environmental microbiology reports

Environmental Microbiology Reports (2014) **6**(6), 776–785 doi:10.1111/1758-2229.12204

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

Yimo Liu,[1] Zheming Wang[1], Juan Liu,[1†] Caleb Levar,[2] Marcus J. Edwards,[3] Jerome T. Babauta,[4] David W. Kennedy,[1] Zhi Shi,[1‡] Haluk Beyenal,[4] Daniel R. Bond,[2] Thomas A. Clarke,[3] Julea N. Butt,[3] David J. Richardson,[3] Kevin M. Rosso,[1] John M. Zachara,[1] James K. Fredrickson[1] and Liang Shi[1]*

[1]*Fundamental & Computational Sciences Directorate, Pacific Northwest National Laboratory, Richland WA 99352, USA.*

[2]*Department of Microbiology, University of Minnesota, St. Paul, MN 55108, USA.*

[3]*Center for Molecular and Structural Biochemistry, School of Biological Sciences and School of Chemistry, University of East Anglia, Norwich NR4 7TJ, UK.*

[4]*The Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, WA 99164, USA.*

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-ombC-omaC-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.

Received 17 June, 2014; accepted 14 August, 2014. *For correspondence. E-mail liang.shi@pnnl.gov; Tel. 509 371 6967; Fax 509 376 1632. Present addresses: †College of Environmental Sciences and Engineering, Peking University, China; ‡Analytical Technology Center, The Dow Chemical Company, Freeport, TX 77541, USA.

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*-Cyts (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 OmcS 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 OmcS reduces Fe(III) oxides, it is believed to be the terminal reductase for this process (Qian *et al*., 2011). OmcB is localized to the exterior surface of the outer membrane (Qian *et al*., 2007). Because it is only partially exposed to the external environment, OmcB 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 OmcB of *G. sulfurreducens* PCA and found that OmcB was part of a trans-outer 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-outer 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 OmcB of *G. sulfurreducens* PCA and found no apparent trans-outer membrane motif in the OmcB sequence, suggesting that by itself OmcB 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; Aklujkar *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-outer 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-omcB/ombC-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*-Cyts. 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-outer 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 OmcB/OmcC-specific antibodies confirmed that the 27-, 76- and 80 kDa bands were OmaB/OmaC, OmcB and OmcC respectively

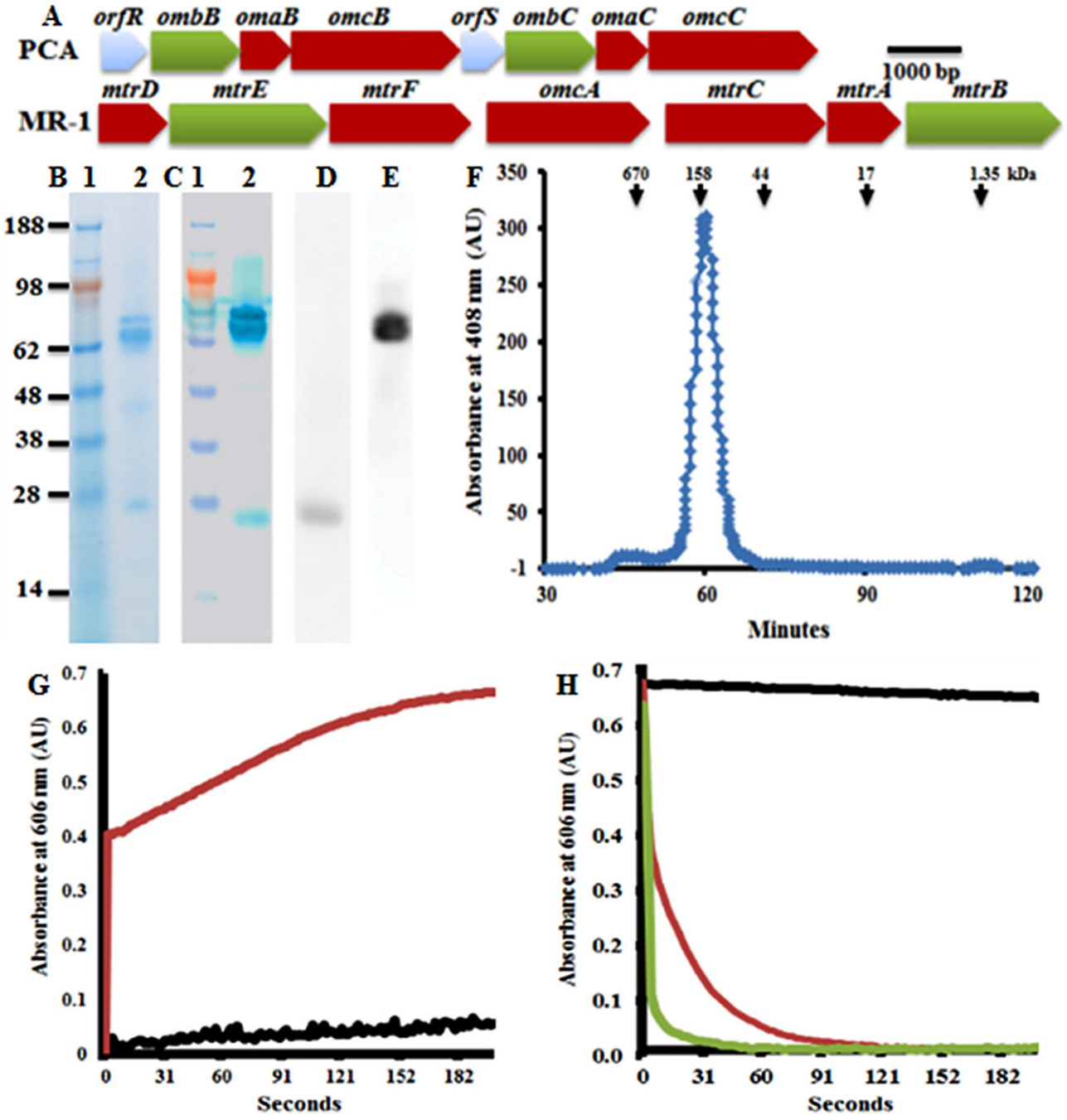

Fig. 1. Identification and characterization of the Pcc protein complexes from *G. sulfurreducens* PCA. (A) Comparison of the *omcB*-associated gene clusters of *G. sulfurreducens* PCA and the *mtr* gene clusters of *S. oneidensis* MR-1.The genes encoding *c*-type cytochromes are labelled in red, while 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 ferrihydrite (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 ferrihydrite, 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 ferrihydrite 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 ($1692.4 \pm 190\ M^{-1}\ S^{-1}$, $n = 3$) was eight times faster than that by ferrihydrite ($211.4 \pm 1.4\ M^{-1}\ S^{-1}$, $n = 3$) (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 cluster 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 on fumarate, as 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 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), Δ*ombB-omaB-omcB* and Δ*ombC-omaC-omcC* completely reduced the 50 mM Fe(III)-citrate within 48 h. However, Δ*ombB-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 Δ*ombB-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 Δ*ombB-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 ferrihydrite, and found that the impacts on ferrihydrite reduction were much greater

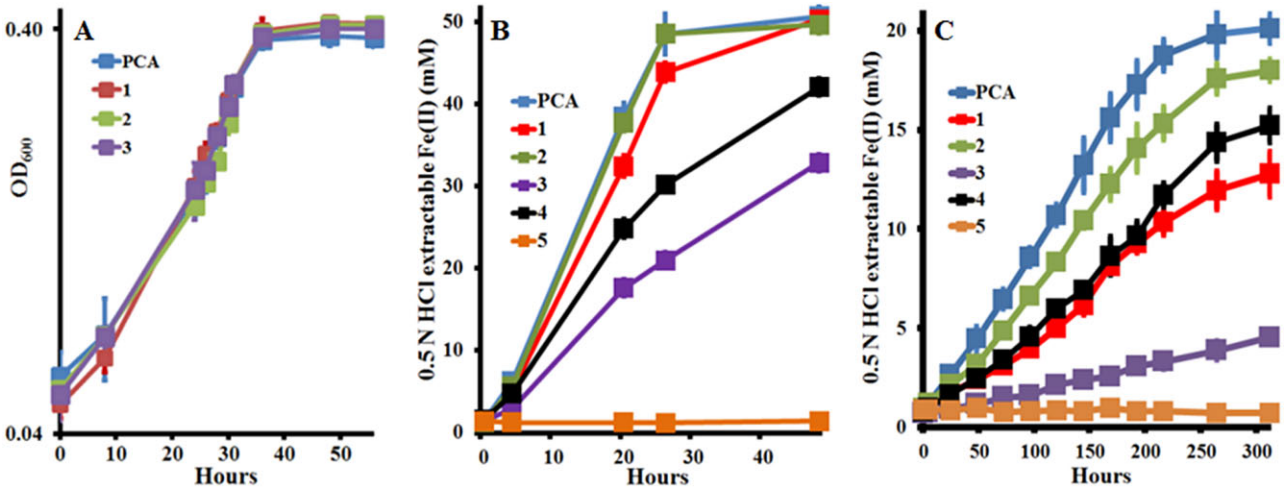

Fig. 2. Characterization of the *omcB*-associated gene clusters of *G. sulfurreducens* PCA.
A. Growth on fumarate.
B. Fe(III)-citrate reduction.
C. Ferrihydrite reduction. The curves are labelled in the same way in (A–C). PCA, *G. sulfurreducens* PCA; 1, Δ*ombB-omaB-omcB*; 2, Δ*ombC-omaC-omcC*; 3, Δ*ombB-omaB-omcB-orfS-ombC-omaC-omcC* (A) or Δ*ombB-omaB-omcB-orfS-ombC-omaC-omcC* + empty vector (B and C); 4, Δ*ombB-omaB-omcB-orfS-ombC-omaC-omcC* complemented with *ombB-omaB-omcB*; 5, no cell control. The values plotted at each time point are the average OD_{600} (A) and 0.5 N HCl extractable Fe(II) (B and C) measured for each strain from triplicate assays, respectively and error bars are standard deviations. The Y-axis of (A) is on logarithmic scale.

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 Δ*ombB-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 Δ*ombC-omaC-omcC* of *G. sulfurreducens* PCA from this study behaved very much like the Δ*omcC* mutant of DL-1 in terms of reducing Fe(III)-citrate and ferrihydrite, the Δ*ombB-omaB-omcB* of this study was affected much less in terms of reduction of Fe(III)-citrate and ferrihydrite relative to the Δ*omcB* 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 Δ*omcB* 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

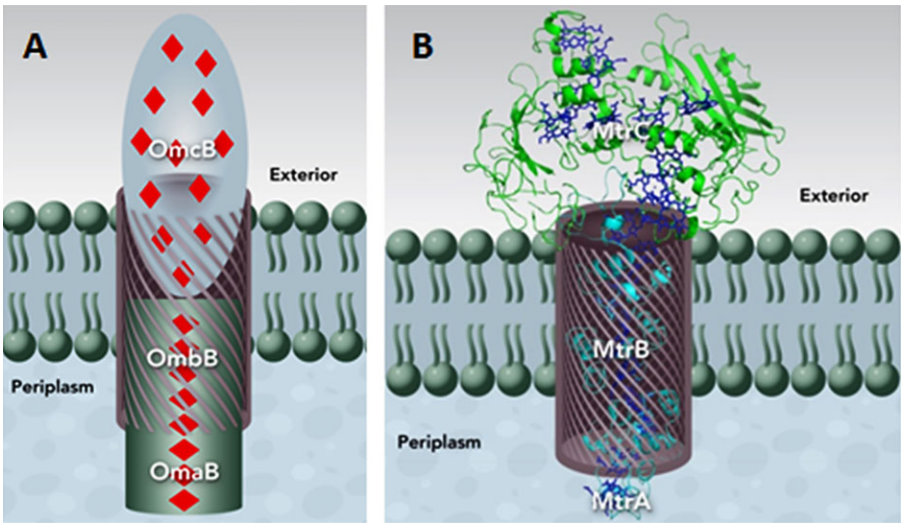

Fig. 3. The proposed Pcc model of *G. sulfurreducens* PCA (A) and MtrABC model of *S. oneidensis* MR-1 (B).
A. Only OmaB, OmbB and OmcB are shown and the heme groups are labelled in red.
B. MtrA and MtrC are modelled based on the structures of NrfB and MtrF, respectively (Clarke *et al*., 2007; 2011), and the heme groups of MtrA and MtrC are labelled in blue.

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 Δ*ombB-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 Δ*omcB* 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. CX_2CH) 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 (diethylaminoethanol) 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 fast protein liquid chromatography systems (GE Healthcare). The procedures for SDS-PAGE, CoomassieBlue (Pierce, Rockford, IL, USA) staining, heme staining, Western blot analysis and measurements of heme content and protein concentration were described previously (Shi *et al.*, 2006; Liu *et al.*, 2012). The mass spectrometry identification of OmbB/OmaC proteins after SDS-PAGE separation was performed by following a published protocol (Shevchenko *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 Δ*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 (Stookey, 1970), and total Fe was determined with inductively coupled plasma emission spectroscopy (Perkin-Elmer, Waltham, MA, USA).

Acknowledgements

This work was supported by the Subsurface Biogeochemical Research program (SBR)/Office of Biological and Environmental Research (BER), U.S. Department of Energy (DOE), and is a contribution of the Pacific Northwest National Laboratory (PNNL) Scientific Focus Area. Y. L. was supported by the Genome Science Program (GSP)/BER (DE-SC0007229). We are grateful for experimental assistance from Dr G. Saalbach (John Innes Centre proteomics facility, UK). A portion of the research was performed at the Environmental Molecular Sciences Laboratory (EMSL), a national scientific user facility sponsored by DOE-BER and located at

PNNL. Pacific Northwest National Laboratory is operated for the DOE by Battelle under contract DE-AC05-76RLO 1830.

References

Aklujkar, M., Coppi, M.V., Leang, C., Kim, B.C., Chavan, M.A., Perpetua, L.A., *et al*. (2013) Proteins involved in electron transfer to Fe(III) and Mn(IV) oxides by *Geobacter sulfurreducens* and *Geobacter uraniireducens*. *Microbiology* **159:** 515–535.

Bagos, P.G., Liakopoulos, T.D., Spyropoulos, I.C., and Hamodrakas, S.J. (2004a) A Hidden Markov Model method, capable of predicting and discriminating beta-barrel outer membrane proteins. *BMC Bioinformatics* **5:** 29.

Bagos, P.G., Liakopoulos, T.D., Spyropoulos, I.C., and Hamodrakas, S.J. (2004b) PRED-TMBB: a web server for predicting the topology of beta-barrel outer membrane proteins. *Nucleic Acids Res* **32:** W400–W404.

Bird, L.J., Bonnefoy, V., and Newman, D.K. (2011) Bioenergetic challenges of microbial iron metabolisms. *Trends Microbiol* **19:** 330–340.

Butler, J.E., Young, N.D., and Lovley, D.R. (2010) Evolution of electron transfer out of the cell: comparative genomics of six *Geobacter* genomes. *BMC Genomics* **11:** 40.

Clarke, T.A., Cole, J.A., Richardson, D.J., and Hemmings, A.M. (2007) The crystal structure of the pentahaem *c*-type cytochrome NrfB and characterization of its solution-state interaction with the pentahaem nitrite reductase NrfA. *Biochem J* **406:** 19–30.

Clarke, T.A., Edwards, M.J., Gates, A.J., Hall, A., White, G.F., Bradley, J., *et al*. (2011) Structure of a bacterial cell surface decaheme electron conduit. *Proc Natl Acad Sci USA* **108:** 9384–9389.

Coppi, M.V., Leang, C., Sandler, S.J., and Lovley, D.R. (2001) Development of a genetic system for *Geobacter sulfurreducens*. *Appl Environ Microbiol* **67:** 3180–3187.

Ding, Y.H., Hixson, K.K., Giometti, C.S., Stanley, A., Esteve-Nunez, A., Khare, T., *et al*. (2006) The proteome of dissimilatory metal-reducing microorganism *Geobacter sulfurreducens* under various growth conditions. *Biochim Biophys Acta* **1764:** 1198–1206.

Edwards, M.J., Hall, A., Shi, L., Fredrickson, J.K., Zachara, J.M., Butt, J.N., *et al*. (2012) The crystal structure of the extracellular 11-heme cytochrome UndA reveals a conserved 10-heme motif and defined binding site for soluble iron chelates. *Structure* **20:** 1275–1284.

Edwards, M.J., Baiden, N.A., Johs, A., Tomanicek, S.J., Liang, L., Shi, L., *et al*. (2014) The X-ray crystal structure of *Shewanella oneidensis* OmcA reveals new insight at the microbe-mineral interface. *FEBS Lett* **588:** 1886–1890.

Emerson, D., Field, E.K., Chertkov, O., Davenport, K.W., Goodwin, L., Munk, C., *et al*. (2013) Comparative genomics of freshwater Fe-oxidizing bacteria: implications for physiology, ecology, and systematics. *Front Microbiol* **4:** 254.

Fredrickson, J.K., Romine, M.F., Beliaev, A.S., Auchtung, J.M., Driscoll, M.E., Gardner, T.S., *et al*. (2008) Towards environmental systems biology of *Shewanella*. *Nat Rev Microbiol* **6:** 592–603.

Hartshorne, R.S., Reardon, C.L., Ross, D., Nuester, J., Clarke, T.A., Gates, A.J., *et al*. (2009) Characterization of an electron conduit between bacteria and the extracellular environment. *Proc Natl Acad Sci USA* **106:** 22169–22174.

Holmes, D.E., Mester, T., O'Neil, R.A., Perpetua, L.A., Larrahondo, M.J., Glaven, R., *et al*. (2008) Genes for two multicopper proteins required for Fe(III) oxide reduction in *Geobacter sulfurreducens* have different expression patterns both in the subsurface and on energy-harvesting electrodes. *Microbiology* **154:** 1422–1435.

Jiao, Y., and Newman, D.K. (2007) The *pio* operon is essential for phototrophic Fe(II) oxidation in *Rhodopseudomonas palustris* TIE-1. *J Bacteriol* **189:** 1765–1773.

Leang, C., and Lovley, D.R. (2005) Regulation of two highly similar genes, *omcB* and *omcC*, in a 10 kb chromosomal duplication in *Geobacter sulfurreducens*. *Microbiology* **151:** 1761–1767.

Leang, C., Coppi, M.V., and Lovley, D.R. (2003) OmcB, a *c*-type polyheme cytochrome, involved in Fe(III) reduction in *Geobacter sulfurreducens*. *J Bacteriol* **185:** 2096–2103.

Leang, C., Adams, L.A., Chin, K.J., Nevin, K.P., Methe, B.A., Webster, J., *et al*. (2005) Adaptation to disruption of the electron transfer pathway for Fe(III) reduction in *Geobacter sulfurreducens*. *J Bacteriol* **187:** 5918–5926.

Leang, C., Qian, X., Mester, T., and Lovley, D.R. (2010) Alignment of the *c*-type cytochrome OmcS along pili of *Geobacter sulfurreducens*. *Appl Environ Microbiol* **76:** 4080–4084.

Liu, J., Wang, Z., Belchik, S.M., Edwards, M.J., Liu, C., Kennedy, D.W., *et al*. (2012) Identification and characterization of MtoA: a decaheme *c*-type cytochrome of the neutrophilic Fe(II)-Oxidizing bacterium *Sideroxydans lithotrophicus* ES-1. *Front Microbiol* **3:** 37.

Lloyd, J.R., Leang, C., Hodges Myerson, A.L., Coppi, M.V., Cuifo, S., Methe, B., *et al*. (2003) Biochemical and genetic characterization of PpcA, a periplasmic *c*-type cytochrome in *Geobacter sulfurreducens*. *Biochem J* **369:** 153–161.

Lovley, D.R. (2006) Bug juice: harvesting electricity with microorganisms. *Nat Rev Microbiol* **4:** 497–508.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004) Dissimilatory Fe(III) and Mn(IV) reduction. *Adv Microb Physiol* **49:** 219–286.

Lovley, D.R., Ueki, T., Zhang, T., Malvankar, N.S., Shrestha, P.M., Flanagan, K.A., *et al*. (2011) *Geobacter*: the microbe electric's physiology, ecology, and practical applications. *Adv Microb Physiol* **59:** 1–100.

Lower, B.H., Yongsunthon, R., Shi, L., Wildling, L., Gruber, H.J., Wigginton, N.S., *et al*. (2009) Antibody recognition force microscopy shows that outer membrane cytochromes OmcA and MtrC are expressed on the exterior surface of *Shewanella oneidensis* MR-1. *Appl Environ Microbiol* **75:** 2931–2935.

Malvankar, N.S., Vargas, M., Nevin, K.P., Franks, A.E., Leang, C., Kim, B.C., *et al*. (2011) Tunable metallic-like conductivity in microbial nanowire networks. *Nat Nanotechnol* **6:** 573–579.

Mehta, T., Coppi, M.V., Childers, S.E., and Lovley, D.R. (2005) Outer membrane *c*-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. *Appl Environ Microbiol* **71:** 8634–8641.

Mehta, T., Childers, S.E., Glaven, R., Lovley, D.R., and Mester, T. (2006) A putative multicopper protein secreted by an atypical type II secretion system involved in the reduction of insoluble electron acceptors in *Geobacter sulfurreducens*. *Microbiology* **152:** 2257–2264.

Okamoto, A., Hashimoto, K., Nealson, K.H., and Nakamura, R. (2013) Rate enhancement of bacterial extracellular electron transport involves bound flavin semiquinones. *Proc Natl Acad Sci USA* **110:** 7856–7861.

Qian, X., Reguera, G., Mester, T., and Lovley, D.R. (2007) Evidence that OmcB and OmpB of *Geobacter sulfurreducens* are outer membrane surface proteins. *FEMS Microbiol Lett* **277:** 21–27.

Qian, X., Mester, T., Morgado, L., Arakawa, T., Sharma, M.L., Inoue, K., *et al*. (2011) Biochemical characterization of purified OmcS, a *c*-type cytochrome required for insoluble Fe(III) reduction in *Geobacter sulfurreducens*. *Biochim Biophys Acta* **1807:** 404–412.

Reardon, P.N., and Mueller, K.T. (2013) Structure of the type IVa major pilin from the electrically conductive bacterial nanowires of *Geobacter sulfurreducens*. *J Biol Chem* **288:** 29260–29266.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., and Lovley, D.R. (2005) Extracellular electron transfer via microbial nanowires. *Nature* **435:** 1098–1101.

Richardson, D.J., Butt, J.N., Fredrickson, J.K., Zachara, J.M., Shi, L., Edwards, M.J., *et al*. (2012) The 'porin-cytochrome' model for microbe-to-mineral electron transfer. *Mol Microbiol* **85:** 201–212.

Rollefson, J.B., Levar, C.E., and Bond, D.R. (2009) Identification of genes involved in biofilm formation and respiration via mini-Himar transposon mutagenesis of *Geobacter sulfurreducens*. *J Bacteriol* **191:** 4207–4217.

Ross, D.E., Ruebush, S.S., Brantley, S.L., Hartshorne, R.S., Clarke, T.A., Richardson, D.J., and Tien, M. (2007) Characterization of protein–protein interactions involved in iron reduction by *Shewanella oneidensis* MR-1. *Appl Environ Microbiol* **73:** 5797–5808.

Schwertman, U., and Cornell, R.M. (2000) *Iron Oxides in the Laboratory: Preparation and Characterization*. Weinheim, Germany: Wiley-VCH.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* **1:** 2856–2860.

Shi, L., Chen, B., Wang, Z., Elias, D.A., Mayer, M.U., Gorby, Y.A., *et al*. (2006) Isolation of a high-affinity functional protein complex between OmcA and MtrC: two outer membrane decaheme *c*-type cytochromes of *Shewanella oneidensis* MR-1. *J Bacteriol* **188:** 4705–4714.

Shi, L., Squier, T.C., Zachara, J.M., and Fredrickson, J.K. (2007) Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem *c*-type cytochromes. *Mol Microbiol* **65:** 12–20.

Shi, L., Deng, S., Marshall, M.J., Wang, Z., Kennedy, D.W., Dohnalkova, A.C., *et al*. (2008) Direct involvement of type II secretion system in extracellular translocation of *Shewanella oneidensis* outer membrane cytochromes MtrC and OmcA. *J Bacteriol* **190:** 5512–5516.

Shi, L., Belchik, S.M., Wang, Z., Kennedy, D.W., Dohnalkova, A.C., Marshall, M.J., *et al*. (2011) Identification and characterization of $UndA_{HRCR-6}$, an outer membrane endecaheme *c*-type cytochrome of *Shewanella* sp. strain HRCR-6. *Appl Environ Microbiol* **77:** 5521–5523.

Shi, L., Rosso, K.M., Zachara, J.M., and Fredrickson, J.K. (2012) Mtr extracellular electron-transfer pathways in Fe(III)-reducing or Fe(II)-oxidizing bacteria: a genomic perspective. *Biochem Soc Trans* **40:** 1261–1267.

Stookey, L. (1970) Ferrozine-a new spectrophotometric reagent for iron. *Anal Chem* **42:** 779–781.

Vargas, M., Malvankar, N.S., Tremblay, P.L., Leang, C., Smith, J.A., Patel, P., *et al*. (2013) Aromatic amino acids required for pili conductivity and long-range extracellular electron transport in *Geobacter sulfurreducens*. *mBio* **4:** e105–e113.

Weber, K.A., Achenbach, L.A., and Coates, J.D. (2006) Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nat Rev Microbiol* **4:** 752–764.

White, G.F., Shi, Z., Shi, L., Dohnalkova, A.C., Fredrickson, J.K., Zachara, J.M., *et al*. (2012) Development of a proteoliposome model to probe transmembrane electron-transfer reactions. *Biochem Soc Trans* **40:** 1257–1260.

White, G.F., Shi, Z., Shi, L., Wang, Z., Dohnalkova, A.C., Marshall, M.J., *et al*. (2013) Rapid electron exchange between surface-exposed bacterial cytochromes and Fe(III) minerals. *Proc Natl Acad Sci USA* **110:** 6346–6351.

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-outer membrane motifs are in red, and predicted long solvent-exposed loops are in blue. The 20 trans-outer 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, Δ*ombB-omaB-omcB*; lane 2, Δ*ombC-omaC-omcC*; lane 3, Δ*ombB-omaB-omcB-orfS-ombC-omaC-omcC*.

B. Complement strain of Δ*ombB-omaB-omcB-orfS-ombC-omaC-omcC* with *ombB-omaB-omcB*. *G. sulfurreducens* PCA cells were used as a positive control. OmaB/OmaC and OmcB/OmcC are indicated. About 10^4 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, Δ*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.