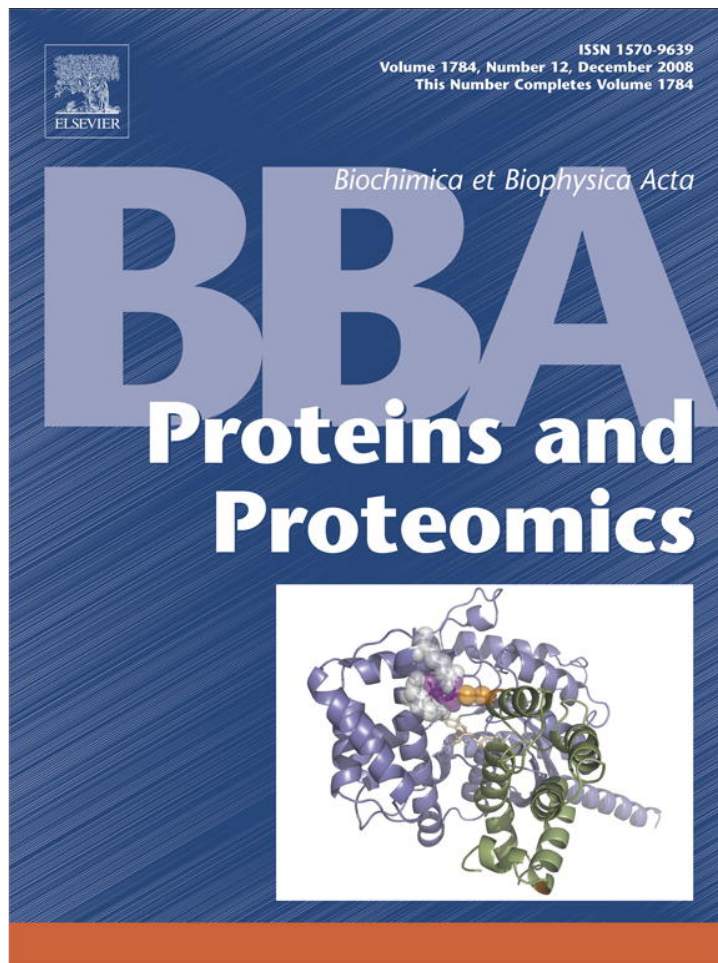


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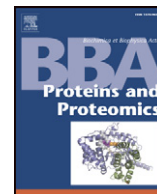
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Proteome of *Geobacter sulfurreducens* grown with Fe(III) oxide or Fe(III) citrate as the electron acceptor

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ABSTRACT

The mechanisms for Fe(III) oxide reduction in *Geobacter* species are of interest because Fe(III) oxides are the most abundant form of Fe(III) in many soils and sediments and *Geobacter* species are prevalent Fe(III)-reducing microorganisms in many of these environments. Protein abundance in *G. sulfurreducens* grown on poorly crystalline Fe(III) oxide or on soluble Fe(III) citrate was compared with a global accurate mass and time tag proteomic approach in order to identify proteins that might be specifically associated with Fe(III) oxide reduction. A total of 2991 proteins were detected in *G. sulfurreducens* grown with acetate as the electron donor and either Fe(III) oxide or soluble Fe(III) citrate as the electron acceptor, resulting in 86% recovery of the genes predicted to encode proteins. Of the total expressed proteins 76% were less abundant in Fe(III) oxide cultures than in Fe(III) citrate cultures, which is consistent with the overall slower rate of metabolism during growth with an insoluble electron acceptor. A total of 269 proteins were more abundant in Fe(III) oxide-grown cells than in cells grown on Fe(III) citrate. Most of these proteins were in the energy metabolism category: primarily electron transport proteins, including 13 c-type cytochromes and PilA, the structural protein for electrically conductive pili. Several of the cytochromes that were more abundant in Fe(III) oxide-grown cells were previously shown with genetic approaches to be essential for optimal Fe(III) oxide reduction. Other proteins that were more abundant during growth on Fe(III) oxide included transport and binding proteins, proteins involved in regulation and signal transduction, cell envelope proteins, and enzymes for amino acid and protein biosynthesis, among others. There were also a substantial number of proteins of unknown function that were more abundant during growth on Fe(III) oxide. These results indicate that electron transport to Fe(III) oxide requires additional and/or different proteins than electron transfer to soluble, chelated Fe(III) and suggest proteins whose functions should be further investigated in order to better understand the mechanisms of electron transfer to Fe(III) oxide in *G. sulfurreducens*.

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1. Introduction

The mechanism for Fe(III) oxide reduction in dissimilatory metal-reducing microorganisms is of interest because this is an important biogeochemical process in a variety of soils and sediments [1–3]. In addition to playing an important role in the oxidation of naturally occurring organic matter [1], Fe(III) reducers can degrade a variety of organic contaminants [4–6]. Furthermore, Fe(III) reduction can affect the fate of a variety of other metals and inorganic nutrients in pristine and contaminated soils and groundwater [6–8].

In many environments in which Fe(III) oxide reduction is an important process, members of the family *Geobacteraceae* are the predominant organisms [9,10]. Previous studies have suggested that, in the absence of exogenous Fe(III) chelators and/or electron shuttles, such as humic substances [11,12], *Geobacter* species have to contact Fe(III) oxides directly in order to reduce them [13]. Biochemical and genetic studies [14–17] have revealed some components likely to be involved in Fe(III) oxide reduction in *Geobacter* species. It has been proposed [10] that electrons generated from central metabolism are probably shuttled across the periplasm via c-type cytochromes such as MacA [18] and PpcA [19] to the outer-membrane cytochromes such as OmcB [20], and OmcS [21]. The final electron transfer to Fe(III) oxides may be via pili, which are conductive and have been shown to serve as nanowires that can extend far beyond the cell's outer membrane [17].

Previous global studies on gene expression [22], and protein abundance [23] have examined *Geobacter* species grown on soluble

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electron acceptors, such as fumarate and Fe(III) citrate due to the ease of cell growth and processing of samples. However, Fe(III) oxides are the most environmentally relevant electron acceptor [1,10]. Recent studies have suggested that proteins that are specifically expressed during growth on Fe(III) oxide play an important role in Fe(III) oxide reduction [15,17,21], but these studies focused on a limited subset of proteins.

In order to obtain a more extensive list of proteins that are more abundant during growth on Fe(III) oxide containing cultures, and thus potentially important in the reduction of Fe(III) oxide, we employed a global proteomic approach in the study reported here. The accurate mass and time (AMT) tag technique for the quantitative identification of peptides results in high confidence and high throughput [24–26] and our previous study demonstrated that it is a useful tool to compare protein expression profiles of *G. sulfurreducens* under various culture conditions [23]. Here we report on a global AMT analysis of protein expression of *G. sulfurreducens* grown on Fe(III) oxide. The results indicate that there is a substantial subset of proteins that are significantly more abundant during growth on Fe(III) oxide than during growth with soluble, chelated Fe(III) as the electron acceptor.

2. Materials and methods

2.1. Chemicals

Dithiothreitol (DTT), iodoacetamide, CHAPS, oxalic acid, ammonium oxalate, and trypsin were purchased from Sigma (St. Louis, MO), protease inhibitor from Roche (Indianapolis, IN), and acetic acid from Promega (Madison, WI).

2.2. Bacterial growth

G. sulfurreducens was grown under anaerobic conditions (N_2/CO_2 : 80/20) at 30 °C in batch cultures as previously described [27]. Acetate (10 mM) served as the electron donor and either synthetic poorly crystalline Fe(III) oxide (100 mmol/l) or Fe(III) citrate (60 mM) was the electron acceptor [28]. The medium supplemented with Fe(III) oxide as electron acceptor was modified by the addition of $MgSO_4$ (30 mg/l) and NaCl (10 mg/l) to maintain better growth.

2.3. Cell harvesting

In order to free cells from the Fe(III) oxide, the Fe(III) oxide was dissolved with oxalate [29]. Cells were harvested with centrifugation at 9000 $\times g$ for 15 min at 4 °C, washed with 50 mM Tris–HCl (pH 8.8) containing 10 mM $MgCl_2$ and protease inhibitors, and stored at –20 °C until further use.

2.4. Protein extraction and peptide preparation for LC-MS/MS

For the global trypsin digestion the cells were resuspended in 4 cell volumes of 50 mM ammonium bicarbonate, pH 7.8. Cells were lysed by bead beating where the cells were mixed with 0.1 mm zirconia/silica beads in a mini-bead beater (Biospec, Bartlesville OK) for 90 s at 4500 rpm. The isolated proteins were dialyzed against 50 mM ammonium bicarbonate, pH 7.8 for one day at 4 °C with two buffer changes. The proteins were treated with trypsin as described elsewhere [30] with the exception of the addition of 1% CHAPS. Samples were cleaned up with strong cation exchange and solid phase extraction (SCX SPE) and the alkylation of samples was carried out by incubation at 20 °C for 30 min with the addition of 10 mM iodoacetamide.

Protein concentrations were determined with a bicinchoninic acid assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

2.5. Capillary LC-MS

The peptide mixtures from both the Fe(III) citrate and Fe(III) oxide growth conditions were separated by an automated in-house designed high resolution reversed phase capillary LC system as described previously [30,31]. The LC system was interfaced to an ion trap MS (LTQ, ThermoFinnigan, San Jose, CA) using electrospray ionization (ESI). For the LC-MS/MS analyses, 10 μg (1 $\mu g/\mu l$) of total peptide material was used. The mass spectrometer operated in a data-dependent MS/MS mode over a full range (400–2000) and a series of seven smaller segmented m/z ranges (400–700, 700–900, 900–1100, 1100–1300, 1300–1500, 1500–1700, 1700–2000) for each sample. For each cycle, the ten most abundant ions from each LC-MS scan were selected for MS/MS analysis using 45% collision energy. The MS/MS spectra were analyzed using the peptide identification software SEQUEST [32] in conjunction with the *G. sulfurreducens* genome from TIGR (<http://www.tigr.org>). Initial peptide identifications (i.e. putative mass and time tags: PMT tags) were based on a minimum cross correlation (Xcorr) score of 1.5 for all peptides identified at least twice in all MS/MS experiments. For peptides only identified once, Xcorr values had to be a minimum of 1.9, 2.2, and 3.5 for charge states of 1+, 2+ and 3+, respectively. All peptides conformed to a tryptic cleavage state on at least one of their termini.

Peptide PMT tags were filtered by subsequent LC-FTICR MS measurements for validation of a more limited set of AMT tags. The AMT tag validation was performed as previously described [24]. The peptide AMT tags identified in at least two of three technical replicate analyses were used and exported into tab-delimited files containing protein reference number, protein annotation, and the integrated LC-MS peak intensity values for the identified peptides (which were used to derive a relative abundance value). The abundance values for each protein in each analysis were transformed into a z-score value (also known as the standard row function) to determine those showing significant changes from their average values. The z-score is obtained using the mean value of each protein across all compared growth conditions, subtracted from each individual protein abundance value and divided by the standard deviation of the values (Supplementary Table). Generally, z-score values between samples were considered significantly different if the difference was at least 1.5 or greater.

2.6. Functional analysis of the proteome

The predicted functions of differentially expressed proteins were checked using a manually curated version (M.A., unpublished) of the *G. sulfurreducens* genome annotation [33], and the unpublished genome annotations of *Geobacter metallireducens* and *Geobacter uraniireducens* (GenBank accession nos. NC_007517 and NC_009483) were queried to determine which proteins are common to all three *Geobacter* species.

3. Results and discussion

G. sulfurreducens was grown with acetate as the electron donor and either Fe(III) oxide or Fe(III) citrate as the electron acceptor. Cells were harvested at late logarithmic phase after 2 and 29 days of growth with Fe(III) citrate and Fe(III) oxide, respectively (Fig. 1). A total of 2991 proteins were detected in cells from these cultures (Supplementary Table). This represents 86% of the genes predicted to encode proteins and is comparable to the percentage of proteins identified in previous studies in which *G. sulfurreducens* was grown under a variety of conditions [23]. Fewer proteins (2464) were detected when Fe(III) oxide served as electron acceptor than in Fe(III) citrate-grown cells (2921). Of the 72 proteins that were exclusively expressed in Fe(III) oxide-grown cells, half were either conserved hypothetical or hypothetical proteins. An additional 197 proteins were detected with higher abundance during growth on Fe(III) oxide than in Fe(III)

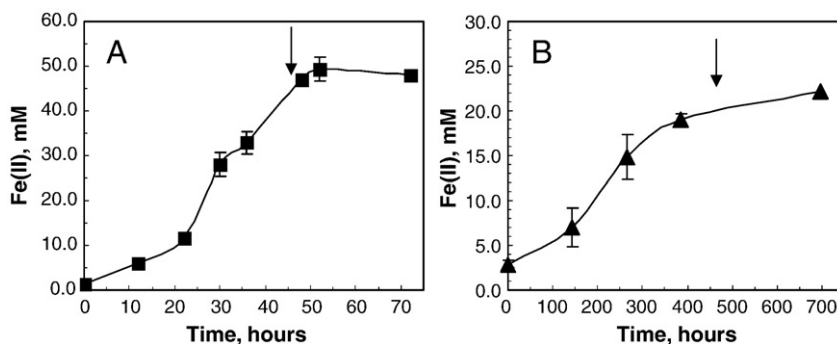


Fig. 1. Time course of growth of *G. sulfurreducens* as indicated by the reduction of Fe(III) in cultures supplemented with acetate as electron donor and Fe(III) citrate (A) or Fe(III) oxides (B) as electron acceptor. Arrows indicate the time of harvesting.

citrate cultures. However, 1875 proteins were less abundant in Fe(III) oxide cultures, which is 76% of the total expressed proteins, consistent with the overall slower rate of metabolism during growth with an insoluble electron acceptor [10].

Of the 269 proteins in higher abundance in Fe(III) oxide-grown cells, most were in the energy metabolism category: primarily electron transport proteins, including 13 *c*-type cytochromes (Fig. 2). This suggests that electron transport to poorly soluble Fe(III) oxide requires additional and/or different proteins than electron transfer to soluble, chelated Fe(III). There were also a substantial number of proteins of unknown function that were more abundant during growth on Fe(III) oxide. Other proteins that were more abundant during growth on Fe(III) oxide included transport and binding proteins, proteins involved in regulation and signal transduction, cell envelope proteins, and enzymes for amino acid and protein biosynthesis, among others (Fig. 2).

3.1. "Nanowires" and *c*-type cytochromes

It has been proposed that the final conduit for electron transfer between the outer surface of *G. sulfurreducens* and Fe(III) oxides is electrically conductive pili that function as "microbial nanowires" [17]. Increased production of pili during growth on Fe(III) oxide compared to growth on Fe(III) citrate was previously noted in transmission electron micrographs [15]. Consistent with this observation, PilA, the

structural pilin protein, was more abundant in cultures grown on Fe(III) oxide than in Fe(III) citrate-grown cultures.

Cytochromes of the *c*-type in *G. sulfurreducens* are of particular interest because of their general abundance and because they have several potential functions. Some *c*-type cytochromes have been proposed to be components of electron transfer to Fe(III) [19–21] and other extracellular electron acceptors, such as electrodes [34], because when the genes for these cytochromes are deleted Fe(III) reduction or electricity production is inhibited. Some of these cytochromes are exposed on the outside of the cell where they may contact extracellular electron acceptors [21,35]. Some *c*-type cytochromes may have regulatory roles [36,37]. Furthermore, the ability of periplasmic and outer-membrane cytochromes to accept electrons from inner membrane electron transfer components may allow these cytochromes to function as a capacitor, permitting continuous electron transfer during brief intervals when *Geobacter* species are not in direct contact with Fe(III) oxides [38].

The AMT tag approach revealed that 79 of the 103 *c*-type cytochromes encoded in the *G. sulfurreducens* genome were expressed during growth on Fe(III) citrate or Fe(III) oxide (Supplementary Table). Of these, 13 *c*-type cytochromes were significantly more abundant in cells grown on insoluble Fe(III) oxide (Table 1); fifty-one were more abundant during growth on Fe(III) citrate (Supplementary Table); whereas the abundance of 15 cytochromes did not show significant differences.

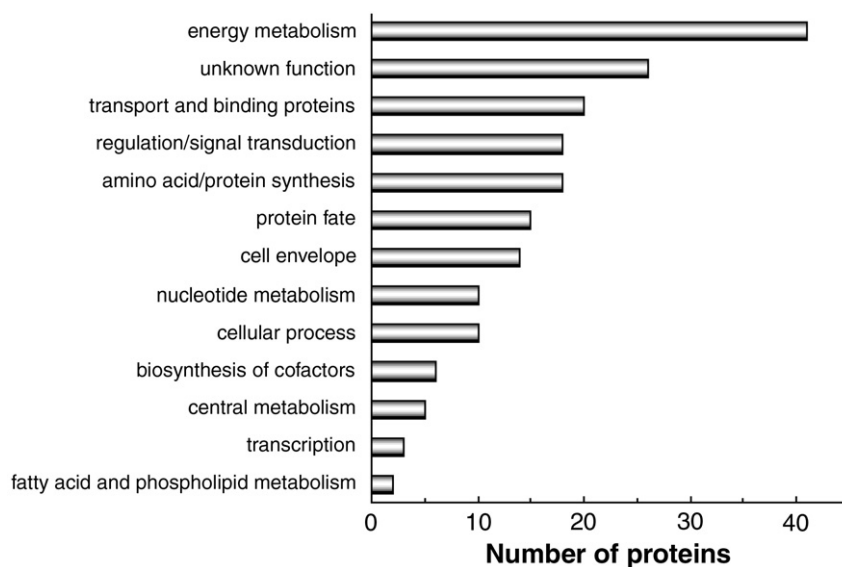


Fig. 2. Number of proteins found with higher abundance in cells from insoluble Fe(III) oxide containing cultures compared to those supplemented with soluble Fe(III) citrate. Proteins were sorted by their function.

Table 1
c-Type cytochromes more abundant during growth with Fe(III) oxides vs. Fe(III) citrate

| GSU number | Name | Number of hemes predicted | Predicted MW (kDa) | Predicted pI | Signal peptide | z-score difference ($Z_{\text{Fe(III) oxide}} - Z_{\text{Fe(III) citrate}}$) |
|------------|------|---------------------------|--------------------|--------------|----------------|--------------------------------------------------------------------------------|
| GSU0068 | | 4 | 18.1 | 8.9 | No | 2.00 |
| GSU0466 | MacA | 2 | 37.3 | 9.2 | Yes | 1.74 |
| GSU0592 | | 12 | 35.8 | 9.3 | No | 1.73 |
| GSU1024 | PpcD | 3 | 9.7 | 9.1 | Yes | 1.71 |
| GSU1334 | | 7 | 49.9 | 6.4 | Yes | 1.54 |
| GSU1761 | | 3 | 50.6 | 8.7 | Yes | 1.83 |
| GSU2210 | | 27 | 75.9 | 8.5 | Yes | 1.62 |
| GSU2504 | OmcS | 6 | 45.4 | 7.2 | Yes | 1.89 |
| GSU2724 | | 15 | 72.0 | 7.4 | Yes | 1.68 |
| GSU2732 | | 8 | 25.8 | 8.9 | Yes | 1.77 |
| GSU2738 | | 8 | 25.8 | 8.9 | Yes | 1.77 |
| GSU2930 | | 4 | 28.8 | 9.8 | Yes | 1.70 |
| GSU2934 | | 10 | 46.7 | 8.8 | Yes | 2.13 |

Previous studies have suggested that differential expression of cytochromes in *G. sulfurreducens* during growth on Fe(III) or electrodes may indicate their specific involvement in electron transport to these electron acceptors [18,21,34,39]. The higher abundance of the outer-membrane cytochrome, OmcS, in cells grown on Fe(III) oxide (Table 1) is consistent with previous studies that have demonstrated that OmcS is required for the reduction of Fe(III) oxide, but not Fe(III) citrate, and that transcript levels for OmcS are higher during growth on Fe(III) oxide than with Fe(III) citrate [21]. OmcT (GSU2503), a homolog of OmcS [21], was not detected in statistically higher abundance during growth on Fe(III) oxide (Supplementary Table). Comparative protein abundance analysis demonstrated that in the Fe(III) oxide growth condition OmcS was on average 40-fold higher in abundance than OmcT (Supplementary Table) suggesting that post-transcriptional modulation of expression may occur because these two adjacent genes are co-transcribed [21]. The relatively low abundance of OmcT is consistent with the previous conclusion that OmcT is not required for Fe(III) oxide reduction.

The c-type cytochrome encoded by GSU1334, which was also more abundant in Fe(III) oxide-grown cells (Table 1), is predicted to be localized in the outer membrane [40]. Cell suspensions of a strain in which GSU1334 was deleted were defective in Fe(III) oxide reduction, but not the reduction of Fe(III) citrate [40]. This suggests that, like OmcS, the GSU1334 cytochrome is specifically involved in Fe(III) oxide reduction.

Another c-type cytochrome that was more abundant in cells grown on Fe(III) oxide was PpcD (Table 1). This cytochrome is one of five closely related small triheme c-type cytochromes (PpcA-E) that have been identified in the genome of *G. sulfurreducens* [33]. PpcA has been shown to be periplasmic [19] and the others are also predicted to have a periplasmic localization by PSORT algorithm (<http://psort.nibb.ac.jp>). Three of these cytochromes: PpcA (GSU0612); PpcB (GSU0364); and PpcD (GSU1024) were detected in both Fe(III) citrate and Fe(III) oxide cultures (Supplementary Table). PpcE (GSU1760) was found only in cultures with Fe(III) citrate. PpcB was more abundant during growth on Fe(III) citrate while the abundance of PpcA was statistically similar under both culturing conditions. Genetic studies have suggested that PpcA is an important component in Fe(III) citrate reduction [19]. PpcD was the only small periplasmic c-type cytochrome which had higher abundance in the Fe(III) oxide supplemented cultures. The possible role of PpcD in Fe(III) oxide reduction should be investigated.

The only other c-type cytochrome that has previously been investigated in detail and was more abundant during growth on Fe(III) oxide was MacA (Table 1). This cytochrome, which is associated with the inner membrane, was initially considered to be essential for Fe(III) reduction because deletion of the *macA* gene inhibited the reduction of Fe(III) [18,40]. However, subsequent studies have revealed that this may be an indirect effect [41]: deletion of the *macA* gene inhibited the expression of the gene for the outer-

membrane c-type cytochrome, OmcB. Expression of *omcB* in the MacA-deficient mutant restored the capacity for Fe(III) reduction.

The functions of the other nine cytochromes that were more abundant during growth on Fe(III) oxide than Fe(III) citrate have not been studied in detail. The cytochromes encoded by GSU0068, GSU0592, GSU2724, GSU2732, GSU2738, GSU2930, and GSU2934 are primarily associated with the outer-membrane fraction, at least when cells are grown with fumarate as the electron acceptor [23]. Thus, they have the potential to interact directly with Fe(III) oxide. The GSU1761 and GSU2210 cytochromes are most abundant in the periplasmic space [23]. Genetic analysis of the function of all of these cytochromes seems warranted in order to determine their potential roles in Fe(III) oxide reduction.

Unlike other outer-membrane c-type cytochromes that have been investigated, OmcB is required for the reduction of Fe(III) citrate as well as Fe(III) oxide [20]. Transcript levels of *omcB* are higher during growth with Fe(III) citrate as the electron acceptor vs. fumarate [22] and during growth on Fe(III) citrate transcript levels increase proportionally with increases in the rate of Fe(III) reduction [39]. At the protein level, OmcB is more abundant in Fe(III) citrate-grown cells than during growth on fumarate [23]. The levels of OmcB were comparable in Fe(III) citrate- and Fe(III) oxide-grown cells, suggesting that OmcB is equally important for the reduction of soluble and insoluble Fe(III).

OmpJ (GSU3304) is the most abundant protein in the outer membrane of *G. sulfurreducens* [14]. This porin-like protein appears to be required for proper processing and transport of outer-membrane proteins, including c-type cytochromes. OmpJ was more abundant in cells grown on Fe(III) oxide, consistent with the greater importance of outer-membrane cytochromes and other electron transfer proteins in Fe(III) oxide reduction [16,17,21]. Earlier studies also showed that two putative multicopper proteins are involved in Fe(III) oxide reduction [16,42]. However, both of them were found with higher abundance in cultures grown on Fe(III) citrate, indicating that not all proteins involved in Fe(III) oxide reduction have higher abundance in the presence of Fe(III) oxide.

3.2. Other electron transfer and metabolic proteins

Growth is slower with Fe(III) oxide than with Fe(III) citrate, and this was reflected by the reduced abundance of many proteins involved in electron transfer and metabolism (Supplementary Table). For example, all enzymes of the tricarboxylic acid cycle, except the 2-oxoglutarate dehydrogenase complex, were less abundant during growth on Fe(III) oxide, as were the proteins of the 2-oxoglutarate:ferredoxin oxidoreductase complex (GSU1467, GSU1468, GSU1469, GSU1470). During growth on Fe(III) oxide, all three aconitate hydratases (GSU0836, GSU1660, GSU2445) were less abundant, as were both succinyl:acetate CoA-transferases (GSU0174, GSU0490) that

provide an alternative to the inefficient succinyl-CoA synthetase reaction [43]. Both isoenzymes of malate oxidoreductase (GSU2308 and GSU1700), which may perform an anaplerotic conversion of pyruvate to malate, were also less abundant on Fe(III) oxides. Sodium/solute symporters that may be responsible for acetate transport (GSU0518, GSU1068, GSU2352) [44] were less abundant during growth on Fe(III) oxide, but the associated conserved hypothetical proteins GSU1069, GSU1071 and GSU2353 were more abundant.

Conversion of acetate to pyruvate in *G. sulfurreducens* is thought to be catalyzed by either a single-polypeptide pyruvate:ferredoxin/flavodoxin oxidoreductase or a pyruvate:ferredoxin oxidoreductase complex. The former (GSU0097) and two subunits of the latter (GSU1860, GSU1862) were less abundant on Fe(III) oxides, consistent with reduced anabolic flux of carbon.

There may be a shift in the pathways for gluconeogenesis that accompanies the slower growth on Fe(III) oxide. *G. sulfurreducens* can synthesize phosphoenolpyruvate from pyruvate by reversible reactions catalyzed by pyruvate carboxylase and phosphoenolpyruvate carboxykinase, or by reactions catalyzed by either phosphoenolpyruvate synthase or pyruvate phosphate dikinase, which are irreversible in the presence of a pyrophosphatase [43]. The reversible reaction enzymes were more abundant during growth on Fe(III) oxide whereas the irreversible reaction enzymes were more abundant during growth on Fe(III) citrate. Also consistent with a lower demand for phosphoenolpyruvate at slower growth rates, the phosphoenolpyruvate-energized phosphotransferase system of solute transport (GSU1881, GSU1883, GSU1885) appeared to be downregulated on Fe(III) oxides.

Almost all enzymes involved in biosynthesis of vitamins and cofactors were less abundant in cells grown on Fe(III) oxides, including those involved in the synthesis of lipoate, biotin, thiamine, pyridoxal phosphate, cobalamin and other porphyrins, coenzyme A, molybdopterin, and nicotinamide and flavin nucleotides. The exception was the greater abundance of the alpha subunit of riboflavin synthase (GSU1689) in Fe(III) oxide-grown cells. Folylpolyglutamate synthase (GSU2368) and the menaquinone biosynthesis enzymes geranylgeranyl pyrophosphate synthase (GSU1317) and 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (GSU2004) were also more highly expressed on Fe(III) oxides.

Many of the enzymes that catalyze the reactions of respiratory metabolism were less abundant during growth on Fe(III) oxides: the cytochrome *b/b₆* complex (GSU1649, GSU1650); the cytochrome *d* ubiquinol oxidase complex (GSU1640, GSU1641); the F₀F₁-type ATP

Table 2
Two-component signaling systems more abundant during growth with insoluble Fe(III) oxides vs. Fe(III) citrate

| GSU number | Name (domains identified) | Found in other <i>Geobacter</i> spp. |
|------------|----------------------------------------------------------------------------------------|---------------------------------------------------------|
| GSU0842 | Sensor histidine kinase response regulator (PAC, HisKA HATPase_c, REC) | <i>G. uraniireducens</i> |
| GSU1220 | Response regulator (GspIIE_N, REC) | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1316 | Response regulator sensor (REC, GAF) | <i>G. uraniireducens</i> |
| GSU1415 | Response regulator, putative (REC) | <i>G. uraniireducens</i> |
| GSU1555 | Sensor histidine kinase response regulator (PAS, PAC, PAS, GAF, HisKA, HATPase_c, REC) | |
| GSU2046 | Response regulator (REC) | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU2144 | Sensor histidine kinase (HAMP, HisKA, HATPase_c) | |
| GSU2444 | Periplasmic substrate-binding histidine kinase (PBPb, HisKA, HATPase_c) | |
| GSU2991 | Sensor histidine kinase (HAMP, HisKA, HATPase_c) | |
| GSU3252 | Sensor histidine kinase (GAF, HisKA, HATPase_c) | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU3437 | Sensor histidine kinase (PAS, PAC, GAF, HisKA, HATPase_c) | |

Table 3
Proteases differentially expressed during growth with insoluble Fe(III) oxides vs. Fe(III) citrate

| GSU number | Name | In other <i>Geobacter</i> spp.? |
|-----------------------------------------------------------------|-----------------------------------------------------------------|---------------------------------------------------------|
| <i>Proteases showing increased expression on Fe(III) oxides</i> | | |
| GSU0856 | Peptidase, M48 family | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0923 | ATP-dependent Lon protease (La) | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0927 | Zinc-dependent peptidase, M16 family | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0969 | Carboxy-terminal processing protease | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1234 | Peptidase, S49 family | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1791 | ATP-dependent Clp protease, ATP-binding subunit ClpX | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1834 | Peptidase, putative | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1873 | Oligoendopeptidase F | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1991 | CAAX amino terminal protease family protein | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU3135 | Lipoprotein signal peptidase | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| <i>Proteases showing decreased expression on Fe(III) oxides</i> | | |
| GSU0304 | Aminopeptidase N | |
| GSU0318 | Peptidase, M48 family | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0330 | Putative serine protease (general secretion pathway protein C?) | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0448 | Metal-dependent amidase/aminoacylase/carboxypeptidase | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0462 | Putative protease | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0499 | Zinc metallopeptidase, M23/M37 family | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0891 | Subtilase domain protein | |
| GSU0896 | Zinc protease TldD, putative modulator of DNA gyrase | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU0928 | Zinc-dependent peptidase, M16 family | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1159 | Intracellular protease, Pfpl family, putative | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1180 | Cell division ATP-dependent Zn protease FtsH | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1267 | Signal peptidase I | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1790 | ATP-dependent Lon protease (La) | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1792 | ATP-dependent Clp protease, proteolytic subunit ClpP | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1809 | Cell division ATP-dependent Zn protease FtsH | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1837 | Zinc metallopeptidase, M23/M37 family | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU1914 | Membrane-associated zinc metalloprotease, putative | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU2021 | Xaa-Pro dipeptidase | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU2060 | PmbA Zn protease, putative | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU2075 | Serine protease, subtilase family | |
| GSU2105 | Predicted ATP-dependent Lon-type protease COG4930 | |
| GSU2196 | Glutamyl aminopeptidase M42 | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU2433 | ATP-dependent protease, putative | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU2835 | Methionine aminopeptidase, type I | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU3193 | ATP-dependent Lon protease (La) | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |
| GSU3360 | Peptidase, M16 family | <i>G. metallireducens</i> , <i>G. uraniireducens</i> |

synthase (except the F₀ C subunit GSU0333, which was more abundant), and the V-type proton-translocating pyrophosphatase (GSU3291). The lower abundance of polyphosphate kinase (GSU3323) is also consistent with reduced energy flow in cultures grown on Fe(III) oxides.

The two NADH dehydrogenase complexes encoded in the genome of *G. sulfurreducens* were expressed under both conditions but the abundance level of different subunits were mixed. Most of the subunits of one NADH dehydrogenase had lower abundance during growth on Fe(III) oxide. For the other complex, five subunits (GSU3443, GSU3441, GSU3439, GSU3431, GSU3430) were less abundant and three (GSU3444, GSU3436, GSU3434) were more abundant during growth on Fe(III) oxide.

Proteins that are generally associated with the use of oxygen as a terminal electron acceptor, including cytochrome *c* oxidase (subunits GSU0219 and GSU0222), were more abundant in the Fe(III) oxide cultures. *G. sulfurreducens* is capable of using oxygen as a terminal electron acceptor [45], but the Fe(III) oxide cultures were grown under strict anaerobic conditions. It may be that the greater stress of growing with Fe(III) oxide resulted in increased expression of the genes for these proteins.

3.3. Other proteins

The *G. sulfurreducens* genome encodes numerous two-component signaling proteins [33]. Eleven of these were more highly expressed during growth on Fe(III) oxides (Table 2), and eighty-eight were less expressed (Supplementary Table). GSU3252 is the only sensor kinase and GSU1220 and GSU2046 are the only response regulators that are common to all three *Geobacter* species examined and were more abundant on Fe(III) oxides, suggesting that they may be important for adaptation to this growth condition. In contrast, thirty-eight two-component signaling proteins shared by all three *Geobacter* species were more abundant on Fe(III) citrate, most of which may be involved in growth rate-related housekeeping processes.

There are six gene clusters in *G. sulfurreducens* encoding chemotaxis-like proteins [46]. Three of these clusters corresponded to proteins that were more abundant during growth on Fe(III) oxide, suggesting the potential importance of the systems during growth under this condition. These included: 1) a putative purine-binding chemotaxis protein (CheW, GSU0297) in the cluster of GSU0297–GSU0293 that was exclusively found during growth on Fe(III) oxide, and GSU0296, a putative chemotaxis sensor; 2) GSU1029, a putative methyl-accepting chemotaxis protein in the cluster GSU1029–GSU1034; and 3) GSU2217, a putative response regulator, and GSU2223, a putative CheY chemotaxis protein in the cluster GSU2224–GSU2211. Chemotaxis is considered to be an important feature of growth on Fe(III) oxides [15].

A remarkable number of proteases were differentially expressed (Table 3): a total of ten with higher expression on Fe(III) oxides and twenty-six with reduced expression. Most are conserved in the three *Geobacter* species examined, indicating that fundamental protease-dependent changes in cell physiology accompany the transition from soluble to insoluble Fe(III) as the terminal electron acceptor.

4. Conclusions

This study suggests that the physiology of cells growing with Fe(III) oxide as the electron acceptor is substantially different than that of cells growing with soluble, Fe(III) citrate. This study not only corroborates some of the previous findings of proteins expressed in higher abundance during growth on Fe(III) oxide, such as pili and some outer-membrane cytochromes, it has identified additional proteins that could be important in Fe(III) oxide reduction. Further investigation of the function of these proteins may aid in better understanding extracellular electron transfer to Fe(III) oxide and the

physiology of *Geobacter* species living in soils and sediments in which Fe(III) oxides are the primary electron acceptor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2008.06.011.

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