

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

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

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21

22 **Abstract**

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

39

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

## 49 Introduction

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

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

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

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

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

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

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

111 **Materials and Methods**

112 **Strains and Media.** Strains and plasmids used in this study are listed in Table S3. *P.*  
113 *aeruginosa* PA14 and *E.coli* strains were routinely cultured on lysogeny broth (LB) solidified with  
114 1.5% agar, or in LB broth at 37°C with shaking. Gentamicin (Gm) was used at 60 µg/ml and  
115 carbenicillin (Cb) at 700 µg/ml for *P. aeruginosa*. Gm was used at 10 µg/mL for *E. coli*. For *P.*  
116 *aeruginosa* phenotypic assays, either M63 (22 mM KH<sub>2</sub>PO<sub>4</sub>, 40 mM K<sub>2</sub>HPO<sub>4</sub>, and 15 mM  
117 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) or M8 (42 mM Na<sub>2</sub>PO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, and 8.5 mM NaCl) minimal salts medium  
118 supplemented with MgSO<sub>4</sub> (1 mM), glucose (0.2%), and casamino acids (CAA; 0.5%), as  
119 indicated. When stated, 1% (v/v) ethanol (200-proof) was added to cooled medium (~50°C) and  
120 equivalent volume of water was added to control cultures. For expression plasmids harboring  
121 pBAD promoter, arabinose was added to the culture as needed (0.02 or 0.05%).

122  
123 **Growth curve of *P. aeruginosa* PA14 wild type in the presence of ethanol.** Growth curve  
124 analysis was performed by diluting *P. aeruginosa* to an OD<sub>600</sub> of ~0.01 in six ml M63 medium  
125 without and with 1% (v/v) ethanol and incubation at 37°C on a roller drum. OD<sub>600</sub> was measured  
126 at specified time points using a Spectronic 20 spectrophotometer. Each sample type was  
127 analyzed in triplicate.

128  
129 **Molecular techniques.** Plasmids were made using previously described homologous  
130 recombination in *Saccharomyces cerevisiae* (39). Plasmids were then extracted from the yeast  
131 using the 'smash and grab' method and electroporated into *E. coli* S-17 cells and confirmed via  
132 colony PCR. *E. coli* with confirmed constructs were then conjugated with the indicated *P.*  
133 *aeruginosa* strain to generate in-frame deletion mutants using allelic replacement as previously  
134 described (39). Exconjugants were selected on solid LB using gentamycin and nalidixic acid

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

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

142  
143 **Swimming motility assays.** Swim assays were performed as previously described (18). Briefly,  
144 M63 medium without and with 1% (v/v) ethanol and solidified with 0.3% agar (swim agar) was  
145 poured into petri plates and allowed to dry at room temperature (~25°C) for ~4 h prior to  
146 inoculation. Sterile tooth picks were used to inoculate bacteria into the center of the agar without  
147 touching the bottom of the plate; liquid cultures grown for 8-16 h were used as inoculum. No  
148 more than four strains were assayed per plate. Plates were incubated upright at 37°C in stacks  
149 of no more than four plates per stack for 16 h; the swim zone diameter was then measured. *P.*  
150 *aeruginosa* wild type was included in each experiment so that mutant phenotypes could be  
151 assessed despite slight day-to-day variation in swim zone diameter. Each strain was inoculated  
152 in four replicates and replicate values were averaged to obtain a final swim zone diameter for  
153 each strain. All strains were assessed on at least three separate days.

154  
155 **Twisting motility assays.** Twisting motility assays were performed with T-agar medium (10g  
156 tryptone, 5g NaCl, and 15g agar in 1L) without and with 1% ethanol in petri plates that were  
157 allowed to dry at room temperature for 24 h prior to inoculation. Sterile toothpicks were used to  
158 inoculate into the agar until the toothpick touched the bottom of the petri plate; liquid cultures  
159 grown for 16 h were used as inoculum. No more than four strains were analyzed per plate and



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

166  
167 **Swarming motility assays.** Swarm assays were performed as previously described (18).  
168 Briefly, M8 medium, without and with 1% ethanol, and with 0.5% agar (swarm agar) was poured  
169 into 60 x 15 mm plates and allowed to dry at room temperature for ~4 h prior to inoculation.  
170 Each plate was inoculated with 0.5 µL of a liquid culture that was grown for 8-16 h, and the  
171 plates incubated face-up at 37°C in stacks of no more than four for 16 h. Each strain was  
172 inoculated in four replicates and was assessed on at least three separate days. Images were  
173 captured using a Canon EOS Rebel T6i camera and images measured for ethanol-dependent  
174 swarm repression.

175  
176 **Reversal rate measurements.** To measure the frequency at which a motile cell changes its  
177 direction, we used a modified version of a method that was previously described (25, 40).  
178 Briefly, overnight liquid cultures were subcultured 1:100 in five mL M63 medium and incubated  
179 at 37°C for 2 h. Once cultures reached exponential phase, they were then diluted 1:1000 in  
180 fresh M63 medium and Ficoll was added to a final concentration of 3% to obtain higher viscosity  
181 conditions that slowed the swimming cells sufficiently to allow the monitoring of reversal rates  
182 and mimic swimming in soft agar. Cells were then exposed to either control medium or medium  
183 containing 1% (v/v) ethanol for 15 min. Two hundred and fifty microliter of treated cell culture  
184 was next gently pipetted into a 35 mm glass bottom MatTek dish and a glass cover slip was

185 placed over the added culture. Four time lapse movies per strain and condition were captured  
186 with dark field using the Nikon Eclipse Ti microscope (Nikon Instruments Inc., Melville, NY)  
187 equipped with a 10X objective, a Hamamatsu ORCA-Flash 4.0 camera and Nikon NIS Elements  
188 AR 4.13.04 64 bit software. Time lapse movies were 8 s in duration with images captured at 20-  
189 25 ms intervals with RAM capture and 50 fps. Fiji ImageJ TrackMate (41) was used to process,  
190 analyze and quantify the reversal rates of 40 cells per movie. Movies were advanced frame by  
191 frame and individual cells were evaluated for the number of times they changed direction within  
192 the field of view and reversal rates were normalized and recorded as reversals per 10 s.

193  
194 **Microscopic agar motility assay.** The population percentages of motile and immobile cells  
195 were calculated in 0.3% swim agar. Overnight liquid cultures were subcultured 1:100 in five mL  
196 M63 medium and incubated at 37°C for 2 h. Once cultures reached exponential phase, they  
197 were then diluted by 1:1000 into freshly-prepared M63 swim agar (0.3%) without and with 1%  
198 (v/v) ethanol cooled to ~45°C. Two hundred and fifty microliters of each agar mixture were  
199 pipetted into a chamber slide (see Fig. 6A) and allowed to solidify and acclimate to treatment for  
200 30 min. Three to four time lapse movies per chamber slide, with two chamber slides per  
201 condition, were captured using the 40X objective on the Nikon Eclipse Ti microscope (Nikon  
202 Instruments Inc., Melville, NY) equipped with a Hamamatsu ORCA-Flash 4.0 camera and Nikon  
203 NIS Elements AR 4.13.04 64 bit software. Time lapse movies were 8 s in duration with images  
204 captured at five ms intervals with RAM capture and 100 fps. Fiji ImageJ TrackMate (41) was  
205 used to process, analyze and quantify the percentage of cells that were motile and immobile for  
206 the entire duration of each movie. Movies were advanced frame by frame and individual cells  
207 were evaluated for movement. All strains were assessed on at least two separate days.

208

209 ***In vivo* cyclic-di-GMP quantification.** C-di-GMP was measured as previously described (5)  
210 with modifications. Overnight liquid cultures were diluted 1:1000 in six mL M63 medium with  
211 either 1% ethanol or an equivalent volume of water and grown at 37°C for 16 h on a roller drum.  
212 Cultures were then adjusted to similar densities (OD<sub>600</sub>) if necessary. Five mL of each culture  
213 was pelleted at 4,500 x g for 15 min at 4°C. Cyclic-di-GMP was extracted by vigorously  
214 suspending the pellet in 250 µL of ice cold extraction buffer (40:40:20 MeOH/Acetonitrile/dH<sub>2</sub>O  
215 and 0.1 N Formic acid, stored at -20°C) and incubating at -20°C for 1 h with tubes positioned  
216 upright. Tubes were then centrifuged briefly prior to transfer of the entirety of each extraction  
217 mix to a pre-weighed ice cold 1.5 mL Eppendorf tube. Cell debris was pelleted at 15,682 x g for  
218 five min at 4°C, 200 µL of the extracted nucleotide was recovered into a clean 1.5 mL ice cold  
219 Eppendorf tube, and samples were each neutralized with 4 µL of 15% NH<sub>4</sub>HCO<sub>3</sub> per 100 µL of  
220 sample. Pellets were dried on high for 1 h and the liquid samples on low overnight using the  
221 Savant Speed Vac SC110. The pellet weights were measured to get sample dry weight and the  
222 dried liquid samples containing the extracted nucleotides were each suspended in 200 µL  
223 HPLC-grade water. Two hundred microliters of each sample was sent to RTSF Mass  
224 Spectrometry and Metabolomics Core at Michigan State University for LC-MS-MS analysis.  
225 Each strain and treatment condition was analyzed in five replicates.

226  
227 **Statistical analysis.** Unpaired Student *t* test, Two-way ANOVA with multiple comparisons, and  
228 One-way ANOVA with multiple comparisons were performed pairwise between the wild type  
229 and each strain, as well as ethanol and control conditions, using the GraphPad Prism 6 software  
230 (GraphPad, La Jolla, CA).

## 231 **Results**

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

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

251  
252 **Ethanol elicits an increase in c-di-GMP levels.** C-di-GMP is an intracellular signaling  
253 molecule that modulates motility (22, 24, 26). When c-di-GMP levels are high, motility is  
254 reduced via multiple mechanisms (6, 21, 22, 45-47). In light of the observed decrease in  
255 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  $\Delta wspR$  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,  $\Delta algD$  and  $\Delta algU$ , 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  $\Delta wspR$  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

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

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

301

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

303 Among the c-di-GMP binding effectors in *P. aeruginosa* are PilZ-domain proteins (47, 48). There  
304 are eight known PilZ-domain proteins in *P. aeruginosa*, and some of these proteins have been  
305 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 ( $\Delta flgZ$  and  $\Delta pilZ$ ; Fig. 3A). A  $\Delta flgZ\Delta pilZ$  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  $\Delta motAB$  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  $\Delta motCD$  mutant displayed a swim zone diameter that was ~90% less than that of

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

333

### 334 **Ethanol-mediated motility repression requires PilY1 and the PilMNOP Type 4 pili**

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

351

### 352 **Type 4 pili are not required for the reduction in swimming motility in response to ethanol.**

353 PilY1 is necessary for T4P activity (55), and thus we sought to determine if PilY1- and  
354 PilMNOP-dependent reduction in flagellar motility by ethanol was due to a decrease in T4P  
355 activity. Two pieces of evidence argue against a role for the T4P in ethanol-mediated effects on  
356 motility. First, a  $\Delta pilA$  mutant (which lacks pili) still showed the same level of motility and



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

361

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

373 The von Willerbrand factor A (vWA) domain of PilY1, depicted in the schematic in Fig.  
374 5A, is necessary for surface-associated swarming motility repression (23). To probe whether the  
375 same domain of PilY1 required for surface-sensing was also necessary for ethanol  
376 responsiveness, we used a strain where a mutated *pilY1* with the vWA domain deleted (*pilY1*-  
377  $\Delta vWA$ ) was placed at the native *pilY1* locus. The *pilY1*- $\Delta vWA$  strain was still responsive to  
378 ethanol-dependent motility repression (25 mm and  $20.5 \pm 0.3$  mm in the absence and presence  
379 of ethanol, respectively ( $p < 0.0001$ ; Fig. 5C). These data indicate that the vWA domain of PilY1  
380 is dispensable for ethanol-mediated swim repression.

381

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  $\mu\text{m}/\text{sec}$  (38, 53, 57) to as low as 15  $\mu\text{m}/\text{sec}$   
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  $4.6 \pm 4.1$  to  $22 \pm 9.1$   
395 reversals/ 10 s ( $p < 0.0001$ ; Fig. S7A). Similarly, the  $\Delta\text{sadC}\Delta\text{gcbA}$ ,  $\Delta\text{pilY1}$ , and  $\Delta\text{pilMNOP}$   
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  $\Delta\text{sadC}\Delta\text{gcbA}$ ) 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  $\Delta pilMNOP$  were statistically different (Fig.  
412 6B). We also noted that in the control cultures,  $\Delta pilMNOP$  was significantly higher than wild type  
413 and  $\Delta pilY1$  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 ( $38 \pm 10.5\%$  motile (control) and  $22 \pm 8.8\%$  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  $\Delta pilMNOP$  mutant, for example, had a similar proportion of motile cells  
419 in the absence and presence of ethanol ( $58 \pm 5.1\%$  and  $56 \pm 3.6\%$ , respectively; Fig. 6B).  
420 Interestingly, the  $\Delta pilY1$ ,  $\Delta pilZ\Delta flgZ$ , and  $\Delta motAB$  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  $\Delta pilY1$  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  $\Delta sadC\Delta gcbA$  double mutant was not significantly different from the wild-type strain ( $46 \pm 6.4\%$   
425 motile (control) and  $29 \pm 6.4\%$  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  $\Delta sadC\Delta gcbA$  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  $\Delta sadC$ ,  $\Delta gcbA$ , and  $\Delta sadC\Delta gcbA$  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

## 447 Discussion

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

466 We also observed an ethanol-dependent increase in global pools of c-di-GMP in  
467 planktonic cells that required the activity of the diguanylate cyclases (DGCs) SadC and GcbA. In  
468 the plate-based swim assay, the  $\Delta sadC\Delta gcbA$  double mutant no longer showed an ethanol-  
469 mediated reduction in swimming motility. However, this double mutant behaved similarly to the  
470 wild type in the microscopic swim assay; we do not fully understand the basis for this  
471 discrepancy. The differences in swim zone diameters may be the consequence of a  
472 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  $\Delta sadC\Delta gcbA$  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  $\beta$ -propeller  
493 structure that shares structural similarity to the quinoxinoprotein 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

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

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

522 To conclude, our findings indicate that ethanol triggers a complex response that  
523 modulates behaviors related to biofilm initiation in order to facilitate the transition from being  
524 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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742 Acad Sci U S A 86:6973-7.



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  $\Delta flgK$  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,  $\Delta sadC$ ,  $\Delta gcbA$ ,  $\Delta wspR$ ,  $\Delta sadC\Delta wspR$ ,  
756  $\Delta gcbA\Delta wspR$ , and  $\Delta sadC\Delta gcbA$  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  $\Delta sadC\Delta gcbA$ , 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,  $\Delta flgZ$ ,  $\Delta PA14\_00130$ ,  
766  $\Delta PA14\_60970$ ,  $\Delta PA14\_27930$ ,  $\Delta PA14\_56180$ ,  $\Delta alg44$ ,  $\Delta PA14\_25420$  and  $\Delta pilZ$  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,  $\Delta pilZ$ ,  $\Delta flgZ$  and  $\Delta pilZ\Delta flgZ$  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,  $\Delta flgK$ ,  $\Delta motAB$  and  $\Delta motCD$  in swim agar  
776 without (grey) and with (black) 1% ethanol after 16 h. Error bars indicate standard deviations,  
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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,  $\Delta pilY1$  and  $\Delta pilMNOP$  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,  $\Delta pilY1$ ,  $\Delta pilY1$  with an empty vector (EV),  
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789 (*pilY1- $\Delta$ vWA*) in an arabinose-inducible expression plasmid in swim agar without (grey) and with  
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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,  $\Delta pilY1$  and  $\Delta pilMNOP$  grown in liquid M63 medium without (grey) and with

794 (black) 1% ethanol for 16 h. Error bars indicate standard deviations, n=8 replicates. \*, *P*-value  
795 <0.05; \*\*\*\*, *P*-value <0.0001 as determined by Two-way ANOVA with multiple comparisons.  
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797 significantly different (*P*-value <0.05).

798

799 **FIG. 6. Ethanol decreases the number of motile cells in swim agar, in a manner**

800 **dependent on PilY1, PilMNOP, FlgZ, PilZ, and MotAB** (A) Schematic of agar motility assay

801 treated with water (control) or 1% ethanol in swim agar. Sample was mixed well then 250  $\mu$ L

802 was pipetted onto a glass slide and staples were used to create a chamber using a glass

803 coverslip. Sample was then incubated at room temperature for 30 min, and then imaged as

804 outlined in the methods. (B) Agar motility assay of *P. aeruginosa* PA14 wild type,  $\Delta sadC\Delta gcbA$ ,

805  $\Delta pilZ\Delta flgZ$ ,  $\Delta motAB$ ,  $\Delta pilY1$  and  $\Delta pilMNOP$  in swim agar without (blue) and with (red) 1%

806 ethanol after 30 min. Three time lapse (8 s) movies of the cells in the agar matrix, for each

807 sample, were captured. Plotted is the average population percentage of the motile

808 subpopulation in each movie. Error bars represent the maximum and minimum data point, n $\geq$ 6

809 replicate movies. Shaded boxes represent the ethanol samples that are significantly different

810 from their controls. \*, *P*-value <0.05; \*\*, *P*-value <0.01; \*\*\*, *P*-value <0.001; ns, not significant as

811 determined by One-Way ANOVA with multiple comparisons.

812

813 **FIG. 7. Ethanol effects on swarming motility repression occur using some surface-**

814 **sensing components as well as components independent of surface sensing.**

815 Representative images of swarming motility assays of (A) *P. aeruginosa* PA14 wild type,  $\Delta pilY1$ ,

816  $\Delta pilY1$  with an empty vector (EV) or with a plasmid that enables arabinose-inducible expression

817 *pilY1* (*PpilY1*) or *pilY1* without a vWA domain (*pilY1*- $\Delta vWA$ ), (B) *P. aeruginosa* PA14 wild type

818 and  $\Delta pilMNOP$ , (C) *P. aeruginosa* PA14 wild type and  $\Delta motAB$ , (D) *P. aeruginosa* PA14 wild

819 type,  $\Delta flgZ$ ,  $\Delta pilZ$ , and  $\Delta pilZ\Delta flgZ$ , and (E) *P. aeruginosa* PA14 wild type,  $\Delta sadC$ ,  $\Delta gcbA$ , and

820  $\Delta sadC\Delta gcbA$  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.

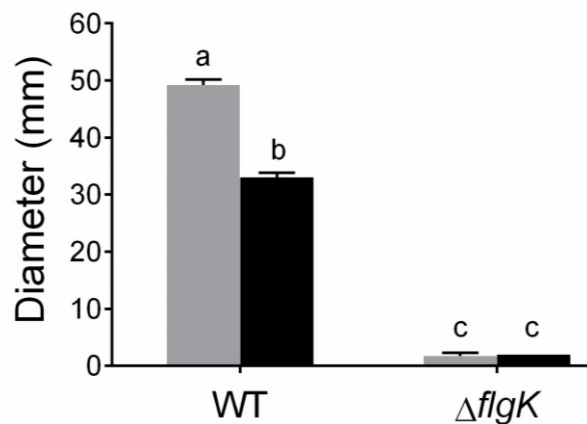
823

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825 swimming *P. aeruginosa* encounters ethanol in a liquid environment, it quickly responds by  
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833 swarming motility.

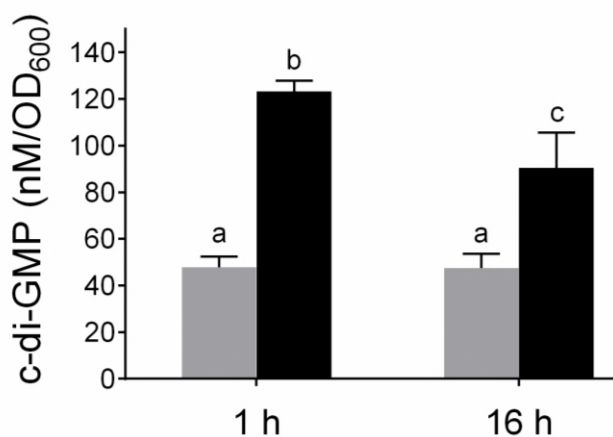
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## Figures and Legends

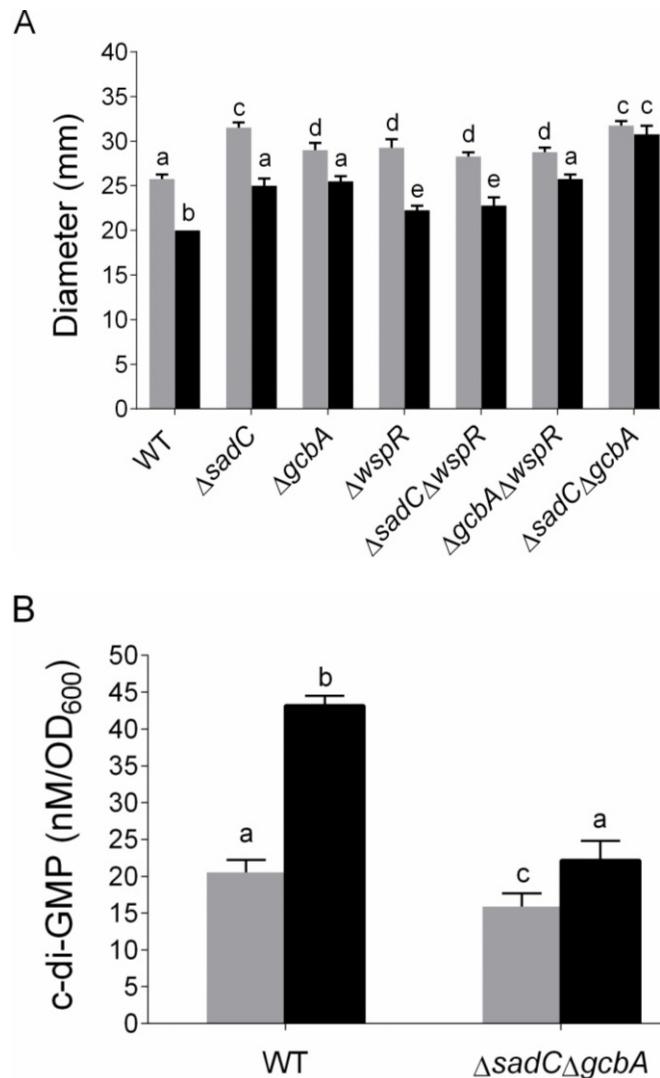
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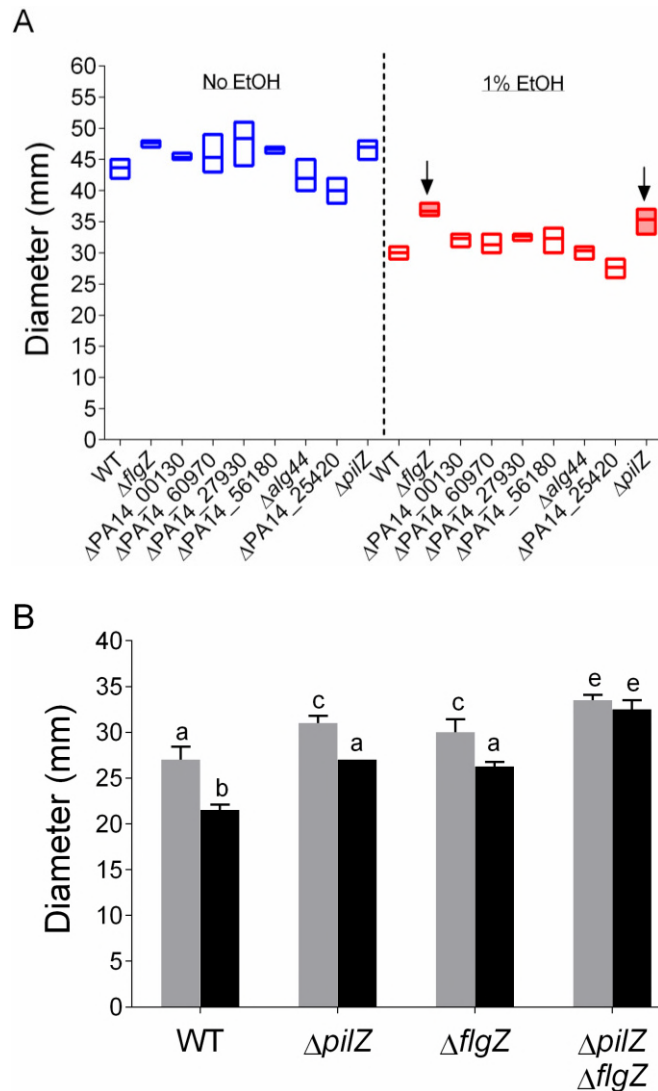
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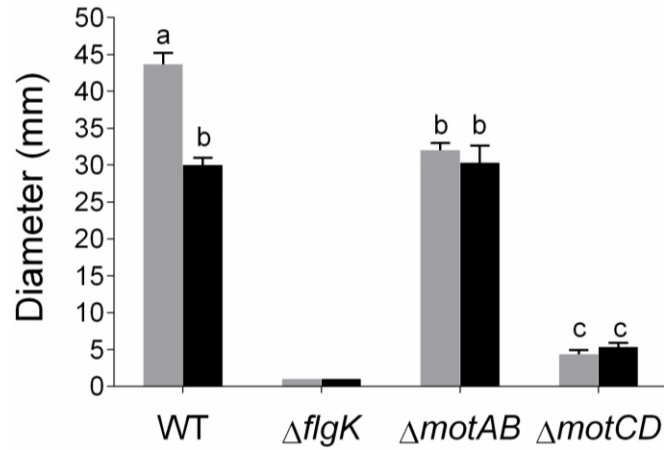
**FIG. 1. Ethanol decreases swim zone diameter and elicits an early and sustained increase in c-di-GMP.** (A) Swim zone diameter of *P. aeruginosa* PA14 wild type and  $\Delta flgK$  in M63 with 0.3% agar (swim agar) without (grey) and with (black) 1% ethanol after 16 h. Bars depict the average swim 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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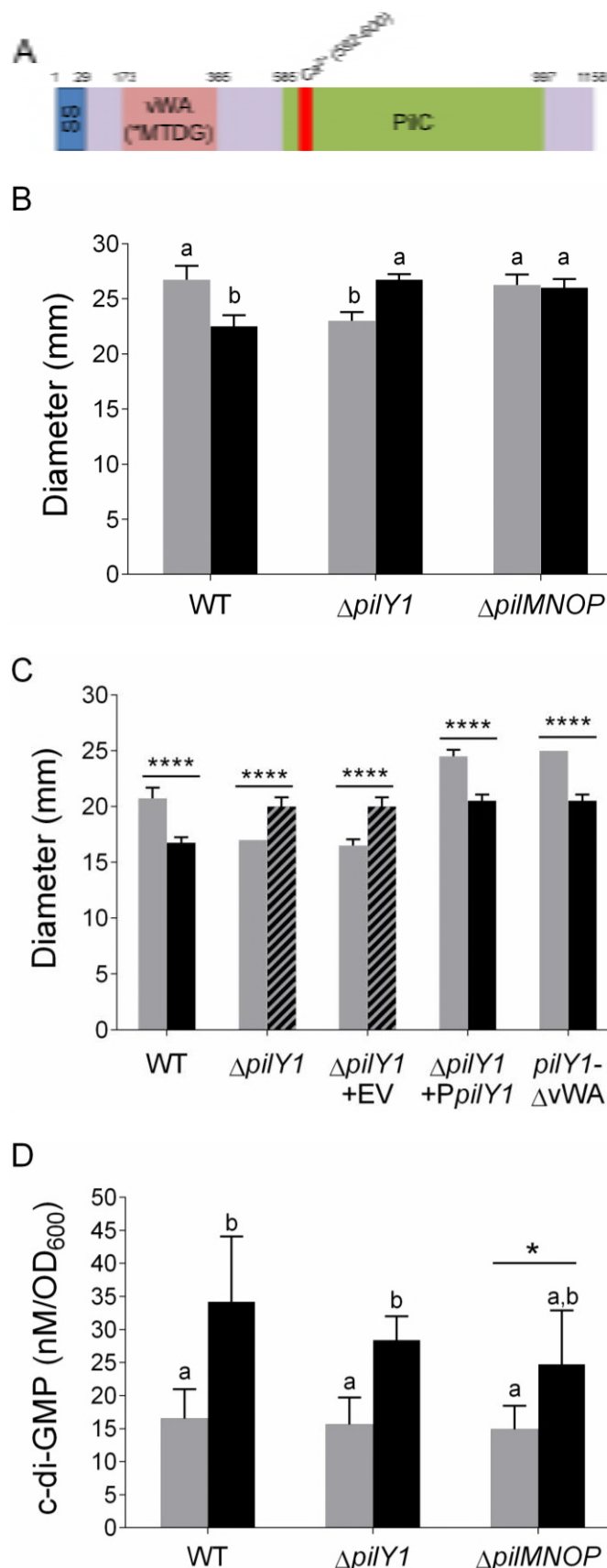


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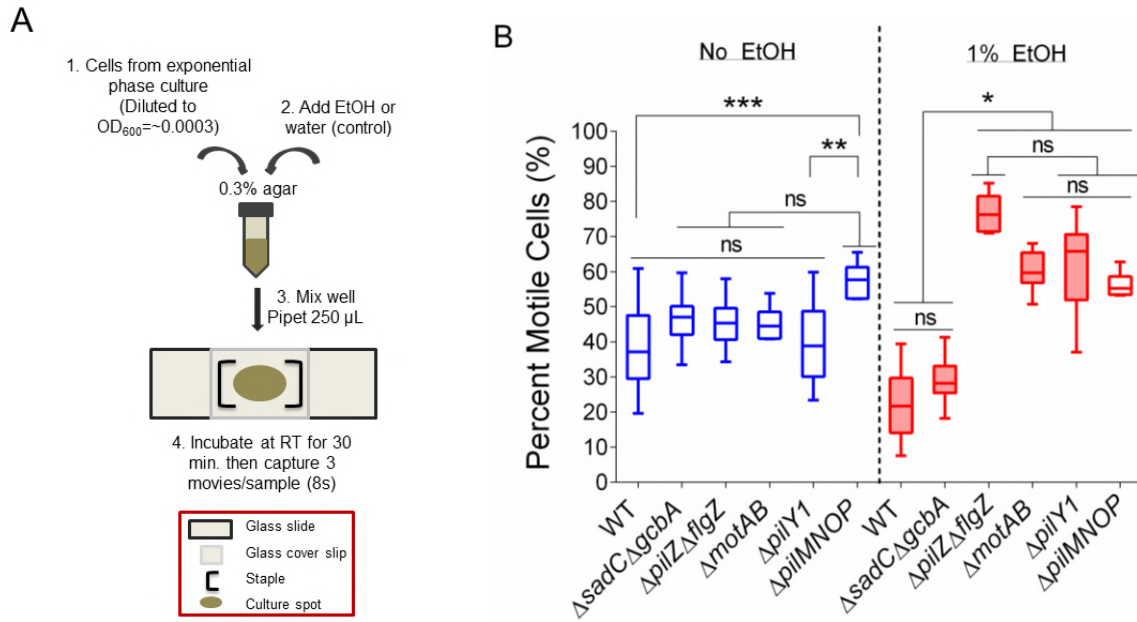
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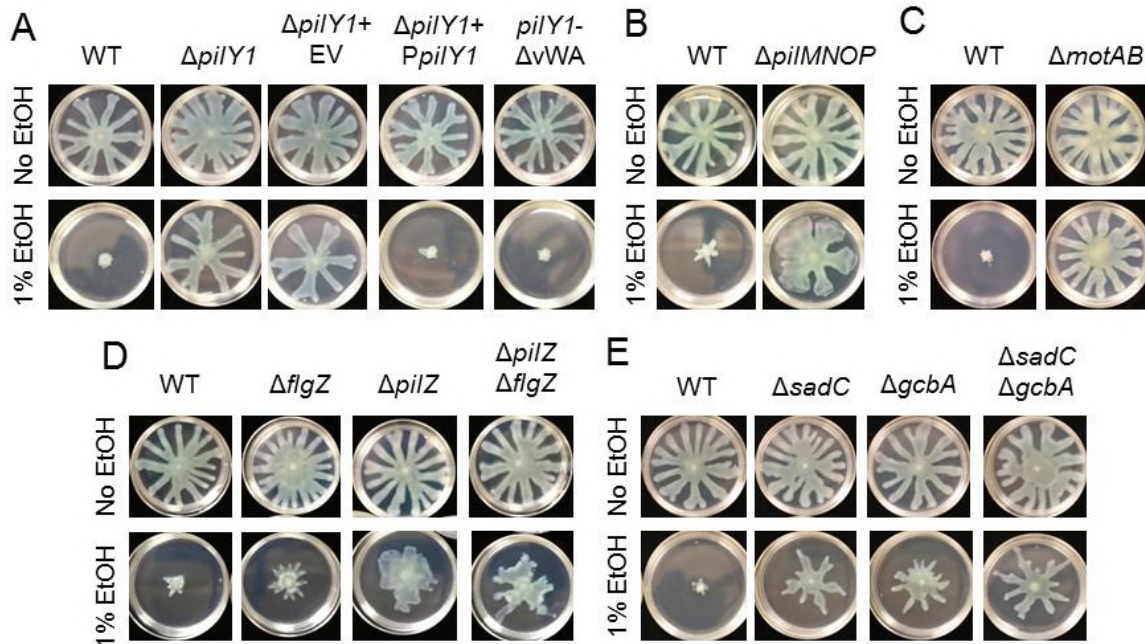
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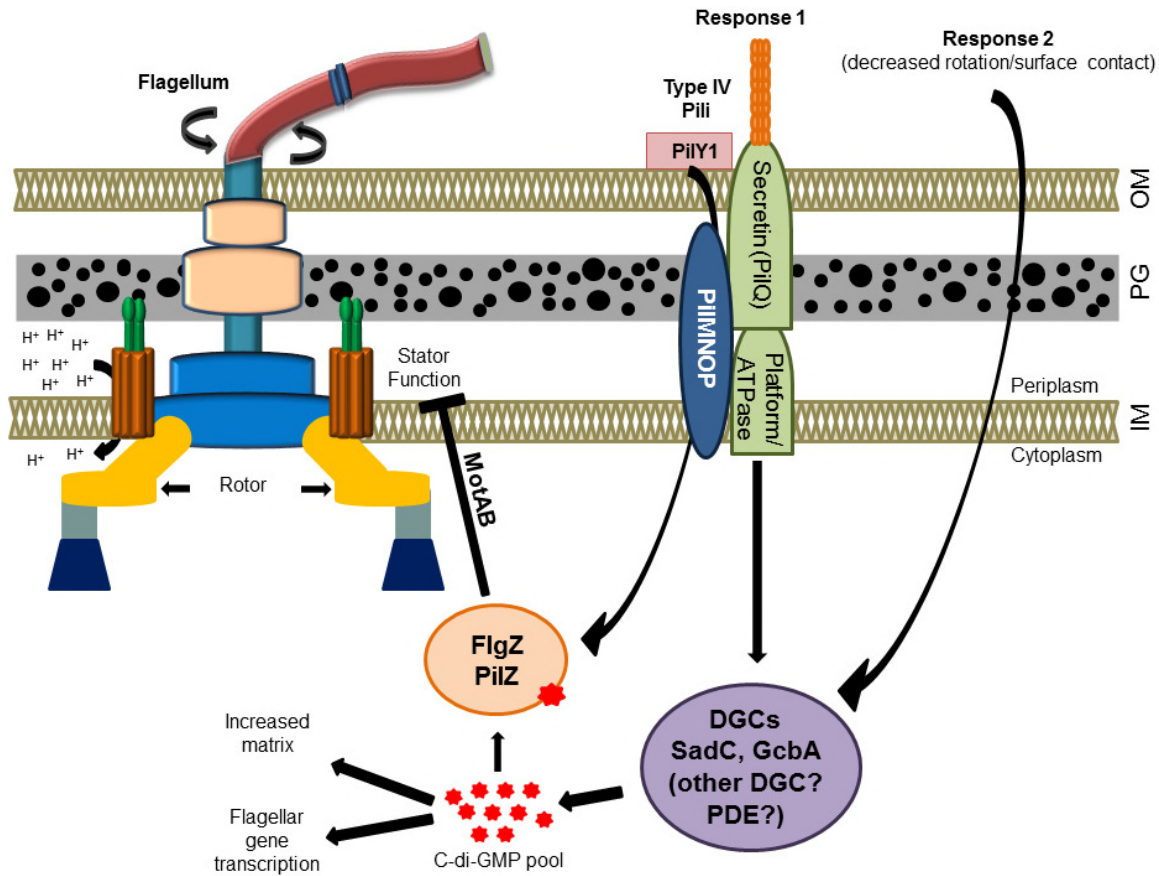
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