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Molecular Analysis of the *In Situ* Growth Rate of Subsurface *Geobacter* Species

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Abstract

Molecular tools that can provide an estimate of the *in situ* growth rate of *Geobacter* species could improve understanding of dissimilatory metal reduction in a diversity of environments. Whole genome microarray analyses of the subsurface isolate, *Geobacter uraniireducens*, grown under a variety of conditions identified a number of genes that are differentially expressed at different specific growth rates. Expression of two genes encoding ribosomal proteins, *rpsC* and *rplL*, were further evaluated with quantitative reverse transcription PCR (qRT-PCR) in cells with doubling times ranging from 6.56 h to 89.28 h. Transcript abundance of *rpsC* correlated best (r^2= 0.90) with specific growth rates. Therefore, expression patterns of *rpsC* were used to estimate specific growth rates of *Geobacter* species during an *in situ* uranium bioremediation field experiment in which acetate was added to the groundwater to promote dissimilatory metal reduction. Initially, increased availability of acetate in the groundwater resulted in higher expression of *Geobacter rpsC* and the increase in the number of *Geobacter* cells estimated with fluorescent *in situ* hybridization compared well with specific growth rates estimated from levels of *in situ rpsC* expression. However, in later phases cell number increases were substantially lower than predicted from *rpsC* transcript abundance. This change coincided with a bloom of protozoa and increased attachment of *Geobacter* species to solid phases. These results suggest that monitoring *rpsC* expression may better reflect the actual rate that *Geobacter* species are metabolizing and growing during *in situ* uranium bioremediation than changes in cell abundance.

Introduction

Understanding how environmental factors control the growth of microorganisms is a major goal in subsurface ecology. However, measuring the growth rate of bacterial populations *in situ* is challenging and approaches focused on enumeration of cells *in situ* are often inaccurate. Not only can unknown proportions of cells be attached to subsurface sediments, cell losses due to predation by protozoa or cell lysis by bacteriophage can also mask true rates of cell multiplication (1, 2).

A number of different techniques have been used to assess microbial growth rates in natural environments. For example, in *situ* growth rates can be estimated from the incorporation of radiolabeled thymidine or leucine into biomass (3, 4), or by the viable plate count technique (5). However, these approaches require sample incubation. Another approach that does not require sample incubation involves monitoring fluorescently labeled cells that are introduced into the subsurface (6, 7). This approach is limited to those strains that can readily be cultivated in the laboratory (8, 9), and studies have shown that labeled cells can become too diluted once substantial growth begins for results to be reliable (7).

In situ growth rates can also be estimated by quantifying the proportion of cells from the environment that are visually dividing with the frequency of dividing cells (FDC) technique (10, 11). This approach assumes that the duration of cell division (T_d) and chromosome replication is constant and identical for all individuals within a population and that all of the cells in a population are metabolically active. However, studies have shown that T_d values and chromosome replication rates can not only vary between different species but even within different strains of the same species (12, 13),

and cells in stationary or death phase will not be as metabolically active as those in logarithmic phase.

A potentially powerful technique for evaluating the physiological status and rates of activity of microorganisms in the subsurface is quantifying key gene transcripts (14). This approach has been particularly successful for monitoring *Geobacteraceae* species, which have physiological features that favor their growth in the subsurface (15-17) and have been found to be the predominant organisms associated with the anaerobic *in situ* bioremediation of groundwater contaminated with organic compounds or uranium (18). In such environments, it has been possible to determine with gene transcript analysis whether *Geobacteraceae* species are limited for major nutrients, such as ammonium and phosphate, as well as nutrients particularly important to *Geobacteraceae* species, such as iron (19-23). Furthermore, this method was used to determine whether *in situ* *Geobacteraceae* were experiencing oxidative or heavy metal stress (20, 24), and to estimate relative rates of *in situ* metabolism (25, 26). Here we report on a method for elucidating *in situ* growth rates of subsurface *Geobacteraceae* populations from gene transcript abundance.

Materials and Methods

Growth of *Geobacter uraniireducens*.

Geobacter uraniireducens strain RF4 (ATCC BAA-1134) was obtained from our laboratory culture collection. Cells were grown with acetate (5 mM) as an electron donor and either fumarate (40 mM), amorphous Fe(III) oxyhydroxide (100 mM), Mn(IV) oxide (20 mM), or sediments collected from a uranium-contaminated aquifer located in Rifle, Colorado, provided as an electron acceptor. All cultures except the sediment incubations

were grown in a bicarbonate-buffered, defined medium (27). All incubations were done in the dark under N_2:CO_2 (80:20).

For sediment incubations, 40 g of heat-sterilized sediments, 6 mL groundwater, and acetate (5 mM) were added to 60 mL serum bottles in an anaerobic chamber under a N_2 atmosphere (28). Cells that served as an inoculum (2%) for sediment incubations were first grown in medium with acetate (5 mM) provided as an electron donor and amorphous Fe(III) oxyhydroxide (100 mM) provided as an electron acceptor. Growing *G. uraniireducens* in chemostats with soluble electron acceptors such as Fe(III) citrate or fumarate as previously described for *G. sulfurreducens* (29) was not feasible because *G. uraniireducens* cannot grow with Fe(III) citrate (30) and fumarate can be fermented when other electron donors are limiting. Therefore, in order to achieve a wide range of specific growth rates, *G. uraniireducens* was grown in batch cultures with acetate as the electron donor and a variety of electron acceptors (sediment Fe(III) oxides; synthetic Fe(III) or Mn(IV) oxides; or fumarate) at various temperatures (Supplementary Material, Figure S1A-D). Cell counts and the exponential growth equation ($\mu = (\ln N_f - \ln N_o)/(t_f - t_0)$, where N_o and N_f are bacterial abundances at the beginning and end points) were used to determine specific growth rates for all pure culture studies of *G. uraniireducens*.

Site and description of field site.

In 2010, a small-scale *in situ* bioremediation experiment was conducted on the grounds of a former uranium ore processing facility in Rifle, Colorado during the months of August-October. This site, designated the Old Rifle site, is part of the Uranium Mill Tailings Remedial Action (UMTRA) program of the U.S. Department of Energy. The test plot was adjacent to a previously studied larger experimental plot at the site (31). The

112 monitoring array consisted of 9 down-gradient and 6 injection wells (Supplementary
113 Material, Figure S2). Groundwater for the experiment was collected from well CD-04.
114 During the field experiment, a concentrated acetate/bromide solution (50/20 mM)
115 mixed with native groundwater was injected into the subsurface to provide approximately
116 5 mM acetate to the groundwater over the course of 30 days as previously described (31,
117 32). Bromide was utilized as a non-reactive tracer.
118 **Analytical techniques**.
119 Acetate and fumarate concentrations were determined with an HP series 1100
120 high-pressure liquid chromatograph (Hewlett Packard, Palo Alto, California) with a Fast
121 Acid Analysis column (Bio-Rad laboratories, Hercules, California), an eluent of 8 mM
122 H_2SO_4 and an absorbance detection at 210 nm as previously described (31).
123 Fe(III) reduction in the Fe(III) oxide grown cultures was monitored by measuring
124 the formation of Fe(II) over time with a ferrozine assay in a split-beam dual-detector
125 spectrophotometer (Spectronic Genosys2; Thermo Electron Corp., Mountain View, CA)
126 at an absorbance of 562 nm after a one hour extraction with 0.5 N HCl (27, 33). Fe(III)
127 reduction in the sediments was monitored by first measuring Fe(II) in the sediments that
128 could be extracted in 0.5 M HCl after a 1 hour incubation with a ferrozine assay (33).
129 The remaining Fe(III) in the sediments that was not HCl-extractable was then converted
130 to Fe(II) by the addition of 0.25 M hydroxylamine) (33). After addition of
131 hydroxylamine, samples were incubated for an additional hour, and then measured with a
132 ferrozine assay. The percentage of Fe(II) in the sediments was then determined by
133 dividing the HCl-extractable Fe(II) by the hydroxylamine extractable Fe(II).

Cell numbers from pure cultures were determined by counting acridine orange stained cells with fluorescence microscopy on a Nikon Eclipse E600 microscope (34).

FISH and DAPI counts

The microbial community was analyzed for *Geobacteraceae* and total bacteria using fluorescent *in situ* hybridization (FISH) and nucleic acid staining with 4',6-diamidino-2-phenylindole (DAPI). The number of *Geobacteraceae* was determined by the number of cells that hybridized to the following probes: GEO3A, GEO3B, and GEO3C (35). Groundwater samples were immediately fixed in a solution consisting of 4% paraformaldehyde/0.5X phosphate buffered saline (PBS; Na_2HPO_4). This solution was vortexed for five seconds before being added to 0.2 µm white polycarbonate filters (GTTP; Millipore, Billerica, MA) and washed with 1% Nonidet (Sigma) solution.

Cells were hybridized for three hours as described previously (36) with a mix of probes GEO3A, GEO3B, and GEO3C in hybridization buffer (0.02 M Tris, 0.9 M NaCl, 0.02% SDS) with a formamide concentration of 30% (v/v) at 46 ºC) (37). Filters were washed twice with wash buffer (0.02 M Tris, 0.1 M NaCl, 5 mM EDTA, 0.01% SDS) and incubated at 48 ºC for 10 minutes. Filters were washed a final time with filtered, autoclaved, distilled water, placed on slides where they were embedded in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and observed with a Nikon epifluorescence microscope. Ten to twenty fields of view for each sample were enumerated; DAPI and FISH were both counted for each field. The DAPI and FISH staining efficiency for Rifle groundwater samples ranged from 68-79% and standard deviations among triplicate counts varied between 10-30% of the average number of cells.

Extraction of RNA from samples

For extraction of RNA from batch cultures, 100 ml of *G. uraniireducens* were first split into 50 ml conical tubes (Falcon), and pelleted by centrifugation at 3,600 x g for 15 min.

RNA was extracted from sediment incubations when 75% of the total iron present in the sediments was reduced to Fe(II). Groundwater and sediment from the incubations (40 g sediment and 6 ml groundwater) were transferred into 50 ml conical tubes (Falcon), and pelleted by centrifugation at 3,600 x g for 15 min. Pellets were then immediately frozen in a dry ice, ethanol bath and stored at –80^{o}C. RNA was extracted from these pellets as previously described (19).

RNA was also extracted from groundwater collected from the U(VI) contaminated aquifer during the bioremediation field experiment. In order to obtain sufficient biomass from the groundwater for RNA extraction, it was necessary to concentrate 15 liters of groundwater by impact filtration on 293 mm diameter Supor membrane disc filters with a pore size of 0.2 µm (Pall Life Sciences), which took about 3 minutes. All filters were placed into whirl-pack bags, flash frozen in a dry ice/ethanol bath, and shipped back to the laboratory where they were stored at –80^{o}C. RNA was extracted from filters as previously described (19).

High quality RNA was extracted from the groundwater, sediment, and batch culture samples. In order to ensure that RNA samples were not contaminated with DNA, PCR amplification with primers targeting the 16S rRNA gene was conducted on RNA samples that had not undergone reverse transcription.

Microarray analysis

The MessageAmp II- Bacteria Kit (Ambion) was used to amplify total RNA (0.5 µg) according to the manufacturers instructions. Amplified RNA was then chemically labeled with Cy3 (for the control or soluble electron acceptor condition) or Cy5 (for the experimental insoluble condition) dye using the MicroMax ASAP RNA Labeling Kit (Perkin Elmer, Wellesley, MA) according to the manufacturer's instructions.

CustomarrayTM 12K oligonucleotide microarrays (Combimatrix, Mukilteo, WA) were designed from the genomic sequence of *G. uraniireducens* (accession number NZ_AAON00000000). A complete record of all oligonucleotide sequences used and raw and statistically treated data files is available in the NCBI Gene Expression Omnibus database (GEO data series GSE10920 and GSE12874).

Results were analyzed via LIMMA mixed model algorithms as previously described (38-41) and multiple oligonucleotide probes were analyzed from each gene (3 or 4 probes per gene). A gene was only considered differentially expressed if at least half of the probes had p values less than or equal to 0.01.

Testing and design of primers

Before primers for quantitative reverse transcription PCR (qRT-PCR) targeting *in situ rpsC* and *proC* mRNA transcripts could be designed, cDNA libraries were constructed with products amplified from RNA extracted from groundwater with the degenerate PCR primer sets, Geo_rpsC200f/640r and Geo_proC55f/460r (Supplementary Material Table S1). These degenerate primers were designed from nucleotide and amino acid sequences extracted from the following *Geobacteraceae* genomes: *G. sulfurreducens*, *G. metallireducens*, strain FRC-32, strain M21, *G. uraniireducens*, *G.*

202 *lovleyi*, and *G. bemidjiensis*. Genome sequence data were obtained from the DOE Joint
203 Genome Institute (JGI) website (www.jgi.doe.gov). Products from these degenerate
204 primer sets were cloned into the TOPO TA cloning vector, version M (Invitrogen,
205 Carlsbad, CA). One hundred plasmid inserts from both the *rpsC* and *proC* cDNA
206 libraries were sequenced with the M13F primer at the University of Massachusetts
207 Sequencing Facility. A *Geobacter rpsC* sequence accounted for 85% of the sequences in
208 the *rpsC* cDNA library and a *Geobacter proC* sequence accounted for 90% of the *proC*
209 cDNA library. Both of these dominant sequences were used to design qRT-PCR primers
210 specific for *in situ Geobacter* species present during bioremediation.
211 All qRT-PCR primers were designed according to the manufacturer's
212 specifications (Applied Biosystems) and had amplicon sizes ranging from100-200 bp.
213 Pure culture primers designed for qRT-PCR only targeted *G. uraniireducens* cells.
214 GU_rpsC178f/245r, GU_rplL66f/136r, and GU_proC263f/367r primer sets were used to
215 quantify *rpsC*, *rplL*, and *proC* mRNA transcripts from *G. uraniireducens* and
216 Geo_rpsC50f/147r and Geo_proC13f/140r targeted *rpsC* and *proC* cDNA made from
217 RNA extracted from groundwater (Supplementary Material Table S1).
218 The reference gene, *proC*, which appears to be constitutively expressed by
219 *Geobacteraceae* species in pure cultures and in the environment (22, 24, 42) and was not
220 differentially expressed in any of the microarray experiments from this study, was
221 selected as an external control. The gene *proC* encodes pyrroline-5-carboxylate
222 reductase, which is involved in arginine and proline metabolism. In order to further
223 confirm the fact that *proC* expression is constitutive, *proC* mRNA transcript abundance
224 was compared to biomass harvested from *G. uraniireducens* cultures grown at four

different temperatures with acetate as the electron donor and fumarate as the electron acceptor. All four of these cultures were harvested at an $OD_{600\ nm}$ of 0.3 (biomass *ca*. 49 mg protein/L), and the number of *proC* mRNA transcripts in these samples only varied from $1.66x10^5$ to $3.34x10^5$.

Expression by one growth-related and one reference gene was monitored in this study and results indicated that mRNA transcripts from these genes could be used to reliably predict *in situ* growth. However, in order to better understand total cell activity, future studies might want to target expression profiles of more genes.

PCR amplification parameters and clone library construction

A DuraScript enhanced avian RT single strand synthesis kit (Sigma) was used to generate cDNA as previously described (19). Previously described parameters were used to amplify genes of interest with degenerate primers (43).

For construction of 16S rRNA gene clone libraries, gene fragments were first amplified by PCR with the following primer sets; 8F (44) with 519R (45) and 338F (46) with 907R (45). PCR products were purified with the Gel Extraction Kit (Qiagen), and clone libraries were constructed with a TOPO TA cloning kit, version M (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Quantification of gene expression by qRT-PCR.

Once the appropriate cDNA fragments were generated by RT-PCR, quantitative PCR amplification and detection were performed with the 7500 Real Time PCR System (Applied Biosystems). Optimal qRT-PCR conditions were determined using the manufacturer's guidelines; 50 cycles of 95˚C for 15 seconds and 60˚C for 1 minute.

Each PCR mixture consisted of a total volume of 25 µL and contained 1.5 µL of the appropriate primers (stock concentrations, 15 µM), 5 ng cDNA, and 12.5 µL Power SYBR Green PCR Master Mix (Applied Biosystems). Standard curves covering 8 orders of magnitude were constructed with serial dilutions of known amounts of purified cDNA quantified with a NanoDrop ND-1000 spectrophotometer at an absorbance of 260 nm.

The qPCR efficiency (95% to 99%) was calculated based on the slope of the standard curve. All qPCR assays had triplicate biological and technical replicates. Thermal cycling parameters consisted of an activation step at 50°C for 2 min, a denaturation step at 95°Cfor 10 min, and 50 cycles at 95°C for 15 s and 60°C for 1 min. This was followed by the construction of a dissociation curve through increasing the temperature from 60°C to 95°C at a ramp rate of 2%. A single predominant peak was observed in the dissociation curve of each gene, supporting the specificity of the PCR product.

Phylogenetic analysis

16S rRNA and functional gene sequences were compared to GenBank nucleotide and protein databases with the blastn and blastx algorithms (47).

The nucleotide sequences of *rpsC*, *proC*, and 16S rRNA genes amplified from the uranium-contaminated aquifer have been deposited in the GenBank database under accession numbers FJ042710-FJ042726.

Results and Discussion

Identification of growth rate related genes with microarray analysis.

G. uraniireducens, an isolate from the Rifle study site, is closely related to *Geobacter* species that predominate during *in situ* uranium bioremediation at the site (30). Whole-genome scale data on transcript abundance during growth with fumarate as

the electron acceptor versus growth on natural Fe(III) oxides in sterile subsurface sediments (20) or laboratory-synthesized Fe(III) and Mn(IV) oxides is available (GEO data series GSE10920 and GSE12874). At its optimal temperature of 30°C, *G. uraniireducens* grew faster with fumarate provided as the electron acceptor than with Fe(III) or Mn(IV) oxides, and even slower in sediments (Table 1).

A number of genes involved in protein synthesis had significantly lower transcript abundance at the slower growth rates (Supplementary Material Tables S2-S4). Of these, fifty-two were less abundant under all three slow-growth conditions. Two of these genes, *rpsC* and *rplL*, which code for ribosomal proteins S3 and L7/L12 (Figure 1) were chosen for further study because their expression levels have also been shown to be linked to specific growth rates in other microorganisms (48-50).

Correlation between specific growth rate and transcript abundance.

In order to achieve an even wider range of growth rates, *G. uraniireducens* was grown at various temperatures with a variety of electron acceptors (sediment Fe(III) oxides; synthetic Fe(III) or Mn(IV) oxides; or fumarate) (Table 1, Supplementary Figure S2). Specific growth rates ranged from 0.007 h^{-1} to 0.106 h^{-1}. The specific growth rate of *G. uraniireducens* increased with temperature in fumarate-grown cultures, but this same increase was not observed in sediment-grown cells. The fact that growth was slower in sediment cultures grown at 30ºC suggests that some factor other than temperature, possibly the need to search for Fe(III) oxide sources (51) can limit growth in sediments. The decrease in specific growth rate in sediments observed at 37 ºC can be explained by the fact that this temperature is above *G. uraniireducens*' optimal growth range (30).

293 Despite the variety of growth conditions, the number of mRNA transcripts from
294 both genes normalized against the number of *proC* transcripts (Table 1) was directly
295 correlated with specific growth rate (Figure 2). The correlation between *rpsC/pro*C and
296 specific growth rate was much better than it was for *rplL/proC* versus specific growth
297 rate (Figure 2).
298 The specific growth rate of *G. uraniireducens* could also be manipulated by
299 controlling the availability of ammonium. Addition of ammonium to cultures that were
300 previously growing under nitrogen-fixing conditions greatly accelerated the specific
301 growth rate (Figure 3A). Increased growth was associated with a dramatic increase in
302 *rpsC* transcript abundance (Figure 3B). Under nitrogen-fixing conditions the specific
303 growth rate was approximately 0.017 h^{-1} which, according to the equation from Fig. 2A
304 (y=511x-5.3), should yield a normalized transcript abundance value of 3.5 and compares
305 well with the value of 3.6 that was observed. These results further suggested that the
306 abundance of *rpsC* could be used to estimate specific growth rates of *G. uraniireducens*
307 under changing environmental conditions.
308 **Evaluation of *in situ* specific growth rate in the subsurface.**
309 The 2010 *in situ* uranium bioremediation field experiment conducted in Rifle
310 provided an excellent opportunity to determine whether the abundance of *rpsC* transcripts
311 might be a good indicator of the relative specific growth rates of *Geobacteraceae* species
312 in the subsurface. As previously observed in acetate-amendment studies at the site (26,
313 31), the addition of acetate resulted in an accumulation of Fe(II) (Figure 4A) and an
314 increase in the number of *Geobacteraceae* species (Figure 4B), consistent with the
315 expected stimulation of dissimilatory metal reduction. In contrast to the increase in

316 *Geobacteraceae* species, there appeared to be little net growth of other microorganisms;
317 as cells labeled with *Geobacteraceae*-specific probes accounted for most of the increase
318 in cells that was detected with DAPI staining (Figure 4B). The relative abundance of
319 *Geobacteraceae* species estimated from analysis of 16S rRNA gene sequences further
320 confirmed the specific stimulation of the growth of *Geobacteraceae* species (Figure 4C
321 and Supplementary Material Table S5). The majority of 16S rRNA gene sequences
322 recovered from the samples after day 9 were *Geobacteraceae* species, specifically
323 *Geobacter bemidjiensis* (Supplementary Material Tables S5 and S6), indicating that the
324 *Geobacteraceae* enumerated with FISH probes were primarily from the genus *Geobacter*.
325 When acetate concentrations in the subsurface started to decline after day 21, Fe(II)
326 concentrations also went down and this was accompanied by a decrease in the number of
327 *Geobacter* cells and their abundance relative to other bacterial species (Figure 4B).
328 Specific growth rates of *in situ Geobacter* species were estimated from *rpsC*
329 transcript abundance. It was assumed that there was a correlation between transcript
330 abundance of *rpsC* in the subsurface isolate, *G. uraniireducens*, and specific growth rate
331 of the *in situ Geobacter* population (Figure 2A). Specific growth rates for the *in situ*
332 *Geobacter* species were calculated using the equation in Figure 2A (y=511x-5.3) and
333 ranged from 0.014 to 0.016 h^{-1} (Figure 4D). These estimated specific growth rates were
334 comparable to the specific growth rate of 0.014 h^{-1} for *G. uraniireducens* from laboratory
335 incubations in acetate-amended sediments.
336 Estimated specific growth rates increased 45% with the addition of acetate,
337 plateaued around day 9, and then started to decline around day 20 to a rate near that
338 observed when acetate amendments were first started (Figure 4D). This same trend was

observed when *Geobacter* cell numbers were monitored over time with FISH. Immediately following the addition of acetate there was a dramatic increase in cell numbers up to day 9 (Figure 4D). Assuming logarithmic growth, the increase in cell numbers in the first 9 days (9.11×10^5 cells/ml) yielded a specific growth rate of 0.0148 h^{-1}, which is comparable to the maximum specific growth rate estimated by *rpsC* transcript numbers during this period (Figure 4D). Between days 9 and 20, the rate of *Geobacter* cell number increase was noticeably slower. The estimated specific growth rate from cell numbers during this phase was 0.0006 h^{-1}, much lower than the specific growth rates of 0.015-0.016 h^{-1} estimated from *rpsC* transcript abundance. After day 20, numbers of *Geobacter* in the groundwater started to decline even though the specific growth rate estimated from *rpsC* transcripts was higher than the specific growth rate at time zero.

A major factor contributing to the discrepancy between estimated specific growth rates and changes in cell number after day 9 may have been a bloom in protozoa grazing on *Geobacter* species. Studies at the Rifle site have shown that protozoa become abundant in the groundwater when dissimilatory metal reduction is stimulated by the addition of acetate (Holmes et al. submitted). The increase in protozoan abundance is significant because protozoa can exploit substantial quantities of bacterial biomass production (52) and grazing can significantly stimulate microbial metabolic activity and growth (53, 54).

Another consideration is that as Fe(III) oxides are depleted at the Rifle site, the *Geobacter* populations shift from one that is ca. 90 % planktonic to one in which most of the cells attach to the sediments (Dar, S. manuscript submitted). The continued decline in

362 the number of planktonic *Geobacter* cells during the later phases of the field experiment
363 may reflect this. Furthermore, there may be other factors contributing to cell loss. The
364 estimated *Geobacter* specific growth rates prior to acetate additions were ca. 70% of the
365 maximum estimated specific growth rates during acetate additions, yet cell numbers were
366 relatively low. Estimates of growth rate in the subsurface are likely to represent an
367 average of a wide range of growth rates of cells exposed to different microenvironments
368 as previously noted in biofilms (55).

369 **Implications**

370 These results suggest that monitoring expression of *rpsC* can provide insight into specific
371 growth rates of subsurface *Geobacter* species, even under situations where predation is
372 equal or higher then production of new cells. The ability to estimate specific growth rates
373 in the subsurface during the bioremediation of metals is particularly important, because
374 the goal may not always be to maximize the specific growth rate or even the overall rate
375 of metal reduction. For example, it may be desirable to maintain an active but slowly
376 respiring population of *Geobacter* species that will effectively reduce U(VI) as it enters
377 the acetate injection zone, without rapidly depleting the Fe(III) oxides needed to maintain
378 their growth in the subsurface.

379 The approach described here may also be useful for monitoring the growth of
380 *Geobacter* species in a diversity of other environments in which they play an important
381 role including anaerobic soils and sediments (18), electrodes harvesting current (56), and
382 some methanogenic environments (18). It seems likely that a similar approach may also
383 be useful for monitoring the growth of other subsurface microorganisms, such as the
384 *Dehalococcoides* species involved in reductive dechlorination (57), or to monitor the

385 growth of microorganisms added to the subsurface to promote bioremediation (58-60).
386 Furthermore, as the ability to predictively model the growth of subsurface
387 microorganisms at the genome-scale advances (16, 17, 61-63), it will be increasingly
388 important to have better methods for monitoring key aspects of microbial physiology,
389 such as growth rate for model validation.
390
391
392 **Figure Legends**
393 Figure 1. Microarray results from experiments comparing three different
394 experimental conditions (*G. uraniireducens* cells grown with Rifle
395 sediment, Fe(III) oxide, or Mn(IV) oxide provided as the electron
396 acceptor) to the control condition (*G. uraniireducens* cells grown with
397 fumarate provided as the electron acceptor) (p value cutoff $\leq$0.01).
398 Transcript levels for both *rpsC* and *rplL* were significantly down-regulated
399 in all three microarray experiments (indicated by negative fold change in
400 the figure). Triplicate biological and duplicate technical replicates were
401 done for all three microarray experiments.
402 Figure 2. The number of (A) *rpsC* and (B) *rplL* mRNA transcripts normalized
403 against the number of *proC* mRNA transcripts expressed by *G.*
404 *uraniireducens* at a wide range of growth rates. Triplicate biological and
405 technical replicates were done for all specific growth rates.
406 Figure 3. (A) The number of *G. uraniireducens* cells in cultures with acetate (5
407 mM) provided as the electron donor and fumarate (40 mM) provided as

the electron acceptor under nitrogen limiting (no NH_4^+) or non-nitrogen limiting (4.67 mM NH_4^+) conditions. (B) The number of *rpsC* mRNA transcripts normalized against the number of *proC* mRNA transcripts expressed by *G. uraniireducens* cells under nitrogen limiting or non-nitrogen limiting conditions. Triplicate biological and technical replicates were done for all samples.

Figure 4. Growth of *Geobacteraceae* in response to acetate availability during the uranium bioremediation field experiment conducted at Rifle in 2010. (A) Acetate and Fe(II) concentrations in groundwater collected during uranium bioremediation, (B) Number of *Geobacteraceae* in the groundwater estimated by direct counts of cells labeled with *Geobacteraceae*-specific FISH probes and total bacterial cells estimated with general DAPI staining, (C) Proportion of *Geobacteraceae* in the groundwater estimated by comparing direct counts of cells labeled with *Geobacteraceae*-specific FISH probes with general DAPI staining of all cells versus proportion of *Geobacteraceae* found in 16S rRNA gene clone libraries, and (D) Number of *Geobacteraceae rpsC* mRNA transcripts normalized against the number of *proC* mRNA transcripts plotted against the number of *Geobacteraceae* cells/mL estimated by FISH in the groundwater. Each point is an average of triplicate determinations, and error bars represent standard deviations. Growth rate constants (μ) were calculated from the following equation: y = 511.35x - 5.2809.

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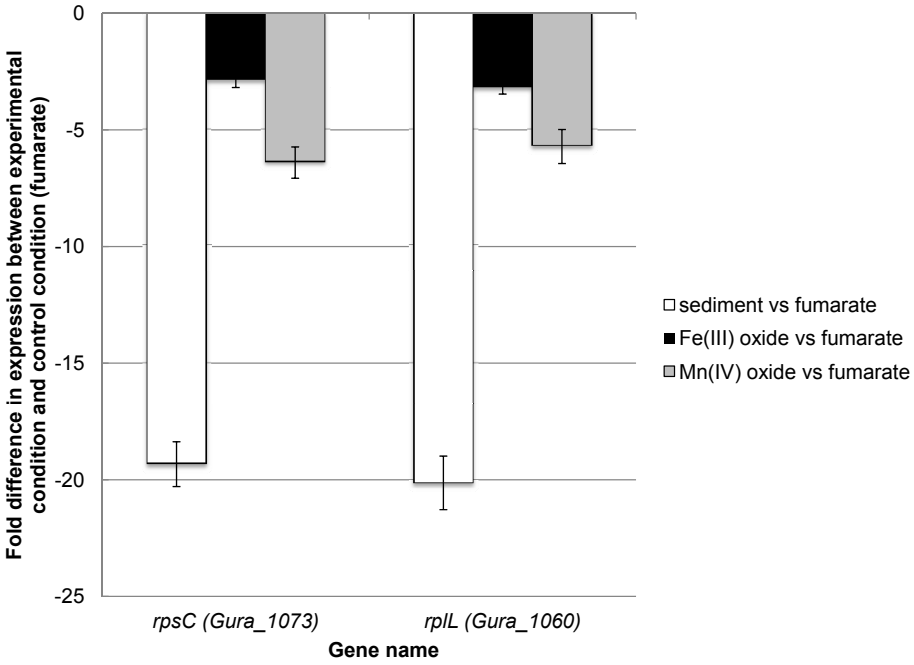


Figure 2

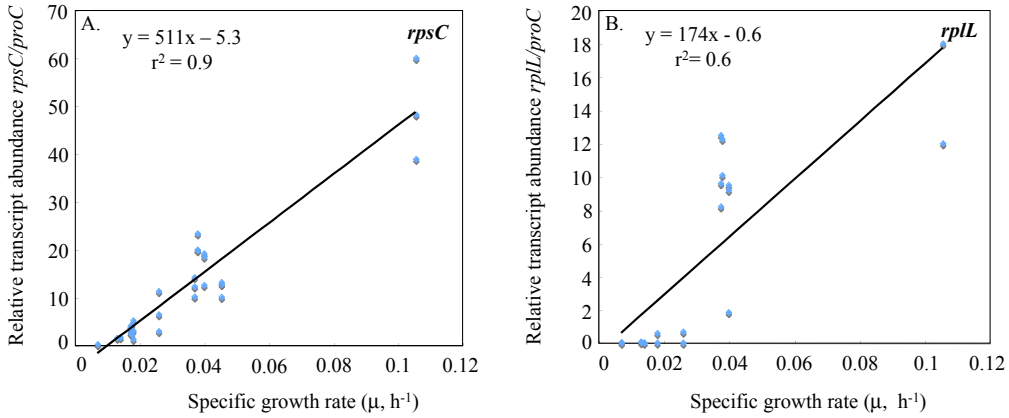
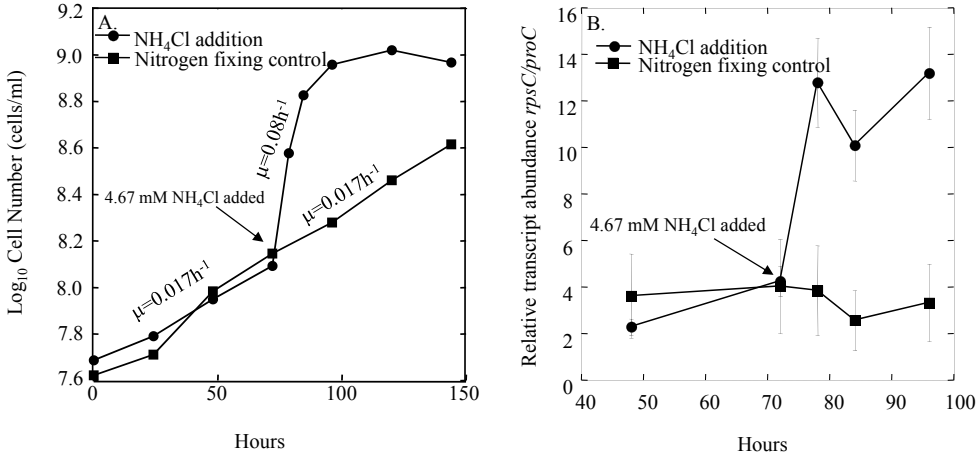


Figure 3



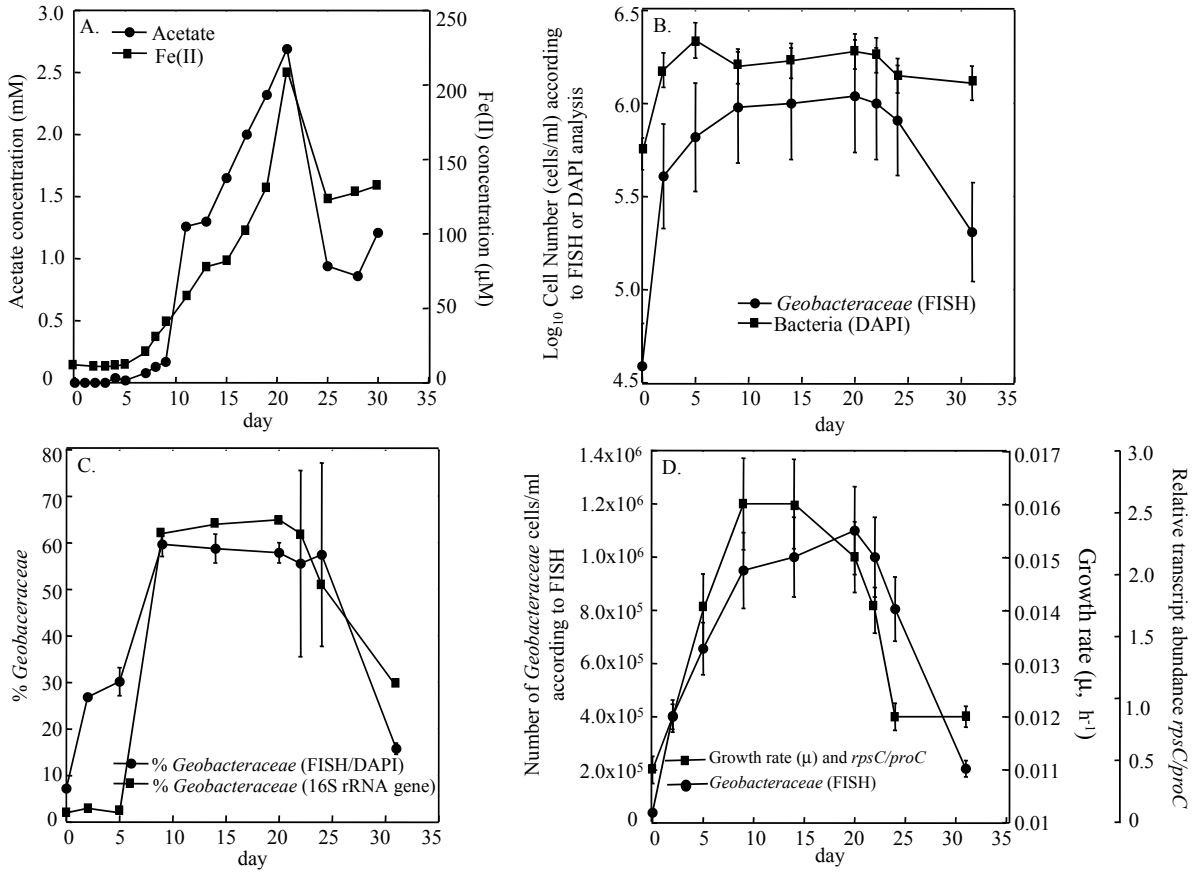


Table 1. Growth rate constants and generation times calculated for *G. uraniireducens* cells growing under a variety of conditions. Electron acceptor and/or temperature were varied.

Electron acceptor	Sediment	Sediment	Sediment	Fe(III)-oxide	Mn(IV)-oxide	Fumarate	Fumarate	Fumarate	Fumarate
Growth temperature	18°C	30°C	37°C	30°C	30°C	10°C	15°C	20°C	30°C
Growth rate constant (μ, h^{-1})	0.014	0.013	0.007	0.037	0.04	0.018	0.026	0.038	0.106
Doubling time (g, h)	49.50	50.10	89.28	18.50	17.40	38.52	26.72	18.28	6.56
rpsC/proC transcript ratios	1.71+/-0.08	1.58+/-0.02	0.26+/-0.11	12.21+/-1.63	16.8+/-2.97	3.22+/1.57	6.93+/-3.40	28.3+/-9.58	55.6+/-17.4
rplL/proC transcript ratios	0.17+/-0.06	0.04+/-0.02	0.30+/-0.21	8.18+/-6.56	10.9+/-6.77	0.02+/-0.01	41.9+/-40.6	4.34+/-2.75	15.3+/-2.72