A trans-outer membrane porin-cytochrome protein complex for extracellular electron transfer by Geobacter sulfurreducens PCA

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Summary
The multi-heme, outer membrane c-type cytochrome (c-Cyt) OmcB of Geobacter sulfurreducens was previously proposed to mediate electron transfer across the outer membrane. However, the underlying mechanism has remained uncharacterized. In G. sulfurreducens, the omcB gene is part of two tandem four-gene clusters, each is predicted to encode a transcriptional factor (OrfR/OrfS), a porin-like outer membrane protein (OmbB/OmbC), a periplasmic c-type cytochrome (OmaB/OmaC) and an outer membrane c-Cyt (OmcB/OmcC) respectively. Here, we showed that OmbB/OmbC, OmaB/OmaC and OmcB/OmcC of G. sulfurreducens PCA formed the porin-cytochrome (Pcc) protein complexes, which were involved in transferring electrons across the outer membrane. The isolated Pcc protein complexes reconstituted in proteoliposomes transferred electrons from reduced methyl viologen across the lipid bilayer of liposomes to Fe(III)-citrate and ferrihydrite. The pcc clusters were found in all eight sequenced Geobacter and 11 other bacterial genomes from six different phyla, demonstrating a widespread distribution of Pcc protein complexes in phylogenetically diverse bacteria. Deletion of ombB-omaB-omcB-orfS-omcC gene clusters had no impact on the growth of G. sulfurreducens PCA with fumarate but diminished the ability of G. sulfurreducens PCA to reduce Fe(III)-citrate and ferrihydrite. Complementation with the ombB-omaB-omcB gene cluster restored the ability of G. sulfurreducens PCA to reduce Fe(III)-citrate and ferrihydrite.

Introduction
Geobacter spp. are abundant Deltaproteobacteria in many sedimentary environments where they can couple oxidation of organic compounds to reduction of oxidized metals, such as Fe(III) oxides (i.e. dissimilatory metal reduction). The dissimilatory metal reduction mediated by Geobacter spp. plays critical roles in global carbon and metal cycles (Lovley et al., 2004; 2011). Because they are poorly soluble in water at circumneutral pH, and in the absence of strong complexing ligands, the solid-phase Fe(III) oxides cannot cross the bacterial outer membrane and so remain external to the bacterial cells. In order for Geobacter spp. to reduce Fe(III) oxides, extracellular electron transfer pathways have evolved in Geobacter spp. to connect intracellular oxidation of organic compounds to the extracellular reduction of Fe(III) oxides. The protein components identified to date for the Geobacter extracellular electron transfer pathways include c-type cytochromes (c-Cyts), multicopper proteins and pilin proteins. They are proposed to transfer electron from the quinone/quinol pool in the inner membrane, across the periplasm and the outer membrane to the Fe(III) oxides directly (Lovley et al., 2004; 2011; Weber et al., 2006; Shi et al., 2007; Bird et al., 2011). Because of their ability to transfer electrons extracellularly, Geobacter spp. has great potential in bioremediation of contaminants, bioenergy production and electrobiosynthesis of valuable chemicals (Lovley et al., 2011).
In *G. sulfurreducens* DL-1, the proteins that are known to be involved in extracellular electron transfer to Fe(III) oxides include a periplasmic c-Cyt PpcA, three outer membrane c-Cys (OmcB, OmcE and OmcS), two outer membrane multi-copper proteins (OmpB and OmpC) and the pilin protein PilA, a key constituent of the electrically conductive *Geobacter* nanowires (Leang et al., 2003; Lloyd et al., 2003; Mehta et al., 2005; 2006; Reguera et al., 2005; Holmes et al., 2008). The exact roles of OmcE, OmpB and OmpC in extracellular reduction of Fe(III) oxides remain unclear. PpcA is believed to transfer electrons across the periplasm (Lloyd et al., 2003), while PilA is required for the long-distance conductivity along the *Geobacter* nanowires with which OmC is physically associated (Reguera et al., 2005; Leang et al., 2010; Malvankar et al., 2011; Reardon and Mueller, 2013; Vargas et al., 2013). Because purified OmC reduces Fe(III) oxides, it is believed to be the terminal reductase for this process (Qian et al., 2011). OmC is localized to the exterior surface of the outer membrane (Qian et al., 2007). Because it is only partially exposed to the external environment, OmC is proposed to be embedded in the outer membrane where it is hypothesized to transfer electrons across the outer membrane (Lovley, 2006; Qian et al., 2007; Lovley et al., 2011).

To investigate the mechanisms by which *Geobacter* spp. transfer electrons across the outer membrane, we further analysed OmC of *G. sulfurreducens* PCA and found that OmC was part of a trans-out membrane porin-cytochrome (Pcc) protein complex that also included a porin-like outer membrane protein and a periplasmic c-Cyt. Together, they transfer electrons across the outer membrane. Genome analyses revealed that Pcc protein complexes are common among *Geobacter* spp. as well as other Gram-negative bacteria from six different phyla, all likely involved in trans-out membrane extracellular electron transfer.

**Results**

**Identification and characterization of the Pcc protein complexes**

To investigate its role in extracellular electron transfer, we first analysed the amino acid sequence of OmC of *G. sulfurreducens* PCA and found no apparent trans-out membrane motif in the OmC sequence, suggesting that by itself OmC is unlikely to be able to transfer electrons across the outer membrane. The omcB gene is part of two tandem four-gene clusters. Each is predicted to encode a transcriptional factor (OrfR/OrfS); an outer membrane protein (OmbB/OmbC); a periplasmic c-Cyt with eight heme-binding motifs (OmaB/OmaC); and an outer membrane c-Cyt with 12 heme-binding motifs (OmcB/OmcC) respectively (Fig. 1A). At the amino acid sequence level, OmbB/OmbC and OmaB/OmaC are 100% identical, respectively, while OrfR/OrfS and OmcB/OmcC are 99% and 71% identical respectively (Leang et al., 2003; Leang and Lovley, 2005; Akuljar et al., 2013). Further analysis of the OmbB/OmbC amino acid sequence with the Hidden Markov Model method predicted that they were porin-like, beta-barrel outer membrane proteins with 20 trans-out membrane motifs (Fig. S1) (Bagos et al., 2004a,b; White et al., 2013).

Comparison of the omcB-associated gene clusters of *G. sulfurreducens* PCA with the mtr (i.e. metal-reducing) gene clusters of another Fe(III)-reducing bacterium *Shewanella oneidensis* MR-1 suggested a possible case of convergent evolution. While the proposed functions and cellular localizations of the proteins encoded by the ombB-omaB-ombB-omaB-omcB-omaC-omcC gene clusters in *G. sulfurreducens* PCA were analogous to that of the mtrC-mtrA-mtrB/mtrD-mtrE-mtrF gene clusters of *S. oneidensis* MR-1, they shared no identity at the amino acid sequence level with exception of heme-binding motifs of the c-Cys. The similarities between their functions and localizations are, however, striking: in *S. oneidensis* MR-1, each mtr three-gene cluster encodes a periplasmic 10-heme c-Cyt (MtrA/MtrD), a porin-like outer membrane protein with 28 predicted trans-out membrane motifs (MtrB/MtrE) and an outer membrane, 10-heme c-Cyt (MtrC/MtrF), respectively (Fig. 1A) (Fredrickson et al., 2008). MtrC is localized on the bacterial cell surface, and MtrABC proteins form a 20-heme complex in the bacterial outer membrane that facilitates electron transfer across the outer membrane (Ross et al., 2007; Fredrickson et al., 2008; Shi et al., 2008; Hartshorne et al., 2009; Lower et al., 2009; Richardson et al., 2012; White et al., 2013). Similar to MtrABC proteins, OmbB/OmbC, OmaB/OmaC and OmcB/OmcC (i.e. porin-cytochrome or Pcc) proteins of *G. sulfurreducens* DL-1 associate with the outer membrane (Ding et al., 2006). These observed similarities between the Pcc and the Mtr proteins suggest that the Pcc proteins may also form complexes for transferring electrons across the bacterial outer membrane.

To test the hypothesis that Pcc proteins form a complex to transfer electrons across the outer membrane, we isolated the Pcc protein complexes from the membrane fraction of *G. sulfurreducens* PCA that was grown with Fe(III)-citrate as the terminal electron acceptor. Analysis of the isolated proteins with Coomassie Blue after SDS-PAGE revealed four bands of proteins with apparent molecular masses of 27, 45, 76 and 80 kDa respectively. Analyses of the same sample by heme staining and OmaB/OmaC- or OmbB/OmbC-specific antibodies confirmed that the 27-, 76- and 80 kDa bands were OmaB/OmaC, OmbB and OmcC respectively.

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Fig. 1. Identification and characterization of the Pcc protein complexes from G. sulfurreducens PCA. (A) Comparison of the omcB-associated gene clusters of G. sulfurreducens PCA and the mtr gene clusters of S. oneidensis MR-1. The genes encoding c-type cytochromes are labelled in red, while those encoding porin-like outer membrane proteins and transcriptional factors are labelled in green and light blue respectively. (B–E) SDS-PAGE analysis of isolated Pcc proteins with Coomassie Blue (B), heme staining (C) and OmaB/OmaC- (D) or OmcB/ OmcC-specific antibodies (E). See Blue Plus 2 Pre-stained standards (Invitrogen, Grand Island, NY, USA) are indicated (lane 1 of B and C). (F) Gel-filtration characterization of isolated Pcc complexes. A representative elution profile of isolated Pcc complexes is shown. The elution times of standard proteins are indicated by arrows. (G) Absorbance change at 606 nm after addition of sodium dithionite to the liposomes without protein (black line) or with 1 nm Pcc proteins (red line). (H) Absorbance change at 606 nm after addition of 200 μM fumarate (black line), Fe(III)-citrate (green line) or ferricydrite (red line) to the Pcc proteoliposomes pre-reduced with sodium dithionite for 5 min.
(Fig. 1B–E). Similar to the porin-like outer membrane protein MtrB of S. oneidensis MR-1 (Hartshorne et al., 2009), the 45 kDa band contained no heme and was weakly stained with Coomassie Blue (Fig. 1B and C). As attempts at generating antibodies specific for OmbB/OmbC were unsuccessful, mass spectrometry analyses confirmed that the 45 kDa band contained OmbB/OmbC (Fig. S2 and Table S1). Gel-filtration analysis of isolated Pcc protein complexes showed that the apparent molecular mass of the isolated complexes was 154 ± 7 kDa (n = 3) (Fig. 1F). The average heme content of isolated Pcc protein complexes was 19.7 ± 0.4 (n = 3). Thus, each of the isolated Pcc complexes is a 20-heme heterotrimer.

To determine whether the Pcc protein complexes transfer electrons across a lipid bilayer, we prepared the Pcc proteoliposomes with encapsulated methyl viologen (MV) (White et al., 2012; 2013). In contrast to the control liposomes lacking Pcc protein complexes that reduced little MV, the addition of sodium dithionite rapidly reduced the MV inside the Pcc proteoliposomes (Fig. 1G). The reduced MV inside the Pcc proteoliposomes was then rapidly re-oxidized by Fe(III)-citrate and ferricydride, a poorly crystalline Fe(III) oxide. Similar to the reduction of MV inside the Pcc proteoliposomes by sodium dithionite, re-oxidation of the reduced MV by Fe(III)-citrate and ferricydride also occurred in two phases: a fast phase at beginning followed by a slower phase. The initial rate of MV oxidation by Fe(III)-citrate (1692.4 ± 190 M⁻¹ S⁻¹, n = 3) was eight times faster than that by ferricydride (211.4 ± 1.4 M⁻¹ S⁻¹, n = 3) (Fig. 1H). These results directly demonstrate the ability of Pcc protein complexes to transfer electrons across a lipid bilayer, similar to our previous findings on the MtrABC complex (Hartshorne et al., 2009; White et al., 2013).

Previous results showed the existence of pcc gene clusters in other Geobacter spp., but no hypothesis was given for the possible functions of this type of gene cluster (Butler et al., 2010). A further survey of sequenced microbial genomes revealed that: (i) in addition to ombB-omaB-omcB and ombC-omaC-omcC, G. sulfurreducens PCA possessed two more pcc gene clusters (Gsu_2724/2725/2726 and Gsu_2642/2643/2644); (ii) all eight sequenced Geobacter genomes contained, at least, one pcc gene cluster; and (iii) the pcc gene clusters also existed in 11 other Gram-negative bacteria that belong to six different phyla, demonstrating a widespread distribution of Pcc protein complexes in phylogenetically diverse bacteria (Table S2). The existence of two additional pcc gene clusters in G. sulfurreducens PCA suggests multiple, parallel pathways for transferring electrons across the outer membrane, which signifies the importance of extracellular electron transfer in the physiology of this microorganism.

Genetic characterization of the omcB-associated gene clusters

To further analyse their roles in extracellular electron transfer, we deleted the ombB-omaB-omcB, ombC-omaC-omcC and ombB-omaB-omcB-orfS-ombC-omaC-omcC gene clusters. After verification of their deletions from the genome (Fig. S3A), we first tested the effects of deleting these gene clusters on the growth of G. sulfurreducens PCA with fumarate as the terminal electron acceptor. Deletion of these omcB-associated gene clusters did not affect the growth of G. sulfurreducens PCA and the mutants were nearly identical under these conditions (Fig. 2A). These results demonstrate that the omcB-associated gene clusters are not required for reduction of fumarate, consistent with previous results for G. sulfurreducens DL-1 (Leang et al., 2003).

We then tested the effects of deleting the omcB-associated gene clusters on the reduction of the soluble Fe(III)-citrate complex. While deletion of ombB-omaB-omcB or ombC-omaC-omcC gene cluster had minor or no impact on the Fe(III)-citrate reduction by G. sulfurreducens PCA, respectively, deletion of the ombB-omaB-omcB-orfS-ombC-omaC-omcC gene clusters significantly decreased the ability of G. sulfurreducens PCA to reduce Fe(III)-citrate. Wild type (wt), ΔombB-omaB-omcB and ΔombC-omaC-omcC completely reduced the 50 mM Fe(III)-citrate within 48 h. However, ΔombB-omaB-omcB-orfS-ombC-omaC-omcC only reduced 32 ± 0.5 (means ± standard deviation, n = 3) mM of Fe(III)-citrate (or 64% of that reduced by wt) after 48 h (Fig. 2B). We also introduced the gene cluster ombB-omaB-omcB into ΔombB-omaB-omcB-orfS-ombC-omaC-omcC and then tested the impact of complementation on the ability of this mutant to reduce Fe(III)-citrate. Heme-staining analysis revealed that heme-containing OmaB and OmcB whose abundances were comparable to that in wt were detected in the complement strain (Fig. S2B). This complementation restored the ability of this mutant to reduce Fe(III)-citrate to the level of 83% of that by the wt. This is consistent with the results that Pcc proteins form complexes and transfer electrons across the lipid-bilayer of the Pcc proteoliposomes and also shows that the observed phenotype of ΔombB-omaB-omcB-orfS-ombC-omaC-omcC in Fe(III)-citrate reduction cannot be attributed to any secondary effect of deleting these gene clusters (Fig. 2B).

We also tested the impacts of deleting the omcB-associated gene clusters on the ability of G. sulfurreducens PCA to reduce ferricydride, and found that the impacts on ferricydride reduction were much greater
than that on Fe(III)-citrate reduction. The deletion of ombB-omaB-omcB, ombC-omaC-omcC and ombB-omaB-omcB-orfS-ombC-omaC-omcC gene clusters decreased the ability of G. sulfurreducens PCA to reduce ferrihydrite by 37%, 11% and 78%, respectively, after 312 h (Fig. 2C). This indicates that both gene clusters are also involved in ferrihydrite reduction in which ombB-omaB-omcB plays a critical role. Similarly, complementation of ΔombB-omaB-omcB-orfS-ombC-omaC-omcC with the ombB-omaB-omcB gene cluster increased the ability of the mutant to reduce ferrihydrite by twofold after 312 h (Fig. 2C).

**Discussion**

Results from this study show for the first time that Pcc proteins form a 20-heme extracellular electron conduit for transferring electrons across the bacterial outer membrane, which is very similar to the MtrABC extracellular electron conduit (Hartshorne et al., 2009; White et al., 2013). Pcc homologues are found in all eight sequenced Geobacter spp. and 11 other bacteria that belong to six different phyla. The observed widespread distribution of Pcc proteins in phylogenetically diverse bacteria suggests a broad application of the Pcc protein complexes in extracellular electron transfer by the Gram-negative bacteria. This finding is very similar to the Mtr proteins whose homologues are found in all characterized metal-reducing Shewanella strains and many other bacteria (Jiao and Newman, 2007; Fredrickson et al., 2008; Shi et al., 2011; 2012; Liu et al., 2012; Emerson et al., 2013).

Results from this study also show for the first time that both ombB-omaB-omcB and ombC-omaC-omcC are involved in reduction of Fe(III)-citrate and ferrihydrite by G. sulfurreducens PCA. This is consistent with the fact that both gene clusters are expressed under the conditions tested, OmaB/OmaC and OmbB/OmbC are 100% identical, respectively, and OmcB and OmcC are 71% identical. Previous results showed that deletion of omcB abolished the ability of G. sulfurreducens DL-1 to reduce Fe(III)-citrate and ferrihydrite, while deletion of omcC had no impact on reduction of these same substrates (Leang et al., 2003). Although the ΔombC-omaC-omcC of G. sulfurreducens PCA from this study behaved very much like the ΔomcC mutant of DL-1 in terms of reducing Fe(III)-citrate and ferrihydrite, the ΔombB-omaB-omcB of this study was affected much less in terms of reduction of Fe(III)-citrate and ferrihydrite relative to the ΔomcB of DL-1. This discrepancy may be attributed to the different adaptability of these two different mutants to compensate for the loss of their respective genes. Previous results showed that the ΔomcB mutant of DL-1 gradually regained the ability to reduce Fe(III)-citrate, but not the ability to reduce ferrihydrite (Leang and Lovley, 2005; Leang et al., 2005). Our results showed the involvement of both ombB-omaB-omcB and ombC-omaC-omcC gene clusters in reducing Fe(III)-citrate and ferrihydrite and...
identification of four pcc gene clusters in the genome of G. sulfurreducens PCA, which clearly demonstrate the existence of multiple and parallel Pcc protein complexes for mediating the electron transfer across the outer membrane. The ΔombB-omaB-omcB of G. sulfurreducens PCA may adapt to different extracellular electron transfer conduits, such as those mediated by other Pcc protein complexes, more quickly and extensively than the ΔomcB of DL-1 to compensate for loss of the ombB-omaB-omcB gene cluster.

Across all identified MtrABC complexes, the amino acid sequences of each individual component (i.e. MtrA, MtrB and MtrC) are conserved (Fredrickson et al., 2008; Shi et al., 2012). All MtrB homologues, including MtoB/PioB of the Fe(II)-oxidizing bacteria, are predicted to possess 28 trans-outer membrane motifs, while all MtrA and MtrC homologues are shown or are predicted to be 10-heme c-Cyts (Jiao and Newman, 2007; Fredrickson et al., 2008; Liu et al., 2012; Shi et al., 2012). Among Pcc complexes, the numbers of predicted trans-outer membrane motifs in the Pcc porin-like outer membrane proteins and typical heme-binding motifs (i.e. CX2CH) in the c-Cyts vary, ranging from 18 to 22 for the trans-outer membrane motifs and 1 to 16 for the heme-binding motifs (Table S2). In MtrABC complex of S. oneidensis MR-1, MtrC can function as the terminal reductase of Fe(III) oxides (Okamoto et al., 2013; White et al., 2013). In G. sulfurreducens DL-1, the outer membrane c-Cyt OmcB is not a terminal reductase of Fe(III) oxide and need to transfer electrons to the terminal reductase OmcS either directly or indirectly via other proteins during reduction of Fe(III) oxides. Likewise, the periplasmic c-Cyts of the Pcc protein complexes also need to receive electrons from other periplasmic c-Cyts to recharge the outer membrane c-Cyts. Lack of sequence conservation among the Pcc c-Cyts indicates that different Pcc protein complexes may interact with different periplasmic and outer membrane c-Cyts for intermolecular electron transfer.

Despite the inherent differences between the Mtr and Pcc protein complexes, our results reveal that the porin-cytochrome protein complex, which was originally identified in S. oneidensis MR-1 (Hartshorne et al., 2009; Richardson et al., 2012), is a common mechanism that is now extended to G. sulfurreducens PCA and likely other bacteria for mediating trans-outer membrane electron transfer reactions. In G. sulfurreducens PCA, the outer membrane protein of the Pcc complex may serve as a sheath through which the c-Cyts may be inserted to provide a heme conduit for contiguous electron transfer across the outer membrane (Fig. 3A), similar to that proposed for MtrB in the MtrABC complex (Fig. 3B) (Hartshorne et al., 2009; Richardson et al., 2012). Indeed, the observed functional and organizational similarities between the Pcc and Mtr proteins are quite remarkable and suggest that these two systems have evolved independently to mediate electron transfer across the bacterial outer membrane by using the same design principle. At this point, only molecular structures of Mtr outer membrane c-Cyts have been determined (Clarke et al., 2011; Edwards et al., 2012; 2014), and detailed structural comparisons await the availability of the high-resolution structures of other Mtr and Pcc proteins as well as the intact protein complexes. As research on microbial extracellular electron transfer continues, we...
expect that additional extracellular electron transfer complexes with designs similar to that of the Pcc and Mtr systems will be identified.

Experimental procedures

Bacterial growth, protein purification and characterization

*Geobacter sulfurreducens* PCA (ATCC 51573) was routinely cultured in the media with 10 mM acetate as an electron donor and 40 mM fumarate as an electron acceptor prior to its growth for protein purification. Twenty grams of wet *G. sulfurreducens* PCA cells grown with 50 mM Fe(III)-citrate as an electron acceptor were used for isolating Pcc protein complexes under the conditions described previously (Shi *et al.*, 2006). The cell pellets were thawed and re-suspended in 100 ml of buffer A [20 mM HEPES, pH 7.8, protease inhibitor (Roche Diagnostic, Indianapolis, IN, USA)]. The cells were lysed by passage through a French press three times at 8000 lb in$^2$. The unbroken cells and debris were removed by centrifugation at 15,000 × g for 30 min. The supernatant was transferred to ultracentrifugation tubes and further centrifuged at 150,000 × g for 1 h. The membrane fraction was solubilized with 50 ml of buffer B (A + 5% Triton X-100) for 17 h. After removing insolubilized material by centrifugation (15,000 × g, 30 min), the supernatant was loaded onto a 2.5 cm (diameter) × 10 cm (height) column of DEAE (diethylaminoethyl) cellulose (Bio-Rad, Hercules, CA, USA) that was pre-equilibrated with buffer C (A + 1% Triton X-100). After washing with 100 ml of buffer C, the column was eluted with 0 to 0.5 M NaCl gradient in buffer C (total, 200 ml). The fractions with OmaB/OmaC and OmcB/OmcC were pooled and concentrated with an Amicon centrifugal filter device (Millipore, Billerica, MA, USA). The concentrated sample was loaded onto a HiLoad 26/60 column of Superdex 200 (GE Healthcare, Piscataway, NJ, USA) pre-equilibrated with buffer C and eluted with buffer C. The fractions with OmaB/OmaC and OmcB/OmcC were pooled and then loaded onto a Mono Q 5/50 GL column (GE Healthcare) pre-equilibrated with buffer C. After washing with 5 ml of buffer C, the column was eluted with 0 to 0.5 M NaCl gradient in buffer C (total, 20 ml). The purified protein complexes were stored at -20°C. The apparent molecular mass of purified Pcc complexes was determined by using a HiLoad 16/60 column of Superdex 200 (GE Healthcare) pre-equilibrated with buffer D (A + 0.2% Triton X-100) and eluted with buffer D. The column was calibrated with the protein standards from Bio-Rad. All the chromatographic columns purchased from GE Healthcare were operated by means of the ÄKTAexplorer system (Amersham Biosciences, Uppsala, Sweden). The mass spectrometry identification of OmbB/OmaC proteins after SDS-PAGE separation was performed by following a published protocol (Shivechenko *et al.*, 2006). The preparation and characterization of Pcc proteoliposomes were similar to that of the Mtr proteoliposomes (Hartshorne *et al.*, 2009; White *et al.*, 2012; 2013). The procedures for searching for and analysing Pcc homologues and predicting porin-like outer membrane proteins were described previously (Bagos *et al.*, 2004a,b; Shi *et al.*, 2012; White *et al.*, 2013).

Antibody generation

Polyclonal antibodies were prepared by Biomatik (Wilmington, DE, USA) with synthesized OmaB/OmaC or OmcB/OmcC polypeptides as antigens (Table S3). After affinity purification with their respectively synthesized polypeptides, the antibodies were characterized with *G. sulfurreducens* PCA and ombB-omaB-omcB-orfS-ombC-omaC-omcC (Fig. S4).

Mutant construction and Fe(III) reductions

*Geobacter sulfurreducens* PCA was also cultured in the media with 10 mM acetate as an electron donor and 40 mM fumarate as an electron acceptor prior to construction of gene cluster replacement mutants. The gene cluster replacement mutants and related complement strain were constructed by using established protocols (Coppi *et al.*, 2001; Leang *et al.*, 2003; Lloyd *et al.*, 2003; Rollefson *et al.*, 2009). All deletion mutants and complement strain were confirmed by polymerase chain reaction. Bacterial strains, plasmids and oligonucleotide primers used in this study are listed in Table S3. Amorphous 2-line ferrihydrite was synthesized (Schwertman and Cornell, 2000) and characterized using transmission electron microscopy (TEM, Jeol JEM 2010 high-resolution TEM, Peabody, MA, USA) and powder X-ray diffraction (XRD, Philips PW 3040/00 X-pert MPD system, Westborough, MA, USA). For Fe(III)-reduction assays, all *Geobacter* strains were pre-cultured in the medium with fumarate as an electrons acceptor. Reduction of 50 mM of Fe(III)-citrate or 2-line ferrihydrite was carried out at 30°C with *Geobacter* cells at starting OD$_{600}$ of 0.05 (Leang *et al.*, 2003; Rollefson *et al.*, 2009). All procedures were performed in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) that was filled with 5% H$_2$, 20% CO$_2$ and 75% N$_2$. The reduced Fe(II) was measured with a ferrozine assay (Stokey, 1970), and total Fe was determined with inductively coupled plasma emission spectroscopy (Perkin-Elmer, Waltham, MA, USA).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. The amino acid sequence of OmbB/OmbC. The N-terminus and predicted short solvent-exposed loops are in green, the predicted trans-outter membrane motifs are in red, and predicted long solvent-exposed loops are in blue. The 20 trans-outter membrane motifs are numbered sequentially, and the numbers are displayed in black and above the polypeptide sequence.

Fig. S2. Mass spectrometry confirmation of OmbB/OmbC proteins. OmbB/OmbC amino acid sequence with peptides identified by tryptic digest MALDI mass spectrometry are highlighted in red (sequence coverage 22%). Observed peaks and corresponding peptides of OmbB/OmbC are detailed in Table S1.

Fig. S3. Verification of gene cluster replacement mutants and related complement strain by heme staining after SDS-PAGE.

A. The gene cluster replacement mutants. Lane 1, ΔombB-ombC-omaB-omaC-omcB-omcC; lane 2, ΔombB-omaB-omaC-omcC; lane 3, ΔombB-omaB-omcB-omfS-omcA-omcC.

B. Complement strain of ΔombB-omaB-omfS-omcA-omcC with ombB-ombB-omaB. G. sulfurreducens PCA cells were used as a positive control. OmaB/OmaA and OmcB/OmcC are indicated. About 10^6 CFU were loaded in each lane.

Fig. S4. Characterization of OmaB/OmaC- or OmcB/OmcC-specific antibodies.

A. OmaB/OmaC-specific antibodies.
B. OmcB/OmcC-specific antibodies. Migration positions of the proteins standards (kDa) are indicated. Affinity purified OmaBC-specific antibodies were used at 1: 10 000 dilutions; while affinity purified OmcBC-specific antibodies were used at 1: 20 000 dilutions. About $10^4$ CFU were loaded in each lane. Lane 1, *G. sulfurreducens* PCA; lane 2, $\Delta$ombB-omaB-omcB-orfS-ombC-omaC-omcC.

**Table S1.** Identified peptide sequences of OmbB/OmbC by mass spectrometry.
**Table S2.** Identified bacterial Pcc homologues$^a$.
**Table S3.** Bacterial strains, plasmids, primers and polypeptides used in this study.