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Engineering a novel c-di-GMP-binding protein for biofilm dispersal

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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 Yjgl) 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 level. 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.

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 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 unfavourable and favourable 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 unfavourable 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 favourable, 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

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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). *Escherichia 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, and 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) (Table S1); 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 described previously (Ueda and Wood, 2009) and measured the concentrations of c-di-GMP

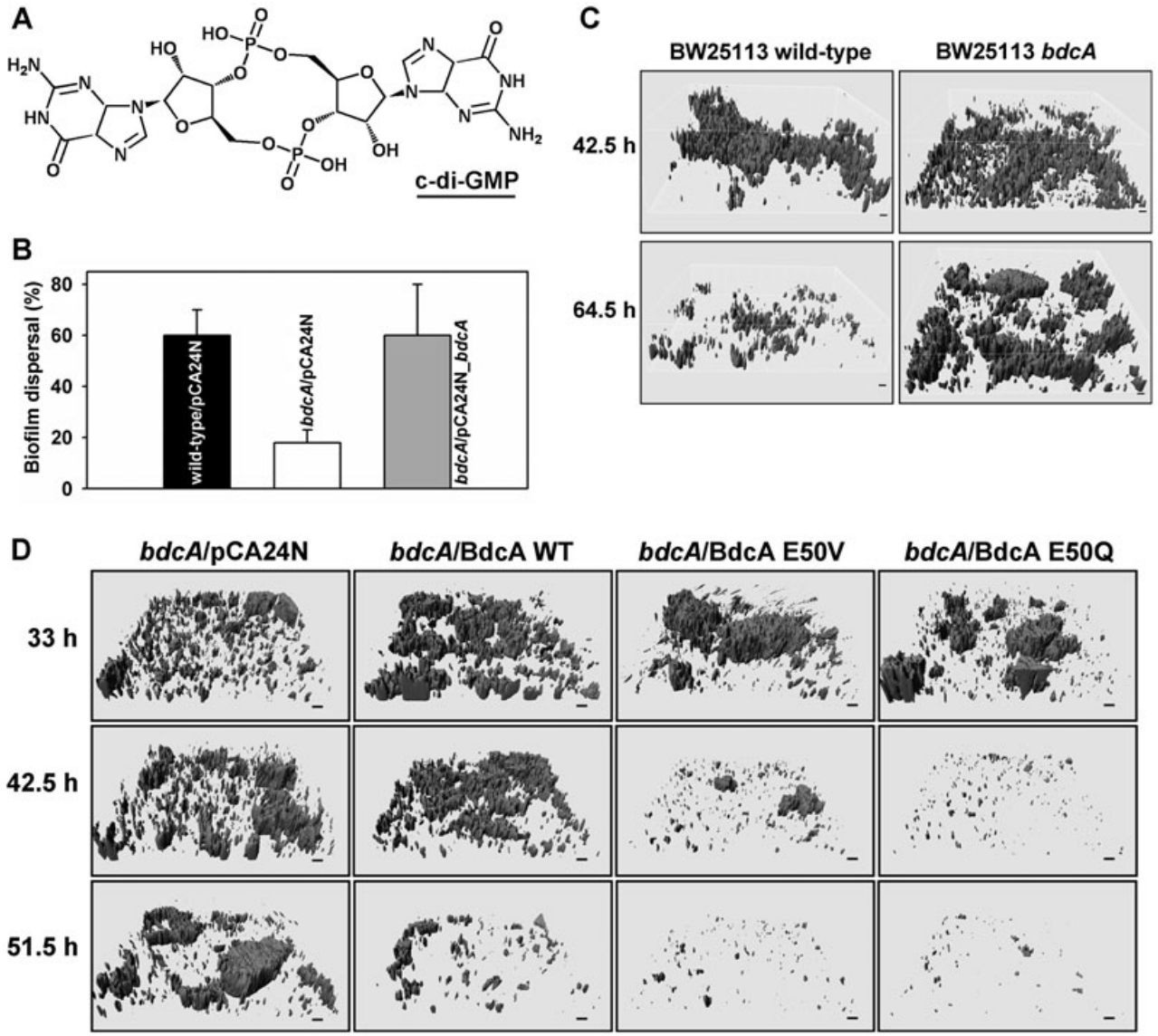

Fig. 1. BdcA increases biofilm dispersal.
A. Chemical structure of c-di-GMP.
B. Relative normalized biofilm dispersal after 42 h with static biofilms formed in 96-well polystyrene plates.
Biofilms were formed with LB and 30 μg ml^{-1} chloramphenicol at 37°C using BW25113/pCA24N, *bdcA*/pCA24N and *bdcA*/pCA24N_*bdcA*. 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 with 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.
C. Representative Imaris images of flow cell biofilms after 42.5 h and 64.5 h of incubation with LB medium.
Each strain has pCM18 for producing GFP to visualize the biofilms, and erythromycin (300 μg ml^{-1}) was added to retain pCM18.
D. Imaris images of flow cell biofilms of BW25113 *bdcA* producing evolved BdcA from pCA24N.
After forming biofilms in LB for 24 h, 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 total). Each strain has the pCM18 plasmid for producing GFP. Chloramphenicol (30 μg ml^{-1}) was used to retain the pCA24N-based plasmids, and erythromycin (300 μg ml^{-1}) was used to retain pCM18. BdcA WT indicates native BdcA, BdcA E50V is the epPCR evolved BdcA, and BdcA E50Q is the best saturation mutant. Scale bars represent 10 μm.

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 eightfold compared with 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

Table 1. Flow cell statistical analysis of biofilms via COMSTAT for biofilms formed in LB at 37°C.

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

For the wild-type strain and the *bdcA* mutant, cultures were grown in LB for 24, 33, 42.5, 51.5 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^{-1} chloramphenicol for 24 h, then *bdcA* expression was induced by adding 0.5 mM IPTG. Data were collected at 33, 42.5 and 51.5 h. BdcA is the native protein, BdcA E50V is the epPCR variant and BdcA E50Q is the saturation mutagenesis variant.

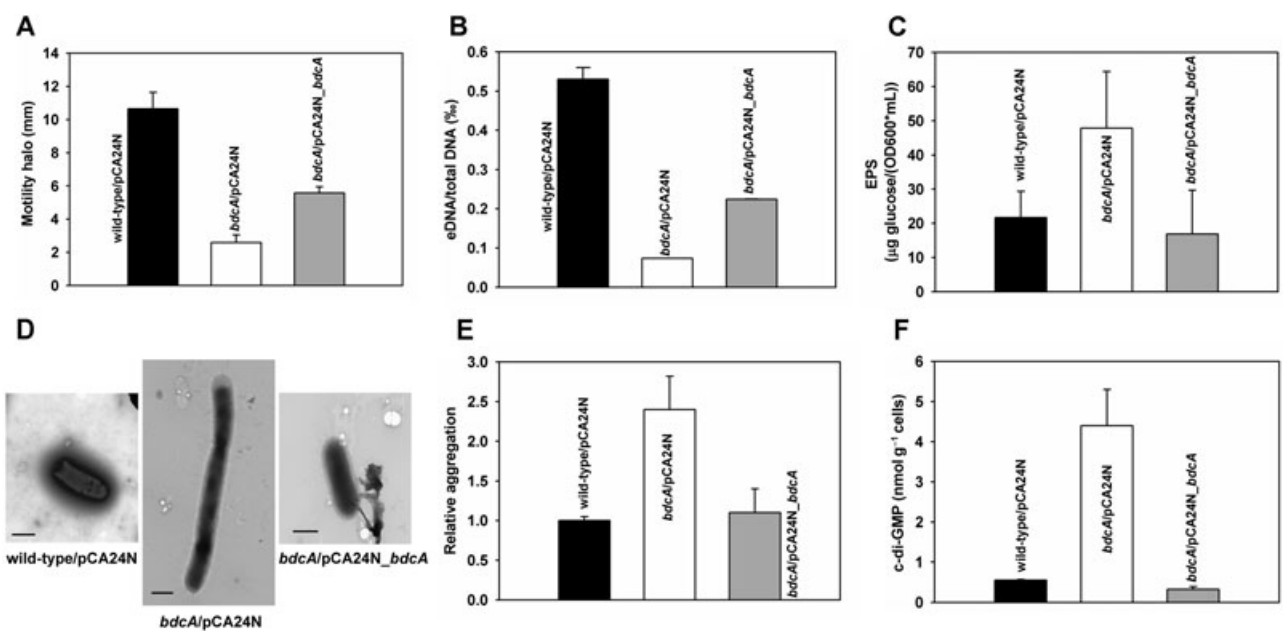

Fig. 2. BdcA binds c-di-GMP to alter swimming, eDNA, EPS, cell morphology and aggregation.
A. Swimming motility after 15 h of growth on motility agar plates.
B. eDNA production after 24 h in 96-well plates.
C. EPS production after 24 h in 96-well plates.
D. Cell length after 24 h in 96-well plates.
E. Cell aggregation after 24 h in 96-well plates.
F. c-di-GMP concentration after 24 h (static cultures).
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^{-1} 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.

motif and since there was no significant change in gene expression in the whole transcriptome for the *bdcA* mutant versus 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 a putative enzyme, it seemed more plausible that BdcA controls c-di-GMP concentrations as a PDE 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 catalysing 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 (Fig. S1). 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 to 24 h). Gene expression of *bdcA* increases with incubation time and is maximum at 8 h (4.9 ± 0.3-fold more compared with 2 h) and decreases at 24 h (2.0 ± 0.2-fold less compared with 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 with the value in the wild-type strain while *bdcA* expression in the *yjgH* mutant was not changed significantly (1.4 ± 0.2 fold versus 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 with 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 with 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 lack 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 (Fig. S2)

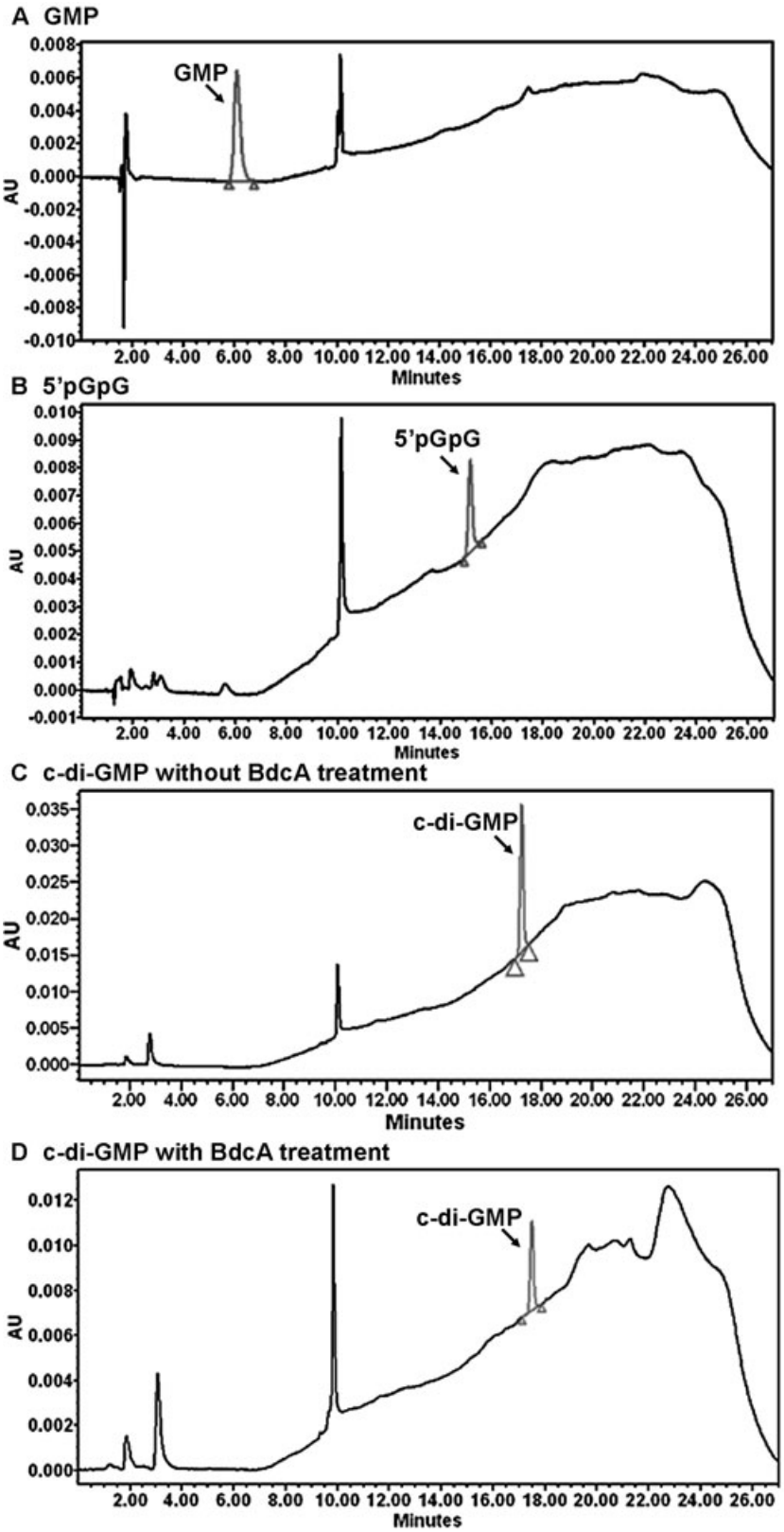

Fig. 3. BdcA decreases free intracellular c-di-GMP.
A. HPLC chromatogram for 10 μM guanosine monophosphate (GMP).
B. HPLC chromatogram for 2.5 μM 5′-phosphoguanylyl-(3′→5′)-guanosine (5′ pGpG) produced by treating 2.5 μM c-di-GMP with 2 μM of known phosphodiesterase YahA for 2 h at 37°C.
C. HPLC chromatogram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

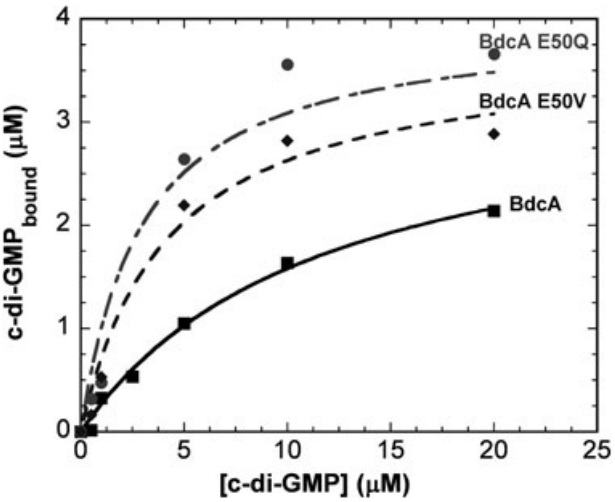

Fig. 4. BdcA binds c-di-GMP. Binding curves for 10 μM BdcA, BdcA E50V and BdcA E50Q with 0.5–20 μM c-di-GMP.

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 QS 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 QS 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 (Fig. S2). BdcA is also smaller (237 aa) than most PDEs. Hence, BdcA does not catalyse c-di-GMP degradation. Intracellular measurements show that BdcA decreases c-di-GMP concentrations, and the *in vitro* studies indicate that 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 pro-

Table 2. *Escherichia coli* strains and plasmids used in this study.

Strain/plasmid	Genotype	Source
Strain		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$	Datsenko and Wanner (2000)
BW25113 *bdcA*	BW25113 *ΔbdcA* 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)

Km^r, Cm^r and Em^r denote kanamycin, chloramphenicol and erythromycin resistance respectively.

gressively 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 biofilm dispersal. Also, *bdcA* shows high sequence conservation with other species with the highest similarity to a homolgue 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 (Fig. S3). 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. *Escherichia 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 T5-*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). Expression of *bdcA* was induced by 0.1 mM IPTG (Sigma, St Louis, MO, USA) unless otherwise indicated.

Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) and 37°C were used for all the experiments. Kanamycin (50 μg ml^{-1}) was used for pre-culturing the isogenic knockouts. Chloramphenicol (30 μg ml^{-1}) was used for the strains harbouring pCA24N and its derivatives, and 300 μg ml^{-1} 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, USA) as described previously (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^{-1} chloramphenicol, then 0.1 mM IPTG was added to the culture to induce *bdcA*. The mixture was shaken at 150 r.p.m. for 1 min and incubated for another 12–23 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 (42 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 described previously (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^{-1} for 2 h, and then fresh medium was added at 10 ml min^{-1}. For the wild-type strain and the *bdcA* mutant, biofilm images were taken after 24, 33, 42.5, 51.5 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, 18.5 and 27.5 h after IPTG addition (i.e. at 33, 42.5 and 51.5 h total). Biofilm images from nine random positions were taken and analysed with Imaris confocal software (Bitplane, Zurich, Switzerland) and COMSTAT confocal software, respectively, as described previously (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 supernatants 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 described previously (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 described previously (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 analyse differential gene expression for the *bdcA* mutant versus 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, USA). After 15 h, the biofilm cells on the glass wool were collected as described previously (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, 4, 8, 15 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, Austin, TX, USA), and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) (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 was higher than the 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^{-1} chloramphenicol and 0.1 mM IPTG for 24 h without shaking. A photodiode array detector (Waters, Milford, MA, USA) 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

The PDE activity was assayed as described previously (Schmidt *et al*., 2005). His-tagged proteins (BdcA, BdcA E50V and BdcA E50Q) were purified with Ni-NTA agarose (Qiagen) and contacted with 5–80 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 analysed 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). Then 85% phosphoric acid was used as an external standard for the chemical shift 0 p.p.m. 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 0.5–20 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 via HPLC (Schmidt *et al*., 2005). BdcA-bound c-di-GMP was recovered using 1.6 μg trypsin (Agilent Technologies, Santa Clara, CA, USA) 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 (Table S2). 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, USA), and 0.3 μM of each primer in 1× 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, USA).

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 (Table S2), 1 μl dNTP mix, 2.5 U *Pfu* polymerase, 30 ng template plasmid, and 1× *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^{-1} BSA]. The PCR program was 95°C for 1 min, followed by 16 cycles of 95°C for 50 s, 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

The qRT-PCR was performed as described previously (Ma and Wood, 2009). Primers used are listed in Table S2 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 modelling

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, USA). 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 and 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 the number of trials as 1000 and random seed as 111.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. BdcA does not catalyse c-di-GMP degradation. ^{31}P NMR spectrum for GMP and for c-di-GMP with and without BdcA E50Q. The 85% phosphoric acid was used as an external standard for the chemical shift at 0 p.p.m.

Fig. S2. Swiss model for evolved BdcA. The E50 residue is indicated in yellow, the E50V variant is indicated in red, and the E50Q variant is indicated in blue. The typical phosphodiesterase for c-di-GMP contains EALXR for coordinating Mg^{2+}, Q/R/D/D for c-di-GMP binding and T/E for catalysis. The degenerate phosphodiesterase domains of BdcA are indicated: EAL in pink as part of the EALXR motif, Q49/D136/D180 in turquoise as part of the Q/R/D/D motif, and E220 in orange as part of the T/E motif. The amino and carboxy termini are marked as N and C.

Fig. S3. Phylogenetic tree of BdcA.

Table S1. Normalized biofilm formation (OD_{540}/OD_{620}) in LB medium at 37°C for mutations in uncharacterized genes that are related to AI-2 transporter TqsA (Herzberg *et al*., 2006).

Table S2. DNA oligonucleotides used in this study. N represents A, T, G or C, while S represents G and C.

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