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The response threshold of *Salmonella* PilZ domain proteins is determined by their binding affinities for c-di-GMP

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Summary

c-di-GMP is a bacterial second messenger that is enzymatically synthesized and degraded in response to environmental signals. Cellular processes are affected when c-di-GMP binds to receptors which include proteins containing the PilZ domain. Although each c-di-GMP synthesis or degradation enzyme metabolizes the same molecule, many of these enzymes can be linked to specific downstream processes. Here we present evidence that c-di-GMP signaling specificity is achieved through differences in affinities of receptor macromolecules. We show that the PilZ domain proteins of *Salmonella* Typhimurium, YcgR and BcsA, demonstrate a 43-fold difference in their affinity for c-di-GMP. Modulation of the affinities of these proteins altered their activities in a predictable manner *in vivo*. Inactivation of *yhjH*, which encodes a predicted c-di-GMP degrading enzyme, increased the fraction of the population that demonstrated c-di-GMP levels high enough to bind to the higher-affinity YcgR protein and inhibit motility, but not high enough to bind to the lower-affinity BcsA protein and stimulate cellulose production. Finally, seven PilZ domain proteins of *Pseudomonas aeruginosa* demonstrated a 145-fold difference in binding affinities, suggesting that regulation of c-di-GMP signaling by binding affinity may be a conserved mechanism that allows organisms with many c-di-GMP-binding macromolecules to rapidly integrate multiple environmental signals into one output.

Keywords

c-di-GMP; PilZ; YcgR; BcsA; c-di-GMP biosensor; *Salmonella*

Introduction

c-di-GMP is a second messenger utilized by many bacteria to integrate environmental information from different inputs in order to direct cellular behavior. c-di-GMP has been implicated in the regulation of many cellular processes, including production of adherence factors and exopolysaccharide, flagellar motility, the cell cycle, virulence for mammals, and antibiotic sensing and resistance (for reviews see (Hengge, 2009, Schirmer & Jenal, 2009,

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Mills *et al.*, 2011)). Diguanylate cyclases (DGCs) generate c-di-GMP by cyclizing two molecules of GTP *via* GGDEF domains, while phosphodiesterases (PDEs) degrade c-di-GMP by the hydrolytic actions of their EAL or HD-GYP domains. Because many GGDEF and EAL domains are physically linked to putative environmental sensing domains, the precise concentration of c-di-GMP is likely tightly controlled by multiple environmental signals (Mills *et al.*, 2011). Signal transduction is accomplished by c-di-GMP binding to specific downstream receptors including proteins containing the PilZ domain, which makes direct contacts with c-di-GMP (Amikam & Galperin, 2006, Ryjenkov *et al.*, 2006, Christen *et al.*, 2007). Many bacterial species encode dozens of c-di-GMP components in their genome (Galperin *et al.*, 2001).

Studies in the last decade have provided insight into the c-di-GMP networks of *Salmonella* Typhimurium. *S. Typhimurium* has three defined processes known to be regulated by c-di-GMP: flagellar-based motility, the generation of curli fimbriae, and cellulose production (Zogaj *et al.*, 2001, Solano *et al.*, 2002, Kader *et al.*, 2006, Ryjenkov *et al.*, 2006, Weber *et al.*, 2006). The genes encoding curli fimbriae are directly up-regulated by the transcription factor CsgD, whose expression is induced by c-di-GMP at temperatures of 30°C or lower through an as-yet unidentified receptor (Ogasawara *et al.*, 2011, Zakikhany *et al.*, 2010, Romling *et al.*, 1998a, Romling *et al.*, 1998b). Like most Enterobacteria, *S. Typhimurium* has two PilZ domain proteins: YcgR, which controls flagellar-based motility, and BcsA, the bacterial cellulose synthase. *Escherichia coli* YcgR has been shown to bind specifically to the flagellar complex to inhibit torque generation and thus decrease motility when it binds to c-di-GMP (Ryjenkov *et al.*, 2006, Boehm *et al.*, 2010, Paul *et al.*, 2010, Fang & Gomelsky, 2010). c-di-GMP stimulates cellulose production presumably by binding to the PilZ domain of BcsA (Zogaj *et al.*, 2001, Solano *et al.*, 2002). Thus, a rise in c-di-GMP concentration results in a decrease in motility (through YcgR) and an increase in cellulose production (through BcsA). Several of the 20 GGDEF/EAL domain proteins of *S. Typhimurium* have been linked to one of these processes. AdrA, a DGC that is dependent on CsgD for its expression, is required for the production of cellulose on LB-agar plates. Under this condition, its loss cannot be complemented by the native expression of any other DGC, even though these are also expressed in cellulose-producing conditions (Romling *et al.*, 2000, Zogaj *et al.*, 2001, Garcia *et al.*, 2004, Solano *et al.*, 2009). Several of these other DGCs have instead been implicated in motility inhibition or CsgD expression, based on studies in *S. Typhimurium* or in *E. coli* (Kader *et al.*, 2006, Pesavento *et al.*, 2008, Boehm *et al.*, 2010, Sanchez-Torres *et al.*, 2011). The PDE that appears to be the most important for maintaining c-di-GMP levels low enough to allow flagellar motility is YhjH (Ko & Park, 2000, Ryjenkov *et al.*, 2006, Paul *et al.*, 2010, Boehm *et al.*, 2010). Like YcgR, YhjH is a member of the flagellar regulon and is expressed from a flagellar class III promoter (Ko & Park, 2000, Frye *et al.*, 2006, Wang *et al.*, 2006).

Although they metabolize the same small, diffusible second messenger molecule, many c-di-GMP synthesizing and degrading enzymes, such as AdrA, can be specifically linked to a particular downstream c-di-GMP-regulated process. These observations have led to the conclusion that c-di-GMP signaling may be functionally compartmentalized (Weber *et al.*, 2006, Pesavento *et al.*, 2008, Hengge, 2009). There are several potential mechanisms for how this compartmentalization could occur. One of these mechanisms involves coordinate

regulation of the expression of c-di-GMP components, in which c-di-GMP metabolizing enzymes would be expressed under the same conditions as their downstream receptors. Indeed, some c-di-GMP components are known to be under the transcriptional control of various signals (Pesavento et al., 2008, Weber et al., 2006); however, others appear to be constitutively expressed, so this mechanism does not adequately account for all observed signaling specificity (Solano *et al.*, 2009). Another proposed model for signaling specificity involves the spatial sequestration of individual c-di-GMP components, such that the generation and degradation of c-di-GMP would act locally on a particular target. Although there is some evidence to support local activity of c-di-GMP metabolizing enzymes in some organisms (Massie *et al.*, 2012, Merritt *et al.*, 2010), the rapid diffusion of c-di-GMP in the bacterial cytoplasm would make it difficult to establish such local pools inside of the bacterial cell in the absence of some kind of compartmentalizing structure (Mills *et al.*, 2011), and there is no firm evidence that such microdomains exist in bacteria. An alternative, though not mutually exclusive, mechanism by which c-di-GMP signaling specificity could be achieved is through the binding affinities of downstream receptors (Hengge, 2009, Ko *et al.*, 2010, Mills *et al.*, 2011), wherein downstream receptor affinity for c-di-GMP would determine which receptors are activated at particular cytoplasmic c-di-GMP levels. Such a mechanism would allow for rapid responses when coupled with the enzymatic ability to alter the concentration of c-di-GMP in response to environmental signals. This hypothesis is supported by the measured affinities of c-di-GMP receptors from a variety of organisms, which have been found to vary considerably (Christen *et al.*, 2006, Benach *et al.*, 2007, Christen *et al.*, 2007, Merighi *et al.*, 2007, Pratt *et al.*, 2007, Hickman & Harwood, 2008, Sudarsan *et al.*, 2008, Duerig *et al.*, 2009, Navarro *et al.*, 2009, Christen *et al.*, 2010, Krasteva *et al.*, 2010, Srivastava *et al.*, 2011, Duvel *et al.*, 2012, Whitney *et al.*, 2012). Differences in binding affinities of receptor proteins could potentially represent a method for c-di-GMP to affect some PilZ domain proteins but not others, thereby selectively activating certain PilZ domain proteins depending on its concentration within the cell.

Based on these observations, we performed a systematic study to measure and compare the c-di-GMP binding affinities of all PilZ domain proteins within two organisms: *Salmonella* Typhimurium, which has two PilZ domain proteins, and *P. aeruginosa*, which has eight (Amikam & Galperin, 2006). We found that the binding affinities of these proteins vary considerably even within the same organism. We then underwent a strategy of site-directed mutagenesis to generate *S. Typhimurium* PilZ domain proteins that have altered affinities but otherwise retain the functions of the native proteins. Assessing the activities of these mutant proteins in phenotypic assays showed that binding affinity is an important determinant for activity. Finally, we demonstrated that inactivation of the EAL domain protein YhjH results in an increase in the percentage of cells that demonstrate a level of c-di-GMP high enough to bind to YcgR but not to BcsA. This work supports the hypothesis that regulation by binding affinity of downstream receptors is a mechanism for the selective activation of c-di-GMP controlled processes.

Results

The binding affinities of *S. Typhimurium* PilZ domain proteins differ by 43-fold

We measured the c-di-GMP binding affinities of PilZ domain proteins by constructing FRET-based c-di-GMP biosensors using these proteins (Fig 1A). Intramolecular FRET is a very sensitive method for detecting changes in protein conformation, such as those that occur upon binding to small molecules (Jares-Erijman & Jovin, 2003, Roy *et al.*, 2008). This method has previously been used to measure the c-di-GMP binding affinity of YcgR in a YPet-YcgR-CyPet fusion protein (Christen *et al.*, 2010, Nguyen & Daugherty, 2005), and to measure affinities of proteins for other second messengers (Miyawaki *et al.*, 1997, Nikolaev *et al.*, 2006, Ohashi *et al.*, 2007, Russwurm *et al.*, 2007, Jager *et al.*, 2012). In this FRET-based method, the conformational change experienced by a PilZ domain protein upon its binding to c-di-GMP manifests as a change in the FRET output emission spectrum. Using this method, we generated a FRET-based biosensor from the BcsA protein in order to compare its c-di-GMP binding properties to those of YcgR. BcsA is predicted to include nine transmembrane helices, a glycosyltransferase domain, and a PilZ domain (InterPro - <http://www.ebi.ac.uk/interpro/>). Making a FRET fusion protein with the entire BcsA protein would not have been possible due to the transmembrane domains, so we used the BcsA PilZ domain as the c-di-GMP-detecting sensor. This biosensor, and the YcgR-based biosensor described above, were purified from *E. coli* BL21(DE3) cells, and their FRET emission spectrum profiles were analyzed in the presence or absence of 40 μ M c-di-GMP using a fluorescence plate reader. For both FRET constructs, the presence of c-di-GMP results in a corresponding decrease in FRET (Fig 1B), suggesting that binding results in an increase in the distance between the amino- and carboxyl-termini, or a change in their dipole-dipole orientation, of these fusion proteins. Thus, c-di-GMP binding to each FRET-based biosensor can be detected by a change in the ratio of YFP emission (535 nm) to CFP emission (480 nm) (Fig 1B).

We used this construct to explore the c-di-GMP binding properties of BcsA. The c-di-GMP binding affinities for both PilZ domain protein based c-di-GMP biosensors were measured by analyzing their FRET profiles in the presence of increasing concentrations of c-di-GMP. This yielded a concentration-responsive curve for c-di-GMP (Fig 1C). We measured the Hill coefficient and the equilibrium binding constant K_d by fitting a line to the concentration-responsive curve (Fig 1C). The Hill coefficient was approximately two for the BcsA PilZ domain, similar to Hill coefficients measured for other PilZ domains (Ko *et al.*, 2010, Ramelot *et al.*, 2007), suggesting binding to two molecules of c-di-GMP and indicating positive cooperativity in c-di-GMP binding (Table S1). Unexpectedly, we observed a Hill coefficient closer to 1.5 for the YcgR biosensor at 25°C, compared to the Hill coefficient previously published of approximately 2 for this protein (Christen *et al.*, 2010); however, as temperature increased, the Hill coefficient approximated 2 (Table S1). The K_d of YcgR for c-di-GMP at 25°C was 191 ± 18 nM, similar to the K_d of 195 nM previously published for this FRET-based biosensor (Christen *et al.*, 2010), and to the K_d of 182 nM of YcgR for c-di-GMP that was determined by UV cross-linking to radiolabeled c-di-GMP (Christen *et al.*, 2007). For BcsA, the binding affinity was 8240 ± 238 nM at 25°C (Table 1). This represents an approximately 43-fold difference in affinity for c-di-GMP between these two proteins.

The c-di-GMP binding affinity of each PilZ domain protein measured was highly dependent on temperature, since the affinity dropped as temperature increased (Fig 1D, Table S1). This indicates that binding is entropically unfavorable, as was also determined for the binding of the *Vibrio cholerae* PilZ domain protein VCA0042 to c-di-GMP (Benach *et al.*, 2007). The disparity in binding affinities between YcgR and BcsA was maintained at every temperature measured (Table S1). Comparison of the binding curves of these two proteins indicates that there exists a range of cellular c-di-GMP concentration that would stimulate binding to YcgR but would not be high enough to stimulate BcsA binding (Fig 1C), and implies that their specific binding affinities may thus be important for their biological functions.

The binding affinities of *P. aeruginosa* PilZ domain proteins span a 145-fold range

Next we compared the binding affinities of PilZ domain proteins of *P. aeruginosa* (Amikam & Galperin, 2006) using the same sensitive FRET-based method. This method has previously been used to measure the affinities of four *P. aeruginosa* PilZ domain proteins (PA0012, PA2799, PA2989, and PA4608) (Christen *et al.*, 2010) (Table 1). We generated FRET-based biosensors to measure the c-di-GMP binding affinities of the remaining PilZ domain proteins (PA2960, PA3353, PA3542, and PA4324). As for the PilZ domain proteins of *S. Typhimurium*, the c-di-GMP-binding affinities of *P. aeruginosa* PilZ domain proteins spanned a sizable range of c-di-GMP concentrations. The highest and lowest c-di-GMP binding affinities measured for *P. aeruginosa* PilZ domain proteins differed by 145-fold (Table 1). The Hill coefficient approximated two for every PilZ domain protein measured, and all measured binding affinities decreased as temperatures increased (Fig S1 and data not shown).

Interestingly, the binding curve for PA3353 suggested two separate binding sites, each binding two molecules of c-di-GMP, that differed by approximately eight-fold, at c-di-GMP concentrations of 88 nM and 732 nM at 25°C (Table 1 and Fig S1). PA3353 has also been measured by another group using surface plasmon resonance to be 262 ± 66 nM (Table 1), although this binding affinity was measured at 20°C (Duvel *et al.*, 2012). We have found that for every °C increase in temperature, the binding affinity decreases by approximately 15% for this protein (Fig S1). Therefore this measurement of 262 ± 66 nM at 20°C would be estimated to be approximately 530 ± 100 nM at 25°C, which is comparable to the lower-affinity site. The published affinity for PA3542, measured using isothermal titration calorimetry, is 8.4 µM (Merighi *et al.*, 2007), which is slightly higher than the affinity of PA3542 measured in this study of 12.7 ± 1.7 µM (Table 1). This difference could be due to differences in measurement methods, since isothermal titration calorimetry is known to be fairly inaccurate in measuring low binding affinities, and cannot accurately resolve affinities of less than approximately 10 µM (Rehm, 2006). No FRET shift was observed for PA2960, suggesting that it does not bind c-di-GMP. This is consistent with a previous study in which PA2960 was shown by UV cross-linking to not bind to c-di-GMP (Merighi *et al.*, 2007), likely because it lacks the RxxxR motif required for binding (Amikam & Galperin, 2006, Christen *et al.*, 2007, Ryjenkov *et al.*, 2006).

To determine how binding affinities of PilZ domain proteins affected their biological output functions, we chose to pursue this study further in *S. Typhimurium*, which has two PilZ domain proteins, both with published phenotypes.

Mutation of the amino acid residue at Position-X alters the affinities of *S. Typhimurium* PilZ proteins for c-di-GMP

The striking variability in c-di-GMP-binding affinities of PilZ domain proteins suggests that binding affinities may be important for the biological phenotypes of these proteins. To test this hypothesis, we employed a strategy of rational, site-directed mutagenesis to alter c-di-GMP binding affinity of the PilZ domain proteins of *S. Typhimurium*. Crystal and NMR studies of PilZ domain proteins in complex with c-di-GMP have elucidated the roles of residues of the binding pocket, especially those of the so-called switch region including the RxxxR motif, in binding to c-di-GMP (Benach et al., 2007, Ko et al., 2010, Shin *et al.*, 2011). Specifically, the presence of an arginine at the position immediately N-terminal of the RxxxR motif, dubbed “Position-X”, has been shown for the *Pseudomonas putida* YcgR homologue, PP4397, to be an important determinant of the affinity for c-di-GMP-binding (Ko et al., 2010). *S. Typhimurium* YcgR also has an arginine at Position-X (arginine-113) (Fig 2A), so we reasoned that mutation of this arginine might decrease binding affinity as it does in PP4397. Indeed, mutation of arginine-113 to alanine resulted in a YcgR variant that bound c-di-GMP with an affinity of 3.2 μ M, which was approximately 17-fold lower than the affinity measured for the wild-type YcgR protein (Fig 2B).

The BcsA PilZ domain has a low binding affinity for c-di-GMP compared to YcgR (8.2 μ M compared to 191 nM), so our goal for BcsA was to increase its affinity. BcsA has a valine at Position-X (Fig 2A). The *V. cholerae* YcgR homologue, VCA0042, also has a large hydrophobic residue, a leucine, in this position. Mutation of this leucine to arginine has been shown to increase the binding affinity of VCA0042 for c-di-GMP by approximately three-fold (Ko et al., 2010). Although BcsA is not similar in sequence to VCA0042 outside of the conserved residues of the binding pocket, we reasoned that mutation of the valine in Position-X of BcsA to an arginine might increase binding affinity in a similar manner as mutation of leucine to arginine in Position-X of VCA0042. Mutating this valine to an arginine increased the affinity of BcsA for c-di-GMP to 4.2 μ M at 25°C, which is roughly two-fold greater than that of the WT protein (Fig 2C). Both the BcsA and YcgR mutant proteins maintained similar fold differences compared to the wild-type proteins from 24°C to 37°C (data not shown). These mutations did not change the wild-type binding stoichiometries of c-di-GMP to the PilZ domain, indicating that the change in affinity was the major determinant of change in downstream function (data not shown). Therefore we were able by site directed mutagenesis to generate PilZ domain proteins with altered binding affinities for c-di-GMP.

A *S. Typhimurium* strain harboring a BcsA protein with increased affinity for c-di-GMP demonstrates an increase in cellulose production at low levels of c-di-GMP

Once we had generated binding affinity mutants of PilZ domain proteins, we needed functional assays to determine the effects of these mutations on phenotype. For BcsA, the bacterial cellulose synthase, activity can be assessed by determining cellulose production,

which is detected by its binding to the fluorescent dye calcofluor. The production of cellulose on LB medium is dependent upon the DGC AdrA, which is expressed at 30°C or lower, but not at 37°C (Zogaj et al., 2001, Solano et al., 2002, Garcia et al., 2004) (Fig 3A). The ability of AdrA-produced c-di-GMP to stimulate cellulose production is not through transcriptional or translational effects, since protein extracts from strains grown at different temperatures demonstrated similar amounts of BcsA protein, and cell lysates prepared from strains grown at 37°C are capable of producing cellulose (Fig S2). Thus, c-di-GMP affects cellulose production on a post-translational level, most likely through its binding to the BcsA PilZ domain. In order to compare cellulose production between strains harboring BcsA variants, we constructed a strain of *S. Typhimurium* in which the level of c-di-GMP can be controlled by the addition of arabinose. This is due to the presence of a DGC, PA1120 from *P. aeruginosa* (Kulasakara et al., 2006), located on the chromosome under the control of the native arabinose-inducible *pBAD* promoter. In this strain, addition of arabinose to the growth media results in induction of the DGC and cellulose production even at 37°C (Fig 3A, Fig S3).

To examine whether the increased-affinity BcsA V695R mutation affects cellulose production, we generated a strain of *S. Typhimurium* that contains the *bcsA-V695R* mutation on the chromosome in the background of the arabinose-inducible DGC strain. We then analyzed differences in cellulose production, as determined by cellulose binding to calcofluor, between the *ara::DGC*, *ara::DGC ΔbcsA*, and *ara::DGC bcsA-V695R* strains at different expression levels of the DGC by varying the arabinose concentration. At low arabinose levels, the *bcsA-V695R* mutant generated reproducibly higher calcofluor fluorescence levels compared to the BcsA wild-type strain (Fig 3B), implying that this strain produces more cellulose at low levels of arabinose than the wild-type strain. Upon addition of higher amounts of arabinose, the amount of cellulose synthesis between the wild-type and *bcsA-V695R* strains was not significantly different, suggesting that the higher DGC levels produce a cellular c-di-GMP concentration above the K_d values for both BcsA V695R mutant and BcsA wild-type enzymes, resulting in saturation and cellulose production by both enzymes (Fig 3C). These data show that the binding affinity of BcsA for c-di-GMP affects its biological function.

A *S. Typhimurium* strain harboring YcgR with a lower binding affinity for c-di-GMP requires higher levels of c-di-GMP to inhibit flagellar motility

Next we needed a functional assay to assess the effect of the YcgR R113A mutation on phenotype. *S. Typhimurium* YcgR has been shown to decrease the swimming halo diameter of colonies on motility agar plates when intracellular levels of c-di-GMP are artificially raised (Ryjenkov et al., 2006). Since the c-di-GMP binding affinity of the YcgR R113A mutant protein is 17-fold less than that of the wild-type protein, we hypothesized that the YcgR R113A protein would require a higher concentration of c-di-GMP than the wild-type YcgR protein in order to inhibit swimming. To test this hypothesis, *S. Typhimurium* strains harboring the DGC PA1120 under the control of an IPTG-inducible *tac* promoter were generated and evaluated. The addition of IPTG results in a decrease in swimming motility for this strain compared to cells that do not harbor the IPTG-inducible DGC, as visualized by a decrease in this strain in the halo diameter on motility agar plates (data not shown). A

ΔycgR deletion mutant does not demonstrate as severe of a decrease in swimming diameter compared to the wild-type strain in the presence of the DGC, indicating a partial relief of c-di-GMP-induced swimming inhibition in this strain (Fig 4A). Expressing YcgR from its native promoter off of a plasmid complemented the *ycgR* mutation (Fig 4A).

To determine whether the YcgR R113A mutation affected swimming, we complemented the *ΔycgR* mutant strain with either the wild-type YcgR protein, or the YcgR R113A mutant protein, and analyzed swimming halo diameter on motility agar plates with different concentrations of IPTG. Complementation with wild-type YcgR partially inhibited swimming even in the absence of IPTG, which could be an overexpression effect of YcgR as has been observed previously (Paul et al., 2010) (Fig 4B). Increasing the IPTG concentration decreased the ability of *S. Typhimurium* complemented with YcgR to swim, indicating that c-di-GMP-bound YcgR was inhibiting motility (Fig 4B). At 20 μM IPTG, the YcgR wild-type protein completely inhibited swimming. Unlike the wild-type YcgR protein, complementation with YcgR R113A had no effect on swimming in the absence of IPTG. Increasing IPTG also decreased motility in the YcgR R113A complemented strain, but 50 μM IPTG was required to completely inhibit swimming by the YcgR R113A mutant protein (Fig 4B). This indicates that the YcgR R113A protein requires a higher concentration of c-di-GMP than the wild-type YcgR protein to inhibit swimming, which is consistent with its lower binding affinity.

Inactivation of *yhjH* results in an increase in the percentage of *S. Typhimurium* cells that demonstrate c-di-GMP levels high enough to bind YcgR

Another way to explore the affinity hypothesis is in the context of inactivation of the PDE YhjH. YcgR is most frequently studied in the context of a *yhjH* mutant background, in which a second mutation in *ycgR* partially relieves the swimming defect that is caused by inactivation of YhjH (Boehm et al., 2010, Paul et al., 2010, Fang & Gomelsky, 2010, Ryjenkov et al., 2006) (Fig 5A). In *E. coli*, cells that harbor a mutation in *yhjH* demonstrate increased c-di-GMP levels compared to wild-type cells (Boehm et al., 2010). However, mutation in *S. Typhimurium* *yhjH* does not appear to result in a significant difference in total c-di-GMP levels in this strain compared to a wild-type strain (Simm et al., 2007), so it is unknown how *yhjH* inactivation results in a motility inhibition in this organism. In order to determine whether the *yhjH* mutation results in a rise in c-di-GMP levels that are high enough to stimulate binding to, and motility inhibition by, YcgR, we analyzed the binding to c-di-GMP of a synthetic YcgR-based FRET biosensor *in vivo*, encoded on the plasmid pYcgR-Spy (Christen et al., 2010) (Fig 5B). Binding of this construct to c-di-GMP can be visualized in live individual cells by determining the FRET/CFP ratios using fluorescence microscopy (Christen et al., 2010). Since this construct is based on YcgR, it allows for an accurate determination of the bound state of YcgR when expressed inside of live cells.

Wild-type *S. Typhimurium*, or an isogenic *ΔyhjH* mutant strain, both harboring pYcgR-Spy, were grown in liquid LB media at 37°C to an OD₆₀₀ of 0.20. At this point, cells were collected and imaged using fluorescence microscopy, and their net FRET (nFRET)/CFP intensity ratios were calculated. Control strains expressing either the DGC PA1120 (*ara::DGC*) or the PDE PA2133 (*ara::PDE*), and pYcgR-Spy, were used to determine the

bound and unbound nFRET/CFP ratios for the YcgR FRET construct in this *in vivo* assay. As expected, these control strains defined homogeneous populations of cells that demonstrated distinct nFRET/CFP ratios (Fig 5C upper panel, Fig S4). Interestingly, in the wild-type strain expressing pYcgR-Spy, a heterogeneous population of c-di-GMP concentrations was observed (Fig 5C, lower panel). This heterogeneity in c-di-GMP concentrations has also been observed previously for other bacterial species, and it is hypothesized that this phenomenon is due to the production of progeny cells after cell division that harbor different c-di-GMP concentrations, and therefore demonstrate different cellular behaviors, in order to promote a diversification strategy that may enhance survival of the bacterial species (Christen et al., 2010). In the wild-type strain, 43.7% of cells demonstrated a nFRET/CFP ratio higher than 0.9, indicative that YcgR was completely unbound to c-di-GMP in these cells. In contrast, in the $\Delta yhjH$ mutant, only 4.8% of cells demonstrated a nFRET/CFP ratio higher than 0.9, a 10-fold decrease compared to wild-type cells (Fig 5C). The remainder of cells demonstrated a nFRET/CFP ratio of 0.9 or lower, indicating that at least some of the YcgR FRET constructs were bound to c-di-GMP in these cells. As demonstrated by Boehm *et al.*, binding of c-di-GMP to only a fraction of the YcgR molecules present in the cell can lead to a decrease in swimming motility, with swimming inhibition increasing as the number of YcgR molecules bound to c-di-GMP in the cell increases (Boehm et al., 2010). Thus it is possible that the $\Delta yhjH$ mutant strain is inhibited for motility because a higher fraction of the YcgR proteins in these cells is bound to c-di-GMP. This experiment demonstrates that mutation of *yhjH* does result in an increase in the percentage of cells with an average level of c-di-GMP that is high enough to bind to the YcgR protein, which is then able to inhibit swimming. However, the change in intracellular c-di-GMP concentration does not appear to be large, which is consistent with previous studies (Simm et al., 2007).

A *S. Typhimurium* strain harboring the lower-affinity *ycgR-R113A* mutation does not demonstrate YcgR- dependent motility inhibition in a $\Delta yhjH$ mutant

Once we had established that mutation in *yhjH* results does affect intracellular c-di-GMP levels enough to be relevant to YcgR, we wanted to assess the effect of the *ycgR-R113A* mutation in the context of a *yhjH* mutant background. To examine this, a $\Delta yhjH$ deletion mutant was generated on a *ycgR* wild-type, *ycgR-R113A* or $\Delta ycgR$ deletion mutant background of *S. Typhimurium*. In this assay, the swimming diameter of the $\Delta yhjH$ mutant was roughly 35% of that observed for the wild-type strain (Fig 6). Swimming was partially restored in the $\Delta yhjH$ mutant by a second deletion in *ycgR* (66% swimming diameter of the wild-type strain). Remarkably, chromosomal replacement of the *ycgR* codon encoding arginine-113 with a codon that encodes alanine resulted in a phenotype practically indistinguishable from the deletion mutant (69% swimming diameter of the wild-type strain) (Fig 6). Thus the YcgR R113A mutant cannot functionally replace YcgR in this experiment (Fig 6). This suggests that the $\Delta yhjH$ mutation causes the intracellular concentration of c-di-GMP to rise high enough to bind to YcgR, but not high enough to bind to the YcgR R113A mutant protein.

yhjH inactivation does not result in c-di-GMP levels that are high enough to bind to BcsA and stimulate cellulose production

Our data indicate that mutation in *yhjH* results in an increase in the fraction of cells that contain c-di-GMP levels that are high enough to bind to the wild-type YcgR protein, but not high enough to bind to the R113A mutant YcgR protein. Since the affinity of the BcsA PilZ domain for c-di-GMP is lower than that of the YcgR R113A protein, we reasoned that Δ *yhjH* mutation would not result in c-di-GMP levels high enough to bind to the BcsA PilZ domain. To test this hypothesis, we expressed a BcsA PilZ FRET- based construct inside of live cells and monitored their nFRET/CFP ratios in a similar manner to that of the YcgR FRET construct (Fig 7A). As for the YcgR FRET construct, *ara::DGC* and *ara::PDE* strains harboring the BcsA PilZ FRET construct were separated into homogeneous populations based on their nFRET/CFP ratios (Fig 7B upper panel, Fig S5). Unlike cells expressing the YcgR FRET construct, wild-type cells expressing the BcsA PilZ construct were homogeneous in their nFRET/CFP ratios (Fig 7A, 7B). The Δ *yhjH* mutant strain did not demonstrate nFRET/CFP ratios that were significantly different than that of the wild-type strain, and in both strains, these ratios matched that of the *ara::PDE* strain, indicating that the BcsA PilZ domain was unbound to c-di-GMP in this assay (Fig 7B). Thus, the Δ *yhjH* mutation does not result in a change in c-di-GMP levels that are large enough to affect the BcsA PilZ construct.

Since no binding of c-di-GMP to the BcsA PilZ domain was observed in the Δ *yhjH* mutant strain, we hypothesized that even though Δ *yhjH* mutation stimulates motility inhibition, it does not result in the production of cellulose. This was observed during growth on solid agar plates (Fig 3A), implying that the increased c-di-GMP concentration that results from Δ *yhjH* deletion does not induce BcsA activity; however, bacterial detection of solid surfaces may result in different levels of c-di-GMP on solid agar plates than in the low-agar motility plate assay (Guvener & Harwood, 2007). Therefore, we analyzed the production of cellulose on motility agar plates under the same conditions at which Δ *yhjH*-dependent motility inhibition is observed. As a control, the *S. Typhimurium* pDGC and Δ *bcsA* pDGC strains were included. Induction of the DGC results in the production of a level of c-di-GMP that completely inhibits motility (Fig 7C upper panel). In the soft-agar motility plate assay, cellulose production was observed in the pDGC strain by its binding to the fluorescent dye calcofluor (Fig 7C lower panel). In contrast, no calcofluor binding was observed, as expected, to the pDGC strain lacking *bcsA*. Although the Δ *yhjH* deletion strain demonstrated an inhibition in swimming motility in this assay, no calcofluor binding in this strain was observed, as the calcofluor fluorescence of this strain was indistinguishable from that of a Δ *yhjH* Δ *bcsA* double mutant (Fig 7C). This demonstrates that the Δ *yhjH* mutation results in motility inhibition but does not stimulate the production of cellulose.

Discussion

In this work, we sought to understand an important question in the field of c-di-GMP signaling: how is specificity between c-di-GMP metabolizing enzymes and their downstream receptors achieved, despite the fact that c-di-GMP is a small, freely diffusible second messenger molecule? To this end, we have explored the validity of the hypothesis

that signaling specificity is achieved through different binding affinities of these receptors for c-di-GMP.

Using the same FRET-based method, we compared the affinities of PilZ domain proteins, which are c-di-GMP downstream receptors, within the same organism and found that they span a large range. For *P. aeruginosa*, this difference is 145-fold (Table 1). This is supported by published binding affinities of three non-PilZ c-di-GMP-binding proteins in *P. aeruginosa*, which also span a large range (Table 1), although it should be noted that the binding affinities of these proteins were not measured at 25°C. In addition, c-di-GMP-binding molecules of *Caulobacter crescentus* and *Vibrio cholerae* also exhibit large differences in binding affinities from the nM to the μM range (Christen *et al.*, 2006, Benach *et al.*, 2007, Christen *et al.*, 2007, Pratt *et al.*, 2007, Sudarsan *et al.*, 2008, Duerig *et al.*, 2009, Krasteva *et al.*, 2010, Srivastava *et al.*, 2011). This suggests that intracellular c-di-GMP concentrations could selectively target certain receptors based on their binding affinities in these organisms. Indeed, measurements of c-di-GMP concentrations in wild-type *P. aeruginosa* under different conditions have resulted in very different c-di-GMP levels (Christen *et al.*, 2010, Malone *et al.*, 2010, Ha *et al.*, 2011, Roy *et al.*, 2012). This is particularly interesting in light of the fact that many DGCs are negatively feedback-inhibited via binding of c-di-GMP to an allosteric I-site, allowing them to maintain homeostasis of c-di-GMP concentrations (Christen *et al.*, 2006). It is attractive to speculate that DGC feedback inhibition may work synergistically with downstream receptor binding affinities, such that some DGCs would produce enough c-di-GMP to “communicate” with receptors that have low c-di-GMP-binding affinity, while other DGCs that bind c-di-GMP with high affinity at the I-site would be limited to activating only receptors with high c-di-GMP binding affinities. Finally, the *P. aeruginosa* PilZ domain protein PA3353 demonstrated the presence of two binding sites in this study. PA3353 might thus act as a bandpass filter, active or inactive only within a narrow range of c-di-GMP levels. Further work is needed to determine the structural basis of the second c-di-GMP binding site in this protein.

For *S. Typhimurium*, the difference in binding affinities of the two PilZ domain proteins is 43-fold (Table 1). Using site-directed mutagenesis of Position-X residues, we generated *S. Typhimurium* PilZ domain proteins that have altered c-di-GMP binding affinities. For YcgR, mutation of arginine-113 to alanine in Position-X likely led to the loss of important H-bond and/or π -stacking interactions with c-di-GMP, thereby reducing binding affinity. For BcsA, the effect of mutation of valine-695 to arginine is less clear. It was hypothesized that the reason why mutation of leucine to arginine in Position-X of VCA0042 caused only a minor increase in binding affinity was because the mutation did not generate any new bonds to c-di-GMP (Ko *et al.*, 2010). If this is also true for the BcsA V695R mutation, this could explain why the effect of Position-X mutation on binding affinity was smaller in BcsA than for YcgR. By using phenotypic assays, these mutations were shown to affect the phenotypes of the proteins in a predictable manner, suggesting that binding affinity is important for the biological functions of these proteins.

This hypothesis is supported by the observation that mutation of *yhjH* results in c-di-GMP levels that are high enough, in most cells, to bind YcgR, but not high enough to bind BcsA, and that this correlates with the activities of these proteins in phenotypic assays. The $\Delta yhjH$

mutation results in a considerable increase in the fraction of the population that contains c-di-GMP levels that are high enough to demonstrate at least partial binding to a YcgR FRET construct (Fig 5). However, a BcsA PilZ FRET construct, which demonstrates a lower affinity for c-di-GMP than YcgR, remained entirely unbound to c-di-GMP in both wild-type and $\Delta yhjH$ mutant strains (Fig 7). This, combined with the observation that the $\Delta yhjH$ mutation does not appear to affect motility inhibition by the lower-affinity YcgR R113A mutant protein (Fig 6), provides evidence that the rise in c-di-GMP levels in the $\Delta yhjH$ mutant is small. This explains why a previous comparison of the total c-di-GMP concentrations between wild-type and $\Delta yhjH$ mutant strains of *S. Typhimurium* did not find a significant difference in c-di-GMP concentration (Simm et al., 2007). Gross measurement of total c-di-GMP concentrations as an average amount from a cell culture would fail to detect a small but significant increase in the free level of c-di-GMP in a fraction of cells. Additionally, the hypothesis that the increase in c-di-GMP levels in a $yhjH$ mutant is small is consistent with our data demonstrating that the $\Delta yhjH$ mutation fails to stimulate cellulose production at 37°C despite the presence of a functional cellulose synthase complex (Fig 3A, Fig 7C, Fig S2). It is possible that in wild-type *S. Typhimurium*, YhjH maintains the level of c-di-GMP close to the K_d for binding YcgR. This is supported by the observed heterogeneity in populations of wild-type cells in which some cells have c-di-GMP levels lower than the K_d of YcgR, while others demonstrate slightly higher c-di-GMP levels (Fig 5). Environmental changes that result in a decrease in YhjH activity could then very quickly result in an increase of c-di-GMP levels that are just above the K_d of YcgR, and are therefore just large enough to be biologically relevant to the YcgR protein, but not to affect BcsA, thus allowing *S. Typhimurium* to modulate its swimming behavior without also investing energy in building an extracellular matrix.

Based on this evidence, we propose the following model to illustrate c-di-GMP signaling regulation in *S. Typhimurium* in rich media based on binding affinity (Fig 8). This model involves the sequential activation of c-di-GMP receptors in *S. Typhimurium* through increasing c-di-GMP levels, which is consistent with the progression of biofilm formation. While the c-di-GMP concentration is maintained at a low level by YhjH, it does not bind to either YcgR or BcsA, resulting in a cell that is motile and does not produce cellulose. Upon sensing appropriate environmental conditions, levels of c-di-GMP rise (perhaps through inactivation of YhjH), which have the immediate impact of decreasing swimming motility through YcgR. This is an important early step in microbial adherence to surfaces since cellular motility must be tightly regulated for appropriate colonization (Sanchez-Torres *et al.*, 2011). Increased c-di-GMP levels induce the expression of CsgD, which would then directly activate the transcription of curli fimbriae, an important attachment and aggregative factor of *S. Typhimurium* (Romling, 2005). CsgD expression also results in the production of AdrA, which, upon sensing appropriate conditions through its environmental sensing domain, raises the intracellular level of c-di-GMP even higher, resulting in c-di-GMP binding to BcsA and stimulating cellulose production, further protecting organisms after attachment. The requirement for a higher level of c-di-GMP to induce cellulose production, but not to activate the production of curli fimbriae, is potentially due the fact that cells that produce cellulose before generating curli fimbriae appear to be deficient in initial attachment to surfaces (Gualdi *et al.*, 2008). There are several lines of evidence that suggest that AdrA

produces a large amount of c-di-GMP inside of the cell. During cellulose-producing conditions, AdrA has been shown to be responsible for producing most of the c-di-GMP present inside of the cell (Kader et al., 2006). AdrA is capable of producing more c-di-GMP than other *Salmonella* DGCs tested. Its overexpression has been found to raise the cellular c-di-GMP level by 10,000-fold, compared to two other *Salmonella* DGCs that have been shown to affect motility and CsgD expression, which raise the c-di-GMP level by no more than 6-fold (Kader et al., 2006).

In conclusion, our data provides evidence to the hypothesis that specificity in c-di-GMP signaling can be achieved through the affinities of downstream receptors. This theory is consistent with environmental signal integration by DGCs and PDEs resulting in the generation of a specific level of intracellular c-di-GMP. The complex regulation of c-di-GMP likely involves various strategies which will be unraveled by future studies into the c-di-GMP signaling networks of many different bacteria.

Experimental Procedures

Bacterial strains

All *Salmonella enterica* serovar Typhimurium strains used in this study were *S.* Typhimurium ATCC 14028s, except for the arabinose-inducible PA1120 strain used to generate Fig 3B, which was constructed on a *S.* Typhimurium LT2 background. For cloning purposes, *Escherichia coli* DH5 α or DH10 β were used (Invitrogen). Chromosomal mutations, insertions, and deletions were generated using the method outlined by Datsenko and Wanner (Datsenko & Wanner, 2000). Antibiotic resistance markers inserted into the genome for the purpose of generating chromosomal mutations were transferred to a genetically unmanipulated strain by P22 transduction, to ensure that the phenotype was due to the intended mutation. Chromosomal deletion mutants were constructed by the substitution of genetic antibiotic resistance cassettes encoding kanamycin, chloramphenicol, or tetracycline resistance, in such a way that deletion did not affect surrounding genes. To insert *PA1120* or *PA2133* into the arabinose locus, a genetic cassette encoding resistance to tetracycline was first inserted into the chromosome replacing the *araBAD* genes. Then, primers were used to amplify *PA1120* or *PA2133* (Kulasakara et al., 2006) from *P. aeruginosa* PAO1 genomic DNA with flanking regions of homology to the *araBAD* locus such that, upon transformation of the PCR product, *PA1120* or *PA2133* replaced the tetracycline resistance cassette. Strains in which the *PA1120* or *PA2133* genes had replaced the tetracycline resistance cassette were then selected by their growth on agar plates containing chlortetracycline, which selects for tetracycline sensitivity (Bochner *et al.*, 1980). *S.* Typhimurium 14028s expressing FLAG-tagged BcsA protein was generated by chromosomal insertion, using the tetracycline selection method described above, of a nucleotide sequence encoding the FLAG tag (DYKDDDDK) immediately 5' of the STOP codon of the *bcsA* gene. This strain was streaked onto calcofluor agar plates along with the wild-type and *bcsA* mutant strains, and it was verified that there was no difference in production of cellulose between the wild-type and BcsA-FLAG strains (Fig S2). *ycgR-R113A* and *bcsA-V695R* chromosomal mutations were generated using the tetracycline selection method described above with oligonucleotides that encoded the single point mutation. All gene manipulations were

designed using the software program Geneious Pro™, and were verified by PCR amplification and sequencing.

Plasmids

The plasmids used for protein expression for *in vivo* phenotypic analysis in this work were generated in a pMMB67EH background encoding ampicillin resistance (Furste *et al.*, 1986). To make pDGC, *PA1120* from *P. aeruginosa* PAO1 was inserted into pMMB67EH under the control of the *tac* promoter, and the pET15B RBS (ACTTTAAGAAGGAGATATCAT) was inserted immediately upstream of the *PA1120* start codon. To make pDGC-YcgR, *ycgR* and its corresponding promoter region (118 bp taken upstream of the *ycgR* start codon) were cloned downstream of *PA1120* in pDGC. To prevent read-through of the *ycgR* gene whenever *PA1120* was induced with IPTG, the transcriptional terminator from phage T4 gene 32 was inserted between the end of *PA1120* and the beginning of the *ycgR* promoter region (Frey & Krisch, 1985). The *ycgR-R113A* mutation was generated in this plasmid background using the Quikchange XL™ kit (Agilent) with primers containing the mutation. To generate plasmids encoding PilZ-based FRET constructs for *in vitro* protein purification, a pET15B plasmid containing the N-terminal His-tagged *yfp-cfp* FRET fusion pair under the control of a *tac* promoter was used as previously described (Christen *et al.*, 2010). PilZ domain proteins were cloned into the SpeI and KpnI sites between the *yfp* and *cfp* genes to generate plasmids that would result in the production of His-tagged, FRET fusion proteins when their expression was induced with IPTG. BcsA of *S. Typhimurium* and PA3542 of *P. aeruginosa* are membrane proteins, so the regions of the genes comprising the PilZ domains were cloned into the FRET construct (encoding amino acid residues 691–791 for BcsA and 299–389 for PA3542). A truncated version of PA3353 was generated lacking the residues corresponding to the first alpha helix (amino acid residues 1–21), since the full-length protein did not demonstrate a change in FRET between the bound and unbound states that was large enough to accurately measure binding affinity. The BcsA V695R and YcgR R113A point mutations were made on the corresponding pET15B fusion construct backgrounds using the Quikchange XL™ kit (Agilent) with primers containing the mutation. pYcgR-Spy, encoding gentamicin resistance and the YcgR FRET-based *in vivo* biosensor, was obtained from Christen *et al.* (Christen *et al.*, 2010). This plasmid contains the gene *mYPet_synthYcgR_mCYPet* under the control of an IPTG-inducible promoter. In this plasmid, the *ycgR* gene, which had been codon-optimized to remove rare codons during expression in *S. Typhimurium*, was cloned between the *mYPet* and *mCYPet* genes in SpeI and ACC651 sites, resulting in mYPet-YcgR-mCYPet fusion protein expression in the presence of IPTG. pBcsA-Spy was constructed by digesting pET15B-BcsAPilZSpy with the restriction enzymes SpeI and ACC651 (FastDigest®, Fermentas) and ligating the released *bcsA* PilZ region into pYcgR-Spy (Rapid DNA Ligation Kit, Fermentas) that had also been digested with SpeI and ACC651, releasing the *ycgR* fragment. All plasmids were designed using the software program Geneious Pro™, and were verified by PCR amplification and sequencing.

Growth Conditions

Unless otherwise stated, cells were grown in Luria Broth at 37°C with shaking, in the presence of antibiotics, arabinose, IPTG, and/or calcofluor (Fluorescence Brightener 28,

Spectrum Chemical MFG) when appropriate. Antibiotic concentrations were: ampicillin, 100 $\mu\text{g ml}^{-1}$; chloramphenicol, 20 $\mu\text{g ml}^{-1}$; gentamicin, 15 $\mu\text{g ml}^{-1}$; kanamycin, 45 $\mu\text{g ml}^{-1}$; tetracycline, 10 $\mu\text{g ml}^{-1}$.

Fluorescence spectra and binding affinity measurements

YPet-PilZ-CyPet fusion proteins were expressed in *Escherichia coli* BL21(DE3) cells and underwent purification through Ni-NTA resin as previously described (Christen et al., 2010). For the affinity measurements of BcsA and YcgR, and their respective affinity mutants, the FRET constructs were further purified by size exclusion chromatography using a Superdex™ 200 10/300 GL column (GE Healthcare). Affinity measurements of PilZ-based FRET constructs were calculated using methods developed by Christen *et al.* (Christen et al., 2010). Briefly, c-di-GMP was added to purified FRET fusion protein in 96-well black plates (Greiner), and the amount of FRET was quantified on an Envision multilabel plate reader (PerkinElmer). Fluorescence spectra were recorded at 24°C using the quad monochromator (excitation at 425 nm, emission scan from 460 to 560 nm in 2 nm intervals) using 50–500 nM biosensor in PBS pH 7.4 with 10% glycerol, 200 mM NaCl, and 5 mM β -mercaptoethanol. Emission spectra were normalized to the total amount of fluorescence emission over the entire scan. Binding affinity was determined by adding increasing concentrations of c-di-GMP and measuring the resulting steady-state FRET/CFP ratio using a filter-based measurement method (CFP excitation filter CWL 430 nm, BW 24 nm; CFP emission filter CWL 485 nm, BW 14 nm; YFP emission filter CWL 535 nm, BW 30 nm, dual dichroic mirror D450/D505 nm) to determine FRET ratios from 24°C to 37°C in 1°C intervals. In the filter-based method, the fraction of FRET biosensor bound to c-di-GMP was calculated for each biosensor by measuring the FRET/CFP ratio at each indicated c-di-GMP concentration, subtracting the unbound FRET/CFP ratio, and dividing this number by the difference between the bound and unbound FRET/CFP ratios. A number of 0 indicates that the FRET biosensor is completely unbound to c-di-GMP, and a number of 1 indicates that the FRET biosensor is completely bound to c-di-GMP. Binding affinities and Hill coefficients were calculated by fitting a curve to the plot of FRET ratio over c-di-GMP concentration in GraphPad Prism™.

Soft-agar motility plate assays

Strains were inoculated as overnight cultures in LB at 37°C with shaking with the appropriate antibiotic (if applicable). The following morning, strains were diluted to OD₆₀₀ of 0.1. 1–1.5 μl were then spotted onto 0.3–0.35% agar LB plates. Plates were incubated at 37°C for 9–13 hours, then scanned using an Epson® Perfection 4990 scanner and imported as TIFF files. The diameter of swimming of each strain was measured by using the magic wand tool in Adobe™ Photoshop™ CS5. The data was analyzed using a Student's two-tailed T test.

Cellulose production

The calcofluor plates used in this work were LB-agar plates containing 50 $\mu\text{g ml}^{-1}$ calcofluor (Fluorescence Brightener 28, Spectrum Chemical MFG). Strains were streaked on calcofluor plates and incubated overnight (37°C) or for 2–3 days (24–30°C). Plates were then analyzed by illumination with an excitation wavelength for of 365 nm unless otherwise

specified using a UVP Bioimaging System[®] equipped with a digital camera for capturing images. For the quantitative calcofluor measurements shown in Fig. 3B, *S. Typhimurium* *ara::DGC*, *ara::DGC bcsA-V695R*, or *ara::DGC ΔbcsA* were incubated overnight at 37°C. The next day, cells were diluted to an OD₆₀₀ of 0.02, incubated at 37°C with shaking until the OD reached 3.3, then diluted to an OD₆₀₀ of 0.0625. 8 μl of each strain were spotted using a multichannel pipettor into each well of a 96-well black plate (Greiner) with 200 μl of calcofluor agar (3 μg ml⁻¹) and the indicated amount of arabinose. After incubation at 37°C for 20 hours, the intensity of calcofluor fluorescence was then quantified by reading the fluorescence values in an ENVISION multilabel reader (PerkinElmer). The data was analyzed using a Student's two-tailed T test.

Measurement of in vivo FRET/CFP ratios in individual live cells

FRET analyses in individual cells were performed using the protocols developed by Christen *et al.* (Christen et al., 2010). To determine FRET/CFP ratios in *S. Typhimurium* 14028s and an isogenic *yhjH* mutant, cells were inoculated into one ml LB with gentamicin and 75 μM IPTG, and incubated at 30°C overnight with shaking. The next day, cells were diluted into 25 ml fresh LB with 15 μg ml⁻¹ gentamicin and 500 μM IPTG at an OD₆₀₀ of 0.02. Cells were incubated at 37°C with shaking for approximately 2 hours until the OD₆₀₀ reached 0.2. To prepare samples for fluorescence microscopy, cells were centrifuged, washed in M63 media (11 mM KH₂PO₄, 20 mM K₂HPO₄, 7.5 mM (NH₄)₂SO₄, 100 mM glycerol, 5 μM Fe²⁺NH₄SO₄, 2 mM MgCl₂, 10 mM NaCl, 15 μg ml⁻¹ gentamicin, 500 μM IPTG), and resuspended in M63 media to an OD of 2.0. M63 media was used in these experiments since LB media demonstrates autofluorescence at the wavelengths used for imaging. 1.5 μl of resuspended cells were then spotted onto 0.75 mm thick agar pads (M63 media with 1% agarose) on prewarmed, prewashed 1 mm microscope slides (Thermo Fisher Scientific) and allowed to dry. A prewarmed number 1.5 glass coverslip (Corning Life Sciences) was applied to the agar pads, and the coverslip was sealed to the slide using a glue gun to prevent liquid evaporation. Fluorescence microscopy images were acquired using a Nikon Eclipse TIE inverted, wide-field microscope prewarmed to 37°C using the same hardware and software as previously published (Christen et al., 2010). Exposure times for the determination of intensities in the FRET, CFP, and YFP channels were 1000, 1300, and 200 ms, respectively, with a conversion gain of 2x and 2x2 binning. For each image, all channels were exported in a TIFF file format by Nikon Elements analytical software and analyzed in ImageJ software as described previously (Christen et al., 2010). The average intensity values for each region of interest were then exported to Microsoft[®] Excel software. Regions of aggregated FRET protein, represented by localized, unphysiologically high intensity values in either the CFP or YFP channels near one pole of the cell were deselected when observed and were not counted in the analysis. Net FRET (nFRET) was calculated by subtracting spectral bleedthrough and direct YFP excitation from the FRET intensity value (Christen et al., 2010, Xia & Liu, 2001). To control for brightness, nFRET was normalized to the intensity of the CFP channel (Vanderklish *et al.*, 2000).

Measurement of cellulose production in swimming agar plates

For determination of cellulose production in swimming agar plates, *S. Typhimurium* strains *ΔyhjH*, *ΔyhjH ΔbcsA*, pDGC, and pDGC *ΔbcsA* were inoculated as described above onto

0.3% agar swimming plates containing 50 $\mu\text{g ml}^{-1}$ calcofluor. To induce production of the DGC PA1120, IPTG had been added to the plate at a concentration of 100 μM . Plates were incubated at 37°C for 12 hours, then scanned using an Epson® Perfection 4990 scanner and imaged using a UVP Bioimaging System© with the excitation wavelength at 254 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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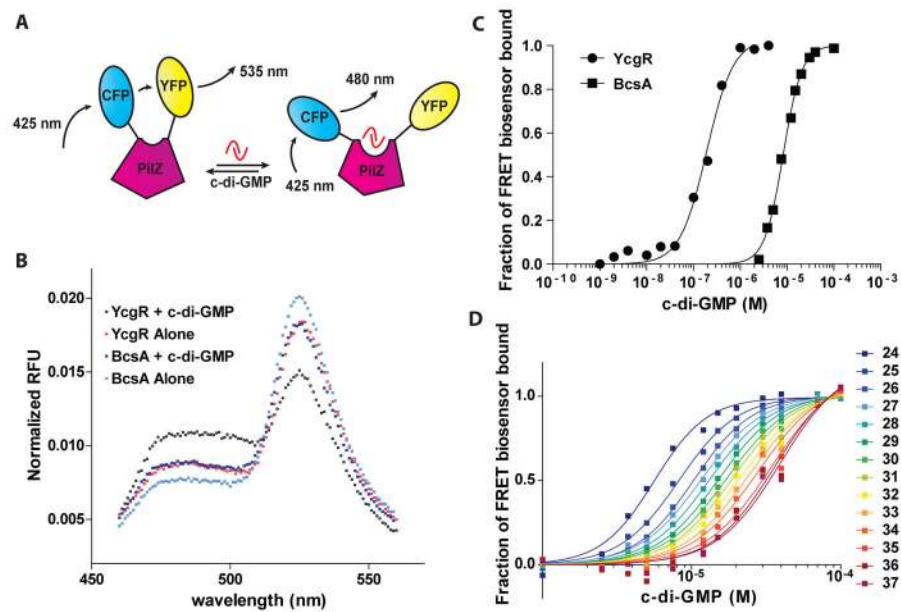


Fig. 1. The binding properties of the BcsA FRET construct for c-di-GMP

A. PilZ domain FRET proteins were generated by the fusion of the fluorophores mYPet (YFP) and mCyPet (CFP) to the termini of a PilZ domain protein. These FRET fusion proteins demonstrate a characteristic FRET signal when exposed to the excitation wavelength of mCyPet (425 nm). Binding of c-di-GMP to the PilZ domain changes the conformation of the PilZ domain protein, which results in a shift in the emission ratio of mYPet (535 nm) to mCyPet (480 nm). In this figure, binding of c-di-GMP results in a decrease in FRET, though c-di-GMP binding can also result in a FRET increase depending on the PilZ domain protein used. B. Normalized fluorescence emission spectra of YcgR and BcsA based FRET biosensors upon excitation of mCyPet at 425 nm *in vitro*. Shown are the emission spectra of the YcgR biosensor in the presence (black circles) and absence (red circles) of 40 μ M c-di-GMP, and the BcsA biosensor in the presence (dark blue squares) and absence (light blue squares) of 40 μ M c-di-GMP at 25°C. For both biosensors, the addition of c-di-GMP results in a decrease in the amount of FRET. C. c-di-GMP binding curves for YcgR (circles) and BcsA (squares) biosensors at 25°C as measured by FRET. The fraction of FRET biosensor bound to c-di-GMP was calculated as follows: Fraction of FRET biosensor bound = (FRET ratio(x) - FRET ratio free) / (FRET ratio bound - FRET ratio free). A number of 0 indicates that the FRET biosensor is completely unbound to c-di-GMP, and a number of 1 indicates that the FRET biosensor is completely bound to c-di-GMP. D. Binding curves of the BcsA biosensor at temperatures ranging from 24°C to 37°C at 1°C intervals as indicated by the legend. Binding affinity decreases as temperature increases.

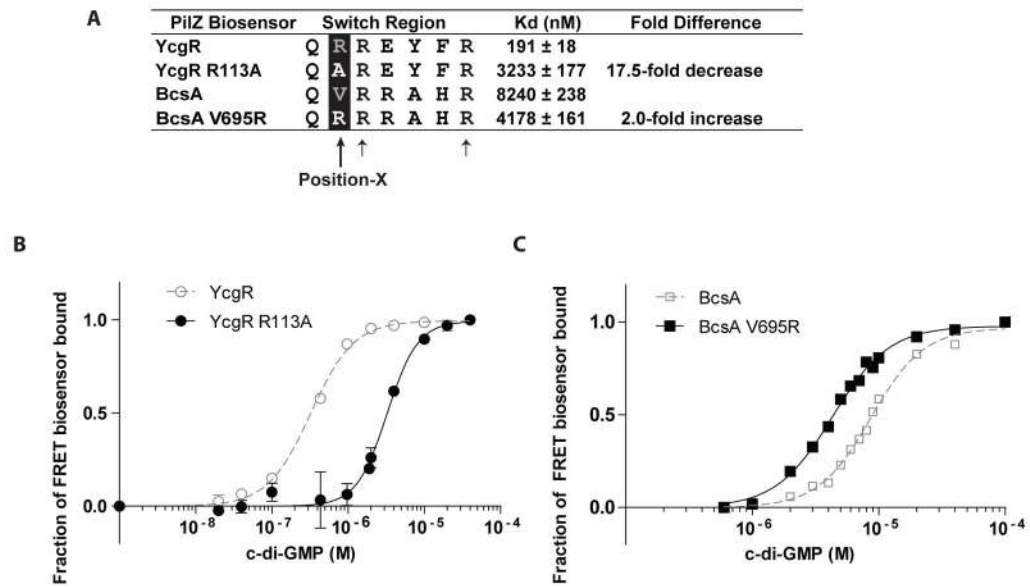


Fig. 2. Mutation of the residue at Position-X alters the c-di-GMP binding affinities of *S. Typhimurium* PilZ domain proteins

A. Table showing the c-di-GMP switch region for YcgR and BcsA. Shown in white are mutated residues. Small arrows show the locations of the invariant arginines of the RxxxR motif. The affinities shown are for proteins measured at 25°C. B. c-di-GMP binding curves for YcgR wild-type (open circles) and YcgR R113A (closed circles) FRET biosensors at 25°C. C. c-di-GMP binding curves for BcsA PilZ wild-type (open squares) and BcsA PilZ V695R (closed squares) FRET biosensors at 25°C.

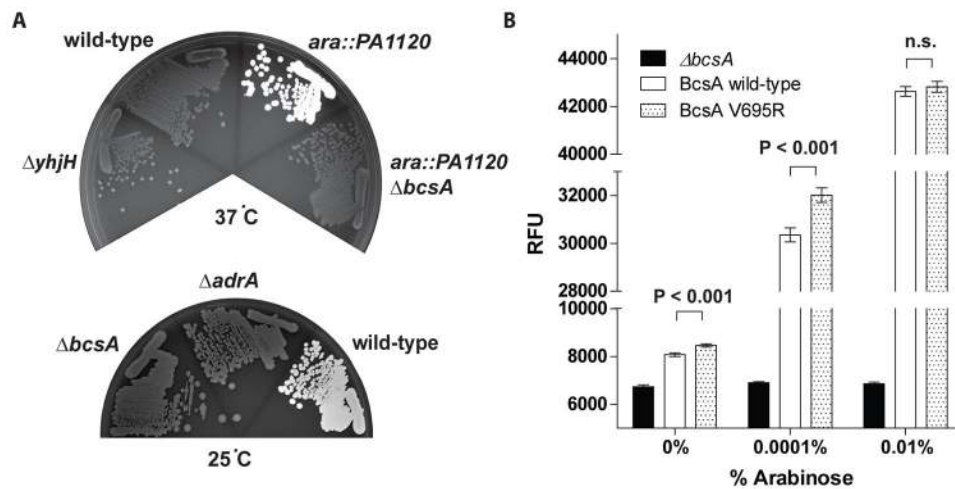


Fig. 3. c-di-GMP binding affinity of *S. Typhimurium* BcsA affects cellulose production *in vivo*
 A. *S. Typhimurium* makes cellulose at 24°C, but not at 37°C, in a manner that is dependent on both AdrA and BcsA. Calcofluor agar plates incubated at either 37°C (top) or 24°C (bottom) with wild-type, $\Delta yhjH$, *ara::DGC*, *ara::DGC* $\Delta bcsA$, $\Delta bcsA$, or $\Delta adrA$ strains of *S. Typhimurium*. *ara::DGC* is a strain that harbors an arabinose-inducible DGC on the chromosome. The plate incubated at 37°C was supplemented with 0.001% arabinose. Plates were exposed to UV light with an excitation wavelength of 365 nm, which is a wavelength that stimulates fluorescence of calcofluor when it is bound to cellulose. White coloring indicates calcofluor binding to cellulose. B. Calcofluor fluorescence at different concentrations of arabinose at 37°C for *ara::DGC* with wild-type *bcsA*, or *ara::PA1120* in which the *bcsA* gene has either been deleted ($\Delta bcsA$) or mutated (BcsA V695R). Shown is the average fluorescence emission intensity at 420 nm, after excitation at 365 nm, of cells growing on 200 μ l agar in individual wells of a 96-well plate in a representative experiment. RFU, relative fluorescence units. n.s. = not significant.

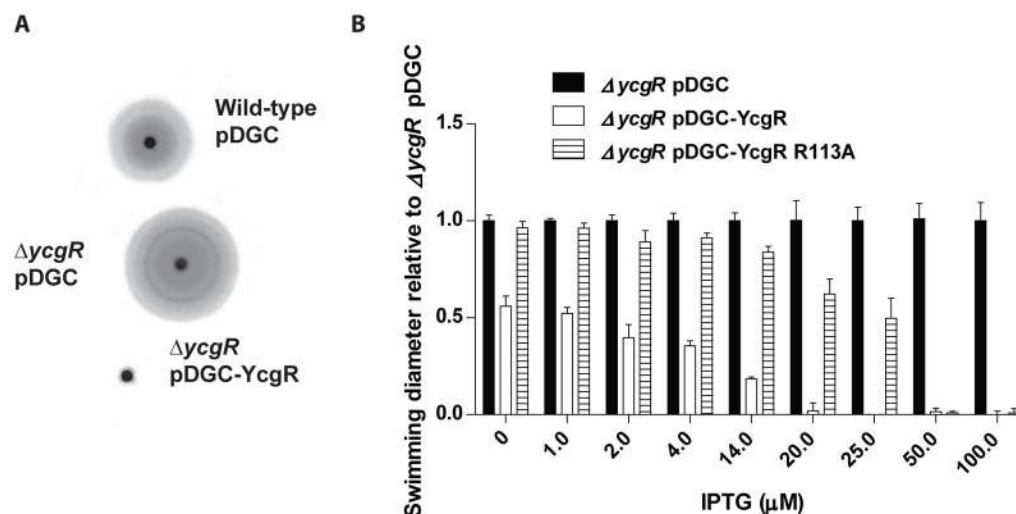


Fig. 4. c-di-GMP binding affinity of *S. Typhimurium* YcgR affects motility inhibition

A. Swimming halos on motility agar plates with 25 μ M IPTG of *S. Typhimurium* strains harboring pDGC or pDGC-YcgR at 37°C for 12 hours. A wild-type strain without exogenous DGC expression was not shown since the swimming diameter of this strain is too large to include and adequately show the differences between strains harboring pDGC. B. Swimming diameters of $\Delta ycgR$ harboring either pDGC-YcgR or pDGC-YcgR R113A relative to the swimming diameter of the uncomplemented $\Delta ycgR$ pDGC strain, at indicated levels of IPTG at 37°C. Both YcgR and YcgR R113A effect a decrease in motility at increasing IPTG levels, but the YcgR R113A mutant protein requires a higher concentration of IPTG to inhibit motility than the wild-type YcgR protein.

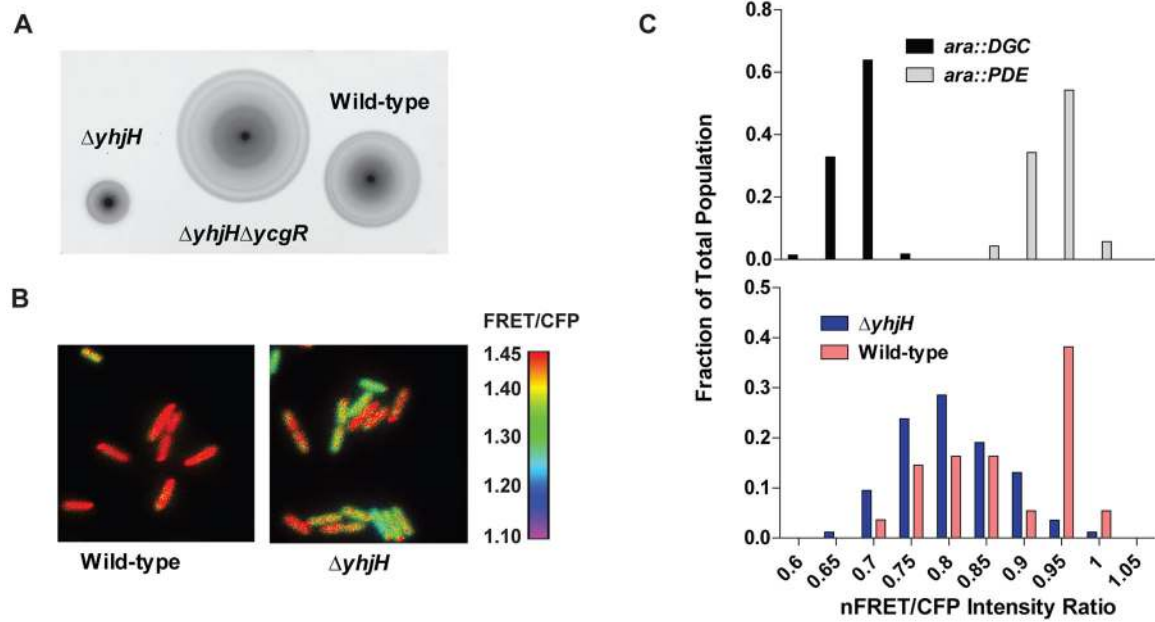


Fig. 5. A *S. Typhimurium* strain harboring a *yhjH* mutation demonstrates a larger proportion of cells with c-di-GMP bound to YcgR than the wild-type strain

A. Swimming halos on motility agar plates of wild-type, $\Delta yhjH$, and $\Delta yhjH\Delta ycgR$ at 37°C.

B. Dual-emission ratio microscopic images (FRET/CFP) of *S. Typhimurium* (above) or a $\Delta yhjH$ mutant (below) expressing pYcgR-Spy. Pseudocolors represent emission ratios (527/480 nm) of the FRET-based biosensor as indicated by the figure legend to the right.

C. Histogram showing the fraction of cells that demonstrate the indicated average nFRET/CFP ratios for either control strains expressing a DGC or a PDE off of an arabinose-inducible promoter (top), or for wild-type or $yhjH$ mutant strains (bottom). nFRET/CFP ratios differ from FRET/CFP ratios since nFRET intensities have been corrected for bleedthrough and fluorescence in the YFP channel. The difference between the wild-type and $\Delta yhjH$ strains is highly significant ($P < 0.0001$). nFRET: net FRET intensity, calculated by subtracting bleedthrough coefficients and intensity of the YFP channel as detailed in Materials and Methods. Over 100 cells were analyzed for each strain.

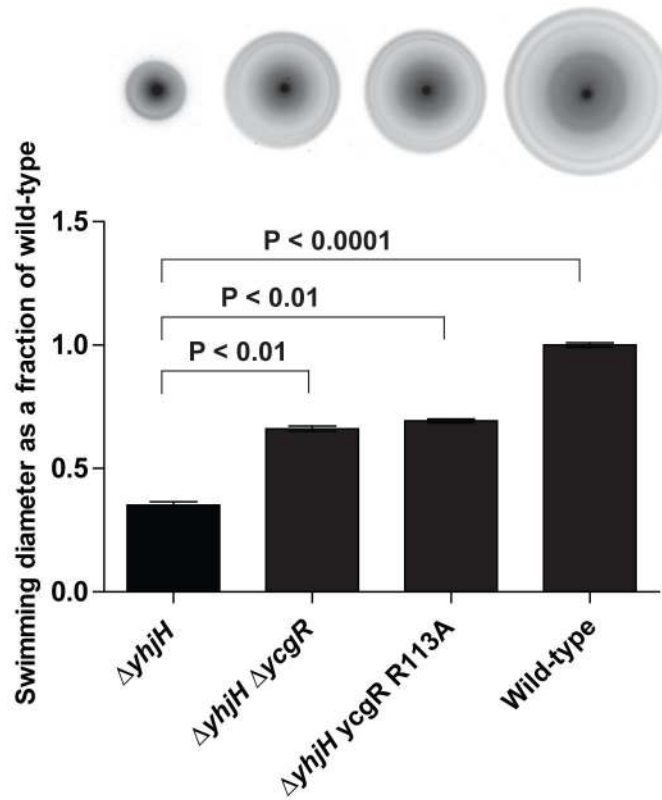


Fig. 6. A *S. Typhimurium* strain harboring the lower-affinity *ycgR-R113A* mutation does not demonstrate YcgR-dependent motility inhibition in a $\Delta yhjH$ mutant

Above: swimming diameters (from left to right) of $\Delta yhjH$, $\Delta yhjH \Delta ycgR$, $\Delta yhjH ycgR-R113A$, and wild-type *S. Typhimurium*, on motility agar plates incubated at 37°C for 9 hours. Below: quantitation of the swimming diameters of indicated *S. Typhimurium* strains compared to the wild-type strain. Statistical significance is indicated.

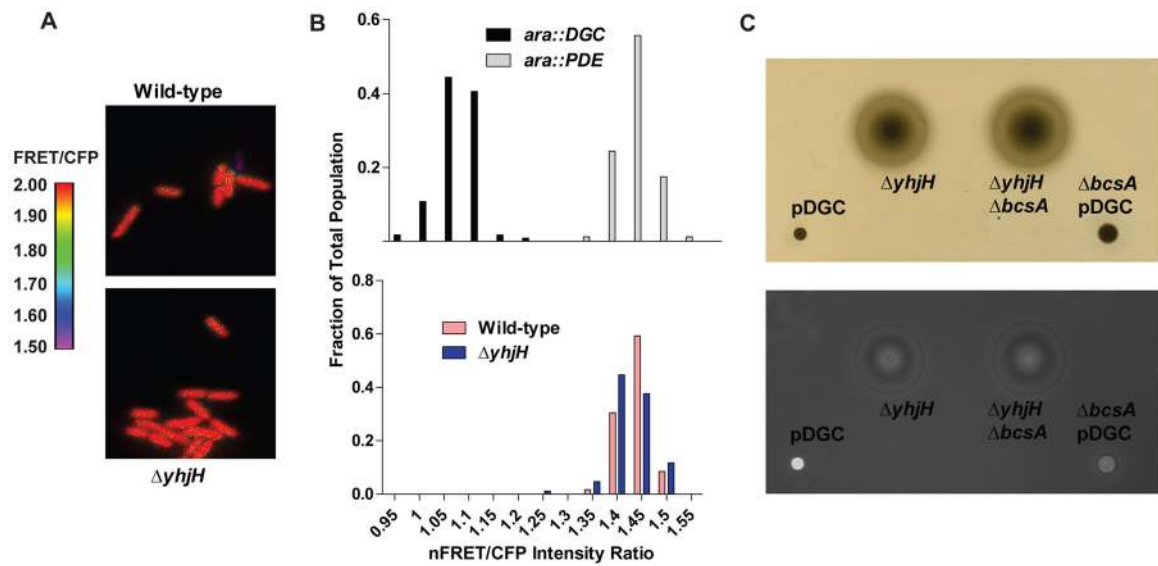


Fig. 7. A *S. Typhimurium* $\Delta yhjH$ strain does not demonstrate c-di-GMP binding to the BcsA PilZ domain or cellulose production

A. Dual-emission ratio microscopic images (FRET/CFP) of wild-type (top) or $\Delta yhjH$ mutant (bottom) *S. Typhimurium* expressing pBcsA-Spy. Pseudocolors represent emission ratios (527/480 nm) of the FRET-based biosensor as indicated by the figure legend to the left. B. Histogram showing the fraction of cells that demonstrate the indicated nFRET/CFP ratios for control strains expressing a DGC or a PDE off of an arabinose-inducible promoter (top) and either wild-type or $\Delta yhjH$ mutant strains (bottom). nFRET/CFP ratios are different than FRET/CFP ratios since nFRET intensities have been corrected for bleedthrough and fluorescence in the YFP channel. nFRET: net FRET intensity, calculated by subtracting bleedthrough coefficients and intensity of the YFP channel as detailed in Materials and Methods. The difference in nFRET/CFP ratios between the wild-type and the $\Delta yhjH$ is not significant ($P > 0.05$). C. Visible light (top) and UV (bottom) images of strains of *S. Typhimurium* on soft- agar motility plates with calcofluor, incubated at 37°C for 2 hours. This plate was supplemented with 100 mM IPTG.

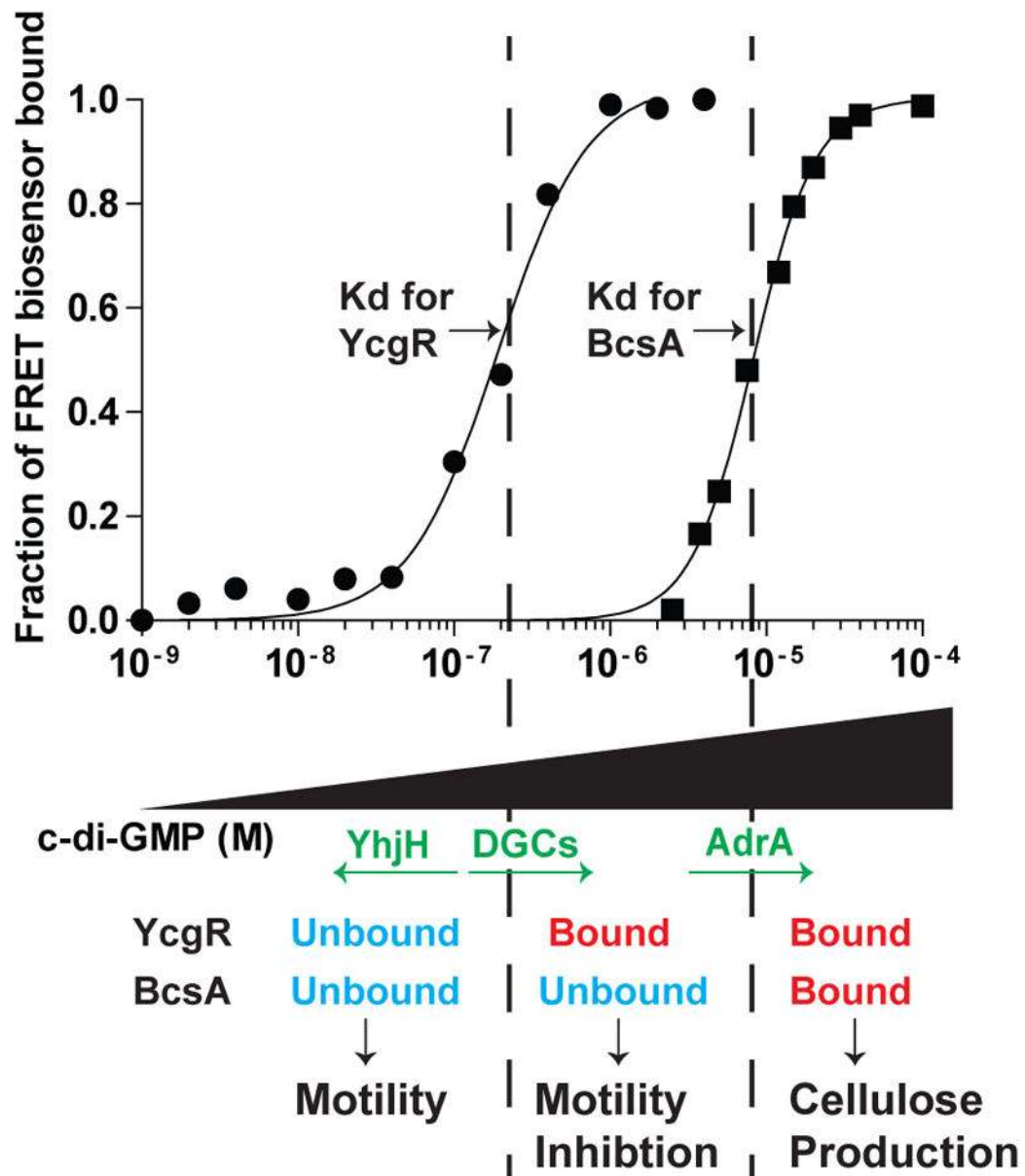


Fig. 8. The binding affinities of YcgR and BcsA for c-di-GMP determines cellular phenotypes
 When cellular c-di-GMP levels are kept low by PDEs such as YhjH, neither PilZ domain protein is bound to c-di-GMP, resulting in a motile cell that does not produce cellulose. As cellular DGCs increase the concentration of c-di-GMP past the K_d for YcgR, YcgR becomes bound to c-di-GMP and thus inhibits motility, even at the levels of c-di-GMP that are not high enough to bind BcsA. Activation of AdrA expression results in enough c-di-GMP being produced to bind to BcsA, and cellulose synthesis occurs.

Table 1

Affinities of c-di-GMP-binding molecules in selected bacteria

Protein	Binding Affinity (nM)		Binding Motif	Temp (°C)	Measurement Method	Reference
	This Work	Published				
<i>Salmonella Typhimurium</i>						
YcgR (STM1798)	191 ± 18	182 ± 29	PiZ	25	FRET, ³³ P-Labeled c-di-GMP	This work, Christen 2007
BcsA (STM3619)	8240 ± 238		PiZ	25	FRET	This work
<i>Pseudomonas aeruginosa</i>						
PA3353 site 1 ^a	88 ± 17		PiZ	25	FRET	This work
PA3353 site 2 ^a	732 ± 122	262 ± 66 ^b	PiZ	25, 20	FRET, surface plasmon res	This work, Duvel 2012
FimX (PA4959)		125	EAL	20	ITC	Navarro 2009
PA2989		288	PiZ	25	FRET	Christen 2010
PA0012		402 ± 39	PiZ	25	FRET	Christen 2010
PA4324		735 ± 474 ^c	PiZ	25	FRET	This work
PeID (PA3061)		1900	RXXD I-Site	20	ITC	Whitney 2012
PA2799		1998 ± 448	PiZ	25	FRET	Christen 2010
PA4608		6126 ± 525	PiZ	25	FRET	Christen 2010
Alg44 (PA3542)		8400	PiZ	25	FRET, ITC	This work, Merighi 2007
FleQ (PA1097)		15000–25000	Unknown	37	³³ P-Labeled c-di-GMP	Hickman 2008
PA2960		DNB ^d	Not Applicable	25	FRET, ITC	This work, Merighi 2007

^aPA3353 binding curves indicate two binding sites^bThe published affinity of this protein was measured at approximately 20°C, corresponding to ~530 nM at 25°C.^cThe dynamic range of FRET for PA4324 between bound and unbound states was small, resulting in a high error for the K_d of this protein^dDoes not bind to c-di-GMP