Novel Regulatory Cascades Controlling Expression of Nitrogen Fixation Genes

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Novel regulatory cascades controlling expression of nitrogen-fixation genes in *Geobacter sulfurreducens*

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**ABSTRACT**

*Geobacter* species often play an important role in bioremediation of environments contaminated with metals or organics and show promise for harvesting electricity from waste organic matter in microbial fuel cells. The ability of *Geobacter* species to fix atmospheric nitrogen is an important metabolic feature for these applications. We identified novel regulatory cascades controlling nitrogen-fixation gene expression in *Geobacter sulfurreducens*. Unlike the regulatory mechanisms known in other nitrogen-fixing microorganisms, nitrogen-fixation gene regulation in *G. sulfurreducens* is controlled by two two-component His–Asp phosphorelay systems. One of these systems appears to be the master regulatory system that activates transcription of the majority of nitrogen-fixation genes and represses a gene encoding glutamate dehydrogenase during nitrogen fixation. The other system whose expression is directly activated by the master regulatory system appears to control by antitermination the expression of a subset of the nitrogen-fixation genes whose transcription is activated by the master regulatory system and whose promoter contains transcription termination signals. This study provides a new paradigm for nitrogen-fixation gene regulation.

**INTRODUCTION**

*Geobacter* species are predominant microorganisms in subsurface environments in which organic compounds such as acetate are oxidized with the reduction of metals including Fe(III) (1,2). *Geobacter* species play a pivotal role in the bioremediation of organic or metal contaminants in subsurface environments (3–9). *Geobacter* species are also considered to play an important part in the biogeochemical cycles in subsurface environments (1,10). One of the factors contributing to the capability of *Geobacter* species to be such effective competitors in a diversity of subsurface environments may be their ability to fix atmospheric nitrogen (11–15). Analyses of transcript abundance demonstrated that *Geobacter* species expressed genes for nitrogen fixation in petroleum-contaminated subsurface sediments and uranium-contaminated groundwater (14,16,17). This suggests that nitrogen fixation was an important process in these environments. Moreover, *Geobacter* species show promise for harvesting electricity from waste organic matter in microbial fuel cells (18–20). *Geobacter* species are often the predominant microorganisms colonizing anodes of microbial fuel cells harvesting electricity from sediments or simulated wastewater (21–25) and the ability to fix nitrogen might aid in growth deep within the thick anode biofilms (26,27) where nutrient access might be limiting. Nitrogen fixation was also demonstrated with *Geobacter* isolates, *Geobacter metallireducens* (12) and *Geobacter sulfurreducens* (13). These *Geobacter* species fix nitrogen gas present in the headspace of the pressure tubes containing liquid medium devoid of fixed nitrogen. The disruption of the *nifD* gene encoding a subunit of nitrogenase eliminated the ability of *G. sulfurreducens* to grow in this medium (13).

Microbial nitrogen fixation is an important component of the global nitrogen cycle. Molecular mechanisms regulating nitrogen-fixation gene expression have been well characterized in some nitrogen-fixing microorganisms (diazotrophs) (28). In the *Proteobacteria* characterized, nitrogen-fixation genes are invariably subject to transcriptional activation by the master regulator of nitrogen fixation, NifA, a member of the enhancer-binding protein (EBP) family, together with the RNA polymerase sigma factor, RpoN (28,29). Transcription initiation by RNA polymerase containing the sigma factor RpoN (RNAP/RpoN) requires a transcription factor EBP (30–33). EBPs typically consist of three domains: an N-terminal sensory (regulatory) domain; a central ATPase domain; and a C-terminal DNA-binding domain. NifA has a GAF domain at the N-terminus.

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The GAF domain is a ubiquitous signaling module known to bind small molecules such as cyclic nucleotide monophosphates, tetrapyrroles and formate (34,35). The expression of the nifA gene is controlled by the two-component His–Asp phosphorelay system in these proteobacterial diazotrophs. For instance, *Klebsiella pneumoniae* utilizes the histidine kinase NtrB and the response regulator NtrC, which is also a member of the EBP family. The two-component His–Asp phosphorelay system is a major scheme in bacterial signal transduction pathways during responses 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 (36). 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.

Although the ability of *Geobacter* species to fix nitrogen appears to be a key to growth in subsurface environments, as described above, little is known about regulatory mechanisms for nitrogen fixation in *Geobacter* species. The genome sequence analyses of *Geobacter* species, unlike other proteobacterial diazotrophs. Here we report novel regulatory cascades controlling gene expression during nitrogen fixation in *G. sulfurreducens* that consist of two two-component His–Asp phosphorelay systems, which regulate transcriptional activation, repression and antitermination of genes involved in nitrogen metabolism.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

*Geobacter sulfurreducens* DL1 (37) was used for genetic and biochemical studies. *Geobacter sulfurreducens* strains were grown anaerobically in NBAF medium (acetate and fumarate as the electron donor and the acceptor, respectively) (11), unless otherwise described. Proper antibiotics were supplemented when necessary. Nitrogen fixation was examined with NH$_4^+$-free NBAF media as described previously (15). Cell growth was monitored by measuring the optical density at 600 nm (OD$_{600}$). *Escherichia coli* DH5Δ (38) was used for plasmid preparation and grown in LB medium (39) supplemented with proper antibiotics when necessary.

**Primer extension assay**

Total RNA was prepared from the wild type and the mutants grown in the media indicated in the figure legends by the hot-phenol method. The primers used in the assays are listed in Supplementary Table S1.

**Construction of EBP expression vectors and purification of EBPs**

DNA fragments encoding EBPs were amplified by PCR with primers listed in Supplementary Table S1. PCR products were digested with restriction enzymes and cloned in pET24b (Novagen). EBPs were overexpressed by Autoinduction system (Novagen) as instructed by the manufacturer and prepared as histidine-tagged proteins at the C-terminus as described previously (40).

**DNA-binding assay**

Promoter regions of genes *gnfK, nifH, glnB, gdhA* and *GSU3409* were used as probes in DNA-binding assays. The DNA fragments containing their promoter regions were prepared by PCR with primers listed in Supplementary Table S1. DNA fragments were labeled with [$\gamma$-32P]ATP by T4 polynucleotide kinase. DNA-binding assays were carried out as described previously (41).

**Footprint assay**

DNA fragments containing the region from nt $-243$ to $-3$ of the *gnfK* promoter or from nt $-223$ to $+66$ of the *gdhA* promoter were amplified with primers listed in Supplementary Table S1 and cloned in the plasmid vector. DNA fragments for the probe were prepared by digesting the plasmid with *SacI* and *XhoI* located in the cloning vector and isolating the fragment with gel electrophoresis. The isolated DNA fragments were then labeled with [$\gamma$-32P]dATP by Klenow fragment of DNA polymerase I. Thus, only the top strand was labeled. DNA-binding reactions were conducted with different amounts of GnfM and DNase I footprint assays were conducted as described previously (41).

**lacZ fusion assay**

For the WT construct of *gnfK*, the promoter region from nt $-243$ to $+32$ of *gnfK* was amplified by PCR with primers $-243T$ and $+32B$. The PCR product was digested with restriction enzymes and cloned into the transcriptional *lacZ* fusion vector, pCMZKT (42). To examine the effects of the binding sites determined by the footprint assays on the promoter activity, the regions from nt $-128$ to $-112$ (BS1) and from $-105$ to $-89$ (BS2) were deleted. For the ΔBS1 construct, the promoter regions from nt $-243$ to $-129$ and from nt $-111$ to $+32$ were amplified by primers $-243T$ and $-129B$, and $-111T$ and $+32B$, respectively, and digested with *XbaI* and *EcoRI*, and *EcoRI* and *BamHI*, respectively. The *XbaI*-*EcoRI* and the *EcoRI*-*BamHI* fragment were ligated and cloned into pCMZKT. For the ΔBS2 construct, the promoter regions from nt $-243$ to $-106$ and from nt $-88$ to $+32$ were amplified by primers $-243T$ and $-106B$, and $-88T$ and $+32B$, respectively, and cloned as described for the ΔBS1 construct. For the ΔBS1ΔBS2 construct, the promoter regions from nt $-243$ to $-129$ and from nt $-88$ to $+32$ were amplified by primers $-243T$ and $-129B$, and $-88T$ and $+32B$, respectively, and cloned as described above.
The plasmids thus constructed were introduced into *G. sulfurreducens* DL1 by electroporation (13).

For the WT construct of *gdhA*, the promoter region from nt –84 to +6 of the *gdhA* gene was prepared by annealing oligonucleotides WT12T and WT12B and oligonucleotides WT34T and WT34B, and by ligating these annealed oligonucleotides. For the M_BS3/BS4 construct, oligonucleotides Mut34T and Mut34B were used instead of WT34T and WT34B. For the ΔBS1/BS2 construct, oligonucleotides WT34T and WT34B were used. These fragments were cloned into pCMZKT. The plasmids thus constructed were introduced into *G. sulfurreducens* DL1 by electroporation (13).

β-Galactosidase activity was measured by the method described and calculated by the formula given by Miller (39). The strains carrying the plasmids were grown in NBAF media supplemented with kanamycin in the presence or absence of NH4Cl. Presented values are averages of two independent experiments.

Promoter regions for testing the transcription termination signals were prepared by oligonucleotides containing an RpoD-dependent like –35/–10 elements without or with the wild type or mutated transcription termination signals from *nifH* (Supplementary Figure S5A). Oligonucleotides were phosphorylated with T4 polynucleotide kinase, annealed and ligated with pCMZKT (42). The resultant plasmids were transformed into *E. coli* and β-galactosidase activity was examined by plating on LB agar media containing kanamycin and X-gal.

**In vitro phosphorylation**

The DNA fragments encoding GnfLK from 103Met to 363Gln of GnfL (GSU1004), GnfM, GnfK (GSU0941) or GnfR (GSU2822) were amplified by PCR with primers listed in Supplementary Table S1. The PCR products were cloned into pET24b (Novagen). The cloned genes were overexpressed by Autoinduction system (Novagen) as instructed by the manufacturer and the proteins were prepared as a histidine-tagged protein at the C-terminus. Purification of these proteins was performed as described previously (40).

**In vitro phosphorylation**

Phosphorylation assays were performed as described previously (40) with modifications. Reactions were performed in the kinase buffer [25 mM Tris–HCl (pH 7.4); 10 mM MgCl2; 5 mM β-mercaptoethanol; 20 μM ATP; 2 μCi [γ-32P]ATP]. GnfLK (20 pmol) was incubated at room temperature for 5 min for the autophosphorylation reaction. GnfM (20 pmol) was then added and further incubated at room temperature for 5 min for the phosphotransfer reaction. GnfK (10 pmol) was incubated at a room temperature for 5 min for the autophosphorylation reaction. GnfR (10 pmol) was then added and further incubated at a room temperature for 5 min for the phosphotransfer reaction. The reactions were stopped by adding the stop solution (10% SDS; 0.4 M Tris–HCl (pH 6.8); 50% glycerol; 0.1 M β-mercaptoethanol; 0.02 % bromphenol blue). The samples were subjected to 10% SDS PAGE and analyzed by autoradiography.

**Overproduction of GnfLK and GnfM in *G. sulfurreducens***

An expression vector was constructed to overproduce GnfLK and GnfM in *G. sulfurreducens*. Oligonucleotides listed in Supplementary Table S1 were phosphorylated, annealed, and ligated with the plasmid pCD341 (43) at the EcoRI site. These oligonucleotides contain a ribosome binding site and an *Nde*I site for the initiation codon preceded by three stop codons in three open reading frames. After confirmation of proper sequence and orientation of the oligonucleotides in pCD341, the expression vector was designated pCDNdeII.

The DNA fragments encoding GnfLK or GnfM were amplified by PCR with primers listed in Supplementary Table S1. The PCR products were digested with *Bgl*II and *Xba*I or *Nde*I and *Xho*I, respectively, and ligated with pET24b (Novagen). For overexpression of GnfLK and GnfM together, after sequence confirmation of the PCR products, the DNA fragments encoding GnfLK or GnfM (with the ribosome binding site from pET24b) were isolated from the plasmids by digestion with *Eco*RI and *Xba*I or *Xho*I and *Hind*III, respectively, and ligated with pCDNdeII in one step. The plasmid containing the DNA fragments encoding GnfLK and GnfM was designated pCDNdeII/GnfLM. For overexpression of GnfM, the DNA fragment encoding GnfM was isolated from the plasmid by digestion with *Nde*I and *Hind*III, and ligated with pCDNdeII. The plasmid containing the DNA fragment encoding GnfM was designated pCDNdeII/GnfM.

Plasmids pCDNdeII, pCDNdeII/GnfLM, and pCDNdeII/GnfM were introduced into *G. sulfurreducens* DL1 by electroporation (13). Overproduction of GnfLK and GnfM was achieved by growing in NBAF media (in the presence of ammonium) supplemented with kanamycin and adding IPTG at a final concentration of 1 mM at a mid-log phase.

**Construction of gnfK or gnfR deletion mutants**

The *gnfK* gene was replaced with a kanamycin resistance gene, such that the coding region for amino acid residues from 13Asp to 164Leu was deleted. Double-crossover homologous recombination was carried out by electroporation (13) with the linear DNA fragment consisting of the kanamycin resistance gene flanked by 0.7-kb DNA fragments containing the upstream and the downstream regions of *gnfK*. These flanking DNA fragments were amplified by PCR with primers listed in Supplementary Table S1. The DNA fragment of the kanamycin resistance gene was amplified by PCR with primers listed in Supplementary Table S1, and pBRR1MCS-2 (44) as a template. The replacement was confirmed by PCR amplification.

The *gnfR* gene was replaced with the kanamycin resistance gene, such that the coding region for amino acid residues from 12Asp to 139Thr was deleted, as described above. The flanking DNA fragments were amplified by PCR with primers listed in Supplementary Table S1. The proper replacement was confirmed by PCR amplification.
RNA-binding assay

RNA fragments CCGAGAUAUUCGUCUGAAGCGC AGGCAAGGGCACCACAAUAAACGGA (wild type, the putative GfR-binding site is indicated by bold letters) and CCGAGAUAUUCGUCUGAAGCGCAGCACUGCU CACUAAUAAACGGA (mutant, mutated positions are underlined) were synthesized by INTEGRATED DNA TECHNOLOGIES. RNA fragments were labeled with [γ-32P]ATP by T4 polynucleotide kinase. After the labeling, the fragments were boiled and kept on ice until used. RNA-binding assays were performed in the binding buffer [50 mM Tris–HCl (pH 7.9); 10 mM MgCl2; 50 mM KCl; 0.1 mM DTT; 0.1 mg/ml BSA; 20 μM ATP]. GfK (1 pmol) was incubated at room temperature for 5 min for the autophosphorylation reaction. GfR (1 pmol) was then added and further incubated at room temperature for 5 min for the phosphotransfer reaction. The 32P-labeled RNA fragments (40 fmol) and yeast tRNA (1 μg) were added and incubated at room temperature for 15 min. The reaction mixtures were subjected to 5% PAGE and analyzed by autoradiography.

Computational tools

Homology search was conducted by NCBI/BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi/). The Genbank accession numbers for the genome sequences of G. sulfurreducens, G. metallireducens and G. uranireducens are AE017180, CP000148 and CP000698, respectively. Sequence alignments were constructed by MAFFT version 6 (http://align.bmr.kyusyu-u.ac.jp/mafft/online/server/). Sequence logos were generated by WebLogo (http://weblogo.Berkeley.edu).

RESULTS

Promoter region of the nitrogen-fixation genes

In order to elucidate the regulation of the genes involved in nitrogen fixation, it is necessary to define the promoter region of the genes. Therefore, transcription initiation sites for genes whose expression was previously shown to be significantly changed during nitrogen fixation in G. sulfurreducens (15) were determined by primer extension assays. Among the genes up-regulated during nitrogen fixation, gnfK (Geobacter nitrogen fixation histidine kinase) and gnfR (Geobacter nitrogen fixation response regulator) encoding a histidine kinase and a response regulator in the two-component system were examined, because they likely play a role in nitrogen-fixation gene regulation (see below). In addition, nifH, which encodes a nitrogenase iron protein, and glnB, which encodes a nitrogen regulatory protein PII, were tested, as they are well known to be involved in nitrogen fixation in other bacteria. Moreover, gdhA encoding glutamate dehydrogenase was investigated, because it was highly down-regulated during nitrogen fixation (15). The expression patterns of these genes were consistent with those found by the transcriptome analysis in that the transcription of gnfK, gnfR, nifH and glnB was activated during nitrogen fixation and gdhA expression was repressed during nitrogen fixation (Figure 1A).

Based on a region upstream of the transcription initiation site, it appears likely that these genes including gdhA have an RpoN-dependent promoter (Figure 1B). RpoN, a subunit of RNA polymerase, recognizes –24 and –12 promoter elements, which contain highly conserved sequences, GG and GC, respectively (45,46). In addition to these genes from G. sulfurreducens, homologues of these genes in other Geobacter species were predicted...
to have RpoN-dependent promoter elements (Supplementary Table S2). Furthermore, the sequence analysis of upstream regions of other up-regulated genes during nitrogen fixation identified by the transcriptome analysis in G. sulfurreducens as well as homologues in other Geobacter species revealed that many of them also have RpoN-dependent promoter elements (Supplementary Table S2). In contrast, gdhA in G. sulfurreducens was the only gene containing −24/−12 promoter elements that was among the genes down-regulated during nitrogen fixation (15). By using these RpoN-dependent promoter elements, the consensus sequence for the −24/−12 promoter elements was determined (Figure 1C). The consensus sequence is similar to other −24/−12 promoter elements in G. sulfurreducens (47) as well as other bacteria (45,46). Therefore, it is likely that the majority of the genes involved in nitrogen fixation are controlled by RpoN in G. sulfurreducens.

Master regulator for the nitrogen-fixation genes

As shown above, the majority of the genes up-regulated during nitrogen fixation appear to have an RpoN-dependent promoter in Geobacter species. Transcription initiation by RNAP/RpoN generally requires an EBP. However, the transcriptome analysis failed to identify a gene encoding an EBP (15). This might be due to the fact that many transcription factors including EBPs are regulated at the level of activity by modulation of the proteins such as phosphorylation and ligand binding. EBPs typically are able to bind specific DNA without modulation, but are incapable of activating transcription initiation with RNAP/RpoN without modulation (30–33).

Because the genes involved in nitrogen fixation are highly conserved in Geobacter species, it is reasonable to speculate that the EBP responsible for the transcription initiation of the nitrogen-fixation genes with an RpoN-dependent promoter is also conserved in Geobacter species. In order to identify the EBP, Geobacter genome sequences were analyzed for EBPs (Supplementary Table S3). The genome sequence of G. sulfurreducens encodes 28 EBPs and 14 of them are highly conserved in both G. metallireducens and G. uranireducens. The 14 EBPs conserved in Geobacter species were then tested for DNA-binding activity to promoters of gnfK, nifH, glnB and gdhA (Figure 2). Only EBP11, termed GnfM (Geobacter nitrogen fixation master regulator), exhibited DNA-binding activity to the promoter region of gnfK, nifH, and glnB as well as to the promoter region of gdhA, which was repressed during nitrogen fixation. To evaluate the specificity of the GnfM binding, the upstream promoter of the GSU3409 gene, which was recently identified as a direct target of Grr1 (EBP6), another EBP in G. sulfurreducens (Ueki and Lovley, a manuscript in preparation), was tested. Only EBP6 (Grr1) showed DNA-binding activity to the promoter region of gnfK, nifH, and glnB and gdhA were tested for DNA-binding activity of the EBPs (0.5 pmol) conserved in Geobacter species (Supplementary Table S3). The upstream promoter region of the GSU3409 gene was used as a control.

DNA-binding domain at the C-terminus (Supplementary Figure S1).

GnfM-binding sites

To determine the binding sites of GnfM, footprint assays were performed, and the region from nt −131 to −91 in the gnfK promoter with respect to the transcription initiation sites was protected from or modified by DNase I in the presence of GnfM (Figure 3A). In addition, the region from nt −77 to +7 in the gdhA promoter with respect to the transcription initiation site was protected from or modified by DNase I in the presence of GnfM (Figure 3B). It is likely that there are two and four GnfM-binding sites in gnfK (Figure 4A) and gdhA (Figure 4B), respectively, as highly similar sequences are found in these identified regions (Figure 4C). The presence of more GnfM-binding sites in gdhA than in gnfK is consistent with the results of the DNA-binding assays in that the GnfM/DNA complex migrated slower with gdhA than with gnfK (Figure 2). The GnfM-binding sites are located upstream of the RpoN-dependent promoter in gnfK, suggesting that GnfM functions as a transcriptional activator. In gdhA, the GnfM-binding sites are located upstream of the RpoN-dependent promoter as well as at the region overlapping the RpoN-dependent promoter, suggesting that GnfM functions as a transcriptional repressor for
When one or both of the GnfM-binding sites were deleted, the activity of the RpoN-dependent promoter were deleted, the activity of the RpoN-dependent promoter was not activated during nitrogen fixation (Figure 5A). These results indicate that the identified GnfM-binding site was not activated during nitrogen fixation (Figure 5A).

Figure 3. GnfM-binding site. Footprint assays were carried out with GnfM and the upstream region of gnfK (A) or gdhA (B). DNA-binding reactions were conducted with GnfM (lane 1, 0 pmol; lane 2, 0.7 pmol; lane 3, 1.3 pmol; lane 4, 2.5 pmol for gnfK; lane 1, 0 pmol; lane 2, 0.3 pmol; lane 3, 0.7 pmol; lane 4, 1.3 pmol; lane 5, 2.5 pmol for gdhA) and probes (0.2 pmol). G, A, T and C represent sequence ladders. The region protected from or modified by DNase I in the presence of GnfM is indicated by a vertical bar.

GdhA. These results are consistent with their expression patterns (Figure 1A). A sequence analysis of upstream regions of the nitrogen-fixation genes with an RpoN-dependent promoter identified similar sequences with those in gnfK and gdhA, which may function as a binding site of GnfM (Supplementary Table S4). A consensus sequence for the GnfM-binding sites was determined (Figure 4D).

To evaluate the effects of the identified GnfM-binding sites on the expression of gnfK and gdhA, lacZ fusion assays were conducted (Figure 5). When one or both of the GnfM-binding sites were deleted, the gnfK promoter was not activated during nitrogen fixation (Figure 5A). These results indicate that the identified GnfM-binding sites were essential for the induction of gnfK expression during nitrogen fixation. When both the GnfM-binding sites (BS1 and BS2) located upstream of the RpoN-dependent promoter were deleted, the activity of the gdhA promoter in the presence of ammonium was similar to that in the absence of ammonium (Figure 5B), indicating that BS1 and BS2 were required for the induction of gdhA expression in the presence of ammonium. In contrast, when the GnfM-binding sites (BS3 and BS4) located at the region overlapping the RpoN-dependent promoter were mutated, the activity of the gdhA promoter was higher both in the presence and absence of ammonium than the activity of the wild-type gdhA promoter (Figure 5B), indicating that BS3 and BS4 were necessary for the repression of gdhA expression. The RpoN-dependent promoter elements were not mutated in this assay (Figure 5B). These results suggest that GnfM functions as a repressor in the absence of ammonium and an activator in the presence of ammonium for gdhA.

The two-component system GnfL/GnfM

In order to assess the physiological function of GnfM, construction of a deletion mutant of the gene encoding GnfM was attempted. However, such a mutant could not be isolated even in the presence of ammonium (data not shown). Therefore, the gnfM gene appears to be essential for growth even in the presence of ammonium. An apparent reason is that GnfM regulates genes involved in nitrogen metabolism not only in the absence of ammonium but also in its presence, such as gdhA as described above. A deletion mutant of the gnfM gene could not be isolated even when the mutant selection medium was supplemented with glutamine and glutamate (data not shown). The results suggest that GnfM regulates other pathway(s) in nitrogen metabolism and/or that the simultaneous inactivation of the genes regulated by GnfM resulting from the deletion of the gnfM gene is deleterious to a cell.

As an alternative to elucidating GnfM function, a strain harboring an expression vector for GnfM was constructed to examine the effects of the overproduction of GnfM on the expression of the nitrogen-fixation genes. GnfM contains a receiver domain at the N-terminus (Supplementary Figure S1) and thus is likely a response regulator of the two-component His–Asp phosphorelay system. In addition, a gene termed gnfL encoding a histidine kinase is located immediately upstream of the gnfM gene on the genome. GnfL appears to have a PAS domain as a sensor with some homology to NtrB homologues (Supplementary Figure S2). A gene encoding the kinase domain of GnfL (GnfLK) was included in the expression vector because the histidine kinase, which becomes autophosphorylated upon sensing a signal, activates the cognate response regulator by phosphorylation, but histidine kinases without the sensor domain are often capable of autophosphorylation independent of their signals and subsequently of phosphorylation of the cognate response regulators (48). In fact, GnfLK had autophosphorylation activity and phosphorylated GnfM in vitro (Figure 6). The short duration (5 min) of this reaction strongly suggests that GnfLK displays specificity to GnfM in this assay. It has been shown that cross-talk observed in vitro likely arises from excesses in reaction time (49,50). Taken together, it is likely that GnfM and GnfL function as a two-component system involved in sensing and responding to nitrogen availability. The expression of gnfK, which is induced during nitrogen fixation, and gdhA, which is repressed during nitrogen fixation, was examined (Figure 7). The expression of gnfK was induced in the presence of ammonium when the two-component system
Figure 4. Promoter regions. (A) gnfK. (B) gdhA. Regions modified in the presence of GnfM in the footprint assays (Figure 3) are highlighted in gray. Similar sequences found in the GnfM-binding sites are indicated by bold letters. Highly conserved dinucleotides GG and GC in RpoN-dependent −24/−12 promoter elements are underlined. Transcription initiation site is indicated by +1. Translation initiation codon is indicated by Met. (C) Alignment of similar sequences found in the GnfM-binding sites. (D) The consensus sequence of the GnfM-binding sites. The consensus sequence was obtained from the alignment of the putative GnfM-binding sites in the nitrogen-fixation genes (Supplementary Table S4) and is presented as a logo.

Figure 5. Effect of the GnfM-binding sites on the expression of gnfK and gdhA. The promoter activity of gnfK (A) and gdhA (B) was analyzed by lacZ fusion assays. The activity is shown as a percentage of the activity of the wild-type promoter in the absence of NH4+ for gnfK and in the presence of NH4+ for gdhA. The location of GnfM-binding sites (BS) is indicated in Figure 4A and B. The binding sites were deleted (Δ) or mutated (M) in the assays. Diagrams of promoter constructs are shown and changes in the binding sites (M_BS3_BS4) are presented.
was analyzed by primer extension assays.

tested for phosphotransfer activity.

for 5 min for the phosphotransfer reaction. The sizes of molecular mass standards are shown in kilodaltons.

GnfM was then added and further incubated at room temperature for 5 min for the autophosphorylation reaction. The sizes of molecular mass standards are shown in kilodaltons.

GnfL/GnfM was overexpressed, whereas the expression of gdhA was repressed under the same conditions. In contrast, when only GnfM was overexpressed, the induction of gnfK expression drastically decreased, suggesting that the phosphorylated form of GnfM is more active in the transcription initiation of gnfK. Moreover, gdhA expression was only slightly repressed by the overexpression of GnfM alone, suggesting that the phosphorylation enhances the activity of GnfM as a transcriptional repressor for gdhA. These results suggest that GnfM functions as a transcription activator for gnfK and a repressor for gdhA and these activities of GnfM are modulated by phosphorylation via GnfL.

Another two-component system in the nitrogen-fixation gene regulation

A potential additional mechanism for the regulation of the genes involved in nitrogen fixation in G. sulfurreducens is regulation via another two-component system. The previous transcriptome analysis for nitrogen fixation indicated that only two genes, gnfK and gnfR, predicted to encode a histidine kinase and a response regulator, respectively, had higher transcript abundance during nitrogen fixation in G. sulfurreducens (15). As shown above, these genes were activated by the master two-component system GnfL/GnfM during nitrogen fixation. This is the first demonstration that the regulatory mechanisms for nitrogen-fixation gene expression consist of two two-component His-Asp phosphorelay systems. gnfK and gnfR are not closely located on the Geobacter genomes. However, purified GnfK exhibited autophosphorylation activity as well as phosphotransfer activity to purified GnfR in in vitro phosphorylation assays (Figure 8A and B). It is likely that the phosphotransfer from GnfK to GnfR is a specific reaction because the reaction time was short (5 min), as described above. These results suggest that GnfK and GnfR function as a two-component system during nitrogen fixation.

Homologues of GnfK and GnfR were found in G. metallireducens and G. uranireducens as well as Pelobacter propionicus. Pelobacter species, which like Geobacter species belong to the δ subdivision of Proteobacteria, are phylogenetically intertwined with Geobacter species (51,52). Therefore, Pelobacter species may also regulate nitrogen-fixation gene expression by the homologues of the two-component GnfK/GnfR system.

Physiological function of GnfK/GnfR

In order to assess the physiological function of the two-component GnfK/GnfR system, deletion mutants of gnfK or gnfR were constructed. The mutant cells grew as well as the wild-type cells when ammonium was provided (Figure 9). In contrast, the deletion mutants exhibited growth defects in the absence of ammonium (Figure 9).
The mutants grew similarly with the wild-type cells until \( \text{OD}_{600} \approx 0.04 \) after being transferred from the ammonium-containing medium to the ammonium-free medium, presumably by utilizing the ammonium transferred with the inoculum or stored inside the cells during growth in the ammonium-containing medium. Subsequent growth of the \( \Delta gnfK \) deletion mutant was slower than the wild-type, and the \( \Delta gnfR \) deletion mutant stopped growing. These results suggest that the GnfK/GnfR system plays an important role during nitrogen fixation in \( G. \text{sulfurreducens} \).

**Premature transcription termination**

An analysis of the amino acid sequence of GnfR suggested that GnfR is a transcription antiterminator, as it has an RNA-binding domain or the ANTAR domain (53) at the C-terminus (Supplementary Figure S3). Furthermore, an analysis of promoter regions of the genes up-regulated during nitrogen fixation identified transcription termination-like sequences in the promoter region of some of the genes such as \( \text{nifH} \), \( \text{nifEN} \), \( \text{nifX} \), \( \text{glnK} \) and \( \text{amtB} \) (Figure 10A, B and Supplementary Figure S4), which are similar to the Rho-independent transcription termination signals that typically consist of a hairpin followed by a T-rich region (Figure 10A, B and C; 54). Therefore, it is likely that premature transcription termination and its antitermination are one of the regulatory mechanisms controlling a subset of the nitrogen-fixation genes that includes the genes regulated by the GnfL/GnfM two-component system and containing a transcription termination signal in their promoter regions (Figure 10A).

To examine the function of these identified transcription termination signals, \( \text{lacZ} \) fusion assays were conducted with artificial promoters that contain an RpoD-dependent like \(-35/\text{-10}\) elements with or without the wild-type or mutated sequences from the identified transcription termination signals in \( \text{nifH} \) (Figure 10D and Supplementary Figure S5). \( \beta\)-Galactosidase activity by the X-gal plate assays indicated that the RpoD promoter was active. In contrast, RpoD-TTS (WT) promoter in which the RpoD promoter was placed upstream of the wild-type transcription termination signals from \( \text{nifH} \) was inactive under the same condition. When the upstream or downstream half of the transcription termination signals from \( \text{nifH} \) was deleted and the RpoD promoter was disrupted, RpoD-TTS (-up) and RpoD-TTS (-down) promoters became active. In addition, when the region predicted to form the stem structure was mutated and thus a stem structure was also disrupted, RpoD-TTS (mut) promoter was active as well. These results suggest that the identified sequences function as transcription termination signals.

**Transcription antitermination**

To assess whether or not the GnfK/GnfR system is involved in transcription antitermination during nitrogen fixation, transcripts of \( \text{nifH} \), which has transcription termination signals, and of \( \text{glnB} \), which does not, were examined by primer extension assays (Figure 11). Both \( \text{nifH} \) and \( \text{glnB} \) appear to be under the control of the RNA polymerase RpoN together with the master two-component GnfL/GnfM system and up-regulated during nitrogen fixation. For \( \text{nifH} \) transcripts, two different primers were used to distinguish prematurely terminated transcripts and readthrough transcripts (Figure 11A). Under nitrogen-fixing conditions, the transcripts of \( \text{nifH} \) were prematurely terminated in the mutant strains in which \( \Delta gnfK \) or \( \Delta gnfR \) was deleted, but not in the wild-type strain (Figure 11B). The expression of \( \text{glnB} \) was not affected by the deletion of \( \Delta gnfK \) or \( \Delta gnfR \) (Figure 11B). These results suggest that premature transcription termination and its antitermination are an important regulatory mechanism controlling the subset of the genes containing transcription termination signals in their promoter among the nitrogen-fixation genes regulated by GnfL/GnfM and that the two-component GnfK/GnfR system is involved in the antitermination of prematurely terminated transcription. It is also possible that the \( \text{nifH} \) mRNA is destabilized by the slow growth conditions imposed by the \( \Delta gnfK \) and \( \Delta gnfR \) deletions. It is noteworthy that primer extension products by the R1 primer increase in intensity in these mutants.

![Figure 9](http://nar.oxfordjournals.org/) Growth under nitrogen-fixation condition. The wild-type (circles) and \( \Delta gnfK \) (triangles) or \( \Delta gnfR \) (squares) mutants were grown in the presence or absence of \( \text{NH}_4^+ \). Growth was monitored by measuring the optical density at 600 nm \( (\text{OD}_{600}) \). Data are representative of three replicate cultures.
RNA binding of GnfR activated by GnfK

As the upstream regions of the sequences forming a hairpin in the premature transcription termination signals contain highly conserved sequences, CAGGCCA NGGCGCC, in the subset of the nitrogen-fixation genes in *G. sulfurreducens* (Figure 10A), it is possible that these conserved sequences serve as a recognition site for GnfR to bind their mRNA and to inhibit the premature transcription termination of these genes. The results shown above suggest that the activity of GnfR is regulated by GnfK via phosphorylation. To test these, RNA-binding assays were conducted in the presence of ATP (Figure 12). GnfR bound the RNA fragment containing the predicted binding site, CAGGCCA NGGCGCC C, from the *nifH* promoter in the presence of GnfK, whereas GnfR was unable to bind the same RNA fragment in the absence of GnfK (Figure 12A).
Nitrogen fixation is a key physiological feature in *Geobacter* species during growth in subsurface environments and bioremediation of contaminated environments. The results presented in this study show that the majority of nitrogen-fixation genes appear to be controlled by the sigma factor RpoN and the two-component system consisting of the histidine kinase GnfL and the response regulator GnfM, which also belongs to the EBP family. Unlike most other bacterial *rpoN* genes, the *G. sulfurreducens* *rpoN* is essential under all conditions tested, including ammonium-sufficient conditions (47). In addition to nitrogen metabolism, RpoN controls genes involved in a wide range of cellular processes such as fumarate respiration, Fe(III) reduction, and pili and flagella biosyntheses in *G. sulfurreducens*. Although most of the up-regulated genes during nitrogen fixation appear to be controlled by RpoN in *G. sulfurreducens*, there are some genes and operons that are up-regulated during nitrogen fixation and appear to be controlled by other sigma factors. For instance, the operon for a putative molybdenum transporter is up-regulated during nitrogen fixation in *G. sulfurreducens* (15). Molybdenum is known to be an important co-factor for nitrogenase as well as other enzymes such as formate dehydrogenase. Genes encoding the molybdenum transporter are regulated by the molybdenum-responsive transcription factor ModE in other bacteria (55). This operon in *G. sulfurreducens* appears to lack an RpoN-dependent promoter and to be regulated by a homologue of ModE, which is encoded by the gene located immediately upstream of the operon on the *G. sulfurreducens* genome (56). In addition, several genes encoding a hypothetical protein are up-regulated during nitrogen fixation and appear to lack an RpoN-dependent promoter. Therefore, it may be possible that genes mainly involved in nitrogen metabolism such as nitrogen fixation genes and *gdhA* are controlled by both RpoN and the GnlL/GnfM system. In contrast, it is likely that genes required for other cellular processes in addition to nitrogen fixation such as molybdenum transporter genes are regulated by other sigma factors and transcription factors in *G. sulfurreducens*.

The expression of *gdhA* was shown to be activated by RpoN and the GlnL/GnfM system in the ammonium-sufficient conditions and repressed during nitrogen fixation in *G. sulfurreducens*. The repression of *gdhA* expression was observed in some bacteria (57,58). However, *gdhA* expression is controlled by the major sigma factor RpoD in other known bacteria (52). In *Klebsiella aerogenes* the NAC (nitrogen assimilation control) protein, which is a member of a LysR transcriptional regulator, represses transcription of the *gdhA* gene under nitrogen-limiting conditions (59). NtrC is known to act as both a repressor and an activator in other bacteria such as *E. coli* (60). Although *gdhA* homologues are conserved in *Geobacter* species and among the EBP homologues in *Geobacter* species only GnfM bound the promoter region of the *gdhA* gene, which has an RpoN-dependent promoter, it is possible that another EBP is involved in the regulation of the *gdhA* gene. Furthermore, it is also possible that another transcription factor plays a role in modulating the activity of GnfM as a repressor and an activator. For instance, integration host factor (IHF) is known to be involved in the transcription mediated by the EBP such as NifA (61). Moreover, phosphorylation of GnfM by GnfL affects the activity of GnfM as a repressor and an activator. Although it is generally thought that phosphorylation is not required for the DNA-binding activity of the EBP family transcription factor (30–33), it has been shown that phosphorylation...
affects DNA-binding activity of NtrC (62,63). In addition, it is known that the expression of ompC and ompF, encoding outer membrane porins, is differentially regulated by the phosphorylation levels of the response regulator OmpR, which is not a member of the EBP family, in the E. coli osmoregulation system (64). Therefore, it is possible that during growth in the ammonium-sufficient conditions, under which the phosphorylated form of GnfM is less abundant, most of GnfM binds the upstream binding sites (BS1 and BS2) in the gdhA promoter that may have higher affinity with GnfM than the downstream binding sites in gdhA (BS3 and BS4) and the binding sites in the nitrogen-fixation genes, resulting in the activation of gdhA expression. In contrast, during growth dependent on nitrogen fixation, under which the phosphorylated form of GnfM is more abundant, GnfM may be able to bind the downstream binding sites in gdhA promoter that are essential for the activation of gdhA. Genes located closer to the RpoN-dependent promoter than typical EBP-binding sites including NtrC bind sites located far upstream of an RpoN-dependent promoter when compared with other types of bacterial transcription factors that activate other sigma factors (30,31,33). However, GnfM-binding sites (BS1 and BS2) in the gdhA promoter that are essential for the activation of gdhA are located closer to the RpoN-dependent promoter than typical EBP-binding sites including the GnfM-binding sites in the gnfK promoter (Figure 4A and B). This difference may also contribute to different activation mechanisms in the expression of gnfK and gdhA.

Another two-component system GnfK/GnfR controls the subset of nitrogen-fixation genes by the transcription antitermination. Transcription of these genes is activated by the GnfL/GnfM system. Although antitermination is found in gene regulation during a variety of cellular processes (65), this is the first demonstration of the involvement of antitermination in nitrogen-fixation gene regulation. Genome sequence analysis revealed that the ANTAR domain is found in other response regulators of the two-component system. However, to our knowledge, GnfR is the first response regulator with the ANTAR domain to be characterized in detail. AmiR, an antiterminator involved in regulating the expression of the aliphatic amidase operon of Pseudomonas aeruginosa (66,67), has an N-terminal domain whose structure is similar to that of the receiver domain of the response regulator (68). However, AmiR is not likely to be a response regulator, because residues typically conserved in the receiver domain are absent from the N-terminal domain of AmiR, most notably a phosphorylation site. Another well-characterized antiterminator, NasR, which has an NIT domain at the N-terminus, is involved in nitrate-responsive transcription antitermination of the nasF operon in Klebsiella oxytoca that is required for nitrate assimilation (69,70). The NIT domain is found in nitrate- and nitrite-sensing receptor components (71). The untranslated region or the leader region containing transcription termination signals has been shown to fold into two different secondary structures in many cases as shown for the NasR-mediated nitrate-responsive transcription antitermination (69,70). Binding of the antiterminator protein to the leader RNA inhibits formation of the transcription terminator structure. It is possible that GnfR functions as an antiterminator in a similar fashion during nitrogen fixation in G. sulfurreducens. GnfR inhibits formation of the transcription terminator structure by binding to the highly conserved sequences, C AGGCAANGGCGCC, located upstream of and at the region overlapping transcription termination signals in leader RNA of the subset of nitrogen-fixation genes.

Although GnfK has a typical histidine kinase domain, it does not have an apparent sensor domain. The kinase activity of the sensor histidine kinase of the two-component system is normally regulated by an environmental signal or stimulus, which is detected by the sensor domain of the histidine kinase (36,48). Therefore, it is likely that GnfK is constitutively active, as suggested by the in vitro phosphorylation assays, and that its expression is regulated at the level of transcription by the GnfL/GnfM system. Therefore, when expressed, GnfK might function as the activator for GnfR regardless of an environmental signal. It is, however, possible that an intracellular signal could regulate the activity of GnfK. For example, the activity of the histidine kinases is dependent on divalent cations (Ueki and Lovley, manuscript in preparation; 48). On the other hand, the response regulator GnfR appears to be regulated at levels of both the expression by transcription via the GnfL/GnfM system and the activity by phosphorylation via GnfK.

The phenotypes of mutants of GnfK and GnfR were somewhat different during growth dependent on nitrogen fixation. This difference may be explained by the fact that some response regulators have been shown to utilize small phosphate-containing molecules such as acetylphosphate as a phospho-donor in the absence of their cognate histidine kinases (72,73). Thus, GnfR might still be active with such small phosphate-containing molecules in the absence of GnfK, but not as efficient as with GnfK. Different phenotypes between the kinase mutant and the response regulator mutant have also been observed in other two-component systems. For instance, in the two-component RegB/RegA system in Rhodobacter capsulatus, disruption of regB encoding a kinase affected photosystem gene expression less dramatically than did disruption of regA encoding a response regulator (74).

In contrast to GnfK, GnfL, the histidine kinase of the master two-component system for the nitrogen-fixation gene regulation in G. sulfurreducens, has a PAS domain as the sensor at the N-terminus and shows some similarity to NtrB homologues (Supplementary Figure S2). The PAS domain is a signaling module known to sense light, oxygen, redox and small molecules (75,76). Oxygen is known to be an important regulatory factor for nitrogen-fixation gene expression due to the oxygen sensitivity of nitrogenase in diazotrophs. As Geobacter species are strictly anaerobic and oxygen has a negative impact on anaerobically growing Geobacter cells (77; Leang, personal communication), critical cellular processes are thought to be sensitive to oxygen in Geobacter species. The transcriptome analysis of G. uranireducens growing
in uranium-contaminated subsurface sediments showed higher levels of transcripts for genes for nitrogen fixation as well as oxidative stress response (13), suggesting that nitrogen fixation is among the critical cellular processes sensitive to oxygen in Geobacter species. NtrB homologues are known to be regulated by the PII regulatory protein GlnB in nitrogen regulation in some bacteria such as Escherichia coli and Klebsiella pneumoniae (16). Geobacter sulfurreducens contains homologues of the PII protein, and genes encoding GlnB and GlnK are up-regulated during nitrogen fixation (15). Therefore, it is possible that GnfL is regulated by GlnB as found in nitrogen regulation in other bacteria.

In summary, G. sulfurreducens appears to utilize novel regulatory mechanisms for the nitrogen-fixation gene expression (Figure 13). The master regulator, the two-component GnfL/GnfM system, appears to activate the transcription of the majority of the nitrogen-fixation genes and repress the transcription of gdhA encoding glutamate dehydrogenase under nitrogen-fixing conditions. In addition, it is likely that the GnfL/GnfM system activates the transcription of gdhA under ammonium-sufficient conditions. The other two-component system, GnfK/GnfR, whose expression is directly activated by the GnfL/GnfM system during nitrogen fixation, appears to control the subset of the nitrogen-fixation genes by the transcription antitermination mechanism. These genes contain transcription termination signals in their promoter and are regulated by the GnfL/GnfM system. These regulatory cascades appear to play a key role in fine-tuning the expression of the nitrogen-fixation genes in G. sulfurreducens to ensure an adequate response to nitrogen availability, as nitrogen fixation is a costly cellular process. These findings provide new insights into regulatory molecular mechanisms in nitrogen-fixation gene expression and important information on how

Figure 13. Model for the regulation of gene expression during nitrogen fixation in G. sulfurreducens. Histidine kinases are presented in red. Response regulators are presented in blue. Phosphorylation is indicated in purple arrows. Transcription activation and repression are indicated in blue arrows and a pink line, respectively. Transcription antitermination is indicated by a green arrow. RNAP represents RNA polymerase.
**Geobacter** species respond to growth conditions in subsurface environments and during *in situ* bioremediation.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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