Gene transcript analysis of assimilatory iron limitation in *Geobacteraceae* during groundwater bioremediation

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Summary

Limitations on the availability of Fe(III) as an electron acceptor are thought to play an important role in restricting the growth and activity of *Geobacter* species during bioremediation of contaminated subsurface environments, but the possibility that these organisms might also be limited in the subsurface by the availability of iron for assimilatory purposes was not previously considered because copious quantities of Fe(II) are produced as the result of Fe(III) reduction. Analysis of multiple *Geobacteraceae* genomes revealed the presence of a three-gene cluster consisting of homologues of two iron-dependent regulators, *fur* and *dtxR (ideR)*, separated by a homologue of *feoB*, which encodes an Fe(II) uptake protein. This cluster appears to be conserved among members of the *Geobacteraceae* and was detected in several environments. Expression of the *fur-feoB-ideR* cluster decreased as Fe(II) concentrations increased in chemostat cultures. The number of *Geobacteraceae* *feoB* transcripts in groundwater samples from a site undergoing *in situ* uranium bioremediation was relatively high until the concentration of dissolved Fe(II) increased near the end of the field experiment. These results suggest that, because much of the Fe(II) is sequestered in solid phases, *Geobacter* species, which have a high requirement for iron for iron-sulfur proteins, may be limited by the amount of iron available for assimilatory purposes. These results demonstrate the ability of transcript analysis to reveal previously unsuspected aspects of the *in situ* physiology of microorganisms in subsurface environments.

Introduction

Poor availability of Fe(III) is known to be a limiting factor in the anaerobic oxidation of organic contaminants coupled to the reduction of Fe(III) in contaminated subsurface sediments. In most subsurface sediments Fe(III) is primarily present as poorly soluble Fe(III) oxides which may be difficult for dissimilatory Fe(III)-reducing microorganisms to access. For example, the addition of chelators, which solubilize Fe(III) from the Fe(III) oxides (Lovley and Woodward, 1996) greatly accelerates the degradation of aromatic hydrocarbons in petroleum-contaminated aquifer sediments (Lovley et al., 1994; 1996a). Soluble electron shuttles, which facilitate electron transfer between the outer surface of dissimilatory Fe(III) reducers and Fe(III) oxides (Lovley et al., 1996b; 1998) promote anaerobic oxidation of aromatic hydrocarbons (Lovley et al., 1996b), MTBE (Finneran and Lovley, 2001) and chlorinated compounds (Bradley et al., 1998). The possibility that the availability of Fe(II) might, under some circumstances, also limit the activity of dissimilatory Fe(III) reducers in subsurface sediments does not appear to have been considered previously, probably because dissimilatory Fe(III) reducers produce substantial quantities of Fe(II) as the result of Fe(III) reduction. However, *Geobacter* species, which molecular studies have shown are the predominant Fe(III)-reducing microorganisms in a diversity of subsurface environments in which Fe(III) reduction is an important process (Anderson et al., 1998; 2003; Roling et al., 2000; 2001; Snoeyenbos-West et al., 2000; Holmes et al., 2002; 2005; 2007; Cummings et al., 2003; Petrie et al., 2003; Istok et al., 2004; North et al., 2004; Chang et al., 2005; Coates et al., 2005; Lin et al., 2005; Mouser et al., 2005; Vrionis et al., 2005; Kovacik et al., 2006), may have exceptionally high assimilatory requirements for iron because of the unusually high abundance of c-type cytochromes in these organisms (Lovley et al., 1993; Methe et al., 2003; Estve-Nunez et al., 2007). Many of these cytochromes have a role in Fe(III) reduction (Leang et al., 2003; Lloyd et al., 2003; Butler et al., 2004; Afkar et al., 2005; Kim et al., 2005; 2006; Mehta et al., 2005; Shi et al., 2007) and thus are considered to be essential for growth and activity in the
The mechanisms of Fe(II) uptake in *Geobacter* species have not been previously investigated in detail, but all of the sequenced *Geobacteraceae* genomes contain a gene for FeoB, which is an Fe(II)-specific membrane-spanning transport protein (Velayudhan *et al.*, 2000).

The other possibility is that during Fe(III) oxide reduction in the subsurface deleteriously high levels of Fe(II) may accumulate. In a number of microorganisms the global, Fe(II)-dependent regulator, Fur, plays an important role in controlling Fe(II) influx into the cell (Kammler *et al.*, 1993; Escolar *et al.*, 1999; Patzer and Hantke, 2001; van Vliet *et al.*, 2002; Wan *et al.*, 2004). In the presence of high concentrations of Fe(II) the Fur protein represses transcription by binding to a specific consensus sequence, the Fur box (Bagg and Neilands, 1987; Coy and Neilands, 1991; Escolar *et al.*, 1999). Fur belongs to a family of related transcriptional repressors, which share similar DNA binding domains and one metal binding site. Other proteins from this family include Per, Zur and Irr, which repress genes involved in oxidative stress, zinc uptake and haem utilization respectively (Baas *et al.*, 1998; Hamza *et al.*, 2000; Patzer and Hantke, 2000; Fuangthong and Helmann, 2003). All of the sequenced genomes of cultured *Geobacteaeae* examined contain homologues of Fur. To date, Fur has only been studied in one dissimilatory Fe(III)-reducing bacterium, *Shewanella oneidensis*, where it appears to be involved in the control of iron uptake, oxidative stress responses, central metabolism and energy production in this organism (Thompson *et al.*, 2002; Wan *et al.*, 2004).

Another group of metal-dependent repressors that are present in *Geobacteraceae* includes DtxR, the dithionite repressor, which is a functional homologue of Fur that is typically found in Gram-positive, G+C-rich organisms (Patzer and Hantke, 2001). DtxR and its homologues, many of which are referred to as IdeR, are iron-dependent repressors that regulate the expression of iron uptake proteins during growth in the presence of excess Fe(II) (Schmitt *et al.*, 1995; Boland and Meijer, 2000; Yellaboina *et al.*, 2004). These proteins are part of a family of related metal-dependent repressors, which includes Fe(II)-, Mn(II)- and Zn(II)-dependent repressors (Que and Helmann, 2000; Hazlett *et al.*, 2003).

Crystallographic studies have revealed that the two repressor groups, Fur and DtxR, have similar tertiary structures (Gonzalez de Peredo *et al.*, 2001). However, there is little conservation between fur and dtxR at the nucleotide level, aside from the presence of a winged helix–turn–helix DNA binding region and an iron binding site.

Recent studies have suggested that it is possible to evaluate the physiological status of *Geobacter* species in subsurface sediments by quantifying levels of transcripts for key metabolic or respiratory genes in groundwater (Holmes *et al.*, 2004; 2005; D.E. Holmes, unpublished). For example, the level of transcripts for *nifD*, a gene involved in nitrogen fixation, can be used to determine if *Geobacter* species are limited for fixed nitrogen (Holmes *et al.*, 2004). Transcript levels for genes for citrate synthase or malate dehydrogenase vary with acetate availability and can be related to rates of metabolic activity (Holmes *et al.*, 2005; D.E. Holmes, unpublished). In order to possibly expand the repertoire of key genes whose expression can be used to diagnose the *in situ* metabolic state of *Geobacter* species in the subsurface and to learn more about the mechanisms by which *Geobacter* species regulate iron uptake and efflux in the environment, the phylogeny and expression patterns of the genes for Fur, FeoB and IdeR were examined, both in pure culture and in subsurface communities.

### Results and discussion

**Phylogenetic analysis of the fur-feoB-ideR gene cluster**

Genes for Fur, FeoB and IdeR were found in all of the *Geobacteraceae* genomes that have been sequenced. These include: *Geobacter sulfurreducens* (Methe *et al.*, 2003), *G. metallireducens*, G. uraniumreducens, *G. bemidjiensis*, *G. lovleyi*, *Geobacter* sp. FRC-32, *Pelobacter propionicus* and *Pelobacter carbinolicus* (sequences available at http://www.jgi.doe.gov). In each case, these genes form a three-gene cluster (Fig. 1). This cluster may be a defining feature of the *Geobacteraceae*. 

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family. The only other bacterium that contains the cluster is *Anaeromyxobacter dehalogenans*. This cluster can be divided into five regions: the *fur* coding region, the intergenic *fur*-*feoB* region, the *feoB* coding region, the intergenic *feoB*-ideR region and the ideR coding region. Degenerate primers designed from the pure culture sequences of this cluster amplified all five regions from genomic DNA extracted from three subsurface environments in which it has previously been shown that Fe(III) reduction is an important process and that *Geobacter* species are the predominant Fe(III)-reducing microorganisms. These included: (i) a uranium contaminated aquifer undergoing bioremediation, (ii) a highway run-off recharge pool exposed to calcium/magnesium acetate and (iii) the Fe(III) reduction zone of a petroleum contaminated aquifer. The gene cluster was also amplified from another microbial population dominated by *Geobacter* species, the community associated with the surface of an electrode harvesting electricity from the sediments of a freshwater pond.

Amino acid sequence comparisons demonstrated that *Geobacteraceae* Fur proteins form a unique phylogenetic clade with *A. dehalogenans* Fur, and are clearly distinct from Fur sequences from other bacterial species (Fig. 2). Analysis of Fur genes amplified from the four different *Geobacteraceae*-dominated environments indicated that Fur sequences from the acetate-impacted aquifer and the surface of the current-harvesting electrode were most similar to *G. sulfurreducens* (93% and 99% similarity respectively); whereas the Fur amino acid sequences detected in the uranium-contaminated aquifer were most similar to *P. propionicus* (90% similarity) and *G. bremensis* (96% similarity); and the Fur amino acid sequence from the petroleum-contaminated sediment is closest to *Geobacter* sp. FRC-32 (98% similarity). The Fur protein appears to be highly conserved across the family, with similarity ranging from 73% to 99% among the *Geobacteraceae* sequences.

Phylogenetic comparisons of FeoB amino acid sequences revealed that *Geobacteraceae* FeoB sequences recovered from all four environments as well as FeoB coding sequences deriving from the *fur-feoB-ideR* clusters of cultured species of *Geobacteraceae* and *A. dehalogenans* formed a distinct clade which also included an *Archeoglobus fulgidus* sequence (Fig. 3, 1). The FeoB sequences amplified from all four environments were most similar to that of *G. bremensis* (88–90% similar). There was a high degree of conservation between the *Geobacteraceae* FeoB sequences in this clade, with similarity among family members ranging from 68% to 99%. A second clade consisted of FeoB homologues from a variety of pure cultures that were not encoded in *fur-feoB-ideR* clusters.

The *Geobacteraceae* IdeR protein sequences also clustered with those of *A. dehalogenans* and *A. fulgidus* (Fig. 4). The sequence amplified from the acetate-impacted groundwater was most similar to *G. sulfurreducens* and *G. uraniumreducens* (93%). One of the sequences amplified from the uranium-contaminated site is most similar to *G. bemidjiensis* (98%) and the other was most closely related to *P. carbinolicus* (85%). All IdeR sequences collected from the surface of the current producing electrode are most similar to *G. lovleyi* (93–94%). The IdeR sequence amplified from the petroleum-contaminated sediment is most similar to *Geobacter* sp. FRC-32 (98%). The IdeR protein is also highly conserved across the *Geobacteraceae*, with similarity values ranging from 68 to 98% when amino acid sequences are compared among the family.

The Fur protein binds with high affinity to a 19 bp inverted repeat known as the Fur box, and Fur box consensus sequences have been defined for a variety of organisms including *Escherichia coli*, *Bacillus subtilis* and the 8-Proteobacteria (Bagn and Neilands, 1987; Escolar et al., 1999; Baichoo and Helmann, 2002; Rodionov et al., 2004). A highly conserved Fur box, similar to that defined for the 8-Proteobacteria (Rodionov et al., 2004), was identified within the *fur-feoB* intergenic region of all cultured *Geobacteraceae* and within the *fur-feoB* intergenic sequences amplified from *Geobacteraceae* present in the four environments analysed in this study (representative sequences from the Rifle field site shown in Fig. 5).

**Expression of fur, feoB and ideR genes in G. sulfurreducens in response to changes in Fe(II) concentrations**

In order to evaluate how the availability of Fe(II) influences the expression of *fur*, *feoB* and *ideR*, *Geobacter sulfurreducens* was grown in chemostats with an Fe(II) concentration of 0.1 μM or 100 μM. Previous studies had determined that the growth was Fe(II)-limited at 0.1 μM whereas 100 μM was optimal for growth in the chemostats (A. Estève-Nunez, unpublished). The number of *fur*, *feoB* and *ideR* mRNA transcripts was determined with quantitative RT-PCR, using *G. sulfurreducens*-specific primers. Transcript levels for all three genes decreased as Fe(II) concentrations went up; whereas expression of *rpoD*, a housekeeping gene that has been shown to be constitutively expressed in *G. sulfurreducens* (Holmes et al., 2004) remained relatively constant (Fig. 6). These results suggest that expression of *fur*, *feoB* and *ideR* is high when the availability of iron limits growth and that expression of these genes is repressed when *G. sulfurreducens* is exposed to high concentrations of Fe(II).

These findings coupled with genetic analysis of *G. sulfurreducens* *fur* and *ideR* (R. O’Neil, unpublished) as well as high similarity to genes that have been intensively investigated in other organisms suggest that the *fur*,

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Fig. 2. Phylogenetic analysis of Geobacteraceae Fur amino acid sequences. Phylogenetic trees were inferred by Neighbor joining using the BioNJ algorithm (Saitou and Nei, 1987; Gascuel, 1997). Bootstrap values were determined for 1000 replicates. Accession numbers are indicated in parentheses after the species name. Alignments can be provided upon request. A. fulgidus was used as the outgroup for construction of the tree.

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Fig. 3. Phylogenetic analysis of Geobacteraceae FeoB amino acid sequences. Phylogenetic trees were inferred by neighbour joining using the BioNJ algorithm (Saitou and Nei, 1987; Gascuel, 1997). Bootstrap values were determined for 1000 replicates. Accession numbers are indicated in parenthesis after the species name. Alignments can be provided upon request. B. bacteriovorus was used as the outgroup for construction of the tree.

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Fig. 4. Phylogenetic analysis of Geobacteraceae IdeR amino acid sequences. Phylogenetic trees were inferred by neighbour joining using the BioNJ algorithm (Saitou and Nei, 1987; Gascuel, 1997). Bootstrap values were determined for 1000 replicates. Accession numbers are indicated in parentheses after the species name. Alignments can be provided upon request. T. acidophilum was used as the outgroup for construction of the tree.
feoB and ideR genes of the Geobacteraceae are likely to have physiological functions related to Fe(II)-dependent transcriptional repression and Fe(II) transport.

**feoB gene expression patterns during uranium bioremediation**

In order to determine whether Geobacteraceae were limited for Fe(II) during in situ uranium bioremediation, expression of the gene for the Fe(II) uptake protein, FeoB, was monitored in groundwater samples collected during the 2005 field experiment at the Rifle, CO site (Fig. 7). As previously reported (Holmes et al., 2007), the introduction of acetate into the subsurface stimulated the growth of Geobacter species, which were the predominant organisms in the groundwater, accounting for as much as 75% of the groundwater 16S rRNA sequences. The same 16S rRNA sequences predominated in the sediments at this site (Holmes et al., 2007).

The increased availability of acetate in the groundwater was initially associated with a slight increase in the concentration of dissolved Fe(II) (Fig. 7). However, dissolved Fe(II) remained fairly stable at c. 40–60 μM during most the field experiment until near the end when it more than doubled (Fig. 7). Geobacteraceae feoB transcript levels were determined with primers that targeted Geobacteraceae feoB sequences that had been previously amplified from the site and were normalized against transcript levels of the constitutively expressed Geobacteraceae housekeeping gene, proC (Holmes et al., 2005). Transcript levels for feoB increased during the initial period when growth of Geobacter species was reported to be highest (Holmes et al., 2007) and remained relatively high during the majority of the field experiment. However, when Fe(II) concentrations increased at the end of the experiment, there was a significant decline in transcripts for the gene, suggesting that transcription had been repressed in response to higher Fe(II) concentrations. These results suggest that the Geobacter species that predominate during in situ uranium bioremediation were limited by Fe(II) availability until near the end of the field experiment. Subsequent whole-genome microarray studies have revealed that expression of feoB was also upregulated in pure cultures of Geobacter species grown in sterilized sediments from the Rifle site (D.E. Holmes, unpublished).

**Implications**

The presence of the fur-feoB-ideR gene cluster in all analysed members of the Geobacteraceae and in four
unique *Geobacteraceae*-dominated environments suggests that expression of these iron-dependent genes is important for survival of *Geobacteraceae* in their natural environments, where Fe(II) levels may fluctuate substantially. Iron limitation is a common phenomenon in aerobic soils of circumneutral pH because iron is almost exclusively in the Fe(III) state and Fe(III) is highly insoluble under those conditions. In contrast, in anoxic environments it might be considered that the production of Fe(II), which is much more soluble, as the result of dissimilatory metal reduction, would provide sufficient iron for assimilatory purposes and that a more important concern would be limiting iron uptake into the cell. However, much of the Fe(II) produced during microbial Fe(III) reduction is recovered in solid phases rather than as soluble Fe(II) (Lovley and Phillips, 1988a; Lovley, 1991; 1993). This is because the Fe(II) forms minerals such as magnetite (Bell et al., 1987; Lovley et al., 1987; Lovley, 2000), sidermite (Bell et al., 1987) and vivianite (Fredrickson et al., 2001). When the structural Fe(III) in clays is reduced, much of the Fe(II) remains in the clay lattice (Kostka et al., 2002; Shelobolina et al., 2007).

*Geobacter* species have an inordinately high requirement for iron for assimilatory purposes because of their high iron-sulfur protein content, primarily c-type cytochromes (Senko and Stolz, 2001; Esteve-Nunez et al., 2007). As iron has to be soluble in order to be taken into the cell for assimilation into proteins there is apparently a competition between geochemical equilibria and *Geobacter* species for the copious quantities of Fe(II) that *Geobacter* species produce during Fe(III) oxide reduction.

These results demonstrate the potential for analysis of key gene transcript levels to diagnose the metabolic status of microorganisms during groundwater bioremediation. The possibility that the availability of iron for assimilatory purposes might limit the growth and activity of *Geobacter* species in subsurface environments was not previously considered. Recent studies have demonstrated that transcript analysis not only can provide information about nutrient status (Holmes et al., 2004; 2005; D.E. Holmes, unpublished), but also environmental stress (P.J. Mouser, unpublished; D.E. Holmes, unpublished) and rates of metabolism (Holmes et al., 2005; D.E. Holmes, unpublished). This approach offers several advantages over environmental proteomic approaches for evaluating the metabolic state of microorganisms during subsurface bioremediation; most notably the ability to quantify transcripts from the small volume of groundwater sample that is typically available. Furthermore, transcripts typically have a much shorter half-life than proteins, thus providing a better diagnosis of current metabolic state.

![Diagram](image-url)
A limitation at the present time is that interpretation of gene transcript levels is qualitative. It is not possible to determine the absolute rates of metabolism or degree of nutrient limitation from one sample in one environment. However, comparisons, over time, or with different amendments within an environment can provide important insights into the relative changes in the metabolic state of the organisms of interest. Application of transcript analysis to other types of bioremediation seems feasible (Lovley, 2003) and, based on the results with Geobacter-driven bioremediation, seems warranted.

Experimental procedures

Bacterial strains and culturing conditions

Geobacter sulfurreducens (ATCC 51573) strain DL1 (Caccavo et al., 1994; Coppi et al., 2001) was cultivated in chemostats as previously described (Esteve-Nunez et al., 2005) in a modified freshwater medium (Lovley and Phillips 1989) containing 27.5 mM fumarate as the electron acceptor, a limiting concentration of acetate (5 mM) as the electron donor and carbon source, and a reduced concentration of monobasic sodium phosphate (0.43 mM) to prevent precipitation during Fe(II) amendment (Fuanthong and Helmann, 2003).

In order to measure gene expression in the presence of 100 μM Fe(II) (added as ferrous ammonium sulfate), chemostats were maintained at a dilution rate of 0.05 h⁻¹ and were harvested for RNA extraction when they reached steady state. To measure expression during iron limitation, cells were grown in sealed chemostat vessels in medium containing the standard mineral mix [3 μM Fe(III)] and then connected to a reservoir containing iron-free medium. Cells were harvested for RNA extraction when the optical density began to drop and the iron concentration in the medium was determined to be 0.1 μM by inductively coupled plasma-atomic emission spectroscopy.

Field sites

Four sites in which Geobacteraceae are the predominant microorganisms were investigated. One was the previously described (Anderson et al., 2003; Holmes et al., 2004; 2007; Vronis et al., 2005) study site in Riffle, CO in which acetate was added to the subsurface to stimulate dissimilatory metal reduction and in situ uranium bioremediation. The samples analysed in this study were collected from well M21 during the previously described 2005 field study (Holmes et al., 2007). A sample was also collected from a petroleum-contaminated aquifer in Berndt, MN (Lovley and Phillips, 1989; Anderson et al., 1998; Rooney-Varga et al., 1999; Holmes et al., 2004; 2005; Lovley, 2005; Nevin et al., 2005). In this sample, the Geobacteraceae accounted for 41% of the 16S rRNA transcript library and were believed to play an important role in the oxidation of aromatic hydrocarbon contaminants coupled to Fe(III) reduction (Holmes et al., 2007). In addition, samples were collected from a calcium magnesium acetate-impacted aquifer in Plymouth, MA, which serves as an analogue for the long-term acetate additions to groundwater that are proposed for in situ uranium bioremediation. In samples from this site, Geobacteraceae accounted for as much as 78% of the 16S RNA gene sequences recovered in clone libraries (Holmes et al., 2005; 2007). Samples were also collected from the surface of a freshwater energy-harvesting electrode in Nantucket, MA (D.E. Holmes, unpublished). Geobacteraceae were the predominant members of this community, accounting for 56% of the 16S RNA gene sequences recovered in clone libraries (Holmes et al., 2005).

Analytical techniques

Fe(III) reduction was monitored by measuring the formation of Fe(II) over time with a ferrozine assay in a split-beam dual-detector spectrophotometer (Spectronic Genosys2; Thermo Electron, Mountain View, CA) at an absorbance of 562 nm after a 1 h extraction with 0.5 N HCl as previously described (Lovley and Phillips, 1987; 1988b). Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was performed as previously described (Kang et al., 2004). Groundwater acetate concentrations were determined with high pressure liquid chromatography as previously described (Anderson et al., 2003; Vronis et al., 2005).

Extraction of mRNA from chemostat cultures

As previously described (Holmes et al., 2005), triplicate chemostat cultures (200 ml) at steady state were transferred to prechilled 50 ml conical tubes and centrifuged at 4000 r.p.m. for 15 min at 4°C. The supernatant was discarded and pellets were flash frozen in an ethanol/dry ice bath and stored at −80°C. The RNeasy Mini Kit (Qiagen, Valencia, CA) was used to extract mRNA from the G. sulfurreducens cell pellets according to the manufacturer's instructions.

Extraction of nucleic acids from environmental samples

Groundwater samples were collected from wells M21 every other day during the previously described in situ uranium bioremediation field experiment (Holmes et al., 2004; 2005). Groundwater was collected for DNA extraction by filtering 1.5 l of groundwater through 0.2 μm pore size Sterivex-GP filters (Millipore, Corporation; Bedford, MA). Prior to DNA extraction, all samples 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°C. Genomic DNA was extracted from the cartridge filters and sediment with the FastDNA SPIN kit (Bio101, Carlsbad, CA) according to the manufacturer's instructions.

Groundwater samples for mRNA analyses were collected as previously described (Holmes et al., 2004; 2005; 2007). Groundwater was concentrated by impact filtration on 293 mm diameter Supor membrane disc filters (Pall Life Sciences). All samples 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°C. RNA was extracted from the frozen filters as previously described (Holmes et al., 2004; 2005; 2007).

DNA was extracted from groundwater from the calcium magnesium impacted aquifer, sediment collected from the
Table 1. Primers used to amplify fur, feoB, ideR and housekeeping genes from members of the Geobacteraceae family.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>geofur160fwd</td>
<td>ATTTGAATACAGCCACGTGTTA</td>
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<tr>
<td>geofof341rev</td>
<td>GCATMTTCTTGCCTTCGCT</td>
</tr>
<tr>
<td>geofof272fwd</td>
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<tr>
<td>geofof42rev</td>
<td>GTWCCGAGATARTTGGAC</td>
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<tr>
<td>geofofB142fwd</td>
<td>GTETCVAATATTCCGGGDS</td>
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<tr>
<td>geofofB1619rev</td>
<td>TATGTTTTGCGRSCCASARRCA</td>
</tr>
<tr>
<td>geofof1961fwd</td>
<td>CCCTGGTSGCCAGGTCTTCT</td>
</tr>
<tr>
<td>geofof339rev</td>
<td>CAGGTGTSGGRTTGGTGAAC</td>
</tr>
<tr>
<td>geoidR226fwd</td>
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<tr>
<td>geoidR511rev</td>
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</tr>
<tr>
<td>sulfideR713fwd</td>
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<td>M21proC156fwd</td>
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</tr>
<tr>
<td>M21proC287rev</td>
<td>ATCGCGCAGCTTCTTCGAGG</td>
</tr>
</tbody>
</table>

Fe(III) reducing zone of the petroleum-contaminated aquifer at Bemidji, MN and the surface of a current harvesting electrode at Nantucket, MA were previously described (Holmes et al., 2005).

Testing and design of primers

Degenerate primers targeting the Geobacteraceae fur-feoB-ideR gene cluster were designed from nucleotide sequences extracted from G. sulfurireducens, G. metallireducens, D. acetoxidans, P. carbinolicus and P. propionicus genomes as previously described (Holmes et al., 2004; 2005; 2007). Preliminary sequence data from G. metallireducens, D. acetoxidans, P. carbinolicus, G. urbanireducens, G. bemidjensis, G. lovleyi, Geobacter sp. FRC-32 and P. propionicus was obtained from the DOE Joint Genome Institute (JGI) website http://www.jgi.doe.gov. The following primer sets were used to amplify the five regions of the fur-feoB-ideR gene cluster: within fur, geofof160fwd and geofof341rev; between fur and feoB, geofof272fwd and geofof42rev; within feoB geofoB, 142fwd and geofoB1639rev; between FeoB and ideR, geofof1861fwd and geofof339rev; and within ideR, geoidR226fwd and geoidR511rev (Table 1).

Primers for quantitative RT-PCR analysis of transcription in G. sulfurireducens were as follows (Table 1). A 101 bp region of ideR was amplified with primers, sulfideR713fwd and sulfideR814rev; a 121 bp region of feoB was amplified with primers, sulfideB11858fwd and sulfideB11979rev; and a 137 bp region of fur was amplified with primers, sulfidefur257fwd and sulfidefur394rev. A 78 bp region from the constitutively expressed housekeeping gene, rpoD, was amplified with previously described primers, rpod1132fwd and rpod1210rev (Holmes et al., 2005).

The dominant feoB sequences amplified from the uranium-contaminated groundwater with degenerate primers were used to design Geobacteraceae-specific primers suitable for quantitative RT-PCR analysis. The following primer pairs amplified a 66 bp region from feoB: M21feoB86fwd and M21feoB152rev (Table 1). Expression of the constitutively expressed housekeeping gene, procC, was also monitored with the following primer set as previously described (Holmes et al., 2007): M21proC156fwd and M21proC287rev.

The specificity of all primer pairs described above was tested by constructing clone libraries of sequences amplified from the uranium-contaminated groundwater. In order to ensure that only Geobacteraceae sequences were amplified, approximately 100 clones from each sequence were sequenced and compared with the GenBank nucleotide and protein databases using the blast and blastx algorithms (Altschul et al., 1990).

Quantification of gene expression by quantitative RT-PCR

A DuraScript enhanced avian RT single strand synthesis kit (Sigma) was used to generate cDNA from fur, ideR, feoB-1, rpoD and procC transcripts as previously described (Holmes et al., 2004). Once the appropriate cDNA fragments were generated by RT-PCR, quantitative RT-PCR amplification and detection were performed with the 7500 Real Time PCR System (Applied Biosystems). Optimal quantitative RT-PCR conditions were determined using the manufacturer’s guidelines. Each PCR mixture consisted of a total volume of 25 µl and contained 1.5 µl of the appropriate primers (stock concentrations, 15 µM), and 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems) as previously described (Holmes et al., 2007).

The following thermocycler parameters were used for G. sulfurireducens quantitative RT-PCR studies: UNG activation step at 50°C for 2 min, followed by a denaturation step at 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 1 min. Quantitative RT-PCR thermocycler parameters for detection of feoB transcripts in the groundwater consisted of 50°C (2 min), 95°C (10 min) and 50 cycles of 95°C (15 s) and 60°C (1 min). Transcripts from procC in the groundwater were quantified using the following parameters: 50°C (2 min), 95°C (10 min) and 50 cycles of 60°C for 1 min. Standard curves were constructed as previously described (Holmes et al., 2004) and covered a range of c. eight orders of magnitude. To verify amplification and correct amplicon sizes, aliquots from real-time PCR were examined in an ethidium bromide-stained 2% agarose gel.

Phylogenetic analysis

Protein sequences were aligned in ClustalX (Thompson et al., 2002) and hypervariable regions were masked. Alignments were imported into PAUP 4.0b10 (Sinauer Associates, Sunderland, MA), where phylogenetic trees were inferred by the neighbour-joining method using the BioNJ algorithm (Saitou and Nei, 1987; Gascuel, 1997). Bootstrap analysis was performed for 1000 replicates. All reported per cent similarity values were determined for global pairwise alignments using the algorithm of Needleman and Wunsch (Needleman and Wunsch, 1970).
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References


