The histidine-containing phosphotransfer protein-B (HptB; PA3345) is an intermediate protein involved in transferring a phosphoryl group from multiple sensor kinases to the response regulator PA3346 in Pseudomonas aeruginosa PA01. The objective of this study was to elucidate the biological significance of the HptB-PA3346 interaction and the regulatory mechanisms thereafter. The transcription profiling analysis of an hptB mutant and its complement strains. Analyzing the swarming phenotype of the P. aeruginosa PAO1 strain complemented with PA3345 indicated that the PA3346 C-terminal region (PA3346C) exhibited with PA3347 individually both in vivo and in vitro. FlgM displaced PA3346C in binding of PA3347 and was then competitively displaced by serine from the PA3347-FlgM complex, forming a phosphorylation-dependent partner-switching system. The phosphorylation of PA3347 in linking the partner-switching system and swarming motility was established by analyzing the swarming phenotype of the PA3347 knock-out mutant and its complement strains.

Two-component signal transduction systems are commonly utilized by bacteria to sense and respond to environmental alterations. A two-component system typically comprises a sensor histidine kinase and a response regulator. After receiving stimuli, the sensors undergo autophosphorylation at a conserved histidine residue before transferring the phosphate group to a response regulator either directly or through an intermediate such as a histidine-containing phosphotransfer (Hpt)2 protein (1, 2). The response regulators are typically multidomain proteins that comprise a conserved receiver and a variable effector domain. The interdomain communication between the regulatory and effector domains of a response regulator shows significant diversity. Removal of the receiver domain may inhibit or constitutively activate the effector domain (3, 4). A more intricate role for response regulators was found in PhyR, which combines a receiver domain with an N-terminal domain that is extremely similar to the $\sigma^{28}$ subunit of RNA polymerase. Unlike other DNA-binding transcriptional regulators, PhyR acts through protein-protein interaction in a partner-switching mechanism where the $\sigma^{28}$ domain of PhyR binds to the anti-$\sigma$ factor NepR. As a result, the original $\sigma$ factor $\sigma^{EcfG}$ is free to transcribe stress-related genes (5).

Partner-switching regulatory systems typically comprise anti-anti-$\sigma$, anti-$\sigma$, and $\sigma$ factors. Dephosphorylation of the anti-anti-$\sigma$ factor by a Ser/Thr protein phosphatase enables its direct interaction with the anti-$\sigma$ factor, rendering it unavailable to bind to the $\sigma$ factor. The $\sigma$ factor is then free to transcribe the downstream genes (6, 7). Once the anti-anti-$\sigma$ is phosphorylated by the Ser/Thr protein kinase activity of the anti-anti-$\sigma$ factor, it dissociates from the anti-$\sigma$ factor, which is then free to bind to the $\sigma$ factor, consequently inhibiting the downstream gene expression. Such a signaling paradigm was first observed in Bacillus subtilis SpoIIE-SpoIIB-SpoIAA, which forms partner switches when regulating sporulation-related $\sigma^{F}$ (8). The other example is Bordetella bronchiseptica BtrU-BtrW-BtrV, which is responsible for regulating a Type III secretion system (9).

Pseudomonas aeruginosa is a Gram-negative, motile bacterium and an opportunistic pathogen known to be the leading cause of numerous acute and chronic nosocomial infections. Our previous studies of P. aeruginosa two-component regulatory systems revealed that following activation by environmental stresses multiple sensor kinases (PA1611, PA1976, PA2824, PA2832, PA2833, and PA2834), phosphate is transferred to the receiver domain of the response regulator. This article contains supplemental Figs. S1–S3, Tables S1–S3, and Videos S1 and S2.)

Background: This study investigates how histidine phosphotransfer protein-B (HptB) regulates Pseudomonas aeruginosa swarming.

Results: HptB regulates the protein phosphatase activity of PA3346, which in turn controls the flagellar gene expression through interaction with PA3347.

Conclusion: Our results reveal a partner-switching mechanism regulating the $\sigma^{28}$-dependent motility genes.

Significance: The interplay between a two-component system and $\sigma^{28}$ has been established.
Partner-switching Mechanism in P. aeruginosa PAO1

TABLE 1
Bacterial strains and plasmids used in this study

| Strains and plasmids | Description | Source/Ref. |
|----------------------|-------------|------------|
| **Bacterial strains** |            |            |
| E. coli              |             |            |
| BL21(DE3)            |             |            |
| XL-1 Blue            |             | Stratagene |
| P. aeruginosa PAO1   |             |            |
| MPA45                | PAO1 ΔhptB  | 11         |
| MJL46                | PAO1 ΔPA3346| 12         |
| MJL47                | PAO1 ΔPA3347| 12         |
| **Plasmids**         |             |            |
| pGEX-5X-1 Ap’        | GST tag protein expression vector | Amersham Biosciences |
| pET30a Km’           | His tag protein expression vector | Novagen |
| pGEX47 Ap’           | a fragment containing entire PA3347 coding region cloned into pGEX-5X-1 | 12 |
| pE3347-SS6A Ap’       | a fragment containing entire PA3347-SS6A coding region cloned into pGEX-5X-1 | 12 |
| pMBH1051 Km’         | a fragment containing entire flgM coding region cloned into pET30a | This study |
| pET11a-link-NFGF Ap’ | plasmid vector designed for fusion of a target protein to the N-terminal fragment of GFP (1–157) | 26, 27 |
| pMRBAD-Z-CGFP Ap’    | plasmid vector that expresses a fusion of an antiparallel leucine zipper peptide to CGFP | 26, 27 |
| pET11a-Z-NFGF Ap’    | plasmid vector that expresses a fusion of an antiparallel leucine zipper peptide to NGFP | 26, 27 |
| pMRBAD-link-CGFP Km’| plasmid vector designed for fusion of a target protein to the C-terminal fragment of GFP (158–238) | 26, 27 |
| pMRBAD-link-NGFP Km’| plasmid vector designed for fusion of a target protein to the N-terminal fragment of GFP | 26, 27 |
| pNBIFC3347 Ap’       | a fragment containing entire PA3347 coding region cloned into pET11a-link-NFGFP with three additional linkers | This study |
| pNBIFC3347M Ap’      | a fragment containing entire PA3347 coding region cloned into pET11a-link-NGFP with three additional linkers | This study |
| pCBIFC3351 Km’       | a fragment containing entire flgM coding region cloned into pMRBAD-link-CGFP | This study |
| pHLBIFC46 Km’        | a fragment containing residues 1214–1716 coding region cloned into pMRBAD-link-CGFP | This study |
| pHL34 Km’            | a fragment containing residues 1212–1713 of PA3347 coding region cloned into pET30a | This study |
| pMBH551 Ap’          | a fragment containing entire flgM coding region cloned into pGEX-5X-1 | This study |
| pMBH3028 Km’         | a fragment containing entire flgA coding region in pET30a | This study |
| pMBH10047 Ap’        | a fragment containing entire PA3347 region in pET100 | This study |
| pHL40 Km’            | a fragment containing entire flgA coding region in pET30a | This study |
| pMMB66EH Ap’         | broad host range expression vector | 52 |
| pMB47 Ap’            | a fragment containing entire PA3347 in pMMB66EH | 12 |
| pMMB47SS6A Ap’       | a fragment containing PA3347-SS6A in pMMB66EH | This study |
| pMMB47SS6D Ap’       | a fragment containing PA3347-SS6D in pMMB66EH | This study |

and RetS) transfer a phosphoryl group to HptB, which then relays the signal to the response regulator PA3346. The phosphorylation on the PA3346 N-terminal receiver domain increases its Ser protein phosphatase activity, leading to dephosphorylation of the potential anti-anti-σ factor PA3347. Although the target phosphorylation site of PA3347 was shown to be Ser-56, the corresponding Ser protein kinase and anti-σ factors remain elusive. The isogenic hptB deletion mutant is defective in swarming, whereas the PA3346 or PA3347 mutant showed increased swarming compared with swarming and other characteristics for the involvement of motility genes in P. aeruginosa PAO1 (10–12).

According to recent information from the Pseudomonas database and the P. aeruginosa Community Annotation Project (PseudoCAP), ~50 flagellum and chemotaxis genes are present in the P. aeruginosa PAO1 genome (13, 14). The checkpoint for flagellum biogenesis is complex, requiring at least two σ factors, RpoN (σ2ν) (15) and FliA (σ2δ) (16). The direct interaction of FliA and the anti-σ factor FlgM in P. aeruginosa was demonstrated using the yeast two-hybrid system, which revealed their role in regulating flagellar biogenesis using a post-translational mechanism (17).

The P. aeruginosa flagellum plays a critical role in virulence as shown by several animal models where the bacteria with flagella were more invasive than flagellum-deficient strains (18). The strain with a deletion at fliC, which codes for the major flagellin in P. aeruginosa, showed a loss of virulence in a pulmonary infection model. These results suggest that the flagellum plays a key role in the P. aeruginosa invasion of epithelial cells (19). Recently, Bordi et al. (20) demonstrated that the HptB signaling pathway is linked to the regulation of the GacA/GacS two-component system by down-regulating the expression of rsyY, a small RNA. Because rsyY is a known regulator of the Type VI secretion system (21), HptB may also negatively regulate protein secretion.

The objective of this study was to explain the regulatory mechanism of HptB-PA3346-PA3347. The microarray analysis of an hptB mutant revealed that a number of the Flia-dependent motility genes were down-regulated, and these results were validated via real time PCR. We also demonstrate that the PA3346 C-terminal region (PA3346C) is a novel Ser/Thr protein kinase that phosphorylates and thereby regulates the activity of PA3347. Our results demonstrate that PA3347 interacts with FlgM and PA3346C in a competitive manner. Overall, these findings indicate that HptB, PA3346, PA3347, FlgM, and FliA are organized into a phosphorylation-dependent partner-switching system that regulates bacterial swarming.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Primers—The bacterial strains and plasmids used in this study are listed in Table 1. The primers used are listed in supplemental Table S1.

Microarray Analysis for Transcriptional Profiling of P. aeruginosa PAO1 and hptB Mutant MPA45—P. aeruginosa PAO1 and hptB mutant strain MPA45 were cultured on swarming plates for 36 h at 30 °C. Bacteria were collected from the edge of swarming bacterial colonies, and the RNA was extracted using a Qiagen RNeasy Midi kit (Qiagen, German-
town, MD). Residual genomic DNA was removed using RQ1 DNAse (Promega, Madison, WI). Approximately 10 μg of the RNA from both *P. aeruginosa* PA01 and the *hpb* knock-out mutant MPA45 was reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen). The resulting cDNA was purified using a Qiagen PCR purification kit and tagged with biotin using the GeneChip® DNA labeling reagent (Affymetrix) according to the manufacturer’s instructions. The labeled cDNA was used to hybridize the Affymetrix GeneChip. Data were normalized by Linear Models for Microarray Data Analysis. Real-time PCR analysis was used to verify whether the sequences were correct and in-frame in these plasmids.

Expression and purification of recombinant proteins—The *Escherichia coli* BL21 (DE3) cells were transformed with the PET-based flgM, *fliA* and *PA3346* clones separately, and gene expression was induced using isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.1 mM with a constant shaking rate of 150 rpm at 20 °C for 16 h. The cells were collected by centrifugation, resuspended in a lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 500 mM NaCl), and disrupted on ice by sonication. After centrifugation at 14,000 rpm at 4 °C for 20 min to remove debris, the clarified supernatant was loaded onto a nickel-charged resin (Ni-Sepharose High Performance, Amersham Biosciences), and the proteins retained in the column were eluted using the elution buffer (50 mM Tris-HCl, pH 8.0, 20 mM glutathione). The purified proteins were dialyzed against PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 50% glycerol, pH 7.4), and the concentration of these proteins was determined via the Bradford method using a Bio-Rad kit.

**In vitro** phosphorylation assay and tandem mass spectrometry analysis—To detect the Ser protein kinase activity, both *PA3347* and *PA3347-S56A*, a non-phosphorylatable amino acid substitution variant of *PA3347*, were incubated individually with *PA3346*C in phosphorylation buffer (50 mM Tris-HCl, 200 mM KCl, 10 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol) and 1 μCi of [γ-32P]ATP at 37 °C for 1 h. The reactions were quenched by adding the same volume of the SDS-PAGE loading dye (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromphenol blue, 10% glycerol, 10% β-mercaptoethanol) and heated at 95 °C for 10 min. The phosphorylation patterns were detected using autoradiography. In **in vitro** phosphorylation for mass spectrometer analysis was performed as described above except that [γ-32P]ATP was replaced with 5 mM unlabeled ATP. The eluted sample was further resolved by SDS-PAGE. In-gel digestion was performed as described previously (25) with the following modifications: destaining was achieved by washing the gel twice with 20 mM...
Partner-switching Mechanism in P. aeruginosa PAO1

\( \text{NH}_3\text{HCO}_3 \), acetonitrile (1:1 mixture) for 15 min at room temperature, and reduction was performed using 10 mm dithiothreitol in 20 mm \( \text{NH}_3\text{HCO}_3 \) for 15 min at 56 °C. Alkylation was performed using 55 mm iodoacetamide in 20 mm \( \text{NH}_3\text{HCO}_3 \) for 20 min in darkness at room temperature. For proteolytic digestion, the gel was treated overnight at 37 °C with 20 μg of sequencing grade trypsin (Promega). The digested sample was sonicated for 10 min in the presence of 1% trifluoroacetic acid, and the supernatant was pooled and subjected to MALDI-TOF mass spectrometer analysis.

**Kinetics of PA3347 Phosphorylation**—Four different concentrations (0.25–2.0 μmol) of PA3347 were incubated with PA3346C (1 μmol) in the presence of 0.1 mm ATP and 2 mm MgSO\(_4\) in a 30-μl volume at 37 °C for 7 min. During this period of time, the PA3347 phosphorylation levels were linear with regard to time. The phosphorylation assay was terminated by adding an equal volume of the SDS loading buffer and boiled for 10 min. The protein was resolved using polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences). The membrane was probed with a phosphospecific mouse monoclonal antibody (Qiagen) followed by a horseradish peroxidase-conjugated secondary antibody. The band was visualized using a NovaREDTM Substrate kit (Vector Laboratories, Burlingame, CA). The phosphorylation intensity was determined by quantifying the immunoblot using ImageJ software, and kinetics analysis was performed using the GraphPad Prism® software.

**GST Pulldown Assay**—The recombinant proteins for testing protein-protein interaction were incubated with 500 μl of a 50% slurry of glutathione-agarose beads in PBS for 2 h at 4 °C either in the absence or presence of 2 mm ADP and 2 mm MgSO\(_4\). The reaction mixture was then centrifuged at 3,000 rpm for 5 min, the precipitated glutathione beads were washed twice with PBS, the protein complex was eluted with the GST elution buffer, and the eluted fraction was concentrated using Amicon Ultra (Millipore, Billerica, MA). The proteins present in the eluted fractions were resolved on a polyacrylamide gel and then transferred onto a PVDF membrane. The membrane was incubated sequentially with a monoclonal antibody against His tag (Merck KGaA) and a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, the target proteins were identified using a NovaRED Substrate kit (Vector Laboratories).

**BiFC Assay**—The BiFC assay was performed as described previously (26, 27) with slight modifications. The positive control plasmid pair was pET11a-Z-NGFP and pMRBAD-Z-CGFP encoding the green fluorescence protein fused with one of the antiparallel leucine zipper tags, respectively. The negative control was the cells harboring pET11a-link-NGFP and pMRBAD-link-CGFP. The interacting partners were introduced into E. coli BL21(DE3), and the transformants were cultured on Luria-Bertani agar containing 50 μg/ml ampicillin, 35 μg/ml kanamycin, 0.1 mm isopropyl \( \beta\)-d-thiogalactopyranoside, and 0.05% arabinose at 20 °C for 72 h. The cells were examined for the presence of green fluorescence by an Olympus BX-51 epifluorescence upright microscope. The images were analyzed using a digital camera and SPOT Advanced Plus Imaging Software (version 4.6).

**Antibody Production and Co-immunoprecipitation**—Recombinant GST–PA3347 protein was used as an immunogen to raise rabbit polyclonal antisera for detecting both PA3347 and PA3347-S56A. The co-immunoprecipitation was performed as described previously (28, 29). Protein A-Sepharose beads (125 μl; Amersham Biosciences) were incubated overnight at 4 °C with 50 μg of rabbit polyclonal PA3347 antibody in 125 μl of Pierce Immunopure Ag/Ab binding buffer (Thermo-Fisher Scientific Inc., Rockford, IL). For the co-immunoprecipitation of PA3347 with His\(_{6}\)-FlgM, beads were incubated with 500 μl of the whole cell lysates from ~10^9 cfu of PAO1 and 200 μg of His\(_{6}\)-FlgM at 4 °C overnight under constant agitation. For the co-immunoprecipitation of GST-PA3347-S56A with His\(_{6}\)-FlgM, beads were incubated with 500 μl of the whole cell lysates of E. coli expressing GST–PA3347-S56A from ~10^9 cfu and 200 μg of His\(_{6}\)-FlgM. In both cases, whole cell lysates alone and His\(_{6}\)-FlgM alone were used as negative controls. The co-immunoprecipitation of PA3347 with His\(_{6}\)-PA3346C was similarly performed. The beads were washed with PBS and boiled in SDS loading dye for 10 min. Western blotting was conducted using the mouse monoclonal anti-His\(_{6}\) antibody (1:2,500) as the primary antibody and the goat anti-mouse IgG antibody conjugated to HRP (1:10,000) as the secondary antibody.

**Competitive Interaction Using GST Pulldown Assay**—To investigate the binding activity of PA3346C versus FlgM on PA3347 in the presence of ATP or ADP, a competition assay was performed as described previously (30) with slight modifications. A constant concentration of PA3347 (25 μg/ml) bound to FlgM (30 μg/ml) was incubated on 100 μl of a 50% GST bead slurry in six separate tubes. Following 2 h of mixing, 2 mM ADP and 2 mM MgSO\(_4\) were added to three microcentrifuge tubes with increasing concentrations of PA3346C (0, 10, and 20 μg/ml). Meanwhile, in three other tubes, 2 mM ATP was added instead of ADP and then incubated for 2 h. In a reciprocal experiment, a constant concentration of PA3347 was bound to PA3346C and incubated with increasing concentrations of FlgM (0, 15, and 30 μg/ml) in the presence of either ATP or ADP. In this study, GST was used as a negative control.

Similarly, to demonstrate that increasing concentrations of FliA (0, 20, and 100 μg/ml) compete to bind with FlgM (30 μg/ml) in a complex comprising PA3347 (30 μg/ml), a competition assay was performed. The GST beads carrying the interacting proteins were washed with PBS extensively and then eluted with the GST elution buffer. The proteins present in the eluents were resolved using SDS-polyacrylamide gel electrophoresis.

**Analysis of Bacterial Swarming**—A swarming assay for P. aeruginosa PAO1 and P. aeruginosa PA3347 mutant MJL47 was performed as reported previously (12). Briefly, the swarming assay medium comprises an M8 salt base (50 mm Na\(_2\)HPO\(_4\), 25 mm KH\(_2\)PO\(_4\), 4 mm NaCl) supplemented with 0.5% Bacto agar, 0.02% glucose, and 10 mM glutamic acid. The bacterial strains that required testing were cultured overnight, transferred to the swarming plate using sterile toothpicks, and then incubated at 37 °C for 36 h. The experiment was performed three independent times.
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Comparison of expression levels of selected motility-related genes in hptB mutant determined by real time quantitative PCR (Q-PCR) and microarray

| Locus index | Gene name | -Fold change for microarray (p value) | -Fold change for Q-PCR (±S.D.) |
|-------------|-----------|--------------------------------------|---------------------------------|
| PA3351      | fliM      | -5.7 (0.070)                         | -10.2 ± 2.6                     |
| PA1092      | fliC      | -3.6 (0.004)                         | -7.7 ± 0.1                      |
| PA1094      | fliD      | -4.5 (0.090)                         | -28.6 ± 1.0                     |
| PA1443      | cheY      | -3.9 (0.020)                         | -5.5 ± 0.2                      |
| PA4915      | NA*       | -8.7 (0.020)                         | -6.3 ± 0.5                      |

* Not available.

RESULTS

Transcriptional Profiling of P. aeruginosa PAO1 and hptB Mutant—To obtain a more comprehensive picture of the physiological functions of the HptB-PA3346-PA3347 signaling system, a Pseudomonas whole genome DNA microarray was used to determine the gene expression profiles of the hptB mutant. The results were compared with those of the wild-type PAO1 strain. This study identified 142 genes with a more than 2-fold increase in expression and 235 genes showing at least a 2-fold decrease in expression. The top 20 up- and down-regulated genes are listed in supplemental Tables S2 and S3, respectively. Approximately half of the genes on the list encode a hypothetical protein. Several genes, including adhA, pldA, stp1, and pilQ that may be associated with bacterial virulence, were also noted. Among the genes up-regulated in the hptB mutant, adhA is known to be important in biofilm formation in P. aeruginosa (31). This notion is consistent with higher biofilm formation in P. aeruginosa known to be important in biofilm formation in B. stearothermophilus and B. bronchiseptica (32). The C-terminal region of PA3346 has a signal receiver domain containing a conserved Asp residue. The middle portion of the protein comprises a protein phosphatase 2C domain, which can dephosphorylate PA3347 (12). Further analysis of the C-terminal region of PA3346 using the BLASTp program revealed significant similarity to several confirmed Ser/Thr protein kinases, including Bacillus stearothermophilus SpoIIAB (39), B. subtilis SpoIIAB (40), B. subtilis RsbW (7), and B. bronchiseptica BtrW (9) (Fig. 1). The amino acid residues and molecular structure of B. stearothermophilus SpoIIAB have been determined and found to be distinct from Hank’s type Ser protein kinases (41) while also exhibiting significant similarities to the ATPases of the Gyrase, Hsp90, Histidine kinase, and MutL superfamily (42). Similar to SpoIIAB and RsbW, the C-terminal region of PA3346 also has N, G1, and G2 boxes responsible for θ/anti-θ antigen binding, Mg²⁺ ion binding, and ADP/ATP binding, respectively (43, 44) (Fig. 1).

PA3346C Is Divalent Cation-dependent Ser/Thr Protein Kinase—If PA3347 is an anti-θ factor antagonist, it is likely to be phosphorylated, and the phosphorylation should influence its interaction with the anti-θ factor. Phosphorylation of PA3347 at Ser-56 using the whole cell extract of P. aeruginosa PAO1 was demonstrated previously through an in vitro phosphorylation assay (12). The presence of a kinase domain at the C-terminal region of PA3346 implies that the protein is probably the kinase in PA3347 phosphorylation. To verify this possibility, the PA3346 C-terminal region ranging from nucleotide position 1222 to 1713 was cloned and overexpressed, and the recombinant protein, designated PA3346C, was used in the in vitro phosphorylation assay with [γ-³²P]ATP as a tracer. As shown in Fig. 2, PA3346C is capable of phosphorylating PA3347 but not PA3347-S56A, the PA3347 variant with an Ala site-directed mutant (supplemental Fig. S2B). Together, the phosphorylation site on PA3347, PA3347 with or without PA3346C treatment was subjected to trypsin digestion followed by MALDI-TOF mass spectrometry. The PA3347 sample treated with PA3346C and ATP yielded two peaks of 1849.997 and 1929.963 Da, respectively (supplemental Fig. S2A), representing unphosphorylated and phosphorylated Ser-56-containing peptides (NATYLDSSALGMLLLLR) of PA3347. However, the phosphorylated Ser-56-containing peptide peak of 1929.963 Da was not observed in the PA3347-S56A site-directed mutant (supplemental Fig. S2B). Together, the results indicate that PA3347 is phosphorylated at Ser-56 by PA3346C. The result is consistent with our previous finding using the whole cell extract to phosphorylate PA3347 (12).
PA3347 Interacts with PA3346C in Vitro—To function as an anti-/H9268 antagonist, PA3347 must interact with PA3346C to perform regulatory activity. Using the GST pulldown assay, both PA3347 and PA3347-S56A were found to form a stable complex with PA3346C but only in the presence of ADP (Fig. 3A), suggesting that ADP stabilizes PA3346C in a conformation favorable for binding with PA3347 (Fig. 3A).

PA3347 Interacts with FlgM in Vitro—The PA3347 deletion mutant MJL47 displayed a hyperswarming phenotype, suggesting an up-regulation of motility-related gene expression (12). In P. aeruginosa PAO1, the anti-/H9268 factor FlgM-encoding gene (PA3351) is located immediately downstream of the hptB-PA3346-PA3347 operon (12). Because FlgM has been shown to interact with the /H9268 factor FliA (α28) to regulate the synthesis of flagella (17), this study examined whether PA3347 regulates the FliA-dependent flagellum activity by interacting with FlgM. As shown in Fig. 3B, the Hisα28-tagged FlgM could be co-eluted with either the GST-tagged PA3347 or the PA3347-S56A proteins, indicating an interaction between the FlgM protein and PA3347 or PA3347-S56A. Binding in the absence of ATP suggests that phosphorylation of PA3347 is not required for the interaction.

PA3347 Interacts with FlgM as Well as with PA3346C in Bimolecular Fluorescence Complementation Assay—To demonstrate that interaction of PA3347 with its binding partners FlgM and PA3346C is also taking place in vivo, a set of plasmids that are stable and express the proteins upon induction in E. coli BL21(DE3) was constructed for the BiFC assay. The cells harboring positive control plasmids gave a strong green fluorescence due to GFP reassembly, whereas negative control cells showed no green fluorescence. As shown in Fig. 4, cells harboring pNBIFC3347 and pCBIFC3351, expressing the NGFP-
PA3347 and CGFP-FlgM, respectively, exhibit green fluorescence, indicating an interaction of PA3347 and FlgM in vivo. A similar study also showed that PA3347-S56A can interact with FlgM. The result further indicates that phosphorylation is not required for PA3347 to interact with FlgM. Consistent with the in vitro protein-protein interaction results, E. coli BL21(DE3)
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Cells harboring pNBIFC3347 and pHLBIFC46, which encodes PA3346C, also showed green fluorescence (Fig. 4).

Co-immunoprecipitation of PA3347 with FlgM and PA3346C Proteins in Vivo—Co-immunoprecipitation analysis showed that recombinant His6-FlgM and His6-PA3346C proteins bound to both the PA3347 expressed in PAO1 and the recombinant GST-PA3347-S56A expressed in E. coli. A control experiment demonstrated that PA3347, PA3347-S56A, the antibody against PA3347, the recombinant His6-FlgM, and His6-PA3346C proteins alone do not bind to Protein A-Sepharose beads (Fig. 5, A and B).

Expression of Both fliA and flgM Is Regulated by PA3347—To determine whether PA3347 is involved in regulating the expression of fliA and flgM, we analyzed the expression of flgM and fliA in the presence and absence of PA3347. Compared with that of the wild type, the expression of flgM and fliA was significantly increased in PA3347 mutant MJLA7 by ~5- and 13-fold, respectively, as determined by quantitative real time PCR. This is possibly because PA3347 deletion results in the partial loss of FlgM function as an anti-σ factor, which results in an increase of FlgM activity to induce fliA and flgM transcription. It is necessary to mention only a partial loss because full loss of FlgM function would bring about uncontrolled filament assembly and loss of motility.

FlgM Competes for Binding to FlgM-PA3347 Complex, and FlgM Competes for Binding to PA3347-PA3346C Complex—To verify that the HptB-PA3346-PA3347 signaling system can modulate flagellum activity through the interaction of FlgM and FliA, a GST pulldown competitive assay was performed. As shown in Fig. 6, increasing concentrations of FliA competitively displaced the binding of PA3347 to FlgM, demonstrating the well known finding that FliA interacts directly with the anti-σ factor FlgM (17).

Elucidation of whether PA3346C, FlgM, and PA3347 can form a stable complex or whether PA3346C and FlgM bind to PA3347 in a competitive manner is critical for understanding the regulatory mechanism exerted by the HptB-mediated signaling pathway. As shown in Fig. 7A, increasing concentrations of FlgM displaced the binding of PA3346C to PA3347 in the presence of ATP. However, displacement was not observed when ADP was used to replace ATP. This finding agrees with the expected partner-switching mechanism that the phosphorylation of the anti-σ antagonist by the Ser protein kinase in the presence of ATP could lead to the dissociation of the complexes, whereas ADP can stabilize the complexes (8, 9, 45). By contrast, the binding of FlgM to PA3347 was not affected by increasing concentrations of PA3346C in the presence of ATP or ADP. This suggests that the binding of FlgM and PA3347 is independent of the phosphorylation state (Fig. 7B).

PA3347 Phosphorylation Is Crucial for P. aeruginosa Swarming—Consistent with a previous finding (12), deleting PA3347 increased the swarming motility, and the introduction of a plasmid that harbored a full-length PA3347 (pM4B47) into MJLA7 could reverse the hyperswarming motility to the parental phenotype (Fig. 8A). More interestingly, a plasmid...
expressing a *PA3347* with an Asp substitution at Ser-56 (*pMMB47S56D*) to mimic a constitutively phosphorylated Ser residue can restore the hyperswarming activity of MJL47 to that of wild-type PAO1. By contrast, the plasmid *pMMB47S56A* producing a non-phosphorylated variant of *PA3347* cannot complement the hyperswarming phenotype of MJL47. Additionally, *pMMB47* can restore the defect in the swarming phenotype of MPA45, whereas *pMMB47S56A* cannot (Fig. 8C). This suggests that the absence of the PA3346 as a kinase in MJL46 prevents the phosphorylation of *PA3347* produced from *pMMB47* and hence formation of a stable complex with FlgM. These findings indicate that phosphorylation at Ser-56 of *PA3347* plays a critical role in regulating flagellum activity.

**DISCUSSION**

The objective of this study was to elucidate the molecular mechanism that leads to flagellum gene regulation by the HptB-PA3346-PA3347 signaling system in *P. aeruginosa* PAO1. The response regulator PA3346 differs from other transcriptional regulators because it is bifunctional, possessing both phosphatase and kinase activities. PA3346C was found to be a homolog of SpoIIAB. SpoIIAB performs the dual function of a Ser protein kinase and an anti-/H9268 factor (46). This study has shown that PA3346C behaves like SpoIIAB, exhibiting a Ser protein kinase activity capable of phosphorylating PA3347 and an anti-/H9268 antagonist binding activity.

This study also demonstrated a direct interaction between the anti-/H9268 factor antagonist PA3347 and the anti-/H9268 factor FlgM both *in vitro* and *in vivo*. The competition assay showed
that the binding of FlgM to PA3347 was not affected by excess PA3346C, indicating that FlgM and PA3346C possibly use a different structural motif to interact with PA3347. However, the FlgM-PA3347 complex can be displaced by excess FliA, suggesting that both PA3347 and FliA may interact with FlgM possibly through the same binding site. Furthermore, the role of PA3347 phosphorylation in regulating swarming motility indicates that the regulation essentially occurs through a partner-switching mechanism. Partner-switching mechanisms for various Gram-positive bacteria have been documented. This type of regulation has been reported more recently for Gram-negative bacteria such as the Type III secretion system regulator BtrU-BtrW-BtrV in *B. bronchiseptica* (43). A conserved partner-switching regulatory system was also found in *Chlamydia trachomatis* (47). The partner-switching mechanism of *P. aeruginosa* may also have a widespread presence in related Gram-negative bacteria, controlling important aspects of bacterial physiology such as swarming.

According to the model established in this study (Fig. 9), HptB modifies the receiver domain of PA3346, altering the balance of phosphatase and kinase activity. In certain growth conditions, this alteration would affect flagellar gene expression through the partner-switching mechanism involving PA3347, FlgM, and FliA as demonstrated in this study. However, because swarming is a complex bacterial behavior, HptB may also affect swarming by other mechanisms. In analogy of SpoI-IAB, the kinase domain of PA3346 (PA3346C) may act as an anti-σ factor to regulate a yet to be identified σ factor. This pathway may participate in cyclic di-GMP level modulation as suggested in a previous study (20, 48) and therefore may explain why the PA3347 deletion mutant shows a hyperswarming phenotype. In addition, this PA3346C-mediated pathway may affect the expression of genes unrelated to flagellum biogenesis that were found in our gene profiling analysis (supplemental Tables S2 and S3). Experiments are being carried out to verify these hypotheses.

Direct observation of the leading edge of the swarming colonies using optical microscopy showed that the wild-type colony edge exhibited vigorous and rapid collective cell movements. By contrast, cells at the leading edge of the *hptB* deletion MPA45 colony were resting (supplemental Videos S1 and S2). The absence of collective movement in the MPA45 strain, causing a defective swarming phenotype, is probably because the flagella were absent or incompletely assembled. To test this hypothesis, flagella were stained in both the wild-type and MPA45 swarming cells and examined under a microscope. Intriguingly, although both the microarray and real time PCR analyses showed that numerous flagellum regulatory and biosynthesis genes were significantly down-regulated in the *hptB* mutant, no apparent difference in the flagellum morphology was observed (supplemental Fig. S3, A and B). The *hptB* mutant cells even swarmed normally in broth with a chemical composition identical to the swarming agar. The twitching motility of the *hptB* mutant was also comparable with the wild-type cells, indicating normal type IV pili. Thus, the precise defects leading to the deficient swarming activity of the *hptB* mutant still require investigation. A component responsible for regulating rotation or supplying energy for flagellum movement is likely defective in the *hptB* mutant cells as also revealed by our gene expression profiling analysis. Such gene types have been
reported to affect the direction of flagellum motor rotation (49, 50) and the swarming motility in Salmonella enterica serovar Typhimurium (51).

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