Published in final edited form as:
Environ Microbiol. 2011 March ; 13(3): 631–642. doi:10.1111/j.1462-2920.2010.02368.x.

Engineering a Novel c-di-GMP-Binding Protein for Biofilm Dispersal

Qun Ma[1], **Zhonghua Yang**[1,2], **Mingming Pu**[1], **Wolfgang Peti**[3], and **Thomas K. Wood**[1,4,5,*]

[1]Department of Chemical Engineering, 220 Jack E. Brown Building, Texas A & M University, College Station, TX 77843-3122

[2]College of Chemical Engineering and Technology, Wuhan University of Science and Technology, Wuhan 430081

[3]Department of Molecular Pharmacology, Physiology, and Biotechnology and Brown University, Providence, RI 02912

[4]Department of Biology, 220 Jack E. Brown Building, Texas A & M University, College Station, TX 77843-3122

[5]Department of Civil Engineering, 220 Jack E. Brown Building, Texas A & M University, College Station, TX 77843-3122

SUMMARY

Bacteria prefer to grow attached to themselves or an interface, and it is important for an array of applications to make biofilms disperse. Here we report simultaneously the discovery and protein engineering of BdcA (formerly YjgI) for biofilm dispersal using the universal signal 3,5-cyclic diguanylic acid (c-di-GMP). The *bdcA* deletion reduced biofilm dispersal, and production of BdcA increased biofilm dispersal to wild-type levels. Since BdcA increases motility and extracellular DNA production while decreasing exopolysaccharide, cell length, and aggregation, we reasoned that BdcA decreases the concentration of c-di-GMP, the intracellular messenger that controls cell motility through flagellar rotation and biofilm formation through synthesis of curli and cellulose. Consistently, c-di-GMP levels increase upon deleting *bdcA*, and purified BdcA binds c-di-GMP but does not act as a phosphodiesterase. Additionally, BdcR (formerly YjgJ) is a negative regulator of *bdcA*. To increase biofilm dispersal, we used protein engineering to evolve BdcA for greater c-di-GMP binding and found that the single amino acid change E50Q causes nearly complete removal of biofilms via dispersal without affecting initial biofilm formation.

Keywords

biofilm dispersal; c-di-GMP; YjgI

INTRODUCTION

Biofilm dispersal is the last stage of biofilm development in which cells detach from the biofilm and disperse into the environment (Kaplan, 2010). The ability to control biofilm formation is important, since bacteria in biofilms are responsible for most infectious diseases (Romero et al., 2008), and biofilms need to be controlled for engineering applications such as biocorrosion (Jayaraman et al., 1999). Dispersal occurs by two mechanisms: active

*Corresponding author: phone: 979-862-1588, Fax: 979-865-6446, Thomas.Wood@chemail.tamu.edu.

dispersal which is initiated by the bacteria themselves and passive dispersal mediated by external forces such as fluid shear and abrasion (Kaplan, 2010).

Biofilm dispersal occurs both in unfavorable and favorable conditions. As biofilms grow, cells inside the biofilm may not be able to access nutrients and may not be able to release toxic compounds quickly, so biofilm dispersal allows cells to escape from the unfavorable conditions. Hence, a change in environmental conditions (e.g., nutrition level and oxygen depletion) can lead to the removal of biofilms (Karatan and Watnick, 2009). For example, *Pseudomonas aeruginosa* biofilms undergo dispersal in response to a sudden decrease/increase of substrates (Hunt et al., 2004; Sauer et al., 2004). Even when conditions are favorable, cells may leave the biofilm and attach to a new surface, where they can make more colonies. Therefore, biofilm dispersal is important for the survival of the species as it allows the bacterial population to expand (Kaplan, 2010). For example, reproducible, periodic dispersal occurs in *Actinobacillus actinomycetemcomitans* (Kaplan, 2010), *Pseudomonas putida* (Gjermansen et al., 2010), and *Serratia marcescens* (Rice et al., 2005). In addition, for many pathogenic bacteria, biofilm dispersal plays a critical role in the transmission of bacteria from environmental reservoirs to human hosts, in the transmission of bacteria between hosts, and in the exacerbation and spread of infection within a single host (Kaplan, 2010).

Biofilms may be dispersed by several mechanisms including by (i) degrading or repressing production of adhesive components in the biofilm matrix, (ii) degrading the substrate on which the biofilm colony is growing, (iii) lysing a subpopulation of cells (e.g., phage-mediated cell lysis), (iv) inducing motility, (v) producing extracellular surfactants such as rhamnolipids, (vi) modulating fimbrial adherence, and (vii) increasing cell division at the outer surface of the biofilm colony (Kaplan, 2010). Proteins that increase biofilm removal upon their production include CsrA which represses synthesis of the adhesion poly-β-1,6-*N*-acetyl-D-glucosamine (Wang et al., 2005) in *Escherichia coli*, although it does not disperse biofilms in the presence of glucose (Jackson et al., 2002). Other proteins have been identified that, upon inactivation, prevent dispersal including DspB of *A. actinomycetemcomitans*, which degrades a linear polymer of *N*-acetylglucosamine (Kaplan et al., 2003), BdlA of *P. aeruginosa*, which decreases adhesiveness by decreasing 3,5-cyclic diguanylic acid (c-di-GMP) (Morgan et al., 2006), and AlpP of *Pseudoalteromonas tunicate*, which kills cells by producing hydrogen peroxide from L-lysine (Mai-Prochnow et al., 2008). In addition, NirS (Barraud et al., 2006) of *P. aeruginosa* produces, via BdlA, nitric oxide that is important for biofilm dispersal although nitric oxide alone removes 63% of biofilms but the combination of nitric oxide and chlorine can remove 85–90% of biofilms (Barraud et al., 2009).

Active dispersal based on motility is regulated by the universal intracellular signal c-di-GMP (Fig. 1A). c-di-GMP is synthesized by diguanylate cyclases, enzymes that are identified by a typical GGDEF motif, from two guanosine-5'-triphosphate molecules and degraded by phosphodiesterases (PDEs) which are characterized by an EAL or a HD-GYP domains (Dow et al., 2006). *E. coli* has 29 putative c-di-GMP related proteins, including 12 proteins with a GGDEF domain, 10 proteins with an EAL domain, and 7 proteins with both domains (Sommerfeldt et al., 2009). As an important intracellular signal, c-di-GMP affects many phenotypes including extracellular polysaccharide (EPS) production, biofilm formation, rugose colony development in *Vibrio vulnificus* (Nakhamchik et al., 2008) and *P. aeruginosa* (Ueda and Wood, 2009), and biofilm formation, cell length, and swimming motility in *E. coli* (Méndez-Ortiz et al., 2006).

In this study, we focused on creating an engineered protein that causes bacteria to disperse. Using a biofilm screen for uncharacterized genes related to biofilm formation and the

transport of the quorum-sensing (QS) signal autoinducer 2 (AI-2) (Herzberg et al., 2006), we identified that YjgI (renamed BdcA for biofilm dispersal via c-di-GMP) is a positive factor for removing biofilms. We explored the mechanism of how BdcA influences biofilm dispersal by a series of phenotype assays and determined that BdcA is a c-di-GMP-binding protein. In addition, we evolved BdcA by random mutagenesis and saturation mutagenesis of *bdcA* to obtain a more effective biofilm dispersal protein. Hence, we evolved the first protein for the enhanced biofilm dispersal, BdcA E50Q, resulting in nearly complete biofilm removal.

RESULTS

BdcA increases biofilm dispersal

To identify proteins that may be used to remove biofilms, we screened biofilm formation with knockout mutants for 32 uncharacterized genes whose expression is altered by a *tqsA* deletion (Herzberg et al., 2006) (Supplementary Table 1); TqsA is the putative exporter of QS signal AI-2, which enhances biofilm formation in *E. coli* (González Barrios et al., 2006). We found that the *bdcA* mutant decreased biofilm dispersal by 3 ± 1 fold compared with the wild-type strain in a static biofilm assay with 96-well polystyrene plates (Fig. 1B), which suggested BdcA controls biofilm removal. To corroborate this biofilm result with a more rigorous assay and to further investigate the function of BdcA under other conditions (e.g., glass surface and continuous flow), we performed a flow cell assay and again found the *bdcA* mutant showed 6 ± 2 fold decreased biofilm dispersal (Fig. 1C and Table 1). The lack of biofilm dispersal was complemented by expressing *bdcA* in trans (Fig. 1B); hence, BdcA increases biofilm dispersal.

BdcA increases cell motility and extracellular DNA (eDNA) while decreasing EPS production, cell size, and cell aggregation

To provide insights into the mechanism by which BdcA increases biofilm dispersal, we tested other biofilm-related phenotypes for the *bdcA* mutant and the *bdcA*-expressing strain. BdcA increased cell swimming motility by 2.2 ± 0.4 fold (Fig. 2A) and eDNA production by 3.05 ± 0.02 fold (Fig. 2B), while it decreased EPS production by 3 ± 2 fold (Fig. 2C), cell size by 2 ± 1 fold (Fig. 2D), and cell aggregation by 2.2 ± 0.7 fold (Fig. 2E). All five phenotypes seen upon producing BdcA are consistent with decreasing c-di-GMP concentrations (D'Argenio and Miller, 2004;Méndez-Ortiz et al., 2006;Nakhamchik et al., 2008;Ueda and Wood, 2010). Furthermore, the deletion of *bdcA* has the opposite effect on all these five phenotypes (Fig. 2), and production of BdcA from a plasmid complemented each of these phenotypes. Hence we hypothesized that BdcA may activate biofilm dispersal by decreasing the c-di-GMP concentrations in *E. coli*.

Importantly, four of the five phenotype changes found here (eDNA production, EPS production, cell aggregation, and cell size) were obtained with cells grown in biofilms. Planktonic cultures did not show differences in eDNA production, cell aggregation, and EPS production (cell size was not tested under this condition). This indicates that the physiological role of BdcA is strongly linked to biofilm formation.

BdcA binds c-di-GMP to control phenotypes

To confirm that BdcA can change the c-di-GMP level, we performed high performance liquid chromatography (HPLC) as previously described (Ueda and Wood, 2009) and measured the concentrations of c-di-GMP upon deleting *bdcA* and upon producing BdcA from plasmid pCA24N_*bdcA* via 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). We found that deleting *bdcA* increased the c-di-GMP concentration 8 fold compared to the wild-

strain and that producing BdcA decreased c-di-GMP concentrations back to the wild-type levels (Fig. 2F). Hence BdcA is directly or indirectly related to c-di-GMP levels in *E. coli*.

There are several ways BdcA may affect c-di-GMP concentrations. However, since BdcA has no DNA-binding motif and since there was no significant change in gene expression in the whole-transcriptome for the *bdcA* mutant vs. the wild-type strain (results not shown), BdcA is not likely to be a regulator that can change the transcription of c-di-GMP-related genes. Furthermore, since BdcA was annotated as putative enzyme, it seemed more plausible that BdcA controlled c-di-GMP concentrations as a phosphodiesterase or as a c-di-GMP-binding protein. Hence, we tested BdcA for PDE activity by purifying the His-tagged protein. HPLC indicated BdcA reduces c-di-GMP; however, we could not detect the traditional c-di-GMP degradation products 5′-phosphoguanylyl-(3′→5′)-guanosine (Rao et al., 2010) and guanosine monophosphate (GMP) (Schmidt et al., 2005) (Fig. 3). Therefore, BdcA decreases the concentration of c-di-GMP probably by binding rather than by catalyzing the degradation of c-di-GMP as a PDE. These results also show that no other effector is required for the binding of c-di-GMP by BdcA.

To prove that BdcA acts as a c-di-GMP-binding protein, not a PDE, we used ^{31}P nuclear magnetic resonance (NMR) spectroscopy since it shows a peak for c-di-GMP whether it is bound or unbound; hence, it may be used to quantify the amount of c-di-GMP after contact with BdcA. Using NMR, we found the amount of c-di-GMP was not altered by treatment with purified BdcA (Supplementary Fig. 1). Furthermore, BdcA was found to have a binding constant of 11.7 μM for c-di-GMP (Fig. 4), and unaltered c-di-GMP was recovered from BdcA after digestion with trypsin. Therefore, BdcA is not a PDE but instead alters phenotypes by changing c-di-GMP concentrations in the cell.

BdcR (YjgJ) regulates *bdcA*

We performed quantitative, reverse transcription-polymerase chain reaction (qRT-PCR) to check the transcription of *bdcA* in biofilms at different time points (from 2 h to 24 h). Gene expression of *bdcA* increases with incubation time and is maximum at 8 h (4.9 ± 0.3 fold more compared to 2 h) and decreases at 24 h (2.0 ± 0.2 fold less compared to 2 h). In addition, to investigate the regulation of *bdcA*, we measured *bdcA* transcription via qRT-PCR for the *yjgJ* mutant (*yjgJ* is upstream of *bdcA*) and the *yjgH* mutant (*yjgH* is downstream of *bdcA*) relative to the wild-type strain with 8 h cultures since this was the maximum for *bdcA* induction. *bdcA* expression in the *yjgJ* mutant was increased 7.3 ± 0.9 fold comparing to the value in the wild-type strain while *bdcA* expression in the *yjgH* mutant was not changed significantly (1.4 ± 0.2 fold vs. the wild-type strain). In addition, YjgJ is predicted to be a putative regulator with a helix-turn-helix motif, which is the most common DNA-binding motif in prokaryotic transcription factors (Ramos et al., 2005). Hence we conclude that the transcription of *bdcA* is time-dependent in biofilms and is controlled by a repressor YjgJ. We renamed the YjgJ as BdcR for its regulation of BdcA.

Protein engineering of BdcA for biofilm dispersal

After we indentified that BdcA reduces c-di-GMP concentrations, we used a novel protein engineering screen, based on higher motility (high motility indicates better dispersal due to reduction of c-di-GMP), to engineer superior biofilm dispersal. This screen was used rather than measuring biofilm removal directly, since it is much more efficient than a comparison of biofilms formed on two plates at two different time points.

Using random *bdcA* mutagenesis and expression of these mutant *bdcA* genes using BW25113 *bdcA*/pCA24N_*bdcA*, we screened ~6000 colonies for enhanced swimming motility. From the motility screen, we obtained 13 alleles with better motility and sequenced

them. Among them, the strain producing BdcA E50V showed 2.8 ± 0.4-fold higher swimming motility and 6 ± 2-fold increased biofilm dispersal in 96-well plates compared to native BdcA. With only one amino acid changed (E50V), the binding constant of BdcA for c-di-GMP was reduced from 11.7 μM (native BdcA) to 4.2 μM (BdcA E50V) (Fig. 4), which indicates the BdcA E50V variant has higher affinity for c-di-GMP.

Since a single mutation randomly placed in codons generates on average only 5.6 out of 19 possible substitutions (Rui et al., 2004), we performed saturation mutagenesis on the codon of E50 and screened for mutants with higher motility than the *bdcA*/BdcA E50V strain. Over 400 colonies were screened to ensure the probability of trying all the possibilities is nearly 100% (Rui et al., 2004). By substituting all 20 amino acids into this position, we obtained the *bdcA*/BdcA E50Q mutant with 2.0 ± 0.2-fold higher swimming motility and 2 ± 1-fold increased biofilm dispersal ability compared to E50V. The binding constant of BdcA E50Q was measured as 3.0 μM (Fig. 4), which is smaller than BdcA E50V, indicating a further improved binding affinity to c-di-GMP.

Improved biofilm dispersal for strains producing the BdcA E50V and BdcA E50Q protein engineering variants was confirmed using the flow cell assay (Fig. 1D and Table 1). After 33 h, biofilm formation was similar for the strains that lacked BdcA, that produce wild-type BdcA, that produce error-prone PCR (epPCR) variant BdcA E50V, and that produce saturation mutagenesis variant BdcA E50Q, indicating the four strains produce initially about the same amounts of biofilm. After IPTG induction for 18.5 h (42.5 h), the strains that produce BdcA E50V and BdcA E50Q showed 3.8 fold and 18-fold less biomass than the strain that produces native BdcA. This trend was consistent after IPTG induction for 27.5 h (51.5 h). Hence, the E50V and E50Q amino acid replacements (Supplementary Fig. 2) dramatically increased the ability of BdcA to cause biofilm dispersal, and biofilm formation is almost completely removed.

DISCUSSION

Protein engineering and recombinant engineering are promising strategies to control biofilm formation, but they have not been applied previously for enhancing biofilm dispersal. The first report for engineering biofilm formation via a genetic circuit used external stress from DNA-damaging agents to control the biofilm formation of *E. coli* (Kobayashi et al., 2004). Previously, we created the first synthetic circuit utilizing quorum-sensing signals to control biofilm formation by manipulating concentrations of the signal indole via toluene *o*-monooxygenase in a consortium of *Pseudomonas fluorescens* and *E. coli* (Lee et al., 2007). In addition, we controlled *E. coli* biofilm formation by evolving the quorum-sensing regulator SdiA; upon addition of the extracellular signal *N*-acylhomoserine lactone, biofilm formation was increased with SdiA variant 2D10, and SdiA variant 1E11 was created that reduced biofilm formation by increasing concentrations of the inhibitor indole (Lee et al., 2009). We also engineered the global regulator H-NS to control biofilm formation via prophage excision and cell death (Hong et al., 2010). These results showed that bacterial biofilm formation may be controlled by manipulating key regulatory proteins and enzymes. Here we created the first engineered protein for biofilm dispersal.

BdcA is predicted to be an oxidoreductase with a Rossmann fold to bind nucleotides (Gherardini et al., 2010). Unlike PDEs, BdcA does not have complete amino acid domains for enzymatic activity as it lacks R of EALXR for coordinating Mg^{2+}, lacks R of Q/R/D/D for c-di-GMP binding, lacks T of the T/E catalytic domain (Sommerfeldt et al., 2009), and lacks completely HD-GYP (Dow et al., 2006) for catalysis (Supplementary Fig. 2). BdcA is also smaller (237 aa) than most PDEs. Hence, BdcA does not catalyze c-di-GMP degradation. Intracellular measurements show that BdcA decreases c-di-GMP

concentrations, and the *in vitro* studies indicate BdcA directly reduces unbound c-di-GMP concentrations. Since ^{31}P NMR spectroscopy showed that c-di-GMP is not degraded, since the usual c-di-GMP degradation products were not detected, and since we recovered fully c-di-GMP from BdcA after trypsin digestion, we conclude that BdcA controls biofilm removal and many other biofilm-related phenotypes (motility, EPS, eDNA, cell length, and aggregation) through its direct binding of c-di-GMP. Also, our estimate is that upon producing BdcA from pCA24N_*bdcA*, BdcA levels are 200 fold greater than c-di-GMP on a molar basis; hence, binding of c-di-GMP by BdcA is reasonable to explain the phenotypes seen.

It is not necessary for BdcA to interact with other proteins to control these phenotypes; for example, by decreasing the concentration of free c-di-GMP, motility is enhanced by releasing the brake on motility via YcgR. YcgR reduces bacterial swimming by binding c-di-GMP and interacting with MotA to act as a brake that limits individual stator complexes (Boehm et al., 2010) as well as interacts with flagellar proteins FliG and FliM in the presence of c-di-GMP to reduce torque (Paul et al., 2010).

c-di-GMP also controls many other vital processes in bacteria; however, the mechanisms by which c-di-GMP affects these phenotypes are diverse and our understanding is not always complete. c-di-GMP riboswitches bind the second messenger to control complex physiological processes such as biofilm formation, virulence gene expression, and persistence of infection in *V. cholerae* (Kulshina et al., 2009). Alternatively, the transcriptional regulator VpsT in *V. cholerae* directly senses c-di-GMP by oligomerizing upon c-di-GMP binding to control extracellular matrix production, motility, and biofilm formation (Krasteva et al., 2010). LapD of *Pseudomonas fluorescens* regulates surface attachment via LapA by binding to intracellular c-di-GMP (Newell et al., 2009). Our results show we have identified a novel class of protein that controls cellular activity (i.e., biofilm dispersal) by binding c-di-GMP.

Without knowing the detailed mechanism about how c-di-GMP affects biofilm formation in *E. coli*, we evolved BdcA to create a better biofilm-dispersing protein. These results provide further evidence of the tight relationship between c-di-GMP and biofilm dispersal. The most important amino acid replacements occurred at the E50 position. By replacing glutamic acid with valine (E50V) and glutamine (E50Q), these one amino acid changes progressively increase the binding affinity of BdcA for c-di-GMP, which subsequently reduces, in a corresponding manner, the intracellular c-di-GMP concentrations. Supporting increased c-di-GMP binding, the E50 position is adjacent to the Q49 position of the remnant Q/R/D/D motif for c-di-GMP binding by PDEs.

With BdcA, we demonstrate that we can control the final stage in biofilm development, dispersal, by performing protein engineering on a single regulator. Therefore, although there are myriad genetic paths leading to biofilm formation, biofilm dispersal may be triggered via a single engineered protein. Furthermore, this work is promising in terms of broad applications since c-di-GMP is utilized by diverse bacteria (D'Argenio and Miller, 2004) and in nearly all these strains it controls motility; hence, reduction of c-di-GMP concentrations may be a universal mechanism for increasing bioiflm dispersal. Also, *bdcA* shows high sequence conservation with other species with the highest similarity to a homolg in *Shigella* sp.(98%). Furthermore, the genera *Klebsiella*, *Salmonella*, *Xanthomonas*, *Citrobacter*, *Sphingopyxis*, *Pantoea*, and *Roseomonas* show *bdcA* sequence conservation above 70%. In addition, 17 other bacteria show over 50% protein sequence identity with BdcA in the *E. coli* BW25113 strain (Supplementary Fig. 3). Thus, BdcA is well conserved in many bacteria and may be used for biofilm dispersal by many strains.

By discovering BdcA and creating the E50V and E50Q variants, we have also obtained important new tools that along with H-NS K57N (evolved to decrease biofilm formation through cell lysis by inducing cryptic prophage Rac) (Hong et al., 2010), SdiA 1E11 (evolved to decrease biofilm formation by increasing indole concentrations) (Lee et al., 2009), and SdiA SD10 (evolved to increase biofilm with the addition of homoserine lactone signals) (Lee et al., 2009), allow the control of biofilm formation for various applications. These applications include decreasing biocorrosion on carbon steel (Jayaraman et al., 1999) and performing biocatalysis for producing biofuels, chemicals, and food additives (Rosche et al., 2009). Therefore our study here shows the feasibility of controlling biofilm development via protein engineering using *E. coli* K-12 as a model organism. Extrapolating these results, one can imagine that with similar strategies, each stage of biofilm development may be controlled by synthetic biology using these and other engineered biofilm proteins. This would allow multi-species biofilms to be formed and dissolved and even controlled temporally and spatially (including at various depths and lengths) to expedite chemical transformations in biofilm reactors.

EXPERIMENTAL PROCEDURES

Bacterial strains, media, growth conditions, and growth rate assay

The strains and plasmids used in this study are listed in Table 2. *E. coli* K-12 BW25113 and its isogenic mutants (Baba et al., 2006) were obtained from the Genome Analysis Project in Japan. Plasmid pCA24N_*bdcA*, carrying *bdcA* under control of the $P_{T5\text{-}lac}$ promoter with tight regulation via the $lacI^q$ repressor, and the empty plasmid pCA24N were also obtained from the Genomic Analysis Project in Japan (Kitagawa et al., 2005). of *bdcA* was induced by 0.1 mM IPTG (Sigma, St. Louis, MO) unless otherwise indicated.

Luria-Bertani (LB) (Sambrook et al., 1989) and 37°C were used for all the experiments. Kanamycin (50 μg/mL) was used for pre-culturing the isogenic knock-outs. Chloramphenicol (30 μg/mL) was used for the strains harboring pCA24N and its derivatives, and 300 μg/mL erythromycin was used for pCM18.

Static biofilms for screening biofilm dispersal

Biofilm formation was assayed in 96-well polystyrene plates using 0.1% crystal violet staining (Corning Costar, Cambridge, MA) as previously described (Fletcher, 1977) with small modifications. Briefly, each well was inoculated with overnight cultures at an initial turbidity at 600 nm of 0.05 and grown without shaking for 19 h with 30 μg/mL chloramphenicol, then 0.1 mM IPTG was added to the culture to induce *bdcA*. The mixture was shaken at 150 rpm for 1 min and incubated for another 12 to 23 h (42 h total). Biofilm formation after 12 h of IPTG induction (31 h total) was used to establish the extent of mature biofilm formation. Biofilm dispersal was determined after 23 h of IPTG induction (43 h total). Comparison of these two values gave the percentage of biofilm dispersal. At least two independent cultures were used for each strain.

Flow cell biofilms and image analysis

The flow cell experiments were performed as previously described (Yang et al., 2008). pCM18 (Hansen et al., 2001) was used to produce the green fluorescent protein (GFP) for imaging each strain. The flow cells were inoculated with cultures at an initial turbidity at 600 nm of 0.05 at a flow rate of 10 mL/min for 2 h, and then fresh medium was added at 10 mL/min. For the wild-type strain and the *bdcA* mutant, biofilm images were taken after 24 h, 33 h, 42.5 h, 51.5 h, and 64.5 h. For *bdcA*/pCA24N, *bdcA*/pCA24N_*bdcA*, *bdcA*/BdcA E50V, and *bdcA*/BdcA E50Q, 0.5 mM IPTG was added to each flow cell system after 24 h, and images were taken 9 h, 18.5 h, and 27.5 h after IPTG addition (i.e., at 32, 42.5, and 51.5

total h). Biofilm images from nine random positions were taken and analyzed with Imaris confocal software (Bitplane, Zurich, Switzerland) and COMSTAT confocal software, respectively, as previously described (Yang et al., 2008).

EPS, swimming motility, aggregation, and eDNA assays

The amount of total EPS was determined as described previously (Zhang et al., 2008) with slight modifications. Briefly, cell cultures grown in 96-well plates for 24 h without shaking were collected and boiled in water for 10 min. The supernatant were then used for an anthrone-H_2SO_4 assay to determine EPS concentrations. This assay was performed with two independent cultures.

Swimming motility was performed as previously described (Sperandio et al., 2002). Single colonies or overnight cultures were used to inoculate the plates. The swimming halo was measured after 15 h. At least five plates were used for each independent culture, and two independent cultures were used for each strain.

Cell aggregation was assayed by comparing the cell turbidity at 600 nm near the surface of cultures in 96-well plates after 24 h of growth without shaking. This assay was performed with two independent cultures.

eDNA was measured as previously described (Ma and Wood, 2009) using cultures grown in 96-well plates for 24 h without shaking. This assay was performed with two independent cultures.

RNA isolation from biofilms

To analyze differential gene expression for the *bdcA* mutant *vs*. the wild-type strain in biofilms, overnight cultures (2.5 mL) of the wild-type strain and the *bdcA* mutant were inoculated into 250 mL of LB medium with 10 g glass wool (Corning Glass Works, Corning, NY). After 15 h, the biofilm cells on the glass wool were collected as previously described (Ren et al., 2004a). To determine temporal *bdcA* expression, overnight cultures of the wild-type strain were inoculated into 130 mL of LB medium with 5 g of glass wool, and biofilm cells were collected from the glass wool after 2 h, 4 h, 8 h, 15 h, and 24 h. For the qRT-PCR for *bdcA* expression with the *yjgJ* deletion and the *yjgH* deletion, overnight cultures of the wild-type strain, the *yjgJ* mutant, and the *yjgH* mutant were inoculated into 130 mL LB medium with 5 g glass wool, and biofilm cells were collected after 8 h incubation. Cell pellets were resuspended in RNA*later* (Ambion Inc., Austin, TX), and total RNA was isolated using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) (Ren et al., 2004a).

Whole-transcriptome analysis

The *E. coli* GeneChip Genome 2.0 array (Affymetrix, P/N 900551) was used, and cDNA synthesis, fragmentation, and hybridizations were performed as described previously (González Barrios et al., 2006). If the gene with the larger transcription rate did not have a consistent transcription rate based on the 11–15 probe pairs (*P*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *P*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and if their fold change is higher than standard deviation for the whole genome (Ren et al., 2004b). The expression data were deposited in the NCBI Gene Expression Omnibus and are accessible through accession number GSE22057.

Quantification of c-di-GMP

c-di-GMP was quantified using HPLC as described previously (Ueda and Wood, 2009). Strains were grown from overnight cultures in 1 L of medium with 30 μg/mL chloramphenicol and 0.1 mM IPTG for 24 h without shaking. A photodiode array detector (Waters, Milford, MA) was used to detect nucleotides at 254 nm after the HPLC separation step. Commercial c-di-GMP (BIOLOG Life Science Institute, Bremen, Germany) was used as the standard. The c-di-GMP peak was verified by spiking the *bdcA*/pCA24N sample with the commercial c-di-GMP. This experiment was performed with two independent cultures.

PDE assay

PDE activity was assayed as previously described (Schmidt et al., 2005). His-tagged proteins (BdcA, BdcA E50V, and BdcA E50Q) were purified with Ni-NTA agarose (Qiagen) and contacted with 5 to 80 μM c-di-GMP in PDE assay buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, pH ~6.0) for 0.5 h and 1 h. The reaction was stopped by heating at 95°C for 5 min after the addition of 10 mM $CaCl_2$. After centrifugation, the c-di-GMP concentration in the supernatant was analyzed by HPLC. Phosphodiesterase YahA from *E. coli* was used as a positive control and to generate the 5′-phosphoguanylyl-(3′→5′)-guanosine (Schmidt et al., 2005) from c-di-GMP. Guanosine monophosphate was obtained from Sigma. These data also provided rough estimates of the binding constants.

^{31}P NMR

BdcA E50Q (5 μM) was incubated with 200 μM c-di-GMP for 8 h in PDE assay buffer. The ^{31}P NMR spectrum was obtained using a Varian INOVA 400 spectrometer and a broad band probe (proton decoupled, acquisition time 1.6 s, first delay 1.0 s, 90° pulse width 8.5 μs, line broadening 2 Hz, and number of transients 2000). 85% phosphoric acid was used as an external standard for the chemical shift 0 ppm. The ^{31}P NMR spectrum for GMP was obtained in the same manner.

c-di-GMP binding assays

Purified His-tagged proteins (10 μM) were incubated with 0.5 to 20 μM c-di-GMP for 0.5 h. Free c-di-GMP and BdcA-bound c-di-GMP were separated using a 10 kDa protein filter unit (Millipore, Cork, Ireland). The amount of free c-di-GMP for each sample was measured with HPLC (Schmidt et al., 2005). BdcA-bound c-di-GMP was recovered using 1.6 μg trypsin (Agilent Technologies, Inc., Santa Clara, CA) for 16 h to remove the protein and release c-di-GMP from BdcA. The released c-di-GMP was measured via HPLC to confirm the c-di-GMP was bound to the protein.

Random mutagenesis and saturation mutagenesis

bdcA expressed from pCA24N_*bdcA* was mutated by epPCR using primers epPCR-f and epPCR-r (Supplementary Table 2). A 100 μL reaction contained 7.5 mM $MgCl_2$, 0.7 mM $MnCl_2$, 1 M betaine, 100 ng template DNA, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 5U *Taq* DNA polymerase (New England Biolabs, Beverly, MA), and 0.3 μM of each primer in 1X epPCR buffer (Sigma). The PCR program was set as 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension step of 72°C for 7 min. The epPCR product was cloned into pCA24N_*bdcA* using restriction enzymes BseRI and HindIII after treating the plasmid with Antarctic phosphatase (New England Biolabs). The ligation mixture was electroporated into BW25113 *bdcA*. Screening based on swimming motility was performed by inoculating single colonies into 150 × 15 mm 0.3% agar plates (1% tryptone, 0.25% NaCl, and 0.3% agar) with autoclaved toothpicks. At least one control sample (*bdcA*/pCA24N_*bdcA*) was used on each plate (no

IPTG was required for induction of *bdcA*). The plates were incubated for 15 h, and colonies with increased motility were selected for a second round of screening based on motility. Plasmids were isolated, re-electroporated into BW25113 *bdcA*, and the best strains were re-tested for motility to ensure the changes in motility were due to mutated *bdcA* rather than chromosomal changes. Biofilm dispersal ability was then tested with these mutants. Over 6000 colonies were screened in this experiment. Plasmids that resulted in improved BdcA activity were sequenced by a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Saturation mutagenesis of *bdcA* at the E50 codon was performed in a 50 μL system with 125 ng of primers BdcASM-f and BdcASM-r (Supplementary Table 2), 1 μL dNTP mix, 2.5 U *Pfu* polymerase, 30 ng template plasmid, and 1X *Pfu* reaction buffer (10 mM KCl, 6 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% triton X-100, and 0.1 mg/mL BSA). The PCR program was 95°C for 1 min, followed by 16 cycles of 95°C for 50 sec, 60°C for 1 min, and 68°C for 6 min, with a final extension step of 68°C for 6 min. The PCR product was treated with 10 U *Dpn* I restriction enzyme (New England Biolabs) for 3 h, and the mixture was directly used for electroporation. PCR Screening was based on motility.

Electron microscopy

Electron microscopy was performed using cultures grown in 96-well plates for 24 h without shaking. Each sample was diluted in LB to a turbidity at 600 nm of 0.2–0.5, stained in ammonium molybdate, and checked under JEOL 1200EX electron microscopy. This assay was performed with two independent cultures.

qRT-PCR

qRT-PCR was performed as previously described (Ma and Wood, 2009). Primers used are listed in Supplementary Table 2 as *bdcA*-rt-f and *bdcA*-rt-r. The housekeeping gene *rrsG* was used as the internal reference, and the annealing temperature was 60°C.

Protein modeling

The three-dimensional model was obtained using the SWISS-MODEL server (http://swissmodel.expasy.org/) (Peitsch, 1995; Arnold et al., 2006; Kiefer et al., 2009) using L-xylulose reductase from *E. coli* (PDB 3d3w), which has 24% sequence identity. The protein image was made with PyMOL (http://pymol.sourceforge.net/).

Phylogenetic tree construction

The phylogenetic tree was constructed with the DNASTAR-Lasergene MegAlign software (DNASTAR, Madison, WI). The Kimura distance formula was used to calculate distance values. The values computed are the mean number of differences per site and fall between 0–1. Zero represents complete identity and 1 represent no identity. The phylogenetic tree scale uses these values multiplied by 100. The bootstrap analysis was performed with number of trials as 1000 and random seed as 111.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (R01 EB003872 and R01 GM089999). We thank the National Institute of Genetics in Japan for providing the Keio and ASKA strains and Dr. Christos Savva in the Microscopy & Imaging Center of Texas A&M University for his help with electron microscopy.

REFERENCES

Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 2006;22:195–201. [PubMed: 16301204]

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2006;2 2006 0008.

Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. J Bacteriol 2006;188:7344–7353. [PubMed: 17050922]

Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, Kjelleberg S. Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. Microb. Biotechnol 2009;2:370–378. [PubMed: 21261931]

Boehm A, Kaiser M, Li H, Spangler C, Kasper CA, Ackermann M, et al. Second messenger-mediated adjustment of bacterial swimming velocity. Cell 2010;141:107–116. [PubMed: 20303158]

D'Argenio DA, Miller SI. Cyclic di-GMP as a bacterial second messenger. Microbiology 2004;150:2497–2502. [PubMed: 15289546]

Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 2000;97:6640–6645. [PubMed: 10829079]

Dow JM, Fouhy Y, Lucey JF, Ryan RP. The HD-GYP domain, cyclic di-GMP signaling, and bacterial virulence to plants. Mol Plant Microbe Interact 2006;19:1378–1384. [PubMed: 17153922]

Fletcher M. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Canadian Journal of Microbiology 1977;23:1–6.

Gherardini PF, Ausiello G, Russell RB, Helmer-Citterich M. Modular architecture of nucleotide-binding pockets. Nucleic Acids Res 2010;38:3809–3816. [PubMed: 20185567]

Gjermansen M, Nilsson M, Yang L, Tolker-Nielsen T. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms: genetic elements and molecular mechanisms. Mol Microbiol 2010;75:815–826. [PubMed: 19602146]

González Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE, Wood TK. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). J Bacteriol 2006;188:305–316. [PubMed: 16352847]

Hansen MC, Palmer RJ Jr, Udsen C, White DC, Molin S. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. Microbiology 2001;147:1383–1391. [PubMed: 11320140]

Herzberg M, Kaye IK, Peti W, Wood TK. YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. J Bacteriol 2006;188:587–598. [PubMed: 16385049]

Hong SH, Wang X, Wood TK. Controlling biofilm formation, prophage excision and cell death by rewiring global regulator H-NS of *Escherichia coli*. Microb. Biotechnol 2010;3:344–356. [PubMed: 21255333]

Hunt SM, Werner EM, Huang B, Hamilton MA, Stewart PS. Hypothesis for the role of nutrient starvation in biofilm detachment. Appl Environ Microbiol 2004;70:7418–7425. [PubMed: 15574944]

Jackson DW, Suzuki K, Oakford L, Simecka JW, Hart ME, Romeo T. Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. J Bacteriol 2002;184:290–301. [PubMed: 11741870]

Jayaraman A, Mansfeld FB, Wood TK. Inhibiting sulfate-reducing bacteria in biofilms by expressing the antimicrobial peptides indolicidin and bactenecin. J. Ind. Microbiol. Biotechnol 1999;22:167–175.

Kaplan JB. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. J Dent Res 2010;89:205–218. [PubMed: 20139339]

Kaplan JB, Ragunath C, Ramasubbu N, Fine DH. Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous β-hexosaminidase activity. J Bacteriol 2003;185:4693–4698. [PubMed: 12896987]

Karatan E, Watnick P. Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiol Mol Biol Rev 2009;73:310–347. [PubMed: 19487730]

Kiefer F, Arnold K, Künzli M, Bordoli L, Schwede T. The SWISS-MODEL Repository and associated resources. Nucleic Acids Res 2009;37:D387–D392. [PubMed: 18931379]

Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H. Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. DNA Res 2005;12:291–299. [PubMed: 16769691]

Kobayashi H, Kærn M, Araki M, Chung K, Gardner TS, Cantor CR, Collins JJ. Programmable cells: interfacing natural and engineered gene networks. Proc Natl Acad Sci U S A 2004;101:8414–8419. [PubMed: 15159530]

Krasteva PV, Fong JC, Shikuma NJ, Beyhan S, Navarro MVAS, Yildiz FH, Sondermann H. *Vibrio cholerae* VpsT regulates matrix production and motility by directly sensing cyclic di-GMP. Science 2010;327:866–868. [PubMed: 20150502]

Kulshina N, Baird NJ, Ferré-D'Amaré AR. Recognition of the bacterial second messenger cyclic diguanylate by its cognate riboswitch. Nat Struct Mol Biol 2009;16:1212–1217. [PubMed: 19898478]

Lee J, Jayaraman A, Wood TK. Indole is an inter-species biofilm signal mediated by SdiA. BMC Microbiol 2007;7:42. [PubMed: 17511876]

Lee J, Maeda T, Hong SH, Wood TK. Reconfiguring the quorum-sensing regulator SdiA of *Escherichia coli* to control biofilm formation via indole and *N*-acylhomoserine lactones. Appl Environ Microbiol 2009;75:1703–1716. [PubMed: 19168658]

Ma Q, Wood TK. OmpA influences *Escherichia coli* biofilm formation by repressing cellulose production through the CpxRA two-component system. Environ Microbiol 2009;11:2735–2746. [PubMed: 19601955]

Mai-Prochnow A, Lucas-Elio P, Egan S, Thomas T, Webb JS, Sanchez-Amat A, Kjelleberg S. Hydrogen peroxide linked to lysine oxidase activity facilitates biofilm differentiation and dispersal in several gram-negative bacteria. J Bacteriol 2008;190:5493–5501. [PubMed: 18502869]

Méndez-Ortiz MM, Hyodo M, Hayakawa Y, Membrillo-Hernández J. Genome-wide transcriptional profile of *Escherichia coli* in response to high levels of the second messenger 3',5'-cyclic diguanylic acid. J Biol Chem 2006;281:8090–8099. [PubMed: 16418169]

Morgan R, Kohn S, Hwang SH, Hassett DJ, Sauer K. BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. J Bacteriol 2006;188:7335–7343. [PubMed: 17050921]

Nakhamchik A, Wilde C, Rowe-Magnus DA. Cyclic-di-GMP regulates extracellular polysaccharide production, biofilm formation, and rugose colony development by *Vibrio vulnificus*. Appl Environ Microbiol 2008;74:4199–4209. [PubMed: 18487410]

Newell PD, Monds RD, O'Toole GA. LapD is a bis-(3',5')-cyclic dimeric GMP-binding protein that regulates surface attachment by *Pseudomonas fluorescens* Pf0-1. Proc Natl Acad Sci U S A 2009;106:3461–3466. [PubMed: 19218451]

Paul K, Nieto V, Carlquist WC, Blair DF, Harshey RM. The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a "backstop brake" mechanism. Mol Cell 2010;38:128–139. [PubMed: 20346719]

Peitsch MC. Protein Modeling by E-mail. Nature biotechnology 1995;13:658–660.

Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, Zhang X, et al. The TetR family of transcriptional repressors. Microbiol Mol Biol Rev 2005;69:326–356. [PubMed: 15944459]

Rao F, See RY, Zhang D, Toh DC, Ji Q, Liang ZX. YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. J Biol Chem 2010;285:473–482. [PubMed: 19901023]

Ren D, Bedzyk LA, Thomas SM, Ye RW, Wood TK. Gene expression in *Escherichia coli* biofilms. Appl Microbiol Biotechnol 2004a;64:515–524. [PubMed: 14727089]

Ren D, Bedzyk LA, Ye RW, Thomas SM, Wood TK. Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*. Biotechnol Bioeng 2004b;88:630–642. [PubMed: 15470704]

Rice SA, Koh KS, Queck SY, Labbate M, Lam KW, Kjelleberg S. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. J Bacteriol 2005;187:3477–3485. [PubMed: 15866935]

Romero R, Schaudinn C, Kusanovic JP, Gorur A, Gotsch F, Webster P, et al. Detection of a microbial biofilm in intraamniotic infection. Am J Obstet Gynecol 2008;198:135.e1–135.e5. [PubMed: 18166328]

Rosche B, Li XZ, Hauer B, Schmid A, Buehler K. Microbial biofilms: a concept for industrial catalysis? Trends Biotechnol 2009;27:636–643. [PubMed: 19783314]

Rui L, Kwon YM, Fishman A, Reardon KF, Wood TK. Saturation mutagenesis of toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4 for enhanced 1-naphthol synthesis and chloroform degradation. Appl Environ Microbiol 2004;70:3246–3252. [PubMed: 15184118]

Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.

Sauer K, Cullen MC, Rickard AH, Zeef LA, Davies DG, Gilbert P. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J Bacteriol 2004;186:7312–7326. [PubMed: 15489443]

Schmidt AJ, Ryjenkov DA, Gomelsky M. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. J Bacteriol 2005;187:4774–4781. [PubMed: 15995192]

Sommerfeldt N, Possling A, Becker G, Pesavento C, Tschowri N, Hengge R. Gene expression patterns and differential input into curli fimbriae regulation of all GGDEF/EAL domain proteins in *Escherichia coli*. Microbiology 2009;155:1318–1331. [PubMed: 19332833]

Sperandio V, Torres AG, Kaper JB. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. Mol Microbiol 2002;43:809–821. [PubMed: 11929534]

Ueda A, Wood TK. Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). PLoS Pathog 2009;5:e1000483. [PubMed: 19543378]

Ueda A, Wood TK. Tyrosine phosphatase TpbA of *Pseudomonas aeruginosa* controls extracellular DNA via cyclic diguanylic acid concentrations. Environ. Microbiol. Reports 2010;2:449–455.

Wang X, Dubey AK, Suzukl K, Baker CS, Babltzke P, Romeo T. CsrA post-transcriptionally represses *pgaABCD*, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. Mol Microbiol 2005;56:1648–1663. [PubMed: 15916613]

Yang X, Ma Q, Wood TK. The R1 conjugative plasmid increases *Escherichia coli* biofilm formation through an envelope stress response. Appl Environ Microbiol 2008;74:2690–2699. [PubMed: 18344336]

Zhang XS, García-Contreras R, Wood TK. *Escherichia coli* transcription factor YncC (McbR) regulates colanic acid and biofilm formation by repressing expression of periplasmic protein YbiM (McbA). ISME J 2008;2:615–631. [PubMed: 18309357]

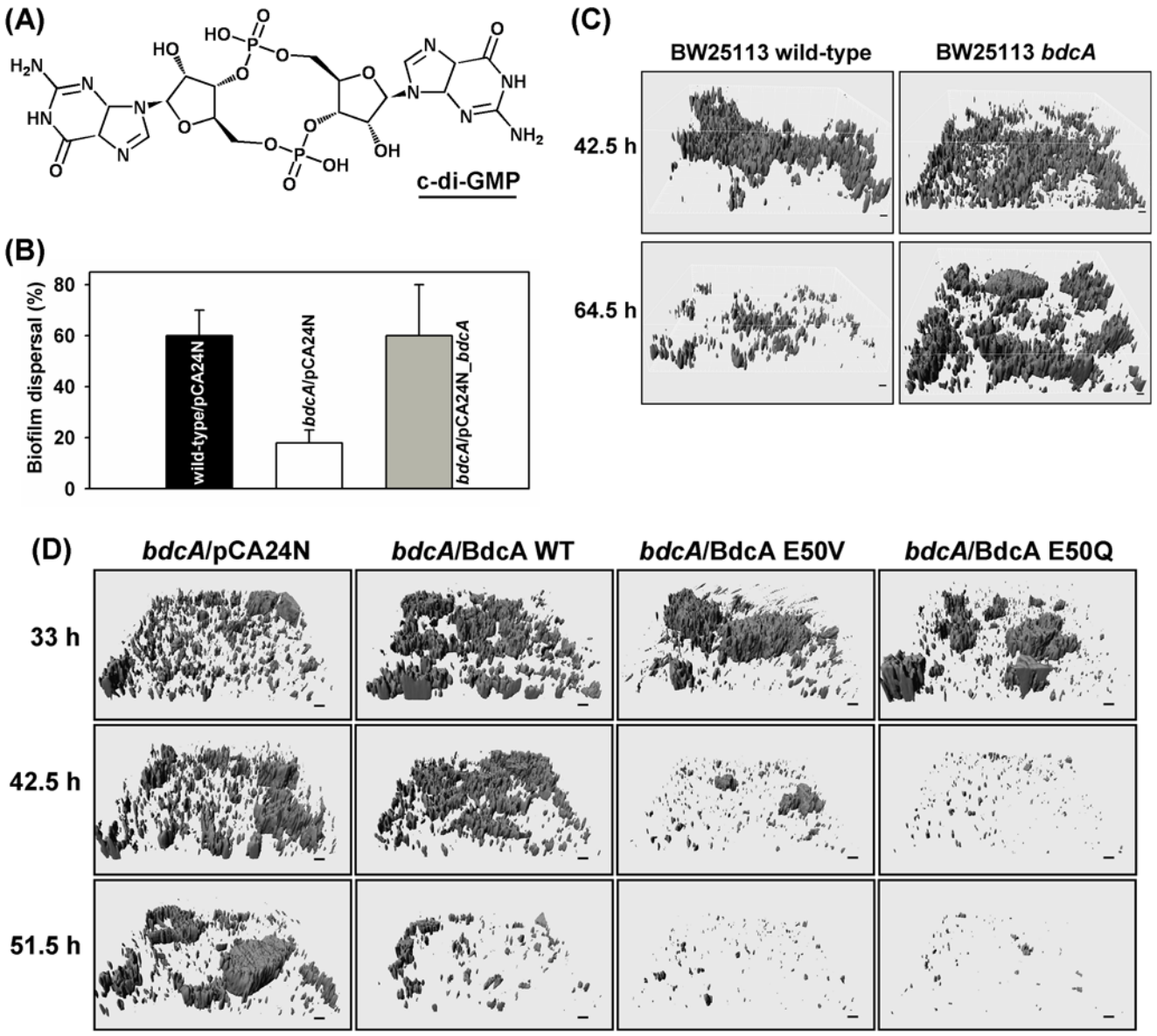

Fig. 1. BdcA increases biofilm dispersal

Chemical structure of c-di-GMP **(A)**. Relative normalized biofilm dispersal after 42 h with static biofilms formed in 96-well polystyrene plates **(B)**. Biofilms were formed with LB and 30 μg/mL chloramphenicol at 37°C using BW25113/pCA24N, *bdcA*/pCA24N, and *bdcA*/pCA24N_*bdcA*. IPTG (0.1 mM) was added to each strain after 19 h of incubation. Biofilm formation after 23 h of IPTG induction (42 h total) is compared to the biofilm formation after 12 h of IPTG induction (31 h total) to obtain biofilm dispersal. Data are the average of 12 replicate wells from two independent cultures, and one standard deviation is shown. Representative Imaris images of flow cell biofilms after 42.5 h and 64.5 h of incubation with LB medium **(C)**. Each strain has pCM18 for producing GFP to visualize the biofilms, and erythromycin (300 μg/mL) was added to retain pCM18. Imaris images of flow cell biofilms of BW25113 *bdcA* producing evolved BdcA from pCA24N **(D)**. After forming biofilms in LB for 24 hr, IPTG (0.5 mM) was added to induce BdcA production for 9 h (33 h total), 18.5 h (42.5 h total), and 27.5 h (51.5 h). Each strain has the pCM18 plasmid for producing

GFP. Chloramphenicol (30 μg/mL) was used to retain the pCA24N-based plasmids, and erythromycin (300 μg/mL) was used to retain pCM18. BdcA WT indicates native BdcA, BdcA E50V is the epPCR evolved BdcA, BdcA E50Q is the best saturation mutant. Scale bars represent 10 μm.

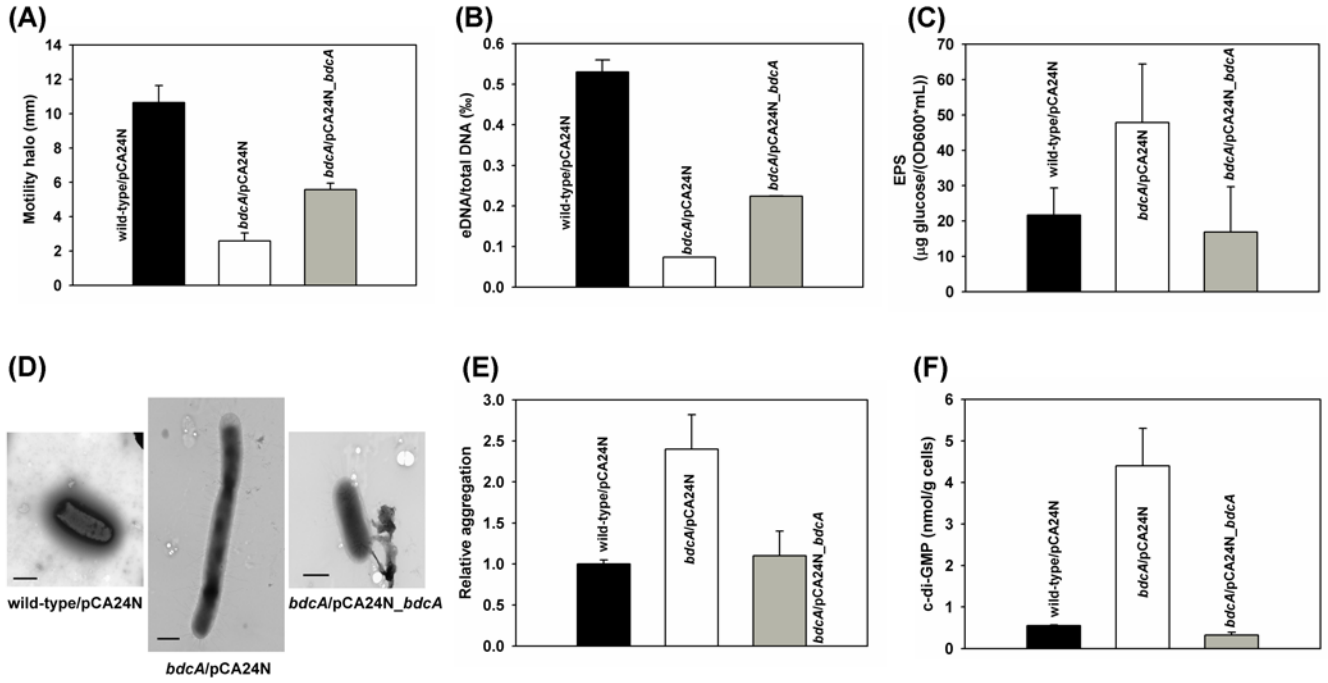

Fig. 2. BdcA binds c-di-GMP to alter swimming, eDNA, EPS, cell morphology, and aggregation

Swimming motility after 15 h of growth on motility agar plates **(A)**. eDNA production after 24 h in 96-well plates **(B)**. EPS production after 24 h in 96-well plates **(C)**. Cell length after 24 h in 96-well plates **(D)**. Cell aggregation after 24 h in 96-well plates **(E)**. c-di-GMP concentration after 24 h (static cultures) **(F)**. All experiments were conducted in LB medium at 37°C. *bdcA* was induced from pCA24N_*bdcA* via 0.1 mM IPTG, and 30 μg/mL chloramphenicol was used to retain the pcA24N-based plasmids. Data are the average of at least two independent cultures, and one standard deviation is shown.

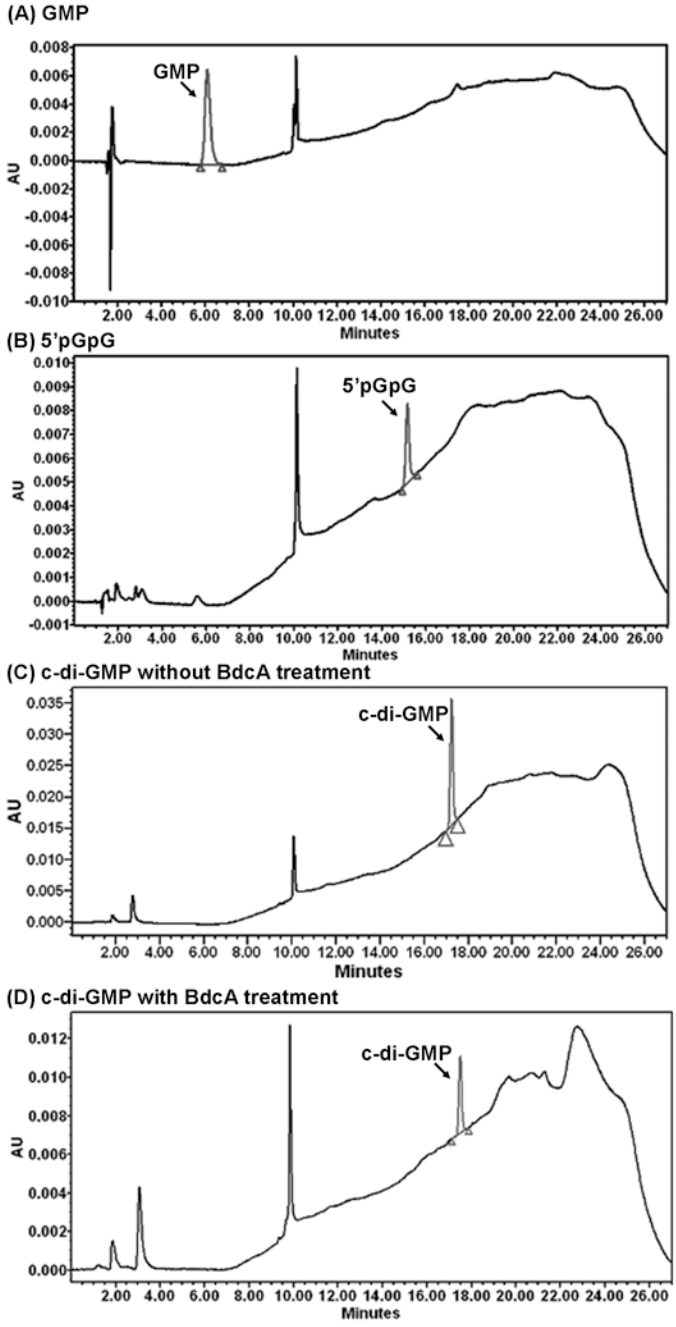

Fig. 3. BdcA decreases free intracellular c-di-GMP

HPLC chromatogram for 10 μM guanosine monophosphate (GMP) **(A)**. HPLC chromatogram for 2.5 μM 5′-phosphoguanylyl-(3′→5′)-guanosine (5'pGpG) produced by treating 2.5 μM c-di-GMP with 2 μM of known phosphodiesterase YahA for 2 h at 37°C **(B)**. HPLC chromatogram for 10 μM c-di-GMP **(C)**. HPLC chromatogram after treating 10 μM c-di-GMP with 5 μM BdcA for 2 h at 37°C **(D)**.

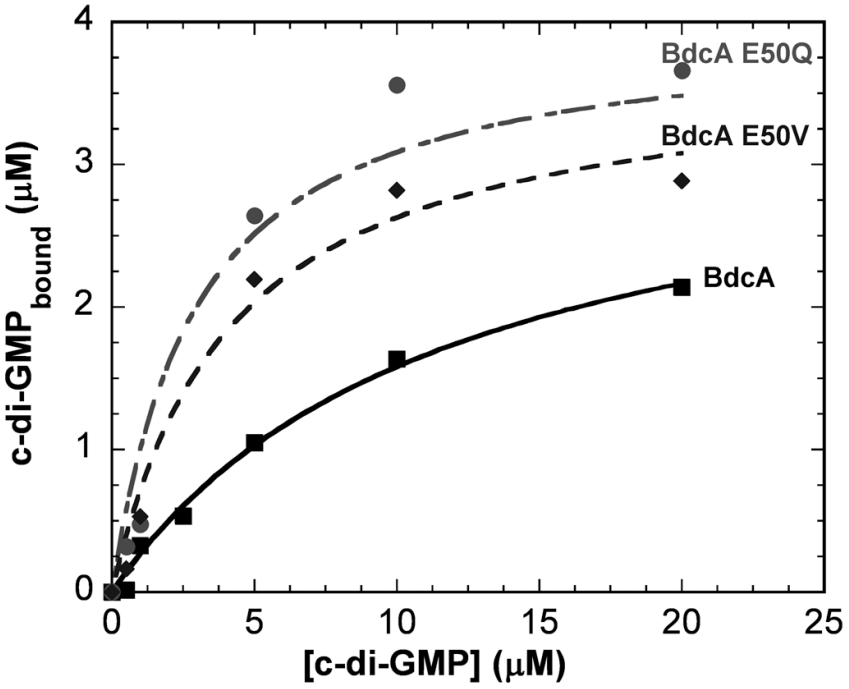

Fig. 4. BdcA binds c-di-GMP

Binding curves for 10 μM BdcA, BdcA E50V, and BdcA E50Q with 0.5 to 20 μM c-di-GMP.

Table 1

Flow cell statistical analysis of biofilms via COMSTAT for biofilms formed in LB at 37°C. For the wild-type strain and the *bdcA* mutant, cultures were grown in LB for 24 h, 33 h, 42.5 h, 51.5 h, and 64.5 h. For *bdcA*/pCA24N, *bdcA*/BdcA, *bdcA*/BdcA E50V, and *bdcA*/BdcA E50Q, cultures were grown in LB with 30 μg/mL chloramphenicol for 24 h, then *bdcA* expression was induced by adding 0.5 mM IPTG. Data were collected at 33 h, 42.5 h, and 51.5 h. BdcA is the native protein, BdcA E50V is the epPCR variant, and BdcA E50Q is the saturation mutagenesis variant.

COMSTAT values	Time	wild-type	*bdcA*	*bdcA*/pCA24N	*bdcA*/BdcA	*bdcA*/BdcA E50V	*bdcA*/BdcA E50Q
Biomass ($\mu m^3/\mu m^2$)	24 h	0.5 ± 0.8	0.4 ± 0.4				
	33 h	2 ± 3	2 ± 4	3 ± 3	3 ± 2	4 ± 3	4 ± 2
	42.5 h	2 ± 4	3 ± 2	4 ± 2	3 ± 1	0.8 ± 0.5	0.17 ± 0.07
	51.5 h	0.4 ± 0.3	2 ± 1	3.7 ± 0.8	1.7 ± 0.8	0.2 ± 0.3	0.16 ± 0.08
	64.5 h	0.3 ± 0.2	3 ± 2				
Surface coverage (%)	24 h	5 ± 2	5 ± 2				
	33 h	6 ± 6	8 ± 3	10 ± 3	13 ± 6	13 ± 7	11 ± 5
	42.5 h	5 ± 5	15 ± 9	13 ± 5	12 ± 4	5 ± 2	2.2 ± 0.7
	51.5 h	3 ± 1	14 ± 6	12 ± 4	10 ± 5	2 ± 1	3.0 ± 0.9
	64.5 h	2.6 ± 0.9	18 ± 9				
Average thickness (μm)	24 h	1 ± 1	0.8 ± 0.8				
	33 h	3 ± 5	3 ± 6	4 ± 4	5 ± 4	5 ± 3	4 ± 2
	42.5 h	4 ± 7	4 ± 3	5 ± 2	4 ± 2	1.1 ± 0.6	0.2 ± 0.1
	51.5 h	0.6 ± 0.4	3 ± 2	4 ± 1	2.3 ± 0.9	0.3 ± 0.4	0.2 ± 0.1
	64.5 h	0.5 ± 0.3	3 ± 2				
Roughness coefficient	24 h	1.89 ± 0.07	1.8 ± 0.1				
	33 h	1.8 ± 0.3	1.7 ± 0.2	1.6 ± 0.1	1.5 ± 0.2	1.5 ± 0.2	1.7 ± 0.1
	42.5 h	1.7 ± 0.3	1.5 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	1.80 ± 0.07	1.92 ± 0.02
	51.5 h	1.89 ± 0.05	1.5 ± 0.1	1.60 ± 0.06	1.6 ± 0.2	1.91 ± 0.06	1.89 ± 0.04
	64.5 h	1.90 ± 0.04	1.5 ± 0.2				

Table 2

E. coli strains and plasmids used in this study. Km^r, Cm^r, and Em^r denote kanamycin, chloramphenicol, and erythromycin resistance, respectively.

Strain/Plasmid	Genotype	Source
Strain		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	(Datsenko and Wanner, 2000)
BW25113 *bdcA*	BW25113 Δ*bdcA* Ω Km^r	(Baba et al., 2006)
Plasmid		
pCA24N	Cm^r; *lacI*^{q}, pCA24N	(Kitagawa et al., 2005)
pCA24N_*bdcA*	Cm^r; *lacI*^{q}, pCA24N $p_{T5\text{-}lac}$::*bdcA*	(Kitagawa et al., 2005)
pCA24N_*yahA*	Cm^r; *lacI*^{q}, pCA24N $p_{T5\text{-}lac}$::*yahA*	(Kitagawa et al., 2005)
pCM18	Em^r; pTRKL2-P_{CP25}RBSII-*gfp*mut3*-T_0–T_1	(Hansen et al., 2001)