University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

US Department of Energy Publications | US Department of Energy

1-1-2011

Identification and Characterization of UndAHRCR-6, an Outer Membrane Endecaheme c-Type Cytochrome of *Shewanella* sp. Strain HRCR-6

Liang Shi
Pacific Northwest National Laboratory, liang.shi@pnnl.gov

Sara M. Belchik
Pacific Northwest National Laboratory

Zheming Wang
Pacific Northwest National Laboratory

David W. Kennedy
Pacific Northwest National Laboratory

Alice C. Dohnalkova
Pacific Northwest National Laboratory

See next page for additional authors

Follow this and additional works at: http://digitalcommons.unl.edu/usdoepub

Part of the Bioresource and Agricultural Engineering Commons

Shi, Liang; Belchik, Sara M.; Wang, Zheming; Kennedy, David W.; Dohnalkova, Alice C.; Marshall, Matthew J.; Zachara, John M.; and Fredrickson, James K., "Identification and Characterization of UndAHRCR-6, an Outer Membrane Endecaheme c-Type Cytochrome of *Shewanella* sp. Strain HRCR-6" (2011). *US Department of Energy Publications.* Paper 181.
http://digitalcommons.unl.edu/usdoepub/181

This Article is brought to you for free and open access by the US Department of Energy at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in US Department of Energy Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors
Liang Shi, Sara M. Belchik, Zheming Wang, David W. Kennedy, Alice C. Dohnalkova, Matthew J. Marshall, John M. Zachara, and James K. Fredrickson

This article is available at DigitalCommons@University of Nebraska - Lincoln: http://digitalcommons.unl.edu/usdoepub/181

This article is a U.S. government work, and is not subject to copyright in the United States.

Applied and Environmental Microbiology, Aug. 2011, p. 5521–5523 Vol. 77, No. 15
0099-2240/11/$12.00 doi:10.1128/AEM.00614-11
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Identification and Characterization of $UndA_{HRCR-6}$, an Outer Membrane Endecaheme *c*-Type Cytochrome of *Shewanella* sp. Strain HRCR-6▽†

Liang Shi,* Sara M. Belchik, Zheming Wang, David W. Kennedy, Alice C. Dohnalkova, Matthew J. Marshall, John M. Zachara, and James K. Fredrickson

Pacific Northwest National Laboratory, Richland, Washington 99352

Received 17 March 2011/Accepted 29 May 2011

$UndA_{HRCR-6}$ was identified from the metal-reducing bacterium *Shewanella* sp. strain HRCR-6. Both *in vivo* and *in vitro* characterization results indicate that $UndA_{HRCR-6}$ is an outer membrane endecaheme *c*-type cytochrome and probably has a key functional role in the extracellular reduction of iron [Fe(III)] oxides and uranium [U(VI)] by *Shewanella* sp. HRCR-6.

In a recent study, 23 *Shewanella* strains were isolated from the Hanford Reach of the Columbia River (HRCR) (M. J. Marshall, D. W. Kennedy, A. E. Plymale, A. C. Dohnalkova, A. S. Beliaev, and J. K. Fredrickson, unpublished data). Analyses of the proteins from the metal-reducing HRCR isolates showed that they were rich in heme-containing proteins, and some of the strains had homologues of the outer membrane (OM) decaheme *c*-type cytochromes (*c*-Cyts) MtrC and OmcA of *Shewanella oneidensis* MR-1 (1, 10; Marshall et al., unpublished). However, little information is available regarding the functions of these *c*-Cyts because their encoding genes were not identified

Gene identification. *Shewanella* isolates HRCR-2, -3, -4, -6, -7, -9, and -14 were selected for PCR screening of possible *mtrC*, *omcA*, and *undA1* (a gene encoding putative an 11-heme *c*-Cyt) homologues with the primers listed in Table S1 in the supplemental material. All tested strains were positive for *mtrC* homologues. While HRCR-2, -4, and -7 were positive for *omcA* homologues, HRCR-3, -6, -9, and -14 were positive for *undA1* homologues (Fig. 1). The *undA1*-like gene of the isolate HRCR-6 was cloned and sequenced by using a PCR-based method (5, 6) (GenBank accession number JF933770). Phylogenetic analysis of the deduced amino acid sequence of the sequenced gene revealed that it was 94% identical to UndA of *S. baltica* OS223 (see Fig. S1 in the supplemental material) (2, 9). Based on these results, this sequenced gene and its protein product were named *undA* and $UndA_{HRCR-6}$, respectively.

FH and uranium [U(VI)] reduction by $UndA_{HRCR-6}$. Ferrihydrite (FH) reduction was conducted in 1,4-piperazinediethanesulfonic acid-buffered M1 medium (pH 7.2) with 20 mM sodium lactate, 10 mM FH as the sole electron acceptor, and 1×10^8 cells ml^{-1} at 30°C with shaking (25 rpm) (4). Under this condition, MR-1 and HRCR-6 began to reduce FH within 4 h. At 48 h, MR-1 and HRCR-6 produced 1.18 ± 0.04 and

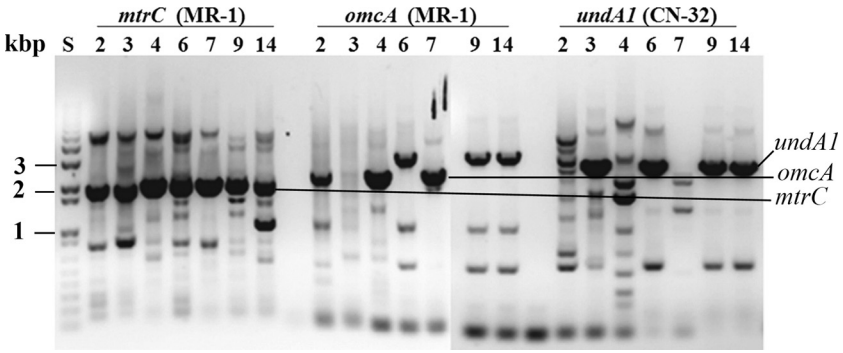

FIG. 1. PCR amplification of the *mtrC*, *omcA*, and *undA1* homologues from the genomes of 7 *Shewanella* HRCR isolates. Agarose gel showing the sizes of DNA standards in kilobase pairs (lane S) and PCR products amplified with the primers based on MR-1 *mtrC* or *omcA* or CN-32 *undA1* from the genomic DNA of HRCR-2 (lane 2), HRCR-3 (lane 3), HRCR-4 (lane 4), HRCR-6 (lane 6), HRCR-7 (lane 7), HRCR-9 (lane 9), and HRCR-14 (lane 14). The PCR products whose sizes were similar to that of MR-1 *mtrC*, MR-1 *omcA*, or CN-32 *undA1* are indicated.

* Corresponding author. Mailing address: Microbiology Group, Pacific Northwest National Laboratory, 902 Battelle Blvd., MSIN: J4-18, Richland, WA 99352. Phone: (509) 371-6967. Fax: (509) 372-1632. E-mail: liang.shi@pnnl.gov.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 7 June 2011.

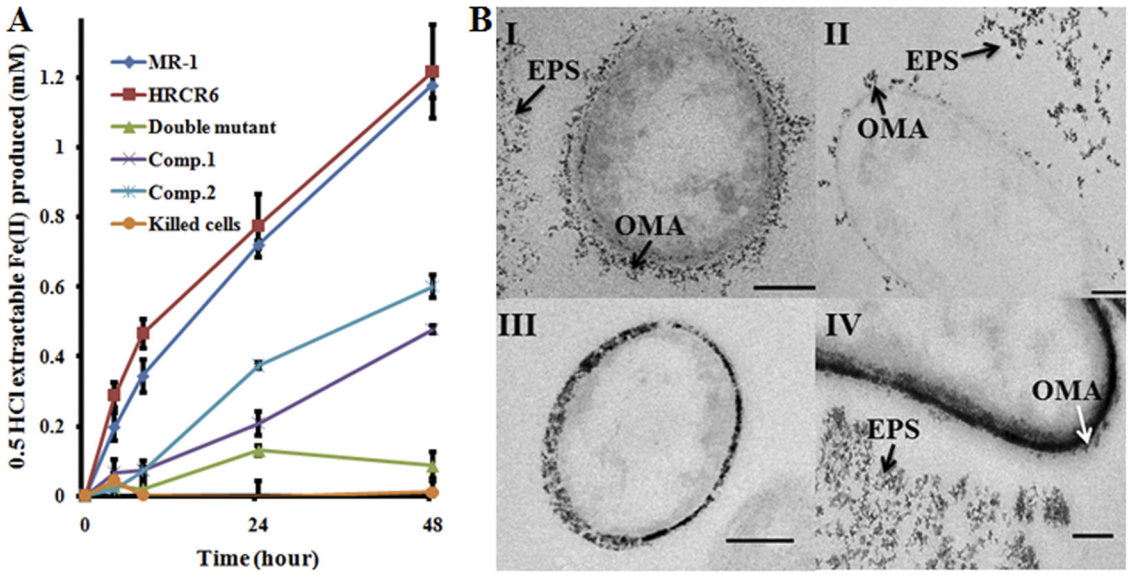

FIG. 2. Complementation of the MR-1 mutant without MtrC and OmcA by $UndA_{HRCR-6}$. (A) Ferrihydrite reduction. Kinetics of Fe(II) formation are shown after reduction of 10 mM ferrihydrite by MR-1, HRCR-6, the Δ*mtrC* Δ*omcA* double mutant with empty vector, and the double mutant complemented with $UndA_{HRCR-6}$ (Comp.1) or MtrC and OmcA (Comp.2). Heat-killed MR-1 cells were used as a negative control. The values reported are the means and standard deviations from triplicate biological measurements. (B) U(VI) reduction. Transmission electron microscopy examination of reduced UO_2 after reduction of 250 μM U(VI) for 48 h by the MR-1 wild type (I), HRCR-6 (II), the Δ*mtrC* Δ*omcA* double mutant with empty vector (III), and the double mutant complemented with $UndA_{HRCR-6}$ (IV). The outer membrane-associated (OMA) and extracellular polymeric substance (EPS)-associated UO_2 are indicated by arrows. Bar = 200 nm.

1.22 ± 0.13 mM iron [Fe(II)] (n = 3), respectively. At the same time, while the MR-1 Δ*mtrC* Δ*omcA* double deletion mutant with an empty vector produced only 0.09 ± 0.04 mM Fe(II) (n = 3), the mutant complemented with $UndA_{HRCR-6}$ (Comp.1) or MtrC and OmcA (Comp.2) produced 0.48 ± 0.01 or 0.6 ± 0.03 mM Fe(II) (n = 3), which was 40% or 50% of that produced by MR-1, respectively. Under the same conditions, little Fe(II) was produced by the heat-killed MR-1 cells (Fig. 2A). These results demonstrate that when expressed in MR-1 cells, $UndA_{HRCR-6}$ is a functional reductase of FH. All strains, plasmids, and primers used in this study are listed in Table S1 in the supplemental material.

Uranium [U(VI)] reduction was performed in 30 mM sodium bicarbonate at pH 7.4, 10 mM sodium lactate, and 250 μM uranyl acetate as the sole electron acceptor and 2×10^8 cells ml^{-1} at 30°C with shaking (25 rpm) (3). MR-1 and HRCR-6 reduced U(VI) by >90% within 48 h. Compared to that of MR-1, deletion of *mtrC-omcA* decreased MR-1's ability to reduce U(VI) by 25%. Complementation of the Δ*mtrC* Δ*omcA* mutant with $UndA_{HRCR-6}$ did not significantly increase the rate of U(VI) reduction by the mutant (data not shown) but did impact the location of the reduction end product, UO_2(s). Examination of the U cell suspensions by transmission electron microscopy revealed that in MR-1- and HRCR-6-mediated incubations, electron-dense materials were associated with the extracellular polymeric substance (EPS) and the cell envelope (Fig. 2BI and II). Previous analysis of these electron-dense particles formed under similar conditions indicated that they were UO_2 (3). In the Δ*mtrC* Δ*omcA* mutant with the empty vector, UO_2 was present mainly in the periplasm (Fig.2 BIII), likely due to transport of U(IV) across the OM, where it can be reduced by periplasmic *c*-Cyts. In comparison, in the mutant complemented with $UndA_{HRCR-6}$, UO_2 was present not only in the periplasm but also in association with the OM and EPS (Fig.2 BIV). Complementation of the Δ*mtrC* Δ*omcA* double deletion mutant with $UndA_{HRCR-6}$, thus, increases UO_2 formation on the cell surface and in the EPS. This supports the prediction that $UndA_{HRCR-6}$, like OmcA and MtrC, facilitates electron transfer to U(VI) externally to the cell and hence is an OM and extracellular protein. Given that MtrC and OmcA contribute only 25% of the total U(VI) reduction under the conditions tested and that $UndA_{HRCR-6}$ only partially complements the mutant in FH

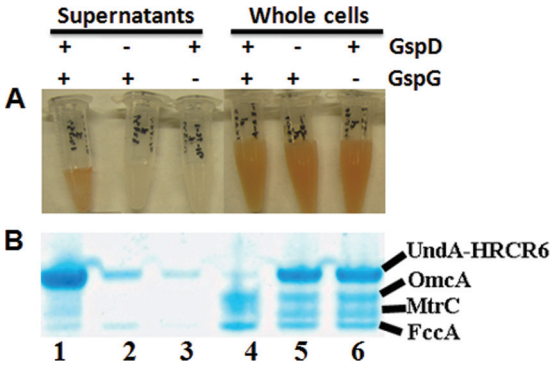

FIG. 3. Influence of the deletion of MR-1 *gspD* and *gspG* on the extracellular release of nonacylated $UndA_{HRCR-6}$. (A) Concentrated supernatants (lanes 1 to 3) and whole cells (lanes 4 to 6) of wild-type MR-1 (lanes 1 and 4), the *gspD* mutant (lanes 2 and 5), and the *gspG* mutant (lanes 3 and 6) in which nonacylated $UndA_{HRCR-6}$ was overexpressed. (B) Heme staining. A total of 1 μg of concentrated supernatant proteins (lanes 1 to 3) and 10 μg of whole-cell lysate proteins (lanes 4 to 6) from the MR-1 wild type (lanes 1 and 4), *gspD* mutant (lanes 2 and 5), and *gspG* mutant (lanes 3 and 6) were separated by SDS-PAGE and then visualized by heme staining.

TABLE 1. Kinetic rate constants for the reduction of Fe(III) complexes by $UndA_{HRCR-6}$ at 25°C

Complex	Rate constant (μM^{-1} s^{-1})[a]	
	First component	Second component
Fe(III)-citrate	0.0736 ± 0.0002 (60)	0.0134 ± 0.0001 (40)
Fe(III)-NTA	0.8078 ± 0.0180 (75)	0.3453 ± 0.0021 (25)
Fe(III)-EDTA	12.336 ± 0.365 (90)	2.5752 ± 0.0269 (10)

[a] The final concentrations were 66.7 μM for the Fe(III) complexes and 3.3 μM for $UndA_{HRCR-6}$. The numbers in parentheses represents the percent reductions.

reduction, an increase in the U(VI) reduction by addition of $UndA_{HRCR-6}$ may be too small to be detected by the U(VI) reduction assay used in this study.

Extracellular secretion of $UndA_{HRCR-6}$ by the MR-1 type II secretion system. Nonacylated $UndA_{HRCR-6}$ was constructed and was then heterologously expressed in MR-1 and in two MR-1 mutants without functional type II secretion systems (T2S) (i.e., Δ*gspD* and Δ*gspG*) under the culture condition in which the electron acceptor was limited and 20 mM lactate was used as the sole carbon and energy source (8). After it was concentrated, supernatant from the MR-1 with $UndA_{HRCR-6}$ cultures was red, while the supernatants of the T2S mutants with $UndA_{HRCR-6}$ were pale yellow (Fig. 3A). A dominant band of heme-containing protein with an apparent molecular mass of ~90 kDa was found in the supernatant of MR-1 with $UndA_{HRCR-6}$ (Fig. 3B). Western blot analysis of the same sample confirmed that this dominant heme-containing protein was $UndA_{HRCR-6}$ (data not shown). Compared to the wild type (wt), much less $UndA_{HRCR-6}$ was found in the culture supernatants of the Δ*gspD* and Δ*gspG* mutants. Conversely, more $UndA_{HRCR-6}$ was found in the whole cells of the Δ*gspD* and Δ*gspG* mutants than in the wt, demonstrating that inactivation of the MR-1 T2S decreases extracellular transport of $UndA_{HRCR-6}$ and increases its accumulation inside the cells (Fig. 3B). Thus, $UndA_{HRCR-6}$ is recognized and secreted extracellularly by the MR-1 T2S.

Purification and characterization of recombinant $UndA_{HRCR-6}$. Recombinant $UndA_{HRCR-6}$ was overexpressed in MR-1 cells. Following ultracentrifugation, $UndA_{HRCR-6}$ was solubilized from membrane fractions with 3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonic acid (CHAPS) and isolated by immobilized metal-ion affinity chromatography, followed by gel filtration chromatography (7) (see Fig. S2 in the supplemental material). Given that $UndA_{HRCR-6}$ (i) is purified from membrane fractions, (ii) possesses a putative lipid-binding site (removal of this site renders the remaining protein water soluble without using CHAPS as a solubilizing regent), and (iii) impacts the formation of UO_2 exterior to the OM, we conclude that $UndA_{HRCR-6}$ is an OM *c*-Cyt. Inductively coupled plasma (ICP) analysis of the purified protein showed that its Fe content was 10.8 μmol per μmol of protein. Results of heme staining confirm that purified $UndA_{HRCR-6}$ is a heme-containing protein (Fig. S2). Furthermore, the $UndA_{HRCR-6}$ polypeptide contains 11 heme-binding motifs. Together, these results suggest that $UndA_{HRCR-6}$ is an endecaheme (i.e., 11-heme) *c*-Cyt. Reduction kinetics of soluble Fe(III) complexed with citrate, nitrilotriacetic acid (NTA), or EDTA by the purified protein were investigated with a stopped-flow method (11). All reactions occurred in the following two stages: a fast stage that was completed in less than 1 s and a slow stage that followed the fast stage (see Fig. S3 in the supplemental material). The second-order rate constants calculated ranged from 12.336 μM^{-1} s^{-1} for the fast reduction of Fe(III)-EDTA to 0.013 μM^{-1} s^{-1} for the slow reduction of Fe(III)-citrate (Table 1). The *in vitro* rate of oxidation of fully reduced $UndA_{HRCR-6}$ by Fe(III)-EDTA was more than 1 order of magnitude higher than the oxidation of either MtrC or OmcA. Consistent with $UndA_{HRCR-6}$ being a facile reductant of complexed Fe(III), its rate of oxidation by Fe(III)-NTA was 2 to 16 times higher than those of MtrC and OmcA. Interestingly, the initial rate of oxidation of $UndA_{HRCR-6}$ by Fe(III)-citrate was 24% of that of MtrC but nearly twice as high as the oxidation of OmcA (11). The different rates among $UndA_{HRCR-6}$, MtrC, and OmcA in reducing these ferric complexes may be attributed to the structural difference among these *c*-Cyts, such as the extra heme group found in the $UndA_{HRCR-6}$ polypeptide.

Conclusions. $UndA_{HRCR-6}$, an OM endecaheme *c*-Cyt of *Shewanella* sp. HRCR-6, was identified and characterized. In MR-1, $UndA_{HRCR-6}$ partially complemented a Δ*mtrC* Δ*omcA* mutant in an FH reduction assay, increased formation of UO_2 associated with the OM and EPS in a U(VI) reduction assay, and is translocated across the OM to the extracellular environment by the T2S. Reduced $UndA_{HRCR-6}$ was also rapidly oxidized by soluble Fe(III) complexes at rates comparable to or higher than those previously reported for MtrC and OmcA. Collectively, these results show that $UndA_{HRCR-6}$ can serve as an extracellular metal reductase in MR-1 and is probably involved in extracellular reduction of Fe(III) oxides and U(VI) by *Shewanella* sp. HRCR-6.

This research was supported by the Subsurface Biogeochemical Research program (SBR)/Office of Biological and Environmental Research (BER), U.S. Department of Energy (DOE). A portion of the research was performed using EMSL, a national scientific user facility sponsored by the DOE-BER and located at Pacific Northwest National Laboratory (PNNL). PNNL is operated for the DOE by Battelle under contract DE-AC05-76RLO 1830.

REFERENCES

1. **Cao, B., et al.** 2011. Extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms: characterization by infrared spectroscopy and proteomics. Environ. Microbiol. **13:**1018–1031.
2. **Fredrickson, J. K., et al.** 2008. Towards environmental systems biology of *Shewanella*. Nat. Rev. Microbiol. **6:**592–603.
3. **Marshall, M. J., et al.** 2006. c-Type cytochrome-dependent formation of U(IV) nanoparticles by *Shewanella oneidensis*. PLoS Biol. **4:**e268.
4. **Reardon, C. L., et al.** 2010. Role of outer-membrane cytochromes MtrC and OmcA in the biomineralization of ferrihydrite by *Shewanella oneidensis* MR-1. Geobiology **8:**56–68.
5. **Shi, L., and W. W. Carmichael.** 1997. *pp1-cyano2*, a protein serine/threonine phosphatase 1 gene from the cyanobacterium *Microcystis aeruginosa* UTEX 2063. Arch. Microbiol. **168:**528–531.
6. **Shi, L., W. W. Carmichael, and P. J. Kennelly.** 1999. Cyanobacterial PPP family protein phosphatases possess multifunctional capabilities and are resistant to microcystin-LR. J. Biol. Chem. **274:**10039–10046.
7. **Shi, L., et al.** 2006. Isolation of a high-affinity functional protein complex between OmcA and MtrC: two outer membrane decaheme c-type cytochromes of *Shewanella oneidensis* MR-1. J. Bacteriol. **188:**4705–4714.
8. **Shi, L., et al.** 2008. Direct involvement of type II secretion system in extracellular translocation of *Shewanella oneidensis* outer membrane cytochromes MtrC and OmcA. J. Bacteriol. **190:**5512–5516.
9. **Shi, L., and W. Zhang.** 2004. Comparative analysis of eukaryotic-type protein phosphatases in two streptomycete genomes. Microbiology **150:**2247–2256.
10. **Turse, J. E., M. J. Marshall, J. K. Fredrickson, M. S. Lipton, and S. J. Callister.** 2010. An empirical strategy for characterizing bacterial proteomes across species in the absence of genomic sequences. PLoS One **5:**e13968.
11. **Wang, Z., et al.** 2008. Reduction kinetics of Fe(III) complexes by outer membrane cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1. Appl. Environ. Microbiol. **74:**6746–6755.