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Young, Hwa; Zhang, Yan; Cheon Lee, Byung; Kim, Jae-Ryong; and Gladyshev, Vadim N., "The selenoproteome of *Clostridium* sp. OhILAs: Characterization of anaerobic bacterial selenoprotein methionine sulfoxide reductase A" (2009). *Vadim Gladyshev Publications*. 85.

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The selenoproteome of *Clostridium* sp. OhILAs: Characterization of anaerobic bacterial selenoprotein methionine sulfoxide reductase A

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Abstract

Selenocysteine (Sec) is incorporated into proteins in response to UGA codons. This residue is frequently found at the catalytic sites of oxidoreductases. In this study, we characterized the selenoproteome of an anaerobic bacterium, *Clostridium* sp. (also known as *Alkaliphilus oremlandii*) OhILA, and identified 13 selenoprotein genes, five of which have not been previously described. One of the detected selenoproteins was methionine sulfoxide reductase A (MsrA), an antioxidant enzyme that repairs oxidatively damaged methionines in a stereospecific manner. To date, little is known about MsrA from anaerobes. We characterized this selenoprotein MsrA which had a single Sec residue at the catalytic site but no cysteine (Cys) residues in the protein sequence. Its SECIS (Sec insertion sequence) element did not resemble those in *Escherichia coli*. Although with low translational efficiency, the expression of the *Clostridium* selenoprotein *msrA* gene in *E. coli* could be demonstrated by ⁷⁵Se metabolic labeling, immunoblot analyses, and enzyme assays, indicating that its SECIS element was recognized by the *E. coli* Sec insertion machinery. We found that the Sec-containing MsrA exhibited at least a 20-fold higher activity than its Cys mutant form, indicating a critical role of Sec in the catalytic activity of the enzyme. Furthermore, our data revealed that the *Clostridium* MsrA was inefficiently reducible by thioredoxin, which is a typical reducing agent for MsrA, suggesting the use of alternative electron donors in this anaerobic bacterium that directly act on the selenenic acid intermediate and do not require resolving Cys residues.

Keywords: MsrA, selenoproteins, oxidoreductase, SECIS elements, thioredoxin

Abbreviations: DTT, dithiothreitol; Msr, methionine sulfoxide reductase; Sec, selenocysteine; SECIS, selenocysteine insertion sequence; Trx, thioredoxin.

INTRODUCTION

Selenocysteine (Sec) is inserted into proteins when UGA (normally a stop codon) is recoded to serve as Sec codon.[1,2] This amino acid is typically found at the catalytic sites of oxidoreductase selenoproteins and is responsible for the high catalytic activity of selenoenzymes when compared with their cysteine (Cys)-containing homologs.[3-6] Selenoproteins are found in all three domains of life: bacteria, archaea, and eukaryotes. The glycine reductase A from *Clostridium sticklandii* was discovered as the first anaerobic selenoprotein.[7,8] A complex machinery is required for translational insertion of Sec in response to UGA codon.[2,9,10] A stem-loop structure in selenoprotein mRNAs, called SECIS (Sec insertion sequence) element, is essential for Sec insertion in both prokaryotes and eukaryotes. SECIS elements are located

immediately downstream of Sec-encoding UGA codons in bacteria, whereas they are present in the 3'-untranslational regions in archaea and eukaryotes. In addition to this *cis*-acting factor, several *trans*-acting factors, such as Sec-specific elongation factor and tRNA^{Sec}, are required for Sec incorporation.

There has been significant progress recently in identifying selenoprotein genes in organisms from bacteria to mammals owing to both a dramatic increase in genomic sequence information and the development of bioinformatics methods that allow searches for selenoprotein genes.[11-16] These methods include searches for (1) SECIS elements; and (2) Sec/Cys pairs in predicted homologs.

Methionine residues are easily oxidized by various reactive oxygen species to methionine sulfoxides; however, the oxidized methionines can be reduced back to methio-

nine by repair enzymes, the methionine sulfoxide reductases (Msrs).[17] Two distinct families of these enzymes, MsrA and MsrB, have evolved for stereospecific reduction of methionine sulfoxide residues in proteins. MsrA is specific for the reduction of the *S*-form of methionine sulfoxide, whereas MsrB is specific for the *R*-form. Msrs are present in most organisms in all three domains of life (they have been even found in anaerobic bacteria), but are absent in some hyperthermophiles and parasites.[18,19] Msrs play a pivotal role in the repair of oxidatively damaged proteins and defend cells from oxidative stress. Therefore, these proteins are viewed as protein repair and antioxidant enzymes.[20-22] These enzymes have been implicated in regulation of physiological and pathological processes such as aging and neurodegenerative diseases.[23,24]

In this study, we characterized the selenoproteome of an anaerobic gram-positive bacterium, *Clostridium sp.* (also known as *Alkaliphilus oremlandii*) OhILAs and found that this bacterium is predicted to be rich in selenoproteins and contains at least 13 selenoproteins, including MsrA. We further experimentally characterized *Clostridium* selenoprotein MsrA. We found that its SECIS element is recognized by the *Escherichia coli* Sec insertion machinery, and that Sec plays a critical role in the catalytic activity of this selenoprotein.

METHODS

Identification of selenoprotein genes in *Clostridium sp.* OhILAs

The complete genome of *Clostridium sp.* OhILAs (NC_009922) was downloaded from the NCBI ftp server (<ftp://ftp.ncbi.nih.gov/genomes/Bacteria/>). We used bSECISearch program developed previously[15] to search this genome for all candidate selenoprotein sequences containing in-frame UGA codons and bacterial SECIS (bSECIS) elements. The resulting dataset was then divided into homologs of previously known selenoproteins and selenoprotein candidates. All candidates were manually analyzed for the location of the UGA codon, occurrence of Sec- and Cys-containing homologs in Sec-utilizing or other organisms, and presence of SECIS elements in Sec-containing homologs. In parallel, independent BLAST homology searches for Sec-containing homologs of all known selenoprotein families were performed using default parameters. Finally, both known and candidate selenoprotein sets were generated. Multiple sequence alignments were performed using ClustalW program[25] with default parameters and visualized with BoxShade program v3.21.

Cloning, expression, and purification of wild-type selenoprotein and its Cys mutant of *Clostridium MsrA*

A coding region of the selenoprotein *msrA* gene was amplified by PCR using *Clostridium sp.* OhILAs genomic

DNA (kindly provided from Dr. John Stolz, Duquesne University, Pittsburgh, USA) and cloned into *NdeI/XhoI* sites of pET21b (Novagen). To amplify the gene, forward (5'-GCGCCATATGGATACCAATCAGAAGTTG-3') and reverse (5'-CGCGCTCGAGTTAATTTCTTCGACGATAG-3') primers were used. The resulting construct, designated pET-CLOS-MsrA, coded for full-length selenoprotein MsrA with a C-terminal His-tag (LEHHH-HHH). A SECIS element was found to be located immediately downstream of the Sec UGA codon (see Figure 4). A Cys mutant form was also generated in which Sec16 was replaced with Cys by site-directed mutagenesis. The resulting construct was named pET-CLOS-MsrA/U16C.

To express selenoprotein MsrA in *E. coli*, the plasmid pET-CLOS-MsrA was transformed into BL21(DE3) cells that also harbored a plasmid pSUABC,[26] which encodes *E. coli selA, selB, and selC*. The transformed cells were grown in LB media containing 4 μ M sodium selenite, 100 μ g/mL ampicillin, and 25 μ g/mL chloramphenicol at 37°C with shaking until optical density at 600 nm reached 0.6-0.8; then 0.2 mM IPTG was added and the cells were further cultured for 4 h at 30°C, harvested, and stored at -20°C until use. A typical expression level of the selenoprotein MsrA was 0.6 μ g/mg crude protein.

The cell pellets were resuspended in the extraction buffer (50 mM sodium phosphate, pH 7.0, 300 mM NaCl, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. The selenoprotein MsrA was further purified using a Talon-metal affinity resin (Clontech) according to the manufacturer's protocol. The eluted proteins were dialyzed against 50 mM sodium phosphate, pH 7.5, and 50 mM NaCl. Typical yield of the purified selenoprotein MsrA was 3-5 μ g from 250 mL culture broth, as estimated by Western blot assays. Although the protein was not pure, this preparation was sufficient to determine kinetic parameters of the enzyme.

To express the Cys mutant form (U16C), BL21(DE3) cells transformed with pET-CLOS-MsrA/U16C were cultivated in LB media containing 100 μ g/mL ampicillin at 37°C with shaking until optical density at 600 nm reached 0.6-0.8, 0.1 mM IPTG was added, and the cells were further cultured for 6 h at 30°C. The cells were harvested and stored at -20°C until use. Procedures for purification of this protein were as described earlier for the selenoprotein form. The purity of the Cys mutant protein was analyzed by SDS-PAGE and found to exceed 95%.

Metabolic labeling with ⁷⁵Se

To prepare cells expressing *Clostridium* selenoprotein MsrA with metabolically labeled ⁷⁵Se, *E. coli* BL21(DE3) cells were transformed with pET-CLOS-MsrA and grown at 37°C in 5 mL LB media containing ampicillin until optical density at 600 nm reached ~0.6. Then, 0.05 mCi of freshly neutralized [⁷⁵Se]selenous acid (specific activity 1000 Ci/mmol, University of Missouri Research Reactor,

Table I. Known and New Selenoproteins Detected in *Clostridium* sp. OhILAs

Protein family	Accession no.	COG/Pfam	Occurrence	Sec position (protein length)
Known selenoproteins				
FdhA ^a	YP_001513778	COG3383	1	349 (891)
SelD ^a	YP_001513274	COG0709	1	16 (346)
MsrA ^a	YP_001513481	COG0225	1	16 (209)
Proline reductase ^a	YP_001511596	pfam07355 (low homology)	1	151 (241)
HesB-like	YP_001511838 (partial sequence)	COG0316	1	32 (95)
Glutaredoxin ^a	YP_001513661	COG0695	1	13 (76)
GrdA ^a	YP_001512500	pfam04723	1	44 (158)
GrdB ^a	YP_001512501	pfam07355	1	350 (435)
New selenoprotein candidates				
Putative anaerobic dehydrogenase ^a	YP_001511765	COG0243	1	257 (1021)
BFD-like (2Fe-2S)-binding domain protein	YP_001512630 (partial sequence)	COG2906 (low homology)	1	11 (162)
Split soret cytochrome c precursor ^a	YP_001512574; YP_001512575	pfam09719 (low homology)	2	83 (223)
				81 (221)
Predicted NADH:ubiquinone oxidoreductase, subunit RnfC	YP_001511593 (partial sequence)	COG4656	1	389 (424)

^a Annotated as selenoproteins in the current genome annotation.

Columbia, USA) was added to the cell culture with 1 mM IPTG for induction. The cells were further grown at 37°C for 5 h, harvested, washed with PBS buffer, and lysed. Cell extracts (40 µg of total protein) were applied to a 10% Bis-Tris gel (Invitrogen), electrophoresed, and transferred onto a PVDF membrane. The ⁷⁵Se radioactivity pattern on the membrane was visualized by using a PhosphorImager (GE Health Care).

Determination of protein concentration

Concentration of recombinant selenoprotein MsrA was determined by western blotting using His antibodies and Cys mutant as an internal standard, followed by quantifying the blot signals with a densitometer. Concentration of purified Cys mutant was determined by Bradford method using a Bio-Rad protein assay reagent (Bio-Rad) and bovine serum albumin as a standard.

MsrA enzyme assay and analysis of kinetics

MsrA activity was measured in the presence of dithiothreitol (DTT) or thioredoxin (Trx) as a reducing agent. In the DTT-dependent reaction, reaction mixture (100 µL) contained 50 mM sodium phosphate, pH 7.5, 50 mM NaCl, 20 mM DTT, 200 µM DABSyl-methionine-S-sulfoxide, and purified (or crude) proteins. In the Trx-dependent reaction, the reaction mixture (100 µL) contained 50 mM sodium phosphate, pH 7.5, 50 mM NaCl, 0.2 mM NADPH, 6.8 µM *E. coli* Trx (Sigma) or human Trx1,[27] 0.8 µM human Trx reductase 1,[27] 200 µM DABSyl-methionine-S-sulfoxide, and purified proteins. The reactions were carried out at 37°C for 30 min and analyzed by HPLC as described previously.[28] A full-length mouse MsrA was purified from *E. coli* as described previously[29] and used for comparison.

K_m and k_{cat} values were determined from Lineweaver-Burk plots using 50-400 µM substrate in the DTT- dependent reaction.

RESULTS AND DISCUSSION

Clostridium sp. OhILAs is a selenoprotein-rich organism

By searching the *Clostridium* sp. OhILAs genome against a set of all known selenoprotein genes, we identified eight selenoproteins in *Clostridium* sp. OhILAs, including formate dehydrogenase alpha subunit (FdhA), which is the most widespread prokaryotic selenoprotein that appears to be responsible for maintaining the Sec utilization trait in sequenced bacteria,[30] and selenophosphate synthetase (SelD), another widespread selenoprotein which is a key component in prokaryotic selenoprotein biosynthesis. Other detected selenoproteins included proline reductase, HesB-like protein, glutaredoxin, glycine reductase selenoproteins A (GrdA) and B (GrdB), and MsrA. Previously, selenoprotein MsrA has only been characterized in a eukaryotic organism, *Chlamydomonas reinhardtii*. [31][32] The selenoproteome of *Clostridium* sp. OhILAs is shown in Table I and all detected selenoprotein sequences are included in the supporting sequence data.

Next, we used bSECISearch to analyze *Clostridium* sp. OhILAs for full complement of selenoprotein genes. In addition to the eight homologs of known selenoproteins, we detected five new selenoproteins that represented four protein families (Table I). The mRNAs of all new selenoproteins contained predicted stable stem-loop structures downstream of Sec-encoding UGA codons that shared common features of bacterial SECIS elements,[15] i.e. a stable stem with a small apical loop containing a G nucleotide located at a certain distance from the Sec UGA co-

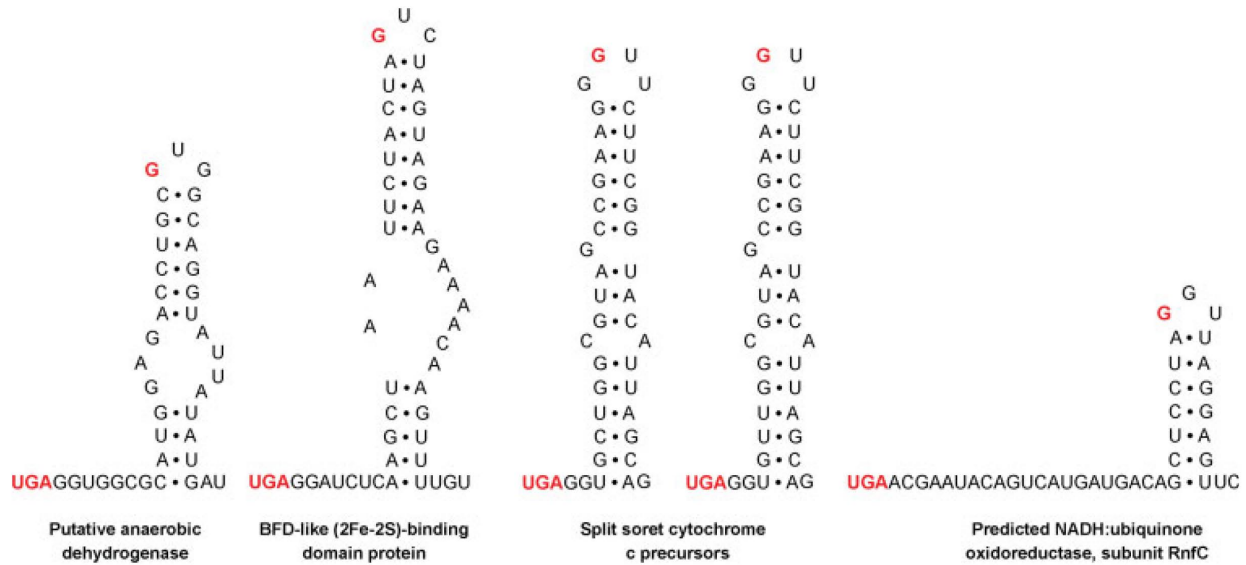


Figure 1. Predicted bacterial SECIS elements in new selenoprotein genes. Only sequences downstream of in-frame UGA codons are shown. In-frame UGA codons and functionally important G nucleotides in the apical loop are shown in red.

don (see Figure 1). No additional common features could be found in those *Clostridium* SECIS elements and all of them were predicted to lack a bulged U which is conserved in *E. coli* SECIS elements. Selenoprotein homologs of the new selenoproteins were also found in several other anaerobic bacteria, such as *Desulfitobacterium hafnicense*, *Alkaliphilus metalliredigens*, and *Clostridium botulinum* (see Figure 2). In addition, conserved Cys-containing homologs could be detected for all new selenoproteins in other organisms. Multiple alignments of new selenoprotein families and their Cys homologs are shown in Figure 2 and highlight conserved Sec/Cys pairs in homologous sequences that support predicted redox functions of these proteins.[33]

Although the functions of new selenoproteins are not clear, they either contained a domain of known function or were homologous to protein families with known functions. However, in some of them Sec was not part of the detected domains, suggesting the presence of functional redox-active sites in the locations outside of known domains. For example, Sec was located in the N-terminal region of BFD-like (2Fe-2S)-binding protein in which the BFD-like domain (COG2906) was present in the C-terminal region. It was also found in a CxxU (x, any amino acid residue; U, Sec) redox motif in the C-terminal region of a predicted NADH:ubiquinone oxidoreductase subunit RnfC, whereas the known RnfC domain (COG4656) was located in the N-terminal region. The other three new selenoproteins (putative anaerobic dehydrogenase and two cytochrome c precursor sequences) were annotated as putative selenoproteins in this genome although the criteria

used in their detection/annotation are not clear. As these sequences have passed the stringent criteria employed by bSECISearch in our study, they should be viewed as excellent candidate selenoproteins.

While this study was prepared for publication, the sequences of three new selenoproteins were released at GenBank database (putative anaerobic dehydrogenase, 158139457; split soret cytochrome c precursor 1, 158320067; split soret cytochrome c precursor 2, 158320068). It is interesting that the Sec positions differed in the predicted NADH:ubiquinone oxidoreductase subunit RnfC family between *Clostridium* sp. OhILAs and several strains of *Clostridium botulinum* (see Figure 2). Taken together, our data suggested that novel Se-dependent pathways are present in this selenoprotein-rich organism. Further experimental verification is needed for the newly identified selenoproteins.

***Clostridium* selenoprotein MsrA sequence**

Msr genes have been found in most organisms, even in anaerobes. The presence of Msr genes in anaerobic bacteria may be due to their roles in protein repair and antioxidant defense when transiently exposed to oxygen. To date, little is known about such anaerobe-based Msrs. We selected the anaerobic bacterial selenoprotein MsrA for further experimental examination. Interestingly, sequence context revealed lack of any Cys residues in this protein. Because Sec corresponded to the catalytic residue, this observation suggested the lack of resolving residues during catalysis (see Figure 3). The catalytic mechanism of

A. Putative anaerobic dehydrogenase

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Clostridium sp. OhILAs      233 RINFAQREVLNSEFGTINFYCHITSTUGGANRP-AWCVLYENGAGEVYKPDLDQDAEFCILYCG
Desulfitobacterium hafniense 230 RTAFAGRFAG-AFGTENNESHGYTUGGSKIP-AGCVLSS---GQGRPTVNHAEFTLYIG
Shewanella baltica        237 RDAITKRRTFNSFGTRNFANHGAYCGNTFRAGAGALLDILKRYAHLKPDWHSEFTLFIG
Aeromonas hydrophila      236 RDHFIKRRTFNSFGTRNFANHGAYCGLSFRWAGAGALLDLEKNAHLKPDWDESFTLFIG
Photobacterium profundum  234 RDDILKRFAPNSEGTRNFGHGSYCGYAFRAGSGALMNDLQKFSHWKPDLDNTEBFLFIG
Vibrio alginolyticus      234 RDITIKRFAPNSYGRNFGHRSYCGYAFRAGSGAFMNDLQKNAHLKPDWNAEYTIIFIG

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B. BFD-like (2Fe-2S)-binding domain protein

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Clostridium sp. OhILAs      1 MSENIMNENCUSQANSTSLVBEENKFCPTCNREGIQVRGITVKHVMVLENLPOVEDDNY
Alkaliphilus metalliredigens 1 MRENKINKNCUGSQIDSTGLVEEKNLCPICNKEGKVKRITLTKHVMVIGSLLETVLDDY
Alkaliphilus metalliredigens 1 MRENKINKNCUGSQMDSTGLVEEKKLCRNCNFBGTVKVTITVKHVMVIGSLLETVLDDY
Campylobacter hominis      1 --MKINNECCGCKTEKPDQIKDN--CPVCNREGISVSKVTVVHVVDDYRNANVNGDQYK
Thermoanaerobacter ethanolicus 1 MGKETLSNYCCCNLGESSCEVEKNNFCPVCEKGGTLVKNITVKHVMVLELITGLDNY
Clostridium phytofermentans 1 MEKETANKCCGSSVCSGSCQTQNNLCSVCGKGGTLVKNITVKHVMVLELITGLDNY

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C. Split soret cytochrome c precursor

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Clostridium sp. OhILAs      51 EFDKFOQPFKVKIDPAKAEARAYTGYKEKGGUGAGVAEEFFGILADE---AGYPFNQIF
Clostridium sp. OhILAs      49 EFDKEQPFKVKVLDPAKVEERALKGKKEKGGUGVGVAAEFPGLADE---VGYFPNQIF
Clostridium sp. OhILAs      61 ERDAPSNPYAKKLDVNTAIEERGHAGYNNKGGCCRGVADAITGQLDSD---AGYPFNQIF
Desulfitobacterium hafniense 12 HCAPESPPFYVQLDEBKAKLDEYAGYG-EKHSYGAFAEAVGQLREK---VGYFTHVF
Anaeromyxobacter dehalogenans 39 GKGLQATPWPAPLDEDATAERAFKGYL-ECHVMYGAFAEAVGQVADK---VGFPTSFV
Shewanella sediminis      44 GALLGANLLAVVELDFEVARAYEYV-EGCOMYGFNAVWKELEAQQHEDENCAATE

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D. Predicted NADH:ubiquinone oxidoreductase, subunit RnfC

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Clostridium sp. OhILAs      326 ERLKRTAAGME-AEVTAEVQCKRRTAFNG----RYRCDLPGVCPGQAEKVFLLKKQGAHV
Alkaliphilus metalliredigens 329 ERLREIANAMG-AEVTAEKCKRVEITNG----RYRCDRPGVCPGQAEKTLSPKKNQIQV
Clostridium botulinum       332 ERLKRTATKMN-AQVAGVVECKNLEYPKKGNGPCKCKTPGEPUGQAAAVMQLKKGDAER
Clostridium botulinum       332 ERLKRTATKMN-AKVAAGVVECKNLEYPKKGNGPCKCKTPGEPUGQAAAVMQLKKGDAER
Clostridium botulinum       332 ERLKRTATKMN-AEVTAEVQCKRVEITNG----ANCKTPGDCPGQVAGVMLKKGDAER
Clostridium botulinum       332 ERLKDIASKMR-AEVTAEVQCKRVEVKG----TYRCKTPGKCPGQAGAVMYLKSFGAER
Clostridium difficile        330 ERMRTATKMGVTDVSVQCKCKACETKG----ALKCENPGHCPGQAKQCEFKKFGAHV
Dorea longicatena           360 ERMREIASKNN-AKVVSQCKCKAIEENKPG--APLRCLEPGNCPGQVKNNDQFKKIKCEY

Clostridium sp. OhILAs      381 ILIGDCEDUNTVMMTAPRLGVVYHSTDHVLKSGSMHKLKPKKQ-----
Alkaliphilus metalliredigens 384 ILIGDCEDUNTVMMTAPRLGVVYHSTDHVLKSGSMHKLKPKKQ-----
Clostridium botulinum       391 ILISNCSDCSNTVMQAPRAKIPVYHHTDHFRTIDYTLTR-----
Clostridium botulinum       391 ILISNCSDCSNTVMQAPRAKIPVYHHTDHFRTIDYTLTR-----
Clostridium botulinum       387 ILISNCSDCSNTVMCGAFNIGIPVYHHTDHFRTIDHILTRRIPIDK-----
Clostridium botulinum       387 ILISNCSDCSNTVMQAPRAKIPVYHHTDHFRTIDYKLRRLPKEKHLK-----
Clostridium difficile        386 ILIGNCSDCSNTVMGSAFKLGGVYHITDHFVRTNHPILRRIKGDVRLKIK---
Dorea longicatena           417 ILIGNCSDCSNTVMASEPKMLKVFHDTDHFVRTNHPILRRSLKVSKEVSQEIFD

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Figure 2. Multiple sequence alignments of new selenoproteins and their Cys homologs. The alignments show Sec-flanking regions in detected selenoproteins. Both selenoprotein sequences detected in *Clostridium* sp. OhILAs and their Sec/Cys-containing homologs that occur in the indicated organisms are shown. Conserved residues are highlighted. Predicted Sec (U) and the corresponding Cys (C) residues in other homologs are shown in red and blue, respectively.

Cys-containing MsrA is well characterized and involves a sulfenic acid intermediate.[34-36] During the reaction, the catalytic Cys attacks the sulfoxide moiety of methionine sulfoxide resulting in sulfenic acid on this residue with a concomitant release of the product, methionine. Then, the sulfenic acid intermediate is rearranged into a disulfide bond by reacting with another Cys, known as resolving (or recycling) Cys, and finally the disulfide bond is reduced by a two-electron reductant. Trx is typically an *in vivo* reducing agent while DTT can be used in the *in vitro* assays. In the case of Sec-containing forms, the selenenic

acid intermediate may form a selenenylsulfide bond with a resolving Cys; however, neither of the previously identified selenoprotein MsrAs possesses conserved Cys that could function as resolving residues.[31] Furthermore, we previously reported that *Chlamydomonas* selenoprotein MsrA does not use resolving Cys.[31]

Expression of *Clostridium* selenoprotein MsrA in *E. coli*

The mechanism of Sec insertion has been well elucidated in *E. coli*. [3,37-39] The SECIS element in mRNA is essential for Sec insertion and is located immediately

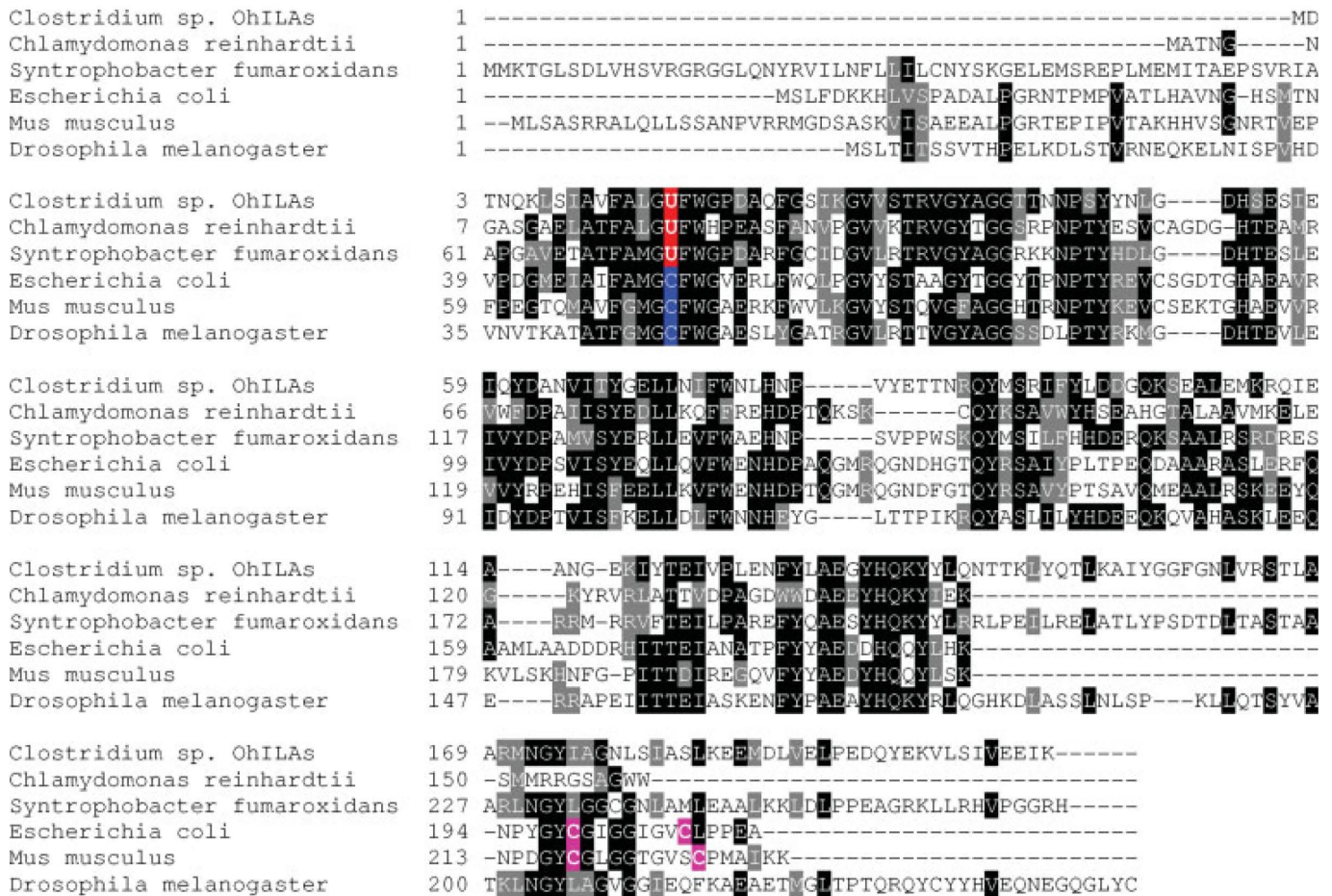


Figure 3. Multiple sequence alignment of MsrAs. Sec (U) residues are highlighted in red in the catalytic GUFW(G/H) motif and the corresponding Cys residues are highlighted in blue. The known resolving Cys residues in the C-terminal region are highlighted in pink. Genbank accession numbers are as follows: *Clostridium* sp. OhILAs, 106894182; *Chlamydomonas reinhardtii*, 23452038; *Syntrophobacter fumaroxidans*, 71547479; *E. coli*, 1790665; *Mus musculus*, 31981013; *Drosophila melanogaster*, 23338220.

downstream of in-frame UGA codons in bacteria. Studies on *E. coli* SECIS elements revealed that only a minimal stem-loop is required for Sec insertion in which a GU sequence on the tip of the apical loop and a bulged U present at the bottom of the stem are essential for Sec insertion (Figure 4, left).[40-42] We recently reported a consensus bacterial SECIS structural model in which a conserved G is present in the apical loop, whereas the bulged U is an *E. coli* adaptation that is missing in most other bacterial SECIS elements.[15] Consistent with this consensus, the SECIS element of *Clostridium msaA* had a G in the apical loop, but not the bulged U in the stem (Figure 4, right). The differences between *E. coli* SECIS elements and the corresponding structures in other bacteria result in barriers in heterologous selenoprotein expression.[43]

To test whether *Clostridium* selenoprotein MsrA can be expressed in *E. coli* as recombinant protein, we metabolically labeled cells transformed with *Clostridium msaA* gene with ^{75}Se . As shown in Figure 5(A), a 25 kDa radioactive band corresponding in size to the calculated mo-

lecular weight of recombinant *Clostridium* selenoprotein MsrA, was detected. We also verified the expression of this protein by western blot analysis [Figure 5(B)]. In addition, enzyme assays revealed that crude extracts from cells expressing the *Clostridium msaA* gene had a 36-fold increased activity compared with that from cells containing an empty vector [Figure 5(C)]. Thus, these data showed that *Clostridium* selenoprotein *msaA* could be expressed in *E. coli*, indicating that its SECIS element is recognized by the *E. coli* Sec insertion machinery. To estimate the efficiency in Sec insertion (UGA readthrough), we analyzed the amounts of wild-type and Cys mutant (U16C) forms of the enzyme in crude extracts by Western blotting using the purified U16C as an internal standard. It should be noted that most of U16C protein (>95%) expressed in BL21(DE3) cells was present in the soluble fraction. The yields of the wild-type selenoprotein and the U16C mutant were 0.6 and 380 $\mu\text{g}/\text{mg}$ crude protein, respectively. Therefore, the translational efficiency of the selenoprotein MsrA was low, estimated to be 0.15% of that of its Cys

variant. The activities of the wild-type selenoprotein and Cys mutant forms in the crude extracts as shown in Figure 5(C) agreed well with those of the purified forms, respectively (Table II).

There have been several reports on heterologous expression of eukaryotic selenoproteins in *E. coli*. [6,26,44,45] Either the entire SECIS element of *E. coli fdhH* (in most studies) or a minimal SECIS stem-loop had to be introduced downstream of UGA codon to express eukaryotic selenoproteins. In *E. coli*, heterologous expression of bacterial selenoproteins that contain their own SECIS elements often fails due to SECIS structure barriers. [46] However, a few successful cases have been previously reported. [47,48] In our study, irrespective of discrepancies in *Clostridium msrA* and *E. coli* SECIS elements, the *Clostridium* selenoprotein *msrA* gene could be expressed in *E. coli*. However, as discussed earlier, the translational efficiency was low (less than 0.2% compared with its Cys variant) even when the cells were co-expressed with *selA*, *selB*, and *selC* genes encoding selenocysteine synthase, SelB and tRNA^{Sec}, respectively. These results suggest a low functionality of the SECIS element of *Clostridium msrA* in the *E. coli* Sec insertion system.

Characterization of *Clostridium* selenoprotein MsrA

To investigate catalytic properties of *Clostridium* MsrA, we purified this protein (tagged at the C-terminus with His) from *E. coli* cells. As shown in Table II, the specific

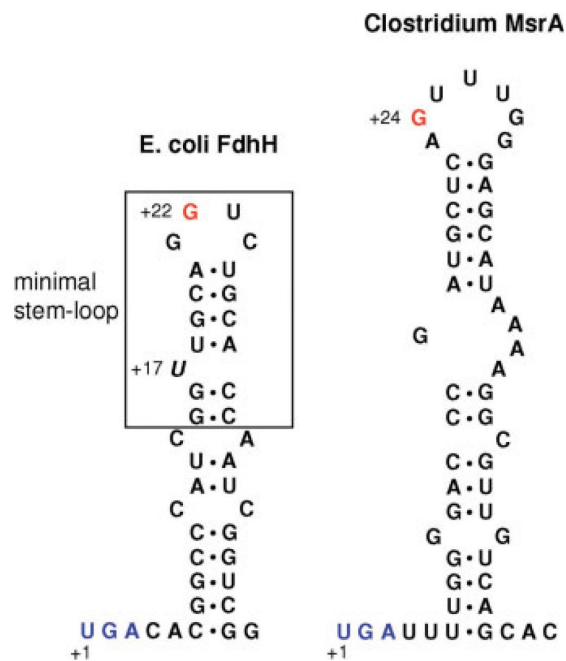


Figure 4. Structures of SECIS elements in *E. coli fdhH* (left) and *Clostridium* sp. OhILAs *msrA* (right). In the *E. coli fdhH* SECIS element, the minimal stem-loop structure is boxed and the bulged U is shown in italics. The *Clostridium msrA* SECIS element lacks the bulged U. Conserved G in the apical loops is shown in red in both SECIS elements. Sec UGA codons are shown in blue.

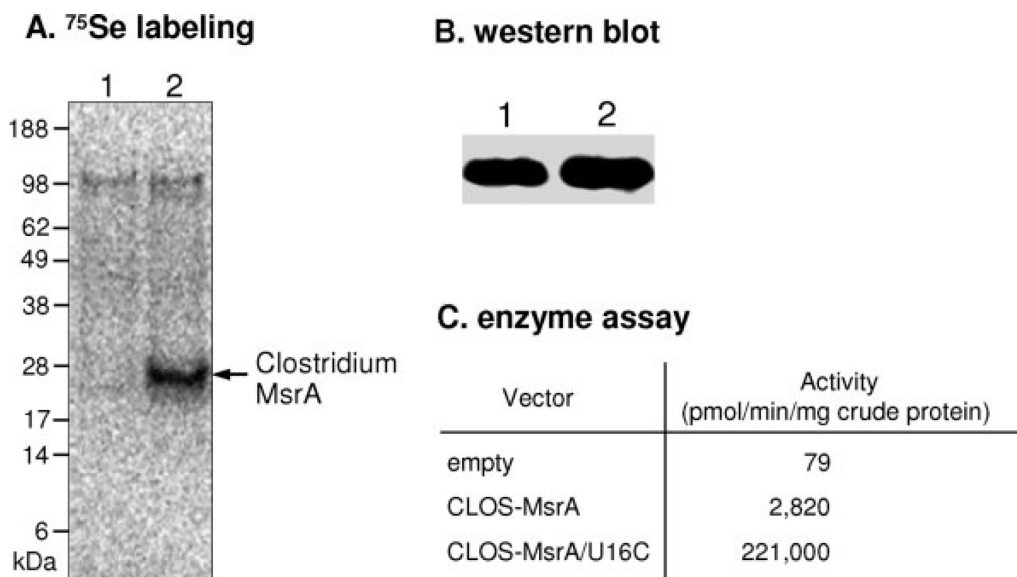


Figure 5. Expression of *Clostridium* selenoprotein MsrA in *E. coli*. (A) ⁷⁵Se metabolic labeling. BL21(DE3) cells transformed with an empty vector (lane 1) or pET-CLOS-MsrA (lane 2) were metabolically labeled with ⁷⁵Se. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane, and visualized with a PhosphorImager. (B) Western blot analysis. Purified Cys mutant (U16C) and crude extracts from cells expressing *Clostridium* selenoprotein MsrA were analyzed using anti-His antibodies. Lane 1, purified U16C; lane 2, wild-type selenoprotein MsrA from cell extracts. (C) Enzyme assays. Crude extracts from cells containing an empty vector, pET-CLOS-MsrA (wild-type), or pET-CLOS-MsrA/U16C (U16C mutant) were prepared, respectively. A total of 200 μg crude protein from control and CLOS-MsrA extracts and 2 μg crude protein of the CLOS-MsrA/U16C extract were used in a DTT-dependent assay in the presence of 200 μM DABSyl-methionine-S-sulfoxide

Table II. Specific Activity and Kinetic Constants of Clostridium MsrA Forms

MsrA form	Specific activity [nmol/(min mg protein)]	K _m (mM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (M ⁻¹ s ⁻¹)
WT	4185 ± 46	10.2 ± 1.2	84 ± 5	8235
U16C	464 ± 14	4.0 ± 0.2	3.8 ± 0.3	950

Purified proteins (90 ng WT and 1 µg U16C) were assayed in the DTT-dependent reaction. The substrate (DABSyl-methionine-S-sulfoxide) concentration used was 200 µM for the determination of specific activity. K_m and k_{cat} values were determined from Lineweaver-Burk plots using 50-400 µM substrate in the presence of DTT. WT, wild-type selenoprotein; U16C, Sec to Cys mutant.

activity of *Clostridium* selenoprotein MsrA was found to be 4185 nmol/(min mg protein) in a DTT-dependent reaction. This value was 9-fold higher than that of its Cys mutant (U16C). The k_{cat} value of the selenoprotein MsrA was 22-fold higher than that of its Cys mutant and the K_m value of the selenoprotein was 2.5-fold higher. Overall, these data show that selenium provides catalytic advantage to *Clostridium* MsrA. Importantly, this enzyme appears to be a highly efficient catalyst with kinetic properties comparable with those of *Chlamydomonas* MsrA characterized previously.[31]

Trx is typically a natural reductant for MsrA. To test if Trx can function as a reductant for *Clostridium* MsrA, we measured Trx-dependent activities of its natural Sec-containing and mutant Cys-containing forms using human Trx1 and Trx reductase 1, and compared the data with the DTT-dependent activities of these MsrA forms. In addition, mouse MsrA served as control because this protein is known as a target of Trx. As shown in Table III, the ratio of Trx- to DTT-dependent activities of mouse MsrA was 0.73, indicating that human Trx1 as well as DTT could efficiently reduce this protein. However, for *Clostridium* selenoprotein MsrA, this ratio was less than 0.01, suggesting that Trx1 was a poor reductant for this selenoprotein. Similarly, the Cys mutant could not be efficiently reduced by Trx1 (the ratio of Trx- to DTT-dependent activity was 0.03). To examine the possibility that human Trx couples poorly to the bacterial selenoprotein MsrA, we carried out the enzyme assay in the presence of bacterial (*E. coli*) Trx. The ratios of Trx- to DTT-dependent activities were consistent with the results for human Trx1; 0.93 for mouse MsrA, 0.016 for *Clostridium* selenoprotein MsrA, and 0.05 for its Cys mutant. Therefore, our data suggested that Trx is not a reducing agent *in vivo* for *Clostridium* MsrA and that unknown physiological reductant(s) must function in the reduction of this enzyme.

Recent studies revealed that thionein (a low-molecular-mass Cys-rich protein) can function as a reducing agent for MsrA and MsrB,[49] and that selenium com-

Table III. Ratios of Trx- to DTT-Dependent Activities

MsrA form	Specific activity [nmol/(min mg protein)]		Ratio of Trx/DTT activity	
	DTT-dependent reaction	Trx-dependent reaction	hTrx1	eTrx
WT	4185 ± 46	34 ± 5	0.008	0.017
U16C	464 ± 14	14 ± 2	0.03	0.05
Mouse MsrA	256 ± 24	188 ± 9	0.73	0.93

Purified proteins (90 ng WT, 1 µg U16C, and 1 µg mouse MsrA) were used in the DTT- and Trx-dependent assays containing 200 µM DABSyl-methionine-S-sulfoxide. hTrx1, human cytosolic Trx; eTrx, *E. coli* Trx; WT, *Clostridium* selenoprotein MsrA; U16C, its Cys mutant.

pounds such as selenocysteamine could reduce mammalian MsrBs.[50] Our previous study on *Chlamydomonas* selenoprotein MsrA and its Cys mutant revealed that the Trx-dependent activities of these enzyme forms were more than 20-fold lower than their DTT-dependent activities.[31] Taken together and combined with the observation that known selenoprotein MsrAs lack conserved resolving Cys residues (or do not have any Cys as in *Clostridium* MsrA),[31] our data on *Chlamydomonas* and *Clostridium* MsrAs suggest that Trx is not a natural reducing agent for the selenenic acid intermediates of selenoprotein MsrAs.

CONCLUSIONS

This study shows that *Clostridium* sp. OhILAs, a gram-positive anaerobic bacterium, is predicted to be a selenoprotein-rich organism that contains 13 selenoproteins, including five that have not been previously described. Interestingly, one of the detected selenoproteins was MsrA, which serves an antioxidant function by catalyzing the reduction of methionine-S-sulfoxide. Little is known about anaerobic bacterial MsrAs. We cloned and expressed this *Clostridium* selenoprotein in *E. coli*. Although its SECIS element did not resemble those in *E. coli*, we could verify the recombinant protein expression by ⁷⁵Se labeling, western blots, and activity assays. Thus, the SECIS element of *Clostridium* selenoprotein *msrA* is functional and recognized by the *E. coli* Sec insertion machinery, although the translational efficiency was found to be low. The enzyme assays and kinetic analyses revealed that the selenoprotein MsrA is much more active than its Cys mutant form, indicating a key contribution of selenium to the catalytic efficiency of this selenoenzyme. Our data also suggest that Trx, a general *in vivo* reducing agent for MsrA, is not a reductant for *Clostridium* selenoprotein MsrA. Further studies will be needed to characterize the detailed reductase steps including determination of the natural reducing agent for this selenoprotein MsrA.

Acknowledgments

We thank Dr. John Stolz (Duquesne University, USA) for providing *Clostridium* sp. OhILAs genomic DNA and Dr. Dmitri Fomenko (University of Nebraska, USA) for help with sequence analyses.

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Supplementary Sequence Data

>Fdha

MAKIMINGESYEKNDITILQACREIGIEIPTLCHDDRLEPHAACRLCIVEIEGVRNLSTSCMKVAEGM
NIQTHSKNVMNTRKELLDLLFSNHPNDCLTCEKSGECKLQDYCYEYGVLGGSYKGEKRNLPIDYSNHFYT
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KVRAWETKKVKTTCSYCGVGCQLNLI VHEDKVI GAEPGRGVPNDGLLCVKGRFANAFINHEERLKTPLIR
KNGVLEEATWDEALGLIVSKVQSTKEKHGANALGGFSSARCTNEENYLFQKLFRAVIGTNNVDHCARLUH
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ADVYLQIKPGTNIALMNGMLNYI I ENDLHDKAYIEERTENFEDVKETVKAYTLERTAQICGVAPEDIAKA
ARMYAASDRAGIYYAMGITQHTTGTHNVI SVSNLALACGNIGKEFAGVNPLRGQNNVQGACDMGALPSDY
PGYQKVFKEPIAEKFEAAWNAKLSREVGLTVSEMIQKAETGDLKFLYIMGENPMVSDPDINHVKHSL ENI
DFLVVQDIFLTETAELADIVLPAASFAEKDGT FVNTERRVQRVRQAI PPVGGAKADWVILMELMNR LGYS
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TKDREYFPI LTTGRILYHYHTRTMTGREEGLNKI VPNSYIEVNDITANKLDLEDGEVVKVSSRRGS IKVA
VKVTDIVDEDVVFIPFHFAQGAANYLTNAQHDPISKI PELKVA AVKLEKIV

>Se1D

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VVDDPYTYGQIAAANSLSDVYAMGGRPLTAMNIVCFPNCLDPKILNQILKGGADKVLESGALLVGGHTVE
DDEPKYGLSITGIVHPKKVWANSTAREGDYILITKPVGLGVLNTAIKADIATKEQYDGA VKVMSMLNKYA
FEALENLDVSACTDITGFGFLGHVYEMAKGSGVSI EIPADKVPLIDGAKELATMGI IPAGMYSNKKHI EN
EVLKEQDIEDAIEDLLYDPQTS GGLLVSI SEKDLDALENLSLIESNGFSVVGRLPRSDYAVYVK

>GrdA

MARFDGKKII IIGDRDGI PGPAIEECLKGTGAEVVFSATECFVUTAAGAMDLENQQRVKDLTEKYGADNV
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>GrdB

MSKKRVVHYINQFFAGIGGEEKADIRPELREGVVGPGMALNTAMNEEAEIVATI ICGDSYFNENLEEAKN
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GTCTRCGATMVKEIERAGIPVVMCTVVPISLTVGANRIVPTVAIPHPLGNPNLEPKEEKALRRKLVEKA
LEALTTEVAGQTVFE

>Proline reductase

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LMVSHGGYDNGDVNKDINCMFPIDRLKELAE EGFIAAAPVHFGFMGGGDQQKFQEETGPEIARRLKSE
GVDVLLTAGUGTCHRS AVIVQRAIEESGIPTII I AALPPVVRQNGTPRAVAPLVPMGANAGAPNDKAMQ
TAIVRDSL IQLVEITSAGKIVQLPYEY TAKV

>HesB-like

MKINLTESAKQEILKQNTKDRGVRIVVASMGSUGPSFSLSLEEATQHDVVKDVNGVKFVIDKMLDNRFDE
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>MsrA

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LLNI FWNLHNPVYETNTRQYMSRI FYLDDGQKSEALEMKRQIEAANGEKIYTEIVPLENFYLAEGYHQY
YLQNTTKLYQTLKAIYGGFGNLVVRSTLAARMNGYIAGNLSIASLKEEMDLVELPEDQYEVKVL SIVEEIK

>Glutaredoxin

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IEELLG

#New

>putative anaerobic dehydrogenase

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RNSITGAYNEGSPVWAPETLADGTLVDQAYPSEEPFRVCSKAKLRGV SMLNNCPTLQVLSDTNYIEIN
ALDAKDFGLKDGQEV MVETPSNKAKGV LK VREGVARGSLGISFGYKWEYGSKDTTIDGKVVKGESIRAV
GIANPLALIDHSVKGYGLSEVTSGTHNRNGIRAKIVPLS

>BFD-like (2Fe-2S)-binding domain protein

MEENIMNRNCUGSQANSTSLVEENKFCPI CNEEGIQVKGITVKHMVIENLI PQVEDDNYLCLSEKCDIV
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NHPTGKCCSPIIQETIDRALGI

>Split solet cytochrome c precursor 1

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ARAYTGYKEKGGUGAGVAEGFFGTLADEAGYPFNQIPAEAFATAAGGYGQATL CGSLGVAAACIGMVTDV
DTQKKIVGELFKWYKTAEFPPQYQPEGLGLKHTVAESILCEDSVGTFMKEQGVAYGDPERKARCAGTAADV
TRKMVELLNETLA

>Split solet cytochrome c precursor 2

MDVKKSI TRKDFLKRAGMSAAGIAMAGGVGGILTACTNAGTASVNSTGTPDKPQWPFKYVKLDPAKVEER
AFKGYKEKGGUGVGVAEGFFGTLADEVGYPFNQIPTEGFTAAAGGYGMATL CGSLGVAAACIGMVTDVDT
QKKIVADLYKWKYKTFEFPQYQPEGLGLKHTVAESVLCEDSVGKFMKEQGVAYGDPERKSR CAGVTADVTR
KMVELLNEALA

>COG4656: Predicted NADH:ubiquinone oxidoreductase, subunit RnfC

MLIKISLKHVGVESKAI VKENEKVKKGQLLAVPDGLGSIHSSVHGIIEKVTSEYVSIKAYS DQSEDYM
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VNTVVLNIETIKNICLAIEKRKPVITKDI TVGGRVKAPKVFL ESPIGYPVSYFIDQCGGYENPHGEIVIG
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FNGRYRCDLPGVCPGQAEKVLFLKKQGAEVIITGTCEDU'NTVMMTAPRLGVPVYHSTDHVL RGSNHKLY
RKKQ