3-21-2014

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Deletion Mutant Library for Investigation of Functional Outputs of Cyclic Diguanylate Metabolism in *Pseudomonas aeruginosa* PA14

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We constructed a library of in-frame deletion mutants targeting each gene in *Pseudomonas aeruginosa* PA14 predicted to participate in cyclic di-GMP (c-di-GMP) metabolism (biosynthesis or degradation) to provide a toolkit to assist investigators studying c-di-GMP-mediated regulation by this microbe. We present phenotypic assessments of each mutant, including biofilm formation, exopolysaccharide (EPS) production, swimming motility, swarming motility, and twitch motility, as a means to initially characterize these mutants and to demonstrate the potential utility of this library.

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium found in a diverse array of environments, ranging from soil to the mammalian host. As an opportunistic bacterial pathogen, individuals with a compromised immune system, epithelial damage, or cystic fibrosis (CF) can succumb to its colonization and infection (1–3). In addition to its arsenal of virulence factors, including exotoxin A, exoenzyme S, phenazines, rhamnolipids, and lipopolysaccharides, *P. aeruginosa* is also capable of switching its life-style from a planktonic growth mode to a surface-associated life-style in response to environmental stimuli. Biofilm-associated infections have garnered significant attention in the medical context due to the increased antibiotic tolerance of these communities (4–6) and their potential as a reservoir of infection in the host (7, 8).

Cyclic diguanylate (c-di-GMP) is a ubiquitous second-messenger molecule capable of regulating a myriad of cellular functions in bacteria. Studies to date have demonstrated numerous functions regulated by this cyclic dinucleotide, including, but not limited to, bacterial physiology (9–14), group behavior (15–21), and virulence (21, 22). The level of c-di-GMP in the cell is contingent on the activity of two classes of enzymes: diguanylate cyclase (DGC) and phosphodiesterase (PDE). DGCs, with a canonical GG(D/E)EF motif, synthesize c-di-GMP from two molecules of GTP (23, 24), while PDEs, with a canonical E(A/E/V)L motif, degrade c-di-GMP to pGpG (24). More recently, proteins with an HD-GYP domain were also shown to demonstrate c-di-GMP phosphodiesterase activity (25). Current models correlate high intracellular c-di-GMP levels to a sessile life-style, or a biofilm state, while low levels of this signal promote motility and/or planktonic growth. Such a simple model likely underestimates the complexity of the c-di-GMP signaling network, as implied by 40 DGCs and PDEs encoded in the genome of *P. aeruginosa* PA14.

A surging interest in c-di-GMP and its functional outputs in *P. aeruginosa* has resulted in a large number of publications focused primarily on forward-genetics approaches (9, 13, 16–18, 26–28). For many, the emergence of a publicly available, nonredundant transposon insertion library in *P. aeruginosa* PA14 (29) proved very useful in advancing studies of this microbe. For example, Kulasakara et al. benefited from this library in their genome-wide analysis of DGC and PDE mutants in *P. aeruginosa* PA14 (30). Although useful, drawbacks of transposon insertion mutants are 2-fold: (i) the potential for partial loss of function at the insertion site and (ii) the possibility of polarity, causing either loss or increased expression of downstream genes.

Here, we constructed a library of in-frame deletion mutants targeting each gene in *P. aeruginosa* PA14 predicted to participate in c-di-GMP synthesis or degradation. We present phenotypic assessments of each mutant, including biofilm formation, exopolysaccharide (EPS) production, swimming motility, swarming motility, and twitch motility, as a means to initially characterize these mutants as well as to demonstrate the potential value of this library to investigators in the field.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Strains, plasmids, and primers used in this study are listed in Tables S1 to S3 in the supplemental material. *Pseudomonas aeruginosa* strain UCBPP-PA14 (abbreviated *P. aeruginosa* PA14) was used in this study. *P. aeruginosa* PA14 and *Escherichia coli* were routinely cultured in lysogeny broth (LB) (31) at 37°C and, when appropriate, solidified with 1.5% agar and/or supplemented with gentamicin (Gm) at 10 μg ml^{-1} (*E. coli*) and 50 μg ml^{-1} (*P. aeruginosa*). Minimal M63 salts (32) supplemented with $MgSO_4$ (1 mM) plus glucose (0.2%) and Casamino Acids (0.5%) or supplemented with $MgSO_4$ (1 mM) plus arginine (0.4%) were used for biofilm assays. Swarming, swimming, and twitching motility medium contained M8 salts (33) with glucose (0.2%), Casamino Acids (0.5%), and $MgSO_4$ (1 mM). Congo red (CR) agar (1.5% agar) medium was the same as that used for swarming, swimming, and twitching motility assays but further supplemented with Congo red (40 μg ml^{-1}) and Coomassie brilliant blue dye (20 μg ml^{-1}), as reported previously (34).

Molecular techniques. Plasmids constructed during the course of this study were prepared by using homologous recombination in *Saccharomyces cerevisiae* (35). All restriction enzymes were obtained from New England BioLabs (Ipswich, MA). Plasmids constructed in yeast were subsequently extracted by a modified "smash-and-grab" method (36) and

Received 27 January 2014 Accepted 16 March 2014
Published ahead of print 21 March 2014
Editor: R. M. Kelly
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00299-14.

doi:10.1128/AEM.00299-14

electroporated into *E. coli* S-17 for confirmation by colony PCR (37). PCR-verified constructs were conjugated into *P. aeruginosa* PA14, as previously reported (15), and exconjugants were selected and counterselected by gentamicin and 5% sucrose, respectively. PCR amplification and subsequent DNA sequencing using primers flanking the site of deletion were performed to verify all resulting mutant candidates.

Biofilm assays. Biofilm formation assays on polyvinylchloride (PVC) plates (VWR Scientific, Waltham, MA) were performed as previously reported (38), with the following minor modifications. Bacterial cultures grown overnight in LB were diluted 1:100 in minimal M63 salts supplemented with $MgSO_4$ (1 mM) and either glucose (0.2%) and Casamino Acids (0.5%) or arginine (0.4%). Plates were incubated overnight at 37°C, followed by staining with 0.1% crystal violet and subsequent solubilization with 30% acetic acid. Solubilized crystal violet was transferred onto a new polystyrene microtiter dish (VWR Scientific, Waltham, MA) and quantified by measuring its optical density at 550 nm. The resulting values were normalized to wild-type (WT) *P. aeruginos*a strain PA14 (positive control) biofilm levels to simplify comparisons across numerous strains.

Congo red assays. Congo red assays were performed as previously described (18, 39). Qualitative assessment of Congo red staining was performed considering coloration (gradation from white to red) compared to WT *P. aeruginosa* strain PA14 (positive control) as well as colony morphology (wrinkly versus smooth). Each strain was tested on at least two different days with 3 to 4 technical replicates per day.

Swimming motility assays. Swimming motility assays were performed as previously reported (40), with the following minor modifications. Swim agar (0.3%) plates were poured and left to dry at room temperature (~25°C) for ~4 h prior to inoculation, with 3 to 4 replicates per strain. Plates were used on the day that they were poured. Each strain was tested on at least three separate days. All swim plates were incubated upright at 37°C for 24 h prior to measurements using ImageJ software (NIH). Swim zones of a strain, measured as the percentage of plate coverage, were normalized to the WT *P. aeruginosa* strain PA14 (positive-control) swim zone on the same plate. The resulting values from each set of replicates were averaged to yield a final swim zone coverage value for the particular strain.

Swarming motility assays. Swarming motility assays were performed as previously reported (40), with the following minor modifications. Swarm agar (0.5%) plates were poured and left to dry at room temperature (~25°C) for ~4 h prior to inoculation. Five to six replicates per strain per trial were tested, and all studies were repeated at least twice on different days. All swarm plates were incubated upright at 37°C for 16 h, followed by 24 h at room temperature. Swarming motility was quantified by using ImageJ software (NIH). Surface coverage was calculated by dividing the area of the swarm by the area of the entire plate, and the values from each replicate were averaged. These values were normalized to values for WT *P. aeruginosa* strain PA14 (positive-control) swarm coverage from the same batch of plates to facilitate comparisons among strains.

It is important to note that even the nonswarming Δ*flgK* mutant is capable of ~10% surface coverage, resulting from the colony formed at the site of inoculation expanding as bacterial cell numbers increase. Thus, strains with swarm coverage of 10 to 20% may indicate no significant change from the negative control.

Twitch motility assays. Twitch motility assays were performed as previously reported (41), with minor modifications, as follows. Twitch motility agar (1.5%) plates contained minimal M8 salts supplemented with $MgSO_4$ (1 mM), glucose (0.2%), and Casamino Acids (0.5%). Cells were stabbed into the bottom of the agar plate by using a toothpick and incubated upright at 37°C overnight, followed by 48 h of incubation at room temperature (~25°C). Each strain was tested on three separate days. After incubation, agar was carefully removed, and the basal surface was stained with 0.1% crystal violet (Sigma, St. Louis, MO) to facilitate visualization of the twitch zones. Twitch zones were determined by using ImageJ software (NIH), by measuring the area covered by the twitch motility zone (stained with crystal violet) and dividing it by the twitch area of WT *P. aeruginosa* PA14 (positive control). The resulting twitch zone values were averaged to yield a final twitch zone value for the particular strain.

Statistical analysis. The two-tailed Student *t* test was performed pairwise, between the wild type and each strain, by using GraphPad Prism software (GraphPad, La Jolla, CA).

RESULTS AND DISCUSSION

Complete library of strains carrying diguanylate cyclase and phosphodiesterase deletion mutations. Analysis of the genome of *P. aeruginosa* PA14 revealed 40 genes with predicted GGDEF, EAL, and HD-GYP domains either singly or in combination (30). These c-di-GMP metabolism proteins can be divided into four subclasses: 16 GGDEF-only, 5 EAL-only, 16 dual-domain GGDEF-EAL, and 3 HD-GYP-only proteins (Fig. 1). It is important to note that while alignments of primary amino acid sequences have been largely successful in determining whether a GGDEF/EAL/HD-GYP domain is either functional or degenerate, definitive conclusions require empirical data. For example, *in vitro* analyses showed minimal DGC activity in a seemingly degenerate GDSIF motif in the PA14_65540 (*fimX*) gene (13) as well as for the PA14_53140 (*rbdA*) gene, which has the canonical GGDEF motif (28).

The presence of other domains in proteins carrying GGDEF/EAL/HD-GYP domains can provide some functional context for a particular protein. Therefore, we first performed an analysis of all 40 c-di-GMP metabolism proteins for putative domains using the publicly available SMART algorithm (42, 43). Of the 40 sequences queried, 19 are predicted to be either exported or membrane bound based on the presence of either a transmembrane (TM) or signal domain(s) at the N-terminal region of the protein. These include 8 GGDEF-only (PA14_20820, PA14_26970, PA14_40570, PA14_49890, PA14_50060 [*roeA*], PA14_53310, PA14_56280 [*sadC*], and PA14_65090), 2 EAL-only (PA14_14530 and PA14_36260), and 9 dual-domain GGDEF-EAL (PA14_03720, PA14_07500, PA14_21190, PA14_37690, PA14_42220 [*mucR*], PA14_53140 [*rbdA*], PA14_56790 [*bifA*], PA14_60870 [*morA*], and PA14_71850) proteins (Fig. 1). We are aware of alternative predictive algorithms, and while such predictions are useful, we emphasize that they are sometimes inconsistent with experimental studies. For example, PA14_66320 (*dipA*) was shown to be an inner membrane or inner membrane-associated protein (26) despite its lack of obvious transmembrane or signal domains (Fig. 1).

The PAS domain was the most prevalent domain identified in our analysis. Observed in 12 different proteins, the number of PAS domains can range from 1 (e.g., PA14_03790) to 4 (e.g., PA14_07500) within a single protein. PAS domains are generally regarded as signal sensors, including signals such as oxygen and redox potential (44, 45). Moreover, PAS domains can also mediate protein-protein interactions (46, 47), perhaps allowing these proteins to participate in larger complexes. Each PAS domain was frequently associated with a downstream PAC domain, with the few exceptions to this organization including PA14_60870 (*morA*), PA14_65540 (*fimX*), and PA14_66320 (*dipA*) (Fig. 1). The PAS-PAC organization is thought to provide structural stability to the PAS domain.

Other domains with putative or known sensor or signal transduction functions are also predicted with high frequency. These domains include REC, HAMP, and CHASE domains. REC domains, typically found in response regulators in two-component signaling systems, are predicted in three GGDEF-only proteins (PA14_16500 [*wspR*], PA14_57140, and PA14_64050), two EAL-

GGDEF only

PA#	PA14#	Predicted domains
0169 (*siaD*)	02110	
0290	03790	
0338	04420	
3702 (*wspR*)	16500	
3343	20820	
3177	23130	
2870	26970	
1851	40570	
1120 (*yfiN*)	49890	
1107 (*roeA*)	50060	
0847	53310	
4332 (*sadC*)	56280	
4396	57140	
4843	64050	
4929	65090	
5487	72420	

EAL only

PA#	PA14#	Predicted domains
3947 (*rocR*)	12810	
3825	14530	
2200	36260	
2133	36990	
- (*pvrR*)	59790	

HD-GYP only

PA#	PA14#	Predicted domains
4108	10820	
2572	30830	
4781	63210	

GGDEF + EAL

PA#	PA14#	Predicted domains
0285	03720	
0575	07500	
3311	21190	
3258	21870	
2567	31330	
2072	37690	
1727 (*mucR*)	42220	
1433	45930	
1181	49160	
0861 (*rbdA*)	53140	
4367 (*bifA*)	56790	
4601 (*morA*)	60870	
4959 (*fimX*)	65540	
5017 (*dipA*)	66320	
5295	69900	
5442	71850	

Legend

Symbol	Domain
	CBS
	GAF
	Unknown function
	CC
	PSB
	Signal
	TM
	CHASE
	HAMP
	HD
	EAL
	GGDEF
	PAC
	PAS
	7TM
	REC
	Repeat

FIG 1 Predicted domains in GGDEF-only, EAL-only, HD-GYP-only, and dual-domain GGDEF-EAL proteins. Based on the SMART algorithm, putative functional domains were identified in each protein (42, 43). Note that the cartoon depiction of each protein is not drawn to scale. The CBS (cystathionine beta-synthase) domain is predicted to serve as a binding site for adenosine and derivatives, as well as playing a regulatory role in regulating enzyme activity; the GAF (cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA) domain is present in cGMP-specific phosphodiesterases, adenylyl and guanylyl cyclases, and phytochromes as well as FhlA, a regulator of nitrogen fixation in bacteria; the CC (coiled-coil) domain is a structural motif in which α-helices intertwine; the PSB (periplasmic substrate binding) domain participates in ligand binding; Signal indicates the protein secretion signal; the TM (transmembrane) domain is the inner membrane transmembrane domain; the CHASE domain (extracellular sensory domain) is a predicted ligand binding domain; the HAMP (histidine kinases, adenylyl cyclases, methyl binding proteins, phosphatases) domain is a signal transduction domain; HD indicates the HD-GYP domain, a domain found in c-di-GMP degrading phosphodiesterases; the EAL (phosphodiesterase) domain is found in c-di-GMP-degrading phosphodiesterases; the GGDEF (diguanylate cyclase) domain is conserved in proteins that produce c-di-GMP from two molecules of GTP; the PAC domain contributes to the PAS domain fold; the PAS domain is a signal transduction domain; the 7TM (7 transmembrane receptors with diverse intracellular signaling modules) domain is a signal transduction domain; the REC (receiver) domain is conserved in response regulators; and Repeat indicates repeat sequences.

only proteins (PA14_12810 [*rocR*] and PA14_59790 [*pvrR*]), two HD-GYP-only proteins (PA14_30830 and PA14_63210), and one dual-domain GGDEF-EAL protein (PA14_65540 [*fimX*]). HAMP and CHASE domains are less common, predicted in three (two GGDEF-only and one dual-domain GGDEF-EAL) and two (one GGDEF-only and one dual-domain GGDEF-EAL) proteins, respectively. Another domain, 7TM, is also thought to be involved in signal transduction. Described as "7 transmembrane receptors with diverse intracellular signaling modules," it is predicted in just one GGDEF-only protein, PA14_65090 (Fig. 1).

The GAF domain (regulatory domain in cyclic GMP-specific phosphodiesterases, adenylyl cyclases, and FhlA) is predicted in two dual-domain GGDEF-EAL proteins (PA14_31330 and PA14_66320 [*dipA*]). For PA14_66320 (*dipA*), binding of cyclic AMP (cAMP) to the GAF domain induced its phosphodiesterase activity (26). Whether the same function applies to PA14_31330 has not yet been explored. Other domains, including CBS (cystathionine beta-synthase) and PSB (periplasmic substrate binding) domains, were predicted in the dual-domain GGDEF-EAL proteins PA14_21870 and PA14_07500, respectively. Functions of CC (coiled-coil) (PA14_63210 and PA14_60870 [*morA*]) and repeat (PA14_02110) domains remain unknown for these proteins (Fig. 1).

Medium-specific impact of DGCs and PDEs on biofilm formation. Given the known role of c-di-GMP in biofilm formation, we tested the biofilm formation of each mutant under two growth conditions used routinely in our laboratory. The first, glucose supplemented with Casamino Acids (glucose+CAA), is the standard assay medium used in most of our previously reported studies (16, 17), and glucose is a common growth substrate used by many groups studying biofilm formation (48–51). The second substrate analyzed is arginine, one of the few carbon sources which *P. aeruginosa* can ferment and which we have shown previously boosts the levels of c-di-GMP by ~4-fold compared to growth on glucose+CAA (52). In all biofilms assays, the wild-type strain and the biofilm-deficient, hyperswarming Δ*sadC* Δ*roeA* double mutant (18) served as controls.

Among the mutants in the GGDEF-only subclass, the majority of mutants formed biofilms at levels ~60 to 70% of those of the wild type, whereas wild-type levels of biofilm formation were observed for strains carrying the ΔPA14_20820, ΔPA14_40570, ΔPA14_53310, ΔPA14_57140, and ΔPA14_65090 mutations. Apart from ΔPA14_20820, these phenotypes are in agreement with previous findings (30). Two mutants, ΔPA14_50060 (*roeA*) and ΔPA14_56280 (*sadC*), formed the weakest biofilms among this group, at roughly 20 to 50% of wild-type levels (Fig. 2A); these observations are in agreement with previous reports of these two well-characterized mutants (17, 18).

Most of the PDE mutants, including both EAL-only and HD-GYP-only subclasses, showed no significant difference from the wild-type strain when biofilm formation was assessed on glucose+CAA medium. In fact, the ΔPA14_30830 and ΔPA14_63210 HD-GYP mutants formed significantly reduced biofilms, at ~50% of wild-type levels (Fig. 2A). Based on the currently accepted model wherein elevated c-di-GMP levels promote biofilm formation, the fact that we observed reduced biofilm formation when we mutated genes coding for putative c-di-GMP-degrading phosphodiesterases is somewhat surprising. The lack of any biofilm-related phenotype among strains mutated for the production of EAL-only proteins (30) and the various phenotypes among strains lacking HD-GYP-only proteins (21) have been reported previously.

The last subclass, strains mutated for dual-domain GGDEF-EAL proteins, consisted of strains that were both weaker and stronger biofilm producers than the wild type. In contrast to *P. aeruginosa* PAO1 carrying a Δ*mucR* mutation, which formed a biofilm equivalent to that formed by the wild type (12), the ΔPA14_42220 (*mucR*) mutant formed the weakest biofilm among mutants in this class, at ~60% of wild-type levels. Strains carrying the ΔPA14_21190, ΔPA14_56790 (*bifA*), and ΔPA14_66320 (*dipA*) mutations, as expected (30), were all hyperbiofilm producers relative to the wild type, ranging from 160 to 300% of wild-type strain levels (Fig. 2A). Strains carrying ΔPA14_56790 (*bifA*) and ΔPA14_66320 (*dipA*) mutations, in particular, were previously reported to demonstrate enhanced biofilm formation (16, 26) and thus serve to validate our findings. Interestingly, mutation of the homolog of the PA14_53140 (*rbdA*) gene was shown to cause a hyperbiofilm phenotype in *P. aeruginosa* PAO1 (28, 30), but mutation of this gene showed wild-type levels of biofilm in the *P. aeruginosa* PA14 background (Fig. 2A). Thus, while there are similarities between strains PAO1 and PA14 of *P. aeruginosa*, these data suggest strain-specific differences in the roles of these proteins in biofilm formation.

As mentioned above, our group has shown that a biofilm medium supplemented with L-arginine (0.4%) promotes c-di-GMP production and, as a consequence, results in more pronounced biofilm formation by *P. aeruginosa* PA14 (52). Under these conditions, L-arginine is utilized as the sole carbon source in the presence of oxygen. Thus, to facilitate observations of minor changes in biofilm formation and potential medium-specific effects of the loss of the DGCs and/or PDEs, we tested the same library of mutants in minimal medium supplemented with arginine.

In general, the GGDEF-only subclass mutants showed negligible effects on biofilm formation in arginine medium. However, two mutants, the previously reported ΔPA14_50060 (*roeA*) and ΔPA14_56280 (*sadC*) mutants (17, 18), showed impaired biofilms, at approximately 40 to 50% of wild-type levels, while the ΔPA14_04420, ΔPA14_20820, ΔPA14_26970, and ΔPA14_64050 mutants produced hyperbiofilms, reaching 110 to 140% of wild-type levels (Fig. 2B).

Similarly, most strains carrying mutations in genes coding for the EAL-only and HD-GYP-only subclasses showed wild-type phenotypes, as shown by ΔPA14_36260, ΔPA14_36990, ΔPA14_59790 (*pvrR*), and ΔPA14_30830 mutant strains. However, noticeably weaker biofilm (ΔPA14_12810 [*rocR*] and ΔPA14_10820) and hyperbiofilm (ΔPA14_14530 and ΔPA14_63210) phenotypes were also observed (Fig. 2B).

The mutations in genes with dual GGDEF-EAL domains also showed various degrees of biofilm formation, with the ΔPA14_42220 (*mucR*) and ΔPA14_60870 (*morA*) mutants showing the weakest biofilm formation in this class of mutants, at 50 to 60% of wild-type levels. In contrast, the ΔPA14_21190, ΔPA14_56790 (*bifA*), ΔPA14_65540 (*fimX*), ΔPA14_66320 (*dipA*), and ΔPA14_71850 mutants produced hyperbiofilms at levels ranging from 110 to 150% of wild-type levels. Other mutants were not significantly different from the wild type, with a few showing a small, but significant, decrease, as seen, for example, with the ΔPA14_31330 mutant (Fig. 2B).

In sum, a total of 16 mutants showed medium-specific changes in biofilm formation. Of these, minimal medium supplemented

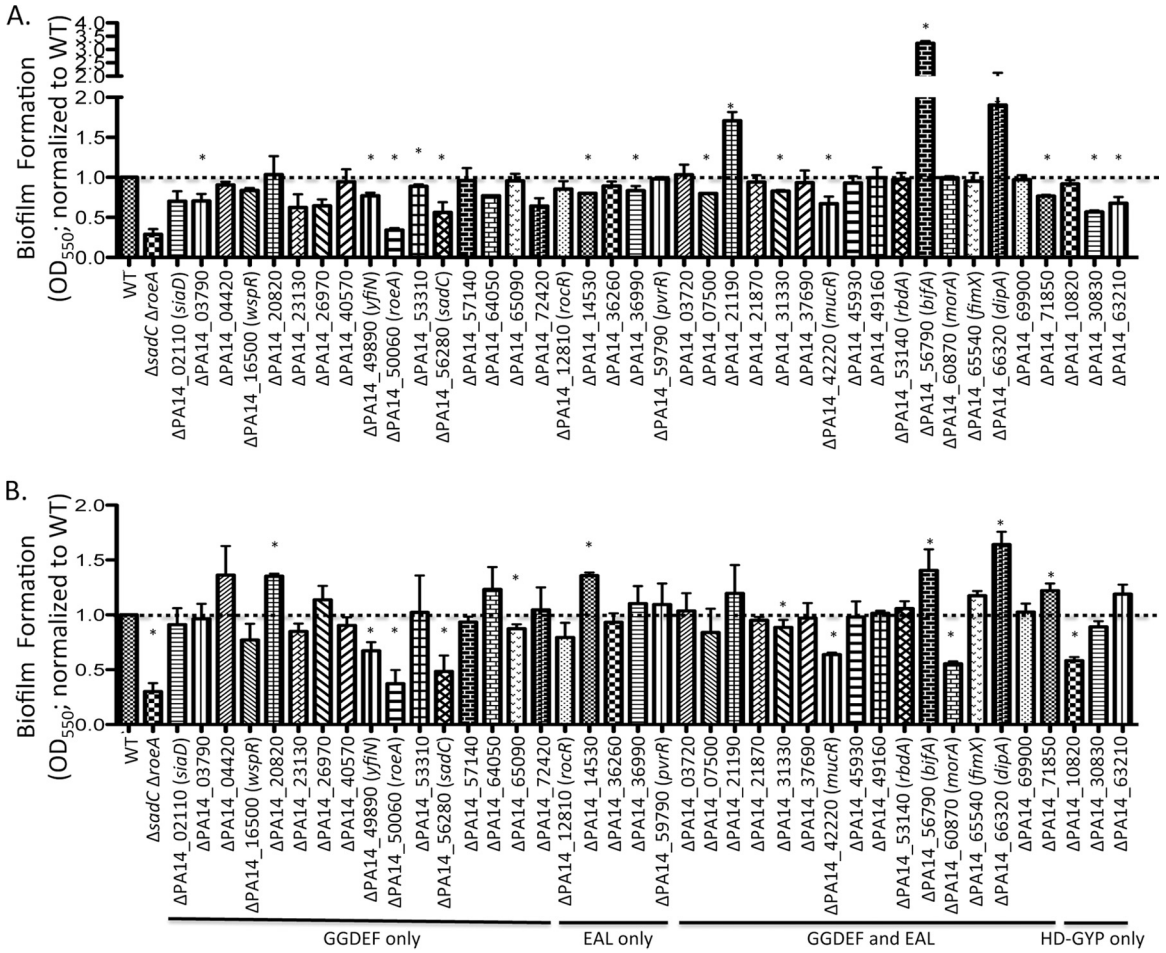

FIG 2 Biofilm formation. Shown is an analysis of biofilm formation by *P. aeruginosa* PA14 strains carrying mutations in genes encoding GGDEF-only, EAL-only, HD-GYP-only, and dual-domain GGDEF-EAL proteins in glucose+CAA (A)- and arginine (B)-supplemented media. The biomass of the attached cells on PVC plates was measured after 24 h of static incubation at 37°C. Final values corresponding to each mutant were normalized to the value for wild-type *P. aeruginosa* strain PA14, which was set to a value of 1, for ease of comparison. OD_{550}, optical density at 550 nm. *, $P < 0.05$.

with arginine increased biofilm formation in 14 of the 16 mutants. The net increase occurred in both hyperbiofilm mutants (e.g., ΔPA14_66320 [*dipA*]) as well as those with weak biofilms (e.g., ΔPA14_03790 and ΔPA14_72420), which is consistent with the previously reported arginine-dependent increase in intracellular c-di-GMP levels (52). Surprisingly, two mutants (ΔPA14_60870 [*morA*] and ΔPA14_10820) showed an arginine-dependent decrease in biofilm formation. It is important to note that the phenotypic differences highlighted in this work are unlikely to be attributed to growth defects, as all strains except the Δ*bifA* strain, which is known to be a hyperbiofilm former, showed no growth defect in glucose minimal medium (see Fig. S1 in the supplemental material).

Effects of c-di-GMP on exopolysaccharide production in *P. aeruginosa*. Extracellular matrix production is typically associated with biofilm formation; exopolysaccharides (EPS) are a critical component of this matrix. In *P. aeruginosa* PA14, two different types of EPS, Pel and alginate, are synthesized, of which Pel has been reported to be a critical factor for biofilm formation *in vitro* (39). Requiring the activities of the PelA to PelG proteins (53, 54), the level of Pel EPS production is typically positively associated with the intracellular pool of c-di-GMP (16, 18). Thus, investigating the impact of mutating genes encoding GGDEF-only, EAL-only, HD-GYP-only, and dual-domain GGDEF-EAL proteins on EPS production is of particular interest.

The qualitative assay used here exploits the ability of Congo red (CR) dye to bind a range of macromolecules, including its high affinity for cellulose in plants and fungi (55, 56). This dye can also bind amyloids (57). CR has been used to identify mutants defective in the production of Pel in the context of pellicle production and biofilm formation in *P. aeruginosa* PA14 (16–18, 39). While acknowledging the potential for CR to stain other macromolecules, a Pel-deficient *P. aeruginosa* PA14 mutant (Δ*pelA*) fails to bind CR (58), resulting in the production of smooth, white colonies, and thus, this mutant serves as a negative control (Fig. 3). In these assays, all strains are scored on a scale from 0 to 3, with the WT being scored as 2, the hyper-CR-binding ΔPA14_56790 (*bifA*) mutant being given a score of 3, and the Pel-deficient Δ*pelA* mutant being assigned a score of 0. The Δ*wspR* mutant, scored as 1, shows an intermediate phenotype between the wild type and the Δ*pelA* mutant (Fig. 3 and Table 1).

There were four mutants that scored "0" (ΔPA14_50060 [*roeA*], ΔPA14_07500, ΔPA14_60870 [*morA*], and ΔPA14_10820) across the four mutant subclasses (Table 1). These mutants

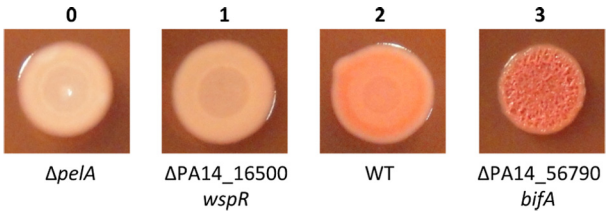

FIG 3 Congo red assays. Shown are representative images of Congo red binding assays for *P. aeruginosa* PA14 and strains carrying mutations in the *pelA*, PA14_16500 (*wspR*), and PA14_56790 (*bifA*) genes. As cited in text, the Δ*pelA* mutant, the ΔPA14_16500 (*wspR*) mutant, *P. aeruginosa* PA14 (wild type), and the ΔPA14_56790 (*bifA*) mutant are representative of scores of 0, 1, 2, and 3 on the score spectrum, respectively (see Table 1 for a complete list). The ΔPA14_56790 (*bifA*) mutant also forms a wrinkly colony morphology, which is indicated by "W" in Table 1.

failed to bind any CR following overnight incubation at 37°C and a lengthy incubation (~2 days) at room temperature. The ΔPA14_50060 (*roeA*) mutant was previously reported to show no CR binding (18). The next class, scored as "1," included eight mutants (ΔPA14_16500 [*wspR*], ΔPA14_20820, ΔPA14_23130, ΔPA14_53310, ΔPA14_36990, ΔPA14_37690, ΔPA14_30830, and ΔPA14_63210) across the subclasses. In particular, diminished CR binding of ΔPA14_16500 (*wspR*) was expected, given its role as a DGC in *P. aeruginosa* (59), and thus, this mutant served as a representative of this class of mutants. We observed 17 mutants with the wild-type phenotype and thus scored them as "2." Finally, there were 11 mutants that bound CR to a greater extent than the wild type (generally after overnight incubation at 37°C) and/or had a wrinkly colony morphology, which we interpret as excessive EPS production (Table 1). The previously reported ΔPA14_56790 (*bifA*) mutant (16) showed strong CR binding and a wrinkly colony morphology and served as the representative of this class of mutants, scored as "3" (Fig. 3 and Table 1). Similarly, the ΔPA14_21190 mutant also formed a wrinkly colony with strong CR binding. In addition to the CR binding score, mutants demonstrating wrinkly colony morphology are indicated with a "W" in Table 1.

Despite the strong association between biofilm formation and EPS production, there was surprisingly little correlation between these two phenotypes. For example, some mutants with low-level CR binding (e.g., ΔPA14_16500 [*wspR*] and ΔPA14_07500) demonstrated minor defects in biofilm formation under both medium conditions (Fig. 2 and Table 1). Conversely, some mutants with high-level CR binding (e.g., ΔPA14_31330 and ΔPA14_69900) formed wild-type biofilms (Fig. 2 and Table 1). However, some strains with high-level CR binding and/or wrinkly colony morphology did yield hyperbiofilm phenotypes, for example, the ΔPA14_21190, ΔPA14_56790 (*bifA*), and ΔPA14_66320 (*dipA*) mutants, as reported previously (16, 26). Thus, EPS production, as measured by CR binding, may not be indicative of the mutant's biofilm formation phenotype.

c-di-GMP impacts flagellar-based swimming motility. The link between c-di-GMP and motility is well documented (60). Thus, we analyzed each mutant for its impact on swimming motility. Motility was assessed by using a standard soft agar (0.3%) plate assay. It is important to note that plate-based motility assays also measure bacterial chemotaxis in addition to motility.

Of the 16 GGDEF-only mutants, 4 showed an increase (ΔPA14_16500 [*wspR*], ΔPA14_23130, ΔPA14_49890 [*yfiN*], and ΔPA14_56280 [*sadC*]) and 4 showed a decrease (ΔPA14_02110 [*siaD*], ΔPA14_03790, ΔPA14_26970, and ΔPA14_72420) in swimming motility compared to the wild type. The remaining mutants showed wild-type swimming motility (e.g., ΔPA14_04420) (Fig. 4).

Wild-type swimming phenotypes were dominant among mutants of the EAL- and HD-GYP-only subclasses (e.g., ΔPA14_12810 [*rocR*] and ΔPA14_36990). However, two mutants, carrying mutations in the PA14_14530 and PA14_10820 genes, showed significant decreases (~30% of wild-type levels) and increases (~140% of wild-type levels) in swimming motility, respectively (Fig. 4).

TABLE 1 Congo red phenotypes of DGC and PDE mutants[a]

Strain	Congo red binding score
WT	2
Δ*pelA*	0
ΔPA14_02110 (*siaD*)	3
ΔPA14_03790	3
ΔPA14_04420	3
ΔPA14_16500 (*wspR*)	1
ΔPA14_20820	1
ΔPA14_23130	1
ΔPA14_26970	3
ΔPA14_40570	2
ΔPA14_49890 (*yfiN*)	2
ΔPA14_50060 (*roeA*)	0
ΔPA14_53310	1
ΔPA14_56280 (*sadC*)	3
ΔPA14_57140	2
ΔPA14_64050	2
ΔPA14_65090	2
ΔPA14_72420	2
ΔPA14_12810 (*rocR*)	2
ΔPA14_14530	3
ΔPA14_36260	2
ΔPA14_36990	1
ΔPA14_59790 (*pvrR*)	2
ΔPA14_03720	2
ΔPA14_07500	0
ΔPA14_21190	3[W]
ΔPA14_21870	2
ΔPA14_31330	3
ΔPA14_37690	1
ΔPA14_42220 (*mucR*)	2
ΔPA14_45930	2
ΔPA14_49160	2
ΔPA14_53140	2
ΔPA14_56790 (*bifA*)	3[W]
ΔPA14_60870 (*morA*)	0
ΔPA14_65540 (*fimX*)	2
ΔPA14_66320 (*dipA*)	3
ΔPA14_69900	3
ΔPA14_71850	2
ΔPA14_10820	0
ΔPA14_30830	1
ΔPA14_63210	1

[a] The scale ranges from 0 to 3, where 0 is equivalent to the negative control (Δ*pelA* mutant), 1 is represented by the Δ*wspR* mutant, 2 is equivalent to the wild type, and 3 represents hyperbinding of Congo red (*bifA* mutant). Samples of each phenotype are shown in Fig. 3. A superscript "W" indicates a mutant that showed a wrinkly colony phenotype.

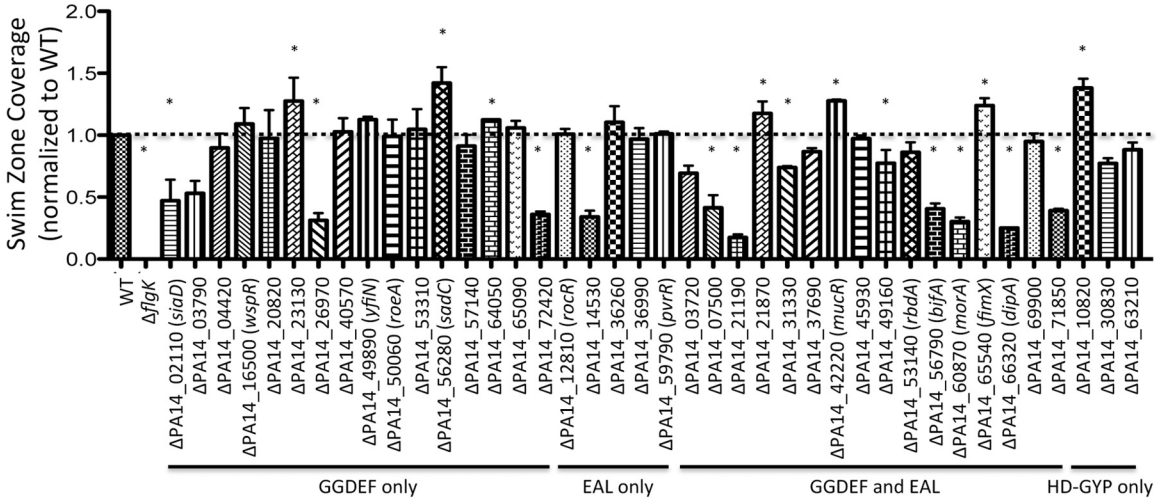

FIG 4 Swimming motility. Shown is an analysis of swimming motility of wild-type *P. aeruginosa* strain PA14 as well as strains carrying mutations in genes encoding GGDEF-only, EAL-only, HD-GYP-only, and dual-domain GGDEF-EAL proteins. The area covered by the swimming motility zone was normalized to that of the wild-type strain, which was set to a value of 1, for ease of comparison. The nonmotile Δ*flgK* mutant served as a negative control. *, $P < 0.05$.

Finally, 11 of the 16 dual-domain GGDEF-EAL subclass mutants showed reduced swimming motility compared to the wild type. Of these, the motility of six mutants (ΔPA14_07500, ΔPA14_21190, ΔPA14_56790 [*bifA*], ΔPA14_60870 [*morA*], ΔPA14_66320 [*dipA*], and ΔPA14_71850) was significantly reduced (<50%) compared to the motility of the wild type. Two mutants (ΔPA14_03720 and ΔPA14_31330) showed ~70% of wild-type levels of motility. In contrast, three mutants (ΔPA14_21870, ΔPA14_42220 [*mucR*], and ΔPA14_65540 [*fimX*]) were hypermotile, at 110 to 120% of wild-type levels (Fig. 4).

As described above, we observed three mutants with a hyperbiofilm phenotype (ΔPA14_21190, ΔPA14_56790 [*bifA*], and ΔPA14_66320 [*dipA*]). These same mutants were impaired in swimming motility (Fig. 4), which is consistent with the general model that high intracellular c-di-GMP levels favor biofilm formation, while low intracellular c-di-GMP levels favor motility. Interestingly, there were two other mutants with similarly impaired swimming motility (e.g., ΔPA14_72420 and ΔPA14_14530) that showed reduced biofilm formation. The complexity underlying c-di-GMP-dependent phenotypes and exceptions to the general model were demonstrated previously (18).

c-di-GMP regulation of swarming motility. *P. aeruginosa* PA14 is capable of a second form of flagellum-dependent motility: swarming. Swarming motility also utilizes rhamnolipid surfactants to move across a semisolid surface and is routinely assayed on 0.5% agar plates. Swarming has also been linked to c-di-GMP in *P. aeruginosa* PA14 (16–18, 61).

Among the 16 GGDEF-only mutants, 5 mutants (ΔPA14_02110 [*siaD*], ΔPA14_03790, ΔPA14_04420, ΔPA14_26970, and ΔPA14_72420) swarmed at ≤50% of wild-type levels (Fig. 5A). Additionally, two mutants (ΔPA14_23130 and ΔPA14_57140) demonstrated a smaller but significant decrease in swarm coverage. In contrast, five other mutants (ΔPA14_16500 [*wspR*], ΔPA14_20820, ΔPA14_49890 [*yfiN*], ΔPA14_50060 [*roeA*], ΔPA14_53310, and ΔPA14_64050) were hyperswarmer strains, showing surface coverages that were 110 to 150% of wild-type levels (Fig. 5A).

Reduction in swarm coverage was observed for three mutants (ΔPA14_14530, ΔPA14_30830, and ΔPA14_63210) of the EAL-only and HD-GYP-only subclasses, with the ΔPA14_30830 mutant showing the highest level of coverage, at 60% of wild-type levels. Three mutants of this class were hyperswarmers (ΔPA14_12810 [*rocR*], ΔPA14_36260, and ΔPA14_10820), with 110% of wild-type swarm coverage. The product of the PA14_12810 (*rocR*) gene was previously linked to Cup fimbria expression, but its contribution to swarming motility was unknown (62, 63).

Mutants with reduced swarm coverage outnumbered hyperswarmers within the dual-domain GGDEF-EAL subclass. In fact, only two mutants in this group (ΔPA14_42220 [*mucR*] and ΔPA14_65540 [*fimX*]) were hyperswarmers, surpassing wild-type surface coverage at 150%. The hyperswarming phenotype of the ΔPA14_42220 (*mucR*) mutant of *P. aeruginosa* PA14 is in contrast to a previous report by Hay et al., wherein Δ*mucR* mutant swarming motility was on par with that of wild-type *P. aeruginosa* strain PAO1 (12). Differences in the strains used and/or medium compositions may explain this discrepancy. Seven mutants of the GGDEF-EAL subclass (ΔPA14_07500, ΔPA14_21190, ΔPA14_53140 [*rbdA*], ΔPA14_56790 [*bifA*], ΔPA14_60870 [*morA*], and ΔPA14_66320 [*dipA*]) showed reduced swarming. The decreased swarming of the ΔPA14_56790 (*bifA*) and ΔPA14_53140 (*rbdA*) mutants is in line with previous reports (16, 28).

In general, weak swarmers (e.g., ΔPA14_02110 [*siaD*] and ΔPA14_07500) are also weak swimmers (Fig. 3). Interestingly, the hyperswarming phenotype (e.g., ΔPA14_50060 [*roeA*] and ΔPA14_53310) did not always translate into a hyperswimming phenotype (Fig. 3). These findings indicate a complex relationship between swimming and swarming motility.

Impact of mutations on pilus-mediated twitching motility. *P. aeruginosa* can also utilize type IV pili (TFP) to move across hard surfaces (≥1% agar) in a process known as twitch motility (41, 64), which is distinct from the two flagellar-dependent modes of motility, swimming and swarming, described above. We investigated the effects of the DGC and PDE mutants on twitching motility. The Δ*pilA* mutant, lacking the type IV pilin (65), served as the negative control (Fig. 6).

A small subset of GGDEF-only mutants (ΔPA14_03790,

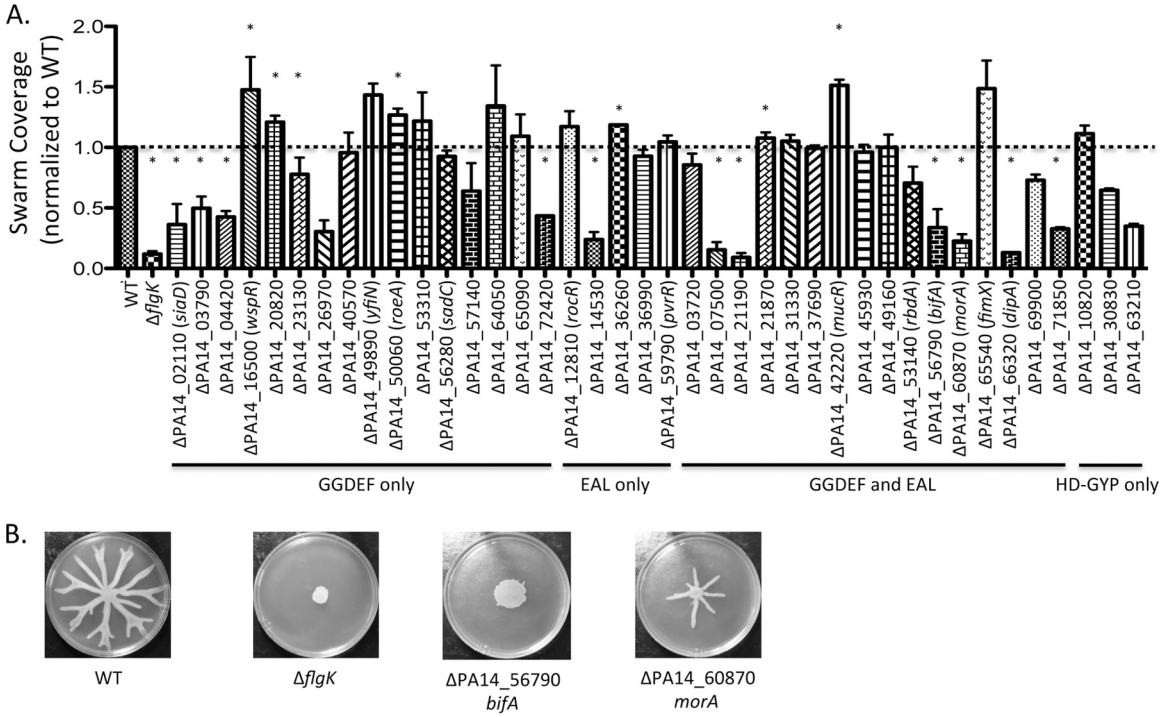

FIG 5 Swarming motility. Shown is an analysis of swarming motility of wild-type *P. aeruginosa* PA14 as well as strains carrying mutations in genes encoding GGDEF-only, EAL-only, HD-GYP-only, and dual-domain GGDEF-EAL proteins. (A) The area covered by the swarm and its tendrils was measured and normalized to that of wild-type *P. aeruginosa* strain PA14, which was set to a value of 1, for ease of comparison. (B) Representative images of swarming motility. Reduced swarm coverage includes reduced tendrils (ΔPA14_60870 [*morA*]) or tendril-less growth of the colony (ΔPA14_56790 [*bifA*]). The nonmotile Δ*flgK* mutant served as a negative control. ∗, $P < 0.05$.

ΔPA14_26970, and ΔPA14_72420) demonstrated reduced twitch zones, at <50% of wild-type levels. Six other mutants (ΔPA14_04420, ΔPA14_23130, ΔPA14_40570, ΔPA14_56280 [*sadC*], ΔPA14_57140, and ΔPA14_64050) also showed statistically significant decreases in their respective twitch zones but to a lesser degree than the previous group of mutants. In comparison, only one mutant (ΔPA14_20820) had a hypertwitch phenotype, with its twitch zone being ~120% of that of the wild type (Fig. 6).

Several mutations of EAL-only and HD-GYP-only genes conferred a reduction in twitch motility. A total of four mutants (ΔPA14_14530, ΔPA14_36260, ΔPA14_30830, and ΔPA14_63210) showed significant decreases in their respective twitch

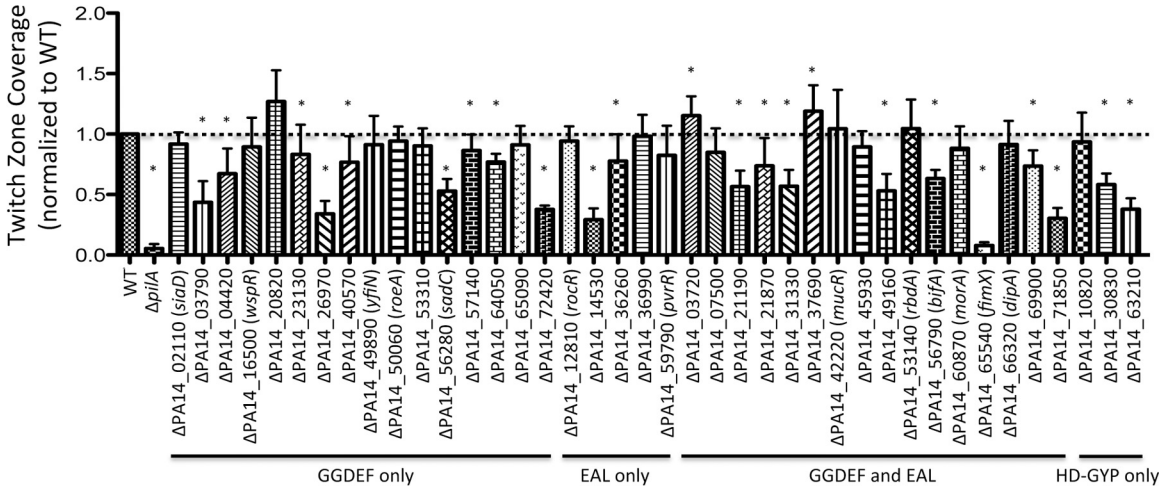

FIG 6 Twitch motility. Shown is an analysis of twitching motility by wild-type *P. aeruginosa* strain PA14 as well as strains carrying mutations in genes encoding GGDEF-only, EAL-only, HD-GYP-only, and dual-domain GGDEF-EAL proteins. Twitch zones were measured and normalized to that of wild-type *P. aeruginosa* strain PA14, which was set to a value of 1, for ease of comparison. The Δ*pilA* mutant served as a negative control. ∗, $P < 0.05$.

zones compared to the wild type. In particular, ΔPA14_14530 and ΔPA14_63210 showed twitch zones that were <50% of that of the wild type (Fig. 6).

Of 16 mutants of the dual-domain GGDEF-EAL class, 8 mutants (ΔPA14_21190, ΔPA14_21870, ΔPA14_31330, ΔPA14_49160, ΔPA14_56790 [*bifA*], ΔPA14_65540 [*fimX*], ΔPA14_69900, and ΔPA14_71850) were statistically significantly impaired for twitch motility, and only 2 (ΔPA14_03720 and ΔPA14_37690) showed larger twitch zones than that of the wild type (Fig. 6). It is also worth noting that our data reproduced a twitch-negative phenotype for a strain carrying PA14_65540 (*fimX*), a known c-di-GMP-dependent regulator of TFP biogenesis in *P. aeruginosa* (13).

Conclusions. Here, we present a library of in-frame deletion mutations targeting the 40 c-di-GMP metabolism proteins identified in the genome of *P. aeruginosa* PA14. Assessment of biofilm formation, swimming motility, swarming motility, twitch motility, and EPS production (via CR binding) was performed by using this mutant library (summarized in Table S4 in the supplemental material). Analysis of these mutants revealed complex relationships among these phenotypes, consistent with the complex nature of the c-di-GMP signaling system. Going forward, this mutant library should serve as a helpful tool for elucidating the role of c-di-GMP metabolism proteins and their regulated pathways.

ACKNOWLEDGMENTS

We thank T. Hampton and K. Price for assisting with statistical analyses and GraphPad Prism software.

This work was supported by NIH grants R01 A1003256 and R01 AI097307 to G.A.O. and by a Rosaline Borison predoctoral fellowship to D.-G.H.

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