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

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Abstract

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

Significance

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

Introduction

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

Materials and Methods

Strains and media. Strains used in this study are listed in **Table 1**. *P. aeruginosa* strain PA14 and derivatives were routinely cultured on lysogeny broth (LB) solidified with 1.5% agar or in LB broth at 37°C with shaking. For *P. aeruginosa* phenotypic assays, either M63 [22 mM KH_2PO_4, 40 mM K_2HPO_4, and 15 mM $(NH_4)_2SO_4$] or M8 (42 mM Na_2PO_4, 22 mM KH_2PO_4, and 8.5 mM NaCl) minimal salts medium supplemented with $MgSO_4$ (1 mM), glucose (0.2%), and casamino acids (CAA; 0.5%) were used as indicated.

Swarming motility assays. Swarm assays were performed as previously described (37). Briefly, M8 medium with 0.5% agar (swarm agar) was poured into 60 x 15 mm plates and allowed to dry at room temperature for ~4 h prior to inoculation. The indicated strains were grown for 8 to 16 h then each was normalized to an OD_{600} of 1. Indicated isolates were then mixed at the indicated ratios in a final volume of 100 µl. Each plate was inoculated with 0.5 µl of the liquid culture mixture and the plates incubated face-up at 37°C in stacks of no more than four plates for 16 h. Each mixture was inoculated in four replicates and was assessed on at least three separate days. Images were captured using a Canon EOS Rebel T6i camera and images assessed for swarm repression.

Swimming motility assays. Swim assays were performed as previously described (37). Briefly, M63 medium solidified with 0.3% agar (soft agar) was poured into petri plates and allowed to dry at room temperature (~25°C) for ~4 h prior to inoculation. Sterile toothpicks were used to inoculate bacterial mixes into the center of the agar without touching the bottom of the plate; liquid cultures grown for 8 to 16 h were used as inoculum. The indicated strains were normalized to an OD_{600} of 1 then were mixed at the indicated ratios in a final volume of 100 µl. No more than four bacterial mixtures were assayed per plate. Plates were incubated upright at 37°C in stacks of no more than four plates per stack for 18 to 20 h.

Twitching motility assays. T-broth [1% tryptone (w/v), 0.5% NaCl (w/v)] medium solidified with 1.5% agar was poured into petri plates and allowed to dry at room temperature (~25°C). Overnight (16 h) cultures were washed once in 1X PBS, resuspended in 1X PBS, and then normalized to an OD_{600} of 1 in 1X PBS. Plates were inoculated by dipping a sterile toothpick into the washed and normalized cultures and then inserting the toothpick into the agar until it touched the bottom of the plate. Plates were then incubated at 37°C for 46 h after which time the agar was removed and the plate incubated in 0.1% crystal violet for 10 min. Plates were then washed three times in water and dried overnight at room temperature. Images of the dried, stained twitch area were taken and twitch diameter was measured.

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

culture was pelleted at 15,682 *x g* for 2 min, and washed once in 1 mL 1X PBS followed by resuspension in 1mL 1X PBS. The OD_{600} of each culture was normalized to 1. The strains to be competed were mixed at the indicated ratios in a final volume of 100 µL and then 0.5 µL of the combined suspension was spotted onto a 0.22 µm polycarbonate filter (Millipore) placed on the surface of a swarm plate in triplicates. Plates were incubated at 37˚C. Filters were then transferred into a 1.5 mL tube and the filter-associated cells were resuspended by adding 1 ml PBS + 0.05% Triton X-100 detergent and agitating the tubes at high speed for 2 minutes using a Genie Disruptor (Zymo). This suspension as well as the inoculum were diluted, spread on LB plates supplemented with 150 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) using glass beads, and incubated at 30˚C until blue colonies were visible (~24h). The number of blue and white colonies per plate were counted and recorded to determine the relative abundance of each strain.

Phage susceptibility assays. Phage susceptibility was analyzed by the cross-streak method or by spotting phage directly onto a lawn of *P. aeruginosa*. For the cross-streak method, P. aeruginosa strains were grown for 16 h in LB before diluting 1:100 in LB and then growing to an OD_{600} of 0.5-0.7. Then, 4 µl of phage DMS3 vir strain was spotted on the side of an LB agar plate and then dragged across the agar surface in a straight line before allowing to absorb into the agar. Once absorbed, 4 µl of each P. aeruginosa strain was spotted at the top of the agar placed and dragged downward in a straight line through the phage line in a straight line. Plates were incubated at

Alternatively, one percent M8 agar plates (60 x15 mm) containing 0.2% glucose (v/v), 0.5% casamino acids (v/v), and 1 mM $MgSO_4$ were prepared and cooled to room temperature. Fifty microliters of *P. aeruginosa* overnight cultures were added to 1 mL of 0.5% warm top agar (M8 medium and supplements). The mixture was gently mixed and quickly poured onto M8 agar plates. Plates were swirled to ensure even spreading of top agar. Once cooled, 2 µl of phage DMS3 vir strain were spotted onto the center of the plate and allowed to dry before incubating plates at 37°C overnight.

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

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

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

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

Δ*vfr* (DH2701;SMC6722)	Unmarked deletion of *vfr*	(6)

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Figure Legends

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 Δ*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 Δ*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 Δ*flgK* (non-motile), Δ*fliC* (non-motile), or Δ*motABCD* (non-motile, paralyzed flagellum) in the indicated ratios on M8 agar.

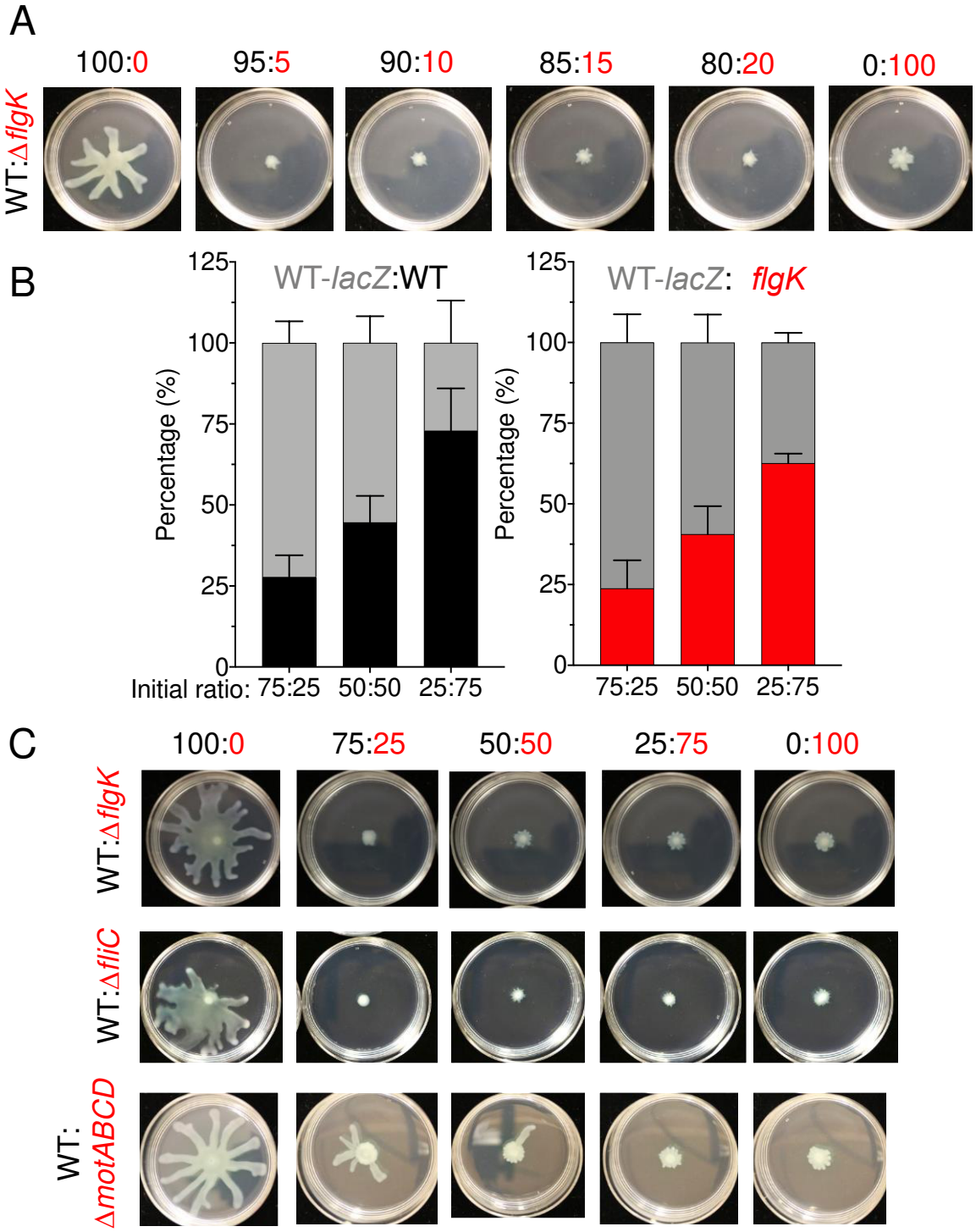
Fig. 2. Non-motile *Pseudomonas* requires T4P to repress flagellum-mediated motility of motile strains on soft agar. A. Representative images of swarm assays for wild-type *P. aeruginosa* strain PA14 (motile, Pel+, T4P+) mixed with Δ*flgK* (non-motile, Pel+, T4P+) or Δ*flgKpilA* (non-motile, Pel+, T4P-) on M8 agar at the indicated ratios. **B.** Representative image showing the interaction between WT (black dot), Δ*flgK* (red dots), and Δ*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 the Δ*flgK* mutant

(functional T4P) mixed with either WT, the Δ*pilA* mutant (lacking T4P function), the Δ*pilT* mutant (no T4P retraction), or the Δ*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 Δ*flgK* (non-motile and Pel+) mixed with WT (motile and Pel+) or Δ*pelA* (motile and Pel-) and of WT mixed with Δ*flgK*Δ*pelA* (non-motile and Pel-) at the indicated ratios on M8 agar. **B.** Representative images of WT, Δ*pilA,* Δ*pelA*, Δ*flgK,* Δ*flgKpilA*, Δ*flgKpelA,* and Δ*flgKpilApelA* grown on Congo Red plates to assess Pel production. WT and derivatives are labeled in black while Δ*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.



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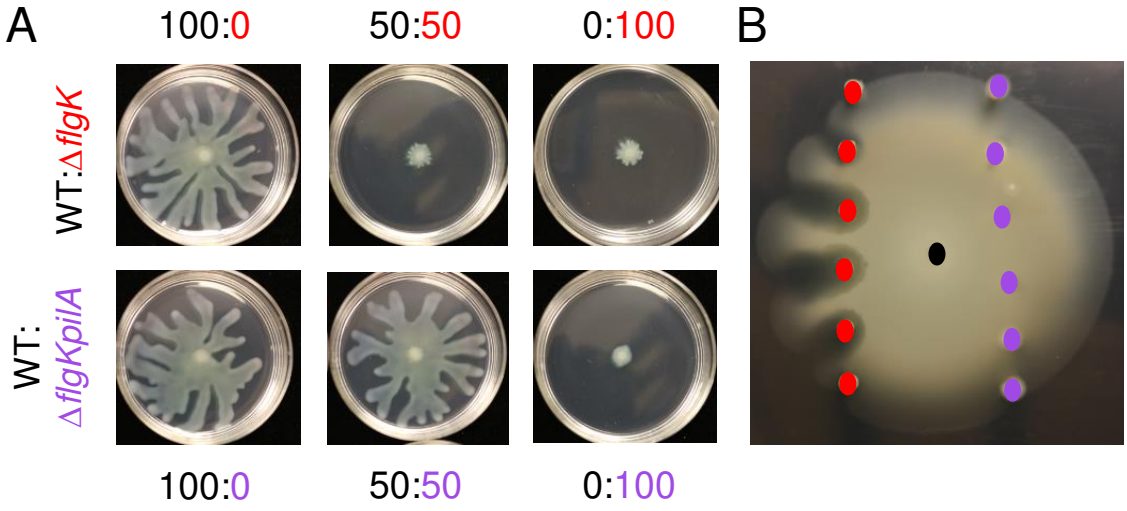

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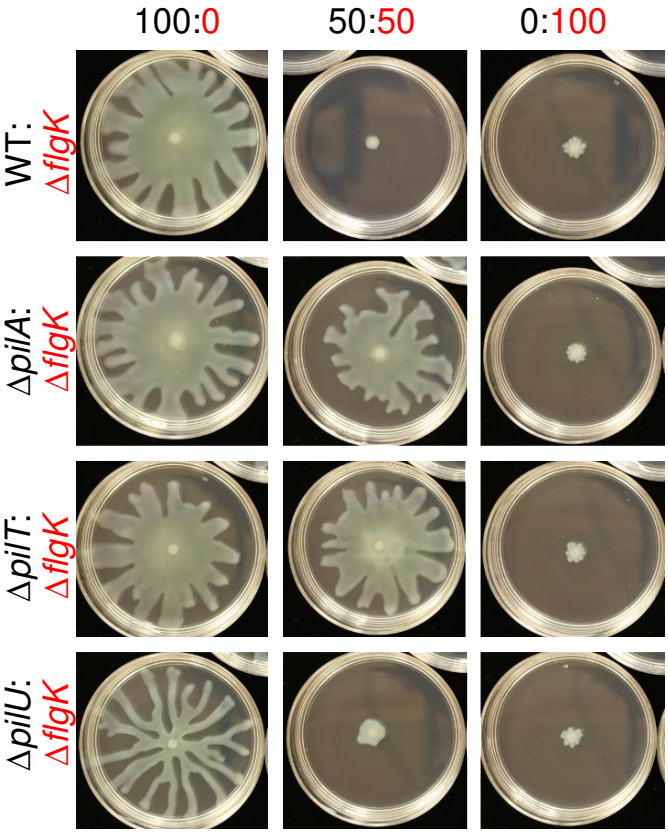

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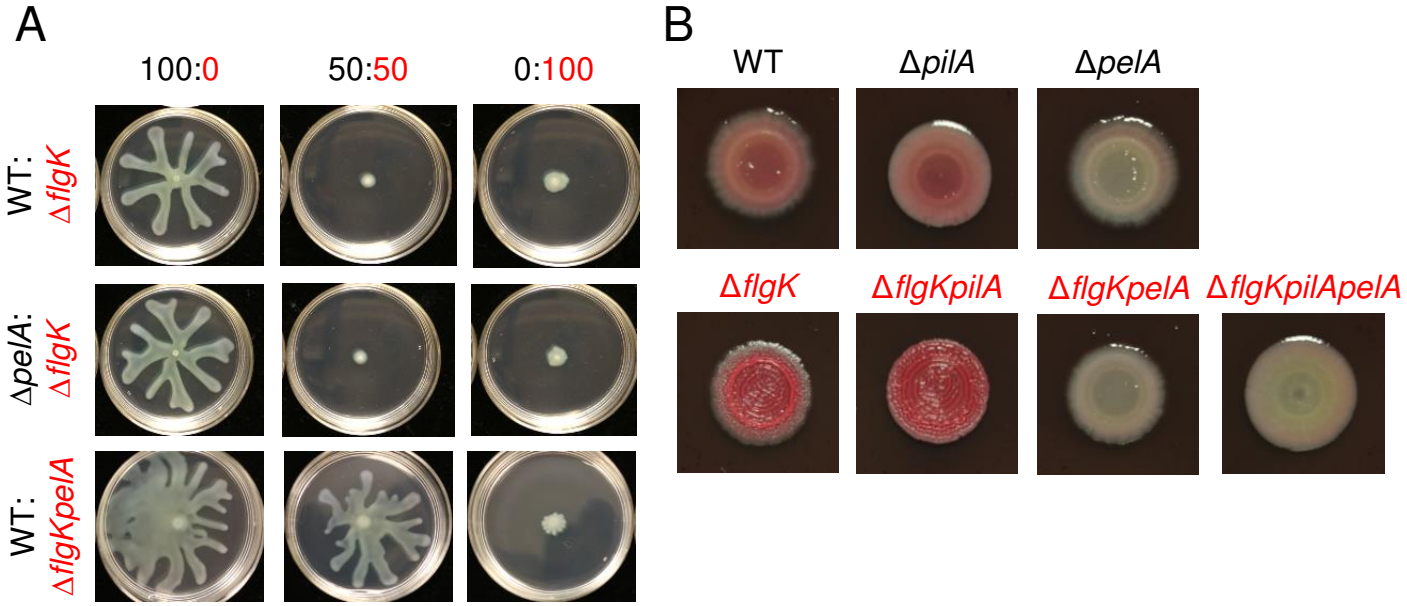

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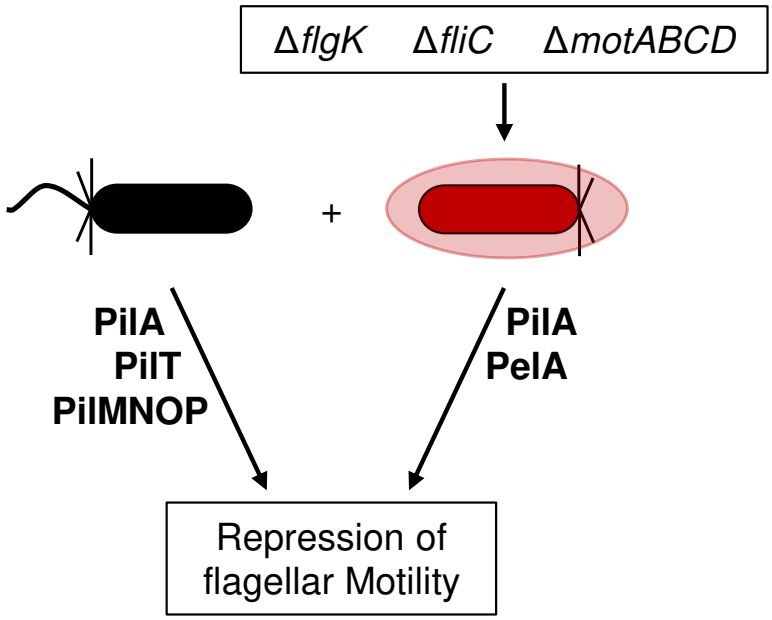

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