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ORIGINAL ARTICLE

Functional environmental proteomics: elucidating the role of a *c*-type cytochrome abundant during uranium bioremediation

Jiae Yun[1], Nikhil S Malvankar[1,2], Toshiyuki Ueki[1] and Derek R Lovley[1]
[1]*Department of Microbiology, University of Massachusetts, Amherst, MA, USA and* [2]*Department of Physics, University of Massachusetts, Amherst, MA, USA*

Studies with pure cultures of dissimilatory metal-reducing microorganisms have demonstrated that outer-surface *c*-type cytochromes are important electron transfer agents for the reduction of metals, but previous environmental proteomic studies have typically not recovered cytochrome sequences from subsurface environments in which metal reduction is important. Gel-separation, heme-staining and mass spectrometry of proteins in groundwater from *in situ* uranium bioremediation experiments identified a putative *c*-type cytochrome, designated *Geobacter* subsurface *c*-type cytochrome A (GscA), encoded within the genome of strain M18, a *Geobacter* isolate previously recovered from the site. Homologs of GscA were identified in the genomes of other *Geobacter* isolates in the phylogenetic cluster known as subsurface clade 1, which predominates in a diversity of Fe(III)-reducing subsurface environments. Most of the *gscA* sequences recovered from groundwater genomic DNA clustered in a tight phylogenetic group closely related to strain M18. GscA was most abundant in groundwater samples in which *Geobacter* sp. predominated. Expression of *gscA* in a strain of *Geobacter sulfurreducens* that lacked the gene for the *c*-type cytochrome OmcS, thought to facilitate electron transfer from conductive pili to Fe(III) oxide, restored the capacity for Fe(III) oxide reduction. Atomic force microscopy provided evidence that GscA was associated with the pili. These results demonstrate that a *c*-type cytochrome with an apparent function similar to that of OmcS is abundant when *Geobacter* sp. are abundant in the subsurface, providing insight into the mechanisms for the growth of subsurface *Geobacter* sp. on Fe(III) oxide and suggesting an approach for functional analysis of other *Geobacter* proteins found in the subsurface.
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Introduction

Geobacter sp. are abundant in various subsurface environments in which Fe(III) reduction is an important biogeochemical process and they can have an important role in the anaerobic bioremediation of groundwater contaminated with metal or organic contaminants (Lovley *et al.*, 2011). The ability of *Geobacter* sp. to reduce soluble U(VI) to poorly soluble U(IV) (Lovley *et al.*, 1991) has been exploited as an *in situ* bioremediation strategy to prevent the migration of uranium in contaminated subsurface environments (Lovley *et al.*, 2011; Williams *et al.*, 2013).

Geobacter sulfurreducens has served as the primary model species for elucidating the physiology of *Geobacter* sp. (Lovley *et al.*, 2011; Mahadevan *et al.*, 2011). Functional genomic analysis of *G. sulfurreducens* has led to molecular tools for diagnosing the physiological status of subsurface *Geobacter* sp. during *in situ* uranium bioremediation based on gene transcript and proteomic approaches (Lovley *et al.*, 2011; Mahadevan *et al.*, 2011). These approaches have been effective in evaluating rates of metabolism and growth, nutrient limitations and stress responses because the genes for these functions are highly conserved among *Geobacter* sp. and gene function could first be elucidated in the genetically tractable *G. sulfurreducens* (Lovley *et al.*, 2011). In contrast, the lack of conservation of outer-surface *c*-type cytochromes within the Geobacteraceae (Butler *et al.*, 2009, 2010) has made it difficult to develop similar molecular strategies to meaningfully interpret the transcriptomic or proteomic data related to the expression of outer-surface *c*-type cytochromes (Chin *et al.*, 2004), which have an essential role in extracellular electron transfer (Lovley, 2011; Lovley *et al.*, 2011).

For example, the multi-heme *c*-type cytochrome, OmcS (Qian *et al.*, 2011), is essential for Fe(III) oxide reduction in *G. sulfurreducens* (Mehta *et al.*, 2005). OmcS is localized on the pili of *G. sulfurreducens* (Leang *et al.*, 2010) that are electrically conductive

Correspondence: T Ueki, Department of Microbiology, University of Massachusetts Amherst, Morrill Science Center IV North, 639 North Pleasant Street, Amherst, MA 01003, USA.
E-mail: tueki@microbio.umass.edu
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(Reguera *et al*., 2005; Malvankar *et al*., 2011, 2014; Malvankar and Lovley, 2014). Multiple lines of evidence have demonstrated that OmcS is not responsible for long-range electron transport along the pili, (Leang *et al*., 2010; Malvankar *et al*., 2011, 2012; Vargas *et al*., 2013; Liu *et al*., 2014). Therefore, the proposed role for OmcS is to facilitate electron transfer from the pili to Fe(III) oxide (Lovley, 2011, 2012).

Further evidence that a mediator for electron transfer from pili to Fe(III) oxide is needed was the finding that magnetite, a product of microbial Fe(III) oxide reduction (Lovley *et al*., 1987; Lovley, 1991), binds to the pili of *G. sulfurreducens* accelerating Fe(III) oxide reduction and restoring the capacity for Fe(III) oxide reduction in the OmcS-deficient mutant (Liu *et al*., 2015). These results demonstrate that other molecules, even non-biological minerals with the capacity for electron transfer, can substitute for OmcS to facilitate electron transfer from pili to Fe(III) oxides.

None of the *Geobacter* sp. in Subsurface Clade I, the phylogenetic group that typically predominates in subsurface environments in which Fe(III) is being actively reduced (Holmes *et al*., 2007), encode an OmcS homolog (Butler *et al*., 2009, 2010). The lack of methods for genetic manipulation of *Geobacter* sp. in Subsurface Clade 1 has hindered the efforts to determine whether there are one or more cytochromes that have a role similar to OmcS in the *Geobacter* sp. that are most abundant during *in situ* uranium bioremediation.

Geobacter sp. contain a large number of *c*-type cytochromes (Lovley *et al*., 2011), but previous proteomic studies of the microbial community during *in situ* uranium bioremediation sites, identified few, if any, *c*-type cytochromes (Wilkins *et al*., 2009, 2013; Callister *et al*., 2010). One reason for this may be the poor conservation of *c*-type cytochromes in *Geobacter* sp. (Lovley *et al*., 2011) and thus a lack of the relevant *c*-type cytochrome gene sequences in databases. Furthermore, it can be difficult to identify *c*-type heme-containing peptides with mass spectrometry, leading to an underrepresentation of heme-containing peptides even in studies of pure cultures (Romine *et al*., 2004; Yang *et al*., 2005; Merkley *et al*., 2012).

The study summarized here was designed to evaluate which cytochromes might be important in extracellular electron transfer during *Geobacter*-catalyzed *in situ* uranium bioremediation and the function of those cytochromes. This was accomplished with environmental proteomic analysis including a biochemical method for the detection of *c*-type heme-containing proteins and the study of cytochrome function via heterologous expression in *G. sulfurreducens.*

Materials and methods

Bacterial strains and growth conditions

G. bemidjiensis BEM (Nevin *et al*., 2005), *G. sulfurreducens* DL1 (Caccavo *et al*., 1994; Coppi *et al*., 2001) and its mutants were routinely grown anaerobically at 30 °C in NBAF media (Coppi *et al*., 2001) unless indicated otherwise. Acetate (10 mM) was added when Fe(III) oxide (100 mmol l^{-1}) was the electron acceptor (Lovley and Phillips, 1987). Kanamycin (200 μg ml^{-1}) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mg ml^{-1}) were added when necessary.

Sampling site description

During the summer of 2007 and 2008, *in situ* uranium bioremediation experiments were conducted as part of the Rifle Integrated Field Research challenge, at the uranium-contaminated aquifer in Rifle, CO, USA. Acetate was added to the groundwater to stimulate the growth of dissimilatory metal-reducing microorganisms (Williams *et al*., 2011). This site, methods for introducing acetate into the subsurface, and groundwater sample collection methods have previously been described in detail (Mouser *et al*., 2009; Wilkins *et al*., 2009; Williams *et al*., 2011). Groundwater samples from wells D05 and D07 of the 2007 field experiment and wells D04 and D07 in the 2008 field experiment were investigated because previous studies suggested that *Geobacter* sp. were abundant at the time points studied (Mouser *et al*., 2009; Wilkins *et al*., 2009; Williams *et al*., 2011).

Identification of c*-type heme-containing proteins*

The proteins from filters that collected groundwater bacteria were extracted as previously described (Yun *et al*., 2011) with the modification that the ultrafiltration at the last step was replaced by adding 200 μl of PPS (protein precipitation solution, MP Biomedicals, Santa Ana, CA, USA) to 1 ml of protein extract. Protein concentration was determined by measuring absorbance at 280 nm prior to the protein precipitation step using dilutions of *G. bemidjiensis* cell extract of known protein concentration. Extracted groundwater protein (6 mg) was analyzed with SDS-PAGE (Bollag *et al*., 1996), and *c*-type heme-containing proteins were visualized by staining using *N, N, N', N'*-tetramethylbenzidine as described previously (Thomas *et al*., 1976; Francis and Becker, 1984). Major bands were selected and excised from the gel after heme-staining, subjected to reduction, alkylation and in-gel digestion (trypsin or Asp-N), and analyzed with liquid chromatography-tandem mass spectrometry (LC/MS/MS) (LCQ Deca ESI Ion Trap, Thermo, Waltham, MA, USA), performed by the UMASS Proteomics and Mass Spectrometry facility (Pazour *et al*., 2005). The raw data were converted to MS/MS spectra peak lists with the LCQ_DTA program (Thermo). The analyzed results were compared against the NCBInr 032810 database with the Mascot algorithm (http://www.matrixscience.com).

Antibody production and western blot analyses
Antibodies were produced against polypeptides that were designed to be specific to the *Geobacter* subsurface *c*-type cytochrome A (GscA) homologs found in *Geobacter* sp., or the RpoA of *Geobacter* subsurface clade 1, because the majority of Geobacteraceae 16 S ribosomal RNA sequences recovered from the uranium-contaminated aquifer clustered in this phylogenetic clade (Holmes *et al.*, 2007). The selected amino acid sequences of the polypeptides were HKIHMGNRLTC for the GscA-specific antibody, and NFPDPEIMRRLRGE for the RpoA-specific antibody. Peptide synthesis and production of the polyclonal antibodies in rabbits against these polypeptides were performed by New England Peptide, LLC (Gardner, MA, USA).

In order to generate purified proteins as standards for western blot analysis, *gscA* and *rpoA* were amplified from the genome of *G. bemidjiensis* with the *gscA* primers gscA_NdeF (5′-ACAAGGAGGACA TATGCAAGGCACG-3′) and gscA_XhoR (5′-GGCGCT TATTGCTCGAGCTGGTGAACC-3′) and the *rpoA* primers RpoAF_Nd (5′-GGGGATCCATATGTATAG AAACTG-3′) and RpoAR_E1_His (5′- AGTCTTGAAT TCTAGTGGTGGTGGTGGTGTTCTTCTTTCT GC TCGCCGC-3′). The amplified PCR products were digested with NdeI and XhoI for *gscA*, or *Nde*I and *Eco*RI for *rpoA* and ligated with pET24b (Novagen, Billerica, MA, USA) treated with the same enzymes. *Escherichia coli* BL21(DE3) (Studier *et al.*, 1990) was used as the host. For expression of GscA, pEC86 was co-transformed to enable constitutive expression of the cytochrome *c* maturation genes (Arslan *et al.*, 1998). Purified His-tagged proteins were obtained using Ni-NTA agarose (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. Western blotting analyses were performed as previously described (Yun *et al.*, 2011).

Diversity analysis
In order to evaluate the phylogenetic diversity of *gscA* during active Fe(III) reduction in the subsurface, DNA was extracted from filter-collected biomass from well D07 on day 15 of the 2007 field study with the FastDNA SPIN kit for soil (MP Biomedicals). Degenerate primers of gscA1840F (5′-TWCWC CGGMRGCCCSWSYTTCCTGMT-3′) and gscA2554R (5′-CGGCRTGGAYRCCGTGRATCADRTCCTT-3′) were designed to amplify the *gscA* homologs from the *in situ* uranium bioremediation site. The 16 S ribosomal RNA gene was amplified with primers 8F (Eden *et al.*, 1991) and 519R (Lane *et al.*, 1985) under the previously determined amplification condition (Anderson *et al.*, 2003). The resultant PCR products purified from a 1% agarose gel were cloned into the TOPO TA vector pCR2.1 (Invitrogen, Waltham, MA, USA). Inserts of the clones were amplified with the M13F and M13R primers, and sequenced with the M13F primer.

DNA sequences of clone libraries were compared with the database in GenBank with BlastN, BlastX (Altschul *et al.*, 1997), or programs in the Greengenes Web site (http://greengenes.lbl.gov) (DeSantis *et al.*, 2006a, b). The translated amino acid sequences of *gscA* clone libraries were aligned using Clustal W (Thompson *et al.*, 1994). Neighbor-joining trees of amino acid sequences were constructed with the MEGA 4.0 (http://www.megasoftware.net) (Tamura *et al.*, 2007) with default parameters. Bootstrap values were obtained from 1000 replicates. The sequences in this study were deposited in GenBank (GscA homologs: KP338821-KP338928, 16 S ribosomal RNA: KP338929-KP338993).

Expression of GscA in omcS*-deficient strain of* G. sulfurreducens
In order to evaluate the function of GscA, an expression plasmid containing *gscA* was constructed. The *gscA* homolog, Gbem_3371 was amplified from the genome of *G. bemidjiensis* with the primers Gbem_3371_EcoRI (5′-CATCTGAATTCAGA CAAGGAGGATTT-3′) and Gbem_3371_HindIIIR (5′-GCTCCCGTTATAAGCTTTTGGATTT-3′), and the amplified PCR product was ligated with pCD341 (Coppi *et al.*, 2001) treated with same enzymes. The correctly cloned plasmid was transformed into the *omcS*-deficient strain of *G. sulfurreducens* (Mehta *et al.*, 2005) by electroporation (Coppi *et al.*, 2001). The expression of GscA in the *omcS*-deleted *G. sulfurreducens* mutant by induction with IPTG was confirmed by SDS-PAGE followed by Coomassie brilliant blue staining (data not shown). The ability of the *G. sulfurreducens* strains to reduce insoluble Fe(III) oxides was evaluated by the determination of Fe(II) concentration with the ferrozine assay (Lovley and Phillips, 1986).

Atomic force microscopy
G. sulfurreducens Δ*omcS*/pCD341 and *G. sulfurreducens* Δ*omcS*/pCD-*gscA* were grown in NBAF liquid medium supplemented with kanamycin and IPTG at 25 °C. Pili expression of cells was verified by negatively staining cells and examining with transmission electron microscopy. These pili-expressing cells were placed on freshly cleaved mica surface and atomic force microscopy was conducted to localize and map pili and any associated cytochrome-like globules aligned along the pili as described previously (Malvankar *et al.*, 2012).

Results and discussion

Identification of c*-type heme-containing proteins from the groundwater at the* in situ *uranium bioremediation site*
Although *c*-type cytochromes are abundant in *Geobacter* sp. that bloom when acetate is added to groundwater at the Rifle, Colorado study site to promote *in situ* uranium bioremediation (Lovley *et al.*, 2011), previous studies of the microbial community that employed a global proteomics

approach, detected few, if any, *c*-type cytochromes (Wilkins *et al.*, 2009, 2013; Callister *et al.*, 2010). As an alternative heme-specific approach, proteins extracted from groundwater samples collected during two *in situ* bioremediation experiments were separated with SDS-PAGE and visualized with heme staining. Two major bands with a molecular weight of > 80 kD and ca. 30 kD were recovered in groundwater samples in which previous studies (Mouser *et al.*, 2009; Wilkins *et al.*, 2011; Williams *et al.*, 2011; Yun *et al.*, 2011) found an abundance of *Geobacter* sp. (Figure 1).

The abundant heme-staining bands from the day 15 sample from well D05 were excised from the heme-stained gel for proteomic analysis. A number of peptides were detected, but no *c*-type heme-containing proteins could be identified with LC/MS/MS when the samples were digested with trypsin, or when the lower band was digested with Asp-N. One possibility for this is that many *c*-type cytochromes in *Geobacter* sp. are poorly conserved (Butler *et al.*, 2009; Butler *et al.*, 2010), and thus there is a high probability that an isolate containing the sequence for this cytochrome is yet to be isolated and sequenced.

However, analysis of the upper band digested with Asp-N identified two peptides (QDANILGAED, score 29 and GDGSYTVIFGKD, score 73) that correspond to the protein encoded by ORF G18_3459 (YP_004200169) in the genome of *Geobacter* sp. M18 (accession no. NC_014973), which was isolated from the uranium bioremediation site at Rifle, CO, USA (Lovley *et al.*, 2011). This protein, annotated as a decaheme *c*-type cytochrome, was designated GscA.

GscA in Geobacter *sp. and groundwater*

The GscA of *Geobacter* sp. M18 (GM18_3459) consists of 1027 amino acids with 10 typical heme-binding motifs (CXXCH) (Figure 2). PSORTb (http://www.psort.org/psortb/) predicted GscA to be extracellular (score, 9.34). SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) analyses predicted a signal peptide exists in GM18_3459 with a cleavage site between amino acids 26 and 27. Topology prediction with TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) suggested that GscA has a transmembrane helix at the N-terminal region. LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/) suggested

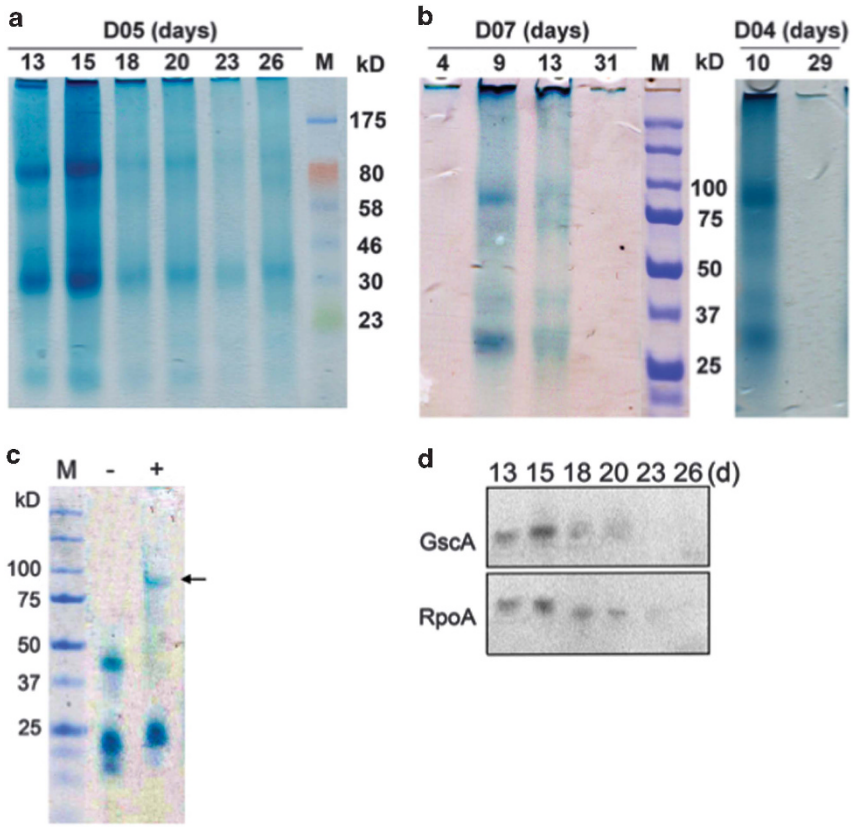

Figure 1 Detection of *c*-type heme-containing proteins during *in situ* uranium bioremediation. The groundwater samples were collected at the Rifle site in 2007 (**a**) and 2008 (**b**). Proteins extracted from the groundwater (6 mg) were separated by SDS-PAGE, and visualized with heme staining. Lane M, molecular weight prestained standards. Previous studies have demonstrated an abundance of *Geobacter* in 2007 D05 samples on days 13 and 15, but not on subsequent days (Yun *et al.*, 2011); in 2008 D07 samples on days 9 and 13, but not day 31 (Yun *et al.*, 2011); and for 2008 D04 samples for day 10, but not day 29 (Wilkins *et al.*, 2011). GscA was expressed in *E. coli* BL21 (DE3) with the plasmid-containing genes for cytochrome maturation proteins (Arslan *et al.*, 1998) and total proteins (10 mg) were separated by SDS-PAGE and visualized by heme staining (**c**). Lane – and + indicate no addition of IPTG and addition of IPTG, respectively. Abundance of GscA and *Geobacter* RpoA in the groundwater of the well D05 in 2007 was analyzed by western blot (**d**).

that GscA has high probability (score, 26.94) to be a lipoprotein.

Amino acid sequences of GscA were compared with the GenBank databases using BlastP (Altschul *et al*., 1997). Within *Geobacter* sp., GM18_3459 homologs include: GM18_3465 of *Geobacter* sp. M18 (YP_004200175, 44% identity), GM21_0875 of *Geobacter* sp. M21 (YP_003020700, 40% identity) and Gbem_3371 (YP_002140162, 39% identity) of *G. bemidjiensis* (Figure 2). These species are closely related to *Geobacter* sp. in Subsurface Clade I that are abundant in many subsurface environments (Holmes *et al*., 2007; Wilkins *et al*., 2009). *G. lovleyi*, which is in the phylogenetic clusters known as Subsurface Clade II (Lovley *et al*., 2011), also contains a GscA homolog (Glov_2292 of *G. lovleyi* strain SZ (YP_001952528, 45% identity). There was no protein with significant similarity to GscA in the genomes of *G. sulfurreducens*, *G. metallireducens*, *G. uraniireducens* or *G. daltonii*. The two GscA homologs of *Geobacter* sp. M18 have ca. 120 more amino acids after the putative signal peptide compared with the other species (Figure 2). Other than *Geobacter* sp., similar proteins were found in *Rhodoferax ferrireducens*, *Candidatus Solibacter usitatus*, *Anaeromyxobacter dehalogenans* and *Shewanella oneidensis* with identities of ~30%.

Geobacter sp. M18 is not well-characterized and is difficult to grow because it can only be grown on Fe (III) oxide, on which it grows very slowly (Lovley *et al*., 2011). In contrast, the closely related *G. bemidjiensis*, which is also a member of the *Geobacter* subsurface clade 1, has been substantially characterized and is readily grown with soluble electron acceptors such as fumarate (Aklujkar *et al*., 2010; Lovley *et al*., 2011; Ueki, 2011; Yun *et al*., 2011). The GscA of *G. bemidjiensis* (Gbem_3371) was successfully expressed in *E. coli*. Heme staining confirmed that it was a *c*-type cytochrome (Figure 1c).

In order to further evaluate the presence of GscA in groundwater samples, an antibody to a peptide conserved in the GscA homologs in Subsurface Clade I *Geobacter* sp. was generated. Western blot analysis with this antibody confirmed the presence of GscA in the groundwater (Figure 1d, Supplementary Information 1), exhibiting the same expression pattern as the predominant heme-staining upper band shown in the original proteomic analysis (Figure 1a). The abundance of GscA tracked with the abundance of *Geobacter* sp. as indicated by the abundance of the housekeeping protein RpoA (Figure 1d, Supplementary Information 1).

Phylogenetic diversity of GscA in the in situ *uranium bioremediation site*

In order to evaluate the diversity of GscA homologs expressed during active growth of *Geobacter* sp. in the subsurface, *gscA* sequences were amplified with degenerate primers from subsurface genomic DNA collected from well D07, at 15 days after the start of acetate injection in 2007. Previous analysis suggested that *Geobacter* sp. were abundant during this time (Williams *et al*., 2011) and analysis of a 16 S ribosomal RNA gene clone library generated with the environmental DNA from this time point revealed that *Geobacter* sp. accounted for ca. 85% of the sequences recovered (Figure 3a). Both heme-staining and western blot analyses demonstrated that GscA homologous proteins were abundant in this sample (Figure 3b).

A total of 108 partial amino acid sequences of GscA homologs were recovered from this groundwater sample (Figure 3c). All the amplified sequences had highest similarity to *Geobacter* GscA homologs. They were distributed into five subclusters (I, II, III, IV and V) with 90% amino acid sequence consensus cutoff for each cluster. The majority (85 of 108) belonged to subcluster I, which includes GM18_3459 of *Geobacter* sp. M18, the protein initially identified with LC/MS/MS analyses. There were no GscA-like sequences that could be grouped with the GscA of *G. lovleyi* (Glov_2292) or GM18_6465, the other GscA homolog of *Geobacter* sp. M18. No GscA homologs of *R. ferrireducens* were detected, consistent with previous findings that *Geobacter* sp. outcompete *Rhodoferax* sp. once acetate is added to the subsurface at the Rifle site (Mouser *et al*., 2009; Zhuang *et al*., 2011). No *Shewanella* GscA homologs were detected, consistent with the general lack of abundance of *Shewanella* sp. in Fe(III)-reducing subsurface environments (Snoeyenbos-West *et al*., 2000).

Expression of GscA compensates for the loss of OmcS in G. sulfurreducens

The lack of a developed genetic system for any of the subsurface clade I *Geobacter* sp. prevented evaluation of the GscA function in the available pure cultures. As an alternative, the possibility that expression of *gscA* might complement the deletion of *omcS* in *G. sulfurreducens* was evaluated because: (1) GscA and OmcS are both outer-surface proteins and (2) the deletion and complementation of *omcS* in *G. sulfurreducens* yields a very clear phenotype for the capacity for Fe(III) oxide reduction (Mehta *et al*., 2005). Therefore, the *gscA* of *G. bemidjiensis* was introduced into the previously described (Mehta *et al*., 2005) *omcS*-deficient strain of *G. sulfurreducens* on a plasmid under the control of an IPTG-inducible promoter. As expected from the previous results (Mehta *et al*., 2005), the *omcS*-deficient strain of *G. sulfurreducens* did not effectively reduce Fe(III) oxide (Figure 4a). However, the strain with the IPTG-inducible *gscA* effectively reduced Fe(III) oxide in the presence of IPTG (Figure 4b). The lag period prior to Fe(III) oxide reduction was slightly longer than the wild-type strain, but the rate of Fe(III) oxide reduction was comparable between the wild-type strain (1.4 mM Fe(II) produced/day) and the *gscA*-induced strain (1.1 mM Fe(II) produced/day)

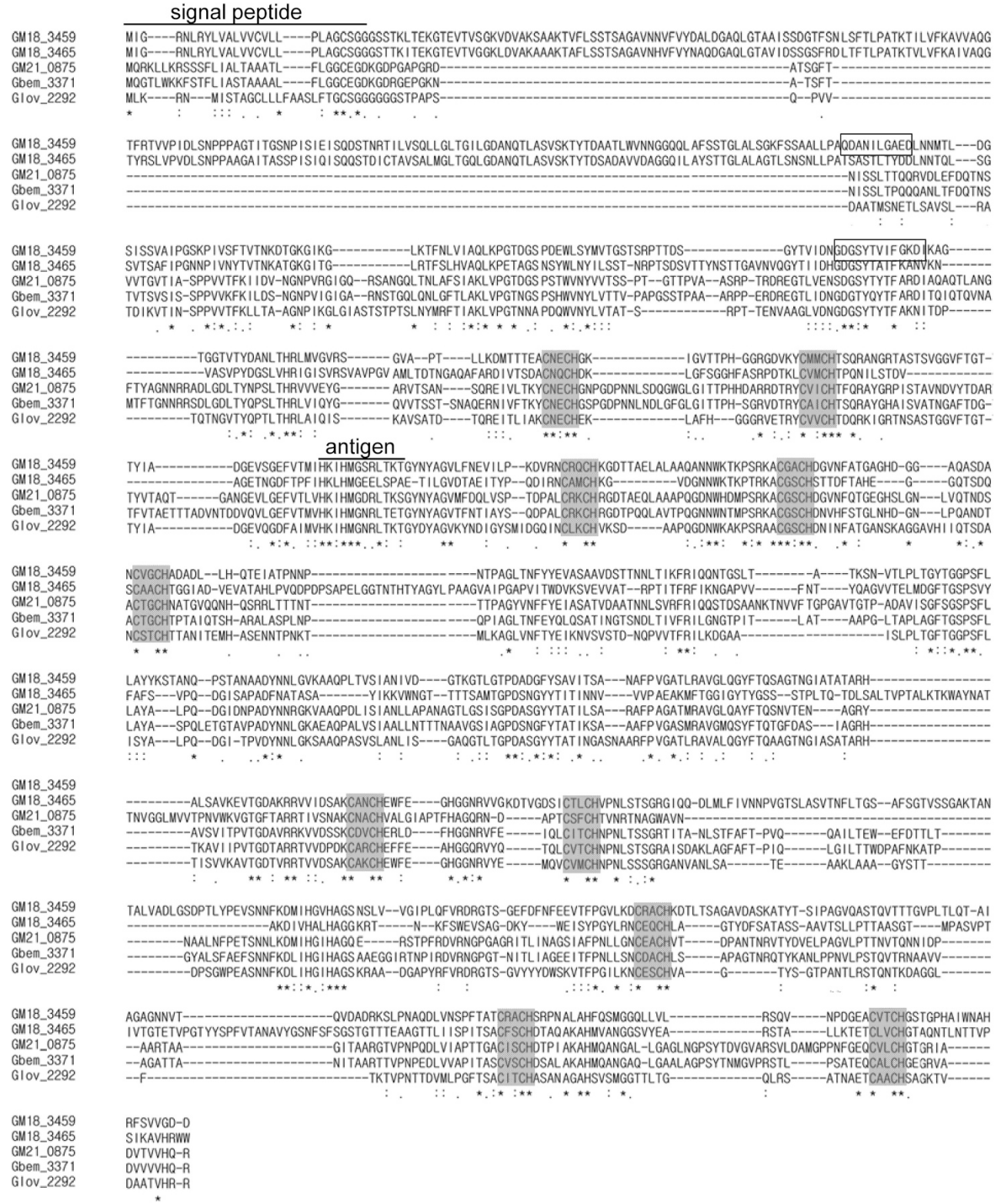

Figure 2 Multiple alignment of GscA homologs in *Geobacter* sp. The identical, strongly similar and weakly similar residues are indicated by asterisks, colons and dots, respectively. The fragments detected by liquid chromatography-tandem mass spectrometry were boxed. The heme-binding motifs were shaded in gray. The putative signal peptide and the antigenic polypeptide used for the antibody production are marked. Alignment was made with Clustal W (Thompson *et al.*, 1994).

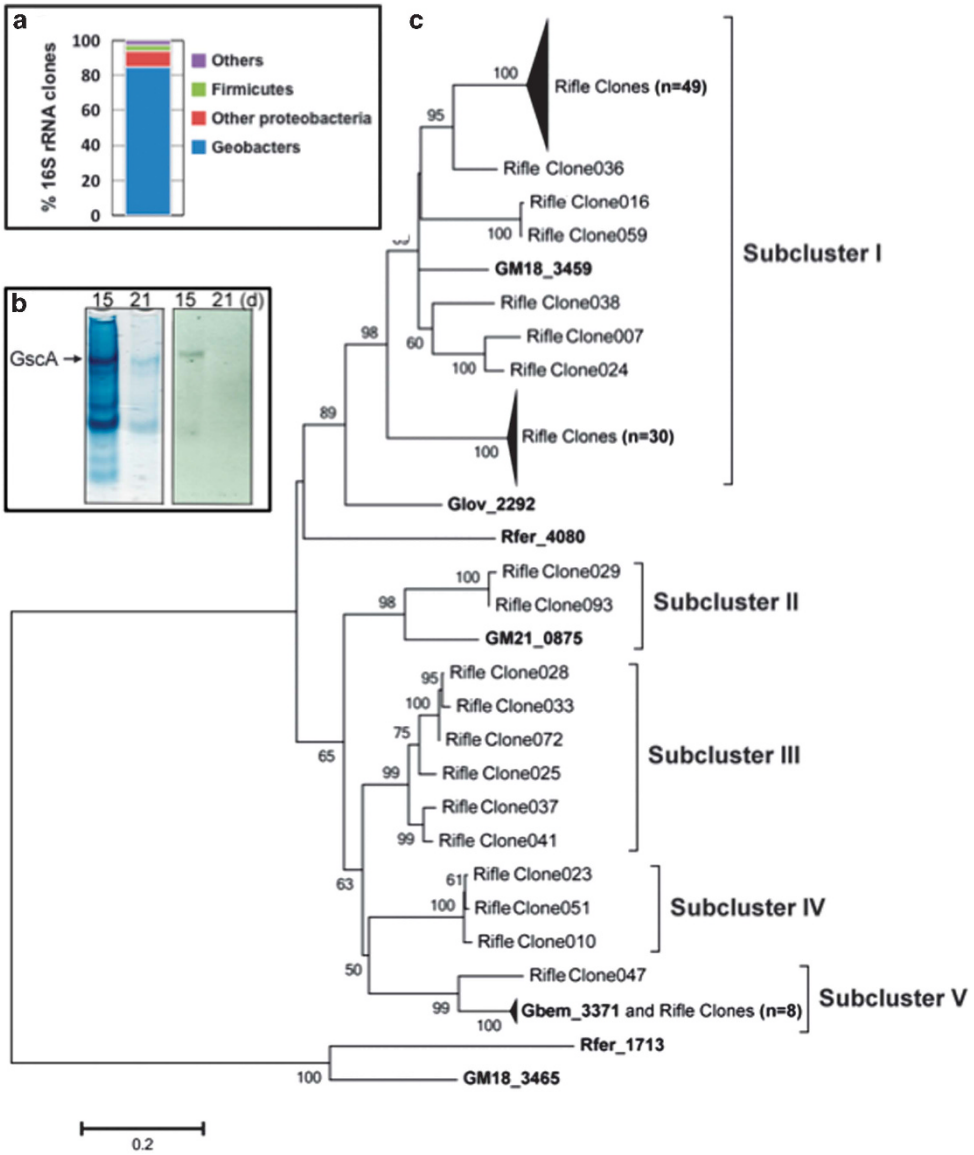

Figure 3 Phylogenetic relationship of GscA. (**a**) Microbial community composition based on a 16 S rRNA clone library generated with the environmental DNA collected from well D07 at day 15 in 2007. (**b**) Abundance of GscA analyzed by the heme-staining method and western blot. (**c**) Comparison of amino acid sequences of GscA recovered from groundwater of Rifle, CO, and those from *Geobacter* isolates and *R. ferrireducens*. The tree was constructed by using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The scale bar represents 0.2 amino acid substitutions per site.

during the linear phase of Fe(III) reduction. The *omcS*-deficient strain with IPTG-inducible *gscA* did not reduce Fe(III) oxide in the absence of the inducer and a strain with the same plasmid, but lacking *gscA*, did not reduce Fe(III) oxide in the presence of IPTG (Figure 4b).

After OmcS, the outer-membrane *c*-type cytochrome OmcB is typically the most abundant outer-surface *c*-type cytochrome when *G. sulfurreducens* is growing on Fe(III) oxide. The *omcB*-deficient strain is unable to reduce Fe(III) (Leang *et al*., 2003; Qian *et al*., 2007). Expression of GscA did not enable the *omcB*-deficient strain to reduce Fe(III), suggesting that the restoration of the capacity for Fe(III) oxide reduction when GscA was expressed in the *omcS*-deficient strain was specifically related to replacing the OmcS function.

In order to further evaluate how the expression of GscA could compensate for the loss of OmcS, the localization of GscA expressed in *G. sulfurreducens* was investigated. Attempts to visualize GscA with immunogold labeling using the antibody developed from a GscA peptide, in a manner similar to that reported for the localization of OmcS (Leang *et al*., 2010), were unsuccessful, presumably because the antibody did not recognize the undenatured protein.

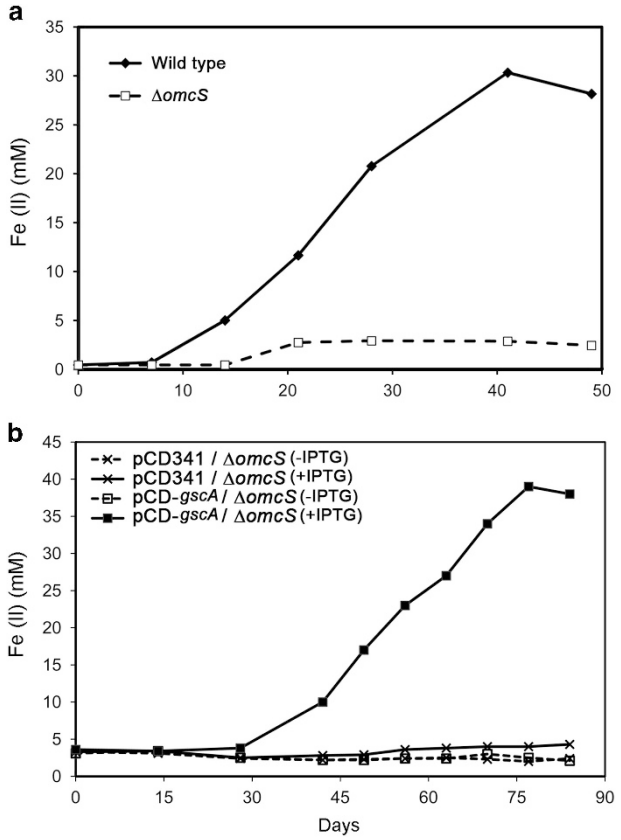

Figure 4 Fe(III) oxide reduction. Fe(III) reducing ability of *G. sulfurreducens* wild-type PCA strain (Wild type) and the *G. sulfurreducens omcS*-deletion mutant (Δ*omcS*) (**a**), or the Δ*omcS* harboring the empty vector (pCD341) and the *gscA* expressing plasmid (pCD-*gscA*) (**b**) was evaluated. IPTG (1 mg ml^{-1}) was added to induce expression of *gscA*. Data shown are a representative of triplicate experiments with similar results.

Atomic force microscopy is an alternative to visualizing OmcS on pili, which does not require antibodies (Malvankar *et al*., 2012). Atomic force microscopy revealed cytochrome-like globules on the pili of the *G. sulfurreducens omcS*-deficient strain expressing GscA (Figure 5a), similar in appearance to previously reported wild-type cells (Malvankar *et al*., 2012). The heights of the globules and the pili were consistent with the known sizes of cytochromes and pili, respectively (Figure 5b). In contrast, the pili of the *omcS*-deficient strain without *gscA* were bare (Figures 5c and d). These results suggest that GscA was localized along the pili and was able to substitute for OmcS to facilitate electron transfer between the pili and Fe(III) oxide.

Implications

These results suggest that one of the most abundant *c*-type cytochromes during *in situ* uranium bioremediation functions in a manner similar to the cytochrome OmcS of *G. sulfurreducens*. The proposed role of OmcS is to facilitate electron transfer from pili to Fe(III) oxides (Lovley, 2011, 2012). The finding that magnetite, which associates with pili, can rescue the ability of *omcS*-deficient *G. sulfurreducens* to reduce Fe(III) oxide demonstrated that there is no specific requirement for OmcS for this electron transfer function (Liu *et al*., 2015). If a mineral can substitute for OmcS then it may not be surprising that another *c*-type cytochrome can also fulfill this function. However, there do not appear to be any other *c*-type cytochromes among the over 100 encoded in the *G. sulfurreducens* genome (Methe *et al*., 2003) that can substitute for OmcS, suggesting that GscA possess some unique properties that facilitate its association with pili. Elucidation of the salient features will require extensive further investigation with approaches such as site-directed mutagenesis of the *gscA* gene sequence expressed in the *omcS*-deficient strain of *G. sulfurreducens*.

The poor conservation of *c*-type cytochromes across *Geobacter* sp. (Butler *et al*., 2009, 2010) suggests that in different *Geobacter* sp. alternative cytochromes are likely to have similar functions. Although genes aligned with *c*-type cytochrome genes on *Geobacter* chromosomes are often highly conserved, the *c*-type cytochrome sequences can vary significantly, suggesting that there is often little selective pressure for specific *c*-type cytochrome structure as long as the capacity for electron transfer inherent in *c*-type cytochromes is retained (Butler *et al*., 2009, 2010).

The apparent role of GscA in Fe(III) oxide reduction and its abundance during *in situ* uranium bioremediation is consistent with the concept that *Geobacter* sp. primarily grow via Fe(III) oxide reduction in uranium-contaminated aquifers (Finneran *et al*., 2002). Although growth via U(VI) reduction is feasible (Lovley *et al*., 1991; Sanford *et al*., 2007), even in uranium-contaminated aquifers the availability of U (VI) (μM concentrations) is typically much less than Fe (III) oxide. In contrast to the specific need for OmcS for Fe(III) oxide reduction in *G. sulfurreducens*, many outer-surface *c*-type cytochromes appear to have the ability to reduce U(VI) (Shelobolina *et al*., 2007; Orellana *et al*., 2013). A similar non-specific reduction of U(VI) by outer-surface cytochromes seems probable for the *Geobacter* sp. that are enriched during *in situ* uranium bioremediation.

In contrast to *G. sulfurreducens* and *G. metallireducens*, which are amenable to genetic manipulation (Coppi *et al*., 2001; Tremblay *et al*., 2012), many of the available isolates from the Subsurface Clade I of *Geobacter* sp. (Holmes *et al*., 2007) are difficult to cultivate on solidified medium, complicating the development of genetic tools. Genes for central metabolism are typically highly conserved among *Geobacter* sp. and thus functional studies from *G. sulfurreducens* have successfully been used to identify target genes or proteins that have been useful in diagnosing the physiological status of *Geobacter* sp. in subsurface environment approaches (Lovley *et al*., 2011; Mahadevan *et al*., 2011). The approach described here of evaluating the function of genes found in Subsurface Clade I *Geobacter* sp.

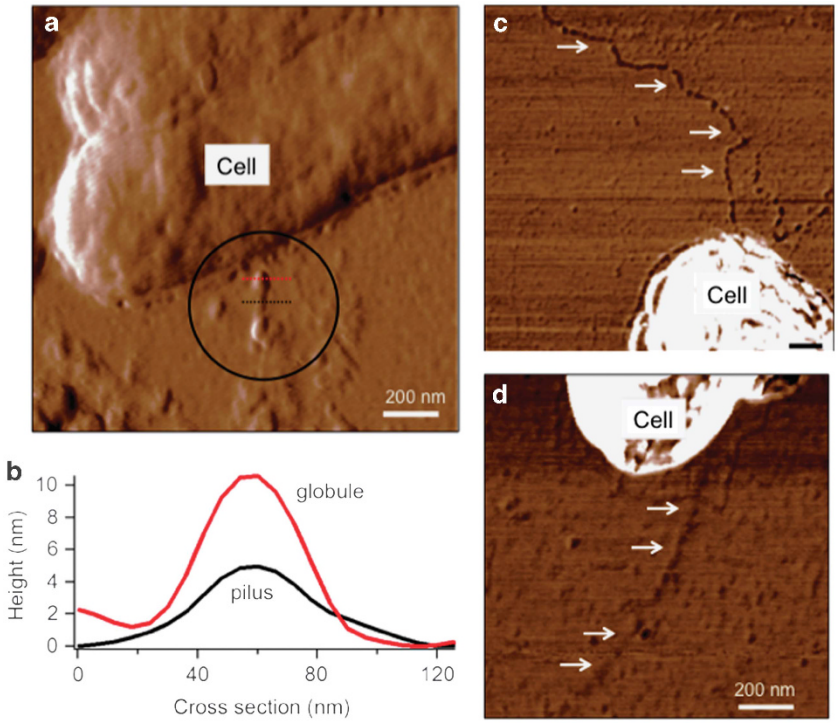

Figure 5 AFM. (**a**) *G. sulfurreducens* Δ*omcS*/pCD-*gscA*. Pilus and associated cytochrome-like globules are shown in a circle. (**b**) Corresponding height profiles at the section indicated in **a**. Black line corresponds to pilus filament whereas red line corresponds to a cytochrome-like globule aligned along the pilus filament. (**c** and **d**) AFM images of bare pili with no associated globules in *G. sulfurreducens* Δ*omcS*/pCD341. All scale bars = 200 nm. Pili are indicated by arrows. The data are a representative of three biological replicates.

by determining the capacity for those genes to restore functions in *G. sulfurreducens* is expected to accelerate the understanding of the physiology and ecology of subsurface *Geobacter* sp.

Conflict of Interest

The authors declare no conflict of interest.

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