Extracellular electron transfer is the key metabolic trait that enables some bacteria to play a significant role in the biogeochemical cycling of metals and in bioelectrochemical devices such as microbial fuel cells. In *Shewanella oneidensis* MR-1, electrons generated in the cytoplasm by catabolic processes must cross the periplasmic space to reach terminal oxidoreductases found at the cell surface. Lack of knowledge on how these electrons flow across the periplasmic space is one of the unresolved issues related with extracellular electron transfer. Using NMR to probe protein–protein interactions, kinetic measurements of electron transfer and electrostatic calculations, we were able to identify protein partners and their docking sites, and determine the dissociation constants. The results showed that both STC (small tetrahaem cytochrome *c*) and FccA (flavocytochrome *c*) interact with their redox partners, CymA and MtrA, through a single haem, avoiding the establishment of stable redox complexes capable of spanning the periplasmic space. Furthermore, we verified that the most abundant periplasmic cytochromes STC, FccA and ScyA (monohaem cytochrome *c*$_1$) do not interact with each other and this is likely to be the consequence of negative surface charges in these proteins. This reveals the co-existence of two non-mixing redox pathways that lead to extracellular electron transfer in *S. oneidensis* MR-1 established through transient protein interactions.

**Key words:** binding site, chemical shift, dissociation constant, electrostatics, periplasmic cytochrome, *Shewanella oneidensis* MR-1.

## INTRODUCTION

Metal-reducing organisms are attracting widespread attention from the scientific and engineering communities due to the key role that they play in the biogeochemical cycling of metals and in novel biotechnological processes for the bioremediation of contaminated sites, sustainable energy production or synthesis of added-value compounds from waste [1–4].

*Shewanella oneidensis* MR-1 is one of the most studied metal-reducing organisms. This Gram-negative γ-proteobacterium is one of the most versatile organisms with respect to the use of terminal electron acceptors [5,6]. In addition to gaseous oxygen it can use soluble compounds, such as fumarate, and also insoluble compounds, such as metal oxides or even electrode surfaces in bioelectrochemical devices [6–8]. Reduction of insoluble compounds requires the delivery of electrons to the cell surface. In Gram-negative bacteria electrons from the quinone pool located in the cytoplasmic membrane must cross the periplasmic space to reach the terminal reductases located in the external membrane [9].

Proceeding outwards, the quinone pool reduces the 21 kDa tetrahaem cytochrome CymA attached to the cytoplasmic membrane that acts as a hub for electron distribution to multiple periplasmic partners [10–12]. Among these, MtrA is a 35 kDa decahaem periplasmic protein that has a definite phenotype in metal reduction in *S. oneidensis* MR-1. It was shown to be 235 Å wide [18], which is too wide for electron transfer to proceed by direct contact between CymA and the MtrCAB–OmcA complex. Therefore MtrA must be able to diffuse within the periplasmic space or additional redox proteins are necessary to establish the transfer of electrons across the periplasmic gap.

The most abundant periplasmic proteins found in anaerobically grown *S. oneidensis* MR-1 are the STC (small tetrahaem cytochrome *c*, also known as CctA), ScyA (monohaem cytochrome *c*$_1$) and the FccA (flavocytochrome *c*) [19,20]. Their genes are up-regulated during extracellular respiration [21], but individual gene deletion mutants of these proteins have thus far failed to confirm a phenotype in metal reduction [15,22,23]. No clear physiological role has been attributed to STC yet [15,24], whereas ScyA is considered to be the electron donor to the dihaem CcpA (cytochrome *c* peroxidase) [25]. FccA is the unique fumarate reductase in *S. oneidensis* MR-1 and it also appears to contribute to metal reduction [26,27].

The identification of the redox partners involved in bridging the periplasmic gap between CymA and MtrCAB–OmcA complex is one of the key remaining issues in the understanding of extracellular metal respiration by *S. oneidensis* MR-1 [28]. Complexes of redox proteins typically have a transient nature, since they need to balance the requirement for fast electron transfer with fast turnover, making their interaction studies more challenging [29]. NMR spectroscopy is exquisitely sensitive to the presence of transient protein–protein interactions and can probe very weak interactions between redox proteins by either

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**Abbreviations used:** CcpA, cytochrome *c* peroxidase; DDM, dodecyl maltoside; FccA, flavocytochrome *c*; HTP, hydroxyapatite; SAXS, small-angle X-ray scattering; ScyA, monohaem cytochrome *c*$_1$; STC, small tetrahaem cytochrome *c*; TB, Terrifi broth.

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Chemical shift perturbation or relaxation enhancement methods [29,30]. NMR has the unique added advantage of allowing the identification of the docking regions between the partners when resonance assignments are available.

In the present study protein–protein interactions involving the most abundant cytochromes from the periplasmic space of S. oneidensis MR-1 were identified and used to propose an organisation for the electron transfer network across the periplasmic space.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

The bacterial strains used in the present study, S. oneidensis LS82 and Escherichia coli LS246, were kindly provided by Dr Liang Shi from the Pacific Northwest National Laboratory (Richland, WA, U.S.A.). S. oneidensis LS82 overexpresses the tetrahaem cytochrome CymA and _E. coli_ LS246 overexpresses the decahaem cytochrome MtrA. The strain _S. oneidensis_ LS82 was grown at 30°C in TB (Terrific broth) containing 50 μg/ml kanamycin in 5 litre Erlenmeyer flasks containing 2 litres of medium at 150 rev./min. The cells were allowed to grow for 24 h before harvesting. The _E. coli_ LS246 strain was grown at 30°C in TB containing 100 μg/ml ampicillin and 35 μg/ml chloramphenicol in 5 litre Erlenmeyer flasks containing 1 litre of medium at 180 rev./min. After reaching an attenuation at 600 nm of approximately 0.6, the cells were induced with 0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside). After induction, cells were allowed to grow under the same conditions for 16 h before harvesting.

**Protein purification**

Cells were harvested by centrifugation at 10000 g for 15 min at 4°C. The cell pellet was washed and resuspended in 20 mM Tris/HCl buffer (pH 7.5) containing a protease inhibitor cocktail (Roche). Cell disruption was obtained by two passages through a French Press at a pressure of 1000 psi (1 psi = 6.9 kPa). Cell debris were removed by centrifugation at 10000 g for 15 min at 4°C. The supernatant was ultracentrifuged at 42 000 rev./min for 1 h at 4°C using a 45 Ti Beckman rotor. The supernatant obtained from this procedure contained the soluble protein fraction and the pellet contained the membrane protein fraction.

The soluble protein fraction obtained from the _E. coli_ LS246 cell growth, containing the MtrA cytochrome, was loaded directly on to a DEAE column (GE Healthcare) previously equilibrated with 20 mM Tris buffer (pH 7.6). A salt gradient from 0 to 1 M NaCl in 20 mM Tris buffer (pH 7.6) was applied and the fraction containing MtrA was eluted at 150 mM NaCl. This fraction was dialysed and loaded on to a Q-Sepharose column, previously equilibrated with 20 mM NaCl. The fraction containing MtrA was eluted at 200 mM NaCl. This fraction was subsequently dialysed and loaded on to a HTP (hydroxyapatite) column (Bio-Rad Laboratories), pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.6). MtrA was eluted with 50 mM of potassium phosphate buffer (pH 7.6).

The soluble protein fraction obtained from the _S. oneidensis_ LS82 cell growth, containing the cytochromes ScyA, STC and FccA, was loaded directly on to a DEAE column previously equilibrated with 20 mM Tris buffer (pH 7.6). A salt gradient from 0 to 1 M NaCl in 20 mM Tris buffer (pH 7.6) was applied and the fractions containing the cytochromes ScyA, FccA and STC were eluted at 70 mM NaCl, 200 mM NaCl and 250 mM NaCl respectively. These fractions were dialysed and loaded separately on to a Q-Sepharose column, equilibrated previously with 20 mM Tris buffer (pH 7.6). Fractions containing the cytochromes ScyA, FccA and STC were eluted at 50 mM NaCl, 150 mM NaCl and 200 mM NaCl respectively. Fractions were loaded separately on to a HTP column, pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.6), after dialysis. Although the fractions containing the cytochromes ScyA and STC did not bind to the column and were eluted in the washout volume, the fraction containing FccA was eluted with 100 mM of potassium phosphate buffer (pH 7.6).

The membrane pellet obtained from the _S. oneidensis_ LS82 cells was homogenized and solubilized in 20 mM Tris buffer (pH 7.6) containing a protease inhibitor cocktail and 4% (w/v) DDM (dodecyl maltoside) at 4°C overnight. The insoluble material was removed by ultracentrifugation at 42 000 rev./min for 1 h at 4°C using a 45 Ti Beckman rotor, and the supernatant was used to purify CymA cytochrome using a procedure reported previously [31].

The chromatographic fractions were routinely analysed by SDS/PAGE (12% gel) and UV–visible spectroscopy to select those containing the protein of interest. The purity of the protein was revealed as a single band in the gel. Fractions containing pure cytochrome samples had a typical absorbance ratio, _A_Soret_/A_L280nm_ larger than 4.5.

**NMR sample preparation and titrations**

The protein samples were freeze-dried twice using 1H2O (99.9 atom%). NMR spectra obtained before and after the freeze-drying were identical, showing that the protein structure was not affected by this procedure. All protein samples were dissolved in 1H2O (99.9 atom%) and contained 20 mM potassium phosphate buffer (pH 7.6) with anionic strength of 100 mM adjusted by the addition of potassium chloride. The cytochrome stock samples had a protein concentration of 1 mM.

NMR experiments were performed at 25°C on a Bruker Avance II 500 MHz NMR spectrometer equipped with a TXI probe. Samples containing 100 μM of one cytochrome were titrated against increasing concentrations of another cytochrome. 1H-1D-NMR spectra were recorded for each addition. All cytochromes were in the fully oxidized state, except the high potential monohaem cytochrome ScyA, which was maintained fully reduced by the addition of 200 μM of ascorbic acid to the buffer.

**Data analysis and binding affinities**

In low-spin paramagnetic haem proteins the methyl substituents at the periphery of the haems show NMR resonances that are well separated from the protein envelope. This makes them convenient targets for chemical shift perturbation measurements. Furthermore, in the case of STC and FccA these signals have been assigned to specific haems in the structure allowing the identification of the docking sites [32,33]. In reduced diamagnetic haems, such as that found in the monohaem cytochrome ScyA, the ring currents of the haem shift the meso protons outside of the protein envelope [34]. Thus the NMR signals from the meso protons of the haem were followed instead.

The NMR spectra were processed and analysed with Bruker TopSpin program. Chemical shifts are reported in p.p.m. and the proton spectra were calibrated using the water signal as the internal reference. Only chemical shift perturbations equal to or larger than 0.025 p.p.m. were considered significant [30]. The chemical shift perturbations (Δ_δcalc_) of the NMR signals from

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a cytochrome (Cyt\(_a\)) resulting from the complex formation with another cytochrome (Cyt\(_b\)) were plotted against the molar ratio (R) of \([\text{Cyt}_b]/[\text{Cyt}_a]\). The data were fitted using least squares minimization to a 1:1 binding model using eqns (1) and (2) [35]:

\[
\Delta \delta_{\text{bind}}^{\infty} = \frac{1}{2} \Delta \delta_{\text{het}}^{\infty} (A - \sqrt{(A^2 - 4R)})
\]

(1)

\[
A = 1 + R = \frac{K_d([\text{Cyt}_b]_0 R + [\text{Cyt}_b]_0)}{[\text{Cyt}_b]_0 [\text{Cyt}_a]_0}
\]

(2)

where \(\Delta \delta_{\text{het}}^{\infty}\) is the maximal chemical shift perturbation of the NMR signals resulting from the complex formation between Cyt\(_a\) and Cyt\(_b\). \(K_d\) is the dissociation constant, \([\text{Cyt}_a]_0\) is the initial concentration of Cyt\(_a\), and \([\text{Cyt}_b]_0\) is the stock concentration of Cyt\(_b\). When several methyl signals belonging to an individual haem were clearly visible, the data obtained for all methyl signals were used to define the dissociation constant. Experimental uncertainty was estimated from the spectral resolution of the NMR acquisition.

Spectroscopic assay of interprotein electron transfer

Electron transfer involving FccA from \textit{S. oneidensis} MR-1 was measured inside an anaerobic glove box (Coy Laboratory, model type B), using a UV–visible spectrophotometer (Shimadzu model UV-1800) to collect spectra in the range of 250–800 nm. A 1-ml cuvette was prepared using 20 mM potassium phosphate buffer (pH 7.6) and 100 mM KCl with each of the target proteins at an approximate concentration of 1 μM. In the case of CymA, DDM (0.05 %) was added to the potassium phosphate buffer to avoid precipitation of the protein. To reduce the target proteins a concentrated solution of sodium dithionite was added in small amounts and the absorbance was monitored at 314 nm to control for excess dithionite that could interfere with the results. A large excess of fumarate was added (approximately 1 mM) and the sample checked for the absence of changes in absorbance at 552 nm. The reaction was initiated by the addition of catalytic amounts of FccA (approximately 1 nM) and spectral changes were monitored over time. Experiments were performed with constant stirring and the temperature was kept at 25°C using an external thermostating bath.

Protein electrostatic surface potential calculations

The structures of STC (PDB code 1M1Q), FccA (PDB code 1D4D) and ScyA (PDB code 1KX2) were used to perform electrostatic surface potential calculations considering a fully oxidised state for STC and FccA and a fully reduced state for ScyA, mimicking the experimental conditions used for studying the protein interactions. Protein and cofactor partial charges were set using the GROMOS 43A1 force field [36]. Electrostatic potential calculations were performed with the MEAD package [37] that solves the Poisson–Boltzmann equation for a given system. The ionic strength used was 0 mM and the internal and external dielectric constants used were 2 and 80 respectively. The electrostatic potential was mapped at the proteins’ surface using PyMOL (http://www.pymol.org).

RESULTS

NMR titrations and binding affinities

NMR spectroscopy is exquisitely sensitive to changes in the chemical environment. When two proteins bind in fast exchange the signals of the nuclei that are close to the binding site suffer a change in position according to the bound fraction. For electron transfer to occur at physiologically relevant rates between cytochromes one haem in the donor and one haem in the acceptor must be in close proximity [39,40]. Thus changes in the chemical shifts of the signals from haems are among the best to monitor protein–protein interactions that are relevant for electron transfer. Figure 1 illustrates this phenomenon by presenting the spectral changes for the 18\(^1\) methyl signal (IUPAC-IUB nomenclature) from haem IV (18\(^1\)CH\(_3\))\(^{IV}\) of STC in the presence of increasing amounts of MtrA. The broadening of the signals arises from the increased fraction of the bound species which is heavier. Primary NMR spectra for the free and bound forms of the cytochromes that display interactions are shown in the Supplementary Online data (at http://www.biochemj.org/bj/449/bj4490101add.htm).

All the signals from STC, FccA and ScyA that are clearly visible in the NMR spectra were analysed, and those that show changes resulting from interactions with putative partner proteins are reported in Figure 2. Table 1 reports all pairs of proteins tested and the \(K_d\) values determined for those that showed interaction. The haem from STC or FccA that has signals showing greater perturbations in the chemical shifts for the 18\(^1\) methyl signal (IUPAC-IUB nomenclature) from haem IV (18\(^1\)CH\(_3\))\(^{IV}\) of STC in the presence of increasing amounts of MtrA. The broadening of the signals arises from the increased fraction of the bound species which is heavier. Primary NMR spectra for the free and bound forms of the cytochromes that display interactions are shown in the Supplementary Online data (at http://www.biochemj.org/bj/449/bj4490101add.htm).

Spectroscopic assay of interprotein electron transfer

The interaction data obtained from the NMR experiments involving FccA were confirmed spectrophotometrically. The fumarate reductase activity of FccA, which is lacking in the other periplasmic cytochromes, was used to measure the re-oxidation of the partner cytochrome by catalytic FccA in the presence of excess fumarate. Figure 3 shows that catalytic amounts of FccA can re-oxidise CymA and MtrA in the presence of excess fumarate, but not
Figure 2  Binding curves of periplasmic cytochromes from *S. oneidensis* MR-1 that show interactions monitored by 1H-NMR

FccA and CymA (A), FccA and MtrA (B), STC and CymA (C), and STC and MtrA (D). The chemical shift perturbations of the haem methyl signals are plotted as a function of the molar ratio of the interacting proteins. Nomenclature is as in Figure 1. The solid lines represent the best global fit to the 1:1 binding model [eqn (1)].

STC or ScyA. In the case of ScyA the initial spectrum was already partially reduced due to the high reduction potential of this monohaem cytochrome.

**Protein electrostatic surface potential characteristics**

Electrostatic potential calculations show that STC, FccA and ScyA have, overall, surfaces displaying negative potential (Figure 4 and Supplementary Movie S1 at http://www.biochemj.org/bj/449/bj4490101add.htm). STC shows very negative potentials in the whole protein with a small exception around the C-terminus fairly distant from the haems. In FccA the haem-containing domain also shows strongly negative surface potentials, whereas in ScyA the protein shows a neutral surface potential, with a neutral zone near the haem edge.

**DISCUSSION**

Of all the aspects of the extracellular electron transfer performed by metal-reducing bacteria it is the organization of the trans-periplasmic redox network that has remained poorly understood. In order to try to unravel this issue, several approaches have been used to study interactions among the numerous periplasmic c-type cytochromes. Although attempts to cross-link these cytochromes have failed, most likely owing to the highly dynamic transient nature of the interactions [24], studies performed with proteins attached to electrodes or other surfaces have been more successful and allowed the identification of some partners [11,44]. However, this approach may lead to false negatives since the orientation of the cytochrome on the surface may prevent the access of partners in physiological orientations.

In order to overcome these limitations, we probed the interactions between periplasmic cytochromes from *S. oneidensis* MR-1 using the perturbation of the chemical shifts of the NMR signals belonging to the most abundant proteins in the periplasmic space (STC, ScyA and FccA) [19,20] as a probe for interaction between these proteins and their physiological redox partners.

NMR docking studies are done in conditions where electron transfer between the interacting partners is not taking place during the measurement. Notwithstanding, the vast body of literature on the subject shows the power of this approach for obtaining physiological and structural insights on the details of interactions between redox partners [29,43]. Furthermore, the relevance of the results for physiological electron transfer in the present study was independently supported by the observation of fumarate reduction only for those proteins that showed interaction with FccA in the NMR data (Figure 3). Table 2 summarizes the results obtained in the present study and places them in the context of the previous information available in the literature.

**CymA** plays a central role in the respiratory chain of *Shewanella* by coupling the oxidation of menaquinone to a broad variety of multihem cytochromes in the periplasmic space [10]. This was also shown by the NMR data obtained in the present study, where CymA displayed interactions with both STC and FccA. The magnitude of the $K_d$ values obtained between CymA and STC or CymA and FccA (Table 1) reveals the formation of weak complexes that prevent the blockage of access to CymA.

<table>
<thead>
<tr>
<th>Cytochrome complex</th>
<th>$K_d$ (μM)</th>
<th>Docking site</th>
</tr>
</thead>
<tbody>
<tr>
<td>FccA and CymA</td>
<td>398 (5)</td>
<td>Haem II</td>
</tr>
<tr>
<td>FccA and MtrA</td>
<td>35 (14)</td>
<td>Haem II</td>
</tr>
<tr>
<td>FccA and STC</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FccA and ScyA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>STC and CymA</td>
<td>250 (17)</td>
<td>Haem IV</td>
</tr>
<tr>
<td>STC and MtrA</td>
<td>572 (5)</td>
<td>Haem IV</td>
</tr>
<tr>
<td>STC and FccA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>STC and ScyA</td>
<td>–</td>
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<td>ScyA and MtrA</td>
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</tbody>
</table>

**Table 1** Pairwise interactions tested for cytochromes found in the periplasm of *S. oneidensis* MR-1

The $K_d$ values were calculated as described in the Materials and methods section and S.E.M. values were calculated from the diagonal elements of the covariance matrix and are shown in parentheses. The haem belonging to STC or FccA displaying the greatest changes is reported. The Roman numerals correspond to the order of haem attachment to the polypeptide chain.
Figure 3  UV–visible spectroscopy of reduced periplasmic cytochromes in the presence of fumarate and catalytic amounts of FccA

Table 2  Pairwise interactions tested for cytochromes found in the periplasm of S. oneidensis MR-1

by the various redox partners. The results also indicate that both STC and FccA interact with the decahaem cytochrome MtrA, where FccA has a stronger affinity for MtrA than STC. Taking into consideration the importance of MtrA in metal respiration, these results indicate that both FccA and STC participate in metal reduction in S. oneidensis MR-1. This conclusion is consistent with gene knockout experiments where STC was shown to play a small role in iron reduction [16,45]. This observation may be a consequence of the stronger affinity of FccA for MtrA that leads to a longer lifetime of the complex and a lower turnover rate of electron transfer relative to STC. Also, knockout of the gene encoding for FccA showed that ferric iron reduction was faster in the deletion mutant than in the wild-type strain [27]. In this context, the absence of clear phenotypes in knockout mutants of Shewanella may be owing to the presence of both cytochromes in the periplasmic space [20,46], as well as some of the other 41 c-type cytochromes encoded in the S. oneidensis MR-1 genome. This allows functional redundancy as previously reported for the decahaem cytochromes [16].

This electron transfer scenario is also favoured by the structural and electrostatic similarities between the haem domains of FccA and STC. The electrostatic calculations show that both proteins have very negative surfaces in the regions that would be relevant for electron transfer. This provides a rationale for the lack of interaction between FccA and STC both in the NMR data and in the electron transfer assay. The two proteins are highly abundant in the periplasmic space and interact with the same partners, but do not exchange electrons with each other. Therefore electrons in the pool of STC molecules in the periplasm will not equilibrate with those in the pool of FccA molecules. Interestingly this occurs in the absence of physical barriers and in the presence of overlapping ranges of redox activity of both proteins [33,47]. Under these conditions it is the tuning of the surface electrostatics of the proteins that serves to maintain segregated electron transfer pathways across the periplasmic space.

Likewise the monohaem cytochrome ScyA, that is also produced in high amounts in the periplasm of Shewanella under oxygen limited conditions [20], also has negative electrostatic surface potentials. It does not interact with STC, FccA or MtrA, showing the presence of a third segregated redox pathway that in this case does not lead to metal reduction. These results lend further support to the proposal that ScyA mediates electron transfer between CymA and the CcpA [25].

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Figure 4  Electrostatic potential mapping on the protein’s surface of the most abundant periplasmic cytochromes of *S. oneidensis* MR-1

STC (PDB code 1M1Q) (A), FccA (PDB code 1D4D) (B) and ScyA (PDB code 1KX2) (C). Electrostatic potentials were calculated (see the Materials and methods section for details) considering a fully oxidized state for STC and FccA and a fully reduced state for ScyA. The Roman numeral corresponds to the order of haem binding to the polypeptide chain.

The present study also enabled, for the first time, the identification of the haems in STC and FccA that are responsible for electron transfer with their periplasmic redox partners. FccA interacts with CymA and MtrA in the vicinity of haem II, whereas interactions between STC and CymA or MtrA occur near haem IV. The region around haem IV from STC displays the strongest negative potentials (Figure 4A), making it a good candidate to interact with a redox partner displaying (presumably) positively charged potentials at the surface. Similarly, the zone corresponding to haem II in FccA is also the one displaying the highest negative potentials (Figure 4B). Unfortunately an experimentally determined structure of CymA does not exist and MtrA has been structurally characterized only by SAXS. Therefore it is not possible presently to determine whether CymA and MtrA contain surface regions of positive potential where they can establish an interaction with their negatively charged redox partners. Furthermore, although STC and FccA bind to CymA and MtrA through different haems, until the structures of CymA and MtrA are available it remains to be discovered whether STC and FccA compete for the same docking positions in CymA and MtrA or if they bind to different positions.

Nonetheless, an important insight on the organization of the transperiplasmic redox network emerges from these results. As had been previously observed for STC with inorganic electron donors and acceptors [48], the entry and exit of electrons appears to occur through the same haem implying that ternary complexes are not formed (Figure 5). Therefore both STC and FccA must reorientate to present the same haem (haem IV in the case of STC and haem II in the case of FccA) to CymA and MtrA as they glide back and forth in the periplasmic space with a movement akin to that of a boomerang.

To conclude, the present study filled important gaps in the knowledge of the interaction network of periplasmic cytochromes of *S. oneidensis* MR-1. The results show for the first time that there are two clearly segregated pathways to transfer electrons across the periplasmic gap to the outer-membrane metal reductases, one involving STC and the other involving FccA. This finding provides a rationale for the lack of clear phenotypes of deletion mutants of these two proteins. The fact that in each of these cytochromes it is the same haem that contacts the donor (CymA) and the acceptor (MtrA), together with the large dissociation constants measured, showed that a stable multi-protein complex capable of bridging the periplasmic gap does not exist. However, these findings raise the question of whether the two pathways involving STC and FccA, that share the same cell compartment, serve any other purpose than the maintenance of redundancy in this essential physiological trait of extracellular electron transfer. Experiments to address this issue in particular in the context of proposals for these proteins as electron storage devices are being planned.
Figure 5 Cartoon depicting the interactions that occur between the most abundant periplasmic cytochromes of S. oneidensis MR-1

The arrows in bold indicate the interactions that occur between the cytochromes and point to the possible docking site. The dissociation constants (K_d) corresponding to each interaction are positioned next to their respective arrow. Interactions that do not occur are represented by a red X in the middle of the arrow. The Roman numerals correspond to the order of haem attachment to the polypeptide chain. Cytochrome representations were made with PyMOL using the structures of STC (PDB code 1M1Q), FccA (PDB code 1D4D), ScyA (PDB code 1KX2) and the SAXS model of MtrA. For MtrC and CymA the models were made with SWISS-MODEL [49,50] using as template the structures of MtrF (PDB code 3PMQ) and NrfH (PDB code 2J7A) respectively.

AUTHOR CONTRIBUTION
Bruno Fonseca and Catarina Paquete planned research, performed experiments and wrote the paper; Sónia Neto and Isabel Pacheco performed experiments; Cláudio Soares performed experiments and wrote the paper; and Ricardo Louro planned research and wrote the paper.

ACKNOWLEDGEMENTS
We thank Dr Liang Shi from the Pacific Northwest National Laboratory (Richland, WA, U.S.A.) for the bacterial strains used in the present study.

FUNDING
This work was supported by the FCT (Fundação para a Ciência e a Tecnologia) [grant numbers SFRH/BD/41205/2007 (to B.M.F.), SFRH/BPD/34591/2007 (to C.M.P.), PTDC/BIA-PRO-098158/2008, MIT-Pt BS-BB/1014/2008 and PEst-OE/EEB/LA0004/2011]. The NMR data were collected at The Portuguese National NMR Network supported by POCI (Programa Operacional Ciência e Inovação) 2010 and the FCT [grant number REDE/1517/RMN/2005].

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29 Reference deleted
SUPPLEMENTARY ONLINE DATA
Mind the gap: cytochrome interactions reveal electron pathways across the periplasm of *Shewanella oneidensis* MR-1
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Figure S1  $^1$H-1D NMR spectral changes of STC in the presence of increasing amounts of MtrA

The methyl group is identified using the IUPAC-IUB nomenclature for haems. The Roman numeral corresponds to the order of haem binding to the polypeptide chain. The R value corresponds to the molar ratio of [MtrA]/[STC]. Boxes show examples of methyl signals without significant chemical shift perturbation ($1^\text{st}$-CH$_3^I$) and with significant changes ($1^\text{st}$-CH$_3^{IV}; 7^\text{th}$-CH$_3^{IV}$).

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Figure S2  $^1$H-$^1$D NMR spectral changes of STC in the presence of increasing amounts of CymA

The methyl group is identified using the IUPAC-IUB nomenclature for haems. The Roman numeral corresponds to the order of haem binding to the polypeptide chain. The $R$ value corresponds to the molar ratio of [CymA]/[STC]. Boxes show examples of methyl signals without significant chemical shift perturbation ($18^1$-CH$_3^I$) and with significant changes ($18^1$-CH$_3^{IV}$, $7^1$-CH$_3^{IV}$).

Figure S3  $^1$H-$^1$D NMR spectral changes of FccA in the presence of increasing amounts of MtrA

The methyl group is identified using the IUPAC-IUB nomenclature for haems. The Roman numeral corresponds to the order of haem binding to the polypeptide chain. The $R$ value corresponds to the molar ratio of [MtrA]/[FccA]. Boxes show examples of methyl signals without significant chemical shift perturbation ($18^1$-CH$_3^I$, $12^1$-CH$_3^{III}$) and with significant changes ($2^1$-CH$_3^{II}$).
Figure S4  $^1$H-1D NMR spectral changes of FccA in the presence of increasing amounts of CymA

The methyl group is identified using the IUPAC-IUB nomenclature for haems. The Roman numeral corresponds to the order of haem binding to the polypeptide chain. The R value corresponds to the molar ratio of [CymA]/[FccA]. Boxes show examples of methyl signals without significant chemical shift perturbation ($1^1$-CH$_3$; $1^2$-CH$_3$) and with significant changes ($2^1$-CH$_3$).

Received 21 September 2012/12 October 2012; accepted 16 October 2012
Published as BJ Immediate Publication 16 October 2012, doi:10.1042/BJ20121467