Isolation, characterization and gene sequence analysis of a membrane-associated 89 kDa Fe(III) reducing cytochrome *c* from *Geobacter sulfurreducens*

Timothy S. MAGNUSON*†[1], Naohito ISOYAMA*†, Allison L. HODGES-MYERSON‡, Gerard DAVIDSON§, Michael J. MARONEY§, Gill G. GEESEY*† and Derek R. LOVLEY‡

*Department of Microbiology, P.O. Box 3520, Montana State University, Bozeman, MT 59717-3520, U.S.A., †Center for Biofilm Engineering, 366 EPS Building, Montana State University, Bozeman, MT 59717-3980, U.S.A., ‡Department of Microbiology, Morrill Science Center, University of Massachusetts, Amherst, MA 01003, U.S.A., and §Department of Chemistry, University of Massachusetts, Amherst, MA 01003, U.S.A.

Geobacter sulfurreducens is capable of anaerobic respiration with Fe(III) as a terminal electron acceptor via a membrane-bound Fe(III) reductase activity associated with a large molecular mass cytochrome *c*. This cytochrome was purified by detergent extraction of the membrane fraction, Q-Sepharose ion-exchange chromatography, preparative electrophoresis, and MonoQ ion-exchange chromatography. Spectrophotometric analysis of the purified cytochrome reveals a *c*-type haem, with no evidence of haem *a*, haem *b* or sirohaem. The cytochrome has an M_r of 89000 as determined by denaturing PAGE, and has an isoelectric point of 5.2 as determined by analytical isoelectric focusing. Dithionite-reduced cytochrome can donate electrons to Fe(III)-nitrilotriacetic acid and synthetic ferrihydrite, thus demonstrating that the cytochrome has redox and thermodynamic properties required for reduction of Fe(III). Analysis using cyclic voltammetry confirmed that the reduced cytochrome can catalytically transfer electrons to ferrihydrite, further demonstrating its ability to be an electron transport mediator in anaerobic Fe(III) respiration. Sequence analysis of a cloned chromosomal DNA fragment revealed a 2307 bp open reading frame (*ferA*) encoding a 768 amino acid protein corresponding to the 89 kDa cytochrome. The deduced amino acid sequence (FerA) translated from the open reading frame contained 12 putative haem-binding motifs, as well as a hydrophobic N-terminal membrane anchor sequence, a lipid-attachment site and an ATP/GTP-binding site. FerA displayed 20% or less identity with amino acid sequences of other known cytochromes, although it does share some features with characterized polyhaem cytochromes *c*.

Key words: electron transport protein, iron reductase, metal respiration.

INTRODUCTION

Dissimilatory iron-reducing bacteria are increasingly recognized as an ecologically and environmentally important group of micro-organisms [1–4]. Progress is being made toward the understanding of their biochemistry and physiology, particularly with the genera *Shewanella* and *Geobacter*, both of which are being studied intensively in terms of the biochemistry and physiology of Fe(III) reduction [2,5–17]. It is currently hypothesized that *c*-type cytochromes play an important role in dissimilatory metal reduction by metal-respiring micro-organisms. The strongest evidence that supports this hypothesis is the thermodynamic and redox properties of the metal-reducing cytochromes that have been characterized thus far [18,19]. One possible model for the electron transport pathway from organic compounds (or hydrogen) to Fe(III) involves a membrane-associated respiratory chain coupled to membrane-bound cytochromes that serves as terminal metal reductases [10,20]. This situation has been demonstrated for Fe(III) and U(VI) reductase activity in *Geobacter sulfurreducens* [7,10,21], and in *Shewanella putrefaciens* [14,15,22–24].

Members of the family Geobacteraceae are of particular interest because of their ability to degrade petroleum hydrocarbons, as well as their ability to reduce radionuclides, such as uranium [21,25]. Representative members of the Geobacteraceae include *G. metallireducens* strain GS-15 and *G. sulfurreducens* strain PCA, both of which are Gram-negative anaerobes capable of using a variety of organic acids and aromatic compounds as electron donors [2]. These organisms share a unique metabolic feature, namely the ability to couple metal [Fe(III), U(VI), and Mn(IV)] reduction with energy transduction and ATP synthesis. A model organism for the study of dissimilatory metal reduction has been *G. sulfurreducens* strain PCA [26]. This organism has the ability to use formate, acetate and hydrogen as electron donors, and can respire on fumarate, Mn(IV) (as MnO_2) and Fe(III) [as both Fe(III)-citrate and insoluble Fe(III) oxyhydroxides] [2,26]. Preliminary research established that the enzyme complex responsible for this process resided in the cell membrane of the organism, and contained a cytochrome *c* component that acts as an electron transport mediator to Fe(III) [10]. The emphasis of the present paper is the purification and characterization of this cytochrome, the cloning and sequencing of its structural gene, and its description as a novel iron-reducing protein.

MATERIALS AND METHODS

Organism and culture conditions

G. sulfurreducens strain PCA (A.T.C.C. 51573) was obtained from our laboratory culture collection. It was cultured under

Abbreviations used: IEF, isoelectric focusing, ORF, open reading frame.

[1] To whom correspondence should be addressed, at the Department of Biological Sciences, Idaho State University, 921 South 8th Avenue, Pocatello, ID, U.S.A. (e-mail magntimo@isu.edu).

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strict anaerobic conditions on modified freshwater medium, containing 20 mM sodium acetate as the electron donor and carbon source, and either 50 mM Fe(III) or 40 mM fumarate as the electron acceptor [26]. *G. sulfurreducens* was mass-cultured in 10-litre glass carboys under N_2/CO_2 (4:1). Late log-phase cells were harvested via centrifugation and the pellet was resuspended in 20 mM Tris/HCl (pH 7.5)/10 % glycerol, and frozen at −80 °C until use for protein preparation.

Purification of the membrane-bound cytochrome *c*

All manipulations were carried out aerobically at 4 °C. Cell suspensions (containing about 10 g wet weight of cell paste) of *G. sulfurreducens* were thawed and deoxyribonuclease I (40 units/ml), ribonuclease A (10 μg/ml) and lysozyme (0.1 mg/ml) were added. The cell suspension was incubated at 37 °C for 30 min, and then disrupted with a French pressure cell at 40000 kPa. After two passes through the cell, the crude cell extract was clarified by centrifugation at 7000 ***g*** for 20 min. The supernatant was removed and centrifuged at 105000 ***g*** for 1 h to pellet the membrane fraction. The supernatant (soluble fraction) was removed and the pellet (membrane fraction) resuspended in buffer A (20 mM Tris/HCl, pH 7.5, 10 % glycerol and 0.1 mg/ml dodecyl β-D-maltoside). The protein concentration of the membrane suspension was determined, and adjusted to 10.0 mg/ml with buffer A. The membrane suspension was then extracted with dodecyl β-D-maltoside at a ratio of 1 mg of detergent/mg of protein. Detergent was added from a 5 % aqueous stock solution. Extraction was carried out for 1 h at 4 °C with stirring, after which insoluble material was pelleted by ultracentrifugation for 1 h at 105000 ***g***. The supernatant (detergent extract) was reserved and the pellet discarded.

Detergent extract (400 mg) was loaded on to a column of Q-Sepharose Fast Flow ion-exchange media (column dimensions 2.5 cm × 40 cm) equilibrated with buffer A. Bound proteins were eluted with a linear gradient of 0–0.6 M NaCl (300 ml total volume). Fractions (5.0 ml) were collected and assayed for Fe(III) reductase activity [10] and cytochrome content (A_{409}). The cytochrome *c* was eluted at approx. 0.3 M NaCl. Fractions containing cytochrome were pooled and concentrated with a Filtron Ultrasette tangential flow ultrafiltration device (Filtron, Northborough, MA, U.S.A.) equipped with a 30 kDa cut-off membrane. The pool from the ion-exchange chromatography step was desalted against 1 × stacking gel buffer (0.125 M Tris/HCl, pH 6.8, 10 % glycerol and 0.5 % *N*-lauroylsarcosine) on a Sephadex G-25 column (2.5 cm × 40 cm), and then resolved on a PrepCell Preparative electrophoresis apparatus (Bio-Rad). A Tris-buffered system [27,28] was used, with some modifications. Stacking and resolving gels contained 0.5 % *N*-lauroylsarcosine and 10 % glycerol. A 4 % acrylamide, 10 mm high × 37 mm diameter stacking gel and 6 % acrylamide, 60 mm high × 37 mm diameter resolving gel was used. Approx. 60–80 mg of protein was loaded on to the gel. Electrophoresis conditions were 20 W constant power for approx. 15 h with cooling. Fractions (6 ml) were collected by elution with buffer A at a flow rate of 0.7 ml/min. Fractions were analysed by non-denaturing gel electrophoresis, and fractions containing cytochrome *c* were pooled and concentrated with a Filtron Ultrasette. The final 'polishing' step in the purification involved ion-exchange chromatography on a MonoQ HR5/5 anion-exchange column (Amersham Pharmacia Biotech). Samples from the preparative electrophoresis step were first desalted against buffer A on a HiPrep Fast Desalting Column (Amersham Pharmacia Biotech) and then loaded on to the MonoQ column (5 mg of protein in 1.0 ml). Bound proteins were eluted with a 20 ml linear gradient of 0–2.0 M NaCl in buffer A. Fractions (1.0 ml) were collected and analysed for presence of 89 kDa cytochrome. Fractions containing the cytochrome were pooled and concentrated, and stored at −80 °C.

Biophysical and biochemical characterization

Proteins were routinely analysed by non-denaturing and denaturing (SDS) PAGE [27,28]. Fractions containing cytochrome *c* were identified by haem staining of SDS/PAGE gels [29]. Analytical isoelectric focusing (IEF) was performed on precast vertical IEF gels (Novex, San Diego, CA, U.S.A) with a pH range of 3–10. IEF standards covered the range 4.45–9.6, and were obtained from Bio-Rad. Purified cytochrome was characterized using UV-visible spectrophotometry using a Shimadzu UV-2401-PC dual-beam spectrophotometer. Pyridine haemochrome analysis was performed as described in [30]. After addition of NaOH and pyridine to protein samples, the oxidized form of the protein was obtained by addition of 3 μl of 0.1 M potassium ferricyanide solution. The spectrum was recorded, and the protein was then reduced by the addition of sodium dithionite from a 0.1 M stock solution prepared in 0.1 M sodium bicarbonate buffer, pH 7.0. The haem *c* content was calculated using the absorption coefficient of 30.1 $mM^{-1} \cdot cm^{-1}$ for the α-peak (reduced) at 550 nm [30]. Extraction and determination of flavin nucleotides was performed by acid extraction and HPLC analysis [31].

Measurement of Fe(III) reducing activity

For measurement of direct reduction of Fe(III) compounds by reduced cytochrome *c*, spectrophotometric and electrochemical methods were employed. For spectrophotometric measurement of Fe(III) reduction by the cytochrome, the protein was diluted to 0.1 mg/ml total protein in buffer A and titrated with small aliquots (2–5 μl) of 0.1 M sodium dithionite until reduction of the *c*-type cytochrome was observed (15–30 μl was typically required for reduction). Aliquots of 0.1 M Fe(III)-nitrilotriacetic acid or 0.1 M ferrihydrite suspension were then added (5–10 μl) and the spectrum was re-recorded. Cyclic voltammetry was performed on a BAS CV-50-S electrochemistry workstation with accompanying data analysis software. A three-electrode (platinum reference, saturated calomel and glassy carbon) anaerobic cell was used, and redox analysis was done using a thin-film membrane entrapment method [18]. Protein was dissolved in electrolyte solution (0.1 M Tris/HCl, pH 7.5) containing 0.1 mg/ml dodecyl maltoside. Parameters for the analysis were: scan rate 10 mV/s, protein concentration 2.0 mg/ml, the gross mid-point potential was estimated by calculating the average of the anodic and cathodic peak potentials ($E_o^{'} = 1/2(E_{pa} + E_{po})$). For experiments measuring electron transfer to ferrihydrite, ferrihydrite suspension [10 % (w/v)] was mixed with the cytochrome at varying concentrations, spotted on to the electrode, and the experiment repeated. Peak potentials and currents were determined by computer analysis, and a second-order rate constant was calculated using peak potentials and currents from a series of experiments with varying scan rates and iron concentrations.

Cloning of the structural gene encoding the 89 kDa cytochrome *c*

Internal amino acid sequences of the 89 kDa cytochrome *c* were obtained by tryptic digest of electroblotted cytochrome followed by automated Edman degradation sequencing [32]. *G. sulfurreducens* chromosomal DNA was prepared by the method of Ausabel et al. [33]. PCR primers were obtained from amino acid

sequences and used to amplify a segment of *G. sulfurreducens* chromosomal DNA. The primers used had the sequences 5′-CKACKGCKCCBTTSTAYCAYAAYAC-3′ (forward) and 5′-CCAGTCBAGGAADATNGTRTTVGG-3′ (reverse). PCR was performed as described in [33]. PCR products were analysed and purified on agarose gels, cloned into the cloning vector pCR-TOPO (Invitrogen), and subsequently sequenced using automated dye-termination methods. Sequences were analysed using Genetics Computer Group software.

In order to clone the gene encoding the 89 kDa cytochrome, a genomic library of *G. sulfurreducens* was constructed using the cosmid vector pDDC1 [34]. Approx. 1000 transfectants were screened by colony hybridization with a probe that was prepared by PCR incorporation of fluorescein-11-dUTP into the 230 bp amplicon. One clone, pCC901 (containing an 11 kb fragment), was used for all further analysis. Sequencing of the 89 kDa cytochrome gene was performed bi-directionally using pCC901 as template. Primers for sequencing were designed using the DNA sequence data from the original PCR product. The DNA was sequenced using an ABI Prism 310 Genetic Analyzer (PE biosystems) at the Thermal Biology Institute sequencing facility at Montana State University. The raw sequence data were assembled using Sequencher 3.1 sequence analysing software (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). Sequence analysis was performed using the Biology Workbench NCSA version 3.2 (http://workbench.sdsc.edu/). Non-redundant GenBank® and SwissProt databases were used for DNA and amino acid sequence comparison.

Materials and chemicals

Gel filtration molecular-size standards were obtained from Sigma. Electrophoresis standards were from Bio-Rad Laboratories. DEAE-Sepharose Fast Flow chromatography media were obtained from Pharmacia Amersham Biotech. All other reagents were of analytical grade.

RESULTS

Purification and characterization of the 89 kDa membrane-associated cytochrome *c*

A combination of chromatographic and electrophoretic steps led to the purification of the large-molecular-mass membrane-bound *c*-type cytochrome from *G. sulfurreducens* (Figure 1). Typical yields were approx. 1 mg of protein per 10 g wet weight of cell mass. The cytochrome is found mainly in the cell membrane (approx. 70 % by mass), although it is also detected in smaller amounts in the soluble fraction. This cytochrome is present in slightly higher amounts in cells grown with Fe(III) than with cultures grown on fumarate (Figure 1), but purification is more difficult with cells from Fe(III)-grown cultures, since they carry a high amount of Fe(II) mineral precipitates. SDS/PAGE analysis confirmed that the final product of the purification is pure, and contains a haem-staining protein band with an M_r of 89000 (Figure 1). Pyridine haemochrome analysis of the cytochrome confirms the presence of haem *c* in the protein, with absorbance maxima (reduced protein) of 413 nm, 534 nm and 550 nm (Figure 2). When haem groups were quantified with the pyridine haemochrome method, a value of 14 haems/mol of protein was obtained. No other redox-active cofactors (FAD, FMN, Fe–S etc.) were detected by chemical or spectrophotometric means. The isoelectric point of the pure protein determined by analytical IEF is 5.2. Spectrophotometric analysis of the oxidation/reduction reactions with Fe(III) is shown in Figure 3. A distinct reoxidation of the cytochrome was observed when

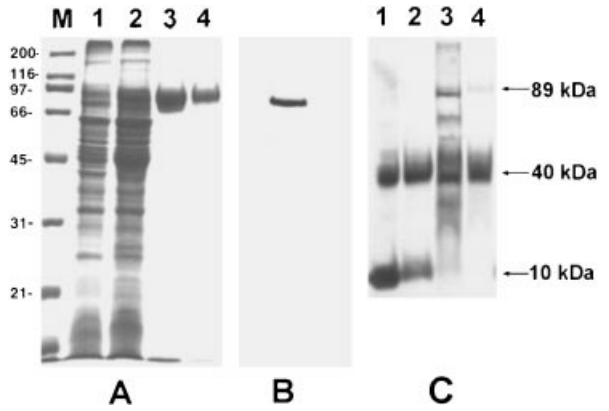

Figure 1 Electrophoretic analysis of cytochromes in *G. sulfurreducens*

(**A**) Denaturing electrophoresis analysis of fractions obtained from the purification of FerA. Lane M, molecular size standards (kDa): lane 1, 5 μg of membrane extract; lane 2, 5 μg of membrane extract; lane 3, 1 μg of PrepCell FerA cytochrome pool; lane 4, 1 μg of MonoQ FerA cytochrome pool. The gel was stained with silver. (**B**) Purified cytochrome *c* (1 μg) stained for haem. (**C**) Denaturing gel electrophoresis of cytochromes found in *G. sulfurreducens* subcellular fractions. Lane 1, soluble fraction from Fe(III)-grown culture; lane 2, soluble fraction from fumarate-grown culture; lane 3, membrane fraction from Fe(III)-grown culture; lane 4, membrane fraction from fumarate-grown culture. The gel was stained for haem *c*, and 40 μg of protein was loaded in each lane. The image was processed using Adobe Photoshop 5.0 for PC.

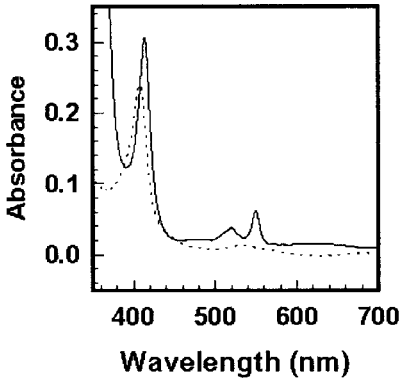

Figure 2 UV-visible pyridine haemochrome spectra of purified FerA in oxidized (broken line) and reduced (solid line) states

Note α and β peaks (reduced) at 550 and 535 nm respectively.

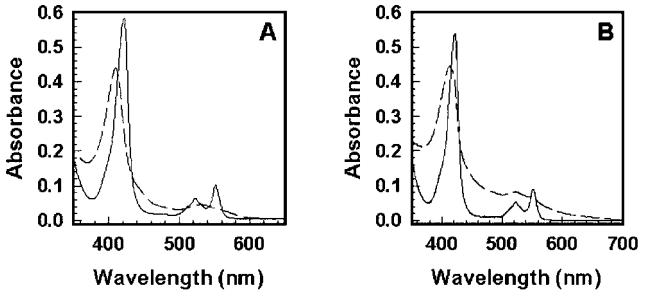

Figure 3 UV-visible redox spectral analysis of electron transfer from reduced FerA to Fe(III) compounds

Broken lines represent oxidized cytochrome; solid lines represent reduced cytochrome. (**A**) Cytochrome with soluble Fe(III)-NTA, (**B**) Cytochrome with insoluble ferrihydrite.

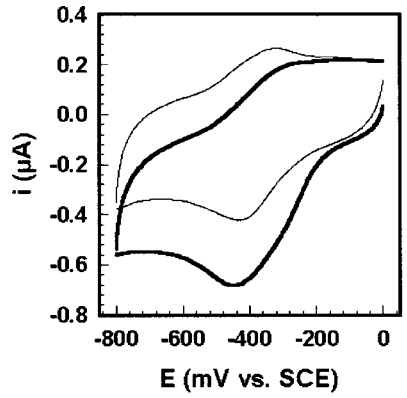

Figure 4 Cyclic voltammograms of FerA without ferrihydrite (thin line) and with 10% (w/v) ferrihydrite suspension (thick line)

Experimental conditions are given in the Materials and methods section.

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        *         20         *         40         *         60         *
MSRKVTKYSAVLAVSLFAAALAGCGSENKEGTVGTGPGGVATVGDTACVQCHSAVVDPLTGESIITQYTR :  70

        80         *        100         *        120         *        140
SFHYSKGVGCEGCHGGGAQHNGVGPLPFPLAGQSEAQIAARCASCHNGVIAPLSSSPNFVNGNHANPFGG : 140

        *        160         *        180         *        200         *
EEAKENLCSRCHSHEGAIFGAQAGFTGDGNILRNAAYQPVYPQDPETFNVMTCATCHQHGGAQRQVFTQI : 210

       220         *        240         *        260         *        280
STAGVPNSRRTVAWDPNRNSINDQYDLCTSCHTVNTMTGTLIGSGNVLQIFTSNAVGSGTKSVTTAPFYH : 280

        *        300         *        320         *        340         *
NTRWFRTLPSTHYDFPESKTTASGTTIEGYVIRRNTANPCFDCHGHEPQTNTRRLAGADRPNTIFLDWGQ : 350

       360         *        380         *        400         *        420
SAHGGKLLQAKVAAAALASSGAAEVDDVMKAGATDATAPGWTHYNWDDTASRGACQRCHTSTGASNFLNN : 420

        *        440         *        460         *        480         *
PAGYDRTGAGNSFTHLAGWTSSNKRSDQNELLYCWGCHTNAGTGELRNPGAITENYAGVNSTSTGTTGTA : 490

       500         *        520         *        540         *        560
VTISYPDIAGSNVCMTCHLGREAGENIKAITDADGILGFVNSHYLAAGGQLFGKTGYEYATRSYAKPTPF : 560

        *        580         *        600         *        620         *
AHDKIGTAAAPGTGTNGPCAGCHMTTPNSHSFLPVTKDGTGAVTAITSTACATCHAGAYALTPEALTAEE : 630

       640         *        660         *        680         *        700
EEYVASLEALKAALAGKGILFFNAHPYFYRDTNANGIGDPGELVSSNAFTNWAGVYGLALWKDVMGAAFN : 700

        *        720         *        740         *        760
ANLLIHDPGGYAHNRFYVKRLIWDSIDFIYDGVLNSDVTAAIDAQVTATRLDSATATAAKAYLGTTRP : 768
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Figure 5 Deduced amino acid sequence of FerA

The lipid attachment site is **double-underlined**, haem-binding sites are **underlined**, and histidine residues are in **bold**; the ATP-GTP-binding site is in **wavy-underline**. Amino acid sequences corresponding to the internal amino acid sequence data from the purified protein are in **dash underline**.

Fe(III) was added to the reduced cytochrome. Electrochemistry experiments estimate a gross redox potential of −190 mV (versus standard hydrogen electrode), based on the average of experimental oxidation and reduction values. Experiments with insoluble Fe(III)-oxides (ferrihydrite) confirm that the cytochrome can transfer electrons to Fe(III), as judged by the characteristic difference between the voltammograms with and without Fe(III) (Figure 4). The specific evidence for electron transfer to ferrihydrite is the diminished reduction peak at −350 mV, and the increased oxidation peak at −420 mV, presumably due to re-oxidation of both the cytochrome and the Fe(II) in solution. Kinetic analysis determined a second-order rate constant of $1 \times 10^3\ M^{-1} \cdot s^{-1}$.

Cloning and sequence analysis of *ferA*, the gene encoding the 89 kDa cytochrome *c*

Screening of a genomic library (approx. 1000 clones) with a probe corresponding to an internal DNA sequence of the gene encoding the cytochrome resulted in the isolation of five positive clones. Of these, a single clone, pCC901, was retained for further study. The clone contained a DNA insert of approx. 11 kb, and the presence of the desired sequence in pCC901 was confirmed by PCR analysis using the same degenerate primers used in initial isolation of the gene fragment. Sequencing was initiated by using the intact cosmid as template with primers designed from the 230 bp amplicon sequence. A primer walking strategy was subsequently used to design further primers for sequencing. Sequence analysis of cosmid pCC901 revealed a 2307 bp open reading frame (ORF) with a putative Shine–Dalgano site (AGGA) located 10 bp upstream from the start codon of the ORF. No known RNA polymerase-binding sites upstream of the ORF were detected, but a possible consensus sequence may exist in the −59 to −33 region. The ORF, designated *ferA*, encodes a protein of 768 amino acids, with a predicted molecular protein mass of 80.5 kDa (Figure 5). Amino acid sequence data from the purified protein exactly matched regions of the deduced amino acid sequence of *ferA*, thus confirming that the gene in fact encodes the 89 kDa cytochrome *c* of *G. sulfurreducens*. The nucleotide sequence of the PCR amplicon generated from the protein sequence-based degenerate primers also matched a portion of the gene sequence, further supporting the conclusion that *ferA* encodes the 89 kDa cytochrome *c*. The deduced amino acid sequence of the FerA protein contains 12 *c*-type haem-binding motifs (CXXCH), as well as a lipid-attachment site and an ATP/GTP-binding site.

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                    *         20         *         40         *         60         *
MtrF  : MNKFASFTTQYSLVL---LIATLLSACGGSDGDDGSPGEPGKP--PAMTISSLNISVDKVAISDGIAQVD :  65
OmcA  : MMKRFNFNTATKAVLGAGLLSLLLTGCGGSDGKDGEDGKPGVVGVNINSTSTLKAKFTNATVDAGKVTVN :  70
MtrC  : --MMNAQKSKIALLLAASAVTMALTGCGGSSGNNGNDGSDGGE--PAGSIQTLNLDITKVSYENGAPMVT :  66
FerA  : ---MSRKVTKYSAVLAVSLFAAALAGCG-SENKEGTVGTGPGGVATVGDTACVQCHSAVVDPLTGESIIT :  66
NrfA  : -------MTKFKLLLAGSLVAIVSMGLLASNINEREKERVALNKTAHSQGIKGKAMSEEWARYYPRQFDS :  63
Hmc   : -----MRNGRTLLRWAGVLAATALIGVGGFWSQGTTKALPEGPGEKRADLIELGAMERFGKLDLPKVAFR :  65
Fcc   : MKKMNLAVCIATLLVGTAGLMGTAVAADNLAEFHVQNQECDSCHTPDGELSNDSLTYENTQCVSCHGTLAE :  70
MtrA  : ----------------------------------------------MKNCLKVKNLLPALTITMAMSAVM :  24
                                                         1
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Figure 6 Alignment of the N-terminal region of outer and periplasmic cytochromes *c* used in anaerobic respiration

OmcA, MtrC, MtrF, outer membrane cytochromes from *S. oniedensis*; MtrA, soluble cytochrome from *S. oniedensis*; Hmc, high-mass cytochrome from *D. vulgaris*; Fcc, fumarate reductase from *S. fridgidimarina*; NrfA, nitrate reductase from *Wollinella succinogenes*. Dark and light shading represent amino acid identity (exact match) and amino acid similarity (similar side chain), respectively.

Amino acid sequence comparisons indicated that FerA had 19.2% identity with MtrA from *S. putrefaciens* MR-1 [35], 19.1% identity with the 16-haem *c*-type cytochrome Hmc from *Desulfovibrio vulgaris* [36] and 15.2% identity with the 9-haem *c*-type cytochrome 9Hcc from *D. desulfuricans* [37]. Alignment of the N-terminal regions of FerA with a variety of membrane and periplasmic respiratory cytochrome is shown in Figure 6. A shared LXXC lipid-attachment motif is seen with all membrane-associated respiratory proteins (MtrA, MtrC, MtrF, OmcA and FerA), but not with soluble cytochromes FccA and NrfA. Haem-binding site distribution seems to be random in FerA, whereas a more clustered pattern exists for OmcA, 9Hcc and Hmc. A Kyte–Doolittle hydrophobicity plot of FerA indicated a strongly hydrophobic region near the N-terminal of the amino acid sequence. Otherwise, hydrophobic and hydrophilic residues were mixed, with no strong evidence for transmembrane spanning regions of higher hydrophobicity.

DISCUSSION

Dissimilatory metal reduction by micro-organisms is believed to be mediated by membrane-bound cytochromes [6,9,11,12,14, 16,17,23]. In *S. putrefaciens*, outer membrane *c*-type cytochromes are thought to be components for the electron transport to Fe(III) [9,11,12,14,16,17] and Mn(IV) [23]. In *G. sulfurreducens*, Fe(III) reductase activity has been purified as an enzyme complex [7,10], which contained a single *c*-type cytochrome. In addition to these findings, it has also been suggested that a small soluble cytochrome *c* may act as an electron shuttle between the membrane respiratory complexes and Fe(III) outside the cell [8]. Upon further study, however, this does not appear to be the case [13]. A recently discovered [21] 40 kDa membrane cytochrome *c* in *G. sulfurreducens* may also play a role in electron transport to Fe(III) and other metals, although this protein has not yet been purified or extensively characterized.

Purification of the Fe(III) reductase activity from *G. sulfurreducens* led to the identification and purification of the 89 kDa cytochrome *c*. Pyridine haemochrome analysis of the purified protein gave a high estimation of the number of haems, possibly due to the fact that polyhaem cytochromes with large amounts of haem are difficult to analyse using this technique. This was the case for the 16-haem cytochrome *c* from *D. desulfuricans* [38], where sequence data were used to definitively determine the number of haems in the protein. It can be concluded from sequence data that the 89 kDa cytochrome of *G. sulfurreducens* has 12 haem *c* moieties. In addition, cofactor analysis did not detect any presence of either FAD or FMN, and spectrophotometric analysis did not reveal any other cofactors. Thus the cytochrome *c* is not similar to the flavocytochrome fumarate reductase from *S. oniedensis*, or any characterized sirohaem bisulphite reductases. Electrochemistry experiments with the 89 kDa cytochrome *c* and ferrihydrite gave very similar results to those seen with cytochrome c_7 from *Desulfuromonas acetoxidans* (E_o' approx. -200 mV) and ferrihydrite [19]. It should be noted that the electrochemistry experiments did not resolve individual mid-point potentials of the haems in the protein, but they did give reliable data regarding the overall redox properties of FerA. Kinetic analysis confirmed that the 89 kDa cytochrome *c* can catalytically reduce insoluble Fe(III) oxides, with the second-order rate constant comparable with those obtained for cytochromes *c* from *Desulfovibrio* spp. and Fe(III) oxides [18]. Based in this evidence, the cytochrome falls into Ambler's Class III [39] cytochrome group, since it is a polyhaemic low-redox-potential cytochrome *c*. This cytochrome shares these features with some *c*-type cytochromes from sulphate-reducing bacteria, as well as other metal-reducing bacteria. It also has the features presumably required for it to be a metal reductase, namely a membrane location, a low redox potential and an ability to reduce Fe(III) compounds [18]. The results of the present study clearly demonstrate that the 89 kDa cytochrome *c* of *G. sulfurreducens* can serve as an electron-transport mediator in the dissimilatory reduction of Fe(III) by this organism.

DNA and amino acid sequences have been reported for polyhaem cytochromes in sulphate-reducing [36,37,40,41] and iron-reducing bacteria [10,16,24]. The present study has characterized the first gene sequence encoding a cytochrome from *G. sulfurreducens*. This gene, designated *ferA*, encodes the 89 kDa *c*-type cytochrome, FerA, which was purified from Fe(III) reductase complex [10]. The lines of evidence supporting this conclusion are: (i) internal amino acid sequence from the purified native protein exactly matches the deduced amino acid sequence from the cloned gene; (ii) the predicted mass of the cytochrome is 80.5 kDa, and the corrected mass including haem and lipoprotein moieties is 86 kDa, which coincides with the experimental molecular mass of 89 kDa for the native protein as determined by SDS/PAGE; and (iii) the predicted isoelectric point of the protein is 5.6, coinciding with the experimental value of 5.2 for the purified protein. In addition, the gene sequence obtained matched a contig in the partially completed *G. sulfurreducens* genome (http://www.tigr.org), further supporting the accuracy of the sequence data. The CXXCH haem-binding motif found in all *c*-type cytochromes was also present in FerA, based on the deduced amino acid sequence of *ferA*. In addition to the haem-binding motifs, computer analysis also predicted a lipid-attachment site, as well as an ATP/GTP-binding site. While the significance of the lipid-attachment site is more obvious (see below), the reason for the existence of an ATP/GTP-binding site is less clear. An ATP/GTP-binding site is not present in any cytochromes supposedly involved in metal reduction, including MtrA, OmcA and Hmc. ATP binding by the cytochrome could play a role in some type of active transport process, perhaps involving Fe(III)/Fe(II). The presence of this site appears to be unique among putative metal-reducing *c*-type cytochromes.

A BLAST search of GenBank®/SwissProt found no DNA base sequence of high similarity to *ferA*. Comparisons using FASTA revealed, however, that FerA is at least partially identical with MtrA, a *c*-type cytochrome presumed to play a role in Fe(III) reduction in *S. putrefaciens* [35]. In contrast with the clustered distribution of haem-binding sites within cytochromes such as the c_3-type cytochromes Hmc and 9Hcc [37,41], the 12 *c*-type haem-binding motifs in FerA were distributed more evenly throughout the protein. These results suggest that FerA does not have high secondary-structural similarity to OmcA or these c_3-type cytochromes. FerA, like OmcA in *S. putrefaciens*, lacks the CXXCHH haem-binding motif found in c_3-type cytochromes such as Hmc and 9Hcc in *D. vulgaris* and *D. desulfuricans* respectively. However, there are a sufficient number of neighbouring histidine residues to participate in hexaco-ordinate bis-histidinyl haem-binding. Hydropathy-plot analysis of FerA revealed a hydrophobic domain at the N-terminus, consistent with a signal peptide/membrane anchor sequence. Previous work [24] indicated that this lipid-attachment site (LXXC) near the N-terminus of the mature protein serves as a hydrophobic anchor for attachment of OmcA to the membrane. This structural feature may serve a similar role for FerA. Secondary-structure prediction programs failed to detect consistently any other transmembrane domains in FerA, however. It thus appears that FerA may be anchored to the membrane via its N-terminus, but the remainder of the protein extends into the aqueous environment outside the outer membrane. In summary, this work

represents the first characterization of a cytochrome *c* involved in dissimilatory Fe(III) reduction in a *Geobacter* isolate. The cytochrome *c*, designated FerA, has physiological, thermodynamic and biochemical properties that allow it to serve as a Fe(III) reductase protein.

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