The GGDEF Domain of the Phosphodiesterase PdeB in *Shewanella putrefaciens* Mediates Recruitment by the Polar Landmark Protein HubP

Florian M. Rossmann, Tim Rick, Devid Mrusek, Lasse Sprankel, Anja K. Dörrich, Tabea Leonhard, Sebastian Bubendorfer, Volkhard Kaever, Gert Bange, Kai M. Thormann

*Justus-Liebig Universität, Department of Microbiology and Molecular Biology, Giessen, Germany*

*LOEWE Center for Synthetic Microbiology (Synmikro), Philipps Universität Marburg, Marburg, Germany*

*Department of Chemistry, Philipps Universität Marburg, Marburg, Germany*

*Research Service Center Metabolomics, Hannover Medical School, Hannover, Germany*

**ABSTRACT**  Bacteria commonly exhibit a high degree of cellular organization and polarity which affect many vital processes such as replication, cell division, and motility. In *Shewanella* and other bacteria, HubP is a polar marker protein which is involved in proper chromosome segregation, placement of the chemotaxis system, and various aspects of pilus- and flagellum-mediated motility. Here, we show that HubP also recruits a transmembrane multidomain protein, PdeB, to the flagellated cell pole. PdeB is an active phosphodiesterase and degrades the second messenger c-di-GMP. In *Shewanella putrefaciens*, PdeB affects both the polar and the lateral flagellar systems at the level of function and/or transcription in response to environmental medium conditions. Mutant analysis on fluorescently labeled PdeB indicated that a diguanylate cyclase (GGDEF) domain in PdeB is strictly required for HubP-dependent localization. Bacterial two-hybrid and *in vitro* interaction studies on purified proteins strongly indicate that this GGDEF domain of PdeB directly interacts with the C-terminal FimV domain of HubP. Polar localization of PdeB occurs late during the cell cycle after cell division and separation and is not dependent on medium conditions. *In vitro* activity measurements did not reveal a difference in PdeB phosphodiesterase activities in the presence or absence of the HubP FimV domain. We hypothesize that recruitment of PdeB to the flagellated pole by HubP may create an asymmetry of c-di-GMP levels between mother and daughter cells and may assist in organization of c-di-GMP-dependent regulation within the cell.

**IMPORTANCE**  c-di-GMP-dependent signaling affects a range of processes in many bacterial species. Most bacteria harbor a plethora of proteins with domains which are potentially involved in synthesis and breakdown of c-di-GMP. A potential mechanism to elicit an appropriate c-di-GMP-dependent response is to organize the corresponding proteins in a spatiotemporal fashion. Here, we show that a major contributor to c-di-GMP levels and flagellum-mediated swimming in *Shewanella*, PdeB, is recruited to the flagellated cell pole by the polar marker protein HubP. Polar recruitment involves a direct interaction between HubP and a GGDEF domain in PdeB, demonstrating a novel mechanism of polar targeting by the widely conserved HubP/FimV polar marker.

**KEYWORDS**  c-di-GMP, cell polarity, flagella, heterogeneity, motility

Research during the last decades has provided compelling evidence that bacterial cells, despite lacking the typical membrane-enclosed organelles common for eukaryotic cells, exhibit a high degree of spatial organization which is crucial for numer-
ous processes in general cellular functions. Rod-shaped bacteria commonly show an asymmetric distribution of cellular components, leading to a distinct polarity which affects processes such as cell cycle regulation, virulence, and diverse aspects of motility and chemotaxis (for recent reviews, see references 1 to 3). To establish cell polarity, many bacteria rely on so-called landmark proteins, which localize to specific compartments and directly or indirectly recruit other molecules. Well-studied landmark protein systems are TipN/TipF, which mediate polarity in *Caulobacter crescentus* (4, 5) or DivIVA in *Streptomyces* and *Bacillus* (6–9).

Another emerging bacterial polar landmark protein is HubP (see Fig. S1 in the supplemental material), which has been demonstrated to affect a wide range of cellular processes in *Vibrio* sp. and *Shewanella* sp. (10, 11). HubP is a transmembrane protein with an N-terminal periplasmic section harboring a peptidoglycan-binding LysM domain that mediates HubP localization to both cell poles and the divisional plane. The cytoplasmic part of HubP is characterized by a number of imperfect amino acid repeats highly enriched in acidic residues, which impart a low overall pI on the protein. A FimV domain, located in the very C-terminal region of HubP, is named after *Pseudomonas aeruginosa* FimV, a protein with features highly similar to those of HubP and also involved in mediating cell polarity (12–17). While the N- and C-terminal regions of HubP from *Vibrio cholerae* and *Shewanella putrefaciens* CN-32 comprising the LysM and FimV domains, respectively, are quite well conserved, *V. cholerae* HubP (VcHubP) and *S. putrefaciens* HubP (SpHubP) differ considerably in protein length, amino acid sequence, and the length and number of repeats (10 in *V. cholerae* and 9 in *S. putrefaciens*). However, in both *Vibrio* sp. and *S. putrefaciens*, HubP is involved in normal chromosome segregation, polar placement of the chemotaxis machinery, and proper assembly and function of the polar flagellar system (10, 11, 18). Bacterial two-hybrid (BACTH) analysis of different sections of VcHubP indicated that the polar landmark directly interacts with ParA1, involved in chromosome segregation (10). A putative direct interaction of HubP was also found for the signal recognition particle (SRP)-like GTPase FlhF and the MinD-like ATPase FlhG, which are determinants of flagellar number, placement, and function. This interaction may account for the aberrant flagellation observed in *V. cholerae* and, in particular, in *Vibrio parahaemolyticus* mutants lacking HubP (10, 18). However, the wide array of phenotypes associated with a deletion of hubP in *V. cholerae* and *S. putrefaciens* strongly suggests that not all potential client proteins of HubP have already been identified. A characterization of *S. putrefaciens* CN-32 hubP mutants with respect to swimming motility revealed a significant decrease in cellular swimming speed and spreading in soft agar that was unlikely to be exclusively attributed to misplacement of the chemotaxis system (11). We therefore hypothesized that other client proteins affecting flagellar functions might be directly or indirectly recruited by HubP. Accordingly, we recently identified another client protein, ZomB, which is crucial for inducing directional switches of the flagellar motor, that directly interacts with HubP and is to be destabilized in its absence (19).

A common means to control flagellum-mediated motility in a wide range of bacterial species is via the second messenger molecule c-di-GMP. c-di-GMP has been demonstrated to affect swimming motility at the level of transcriptional control (20–24), flagellar assembly (25), motor function (26–32), and chemotaxis (33–36). This second messenger is synthesized from two molecules of GTP by diguanylate cyclases (DGCs) with a signature GGDEF domain and hydrolyzed by phosphodiesterases (PDEs) characterized by an EAL or an HD-GYP domain (reviewed in references 37 and 38). Commonly, these domains are found in multidomain proteins together with various sensor input domains, and many bacteria harbor a plethora of proteins with potential DGC or PDE activity. Spatial sequestration of DGCs or PDEs with respect to flagellum-mediated motility has been demonstrated for *C. crescentus* (39), in which EAL domain-containing protein TipF orchestrates the polarization of flagellar morphogenesis in response to c-di-GMP levels, and for *P. aeruginosa* (40). In the latter species, the PDE Pch (also named DipA [41]) is polarly recruited by the chemotaxis histidine kinase CheA. The activity of Pch depends on the distribution of the chemotaxis cluster as well as the
phosphorylation state and, hence, the activity of CheA and therefore mediates a broad heterogeneity in c-di-GMP levels within the bacterial population.

In *S. putrefaciens* CN-32, 51 proteins with a putative role in synthesis and degradation of c-di-GMP can be identified. None of these proteins has so far been directly implicated in affecting flagellum-mediated motility. However, in the close relative *Shewanella oneidensis* MR-1, the phosphodiesterase PdeB was shown to inversely regulate swimming motility and biofilm formation (42). PdeB is a membrane-located multidomain protein containing an N-terminal periplasmic domain followed by a cytoplasmic HAMP domain, a Per-Arnt-Sim (PAS) sensor domain, and a GGDEF-EAL domain pair at the C terminus. In vitro activity measurements of the purified cytoplasmic section of the protein showed that PdeB is a phosphodiesterase, and characterization of the corresponding substitution mutant revealed that this activity is absolutely crucial for function. Notably, PdeB has an effect on swimming only in complex medium; however, this could not yet be attributed to one or more specific signals (42). Here, we show that the ortholog of PdeB in *S. putrefaciens* CN-32 is localized to the flagellated cell pole and that this localization is strictly dependent on the polar landmark protein HubP. We demonstrate that PdeB polar recruitment is mediated by direct interactions between the GGDEF domain of PdeB (GGDEFPdeB) and the C-terminal FimV (FimVHubP) domain of HubP (FimVHubP). The results provide novel insights into how HubP may recruit effector molecules to a specific cellular compartment, the cell pole.

RESULTS

The phosphodiesterase PdeB affects swimming of *S. putrefaciens* CN-32. Earlier transposon mutagenesis and screening for defects in swimming motility (19) identified Sputcn32_3405 as a positive determinant for spreading in soft agar. This gene encodes a potential ortholog of *S. oneidensis* MR-1 PdeB (42) as the two proteins share the same domain organization and have 79% identity and 87% similarity at the amino acid level for the complete protein. In both species, the respective genes reside in the same genetic context, and loss of PdeB results in a pronounced phenotype in *S. oneidensis* MR-1 with respect to flagellum-mediated swimming (42). Correspondingly, motility assays on soft-agar plates demonstrated that mutants lacking Sputcn32_3405 exhibited significantly decreased lateral expansion on complex medium but not on mineral medium, and a similar phenotype was observed when the EAL domain was mutated by targeted substitution (E637A) to eliminate the phosphodiesterase activity (Fig. 1A). Accordingly, in a ΔSputcn32_3405 strain, the c-di-GMP concentration was significantly higher (about 20 pmol/mg protein) than wild-type levels (about 12.5 pmol/mg protein) (Fig. 1D). From this we concluded that Sputcn32_3405 is the *S. oneidensis* MR-1 PdeB ortholog in *S. putrefaciens* CN-32, and we refer to this protein as PdeB accordingly.

c-di-GMP has been shown to affect flagellum-mediated motility at various levels. We therefore determined whether loss of PdeB affects flagellar function and/or production. In contrast to *S. oneidensis* MR-1, *S. putrefaciens* CN-32 harbors two distinct flagellar systems, a single primary polar flagellum typical for *Shewanella* sp. and one or more lateral flagella. As both contribute to swimming through soft agar when complex medium is provided (43, 44), we determined if loss of PdeB specifically affects one or both of the two flagellar systems. To this end, a pdeB mutation was introduced into *S. putrefaciens* strains that lack the genes encoding the flagellins of the lateral or the polar system (ΔflaAB2 or ΔflaAB1, strain, respectively) and that are therefore unable to produce the respective flagellar filament. The additional loss of PdeB further decreased the lateral expansion of both ΔflaAB2 and ΔflaAB1 strains significantly (Fig. 1B; see also Fig. S2A in the supplemental material); the ΔpdeBΔflaAB1 strain was almost nonmotile on soft-agar plates. Thus, both flagellar systems depend on the presence of PdeB for normal activity.

To determine a potential effect of the pdeB deletion on expression of the flagellar genes, we employed chromosomal transcriptional fusions of the flagellin-encoding genes flaB1 (for the polar system) and flaA2 (for the lateral system) to luxCDABE (45). These genes are among the latest to be activated during the flagellar regulatory
cascade. In the absence of \( pdeB \), there was no significant difference in expression levels of the polar \( flaB_1 \), while expression of lateral \( flaA_2 \) was significantly decreased (Fig. S2B).

Correspondingly, quantification of polar and lateral hook structures as a measure of actual flagellar production and assembly showed that, compared to wild-type cells, a similar number of PdeB mutants had a polar hook structure (about 55%) (Fig. 1C). In contrast, the number of cells with lateral flagellar hooks dropped by almost half (wild type, ~26%; \( \Delta pdeB \) strain, ~13%) (Fig. 1C). Under the conditions tested, the wild-type and \( \Delta pdeB \) mutant cells had highly similar swimming speeds (about 40 \( \mu \text{m} \cdot \text{s}^{-1} \)) and rates of directional switches (~0.24 turns per track), indicating that the absence of PdeB had little or no effect on the general function of the flagellar motor with respect to torque production and chemotaxis. However, we observed a drop in the population of actively moving cells (wild type, ~60%; \( \Delta pdeB \) strain, 30%).
Taken together, the results suggest that the phosphodiesterase PdeB affects the production of the lateral S. putrefaciens CN-32 flagellar system. In contrast, formation of the main polar flagellar system and its general function are not affected; however, an increased number of cells does not seem to rotate the existing flagellum in the absence of PdeB. Notably, PdeB appears to affect swimming motility only under conditions of complex medium.

**PdeB is recruited to the flagellated cell pole in dependence of HubP.** To determine the production and potential spatiotemporal organization of PdeB within the cells, we generated a hybrid gene encoding a C-terminal fusion of PdeB to a superfolder green fluorescent protein sfGFP (pdeB-sfgfp), which we integrated into the chromosome where it replaced the native gene. PdeB-sfGFP was stably produced and supported flagellum-mediated spreading through soft agar, albeit to a slightly lesser extent than wild-type PdeB (Fig. S2C to E). Fluorescence microscopy revealed that PdeB-sfGFP localized to the cell pole in 73% ± 5% of the population in both complex and mineral media (Fig. 2A). Colocalization with fluorescently labeled FliM1-mCherry (observed in 71% ± 3% of the cells), a component of the polar flagellar C ring, occurred in 87% ± 4% of the cells in which either PdeB-sfGFP or FliM1-mCherry was present, indicating that PdeB localizes to the flagellated cell pole, which also harbors the chemotaxis machinery (Fig. S3A).

Previous studies have shown that in P. aeruginosa, the PDE DipA or Pch is recruited to the flagellated cell pole by the chemotaxis histidine kinase CheA (40). Similar to S. putrefaciens PdeB, P. aeruginosa DipA/Pch also contains a C-terminal GGDEF-EAL domain and an upstream PAS sensor domain; however, Pch is predicted to be a cytoplasmic protein and possesses an additional GAF sensor domain in the N-terminal region (41). In S. putrefaciens, the chemotaxis cluster is also localized to the flagellated cell pole (11). Therefore, despite the differences in sequence and domain organization, PdeB may similarly be recruited by the chemotaxis machinery. To investigate whether the chemotaxis and/or flagellar complexes are required for PdeB localization in S. putrefaciens CN-32, we performed localization studies of PdeB-sfGFP in a strain in which the complete gene cluster encoding the primary flagellar gene cluster and the major components of the chemotaxis system, including CheA, was deleted (44). Also, in the absence of the flagellar basal body and the chemotaxis cluster, PdeB-sfGFP localized to the cell pole to a similar extent as in the wild type (Fig. 2A).

We then determined whether polar localization of PdeB-sfGFP might depend on the polar landmark protein HubP, which recruits the chemotaxis cluster to the appropriate...
Localization studies and Western immunoblotting of PdeB-sfGFP in a strain lacking HubP (ΔhubP pdeB-sfGFP strain) revealed that in the absence of HubP, PdeB-sfGFP is normally and stably produced (Fig. S3B) but loses its polar localization pattern (Fig. 2B). In S. putrefaciens CN-32, HubP localizes to both cell poles in a major cluster at the flagellated cell pole and in a minor cluster at the opposite cell pole. During cell growth and division, the minor HubP cluster enlarges, and when the cells start to divide, HubP is also recruited to the division plane to reform the small cluster at the new cell pole after completion of cell division and fission (11). Time-lapse microscopy revealed that PdeB-sfGFP appeared at the cell pole rather late during the cell cycle after completion of cell division and cell fission (Fig. S3C). Correspondingly, PdeB did not localize to the division plane or to both cell poles.

The results showed that PdeB localizes to the flagellated cell pole irrespective of the medium conditions. Localization occurs late during the cell cycle. PdeB polar recruitment depends on the polar landmark protein HubP but occurs independently of the flagellar or chemotaxis system, strongly indicating that PdeB is localized in a different fashion than DipA/Pch in P. aeruginosa.

**The GGDEF domain of PdeB is required for polar localization.** The polar localization of PdeB raised the question of the mechanism by which the protein is recruited. We therefore conducted a domain analysis on PdeB to identify the section or activity of the protein required for polar recruitment. To this end, we introduced in-frame deletions or substitutions into chromosomal pdeB-sfGFP genes that gave rise to PdeB-sfGFP variants in which single domains or several domains were missing or in which residues presumably required for activity were substituted for appropriately (Fig. 3C). Production of the modified proteins was determined by Western immunoblotting, and all variants were found to be stably produced at levels similar to the level of the wild-type protein (Fig. S4). The corresponding S. putrefaciens mutant strains were then
used for protein localization studies by fluorescence microscopy. We found that exclusively all PdeB variants lacking the GGDEF domain lost polar localization (Fig. 3A and Fig. S5A), while all other mutants retained robust polar localization patterns. This indicated that the presence of the domain, its potential c-di-GMP binding, or the potential DGC activity of this domain mediates polar recruitment of PdeB.

To further investigate this, we introduced substitutions into the GGDEF signature motif (PdeBD508A/E509A) that would render the domain unable to bind c-di-GMP (46, 47). The corresponding mutant protein still localized to the cell pole (62%) (Fig. S5A), suggesting that the presence rather than a potential DGC activity of the GGDEF domain is required for PdeB polar targeting. This finding also implied that the GGDEF domain alone might be recruited to the cell pole. We therefore constructed a plasmid from which a GGDEFPdeB-sfGFP fusion could be stably produced upon induction (Fig. S4), and the plasmid was introduced into the S. putrefaciens CN-32 ΔpdeB mutant strain. We observed that ectopically produced GGDEFPdeB-sfGFP readily localized to the cell pole in the presence, but not in the absence, of HubP (Fig. 3A and Fig. S5B), suggesting that the presence of the GGDEF domain is crucial and likely sufficient for HubP-dependent polar recruitment of PdeB. To further test this, we constructed a PdeB variant in which we replaced the native GGDEF domain with that of Sputcn32_2830, another transmembrane DGC/PDE in S. putrefaciens. The variant was stably produced, but no polar localization could be observed (Fig. S6), demonstrating that polar targeting of PdeB by HubP cannot be mediated by any GGDEF domain. In addition, the variant had a motility phenotype comparable to that of a PdeB deletion, suggesting that the protein either lost its in vivo function or that polar localization is required for full activity.

HubP has been demonstrated to also directly or indirectly affect flagellum-mediated motility in S. putrefaciens, e.g., by recruitment of the chemotaxis cluster and stabilizing the motor-affecting protein ZomB (11, 19) and likely other, yet unidentified, factors. To determine if HubP and PdeB swimming phenotypes are additive, we determined flagellum-mediated spreading in mutants lacking HubP, PdeB, or both (Fig. S7). In the wild-type background with both flagellar systems present, deletion of hubP or pdeB had highly similar effects and reduced spreading to about 40% to 50% of that of wild-type cells. The double deletion had an additive effect, indicating that both proteins mediate different aspects of swimming. In contrast, for cells equipped with the lateral flagellar system only (ΔflaA_2ΔflaB_2 strain), which is not addressed by the chemotaxis system or ZomB (19, 43) and for which, so far, no direct effect mediated by HubP is reported, no such additive effect was observed. The absence of HubP also reduced spreading to about 70% of that of the wild type and did not further reduce spreading by ΔpdeB mutants (both at about 50% of that of wild-type cells), indicating that for the lateral flagellar system, PdeB is epistatic to HubP. That the hubP deletion on the lateral system is mediated through partial loss of PdeB activity is possible, but this remains unclear.

HubP directly interacts with GGDEF<sub>PdeB</sub>. The previous results showed that HubP directly or indirectly recruits PdeB to the cell pole and that the GGDEF domain of PdeB has a crucial role for polar targeting of the full PdeB protein. We therefore determined if polar recruitment of GGDEF<sub>PdeB</sub> by HubP is mediated through direct protein-protein interactions. To this end, we employed bacterial two-hybrid (BACTH) analysis on GGDEF<sub>PdeB</sub> against the cytoplasmic repeat segment and the C-terminal FimV domain of HubP. The results indicated a pronounced direct interaction between GGDEF<sub>PdeB</sub> and the FimV domain when the two adenylate cyclase fragments (T25 and T18) were fused to the N terminus of GGDEF<sub>PdeB</sub> and the C terminus of FimV independent of the protein stoichiometries, which may be due to the orientation of the interaction of GGDEF<sub>PdeB</sub> and FimV. No interaction of GGDEF<sub>PdeB</sub> was seen with the repeat region of HubP (Fig. S8A). To rule out that interaction occurs with any GGDEF domain, we repeated the BACTH assay with the GGDEF domain of Sputcn32_0099; no interaction was observed (Fig. S8C).

To corroborate this finding, interaction of the two domains was determined using purified proteins. To this end, GGDEF<sub>PdeB</sub> was heterologously overproduced with an
N-terminal fusion to *Escherichia coli* maltose-binding protein E (MBP) and a C-terminal His tag (MBP-GGDEFPdeB-His; ~64 kDa) and subsequently purified. FimV was similarly produced fused with an N-terminal FLAG tag and a C-terminal His tag (FLAG-FimV-His; ~18 kDa). These proteins were then used for coimmunoprecipitation experiments (Fig. S8B). We found that FLAG-FimV-His was retained only when MBP-GGDEFPdeB-His, but not just MBP, was used as bait, strongly indicating specific interaction between the two domains. Furthermore, a mixture of both purified proteins separated by gel filtration eluted as a defined complex containing both MBP-GGDEFPdeB-His and FimV-His at a predicted molecular mass of about 150 kDa, which would correspond to an MBP-GGDEFPdeB-His/FimV-His ratio of 2:1 (Fig. 4A). Taken together, the results strongly indicate that polar interaction of PdeB and HubP involves direct interaction of the GGDEF and FimV domains. Accordingly, the polar localization of PdeB-sfGFP in a mutant bearing a HubP variant lacking the FimV domain was significantly decreased from 73% to 38% (*P* < 0.01). However, as some PdeB-sfGFP still localized to the cell pole, the protein might to some extent also directly or indirectly interact with other domains of HubP.

The physical interaction of GGDEFPdeB and FimVHubP suggested that the activity of PdeB may be affected by HubP. We therefore purified the cytoplasmic part of PdeB (PdeBcyt) fused N-terminally to MBP (42) and determined the in vitro PDE activity in the presence and absence of purified FLAG-FimV-His. PDE activity of MBP-PdeBcyt was readily detected (Fig. 4B). However, we did not observe a difference in the rate of c-di-GMP degradation in the presence or absence of FimV, indicating that interaction with FimV does not affect c-di-GMP turnover by PdeB in vitro. Accordingly, we found that the overall c-di-GMP level in the absence of HubP was not significantly different from that of wild-type cells, suggesting that PdeB-HubP interaction does not affect PDE activity of PdeB in vivo, at least not at the whole-cell level (Fig. 1D).

**DISCUSSION**

For a number of different bacterial species, FimV/HubP proteins are important factors for cell polarity. Although not essential for cell growth and division, they were shown to be involved in a number of diverse processes ranging from localization of chemotaxis clusters to normal chromosome segregation and mediating normal placement, assembly, and activity of motility or adhesion structures such as type IV pili and flagella (10–18, 48, 49). HubP is generally thought to mediate these functions by directly or indirectly recruiting the corresponding systems to the cell pole; however,
rather few proteins directly interacting with HubP have been identified so far. Here, we show that, in S. putrefaciens CN-32, HubP directly recruits a membrane sensor DGC/PDE hybrid protein, PdeB, to the flagellated cell pole.

**HubP recruits PdeB to the flagellated cell pole via the GGDEF \(_{\text{PdeB}}\) domain.** HubP-mediated polar localization specifically requires the GGDEF domain of PdeB. The involvement of GGDEF domains for specific targeting was previously shown for Caulobacter crescentus PopA, a protein which is involved in cell cycle control, and P. aeruginosa GbcC, which is involved in biofilm formation (50, 51). While the direct interaction partner interface for recruitment of PopA to the C. crescentus cell pole via the GGDEF domain remains to be identified, the GGDEF domain of GbcC is recognized by an EAL domain in the protein LapD, a c-di-GMP-responsive inner membrane domain. Here, we demonstrated by BACTH and in vitro protein-protein interaction studies that in S. putrefaciens direct interaction occurs between GGDEFPdeB and the FimV domain, which is located within the very C-terminal region of HubP. This C-terminal FimV domain is highly conserved among the FimV/HubP orthologs of different bacterial species. It is predicted to harbor a tetratricopeptide (TPR) protein-protein interaction motif (13), and the domain’s structure has been solved for P. aeruginosa FimV (14).

Although the FimV domain represents only a small C-terminal section of FimV/HubP proteins, it appears to be important for many of the processes mediated by these proteins. In P. aeruginosa the FimV C-terminal domain interacts with FimL, a protein involved in regulation of intracellular cAMP levels and normal type 4 pilus assembly (14, 15). The FimV domain of Vibrio cholerae HubP directly interacts with ParA1, required for normal segregation of chromosome 1, as shown by BACTH and coexpression studies (10). The same study strongly suggests that also the flagellar regulators FlhF and FlhG directly contact VcHubP via this C-terminal domain. Thus, the FimV domain appears to be capable of mediating contact with a diverse array of binding partners, and further studies will be required to elucidate the underlying molecular basics. Another interesting observation was that PdeB variants affected in the PDE activity (ΔEAL and E637A) displayed pronounced localization patterns in almost 90% of all cells, i.e., more than in wild-type PdeB, suggesting that the localization of PdeB may respond to the protein’s activity.

**The two S. putrefaciens flagellar systems are differently affected by a loss of PdeB.** Although S. putrefaciens harbors numerous proteins predicted to contribute to c-di-GMP production and turnover, PdeB has a huge impact on cellular c-di-GMP levels. In Shewanella, PdeB is a major mediator of motility and biofilm formation in response to environmental signals (42; also this work). Our results indicate that in the absence of PdeB, S. putrefaciens produces the same amount of polar flagella, and, accordingly, expression of the corresponding genes is not affected. This is in agreement with transcriptome analysis of a pdeB mutant in S. oneidensis MR-1, which also did not reveal any effect on polar flagellar gene transcription (42). In contrast to the main polar flagellar system, transcription of the secondary lateral flagellar system was significantly reduced in ΔpdeB mutants, and, accordingly, spreading in soft agar, which is assisted by the lateral filaments, was drastically reduced. An effect of the c-di-GMP concentration at the level of transcription is in accordance with earlier reports showing that the master regulator of flagellar synthesis, FlrA/FleQ, responds to cellular c-di-GMP levels in P. aeruginosa, V. cholerae, and S. putrefaciens (20, 24, 52, 53). Notably, S. putrefaciens CN-32 harbors two FlrA orthologs, one for each flagellar system, and further studies will show if and how the two FlrA regulators respond to changes in c-di-GMP levels.

Although the polar flagellum was normally produced, the number of actively swimming cells was reduced by approximately 50%. The cells that were actively swimming displayed a number of turns highly similar to those of wild-type cells; thus, chemotaxis appears not to be affected. For a number of bacterial species, such as E. coli, P. aeruginosa, or Bacillus subtilis, c-di-GMP-responsive flagellar brakes have been described previously (27–29, 31, 36). Upon c-di-GMP binding, these proteins interact with the flagellar motor to slow down the rotation and swimming speed. However, in S. putrefaciens we observed no decrease in swimming speed in the absence of PdeB, as
would be expected if the *S. putrefaciens* CN-32 motor would respond to such a c-di-GMP-dependent YcgR-like flagellar motor brake. Thus, these findings suggest a flagellar on/off switch rather than a velocity control for the *S. putrefaciens* polar flagellum, such as a motor clutch mechanism similar to that described for *Bacillus subtilis* EspA (54). The mechanism underlying the effect of c-di-GMP on the polar and lateral flagellar motors of *S. putrefaciens* requires further studies.

**Consequence of polar recruitment of PdeB.** The major question remaining is why HubP recruits PdeB to the flagellated cell pole. This is not easy to address as both the presence of the FimVHubP domain as well as the GGDEF_PdeB domain are required for the function of the corresponding proteins with respect to mediating effects on swimming motility. Therefore, PdeB cannot be simply and functionally detached from HubP and the cell pole to investigate the corresponding effects. One potential consequence of the interaction may be that HubP affects PdeB function. Although our *in vitro* analyses did not show such a function, it cannot be ruled out that this is different in an *in vivo* setting when PdeB perceives its signals, which are also yet unknown, during growth in complex medium. However, we also found that in a mutant lacking HubP, the c-di-GMP levels are only marginally different from those in wild-type cells, which would argue against such a role. In *P. aeruginosa*, recruitment to the flagellated pole of the PDE Pch by the chemotaxis kinase CheA generates an asymmetry in c-di-GMP levels between the cell bearing the flagellum and the new cell still in the process of flagellar assembly (40). Thus, the already flagellated cell has a lower level of c-di-GMP, resulting in more active movement than that of the nonflagellated cell. Since PdeB occurs only at the designated cell pole rather late after cell division and fission, such an asymmetry in c-di-GMP levels through PdeB in *Shewanella* is similarly conceivable and is, for the time being, the most obvious explanation for the observed polar recruitment.

Although our data on the lateral flagellar system strongly indicate that PdeB affects processes away from the cell pole, it may be speculated further that polar recruitment of PdeB to the flagellated pole of *S. putrefaciens* creates a local environment of c-di-GMP depletion to also affect processes other than flagellum-mediated motility. Such a function of localized c-di-GMP pools has been suggested to occur during regulation of polarity and chromosome replication in *C. crescentus* (55). In addition, recent studies on *E. coli* have provided evidence that proteins involved in c-di-GMP synthesis and turnover are organized into larger modules of complex hierarchical protein interaction networks to coordinate a major physiological output (56). It can be postulated that also in *S. putrefaciens* the many proteins potentially involved in c-di-GMP synthesis and turnover are similarly arranged in such modules. It is therefore possible that PdeB is only part of a polar regulatory cluster recruited by HubP to integrate external and internal signals into cellular c-di-GMP levels; however, this and the potential corresponding components and signals remain to be shown.

**MATERIALS AND METHODS**

**Strains, growth conditions, and media.** *Escherichia coli* strains were routinely grown in LB medium at 37°C if not indicated otherwise. *S. putrefaciens* CN-32 strains were cultivated in LB medium, lactate medium (LM) (10 mM HEPES, pH 7.5, 200 mM NaCl, 0.02% yeast extract, 0.01% peptone, 15 mM lactate), or 4M mineral medium (50 mM lactate, 25 mM HEPES, 150 mM NaCl, 1.27 mM K,HPO4, 0.73 mM KH2PO4, 9 mM (NH4)2SO4, 0.5 mM MgSO4, 485 μM CaCl2, 67.2 μM Na2EDTA, 1.3 μM MnSO4, 5.4 μM FeCl3, 5 μM CoCl2, 1 μM ZnSO4, 0.2 μM CuSO4, 56.6 μM H3BO3, 3.9 μM Na2MoO4, 5 μM NiCl2, 1.5 μM Na2SeO3, pH 7.4) at 30°C. When appropriate, media were supplemented with 50 μg ml−1 kanamycin or 10% (wt/vol) sucrose. Cultures of the *E. coli* conjugation strain WM3064 were supplemented with 2.6-diaminopimelic acid (DAP) to a final concentration of 300 μM. For plates, the appropriate medium was supplemented with 1.5% (wt/vol) agar.

**Strain constructions.** Strains and plasmids used in this study are summarized in Tables S1 and S2 in the supplemental material. DNA preparations and manipulations were carried out using appropriate kits (VWR International GmbH, Darmstadt, Germany) and enzymes (Fermentas, St Leon-Rot, Germany). The corresponding oligonucleotides (Sigma-Aldrich, Taufkirchen, Germany) used for cloning are listed in Table S3. Plasmids were constructed using standard restriction-ligation techniques or the Gibson assembly method (57). Plasmid delivery to *S. putrefaciens* CN-32 was performed by conjugation from *E. coli* WM3064.

To generate the strains deleted in genes or certain gene regions, 500- to 600-bp flanking regions were amplified and fused. For C-terminal fluorescent fusions of PdeB and its variants, flanking regions were amplified as described previously (58).

To generate the strains deleted in genes or certain gene regions, 500- to 600-bp flanking regions were amplified and fused. For C-terminal fluorescent fusions of PdeB and its variants, flanking regions were amplified as described previously (58).
upstream and downstream of the position were fused to a PCR product encoding one copy of a flexible GS linker and GFP. Delivery into S. putrefaciens occurred by markerless sequential crossover into the native site of the genes using pHTPS-138-R6K (58). To complement in-frame deletion mutants, the mutated locus was exchanged with the wild-type gene using the same sequential crossover approach. The same technique was also used to construct PdeB harboring the GGDEF domain of Sputcn32-2830, another transmembrane DGC/PDE. To this end, the corresponding fragment was introduced into the genomic ΔGGDEF deletion mutant of pdeB to replace the original GGDEF domain (residues 417 to 598) with that of Sputcn32-2830 (aa 237 to 410). Point mutations were introduced by the same method using the respective primer pair. Correct insertions or deletions were verified by PCR. Production levels and stability of fusion proteins were checked by immunofluorescence approaches and appropriate phenotypic analysis. In order to express a GFP-tagged version of the GGDEF domain of PdeB in S. putrefaciens, a DNA product encoding the GGDEF domain (residues 417 to 598 plus residues 846 to 859) was fused to sfGFP and cloned into the inducible overexpression plasmid pBTOK (11). The plasmid was then conjugated into CN-32 ΔpdeB. Background expression was already sufficient for localization studies. After 45 min the culture was harvested for subsequent fluorescence microscopy and Western blotting. For overproduction of the C-terminal FimV core of HubP (residues 970 to 1097) and the GGDEF domain of PdeB (residues 417 to 598 plus residues 846 to 859), the corresponding DNA segments were amplified from S. putrefaciens CN-32 genomic DNA. The sequence of a FLAG tag (3 copies) was fused N-terminally to FimV-C, and a MBP tag was fused N-terminally to the GGDEF domain of PdeB. The resulting PCR fragments were cloned into pET24a (Novagen) via Gibson assembly.

To construct a plasmid for lux-based transcriptional reporter fusions, gene fragments of the polar main flagellin flaB, and the lateral main flagellin flaA, were amplified using appropriate primers, yielding DNA fragments framed by EcoRI and PspOMI, which were subsequently cloned into pNPTS138-R6KT-lux(44). The resulting plasmids were conjugated into S.putrefaciens wild-type and ΔpdeB strains and integrated into the chromosome by a single-crossover event of the full vector.

Expression analysis by lux fusions. Transcriptional reporter assays using fusions to luxCDABE were carried out essentially as described in Bubendorfer et al. (44). Briefly, cells from overnight cultures were diluted in fresh LB medium to an optical density at 600 nm (OD$_{600}$) of 0.02 and grown to an OD$_{600}$ of 0.5. A total of 160 μl of the suspension was then transferred into a well of a white 96-well polypropylene microtiter plate (Greiner, Germany). Luminescence emission was measured using a Tecan Infinite M200 plate reader (Tecan, Switzerland) and correlated with the OD$_{600}$ determined at the corresponding time point. Measurements were performed in 4-fold repeats, and each experiment was conducted in biological triplicates.

Fluorescence microscopy. Prior to microscopy, strains and microorganisms were cultivated overnight in LB medium and subcultured in LB medium until they reached the exponential growth phase (OD$_{600}$ of ~0.2). Three microliters of culture was spotted on an agarose pad (LM medium solidified with 1% [wt/vol] agarose). Fluorescence images were recorded using a Leica DMI 6000 B inverse microscope (Leica, Wetzlar, Germany) equipped with an sCMOS camera and an HCX PL APO 100×/1.4 numerical-aperture objective using VisiView software (Visitron Systems, Puchheim, Germany). Images were further processed using ImageJ (to adjust contrast, change grayscale to color, define representative areas for display, and add scale bars) and Adobe Illustrator CS6 (to add an outline of cells where required and to create the final assembly of panels). Cells from at least three independent cultures were imaged. For quantification, at least 150 cells were scored for the truncation experiments (Fig. S5); for all other experiments, at least 350 cells were scored.

Flagellar hook staining. Fluorescent staining of flagellar hook structures (FlagE1/FlagE2-Cys) (59) was essentially carried out on exponentially growing cells as previously described (11, 60) using Alexa Fluor 488-maleimide (Molecular Probes, Life Technologies) prior to microscopy.

Fluorescence microscopy. Prior to microscopy, strains and microorganisms were cultivated overnight in LB medium and subcultured in LB medium until they reached the exponential growth phase (OD$_{600}$ of ~0.2). Three microliters of culture was spotted on an agarose pad (LM medium solidified with 1% [wt/vol] agarose). Fluorescence images were recorded using a Leica DMI 6000 B inverse microscope (Leica, Wetzlar, Germany) equipped with an sCMOS camera and an HCX PL APO 100×/1.4 numerical-aperture objective using VisiView software (Visitron Systems, Puchheim, Germany). Images were further processed using ImageJ (to adjust contrast, change grayscale to color, define representative areas for display, and add scale bars) and Adobe Illustrator CS6 (to add an outline of cells where required and to create the final assembly of panels). Cells from at least three independent cultures were imaged. For quantification, at least 150 cells were scored for the truncation experiments (Fig. S5); for all other experiments, at least 350 cells were scored.

Determination of swimming speed. Cells of S. putrefaciens CN-32 and S. putrefaciens CN-32 ΔpdeB from overnight cultures were used to inoculate LB medium to an OD$_{600}$ of 0.05 and cultivated for 3 to 4 h to an OD$_{600}$ of ~0.2. An aliquot of each culture was placed under a coverslip fixed by four droplets of silicone to create a space of 1- to 2-mm width. Movies of 12 s (157 frames) were taken with an inverse microscope (for specification, see above). Speeds of at least 200 cells per strain were determined using the MTrackJ plug-in of ImageJ. For determination of motile/nonmotile cells within the wild-type and ΔpdeB populations, at least 380 cells per strain were examined. At least three independent assays were scored.

Determination of in vivo protein-protein interactions by BACTH assay. To perform interaction studies, a bacterial adenylate cyclase two-hybrid BACTH system kit (61) (Euromedex) was used. According to the required procedure, the GGDEF domain of PdeB (residues 41 to 598 plus 846 to 859), the FimV domain of HubP (residues 970 to 1097), and the 10 amino acid repeats of HubP (residues 370 to 1009) were fused to the T25 and T18 fragments of Cy3A from Bordetella pertussis. To obtain N- and C-terminal fusions, as well as high and low expression levels of the proteins of interest, the plasmids pUT18, pUT18C, pKT25, and pKNT25 were used. The plasmids were then transformed into the E. coli BTH101 reporter strain in all eight possible combinations. The pKNT25-zip and pUT18C-zip plasmids were used as positive
controls, while the empty vectors were used as negative controls. The cells were then grown overnight at 37°C in LB medium containing the appropriate antibiotics. Following this, 10 μl of each culture was spotted onto LB agar plates containing isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 80 μg ml⁻¹), kanamycin (50 μg ml⁻¹), and ampicillin (100 μg ml⁻¹). The samples were then incubated again at 30°C until they were visibly blue.

**Protein production and purification.** For protein production, *E. coli* BL21(DE3) (New England Biolabs, Frankfurt, Germany) cells carrying the pET24C overexpression plasmids were grown in LB medium, supplemented with kanamycin (50 μg ml⁻¹), to an OD₆₀₀ of 0.3. After induction with IPTG (0.1 mM) or β-(+)-lactose-mono-hydrate (12.5 g liter⁻¹), the cultures were incubated overnight at 15°C under rigorous shaking. After cells were harvested by centrifugation (5,000 rpm, 20 min, 4°C), the pellet was resuspended in HEPES buffer (20 mM HEPES, pH 8.0, 250 mM NaCl, 20 mM MgCl₂, and 20 mM KCl) for pulldown experiments or in Tris-HCl buffer (50 mM Tris-HCl, 250 mM NaCl, 25 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.01% sodium azide, 5% glycerol, pH 7.5) for degradation assay, supplemented with 40 mM imidazole (buffer A). Subsequently, the cell suspension was lysed by sonication (Bandelin Sonoplus), cell debris was removed by centrifugation (20,000 rpm, 30 min, 4°C), and the supernatant was filtered and loaded on a 1-ml or 5-ml HisTrap column (GE Healthcare) equilibrated with the corresponding protein buffers. Protein concentration was determined by a spectrophotometer (NanoDrop Lite, Thermo Scientific). For storage, the glycerol concentration was elevated to 20%, and the protein was snap-frozen in liquid nitrogen and stored at 20°C.

**Protein pulldown experiments.** To perform pulldown assays, the proteins of interest were purified, and a His-tagged MBP was used as a negative control. The MBP-tagged GGDEF domain of PdeB and the MBP control were diluted with SEC buffer (20 mM HEPES-Na [pH 7.5], 200 mM NaCl, 20 mM MgCl₂, and 20 mM KCl), and 5 nmol was incubated overnight with amylose resin beads (NEB) at 4°C. The samples were then washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany). The beads were washed with SEC buffer and subsequently incubated with 150 nmol of the purified FimV (Bandelin Sonoplus, Germany).
Quantification of cyclic dinucleotides by LC-MS/MS. The chromatographic separation was performed on an LC-10AD series high-performance liquid chromatography (HPLC) system (Shimadzu) as described previously (64). Cyclic dinucleotide detection and quantification were performed on an API 4000 triple quadrupole mass spectrometer equipped with an electrospray ionization source (AB Sciex) using selected reaction monitoring (SRM) analysis in positive-ionization mode. The most intense SRM transitions ("quantifiers") were used to quantify the compound of interest, whereas "identifier" SRM transitions were monitored as confirmatory signals.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/ JB.00534-18.

SUPPLEMENTAL FILE 1, PDF file, 2.3 MB.

ACKNOWLEDGMENTS
This study was supported by the Deutsche Forschungsgemeinschaft within the framework of priority program SPP1879 to K.M.T. and G.B. We are grateful to Ulrike Ruppert for excellent technical support. We declare that we have no conflict of interests.

REFERENCES
April 2019 Volume 201 Issue 7 e00534-18 www.jb.asm.org

Rossmann et al. Journal of Bacteriology

Pseudomonas regulates swimming motility and biofilm formation in Shewanella oneidensis (PA5017) is essential for increasing the directional persistence of swimming. Proc Natl Acad Sci U S A 111:11485–11490. https://doi.org/10.1073/pnas.1405820111.


