

1 **Title:** Twitching cells use a chemoreceptor to detect bacterial competitors

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12 **Abstract:**

13 Bacteria live in cosmopolitan communities, where the ability to sense and respond to interspecies and
14 environmental signals is critical for survival. We previously showed the pathogen *Pseudomonas aeruginosa* detects
15 secreted peptides from bacterial competitors and navigates interspecies signal gradients using pilus-based motility.
16 Yet, it remained unknown whether *P. aeruginosa* utilizes a designated chemosensory system for this behavior.
17 Here, we performed a comprehensive genetic analysis of a putative pilus chemosensory system to reveal behaviors
18 of mutants that retain motility, but are blind to interspecies signals. The enzymes predicted to methylate (PilK) and
19 demethylate (ChpB) the putative pilus chemoreceptor, PilJ, are necessary for cells to control the direction of
20 migration. While these findings implicate PilJ as a *bona fide* chemoreceptor, such function had yet to be
21 experimentally defined, as PilJ is essential for motility. Thus, we constructed systematic genetic modifications of
22 PilJ and found that without the predicted ligand binding domains or methylation sites cells lose the ability to detect
23 competitor gradients, despite retaining pilus-mediated motility. Collectively, this work uncovers the chemosensory
24 nature of PilJ, providing insight into chemotactic interactions necessary for bacterial survival in polymicrobial
25 communities and revealing putative pathways where therapeutic intervention might disrupt bacterial
26 communication.

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Introduction

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Microbes often exist in complex, dynamic environments and have evolved sophisticated systems to perceive and respond to the outside world. Because they commonly reside in multispecies communities, bacteria experience gradients of nutrients, metabolites, and secretions generated by neighboring cells. Gradients are particularly steep in surface-attached biofilm communities and ecological theory predicts bacteria must sense and respond to competitor and cooperator signals to thrive in such complex environments (Foster & Bell, 2012; Oliveira et al., 2016).

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In line with this hypothesis, we recently reported that *Pseudomonas aeruginosa* is attracted to gradients of secreted factors from other microbial species (Limoli et al., 2019). *Pseudomonads* are opportunistic bacteria found in polymicrobial communities in soil, wounds, and chronic lung infections, such as those in people with cystic fibrosis (Limoli & Hoffman, 2019; Tashiro et al., 2013). *P. aeruginosa* is frequently co-isolated with *Staphylococcus aureus* from cystic fibrosis respiratory samples and coinfections can persist for decades (Deleon et al., 2014; Gabrilska & Rumbaugh, 2015; Hotterbeekx et al., 2017; Limoli & Hoffman, 2019). Coinfection is also associated with pulmonary decline; thus, understanding ecological competition between these organisms may provide insight into patient outcomes (Hubert et al., 2013; Limoli et al., 2016; Maliniak et al., 2016).

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Accordingly, *in vitro* studies have documented interspecies interactions between *P. aeruginosa* and *S. aureus* leading to reciprocal enhancement of antibiotic tolerance, production of virulence factors, and ability to alter host immune cell responses, further supporting clinical observations (Limoli & Hoffman, 2019; Orazi et al., 2020; Orazi & O'Toole, 2017; Orazi et al., 2019). Additionally, these data suggest each species may sense a secreted signal from the other, which instigates a competitive or cooperative response through alteration of their virulence arsenals, a model supported by differential regulation of specific *P. aeruginosa* virulence pathways in response to *S. aureus* exoproducts (Kvich et al., 2022; Zarrella & Khare, 2022). Remarkably, *P. aeruginosa* and *S. aureus* have been shown to form mixed microcolonies when cocultured on bronchial epithelial cells (Orazi & O'Toole, 2017). One possible explanation for the formation of mixed communities is that *P. aeruginosa* and *S. aureus* may be initially attracted to one another through detection of secreted interspecies signals. Such attraction has the potential to facilitate formation of blended microcolonies or microbial competition, depending on the environmental conditions.

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Supporting this model, *P. aeruginosa* senses secreted *Staphylococcal* peptide toxins referred to as phenol soluble modulins (PSMs) and responds with directed motility towards the increasing PSM concentration gradient, mediated by the type IV pilus (TFP) (Limoli et al., 2019). With the identification of a putative interspecies chemoattractant for pilus-based motility, we hypothesized that *P. aeruginosa* uses a chemosensory pathway to move towards *S. aureus*. TFP-mediated motility, or twitching motility, occurs through the grappling hook activity of the pilus, which undergoes episodes of extension, substrate attachment and retraction which pulls the cell body along the surface (Burrows, 2012). The direction of twitching motility is thought to be controlled by preferential extension of pili at the pole facing the direction of movement, referred to as the leading pole. Cells are predicted to change direction by extending pili from the opposite pole, reversing the direction of cellular movement along the long axis of the cell body and so swapping leading poles (Kühn et al., 2021). However, whether modulation of reversal frequency is necessary and

66 sufficient to bias the movement of twitching cells towards a chemoattractant, similar to the run-and-tumble or run-
67 reverse-turn strategies used in flagella-mediated chemotaxis, remains unknown (Qian et al., 2013). While planktonic
68 swimming cells smoothly sample gentle chemoattractant gradients by typically traveling a full body length or more
69 between tumbling events, surface-associated twitching motility is hundreds to thousands of times slower and steep,
70 varying gradients characterize the chemotactic landscape (Berg & Brown, 1972; Patteson et al., 2015). Thus,
71 twitching cells experience less certain chemotactic signals (Carabelli et al., 2020; Hook et al., 2019; Oliveira et al.,
72 2016). Thus, we predict that additional parameters need to be considered to fully understand how a twitching
73 community biases directional movement. Indeed, even simple models of twitching motility are known to produce
74 complex dynamics (Nagel et al., 2020).

75

76 While TFP-mediated chemotaxis has not been thoroughly dissected in *P. aeruginosa*, prior work has described
77 chemotactic roles for the two proteins of the putative pilus chemosensory system, Pil-Chp (Kühn et al., 2021;
78 Oliveira et al., 2016). Namely, the predicted response regulators, PilG and PilH, are thought to control reversals
79 and increase levels of the intracellular second messenger cyclic adenosine monophosphate (cAMP) through PilG
80 direct activation of the adenylate cyclase CyaB (Fulcher et al., 2010; Kühn et al., 2021; Oliveira et al., 2016; Persat
81 et al., 2015). cAMP controls a large arsenal of virulence factors targeting both eukaryotic and prokaryotic cells, as
82 well as multiple modes of motility through activation of the virulence response transcriptional regulator (Vfr)
83 (Wolfgang et al., 2003). However, whether cAMP is also necessary to transduce the detection of interspecies
84 attractants to modulate directional motility has not been investigated.

85

86 In addition, Pil-Chp includes homologous proteins to a majority of the Chel flagella chemotaxis system in *P.*
87 *aeruginosa* (Matilla et al., 2021; Sampedro et al., 2015). However, unlike the 24 Chel-associated chemoreceptors,
88 referred to as methyl-accepting chemotaxis proteins (MCPs), Pil-Chp only has one MCP, called PilJ. This putative
89 pilus chemoreceptor differs from the flagella systems, in that PilJ is uniquely essential for twitching motility and
90 possesses low protein sequence similarity in both the ligand binding domain (LBD) and cytoplasmic signaling
91 domains with other MCPs (Delange et al., 2007; Matilla et al., 2021). The chemoreceptor for this system and the
92 chemotactic role of the rest of the pathway has yet to be fully interrogated.

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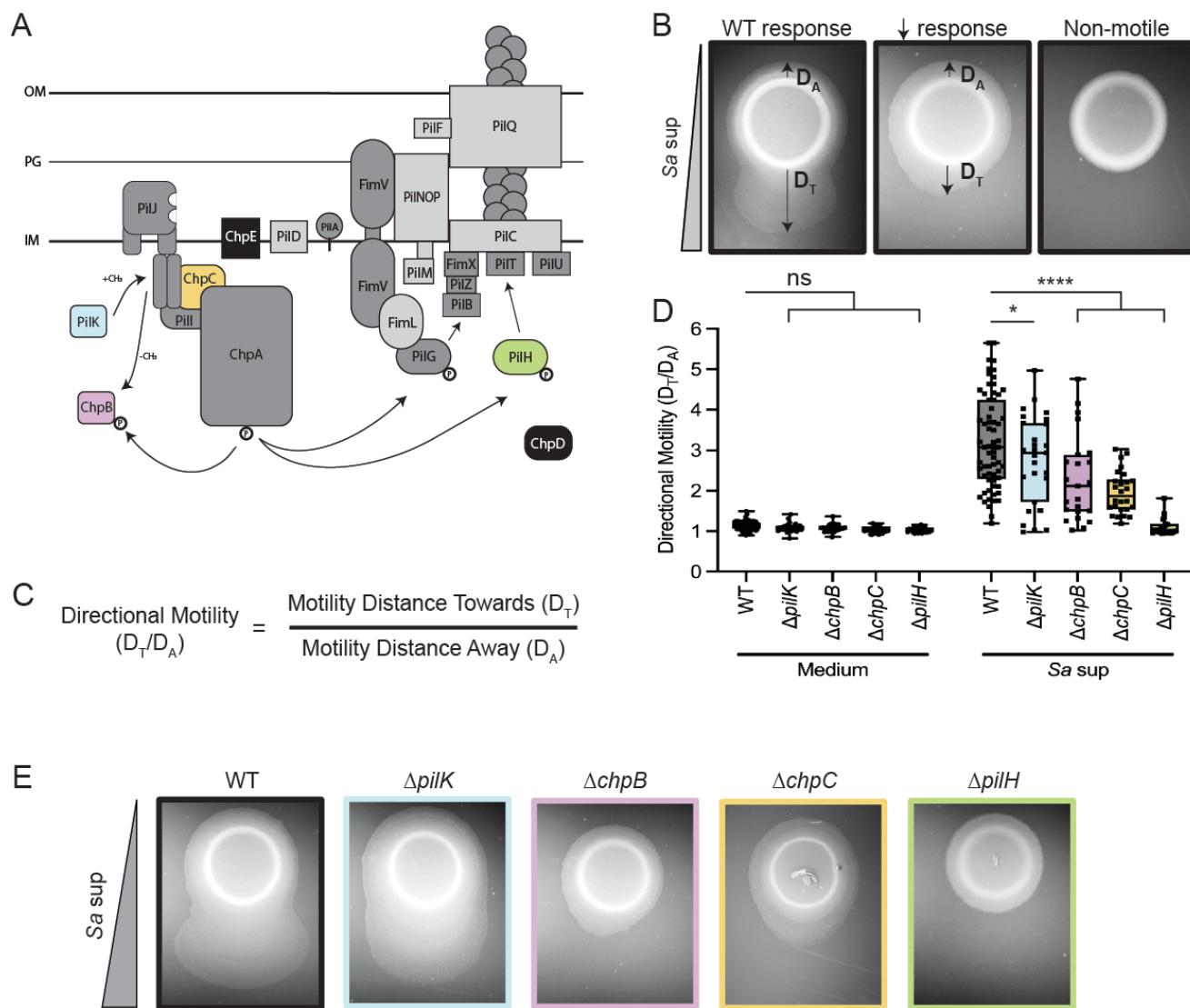
94 To determine the contribution of the remaining proteins encoded within the Pil-Chp pathway, we systematically
95 deleted these genes and identified those mutants that retain twitching yet are unable to bias movement up a gradient
96 of *S. aureus* secreted factors. From this analysis, six genes fit these criteria: *pilK*, *chpB*, *chpC*, *pilH*, *cyaB*, and *cpdA*.
97 ChpC is a CheW-like linker protein and PilH is a response regulator, while CyaB and CpdA are enzymes that
98 synthesize and degrade cAMP, respectively. PilK and ChpB are proteins that control chemoreceptor adaptation
99 through methylation of the Pil-Chp MCP PilJ, implicating a role for PilJ as a chemoreceptor for interspecies signals.
100 Accordingly, PilJ mutations in the key regions that define an MCP, including the LBD for sensing interspecies signals
101 and methylation sites for chemoreceptor adaptation, revealed PilJ is necessary for *P. aeruginosa* to perceive and
102 bias movements towards *S. aureus*. Quantification of cAMP in single cells also reveals that cAMP levels rise in *P.*
103 *aeruginosa* during chemotaxis towards *S. aureus*; yet cAMP increases are not strictly due to enhanced twitching

104 motility. Collectively, these results define a novel chemosensory role for PilJ and the Pil-Chp system to sense and
105 respond to interspecies signals.

106 **Results: The Pil-Chp system controls *P. aeruginosa* attraction to *S. aureus* peptides**

107 The directional nature of *P. aeruginosa* movement up a gradient of *S. aureus* secreted peptides suggests a role for
108 a chemosensory network. We hypothesize that Pil-Chp controls a chemotaxis-like TFP-mediated response by *P.*
109 *aeruginosa* towards *S. aureus*. To test this hypothesis, we systematically deleted genes in several components of
110 the Pil-Chp pathway to identify mutants that retain twitching motility, but show diminished directional response to *S.*
111 *aureus*, using a macroscopic directional motility assay (Figure 1A, B) (Kearns et al., 2001; Limoli et al., 2019).
112 Growth medium or cell-free *S. aureus* supernatant was spotted on top of an agar plate and allowed to diffuse for 24
113 hours to form a gradient. *P. aeruginosa* was then spotted at a distance from the gradient and allowed to respond to
114 each gradient for 36 hours before imaging the plates and quantifying the directional motility ratio (Figure 1C). Four
115 of the 16 pilus mutants retain twitching motility, but reduced directional motility up a gradient of *S. aureus* secreted
116 signals (Figure 1D, E). The remaining mutants either phenocopy wildtype or are non-motile (Figure 1A,B). Mutants
117 with reduced ability to respond include genes that encode for the predicted methyltransferase PilK and
118 methylesterase ChpB. The other two have modification of the chemoreceptor ChpC, which is a CheW-like linker
119 protein that connects the chemoreceptor and kinase, and PilH, which is a response regulator that is predicted to
120 regulate pilus retraction (Darzins, 1994, 2006; Whitchurch et al., 2004). These four mutants indicate that *P.*
121 *aeruginosa* uses the Pil-Chp system for a pilus-mediated chemotaxis response to *S. aureus*. Yet how these
122 chemotaxis-deficient mutants control *P. aeruginosa* attraction towards *S. aureus* is not precisely defined by
123 macroscopic directional motility assays.

124



125
126 **Figure 1: The Pil-Chp system controls *P. aeruginosa* attraction to *S. aureus* peptides.** (A) Schematic of the
127 putative type IV pilus (TFP) Pil-Chp chemosensory pathway. When the colored proteins are deleted, cells retain
128 TFP motility, but show reduced pilus-mediated response to the gradient of *S. aureus* secreted factors. Mutants for
129 the proteins highlighted in black retain TFP motility and wildtype levels of response up a gradient of *S. aureus*
130 secreted factors. When the proteins highlighted in dark gray are deleted, the cells are non-motile. Proteins
131 highlighted in light gray were not tested here, but have been previously reported to lack TFP motility. Representative
132 images for each response type are shown in (B). (C) Response measured by calculating the ratio of directional
133 motility up the gradient of *S. aureus* secreted factors. The equation for calculating directional motility is shown. (D)
134 Directional motility of *pil-chp* mutant candidates that retain TFP motility, but show reduced response to *S. aureus*
135 supernatant and representative directional motility images for the wildtype and each mutant shown in (E). Directional
136 motility for at least three biological replicates, each containing a minimum of four technical replicates are shown
137 and statistical significance determined with a two-way ANOVA followed by Dunnett's multiple comparisons test. ***
138 indicates $p < 0.0001$; * indicates $p < 0.05$; ns indicates no statistically significant difference in directional motility
139 compared to wildtype *P. aeruginosa*.

140 **Results: Methyl modification proteins for chemotaxis adaption are necessary for full directional TFP-
141 mediated motility towards *S. aureus***

142 Since both predicted adaption proteins for chemoreceptor methylation modification are necessary for full response
143 to *S. aureus* peptides at the community level, we next investigated the behavior of each mutant at the single-cell
144 level to uncover how each mutant is unable to correctly bias the direction of movement. PilK is predicted to
145 methylate, while ChpB is predicted to demethylate, PilJ (Figures 1A, 2A). For other MCPs, the addition and removal
146 of methyl groups to a chemoreceptor, facilitates intracellular signal transmission to the downstream kinase to shift
147 activity to an 'ON' or 'OFF' state, while also inducing conformational changes to the ligand binding region of the
148 MCP that alters its sensitivity to signals as the bacterial cell moves up or down a gradient (Parkinson et al., 2015).
149 Higher levels of MCP methylation, facilitated by PilK, are thus expected to shift kinase activity ON and subsequently
150 increase Pil-Chp control of pilus extension and retraction, while hydrolysis of methylated sites by ChpB is expected
151 to shift kinase activity towards an OFF state, with a reduction in controlled pilus dynamics (Figure 2A).

152

153 Based on the information above, we predicted that cells lacking PilK would have low methylated PilJ, while those
154 lacking ChpB would have highly methylated PilJ. Thus, both are predicted to be unable to properly transmit
155 intracellular signals to control activity of kinase ChpA and subsequently the response regulators, ultimately resulting
156 in altered control over biased extension and retraction events for motility (Figure 2A). Macroscopically, the *chpB*
157 mutant, which presumably has high methylation and thus a shifted-ON kinase state, exhibits a decrease in
158 directional movement up the *S. aureus* secreted factor gradient which is restored when *chpB* is complemented
159 (Figure 1, Figure 2 – figure supplement 1). To uncover how $\Delta chpB$ fails to move fully towards *S. aureus* signals, we
160 evaluated single-cell TFP-mediated motility behaviors of $\Delta chpB$ in the absence and presence of *S. aureus*.
161 Compared to wildtype *P. aeruginosa*, in both monoculture and coculture, the $\Delta chpB$ mutant exhibits earlier motility
162 away from the growing microcolony, with groups of motile cells migrating outwards in all directions (Videos 1-4). In
163 coculture with *S. aureus*, some cells migrate towards *S. aureus*; however, similar numbers of cells move in the
164 opposite directions, suggesting $\Delta chpB$ cells are unable to bias movements towards the interspecies signals, unlike
165 wildtype, which shows stronger apparent bias with more cells moving towards *S. aureus* (Video 4). To quantify
166 these behaviors, we determined the direction of motion for each *P. aeruginosa* cell in relation to the position of *S.*
167 *aureus* cells. Rose graphs of the principal angles of motility for each *P. aeruginosa* cell trajectory, reveal that most
168 wildtype cells bias their collective direction of movement towards *S. aureus*, whereas $\Delta chpB$ cells are prone to move
169 both towards and away from *S. aureus* (Figure 2B, inset).

170

171 To determine the aspects of *P. aeruginosa* movement that contribute to biased motion towards *S. aureus*, the
172 dynamics were quantified by the mean-squared displacement (MSD), a measure of the typical distance traveled
173 over a given time Δt (Figure 2B). While MSD measurements commonly assume isotropic motion, we decomposed
174 microscopic cell migration into components parallel (||) and perpendicular (\perp) to each trajectory's principal direction.
175 While wildtype cells travel comparable distances in the parallel and perpendicular directions, only significantly
176 different at the longest lag times, they have different dynamics in the two directions (Figure 2B, left). Perpendicular
177 to the principal direction, the cells perform an unbiased random walk, which is quantified by fitting the anomalous
178 diffusion exponent (Methods and Materials; Eq 4) and finding $\alpha_{\perp} \approx 1.1$, which is close to the expected value of 1 for

179 diffusive dynamics. However, in the parallel direction, the anomalous exponent $\alpha_{||}=1.3$. This larger value suggests
180 that cells tend to move with more self-directed, propulsive transport in the parallel direction.
181
182 In comparison to wildtype, cells lacking ChpB exhibit markedly different MSD profiles. $\Delta chpB$ cells have significantly
183 larger displacements in the parallel direction than perpendicular (Figure 2B, middle). However, this increased
184 microscopic motion does not translate into directional motility, since the anomalous diffusion exponent is near unity,
185 $\alpha_{||}=1.1$, which suggests diffusive random walk dynamics. The combination of increased MSD but loss of self-
186 directed, propulsive transport compared to wildtype is consistent with ChpA kinase activity more often in the ON
187 state. Given the predicted chemotactic role for Pil-Chp, one possible explanation for reduced bias towards *S. aureus*
188 is that $\Delta chpB$ cells have increased rates of cellular reversals. Reversing, or changing the direction of type IV pilus-
189 mediated movements, is thought to require switching of the leading and lagging poles. These cellular reversals also
190 enable bacteria to bias the direction of type IV pilus-mediated movement up an increasing concentration gradient
191 of chemoattractant (Oliveira et al., 2016). However, single-cell tracking analyses under these conditions did not
192 show statistically significantly different reversal dynamics between $\Delta chpB$ and wildtype. Therefore, if pole switching
193 reversals do not explain how cells can perform TFP-mediated chemotaxis towards interspecies signals, then how
194 are cells able to bias their movements up a gradient?
195
196 To understand how directional motility arises from the microscopic dynamics, we considered the displacement
197 distribution function, which gives the probability that a cell moves a given displacement over a given lag time (Figure
198 2 - figure supplement 3). The probability density functions of displacements made by *P. aeruginosa* cells are
199 dominated by a narrow peak of submicron jiggling motion. While imaging introduces narrow Gaussian noise (Figure
200 2 - figure supplement 4A), the submicron peak is observed to be wider and exponentially distributed (Figure 2 -
201 figure supplement 4B). Neither jiggling, nor rare-but-large steps, show substantial differences parallel or
202 perpendicular to the principal direction of motion for wildtype cells. The dynamics leading to directional motility in
203 wildtype cells become apparent once the trajectories are divided into subpopulations of “movers” and “resters” (see
204 Methods and Materials). While rester-designated cells exhibit displacement distributions with only jiggling, movers
205 possess a pair of non-zero sharp-shoulder peaked step sizes in the direction parallel to the principal angle (Figure
206 2C, red regions). These peaks represent a well-defined step size of $0.69 \pm 0.01 \mu\text{m}$ at the shortest lag times. For
207 wildtype cells, these sharp peaks are found at step sizes of nearly one micron for all lag times and exist in both the
208 parallel and perpendicular directions but are entirely absent in resters. The peak step size is larger in the parallel
209 direction than the perpendicular direction (Figure 2 - figure supplement 3, top row), explaining the anisotropic motion
210 and single-cell directional motility, but not bias towards *S. aureus*. The bias occurs because the symmetry between
211 the forward and backward peaks breaks with increasing lag time, with the forward (positive, parallel) peak shifting
212 to slightly larger step sizes and the backward (negative, antiparallel) peak shifting to slightly smaller values and
213 eventually vanishing at the largest lag times (Figure 2C, left, inset). The probability distributions at different time
214 lags reveal the microscopic basis of the directed motility of wildtype *P. aeruginosa* towards *S. aureus* and the
215 difference between these peak step sizes explains the superdiffusive anomalous exponent. Comparison of wildtype
216 and $\Delta chpB$ step sizes show that both strains exhibit the dominant submicron jiggling peak with no selected direction
217 much of the time (Figure 2C). However, the peak step size can no longer be distinguished in $\Delta chpB$ (Figure 2C,

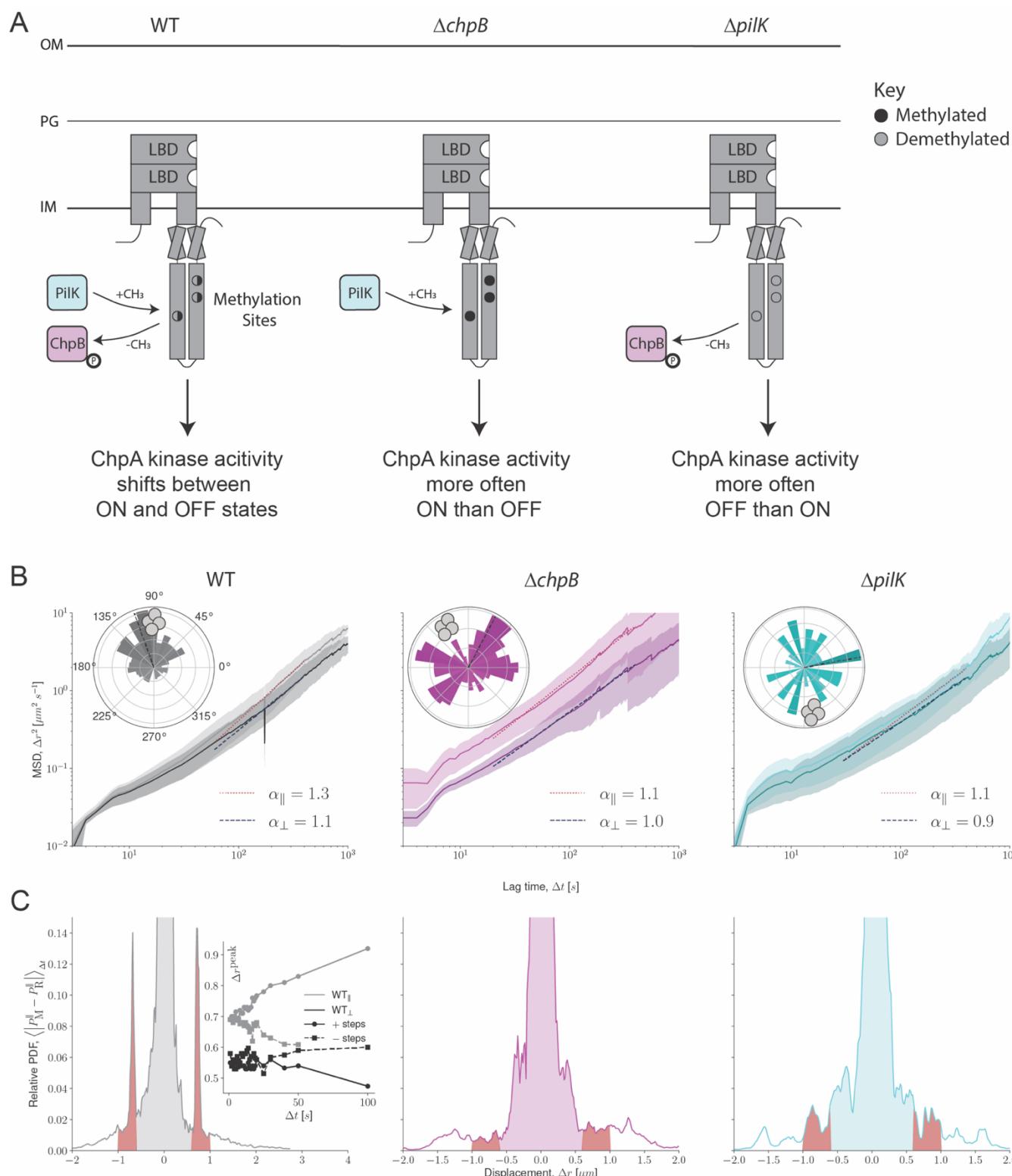
218 middle, red region). This suggests an apparent loss of directional TFP-mediated dynamics and is consistent with
219 the reduced macroscopic directional motility observed in $\Delta chpB$ (Figure 1D, E).
220

221 Given that reduced pilus-mediated chemotaxis of $\Delta chpB$ can be explained by a loss of the peak step size in the
222 parallel direction, we next live-imaged $\Delta pilK$ and tracked cells to evaluate whether $\Delta pilK$ exhibits similar reduction
223 in bias, thus explaining the modest macroscopic chemotaxis deficiency. Observations of $\Delta pilK$ in monoculture and
224 coculture with *S. aureus* show similar, but bimodal phenotypes. While 50% of $\Delta pilK$ microcolonies imaged show
225 hypermotile single-cells or small packs of cells moving outwards in all directions from the growing microcolony,
226 regardless of where *S. aureus* is in coculture, the other 50% of imaged $\Delta pilK$ microcolonies do not show any motility
227 (Figure 2 – figure supplement 2, Videos 5-8). When motile, $\Delta pilK$ cells disperse from the microcolony later than
228 $\Delta chpB$ but earlier than wildtype (Video 6). Compared to $\Delta chpB$ cells that tend to move as clusters of cells in tendril-
229 like patterns away from the microcolony, $\Delta pilK$ cells more frequently move in smaller groups or strictly as single
230 cells and spread outwards radially (Videos 6 and 8). Regardless, complementation of the $\Delta pilK$ mutant restores
231 chemotaxis response to wildtype levels (Figure 2 – figure supplement 1). The tracking analyses show that $\Delta pilK$
232 MSD is comparable to wildtype but the parallel anomalous exponent $\alpha_{||}=1.1$ is closer to the diffusive random walk
233 value of $\Delta chpB$ (Figure 2B, right). Similarly, like $\Delta chpB$, $\Delta pilK$ has largely lost the peak step size (Figure 2C, right).
234 Compared to $\Delta chpB$, the remnant of the peak step size may be discerned (Figure 2C, right, red region); however,
235 they are indefinite and the larger step size of $1.57 \pm 0.02 \mu\text{m}$ at the shortest lag times may also be present (Figure
236 2 - figure supplement 3, middle row).
237

238 Collectively, the bimodal nature of $\Delta pilK$ motility and phenotype of $\Delta chpB$ suggest that *P. aeruginosa* cells can lose
239 control of directional pilus-mediated motility dynamics in two ways. Cells with too much ChpA kinase activity, such
240 as $\Delta chpB$, move far without precise control over direction and resulting in persistent movement in the initial direction
241 of movement, resulting in larger displacement than wildtype. In contrast, cells with loss of PilJ-mediated ChpA
242 kinase activity, like $\Delta pilK$, are unable to control pilus dynamics and thus are more susceptible to environmental
243 conditions, which leads to either the inability to move or uncontrollable, short movements away from the
244 microcolony. This is further exemplified for $\Delta pilK$ when multiple microcolonies from four separate cultures were
245 simultaneously imaged and retain the bimodal distribution of motility (Figure 2 – figure supplement 2). Whether
246 persisting longer or spreading, neither $\Delta chpB$ nor $\Delta pilK$ can chemotax towards interspecies signals, supporting the
247 model that proper methylation and demethylation of chemoreceptor PilJ is an essential component of pilus-mediated
248 chemotaxis.
249

250 We previously reported that PilJ was not necessary for *P. aeruginosa* to bias movement towards *S. aureus* (Limoli
251 et al., 2019). However, these data reveal a role for two enzymes predicted to modify PilJ; thus, we revisited the
252 necessity of PilJ in interspecies signaling here. One hypothesis for the prior observations was that motile $\Delta pilJ$ cells
253 were using flagella-mediated motility, which obscured the pilus-mediated defect. To test this hypothesis, we
254 generated a mutant lacking both *pilJ* and *flgK*, the flagellar hook; therefore, this mutant was not able to use flagella-
255 mediated motility (O'Toole & Kolter, 1998). Live-imaging shows that $\Delta pilJ \Delta flgK$ cells are non-motile and thus fully

256 non-responsive to *S. aureus*, suggesting that previously it was flagella-mediated motility interfering with evaluation
257 of pilus-mediated response to *S. aureus* (Figure 2 – video 1). Additionally, as shown prior with a $\Delta pilA \Delta flgK$ mutant,
258 which similarly lacked functional pili and flagella, $\Delta pilJ \Delta flgK$ cells are unable to remain in a clustered microcolony
259 at later time points (Figure 2 – video 1) (Limoli et al., 2019). To test that $\Delta chpB$ and $\Delta pilK$ phenotypes at the single-
260 cell level were not due to flagella-mediated motility as well, we generated $\Delta chpB \Delta flgK$ and $\Delta pilK \Delta flgK$ mutants and
261 live-imaged each in monoculture. Both of these mutants phenocopy the behaviors of their respective parental single
262 mutant strains (Figure 2 – videos 2-4).

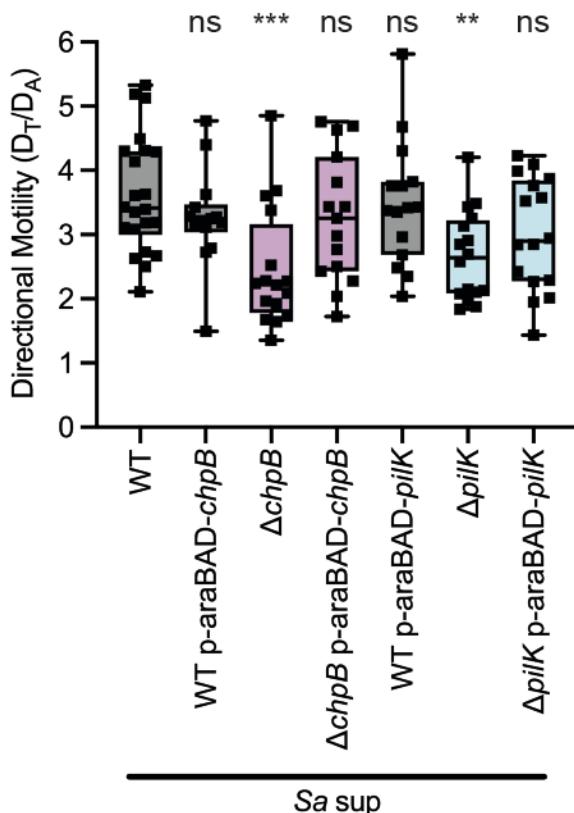


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264 **Figure 2. Methyl modification proteins for chemotaxis adaption are necessary for full directional TFP-**
 265 **mediated motility towards *S. aureus*.** (A) Schematic of *P. aeruginosa* MCP protein PilJ and methyl modification
 266 proteins PilK and ChpB. The predicted methylation sites with cytoplasmic domain of PilJ are represented by the
 267 black and gray circles. In wildtype, PilJ is predicted to undergo methylation and demethylation; thus, ChpA kinase
 268 activity switches between ON and OFF states (left). In the absence of *chpB*, PilJ is expected to have high

269 methylation (filled, black circles) and therefore ChpA kinase activity shifted ON (middle). In the absence of *pilK*, PilJ
270 is expected to have low methylation (empty, gray circles) and therefore ChpA kinase activity shifted OFF (right). (B)
271 Mean-squared displacements (MSD) for the parallel (||) and perpendicular (⊥) directions of wildtype, $\Delta chpB$, and
272 $\Delta pilK$. The anomalous diffusion exponent (α) for each MSD is shown. Insets show the rose graphs of the principal
273 angle of motility for each cell trajectory relative to starting position for *P. aeruginosa* wildtype, $\Delta chpB$, and $\Delta pilK$ in
274 coculture with *S. aureus*. Position of *S. aureus* relative to the center of the *P. aeruginosa* microcolony is represented
275 by the gray cocci on the perimeter. Trajectory angles are shown by colored vectors with the average angle of all
276 trajectories represented by the dotted black line. Larger vectors indicate more cells for the given principal angle.
277 One rose graph representative of at least three replicates is shown. (C) Relative distributions of parallel component
278 step displacements (Δr) of wildtype, $\Delta chpB$, and $\Delta pilK$ cells. Relative distributions are the absolute difference
279 between probability density functions (PDF) of movers and resters, averaged over lag times $\Delta t \leq 20$ seconds. The
280 red regions highlight the non-zero sharp-shouldered peak step size between $0.6 \mu\text{m} \leq |\Delta r| \leq 1.0 \mu\text{m}$. The inset
281 shows the location of the peak step size for wildtype movers over increasing lag times for forward and backwards
282 steps in both parallel and perpendicular directions.

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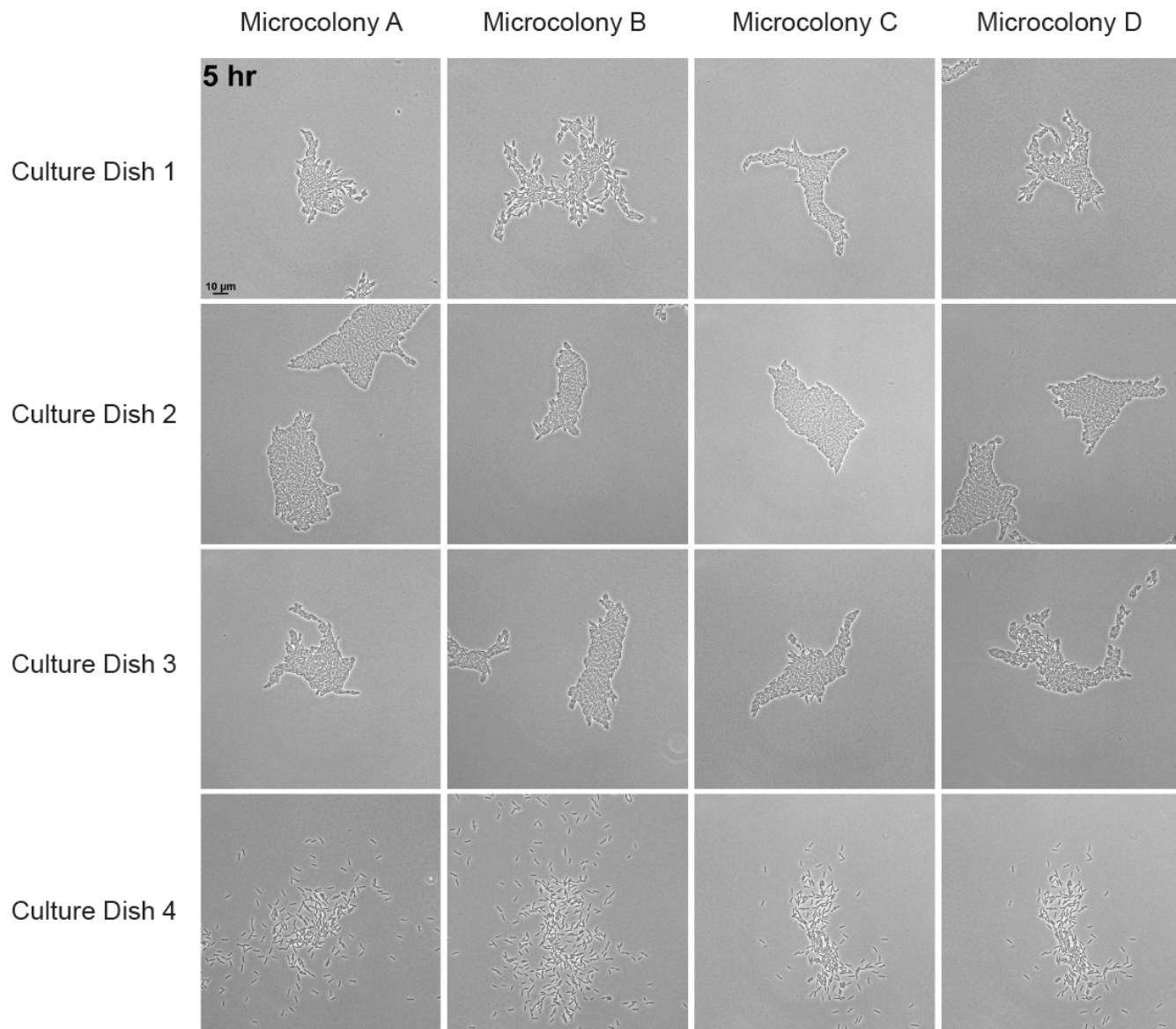


285

286 **Figure 2 – Figure supplement 1. Complementing $\Delta pilK$, and $\Delta chpB$ restores pilus-mediated chemotaxis.**
287 Directional motility towards *S. aureus* secreted factors of wildtype, $\Delta pilK$, and $\Delta chpB$ with and without
288 complementing plasmids carrying arabinose-inducible copies of *pilK* or *chpB*. Complemented strains were induced
289 with 0.2% arabinose; however, phenotypes were the same in the absence of induction. Directional motility for at
290 least four biological replicates, each containing a minimum of three technical replicates are shown. Statistical

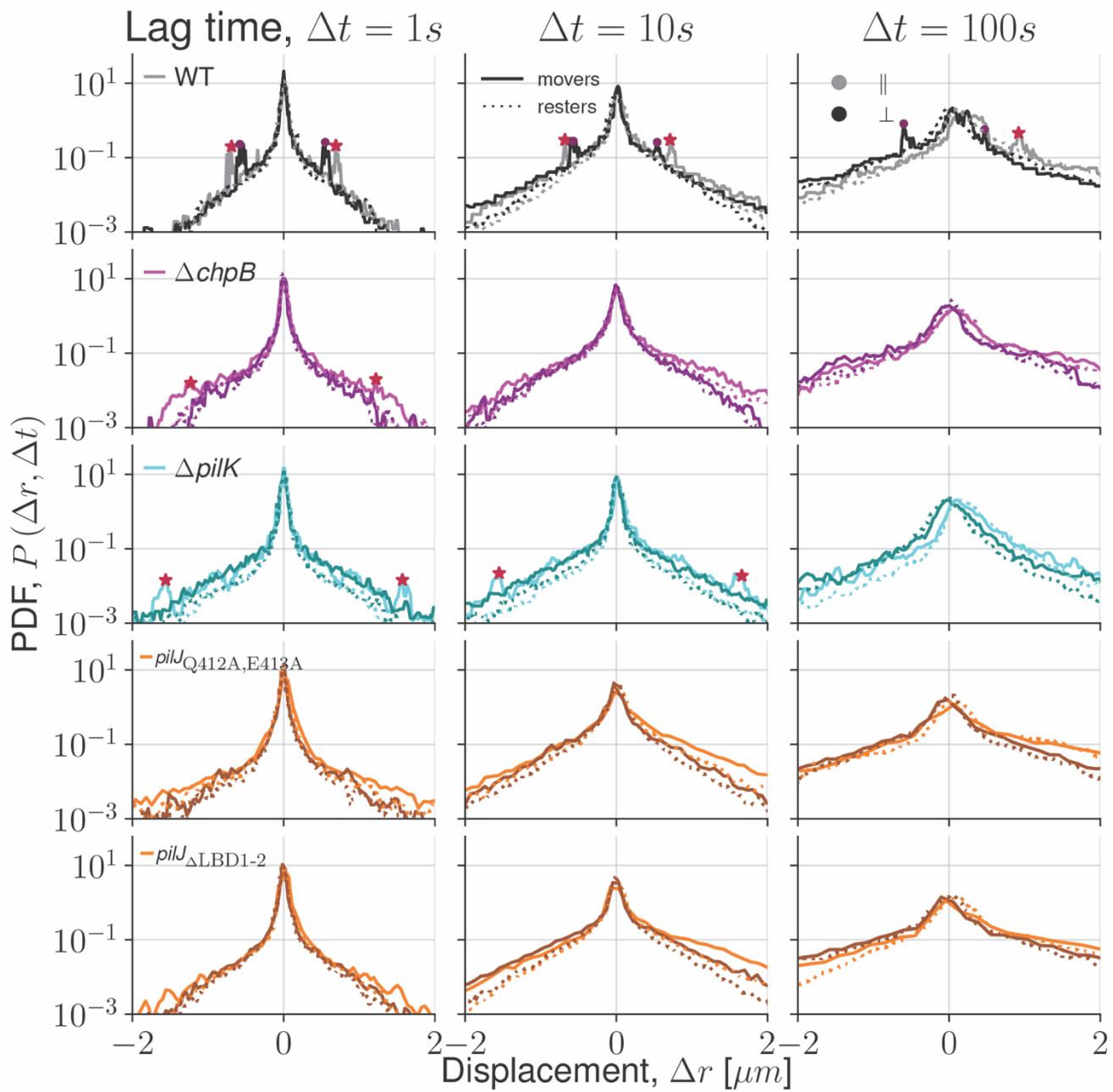
291 significance was determined with a one-way ANOVA followed by Dunnett's multiple comparisons test. *** indicates
292 p< 0.001; ** indicates p<0.01; ns indicates no statistically significant difference.

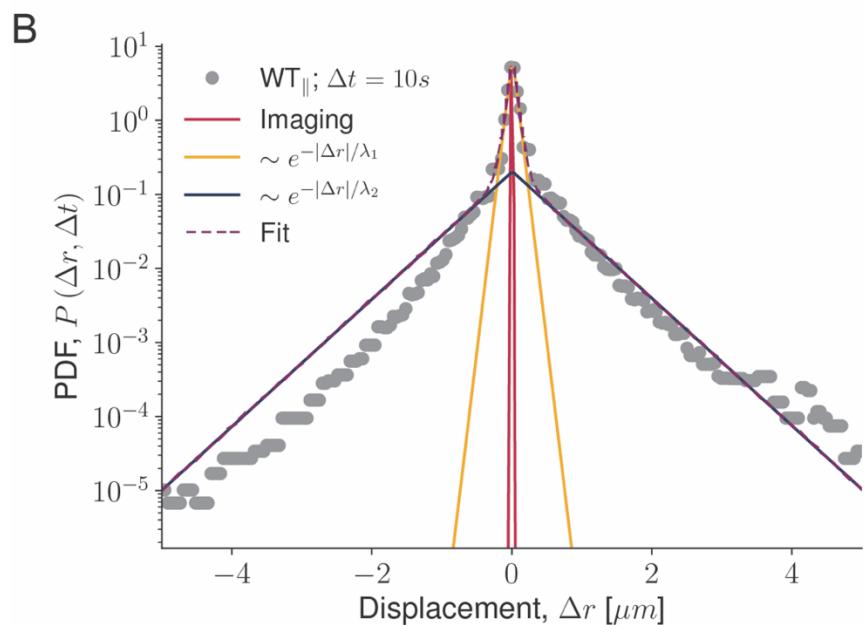
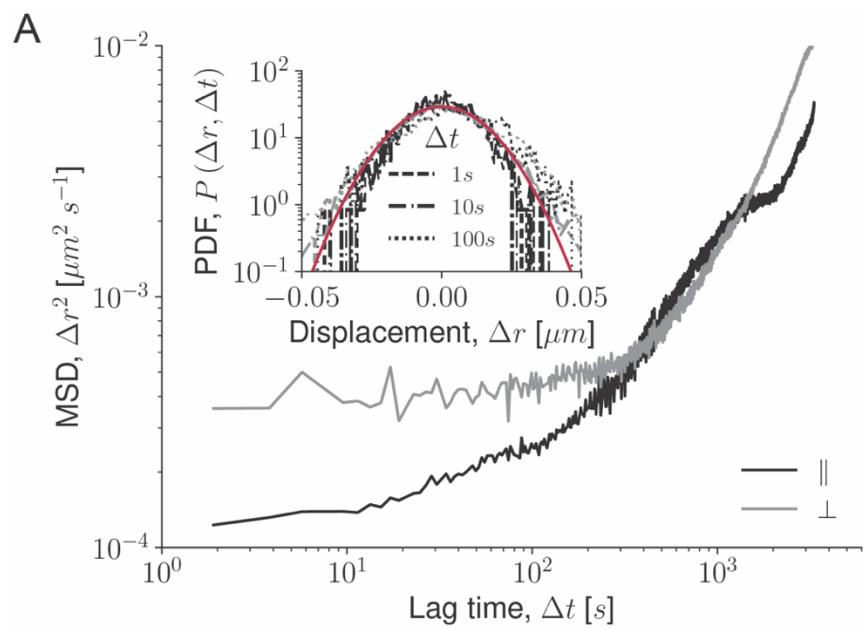
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295 **Figure 2 – Figure supplement 2. *P. aeruginosa* Δ pilK has bimodal pilus-mediated motility.** Separate Δ pilK
296 cultures plated onto four individual experimental dishes and four fields of view in each culture dish were
297 simultaneously imaged at 5 hours post-inoculation. Agarose pads were made from the same media and dried under
298 the same conditions at the same time. A range in motility phenotypes are seen between all microcolonies imaged.





306

307 **Figure 2 – figure supplement 4. Dynamics due to imaging errors.** (A) Mean-squared displacements (MSD) for
 308 the parallel (\parallel) and perpendicular (\perp) directions for dust particles used to measure noise in the imaging. Inset shows
 309 the particle-displacement probability density function (PDF). The PDF is a narrow noise peak, that is fit to a
 310 Gaussian distribution (solid red line) but is non-diffusive, as it does not broaden in time. (B) PDF of the total cell
 311 step displacements (Δr), regardless of principal direction, for wildtype cells at a lag time of $\Delta t = 10$ seconds. The PDF
 312 is composed of a narrow peak of small displacements (jiggling) and long tails of large-but-rare displacements. The
 313 narrow peak cannot be explained by imaging uncertainty (solid red curve) and is better described by a Laplace
 314 distribution (Eq 7), as are the long tails.

315

316

317 **Video 1.** *P. aeruginosa* wildtype in monoculture. Duration 3.5 hr. 3 hr post-inoculation. Acquisition interval 1 sec.
318 Output interval every 40th frame at 50ms/frame.
319
320 **Video 2.** *P. aeruginosa* wildtype in coculture. Duration 1 hr 45 min. 3 hr post-inoculation. Acquisition interval 1 sec.
321 Output interval every 40th frame at 50ms/frame.
322
323 **Video 3.** *P. aeruginosa* $\Delta chpB$ in monoculture. Duration 2 hr. 3 hr post-inoculation. Acquisition interval 1 sec. Output
324 interval every 40th frame at 50ms/frame.
325
326 **Video 4.** *P. aeruginosa* $\Delta chpB$ in coculture with *S. aureus* wildtype. Duration 3 hr. 2 hr post-inoculation. Acquisition
327 interval 1 sec. Output interval every 40th frame at 50ms/frame.
328
329 **Video 5.** *P. aeruginosa* $\Delta pilK$ in monoculture. Growing microcolony cells. Duration 3 hr. 3 hr post-inoculation.
330 Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.
331
332 **Video 6.** *P. aeruginosa* $\Delta pilK$ in monoculture. Hypermotile cells. Duration 3 hr. 3 hr post-inoculation. Acquisition
333 interval 1 sec. Output interval every 40th frame at 50ms/frame.
334
335 **Video 7.** *P. aeruginosa* $\Delta pilK$ in coculture with *S. aureus* wildtype. Growing microcolony cells. Duration 4 hr. 2 hr
336 post-inoculation. Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.
337
338 **Video 8.** *P. aeruginosa* $\Delta pilK$ in coculture with *S. aureus* wildtype. Hypermotile cells. Duration 4 hr. 2 hr post-
339 inoculation. Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.
340
341 **Figure 2 – video 1.** *P. aeruginosa* $\Delta pilJ \Delta flgK$ in coculture with *S. aureus* wildtype. Duration 1 hr 15 min. 3 hr post-
342 inoculation. Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.
343
344 **Figure 2 – video 2.** *P. aeruginosa* $\Delta chpB \Delta flgK$ in coculture with *S. aureus* wildtype. Duration 3 hr. 3 hr post-
345 inoculation. Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.
346
347 **Figure 2 – video 3.** *P. aeruginosa* $\Delta pilK \Delta flgK$ in coculture with *S. aureus* wildtype. Growing microcolony cells.
348 Duration 2.5 hr. 3 hr post-inoculation. Acquisition interval 2 sec. Output interval every 20th frame at 50ms/frame.
349
350 **Figure 2 – video 4.** *P. aeruginosa* $\Delta pilK \Delta flgK$ in coculture with *S. aureus* wildtype. Hypermotile cells. Duration 2.5
351 hr. 3 hr post-inoculation. Acquisition interval 2 sec. Output interval every 20th frame at 50ms/frame.

352 **Results: Methyl modification of PilJ is necessary for TFP-mediated chemotaxis response to *S. aureus***

353 The necessity of PilK and ChpB for directional twitching motility suggests that, like other bacterial chemoreceptors,
354 methylation levels of PilJ influence the downstream signaling cascade; yet, number and location of the methylation
355 sites in PilJ are unknown. We hypothesized that PilJ contained at least one methylation site for chemoreceptor
356 adaptation. To investigate this hypothesis, we first searched the amino acid sequence of PilJ for the conserved ten
357 amino acid methylation motif. This motif, [A/S/T/G]-[A/S/T/G]-X-X-[E/Q]-[E/Q]-X-X-[A/ S/T/G]-[A/S/T/G], has a pair
358 of glutamine and/or glutamate residues at the center (Alexander & Zhulin, 2007; Ortega et al., 2017; Salah Ud-Din
359 & Roujeinikova, 2017; Terwilliger et al., 1986). In other MCPs, one of these residues is methylated by the
360 methyltransferase and demethylated by the methylesterase. Examination of PilJ reveals two motifs with an exact
361 match to the conserved sequence and one motif that shares nine of the ten conserved amino acids. This suggests
362 PilJ has three potential methylation sites in the predicted cytoplasmic region (Figure 3A, black and pale orange
363 circles; Figure 3 – figure supplement 1). The identified methylation sites are located at residues Q412/E413,
364 Q623/Q624, and Q639/E640.

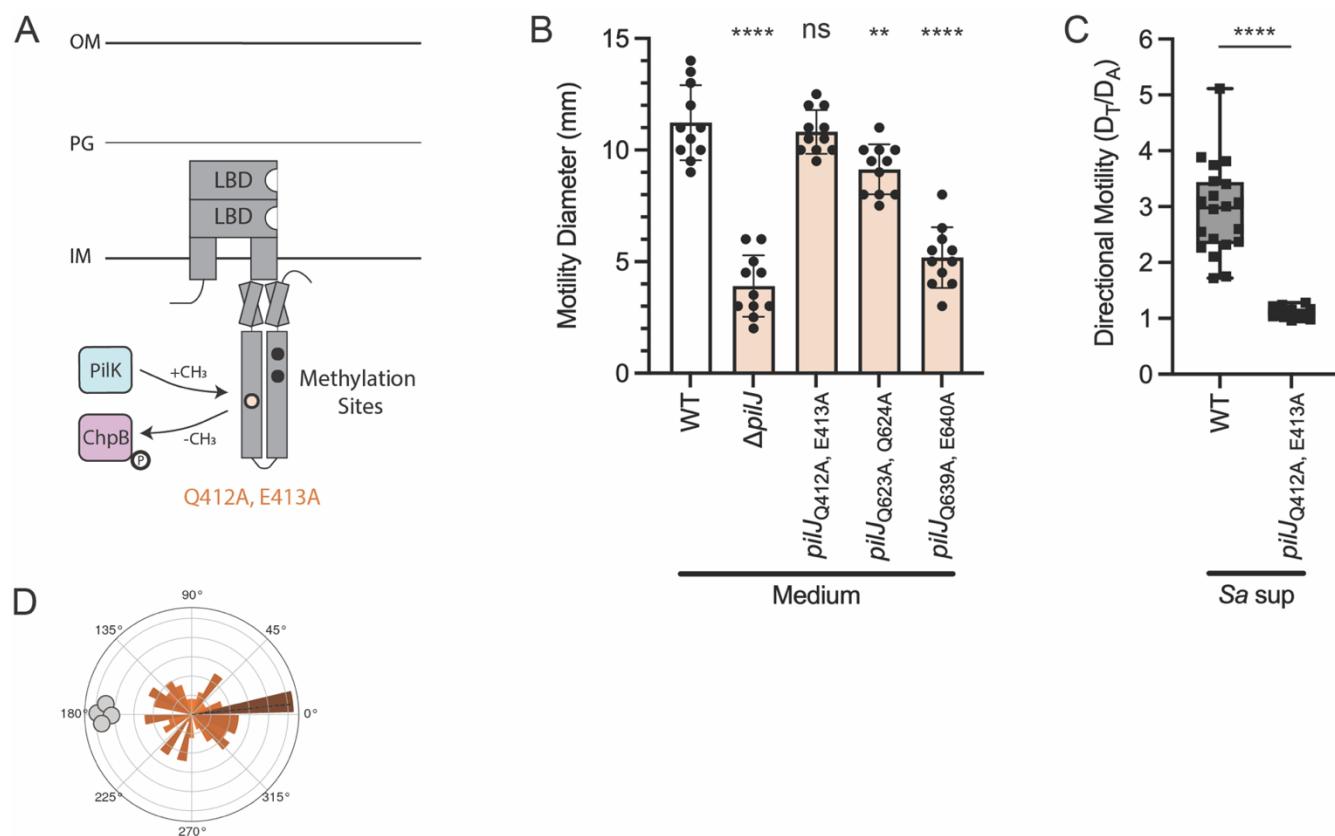
365

366 To experimentally determine whether the methylation sites were necessary for TFP-mediated chemotaxis, we next
367 generated a mutation in each methylation site by substituting the glutamine/glutamate residue pair to an
368 alanine/alanine pair. Given most other *pilJ* mutants exhibit severely diminished twitching motility, we first compared
369 the ability of each PilJ methylation site mutant to twitch in a standard subsurface motility assay (Turnbull &
370 Whitchurch, 2014). Briefly, *P. aeruginosa* was inoculated at the plastic-agar interface and allowed to move prior to
371 measuring the diameters of motility. Only the *pilJ*_{Q412A, E413A} mutant retains full wildtype levels of motility, while
372 *pilJ*_{Q623A, Q624A} has moderate-but-significant reduction in motility and *pilJ*_{Q639A, E640A} is severely diminished in twitching
373 motility (Figure 3B). As the only methylation site mutant that retains wildtype levels of pilus-mediated motility, the
374 next question is whether *pilJ*_{Q412A, E413A} exhibits reduced attraction towards *S. aureus*. In the macroscopic directional
375 motility assay, *pilJ*_{Q412A, E413A} demonstrates significant loss of migration up the gradient of *S. aureus* supernatant
376 (Figure 3C). The other methylation site mutants also display attenuated chemoattraction to *S. aureus*; yet, it is
377 unclear how much of this reduced response is due to motility defects versus deficiency in pilus-mediated chemotaxis
378 (Figure 3 – figure supplement 2). To confirm that the migration phenotype of *pilJ*_{Q412A, E413A} can be restored with full-
379 length *pilJ*, the mutant and wildtype strains were complemented with a plasmid containing a GFP-tagged copy of
380 wildtype *pilJ* under control of an arabinose-inducible promoter (Figure 3 – figure supplement 3). This allows for
381 visualization of PilJ in the cells, which show the expected bipolar localization (Figure 3 – figure supplement 3A).
382 Additionally, the complemented mutant strain shows levels of directional response similar to wildtype harboring the
383 complementation plasmid (Figure 3 – figure supplement 3B). Due to the GFP tag, some PilJ signaling may be
384 diminished leading to the lower interspecies signal response in the complemented strains.

385

386 Next, we live-imaged the *pilJ*_{Q412A, E413A} mutant in monoculture and observe that it moves earlier than wildtype, Δ *pilK*,
387 or Δ *chpB*, with single-cell or small groups of two to three cells traveling together (Video 9). Furthermore, *pilJ*_{Q412A,}
388 _{E413A} does not form a microcolony; rather, the cells begin to twitch and move apart from each other at very low cell
389 density, typically before there are \sim 10 cells present. This behavior is recapitulated in the presence of *S. aureus*,
390 with *pilJ*_{Q412A, E413A} additionally showing no bias in movement towards *S. aureus* (Figure 3D, Video 10). The *pilJ*_{Q412A,}

391 E_{413A} mutant cells do become elongated after a few hours compared to wildtype. This slight cell division defect is
392 likely due to the high cAMP levels in $pilJ_{Q412A, E413A}$ (see Figure 5). Live-imaging of a $pilJ_{Q412A, E413A} \Delta flgK$ mutant in
393 coculture with *S. aureus* phenocopies the parental $pilJ_{Q412A, E413A}$ (Figure 3 – video 1). Together these data identify
394 the methylation sites of PilJ and show that methyl modification of these sites is important for chemotaxis signaling.



395

396 **Figure 3. Methyl modification of PilJ is necessary for TFP-mediated chemotaxis response to *S. aureus*.** (A) 397 Schematic of PilJ with cytoplasmic methylation sites represented as black circles. The methylation site Q412, E413, 398 whose mutant retains wildtype motility and is further studied for chemotaxis response, is highlighted in pale orange. 399 (B) Twitching motility diameters of *P. aeruginosa* wildtype and methylation mutants. Motility diameters were 400 analyzed by a one-way ANOVA followed by Dunnett's multiple comparisons test. **** indicates $p < 0.0001$; ** 401 indicates $p < 0.01$; ns indicates no statistically significant difference. (C) Migration towards *S. aureus* secreted factors 402 of *P. aeruginosa* wildtype and methylation mutant *pilJ*_{Q412A, E413A}. Directional motility measurements were 403 analyzed with an unpaired t-test. **** indicates $p < 0.0001$. Motility diameters and directional motility are shown for at 404 least three biological replicates each containing a minimum of four technical replicates. (D) Rose graph of the principal 405 angle of motility for each cell trajectory relative to starting position for *P. aeruginosa* *pilJ*_{Q412A, E413A} in 406 coculture with *S. aureus*. Position of *S. aureus* relative to the center of the *pilJ*_{Q412A, E413A} cells is 407 represented by the gray cocci on the perimeter. Trajectory angles are shown by colored vectors with the average angle of all 408 trajectories represented by the dotted black line. Larger vectors indicate more cells for the given principal angle. One rose 409 graph representative of at least three replicates is shown.

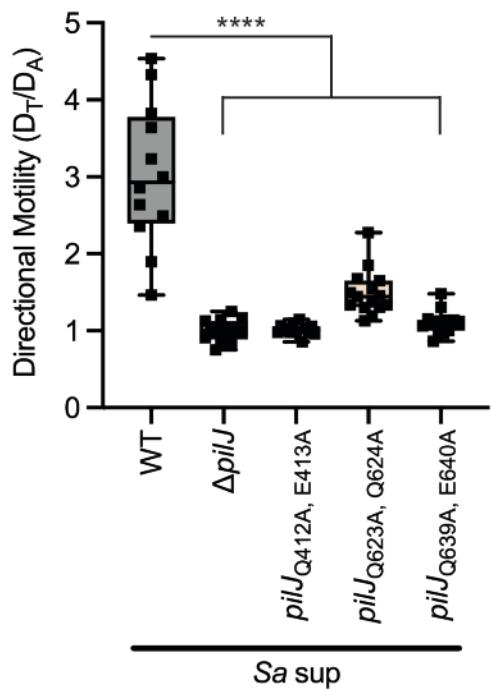
> PilJ (PA14_05360)

MKKINAGNLFAGMRSSVIAGLFIVLIVSIVLLFANFAYLNTQSNHDKQYIGHAGELRVLSQRIAKNATEAAA
GKGEAFKLLKDARNDFEKRNILVNGDESTSPPSPEAVKPQMDVVQQDWGGLRKNADSILASEQTVLSLHQV
ASTLAETIPQLQVEYEEVVDILLENGAPADQVAQROSLLAERILGSVNKVLAGDENSQAAADSFGRDASLF
GRVLKGMQEGNAAMSISKVTNAEAVDRLNEIAELFEFVSGSVDEILETSPDLFQVREAANNIFSVSQTL
SQLADGFENLAGGRSINLFAGYVLGALALASIILIGLVMVRETNRRLAETAEKNDRNQAAILRLLDEIADLAD
GDLTVAAVTEDFTGAIADSINYSIDQLRELVETINQTAQVQAAAAQETOSTAMH LAEASEHQAQEIAGASAA
INEMAVSIDQVSANASESSAVAERSVAIANKGNEVVHNTITGMDNIREQIQDTSKRIKRLGE
INDIADQTNILALNAAIQASMAGDAGRGA
EVVRGARLAQDAGVALEEIEKVS
NLAKMASEMRSVSGFKLPEGVEQA

410

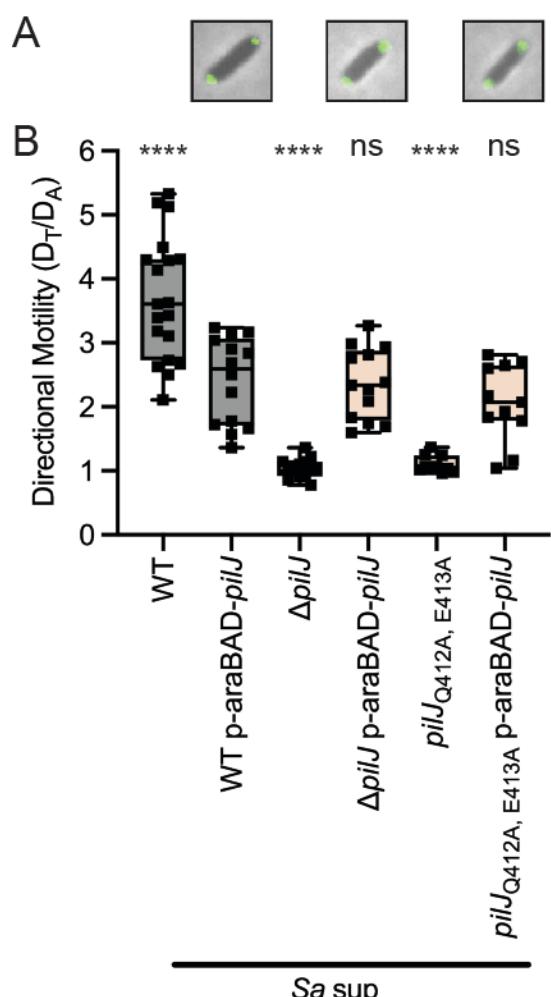
411 **Figure 3 – Figure supplement 1. Methylation sites of PilJ.** Amino acid sequence of *P. aeruginosa* PA14 PilJ
412 (PA14_05360) with conserved MCP methylation motifs highlighted in pale orange and predicted methyl modification
413 glutamate/glutamine residue pairs in bold.

414



415

416 **Figure 3 – Figure supplement 2. Mutation of any cytoplasmic PilJ methylation site reduces *P. aeruginosa***
417 **attraction to interspecies signals.** Directional motility of *P. aeruginosa* wildtype and methylation mutants $pilJ_{Q412A}$,
418 $pilJ_{Q623A, Q624A}$, and $pilJ_{Q639A, E640A}$ in the presence of a gradient of *S. aureus* supernatant. Directional motility for
419 at least four biological replicates, each containing a minimum of three technical replicates are shown and statistical
420 significance was determined with a one-way ANOVA followed by Dunnett's multiple comparisons test. **** indicates
421 $p < 0.0001$.



422

423 **Figure 3 – Figure supplement 3. Complemented $pilJ_{Q412A, E413A}$ with full-length PilJ displays bipolar PilJ**

424 localization and restoration of TFP-mediated directional motility. (A) Representative *P. aeruginosa* cells with

425 bipolarly localized GFP-tagged PilJ. (B) Directional motility towards *S. aureus* secreted factors of wildtype or

426 *pilJ*_{Q412A, E413A} with and without complementing plasmids carrying arabinose-inducible copy of wildtype *pilJ*.

427 Complemented strains were induced with 0.2% arabinose; however, phenotypes were the same in the absence of

428 induction. Directional motility for at least three biological replicates, each containing a minimum of three technical

429 replicates are shown and statistical significance was determined with a one-way ANOVA followed by Dunnett's

430 multiple comparisons test to compare each strain to wildtype *P. aeruginosa* carrying p-araBAD-*pilJ*. **** indicates

431 $p < 0.0001$; ns indicates no statistically significant difference.

432

433

434 **Video 9.** *P. aeruginosa* $pilJ_{Q412A, E413A}$ in monoculture. Duration 2 hr 40 min. 2 hr 20 min post-inoculation. Acquisition

435 interval 1 sec. Output interval every 40th frame at 50ms/frame.

436

437 **Video 10.** *P. aeruginosa* $pilJ_{Q412A, E413A}$ in coculture with *S. aureus* wildtype. Duration 3 hr. 2 hr post-inoculation.

438 Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.

439

440 **Figure 3 – video 1.** *P. aeruginosa* *pilJ*_{Q412A, E413A} Δ *flgK* in coculture with *S. aureus* wildtype. Duration 3.5 hr. 1 hr
441 post-inoculation. Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.
442

443 **Results: The ligand binding domains of PilJ are required for pilus-mediated chemotaxis but not twitching**
444 **motility**

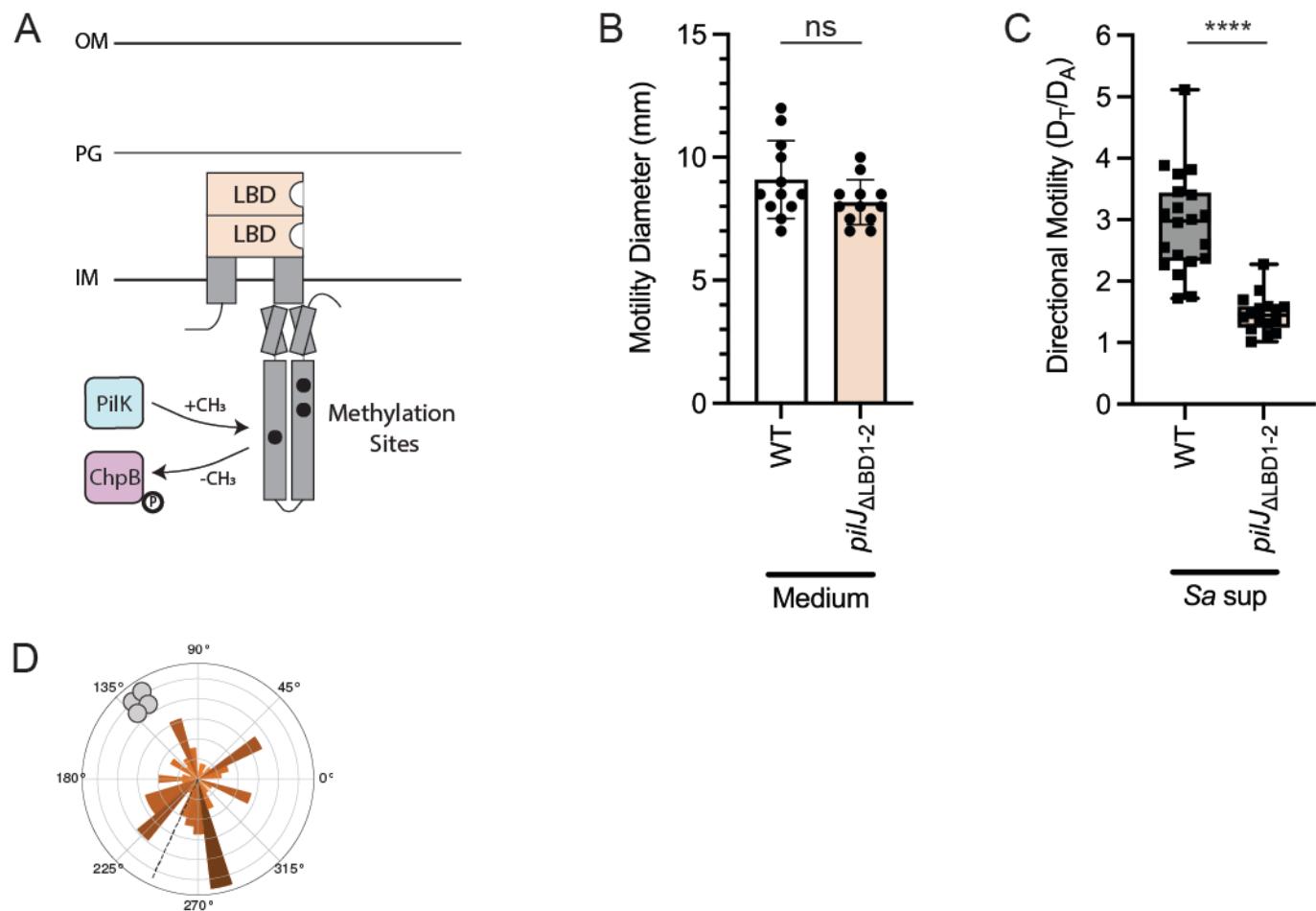
445 Given methylation adaptation is necessary to bias movement towards *S. aureus* but not twitching, we next asked if
446 the LBDs of *P. aeruginosa* PilJ are also required for directing motility. We generated a mutant lacking the periplasmic
447 portion containing both predicted PilJ LBDs (residues 39-303), based on the predictions by Martín-Mora et al. and
448 confirmed by AlphaFold, yet kept the transmembrane domains and entire cytoplasmic region intact, which we now
449 refer to as *pilJ*_{ΔLBD1-2} (Figure 4A, LBDs in pale orange) (Martin-Mora et al., 2019).

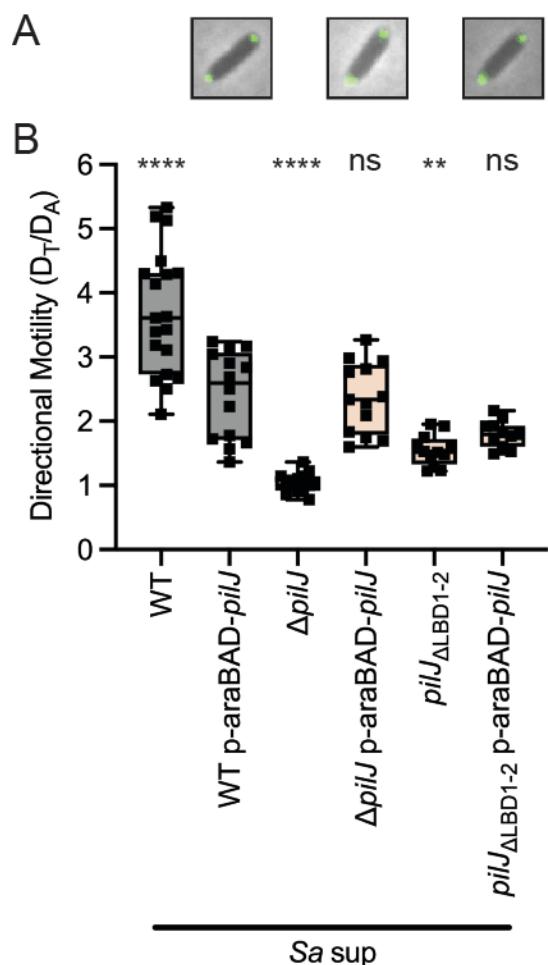
450

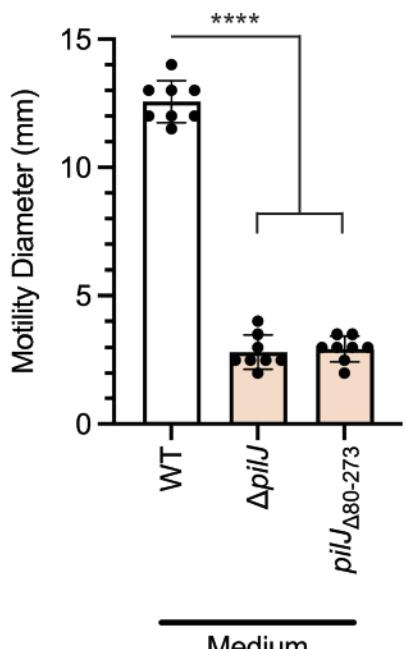
451 We first tested whether *pilJ*_{ΔLBD1-2} retained any twitching motility using the subsurface twitching assay, as described
452 above, and found that loss of the LBDs does not reduce the ability to twitch (Figure 4B). Since *pilJ*_{ΔLBD1-2} is able to
453 twitch to wildtype levels, we next tested the extent that it could chemotax up a gradient of *S. aureus* secreted factors.
454 Despite being able to twitch, *pilJ*_{ΔLBD1-2} loses nearly all ability to move directionally towards *S. aureus* secreted
455 factors (Figure 4C). When complemented with the same *pilJ-gfp* plasmid described above, *pilJ*_{ΔLBD1-2} exhibits bipolar
456 localization of PilJ; however, only partial restoration of interspecies signal response is observed, despite statistical
457 similarity to wildtype with the complement plasmid (Figure 4 – figure supplement 1). Chemoreceptors are typically
458 grouped in arrays of trimer-of-dimer units with each unit signaling downstream to a kinase (Parkinson et al., 2015).
459 Therefore, despite proper localization, the combination of mutant and GFP-tagged wildtype copies of PilJ for this
460 particular strain may lead to trimers-of-dimers with inefficient signaling for complete directional response (i.e., the
461 *pilJ*_{ΔLBD1-2} allele is partially dominant).

462

463 Following examination of community level movement and response, we performed live-imaging of *pilJ*_{ΔLBD1-2} in
464 monoculture to determine how loss of the LBDs impacted single-cell movements. The *pilJ*_{ΔLBD1-2} mutant displays
465 increased and earlier motility, with cells lacking microcolony formation seen by the wildtype (Video 11). Unlike the
466 methylation PilJ mutant, *pilJ*_{ΔLBD1-2} tends to move as groups of cells, with trajectories curving in wide loops (Video
467 11). These behaviors are echoed in coculture with *S. aureus*, with *P. aeruginosa* *pilJ*_{ΔLBD1-2} appearing to show no
468 bias towards *S. aureus* microcolonies (Figure 4D, Video 12). This behavior is also phenocopied by a *pilJ*_{ΔLBD1-2} Δ *flgK*
469 mutant (Figure 4 – video 1). Collectively, these data establish a role for the LBDs of PilJ in control of response to
470 interspecies signals, while also showing they are not essential for general pilus-mediated motility.







Medium

493

494 **Figure 4 – figure supplement 2. PilJ mutant lacking a portion of the periplasmic region is non-motile.**

495 Twitching motility diameters of *P. aeruginosa* wildtype, $\Delta p\text{ilJ}$, and a *pilJ* mutant lacking amino acids 80-273 ($p\text{ilJ}_{\Delta 80-273}$). Macroscopic motility measurements are shown for two biological replicates, each containing four technical 496 replicates and statistical significance was determined with a one-way ANOVA followed by Dunnett's multiple 497 comparisons test. **** indicates $p < 0.0001$.

498

499

500

501 **Video 11.** *P. aeruginosa* $p\text{ilJ}_{\Delta LBD1-2}$ in monoculture. Duration 2 hr 40 min. 2 hr 20 min post-inoculation. Acquisition 502 interval 1 sec. Output interval every 40th frame at 50ms/frame.

503

504 **Video 12.** *P. aeruginosa* $p\text{ilJ}_{\Delta LBD1-2}$ in coculture with *S. aureus* wildtype. Duration 2 hr. 3 hr post-inoculation. 505 Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.

506

507 **Figure 4 – video 1.** *P. aeruginosa* $p\text{ilJ}_{\Delta LBD1-2}$ $\Delta f\text{lgK}$ in coculture with *S. aureus* wildtype. Duration 3 hr. 2 hr post- 508 inoculation. Acquisition interval 1 sec. Output interval every 40th frame at 50ms/frame.

509 **Results: cAMP changes during pilus-mediated chemotaxis independent of TFP activity**

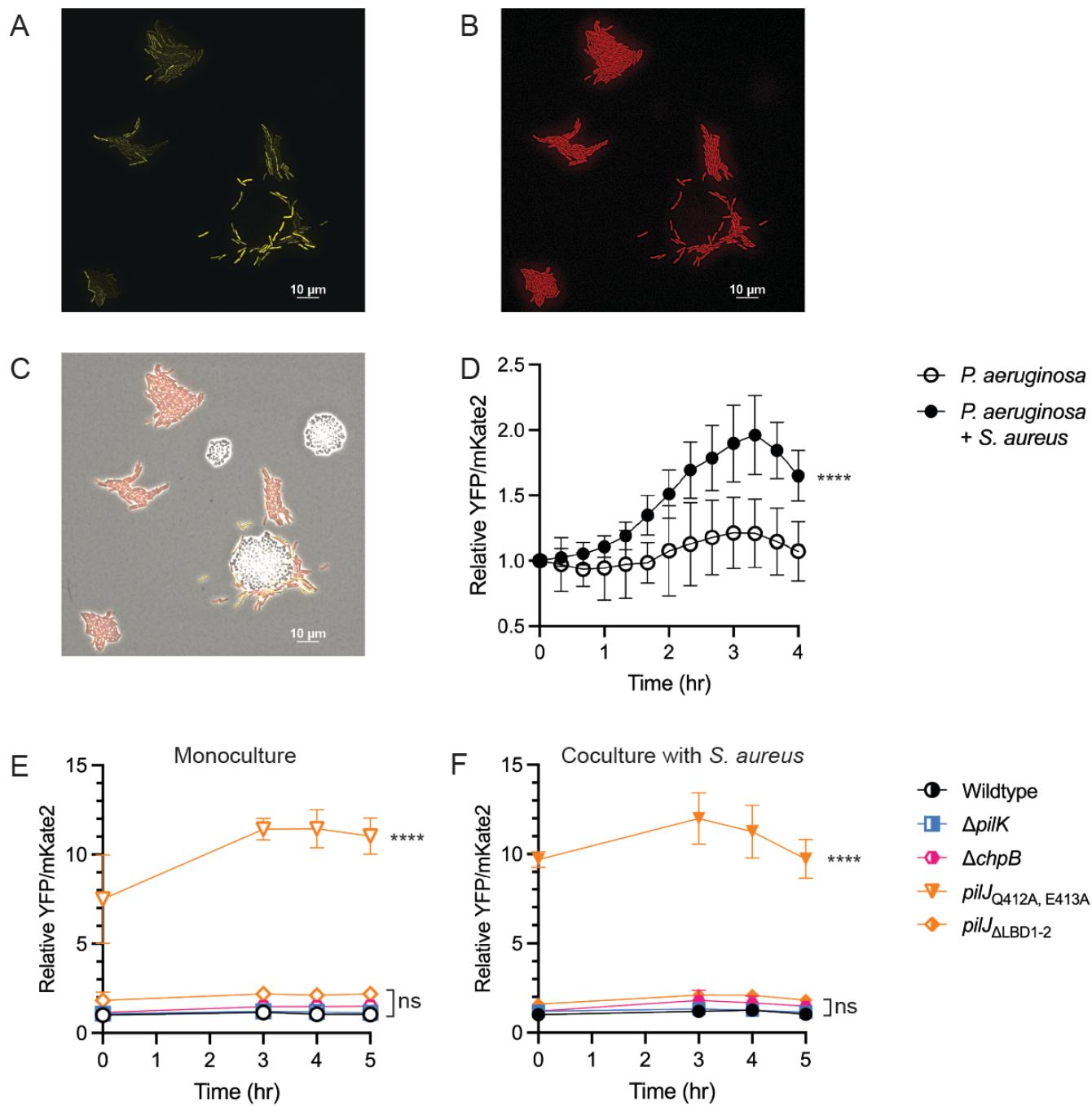
510 Intracellular levels of the second messenger cAMP and Pil-Chp activity are connected. As signal transduction
511 through Pil-Chp increases, the response regulator PilG directly activates the adenylate cyclase, CyaB, necessary
512 for cAMP synthesis (Fulcher et al., 2010). In turn, cAMP indirectly increases transcriptional expression of *pil-chp*
513 generating a positive feedback loop between the two systems (Fulcher et al., 2010; Wolfgang et al., 2003).

514

515 Due to the role of Pil-Chp in perception and reaction to *S. aureus*, we asked whether *P. aeruginosa* increased cAMP
516 levels during interactions with *S. aureus*. To answer this question, we live-imaged a previously established cAMP
517 reporter strain *P. aeruginosa* wildtype PAO1 carrying a cAMP-responsive promoter, P_{xphA} , fused to *yfp* and a
518 constitutively expressed promoter P_{rpoD} , fused to *mKate2* (Persat et al., 2015). Kinetic cAMP levels were measured
519 in individual cells in monoculture or coculture with *S. aureus*, with YFP normalized to mKate2 fluorescence. cAMP
520 is known to be heterogenous amongst cells in a population. In wildtype *P. aeruginosa* monoculture, this
521 heterogeneity is observed, but with minor changes in cAMP levels over time. However, in coculture, cAMP
522 increases, particularly in cells that move towards and surround *S. aureus* (Figure 5A-D). This suggests that
523 response to *S. aureus* is associated with increases in cAMP. Furthermore, when community level response of *P.*
524 *aeruginosa* lacking either CyaB or the cAMP phosphodiesterase, CpdA, was examined, *P. aeruginosa* cannot fully
525 move up a gradient of *S. aureus* supernatant, despite retaining twitching motility (Figure 5 – figure supplement 1).
526 These data suggest that *P. aeruginosa* controls cAMP levels for proper TFP-mediated response to interspecies
527 signals.

528

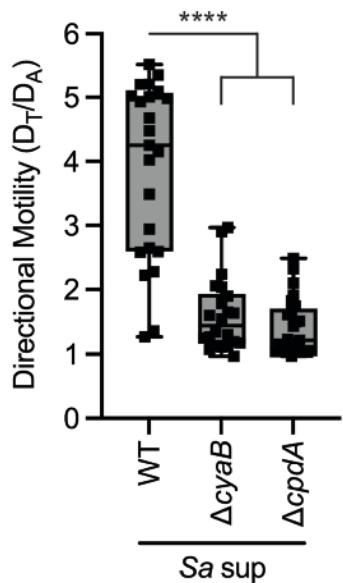
529 Next, we asked how cAMP levels compared between response-deficient mutants and the parental *P. aeruginosa*
530 (PA14). While reporter levels in PA14 are lower in comparison to PAO1, a similar trend is observed. Each mutant
531 shows at least some increase in cAMP relative to wildtype cAMP levels in monoculture or coculture, though not
532 significant for $\Delta pilK$ and $\Delta chpB$. The $pilJ_{\Delta LBBD1-2}$ mutant exhibits an approximately two-fold increase in cAMP over
533 time compared to wildtype; whereas, $pilJ_{\Delta Q412A, E413A}$ exhibits a ten-fold increase, independent of the presence of *S.*
534 *aureus* (Figure 5E, F). These data suggest that cAMP levels are determined by chemoreceptor-mediated signaling,
535 although the degree to which cAMP increases does not always directly correlate with the extent of increased motility.



536

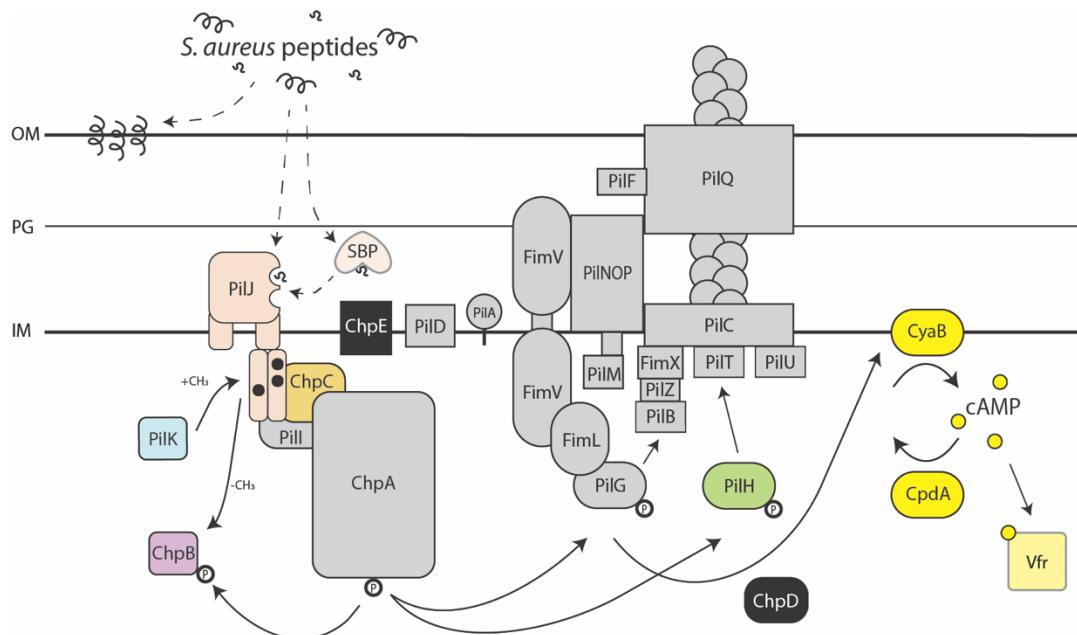
537 **Figure 5. cAMP changes during pilus-mediated chemotaxis independent of TFP activity.** (A-D) Intracellular
 538 levels of *P. aeruginosa* cAMP measured in monoculture and coculture with *S. aureus* using a *P. aeruginosa* PAO1
 539 strain carrying a reporter with the cAMP-responsive promoter *PxphA* transcriptionally fused to *yfp* and constitutively
 540 expressed promoter *PrpoD* fused to *mKate2* for normalization. Representative coculture images at t = 3.5 hr for the
 541 YFP (A) TxRed (B), and merged channels (C). cAMP levels of *P. aeruginosa* PA14 *pil-chp* mutants in monoculture
 542 (E) and coculture with *S. aureus* (F) using the cAMP reporter. cAMP levels were monitored by dividing YFP by
 543 *mKate2* fluorescence intensity for each time point and normalizing intensity to wildtype cAMP at t = 0 hr. cAMP
 544 levels are shown for at least four microcolonies per condition and were compared using either multiple unpaired t-
 545 tests or a one-way ANOVA followed by Dunnett's multiple comparison's test. **** indicates p<0.0001; ns indicates
 546 no statistically significant difference.

547



548

549 **Figure 5 – figure supplement 1. Enzymes that control cAMP levels are necessary for pilus-mediated**
550 **chemotaxis.** Directional motility towards *S. aureus* secreted factors of $\Delta cyaB$ and $\Delta cpdA$. At least three biological
551 replicates, each containing a minimum of three technical replicates are shown and statistical significance was
552 determined with a one-way ANOVA followed by Dunnett's multiple comparisons test. **** indicates $p < 0.0001$.



554 **Figure 6. Model of TFP-mediated interspecies chemotaxis.** *S. aureus* peptide signals (black) are sensed by *P.*
555 *aeruginosa* Pil-Chp chemoreceptor PilJ (pale orange) ligand binding domains and transmitted to histidine kinase
556 ChpA (gray), likely through the linker protein ChpC (gold). The kinase state modulates the activity of response
557 regulators PilG (gray) and PilH (green), which coordinate TFP extension and retraction events that bias movements
558 towards the interspecies signals. Adaption of PilJ through modification of the methylation sites by enzymes PilK
559 (blue) and ChpB (purple), is also required for proper chemotactic response. Chemotaxis through the Pil-Chp system
560 also leads to increases in cAMP (yellow) and depends on careful regulation of these cAMP levels by enzymes CyAB
561 and CpdA (yellow). cAMP activates Vfr for upregulation of virulence factors, including the Pil-Chp system. The
562 chemoattractant peptide signals sensed by PilJ may be full-length *S. aureus* PSMs (black helices) or oligopeptide
563 cleavage products (black squiggles). Additionally, these peptides, whether whole or cleaved, could directly bind PilJ
564 or indirectly activate PilJ via an interaction with a periplasmic solute binding protein (SBP). Alternatively, *S. aureus*
565 peptides may embed into the *P. aeruginosa* membrane, causing transient membrane stress and activating Pil-Chp.
566 The solid arrows indicate previously described interactions and the dashed arrows indicate hypothetical pathways
567 that will be tested in future studies. OM = outer membrane, PG = peptidoglycan, IM = inner membrane.

568

Discussion

569

While the Pil-Chp system has been studied for its roles in twitching motility and cAMP regulation, less work has explored the additional roles of the system in TFP-mediated chemotaxis. Furthermore, most studies have primarily investigated PilJ through a non-motile complete-deletion mutant, which allowed little understanding of the domains of PilJ that determine chemoreceptor activity. Here, we utilized domain-specific mutations of PilJ to evaluate single-cell and community level behaviors that define the importance of PilJ to sense and relay interspecies signals to pilus response regulators. These observations show *P. aeruginosa* is able to move towards interspecies signals through a novel TFP-mediated chemotaxis mechanism and that PilJ does have the necessary components to serve as a MCP for signal sensation, transmission, and adaptation (Figure 6). To our knowledge, this is the first study to generate mutants in the LBDs and methylation sites of PilJ to define their contribution to chemotactic regulation.

578

579

While flagella-mediated chemotaxis of swimming bacteria has been extensively characterized, there is comparatively little insight into bacterial chemotaxis on a surface. Despite overall sequence similarity of PilJ in the cytoplasmic region to other *P. aeruginosa* MCPs, the low similarity of the periplasmic region containing the LBDs has led to several questions about the function of this putative pilus MCP. First, what ligands does PilJ bind? Martin-Mora et al. previously investigated the only other *P. aeruginosa* MCP containing a PilJ LBD, McpN, which was shown to bind nitrate (Martin-Mora et al., 2019). Evaluation of the McpN and PilJ ligand binding pocket motifs showed little conservation between the two MCPs and PilJ lacked nitrate binding ability (Martin-Mora et al., 2019). If PilJ does not bind nitrate, then what signals can it bind? Persat et al. showed that PilJ can interact with the pilus monomer PilA in the periplasmic regions of each protein and proposed this interaction regulates mechanosensing (Persat et al., 2015), although recent data argues against this model (Kuchma & O'Toole, 2022). *P. aeruginosa* has a second non-conventional chemosensory system for surface sensing, called Wsp. Recently, it was shown that the Wsp system is more broadly a membrane stress detection system and surfaces are just one of many membrane stressors that the receptor WspA detects (O'Neal et al., 2022). In eukaryotic cells, phenol soluble modulins (PSMs) are known to form a membrane-perturbing pore; thus, PilJ may also perceive and transduce interspecies peptide-induced membrane stress (Figure 6) (Verdon et al., 2009).

594

595

If PilJ binds a conventional chemoattractant signal, full length PSMs are unlikely to interact directly with the LBD, due to their size and amphipathic secondary structure. Nolan et al. identified that *P. aeruginosa* exhibits increased twitching in the presence of environmental signals, such as tryptone, mucins, or bovine serum albumin (Nolan et al., 2020). This response required *P. aeruginosa* protease activity, presumably to cleave environmental factors into smaller signals (Nolan et al., 2020). While this group did not study the chemotactic nature of these compounds, they do suggest that the increased twitching response is PilJ-mediated. This indicates that PilJ may sense a broad range of environmental signals. It is thus plausible that the *S. aureus* peptides, which are shown to elicit a PilJ-dependent chemotaxis response here, are indeed a chemoattractant signal. Yet, it is still unclear how PSMs may gain access to and bind the periplasmic LBDs of PilJ and thus act as a traditional chemoattractant signal for activation of downstream signaling. Therefore, protease-cleavage of interspecies peptides may be necessary to fragment PSMs into signal-sized peptides for either direct or indirect PilJ binding, in conjunction with earlier observations that *P. aeruginosa* chemotaxes towards di- or tripeptides rather than larger oligopeptides (Kelly-

607 Wintenberg & Montie, 1994). Another possibility is that the peptides bind PilJ indirectly, perhaps through a solute
608 binding protein, as previously reported for chemoattractant inorganic indirectly binding chemoreceptor CtpL through
609 the mediating solute binding protein PtsS (Matilla et al., 2021; Rico-Jiménez et al., 2013). Investigation of these
610 hypotheses, including a role for membrane stress, is currently underway (Figure 6).

611

612 Jansari et al. previously reported that their *pilJ* mutant retained some twitching motility, but had diminished response
613 to phosphatidylethanolamine (Jansari et al., 2016). In our current study, chemoattraction to
614 phosphatidylethanolamine by wildtype *P. aeruginosa* was not observed and therefore chemotaxis towards
615 phosphatidylethanolamine by the *pilJ* mutants generated here could not be determined. Jansari et al. generated a
616 *pilJ* mutant lacking residues 74-273 (Jansari et al., 2016). Examination of a similar mutant lacking residues 80-273
617 in the present investigation shows that *P. aeruginosa pilJ*_{Δ80-273} similarly has reduced twitching; yet, this defect
618 unfortunately yields insufficient motility to measure reduced attraction to *S. aureus* and, thus, could not be evaluated
619 here (Figure 4 – figure supplement 2). However, the mutant lacking both PilJ domains (residues 39-303; *pilJ*_{ΔLBD1-2})
620 retains near wildtype levels of motility and allows for visualization of response deficiency at both the community and
621 single-cell levels. These observations show that without the PilJ LBDs, *P. aeruginosa* is unable to bias motility
622 towards interspecies signals, which further supports that the periplasmic portion of PilJ is not essential for twitching
623 motility but is important for sensing signals—whether they are chemoattractants or surface signals. This suggests
624 PilJ has evolved to coordinate TFP motility in response to several environmental factors. It remains unknown,
625 however, why *P. aeruginosa* PilJ contains two pilJ LBDs and whether they bind ligands cooperatively or
626 independently, or if each has a designated role for particular chemo- or surface-sensing signals.

627

628 Little was previously known about the methylation sites on PilJ, which are typically required for chemotaxis
629 adaptation. While bacterial chemoreceptors have a conserved motif for methylation sites, potential motifs on PilJ
630 had not been identified. This work is the first to identify three likely residue pairs for methyl modification on PilJ,
631 further characterizing the chemoreceptor. In other bacterial chemoreceptors, mutation of each methylation site on
632 a chemoreceptor does not lead to the same phenotype (Astling et al., 2006). Thus, it is reasonable that mutations
633 in each PilJ methylation site differentially influence the conformation of PilJ signaling domains and lead to the three
634 different macroscopic motility behaviors described here (Figure 3B; Figure 3 – figure supplement 2). While live-
635 imaging was only performed on the first methylation site, this was sufficient to show changes in these sites can
636 dramatically alter *P. aeruginosa* chemotaxis (Video 9).

637

638 Increases in *P. aeruginosa* cAMP are commonly associated with surface sensing and Pil-Chp activity. It is further
639 established that while PilG activates CyaB for cAMP production, cAMP in turn increases expression of *pil-chp* genes
640 (Fulcher et al., 2010; Wolfgang et al., 2003). However, previous reports focused on Pil-Chp activity in terms of
641 twitching motility; thus, a link between chemotactic Pil-Chp activity and cAMP levels had been ambiguous. While
642 each mutant shown here has varying levels of motility, all are increased in both motility and cAMP levels relative to
643 wildtype; yet, there is not a direct correlation between mutants that have increased motility and the degree to which
644 cAMP is increased. Only *pilJ*_{Q412A, E413A} showed much higher levels of cAMP at nearly 10-fold the amount as wildtype.

645 While the precise role of cAMP signaling in chemotaxis is unclear, studies are currently underway to further
646 interrogate this signaling pathway.

647

648 Dissection of Pil-Chp and its role in chemotaxis towards interspecies signals has broadened understanding of a
649 unique bacterial chemosensory system that may be utilized for bacterial communication and survival in complex,
650 polymicrobial environments. During *P. aeruginosa*-*S. aureus* coinfections, such as those in cystic fibrosis airways,
651 patients often succumb to worse clinical outcomes than their counterparts who are only infected by one organism
652 (Limoli & Hoffman, 2019). Furthermore, once coinfected, patients tend to stay infected by both organisms for several
653 years (Fischer et al., 2021). Such stable, long-term polymicrobial infections may be enhanced by the chemical and
654 physical interactions between species seen here. With this knowledge of how bacteria can sense their respective
655 secreted factors, new therapeutic strategies targeting this system may provide the opportunity to break
656 communication between species and prevent these detrimental interactions thereby eliminating infections and
657 consequently improve patient outcomes.

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional Information
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 wildtype (WT)	PMID: 7604262	SMC232 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 Δ pilA	PMID: 20233936	SMC3782 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 Δ pilJ	PMID: 18178737	SMC2992 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 pilJ _{ΔLBD1-2}	This study	PADHL496	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 pilJ _{Δ80-273}	This study	PADHL497	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 pilJ _{Q412A, E413A}	This study	PADHL498	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 pilJ _{Q623A, Q624A}	This study	PADHL559	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 pilJ _{Q639A, E640A}	This study	PADHL560	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 Δ pilK		SMC8426 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 chpA::Tn		PA14 Ordered Transposon Library	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 Δ chpB	PMID: 17337585	SMC2990 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 Δ chpC	This study	PADHL445	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 Δ chpD	This study	PADHL459	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 Δ chpE	This study	PADHL460	See Methods and Materials

(<i>Pseudomonas aeruginosa</i>)				
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>pilL</i> ::Tn		PA14 Ordered Transposon Library	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔpilG</i>		SMC4375 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔpilH</i>		SMC4376 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>pilB</i> ::Tn		PA14 Ordered Transposon Library	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔpilT</i>	PMID: 36073942	SMC7302 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔpilU</i>	PMID: 36073942	SMC7304 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔfimX</i>		SMC6290 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔpilZ</i>	PMID: 27114465	SMC5569 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔfimV</i>		SMC6726 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔflgK</i>	PMID: 9791175	SMC5845 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔpilJ ΔflgK</i>	This study	PADHL266	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>pilJ_{ΔLBD1-2} ΔflgK</i>	This study	PADHL514	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>pilJ_{Q412A, E413A} ΔflgK</i>	This study	PADHL515	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔpilK ΔflgK</i>	This study	PADHL392	See Methods and Materials

Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 $\Delta chpB \Delta flgK$	This study	PADHL393	See Methods and Materials
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 WT + pMQ80- P_{araBAD} - <i>pilJ</i>		SMC6793 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 $\Delta pilJ$ + pMQ80- P_{araBAD} - <i>pilJ</i>		SMC6790 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>pilJ_{ALBD1-2}</i> + pMQ80- P_{araBAD} - <i>pilJ</i>	This study	PADHL549	See Methods and Materials; Plasmid obtained from George O'Toole (Dartmouth)
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>pilJ_{Q412A, E413A}</i> + pMQ80- P_{araBAD} - <i>pilJ</i>	This study	PADHL551	See Methods and Materials; Plasmid obtained from George O'Toole (Dartmouth)
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 WT + pMQ80- P_{araBAD} - <i>pilK</i>		SMC6794 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 $\Delta pilK$ + pMQ80- P_{araBAD} - <i>pilK</i>	This study	PADHL537	See Methods and Materials; Plasmid obtained from George O'Toole (Dartmouth)
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 WT + pMQ80- P_{araBAD} - <i>chpB</i>		SMC6795 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 $\Delta chpB$ + pMQ80- P_{araBAD} - <i>chpB</i>		SMC6791 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PAO1 WT + pPxhpA-yfp <i>PrpoD-mKate2</i>	PMID: 26041805	SMC7431 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 WT + pPxhpA-yfp <i>PrpoD-mKate2</i>		SMC7438 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 $\Delta pilJ$ + pPxhpA-yfp <i>PrpoD-mKate2</i>	This study	PADHL501	See Methods and Materials; Plasmid obtained from George

				O'Toole (Dartmouth)
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>pilJ</i> _{ALBD1-2} + pPxhpA-yfp <i>PrpoD-mKate2</i>	This study	PADHL530	See Methods and Materials; Plasmid obtained from George O'Toole (Dartmouth)
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>pilJ</i> _{Q412A, E413A} + pPxhpA-yfp <i>PrpoD-mKate2</i>	This study	PADHL531	See Methods and Materials; Plasmid obtained from George O'Toole (Dartmouth)
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔpilK</i> + pPxhpA-yfp <i>PrpoD-mKate2</i>	This study	PADHL508	See Methods and Materials; Plasmid obtained from George O'Toole (Dartmouth)
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔchpB</i> + pPxhpA-yfp <i>PrpoD-mKate2</i>	This study	PADHL509	See Methods and Materials; Plasmid obtained from George O'Toole (Dartmouth)
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔcyaB</i>	PMID: 25626906	SMC6852 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>ΔcpdA</i>	PMID: 25626906	SMC6851 (O'Toole Strain Collection)	
Strain, strain background (<i>Pseudomonas aeruginosa</i>)	PA14 <i>Δvfr</i>	PMID: 25626906	SMC6722 (O'Toole Strain Collection)	
Strain, strain background (<i>Staphylococcus aureus</i>)	USA300 LAC JE2 (WT)	PMID: 23404398	USA300 CA-Methicillin resistant strain LAC without plasmids	Obtained from Ambrose Cheung (Dartmouth)
Strain, strain background (<i>Escherichia coli</i>)	DH5 α	New England Biolabs	C2987H	
Strain, strain background (<i>Escherichia coli</i>)	S17 λ pir	Life Technologies		
Recombinant DNA reagent	pEXG2-Tc			Obtained from Timothy Yahr

				(University of Iowa)
Commercial assay or kit	Gibson Assembly Cloning Kit	New England Biolabs	E5510S	
Commercial assay or kit	QIAquick PCR Purification Kit	Qiagen	28104	
Commercial assay or kit	QIAquick Gel Purification Kit	Qiagen	28704	
Commercial assay or kit	Gentra Puregene Yeast/Bac Kit	Qiagen	Discontinued	
Commercial assay or kit	QIAprep Spin Miniprep Kit	Qiagen	27104	
Software, algorithm	NIS-Elements AR	Nikon	Version 5.20.01 64-bit	
Software, algorithm	ZEN	Zeiss	Version 2.6	
Software, algorithm	Fiji	ImageJ	Version 2.0.0	
Software, algorithm	GraphPad Prism	GraphPad Software	Version 9.3.1	
Software, algorithm	Python	Python Software Foundation	Version 3.10	

659
660
661

Table 1. Oligonucleotides used in this study.

Name	Sequence 5' – 3'	Reference
pilJ_upF-pEXG2-Tc	CTTCTGCAGGTCGACTCTAGATGACCAAGGACGCCGAGACC	This study oDHL198
pilJ_dnR-pEXG2-Tc	TAAGGTACCGAATTCGAGCTCCACATGCCAGCGAGTAGGGT	This study oDHL199
pilJ-LBD_upR	ATGCTACGCCGCCGGCAAAGTTGGCGAACAGCAA	This study oDHL225
pilJ-LBD_dnF	TCGCCAACTTGCCGGGGCGTAGCATCAACC	This study oDHL226
pilJ-Q412A-E413A_upR	GTTAGTTGATCGAGTCGGCGATGGC	This study oDHL246
pilJ-Q412A-E413A_dnF	CAGGCGCAGGAAATCGCCG	This study oDHL247
pilJ-Q412A-E413A-Gene Block	ACTCGATCAACTACTCCATCGACCAAGCTCCGCGAACTGGTGGAG ACCATCAACCAAGACCGCCGTGCAGGTGGCCGCAGCGGCCGCG GCAACCCAGTCCACCGCGATGCACCTGGCGAAGCCTCCGAGC ACCAGGCGCAGGAAAT	This study oDHL249
pilJ-Q623A-Q624A/Q639A-E640A_upR	CTCGATCTCCTCCAGGGCC	This study oDHL262
pilJ-Q623A-Q624A/Q639A-E640A_dnF	GCAGGCCTGAGCATAGGCG	This study oDHL263
pilJ-Q623A-Q624A-Gene Block	TGGAGGAGATCGAGAAGGTATCCAAGACCCCTCGCGGCCTGAT CCAGAACATCTCCAACGCCGCCGT GC GG C G CATCGTCGCC GCCACATTCCAACACCATGAACGTCATTCAAGGAGATCACCTC GCAGACCTCCGCCGTACCACGCCACCGCGCGGAGCATCGG CAACCTGGCGAAGATGGCGAGCGAGATGCGCAACTCGGTATCC GGCTCAAACGTGCCGGAGGGCGTGGAGCAGGCCTGAGCATAG GCG	This study oHDL259
pilJ-Q639A-E640A-Gene Block	TGGAGGAGATCGAGAAGGTATCCAAGACCCCTCGCGGCCTGAT CCAGAACATCTCCAACGCCGCCGT GC GG C G CATCACCTC GCAGACCTCCGCCGTACCACGCCACCGCGCGGAGCATCGG CAACCTGGCGAAGATGGCGAGCGAGATGCGCAACTCGGTATCC	This study oHDL260

	GGCTTCAAACTGCCGGAGGGCGTGGAGCAGGCCTGAGCATA GCG	
pilJ-80-273_upR	GCCGCTTCCCGGACGAACGCCTGCCCTGCC	This study oDHL227
pilJ-80-273_dnF	AGGGCGAGGCCTCGTCCGGGAAGCGGCGAAC	This study oDHL228

662

663 **Methods and Materials**

664

665 **Bacterial strains and culture conditions:** *P. aeruginosa* PA14 or PAO1 and *S. aureus* JE2 strains were cultured
666 in tryptic soy broth (TSB; Becton Dickenson) or M8 minimal media broth supplemented with 0.2% glucose and 1.2%
667 tryptone (M8T) with aeration at 37°C. The following antibiotics were added for *P. aeruginosa* cultures when
668 appropriate: carbenicillin (200 µg/mL), gentamicin (30 µg/mL), tetracycline (100 µg/mL). *Escherichia coli* strains for
669 cloning were cultured in lysogeny broth (LB; 1% tryptone, 0.5% yeast extract, 1% sodium chloride). The following
670 antibiotics were added for *E. coli* when appropriate: ampicillin (100 µg/mL), gentamicin (15 µg/mL), tetracycline (12
671 µg/mL). All strains used in this study can be found in the Key Resources Table.

672

673 **Generating *P. aeruginosa* mutants:** *P. aeruginosa* mutants in this study were generated by allelic exchange at
674 the native site in the chromosome using Gibson Assembly with a pEXG2-Tc vector containing the DNA mutation
675 between restriction sites SacI and XbaI (Hmelo et al., 2015). Mutant constructs were generated by PCR amplifying
676 ~1 kb DNA fragments upstream and downstream of the gene or region of interest while substitution mutants were
677 generated by synthesis of a DNA fragment gene block containing the correct codon change (Integrated DNA
678 Technologies, Coralville, IA, USA). Assembled vectors were transformed into *E. coli* DH5 α , then into *E. coli* S17 for
679 conjugation into *P. aeruginosa*. Correct mutations in *P. aeruginosa* were verified with PCR and Sanger sequencing.

680 *P. aeruginosa* Δ pilK, Δ chpB, Δ pilJ deletion mutants and pilJ LBDs and methylation site mutants were complemented
681 by electroporating the respective mutant with expression vector pMQ80 containing the full-length gene under control
682 of the arabinose-inducible P_{BAD} promoter and fused to a C-terminal GFP tag(Shanks et al., 2006). All
683 oligonucleotides used to generate *P. aeruginosa* mutants can be found in Table 1.

684

685 **Macroscopic coculture twitching chemotaxis assay:** Motility experiments were performed as previously
686 described (Kearns et al., 2001; Limoli et al., 2019; Miller et al., 2008). Buffered agar plates (recipe: 10 mM Tris, pH
687 7.6; 8 mM MgSO₄; 1 mM NaPO₄, pH 7.6; and 1.5% agar) were poured and allowed to solidify for 1 hour prior to
688 incubation for 16 hours at 37°C and 22% humidity. After solidifying, 4 µL of either growth medium (TSB) or cell-free
689 supernatant derived from an overnight culture of *S. aureus* at OD₆₀₀ 5.0 and filter sterilized with a 0.22 µm filter were
690 spotted on the surface of the plate and allowed to diffuse for 24 hours at 37°C and 22% humidity to establish a
691 gradient. *P. aeruginosa* cultures were incubated overnight in TSB with aeration at 37°C, subcultured 1:100 in TSB
692 the following morning, then standardized to OD₆₀₀ 12.0 in 100 µL of 1 mM MOPS buffer supplemented with 8 mM
693 MgSO₄ prior to inoculating 1 µL on the surface of the plate at five mm from the center of the gradient. Plates were
694 incubated in a single layer, agar-side down, for 24 hours at 37°C with 22% humidity, followed by an additional 16
695 hours at room temperature prior to imaging the motility response of *P. aeruginosa*. Images were captured using a
696 Zeiss stereoscope with Zeiss Axiocam 506 camera and directional motility ratios were calculated in Fiji before
697 graphing and performing statistical analysis in GraphPad Prism.

698

699 **Macroscopic coculture subsurface twitching assay:** The assay was modified from a previous protocol, as shown
700 before (Limoli et al., 2019; Turnbull & Whitchurch, 2014). Prior to pouring plates, 200 µL of either growth medium
701 (TSB) or cell-free supernatant derived from an overnight culture of *S. aureus* at OD₆₀₀ 2.0 and filter sterilized with a

702 0.22 μm filter were spread on the bottom of a 120 mm square petri plate. Tryptic soy agar (1.5%) was then poured
703 into the plates and allowed to dry for 4.5 hours at room temperature. *P. aeruginosa* cultures were incubated
704 overnight in TSB with aeration at 37°C, subcultured 1:100 in TSB the following morning, then standardized to OD₆₀₀
705 2.0 in 1 mL TSB. A sterile toothpick was dipped into the standardized culture, then stabbed to the bottom of the
706 agar plate. Plates were incubated in a single-layer, agar-side down, at 37°C with 22% humidity for 24 hours, followed
707 by an additional 24 hours at room temperature. Following incubation, the agar was removed from the plates and
708 the motility diameters were measured in mm. Diameter measurements were graphed and analyzed in GraphPad
709 Prism.
710

711 **Live-imaging and tracking of *P. aeruginosa* directional response to *S. aureus*:** To visualize single-cell motility
712 behavior and measure cAMP in individual cells, *P. aeruginosa* cells were imaged using a previously described
713 method (Limoli et al., 2019; Yarrington et al., 2020). *P. aeruginosa* and *S. aureus* were grown in M8T medium
714 overnight at 37°C with aeration, subcultured the next day in fresh M8T and grown to mid-log phase at 37°C with
715 aeration. Cultures were standardized to OD₆₀₀ of 0.015 to 0.03 for *P. aeruginosa* or 0.05 to 0.1 for *S. aureus*. For
716 coculture experiments, *P. aeruginosa* and *S. aureus* were mixed 1:1. One μL of mono- or coculture cells were
717 inoculated onto a 10 mm diameter glass coverslip in a 35 mm dish before placing an agarose pad on top. Pads
718 were made by pipetting 920 μL of M8T with 2% molten agarose into a 35 mm dish containing a 10 mm diameter
719 mold and drying uncovered for 1 hour at room temperature, followed by 1 hour 15 minutes at room temperature
720 covered with a lid, then 1 hour at 37°C before transferring the pad onto the inoculated coverslip. Time-lapse imaging
721 was performed with an inverted Nikon Ti2 Eclipse microscope, 100x oil objective (1.45 NA), and Andor Sona
722 camera. Phase contrast images were acquired every 20 minutes for 2 hours, then every 1 second for 3-4 hours
723 with 100 ms exposure and 20% DIA LED light. Fluorescent images were acquired every 20 minutes with TxRed
724 images taken at 100 ms exposure and 20% Sola fluorescent light and YFP imaged at 25 ms exposure and 20%
725 Sola fluorescent light.
726

727 Images were analyzed using Nikon NIS-Elements AR software. To automatically track movements of single cells,
728 bacterial cells in the phase channel were first converted into binary objects by thresholding to the dark bacterial
729 cells. The Tracking Module in NIS-Elements was then used to form trajectories for all binary objects. Specifically,
730 the following parameters were used: objects have a minimum area of 1 μm^2 , track with random motion model, find
731 center of object based on area, no maximum speed limit of each object track, allow new tracks after the first frame
732 in file and track objects backwards to previous frames. Tracks were allowed up to 60 gaps (frames) per track and
733 any tracks with less than 180 frames were automatically removed from the final trajectories. Trajectories were
734 exported and analyzed in Python using the parameters described below.
735

736 **Principal direction of single-cell trajectories:** The gyration tensor, commonly employed in polymer physics
737 determines the principal direction of each single-cell trajectory *i* (Kim & Baig, 2016). The shape of each random
738 walk is described by the gyration tensor

$$739 \mathbf{G}_i = \frac{1}{N} \sum_t^N (\vec{r}(t) - \vec{r}_i^{cm}) \otimes (\vec{r}(t) - \vec{r}_i^{cm}),$$

740

(1)

741 for a trajectory of N time steps each at position $\vec{r}(t)$ at time t and center of mass position \vec{r}_i^{cm} . Eigendecomposition
742 of the gyration tensor determines the largest eigenvalue and the associated eigenvector \hat{e}_i^{\parallel} is identified as the
743 principal direction of motion for the i^{th} trajectory. The sign of \hat{e}_i^{\parallel} is chosen by setting the direction of motion parallel
744 to the end-to-end vector. The principal direction is determined without any information about the location of *S.*
745 *aureus* colonies or other *P. aeruginosa* cells. Rose graphs shown in Figures 2-4 present histograms of \hat{e}_i^{\parallel} .

746

747 **Directed Mean-Squared Displacement:** The mean-squared displacement (MSD)

748

$$\Delta r^2(\Delta t) = \langle \Delta \vec{r}(\Delta t; t) \cdot \Delta \vec{r}(\Delta t; t) \rangle$$

749

(2)

750 of displacement vectors $\Delta \vec{r}(\Delta t; t) = \vec{r}(t + \Delta t) - \vec{r}(t)$ is a measure of the distance gone after a lag time Δt , where
751 the average $\langle \cdot \rangle$ is the ensemble average over all trajectories i and all times t within that trajectory. While the MSD
752 quantifies the degree of motion, it assumes isotropic dynamics and so does not discern between any potential
753 directionality in microbial motion. Quantifying the degree of motion parallel and perpendicular to the principal
754 direction requires decomposing the movement into components parallel and perpendicular to each principal
755 direction \hat{e}_i^{\parallel} . The displacement in the principal direction is $\Delta \vec{r}_{\parallel} = (\Delta \vec{r} \cdot \hat{e}_i^{\parallel}) \hat{e}_i^{\parallel}$ and the orthogonal direction is $\Delta \vec{r}_{\perp} =$
756 $\Delta \vec{r} - \Delta \vec{r}_{\parallel}$. From these, the parallel and perpendicular MSD are

757

$$\Delta r_{\parallel}^2(\Delta t) = \langle \Delta \vec{r}_{\parallel} \cdot \Delta \vec{r}_{\parallel} \rangle$$

758

$$\Delta r_{\perp}^2(\Delta t) = \langle \Delta \vec{r}_{\perp} \cdot \Delta \vec{r}_{\perp} \rangle.$$

759

(3)

760 To characterize the MSDs, the anomalous exponent α defined by the power law

761

$$\Delta r^2(\Delta t) \sim \Delta t^{\alpha}.$$

762

(4)

763 Exponents of $\alpha = 1$ describe diffusive dynamics, while $\alpha < 1$ represents subdiffusive motion and $\alpha > 1$
764 superdiffusive.

765

766 **Displacement probability distributions:** The MSD can be deceiving since it is known that a broad class of
767 diffusive dynamics exist in soft matter, biological, and complex systems for which the dynamics are “Brownian yet
768 non-Gaussian” (Chechkin et al., 2017; Metzler, 2020). In such systems, the MSD appears diffusive with anomalous
769 exponent $\alpha = 1$ but the probability density function (PDF) of steps $P(\Delta r; \Delta t)$ is non-Gaussian, a traditional
770 assumption for Brownian motion. The probability of finding a bacterium Δr after some lag time Δt is called the van
771 Hove self-correlation function and it has proven useful in understanding the dynamics of simulations of twitching
772 bacteria (Nagel et al., 2020). The formal definition of the van Hove self-correlation function is

773

$$P(\Delta\vec{r}; \Delta t) = \frac{1}{N} \left\langle \sum_{j=1}^N \delta(\Delta\vec{r} - [\vec{r}(t + \Delta t) - \vec{r}(t)]) \right\rangle. \quad (5)$$

774

775 To consider the probability that *P. aeruginosa* cells move a given distance towards *S. aureus* colonies, the van
776 Hove function is found for steps parallel or orthogonal to the principal direction of motion, $\Delta\vec{r}_{\parallel}$ and $\Delta\vec{r}_{\perp}$ respectively.
777 There are two probability density functions of particular interest here.

778

779 (i) The first is a Gaussian diffusive distribution

780

$$P_G(\Delta r; \Delta t) = \frac{1}{(4\pi D \Delta t)^{1/2}} \exp\left(-\frac{\Delta r^2}{4D \Delta t}\right), \quad (6)$$

781

782 for a random process with diffusion coefficient D . This form can be scaled in time to collapse the distribution to
783 $\tilde{P}_G(\Delta r) \sim e^{-\Delta\tilde{r}^2}$, where $\tilde{P}_G = \Delta t^{1/2} P_G$ and $\Delta\tilde{r} = \Delta t^{-1/2} \Delta r$. Thus, observing a Gaussian distribution at only one lag
784 time is insufficient for determining Fickian diffusivity. An example of this is the microscopy imaging noise (Figure 2
785 – figure supplement 4A): although the step size distributions are Gaussian, they do not scale in time for lag times
786 $\Delta t < 300s$, a distinct indication that this is a measure of the tracking noise and not diffusion of the dust particle.

787

788 (ii) The second probability density functions of interest is a Laplace distribution

789

$$P_L(\Delta r; \Delta t) = \frac{1}{2\lambda} \exp\left(-\frac{|\Delta r|}{\lambda}\right), \quad (7)$$

790

791 for a decay length λ . Laplace distributions have longer tails than Gaussian distributions and have emerged as a
792 canonical example of non-Gaussian functions that lead to Brownian MSDs (Chechkin et al., 2017; Metzler, 2020).
793 If the decay length scales with lag time as $\lambda = (\langle D \rangle \Delta t)^{1/2}$ for an average diffusivity $\langle D \rangle$ then the MSD scales
794 diffusively. In this case, the distributions can be collapsed with the same scaling as the Gaussian distributions,
795 $\tilde{P}_L(\Delta r) \sim e^{-|\Delta\tilde{r}|}$, where $\tilde{P}_L = \Delta t^{1/2} P_L$ and $\Delta\tilde{r} = \Delta t^{-1/2} \Delta r$.

796

797 The probability density distributions of step sizes are typically dominated by a sharp narrow peak of highly-likely
798 small step sizes, which represents jiggling and long tails of rare-but-large step sizes (Figures 2-4) (Kühn et al.,
799 2021). The long tails are primarily exponential. It is tempting to think that the narrow peak of small steps sizes
800 represents the imaging uncertainty. However, we find that the standard deviation of the imaging distribution is
801 $1.4 \times 10^{-2} \mu\text{m}$ (Figure 2 – figure supplement 4A), narrower than the width of the primary van Hove peak (Figure 2 –
802 figure supplement 4B). Indeed, the narrow peak is not Gaussian at all, but rather better fit by a second Laplace
803 distribution. Thus, the displacement probability density distributions are well described as double Laplace functions
804 (Figure 2 – figure supplement 4B).

805

806 **Identifying subpopulations of persistent movers and resters:** Qualitative assessment of the microscopy data
807 makes it apparent that *P. aeruginosa* cells possess two dynamic modes:

- 808 1. Persistent “Resters”: Colony-associate *P. aeruginosa* cells do not exhibit an active exploratory motion.
809 Instead, the motion of these cells is composed of small ‘jiggling’ and expansion due to colony growth.
- 810 2. Persistent “Movers”: These are cells that have left the colony to actively move through the surroundings,
811 either as individuals or in multi-cell rafts. Like “resters”, these “movers” exhibit small jittering motion but also
812 intermittently persistent motion. The intermittency of the motion can at times have a run-reversal-type or a
813 run-rest-type character, but a “mover” is not simply a continually moving bacterium.

814 Both of these dynamic modes are composed of small jittery motion and larger motions, which makes it difficult to
815 algorithmically separate the cells into subpopulations of movers and resters.

816

817 To disentangle the subpopulations, we consider the velocity-velocity correlation function $C_i^{vv}(\Delta t)$ for lagtime Δt for
818 each bacterium i averaged over start times. The velocity autocorrelation function takes into account both the
819 direction and speed of the bacteria. Rather than averaging the correlation function over all possible start times within
820 trajectory i , we employ a rolling velocity autocorrelation

$$821 C_i^{vv}(\Delta t; t) = |\langle \vec{v}(t + \tau) \cdot \vec{v}(t + \tau + \Delta t) \rangle_{\tau \leq T}|, \quad (8)$$

822

823 where t is each time point in the trajectory, T is the rolling window duration, τ is every possible starting time within
824 the rolling window and the average $\langle \cdot \rangle_{\tau \leq T}$ is over all starting times. Since run-reversal dynamics are likely to be an
825 aspect of the dynamics, we consider the absolute value of the velocity autocorrelation. If the duration T is too short
826 then the correlation functions are overly noisy but if it is too long then instances of correlated motion is smeared
827 out. To assess the immediate degree of correlation in motion, the correlation function is averaged over the duration
828 to produce a correlation constant $c_{vv}^T(t) = \langle C_i^{vv}(\Delta t; t) \rangle_{\Delta t \leq T}$. The correlation constant acts as a signal of immediately
829 persistent motion, with persistent resters showing near-zero $c_{vv}^T(t)$ and movers having significantly larger values
830 above a cutoff c^* . While $c_{vv}^T(t)$ matches our expectations from qualitative observations of the microscopy movies,
831 false positive instances of colony-associated cells occur. Thus, the signal is weighted by the behavior of neighboring
832 cells

$$833 s(t; T, c^*, R) = \langle \Theta(c_{vv}^T(t) - c^*) \rangle_{r \leq R}, \quad (9)$$

834

835 where $\Theta(\cdot)$ is the Heaviside step function and the average is over all neighbouring cells in the vicinity of $r \leq R$.
836 Finally, the neighbor-weighted signal s is given a cutoff s^* , above which cells are identified as “persistent movers”
837 and below which they are “persistent resters.” The parameters are chosen to be $T = 30s$, $c^* = 0.01$, $R = 6\mu\text{m}$ and
838 $s^* = 0.75$ for this study. Due to the intermittent nature of the twitching dynamics, once a bacterium has been
839 identified as a mover, it keeps a mover-designation until the trajectory is lost.

840

841 **Quantification of intracellular cAMP:** To quantify cAMP in individual cells, time-lapse imaging was performed with
842 *P. aeruginosa* cells carrying the *PxphA-yfp* *PrpoD-mKate2* dual fluorescent reporter(Persat et al., 2015).

843 Thresholding of bacterial cells in the red channel to the constitutively expressed *PrpoD-mKate2* fluorescence
844 generated binary objects. The fluorescence of these binaries was then measured in the YFP channel for levels of
845 *PxphA-yfp* expression. For total cAMP in a frame at a given time point, the ratio of YFP over mKate2 intensity for
846 each bacterial cell was calculated, then summed with all other bacterial cells. For normalization, the total
847 YFP/mKate2 ratio from all objects in the frame was normalized to the average area of all binary objects in the same
848 frame. The ratios for each time point were then graphed and analyzed in GraphPad Prism.
849

850 **Acknowledgements:** This work was supported by funding from the CFF Postdoc-to-Faculty Transition Award
851 (LIMOLI18F5), CFF RDP Junior Faculty Recruitment Award (LIMOLI19R3), NIH (R35GM142760), CFF Student
852 Traineeship Award (YARRIN21H0), and the European Research Council (ERC) under the European Union's
853 Horizon 2020 research and innovation programme (Grant agreement No. 851196). We thank Drs. George O'Toole
854 and Sherry Kuchma for *P. aeruginosa* and *E. coli* bacterial strains. We also thank Dr. J. Muse Davis for use of the
855 stereoscope and Dr. Timothy Yahr for the pEXG2-Tc cloning vector. We are grateful to George O'Toole and
856 members of the Limoli Lab for thoughtful discussions and feedback on the manuscript.

857
858 **Competing interests:** The authors have no competing interests to declare.

859

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