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Genome-wide gene regulation of biosynthesis and energy generation by a novel transcriptional repressor in *Geobacter* species

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ABSTRACT

Geobacter species play important roles in bioremediation of contaminated environments and in electricity production from waste organic matter in microbial fuel cells. To better understand physiology of *Geobacter* species, expression and function of citrate synthase, a key enzyme in the TCA cycle that is important for organic acid oxidation in *Geobacter* species, was investigated. *Geobacter sulfurreducens* did not require citrate synthase for growth with hydrogen as the electron donor and fumarate as the electron acceptor. Expression of the citrate synthase gene, *gltA*, was repressed by a transcription factor under this growth condition. Functional and comparative genomics approaches, coupled with genetic and biochemical assays, identified a novel transcription factor termed HgtR that acts as a repressor for *gltA*. Further analysis revealed that HgtR is a global regulator for genes involved in biosynthesis and energy generation in *Geobacter* species. The *hgtR* gene was essential for growth with hydrogen, during which *hgtR* expression was induced. These findings provide important new insights into the mechanisms by which *Geobacter* species regulate their central metabolism under different environmental conditions.

INTRODUCTION

Geobacter species can play an important role in the bioremediation of groundwater contaminated with organics or metals (1–7) and are one of the most effective microorganisms in converting organic compounds to

electricity in microbial fuel cells (8–11). Studies on the physiology of *Geobacter* species have primarily focused on *Geobacter sulfurreducens* because it has the key hall mark physiological characteristics of *Geobacter* species (12), including the ability to completely oxidize organic acids to carbon dioxide with electron transfer to extracellular electron acceptors such as Fe(III) oxides (13–15), toxic metals (16), humic substances (17) and electrodes (18,19). In addition to organic compounds, *Geobacter* species can utilize hydrogen as an electron donor to generate energy for growth (12,20,21).

The tricarboxylic acid (TCA) cycle is the main pathway for oxidation of organic compounds for energy conservation in *G. sulfurreducens* and serves to synthesize a diversity of precursor metabolites for biosynthetic reactions (22,23). Citrate synthase is a key TCA cycle enzyme. Analysis of the *G. sulfurreducens* genome revealed only one homologue of the citrate synthase gene, termed *gltA* (24), which encodes the protein responsible for citrate synthase activity (25). Surprisingly, the citrate synthases of *G. sulfurreducens* as well as other members of *Geobacter* species show higher sequence similarity to eukaryotic citrate synthases than to the majority of prokaryotic citrate synthases (24–26).

The production of citrate synthase in *Geobacter* species appears to be highly regulated. For example, cells grown with hydrogen as the electron donor had much lower citrate synthase activities than cells grown on acetate (25). Transcript abundance of *gltA* directly correlated with the rates of Fe(III) reduction in chemostats or the rates of electron transfer to electrodes in microbial fuel cells (26).

Here we report on one of the mechanisms by which the expression of *gltA* and other genes encoding proteins important for central metabolism is regulated in *Geobacter* species. The results suggest that a novel transcriptional repressor plays an important role in controlling the expression of these genes.

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MATERIALS AND METHODS

Bacterial strains and growth conditions

Genetic and biochemical studies were conducted with *G. sulfurreducens* strain DL1 (12). *Escherichia coli* DH5 α (27) was used for plasmid preparation and grown in LB medium (28) supplemented with antibiotics, when necessary.

Growth studies on *G. sulfurreducens* were carried out in 27-ml pressure tubes containing 10 ml of either donor-free fumarate medium (NBF) or donor-free Fe(III) citrate medium (FWFC) as described previously (20). Acetate was included as the electron donor at a concentration of 15 or 10 mM in NBF or FWFC medium, respectively. Lactate was included as the electron donor at a concentration of 20 mM in NBF medium. When hydrogen was used as the electron donor, 10 ml of hydrogen gas was injected into the headspace, resulting in an initial headspace composition of 37% H₂: 12.6% CO₂: 50.4% N₂ at a total pressure of ca. 1.61×10^3 Pa, and media were supplemented with acetate or lactate as a carbon source at a concentration of 4 or 1 mM in NBF or FWFC medium, respectively.

Analytical techniques

Growth of cells in media containing fumarate as the electron acceptor was monitored by measuring the optical density at 600 nm (OD₆₀₀). The number of cells in cultures containing Fe(III) as the electron acceptor was determined by acridine orange staining with epifluorescence microscopy (15). The concentrations of Fe(II) were determined by the ferrozine assay (29).

Western blot analysis

Geobacter sulfurreducens DL1 was grown in media containing electron donors and acceptors indicated in Figure 1A. Cell extracts were prepared with the reagent B-PER (Pierce Biotechnology) as recommended by the manufacturer. Cell extracts were loaded on SDS-PAGE. Western blot analyses were carried out with antisera prepared by Sigma-Genosys against the peptide, TPMLEKWAEEGGRK, from amino acid residues 427–440 of the citrate synthase of *G. sulfurreducens*.

Primer extension assay

Total RNA was prepared from *G. sulfurreducens* strains grown in media containing electron donors and acceptors indicated in figure legends. The sequences of primers used in the assays were 5'TCGATAATGACCTTGCCGAACCTCC3' (*gltA*), 5'CACATCAATTTCCGTCCAGATAATC3' (*icd*), 5'CTTCCCACAGAACTCGTGAAC3' (*frdC*), 5'CGGTTATCGACGATTGTTCTTCC3' (*sfrB*), 5'CCAGGACAATGATTGGCAGGTATAC3' (*nuoA*), 5'CCTGAA TAACAAAGGCAAGATC3' (*atpG*), 5'ATGATGGCATCGCGAATGGTTTC3' (*gntR*) and 5'TCATGGAGATAACGTTGTAC3' (*hgtR*).

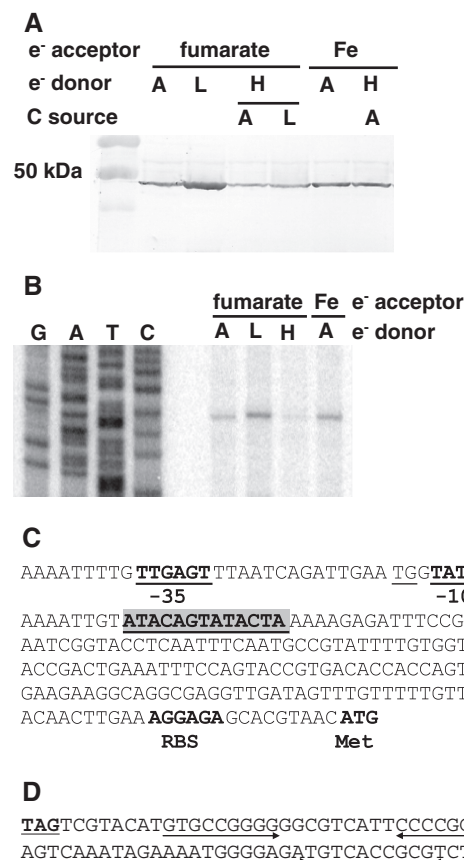


Figure 1. Regulation of the citrate synthase. (A) Expression of the citrate synthase protein. Cell extracts were prepared from *G. sulfurreducens* DL1 grown in media containing acetate (A), lactate (L), or hydrogen (H) as the electron donor and fumarate or Fe(III) as the electron acceptor and analyzed by western blot analysis. When hydrogen was used, acetate (A) or lactate (L) was included as the carbon (C) source. (B) Expression of the *gltA* transcript. Total RNA was prepared from *G. sulfurreducens* DL1 grown in media as described above. G, A, T and C represent sequence ladders generated by the same primer used in the primer extension assays. (C) Promoter region of *gltA*. The putative $-35/-10$ and extended -10 elements are underlined. The 5' end of *gltA* mRNA (transcription initiation site) is indicated by a bold letter with +1. The binding site for the repressor identified in Figure 2B is highlighted in grey with bold letters. The putative ribosome binding site (RBS) is indicated by bold letters. The initiation codon is indicated by bold letters with Met. (D) Transcription termination of *gltA*. The termination codon is indicated by bold letters with an underline. Two putative transcription termination signals are indicated by arrows.

Construction of mutants

The *gltA* gene was replaced with a kanamycin resistance gene, such that the coding region from amino acid residues 6Thr to 429Met was deleted. Double-crossover homologous recombination was carried out by electroporation (30) with the linear DNA fragment consisting of the kanamycin resistance gene flanked by 0.7 kbp DNA fragments containing the up- and the downstream regions of the *gltA* gene. These flanking DNA fragments were amplified by PCR with primers, 5'TCGGATCCGTCGC ACTCTGGCTGAGGAGC3' and 5'TCGAATTCCTTG AGTGCCATGTTAC3' (*Bam*HI and *Eco*RI sites are

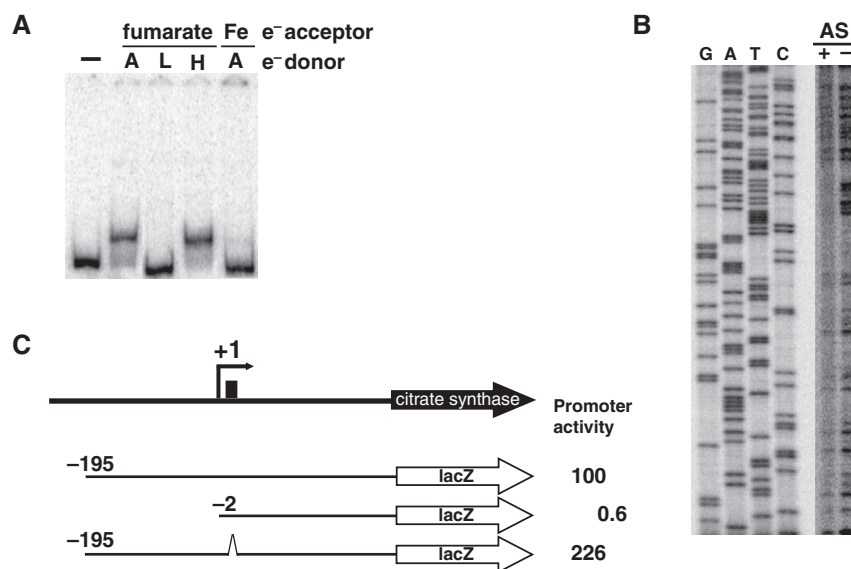


Figure 2. Transcriptional regulation. (A) DNA-binding assay. Cell extracts were prepared from cultures grown in media as described in Figure 1A. The probe contained the promoter region from nt -127 to +153 of *gltA*. '—' indicates the DNA-binding assay without cell extracts. (B) Footprint assay. The ammonium sulfate fraction (AS) was prepared from cultures with acetate and fumarate as the electron donor and the acceptor, respectively. The probe contained the promoter region from nt -49 to +153 of *gltA*. '—' and '+' indicate footprint assays without or with AS, respectively. G, A, T and C represent sequence ladders. (C) *lacZ* fusion assay. The promoter regions from nt -195 to +232, from nt -2 to +232, and from nt -195 to +232 without the repressor binding site in *gltA* were tested. The activity is shown as a percentage of the activity of the promoter from nt -195 to +232.

underlined) and, 5'TCAAGCTTGAGAAGTGGGCGGAA3' and 5'TCTCTAGACGACGACAAGAAGCTCTTAC3' (*Hind*III and *Xba*I sites are underlined), respectively. The DNA fragment of the kanamycin resistance gene was amplified by PCR with primers, 5'GCATGAGAATTCCTGACGGAACAGCGGGAAGTCCAGC3' and 5'GCTATGAAGCTTTTCATAGAAGGCGGCGGTGGAATCGAA3' (*Eco*RI and *Hind*III sites are underlined), and pBBR1MCS-2 (31) as a template. These PCR products were digested with restriction enzymes, ligated and cloned in the plasmid. The sequences of these cloned fragments were confirmed by DNA sequencing. After electroporation, kanamycin-resistant transformants were isolated and inoculated in NBF medium supplemented with 4mM acetate and 200μg/ml kanamycin in the presence of hydrogen. The replacement was confirmed by PCR amplification.

The *hgtR* gene was replaced with the kanamycin resistance gene, such that the coding region from amino acid residues 4Met to 59Gln was deleted, as described above. The oligonucleotides used to amplify DNA fragments for double-crossover homologous recombination were 5'TCTCTAGAGGGCTGGTTCCGCGAGGATC3' and 5'TCGAATTCTTCCCATGTCTGGTATC3' for the upstream region and 5'TCAAGCTTAGAAGGCCGCTGATTTTCAC3' and 5'TCTCTCGAGGAAGTGCTGGAGAGCTATGAC3' for the downstream region.

Preparation of cell extracts and fractionation by ammonium sulfate

Geobacter sulfurreducens DL1 was grown in media containing electron donors and acceptors indicated in Figure 2A. Cells were harvested by centrifugation,

resuspended in TGED buffer (10mM Tris-Cl, pH 7.9; 10% glycerol; 0.1mM EDTA; 0.1mM DTT) supplemented with protease inhibitors (Roche), and disrupted by sonication. Cell extracts were prepared by centrifugation after the sonication. For a DNA-binding assay, the cell extracts thus prepared were used. For a footprint assay, the cell extracts were further fractionated by ammonium sulfate. The DNA-binding activity was detected in the fraction of 40–65% ammonium sulfate saturation (data not shown). The precipitations by 40–65% ammonium sulfate saturation were resuspended in TGED buffer supplemented with the protease inhibitors.

DNA-binding and footprint assays

DNA fragments containing the promoter regions were amplified by PCR with oligonucleotide primers, 5'TCTCTAGACTTGACCGAGGTGAAACGTC3' and 5'TCGGATCCTTCTTCCCCCTCCTTCCAC3' (*gltA*), 5'TCTCTAGATGTCCATTCCGCGGACCTC3' and 5'TCGGATCCTGAATAACAAAGGCAAGATC3' (*atp*), 5'TCTCTAGAGCTGTGAGCATTGGCGATTTC3', 5'TCGGATCCAGGACAATGATTGGCAGGTATAC3' (*nuo*), and 5'TCTCTAGATTGATCCCACGGGCGGCGAC3' and 5'TCGGATCCATGATGGCATCGCGAATGGTTTC3' (*gntR*) (*Xba*I and *Bam*HI sites are underlined). The PCR products were digested with *Xba*I and *Bam*HI and cloned into a plasmid. For a probe in DNA-binding assays, DNA fragments were prepared by digesting the plasmid with *Xba*I and *Bam*HI and isolating the fragment by agarose gel. The isolated DNA fragments were then labeled with [α -³²P]dCTP by Klenow fragment of DNA polymerase I. For a probe in footprint assays, DNA fragments were prepared by digesting the plasmid

with *Sac*II and *Xho*I located in the plasmid vector and isolating the fragment by agarose gel. The isolated fragments were labeled with [α - 32 P]dCTP by Klenow fragment of DNA polymerase I. Therefore, only the leading strand was labeled.

The reactions and analyses of DNA-binding and footprint assays were performed as described previously (32).

lacZ fusion assay

A vector for a transcriptional *lacZ* fusion assay was constructed by inserting the *Eco*RI-*Hind*III fragment containing the kanamycin resistance gene, the *Eco*RI-*Bam*HI fragment containing transcription termination signals and cloning sites, and the *Bam*HI-*Sal*I fragment containing the *lacZY* genes into *Hind*III and *Xho*I sites of pCM66 (33). The resultant plasmid was named pCMZKT. The promoter regions from nucleotide (nt) -195 or -2 to +232 of the *gltA* gene were amplified by PCR with primers -195T; 5'TCTCTAGACATTCTCGGACCAGGAAC3', or -2T; 5'TCTCTAGAGTAAAATTGTATACAGTATACTA3', respectively, and +232B; 5'TCGGATCCTTGAGTGCCATGTTAC3' (*Xba*I and *Bam*HI sites are underlined). The PCR products were digested with *Xba*I and *Bam*HI and cloned into pCMZKT. To examine the effects of the binding site determined by the footprint assay on the promoter activity, the region from nt +11 to +23 containing the binding site was deleted. The promoter regions from nt -195 to +10 and from nt +24 to +232 were amplified by primers -195T and +10B; 5'TCGAATTCTACAATTTTACTGTAAAGCCTTA3', and +24T; 5'TCGAATTCAAAGAGATTTCGCCGTTTTAC3' and +232B, respectively (*Eco*RI site is underlined), and digested with *Xba*I and *Eco*RI, and *Eco*RI and *Bam*HI, respectively. The *Xba*I-*Eco*RI fragment and the *Eco*RI-*Bam*HI fragment were ligated and cloned into pCMZKT. The plasmids thus constructed were introduced into *G. sulfurreducens* DL1 by electroporation (30).

β -Galactosidase activity was measured by the method described by Miller (28) with modifications. The strains carrying the plasmids were grown in NBAF medium supplemented with kanamycin and the cells were harvested at the log phase ($OD_{600} = 0.3$ nm) by centrifuging 1 ml of the cultures. The cells were resuspended in 1 ml of the Z buffer and the OD_{600} of samples was measured. The rest of the procedure was same as the method described by Miller.

Construction of expression vectors for transcription factors

DNA fragments encoding a putative transcription factor were amplified by PCR with primers: 5'TCTCATATGGA CGATACAAATCCCAGATC3' and 5'TCTCTCGAG GTCCCGGTATAGAGAACC GCCAC3' (GSU0079), 5'TCTCATATGACCAAGCGGACTGTCGCA3' and 5'TCTCTCGAGTGCCGCGCGGCCCGCCGTCGG CCATGA3' (GSU0175), 5'TCTCATATGGATAACCTT GCCGAACACA3' and 5'TCTCTCGAGCCGGACC ACCTCGAACGTTTTCA3' (GSU0366), 5'TCTCATAT GGCGAAGAAGGACAAATCCGAA3' and 5'TCTCT CGAGCCGATGAAAGCCGAGCTTCATGGATA3' (GSU0514), 5'TCTCATATGAAGCTCTCAACGAAAA

GCCGGTA3' and 5'TCTCTCGAGGATAAAGTACAT GAACCGGTGATC3' (GSU0534), 5'TCTCATATGAA CGAGCTTCGCGAAAAGGATGAATC3' and 5'TCT CTCGAGCGGATGTTCTCCCTGATTGAC3' (GSU0625), 5'TCTCATATGACGAGGGAAAAAGC TCATAC3' and 5'TCTCTCGAGCAGCTTTGCGTAG CCAAACTTC3' (GSU1072), 5'TCTCATATGATATCG AAAAAGACAAAATAC3' and 5'TCTCTCGAGGATC GTGTAAAAAGCACTCCGTC3' (GSU1345), 5'TCTCATATGAACTGTAATATAGGACATGC3' and 5'TCTCTCGAGCGGCTTGCTGTACGAGGGGC CGTC3' (GSU1419), 5'TCTCATATGGTGGCGGAGG AGACCCCGGACA3' and 5'TCTCTCGAGTTGCAGT TGTTTTTTCAGCAATTC3' (GSU1522), 5'TCTCATA TGATGGAATTAACCCGGA3' and 5'TCTCTCGAG TTTCGTGGTAGCGGCCAGCTC3' (GSU1639), 5'TCTCATATGATCCGCCCCGGCGTTTCATC3' and 5'TCTCTCGAGGATGTCGAACATCACGGGCCA 3' (GSU1702), 5'TCTCATATGGCGCCGACATTATTG GAATTGAC3' and 5'TCTCTCGAGGGCAGTAGCTT TCTTGCGCGAGTA3' (GSU1863), 5'TCTCATATG ACCACCGTGAAATTATC3' and 5'TCTCTCGAGGT CGATAAACACCGCGTCC3' (GSU2033), 5'TCTCAT ATGCGACACGACAAATTCGCA3' and 5'TCTCTCG AGGCATGGTCCGTGCCCGAGCTTC3' (GSU2354), 5'TCTCATATGAACCTCAAGCAGCTCGAAGTA3' and 5'TCTCTCGAGGGCGAGCCGGGTTCCGGTCA GTTC3' (GSU2523), 5'TCTCATATGAAGATCGGCG AGCGGTTGAA3' and 5'TCTCTCGAGGTAATCAAA TGTCGGCGGGGTCAC3' (GSU2540), 5'TCTCATAT GAGACTGTGACGCGCGCCCA3' and 5'TCTCTCG AGTTGATCCTCACTCTCGCCGGGCCGTC3' (GSU2571), 5'TCTCATATGGAAATGGAAGAATTTCG ACAA3' and 5'TCTCTCGAGGCCGAGCAGCTCGAT GAGC3' (GSU2625), 5'TCTCATATGTCGAAGCTGG AAAACCGGGAC3' and 5'TCTCTCGAGAGAATGA ACATTCTTCTTAGCCA3' (GSU2698), 5'TCTCATA TGATTCTGCCACGACCTCTTTAC3' and 5'TCTCTC GAGTTCCCCGGCAAGCACC GGCCAGCCGCTC3' (GSU2716), 5'TCTCATATGGATTTGATCCTTCTTAA TAC3' and 5'TCTCTCGAGCCGGGTGCGCCGGGAG GAGAAC3' (GSU2941), 5'TCTCATATGAAACAGGC GGCCCGTTTATTC3' and 5'TCTCTCGAGGGCGCA ACGATTTCCCTGACC3' (GSU2952), 5'TCTCATATG AAACAGGCGGCCCGTTTATTC3' and 5'TCTCTCG AGGGCGCAACGATTTCCCTGACC3' (GSU2980), 5'TCTCATATGCAGAAAAAAGACAAGTCAGACTA C3' and 5'TCGAATTCAGACCCGCGGTAGGAGCG GCATAC3' (GSU3109), 5'TCTCATATGACCGAGTA CAATATC3' and 5'TCTCTCGAGACGGGTGACTAC GGCGAGAAC3' (GSU3298), 5'TCTCATATGGAAAG TGCGCGAAAC3' and 5'TCTCTCGAGCAGCTTCAC GTTCCGCCAGAC3' (GSU3324), 5'TCTCATATGGG AAGAAAGAGAGAAAAATC3' and 5'TCTCTCGAGG GCGGCCTTCTGGTCTCCGGGTTGAGAC3' (GSU 3364, HgtR), 5'TCTCATATGAAAAAACCGATGGAG AAGCAC3' and 5'TCTCTCGAGGATATCAAGTTGC AGGGCAGCA3' (GSU3370), and 5'TCTCATATGGA CATCAGGGACATCCTC3' and 5'TCTCTCGAGGGC CGGGCGCCTCCCGGCGAGCGGCCTTCA3' (GSU 3421) (*Nde*I, *Xho*I and *Eco*RI sites are underlined). PCR

products were digested with *Nde*I and *Xho*I or *Eco*RI and cloned in pET24b (Novagen). The transcription factors were prepared as histidine-tagged proteins at the C-terminus. The overexpression of transcription factors was achieved by Autoinduction system (Novagen) as instructed by the manufacturer. Purification was carried out as described previously (32).

Construction of the HgtR overexpressing strain

The DNA fragment encoding HgtR was amplified by PCR with primers 5'TCTCATATGGGAAGAATGAGAGAAATC3' and 5'TCGAATTCAGGCGGCCTTCTGGTC TCCGGGTTGAGAC3' (*Nde*I and *Eco*RI sites are underlined). PCR products were digested with *Nde*I and *Eco*RI, and ligated with pET24b (Novagen). After sequence confirmation of the PCR products, the DNA fragment encoding HgtR was isolated by digesting the plasmid with *Nde*I and *Eco*RI and ligated with pCDNdeII (Ueki and Lovley, unpublished data). The plasmid containing the DNA fragment encoding HgtR was designated pCDNdeII/HgtR. Plasmids pCDNdeII and pCDNdeII/HgtR were introduced into *G. sulfurreducens* DL1 by electroporation (30). Overproduction of HgtR was achieved by growing in NBAF media supplemented with kanamycin and IPTG at a final concentration of 1 mM.

Computational tools

To identify a putative repressor binding site, genome sequences of *Geobacter* species reported in NCBI Microbial Genomes (www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html) were analyzed with the sequence 5'TTGTATACAGTATACTAA3' by BLAST within the each genomic resource site. Homology search was conducted by NCBI/BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence alignments were constructed by MAFFT version 6 (<http://align.bmr.kyusyu-u.ac.jp/mafft/online/server/>).

RESULTS

Expression and function of the citrate synthase

In order to better understand the regulation of the expression of *gltA*, the gene encoding citrate synthase, the abundance of *gltA* transcripts and the citrate synthase protein was compared under a diversity of growth conditions. Western blot analysis revealed that the abundance of the citrate synthase protein was lowest when hydrogen was the electron donor and fumarate was the electron acceptor (Figure 1A). Of the other conditions evaluated, growth with lactate as the electron donor and fumarate as the electron acceptor yielded somewhat higher levels of the citrate synthase protein. Primer extension assays showed that the abundance of *gltA* transcripts followed a similar pattern (Figure 1B), suggesting that the expression of *gltA* was primarily regulated at the level of transcription.

The putative $-35/-10$ promoter elements assigned on the basis of the 5' end of the mRNA sequence in the primer extension assays (Figure 1C) show similarity to

Table 1. Growth of the *gltA* deletion mutant

Electron donor	Electron acceptor	Carbon source	Growth
Acetate	Fumarate	Acetate	No
Lactate	Fumarate	Lactate	No
Acetate	Fe(III)	Acetate	No
Hydrogen	Fumarate	Acetate	Yes
Hydrogen	Fumarate	Lactate	Yes
Hydrogen	Fe(III)	Acetate	No

those of *E. coli* RpoD-dependent promoters (34,35) and to those of *G. sulfurreducens* RpoD/RpoS-like promoters (36). The promoter region contains the putative extended -10 element (37,38). Putative transcription termination signals were found downstream of the translation termination codon (Figure 1D). These results suggest that *gltA* is monocistronic.

A *gltA* deletion mutant was isolated with hydrogen as the electron donor and fumarate as the electron acceptor with acetate as the carbon source. Lactate could be substituted for acetate as the carbon source (Table 1). The mutant was unable to grow with organic acids such as acetate and lactate as the electron donor. This is expected because *gltA* encodes the only citrate synthase in *G. sulfurreducens* (24), and citrate synthase is required for the entry of acetyl-CoA derived from organic acids into the TCA cycle (39). The mutant also failed to grow with Fe(III) as the electron acceptor even in the presence of hydrogen. Therefore, the citrate synthase was required for all growth conditions other than with hydrogen as the electron donor and fumarate as the electron acceptor, consistent with the low expression of *gltA* under this growth condition.

Citrate synthase also plays critical roles in growth on organic acids in other microorganisms. For instance, the absence of the citrate synthase influences the growth rate on propionate, as a citrate synthase-negative strain of *E. coli* grows significantly more slowly than its parent strain (40). This citrate synthase mutant can still grow on propionate because *E. coli* possesses 2-methylcitrate synthase, which is induced during growth on propionate (40). In *Saccharomyces cerevisiae*, which contains two citrate synthase isozymes, mitochondrial and cytosolic, a deletion of the mitochondrial citrate synthase causes failure in growth on acetate, while the absence of the cytosolic citrate synthase does not affect growth on acetate (41).

Transcriptional regulation of *gltA* expression

The possibility that the expression of *gltA* is transcriptionally regulated was further investigated. A DNA-binding assay performed with the *gltA* promoter region as a probe detected DNA-binding activity in cell extracts prepared from cultures grown with fumarate as the electron acceptor and acetate or hydrogen as the electron donor (Figure 2A), the growth conditions under which the transcript abundance of *gltA* was low (Figure 1B). In contrast, the DNA-binding activity was undetectable or extremely low, under the other growth

conditions in which the transcript abundance of *gltA* was higher. These results suggested that a transcriptional repressor was involved in the gene regulation.

To determine the binding site for the putative transcriptional repressor, a footprint assay was performed (Figure 2B). The region from nt +11 to +23 with respect to the transcription initiation site was protected from DNase I in the presence of the ammonium sulfate fraction prepared from cell extracts (see also Figure 1C for the sequence). This repressor-binding site shows the dyad symmetry.

To examine the effects of this region on the promoter activity, a *lacZ* fusion assay was carried out (Figure 2C). When the promoter region up to nt -195 with respect to the transcription initiation site was included, there was high β -galactosidase activity. However, no activity was observed when only the region up to nt -2 was included. Deleting the binding site from the promoter fused to the *lacZ* gene doubled β -galactosidase activity over that of the wild-type promoter. These results further indicate that a transcription factor repressed the expression of *gltA* by binding the promoter region from nt +11 to +23.

Other genes involved in metabolism

Searching the genome of *G. sulfurreducens* for the transcriptional repressor binding site sequence of *gltA* (Figure 1C) revealed that this sequence is also located upstream of the genes encoding aconitase B (*acnB*), isocitrate dehydrogenase (*icd*), 2-oxoglutarate:ferredoxin oxidoreductase ferredoxin subunit (*oorD*), succinyl:acetyl CoA transferase (*ato*), succinate dehydrogenase/fumarate reductase cytochrome *b* subunit (*frdC*), ATP synthase F0 β' subunit (*atpG*), nucleoside diphosphate sugar epimerase (*nad*), NADH dehydrogenase I A subunit (*nuoA*), soluble Fe(III) reductase β' subunit (*sfrB*), sugar phosphate isomerase/epimerase (*iolE*) and a transcription factor (*gntR*) in the GntR family (Figure 3A). These putative binding sites also show the dyad symmetry. These genes, except for the transcription factor, appear to encode proteins involved in biosynthesis and energy generation, such as the TCA cycle and electron transport systems. It appears that some of these identified genes (*acnB*, *icd*, *oorD*, *frdC*, *atpG*, *sfrB*, *nad* and *nuoA*) are the first gene in an operon and others (*ato*, *fumA*, *iolE* and *gntR*) are monocistronic. For example, the *frdC* gene is co-transcribed with *frdA* and *frdB*, the genes encoding the other two subunits of the succinate dehydrogenase/fumarate reductase complex (42). An analysis of the genome sequence suggested that the *icd* gene for isocitrate dehydrogenase is in an operon with the *mdh* gene encoding malate dehydrogenase. The *oorD* gene encoding a subunit of 2-oxoglutarate:ferredoxin oxidoreductase appears to be in an operon with the genes *oorA*, *oorB* and *oorC* which encode three other subunits of 2-oxoglutarate:ferredoxin oxidoreductase. It is likely that the *atpG* gene is the first gene of the operon encoding other subunits of ATP synthase F0F1. The *nuoA* gene appears to be the first gene of the operon encoding other subunits of NADH

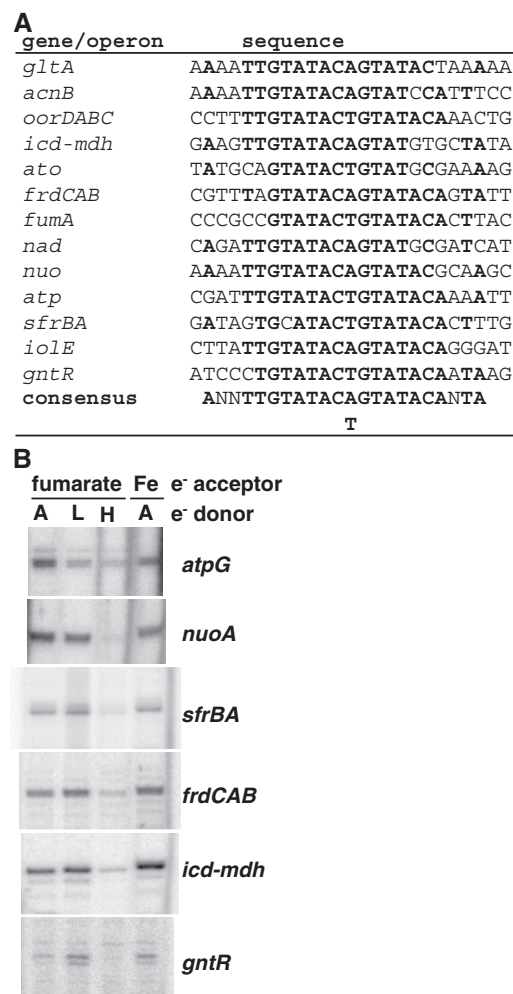


Figure 3. Genes containing the repressor binding site. (A) DNA sequences similar to the repressor binding site in *gltA*. Nucleotides conserved in more than six genes are indicated in bold. (B) Expression of genes containing a putative repressor binding site. Expression was analyzed by primer extension assays as described in Figure 1B.

dehydrogenase I. It appears likely that the *sfrB* gene composes an operon with the *sfrA* gene (43).

All of the genes evaluated (*atpG*, *nuoA*, *sfrB*, *frdC*, *icd* and *gntR*) had lower transcript abundance during growth with hydrogen as the electron donor and fumarate as the electron acceptor (Figure 3B), similar to the results with *gltA* (Figure 1B). The genes involved in the TCA cycle, *frdC* and *icd*, as well as *sfrB* and *gntR* had expression patterns similar to *gltA* under other growth conditions (Figure 3B). However, *atpG* and *nuoA* had transcript abundances during growth on acetate with fumarate as the electron acceptor that were as high as or higher than during growth on lactate. This contrasts with the higher transcript abundance during growth on lactate for the other genes.

Primer extension assays indicated that the putative repressor binding sites are located at the downstream region of the transcription initiation site, the region overlapping the transcription initiation site, or the

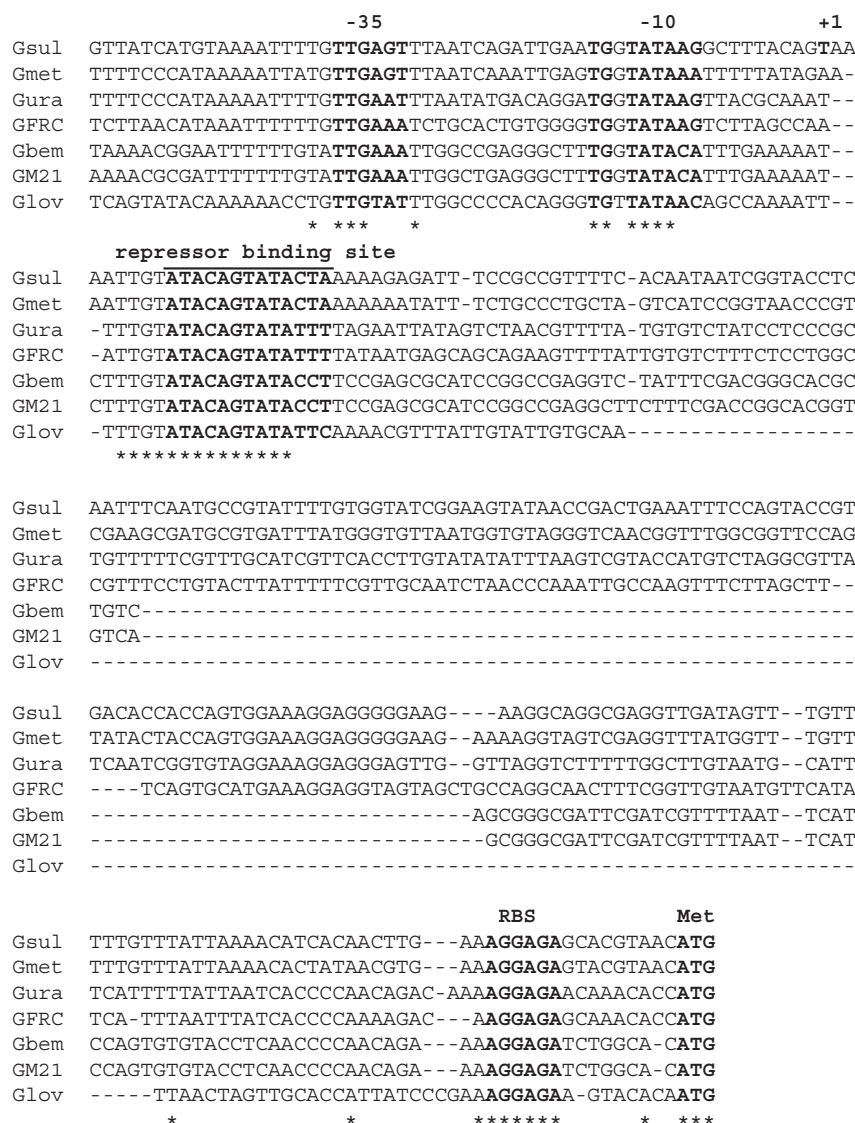


Figure 4. Comparison of the promoter regions of *gltA* from *Geobacter* species. Nucleotides conserved in all species are indicated by asterisks. The putative -35/-10 and extended -10 elements are indicated in bold. The putative transcription initiation site for the *G. sulfurreducens* *gltA* is indicated by a bold letter with +1. The binding site for the repressor is indicated by bold letters. The putative RBS is indicated by bold letters. The initiation codon is indicated by bold letters with Met. Gsul; *G. sulfurreducens*, Gmet; *G. metallireducens*, Gura; *G. uraniireducens*, GFRC; *Geobacter* sp. FRC-32, Gbem; *G. bemidjensis*, GM21; *Geobacter* sp. M21, Glov; *G. lovleyi*.

region overlapping the -35/-10 promoter elements (data not shown). These results suggest that the transcriptional repressor inhibits RNA polymerase from binding to the -35/-10 promoter elements or from elongating transcripts.

Searching the genomes of other *Geobacter* species demonstrated that the transcriptional repressor binding site sequence in the *gltA* promoter region identified in *G. sulfurreducens* is conserved within the genus (see Figure 4 for the *gltA* genes, data not shown for others). These results suggest that this transcriptional repressor is a regulator for the genes involved in biosynthesis and energy generation in *Geobacter* species.

Identification of the transcriptional repressor

In order to identify the transcriptional repressor, putative transcription factors encoded in the *G. sulfurreducens*

genome were analyzed. Among 151 putative transcription factors from *G. sulfurreducens*, 81 are highly conserved in both *G. metallireducens* and *G. uraniireducens*, the other *Geobacter* species for which other complete genome sequences were available at the time of analysis. Putative transcription factors that appeared to be involved in regulatory functions, such as a heat-shock responsive repressor, SOS responsive factors and RNA-binding proteins, were excluded from the subsequent assays. Homologues of enhancer-binding proteins were also excluded because they are typically involved in activation of transcription initiation by RNA polymerase containing the alternative sigma factor RpoN (44–46) whereas the genes regulated by the transcriptional repressor are likely transcribed by RNA polymerase containing the major sigma factor RpoD, as described above. This left 30 putative transcription factors which were selected for the further study.

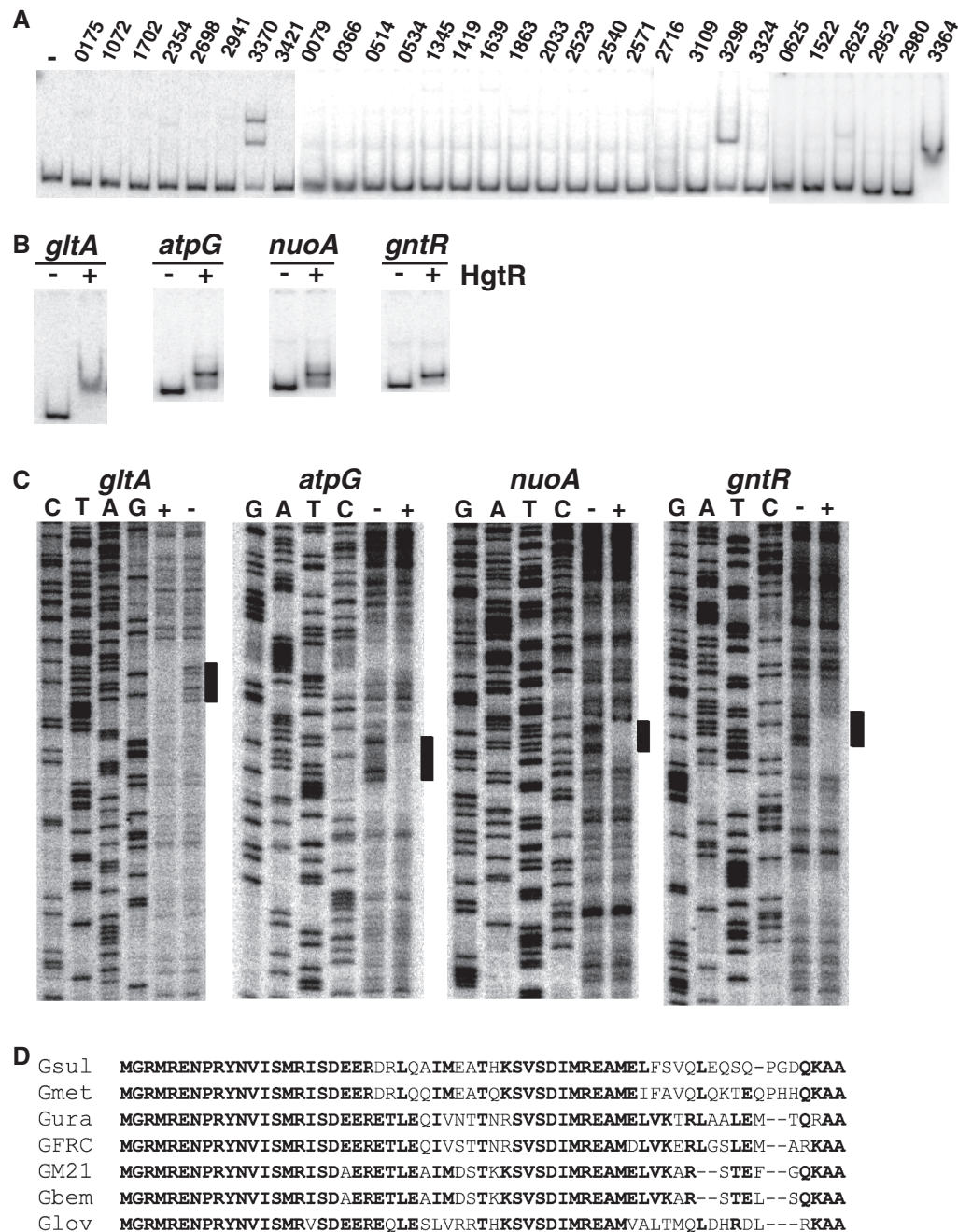


Figure 5. Identification of the repressor. (A) DNA-binding assay with transcription factors conserved in *Geobacter* species. DNA-binding assays were conducted with the probe containing the promoter region of *gltA* and the transcription factors. (B) DNA-binding assay with HgtR. Purified HgtR and promoters of *gltA*, *atpG*, *nuoA* and *gntR* were used in the DNA-binding assays. '–' and '+' indicate the absence and presence of HgtR in DNA-binding assays, respectively. (C) Footprint assay. Purified HgtR and promoter regions of *gltA*, *atpG*, *nuoA* and *gntR* were used in the DNA-binding reactions. '–' and '+' indicate the absence and presence of HgtR in the DNA-binding reactions, respectively. G, A, T and C represent sequence ladders. (D) Comparison of HgtR homologues in *Geobacter* species. Residues conserved in more than three species are indicated in bold.

Of the 30 putative transcription factors evaluated, GSU3370, GSU3298 and GSU3364 bound the *gltA* promoter region (Figure 5A). However, GSU3370 showed a different binding pattern from that observed with the *G. sulfurreducens* cell extracts (Figure 2A), while GSU3298 did not bind the promoters of *atp*, *nuo* and *gntR* (data not shown). Thus, it is unlikely that GSU3370 or GSU3298 was the repressor. The ability

of GSU3364 to bind the promoter regions of *atpG*, *nuoA* and *gntR*, three other genes predicted to have a repressor-binding site (Figure 3A), was evaluated and GSU3364 bound all the three promoters (Figure 5B). Footprint assays demonstrated that GSU3364 bound specifically the promoter regions of *gltA*, *atpG*, *nuoA* and *gntR* (Figure 5C) in the same region expected from the studies outlined above for *gltA* (Figure 2B) and *atpG*,

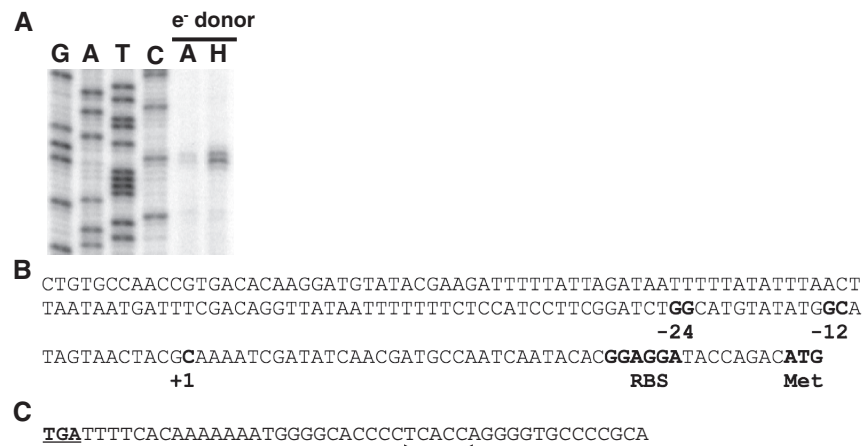


Figure 6. Regulation of *hgtR*. (A) Expression of *hgtR*. Total RNA was prepared from *G. sulfurreducens* DL1 grown in media containing acetate (A) or hydrogen (H) as the electron donor and fumarate as the electron acceptor and analyzed by a primer extension assay. G, A, T and C represent sequence ladders generated by the same primer used in the primer extension assays. (B) The promoter region of *hgtR*. The RpoN-dependent -24/-12 promoter elements are indicated in bold. The 5' end of mRNA (transcription initiation site) is indicated in bold with +1. The putative RBS is underlined. The initiation codon is indicated by bold letters with Met. (C) Transcription termination of *hgtR*. The termination codon is indicated by bold letters with an underline. A putative transcription termination signal is indicated by arrows.

nuoA and *gntR* (Figure 3A). Therefore, GSU3364, which was termed hydrogen-dependent growth transcriptional repressor (HgtR), is likely the transcriptional repressor. Although GSU3298 and GSU3370 are not the repressor that binds the sequence identified by the footprint assay (Figure 2B), it is possible that these transcription factors are involved in the regulation of *gltA* under different growth conditions. Sequence analysis suggests that putative DNA-binding domains of GSU3298 and GSU3370 are similar to those of the XRE and GntR family, respectively, while their putative regulatory (sensor) domains do not exhibit high similarity to known domains (data not shown). Their roles in the regulation of *gltA* remain to be examined.

HgtR is a relatively small protein consisting of 62 amino acid residues (Figure 5D). As expected, HgtR is conserved in all *Geobacter* species whose genome sequences are available (Figure 5D). Although the homologues of HgtR in some of other *Geobacter* species were annotated as CopG-like DNA-binding protein, the amino acid sequences of HgtR homologues showed no similarity to known proteins.

Expression and function of HgtR

Primer extension assays demonstrated that the expression of *hgtR* was higher when hydrogen served as the electron donor than in acetate-grown cells (Figure 6A). This is consistent with the previous results that the genes on which the transcriptional repressor acts were expressed at lower levels during growth on hydrogen.

The highly conserved sequences, GG and GC, of the -24/-12 promoter elements recognized by RpoN (44-46) were identified (Figure 6B). A transcription termination-like signal was found at the downstream of the translation termination codon (Figure 6C), suggesting that *hgtR* is monocistronic.

Table 2. Growth of the *hgtR* deletion mutant

Electron donor	Electron acceptor	Carbon source	Growth
Acetate	Fumarate	Acetate	Yes
Acetate	Fe(III)	Acetate	Yes
Hydrogen	Fumarate	Acetate	No
Hydrogen	Fe(III)	Acetate	No

When the gene for HgtR was deleted, the mutant was unable to grow with hydrogen as the electron donor and either fumarate or Fe(III) as the electron acceptor (Table 2). Transcript levels of *gltA* and *nuoA* were examined during growth with either acetate or hydrogen as the electron donor and fumarate as the electron acceptor. Their transcript levels were much higher in the *hgtR* mutant than the wild-type during growth both in the absence and presence of hydrogen (Figure 7A). It should be noted that the *hgtR* mutant grew with acetate as the electron donor as well as the carbon source even in the presence of hydrogen whereas the wild-type cells grew with hydrogen as the electron donor and acetate as the carbon source in the presence of hydrogen. This suggests that HgtR represses the expression of *gltA* and *nuoA* during growth with acetate as the electron donor as well as during growth with hydrogen as the electron donor, which is consistent with the results showing that the activity of HgtR was detected in the culture under these conditions (Figure 2A). Moreover, this aberrant gene expression caused by the deletion of *hgtR* made the *hgtR* mutant cells unable to adapt to growth with hydrogen.

To further evaluate the function of HgtR, a plasmid containing *hgtR* was introduced into *G. sulfurreducens* and HgtR was overexpressed. The overexpression of HgtR resulted in repression of *gltA* expression (Figure 7B). This is consistent with the conclusion that HgtR is the repressor for *gltA*.

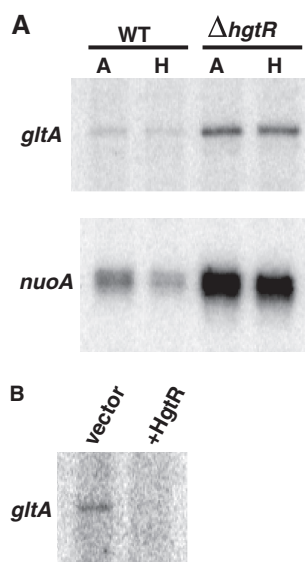


Figure 7. Effects of deletion and overexpression of HgtR. (A) Expression of *gltA* and *nuoA* in the *hgtR* deletion mutant. Total RNA was prepared from the wild-type (WT) and the *hgtR* deletion mutant ($\Delta hgtR$) grown in media containing acetate (A) or hydrogen (H) as the electron donor and fumarate as the electron acceptor in the presence of acetate as the carbon source and analyzed by a primer extension assay. (B) Expression of *gltA* in the strain overexpressing HgtR. Total RNA was prepared from strains harboring the vector or the HgtR expression vector and analyzed by a primer extension assay.

DISCUSSION

These results suggest that HgtR is a global transcriptional regulator that represses a diversity of genes involved in biosynthesis and energy generation in *Geobacter* species during hydrogen-dependent growth. Proper differential expression of this suite of genes appears to be important for growth with acetate or hydrogen as the electron donor. In addition to *gltA*, many of the other genes that were predicted to have an HgtR-binding site, such as malate dehydrogenase, fumarate reductase, succinate dehydrogenase and *sfrAB*, are required for growth with acetate as the electron donor, but not for growth on hydrogen (42,43,47). In contrast, mutants deficient in these genes can grow with hydrogen as the electron donor. Previous studies have demonstrated that levels of expression of genes such as citrate synthase and malate dehydrogenase that are under the transcriptional control by HgtR can be related to the rates of electron transfer to Fe(III) and electrodes in cultures of *G. sulfurreducens* as well as the *in situ* metabolic rates of subsurface *Geobacter* species during the bioremediation of uranium-contaminated groundwater (26,48). Therefore, HgtR is likely to play an important role in optimizing the growth of *Geobacter* species in a range of environments.

Regulation of the expression of citrate synthase via transcriptional repression has been noted previously in other microorganisms. For example, the ArcA two-component response regulator controls the expression of *gltA* in *E. coli* in response to anaerobiosis and carbon supply (49,50). The expression of *citZ* encoding the major citrate synthase in *Bacillus subtilis* is under the control of

catabolite repression by CcpA and CcpC, a member of the LacI/GalR family and the LysR family of transcriptional regulators, respectively (51,52). As with HgtR, ArcA (53), CcpA and CcpC (54,55) are global transcriptional regulators in these bacteria and also control other genes involved in biosynthesis and energy generation.

The amino acid sequences of the HgtR homologues showed no homology to known proteins. The only predicted domain from the structure prediction of the HgtR homologues is a DNA-binding domain, which was predicted to form a structure similar to those of the ribbon-helix-helix (RHH) transcription factor superfamily (Aklujkar, personal communication). The RHH transcription factors share a conserved three dimensional structure, but their amino acid sequences are diversified (56).

The molecular mechanism of hydrogen-dependent gene regulation was previously characterized in the aerobic hydrogen oxidizer, *Ralstonia eutropha*, in which a regulatory [NiFe]-hydrogenase acts as a hydrogen receptor and regulates the two-component system consisting of HoxJ and HoxA that controls the gene expression (57). The increase in *hgtR* transcripts during growth with hydrogen suggests that the expression of *hgtR* is regulated at the level of transcription and the transcription of *hgtR* appears to be mediated by the sigma factor RpoN (Figure 6). Unlike most of other bacterial *rpoN* genes, the *G. sulfurreducens* *rpoN* is essential under the all conditions tested (58). In *G. sulfurreducens*, RpoN controls the expression of genes involved in a wide range of cellular processes such as fumarate respiration, Fe(III) reduction, nitrogen fixation, and pili and flagella biosyntheses (58). The genome sequence analysis identified the gene located at the upstream of *hgtR*, which encodes a putative enhancer-binding protein (EBP) that has a domain similar to the iron only hydrogenase large subunit (data not shown). HgtR does not have an apparent regulatory (sensor) domain. Therefore, it is possible that the EBP senses hydrogen and activates the transcription of *hgtR*. This type of EBP has not been identified in other bacteria. A gene encoding a homologue of the transcription factor GntR appears to be a target of HgtR (Figure 5), and thus the expression of other genes is likely to be affected during growth with hydrogen. Taken together, it is likely that metabolic genes are controlled by novel transcriptional regulatory cascades in *Geobacter* species.

It is likely that transcriptional regulation by HgtR represents only one of several levels of regulation of citrate synthase. For example, the promoter regions of *gltA*, *ato*, *frdCAB* and *icd-mdh* contain a long 5' untranslated region, which was predicted to form a secondary structure that appears to play a role in translation of their mRNA (data not shown), providing the possibility for further regulation at the post-transcriptional level. Furthermore, western blot analysis showed that the migration of the *G. sulfurreducens* citrate synthase in SDS-PAGE gel was affected by heat-denaturation, suggesting that heat-sensitive modulation occurred at the post-translational level (Yun, personal communication). Phosphorylation is a means of regulating citrate synthase in *Tetrahymena* (59)

and isocitrate dehydrogenase, another enzyme in the TCA cycle, in some bacteria (60–62).

In summary, our findings provide important new insights into the mechanisms by which *Geobacter* species regulate, and presumably optimize, their central metabolism under different environmental conditions. Further research into the environmental cue(s) controlling the expression of HgtR and additional mechanisms for regulation of central metabolism is warranted and is underway.

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