Highly conserved genes in *Geobacter* species with expression patterns indicative of acetate limitation

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Analysis of the genome of *Geobacter sulfurreducens* revealed four genes encoding putative symporters with homology to ActP, an acetate transporter in *Escherichia coli*. Three of these genes, *aplA*, *aplB* and *aplC*, are highly similar (over 90 % identical) and fell within a tight phylogenetic cluster (Group I) consisting entirely of *Geobacter* homologues. Transcript levels for all three genes increased in response to acetate limitation. The fourth gene, *aplD*, is phylogenetically distinct (Group II) and its expression was not influenced by acetate availability. Deletion of any one of the three genes in Group I did not significantly affect acetate-dependent growth, suggesting functional redundancy. Attempts to recover mutants in which various combinations of two of these genes were deleted were unsuccessful, suggesting that at least two of these three transporter genes are required to support growth. Closely related Group I *apl* genes were found in the genomes of other *Geobacter* species whose genome sequences are available. Furthermore, related genes could be detected in genomic DNA extracted from a subsurface environment undergoing *in situ* uranium bioremediation. The transporter genes recovered from the subsurface were most closely related to Group I *apl* genes found in the genomes of cultured *Geobacter* species that were isolated from contaminated subsurface environments. The increased expression of these genes in response to acetate limitation, their high degree of conservation among *Geobacter* species and the ease with which they can be detected in environmental samples suggest that Group I *apl* genes of the *Geobacteraceae* may be suitable biomarkers for acetate limitation. Monitoring the expression of these genes could aid in the design of strategies for acetate-mediated *in situ* bioremediation of uranium-contaminated groundwater.

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INTRODUCTION

Rational design of groundwater bioremediation strategies requires information on the *in situ* physiological status of the micro-organisms in the subsurface (Lovley, 2003; Lovley *et al.*, 2008). Such knowledge will make it possible to tailor amendments to groundwater to optimize bioremediation processes of interest. For example, the addition of electron donors, such as acetate, to stimulate dissimilatory metal reduction has proven to be an effective strategy for promoting the reductive precipitation of toxic metals, thereby immobilizing them and preventing their further migration through the subsurface. In the case of uranium, soluble U(VI) is microbially reduced to insoluble U(IV) (Amos *et al.*, 2007; Anderson *et al.*, 2003; Cummings

et al., 2003; Istok *et al.*, 2004; Luo *et al.*, 2007; N'Guessan *et al.*, 2008; North *et al.*, 2004; Petrie *et al.*, 2003; Sanford *et al.*, 2007; Vrionis *et al.*, 2005). In most instances, *Geobacter* species have been identified as the primary U(VI)-reducing micro-organisms during active uranium precipitation (Anderson *et al.*, 2003; Holmes *et al.*, 2002, 2007; Sanford *et al.*, 2007). While in some cases adding limiting nutrients to optimize maximum growth rates is the preferred option, in other instances it may actually be preferable to design strategies in which rates of metabolism are regulated to suit particular needs. During most initial field trials on acetate-driven *in situ* uranium bioremediation, relatively high concentrations of electron donor were added to stimulate rapid growth of *Geobacter* species. However, rapid growth of *Geobacter* species is associated with rapid depletion of Fe(III) oxides, the primary electron acceptor for growth of *Geobacter* species in uranium-contaminated subsurface sediments (Finneran *et al.*, 2002; Holmes *et al.*, 2002, 2004a). As Fe(III) oxides are depleted,

Abbreviation: qRT-PCR, quantitative real-time polymerase chain reaction.

A supplementary table of the primers used for qRT-PCR is available with the online version of this paper.

sulfate-reducing micro-organisms that are less effective U(VI) reducers than *Geobacter* species become prevalent. Therefore, it may be more desirable to fine tune acetate additions so that the growth of *Geobacter* species and U(VI) reduction are stimulated, yet remain acetate-limited, thereby slowing the depletion of Fe(III) oxides.

Insights into some aspects of the *in situ* physiological status of *Geobacter* species in subsurface environments have been derived from analysis of transcript levels for key genes (Holmes *et al.*, 2004a, b, 2005). High levels of *nifD* transcripts suggested that *Geobacter* species were limited for fixed nitrogen in aquifer sediments, and fixed atmospheric nitrogen (Holmes *et al.*, 2004b). Transcript levels for genes involved in central metabolism were related to rates of metabolism in the subsurface (Holmes *et al.*, 2005). In addition, differential expression of two genes encoding multi-copper containing proteins was related to differences in growth rates in the subsurface (Mehta *et al.*, 2006). In each case, studies of gene expression in the environment were preceded by genome-scale analysis of the metabolic processes of interest in pure cultures, with the primary focus on *Geobacter sulfurreducens* because of the availability of a complete genome sequence (Methé *et al.*, 2003), a genetic system (Coppi *et al.*, 2001; Lloyd *et al.*, 2003), and a genome-scale metabolic model (Mahadevan *et al.*, 2006).

We hypothesized that levels of expression of genes for acetate transporters in *Geobacter* species might be indicative of the availability of acetate to these organisms. To our knowledge, the only acetate transport system described to date is *Escherichia coli* ActP, a membrane permease highly specific for short-chain aliphatic monocarboxylates, which belongs to the sodium-solute symporter family (Gimenez *et al.*, 2003). The expression of the operon that includes the *actP* gene was upregulated in acetate-grown versus glucose-grown *E. coli* (Oh *et al.*, 2002). Here we report that genes with high homology to *actP* are highly conserved in the genomes of cultured *Geobacter* species as well as in uncultured *Geobacter* species that predominate during *in situ* uranium bioremediation. In addition, these genes are highly expressed in acetate-limited *Geobacter sulfurreducens* in continuous cultures. These characteristics make these transporter genes uniquely suited to act as phylogenetic and physiological markers of *Geobacter*-dominated communities in acetate-driven bioremediation processes.

METHODS

Bacterial strains and culturing conditions. *Geobacter sulfurreducens* strain DL1 (ATCC 51573) (Coppi *et al.*, 2001) was obtained from our laboratory culture collection and used to construct strains DLCR1 (*apLB::kn*), DLCR2 (*apLA::kn*) and DLCR3 (*apLC::kn*) as described below. Strains were cultured under strict anaerobic conditions at 30 °C in a N₂/CO₂ (80%:20%) atmosphere as previously described (Caccavo *et al.*, 1994) in either freshwater medium (Lovley & Phillips, 1988) or NBAF medium (Coppi *et al.*, 2001). When noted,

kanamycin was added at 50 µg ml⁻¹. For the growth curves, freshwater medium containing 10 mM acetate and 56 mM ferric citrate was used. Continuous culture was carried out in freshwater medium as previously described (Esteve-Núñez *et al.*, 2005) in acetate-limiting conditions (5 mM acetate, 30 mM fumarate) or fumarate-limiting conditions (10 mM acetate, 10 mM fumarate). The dilution rate was 0.05 h⁻¹.

Determination of acetate consumption in resting cell suspensions. Cell suspensions were prepared by harvesting 800 ml of late-exponential-phase cultures of DL1 and DLCR3 grown in freshwater medium (20 mM acetate, 56 mM ferric citrate). All manipulations were performed in an anaerobic chamber. The cultures were centrifuged at 4300 g at 4 °C, washed with anoxic isotonic basal wash medium (BWM) (Leang *et al.*, 2003) and resuspended in pressure tubes with BWM to OD₆₀₀=2. The tubes were placed at 30 °C and amended with 10 ml of hydrogen and 30 mM ferric citrate. The reactions were started by the addition of 2 mM acetate. Aliquots were taken at appropriate times, filtered and diluted. Acetate concentration was determined by HPLC. The results were normalized to the total protein concentration of the cell suspension.

Construction of acetate-permease-like (*apl*) mutants via single-step gene replacement. Single-step gene replacement was performed as previously described (Coppi *et al.*, 2001; Lloyd *et al.*, 2003). The sequences of all primers used for the construction and screening of strains DLCR1, DLCR2 and DLCR3 are listed in Table 1. To create a linear DNA fragment for the construction of mutant DLCR1 (*apLB::kn*) three primary fragments were generated independently by PCR. The first fragment was amplified from DL1 chromosomal DNA using primers 1070-1 and 1070-2. The middle fragment containing a kanamycin-resistance cassette was amplified from plasmid pBBR1MCS-2 (Kovach *et al.*, 1995) with hybrid primers 1070-3Kn and 1070-4Kn. The third fragment was amplified from DL1 chromosomal DNA using primers 1070-5 and 1070-6. PCR conditions were as follows: 5 cycles at 95 °C, 30 s; 50 °C, 45 s; 72 °C, 1 min; followed by 30 additional cycles at 95 °C, 30 s; 62 °C, 45 s; 72 °C, 1 min. All reactions were preceded by a 5 min incubation at 95 °C during which the *Taq* polymerase was added ('hot start') and followed by a 10 min extension period at 72 °C. The amplified fragments were gel-purified and joined by recombinant PCR. The resulting linear fragment was amplified with distal primers 1070-1 and 1070-6. PCR conditions during these two steps were as described above except that an extension time of 3 min at 72 °C was employed. A similar strategy was employed to make the other two mutants. For construction of DLCR2 (*apLA::kn*), the first fragment was amplified from DL1 chromosomal DNA using primers 1068-1 and 1068-2; the middle segment containing a kanamycin-resistance cassette was amplified from plasmid pBBR1MCS-2 (Kovach *et al.*, 1995) with hybrid primers 1068-3Kn and 1068-4Kn; and the third fragment was amplified from DL1 chromosomal DNA with primers 1068-5 and 1068-6. For construction of DLCR3 (*apLC::kn*), the first fragment was amplified from DL1 chromosomal DNA using primers 2352-1 and 2352-2; the middle segment containing a kanamycin-resistance cassette was amplified from plasmid pBBR1MCS-2 (Kovach *et al.*, 1995) with hybrid primers 2352-3Kn and 2352-4Kn; and the third fragment was amplified from DL1 chromosomal DNA with primers 2352-5 and 2352-6.

For the construction of *apLB-apLA* and *apLB-apLC* double mutants, hybrid primers 1068-3Kn and 1068-4Kn were modified to amplify a gentamicin-resistance cassette, and 2352-3Kn and 2352-4Kn were modified to amplify a chloramphenicol-resistance cassette (sequences not shown). Linear recombinant DNA fragments were constructed as described above and used to transform DLCR1 (*apLB::kn* mutant) and DL1 competent cells. Similarly, linear fragments containing the *apLB::kn* or *apLC::cm* constructs were used to transform DL1 and

Table 1. Oligonucleotides used in this work

Oligonucleotide	Coordinates*/use	Sequence (5'–3')
1068-1	1163503	GCAACATTGCCATTTACAGC
1068-2	1164006	CATCGGGTTCATTCCCTCCTT
1068-3Kn	Hybrid primer	AAGGAGGAATGAACCCGATGAGCTACTGGGCTATCTGGACAA
1068-4Kn	Hybrid primer	GACCTTCCCCCAGAGTGTAACATCGCTTGGTCGGTCATTTTC
1068-5	1166017	GTTACACTCTGGGGGAAGGTC
1068-6	1166521	CGTCCCCATAAACAATCAGG
1070-1	1160465	GTTGCCGTTGATTTTCGAC
1070-2	1161101	CACAGACCTCCTTCCTATTTG
1070-3Kn	Hybrid primer	CAAAATAGGAAGGAGGTCTGTGAGCTACTGGGCTATCTGGACAA
1070-4Kn	Hybrid primer	CAAACGAGGGTATGAAACAGATCGCTTGGTCGGTCATTTTC
1070-5	1163161	CTGTTTCATACCCTCGTTTG
1070-6	1164003	CGGGTTCATTCTCCTTAC
2352-1	2573415	GTGCCATCGTTCAGGGCTGTG
2352-2	2573996	CAGCGAAGGCGAACGAGCCC
2352-3Kn	Hybrid primer	GGGCTCGTTCGCCTTCGCTGAGCTACTGGGCTATCTGGACAA
2352-4Kn	Hybrid primer	GCTACCCCTCCTGTCACATAATCGCTTGGTCGGTCATTTTC
2352-5	2575991	TATGTGACAGGAGGGGGTAGC
2352-6	2576516	CTATCCTGACCATCGCCTACG
2352-Rchk	2576491	CACGAAAGTGGATAACAGGC
KnF	Confirm <i>aplC</i> mutant genotype	ATTGCACGCAGGTTCTCC
422Fw	Degenerate primer	TSTCSGCNGCCWCNTTCCT
660Rv	Degenerate primer	TTNCCBGCVCNCCATCTG

*Coordinates refer to the 5' end in the *G. sulfurreducens* genome.

aplA::gm mutants. Hybrid primers 1068-3Kn and 1070-4Kn were used to construct a recombinant fragment to replace the whole GSU1068–GSU1070 region.

Electroporation and mutant isolation were carried out as previously described (Coppi *et al.*, 2001; Lloyd *et al.*, 2003) except that the recovery medium and plates were supplemented with 10 mM pyruvate and H₂. One colony of each of the mutants was selected as representative for further analysis. In order to confirm their genotypes, the mutant and wild-type strains were screened with combinations of primers which annealed outside and inside the mutagenic constructs and thus were expected to yield amplicons only in specific mutants. Primers 1070-1/1070-4Kn and 1070-3Kn/1070-6 were used to confirm the presence of the *aplB::kn* mutation in strain DLCR1. Primers 1068-1/1068-4Kn and 1068-3Kn/1068-6 were used to confirm the presence of the *aplA::kn* mutation in strain DLCR2. Primers KnF and 2532R-chk were employed to confirm the *aplC* mutation in strain DLCR3. As expected, bands of the correct sizes were obtained from all the mutants, but not from the wild-type (not shown).

Analytical techniques. Growth of fumarate cultures was assessed by measuring OD₆₀₀ in pressure tubes (1.5 cm path) with a Genesys 2 spectrophotometer (Spectronic Instruments). Fe(II) concentration was determined with the ferrozine assay as previously described (Lovley & Phillips, 1986). Protein concentrations were determined by the bicinchoninic acid method with BSA as standard (Smith *et al.*, 1985). The organic acid content of the medium in the resting cell suspension experiment was determined by HPLC using an LC-10AT high-pressure liquid chromatograph (Shimadzu) equipped with an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad). Organic acids were eluted in 8 mM H₂SO₄ and quantified with an SPD-10VP UV detector (Shimadzu) set at 215 nm.

Nucleic acid manipulations. Genomic DNA preparations, RNA extractions and gel extractions were carried out using Qiagen Genome-tip 100G, RNeasy Mini and Qiaquick Gel Extraction kits respectively.

Quantitative real-time PCR (qRT-PCR). Cells from steady-state chemostats were harvested by centrifugation and pooled for RNA extraction. Total RNA was extracted as described above. A reverse transcription reaction was performed to synthesize single-stranded cDNA from approximately 1 µg total RNA from each sample in a 100 µl reaction volume using Taqman Reverse Transcription Reagents (Applied Biosystems). Reactions were performed in duplicate for each gene tested. The resulting cDNA was subsequently used as template for real-time PCR using the SYBR Green PCR Master Mix (Applied Biosystems) and primers suitable for qRT-PCR amplification that were designed using Primer3 (Rozen & Skaletsky, 2000) software. These gene-specific primers are listed in Supplementary Table S1, available with the online version of this paper. Forward and reverse primers were added to the reaction at a final concentration of 200 nM along with 1 µl of the cDNA reaction. The incorporation of SYBR Green dye into the PCR products was detected in real time on the ABI Prism 7900HT Sequence Detection System. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Values of approximately 1 reflect little or no significant change in expression.

Phylogenetic analysis of acetate permease-like proteins from sequenced genomes. The amino acid sequences of the sodium-solute symporters were aligned with CLUSTAL_X software (Thompson *et al.*, 1997). Aligned sequences were imported into PAUP 4.0b10 to construct the phylogenetic tree (Swofford, 1998). Distances were determined using distance-based algorithms (neighbour-joining) (Saitou & Nei, 1987). Bootstrap values were obtained from 100

replicates. Preliminary sequence data from *Geobacter metallireducens*, *G. uraniireducens*, *G. bemidjensis*, *G. lovleyi* and FRC-32 were obtained from the DOE JGI website (<http://www.jgi.doe.gov>).

Design of degenerate primers for detection of *Geobacter* Group I *apl* genes in environmental samples. Nucleotide sequences of Group I *apl* genes from all available *Geobacter* genomes were aligned. Conserved regions were identified and used to design a pair of degenerate primers, 422Fw and 660Rv (Table 1), that produced a 222 bp amplicon. To verify that these primers targeted the Group I *apl* genes of the *Geobacter* species, they were tested using genomic DNA from *G. sulfurreducens*, *G. metallireducens*, *G. uraniireducens*, *G. bemidjensis* and *G. lovleyi* as templates. The PCR conditions used were: 95 °C, 3 min; (95 °C, 30 s; 60 °C, 45 s; 72 °C, 90 s), 30 cycles; 72 °C, 10 min – except for *G. bemidjensis*, where annealing temperature was 54 °C.

Environmental DNA sampling and extraction. The sample analysed was collected from groundwater extracted from well M21 of a uranium-contaminated aquifer undergoing acetate-stimulated bioremediation in Rifle, Colorado, USA (Holmes *et al.*, 2005). The sample corresponds to the M21 well of the 2005 study, on the 14th day of sampling, when the *Geobacter* content of the microbial community was 80 % (Holmes *et al.*, 2007). Cells were collected from groundwater using Sterivex filters (Millipore). Following cell collection, the filters were flash-frozen in a dry-ice/ethanol bath, shipped back to the laboratory at –20 °C and stored at –80 °C for further use. DNA was extracted from half of the filter using Bio 101 FastDNA soil kits (MP Biomedicals).

Clone library construction and diversity analysis. The acetate permease-like gene fragment was amplified from environmental DNA from well M21 using the degenerate primers and PCR conditions described above. The PCR products were subjected to electrophoresis on a 2 % agarose gel and the 222 bp amplicon was excised and purified. The purified PCR products were cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Ninety-six clones were picked and sequenced with the M13F and M13R primers at the University of Massachusetts Sequencing Facility. Neighbour-joining phylogenetic trees of nucleotide acid sequences with bootstrap values based on 100 replicates were constructed with MEGA 3 (Kumar *et al.*, 2004). Rarefaction curves for determination of diversity coverage were calculated using DOTUR software (Schloss & Handelsman, 2005) (not shown). The 16S rRNA genes were amplified

from environmental DNA with primers 8F (Eden *et al.*, 1991) and 519R (Lane *et al.*, 1985).

RESULTS AND DISCUSSION

Homologues of an *E. coli* acetate transporter in *Geobacter* species

Protein BLAST analysis revealed four homologues of the *E. coli* acetate permease, ActP, in the *G. sulfurreducens* genome. GSU1068, GSU1070, GSU2352 and GSU0518 were designated AplA, AplB, AplC and AplD, respectively, for acetate permease-like, and had previously been annotated as a sodium-solute symporter (Méthé *et al.*, 2003). Three of these, AplA, AplB and AplC, were highly similar to each other, with over 90 % identity at the amino acid level, and also shared 46 % identity with ActP of *E. coli*. The three homologues are 659 aa proteins with 13 predicted transmembrane segments and a signal peptide; their predicted molecular mass is ~61 kDa (<http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=ggs>). The physical arrangement of each of these genes in the chromosome suggests that each is in a two-gene operon that also contains an ORF encoding a conserved hypothetical protein (GSU1069, GSU1071 and GSU2353) (Fig. 1). Predicted promoter and terminator structures are located immediately upstream of the conserved hypothetical protein and downstream of the putative transporter sequences respectively. AplD is a less similar fourth paralogue, with an average 24 % identity to AplA, AplB and AplC and 30 % identity to ActP. It constitutes a 508 aa protein, also with 13 predicted transmembrane segments. Its chromosomal arrangement differs from its counterparts; its preceding hypothetical protein, GSU0519, does not share homology with GSU1069, GSU1071 or GSU2353. Furthermore, the ORFs corresponding to GSU0518 and GSU0519 overlap by 7 bp (Fig. 1).

Fig. 1. Physical arrangement of *apl* genes in the genome of *G. sulfurreducens*. Upper bars represent the deleted portions of the genome that were replaced by a kanamycin-resistance cassette in each of the three single mutants. Note that the coding sequences of GSU0518 and GSU0519 overlap by 7 bp. Operons, promoters and terminators were predicted using the commercial version of the FGENES-B software package (Softberry Inc.) as previously described (Yan *et al.*, 2004).

Phylogenetic analysis of Apl proteins in the family *Geobacteraceae* and other acetate-oxidizing bacteria

Putative acetate transporter homologues are widely conserved among acetate-oxidizing bacteria. Phylogenetic analysis indicated that these proteins can be divided into two distinct clades designated Group I and Group II (Fig. 2). It is worth noting that all members of the genus *Geobacter* whose genomes have been sequenced possess multiple Group I Apl homologues and a single Group II Apl homologue, including *G. metallireducens*, *G. lovleyi*, *G. uraniireducens*, *G. bemidjiensis* and *Geobacter* strain

FRC-32. In the case of *G. sulfurreducens*, AplA, AplB and AplC belong to Group I, while AplD belongs to Group II. All *Geobacter* Group I Apl sequences cluster in a tight, well-supported clade, with identities at or above 62 % and similarities at or above 79 %. Apl sequences from *Geobacter* spp. are more closely related to those of Firmicutes than they are to Apl sequences of other Proteobacteria, such as *Rhodospirillum rubrum* (Betaproteobacteria) or *Escherichia coli* (Gammaproteobacteria).

Group II also contains a separate *Geobacter* clade that includes the single Apl homologue from *Desulfuromonas acetoxidans*, a marine Fe(III)-reducing and acetate-oxidizing

Fig. 2. Phylogenetic analysis of sodium-solute symporters from *Geobacteraceae* and other acetate-oxidizing organisms. The phylogenetic tree was inferred from protein sequences by neighbour-joining (Saitou & Nei, 1987) with bootstrap values based on 100 replicates. The glucose symporter from *V. parahaemolyticus* was used as an outgroup. The characterized acetate transporter of *E. coli*, ActP, and *apl* genes from *G. sulfurreducens* are indicated in bold type.

member of the family *Geobacteraceae*. Other members from the *Deltaproteobacteria* (*Desulfovibrio vulgaris*) and *Gammaproteobacteria* (*Shewanella oneidensis*) possess putative acetate transporter homologues from Group II. Notably, no *Apl* homologues were found in *Pelobacter* species. These organisms are phylogenetically intertwined with acetate-oxidizing *Geobacteraceae*, but are unable to use acetate as an electron donor (Haveman *et al.*, 2006; Lovley *et al.*, 1995; Schink, 1984).

Expression in response to acetate limitation

In order to determine whether acetate limitation affected the expression of the *apl* genes, the ratio of transcript levels was quantified in continuous cultures of *G. sulfurreducens* grown under electron-donor-limiting or electron-acceptor limiting conditions (acetate and fumarate, respectively) by qRT-PCR. The expression of the Group I genes, *aplA*, *aplB* and *aplC*, was 6–9-fold higher under acetate-limiting conditions relative to fumarate-limiting conditions (Fig. 3). The transcript levels of each of the small conserved hypothetical proteins upstream each of the *apl* genes (GSU1069, GSU1071 and GSU2353) were also higher under acetate-limited conditions, consistent with the predicted operon structure. In contrast, transcript levels for flanking genes that were not predicted to be within the same operons (GSU1067, GSU1072 and GSU2354) were similar under both growth conditions (Fig. 3). Transcript levels for the Group II transporter, *aplD*, were not affected by either acetate or fumarate limitation.

These results were in agreement with microarray analyses of electron-donor-limiting versus electron-acceptor-limiting conditions. In *G. sulfurreducens*, transcription of the three members of Group I was upregulated under acetate-limited conditions, while the single member from Group II displayed no change in expression (A. Esteve-Núñez, unpublished). In *G. metallireducens*, transcription

of Gmet793, a closely related homologue of Group I with identities to GSU1068, GSU1070 and GSU2352 ranging from 89 % to 94 % (Fig. 2), was upregulated in acetate-limiting conditions. Transcription of Gmet3031, which belongs to Group II, was not affected (Didonato *et al.*, 2006). These results suggest that high levels of expression of Group I genes are indicative of acetate limitation, which is also consistent with their proposed role as acetate transporters. The lack of increased expression of Group II genes does not necessarily rule out a role in acetate transport, as constitutive expression of one of multiple acetate transporters might not be maladaptive.

Effect of gene deletion

Because of their apparent role in acetate uptake, the potential function of the Group I *apl* genes was further characterized by genetic analysis. Single mutants in which *aplA*, *aplB* or *aplC* was deleted were constructed by replacing each gene with a kanamycin-resistance cassette (Fig. 1). The acetate recovery and selection medium included an alternative electron donor (hydrogen) and carbon source (10 mM pyruvate) in order to enable growth should the mutant be incapable of uptaking acetate.

All the strains with a single mutation grew as well as the wild-type in a medium in which acetate was the electron donor and fumarate was the electron acceptor (Fig. 4a). When ferric citrate was used as electron acceptor, *aplA* and *aplB* mutants reduced Fe(III) at the same rate as the wild-type. The *aplC* mutant exhibited a diminished rate of Fe(III) reduction (Fig. 4b), but the biomass yield was similar to the wild-type (21.9 ± 0.7 mg total protein l^{-1}). In all strains, the acetate consumption profile during growth on ferric citrate matched the growth rate (Fig. 4c). The slow-growth phenotype of the *aplC* mutant during growth on ferric citrate was studied by measuring the acetate consumption of this mutant and the wild-type

Fig. 3. Upregulation of *G. sulfurreducens* Group I *apl* genes under acetate-limiting conditions. qRT-PCR was performed with the SYBR Green PCR Master kit (Applied Biosystems). Relative expression ratios (limiting acetate versus limiting fumarate) were statistically analysed by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The shading of the bars corresponds to that used in Fig. 1.

Fig. 4. (a) Growth of wild-type (DL1) and *apI* mutant strains in 20 mM acetate with 40 mM fumarate as electron acceptor (NBAF medium); (b) Fe(III) reduction of wild-type and *apI* mutants in 5 mM acetate, 56 mM ferric citrate (freshwater medium). Inocula (5 %) were exponential-phase acetate : fumarate cultures in both cases. Each determination represents the mean $\pm$ SD of triplicate cultures (error bars not shown where smaller than symbols). (c) Acetate concentration in freshwater medium during growth of wild-type and mutant strains in Fe(III) citrate. (d) Acetate consumption in resting cell suspensions of wild-type (DL1) and the *apIC* mutant prepared from cells grown in freshwater medium with 20 mM acetate and 56 mM Fe(III) citrate.

strain in resting cell suspensions, i.e. in growth-independent conditions with ferric citrate as electron acceptor. Interestingly, the rate of acetate consumption by the *apIC* mutant was comparable to that of the wild-type under these conditions (Fig. 4d). This indicates that the effect of the *apIC* mutation during growth on ferric citrate may not have been directly related to acetate uptake and thus it was not further investigated.

Given the high degree of similarity among the Group I *apI* genes, the fact that single mutants in these genes are not impaired in growth via acetate may be due to functional redundancy. In order to investigate this possibility, the mRNA levels of the remaining *apI* genes were measured in each of the single mutants and compared to those of the wild-type (Fig. 5). In the case of the *apIB* mutant, the expression of *apIA* was upregulated. Conversely, *apIB* was upregulated in the *apIA* mutant. In both cases, the levels of *apIC* and *apID* remained constant. In the case of the *apIC* mutant, only *apIB* was upregulated. These data suggest that compensatory changes in *apI* expression occur in *G. sulfurreducens*.

The construction of double knockouts of Group I genes using different antibiotic markers for each *apI* gene was

attempted in order to isolate and study the function of single *apI* genes. However, the introduction of *apIA::gm* or

Fig. 5. Ratio of *apI* transcript levels in three Group I *apI* single mutants relative to wild-type. qRT-PCR was performed with the SYBR Green PCR Master kit (Applied Biosystems). Relative expression ratios (wild-type versus mutant) were statistically analysed by the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

aplC::cm alleles was successful in DL1, but not in *aplB::kn* mutants (DLCR1). Similarly, introduction of *aplC::cm* and *aplB::kn* markers in *aplA::gm* mutants failed as well. It is worth mentioning here that the sequential introduction of antibiotic-marked mutations in *G. sulfurreducens* has been successful in other studies (Risso *et al.*, 2008; Segura *et al.*, 2008). To attempt to overcome this problem we tried a different strategy whereby the whole chromosomal region comprising *aplA* and *aplB* was replaced with a single antibiotic marker via a single step as previously described (Kim *et al.*, 2006). However, this effort also proved ineffective. This suggests that at least two of these three transporter genes are required to support growth.

Complementation of an *E. coli* strain lacking the ActP protein could not be accomplished because the cloned Group I members could not be functionally expressed (data not shown).

Thus, although the sequence similarity to the known acetate transporter in *E. coli* and the increased expression of the Group I transporter genes under acetate-limiting conditions suggest that these genes might be involved in acetate transport, it has not yet been possible to definitively prove this with a genetic approach. Similar difficulties were encountered during the study of σ^{54} , which could not be knocked out and was subsequently determined to be an essential gene (C. Leang, unpublished).

Detection of *apl* genes in a field sample collected during *in situ* acetate-mediated uranium bioremediation

Group I *apl* genes represent valid phylogenetic and physiological biomarkers due to their high degree of conservation among *Geobacter* species and their increased transcription in response to acetate limitation (Figs 2 and 3). Monitoring the expression of these genes may be an effective strategy for determining when acetate availability is limiting the growth and activity of *Geobacter* species in subsurface environments. This approach could be particularly helpful during *in situ* uranium bioremediation, when acetate is added to promote the reduction of soluble U(VI) to insoluble U(IV) (Anderson *et al.*, 2003; Lovley *et al.*, 1991; N'Guessan *et al.*, 2008) and fine tuning of acetate amendment is necessary to improve the efficiency of the process. As a preliminary step towards implementing this strategy, it was necessary to determine the feasibility of detecting *apl* genes in *Geobacter*-dominated subsurface environments.

Since the Group I genes are highly conserved among *Geobacter* species (Fig. 1), degenerate PCR primers 422Fw and 640Rv were designed based on a sequence alignment of Group I sequences of *Geobacter* species spanning a particularly well-conserved region of 222 bp. This primer pair was tested by successfully amplifying *apl* fragments using DNA from each *Geobacter* species available in our culture collection as template (data not shown).

The environmental sample analysed in this study consisted of groundwater collected from a uranium-contaminated aquifer located in Rifle, Colorado, on the 14th day of an acetate-stimulated bioremediation field study (Anderson *et al.*, 2003). *Geobacteraceae* 16S rRNA gene sequences accounted for 80 % of the microbial community in this sample (Holmes *et al.*, 2007), and six different *Geobacter* 16S rRNA phylotypes were detected (Fig. 6a). Genomic DNA from this sample was also used to construct an *apl* clone library with the degenerate primers described above (Fig. 6b). Degenerate primers amplified six unique *apl* sequences that were all *apl* Group I homologues closely related to *G. uraniireducens* and *G. bemidjiensis*. This is significant because these two organisms are members of a clade of *Geobacter* species that predominate in a diversity of subsurface environments in which dissimilatory metal reduction is an important process (Holmes *et al.*, 2007).

Implications

The results indicate that *Geobacter* species contain multiple copies of *apl* genes, and that, at least in *G. sulfurreducens* and *G. metallireducens*, those in the phylogenetic cluster designated Group I are more highly expressed when growth is limited by acetate availability. The presence of multiple copies of putative acetate transporters in the genomes of the *Geobacter* species suggests not only functional redundancy, but also an adaptation to the nutrient-deprived status that micro-organisms often encounter in their natural environment. Such gene redundancy is common for essential metabolic pathways in this specialized organism (Mahadevan *et al.*, 2006; Segura *et al.*, 2008). Although the role of the putative acetate transporters could not be definitively confirmed with genetic approaches, the finding that the Group I transporters are more highly expressed under acetate-limiting conditions suggests that the expression of these genes may aid in diagnosing the metabolic state of *Geobacter* species in subsurface environments. Expression of genes related to other metabolic functions such as oxidation of acetate via the tricarboxylic acid cycle (Holmes *et al.*, 2005, 2007), nitrogen fixation (Holmes *et al.*, 2004b) and iron assimilation (O'Neil *et al.*, 2008) by the natural community of *Geobacter* species that predominates during *in situ* uranium bioremediation has made it possible to gain insights into rates of metabolism and factors limiting the growth of *Geobacter* species during bioremediation. The finding that genes highly similar to the Group I transporter genes in *Geobacter* pure cultures are also present in the *Geobacter* species that are abundant during *in situ* uranium bioremediation suggests that monitoring the expression levels of Group I genes in the subsurface may aid the optimization of acetate-mediated bioremediation processes. Preliminary data from field studies indicate that *apl* genes can be detected in mRNA from environmental samples from *Geobacter*-dominated field sites undergoing *in situ* uranium bioremediation, and that there is a correlation between expression levels and

Fig. 6. (a) Phylogenetic tree constructed by maximum-parsimony analysis comparing *Geobacter* 16S rRNA gene sequences detected in groundwater collected from a uranium-contaminated aquifer in Rifle, Colorado, undergoing bioremediation to 16S rRNA gene sequences from other *Geobacteraceae* isolates. Bootstrap values were obtained from 100 replicates, and *Desulfovibrio profundus* and *Desulfobulbus propionicus* were used as outgroups. (b) Analysis of clone library of *apl* genes amplified from the sample described above. Partial sequences corresponding to the 222 bp amplicon from cultured *Geobacter* and other reference organisms were included for comparison. The phylogenetic tree was inferred from nucleotide acid sequences by neighbour-joining (Saitou & Nei, 1987), with bootstrap values based on 100 replicates, using MEGA 3 software (Kumar *et al.*, 2004).

acetate availability (H. Elifantz, unpublished results). This information could be used to guide the necessary adjustments to groundwater acetate conditions to ensure that acetate availability is not limiting or, if desired, to restrict acetate inputs to slow down the rate of metal reduction and ensure that U(VI)-reducing *Geobacter* species remain as dominant components of the subsurface microbial community.

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