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Evidence for Cyclic Di-GMP-Mediated Signaling in *Bacillus subtilis*

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Cyclic di-GMP (c-di-GMP) is a second messenger that regulates diverse cellular processes in bacteria, including motility, biofilm formation, cell-cell signaling, and host colonization. Studies of c-di-GMP signaling have chiefly focused on Gram-negative bacteria. Here, we investigated c-di-GMP signaling in the Gram-positive bacterium *Bacillus subtilis* by constructing deletion mutations in genes predicted to be involved in the synthesis, breakdown, or response to the second messenger. We found that a putative c-di-GMP-degrading phosphodiesterase, YuxH, and a putative c-di-GMP receptor, YpfA, had strong influences on motility and that these effects depended on sequences similar to canonical EAL and RxxxR—D/NxSxxG motifs, respectively. Evidence indicates that YpfA inhibits motility by interacting with the flagellar motor protein MotA and that *yuxH* is under the negative control of the master regulator Spo0A∼P. Based on these findings, we propose that YpfA inhibits motility in response to rising levels of c-di-GMP during entry into stationary phase due to the downregulation of *yuxH* by Spo0A∼P. We also present evidence that YpfA has a mild influence on biofilm formation. *In toto*, our results demonstrate the existence of a functional c-di-GMP signaling system in *B. subtilis* that directly inhibits motility and directly or indirectly influences biofilm formation.

The biological activity of cyclic di-GMP (c-di-GMP) was discovered in *Gluconacetobacter xylinus* as a small-molecule cofactor involved in regulation of cellulose biosynthesis (42). More recently, c-di-GMP-mediated regulation has been found in many bacterial species, where it has been shown to be primarily involved in regulating the switch from a free-living, planktonic state to a multicellular (biofilm) state (12, 22, 26, 41). In some bacteria, c-di-GMP signaling also influences the expression of virulence genes, host colonization, and cell-cell communication (16, 44, 53, 54).

c-di-GMP is synthesized by diguanylate cyclases (DGCs) from two molecules of GTP (Fig. 1A) (3, 14, 49). Members of the DGC protein family usually contain a so-called GGDEF domain, which refers to a conserved 5-amino-acid motif (3). Breakdown of c-di-GMP into pGpG is carried out by phosphodiesterases (PDEs) (Fig. 1A). PDE family proteins also contain conserved motifs, either EAL or, less commonly, HD-GYP (15, 43, 47, 49). The number of predicted GGDEF and EAL domain-containing proteins varies dramatically among bacteria, from as few as one to dozens. For example, *Mycobacterium smegmatis* is predicted to have a single bifunctional protein with both GGDEF and EAL domains. This protein was shown to have both DGC and PDE activities *in vitro* (7). In contrast, the genome of *Vibrio cholerae* is predicted to encode more than 60 GGDEF and EAL/HD-GYP domain proteins and other proteins likely involved in c-di-GMP signaling (5).

Many DGCs and PDEs also contain separate domains predicted to be involved in signaling sensing, making these multidomain enzymes part of the multicomponent signal-sensing systems (38, 58). These separate domains also provide a link between environmental signals and c-di-GMP signaling, suggesting a broader role of c-di-GMP in responding to environmental cues. Examples of such multidomain proteins include *Borrelia burgdorferi* protein Rrp1, *V. cholerae* PDE protein VieA, *Pseudomonas aeruginosa* DGC protein WspR and PDE protein SadR, and *Caulobacter crescentus* DGC protein PleD (24, 38, 40, 57). Sensing of environmental signals by these proteins is achieved either by a transmembrane sensor domain that responds to external stimuli or by a separate histidine kinase that phosphorylates a response regulator domain within the multidomain protein, as in the case of PleD of *C. crescentus* (38).

c-di-GMP is believed to bind to cellular effectors in order to exert its regulatory function. Several classes of effectors have been described in various bacteria (1, 32, 52). One of them is the so-called PilZ domain family. Most members of this family have a conserved motif, RxxxR—D/NxSxxG (1). Conserved residues in this motif are proposed to be critical in interacting with the c-di-GMP ligand, according to the recently published X-ray crystal structures of PlzD and PA4608, two PilZ domain proteins from *V. cholerae* and *P. aeruginosa*, respectively (4, 21, 48).

The second major class of c-di-GMP effectors are riboswitches (50, 52), which are domains in mRNAs that control gene expression in response to changing concentrations of their target ligand (11). Previous studies have revealed a conserved RNA motif called GEMM that has been shown to bind c-di-GMP as a ligand (60). These GEMM motifs have been found to reside within the 5′-untranslated region (5′-UTR) of mRNAs for genes encoding DGC and PDE proteins and of mRNAs for other genes shown to be regulated by c-di-GMP (60). The c-di-GMP-sensing riboswitch is predicted to be widely distributed in bacteria, making it a common regulatory mechanism in c-di-GMP signaling (52). In addition to the above two classes of c-di-GMP receptors, degenerate DGCs and PDEs can also function as c-di-GMP effectors. These degenerate proteins bind c-di-GMP, but for regulatory purposes.

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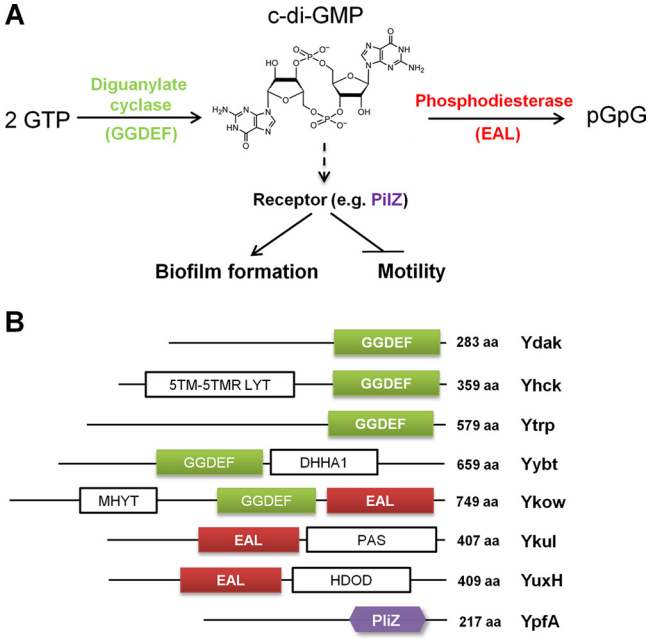

FIG 1 Genes predicted to be involved in c-di-GMP signaling in *B. subtilis*. (A) Schematic representation of c-di-GMP-mediated regulation of motility and biofilm formation in bacteria. (B) Domain composition and organization of eight putative c-di-GMP signaling proteins in *B. subtilis*, including four GGDEF domain proteins, two EAL domain proteins, one protein with both a GGDEF and an EAL domain, and a PilZ domain protein. GGDEF domains are shown in green, and EAL domains are shown in red. The predicted PilZ domain in YpfA is shown in purple. Additional domains predicted by Pfam are shown in white, and their annotations are as follows: 5TM-5TMR LYT, transmembrane region of the 5TM-LYT (5-transmembrane receptors of the LytS-YhcK type); DHHA1, DHH subfamily 1 members; MHYT, N-terminal triplet tandem repeat in bacterial signaling proteins; PAS, functions as a signal sensor; HDOD, HD/PDEase superfamily.

There are also examples of transcription factors containing domains that can bind c-di-GMP and that can function as c-di-GMP effectors, regulating target genes in response to changing levels of c-di-GMP (23, 32).

Much of the past work on c-di-GMP signaling focused on Gram-negative bacteria, such as *Escherichia coli*, *V. cholerae*, *C. crescentus*, and *Pseudomonas* spp. in which the second messenger was shown to be involved in regulating motility, biofilm formation, and control of the cell cycle (8, 17, 32, 36, 37). In Gram-positive bacteria, few studies have investigated the presence and significance of c-di-GMP signaling (9, 39). In one study (9), genes encoding putative DGCs or PDEs from the Gram-positive bacterium *Clostridium difficile* were expressed in *V. cholerae*, where they were shown to affect motility and biofilm formation. Very recently, Purcell et al. (39) measured c-di-GMP levels in *C. difficile* and presented evidence that elevated levels of the second messenger inhibited motility.

In this work, we systematically investigated the function of genes predicted to be involved in c-di-GMP signaling in motility and biofilm formation in the model Gram-positive bacterium *B. subtilis*. Our results indicate that *B. subtilis* possesses a functional c-di-GMP signaling system that contributes to the control of swimming and swarming and that influences biofilm formation.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *B. subtilis* strains were routinely cultured in Luria-Bertani (LB) medium or TY medium (LB medium supplemented with 10 mM $MgSO_4$ and 100 μM $MnSO_4$). For pellicle formation or colony development, the biofilm-inducing medium MSgg (10) or 2× SGG (30) was used, and a previously published protocol was followed (13). Strains used in this study are listed in Table S1 of the supplemental material. Antibiotics were added, when necessary, at the following concentrations: 100 μg/ml spectinomycin, 10 μg/ml tetracycline, 5 μg/ml chloramphenicol, 0.5 μg/ of erythromycin, 2.5 μg/ml lincomycin, and 10 μg/ml kanamycin for *B. subtilis* strains. Chemicals were purchased from Sigma.

Strain constructions. All insertion/deletion mutations were generated by long-flanking PCR mutagenesis (59). Transfer of antibiotic cassette-marked deletion mutations or reporter fusions among different *B. subtilis* strains was conducted by using SPP1 phage-mediated transduction (61). Other routine molecular manipulation techniques followed published protocols (46). All plasmids constructed and used in this study are described in Table S2 of the supplemental material. All primers used in the construction of strains and plasmids are listed in Table S3 of the supplemental material.

To complement the Δ*yuxH* mutation with a wild-type (WT) copy of *yuxH*, the promoter sequence and the open reading frame of *yuxH* were amplified by PCR using chromosomal DNA of the wild-type *B. subtilis* strain NCIB 3610 (abbreviated as 3610 here) (see Table S1 in the supplemental material) as the template and primers P_{yuxH}-F1 and yuxH-R1. The PCR products were cloned into the integration plasmid pDG1662 (20), resulting in the recombinant plasmid pCY60. To complement the Δ*yuxH* mutation with the two putative EAL domain mutants of *yuxH*, overlapping PCR (25) was carried out to introduce codon substitutions in *yuxH*. In brief, two DNA fragments were amplified by PCR, one covering from the 5′ end of the promoter region of *yuxH* to the middle of the *yuxH* coding region and the other covering from the middle to the 3′ end of *yuxH*. The primers used to introduce mutations in the first putative catalytic motif, ^{20}ELL^{22}, were the pairs P_{yuxH}-F1/yuxH-M1-R and yuxH-M1-F/yuxH-R1. The P_{yuxH}-F1/yuxH-M2-R and yuxH-M2-F/yuxH-R1 primer pairs were used to introduce codon substitutions in the second ^{88}EIL^{90} motif. The two PCR products were joined by overlapping PCR, and the PCR products containing the mutant *yuxH* alleles were similarly cloned into the plasmid pDG1662, resulting in the recombinant plasmids pCY58 and pCY59 (see Table S2 in the supplemental material), respectively.

To compare expression of the *yuxH* gene in the wild type and the *spo0A* mutant, the regulatory region of *yuxH* was amplified by PCR using 3610 chromosomal DNA as the template and primers P_{yuxH}-F1 and P_{yuxH}-R1. The PCR products were cloned into the plasmid pDG268 (2) to make a P_{yuxH}-*lacZ* transcriptional fusion, resulting in the recombinant plasmid pCY100.

To create PilZ domain mutants of YpfA, the wild-type *ypfA* gene was amplified by PCR using 3610 chromosomal DNA as the template and primers B2ypfA-F and B2ypfA-R1. The PCR products were cloned into pKNT25 (27), resulting in the recombinant plasmid pCY283. Codon substitutions in *ypfA* were subsequently generated by using a QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) with pCY283 as the plasmid template and corresponding primers (listed in Table S3 of the supplemental material). Codon substitutions in all of the above recombinant plasmids were confirmed by DNA sequencing.

To overproduce wild-type or mutant YpfA proteins in *B. subtilis*, we cloned the wild type or the mutant alleles of *ypfA* into the plasmid pDR111 (28), which contains an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *hyspank* promoter. To do so, genes of *ypfA* (WT and mutants) were first amplified by PCR using pCY283 or derivatives of pCY283 that contained mutant alleles of *ypfA* as the templates and primers ypfA-F and ypfA-R. The PCR products were then cloned into pDR111 (28), generat-

ing a series of recombinant plasmids listed in Table S2 of the supplemental material.

All recombinant plasmids were first introduced into the *B. subtilis* laboratory strain PY79 by genetic transformation (19). A double-crossover recombination of the DNA sequences at the *amyE* locus on the chromosome of PY79 was confirmed. The DNA fragments at *amyE* were then introduced into 3610 or its derivatives by SPP1 phage-mediated transduction.

Assays of swarming motility. To assay swarming motility of *B. subtilis*, 1-ml aliquots of cells that were grown in LB broth to mid-log phase at 37°C were collected, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4), and resuspended in 100 μl PBS. Swarming agar plates (LB solidified with 0.7% agar) were dried for 20 min in a laminar flow hood before use. Ten microliters of the cell suspension was spotted at the center of the plates, and plates were dried for another 10 min and incubated at 37°C for 5 h. The diameter of the swarming zone was later measured.

We also developed a method to better visualize the swarming zones of cells on the plates. In brief, after incubation at 37°C for several hours, the swarming plates (including those inoculated with either wild-type or mutant cells) were removed from incubation at the time when the surface of the plates inoculated with wild-type cells had just been fully covered by the swarming cells (normally 4.5 to 5 h after incubation at 37°C with humidity control). Those plates were immediately dried in a laminar flow hood for 1 h at room temperature to stop swarming (by reducing the humidity). One hour after being dried in the hood, the plates were taken out and incubated on a regular bench for another 12 h at room temperature to allow cell growth (but no further swarming) before imaging. In some assays, as indicated, the swarming plates were incubated for 20 h at 37°C before being treated similarly for imaging. This method allowed us to clearly visualize the swarming zone (due to cell growth) without altering its size.

Bacterial two-hybrid assays. Bacterial two-hybrid assays were performed similarly to what was described previously (27). The coding region of *ypfA* (excluding the stop codon) was amplified by PCR using primers B2ypfA-F and B2ypfA-R1. The PCR products were digested with BamHI and EcoRI and were cloned into the plasmid pKNT25 (27), resulting in pCY283. As a control, the *yabK* gene was amplified by PCR using *B. subtilis* 3610 chromosomal DNA as the template and primers B2yabK-F and B2yabK-R, and it was then cloned into pKNT25, generating the recombinant plasmid pCY301. The coding sequences of *motA* and *fliG* (excluding the stop codon) were similarly amplified by PCR using the primer pairs B2motA-F and B2motA-R for *motA* and B2fliG-F and B2fliG-R for *fliG*. The PCR product of *motA* was digested with BamHI and EcoRI and cloned into plasmid pCH363 (27), resulting in pCY302. Similarly, the PCR product of *fliG* was digested with KpnI and EcoRI and cloned into the same sites of pCH363, generating pCY303.

To introduce recombinant plasmids into the *E. coli* host strain BTH101, 5 μl of each pair of the recombinant plasmids was mixed with 100 μl of chemically competent cells of BTH101. Samples were incubated at 4°C for 30 min and then heat shocked at 42°C for 90 s. An 800-μl volume of LB broth was added to the heat-shocked cells, and cells were incubated with shaking for 1 h at 37°C. Cells were concentrated and spread on LB plates supplemented kanamycin (25 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). Plates were incubated overnight at 37°C. Single colonies were picked and grown at 37°C in LB broth plus kanamycin (25 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) with vigorous shaking. Five-microliter aliquots of cells (optical density at 600 nm [OD_{600}], 1.0) were spotted on LB plates supplemented with 40 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 500 μM IPTG, 100 μg ml^{-1} ampicillin, and 25 μg ml^{-1} kanamycin. Plates were incubated for 48 h at 23°C before imaging.

Protein pulldown and MS analysis. CY158 (Δ*yuxH amyE*::*gst-ypfA*) and CY182 (Δ*yuxH amyE*::*gst*) cells were grown in LB broth at 37°C to an OD_{600} of 0.5. IPTG was then added to the cultures at a final concentration of 500 μM. Cells were incubated for two more hours at 37°C, harvested at 5,000 rpm for 5 min, and washed once with cold PBS buffer. Cell pellets were resuspended in 10 ml sucrose buffer (500 mM sucrose, 20 mM $MgCl_2$, 10 mM KPO_4 [6.2 ml/liter of 1 M K_2HPO_4 and 3.8 ml/liter of 1 M KH_2PO_4 in 100 ml double-distilled H_2O], and 0.1 mg ml^{-1} freshly prepared lysozyme) and incubated at 37°C for 20 min. After incubation, cells were spun down again at 5,000 rpm for 10 min, and cell pellets were resuspended in 10 ml binding buffer (25 mM Tris [pH 8.0], 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM dithiothreitol, and 10% glycerol). Cells were then disrupted by using a French press minicell at 30 KSI (two shots), and supernatants were collected after centrifugation at 15,000 rpm for 5 min. Glutathione-Sepharose beads (GE Healthcare) were washed with PBS buffer at five times the column bed volume before sample loading. Supernatants described above were then loaded into the column filled with glutathione-Sepharose beads, and samples were incubated overnight at 4°C with rotating. The next day, samples were passed through the column, flowthrough was discarded, and the beads were washed with binding buffer five times. Proteins were then eluted with elution buffer (50 mM Tris-HCl, 25 mM glutathione; pH 8.0). The eluted protein samples were size fractionated on a 12% SDS-PAGE gel and subsequently applied for mass spectrometry (MS) analysis. MS analysis was performed at the Harvard FAS Center for Systems Biology.

RESULTS

Genes predicted to be involved in c-di-GMP signaling in *B. subtilis*. According to the Signal Census Database (http://www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html), which predicts signal transduction proteins encoded in the genomes of more than 500 bacterial and archaeal species, the genome of *B. subtilis* is predicted to encode four GGDEF domain proteins (YdaK, YhcK, YtrP, and YybT), two EAL domain proteins (YkuI and YuxH), and one protein containing both a GGDEF and an EAL domain (YkoW) (Fig. 1B). Predicting protein receptors for the c-di-GMP molecule is more difficult, because these receptors are structurally diverse and many of them lack conserved motifs. Several so-called PilZ domain proteins have been demonstrated biochemically and structurally to bind, and function as a protein receptor for, c-di-GMP (4, 21, 45). *B. subtilis* contains one predicted PilZ domain protein, YpfA. The YpfA protein contains a cluster of residues (RxxxR—D/NxSxxG) that are highly conserved among PilZ family members and have been shown to play an important role in recognition of c-di-GMP (45).

To investigate the role of c-di-GMP signaling in *B. subtilis*, we constructed insertion/deletion mutations for each of the genes predicted to be involved in c-di-GMP signaling (see Materials and Methods). We also made double and triple mutants for those genes, since it had been shown for other bacteria that many GGDEF domain or EAL domain proteins are functionally redundant (9, 37). We succeeded in making null mutations for all of the genes except for *ydaK*. *ydaK* is the second gene in a putative five-gene operon (*ydaJKLMN*) and is predicted to encode a GGDEF domain protein. We were also unsuccessful in creating insertion/deletion mutations for other individual members of the same operon and for the entire operon. Therefore, we speculate that the *ydaJKLMN* operon is essential. If so, it is surprising that this was not reported previously.

YuxH and YpfA are involved in regulating motility. Previous work in Gram-negative bacteria has shown that c-di-GMP signaling controls switching between free-living and multicellular states (22, 26, 41). We therefore asked whether mutations in the predicted c-di-GMP signaling genes might influence motility in *B. subtilis*. We tested single and triple mutants for swarming motility

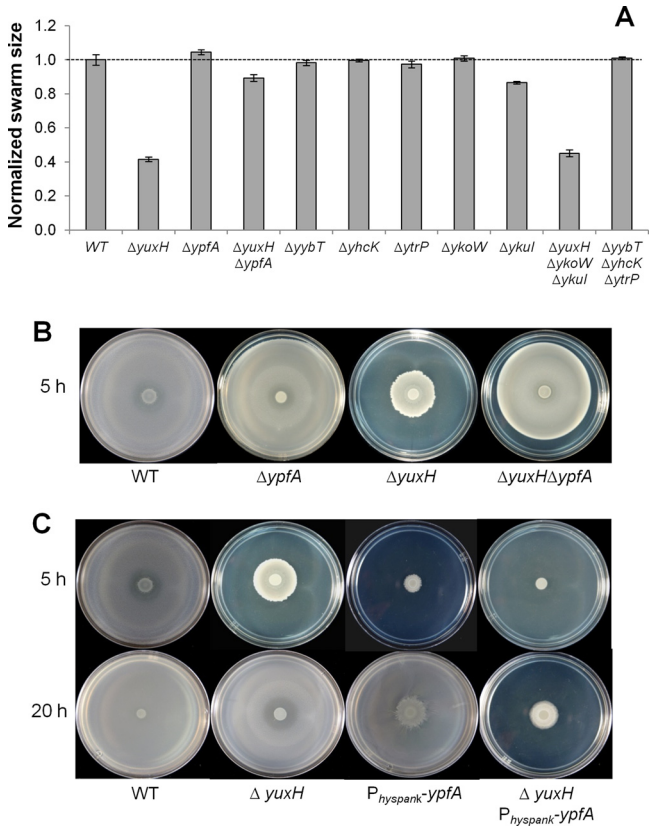

FIG 2 EAL domain protein YuxH and PilZ domain protein YpfA regulate swarming motility. (A) Assays of swarming motility by various single and triple mutants of putative c-di-GMP signaling genes. The sizes of the swarming zones on the plates inoculated with the mutants were normalized against that of the wild type. The results showed that the mutant of YuxH for a putative phosphodiesterase (CY9) and the triple mutant of YuxH YkoW YkuI (CY25) were severely impaired for swarming motility. All values are the averages of three replicates. (B) The severe defect in swarming motility by the *yuxH* mutant was substantially rescued by a second mutation in *ypfA*. Swarming zones of the wild type, Δ*ypfA* (CY3), Δ*yuxH* (CY9), and Δ*yuxH* Δ*ypfA* double mutant (CY30) were visualized after incubation of the swarming plates at 37°C for 5 h and at room temperature for another 12 h (see Materials and Methods). (C) YpfA overexpression had a strong, negative effect on swarming motility. No swarming motility was observed after 5 h of incubation in the *ypfA*-overexpressing strain (CY84). The inhibitory effect was even greater in a *yuxH* mutant strain overexpressing YpfA (CY85), in that the strain did not initiate swarming even at 20 h postinoculation.

on 0.7% LB agar plates. The ability of a tested strain to swarm was measured by the diameter of the swarming zone on the agar plate. *B. subtilis* 3610, the parent strain, was known to be highly proficient in swarming motility (29). None of the mutants lacking the GGDEF domain proteins was impaired for swarming motility, including the Δ*yybT* Δ*yhcK* Δ*ytrP* triple mutant (Fig. 2A). On the other hand, the mutant lacking one of the EAL domain proteins, YuxH, did show a clear defect in swarming motility, with an approximate 60% reduction in the diameter of the swarming zone compared to that of the wild type at 5 h postinoculation (Fig. 2A and B). Since YuxH is an EAL domain protein and a putative phosphodiesterase thought to degrade c-di-GMP molecules, the above result implies that in the *yuxH* mutant, elevated levels of c-di-GMP inhibited swarming motility. This result is also consistent with findings from other bacteria, in which elevated levels of c-di-GMP almost ubiquitously inhibit motility (6, 33, 49). The triple mutant (Δ*yuxH* Δ*ykuI* Δ*ykoW*) that lacked all three EAL domain proteins behaved similarly to the *yuxH* single mutant (Fig. 2A), suggesting that the other two EAL domain proteins (individually or collectively) do not contribute significantly to the regulation of swarming motility under the tested conditions. We do not know why none of the GGDEF domain protein mutants had a clear phenotype on swarming motility. Since we were not successful in making a null mutation in the GGDEF domain-encoding gene *ydaK*, it is possible that YdaK is the most important diguanylate cyclase that synthesizes c-di-GMP in *B. subtilis*. It is also possible that there are enzymes in *B. subtilis* that are capable of making c-di-GMP molecules but that do not resemble a typical GGDEF domain protein.

A null mutation in the gene encoding the putative c-di-GMP receptor YpfA had a very mild but reproducible effect in stimulating swarming motility (Fig. 2A). This was seen in time course experiments in which *ypfA* mutant cells were seen to swarm slightly faster than wild-type cells. (This difference is not apparent in Fig. 2B because the image was taken at a time when the wild type had already fully covered the surface of the plate.) Interestingly, however, Δ*ypfA* was epistatic to Δ*yuxH*, in that the double mutant Δ*yuxH* Δ*ypfA* was clearly less defective in swarming motility than the Δ*yuxH* single mutant (Fig. 2A and B). Our interpretation of this result is that YpfA negatively regulates swarming motility by acting downstream of YuxH in the c-di-GMP signaling pathway. If so, we predict that overproduction of YpfA would block swarming motility. To test that idea, we constructed a $P_{hyspank}$-*ypfA* fusion and introduced it into strain 3610 to allow YpfA to be expressed from an IPTG-inducible promoter (*hyspank*) (see Materials and Methods). Indeed, and as shown in Fig. 2C, overproduction of YpfA upon the addition of IPTG strongly inhibited motility; swarming by the YpfA overproducing strain did not commence until 5 h postinoculation, by which time wild-type cells had covered the entire plate (Fig. 2C). Eventually, the overproducing cells initiated swarming and covered the entire plate (by 20 h postinoculation) (Fig. 2C). Furthermore, in the *yuxH* mutant background, YpfA overproduction had an even greater inhibitory effect, with swarming not initiated even after 20 h postinoculation (Fig. 2C).

We also tested whether YuxH and YpfA play a role in regulating swimming motility. To do so, we applied time-lapse phase-contrast microscopic analysis. The *yuxH* mutant cells that harbored the $P_{hyspank}$-*ypfA* fusion (CY85) were grown in an LB shaking culture to an OD_{600} of about 0.3. IPTG was added to the culture. After 30 min of incubation, cells were analyzed under phase-contrast microscopy (see the description of our methods in the supplemental material). pfA^{OE} with IPTG (see Movie S1 in the supplemental material) demonstrated that overproduction of YpfA in the *yuxH* mutant background rapidly blocked swimming by the cells: 30 min after addition of IPTG, swimming was almost completely blocked. The block was due to overproduction of YpfA, since in the sample without IPTG treatment ($ypfA^{OE}$ without IPTG) (see Movie S2 in the supplemental material), swimming of the same cells was not prevented. *In toto*, our results suggest that YuxH, a putative phosphodiesterase, and YpfA, a putative receptor for c-di-GMP, are involved in regulating *B. subtilis* motility.

YuxH is likely a phosphodiesterase, and its synthesis is regulated by Spo0A~P. YuxH is predicted to be a phosphodiesterase

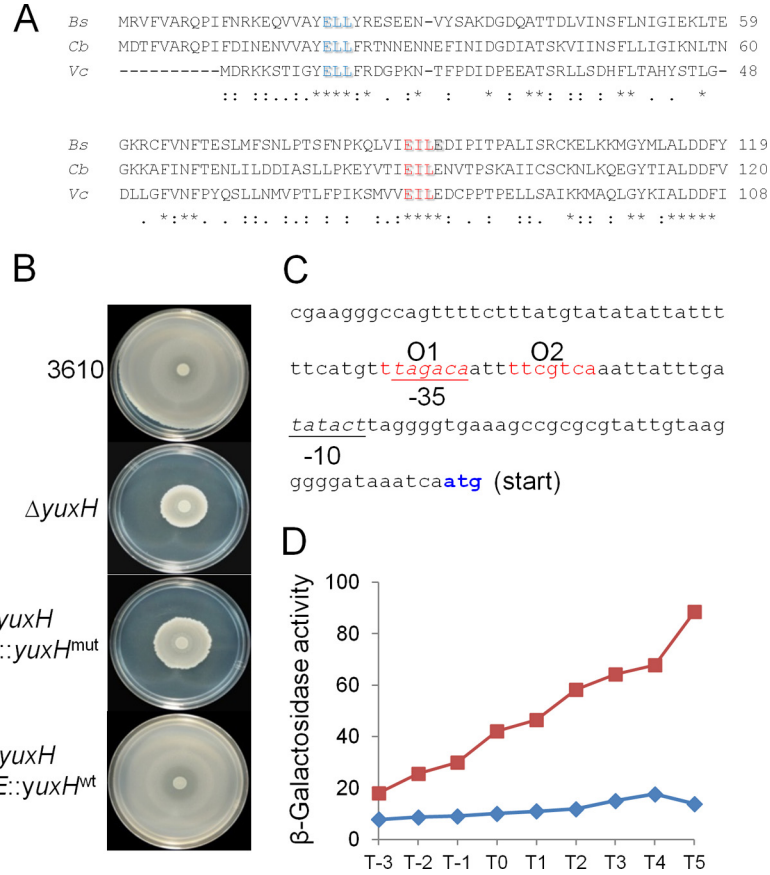

FIG 3 Conserved residues in the EAL domain of YuxH are critical for its activity, and *yuxH* is negatively regulated by Spo0A~P. (A) Partial sequence alignment of YuxH of *B. subtilis* with homologs of YuxH from *C. difficile* and *V. cholerae*. Conserved EAL-like motifs are highlighted in blue and red. The sequence alignment was created using Clustal X 2.0 (56). (B) Swarming plates inoculated with the wild type, the *yuxH* mutant (CY9), or the *yuxH* mutant complemented with wild-type *yuxH* at *amyE* (CY87) or with a mutant allele of *yuxH* (^{88}EIL^{90}>^{88}EDA^{90}) at *amyE* (CY89). (C) The regulatory region of *yuxH* contains two imperfect Spo0A~P binding sites (TTAGACA and TTCGTCA [highlighted in red]), which overlap the putative −35 region of the σ^A-dependent promoter (putative −35 and −10 regions are underlined). (D) Expression levels of the P_{yuxH}-*lacZ* fusion in the wild type (blue diamonds; CY110) and the *spo0A* mutant (red squares; CY121) were determined based on β-galactosidase activities in DS medium. T_0 represents start of stationary growth of cells.

capable of degrading c-di-GMP, since bioinformatic analysis revealed the conserved 3-amino-acid motif EAL in the protein. To investigate the significance of the EAL motif in YuxH, we performed sequence alignment between the YuxH protein from *B. subtilis* and homologous proteins from *Clostridium difficile* and *V. cholerae* (Fig. 3A). The sequence alignment revealed two putative EAL motifs: ^{20}ELL^{22} and ^{88}EIL^{90}. Both motifs were highly conserved in the other homologs (Fig. 3A). Note that the alanine residue in the EAL motif is sometimes degenerate and can be replaced by branched amino acids, such as leucine, valine, and isoleucine. To determine which (or both) of the two motifs is the functional and the putative catalytic site for the phosphodiesterase activity, we replaced the residues in ^{20}ELL^{22} and ^{88}EIL^{90} with either AEL or EDA by site-directed mutagenesis, resulting in four mutant alleles of *yuxH* (see Materials and Methods). We then introduced wild-type or mutant alleles of *yuxH* at an ectopic locus (*amyE*) of the *yuxH* null mutant and tested whether the ectopic copy of *yuxH* (WT or mutant) could complement Δ*yuxH*. A swarming assay for the *yuxH* complementation strains was similarly performed on 0.7% LB agar plates at 37°C. The results showed that the wild-type copy of *yuxH* at the ectopic locus restored swarming motility in the *yuxH* null mutant to a level similar to that of strain 3610 (Fig. 3B). The mutant copies of *yuxH* with amino acid substitutions at the ^{88}EIL^{90} motif failed to complement Δ*yuxH*, since the complementation strains were still severely defective in swarming motility (Fig. 3B shows the ^{88}EIL^{90} → ^{88}EDA^{90} substitution). The mutant copies of *yuxH* with amino acid substitutions at ^{20}ELL^{22} almost fully restored the swarming motility, exhibiting only a slight defect compared to the wild-type complementation (data not shown). This result suggested that ^{88}EIL^{90} is the functional EAL motif in YuxH. The same could be true for the homologous proteins from *C. difficile* and *V. cholerae*, whose functional domains have not been determined by genetic analysis.

Many EAL domain proteins also contain separate domains, such as PAS, which mediate signal sensing (e.g., YkuI in Fig. 1B). This allows regulation of the phosphodiesterase activity at a post-translational level by another cellular signal (55, 58). For YuxH, however, no such signal-sensing domain was identified. We therefore postulated that *yuxH* might be chiefly regulated at the level of gene expression. Analysis of the promoter sequence of the *yuxH* gene (Fig. 3C) revealed a putative σ^A-dependent promoter, with the −35 region (TTGACA) perfectly matching the consensus and the −10 region (TATACT) bearing one mismatch from the con-

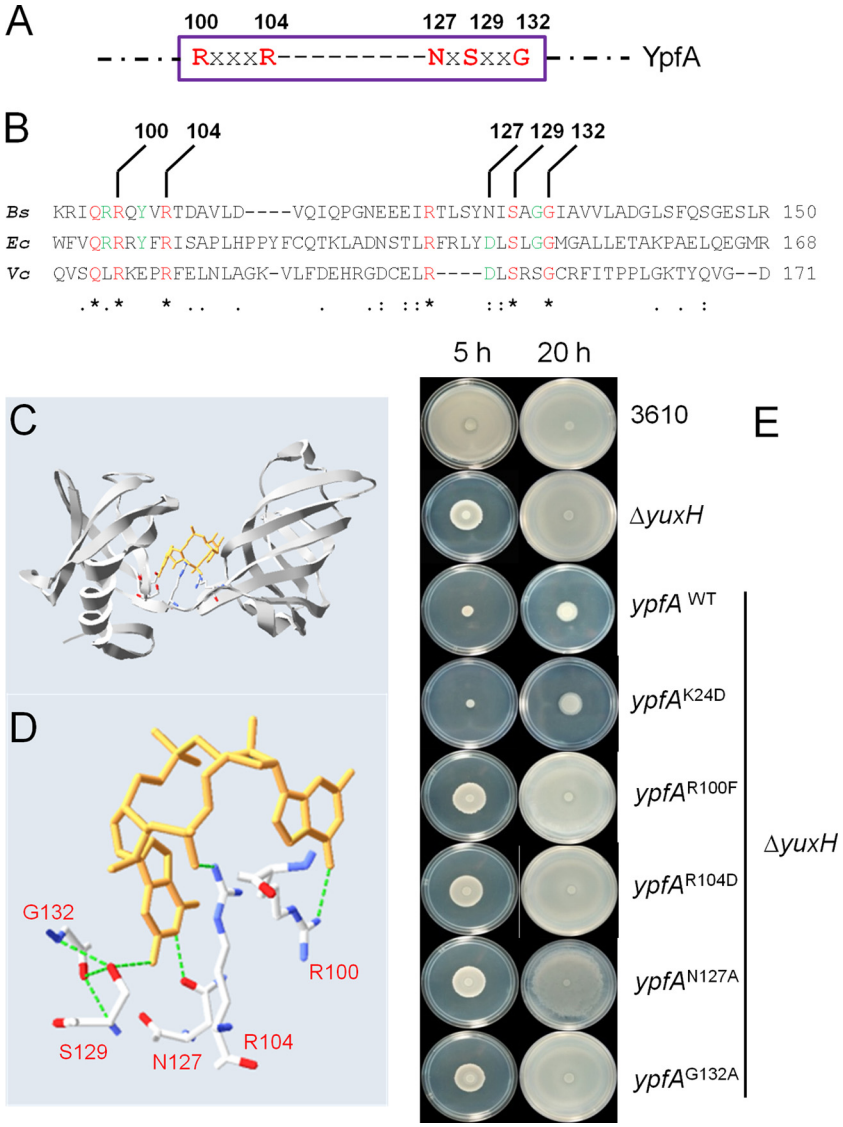

FIG 4 Conserved PilZ domain residues in YpfA are critical for regulating motility. (A) Schematic showing conserved amino acid residues in the putative PilZ domain in YpfA. Conserved residues of the PilZ domain in YpfA, the putative c-di-GMP binding sites, are highlighted in red, and on the top their positions in the protein are marked. (B) Partial sequence alignment among YpfA of *B. subtilis*, PlzD of *V. cholerae*, and YcgR of *E coli*. Conserved residues in all three PilZ domains are highlighted in red. (C) The X-ray crystal structure of PlzD of *V. cholerae* complexed with c-di-GMP (4). The PlzD protein is shown in light gray, and the c-di-GMP molecule is shown in yellow. (D) Predicted interactions between c-di-GMP and conserved residues in PlzD. (E) The swarming phenotype of a *yuxH* mutant that overproduced YpfA, bearing the indicated mutations in the conserved residues in the PilZ domain.

sensus sequence (TATAAT). The sequence analysis also revealed two imperfect Spo0A~P binding sites (TTAGACA and TTCGTCA). Each site contained one mismatch from the consensus sequence, the so-called 0A box (TTCGACA) (35). The two imperfect 0A boxes overlap the −35 region of the putative σ^A-dependent promoter, indicating that transcription of *yuxH* might be negatively regulated by Spo0A~P. To investigate this possibility, we fused the regulatory region of *yuxH* to the *lacZ* gene, creating a P_{yuxH}-*lacZ* reporter fusion, and then introduced the reporter to the wild-type strain and the *spo0A* mutant, resulting in CY110 and CY121, respectively. β-Galactosidase production by CY110 and CY121 was measured in Difco sporulation (DS) medium. The results in Fig. 3D show that expression of the P_{yuxH}-*lacZ* fusion remained at a low level in wild-type cells but markedly increased in the *spo0A* null mutant, confirming that *yuxH* is under the negative control of Spo0A~P. Our results confirmed and reinforced the conclusions of a previous report in which Spo0A~P was shown to bind to the regulatory region of *yuxH* in gel mobility shift assays (35).

Conserved PilZ domain residues in YpfA are critical for regulating motility. We inferred (see above) that YpfA is a c-di-GMP binding protein, because it contains a motif, RxxxR—D/NxSxxG, that is characteristic of so-called PilZ family proteins (Fig. 4A) (1). This motif has been shown to be essential for the function of the *V. cholerae* PliZ domain protein PlzD (4) and the *E. coli* PilZ domain protein YcgR (45). That the putative PilZ domain of YpfA is similar to the corresponding PilZ domains of PlzD and YcgR was reinforced by the sequence alignment of Fig. 4B.

The X-ray crystal structure of PlzD from *V. cholerae* complexed with c-di-GMP was recently solved (4). In the crystal structure, the c-di-GMP molecule is embedded between two subdomains of PlzD, interacting extensively with a region containing most of the highly conserved PilZ domain residues (Fig. 4C). A further examination of the crystal structure revealed that four (R124, R128, D150, and G155) of the five most highly conserved residues in the PilZ domain proteins directly interact with c-di-GMP through hydrogen bonds and other types of interactions. Although the fifth residue, S152, does not directly contact c-di-GMP, it may be important in helping to position G155, which interacts directly with c-di-GMP (Fig. 4D).

To assess the functional significance of these residues in YpfA, we created amino acid substitutions for each of the conserved PilZ domain residues in YpfA. We then overproduced the proteins in the *yuxH* null mutant to see whether the mutant proteins could block motility. The results (Fig. 4E) showed that none of the mutant proteins retained the ability to block swarming motility. As a control, YpfA^{K24D}, which contained a substitution outside the PilZ domain, retained the capacity to inhibit motility. These results show that the conserved residues are critical for activity and reinforce the idea that YpfA is a PliZ domain protein and likely a receptor for c-di-GMP.

YpfA likely targets the flagellar motor protein MotA in regulating motility. Based on our results, we propose that YpfA negatively regulates motility, presumably in response to increasing levels of c-di-GMP during growth transition. But what is the target(s) of YpfA in regulating motility? We took two different approaches to address this question. In the first approach, we picked two candidate proteins, MotA and FliG. The reason why we picked these two proteins was because both MotA and FliG are part of the inner base of the flagellar motor that drives motility, and previous work had shown that the *E. coli* and *Salmonella* PilZ domain proteins YcgR, homologs of YpfA, blocked motility by directly interacting with MotA or FliG (three independent studies identified two different targets of YcgR [8, 18, 37]). To test whether YpfA interacts with MotA or FliG in *B. subtilis*, we first carried out bacterial two-hybrid experiments (27). Full-length YpfA was fused to the N-terminal half of the adenylate cyclase protein, whereas MotA or FliG was fused to the C-terminal half of the adenylate cyclase (and vice versa). The two fusion proteins were expressed in an *E. coli* host strain (see Materials and Methods). If YpfA and MotA (or FliG) interact with each other, they will bring together the two complementary halves of the adenylate cyclase, resulting in cyclic AMP (cAMP) synthesis and expression of β-galactosidase from a cAMP-dependent promoter (27). Thus, protein-protein interactions will give rise to a blue colony from cells expressing the two fusion proteins on a medium that contains the chromogenic substrate X-Gal. As shown in Fig. 5A, an interaction (blue colony) was only seen for the YpfA/MotA pair, indicating that YpfA and MotA interact with each other. In the same assay, we also used both vector only and a nonrelevant protein, YabK, as controls. Neither of them gave a positive result (Fig. 5A).

To further investigate whether the putative c-di-GMP binding motif in YpfA is critical for the YfpA-MotA interaction, we tested five YpfA point mutants that we previously built in bacterial two-hybrid assays similar to that described above. Our results showed that four out of the five YpfA point mutants failed to interact with MotA (Fig. 5B). The fifth one, YpfA^{K24D}, which was previously shown to be fully functional and able to block motility when over-

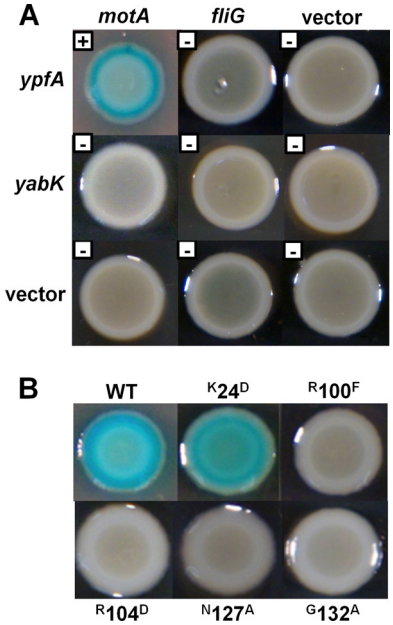

FIG 5 YpfA interacts with MotA for motility inhibition. (A) Bacterial two-hybrid assays were applied to test the interactions between YpfA and the putative targets MotA and FliG. YabK and vector only were used as controls. Five-microliter culture aliquots of *E. coli* BTH101 cells harboring derivatives of pKNT25 and pCH363 with cloned genes were spotted on plates supplemented with X-Gal, and colonies were visualized and imaged after 48 h of incubation at 23°C. A blue colony indicates strong interactions between the two fusion proteins, while a white colony implies no interactions. (B) The bacterial two-hybrid assays showed that mutations in the putative c-di-GMP binding sites in YpfA blocked interactions between YpfA and MotA. YpfA^{K24D} was used as a control and was shown previously to be fully functional.

expressed, was able to interact with MotA, and interactions gave rise to the blue colony (Fig. 5B). These results suggest that the four conserved PilZ domain residues are critical for YpfA-MotA interactions.

In a second approach, we constructed a *B. subtilis* strain that expresses GST-YpfA fusion proteins (YpfA fused to the carboxyl terminus of GST) from an IPTG-inducible promoter in a *yuxH* mutant background (see Materials and Methods). This fusion protein was functional, since overproduction of the fusion protein completely inhibited motility (data not shown). We then prepared total protein lysate from the engineered *B. subtilis* strain grown in LB shaking culture to late exponential phase (OD_{600}, 1.0) and purified GST-YpfA fusion proteins by using affinity resin for GST. Cells expressing GST only were used as a control. The purified protein samples were size fractionated on a 12% SDS-PAGE gel and subsequently analyzed by MS analysis. The MS analysis showed that MotA (and MotB, but not FliG) was among several dozen abundant proteins that copurified with GST-YpfA (data not shown). This was consistent with the results from the bacterial two-hybrid assays. Interestingly, among the several dozen proteins that copurified with GST-YpfA, the most abundant ones were the flagellin subunit (Hag) and the surfactin biosynthesis proteins (SrfAA, SrfAB, and SrfAC) (data not shown). In the future, it will be interesting to test whether c-di-GMP signaling also regulates surfactin production.

Effect of c-di-GMP signaling on biofilm formation. Because c-di-GMP signaling is also known to regulate biofilm formation

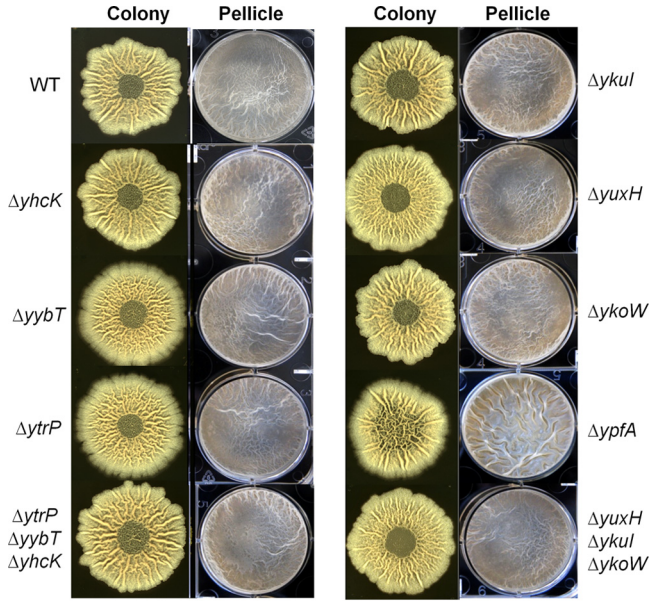

FIG 6 Effects of mutations in c-di-GMP signaling genes on biofilm colony morphology and pellicle formation. For biofilm colony development, individual colonies of the indicated mutants were grown on MSgg agar plates for 3 days at 23°C before imaging. Pellicles were formed in 6-well microtiter plates for 3 days at 23°C before imaging, with each well filled with 9 ml of MSgg liquid medium and inoculated with the indicated mutant cells.

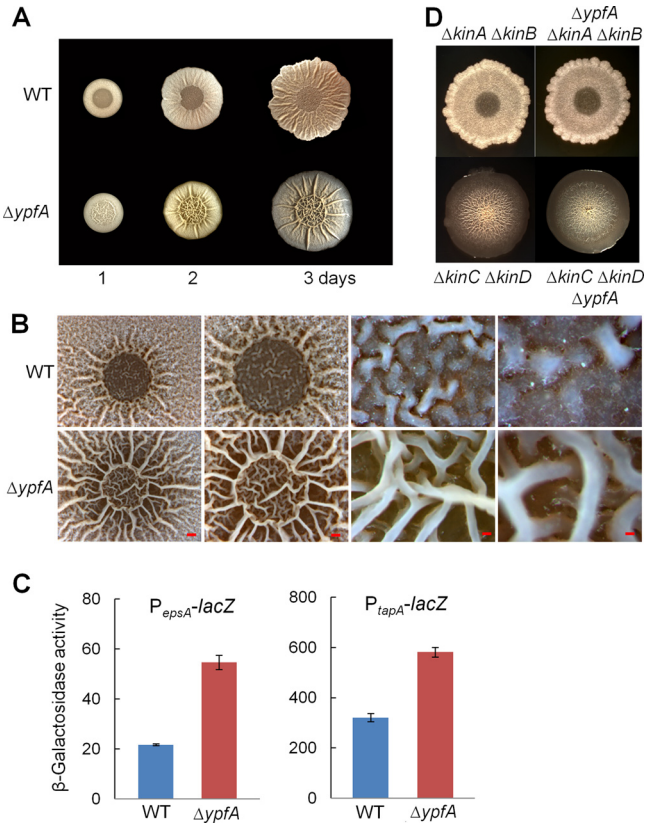

FIG 7 The *ypfA* mutant forms robust central wrinkles in biofilm colonies. (A) Colony morphology of the Δ*ypfA* mutant (CY3) and the wild type (3610) on MSgg agar plates was recorded every 24 h over a period of 72 h. (B) Details of the central parts of the biofilm colonies formed by the wild type and the *ypfA* mutant (CY3) on MSgg agar plates 3 days after inoculation were captured by using Zeiss Lumar stereomicroscope. Bars, in panels from left to right, represent 500, 300, 100, and 50 μm, respectively. (C) Expression levels of the two biofilm matrix operons (*epsA-O* and *tapA*) in the wild type and the *ypfA* mutant as measured by β-galactosidase activities. Reporter strains were grown to an OD_{600} of 1.0 in MSgg broth and assayed for β-galactosidase activities. Strains used in the assays were YC110 and CY437 for the wild type and the *ypfA* mutant bearing P_{epsA}-*lacZ* and strains YC121 and CY438 for the wild type and the mutant bearing P_{tapA}-*lacZ*. (D) YpfA acts upstream of KinA and KinB in regulating central winkle formation of the colony. Shown are colony morphologies of *kinA kinB* (RL4573), *kinC kinD* (RL5273), *kinA kinB ypfA* (CY230), and *kinC kinD ypfA* (CY231) on MSgg agar plates after 3 days of incubation at 23°C.

(16, 32, 33), we next asked whether mutations in the c-di-GMP signaling genes affected biofilm formation by *B. subtilis*. We tested for biofilm formation both as colonies on agar and as pellicles in liquid in biofilm-inducing medium (MSgg). Biofilms were visualized after 3 days of incubation at 23°C (Fig. 6). None of the single or triple mutants lacking the GGDEF or EAL domain proteins showed altered biofilm phenotypes compared to those formed by the wild type (Fig. 6).

On the other hand, the mutant of the *ypfA* gene, which encodes the putative c-di-GMP receptor, demonstrated elevated robustness in biofilm colony morphology, especially in the central area of the colony, and exhibited hyperwrinkles in floating pellicles (Fig. 6). A time course experiment showed that the *ypfA* mutant started to exhibit wrinkles in the central area of the colony more rapidly and more robustly than did the wild type (Fig. 7A and B). We attributed the increased robustness of the mutant to heightened matrix production, because both matrix operons (the *epsA-epsO* operon [herein designated *epsA-O*] and *tapA*) were upregulated compared to the wild type (Fig. 7C).

We previously showed that the histidine kinase pair KinA and KinB controls the inner region of the biofilm colony, whereas the KinC and KinD pair acts on the outer and younger region of the biofilm colony (34). As a result, a *kinA kinB* double mutant exhibits a relatively featureless central area, whereas the *kinC kinD* double mutant behaves in the opposite manner, exhibiting a relatively smooth outer zone while retaining a robust central part (Fig. 7D) (34). Because the *ypfA* mutation affected the central zone, we asked whether YpfA and KinA and KinB are in the same regulatory pathway. To test that, we built the triple mutants *ypfA kinA kinB* and *ypfA kinC kinD*. The *ypfA kinA kinB* triple mutant did not exhibit the robust central wrinkles (Fig. 7D) characteristic of the *ypfA* single mutant (Fig. 7B). This result indicates that YpfA acts upstream of KinA and KinB in regulating central winkle formation of the colony.

The *ypfA* mutant exhibited delayed biofilm disassembly. Pellicles lose their robustness and disassemble over time (31). Peak architectural complexity is seen after about 3 days of incubation at room temperature. Robustness starts to decrease after day 4, and over a period of 1 to 2 weeks the pellicles gradually lose their architectural complexity or even completely disassemble (Fig. 8A). Disassembly of the pellicles was also accompanied by production of a dark brown pigment(s), whose chemical composition was unknown and not determined (Fig. 8A). In contrast, although pellicle formation was comparable to the wild type for the Δ*ypfA* mutant, disassembly of the pellicles was significantly delayed. At 2 weeks postinoculation, pellicles formed by the Δ*ypfA* mutant cells remained fresh and robust, and much less production of the dark

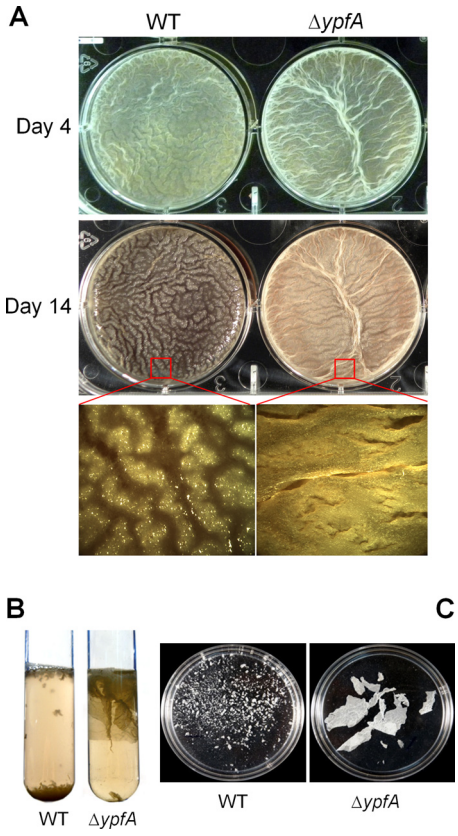

FIG 8 The *ypfA* mutation delays biofilm disassembly. (A) Pellicles formed by the wild type (3610) and the *ypfA* mutant (CY3) in MSgg liquid medium at day 4 (top panels) and day 14 (middle panels) postinoculation at 23°C. Details of the day 14 pellicles (lower panels) were also examined by using a stereomicroscope at ×12 magnification. (B) Day 4 pellicles formed by the wild type or the *ypfA* mutant cells were subjected to vortexing. The *ypfA* mutant pellicles remained as large pieces and floated to the air-liquid interface in the test tube after vortexing, whereas the wild-type pellicles broke into smaller pieces and sunk to the bottom of the test tube after vortex treatment. (C) Similar to the results shown in panel B, pellicles formed by the wild-type and the *ypfA* mutant cells after vortex treatment were compared in petri dishes.

brown pigment was observed (Fig. 8A). As a further test of robustness, we subjected 4-day-old pellicles to vortexing. Pellicles formed by the wild type were readily disrupted into smaller pieces, with the pieces sinking to the bottom of the test tube (Fig. 8B and C). In contrast, the same vortex treatment did not break the pellicles formed by the *ypfA* mutant: these pellicles remained as large pieces and floated to the air-liquid interface (Fig. 8B and C). Two possible interpretations for these results are that enhanced matrix production by the *ypfA* mutant increased the structural integrity of the pellicles, or that the mutation interfered or delayed previously described biofilm disassembly mechanisms.

DISCUSSION

Previous studies of c-di-GMP signaling have mainly focused on Gram-negative bacteria, such as *E. coli*, *P. aeruginosa*, and *V. cholerae*. Here we have provided evidence for a functional c-di-GMP signaling pathway in a Gram-positive bacterium, *B. subtilis*, which is predicted to contain eight c-di-GMP signaling genes. We constructed deletion mutations for all but one (*ydaK*) of the predicted c-di-GMP signaling genes and studied the effects of these mutations on motility and biofilm formation in *B. subtilis*. Our results showed that the EAL domain protein YuxH and the PilZ domain protein YpfA have a pronounced effect on motility. YpfA also seemed to influence biofilm formation and pellicle disassembly. *In toto*, our findings support the idea that *B. subtilis* has a functional c-di-GMP signaling pathway that controls motility and, directly or indirectly, influences biofilm formation. We have not, however, directly demonstrated the presence of c-di-GMP in *B. subtilis*, although we note that very recently, biochemical evidence for the presence of the second messenger was obtained for the Gram-positive bacterium *C. difficile* (39).

Unlike many other EAL domain proteins, which often contain separate domains for signal sensing and for posttranslational regulation, YuxH does not have a separate regulatory domain. Therefore, it is unclear whether YuxH activity is regulated at the posttranslational level. We have, however, found that YuxH is regulated at the level of the expression of its gene, which is under the negative control of the master regulator Spo0A∼P. Because phosphorylation of Spo0A is regulated by a network of histidine kinases and phosphatases in response to a variety of environmental signals (51), our findings provide a potential link between c-di-GMP signaling and environmental signaling. We hypothesize that rising Spo0A∼P levels at the onset of stationary phase repress *yuxH*, causing c-di-GMP levels to rise. We further hypothesize that YpfA, in response to the increasing levels of c-di-GMP, blocks motility by directly interacting with the motor protein MotA. Homologs of the EAL domain protein YuxH are found in both Gram-positive bacteria, such as *Bacillus* and *Clostridium* spp., and in Gram-negative bacteria, such as *V. cholerae* (Fig. 3B; Y. Chen, unpublished observation). It will be interesting to find out how these homologs are controlled in other bacteria.

None of our mutants of the GGDEF domain proteins had a conspicuous effect on motility, including a triple mutant (Δ*ytrP* Δ*yhcK* Δ*yybT*). This observation is not out of keeping with what has been seen in Gram-negative bacteria for which single and multiple mutations of genes encoding GGDEF domain proteins often do not have a strong phenotype, likely due to functional redundancy among the encoded proteins. For example, *E. coli* is predicted to have genes for about 10 GGDEF proteins, none of which when mutated individually or in a quadruple mutant exhibited a strong phenotype (8).

Our findings implicate YpfA as a principal mediator of c-di-GMP signaling in *B. subtilis*. YpfA is predicted to be a PilZ domain protein and a putative c-di-GMP receptor. A Δ*ypfA* mutation by itself did not have a strong effect on motility, but the mutation substantially reversed the motility defect caused by Δ*yuxH*, implying that YpfA could mediate the effect of increasing levels of c-di-GMP. In parallel, overexpression of YpfA from an inducible promoter had a strong, negative effect on motility. We further hypothesize that YpfA inhibits motility by targeting the flagellar motor protein MotA. This is supported by two lines of evidence: first, bacterial two-hybrid assays showed that MotA and YpfA interact strongly, and second, MotA copurified with a GST-YpfA fusion protein. Inhibition of motility by YpfA was dependent on the PilZ domain, as amino acid substitution mutants of the conserved residues eliminated motility inhibition activity. This is also consistent with what was previously reported for the PilZ domain protein YcgR in *E. coli* (45) and with X-crystal structure analysis of the PilZ domain protein PlzD from *V. cholerae* (4). Most of the

conserved PilZ domain residues in PlzD seem to be in contact with the c-di-GMP ligand (4).

The Δ*ypfA* mutation also had effects on biofilm formation, increasing biofilm robustness and delaying pellicle disassembly. The former phenotype is likely due to heightened production of the extracellular matrix through enhanced expression of the matrix operons. Enhanced matrix production might also account for the delay in biofilm disassembly. Alternatively, Δ*ypfA* might interfere with previously described biofilm disassembly mechanisms (31). How the Δ*ypfA* mutation upregulates matrix production is unknown. Our work revealed that key features in the c-di-GMP signaling pathway are conserved between Gram-positive and Gram-negative bacteria. For example, in *E. coli*, deletion of the c-di-GMP phosphodiesterase gene *yhjH* impairs swimming and swarming, and the defect in motility is largely rescued by deletion of the gene encoding the PilZ domain protein YcgR (37). Further, YcgR was shown to directly interact with MotA (although separate studies indicated that YcgR inhibited motility by interacting with another flagellar motor protein FliG [8, 18, 37]). These findings are very similar to what we have observed for *B. subtilis*.

Finally, and unexpectedly, we were unable to create a null mutation in the gene encoding the GGDEF domain protein YdaK. *ydaK* is the second gene in a putative five-gene operon, *ydaJKLMN*. Since we also failed to construct null mutations for other genes in the same operon and for the entire operon, we speculate that this operon is essential for viability in *B. subtilis* (at least under our experimental conditions). Several proteins encoded by genes in the operon are predicted to be membrane associated or membrane spanning, and at least one of them seems to resemble a glycosyl-transferase (Y. Chen et al., unpublished observation). It is worth noting that the GGDEF motif of YdaK has a relatively poor match to the consensus. Therefore, it is possible that YdaK does not actually synthesize c-di-GMP in *B. subtilis*. Rather, and conceivably, YdaK may be a regulatory protein that responds to c-di-GMP by controlling the activities of the products of other genes in the operon.

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