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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Abstract

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

Importance

Bacteria can switch between sessile and motile lifestyles and c-di-GMP is a crucial second messenger for this decision. They present several proteins that can synthetize, degrade and sense this molecule and it is intriguing why so many apparently redundant proteins are needed. An idea is that some of these proteins may act at specific subcellular locations, leading to the formation of localized c-di-GMP pools that may control the assembly and function of polar-localized machineries such as pili and flagella. We found that DgcP, a c-di-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

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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 DcgP

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 Δ*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 Δ*dgcP* strains (**Fig. 2A**). This

132 localization is lost when DgcP-msfGFP is expressed in a Δ*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, includind 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. T**he 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 Δ*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 Δ*dgcP* (**Fig. 3B** and **C**). In LB, Δ*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, Δ*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

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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 Δ*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　Δ*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 Δ*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 Δ*dgcP* mutation, but DcgP-msfGFP is not able to increase biofilm formation or decrease

188   swimming motility in the Δ*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 Δ*dgcP* and Δ*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 Δ*dgcP*, but only fourfold in the Δ*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 .

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Discussion

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

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

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

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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 $MgSO_4$, 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 TM 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

13

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 $O.D_{600} = 0.05$ in M63 medium

301 and transferred to a 24-well plate where silicon slides (2×1 cm^2) 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_2 flow and subsequently treated with O_2 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

14

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 β-galactosidase assays. Cells were

319   grown on MacConkey plates for 96 hours and they were scrapped and suspended in 1mL of

320   PBS. 100 µL were used in the classical β-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 (~25°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 µl 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 µl of stationary phase-grown cultures were inoculated at 1%

332   agar plates of tryptone broth (10 g/L) containing Congo red (40 µg/mL) and Coomassie

333   brilliant blue (20 µg/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 ($2^{-\Delta\Delta Ct}$) 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 ΔΔCt.

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 µg/mL FM4-64 (Invitrogen). For phase contrast microscopy, a Plan APO λ

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 $O.D._{600}$ = 1. Cells were collected by centrifugation resuspended in 500 µL 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 $KHCO_3$. The nucleotide extracts

361 were centrifuged again and the supernatants were stored at –80 °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^{-1}. 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−1).

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

17

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).

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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 β-galactosidase activity as a reporter (**D**).

585 **Figure 2. DgcP localizes at the cell poles in a FimV-dependent manner.** msfGFP was

586 fusioned 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 Δ*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 Δ*dgcP* strains were

593 inoculated at OD600=0.05 in 48 well polystyrene plates in the media shown and kept at

594 30°C for 16h 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.

28

599   **Figure 4. Δ*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 Δ*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 Δ*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   Δ*dgcP* and Δ*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 ± 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 Δ*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 elipses; 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-helixes (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.

A

1   501   671

B

Long cytoplasmic region 488- 907 (II)

Prey fragment 658 - 695 (I)

1   924

Short cytoplasmic region 644 - 785 (III)

C

pKT25_DgcP + Prey

pKT25_dgcP + pUT18 fimV Long

pKT25_dgcP + pUT18 fimV Short

pKT25 + pUT18 fimV Long

D

β-Gal (Miller Units)

pKT25_dgcP + pUT18 fimV Long

pKT25_dgcP + pUT18 fimV Short

pKT25_dgcP + pUT18

pKT25 + pUT18 fimV Long

pKT25 + pUT18 fimV Short

pKT25 + pUT18

A

Biofilm

B

LB Biofilm

C

16 Hours Biofilm

PA14 | ΔdgcP

ΔdgcP/pJN105 | ΔdgcP/pdgcP

D

72 Hours Biofilm

PA14 | ΔdgcP

A

B **Twitching**

C

PA14   ΔdgcP   ΔfimV

D   PA14   ΔdgcP

60 min

180 min

300 min

A Biofilm

Legend: pJN105, pDgcP, pDgcP-GGAAF, pDgcP-Cterm, pWspR

Strains: PA14, ΔdgcP, ΔfimV

OD_{595nm}

B Swimming

Legend: pJN105, pDgcP, pDgcP-GGAAF, pDgcP-Cterm, pWspR

Diameter (mm)

Strains: PA14, ΔdgcP, ΔfimV

C c-di-GMP levels

Relative c-di-GMP levels

Strains: PA14/pJN105, PA14/pDgcP, FimV/pJN105, FimV/pDgcP, PA14/Cterm

D *cdrA* levels

Legend: pJN105, pDgcP

mRNA Levels

Strains: PA14, ΔdgcP, ΔfimV

E

pET-FimV

pET-DgcP

pET-DgcP/FimV