Shewanella oneidensis MR-1, a gammaproteobacterium with respiratory versatility, forms biofilms on mineral surfaces through a process controlled by the cyclic dinucleotide messenger c-di-GMP. Cellular concentrations of c-di-GMP are maintained by proteins containing GGDEF and EAL domains, which encode diguanylate cyclases for c-di-GMP synthesis and phosphodiesterases for c-di-GMP hydrolysis, respectively. The S. oneidensis MR-1 genome encodes several GGDEF and EAL domain proteins (50 and 31, respectively), with a significant fraction (~10) predicted to be multidomain (e.g., GGDEF-EAL) enzymes containing an additional Per-Arnt-Sim (PAS) sensor domain. However, the biochemical activities and physiological functions of these multidomain enzymes remain largely unknown. Here, we present genetic and biochemical analyses of a predicted PAS-GGDEF-EAL domain-containing protein, SO0437, here named PdeB. A pdeB deletion mutant exhibited decreased swimming motility and increased biofilm formation under rich growth medium conditions, which was consistent with an increase in intracellular c-di-GMP. A mutation inactivating the EAL domain also produced similar swimming and biofilm phenotypes, indicating that the increase in c-di-GMP was likely due to a loss in phosphodiesterase activity. Therefore, we also examined the enzymatic activity of purified PdeB and found that the protein exhibited phosphodiesterase activity via the EAL domain. No diguanylate cyclase activity was observed. In addition to the motility and biofilm phenotypes, transcriptional profiling by DNA microarray analysis of biofilms of pdeB (in-frame deletion and EAL) mutant cells revealed that expression of genes involved in sulfate uptake and assimilation were repressed. Addition of sulfate to the growth medium resulted in significantly less motile pdeB mutants. Together, these results indicate a link between c-di-GMP metabolism, S. oneidensis MR-1 biofilm development, and sulfate uptake/assimilation.

PdeB, a Cyclic Di-GMP-Specific Phosphodiesterase That Regulates Shewanella oneidensis MR-1 Motility and Biofilm Formation

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Shewanella oneidensis MR-1, a gammaproteobacterium with respiratory versatility, forms biofilms on mineral surfaces through a process controlled by the cyclic dinucleotide messenger c-di-GMP. Cellular concentrations of c-di-GMP are maintained by proteins containing GGDEF and EAL domains, which encode diguanylate cyclases for c-di-GMP synthesis and phosphodiesterases for c-di-GMP hydrolysis, respectively. The S. oneidensis MR-1 genome encodes several GGDEF and EAL domain proteins (50 and 31, respectively), with a significant fraction (~10) predicted to be multidomain (e.g., GGDEF-EAL) enzymes containing an additional Per-Arnt-Sim (PAS) sensor domain. However, the biochemical activities and physiological functions of these multidomain enzymes remain largely unknown. Here, we present genetic and biochemical analyses of a predicted PAS-GGDEF-EAL domain-containing protein, SO0437, here named PdeB. A pdeB deletion mutant exhibited decreased swimming motility and increased biofilm formation under rich growth medium conditions, which was consistent with an increase in intracellular c-di-GMP. A mutation inactivating the EAL domain also produced similar swimming and biofilm phenotypes, indicating that the increase in c-di-GMP was likely due to a loss in phosphodiesterase activity. Therefore, we also examined the enzymatic activity of purified PdeB and found that the protein exhibited phosphodiesterase activity via the EAL domain. No diguanylate cyclase activity was observed. In addition to the motility and biofilm phenotypes, transcriptional profiling by DNA microarray analysis of biofilms of pdeB (in-frame deletion and EAL) mutant cells revealed that expression of genes involved in sulfate uptake and assimilation were repressed. Addition of sulfate to the growth medium resulted in significantly less motile pdeB mutants. Together, these results indicate a link between c-di-GMP metabolism, S. oneidensis MR-1 biofilm development, and sulfate uptake/assimilation.

the bacterial second messenger cyclic di-GMP (c-di-GMP) has become the focus of intense research for its role in controlling biofilm formation, motility, virulence, and cell cycle progression (1–13). The modes of its regulatory activity are diverse, and this regulation can occur at the transcriptional (e.g., FleQ [14]), post-transcriptional (e.g., GEMM riboswitch [15]), or posttranslational (e.g., cellulose synthase [16–18]) level, via binding to specific effector proteins whose (regulatory) activity is modulated by c-di-GMP. Thus far, most physiological processes that have been demonstrated to be regulated by c-di-GMP do not control essential cellular functions, and no control of central metabolic processes has been reported.

C-di-GMP is synthesized by diguanylate cyclases (DGC), characterized by a canonical GGDEF amino acid sequence motif and by condensation of two molecules of GTP, and then hydrolyzed by phosphodiesterases (PDE), characterized by conserved EAL or HD-GYP amino acid sequence motifs to GpG or two molecules of GMP, respectively (7, 19–22). Based on the amino acid sequences conserved in DGCs and PDEs, bacterial genomes are predicted to have highly variable numbers of genes encoding these enzymes, and their abundance appears to roughly correlate with genome size and environmental versatility of the microorganism (23, 24). The complexity of the c-di-GMP regulatory network is indicated further by the presence of multiple multidomain proteins containing both GGDEF and EAL or HD-GYP domains, as well as additional sensor domains, such as Che, Per-Arnt-Sim (PAS), and NIT domains (23). The presence of the associated sensor domains suggests that c-di-GMP signaling targets physiological processes other than those currently known. When both GGDEF and EAL domains are present, one of the two domains is often either inactive or acts as a regulatory domain that changes the activity of the other domain upon allosteric activation by GTP or c-di-GMP (6, 25). For example, the GGDEF domain of PdeA from Caulobacter crescentus binds GTP and allosterically activates the EAL domain; the GGDEF domain in this protein has the slightly altered amino acid sequence GEDEF and is incapable of catalyzing the formation of c-di-GMP (25).

Shewanella oneidensis MR-1 is a Gram-negative gammaproteobacterium with respiratory versatility that is of considerable interest for applications in bioremediation and microbial fuel cells, partly because of its ability to form biofilms on mineral surfaces (26–28). Biofilm formation in S. oneidensis MR-1, which has been studied in detail, is also controlled by c-di-GMP (29–31), but little is known about the metabolism and targets of c-di-GMP in this microorganism in general. The S. oneidensis MR-1 genome is predicted to encode several GGDEF- and EAL-domain proteins (50 and 31, respectively), with a significant fraction predicted to be multidomain (e.g., GGDEF-EAL domain-containing) enzymes. Ten of those multidomain proteins contain additional Per-Arnt-Sim (PAS) sensory domains, which are important signaling modules previously shown to respond to changes in environmental or
cellular cues, including light, redox state, and oxygen in a wide range of organisms. Alternatively, PAS domains may mediate protein-protein interactions or bind other small ligands (32, 33). In this work, we describe the characterization of the PAS-GGDEF-EAL protein SO0437, renamed PdeB (for Phosphodiesterase Biofilm). We demonstrate that PdeB is a c-di-GMP-hydrolyzing protein-protein interaction mutant. The ΔpdeB deletion mutant was constructed as previously described (34). Briefly, the upstream and downstream regions of the pdeB open reading frame were PCR amplified from wild-type (WT; AS579) genomic DNA and subsequently joined using overlap extension PCR. The fusion product was ligated into pDS3.0 via the Smal restriction site and transformed into E. coli S17-λpir. The resulting plasmid, pDS3.0-ΔpdeB, was also verified by sequencing.

Following confirmation, pDS3.0-ΔpdeB was transformed into AS579 through biparental mating on an LB agar plate. After 8 h of incubation, the mating mix was resuspended in 4M liquid media and subsequently plated on 4M agar plates containing 5 µg/ml gentamicin. Colonies were screened for integration of pDS3.0-ΔpdeB into the chromosome using PCR primers flanking the recombination region. The strain with pDS3.0-ΔpdeB integrated into the chromosome was grown in LB medium (without NaCl) and then plated on LB (without NaCl) agar plates supplemented with 10% sucrose. The resulting colonies were patched onto LB plates containing 10 µg/ml gentamicin to confirm the loss of plasmid. The gene deletion was confirmed by PCR and DNA sequencing.

To complement the mutant, the wild-type gene was reintroduced at the chromosomal pdeB locus by gene replacement as an exact restoration of the original gene. This was done similarly to the method described above, except that the pdeB wild-type gene and its flanking regions were cloned into pDS132, resulting in pDS32-ΔpdeB (35). The mating was performed using E. coli strain WM3064, and the mating mix was plated on LB plates containing 15 µg/ml chloramphenicol to select for integration of the plasmid into the chromosome.

The pdeB EAL domain mutant (pdeBmut; E634A) was constructed by generating the mutation in pDS132-ΔpdeB via QuikChange site-directed mutagenesis (Agilent). The mutated pdeB allele was then introduced into the ΔpdeB mutant at the pdeB locus, as described for the complementation strain used above.

For biofilm studies, the strains were labeled with constitutively expressed green fluorescent protein (GFP) using the Tn7 delivery system as previously described (30). Briefly, GFP-expressing strains were constructed by triparental mating of the S. oneidensis MR-1 strain with AS262 and AS392 harboring the Tn7-egfp plasmid. The resulting gentamicin-resistant strains exhibited growth and biofilm phenotypes similar to those of the untagged strains.

**MATERIALS AND METHODS**

**Growth conditions and media.** The strains used in this study are summarized in Table 1. *Escherichia coli* and *S. oneidensis* MR-1 strains were grown at 37 and 30°C, respectively.

Construction of the mutants was carried out in Luria-Bertani (LB) or minimal medium (4M) | 485 µM CaCl₂ | 2H₂O, 5 µM CoCl₂, 0.2 µM CuSO₄ · 5H₂O, 57.5 µM H₂O₂, 1.27 mM K₃HPO₄, 0.73 mM KH₂PO₄, 1.0 mM MgSO₄ · 7H₂O, 1.3 mM MnSO₄, 67.2 µM Na₂EDTA, 3.9 µM Na₃MoO₄ · 2H₂O, 1.5 µM Na₂SeO₃, 150 mM NaCl, 2 mM NaHCO₃, 5 µM NiCl₂ · 6H₂O, 1 µM ZnSO₄, 9 mM (NH₄)₂SO₄, 20 mM lactate, and 5 mM HEPES (pH 7.4).

Swim plate experiments were carried out in LB or lactate medium (LM) (0.02% [wt/vol] yeast extract, 0.01% [wt/vol] peptone, 10 mM HEPES [pH 7.4], 10 mM NaHCO₃, 0.5 mM lactate) plates solidified with 0.25% (wt/vol) agar. All swim plate experiments were conducted in quadruplicate.

Flow chamber-grown biofilm experiments were carried out as previously described (30) in LM. All biofilm characterizations were conducted in triplicate.

Samples collected for microarray and reverse transcription-quantitative PCR (RT-qPCR) analyses were collected from flow chamber-grown biofilms (described below).

*E. coli* cultures for protein expression were carried out in maximal induction medium (MIM) | 3.2% tryptone, 2% yeast extract, 33.5 mM Na₂HPO₄ · 17.5 mM KH₂PO₄, 5.6 mM NaCl, 18.7 mM NH₄Cl, 1 mM MgSO₄ · 0.1 mM CaCl₂ | 44.

**Strain construction in S. oneidensis MR-1.** All genetic work was carried out according to standard protocols. Kits for isolation and/or purification of DNA were obtained from Promega, and enzymes were purchased from New England BioLabs (NEB). Table 1 describes strains used; also see Table S1 in the supplemental material for plasmids used and Table S2 for primers used in this study.

**TABLE 1 Strains used in this study**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Construct*</th>
<th>Relevant genotype or description</th>
<th>Reference or source</th>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α-λpir</td>
<td>q80ΔlacZΔM15 ΔlacZYA-argF</td>
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<tr>
<td>WM3064-λpir</td>
<td>thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-1360 Δ(araBAD)567 ΔdualA1341::erm pir</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>S17-λpir</td>
<td>thi pro recA hsdR (RP4-2C:mKm::Tn7)λpir; Tcp Smr⁷</td>
<td></td>
<td>43</td>
</tr>
<tr>
<td>Tuner(DE3)pLysS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS977</td>
<td>PdeB⁺</td>
<td>pLIC-SO0437 in Tuner(DE3)pLysS</td>
<td>This work</td>
</tr>
<tr>
<td>AS978</td>
<td>PdeBmut⁺</td>
<td>pLIC-SO0437(E364A) in Tuner(DE3)pLysS</td>
<td>This work</td>
</tr>
<tr>
<td>AS262</td>
<td>E. coli S17-λpir with pUX-BF13 (helper plasmid for Tn7 transposon); Amp⁷</td>
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<td>44</td>
</tr>
<tr>
<td>AS392</td>
<td>E. coli S17-λpir with pGP704-mTn7-egfp; Genr</td>
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<td><strong>S. oneidensis</strong></td>
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<td></td>
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<tr>
<td>AS579</td>
<td>WT</td>
<td>Shewanella oneidensis MR-1 (PNNL strain)</td>
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</tr>
<tr>
<td>AS979</td>
<td>ΔpdeB</td>
<td>AS579 ΔpdeB (markerless in-frame deletion)</td>
<td>This work</td>
</tr>
<tr>
<td>AS980</td>
<td>pdeB⁺</td>
<td>AS579 ΔpdeB plus pdeB⁺ (chromosomal replacement)</td>
<td>This work</td>
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<tr>
<td>AS981</td>
<td>pdeBmut⁺</td>
<td>AS579 ΔpdeB plus pdeBmut⁺ (E634A)</td>
<td>This work</td>
</tr>
<tr>
<td>AS982</td>
<td>WT</td>
<td>AS979 chromosomally tagged with GFP; Genr</td>
<td>This work</td>
</tr>
<tr>
<td>AS983</td>
<td>ΔpdeB</td>
<td>AS979 chromosomally tagged with GFP; Genr</td>
<td>This work</td>
</tr>
<tr>
<td>AS984</td>
<td>pdeBmut⁺</td>
<td>AS981 chromosomally tagged with GFP; Genr</td>
<td>This work</td>
</tr>
</tbody>
</table>
| * An asterisk indicates a protein.
Image acquisition and processing. Microscopic visualization of biofilms was performed on an upright Zeiss LSM510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) equipped with the following objectives: x10 magnification, 0.3-W Plan-Neofluar; x20 magnification, 0.5-W Achromplan; and x40 magnification, 1.2-W C-Apochromat. Biofilm parameters, such as biofilm mass and average biofilm thickness, were quantified with the COMSTAT program (36). Image data obtained were further processed by using the IMARIS software package (Bitplane AG, Zürich, Switzerland).

Expression and purification of MBP-His\(_f\)_fusion protein. The pLIC-HMK vector (gift from J. Berger) was used to express PdeB protein (residues 260 to 856) and its EAL domain mutant (E634A) form as fusion proteins with an N-terminal maltose-binding protein (MBP) tag and a C-terminal His\(_f\) tag. Site-directed mutations in PdeB were generated using QuikChange site-directed mutagenesis (Agilent). The sequence changes were confirmed by DNA sequencing.

The pLIC-pdeB plasmid was transformed into E. coli Tuner(DE3)pLysS cells (EMD Chemicals) for protein expression. A single colony was used to inoculate LB medium containing 50 \(\mu\)g/ml kanamycin and then grown with shaking at 37°C. After overnight growth, two shaker flasks containing 1 liter MIM containing 0.5% glucose and 50 \(\mu\)g/ml kanamycin were inoculated with 10 ml of the overnight culture. The culture was grown at 250 rpm at 37°C to an optical density at 600 nm (OD\(_{600}\)) of 0.3. The cultures then cooled to 16°C, and expression was induced by the addition of 0.5 mM isopropyl-\(\beta\)-1-thiogalactopyranoside (IPTG). The cultures were shaken for another 18 hours before the cells were harvested by centrifugation. The cells were then resuspended in buffer A (10 ml of 50 mM Na\(_2\)HPO\(_4\) [pH 8.0], 300 mM NaCl, 5 mM \(\beta\)-mercaptoethanol [BME], and 5% glycerol) containing 100 \(\mu\)M phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and DNase I before being stored at −80°C.

Nickel and amylase affinity chromatography were used for protein purification. The frozen cell suspensions were thawed, lysed with a French pressure cell press (SLM-Aminco), and pelleted at 140,000 \(\times\) g for 45 min. After centrifugation, the supernatant was applied to a 1-ml column of nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen) equilibrated with buffer A. The column was washed with five volumes of buffer A and one column volume of buffer A containing 40 mM imidazole and 1 M NaCl. Bound protein was eluted with 500 mM imidazole in buffer A (buffer B). The eluted protein was then applied to a 1-ml column of amylose resin (New England BioLabs) equilibrated with buffer B. The column was washed with five volumes of buffer B and five column volumes of buffer C (10 mM morpholinepropanesulfonic acid [MOPS]; pH 7.6, 150 mM NaCl, 10 mM Mg\(_2\)Cl, and 10% glycerol) containing 5 mM BME. Bound protein was eluted with 15 mM maltose in buffer C. The eluted protein was concentrated to 1 ml using a Vivaspin-6 10K filter (Vivasience) before it was buffer exchanged with buffer D (10 ml of the overnight culture of S. oneidensis MR-1) biofilms using TRIzol reagent (Invitrogen). The culture was grown at 250 rpm at 37°C to an optical density at 600 nm (OD\(_{600}\)) of 0.3. The cultures then cooled to 16°C, and expression was induced by the addition of 0.5 mM isopropyl-\(\beta\)-1-thiogalactopyranoside (IPTG). The cultures were shaken for another 18 hours before the cells were harvested by centrifugation. The cells were then resuspended in buffer A (10 ml of 50 mM Na\(_2\)HPO\(_4\) [pH 8.0], 300 mM NaCl, 5 mM \(\beta\)-mercaptoethanol [BME], and 5% glycerol) containing 100 \(\mu\)M phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and DNase I before being stored at −80°C.

Radioactively labeled c-di-GMP was enzymatically synthesized from \([\alpha,\beta\rceil^32P\rceil\)GTP using the TM1788 DGC from T. maritima (tDGC), which was expressed and purified as previously described (37). Reaction mixtures incubated for 3 hours at 30°C were boiled for 5 min and centrifuged to remove the precipitated tDGC, and the supernatant was applied to a 10K Ultrafree 0.5-ml filter (Millipore) to remove any leftover protein. The flowthrough was then used in PDE activity assays as described above.

RNA purification and cDNA synthesis. Total RNA was purified from 24-h S. oneidensis MR-1 biofilms using TRizol reagent (Invitrogen). Cells from L-M-grown flow chamber biofilms were harvested and lysed in TRizol, and total RNA was isolated according to the recommendations of the manufacturer. DNA was digested with amplification-grade DNase I (Invitrogen). The digests were cleaned using the RNeasy Mini kit (Invitrogen), and the integrity of the RNA samples was evaluated using a 2100 Bioanalyzer (Agilent). cDNA was synthesized using the RevertAid first-strand cDNA synthesis kit and random hexamer primer (Fermentas).

Gene expression profiling. Labeling of cDNA with cy3-dUTP or cy5-dUTP dyes (Invitrogen) and microarray hybridization were performed according to the Agilent gene expression oligonucleotide microarray protocol (two-color microarray-based gene expression analysis; Agilent Technologies). A dye swap was performed to account for dye incorporation artifacts. Competitive cDNA hybridization was performed on an Agilent 44,000-probe custom oligonucleotide DNA microarray. Each gene in the S. oneidensis MR-1 genome was represented by three unique probes, for a total of 13,180 60-mer probes, representing 4,648 genes. Microarrays were scanned with a GenePix 400A instrument (Axon Instruments) using GenePix 5.0 software. Normalization was executed with the Agilent feature extraction software. Microarray data were analyzed using GeneSpring software (Agilent) and filtering based on \(P < 0.05\) and a fold change (FC) of 2 or greater. Data are based on one microarray experiment performed with three biological replicates each of the wild type (WT) and the \(\Delta pdeB\) mutant.

qPCR. Quantitative analyses of transcripts were carried out with an IQ SYBR green supermix (Bio-Rad) and iCycler IQ real-time PCR detection system (Bio-Rad). The genes examined and primers used are listed in Table S1 in the supplemental material. Samples were analyzed in triplicate. Data analysis was performed as previously described (38). SO0011 (gyrB) and SO1126 (dnaK) were used for normalization; data for gyrB are shown (see Table 3).

RESULTS AND DISCUSSION

A gene search (Pfam entries GGDEF, EAL, and PAS) against the S. oneidensis MR-1 genome database (Joint Genome Institute Integrated Microbial Genomes; as of May 2011) identified a gene, SO0437, which we name here pdeB, as encoding a putative diguanylate cyclase/phosphodiesterase (DGC/PDE) with a PAS sensor domain (Swiss-Prot) (Fig. 1A). Primary amino acid sequence analysis indicated that both GGDEF and EAL domains appeared to be intact and that the protein also contained two transmembrane domains (positions 7 to 29 and 230 to 252; TMHMM2), suggesting that PdeB is a transmembrane protein.

In order to gain insights into the in vivo function of PdeB, an in-frame chromosomal deletion of pdeB (\(\Delta pdeB\) mutant; AS979) was constructed. We observed no difference in growth rate between the \(\Delta pdeB\) and wild-type (WT) strains in either rich or minimal medium (data not shown). However, the \(\Delta pdeB\) mutant was observed to be less motile than the WT when grown on rich agar plates (Fig. 2A and C), but this was not observed on minimal media (Fig. 2B). The swim diameter of the mutant was ~50% that of the WT. This motility phenotype was rescued when a wild-type copy of the pdeB allele was reintroduced at the chromosomal locus by gene replacement (pdeB\(^{+}\)) (Fig. 2). We also analyzed the \(\Delta pdeB\)
mutant under hydrodynamic biofilm conditions. As Fig. 3 shows, the ΔpdeB mutant formed thicker biofilms with approximately twice as much biomass as the WT when grown under semirich medium conditions (Table 2). These observations suggest that a pdeB deletion mutant results in an increase in intracellular c-di-GMP concentration. An increase has been previously shown to result in thicker S. oneidensis MR-1 biofilms (29).

The motility and biofilm phenotypes of the ΔpdeB mutant suggested that there was an increase in intracellular c-di-GMP concentration in S. oneidensis MR-1. In order to determine the domain(s) of the PdeB protein that may contribute to this intracellular increase in c-di-GMP, we examined whether PdeB showed PDE and/or DGC activity in vitro and whether the ΔpdeB mutant phenotype was due to a c-di-GMP-related activity of PdeB. Briefly, a truncated form of PdeB (residues 260 to 856) containing the PAS, GGDEF, and EAL domains but lacking the transmembrane segment was expressed and purified with an N-terminal MBP tag as well as a C-terminal His6 tag (MBP-PdeB(260-856)-His6) (Fig. 1B). The MBP tag allowed for higher-yield expression of soluble protein and purification by amyllose-affinity chromatography, whereas the C-terminal His6 tag allowed for purification by nickel-affinity chromatography. All protein constructs made in this study were purified to >95% homogeneity as assessed by SDS-PAGE (data not shown). DGC and PDE activities were assayed by incubating the purified protein with radioactively labeled substrates (e.g., [α-32P]-GTP and [32P]c-di-GMP) and then by separating the reaction products on a TLC plate. Incubation of PdeB with [α-32P]GTP did not result in formation of a detectable (limit, ~2 × 10^{-15} mol) TLC spot corresponding to c-di-GMP or pGpG (data not shown), suggesting that the purified protein did not carry DGC activity. In contrast, after a 30-min incubation for assaying PDE activity using [32P]c-di-GMP as the substrate, >50% of the radioactivity was recovered at a spot corresponding to pGpG, indicating that PdeB exhibited PDE activity in vitro (Fig. 4). We estimate the specific activity of this PdeB construct to be approximately 0.6 μmol c-di-GMP hydrolyzed per mg protein per min. Additionally, a PdeB protein carrying an E634A mutation in the EAL domain, MBP-PdeB(E634A)-His6, was also purified and assayed for PDE activity. As shown in Fig. 4, the alanine substitution for the glutamate residue in the EAL motif was sufficient to eliminate the in vitro PDE activity of PdeB. No DGC activity was observed with this protein (data not shown). Together, these biochemical data demonstrate that PdeB has PDE activity and likely acts as a phosphodiesterase in vivo. Although amino acid sequence comparisons to other biochemically characterized DGGs suggest that PdeB had an intact GGDEF domain, no in vitro DGC activity was observed even when the EAL domain was inactivated. It is possible that the GGDEF domain of PdeB has no catalytic role but a regulatory role, and that it is involved in the allosteric regulation of the EAL domain. It is also possible that the removal of the N-terminal transmembrane portion of the protein and/or the addition of the N-terminal MBP tag resulted in the loss or inhibition of DGC activity. Further experiments would be required to resolve this issue.

In order to determine whether the observed PDE activity of PdeB was relevant to the observed in vivo ΔpdeB mutant phenotypes, we also constructed the corresponding EAL mutant (pdeBeal) (AS981; GFF-tagged pdeBeal, AS984) and compared its
swimming motility and biofilm phenotypes to those of the ΔpdeB mutant. The mutated allele was introduced at the chromosomal locus of the ΔpdeB mutant through homologous recombination. The pdeBmutant displayed both swimming motility and biofilm phenotypes that were similar to those of the ΔpdeB mutant (Fig. 2 and 3), demonstrating that all of these observed phenotypes were consistent with PdeB acting as a phosphodiesterase in vivo.

In addition to the EAL and GGDEF domains, PdeB also contains a predicted PAS sensor domain. Many GGDEF and EAL domains are associated with putative sensor domains for perceiving various environmental signals (23). However, the characterization of these sensor domains and their associated signals has yet to be examined in S. oneidensis MR-1. Found in all domains of life, PAS domains are best known for perceiving changes in light, oxygen concentration, and redox potential via a linked flavin or heme cofactor (32, 39). However, sequence analyses of the PAS domain associated with PdeB suggest that it contained no conserved residues for binding either a flavin or heme cofactor (32, 39). Indeed, no flavin or heme was found associated with the purified PdeB protein, and no differences in motility and biofilm phenotype were observed in the pdeB mutant under aerobic and anaerobic growth conditions.

Therefore, in order to identify potential environmental signals that PdeB may perceive, we also examined the global transcriptional profiles of ΔpdeB mutant and wild-type cells in biofilms under semirich growth conditions by competitive hybridization of total labeled RNA using custom-designed DNA microarrays. Briefly, total RNA was extracted from 24-h biofilms of each strain, reverse transcribed, and subjected to microarray analysis. The DNA microarray included three probes targeting each and every gene of the S. oneidensis MR-1 genome. While most genes in the mutant did not show a significant (FC, >2; P < 0.05) difference in expression, we found that a set of genes related to sulfate uptake and assimilation was differentially expressed in the ΔpdeB mutant relative to that of the WT (see Table S2 in the supplemental material). These genes include those that encode some of the subunits of the major sulfate ABC transporter (cysT-2 and cysP) required for uptake of sulfate, as well as several enzymes that catalyze the activation of sulfate and its assimilation into cysteine (cysK and cysM, cysteine synthase subunits A and B; cysN and cysD, sulfate adenylyltransferase subunits 1 and 2) (see Fig. S1 in the supplemental material).

We also used quantitative PCR analyses to accurately determine that these genes were downregulated (~2-fold) in the ΔpdeB mutant versus the WT strain (Table 3). The expression of these genes was also measured in the pdeBmutant. Interestingly, we observed similar differences in expression between ΔpdeB and pdeBmutant biofilms, suggesting that c-di-GMP metabolism and sulfate uptake and assimilation are linked (Fig. 3 and 5 and Table 3).

Because we observed the downregulation of genes involved in sulfate transport/assimilation from cells harvested from ΔpdeB and pdeBmutant biofilms, we hypothesized that PdeB activity is controlled by sulfate. In order to test this, we examined the swimming motility phenotype of the mutants when the growth medium was amended with additional sulfate (e.g., Na2SO4 and NH4SO4). Sulfate was added to semirich medium (LM) instead of limiting it in defined minimal medium (4M), because no detectable swimming motility phenotype was observed in 4M minimal medium, which contained ~9 to 10 mM sulfate (Fig. 2B). Swimming motility was tested in the WT, the ΔpdeB mutant, the mutant complemented with the WT allele (pdeB+), and the pdeBmutant. Sulfate (40 mM Na2SO4 or NH4SO4) was added to the swim plates and the relative difference between the WT and mutant motility was measured, where relative difference represents the difference between the WT and mutant swimming diameters divided by the WT swimming diameter. In the absence of additional sulfate, the swimming diameters of the ΔpdeB mutant and

TABLE 2 COMSTAT analysis of S. oneidensis MR-1 biofilms

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biomass (μm²/μm²)</th>
<th>Thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.15 ± 1.46</td>
<td>10.18 ± 2.43</td>
</tr>
<tr>
<td>ΔpdeB mutant</td>
<td>13.81 ± 1.20</td>
<td>22.77 ± 3.13</td>
</tr>
<tr>
<td>pdeBmutant</td>
<td>12.33 ± 1.59</td>
<td>21.35 ± 3.27</td>
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</tbody>
</table>

* Results were quantified from images taken from three flow chamber replicates. Errors indicate standard deviations.

TABLE 3 Summary of quantitative PCR results

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene</th>
<th>FC in WT</th>
<th>pdeBmutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO2903</td>
<td>cysK</td>
<td>0.66 ± 0.03</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>SO3598</td>
<td>cysM</td>
<td>0.52 ± 0.03</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>SO3599</td>
<td>cysP</td>
<td>0.65 ± 0.05</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>SO3727</td>
<td>cysD</td>
<td>0.39 ± 0.04</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>SO4653</td>
<td>cysT-2</td>
<td>0.27 ± 0.02</td>
<td>0.27 ± 0.02</td>
</tr>
</tbody>
</table>

* Samples were grown in biofilms with LM media for approximately 24 h before harvest.

* Results represent averages from triplicate samples. FC represents fold change in gene expression in the mutant normalized to that of the WT. Errors indicate standard deviations.
Thus far, it was unclear what the signal input (if any) into PdeB was, and to what degree the PAS domain was involved in sensing that signal. As stated earlier, we did not find evidence for the presence of a heme or flavin cofactor bound to the PAS domain of PdeB during purification, and the amino acid sequence of the PAS domain did not contain predicted heme- or flavin-binding residues. Therefore, we tested whether addition of sulfate (1:1 or 1:10 protein-to-Na₂SO₄ ratio) to the in vitro PDE assay changed the PDE activity of PdeB; notably, no change in overall specific activity was observed (data not shown). It is possible that ligand binding to the PAS domain requires the presence of the N terminus of PdeB that was not present in our purified construct (PdeB residues 260 to 856). It is also possible that another intermediate (e.g., from the sulfate assimilation pathway) is responsible for regulating the enzymatic activity of PdeB. Alternatively, the PAS domain may not bind a ligand but rather mediate protein-protein interactions (40).

In summary, we have identified a c-di-GMP-hydrolyzing enzyme, PdeB, whose phosphodiesterase activity was involved in S. oneidensis MR-1 biofilm formation. Furthermore, biochemical and genetic experiments also suggest that PdeB was linked to the regulation of sulfate transport and assimilation. Together, these observations indicate that c-di-GMP metabolism is linked not only to S. oneidensis MR-1 biofilm development but also to sulfate transport and assimilation. However, the details of the signaling pathways involved remain to be elucidated. To our knowledge, regulation of the sulfate transport and assimilation genes is a previously unknown target of c-di-GMP signaling and biofilm formation.

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References


