

Biochemical and genetic characterization of PpcA, a periplasmic *c*-type cytochrome in *Geobacter sulfurreducens*

Jon R. LLOYD¹, Ching LEANG, Allison L. HODGES MYERSON, Maddalena V. COPPI, Stacey CUIFO, Barb METHE, Steven J. SANDLER and Derek R. LOVLEY

Department of Microbiology, University of Massachusetts, Amherst, MA 01003, U.S.A.

A 9.6 kDa periplasmic *c*-type cytochrome, designated PpcA, was purified from the Fe(III)-reducing bacterium *Geobacter sulfurreducens* and characterized. The purified protein is basic (pI 9.5), contains three haems and has an N-terminal amino acid sequence closely related to those of the previously described trihaem *c*₇ cytochromes of *Geobacter metallireducens* and *Desulfuromonas acetoxidans*. The gene encoding PpcA was identified from the *G. sulfurreducens* genome using the N-terminal sequence, and encodes a protein of 71 amino acids (molecular mass 9.58 kDa) with 49% identity to the *c*₇ cytochrome of *D. acetoxidans*. In order to determine the physiological role of PpcA, a knockout mutant was prepared with a single-step recombination method. Acetate-dependent Fe(III) reduction was significantly inhibited in both growing cultures and cell suspensions of the mutant. When *ppcA* was expressed *in trans*, the full capacity for Fe(III)

reduction with acetate was restored. The transfer of electrons from acetate to anthraquinone 2,6-disulphonate (AQDS; a humic acid analogue) and to U(VI) was also compromised in the mutant, but acetate-dependent reduction of fumarate was not altered. The rates of reduction of Fe(III), AQDS, U(VI) and fumarate were also the same in the wild type and *ppcA* mutant when hydrogen was supplied as the electron donor. When taken together with previous studies on other electron transport proteins in *G. sulfurreducens*, these results suggest that PpcA serves as an intermediary electron carrier from acetate to terminal Fe(III) reductases in the outer membrane, and is also involved in the transfer of electrons from acetate to U(VI) and humics.

Key words: dissimilatory Fe(III) reduction, electron transfer, iron respiration.

INTRODUCTION

Dissimilatory Fe(III) reduction is likely to have been an early form of microbial respiration on Earth, and remains one of the most geochemically significant processes in sedimentary environments [1]. Mechanisms for electron transport in the family *Geobacteraceae* in the delta subclass of the *Proteobacteria* are of particular interest, because molecular analysis of microbial communities in a geographical and geochemical diversity of sedimentary environments has demonstrated that microorganisms in this family predominate when Fe(III) reduction is an important terminal electron-accepting process [1–3]. Previous biochemical studies on electron transport in the *Geobacteraceae* have focused on *Desulfuromonas acetoxidans* [4–7], *Geobacter metallireducens* [8,9] and *Geobacter sulfurreducens* [10–13]. However, *G. sulfurreducens* is currently the organism of choice because both the whole genome sequence (<http://www.tigr.org>) and a genetic system [14] are available for this organism.

Low-redox-potential *c*-type cytochromes are thought to play an important role in Fe(III) reduction, not only in the *Geobacteraceae* [10–13,15], but also in other well studied Fe(III)-reducing micro-organisms such as *Shewanella* species [16,17]. One class of *c*-type cytochromes of particular interest in the *Geobacteraceae* is the low-molecular-mass periplasmic *c*₇ cytochromes. The *c*₇ family of cytochromes comprise the smallest representatives of the multihem cytochrome *c*₃ superfamily [18], the best studied example of which is the cytochrome *c*₇ of the sulphur- and Fe(III)-reducing bacterium *D. acetoxidans*. The cytochrome *c*₇ of *D. acetoxidans* is a small soluble trihaem protein (68 amino acids; 9.1 kDa [5,19]) that reduces polysulphide

in vitro and is thought, therefore, to play a direct role in the reduction of elemental sulphur *in vivo* [20]. Alignment with the sequences of tetrahaem *c*₃ cytochromes from sulphate-reducing bacteria of the genus *Desulfovibrio* have suggested that the cytochrome *c*₇ of *D. acetoxidans* is structurally similar to cytochrome *c*₃, but lacks haem 2 of the protein. This has been confirmed in NMR-based studies [5] and by a new crystal structure of cytochrome *c*₇ [7].

A trihaem cytochrome *c*₇ of molecular mass 9.68 kDa was also purified from *G. metallireducens* and was proposed to play a role in electron transfer to Fe(III) [8,9]. It was thought unlikely that the cytochrome *c*₇ was the terminal reductase in this organism, however, because the protein was unable to reduce Fe(III) *in vitro* [8,9]. In contrast, a *c*-type cytochrome of similar mass (9.57 kDa) purified from *G. sulfurreducens* was able to reduce Fe(III) *in vitro* [12]. It was suggested that this cytochrome was released into the environment, where it could serve as a soluble electron shuttle between the cell and insoluble Fe(III) oxides [12]. This hypothesis was questioned in a subsequent study, which reported that the 9.57 kDa protein was not the dominant *c*-type cytochrome secreted by *G. sulfurreducens*, and nor did it function as an electron shuttle between whole cells and Fe(III) oxides [10]. Thus it is clear that there is considerable debate on the precise physiological role of cytochrome *c*₇ in the *Geobacteraceae*.

Here we report on biochemical and genetic analysis of the small periplasmic *c*-type cytochrome in *G. sulfurreducens*. Since the *G. sulfurreducens* genome contains genes for over 100 *c*-type cytochromes (<http://www.tigr.org>), many of them of low molecular mass and potentially periplasmic, we have designated this first cytochrome to be genetically evaluated as PpcA. The results

Abbreviations used: AQDS, anthraquinone 2,6-disulphonate; MALDI, matrix-assisted laser-desorption ionization.

¹ To whom correspondence should be addressed, at present address: The Williamson Centre for Molecular Environmental Science, The Department of Earth Sciences, The University of Manchester, Manchester M13 9PL, U.K. (e-mail jrlloyd@fs1.ge.man.ac.uk).

The nucleotide sequence data for *ppcA* have been submitted to the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence databases under accession number AF505790.

suggest that the physiological function of PpcA is to serve as an intermediary electron carrier between electron donors such as acetate, which are metabolized in the cytoplasm, and Fe(III) reductases in the outer membrane.

MATERIALS AND METHODS

Organism and culture conditions

G. sulfurreducens (A.T.C.C. 51573) was obtained from our laboratory culture collection. It was grown at 30 °C under anaerobic conditions in a modified freshwater medium as described previously [10]. Acetate (20 mM) and fumarate (40 mM) were supplied as the electron donor and the electron acceptor respectively, unless stated otherwise. Cells were manipulated under an atmosphere of N₂/CO₂ (80:20) at all times.

Protein purification

Cells were harvested in late exponential phase by centrifugation and resuspended for 45 min at 37 °C in 50 mM Hepes buffer (pH 7.0) containing lysozyme (0.1 mg · ml⁻¹), DNase (40 units · ml⁻¹) and glycerol (10%, v/v). Cells were then broken in a French press at 86 250 kPa (12 500 lbf/in²) (two passages); the cell debris was removed by centrifugation (5000 g; 20 min) and the supernatant was clarified further using an ultracentrifuge (100 000 g; 1 h). The 9.6 kDa *c*-type cytochrome was purified from this final supernatant using FPLC with gel filtration, cation exchange and metal affinity columns (all from Amersham Pharmacia Biotech, Uppsala, Sweden). The FPLC system was fitted with two UV-M II spectrophotometric detectors (Amersham Pharmacia Biotech) with filters at 280 nm and 405 nm, allowing simultaneous monitoring of the concentrations of protein and *c*-type cytochrome respectively in column effluents. In the first step, cytochrome *c*₇ was separated from larger proteins using a Sephacryl S-300 HR gel filtration column and phosphate buffer (25 mM, pH 6.5). Fractions containing cytochrome *c*₇ were then passed over an SP-Sepharose cation exchange column in the same buffer, and eluted using a salt gradient (0–1 M NaCl in 25 mM phosphate buffer, pH 6.5). Cytochrome *c*₇ was eluted with 150 mM NaCl. A final purification step utilized Fe(III) immobilized metal affinity chromatography [21]. A HiTrap chelating column was charged with 20 mM iron(III) chloride and rinsed with several column volumes of 25 mM phosphate buffer (pH 6.5) containing 5 mM imidazole. Samples containing cytochrome *c*₇ were applied to the column in the same buffer, and eluted using a gradient of imidazole (5–500 mM). The protein was eluted with 250 mM imidazole and was purified to homogeneity as determined by SDS/PAGE in combination with Coomassie Blue staining.

Protein analysis

Proteins were analysed by SDS/PAGE, using a 15% (w/v) polyacrylamide gel buffered with tricine [10]. Proteins were separated using a Hoefer Mighty Small II SE250/SE260 electrophoresis system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) run at 40 mA per gel. Proteins were stained with Coomassie Blue, and also for haem using the method of Goodhew et al. [22]. Protein molecular mass standards were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Cytochrome *c*₇ was also analysed using pre-cast isoelectric focusing gels (Novex, San Diego, CA, U.S.A.) with a pH range between 3 and 10. Protein standards (pI range 4.45–9.6) were also obtained from Bio-Rad. The molecular mass of the protein was determined by gel filtration chromatography using an FPLC system fitted with a Superdex 75 HR10/30 column (Pharmacia Biotech AB). The buffer used was 50 mM Tris/HCl (pH 7.5) containing 100 mM

KCl and 5% (v/v) glycerol. Protein standards (Sigma GF70) ran from 6.5 to 66 kDa molecular mass. The purified protein was also blotted on to PVDF membranes and the N-terminal sequence was obtained by automated Edman degradation sequencing using an ABI/Perkin Elmer Procise 494 protein sequencer.

UV-visible spectra were recorded using a Shimadzu UV2401-PC dual-beam spectrophotometer (Shimadzu, Baltimore, MD, U.S.A.). The range of electron acceptors reduced by cytochrome *c*₇ was determined using a spectrophotometric assay. Samples of the purified cytochrome (50 μl; approx. 1 mg · ml⁻¹ protein) were added to aliquots of bicarbonate buffer (2 ml) in an anaerobic cuvette sealed with a butyl stopper. Sodium dithionite (50 μl; 25 mM) was added through the stopper, using a syringe fitted with a needle, to reduce the cytochrome. The spectrum of the dithionite-reduced cytochrome was recorded between 380 nm and 580 nm, and the potential electron acceptor was added from an anoxic stock solution (250 μl from a 5 mM stock). Fe(III)-nitrioloacetic acid, U(VI), Cr(VI) and menadione bisulphate were tested, with anaerobic stocks of water added to cuvettes in control experiments. The disappearance of the α and β peaks at 551 nm and 522 nm respectively, indicative of reduced *c*-type cytochrome, demonstrated transfer of electrons from the protein to the electron acceptor. Pyridine haemochrome spectra were also recorded as described previously, and used to quantify haem *c* [23].

The midpoint redox potential of cytochrome *c*₇ was measured by cyclic voltammetry, using a method adapted from [13]. A BAS CV-50-S electrochemistry work station fitted with an anaerobic cell containing three electrodes (platinum reference, saturated calomel and glassy carbon) was used. An approx. 5 μl sample of the protein (concentration 1 mg/ml) was immobilized on the glassy electrode surface by membrane entrapment [24]. The electrolyte in the anaerobic cell was 0.1 M Tris/HCl, pH 7.5, and samples were scanned between 200 and –800 mV at a scan rate of 10 mV/s. The midpoint potential was calculated from the average of the anodic and cathodic peak potentials [$E'_0 = 1/2(E_{pa} + E_{pc})$].

Polyclonal antibodies were produced against cytochrome *c*₇ in an adult New Zealand White rabbit [25]. The cytochrome was purified to near homogeneity using FPLC as described above, with an additional step employing preparative SDS/PAGE to purify the protein further prior to injection into the rabbit. Western blotting was performed using the method described in [26], with transfer to a PVDF-Plus Transfer membrane (MSI Inc., Westboro, MA, U.S.A.) using a Hoefer TE Series Transphor Electrophoresis Unit (Amersham Pharmacia Biotech) for 1 h at 100 V. Detection of cytochrome *c*₇ was achieved using a Protein Detector Western Blot Kit (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, U.S.A.).

Analysis of the *ppcA* gene

The *ppcA* gene was identified by searching the *G. sulfurreducens* genome database on The Institute for Genomic Research web site (<http://www.tigr.org>) with the N-terminal sequence of PpcA using BLAST software and verified using PCR analysis. Sequences were analysed using the Genetic Computer Group Software package (Genetics Computer Group, Madison, WI, U.S.A.) and the SignalP program (V1.1; www.cbs.dtu.dk/services/SignalP).

Disruption of *ppcA*

A linear DNA fragment containing a kanamycin-resistance marker flanked by the upstream and downstream sequences of the *ppcA* gene was generated by PCR (Figure 1). The flanking

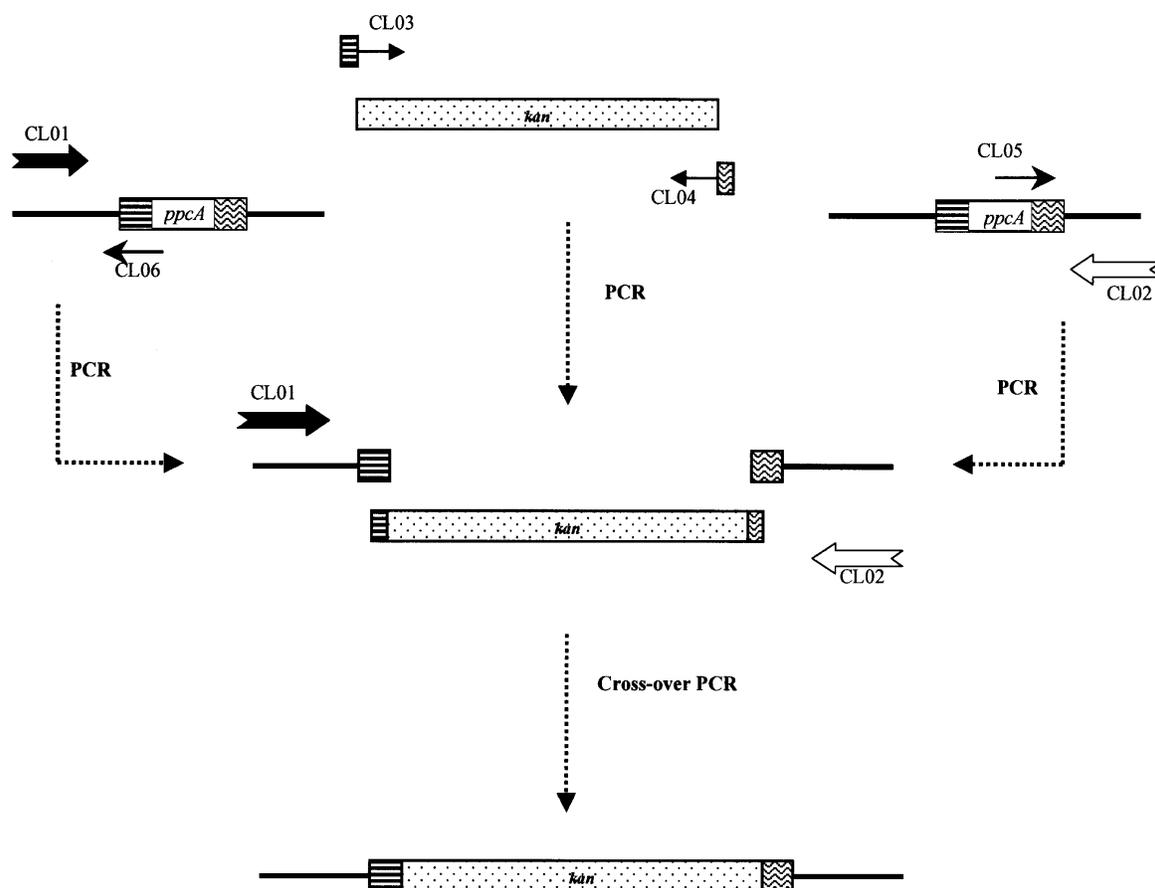


Figure 1 Recombinant PCR protocol used to disrupt the gene encoding cytochrome c_7

(A) Three DNA fragments were amplified independently: a kanamycin-resistance cassette, and the upstream and downstream sequences of *ppcA*. Primers CL03 and CL04 were designed so that their products hybridized with either end of the upstream and downstream sequences of *ppcA*. (B) The three PCR products were combined as templates for recombinant PCR to generate a linear fragment (2.2 kb) containing the kanamycin gene flanked by upstream and downstream sequences from *ppcA*. The final product was used directly for gene replacement following electroporation into *G. sulfurreducens*.

Table 1 PCR primers used in the present study

Italics represent complementary sequences to CL06 (in CL03) and to CL05 (in CL04).

Name	Sequence (5' → 3')	Usage
CL01	CCGAGACTATTCTGCCGG	} PCR amplification of sequence upstream of gene for cytochrome c_7 (−411 to +78)
CL06	GAGGACGATGTCGTCGGC	
CL02	GGTACATATCAAAGAAGCC	} PCR amplification of sequence downstream of gene for cytochrome c_7 (+163 to +693)
CL05	CGGCAAAGAGATGGCTCATGGC	
CL03	<i>GCCGCCGACGACATCGTCTCACCTGGGATGAATGTCAGCTAC</i>	} PCR amplification of the <i>kan^R</i> gene from pBBR1MCS-2 with homologous overhangs to CL06 and CL05 respectively
CL04	<i>GCCATGAGCCATCTCTTGCCGAGAAGGCGCGGTGGAATCG</i>	

region of the linear DNA fragment promoted replacement of the functional gene in the chromosome of *G. sulfurreducens* via homologous recombination. The linear DNA fragment was generated by preparing three PCR products. The first fragment encoded the upstream sequence of the gene for cytochrome c_7 (−411 to 78; +1 at A of the start codon), the second the downstream sequence of the target gene (+163 to +693; cytochrome c_7 ends at +276) and the third the kanamycin-resistance marker (1.1 kb [14]). Primers used in the present work are listed in Table 1. The primers CL03 and CL04 were used

to amplify the kanamycin-resistance marker; the first 21mer of CL03 is complementary to CL06 and the first 22mer of CL04 is complementary to CL05. The PCR products containing the two fragments of the gene for cytochrome c_7 (upstream and downstream) overlapped with either end of the third PCR product carrying the kanamycin-resistance marker.

The three PCR products were gel purified and used as templates for recombinant PCR to assemble a 2.2 kb linear DNA fragment. For the first 15 cycles of the recombinant PCR reaction, no primers were added, facilitating formation of a full-length 2.2 kb

fragment containing the kanamycin-resistance marker flanked by the two sequences from the gene encoding cytochrome c_7 . In the second step of the recombinant PCR reaction (30 cycles), distal primers (CL01 and CL02) were added to amplify the 2.2 kb fragment. The final PCR product (2.2 kb) was gel purified and resuspended in $0.5 \times$ TE buffer [27] at a final concentration of $1 \mu\text{g}/\mu\text{l}$ DNA for introduction into electrocompetent cells of *G. sulfurreducens*. The PCR programme was as follows; 96°C for 40 s, followed by 15 or 30 cycles of 96°C for 40 s, 47°C for 1 min and 72°C for 2 min, and a final extension step at 72°C for 10 min. In the first step of the recombinant PCR reaction, equal amounts of the three templates ($15 \text{ ng}/\mu\text{l}$) were added in a total volume of $50 \mu\text{l}$, and the reaction was performed as above for 15 cycles. The product of the primerless PCR reaction was used directly as a template in the second step of the recombinant PCR for an additional 30 cycles. The total volume for this step was $100 \mu\text{l}$, with distal primers added at a final concentration of 200 nM. Qiagen *Taq* DNA polymerase (Qiagen Inc., Valencia, CA, U.S.A.) was used for all PCR amplifications.

Electrocompetent cells were prepared as described previously [14], and transformed with $1 \mu\text{g}$ of the 2.2 kb recombinant PCR product [14]. Transformed cells were incubated at 30°C in NBAFYE medium [14] overnight, and then plated on to NBAFYE agar supplemented with $200 \mu\text{g}/\text{ml}$ kanamycin. Plates were incubated at 30°C under a modified atmosphere of 7% H_2 , 10% CO_2 and 83% N_2 . Kanamycin-resistant colonies were tested for insertion of the kanamycin-resistance marker into the gene for cytochrome c_7 by PCR and Southern blot analysis. One of the same-genotype mutants was chosen as a representative, and was designated as strain DL3.

DNA manipulations

Genomic DNA of *G. sulfurreducens* was prepared using a G NOME DNA kit (Bio101 Inc., Vista, CA, U.S.A.) and PCR products were gel purified and cleaned using a QIAquick gel extraction kit (Qiagen Inc.). Other protocols for DNA manipulations were as described in Sambrook et al. [27]. Southern blot analysis was also carried out as described in [27], with a probe prepared using the Multiprime DNA Labeling System kit (Amersham Pharmacia Biotech Inc.) with [^{32}P]dCTP (New England Nuclear, Boston, MA, U.S.A.).

Analytical techniques

Fe(II) was measured using a ferrozine-based colorimetric assay as described previously [28]. U(VI) was measured using a kinetic phosphorescence analyser [29]. Protein concentrations were determined using the bicinchoninic acid method [30]. Fumarate concentrations in culture supernatants were measured using a Shimadzu LC-6A HPLC system (Shimadzu, Baltimore, MD, U.S.A.), fitted with a Bio-Rad Fast Acid Analysis Column. Cells were removed using a Microfuge (13000 g ; 5 min) prior to analysis. The reduction of anthraquinone 2,6-disulphonate (AQDS) was monitored spectrophotometrically at 450 nm. Cells were counted using Acridine Orange staining and epifluorescence microscopy, as described previously [28]. Determinations of protein concentration, Fe(III), U(VI), fumarate, AQDS and cell counts were in triplicate, and the experimental error was within 5% of the mean throughout.

RESULTS

Purification of PpcA

The 9.6 kDa PpcA cytochrome was purified to near homogeneity from the soluble fraction of *G. sulfurreducens* by FPLC using a

Table 2 Enrichment of cytochrome c_7 during purification from the soluble fraction of *G. sulfurreducens*

IMAC, immobilized metal affinity chromatography. The Horio–Kamen index is calculated as $(A_{552} - A_{570})_{\text{reduced}}/A_{280} \text{ oxidized}$.

Purification step	A_{408}/A_{280}	Horio–Kamen index
Soluble fraction	0.27	0.041
Sephacryl S-300 (gel filtration)	0.41	0.045
SP-Sepharose (cation exchange)	9.39	1.97
HiTrap chelating [Fe(III)-IMAC]	12.8	3.16

novel combination of gel filtration, cation exchange and immobilized metal [Fe(III)] affinity columns (Table 2). Purification using immobilized Fe(III) affinity columns may be surprising, given that direct interactions between PpcA and Fe(III) are not expected *in vivo* due to the periplasmic location of the protein and the low solubility of Fe(III). This final step was, however, both effective and preferable to the hydrophobic interaction column used previously [12], which required the use of a high-salt buffer. The PpcA protein passed through ultrafiltration cartridges (molecular mass cut-off 5 kDa) in the high-salt buffer, which prevented use of the cartridges for concentrating the purified protein.

The purity of the PpcA preparations was monitored throughout the purification procedure by calculating the ratio of the absorbance values at 408 nm and 280 nm, and also by determining the Horio–Kamen index of the different preparations ($A_{552} - A_{570}$ of dithionite-reduced preparations, divided by the A_{280} of the air-oxidized protein; [31]) (Table 2). The values of 12.8 and 3.16 respectively recorded for the cytochrome-containing fraction eluted from the final Fe(III) metal affinity chromatography step were indicative of a highly purified cytochrome preparation [8,31]. The purity of this preparation was confirmed by SDS/PAGE analysis, with a single band of molecular mass 10 kDa apparent when stained using Coomassie Blue (results not shown), which was in agreement with the molecular mass of 9.57 kDa determined by matrix-assisted laser-desorption ionization (MALDI) MS in a previous study [12]. A single band of similar electrophoretic mobility was apparent when the gel was stained using a cytochrome-specific haem stain (results not shown). Gel filtration studies of the purified protein using an FPLC system fitted with a Superdex 75 column gave an apparent molecular mass of 11.7 kDa, suggesting that PpcA was present as a monomer, similar to the 9.1 kDa cytochrome c_7 of *D. acetoxidans* [5].

c -type cytochromes have also been obtained at sufficient purity for N-terminal sequence analysis from the soluble fraction of other Gram-negative Fe(III)-reducing bacteria using the combination of gel filtration and cation exchange columns described for the first two steps in the purification of the 9.6 kDa cytochrome from *G. sulfurreducens*. These organisms include *Geobacter 'humireducens'* [32], *Geovibrio ferrireducens* [33] and *Geothrix fermentans* [34].

Biochemical characterization of PpcA

Analysis of pyridine haemochrome spectra confirmed the presence of pyridine haemochrome c with a characteristic absorbance maximum at 550 nm, which was used to calculate a value of 2.9 haem groups per molecule of protein using the method described in [23]. Analysis of the protein by analytical isoelectric focusing gel electrophoresis demonstrated that the protein was basic, with an isoelectric point of approx. 9.5. The midpoint redox potential

	1				5					10				15			
<i>Geobacter sulfurreducens</i>	ALA	ASP	ASP	ILE	VAL	LEU	LYS	<u>ALA</u>	<u>LYS</u>	<u>ASN</u>	<u>GLY</u>	ASP	VAL	LYS	PHE	PRO	HIS
<i>Geobacter humireducens</i>	ALA	ASP	SER	VAL	VAL	TYR	PRO	<u>ALA</u>	<u>LYS</u>	<u>ASN</u>	<u>GLY</u>	ASN	VAL	THR	PHE	(N)	HIS
<i>Geobacter metallireducens</i>	ALA	ASP	GLU	LEU	THR	PHE	LYS	<u>ALA</u>	<u>LYS</u>	<u>ASN</u>	<u>GLY</u>	ASP	VAL	LYS	PHE	PRO	HIS
<i>Desulfuromonas acetoxidans</i>	ALA	ASP	VAL	VAL	THR	TYR	GLU	ASN	<u>LYS</u>	<u>LYS</u>	<u>GLY</u>	ASN	VAL	THR	PHE	ASP	HIS

Figure 2 Alignment of N-terminal amino acid sequences of c_7 cytochromes of *G. sulfurreducens* and *G. humireducens* (the present study), *G. metallireducens* [8,9] and *D. acetoxidans* [6]

Amino acid residues conserved among all proteins are in bold, and those conserved among the proteins from the *Geobacter* species are underlined.

1	TGCATGTGCTGTATAAAAGGTTGCGTTTTCATCAACCTGTTAGAAAGGGTAAQAATCAC RBS
61	ATGAAAAGGTTATGCTTCTCTCGCGTGTCCGTATTCTGCGCCGGCTCGCCTTTGGCC M K K V I A S L A L S V F C A G L A F A
121	GCCGACGACATCGTCTCAAGGCCAAGAAGCGTGATGTGAAGTTCCCGCAAGGCCCCAC A D D I V L K A K N G D V K F P H K A H
181	CAGAAGGCTGTTCCCGACTGTAAGAAGTGCCACGAGAAAGCCCGGCAAGATCGAGGGC Q K A V P D C K K C H E K G P G K I E G
241	TTGGCAAAGAGATGGCTCATGGCAAGGGTGCACGAGGGTGCACGAAGAAATGAAGAAG F G K E M A H G K G C K G C H E E M K K
301	GGCCGACGAAGTCCGCGAGTGCCCAAGAAGTAATGATGATTGACGTTACTTCACTG G P T K C G E C H K K * * *
361	GAGGTGCACCGAAGGATAACAGAACTTACTGTTATCCCTTTTCTTGACCGCTAAAATA Terminator loop

Figure 3 DNA sequence of the gene encoding cytochrome c_7 and inferred amino acid sequence of the mature protein

A hydrophobic signal peptide is present at the N-terminus of the protein (italicized). Underlined amino acids indicate those determined by N-terminal sequencing of purified cytochrome c_7 . Three haem-binding motifs (CXXCH) are indicated in bold, with three additional histidine residues (positions 17, 20 and 47 of the mature protein) present as the sixth axial ligands for the haems. A putative operator/promoter region is underlined in front of the gene for cytochrome c_7 , and a terminator loop characteristic of Rho-independent transcription terminator signals [36] is also underlined 29 bp behind the last of three stop codons (*).

of the protein was measured at -169.5 mV by scanning voltammetry (versus a standard hydrogen electrode), which was in good agreement with a value measured by redox titration (-167 mV; [12]). Consistent with this low redox potential, dithionite-reduced cytochrome was oxidized by Fe(III)-NTA, U(VI), Cr(VI) and the humic acid analogue menadione bisulphate, confirming earlier results obtained using Fe(III) [10,12], and extending the range of electron acceptors known to be reduced by the protein. However, nitrite was not reduced effectively by PpcA.

The N-terminal sequence of the protein shared considerable identity with the N-terminal sequences of trihaem c_7 cytochromes purified from other members of the family *Geobacteraceae* (Figure 2). These include a 9.7 kDa cytochrome c_7 purified from *Geobacter metallireducens* [8,9], a cytochrome c_7 purified from *Desulfuromonas acetoxidans* [6,19], and a 10 kDa cytochrome c_7 purified from the soluble fraction of *Geobacter humireducens* using a combination of gel filtration and

cation exchange chromatography (the present study). There was 41% identity (7 out of 17 amino acid residues) shared between all sequences, including alanine/aspartic acid residues conserved at the N-terminal region indicative of a signal peptide cleavage site [35], and a histidine residue at position 17, which forms the sixth axial ligand of haem 1 in the *D. acetoxidans* cytochrome c_7 [6]. Additional amino acid residues (alanine and asparagine residues at positions 8 and 10) were conserved among the *Geobacter* cytochrome c_7 sequences. There were no similarities between the N-terminal sequences of the small soluble c -type cytochromes purified from the phylogenetically distinct Fe(III)-reducing bacteria *Geovibrio ferrireducens* and *Geothrix fermentans* and those of the c_7 cytochromes shown in Figure 2.

Sequence analysis of *ppcA*

The N-terminal sequence of the 9.6 kDa cytochrome c_7 was used to identify the corresponding gene (Figure 3) from the preliminary genome sequence of *G. sulfurreducens* on the TIGR website (<http://www.tigr.org>) using BLAST software. Consistent with the periplasmic location of PpcA [10,12], analysis of the translated protein using the SignalP program (www.cbs.dtu.dk/services/SignalP) identified a 20-amino-acid hydrophobic signal peptide at the N-terminus of the protein (Figure 3; italicized). The predicted cleavage site was between amino acids 20 and 21 (Ala-Ala), which would generate a mature protein with an N-terminal sequence identical to that obtained for the purified protein (underlined in Figure 3). Three haem-binding motifs (CXXCH) were present in the deduced amino acid sequence (bold in Figure 3), in agreement with the three haems measured from the pyridine haemochrome spectra. Three additional histidine residues were also present, presumably providing the sixth axial ligands for the haems (at positions 17, 20 and 47 of the mature protein). The mature protein was 71 amino acids long, with a molecular mass of 9.58 kDa, including the three haem groups, which is in good agreement with the mass of 9.57 kDa measured by MALDI MS [12]. Further evidence that we had identified the gene encoding the cytochrome that had been purified was the observation that the calculated pI of the mature protein was 9.44, also in excellent agreement with the value measured by analytical isoelectric focusing. A Kyte-Doolittle hydrophobicity plot of PpcA (not shown) confirmed that the mature protein was hydrophilic, in keeping with its proposed location in the periplasm [10,12]. Hydrophobic amino acids were associated only with the signal peptide at the N-terminus of the protein. Finally, there was a highly conserved operator/promoter region (-35 TTGACA located at -71 and -10 TATA located at -49) located approx. 50 bp upstream of the cytochrome c_7 gene, and a terminator loop characteristic of Rho-independent transcription terminator signals 29 bp downstream of the last of

PpcA	<i>G. sulfu</i>	-----ADDIVLKAK-----NGDVKFPKHAHQKAVPD	CKKCH	EKGPGKIEG--	FGKEMAHGKG	50			
Cyt C7	<i>D. aceto</i>	-----ADVVTYENK-----KGNVTFDHKAHAELG	CDACH	EGTPAKIA--	IDKSAHKDA	48			
Cyt C3	<i>D. desul</i>	-----VDAPADMVVKAPAGAK-----VTKAPVAFSHKGHASMD	CKTCH	HKWDGAGAIQP	CQASGCHANT	59			
Cyt C3	<i>D. salex</i>	-----VDAPADMVVKAPAGAK-----MTKAPVDFSHKGHAAALD	CTKCH	HKWDGKAIEVKK	CSAEGCHVBT	59			
C3 (26)	<i>D. desul</i>	-----ETFEIPESVTMSPKQFEGYTPKKGDVTFNHASHMDIA	CQOCH	HTVPDITYTIES	CMTEGCHDNI	63			
OmcA	<i>S. oneid</i>	DVVRPHVLPRIYAYIQDQPRFRKWNQTDNSAAEKRRAI	IDTAK	CSGCH	NKEIVHYDNG--VNCQACH	TPD 589			
PpcA	<i>G. sulfu</i>	-----	CKGCH	EEMKK	-----GPTK	CGECH	KK-----	71	(71 total)
C7	<i>D. aceto</i>	-----	CKTCH	KSNM	-----GPTK	CGGCH	IK-----	68	(68 total)
C3	<i>D. desul</i>	ESKKGDDSD--FYMAFHERK-SEKS	CVGCH	KSMKK	-----GPTK	CTECH	PKN-----	102	(102 total)
C3	<i>D. salex</i>	-SKKGGKSTPKFYSAFHS-K-SDIS	CVGCH	KALKKA	-----TGPTK	CGDCH	PKKK-----	106	(106 total)
C3 (26)	<i>D. desul</i>	KERTEISS--VYRTFHHTTKDSEKS	CVGCH	RELKRRQGPSDAPLA	-----CNSCH	VQ-----		111	(111 total)
OmcA	<i>S. oneid</i>	KGLKTDNTYPGKVPKPTSAFWAKHASEGHYLYAGVQSGTVLKT			-----CATCH	TADKSNVVTGI		649	(734 total)

Figure 4 Alignment of cytochrome c_7 , PpcA with other c -type cytochromes identified by BLAST analysis

The numbers on the right indicate the relative numbering of the residues within each protein. The total number of residues in each protein is shown in parentheses. Haem-binding motifs are boxed. Asterisks mark the conserved histidines that are the sixth axial ligands to the haems (above alignments for *Geobacter* and *Desulfuromonas* sequences, and below alignments for *Desulfovibrio* sequences). Sequences and accession numbers are as follows: Cyt C7 *D. aceto*, *Desulfuromonas acetoxidans* cytochrome c_7 (Swiss-Prot P00137 [19]); Cyt C3 *D. desul*, *Desulfovibrio desulfuricans* cytochrome c_3 (Swiss-Prot P00134 [19]); C3 *D. salex*, *Desulfovibrio salexigens* cytochrome c_3 (Swiss-Prot P00135 [37]); C3(26) *D. desul*, *Desulfovibrio desulfuricans* 26 kDa cytochrome c_3 (Swiss-Protein P38554 [38]); OmcA *S. oneid*, *Shewanella oneidensis* 83 kDa c -type outer membrane cytochrome (GenBank AAC29030 [39]). Alignments were produced using ClustalW.

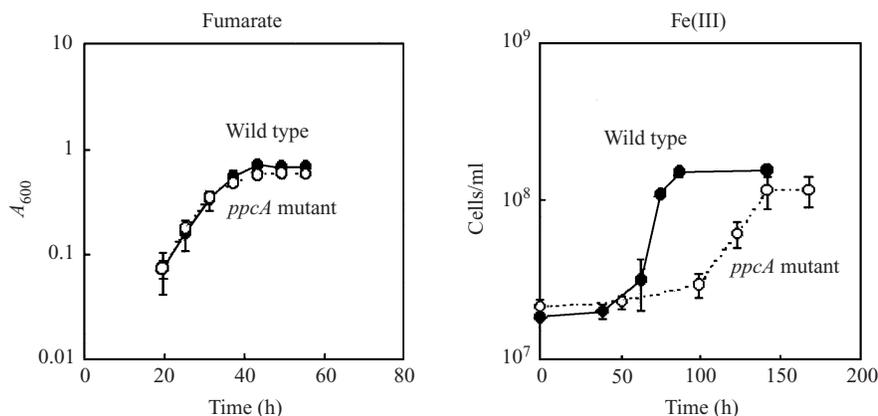


Figure 5 Growth of wild-type *G. sulfurreducens* (●) and of a mutant unable to synthesize cytochrome c_7 (○) using 40 mM fumarate (left) or 55 mM Fe(III) citrate (right) as electron acceptor

Acetate (20 mM) was provided as the electron donor.

three stop codons [36], which may suggest that the gene encoding cytochrome c_7 is not part of an operon.

BLAST analysis of the deduced amino acid sequence of PpcA identified several related c -type cytochromes from other Gram-negative bacteria (Figure 4). The closest identity was with the c_7 cytochrome of *D. acetoxidans* [6,19]; 49% identity, 33/68 residues). The positions of the haem-binding motifs, and the histidine residues required for axial binding of the haem groups, were conserved in the *G. sulfurreducens* and *D. acetoxidans* cytochromes (Figure 4). There was also significant identity between the haem-binding motifs of the cytochrome c_7 of *G. sulfurreducens* and those of other low-molecular-mass soluble cytochromes purified from various *Desulfovibrio* species [19,37,38]. Finally, very weak identity was noted between cytochrome c_7 and OmcA, an 83 kDa decahaem outer-membrane cytochrome identified in *Shewanella oneidensis* MR-1 [39]. This was also the closest match identified for the *Shewanella oneidensis* MR-1 genome (at <http://www.tigr.org>) using a BLAST search with the complete amino acid sequence of cytochrome c_7 .

Physiological role of PpcA

In order to determine the physiological role of PpcA, a mutant was constructed in which part of the *ppcA* gene had been deleted as well as disrupted with a kanamycin-resistance marker. Analysis of the mutant (strain DL3) by PCR and Southern blotting confirmed that *ppcA* had been disrupted by insertion of the kanamycin-resistance marker. Furthermore, when the soluble fraction was assayed by Western blotting using polyclonal antisera raised against purified PpcA, the protein was readily detected in the wild-type strain, but not in the mutant (results not shown).

The growth rate (doubling time 6 h) and cell yield of the *ppcA* mutant were similar to those of the wild-type strain when grown in medium containing acetate as electron donor and fumarate as electron acceptor (Figure 5). The growth rate of the mutant was, however, decreased significantly when Fe(III) was the electron acceptor (Figure 5). Under these conditions, the lag period was extended in the mutant, and once growth was initiated the

Table 3 Rates of reduction of fumarate, Fe(III) citrate, the humic acid analogue AQDS and U(VI) by cells of wild-type *G. sulfurreducens* and a mutant unable to synthesize cytochrome c_7

Hydrogen or acetate was supplied as the electron donor. Cells were pre-grown using acetate (20 mM) and fumarate (40 mM) as the electron donor and the electron acceptor respectively.

Electron acceptor	Reduction (mmol · h ⁻¹ · g dry weight biomass ⁻¹)			
	Hydrogen		Acetate	
	Wild type	Mutant	Wild type	Mutant
Fumarate	53	50	21	20
Fe(III)	5.2	5.2	4.6	2.7
AQDS	2.1	2.2	2.0	0.1
U(VI)	0.57	0.44	0.56	0.11

doubling time for the mutant (42 h) was 4-fold longer than for the wild type. Furthermore, the final cell yield was significantly lower in the mutant (Figure 5). The impact of the deletion mutation on Fe(III) reduction could be attributed to the lack of PpcA, since when *ppcA* was expressed *in trans* the capacity for Fe(III) reduction was restored (results not shown).

Since growth studies offer the mutant an opportunity to adapt to the mutation by expressing alternative pathways, further analyses were conducted in short-term studies with washed cells suspended in a buffer that did not permit growth. As seen in growth studies, the rate of fumarate reduction was the same in both the wild-type and mutant strains (Table 3), irrespective of the electron donor supplied. Fe(III) reduction was inhibited by over 40% in the *ppcA* mutant with acetate, but not hydrogen, as the electron donor (Table 3).

U(VI) represents another environmentally significant electron acceptor for *Geobacter* species [40]. Whereas the deletion mutation had little impact on U(VI) reduction in cell suspensions with hydrogen as the electron donor, U(VI) reduction was inhibited when acetate was the electron donor (Table 3). *Geobacter* species are also able to use humic acids as electron acceptors [41]. Again the deletion mutation had no impact on the reduction of the humic acid analogue AQDS in cell suspensions with hydrogen as the electron donor, but reduction of AQDS was inhibited when acetate was supplied as the electron donor (Table 3).

DISCUSSION

The present results demonstrate that the periplasmic 9.6 kDa *c*-type cytochrome of *G. sulfurreducens*, designated PpcA, is a member of the cytochrome c_7 family, and suggest that PpcA functions as an intermediary electron carrier in electron transport from acetate to Fe(III) reductases in the outer membrane. PpcA also plays a role in electron transfer to other electron acceptors, including U(VI) and humics. Although biochemical studies have previously suggested a potential role in metal reduction for various *c*-type cytochromes from *G. sulfurreducens* and the closely related *G. metallireducens* [8–13], the present study represents the first evidence that a *c*-type cytochrome is involved in metal reduction in these organisms *in vivo*. The results also suggest that portions of the electron transport pathways for the reduction of Fe(III), U(VI) and humics with acetate as the electron donor differ significantly from the route for electron transfer to these electron acceptors from hydrogen.

Role of PpcA in the reduction of Fe(III)

All of the available evidence suggests that *G. sulfurreducens* reduces Fe(III) at the outer cell surface [8,11,13,29]. If so, the periplasmic location of PpcA precludes it from functioning as the terminal Fe(III) reductase, even though it can reduce Fe(III) *in vitro*. Further evidence that PpcA is not the terminal Fe(III) reductase is that the *ppcA* mutant reduced Fe(III) with hydrogen as the electron donor as well as did the wild type. In contrast, the *ppcA* mutant was significantly impaired in Fe(III) reduction with acetate as the electron donor. In order to reduce Fe(III) with electron donors such as acetate that are metabolized within the cytoplasm, *G. sulfurreducens* requires a mechanism to transfer electrons across the periplasm. This is the likely function of PpcA. The reason that PpcA is not required for Fe(III) reduction with hydrogen as the electron donor is that the hydrogenases that are involved in hydrogen oxidation coupled to Fe(III) reduction are in the periplasm. Fumarate reduction, which is normally catalysed by fumarate reductase located in the cytoplasmic membrane [42] and has been localized to the membrane fraction of *G. sulfurreducens* [43], should not require a periplasmic electron carrier, explaining the lack of impact of the *ppcA* mutation on fumarate reduction with either electron donor.

Further evidence for a specific role for PpcA in electron transfer to Fe(III) with acetate as the electron donor comes from the distribution of cytochromes closely related to PpcA in the *Geobacteraceae*. The four acetate-oxidizing, Fe(III)-reducing *Geobacteraceae* that have been examined, *G. sulfurreducens* [10,12], *G. metallireducens* [8,9], *D. acetoxidans* [5,19] and *G. humireducens* (the present study) all contain a similar c_7 -type cytochrome. However, *Pelobacter* species, which are phylogenetically intertwined with the *Geobacter* and *Desulfuromonas* species in the *Geobacteraceae*, and which can reduce Fe(III) with hydrogen (but not acetate) as the electron donor, do not contain *c*-type cytochromes [44].

The fact that the *ppcA* mutant still retained some ability to reduce Fe(III) with acetate as the electron donor suggests that other electron carriers may shuttle electrons across the periplasm to the electron carriers involved in Fe(III) reduction in the outer membrane. Indeed, there was a significant lag phase before the mutant started to grow at a decreased rate on Fe(III). It is possible that this period represents the time required for induction of alternative mechanisms for electron transfer across the periplasm. This clearly warrants further attention. However, the inhibition of both growth on Fe(III) and the rate of acetate-dependent Fe(III) reduction in pre-grown cells demonstrates the important role of PpcA in electron transport to Fe(III).

Role of PpcA in the reduction of U(VI) and humics

The finding that acetate-dependent U(VI) reduction was inhibited in the *ppcA* mutant demonstrates that PpcA plays a significant role in U(VI) reduction with this electron donor. However, interpretation of the specific role of PpcA in U(VI) reduction is hampered by a lack of complete understanding of the site of U(VI) reduction. Although it has been assumed that much of the U(VI) reduction in *Geobacter* species takes place at the outer membrane, since most of the U(IV) precipitate that is formed is extracellular, selective removal of outer-membrane cytochromes required for the reduction of Fe(III) by treatment of whole cells of *G. sulfurreducens* with protease had little effect on U(VI) reduction by this organism [29]. In addition U(IV) precipitates have also been documented recently within the periplasmic space, suggesting that some U(VI) may be reduced in the periplasm [29]. Thus although it is conceivable that PpcA could

function as an intermediary electron carrier for transfer of electrons to electron carriers involved in U(VI) reduction in the outer membrane, it could also serve as the reductase for U(VI) in the periplasm.

Biochemical studies [45] have suggested that the c_3 cytochrome of *Desulfovibrio* species, which is structurally related to PpcA [5,7], functions as a periplasmic U(VI) reductase. Thus it would not be surprising if PpcA could perform a similar function. However, PpcA cannot be the only U(VI) reductase in *G. sulfurreducens*, as indicated by the fact that when hydrogen was provided as an electron donor the *ppcA* mutant reduced U(VI) as well as did the wild type. Further investigations into the mechanisms of U(VI) reduction, involving generating hydrogenase mutants and mutants defective in the production of various outer-membrane proteins, are under way, and should help to further define the role of PpcA in U(VI) reduction. These mutants will also be useful in further dissecting the role of PpcA in the transfer of electrons from acetate to humics. As with U(VI), the *ppcA* mutant was unable to couple the oxidation of acetate to the reduction of the humic analogue AQDS, but was able to reduce AQDS when hydrogen was supplied as the electron donor. However, as hydrogenases are known to reduce AQDS directly [46], we cannot assume that additional proteins were involved in electron transfer from the hydrogenase to AQDS. If this is true, it is conceivable that PpcA may also transfer electrons directly to AQDS in the periplasm. The same could also be true for humic materials able to traverse the outer membrane.

Comparison with other Fe(III)-reducing micro-organisms

Phylogenetically distinct acetate-oxidizing, Fe(III)-reducing micro-organisms, such as *Geothrix fermentans* and *Geovibrio ferrireducens*, also contain soluble, low-molecular-mass cytochromes, but these are not closely related to PpcA of *G. sulfurreducens*. Similarly, *Shewanella oneidensis* MR-1 lacks a homologue of PpcA in its genome, but there are alternative c -type cytochromes that may form part of an electron transfer chain across the periplasm to the outer membrane in *Shewanella* species. Candidates for this role include a 21 kDa tetrahaem c -type cytochrome [16] and the decahaem c -type cytochrome MtrA in *Shewanella oneidensis* MR-1 [47], as well as a 11.8 kDa tetrahaem cytochrome c_3 in *S. frigidimarina* [17]. The apparent lack of proteins with high sequence identity with PpcA in Fe(III)-reducing bacteria outside the family *Geobacteraceae*, including *S. oneidensis*, is consistent with the emerging pattern that phylogenetically distinct Fe(III)-reducing micro-organisms may have distinctly different mechanisms for Fe(III) reduction [48].

Implications for environmental studies

Acetate is the most important electron donor for Fe(III) reduction in many sedimentary environments [49], and acetate is the preferred electron donor for addition to subsurface environments in order to promote dissimilatory metal reduction for the remediation of uranium contamination [2,50]. *Geobacteraceae* are the predominant Fe(III)-reducing micro-organisms in many sedimentary environments [1–3]. Thus monitoring of the distribution of *ppcA* in environmental samples may provide an indication of the number of acetate-oxidizing *Geobacteraceae* present in the sediments of interest. Furthermore, the level of *ppcA* might be expected to provide an indication of the metabolic activity of these organisms. Studies to further evaluate these possibilities are under way.

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