Cyclic-di-GMP-Mediated Repression of Swarming Motility by *Pseudomonas aeruginosa*: the *pilY1* Gene and Its Impact on Surface-Associated Behaviors[▽]

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The intracellular signaling molecule cyclic-di-GMP (c-di-GMP) has been shown to influence surface-associated behaviors of *Pseudomonas aeruginosa*, including biofilm formation and swarming motility. Previously, we reported a role for the *bifA* gene in the inverse regulation of biofilm formation and swarming motility. The *bifA* gene encodes a c-di-GMP-degrading phosphodiesterase (PDE), and the Δ*bifA* mutant exhibits increased cellular pools of c-di-GMP, forms hyperbiofilms, and is unable to swarm. In this study, we isolated suppressors of the Δ*bifA* swarming defect. Strains with mutations in the *pilY1* gene, but not in the pilin subunit *pilA* gene, show robust suppression of the swarming defect of the Δ*bifA* mutant, as well as its hyperbiofilm phenotype. Despite the ability of the *pilY1* mutation to suppress all the c-di-GMP-related phenotypes, the global pools of c-di-GMP are not detectably altered in the Δ*bifA* Δ*pilY1* mutant relative to the Δ*bifA* single mutant. We also show that enhanced expression of the *pilY1* gene inhibits swarming motility, and we identify residues in the putative VWA domain of PilY1 that are important for this phenotype. Furthermore, swarming repression by PilY1 specifically requires the diguanylate cyclase (DGC) SadC, and epistasis analysis indicates that PilY1 functions upstream of SadC. Our data indicate that PilY1 participates in multiple surface behaviors of *P. aeruginosa*, and we propose that PilY1 may act via regulation of SadC DGC activity but independently of altering global c-di-GMP levels.

Pseudomonas aeruginosa forms surface-attached communities known as biofilms, and this microbe is also capable of surface-associated motility, including twitching and swarming. The mechanism by which cells regulate and coordinate these various surface-associated behaviors, or how these microbes transition from one surface behavior to another, has yet to be elucidated. Given that *P. aeruginosa* is capable of such diverse surface-associated lifestyles, this Gram-negative organism serves as a useful model to address questions regarding the regulation of surface-associated behaviors.

Recent studies indicate that biofilm formation and swarming motility by *P. aeruginosa* are inversely regulated via a common pathway (12, 27, 37). Important factors that influence early biofilm formation by *P. aeruginosa* strain PA14 include control of flagellar motility and the robust production of the Pel exopolysaccharide (EPS). Swarming occurs when cells move across a hydrated, viscous semisolid surface, and like biofilm formation, flagellar function is important for this surface-associated motility. Additionally, swarming requires production of rhamnolipid surfactant acting as a surface-wetting agent (25, 58). In contrast to biofilm formation, swarming motility is enhanced in strains which are defective for the production of Pel EPS (12).

The inverse regulation of biofilm formation and swarming motility is reminiscent of the regulation of sessile and motile behaviors that occurs in a wide range of bacterial species via the intracellular signaling molecule cyclic-di-GMP (c-di-GMP) (17, 24, 50, 51, 56). High levels of this signaling molecule promote sessile behaviors and inhibit motility, whereas low levels of c-di-GMP favor motile behaviors (8, 9, 22, 56). Recently, we reported that the BifA phosphodiesterase, which catalyzes the breakdown of c-di-GMP, inversely regulates biofilm formation and swarming motility (27). In addition, Merritt et al. reported that SadC, a diguanylate cyclase (DGC) which synthesizes c-di-GMP, participates with BifA to modulate cellular c-di-GMP levels and thus regulate biofilm formation and swarming motility (37).

Consistent with a role for BifA as a c-di-GMP phosphodiesterase, Δ*bifA* mutants exhibit increased cellular pools of c-di-GMP relative to the wild type (WT) (27). Phenotypically, Δ*bifA* mutants form hyperbiofilms and are unable to swarm. The hyperbiofilm phenotype of the Δ*bifA* mutant results largely from increased synthesis of the *pel*-derived polysaccharide; that is, the Δ*bifA*Δ*pel* double mutant shows a marked decrease in biofilm formation compared to the Δ*bifA* mutant (27). Interestingly, elevated Pel polysaccharide production alone is not sufficient to explain the swarming defect of the Δ*bifA* mutant, as the Δ*bifA*Δ*pel* double mutant recovers only minimal swarming ability (27). These data indicate that high levels of c-di-GMP inhibit swarming motility in a largely Pel-independent manner.

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To better understand how elevated c-di-GMP levels in the cell inhibit swarming motility, we exploited the swarming defect of the Δ*bifA* mutant, and using a genetic screen, we identified suppressors in the Δ*bifA* background that restored the ability to swarm. Here we report a role for the PilY1 protein in repression of swarming motility in the Δ*bifA* mutant background. Our data are consistent with a model in which PilY1 functions upstream of the c-di-GMP diguanylate cyclase SadC to regulate swarming motility by *P. aeruginosa*.

MATERIALS AND METHODS

Strains and media. The strains, plasmids, and primers used in this study are listed in Table 1. The *P. aeruginosa* PA14 and *Escherichia coli* DH5α and S17-1 λpir strains were routinely cultured on lysogeny broth (LB) medium, which was solidified with 1.5% agar when necessary. Gentamicin (Gm) was used at from 25 to 50 μg/ml for *P. aeruginosa* and at 10 μg/ml for *E. coli*. Ampicillin (Ap) was used at 150 μg/ml and nalidixic acid (NA) at 20 μg/ml for *E. coli*. For all phenotypic assays with *P. aeruginosa*, M63 minimal salts medium supplemented with MgSO₄ (1 mM), glucose (0.2%), and Casamino Acids (CAA) (0.5%) was used, except where noted. For expression plasmids harboring the pBAD promoter, arabinose was added to cultures at a 0.2% final concentration, unless noted otherwise.

Saccharomyces cerevisiae strain InvSc1 (Invitrogen), used for plasmid construction via *in vivo* homologous recombination, was grown with yeast extract-peptone-dextrose (1% Bacto yeast extract, 2% Bacto peptone, and 2% dextrose) (55). Selections with InvSc1 were performed using synthetic defined agar-uracil (catalog no. 4813-065; Qbiogene).

mariner **mutagenesis of the Δ***bifA* **mutant.** The donor strain *E. coli* S17-1 λ pir carrying the pBT20 plasmid and the *P. aeruginosa* Δ*bifA* mutant recipient strain were conjugated as follows. From overnight LB-grown cultures (with the appropriate antibiotic), 1 ml of each strain was centrifuged to pellet the cells. Cell pellets were washed twice with LB and resuspended in 100 μl of fresh LB. The cell suspensions of each strain were then mixed, and 60 μl of the mixture was plated to two LB plates and the conjugation was allowed to proceed for 1 h at 30°C. The conjugation products were harvested, resuspended in 100 μl of LB, and then plated to LB agar plates containing Gm, as well as NA to select against *E. coli* growth. Libraries of mutants were generated by inoculating colonies into 96-well plates containing LB medium supplemented with Gm, and plates were incubated overnight at 37°C.

Mutant strains in library plates were screened for recovery of swarming motility by inoculating swarm motility plates (see below) using a multichannel pipette to deliver cells from the wells of the plate to the swarm plate. WT and Δ*bifA* mutant controls were included on each plate. Plates were incubated at 37°C for 12 to 16 h, and mutant strains were assessed for swarming motility.

Approximately 5,500 *mariner* transposon mutants in the Δ*bifA* mutant background were screened. DNA sequences flanking the transposon insertions in mutants of interest were determined using an arbitrary PCR method, as described previously (11, 43). PCR products were sequenced at the Molecular Biology and Proteomics Core at Dartmouth College. The resulting DNA sequences were aligned to the PA14 genomic sequence using the NCBI BLAST program.

Construction of in-frame deletion mutants and plasmids. The Δ*bifA* Δ*pelA* double mutant was constructed by deletion of *bifA* in the Δ*pelA* mutant (provided by R. Kolter) using the previously described pEX18Gm-*bifA* construct (27). The *pilY1* deletion construct was generated using primers *pilY1* KO-1 with *pilY1* KO-2 and *pilY1* KO-3 with *pilY1* KO-4, and these 1-kb fragments were cloned into the pMQ30 vector via homologous recombination in the yeast *S. cerevisiae* InvSc1 as described previously (55). The resulting pMQ30-*pilY1* vector was used to transform *E. coli* S17-1 and introduced into the *P. aeruginosa* WT and Δ*bifA* strain backgrounds by conjugation. Integrants were isolated on Gm and NA and subjected to sucrose selection. The resolved integrants were confirmed by PCR using primers *pilY1* KO conf 5′ and 3′. The resulting internal deletion in *pilY1* removes approximately 3.4 kb, resulting in a deletion from amino acid 26 to 1158 (up to the stop codon) of the 1158-amino-acid full-length PilY1 protein.

The pMQ30-*pilA* knockout construct was generated using primers *pilA* KO-1 with *pilA* KO-2 and *pilA* KO-3 with *pilA* KO-4, and the PCR fragments were cloned into pMQ30 by homologous recombination in yeast. Construction of the *pilA* deletion mutation using pMQ30-*pilA* in *P. aeruginosa* proceeded as described above and was confirmed by PCR using the *pilA* KO conf 5′ and *pilA* KO conf 3′ primers. The resulting internal deletion in *pilA* removes approximately

450 base pairs, resulting in a deletion of amino acids 5 to 157 of the 179-amino-acid full-length PilA protein. The Δ*bifA* Δ*pilY1* Δ*pilA* triple mutant was constructed by deletion of the *pilA* gene in the Δ*bifA* Δ*pilY1* double mutant.

The *wspR* deletion was constructed using the pEX19Gm-*wspR* plasmid (provided by C. Harwood). The construct was introduced into WT *P. aeruginosa*, and deletion mutants were isolated as described above.

The pPilY1 complementation construct was generated by PCR amplification using the high-fidelity Phusion polymerase (Finnzyme, Espoo, Finland). Primer *pilY1* comp 5′ was used with *pilY1*-C6His B and *pilY1* comp 3′ was used with *pilY1*-C6His A, and these fragments were cloned into pMQ80 by homologous recombination in yeast to generate an arabinose-inducible C-terminally His-tagged version of the *pilY1* gene.

Deletion of the PilC domain from the *pilY1* gene was performed by PCR amplification using Del-PilC 5′ with the *pilY1* comp 5′ primer and Del-PilC 3′ with the *pilY1* comp 3′ primer and using the pPilY1 construct as the template for these reactions to incorporate the C-terminal His tag. These PCR fragments were cloned into pMQ80 in yeast.

Mutagenesis of *pilY1*. (i) Random PCR mutagenesis of *pilY1*ΔPilC. Using the pPilY1ΔPilC construct as a template for PCR, linear DNA fragments comprised of overlapping halves of the *pilY1*ΔPilC mutant coding region (~1,200 bp each) were amplified by PCR using Phusion polymerase (Finnzyme). The primers p730 and PCRmut1Rev were used to amplify the N-terminal half of the *pilY1*ΔPilC gene, and primers PCRmut2For and p883 were used to amplify the C-terminal half of the gene. These linear DNA fragments served as templates for the subsequent mutagenic PCR, which was performed as reported previously (10), with the following modification: 0.125 mM MnCl was added (instead of 0.5 mM) to lower the frequency of mutations to ~2 mutations per 1,200 bp (as determined by sequencing a number of PCR products at both MnCl concentrations).

Mutagenized PCR products were gel purified using the Qiagen gel extraction kit. To reconstruct the intact *pilY1*ΔPilC gene, a mutated N-terminal fragment was paired with a nonmutated C-terminal region of the gene (and vice versa) for cloning together into the pMQ80 vector via homologous recombination in yeast. This also served to focus sequencing of the mutations generated by mutagenesis to only one half of the *pilY1*ΔPilC gene. The resulting plasmids were purified and electroporated first into *E. coli* DH5α and then into PA14 for greater efficiency. The resulting colonies were picked into a 96-well plate to grow overnight at 37°C in LB medium supplemented with Gm. The resulting cultures, as well as the control strains (WT carrying either vector alone, pPilY1ΔPilC or pPilY1), were then inoculated onto swarm motility plates containing 0.2% arabinose using a multichannel pipette. Strains carrying mutated versions of pPilY1ΔPilC that lost swarming inhibition were subjected to dot blot analysis (described below) to check for protein stability. Strains expressing stable PilY1ΔPilC proteins were retested by repeating the plasmid isolation, transforming PA14, and again testing for swarming. Mutants were sequenced with the primers p730, p883, pPCRmut seq1, and pPCRmut seq2.

(ii) Site-directed mutagenesis of *pilY1*. To identify regions for mutagenesis, PilY1 homologs were identified by BLAST and aligned with the PA14 PilY1 protein using ClustalW2. Regions of amino acid conservation were identified, and two such regions were targeted for mutagenesis. The threonine at position 270 was changed to alanine by site-directed mutagenesis using primer pTPLmutF with *pilY1*-comp 5′ and primer pTPLmutR with *pilY1*-comp 3′. Amino acids D310 and G311 were both replaced with alanines via the same method, using primer MTDGmutF with *pilY1*-comp 5′ and primer MTDGmutR with *pilY1*-comp 3′. Products were cloned into the pMQ80 vector via yeast-based homologous recombination and transformed into PA14. The mutations were verified by sequencing using primers p730 and pPCRmut1Rev. Strains expressing these mutant versions of PilY1 were tested in swarming assays as described below.

Motility assays. Swim (0.3% agar) and twitch (1.5% agar) motility plates consisted of M63 medium supplemented with glucose, MgSO₄ and CAA. Swim and twitch assays were performed as previously reported (43, 61). Swarm motility plates were comprised of M8 medium supplemented with MgSO₄, glucose, and CAA and solidified with 0.5% agar. For testing individual strains, 2 μl of LB-grown overnight cultures was inoculated onto the surface of the swarm plates and incubated for 12 to 16 h at 37°C. For screening large numbers of strains, cells from overnight cultures grown in 96-well plates were transferred with a multichannel pipette to the surface of swarm plates.

Biofilm formation assays. Biofilm formation in 96-well microtiter plates was assayed and quantified as previously described (13, 42). All biofilm assays were performed using M63 minimal medium supplemented with glucose, MgSO₄, and CAA.

CR binding assays. Congo red (CR) binding assays were performed as reported previously (12, 19, 20).

TABLE 1. Strains, plasmids, and primers

Strain, plasmid, or primer	Relevant genotype, description, or sequence[a]	Source or reference
E. coli strains		
DH5α	*supE44* Δ*lacU169*(φ80*lacZ*ΔM15) *hsdR17 thi-1 relA1 recA1*	Life Technologies
S17-1 (λpir)	*thi pro hsd*R⁻M⁺ Δ*recA* RP4-2::TcMu-Km::Tn7	57
P. aeruginosa strains		
PA14	Wild type	47
PA14 Δ*bifA*	PA14 with unmarked deletion of *bifA*	27
PA14 Δ*pelA*	PA14 with unmarked deletion of *pelA*	19
PA14 Δ*bifA* Δ*pelA*	PA14 Δ*pelA* with unmarked deletion of *bifA*	This study
PA14 Δ*bifA pilY1*::*Mar* 57E12	PA14 Δ*bifA* with *mariner* insertion in *pilY1*; Gmʳ	This study
PA14 Δ*bifA pilY1*::*Mar* 68C1	PA14 Δ*bifA* with *mariner* insertion in *pilY1*; Gmʳ	This study
PA14 Δ*bifA pilY1*::*Mar* 52H5	PA14 Δ*bifA* with *mariner* insertion in *pilY1*; Gmʳ	This study
PA14 Δ*bifA* Δ*sadA*::*Mar* 51G2	PA14 Δ*bifA* with *mariner* insertion in *sadA*; Gmʳ	This study
PA14 Δ*bifA* Δ*pvrS*::*Mar* 57B1	PA14 Δ*bifA* with *mariner* insertion in *pvrS*; Gmʳ	This study
PA14 Δ*bifA* Δ*pvrS*::*Mar* 39D11	PA14 Δ*bifA* with *mariner* insertion in *pvrS*; Gmʳ	This study
PA14 Δ*bifA* Δ*pvrS*::*Mar* PA14 23H6	PA14 Δ*bifA* with *mariner* insertion in *pvrS*; Gmʳ	This study
Δ*bifA* Δ*pvrS*::*Mar* PA14 58A9	PA14 Δ*bifA* with *mariner* insertion in *pvrS*; Gmʳ	This study
Δ*bifA* Δ*pvrS*::*Mar* PA14 39H1	PA14 Δ*bifA* with *mariner* insertion in *pvrS*; Gmʳ	This study
PA14 Δ*pilY1*	PA14 with unmarked deletion of *pilY1*	This study
PA14 Δ*bifA* Δ*pilY1*	PA14 Δ*bifA* with unmarked deletion of *pilY1*	This study
PA14 Δ*pilA*	PA14 with unmarked deletion of *pilA*	This study
PA14 Δ*bifA* Δ*pilA*	PA14 Δ*bifA* with unmarked deletion of *pilA*	This study
PA14 Δ*bifA* Δ*pilY1* Δ*pilA*	PA14 Δ*bifA* Δ*pilY1* with unmarked deletion of *pilA*	This study
PA14 Δ*sadC*	PA14 with unmarked deletion of *sadC*	37
PA14 Δ*wspR*	PA14 with unmarked deletion of *wspR*	This study
PA14 Δ*PA1107*	PA14 with unmarked deletion of *PA1107*	This study
Plasmids		
pEX18-*bifA*	Vector for knockout of *bifA* gene; Gmʳ	27
pMQ72	Shuttle vector for cloning in yeast and for arabinose-inducible gene expression; Gmʳ	55
pRapA	*P. fluorescens rapA* gene cloned in pMQ72; Gmʳ	38
pBT20	Vector carrying *mariner* transposon; Apʳ Gmʳ (marker on transposon)	30
pMQ30	Shuttle vector for yeast cloning and Gram-negative allelic replacement; Gmʳ	55
pMQ30-*pilY1*	Vector for knockout of *pilY1* gene; Gmʳ	This study
pMQ30-*pilA*	Vector for knockout of *pilA* gene; Gmʳ	This study
pEX19Gm-*wspR*	Vector for knockout of *wspR* gene; Gmʳ	22
pMQ80	Shuttle vector for cloning in yeast and for arabinose-inducible gene expression; Gmʳ	55
pPilY1His	His-tagged *pilY1* gene cloned in pMQ80; Gmʳ	This study
pSadC	HA-tagged *sadC* gene cloned in pMQ72; Gmʳ	37
pPilY1HisΔPilC	His-tagged *pilY1* gene lacking the PilC domain in pMQ80; Gmʳ	This study
pPilY1His-MTAA	His-tagged *pilY1* gene with D309A/G310A mutations in pMQ80; Gmʳ	This study
Primers		
pilY1 KO-1	tgtaaaacgacggccagtgccaagcttgcatgcctgACGGTGTCTGCTTGCGCTATC	
pilY1 KO-2	*GACGTAAGGGGTTCATGTTC*AAGAGGCGAACAGGATGCTCAG	
pilY1 KO-3	CTGAGCATCCTGTTCGCCTCT*TGAACATGAACCCCTTACGTC*	
pilY1 KO-4	ccatgattacgaattcgagctcggtacccggggatccCAGCCCCAACAGTTCCAACTC	
pilY1 KO conf 5′	AGACTATCGCTGCGGCAACTG	
pilY1 KO conf 3′	CAGGATGCCGTGAAGGAACTG	
pilY1 comp 5′	tctccatacccgtttttttgggctagcgaattcgaaggagatatacatATGATCCACCAGATTACC	
pilY1 comp 3′	tcctttactcatatgtatatctccttccccgggtaccCTGCCCATCGACTACTACCAG	
pilY1-C6His A	AGGGGGTTCATGTTC**GTGGTGATGGTGGTGGTG**TTTCTCCTCGACGACCCG	
pilY1-C6His B	GTCGAGGAGAAA**CACCACCACCATCACCAC**TGAACATGAACCCCTTACGTC	
pilA KO-1	tgtaaaacgacggccagtgccaagcttgcatgcctgTCGATGGGACTTGGTCTTCGAC	
pilA KO-2	*GGCAGGCATATTGCGGTCC*TGAGCTTTCATGTATCTCTC	
pilA KO-3	GAGAGATACATGAAAGCTCAGG*ACCGCAATATGCCTGCC*	
pilA KO-4	ccatgattacgaattcgagctcggtacccggggatccCCTGACCAAGGATGTCAG	
pilA KO conf 5′	GATCCGCATCACGATCTTCTC	
pilA KO conf 3′	GAACTCAGCCTGGACGACATG	
pvrR RT 5′	GTCGAAGATCAGCCGTTTCAG	
pvrR RT 3′	GTCGAACCTGTCCTGCTTCAG	
sadR RT 5′	TCATTCGGTGATCCTCAGCAG	
sadR RT 3′	GAGGTCGCCGAGAAAATTGAG	
pelA RT 5′	GCTACGTGCCGTTCGTCAGCACC	
pelA RT 3′	CAGGCCGCCGAGGTAGACGTG	
pilY1 RT 5′	TGCACCCTGAGCATCCTGTTC	
pilY1 RT 3′	GGCCATACTGCCTGAGTCATC	
Del-PilC 5′	*CGGGTTTTGGCTAATAAGCCG*CCAAGTGGCTAACTGACTTCC	
Del-PilC 3′	GGAAGTCAGTTAGCCACTTGG*CGGCTTATTAGCCAAAACCCG*	
MTDGmutF	AGCTATCACATCATGATGACCGCCGCCATCTGGAACGGTCGGAACGTC	
MTDGmutR	GACGTTCCGACCGTTCCAGATGGCGGCGGTCATCATGATGTGATAGCT	
TPLmutF	AACAGCCCGGCCAGCGGCGGTGCTCCTCTGCATGCGGCTCTTG	
TPLmutR	CAAGAGCCGCATGCAGAGGAGCACCGCCGCTGGCCGGGCTGTT	
PCRmut1 Rev	GAGCGAATAGGAAAGACCTAG	
P730	GCAACTCTCTACTGTTTCTCC	
PCRmut2 For	ACTTGGCAACACATGGTCAAC	
P883	AGAATTGGGACAACTCCAGTG	
PCRmut seq1	CCTGGCTTTCAAATACTGGAC	
PCRmut seq2	AGTCTCCACGAGAGTTGATAG	

[a] In primer sequences, lowercase letters indicate sequence homology to the cloning vector, uppercase letters indicate a *Pseudomonas* gene-specific sequence, boldface indicates a His tag sequence, and italics indicate a 5′ sequence flanking a deletion mutation.

qRT-PCR. Strains were diluted from LB-grown overnight cultures 1:100 into M63 minimal medium supplemented with glucose, $MgSO_4$, and CAA and grown to an optical density at 600 nm (OD_{600}) of 0.6. RNA was isolated, and cDNA was prepared as previously described (28). Quantitative reverse transcription-PCR (qRT-PCR) was performed using an ABI 7500 Fast System and analyzed using ABI Fast System software version 1.4. Expression levels were quantified in picograms of input cDNA using a standard curve method for absolute quantification, and these values were normalized to *rplU* expression. Results shown are based on the average from two independent experiments with three replicates per sample. The primers used are listed in Table 1.

In vivo quantification of c-di-GMP. (i) Whole-cell labeling. Whole-cell [^{32}P]orthophosphate labeling, acid extraction, and two-dimensional (2D) thin-layer chromatography (TLC) analysis of c-di-GMP for quantification were performed as reported previously (22, 38).

(ii) Mass spectrometry. Cultures were grown in LB for 13 h before being back diluted into 50 ml of M63-glucose-CAA medium to an OD_{600} of 0.05. Subcultured strains were grown with shaking at 37°C for ~4 h until the OD_{600} was 0.4, at which point 40 ml of cultured cells was pelleted by centrifugation for 3 min at $10,000 \times g$ and 25°C. The culture broth supernatant was discarded, and 250 µl of cold (−20°C) extraction solvent (methanol-acetonitrile-water at a volumetric ratio of 40:40:20, acidified with formic acid to final concentration of 0.1 N) was immediately added to the cell pellet to quench metabolism and initiate c-di-GMP extraction. Extraction was allowed to proceed for 30 min at −20°C. The mixture was then transferred to a 1.5-ml Eppendorf tube and microcentrifuged for 5 min at 4°C and maximum speed (13,000 rpm). The supernatant was set aside in a fresh tube on ice. The cell pellet was then resuspended in an additional 125 µl of extraction solvent, and the mixture was incubated at −20°C for 15 min and then microcentrifuged as above. The resulting supernatant was combined with the prior one, and the combined supernatants were microcentrifuged again to ensure complete removal of debris. The final solution was then neutralized by addition of 4 µl of 15% $(NH_4)_2HCO_3$. Note that the extracts were collected from pelleted cells; thus, although our results were reproducible across multiple independent experiments, they may be affected by metabolic events occurring during centrifugation.

The cellular extracts were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Finnigan TSQ Quantum DiscoveryMax triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled with an LC-20AD high-pressure liquid chromatography (HPLC) system (Shimadzu, Columbia, MD). The mass spectrometry parameters were as follows: spray voltage, 3,000 V; nitrogen as the sheath gas at 30 lb/in^2 and auxiliary gas at 10 lb/in^2; argon as the collision gas at 1.5 millitorr; and capillary temperature, 325°C. Reversed-phase liquid chromatography separation was achieved on a Synergi Hydro-RP column (4-µm particle size, 150 by 2 mm; Phenomenex, Torrance, CA). Solvent A was 10 mM tributylamine plus 15 mM acetic acid in 97:3 water-methanol. Solvent B was methanol. The gradient was as follows: $t = 0$, 0% solvent B; $t = 5$ min, 0% solvent B; $t = 10$ min, 20% solvent B; $t = 20$ min, 20% solvent B; $t = 35$ min, 65% solvent B; $t = 38$ min, 95% solvent B; $t = 42$ min, 95% solvent B; $t = 43$ min, 0% solvent B; and $t = 50$ min, 0% solvent B. Each sample had a running time of 50 min. Other liquid chromatography parameters were as follows: autosampler temperature, 4°C; column temperature, 25°C; injection volume, 20 µl; and solvent flow rate, 200 µl/min.

The nucleotide c-di-GMP was detected by using selected reaction monitoring (SRM) in negative ionization mode. The scan time for each SRM was 0.1 s, with a scan width of 1 *m/z*. The LC-MS/MS parameters for c-diGMP were as follows: parent ion *m/z* 689 forms product ion *m/z* 344 at a collision energy of 32 eV, eluting at a retention time of 32.1 min.

Assessment of PilY1 protein expression and stability. (i) SDS-PAGE and Western blotting. Strains were cultured overnight at 37°C in LB and then diluted 1:100 into 5 ml of M63 minimal medium with glucose, $MgSO_4$, and Casamino Acids (and arabinose at the indicated concentrations where necessary) and grown to an OD_{600} of 0.6. Cells were centrifuged at $16,000 \times g$ to pellet them, the supernatants were removed, and the pellets were resuspended in 1× sodium dodecyl sulfate (SDS) gel loading buffer with 100 mM dithiothreitol (DTT). Samples were boiled for 10 min and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) in a 7.5% polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were transferred to a nitrocellulose membrane and probed with either PilY1 antiserum to detect the PilY1 protein or an anti-penta-His antibody (Qiagen, Valencia, CA) to detect the His-tagged version of PilY1 where appropriate.

Western blots were developed with the Western Lightning ECL detection kit (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions.

(ii) Dot blot analysis. Strains carrying mutated versions of pPilY1ΔPilC that lost swarming inhibition when expressed in the WT were cultured overnight at

37°C in 96-well plates containing LB supplemented with Gm and then subcultured 1:100 in M63 minimal medium supplemented with glucose, $MgSO_4$ and CAA, and 0.2% arabinose and grown for another 6 h. The cultures were combined with 6x SDS protein loading buffer and boiled for 10 min, and 5 µl of each sample was spotted onto a nitrocellulose membrane and allowed to dry. Membranes were probed with the anti-penta-His antibody (Qiagen), and antibodies were detected in the same manner described above for Western blots.

Mutants of interest were further examined by SDS-PAGE to resolve and specifically detect the PilY1 variant, as described above.

PilY1 cellular localization experiments. Cell fractionations were performed as previously reported (27, 40) with modifications. LB-grown overnight cultures were diluted (1:100) into M63 supplemented with glucose, $MgSO_4$, and CAA and grown for ~5 h (OD_{600} of ~0.6) at 37°C with shaking. Bacterial cells were harvested by centrifugation at $5,520 \times g$ for 15 min at 4°C. Thirty milliliters of culture supernatant for each strain was collected and filtered through a 0.22-µm filter and concentrated approximately 300-fold using Amicon Ultra-15 filter devices (10,000-molecular-weight cutoff [MWCO]) (Millipore Corporation, Billerica, MA) according to the manufacturer's instructions. The resulting fractions were designated the supernatant (Sup).

The harvested cell pellets were resuspended in Tris-buffered saline (5 ml). Cell suspensions were vortexed vigorously for 2 min, followed by centrifugation at $4,400 \times g$. The supernatants were collected, filtered through a 0.22-µm filter, and concentrated approximately 100-fold using the Amicon Ultra-15 (10,000-MWCO) filter devices according to the manufacturer's instructions. The resulting supernatant was designated the cell-associated (CA) fraction, as defined previously (38).

The cell pellets were then resuspended in buffer A (200 mM Tris-HCl [pH 7.5], 20 mM EDTA [pH 8.0]) with 1× Complete protease inhibitors [Roche Diagnostics Corp., Indianapolis, IN]) and lysed in a French pressure cell. Samples were centrifuged at $9,300 \times g$ for 10 min at 4°C to remove unbroken cells, and supernatants were collected as whole-cell lysates. Samples were further fractionated to isolate the inner and outer membrane fractions, as described previously (27).

For Western blots of cellular fractions, samples from each fraction were normalized to the same concentration, and equivalent amounts of total protein were mixed with a 2× SDS loading buffer containing dithiothreitol (200 mM). Concentrations of total protein in each fraction were determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Samples were resolved by SDS-PAGE using 7.5% polyacrylamide gels (Bio-Rad). Proteins were transferred to a nitrocellulose membrane and probed with one of four antibodies: (i) PilY1 antiserum to detect the PilY1 protein (41), (ii) anti-penta-His antibody (Qiagen, Valencia, CA) to detect His-tagged PilY1 where appropriate, (iii) SecY antiserum (23) to detect the inner membrane protein SecY (1), and (iv) antiserum to detect the outer membrane protein OprF (7, 21, 34). Western blots were developed as described above.

RESULTS

A Δ*bifA* mutant does not swarm, likely due to high cellular c-di-GMP pools. In previous studies, we reported that the *bifA* gene (*PA4367*) encodes a phosphodiesterase (PDE) that is able to degrade c-di-GMP and influence surface-associated behaviors of *Pseudomonas aeruginosa* (27). Our studies showed that deletion of the *bifA* gene leads to an increase in cellular c-di-GMP pools and that the Δ*bifA* mutant exhibits a hyper-biofilm phenotype as well as a complete loss of swarming motility (27).

We predicted that the nonswarming phenotype of the Δ*bifA* mutant results from the enhanced production of the *pel*-derived polysaccharide. However, a *pelA* mutation in the Δ*bifA* mutant background resulted in only a minor improvement in the swarming defect, indicating that excess Pel polysaccharide production is not the primary contributor to the Δ*bifA* swarming impairment (Fig. 1A). This finding is consistent with a previous report from our group (27). Thus, we postulated that high levels of c-di-GMP inhibit swarming motility of the Δ*bifA* mutant via one or more Pel-independent factors.
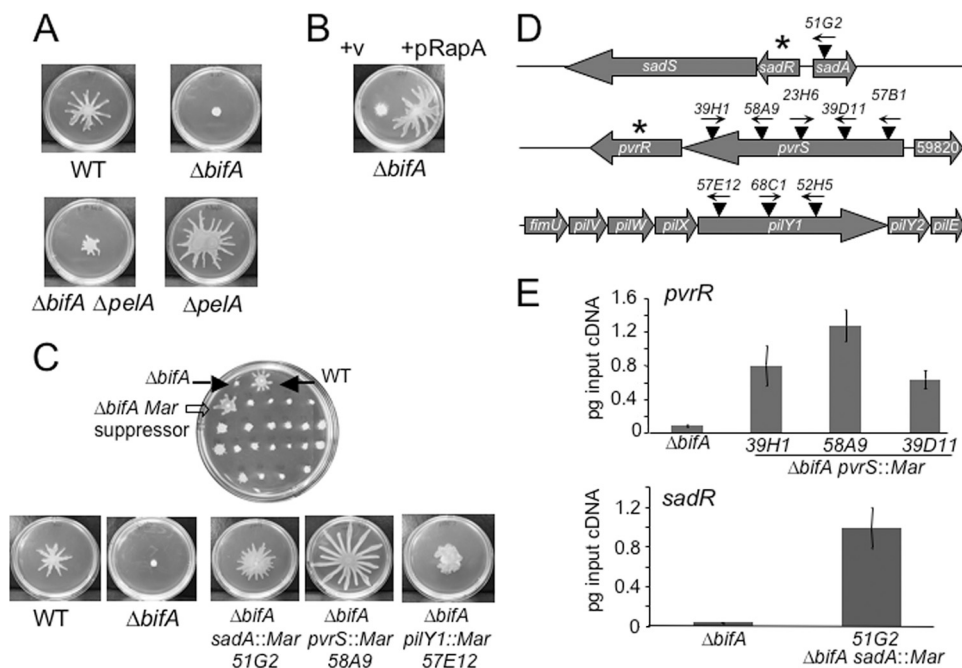
FIG. 1. Genetic analyses of the Δ*bifA* swarming defect. (A) Impact of Pel polysaccharide on the Δ*bifA* swarming impairment. Representative images of swarms formed by the WT, the Δ*bifA* mutant, the Δ*bifA* Δ*pelA* double mutant, and the Δ*pelA* mutant swarms after 16 h at 37°C on 0.5% swarming agar are shown. (B) Impact of expression of the RapA PDE on swarming motility of the Δ*bifA* mutant. Representative images of swarms of the Δ*bifA* mutant carrying the pMQ72 vector control (left) and pMQ72 expressing the RapA c-di-GMP phosphodiesterase (right) are shown. The plates contained 0.2% (wt/vol) arabinose and were incubated for 16 h at 37°C. (C) Genetic screen for suppressors of the Δ*bifA* swarming impairment. The top image shows a swarm plate from the screen of Δ*bifA mariner* transposon mutants, with filled arrows indicating the WT control (top right) and the Δ*bifA* mutant (top left). The open arrow indicates a candidate Δ*bifA mariner* suppressor mutant. The lower panel shows typical images of swarms of the WT, the Δ*bifA* mutant, and the Δ*bifA sadA*::*Mar*, Δ*bifA pvrS*::*Mar*, and Δ*bifA pilY1*::*Mar* suppressor mutants. The plates were incubated for 16 h at 37°C. (D) Genetic loci of Δ*bifA* swarm suppressors. The schematic diagram shows the approximate locations of *mariner* transposon mutations (▼) in the *sadA*, *pvrS*, and *pilY1* genes. Arrows above transposons indicate the direction of transcription from the *mariner* P$_{tac}$ promoter. Asterisks denote genes encoding predicted c-di-GMP phosphodiesterases. (E) qRT-PCR analysis of *pvrR* expression in Δ*bifA pvrS*::*Mar* suppressor mutants (top panel) and *sadR* expression in the Δ*bifA sadA*::*Mar* suppressor mutant (bottom panel) relative to the Δ*bifA* single mutant. Expression is plotted as picograms input cDNA for each strain. Error bars indicate standard deviations.

To first confirm that the accumulation of excess c-di-GMP does, in fact, inhibit swarming motility in the Δ*bifA* mutant, we tested whether expression of a nonendogenous PDE in the Δ*bifA* mutant could rescue the swarming defect. We expressed the *Pseudomonas fluorescens rapA* gene, encoding a PDE previously shown to possess c-di-GMP-degrading activity (38), under the control of the arabinose-inducible pBAD promoter. As shown in Fig. 1B, expression of pRapA in the Δ*bifA* mutant background is able to rescue the swarming defect of the Δ*bifA* mutant. These results indicate that degradation of c-di-GMP in the Δ*bifA* mutant is able to restore swarming motility and supports the hypothesis that excess c-di-GMP inhibits swarming motility in the Δ*bifA* mutant.

Identification of suppressors of the Δ*bifA* swarming motility defect. To better understand how c-di-GMP negatively affects swarming motility, we sought to identify those factors required for swarming inhibition in the Δ*bifA* mutant. To this end, we performed *mariner* transposon mutagenesis of the Δ*bifA* mutant and screened for restoration of swarming motility. We screened approximately 5,500 *mariner* transposon mutants in the Δ*bifA* mutant background on swarm agar plates (Fig. 1C, top panel) and isolated suppressor strains with mutations in four different genes.

We mapped these transposon mutations in the suppressors to *sadA*, *pvrS*, *pilY1*, and *sadB* (Fig. 1D). The swarm phenotypes of three representative Δ*bifA* suppressor mutants are shown in Fig. 1C, bottom panel. The Δ*bifA sadA*::*Mar* mutant exhibits a pattern of swarming motility distinct from that of the WT, with shorter and more numerous tendrils. We isolated five independent *pvrS* mutants as suppressors of the Δ*bifA* swarming defect (Fig. 1D). For the representative Δ*bifA pvrS*::*Mar* mutant (58A9) shown in Fig. 1C, the extent of swarming of the mutant is greater than that of the WT. The remaining four *pvrS*::*Mar* mutants suppress the Δ*bifA* mutant phenotype to various degrees, which likely reflects the different positions of the transposon insertion in each mutant (see below). The three independent Δ*bifA pilY1*::*Mar* suppressor mutants isolated exhibit an unusual swarm morphology relative to the WT in that the mutants lack distinct tendril formation and instead show a more uniform swarming pattern radiating from the origin of inoculation (Fig. 1C). The fourth gene, *sadB*, was previously shown to be required for inhibition of swarming by the Δ*bifA* mutant (27). Given that mutations in the *sadB* gene completely suppress the swarming defects of the Δ*bifA* mutant, the isolation of *sadB* mutants served to validate the screen.

Enhanced expression of genes encoding putative PDEs suppresses the Δ*bifA* swarming defect. Of the genes identified as suppressors in this screen, two genes, *sadA* and *pvrS*, are lo-

cated directly adjacent to genes that code for proteins containing EAL motifs and thus are predicted to encode c-di-GMP phosphodiesterases (*sadR* and *pvrR*, respectively) (Fig. 1D). The *sadA* gene (also known as *rocA*) encodes a response regulator that has been previously shown, together with the adjacent genes, *sadR* and *sadS*, to be involved in biofilm formation as well as regulation of Cup fimbrial gene expression (28, 30). The *pvrS* gene encodes a sensor histidine kinase and is adjacent to the response regulator gene *pvrR*, which has been shown to influence the frequency of formation of antibiotic-resistant small-colony variants of *P. aeruginosa* (18).

Given the proximity of the transposon insertions in *sadA* and *pvrS* to a PDE-encoding gene and the fact that overexpression of the RapA PDE in the Δ*bifA* mutant is able to rescue the swarming defect, we hypothesized that the phenotypic suppression of the Δ*bifA* swarming defect observed for the Δ*bifA sadA*::*Mar* and Δ*bifA pvrS*::*Mar* mutants was due to enhanced expression of *sadR* and *pvrR* rather than to inactivation of either *sadA* or *pvrS*. This also seemed a reasonable prediction based on the fact that the *mariner* transposon used in these studies harbors an outward-directed Ptac promoter and has been shown in previous studies to enhance expression of genes adjacent to transposon insertion sites (30). Furthermore, in a recent study, overexpression of *pvrR* led to a reduction in intracellular c-di-GMP levels, with a corresponding impact on c-di-GMP-sensitive phenotypes (36).

As shown in Fig. 1D, the Ptac promoter for several of the transposon insertion alleles is oriented toward the adjacent PDE-encoding gene. In cases where the opposite orientation is found, it is possible that the promoter driving expression of the *aacC1* gene, encoding resistance to gentamicin, is able to suitably activate transcription of adjacent genes. To address the possibility that *sadR* and *pvrR* are overexpressed in these mutants, we performed qRT-PCR to examine expression of the *pvrR* gene in several of the Δ*bifA pvrS*::*Mar* mutants, as well as expression of the *sadR* gene in the Δ*bifA sadA*::*Mar* 51G2 mutant (Fig. 1E). The results show that expression of the *pvrR* gene is elevated in each of the Δ*bifA pvrS*::*Mar* mutants, ranging from 8-fold to 17-fold higher than that in the Δ*bifA* mutant (Fig. 1E, top panel). Likewise, expression of the *sadR* gene in the Δ*bifA sadA*::*Mar* 51G2 mutant is elevated approximately 30-fold relative to that in the Δ*bifA* single mutant (Fig. 1E, bottom panel).

To further confirm that inactivation of either the *sadA* or *pvrS* gene is not responsible for suppression of the Δ*bifA* swarming impairment, we tested the Δ*bifA* Δ*sadA* double mutant and a Δ*bifA* mutant containing a single crossover insertion in the *pvrS* gene and found that they are indistinguishable from the Δ*bifA* single mutant in swarming motility, CR binding, and biofilm formation assays (data not shown). Taking these results together with the data from the RT-PCR experiments, we infer that suppression of the swarming motility defect exhibited by the Δ*bifA sadA*::*Mar* and Δ*bifA pvrS*::*Mar* mutants occurs via overexpression of the adjacent PDE-encoding genes, *sadR* and *pvrR*, respectively. These data are consistent with our observation that overexpression of a heterologous PDE (i.e., RapA) also restores swarming to a Δ*bifA* mutant (Fig. 1B).

Mutations in the *pilY1* gene suppress multiple Δ*bifA* mutant defects. We isolated three independent *pilY1* mutants as suppressors of the Δ*bifA* swarming defect (Fig. 1D). The *pilY1*

gene encodes a protein involved in type IV pilus biogenesis and twitching motility, a form of surface-associated motility of *P. aeruginosa* that is distinct from swarming motility. While studies from our laboratory and others have previously shown that pili are not absolutely required for swarming motility by this bacterium (49, 58), it has been reported that pili participate in the patterning of swarming motility (31).

To better understand the potential role of the *pilY1* gene in c-di-GMP-mediated swarming suppression, we further characterized the ability of Δ*bifA pilY1*::*Mar* suppressors to affect each of the Δ*bifA* mutant phenotypes resulting from the accumulation of c-di-GMP. We observed that Δ*bifA pilY1*::*Mar* mutants show markedly decreased biofilm formation (Fig. 2A, bottom row, and B) and CR binding (a measure of Pel polysaccharide production) relative to those of the Δ*bifA* single mutant (Fig. 2A, middle row). Furthermore, we generated an in-frame deletion of the *pilY1* gene and observed the same suppression of these Δ*bifA* mutant phenotypes as we observed for the *mariner* suppressor mutants (Fig. 2A, fourth column).

Given that the *pilY1* gene maps to a region containing other pilus-related genes and has been shown to be cotranscribed as a unit with members of this locus (*fimU-pilVWXY1Y2E*) (6) (Fig. 1D), we assessed whether suppression of the Δ*bifA* mutant defects was due to inactivation of *pilY1* alone, or possibly due to polar effects of the Δ*pilY1* mutation, by generating a His-tagged version of the *pilY1* gene on a multicopy plasmid under the control of the pBAD promoter. Introduction of this construct (pPilY1His) into the Δ*bifA* Δ*pilY1* mutant background restored the hyperbiofilm and hyper-CR binding phenotypes as well as swarming inhibition to the same extent observed for the Δ*bifA* single mutant (Fig. 2A and B). This and all complementation studies with pPilY1His were performed in the absence of added arabinose and thus rely upon the basal expression of the pBAD promoter to drive PilY1 production. These results indicate that mutation of *pilY1* alone is responsible for the observed suppression of the Δ*bifA* mutant phenotypes.

Finally, we also constructed a Δ*pilY1* single mutant strain. This strain is defective for twitching motility (not shown), has reduced CR binding, and shows both increased swarming motility (a 2-fold increase in percent surface coverage of the swarm plate) compared to the WT and an altered swarming pattern. Thus, the Δ*pilY1* single mutant does have a small but reproducible positive impact on swarming motility, consistent with a role for PilY1 in repressing swarming motility.

Loss of type IV pili is not sufficient for full suppression of Δ*bifA* defects. Both the Δ*pilY1* and Δ*bifA* Δ*pilY1* mutants fail to twitch in twitching motility assays, and we can complement these twitching defects using the *pilY1* complementation construct described above (data not shown). Given that loss of *pilY1* function leads to disruption of type IV pilus biogenesis, it is formally possible that suppression of the Δ*bifA* mutant defects is due to loss of surface piliation and/or twitching motility. To test this possibility, we introduced a deletion mutation of the *pilA* gene, which encodes the major pilin subunit of *P. aeruginosa*, into the Δ*bifA* mutant background and assessed the suppression of each of the Δ*bifA* mutant phenotypes in this Δ*bifA* Δ*pilA* double mutant compared to the Δ*bifA* Δ*pilY1* double mutant (Fig. 2A and B).

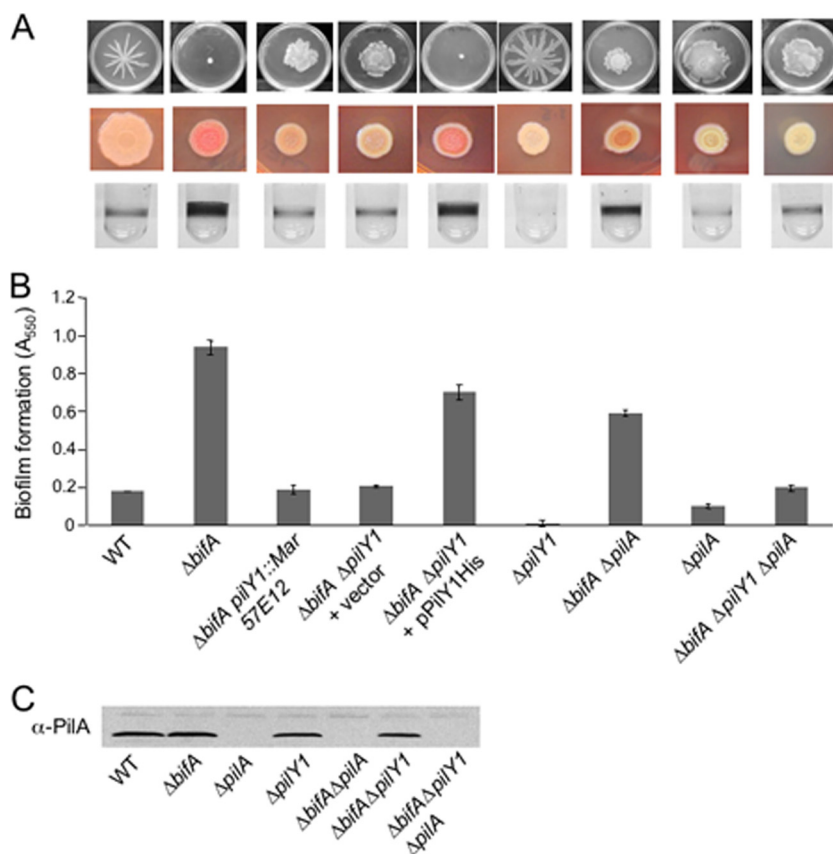The data show that elimination of *pilA* function in the Δ*bifA*

FIG. 2. Phenotypes of the *bifA* suppressor mutants. (A) *pilY1* mutations suppress multiple defects of the Δ*bifA* mutant. Top panel, typical swarm images for (left to right) the WT, the Δ*bifA* mutant, a representative Δ*bifA pilY1*::*Mar* mutant (57E12), the Δ*bifA* Δ*pilY1* double mutant carrying either empty vector or the pPilY1His complementing plasmid, and the Δ*pilY1* mutant. Also shown are swarm images of the Δ*bifA* Δ*pilA* double mutant, the Δ*pilA* single mutant, and the Δ*bifA* Δ*pilA* Δ*pilY1* triple mutant. The plates were incubated for 16 h at 37°C. The middle panel shows CR binding for each strain. CR assay plates were incubated for 24 h at 37°C followed by 48 h at room temperature. The bottom panel shows representative wells of a 96-well dish from a biofilm assay for each strain. Strains were grown in M63 with glucose, MgSO₄, and CAA for 24 h at 37°C prior to crystal violet staining. (B) Quantification of crystal violet-stained biofilms. Crystal violet was solubilized in 30% glacial acetic acid, and the absorbance was measured at 550 nm. Error bars indicate standard deviations. (C) Western blot analysis showing PilA protein in crude lysates prepared from the indicated strain.

mutant background does lead to partial suppression of the swarming, CR binding, and biofilm formation phenotypes. However, the suppression observed for the Δ*bifA* Δ*pilA* mutant is not nearly as robust as that observed for the Δ*bifA* Δ*pilY1* double mutant in each of the assays tested. These results indicate that it is not loss of surface pili *per se* that leads to suppression of the Δ*bifA* mutant defects but that loss of PilY1 function in particular is responsible for full suppression of the Δ*bifA* mutant defects.

Although the Δ*bifA* Δ*pilY1* and the Δ*bifA* Δ*pilA* double mutants both lack functional surface pili (as determined by the twitching motility assay), they differ in that the Δ*bifA* Δ*pilY1* mutant produces pilin whereas the Δ*bifA* Δ*pilA* mutant does not (Fig. 2C). To control for the possibility that a potential buildup of pilin in cells of the Δ*bifA* Δ*pilY1* mutant contributes to suppression of the swarming defect, we constructed a Δ*bifA* Δ*pilY1* Δ*pilA* triple mutant and found no difference in the swarming, biofilm, and CR binding phenotypes compared with those of the Δ*bifA* Δ*pilY1* mutant (Fig. 2A to C). Together, these data indicate that it is loss of PilY1 function and not loss of pili that suppresses the Δ*bifA* swarming defect.

Global c-di-GMP pools are not detectably altered by deletion of the *pilY1* gene. Deletion of the *pilY1* gene in the Δ*bifA* mutant background suppresses all of the c-di-GMP-related phenotypes of the Δ*bifA* mutant, suggesting that such a mutation might lead to a decrease in the cellular pools of c-di-GMP in the Δ*bifA* mutant. To test this notion, we examined levels of c-di-GMP in Δ*bifA* and Δ*bifA* Δ*pilY1* mutant cells grown in the presence of radiolabeled inorganic phosphate, followed by nucleotide extraction, analysis by 2D TLC, and autoradiography (22, 38).

Inspection of the two-dimensional TLC autoradiographs revealed no detectable difference in the levels of c-di-GMP between the two strains (Fig. 3A). Quantification of c-di-GMP and normalization to total $^{32}P_i$ incorporation (for which there is no significant difference between the two strains [$P = 0.55$, $n = 3$]) showed no significant difference between the Δ*bifA* single mutant and Δ*bifA* Δ*pilY1* double mutant ($P > 0.05$) (Fig. 3B). These data suggest that the *pilY1* mutation has little impact on the global levels of c-di-GMP in cells of the Δ*bifA* mutant.

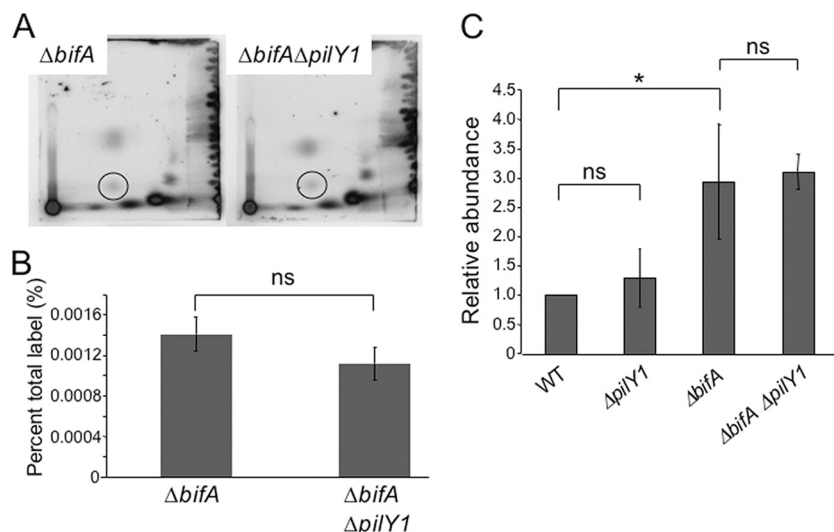To further investigate c-di-GMP cellular pools, we used liq-

FIG. 3. Quantification of global c-di-GMP pools. (A) Shown are autoradiographs of representative two-dimensional TLC plates used to separate [^{32}P]orthophosphate-labeled, acid-extracted whole-cell extracts prepared from the $\Delta bifA$ mutant and the $\Delta bifA$ $\Delta pilY1$ double mutant. The circles indicate the position of c-di-GMP. (B) Quantification of c-di-GMP levels determined by analyzing autoradiographs using the Storm 860 and ImageQuant software (v5.1). The graph shows a representative experiment with results for three replicates of each strain plotted as a percentage of the total ^{32}P label incorporated into c-di-GMP. ns, no statistically significant difference between the $\Delta bifA$ mutant and the $\Delta bifA$ $\Delta pilY1$ double mutant using the criterion of a P value of <0.05. (C) Quantification of c-di-GMP levels as measured using liquid chromatography-tandem mass spectrometry. The graph depicts the relative abundance of c-di-GMP for three replicates. ∗, statistically significant difference; ns, no significant difference (using the criterion of a P value of <0.05 by the t test). Error bars indicate standard deviations.

uid chromatography-mass spectrometry (LC-MS) to quantify the levels of c-di-GMP in these strains. Strains were grown to OD_{600} of ≈ 0.6 prior to acid extraction of nucleotides, followed by LC-MS analysis. Consistent with our previous studies, the $\Delta bifA$ mutant accumulates approximately 3-fold more c-di-GMP than the WT (Fig. 3C) (27). Consistent with our observations using the whole-cell labeling method described above, mutation of $pilY1$ has no detectable impact on c-di-GMP levels in either the WT or the $\Delta bifA$ mutant background.

Expression of the *pel* polysaccharide genes is not altered by deletion of the *pilY1* gene. Mutation of the *pilY1* gene in the $\Delta bifA$ mutant background suppresses the hyper-CR binding phenotype of the $\Delta bifA$ mutant and thus abrogates the increased production of Pel polysaccharide. It is plausible that *pilY1* affects Pel polysaccharide production by influencing *pel* gene expression, and if so, we would predict that *pel* gene expression is reduced in the $\Delta bifA$ $\Delta pilY1$ mutant relative to the $\Delta bifA$ single mutant. Thus, we assessed *pel* gene expression in the WT, $\Delta bifA$ mutant, and $\Delta bifA$ $\Delta pilY1$ mutant backgrounds. Consistent with our previous studies (27), we observe no statistically significant difference (using the criterion of a P value of <0.05 [$n = 3$]) in *pelA* expression between the WT (0.012 ± 0.001 pg input cDNA) and the $\Delta bifA$ mutant (0.013 ± 0.004 pg input cDNA). Furthermore, our data show that the *pilY1* mutation confers no significant change in *pelA* gene expression in the $\Delta bifA$ $\Delta pilY1$ (0.01 ± 0.002 pg input cDNA) mutant relative to the $\Delta bifA$ single mutant. These data indicate that excess Pel polysaccharide production in the $\Delta bifA$ mutant is likely not due to a *pilY1*-mediated increase in *pel* transcription.

Enhanced expression of *pilY1* represses swarming in a pilus-independent manner. The identification of the *pilY1* gene as a suppressor of the $\Delta bifA$ swarming defect indicates that PilY1 is

important for inhibition of swarming motility in the $\Delta bifA$ mutant. Additionally, a *pilY1* single mutant is a hyperswarmer relative to the WT (Fig. 2A), suggesting that the *pilY1* gene can repress swarming in the WT. Based on these observations, we assessed whether the *pilY1* gene could act as a repressor of swarming when expressed from a multicopy plasmid in the WT background.

As shown in Fig. 4A (left panel), arabinose-induced expression of the pPilY1His plasmid was indeed able to repress swarming motility by the WT. Based on the swarm suppression results with the $\Delta bifA$ $\Delta pilA$ mutant above indicating that the pilus might affect *pilY1* function, we next asked whether *pilY1*-mediated repression of swarming required functional pili. As shown in Fig. 4A (right panel), overexpression of *pilY1* in the $\Delta pilA$ mutant was still able to repress swarming motility. These results further confirm that the *pilY1* gene is able to function as a repressor of swarming motility and indicate that pilin production is not required for this effect.

Expression of *pilY1* in a $\Delta bifA$ mutant. Our data thus far suggest that PilY1 functions to repress swarming motility. Thus, one possibility is that levels of c-di-GMP modulate *pilY1* gene expression and that the high levels of c-di-GMP in a $\Delta bifA$ mutant could enhance *pilY1* expression, thus providing an explanation for the loss of swarming motility upon loss of BifA function. To address this hypothesis, we examined the levels of *pilY1* gene expression in the WT and $\Delta bifA$ mutant backgrounds by qRT-PCR. Expression of the *pilY1* gene is not different in the $\Delta bifA$ mutant and the WT, suggesting that elevated levels of c-di-GMP do not affect *pilY1* transcription (Fig. 4B).

To address whether c-di-GMP affects *pilY1* expression in a posttranscriptional manner, we examined whether levels of PilY1 protein are altered in the $\Delta bifA$ mutant relative to the
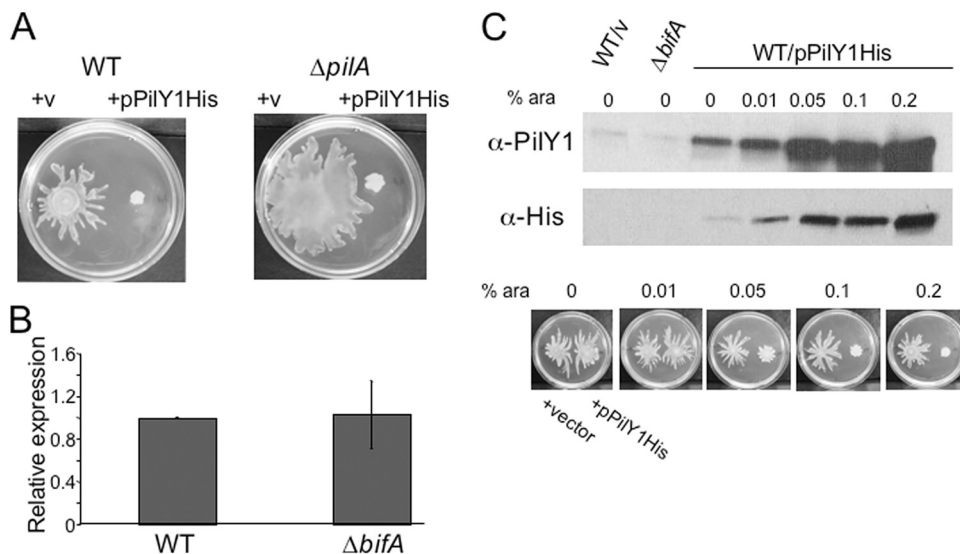
FIG. 4. Increased expression of PilY1 represses swarming motility. (A) Enhanced expression of *pilY1* represses swarming in a pilus-independent manner. Shown are representative images of the WT (left panel) and the Δ*pilA* mutant (right panel) carrying either empty vector or the pPilY1His arabinose-inducible expression plasmid. Swarm plates contained 0.2% arabinose and were incubated for 16 h at 37°C. (B) qRT-PCR analysis of *pilY1* expression in the Δ*bifA* mutant relative to the WT. Expression data were normalized to the WT, and the results shown are the average from two independent experiments. Error bars indicate standard deviations. (C) Assessment of PilY1 levels and impact on swarming phenotype. Shown in the upper panels are Western blots showing PilY1 protein levels in the WT carrying empty vector, the Δ*bifA* mutant, and the WT carrying the pPilY1His plasmid. Concentrations of arabinose (wt/vol) added during culturing of each sample are indicated. PilY1 protein was detected using anti-PilY1 antibodies (top panel) or anti-His antibody to detect His-tagged PilY1 (middle panel) expressed from the pPilY1His plasmid. Shown in the lower panel are swarm plates containing arabinose at the indicated concentrations. On the left side of each plate is the WT carrying the empty vector control, and on the right is the WT carrying pPilY1His.

WT using a polyclonal PilY1 antibody. As shown on a representative Western blot in Fig. 4C (top panel), the levels of PilY1 protein in the WT (lane 1) and the Δ*bifA* mutant (lane 2) are comparable. Quantification of PilY1 protein levels on a Western blot reveals no significant difference between the WT and Δ*bifA* mutant backgrounds ($P = 0.78$, $n = 3$ replicates each), indicating that c-di-GMP exerts no detectable posttranscriptional effects on PilY1 levels.

To further assess whether the levels of PilY1 in the Δ*bifA* background would be sufficient to repress swarming, we utilized the pPilY1 arabinose-inducible pMQ80 construct to titrate the levels of PilY1 in the WT and determine the impact on swarming motility. When the *pilY1* gene is expressed from the pPilY1His plasmid in the WT strain (WT/pPilY1His) in the absence of arabinose (Fig. 4C, lane 3), we observe a 13-fold increase in PilY1 protein levels relative to PilY1 levels in the Δ*bifA* mutant (lane 2), as detected using the PilY1 antibody (top panel). Importantly, we do not observe an impact on swarming motility (bottom left panel) under these conditions, indicating that this increase in PilY1 above the levels in the Δ*bifA* mutant is not sufficient to repress swarming motility of the WT.

For robust repression of WT swarming motility, arabinose was added to a final concentration of 0.2%. Under these conditions, a substantial induction of PilY1 protein expression occurs (Fig. 4C, lane 7) and swarming motility of the WT strain is repressed (bottom right panel). Using intermediate arabinose concentrations of between 0 and 0.2%, we can estimate a minimum level of PilY1 protein that is sufficient to repress swarming in the WT. At 0.01% arabinose, we still do not

reliably observe swarming repression at levels of PilY1 that are ~15-fold above that in the Δ*bifA* mutant. However, we begin to observe swarming repression with the addition of 0.05% arabinose, which results in PilY1 levels that are ~30-fold higher than those in the Δ*bifA* mutant. Together, these data argue that swarming repression in the Δ*bifA* mutant is not primarily due to increased expression of the PilY1 protein.

PilY1 functions genetically upstream of the DGC SadC and specifically requires SadC, but not other DGCs, for repression of swarming motility. Previous work in our laboratory has shown that the SadC protein, a diguanylate cyclase (DGC), functions in a genetic pathway with the BifA protein to modulate levels of c-di-GMP in the cell and thereby influence both biofilm formation and swarming motility (37). As shown in those studies, mutating the *sadC* gene in the Δ*bifA* mutant background led to suppression of the Δ*bifA* mutant swarming motility defect, as well as partial suppression of the hyperbiofilm formation and CR binding phenotypes (37), results that are similar to those observed for the Δ*bifA* Δ*pilY1* double mutant. Furthermore, overexpression of the *sadC* gene in the WT leads to inhibition of swarming motility as well as stimulation of biofilm formation and CR binding (37). Given the similarities between the effects of the *sadC* and *pilY1* genes on surface-associated behaviors, we wondered whether these two genes might function together in a pathway to regulate biofilm formation and swarming motility. To determine the potential epistatic relationship between the *pilY1* and *sadC* genes, we first asked whether the *sadC* gene is required for PilY1-mediated repression of swarming. When overexpressed in the Δ*sadC* mutant, pPilY1His fails to repress swarming, indicating
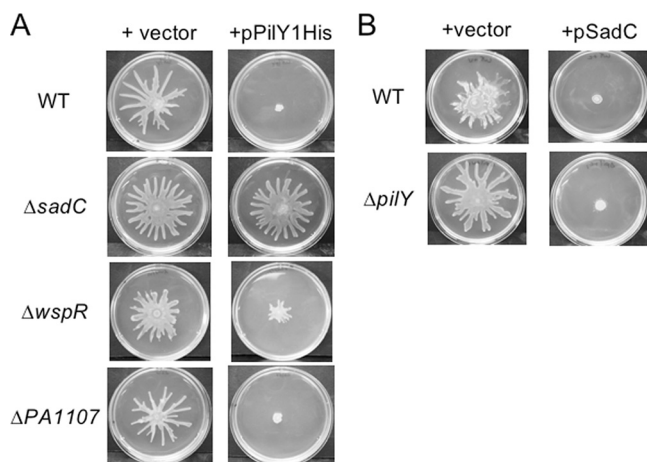
FIG. 5. PilY1 functions genetically upstream of the SadC diguanylate cyclase. (A) Shown are representative swarms for the WT and the Δ*sadC*, Δ*wspR*, and Δ*PA1107* DGC mutants carrying either the empty vector (left column) or the pPilY1His plasmid (right column). Plates contained arabinose at a concentration of 0.2%. (B) Swarms of the WT and Δ*pilY1* mutant when carrying either the vector (left column) or pSadC expression plasmid (right column). Plates contained arabinose at a concentration of 0.2%.

that *sadC* functions downstream of *pilY1* in the repression of swarming (Fig. 5A, second row).

Given that there are 16 predicted DGCs encoded by the *P. aeruginosa* PA14 genome, we assessed whether this requirement by *pilY1* for a downstream DGC was specific for SadC or whether other DGCs might also fulfill this function. In particular, we tested two additional DGC mutants for their impacts on *pilY1*-mediated swarming repression. The *wspR* gene encodes a CheY-like response regulatory component of a chemosensory signal transduction pathway (16). The WspR protein contains a GGDEF domain and was shown to possess DGC activity and to be required for the hyperbiofilm phenotype of a *wspF* mutant (16, 22). The *PA1107* gene also encodes a GGDEF domain-containing protein and was shown previously to possess DGC activity and to stimulate hyperbiofilm formation upon overexpression (29). As shown in Fig. 5A, enhanced expression of pPilY1His is still able to repress swarming in both the Δ*wspR* and Δ*PA1107* mutant backgrounds, indicating a specific interaction between the *pilY1* gene and *sadC*-encoded DGC in the repression of swarming.

To further confirm that the *pilY1* gene functions upstream of the *sadC* gene in a genetic pathway, we performed the reciprocal experiments to those described above. That is, we overexpressed the *sadC* gene in the Δ*pilY1* mutant to determine whether *pilY1* function is required for repression of swarming by *sadC*. Using an arabinose-inducible hemagglutinin (HA)-tagged version of the *sadC* gene (pSadC), we found that *sadC* overexpression was still able to repress swarming to the same extent in the Δ*pilY1* mutant background as in the WT (Fig. 5B), indicating that *pilY1* likely functions upstream of *sadC* in swarm repression.

Cellular localization of PilY1. According to the signal peptide prediction program SignalP, the PilY1 protein harbors an N-terminal signal peptide resembling a Sec secretion system signal peptide for transport across the inner membrane. The

bacterial localization prediction tool PSORTB predicts that the PilY1 protein localizes to the outer membrane and also is secreted to the extracellular environment. Consistent with these predictions, previous studies using the *P. aeruginosa* PAK strain showed that PilY1 was found in the insoluble membrane fraction as well as the pilus-associated extracellular shear fraction (3). Results from a *phoA* fusion screen to identify exported proteins in *P. aeruginosa* PAO1 indicated that PilY1 is transported across the cytoplasmic membrane (32).

To determine the cellular localization of PilY1 in *P. aeruginosa* strain PA14, we performed cellular fractionations of WT cells and detected PilY1 using a polyclonal PilY1 antibody. In WT cells, we observe PilY1 in the whole-cell (WC) lysate and in the cytosolic (Cyt) fraction as well as in the total membrane (TM) and inner membrane (IM) fractions (Fig. 6). We do not detect PilY1 in the outer membrane (OM) fraction. Given predictions that PilY1 is a secreted protein, we tested whether PilY1 was present in the supernatant (Sup) fraction and the so-called cell-associated (CA) fraction, which refers to proteins weakly bound to the cell surface (see Materials and Methods for details). We can detect PilY1 in the supernatant after concentrating these samples approximately 300-fold. However, we are unable to detect PilY1 in the CA fractions.

The integrity of the inner and outer membrane fractions was confirmed by Western blotting using antibodies to SecY, an inner membrane protein (1), and OprF, an outer membrane protein (7, 21, 34).

Based on our findings indicating that loss of pilin production by mutation of *pilA* partially suppresses the Δ*bifA* swarming defect, we hypothesized that pilin production and/or pilus formation might affect PilY1 cellular localization. Thus, we tested whether PilY1 localization is altered in a Δ*pilA* mutant relative to the WT. As shown in Fig. 6 (compare lane 3 to lane 1), we observe that the supernatant fraction of the Δ*pilA* mutant lacks detectable PilY1 relative to the WT. The remaining cellular fractions contain levels of PilY1 that are comparable in the Δ*pilA* mutant and the WT. These data suggest that loss of pilin (and/or pilus formation) affects localization of PilY1 to the supernatant fraction. Furthermore, it is plausible that the absence of PilY1 from the supernatant fraction of the Δ*pilA* mutant contributes to the partial suppression of swarming observed in the Δ*bifA* Δ*pilA* double mutant. However, we cannot exclude the possibility that loss of surface pili alone contributes to the partial swarming restoration observed for the Δ*bifA* Δ*pilA* double mutant.

Structure/function analysis of PilY1. To better understand the potential mechanism by which PilY1 represses swarming motility, we undertook a structure/function analysis of the protein. A schematic diagram of the predicted PilY1 structure is shown in Fig. 7A. At the N terminus, the PilY1 protein has a Sec secretion signal for transport across the inner membrane. Besides other PilY1 homologs from various species, the remainder of the N-terminal region bears little resemblance to other proteins and lacks any obvious protein motifs. However, a 200-amino-acid region within the N terminus shows weak similarity to the von Willebrand factor A (VWA) domain. Most VWA domains that have been well characterized to date belong to extracellular eukaryotic proteins, including cell adhesion and extracellular matrix (ECM) proteins, and are involved in protein-protein interactions (14, 15, 59). However,
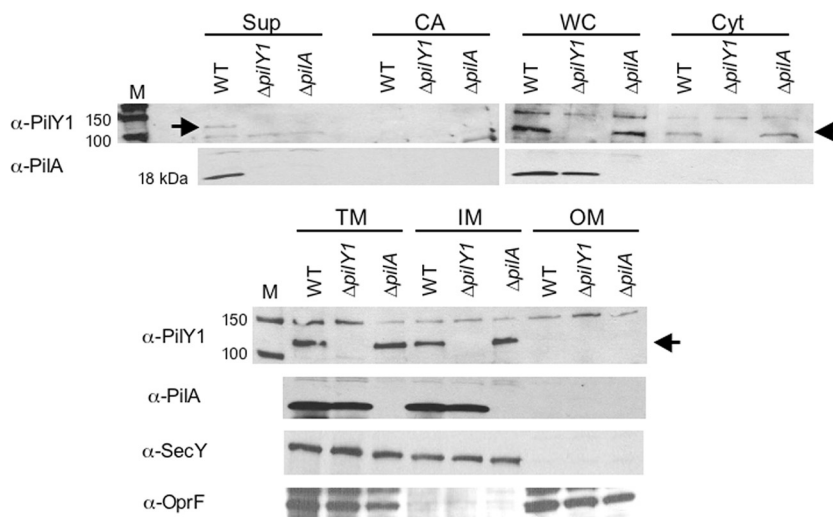
FIG. 6. PilY1 cellular localization. Cellular fractions of the WT, Δ*pilY1* mutant, and Δ*pilA* mutant were separated by SDS-PAGE. Fractions are indicated as supernatant (Sup), cell-associated (CA), whole-cell (WC), soluble cytoplasmic (Cyt), total membrane (TM), inner membrane (IM), and outer membrane (OM) fractions. The cell-associated fraction refers to proteins weakly associated with the cell surface that can be released by brief vortexing. Western analysis was performed using the following antibodies as indicated: anti-PilY1, anti-SecY, anti-OprF or anti-PilA antibody. SecY (~50 kDa) served as a control for inner membrane localization, and OprF (~37 kDa) served as an outer membrane marker. The protein size marker lanes are indicated (M), with sizes in kDa. The arrow indicates the position of the PilY1 protein at ~110 kDa. The molecular mass of WT PilY1 remains constant across all cellular fractions, as confirmed by SDS-PAGE and Western blotting (not shown).

many intracellular eukaryotic proteins with a diverse array of functions, including transcription, DNA repair, and proteolysis, also contain VWA domains (62). Recently, VWA domains in prokaryotic proteins have also been identified (45), although the precise role of this domain in these proteins has not yet been established, with the exception of a recent study showing a role for the VWA domain of the *Streptococcus agalactiae* PilA protein, a pilus-associated adhesin, in adherence of this organism to epithelial cells (26).

The most obvious domain of PilY1 lies within the C-terminal half of this protein, which shares similarity (24% identity and 46% similarity) with the C terminus of the PilC proteins of pathogenic *Neisseria* species. PilC of *Neisseria* has been shown to associate with the bacterial cell surface, including localization to the tip of the pilus, and is required for bacterial adherence to epithelial cells (39, 46, 52, 53). A recent crystal structure-based analysis of the PilC-like domain of PilY1 has shown this domain to be essential for regulating pilus extension and retraction mechanisms involved in twitching motility in a calcium-dependent manner (41).

To test the functions of these various PilY1 domains in swarming inhibition as well as twitching motility, we generated deletion constructs of the *pilY1* gene (Fig. 7A) and expressed the His-tagged PilY1 variants from each of these constructs in the WT background. However, all of the deletion constructs except PilY1ΔPilC produced proteins that appeared to be unstable relative to full-length PilY1His protein as assessed by Western blotting (not shown), making it difficult to draw any firm conclusions regarding the functions of these domains.

Thus, we proceeded to characterize the PilY1ΔPilC construct. The results show that the PilY1ΔPilC protein is able to inhibit swarming motility as well as the full-length PilY1 protein, indicating that the PilC-like region of the PilY1 protein is dispensable for swarming inhibition (Fig. 7B). Expression of

the PilY1ΔPilC protein in the WT background has no impact on twitching motility. In complementation assays, expression of the PilY1ΔPilC protein is unable to complement the twitching defect of the Δ*pilY1* mutant (not shown), a result consistent with recent findings regarding the essential role of this domain in twitching motility (41).

Given that the N-terminal half of the PilY1 protein is sufficient to inhibit swarming motility, we focused on this region for further mutagenesis experiments. We first undertook a random PCR mutagenesis approach by mutagenizing the PilY1ΔPilC construct and screening for loss of swarming inhibition when expressed in the WT background. We screened approximately 1,000 strains expressing mutant variants of PilY1, and about 30% of those mutants recovered swarming motility. However, upon closer investigation using Western dot blot analysis, we found that only 3% of these swarmer strains produced a detectable PilY1ΔPilC protein, indicating that the vast majority of the mutations were likely causing PilY1 protein instability. Furthermore, when those constructs making apparently stable PilY1ΔPilC protein were retransformed into WT *P. aeruginosa*, the swarming phenotypes were not reproducible, indicating that the mutation in the original mutant strains was not linked to the plasmid. Thus, we chose to take a more directed mutagenesis approach.

Alignment of the PilY1 protein across pseudomonads as well as other bacterial species using the ClustalW2 sequence alignment tool indicated there were a number of highly conserved amino acid residues throughout the N terminus of the protein, and we used this alignment as a guide to generate site-directed mutations. A subset of the highly conserved amino acids found in the alignment fall within the putative VWA-like region of PilY1. The alignment of PilY1 proteins spanning two of the conserved sites within the VWA-like region is shown in Fig. 7D.
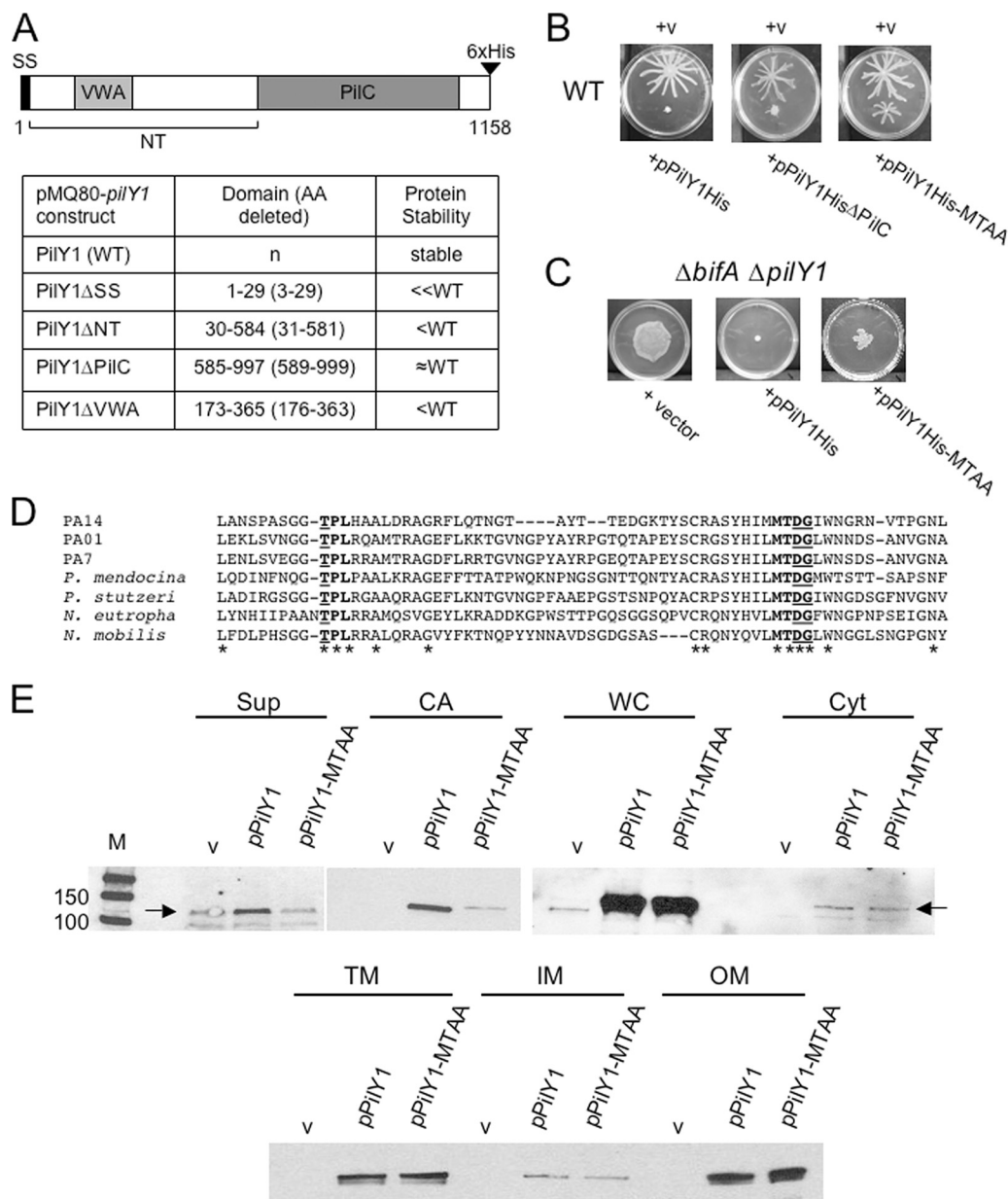
FIG. 7. Structure/function analysis of PilY1. (A) A schematic diagram of the PilY1 protein. Numbers indicate amino acids, and predicted regions of PilY1 are denoted as follows: signal sequence (SS), N terminus (NT), von Willebrand factor A (VWA), and the domain with similarity to PilC of *Neisseria* species (PilC). The table lists the *pilY1* deletion constructs made in the pMQ80 vector (left column), the amino acids deleted from PilY1 for each construct (middle column), and the protein stability (right column) for each of the expressed proteins relative to the WT protein as assessed by Western blot analysis using the anti-His antibody. WT PilY1 and all PilY1 variants are C-terminally His tagged as indicated in the diagram. (B) Representative images of swarms for the WT carrying either the vector (v), pPilY1His (left), pPilY1HisΔPilC (middle), or the pPilY1His-MTAA mutant construct (right). Plates contained 0.2% arabinose and were incubated for 16 h at 37°C. (C) Partial complementation of the Δ*bifA* Δ*pilY1* mutant by pPilY1His-MTAA. Shown are images of swarms for the Δ*bifA* Δ*pilY1* mutant carrying the vector (left), pPilY1His (middle), or pPilY1His-MTAA (right). Plates contained 0.2% arabinose and were incubated 16 h at 37°C. (D) CLUSTALW-generated amino acid sequence alignment of the PilY1 protein from representative strains listed on the left: *P. aeruginosa* strains PA14, PAO1, and PA7; *Pseudomonas mendocina*, *Pseudomonas stutzeri*, *Nitrosomonas eutropha*, and *Nitrococcus mobilis*. Asterisks indicate identical amino acids conserved across the PilY1 proteins shown in the alignment. Bold indicates conserved regions ("TPL" and "MTDG"), with underlined amino acids mutated to alanine residues. (E) Western blot analysis showing cellular localization of PilY1 in the WT carrying either the vector, pPilY1, or pPilY1-MTAA. Cellular fractions are as follows: supernatant (Sup), cell-associated (CA), whole-cell (WC), soluble cytoplasmic (Cyt), total membrane (TM), inner membrane (IM), and outer membrane (OM) fractions. Fractions were separated by SDS-PAGE, and Western blots were probed with the anti-PilY1 polyclonal antibody. The protein size marker (M) is indicated on the top left panel with sizes in kDa. Note that the PilY1 protein expressed from its native promoter can be detected in the TM and IM fractions (Fig. 6) but is not observed in those fractions in this figure due to the shorter exposure times used to detect the PilY1His protein expressed from the arabinose-inducible plasmid. Furthermore, endogenously expressed PilY1 was not detected in the OM fraction (Fig. 6) but can be detected when PilY1 is overexpressed from this plasmid.

To determine whether either of these conserved regions is important for PilY1 function in swarming repression, we changed the threonine in the conserved "TPL site" to an alanine in one construct and changed the aspartic acid and glycine in the conserved "MTDG site" to alanine residues in a second construct. We then overexpressed these His-tagged mutant PilY1 proteins (PilY1-APL and PilY1-MTAA) in the WT background and assessed swarming motility. As shown in Fig. 7B (right panel), expression of the PilY1-MTAA mutant protein led to only partial swarming inhibition relative to the complete inhibition observed for the WT protein (left panel), whereas the PilY1-APL mutant protein behaved like the WT in regard to swarming inhibition (data not shown). The stability of the PilY1-MTAA protein was comparable to that of the WT PilY1 protein as assessed by Western blotting (Fig. 7E). These data indicate a potential role for the conserved aspartic acid and glycine amino acids in swarming inhibition by PilY1. Furthermore, these data are consistent with our observations showing that the PilY1-MTAA construct only partially complements the swarming phenotype of the Δ*bifA* Δ*pilY1* double mutant (Fig. 7C). Similarly, the PilY1-MTAA construct only partially complements the twitching phenotype of the Δ*pilY1* mutant (not shown).

Our observations above indicate that the PilY1-MTAA mutant protein shows only partial function in swarming inhibition. To further investigate this, we determined whether the cellular localization of PilY1-MTAA differs from that of WT PilY1 when overexpressed in the WT background. When expression is induced with arabinose, we observe elevated levels of WT PilY1 in all of the cellular fractions relative to the WT carrying vector alone (Fig. 7E). In contrast, the levels of PilY1-MTAA in the Sup and CA fractions are greatly reduced relative to those fractions containing WT PilY1. In all other cellular fractions, the levels of PilY1-MTAA are comparable to those of the WT PilY1. These data indicate that localization of the PilY1-MTAA mutant protein is altered in both the Sup and CA fractions and suggest that the mutated amino acids may affect proper extracellular localization of the PilY1-MTAA protein. Taken together with the results showing an absence of PilY1 in the Δ*pilA* mutant Sup fraction relative to the WT, these data support the notion that extracellular localization of PilY1 may contribute to its function in swarming inhibition.

DISCUSSION

The mechanism(s) by which the intracellular signaling molecule c-di-GMP negatively affects swarming motility of *P. aeruginosa* is not well understood. In this study, we chose to address this question using a genetic screen to identify suppressors of a mutant that is defective for swarming motility, a defect that we show is most likely due to excess accumulation of c-di-GMP (Fig. 1). In previous work, we reported that the *bifA* gene encodes a c-di-GMP-degrading phosphodiesterase and that the Δ*bifA* mutant accumulates elevated levels of c-di-GMP relative to the WT, forms hyperbiofilms, and is completely defective for swarming motility (27). Here we demonstrate that the swarming defect of the Δ*bifA* mutant can be rescued by heterologous expression of the RapA c-di-GMP phosphodiesterase from *P. fluorescens*, thereby supporting the hypothesis that elevated c-di-GMP levels repress swarming by

P. aeruginosa and demonstrating a proof of concept for the Δ*bifA* suppressor screen to identify factors that participate in c-di-GMP-mediated swarming repression.

As further validation for c-di-GMP-mediated repression of swarming and the utility of our screen, we isolated several Δ*bifA* suppressor strains with transposon mutations that lead to the overexpression of two different genes predicted to encode c-di-GMP PDEs, namely, SadR and PvrR, which have both been shown to be associated with c-di-GMP-degrading activity (29, 36, 48). From these results, we surmise that increased expression of these genes leads to an increase in PDE activity in the Δ*bifA* mutant, and presumably decreased c-di-GMP levels, with subsequent restoration of swarming motility.

Using this suppressor screen, we also found that *pilY1* mutants suppressed the loss of swarming motility of the Δ*bifA* mutant, and we present data showing that the PilY1 protein, a component of the pilus biogenesis machinery, is able to repress swarming motility. That is, in addition to suppressing the swarming impairment of the Δ*bifA* mutant, a Δ*pilY1* single mutant is a hyperswarmer relative to the WT (Fig. 2). Furthermore, overexpression of PilY1 in the WT background represses swarming motility (Fig. 4).

When interpreting data involving overexpression of a gene, caution is warranted, given that the elevated levels of PilY1 observed in our studies might not be physiologically relevant and may give rise to misleading secondary effects. However, our observations that PilY1 overexpression requires the DGC SadC to exert its effects on swarming, but not other DGCs with known roles in regulating surface behaviors, argue for a level of specificity that one would not expect if PilY1 overexpression led to nonspecific effects on the cell. PilY1 overexpression has no impact on twitching motility, which also argues against a broad cellular impact caused by PilY1 overexpression. Given these data, we postulate that overexpression of PilY1 allows for activation of this specific SadC-dependent signaling pathway in the absence of a putative input signal, and as such, it is a useful tool to determine what additional factors might be required for PilY1-mediated swarming repression. One set of candidate factors includes the minor pilins encoded at the *pilY1* locus itself, namely, the *fimU*, *pilV*, *pilW*, *pilX*, *pilY2*, and *pilE* products. The minor pilins are required for pilus biogenesis, but little is known about their precise roles in pilus assembly and function (3, 4, 5, 54, 63). Given that *pilY1* and the minor pilin genes are cotranscribed as an operon (6), it is tempting to speculate that the minor pilins not only are involved in pilus assembly/function but also play a role, together with PilY1, in swarming repression.

Recent studies provide evidence that *pilY1* gene expression may be differentially regulated under different environmental conditions. The *pilY1* operon has been shown to be under the control of the AlgR transcriptional regulator (6, 33), and that AlgR-dependent regulation of this operon is growth phase specific (33). Interestingly, expression of a *pilY1* luciferase gene fusion was found to be induced in the presence of subinhibitory concentrations of aminoglycoside antibiotics (35). However, our observations indicate that *pilY1* expression is not altered at least under conditions of elevated c-di-GMP in the Δ*bifA* mutant. Furthermore, our data examining the cellular localization of PilY1 suggest that it is not necessarily the total abundance but rather the localization of PilY1 to a particular subcellular

fraction that may be important for its role in swarming repression. More specifically, mutations that decrease the extracellular localization of PilY1 correlate with a decrease in repression of swarming motility. For example, mutation of the *pilA* gene, encoding the structural subunit of the pilus, leads to a reduction in the supernatant levels of PilY1 relative to those in the WT, which correlates with a partial suppression of the Δ*bifA* swarming impairment. However, functional pili are not absolutely required for PilY1-mediated repression of swarming. Furthermore, a stable PilY1 mutant protein containing alanine substitutions of two amino acids in a conserved region of the putative VWA domain (PilY1-MTAA) shows decreased levels in both the cell-associated and supernatant fractions relative to WT PilY1, and this mutant protein is unable to fully complement the Δ*bifA* Δ*pilY1* double mutant for its swarming phenotype, nor is it able to repress swarming as well as WT PilY1 when overexpressed in the WT background. Together, these data suggest that extracellular secretion and proper localization of PilY1 may be important for swarming repression.

Mutation of the *pilY1* gene in the Δ*bifA* background not only suppresses the swarming defect but also eliminates both hyper-biofilm formation and hyper-CR binding, thus affecting all of the c-di-GMP-sensitive phenotypes of the Δ*bifA* mutant. Based on these data, we predicted that cellular c-di-GMP levels in the Δ*bifA* Δ*pilY1* double mutant would be reduced relative to those in the Δ*bifA* single mutant. However, using two different methods to measure *in vivo* c-di-GMP levels, we found no difference in the pools of c-di-GMP between these two strains. It is formally possible that PilY1 exerts its effects via a c-di-GMP-independent mechanism, although the experiments with SadC argue against such a model. An alternative possibility to explain our results is the notion that PilY1 does not affect the overall global levels of c-di-GMP but rather exerts a more localized or specific effect on this signaling molecule and that changes in such a local pool of c-di-GMP would be difficult to detect when measuring global pools of this signal using current methods. Indeed, our data do suggest that PilY1 might affect the activity of SadC but not other DGCs, and as such they are consistent with the possibility of SadC-mediated localized alterations in the c-di-GMP level.

This notion of "local action" has been postulated to explain how c-di-GMP can specifically modulate a particular cellular process, given that the genomes of many bacterial species encode a large number of both DGCs and PDEs (24). That is, rather than a common global pool of signaling molecule regulating cellular behaviors, a local pool would be generated, controlled, and/or responded to by a subset of spatially localized enzymes and effectors to influence a cellular process. This model seems particularly compelling for *Caulobacter crescentus*, for example, where the DGC activity of PleD is spatially restricted to the cell pole where it controls the swarmer-to-stalked cell transition during cell differentiation (2, 44, 60), but it may represent a more general theme of c-di-GMP signaling in many bacterial species (24). In the case of PilY1, it may be that the specificity we observe between PilY1 and the SadC DGC enables local control of c-di-GMP levels to specifically influence swarming motility of *P. aeruginosa*.

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