Ethanol decreases *Pseudomonas aeruginosa* flagellar motility through a cyclic-di-GMP- and stator-dependent pathway

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Running Head: Ethanol Decreases *P. aeruginosa* Flagellar Motility

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Abstract

Pseudomonas aeruginosa frequently encounters microbes that produce bioactive metabolites including ethanol. At concentrations that do not affect growth, we found that ethanol reduces *P. aeruginosa* motility by 30% in a swim agar assay and this decrease is accompanied by a 2.5-fold increase in levels of cyclic diguanylate (c-di-GMP), a second messenger that represses motility, in planktonic cells. A screen of mutants lacking genes involved in c-di-GMP metabolism identified SadC and GcbA as diguanylate cyclases involved in swim zone reduction by ethanol and ethanol-induced c-di-GMP production. The reduction of swimming in response to ethanol also required the stator set, MotAB, two PilZ-domain proteins (FlgZ and PilZ), PilY1- a proposed surface-sensing protein, and PilMNOP, which comprises the pilus alignment complex and these proteins have been previously implicated in the control of motility on agar surfaces. Microscopic analysis of the fraction of quiescent cells in swim medium showed that ethanol decreased the portion of motile cells in the wild type, but had opposite effects in the Δ*pilY1*, Δ*pilMNOP*, Δ*motAB*, and Δ*pilZ*Δ*flgZ* mutants. Together, these data indicate ethanol induces a regulated change in motility in planktonic cells at concentrations similar to those produced by other microbes. We propose that this ethanol-responsiveness may contribute to the co-localization of *P. aeruginosa* with ethanol-producing microbes.

40 **Importance**

41 Ethanol is an important, biologically active molecule produced by many bacteria and fungi. It
42 has also been identified as a potential marker for disease state in cystic fibrosis. In line with
43 previous data that show that ethanol promotes biofilm formation by *Pseudomonas aeruginosa*,
44 here we report that ethanol also induces cyclic-di-GMP levels in planktonic cells and reduces
45 swimming motility using some of the same proteins involved in surface sensing. We propose
46 that these data may provide insight into how microbes can influence *P. aeruginosa* localization
47 and surface association in the context of infection and in other polymicrobial settings.

48

Introduction

Ethanol, in addition to being a common fermentation product and a carbon source, can also serve as a signaling molecule for many microbes. For example, fungal gardens formed as part of a symbiosis between ambrosia beetles and their fungal symbionts, *Ambrosiella* and *Raffaelea*, are preferentially localized to sites with higher ethanol (1). In the parasite *Toxoplasma gondii*, low concentrations of ethanol (<200 mM or 1.2%) facilitates an increase in the second messenger inositol 1, 4, 5-triphosphate, resulting in increased intracellular calcium and increased host colonization (2, 3). In *Acinetobacter baumannii*, a Gram-negative opportunistic pathogen, ethanol causes an increase in virulence and an increase in carbohydrate leading to biofilm formation and repression of motility through mechanisms not yet described (4). In *Pseudomonas aeruginosa*, exogenous ethanol and ethanol produced by the fungus *Candida albicans* alters phenazine production and promotes biofilm formation on plastic and airway cells (5). Ethanol leads to increased Pel matrix production and decreased surface motility, two factors that are necessary for biofilm formation and maturation (5-7).

The many responses to ethanol are not surprising considering its frequent production as a fermentation product by many bacteria and fungi, including in host infection settings. One example where microbial ethanol is detected is in the polymicrobial lung infections associated with cystic fibrosis (CF), a genetic disorder that results in an accumulation of thick mucus in the airways (8-10). In addition to *P. aeruginosa*, CF lung infections often contain other microbes, many of which are capable of producing ethanol (11). Metabolomic and NMR studies examining the bronchoalveolar lavage fluid (BALF) and exhaled breath condensate (EBC) of patients with CF indicate that volatiles such as ethanol are present in the CF lung at varying amounts depending on the state of disease (stable vs. during exacerbation), and therefore may serve as biomarkers of disease (8, 9, 12, 13).

Previous studies found that in surface-associated *P. aeruginosa*, ethanol stimulates an increase in the level of the second messenger, cyclic-di-GMP (c-di-GMP), in part through WspR,

a diguanylate cyclase (DGC) (5). WspR is among the forty enzymes in *P. aeruginosa* thought to metabolize c-di-GMP, including other DGCs (14), c-di-GMP-degrading phosphodiesterases (PDEs) (15-17), and proteins that possess both activities. In *P. aeruginosa* and other pseudomonads, c-di-GMP metabolic enzymes have additional domains (e.g. PAS, REC, HAMP, CAHCE and GAF) that can sense external stimuli or promote protein-protein interactions in order to modulate enzyme activities at appropriate times (18-20), or in response to specific cues (19). In addition to the c-di-GMP metabolic enzymes, *P. aeruginosa* has thirteen effector proteins that bind c-di-GMP at various affinities to affect many behaviors including biofilm formation and motility (21, 22).

One of the roles of high c-di-GMP in *P. aeruginosa* is the down-regulation of flagellar motility (5, 7, 22-26). In pseudomonads and other bacteria, motility repression occurs in multiple ways, including (i) obstruction of the flagellum by exopolysaccharides (27, 28), (ii) transcriptional down-regulation of flagellar gene expression (26, 29), (iii) loss of flagellar rotation by c-di-GMP-bound effector proteins and their interactions with flagellum motor components (26, 30, 31), (iv) sequestration of flagellar motor proteins by c-di-GMP-bound effectors (22, 24), and (v) inhibition of flagellar rotation switching (clockwise vs. counterclockwise) (25, 32, 33). In Gram-negative bacteria, the flagellar motor is composed of two structures, the rotor (FliG, FliM, and FliN), which determines clockwise or counterclockwise rotation (the switch complex), and the stator (MotA and MotB), which generates torque for flagellar rotation powered by proton motive force (34-36). Pseudomonads have a second stator set (MotCD) that is incorporated into the stator complex to facilitate optimal motor function (22, 24, 37, 38). In *P. aeruginosa* the two stator sets have distinct roles: MotAB is required to reduce swarming motility when c-di-GMP levels are high, while MotCD is critical for promoting swimming and swarming motility (24, 37).

In the present study, we outline a pathway by which 1% ethanol represses motility in planktonic *P. aeruginosa* cells. Decreased swimming motility in cells exposed to ethanol is accompanied by a sustained increase in global cellular c-di-GMP pools. Genetic screens found

two DGCs, SadC and GcbA, two PilZ-domain effector proteins (FlgZ and PilZ), and the MotAB stator set as components required for ethanol-dependent motility repression. In addition, PilY1, and the PilMNOP proteins, components of the type 4 pili (T4P) machinery that are involved in surface sensing, were also required for the ethanol response in the swim agar assay. Ethanol decreased the portion of motile cells in the wild type by microscopic analysis, but mutants blind to the effects of ethanol (Δ*pilZ*Δ*flgZ,* Δ*motAB,* Δ*pilY1*, and Δ*pilMNOP*) had an opposite response, meaning cells were more motile in ethanol. Taken together with our previous studies (5), we propose that ethanol, a common metabolite produced by microbes, acts as a signal to rapidly repress *P. aeruginosa* swimming motility in planktonic cells, and thus potentiate biofilm initiation.

Materials and Methods

Strains and Media. Strains and plasmids used in this study are listed in Table S3. *P. aeruginosa* PA14 and *E.coli* strains were routinely cultured on lysogeny broth (LB) solidified with 1.5% agar, or in LB broth at 37^{o}C with shaking. Gentamicin (Gm) was used at 60 µg/ml and carbenicillin (Cb) at 700 µg/ml for *P. aeruginosa.* Gm was used at 10 µg/mL for *E. coli*. 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%), as indicated. When stated, 1% (v/v) ethanol (200-proof) was added to cooled medium (~50^{o}C) and equivalent volume of water was added to control cultures. For expression plasmids harboring pBAD promoter, arabinose was added to the culture as needed (0.02 or 0.05%).

Growth curve of *P. aeruginosa* PA14 wild type in the presence of ethanol. Growth curve analysis was performed by diluting *P. aeruginosa* to an OD_{600} of ~0.01 in six ml M63 medium without and with 1% (v/v) ethanol and incubation at 37^{o}C on a roller drum. OD_{600} was measured at specified time points using a Spectronic 20 spectrophotometer. Each sample type was analyzed in triplicate.

Molecular techniques. Plasmids were made using previously described homologous recombination in *Saccharomyces cerevisiae* (39). Plasmids were then extracted from the yeast using the 'smash and grab' method and electroporated into *E. coli* S-17 cells and confirmed via colony PCR. *E. coli* with confirmed constructs were then conjugated with the indicated *P. aeruginosa* strain to generate in-frame deletion mutants using allelic replacement as previously described (39). Exconjugants were selected on solid LB using gentamycin and nalidixic acid

followed by counterselection on 5% sucrose. PCR amplification and DNA sequencing, using primers that flanked the site of deletion, were used to confirm all resulting mutants.

For arabinose-inducible complementation, the gene being complemented was expressed on either pMQ80 (60 µg/ml gentamycin) or pDPM73 (700 µg/ml carbenicillin) plasmid backbones. Confirmed constructs were electroporated into the indicated *P. aeruginosa* strains, selecting for the appropriate antibiotic resistance marker. Arabinose (0.02 or 0.05%) was added to the medium and complementation was confirmed via the indicated phenotypic assay.

Swimming motility assays. Swim assays were performed as previously described (18). Briefly, M63 medium without and with 1% (v/v) ethanol and solidified with 0.3% agar (swim agar) was poured into petri plates and allowed to dry at room temperature (~25^{o}C) for ~4 h prior to inoculation. Sterile tooth picks were used to inoculate bacteria into the center of the agar without touching the bottom of the plate; liquid cultures grown for 8-16 h were used as inoculum. No more than four strains were assayed per plate. Plates were incubated upright at 37^{o}C in stacks of no more than four plates per stack for 16 h; the swim zone diameter was then measured. *P. aeruginosa* wild type was included in each experiment so that mutant phenotypes could be assessed despite slight day-to-day variation in swim zone diameter. Each strain was inoculated in four replicates and replicate values were averaged to obtain a final swim zone diameter for each strain. All strains were assessed on at least three separate days.

Twitching motility assays. Twitching motility assays were performed with T-agar medium (10g tryptone, 5g NaCl, and 15g agar in 1L) without and with 1% ethanol in petri plates that were allowed to dry at room temperature for 24 h prior to inoculation. Sterile toothpicks were used to inoculate into the agar until the toothpick touched the bottom of the petri plate; liquid cultures grown for 16 h were used as inoculum. No more than four strains were analyzed per plate and

six replicate plates were included in each experiment. Plates were incubated in inverted stacks of four at 37°C for 40 h. To visualize the twitch zone, a spatula was used to gently ease the agar out of the petri plates and two mL of 0.1% (w/v) crystal violet in water was added to each plate and allowed to stand for 10 min. The crystal violet was removed and the plates rinsed with water and allowed to air dry. Twitch zone diameter was measure and recorded. All strains were assessed on at least three separate days.

Swarming motility assays. Swarm assays were performed as previously described (18). Briefly, M8 medium, without and with 1% ethanol, and 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. Each plate was inoculated with 0.5 µL of a liquid culture that was grown for 8-16 h, and the plates incubated face-up at 37°C in stacks of no more than four for 16 h. Each strain 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 measured for ethanol-dependent swarm repression.

Reversal rate measurements. To measure the frequency at which a motile cell changes its direction, we used a modified version of a method that was previously described (25, 40). Briefly, overnight liquid cultures were subcultured 1:100 in five mL M63 medium and incubated at 37°C for 2 h. Once cultures reached exponential phase, they were then diluted 1:1000 in fresh M63 medium and Ficoll was added to a final concentration of 3% to obtain higher viscosity conditions that slowed the swimming cells sufficiently to allow the monitoring of reversal rates and mimic swimming in soft agar. Cells were then exposed to either control medium or medium containing 1% (v/v) ethanol for 15 min. Two hundred and fifty microliter of treated cell culture was next gently pipetted into a 35 mm glass bottom MatTek dish and a glass cover slip was

placed over the added culture. Four time lapse movies per strain and condition were captured with dark field using the Nikon Eclipse Ti microscope (Nikon Instruments Inc., Melville, NY) equipped with a 10X objective, a Hamamatsu ORCA-Flash 4.0 camera and Nikon NIS Elements AR 4.13.04 64 bit software. Time lapse movies were 8 s in duration with images captured at 20-25 ms intervals with RAM capture and 50 fps. Fiji ImageJ TrackMate (41) was used to process, analyze and quantify the reversal rates of 40 cells per movie. Movies were advanced frame by frame and individual cells were evaluated for the number of times they changed direction within the field of view and reversal rates were normalized and recorded as reversals per 10 s.

Microscopic agar motility assay. The population percentages of motile and immobile cells were calculated in 0.3% swim agar. Overnight liquid cultures were subcultured 1:100 in five mL M63 medium and incubated at 37^{o}C for 2 h. Once cultures reached exponential phase, they were then diluted by 1:1000 into freshly-prepared M63 swim agar (0.3%) without and with 1% (v/v) ethanol cooled to ~45^{o}C. Two hundred and fifty microliters of each agar mixture were pipetted into a chamber slide (see Fig. 6A) and allowed to solidify and acclimate to treatment for 30 min. Three to four time lapse movies per chamber slide, with two chamber slides per condition, were captured using the 40X objective on the Nikon Eclipse Ti microscope (Nikon Instruments Inc., Melville, NY) equipped with a Hamamatsu ORCA-Flash 4.0 camera and Nikon NIS Elements AR 4.13.04 64 bit software. Time lapse movies were 8 s in duration with images captured at five ms intervals with RAM capture and 100 fps. Fiji ImageJ TrackMate (41) was used to process, analyze and quantify the percentage of cells that were motile and immobile for the entire duration of each movie. Movies were advanced frame by frame and individual cells were evaluated for movement. All strains were assessed on at least two separate days.

***In vivo* cyclic-di-GMP quantification**. C-di-GMP was measured as previously described (5) with modifications. Overnight liquid cultures were diluted 1:1000 in six mL M63 medium with either 1% ethanol or an equivalent volume of water and grown at 37^{o}C for 16 h on a roller drum. Cultures were then adjusted to similar densities (OD_{600}) if necessary. Five mL of each culture was pelleted at 4,500 x g for 15 min at 4^{o}C. Cyclic-di-GMP was extracted by vigorously suspending the pellet in 250 µL of ice cold extraction buffer (40:40:20 MeOH/Acetonitrile/dH_2O and 0.1 N Formic acid, stored at -20^{o}C) and incubating at -20^{o}C for 1 h with tubes positioned upright. Tubes were then centrifuged briefly prior to transfer of the entirety of each extraction mix to a pre-weighed ice cold 1.5 mL Eppendorf tube. Cell debris was pelleted at 15,682 x g for five min at 4^{o}C, 200 µL of the extracted nucleotide was recovered into a clean 1.5 mL ice cold Eppendorf tube, and samples were each neutralized with 4 µL of 15% NH_4HCO_3 per 100 µL of sample. Pellets were dried on high for 1 h and the liquid samples on low overnight using the Savant Speed Vac SC110. The pellet weights were measured to get sample dry weight and the dried liquid samples containing the extracted nucleotides were each suspended in 200 µL HPLC-grade water. Two hundred microliters of each sample was sent to RTSF Mass Spectrometry and Metabolomics Core at Michigan State University for LC-MS-MS analysis. Each strain and treatment condition was analyzed in five replicates.

Statistical analysis. Unpaired Student *t* test, Two-way ANOVA with multiple comparisons, and One-way ANOVA with multiple comparisons were performed pairwise between the wild type and each strain, as well as ethanol and control conditions, using the GraphPad Prism 6 software (GraphPad, La Jolla, CA).

Results

Ethanol represses *P. aeruginosa* PA14 swimming motility independently of catabolism and without reducing growth rate. Exogenous ethanol stimulates *P. aeruginosa* biofilm behaviors, including attachment to glass and plastic, pellicle formation, and microcolony formation on airway cells, in part through stimulation of Pel extracellular matrix production (5, 42). Although *P. aeruginosa* can catabolize ethanol (43, 44), ethanol catabolism is not required for these phenotypes (5) indicating that the ethanol was acting as a signal or stimulus that modulates *P. aeruginosa* biofilm-related behaviors.

To further characterize the response to non-inhibitory concentrations of ethanol, we assessed ethanol effects on flagellar motility using a swim agar assay. We observed that *P. aeruginosa* strain PA14 wild type had a 33% smaller swim zone diameter in the presence of 1% ethanol when compared to the control cultures (49 mm versus 33 mm; $p<0.0001$) (Fig. 1A). A Δ*flgK* mutant that lacks a flagellum is non-motile and served as a reference strain (Fig. 1A). This reduction in swim zone diameter was not a result of differences in growth as *P. aeruginosa* strain PA14 wild type had similar growth rates in this medium in the absence or presence of 1% ethanol (Fig. S1A). Furthermore, the reduction in swim zone diameter also occurred independently of ethanol catabolism as a Δ*exaA* mutant, which cannot grow with ethanol as a carbon source (5), still showed motility repression when ethanol was added to the medium (Fig. S1B). These data indicated that the ethanol-dependent motility repression observed was not a result of ethanol metabolism or a change in the rate of growth.

Ethanol elicits an increase in c-di-GMP levels. C-di-GMP is an intracellular signaling molecule that modulates motility (22, 24, 26). When c-di-GMP levels are high, motility is reduced via multiple mechanisms (6, 21, 22, 45-47). In light of the observed decrease in swimming motility (Fig. 1A), we examined the effects of ethanol on c-di-GMP levels in planktonic

256 cells after 1 h and 16 h of growth in medium with 1% ethanol. We found that ethanol caused a
257 2.6- and 1.9-fold increase ($p<0.0001$) in c-di-GMP at 1 h and 16 h, respectively (Fig. 1B).
258
259 **Ethanol-dependent motility repression is not due to increased Pel and alginate matrix**
260 **production**. Increased c-di-GMP signals have been associated with an increase in alginate and
261 Pel matrix production in *P. aeruginosa* (6, 7). Although ethanol activates WspR-dependent
262 production of Pel polysaccharide matrix (5), neither WspR nor PelA was required for the
263 reduction in swim zone diameter in the presence of ethanol, with a 36.4% and 31.6%
264 ($p<0.0001$) decrease in their swim zones, respectively (Figs. S2A-B). We did note that the
265 Δ*wspR* mutant had a slightly larger swim zone diameter in control conditions (Fig. S2B).
266 Alginate was also not required for a reduction in swim zone diameter in the presence of ethanol,
267 as two mutants defective in alginate production, Δ*algD* and Δ*algU*, also had similar levels of
268 swim zone reduction (26.7% and 32.6% decrease ($p<0.0001$), respectively, as the wild type
269 (Fig. S3). These data indicate ethanol-dependent motility repression occurred independently of
270 matrix production.
271
272 **A screen of proteins that contribute to c-di-GMP metabolism reveal multiple enzymes**
273 **involved in the ethanol response.** While WspR was found to be required for increased c-di-
274 GMP in response to ethanol in surface-associated cells (5), the Δ*wspR* mutant still showed
275 increased c-di-GMP in planktonic cells in medium with ethanol compared to control medium
276 (Fig. S2C). This suggests that other enzymes are involved in the response to ethanol in
277 planktonic cells. Thus, we screened the collection of the reported *P. aeruginosa* PA14 in-frame
278 deletion mutant library containing mutants lacking each of the 40 known c-di-GMP metabolizing
279 enzymes (18) to identify the gene(s) involved in the ethanol-dependent motility repression. Our
280 primary focus was on mutants that (i) had a swim zone greater than or equal to that of the wild

type in control conditions, and (ii) had showed less of a reduction in swim zone diameter when ethanol was present in the medium. Using these criteria, analysis of the data from three independent screens of the mutant collection identified SadC and GcbA as the most promising candidates (Table. S1); data for mutants with swim zone sizes smaller than wild type under control conditions are provided (Table S2), but not pursued as part of these studies. The differences in the magnitude of the effect of ethanol on swim zone size in the Δ*gcbA* and Δ*sadC* single mutants compared to the wild type were small (Fig. 2A and Fig. S4A-B), but could be complemented with the wild-type *gcbA* and *sadC* genes, respectively, in *trans* (Fig. S4A-B). Both SadC and GcbA have been reported to impact c-di-GMP levels (7, 14, 25). Deletion of *wspR* in combination with either *sadC* or *gcbA* did not enhance the resistance of the effects of ethanol on motility (Fig. 2A).

The effects of SadC and GcbA on changes in motility in response to ethanol were additive as the Δ*sadC*Δ*gcbA* double mutant showed no significant difference in swim zone diameter between medium without and with ethanol (Fig. 2A). The Δ*sadC*Δ*gcbA* mutant also had lower levels of c-di-GMP in planktonic cultures, both in the absence and presence of ethanol, when compared to wild type in the same conditions (Fig. 2B). While the wild type showed 2.1-fold higher levels in c-di-GMP in ethanol-grown cells, the Δ*sadC*Δ*gcbA* mutant only showed a 1.4-fold difference. The small but significant increase that remained in the Δ*sadC*Δ*gcbA* mutant upon growth with ethanol suggests that other enzymes may contribute to changes in cellular c-di-GMP pools when ethanol is present.

Ethanol induced motility repression requires two PilZ-domain proteins, FlgZ and PilZ.

Among the c-di-GMP binding effectors in *P. aeruginosa* are PilZ-domain proteins (47, 48). There are eight known PilZ-domain proteins in *P. aeruginosa,* and some of these proteins have been shown to mediate changes in motility and/or biofilm formation (22, 48). Given that ethanol

306 stimulates c-di-GMP production and motility regulation, we assessed whether one or more of
307 these PilZ-domain proteins might be involved in ethanol-dependent motility repression.
308 In the absence of ethanol, all eight mutants and the wild type had swim zone diameters
309 that were similar (Fig. 3A). While six of the mutants phenocopied the wild type, two mutants
310 displayed significantly greater swimming motility than that observed for the wild-type strain in
311 the presence of ethanol (Δ*flgZ* and Δ*pilZ*; Fig. 3A). A Δ*flgZ*Δ*pilZ* double mutant had the same
312 level of motility in the presence and absence of ethanol, and thus did not show ethanol-
313 dependent motility repression (Fig. 3B). Interestingly, both PilZ and FlgZ were shown previously
314 to be involved in the repression of swarming motility on agar surfaces in a *P. aeruginosa* strain
315 that had high levels of c-di-GMP due to the absence of a phosphodiesterase, and to regulate
316 flagellar motility in other species (22, 27, 49, 50). Together, these data indicated that PilZ and
317 FlgZ play partially redundant roles in ethanol-dependent motility repression.
318
319 **Ethanol-dependent motility repression is mediated via the MotAB stator set.** Since the
320 PilZ-domain proteins PilZ and FlgZ have been linked to c-di-GMP-dependent decreases in
321 motility mediated by flagellar stators in *P. aeruginosa* and other species (22, 24, 28, 51, 52), we
322 postulated that flagellar stators may also be involved in the response to ethanol. *P. aeruginosa*
323 has two stator sets, MotAB and MotCD (38, 53). Specifically, FlgZ, upon c-di-GMP binding, has
324 been implicated in the sequestration of flagellar motor protein MotC and mislocalization of MotD,
325 which results in loss of motility due to increased incorporation of MotAB which cannot support
326 swimming in many environments such as on swarm agar (22, 24).
327 In line with the hypothesis that PilZ-domain proteins interact with flagellar stators to
328 reduce motility in the presence of ethanol, the Δ*motAB* mutant had no observable change in
329 motility in the presence of ethanol versus control cultures (Fig. 4). Also consistent with previous
330 reports, the Δ*motCD* mutant displayed a swim zone diameter that was ~90% less than that of

wild-type cells grown in the absence of ethanol (24) (Fig. 4). Overall, these data support the conclusion that the MotAB stator set is required for ethanol-dependent swim repression.

Ethanol-mediated motility repression requires PilY1 and the PilMNOP Type 4 pili alignment complex. PilY1 has been shown to be a surface-sensing protein required for decreased motility and stimulation of biofilm pathways in cells upon contact with a surface (23, 54) (Fig 5A). PilY1, in conjunction with the type 4 pili (T4P) alignment complex, PilMNOP (54), functions upstream of SadC (23), FlgZ (22) and the MotAB stator (23) to regulate swarming motility in *P. aeruginosa* by controlling the production of and the response to c-di-GMP. Thus, we tested the roles of PilY1 and PilMNOP in ethanol-dependent swimming motility repression. In contrast to the wild-type strain, the Δ*pilY1* mutant did not show decreased motility when ethanol was added to the medium (Fig. 5B). Instead, the Δ*pilY1* mutant showed a reproducible and significant increase in the swim zone diameter when ethanol was added to the medium (Fig. 5B), and a wild-type copy of the *pilY1* gene complemented this phenotype (Fig. 5C). Moreover, a mutant lacking *pilMNOP* showed no ethanol-dependent reduction in swimming motility (Fig. 5B). Though PilY1 and PilMNOP were required for the motility decrease in the presence of ethanol, they were not required for the stimulation of global c-di-GMP levels in planktonic cells (Fig. 5D). These data indicate that PilY1 and PilMNOP are involved in the decreased motility caused by ethanol, and suggest that this may be independent of changing global pools of c-di-GMP.

Type 4 pili are not required for the reduction in swimming motility in response to ethanol. PilY1 is necessary for T4P activity (55), and thus we sought to determine if PilY1- and PilMNOP-dependent reduction in flagellar motility by ethanol was due to a decrease in T4P activity. Two pieces of evidence argue against a role for the T4P in ethanol-mediated effects on motility. First, a Δ*pilA* mutant (which lacks pili) still showed the same level of motility and

responsiveness to ethanol (Fig. S5A) when compared to wild-type cells. Secondly, ethanol did not reduce twitching motility in wild-type cells (Fig. S5B); rather, a small but significant increase in twitch zone diameter was observed in cultures with ethanol. These data suggest that the ethanol effects on swimming motility are not due to changes in T4P function.

Previously described elements involved in PilY1 activation were dispensable for the ethanol-dependent reduction in motility. We sought to determine if previously described factors involved in PilY1 activation were involved in the ethanol response. Previous studies had shown that *pilY1* transcription is regulated by PilJ, a component of the Pil-Chp pathway, in response to surface engagement (54) through stimulation of cAMP production (56) upon surface contact (54). The *cyaAB* genes, which encode adenylate cyclases responsible for cAMP production by *P. aeruginosa,* were also implicated in PilY1 activation (54). We found that the Δ*pilJ* and Δ*cyaAB* mutants, though hyper motile in control cultures, both exhibited motility repression in response to ethanol ($p<0.0001$, Fig. S6). These data indicated that the upstream cAMP signal previously shown to be required for *pilY1* transcription, upon surface contact, was not required for the PilY1-dependent changes in motility in response to ethanol.

The von Willerbrand factor A (vWA) domain of PilY1, depicted in the schematic in Fig. 5A, is necessary for surface-associated swarming motility repression (23). To probe whether the same domain of PilY1 required for surface-sensing was also necessary for ethanol responsiveness, we used a strain where a mutated *pilY1* with the vWA domain deleted (*pilY1*-ΔvWA) was placed at the native *pilY1* locus. The *pilY1*-ΔvWA strain was still responsive to ethanol-dependent motility repression (25 mm and 20.5 ± 0.3 mm in the absence and presence of ethanol, respectively ($p<0.0001$; Fig. 5C). These data indicate that the vWA domain of PilY1 is dispensable for ethanol-mediated swim repression.

382 **Increased flagellar reversal frequency in the presence of ethanol does not cause ethanol-**
383 **dependent motility repression.** *P. aeruginosa* is a monotrichous flagellated bacterium whose
384 flagellar motility is governed by a run-reverse pattern (32, 57) rather than the run-tumble pattern
385 in organisms with peritrichous flagella like *E. coli* (58). *P. aeruginosa* directional movement is
386 due to a change in the flagellar rotation (clockwise or counterclockwise) and this rotation change
387 is called a 'reversal'. The frequency of reversals can impact the area covered since *P.*
388 *aeruginosa* must slow its normal speed from 40-55 µm/sec (38, 53, 57) to as low as 15 µm/sec
389 immediately before a reversal (57). To determine if the decrease in swim zone diameter in
390 ethanol was a result of a change in reversal frequencies, this parameter was measured in the
391 absence and presence of 1% ethanol.
392 Cells from mid-exponential phase cultures were treated with ethanol for 15 min prior to
393 measurement of the reversal frequency. *P. aeruginosa* strain PA14 wild type showed a 4.8-fold
394 increase in its reversal frequency in the presence of ethanol, going from 4.6 ± 4.1 to 22 ± 9.1
395 reversals/ 10 s ($p<0.0001$; Fig. S7A). Similarly, the Δ*sadC*Δ*gcbA,* Δ*pilY1,* and Δ*pilMNOP*
396 mutants also showed significant 2.8-fold, 3.4-fold, and 2.7-fold ($p<0.0001$) increases in reversal
397 frequencies upon the inclusion of ethanol in the medium (Fig. S7A-B). There were no significant
398 differences between the wild type and the mutants in either the control (except for
399 Δ*sadC*Δ*gcbA*) or ethanol conditions (Fig. S7A-B). These data indicated that in the presence of
400 ethanol, *P. aeruginosa* had a higher rate of flagellar reversals than in control conditions, but this
401 change did not account for the SadC/GcbA-, PilY1-, or PilMNOP-dependent suppression of
402 motility in the presence of ethanol.
403
404 **Ethanol rapidly increases the sub-population of immobile cells in swim agar, in a PilY1-,**
405 **PilMNOP-, FlgZ-, PilZ-, and MotAB-dependent manner.** We next observed the behavior of
406 single cells in swim agar in the absence and presence of 1% ethanol in order to better
407 understand how ethanol affected the macroscopic swim zone size. To do this experiment, we

408 exposed exponentially growing cells to swim agar without and with ethanol for 30 min followed
409 by the acquisition of 8 s time-lapse movies to visualize cellular behavior as outlined in Fig. 6A.
410 We first noted that when the fraction of motile cells in the control and ethanol-treated
411 samples were compared for each mutant, all except Δ*pilMNOP* were statistically different (Fig.
412 6B). We also noted that in the control cultures, Δ*pilMNOP* was significantly higher than wild type
413 and Δ*pilY1* in the same condition (Fig. 6B). For *P. aeruginosa* PA14 wild type cells swimming in
414 swim agar, we observed a decrease in the fraction of motile cells in the presence of ethanol
415 within the 8 s time interval analyzed (38 ± 10.5% motile (control) and 22 ± 8.8% motile (ethanol);
416 $p \leq 0.05$; Fig. 6B). In contrast, most of the mutants that did not show a smaller swim zone in
417 response to ethanol also did not show a reduction in the fraction of motile cells when ethanol
418 was in the medium. The Δ*pilMNOP* mutant, for example, had a similar proportion of motile cells
419 in the absence and presence of ethanol (58 ± 5.1% and 56 ± 3.6%, respectively; Fig. 6B).
420 Interestingly, the Δ*pilY1*, Δ*pilZ*Δ*flgZ*, and Δ*motAB* cells showed an increase in the fraction of
421 motile cells in the presence of ethanol (Fig. 6B) which also mirrored the observation that the
422 Δ*pilY1* strain had a larger swim zone size in the presence of ethanol (Fig. 5B). Of the mutants
423 that were resistant to the effects of ethanol in the macroscopic swim zone assay, only the
424 Δ*sadC*Δ*gcbA* double mutant was not significantly different from the wild-type strain (46 ± 6.4%
425 motile (control) and 29 ± 6.4% motile (ethanol); Fig. 6B). These data suggest that in response to
426 ethanol, *P. aeruginosa* exhibits an increase in the periods of immobility or decrease in the
427 fraction of cells swimming in the swim agar, and that this response is dependent on PilY1,
428 PilMNOP, FlgZ, PilZ, and MotAB proteins. The observation that the Δ*sadC*Δ*gcbA* double mutant
429 behaves like the wild type in the microscopic assay may suggest functional redundancy with
430 other c-di-GMP enzymes or that c-di-GMP levels affect motility by a mechanism distinct from
431 that of the PilY1-PilMNOP-FlgZ/PilZ-MotAB pathway.
432

433 **Ethanol inhibits swarming motility.** In addition to its effects on planktonic cells and motility in
434 swim agar, ethanol also inhibits flagellum-dependent swarming motility on agar surfaces (Fig.
435 7A) (5). We found that PilY1, the PilMNOP alignment complex, the PilZ and FlgZ proteins, and
436 the MotAB stators, which were all required to increase the fraction of sessile cells in medium
437 with ethanol, were also necessary for full suppression of flagellar-mediated swarming motility on
438 the surface of 0.5% agar in the presence of ethanol (Fig. 7A-D). Furthermore, while the vWA
439 domain of PilY1 has been shown to be important for surface-sensing (23), we found that this
440 domain was not required for ethanol-mediated repression of swarming motility (Fig. 7A).
441 Consistent with our observation that the Δ*sadC*, Δ*gcbA*, and Δ*sadC*Δ*gcbA* mutants were
442 resistant to the effects of ethanol on motility in the swim agar assay, these mutants were also
443 less responsive to ethanol in the surface-associated swarming motility assay (Fig. 7E).
444 Together, these data suggest that common factors are involved in the repression of flagellar
445 motility in planktonic cells as well as in cells on a surface.
446

Discussion

Here we present a model (Fig. 8) in which ethanol leads to decreased flagellar motility in *P. aeruginosa*. The down-regulation of flagellar motility by ethanol is in line with our previous work and the work of others that together shows that ethanol (1%) increases biofilm formation on abiotic and biotic surfaces (5, 42). We identified a pathway that involves PilY1, the T4P alignment complex (PilMNOP), two PilZ-domain proteins (FlgZ and PilZ) and the stator MotAB, all of which are required for ethanol-mediated down-regulation of swimming motility in both macroscopic and microscopic swim motility assays and for flagellar-mediated swarming motility on a surface. Microscopic observations of cells in swim agar, a medium that is widely used to assess chemotaxis and swimming motility (18, 59-61), showed that ethanol decreased the fraction of cells that were motile during the image capture period and that this change in behavior required all of the components of the PilY1 pathway outlined above. This same PilY1/PilMNOP/PilZ/FlgZ/MotAB pathway was shown previously to play a key role in surface sensing and early biofilm formation by *P. aeruginosa* (22-24, 54). FlgZ, a homolog of *E. coli* YcgR, has been shown to regulate flagellar motility by directly interacting with the flagellar motor proteins, thereby behaving like a ‘brake’ for flagellar rotation (22, 51, 62). The finding that ethanol reduces flagellar motility in cells in the soft agar suspension may provide more insight into the mechanism by which external or membrane-localized signals transduced by PilY1 and PilMNOP cause FlgZ, and perhaps PilZ, to act to affect flagellar rotation.

We also observed an ethanol-dependent increase in global pools of c-di-GMP in planktonic cells that required the activity of the diguanylate cyclases (DGCs) SadC and GcbA. In the plate-based swim assay, the Δ*sadC*Δ*gcbA* double mutant no longer showed an ethanol-mediated reduction in swimming motility. However, this double mutant behaved similarly to the wild type in the microscopic swim assay; we do not fully understand the basis for this discrepancy. The differences in swim zone diameters may be the consequence of a combination of factors that influence flagellar motility in different ways over the course of hours,

473 while the short-term microscopic assay may only assess a subset of the early effects of ethanol.
474 Previous studies have highlighted the distinct roles that SadC and GcbA play during the
475 different stages of biofilm formation. GcbA, for example, was implicated in c-di-GMP production
476 only in planktonic cells or cells initiating biofilm formation or dispersing from a mature biofilm
477 (21, 25, 63). SadC, on the other hand, is implicated in biofilm initiation and maturation (7, 21). It
478 is also important to note that the Δ*sadC*Δ*gcbA* double mutant still showed a significant, albeit
479 reduced, increase in c-di-GMP in the presence of ethanol (Fig. 2B), and thus other c-di-GMP
480 metabolizing enzymes may be involved in ethanol-mediated swimming repression, a finding
481 consistent with our initial genetic screen. In addition, c-di-GMP metabolizing enzymes can affect
482 target protein activities either by not altering global pools of c-di-GMP (local signaling) or by
483 increasing global levels of c-di-GMP by overexpression of a DGC (6, 7, 23, 25) or disruption of a
484 PDE (6, 22, 23). It is also possible that the status of global c-di-GMP is important and may alter
485 how *P. aeruginosa* responds to ethanol. Future studies will dissect the contributions of other c-
486 di-GMP metabolic activities on ethanol-induced effects on motility and other c-di-GMP controlled
487 processes.

488 A key question is how does *P. aeruginosa* PilY1 and PilMNOP, which are localized to
489 the membrane and extracytoplasmic space, contribute to the repression of motility in the
490 presence of ethanol? We showed that the N-terminal vWA domain of PilY1 is dispensable for
491 responding to ethanol, implicating the C-terminal domain of this protein as key for the observed
492 ethanol response. The C-terminus of the PilY1 protein has a seven-bladed, modified β-propeller
493 structure that shares structural similarity to the quinohemoprotein alcohol dehydrogenase from
494 *Comamonas testosteroni* (55). An attractive hypothesis is that PilY1 has the ability to bind
495 ethanol or the co-factor required for its catabolism. Alternatively, alcohols such as ethanol have
496 been implicated in membrane perturbation (64-66). For example, in *E. coli*, proteomics analysis
497 during ethanol stress, using 4% (684 mM) ethanol, revealed an induction of the general stress
498 response, a 15% increase in membrane fluidity, and the induction of mechanosensitive

channels that are active during osmotic stress (66). In another publication, Cao *et. al*. also showed that in *E. coli*, the effect of 2.5-5% (428-855 mM) ethanol resulted in increased ROS stress, reduced peptidoglycan, and a decrease in the proton gradient that might be explained by increased membrane fluidity (65). Cao *et. al.* also showed that evolution of *E. coli* on ethanol resulted in the correction of all the transient changes listed above, due to increased mutation rates (65). It is possible that this membrane perturbation also occurs in *P. aeruginosa* upon ethanol exposure and might induce structural changes to proteins associated with the cell membrane (PilY1, PilMNOP and SadC) that would then play a role in their activation. It is also possible that there are unknown regulators that are activated by ethanol and induce the activity of the pathway described above.

We also found that *P. aeruginosa* flagellar reversal frequency was significantly increased in the presence of ethanol, but this response did not depend on any of the proteins that we tested. Previous studies have indicated that an increase in reversal frequency increases the cell's ability to move more efficiently through soft agar (7, 67). Additionally, *P. aeruginosa* and related bacteria that utilize a run-reverse-turn trajectory, spend equal time going clockwise or counterclockwise with variation in their pause duration in order to turn at different angles to maximize space exploration (32). Since chemotaxis involves the modulation of reversal frequencies (57), these data may suggest that ethanol also affect chemotaxis in *P. aeruginosa*. More work is required to determine if ethanol influences positive or negative chemotactic pathways. Together, our data suggest that, while ethanol reduces the fraction of motile *P. aeruginosa* cells within a given time interval, these motile cells can navigate a viscous environment more efficiently in order to remain in the local space of the ethanol-producing microbes.

To conclude, our findings indicate that ethanol triggers a complex response that modulates behaviors related to biofilm initiation in order to facilitate the transition from being motile to being sessile. Therefore, the effects of ethanol on microbes at concentrations much

525 lower than those used for the purpose of sterilization is of interest in the context of biofuel
526 production, microbial remediation of industrial waste, and the activity of naturally occurring
527 communities in the environment and those in association with humans. Future studies will
528 determine if ethanol's effects on *P. aeruginosa* motility contributes to the stimulation of biofilm
529 formation and if the effects of ethanol on motility and biofilm formation in other Gram-negative
530 species, like *Acinetobacter baumannii* (4), occurs through a common pathway.
531

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743 **Figure Legends**

744 **FIG. 1. Ethanol decreases swim zone diameter and elicits an early and sustained increase**
745 **in c-di-GMP**. (A) Swim zone diameter of *P. aeruginosa* PA14 wild type and Δ*flgK* in M63 with
746 0.3% agar (swim agar) without (grey) and with (black) 1% ethanol after 16 h. Bars depict the
747 average swim zone diameter (n=4 replicates). Error bars indicate standard deviations. (B)
748 Quantification of c-di-GMP levels in *P. aeruginosa* PA14 grown in liquid M63 after 1 h and 16 h
749 exposure to 1% ethanol (black) or medium with no ethanol (grey). Bars depict the average of
750 normalized values (n=5 replicates). Error bars indicate standard deviations. Same letters are not
751 significantly different and different letters are significantly different (P<0.05) as determined by
752 Two-way ANOVA with multiple comparisons.

753

754 **FIG. 2. Ethanol effects on motility require two diguanylate cyclases, SadC and GcbA**. (A)
755 Swim zone diameter of *P. aeruginosa* PA14 wild type, Δ*sadC*, Δ*gcbA,* Δ*wspR*, Δ*sadC*Δ*wspR,*
756 Δ*gcbA*Δ*wspR*, and Δ*sadC*Δ*gcbA* in swim agar without (grey) and with (black) 1% ethanol
757 measured at 16 h. Error bars indicate standard deviations, n=4 replicates. (B) Quantification of
758 c-di-GMP levels in *P. aeruginosa* PA14 wild type and Δ*sadC*Δ*gcbA*, grown in liquid M63
759 medium without (grey) and with (black) 1% ethanol for 16 h. Error bars indicate standard
760 deviations, n=5 replicates. Common letters indicate no significant differences, different letters
761 indicate significant differences (P-value <0.05) as determined by Multiple t-test corrected using
762 the Holm-Sidak method (A) or One-way ANOVA with multiple comparisons (B).

763

764 **FIG. 3. PilZ domain proteins, PilZ and FlgZ, are required for ethanol effects on swim zone**
765 **diameter**. (A) Swim zone diameter for *P. aeruginosa* PA14 wild type, Δ*flgZ*, ΔPA14_00130*,*
766 ΔPA14_60970*,* ΔPA14_27930*,* ΔPA14_56180*,* Δ*alg44,* ΔPA14_25420 and Δ*pilZ* in swim agar
767 without (grey) and with (black) 1% ethanol after 16 h. Black arrows indicate the candidate
768 mutants of interest (shaded) that were least responsive to ethanol. (B) Swim zone diameter of

769 *P. aeruginosa* PA14 wild type, Δ*pilZ,* Δ*flgZ* and Δ*pilZ*Δ*flgZ* in swim agar without (grey) and with
770 (black) 1% ethanol after 16 h. Error bars indicate standard deviations, n=4 replicates. Common
771 letters indicate samples that are not significantly different, different letters indicate significant
772 differences (*P*-value <0.05) as determined by Two-way ANOVA with multiple comparisons.
773
774 **FIG. 4. Ethanol effects on swimming motility require the MotAB flagellar stator set**. Swim
775 zone diameter of *P. aeruginosa* PA14 wild type, Δ*flgK*, Δ*motAB* and Δ*motCD* in swim agar
776 without (grey) and with (black) 1% ethanol after 16 h. Error bars indicate standard deviations,
777 n=4 replicates. Same letters indicate samples that are not significantly different and different
778 letters have significant differences (*P*-value <0.05) as determined by Multiple t-test corrected
779 using the Holm-Sidak method.
780
781 **FIG. 5. PilY1 and PilMNOP are necessary for ethanol effects on swim zone diameter but**
782 **not ethanol-dependent c-di-GMP increase.** (A) Schematic of the PilY1 protein showing the
783 amino acid positions of the signal sequence (SS), von Willerbrand A factor domain (vWA),
784 calcium binding domain (red), and the PilC domain (green). (B) Swim zone diameter of *P.*
785 *aeruginosa* PA14 wild type, Δ*pilY1* and Δ*pilMNOP* in swim agar without (grey) and with (black)
786 1% ethanol measured after 16 h. Error bars indicate standard deviations, n=4 replicates. (C)
787 Swim zone diameter of *P. aeruginosa* PA14 wild type, Δ*pilY1*, Δ*pilY1* with an empty vector (EV),
788 with a plasmid-borne *pilY1* (P*pilY1*), or with a plasmid-borne *pilY1* with the vWA domain deleted
789 (*pilY1*-ΔvWA) in an arabinose-inducible expression plasmid in swim agar without (grey) and with
790 (black) 1% ethanol measured after growth for 16 h. 0.05% arabinose was added to the medium.
791 Error bars indicate standard deviations, n=4 replicates. Hashed bars indicate mutants that swim
792 more in ethanol than in control cultures. (D) Quantification of c-di-GMP levels in *P. aeruginosa*
793 PA14 wild type, Δ*pilY1* and Δ*pilMNOP* grown in liquid M63 medium without (grey) and with

(black) 1% ethanol for 16 h. Error bars indicate standard deviations, n=8 replicates. *, *P*-value <0.05; ****, *P*-value <0.0001 as determined by Two-way ANOVA with multiple comparisons. Same letters indicate no significant differences, different letters indicate samples that are significantly different (*P*-value <0.05).

FIG. 6. Ethanol decreases the number of motile cells in swim agar, in a manner dependent on PilY1, PilMNOP, FlgZ, PilZ, and MotAB (A) Schematic of agar motility assay treated with water (control) or 1% ethanol in swim agar. Sample was mixed well then 250 µL was pipetted onto a glass slide and staples were used to create a chamber using a glass coverslip. Sample was then incubated at room temperature for 30 min, and then imaged as outlined in the methods. (B) Agar motility assay of *P. aeruginosa* PA14 wild type, Δ*sadC*Δ*gcbA,* Δ*pilZ*Δ*flgZ,* Δ*motAB,* Δ*pilY1* and Δ*pilMNOP* in swim agar without (blue) and with (red) 1% ethanol after 30 min. Three time lapse (8 s) movies of the cells in the agar matrix, for each sample, were captured. Plotted is the average population percentage of the motile subpopulation in each movie. Error bars represent the maximum and minimum data point, n≥6 replicate movies. Shaded boxes represent the ethanol samples that are significantly different from their controls. *, *P*-value <0.05; **, *P*-value <0.01; ***, *P*-value <0.001; ns, not significant as determined by One-Way ANOVA with multiple comparisons.

FIG. 7. Ethanol effects on swarming motility repression occur using some surface-sensing components as well as components independent of surface sensing. Representative images of swarming motility assays of (A) *P. aeruginosa* PA14 wild type, Δ*pilY1*, Δ*pilY1* with an empty vector (EV) or with a plasmid that enables arabinose-inducible expression *pilY1* (P*pilY1*) or *pilY1* without a vWA domain (*pilY1*-ΔvWA), (B) *P. aeruginosa* PA14 wild type and Δ*pilMNOP,* (C) *P. aeruginosa* PA14 wild type and Δ*motAB,* (D) *P. aeruginosa* PA14 wild type, Δ*flgZ*, Δ*pilZ,* and Δ*pilZ*Δ*flgZ,* and (E) *P. aeruginosa* PA14 wild type, Δ*sadC*, Δ*gcbA,* and

820 Δ*sadC*Δ*gcbA* in M63 medium with 0.5% agar (swarm agar) without and with 1% ethanol and
821 grown for 16 h. Images are representative of observed phenotypes, n=4 per experiment and
822 each experiment was performed 3-5 times.
823
824 **FIG. 8. Model for the effects of ethanol on *Pseudomonas aeruginosa* motility.** When free
825 swimming *P. aeruginosa* encounters ethanol in a liquid environment, it quickly responds by
826 changing its motility. One response (1) occurs via PilY1 and the alignment complex (PilMNOP).
827 This motility repression requires two PilZ domain proteins, PilZ and FlgZ, as well as flagellar
828 stator set, MotAB. We propose that this signal causes the flagellar machinery to 'brake',
829 resulting in a decrease in the number of cells that are motile. The second response (2) involves
830 a SadC and GcbA c-di-GMP production-dependent change that may also involve other c-di-
831 GMP metabolic proteins. C-di-GMP may further activate the PilZ domain proteins. Together,
832 these responses repress flagellar motility in swim agar conditions and a soft agar that supports
833 swarming motility.
834

Figures and Legends

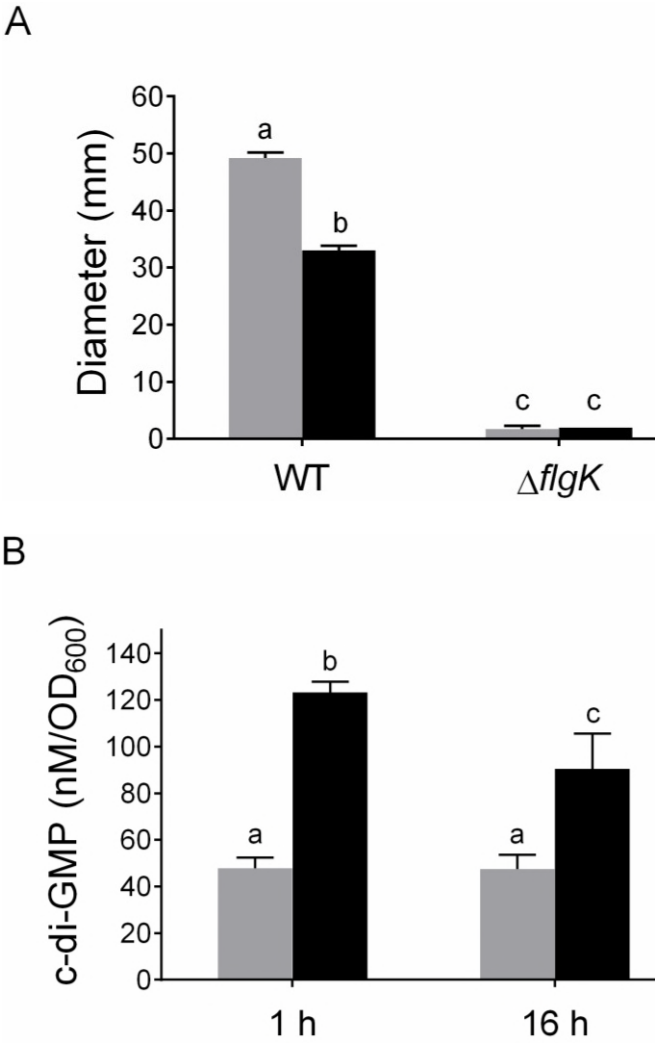
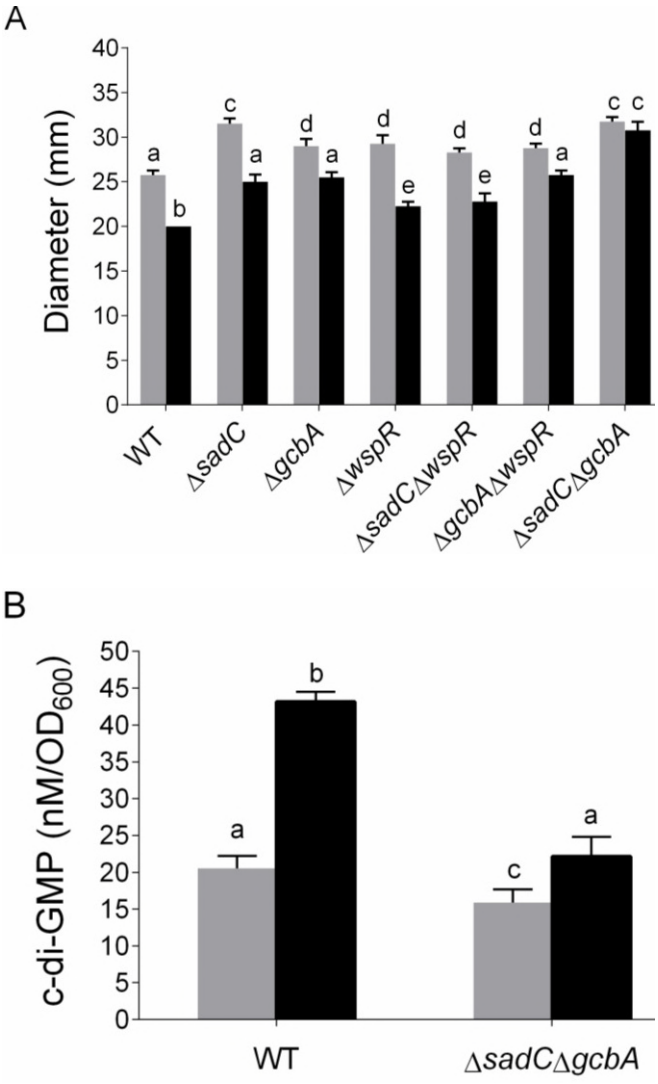


FIG. 1. Ethanol decreases swim zone diameter and elicits an early and sustained increase in c-di-GMP. (A) Swim zone diameter of *P. aeruginosa* PA14 wild type and Δ*flgK* in M63 with 0.3% agar (swim agar) without (grey) and with (black) 1% ethanol after 16 h. Bars depict the average swim zone diameter (n=4 replicates). Error bars indicate standard deviations. (B) Quantification of c-di-GMP levels in *P. aeruginosa* PA14 grown in liquid M63 after 1 h and 16 h exposure to 1% ethanol (black) or medium with no ethanol (grey). Bars depict the average of normalized values (n=5 replicates). Error bars indicate standard deviations. Same letters are not significantly different and different letters are significantly different ($P<0.05$) as determined by Two-way ANOVA with multiple comparisons.



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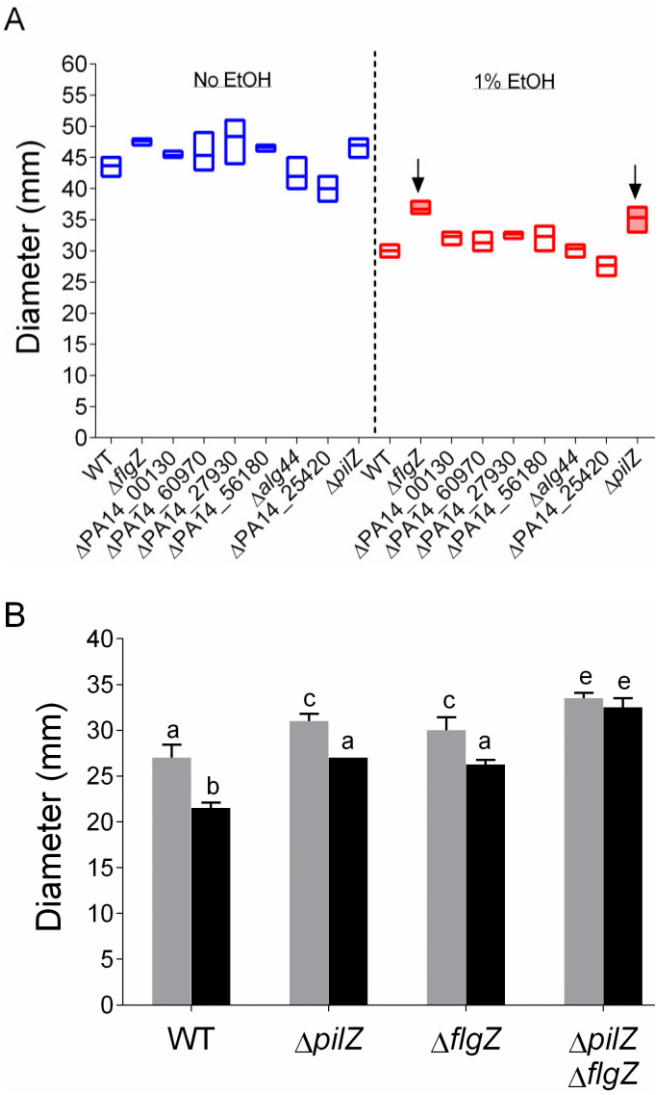

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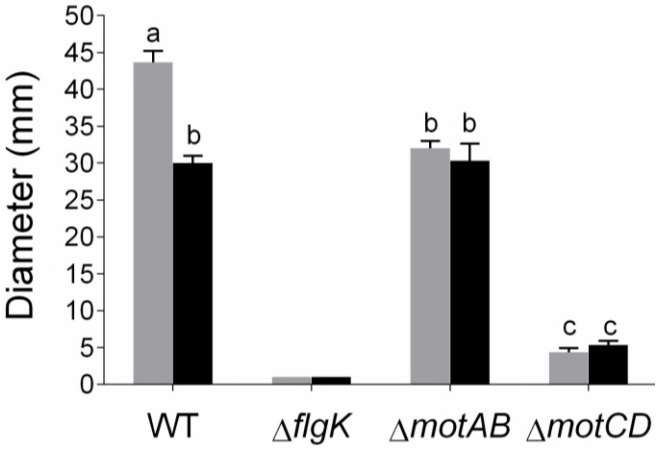

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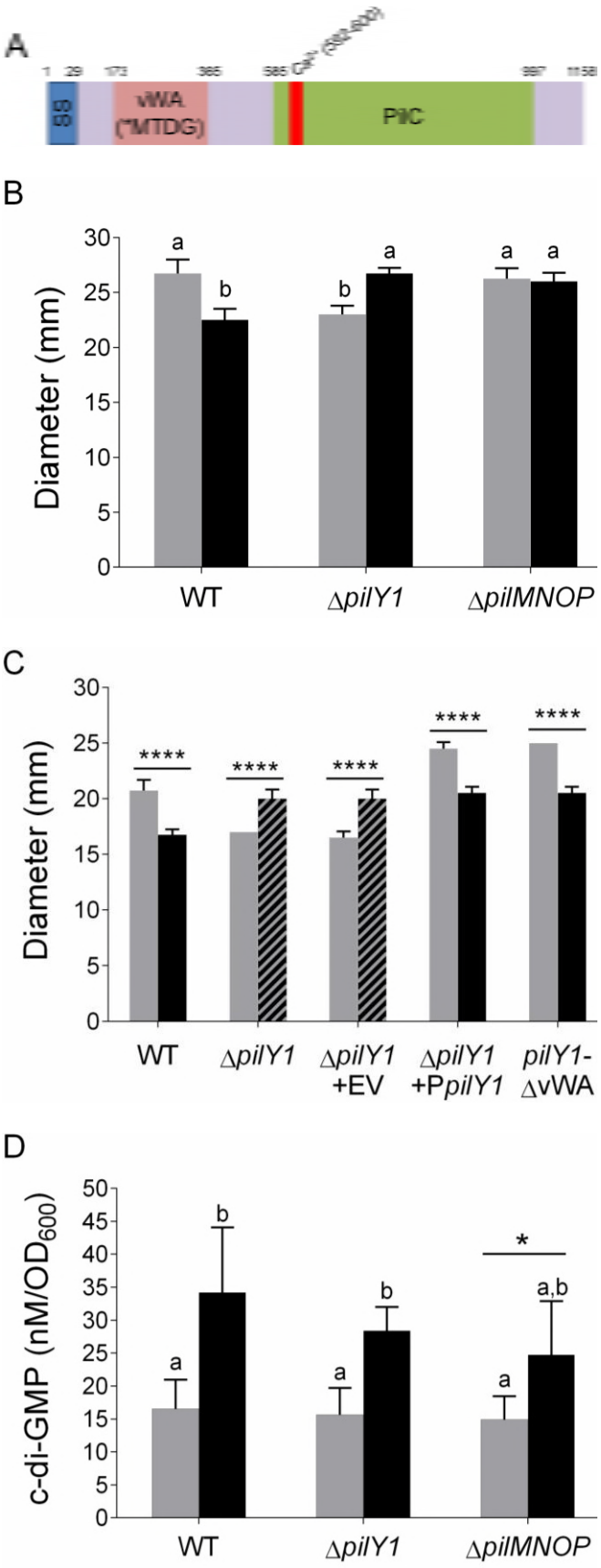

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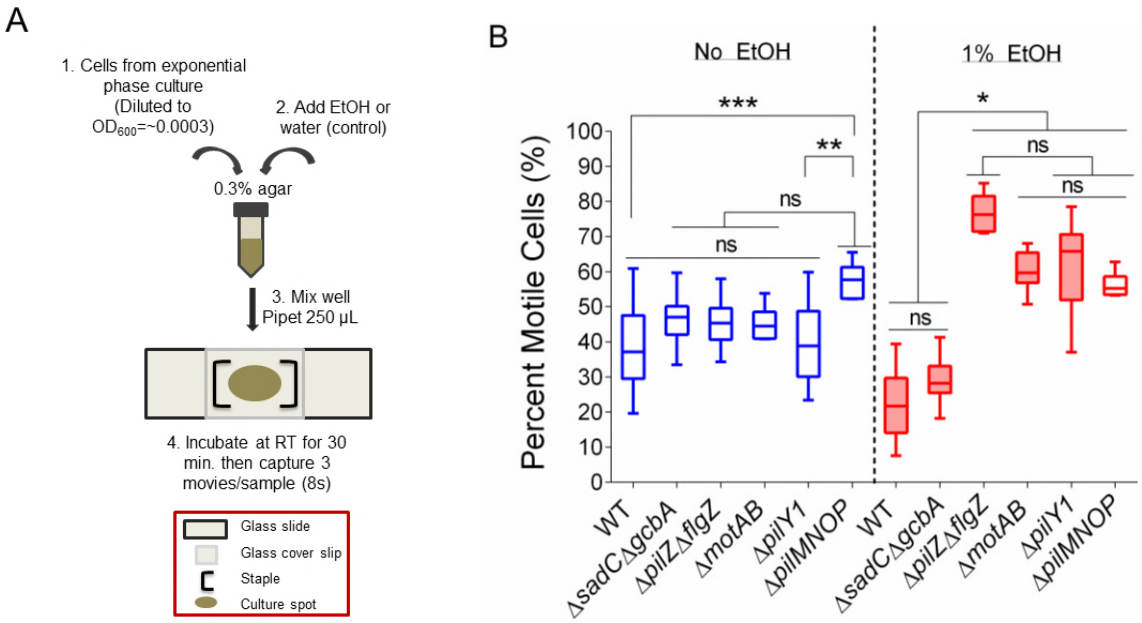

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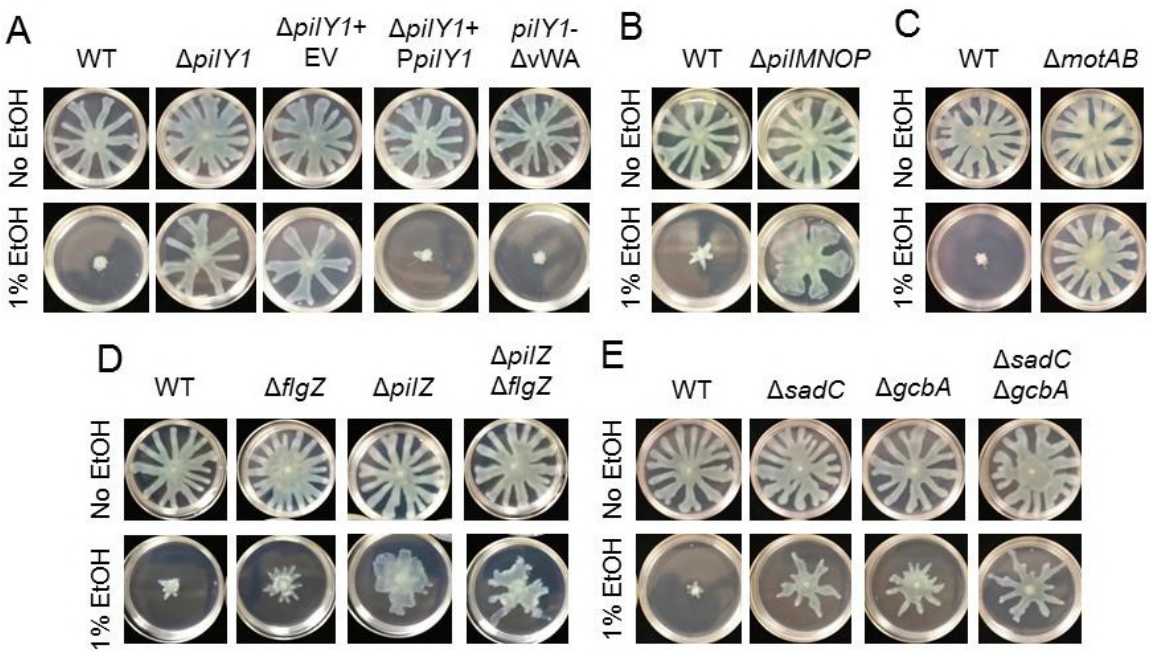

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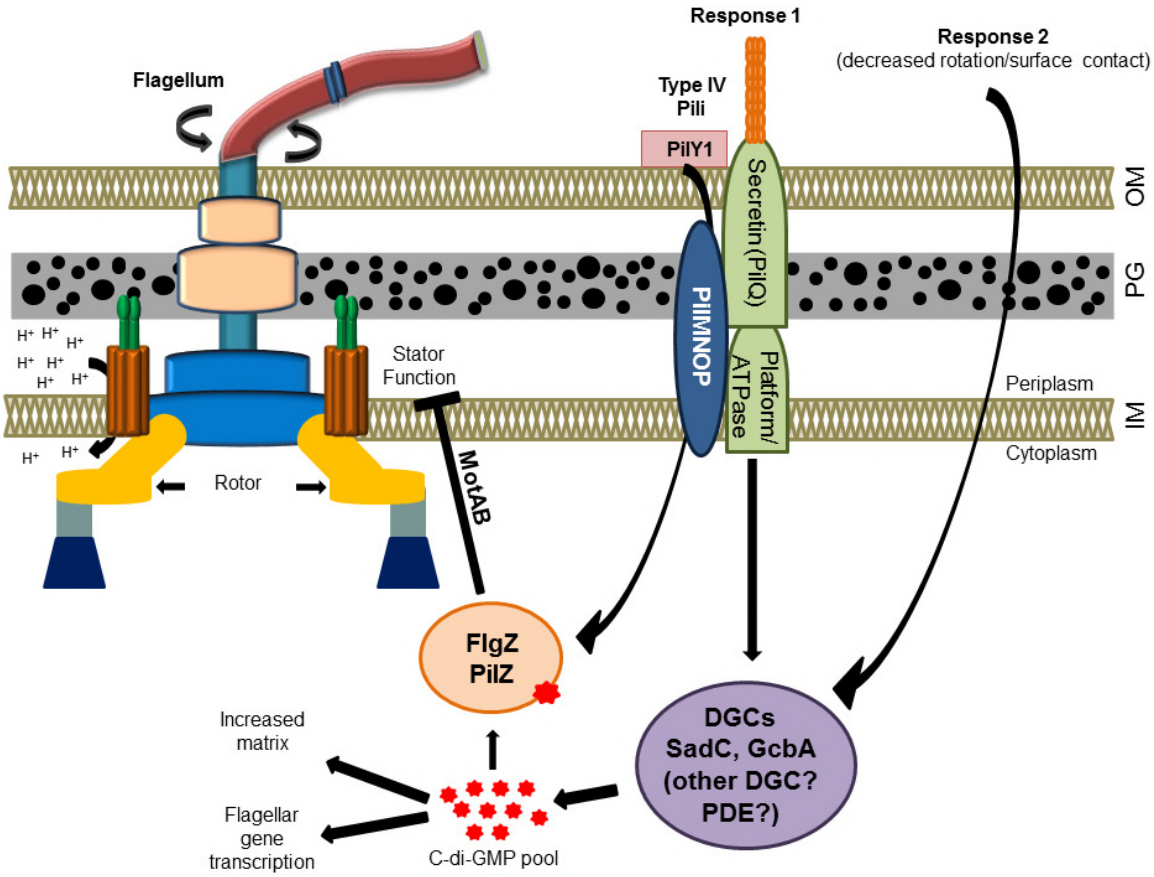

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