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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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Enzymes that make c-di-GMP are part of a complex network, with LapD as a central receptor for DGCs to interact with (Fig. 4A). It is highly unlikely that the twelve DGCs that make up this DGC network all interact with LapD without regulation to control signaling specificity. A more likely scenario could be that ligands are used as a signal to regulate signaling of a DGC to interact with LapD and outcompete with one another. It is also possible that other ligand-mediated mechanisms exist, such as a ligand-enhanced homodimerization of a DGC. Further characterizing and identifying what small molecules DGCs respond to could provide insight into how c-di-GMP signaling specificity is regulated within this complex signaling network. For example, 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. Our work provides insight of a ligand-based mechanism of how signaling specificity occurs among a complex network of enzymes and receptors that make, break, and bind c-d-GMP.

Material and Methods

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

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

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

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

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

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

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

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Literature Cited.

1. O'Toole GA, Kolter R (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: A genetic analysis. *Mol Microbiol* 28: 449-461.
2. Pruss BM, Besemann C, Denton A, Wolfe AJ (2006) A complex transcription network controls the early stages of biofilm development by *Escherichia coli*. *J Bacteriol* 188(11): 3731-3739.
3. Römling U, Gomelsky M, Galperin MY (2005) c-di-GMP: The dawning of a novel bacterial signaling system. *Mol Microbiol* 57(3): 629-639.
4. Baker AE, Diepold A, Kuchma SL, Scott JE, Ha DG, Orazi G, Armitage JP, O'Toole GA (2016) A PilZ domain protein FlgZ mediates cyclic-di-GMP-dependent swarming motility control in *Pseudomonas aeruginosa*. *J Bacteriol* 198(13): 1837-1846.
5. Ross P, Weinhouse H, Aloni Y, Michaeli D, Weinberger-Ohana P, Mayer R, Braun S, de Vroom E, van der Marel GA, van Boom JH, Benziman M (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanlyic acid. *Nature* 325(6101): 279-281.

6. Merrit JH, Brothers KM, Kuchma SL, O'Toole HA (2007) SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J Bacteriol* 189(22): 8154-8164.
7. Monds RD, Newell PD, Gross RH, O'Toole GA (2007) Phosphate-dependent modulation of c-di-GMP levels regulates *Pseudomonas fluorescens* Pf0-1 biofilm formation by controlling secretion of the adhesin LapA. *Mol Microbiol* 63(3): 659-679.
8. Martínez-Gil M, Ramos-González MI, Espinosa-Urgel M (2014) Roles of cyclic di-GMP and the Gac system in transcriptional control of the genes coding for *Pseudomonas putida* adhesins LapA and LapF. *J Bacteriol* 196(8): 1484-1495.
9. Hinsa SM, Espinosa-Urgel M, Ramos JL, O'Toole HA (2003) Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Mol Microbiol* 49: 905-918.
10. Newell PD, Monds RD, O'Toole GA (2009) LapD is a bis-(3'-5')-cyclic dimeric GMP-binding protein that regulates surface attachment by *Pseudomonas fluorescens* Pf0-1. *Proc Natl Acad Sci USA* 106(9): 3461-3466.
11. Navarro MV, Newell PD, Krasteva PV, Chatterjee D, Madden DR, O'Toole GA, Sondermann HA (2011) Structural basis for c-di-GMP-mediated inside-out signaling controlling periplasmic proteolysis. *PLoS Biol* 9(2): e1000588. doi: 10.1371.
12. Dahlstrom KM, Giglio KM, Collins AJ, Sondermann H, O'Toole GA (2015) Contribution of physical interactions to signaling specificity between a diguanylate cyclase and its effector. *mBio* 6(6):e01978-15. doi:10.1128/mBio.01978-15.
13. Anantharamn V, Aravind L (2000) Cache – a signaling domain common to animal Ca(2+)-channel subunits and a class of prokaryotic chemotaxis receptors. *Trends Biochem Sci* 25(11): 535-537.
14. Upadhyay AA, Fleetwood AD, Adebali O, Finn RD, Zhulin IB (2016) Cache domains that are homologous to, but different from PAS domains comprise the largest superfamily of extracellular sensors in prokaryotes. *PLoS Comput Biol* 12(4): e1004862. doi: 10.1371/journal.pcbi.1004862.
15. Zhang Z, Hendrickson WA (2010) Structural characterization of the predominant family of histidine kinase sensor domains. *J Mol Biol* 400(3): 335-353.
16. Boyd CD, Chatterjee D, Sondermann H, O'Tool GA (2012) LapG, required for modulating biofilm formation by *Pseudomonas fluorescens* Pf0-1, is a calcium-dependent protease. *J Bacteriol* 194(16): 4406-4414.
17. Newell PD, Yoshioka S, Hvorecny KL, Monds RD, O'Toole GA (2011) Systematic analysis of diguanylate cyclases that promote biofilm formation by *Pseudomonas fluorescens* Pf0-1. *J Bacteriol* 193(18): 4685-4698.
18. Chen Y, Cao S, Chai Y, Clardy J, Kolter R, Guo JH, Losick R (2012) A *Bacillus subtilis* sensor kinase involved in triggering biofilm formation on the roots of tomato plants. *Mol Microbiol* 85(3): 418-430.

19. Chan C, Paul R, Samoray D, Amiot NC, Giese B, Jenal U, Schirmer T (2004) Structural basis of activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci USA* 101(49): 17084-17089.
20. Ryjenkov DA, Tarutina M, Moskvin OV, Gomelsky M (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J Bacteriol* 187(5): 1792-1798.
21. Merritt JH, Brothers KM, Kuchma SL, O'Toole GA (2007) SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J Bacteriol* 189(22): 8154-8164.
22. Merritt JH, Ha DG, Cowles KN, Lu W, Morales DK, Rabinowitz J, Gitai Z, O'Toole GA (2010) Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. *Mbio* 1(4): e00183-10. doi:10.1128/mBio. 00183-10.
23. Cooley RB, O'Donnell JP, Sondermann H (2016) Coincidence detection and bi-directional transmembrane signaling control a bacterial second messenger receptor. *Elife* 5: e21848 doi: 10.7554/eLife.21848.
24. Haas D, Défago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* 3(4): 307-319.
25. Jones DL (1998) Organic acids in the rhizosphere – a critical review. *Plant Soil* 205: 25-44.
26. de Weert S, Vermeiren H, Mulders IH, Kuiper I, Hendrickx N, Bloemberg GV, Vanderleyden J, De Mot R, Lugtenberg BJ (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant Microbe Interact* 15(11): 1173-1180.
27. Brewster JL, McKellar JL, Finn TJ, Newman J, Peats TS, Gerth ML (2016) Structural basis for ligand recognition by a Cache chemosensory domain that mediates carboxylate sensing in *Pseudomonas syringae*. *Sci Rep* 6:35198. doi: 10.1038/srep35198.
28. Shanks RMA, Calazza NC, Hinsa SM, Toutain CM, O'Toole GA (2006) *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of gram-negative bacterial genes. *Appl Environ Microbiol* 72(7): 5027-5036.
29. Bascom-Slack CA, Dawson D (1998) A physical assay for detection of early meiotic recombination intermediates in *Saccharomyces cerevisiae*. *Mol Gen Genet* 258: 512-520.
30. Monds RD, Newell PD, Schwartzman JA, O'Toole GA (2006) Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0-1. *Appl Environ Microbiol* 72(3): 1910-1924.
31. Newell PD, Boyd CD, Sodermann H, O'Toole GA (2011) A c-di-GMP effector system control cell adhesion by inside-out signaling and surface protein cleavage. *PloS Biol* 9(2): e1000587. doi: 10.1371/journal.pbio.1000587.
32. Karimova G, Pidoux J, Ullmann A, Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci USA* 95: 5752-5756.
33. Shultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: Identification of signaling domains. *Proc Natl Acad Sci USA* 95: 5857-5864.

399 34. Ulrich LE, Zhulin IB (2010) The MiST2 database: a comprehensive genomics resource on
400 microbial signal transduction. *Nucleic Acids Res* 38: D401-407 doi:10.1093/nar/gkp940.
401 35. Simon R, Preifer U, Puhler A (1983) A broad host range mobilization system for *in vivo*
402 genetic engineering: transposon mutagenesis in gram negative bacteria. *Nat Biotechnol*
403 1: 784-791.

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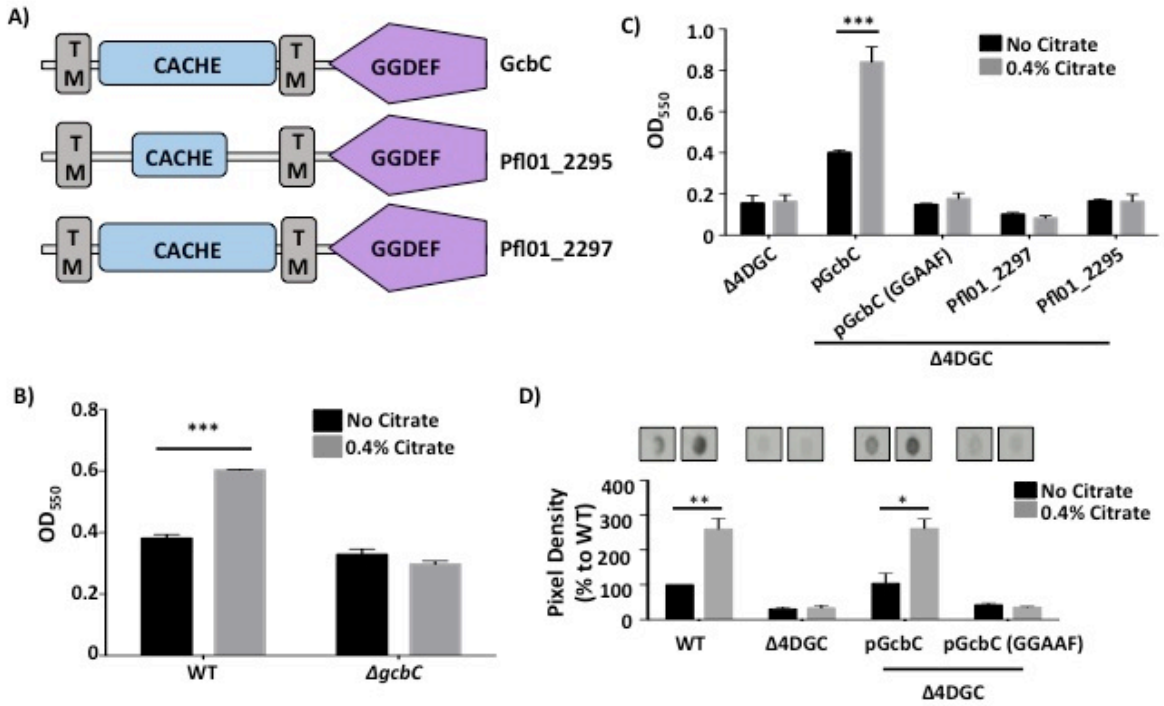
406 **Figures**



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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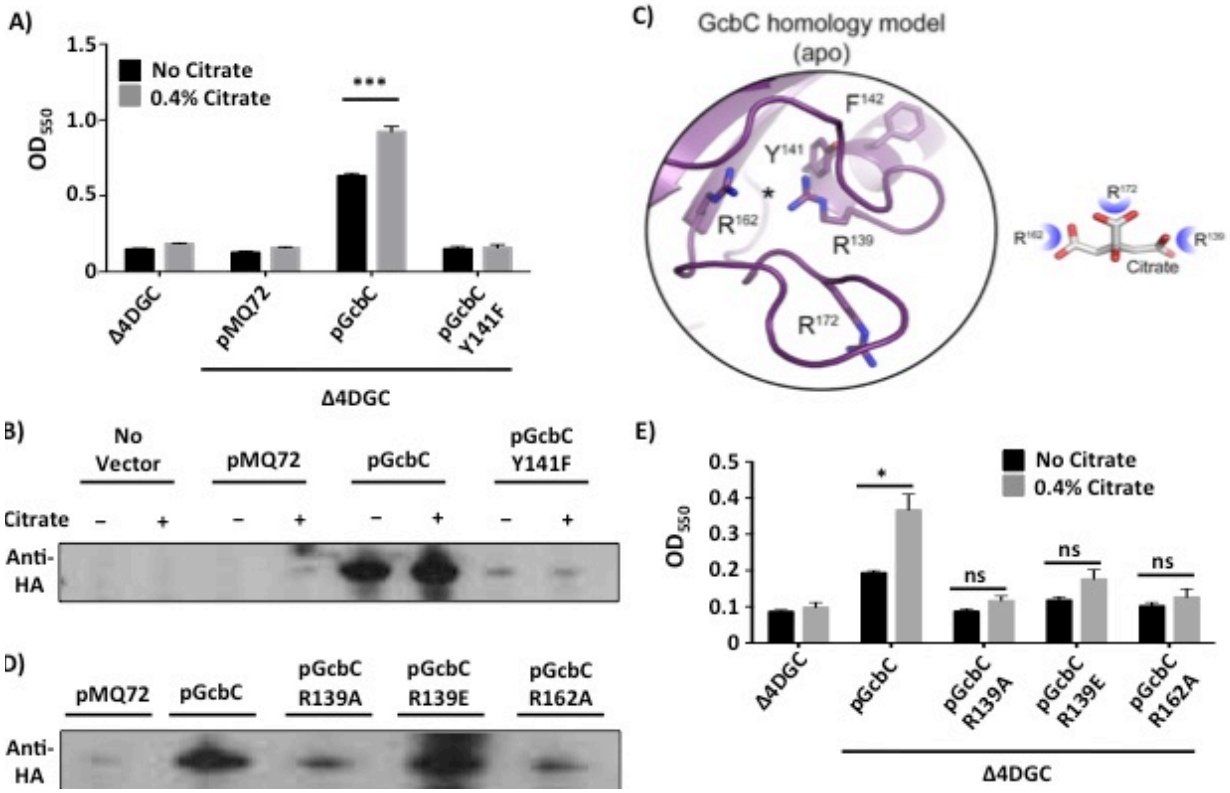

Fig. 2. Effects of CACHE domain mutations on biofilm formation. (A) Quantitation of the effect of CACHE domain mutant, GcbC Y141F, on biofilm formation in the presence and absence of citrate. (B) Western blot to assess the relative stability of GcbC-HA and GcbC-Y141F-HA in the presence and absence of 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.

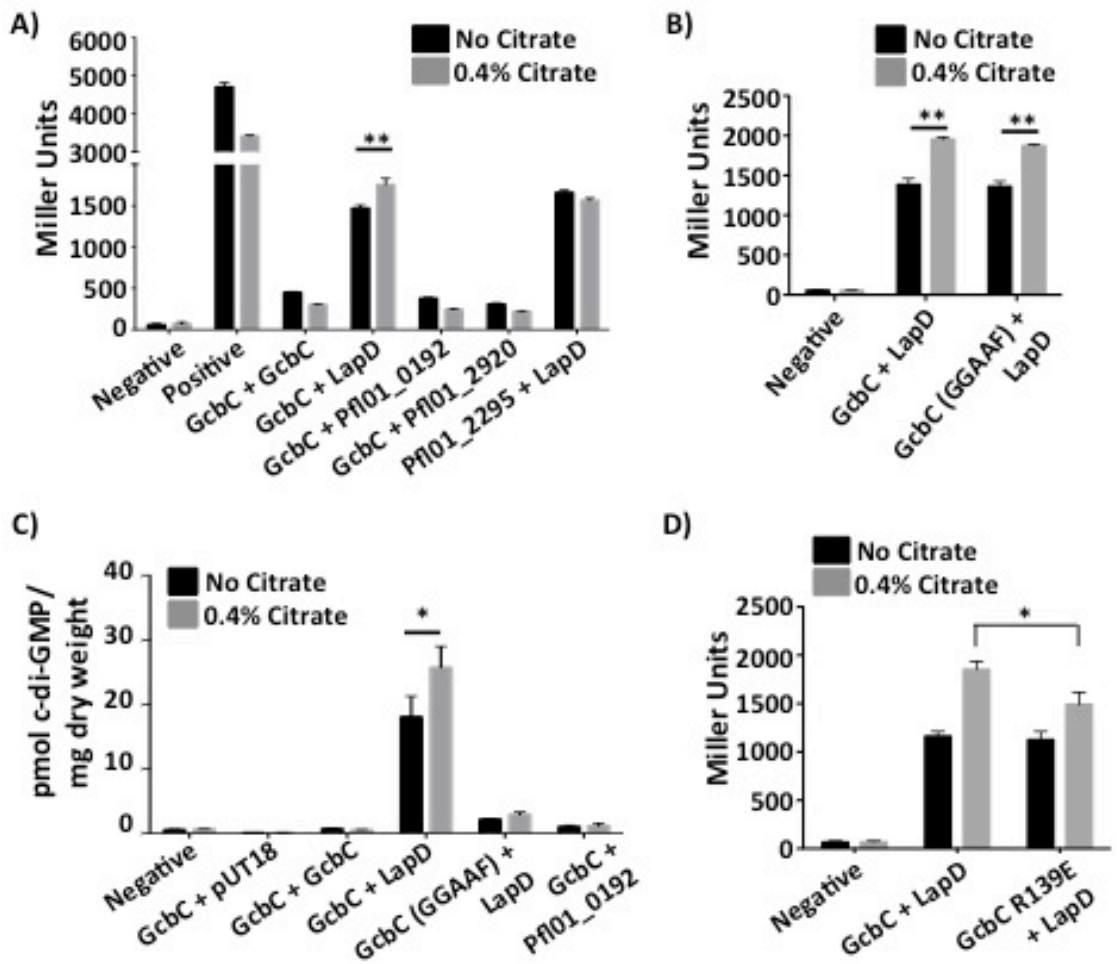

Fig. 3. Citrate-mediated biofilm enhancement. (A) The effect of citrate on interaction with the indicated proteins is compared by the B2H assay. Pfl01_2295 = CACHE domain-containing DGC; Pfl01_0192 = GGDEF and EAL domain-containing protein; Pfl01_2920 = phosphodiesterase. Briefly, the proteins of interest 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.

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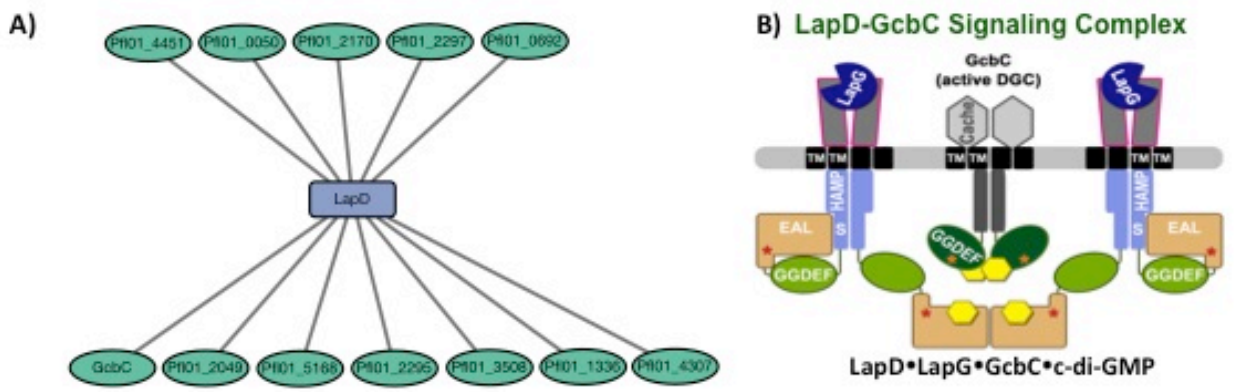

453 **Fig. 4. LapD interacts with multiple DGCs**. (A) Interaction map of DGCs that interact with LapD
454 by 20 hours by bacterial-two-hybrid assay. Interaction map constructed using XXXXX. (B) A
455 model of a GcbC, LapD, LapG signaling "basket" complex (23).