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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-type cytochromes. The c-type family of cytochromes comprise the smallest representatives of the multihaem cytochrome c family superfamily [18], the best studied example of which is the cytochrome c553 of the sulphur- and Fe(III)-reducing bacterium D. acetoxidans. The cytochrome c553 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 vitro [20]. Alignment with the sequences of tetrahaem c-type cytochromes from sulphate-reducing bacteria of the genus Desulfovibrio have suggested that the cytochrome c553 of D. acetoxidans is structurally similar to cytochrome c553, but lacks haem 2 of the protein. This has been confirmed in NMR-based studies [5] and by a new crystal structure of cytochrome c553 [7].

A trihaem cytochrome c553 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 c553 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 c553 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

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 c553 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 c553 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.

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

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

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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 harvested 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 86250 kPa (12500 lbf/in²) (two passages); the cell debris was removed by centrifugation (5000 g; 20 min) and the supernatant was clarified further using an ultracentrifuge (100000 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 c₇ 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 c₇ 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 c₇ 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 c₇ 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 [10]. Acetate (20 mM) and fumarate (40 mM) were supplied as the electron donor and the electron acceptor, respectively, unless stated otherwise. Cells were harvested under an atmosphere of N₂/CO₂ (80:20) at all times.

**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
Periplasmic cytochrome of *Geobacter sulfurreducens*

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

(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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL01</td>
<td>CCGAGACTATTCTGCCGG</td>
<td>PCR amplification of sequence upstream of gene for cytochrome c (_1) (-411 to +78)</td>
</tr>
<tr>
<td>CL06</td>
<td>GAGGACGATGTCGTCGGC</td>
<td>(\text{CL03})</td>
</tr>
<tr>
<td>CL02</td>
<td>GGTACATATCAAAGAAGCC</td>
<td>PCR amplification of sequence downstream of gene for cytochrome c (_1) (+163 to +693)</td>
</tr>
<tr>
<td>CL05</td>
<td>CGGCAAAGAGATGGCTCATGGC</td>
<td>PCR amplification of sequence downstream of gene for cytochrome c (_1) (+163 to +693)</td>
</tr>
<tr>
<td>CL03</td>
<td>GCCGCCGACGACATCGTCCTCCTGGGATGAATGTCAGCTAC</td>
<td>PCR amplification of the <em>kan²</em> gene from <em>pBBR1MCS-2</em> with homologous overhangs to CL06 and CL05 respectively</td>
</tr>
<tr>
<td>CL04</td>
<td>GCCATGAGCCATCTCTTTGCCG</td>
<td>PCR amplification of sequence upstream of gene for cytochrome c (_1) (-411 to +78)</td>
</tr>
</tbody>
</table>

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\(_1\) (-411 to 78; +1 at A of the start codon), the second the downstream sequence of the target gene (+163 to +693; cytochrome c\(_1\) 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\(_1\) (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 $c_\text{c}$. 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 $\mu$g/ $\mu$L DNA for introduction into electrocompetent cells of $G. \text{sulfurreducens}$. 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 ng/ $\mu$L) were added in a total volume of 50 $\mu$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 $\mu$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 $\mu$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 $\mu$g/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 $c_\text{c}$ 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 $G. \text{sulfurreducens}$ 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 $\times$ 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 $G. \text{sulfurreducens}$ by FPLC using a 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 ($A_{280} - A_{250}$/ $A_{280}$; [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 $c_\text{c}$ of $D. \text{acetoxidans}$ [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 $G. \text{sulfurreducens}$. These organisms include Geobacter ‘humireducens’ [32], Geovibrio ferrireducens [33] and Geolithrix 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

Table 2  Enrichment of cytochrome $c_\text{c}$ during purification from the soluble fraction of $G. \text{sulfurreducens}$

<table>
<thead>
<tr>
<th>Purification step</th>
<th>$A_{280}/A_{250}$</th>
<th>Horio–Kamen index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble fraction</td>
<td>0.27</td>
<td>0.041</td>
</tr>
<tr>
<td>Sephacyr S-300 (gel filtration)</td>
<td>0.41</td>
<td>0.045</td>
</tr>
<tr>
<td>SP-Sepharose (cation exchange)</td>
<td>9.39</td>
<td>1.97</td>
</tr>
<tr>
<td>HiTrap chelating [Fe(III)-IMAC]</td>
<td>12.8</td>
<td>3.16</td>
</tr>
</tbody>
</table>

IMAC, immobilized metal affinity chromatography. The Horio–Kamen index is calculated as $(A_{280} - A_{250})/A_{280}$ oxidized.
A hydrophobic signal peptide is present at the N-terminus of the protein (italics). Underlined amino acids indicate those determined by N-terminal sequencing of purified cytochrome c7. 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 c7.

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The mature protein was 71 amino acids long, with a molecular mass of 9.58 kDa, including the three haems of the protein. A Kyte–Doolittle hydrophylicity plot of PpcA (not shown) confirmed that the mature protein was hydrophilic, in keeping with its proposed periplasmic location [10, 12]. Hydrophobic amino acids were associated only with the signal peptide at the N-terminus of the mature protein.
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**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-Prot 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.

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

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 $c_3$ 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

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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-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(VI) 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(VI) 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

### 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

<table>
<thead>
<tr>
<th>Reduction (mmol·h⁻¹·g dry weight biomass⁻¹)</th>
<th>Hydrogen</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron acceptor</td>
<td>Wild type</td>
<td>Mutant</td>
</tr>
<tr>
<td>Fumarate</td>
<td>53</td>
<td>50</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>5.2</td>
<td>2.2</td>
</tr>
<tr>
<td>AQDS</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>U(VI)</td>
<td>0.57</td>
<td>0.44</td>
</tr>
</tbody>
</table>

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.

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 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.
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 \( c \) cytochrome of *Desulfuromonas acetoxidans* 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. sulfurireducens*, 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 *Geotrichum fermentans* and *Geovibrio ferrireducens*, also contain soluble, low-molecular-mass cytochromes, but these are not closely related to PpcA of *G. sulfurireducens*. 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 \( c \) 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 dissipilatory 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 *ppc* \(_4\) 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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