

The Diguanylate Cyclase GcbA Facilitates *Pseudomonas aeruginosa* Biofilm Dispersion by Activating BdlA

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Biofilm dispersion is a highly regulated process that allows biofilm bacteria to respond to changing environmental conditions and to disseminate to new locations. The dispersion of biofilms formed by the opportunistic pathogen *Pseudomonas aeruginosa* is known to require a number of cyclic di-GMP (c-di-GMP)-degrading phosphodiesterases (PDEs) and the chemosensory protein BdlA, with BdlA playing a pivotal role in regulating PDE activity and enabling dispersion in response to a wide array of cues. BdlA is activated during biofilm growth via posttranslational modifications and nonprocessive cleavage in a manner that is dependent on elevated c-di-GMP levels. Here, we provide evidence that the diguanylate cyclase (DGC) GcbA contributes to the regulation of BdlA cleavage shortly after initial cellular attachment to surfaces and, thus, plays an essential role in allowing biofilm cells to disperse in response to increasing concentrations of a variety of substances, including carbohydrates, heavy metals, and nitric oxide. DGC activity of GcbA was required for its function, as a catalytically inactive variant could not rescue impaired BdlA processing or the dispersion-deficient phenotype of *gcbA* mutant biofilms to wild-type levels. While modulating BdlA cleavage during biofilm growth, GcbA itself was found to be subject to c-di-GMP-dependent and growth-mode-specific regulation. GcbA production was suppressed in mature wild-type biofilms and could be induced by reducing c-di-GMP levels via overexpression of genes encoding PDEs. Taken together, the present findings demonstrate that the regulatory functions of c-di-GMP-synthesizing DGCs expand beyond surface attachment and biofilm formation and illustrate a novel role for DGCs in the regulation of the reverse sessile-motile transition of dispersion.

The process of biofilm dispersion represents an important and highly regulated phenotypic switch that allows cells residing within a biofilm to respond to changing conditions outside and within biofilm structures and to evade environmental stresses. Dispersion occurs in response to a wide array of signals, with dispersion-inducing conditions ranging from directly sensed environmental cues to self-synthesized signaling molecules (1–6). Biofilm dispersion in various bacterial species, including *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Pseudomonas fluorescens*, is tightly linked to nutrient availability, with both elevation and reduction in available nutrients, such as carbon and nitrogen sources, having been previously reported to trigger the process (1, 2, 7–12). Additional environmental dispersion triggers include temperature changes (13), oxidative and nitrosative stress (4, 14, 15), oxygen limitation (16, 17), and iron availability (18). More recently, the accumulation of D-amino acids has been found to trigger dispersion of biofilms formed by *Bacillus subtilis* and to prevent biofilm formation by *P. aeruginosa* and *Staphylococcus aureus*, with various reports suggesting that the mechanism of action may be based on specific effects on amyloid fibers or protein biofilm matrix components or general effects on protein synthesis and growth (19–23). Biofilm bacteria also control their own dispersion fate through the use of self-synthesized signaling molecules, such as *cis*-unsaturated fatty acids, including the *Xanthomonas campestris* diffusible signal factor (DSF) and the *P. aeruginosa cis*-2-decenoic acid (*cis*-DA) (3, 24, 25). These factors may have cross-species or even cross-kingdom activity, with *P. aeruginosa cis*-DA also triggering dispersion of biofilms formed by *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Candida albicans* (3, 26).

With such a wide array of reported dispersion triggers, it has become clear that the regulation of biofilm dispersion involves the

integration of sensory inputs into intracellular signal transduction that leads to a phenotypic switch enabling bacterial cells to leave the surface-associated biofilm and to disseminate to new locations. Surface attachment and biofilm development have long been established to be dependent on the modulation of the messenger molecule cyclic di-GMP (c-di-GMP), with increases in c-di-GMP levels correlating with surface attachment, aggregation, and biofilm formation (27–29). c-di-GMP regulates the motile-sessile transitions by binding and controlling the activities of effectors such as FleQ, PilZ domain-containing proteins and riboswitches, which in turn regulate growth-mode-specific gene expression patterns and phenotypes (30–35). Mounting evidence has suggested that biofilm dispersion, the reverse transition from surface-associated to planktonic growth, is also dependent on c-di-GMP regulation (1, 4, 17, 36), with a new question arising of how different dispersion-inducing cues are translated into c-di-GMP modulation. The cellular levels of c-di-GMP are controlled by the opposing activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which synthesize and degrade c-di-GMP, respectively. The genomes of various bacterial species encode multiple DGCs and PDEs, characterized by the presence of

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drastically different sensory domains, which allow *c*-di-GMP-dependent regulation of distinct phenotypic outputs in response to specific diverse signals. However, the integration of diverse signals into *c*-di-GMP modulation to elicit a dispersion response remains poorly characterized.

Recent evidence has suggested that *P. aeruginosa* biofilms, which can disperse in response to changing carbon source concentrations, ammonium chloride, heavy metals, and nitric oxide (NO) (1, 4, 12, 14, 15), exhibit some specificity for sensing of various dispersion-inducing conditions, with distinct *c*-di-GMP-modulating proteins required for the dispersion response to specific cues. For instance, the membrane-bound PDE RbdA, harboring Per-Arnt-Sim (PAS/PAC) sensory domains, was found to be required for the dispersion response to low-oxygen conditions, with the PAS domain specifically found to be involved in hypoxia sensing (17). Two membrane-bound PDEs, MucR and NbdA, have recently been shown to be required for NO-mediated biofilm dispersion (15). These PDEs contain transmembrane MHYT (Met-His-Tyr-Thr) domains, proposed to sense diatomic gases through protein-bound copper ions, with these domains likely facilitating *c*-di-GMP degradation by NbdA and/or MucR to enable dispersion in response to NO (15).

Interestingly, in addition to producing distinct proteins for translating specific dispersion-inducing cues into *c*-di-GMP modulation, *P. aeruginosa* also harbors proteins that appear to be central to the regulatory cascade and required for dispersion regardless of inducing signals. The membrane-associated active PDE DipA, harboring sensory GAF and PAS domains, is required for biofilm dispersion in response to carbohydrates, heavy metals, ammonium chloride, and NO (36). Such a dominant role in dispersion was also previously reported for the methyl-accepting chemotaxis protein (MCP) BdlA, which harbors two PAS domains (PASa and PASb) and a signal transduction/methyl-accepting chemotaxis domain (Tar). As opposed to the PDEs DipA, RbdA, NbdA, and MucR, BdlA does not possess any domains associated with modulation of *c*-di-GMP levels. However, it appears to indirectly regulate *c*-di-GMP levels in order to enable the process of dispersion, with inactivation of BdlA elevating the total *c*-di-GMP levels of biofilm cells and the production of a constitutively active BdlA-G31A variant (having a change of Gly to Ala at position 31) elevating overall cellular PDE activity levels (12, 37). Further evidence suggests that BdlA regulates biofilm cellular PDE activity and *c*-di-GMP levels via direct interaction with at least two of the dispersion-specific PDEs, DipA and RbdA, and potentially, via regulation of DipA protein levels (37). Recent evidence also suggests a feedback regulation between *c*-di-GMP levels and BdlA function. While BdlA regulates cellular *c*-di-GMP abundance, activating modifications of BdlA structure and function are, in turn, dependent on elevated *c*-di-GMP levels. Specifically, BdlA function in activating dispersion was found to require posttranslational modifications in the form of phosphorylation at the tyrosine-238 (Y238) residue, followed by an unusual nonprocessive cleavage of the MCP at the methionine-130 (M130) residue into two peptides (PASa and PASbTar) (see Fig. 1), with the two truncated peptides interacting to enable the dispersion response (38). Correlating with its role in regulating dispersion of biofilm cells, phosphorylation and cleavage of BdlA did not occur in planktonic cells (38). Instead, the modifications were found to be biofilm specific and to require elevated *c*-di-GMP levels following the transition to surface-associated growth (38). Moreover, manipu-

lating the total cellular *c*-di-GMP levels could artificially control cleavage of BdlA. Overexpression of the gene encoding the dispersion-associated PDE DipA significantly reduced BdlA cleavage during biofilm growth (38). In contrast, while BdlA is detectable only in its intact form in planktonic cells, plasmid-borne overexpression of the gene PA4843 triggered BdlA cleavage in planktonic cells (38). PA4843 encodes the recently characterized active DGC GcbA (also identified as AdcA), which was found to be involved in the regulation of the transition from planktonic to surface-associated growth (39, 40). Although BdlA plays a pivotal and central role in enabling *P. aeruginosa* biofilm dispersion in response to a variety of environmental cues, the system contributing to the elevation of *c*-di-GMP levels responsible for BdlA posttranslational modification and activation is currently not known. Thus, given our previous findings, we presently asked whether the DGC GcbA specifically contributes to the elevation of *c*-di-GMP levels required for the cleavage and activation of BdlA upon transition to surface-associated growth and investigated the potential role of GcbA in biofilm dispersion.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strain PAO1 was used as the parental strain. All planktonic strains were grown in Lennox broth (LB; BD Biosciences) or Vogel-Bonner minimal medium (VBMM) (49, 50) at 220 rpm in the absence or presence of 0.1 to 1.0% arabinose. Antibiotics were used at the following concentrations: 50 to 75 μ g/ml gentamicin, 60 μ g/ml tetracycline, and 250 μ g/ml carbenicillin for *P. aeruginosa* and 100 μ g/ml ampicillin, 20 μ g/ml gentamicin, and 20 μ g/ml tetracycline for *E. coli*.

Strain construction. Complementation and overexpression were accomplished by conjugating into *P. aeruginosa* strains the pJN105 vector harboring the respective genes under the control of the arabinose-inducible P_{BAD} promoter (45). In order to assess the native protein production levels of GcbA and BdlA, the chromosomal integration vectors pCTX-*gcbA*-V5/His₆ and pCTX-*bdlA*-V5/His₆ (38, 39), harboring constructs encoding the respective C-terminally V5/His₆-tagged proteins under the control of the native promoters, were introduced into the indicated *P. aeruginosa* strains via conjugation. The resulting strains (PAO1:: P_{bdlA} -*bdlA*-V5/His₆, PAO1 Δ *gcbA*:: P_{bdlA} -*bdlA*-V5/His₆, PAO1:: P_{gcbA} -*gcbA*-V5/His₆, and PAO1 Δ *gcbA*:: P_{gcbA} -*gcbA*-V5/His₆) contained chromosomally encoded *gcbA*-V5/His₆ or *bdlA*-V5/His₆ under the control of the respective native promoter at the *attB* site. Site-directed mutagenesis for the construction of the GcbA-GGAFF variant was accomplished by using the GeneArt site-directed mutagenesis kit (Life Technologies) according to the manufacturer's protocol using the primer pair *gcbA*-GGAFF456-for (CGGCCGCTATGGCGGCAGCGTTCGCGGTGGTCTCTTC)/*gcbA*-GGAFF456-rev (GAAGGACCACGGCGAACGCTGCGCCGCCATAGCGGCCG). The identities of vector inserts and site-directed mutations were confirmed by PCR and sequencing.

Biofilm formation. Biofilms were grown in a continuous flow tube reactor system (1-m lengths of size 14 silicone tubing, Masterflex; Cole Parmer, Inc.) in 20-fold-diluted LB medium or VBMM in the presence of 0.1% arabinose at 22°C for up to 144 h at a flow rate of 0.2 ml/min. For visualization of biofilm architecture, biofilms were grown under similar conditions in flow cell systems (BioSurface Technologies). Biofilm architecture was visualized via confocal laser scanning microscopy (CLSM) using a Leica TCS SP5 confocal microscope and the BacLight Live/Dead viability kit (Life Technologies). The CLSM images were processed using LAS AF software, version 2.4.1. Quantitative analysis of biofilm architecture was accomplished using MATLAB with the COMSTAT software package (51).

Biofilm dispersion assays. For biofilm dispersion assays, biofilms were cultivated in a once-through continuous flow tube reactor system at

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference(s) or source
<i>Escherichia coli</i> DH5 α	F ⁻ ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44 thi-1 gyrA96 relA1 tonA</i>	Life Technologies
<i>P. aeruginosa</i> strains		
PAO1	Wild type	B. H. Holloway
PAO1 Δ <i>gcbA</i>	<i>gcbA</i> (PA4843) allelic gene replacement in PAO1	39
PAO1 Δ <i>dipA</i>	PA5017::IS <i>phoA</i> /hah Tet ^r	36
PAO1 Δ <i>bdla</i>	Δ <i>bdla</i> in PAO1 Km ^r	12
PAO1 Δ <i>wspF</i>	<i>wspF</i> allelic exchange in PAO1	41
PAO1 Δ <i>siaD</i>	PA0169::IS <i>phoA</i> /hah Tet ^r	42
PAO1 Δ <i>wspR</i>	<i>wspR</i> allelic exchange in PAO1	43
PAO1::P _{<i>bdla</i>} - <i>bdla</i> -V5/His ₆	Chromosomal expression of <i>bdla</i> -V5/His ₆ in PAO1	38
PAO1 Δ <i>gcbA</i> ::P _{<i>bdla</i>} - <i>bdla</i> -V5/His ₆	Chromosomal expression of <i>bdla</i> -V5/His ₆ in PAO1 Δ <i>gcbA</i>	This study
PAO1::P _{<i>gcbA</i>} - <i>gcbA</i> -V5/His ₆	Chromosomal expression of <i>gcbA</i> -V5/His ₆ in PAO1	This study
PAO1 Δ <i>gcbA</i> ::P _{<i>gcbA</i>} - <i>gcbA</i> -V5/His ₆	Chromosomal expression of <i>gcbA</i> -V5/His ₆ in Δ <i>gcbA</i>	This study
Plasmids		
pRK2013	Helper plasmid for triparental mating; <i>mob tra</i> Km ^r	44
pJN105	Arabinose-inducible gene expression vector; pBRR-1 MCS <i>araC</i> -P _{BAD} Gm ^r	45
pMJT-1	<i>araC</i> -P _{BAD} cassette of pJN105 cloned into pUCP18; Amp ^r (carbapenem resistant)	46
pJN- <i>gcbA</i>	C-terminally V5/His ₆ -tagged <i>gcbA</i> cloned into pJN105 at XbaI	39
pCTX- <i>gcbA</i> -V5/6 \times His	C-terminally V5/His ₆ -tagged <i>gcbA</i> gene and upstream 500 bp cloned into pMini-CTX	39
CTX-P <i>bdla</i> - <i>bdla</i> -V5/6 \times His	C-terminally V5/His ₆ -tagged <i>bdla</i> gene and upstream 500 bp cloned into pMini-CTX	38
pJN- <i>gcbA</i> (GGAAF)	C-terminally V5/His ₆ -tagged <i>gcbA</i> with mutation to convert GGEEF motif to GGAAF cloned into pJN105	This study
pJN-PASa	PASa-encoding segment of <i>bdla</i> cloned into pJN105	38
pMJT- <i>bdla</i> -PASbTarH	C-terminally V5/His ₆ -tagged PASaTar of <i>bdla</i> cloned into pMJT-1	38
pJN- <i>bdla</i> -V5/6 \times His	C-terminally V5/His ₆ -tagged <i>bdla</i> cloned into pJN105	37
pJN- <i>dipA</i> -V5/6 \times His	C-terminally V5/His ₆ -tagged <i>dipA</i> cloned into pJN105	36
pJN-PA2133	PA2133 cloned into pJN105	43
pJN- <i>bdla</i> -G31A-His	C-terminally His ₆ -tagged <i>bdla</i> with G31A mutation cloned into pJN105	37
pMJT- <i>bdla</i> -HA	C-terminally HA-tagged <i>bdla</i> cloned into pMJT-1	This study
pJN- <i>bfiS</i>	C-terminally V5/His ₆ -tagged <i>bfiS</i> cloned into pJN105	47, 48

22°C for 120 h. After 120 h of biofilm growth, biofilm dispersion was induced by the sudden addition of L-glutamate (18 mM) or mercury chloride (2 mM) to the growth medium as previously described (12). In addition, 500 μ M sodium nitroprusside (SNP) was used as a source of nitric oxide (4). Following the addition of one of the dispersion-inducing substances, the optical density of the biofilm effluents, collected at 1-min intervals, was assessed by spectrophotometry, with dispersion events indicated by an increase in the turbidity measured at 600 nm.

Immunoblot analysis and pulldown assays. The abundance of V5/His₆-tagged GcbA or BdlA present in *P. aeruginosa* cells was assessed by SDS-PAGE and immunoblot analysis. Total cell extracts from planktonic and biofilm cells, harvested by centrifugation for 5 min at 16,000 \times g and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.3 mg phenylmethanesulfonyl fluoride, were obtained by sonication on ice with six 10-s bursts at 4 W, followed by centrifugation for 5 min at 21,200 \times g to pellet cell debris and unbroken cells. The protein concentrations were determined using a modified Lowry assay (Thermo Scientific) and bovine serum albumin as a standard. The samples (15 μ g) were resolved on an 11% polyacrylamide gel and subsequently transferred onto polyvinylidene difluoride (PVDF) membrane using a TurboTransblot apparatus (Bio-Rad). Western blots were probed with anti-V5-horseradish peroxidase (HRP) or anti-V5 antibody (Invitrogen) and antihemagglutinin (anti-HA) antibody (Covance). When necessary, a secondary anti-mouse IgG antibody (Cell Signaling Technologies) was used. The blots were subsequently developed using Immuno-Star WesternC chemiluminescence reagents (Bio-Rad). Following transfer, SDS-PAGE gels were Coomassie stained to ensure equal loading. Densitometry was performed using ImageJ software.

Pulldown assays were used to assess the interactions between BdlA and GcbA, DipA, or BfiS in total protein cell extracts of cells coproducing C-terminally V5/His₆-tagged GcbA, DipA, or BfiS and C-terminally HA-tagged bait BdlA protein (PAO1/pMJT-*bdla*-HA/pJN-*gcbA*, PAO1/pMJT-*bdla*-HA/pJN-*dipA*, or PAO1/pMJT-*bdla*-HA/pJN-*bfiS*). Following immunoprecipitation of HA-tagged proteins using immobilized anti-HA antibody (Covance), immunoprecipitation eluates were separated by SDS-PAGE and assessed by immunoblot analysis for the presence of V5/His₆-tagged prey proteins using anti-V5-HRP antibodies (Life Technologies). Pulldown assays were carried out using 200 μ g protein from cellular extracts. Anti-HA and anti-V5 antibodies were used for immunoprecipitation at 2 μ g/ml and immunoblotting at 0.2 μ g/ml.

Phosphoprotein enrichment and detection. Analysis of phosphorylated BdlA-V5/His₆ levels in protein extracts obtained from wild-type PAO1 or Δ *gcbA* mutant cells grown as biofilms for 144 h was accomplished using phosphoprotein purification via metal oxide affinity chromatography (MOAC) essentially as described by Wolschin and colleagues (52). MOAC has been demonstrated by Krüger et al. to result in up to 20-fold enrichment of phosphoproteins and to approach 100% specificity (53). Briefly, 750 μ g of total protein cell extract was diluted with MOAC incubation buffer (30 mM morpholineethanesulfonic acid [MES], 0.2 M potassium glutamate, 0.2 M sodium aspartate, 0.25% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 8 M urea) to a final volume of 1.5 ml and subsequently incubated for 30 min at 4°C in the presence of 80 mg of aluminum hydroxide. Unbound proteins were removed via six 1-min washes of the aluminum hydroxide slurry with 1.5 ml of incubation buffer at 16,000 \times g and 4°C. Then, phosphoproteins were eluted from the slurry using 100 mM potassium pyrophos-

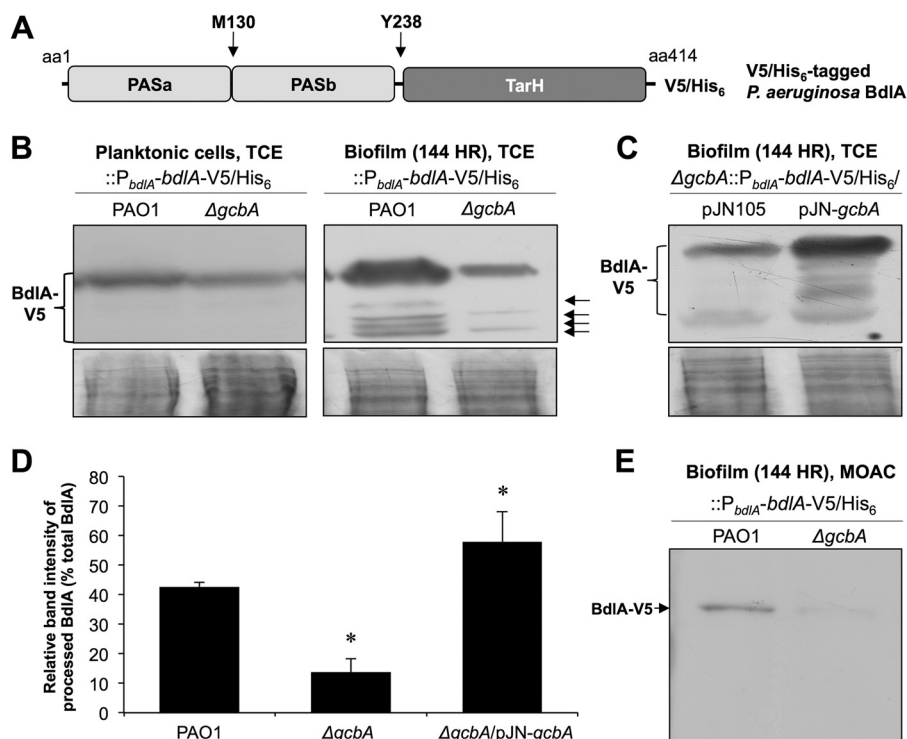


FIG 1 The DGC GcbA is required for cleavage of BdlA during biofilm growth. (A) Domain structure of BdlA. TarH, conserved chemoreceptor domain found in Tar (taxis toward aspartate and related amino acids); PAS, Per-Arnt-Sim sensory domain. Arrows indicate the methionine-130 (M130) putative ClpP cleavage site and tyrosine-238 (Y238) phosphorylation site. V5/His₆ indicates the C-terminal location of the V5 epitope–6-histidine tag of the BdlA construct used in this study. (B) Detection with anti-V5 antibody of chromosomally encoded C-terminally V5/His₆-tagged BdlA in total cell extracts (TCE) of planktonic or biofilm (144-h-old) wild-type PAO1 or ΔgcbA mutant cells. Arrows indicate detected cleavage products of BdlA. (C) Detection with anti-V5 antibody of chromosomally encoded C-terminally V5/His₆-tagged BdlA in total cell extracts of 144-h-old biofilm ΔgcbA mutant cells harboring arabinose-inducible gcbA in the pJN105 vector or the empty vector control. Panels below immunoblot images (B and C) are Coomassie-stained gels after transfer to ensure equal TCE protein loading. (D) ImageJ software densitometry analysis was used to determine the relative fraction of processed BdlA, reported as percentage of band intensity of processed BdlA relative to intensity of all detectable BdlA bands. Error bars represent standard deviations. *, significantly different from the results for PAO1 cells ($P < 0.05$). (E) Detection with anti-V5 antibody of chromosomally encoded C-terminally V5/His₆-tagged BdlA in metal oxide chromatography (MOAC)-enriched phosphoproteomes of biofilm (144-h-old) wild-type PAO1 or ΔgcbA mutant cells. All experiments were performed in triplicate. Representative images are shown.

phate and 8 M urea, precipitated and desalted by methanol-chloroform precipitation, and subsequently vacuum dried. Samples were then analyzed by SDS-PAGE, followed by the detection of C-terminally tagged BdlA-V5/His₆ by immunoblotting with anti-V5 antibodies.

qRT-PCR. Quantitative real-time reverse transcription-PCR (qRT-PCR) was used to determine gene expression levels using 2 μg of total RNA isolated from planktonic, biofilm, and dispersed cells of wild-type *P. aeruginosa* and mutant strains. Planktonic cells were grown in LB medium for 6 h starting with initial cultures adjusted to an optical density at 600 nm (OD₆₀₀) of ~0.03. Biofilms were grown in continuous flow tube reactors as outlined above. Cells dispersing from biofilms following the induction of dispersion as described above were collected directly into RNeasy Protect (Qiagen). Isolation of mRNA and cDNA synthesis were carried out as previously described (54–57). qRT-PCR was performed using the Bio-Rad CFX Connect real-time PCR detection system and SsoAdvanced SYBR green supermix (Bio-Rad) with oligonucleotide pairs PA4843RTf/PA4843RTr (39) and bdIA-RT-for (CTACGCGCAATCGGAAGAC)/bdIA-RT-rev (GGACATTGCCGTCGAGGTC). *mreB* (39) was used as a control. Quantitation of relative transcript levels was accomplished using the CFX Manager Software (Bio-Rad), by first normalizing the transcript abundances (based on the threshold cycle [C_T] value) to that of *mreB* and then determining transcript abundance ratios. Melting curve analyses were employed to verify amplification of specific single products. The qRT-PCR analysis was performed using at least three biological replicates per strain.

Determination of phosphodiesterase activities. The phosphodiesterase activities of total cell extracts of biofilm cells harboring pJN-*dipA* or pJN-PA2133 were determined using the synthetic chromogenic substrate bis(*p*-nitrophenyl) phosphate (bis-pNPP) (Sigma-Aldrich) essentially as previously described (36, 58, 59), by measuring the release of *p*-nitrophenol (pNP) at 405 nm. An extinction coefficient for *p*-nitrophenol of $1.78 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was used. Reaction mixtures without the addition of cell extracts were used as controls to account for any nonenzymatic bis-pNPP hydrolysis.

Statistical analysis. All statistical analyses were performed in Microsoft Excel using a two-tailed Student's *t* test, assuming equal variance, or single-factor analysis of variance (ANOVA).

RESULTS

GcbA contributes to biofilm-specific posttranslational modifications of BdlA. Biofilm-specific processing of BdlA has been shown to result in up to four cleavage products in biofilm PAO1 cells, with the predominant products of C-terminally tagged BdlA corresponding in size to a BdlA variant lacking the N-terminal PASa domain (Fig. 1A). However, at least one of the products was still detectable for a BdlA variant with an M130I substitution at the putative ClpP cleavage site (38). Considering that artificially induced overexpression of *gcbA* triggered BdlA cleavage under planktonic growth conditions (38), we asked whether GcbA also

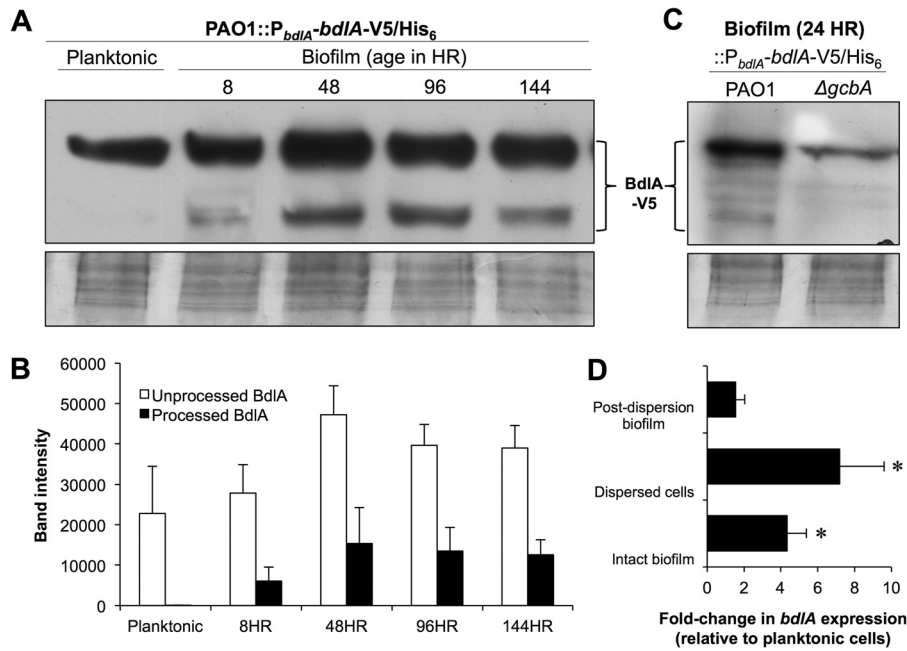


FIG 2 Cleavage of BdlA is initiated in *P. aeruginosa* cells following initial attachment to surfaces. (A) Detection of chromosomally encoded, C-terminally V5/His₆-tagged BdlA in total cell extracts of *P. aeruginosa* PAO1 cells grown planktonically or as biofilms for 8, 48, 96, and 144 h by immunoblot analysis using anti-V5 antibody. (B) ImageJ software densitometry analysis of intact BdlA and BdlA cleavage products detected in total cell extracts of *P. aeruginosa* PAO1 cells grown planktonically or as biofilms for 8, 48, 96, and 144 h. (C) Detection with anti-V5 antibody of chromosomally encoded, C-terminally V5/His₆-tagged BdlA in total cell extracts of planktonic or attached (24 h) wild-type PAO1 or $\Delta gcbA$ mutant cells. (A, C) Panels below immunoblot images are Coomassie-stained gels after transfer to ensure equal TCE protein loading. (D) Relative transcript abundances of *bdlA* in *P. aeruginosa* PAO1 144-h-old biofilm cells (Intact biofilm), cells dispersed from 144-h-old biofilms in response to a changed glutamate concentration in the medium (Dispersed cells), and cells remaining within the biofilm following dispersion (Post-dispersion biofilm). Transcript abundance is reported relative to the abundance in planktonically grown PAO1 cells. (B, D) Error bars represent standard deviations. *, significantly different from expression in planktonically grown PAO1 cells ($P < 0.05$). All experiments were performed in triplicate, and representative images are shown.

contributes to the activation of BdlA during biofilm growth. We therefore monitored the native production and processing levels of BdlA using a chromosomally encoded C-terminally V5–His₆-tagged BdlA (BdlA-V5/His₆) construct introduced into wild-type PAO1 or the isogenic *gcbA* mutant strains. Consistent with previous observations, no cleavage of BdlA was observed in either strain grown planktonically, and the BdlA levels were similar in both strains (Fig. 1B). However, when anti-V5 immunoblotting was used to assess BdlA-V5/His₆ abundance in cells grown for 144 h as biofilms, the levels of detectable C-terminal cleavage products of BdlA in biofilms formed by $\Delta gcbA$ cells were significantly reduced relative to the levels in biofilms formed by PAO1 cells (Fig. 1B and D). Moreover, $\Delta gcbA$ biofilm cells were found to lack at least two of the cleavage products detectable in the wild-type biofilm cells (Fig. 1B). Complementation ($\Delta gcbA/pJN-gcbA$) restored the cleavage of BdlA to wild-type levels (Fig. 1C and D).

BdlA processing has previously been demonstrated to correlate with elevated protein phosphorylation levels and to require the phosphorylation of the BdlA tyrosine-238 residue (38). Correlating with the reduced BdlA processing levels, inactivation of *gcbA* resulted in significantly reduced levels of phosphorylated BdlA detected in biofilm cells (Fig. 1E). Taken together, these findings suggested that GcbA contributes to the posttranslational modification and activation of BdlA during biofilm growth.

GcbA contributes to BdlA levels and cleavage upon initial transition to surface-associated growth. GcbA was recently shown to make a greater contribution to c-di-GMP levels in

planktonic cells and those undergoing early attachment stages than it does in cells of developed biofilms, with GcbA not affecting the overall c-di-GMP levels of biofilm cells (39). Given that GcbA affected BdlA processing in established *P. aeruginosa* biofilms, we therefore asked whether BdlA cleavage is initiated during early biofilm formation and, if so, whether BdlA processing is GcbA dependent. Using the chromosomally encoded BdlA-V5/His₆ construct and anti-V5 immunoblotting, we monitored BdlA processing in *P. aeruginosa* cells at 8, 48, 96, and 144 h following attachment. These time points were chosen because they were previously demonstrated to correspond to the following stages of biofilm development: initial surface attachment, transition to irreversible surface-associated growth, early biofilm maturation, and late biofilm maturation (57, 60). While no cleavage of BdlA was detected in cells grown planktonically, processing of the protein was detectable as early as 8 h following initial attachment (Fig. 2A and B). Moreover, cleavage of BdlA appeared to reach a steady-state level by 48 h of biofilm growth (Fig. 2A and B).

To determine the contribution of GcbA to cleavage of BdlA during early stages of biofilm formation, BdlA processing was assessed following 24 h of surface-associated growth. This time point was chosen to ensure that the observed effects were not due to differences in the amounts of attached biomass, as inactivation of *gcbA* was previously demonstrated to affect attachment following 8 h but not 24 h of growth (39). Under the conditions tested, $\Delta gcbA$ cells exhibited significantly reduced levels of BdlA processing relative to the levels in the wild-type PAO1 cells (Fig. 2C).

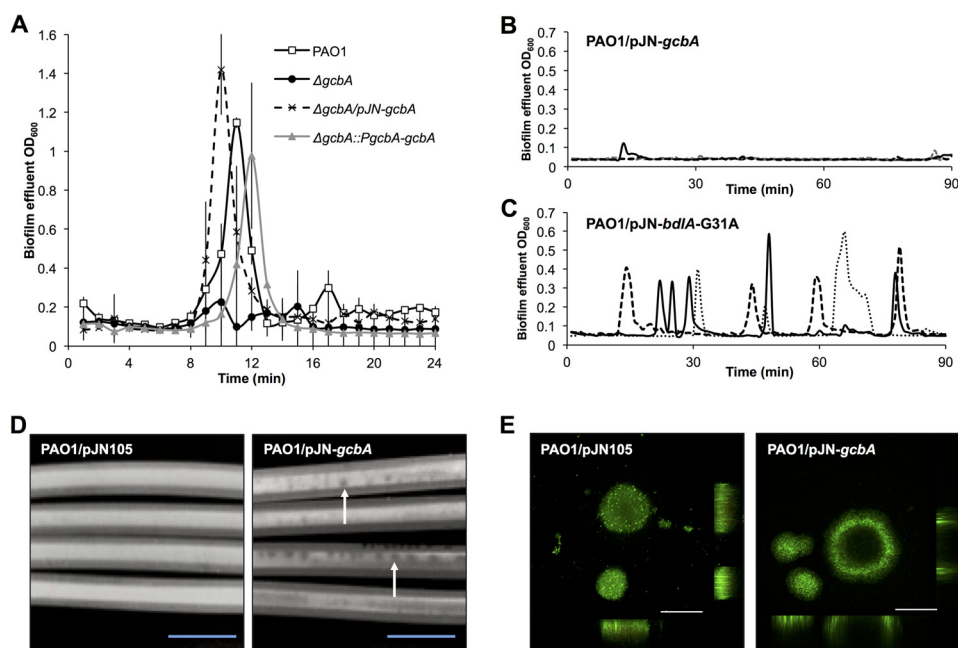


FIG 3 GcbA contributes to the regulation of biofilm dispersion. (A to C) Biofilms were grown for 120 h in tube reactors prior to exposure to dispersion-inducing conditions, at which time the biofilm effluents were monitored for dispersion events, as evidenced by an increase in turbidity measured at 600 nm. (A) Inactivation of *gcbA* renders *P. aeruginosa* dispersion deficient in response to a sudden change in the glutamate concentration in the medium. Error bars indicate standard deviations. (B, C) Detection of dispersion following induction of *gcbA* or *bdIA*-G31A gene expression. Expression of *gcbA* or *bdIA*-G31A was under the control of the arabinose-inducible P_{BAD} promoter, and induction of gene expression was accomplished by adding arabinose to the growth medium. Representative dispersion data of three independent biofilm replicates, indicated by dotted, dashed, and solid lines, are shown. Dispersion was detected upon the addition of arabinose to the PAO1/pJN-*bdIA*-G31A strain (C) but not to the PAO1/pJN-*gcbA* strain (B). (D) Photographs of 144-h-old biofilms formed on the interior surface of silicone tubing by *P. aeruginosa* cells overexpressing *gcbA* (PAO1/pJN-*gcbA*) or harboring the respective vector control (PAO1/pJN105). The confluent white coverage within the tubing is indicative of accumulated bacterial biofilm biomass, and the darker spots are indicative of a lack of biofilm biomass, both of which are observable by the naked eye without the aid of a microscope. Arrows indicate examples of voids in the biofilm cellular layers. Bars = 1 cm. (E) CLSM images of PAO1/pJN105 and PAO1/pJN-*gcbA* cells grown as biofilms under flowing conditions in flow cells for 144 h and stained with the Live/Dead BacLight viability stain. Bars = 100 μ m. All experiments were performed in triplicate, and representative images are shown.

Taken together, these findings indicated that BdlA is cleaved soon after initial attachment in a manner dependent on GcbA.

***bdIA* is transcriptionally regulated independently of GcbA during biofilm growth.** The immunoblot analyses of BdlA protein production and cleavage (Fig. 1 and 2) indicated that processing may correlate positively with the overall abundance of BdlA. This prompted us to ask whether BdlA is modulated at a transcriptional level in a growth mode-dependent manner and, if so, whether the transcriptional regulation of *bdIA* is affected by GcbA. qRT-PCR analysis revealed that *bdIA* transcript abundances increase approximately 4-fold in biofilm cells relative to the levels in their planktonic counterparts (Fig. 2D). The *bdIA* transcript levels increased even further in cells dispersing from biofilms in response to an increased glutamate concentration in the medium, and the levels decreased in biofilm cells remaining attached to the surface following dispersion (Fig. 2D). In contrast, however, no differences were observed in the relative transcript abundances of *bdIA* in Δ *gcbA* and wild-type PAO1 cells grown planktonically (fold change, 1.10 ± 0.6 [mean \pm standard deviation]), for Δ *gcbA* versus PAO1) or as biofilms (fold change, 1.18 ± 0.4). Moreover, overexpression of *gcbA* did not affect *bdIA* expression, as PAO1/pJN-*gcbA* demonstrated fold changes of 1.18 ± 0.3 and 0.8 ± 0.3 in *bdIA* transcript abundance relative to the levels in the PAO1/pJN105 vector control under planktonic and biofilm growth conditions, respectively. These findings suggested that, while GcbA

contributes to the translational and/or posttranslational regulation of BdlA, *bdIA* transcript levels are regulated in a manner dependent on the mode of growth but not on GcbA.

GcbA is required for nutrient-induced biofilm dispersion but does not directly trigger dispersion. The dispersion of *P. aeruginosa* biofilm cells in response to changing environmental conditions was previously shown to require not only the presence of BdlA but also its posttranslational modification and cleavage, with lack of BdlA processing correlating with impaired nutrient-induced dispersion in response to an increasing extracellular glutamate concentration (12, 38). Considering the contribution of GcbA to BdlA cleavage and phosphorylation, the role of this DGC in nutrient-induced biofilm dispersion in response to a change in the glutamate concentration in the medium was assessed. Following 120 h of growth, biofilms formed by wild-type PAO1 and Δ *gcbA* cells were exposed to 18 mM glutamate added to the growth medium, and the biofilm effluents were monitored for cellular release as a result of dispersion events. While biofilms formed by the wild-type PAO1 strain dispersed in response to a change in the concentration of glutamate, as evidenced by a significant increase in the optical density of the biofilm effluent, biofilms formed by the isogenic *gcbA* mutant did not disperse (Fig. 3A). Complementation of the *gcbA* mutant strain with a chromosomally encoded *gcbA* (Δ *gcbA*:: P_{gcbA} -*gcbA*), as well as multicopy expression of *gcbA* under the control of the P_{BAD} promoter in the

TABLE 2 COMSTAT analysis of PAO1, PAO1 $\Delta gcbA$, PAO1/pJN105, and PAO1/pJN-*gcbA* biofilms^a

Strain	Total biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Substratum coverage (%)	Avg thickness (μm)	Roughness coefficient	Maximum thickness (μm)
PAO1	7.27 (± 1.85)	22.27 (± 6.90)	7.61 (± 2.15)	1.41 (± 0.15)	42.13 (± 8.93)
PAO1 $\Delta gcbA$	9.28 (± 3.17)	40.88 (± 16.36)*	9.82 (± 3.46)	1.02 (± 0.24)	45.33 (± 9.19)
PAO1/pJN105	6.16 (± 2.85)	14.08 (± 4.15)	6.22 (± 3.04)	1.68 (± 0.09)	48.43 (± 9.30)
PAO1/pJN- <i>gcbA</i>	3.78 (± 3.29)*	8.18 (± 5.84)*	3.95 (± 3.59)*	1.81 (± 0.14)	45.43 (± 14.24)

^a COMSTAT analysis was carried out on 144-h-old biofilms grown in triplicate, using at least 6 images per replicate. Values are means (\pm standard deviation). *, significantly different from the results for wild-type biofilms ($P \leq 0.01$).

pJN105 vector ($\Delta gcbA/pJN-gcbA$), restored biofilm dispersion in response to glutamate (Fig. 3A). It can be argued that the impaired dispersion response of biofilms formed by $\Delta gcbA$ is the result of reduced c-di-GMP levels and/or biomass accumulation. However, inactivation of *gcbA* has previously been reported not to correlate with reduced biofilm cellular c-di-GMP levels (39). Moreover, in agreement with previous reports (39) and as presently confirmed (Table 2), inactivation of *gcbA* does not result in a lack of mature biofilm architecture or reduced biomass accumulation at the later stages of biofilm development (Table 2). Interestingly, however, greater substratum coverage was observed in biofilms formed by $\Delta gcbA$ cells (Table 2), potentially resulting from the impaired dispersion response.

With evidence suggesting that glutamate-induced biofilm dispersion requires GcbA, we next assessed whether the induction of *gcbA* expression in established biofilms could trigger a rapid dispersion response. This reasoning was based on previous findings demonstrating that the production of the BdlA-G31A variant results in a hyperdispersive phenotype and that arabinose induction of *bdlA-G31A* expression triggers dispersion in established biofilms (37). Biofilms of PAO1 harboring *gcbA* under the control of the arabinose-inducible P_{BAD} promoter in the pJN105 vector (PAO1/pJN-*gcbA*) were grown in a minimal medium in the absence of arabinose under flowing conditions. Following 120 h of growth, arabinose was added to the medium to induce *gcbA* expression, and the effluent was monitored for an increase in optical density, indicative of dispersion events. Strain PAO1/pJN-*bdlA-G31A*, harboring pJN105 with a gene encoding BdlA-G31A, the constantly “on” variant of BdlA (37), was used as a positive control. In accord with previous findings, exposure to arabinose triggered dispersion events in biofilms formed by PAO1/pJN-*bdlA-G31A* (Fig. 3C). The induction of *gcbA* expression, however, did not induce dispersion within a similar time frame (Fig. 3B), indicating that GcbA by itself does not directly trigger the dispersion response.

We next assessed whether overexpression of *gcbA* affects biofilm formation. Despite the fact that *gcbA* overexpression has been previously found to induce a hyperaggregative phenotype in liquid culture and to promote initial attachment to a surface (39), continuous induction of *gcbA* during biofilm growth in PAO1/pJN-*gcbA* resulted in the formation of biofilms with visibly reduced biomass compared to biofilms formed by the vector control PAO1/pJN105 (Fig. 3D and E). While the wild-type vector control strain formed confluent biofilms with uniform surface coverage, the PAO1/pJN-*gcbA* strain instead formed biofilms that were characterized by patchy coverage of the substratum and the presence of voids visible without the aid of a microscope (Fig. 3D). CLSM analysis confirmed reduced levels of biomass accumulation in the biofilms formed by PAO1/pJN-*gcbA* relative to the amounts

formed by the vector control (Fig. 3E, Table 2). The CLSM analysis also revealed increased instances of hollowed-out cellular clusters, indicative of dispersion events, suggesting that multicopy expression of *gcbA* reduced biomass accumulation not by interfering with biofilm formation but rather by triggering or facilitating dispersion events (Fig. 3E).

DGC activity of GcbA is required for BdlA cleavage and biofilm dispersion. Elevated levels of c-di-GMP correlate with the sessile, biofilm mode of growth, while low levels of c-di-GMP generally correspond with free-swimming, motile growth away from the surface (61). Furthermore, the sessile-motile transition of dispersion has been linked to the activity of PDEs in reducing the intracellular levels of c-di-GMP (4, 12, 15, 17, 36, 37). Therefore, the observation of a DGC being required for biofilm dispersion was rather unexpected. GcbA is composed of a response regulator receiver domain and a GGDEF cyclase domain, with a conserved active-site GGEEF motif (39). Considering that response regulator receiver domains harbor conserved phosphorylation sites and participate in phosphotransfer events with other proteins to form signaling cascades (62), we asked whether GcbA-mediated regulation of BdlA cleavage and dispersion is dependent on the GcbA signaling domain rather than on its enzymatic activity. However, a GcbA variant with the GGEEF cyclase active-site motif replaced with GGAAF (GcbA-GGAAF), while soluble and produced at levels comparable to those of the wild-type protein (Fig. 4A), was unable to restore the BdlA processing (Fig. 4B and C) and the dispersion phenotype of $\Delta gcbA$ biofilms to wild-type levels (Fig. 4D), suggesting that GcbA DGC activity is required for the dispersion response. In contrast, multicopy expression of wild-type *gcbA* in $\Delta gcbA$ cells restored BdlA processing during biofilm growth to wild-type levels (Fig. 4B and C) and rescued the dispersion-deficient phenotype (Fig. 4D).

To test whether any decrease in the general c-di-GMP pool or in DGC activity previously associated with biofilm formation would affect the dispersion response, we also assessed the ability of biofilms formed by mutants in which the DGC-encoding genes *wspR* and *siaD* were inactivated to disperse in response to glutamate. In contrast to biofilms formed by the $\Delta gcbA$ mutant, those formed by *wspR* and *siaD* mutants were capable of responding to a changed glutamate concentration via a dispersion response, as evidenced by increasing optical densities of the biofilm effluents (Fig. 4E). These results suggested specificity for the role of GcbA in biofilm dispersion and that the c-di-GMP synthesis catalyzed by GcbA likely contributes to the regulation of biofilm dispersion, potentially via processing of BdlA.

***gcbA* expression and GcbA production are suppressed during biofilm growth and induced upon dispersion.** Previous findings demonstrated GcbA to contribute to the cellular c-di-GMP levels of planktonic but not biofilm cells (39). Our present find-

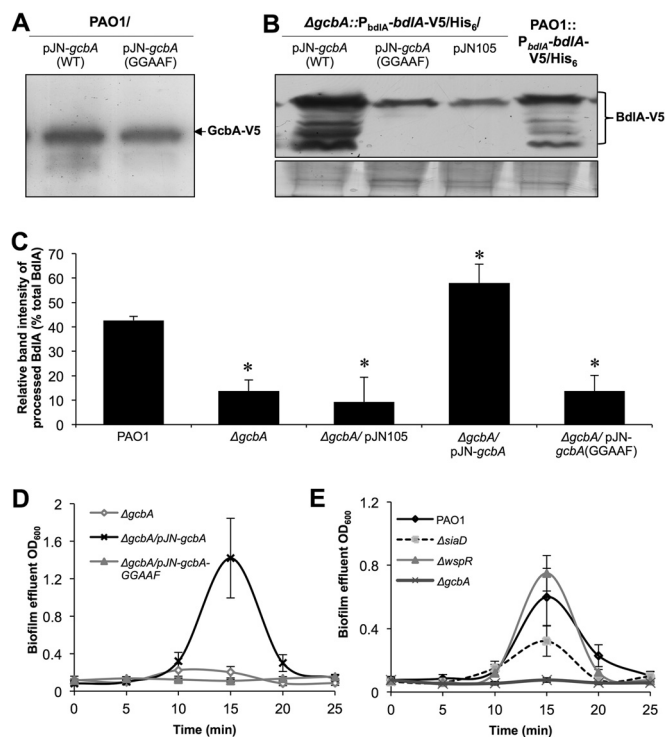


FIG 4 GcbA DGC activity is required for biofilm dispersion and BdlA processing. (A) The GcbA-GGAAAF variant is produced in a soluble manner, as revealed by anti-V5 immunoblot analysis of total cell extracts of PAO1 cells producing C-terminally V5/His₆-tagged wild-type GcbA or GcbA-GGAAAF. (B) Detection of chromosomally encoded C-terminally V5/His₆-tagged BdlA in total cell extracts of biofilms (144-h-old) formed by wild-type PAO1 or $\Delta gcbA$ mutant cells complemented with wild-type *gcbA* or *gcbA*-GGAAAF cloned into the pJN105 vector. $\Delta gcbA$ cells harboring the empty pJN105 vector were used as negative controls. Panel below immunoblot image is Coomassie-stained gel after transfer to ensure equal TCE protein loading. (C) ImageJ software densitometry analysis was used to determine the relative fraction of processed BdlA, reported as the percentage of the band intensity of processed BdlA relative to the intensity of all detectable BdlA bands. Error bars represent standard deviations. *, significantly different from results for PAO1 ($P < 0.05$). (D) Complementation of $\Delta gcbA$ cells with wild-type *gcbA* but not *gcbA*-GGAAAF restores dispersion of 120-h-old tube reactor-grown biofilms in response to an increased glutamate concentration in the medium. (E) Inactivation of the DGC-encoding genes *wspR* and *siaD* does not abrogate the dispersion response of *P. aeruginosa* biofilm cells triggered by increased glutamate concentration. Error bars denote standard deviations. All experiments were performed in triplicate.

ings of GcbA participating in the regulation of BdlA cleavage in biofilm cells soon after attachment further prompted us to ask whether GcbA is regulated in a growth mode-specific manner. The transcript levels of *gcbA*, as revealed by qRT-PCR analysis, were found to be highest in planktonic cells, with an 8-fold increase relative to the transcript levels in biofilm cells (Fig. 5A). In contrast, *gcbA* expression was found to be significantly reduced in cells remaining attached to the surface after dispersion relative to its expression in biofilm cells prior to dispersion (Fig. 5A).

In order to assess the native production levels of the GcbA protein, we used a chromosomally encoded V5/His₆-tagged GcbA (GcbA-V5/His₆) construct introduced into the wild-type PAO1 strain (PAO1::P_{*gcbA*}-*gcbA*-V5/His₆). In accord with the qRT-PCR data, GcbA protein production was significantly reduced in biofilm cells, with GcbA in biofilms grown for 144 h not detectable via

the immunoblotting methods utilized (Fig. 5B). The observation of GcbA downregulation in biofilm cells relative to its expression in their planktonic counterparts was somewhat unexpected considering the contribution of the DGC to BdlA processing during biofilm growth. Given that GcbA appeared to contribute to BdlA processing shortly after attachment, the GcbA protein levels were also assessed in initially attached cells following 24 h of growth at the surface. In contrast to 144-h-old biofilms, however, the GcbA levels in 24-h attached cells were similar to those observed for planktonic cells (Fig. 5B).

GcbA production can be induced via reduction of cellular c-di-GMP levels. As both *gcbA* transcript and GcbA protein levels were found to be higher in free-floating cells, which are associated with elevated PDE activity and reduced c-di-GMP levels, the effect of cellular c-di-GMP levels on GcbA production was subsequently assessed. We reasoned that, if GcbA production is c-di-GMP dependent, controlling the c-di-GMP levels could modulate the abundance of GcbA. Specifically, we asked whether GcbA production in biofilm cells could be induced or enhanced by overexpressing PDE-encoding genes. Two genes encoding previously confirmed active PDEs, PA2133 (43) and *dipA* (36), were selected to test this. Multicopy expression of *dipA* has previously been shown to reduce c-di-GMP levels 2-fold relative to its levels in wild-type biofilm cells (36), while overexpression of PA2133 has been shown to reduce c-di-GMP below detectable levels (43). The expression of either *dipA* or PA2133 induced GcbA production in biofilm cells to detectable levels (Fig. 5C to E). However, despite exhibiting higher total cellular PDE activity (Fig. 5F), PAO1/pJN-PA2133 biofilm cells harbored GcbA at levels lower than those observed in PAO1/pJN-*dipA* biofilm cells (Fig. 5C to E). Considering that dispersion has been previously found to require DipA but not PA2133 (36), these findings suggested a specificity of the c-di-GMP-dependent regulation of GcbA induction by demonstrating that a dispersion-specific PDE has a more profound effect on GcbA abundance.

In order to assess whether elevated c-di-GMP levels could reduce GcbA levels in planktonically growing cells, a *wspF* mutant, which is characterized by constitutive phosphorylation and activation of the DGC WspR (43), was used. However, no decrease in GcbA production was observed in $\Delta wspF$ cells relative to its production in wild-type PAO1 cells, with inactivation of *wspF* resulting in a statistically insignificant fold increase of 2.1 ± 1.2 in GcbA levels (Fig. 5G).

In order to assess whether conditions associated with surface-attached growth, other than elevated c-di-GMP levels, could account for suppression of GcbA abundance, GcbA levels were assessed in planktonic cells exposed to conditions of increasing viscosity. While the addition of 3% Ficoll had no effect on GcbA production, increasing the viscosity further by the addition of 15% Ficoll, a concentration previously suggested to mimic conditions of swarming motility and initiation of biofilm growth (63), correlated with a $20.5\% \pm 9.9\%$ reduction of detectable GcbA abundance relative to the GcbA levels observed in the absence of Ficoll (Fig. 5H). Taken together, these findings suggested that, while GcbA production is suppressed upon transition to biofilm growth, potentially due to sensing of surface growth and reduced motility, GcbA is upregulated upon the return to free-swimming growth via a dispersion-specific reduction in the cellular c-di-GMP levels.

BdlA interacts with and requires GcbA to induce dispersion. The MCP BdlA and the PDE DipA have recently been suggested to

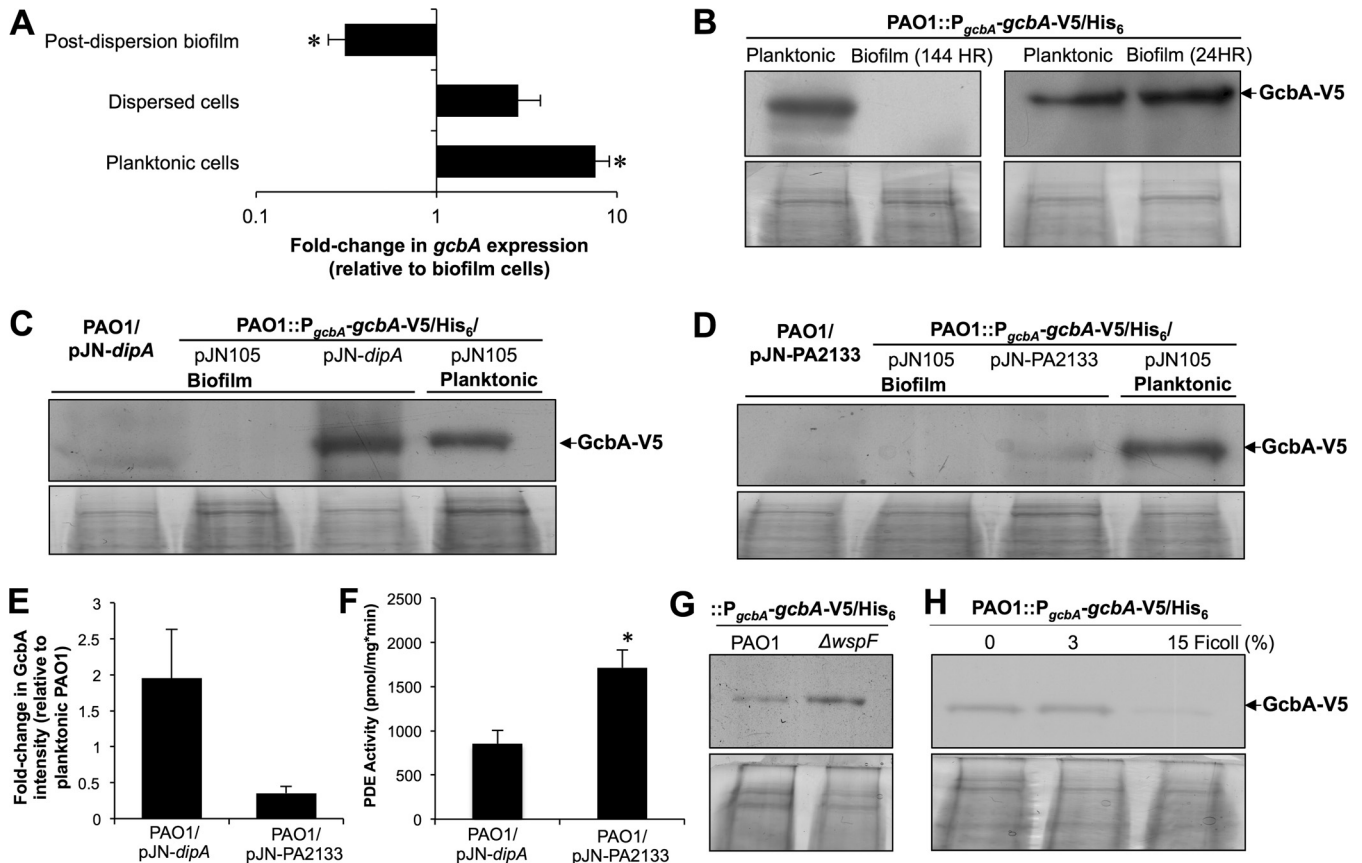


FIG 5 GcbA is regulated in a growth mode- and c-di-GMP-dependent manner. (A) Relative transcript abundances of *gcbA* in *P. aeruginosa* PAO1 exponential-phase planktonic cells, cells dispersed from 144-h-old biofilms in response to a changed in the glutamate concentration in the medium (Dispersed cells), and cells remaining within the biofilm following dispersion (Post-dispersion biofilm). Transcript abundance is reported relative to that of PAO1 cells grown as biofilms for 144 h. Error bars denote standard deviations. *, significantly different from expression in 144-h PAO1 biofilm cells ($P < 0.05$). (B) Detection with anti-V5 antibody of chromosomally encoded C-terminally V5/His₆-tagged GcbA in total cell extracts of planktonic or biofilm (24- or 144-h-old) PAO1 cells. (C, D) Overexpression of genes encoding the PDEs DipA (C) and PA2133 (D) induced production of GcbA to detectable levels in *P. aeruginosa* cells grown as biofilms, as revealed by anti-V5 immunoblot analysis of chromosomally encoded C-terminally V5/His₆-tagged GcbA. Planktonically grown cells harboring the empty pJN105 vector were used as positive controls. (E) ImageJ software densitometry analysis was used to determine the relative abundance of GcbA in PAO1/pJN-*dipA* or PAO1/pJN-PA2133 biofilm cells with respect to GcbA levels detected in PAO1 planktonic cells. Here, PAO1 refers to PAO1::P_{*gcbA*}-*gcbA*-V5/His₆. (F) Total cellular PDE activity of cell extracts obtained from *P. aeruginosa* PAO1 biofilms overexpressing *dipA* or PA2133 was determined using the synthetic chromogenic substrate bis(*p*-nitrophenyl) phosphate (bis-pNPP) and measuring the release of *p*-nitrophenol (pNP) at 405 nm. Error bars denote standard deviations. *, significantly different from results for PAO1 biofilms overexpressing *dipA* ($P < 0.05$). (G) Detection with anti-V5 antibody of chromosomally encoded C-terminally V5/His₆-tagged GcbA in total cell extracts of planktonically grown PAO1 and isogenic Δ*wspF* mutant cells. (H) Increasing viscosity leads to reduced GcbA levels, as determined via anti-V5 antibody detection of chromosomally encoded C-terminally V5/His₆-tagged GcbA in total cell extracts of planktonically grown PAO1 in the presence of increasing concentrations of Ficoll. All experiments were performed in triplicate. Panels below immunoblot images (B, C, D, G, H) are Coomassie-stained gels after transfer to ensure equal TCE protein loading.

participate in a signaling network regulating dispersion in *P. aeruginosa*: while evidence suggested that BdlA interacts with and activates the activity of DipA, DipA has been shown to reduce c-di-GMP to enable dispersion and, also, reduce BdlA processing in biofilms (36–38). Considering the present observations of GcbA being required for biofilm dispersion, we asked whether GcbA also interacts with BdlA. The interaction of BdlA and GcbA in *P. aeruginosa* cells was assessed using a PAO1 strain harboring HA-tagged BdlA (BdlA-HA) and V5/His₆-tagged GcbA (GcbA-V5/His₆). DipA-V5/His₆ (37) and BfiS-V5/His₆ were used as prey proteins for positive- and negative-control assays, respectively (Fig. 6A). Following the enrichment of BdlA-HA by immunoprecipitation using anti-HA antibodies, the presence of copurifying V5/His₆-tagged proteins was assessed using anti-V5 immunoblotting. BfiS-V6/His₆ did not copurify with BdlA-HA, while in accord

with previous observations, BdlA-HA interacted with DipA-V5/His₆ (Fig. 6A) (37). While no GcbA-V5/His₆ was isolated in the absence of BdlA-HA, the protein was detected as copurifying with BdlA-HA in total protein extracts derived from PAO1 cells grown planktonically (not shown) or as biofilms (Fig. 6A), suggesting that GcbA interacts, at least indirectly, with BdlA. Considering that BdlA was found to interact with both DipA and GcbA, it is likely that GcbA may also interact with DipA.

The findings of BdlA-GcbA interaction suggested that GcbA plays a potential role within the BdlA-dependent dispersion network. Subsequently, a cross-complementation analysis was carried out to determine the hierarchical organization of GcbA with respect to BdlA. While the expression of *gcbA* and *bdlA* rescued the dispersion phenotypes of the respective mutants, multicopy expression of *gcbA* in Δ*bdlA* (Δ*bdlA*/pJN-*gcbA*) biofilm cells failed to

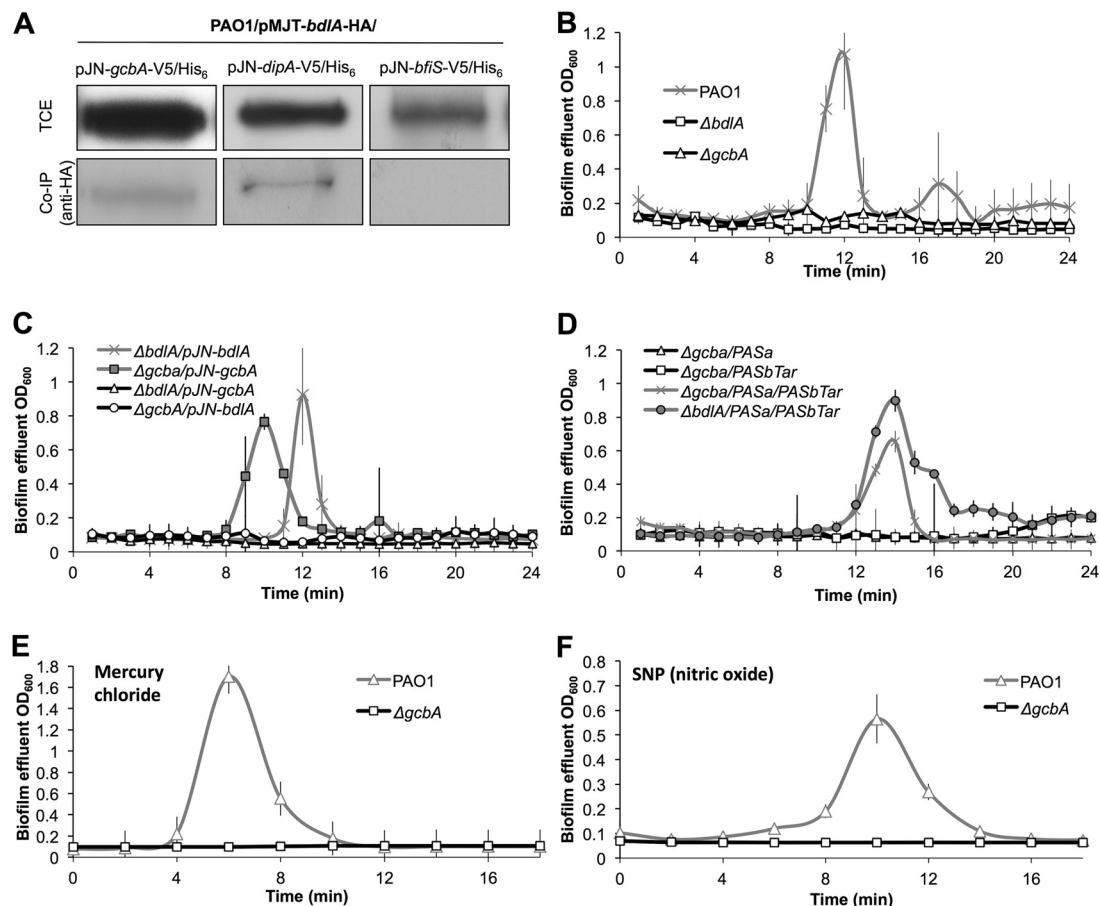


FIG 6 GcbA is central to the *P. aeruginosa* biofilm dispersion response. (A) BdlA interacts with GcbA but not with the negative control BfiS as revealed by *in vivo* pulldown assays and subsequent immunoblotting analysis. Immunoprecipitation using anti-HA antibody and C-terminally HA-tagged BdlA as bait was followed by anti-V5 antibody detection of indicated C-terminally V5/His₆-tagged prey proteins. Co-IP, coimmunoprecipitation. (B to D) The ability of 120-h-old biofilms to initiate dispersion in response to an increased glutamate concentration in the medium was assessed for wild-type PAO1 and isogenic *bdlA* and *gcbA* mutants (B), $\Delta gcbA$ or $\Delta bdlA$ mutants exhibiting multicopy expression of *gcbA* or *bdlA* (C), and the indicated mutant strains exhibiting multicopy expression of constructs encoding truncated peptides of BdlA corresponding to its PASa and PASbTar domains (D). (E, F) Induction of dispersion in response to mercury chloride (E) or nitric oxide (source, sodium nitroprusside [SNP]) (F) was assessed using 120-h-old biofilms of the wild-type PAO1 and isogenic *gcbA* mutant strains. For all assays, dispersion events were detected as increases in the biofilm effluent optical density. Error bars denote standard deviations. All experiments were performed in triplicate.

restore the dispersion response (Fig. 6B and C). We subsequently reasoned that, if GcbA is involved in the regulation of BdlA levels but not of BdlA cleavage, multicopy expression of *bdlA* will restore the dispersion response of biofilms formed by $\Delta gcbA$ to wild-type levels. However, if GcbA is responsible for regulating the post-translational modification and activation of BdlA, overexpression of *bdlA* will not rescue the *gcbA* dispersion-deficient phenotype. In agreement with our findings indicating GcbA to specifically modulate posttranslational modification of BdlA, multicopy expression of *bdlA* in $\Delta gcbA$ biofilm cells did not restore biofilm dispersion in response to an increased glutamate concentration in the medium (Fig. 6C).

The $\Delta gcbA$ dispersion-deficient phenotype can be rescued by separately encoded truncated domains of BdlA. Our data demonstrated that inactivation of GcbA impaired the biofilm-specific cleavage of BdlA, which correlated with an impaired biofilm dispersion response. Considering previous findings highlighting the importance of BdlA processing in enabling the dispersion response, we next asked whether the impairment in BdlA cleavage is

specifically responsible for the dispersion-deficient phenotype of $\Delta gcbA$ biofilm cells. Specifically, we tested whether *in trans* expression of separately encoded PASa and PASbTar domains of BdlA is capable of rescuing the dispersion-deficient phenotype of *gcbA* mutant biofilms, in a manner similar to that previously observed for *bdlA* mutant biofilms (38). The expression of constructs encoding BdlA-PASa or BdlA-PASbTar ($\Delta gcbA$ /PASa or $\Delta gcbA$ /PASbTar) in $\Delta gcbA$ cells did not restore dispersion in response to an increased glutamate concentration to wild-type levels (Fig. 6B and D). In contrast, coproduction of the separately encoded BdlA peptides PASa and PASbTar rescued the dispersion phenotype of $\Delta gcbA$ biofilm cells to the levels observed for the wild-type or $\Delta bdlA$ /PASa/PASbTar (Fig. 6B and D), suggesting that BdlA functions downstream from GcbA.

GcbA is required for the regulation of responses to various dispersion-inducing cues. The regulatory network containing BdlA and DipA regulates biofilm dispersion in response to a variety of substances, including carbohydrates (e.g., glutamate and succinate), heavy metals (e.g., silver nitrate and mercury chlo-

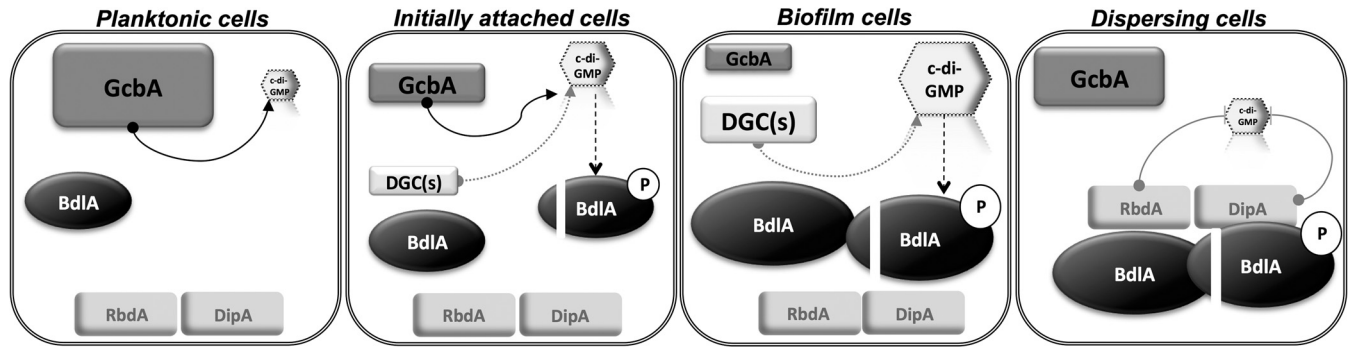


FIG 7 Proposed model of GcbA-mediated modulation of the BdlA-dependent biofilm dispersion regulatory network. GcbA and BdlA are required for biofilm dispersion in response to changes in the extracellular environment, with the levels of the two proteins regulated inversely during motile-sessile and sessile-motile transitions in a manner dependent on c-di-GMP levels. GcbA, produced and active predominantly in planktonic and initially attached cells, synthesizes c-di-GMP (indicated by solid black line) to promote the transition from reversible to irreversible surface attachment and to contribute to the posttranslational modification and cleavage of BdlA in initially attached cells through an as-yet-uncharacterized mechanism (indicated by black dashed line). Other, unidentified DGC(s), which may or may not be produced in a growth-mode specific manner, potentially act in concert with GcbA to contribute to the c-di-GMP pool regulating the processing of BdlA, as indicated by the gray dotted line. Concomitant with initiation of attachment and activation of BdlA processing, conditions of elevated viscosity and reduced cellular motility lead to a reduction in GcbA abundance during subsequent biofilm growth. Following activation via cleavage, BdlA within biofilm cells directly interacts with the PDEs RbdA and DipA, likely modulating their activity to reduce c-di-GMP levels following the sensing of dispersion-inducing conditions. Direct reductions in c-di-GMP levels facilitated by RbdA and DipA are indicated by solid gray lines. During the dispersion process, DipA functions to reduce c-di-GMP levels to facilitate the sessile-motile transition and to induce GcbA production, thus activating a mechanism that will allow the newly detached cells to attach to a surface in new locations. The relative sizes of symbols indicating proteins and c-di-GMP in the model correlate with their relative abundances in the cell during the indicated growth mode.

ride), and nitric oxide, with inactivation of either *bdlA* or *dipA* abrogating the dispersion response to all of these cues (12, 36). Considering that GcbA was found to be necessary for the activation of BdlA and for induction of dispersion in response to a change in the glutamate concentration, we next assessed the ability of Δ *gcbA* biofilm cells to respond to heavy metals and nitric oxide. With biofilms formed by the wild-type PAO1 used as a positive control, inactivation of *gcbA* was found to abrogate biofilm dispersion in response to mercury chloride and nitric oxide (Fig. 6E and F). These findings suggested that, similar to BdlA and DipA, GcbA is required for enabling dispersion in response to a wide variety of extracellular cues.

DISCUSSION

The response of *P. aeruginosa* biofilms to various dispersion-inducing conditions requires the reduction of cellular c-di-GMP levels via the activity of a complex regulatory network that to date has been found to contain the MCP BdlA and the c-di-GMP-degrading PDEs DipA, RbdA, NbdA, and MucR (12, 15, 17, 36–38). Here, we provide evidence that the catalytically active c-di-GMP-synthesizing DGC GcbA (39) is required for biofilm dispersion by regulating the posttranslational processing of BdlA. Considering that dispersion has been associated with reduction of c-di-GMP levels and that a DGC has not been previously demonstrated to be required for dispersion, the present findings were somewhat unexpected and raised a number of vital questions. For one, how does a DGC contribute to biofilm dispersion, a process that is associated with decreases in c-di-GMP levels? Previous findings have suggested that BdlA, which directly interacts with and potentially regulates the activities of the PDEs DipA and RbdA, plays a central role in the dispersion response, being required for biofilm cells to respond to diverse classes of dispersion-inducing cues, including carbohydrates, ammonium chloride, heavy metals, and NO (37, 38). While indirectly serving to regulate c-di-GMP levels to enable the dispersion response, BdlA post-

translational modification and activation, in turn, are dependent on elevated c-di-GMP levels following the initiation of surface-associated growth. In the present work, we demonstrate that the DGC GcbA contributes to the cleavage of BdlA in *P. aeruginosa* cells following initial attachment to a surface and, consequently, indirectly plays a role in enabling the regulation of biofilm dispersion, demonstrating a novel role for a DGC in the regulation of not only motile-sessile but also sessile-motile transitions (Fig. 7). We furthermore demonstrate that the levels of GcbA and BdlA are inversely regulated during motile-sessile and sessile-motile transitions in a manner that is dependent, at least in part, on cellular c-di-GMP levels (Fig. 7). The DGC GcbA, produced and active predominantly in planktonic and initially attached cells, synthesizes c-di-GMP to promote the transition from reversible to irreversible surface attachment and to contribute to the cleavage of BdlA in initially attached cells. Concurrent with the initiation of surface-associated growth and activation of BdlA processing, conditions of high viscosity limiting cellular motility lead to a reduction in the detectable levels of GcbA during subsequent biofilm growth (Fig. 7).

Based on our previous findings, within biofilm cells, BdlA directly interacts with the PDEs RbdA and DipA and likely contributes to the regulation of their activity to reduce c-di-GMP levels following sensing of dispersion-inducing conditions (12, 36–38). During the dispersion process, the DipA-dependent reductions in cellular c-di-GMP levels not only facilitate the change in cellular physiology that allows biofilm cells to undergo the sessile-motile transition but also induce GcbA production, thereby activating a system that will allow the newly detached cells to attach to a surface in new locations (Fig. 7).

Another important question was raised by the results of the present study: how does a DGC that has previously been found to play a specific role in enabling surface attachment and to have no effect on biofilm formation contribute to biofilm-specific regulation of BdlA? GcbA was recently found to promote initial attach-

ment to the surface through the regulation of a very specific phenotypic output (39). While not affecting extracellular polymeric substance (EPS) production, GcbA was found to facilitate the transition from reversible to irreversible attachment to a surface via regulation of flagellum-associated motility and flagellar reversal rates in a manner independent of surface hardness or previously characterized mechanisms, including FleQ, flagellar stators, chemotaxis IV gene cluster, and SadC and SadB (39). Although the present findings ascribe an additional role to GcbA, they do not necessarily contradict the previous findings of GcbA carrying out a specific role during initial surface attachment. This is because BdlA is processed soon after initial surface contact, with cleavage products detectable as early as following 8 h of surface-associated growth, with *gcbA* inactivation impairing BdlA cleavage in initially attached and developed biofilm cells. It is thus possible to suggest that, rather than being directly activated by GcbA-synthesized c-di-GMP, BdlA processing is potentially induced via sensing of reduced motility or of surface contact following GcbA-mediated reduction of flagellar reversals, motility, and surface contact and attachment. The question of the effects of EPS production, motility, and surface contact sensing on the initiation of BdlA processing, as well as the suppression of GcbA levels, will be the focus of future investigations.

Our present findings underscore the importance of the question of localized c-di-GMP pools regulating specific phenotypic outputs versus total cellular c-di-GMP levels regulating overall cellular behavior and determining the physiology and mode of growth of the cells. Specificity of c-di-GMP regulation was suggested by the observation of DipA having a more profound effect than the more active PDE PA2133 on the induction of GcbA production in biofilm cells (Fig. 5C to F). In contrast, the observations of GcbA playing a role in the regulation of both surface attachment and biofilm dispersion may be viewed as contradicting the concept of distinct c-di-GMP pools regulating specific phenotypic outputs. Furthermore, it is important to note that inactivation of *gcbA* did not completely abrogate BdlA processing, as smaller amounts of BdlA cleavage products were detectable in *gcbA* biofilms and at significantly lower levels than in wild-type biofilms. It is important to point out that previous findings have indicated that at least one of the cleavage products is still detectable for a BdlA variant with an M130I substitution at the putative ClpP cleavage site (38). However, the present findings may also suggest that GcbA potentially works in concert with other DGCs to regulate BdlA cleavage (Fig. 7). However, despite the possibility of multiple DGCs contributing to the activation of signaling systems enabling biofilm dispersion, the process does exhibit some specificity, as inactivation of the DGCs WspR and SiaD did not abrogate the dispersion response (Fig. 4E).

The finding of BdlA processing being activated soon following the transition to surface-associated growth may have important implications for our understanding of the regulation of dispersion mechanisms; specifically, the difference between environmentally and self-triggered or native dispersion. Native biofilm dispersion, mediated by the signaling molecule *cis*-DA, occurs when bacterial cells within biofilm microcolonies become motile and evacuate the structures through an opening into the bulk fluid, leaving behind a void within the microcolony (3, 60). Previous observations have indicated that native biofilm dispersion depends on the developmental stage of the biofilm and/or the size of the biofilm microcolonies (3, 60). Moreover, the findings of native biofilm

microcolony dispersion requiring a minimum diameter of 40 μm and a minimum thickness of 10 μm suggested that the accumulation of the self-produced intracellular signaling molecule provides a way for biofilm cells to control their own accumulation and density (3, 60). In contrast, the dispersion process in response to changes in the extracellular environment is likely not dependent on cell density or microcolony formation. This is based on our finding that BdlA, a central modulator of these types of dispersion events, is activated via posttranslational processing soon after initial attachment and, thus, prior to initiation of biofilm maturation, significant biomass accumulation, or microcolony formation. Evidence for this is also provided by previous microscopic observations of nutrient-induced dispersion occurring within and outside microcolonies, as well as by findings of nutrient-induced dispersion not being affected in certain biofilms (such as those formed by ΔflgB or ΔpilA mutant strains or the *dipA*-overexpressing PAO1) that were characterized by significantly reduced biomass accumulation and thicknesses comparable to those of wild-type cells at the initial attachment stages of biofilm development (12, 37, 54, 57, 60). Together with these findings, the present results suggest that attached cells are likely able to respond to changing environmental conditions in a manner independent of biomass accumulation and microcolony size, with GcbA contributing to the activation of BdlA and mechanisms for sensing and relaying dispersion-inducing cues soon after the initial surface attachment.

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