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Progress Toward Genome-Scale Monitoring of *In Situ* Gene Expression During Uranium Bioremediation and Electricity Harvesting

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The first two goals of our Genomics:GTL project are: 1) to determine the genome sequences of the *Geobacteraceae* that predominate during *in situ* bioremediation of uranium-contaminated waters and on the surface of electrodes harvesting electricity from waste organic matter and 2) to comprehensively determine genome-wide patterns of the expression of these *Geobacteraceae* genes in the environments of interest. This data will be used to modify *in silico* models of *Geobacteraceae*, that are initially being developed with data from well-studied pure cultures of *Geobacteraceae* so that these models will be able to more accurately predict the growth and metabolism under a variety of conditions in subsurface environments or on electrodes.

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Substantial progress was made in 2004 in field-scale genomic studies of *in situ* uranium bioremediation and harvesting electricity from aquatic sediments. The *in situ* uranium bioremediation studies were conducted at the DOE-NABIR UMTRA field study site in Rifle, Colorado. Acetate was added to the groundwater in order to stimulate the activity of dissimilatory metal-reducing microorganisms. As previously observed, uranium was rapidly removed from the groundwater as soluble U(VI) was reduced to insoluble U(IV). This is important because it demonstrates the ability to conduct reproducible field experiments at the site.

The composition of the microbial community and groundwater chemistry were monitored daily during the 27 day experiment. The expression of a suite of key genes was monitored every other day. Furthermore, about every 5 days large quantities of groundwater (> 500 liters) were collected for analysis of the genome sequences of the predominant microorganisms. This level of molecular analysis of a subsurface environment is unprecedented.

Analysis of 16S rRNA gene sequences demonstrated that within 13 days *Geobacteraceae* increased from less than 5% of the microbial community when acetate was first injected to over 99% of the community. Quantitative analysis of multiple highly conserved *Geobacteraceae* genes indicated that the number of *Geobacteraceae* increased more than 4 orders of magnitude in this short time. Furthermore, the diversity of *Geobacteraceae* was extremely low. For example, during the height of uranium removal, 82% of the *Geobacteraceae* had 16S rRNA gene sequences that were 97.5-100% identical with 35% having an identical sequence within sequencing error. This is an incredible enrichment of closely related microorganisms in a natural environment. The finding that field experiments can be reproducibly conducted at the Rifle site and that the environment is highly enriched in a small cluster of highly related organisms demonstrates that this environment is ideal for the genome-enabled *in silico* environmental modeling we have proposed.

Furthermore, as the result of our detailed investigations of pure culture *Geobacteraceae* genomes in the first two years of our Genomics:GTL project, it was possible to use newly discovered *Geobacteraceae*-specific gene sequences to conduct detailed studies on levels of *in situ* gene expression in the subsurface. For example, from the analysis of the six available *Geobacteraceae* genomes it was possible to identify *Geobacteraceae*-specific sequences for unique genes such as OmpB, a unique *Geobacteraceae*-specific outer-membrane protein and GltA, a eukaryotic-like citrate synthase unique to *Geobacteraceae*, as well as for genes involved in nutrient uptake and a diversity of housekeeping genes.

Studies on expression of *gltA* in chemostat cultures demonstrated a positive correlation between rates of acetate metabolism and levels of *gltA* transcripts, suggesting that levels of *gltA* transcripts might provide an indication of rates of metabolism. In the field experiment there was a remarkable correspondence between acetate levels in the groundwater and levels of *gltA* transcripts. As acetate rose *gltA* transcript levels increased. Both acetate and *gltA* transcript levels dropped during a rain event that diluted the acetate with rainwater recharge, and then *gltA* levels increased concurrent with a renewed increase in acetate over time. This contrasted with the constant expression, relative to total RNA, of *Geobacteraceae* housekeeping genes such as *recA*, *rpoD*, and *proC*. The pattern of expression of *ompB* was similar to that of the housekeeping genes, consistent with pure culture results indicating that this gene is constitutively expressed and its transcript levels are not correlated with rates of metabolism. Expression of *Geobacteraceae nifD*, which encodes for a portion of the nitrogenase complex, followed a pattern similar to that of *gltA*. This provided further evidence that the metabolism of the *Geobacteraceae* was controlled by the availability of acetate and also demonstrated that the growth of *Geobacteraceae* during bioremediation was limited by the availability of fixed nitrogen.

These results demonstrate that high quality mRNA can be recovered from the subsurface and used to monitor the activity and metabolic state of *Geobacteraceae* during in *situ* uranium bioremediation. In order to monitor gene expression on a genome-wide basis a microarray approach is required. In order to develop this technique, pure cultures of *Geobacter metallireducens* were inoculated into sterilized sediments from the Rifle site which were amended with acetate in order to simulate growth of *Geobacter* species during *in situ* uranium bioremediation. High quality mRNA in sufficient quantities for microarray analysis could be extracted from the sediments and are currently being analyzed with a whole-genome microarray.

In order to construct microarrays that represent the genomes of the *Geobacteraceae* that predominate in the subsurface the genome sequences of these organisms are being determined with three approaches. Isolates with 16S rRNA gene sequences that are identical to those that predominate during *in situ* uranium bioremediation have been recovered and their genomes are being sequenced. High quality genomic DNA was extracted from the subsurface during the 2004 field experiment and is being used to construct both BAC and small insert libraries in order to obtain additional sequence from any *Geobacteraceae* that might not be isolated. Furthermore, samples have been preserved for single-cell genome sequencing. Sequence data from all three approaches should be available at the time of the meeting. This sequence data will be used to construct arrays for genome-scale analysis of gene expression using mRNA extracted for this purpose in the 2004 field experiment.

Field experiments on electricity harvesting were carried out in freshwater and marine sediments at the UMASS field station on Nantucket Island as well as with a swine waste digestor. Electrodes harvesting energy were highly enriched with *Geobacteraceae* of low diversity and representatives of the predominant *Geobacteraceae* were recovered in culture. Studies on gene expression and sequencing genomic DNA similar to those described above for the uranium bioremediation field experiment were carried out and will be presented.

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Integrating Phenotypic and Expression Data to Characterize Metabolism in *G. sulfurreducens*

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Geobacteraceae have been shown to be important in bioremediation of uranium contaminated subsurface environments, and in harvesting electricity from waste organic matter. These applications are intricately linked to cellular metabolism, and hence, motivating the need to understand metabolism in these metal reducing bacteria. An iterative approach of mathematical modeling followed by experimentation was adopted to understand metabolism in these organisms.

A genome-scale metabolic model has been developed using the constraint-based modeling approach. Model-based analysis has revealed significant insights on the effect of global proton balance on the physiology of *G. sulfurreducens* and has provided explanation for the reduced yields during Fe (III) reduction. In addition, the comparison of the model predictions of the flux distributions with gene

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expression data was valuable in elucidating the function of genes putatively annotated as encoding for NADPH dehydrogenase. The *in silico* analysis of the energetics of menaquinone secretion indicated a substantial reduction in the growth rate and suggested an explanation for why *Geobacteraceae* predominate over other bacteria that require such electron shuttles. The initial metabolic model provided important physiological and ecological insights on the metabolism of *Geobacteraceae*. However, the analysis of metabolism revealed several redundant pathways in central metabolism around acetate utilization and pyruvate metabolism.

In order to further understand the role of these redundant pathways and their contribution to the overall robustness of metabolism, a combined computational and experimental approach was utilized to unravel the activity of the redundant pathways under different environmental conditions. The computational analysis of the metabolic network identified all the conditionally dependent metabolic pathways. A series of metabolic mutants in pyruvate oxidoreductase (POR), malate dehydrogenase, phosphoenol pyruvate carboxykinase (PPCK), phosphotransacetylase, was designed based on the computational analysis to resolve the activity of the redundant pathways. These mutants were characterized phenotypically under different growth conditions and the experimental data was compared with model predictions. This comparison revealed several interesting aspects of how central metabolism is regulated: POR is the only mechanism for the synthesis of pyruvate from acetate, PPCK is essential for growth on Fe(III) suggesting a potential for this enzyme to be regulated during Fe(III) reduction. These studies indicated the importance of incorporating the mechanism corresponding to the regulation of metabolism to refine the conceptual and *in silico* model.

Gene expression data corresponding to several environmental and genetic perturbations in *G. sulfurreducens* represents information that captures the activity of the regulatory network. Hence, gene expression data derived from several experiments were processed and assembled for further analysis. This expression data was filtered and then clustered based on expression similarities to identify co-expressed genes across the different perturbations. This was followed by sequence analysis including the searching the upstream regions of these co-expressed genes and operons for known transcription factor binding sites, and aligning the upstream regions to identify motifs that correspond to novel sites. This analysis revealed several potential regulatory interactions including a mechanism for regulating heat shock response, and motifs for regulation of sulfate metabolism. Further analysis with additional expression data that incorporates metabolic perturbations is expected to derive regulatory constraints for the metabolic model.

These studies reveal the potential of a combined computational and experimental strategy to iteratively characterize metabolism and the associated regulatory network. Such highly refined conceptual and in silico models of cellular metabolism will be important to design and optimize efficient strategies for bioremediation and harvesting energy from organic substrates.

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Novel Regulatory Systems and Adaption of Some Well-Known Systems Controlling Respiration, Growth, and Chemotaxis of *Geobactor* Species

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The goal of our Genomics:GTL project is to be able to predicatively model the growth and activity of the *Geobacter* species that predominate during *in situ* bioremediation of uranium and on the surface of energy-harvesting electrodes in order to better understand these processes and to have the ability to predict the likely outcome of optimization strategies. This requires an understanding not only of the physiological capabilities of *Geobacter* species, but also of how the expression of those physiological capabilities is regulated under various environmental conditions. At last year's meeting we reported on elucidation of global transcriptional regulatory systems in *G. sulfurreducens*, such as the RpoS, RpoE, and Fur regulons. In the past year studies have focused on genome-scale computational and microarray analysis of transcriptional regulation as well as more in depth studies on the regulation of expression of genes known to encode for proteins important in extracellular electron transfer to metals and electrodes.

One of the most surprising findings was that some of the *c*-type cytochromes in *G. sulfurreducens* have a regulatory function and play a role in regulating the production of other *c*-type cytochromes via either transcriptional or post-translation regulatory functions. For example, the *omcF* gene is predicted to encode for a small outer-membrane mono-heme *c*-type cytochrome. Thus, its function as a potential electron transfer protein was evaluated. An OmcF-deficient mutant was deficient in its ability to reduce Fe(III) and this was associated with an absence of the outer-membrane *c*-type cytochrome, OmcB, which is known to be required for Fe(III) reduction. Reverse transcriptase PCR and northern blot analysis revealed that the *omcB* was not transcribed in the OmcF-deficient mutant. Expression of *omcF in trans* restored the expression of *omcB* as well as the ability of the mutant to reduce Fe(III). These results suggest that OmcF may play a role in transcriptional regulation of *omcB*. Deletion of another outer-membrane *c*-type cytochrome gene, designated *omcG*, which is predicted to contain 13 hemes, also greatly diminished levels of OmcB. However, unlike the *omcF* mutant, *omcB* transcription was not affected. These results indicate that OmcG is specifically involved in either the modification, stabilization, or maturation of OmcB. These are the first reports of cytochromes that are necessary for electron transfer to metals, but not directly involved in electron transfer process. Their more likely role is to serve as sensors that regulate cytochrome expression.

Expression of *omcB* is also controlled by RpoS and Rel_{Gsu}, the *G. sulfurreducens* homolog of RelA, which are important in response to growth under nutrient-limited or stressful conditions. Another transcriptional regulator of *omcB* expression appears to be the product of the gene *ofrR,* which is immediately upstream of the operon that includes *omcB*. Levels of *omcB* transcripts increased orders of magnitude in response to a limitation in electron-acceptor availability or as rates of growth on Fe(III) increased. In a similar manner, expression of *omcS*, which encodes for an outer-membrane *c*-type cytochrome that is required for electricity production is regulated via multiple mechanisms.

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Microarray studies have identified two-component systems that subsequent genetic studies have demonstrated control cytochrome production in response to changing environmental conditions. These results demonstrate that extracellular electron transfer is highly regulated in *G. sulfurreducens*. As outlined in a companion abstract, it has recently been determined that the pili of *G. sulfurreducens* function as nanowires that are required for electron transfer to Fe(III) oxides. Genome-scale studies of the regulation of pilin formation suggested that expression of *pilA*, the gene for the structural pilin protein, is regulated in response to electron acceptor availability, as well as redox and nutrient status. For example, levels of *pilA* transcripts were significantly higher in mutants in which one of the two Fnr-like genes was deleted or when cells were grown under electron-acceptor limiting conditions. Deleting the rel_{Gsu} lowered *pilA* transcript levels. Genome analysis suggests that *pilA* expression is also controlled by a two component regulatory system and the sigma factor, RpoN. Additional mechanisms for pilin production will be reported.

It is expected that regulation of cell behavior in the form of chemotaxis plays an important role in the predominance of *Geobacter* species in subsurface environments. Previous studies have demonstrated that chemotaxis to iron is an important aspect of the reduction of Fe(III) oxides by *Geobacter* species. The genome sequence of *G. sulfurreducens*, contains multiple homologs of chemotaxis genes, including cheW (10), cheA (4), cheY (7), cheR (5), cheB (3), cheC (3), cheD (3) and cheV (1). This contrasts with the genome of *E. coli* which only contains a single copy of a subset of these genes. In order to elucidate factors controlling chemotaxis in *Geobacter* species the gene for a methyl-accepting chemotaxis protein (MCP), from *Geobacter metallireducens* was expressed in a strain of *E. coli* (HCB429) that lacks MCPs. This restored chemotaxis-like in the *E. coli* strain grown in soft agar. Evaluating the function of *Geobacter* chemotaxis proteins in *E. coli* shows promise as a versatile high throughput approach.

Computational analyses that integrated whole genome analyses, comparative genomics, and gene expression microarray data have identified thousands of sites potentially related to gene regulation in *Geobacter* species. A comprehensive resource has been developed that provides the predicted operon organization of the genomes and contig assemblies of *Geobacter* species, potential transcriptional regulatory motifs in the upstream regions of every predicted operon, the results of bi-directional similarity comparisons between *E. coli* regulatory proteins and proteins of *G. sulfurreducens*, and the genome locations of predicted transcription regulatory elements, palindromic motifs, and conserved bacterial elements. This is an invaluable resource for ongoing experimental studies of regulation of respiration and more recently initiated studies on regulation of central metabolism. As will be detailed at the meeting, many of the binding sites for transcriptional regulators that were predicted from analysis of multiple whole-genome gene expression studies with microarrays are in agreement with sequence-based predictions and with experimental results.

Details will also be provided on studies of other forms of regulation such as the response to oxidative stress and the coordinated regulation of the expression of central metabolism and respiratory genes under conditions simulating those expected during *in situ* uranium bioremediation as well as continued studies of the global regulatory systems first described at last years meeting.

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Nanowires, Capacitors, and Other Novel Electron Transfer Mechanisms in *Geobacter* Species Elucidated from Genome-Scale Investigations

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Molecular ecology studies have demonstrated that *Geobacteraceae* are the predominant microorganisms involved in *in situ* bioremediation of uranium-contaminated groundwater and on the surface of electrodes harvesting electricity from waste organic matter. However, there has been little information on how these organisms transfer electrons outside the cell onto insoluble electron acceptors, such as metals and electrodes, or alternative mechanisms for respiration.

Insoluble Fe(III) oxides are the primary electron acceptor supporting the growth of *Geobacter* species in subsurface environments, including during uranium bioremediation. Previous studies on electron transfer to Fe(III) oxides in metal-reducing microorganisms have primarily focused on outer-membrane *c*-type cytochromes functioning as the terminal electron carriers that transfer electrons onto Fe(III) oxides. However, analysis of the genomes of two *Pelobacter* species, which are also members of the *Geobacteraceae* indicated that these organisms lacked genes for the outer-membrane cytochromes that are prevalent in *Geobacter* species. Yet, *Pelobacter* species can reduce Fe(III) oxide. If it is assumed that the same mechanism for extracellular electron transfer to Fe(III) oxide is conserved within the *Geobacteraceae*, then these results suggested that outer-membrane *c*-type cytochromes are not the Fe(III) oxide reductase.

Comparison of the available *Geobacteraceae* genomes indicated that the genes for pili, are highly conserved and expression studies indicated that PilA, the structural pilin protein, was expressed during growth on Fe(III) oxide, but not soluble, chelated Fe(III). A mutant of *Geobacter sulfurreducens* in which *pilA* was deleted could reduce soluble electron acceptors, including Fe(III) citrate, as well as wild-type but could not reduce Fe(III) oxide. Complementation with a functional *pilA* restored the capacity for Fe(III) oxide reduction. Based on the role of pili in other organisms, it was hypothesized that the pili were required for *G. sulfurreducens* to attach to Fe(III) oxides, but the *pilA* mutant attached to Fe(III) oxide as well as the wild-type, suggesting a novel role for pili in Fe(III) oxide reduction. Conducting-probe atomic force microscopy revealed that the pili were highly conductive. In contrast, non-pilin proteins had no detectable conductivity and in instances in which the non-pilin proteins covered the pili filaments, they insulated the pili from the conductive tip. No conductivity was detected in the pili of *Shewanella oneidensis*, which, unlike *Geobacter* species, does not need to contact Fe(III) oxides in order to reduce them. These results suggest that the pili of *G. sulfurreducens* serve as biological nanowires, transferring electrons from the cell surface to the surface of Fe(III) oxides. Electron transfer via pili suggests possibilities for other unique cell-surface and cell-cell interactions, and for bioengineering of novel conductive materials.

The finding that pili, rather than *c*-type cytochromes, are likely to be responsible for the final electron transfer to Fe(III) oxides leads to the question of why *c*-type cytochromes are so abundant in

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Geobacter species, both in quantity and number of genes in the genomes. Some *c*-type cytochromes, such as the small, periplasmic PpcA, which are highly conserved in *Geobacter* and *Pelobacter* genomes, may be intermediaries in electron transfer to Fe(III). However, for many outer-membrane cytochromes, there is little similarity in gene sequences in even closely related *Geobacter* species. Whole genome gene microarray and proteomic studies revealed much higher expression of multiple cytochrome genes under electron-acceptor limiting conditions. Further investigation suggested that the cytochromes behave as a capacitor capable of accepting electrons from energy-generating electron transfer reactions in the inner membrane and storing these electrons until a suitable electron acceptor is available. This explains how *Geobacter* species are able to thrive in subsurface environments in which insoluble Fe(III) oxides are heterogeneously dispersed because the capacitor cytochromes permit continued electron transfer during the search for new Fe(III) oxides followed by discharge to the Fe(III) oxide once a suitable source is found.

Surprisingly, the *pilA* mutant produced electricity as well as the wild-type, suggesting that electron transfer to electrodes proceeded via different mechanisms than electron transfer to Fe(III) oxides. Global analysis of gene expression in *G. sulfurreducens* with a whole-genome DNA microarray indicated that the outer-member *c*-type cytochrome gene, *omcS*, and its co-transcribed homolog, *omcT*, were the only genes coding for likely electron transfer proteins that were consistently up-regulated during growth on electrodes versus growth with Fe(III) as the electron acceptor. Quantitative PCR demonstrated that mRNA levels for these cytochromes increased as the amount of current harvested with the electrode increased. When *omcS* and *omcT* were deleted, current production decreased to ca. a third of the wild type and the potential of the anode went from –0.5 V in the wild type to only –0.15 in the mutant. Complementation of the *omcS* gene in the mutant restored current production and anode potential to values comparable to wild type. These results suggest that OmcS is likely to be the primary protein mediating electrical contact between the cell and the electrode surface. This finding offers several possibilities for engineering electrode surfaces and/or microorganisms to improve the function of microbial fuel cells.

Fumarate is an electron acceptor in some *Geobacter* species, such as G. *sulfurreducens*, but not in others, such as *G. metallireducens*. Yet the genomes of both organisms contained what appeared to be a heterotrimic type of fumarate reductase, frdCAB, homologous to the fumarate reductase of *Wolinella succinogenes*. Mutation of the putative catalytic subunit in *G. sulfurreducens* resulted in a strain that lacked fumarate reductase activity and was unable to respire fumarate. Furthermore, the mutant strain could not grow with acetate as the electron donor, regardless of electron acceptor, and lacked succinate dehydrogenase activity. Oxidation of acetate coupled to Fe(III) reduction was possible in the mutant strain if exogenous fumarate was provided, as fumarate could be converted to succinate through TCA cycle reactions and excreted. Highly similar genes were present in *Geobacter metallireducens*, which cannot respire fumarate. When a putative dicarboxylic acid transporter from *G. sulfurreducens* was expressed in *G. metallireducens*, growth with fumarate as the sole electron acceptor was possible. These results demonstrate that, unlike previously described organisms, *Geobacter* species use the same enzyme for both fumarate reduction and succinate oxidation in vivo. This also represents the first example of genetic engineering of a *Geobacter* species for novel respiratory abilities.

Significant progress was also made in genome-enabled studies of oxygen respiration and novel outer-membrane proteins that were first reported at last year's meeting and updates will be provided.

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Continued Progress in the use of Microarray Technology to Predict Gene Regulation and Function in *Geobacter sulfurreducens*

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Geobacter species represent a rare example in environmental microbiology in which microorganisms closely related to those which predominate in the environment and carry out environmental processes of interest can be readily cultivated in the laboratory. Molecular analyses designed to avoid culture bias, have demonstrated that microorganisms in the family *Geobacteraceae* are the dominant dissimilatory metal-reducing microorganisms in subsurface environments in which organic contaminants are being degraded with the reduction of Fe(III) and in aquatic sediments where dissimilatory metal reduction is important. In addition to their importance in global carbon, nutrient and metal cycles interest in *Geobacter* spp. stems from their potential as agents of bioremediation and capacity to create electricity.

Since completion of the *G. sulfurreducens* genome sequence, global gene expression profiling has been undertaken through the application of microarray technology. Experiments for querying whole genome PCR-based arrays currently being pursued include the examination of wild type *G. sulfurreducens* gene expression profiles under relevant physiological conditions and the testing of mutant strains in which a selected gene has been knocked out versus their wild type counterpart. Various data mining techniques including cluster analyses and analysis of variance are being employed to examine results from individual experiments and collectively across multiple experiments. These efforts have provided new insights into *Geobacter* physiology and regulatory networks.

For example, cells grown with chelated Fe(III) as the electron acceptor had higher levels of transcripts for *omcB* (GSU2737), an outer-membrane *c*-type cytochrome that is essential for Fe(III) reduction. Several other *c*-type cytochrome genes also appeared to be up regulated including a putative *c*-type cytochrome (GSU1334) which based on current genome comparative analyses is unique to *Geobacter* lineages. A substantial proportion (30%) of the significantly expressed genes during Fe(III) reduction were genes of unknown function, or hypothetical proteins. These results suggest differences in the physiology of Fe(III) reduction among microorganisms which perform this metabolic process. An unexpected result was significantly higher levels of transcripts for genes which have a role in metal efflux, potentially suggesting the importance of maintaining metal homeostasis during release of soluble metals when reducing Fe(III). This includes at least six transporter genes that are members of the resistance-nodulation-division (RND) superfamily of efflux transporters such as representatives of the transmembrane spanning heavy metal efflux pump *czc*A family (GSU0830, GSU1332) and one gene encoding for the membrane fusion protein of the *czc*B family (GSU0829). In contrast, transcript levels for other members of the *czc*A and *czc*B families in the *G. sulfurreducens* genome (GSU2135, GSU2136, GSU3400) were comparable during growth on Fe(III) and fumarate. This suggests that these *czc* family members are paralogs with different physiological roles and regulation. Common themes appearing across multiple experiments include the importance of transporter expression and the expression of a group of genes related to protein folding which for example are down regulated under conditions such as growth of *Geobacter* as a biofilm and with Fe(III) as an electron acceptor, in contrast they are up regulated in a mutant strain in which the *rpo*E sigma factor has been knocked out.

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The next phase of the microarray component is building upon these previous successes while extending the flexibility and power of this technique. One example is the adaptation of methods to effect linear amplification of total RNA. Development of this protocol is important to producing high quality hybridizations from samples where the quantity of RNA that can ultimately be obtained from that sample is limited. High quality microarray hybridizations typically require several micrograms of total RNA per replicate. In order to obtain sufficient statistical power for meaningful analyses of microarray data it is necessary to replicate both biological samples as well as within sample replication (technical replication). Cell growth under conditions such as on poorly crystalline iron oxide media (an environmentally relevant growth condition of *G. sulfurreducens*) produces less total RNA upon extraction which can potentially hamper microarray efforts. Linear amplification (as opposed to geometric amplification with traditional PCR) of the total RNA allows the production of sufficient RNA quantities representative of the original proportions of the mRNA transcript population to facilitate the necessary replication of hybridizations to ensure a meaningful outcome post data analysis. The protocol described briefly here is currently being successfully tested on the *G. sulfurreducens* microarray and is a modification of the work of the classic "Eberwine" T7-Amplification method.

Amplified sense RNA is produced using random hexamers in a standard manner for first strand cDNA synthesis to create antisense cDNA. This product (antisense cDNA) is now used as the template in a second strand synthesis along with random nonamers to which a viral T3 promoter is attached. This results in a double-stranded cDNA in which the T3 promoter has been incorporated into the second strand. This second strand is in the sense orientation. An in vitro transcription (IVT) reaction can then be used to transcribe copious quantities of sense RNA from the T3 promoter sites. The resulting IVT product now serves as the template for standard cDNA synthesis and indirect fluorescent (Cy-Dye) labeling. These resulting targets can be used for hybridization to both PCR and oligonucleotide-based arrays.

One example where linear amplification has been applied is to the examination of gene expression patterns of *G. sulfurreducens* when grown using (insoluble) iron oxide as a sole electron acceptor. An overall improvement in hybridization intensity was realized with targets prepared from linear amplified RNA in comparison to unamplified RNA targets. Further, quantitative RT-PCR of a subset of genes from each RNA population (amplified and unamplified) have revealed that the trend of each gene (either significant elevation, depression or no change in gene expression) was the same in both RNA populations. The use of this linear RNA amplification technique in future will include the examination of global gene expression patterns from RNA extracted directly from environments in which *Geobacter* spp. are dominant members of the microbial community.