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Title: Ligand-Mediated Biofilm Formation via Enhanced Physical Interaction Between a Diguanylate Cyclase and Its Receptor

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Abstract. The second messenger, cyclic dimeric GMP (c-di-GMP) regulates biofilm formation and surface attachment for many bacteria. Biofilm formation of *Pseudomonas fluorescens* Pf0-1 is controlled by the transfer of c-di-GMP to the inner-membrane protein, LapD. LapD-bound c-di-GMP in turn inhibits proteolytic cleavage of the adhesin LapA by the periplasmic protease LapG, which allows localization of LapA to the cell surface and thereby promotes biofilm formation. LapD is central to a complex network of c-di-GMP-mediated biofilm formation. In this study, we examine how signaling specificity of c-di-GMP by a DGC is achieved by small ligand sensing through characterization of the calcium channel chemotaxis (CACHE) receptor of the DGC, GcbC. We provide evidence that biofilm formation is enhanced by the environmentally relevant organic acid citrate, in a GcbC-dependent manner through enhanced LapA localization to the cell surface. In the presence of citrate, the GcbC shows enhanced physical interaction with LapD. GcbC also shows increased c-di-GMP production when LapD is present, and this enhanced GcbC-LapD interaction by citrate further drives c-di-GMP synthesis. Given that LapD can interact with a dozen different DGCs of *P. fluorescens*, many of which have ligand-binding domains, the ligand-mediated enhanced signaling of LapD-GcbC described here is likely a conserved mechanism of signaling in this network.

Significance Statement. Biofilm formation is a highly regulated event controlled by c-di-GMP signaling. In many bacteria, dozens of DGCs produce this dinucleotide signal, however it is unclear how undesired cross-talk is mitigated in the context of this soluble signal, and how c-di-GMP signaling is regulated in the context of environmental inputs. Here we show a ligand-mediated mechanism of signaling specificity whereby citrate enhances catalysis of the c-di-GMP synthesizing diguanylate cyclase (DGC) via increased physical interaction with its cognate receptor. We envision a scenario wherein a "cloud" of weakly interacting DGCs can increase specific interaction with their receptor in response to appropriate environmental signals, concomitantly boosting c-di-GMP production, ligand-specific signaling and biofilm formation.

Text

For most bacteria, surface attachment and biofilm formation is a highly regulated event (1, 2). The bacterial intracellular second messenger, cyclic dimeric GMP (c-di-GMP) regulates biofilm formation by regulating a diversity of biofilm-relevant outputs, including surface attachment (3), flagellar motility (4), extracellular polysaccharide production (5, 6), adhesin localization (7), and transcriptional control of pathways important for attachment (8). An important research theme has been the focus on c-di-GMP signaling specificity in the context of microbes that can have >50 proteins that make, degrade and bind this second messenger.

Biofilm formation by *Pseudomonas fluorescens* Pf0-1 occurs when the adhesin LapA localizes to the cell surface (9). LapA is maintained on the cell surface when the inner membrane protein

58 LapD binds c-di-GMP, which in turn inhibits cleavage of LapA by the periplasmic protease LapG
59 (10, 11). The c-di-GMP-bound LapD sequesters LapG, thus this protease is not available to
60 target the N-terminal cleavage site of LapA (11). One example of how c-di-GMP is specifically
61 transferred to the LapD receptor is by physical interaction with a diguanylate cyclase (DGC)
62 (12). The DGC called GcbC has been shown to physically interact with LapD, a c-di-GMP
63 receptor located in the inner membrane, utilizing a surface exposed α-helix of the GGDEF
64 domain on GcbC and the surface exposed α-helix of the EAL domain of LapD (12). This direct
65 interaction model was proposed as one means to confer signaling specificity.

66 GcbC is an inner membrane protein that contains a calcium channel chemotaxis receptor
67 (CACHE) located N-terminal to its GGDEF domain. CACHE domains are responsible for small
68 ligand sensing (13). Many signal transduction proteins, including DGCs and histidine kinases,
69 contain CACHE domains (14). GcbC, along with five other DGCs encoded on the *P. fluorescens*
70 Pf0-1 genome (Pfl01_2295, Pfl01_2297, Pfl01_1336, Pfl01_3550, and Pfl01_3800), contains a
71 CACHE domain located N-terminally to the GGDEF domain, suggesting that these six DGCs are
72 capable of sensing and responding to small ligands. The predicted domain organization of
73 GcbC, Pfl01_2295, and Pfl01_2297 are shown in Figure 1A.

74 In this study, we analyze the CACHE domain of GcbC and identify the environmentally relevant
75 organic acid citrate as a ligand for this DGC. We show citrate-enhanced physical interaction
76 with GcbC and LapD, and that this enhanced interaction promotes increased c-di-GMP synthesis
77 by GcbC, thereby promoting biofilm formation. We propose that ligand-mediated
78 enhancement of DGC interactions with a receptor could serve as a general mechanism to
79 confer specificity to this complex signaling network.

80

81 **Results**

82 **Citrate-mediated Biofilm Enhancement is Dependent on GcbC Activity.** We identified a CACHE
83 domain in the periplasmic loop of GcbC (Figure 1A). To identify small molecules that the CACHE
84 domain of GcbC may bind, we performed a CLUSTAL alignment of the amino acid sequence of
85 CACHE domains of known structure (15). GcbC showed the highest amino acid similarity to
86 rpHK1S-Z16 (PDB ID: 3LIF) of *Rhodopseudomonas palustris* with 31% identity (Fig. S1). When
87 crystallized, the CACHE domain of rpHK1S-Z16 was bound to citrate and methyl-2,4-
88 pentanediol, two small ligands recruited from the crystallization cocktail (15).

89 In a previous study, citrate was shown to act as a calcium chelator thus inhibiting proteolytic
90 cleavage of LapA by LapG, and thereby stimulating biofilm formation (16). However, in a *lapG*
91 mutant, citrate was capable of further stimulating biofilm levels (16) thus suggesting there was
92 a second role for citrate in promoting biofilm formation, a conclusion supported by the putative
93 small molecule-binding sites present in GcbC. To investigate this point, we tested the ability of
94 citrate to promote biofilm formation by *P. fluorescens* Pf0-1. Citrate-mediated enhancement of
95 biofilm formation observed for the wild-type (WT) strain was abolished in the *gcbC* mutant (Fig.

96 1B), suggesting that citrate-mediated enhancement of biofilm formation is dependent on the
97 presence of GcbC. We selected Pfl01_2295 and Pfl01_2297 to serve as controls to determine if
98 other CACHE domains also respond to citrate to promote biofilm formation. We used a low
99 biofilm forming *P. fluorescens* Pf0-1 strain, lacking four DGCs, referred to as Δ4DGC, which
100 shows minimal biofilm formation; the four DGCs deleted in the Δ4DGC strain, identified
101 previously, are *gcbA*, *gcbB*, *gcbC*, and *wspR* (17). Of these three CACHE domain-containing
102 DGCs expressed in the Δ4DGC mutant background (GcbC, Pfl01_2295, and Pfl01_2297), only
103 the strain expressing GcbC responded to citrate with increased biofilm formation (Fig. 1C).
104 GcbC synthesizes c-di-GMP via the GGDEF motif and mutation of this motif to GGAAF eliminates
105 synthesis of c-di-GMP but this mutant variant is stably expressed (17). When GcbC-GGAAF was
106 expressed in the Δ4DGC mutant background, biofilm formation was abolished and notably,
107 citrate-mediated biofilm formation was also abolished (Fig. 1C).

108 Importantly, citrate enhanced biofilm formation is dependent on LapA (Fig. S2), indicating that
109 citrate acts via the known LapD-LapG-LapA pathway. We used *P. fluorescens* strains containing
110 a HA-tagged LapA variant to detect the amount of LapA at the cell surface as a function of the
111 presence of citrate, as reported (7,10). In a WT *P. fluorescens* strain, citrate caused a 159%
112 increase in LapA pixel density, which suggests a higher abundance of LapA at the cell surface in
113 the presence of citrate (Fig. 1D). Only when GcbC was present and catalytically active did
114 citrate cause an increase in cell surface-associated LapA (Fig. 1D). Taken together, these data
115 show that citrate-mediated stimulation of biofilm formation in WT *P. fluorescens* Pf0-1 requires
116 the active diguanylate cyclase GcbC, and is associated with enhanced cell surface LapA.

117

118 **The Putative Ligand Binding Site of the CACHE Domain is Important for GcbC-mediated**
119 **Biofilm Formation.** CACHE domains are ubiquitous, extracellular, ligand-binding domains (13,
120 14). In a previous study, the RXYF motif was found to be the most conserved feature among the
121 characterized CACHE domains (15). The study found that the X residue is either negatively
122 charged or polar, and the tyrosine residue of the RXYF motif points towards the ligand-binding
123 site (15). A mutation of the RXYF motif of the CACHE domain of KinD, a histidine kinase in
124 *Bacillus subtilis*, caused this microbe to lose its ability to respond to root exudates, forming
125 reduced biofilm levels on tomato roots compared to a WT strain. (18). Thus, we predicted that
126 a mutation within the RXYF motif of GcbC (Fig. S1) would result in impairment of citrate-
127 dependent biofilm formation when expressed in a Δ4DGC mutant background. We mutated
128 the tyrosine residue of the RXYF motif to a phenylalanine (Y141F) in a HA-tagged variant of
129 GcbC (17) and expressed GcbC-Y141F-HA in the Δ4DGC mutant. Mutation of the RXYF motif
130 resulted in the inability of GcbC to promote biofilm formation or respond to citrate (Fig. 2A).
131 We assessed the stability of the GcbC-Y141F variant, which showed a 70% reduction in level
132 compared to WT GcbC (Fig. 2B).

133 We sought to identify the putative site where citrate might bind to GcbC using the known
134 structures of CACHE domains (Fig. 2C, left; template PDB ID 3LIB.). Like in the other CACHE
135 domains, the tyrosine residue of the RXYF motif of GcbC was predicted to point towards the
136 ligand-binding site (Fig. 2C, Fig, S3). Furthermore, based on the CACHE domain model of GcbC,
137 the amino acids R139, R162, and R172 were predicted to shape the predicted ligand-binding
138 site (Fig. 2C, right). Based on the model, we predict that three arginine residues can coordinate
139 the three carboxylic acid groups of citrate (Fig. 2C, left). R139 is also part of the RXYF motif.
140 Each of the arginine residues forming the putative ligand site were mutated and introduced
141 into the Δ4DGC mutant background. Mutating R172 (R172A and R172E) resulted in instability of
142 GcbC (Table S1), however GcbC-R139A, GcbC-R139E, and GcbC-R162A were detected by
143 Western blot, with GcbC-R139E variant present at ~WT levels (Fig. 2D). The strain carrying
144 GcbC-R139E variant did not show a significant enhancement of biofilm formation in the
145 presence of citrate (Fig. 2E). Together, our data indicate that the RXYF motif and a putative
146 citrate-binding arginine triad are critical for GcbC-dependent, citrate-mediated enhancement of
147 biofilm formation. We further expanded our search for important conserved residues within
148 the CACHE domain that were predicted based on alignments with other CACHE domain
149 proteins, however the other twenty-seven mutant proteins we constructed were unstable
150 (Table S1).

151

152 **Citrate-mediated Interaction of GcbC with LapD Enhances Synthesis of c-di-GMP.** In our
153 published model, GcbC mediates biofilm formation by transferring c-di-GMP to LapD through
154 physical interaction of the $\alpha5^{GGDEF}$ helix of GcbC with the $\alpha2^{EAL}$ helix of LapD (12). We asked
155 whether citrate might exert its effect of stimulating biofilm formation via stabilization of the
156 LapD-GcbC signaling complex. To test if citrate bolstered GcbC-LapD interaction, we exploited
157 the bacterial two-hybrid system used to initially demonstrate interaction between these
158 proteins. We did observe a modest but significant enhancement of LapD-GcbC interaction in
159 the presence of citrate. No such enhancement was observed for the control interactions: LapD-
160 Pfl01_2295, or GcbC with two other LapD-like dual domain proteins (Fig. 3A). Citrate also did
161 not enhance GcbC-GcbC dimerization (Fig. 3A), which is perhaps expected, as dimerization is
162 required for diguanylate cyclase activity (19, 20). Further, the catalytically inactive variant of
163 GcbC still showed citrate-stimulated interaction (Fig. 3B).

164 We further explored whether citrate could also enhance the diguanylate cyclase activity of
165 GcbC. We used the bacterial two-hybrid (B2H) plasmids and strains to express GcbC and LapD
166 outside of their native context, and to better focus on how the interaction of these two
167 proteins might specifically impact GcbC's activity. The activity of GcbC was assessed by
168 measuring the level of c-di-GMP extracted from the indicated strains. The level of c-di-GMP
169 measured in the *E. coli* strain carrying the vector controls was <2 pmol c-di-GMP/mg dry
170 weight. This low background of c-di-GMP provided a useful tool to measure differences in c-di-
171 GMP levels derived from GcbC in the presence and absence of citrate. GcbC alone did not

172 synthesize detectable levels of c-di-GMP and citrate did not promote c-di-GMP production (Fig.
173 3C). Co-expression of GcbC with LapD resulted in ~ 20 pmol c-di-GMP/mg dry weight, and the
174 level of c-di-GMP was significantly increased upon addition of citrate (Fig. 3C). The increase in c-
175 di-GMP required catalytically active GcbC, and was specific to GcbC interacting with LapD (Fig.
176 3C). Pfl01_0192 is a dual domain protein and was shown to interact weakly with GcbC (Fig. 3A),
177 but background levels of c-di-GMP were detected when GcbC and Pfl01_0192 were co-
178 expressed +/- citrate (Fig. 3C).

179 Given that stability of GcbC-R139E was equivalent to WT GcbC levels (Fig. 2D), we tested for
180 interaction of GcbC-R139E with LapD. Mutation of the arginine residue in the RXYF motif did
181 not affect basal GcbC-LapD interaction. However, citrate-enhanced interaction of GcbC-R139E-
182 LapD interaction was significantly reduced (but not eliminated) compared to citrate-enhanced
183 WT GcbC-LapD interaction (Fig. 3D). Thus, our data indicate that LapD-GcbC interaction
184 enhances c-di-GMP production, and the addition of citrate stimulates both interation of these
185 proteins and c-di-GMP synthesis, likely via the CACHE domain of GcbC.

186

187 **The CACHE Domain Participates in GcbC-LapD Interaction**. Thus far, we showed the CACHE
188 domain to be important for citrate-mediated biofilm formation via increasing GcbC-LapD
189 interaction and enhance GcbC activity. In a previous study (12), the four point mutations,
190 E477A, Q478A, F481A, K485A (Quad Alanine) were introduced to the surface-exposed $\alpha5^{GGDEF}$
191 helix of GcbC and expressed in a Δ4DGC mutant background, which resulted in reduced biofilm
192 formation of *P. fluorescens* due to reduced interaction with LapD (12). However, the GcbC-
193 Quad Alanine mutant, expressed in the Δ4DGC mutant background still showed a significant
194 citrate-mediated enhancement of biofilm formation (Fig. S4A), thus suggesting the possibility of
195 a second interface of GcbC-LapD interaction that is enhanced by citrate.

196 We next assessed whether the CACHE domain was required for citrate-enhanced interaction
197 between GcbC and LapD. The two transmembrane domains plus the periplasmic portion of
198 GcbC showed only a modest level of dimerization with full length GcbC, which was not
199 stimulated by citrate (Fig. S4B). Importantly, the periplasmic domain of GcbC interacted with
200 LapD at a level similar to full length GcbC, but citrate-enhanced interaction was abolished (Fig.
201 S4B). Together, these data indicated that CACHE domain is required but not sufficient for
202 citrate-mediated, enhanced interaction of LapD and GcbC; however, this domain appears
203 responsible for basal LapD-GcbC interaction.

204

205 **LapD Interacts With Numerous DGCs.** We next explored if the mechanism we defined for GcbC-
206 LapD interactions might apply to any of the other 20 DGCs in *P. fluorescens*. We found that
207 LapD is a central hub of DGC interaction; LapD interacts with a dozen different DGCs in a
208 pairwise bacterial two-hybrid assay (Fig. 4A). Included among the twelve DGCs that interact

209 with LapD are Pfl01_2295 and Pfl01_2297, and the putative SadC homolog, Pfl01_4451 (Fig.
210 4A). SadC is a DGC identified for its role in the early stages of biofilm development in *P.*
211 *aeruginosa* (21), and the Δ*sadC* mutant of *P. aeruginosa* shows approximately a 50% reduction
212 of global, cellular c-di-GMP levels compared to WT PA14 (22). We have not yet identified
213 ligands that enhance interactions and/or activity of these other DGCs.

214

215 **Discussion**. A key open question relating to c-di-GMP signaling is understanding how specificity
216 of an output is mediated in the context of up to dozens of enzymes or receptors making,
217 breaking and binding this dinucleotide. Here, our data suggest that an extracellular ligand can
218 modulate the activity of a DGC and do so via interaction with its cognate receptor. Our data are
219 consistent with the model that citrate binding to the CACHE domain of GcbC enhances
220 interaction with LapD. We propose the increased interaction between GcbC and LapD to have
221 two important consequences: stabilization of a complex that allows direct transfer of the GcbC-
222 generated c-di-GMP signal to the LapD receptor, and equally importantly, activation of the DGC
223 activity of GcbC. Indeed, when expressed in a heterologous system, GcbC showed almost no
224 capacity to synthesize c-di-GMP; it is only when co-expressed with LapD that a significant
225 increase in c-di-GMP synthesis above background was detected. An additional boost in cyclic
226 nucleotide production was measured when citrate was added to the medium in a strain co-
227 expressing GcbC and LapD, indicating that the enhanced GcbC-LapD interaction also enhanced
228 DGC activity. Furthermore, recent work from the Sondermann lab (23) proposed a model
229 where GcbC and LapD, along with LapG, form a large signaling complex that facilitates LapD-
230 GcbC interactions (Fig. 4B). Thus, our data supports a model for a ligand-based mechanism to
231 enhance signaling specificity by c-di-GMP in the context of a large signaling complex.

232 We have identified citrate as a potential ligand that binds to GcbC via its CACHE domain. There
233 is an abundance of CACHE domains present on signal transduction proteins (14), including
234 proteins involved in c-di-GMP signaling, and yet, the role of CACHE domains fused to DGCs is
235 poorly understood. Our data show that citrate is a potential ligand for the CACHE domain
236 associated with GcbC. *P. fluorescens*, a plant symbiont, forms biofilms on tomato roots and the
237 root exudates contain organic acids including citrate (24, 25, 26). It is possible, however, that
238 GcbC senses other organic acids. For example, in *P. syringe* pv. *actinidiae*, the CACHE domain
239 PscD (PDB ID: 5G4Z) binds glycolate, acetate, propionate, and pyruvate (27). It is also possible
240 that different ligands sensed via the CACHE domain of GcbC can dictate which proteins interact
241 with this DGC (Fig. 4A). Together, our data indicate that extracellular ligands, via their ability to
242 modulate protein-protein interactions and/or DGC activity, can modulate c-di-GMP signaling
243 specificity. Given the large number of ligand-binding domains associated with c-di-GMP-
244 metabolizing proteins, the data presented here could represent a general means of regulating
245 c-di-GMP-controlled outputs by enhancing the signaling between c-di-GMP-metabolizing
246 enzymes and their effectors.

247 Enzymes that make c-di-GMP are part of a complex network, with LapD as a central receptor for
248 DGCs to interact with (Fig. 4A). It is highly unlikely that the twelve DGCs that make up this DGC
249 network all interact with LapD without regulation to control signaling specificity. A more likely
250 scenario could be that ligands are used as a signal to regulate signaling of a DGC to interact with
251 LapD and outcompete with one another. It is also possible that other ligand-mediated
252 mechanisms exist, such as a ligand-enhanced homodimerization of a DGC. Further
253 characterizing and identifying what small molecules DGCs respond to could provide insight into
254 how c-di-GMP signaling specificity is regulated within this complex signaling network. For
255 example, we envision a scenario wherein a "cloud" of weakly interacting DGCs can increase
256 specific interaction with their receptor in response to appropriate environmental signals,
257 concomitantly boosting c-di-GMP production, ligand-specific signaling and biofilm formation.
258 Our work provides insight of a ligand-based mechanism of how signaling specificity occurs
259 among a complex network of enzymes and receptors that make, break, and bind c-d-GMP.

260

261 **Material and Methods**

262 **Strains and Media.** Bacterial strains used in this study are listed in Table S2, and were cultured
263 and maintained in lysogeny broth (LB) or on 1.5% agar LB plates. *P. fluorescens* was grown at
264 30°C and *E. coli* was grown at 37°C. *E. coli* S17-1-λ-pir was used for maintenance and transfer of
265 plasmids. *Saccharomyces cerevisiae* strain InvSc1 was used for plasmid modification as
266 described previously (28, 29). K10T-1 medium was prepared as described previously (30).
267 Sodium citrate was added to 1.5% agar LB plates and K10T-1 media to a final concentration of
268 13.6 mM (0.4% wt/vol) for all the experiments described. The following antibiotics were used
269 as indicated: gentamycin (15µg/mL for *E. coli*, 30µg/mL for *P. fluorescens*), kanamycin (50µg/mL
270 for *E. coli*), and carbenicillin (50µg/mL for *E. coli*).

271 **Biofilm Assay.** Biofilm assays were performed as described previously (1). *P. fluorescens* Pf0-1
272 strains were incubated in K10T-1 medium with and without 0.4% sodium citrate, as indicated,
273 for 6 hours at 30°C. Biofilms were stained with 0.1% crystal violet, washed with water and then
274 solubilized with a 45% methanol, 45% dH_2O, and 10% glacial acetic acid solution. The optical
275 density (OD) of the solubilized crystal violet solution was measured at 550 nm to determine the
276 amount of biofilm formed.

277 **Dot Blot LapA Localization Assay.** Localization of LapA to the cells surface was measured using
278 a HA-tagged LapA variant integrated into the chromosome of *P. fluorescens* as described
279 previously (7, 10, 31). Overnight LB-grown cultures were subcultured in 5mL K10T-1 medium in
280 the presence and absence of 0.4% sodium citrate for 6 hours at 30°C. Samples were normalized
281 by the lowest OD_{600} value.

282 **Bacterial Two-Hybrid Assay.** Bacterial two-hybrid assays were performed using *E. coli* BTH101
283 cells based on a previously described system (32). Briefly, ~100ng of each bacterial two hybrid

284 plasmid was cotransformed into *E. coli* BTH101 by electroporation. *E. coli* BTH101 cells were
285 incubated on LB agar supplemented with 50µg/mL kanamycin, 50µg/mL carbenicillin, and
286 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 hours at 30°C. At 24 hours, either β-
287 galactosidase or c-di-GMP levels were quantified as described below. β-galactosidase assays
288 were performed as exactly described previously (12) to quantify the extent of protein-protein
289 interaction. β-galactosidase levels are presented in Miller Units.

290 **c-di-GMP Quantification Assay.** c-di-GMP was extracted from *E. coli* BTH101 cells after
291 incubation on LB agar plates at 30°C for 24 hours. The cells were scraped from the plate surface
292 with 1 mL of dH_2O, then pelleted and resuspended in 0.250 mL nucleotide extraction buffer
293 (40% methanol, 40% acetonitrile, 20% dH_2O, and 0.1N formic acid), followed by incubation at -
294 20°C for 1 hour. Cells were pelleted again and the reaction was neutralized by transfer of 0.2
295 ml nucleotide extract to 8µl of 15% NH_4CO_3. Nucleotide extracts were vacuum-dried and
296 resuspended in 0.2 mL HPLC grade H_2O. c-di-GMP concentration was analyzed by liquid
297 chromatography-mass spectrometry and compared to a standard curve of known c-di-GMP
298 concentration, as reported (12). The moles of c-di-GMP were normalized to the dry weight of
299 the cell pellet from which the nucleotides were extracted.

300 **Statistical Analysis.** Student's t-test was used to test for statistical significance. $P < 0.05$; *, $P <$
301 0.01; **, $P < 0.001$; ***

302

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304

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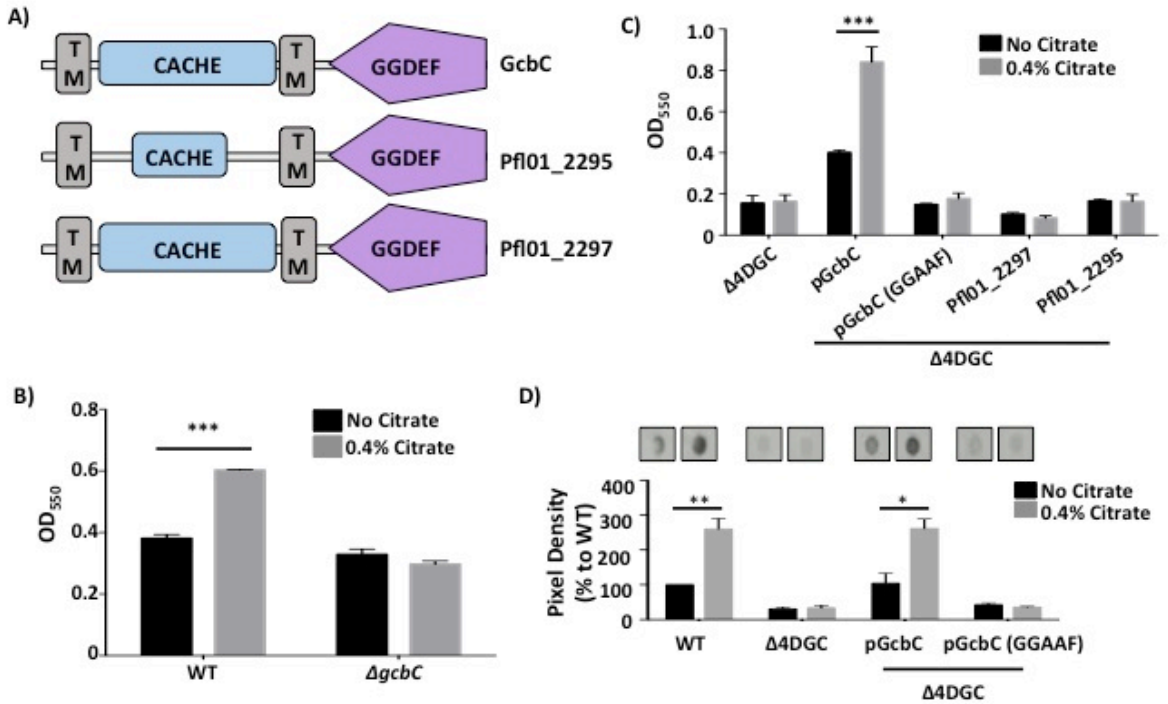
406 **Figures**



407 **Fig. 1. Citrate stimulates biofilm formation via GcbC.** (A) Predicted domain organization of
408 GcbC, Pfl01_2295, and Pfl01_2297 as predicted by SMART (33). The domains are indicated in
409 each block. TM = transmembrane domain. Analysis of Pfl01_2295 by MiST2 (34) predicts a
410 second transmembrane domain. (B) Biofilm formation by WT *P. fluorescens* and the Δ*gcbC*
411 mutant in the presence and absence of 0.4% citrate (n = 3; ± SD). (C) Biofilm formation by the
412 indicated strains +/- citrate. In this panel, the Δ4DGC mutant background is used, with the WT
413 GcbC and the catalytically inactive variant (GGAAF) introduced into this mutant on plasmids.
414 Pfl01_2297 and Pfl01_2295 are two other CACHE-domain containing diguanylate cyclases in *P.*
415 *fluorescens* that serve as controls. (D) Quantification of cell-surface levels of LapA in the
416 presence and absence of 0.4% citrate. Representative blots are shown. For panels B, C, and D,
417 the values shown are an average of 3 replicates (± SD); horizontal black bars indicate a P value
418 of either <0.05 (*), <0.01 (**), or <0.001 (***) with a student's t-test compared to the condition
419 without citrate comparing each strain without versus with citrate.

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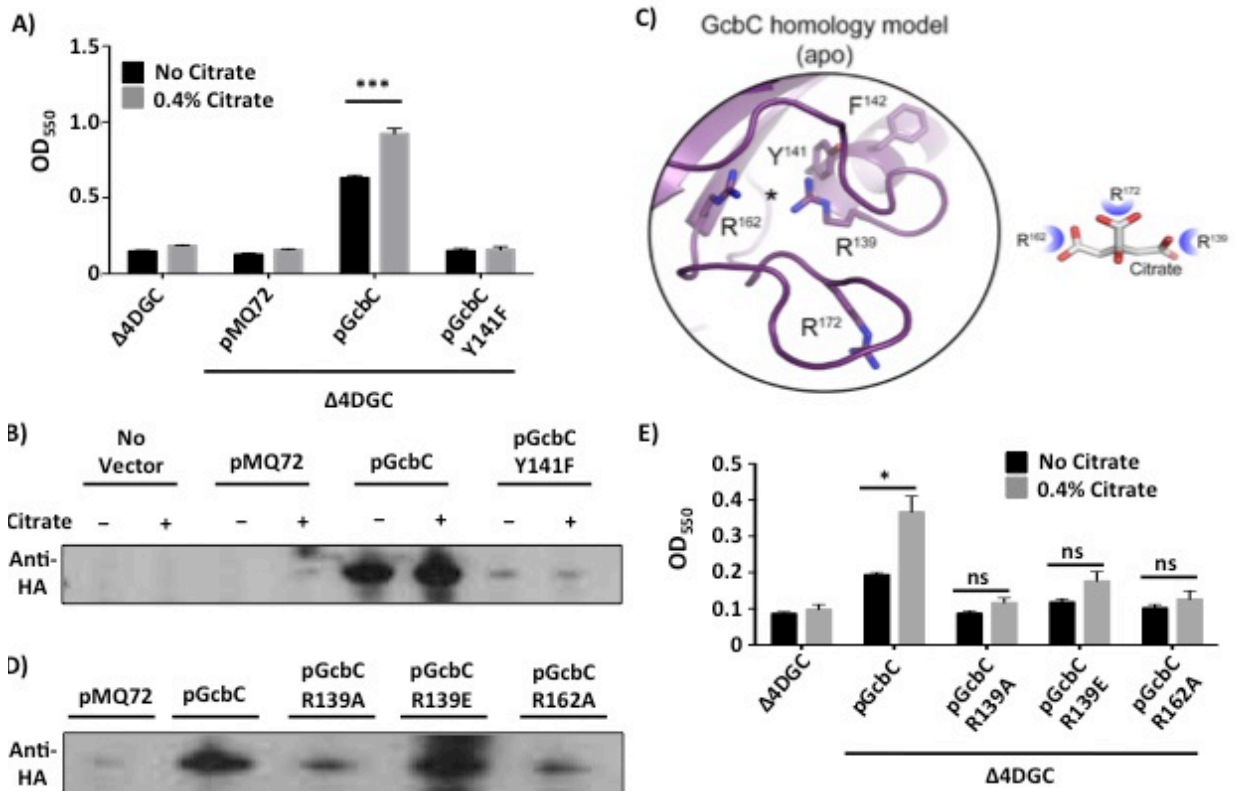

Fig. 2. Effects of CACHE domain mutations on biofilm formation. (A) Quantitation of the effect of CACHE domain mutant, GcbC Y141F, on biofilm formation in the presence and absence of citrate. (B) Western blot to assess the relative stability of GcbC-HA and GcbC-Y141F-HA in the presence and absence of citrate. (C) Homology model of the CACHE domain of GcbC based on rpHK1S-Z16 (PDB ID: 3LIF) and vpHK1S-Z8 (PDB ID: 3LID) (see Figure S3) to identify the putative ligand-binding site. (D) Western blot assessment of the relative stability of GcbC-R139A-HA, GcbC-R139E-HA and GcbC-R162A-HA. (E) Quantitative analysis of biofilm formation by strains expressing the indicated CACHE domain mutants in the presence and absence of citrate. For panels A and E, data shown is the average of three replicates (± SD); horizontal black bars indicate a P value of either <0.05 (*) or <0.001 (***) with a student's t-test comparing each strain without versus with citrate.

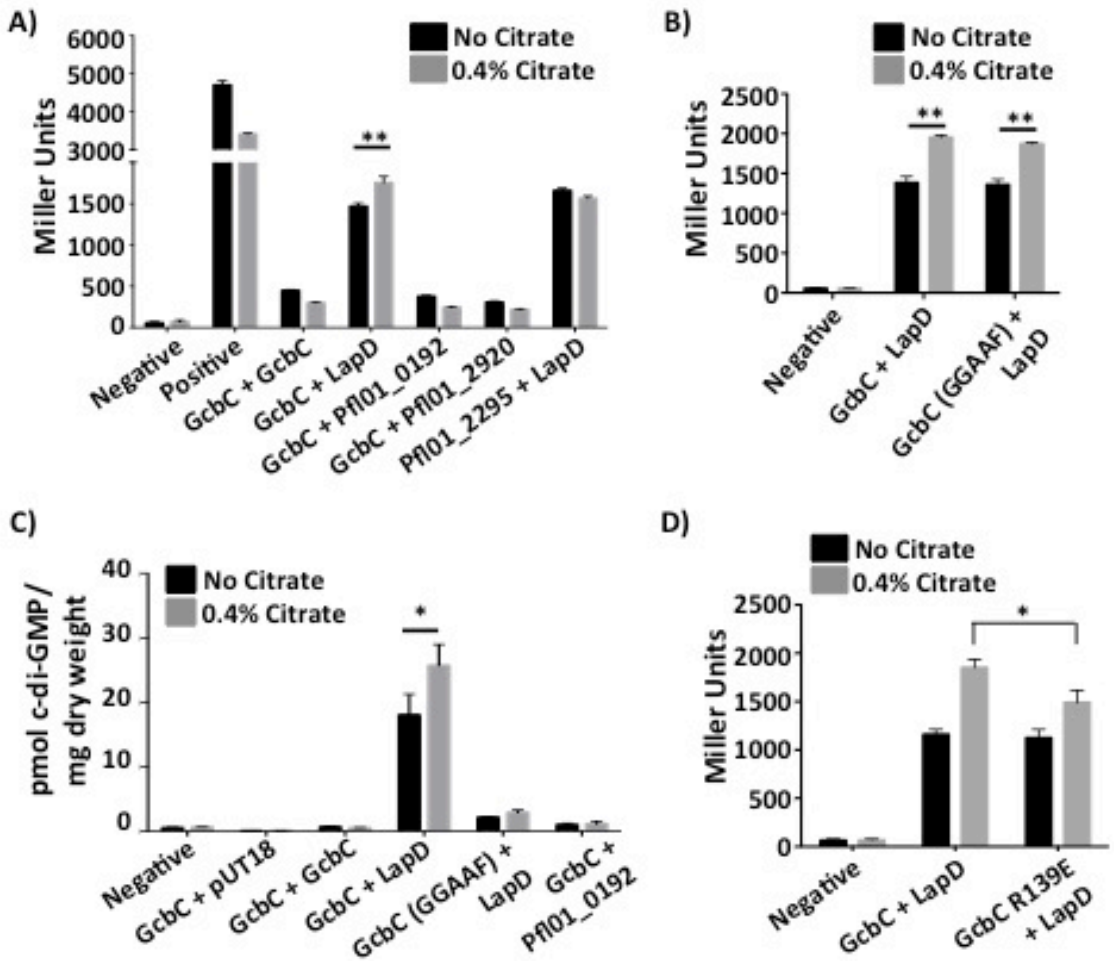

Fig. 3. Citrate-mediated biofilm enhancement. (A) The effect of citrate on interaction with the indicated proteins is compared by the B2H assay. Pfl01_2295 = CACHE domain-containing DGC; Pfl01_0192 = GGDEF and EAL domain-containing protein; Pfl01_2920 = phosphodiesterase. Briefly, the proteins of interest are fused to two halves of the catalytic domain of an adenylate cyclase, T25 and T18, from *Bordetella pertussis* are transformed into *E. coli* BTH101 cells (32). Plasmids pKT25 and pUT18C are the vector-only controls. If the two proteins interact, the adenylate cyclase activity is reconstituted, promoting cAMP synthesis, which in turn activates transcription of *lacZ*. The β-galactosidase activity is assessed using X-gal as a substrate, which serves as a read out for the degree of interaction (32). Citrate did not enhance interaction of the vectors or of the positive control (GCN4 leucine zipper protein). Citrate only enhanced the interaction between LapD and GcbC. (B) The effect of catalytic activity of GcbC on interaction with LapD were compared in the presence and absence of 0.4% citrate. (C) c-di-GMP production in *E. coli* BTH101 was determined for strains carrying the indicated proteins. Only co-expression of LapD-GcbC showed appreciable accumulation of c-di-GMP, which was stimulated by added citrate. (D) The effect of R139E CACHE mutation on interaction with LapD in the presence and absence of citrate. For panels B, C, D, and E, all experiments were performed in triplicate (± SD), with an indicated P value of either <0.05 (*) or <0.01 (**) with a student's t-test comparing each strain without versus with citrate.

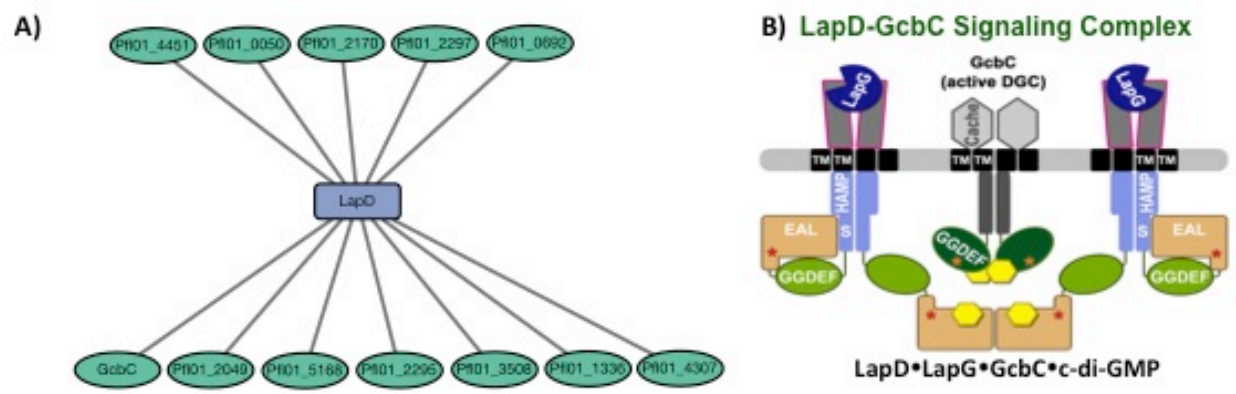

Fig. 4. LapD interacts with multiple DGCs. (A) Interaction map of DGCs that interact with LapD by 20 hours by bacterial-two-hybrid assay. Interaction map constructed using XXXXX. (B) A model of a GcbC, LapD, LapG signaling "basket" complex (23).