The Inhibitory Site of a Diguanylate Cyclase Is a Necessary Element for Interaction and Signaling with an Effector Protein

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ABSTRACT

Many bacteria contain large cyclic diguanylate (c-di-GMP) signaling networks made of diguanylate cyclases (DGCs) and phosphodiesterases that can direct cellular activities sensitive to c-di-GMP levels. While DGCs synthesize c-di-GMP, many DGCs also contain an autoinhibitory site (I-site) that binds c-di-GMP to halt excess production of this small molecule, thus controlling the amount of c-di-GMP available to bind to target proteins in the cell. Many DGCs studied to date have also been found to signal for a specific c-di-GMP-related process, and although recent studies have suggested that physical interaction between DGCs and target proteins may provide this signaling fidelity, the importance of the I-site has not yet been incorporated into this model. Our results from Pseudomonas fluorescens indicate that mutation of residues at the I-site of a DGC disrupts the interaction with its target receptor. By creating various substitutions to a DGC’s I-site, we show that signaling between a DGC (GcbC) and its target protein (LapD) is a combined function of the I-site-dependent protein-protein interaction and the level of c-di-GMP production. The dual role of the I-site in modulating DGC activity as well as participating in protein-protein interactions suggests caution in interpreting the function of the I-site as only a means to negatively regulate a cyclase. These results implicate the I-site as an important positive and negative regulatory element of DGCs that may contribute to signaling specificity.

IMPORTANCE

Some bacteria contain several dozen diguanylate cyclases (DGCs), nearly all of which signal to specific receptors using the same small molecule, c-di-GMP. Signaling specificity in these networks may be partially driven by physical interactions between DGCs and their receptors, in addition to the autoinhibitory site of DGCs preventing the overproduction of c-di-GMP. In this study, we show that disruption of the autoinhibitory site of a DGC in Pseudomonas fluorescens can result in the loss of interactions with its target receptor and reduced biofilm formation, despite increased production of c-di-GMP. Our findings implicate the autoinhibitory site as both an important feature for signaling specificity through the regulation of c-di-GMP production and a necessary element for the physical interaction between a diguanylate cyclase and its receptor.

A major method of intracellular signaling in bacteria revolves around the second messenger cyclic diguanylate (c-di-GMP). c-di-GMP is produced by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs) in networks that may range from one or a few such proteins to several dozen (1). Effector proteins then sense c-di-GMP by binding it and promote some specific cellular action as a result. Importantly, many DGCs studied to date promote only one or a small number of such processes (2–6), indicating that there must be mechanisms in place that allow for specificity in signaling among the many members of a c-di-GMP network.

A key regulatory element in signaling specificity is the autoinhibitory site (I-site) found on many DGCs that locks the enzyme in an inactive state when bound to c-di-GMP. Many, but not all, DGCs have a characteristic I-site that provides this kind of negative-feedback regulation, which has been described as characteristic of systems that require fast up-kinetics in their enzymatic reactions (7). Studies focused on disrupting the I-site have previously found that deregulation of a DGC leads to increased production of c-di-GMP and a concomitant increase in c-di-GMP-related phenotypes, including increases in biofilm formation and decreases in motility (7, 8). Additionally, effectors have been thought to provide one mechanism of signaling specificity by differentially activating at various levels of c-di-GMP, as demonstrated in Salmonella enterica serovar Typhimurium (9), with a complementary study identifying various response curves for c-di-GMP-dependent processes in Caulobacter crescentus (10). The apparent function of the I-site is therefore 2-fold. First, the I-site allows for a rapid enzymatic response to changing environmental factors in that the rate of c-di-GMP synthesis can be manipulated without having to translate or degrade a given DGC. Second, the I-site can limit the total amount of c-di-GMP available to be read by effector proteins, providing one level of signaling regulation.

Recently, a theme that has emerged in signaling specificity for large c-di-GMP networks is physical interactions between DGCs, PDEs, and effectors (4, 11). By using physical proximity, DGCs can successfully direct their signal to a specific effector target. It
has remained an open question to what extent, if any, this mode of c-di-GMP regulation is intertwined with the I-site-mediated control of DGC activity to govern signaling fidelity.

One example of signaling specificity achieved by physical interaction is the regulation of biofilm formation by the inner membrane proteins GcbC and LapD in *Pseudomonas fluorescens*. GcbC is a DGC that signals to the effector LapD. When bound to c-di-GMP, LapD changes conformation to sequester a periplasmic protease called LapG, thus allowing the large adhesin LapA to accumulate on the cell surface and thereby promoting biofilm formation (12–14). A previous study from our laboratory used a mutagenic screen to identify a string of amino acids on the surface of GcbC that appears to interact with a surface-exposed α-helix of LapD (11). While this result paints a partial picture of how these two proteins interact, the residues identified to date are not sufficient to account for the entirety of the interaction observed between these two proteins.

In this study, we demonstrate that mutations made to residues in the I-site of GcbC disrupt the interaction with LapD. Furthermore, we find that I-site mutations that disrupt the interaction between GcbC and LapD fall into two groups. One group of mutations to the I-site causes large spikes in c-di-GMP production, resulting in the typical phenotype of an increase in biofilm formation. The other group of mutations to the I-site results in reduced biofilm formation despite similar, or modestly increased, c-di-GMP levels compared to those of the wild-type strain. These results suggest an important role for the I-site of GcbC in controlling c-di-GMP production as well as directly or indirectly mediating physical interaction with LapD. Collectively, this study reinforces a model whereby the activation of some effector proteins is a combined function of c-di-GMP production and physical interaction and adds to it evidence that the I-site is relevant to mediating both of these processes.

**MATERIALS AND METHODS**

**Strains and media.** *P. fluorescens* BTH101, *Pseudomonas aeruginosa* PA14, and *Escherichia coli* BTH101 were used in this study (see Table S1 in the supplemental material). *E. coli* S17-1 was used for cloning (15). *P. aeruginosa* and *E. coli* were grown at 37°C except where indicated in the text. *P. fluorescens* was grown at 30°C. All overnight growth was done with lysogeny broth (LB), and all plates contained 1.5% agar. The expression vector pMQ72 was used for all biofilm, Congo red, and c-di-GMP quantification assays and was induced with arabinose as described below. The expression vectors pUT18 and pKNT25 were used for bacterial two-hybrid (B2H) experiments and were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Gentamicin (Gm) was used in conjunction with pMQ72 at 30 μg/ml for *P. fluorescens* on solid agar and 15 μg/ml in liquid and at 50 μg/ml for *P. aeruginosa*, Kanamycin (Kn) and carbenicillin (Cb) were used at concentrations of 50 μg/ml in conjunction with pKNT25 and pUT18, respectively. All subculturing was the result of picking a single *P. fluorescens* colony and growing the culture overnight in 4 ml of LB with the appropriate antibiotics before inoculating the subculture at a 1:75 ratio. Subcultures of 5 ml were grown on a rotating wheel, while subcultures of 45 ml were grown in 250-ml flasks on a platform shaker at 230 rpm (see Table S1 in the supplemental material for a complete list of strains and plasmids, and see Table S2 in the supplemental material for a list of primers used in this study).

**Bacterial two-hybrid studies.** *E. coli* BTH101 cells were coelectroporated with 50 ng each of plasmids pUT18 and pKNT25. Cells were recovered in LB at 37°C for 1 h and plated at a 1:4 dilution onto LB plates amended with Kn, Cb, and IPTG. Plates were incubated at 30°C for 15 h, and colonies were recovered from the plates by scraping. Recovered cells were diluted to an optical density (OD) at 600 nm of ~0.5, and a β-galactosidase assay was conducted as described previously (11), using a previously reported system (16). Briefly, cells were permeabilized by using chloroform and SDS followed by vortexing and a 5-min incubation at 30°C. Eight hundred micrograms of *ortho*-nitrophenyl-β-galactoside (ONPG) was added to each sample, followed by another 5-min incubation at 30°C. The reaction was stopped by using 500 μl of 1 M Na2CO3, and the optical density was read at 420 and 550 nm to calculate Miller units. Each biological replicate represents an independent cotransformation of BTH101 cells.

**Congo red assay.** *Pseudomonas aeruginosa* PA14 was transformed with various GcbC alleles on the pMQ72 backbone. One milliliter of a culture grown overnight was washed twice with 1 ml 20% sucrose, before a final resuspension in 100 μl of 20% sucrose, after which the desired plasmid was added to 40 μl of the washed sample and electroporated, following a 1-h recovery period in LB at 37°C. The resulting transformants were picked from selective agar and grown overnight in LB cultures supplemented with Gm, and 2.5 μl of the culture was spotted onto 1.0% agar minimal medium plates amended with Congo red dye. Plates were grown at 30°C for 36 h.

**Biofilm assay.** A biofilm assay was conducted as described previously (17). Briefly, strains of *P. fluorescens* were grown overnight in 4 ml of liquid LB supplemented with Gm from LB agar plates. Samples were normalized to the cell optical density, and 1.5 μl of the normalized culture was added to 100 μl of K10T-1 minimal medium (6) in a 96-well U-bottom microtiter plate (Costar). Plates were incubated inside a humidified chamber at 30°C for 6 h, after which the supernatant was decanted from the wells and stained for 20 min with 125 μl of 0.1% crystal violet, followed by three washes in distilled water and drying overnight. Wells were then photographed and dissolved for quantification by using 150 μl of a destain solution (45:45:10 dilution of water-methanol-glacial acetic acid). One hundred twenty-five microliters was transferred to a flat-bottomed 96-well plate to read the OD at 550 nm.

**Quantification of c-di-GMP.** Strains of *P. fluorescens* were struck on LB plates supplemented with Gm, and colonies were picked for growth overnight in liquid LB supplemented with Gm. At 16 h, 75 μl of the culture grown overnight was added to 5 ml of K10 minimal medium supplemented with 0.2% arabinose. The subcultures were aerated and grown for 6 h at 30°C. Samples were then pelleted, and nucleotides were extracted by using 0.5 ml of extraction buffer (40:40:20 dilution of methanol-acetonitrile–double-distilled water [ddH2O] plus 0.1 N formic acid) at −20°C for 1 h as described previously (18). Samples were then centrifuged, and 8 μl of NH4HCO3 was added to the supernatant, followed by drying under a vacuum to dryness. Samples were resuspended in 200 μl of high-performance liquid chromatography (HPLC)-grade water. Samples were analyzed by liquid chromatography–mass spectrometry (LC-MS) via the Michigan State University Mass Spectrometry Facility and compared to a standard curve of a known c-di-GMP concentration, and results were normalized to the dry weight of the bacterial pellets that generated the extractions. Each biological replicate represents a separate colony picked from the struck-out plate.

**Western blot analysis.** Cultures of *P. fluorescens* grown overnight were subcultured at a 1:75 dilution in 45 ml of K10 minimal medium supplemented with 0.1% arabinose. Subcultures were shaken for 6 h and pelleted by centrifugation, followed by resuspension in lysis buffer (20 mM Tris [pH 8.0] and 25 mM MgCl2) and sonication. Sample values were normalized to the protein concentration via a bicinchoninic acid (BCA) assay, and 6 μg of the protein sample was loaded onto a 10% polyacrylamide gel. Blotting was conducted with an antihemagglutinin (anti-HA) antibody (Covance) at 4°C overnight in 3% bovine serum albumin (BSA).

**Statistics.** Error bars throughout the study represent standard deviations, and *P* values were calculated by using a two-tailed *t* test.
RESULTS

The I-site of GcbC is necessary for interaction with LapD. A previous study from our laboratory reported the structure for the GGDEF domain of the P. fluorescens diguanylate cyclase GcbC (11). Using this crystal structure, we are able to verify that GcbC likely has a primary inhibitory site (Fig. 1) with the previously characterized RXXD motif (7, 19). The RXXD motif is found at residues R410 to D413 of GcbC. Notably, R409 also makes contact with a c-di-GMP molecule; the residue at position 409 is not highly conserved among DGCs, and it is somewhat unusual for it to participate in c-di-GMP binding. In addition to the primary I-site, GcbC also contains a secondary I-site at position R366 that completes GcbC’s regulatory c-di-GMP binding site (Fig. 1). In our crystal structure of GcbC bound to c-di-GMP, two molecules of c-di-GMP were found to be bound between the primary and secondary I-sites of this inactive dimer (11), following a convention described previously for other DGCs known to contain both primary and secondary I-sites (19–23). Finally, three more residues were found to coordinate the formation of the I-site pocket where residue R363 of each monomer of GcbC forms a polar contact with residues E360 and E429 of the other GcbC monomer within the dimer complex of this protein (Fig. 1).

This crystal structure was previously used to understand how the diguanylate cyclase GcbC physically interacts with the effector protein LapD in order to promote biofilm formation. Specifically, a mutagenic bacterial two-hybrid (B2H) screen was conducted to identify alleles of GcbC that failed to interact with LapD (11). In this assay, wild-type versions of GcbC and LapD interacted, as judged by the blue color formation on medium supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (see Materials and Methods for details of the B2H assay). E. coli cells containing the wild-type allele of the lapD gene and a mutagenized library of gcbC alleles were screened on plates supplemented with X-Gal, and colonies that failed to turn blue were identified. The gcbC-containing plasmids from these colonies were isolated and sequenced, yielding 15 isolates carrying mutations in the gcbC gene that failed to interact normally with LapD (11). While some of these isolates provided information that led to the identification of a string of amino acids on a surface-exposed α-helix of GcbC that contacts a similarly surface-exposed α-helix of LapD, not all mutations mapped to these helical regions. Furthermore, the previously identified residues on these surface-exposed α-helices of GcbC and LapD were not sufficient to fully account for binding between these two proteins (11).

Suspecting that the interaction between GcbC and LapD may depend upon additional structural elements, we reanalyzed the mutagenic data set using the crystal structure of GcbC. A surprising finding was the number of isolates that implicated the GcbC I-site as a necessary element of interaction. Of the original 15 mutants isolated in our screen, 7 had mutations that mapped near the I-site of GcbC (Table 1). Two isolates with mutations near the primary I-site were found, R402W and I404T, and five isolates with mutations in the vicinity containing the secondary I-site residues that interact in the inhibited GcbC GGDEF domain dimer were identified, including Q355R, S356P, and W361R (Fig. 1B and C). The Q355R and W361R mutations were found twice each, suggesting that these specific mutations are responsible for the observed phenotype among the multiple mutations found in the isolates.

FIG 1 Architecture of the GcbC I-site. (A) Model for negative-feedback inhibition of GcbC by c-di-GMP. The yellow circles and asterisks denote the active and inhibitory sites, respectively, on the GGDEF domain. (B) The crystal structure of GcbC shows a dimer of the GGDEF domains binding two molecules of c-di-GMP at the canonical I-site located at the GcbC dimer interface. GcbC contains a primary I-site with the canonical R410XXD413 motif, which in GcbC is extended by another arginine residue at position 409, and a secondary I-site that uses a single arginine residue (R366). Primary and secondary I-sites are located on adjacent dimers, with a c-di-GMP dimer bridging the interface. Additionally, the GcbC dimer uses a protein-protein interface made up of a series of glutamic acid and arginine residues. The catalytic residues are highlighted in yellow. The GcbC crystal structure was originally reported by Dahlstrom et al. (11). IM, inner membrane; TM, transmembrane. (C) Mutations predicted to disrupt the I-site or GcbC domain dimers listed in Table 1 are mapped onto the crystal structure of GcbC as cyan spheres. Most substitutions identified in this screen appear to disrupt the ability of GcbC to form an inactive dimer by preventing the I-site from being occupied by c-di-GMP.
antibody was used to detect GcbC.

Concentrations, and 6 h at 30°C, followed by staining with 0.1% crystal violet. Error bars represent standard deviations of data from four biological replicates made up of eight technical replicates per biological replicate. **, ***.

The mutations identified could feasibly disrupt the I-site through structural changes or via the result of substitutions that directly interfere with a nearby residue’s ability to bind c-di-GMP, which led us to hypothesize that the I-site of GcbC is necessary for the interaction with the effector LapD. However, because most isolates from our screen contained multiple mutations, and because the exact residues mutated could not be directly shown to impact the GcbC I-site, we made targeted, nonconservative substitutions of several canonical I-site residues to verify by bacterial two-hybrid assays that disruption of the I-site residues results in a decrease in the interaction between GcbC and LapD.

The use of the B2H assay was previously validated for the GcbC-LapD interaction through a coprecipitation analysis (11); thus, we used this method to assess the impact of the directed I-site mutations on the interaction of these two proteins. Three I-site substitutions that resulted in a reduced interaction with LapD were initially identified in GcbC. GcbC-E429R demonstrated a modest, nonsignificant reduction in the interaction with LapD, while GcbC proteins with mutations E360R and D413K showed an almost complete loss of the interaction with LapD (Fig. 2A).

Residues E360 and E429 are engaged in a protein-protein interface between two monomers of GcbC, which is a prerequisite to form the inactive, c-di-GMP-bound dimer, while D413 is part of the primary I-site and binds c-di-GMP directly. The decrease in the interaction between LapD and GcbC associated with these changes to I-site residues in GcbC suggests that disruption of either GcbC’s ability to form the I-site or the I-site’s ability to bind c-di-GMP leads to a loss of the interaction with LapD. Importantly, a previous study demonstrated that neither GcbC’s ability to produce c-di-GMP nor LapD’s ability to bind c-di-GMP was required for protein-protein interactions, eliminating the catalytic state of GcbC as a confounding factor for the interaction between these two proteins (11).

**I-site mutations in GcbC result in decreased biofilm formation.** While disruption to the I-site of GcbC led to an apparent loss of the interaction with LapD, we wanted to directly test the impact that these mutations would have on the functional relationship between these two proteins in terms of their ability to govern biofilm formation. We therefore set out to test biofilm formation when GcbC has a mutated I-site using the mutated alleles described above. To this end, we built HA-tagged versions of the wild-type and mutant versions of GcbC on a plasmid and expressed these constructs in a background of P. fluorescens lacking its native DGC subnetwork that normally governs biofilm formation, referred to as the ΔDGC strain. In this way, the wild-type or mutant version of GcbC would be the sole DGC responsible for promoting biofilm formation under our assay conditions (6).

We found that in each case examined, variants of GcbC mutated in the I-site resulted in a significant decrease in biofilm formation compared to that of wild-type GcbC (Fig. 2B), suggesting that the loss of the physical interaction between GcbC and LapD as a result of the disrupted I-site is physiologically important for the in vivo functionality of these two proteins. To confirm that the

**TABLE 1** GcbC mutations that disrupt interactions between GcbC and LapD in a bacterial two-hybrid screen

<table>
<thead>
<tr>
<th>Isolate</th>
<th>GcbC mutation(s)</th>
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<tbody>
<tr>
<td>1</td>
<td>I179T, N244Y, Q355R</td>
</tr>
<tr>
<td>2</td>
<td>M153V, S212G, H236N, I404T</td>
</tr>
<tr>
<td>3</td>
<td>L88Q, W361R, A482T</td>
</tr>
<tr>
<td>4</td>
<td>E209C, S235P, H390Q</td>
</tr>
<tr>
<td>6</td>
<td>W361R</td>
</tr>
<tr>
<td>7</td>
<td>Q355R</td>
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</tbody>
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The mutation in each sequenced allele of GcbC predicted to disrupt the I-site is highlighted in boldface type.

*FIG 2* Disruption of the GcbC I-site leads to loss of interaction with LapD and loss of biofilm formation. (A) Bacterial two-hybrid experiment measuring interactions between LapD and several variants of GcbC. *E. coli* BTH101 cells were grown on selective medium at 30°C for 15 h, scraped from the plate, and analyzed for β-galactosidase production. Error bars represent standard deviations of data from three biological replicates made up of three technical replicates per biological replicate. ***, P < 0.001 (compared to wild-type GcbC). (B) The same GcbC mutations as those described above for panel A were tested for their ability to promote biofilm formation by *P. fluorescens*. Strains were cultured overnight, inoculated into a 96-well plate of K10 minimal medium, and grown for 6 h at 30°C, followed by staining with 0.1% crystal violet. Error bars represent standard deviations of data from four biological replicates made up of eight technical replicates per biological replicate. **, P < 0.01; ***, P < 0.001 (compared to wild-type GcbC). (C) The strains used for panel B were subcultured in a shaking flask for 6 h in 45 ml of K10 minimal medium, and cells were lysed by sonication. Concentrations in whole-cell lysates were normalized to total protein concentrations, and 6 μg of protein was analyzed by Western blotting to evaluate the protein stability of wild-type GcbC and its mutant variants. An anti-HA antibody was used to detect GcbC.
FIG 3 Mutations to the I-site of GcbC disrupt c-di-GMP regulation. (A) Wild-type (WT) GcbC and its mutant variants were heterologously expressed in P. aeruginosa PA14. Strains were grown overnight at 37°C, and 2.5 μl was spotted onto a minimal medium plate containing Congo red dye. Expression was induced with 0.1% arabinose. Colony biofilms were grown at 30°C for 36 h and photographed. Stronger red coloration indicates more c-di-GMP production. Note the redder tint of the strain expressing GcbC(D413K) and the darker red coloration of strains expressing GcbC(E360R) and GcbC(E429R) than of the strain expressing wild-type GcbC. (B) The same P. fluorescens strains used for the biofilm assay described in the legend to Fig. 2B were subcultured in rotating tubes for 6 h and pelleted. Nucleotide extraction was performed on the pellets, and the resulting organic phase was analyzed by mass spectrometry (see Materials and Methods). Expression was induced with 0.2% arabinose. Error bars represent standard deviations of data from three biological replicates. *, P < 0.05 (compared to wild-type GcbC).

Mutant versions of GcbC are stably expressed, whole-cell lysates normalized by protein concentration were examined by Western blotting. In each case, the mutant variants of GcbC appeared to be stably expressed at a level comparable to that of wild-type GcbC (Fig. 2C), indicating that protein instability is not responsible for the loss of biofilm formation observed.

Previous studies suggested that disruption of a DGC’s I-site leads to an inability to form the inactive dimer conformation (22) and that a DGC will still readily adopt its active dimer conformation, leading to c-di-GMP overproduction (8). However, we could not immediately rule out the possibility that the mutant variants of GcbC experienced a loss of the ability to produce c-di-GMP; thus, their catalytic ability is assessed below.

Disruption of the I-site of GcbC leads to deregulation of c-di-GMP production. Our initial results showing that mutation of the I-site of GcbC reduced biofilm formation was somewhat surprising, given that other studies have found that I-site disruption leads to the overproduction of c-di-GMP and enhanced biofilm formation relative to proteins with functional I-sites (7, 8). To analyze the impact of these I-site mutations on c-di-GMP levels, we employed two different assays. First, we overexpressed each allele of GcbC heterologously in Pseudomonas aeruginosa PA14 and grew the resulting transformants on minimal medium supplemented with Congo red. This assay has been shown to detect c-di-GMP production via c-di-GMP-dependent Pel polysaccharide production; this polysaccharide binds to the Congo red dye, resulting in a red colony. P. aeruginosa was used for this experiment due to its ability to produce Pel in response to c-di-GMP, which is a process that P. fluorescens lacks. Expression of GcbC produces a very light red color compared to that produced by wild-type P. aeruginosa (Fig. 3A). The D413K substitution in the I-site produced a somewhat darker red tint compared to that produced by wild-type GcbC. Furthermore, the E360R and E429R substitutions produced very pronounced red colors, consistent with elevated levels of c-di-GMP production (Fig. 3A).

We next wanted to test c-di-GMP production in vivo to determine the relative amounts of c-di-GMP produced in P. fluorescens carrying wild-type GcbC or the I-site mutant variants. We again grew each strain in minimal medium, overexpressed each allele of GcbC, performed nucleotide extraction on the resulting cells, and analyzed the resulting samples using mass spectrometry for c-di-GMP levels normalized to total cellular dry weight. We saw similar levels of c-di-GMP produced by the wild-type strain and the ΔDGC strain expressing GcbC from a plasmid (Fig. 3B). We further saw a significant reduction in c-di-GMP levels for the ΔDGC strain lacking its native DGC subnetwork compared to the ΔDGC strain expressing GcbC from a plasmid. Conversely, the strain carrying plasmid pGcbC-GGAAF, wherein the catalytic residues are mutated to prevent c-di-GMP production, as reported previously (6), showed no increase in c-di-GMP production compared to that of the ΔDGC strain (Fig. 3B). The D413K mutant produced a level of c-di-GMP similar to that of wild-type GcbC (Fig. 3B). The E429R mutant showed a small but nonsignificant increase in c-di-GMP levels compared to that of the strain expressing wild-type GcbC, while the E360R mutant showed a significant increase in c-di-GMP production compared to that of wild-type GcbC, mirroring the results found in the Congo red assay (Fig. 3B). Taken together, these results suggest that these mutations made to the I-site of GcbC do not result in lower levels of biofilm formation due to lower c-di-GMP production and that the individual mutations result in levels of c-di-GMP production ranging from 1 to 1.6 times that of wild-type GcbC.

Targeted I-site mutations result in elevated levels of biofilm formation. For two of the I-site mutants that we constructed (E429R and E360R), there appears to be reduced biofilm formation despite elevated levels of c-di-GMP production. Interestingly, previous studies found that disruptions of DGC I-sites result in an increase in the c-di-GMP level and a concomitant increase in c-di-GMP-regulated processes. Given that our results show that a DGC I-site mutant that produces more c-di-GMP is less able to stimulate biofilm formation, we wanted to better understand the relationship between the necessity of physical interaction for productive signaling and the amount of c-di-GMP produced.

The mutations to the I-site that we have tested up to this point created relatively modest (<2-fold) increases in total cellular c-di-GMP levels. Inspection of the crystal structure of GcbC indicated that residue D413 coordinates the base of only one of the c-di-
GMP molecules of the intercalated c-di-GMP dimer bound at the I-site. Nearby residues R409 and R410 coordinate the same base as D413, but these arginine residues are also involved in Φ-stacking interactions involving the entire c-di-GMP dimer (Fig. 1B). Hence, one would expect mutations to the D413 residue to produce modest effects, and it is expected that mutations at positions R409 and R410 would similarly lack dramatic deregulation effects given that these residues are in part functionally redundant. Residues E429 and E360 each contribute to protein-protein interactions between GcbC protomers in the dimer, again having overlapping functions with each other, possibly reducing the impact of either mutation alone. We hypothesized that if we made more severe disruptions to the I-site of GcbC, we would create a DGC that overproduced c-di-GMP to a greater degree. Previous studies established that mutations at the secondary I-site also yielded a more active DGC (21). We therefore selected residues R366 and R363 for mutagenesis studies. R366 is the sole residue in the secondary I-site of GcbC contacting c-di-GMP, and this residue engages in interactions with both c-di-GMP molecules, suggesting that its disruption would have a profound effect on c-di-GMP binding and DGC regulation (Fig. 1B). R363 was chosen as it is intimately involved in GcbC protein-protein interactions, similar to residues like E360 and E429 (Fig. 1B). In fact, R363 interacts directly with both E360 and E429, rendering this position ideal for a targeted mutation predicted to severely interfere with the formation of an inactive, c-di-GMP binding-competent conformation. We therefore tested R366E and R363E mutant variants of GcbC for their impact on LapD-GcbC protein-protein interactions and biofilm disruption.

Since our above-described results suggest that the ability to form the I-site is necessary for GcbC to interact with LapD, we hypothesized that these mutations would result in minimal interactions with LapD. When tested by bacterial two-hybrid assays, a significant reduction in the interaction between these mutant versions of GcbC and the wild-type LapD protein was observed (Fig. 4A). We next tested these GcbC mutants for their ability to promote biofilm formation in vivo from the same expression construct used previously. Both the R366E and R363E mutants resulted in a significant increase in biofilm formation, suggesting that more severe disruptions to the I-site of GcbC result in the expected finding of increased c-di-GMP production resulting in increased biofilm formation (Fig. 4B). Finally, we again tested these mutant proteins for expression stability by Western blotting; each mutant appeared to be as stably expressed as wild-type GcbC (Fig. 4C).

Mutations R366E and R363E at the I-site of GcbC result in a significant increase in c-di-GMP production. We next wanted to verify that the reason why the R366E and R363E mutations behave more like other previously reported I-site mutations was in fact due to an increase in c-di-GMP production. To this end, we again used the Congo red assay by heterologously expressing each mutant construct in P. aeruginosa PA14. We found that the colony biofilms produced by the mutant variants of GcbC were considerably more red and wrinkly than the wild-type GcbC-expressing colonies, indicating much higher levels of c-di-GMP production (Fig. 5A). To confirm c-di-GMP overexpression in vivo in P. fluorescens, we subcultured strains expressing each construct and again performed nucleotide extraction, which was analyzed by mass spectrometry. We found that strains carrying either the R366E or R363E mutation produced levels of c-di-GMP that were ~16 and 20 times higher, respectively, than those in strains expressing wild-type GcbC (Fig. 5B). These results suggest that more severe mutations at the I-site of GcbC result in higher levels of c-di-GMP production than those resulting from mutation of I-
DGC I-Site Mediates Interaction with Its Receptor

FIG 5 Mutations made to nonredundant residues in the I-site of GcbC correspond with greatly increased levels of c-di-GMP production. (A) To determine if the increases in biofilm formation shown in Fig. 4B are the result of increased c-di-GMP levels, Congo red assays were used as an indication of c-di-GMP production. Mutant and wild-type versions of GcbC were heterologously expressed in P. aeruginosa PA14 and grown on Congo red plates supplemented with 0.1% arabinose. Colonies were grown for 36 h at 30°C. Note both the redness and wrinkling features of the R366E and R363E mutations, indicating high levels of c-di-GMP production. (B) The strains used for the biofilm assay described in the legend to Fig. 4B were subcultured and subjected to nucleotide extraction followed by quantification by mass spectrometry. Expression was induced with 0.2% arabinose. Error bars represent standard deviations of data from three biological replicates. ***P < 0.005 (compared to wild-type GcbC). Note that the results for the wild-type, ΔDGC, pGcbC, and pGcbC GGAAF strains are the same as those shown in Fig. 3C and were also included here for ease of comparison.

DISCUSSION

Physical interaction is an emerging theme in DGC signaling specificity. In E. coli, the DGC YdaM has been shown to physically interact with and stimulate the Mer-like transcription factor MlrA (4). Additionally, the DGC HmsT and the PDE HmsP colocalize to impact biofilm development in Yersinia pestis (24). In this study, we demonstrated that GcbC must be able to form an intact autoinhibitory site in order to properly interact with and functionally signal to the c-di-GMP receptor LapD. We determined that nearly any kind of disruption to GcbC’s I-site, be it to the primary I-site, the secondary I-site, or residues supporting the dimerization of inactive, c-di-GMP-bound GcbC, results in a decrease in the interaction with LapD. We further demonstrated that while mutations in the I-site of GcbC predicted to result in a loss of binding of this dinucleotide based on data from previous studies (7, 19) resulted in canonical increases in c-di-GMP levels and biofilm formation, mutation of I-site residues with predicted less severe c-di-GMP binding defects resulted in reduced biofilm formation despite the production of equal or larger amounts of c-di-GMP than those of the wild-type DGC. We also note that interaction between GcbC and LapD appears to be very sensitive to any change made to the GcbC I-site, likely causing the loss of specific signaling between this DGC and its effector. As we make mutations with a greater impact on I-site function, more c-di-GMP is produced, which correlates with greater promotion of biofilm formation. This increase in biofilm formation may occur by activating other targets, including motility regulators, or may directly compensate for the loss of the GcbC-LapD interaction through the production of very high levels of c-di-GMP. We further note that there does not appear to be any correlation between the amount of c-di-GMP produced and the ability of GcbC to interact with LapD. Our data suggest a model whereby, in addition to its known role in regulating c-di-GMP output, the I-site regulates signaling specificity in that it is required for GcbC to functionally interact with its receptor.

An important question remaining is how the I-site of GcbC physically contributes to the protein-protein interaction of GcbC and LapD. While our results support a model that makes the formation of a functional I-site necessary for the GcbC-LapD interaction, it is not immediately apparent how these residues are contributing to the interaction. One possibility is that GcbC must enter its autoinhibited conformation in order to appropriately interact with LapD. If this model were correct, it would strongly tie the regulation of the cyclase activity by c-di-GMP to the interaction with its effector. Perhaps, linking such control of DGC activity to receptor interaction allows the tuning of enzymatic activity or product availability to the level necessary to signal specifically to LapD without signaling to other potential targets, thus providing one possible explanation for how dozens of cyclases may signal simultaneously in the same cell without significant cross talk. Alternatively, mutation of I-site residues may simply disrupt larger structural elements, resulting in more gross structural changes that would impact the GcbC-LapD interaction; while possible, we find such a scenario unlikely. Every independent, I-site-related mutation that we tested appeared necessary for the full interaction between GcbC and LapD. Furthermore, the mutant proteins are expressed at levels similar to those of the wild-type form, lacking any sign of global protein instability. Additionally, the active dimer of GcbC appears to be able to form successfully in each I-site mutant that we created, as evidenced by the normal or elevated levels of c-di-GMP production. Thus, it appears that the inability to adopt the autoinhibited state, rather than a general structural defect, is likely responsible for the observed loss of the interaction between GcbC1-site mutants and LapD. We also cannot rule out the possibility that c-di-GMP at the I-site is itself part of the interaction interface.

Furthermore, we note that the I-site is on the opposite face of GcbC, as is the α5GGDEF helix, previously shown to bind LapD’s EAL domain (11). If these two elements work synergistically, it is possible that the I-site also targets the EAL domain of LapD, which
may indicate that only a catalytically quieted version of GcbC is able to bind LapD under some conditions. Alternatively, disruption of the I-site may abolish the GcbC-LapD interaction by causing other interacting residues, such as those in the α5GDEG helix, to come out of register with their interface partners in LapD, although we note that no previous mutations or combination thereof have had as profound an impact on the GcbC-LapD interaction as some of the I-site mutants. Intriguingly, the GcbC-LapD interaction has been shown to endure regardless of the activation state of LapD (11), allowing for the possibility that different surfaces of GcbC are in play and operate independently of one another depending on the differential structural conformation taken by active versus inactive LapD (25). A final possibility is that some of the I-site residues or their neighbors are physically contacting a surface on LapD, which may itself resemble the points of contact that these residues make with c-di-GMP. Crystallographic studies that focus on a GcbC-LapD cocystal structure will be necessary to sort among these and other possibilities.

Our study calls for renewed attention to the I-site as an important regulatory element for DGCs. In the case of GcbC, the I-site appears to function as both a regulatory mechanism to control c-di-GMP output and a structural element that allows interaction between GcbC and LapD. Our results also highlight that the physical interaction between a DGC and an effector protein can be an important part of proper signaling and that the ability to produce, or mildly overproduce, c-di-GMP cannot always make up for the loss of physical interactions. In an effort to replicate the concept that a disrupted inhibitory site will lead to an increase in the c-di-GMP-dependent phenotype being studied, we constructed the R363E and R366E mutants, which resulted in >15-fold increases in c-di-GMP pools, and demonstrated that this large increase in production of the cyclic dinucleotide is sufficient to overcome the loss of interaction. Intriguingly, despite an apparent increase in c-di-GMP production for the R363E and R366E mutations of over an order of magnitude compared to that of the wild type (Fig. 5B), we observed only an ~40% increase in biofilm formation compared to that of the wild-type strain. This observation underscores the importance of physical interaction to the potency of signaling between this DGC-effector pair while raising questions about how PDEs may be involved in keeping distal DGCs from impacting effectors. Together, the R363E and R366E substitutions demonstrate that choosing mutations to make to a DGC’s inhibitory site should be undertaken with care, as very severe disruptions may result in exaggerated phenotypes that lack biological meaning. On the other hand, we note that dramatically increased global levels of c-di-GMP overcome the loss of specific DGC-effector interactions to promote biofilm formation. While the impact of high c-di-GMP levels may not have physiological relevance in the case of GcbC, it leaves open the possibility for global levels of c-di-GMP overriding local DGC signaling events, potentially providing some of the other DGCs or PDEs with a means to regulate biofilm formation in P. fluorescens that does not require interaction with LapD. For example, expression of the RapA phosphodiesterase reduced LapD-dependent biofilm formation, but RapA does not appear to interact with the receptor based on B2H assays (26; A. J. Collins and G. A. O’Toole, unpublished data).

Finally, the differing sets of results that we produced with targeted mutations to the I-site of GcbC underscore the need to interpret observations regarding perturbing the DGC’s I-site with care. Our results show that some mutations to the I-site of a DGC result in exaggerated phenotypes as a consequence of large (order-of-magnitude) increases in c-di-GMP levels. This finding suggests that the role of an I-site is to reduce the signal that a DGC is broadcasting to its receptor or other noncognate receptors. However, our results also suggest that the I-site may have an additional function to affirmatively associate a DGC’s signal to its target and thus provide true modulation to the DGC-effector signaling event. Future studies will be necessary to determine if the I-sites of other DGCs provide similar functionality with effector proteins or if there may be some interplay between DGCs and PDEs utilizing I-sites.

Additionally, many DGCs lack a traditional I-site altogether, and it is largely unknown how these enzymes regulate themselves or specify an effector, although one recent study suggested an alternative inhibitory state that such DGCs may enter (27). While it is possible that proteins with primary inhibitory sites using the RXXD motif favor specificity through interaction, there is at least one example of a DGC lacking an I-site interacting with LapD in P. fluorescens (Collins and O’Toole, unpublished). This result suggests that some DGCs contribute to the global pool of c-di-GMP and are subject to other modes of regulation, while other DGCs, such as GcbC, engage in more localized signaling.

Our results on the role of the I-site in the ability of GcbC to effectively interact with and signal to LapD add to work that demonstrates physical interaction as a mechanism of signaling specificity between DGCs and their effectors. Collectively, our work provides a model that suggests that protein complexes can exist in large c-di-GMP networks and that the regulatory state of a given DGC may be integrated with the proximity of its effector to modulate its signal. The finding that a canonical regulatory site of a DGC is involved in effector signaling reinforces the notion that these systems are highly organized and provides a focal point for future studies aimed at signaling specificity in c-di-GMP networks.

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