

1 **c-di-GMP-linked phenotypes are modulated by the interaction between a diguanylate**  
2 **cyclase and a polar hub protein**

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14 Running title: FimV localizes and modulates DgcP activity

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## 22 **Abstract**

23 c-di-GMP is a major player in the decision between biofilm and sessile lifestyles.  
24 Several bacteria present a large number of c-di-GMP metabolizing proteins, thus a fine-tuning  
25 of this nucleotide levels may occur. It is hypothesized that some c-di-GMP metabolizing  
26 proteins would provide the global c-di-GMP levels inside the cell whereas others would  
27 maintain a localized pool, with the resulting c-di-GMP acting at the vicinity of its production.  
28 Although attractive, this hypothesis was yet to be proven in *Pseudomonas aeruginosa*. We  
29 found that the diguanylate cyclase DgcP interacts with the cytosolic region of FimV, a  
30 peptidoglycan-binding protein involved in type IV pilus assembly. Moreover, DgcP is located  
31 at the cell poles in wild type cells, but scattered in the cytoplasm of cells lacking FimV.  
32 Overexpression of DgcP leads to the classical phenotypes of high c-di-GMP levels (increased  
33 biofilm and impaired motilities) in the wild-type strain, but not in a  $\Delta fimV$  background.  
34 Therefore, our findings strongly suggest that DgcP is regulated by FimV and may provide the  
35 local c-di-GMP pool that can be sensed by other proteins at the cell pole, bringing to light a  
36 specialized function for a specific diguanylate cyclase.

## 37 **Importance**

38 Bacteria can switch between sessile and motile lifestyles and c-di-GMP is a crucial second  
39 messenger for this decision. They present several proteins that can synthesize, degrade and  
40 sense this molecule and it is intriguing why so many apparently redundant proteins are  
41 needed. An idea is that some of these proteins may act at specific subcellular locations,  
42 leading to the formation of localized c-di-GMP pools that may control the assembly and  
43 function of polar-localized machineries such as pili and flagella. We found that DgcP, a c-di-  
44 GMP synthesizing enzyme, interacts with the type IV pili hub located at the cell poles, and this

45 interaction modulates c-di-GMP-related phenotypes. Our findings support the hypothesis that  
46 c-di-GMP acts on targets near the spot where it is produced.

## 47 **Introduction**

48 Over the past decades, (3'-5')-cyclic diguanylic acid (c-di-GMP) has been  
49 characterized as an important second messenger in bacteria. The concentration of c-di-GMP  
50 within the cell is associated with cellular behavior: high c-di-GMP levels are linked to biofilm  
51 formation and low levels to the motile planktonic lifestyle (1, 2). This molecule is synthesized  
52 from GTP by a class of enzymes known as diguanylate cyclases (DGC) bearing a conserved  
53 GGDEF domain (3). The c-di-GMP hydrolysis reaction is performed by phosphodiesterases  
54 (PDE) with EAL or HD-GYP domains, which cleave c-di-GMP to pGpG or GMP, respectively  
55 (4, 5). Multiple genes coding for the c-di-GMP-metabolizing proteins are found in a variety of  
56 bacterial genomes. A puzzling question in the study of c-di-GMP signaling is how the bacterial  
57 cell integrates the contributions of multiple c-di-GMP-metabolizing enzymes to mediate its  
58 cognate functional outcomes. Merritt and collaborators showed that the *P. aeruginosa*  
59 phenotypes controlled by two different DGC have discrete outputs despite the same level of  
60 total intracellular c-di-GMP (6). These data support the model in which localized c-di-GMP  
61 signaling contributes to the action of proteins involved in the synthesis, degradation, and/or  
62 binding to a downstream target (2). Studies of c-di-GMP signaling regulation during the  
63 swarmer to stalked-cell transition in *Caulobacter crescentus* also supports this hypothesis. In  
64 this dimorphic bacterium, PleD is a DGC that is inactive in swarmer cells and is activated  
65 during the swarmer-to-stalked cell differentiation (7, 8). Activation of PleD is coupled to its  
66 subcellular localization at the stalk pole, suggesting that PleD activates nearby downstream  
67 effectors involved in pole remodeling (9). Opposite to PleD, the EAL domain protein TipF

68 localizes at the swarmer pole, where it contributes to the proper placement of the motor  
69 organelle in the polarized predivisional cell (10).

70 Even though a large body of research on c-di-GMP regulation in *P. aeruginosa* is  
71 available, it is still unclear whether compartmentalization of c-di-GMP signaling components is  
72 required to mediate an appropriate c-di-GMP signal transduction. The genome of *P.*  
73 *aeruginosa* strain PA14 presents forty genes coding for proteins associated with c-di-GMP  
74 metabolism (11, 12). Some of these proteins were already characterized and a few of them  
75 present a specific localization within the cell. For instance, the DGC WspR is associated to  
76 contact-dependent response to solid surfaces. Activation of the Wsp system by contact leads  
77 to the formation of subcellular clusters of WspR followed by synthesis of c-di-GMP, increasing  
78 exopolysaccharide production and biofilm formation (13). The DGC SadC is a central player in  
79 Gac/Rsm-mediated biofilm formation (14) and influences biofilm formation and swarming  
80 motility via modulation of exopolysaccharide production and flagellar function (15). Recently, it  
81 was demonstrated that SadC activity is promoted by membrane association, with the  
82 formation of active DGC oligomers (16). The PDE DipA/Pch is essential for biofilm dispersion  
83 (17) and promotes c-di-GMP heterogeneity in *P. aeruginosa* population (18). This PDE is  
84 partitioned after cell division and is localized to the flagellated cell pole by the chemotaxis  
85 machinery. This asymmetric distribution during cell division results in a bimodal distribution of  
86 c-di-GMP (18).

87 Previously, we demonstrated that PA14\_72420 is an enzymatically active DGC that  
88 increased imipenem fitness (19). Although this protein has been the subject of recent  
89 publications that named it as DgcP (20), its molecular function has not yet been addressed.  
90 Thus, we decided to pursue its role by seeking for DgcP interaction partners that could  
91 participate in the same signaling pathway. DgcP was found to interact with the inner

92 membrane protein FimV, which has a regulatory role in type IV pilus (T4P) function.  
93 Moreover, we determined that DgcP localizes to cell poles in a FimV-dependent manner and  
94 is more active when the FimV protein is present. We suggest that the DgcP regulation by  
95 FimV may provide a local c-di-GMP pool at the cell pole, making this second messenger  
96 available for the c-di-GMP binding proteins that may regulate the machineries associated with  
97 the cell motility, such as the flagellum and pili.

## 98 **Results**

99 **DgcP interacts with FimV.** One of the interesting paradoxes of signal transduction by  
100 c-di-GMP is the redundancy of DGCs and PDEs in bacterial cells. It has been shown that  
101 distinct phenotypes are controlled by specific DGCs or PDEs (2, 6). The specificity of DGCs  
102 and PDEs has been proposed to be related to protein localization, allowing the regulation of  
103 subcellular pools of c-di-GMP close to a target receptor (6, 21). Therefore, we sought, using  
104 bacterial two-hybrid system, for interaction partners of DgcP that could give a hint of DgcP  
105 localization and function. The bait plasmid pKT25\_*dgcP* was constructed with the complete  
106 *dgcP* coding region (**Fig. 1A**) and co-transformed in BTH101 *E. coli* cells with a *P. aeruginosa*  
107 fragment prey library cloned in the pUT18C plasmid (22). About 100,000 co-transformants  
108 were obtained and 40 positive red colonies identified. All positives clones were retested by  
109 individually transforming different pUT18 derivative preys and the pKT25\_*dgcP* or pKT25  
110 empty vector plasmids into BTH101 cells. From the 40 clones initially obtained, 26 were  
111 confirmed and the inserts were further identified by DNA sequencing. Only two clones  
112 presented *in frame* inserts, one of them corresponding to a short peptide fragment (36 amino  
113 acids) of the cytoplasmic, C-terminal portion of FimV (**Fig. 1B**, red box). FimV is a large  
114 protein (924 amino acids, 97 kDa) containing a periplasmic domain with a peptidoglycan-  
115 binding LysM motif connected via a single transmembrane segment to a highly acidic

116 cytoplasmic domain with three predicted protein-protein interaction tetratricopeptide repeat  
117 (TPR). FimV is part of the T4P secretion machinery in *Pseudomonas* (23) and is required for  
118 localization of some T4P assembly components to the cell pole (24). As T4P is important for  
119 initial cell attachment and the  $\Delta dgcP$  mutant is impaired in this trait (20), we decided to further  
120 investigate the interaction of FimV with DgcP. Two constructions, one containing the FimV  
121 cytoplasmic region and the other, a smaller cytoplasmic fragment were cloned into pUT18  
122 plasmid (**Fig. 1B**). After co-transformation of each of these plasmids with pKT25\_*dgcP*, we  
123 confirmed that DgcP interacts with the cytoplasmic region of FimV (**Fig. 1C and D**). Stronger  
124 interactions were observed with the constructs containing the prey fragment or the full  
125 cytoplasmic region.

126 **DgcP localizes at the cell poles.** Type IV pili are surface appendages that localize at  
127 the cell poles in *P. aeruginosa*. There are several polar localized proteins involved in the  
128 assembly and regulation of the T4P system and FimV is one of them (23, 25, 26). Due to its  
129 interaction with FimV, we asked whether DgcP would also localize at the cell poles. Indeed,  
130 when cells overexpresses the fusion protein DgcP-msfGFP, polar fluorescent foci were  
131 observed in virtually 100% of both PA14 (not shown) and  $\Delta dgcP$  strains (**Fig. 2A**). This  
132 localization is lost when DgcP-msfGFP is expressed in a  $\Delta fimV$  mutant, where the  
133 fluorescence is scattered throughout the cells (**Fig. 2B and C**). To narrow the DgcP region  
134 important to the interaction, we use two different DgcP-msfGFP fusions, one containing the  
135 first 199 amino acids (N-ter-DgcP-msfGFP) and the other the last 461 amino acids (C-ter-  
136 DgcP-msfGFP) (**Fig. 2A**). The C-terminal fusion fluorescence was scattered in the cells (not  
137 show) and cells overexpressing the N-ter-DgcP-msfGFP presented a growth defect,  
138 precluding its analysis. Therefore, we cannot determine precisely which region of DgcP is  
139 important to polar localization.

140 As the DgcP C-terminal construct is not localized and there are no predicted domains  
141 in the N-terminal region, we aligned 85 sequences of putative DgcP orthologues from 75  
142 species, including 53 Pseudomonadaceae and 32 other proteobacteria, such as Vibrionales  
143 and Alteromonadales (Table S2). We found that the N-terminal region is composed by two  
144 separate conserved segments (**Fig. S1**) and both or one of them may be responsible for  
145 interaction with FimV, which homologues are present in all those bacterial genomes.

146 **DgcP plays a role in biofilm formation.** The role of DgcP in biofilm formation has  
147 been investigated by different groups under different conditions. Kulasekara and collaborators  
148 showed that mutation in DgcP abolished biofilm formation in LB medium (11) and Ha and  
149 collaborators showed that a *dgcP* mutation did not affect biofilm formation in M63 minimum  
150 medium (27). Aragon and collaborators demonstrated that deletion of *dgcP* orthologues in  
151 *Pseudomonas savastanoi* pv. *savastanoi* and *P. aeruginosa* PAK indeed decreased biofilm  
152 formation in LB (20). Here, we confirmed that the PA14  $\Delta$ *dgcP* mutant is impaired in biofilm  
153 formation in the rich medium LB, but minor differences were observed in minimal M63  
154 medium (**Fig. 3A**). These results are in agreement with the differences observed by the two  
155 previous studies (11, 27). The expression in trans of *dgcP* restores the phenotype of biofilm  
156 defect on  $\Delta$ *dgcP* (**Fig. 3B** and **C**). In LB,  $\Delta$ *dgcP* was not able to form a biofilm and just a few  
157 adherent cells were observed by confocal laser scanning microscopy (CLSM) after 16h post-  
158 inoculation, while the wild type PA14 biofilm was mature at this time point (**Fig. 3C**). At 72  
159 hours,  $\Delta$ *dgcP* biofilm was thin and undifferentiated (**Fig. 3D**). As FimV is also involved in  
160 biofilm formation (**Fig. 5**), this is another indication that they have complementary roles in the  
161 cells, probably related to the T4P function.

162 **DgcP has a role in twitching motility.** *P. aeruginosa* utilizes T4P to move across solid  
163 surfaces in a process known as twitching motility. As T4P is regulated by FimV, we decided to

164 investigate if DgcP is important for twitching. A small portion of the outer edge of the bacterial  
165 streak was taken and stabbed into the bottom of the agar plate or placed on a thin layer of  
166 solidified media and covered with a glass coverslip. Cells were incubated and active colony  
167 expansion occurred at the interstitial interface. Twitching motility was analyzed by staining the  
168 plates with crystal violet after 16h (**Fig. 4A** and **B**) or by phase contrast microscopy after 4  
169 hours of colony expansion (**Fig. 4C**). As expected, the  $\Delta dgcP$  mutant presented decreased  
170 twitching motility with a less defined structure whereas PA14 presented a well-defined lattice-  
171 like network. The *fimV* mutant was not able to perform the twitching motility (**Fig. 4**), as  
172 expected. Initiation of biofilm formation was also analyzed after five hours of adhesion of cells  
173 on a silicon slide at the air-liquid interface. The cultures were adjusted to an  $OD_{600} = 0.05$  in  
174 M63 minimum medium supplemented with glucose and casamino acids. The silicon slide was  
175 placed upright in a culture tube and cells at the air-liquid interface were analyzed by field  
176 emission scanning electron microscope (FESEM), after different time points (**Fig. 4D**). PA14  
177 early biofilm presented an irregular architecture related to the motility of the initial adhering  
178 cells, but only round microcolonies that did not expand on the surface were observed for the  
179  $\Delta dgcP$  mutant (**Fig. 4D**). These results show that DgcP is important to early stages of biofilm  
180 formation and twitching motility.

181 **DgcP activity is FimV dependent.** Here we observed that the FimV protein localizes  
182 the diguanylate cyclase DgcP at the cell pole (**Fig. 2**). Thus, we asked whether DgcP activity  
183 could be regulated by FimV. To answer this question, we overexpressed the DgcP-msfGFP in  
184 the  $\Delta fimV$  mutant and analyzed the phenotypes related to c-diGMP. Overexpression of the  
185 DGCs DgcP and WspR fused to msfGFP in PA14 increases biofilm formation and decreases  
186 swimming motility, indicating that these fusions are functional. Both DGCs also complement  
187 the  $\Delta dgcP$  mutation, but DcgP-msfGFP is not able to increase biofilm formation or decrease



188 swimming motility in the  $\Delta fimV$  background, suggesting that it needs FimV for full activity. This  
189 is not observed for WspR-msfGFP, which has the same effect with or without FimV in the  
190 cells. Overexpression of the C-terminal portion of DgcP (pDgcP-Cterm) that does not localize  
191 to the cell poles has no effect in biofilm formation and swimming motility (**Fig. 5A and B**) in all  
192 strains tested, even though there is an increase in overall c-di-GMP levels (**Fig. 5C**),  
193 suggesting that localization of the diguanylate cyclase activity is important for those  
194 phenotypes. Moreover, overexpression of a mutated DgcP in the diguanylate cyclase motif  
195 (GGEEF to GGAAF) decreases biofilm formation in the wild type PA14, but has no effect in  
196 both  $\Delta dgcP$  and  $\Delta fimV$  backgrounds (**Fig. 5A and B**). DGCs are dimeric proteins therefore  
197 the GGAAF mutation may act as a negative dominant on the wild type DgcP.

198 CdrA is a extracellular protein considered as a scaffold for the biofilm extracellular  
199 matrix and transcription of *cdrA* is c-di-GMP-dependent, via FleQ (28) and it is widely used as  
200 a reporter of c-di-GMP levels (29). Overexpression of DgcP-msfGFP leads to ~10-fold  
201 increased *cdrA* mRNA levels in PA14 and  $\Delta dgcP$ , but only fourfold in the  $\Delta fimV$  strain (**Fig.**  
202 **5D**).The quantitation of c-di-GMP agrees with the *cdrA* expression levels (**FIG. 5C**).  
203 Exopolysaccharide (EPS) is also an indication of c-di-GMP levels in several bacteria (30, 31).  
204 DgcP and FimV were overexpressed alone or in combination in *Escherichia coli* cells and the  
205 production of EPS was assessed in Congo red plates. FimV overexpression does not result in  
206 EPS production, but colonies overexpressing DgcP have a pale tint. When both proteins are  
207 overexpressed together, EPS production increases, resulting in pink colonies (**Fig. 5E**).  
208 Altogether, our results corroborate the hypothesis that the polar localization of DgcP by FimV  
209 also regulates its activity and that DgcP may contribute to a local c-di-GMP pool .

210

211

## 212 Discussion

213 Recently, Aragon and collaborators showed that DgcP is a well conserved DGC protein  
214 in Pseudomonads related to plant and human infections (20). Previously, we showed that  
215 overexpression of this protein alters biofilm formation, swimming and swarming motilities as  
216 well as imipenem fitness, due to reduced levels of OprD (19). However, we could not  
217 conclude that those phenotypes were specifically related to the physiological role of DgcP,  
218 because it was assumed that the overexpression of a DGC increases the global c-di-GMP  
219 levels. Herewith, we used protein-protein interactions, characterization of a deletion mutant  
220 and protein localization to look for the specific function of DgcP.

221 *P. aeruginosa* possesses polar T4P which are used for twitching motility and adhesion  
222 (32), essential traits for mature biofilm architecture. Assembly of T4P at normal conditions  
223 requires FimV (23), which shares similar domain organization with *Vibrio cholerae* HubP.  
224 These proteins, despite low overall sequence similarity, present a conserved N-terminal  
225 periplasmic domain required for polar targeting, and a highly variable C-terminal acidic  
226 cytoplasmic region, implicated in protein-protein interactions. HubP is required for polar  
227 localization of the chromosomal segregation and chemotactic machineries (33, 34). We found  
228 that DgcP is present only at the cell poles and that this pattern is dependent on FimV. Thus,  
229 we hypothesized that DgcP localization is important for the formation of a localized c-di-GMP  
230 pool at the cell poles that would assist the assembly and/or function of the T4P apparatus or  
231 other pole-localized organelles (**Fig. 6**). We suggest that DgcP could be one of the sources of  
232 c-di-GMP required for pilus biogenesis in *P. aeruginosa* and probably in other gamma-  
233 proteobacteria that carry DgcP and FimV homologs.

234 Assembly of *P. aeruginosa* T4P at normal conditions requires FimX, a polarly localized  
235 c-di-GMP binding protein (35) that has degenerate DGC and PDE domains and seems to be

236 enzymatically inactive (36). It is possible that binding of c-di-GMP to the EAL domain of FimX  
237 implicates it as an effector protein rather than a PDE. The FimX homolog in *Xanthomonas citri*  
238 interacts with a PilZ protein required for surface localization and assembly of pilin, but does  
239 not bind c-di-GMP (37). *X. citri* PilZ subsequently interacts with PilB, an ATPase required for  
240 T4P polymerization. This cascade of protein-protein interactions likely conveys the presence  
241 of c-di-GMP to PilB, inactivating it and turning motility off (38, 39). Remarkably, suppressor  
242 mutations in a *P. aeruginosa fimX* mutant that restored T4P biogenesis and partially restored  
243 twitching motility also increased c-di-GMP levels. However, the suppressor mutant cells  
244 presented peritrichously localized pili (35), indicating that a more specific source of c-di-GMP  
245 would be needed for the correct assembly of the machinery at the cell poles. Similarly, a *P.*  
246 *aeruginosa* PilZ domain protein is also involved in the positive regulation of T4P-based  
247 twitching motility and does not bind to c-di-GMP (32), suggesting a conserved mechanism.  
248 Cumulatively, these findings imply that the molecular mechanisms of pilus protrusion and  
249 retraction are regulated by local fluctuations of c-di-GMP levels. Other polar localized  
250 structures, such as flagella and the chemotactic machinery also bind directly or indirectly to c-  
251 di-GMP (40, 41), and DgcP may have a function in those mechanisms as well, but further  
252 work would be needed to uncover such roles.

253 *P. aeruginosa* T4P was demonstrated to be important not only to attach and move, but  
254 also to sense mechanical features of the environment. T4P sensing on solid surface  
255 increases its extension and retraction frequencies and cAMP production, leading to the  
256 upregulation of the cAMP/Vfr-dependent pathway (42). Recently, FimV was associated with  
257 this process by interaction with FimL, a scaffold protein that connects T4P with the Chp  
258 chemosensory system via interaction with PilG and FimV (25, 26). FimV is also involved in the  
259 transcriptional upregulation of *pilY1*, and PilY1 increases the SadC diguanylate cyclase

260 activity upon surface contact (43). However, SadC presents localized foci at the poles, in the  
261 middle of cells and between these two locations (16), suggesting that it may have a broader  
262 role. Thus, we suggest that an outside signal could be sensed by T4P and transduced by  
263 FimV as described for SadC, resulting in the direct and localized activation of c-di-GMP  
264 production by DgcP. The finding that FimV-DgcP interact at the poles is an important step  
265 towards the understanding of how c-di-GMP localized pools are formed, controlling the spatial  
266 activity of target proteins.

267

## 268 **Experimental procedures**

269 **Bacterial strains, plasmids and growth conditions.** The bacterial strains and  
270 plasmids used in the study are described in the Supplementary **Table S2**. For routine cell  
271 cultures, bacteria were grown aerobically in Luria–Bertani (LB) broth or LB agar at 37 or 30°C.  
272 Ampicillin (100 µg/ ml), kanamycin (50 µg/ml) or gentamicin (10 µg/ml) were added to  
273 maintain the plasmids in *E. coli*. Carbenicillin (300 µg/ ml), kanamycin (250 µg/ml) or  
274 gentamicin (50 µg/ml) were added to maintain the plasmids in *P. aeruginosa*. For the pJN105  
275 related constructs (**Table S1**), arabinose was added to cultures at 0.2 % final concentration.  
276 Both M8 (44) and M63 (45) minimal salt media were supplemented with 1mM MgSO<sub>4</sub>, 0.2 %  
277 glucose and 0.5 % casamino acids (CAA). To visualize bacterial two-hybrid interactions on  
278 solid medium, MacConkey indicator medium (Difco) supplemented with 1 % maltose and 100  
279 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside), herein designated MacConkey medium,  
280 was used.

281 **General molecular techniques.** DNA fragments were obtained by PCR using Q5 DNA  
282 polymerase (NEB). Oligonucleotide primers were purchased from Life Technologies and the  
283 sequences are listed in **Table S1**. PCR products of the expected sizes were purified from gels  
284 using GeneJET™ Gel Extraction Kit (Thermo Scientific), cloned using the SLIC method (46)  
285 and transformed into *E. coli* DH5α (**Table S1**). Plasmid purification was performed with  
286 GeneJET Plasmid Miniprep kit (Thermo Scientific). Sequencing was carried out using the Big  
287 Dye terminator cycle sequencing kit (Applied Biosystems) using the facility of the  
288 Departamento de Bioquímica, IQ-USP (SP, Brazil).

289 To construct unmarked in-frame deletions, the upstream and downstream regions of  
290 the target gene were amplified and cloned into the pEX18Ap (*fimV*) or pKNG (*dgcP*). The  
291 resulting constructs were used to delete target genes on wild type PA14 genome by

292 homologous recombination. To construct the pDgcP plasmid the *dgcP* coding region was  
293 cloned in frame with a synthetic *msfGFP* gene into the pJN105 plasmid. The *msfGFP* codes  
294 for a N-terminal 40 amino acids spacer and a C-terminal monomeric super fold GFP. All  
295 vectors and constructs are described in more detail in **Table S1**.

296 **Biofilm assays.** Three different biofilm assays were performed. The microtiter dish  
297 biofilm formation assay was performed as described (47). The biofilms observed by confocal  
298 laser scanning microscopy (CLSM) were grown in 8-well chamber slides and stained with  
299 DAPI as described (48) and imaged using a Zeiss - LSM 510-Meta. For the early stages of  
300 biofilm formation on silicon wafers, cultures were adjusted to an O.D<sub>600</sub> = 0.05 in M63 medium  
301 and transferred to a 24-well plate where silicon slides (2×1 cm<sup>2</sup>) were placed upright in each  
302 well. Before using the silicon substrates, they were previously cleaned by ultra-sonication for  
303 a period of 15 min each in acetone, isopropanol and distilled water, respectively. Slides were  
304 dried under N<sub>2</sub> flow and subsequently treated with O<sub>2</sub> plasma at 100 mTorr for 15 min (720 V  
305 DC, 25 mA DC, 18 W; Harrick Plasma Cleaner, PDC-32G). After 60, 180 or 300 minutes the  
306 slides were rinsed three times with water, fixed with 4% paraformaldehyde for 1h and  
307 analysed by field-emission scanning electron microscopy (FESEM; model F50, FEI Inspect)  
308 operated at 2 keV. Prior to examination, samples were coated with sputtered gold to prevent  
309 electrical charging.

310 **High-throughput two-hybrid assays.** PAO1 two-hybrid library (22) was tested against  
311 the pKT25\_DgcP bait. Basically, 25–50 ng of pUT18 library was transformed into BTH101  
312 cells carrying the pKT25\_DgcP vector and plated on MacConkey medium for 48–96 h at 30  
313 °C. Red colonies were picked up and restreaked on MacConkey plates. The positive colonies  
314 were cultivated in liquid medium, and plasmids were isolated and further analyzed. The  
315 candidate preys were retested individually for interaction with the bait by retransforming

316 pUT18 derivative prey and pKT25 bait plasmids into BTH101 cells and also the pUT18  
317 derivative preys and the pKT25 empty vector. The interaction was evaluated by the color of  
318 spotted co-transformants on MacConkey plates and  $\beta$ -galactosidase assays. Cells were  
319 grown on MacConkey plates for 96 hours and they were scrapped and suspended in 1mL of  
320 PBS. 100  $\mu$ L were used in the classical  $\beta$ -galactosidase assay (49).

321 **Twitching assay.** Macroscopic twitching assay was performed as described (27) with  
322 minor modifications. Briefly, a colony was picked with a toothpick and stabbed at the bottom  
323 of a plate containing M8 medium supplemented with 1 mM  $MgSO_4$ , 0.2 % glucose, 0.5 %  
324 casamino acids and 2.0 % agar. The plates were incubated upright at 37°C overnight,  
325 followed by 48 h of incubation at room temperature ( $\sim 25^\circ C$ ). Next, the agar was removed,  
326 and the bacteria were stained with 0.1% crystal violet. Microscopic twitching assay was  
327 performed as described (50).

328 **Swimming assay.** Swimming assays were performed by inoculating 5  $\mu$ L of a  
329 stationary phase-grown liquid cultures in M8 with 0.3% agar that were incubated for 16 h at  
330 30°C in a plastic bag to maintain the humidity constant (51).

331 **Congo red assay.** 5  $\mu$ L of stationary phase-grown cultures were inoculated at 1%  
332 agar plates of tryptone broth (10 g/L) containing Congo red (40  $\mu$ g/mL) and Coomassie  
333 brilliant blue (20  $\mu$ g/mL). The plates were incubated for 16 h at 30°C and then for 96 hours at  
334 room temperature.

335 **qRT-PCR.** For qRT-PCRs, total RNA was extracted with Trizol (Invitrogen), treated  
336 with DNase I (Thermo Scientific, Waltham, MA, USA) and used for cDNA synthesis with  
337 Improm II (Promega) or Superscript III (Invitrogen) and hexamer random primers (Thermo  
338 Scientific). cDNA was then amplified with specific primers using Maxima SYBRGreen/ROX

339 qPCR Master Mix (Thermo Scientific) and the 7300 Real Time PCR System (Applied  
340 Biosystems). *nadB* was used as internal control for normalization of total RNA levels (52). The  
341 relative efficiency of each primer pair was tested and compared with that of *nadB* and the  
342 threshold cycle data analysis ( $2^{-\Delta\Delta C_t}$ ) was used (53). All reactions were performed in  
343 triplicates, the assays were repeated at least twice using independent cultures and the results  
344 of one representative experiment are shown, with average values of technical triplicates and  
345 error bars representing standard deviation of  $\Delta\Delta C_t$ .

346 **Fluorescence and light microscopy.** To verify the localization of DgcP\_msfGFP  
347 fusions, fluorescence microscopy was performed using a Nikon Eclipse TiE microscope  
348 equipped with a 25-mm SmartShutter and an Andor EMCCD i-Xon camera. For fluorescence  
349 microscopy and bright field microscopy, a Plan APO VC Nikon 100X objective (NA = 1.4) and  
350 a Plan Fluor Nikon 40X objective (NA = 1.3) were used. For membrane staining, cells were  
351 treated with 50  $\mu\text{g}/\text{mL}$  FM4-64 (Invitrogen). For phase contrast microscopy, a Plan APO  $\lambda$   
352 OFN25 Nikon 100X objective (NA = 1.45) was used. All microscopy assays were performed  
353 with immobilized cells on 25 % LB pads with 1.5% agarose. Image analyses were performed  
354 using the ImageJ (54) and MicrobeJ (55) softwares.

355 **c-di-GMP extraction and quantification.** c-di-GMP was extracted as described by  
356 (56) with minor modifications. 50 mL of cultures were grown in M8 medium at 37°C and 200  
357 rpm until reach  $\text{O.D.}_{600} = 1$ . Cells were collected by centrifugation resuspended in 500  $\mu\text{L}$  of  
358 M8 medium with 0.6 M perchloric acid. The tubes were incubated on ice for 30 minutes and  
359 then centrifuged at 20000  $g$  for 10 minutes. The pellets were used for protein quantitation and  
360 the supernatants were neutralized with 1/5 volume 2.5 M  $\text{KHCO}_3$ . The nucleotide extracts  
361 were centrifuged again and the supernatants were stored at  $-80^\circ\text{C}$ . High-performance liquid  
362 chromatography (LC) was performed using the 1200 Infinity LC System (Agilent) that consists



363 of a degasser, a quaternary pump, a thermostated autosampler (4°C) and a temperature  
364 (30°C)-controlled column compartment. This system was coupled to an 3200 Qtrap LC-  
365 MS/MS system equipped with an Electrospray Ionization source (ESI) (AB Sciex, USA).  
366 Analyst 1.4.2 software (AB Sciex, USA) was used to operate the equipment and calculate c-  
367 di-GMP concentrations.

368 Samples (injection of 10 µL) were separated by a Phenomenex Synergi Hydro-RP  
369 column (150 × 2 mm, 4 µm) using 0.1% formic acid in 15 mM ammonium acetate as mobile  
370 phase A and MeOH as mobile phase B at a flow rate of 0.3 mL min<sup>-1</sup>. The gradient program  
371 was 0 min 2 % B, 0.5 min 2 % B, 4.5 min 30 % B, 6.0 min 80 % B, 7.0 min 80% B, 7.01 min  
372 2% B and 14 min 2 % B. For quantification of c-di-GMP, the tandem mass spectrometry  
373 method multiple reaction monitoring (MRM) was used in negative mode. The following  
374 parameters were set: nebulizer, heated auxiliary and curtain gases (nitrogen) at 20, 30, 10,  
375 respectively; Turbo IonSpray voltage and temperature at -3,800 V and 250 °C, respectively;  
376 MRM transition (in m/z) 689.1 → 344.2 with a dwell time of 200 ms per transition; collision  
377 energy (CE) at -45 eV; and declustering potential at -53 V. An external standard curve was  
378 prepared for c-di-GMP in the MRM mode. The stock solution was diluted and the c-di-GMP  
379 peak area plotted against the nominal concentrations (16 to 2,000 ng mL<sup>-1</sup>).

380 **Multiple Sequence Alignment and secondary structure prediction.** Reciprocal  
381 best-hit search was performed as described before (Kohler et al., 2015). Briefly, the Kyoto  
382 Encyclopedia of Genes and Genomes (KEGG) database was initially used to search open-  
383 reading frames showing highest identity against DgcP (GenBank: ABJ14873.1). This search  
384 resulted in a list that was used to reciprocal search its highest identity orthologue in *P.*  
385 *aeruginosa* UCBPPA14 genome. In both searches the threshold for Smith-Waterman score  
386 was 100, and the resulting best-best hit was filtered using a minimum 20% identity comparing

387 the identified homologue against the DgcP. A list of all organisms that displays a DgcP  
388 homologue are listed in Supplemental Table S1. Redundant sequences were filtered using  
389 CD-Hit (Li and Godzik, 2006) with a maximum identity of 90% (Supplemental Table S1). A  
390 multiple sequence alignment was performed using MUSCLE software (Edgar, 2004). The  
391 multiple sequence alignment was then visualized in the jalview (Waterhouse et al., 2009) and  
392 secondary structure analysis predicted with JPred (Drozdetskiy et al., 2015).

### 393 **Acknowledgments.**

394 We would like to thank A. Bisson-Filho for kindly providing the msGFP synthetic gene,  
395 D. Schechtman and M. Navarro for carefully reading the manuscript. We are also in debt with  
396 F. Gueiros-Filho, L. Zambotti-Villela and M.A. Cotta for assistance with fluorescence  
397 microscopy, mass spectrometry and electron microscopy, respectively. We acknowledge the  
398 National Nanotechnology Laboratory (LNNano, CNPEM) for granting access to the electron  
399 microscopy facilities.

400 Conceived and designed the experiments: GGN, CB and RLB. Performed the FESEM  
401 experiments: JHM. Performed two-hybrid screening: GGN and CB. Performed fluorescence  
402 and light microscopy: GGN and AAP. General molecular procedures: GGN, GHK, ALB and  
403 TOP. HPLC-MS/MS: ES. Contributed reagents/materials/analysis tools: RLB, CB, PC. Wrote  
404 the paper: GGN, RLB.

405 G.G.N. is supported by São Paulo Research Foundation (FAPESP) grant numbers:  
406 2013/02375-1 and 2014/02381-4.

407 R.L.B. is partially supported by National Council for Scientific and Technological  
408 Development (CNPq 307218/2014-7) a São Paulo Research Foundation (FAPESP  
409 2014/05082-8).grant allowed the work in RLB laboratory.

410 C.B. is supported by the ANR grants REGALAD ANR-14-CE09-0005-02.

411 The authors declare that there is no conflict of interest.

412

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576

## 577 **Figure Legends**

578 **Figure 1. DgcP interacts with FimV.** Schematic diagrams of DgcP (**A**) and FimV (**B**),  
579 showing the GGDEF domain of DgcP; the transmembrane region (TM), TPR motifs and the  
580 LysM domain of FimV. The red box in FimV corresponds to the prey fragment and lines show  
581 the regions cloned to confirm the interaction. The *E. coli* host strain BTH101 was  
582 cotransformed with pKTA25\_DgcP (full length) and pUT18\_FimV constructs, as indicated in  
583 the figure; the interactions were observed in MacConkey plates as red colonies (**C**) or  
584 measured using the  $\beta$ -galactosidase activity as a reporter (**D**).

585 **Figure 2. DgcP localizes at the cell poles in a FimV-dependent manner.** msfGFP was  
586 fused to DgcP full-length (1-670), its N-terminal (1-199) or the C-terminal (209-670)  
587 regions, as depicted (A). The full length protein localizes at the cell poles in the  $\Delta dgcP$   
588 background (B, top panels), but deletion of *fimV* leads to a loss of localization (B, bottom  
589 panels). The intensity of the GFP fluorescence was measured in 300 cells of each strains and  
590 a heat map of DgcP\_msfGFP localization was obtained with MicrobeJ, as described in  
591 Material and Methods (**C**).

592 **Figure 3. Mutation in *dgcP* affects biofilm formation.** PA14 and the  $\Delta dgcP$  strains were  
593 inoculated at OD<sub>600</sub> = 0.05 in 48 well polystyrene plates in the media shown and kept at  
594 30°C for 16 h without agitation. The medium was discarded and adherent cells were washed  
595 and stained with 1% crystal violet, washed and measured at OD = 595 nm (**A**). The same  
596 procedure was carried out for the strains overexpressing DgcP in LB with 0.2% arabinose (**B**).  
597 3D pictures resulting from CLSM after 16 h (**C**) and 72 h (**D**) of biofilm formation in LB at 30°C  
598 in an 8-well Lab-tek chambered coverglass system.

599 **Figure 4.  $\Delta dgcP$  mutant presents defects related to surface behaviors.** Cells were  
600 stabbed into the bottom of an agar plate by using a toothpick and incubated upright at 37°C  
601 overnight, followed by 48 h of incubation at room temperature, the medium was discarded and  
602 adherent cells stained with crystal violet (**A**). Diameter of the twitching colonies was measured  
603 in triplicates (**B**). Light microscopy images of PA14 and  $\Delta dgcP$  twitching colonies. Interstitial  
604 biofilms formed at the interface between a microscope slide coated in solidified nutrient media  
605 (Gelzan Pad) after four hours of colony expansion. The  $\Delta fimV$  mutant was used as a negative  
606 control of twitching (**C**). A silicon slide was placed upright in a culture tube and after the  
607 different time points cells at the air-liquid interface were washed, fixed and the spread of cells  
608 during the initial stages of biofilm formation was observed by FESEM (**D**).

609 **Figure 5. DgcP activity is FimV dependent.** Full-length wild type DgcP (pDgcP) or DgcP  
610 mutated in its GGDEF domain (pDgcP-GGAAF), only DgcP C-terminal wild-type region  
611 (pDgcP-Cterm) and WspR (pWspR) were overexpressed from the pJN105 vector in PA14,  
612  $\Delta dgcP$  and  $\Delta fimV$  backgrounds. Biofilm (**A**), swimming motility (**B**), c-di-GMP quantitation (**C**)  
613 and *cdrA* mRNA relative levels (**D**) were assayed. FimV, DgcP or both were expressed in *E.*  
614 *coli* and the EPS production was assessed in Congo red plates (**E**). Data are the means  $\pm$  SD  
615 from three replicates. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$

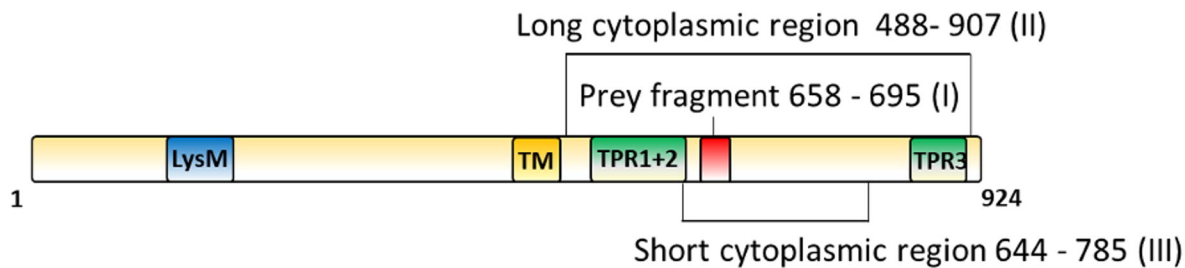
616 **Figure 6. Model of FimV-dependent localization and activity of DgcP.** In the PA14 wild  
617 type strain, DgcP is located at the pole due to interaction with FimV and may contribute to a  
618 local c-di-GMP pool (left). In a  $\Delta fimV$  background, DgcP is scattered in the cytoplasm and  
619 may have a small contribution to the global c-di-GMP pool (right). FimV, blue boxes; DgcP,  
620 green ellipses; c-di-GMP, red dots; nucleoid, blue dashed line; T4P, orange wavy lines and  
621 flagella, dark green lines.

622 **Figure S1. Multiple Sequence Alignment (MSA) and secondary structure prediction of**  
623 **DgcP homologues.** The MSA was generated using Muscle and edited in Jalview. The amino  
624 acids residues are colored using the Clustal X colour scheme. Secondary structure prediction  
625 was generated using JPred and is displayed as alpha-helices (red cylinders) and beta-sheets  
626 (green arrows) on the top of the alignment. Horizontal black bars represent the two putative  
627 N-terminal domains and the blue bar shows the GGDEF C-terminal domain. The orange  
628 vertical bar on the left point to *Pseudomonas* spp. and the purple bar groups other bacteria,  
629 as listed in Table S1.

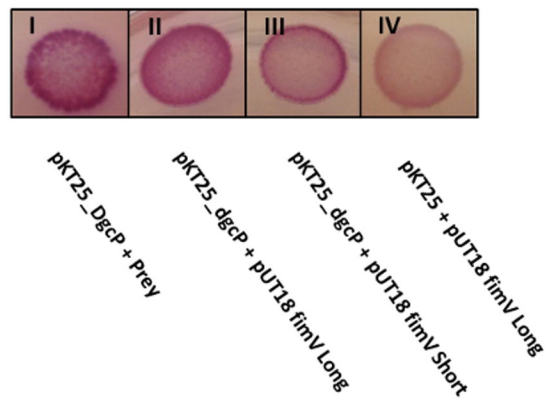
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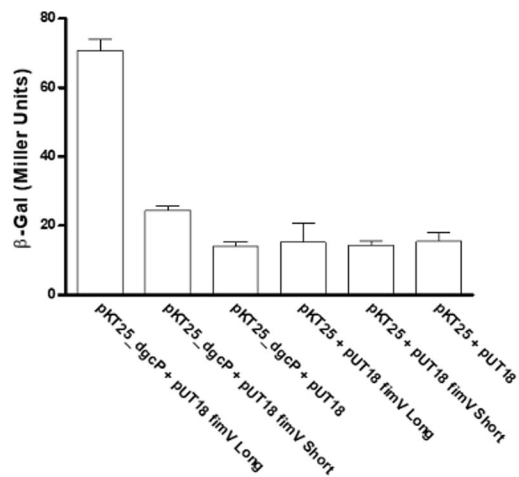
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C



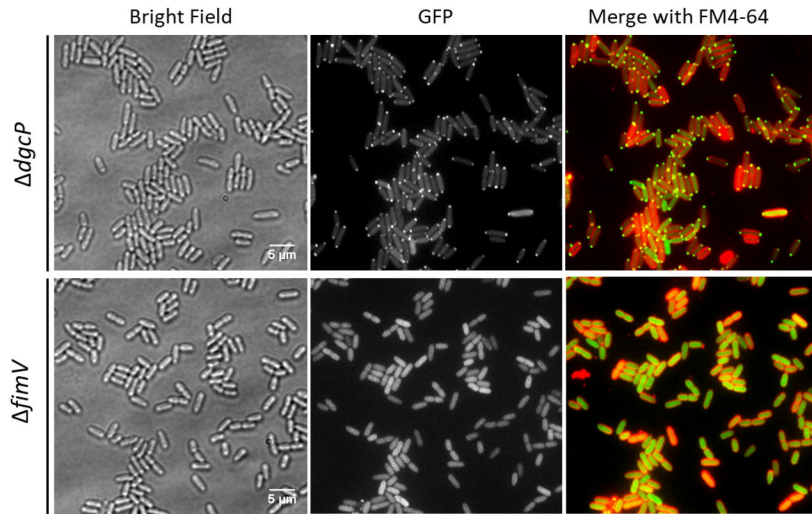
D



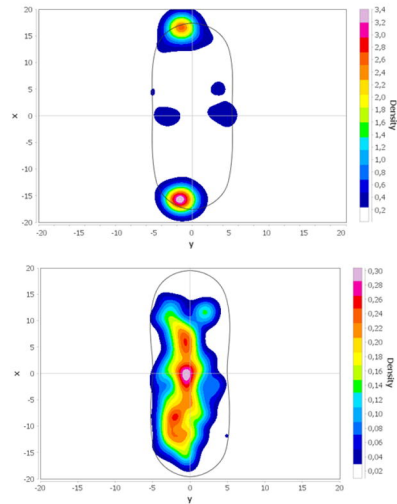
A



B

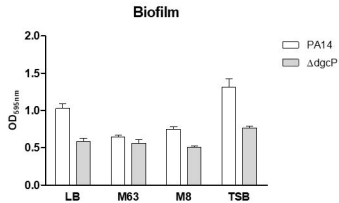


C

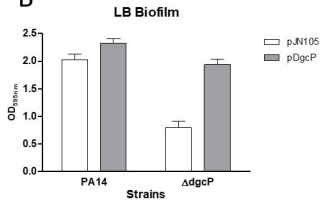




A

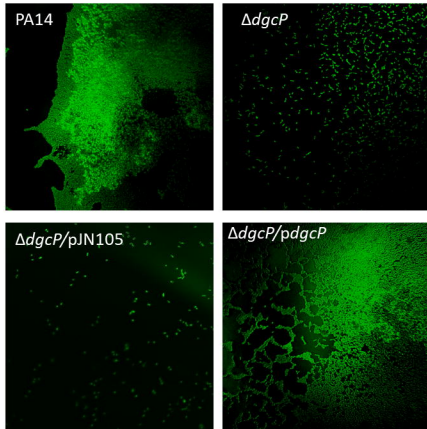


B



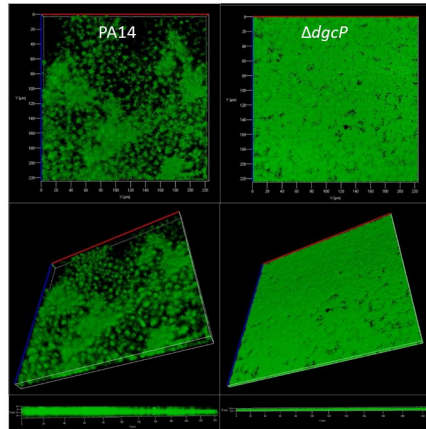
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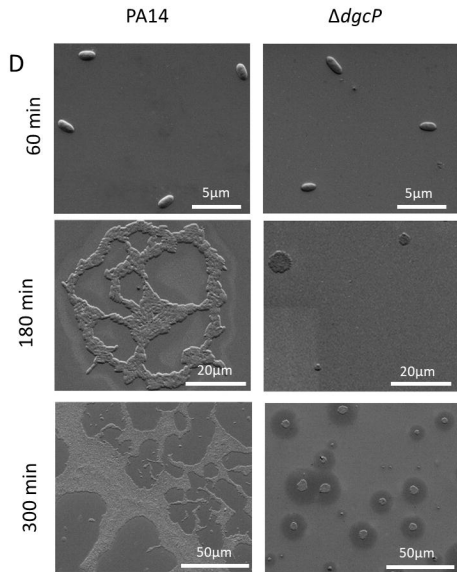
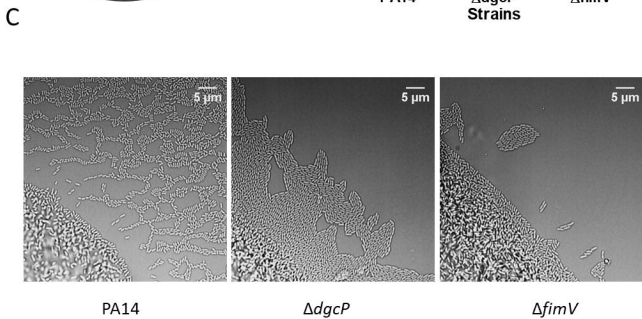
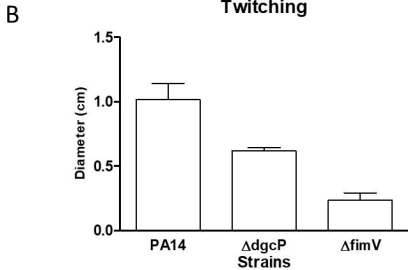
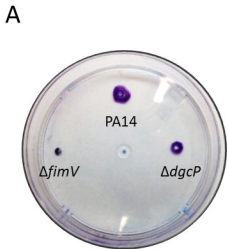
16 Hours Biofilm



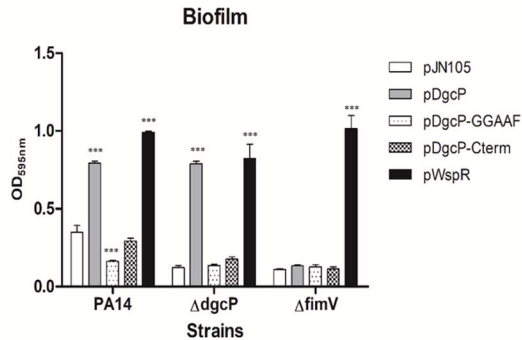
D

72 Hours Biofilm

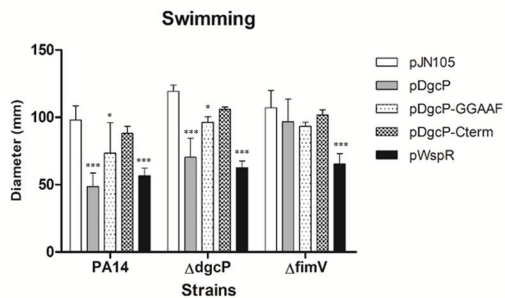




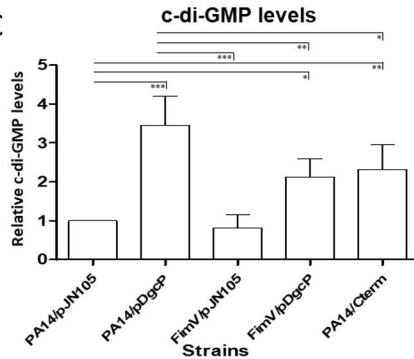
A



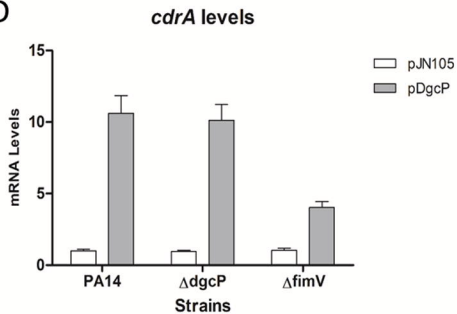
B



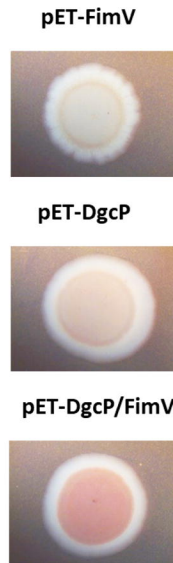
C



D

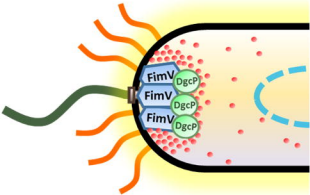


E



↑ Biofilm

PA14



↓ Biofilm

$\Delta fimV$

