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In Vivo Identification of the Outer Membrane Protein OmcA–MtrC Interaction Network in *Shewanella oneidensis* MR-1 Cells Using Novel Hydrophobic Chemical Cross-Linkers

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Abstract

Outer membrane (OM) cytochromes OmcA (SO1779) and MtrC (SO1778) are the integral components of electron transfer used by *Shewanella oneidensis* for anaerobic respiration of metal (hydr)oxides. Here the OmcA–MtrC interaction was identified in vivo using a novel hydrophobic chemical cross-linker (MRN) combined with immunoprecipitation techniques. In addition, identification of other OM proteins from the cross-linked complexes allows first visualization of the OmcA–MtrC interaction network. Further experiments on *omcA* and *mtrC* mutant cells showed OmcA plays a central role in the network interaction. For comparison, two commercial cross-linkers were also used in parallel, and both resulted in fewer OM protein identifications, indicating the superior properties of MRN for identification of membrane protein interactions. Finally, comparison experiments of in vivo cross-linking and cell lysate cross-linking resulted in significantly different protein interaction data, demonstrating the importance of in vivo cross-linking for study of protein–protein interactions in cells.

Keywords

protein interactions; Protein Interaction Reporter; PIR; electron transport; metal ion reduction; membrane proteins; FTICR-MS; outer membrane cytochrome C; OmcA; OmcB; MtrC; SO-0404

Introduction

Shewanella oneidensis MR-1, a facultatively anaerobic bacterium, is able to use a wide range of terminal electron acceptors during anaerobic respiration. The metal reducing activity of *S. oneidensis* provides an alternative strategy for remediation of the sites contaminated with toxic metals such as uranium, technetium, and chromium.[1–3] A greater understanding of the underlying mechanisms of this metal reducing activity will enable utilization of *S.*

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oneidensis in bioremediation, bioenergy production, or other areas of biotechnology that can benefit from electron transfer.

Because of their cell surface location,[4] outer membrane (OM) decaheme *c*-type cytochromes OmcA (SO1779) and MtrC (SO1778) play critical roles in the reduction of extracellular electron acceptors. Deletion of the genes impairs the ability of *Shewanella* to reduce Fe(III) and Mn(VI) oxides.[4–12] Purified OmcA and MtrC are functional metal reductases with the ability to bind and reduce solid metal oxide such as hematite.[8,13] In addition, OmcA and MtrC are directly involved in extracellular reduction of uranyl carbonate complexes to uraninite.[14] A previous study showed that MtrC was copurified with recombinant OmcA, and purified OmcA and MtrC formed a stable complex in vitro.[13] Using "in vivo" cross-linking by formalde-hyde and Western blot, the OmcA–MtrC interaction has also been reported.[15] Despite recent advances made in understanding the roles of OmcA and MtrC in reduction of metals, the other components of the OmcA/MtrC-mediated electron transfer pathway have yet to be identified.

Mapping protein–protein interaction networks in vivo is crucial for understanding the nature of biological processes at the systems-level. However, determination of protein interactions in native living systems is largely an unmet challenge for today's technology. The difficulties of this type of analysis stem from the fact that the primary physical property that must be detected is the close proximity of interacting partners. Often times, this is difficult to detect even for specific isolated interactions. For large-scale determinations, this becomes improbable, if not impossible. When specific antibodies are available, immunoprecipitation of the target protein along with its noncovalent binding protein partners, i.e., coimmunoprecipitation (co-IP), has been a commonly used technique for identifying potential interacting proteins surrounding the target proteins.[16–21] Co-IP methods have generated large-scale protein interaction data in yeast, mammalian, and many other organisma, and the validation of many of these results with orthogonal methods confirms the utility of these methods. However, prior to analysis, co-IP-related methods require cell lysis during which the native environment is disrupted, and nonspecific binding to the antigen may occur resulting in false identification of protein–protein interactions. In fact, nonspecific interactions are one of the most challenging impediments to nearly every protein interaction determination.

Chemical cross-linking coupled with immunoprecipitation provides an alternative strategy for in vivo identification of protein–protein interactions,[22–36] which has been extensively reviewed.[37–39] Cross-linking reactions can be carried out with intact cells and chemically "freeze" protein–protein interactions with stable covalent bonds that allow subsequent purification steps to be carried out under much harsher or more stringent conditions. Consequently, nonspecific binding can be reduced considerably. In addition, immunoprecipitation in conjunction with cross-linking is well suited for investigating the interactions of membrane proteins. Isolation and purification of membrane proteins usually requires use of detergents that can sometimes disrupt interactions among membrane proteins. Thus, stabilization of the complexes with cross-linkers prior to immunoprecipitation of membrane proteins significantly increases the chances of identification of the proteins bound to the antigens.

In this study, we report the development and application of a novel type of cross-linker coupled with immunoprecipitation techniques to specifically identify OM protein–protein interactions. OmcA and MtrC interactions were targeted in this study due to the critical importance of these proteins in the electron transport pathway of *S. oneidensis*. For comparison, two commercial cross-linkers, DSS and DST, were also applied in parallel. We previously reported a new family of cross-linkers, from a class of reagents that we call protein interaction reporter (PIR) molecules, with the incorporation of tunable spacer chain chemistry, mass spectrometry-

cleavable bonds, affinity tag, and hydrophobic groups.[40,41] We further demonstrated that the hydrophobic PIR cross-linkers preferentially label membrane proteins.[42] However protein–protein interactions identified in vivo with PIR cross-linkers have not yet been reported. This research illustrates that novel hydrophobic PIR cross-linkers allow identification of more OM protein interactions as compared to DSS and DST, indicating hydrophobicity of the PIR cross-linker is advantageous for mapping OM protein–protein interactions. Collectively, these cross-linking studies not only showed that OmcA and MtrC closely interact with each other in cells but also revealed the identities of many other OM proteins that are likely components of a larger OmcA–MtrC extracellular electron transfer complex or interaction network.

Experimental Procedures

Chemical Cross-Linkers

The novel hydrophobic PIR cross-linker, MRN (Figure 1), was synthesized using solid phase peptide synthesis methodology. First, methionine was coupled to HMPB-MBHA (4-hydroxymethyl-3-methoxyphenoxybutyric acid) resin. Then, the lysine in the form of Fmoc-Lys-ε-Fmoc was coupled to methionine and formed a branch point for the cross-linker. Finally, the Rink groups, succinic anhydride (SA), and *N*-hydroxysuccinimides (NHS) were coupled as described previously.[41] The crude product was cleaved using either 0.5 or 1.0% TFA in chloroform and then neutralized with pyridine. The chloroform and TFA pyridine salts were removed under a vacuum. The crude product was purified using reversed-phase HPLC, and the final product had a purity of approximately 90%.

Two commercial cross-linkers (Pierce, Rockford, IL), disuccinimidyl suberate (DSS) and disuccinimidyl tartarate (DST), were used to label *S. oneidensis* cells without further purification (Figure 1).

All three chemical cross-linkers contain two *N*-hydroxysuccinamide (NHS) esters, which react with primary amine groups in proteins and form stable covalent bonds. DSS, DST, and MRN have a maximum spacer arm length of 11.4, 6.4, and 40 Å, respectively. DST is soluble both in water and in organic solvents. DSS and MRN can only be dissolved in organic solvents such as DMSO or DMF. Thus, DST is less hydrophobic than DSS and MRN. HPLC was used to further characterize the hydrophobicity of DSS and MRN. The retention time of MRN is 50% higher than that of DSS under the same LC conditions, which indicates significantly higher hydrophobicity of MRN as compared to DSS (data not shown).

Cell Culture

S. oneidensis MR-1 cells, ATCC 700550, were obtained from American Type Culture Collection (Manassas, VA) and maintained in Luria–Bertani (LB) medium. A single colony was transferred with an inoculating loop to 40 mL LB broth and was incubated overnight at room temperature with rotary shaking (100 rpm). An amount of 4 mL of the overnight culture was transferred to 200 mL fresh LB in a 1 L flask and incubated with a rotary shaker at 100 rpm at room temperature for 8 h. Cells were harvested by centrifuging at 3000*g* for 20 min. Cell pellets were washed 4 times with 40 mL of ice-chilled PBS buffer (150 mM sodium phosphate, 100 mM NaCl, pH 7.5).

OmcA and MtrC mutant cells of *S. oneidensis* MR-1 were generated as described previously[14,43] and were cultured under the same conditions as wild type cells.

Cross-Linking Labeling and Cell Lysis

After the last wash, about 1×10^7 cells were suspended in 1 mL of PBS buffer, and the cross-linkers were added to the suspended cell pellets to produce a final concentration of 1 mM. The

cross-linking reaction was carried out at 4 °C for 1 h and quenched by 1 M ammonium bicarbonate. Extensive washing steps with PBS after cross-linking reactions were used to eliminate most nonspecific contamination as reported previously.[42] Then, cells were lysed in 0.1% NP-40 PBS solution by sonication for 2 min. The cell lysates were centrifuged at 15 000 *g* at 4 °C for 45 min. The pellets were discarded, and supernatants were collected in a clean tube. The protein concentration of the supernatants was determined by a Bradford Assay (Bio-Rad, Hercules, CA). Cell-lysate cross-linking experiments were performed under the same experimental conditions as in vivo cross-linking except that the cross-linkers were added after cells were lysed.

Immunoprecipitation (IP), SDS-PAGE, In-Gel Digestion, and Western Blot Analysis

Recombinant MtrC or OmcA were individually purified from *S. oneidensis* cells,[13] and respective polyclonal antibodies toward each protein were commercially generated and purified by affinity chromatography (BioSyn-thesis, Inc., Lewisville, TX). Antibody specificities were validated by immunoblot analysis of *S. oneidensis* cell lysate (Supplementary Figure 5).

An amount of 10 μg of OmcA or MtrC antibody was added to 400 μL of cross-linker-labeled cell lysate, and the reaction was incubated at 4 °C overnight. An amount of 50 μL of immobilized protein G gel slurry (Pierce, Rockford, IL) was added to antigen–antibody complex solution, and the reaction was incubated for 2 h with gentle mixing at room temperature. Then, the protein G beads were washed twice with 500 μL of IP buffer (25 mM Tris, 150 mM NaCl, pH 7.2) and once with 25 mM Tris buffer (pH 7.2). After the last wash, protein G beads were incubated with 50 μL of SDS-PAGE sample loading buffer (Bio-Rad, Hercules, CA) for 5 min at 95 °C. The supernatant was collected after centrifugation (2500*g*) and then analyzed by 8% SDS-PAGE. Protein bands were visualized with Coo-massie Blue R250 (Bio-Rad, Hercules, CA). In-gel digestion was performed as described previously.[42]

For Western blot analysis, the eluents from Protein G beads were separated by 8% SDS-PAGE gel and then transferred to a nitrocellulose membrane (Whatman, Sanford, ME) with a Trans-Blot semidry transfer cell (BioRad, Hercules, CA). After transferring, the membranes were blocked with 5% nonfat milk in TBS overnight at 4 °C followed by incubation with the primary antibody anti-OmcA/anti-MtrC at 1:5000 dilution for 1 h at room temperature. Finally, the membranes were probed by HRP-conjugated secondary antibody and chemiluminescence peroxidase substrate (Sigma, St. Louis, MO).

LC/MS/MS and Protein Identification

LC/MS/MS analysis of in-gel digest was performed using an ion trap mass spectrometer (Esquire HCT, Bruker Daltonics, Billerica, MA) equipped with a nano ESI source and nano HPLC systems (Ultimate, Dionex, Sunnyvale, CA). Data analysis was performed with Bruker Daltonics *DataAnalysis* software (version 3.1). Protein identification was carried out by searching against the complete genome database of *S. oneidensis* MR-1 (4,854 ORFs) downloaded from The Institute for Genomic Research (TIGR) (http://www.tigr.org/), using Mascot[44] (version 2.1.0, MatrixScience Ltd., London) licensed in house. Database search parameters were used as previously reported;[42] briefly: enzyme, trypsin; allowed missed cleavages up to 3; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide tolerance, 1.6 Da; and MS/MS tolerance, 0.8 Da. The auto hits option was selected to allow reporting of all the protein hits with the probability-based Mowse scores that exceeded their thresholds ($p < 0.05$), indicating significance above the 95% confidence level. The search output results were further filtered using more stringent MudPIT scoring and an ion score cutoff of 0.05, which removed all the peptides with expected values (E) > 0.1. All proteins identified with more than 2 unique peptides which had a minimal total peptide–ion score of 45 were

accepted as true identifications without additional manual spectral inspection. There is one exception, which is that the major outer membrane lipoprotein (SO1295) which has a mass of 9 kDa was identified by a single peptide with high score (>78) in all experiments. To evaluate false positive identification, searches were also performed against all mammalia of MSDB, which contains 280 595 sequences. In most searches, only trypsin and various forms of keratins (common contamination during in-gel digestion) were identified with significance; other searches resulted in no significant protein hits. Protein interaction visualization was carried out using Cytoscape (version 2.4.0) downloaded from http://www.cytoscape.org/.[45]

Results

Anti-OmcA and Anti-MtrC Immunoprecipitation

The structures of three cross-linkers, MRN, DSS, and DST, are shown in Figure 1. Cells are harvested, washed, and then reacted with each cross-linker separately. After IP from cross-linked cell lysates, five types of proteins were expected to be eluted from protein G beads, i.e., (i) antigen (either OmcA or MtrC), (ii) proteins that bind with antigen in strong noncovalent bonds (co-IP products), (iii) cross-linked protein complexes with a component of antigen, (iv) nonspecific binding proteins, and (v) IgGs. As shown in Figure 2, the antigen (either OmcA or MtrC) band appeared at the expected molecular weight (MW) of ~75 kD in every sample. These bands were excised, and the presence of antigens was confirmed by LC/MS/MS following in-gel digestion (Supplementary Table 1). The bands at ~50 kD were identified to be IgGs. Other bands which appeared in both control and labeled samples were the results of co-IP and/or nonspecific binding. Some of these bands were also selected for protein identification (Supplementary Figure 1 and Supplementary Table 1). It is worth noting that in all labeled samples there were bands above a MW of 250 kD which were not observed in the control samples. These high MW bands are likely to be cross-linked protein complexes with either OmcA or MtrC. To further verify this, we investigated the protein mass distribution of the entire genome database in *S. oneidensis* cells and plotted protein counts vs MW (Supplementary Figure 2). This histogram shows most proteins have a MW between 10 and 100 kD, and only four proteins have a MW of about 250 kD (SO0189, SO1602, SO4149, and SO4317). This further suggests that the bands above 250 kD can only be from cross-linked proteins.

Identification of Cross-Linked Complexes

The stained regions that appeared above 250 kD for labeled cells and the corresponding blank regions for unlabeled cells (Figure 2) were excised and subjected to in-gel digestion and LC/MS/MS. No proteins were identified with significant Mascot scores for unlabeled cells. The Mascot scores of identified proteins for labeled cells are reported in Table 1. In most cases, both OmcA and MtrC were identified with the highest Mascot scores, especially those cross-linked by MRN and DSS. This provides strong evidence of close interaction between OmcA and MtrC in *S. oneidensis* cells and is consistent with the results that were obtained previously from in vitro or in vivo measurements.[13,15] As for DST-labeled samples, MtrC was identified in the anti-OmcA IP; however, OmcA was not identified in the anti-MtrC IP. In addition to OmcA and MtrC, a few other OM proteins, as well as some periplasmic, inner membrane, and cytoplasmic proteins, were identified and are listed in Table 1.

Western Blot Analysis to Confirm OmcA–MtrC Interaction

To further verify OmcA and MtrC interaction, anti-OmcA and anti-MtrC Western blot analyses were done with MtrC and OmcA IP eluents, respectively, which showed a strong contrast between labeled and unlabeled samples (Figure 3). Both Western blot images show their corresponding antigen bands, either OmcA or MtrC, that appear near 75 kDa MW, resulting from copurification from MtrC or OmcA IP. More importantly, the cross-linked MtrC/OmcA

complexes >250 kDa only appeared in MRN labeled cells, which confirms the OmcA–MtrC interaction.

In Vivo Cross-Linking on *omcA* and *mtrC* Mutant Cells

To further investigate protein–protein interactions among OmcA, MtrC, and other OM proteins, in vivo cross-linking as well as anti-OmcA and anti-MtrC IP experiments were carried out with *mtrC* and *omcA* mutant cells, respectively. SDS-PAGE images from MRN and DSS labeled mutant cell samples appeared similar to that of wild type cells except that the observed intensity of the cross-linking complex band above 250 kDa from *omcA* mutant cells was lower (Supplementary Figure 3). All cross-link-specific bands were excised and subjected to protein identification (Table 2). OmcA and MtrC were not identified in corresponding immunoprecipitated cross-linked complexes from either mutant cells, as one might expect since their genes are deleted. As for the *mtrC* mutant, four OM proteins (SO0404, SO1429, SO3099, and SO2001), one inner membrane protein (SO1825), and two cytosolic proteins (SO1930, SO4749) were missing as compared to the results from experiments with wild type cells. In strong contrast, however, all the proteins except for the major outer membrane lipoprotein (SO1295) and translation elongation factor Tu (SO0217) were not observed in cross-linking results from anti-MtrC IP experiments with *omcA* deletion mutant cells. In fact, this striking change was observed in both MRN and DSS experiments.

Identification of Cross-Linked Protein Complexes from Cell-Lysate Cross-Linking

Protein–protein interactions can occur transiently, and sometimes only a few amino acids of proximal proteins are required for the interactions. The local environments close to the interaction sites play a critical role for the stability of noncovalent protein interactions. When the environment is changed, such as during cell lysis, the original interactions may be disturbed and new interactions may occur, some of which may hold no physiological relevance. However, to the best of our knowledge, no comparison has been made to determine the degrees of difference between the protein–protein interactions measured from intact cells cross-linked prior to cell lysis with those observed when cross-linking was performed after cell lysis. To investigate this issue, we performed similar anti-OmcA IP experiments with cell lysates that were cross-linked with MRN and DSS immediately after lysis.

As with in vivo cross-linking experiments, we observed high mass gel bands (MW > 250 kD) in these cell-lysate cross-linked samples that were not present in control samples (Supplementary Figure 4). These high MW bands were excised for protein identification, and the results are summarized in Table 3. In contrast to the results obtained with in vivo cross-linking, IP experiments with DSS resulted in identification of many more cross-linked proteins from cell lysate application as compared with MRN. Furthermore, a large number of cytosolic proteins were identified with significantly high Mascot scores, indicating their high abundance and/or high reactivity with cross-linkers.

Discussion

To identify OmcA and MtrC interactions and their interaction networks, we have developed an in vivo cross-linking strategy that includes novel hydrophobic compounds which facilitate cross-linking proteins within cell membranes. Analysis of the results presented in Table 1 shows that for both anti-OmcA and anti-MtrC IP cross-linking with MRN resulted in more identified proteins than that with DSS or DST. More importantly, the identified OM proteins resultant from DSS and DST experiments are a subset of those identified using MRN, indicating superior cross-linking properties of MRN for membrane protein interaction identification as compared with either DSS or DST. All three cross-linkers are cell membrane permeable. However, MRN is larger in size and much more hydrophobic than DSS and DST. Thus, MRN

may penetrate cell membranes at a different rate than DSS or DST and may remain closer to hydrophobic membranes after cell penetration. The different penetration rate of MRN may also allow more reaction time with membrane proteins.

As shown in Figure 1, MRN has much longer spacer chain than DSS and DST. However, hydrophobicity of MRN seems to play a more important role than spacer length for enhanced labeling efficiency of OM proteins. This observation is supported by comparison of the results of in vivo cross-linking and cell lysate cross-linking experiments. Anti-OmcA IP using DSS resulted in identification of more proteins from cell lysate cross-linking (21 proteins) as opposed to those identified from in vivo cross-linking (9 proteins). On the other hand, anti-OmcA IP using MRN resulted in fewer cross-linked protein identifications in cell lysates (9 proteins) than was observed with intact cells (16 proteins). This further reflects the selectivity of the hydrophobic property of the cross-linkers for labeling OM proteins in cells. If MRN length were the dominant difference, one would expect proportionately more cross-linked proteins from cell lysate experiments, rather than fewer proteins. DSS is less hydrophobic than MRN and more soluble in cell lysates; thus, DSS exhibits good cross-linking activity with soluble proteins in cell lysates and likely results in higher cross-linked protein concentration than MRN (Supplementary Figure 4), whereas MRN works better under the hydrophobic environment of cell membranes leading to detection of more cross-linked membrane proteins in cells. It should also be noted that other PIR compounds we have synthesized with similar overall size as compared to MRN, but with decreased hydrophobic properties, do not provide the same performance for OM protein interaction identification as we observe with MRN (data not shown). Therefore, the novel PIR cross-linker, MRN, with increased hydrophobic properties allows greater capability of OM protein–protein interaction identification. In contrast, in vivo cross-linking experiments with DST failed to result in identification of the OmcA–MtrC interaction in the anti-MtrC IP experiment, most likely due to its weak hydrophobicity (Table 1). In all experiments, a few cytosolic proteins were identified which could result from cross-linker labeling after cell membrane penetration. However, we do not eliminate the possibility of nonspecific contamination of highly abundant proteins. For example, the translation elongation factor Tu has previously been observed as a contaminant in other proteomic studies.[13,42]

After cell lysis, proteins are extracted into the buffer solution. In contrast to the native environment of intact cells, protein conformation and its interacting partners may change dramatically in the cell lysate solution. Accordingly, cross-linked protein identities from cell-lysate cross-linking appear significantly different from those observed with in vivo cross-linking, as is indicated in the results shown in Table 1 and Table 3. For example, cell-lysate cross-linking experiments resulted in identification of mostly cytosolic proteins (18 out of 21 using DSS, and 7 out of 9 using MRN), while in vivo cross-linking experiments identified more membrane proteins (7 out of 9 using DSS, and 13 out of 16 using MRN). Interestingly, MtrC was not identified from either DSS or MRN cell-lysate labeling experiments, indicating either OmcA and MtrC interaction was interrupted or the cross-linking became less efficient after the cells were lysed. This comparison is further illustrated in the Venn diagram (Figure 4). Few proteins (3 using DSS and 4 using MRN) were identified both from cell-lysate and in vivo cross-linking including the antigen protein itself, OmcA. Copurified proteins identified from unlabeled cell lysates can be considered as traditional IP results (Supplementary Figure 1 and Supplementary Table 1). Both OmcA and MtrC were identified from unlabeled control samples at band B2 in Supplementary Figure 1 further supporting the identified OmcA and MtrC interaction from this work and that of others.[13,15] However the IP of unlabeled samples also resulted in many other proteins that were different from those cross-linked proteins identified from in vivo cross-linking. Traditional co-IP experiments for identifying protein–protein interactions can only be applied after cells are lysed. Our results demonstrate that the interruption of living cells can change protein–protein interactions dramatically, especially for

membrane proteins. Therefore, caution must be exercised when interpreting results from co-IP experiments or cell-lysate cross-linking experiments. Furthermore, this study also demonstrated the advantages of cross-linking methods which can provide snap-shots of the native protein–protein interactions in vivo.

OmcA and MtrC have been shown to be exposed on the outer membranes of *S. oneidensis* cells to allow direct electron transfer to extracellular electron acceptors during anaerobic respiration. Four identified proteins in Table 1 may be involved in either OmcA or MtrC transport across the OM or are components of a large protein complex that facilitates extracellular electron transport. Based on the identified proteins in Table 1, an OmcA–MtrC interaction network map was constructed with Cytoscape (Figure 5a). In addition to OmcA and MtrC, three OM proteins including the OmpA family protein (SO3545), the outer membrane porin (SO3896), and the major outer membrane lipoprotein (SO1295) were identified by cross-linking and immunoprecipitation of OmcA or MtrC with all three chemical cross-linkers. The first two OM proteins are predicted to be transmembrane β-barrel proteins,[46,47] and the major outer membrane lipoprotein (SO1295) is known as one of the most abundant OM proteins in another Gram-negative bacterium, *E. coli*,[48] that, like *S. oneidensis*, is a member of the γ-proteobacteria. The other five OM proteins, putative lipoprotein (SOa0110), hypothetical protein (SO0404), anaerobic DMSO reductase (SO1429), putative long chain fatty acid transport (SO3099), and 5-nucleotidase (SO2001), were only identified from MRN experiments presumably due to their more hydrophobic localization environment. Among all the identified OM proteins, the putative lipoprotein (SOa0110) as well as three other proteins, SO1295, SO3896, and SO3545, were identified in both anti-OmcA and anti-MtrC IP experiments, and the other four proteins (SO0404, SO1429, SO3099, and SO2001) were only identified in anti-OmcA IP experiments. One periplasmic protein (SO1824), two inner membrane proteins (SO1825, SO3286), and five cytosolic proteins (SO4749, SO1930, SO4747, SO0217, and SO0237) were identified as shown in Table 1. Identified cytosolic proteins could be the result of cross-linker penetration, nonspecific binding, and indirect binding through formation of larger complexes as discussed previously.[42] Furthermore, results from in vivo cross-linking with *omcA* and *mtrC* deletion mutant cells strongly suggest many of the identified protein interactions, except for the major outer membrane lipoprotein (SO1295), are mediated by OmcA. Figure 5b and 5c illustrate the Cytoscape visualization of the subset of interactions observed from the *mtrC* and *omcA* deletion mutant cell experiments. In both cases, four OM proteins (SO0404, SO1429, SO3099, and SO2001) were missing as compared to the results from experiments with wild type cells and appear in gray in the figures, which suggests that both OmcA and MtrC are required to maintain these interactions. However, OmcA and MtrC could possibly play different roles in maintaining these interactions. Several cross-linked OM proteins (SOa0110, SO1295, SO3896, and SO3545) that were identified with high scores for wild type cells were also identified with the use of *mtrC* deletion mutant cells (except for MtrC). In addition, one inner membrane protein (SO1825) and two cytosolic proteins (SO1930, SO4749) were also missing for *mtrC* mutant cells (Figure 5b). In strong contrast, however, all proteins except for the major outer membrane lipoprotein (SO1295) and the translation elongation factor Tu (SO0217) were not observed in cross-linking results from anti-MtrC IP experiments with *omcA* deletion mutant cells, as illustrated in Figure 5c. It should be noted that translation elongation factor Tu (SO0217) has been reported as a contamination in other proteomic studies.[13,42] These results strongly suggest that deletion of OmcA from cells has a strong influence on the interaction network of MtrC. A possible explanation for this observation is that the binding stoichiometry for the OmcA–MtrC interaction in cell membranes may involve more than one OmcA molecule per MtrC molecule, as observed in vitro by Shi et al.[13] If this were the case, then one might expect that multiple OmcA molecules might occupy most interaction sites on MtrC. Therefore, MtrC might form cross-linking complexes with other OM proteins through the mediation of OmcA. The comprehensive protein complex network involved in electron transfer in *S. oneidensis* cells is still far from

being fully determined, and most interacting protein components remain unknown. Nonetheless, our initial characterization of the OmcA–MtrC network provides a critical first step to help unveil the interaction map and further understand electron transfer mechanisms within *S. oneidensis* cells.

In summary, the OmcA–MtrC interaction within *S. oneidensis* MR-1 cells was identified in vivo with cross-linking and IP methods. The network surrounding the OmcA–MtrC interaction core was further characterized, and identification of other proteins involved in this network could help decipher the unique electron transfer and metal reduction mechanisms associated within *S. oneidensis* cells. Membrane proteins have presented great challenges for the identification of protein–protein interactions due to their strong hydrophobicity. Our study demonstrates that hydrophobic PIR cross-linkers preferentially label membrane proteins, in particular, OM proteins. Therefore, employing chemical cross-linkers with added hydrophobicity coupled with immunoprecipitation presents a practical strategy for mapping protein–protein interactions in cell membranes.

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Supplementary Material

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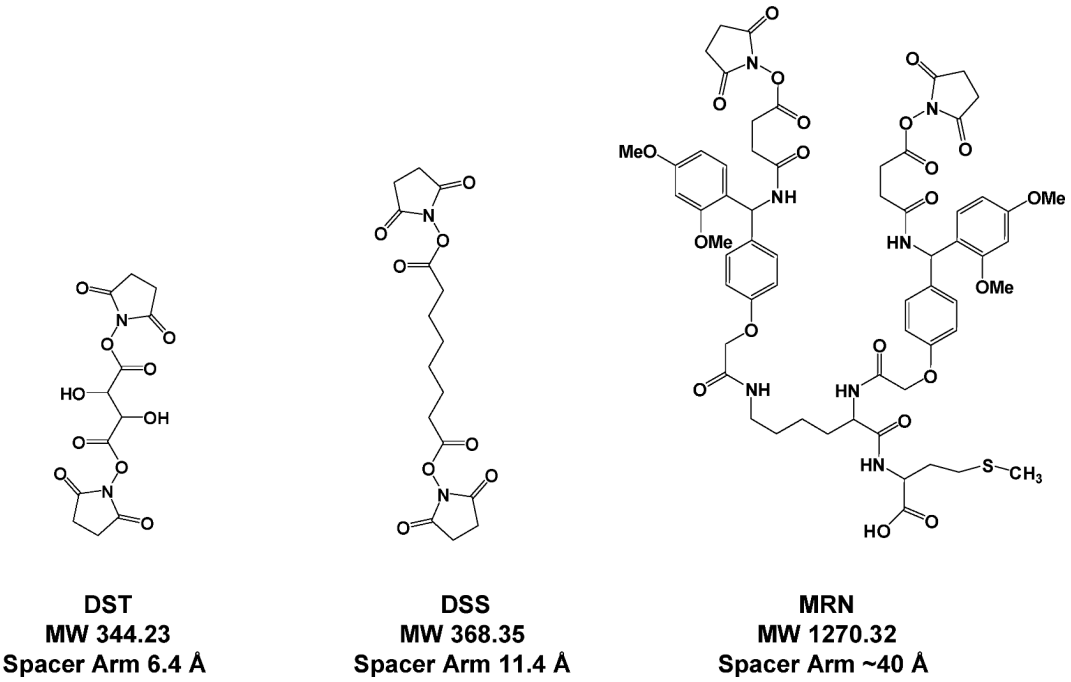

Figure 1.
Structures of the novel hydrophobic PIR cross-linker, MRN, and two commercial cross-linkers, DSS and DST.

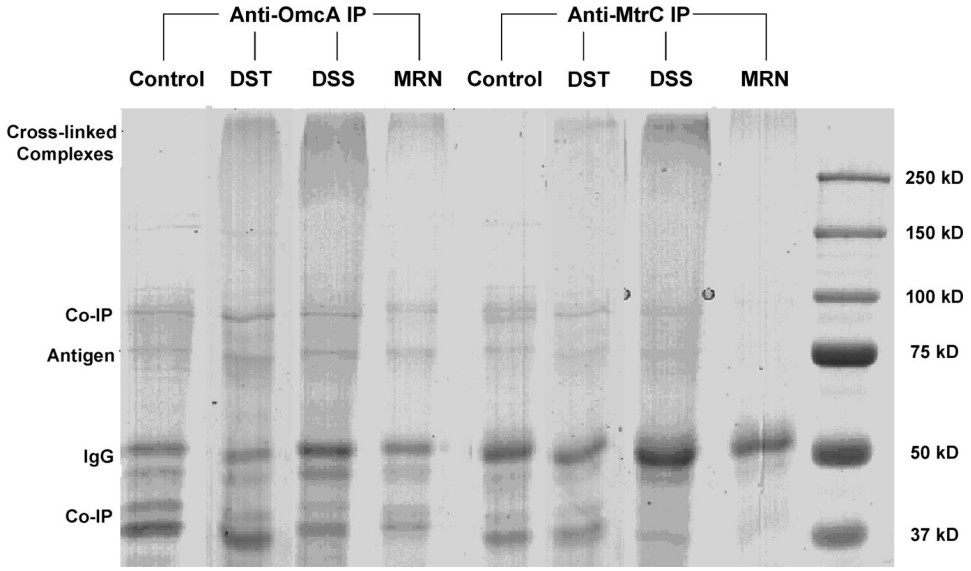

Figure 2.
SDS-PAGE analysis of the proteins immunoprecipitated with the OmcA or MtrC antibody from in vivo cross-linked cells by DST, DSS, or MRN. IP of uncross-linked cells served as a control.

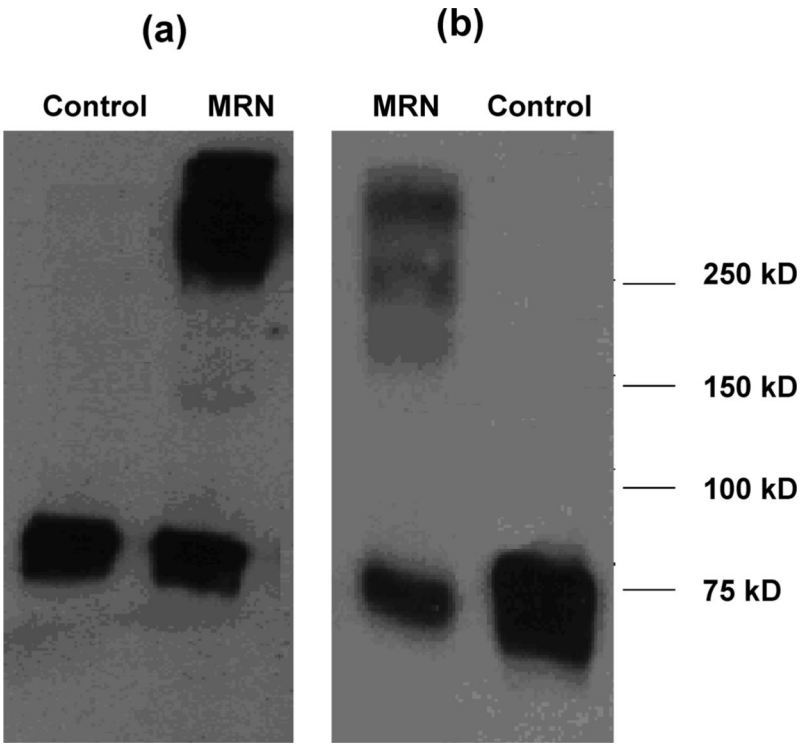

Figure 3.
(a) Anti-OmcA Western blot analysis after anti-MtrC immunoprecipitation. (b) Anti-MtrC Western blot analysis after anti-OmcA immunoprecipitation.

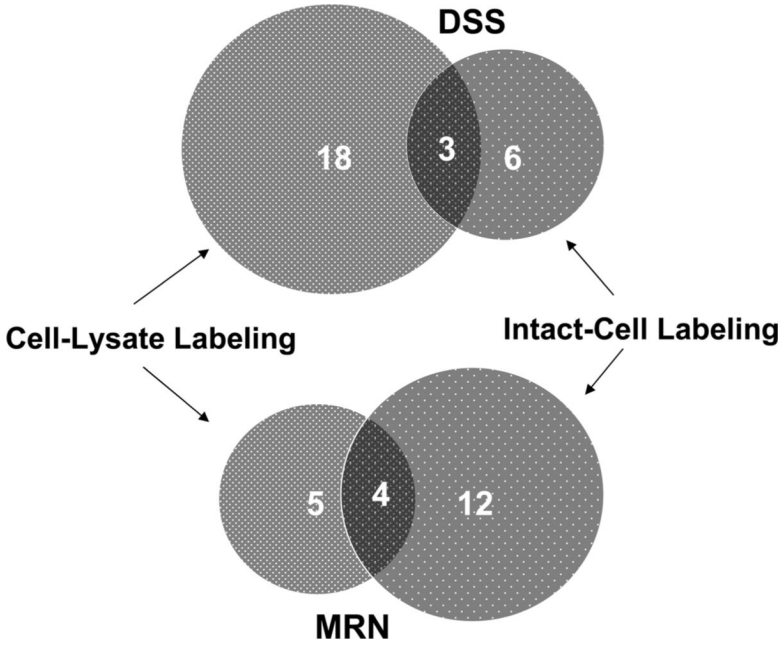

Figure 4.

Venn-diagram analysis on cross-linked protein numbers of cell-lysate labeling and intact-cell labeling with anti-OmcA IP using DSS and MRN. The numbers in the overlaid area are the numbers of proteins that were identified in both cell-lysate labeling and intact-cell labeling.

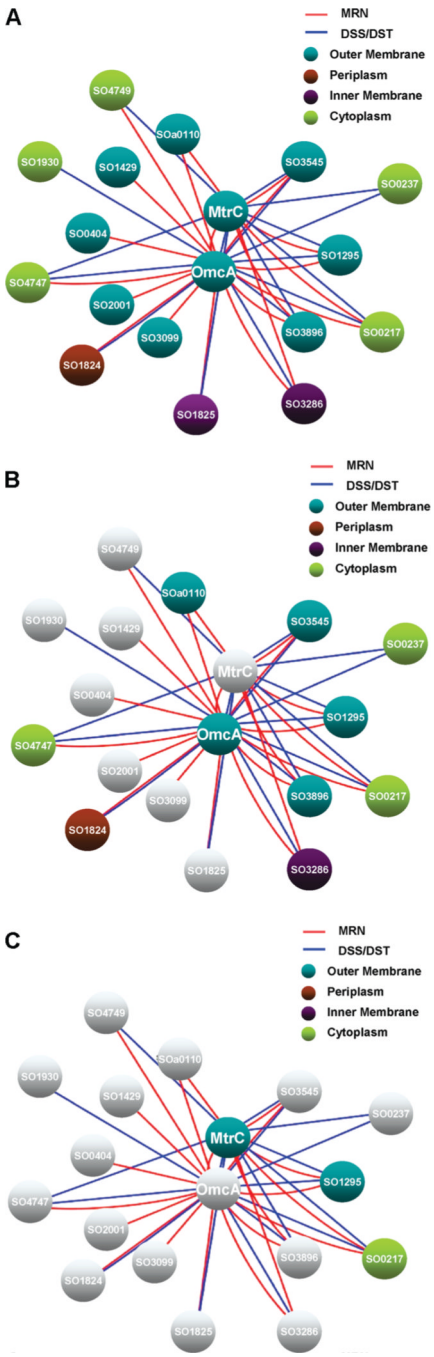

Figure 5.
Cytoscape maps of the OmcA–MtrC interaction network based on in vivo cross-linking and IP results: (a) of wild type *S. oneidensis* MR-1 cells; (b) of *mtrC* mutant cells; (c) of *omcA* mutant cells.

Table 1

Identified Cross-Linked Proteins from > 250 kD Gel Bands in Anti-OmcA and Anti-MtrC IP of MRN, DSS, and DST in Vivo Labeling[a]

	Anti-OmcA IP			Anti-MtrC IP					MW
gene	MRN	DSS	DST	MRN	DSS	DST	annotation	category[b]	(kDa)
SO1779	789	432	523	811	132		decaheme cytochrome c, OmcA	OM	79
SO1778	378	49	118	343	307	136	decaheme cytochrome c, MtrC	OM	72
SOa0110	253			322			lipoprotein, putative	OM	145
SO3545	228	45	376	123		189	OmpA family protein	OM	40
SO3896	149	67		90		97	outer membrane porin, putative	OM	39
SO1295	126	78	202	153	96	153	major outer membrane lipoprotein	OM	9
SO0404	112						hypothetical protein	OM	129
SO1429	58						anaerobic dimethyl sulfoxide reductase	OM	91
SO3099	57						long chain fatty acid transport, putative	OM	47
SO2001	46						5-nucleotidase	OM	61
SO1824	134	51					conserved hypothetical	P	25
SO1825	174	103			87		MotA/TolQ/ExbB proton channel protein	IM	48
SO3286	105		76	126			cytochrome c ubiquinol oxidase	IM	58
SO4747	100						ATP synthase F1, beta subunit	C	49
SO0217	79	94	231	91	146	120	translation elongation factor Tu	C	43
SO4749	54						ATP synthase F1, alpha subunit	C	55
SO0237		141			125		ribosomal protein S3	C	25
SO1930			72				2-oxoglutarate dehydrogenase, sucA	C	105

[a] The numbers in the table are the Mascot scores.

[b] OM denotes outer membrane; P denotes periplasmic; IM denotes inner membrane; and C denotes cytoplasmic.

Table 2

Identified Cross-Linked Proteins from > 250 kD Gel Bands in Anti-OmcA and Anti-MtrC IP of MRN and DSS in Vivo Labeling on MtrC and OmcA Mutant Cells, Respectively[a]

	anti-OmcA		anti-MtrC				
	ΔMtrC		ΔOmcA				MW
gene	MRN	DSS	MRN	DSS	annotation	category[b]	(kDa)
SO1779	971	476			decaheme cytochrome c, OmcA	OM	79
SO1778			333	312	decaheme cytochrome c, MtrC	OM	72
SOa0110	308				lipoprotein, putative	OM	145
SO3545	472	149			OmpA family protein	OM	40
SO3896	100	125			outer membrane porin, putative	OM	39
SO1295	183	153	179	106	major outer membrane lipoprotein	OM	9
SO1824	69				conserved hypothetical	P	25
SO3286	68				cytochrome c ubiquinol oxidase	IM	58
SO1490				306	alcohol dehydrogenase II, AdhB	C	40
SO4747	108				ATP synthase F1, beta subunit	C	49
SO0217	82			179	translation elongation factor Tu	C	43
SO0237		105			ribosomal protein S3	C	25
SO0314				57	ornithine decarboxylase	C	82

[a] The numbers in the table are the Mascot scores.

[b] OM denotes outer membrane; P denotes periplasmic; IM denotes inner membrane; and C denotes cytoplasmic. ΔMtrC denotes *mtrC* mutant, and ΔOmcA denotes *omcA* mutant.

Table 3

Identified Cross-Linked Proteins from >250 kD Gel Bands in Anti-OmcA IP of MRN and DSS Cell-Lysate Labeling[a]

gene	DSS	MRN	annotation	category[b]	MW (kDa)
SO0225	1928		DNA-directed RNA polymerase, rpoC	C	156
SO0224	1431	74	DNA-directed RNA polymerase, rpoB	C	150
SO1490	987		alcohol dehydrogenase II, AdhB	C	40
SO0229	928	359	translation elongation factor Tu, TufA	C	43
SO0217	888[c]	367[c]	translation elongation factor Tu, TufB	C	43
SO4749	467		ATP synthase F1, alpha subunit	C	55
SO1142	321	88	carbamoyl-phosphate synthase, CarB	C	119
SO4747	238	45[c]	ATP synthase F1, beta subunit	C	50
SO2638	170		leucine dehydrogenase, Ldh	C	37
SO0314	161	130	ornithine decarboxylase, SpeF	C	82
SO3032	160		NTP-dependent alcaligin synthetase, AlcC	C	69
SO1679	120		branched-chain acyl-CoA dehydrogenase	C	42
SOA0112	117		lipoprotein, putative	OM	146
SO1521	100		conserved hypothetical protein	C	103
SO3532	91		isoleucyl-tRNA synthetase, IleS	C	106
SO0021	86		fatty oxidation complex, alpha subunit	C	77
SO0256	81		DNA-directed RNA polymerase, RpoA	C	36
SO4030	73		excinuclease ABC, A subunit, UvrA	C	106
SO1295	68[c]	138[c]	major outer membrane lipoprotein	OM	9
SO3440	56		enolase, Eno	C	42
SO1779	41[c]	140[c]	OmcA	OM	80
SO3542		55	conserved hypothetical protein	C	90

[a] The numbers in the table are the Mascot scores.

[b] C denotes cytoplasmic, and OM denotes outer membrane.

[c] Indicates that those proteins were also identified from on-cell labeling.