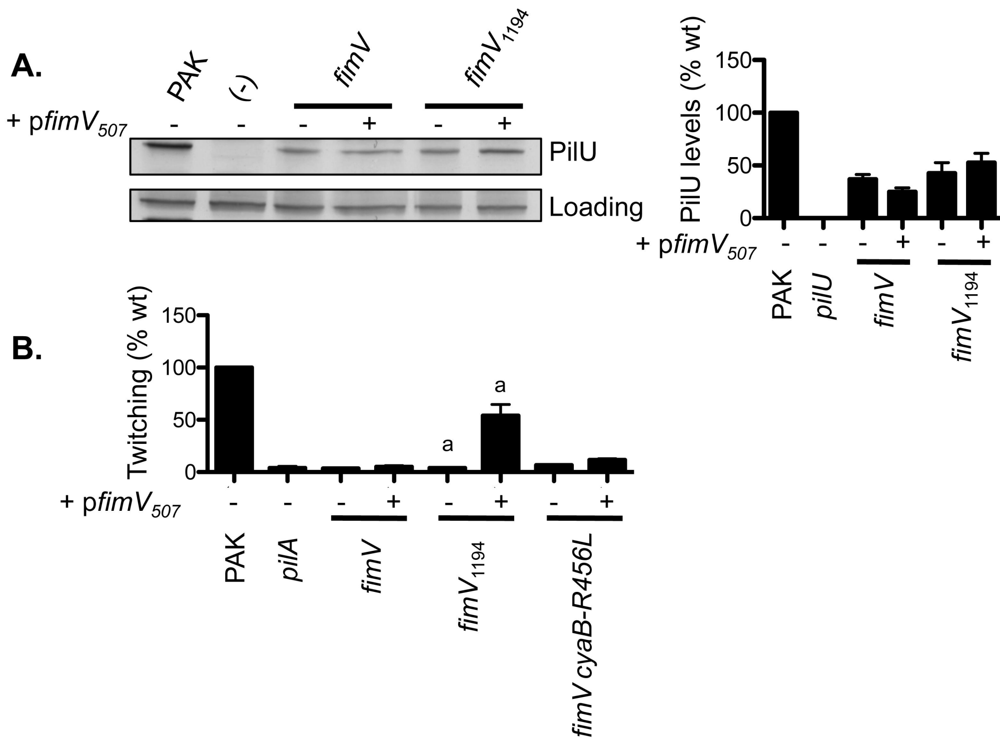
0 100 200

0 50 100 150

p*fimV* - - - + - + - + - +

PAK (-) *fimV* *fimV*$_{1194}$ *fimV*$_{LysM}$ *fimV cyaB-R456L*

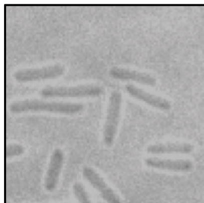
a

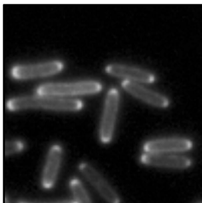


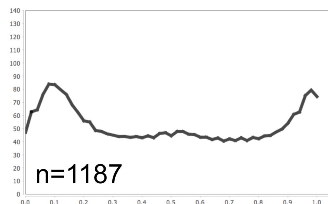
A.
PAK
(-)
fimV
fimV1194
+ pfimV507
-
-
-
+
-
+
PilU
Loading
PilU levels (% wt)
150
100
50
0
+ pfimV507
-
-
-
+
-
+
PAK
pilU
fimV
fimV1194
B.
Twitching (% wt)
150
100
50
0
a
a
+ pfimV507
-
-
-
+
-
+
-
+
PAK
pilA
fimV
fimV1194
fimV cyaB-R456L

Brightfield YFP-PilS

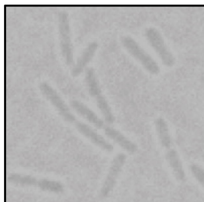
PAK

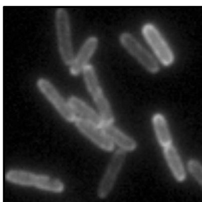


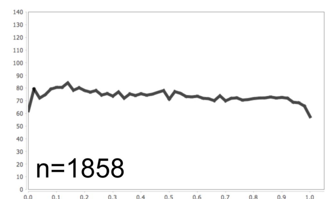




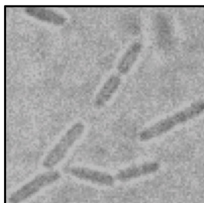
fimV

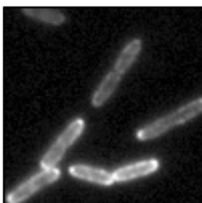


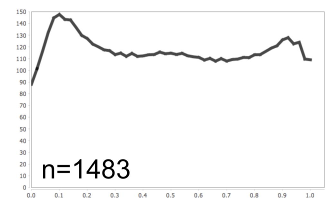




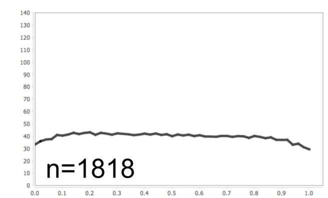
fimV$_{LysM}$



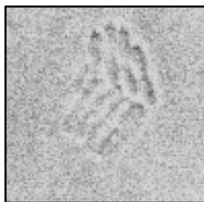


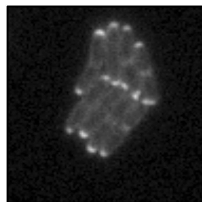


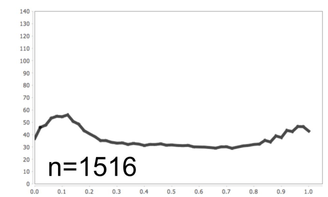
fimV$_{1194}$



pilG







Pixel intensity

Position

A.

PAK fliC
fimL fimV pilG
cpdA cpdA fimL

cpdA fimV cpdA pilG
pilH fimL pilH fimV pilH

B.