

Involvement of *Geobacter sulfurreducens* SfrAB in acetate metabolism rather than intracellular, respiration-linked Fe(III) citrate reduction

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A soluble ferric reductase, SfrAB, which catalysed the NADPH-dependent reduction of chelated Fe(III), was previously purified from the dissimilatory Fe(III)-reducing micro-organism *Geobacter sulfurreducens*, suggesting that reduction of chelated forms of Fe(III) might be cytoplasmic. However, metabolically active spheroplast suspensions could not catalyse acetate-dependent Fe(III) citrate reduction, indicating that periplasmic and/or outer-membrane components were required for Fe(III) citrate reduction. Furthermore, phenotypic analysis of an SfrAB knockout mutant suggested that SfrAB was involved in acetate metabolism rather than respiration-linked Fe(III) reduction. The mutant could not grow via the reduction of either Fe(III) citrate or fumarate when acetate was the electron donor but could grow with either acceptor if either hydrogen or formate served as the electron donor. Following prolonged incubation in acetate : fumarate medium in the absence of hydrogen and formate, an 'acetate-adapted' SfrAB-null strain was isolated that was capable of growth on acetate : fumarate medium but not acetate : Fe(III) citrate medium. Comparison of gene expression in this strain with that of the wild-type revealed upregulation of a potential NADPH-dependent ferredoxin oxidoreductase as well as genes involved in energy generation and amino acid uptake, suggesting that NADPH homeostasis and the tricarboxylic acid (TCA) cycle were perturbed in the 'acetate-adapted' SfrAB-null strain. Membrane and soluble fractions prepared from the 'acetate-adapted' strain were depleted of NADPH-dependent Fe(III), viologen and quinone reductase activities. These results indicate that cytoplasmic, respiration-linked reduction of Fe(III) by SfrAB *in vivo* is unlikely and suggest that deleting SfrAB may interfere with growth via acetate oxidation by interfering with NADP regeneration.

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Abbreviations: AQDS, anthraquinone 2,6-disulfonate; FNOR, ferredoxin oxidoreductase; QRT-PCR, quantitative RT-PCR. The Array Express (www.ebi.ac.uk/arrayexpress/) accession number for the microarray data for this paper is E-TIGR-130.

Three supplementary figures, and supplementary tables of the primers used in QRT-PCR and genes that are up- or downregulated in the acetate-adapted SfrAB-null strain during growth in chemostats in acetate : fumarate medium are available with the online version of this paper, together with an overview and additional discussion of the microarray analysis.

INTRODUCTION

Geobacter species are dissimilatory Fe(III)-reducing *Deltaproteobacteria* that have evolved the ability to transfer electrons across the outer membrane and onto a variety of electron acceptors that are too large to enter the cell, including insoluble Fe(III) and Mn(IV) oxides (Lovley *et al.*, 2004; Reguera *et al.*, 2005), the quinone groups of humic acids (Lovley *et al.*, 1996; Scott *et al.*, 1998), and the anodes of microbial fuel cells (reviewed by Lovley, 2006). In addition, *Geobacter* species can reduce soluble electron

acceptors that have the potential to enter the periplasm and/or cytoplasm. These include fumarate, low-molecular-mass electron-shuttling compounds such as anthraquinone 2,6-disulfonate (AQDS), the radionuclides U(VI) and Tc(VII), and a variety of chelated forms of Fe(III) (Lovley *et al.*, 2004).

Valuable insights into the electron transport pathways to Fe(III) have been gained through the study of two *Geobacter* species, *Geobacter metallireducens* (Lovley *et al.*, 1993) and *Geobacter sulfurreducens* (Caccavo *et al.*, 1994), for which a complete genome sequence and a genetic system are available (Coppi *et al.*, 2001; Methé *et al.*, 2003). Due to technical considerations, chelated forms of Fe(III), most often Fe(III) citrate and Fe(III) chelated with nitrilotriacetic acid (Fe(III)-NTA), are frequently used for laboratory studies addressing Fe(III) reduction by *Geobacter* species (Butler *et al.*, 2004; Ding *et al.*, 2006; Esteve-Núñez *et al.*, 2005; Kim *et al.*, 2005, 2006; Leang *et al.*, 2003; Lloyd *et al.*, 2003; Methé *et al.*, 2005; Núñez *et al.*, 2004, 2006). An understanding of the similarities and differences between the electron transport pathways to Fe(III) citrate and insoluble Fe(III) oxides is, therefore, crucial for determining the environmental relevance of these studies.

The reduction of Fe(III) oxides clearly occurs at the cell surface (Lovley *et al.*, 2004; Nevin & Lovley, 2000) and involves a variety of outer-membrane components, including cytochromes (Mehta *et al.*, 2005), a putative multicopper protein (Mehta *et al.*, 2006) and pili, which are postulated to act as electrically conductive nanowires extending the Fe(III)-oxide reducing capacity of *Geobacter* species beyond the cell surface (Reguera *et al.*, 2005). In contrast, the subcellular localization of the reduction of chelated forms of Fe(III) is not known. Genetic studies conducted in *G. sulfurreducens* have implicated several periplasmic and outer-membrane cytochromes in Fe(III) citrate reduction (Butler *et al.*, 2004; Kim *et al.*, 2005, 2006; Leang *et al.*, 2003; Lloyd *et al.*, 2003), suggesting that the reduction of Fe(III) citrate might occur at the cell surface. Biochemical studies performed in *G. sulfurreducens* have provided conflicting information regarding the site of Fe(III) chelate reduction. Purification of SfrAB, a two-subunit complex capable of catalysing the NADPH-dependent reduction of Fe(III)-NTA, from the soluble fraction (Kaufmann & Lovley, 2001) suggested that Fe(III) chelates might be reduced within the cytoplasm. Membrane-bound, multi-subunit complexes with the capacity to catalyse NADH-dependent Fe(III)-NTA reduction (Gaspard *et al.*, 1998; Magnuson *et al.*, 2000) have also been isolated, one of which was reported to be associated the outer-membrane fraction (Gaspard *et al.*, 1998).

Both the subcellular localization of Fe(III) citrate reduction and the physiological role of SfrAB were addressed in this study. The results of this work are consistent with Fe(III) citrate reduction occurring outside the cytoplasm, and suggest that SfrAB is involved in acetate metabolism and

does not participate directly in the reduction of Fe(III) chelates, as was previously proposed (Kaufmann & Lovley, 2001).

METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain DH5 α [*supE44* ϕ 80*lacZ* Δ M15 Δ (*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17 phoA supE44 thi-11 gyrA96 relA1* λ] (Woodcock *et al.*, 1989) was used for routine DNA manipulations. *G. sulfurreducens* strain DL1, a wild-type derivative of ATCC 51573 (Caccavo *et al.*, 1994; Coppi *et al.*, 2001), was obtained from our laboratory culture collection. During growth of *G. sulfurreducens* strains in batch cultures, cells were cultivated anaerobically at 30 °C under an N₂:CO₂ (80:20) atmosphere in two defined liquid freshwater media, NBF and FWFC, which contained 40 mM fumarate or 55 mM Fe(III) citrate, respectively, as the electron acceptor (Lovley & Phillips, 1988; Coppi *et al.*, 2001, 2004). Acetate (1–15 mM), formate (40 mM), cysteine (1 mM), and antibiotics (200 μ g kanamycin ml⁻¹ and/or 400 μ g streptomycin ml⁻¹) were added from concentrated stock solutions as needed. Unless otherwise indicated, cysteine was routinely added to all fumarate media as a reductant.

Culture in the presence of hydrogen was accomplished by injecting 10 ml hydrogen gas into the headspace of 27 ml pressure tubes containing 10 ml medium, resulting in a headspace composition of 37 % H₂, 12.6 % CO₂, 50.4 % N₂ at a total pressure of $\sim 1.61 \times 10^5$ Pa. Hydrogen-containing tubes were incubated horizontally at 30 °C with constant shaking at 100 r.p.m.

Continuous culture of wild-type and mutant strains was performed as previously described (Esteve-Núñez *et al.*, 2005). Briefly, cells were cultured under an N₂:CO₂ (80:20) atmosphere at 30 °C and a dilution rate of 0.05 h⁻¹ in a working volume of 200 ml antibiotic- and reductant-free freshwater medium (Lovley & Phillips, 1988) containing 27.5 mM fumarate as the electron acceptor and a limiting concentration of acetate (5 mM) as electron donor and carbon source.

Preparation of whole-cell and spheroplast suspensions. All buffers were prepared using strict anaerobic techniques and all manipulations were carried out at 30 °C in an anaerobic chamber containing an atmosphere of 7 % H₂ and 10 % CO₂ balanced with N₂. Intact-cell and spheroplast suspensions were prepared in parallel from two 500 ml aliquots derived from a single, mid-exponential, acetate:fumarate (15 mM:40 mM) culture. Intact-cell suspensions were prepared as previously described (Coppi *et al.*, 2004; Leang *et al.*, 2003). Because previously published methods for the production of *G. sulfurreducens* spheroplasts (Galushko & Schink, 2000; Kaufmann & Lovley, 2001) failed to yield homogeneous spheroplast preparations reproducibly, the spheroplast preparation protocol of Witholt *et al.* (1976) was adapted for use in *G. sulfurreducens*. Intact cells were harvested by centrifugation for 15 min at 6000 g and resuspended in ~ 100 ml spheroplast wash medium, consisting of (g l⁻¹): 0.42 KH₂PO₄, 0.22 K₂HPO₄, 0.38 KCl, 4.96 NaCl, 1.8 NaHCO₃ and 0.5 Na₂CO₃. The washed cells were then pelleted by centrifugation at 6000 g for 6 min and resuspended in 30 ml spheroplast wash medium containing 350 mM sucrose. Following another 6 min centrifugation at 6000 g, the cells were resuspended in 10 ml 250 mM Tris/HCl (pH 7.5), and this was designated time zero. After 1 min of incubation at 30 °C, 1 ml 500 mM EDTA (pH 8.0) was added, followed by the addition of 10 ml 700 mM sucrose at 2 min, 150 mg lysozyme at 3.5 min, and 20 ml water at 4 min to induce osmotic shock. Immediately following the osmotic shock, spheroplasts were harvested by centrifugation at 20 000 g for 10 min. The supernatant (periplasmic fraction) was reserved for further analysis, and the

spheroplasts were resuspended in 5 ml spheroplast wash medium containing 350 mM sucrose.

Acridine orange staining and epifluorescence microscopy of whole-cell and spheroplast preparations as well as transmission electron microscopy of spheroplast thin sections were performed as previously described (Afkar *et al.*, 2005; Lovley & Phillips, 1988). A cytoplasmic fraction was prepared by subjecting spheroplast suspensions to two passes through a French pressure cell at 40 000 kPa followed by low-speed centrifugation at 20 000 *g* for 5 min at 4 °C to remove intact spheroplasts and debris, and high-speed centrifugation at 100 000 *g* for 45 min at 4 °C to remove membranes.

Resting intact-cell and spheroplast suspension assays. Cell suspension assays were carried out in an isotonic buffer (basal wash medium; Leang *et al.*, 2003) as previously described (Coppi *et al.*, 2004). Spheroplast suspension assays were performed identically except for the substitution of the basal wash medium with spheroplast wash buffer containing 350 mM sucrose (see above). Suspensions were provided with 20 mM Fe(III) citrate, 10 mM fumarate or 1 mM AQDS as the electron acceptor and were incubated in either the absence or presence of 20 mM acetate.

DNA extraction and manipulation. *G. sulfurreducens* genomic DNA was extracted using the MasterPure complete DNA & RNA Purification kit (Epicentre Technologies) or the Genome DNA kit (Bio 101). Taq DNA polymerase (Qiagen) was used for all PCR amplifications. Plasmid purification, PCR product purification and gel extractions were performed with the following kits: the QIAprep Spin Miniprep kit, the Qiagen Plasmid Midi kit, the QIAquick PCR Purification kit, and the QIAquick Gel Extraction kit (Qiagen). Ligations, transformations into *E. coli*, Southern blotting and other routine DNA manipulations were carried out according to the methods outlined in Sambrook *et al.* (1989). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Radiolabelled probes for Southern analysis were labelled with the NEBlot kit (New England Biolabs) and [α -³²P]dCTP (Perkin Elmer) according to the manufacturer's instructions.

Construction of an SfrAB-null (Δ sfrAB::kan) strain. SfrAB is encoded by a two-gene cluster (*sfrBA*), consisting of the *sfrB* gene followed by a 126 bp intergenic region and the *sfrA* gene (Kaufmann & Lovley, 2001; Methé *et al.*, 2003). In order to disrupt the *sfrBA* cluster by homologous recombination, a linear DNA fragment, consisting of a kanamycin resistance cassette flanked by the first 0.58 kb of the *sfrB* gene and the last 0.54 kb of the *sfrA* gene, was constructed. Insertion of this fragment into the chromosome via a double recombination event would result in replacement of 78 % of the *sfrBA* cluster (the final 72 % of the *sfrB* gene, the intergenic region, and the initial 80 % of the *sfrA* gene) with the kanamycin resistance cassette.

The 0.58 kb *sfrB* and 0.54 kb *sfrA* fragments were amplified from *G. sulfurreducens* genomic DNA with primer combinations FR01 and FR02 (5'-GCGAAGGTCTGAACGGAGG-3' and 5'-CGCGGATGATCAAGCG-3') and FR03 and FR04 (5'-GAGTCGCTTGATATCATCCGCAACGCCGTGAGGACTGGG-3' and 5'-CGATTGTTCCGCCGAATCAAGC-3'), respectively (*EcoRV* sites indicated in bold). Using the amplification conditions described by Lloyd *et al.* (2003) and an annealing temperature of 50 °C, the two fragments were fused by recombinant PCR and the resultant 1.12 kb fusion product was amplified with distal primers, FR01 and FR04. The fusion product was subsequently inserted into the pGem-T Easy vector (Promega) according to the manufacturer's instructions, and the resultant plasmid was cleaved with *EcoRV*, which cut at the junction of the *sfrB* and *sfrA* fragments. A kanamycin resistance cassette was amplified from pBBR1-MCS2 (Kovach *et al.*, 1994) with primers KanEcoRV and KanR (5'-CCCGATATCGGATGAATGTCAGC-3'

and 5'-CCCGATATCGCGGTGGAATCG-3', *EcoRV* sites indicated in bold), treated with *EcoRV* and inserted at the *EcoRV* site at the junction of the *sfrB* and *sfrA* fragments. A plasmid containing the kanamycin resistance cassette in the same orientation as the fragments of the *sfrB* and *sfrA* coding regions was identified and designated pTfr. The final 2.3 kb linear fragment used for disruption of the *sfrBA* cluster was excised from pTfr with *EcoRI*.

Electroporation and mutant isolation were performed as previously described (Coppi *et al.*, 2001; Lloyd *et al.*, 2003). Kanamycin-resistant colonies were recovered on NBAF-YE plates (Coppi *et al.*, 2001) supplemented with 200 μ g kanamycin ml⁻¹, purified by streaking, and inoculated into pressure tubes containing 10 ml kanamycin-containing acetate:fumarate medium and 10 ml hydrogen gas in the headspace. The genotype of these kanamycin-resistant strains was determined by Southern blotting and by screening with PCR primers FR01 and FR04, which yield differently sized amplicons, 2.3 and 4.7 kb, respectively, for the SfrAB-null and wild-type strains. During Southern blot analysis, genomic DNA from wild-type *G. sulfurreducens* and various kanamycin-resistant isolates was digested with *Bgl*III, and blots were probed with the 2.3 kb fragment of pTfr used for homologous recombination. The presence and placement of the Δ sfrAB::kan mutation was confirmed by detection of two fragments (1.2 kb and 1.9 kb) in kanamycin-resistant isolates versus a single radiolabelled fragment (5.5 kb) in genomic DNA from the wild-type strain (data not shown). One of the mutants was selected as the representative SfrAB-null strain.

Because a large segment of the *sfrBA* cluster had been replaced with the kanamycin resistance cassette in the SfrAB-null strain, reversion to wild-type by excision of the antibiotic cassette was not possible, rendering further selection with antibiotics unnecessary. The SfrAB-null strain was subsequently cultured in the absence of antibiotics and screened routinely for wild-type contamination with primers FR01 and FR04 (described above) as well as primers FR01 and FRWT (5'-GCACCATGATGTTGACCGAG-3'), which amplify sequences from the wild-type but not the SfrAB-null strain.

Construction of an SfrAB expression vector and complementation of the acetate-adapted SfrAB-null strain. A plasmid from a *G. sulfurreducens* genomic DNA library containing the *sfrBA* cluster was obtained from Barbara Methé at the Institute for Genomic Research (Rockville, MD, USA). The *sfrBA* cluster was excised from this plasmid with the restriction enzymes *Bsi*WI and *Bst*BI, treated with Klenow fragment and inserted into the *Sma*I site of pCDS (Coppi *et al.*, 2001) to generate the expression vector pCDSsfrAB. The acetate-adapted SfrAB-null strain was made electrocompetent and transformed with pCDSsfrAB as previously described (Coppi *et al.*, 2001). Streptomycin-resistant colonies were recovered on NBAF-YE plates supplemented with 400 μ g streptomycin ml⁻¹. The resulting streptomycin-resistant colonies were inoculated into liquid medium containing 400 μ g streptomycin ml⁻¹ and screened for the presence of the plasmid by PCR amplification of a vector-specific fragment with the universal M13-reverse primer and primer FRWT and by isolating plasmid DNA and digesting it with *Eco*RI. Amplification conditions were 3 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C, and a final 10 min extension at 72 °C. One plasmid-containing, SfrAB-null strain was selected for further study; this strain was cultured in medium supplemented with streptomycin at all times.

Enzymic assays. Membrane and soluble fractions for enzymic assays were prepared from batch or chemostat cultures under anaerobic conditions as described by Kaufmann & Lovley (2001) except that intact cells were washed in 50 mM HEPES (pH 7.0), 1 mM MgSO₄ and resuspended in 50 mM Tris/HCl (pH 7.5), 1 mM MgSO₄ prior to lysis in the French pressure cell. All enzymic assays were carried out

at 30 °C using strict anaerobic techniques, except for measurement of malate dehydrogenase activity, which was performed aerobically. Malate dehydrogenase activity was determined by monitoring the oxidation of NADH at 340 nm ($\epsilon=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) in the presence of oxaloacetate (Reeves *et al.*, 1971). Pyruvate-ferredoxin oxidoreductase and 2-oxoglutarate-ferredoxin oxidoreductase activity were determined by monitoring pyruvate- or oxoglutarate-dependent reduction of benzyl viologen at 578 nm ($\epsilon=8.6 \text{ mM}^{-1} \text{ cm}^{-1}$) in a modified form of the assay buffer used by Brandis-Heep *et al.* (1983), which consisted of 100 mM Tricine/NaOH (pH 8.5), 0.05 mM coenzyme A, 0.1 mM thiamine pyrophosphate, 5 mM MgCl₂, 5 mM β -mercaptoethanol, 1 mM benzyl viologen and either 10 mM 2-oxoglutarate or 10 mM pyruvate. NADPH-dependent reduction of Fe(III)-NTA was determined by monitoring the formation of an Fe(II)-ferrozine complex at 562 nm ($\epsilon=28 \text{ mM}^{-1} \text{ cm}^{-1}$) as previously described (Kaufmann & Lovley, 2001). NADPH-dependent reduction of benzyl viologen and AQDS reduction were determined in a reaction buffer containing Tris/HCl (pH 7.5), 0.2 mM NADPH and either 1 mM benzyl viologen or AQDS by monitoring the reduction of benzyl viologen at 578 nm ($\epsilon=8.6 \text{ mM}^{-1} \text{ cm}^{-1}$) or that of AQDS at 436 nm ($\epsilon=3.5 \text{ mM}^{-1} \text{ cm}^{-1}$).

Microarray analysis of global gene expression. A description of the microarray experiment and relevant data in MIAME format have been deposited in the Array Express repository (www.ebi.ac.uk/arrayexpress/; accession number E-TIGR-130). The methods used for cell harvesting, RNA preparation, microarray design and microarray hybridization have been previously described (DiDonato *et al.* 2006; Methé *et al.*, 2005; Núñez *et al.*, 2006). Briefly, total mRNA was isolated from three pairs of identically treated steady-state chemostat cultures of the wild-type and acetate-adapted SfrAB-null strains (biological replicates). cDNA was prepared from 5 μg total RNA from each member of a biological replicate and labelled with either cyanine3 or cyanine5, then mixed and hybridized to the microarray. A total of 22 replicate hybridizations were carried out, six for the first biological replicate and eight for each of the remaining two.

Signal intensities from the replicate hybridizations could not be treated as independent values, because the variation among signal intensities for replicate hybridizations performed on RNA extracted from the individual biological replicates was significantly smaller than the variation among all of the replicate hybridizations. Differentially expressed genes were, therefore, identified by combining two independent statistical approaches. In the first, signal intensities from the replicate hybridizations for each of the three biological replicates were analysed separately using significance analysis of microarrays (SAM; Tusher *et al.*, 2001) as previously described (Méthé *et al.*, 2005). Only the genes (147) that were identified by SAM in at least two out of three biological replicates were considered to be differentially expressed. Differentially expressed genes were also identified by linear models with B statistics by applying the LIMMA statistical software package (Smyth, 2005; <http://bioinf.wehi.edu.au/limma/>). Specifically, for each gene, a linear model with fixed biological replicate effects was used to estimate the average log gene expression ratio of all replicates. The moderated *t*-statistics for the estimate (standard error moderated based on the empirical Bayes shrinkage), the log-odd of differential expression (the B statistic), and the false-discovery-rate-adjusted *P*-value were then computed. A total of 165 differentially expressed genes with adjusted *P*-values $<1 \times 10^{-7}$ and *B*-values greater than 10.0 were identified using this procedure. There was considerable overlap (90%) between the results of the two statistical analyses.

Quantitative reverse transcriptase PCR (QRT-PCR). Cells from three steady-state, wild-type or *sfrAB::kan* acetate: fumarate chemostats were harvested by centrifugation and pooled for RNA extraction. Total RNA was extracted with the RNeasy kit (Qiagen Inc) and any residual DNA was removed via an on-column RNase treatment,

performed according to the manufacturer's instructions. cDNA was synthesized with the Superscript I RNaseH reverse transcriptase and random primers (Invitrogen) and QRT-PCR was performed with the SYBR Green PCR Master kit (Applied Biosystems). Relative expression levels were calculated by the $2^{-\Delta\Delta\text{CT}}$ method (Livak & Schmittgen, 2001). Gene-specific primers utilized for QRT-PCR are listed in Supplementary Table S1, available with the online version of this paper.

Analytical techniques. Protein concentrations were determined by the bicinchoninic acid method with BSA as a standard (Smith *et al.*, 1985). Cell densities were determined by acridine orange staining and epifluorescence microscopy (Lovley & Phillips, 1988). Fe(II) concentrations were determined with the ferrozine assay (Lovley & Phillips, 1988). Turbidity was monitored by placing pressure tubes (path length 1.5 cm) directly into a Genesys 2 spectrophotometer (Spectronics Instruments). Organic acids produced during spheroplast and cell suspension studies were separated, with a Fast Acid Analysis column (Bio-Rad) and a mobile phase of 5 mM sulfuric acid on an HP series 1100 high pressure liquid chromatograph equipped with a UV detector, and quantified at 215 nm.

RESULTS AND DISCUSSION

Fe(III) reduction by spheroplasts

Suspensions of *G. sulfurreducens* spheroplasts were prepared in order to investigate the subcellular localization of Fe(III) citrate reduction. The homogeneity and integrity of these suspensions was confirmed by phase-contrast and transmission electron microscopy (Fig. 1a) as well as by comparison of the amount of malate dehydrogenase activity present in a lysed spheroplast suspension ($7.44 \mu\text{mol mg}^{-1} \text{ min}^{-1}$) with that in the periplasmic fraction that was collected during spheroplast preparation ($0.002 \mu\text{mol mg}^{-1} \text{ min}^{-1}$).

With acetate serving as the electron donor, the spheroplasts reduced fumarate, an intracellular electron acceptor (Butler *et al.*, 2006), at a rate comparable to that of intact cells for up to 3 h (Fig. 1b) but reduced Fe(III) citrate at less than 5% of the rate of intact cells (Fig. 1c). This result indicated a requirement for periplasmic and/or outer-membrane components for the acetate-dependent reduction of Fe(III) citrate and was in agreement with previous studies, in which deletion of genes coding for a variety of periplasmic or outer membrane proteins impaired Fe(III) citrate reduction (Afkhar *et al.*, 2005; Butler *et al.*, 2004; Kim *et al.*, 2005, 2006; Leang *et al.*, 2003; Lloyd *et al.*, 2003). Spheroplasts also failed to reduce AQDS with acetate serving as the electron donor (Fig. 1d).

Construction and phenotypic analysis of an SfrAB-null strain

The evidence described above, which was consistent with reduction of soluble Fe(III) outside the cytoplasm, did not support the hypothesis that SfrAB might function as a cytoplasmic Fe(III) reductase (Kaufmann & Lovley, 2001). In order to further evaluate the physiological function of SfrAB, an SfrAB-null strain ($\Delta\text{sfrAB}::\text{kan}$) was constructed

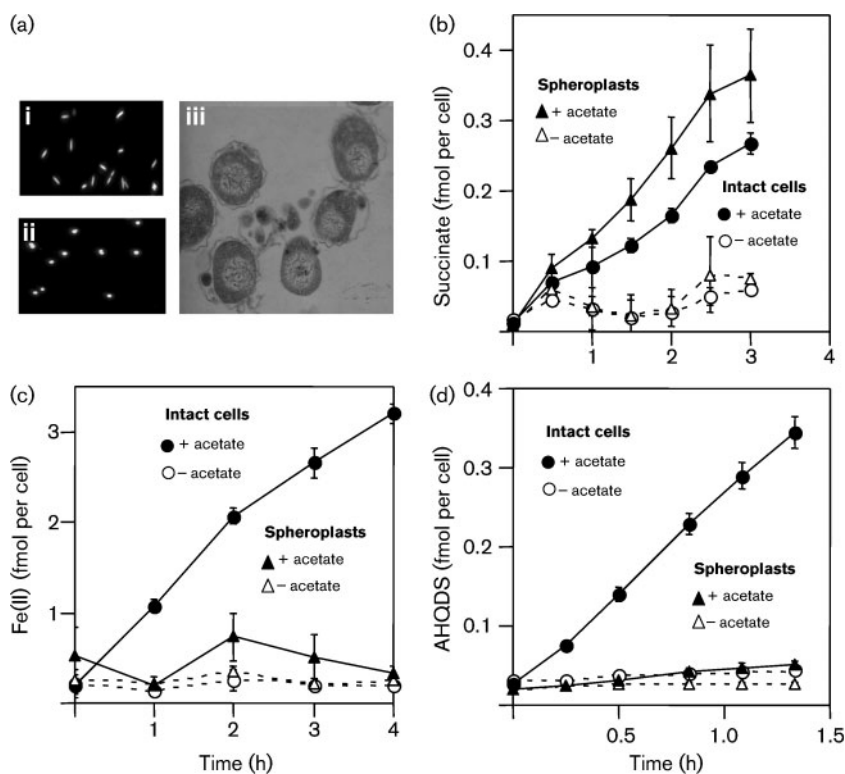


Fig. 1. Properties of *G. sulfurreducens* spheroplasts. (a) Visual characterization of intact cell and spheroplast suspensions. Acridine orange staining of (i) intact cells and (ii) spheroplast suspensions, and (iii) transmission electron microscopy of thin sections of spheroplasts. (b–d) Acetate-dependent reduction of (b) fumarate, (c) Fe(III) citrate and (d) AQDS by intact cell and spheroplast suspensions. Data are means \pm SD of determinations for triplicate incubations.

by replacing 78% of the *sfrBA* cluster with a kanamycin-resistance cassette by homologous recombination.

Wild-type *G. sulfurreducens* can utilize acetate as both an electron donor and a carbon source (Caccavo *et al.* 1994) but cannot exploit either citrate or fumarate for either of these purposes (Esteve-Núñez *et al.*, 2005; Galushko & Schink, 2000). Both the wild-type and the SfrAB-null strains were able to grow in Fe(III) citrate medium when either hydrogen or formate was provided as the electron donor (Fig. 2b, c) and a small amount of acetate was provided as a carbon source (1 mM; Coppi *et al.*, 2004). In contrast, only the wild-type strain could grow in Fe(III) citrate medium when acetate was the sole electron donor and carbon source present (Fig. 2a). A similar phenotype was observed when fumarate served as the electron acceptor (data not shown). The SfrAB-null strain failed to grow in the presence of acetate and fumarate unless an additional electron donor, either hydrogen or formate, was present. These results suggested that SfrAB was not required for Fe(III) citrate reduction, but was necessary for acetate metabolism, most probably acetate oxidation via the tricarboxylic acid (TCA) cycle (Galushko & Schink, 2000). Like the wild-type strain (Coppi *et al.*, 2004), the SfrAB-null strain could still utilize acetate as a carbon source during growth via hydrogen- and formate-dependent Fe(III) reduction.

A specific role for SfrAB in acetate oxidation was also supported by comparative genomic analyses. SfrAB-encoding gene clusters are present exclusively in the

genomes of acetate-oxidizing members of the *Geobacteraceae*, and are notably absent from the genomes of *Geobacteraceae* belonging to the genus *Pelobacter*, which can assimilate acetate but cannot use it as an electron donor (Schink, 1984).

Phenotypic characterization of an SfrAB-null strain with the capacity to grow in acetate:fumarate medium

Two to three weeks after a 1% transfer of the SfrAB-null mutant from H₂/acetate/fumarate medium into acetate:fumarate medium lacking hydrogen and antibiotics, growth of the SfrAB-null strain was detected. Following this adaptation period, the SfrAB-null strain was able to grow in acetate:fumarate medium with a doubling time and maximum yield that were $118 \pm 13\%$ and $81 \pm 1.5\%$, respectively, of those of the wild-type (Fig. 3a). This change in the phenotype of the SfrAB-null strain was stable and was not due to cross-contamination with the wild-type strain (determined by PCR screening with primers FR01 and FRWT) or to reversion of the gene disruption (determined by PCR screening with primers FR01 and FR04; data not shown). The SfrAB-null strain with the capacity to utilize acetate as an electron donor was designated 'acetate-adapted'.

Surprisingly, the acetate-adapted SfrAB-null strain was unable to grow when transferred into acetate:Fe(III) citrate medium (Fig. 3b). Fe(III) reduction by the acetate-adapted SfrAB-null strain was undetectable for up

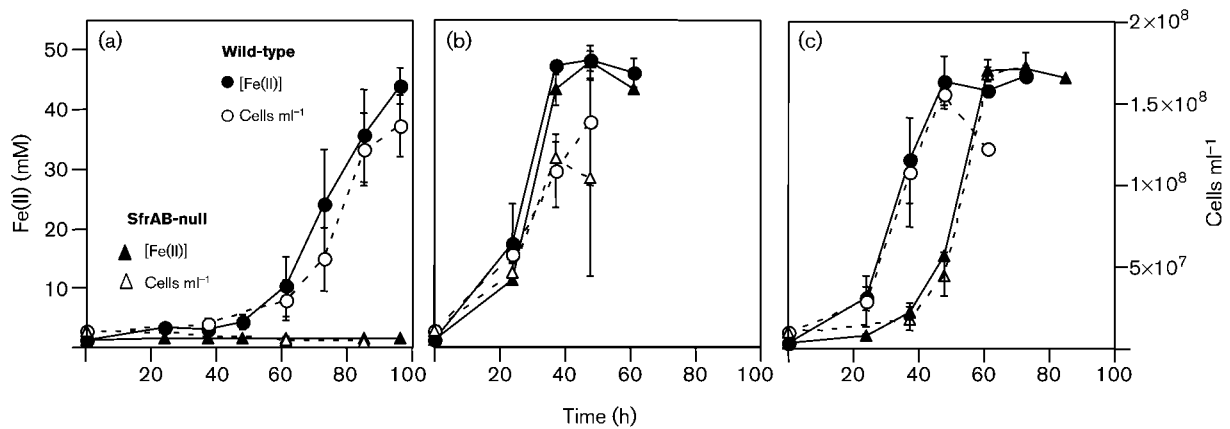


Fig. 2. Growth of wild-type and SfrAB-null strains in Fe(III) citrate medium containing 1 mM acetate as a carbon source and either (a) additional acetate (9 mM), (b) hydrogen (60 kPa) or (c) formate (40 mM) as the electron donor. Exponential-phase cultures growing in acetate:fumarate medium under an N₂:CO₂ atmosphere supplemented with 60 kPa hydrogen were pelleted and resuspended in donor-free freshwater Fe(III) citrate medium prior to inoculation for growth studies. Data are means ± SD of triplicate cultures.

to 2 weeks, whereas the wild-type strain completely reduced the Fe(III) in the growth medium within 48 h.

The slight reduction in the growth rate and yield of the acetate-adapted SfrAB-null strain in acetate:fumarate medium relative to the wild-type and the inability of the strain to grow in acetate:Fe(III) citrate medium suggested that the ‘acetate-adapted’ strain had only partially compensated for the loss of SfrAB and could not metabolize acetate at the wild-type rate. Growth of *G. sulfurreducens* via Fe(III) citrate reduction requires a faster rate of acetate oxidation than growth via fumarate reduction. During growth of wild-type *G. sulfurreducens* in chemostats, the rate of acetate uptake was approximately fourfold higher when Fe(III) citrate served as the electron acceptor than

when fumarate served as the acceptor (Esteve-Núñez *et al.*, 2005). In addition, *in silico* simulations of central metabolism indicated that, in order to achieve equivalent growth rates, *G. sulfurreducens* must oxidize acetate three times faster during growth with Fe(III) citrate relative to growth with fumarate (Mahadevan *et al.*, 2006).

The adaptation phenomenon was reproducible, and the length of the lag phase prior to growth in the absence of hydrogen was inversely proportional to the size of the inoculum. The mechanism by which the SfrAB-null strains developed the ability to grow on acetate was not investigated further. An independently isolated acetate-adapted strain was phenotypically and biochemically characterized, with results comparable to those obtained

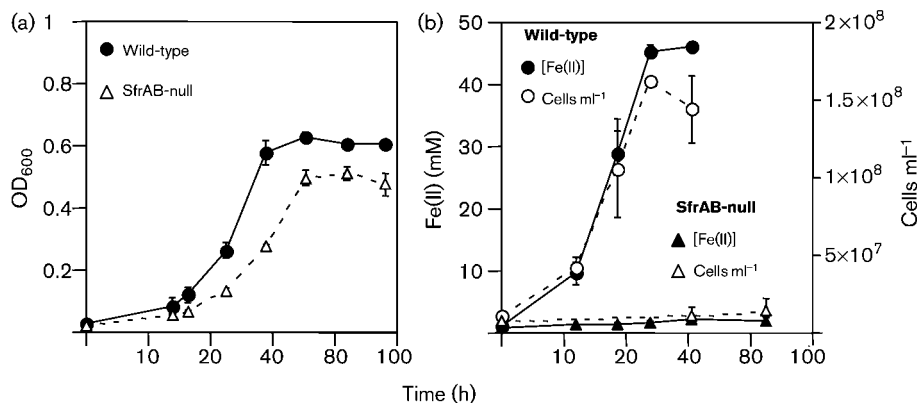


Fig. 3. Growth of the acetate-adapted SfrAB-null and wild-type strains in (a) acetate:fumarate (10:40 mM) and (b) acetate:Fe(III) citrate (10:55 mM) media. Exponential-phase acetate:fumarate cultures of wild-type and acetate-adapted SfrAB-null cultures were washed once in either acetate:fumarate or acetate:Fe(III) citrate medium prior to inoculation. Data are means ± SD of triplicate cultures.

for the strain described above (see Supplementary Figs S1 and S2, available with the online version of this paper).

Biochemical characterization of membrane and soluble fractions of the wild-type and acetate-adapted SfrAB-null strains

In order to gain additional insight into the physiological function of SfrAB, the acetate-adapted SfrAB-null strain was cultured in chemostats at an intermediate growth rate (0.05 h^{-1}) in acetate-limited freshwater fumarate medium (5 mM acetate:27.5 mM fumarate), and cells were harvested for biochemical analysis and mRNA extraction. During growth in chemostats, the cell density of the acetate-adapted SfrAB-null strain was 31 % lower than that of the wild-type ($2.88 \pm 0.30 \times 10^8$ vs $3.78 \pm 0.88 \times 10^8$ cells ml^{-1}), whereas the metabolism of acetate and fumarate by the two strains was similar. Acetate was undetectable in the culture media of both strains, and the levels of malate (9.4 ± 0.7 vs 8.3 ± 0.3 mM), succinate (14.0 ± 0.7 vs 14.6 ± 0.6 mM) and fumarate (5.0 ± 0.1 vs 5.6 ± 1 mM) were also comparable (data are means \pm SD of triplicate chemostats).

Biochemical analysis of membrane and soluble fractions from chemostat-grown cells (Table 1) demonstrated that elimination of SfrAB greatly decreased the amount of two enzymic activities previously associated with SfrAB, the NADPH-dependent reduction of Fe(III)-NTA and benzyl viologen (Kaufmann & Lovley, 2001). A third enzymic activity, NADPH-dependent reduction of AQDS, was also greatly reduced in the SfrAB-null strain (Table 1). Although SfrAB was previously reported to be a cytoplasmic complex, with 98 % of the activity found in the cytoplasmic fraction (Kaufmann & Lovley, 2001), in this study 36 %, 41 % and 57 % of the amount of NADPH-dependent Fe(III)-NTA, benzyl viologen and AQDS reductase activity, respectively, were associated with the membrane fraction (Table 1). This discrepancy did not appear to be due to non-specific contamination of the membrane fraction, as only 0–0.5 % of the activity of three cytoplasmic marker enzymes – pyruvate-ferredoxin

oxidoreductase, oxoglutarate ferredoxin-oxidoreductase and malate dehydrogenase – were detected in the membrane fraction. The cause of the discrepancy is not known. Previous failure to detect SfrAB activity in the membrane fraction may have been due to oxygen contamination or to subtle differences in the procedures used to prepare the membrane fractions. In this study, the membrane and soluble fractions were prepared from intact cells and 1 mM magnesium was added to both the wash and lysis buffers, whereas in the previous study (Kaufmann & Lovley, 2001) the membrane and soluble fractions were prepared from spheroplasts, and neither the spheroplast wash buffer nor the lysis buffer contained magnesium.

Expression of SfrAB from the low-copy-number vector pCDS (Coppi *et al.*, 2001; Leang *et al.*, 2003) resulted in an 8.2-fold increase in the amount of NADPH-dependent Fe(III)-NTA reductase (0.041 ± 0.001 vs 0.005 ± 0.002 $\mu\text{mol mg}^{-1} \text{ min}^{-1}$) and a 13-fold increase in the NADPH-dependent Fe(III)-NTA reductase (0.038 vs 0.507 ± 0.021 $\mu\text{mol mg}^{-1} \text{ min}^{-1}$) activity present in the soluble fraction of the acetate-adapted SfrAB null strain. Data are mean \pm SD of triplicate determinations from a soluble fraction prepared from a single mid-exponential culture. The activity of the membrane fraction was not determined.

In summary, deletion of SfrAB caused a dramatic decrease (≥ 98 %) in the NADPH-dependent Fe(III)-NTA, viologen and AQDS reductase activity detected in acetate:fumarate-grown *G. sulfurreducens* and this decrease was partially reversed by expression of the *sfrBA* cluster *in trans*. These results suggest that the majority of the Fe(III)-NTA, viologen and AQDS reductase activity detected in acetate:fumarate-grown *G. sulfurreducens* may be attributable to SfrAB. In addition, detection of a substantial fraction of these activities in the membrane fraction of wild-type *G. sulfurreducens* indicates that SfrAB may be peripherally membrane-associated, rather than cytoplasmic, as was previously reported (Kaufmann & Lovley, 2001).

Sustained acetate oxidation is dependent upon the continuous oxidation of intracellular electron acceptors

Table 1. Enzymic characterization of the membrane and soluble fractions of the wild-type and SfrAB-null strains

Enzyme	Specific activity ($\mu\text{mol mg}^{-1} \text{ min}^{-1}$)*			
	Wild-type/soluble fraction	Wild-type/membrane fraction (% total activity)	SfrAB-null/soluble fraction	SfrAB-null/membrane fraction
NADPH-dependent Fe(III)NTA reductase	0.23 ± 0.02	0.28 ± 0.05 (35.97 %)	0.005 ± 0.002	0.004 ± 0.003
NADPH-dependent benzyl viologen reductase	2.05 ± 0.05	3.67 ± 0.05 (40.95 %)	0.038 ± 0.01	0.00
NADPH-dependent AQDS reductase	0.087 ± 0.00	0.254 ± 0.023 (57.5 %)	0.00	0.00
Malate dehydrogenase (NADH dependent)	18.62 ± 1.08	0.18 ± 0.01 (0.46 %)	14.28 ± 0.21	0.08 ± 0.02
Oxoglutarate-ferredoxin oxidoreductase	1.50 ± 0.02	0.00 (0.00 %)	1.99 ± 0.20	0.00
Pyruvate-ferredoxin oxidoreductase	1.86 ± 0.16	0.02 ± 0.00 (0.51 %)	1.82 ± 0.13	0.01 ± 0.00

*Data are means \pm SD of triplicate determinations.

including NADPH, as failure to regenerate NADP at an adequate rate would increase the NADPH:NADP ratio and block and/or reverse NADP-dependent reactions including the isocitrate dehydrogenase reaction of the TCA cycle. Inhibition of respiratory growth due to excess NADPH accumulation has been documented in yeast (Boles *et al.* 1993; Fiaux *et al.* 2003; Nissen *et al.*, 2001) and in a strain of *E. coli* lacking the cytoplasmic transhydrogenase UdhA (Sauer *et al.*, 2004). UdhA catalyses the oxidation of NADPH, with NAD serving as the electron acceptor, and is essential for growth of *E. coli* under two conditions in which the rate of NADPH production exceeds its consumption: growth on glucose in the absence of the phosphoglucose isomerase and growth in minimal medium containing acetate as the sole electron donor and carbon source (Canonaco *et al.*, 2001; Sauer *et al.*, 2004). Thus, one possible explanation for the phenotype of the SfrAB-null strain is that SfrAB serves as a major route for NADP regeneration in *G. sulfurreducens*. If this were the case, then eliminating SfrAB would elevate the NADPH:NADP ratio and negatively impact acetate oxidation via the TCA cycle. Further research will be required to confirm this hypothesis.

Microarray analysis of the acetate-adapted SfrAB-null strain

Because identifying genes involved in compensating for the absence of SfrAB might provide additional insights into its physiological function, microarray analysis comparing global gene expression in chemostat cultures of the acetate-adapted SfrAB-null and wild-type strains was performed. RNA for this analysis was isolated from an independent set of triplicate acetate:fumarate chemostats cultured under the conditions described above. Only those genes which were determined to be differentially expressed in the acetate-adapted SfrAB mutant by two statistical methods, SAM (Tusher *et al.*, 2001) and LIMMA (Smyth, 2005), and had a fold change in expression of >1.5 or <-1.5 were considered to be potentially biologically significant. This group of 91 genes consisted of 38 upregulated and 53 downregulated genes (see Supplementary Tables S1 and S2, available with the online version of this paper) and included 25 genes of unknown function as well as the *sfrA* and *sfrB* genes, the majority of which (70–80%) had been substituted with a kanamycin resistance cassette in the SfrAB-null strain. An overview of these genes and their characteristics is given in the supplementary material available with the online version of this paper (Fig. S3 and Overview and Additional Discussion of Microarray Analysis).

In order to assess the validity of the microarray analysis, the differential expression of 14 genes, including both potentially biologically significant genes and genes predicted to be encoded in operons with such genes, was evaluated by QRT-PCR (Table 2, shown in bold). The RNA used for this analysis was prepared from an independent set of chemostats grown approximately 2 months later than

those used for the microarray analysis, specifically those utilized for the biochemical analysis of the membrane and soluble fractions. Differential expression of all but two genes (GSU2706 and GSU1705) was confirmed (Table 2). The biological significance of the 14 genes was evaluated further by comparing their expression in wild-type and SfrAB-null cultures that had been cultured exclusively in the presence of hydrogen (unadapted SfrAB-null), a condition in which SfrAB is not essential for growth. Ten genes were either not differentially expressed or exhibited a lower level of differential expression during growth in the presence of hydrogen (Table 2, acetate-adapted vs unadapted SfrAB-null). This result suggested that many of the genes identified via microarray analysis might play a role in either compensating for or responding to physiological changes caused by the absence of SfrAB during growth via acetate oxidation.

GSU3057 and GSU3058 were among the most highly upregulated genes identified by microarray analysis. Upregulation of GSU3058 was confirmed by QRT-PCR (Table 2), and upregulation of GSU3057 was confirmed by Northern analysis, which also revealed a transcript size that was consistent with cotranscription of GSU3057 and GSU3058 (data not shown). GSU3057 and GSU3058 were previously annotated as a homotetrameric NADPH-dependent glutamate synthase and a putative dihydroorotate electron transfer subunit, respectively (Methé *et al.*, 2003). However, no NADPH-dependent glutamate synthase activity could be detected in either wild-type or acetate-adapted SfrAB-null extracts, and GSU3057 and GSU3058 were found to be 65.6% and 65.2% similar, respectively, to the α and β subunits of the NADPH-dependent ferredoxin oxidoreductase (FNOR) of *Pyrococcus furiosus* (Ma & Adams, 1994, 2001; Schut *et al.*, 2003). Phylogenetic analysis of GSU3057 and related proteins, including SfrB, which is 36% similar, clearly indicated that GSU3057 was more closely related to the α subunit of *P. furiosus* FNOR than to the small subunits of the characterized glutamate synthases (Fig. 4). Thus GSU3057 and GSU3058 are likely to encode an FNOR, and were reannotated accordingly (Table 2).

Northern analysis (data not shown) and QRT-PCR (Table 2) indicated that the putative FNOR was not differentially expressed in the unadapted SfrAB-null strain growing in the presence of hydrogen, which is consistent with a specific role for the FNOR in compensating for the absence of SfrAB during growth via acetate oxidation. If SfrAB does, in fact, serve as a major pathway for NADP regeneration during growth of *G. sulfurreducens* via acetate oxidation, the putative FNOR may serve as an alternative route for NADP regeneration and thus play a key role in adaptation of the SfrAB-null strain.

Although the *P. furiosus* FNOR, like SfrAB, also catalyses NADPH-dependent benzyl viologen reduction (Ma & Adams, 1994, 2001; Schut *et al.*, 2003), the amount of NADPH-dependent benzyl viologen reductase activity in the soluble fraction of the acetate-adapted SfrAB-null strain was less than 1% of the activity present in the

Table 2. Differential expression of selected putative operons in the SfrAB-null mutant

Putative operon*	Gene: annotation	Relative fold change in expression†		
		Acetate-adapted SfrAB-null vs wild-type during growth in acetate: fumarate chemostats (expt 1, whole-genome microarray)	Acetate-adapted SfrAB-null vs wild-type during growth in acetate: fumarate chemostats (expt 2, QRT-PCR)	Unadapted SfrAB-null vs wild-type during growth in acetate: fumarate batch cultures in the presence of H ₂ (QRT-PCR)
ATP synthase 1	GSU0108: putative ATP synthase F ₀ , B' subunit	1.44‡	NA	NA
	GSU0109 (<i>atpF</i>): ATP synthase F ₀ , B subunit	1.48	NA	NA
	GSU0110 (<i>atpH</i>): ATP synthase F ₁ , δ subunit	1.54	NA	NA
	GSU0111 (<i>atpA</i>): ATP synthase F₁, α subunit	1.35‡	3.93	-1.05
	GSU0112 (<i>atpG</i>): ATP synthase F ₁ , ?γ subunit	ND	NA	NA
	GSU0113 (<i>atpD</i>): ATP synthase F ₁ , β subunit	1.44‡	NA	ND
	GSU0114 (<i>atpC</i>): ATP synthase F ₁ , ε? subunit	ND	NA	ND
	ATP synthase 2	GSU0333 (<i>atpE</i>): ATP synthase F ₀ , C subunit	1.67	NA
GSU0334 (<i>atpF</i>): ATP synthase F ₀ , A subunit		ND	NA	NA
Formate dehydrogenase	GSU0777 (<i>fdhG</i>): catalytic subunit	1.31‡	NA	NA
	GSU0778 (<i>fdnH</i>): iron-sulfur subunit	1.46	1.92	1.46
	GSU0779: integral membrane subunit	1.6‡	NA	NA
	GSU0780 (<i>fdhD</i>): accessory protein	ND	NA	NA
Respiratory hydrogenase	GSU0782 (<i>hybS</i>): small subunit	ND	NA	NA
	GSU0783 (<i>hybA</i>): iron-sulfur subunit	1.65	NA	NA
	GSU0784 (<i>hybB</i>): integral membrane subunit	ND	NA	NA
	GSU0785 (<i>hybL</i>): large subunit	1.62	2.89	1.44
	GSU0786 (<i>hybP</i>): maturation protease	ND	NA	NA
Monocistronic transcription unit	GSU1024 (<i>ppcD</i>) periplasmic trihaem cytochrome <i>c</i>	1.73	NA	NA
Sodium/monocarboxylate symporter	GSU1068 (<i>actP</i>): sodium/monocarboxylate symporter	-1.58	-2.05	1.08
	GSU1069: conserved hypothetical protein	-1.4‡	NA	NA
Monocistronic transcription unit	GSU1379 (<i>fur</i>): ferric uptake regulator	-2.11	-1.76	-1.64
Geopilin/unknown function	GSU1496 (<i>pilA</i>): geopilin structural subunit	6.83	1.54	-6.87
	GSU1497: hypothetical protein	6.36	NA	NA
	GSU1498: putative membrane protein	5.13	NA	NA
	GSU1499: putative membrane protein	2.59	NA	NA
	GSU1500: hypothetical protein	1.9	NA	NA

Table 2. cont.

Putative operon*	Gene: annotation	Relative fold change in expression†		
		Acetate-adapted SfrAB-null vs wild-type during growth in acetate: fumarate chemostats (expt 1, whole-genome microarray)	Acetate-adapted SfrAB-null vs wild-type during growth in acetate: fumarate chemostats (expt 2, QRT-PCR)	Unadapted SfrAB-null vs wild-type during growth in acetate: fumarate batch cultures in the presence of H ₂ (QRT-PCR)
Pantothenate biosynthesis	GSU1705 (<i>panB</i>): 3-methyl-2-oxobutanoate hydroxymethyltransferase	-1.45	-1.01	-1.64
	GSU1706 (<i>panC</i>): pantoate- β -alanine ligase	-1.65	NA	NA
	GSU1707: group II decarboxylase	-1.50	NA	NA
Monocistronic transcription unit	GSU2076: cytochrome <i>c</i>	1.6	NA	NA
Monocistronic transcription unit	GSU2490: carboxylate exchanger of unknown specificity	2.19	20.25	1.17
Outer-membrane cytochrome§	GSU2503 (<i>omcT</i>): outer-membrane <i>c</i> -type cytochrome	ND	NA	NA
	GSU2504 (<i>omcS</i>): outer-membrane <i>c</i> -type cytochrome	4.97	NA	NA
Monocistronic transcription unit	GSU2706: phosphate acetyl transferase	-1.55	1.33	-1.05
OmcB operon	GSU2737: <i>omcB</i>, cytochrome <i>c</i> family protein	2.43	1.52	-11.20
	GSU2738 (<i>orf2-1</i>): cytochrome <i>c</i> family protein	ND	NA	NA
	GSU2739 (<i>orf1-1</i>): hypothetical protein	2.2	NA	NA
NADPH-dependent ferredoxin oxidoreductase	GSU3057: <i>fnpA</i> , α subunit	5.84	NA	NA
	GSU3058: <i>fnpB</i>, β subunit	2.85	5.39	-1.07
Chorismate biosynthesis/ unknown	GSU3141: hypothetical protein	2.91	NA	NA
	GSU3142: <i>aroF</i>, phospho-2-dehydro-3-deoxyheptonate aldolase	3.02	3.70	-1.04
Amino acid ABC transporter	GSU3401: periplasmic amino acid binding protein	7.09	2.61	1.25
	GSU3402: hypothetical protein	ND	NA	NA
	GSU3403: hypothetical protein, potential porin	5.5	NA	NA
	GSU3404: ATP-binding protein	4.25	NA	NA
	GSU3405: permease	1.86	NA	NA
	GSU3406: periplasmic amino acid binding protein	7.45	2.13	1.05

*Operon predictions are based on homology to characterized operons or using the FGENESB software (www.softberry.com) as previously described (Yan *et al.*, 2004).

†Relative fold changes for whole-genome microarray data were estimated via LIMMA analysis as described in Methods. ND indicates that statistically significant differential expression was not detected in the microarray. For QRT-PCR data, relative fold changes in expression were determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and are the mean of triplicate determinations. NA indicates that QRT-PCR analysis was not performed for a particular gene.

‡These genes did not make the stringent statistical and fold-change cutoffs for differential expression described in Methods but were identified as differentially expressed by one of the two statistical approaches that were employed, either LIMMA or SAM.

§*omcS* can be transcribed either monocistronically or in conjunction with *omcT* (Mehta *et al.*, 2005).

||The *omcB* operon has been experimentally confirmed (Leang & Lovley, 2005).

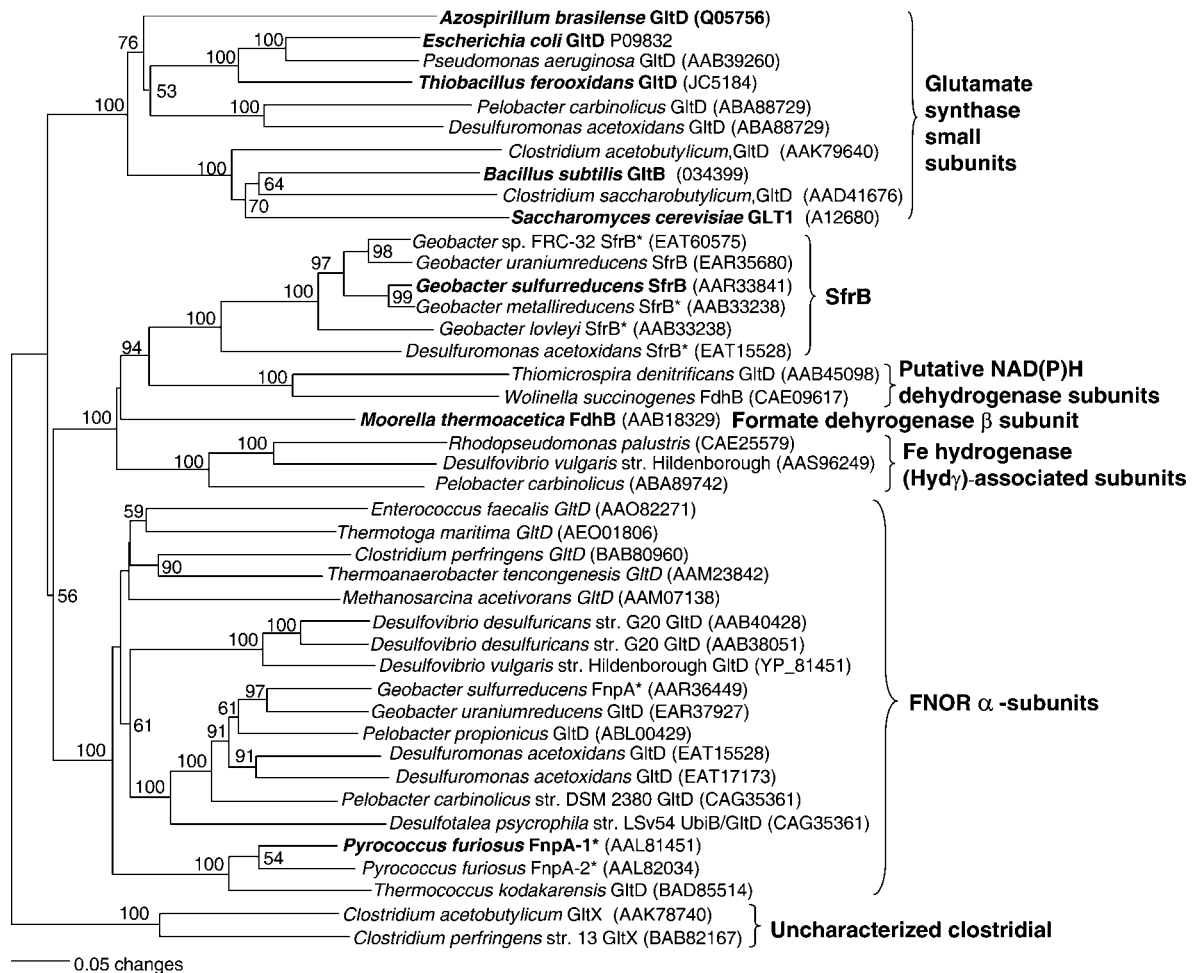


Fig. 4. Phylogenetic analysis of NADPH-oxidizing subunits related to SfrB and GSU3057. Distances and branching order were determined for protein sequences by the neighbour-joining method (Saitou & Nei, 1987) using the BioNJ algorithm (Gascuel, 1997) as previously described (Coppi, 2005), and bootstrap values were determined for 100 replicates. Biochemically and/or genetically characterized genes are indicated in bold, accession numbers are in parentheses and * indicates that the gene symbol was assigned by the authors. The majority of the proteins included in this phylogenetic tree are annotated as glutamate synthase small subunits in the NCBI database regardless of their degree of homology to characterized proteins (Stutz & Reid, 2004) or the identity of neighbouring genes. The genes for all proteins within the glutamate synthase small subunit cluster are adjacent to genes homologous to characterized glutamate synthase large subunit genes, with the exception of GLT1 of *Saccharomyces cerevisiae*, in which the large and small subunits constitute distinct domains within a single peptide. Likewise the genes for all members of the SfrB and FNOR α subunit clusters are adjacent to *sfrA* and FNOR β subunit homologues, respectively. Members of the Fe-hydrogenase (Hyd_γ)-associated subunit cluster are encoded adjacent to homologues of the putative Fe-hydrogenase of *Desulfovibrio vulgaris*, Hyd_γ , whereas the putative NAD(P)H dehydrogenase subunits are encoded in clusters that contain multiple genes that are homologous to the various subunits of NADH dehydrogenases.

wild-type strain. The apparent lack of FNOR activity in the adapted mutant despite a three- to sixfold increase in the level of its putative transcripts could have several possible explanations, including insufficient expression despite elevated mRNA levels, post-transcriptional regulation, or differences in the activity, stability or substrate specificity of the putative *G. sulfurreducens* FNOR relative to that of *P. furiosus*. Alternatively, GSU3057 and GSU3058 may not encode a FNOR at all.

Additional study will clearly be required to elucidate the substrate specificity and physiological function of the complex consisting of GSU3057 and GSU3058. Nevertheless, the fact that two of the most highly upregulated genes in the acetate-adapted SfrAB-null strain appear to encode a complex with NADPH oxidoreductase activity is consistent with a role for SfrAB in influencing NADP concentrations, which in turn would affect acetate oxidation via the TCA cycle.

Several genes involved in energy generation and amino acid uptake and biosynthesis were also upregulated in the 'acetate-adapted SfrAB-null strain'. These included a putative polar and branched-chain amino acid ABC transporter, an enzyme catalysing a critical step in aromatic amino acid biosynthesis, the operons encoding the F_1F_0 ATPase and a variety of redox-active proteins, including the structural subunit of the electroconductive pili of *G. sulfurreducens* (Reguera *et al.*, 2005) (see Table 2 and the supplementary Overview and Additional Discussion of Microarray Analysis for additional details). Because both energy generation and amino acid biosynthesis are dependent upon acetate oxidation via the TCA cycle (Galushko & Schink, 2000; Mahadevan *et al.*, 2006; Reitzer, 2006), upregulation of these genes may be an indication that flux through the TCA cycle was insufficient to fulfil both the energetic and biosynthetic needs of the acetate-adapted SfrAB-null strain. Many of these genes were not upregulated during growth of the unadapted SfrAB-null strain on hydrogen (Table 2), which is consistent with a specific role for these genes in growth on acetate in the absence of SfrAB.

Implications

This study indicates that although SfrAB was purified as an Fe(III) reductase and catalyses NADPH-dependent Fe(III) reduction *in vitro* (Kaufmann & Lovley, 2001), it does not function as an Fe(III) reductase *in vivo*. Intact and metabolically active spheroplasts could not carry out acetate-dependent Fe(III) citrate reduction, indicating that proteins beyond the cytoplasm and cytoplasmic surface of the inner membrane, where SfrAB is likely to be found, are required for Fe(III) citrate reduction. In addition, characterization of an SfrAB-null strain indicated that SfrAB was involved in acetate metabolism. Immediately following isolation, the SfrAB-null strain was unable to grow in medium containing acetate as the sole electron donor and carbon source but retained the ability to grow with the alternative electron donors hydrogen and formate if a small amount of acetate was provided as a carbon source. Although the SfrAB-null strain eventually developed the ability to grow on acetate with fumarate as the electron acceptor, growth with Fe(III) citrate as the electron acceptor, which requires faster rates of acetate oxidation, was not achieved.

The lack of a direct role for SfrAB in Fe(III) citrate reduction is not surprising given that *Geobacter* species normally grow in subsurface environments where the majority of Fe(III) is in the form of insoluble Fe(III) oxides (Amonette, 2002; Lovley *et al.*, 2004) and that they possibly synthesize a variety of specialized proteins and structures for exploiting extracellular electron acceptors, including electroconductive pili (Mehta *et al.*, 2005, 2006; Reguera *et al.*, 2005). The purification and characterization of SfrAB as an Fe(III) reductase took place before a genetic system for analysing gene function in *G. sulfurreducens* was readily available. In the search for Fe(III) reductases, the genetic

approach may be superior to a strictly biochemical approach because many enzymes, particularly those with flavin cofactors or flavin-reductase activity, may non-specifically reduce Fe(III) *in vitro* (Filisetti *et al.*, 2005; Fischer *et al.*, 2002; Petrat *et al.*, 2003; Schroder *et al.*, 2003). In light of these results, previous studies describing the purification of membrane-bound complexes that had NADH-dependent Fe(III) reductase activity and contained both cytochromes and flavin cofactors (Gaspard *et al.*, 1998; Magnuson *et al.*, 2000) should be interpreted with caution.

Differences in gene expression between the acetate-adapted SfrAB-null and wild-type strains suggested that knocking out SfrAB caused substantial changes in the metabolic state of *G. sulfurreducens*. The apparent upregulation of a putative NADPH-dependent ferredoxin oxidoreductase in the acetate-adapted SfrAB-null strain suggested that SfrAB might play a role in regulating the intracellular NADPH:NADP ratio, as did the discovery that deletion of SfrAB caused a dramatic decrease in the amount of NADPH-dependent benzyl viologen reductase activity in *G. sulfurreducens*. Because continuous regeneration of NADP is critical for flux through the TCA cycle, it is possible that deleting SfrAB impacted acetate metabolism by perturbing the NADPH:NADP ratio. Given that acetate fuels the growth of *Geobacter* species in many soils and sediments (Lovley *et al.*, 2004), and homologues of SfrAB are encoded in the genomes of all acetate-oxidizing *Geobacter* species sequenced to date, further investigation of the role of SfrAB in acetate metabolism is warranted.

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