

A Cyclic di-GMP-binding Adaptor Protein Interacts with Histidine Kinase to Regulate Two-component Signaling^{*S}

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The bacterial messenger cyclic di-GMP (c-di-GMP) binds to a diverse range of effectors to exert its biological effect. Despite the fact that free-standing PilZ proteins are by far the most prevalent c-di-GMP effectors known to date, their physiological function and mechanism of action remain largely unknown. Here we report that the free-standing PilZ protein PA2799 from the opportunistic pathogen *Pseudomonas aeruginosa* interacts directly with the hybrid histidine kinase SagS. We show that PA2799 (named as HapZ: histidine kinase associated PilZ) binds directly to the phosphoreceiver (REC) domain of SagS, and that the SagS-HapZ interaction is further enhanced at elevated c-di-GMP concentration. We demonstrate that binding of HapZ to SagS inhibits the phosphotransfer between SagS and the downstream protein HptB in a c-di-GMP-dependent manner. In accordance with the role of SagS as a motile-sessile switch and biofilm growth factor, we show that HapZ impacts surface attachment and biofilm formation most likely by regulating the expression of a large number of genes. The observations suggest a previously unknown mechanism whereby c-di-GMP mediates two-component signaling through a PilZ adaptor protein.

Cyclic di-GMP (c-di-GMP)⁴ has emerged as a central regulator in many environmental and pathogenic bacteria. High c-di-GMP concentration generally correlates with the sessile lifestyle and formation of surface-associated biofilm. The cellular concentration of c-di-GMP oscillates with environmental changes through the opposing activities of diguanylate cyclase (DGC) and c-di-GMP phosphodiesterase (PDE) proteins. C-di-

GMP exerts its biological effect by binding to riboswitches and a wide variety of protein effectors. PilZ domain proteins are the first discovered c-di-GMP-binding proteins and arguably the most prevalent ones (1). Apart from a small number of atypical PilZ proteins that lack c-di-GMP binding capability, PilZ proteins bind c-di-GMP with a remarkably wide range of binding affinities to regulate different pathways over a wide c-di-GMP concentration range (2, 3). More than half of the PilZ proteins encoded by bacterial genomes are free-standing PilZ proteins that consist of a single PilZ domain of 90–130 residues. The remaining PilZ proteins are didomain or multidomain proteins that contain a PilZ domain and other functional domains. Recent studies have yielded insight into the function and mechanism of several didomain or multidomain PilZ proteins, including the “backstop brake” YcgR protein from *E. coli*, the cellulose synthase subunit BcsA from *Rhodobacter sphaeroides*, the transcriptional regulator MrkH from *Klebsiella pneumoniae*, the alginate-biosynthesizing protein Alg44 from *P. aeruginosa* and the chemotaxis receptor Tlp1 from *Azospirillum brasiliense* (4–12). A common theme emerged from these studies is that the binding of c-di-GMP to the PilZ domain triggers an intra-protein conformational change to modulate the enzymatic activity or binding property of output domains. In contrast, the molecular mechanisms for the free-standing PilZ proteins remain less known despite that several of them have been implicated in bacterial pathogenesis. It was found that the free-standing PilZ proteins from *Xanthomonas campestris* pv. *campestris* and *Xanthomonas oryzae* pv. *Oryzae* mediate motility, virulence, and extracellular enzyme production (13, 14). It was also found that the sole PilZ protein PlzA in *Borrelia burgdorferi* regulates motility and virulence by controlling gene expression (15, 16). Although it is widely hypothesized that free-standing PilZ proteins function as c-di-GMP adaptors by binding to their protein partners in a c-di-GMP-dependent fashion, experimental evidence supporting this hypothesis is still lacking. This hypothesis was even challenged when a PilZ protein from *X. campestris* pv. *Campestris* was found to interact with its protein partners in a c-di-GMP-independent manner (17).

The opportunistic pathogen *P. aeruginosa* is notorious for its ability to form multidrug-resistant biofilm on abiotic and biotic surfaces. *P. aeruginosa* harbors an extensive c-di-GMP signaling network that controls the switch between the motile and

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⁴The abbreviations used are: c-di-GMP, cyclic di-GMP; DGC, diguanylate cyclase; PDE, c-di-GMP phosphodiesterase; HapZ, histidine kinase-associated PilZ; REC, phosphoreceiver.

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sessile life styles and regulates the formation and dispersal of biofilm. The *c*-di-GMP network of *P. aeruginosa* PAO1 consists of a large number of DGC and PDE proteins and at least 12 *c*-di-GMP binding effector proteins. The 12 effectors include FleQ, PelD, FimX, BrlR, PA4395, and seven PilZ-domain-containing proteins. The transcriptional regulator FleQ controls flagellar biosynthesis and the production of extracellular polysaccharides Pel and Psl; whereas the second transcription regulator BrlR is a multidrug transport activator associated with high-level antibiotic resistance (18–22). PelD and FimX control the production of the Pel exopolysaccharide (EPS) and type IV pili biosynthesis, respectively (23–27). The *c*-di-GMP-binding PA4395, a member of the YajQ family proteins, interacts with a transcriptional regulator for controlling gene transcription and virulence (28). The multi-domain protein Alg44 contains a *c*-di-GMP-binding PilZ domain to regulate the biosynthesis of the EPS alginate (10). Besides the six proteins with known function, there are six *c*-di-GMP-binding PilZ proteins whose functions still remain unknown. Among the six PilZ proteins, PA0012, PA2799, PA4324, and PA4608 are free-standing PilZ proteins and PA3353 and PA2989 are didomain proteins, with PA3353 as the homologue of YcgR from *Escherichia coli* (5). Elucidating the physiological role of the six PilZ proteins will substantially advance our understanding of *c*-di-GMP signaling in *P. aeruginosa*. As a step toward this goal, here we report the finding that PA2799 interacts with a sensor histidine kinase in a *c*-di-GMP-dependent manner. We show that PA2799 plays a role in early and late stages of biofilm formation by regulating the expression of a large number of genes. The results suggest a novel mechanism used by *c*-di-GMP to regulate two-component signaling through a PilZ adaptor and establish PA2799 as a new regulatory factor in biofilm formation.

Results

PA2799 Interacts with the Phosphoreceiver (REC) Domain of the Hybrid Histidine Kinase SagS—We postulated that free-standing PilZ proteins function as *c*-di-GMP adaptor proteins by binding to their protein targets. To identify the protein partner of PA2799, a customized bacterial two-hybrid screening system was created with a high-coverage *P. aeruginosa* genomic DNA library. The *P. aeruginosa* PAO1 genomic DNA library used for the bacterial two-hybrid screening was constructed by using the pTRG vector and two engineered pTRG vectors (see “Materials and Methods”) to maximize the number of open reading frames (ORFs) covered by the library. The high-coverage genomic library was designed to contain DNA fragments of 1–3 kb in length (supplemental Table S1 and Fig. S1). The bait plasmid was constructed by fusing the PA2799 gene to the bacteriophage λ repressor protein gene. The screening was performed by using PA2799 as the bait to probe for the prey protein encoded by the *P. aeruginosa* genomic DNA library. After several rounds of optimization to reduce the number of false-positive colonies, the screening yielded consistent results with more than two third of the prey plasmids containing a DNA fragment from the gene PA2824 (Fig. 1, A and B). While most of the other prey plasmids were found to be false positives that contain shifted ORFs, several clones contain a DNA frag-

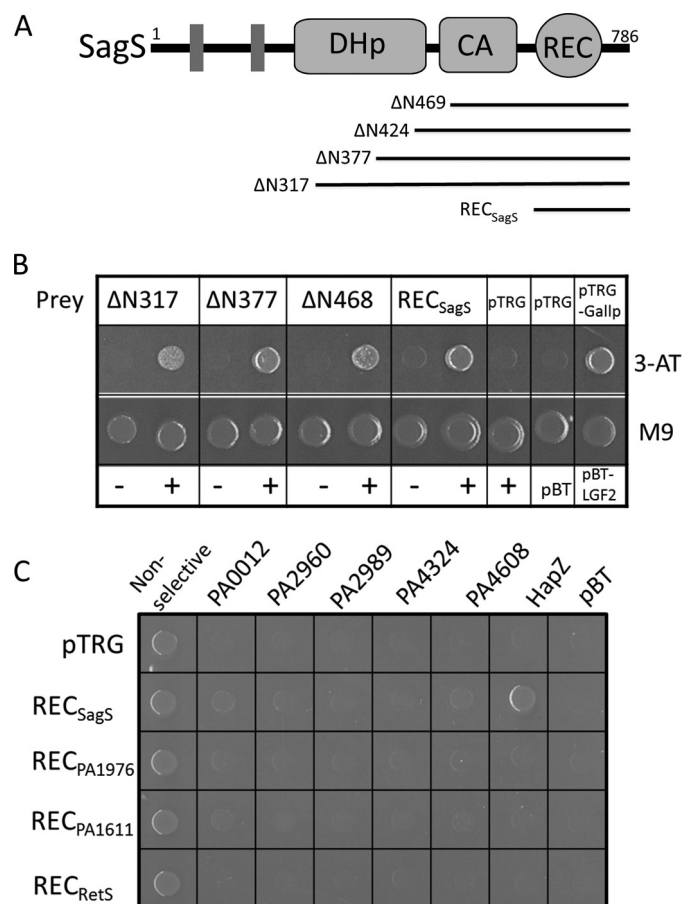


FIGURE 1. PA2799 (or HapZ) interacts with the phosphoreceiver domain of SagS as demonstrated by bacterial two-hybrid assay. A, domain organization of the hybrid histidine kinase SagS (DHP: dimerization and histidine phosphotransfer domain; CA: catalytic and ATP-binding domain; REC: phosphoreceiver domain). The fragments found to interact with PA2799 are represented by bars. B, colonies from bacterial two-hybrid assay indicate the interaction between PA2799 and various SagS constructs (ΔN317 denotes the construct that lacks the N-terminal 317 residues; – denotes empty pBT vector; + denotes pBT-HapZ vector). C, specific bacterial two-hybrid assays demonstrate the specific interaction between PA2799 (or HapZ) and SagS.

ment that encodes a portion of the diguanylate cyclase WspR that encompasses the entire GGDEF domain. However, no interaction was detected between PA2799 and the full-length WspR in the subsequent specific two-hybrid binding assay.

The gene PA2824 codes for the protein SagS, a membrane-anchored hybrid histidine kinase that contains a sensor domain, a dimerization and histidine phosphotransfer (DHP) domain, a catalytic and ATP-binding (CA) domain and a phosphoreceiver (REC) domain (Fig. 1A). To validate the protein-protein interaction and to test whether the C-terminal REC domain of SagS is sufficient for maintaining the interaction with PA2799, we performed specific bacterial two-hybrid binding assays using a construct that contains the free-standing REC_{SagS} domain. As evidenced by the robust growth of the colonies on selective medium (Fig. 1B), the interaction between PA2799 and the REC_{SagS} domain was readily detected to suggest that PA2799 is likely to interact with SagS through the REC domain. As the first PilZ protein found to interact with a histidine kinase, we renamed PA2799 as HapZ (Histidine kinase-associated PilZ protein).

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The Protein-Protein Interaction between HapZ and SagS Is Specific—The histidine-containing phosphotransfer protein-B (HptB) is known as one of the downstream and phosphoryl-receiving partners of SagS (29). In addition to SagS, three additional sensor histidine kinases (PA1976, PA1611, and PA4856 or RetS) can also interact with HptB for phospho-transfer (29–32). On the other hand, *P. aeruginosa* PAO1 contains several PilZ proteins in addition to HapZ. To test the specificity of the interaction between SagS and HapZ, prey plasmids that encode the REC domains of PA1976, PA1611, and PA4856 and bait plasmids that encode the PilZ proteins were constructed for a “cross-binding” assay. Bacterial two-hybrid assays showed that only the interaction between HapZ and REC_{SagS} could be detected (Fig. 1C), suggesting that HapZ is likely the only PilZ protein that interacts with SagS and that the interaction between HapZ and SagS is highly specific. Such specific protein recognition between the PilZ adaptors and its protein partners could be crucial for preventing crosstalk among different signaling pathways.

***c*-di-GMP Enhances the Protein-Protein Interaction between HapZ and SagS**—It is known that HapZ binds *c*-di-GMP with a dissociation constant (K_d) of 2.0 μ M (2). To further validate the interaction between HapZ and REC_{SagS} and examine the effect of *c*-di-GMP on SagS-HapZ interaction, co-immunoprecipitation (Co-IP) assays were performed in *P. aeruginosa* PAO1 cell lysate. Plasmids were constructed to express hemagglutinin (HA)-tagged HapZ and 6 \times His-tagged REC_{SagS}. We found that HA-HapZ could be co-immunoprecipitated with 6 \times His-REC_{SagS} to confirm the interaction between SagS and HapZ (Fig. 2, A and B). The Co-IP assays also showed that the SagS-HapZ interaction is enhanced by *c*-di-GMP in a dose-dependent manner within the 0–20 μ M range (Fig. 2B). Little enhancement was observed above 20 μ M *c*-di-GMP, which is consistent with the binding affinity ($K_d = 2.0 \mu$ M) of HapZ for *c*-di-GMP. The structurally related nucleotides GMP, cGMP, and *c*-di-AMP had little effect on SagS-HapZ interaction, indicating that the enhancing effect of *c*-di-GMP is rather specific. The enhancing effect of *c*-di-GMP on SagS-HapZ interaction is reminiscent of YcgR, a didomain PilZ protein that interacts with the switching complex of flagellar motor in *E. coli* (4, 5).

PilZ proteins bind *c*-di-GMP by using several conserved residues from the β -barrel and the flexible N-terminal loop (7, 11, 33). Sequence alignment and structural modeling suggests that PA2799 is likely to bind *c*-di-GMP using the same set of residues from both the β -barrel and the 22-residue N-terminal loop (Fig. 2, C and D). As indicated by the barely visible colony, the protein-protein interaction seemed to be significantly weaker between REC_{SagS} and the HapZ_R12A mutant that lacks the conserved *c*-di-GMP-binding residue Arg12 (Fig. 2E). Likewise, the interaction also seemed to be weaker between REC_{SagS} and the HapZ_Δ21aa construct that lacks the entire N-terminal loop. The Co-IP and two-hybrid assay results strongly suggest that HapZ and SagS directly interact with each other, and that the binding of *c*-di-GMP to HapZ enhances the protein-protein interaction.

Unfortunately, our further attempts to characterize the SagS-HapZ interaction and quantify the binding affinity using isothermal titration calorimetry (ITC) and nuclear magnetic

resonance (NMR) spectroscopy was not successful because of the tendency of HapZ and its variants to form insoluble aggregate at high concentration (>100 μ M), which is required for the *in vitro* binding assays.

HapZ Blocks the Phosphotransfer between SagS and the Downstream Protein HptB in a *c*-di-GMP-dependent Manner—It has been known that SagS initiates a phosphorelay cascade in *P. aeruginosa* with HptB and the bifunctional PA3346 protein as the immediate downstream protein partners (Fig. 3A) (31, 32). PA3346 possesses either kinase or phosphatase activity, depending on the phosphorylation state of the protein. The observed SagS-HapZ interaction hints that HapZ can potentially regulate the SagS two-component signaling pathway by directly binding to SagS. We performed *in vitro* phosphorylation assay to investigate how HapZ and *c*-di-GMP affect the phosphotransfer between SagS and HptB. When the 6 \times His-tagged recombinant proteins SagS_{246–786} and HptB (1:1 ratio) were incubated in the presence of [γ -³²P]ATP, phosphorylation of HptB was observed readily (Fig. 3B). When we included freshly prepared HapZ in the reaction mixture at a SagS/HptB/HapZ ratio of 1:1:1, the phosphorylation of HptB was suppressed, with the final amount of phosphorylated HptB reduced by ~40%. This observation supports the view that HapZ can interact with SagS even in the absence of *c*-di-GMP. Nonetheless, addition of *c*-di-GMP further decreased the phosphorylation level of HptB in a dose-dependent manner till the [*c*-di-GMP] reached about 50 μ M (Fig. 3B). The effect of *c*-di-GMP is specific because the enhancing effect was not observed for GMP and other nucleotides (Fig. 2C). *C*-di-GMP did not seem to have a significant effect on HptB phosphorylation when HapZ was replaced by the HapZ_R12A/R16A double mutant that is likely to have a much weaker affinity for *c*-di-GMP (Fig. 3D), suggesting that *c*-di-GMP exerts its effect through HapZ. The different effect of HapZ and the double mutant is unlikely due to different protein stability as the recombinant mutant protein seemed to exhibit similar fold efficiency (in *E. coli*) and *in vitro* stability (estimated by the decrease of the $A_{280 \text{ nm}}$ of protein solutions at ambient temperature). Note that although a small sub-family of histidine kinases has been reported to be able to bind *c*-di-GMP directly (34), SagS is unlikely to bind *c*-di-GMP due to the absence of *c*-di-GMP binding residues (supplemental Fig. S2). Taken together, the phosphorylation assays indicate that HapZ can bind to SagS to inhibit phosphorelay, and that the inhibition is likely to be further enhanced at high *c*-di-GMP concentration.

HapZ Is Involved in Surface Attachment and Biofilm Formation—*P. aeruginosa* is notorious for its ability to form antibiotic-resistant biofilm on abiotic and biotic surfaces. SagS, which stands for surface attachment and growth sensor hybrid, is known to control surface attachment and development of *P. aeruginosa* biofilm (29). If HapZ interacts with SagS in the cell, HapZ is likely to affect surface attachment and biofilm development as well. By using biofilm-growing flow cells and a confocal microscope, we examined how the deletion and overexpression of *hapZ* affects the formation of *P. aeruginosa* PAO1 biofilm. As shown in Fig. 4A, PAO1 formed the characteristic mushroom-shaped biofilm after 5 days of growth; whereas the Δ *hapZ* mutant strain exhibited significant delay in early surface

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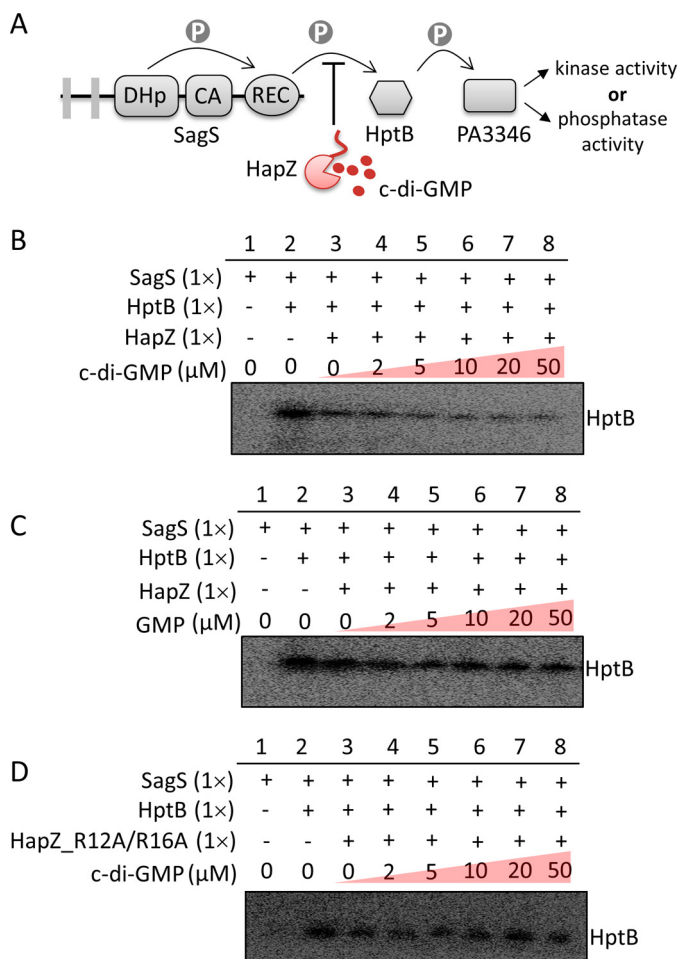


FIGURE 3. HapZ inhibits the phosphotransfer between SagS and HptB and the inhibition is further enhanced *c*-di-GMP. *A*, schematic illustration of the phosphorelay between SagS and downstream signaling proteins HptB and PA3346. *B*, effect of HapZ and *c*-di-GMP on the phosphorylation of HptB by SagS. The cytoplasmic portion of SagS (SagS_{246–786}) and [³²P]ATP were used for the assays. Detailed reaction conditions are described in “Materials and Methods.” *C*, effect of GMP on the phosphorylation of HptB. *D*, effect of *c*-di-GMP on the phosphorylation of HptB in the presence of the HapZ_{R12A/R16A} double mutant instead of HapZ.

was not affected (data not shown). These observations indicate that HapZ impacts both surface attachment and biofilm development.

To further confirm the role of HapZ in initial surface attachment during early biofilm formation, we conducted a competitive biofilm assay with mixed *P. aeruginosa* populations. When the cyan fluorescence protein (CFP)-tagged PAO1 strain was co-cultured with the YFP-tagged Δ *hapZ* strain in flow cells, the final biofilm was dominated by PAO1 (Fig. 4*B*), confirming that the Δ *hapZ* strain was impaired in surface attachment. In contrast, the matured biofilm was dominated by the *hapZ*⁺ strain when PAO1 was co-cultured with YFP-tagged *hapZ*⁺ strain, suggesting that the *hapZ*⁺ strain is more competent than PAO1 in surface attachment. These observations suggest that HapZ is involved in the processes of surface attachment and biofilm development, which further strengthens the functional relationship between SagS and HapZ.

HapZ Mediates Swarming and Twitching Motility—Flagella-mediated swimming and swarming motility are indispensable

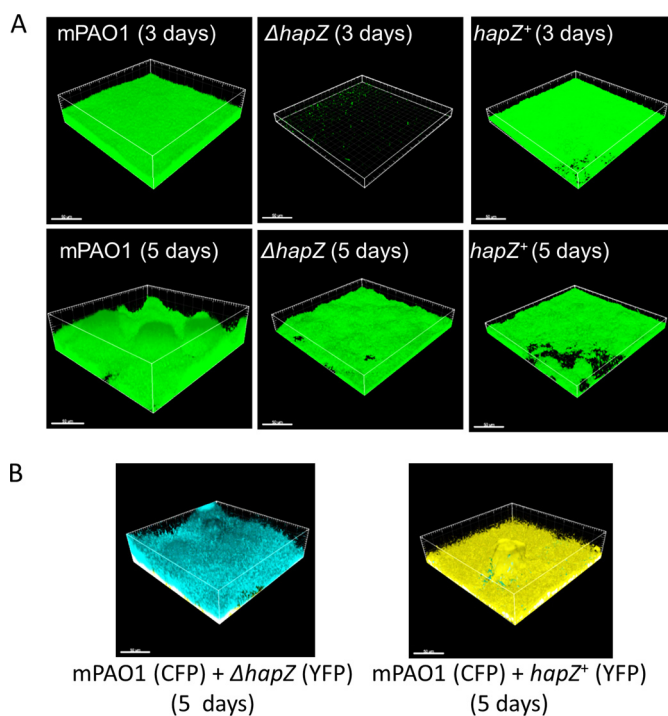


FIGURE 4. HapZ plays a regulatory role in surface attachment and biofilm development. *A*, flow cell biofilm assay for PAO1 and the two mutants. The biofilms of PAO1, the deletion (Δ *hapZ*) and overexpression (*hapZ*⁺) mutants were grown in flow cells side-by-side and continuously monitored by confocal microscope without disrupting the flows. Confocal scanning laser microscopy micrographs show surface attachment of bacterial cells and formation of biofilm at the end of day 3 and day 5. Scale bars (50 μm). *B*, confocal scanning laser microscopy micrographs of the biofilm formed by the co-cultured PAO1 and Δ *hapZ* strain and co-cultured PAO1 and *hapZ*⁺ strains.

for *P. aeruginosa* biofilm formation. We found that the deletion and overexpression of *hapZ* affects swarming, but not swimming motility. The Δ *hapZ* mutant exhibited a hyper-swarming phenotype and the phenotype could be reversed by HapZ overexpression (Fig. 5*A*). Suppression of swarming by HapZ was also demonstrated by the further subdued swarming activity of *hapZ*⁺, suggesting that the swarming phenotype of Δ *hapZ* is indeed due to the low level of *hapZ*. The hyper-swarming phenotype of Δ *hapZ* could not be reversed by the complementation with the *hapZ*_{Δ21} construct that lacks the N-terminal loop, which confirms the importance of *c*-di-GMP binding for the function of HapZ. A close inspection of the swarming plate revealed large zones of fluid surrounding the swarming tendrils of the Δ *hapZ* cells. Since the zone of fluid is known to be caused by the production of rhamnolipids (35), we reasoned that the increased swarming for Δ *hapZ* mutant is, at least partially, caused by an increased production of rhamnolipids. To test this, we quantified the amount of rhamnolipid produced by PAO1 and the two mutants by emulsification activity assay. Indeed, increased rhamnolipid production was observed for Δ *hapZ* whereas decreased rhamnolipid production was observed *hapZ*⁺ (Fig. 5*B*).

Apart from swimming and swarming, type IV pili-dependent twitching motility is also known to be crucial for the formation of *P. aeruginosa* biofilm (36). Our twitching motility assay suggests that HapZ affects twitching motility. The Δ *hapZ* mutant showed increased twitching zone and the enhanced twitching

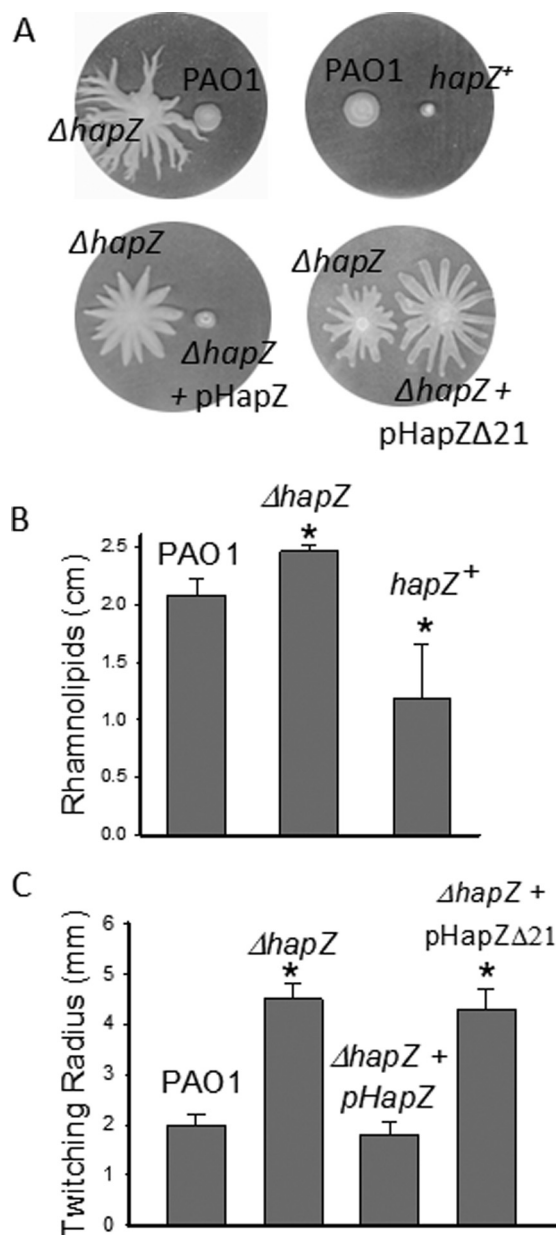


FIGURE 5. HapZ impacts swarming and twitching motility. *A*, analysis of swarming motility of PAO1 and mutants on semi-hydrated swarming plates. *B*, altered production of rhamnolipids for the $\Delta hapZ$ and $hapZ^+$ strains as measured by emulsification activity assay. *C*, analysis of twitching motility of PAO1 and mutant strains by comparing the radius of twitching zones.

could be suppressed by the overexpression of HapZ, but not by the overexpression of HapZ_Δ21 (Fig. 5C). It is already known that *c*-di-GMP can regulate twitching motility through the effector FimX in *P. aeruginosa* (23). The results described here suggest that HapZ is another effector through which *c*-di-GMP mediates twitching motility.

HapZ affects the expression of a large number of genes related to quorum-sensing (QS), motility and other functions (69). SagS and HptB-associated signaling pathways are known to control gene expression (32). We performed transcriptomic analysis by RNA-Seq for PAO1, $\Delta hapZ$, $\Delta sagS$, and the complementation strain ($\Delta hapZ + pHapZ$) to further establish the relationship between SagS and HapZ and to identify the genes under the control of HapZ (32, 37).

For the cells collected at stationary growth stage, the gene expression profile of the $\Delta hapZ$ strain differs substantially from that of PAO1 (Fig. 6A). The gene expression pattern could be restored by HapZ complementation, suggesting the differential gene expression is due to a change in HapZ level. Compared with PAO1, a total of 94 genes of $\Delta hapZ$ exhibited statistically significant alteration in mRNA level (Fig. 6B). Among the 61 genes showing up-regulation (>2.5 fold-change) (Fig. 6, D and E; supplemental Tables S4 and S6), some are already implicated in motility, virulence and biofilm formation. The greatest increases were observed for the genes *lecA* (46-fold) and *lecB* (17-fold), which encodes two lectins that are implicated in cell surface adhesion. *LecB* is also essential for biofilm development because the *lecB*-deficient strain is impaired in biofilm formation (38). Pseudomonas quinolone signal (PQS) is a mediator of motility, biofilm formation, population structure and survival of *Pseudomonas* in hostile environment (39, 40). Several genes (*pqsA*, *B*, *C*, *D*, and *E*) involved in the biosynthesis of PQS are up-regulated in the $\Delta hapZ$ mutant by 4–11-fold. The expression levels of the rhamnolipid biosynthetic genes *rhlA* (9.4-fold), *rhlB* (4.4-fold), and *rhlI* (2.3-fold) are also up-regulated, which is consistent with the hyper-swarming phenotype and stimulated production of rhamnolipid of $\Delta hapZ$. Elevated expression levels were also observed for the genes that encode the MexG/H/I-OpmD efflux pump (3–8 fold), elastase (*lasB*, 6.6-fold), and three genes (*glcD/E/F*) from the central metabolic glycolate pathway. The altered expression levels of the QS-controlled *lasB*, *rhlA* and *pqsA* were further validated for $\Delta hapZ$ by using promoter-*gfp*-fusion reporters. Quantification of GFP fluorescence showed elevated transcription levels for the three QS-controlled genes for $\Delta hapZ$ (Fig. 6C). Meanwhile, a total of 33 genes exhibited decreased expression levels in $\Delta hapZ$ (Fig. 6, F and G; supplemental Tables S5 and S7). Among the annotated genes, *mexE*, *mexF* and *oprN* code for the MexEF-OprN efflux pump and *mexS* codes for an associated regulator that has been implicated in biofilm formation, antibiotic resistance, and virulence (41). The expression levels of *ibpA* and *ampDh3*, which encode a small heat shock protein and an antibiotic resistance protein, respectively, are also lower in $\Delta hapZ$.

Compared to the 94 genes of $\Delta hapZ$ that showed different expression from PAO1, a total of 105 genes exhibited altered expression levels in $\Delta sagS$ (supplemental Tables S4, S5, S8, and S9). The regulons of SagS and HapZ overlap with a total of 64 co-regulated genes (Fig. 6B). Notably, among the 64 genes, 44 are up-regulated and 20 are down-regulated, and none of the genes showed opposite change in $\Delta sagS$ and $\Delta hapZ$. The 44 up-regulated genes shared by $\Delta sagS$ and $\Delta hapZ$ include *lasB*, *lecA/B*, *rhlA/B*, *mexG/H/I*, *opmD*, *pqsA-E*, *glcD/E/F*, and other unannotated genes (Fig. 6D). The most notable down-regulated genes shared by the $\Delta sagS$ and $\Delta hapZ$ are the efflux pump genes *mexE/F*, *oprN*, and several other unannotated genes (Fig. 6F). Sauer *et al.* recently reported that inactivation of *sagS* altered the expression of *mexA/B*, *oprM*, *mexE/F* and *oprN* genes (20). The observed changes in the expression level for these genes for the $\Delta sagS$ strain are consistent with their observations. Taken together, the gene expression studies show that both

of biofilm formation by regulating a myriad of cellular functions (45). It has been suggested that SagS acts as a motile switch during early stage of biofilm formation to control surface attachment (29, 37). In accordance with the role of SagS, we found that HapZ affects the ability of *P. aeruginosa* cells in surface attachment. Ability to swarm on solid or semi-solid surface is one of the major factors that affect surface attachment; and suppression of swarming motility to create a non-motile subpopulation is crucial for initial surface attachment during biofilm formation (46, 47). Our study indicates that swarming motility is likely one of the functions controlled by HapZ to impact surface attachment. The thin biofilm formed by the Δ *hapZ* mutant is partially due to enhanced swarming motility that prevents cells from initiating surface attachment. The ability of *P. aeruginosa* to swarm is dependent on many factors (35, 48). The HapZ-dependent pathway is likely to mediate swarming motility by affecting the production of QS signals, rhamnolipids and likely other factors.

In addition to its role in surface attachment, our data suggests that HapZ is actively involved in the process of biofilm development or maturation. Development of robust *P. aeruginosa* biofilm microcolonies following surface attachment requires phenotypic changes in swarming and twitching motility, EPS biosynthesis and export, QS activity, rhamnolipid production and central metabolism. The transition from surface attachment stage to maturation stage during biofilm formation is accompanied by extensive shift in gene expression (46). Such shift in gene expression is vital for biofilm maturation as the surface-attached cells need to build extracellular matrix and grow microcolonies vertically. The lack of microcolonies for the *hapZ*⁺ mutant that is highly efficient in surface attachment indicates that a high level of HapZ impedes the shift from a “surface attachment phenotype” to a “maturation phenotype” in biofilm formation. HapZ and its associated pathways are likely to contribute to biofilm development by controlling multiple factors and pathways. Most notably, our data suggest that HapZ controls the expression of many genes that are under the control of the three QS systems (Las, Rhl, and PQS) of *P. aeruginosa*. As the three QS systems have all been implicated in biofilm development (49–51), it is likely for HapZ to impact biofilm development by regulating quorum-sensing activity. Secondly, SagS is a key regulator in the formation of highly antimicrobial tolerant biofilm microcolonies (20). HapZ is likely to contribute to the development of resistant biofilm because it mediates the expression of genes that encode several major efflux pumps and metabolic enzymes. Thirdly, as twitching motility is essential for the formation of structured biofilm microcolonies (52), HapZ may also participate in biofilm development by controlling twitching motility.

SagS is part of a highly complex two-component signaling network composed of multiple sensor kinases, response regulators, connectors, and transcriptional regulators. SagS can initiate phosphorelay cascades through both HptB-dependent and -independent pathways (29, 31, 32, 37, 53). Our current understanding of this intricate two-component signaling network is far from complete. What is clear now is that this complex two-component signaling network controls the expression of a large number of genes to coordinate the timing of surface

attachment and biofilm development, with the pathways activated or deactivated at different stages during biofilm formation (37, 54–57). Integration of HapZ in the two-component signaling network presumably allows the phosphorelay cascades to be regulated by additional inputs sensed by *c*-di-GMP pathways. It was presumed that the input signal sensed by the sensor domain of SagS triggers the SagS-controlled motile-to-sessile switching. The current study raises the possibility that the switching is triggered by a change in cellular *c*-di-GMP level instead. Moreover, SagS has been suggested to regulate gene expression by interacting with the histidine kinase BfiS and controlling its phosphorylation (37). It is plausible that *c*-di-GMP and HapZ may also mediate this HptB-independent pathway to impact gene expression. This may be the reason why the deletion of *sagS* and *hapZ* did not have opposite effect on gene expression, as one would infer from the observation that HapB inhibits the phosphotransfer between SagS and HptB. As a step toward fully unraveling this intricate two-component signaling network, the current study unveils the unexpected involvement of *c*-di-GMP and HapZ and sets the stage for investigating how *c*-di-GMP controls various stages of biofilm formation through this signaling network.

The involvement of *c*-di-GMP in two-component signaling is a common phenomenon, as evidenced by the fact that many bacterial genomes contain a large number of genes that encode response regulators containing *c*-di-GMP-synthesizing (GGDEF) and degrading (EAL or HD-GYP) domains (58–63). By using the *c*-di-GMP-synthesizing or degrading response regulators, the environmental inputs sensed by histidine kinases can be translated into oscillation of cellular *c*-di-GMP concentration. One of the findings from recent studies is that *c*-di-GMP can also control the activity and function of histidine kinases to regulate two-component signaling. For example, the bifunctional histidine kinase CckA involved in cell cycle control in *C. crescentus* binds *c*-di-GMP directly in the catalytic- and ATP-binding domain for regulating the kinase and phosphatase activities (34). Another example is the orphan hybrid histidine kinase SgmT from *Myxococcus xanthus*. SgmT has a C-terminal non-enzymatic GGDEF domain that functions as a *c*-di-GMP-binding effector. The binding of *c*-di-GMP to the GGDEF domain is crucial for the spatial sequestration, and likely cellular function of SgmT (64). The current study suggests that another way for *c*-di-GMP to control histidine kinase is by using a discrete PilZ adaptor. In principle, the use of PilZ adaptors has the advantage of allowing *c*-di-GMP to control multiple pathways through highly specific kinase-adaptor interaction. Because PilZ proteins exhibit a wide range of binding affinity for *c*-di-GMP (23), the effect of *c*-di-GMP on a specific pathway can be “felt” only when the *c*-di-GMP concentration reaches a certain threshold. It is also conceivable that evolving a discrete PilZ adaptor to regulate histidine kinase is easier than creating a *c*-di-GMP-binding site in the catalytic domain of histidine kinases because of fewer evolutionary constricts. The ability to rapidly evolve new *c*-di-GMP-binding PilZ adaptors for two-component signaling pathways may be crucial for the survival of such highly adaptable bacteria as *P. aeruginosa*.

Function of *c*-di-GMP-binding PilZ Protein

Materials and Methods

Construction of *P. aeruginosa* Genomic DNA Libraries—For genomic library construction, the BamHI site was used for vector digestion and Sau3AI site for gDNA partial digestion. The vector pTRG of the BacterioMatch II Two-Hybrid system (Stratagene) was used for prey library construction. Three independent genomic DNA libraries were constructed by using the vector pTRG, and the engineered pTRG-derived pTRG-A2 and pTRG-AC1. The plasmids pTRG-A2 and pTRG-AC1 were constructed by inserting the nucleotides “A” and “AC” between the 3′-end of the RNAP- α (992 bp) gene and the BamHI site (993 bp) of, respectively. The insertion resulted in a shift of the polylinker site by +1 or +2 nucleotides. The vectors pTRG, pTRG-A2 and pTRG-AC1 were digested by BamHI and dephosphorylated with phosphatase. The vector digestion mixture was extracted with phenol: chloroform, precipitated, and resuspended into 1 \times TE buffer. The genomic DNA of *P. aeruginosa* strain PAO1 was isolated using the chloroform-cetyltrimethylammonium bromide extraction method, and partially digested by Sau3AI. The randomly digested fragments ranging in size from 1,000 to 3,000 bp were purified using NucleoSpin[®] Gel and PCR Clean-up kit (MACHEREY-NEGEL). Gel-purified DNA fragments were ligated to the linearized and dephosphorylated pTRG, pTRG-A2, or pTRG-AC1 vectors. The resulting ligation mixtures were precipitated and resuspended into 1 \times TE buffer, followed by transformation into *E. coli* XL1-Blue MRF′ Kan competent cells using a standard electroporation procedure. The three libraries were collected and pooled separately as the prey libraries and stored in -80°C freezer.

Bacterial Two-hybrid Screening—The plasmid pBT-PA2799 was used as the bait to probe the genomic DNA libraries using the BacterioMatch II Two-Hybrid system (Stratagene). The plasmids extracted from the cells harboring the library fragments were co-transformed into the reporter strain with the bait plasmid by electroporation. The co-transformed cells were grown on M9⁺ His-deficient medium containing 5 mM 3-AT for 48–72 h at 30 $^{\circ}\text{C}$. Colonies that grew on these plates were selected as positive colonies. The equivalent of 1 \times 10⁶ colonies were screened, as assessed by plating the transformed cells on M9⁺ His-deficient medium. Positive colonies were subsequently picked and re-streaked on M9⁺ His-deficient medium containing 5 mM 3-AT and 12.5 $\mu\text{g}/\text{ml}$ streptomycin. The colonies that grew on the double selection medium were cultured in liquid medium, and used for colony PCR with pTRG F/R pairs to amplify the insert from the library plasmid. The positive colonies were grown for the preparation of plasmids for sequencing. For the specific two-hybrid binding assay, the bait and prey plasmids were co-transformed and the co-transformed mixtures were plated onto non-selective medium and incubated at 37 $^{\circ}\text{C}$ overnight. The growth of the selected co-transformed clones was verified by dotting on the selective medium by 37 $^{\circ}\text{C}$ overnight incubation. Normal growth on the selective screening medium was considered as an indicator of positive protein-protein interaction.

Co-immunoprecipitation (Co-IP) Assay—To validate the interaction between HapZ and REC_{Sags} in *P. aeruginosa* by

Co-IP assay, the two plasmids pUCP-HA-HapZ, pUCP-HA-HapZ/6 \times His-REC_{Sags} (supplemental Table S2) were constructed and transformed into PAO1. The strain harboring pUCP-HA-HapZ consistently expressed protein HA-HapZ while the strain harboring pUCP-HA-HapZ/6 \times His-REC_{Sags} produced both HA-HapZ and 6 \times His-REC_{Sags}. The bacterial strains were cultured in LB containing 250 $\mu\text{g}/\text{ml}$ carbenicillin at 37 $^{\circ}\text{C}$ overnight with shaking and subsequently sub-cultured to an OD₆₀₀ of 1.0 at 37 $^{\circ}\text{C}$. Cells were harvested by centrifugation (13,000 $\times g$ for 10 min) and lysed with ice-cold cell lysis 1 \times PBS buffer (10 mM NaP_i (pH 7.4), 137 mM NaCl, and 2.7 mM KCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EDTA and protease inhibitor mixture (Roche)). The total cell lysates were clarified by centrifugation at 15,000 $\times g$ for 10 min, and the supernatant was pre-clarified with protein G beads after being filtered by Minisart[®] high flow Syringe Filters. Lysates was subsequently immunoprecipitated with the protein A-Sepharose beads (EZview[™] Red Anti-HA Affinity Gel, Sigma-Aldrich). Each immunoprecipitation sample containing 800 μl of lysate, 20 μl of the prewashed beads with different concentrations of nucleotides (*c*-di-GMP, GMP or cGMP) was incubated for 1 h at 4 $^{\circ}\text{C}$. The beads were collected by centrifugation and washed six times with lysis buffer at 4 $^{\circ}\text{C}$, and then the immunoprecipitated proteins were diluted with 2 \times sample buffer and subjected to SDS-PAGE and Western blotting with HA antibody.

Cloning, Expression, and Purification of Recombinant Proteins—The recombinant proteins HapZ, HptB, and SagS_{246–786} were expressed using *E. coli* Rosetta competent cells. Transformed cells were grown at 37 $^{\circ}\text{C}$ to OD₆₀₀ of 0.6 in LB medium containing 50 $\mu\text{g}/\text{ml}$ kanamycin and induced for protein expression. Protein expression was induced using 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) (16 $^{\circ}\text{C}$). After 16 h, cells were harvested by centrifugation and resuspended in phosphate buffer, pH 8.0 and lysed using French Press homogenizer at 15,000 psi. The cells were then centrifuged at 25,000 rpm for 30 min, and supernatants were filtered and loaded onto a nickel-charged column equilibrated with KP_i buffer (pH 8.0). The column was washed sequentially using W1 (KP_i buffer, pH 8.0 + 20 mM imidazole) and W2 (KP_i buffer, pH 8.0 + 50 mM imidazole) buffer. The 6 \times His-tagged proteins were eluted from the column using elution buffers (KP_i buffer, pH 8.0) containing increasing concentrations of imidazole (100, 200, and 300 mM) and were analyzed using SDS-PAGE. The fractions containing the desired proteins were pooled with a purity > 95%, desalted using PD-10 column (GE Healthcare) and concentrated using Amicon concentrator (10 kDa or HptB and SagS_{246–786} and 5 kDa for HapZ) at 4,000rpm at 4 $^{\circ}\text{C}$. The concentrations of the proteins were determined by Bradford method and were aliquot and stored at -80°C with the exception of HapZ, which must be used without storage due to its propensity to aggregation.

In Vitro Phosphorylation Assay—*C*-di-GMP was prepared using a thermophilic diguanylate cyclase as described previously (65, 66). Phosphorelay between SagS and HptB was examined by performing *in vitro* phosphorylation assay with 6 \times His-tagged recombinant proteins. The reaction mixture that contains SagS_{246–786} and HptB (1:1 ratio), [³²P]ATP, and MgCl₂ was incubated at 25 $^{\circ}\text{C}$ for 30 min in reaction buffer,

which is composed of 100 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1 μCi of [γ -³²P]ATP (about 111 TBq/mmol, 10 mCi/ml, 3.33 μM, 3,000 Ci/mmol). Phosphorylation of HptB was visualized by running 15% SDS-polyacrylamide gel electrophoresis and autoradiography. The effect of HapZ and c-di-GMP on HptB phosphorylation was examined by incubating the corresponding components with the reaction mixture.

Biofilm Formation in Flow-cell—The *P. aeruginosa* strains were tagged with GFP reporter by inserting the mini-Tn7-eGFP-Gmr, mini-Tn7-eCFP-Strept and mini-Tn7-eYFP-Strept cassette. Biofilms were grown in flow chambers with individual channel dimensions of 1 × 4 × 40 mm. The flow chambers were inoculated by injecting 350 μl overnight culture diluted to an OD_{600 nm} of 0.01 into each flow channel using a small syringe. After inoculation, the flow channels were left without flow for 1 h, after which medium flow was started using a Watson Marlow 205S peristaltic pump. The mean flow velocity in the flow-chambers was 0.2 mm s⁻¹. All microscopic observations and image acquisitions were done with a Zeiss LSM780 confocal laser scanning microscope (CLSM). Data were analyzed to generate the simulated three-dimensional images using the IMARIS software package (Bitplane AG) (67).

Transcriptome Analysis by RNA-Seq—Colonies of *P. aeruginosa* PAO1 and the three mutant strains were grown overnight in ABTGC medium at 37 °C. The cultures were diluted to a starting OD_{600 nm} of 0.01, and 1 ml of culture was added to a well of a 24-well plate. The plate was sealed with parafilm and incubated at 37 °C to reach stationary phase. The cultures were mixed immediately with 2 volumes of RNaprotect[®] Bacteria Reagent (Qiagen). After a 5-min incubation at room temperature, samples were centrifuged, and the pellets were stored at -80 °C. Bacterial cells were treated with lysozyme and total RNA was extracted with RNeasy Mini Purification kit (Qiagen). Removal of DNA was carried out by on-column DNase digestion with the RNase-free DNase Set (Qiagen). The integrity of total RNA and DNA contamination was assessed and the 16S, 23S, and 5S rRNAs were removed by using the Ribo-Zero[™] Magnetic Kit (Epicenter). Gene expression analysis was performed in duplicate by RNA sequencing. The rRNA-depleted RNA was fragmented to 200–300-bp fragments, and the first and second strand cDNA were synthesized, followed by end repair and adaptor ligation. The libraries were sequenced using the Illumina HiSeq2000 platform with a paired-end protocol and read lengths of 100 nt. The RNA-Seq data are available in the NCBI GEO Short Read Archives (SRA No: SAMN02369270). The sequence reads were assembled and analyzed in RNA-Seq and the expression analysis application of CLC genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark). The following criteria were used to filter the unique sequence reads: minimum length fraction of 0.9, minimum similarity fraction of 0.8, and maximum number of two mismatches. Data were normalized by calculating the reads per kb per million mapped reads for each gene (68). ANOVA and *t* test were performed on transformed data to identify the genes with significant changes in expression (*p* < 0.05, fold change > 2.5).

Swarming and Twitching Motility Assay—Swarming motility was analyzed on 0.5% agar M8 salt plates supplemented with 0.2% glucose, 2 mM MgSO₄, and 0.5% casamino acid. 1.5 μl of

cell culture grown at 37 °C overnight in LB medium were dotted on freshly prepared swarming plates and incubated for 16 h at 37 °C. The PAO1 strain was included as a control on each swarming plate. For twitching motility assay, PAO1 cells were stab inoculated with a toothpick through a thin Difco LB agar (3 mm, 1% Difco granulated agar) layer to the bottom of the Petri dish. After overnight growth at 37 °C, the zone of twitching was visualized by staining with Coomassie Brilliant Blue R250, and the radius of the zone was measured.

Quantification of Rhamnolipid Production—The emulsification activity assay was modified from as previously described (69). 1 ml of filtered overnight culture of the different *P. aeruginosa* strains was mixed with 1 ml of hexadecane in a glass tube. The mixture was vortexed rigorously for 2 min, and was allowed to stand for 2 h in room temperature. Emulsification activity was measured as the height of emulsion layer divided by the total height of the mixture. Experiments were performed in triplicate, and the results are shown as the mean ± S.D.

Gene Expression Reporter Fusion Assay—Overnight cultures of *P. aeruginosa* PAO1 and Δ*hapZ* strains carrying the different QS report fusions (*P_{lasB}-gfp* (69), *P_{rhlA}-gfp* (70), and *P_{pqsA}-gfp* (71)) were diluted 100 times in ABTGC medium. 200 μl of each diluted culture was added to each wells of a 96-well plate. GFP fluorescence and observance at 600 nm was monitored overnight using a microplate reader (Tecan Infinite 2000). Experiments were performed in six replicates, and the results are shown as the mean ± S.D.

Author Contributions—Z. X. L. designed the study and wrote the paper with the assistance of L. X., P. V., and Y. L. The two-hybrid screening, colony morphology, and motility experiments were performed by L. X., P. V., Y. D., L. X., R. Y., L. Z. The *in vitro* phosphorylation and recombinant protein preparation were performed by L. X., P. V., and G. L. Y. The flow-cell biofilm, RNA-Seq, 3D-SIM, and fluorescent reporter assays were designed and performed by L. Y., Y. L., and Y. D.

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