RESEARCH LETTER

Involvement and specificity of Shewanella oneidensis outer membrane cytochromes in the reduction of soluble and solid-phase terminal electron acceptors

Clemens Bücking1, Felix Popp1, Sven Kerzenmacher2 & Johannes Gescher1

1Department of Microbiology, Institute for Biology II, University of Freiburg, Freiburg, Germany; and 2Laboratory for MEMS Applications, Department of Microsystems Engineering–IMTEK, University of Freiburg, Freiburg, Germany

Correspondence: Johannes Gescher, Institut Biologie II, Mikrobiologie, Universität Freiburg, Schänzlestr. 1, D-79104 Freiburg, Germany. Tel.: +49 761 203 2685; fax: +49 761 203 2626; e-mail: johannes.gescher@biologie.uni-freiburg.de

Received 18 February 2010; revised 26 February 2010; accepted 27 February 2010. Final version published online 30 March 2010. DOI:10.1111/j.1574-6968.2010.01949.x

Editor: Jörg Simon

Keywords
Shewanella oneidensis; dissimilatory iron reduction; c-type cytochromes; MtrC; OmcA; MtrF.

Abstract
The formation of outer membrane (OM) cytochromes seems to be a key step in the evolution of dissimilatory iron-reducing bacteria. They are believed to be the endpoints of an extended respiratory chain to the surface of the cell that establishes the connection to insoluble electron acceptors such as iron or manganese oxides. The gammaproteobacterium Shewanella oneidensis MR-1 contains the genetic information for five putative OM cytochromes. In this study, the role and specificity of these proteins were investigated. All experiments were conducted using a markerless deletion mutant in all five OM cytochromes that was complemented via the expression of single, plasmid-encoded genes. MtrC and MtrF were shown to be potent reductases of chelated ferric iron, birnessite, and a carbon anode in a microbial fuel cell. OmcA-producing cells were unable to catalyze iron and electrode reduction, although the protein was correctly produced and oriented. However, OmcA production resulted in a higher birnessite reduction rate compared with the mutant. The presence of the decaheme cytochrome SO_2931 as well as the diheme cytochrome SO_1659 did not rescue the phenotype of the deletion mutant.

Introduction
Dissimilatory metal-reducing bacteria have been investigated intensively since the late 1980s. One important model organism for the biochemical elucidation of metal-reducing processes is Shewanella oneidensis. Electron transfer to insoluble metal oxides at the cell surface was shown to be mostly dependent on a c-type cytochrome-based conductive interprotein connection between the quinone pool within the cytoplasmic membrane and the insoluble terminal electron acceptor located at the outer membrane (OM) (Shi et al., 2007).

The final reduction is catalyzed by c-type cytochromes that are attached to the OM by a lipid anchor. In addition to this catalysis of a direct electron transfer to metal oxides (Shi et al., 2007; Wang et al., 2008), other possible functions have also been ascribed to OM cytochromes, including adhesion to mineral particles (Xiong et al., 2006; Lower et al., 2007; Coursolle et al., 2009) and interaction with shuttling compounds (Lies et al., 2005; Marsili et al., 2008). Many studies on the role of OM cytochromes have been published to date. Surprisingly, it is still a matter of ongoing research to assign specific functions to independent proteins. This situation might in part be attributed to the conceivable functional redundancy of these proteins and c-type cytochromes in general (Dobbin et al., 1999; Myers & Myers, 2003b).

The aim of this study was the characterization and comparison of reductase activities of individual OM cytochromes. For this purpose, an S. oneidensis deletion mutant deficient in all five OM cytochromes (Meyer et al., 2004) was generated to avoid data acquisition that is at least partly affected by a potential low level or upregulated production of proteins with overlapping activities. Subsequently, individually tagged proteins were produced in this background and the activity of complemented strains to reduce soluble and insoluble electron acceptors was tested.

Materials and methods

Growth conditions and media
All the microorganisms used in this study are listed in Table 1. Escherichia coli strains were grown in Luria–Bertani (LB)
medium at 37 °C. *Saccharomyces cerevisiae* InvSc1 was grown on YPD medium and was selected for transformants on uracil-free medium (Clontech, Mountain View). *Shewanella oneidensis* strains were grown aerobically at 30 °C in an LB medium or anaerobically in a mineral medium, as described elsewhere (Schuetz et al., 2009). If not mentioned, disodium-fumarate (100 mM) was used as an electron acceptor. If necessary, kanamycin (25 or 50 µg mL⁻¹) was added to the medium.

For growth experiments with birnessite as an electron acceptor, 2.5 mM birnessite was added to the mineral medium that was supplemented with 0.1 mM arabinose. Birnessite was prepared as described earlier (Burdige & Nealon, 1985). Manganese reduction was determined in two independent cultures using leucoberbelin blue (Booger & de Vriend, 1987).

### Construction of a markerless *S. oneidensis* \(\Delta\text{OMC} \) strain

*Saccharomyces cerevisiae*-based cloning according to Shanks et al. (2006) was used to combine three fragments into suicide plasmid pMQ150 (accession no. EU546823): two 500-bp regions flanking the upstream and downstream regions of \(\text{mtrD} \) and \(\text{mtrC} \), respectively, and one fragment containing \(\text{PBAD} \) and the \(\text{araC} \) gene. The fragments were amplified (primers 1–2, 3–4, 5–6; see Table 2) and contained overlapping regions to the vector and to the adjacent fragment. The three fragments and the BamHI and the SalI linearized vector were transformed into *S. cerevisiae*. The resulting suicide plasmid was used for mutagenesis of *S. oneidensis* MR-1, resulting in strain JG53 (Table 1). Subsequently, genes *SO_2931* and *SO_1659* were deleted using the same technique (fragments were amplified with primers 7–14; Table 2).

### Cloning and production of OM cytochromes

Gene *SO_2931*\_strept was cloned into pBAD202 via TOPO cloning (Invitrogen, Karlsruhe, Germany). The gene was amplified using primers 15 and 16 and was thereby modified to contain an NcoI restriction site and the sequence for a C-terminal strep-tag. His-patch thioredoxin was excised from the vector by cleavage with NcoI and subsequent religation. This vector was used for cloning of the other OM cytochrome genes after NcoI/PmeI restriction digest. The genes were PCR amplified using 5′ primers (primers 17, 19, 21, 23) containing a BspHI site and 3′ primers with a PmeI site and a sequence for a C-terminal strep-tag (primers 18, 20, 22, 24; Table 2). For strain JG162, *omcA* was amplified with primers 21 and 26 containing no strep-tag sequence.

### Membrane preparation, SDS-PAGE, heme staining, and Western blotting

Membrane fractions were prepared as described elsewhere (Schuetz et al., 2009). Protein concentrations were determined using the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard. For the quantification of protein concentrations in cell suspensions, 0.2 mM NaOH was added to the suspensions before a 10-min incubation at 95 °C.

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### Table 1. Yeast and bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
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<tr>
<td>JG152</td>
<td><em>E. coli</em> DH5α λ(pir)</td>
<td>F–[argF-lac]169 Δ[800diazCZ88(AM15) glnV44(AS) rfbD1 gynA96(NalR) recA1 endA1 spoT1 thi-1 hsdR17 deoR2 pir+ MATa/MATα leu2-3,112 trp1-14897 trp1-1897 ura3-52 His3Δ1 his3Δ1</td>
<td>Stalker et al. (1992)</td>
</tr>
<tr>
<td>JG26</td>
<td><em>S. cerevisiae</em> InvSc1</td>
<td>Wild type</td>
<td>Invitrogen</td>
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<td>JG7</td>
<td><em>S. oneidensis</em> MR-1</td>
<td>Δ(mtrD-mtrC)</td>
<td>Venkateswaran et al. (1999)</td>
</tr>
<tr>
<td>JG132</td>
<td><em>S. oneidensis</em> MR-1ΔMC (OM cytochrome deletion mutant)</td>
<td>Δ(mtrD-mtrC) ΔSO_2931 ΔSO_1659, 3100633 Δ(arac, PBAD)</td>
<td>This work</td>
</tr>
<tr>
<td>JG137</td>
<td><em>S. oneidensis</em> MR-1ΔMC pBADomcAΔSO_2931</td>
<td>Δ(mtrD-mtrC) ΔSO_2931 ΔSO_1659, 3100633 Δ(arac, PBAD)</td>
<td>This work</td>
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<td>JG138</td>
<td><em>S. oneidensis</em> MR-1ΔMC pBADmtrCΔSO_2931</td>
<td>Δ(mtrD-mtrC) ΔSO_2931 ΔSO_1659, 3100633 Δ(arac, PBAD)</td>
<td>This work</td>
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<tr>
<td>JG139</td>
<td><em>S. oneidensis</em> MR-1ΔMC pBADmtrFΔSO_2931</td>
<td>Δ(mtrD-mtrC) ΔSO_2931 ΔSO_1659, 3100633 Δ(arac, PBAD)</td>
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<td>Δ(mtrD-mtrC) ΔSO_2931 ΔSO_1659, 3100633 Δ(arac, PBAD)</td>
<td>This work</td>
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<tr>
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<td><em>S. oneidensis</em> MR-1ΔMC pBADSO_2931ΔSO_1659</td>
<td>Δ(mtrD-mtrC) ΔSO_2931 ΔSO_1659, 3100633 Δ(arac, PBAD)</td>
<td>This work</td>
</tr>
<tr>
<td>JG162</td>
<td><em>S. oneidensis</em> MR-1ΔMC pBADomcA</td>
<td>Δ(arac, PBAD)</td>
<td>Schuetz et al. (2009)</td>
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<td>JG50</td>
<td><em>S. oneidensis</em> MR-1mutations pBADmtrAΔSO_2931</td>
<td>Δ(arac, PBAD)</td>
<td>Schuetz et al. (2009)</td>
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Proteins were separated on polyacrylamide gels according to Laemmli (1970). Heme proteins were visualized by peroxidase staining (Thomas et al., 1976). Proteins containing a C-terminal strep-tag were detected on a Western blot using a primary strep-tag antibody (Qiagen, Hilden, Germany) and a secondary horseradish peroxidase-labeled antibody. The blot was developed using the Ace-glow detection system and was quantified using the Image Lab software (Biorad, Munich, Germany).

**Cell surface exposure of OM cytochromes**

Surface exposure of OM cytochromes was detected using a proteinase K digest as described by Myers & Myers (2003a), with slight modifications. The duration of incubation was increased to 60 min. The incubation temperature was set to 37 °C. As positive controls for cell surface exposure, strains JG137 and JG138, producing OmcAstrep and MtrCstrep were used; as a control for OM integrity under the incubation conditions, the periplasmic c-type cytochrome MtrA containing an N-terminal strep-tag (MtrAstrept) was produced in an *S. oneidensis* ΔmtrA background (Schuetz et al., 2009).

**Cell suspension assays**

Cells were grown anaerobically overnight in minimal media with fumarate as an electron acceptor. At an OD$_{578}$ nm of ~0.2, 0.1 mM arabinose was added to induce OM cytochrome and MtrA/MtrB production. After 4 h of production, cells were harvested and washed twice with mineral media without fumarate and lactate and then resuspended in HEPES buffer (100 mM, pH 7.5) containing 50 μM MgCl$_2$ to obtain a final OD$_{578}$ nm between 3 and 5. All further measurements were performed in independent duplicates in an anaerobic glove box. Specific reduction rates were obtained by normalization to the protein content of the cell suspension. Fifty microliters of the cell suspension was pipetted in a well of a microtiter plate. The assay was started through the addition of 150 μL of a solution containing 10 mM lactate and 10 mM ferric citrate. At different time points (0–30 min), the reaction was stopped by the addition of 100 μL 3 M HCl. The Fe$^{2+}$ concentration of the samples was determined using the ferrozine reagent (Viollier et al., 2000).

**Microbial fuel cell (MFC) experiments**

The MFC setup used in this study features an anode and cathode chamber with a working volume of 8 mL each, separated by a Nafion-117 membrane (Quintech, Göppingen; Kloke et al., 2010). A saturated calomel reference electrode (SCE) was separated from the anode compartment by another Nafion membrane. Electrodes were made of graphite felt cubes (Alpha Aesar, Karlsruhe). The whole setup was connected to a potentiostat (Pine Instruments, Grove City). The standard measurement protocol consisted of two phases: after a conditioning period with a constant current flux over 5 h (0.3 μA/cm$^2$), MFC cultures were subjected to a continuous increase in current density at a rate of 1.1 μA/cm$^2$·h$^{-1}$ over 45 h (*current sweep* phase). The anode compartment was continuously flushed with nitrogen gas to maintain anoxic conditions. Additional terminal electron acceptors were not added.

### Table 2. Primers used in this study (5′–3′)

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<td>22</td>
<td>AAGCATTGCAATTCGAGCTGCGATTTGAAACATGTTATCGT AAGGAGTGG</td>
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Results

Preparation of the OM cytochrome-deficient mutant

A markerless multideletion mutant in all annotated OM cytochromes of *S. oneidensis* was constructed to generate a strain platform that allows for analysis of OM cytochrome activity without the potential detection of redundant activities from similar proteins. We will refer to this mutant as ΔOMC. Genes coding for MtrE, MtrC, and OmcA were deleted in one step. This deletion led to further excision of *mtrD* and *mtrE* from the chromosome. The genes for the decaheme c-type cytochrome SO_1659 and the diheme cytochrome SO_2931 were deleted subsequently. The presence of MtrA and MtrB was shown to be a requirement for metal reduction by *S. oneidensis* (Bretschger et al., 2007). Hence, possible effects of the removal of genes ranging from *mtrF* to *mtrC* on the expression of *mtrA* and *mtrB* were circumvented by the concomitant introduction of an arabinose-inducible promoter and the *araC* repressor.

Cloning and production of OM cytochromes in the ΔOMC mutant

Genes coding for OM cytochromes from *S. oneidensis* were cloned separately into plasmid pBAD202 to assign specific functions to these proteins in further experiments. The sequence information for a C-terminal strep-tag was added to allow for the specific detection of the proteins produced. The relative amounts of the produced OM cytochromes were quantified via immunodetection of the added strep-tag epitope (Fig. 1a). OmcA production resulted in the strongest strep-tag derived signal compared with all other OM cytochromes produced (Fig. 1c). Signals resulting from MtrCstrep and MtrFstrep production were detected in similar quantities, which indicates similar production levels. In contrast, the production of SO_1659strep and SO_2931strep seems to be strongly reduced compared with the other three OM cytochromes.

Surface exposure of OM cytochromes

Proteinase K assays according to Myers & Myers (2003a) were performed to investigate whether the proteins are oriented toward the periplasm or the surrounding media (Fig. 2). Detection was based on the added strep-tag epitope. A control reaction using production of a strep-tagged MtrA protein that is localized to the periplasm was performed, to ensure that the assay conditions did not interfere with cell integrity. Localization of OmcA and MtrC to the cell surface was already shown by other research groups (Myers & Myers, 2003a; Shi et al., 2008). Hence, MtrCstrep and OmcAstrep were used as proteinase K-degradable control proteins. As Fig. 2 shows, OmcAstrep, MtrCstrep, MtrFstrep, SO_1659strep, and SO_2931strep were loaded. The single values varied within a range of not > 5%. The OmcA signal intensity was set to 100%.

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**Fig. 1.** Relative quantification of the OM cytochromes produced. SDS-PAGE gels were loaded with membrane fractions prepared from *Shewanella oneidensis* ΔOMC strains grown anaerobically on fumarate and induced with 0.1 mM arabinose to produce single OM cytochromes. (a) Western blot of cells producing OmcA_{strep} (lanes 1 and 2), MtrC_{strep} (lanes 3 and 4), MtrF_{strep} (lanes 5 and 6), SO_1659_{strep} (lanes 7 and 8), and SO_2931_{strep} (lanes 9 and 10). Three micrograms of the membrane fraction was loaded in lanes 1, 3, 5, and 7. Two micrograms was loaded in lanes 2, 4, 6, and 8. Lanes 9 and 10 contain 8 and 6 µg membrane fractions, respectively. (b) For a series of control experiments, the native form of OmcA was produced. The protein was detected using heme activity staining. Twenty-five micrograms of the membrane fractions from cells producing either OmcA (lane 1) or OmcA_{strep} (lane 2) were loaded. (c) Relative quantification of the detected hpr-derived signals. Mean values from two independent quantifications are shown. The single values varied within a range of not > 5%. The OmcA signal intensity was set to 100%.
and the decaheme cytochrome SO_1659strp are clearly hydrolyzed by the proteinase. Diheme SO_2931strp does not seem to be surface exposed or is not available for proteinase activity.

Reduction of ferric citrate

Cell suspension assays showed that only the production of MtrCstrp and MtrFstrp could partly rescue the mutant phenotype for ferric citrate reduction (Fig. 3a and b). MtrFstrp production resulted in a 1.2-fold accelerated ferric citrate reduction rate compared with the MtrCstrp-producing strain.

Surprisingly, the presence of OmcAstrp did not lead to increased ferric iron reduction rates compared with the ΔOMC mutant. To exclude the possible effects of the strep-tag epitope on protein activity, control experiments with the native form of omcA in the same vector backbone were performed. Production of the native form of OmcA was shown via heme activity staining (Fig. 1b). Still, even the presence of the native form of OmcA did not lead to an altered phenotype compared with the ΔOMC mutant.

Birnessite reduction

Birnessite was used to study the effect of OM cytochrome production on the reduction of manganese oxides. Interestingly, the complementation pattern did not resemble the results from the reduction experiments with ferric citrate (Fig. 3c). Although MtrFstrp and MtrCstrp production markedly increased the ability of the ΔOMC mutant to reduce Mn⁴⁺ (53 ± 1.8% Mn⁴⁺ reduction after 50 h compared with the wild type), an effect of OmcA and OmcAstrp production (30% Mn⁴⁺ reduction after 50 h compared with the wild type) was also detectable (Fig. 3c). The production of the diheme cytochrome SO_2931strp and the decaheme cytochrome SO_1659strp did not lead to birnessite reduction rates that differed from the ΔOMC mutant. Still, these three strains exhibited a low-level reduction capability (Fig. 3c).
Anode reduction

MFCs represent another form of a solid terminal electron acceptor (Logan, 2009). Each bacterial strain displayed a characteristic $U-I$ curve (Fig. 4a). Common to all MFC cultures was a steep increase in potential at the beginning of the current sweep, followed by a region where potentials increased more linearly in response to higher currents. In this region, bacterial cells behaved analogous to Ohmic resistances. At higher current fluxes, another rapid increase in potential was observed, and above these currents, all $U-I$ curves merged into one common line that presumably results from hydrolysis of the base electrolyte. The current density at which bacteria failed to provide sufficient quantities of electrons to sustain a given current flux represents a characteristic feature of each mutant strain. To simplify comparison between performances of different bacterial strains in current sweep experiments, the limiting current density (LCD) was defined as current flux beyond which the measured anode potential first exceeded 512 mV vs. SCE (Fig. 4b), which roughly corresponds to the potential range where the $U-I$ curves of all strains exhibit the second striking rise in potential. The $\Delta$OMC mutant showed a 75% reduced LCD value compared with the wild type and could be rescued to a small degree by the production of MtrFstrep (Fig. 4a). The presence of MtrCstrep, by contrast, exerted a more significant effect. The LCD values of the other strains were similar to the $\Delta$OMC mutant and are therefore not shown.

Discussion

Elucidation of metal-reducing processes and the underlying cellular network in *S. oneidensis* is a puzzling subject due to the functional overlap of key components (Myers & Myers, 2003b; Bretschger et al., 2007). The focus of this study was to analyze the activity of single OM cytochromes in an *in vivo* context and to examine the phenotype of a mutant deficient in all of these proteins. It was not the purpose of this study to attain wild-type reduction rates with complemented strains, which would most probably necessitate the production of two or more OM cytochromes, but rather to establish a reliable test platform for OM cytochrome activities.

To analyze the activity and specificity of the different OM cytochromes, we compared electron transfer to metals and an anode surface. The reduction of an anode is as surface limited as the reduction of an insoluble metal. However, anode reduction experiments can provide an additional set of information due to the possibility to change the rate of electron abstraction from the anode surface and thus the potential.

MtrC and MtrF

The reduction experiments conducted showed that MtrCstrep and MtrFstrep could partly rescue the $\Delta$OMC phenotype, while the production of other OM cytochromes resulted only in minor effects, if at all. A central role of MtrC in metal reduction is in agreement with earlier results (Beliaev et al., 2001; Myers & Myers, 2001) and might reflect the recently discovered capability of a complex of MtrC, with the $\beta$-barrel protein MtrB and the decaheme cytochrome MtrA, to transport electrons over a liposome membrane and hence most probably also over the OM of *S. oneidensis* cells (Hartshorne et al., 2009).

$mtrF$ is part of a gene cluster that includes with $mtrD$ and $mtrE$ genes that are highly similar to $mtrA$ and $mtrB$ (McLean et al., 2008). We could show that MtrFstrep is a functional reductase that has, under several conditions, an
even accelerated activity compared with MtrC<sub>strep</sub>. McLean et al. (2008) speculate that the mtr<sup>DEF</sup> gene cluster could encode a reductase that is active under oxic or suboxic conditions and might have a function in reduction-based detoxification of radionuclides. The experiments presented here underline at least that MtrF is a reductase that could have this hypothetical function.

The relative reduction activities of MtrF<sub>strep</sub> compared with MtrC<sub>strep</sub> follow the same pattern for all electron acceptors, except for an electrode in an MFC. Here, the LCD of MtrF<sub>strep</sub>-producing cells is only 46% compared with the LCD achieved with MtrC<sub>strep</sub>-producing cells. Therefore, we hypothesize that MtrF<sub>strep</sub> might be not as well connected to the periplasmic electron pool, which could be due to a reduced capability of forming a complex with MtrA and MtrB. This interprotein electron transfer might not be rate limiting under mineral-reducing conditions, but could become important when a certain current is applied to the MFC.

### OmcA

OmcA production did not lead to accelerated reduction rates compared with the ΔOMC mutant in ferric iron reduction assays. This effect does not seem to be due to the reported partial mislocalization of OmcA in a ΔmtrC mutant (Myers & Myers, 2001) since proteinase K assays clearly demonstrated the surface exposure of OmcA in the ΔOMC mutant. OmcA is part of the core proteins that can be found in ferric iron-reducing <i>S. oneidensis</i> cells (Shi et al., 2007). We hypothesize that OmcA is an in vivo ferric iron reductase that is dependent on electron transport by another OM cytochrome. This cytochrome would most probably be MtrC. However, we cannot explain how OmcA might be linked to the periplasmic electron pool under manganese-reducing conditions. Still, our data, which indicate a function of OmcA under manganese-reducing conditions, are in line with the results obtained previously by Myers & Myers (2001, 2003b).

### ΔOMC mutant

Interestingly, a low-level reduction of birnessite and an anode surface were observed for the ΔOMC mutant. This could be due to the production of endogenous shuttling components. Still, our data indicate that if electron shuttles are the reason for this reduction, they are at least in part not dependent on the interaction with OM cytochromes and therefore seem to be OM permeable.

### Acknowledgements

The authors thank Prof. Fuchs and Prof. Majzlan for fruitful discussions. J.G. is indebted to the LANDESSTIFTUNG Baden-Württemberg and the German Science Foundation (DFG) for facilitating the analysis entailed in this article.

### References


