Identification of a Diguanylate Cyclase That Facilitates Biofilm Formation on Electrodes by *Shewanella oneidensis* MR-1

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**ABSTRACT** In many bacteria, cyclic diguanosine monophosphate (c-di-GMP), synthesized by diguanylate cyclase (DGC), serves as a second messenger involved in the regulation of biofilm formation. Although studies have suggested that c-di-GMP also regulates the formation of electrochemically active biofilms (EABFs) by *Shewanella oneidensis* MR-1, DGCs involved in this process remained to be identified. Here, we report that the SO_1646 gene, hereafter named *dgcS*, is upregulated under medium flow conditions in electrochemical flow cells (EFCs), and its product (DgcS) functions as a major DGC in MR-1. **In vitro** assays demonstrated that purified DgcS catalyzed the synthesis of c-di-GMP from GTP. Comparisons of intracellular c-di-GMP levels in the wild-type strain and a *dgcS* deletion mutant (**ΔdgcS** mutant) showed that production of c-di-GMP was markedly reduced in the **ΔdgcS** mutant when cells were grown in batch cultures and on electrodes in EFCs. Cultivation of the **ΔdgcS** mutant in EFCs also revealed that the loss of DgcS resulted in impaired biofilm formation and decreased current generation. These findings demonstrate that MR-1 uses DgcS to synthesize c-di-GMP under medium flow conditions, thereby activating biofilm formation on electrodes.

**IMPORTANCE** Bioelectrochemical systems (BESs) have attracted wide attention owing to their utility in sustainable biotechnology processes, such as microbial fuel cells and electrofermentation systems. In BESs, electrochemically active bacteria (EAB) form biofilms on electrode surfaces, thereby serving as effective catalysts for the interconversion between chemical and electric energy. It is therefore important to understand mechanisms for the formation of biofilm by EAB grown on electrodes. Here, we show that a model EAB, *S. oneidensis* MR-1, expresses DgcS as a major DGC, thereby activating the formation of biofilms on electrodes via c-di-GMP-dependent signal transduction cascades. The findings presented herein provide the molecular basis for improving electrochemical interactions between EAB and electrodes in BESs. The results also offer molecular insights into how *Shewanella* regulates biofilm formation on solid surfaces in the natural environment.

**KEYWORDS** cyclic di-GMP, diguanylate cyclase, biofilm, electrochemically active bacteria, flow cell

Bioelectrochemical systems (BESs) are engineered systems that utilize electrochemical interactions between electrochemically active bacteria (EAB) and electrodes (1). Potential applications of BESs include microbial fuel cells (MFCs) and electrofermentation systems, and these are expected to be used for electricity generation from wastewater and bioproduction of value-added chemicals, respectively (2–4). In BESs, EAB form biofilms during growth on electrode surfaces, thereby functioning as effective catalysts for the interconversion between electric and chemical energy (5). It is also known that the biofilms facilitate the long-range transfer of electrons from EAB cells to electrodes by association with self-secreted redox-active compounds, such as...
cytochromes (6, 7) and flavins (8). The roles of biofilms formed by EAB (i.e., electrochemically active biofilms [EABFs]) are considered particularly important in practical BESs that are assumed to be operated under a continuous flow of electrolytes (e.g., wastewater and culture medium), since planktonic microbes are washed out from these systems (9). It is therefore expected that understanding the mechanisms behind EABF formation would provide insights into the development of high-performance BESs.

Extensive microscopic and electrochemical measurements have been performed to characterize EABFs formed by model EAB, such as *Geobacter sulfurreducens* PCA (10–12) and *Shewanella oneidensis* MR-1 (13–15). However, limited information is available on how EAB regulate EABF formation on electrode surfaces. To explore genes involved in the EABF formation by *S. oneidensis* MR-1, we previously cultivated this strain in electrochemical flow cells (EFCs) and examined the transcriptional profiles of the electrode-associated cells grown in the presence and absence of the flow of medium (16). It was revealed that under the medium flow conditions, MR-1 significantly upregulated the expression of a putative diguanylate cyclase (DGC) gene, SO_1646, as well as other genes related to cell motility and signal transduction (16). DGCs catalyze the synthesis of cyclic diguanosine monophosphate (c-di-GMP), a key second messenger involved in the regulation of biofilm formation in bacteria (17). It is therefore suggested that MR-1 expresses SO_1646 under the flow of medium, thereby facilitating the formation of biofilms on electrodes via c-di-GMP-dependent signal transduction cascades.

The concentration of c-di-GMP in bacterial cells depends on the activities of two enzymes, DGC and phosphodiesterase (PDE) (18). DGCs contain GGDEF domains that catalyze the synthesis of c-di-GMP from two GTP molecules, while PDEs contain EAL or HD-GYP domains that catalyze the hydrolysis of c-di-GMP into 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) or two GMP molecules (19). Bifunctional proteins that have both DGC and PDE activities are also present (20, 21). The genome of MR-1 encodes 51 GGDEF domain-containing proteins, including 20 hybrid proteins containing both GGDEF and EAL domains (22). Although the physiological functions of these GGDEF domain-containing proteins are largely unknown, a previous study reported that the overexpression of a DGC gene that originated from *Escherichia coli* in MR-1 resulted in enhanced biofilm formation on electrodes and increased current generation in MFCs (23). It has also been reported that the disruption of a PDE gene, *pdeB*, in MR-1 facilitates biofilm formation on glass surfaces in aerobically operated flow cells (24). These observations suggest that c-di-GMP plays important roles in the regulation of biofilm formation by MR-1, although roles of its endogenous DGCs in the formation of electrode-associated biofilms remain to be elucidated.

Based on the above-mentioned knowledge, we hypothesized that MR-1 regulates biofilm formation on electrodes via c-di-GMP-dependent signal transduction cascades, in which the gene product of SO_1646, hereafter named DgcS (for diguanylate cyclase in *S. oneidensis*), plays a key role. To address this hypothesis, in vitro and in vivo experiments were conducted in this study to investigate the involvement of DgcS in c-di-GMP synthesis and EABF formation by MR-1.

**RESULTS**

Transcriptional regulation of *dgcS*. Our previous transcriptome analyses for MR-1 cells grown in EFCs found that the expression of the SO_1646 gene (*dgcS*) was upregulated in the presence of medium flow (16). To verify this result, we performed quantitative reverse transcription (RT)-PCR analysis to compare the expression levels of this gene in MR-1 cells grown in EFCs operated under medium flow and nonflow conditions (see Fig. S1 in the supplemental material). The results confirmed that the presence of medium flow significantly upregulated the expression of *dgcS*, supporting the hypothesis that this gene is involved in the formation of electrode-associated biofilms by MR-1 cells exposed to medium flow. Given that a σ^54-dependent promoter-like
sequence is present at the upstream region of \textit{dgcS} (25) and that the downstream \textit{tilS} (SO_1645) gene is arranged in the opposite direction, it is suggested that \textit{dgcS} is transcribed as a single gene from the upstream promoter. It is known that the activities of many DGC proteins are controlled by N-terminal sensor domains, such as PAS, GAF, CHASE, and HAMP domains (26). However, \textit{in silico} domain prediction using the Simple Modular Architecture Research Tool (SMART) (27) showed that the DgcS protein contains no known domain other than a C-terminal GGDEF domain (Fig. 1A). Taken together, these findings suggest that transcriptional regulation plays an important role in controlling the activity of DgcS, although the involvement of posttranscriptional regulation cannot be excluded.

\textbf{In vitro enzyme assay for DgcS.} To examine if DgcS is a DGC, we conducted \textit{in vitro} assays using purified DgcS and examined the synthesis of c-di-GMP from GTP. C-terminally histidine-tagged DgcS (C-ht-DgcS) was expressed in recombinant \textit{Escherichia coli} cells and purified by nickel affinity chromatography (Fig. 1B). The purified protein was incubated in a reaction mixture containing GTP, and the consumption of GTP and the production of c-di-GMP were analyzed by high-performance liquid chromatography (HPLC). Control experiments using crude (nonpurified) extracts from the C-ht-\textit{dgcS}-expressing \textit{E. coli} cells were also performed. The HPLC analysis revealed that synthesis of c-di-GMP, along with GTP consumption (data not shown), occurred in the presence of purified DgcS. The specific activity of purified DgcS was 53 times higher than that of the crude extract (Fig. 1C), demonstrating that DgcS functions as a DGC that catalyzes the synthesis of c-di-GMP from GTP.

\textbf{Contribution of DgcS to intracellular c-di-GMP synthesis.} To evaluate the contribution of DgcS to \textit{in vivo} c-di-GMP synthesis, a \textit{dgcS} deletion mutant of MR-1 (Δ\textit{dgcS} mutant) and its complemented strain (Δ\textit{dgcS}-C strain) were constructed (Table 1). A plasmid expressing an anaerobic fluorescent protein (AFP) (evoglow-Bs2; evocatal GmbH, Düsseldorf, Germany) (28) had been introduced into these strains for subsequent biofilm observation in EFCs. When these mutant strains were cultivated in test tubes containing lactate minimal medium (LMM), they showed growth similar to that of a wild-type control strain (MR-1c) (Table 1) (Fig. S2), suggesting that the loss of DgcS does not affect the growth of MR-1 in batch cultures. These strains were cultivated in LMM until the stationary growth phase and analyzed for intracellular c-di-GMP contents using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 2). Analyses revealed that the c-di-GMP content in the Δ\textit{dgcS} mutant was approximately 35\% of that in MR-1, and that this decrease was restored in the Δ\textit{dgcS}-C strain. These results demonstrated that among multiple GGDEF domain-containing proteins encoded in the genome, DgcS served as a major DGC that affected global c-di-GMP levels in MR-1 cells.
Involvement of DgcS in biofilm formation on electrodes. To examine the involvement of DgcS in EABF formation and current generation in BESs, EFCs were inoculated with MR-1c or the \(D_{dgcS}\) mutant and operated under a medium flow condition at a working electrode (WE) potential of +0.4 V (versus the standard hydrogen electrode [SHE]) until the current generation by both strains became stable (for 168 h).

Measurement of currents (Fig. 3) revealed that the initial increase in current generation by the \(D_{dgcS}\) mutant was slower than that for MR-1c and that the maximum current density achieved by the \(D_{dgcS}\) mutant (0.97 \(\mu A/cm^2\)) was approximately 71% of that for MR-1c (1.36 \(\mu A/cm^2\)). These results demonstrate that the loss of DgcS results in decreased current generation in EFCs.

Figure 4A shows typical confocal laser scanning microscopy (CLSM) images for WE-associated and planktonic cells in EFCs when the growth of WE-associated cells reached a steady state (168 h after the start of operation). As observed in previous studies, MR-1c formed a thin, flat biofilm on the WE surface (Fig. 4A). However, the \(\Delta dgcS\) mutant appeared to form a thicker and sparser biofilm than MR-1c, as seen in the side (x-z) view of Fig. 4A. In the \(\Delta dgcS\) mutant-inoculated EFC, the number of cells in the lower zone (at 3 \(\mu m\) from the WE surface; \(z = 3 \mu m\)) was smaller than that in the MR-1c-inoculated EFC, while the number of cells in the upper zone (\(z = 10 \mu m\)) was larger (Fig. 4A). This trend was confirmed by analyzing the vertical distribution of fluorescent intensities in the CLSM images (Fig. 4B). To quantify the biomass attached to WE, WE-associated MR-1c and \(\Delta dgcS\) cells were collected from EFCs operated for

![FIG 2](Image)

**FIG 2** Intracellular c-di-GMP contents in *S. oneidensis* derivatives. Cells were grown under aerobic conditions and harvested at the stationary growth phase. Error bars represent standard deviations calculated from the results of three independent experiments. Asterisks indicate a statistically significant difference (\(P < 0.05\); one-way ANOVA followed by LSD test; ns, not significant).

**TABLE 1** Bacterial strains and plasmids used in this study

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<td>Donor strain for conjugation; lacT &amp; rnap3 (\Delta lacZ4787) (\Delta)araBAD (\Delta)hsdR514 (\Delta)trfA4787 (\Delta)lacZ4787 (\Delta)araBAD (\Delta)lacZ4787 (\Delta)hsdR514 (\Delta)trfA4787 (\Delta)lacZ4787 (\Delta)araBAD (\Delta)lacZ4787 (\Delta)hsdR514 (\Delta)trfA4787</td>
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<td>Cloning vector; Ap’</td>
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168 h, and their protein contents were determined (Fig. 4C). The analysis revealed that the protein content in \(\Delta dgcS\) biofilm was significantly lower than that in MR-1\(_c\) biofilm. These results demonstrate that the \(\Delta dgcS\) mutant exhibits an impaired ability to form biofilms in EFCs. When the maximum current densities achieved by the

**FIG 3** Time courses of current generation by MR-1\(_c\) and the \(\Delta dgcS\) mutant in EFC. Results and error bars represent the means and standard deviations, respectively, calculated from the results of three independent experiments.

**FIG 4** Characterization of the \(\Delta dgcS\) mutant in EFC. (A) Representative CLSM images of MR-1\(_c\) and the \(\Delta dgcS\) mutant grown in EFC. Vertical (x-z) and horizontal (x-y) images were obtained from EFCs operated for 168 h. Horizontal (x-y) images were obtained at heights of 3 \(\mu\)m and 10 \(\mu\)m from the WE surface (\(z=3\) \(\mu\)m and 10 \(\mu\)m, respectively). (B) Vertical distribution of AFP signal intensities in CLSM images obtained from EFCs operated for 168 h. (C to G) Protein contents (C), maximum current density normalized to protein contents (D), intracellular c-di-GMP contents (E), polysaccharide contents (F), and ratios of polysaccharide contents to protein contents (G) in WE-associated MR-1\(_c\) and \(\Delta dgcS\) biofilms. Cells were grown in EFCs for 168 h. In all graphs, asterisks indicate a statistically significant difference (\(P<0.05\); Student’s t test; ns, not significant). Error bars represent standard deviations calculated from the results of three independent experiments.
ΔdgCS mutant and MR-1, were normalized by their protein content in WE-associated biofilms, the normalized values were not significantly different between these strains (Fig. 4D), suggesting that the loss of DgcS does not affect current generation per cell. We therefore concluded that the decreased current generation observed for the ΔdgCS mutant (Fig. 3) was attributable to the impaired ability of this mutant to physically associate with WE. We also determined the intracellular c-di-GMP contents in WE-associated MR-1 and ΔdgCS cells. The results confirmed that the ΔdgCS mutant contained a lower level of c-di-GMP than did MR-1, (Fig. 4E), although, for unknown reasons, the c-di-GMP contents in the WE-associated cells were significantly lower than those in aerobically grown cells (Fig. 2). Taken together, these findings indicate that DgcS facilitated biofilm formation on electrodes by activating c-di-GMP-dependent signal transduction.

It is well known that c-di-GMP-dependent signal transduction cascades regulate the synthesis of extracellular polysaccharides, thereby modulating the ability of bacterial cells to associate with solid surfaces (29). We therefore determined the extracellular polysaccharide content of WE-associated biofilms formed by the ΔdgCS mutant and MR-1, to examine how DgcS facilitates biofilm formation on electrodes. As shown in Fig. 4F, the ΔdgCS biofilm contained a lower level of polysaccharides than the MR-1, biofilm. Notably, when polysaccharide contents were normalized by protein contents in WE-associated biofilms, the polysaccharide/protein ratio was lower in the ΔdgCS mutant than in MR-1, (Fig. 4G), indicating that the ΔdgCS mutant produced less extracellular polysaccharides per cell than MR-1. These results indicate that c-di-GMP production catalyzed by DgcS activated the synthesis of extracellular polysaccharides, thereby facilitating the association of MR-1 cells with electrodes.

**DISCUSSION**

Here, we found that MR-1 activates biofilm formation on electrodes via the DgcS-dependent c-di-GMP signaling cascades. Our results indicate that DgcS catalyzes the production of c-di-GMP, which induces the synthesis of extracellular polysaccharides and facilitates the association of MR-1 cells with electrode surfaces (Fig. 1 and 4). Notably, a recent study reported that an elevated c-di-GMP level in MR-1 cells by the overexpression of a heterologous DGC gene resulted in increased expression of c-type cytochrome genes involved in extracellular electron transfer (EET) (30). In the present study, however, we observed that current generation normalized by the biomass of WE-associated cells was not significantly different between the wild type and the ΔdgCS mutant (Fig. 4D), suggesting that the loss of DgcS does not affect the EET activity of MR-1 cells. We consider that the intracellular level of c-di-GMP in ΔdgCS is sufficient for the expression of EET-related genes, while it is lower than the threshold level at which the biosynthesis of extracellular polysaccharides is promoted.

In *Pseudomonas aeruginosa*, c-di-GMP regulates biofilm formation by binding to a σ54-dependent transcriptional regulator, FleQ, that activates the expression of biofilm-related genes, including those involved in the biosynthesis of extracellular polysaccharides (31, 32). A homolog of FleQ, referred to as FlrA in MR-1, is found in many members of the genus *Shewanella*, suggesting that similar mechanisms are also present in members of this genus. In support of this idea, a recent study has reported that a spontaneous MR-1 mutant in which the flrA gene is disrupted by an insertion sequence exhibited impaired biofilm formation on electrodes (33). Previous studies have also shown that FlrA in *Shewanella* regulates the expression of genes for the synthesis of flagella (34) and an adhesin protein, BpfA (35). It is therefore possible that these biofilm-related factors other than extracellular polysaccharides are also involved in EABF formation by MR-1. It is also known that c-di-GMP directly binds to polysaccharide biosynthetic enzymes, such as the glycosyltransferase BcsA, thereby regulating biofilm formation at the posttranslational level (36).
Furthermore, previous studies have suggested that the Mxda protein containing a degenerate GGDEF (NVDEF) motif functions as a c-di-GMP receptor and interacts with the Mxdb glycosyltransferase, thereby activating the biosynthesis of extracellular polysaccharides in MR-1 (37, 38). These studies provide information for predicting downstream processes of a DgcS-dependent c-di-GMP signaling cascade, and further studies will be conducted to investigate the involvement of these biofilm-related factors in EABF formation by MR-1.

The genome of MR-1 encodes 51 GGDEF domain-containing proteins, of which only a few have been functionally characterized. For instance, a recent study has reported that the GGDEF-containing proteins PdgA and PdgB play essential roles in the formation of pellicles, floating biofilms formed at the air-liquid interface (38). Although enzymatic activities of these proteins have not been examined in vitro, evidence obtained from in vivo experiments has suggested that they serve as DGCs (38). It is noteworthy that the study also suggests that PdgA and PdgB specifically function for pellicle biogenesis but not for biofilm formation on solid surfaces (38).

These observations, together with the findings in the present study, suggest that MR-1 utilizes multiple DGCs to specifically regulate the formation of solid surface-associated biofilms and pellicles according to environmental conditions. In relation to this idea, recent studies have provided evidence suggesting that local signaling based on direct interaction between DGCs and c-di-GMP receptor proteins regulates specific cellular processes (39, 40). In contrast to these observations, the present study demonstrates that the loss of DgcS results in a significant decrease in the overall intracellular level of c-di-GMP in MR-1 grown in EFCs (Fig. 4E). We therefore suggest that DgcS globally activates c-di-GMP-dependent signal transduction pathways that are involved in biofilm formation on electrodes. It is also notable that the decrease in the c-di-GMP content was also observed in ΔdgcS cells grown under batch culture conditions, where most cells were planktonic (Fig. 2). One possible explanation for this result is that DgcS is activated in response to hydrodynamic signals, such as changes in shear force (41), given that cells are exposed to fluid (medium) flows in both EFCs and shaking cultures. However, considering that medium flow can cause changes in many environmental factors, such as shifts in local nutrient concentrations, identification of an environmental factor(s) that functionally activates DgcS requires additional investigation.

We previously reported that MR-1 uses the extracytoplasmic function (ECF) sigma factor encoded by so_3096 to activate the expression of many genes, including the dsbD genes involved in cytochrome c maturation, under medium flow conditions (16). It is not likely, however, that this ECF sigma factor is involved in the transcriptional regulation of dgcS, because the disruption of SO_3096 did not significantly alter the transcription of dgcS (16). We therefore consider that there may exist an unknown transcription regulator(s) that activates the expression of this gene. We expect that the development of a comprehensive understanding of the DgcS-mediated signaling cascades will provide vital information in regard to EABF formation and current generation by Shewanella oneidensis MR-1.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. S. oneidensis strains were cultivated at 30°C in lysogeny broth (LB) medium or lactate minimal medium (LMM) containing 15 mM D-lactate as the sole carbon and energy source (15). For aerobic cultivation, LB medium or LMM in test tubes was inoculated with an S. oneidensis strain and incubated by reciprocal shaking at 180 rpm. The optical density at 600 nm (OD600) was measured using a mini photo 518R photometer (Taitec, Tokyo, Japan). E. coli strains were cultivated in LB medium at 37°C. The E. coli mating strain (WM6026), required 100 μg/ml 2,6-diaminopimelic acid for growth. When necessary, 50 μg/ml kanamycin (Km), 15 μg/ml gentamicin (Gm), or 100 μg/ml ampicillin (Ap) was added to culture media. Agar plates contained 1.6% Bacto agar (Difco, Franklin Lakes, NJ).

Operation of EFCs and CLSM of biofilms. The setup and operation of EFCs were performed according to methods described previously (16). Briefly, a three-electrode EFC comprising a graphite plate WE (68 by 140 mm; IG-70; Toyo Tanso, Osaka, Japan), an Ag/AgCl reference electrode (HX-R5; Hokuto Denko,
Tokyo, Japan), and a titanium mesh counter-electrode (25 by 25 mm, 100 mesh, 8 pieces; Nilaco, Tokyo, Japan) was filled with LMM and inoculated with an S. oneidensis strain. The electrodes were connected to a potentiostat (VMP3; Bio-Logic Science Instruments, Seyssinet-Pariset, France), and current was measured at a WE potential of +0.4 V (versus SHE). For EFC operation under medium flow conditions, LMM was continuously supplied from a reservoir tank to the EFC using a peristaltic pump at a constant linear velocity of 5.5 mm min$^{-1}$ (a flow rate of 1.38 ml min$^{-1}$). The medium in the reservoir tank was constantly purged with high-purity nitrogen gas to maintain anaerobic conditions. During operation, the EFC was put in an incubator at 30°C. CLSM was performed using an FV1200 (BX61WI configuration) microscope system (Olympus, Tokyo, Japan) according to a method described previously (16). Biofilms expressing AFP were observed at an excitation wavelength of 473 nm.

RNA extraction and quantitative RT-PCR. RNA extraction from WE-attached MR-1 cells and subsequent quantitative RT-PCR were performed according to methods described previously (16). Briefly, EFCs inoculated with MR-1 cells were operated for 72 h under the medium flow condition or for 48 h under the nonflow condition until current generation became stable along with the growth of WE-associated cells. Total RNA was extracted from the WE-attached cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) and was subsequently purified using an RNeasy mini kit and an RNase-free DNase set (Qiagen, Valencia, CA). The quantity and quality of the purified RNA were checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and an Agilent 2100 bioanalyzer with RNA 6000 pico reagents and RNA pico chips (Agilent Technologies, Santa Clara, CA). For quantitative RT-PCR, cDNA was synthesized from total RNA using Superscript III reverse transcriptase (Thermo Fisher Scientific) and random primers (Thermo Fisher Scientific). Quantitative PCR was performed using the Applied Biosystems StepOnePlus real-time PCR system (Thermo Fisher Scientific), Power SYBR green PCR master mix (Thermo Fisher Scientific), and the specific primers listed in Table S1 in the supplemental material. The specificity of the quantitative PCR was verified by dissociation curve analysis. Expression levels of target genes were normalized based on expression levels of the 16S rRNA gene.

Construction of plasmids and mutants. The in-frame disruption of the dgcS gene in MR-1 was performed using a two-step homologous recombination method with the suicide plasmid pSMV-10 (42) (Table 1) as described previously (43). Briefly, a 1.6-kb fusion product, consisting of upstream and downstream sequences of the dgcS (SO_1646) gene joined by an 18-bp linker sequence, was constructed by overlap extension PCR (44) using primers listed in Table S1 in the supplemental material and Phusion high-fidelity DNA polymerase (New England Biolabs, Beverly, MA). The amplified fusion product was ligated into the SpeI site of pUC18 (TaKaRa, Tokyo, Japan), and the resultant plasmid, pSMV-dgcS (Table 1), was introduced into MR-1 by electropermeabilization. Double-crossover mutants in which the dgcS gene was disrupted in-frame were isolated on LB plates containing 10% (wt/vol) sucrose, and one representative mutant strain was selected and transformed with the AFP-expressing plasmid pMElacZ-AFP (15) and the control vector pBBR1MCS-2 (45) (Table 1) by electroporation (46) and filter mating, respectively. The resultant mutant strain was designated the ΔdgcS mutant (Table 1). The control MR-1 strain transformed with pMElacZ-AFP and pBBR1MCS-2 was designated MR-1c (Table 1).

To construct the dgcS-expressing plasmid pBBR-dgcS (Table 1), the DNA fragment consisting of the dgcS gene and its 500-bp upstream region was PCR amplified using primers listed in Table S1. The amplified product was cloned into the Smal site of pUC18 (TaKaRa, Tokyo, Japan), and sequencing confirmed that no error occurred in the PCR amplification. The insert was digested with NsiI and Kpnl and cloned into the corresponding sites of pBBR1MCS-2 (45) (Table 1) to yield pBBR-dgcS. The ΔdgcS derivative transformed with pBBR-dgcS instead of pBBR1MCS-2 was designated the ΔdgcS-C strain (Table 1).

Purification of DgcS. To construct a plasmid expressing C-ht-DgcS, the dgcS gene was amplified from the total DNA of MR-1 using primers listed in Table S1. The amplified product was digested with Ncol and HindIII and cloned into the corresponding sites of the histidine tag expression vector pET-28a(+) (Novagen, Madison, WI). The resultant plasmid, pET-C-ht-dgcS (Table 1), was introduced into E. coli BL21(DE3). Cells carrying the plasmid were grown in 300-ml baffled Erlenmeyer flasks containing 100 ml 2x YT medium supplemented with Km at 37°C. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM when the OD$_{600}$ reached 0.5. After further cultivation for 16 h, the cells were harvested by centrifugation and suspended in 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 20 mM imidazole. The cell suspension was subjected to ultrasonication with an S4000 sonicator (Misonix, Farmingdale, NY) and was neutralized to pH 7.0 using acetic acid. The sample solution was diluted with an RNeasy mini kit and an RNase-free DNase set (Qiagen, Valencia, CA) and was subsequently purified using an RNeasy mini kit and an RNase-free DNase set (Qiagen, Valencia, CA). The quantity and quality of the purified RNA were checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and an Agilent 2100 bioanalyzer with RNA 6000 pico reagents and RNA pico chips (Agilent Technologies, Santa Clara, CA). For quantitative RT-PCR, cDNA was synthesized from total RNA using Superscript III reverse transcriptase (Thermo Fisher Scientific) and random primers (Thermo Fisher Scientific). Quantitative PCR was performed using the Applied Biosystems StepOnePlus real-time PCR system (Thermo Fisher Scientific), Power SYBR green PCR master mix (Thermo Fisher Scientific), and the specific primers listed in Table S1 in the supplemental material. The specificity of the quantitative PCR was verified by dissociation curve analysis. Expression levels of target genes were normalized based on expression levels of the 16S rRNA gene.

In vitro DGC activity assay. For the DGC activity assay, 50 μg of purified C-ht-DgcS was mixed with 200 nmol GTP in 600 μl reaction buffer (50 mM Tris-HCl, 300 mM NaCl, and 20 mM MgCl$_2$; pH 8.0). The reaction mixture was incubated at 30°C for 15 min, and the reaction was stopped by heating at 95°C for 10 min. The consumption of GTP and the production of c-di-GMP were analyzed using an HPLC system (1100 series; Agilent Technologies) equipped with a Zorbax SB-Aq column (4.6 mm by 150 mm, 5 μm; Agilent Technologies) as described previously (47). Briefly, the reaction mixture was diluted with an HPLC mobile phase consisting of 20 mM triethyl ammonium bicarbonate buffer and 9% (vol/vol) methanol and was neutralized to pH 7.0 using acetic acid. The sample solution was filtered using a membrane filter and loaded onto the HPLC system. The HPLC conditions were as follows: flow rate, 1 ml min$^{-1}$; column temperature, 30°C; and detection wavelength, 254 nm. The column was equilibrated with 0.1 M sodium citrate buffer (pH 4.5) and was eluted with 0.1 M sodium citrate buffer (pH 4.5) and 50% methanol at a flow rate of 1 ml min$^{-1}$. The elution of GTP was monitored at 254 nm.
filter unit (0.20-μm pore size, DISMIC-25HP; Advantec, Tokyo, Japan) and injected into the HPLC column.

Isocratic elution of GTP and c-di-GMP was achieved by chromatography using the above-mentioned mobile phase at an elution rate of 0.2 ml/min. Quantitative analysis was performed using a UV detector (Agilent Technologies) set at 243 nm and commercially available c-di-GMP (Sigma-Aldrich, St. Louis, MO) as a standard.

**Extraction and quantification of intracellular c-di-GMP.** Intracellular c-di-GMP in *S. oneidensis* strains was extracted and quantified according to methods described previously (48) with modifications. *S. oneidensis* cells were grown under aerobic conditions in test tubes containing LMM until the stationary growth phase or under anaerobic medium conditions in EFCs until the growth of WE-associated cells reached a steady state. The aerobically grown or WE-associated cells were suspended in 300 µl of an ice-cold extraction solvent consisting of acetonitrile-methanol-water (40:40:20 [vol/vol/vol]) containing 30 pmol cyclic 3′,5′-xanthosine monophosphate (cXMP) as an internal standard. The cell suspension was incubated at 4°C for 15 min and then heated to 95°C for 10 min. After cooling, the cell suspension was centrifuged at 20,000 × g for 5 min to separate the insoluble (pellet) and soluble (supernatant) fractions. The extraction procedure from the insoluble fraction was repeated twice, omitting the heat treatment, using 200 µl of the extraction solvent without cXMP. The solvent of the combined supernatants (700 µl) was evaporated under vacuum using a centrifugal concentrator (VC-155; Taitec).

Analyses of extracts were conducted by liquid chromatography-electrospray ionization-tandem mass spectrometry in positive ionization mode (LC-ESI(+)-MS/MS) by using a Waters 2690 separation module (Waters Corp., Milford, MA) in-line with a Waters 2998 photodiode array detector that was interfaced with a Quattro Ultima triple-stage quadrupole mass spectrometer (Waters-Micromass, Manchester, UK). Sample aliquots of 20 µl were analyzed on a Cosmosil SC-35AR-II column (4.6 mm inside diameter by 250 mm; Shimadzu, Kyoto, Japan) that was in-line with a Security Guard cartridge system pre-column fitted with a wide-pore C18 cartridge (Phenomenex, Torrance, CA, USA). Compounds were eluted at a flow rate of 0.3 ml min⁻¹ via a gradient that began at a ratio of 97% water to 3% methanol, which was changed to a ratio of 25% water to 75% methanol over a period of 30 min. Conditions were returned to a ratio of 97% water to 3% methanol and held through 60 min. Sample components were delivered to the mass spectrometer by electrospray, whereby nitrogen gas was used as the nebulizing gas. The ion source temperature was 130°C, the desolvation temperature was 360°C, and the cone voltage was operated at 35 V. Nitrogen gas was also used as the desolvation gas, at 600 liters/h, and the cone gas, at 60 liters/h. Argon gas (99.9999%) was used as the collision cell gas. MS analyses were conducted in single reaction monitoring (SRM) mode by monitoring c-di-GMP and cXMP at the m/z transitions 691.152 and 347.153, respectively. The collision cell energies utilized for these transitions were 61 eV and 29 eV, respectively. The c-di-GMP content of cells was normalized based on their total protein content determined using a Micro BCA protein assay kit.

**Quantification of extracellular polysaccharides and proteins in biofilms.** WE-associated biofilms were recovered from EFCs operated for 168 h. Polysaccharides and proteins in the biofilms were determined using a phenol-sulfuric acid method (49) and a Micro BCA protein assay kit, respectively, as described previously (15).

**Statistical and in silico analysis.** Data were statistically evaluated using Student’s t test or one-way analysis of variance (ANOVA), followed by Fisher’s least significant difference (LSD) test using js-STAR 2012 (http://www.kisnet.or.jp/nappa/software/star/). The domain structure of DgcS was predicted using the SMART program (27).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.**

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A.M. carried out the majority of the experimental work and drafted the manuscript. R.K. supported EFC operation and CLSM observation. R.A.K. performed LC-MS/MS analyses and manuscript editing. A.K. designed the study and drafted the manuscript. K.W. supervised the study and performed manuscript editing. All authors read and approved the final manuscript.

We have no conflicts of interest to declare.

**REFERENCES**


