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Role of RelGsu in Stress Response and Fe(III) Reduction in Geobacter sulfurreducens

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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 relGsu, 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 RelGsu in G. sulfurreducens, a relGsu mutant was constructed and characterized, and ppGpp levels were monitored under various conditions in both the wild-type and relGsu 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 relGsu mutant. The relGsu 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 relGsu 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 RelGsu 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 its 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 RelGsu, 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.
MATERIALS AND METHODS

Bacterial strains, plasmids, and culturing conditions. Escherichia coli strain DH5α (top10 F’ lacIq proAB 2Δ(lacI qz808 hisD20 auxC28 thi-1 recA1 endA1 galU galK rpsL15 thi-1 relA1) (84) was used for DNA manipulations. Strain DLLD1 (relA1 supE44 lacU169 Δ[hal]) (gift from Mercian Corporation, Japan) was used for all PCR amplifications. Unless otherwise stated, DNA manipulations were carried out as outlined by Sambrook et al. (70).

DNA manipulations and reagents. G. sulfurreducens genomic DNA was extracted with a Masterpure complete DNA and RNA purification kit (Epicentre 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-indoxyl phosphate) substrate according to the manufacturer’s instructions. Taq DNA polymerase (QIAGEN, Inc., Valencia, CA) 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 RelA<sup>+</sup>-deficient strain via single-step gene replacement. To construct a RelA<sup>-</sup>-deficient mutant, recombinant PCR (PCR) was used to construct a linear DNA fragment containing of a kanamycin resistance cassette flanked by homologous sequence from the 5’ and 3’ ends of the relA gene. Three primary PCRs were carried out: (i) amplification of the 5’ end of the linear fragment with primers RelA1 (GGT GCT GGA TGC GGT TTC) and RelA2 (GGA CCT TGC CAG AGT AGA CA), (ii) amplification of the kanamycin resistance cassette from pBBR1MCS-2 (36) with primers RelA3 (GTC TAG TGT GCA AAC GAT CAC TGG GAT GAA TGT CAG CTA C) and RelA4 (GCG CAG AGC AAA CTC GGA AGG CCG CGG TGG AAT CG), and (iii) amplification of the 3’ end of the linear fragment with primers RelA5 (CGG AGT TTC CTG TCA CAT) and RelA6 (GTC CAT TAC GCG CAT). 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 RelA1 and RelA6. 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 Co et al. (17) and Lloyd et al. (41). One of the resulting mutants was chosen as the representative strain.

Expression of relA<sub>in</sub> in <i>trans</i>. The complete coding sequence for relA<sub>in</sub> was amplified using primers containing either an EcoRI (GGATCAATCC CTA TCT TCA TGC TGC TCC) or a BamHI (GGATCCATTT CCT AGT AAG ACC ATC ACC 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 relA<sub>in</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 starvation experiments and quantitation of guanosine phosphates. Wild-type and RelA<sup>-</sup>-deficient strains of <i>G. sulfurreducens</i> 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 cultures of NBF and NBAF were required to obtain Plasmid. 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 0.45-μm 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-μm by 4.6-mm Partisil SAX 10-μm column (Alltech, Deerfield, IL) as described by Jones et al. (35), using an LC-10AFT Inertsil-liquid chromatograph (Shimadzu, Kyoto, Japan) and a 20-μl injection volume. Immediately prior to injection, samples were resuspended in either 400 μl or 500 μl 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(II)-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).

DNA 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 DNA, cells were mechanically disrupted using a FastPrep instrument (Qibioine, Inc., Irvine, CA) with lysing matrix B (Qibioine) 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 RNAeasy minicolumns (QIAGEN, Inc., Valencia, CA), and the purity of total RNA was assessed by agarose 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 RelA<sup>-</sup>-deficient strains. DNA microarray hybridization and data analyses were performed as described previously (51). Briefly, a total of 5 μg 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 μg of cDNA with greater than 200 pmol, respectively, of each dye molecule incorporated per microgram of cdNA synthesized. Triplet 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-μm resolution using an Axon 400B 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). 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 removal and a smooth parameter of 0.25. All intensity values were corrected for background by subtracting from each array mean value less than two times the standard deviation 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 dyees) performed by order of complete 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 μg of total RNA in a 100-μl 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 μl 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-carboxy-X-rhodamine) passive reference dye was used in factor to for sheep 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. All reactions were performed using 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.
Microarray data accession numbers. Descriptions of the microarray experiments, quantitation data, and array design have been deposited into ArrayExpress (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 RelGsu, 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

RelGsu protein and operon characteristics. A RelA/SpoT homolog was identified in the Geobacter sulfurreducens genome and designated relGsu. RelGsu 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, RelGsu 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 RelGsu are present in other Geobacter species, including Geobacter metallireducens, with 95.8% similarity, Geobacter uranireducens, 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 δ-proteobacteria, such as Mysococcus 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 δ-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 relGsu operon and domain architecture of RelGsu protein.

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

<table>
<thead>
<tr>
<th>Gene designation</th>
<th>Gene product</th>
<th>Primer name</th>
<th>Primer sequence (5′–3′)</th>
</tr>
</thead>
</table>
| GSU0466          | Cytochrome c 
peroxidase | RT.ORF00777_F | CACCATGCCTACTCTCCACT |
|                  |              | RT.ORF00777_R | GTTGTGAGGAACTGACAA      |
| GSU1346          | Sulfate ABC 
transporter, periplasmic 
sulfate-binding protein | RT.ORF02287_F | GATGTGGTACCTCGGACT |
|                  |              | RT.ORF02287_R | GAGGTTGAGGGGGAGCTGTT    |
| GSU1496          | Pilin domain 
protein          | RT.ORF02545_F | CCAAACACAGCAGCAGAAAG   |
|                  |              | RT.ORF02545_R | GCAGCGAGAATACCGATGAT    |
| GSU2409          | Heat shock protein, Hsp20 family | RT.ORF03975_F | TGAAGAGACAGGTCACGCA     |
|                  |              | RT.ORF03975_R | TGACTACCGAGGGTTCCTTC    |
| GSU2504          | Cytochrome c family protein | RT.ORF04142_F | CAAACCTGGCATAGCAGTC     |
|                  |              | RT.ORF04142_R | CCATAGTAGGCAGCGGTCCAT   |
| GSU2737          | Polyheme membrane-associated cytochrome c | RT.ORF04536_F | GACACCGTCTACCAAGAAACA   |
|                  |              | RT.ORF04536_R | GTCCCGAGTTGCTAGCAAGGA   |
| GSU2813          | Cytochrome c 
peroxidase | RT.ORF04662_F | TCGCAACACATGCGAAGG     |
|                  |              | RT.ORF04662_R | TGTAGGAAAGACGGTAGG      |
| GSU2814          | Ruberythrin   | RT.ORF04665_F | CAAGCGCTTTTCTAAGTCC    |
|                  |              | RT.ORF04665_R | AGAGGTCGGAAATGCTCTCA    |
| GSU2821          | Nitrogenase iron protein | RT.ORF04677_F | AAGCTCGCCATCAGATGAT     |
|                  |              | RT.ORF04677_R | GCTTGTCGGGAGGAGAATAC    |
| GSU2839          | Ribosomal protein L30 | RT.ORF04707_F | ATATCGGGAGCAGCAAAG     |
|                  |              | RT.ORF04707_R | CCCAGTGTCGAAGGGGATG     |
| GSU2875          | Ribosomal protein S9 | RT.ORF04761_F | CATCGCGACGCGATAACC     |
|                  |              | RT.ORF04761_R | CCGTACTTTTTTCCGCTCTT    |
relGsu 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); relGsu; and endoribonuclease liver perchloric acid-soluble protein (Fig. 1). The structure of this putative operon is similar to spoT operons of several γ-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 δ-proteobacteria, such as Desulfovibrion 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 μl 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 RelGsu-deficient mutant. A relGsu mutant was constructed by homologous recombination (see Materials and Methods). Neither ppGpp nor ppGp levels, in a 20-μl 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 relGsu mutant cannot produce significant levels of ppGp(p), consistent with RelGsu being involved in the production of both compounds in G. sulfurreducens.

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 relGsu mutant. Wild-type and mutant cells were grown in NBAF medium to mid-log phase, harvested by centrifugation, and transferred to FW medium with 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 relGsu mutant (ΔrelGsu) in media lacking acetate or in media lacking ammonia. DCW, dry cell weight.

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.
The relGsu 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 (OD600 of 0.86) than the wild type (OD600 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 relGsu 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 relGsu 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 relGsu 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 relGsu mutant failed to aggregate and grew as a uniform suspension (Fig. 5B).

The relGsu 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 relGsu 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 relGsu mutant versus the wild type. To identify genes that were differentially regulated in the absence of RelGsu activity, levels of gene expression in relGsu 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 relGsu 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-
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 *relGsu* 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, 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 in *G. sulfurreducens* can play a role in the response to nutrient deprivation and oxidative stress 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-
trient deprivation, as was seen with *G. sulfurreducens*. However, in most organisms, a pentaphosphate derivative, ppGpp, was commonly found along with ppGpp (13, 14, 28, 31). Our experiments did not detect ppGpp 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-
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 RelGsu is comparable to that of RelA/SpoT homologs in other organisms. In some ways, the role of RelGsu 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 relGsu mutant suggest a role for RelGsu in inducing slow growth. Comparison of levels of gene expression between the RelGsu-deficient mutant and the wild type during stationary phase demonstrated that transcript levels for multiple genes involved in protein synthesis were higher in the relGsu mutant. These genes included those encoding ribosomal proteins, RNA 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 RelGsu, 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 RelGsu was reintroduced. These data are consistent with a role for RelGsu and ppGp(p) in slowing growth rate in response to nutrient deficiency.

Interestingly, the increased growth seen in the relGsu 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 Myxococcus 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 relGsu gene increased an oxidative stress-dependent growth lag in medium lacking the reductant cysteine (Fig. 5). Furthermore, RelGsu 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 relGsu 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 relGsu 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 RelGsu and ppGpp in oxidative stress response in G. sulfurreducens.

Role of RelGsu in regulating Fe(III) respiration. The results also indicate an important role for RelGsu in regulating key genes required for Fe(III) reduction. The relGsu mutant was severely limited in its ability to reduce Fe(III), and the capacity for Fe(III) reduction was restored when relGsu was expressed in trans (Fig. 6). Transcript levels for many electron transport genes were lower in the relGsu mutant. These included genes for several c-type cytochromes known to be necessary for optimal Fe(III) reduction, as well as the electricity conductive pilus 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 RelGsu 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.

REFERENCES