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# Periplasmic Electron Transfer via the *c*-Type Cytochromes MtrA and FccA of *Shewanella oneidensis* MR-1[▽]

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**Dissimilatory microbial reduction of insoluble Fe(III) oxides is a geochemically and ecologically important process which involves the transfer of cellular, respiratory electrons from the cytoplasmic membrane to insoluble, extracellular, mineral-phase electron acceptors. In this paper evidence is provided for the function of the periplasmic fumarate reductase FccA and the decaheme *c*-type cytochrome MtrA in periplasmic electron transfer reactions in the gammaproteobacterium *Shewanella oneidensis*. Both proteins are abundant in the periplasm of ferric citrate-reducing *S. oneidensis* cells. In vitro fumarate reductase FccA and *c*-type cytochrome MtrA were reduced by the cytoplasmic membrane-bound protein CymA. Electron transfer between CymA and MtrA was 1.4-fold faster than the CymA-catalyzed reduction of FccA. Further experiments showing a bidirectional electron transfer between FccA and MtrA provided evidence for an electron transfer network in the periplasmic space of *S. oneidensis*. Hence, FccA could function in both the electron transport to fumarate and via MtrA to mineral-phase Fe(III). Growth experiments with a Δ*fccA* deletion mutant suggest a role of FccA as a transient electron storage protein.**

Dissimilatory metal reduction is a microbial respiratory process in which the reduction of an oxidized metal is coupled to energy conservation under anoxic conditions (10). The most abundant redox active metal in the Earth's crust is iron. Unlike other terminal electron acceptors, Fe(III) which is found in soil or sediments occurs as solid-phase crystalline iron oxides. Therefore, dissimilatory Fe(III)-reducing microorganisms need to access an insoluble electron acceptor by an extended respiratory electron transport chain. The composition of such an extended electron transport chain and the molecular interaction of its components have been intensely studied in the Fe(III)-reducing bacteria *Shewanella* and *Geobacter* (10, 25).

So far, five proteins have been shown to play an essential role in the electron transport to extracellular Fe(III) in *Shewanella oneidensis*. The tetraheme *c*-type cytochrome CymA, which is localized to the outer surface of the cytoplasmic membrane, catalyzes the transfer of electrons from menaquinol to various periplasmic electron-accepting proteins, including the fumarate reductase FccA (13, 14, 21). CymA appears to function as the entry point of an electron switch for several electron transport pathways, since a *cymA* mutant cannot grow with dimethyl sulfoxide (DMSO), nitrate, nitrite, fumarate, or ferric iron as a terminal electron acceptor (20, 21). In the outer membrane the two decaheme cytochrome proteins OmcA and OmcB were shown to directly transfer electrons to different insoluble Fe(III) oxides, including hematite (2, 25, 29). These two proteins form a high-affinity complex that catalyzes the electron transfer to ferric nitrilotriacetic acid (NTA) faster than these enzymes do separately (24). Pitts et al. and Ross et al. demonstrated that MtrB and OmcB form a protein complex with MtrA, which is a periplasmic decaheme *c*-type cytochrome (17, 18). Mutants defective in *mtrA* are unable to reduce ferric iron (3). The outer membrane protein MtrB seems to be crucial for correct localization of OmcA and OmcB to the outer membrane (1, 15, 18). Based on the topology of these redox-active proteins and the functions demonstrated experimentally, it is speculated that electrons are transferred from CymA via MtrA to OmcA and OmcB. However, such direct electron transfer between all components and how electrons are transferred between the cytoplasmic membrane CymA and outer membrane OmcA and -B has not been shown so far.

The aim of this study was to elucidate periplasmic electron transfer reactions between CymA and OmcA and -B. We pursued a novel approach, since past genetic experiments uncovered a significant level of redundancy in the activities and specificities of periplasmic *c*-type cytochromes, which is further obscured by compensatory transcriptional regulation (7, 11, 12). We characterized the functions of two abundant periplasmic *c*-type cytochromes that are present under ferric iron-reducing conditions using in vitro enzyme assays and heterologous expression experiments. Our results revealed electron transfer interactions between periplasmic MtrA and FccA and provide the basis for the characterization of a highly dynamic periplasmic electron transport network.

## MATERIALS AND METHODS

**Materials.** Chemicals and biochemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany), Roth (Karlsruhe, Germany), and Promega (Mannheim, Germany). Fast protein liquid chromatography equipment was from Qiagen (Hilden, Germany) and GE (Freiburg, Germany). Enzymes were purchased from New England Biolabs (Frankfurt, Germany).

**Microorganisms and plasmids.** *Shewanella oneidensis* MR-1 was used for expression of $FccA_{Strep}$ and analysis of *c*-type cytochrome identity and abundance (28). *Escherichia coli* strain TB1 was used for expression of *c*-type cyto-

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TABLE 1. Bacterial strains used in this study

| Species and strain | Relevant genotype | Reference or source |
|---|---|---|
| *Shewanella oneidensis* | | |
| JG7 (MR-1) | Wild-type strain | 26 |
| JG55 | JG7 Δ*mtrB* | This study |
| JG69 | JG7 Δ*mtrB*/pBAD*mtrB*$_{Strep}$ | This study |
| JG162 | JG7 Δ*mtrA* | This study |
| JG207 | JG7 Δ*fccA* | This study |
| *E. coli* | | |
| JG38 (K-12 TB1) | F$^-$ *ara* Δ(*lac-proAB*) [ϕ*80dlac* Δ(*lacZ*)*M15*] *rpsL* (Str$^r$) *thi hsdR* | New England BioLabs |
| JG92 | TB1/pBAD*fccA*$_{Strep}$, pEC86 | This study |
| JG48 | TB1/pBAD*mtrA*$_{Strep-His}$ | This study |
| JG93 | TB1/pBAD*mtrA*$_{One-Strep}$, pEC86 | This study |
| AS427 | DH5αZI Δ(*napC-F*) | 9 |
| AS457 | DH5αZI Δ(*napC-F*) Δ(*frdA-D*) 4380508::(P$_{tet}$ *cymA*)/pEC86 | 9 |
| JG146 | DH5αZI Δ(*napC-F*) Δ(*frdA-D*) 4380508::(P$_{tet}$ *cymA-mtrA*)/pEC86 | This study |

chromes. *E. coli* DH5αZI was used for all mutation and synthetic biology experiments (Table 1). Plasmid pEC86 containing the cytochrome maturation genes from *E. coli* was provided by Linda Thony-Meyer (26). The plasmids pASK-IBA44 and pEXPR-IBA105 were purchased from IBA (Göttingen, Germany). Plasmid pBAD202 was purchased from Invitrogen (Karlsruhe, Germany).

**Growth conditions and media.** *E. coli* strains were grown in LB medium or anoxically in M9 minimal medium (47.8 mM $Na_2HPO_4$, 22 mM $KH_2PO_4$, 9.2 mM NaCl, 18.7 mM $NH_4Cl$) supplemented with 485 μM $CaCl_2$, 1 mM $MgSO_4$, trace elements (5 μM $CoCl_2$, 0.2 μM $CuSO_4$, 57 μM $H_3BO_3$, 5.4 μM $FeCl_2$, 1.3 μM $MnSO_4$, 67.2 μM $Na_2EDTA$, 3.9 μM $Na_2MoO_4$, 1.5 μM $Na_2SeO_4$, 5 μM $NiCl_2$, and 1 μM $ZnSO_4$), 1.5 g liter$^{-1}$ Casamino Acids, and 15 μM thiamine hydrochloride. The provided carbon source in all *E. coli* minimal medium growth experiments was 0.5% (wt/vol) glycerol. DMSO was used as electron acceptor at a concentration of 70 mM. For comparative growth experiments with 10 mM ferric NTA as an electron acceptor, *E. coli* cells were grown in mineral medium (see below) supplemented with 1.5 g liter$^{-1}$ Casamino Acids, 1 mM $MgSO_4$, 485 μM $CaCl_2$, trace elements, and 15 μM thiamine hydrochloride. *S. oneidensis* strains were grown in LB medium or anoxically in mineral medium with 50 mM lactate as a carbon source and 50 mM ferric citrate, 50 mM fumarate, or 30 mM DMSO as a terminal electron acceptor. Mineral medium [1.27 mM $K_2HPO_4$, 0.73 mM $KH_2PO_4$, 5 mM sodium HEPES, 150 mM NaCl, 9 mM $(NH_4)_2SO_4$] was supplemented with 1 mM $MgSO_4$, 485 μM $CaCl_2$, and trace elements (see above). If necessary, ampicillin (100 μg ml$^{-1}$), kanamycin (50 μg ml$^{-1}$), or chloramphenicol (30 μg ml$^{-1}$) was added to the medium.

**Cell fractionation.** The periplasmic fraction of anoxically grown *S. oneidensis* cells was prepared as described by Pitts et al. with polymyxin B (5, 17). After polymyxin B treatment, the cells were centrifuged at 15,000 × *g* and 4°C for 45 min. The supernatant comprised the periplasmic fraction. The pellet was resuspended in 1 volume of 0.1 M HEPES (pH 7.5) containing 0.1 mg ml$^{-1}$ DNase I and passed through a French pressure cell at 137 MPa. Unbroken cells were removed by two consecutive centrifugations at 3,000 × *g* and 4°C for 10 min. The supernatant was centrifuged at 208,000 × *g* and 4°C for 90 min. The pellet containing the cell membranes was resuspended in 0.1 M HEPES (pH 7.5). The method was evaluated using the following three different assays.

(i) Cellular fractions of a Δ*mtrB* mutant expressing MtrB with a C-terminal Strep-tag (IBA) were used to monitor possible outer membrane contamination within the periplasmic fraction. The fusion protein was detected on a Western blot using a primary Strep-tag antibody (Qiagen, Hilden, Germany) and a secondary alkaline phosphatase-labeled antibody. The blot was developed using an alkaline phosphatase conjugate substrate kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions.

(ii) NADH dehydrogenase activity was measured as described by Osborn et al. to detect cytoplasmic membrane contaminations in the periplasmic fraction (16).

(iii) The lipid A content of membrane and periplasmic fractions was measured to detect possible outer membrane contamination in the periplasmic fraction. Lipid A was quantified using the chromogenic *Limulus* amoebocyte lysate endotoxin assay kit according to the manufacturer's instructions (GenScript, NY).

**Cloning and expression of FccA$_{Strep}$, MtrB$_{Strep}$, and MtrA$_{One-Strep}$.** *fccA* was cloned into pBAD202 via TOPO cloning according to the manufacturer's instructions. The gene was amplified using primers 1 and 2 and was thereby modified to contain the sequence for a C-terminal Strep-tag (Table 2). For expression of *fccA*$_{Strep}$ in *S. oneidensis*, cells were grown in LB medium to an optical density at 600 nm of 0.6 and then induced by addition of 10 mM arabinose to the medium. Cells were harvested 4 h after induction. *mtrB* was cloned into pBAD202 using primers 3 and 4 (Table 2). Primer 3 contained the sequence coding for an NcoI cleavage site. Primer 4 contained the sequence coding for a C-terminal Strep-tag and a HindIII cleavage site. PCR fragments were cleaved and cloned into pBAD202. The resulting plasmid was transformed into *S. oneidensis* MR1 Δ*mtrB*. Heterologous protein expression was induced by addition of 1 mM arabinose to the medium. *mtrA* was first cloned into pASK-IBA44 using primers 5 and 6 (Table 2). Primer 5 contained the sequence coding for a BsaI cleavage site and the 3′region adjacent to the sequence coding for the native Sec leader peptide of *mtrA*. The reverse primer also contained a BsaI site. The PCR fragment was cleaved and cloned into pASK-IBA44. This led to the 5′ addition of the *ompA* leader sequence for Sec system transfer into the periplasm. The sequence coding for a His tag was inserted between the Strep-tag and *mtrA* via inverse PCR using primers 7 and 8 (Table 2). *mtrA*$_{Strep-His}$ was cloned via TOPO cloning into pBAD202 with primers 9 and 10 to increase protein yields (pBAD*mtrA*$_{Strep-His}$) (Table 2). Since purification of the expressed protein using the N-terminal Strep-tag was not possible, we replaced the single Strep-tag of *mtrA*$_{Strep-His}$ with a One-Strep-tag using the In-Fusion 2.0 Dry-Down PCR cloning kit from Clontech. The vector was digested with PmeI and NheI and fused with the amplified One-Strep-tag (PCR with primers 11 and 12) and the amplified *mtrA* (PCR with primers 13 and 14) (Table 2). The resulting plasmid (pBAD*mtrA*$_{One-Strep}$) was transformed into *E. coli* TB1 pEC86. For heterologous protein expression, cells were grown at 37°C to an optical density at 600 nm of 0.7 and thereafter induced by addition of 10 mM arabinose to the medium. After induction, the growth temperature was decreased to 22°C. Heterologous protein expression was induced for 5 h.

**Purification of fusion proteins.** Cells were harvested via centrifugation at 3,000 × *g* and 4°C for 10 min, resuspended in 0.1 M HEPES (pH 7.5) containing 0.1 mg ml$^{-1}$ DNase I, and passed through a French pressure cell at 137 MPa. The broken cells were spun at 208,000 × *g* and 4°C for 90 min. MtrA$_{One-Strep}$ and FccA$_{Strep}$ were both purified using a 10 ml Strep-tactin macroprep column (IBA, Göttingen, Germany). The purifications were performed according to the manufacturer's instructions.

**Construction of an *E. coli cymA-mtrA* strain.** *S. oneidensis mtrA* was amplified using primers 15 and 16 (Table 2). The resulting fragment was cleaved with BamHI and cloned into pJG1, resulting in plasmid pJG2. Integration of the fragment containing the *cymA-mtrA* operon into the genome of AS427 was performed as described before (9).

**Construction of a markerless *mtrA*, *mtrB*, and *fccA* deletion in *S. oneidensis*.** Regions of 500 bp flanking *mtrA* (primers 17 to 20), *mtrB* (primers 21 to 24), and *fccA* (primers 25 to 28) were amplified (Table 2). The resulting fragments contained regions overlapping with the vector pMQ150 (R. M. Shanks, unpublished data) and with themselves. The suicide plasmid pMQ150 was cleaved with BamHI and SalI. The two fragments and the vector were combined in *Saccharomyces cerevisiae* as described by Shanks et al. (23). Markerless deletions were later introduced as described elsewhere (27).

**Enzyme assays with *E. coli* membrane fractions.** AS457 cells were grown anoxically in M9 minimal medium with 70 mM DMSO as a terminal electron acceptor. The *cymA*-containing operon was induced with 0.43 mM anhydrotet-

TABLE 2. Primers used in this study

| Primer | Sequence (5′ → 3′) |
|---|---|
| 1 | CACCTAAGAAGGAGATATACATCCCATGTTCACAAGAAAGATTCAAAAAACAGC |
| 2 | TTATTTTTCGAACTGCGGGTGGCTCCAAGCGCTATTATCTTTAGCGAATTTAGCGGCAG |
| 3 | CATGCCATGGATGAAATTTAAACTCAATTT |
| 4 | GGGAAGCTTTTATTTTTCGAACTGCGGGTGGCTCCAGGCGCCGAGTTTGTAACTCATGCT |
| 5 | ATGGTAGGTCTCAGCGCCTCGAAGTGGGATGAGAAAATGACG |
| 6 | ATGGTAGGTCTCAGGCCTTAGCGCTGTAATAGCTTGCCAGATG |
| 7 | GCCCATCACCATCACCATCACGGCGCCTCGAAGTGGGATGAGAAAATG |
| 8 | CGAGGCGCCGTGATGGTGATGGTGATGGCCTTTTTCGAACTGCGGG |
| 9 | CACCTAAGAAGGAGATATACATCCCATGAAAAAGACAGCTATCGCG |
| 10 | CTATTAGCGCTGTAATAGCTTG |
| 11 | TAGCGCAGGCCGCTAGCTGGAGCCACCCGC |
| 12 | GGCGCCTTTTTCGAACTGCGG |
| 13 | TTCGAAAAAGGCGCCGGCGGATCGAAGTGGGATGAGAAA |
| 14 | AAGCTGGAGACCGTTTAAACTCAATGGTGATGGTGATGATGACC |
| 15 | ACGGGATCCAGAAGGAGACCCTATTATGAAGAACTGCCTAAAAATGAAAAAC |
| 16 | CGGGATCCCTTAGCGCTGTAATAGCTTG |
| 17 | GTTTAAATTTCATTTTCTCGTCGGCTTCCCAATTTGTCCCAAC |
| 18 | GTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGCTATCACACACCCAAGATGC |
| 19 | GAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTAGGCATTGAGGCTGTCCATCA |
| 20 | GACGAGAAAATGAAATTTAAAC |
| 21 | GCCCGCAGAGGCGGGCTTTTGAGCATATGAGGCAAATGGATTTCTCGTCTCCTTAGCGC |
| 22 | GTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGTCAATGGGGTGGCGGATGAAC |
| 23 | GTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGTCAATGGGGTGGCGGATGAAC |
| 24 | TCCATTTGCCTCATATGCTC |
| 25 | GTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGTTAGACTTAACCCCTCAACACCA |
| 26 | AGTTGTTCCTCCGCCGTACAT |
| 27 | GAAATGTACGGCGGAGGAACAACTTATCAATAGGTTCTAGGTTAATGG |
| 28 | GAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACGGCGACTAAGTCGACATTGTTA |

racycline. As a control, AS457 was grown under the same conditions without inducer. Cell membranes were prepared as described above. Membrane integrity was tested using NADH dehydrogenase activity, which was measured photometrically as described by Osborn et al. (16). All further steps were performed under anoxic conditions at 30°C. The reaction mixture (total volume of 500 μl) contained 0.1 M HEPES (pH 7.5), 10 mM glycerol-3-phosphate, and 10 μl or 20 μl membrane fraction (1.53 mg protein $ml^{-1}$). The reactions were started by the addition of 1.5 nmol $MtrA_{One\text{-}Strep}$ or $FccA_{Strep}$, and the cytochrome reduction was observed at 552 nm using a Cary 100 spectrophotometer (Varian, Darmstadt, Germany). The measured rates in independent experiments varied in a range of less than 10%.

**Ferric NTA reduction by *E. coli* cells expressing MtrA and CymA.** *E. coli* strains AS457 and JG146 were grown anoxically in mineral medium with 10 mM ferric NTA as a terminal electron acceptor and 50 mM glycerol as an electron donor and carbon source. At various time points, samples (100 μl) were taken and the reactions were stopped by the addition of 33 μl of 2 N HCl. The ferrous iron concentration was measured using the ferrozine assay as described by Ruebush et al. (19).

**Direct electron transfer from $MtrA_{One\text{-}Strep}$ to FccA.** All steps were performed under anoxic conditions at 30°C. The assay mixture (total volume of 1 ml) contained 0.1 M HEPES (pH 7.5), 1.2 nmol MtrA, 1 mM dithionite, 10 mM fumarate, and 120 pmol $FccA_{Strep}$. First, $MtrA_{One\text{-}Strep}$ was reduced by dithionite. After the addition of 10 μmol fumarate, the reduction status of the protein was recorded for 10 min with scans from 300 to 600 nm. $MtrA_{One\text{-}Strep}$ remained reduced for the whole preincubation time. The addition of $FccA_{Strep}$ led to a fast reoxidation of $MtrA_{One\text{-}Strep}$, which was recorded at 552 nm using a Cary 100 spectrophotometer (Varian, Darmstadt, Germany). The measured rates in independent experiments varied in a range of less than 10%.

**Enzyme assays with *S. oneidensis* membrane fractions.** *S. oneidensis* wild-type and Δ*mtrA* mutant cells were grown anoxically in minimal medium with 50 mM fumarate as the sole electron acceptor. Membrane fractions of these cells were prepared as described above. All further steps were performed under anoxic conditions at 30°C. The reaction mixture (total volume of 500 μl) contained 0.1 M HEPES (pH 7.5), 1 mM, hematite and 15 or 25 μl of the two membrane fractions. $FccA_{Strep}$ (200 μl; 400 pmol) was reduced with 50 μl of 1 mM dithionite. The reduced FccA was added to the reaction mixture, and the reoxidation of FccA was recorded via scanning from 300 to 600 nm using a Cary 100 spectrophotometer (Varian, Darmstadt, Germany). The measured rates in independent experiments varied in a range of less than 10%.

**Quantification of cells and ferrous iron during growth experiments with hydrous ferric oxide as a terminal electron acceptor.** *S. oneidensis* strains JG7 and JG207 were grown under anoxic conditions in mineral medium containing 30 mM DMSO as an electron acceptor and 25 mM lactate as an electron donor. In mid-logarithmic growth phase, cells were washed with mineral medium without an electron donor or acceptor and transferred to medium containing 10 mM hydrous ferric oxide (22) as a terminal electron acceptor and 25 mM lactate as an electron donor and carbon source. At various time points, samples (1,200 μl) were taken; 100 μl of these samples was used for measuring the ferrous iron concentration as described by Ruebush et al. (19), and 1,000 μl was centrifuged for 5 min at 10,000 × *g*. The pellet was washed with 0.75 M HCl. The cells were fixed with 750 μl of 4% (wt/vol) paraformaldehyde and 250 μl of phosphate-buffered saline (PBS) (pH 7). After a 12-h incubation at 4°C, cells were washed two times with PBS (pH 7) and resuspended in a 1:1 mixture of 99% ethanol and PBS (pH 7). Cells were counted in a Neubauer counting chamber.

**Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of protein.** Polyacrylamide (10%) gel electrophoresis was performed by the Laemmli method (6). Proteins were visualized by Coomassie blue staining. Gels were examined for the presence of *c*-type cytochromes by heme-linked peroxidase staining as described previously (9).

**Determination of protein.** Protein was determined by the method of Bradford using bovine serum albumin as standard (6).

**Peptide mass fingerprinting of excised heme-stained protein bands.** Periplasmic fractions from *S. oneidensis* cells grown with ferric citrate as the sole electron acceptor and lactate as an electron donor were loaded on a 10% SDS-polyacrylamide gel. Heme-containing proteins were stained as described above. Bands at 32, 35, and 62 kDa were excised from the gel. Peptide mass fingerprinting using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry after in-gel protein cleavage was performed by Toplab (Munich, Germany).

## RESULTS

**Abundance of MtrA and FccA in the periplasm of *S. oneidensis* cells grown under ferric citrate-reducing conditions.** Three abundant *c*-type cytochrome proteins with molecular masses of 62, 35, and 32 kDa were identified in the periplasm

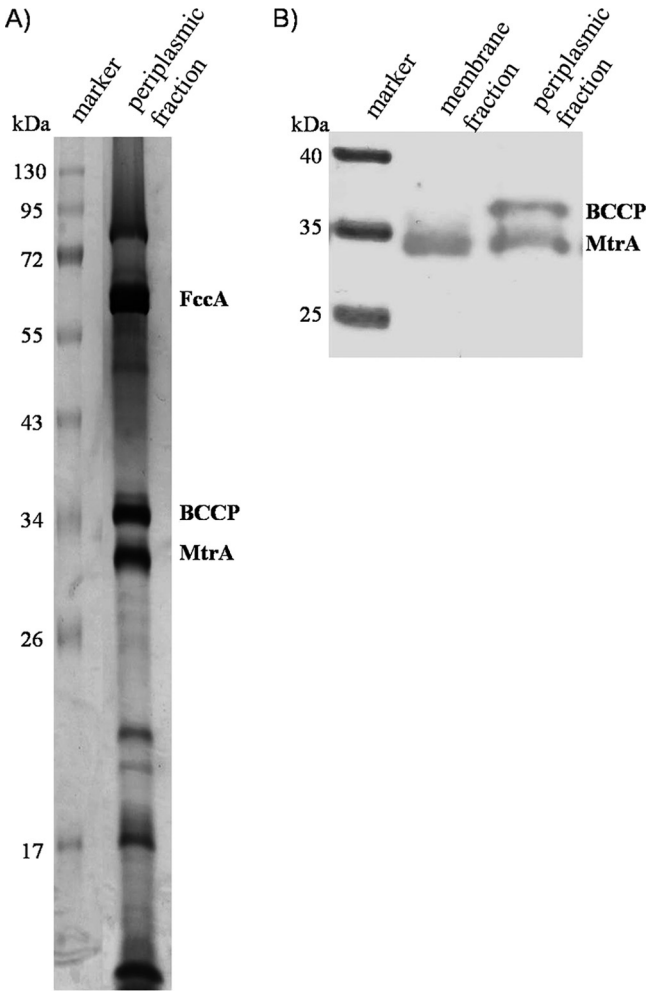


FIG. 1. (A) Heme staining of a periplasmic fraction from ferric iron-grown *S. oneidensis* cells. Heme-containing bands at 62, 35, and 32 kDa were excised from the gel and identified via mass spectrometry. The signals correspond to FccA, bacterial cytochrome *c* peroxidase (BCCP), and MtrA. (B) Relative amounts of MtrA in periplasmic and membrane fractions of *S. oneidensis* cells grown under ferric citrate-reducing conditions. A total of 3.6 g of wet cell mass was separated into a periplasmic (89 mg total protein) and membrane-bound (25 mg total protein) protein pools, and 140 μg of the periplasmic fraction and 40 μg of the membrane fraction were loaded on an SDS-polyacrylamide gel. Relative amounts of MtrA in the two fractions were quantified using heme signal intensity detection.

of ferric citrate-grown *S. oneidensis* cells (Fig. 1A). Mass spectrometric analysis of the heme-containing protein bands identified the 62-kDa protein as FccA (30% fragment coverage with 19 peptide hits), the 35-kDa protein as bacterial cytochrome *c* peroxidase (52% fragment coverage with 18 peptide hits), and the 32-kDa protein as MtrA (21% fragment coverage with 8 peptide hits). MtrA was previously shown to be necessary for ferric iron reduction in *S. oneidensis* (3) and might mediate the electron transfer to outer membrane cytochromes (18). While previous experiments showed that MtrA is associated exclusively with the membrane fraction (18), in the experiments presented here about 46% of the total MtrA pool was localized to the periplasmic protein fraction, while the other 54% was present in the membrane fraction (Fig. 1B). Three different control reactions were performed to exclude the possibility that the presence of MtrA in periplasmic fractions is due to a carryover of membranes. *S. oneidensis* Δ*mtrB* cells expressing the outer membrane protein MtrB with a Strep-tag epitope ($MtrB_{Strep}$) from an inducible plasmid were subjected to the same separation procedure used above. $MtrB_{Strep}$ was detected by immunostaining using a primary Strep-tag antibody. Positive signals were found only in the membrane and not in the periplasmic fraction. Furthermore, NADH dehydrogenase activity was measured to detect possible cytoplasmic membrane contamination in the periplasmic fraction. No activity was detectable in the periplasmic fraction, while the membrane fraction catalyzed NADH oxidation with a rate of 150 nmol $min^{-1}$ $mg^{-1}$. Finally, the lipid A contents of the membrane and periplasmic fractions were measured as another way to detect the level of membrane contamination in the periplasmic fraction. We detected 24.5 mg of lipid A per gram of wet cells in the membrane fraction. In contrast, the periplasmic fraction contained only 1.8 mg lipid A per gram of wet cells.

**CymA-catalyzed reduction of MtrA.** As we found abundant MtrA protein in the periplasm of *S. oneidensis*, we wondered whether MtrA interacts with and can be reduced directly by CymA. In previous experiments it was shown that in an in vitro assay CymA can be reduced by *E. coli* membrane fractions using glycerol-3-phosphate as an electron donor (9). To test for an electron transfer between CymA and MtrA, this assay was extended by limiting the concentration of CymA to catalytic amounts (15.3 to 30.6 μg of total membrane fraction protein containing CymA) and by adding purified $MtrA_{One\text{-}Strep}$ (1.5 nmol). A reduction of hemes observed in this spectrophotometric assay at 552 nm could be due only to the reduction of $MtrA_{One\text{-}Strep}$, since the amount of reduced CymA was too small to cause a change of absorbance. Therefore, the modified assay tested whether CymA was necessary for mediating the reduction of MtrA with electrons derived from glycerol-3-phosphate oxidation.

One-Strep-tagged $MtrA_{One\text{-}Strep}$ was purified from *E. coli* strain JG93 also containing cytochrome maturation genes in a single chromatography step using a Strep-tag affinity column (Fig. 2). CymA-containing membranes were prepared from *cymA*-expressing *E. coli* strain AS457. Expression of *cymA* was induced upon addition of 0.43 mM anhydrotetracycline. Membrane fractions of the induced strain were prepared, and catalytic amounts of these membrane fractions were added to an anoxic cuvette with 10 mM glycerol-3-phosphate as an electron donor. After addition of 1.5 nmol purified $MtrA_{One\text{-}Strep}$, a reduction rate of 121 nmol $min^{-1}$ $mg^{-1}$ membrane protein was recorded. No reduction of $MtrA_{One\text{-}Strep}$ was observed when membrane fractions of uninduced AS457 were added, indicating that MtrA reduction is mediated by CymA.

The same set of experiments was conducted with purified $FccA_{Strep}$ (Fig. 2). Also here, membranes from uninduced cells of AS457 did not reduce $FccA_{Strep}$ at a detectable rate, whereas membrane fractions of induced AS457 cells reduced $FccA_{Strep}$ at a rate of 85 nmol $min^{-1}$ $mg^{-1}$ membrane protein.

To account for potential differences in the catalytic properties of induced and uninduced membrane fractions of AS457, the specific NADH oxidation rate was determined as a surrogate for the electron transfer properties of the membranes.

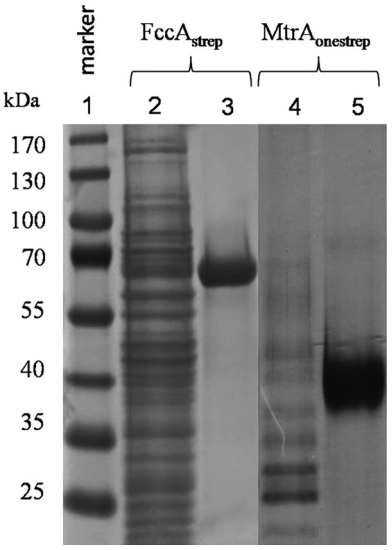


FIG. 2. Purification of FccA and MtrA. Eight micrograms of purified protein was loaded. Lane 1, marker; lane 2, crude extract of *S. oneidensis* cells expressing $FccA_{Strep}$; lane 3, purified $FccA_{Strep}$; lane 4, crude extract of *E. coli* cells expressing $MtrA_{One\text{-}Strep}$; lane 5, purified $MtrA_{One\text{-}Strep}$.

Membranes from uninduced and induced cells catalyzed NADH oxidation at 99 and 104 nmol $min^{-1}$ $mg^{-1}$ membrane protein, respectively, suggesting that the presence of CymA, and not a difference in reduction capacities of the membranes, was the cause of $MtrA_{One\text{-}Strep}$ reduction.

**Enhanced ferric NTA reduction in *E. coli* cells expressing MtrA and CymA.** Pitts et al. showed that MtrA not only is a periplasmic electron transfer protein but also is a reductase of chelated ferric iron (17). To test whether electron transfer between CymA and MtrA also operates under in vivo Fe(III) reduction conditions, we used a comparative growth experiment with isogenic *E. coli* strains expressing either *cymA* (AS457) or an operon containing *cymA* and *mtrA* (JG146) (9). Both strains were grown with glycerol as an electron donor and Fe(III)-NTA as an electron acceptor. As Fig. 3 shows, the *cymA*- and *mtrA*-expressing strain JG146 reduced ferric NTA 1.5-fold faster than AS457. We hypothesize that the higher reduction rate is due to expression of MtrA, which leads to a second ferric NTA reduction site that is directly connected to CymA.

**Direct electron transfer between MtrA and FccA.** Interestingly, FccA is the most abundant periplasmic *c*-type cytochrome protein even under ferric iron-reducing conditions (Fig. 1), and FccA was shown previously to be directly reduced by CymA (20). Thus, we wondered whether FccA has another role during Fe(III) reduction besides its function in fumarate reduction. Since both MtrA and FccA interact with CymA, we tested whether FccA and MtrA can directly interact and reciprocally transfer electrons in the absence of CymA. FccA has fumarate reductase activity, whereas MtrA does not. Hence, we tested in in vitro experiments whether reduced purified $MtrA_{One\text{-}Strep}$ can provide electrons for fumarate reduction via purified $FccA_{Strep}$. To an anoxic cuvette containing reaction buffer we added 1.2 μmol $MtrA_{One\text{-}Strep}$, which was subsequently reduced upon addition of 50 μmol dithionite. Fumarate was subsequently added to a final concentration of 10 mM to the reduced $MtrA_{One\text{-}Strep}$, and the oxidation state of the $MtrA_{One\text{-}Strep}$ was followed spectrophotometrically at 552 nm (Fig. 4). $MtrA_{One\text{-}Strep}$ remained reduced for a time period of at least 10 min. Thereafter, $FccA_{Strep}$ (120 pmol) was added, and after a short lag a rapid oxidation of $MtrA_{One\text{-}Strep}$ was observed at a maximum rate of 1.16 μmol $min^{-1}$ $mg^{-1}$ $MtrA_{One\text{-}Strep}$ (Fig. 4). This oxidation did not occur when fumarate was omitted from the reaction mixture.

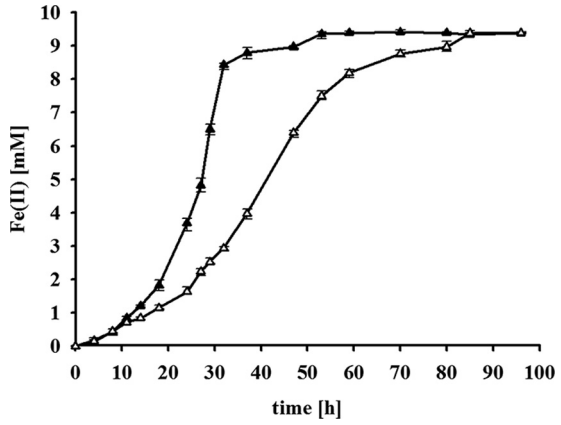


FIG. 3. Fe(III)-NTA reduction of strains JG146, expressing CymA and MtrA (▲), and AS457, expressing CymA only (△). Ferric NTA (10 mM) was added to mineral medium as the sole electron acceptor, while glycerol (50 mM) was used as a carbon and electron source. Both strains were induced with 0.43 mM anhydrotetracycline.

**MtrA-dependent electron transfer from FccA to hematite.** We then tested in an in vitro assay whether FccA is involved in insoluble ferric oxyhydroxide reduction via MtrA. In *S. oneidensis* MR-1, about 50% of the total cellular MtrA is present in a complex with MtrB and OmcB (see above). This complex has goethite- and ferrihydrite-reducing activity (18). Therefore, we

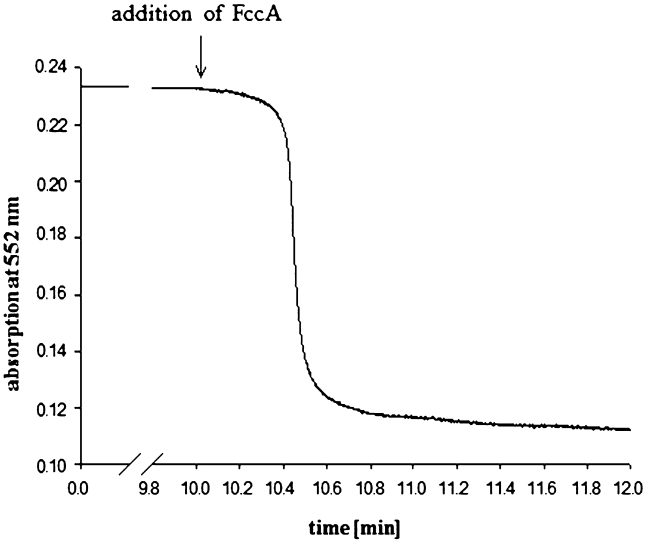


FIG. 4. FccA-mediated electron transfer from MtrA to fumarate. MtrA (1.2 μmol) was reduced with dithionite and incubated for 10 min with fumarate. Addition of 120 pmol FccA (arrow) caused a rapid reoxidation of MtrA.

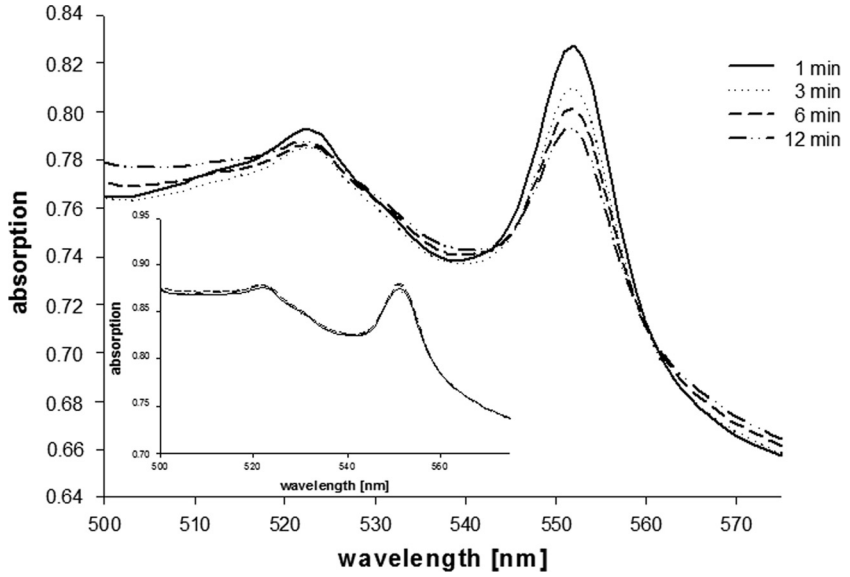


FIG. 5. Electron transfer from reduced FccA to hematite nanoparticles via outer membrane complexes. The main figure shows a scanning kinetics experiment with *S. oneidensis* wild-type membrane fractions, while the inset shows the same experiment with membranes from a Δ*mtrA* mutant.

tested whether reduced FccA could transfer electrons to ferric oxyhydroxides via this outer membrane complex. *S. oneidensis* membrane fractions were prepared from wild-type and Δ*mtrA* cells that were grown with fumarate as a terminal electron acceptor. Four hundred picomoles of sodium dithionite-reduced $FccA_{Strep}$ was added to a cuvette and thereafter mixed with 1 mM hematite particles. The oxidation state of the hemes of $FccA_{Strep}$ was followed via continuous scanning kinetic measurements between 380 and 600 nm. $FccA_{Strep}$ remained reduced for at least 10 min. Subsequently, catalytic amounts of the membrane fraction of wild-type cells (49 to 81 μg in the assay) or Δ*mtrA* cells (68 to 113 μg in the assay) were added. Addition of wild-type membranes resulted in an oxidation of $FccA_{Strep}$ at a rate of 3.5 nmol $min^{-1}$ $mg^{-1}$ membrane protein (Fig. 5). No oxidation was observed when the membrane fraction of Δ*mtrA* cells was added.

**Phenotype of *S. oneidensis* Δ*fccA* cells under ferric iron-reducing conditions.** If FccA can be part of an electron transport chain to ferric iron, its deletion could cause a detectable phenotype. Hence, *S. oneidensis* Δ*fccA* and wild-type cells were pregrown in minimal medium containing DMSO as terminal electron acceptor. At mid-logarithmic growth phase, cells were transferred to minimal medium containing hydrous ferric oxide as terminal electron acceptor (Fig. 6). At all time points tested,

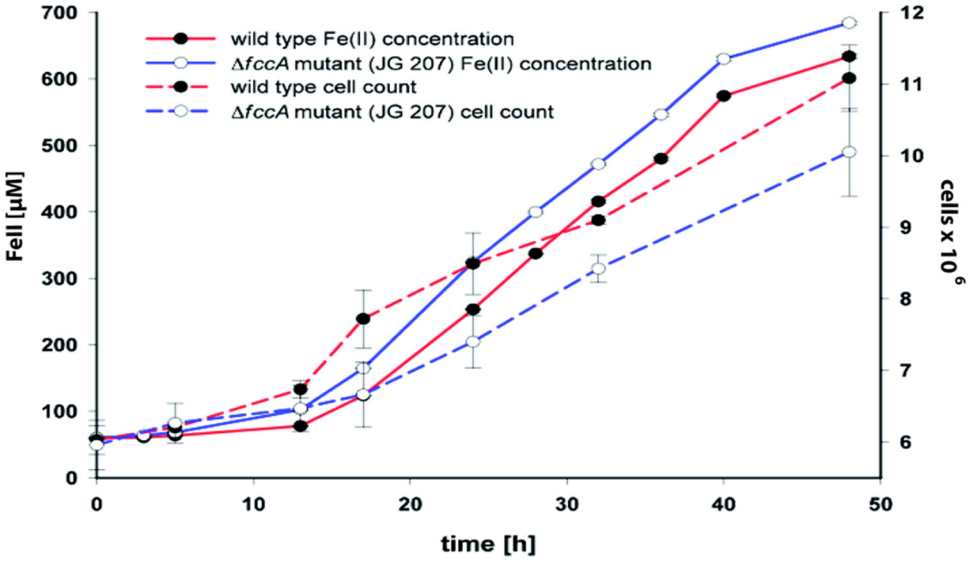


FIG. 6. Comparative growth experiments with wild-type *S. oneidensis* and the Δ*fccA* mutant. The growth medium contained 10 mM hydrous ferric oxide and 5 mM lactate. Error bars indicate standard deviations.

Δ*fccA* mutant cells reduced ferric iron faster than the wild type. In contrast, cell counts revealed that the wild type was able to build up biomass on the same level or even more than the Δ*fccA* mutant. When *S. oneidensis* Δ*fccA* was complemented with *fccA* expressed from a plasmid, iron reduction and cell growth resembled those of the wild type again (data not shown). Since a decrease in the rate of reduction of ferric iron is not coupled to a decrease in cell growth in the wild type, we conclude that FccA can act as a transient electron storage protein that is filled with electrons via reaction kinetics that are faster than electron transfer to an extracellular electron acceptor. This electron storage protein could allow for an initial growth rate higher than that of the Δ*fccA* mutant.

## DISCUSSION

In this study, we conducted in vitro experiments to examine electron transport properties of *c*-type cytochromes that could play physiological roles in the electron transfer between cytoplasmic membrane CymA and the outer membrane cytochromes in *S. oneidensis* MR-1. While determining the abundance of multiheme proteins in the membrane and periplasmic fractions, we were surprised to find 46% of the total MtrA protein in the periplasmic fraction of *S. oneidensis* cells (Fig. 1B). Using an osmotic shock method, which is different from the polymyxin B treatment used here, Ross et al. previously reported that MtrA was present only in the membrane fraction (18). The osmotic shock method is possibly gentler than polymyxin B treatment, but method evaluation with different assays revealed the suitability of the method.

Our subsequent in vitro experiments demonstrated a direct electron transfer between CymA and MtrA, suggesting that MtrA resembles a key periplasmic electron transfer component. Integration of our results with data published by Ross et al. (18) showing a complex of MtrA, MtrB, and OmcB at the outer membrane leads to the hypothesis that MtrA might have a dual role in periplasmic electron transfer. MtrA could interact directly with CymA at the periplasmic membrane and transport the electrons to the outer membrane. There, it could react with an MtrA/MtrB/OmcB complex or build a new complex with an MtrB/OmcB module. Our data furthermore indicate that MtrA in the three-protein complex can abstract electrons from other periplasmic *c*-type cytochromes, such as FccA. The role of MtrB within this MtrA/MtrB/OmcB complex could be to bring the two partners MtrA and OmcB in close contact and therefore allow for a rapid electron transfer over the outer membrane and between these two proteins.

Perhaps more surprising was the finding of FccA as the most abundant *c*-type cytochrome in the periplasmic fraction of ferric iron-reducing *S. oneidensis* cells. The fumarate reductase FccA is a tetraheme flavocytochrome that was previously shown to accept electrons directly from CymA to reduce fumarate (20). Because of its unexpected abundance, we reexamined its potential role in electron transfer to the outer membrane under Fe(III)-reducing conditions. We found in in vitro experiments a rapid, reversible electron transfer between MtrA and FccA (Fig. 4). We also provided evidence for an electron transfer between FccA and an MtrA/MtrB/OmcB outer membrane complex (Fig. 5). This electron transfer was not detectable in the absence of MtrA, again indicating a key role of MtrA in the membrane-spanning electron transfer to OmcB and OmcA. Therefore, under Fe(III)-reducing conditions, FccA could accept electrons from CymA and transfer them to MtrA. MtrA, in turn, could then transfer the electrons to OmcB. The highly abundant FccA could function as a periplasmic "overflow" buffer of electrons. Evidence for this role is provided in the comparative growth experiments that we conducted. Here Δ*fccA* cells reduced more ferric iron than the wild type, but this reduction was not coupled to an accelerated growth. On the contrary, wild-type cell counts were always higher than Δ*fccA* cell counts. Hence, we hypothesize that under conditions where electron transfer reactions to the terminal electron acceptor at the outer membrane are slow, FccA takes up electrons, either directly from CymA or mediated by other periplasmic *c*-type cytochromes such as MtrA. For a certain time, this electron uptake could ensure that the respiratory chain is not stalled due to the limiting terminal electron transfer reactions and the subsequently reduced electron transfer proteins. This strategy would also allow *Shewanella* cells to use a carbon and electron source even if no terminal electron acceptor is present, since the transient electron storage would act as an intermediate electron acceptor. Along these lines, it can be speculated that the realistic physiological function of FccA under environmental conditions might be to act as electron storage protein rather than as a fumarate reductase, since the availability of exogenous fumarate is usually low (4). This hypothesis would be in line with data from Butler et al. showing that *Geobacter sulfurreducens* does contain a fumarate reductase but that the physiological role of this enzyme is to act as a succinate dehydrogenase in the tricarboxylic acid cycle (4).

A highly dynamic network formed via periplasmic *c*-type cytochromes could promote a rapid dispatch of transiently stored electrons to various electron acceptors. We showed here a connection between the electron transport chains to fumarate and ferric iron. When cells are in contact with extracellular Fe(III) oxides, electrons derived from CymA are transferred via MtrA to OmcB, which is oxidized by the Fe(III) oxide. In the absence of such an insoluble electron acceptor, oxidation of OmcB, and consequently of MtrA, is blocked, and electrons are transferred from CymA either directly or indirectly, via MtrA, to the transiently electron-storing FccA. FccA can be rapidly reoxidized by MtrA when MtrA is oxidized by outer membrane OmcB once cells are contacting Fe(III) oxides again. In the presence of fumarate, a direct oxidation of FccA by fumarate would bypass the outer membrane electron transfer. This kind of connection could also exist to electron transport chains to other possible terminal electron acceptors such as nitrate, DMSO, or manganese oxides. This idea of an electron transport network is in line with data recorded by Firer-Sherwood et al. showing that at least the periplasmic *c*-type cytochromes CymA, CctA, and MtrA and the outer membrane cytochromes OmcB and OmcA have redox potentials that would allow for rapid interprotein electron transfer (8). Such a network could also explain the partial or general lack of phenotypes observed in mutants defective in periplasmic *c*-type cytochromes such as STC, SO3300, and SO3420 (3).

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