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]), posttranscriptional (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 pGpG 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

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TABLE 1 Strains used in this study

Bacterial strain	Construct ^a	Relevant genotype or description	Reference or source
<i>E. coli</i>			
DH5α-λpir		φ80dlacZΔM15 Δ(<i>lacZYA-argF</i>)	41
WM3064-λpir		<i>thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-1360 Δ(araBAD)567 ΔdapA1341::[erm pir]</i>	42
S17-λpir		<i>thi pro recA hsdR [RP4-2Tc::Mu-Km::Tn7]λpir; T^r Sm^r</i>	43
Tuner(DE3)pLysS		F [−] <i>ompT hsdSB(r_B[−] m_B[−]) gal dcm lacY1(DE3)</i> pLysS; Cam ^r	EMD Biosciences
AS977	PdeB*	pLIC-SO0437 in Tuner(DE3)pLysS	This work
AS978	PdeB ^{calx}	pLIC-SO0437(E364A) in Tuner(DE3)pLysS	This work
AS262		<i>E. coli</i> S17-λpir with pUX-BF13 (helper plasmid for Tn7 transposon); Amp ^r	44
AS392		<i>E. coli</i> S17-λpir with pGP704-mTn7-gfp; Gen ^r	44
<i>S. oneidensis</i>			
AS579	WT	<i>Shewanella oneidensis</i> MR-1 (PNNL strain)	45
AS979	Δ <i>pdeB</i>	AS579 Δ <i>pdeB</i> (markerless in-frame deletion)	This work
AS980	<i>pdeB</i> ⁺	AS579 Δ <i>pdeB</i> plus <i>pdeB</i> ⁺ (chromosomal replacement)	This work
AS981	<i>pdeB</i> ^{cal}	AS579 Δ <i>pdeB</i> plus <i>pdeB</i> ^{cal} (E634A)	This work
AS982	WT	AS579 chromosomally tagged with GFP; Gen ^r	This work
AS983	Δ <i>pdeB</i>	AS979 chromosomally tagged with GFP; Gen ^r	This work
AS984	<i>pdeB</i> ^{cal}	AS981 chromosomally tagged with GFP; Gen ^r	This work

^a An asterisk indicates a protein.

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 protein). We demonstrate that PdeB is a c-di-GMP-hydrolyzing phosphodiesterase that affects *S. oneidensis* MR-1 biofilm formation and is linked to the regulation of sulfate uptake and assimilation.

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 μM H₃BO₃, 1.27 mM K₂HPO₄, 0.73 mM KH₂PO₄, 1.0 mM MgSO₄ · 7H₂O, 1.3 μM MnSO₄, 67.2 μM Na₂EDTA, 3.9 μM Na₂MoO₄ · 2H₂O, 1.5 μM Na₂SeO₄, 150 mM NaCl, 2 mM NaHCO₃, 5 μM NiCl₂ · 5H₂O, 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 KH₂HPO₄, 5.6 mM NaCl, 18.7 mM NH₄Cl, 1 mM MgSO₄, 0.1 mM CaCl₂).

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.

AS579 served as the parental strain for construction of the *pdeB* deletion mutant. The Δ*pdeB* 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 SmaI restriction site and transformed into *E. coli* S17-λpir. The resulting plasmid, pDS3.0-Δ*pdeB*, was also verified by sequencing.

Following confirmation, pDS3.0-Δ*pdeB* 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-Δ*pdeB* into the chromosome using PCR primers flanking the recombination region. The strain with pDS3.0-Δ*pdeB* 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 pDS132-*pdeB* (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 (*pdeB*^{cal} mutant; E634A) was constructed by generating the mutation in pDS132-*pdeB* via QuikChange site-directed mutagenesis (Agilent). The mutated *pdeB* allele was then introduced into the Δ*pdeB* mutant at the *pdeB* locus, as described for the complementation strain described 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.

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: $\times 10$ magnification, 0.3-W Plan-Neofluar; $\times 20$ magnification, 0.5-W Achroplan; and $\times 40$ 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-His₆ 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 His₆ 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 μ 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 μ 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 (OD₆₀₀) of 0.3. The cultures then were cooled to 16°C, and expression was induced by the addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The cultures were shaken for another 18 h before the cells were harvested by centrifugation. The cells were then resuspended in buffer A (10 ml of 50 mM Na₂HPO₄ [pH 8.0], 300 mM NaCl, 5 mM β -mercaptoethanol [BME], and 5% glycerol) containing 100 μ M phenylmethylsulfonyl fluoride (PMSF), 1 mM benzimidazole, and DNase I before being stored at -80°C .

Nickel and amylose 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 column 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 column volumes of buffer B and five column volumes of buffer C (10 mM morpholinepropanesulfonic acid [MOPS; pH 7.6], 150 mM NaCl, 10 mM MgCl₂, 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 Vivaspinn-6 10K filter (Vivascience) before it was buffer exchanged with buffer C and 5 mM dithiothreitol (DTT) using a PD-10 column (GE Healthcare), after which it was stored at -80°C . The purity of the protein was assessed by SDS-PAGE.

Diguanylate cyclase and phosphodiesterase assays. Assays were carried out in buffer C containing 1 mM DTT. Diguanylate cyclase (DGC) and phosphodiesterase (PDE) activities were assessed by incubating enzyme with α -³²P-labeled GTP ([α -³²P]GTP; Perkin-Elmer) and α -³²P-labeled c-di-GMP (see below for preparation), respectively, at 30°C. The reactions were quenched at various time points with 12.5 mM EDTA (pH 8.0) and/or boiled for 2.5 min and then centrifuged before 1 μ l of the supernatant was spotted onto polyethyleneimine-cellulose thin-layer chromatography plates (PEI-TLC; Merck KGaA, Darmstadt, Germany). The PEI-TLC plates were developed in a 1.5:1.0 mixture of 1.5 M KH₂PO₄ (pH 3.6) and 4.0 M (NH₄)₂SO₄ (pH 3.6), air dried, exposed to a Phosphor-Imager screen (GE Healthcare), and scanned on a Typhoon Imager (GE Healthcare). Under these conditions, pGpG migrates with an R_f (ratio of the distance traveled by the analyte to the distance of the solvent front) of 0.34 and c-di-GMP with an R_f of 0.18. The spot intensities were quantified using ImageQuant, version 5.2 (Molecular Dynamics).

Radioactively labeled c-di-GMP was enzymatically synthesized from [α -³²P]GTP using the TM1788 DGC from *T. maritima* (tDGC), which was expressed and purified as previously described (37). Reaction mixtures incubated for 3 h 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 LM-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 segments (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 $\sim 50\%$ 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 $\Delta pdeB$

FIG 1 Forms of PdeB used in this study. (A) Predicted domain structure of PdeB (Swiss-Prot). The small black rectangles represent the predicted transmembrane regions (THMM). (B) The PdeB(260-856) purified protein (MBP, maltose-binding protein). (C) Mutant version of PdeB with PDE activity inactivated (PdeB^{E634A}, E634A). The black triangle represents a mutated amino acid residue.

mutant under hydrodynamic biofilm conditions. As Fig. 3 shows, the $\Delta pdeB$ 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 $\Delta pdeB$ 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 $\Delta pdeB$ 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^{E634A}-His₆) (Fig. 1B). The MBP tag allowed for higher-yield expression of soluble protein and purification by amylose-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. Incuba-

tion of PdeB with [α -³²P]GTP did not result in formation of a detectable (limit, $\sim 2 \times 10^{-18}$ 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 μ mol 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 DGCs 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* $\Delta pdeB$ mutant phenotypes, we also constructed the corresponding EAL mutant (PdeB^{E634A}, AS981; GFP-tagged PdeB^{E634A}, AS984) and compared its

FIG 2 Swimming motility patterns for $pdeB$ mutants and the wild type. Swimming motility of WT (top) and $\Delta pdeB$ mutant (bottom) cultures were assessed in LB (A) or 4M plus 20 mM fumarate (B). Plates contained 0.25% agar. LB cultures were incubated at 30°C for 48 h, and 4M cultures were incubated anaerobically at room temperature for 48 h. (C) LM swim plate incubated at 30°C for 48 h.

FIG 3 Biofilms of $\Delta pdeB$ and PdeB^{E634A} mutants. Biofilms were grown in hydrodynamic flow chambers with LM for 15 to 16 h at 30°C before imaging. White bars represent 100 μ m. Quantification of biofilms is shown in Table 2.

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

Strain	Biomass ($\mu\text{m}^3/\mu\text{m}^2$)	Thickness (μm)
WT	7.15 ± 1.46	10.18 ± 2.43
$\Delta pdeB$ mutant	13.81 ± 1.20	22.77 ± 3.13
$pdeB^{\text{eal}}$ mutant	12.33 ± 1.59	21.35 ± 3.27

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

swimming motility and biofilm phenotypes to those of the $\Delta pdeB$ mutant. The mutated allele was introduced at the chromosomal locus of the $\Delta pdeB$ mutant through homologous recombination. The $pdeB^{\text{eal}}$ mutant displayed both swimming motility and biofilm phenotypes that were similar to those of the $\Delta pdeB$ 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 and was unlikely to be involved in sensing changes in oxygen or redox (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 transcrip-

TABLE 3 Summary of quantitative PCR results^a

Locus	Gene	FC in ^b :	
		$\Delta pdeB$ mutant	$pdeB^{\text{eal}}$ mutant
SO2903	<i>cysK</i>	0.66 ± 0.03	0.65 ± 0.04
SO3598	<i>cysM</i>	0.52 ± 0.03	0.49 ± 0.03
SO3599	<i>cysP</i>	0.65 ± 0.05	0.47 ± 0.04
SO3727	<i>cysD</i>	0.39 ± 0.04	0.32 ± 0.03
SO4653	<i>cysT-2</i>	0.27 ± 0.02	0.27 ± 0.02

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

^b 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.

tional profiles of $\Delta pdeB$ 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 $\Delta pdeB$ 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 $\Delta pdeB$ mutant versus the WT strain (Table 3). The expression of these genes was also measured in the $pdeB^{\text{eal}}$ mutant. Interestingly, we observed similar differences in expression between $\Delta pdeB$ and $pdeB^{\text{eal}}$ mutant 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 $\Delta pdeB$ and $pdeB^{\text{eal}}$ mutant 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., Na_2SO_4 and NH_4SO_4). 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 $\Delta pdeB$ mutant, the mutant complemented with the WT allele ($pdeB^+$), and the $pdeB^{\text{eal}}$ mutant. Sulfate (40 mM Na_2SO_4 or NH_4SO_4) 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 $\Delta pdeB$ mutant and

FIG 4 Cyclic di-GMP-specific phosphodiesterase activity of PdeB. [³²P]c-di-GMP was incubated with (1 μM) or without MBP-PdeB(260-856)-His₆ at 30°C for 30 min before 1 μl of sample was resolved on a PEI-cellulose TLC plate. —, no enzyme; PdeB, MBP-PdeB(260-856)-His₆; PdeB^{eal}, E634A mutant.

FIG 5 Effect of sulfate addition on swimming motility of $\Delta pdeB$ and $pdeB^{eal}$ mutants. Swimming motility was tested on LM (A) and LM supplemented with 40 mM Na₂SO₄ (B). (C) The relative difference in diameter compared to that of the WT was calculated. Relative difference is the diameter of the mutant minus the diameter of the WT, divided by the average diameter of the mutant and WT. Black, no sulfate; gray, 40 mM NH₄SO₄; white, 40 mM Na₂SO₄.

the $pdeB^{eal}$ mutant were about 85% of that of the WT (Fig. 5A and C). However, in the presence of additional sulfate, the swimming diameters of the $\Delta pdeB$ mutant and the $pdeB^{eal}$ mutant were only about 50% of that of the WT (Fig. 5B and C), indicating that the motility phenotype was exacerbated by the addition of sulfate. Because the swimming motility phenotype was observed only in undefined growth media (LM and LB), it is possible that sulfate, in conjunction with other organic sulfur compounds, contributed to the swimming defect. Although the concentration of sulfate used in this assay was relatively high and nonphysiological, this concentration did not affect growth rate (data not shown), indicating that the observed effect is not due to an unspecific inhibition by sulfate.

In addition to the swimming motility assay, we also investigated the effect of sulfate addition on biofilms of $\Delta pdeB$ and $pdeB^{eal}$ mutant cells. Unfortunately, the results for these experiments were inconclusive (data not shown). Additional salt in the growth medium results in fragile *S. oneidensis* MR-1 biofilms, which easily fell apart under hydrodynamic flow conditions, thereby making it difficult to obtain reproducible results by COMSTAT analysis.

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

- Boehm A, Steiner S, Zaehring F, Casanova A, Hamburger F, Ritz D, Keck W, Ackermann M, Schirmer T, Jenal U. 2009. Second messenger signalling governs *Escherichia coli* biofilm induction upon ribosomal stress. *Mol. Microbiol.* 72:1500–1516.
- Cotter PA, Stibitz S. 2007. C-di-GMP-mediated regulation of virulence and biofilm formation. *Curr. Opin. Microbiol.* 10:17–23.
- Dow JM, Fouhy Y, Lucey JF, Ryan RP. 2006. The HD-GYP domain, cyclic di-GMP signaling, and bacterial virulence to plants. *Mol. Plant Microbe Interact.* 19:1378–1384.
- Fineran PC, Williamson NR, Lilley KS, Salmond GP. 2007. Virulence and prodigiosin antibiotic biosynthesis in *Serratia* are regulated pleiotropically by the GGDEF/EAL domain protein, PigX. *J. Bacteriol.* 189:7653–7662.
- Jenal U. 2004. Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? *Curr. Opin. Microbiol.* 7:185–191.
- Jenal U, Malone J. 2006. Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu. Rev. Genet.* 40:385–407.
- Paul R, Weiser S, Amiot NC, Chan C, Schirmer T, Giese B, Jenal U. 2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes Dev.* 18:715–727.
- Romling U, Amikam D. 2006. Cyclic di-GMP as a second messenger. *Curr. Opin. Microbiol.* 9:218–228.

9. Romling U, Gomelsky M, Galperin MY. 2005. C-di-GMP: the dawning of a novel bacterial signalling system. *Mol. Microbiol.* 57:629–639.
10. Ryan RP, Fouhy Y, Lucey JF, Dow JM. 2006. Cyclic di-GMP signaling in bacteria: recent advances and new puzzles. *J. Bacteriol.* 188:8327–8334.
11. Ryan RP, Fouhy Y, Lucey JF, Jiang BL, He YQ, Feng JX, Tang JL, Dow JM. 2007. Cyclic di-GMP signalling in the virulence and environmental adaptation of *Xanthomonas campestris*. *Mol. Microbiol.* 63:429–442.
12. Tamayo R, Pratt JT, Camilli A. 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu. Rev. Microbiol.* 61:131–148.
13. Wolfe AJ, Visick KL. 2008. Get the message out: cyclic-di-GMP regulates multiple levels of flagellum-based motility. *J. Bacteriol.* 190:463–475.
14. Hickman JW, Harwood CS. 2008. Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Mol. Microbiol.* 69:376–389.
15. Sudarsan N, Lee ER, Weinberg Z, Moy RH, Kim JN, Link KH, Breaker RR. 2008. Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* 321:411–413.
16. Amikam D, Galperin MY. 2006. PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* 22:3–6.
17. Ross P, Weinhouse H, Aloni Y, Michaeli D, Weinberger-Ohana P, Mayer R, Braun S, de Vroom E, van der Marel GA, van Boom JH, Benziman M. 1987. Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* 325:279–281.
18. Weinhouse H, Sapir S, Amikam D, Shilo Y, Volman G, Ohana P, Benziman M. 1997. C-di-GMP-binding protein, a new factor regulating cellulose synthesis in *Acetobacter xylinum*. *FEBS Lett.* 416:207–211.
19. Ryjenkov DA, Tarutina M, Moskvina OV, Gomelsky M. 2005. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J. Bacteriol.* 187:1792–1798.
20. Christen M, Christen B, Folcher M, Schauer A, Jenal U. 2005. Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J. Biol. Chem.* 280:30829–30837.
21. Schmidt AJ, Ryjenkov DA, Gomelsky M. 2005. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. *J. Bacteriol.* 187:4774–4781.
22. Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He YW, Zhang LH, Heeb S, Camara M, Williams P, Dow JM. 2006. Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. *Proc. Natl. Acad. Sci. U. S. A.* 103:6712–6717.
23. Galperin MY, Nikolskaya AN, Koonin EV. 2001. Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol. Lett.* 203:11–21.
24. Seshasayee AS, Fraser GM, Luscombe NM. 2010. Comparative genomics of cyclic-di-GMP signalling in bacteria: post-translational regulation and catalytic activity. *Nucleic Acids Res.* 38:5970–5981.
25. Christen B, Christen M, Paul R, Schmid F, Folcher M, Jenoe P, Meuwly M, Jenal U. 2006. Allosteric control of cyclic di-GMP signaling. *J. Biol. Chem.* 281:32015–32024.
26. Gorby YA, Yanina S, McLean JS, Rosso KM, Moyle D, Dohnalkova A, Beveridge TJ, Chang IS, Kim BH, Kim KS, Culley DE, Reed SB, Romine MF, Saffarini DA, Hill EA, Shi L, Elias DA, Kennedy DW, Pinchuk G, Watanabe K, Ishii S, Logan B, Nealson KH, Fredrickson JK. 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proc. Natl. Acad. Sci. U. S. A.* 103:11358–11363.
27. Hau HH, Gralnick JA. 2007. Ecology and biotechnology of the genus *Shewanella*. *Annu. Rev. Microbiol.* 61:237–258.
28. Marsili E, Baron DB, Shikhar ID, Coursolle D, Gralnick JA, Bond DR. 2008. *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proc. Natl. Acad. Sci. U. S. A.* 105:3968–3973.
29. Thormann KM, Duttler S, Saville RM, Hyodo M, Shukla S, Hayakawa Y, Spormann AM. 2006. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. *J. Bacteriol.* 188:2681–2691.
30. Thormann KM, Saville RM, Shukla S, Pelletier DA, Spormann AM. 2004. Initial phases of biofilm formation in *Shewanella oneidensis* MR-1. *J. Bacteriol.* 186:8096–8104.
31. Saville RM, Dieckmann N, Spormann AM. 2010. Spatiotemporal activity of the *mshA* gene system in *Shewanella oneidensis* MR-1 biofilms. *FEMS Microbiol. Lett.* 308:76–83.
32. Taylor BL, Zhulin IB. 1999. PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.* 63:479–506.
33. Zhulin IB, Taylor BL, Dixon R. 1997. PAS domain S-boxes in Archaea, Bacteria and sensors for oxygen and redox. *Trends Biochem. Sci.* 22:331–333.
34. Thormann KM, Saville RM, Shukla S, Spormann AM. 2005. Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. *J. Bacteriol.* 187:1014–1021.
35. Philippe N, Alcaraz JP, Coursange E, Geiselmann J, Schneider D. 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. *Plasmid* 51:246–255.
36. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, Molin S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146(Part 10):2395–2407.
37. Rao F, Pasunooti S, Ng Y, Zhuo W, Lim L, Liu AW, Liang ZX. 2009. Enzymatic synthesis of c-di-GMP using a thermophilic diguanylate cyclase. *Anal. Biochem.* 389:138–142.
38. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 25:402–408.
39. Henry JT, Crosson S. 2011. Ligand-binding PAS domains in a genomic, cellular, and structural context. *Annu. Rev. Microbiol.* 65:261–286.
40. Anantharaman V, Koonin EV, Aravind L. 2001. Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains. *J. Mol. Biol.* 307:1271–1292.
41. Miller VL, Mekalanos JJ. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* 170:2575–2583.
42. Saltikov CW, Newman DK. 2003. Genetic identification of a respiratory arsenate reductase. *Proc. Natl. Acad. Sci. U. S. A.* 100:10983–10988.
43. Simon R, Priefer U, Puhler A. 1983. A broad host range mobilization system for *in vivo* genetic engineering—transposon mutagenesis in Gram-negative bacteria. *Bio-Technol.* 1:784–791.
44. Muller J, Miller MC, Nielsen AT, Schoolnik GK, Spormann AM. 2007. *vpsA*- and *luxO*-independent biofilms of *Vibrio cholerae*. *FEMS Microbiol. Lett.* 275:199–206.
45. Myers CR, Nealson KH. 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science* 240:1319–1321.