

1 **Non-motile subpopulations of *Pseudomonas aeruginosa* repress flagellar motility**
2 **in motile cells through a type IV pili- and Pel-dependent mechanism**

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15 Running Head: Non-motile *P. aeruginosa* represses flagellar swarming motility

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23 microbe interaction

24 **Abstract**

25 The downregulation of *P. aeruginosa* flagellar motility is a key event in biofilm formation,
26 host-colonization, and the formation of microbial communities, but the external factors
27 that repress motility are not well understood. Here, we report that under swarming
28 conditions, motility can be repressed by cells that are non-motile due to the absence of
29 a flagellum or flagellar rotation. Non-motile cells, due to mutations that prevent either
30 flagellum biosynthesis or rotation, present at 5% of the total population suppressed
31 swarming of wild-type cells under the conditions tested in this study. Non-motile cells
32 required functional type IV pili and the ability to produce the Pel exopolysaccharide to
33 suppress swarming by the motile wild type. In contrast, motile cells required only type IV
34 pili, but not Pel production, in order for swarming to be repressed by non-motile cells.
35 We hypothesize that interactions between motile and non-motile cells may enhance the
36 formation of sessile communities including those involving multiple genotypes,
37 phenotypically-diverse cells, and perhaps other species.

38

39 **Significance**

40 Our study shows that, under the conditions tested, a small population of non-swarming
41 cells can impact the motility behavior of the larger population. The interactions that lead
42 to the suppression of swarming motility require type IV pili and a secreted
43 polysaccharide, two factors with known roles in biofilm formation. These data suggest
44 that interactions between motile and non-motile cells may enhance the transition to
45 sessile growth in populations and promote interactions between cells with different
46 genotypes.

47 **Introduction**

48 Microbial localization through processes other than cell-division are critical for the
49 formation of spatially structured populations and communities. Thus, motility and its
50 regulation by diverse chemical and physical stimuli are major drivers of intraspecies and
51 interspecies microbial interactions (1, 2). Previously, we found that exogenous ethanol,
52 a common product secreted by many microbes, markedly reduced *Pseudomonas*
53 *aeruginosa* swimming and swarming at the population level, but only decreased the
54 motile fraction of cells in the population from an average of 38% to 22% (3). Prompted
55 by this finding, we sought to determine the impact of a subpopulation of non-motile cells
56 on the motility of the larger motile *P. aeruginosa* population.

57 During *P. aeruginosa* biofilm formation, motile cells are recruited to microcolonies
58 of sessile cells via a combination of processes driving colocalization, and these
59 processes may differ between strains with distinct early-biofilm forming strategies (4, 5)
60 or in response to different experimental conditions. For *P. aeruginosa* strain PA14,
61 retractile type IV pili (T4P) participate in surface sensing, which results in the
62 upregulation of cAMP and the subsequent induction of cyclic-di-GMP (6-8); flagellar
63 motility is then down-regulated in order to facilitate surface attachment (6, 7, 9, 10)
64 followed by Pel exopolysaccharide matrix production (11), which can connect cells to
65 one another (10, 12). Both matrix production and T4P function have been shown to
66 participate in motility repression (10, 12-14) suggesting that matrix materials may
67 mediate physical interactions between neighboring cells. T4P also mediate cell-cell
68 interactions in swarms (15).

69 In this study, we show that non-motile cells when present at 5 to 75% of the total
70 inoculum resulted in repression of swarming by WT motile cells. To repress motility,
71 non-motile cells required the ability to produce Pel matrix, and both motile and non-
72 motile cells required functional T4P for this interaction. These data have implications for
73 factors that contribute to population-level behaviors and intra- and inter-species
74 interactions. Interactions that promote cell-cell interactions may be relevant in situations
75 where non-motile and motile cells can be found together such as in cystic fibrosis (CF)-
76 associated lung infections (16-18), wounds (19), and ear infections (17).

77

78 **Results**

79 **The presence of non-motile cells represses swarming motility by *P. aeruginosa***
80 **strain PA14.** To explore the effects of a subpopulation of non-motile cells on swarming
81 motility, mixes containing different proportions of motile *P. aeruginosa* strain PA14 wild-
82 type (WT) cells and non-motile $\Delta flgK$ mutant cells (which lack the flagellar hook protein)
83 were plated on 0.5% M8 agar, which supports swarming motility by the WT strain. The
84 percent of $\Delta flgK$ mutant cells ranged from 5% to 75% in these mixes, and single strains
85 were included as controls. As expected, cultures with 100% WT swarmed readily while
86 cultures with 100% $\Delta flgK$ showed no swarming (**Fig. 1A**). In agreement with published
87 literature, the addition of non-motile $\Delta flgK$ blocked WT swarming (20), although, we
88 found that swarming motility was completely repressed by the addition of non-motile
89 cells at 5% to 75% of the total population under the conditions tested (**Fig. 1A and Fig.**
90 **1C**).

91 To determine if the lack of swarming in WT: Δ *flgK* co-cultures was due to faster
92 growth of the non-motile cells compared to the WT, we competed the WT or Δ *flgK*
93 mutant against a WT strain tagged with a *lacZ* reporter at different ratios atop a 0.22 μ m
94 filter placed on 0.5% M8 agar. After 16 h, the colony was disrupted and colony forming
95 units (CFUs) for each strain were enumerated on blue/white screening plates that
96 contained X-Gal. At all ratios tested, neither untagged WT nor the Δ *flgK* mutant could
97 outgrow the *lacZ*-expressing WT strain (**Fig. 1B**), indicating that the lack of swarming
98 observed in WT: Δ *flgK* mixes was not due to increased growth of non-motile cells and
99 thus overgrowth of the population. Rather, our data suggest that a subpopulation of
100 non-motile cells inhibits motility of the larger flagellated WT population.

101 Using conditions in which the non-motile strain comprised 25%, 50%, or 75% of
102 the population, we also found that, like the Δ *flgK* mutant, the Δ *fliC* mutant, another non-
103 motile strain that lacks the flagellin protein used to make the flagellar filament, inhibited
104 WT motility at all ratios tested (**Fig. 1C**). Similarly, the Δ *motABCD* mutant, which
105 produces a flagellum that is unable to rotate due to the absence of stators, repressed
106 motility of the WT; although, in comparison to Δ *flgK* and Δ *fliC*, the Δ *motABCD* mutant
107 was slightly less effective at repressing WT swarming (**Fig. 1C**). Together, these data
108 suggest that the absence of flagellar motility in a subpopulation of cells is sufficient to
109 suppress WT swarming in co-culture.

110

111 **T4P are required for non-motile cells to affect swarming by motile cells.** Several
112 studies have shown that *P. aeruginosa* strains that are deficient in T4P tend to have a
113 hyper-swarming phenotype (21-23). Additionally, *P. aeruginosa* cells have been shown

114 to interact via their T4P to form close cell associations that facilitate directional
115 swarming (15). Consistent with published results (4, 24, 25), the $\Delta flgK$ mutant has
116 functional T4P as evidenced by the formation of a large twitching motility zone using the
117 agar-plastic interface assay (**Fig. S1A** and **Fig. S2**). While both the WT and $\Delta flgK$
118 formed twitch zones that were significantly larger than those formed by the T4P-null
119 $\Delta pilA$ mutant, the $\Delta flgK$ twitch zone was ~25% smaller than that formed by the WT
120 ($p < 0.05$; **Fig. S2A**). Using $\Delta flgK$ and $\Delta flgK pilA$ strains, we found that *pilA*, which
121 encodes the major pilin component of T4P, was required for the $\Delta flgK$ mutant to
122 suppress WT swarming on 0.5% M8 agar (**Fig. 2A**). In a similar assay using 0.3% M63
123 agar, which supports both flagellum-mediated swimming and swarming, we found that
124 inoculated spots of $\Delta flgK$ cells (**Fig. 2B, red dots**) decreased the local expansion of the
125 motile WT population, while spots of the $\Delta flgK pilA$ mutant (**Fig. 2B, purple dots**) did
126 not. In contrast to the effects of non-motile $\Delta flgK$ cells on flagellum-mediated motility,
127 the $\Delta flgK$ mutant did not alter T4P-dependent twitching motility of the WT in a 50:50
128 ratio when compared to the WT alone (**Fig. S2B**). Therefore, the data show that T4P
129 are required for non-motile cells to inhibit flagellum-mediated motility of WT cells in co-
130 culture.

131

132 **Functional T4P in motile cells are required for non-motile cells to repress**
133 **swarming in co-culture.** With the observation that T4P are required for non-motile
134 cells to suppress flagellum-mediated swarming motility by the WT, we determined if this
135 interaction also required T4P function for the motile cells. We analyzed the ability of
136 $\Delta flgK$ to repress swarming when co-cultured with the following mutants: $\Delta pilA$, which

137 lacks T4P; $\Delta pilMNOP$, which lacks the alignment complex required for T4P function (26)
138 and cyclic-di-GMP signaling (27); and both $\Delta pilT$ and $\Delta pilU$, which lack either of the
139 ATPases that mediate T4P retraction (28). Strains lacking *pilA*, *pilMNOP*, *pilT*, and *pilU*
140 were all defective for twitching motility (**Fig. S1** and **Fig. S2**).

141 The swarming motility of strains lacking *pilA* or *pilMNOP* (both of which are
142 twitching defective) or *pilT* (no T4P retraction) was no longer inhibited by the $\Delta flgK$
143 mutant in co-culture (**Fig. 3** and **Fig. S3**). Interestingly, the swarming motility of the
144 $\Delta pilU$ mutant was still inhibited by the $\Delta flgK$ mutant in co-culture (**Fig. 3**). While PilT is
145 the main retractile ATPase, PilU has an accessory role in retraction; the $\Delta pilT$ mutant
146 completely lacks pilus retraction (29, 30), while the $\Delta pilU$ mutant has reduced retraction
147 but still retains some function (30). These differences were confirmed by the fact that
148 the WT and $\Delta pilU$ were sensitive to phage DMS3 infection, but the $\Delta pilA$ and $\Delta pilT$
149 mutants are resistant to infection (**Fig. S1B**). Together, these data indicate that motile
150 cells likely need to have functional T4P in order for the non-motile subpopulation to
151 inhibit the swarming motility of the WT strain when grown in co-culture.

152 *P. aeruginosa* T4P participate in a cAMP-dependent signaling pathway (see **Fig.**
153 **S4A** for pathway) that involves FimS, PilJ, CyaAB adenylate cyclases, and the cAMP-
154 binding transcription factor Vfr. We found that this pathway was not required for the
155 repression of WT motility by the non-motile $\Delta flgK$ mutant (**Fig. S4B**) using published
156 mutants lacking components of the cAMP signaling pathway ($\Delta pilJ$, $\Delta fimS$, $\Delta cyaAB$, and
157 Δvfr) that retain expression of functional T4P as evidenced by their sensitivity to phage
158 DMS3 (**Fig. S4C**). Taken together, the data show that motile WT cells require functional

159 T4P, but not the cAMP-dependent surface-sensing pathway, in order for non-motile
160 cells to be able to repress swarming in co-culture.

161

162 **Non-motile *P. aeruginosa* requires Pel matrix production to repress swarming**
163 **motility in the motile population.** We next explored whether Pel matrix production
164 played a role in swarming repression. We found that swarming by the $\Delta pelA$ mutant,
165 which is a mutant capable of robust swarming but lacks Pel production, was repressed
166 by $\Delta flgK$ in co-culture like the WT (**Fig. 4**). In contrast, $\Delta flgKpelA$ did not repress WT
167 swarming (**Fig. 4A**). Interestingly, both $\Delta flgK$ and $\Delta fliC$, which repress WT swarming
168 (**Fig. 1C**), had increased Pel production (**Fig. 4B** and **Fig. S5**). Together, these data
169 show that the non-motile strain needs to produce Pel matrix to repress swarming
170 motility, but the motile strain does not. Additionally, while T4P were required by the
171 $\Delta flgK$ mutant to suppress swarm motility, T4P were not required for the observed
172 increase in Pel production in the $\Delta flgK$ mutant background as evidenced by Congo Red
173 binding (**Fig. 4B**).

174

175 **DISCUSSION**

176 Based on the data presented here we propose a model (**Fig. 5**) whereby a
177 subpopulation of non-motile cells in a population limit swarming motility of the entire
178 population. Our analyses revealed that non-motile cells required T4P and Pel
179 polysaccharide to inhibit the larger swarming population, and that motile cells required
180 functional T4P but not the Vfr/cAMP signaling system to respond to the non-motile
181 population. These findings in regards to T4P and Pel are consistent with the recruitment

182 of cells to microcolonies during biofilm development and aggregate formation, which
183 has been reported in numerous contexts (e.g. (4)).

184 The involvement of T4P in swarming cells was previously reported by Anyan et
185 al. (15) who reported that T4P impact cell-cell interactions in swarms in ways that limit
186 the movement of cells away from the population front. T4P mutants have been shown to
187 have increased swarming motility, suggesting that the intercellular T4P interactions that
188 we report between motile and non-motile genotypes may also be occurring in single-
189 strain swarms in which all cells have the potential for flagellar motility (22, 23). Future
190 studies will determine if T4P play direct roles in cell-cell interactions or indirect roles in
191 structuring the community.

192 The WT and motile $\Delta pelA$ mutant responded similarly to non-motile cells while
193 $\Delta flgKpelA$ was no longer able to repress motility suggesting that not all cells need to
194 produce exopolysaccharide matrix to repress swarming. Our findings that Pel
195 production is only necessary in the non-motile subpopulation is supported by published
196 data showing that ethanol not only decreases *P. aeruginosa* flagellar motility (3), but
197 increases Pel production (31). Pel has been suggested to repress motility via the steric
198 hinderance of flagella (3, 32), but further studies are needed to test this hypothesis. The
199 involvement of Pel in the interaction between motile and non-motile cells is particularly
200 interesting in light of recent work by Whitfield et al. (33) which found that the Pel
201 biosynthetic locus is widespread across Gram-positive and Gram-negative bacteria.
202 Future studies will determine if Pel is specifically detected by a T4P-dependent
203 mechanism, or if other matrix materials play a similar role.

204 Boyle et al. (20) previously reported that $\Delta flgK$ was capable of repressing
205 swarming by WT strain PA14 when $\Delta flgK$ was added 5:1 or at 80% of the population,
206 while we show that $\Delta flgK$ at 5% of the population represses WT swarming. Our
207 previously published findings (3) also showed that while strain PA14 was capable of
208 swarming, an average of 38% of flagellated cells were motile at any given time.
209 Furthermore, we showed that ethanol strongly repressed *P. aeruginosa* swarming
210 motility by reducing the motile fraction of the population from 38% to 22% (3). Overall,
211 the data show that motility of individual flagellated WT cells is heterogeneous within a
212 population, but the addition of non-flagellated cells represses population-wide motility.
213 These findings suggest that the number of non-flagellated cells necessary to repress
214 WT motility may vary due to experimental conditions.

215 In the studies here, we seed a low percentage of non-swarming cells into a
216 swarming population, which is reminiscent of the situations where there are genetically
217 heterogeneous populations observed in the CF airway or other chronic infections (18,
218 34, 35) as well as during normal environmental growth (35, 36). Additionally, clinical *P.*
219 *aeruginosa* isolates from the CF lung have diverse motility phenotypes (18). This
220 diversity in motility is even observed within a single patient (18). We hypothesize that
221 such a mechanism promotes interspecies and intraspecies interactions. The strong
222 implication of these findings is that in a mixed population with a subpopulation of non-
223 motile cells, the entire population can be brought to a halt. Similarly, within a population
224 in which a significant number of cells have stopped swimming or swarming, the further
225 addition of even just a few non-motile cells can suppress motility for the entire
226 population (i.e., see the ethanol studies mentioned above). As *P. aeruginosa*

227 populations inherently have some number of non-motile or inactive cells (3), future
228 studies are required to determine how strain background or mutant genotype affects
229 phenotypic heterogeneity among isogenic cells.

230

231 **Materials and Methods**

232 **Strains and media.** Strains used in this study are listed in **Table 1**. *P. aeruginosa* strain
233 PA14 and derivatives were routinely cultured on lysogeny broth (LB) solidified with 1.5%
234 agar or in LB broth at 37°C with shaking. For *P. aeruginosa* phenotypic assays, either
235 M63 [22 mM KH₂PO₄, 40 mM K₂HPO₄, and 15 mM (NH₄)₂SO₄] or M8 (42 mM Na₂PO₄,
236 22 mM KH₂PO₄, and 8.5 mM NaCl) minimal salts medium supplemented with MgSO₄ (1
237 mM), glucose (0.2%), and casamino acids (CAA; 0.5%) were used as indicated.

238

239 **Swarming motility assays.** Swarm assays were performed as previously described
240 (37). Briefly, M8 medium with 0.5% agar (swarm agar) was poured into 60 x 15 mm
241 plates and allowed to dry at room temperature for ~4 h prior to inoculation. The
242 indicated strains were grown for 8 to 16 h then each was normalized to an OD₆₀₀ of 1.
243 Indicated isolates were then mixed at the indicated ratios in a final volume of 100 µl.
244 Each plate was inoculated with 0.5 µl of the liquid culture mixture and the plates
245 incubated face-up at 37°C in stacks of no more than four plates for 16 h. Each mixture
246 was inoculated in four replicates and was assessed on at least three separate days.
247 Images were captured using a Canon EOS Rebel T6i camera and images assessed for
248 swarm repression.

249

250 **Swimming motility assays.** Swim assays were performed as previously described
251 (37). Briefly, M63 medium solidified with 0.3% agar (soft agar) was poured into petri
252 plates and allowed to dry at room temperature (~25°C) for ~4 h prior to inoculation.
253 Sterile toothpicks were used to inoculate bacterial mixes into the center of the agar
254 without touching the bottom of the plate; liquid cultures grown for 8 to 16 h were used as
255 inoculum. The indicated strains were normalized to an OD₆₀₀ of 1 then were mixed at
256 the indicated ratios in a final volume of 100 µl. No more than four bacterial mixtures
257 were assayed per plate. Plates were incubated upright at 37°C in stacks of no more
258 than four plates per stack for 18 to 20 h.

259

260 **Twitching motility assays.** T-broth [1% tryptone (w/v), 0.5% NaCl (w/v)] medium
261 solidified with 1.5% agar was poured into petri plates and allowed to dry at room
262 temperature (~25°C). Overnight (16 h) cultures were washed once in 1X PBS,
263 resuspended in 1X PBS, and then normalized to an OD₆₀₀ of 1 in 1X PBS. Plates were
264 inoculated by dipping a sterile toothpick into the washed and normalized cultures and
265 then inserting the toothpick into the agar until it touched the bottom of the plate. Plates
266 were then incubated at 37°C for 46 h after which time the agar was removed and the
267 plate incubated in 0.1% crystal violet for 10 min. Plates were then washed three times in
268 water and dried overnight at room temperature. Images of the dried, stained twitch area
269 were taken and twitch diameter was measured.

270

271 **Competition experiments.** Competition assays were performed to determine relative
272 growth of selected *P. aeruginosa* strains. Strains were grown overnight and 1 ml of

273 culture was pelleted at 15,682 x g for 2 min, and washed once in 1 mL 1X PBS followed
274 by resuspension in 1mL 1X PBS. The OD₆₀₀ of each culture was normalized to 1. The
275 strains to be competed were mixed at the indicated ratios in a final volume of 100 µL
276 and then 0.5 µL of the combined suspension was spotted onto a 0.22 µm polycarbonate
277 filter (Millipore) placed on the surface of a swarm plate in triplicates. Plates were
278 incubated at 37°C. Filters were then transferred into a 1.5 mL tube and the filter-
279 associated cells were resuspended by adding 1 ml PBS + 0.05% Triton X-100 detergent
280 and agitating the tubes at high speed for 2 minutes using a Genie Disruptor (Zymo).
281 This suspension as well as the inoculum were diluted, spread on LB plates
282 supplemented with 150 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-
283 Gal) using glass beads, and incubated at 30°C until blue colonies were visible (~24h).
284 The number of blue and white colonies per plate were counted and recorded to
285 determine the relative abundance of each strain.

286

287 **Phage susceptibility assays.** Phage susceptibility was analyzed by the cross-streak
288 method or by spotting phage directly onto a lawn of *P. aeruginosa*. For the cross-streak
289 method, *P. aeruginosa* strains were grown for 16 h in LB before diluting 1:100 in LB and
290 then growing to an OD₆₀₀ of 0.5-0.7. Then, 4 µl of phage DMS3 vir strain was spotted on
291 the side of an LB agar plate and then dragged across the agar surface in a straight line
292 before allowing to absorb into the agar. Once absorbed, 4 µl of each *P. aeruginosa*
293 strain was spotted at the top of the agar placed and dragged downward in a straight line
294 through the phage line in a straight line. Plates were incubated at

295 Alternatively, one percent M8 agar plates (60 x15 mm) containing 0.2% glucose
296 (v/v), 0.5% casamino acids (v/v), and 1 mM MgSO₄ were prepared and cooled to room
297 temperature. Fifty microliters of *P. aeruginosa* overnight cultures were added to 1 mL of
298 0.5% warm top agar (M8 medium and supplements). The mixture was gently mixed and
299 quickly poured onto M8 agar plates. Plates were swirled to ensure even spreading of
300 top agar. Once cooled, 2 µl of phage DMS3 vir strain were spotted onto the center of
301 the plate and allowed to dry before incubating plates at 37°C overnight.

302

303 **Congo red assay.** Cultures were grown in 5 ml LB for 16 h (overnight) at 37°C with
304 rolling. Overnight cultures were washed once and resuspended in sterile dH₂O.
305 Washed cells were spotted (3 µl) onto Congo Red plates (1% tryptone [w/v], 1.5% agar,
306 40 µg/ml Congo Red, 20 µg/ml Coomassie Blue). Plates were grown for 16 h at 37°C
307 and then moved to room temperature for 3 days to allow for color development.

308

309 **Statistical analysis.** One-way ANOVA with multiple comparisons were performed
310 pairwise between all isolates and mixtures using the GraphPad Prism 6 software
311 (GraphPad, La Jolla, CA).

312

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320 mutant and Christine Toutain for construction of the *motABCD* deletion strain.
321

322 **Table 1. Strains used in this study**

Strains (laboratory strain #)	Relevant genotype, description, or sequence	Source or reference
<i>P. aeruginosa</i> PA14		
strains		
PA14 (DH123)	<i>P. aeruginosa</i> WT	(38)
WT att:: <i>lacZ</i> (DH22)	WT with constitutive expression of <i>lacZ</i>	(39)
Δ <i>flgK</i> (DH1075)	<i>flgK</i> ::Tn5B30(Tc), non-motile	(4)
Δ <i>fliC</i> (DH3543)	Unmarked deletion of <i>fliC</i>	This study
Δ <i>motABCD</i> (SMC7230)	Unmarked deletion of <i>motABCD</i> with pMQ72 EV	This study
Δ <i>pilMNOP</i> (DH2705)	Unmarked deletion of <i>pilMNOP</i>	(6)
Δ <i>pilA</i> (DH2636)	Unmarked deletion of <i>pilA</i>	(22)
Δ <i>pelA</i> (DH97/SMC2893)	Unmarked deletion of <i>pelA</i>	(40)
Δ <i>flgKpelA</i> (DH3541)	Unmarked deletion of <i>flgK</i> and <i>pelA</i>	(11)
Δ <i>flgKpilA</i> (DH3539)	Unmarked deletion of <i>flgK</i> and <i>pilA</i>	(11)
Δ <i>flgKpilApelA</i> (DH3542)	Unmarked deletion of <i>flgK</i> , <i>pilA</i> , and <i>pelA</i>	(11)
Δ <i>pilT</i> (DH3591/SMC7302)	Unmarked deletion of <i>pilT</i>	(41)
Δ <i>pilU</i> (DH3592/SMC7304)	Unmarked deletion of <i>pilU</i>	(41)
Δ <i>pilJ</i> (DH2637/SMC2992)	Unmarked deletion of <i>pilJ</i>	(42)
Δ <i>fimS</i> (SMC6967)	Unmarked deletion of <i>fimS</i>	(6)
Δ <i>cyaAB</i> (DH2648/SMC6707)	Unmarked deletion of <i>cyaAB</i>	(6)

Δvfr (DH2701;SMC6722)

Unmarked deletion of *vfr*

(6)

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- 454

455 **Figure Legends**

456 **Fig. 1. Motility heterogeneity represses swarming motility independent of**

457 **competition. A.** Representative images from swarm assay of WT *P. aeruginosa* strain

458 PA14 (motile) mixed with $\Delta flgK$ (non-motile) at the indicated ratios on M8 agar. In this

459 and all figure panels in the manuscript, WT and derivatives are labeled in black, while

460 non-motile mutants are labeled in red. **B.** Competition growth assay between WT PA14

461 tagged with a *lacZ* reporter (gray) and either an untagged WT strain (black) or $\Delta flgK$

462 mutant (red). Error bars represent the standard deviation between the replicate values.

463 Statistical analysis using one-way ANOVA showed no difference between input and

464 output for each ratio analyzed. **C.** Representative images from swarm assay of WT

465 PA14 (motile) mixed with $\Delta flgK$ (non-motile), $\Delta fliC$ (non-motile), or $\Delta motABCD$ (non-

466 motile, paralyzed flagellum) in the indicated ratios on M8 agar.

467

468 **Fig. 2. Non-motile *Pseudomonas* requires T4P to repress flagellum-mediated**

469 **motility of motile strains on soft agar. A.** Representative images of swarm assays for

470 wild-type *P. aeruginosa* strain PA14 (motile, Pel+, T4P+) mixed with $\Delta flgK$ (non-motile,

471 Pel+, T4P+) or $\Delta flgKpilA$ (non-motile, Pel+, T4P-) on M8 agar at the indicated ratios. **B.**

472 Representative image showing the interaction between WT (black dot), $\Delta flgK$ (red dots),

473 and $\Delta flgKpilA$ (purple dots) for swimming motility in soft agar (0.3% M63 agar) after 42 h

474 incubation. The colored dots indicate the points of inoculation for the respective strains.

475

476 **Fig. 3. Motile *Pseudomonas* requires functional T4P to be repressed by the non-**

477 **motile subpopulation.** Representative images from swarm assays of the $\Delta flgK$ mutant

478 (functional T4P) mixed with either WT, the $\Delta pilA$ mutant (lacking T4P function), the $\Delta pilT$
479 mutant (no T4P retraction), or the $\Delta pilU$ mutant (reduced T4P retraction) at the indicated
480 ratios on M8 agar.

481

482 **Fig. 4. Pel matrix is only required by the non-motile *Pseudomonas* subpopulation**

483 **in order to repress overall motility. A.** Representative images from swarm assays of

484 $\Delta flgK$ (non-motile and Pel+) mixed with WT (motile and Pel+) or $\Delta pelA$ (motile and Pel-)

485 and of WT mixed with $\Delta flgK\Delta pelA$ (non-motile and Pel-) at the indicated ratios on M8

486 agar. **B.** Representative images of WT, $\Delta pilA$, $\Delta pelA$, $\Delta flgK$, $\Delta flgKpilA$, $\Delta flgKpelA$, and

487 $\Delta flgKpilApeIA$ grown on Congo Red plates to assess Pel production. WT and derivatives

488 are labeled in black while $\Delta flgK$ and derivatives are labeled in red.

489

490 **Fig. 5. Summary model describing the components needed for repression of**

491 **motility by non-motile cells in *P. aeruginosa* strain PA14.** We show that a

492 subpopulation of non-motile *P. aeruginosa* cells (red) in co-culture with motile cells

493 (black) impedes flagellum-mediated swarming motility in *P. aeruginosa* cells in a manner

494 that is dependent on retractable T4P. We also show that Pel matrix, only from the non-

495 motile strain, is required. PilU, PilJ, FimS, CyaAB, and Vfr were not required for

496 repression of motility. These data may indicate ways that *P. aeruginosa* cells come

497 together during biofilm formation.

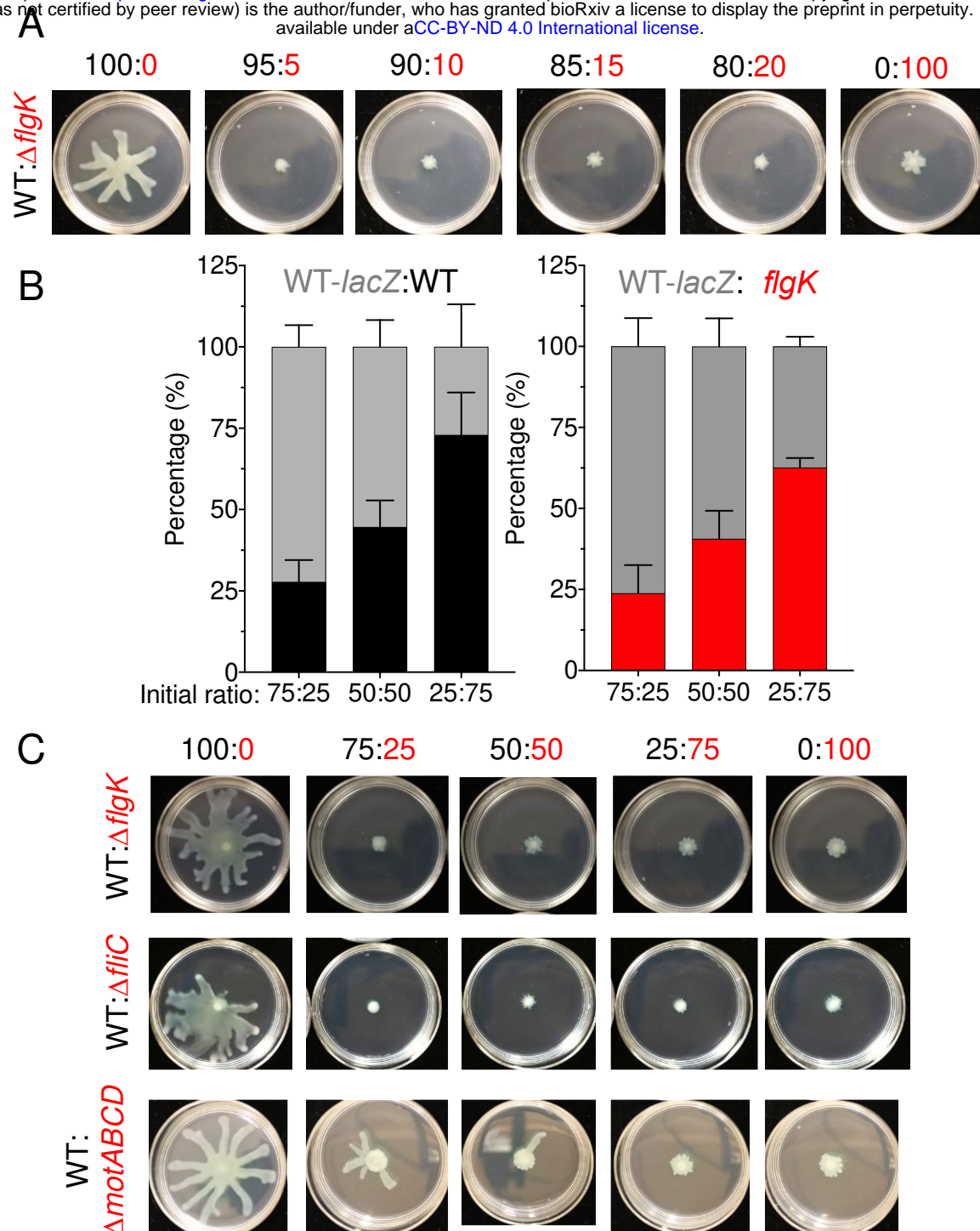


Fig. 1. Motility heterogeneity represses swarming motility independent of competition. **A.** Representative images from swarm assay of WT *P. aeruginosa* strain PA14 (motile) mixed with $\Delta flgK$ (non-motile) at the indicated ratios on M8 agar. In this and all figure panels in the manuscript, WT and derivatives are labeled in black, while non-motile mutants are labeled in red. **B.** Competition growth assay between WT PA14 tagged with a *lacZ* reporter (gray) and either an untagged WT strain (black) or $\Delta flgK$ mutant (red). Error bars represent the standard deviation between the replicate values. Statistical analysis using one-way ANOVA showed no difference between input and output for each ratio analyzed. **C.** Representative images from swarm assay of WT PA14 (motile) mixed with $\Delta flgK$ (non-motile), $\Delta fliC$ (non-motile), or $\Delta motABCD$ (non-motile, paralyzed flagellum) in the indicated ratios on M8 agar.

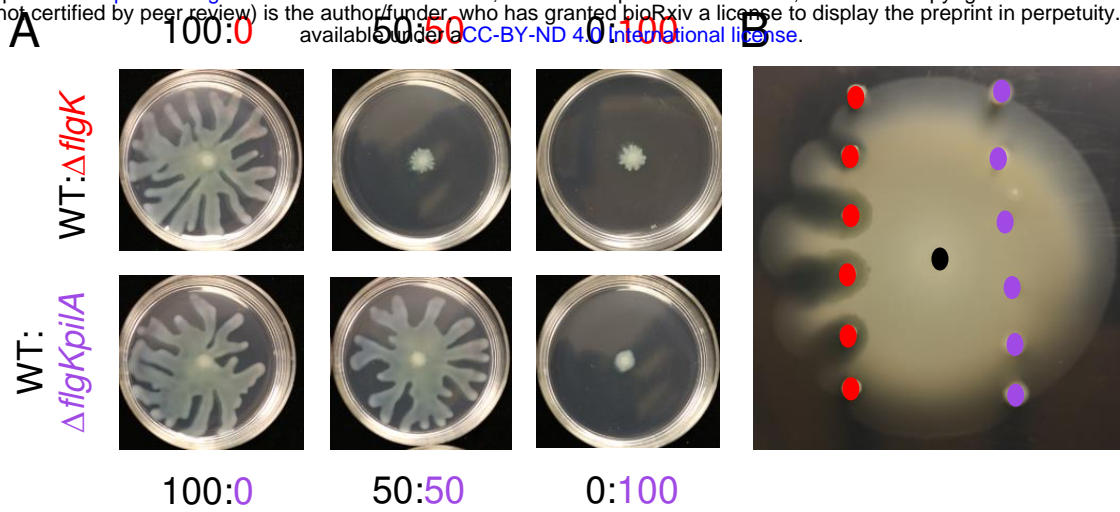


Fig. 2. Non-motile *Pseudomonas* requires T4P to repress flagellum-mediated motility of motile strains on soft agar. A. Representative images of swarm assays for wild-type *P. aeruginosa* strain PA14 (motile, Pel+, T4P+) mixed with $\Delta flgK$ (non-motile, Pel+, T4P+) or $\Delta flgKpilA$ (non-motile, Pel+, T4P-) on M8 agar at the indicated ratios. **B.** Representative image showing the interaction between WT (black dot), $\Delta flgK$ (red dots), and $\Delta flgKpilA$ (purple dots) for swimming motility in soft agar (0.3% M63 agar) after 42 h incubation. The colored dots indicate the points of inoculation for the respective strains.

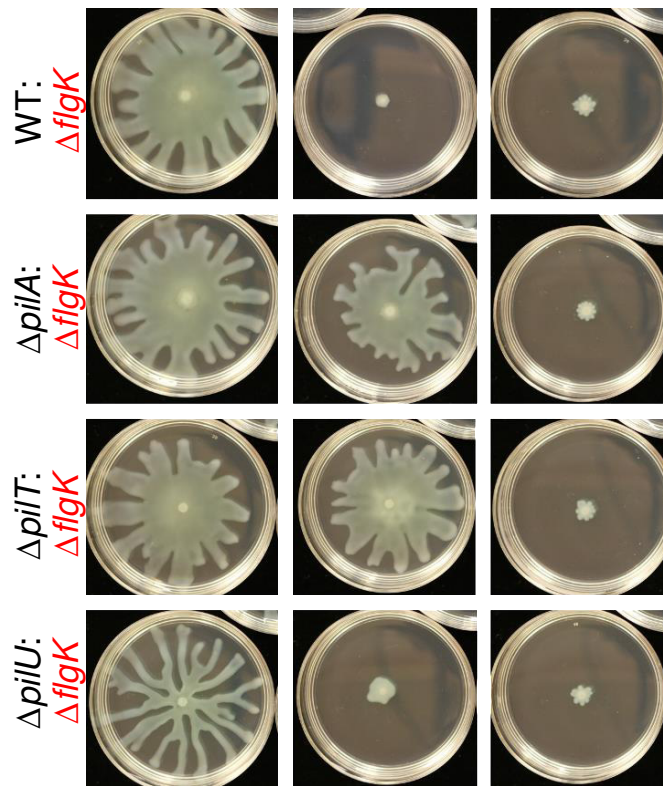


Fig. 3. Motile *Pseudomonas* requires functional T4P to be repressed by the non-motile subpopulation. Representative images from swarm assays of $\Delta flgK$ mutant (functional T4P) mixed with either WT, the $\Delta pilA$ mutant (lacking T4P function), the $\Delta pilT$ mutant (no T4P retraction), or the $\Delta pilU$ mutant (reduced T4P retraction) at the indicated ratios on M8 agar.

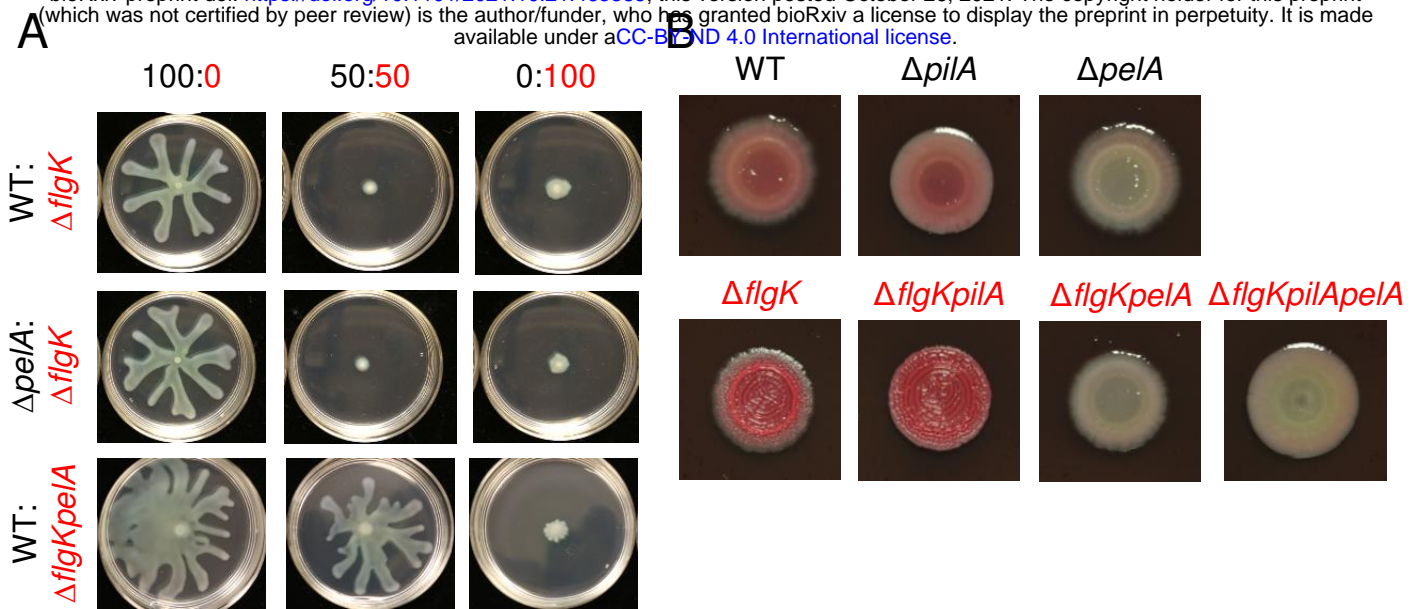


Fig. 4. Pel matrix is only required by the non-motile *Pseudomonas* subpopulation in order to repress overall motility. **A.** Representative images from swarm assays of $\Delta flgK$ (non-motile and Pel+) mixed with WT (motile and Pel+) or $\Delta pelA$ (motile and Pel-) and of WT mixed with $\Delta flgK\Delta pelA$ (non-motile and Pel-) at the indicated ratios on M8 agar. **B.** Representative images of WT, $\Delta pilA$, $\Delta pelA$, $\Delta flgK$, $\Delta flgKpilA$, $\Delta flgKpelA$, and $\Delta flgKpilApelA$ grown on Congo Red plates to assess Pel production. WT and derivatives are labeled in black while $\Delta flgK$ and derivatives are labeled in red.

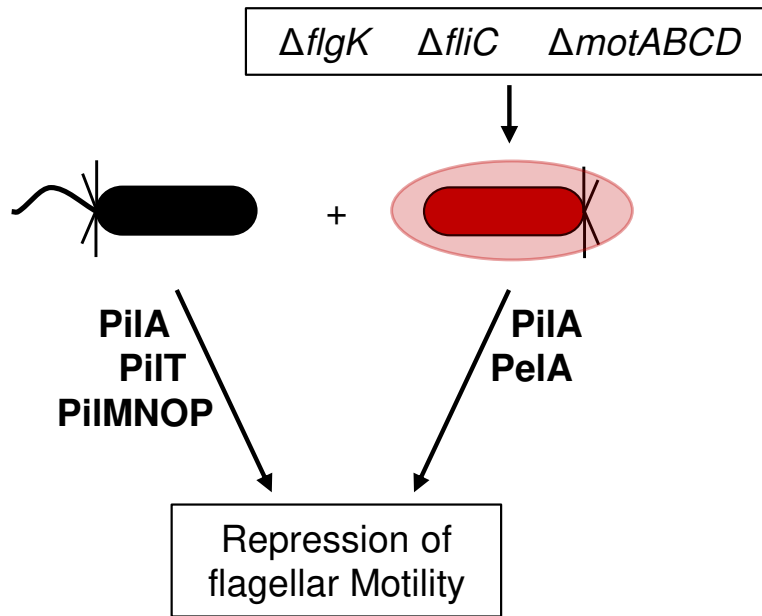


Fig. 5. Summary model describing the components needed for repression of motility by non-motile cells in *P. aeruginosa* strain PA14. We show that a subpopulation of non-motile *P. aeruginosa* cells (red) in co-culture with motile cells (black) impedes flagellum-mediated swarming motility in *P. aeruginosa* cells in a manner that is dependent on retractable T4P. We also show that Pel matrix, only from the non-motile strain, is required. PilU, PilJ, FimS, CyaAB, and Vfr were not required for repression of motility. These data may indicate ways that *P. aeruginosa* cells come together during biofilm formation.