

## Role of Rel<sub>Gsu</sub> in Stress Response and Fe(III) Reduction in *Geobacter sulfurreducens*<sup>∇†</sup>

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*Geobacter* species are key members of the microbial community in many subsurface environments in which dissimilatory metal reduction is an important process. The genome of *Geobacter sulfurreducens* contains a gene designated *rel<sub>Gsu</sub>*, which encodes a RelA homolog predicted to catalyze both the synthesis and the degradation of guanosine 3',5'-bispyrophosphate (ppGpp), a regulatory molecule that signals slow growth in response to nutrient limitation in bacteria. To evaluate the physiological role of Rel<sub>Gsu</sub> in *G. sulfurreducens*, a *rel<sub>Gsu</sub>* mutant was constructed and characterized, and ppGpp levels were monitored under various conditions in both the wild-type and *rel<sub>Gsu</sub>* mutant strains. In the wild-type strain, ppGpp and ppGp were produced in response to acetate and nitrogen deprivation, whereas exposure to oxygen resulted in an accumulation of ppGpp alone. Neither ppGpp nor ppGp could be detected in the *rel<sub>Gsu</sub>* mutant. The *rel<sub>Gsu</sub>* mutant consistently grew to a higher cell density than the wild type in acetate-fumarate medium and was less tolerant of oxidative stress than the wild type. The capacity for Fe(III) reduction was substantially diminished in the mutant. Microarray and quantitative reverse transcription-PCR analyses indicated that during stationary-phase growth, protein synthesis genes were up-regulated in the *rel<sub>Gsu</sub>* mutant and genes involved in stress responses and electron transport, including several implicated in Fe(III) reduction, were down-regulated in the mutant. The results are consistent with a role for Rel<sub>Gsu</sub> in regulating growth, stress responses, and Fe(III) reduction in *G. sulfurreducens* under conditions likely to be prevalent in subsurface environments.

Members of the family *Geobacteraceae* carry out a number of important processes in sedimentary environments, but little is known about the mechanisms regulating their metabolism in response to the environmental stresses typical of the subsurface. The hallmark physiological characteristic of *Geobacteraceae* is their ability to oxidize organic compounds with the reduction of extracellular electron acceptors, such as Fe(III) and Mn(IV) oxides (45), U(VI) (46), humic substances (43), and electrodes (6). *Geobacteraceae* are important not only in the anaerobic oxidation of naturally occurring organic matter coupled to Fe(III) reduction (44) but also in the degradation of organic contaminants in subsurface environments (40, 67, 68) and are useful agents in uranium-contaminated subsurface environments (1, 59, 79).

*Geobacteraceae* are likely to face suboptimal concentrations of electron donors (1) and nutrients (33), as well as other stresses, such as heavy metals and toxic organics, in subsurface environments. Many bacterial stress response systems that have been identified and characterized for other organisms are encoded in the genome of the *Geobacteraceae* model species, *Geobacter sulfurreducens* (52). These include regulatory genes involved in oxidative stress response (RpoS and PerR), heat

shock (RpoH), and metal homeostasis (Fur, Zur, and IdeR), as well as many two-component regulatory system genes (52). Studies have confirmed a role for RpoS in stationary-phase survival and oxidative stress response in *G. sulfurreducens* (60), and preliminary studies of several other regulators suggest their roles are analogous to those found in other organisms.

Another well-known response of microorganisms to suboptimal growth conditions is the stringent response (7, 13, 16, 34, 47). In the stringent response, guanosine 3',5'-bispyrophosphate (ppGpp) and, in some species, triphosphate (21, 35, 62) and pentaphosphate (14, 20, 28) derivatives of this molecule are produced in response to nutrient limitation. These stringent factors interact with RNA polymerase to influence transcription of various genes. The hallmark of the stringent response is the down-regulation of stable RNA molecules and translation machinery, but this response also includes the up-regulation of stress response genes (26, 76). Intracellular levels of ppGpp are regulated by two enzyme activities that act to synthesize and degrade the molecule in response to various triggers in the cell. In *Escherichia coli*, synthesis and degradation of ppGpp are catalyzed by two distinct but homologous proteins, RelA and SpoT (13). Several organisms, including many proteobacteria and gram-positive organisms, contain a single *rel*-like gene that is predicted to perform both enzyme activities (31, 49, 54, 80, 81).

Here we present evidence that *G. sulfurreducens* has a single protein, designated Rel<sub>Gsu</sub>, for controlling levels of ppGp(p) and that this activity plays an important role in regulating the expression of genes necessary not only for adapting to environmental stress but also for Fe(III) reduction.

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culturing conditions.** *Escherichia coli* strain DH5 (*supE44 lacU169  $\phi$ 80 lacZ M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) (84) was used for DNA manipulations. Strain DLLD1 (*rel<sub>Gsu</sub>::kan*) was produced through targeted gene disruption of *Geobacter sulfurreducens* strain DL1 (ATCC 51573) (11, 17). *G. sulfurreducens* strains were routinely cultured anaerobically in acetate-fumarate (NBAF) medium or acetate-Fe(III) citrate medium in batch culture as previously described (17). The plasmid pBBR1MCS-2 (36) was obtained from Michael Kovach.

**DNA manipulations and reagents.** *G. sulfurreducens* genomic DNA was extracted with a MasterPure complete DNA and RNA purification kit (Epicenter Technologies, Madison, WI). Plasmid purification, PCR product purification, and gel extractions were performed with the following kits: a QIAprep Spin miniprep kit, a QIAquick PCR purification kit, and a QIAquick gel extraction kit (QIAGEN, Inc., Valencia, CA). Transformations into *E. coli* and other routine DNA manipulations were carried out as outlined by Sambrook et al. (70). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Ligations were carried out using either a rapid DNA ligation kit (Roche Applied Science, Basel, Switzerland) or a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Southern blotting was performed as previously described (17), and hybridization and detection were performed with a Roche Applied Science digoxigenin-labeling and digoxigenin nucleic acid detection kit using the chromogenic method and nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) substrate according to the manufacturer's instructions. *Taq* DNA polymerase (QIAGEN, Inc.) was used for all PCR amplifications. Unless otherwise stated, chemicals were reagent grade or better and were purchased from Sigma Chemical Co. (St. Louis, MO).

**Construction of *Rel<sub>Gsu</sub>*-deficient strain via single-step gene replacement.** To construct a *Rel<sub>Gsu</sub>*-deficient mutant, recombinant PCR (55) was used to construct a linear DNA fragment consisting of a kanamycin resistance cassette flanked by homologous sequence from the 5' and 3' ends of the *rel<sub>Gsu</sub>* gene. Three primary PCRs were carried out: (i) amplification of the 5' end of the linear fragment with primers RelA.1 (GGT GCT GGA TGC GGT TTC) and RelA.2 (GGA CCT TTG CAC AGT AGA C), (ii) amplification of the kanamycin resistance cassette from pBBR1MCS-2 (36) with primers RelA.3 (GTC TAC TGT GCA AAG GTC C/ACC TGG GAT GAA TGT CAG CTA C) and RelA.4 (CGT GAC AGG AAA CTC GG/AGA AGG CGG CGG TGG AAT CG), and (iii) amplification of the 3' end of the linear fragment with primers RelA.5 (CCG AGT TTC CTG TCA CG) and RelA.6 (GTC CAT TAC GCG CAT CG). Following recombinant PCR with the three primary PCR products serving both as templates and as primers, the final fragment was amplified with the distal primers RelA.1 and RelA.6. PCR conditions were as follows: 94°C for 15 s, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, and a final 10-min extension step at 72°C. The same amount of primer (20 pmol) was used for each reaction. Electroporation, mutant isolation, and genotype confirmation were performed as described by Coppi et al. (17) and Lloyd et al. (41). One of the resulting mutants was chosen as the representative strain.

**Expression of *rel<sub>Gsu</sub>* in trans.** The complete coding sequence for *rel<sub>Gsu</sub>* was amplified using primers containing either an EcoRI (GCATGAATTC CAA TCT CTT TCA TGC TCC) or a BamHI (GTATGGATCC GCT AAT CAC AG CAC TCC) site. PCR amplification conditions were the same as those described above. The amplicon was digested with BamHI and EcoRI and inserted into the corresponding sites of the broad-host-range expression vector pRG5 (9). The insert was then sequenced to screen for PCR artifacts. Following electroporation of the *rel<sub>Gsu</sub>* mutant strain with the appropriate vector, spectinomycin-resistant transformants were isolated. The simultaneous presence of complementation vector and the original mutation in the resulting strain was confirmed by PCR screening and plasmid isolation.

**Nutrient shutdown experiments and quantitation of guanosine phosphates.** Wild-type and *Rel<sub>Gsu</sub>*-deficient strains of *G. sulfurreducens* were initially cultured in NBAF medium (17) supplemented with 1 mM cysteine. Log-phase (optical density at 600 nm [OD<sub>600</sub>] of 0.4 to 0.5) acetate-fumarate cultures (100, 300, or 500 ml) were harvested by centrifugation, washed, and resuspended in 500 ml freshwater acetate-fumarate (FWAF) medium (17) containing either acetate (15 mM) and ammonium (6 mM) or lacking either acetate or ammonium. FWAF differs from NBAF mainly in buffering capacity, trace element content, and fumarate concentration (27.7 mM for FWAF versus 40 mM for NBAF). Initial culturing in NBAF was required to obtain sufficient biomass for detection of guanosine phosphates. FWAF medium was used for subsequent steps, because use of this medium resulted in a cleaner lysate and significantly prolonged the life of the column used to quantitate guanosine phosphate content. At 20-min intervals, 100-ml aliquots of the various cultures were filtered through a 90-mm

Millipore nitrocellulose prefilter (Bedford, MA) and then extracted in 15 ml of 1 N formic acid as previously described (25). Extracts were freeze-dried using a Labconco lyophilizer (Kansas City, KS) and stored at -20°C.

Guanosine phosphate derivatives (ppGp and ppGpp) were quantitated by high-pressure liquid chromatography (HPLC) on a 250-mm by 4.6-mm Partisil SAX 10- $\mu$ m column (Alltech, Deerfield, IL) as described by Jones et al. (35), using an LC-10AT high-pressure liquid chromatograph (Shimadzu, Kyoto, Japan) and a 20- $\mu$ l injection volume. Immediately prior to injection, samples were resuspended in either 400  $\mu$ l or 500  $\mu$ l H<sub>2</sub>O. ppGpp standards were obtained from TriLink Biotechnologies (San Diego, CA), and ppGp standards were a gift from Mercian Corporation, Japan.

**Analytical techniques.** Growth of fumarate cultures was assessed by measuring turbidity at 600 nm. Fe(II) concentrations were determined by ferrozine assay (45). Cell densities of Fe(III)-grown cultures were determined by epifluorescence microscopy using acridine orange staining (45). The protein content of cell fractions was determined by the bicinchoninic acid method, with bovine serum albumin as the standard (74). Pairwise alignments were performed using the Needleman and Wunsch algorithm (57).

**RNA isolation.** Cells were harvested as previously described (51). Briefly, cultures were centrifuged at 4°C for 15 min and pellets were flash frozen and stored at -80°C. To extract total RNA, cells were mechanically disrupted using a FastPrep instrument (Qbiogene, Inc., Irvine, CA) with lysing matrix B (Qbiogene) and nucleic acids were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), a monophasic solution of phenol and guanidine isothiocyanate. Residual DNA was removed using RNase-free DNase (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. The treated RNA was subsequently cleaned and concentrated with RNeasy minicolumns (QIAGEN, Inc., Valencia, CA). Quality of total RNA was assessed by agarose-formaldehyde gel electrophoresis, and the concentration was determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE) (51).

**DNA microarray hybridization and data analysis.** Total RNA was isolated from three sets of identically treated, early-stationary-phase, 100-ml NBAF batch cultures of both the wild-type and the *Rel<sub>Gsu</sub>*-deficient strains. DNA microarray hybridization and data analyses were performed as described previously (51). Briefly, approximately 5  $\mu$ g of total RNA was used for indirect labeling with either cyanine 3 or cyanine 5 (Cy3/Cy5) fluorescent dyes, leading to production of approximately 4 to 5  $\mu$ g of cDNA with greater than 200 pmol, respectively, of each dye molecule incorporated per microgram of cDNA synthesized. Triplicate control and treatment stationary-phase cultures were extracted for each experiment so that extracted RNA could be paired to produce three biological replicates from which hybridizations could be repeated (technical replicates). Following hybridizations, slides were promptly scanned at a 10- $\mu$ m resolution using an Axon 4000B scanner with GenePix 4.0 software.

Processing of 16-bit TIFF images from hybridized arrays was done using the TIGR TM4 package ([www.tigr.org/software](http://www.tigr.org/software)). Intensity values for Cy3 and Cy5 channels were obtained using TIGR-Spotfinder software. Normalization was performed using the LOWESS algorithm available in TIGR-MIDAS using block mode and a smooth parameter of 0.33. All intensity values less than two times greater than background were removed from subsequent analysis, and replicate reporter intensities on one slide (one technical replicate) were reduced to a single value by computing the geometric mean. Four hybridizations were performed from each of three biological replicate stationary-phase pairs (control and treatment). Half of the technical replicate dye labelings were dye swaps (flip dyes) performed as part of overall quality assurance.

**Measurement of relative transcript levels using quantitative reverse transcription-PCR (RT-PCR).** Total RNA was isolated as described in "RNA isolation" above. Single-stranded cDNA was generated by the reverse transcription of 2  $\mu$ g of total RNA in a 100- $\mu$ l reaction volume using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The cDNA was then subjected to quantitative PCR using SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Forward and reverse primers were added to the reaction mixture at a final concentration of 200 nM along with 1  $\mu$ l of the cDNA reaction mixture. The incorporation of SYBR green dye into the PCR products was detected in real time on an ABI Prism 7900HT sequence detection system. ROX (6-carboxyl-X-rhodamine) passive reference dye was used to factor in well and pipetting variability. The incorporation of SYBR green resulted in the determination of the cycle threshold, which identifies the PCR cycle at which exponential growth of the PCR products begins. Standard curves were established for each cDNA sample being analyzed by use of primers for a gene, DNA polymerase III, beta subunit (GSU0001), showing unchanged expression levels in prior microarray analyses. The standard curves were normalized to each other through the control gene (GSU0001), and quantitation was subsequently determined. Primers used for amplification are listed in Table 1.

TABLE 1. Sequences of primers used in quantitative RT-PCR

Gene designation	Gene product	Primer name	Primer sequence (5'-3')
GSU0466	Cytochrome <i>c</i> <sub>551</sub> peroxidase	RT_ORF00777_F RT_ORF00777_R	CACCATGCCCTACTTCCACT GGTGTGAGGAACGTGACAA
GSU1346	Sulfate ABC transporter, periplasmic sulfate-binding protein	RT_ORF02287_F RT_ORF02287_R	GATGTGGTTACCCTGGCACT GAGGTGTAGGGGAGCTGTT
GSU1496	Pilin domain protein	RT_ORF02545_F RT_ORF02545_R	CCAACACAAGCAGCAAAAAAG GCAGCGAGAATACCGATGAT
GSU2409	Heat shock protein, Hsp20 family	RT_ORF03975_F RT_ORF03975_R	TGAAGAGACACGGTCAAACG TGACATCCAGGGTTTCCTC
GSU2504	Cytochrome <i>c</i> family protein	RT_ORF04142_F RT_ORF04142_R	CAACCTGGCATAACGAGTTCA CCATAGTAGGCAGCGGTCAT
GSU2737	Polyheme membrane-associated cytochrome <i>c</i>	RT_ORF04536_F RT_ORF04536_R	GACACGGTCAACCAGAACAA GGTCCCAGTTTACGACAGGA
GSU2813	Cytochrome <i>c</i> <sub>551</sub> peroxidase	RT_ORF04662_F RT_ORF04662_R	TTCGACAACATGGCAAAGG TGTCGAGGAAAAGCTTGAGG
GSU2814	Rubryerythrin	RT_ORF04665_F RT_ORF04665_R	CAAGCGCTTTTTCAAGTTCC AGAGGTCGGAATGCTCTTCA
GSU2821	Nitrogenase iron protein	RT_ORF04677_F RT_ORF04677_R	AAGCTCGGCACTCAGATGAT GCTTGTGCTCGGGAGAATAC
GSU2839	Ribosomal protein L30	RT_ORF04707_F RT_ORF04707_R	ATATCGGGACGACCAGCAAG CCCACGATTTCCAGGAGTGTT
GSU2875	Ribosomal protein S9	RT_ORF04761_F RT_ORF04761_R	CATCAAGCACGGCATAACC CCGTACTTTTTCCGCTCCTT

**Microarray data accession numbers.** Descriptions of the microarray experiments, quantitation data, and array design have been deposited into Array-Express ([www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress)) and have been assigned accession numbers A-TIGR-20 and E-TIGR-5000.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the proteins described in this report are as follows: for Rel<sub>Gsu</sub>, accession number AAR35612; for *Geobacter metallireducens* Rel, accession number ABB32550.1; for *Pelobacter propionicus* Rel, accession number ZP\_00677757.1; and for *Pelobacter carbinolicus* Rel, accession number ABA88536.1.

## RESULTS

**Rel<sub>Gsu</sub> protein and operon characteristics.** A RelA/SpoT homolog was identified in the *Geobacter sulfurreducens* genome and designated rel<sub>Gsu</sub>. Rel<sub>Gsu</sub> appears to consist of four domains, each of which is homologous to those commonly found in bifunctional RelA/SpoT proteins (Fig. 1). These domains include a RelA/SpoT domain, which is the source of ppGpp synthetase activity (4, 31, 48, 49); a metal-dependent hydrolase domain implicated in hydrolysis of ppGpp (3, 4, 31,

48, 49); an ACT (aspartokinase, chorismate mutase, TyrA, or prephenate dehydrogenase) domain (2, 72), considered to have a regulatory role involving amino acid binding; and a TGS (ThrRS, GTPase, and SpoT) domain, predicted to function in nucleotide binding (2, 71, 72, 82) (Fig. 1). In contrast, most monofunctional RelA homologs lack the hydrolase domain, and SpoT homologs lack the ACT domain (NCBI, CDART [<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>]). In addition, this homolog is more similar to bifunctional RelA/SpoT proteins (5, 48, 81), such as that found in *Bacillus subtilis*, with which it shares 69% similarity (81), than to individual RelA and SpoT proteins of *E. coli* (53), with which it shares 58% and 59% similarity, respectively. Thus, Rel<sub>Gsu</sub> is likely to catalyze both the synthesis and the hydrolysis of ppGpp in response to multiple signals in the cell.

Genes with high similarity to Rel<sub>Gsu</sub> are present in other *Geobacter* species, including *Geobacter metallireducens*, with 95.8% similarity, *Geobacter uraniumreducens*, with 93.3% similarity, *Pelobacter propionicus*, with 89.7% similarity, and *Pelobacter carbinolicus*, with 77.5% similarity. Homologs with high percent similarity are also found in other  $\delta$ -proteobacteria, such as *Myxococcus xanthus*, with 72.9% similarity (28), and *Desulfovibrio* species, with 69 to 71% similarity (29). Because all of these genes appear to show high homology to a predicted bifunctional RelA/SpoT protein, it suggests that  $\delta$ -proteobacteria utilize a bifunctional RelA/SpoT protein for the regulation of ppGpp levels rather than the two different proteins, RelA and SpoT, found in many other bacteria.

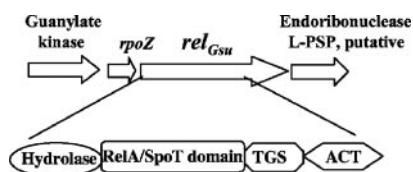


FIG. 1. Structure of putative rel<sub>Gsu</sub> operon and domain architecture of Rel<sub>Gsu</sub> protein.

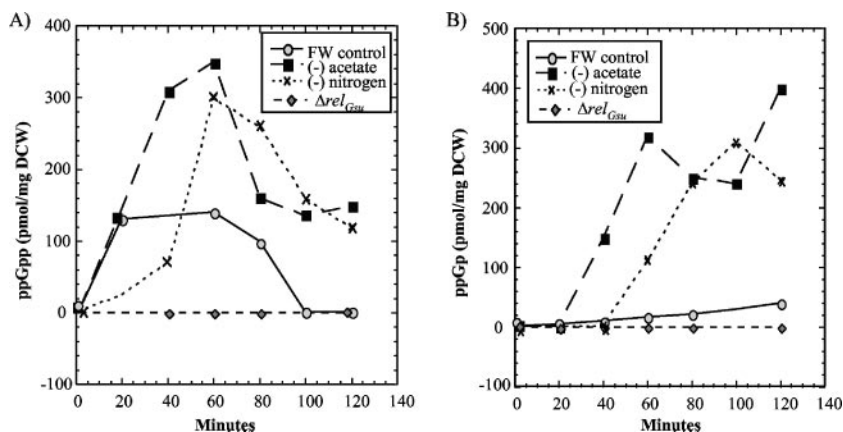


FIG. 2. Production of ppGp(p) in response to nutrient deprivation. Effect of nutrient deprivation on accumulation of (A) ppGpp and (B) ppGp in wild-type *G. sulfurreducens* and the *rel<sub>Gsu</sub>* mutant. Wild-type and mutant cells were grown in NBAF medium to mid-log phase, harvested by centrifugation, and transferred to FW medium containing acetate and ammonium (FW control), FW medium without acetate [(-) acetate], or FW medium without ammonium [(-) nitrogen]. All wild-type data points represent averages of duplicate experiments. No ppGp(p) was detected in the *rel<sub>Gsu</sub>* mutant ( $\Delta rel_{Gsu}$ ) in media lacking acetate or in media lacking ammonia. DCW, dry cell weight.

*rel<sub>Gsu</sub>* is predicted to be in an operon with four open reading frames (86). The genes include guanylate kinase; the RNA polymerase omega subunit *rpoZ*, which has recently been determined to be necessary for ppGpp binding to RNA polymerase (78); *rel<sub>Gsu</sub>*; and endoribonuclease liver perchloric acid-soluble protein (Fig. 1). The structure of this putative operon is similar to *spoT* operons of several  $\gamma$ -proteobacteria, such as *Shewanella oneidensis* and *Pseudomonas aeruginosa*, and is conserved throughout the *Geobacteraceae* (<http://microbesonline.org>). Similar gene clusters are not found in the genomes of other families of  $\delta$ -proteobacteria, such as *Desulfovibrio* spp., in which the *rel* gene lies in a putative operon with an ABC dipeptide transport protein (<http://microbesonline.org>).

**Production of guanosine phosphate derivatives ppGpp and ppGp in response to nutrient deprivation and oxidative stress.** In order to evaluate whether nutrient limitation triggered accumulation of guanosine phosphates in *G. sulfurreducens*, levels of ppGpp and its derivatives were measured during growth in nutrient-deprived conditions. Mid-log-phase NBAF cultures were washed and resuspended in FW medium containing fumarate but no acetate. This resuspension medium differed from the growth medium, containing less buffering capacity and fewer trace minerals, as described in Materials and Methods, likely resulting in a short-term increase in detectable guanosine phosphates even in the presence of acetate (Fig. 2A, FW control). However, levels of ppGpp were substantially higher in the absence of acetate (Fig. 2A), and levels of ppGp, a second guanosine phosphate derivative detected in these cultures, also increased in the absence of acetate (Fig. 2B). Omitting fixed nitrogen, in the form of ammonium, from the resuspension medium also resulted in elevated levels of ppGpp and ppGp (Fig. 2). ppGpp and ppGp were the only two guanosine phosphate derivatives detected in this analysis. A derivative that is commonly found in other organisms, pppGpp (14, 28), was not detected.

To examine ppGp(p) levels induced during other stress responses, ppGp(p) was measured during oxidative stress by exposing cells to oxygen. Control cultures growing in NBAF medium typically had concentrations of ppGpp of less than 10

pmol/mg cells (dry weight) (Fig. 3), while levels of ppGp in 20  $\mu$ l of injected cell extract were never above the HPLC detection limit of 3 pmol (data not shown). When 6% oxygen was added to the headspace of mid-log-phase NBAF cultures, ppGpp accumulated (Fig. 3), but ppGp remained undetectable (data not shown), indicating that ppGpp is the only guanosine phosphate derivative produced at detectable levels in *G. sulfurreducens* in response to oxidative stress.

**Phenotypic characterization of a *Rel<sub>Gsu</sub>*-deficient mutant.** A *rel<sub>Gsu</sub>* mutant was constructed by homologous recombination (see Materials and Methods). Neither ppGpp nor ppGp levels, in a 20- $\mu$ l injection volume, were detected above the HPLC detection limit of 14 pmol or 3 pmol, respectively, in mutant cultures subjected to the same nutrient-deprived conditions that yielded accumulation of ppGpp and ppGp in the wild-type strain (Fig. 2). This result indicates that the *rel<sub>Gsu</sub>* mutant cannot produce significant levels of ppGp(p), consistent with *Rel<sub>Gsu</sub>* being involved in the production of both compounds in *G. sulfurreducens*.

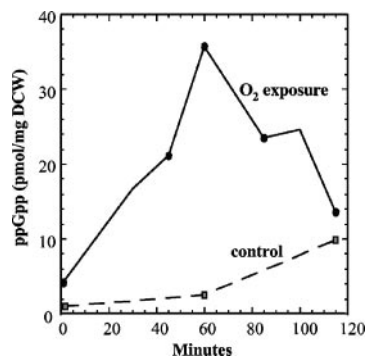


FIG. 3. Production of ppGpp in response to oxidative stress. Wild-type cells were grown in NBAF medium to mid-log phase, and then 6% oxygen was added to the headspace. Samples were taken every 20 min after the addition of oxygen. Detection of ppGp(p) was monitored using HPLC as described in Materials and Methods. Production of ppGp was not detected. Data points are representative values from duplicate experiments. DCW, dry cell weight.

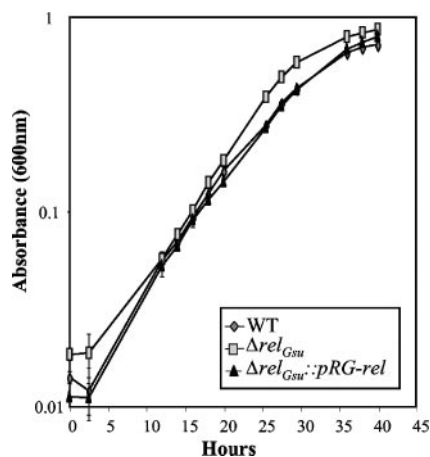


FIG. 4. Growth of the  $rel_{Gsu}$  mutant in acetate-fumarate medium. Wild-type (WT),  $\Delta rel_{Gsu}$  mutant, and complemented  $\Delta rel_{Gsu}$  ( $\Delta rel_{Gsu}::pRG-rel$ ) strains were grown in NBAF medium. Values represent the means  $\pm$  standard errors of triplicate cultures.

The  $rel_{Gsu}$  mutant cells grew at a rate similar to that of wild-type cells during exponential growth in NBAF medium (Fig. 4). However, the mutant consistently achieved a higher cell density ( $OD_{600}$  of 0.86) than the wild type ( $OD_{600}$  of 0.72), and this higher cell density was most apparent when the cells were approaching and entering stationary phase (Fig. 4). Complementing the mutant with the  $rel_{Gsu}$  gene expressed in *trans* restored the wild-type growth pattern (Fig. 4).

Cysteine (1 mM) is typically added as a reductant to NBAF medium because it reduces the length of the lag phase and increases the maximum density of the cultures. Although the  $rel_{Gsu}$  mutant grew at least as well as the wild type in the typical NBAF medium (see above), when cysteine was omitted, raising the redox potential of the medium, the mutant cells had a lag phase that was nearly double that of the wild-type cells (Fig.

5A). Cells of the complemented strain had a lag phase in cysteine-free medium comparable to that of the wild-type cells (Fig. 5A).

Further evidence for an impaired oxidative stress response in  $rel_{Gsu}$  mutants became evident when cysteine-free cultures were grown to stationary phase after the introduction of air into the headspace. The wild-type strain was capable of growth in the presence of 2% air but formed distinct aggregates, a phenomenon seen previously and believed to be an adaptive response to oxidative stress in *G. sulfurreducens* (60) (Fig. 5B). Under the same conditions, the  $rel_{Gsu}$  mutant failed to aggregate and grew as a uniform suspension (Fig. 5B).

The  $rel_{Gsu}$  mutant also demonstrated defects in Fe(III) reduction, reducing Fe(III) nearly threefold more slowly than the wild type in medium with acetate as the electron donor and Fe(III) citrate as the electron acceptor. The  $rel_{Gsu}$  mutant cell yield was also only 60% of that seen with the wild type (Fig. 6). The complemented strain reduced Fe(III) at wild-type rates.

**Microarray analysis of the *G. sulfurreducens*  $rel_{Gsu}$  mutant versus the wild type.** To identify genes that were differentially regulated in the absence of Rel $_{Gsu}$  activity, levels of gene expression in  $rel_{Gsu}$  mutant and wild-type cultures grown to stationary phase in NBAF medium supplemented with cysteine were compared using a whole-genome microarray. Stationary-phase cells were harvested after approximately 35 h of growth, when cell density increases slowed. A subset of genes that were differentially expressed in the microarray analysis were subsequently validated using quantitative RT-PCR (51). Stationary-phase cells were chosen for this analysis because depletion of nutrients that occurs during this stage has been shown to induce Rel activity in other organisms (7, 16) and because growth characteristics were shown to differ between the wild type and the  $rel_{Gsu}$  mutant during this stage (Fig. 4). For microarray results, differentially expressed genes were identified by the application of the SAM algorithm (77) to replicate hybridizations performed with RNA from individual identi-

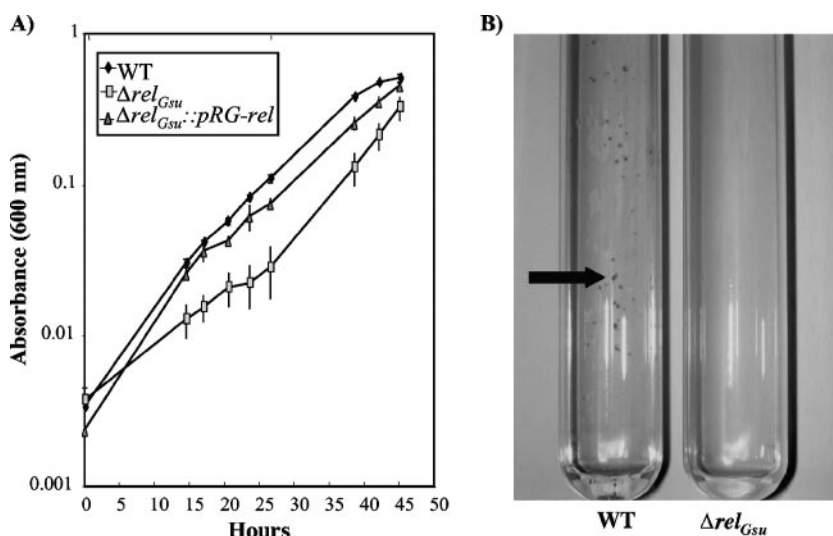


FIG. 5. Growth of the  $rel_{Gsu}$  mutant under oxidative stress. (A) Wild-type (WT),  $\Delta rel_{Gsu}$  mutant, and complemented  $\Delta rel_{Gsu}$  ( $\Delta rel_{Gsu}::pRG-rel$ ) strains were grown in NBAF medium without cysteine. Values represent means  $\pm$  standard errors of triplicate cultures. (B) Effect of exposure to air on wild-type and  $\Delta rel_{Gsu}$  strains. Strains were inoculated into pressure tubes containing 2% air in the headspace and allowed to grow to stationary phase. The arrow indicates aggregates present in the wild-type culture that were absent in the  $\Delta rel_{Gsu}$  culture.

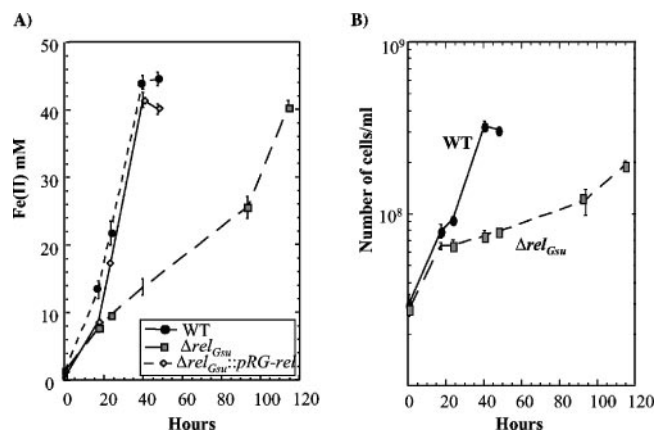


FIG. 6. Growth of the wild-type (WT),  $rel_{Gsu}$  mutant, and complemented  $\Delta rel_{Gsu}$  ( $\Delta rel_{Gsu}::pRG-rel$ ) strains in acetate-Fe(III) citrate medium. (A) Fe(III) citrate reduction and (B) cell growth. Data are means  $\pm$  standard errors of triplicate cultures.

cally treated biological samples. Genes showing significant differential expression under these criteria, as defined by Methé et al. (51), were then further selected according to whether differential expression occurred in at least two biological replicates. Application of these criteria resulted in identification of 332 genes that were differentially expressed in the  $rel_{Gsu}$  mutant during stationary-phase growth. Of the genes found to be differentially expressed, 162 genes were up-regulated, and 170 genes were down-regulated (see Tables S1 and S2 in the supplemental material). As expected,  $rel_{Gsu}$  was among the down-regulated genes.

The differentially expressed genes were assigned to 12 functional categories based on the published annotation of the *G. sulfurreducens* genome (52) (Fig. 7; also see Tables S1 and S2 in the supplemental material). The largest category of differentially expressed genes consisted of genes of unknown function, including both hypothetical genes and conserved genes of unknown function. The next-largest category of differentially expressed genes included those involved in electron transport. The majority of these genes were down-regulated in the mutant (Table 3; Fig. 7). Another large category of differentially expressed genes included those involved in protein biosynthesis, which were primarily up-regulated in the mutant (see Table S1 in the supplemental material). These genes included many encoding proteins involved in sulfur assimilation, which were highly up-regulated, as well as ribosomal proteins and tRNA synthetases. Four genes from this group were confirmed to be up-regulated in the mutant by use of quantitative RT-PCR (Table 2). Many signaling and transport genes were also differentially regulated under these conditions (see Tables S1 and S2 in the supplemental material). Many genes involved in stress response were down-regulated in the mutant, including a universal stress protein (56), an RND efflux transporter (58), and desulfoferredoxin (42), as well as several redox proteins implicated in combating oxidative stress, such as cytochrome  $c_{551}$  peroxidase (12, 73), Hcp1, or prismane (8), and rubrerythrin (75) (see Table S2 in the supplemental material). Cytochrome  $c_{551}$  peroxidase and rubrerythrin were confirmed by quantitative RT-PCR to be down-regulated in the mutant (Table 2).

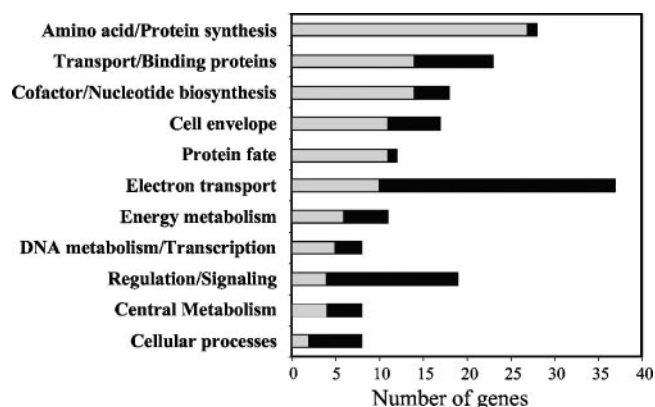


FIG. 7. Functional categorization of genes that are differentially expressed in the  $rel_{Gsu}$  mutant in stationary-phase NBAF cultures, identified by whole-genome microarray analysis as described in Materials and Methods. Functional assignments were obtained from the *G. sulfurreducens* genome page at the TIGR website (<http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=ggs>). Genes of unknown function constituted the largest group of differentially expressed genes and included 54 up-regulated genes and 128 down-regulated genes. This functional group was not included in the figure in order to better compare the remaining groups. Gray bars indicate up-regulated genes, while black bars indicate down-regulated genes.

The large number of differentially expressed genes involved in energy metabolism was of particular interest, as this process is significant to *Geobacter* physiology. A greater number of these genes were down-regulated than up-regulated in the  $rel_{Gsu}$  mutant. Up-regulated genes included a putative NADH dehydrogenase operon, an ATP synthase operon, and several uncharacterized *c*-type cytochromes (see Table S1 in the supplemental material). Down-regulated genes included those encoding *c*-type cytochromes known to be involved in electron transport to Fe(III), such as OmcB (37), OmcS (32, 50), and MacA (10), as well as the outer membrane nanowire component PilA (66) (Table 3). In addition, other respiratory genes were down-regulated in the mutant, such as those encoding HyB hydrogenase, ferredoxin, cytochrome *d* ubiquinol oxidase, cytochrome *b*, and cytochrome *c* oxidase, as well as many uncharacterized *c*-type cytochromes (Table 3). OmcB, OmcS, MacA, and PilA were all confirmed by quantitative RT-PCR to be down-regulated in the mutant (Table 2).

## DISCUSSION

These results demonstrate that Rel $_{Gsu}$ , the RelA/SpoT homolog in *G. sulfurreducens*, plays a role in the response of *G. sulfurreducens* to nutrient deprivation and oxidative stress, conditions often present in subsurface environments where these organisms are found. In addition, this study illustrates that the *G. sulfurreducens* stringent response regulates Fe(III) reduction, the primary mode of respiration for *Geobacteraceae* in their environment. The fact that a similar Rel homolog is found in all other members of the *Geobacteraceae* suggests that ppGpp and ppGp may be important regulators of growth and respiration in the subsurface *Geobacteraceae* community.

**Guanosine phosphate derivatives in *G. sulfurreducens*.** Several previous studies of other organisms have found two guanosine phosphate derivatives produced in response to nu-

TABLE 2. Differential expression of genes, shown by both quantitative RT-PCR and microarray analyses

Gene category	Locus	Annotation	Fold change <sup>a</sup>	
			qRT-PCR	Microarray
Up-regulated in the <i>rel<sub>Gsu</sub></i> mutant	GSU2409	Heat shock protein, Hsp20 family	6.1	2.3
	GSU2839	Ribosomal protein L30	2.5	2.4
	GSU2875	Ribosomal protein S9	9.0	1.8
	GSU1346	Sulfate ABC transporter, periplasmic sulfate-binding protein	8.7	9.2
Down-regulated in the <i>rel<sub>Gsu</sub></i> mutant	GSU1496	Pilin domain protein; PilA	-65.6	-3.0
	GSU2504	Cytochrome <i>c</i> family protein; OmcS	-125.7	-2.3
	GSU2737	Polyheme membrane-associated cytochrome <i>c</i> ; OmcB	-4.2	-2.6
	GSU0466	Cytochrome <i>c</i> <sub>551</sub> peroxidase; MacA	-4.4	-2.9
	GSU2814	Rubrerhythrin	-13.2	-2.8
	GSU2813	Cytochrome <i>c</i> <sub>551</sub> peroxidase	-42.1	-2.1
Showing no change	GSU2821	Nitrogenase iron protein	-1.2	-1.1

<sup>a</sup> Relative changes (*n*-fold) in expression were determined by quantitative RT-PCR (qRT-PCR) via the absolute quantification method (83). Data are means of triplicate determinations.

trient deprivation, as was seen with *G. sulfurreducens*. However, in most organisms, a pentaphosphate derivative, pppGpp, was commonly found along with ppGpp (13, 14, 28, 31). Our experiments did not detect pppGpp but instead detected a

triphosphate derivative that is rarely seen with other organisms (Fig. 2). The rarity of ppGp may simply be a consequence of the methods used for detection, as the most commonly used method, thin-layer chromatography, does not allow differenti-

TABLE 3. Down-regulation of energy metabolism genes in the  $\Delta rel_{Gsu}$  mutant

Locus	Annotation <sup>a</sup>	Mean log ratio <sup>b</sup>	Fold change
GSU3439	NADH dehydrogenase I, G subunit	-0.75 ± 0.06	-1.68
GSU1496	Pilin domain protein; PilA (66)	-1.56 ± 0.21	-2.96
GSU1612	Phosphoglycerate mutase	-0.84 ± 0.11	-1.79
GSU0220	Cytochrome <i>c</i> oxidase, subunit III	-0.89 ± 0.10	-1.85
GSU0069	Oxidoreductase, Fe-S cluster-binding subunit	-0.96 ± 0.23	-1.94
GSU1649	Cytochrome <i>b/b</i> <sub>6</sub>	-1.02 ± 0.24	-2.03
GSU0784	Nickel-dependent hydrogenase, membrane protein; HybB (19)	-1.03 ± 0.15	-2.04
GSU2813	Cytochrome <i>c</i> <sub>551</sub> peroxidase	-1.05 ± 0.16	-2.07
GSU2883	Cytochrome <i>c</i> family protein	-1.05 ± 0.22	-2.07
GSU2882	Cytochrome <i>c</i> family protein	-1.08 ± 0.37	-2.11
GSU2449	2-Oxoglutarate dehydrogenase, E1 component	-1.08 ± 0.37	-2.12
GSU0068	Cytochrome <i>c</i> family protein	-1.09 ± 0.29	-2.12
GSU0070	Oxidoreductase, membrane subunit	-1.10 ± 0.35	-2.14
GSU3257	Glycogen synthase	-1.10 ± 0.06	-2.15
GSU1648	Cytochrome <i>c</i> family protein	-1.13 ± 0.29	-2.18
GSU2732	Cytochrome <i>c</i> family protein	-1.13 ± 0.26	-2.19
GSU2811	Cytochrome <i>c</i> Hsc	-1.15 ± 0.11	-2.22
GSU0510	Fe(III) reductase, beta subunit	-1.16 ± 0.29	-2.23
GSU2504	Cytochrome <i>c</i> family protein; OmcS	-1.18 ± 0.09	-2.27
GSU3187	Ferredoxin family protein	-1.36 ± 0.12	-2.56
GSU1641	Cytochrome <i>d</i> ubiquinol oxidase, subunit II	-1.37 ± 0.45	-2.59
GSU2737	Polyheme membrane-associated cytochrome <i>c</i> ; OmcB (37, 38)	-1.37 ± 0.06	-2.59
GSU1606	Ribose 5-phosphate isomerase B, putative	-1.43 ± 0.22	-2.69
GSU0911	Iron-sulfur cluster-binding protein	-1.45 ± 0.65	-2.74
GSU2814	Rubrerhythrin	-1.48 ± 0.28	-2.79
GSU0466	Cytochrome <i>c</i> <sub>551</sub> peroxidase; MacA (10)	-1.54 ± 0.58	-2.91
GSU1397	Cytochrome <i>c</i> family protein, putative	-1.71 ± 0.19	-3.27
GSU0357	Cytochrome <i>c</i> family protein	-1.79 ± 0.56	-3.46
GSU2748	Cytochrome <i>c</i> family protein, putative	-1.87 ± 0.58	-3.66
GSU0674	Prismane protein	-1.89 ± 0.33	-3.70
GSU1024	Cytochrome <i>c</i> <sub>3</sub> ; PpcD (64)	-1.94 ± 0.30	-3.83
GSU2731	Polyheme membrane-associated cytochrome <i>c</i> ; OmcC (37, 38)	-2.01 ± 0.66	-4.03

<sup>a</sup> Some annotations are followed by gene product designations and references for characterized genes.

<sup>b</sup> Mean log<sub>2</sub> ratios represent the averages of eight replicates, i.e., four technical replicates performed on two biological replicates.

ation between different guanosine triphosphates. The HPLC-based technique used in this study allows distinction between ppGp and GTP, and both ppGpp and ppGp compounds have been detected in other studies using this technique (21, 35). It remains to be investigated how these individual stringent factors might exert specific effects on *G. sulfurreducens*.

**Role of Rel<sub>Gsu</sub> is comparable to that of RelA/SpoT homologs in other organisms.** In some ways, the role of Rel<sub>Gsu</sub> in *G. sulfurreducens* appears to be similar to that in other organisms. For example, in many bacteria, the stringent response slows growth in response to nutrient deprivation (7, 16). Both microarray and phenotypic analyses of the *rel<sub>Gsu</sub>* mutant suggest a role for Rel<sub>Gsu</sub> in inducing slow growth. Comparison of levels of gene expression between the Rel<sub>Gsu</sub>-deficient mutant and the wild type during stationary phase demonstrated that transcript levels for multiple genes involved in protein synthesis were higher in the *rel<sub>Gsu</sub>* mutant. These genes included those encoding ribosomal proteins, tRNA synthetases, chaperones, and enzymes involved in amino acid biosynthesis. In addition, several genes involved in nucleotide and cell membrane biosynthesis were also higher in the mutant (see Table S1 in the supplemental material). These results are comparable to results from microarray analyses of the stringent response in other organisms (22, 23, 69). In addition, in the absence of Rel<sub>Gsu</sub>, *G. sulfurreducens* reached higher cell densities before the onset of stationary phase than the wild type (Fig. 4), suggesting the wild-type decrease in growth rate upon nutrient deprivation may be Rel dependent. Mutant cells subsequently exhibited wild-type growth characteristics when Rel<sub>Gsu</sub> was reintroduced. These data are consistent with a role for Rel<sub>Gsu</sub> and ppGp(p) in slowing growth rate in response to nutrient deficiency.

Interestingly, the increased growth seen in the *rel<sub>Gsu</sub>* mutant is not commonly found in Rel mutants from other bacteria. Several other microorganisms in which Rel activity was knocked out and no ppGpp was produced demonstrated growth defects. In *E. coli*, a double RelA/SpoT mutant demonstrated slower growth than the wild type did (30). In *Mycobacterium xanthus*, loss of ppGpp, caused by a Rel mutation, resulted in developmental arrest (28). One example where the loss of ppGpp resulted in faster growth was reported for *Mycobacterium tuberculosis* (65). An *M. tuberculosis* Rel mutant had a higher growth rate as well as a higher cell yield when grown under certain growth conditions, but the mutant showed growth defects under several other conditions (65). As it is widely understood that production of ppGpp induces slow growth in bacteria (7, 13, 16), it follows that the lack of ppGpp in a Rel mutant might lead to increased growth rates. More observations of slower growth, as opposed to faster growth, caused by *rel* mutations can be explained by findings that ppGpp and *rel* signaling systems affect many cellular processes, and defects in these other processes, due to altered ppGpp levels, may result in defects in growth.

In addition to slowing growth, the stringent response is known to increase stress tolerance in other organisms (24, 26, 39, 85). In *G. sulfurreducens*, ppGpp was produced in response to oxygen exposure (Fig. 3), and deleting the *rel<sub>Gsu</sub>* gene increased an oxidative stress-dependent growth lag in medium lacking the reductant cysteine (Fig. 5). Furthermore, Rel<sub>Gsu</sub> deficiency inhibited a cell aggregation response found in the

presence of oxygen that occurs in the wild type under the same conditions and is believed to be an adaptive response to oxidative stress (60) (Fig. 5). Transcript levels for several genes considered to be involved in oxidative stress response were lower in *rel<sub>Gsu</sub>* mutant cells than in wild-type cells (Tables 2 and 3; also see Table S2 in the supplemental material). In addition, there was a substantial overlap between the *rel<sub>Gsu</sub>* regulon, determined with the microarray analysis reported here, and the regulon of RpoS (61), a well-characterized sigma factor shown to function in stress response in many organisms, including in *G. sulfurreducens* (60). Together, these findings are consistent with a role for Rel<sub>Gsu</sub> and ppGpp in oxidative stress response in *G. sulfurreducens*.

**Role of Rel<sub>Gsu</sub> in regulating Fe(III) respiration.** The results also indicate an important role for Rel<sub>Gsu</sub> in regulating key genes required for Fe(III) reduction. The *rel<sub>Gsu</sub>* mutant was severely limited in its ability to reduce Fe(III), and the capacity for Fe(III) reduction was restored when *rel<sub>Gsu</sub>* was expressed in *trans* (Fig. 6). Transcript levels for many electron transport genes were lower in the *rel<sub>Gsu</sub>* mutant. These included genes for several *c*-type cytochromes known to be necessary for optimal Fe(III) reduction, as well as the electricity conductive pili that are essential for Fe(III) oxide reduction (66) (Tables 2 and 3).

The stringent response has often been found to mediate responses that are related to diverse cellular processes specific to an individual organism's growth requirements. This is the case for *Myxococcus xanthus*, where A-factor production and fruiting body formation are under stringent control (28), and for several pathogenic bacteria, where virulence is under stringent control (27, 39, 63). However, to our knowledge the results presented here are the first indication that the stringent response results in the increased expression of genes involved in respiration. In fact, studies of transient growth arrest and stationary-phase growth in *E. coli* have suggested that aerobic electron transport chain gene expression decreases under stringent control (15).

**Conclusions.** These results suggest that Rel<sub>Gsu</sub> activity regulates the expression of genes involved in Fe(III) reduction, as well as responses to several common environmental stresses in *G. sulfurreducens*. Thus, the stringent response is likely to play an important role in balancing growth in subsurface environments where nutrients are low and stresses are high. Further examination of this response may provide insight into strategies for optimizing practical applications of *Geobacter* species during bioremediation of subsurface contaminants and in harvesting energy from the environment.

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