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Identification of Multicomponent Histidine-Aspartate Phosphorelay System Controlling Flagellar and Motility Gene Expression in *Geobacter* Species*[S]

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Background: Motility is an important environmental response, but flagellar regulation has been studied only in a few model microorganisms.

Results: Essential regulators in the two-component His-Asp phosphorelay system were identified for flagella/motility in *Geobacter*.

Conclusion: A novel multicomponent His-Asp phosphorelay system controls flagella/motility in *Geobacter*.

Significance: Elucidating the mechanisms of signal transduction systems for flagella/motility advances the understanding of cellular responses to environments.

***Geobacter* species play an important role in the natural biogeochemical cycles of aquatic sediments and subsurface environments as well as in subsurface bioremediation by oxidizing organic compounds with the reduction of insoluble Fe(III) oxides. Flagellum-based motility is considered to be critical for *Geobacter* species to locate fresh sources of Fe(III) oxides. Functional and comparative genomic approaches, coupled with genetic and biochemical methods, identified key regulators for flagellar gene expression in *Geobacter* species. A master transcriptional regulator, designated FgrM, is a member of the enhancer-binding protein family. The *fgrM* gene in the most studied strain of *Geobacter* species, *Geobacter sulfurreducens* strain DL-1, is truncated by a transposase gene, preventing flagellar biosynthesis. Integrating a functional FgrM homolog restored flagellar biosynthesis and motility in *G. sulfurreducens* DL-1 and enhanced the ability to reduce insoluble Fe(III) oxide. Interrupting the *fgrM* gene in *G. sulfurreducens* strain KN400, which is motile, removed the capacity for flagellar production and inhibited Fe(III) oxide reduction. FgrM, which is also a response regulator of the two-component His-Asp phosphorelay system, was phosphorylated by histidine kinase GHK4, which was essential for flagellar production and motility. GHK4, which is a hybrid kinase with a receiver domain at the N terminus, was phosphorylated by another histidine kinase, GHK3. Therefore, the multicomponent His-Asp phosphorelay system appears to control flagellar gene expression in *Geobacter* species.**

Motility is considered to be an important attribute that permits *Geobacter* species to effectively compete for Fe(III) in subsurface environments. The current model for Fe(III) reduction by *Geobacter* species in the subsurface (1–3) is that *Geobacter* species are primarily planktonic when electron donors are plentiful. This is because Fe(III) minerals are heterogeneously dispersed in soils and sediments, and thus, once the Fe(III) in one microenvironment is reduced, *Geobacter* species must search for another source of Fe(III). This contrasts with reduction of soluble electron acceptors, which will continuously diffuse toward a zone in which they are being depleted. It is speculated that *Geobacter* species are able to continue respiration in the planktonic state because the abundant *c*-type cytochromes function as capacitors (4, 5), accepting electrons derived from electron donor oxidation as the cells transition between Fe(III) sources. Once a new Fe(III) source is located, the electrons are discharged, presumably via conductive pili (5, 6) and associated cytochromes (7).

Genes for flagellar production are highly conserved in the available genomes of *Geobacter* species, further suggesting the importance of motility (8, 9). The observation that *Geobacter metallireducens* produces flagella during growth on Fe(III) oxide, but not soluble chelated Fe(III) (10), suggests that flagellar expression is regulated based on the physiological status of the cell.

Despite the importance of motility to *Geobacter* species, there is no information on the molecular mechanisms that control the expression of flagella. Until recently, investigations on motility were stymied by the fact that *Geobacter sulfurreducens* strain DL-1, the only strain of *Geobacter* species that could be genetically manipulated (11), does not produce flagella (12), despite possessing the full complement of genes for flagellar production found in other *Geobacter* species (8, 9, 13). However, *G. sulfurreducens* strain KN400, which was recovered from electrodes poised at low potential (14), does produce flagella. The genome of this strain is available (15), and it can be genetically manipulated with the

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[S] This article contains supplemental Figs. S1–S6, Tables S1 and S2, and additional references.

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same procedures that are commonly employed with *G. sulfurreducens* strain DL-1.

In other bacteria, a highly regulated transcriptional hierarchy controls expression of flagellar genes to ensure that flagella are assembled in a highly ordered manner (16–18). However, the mechanisms for transcriptional control vary. For example, in Enterobacteriaceae with lateral flagella, the master regulator FlhDC is at the top of the regulatory hierarchy (19). FlhDC activates flagellar genes whose expression is dependent on the RNA polymerase σ factor RpoD. In contrast, in Pseudomonadaceae and Vibrionaceae, which have a polar flagellum, the master regulators are FleQ (20) and FlrA (21), respectively. FleQ and FlrA are members of the enhancer-binding protein (EBP)[2] family. EBPs typically consist of three domains: an N-terminal sensory (regulatory) domain, a central AAA/σ^{54} activation domain, and a C-terminal DNA-binding domain (22, 23). The N-terminal domains of FleQ and FlrA show some similarity to the receiver domain of the response regulator of the two-component His-Asp phosphorelay system but lack highly conserved residues such as a phosphorylation site (20, 21). Thus, they are not likely to be response regulators. The two-component His-Asp phosphorelay system is known to be an important molecular signaling device for sensing and responding to environmental changes. The two-component system typically consists of a sensor histidine kinase, which senses environmental signals, and a response regulator, which often exerts gene expression necessary for the adaptation (24). The sensor histidine kinase autophosphorylates its own histidine residue upon signal sensing and subsequently transfers the phosphoryl group to the aspartate residue in the receiver domain of the response regulator. Phosphorylation of the receiver domain results in the activation of the effector (output) domain of the response regulator. FleQ and FlrA activate flagellar genes whose expression is RpoN-dependent, consistent with the fact that EBPs generally promote transcription of RpoN-dependent genes (22, 23). It has previously been suggested that the expression of some flagellar genes in *G. sulfurreducens* may be RpoN-dependent (25, 26).

Another σ factor, FliA, is involved in the expression of additional flagellar genes in some microorganisms (16–18). The master regulator FlhDC in Enterobacteriaceae or FlrA in Vibrionaceae controls expression of *fliA* (19). In contrast, in Pseudomonadaceae, *fliA* expression is independent of the master regulator FleQ (27). Genome sequence analysis revealed a homolog of FliA, but no homolog of FlhDC, FleQ, or FlrA in *Geobacter* species (8, 9, 13).

Here, we report on studies with *G. sulfurreducens* that identify the multicomponent His-Asp phosphorelay system regulating flagellar gene expression in *Geobacter* species. This is an important step in identifying the environmental cues controlling flagellar expression and motility in *Geobacter* species.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—G. sulfurreducens strains DL-1 (12) and KN400 (14) were grown anaerobically in NBAF medium (acetate and fumarate as the electron donor and acceptor, respectively) or FWA medium (acetate as the electron donor) containing Fe(III) citrate or Fe(III) oxide as the electron acceptor (11). The medium was supplemented with the appropriate antibiotics when necessary.

Cell growth was monitored by measuring the absorbance at 600 nm when fumarate served as the electron acceptor. The concentrations of Fe(II) in the cultures containing Fe(III) as the electron acceptor were determined by the FerroZine assay (28). Swimming motility was examined on soft agar (0.3%) plates by spotting 10-μl cultures. The FWA medium containing Fe(III) citrate for soft agar plates was supplemented with 0.0008% (w/v) $CaCl_2$ and 0.002% (w/v) $MgSO_4$. Plate manipulations were conducted at 30 °C in an anaerobic glove box containing a $N_2/CO_2/H_2$ (83:10:7) atmosphere. *Escherichia coli* DH5α (29) was used for plasmid preparation and grown in LB medium (30) supplemented with the appropriate antibiotics when necessary.

DNA Binding Assay—The DNA fragment containing the *flhA* promoter region used as a probe was prepared by PCR with primers flhA-pro1 and flhA-pro2 (supplemental Table S1) and labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. DNA binding assays with EBPs were carried out as described previously (31). The *fgrM* gene from the KN400 strain was amplified by PCR with primers fgrM-DB1 and fgrM-DB2 (supplemental Table S1) and cloned in pET24b (Novagen). FgrM protein was prepared with a histidine tag at the C terminus as described previously (32).

Integration of fgrM Gene from KN400 Strain into DL-1 Strain—The *fgrM* gene from the KN400 strain was amplified by PCR with primers fgrM-int1 and fgrM-int2 (supplemental Table S1) and cloned in pET24b. The resultant plasmid was integrated into the chromosome of the *G. sulfurreducens* DL-1 strain (supplemental Fig. S1*A*) by electroporation (11).

RT-PCR—Total RNA was prepared from *G. sulfurreducens* DL-1 strains grown in NBAF medium. cDNA was prepared using reverse transcriptase with primers fgrM-RTPCR1 (*fgrM*), flhA-RTPCR1 (*flhA*), cheW1-RTPCR1 (*cheW1*), and dcuB-RTPCR1 (*dcuB*) (supplemental Table S1). cDNA thus prepared was amplified by PCR with primers fgrM-RTPCR1 and fgrM-RTPCR2 (*fgrM*), flhA-RTPCR1 and flhA-RTPCR2 (*flhA*), cheW1-RTPCR1 and cheW1-RTPCR2 (*cheW1*), and dcuB-RTPCR1 and dcuB-RTPCR2 (*dcuB*) (supplemental Table S1). The amplified DNA fragments were analyzed by agarose gel electrophoresis, followed by staining with EtBr.

Interruption of fgrM Gene in KN400 Strain—The internal region of the *fgrM* gene from the KN400 strain was amplified by PCR with primers fgrM-int3 and fgrM-int4 (supplemental Table S1) and cloned in pBluescript II KS(−) (Stratagene). The resultant plasmid was integrated into the chromosome of the KN400 strain by electroporation (11), and the *fgrM* gene was interrupted (supplemental Fig. S1*B*).

Transmission Electron Microscopy—Cells were grown in NBAF liquid medium. The cells were placed on 400-mesh carbon-coated copper grids, incubated for 2 min, and then stained with 2% uranyl acetate. Cells were observed using a JOEL 100 transmission electron microscope at an accelerating voltage of

[2] The abbreviation used is: EBP, enhancer-binding protein.

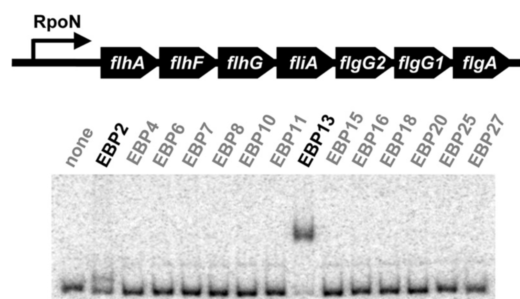

FIGURE 1. **Identification of transcription factor involved in regulation of flagellar gene expression in *G. sulfurreducens* DL-1.** DNA binding assays were conducted with the promoter region of the *flhA* operon containing *flhA*, *flhF*, *flhG*, *fliA*, *flgG2*, *flgG1*, and *flgA* (*upper*). EBPs conserved in *Geobacter* species were tested for their DNA-binding activity for the *flhA* promoter (*lower*).

80 kV. Images were taken digitally using MaxIm DL software and analyzed using ImageJ (rsbweb.nih.giv/ij/index.html).

Construction of Deletion Mutants of Histidine Kinases—Genes for GHK1–8 (Geobacter histidine kinase) were replaced with a spectinomycin resistance gene. Double-crossover homologous recombination was carried out by electroporation with the linear DNA fragment consisting of the spectinomycin resistance gene flanked by 0.7-kilobase pair DNA fragments containing the upstream and downstream regions of the kinase domains. These flanking DNA fragments were amplified by PCR with the primers listed in supplemental Table S1. The DNA fragment of the spectinomycin resistance gene was amplified by PCR with primers Sp-fwd and Sp-rev (supplemental Table S1) using pSJS985 (33) as a template. The replacement was confirmed by PCR amplification.

In Vitro Phosphorylation Assays—The DNA fragments encoding GHK3-K (Ala^{226}–Gln^{499}), GHK4-R (Met^{1}–Glu^{124}), GHK4-K (Met^{424}–Arg^{727}), and FgrM-R (Met^{1}–His^{121}), where "K" is kinase domain and "R" is receiver domain, were amplified by PCR with the primers listed in supplemental Table S1. The PCR products were cloned into pET24b. The cloned genes were overexpressed using an autoinduction system (Novagen) as instructed by the manufacturer, and the proteins were prepared with a histidine tag at their C termini. Purification of these proteins was performed as described previously (34). *In vitro* phosphorylation assays were performed with [γ-^{32}P]ATP as described previously (31). Kinases (10 pmol) were incubated at room temperature for 5 min for the autophosphorylation reaction. Response regulators (10 pmol) were then added and further incubated at room temperature for 5 min for the phosphotransfer reaction.

RESULTS

Master Regulator for Flagellar Gene Expression—Sequence analysis identified genes involved in flagellar biosynthesis and motility that were predicted to be controlled by RpoN (supplemental Fig. S2*A*) or FliA (supplemental Fig. S2*B*). Among them, the *flhA* operon appears to be a key to flagellar gene regulation in *Geobacter* species, as it contains the gene encoding FliA (Fig. 1).

RpoN is known to require an EBP for transcription initiation (22, 23), suggesting an additional level of regulation of expression of the flagellar genes. Therefore, the 14 previously described EBPs that are conserved in the genomes of *G. sulfurreducens* and two flagellum-producing species, *G. metallireducens* and *Geobacter uraniireducens* (31), were tested for their ability to bind the *flhA* promoter region of *G. sulfurreducens* DL-1 (Fig. 1). EBP2 and EBP13 both bound this region (Fig. 1). It should be noted that the EBP2-*flhA* promoter complex migrated much faster than the EBP13-*flhA* promoter complex in the DNA binding assay because, as described below, EBP2 is smaller than EBP13. The DNA-binding activity of EBP2 and EBP13 appears to be specific, as EBP2 and EBP13 did not bind other promoters known to be involved in other cellular functions such as nitrogen fixation (31).

EBP2 in *G. sulfurreducens* DL-1 has a DNA-binding domain but lacks the regulatory domain and most of the AAA/σ^{54} activation domain, which are typically found in the EBP family and necessary for transcription initiation with RpoN. In contrast, EBP2 homologs in other *Geobacter* species have all three domains (supplemental Figs. S3 and S4). The EBP2 homologs appear to have a receiver domain of the response regulator in the two-component His-Asp phosphorelay system as the regulatory domain. In *G. sulfurreducens* DL-1, a gene (GSU0298) encoding a regulatory domain and an AAA/σ^{54} activation domain that are homologous to those of the EBP2 homologs in other *Geobacter* species is located upstream of a gene encoding a transposase that is located upstream of the *EBP2* gene (supplemental Fig. S3). Thus, it appears that insertion of a transposase might have disrupted the *EBP2* gene, which could have the consequence of preventing transcription of key flagellar genes.

In contrast to the *EBP2* gene of *G. sulfurreducens* DL-1, the EBP2 homolog of *G. sulfurreducens* KN400, which produces flagella, contains all three domains (supplemental Fig. S3). The EBP2 homolog of *G. sulfurreducens* KN400 bound the *flhA* promoter region from the DL-1 strain in the DNA binding assay (Fig. 2*B*).

To assess the effects of the *EBP2* gene from the KN400 strain on flagellar gene expression in the DL-1 strain, it was integrated into the chromosome of the DL-1 strain. The expression of the KN400 *EBP2* gene in the DL-1 strain was confirmed with a RT-PCR assay (Fig. 2*C*). The integration of the KN400 *EBP2* gene induced the expression of the *flhA* gene and other flagellum-related genes (Fig. 2*C*).

Furthermore, the expression of genes such as the *cheW* operon containing chemotaxis genes, which appears to be controlled by FliA (Fig. 2*A* and supplemental Fig. S2*B*), was also activated by the integration of the KN400 *EBP2* gene in *G. sulfurreducens* DL-1 (Fig. 2*C*). In contrast, the expression of the dicarboxylic acid transporter gene *dcuB*, which has RpoN-dependent promoter elements (25), was not affected (Fig. 2*C*). These results suggest that the *EBP2* gene is malfunctioning in the wild-type *G. sulfurreducens* DL-1 strain and that EBP2 serves as a master regulator for flagellar gene expression because EBP2 not only directly regulated the expression of flagellar genes such as *flhA* and *fliA* but also influenced other flagellum-related genes, including *cheW1*, that are likely to be regulated by FliA (Fig. 2*A*). Therefore, EBP2 has been termed FgrM (flagellar gene regulator, master).

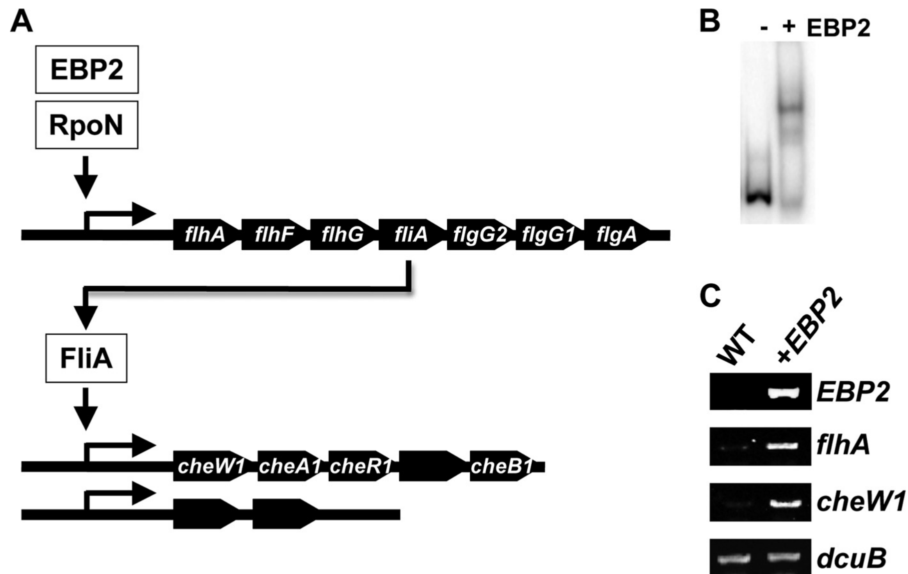

FIGURE 2. **Induction of flagellum-related genes by KN400 EBP2 in *G. sulfurreducens* DL-1.** *A*, model for regulatory cascades in flagellar gene expression in *Geobacter* species. *B*, DNA-binding activity. EBP2 from the KN400 strain was tested with the DNA fragment containing the *flhA* promoter region from the DL-1 strain in DNA binding assays. *C*, expression of flagellum-related genes. Expression of *EBP2*, *flhA*, and *cheW1* was examined by RT-PCR assays in the wild-type DL-1 strain (*WT*) and the DL-1 strain containing the complete *EBP* gene (+*EBP2*). As a control, the expression of the *dcuB* gene was examined.

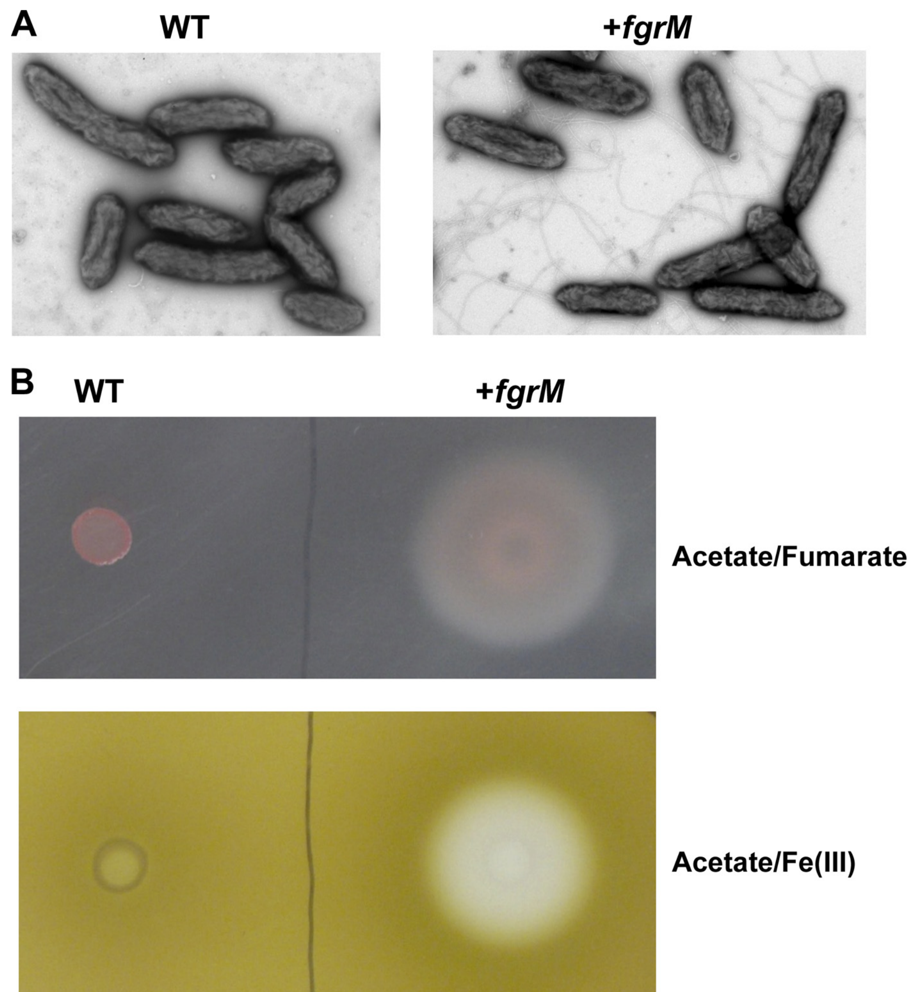

FIGURE 3. **Restoration of flagellar biosynthesis and motility by complete *fgrM* gene in *G. sulfurreducens* DL-1.** *A*, flagellar biosynthesis. The wild-type DL-1 cells (*WT*) and the DL-1 cells containing the complete *fgrM* gene (+*fgrM*) were grown in NBAF liquid medium and observed under a transmission electron microscope. *B*, motility. The wild-type DL-1 cells and the DL-1 cells containing the complete *fgrM* gene were spotted on agar (0.3%) plates containing NBAF or FWA medium supplemented with Fe(III) citrate.

Restoration of Flagellar Biosynthesis—Induced expression of the flagellar genes following the introduction of a complete *fgrM* gene was accompanied by production of flagella (Fig. 3*A*) and motility that could be observed on soft agar plates (Fig. 3*B*). When fumarate was used as the electron acceptor, periodic concentric rings were formed on the soft agar plate by the flagellum-proficient DL-1 strain (Fig. 3*B*). There was a larger zone of Fe(III) reduction on the soft agar plates containing Fe(III) citrate as the electron acceptor with the flagellum-proficient DL-1 strain than with the wild-type DL-1 strain (Fig. 3*B*). These results indicate that the repaired flagellar biosynthesis restored motility in the DL-1 strain.

Introduction of the uninterrupted *fgrM* gene impacted on growth with various electron acceptors. For example, the initial transfer of *G. sulfurreducens* DL-1 from medium with fumarate as the electron acceptor to medium with Fe(III) oxide as the electron acceptor typically resulted in a long lag period, which was significantly diminished in the strain with the uninterrupted *fgrM* gene (Fig. 4*A*). In contrast, the DL-1 strain with the uninterrupted *fgrM* gene reduced Fe(III) more slowly than the wild-type DL-1 strain in medium with soluble Fe(III) citrate as the electron acceptor (Fig. 4*B*) and grew slightly slower and reached a lower final culture density than the wild-type DL-1 strain with the soluble fumarate electron acceptor (Fig. 4*C*).

To further evaluate the FgrM function, the uninterrupted *fgrM* gene in *G. sulfurreducens* KN400 was interrupted. Interruption of the *fgrM* gene resulted in a strain that did not express *flhA* or *cheW1* (Fig. 5*A*), no longer produced flagella (Fig. 5*B*), was nonmotile (Fig. 5*C*), and was impaired in its capacity for Fe(III) oxide reduction (Fig. 5*D*). These results further support the conclusion that FgrM is the master regulator for flagellar gene expression in *Geobacter* species.

Identification of Histidine Kinase Involved in Motility and Flagellar Biosynthesis—FgrM appears to belong to a response regulator of the two-component His-Asp phosphorelay system (supplemental Fig. S4), and thus, it is likely that a sensor histidine kinase regulates the activity of FgrM by phosphorylation. Genes for a two-component system are often co-localized in the same operon in other bacteria, but this is often not the case in *Geobacter* species (8, 9, 13). No genes for a putative sensor histidine kinase are found in the region near the *fgrM* gene in the genomes of *Geobacter* species. The genomes of *Geobacter* species encode an unusually large number of genes for two-component signaling proteins (8, 9, 13). A histidine kinase for FgrM is likely conserved in *Geobacter* species, as for FgrM. To identify such a histidine kinase, eight histidine kinases (GHK1–8) highly conserved in *Geobacter* species were selected from 95 putative histidine kinases encoded in the genome of *G. sulfurreducens* DL-1 by analyzing amino acid sequences of all of the putative histidine kinases (supplemental Table S2). These histidine kinases were inactivated in the DL-1 strain containing the uninterrupted *fgrM* gene, and the motility of these mutants was examined on a soft agar plate. The GHK4 and GHK5 mutants did not exhibit motility, whereas other mutants did (Fig. 6*A*). The GHK5 mutant became motile when incubated for a longer period on the soft agar plate, whereas the GHK4 mutant still did not (supplemental Fig. S5). The lack of motility of the GHK4 mutant was likely due to the absence of flagella (Fig. 6*B*). In contrast, the GHK5 mutant produced flagella (Fig. 6*B*). It is likely that GHK4 was involved in transcription of genes for flagella and motility, as transcripts for *flhA* and *cheW1* were not detected in the GHK4 mutant (Fig. 6*C*). These results suggest that GHK4 is involved in the transcriptional activation of genes for flagella and motility.

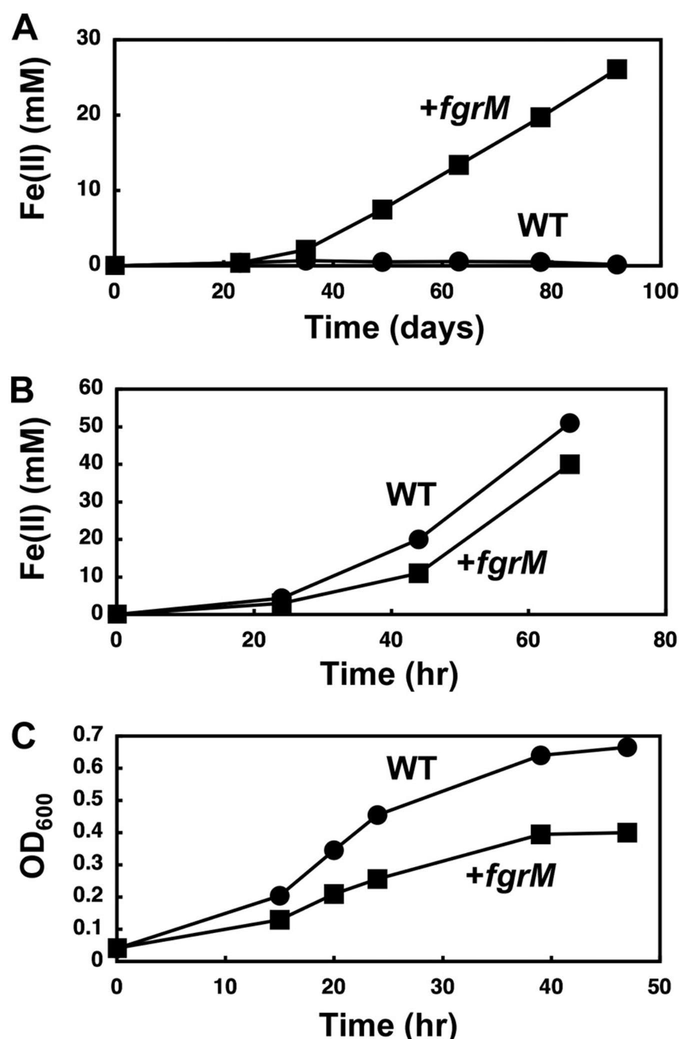

FIGURE 4. **Effects of flagellar biosynthesis on growth with soluble or insoluble electron acceptors.** *A*, insoluble Fe(III) oxide. The wild-type DL-1 strain (*WT*) and the DL-1 strain containing the complete *fgrM* gene (+*fgrM*) were grown in FWA liquid medium containing Fe(III) oxide. Reduction of Fe(III) was monitored by measuring the concentration of Fe(II). *B*, soluble Fe(III) citrate. The wild-type DL-1 strain and the DL-1 strain containing the complete *fgrM* gene were grown in FWA liquid medium containing Fe(III) citrate. Reduction of Fe(III) was monitored by measuring the concentration of Fe(II). *C*, fumarate. The wild-type DL-1 strain and the DL-1 strain containing the complete *fgrM* gene were grown in NBAF liquid medium. Growth was monitored as A_{600}.

His-Asp Phosphorelay—To elucidate the relationship between GHK4 and FgrM in the regulation of flagellar gene expression, the possibility of GHK4 and FgrM functioning as a two-component system was assessed because mutants of GHK4 or FgrM exhibited similar phenotypes, no expression of genes for flagella and motility, no flagellar biosynthesis, and lack of motility, as shown above. The kinase domain of GHK4 (GHK4-K) and the receiver domain of FgrM (FgrM-R) were

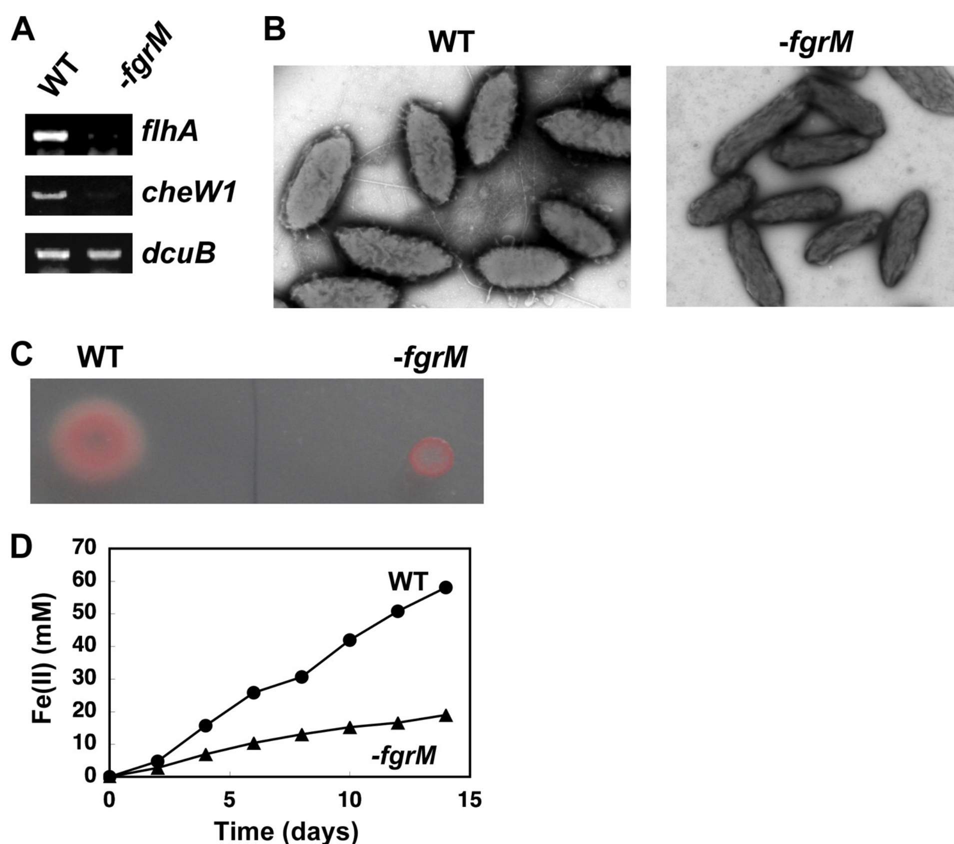

FIGURE 5. **Effects of interruption of *fgrM* gene.** *A*, expression of flagellum-related genes. Expression of *flhA* and *cheW1* was examined by RT-PCR assays in the wild-type KN400 strain (*WT*) and the KN400 strain containing the interrupted *fgrM* gene (−*fgrM*). As a control, the expression of the *dcuB* gene was examined. *B*, flagellar biosynthesis. The wild-type KN400 cells and the KN400 cells containing the interrupted *fgrM* gene were grown in NBAF liquid medium and observed under a transmission electron microscope. *C*, Motility. The wild-type KN400 cells and the KN400 cells containing the interrupted *fgrM* gene were spotted on agar (0.3%) plates containing NBAF medium. *D*, reduction of insoluble Fe(III) oxide. The wild-type KN400 strain and the KN400 strain containing the interrupted *fgrM* gene were grown in FWA liquid medium containing Fe(III) oxide. Reduction of Fe(III) was monitored by measuring the concentration of Fe(II).

purified and examined by *in vitro* phosphorylation assays. GHK4-K showed autophosphorylation activity and phosphorylated FgrM-R (Fig. 7*A*). These results suggest that GHK4 and FgrM function as a two-component system.

GHK4 has a domain homologous to the receiver domain at the N terminus (Fig. 7*B*). The gene for GHK3 is located immediately upstream of the gene for GHK4 on the genome, and GHK3 is also conserved in *Geobacter* species (supplemental Table S2). Thus, it seemed possible that GHK3 might be the kinase for GHK4. To evaluate this possibility, GHK3-K and GHK4-R were purified and examined by *in vitro* phosphorylation assays. GHK3-K exhibited autophosphorylation activity as well as phosphotransfer activity to GHK4-R (Fig. 7*C*), suggesting that GHK3 is involved in the regulatory network containing GHK4 and FgrM. Therefore, it is likely that the His-Asp phosphorelay system mediated by GHK3, GHK4, and FgrM controls flagellar gene expression in *Geobacter* species.

DISCUSSION

In this study, we identified important regulators for the synthesis of flagella in *Geobacter* species. The FgrM master transcriptional regulator for the synthesis of flagella in *Geobacter* species is an RpoN-dependent EBP, as found in other bacteria such as Pseudomonadaceae and Vibrionaceae (16–18). However, unlike these other bacterial EBPs, FgrM appears to be a response regulator of the two-component His-Asp phosphorelay system and to be activated via phosphorylation by the histidine kinase GHK4, which is also essential for the expression of genes for flagella and motility. GHK4 has a receiver domain at the N terminus and was phosphorylated *in vitro* by GHK3, another histidine kinase conserved in *Geobacter* species. This suggests that GHK4 is regulated by GHK3. However, the GHK3 mutant was able to produce flagella and was motile (Fig. 6, *A* and *B*), unlike the GHK4 and FgrM mutants. The role GHK3 plays in the regulation of flagellar gene expression remains to be elucidated. A putative sensor domain of GHK3 shows similarity to the PAS domain, whereas that of GHK4 shows similarity to the PAS and GAF domains (supplemental Table S2). However, they are not highly homologous to known proteins. The PAS domain is a signaling module known to sense light, oxygen, redox, and small molecules (35, 36). The GAF domain is a ubiquitous signaling module known to bind small molecules such as cyclic nucleotide monophosphates, tetrapyrroles, and formate (37, 38). Characterization of these domains should provide critical information for an environmental cue that regulates flagellar biosynthesis. It appears likely that GHK5 is involved in motility, but not in flagellar biosynthesis (Fig. 6, *A* and *B*). Elucidation of the function of GHK5 would further advance the

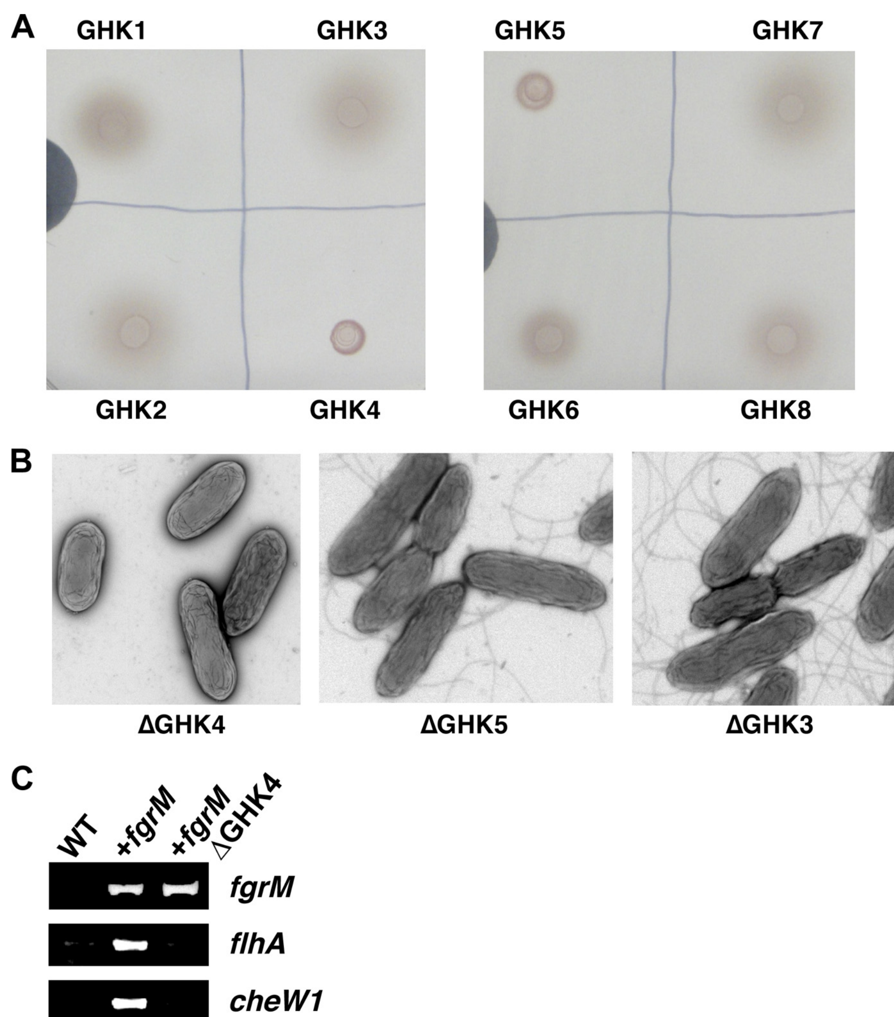

FIGURE 6. **Identification of histidine kinase involved in flagellar biosynthesis.** *A*, motility. Mutants were spotted on agar (0.3%) plates containing NBAF medium. *B*, flagellar biosynthesis. Mutants were grown in NBAF liquid medium and observed under a transmission electron microscope. *C*, expression of flagellum-related genes. Expression of *fgrM*, *flhA*, and *cheW1* was examined by RT-PCR assays in the wild-type DL-1 strain (*WT*), the DL-1 strain containing the complete *fgrM* gene (+*fgrM*), and the DL-1 strain containing the complete *fgrM* gene but lacking the *GHK4* gene (+*fgrM*Δ*GHK4*).

understanding of the regulatory network in motility of *Geobacter* species.

The transcriptional regulatory system for the synthesis of flagella in *Geobacter* species also appears to employ the σ factor FliA, as found in other bacterial systems (16–18). It is likely that expression of the *fliA* gene is under the control of FgrM in *Geobacter* species, as it is in other bacteria such as Enterobacteriaceae and Vibrionaceae. Predicted target genes of FliA include genes for the final steps of flagellar assembly, as well as the chemotaxis signaling system, including chemoreceptors (methyl-accepting chemotaxis proteins). The presence of four *cheA* genes in the *G. sulfurreducens* genome suggests that *G. sulfurreducens* possesses multiple chemotaxis systems or homologs (26). The Che1 system appears to be involved in motility in *G. sulfurreducens*, as it is likely to be under the control of the transcriptional regulatory system mediated by FgrM and FliA. These findings will make it possible to study chemotaxis in *Geobacter* species in a more systematic manner. In addition to FgrM, RpoN, and FliA, there may be additional transcription factors involved in the regulation of flagellar biosynthesis in *Geobacter* species. EBP13 also bound the *flhA* promoter region in the DNA binding assay, but its function remains to be investigated.

The interruption of the *fgrM* gene in *G. sulfurreducens* DL-1 by insertion of a transposase must be a relatively recent phenomenon, as the KN400 strain of the same species contains an uninterrupted gene, as does the closely related *G. metallireducens*. A similar interruption of the *fgrM* homolog by a transposase is found in *Pelobacter propionicus* (supplemental Fig. S6), which is phylogenetically intertwined with *Geobacter* species (39, 40).

G. sulfurreducens DL-1 was enriched and isolated with soluble chelated Fe(III) (12), whereas *G. metallireducens* was enriched and purified by dilution in medium with insoluble Fe(III) oxide (41). *G. metallireducens* cells specifically produce flagella when grown on insoluble Fe(III), but not soluble Fe(III) citrate (10). As shown here, expressing the *fgrM* gene decreased the rate of growth on soluble electron acceptors, suggesting that the original method for enriching and isolating *G. sulfurreducens* may have selected for a strain that did not express *fgrM*. Further investigation into the mechanisms by which environmental signals and regulatory

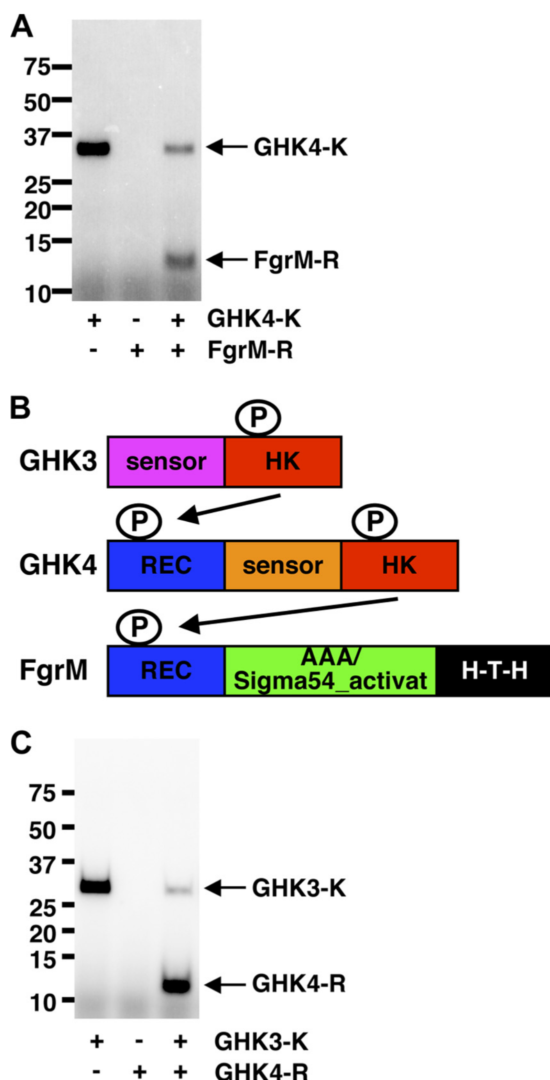

FIGURE 7. **His-Asp phosphorelay.** *A*, *in vitro* phosphorylation assay with GHK4-K and FgrM-R. GHK4-K and FgrM-R were tested for autophosphorylation and phosphotransfer activity *in vitro*. The molecular mass standards are shown in kilodaltons. *B*, model for phosphorelay. *HK*, histidine kinase; *REC*, receiver domain; *AAA/Sigma54_activat*, AAA/σ^{54} activation domain. *C*, *in vitro* phosphorylation assay with GHK3-K and GHK4-R. GHK3-K and GHK4-R were tested for autophosphorylation and phosphotransfer activity *in vitro*. The molecular mass standards are shown in kilodaltons.

proteins control flagellar gene expression and other potential phenotypic impacts of flagellar expression in *Geobacter* species is warranted.

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