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# The Tetraheme Cytochrome CymA Is Required for Anaerobic Respiration with Dimethyl Sulfoxide and Nitrite in *Shewanella oneidensis*[†]

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ABSTRACT: The tetraheme *c*-type cytochrome, CymA, from *Shewanella oneidensis* MR-1 has previously been shown to be required for respiration with Fe(III), nitrate, and fumarate [Myers, C. R., and Myers, J. M. (1997) J. *Bacteriol. 179*, 1143−1152]. It is located in the cytoplasmic membrane where the bulk of the protein is exposed to the periplasm, enabling it to transfer electrons to a series of redox partners. We have expressed and purified a soluble derivative of CymA (CymA$_{sol}$) that lacks the N-terminal membrane anchor. We show here, by direct measurements of electron transfer between the purified proteins, that CymA$_{sol}$ efficiently reduces *S. oneidensis* fumarate reductase. This indicates that no further proteins are required for electron transfer between the quinone pool and fumarate if we assume direct reduction of CymA by quinols. By expressing CymA$_{sol}$ in a mutant lacking CymA, we have shown that this soluble form of the protein can complement the defect in fumarate respiration. We also demonstrate that CymA is essential for growth with DMSO (dimethyl sulfoxide) and for reduction of nitrite, implicating CymA in at least five different electron transfer pathways in *Shewanella*.

The proteobacterium *Shewanella oneidensis* MR-1 (formerly *Shewanella putrefaciens* MR-1) has attracted considerable interest due to its potential applications in bioremediation and metal leaching (*1*, *2*). *S. oneidensis* MR-1 respires with an unusually wide range of electron acceptors such as fumarate, nitrate, nitrite, TMAO,[1] DMSO, sulfite, thiosulfate, and elemental sulfur (*1*, *3*). Unusually, it is also capable of coupling the reduction of metal oxides to the oxidation of organic carbon (*4*). U(VI) and Tc(VI) are reduced to insoluble forms, presenting the possibility of using *Shewanella* for bioremediation of these heavy metals. Fe(III) and Mn(IV) form insoluble oxides and oxyhydroxides; hence reduction of these substrates presents a particular problem of access to any electron transfer chain. Interestingly, anaerobically grown *Shewanella* produces several multiheme *c*-type cytochromes that are localized to the outer membrane (*5*), and these may direct electrons from the cytoplasm to insoluble metal oxides at the outer membrane (*6*).

The genome sequence of *S. oneidensis* MR-1 has allowed the identification of 39 reading frames encoding *c*-type cytochromes (www.tigr.org) (*7*). Several of these are readily identified as homologues of well-characterized *c*-type cytochromes from other species, such as cytochrome $c_1$, a pentaheme nitrite reductase (NrfA), an oxidase of the cytochrome *cbb*$_3$ family, and CymA, a member of the NapC/NirT family.

CymA is a membrane-bound *c*-type cytochrome with a calculated molecular mass of 20.8 kDa (*8*). It contains four low-spin hemes each with bishistidine axial ligation (*9*). CymA possesses the common structural features of the NapC/NirT family, which are an N-terminal transmembrane α-helical anchor and a globular tetraheme periplasmic domain. Simon et al. have demonstrated in liposomal membrane experiments that NrfH, a member of the NapC/NirT family, mediates electron transfer between 2,3-dimethyl-1,4-naphthoquinol and NrfA, the periplasmic nitrite reductase from *Wolinella succinogenes* (*10*). Analysis of the genome sequence revealed that *S. oneidensis* MR-1 possesses a reading frame similar to that of the NrfA nitrite reductase, but no NrfH homologue was found other than CymA. The *cym*A knockout experiments of Myers and Myers (*8*) provided evidence that CymA is essential in the reduction of nitrate, iron(III), manganese(IV), and fumarate but not for the reduction of TMAO, indicating that CymA plays a key role in delivering electrons from the quinone pool to a number of terminal reductases.

Many *c*-type cytochromes are peculiar to *Shewanella*, such as the soluble, periplasmic fumarate reductase (*11*, *12*). Fumarate reductase is the terminal enzyme in a pathway that leads to the two-electron reduction of fumarate to succinate. Fumarate respiration is widespread in bacteria, and systems for quinol-dependent fumarate reduction in *Escherichia coli* and *W. succinogenes* have been well characterized (*13*, *14*). In these organisms the quinol:fumarate reductase (QFR) consists of three or four subunits: a catalytic subunit (FrdA), an electron-delivering protein with three iron−sulfur centers

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[1] Abbreviations: CymA$_{sol}$, soluble form of membrane-associated CymA; MGD, molybdopterin guanine dinucleotide; TMAO, trimethylamine *N*-oxide; DMSO, dimethyl sulfoxide; Fcc$_3$, flavocytochrome $c_3$.

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Table 1: Bacterial Strains and Plasmids Used in This Work

| strain or plasmid | description | source |
|---|---|---|
| *S. oneidensis* MR-1 | wild-type isolate from Lake Oneida (formerly *S. putrefaciens*) | *3* |
| MR-1A | rifampicin-resistant mutant of MR-1 | lab stock |
| SR-536 | MR-1A derivative, menB::Tn5, rif$^r$, kan$^r$ | *19* |
| CMTn-1 | transposon mutant of MR-1A unable to use Fe(III), nitrate, and fumarate as terminal electron acceptors rif$^r$, kan$^r$ | *8* |
| CS21a | MR1-A derivative hosting pRLSK4 | this work |
| CS21c | CMTn-1 derivative hosting pRLSK4 (rif$^r$, kan$^r$, Sm$^r$) | this work |
| *E. coli* TG1 | *supE hsdΔ5 thi (Δlac-proAB) F′[traΔpro proAB*$^+$ *lacI*$^q$ *lacZΔM15]* | lab stock |
| *E. coli* SM10 | *thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu* kan$^r$ | lab stock |
| pGEM-T | 3 kb PCR cloning vector, amp$^r$ | Promega |
| pDA12 | pGEM-T harboring MR-1 *cym*A gene | D. Atanasiu |
| pKK223-3 | expression vector, amp$^r$ | Pharmacia |
| pSPEX-1 | pKK223-3 harboring *S. frigidimarina* NCIMB400 *fcc*A gene | lab stock |
| pRLSK3 | pGEM-T with *fcc*A′-*cym*A construct | this work |
| pMMB503EH | 9.9 kb expression vector, Sm$^r$ | *20* |
| pRLSK4 | pMMB503EH harboring *fcc*A′-*cym*A construct | this work |

(FrdB), and either one or two membrane-attached subunits that interact in some cases via a *b*-type heme with the quinone pool (FrdCD). In *Shewanella* a different arrangement is found involving a soluble, periplasmic fumarate reductase, Fcc$_3$. This enzyme has been well characterized in terms of the reaction mechanism including electron and proton delivery pathways to the active site. The crystal structure reveals that Fcc$_3$ consists of FAD-binding and catalytic domains, which are conserved in FrdA, and a cytochrome domain, which is similar in function to the Fe-S subunit of FrdB, though structurally unrelated.

Electrons are transferred to the membrane-bound fumarate reductases directly from menaquinol in the cytoplasmic membrane. In *Shewanella,* the membrane-associated tetraheme *c*-type cytochrome, CymA, has been implicated in electron transfer between quinols and the periplasmic fumarate reductase (*15*). This reaction may involve direct electron transfer from CymA to the soluble fumarate reductase or could be mediated by another, unidentified redox protein. The *Shewanella* fumarate reductase, flavocytochrome $c_3$, has been extensively characterized, and the crystal structure and reaction mechanism have been determined in detail (*11*, *16*−*18*). Electrons enter fumarate reductase through a tetraheme *c*-type cytochrome domain and are transferred to the FAD at the active site for substrate reduction. In this paper we show direct electron transfer between CymA and fumarate reductase and demonstrate that CymA also mediates electron transfer in the pathways leading to reduction of nitrite and DMSO.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains, Plasmids, Media, and Growth Conditions.* Bacterial strains and plasmids used and constructed in this work are listed in Table 1. *E. coli* was routinely grown on LB medium at 37 °C. *S. oneidensis* MR-1 strains were cultivated on LB medium at 23 °C. For phenotypic studies *S. oneidensis* MR-1 strains were grown on minimal medium (*3*) supplemented with 100 mM sodium lactate (pH 7.4) and the following electron acceptors: fumarate (50 mM), DMSO (50 mM), and KNO$_2$ (250 $\mu$M). Prior to inoculation the medium was deoxygenated by bubbling with N$_2$ gas for 30 min. Antibiotics were added to plates and liquid media at the following concentrations: 100 $\mu$g mL$^{-1}$ ampicillin, 10 $\mu$g mL$^{-1}$ rifampicin, 50 $\mu$g mL$^{-1}$ streptomycin, and 50 $\mu$g mL$^{-1}$ kanamycin. No antibiotics were added to media for phenotypic studies.

*Construction of the Soluble Form of CymA (CymA$_{sol}$).* Figure 1 summarizes all of the PCR steps involved in the generation of CymA$_{sol}$. The vector pSPEX-1 was used as a template in a first PCR performed with the mutagenic primers 1 and 2 (Table 2). A single band of the expected size of 0.68 kb was purified from an ultrapure agarose gel. PCR 2 was performed using pDA12 as a template and primers 3 and 4. PCR 2 produced a single expected band of 0.48 kb and was further purified as PCR product 1. In a third PCR the two overlapping fragments were fused together using primers 1 and 4. The amplified product of 1.16 kb was subsequently cloned into pGEM-T to form pRLSK3. The *Eco*RI and *Bam*HI restriction sites were used to excise the *fcc*A′-′*cym*A coding sequence and transfer it into the broad host-range expression vector pMMB503EH (*20*) to form pRLSK4. This vector contains the *tac* promoter and the lacI$^q$ gene, allowing induction of recombinant protein expression by IPTG. This plasmid, pRLSK4, was then transferred by conjugation from the donor strain *E. coli* SM10 to *S. oneidensis* MR-1A (CS21a) and CMTn-1 (CS21c). The transconjugants were selected on LB plates containing rifampicin, streptomycin, and kanamycin in the case of CS21c.

*Purification of CymA$_{sol}$ and Flavocytochrome $c_3$ from S. oneidensis MR-1. S. oneidensis* MR-1 CS21a was grown microaerobically to an OD$_{600}$ of ∼0.7, induced with 0.5 mM IPTG, and incubated for 18 h at 23 °C. Cells were harvested, and a periplasmic extraction, including an osmotic shock and EDTA treatment, was performed. The periplasmic extract was loaded directly onto a weak anion exchanger (DE52, Whatman), and a fraction containing CymA$_{sol}$ and Fcc$_3$ was eluted with 0.1 M NaCl in 50 mM Tris, pH 8.0. The DE52 resin was equilibrated prior to loading with 50 mM Tris, pH 8.0. In the next step a Q-Sepharose column (Pharmacia Biotech), equilibrated with 10 mM Tris, pH 7.5, was used to separate CymA$_{sol}$ and Fcc$_3$ using a salt gradient from 0 to 1.0 M NaCl in 10 mM Tris, pH 7.5, at a flow rate of 3 mL/min. CymA$_{sol}$ eluted over a range of salt concentrations whereas Fcc$_3$ eluted at 200 mM NaCl. Size exclusion chromatography with Superdex 75 and 200 columns (Pharmacia Biotech) was used as a final step to purify CymA$_{sol}$ and Fcc$_3$ at a flow rate of 2 mL/min. Both columns were equilibrated with 1.5 column volumes of running buffer and 10 mM Tris, pH 8.0. Pure protein was obtained with a yield of approximately 0.5 mg/L of culture.

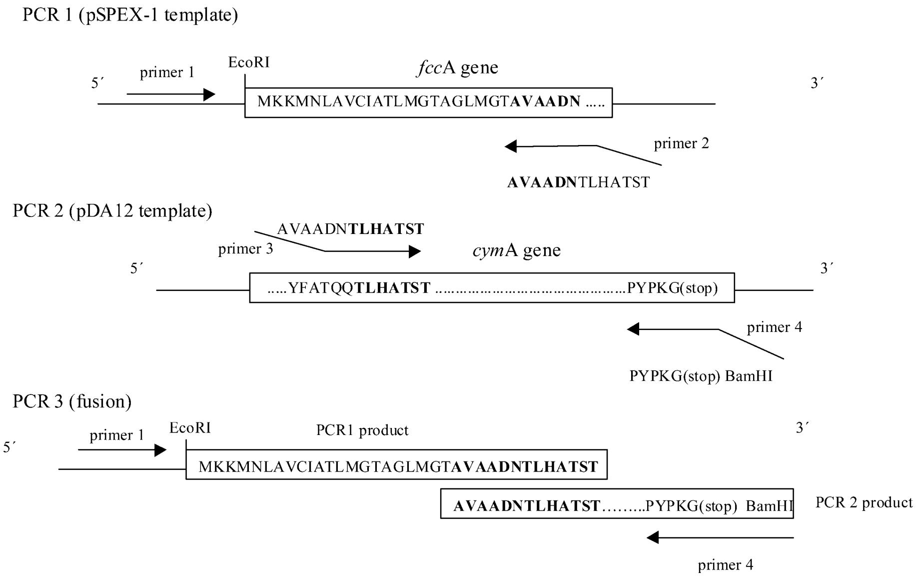

FIGURE 1: Construction of CymA$_{sol}$. DNA encoding the signal sequence from flavocytochrome $c_3$ and approximately 500 bp upstream from this was amplified from pSPEX-1 using primers 1 and 2. The sequence encoding all of CymA except the N-terminal anchor sequence was amplified from pDA12 using primers 3 and 4. A second PCR using the products of the first two reactions as template was performed with primers 1 and 4 to generate an in-frame fusion of the Fcc$_3$ signal sequence with the amplified CymA sequence. The sequence encoding the fusion protein was subsequently transferred to the expression vector pMMB503EH using the *Eco*RI and *Bam*HI sites located immediately before the start codon and after the stop codon, respectively.

Table 2: Primer Sequences

| primer | sequence (5′ to 3′) |
|---|---|
| 1 | CTGACTGGGTTGAAGGCTCTCAAGG |
| 2 | CGACAACGCCGACTATTGTGAAATGTACGCTGTTC |
| 3 | GCTGTTGCGGCTGATAACACTTTACATGCGACAAG |
| 4 | CACGGATCCCTTATCCTTTTGGATAGG |

*Kinetic Analysis of Electron Transfer from CymA$_{sol}$ to Flavocytochrome $c_3$ (Fcc$_3$).* The fumarate-dependent reoxidation of reduced CymA$_{sol}$ was monitored at 552 nm. All experiments were carried out in 5 mm path-length cuvettes with a Shimadzu UV-PC 1201 spectrophotometer contained in a Belle Technology glovebox ($O_2$ < 5 ppm) at 25 °C. The assay buffer contained 0.5 M NaCl and 50 mM Tris, pH 7.2. CymA$_{sol}$ was added to give a range of concentrations (0−40 $\mu$M protein), and fumarate was present at a concentration of 5 mM. CymA$_{sol}$ was reduced completely by addition of sodium dithionite, and the reaction was initiated by addition of the enzyme (Fcc$_3$) to a concentration of 1.76 nM.

*Miscellaneous Procedures.* Protein purification was routinely followed by SDS−PAGE according to Laemmli (*21*). Gels were stained for peroxidase activity using 3,3′,5,5′-tetramethylbenzidine to reveal the presence of *c*-type cytochromes (*22*). $NO_2^-$ was quantified by means of the colorimetric Griess assays. The formation of a stable azo compound was monitored at 540 nm (World Precision Instruments).

## RESULTS

*Expression and Characterization of CymA$_{sol}$.* Since Fcc$_3$ lacks a membrane-anchoring subunit and CymA, a membrane-bound tetraheme *c*-type cytochrome, is essential for fumarate respiration in *Shewanella,* we recently proposed that Fcc$_3$ interacts directly with CymA (*15*). To test this, we have constructed a soluble form of CymA, named CymA$_{sol}$. A PCR approach (Figure 1) was used to replace the hydrophobic N-terminus of CymA with the signal sequence of the periplasmic Fcc$_3$ plus the first three amino acids of mature Fcc$_3$ (ADN) to ensure cleavage of the signal sequence. CymA$_{sol}$ was purified, and the identity of the purified protein was confirmed by mass spectrometry and N-terminal sequencing. The mass determined by electrospray mass spectrometry was 19760 ±10 Da compared with the calculated mass of 19752 Da. The N-terminal sequencing showed that the signal sequence was indeed removed and the first five amino acids were ADNTL, as expected. Redox potentiometry of CymA$_{sol}$ by conventional methods (*23*) showed that the heme groups titrated at low potential, but potentials for individual hemes could not be resolved. Data obtained from repeated redox titrations, using dithionite and ferricyanide, respectively, as the reducing and oxidizing agents, could be fitted to two pairs of hemes having midpoint reduction potentials of $-175 \pm 5$ and $-261 \pm 5$ mV (*15*). Figure 2 summarizes the reduction potentials of all the proposed redox centers involved in fumarate reduction in *Shewanella*.

To determine whether electron transfer between CymA and Fcc$_3$ is direct, we performed a series of assays monitoring the fumarate-dependent oxidation of reduced CymA$_{sol}$ under anaerobic conditions. Figure 3 shows the CymA$_{sol}$ concentration-dependent rate of turnover profile. Observed rate constants, $k_{obs}$, were measured at different CymA$_{sol}$ concentrations, and experiments repeated with different batches of protein showed similar curves. The initial linear increase of observed rate constants could be fitted to a second-order rate constant of $19 \pm 1$ $\mu M^{-1}$ $s^{-1}$ (dotted line). The observed rate constants peaked at a CymA$_{sol}$ concentration of 20 $\mu$M. Interestingly, observed rate constants dropped at CymA$_{sol}$ concentrations higher than 20 $\mu$M. The deviation did not fit to a Michaelis−Menten curve and could not be explained either by reversible dimerization of the substrate CymA$_{sol}$ to form an inactive complex or by a second inhibitory binding site on Fcc$_3$. Similar profiles were found in low salt buffer

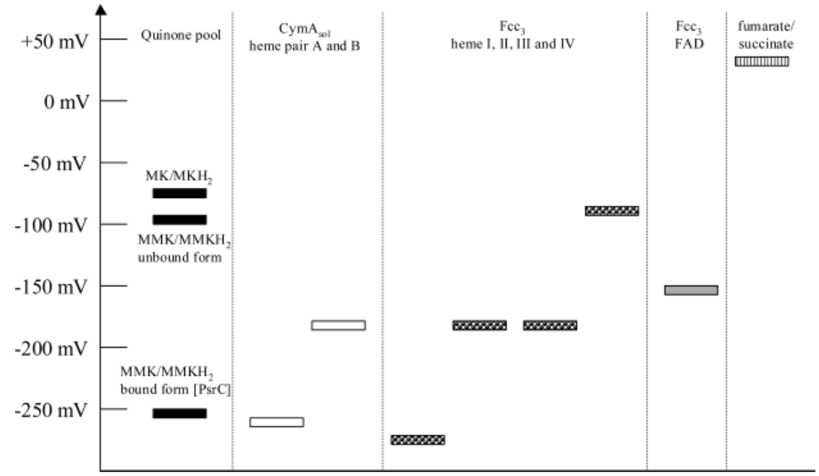

FIGURE 2: Thermodynamic profile of fumarate reduction in *Shewanella*. The reduction potentials of each of the redox centers implicated in fumarate reduction, i.e., in CymA and in Fcc$_3$, are indicated (*15*, *24*). All of these fall well below the potential of the fumarate/succinate couple. The nature and potential of the quinol reductant for CymA are unknown, but the reduction potentials for menaquinone (MK) and methylmenaquinone (MMK) are shown, along with the value reported for MMK bound to polysulfide reductase (*29*).

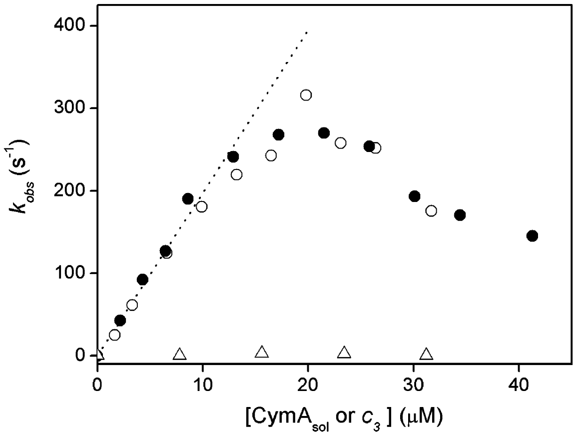

FIGURE 3: Electron transfer from CymA$_{sol}$ to Fcc$_3$. The oxidation of CymA$_{sol}$ was followed spectrophotometrically at constant Fcc$_3$ but varying CymA$_{sol}$ concentrations. The observed electron transfer rate constant ($k_{obs}$) is plotted against the concentration of CymA$_{sol}$ for each of two separate batches of protein (closed circles, batch 1; open circles, batch 2). The reaction of Fcc$_3$ with *Shewanella* cytochrome $c_3$ was followed under the same conditions (triangles).

(50 mM NaCl, 50 mM Tris, pH 7.2) and in the presence of a 10-fold higher Fcc$_3$ concentration (data not shown). The reaction between CymA$_{sol}$ and Fcc$_3$ is specific since no turnover was observed with *Shewanella* cytochrome $c_3$, a small low-potential tetraheme periplasmic protein (*25*), despite the reaction being thermodynamically favorable.

*Complementation of Respiratory Defects by Soluble CymA.* Previous studies have shown that CymA is essential for fumarate respiration in *Shewanella* (*8*). Phenotypic studies were designed to determine whether CymA$_{sol}$ could complement the CMTn-1 strain, which lacks endogenous CymA, and restore the wild-type phenotype. Minimal medium was supplemented with lactate as an electron donor and fumarate as the electron acceptor. The data presented in Figure 4A verify that the *cym*A knockout strain, CMTn-1 (*8*), is unable to grow anaerobically with fumarate whereas the wild-type MR-1 strain grows rapidly. CMTn-1 harboring the CymA$_{sol}$ expression vector (strain CS21c) failed to grow under noninducing conditions, but in the presence of IPTG this strain not only grew but surprisingly grew faster and to a higher final density than the wild type. Similar results were obtained with ferric citrate as the terminal electron acceptor (data not shown).

CymA is involved in respiration not only with fumarate but also with Fe(III) and nitrate (*8*). The protein is not required for TMAO respiration (*8* and our data not shown), but we examined the possibility that it may be involved in respiration with DMSO and nitrite. We tested the ability of the CMTn-1 knockout strain to grow with DMSO as the sole electron acceptor (Figure 4B) and to utilize nitrite (Figure 4C). In both cases the mutant is defective, but the defect is complemented by expression of CymA$_{sol}$. Growth on DMSO was indistinguishable from growth of wild-type MR-1 whereas nitrite was consumed only about half as fast by the complemented mutant compared with MR-1.

During our complementation studies we also tested a menaquinol-deficient derivative of MR-1 (SR536). Saffarini et al. (*19*) isolated and characterized SR536 as having a transposon insertion in *menB*, a component of the menaquinol biosynthesis operon. SR536 behaved in all cases like the *cym*A knockout strain (Figure 4).

## DISCUSSION

Although the route of electron transfer in membrane-bound fumarate reductases is well-defined, much is still to be elucidated in the case of the soluble flavocytochrome $c_3$. It has been speculated that the membrane-bound *c*-type cytochrome CymA functions as the physiological electron donor to the periplasmic fumarate reductase and therefore replaces FrdCD. In previous work with a *cym*A knockout strain, Myers et al. (*8*) showed the importance of CymA for respiration on nitrate, fumarate, and Fe(III). Our studies with the soluble form of CymA indicate that CymA$_{sol}$ and flavocytochrome $c_3$ interact directly. An observed second-order rate constant of 19 $\mu M^{-1}$ $s^{-1}$ demonstrates the high efficiency of electron transfer between these partners in vitro.

As expected from thermodynamic considerations, the reduction potentials of the hemes in CymA are significantly lower than that of the fumarate/succinate couple, with the heme and flavin prosthetic groups of Fcc$_3$ generally lying intermediate to these. It is therefore simple to envisage thermodynamically favorable electron transfer from CymA

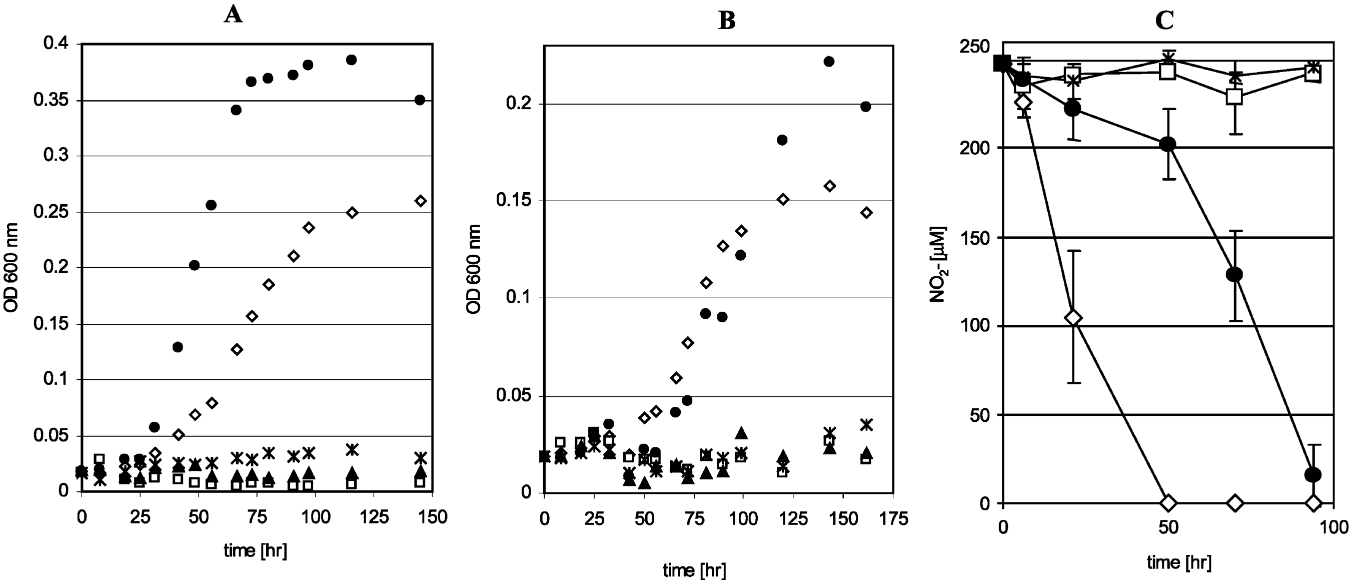

FIGURE 4: Phenotypic analysis of the *cym*A knockout strain and complementation with $CymA_{sol}$. Growth with fumarate (A) or DMSO (B) as sole electron acceptor was followed by measuring the optical density at 600 nm ($OD_{600}$). The ability to reduce nitrite in vivo was determined by measuring residual $NO_2^-$ in the growth medium (C). The following strains were used: MR-1A (◇), CMTn-1 (□), CS21c (▲, without induction; ●, after induction with 0.5 mM IPTG), and SR536 (×).

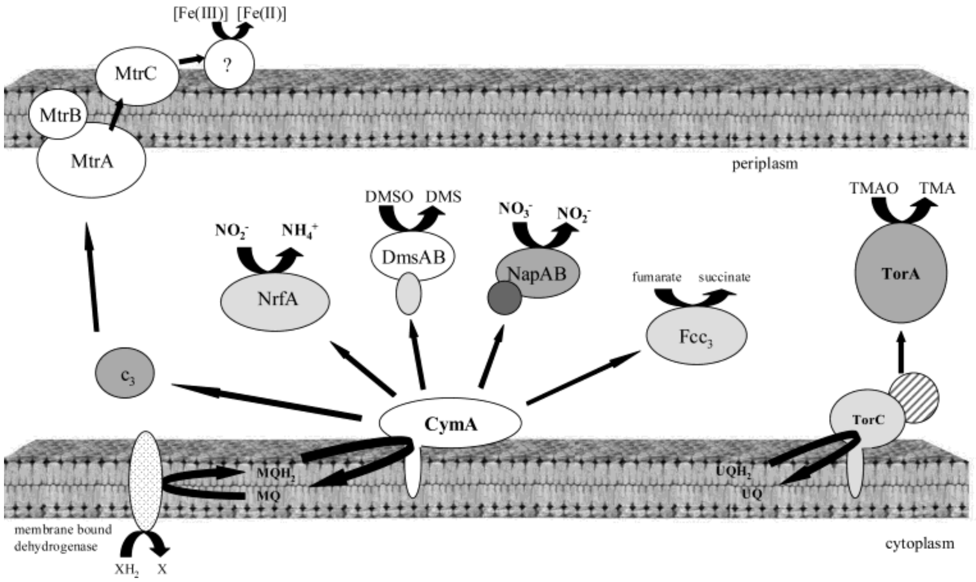

FIGURE 5: Key position of CymA in anaerobic respiration. Electron flow is indicated by the arrows with CymA in the cytoplasmic membrane involved in transfer to Fe(III) at the outer membrane, using cytochrome $c_3$ as a periplasmic electron shuttle (*23*), and in electron transfer to four periplasmic reductases. Direct electron transfer between quinols and CymA has not been demonstrated, but no evidence exists for a requirement for further redox proteins in this part of the pathway.

via the fumarate reductase to fumarate. In contrast, the reduction of CymA by menaquinol is, in principle, an uphill reaction. It is possible that CymA is reduced by one of the lower potential menaquinone derivatives, e.g., methylmenaquinone. MMK-7, a methylated menaquinone derivative, has been purified from *Shewanella* membranes (*27*), and the methyl group in the aromatic ring of naphthoquinones, such as menaquinone, has been shown to result in a lowering of the reduction potential (*28*). It has been postulated that in the *W. succinogenes* polysulfide reductase system a bound form of MMK-6 could have a reduction potential of −260 mV compared to −90 mV in the unbound state (*29*).

Our phenotypic studies have shown that $CymA_{sol}$ can complement the *cym*A knockout strain in vivo. This leads to interesting speculation on the role of the N-terminal anchor. It is possible that $CymA_{sol}$ associates with the membrane despite the absence of the anchor sequence. Presumably it must do so at least transiently to allow electron transfer from the quinol, though we do not know how it interacts either with lipid or with a membrane protein. We have demonstrated that $CymA_{sol}$ interacts directly with the fumarate reductase. It has not been shown whether CymA is reduced directly by quinol, though this is quite likely. If so, then the pathway resulting in the reduction of fumarate consists only of the membrane-located quinol, membrane-bound CymA, and the periplasmic flavocytochrome $c_3$.

CymA was previously shown to be required for respiration with fumarate, nitrate, and Fe(III) but not TMAO (*8*). Further examination of the *cym*A knockout strain has shown that this protein is also an essential component in the electron transfer pathway to DMSO and nitrite.

*Shewanella massilia* has a well-characterized TMAO-inducible operon (torECAD) which encodes the components needed for respiration with TMAO (*30*). A similar operon is found in the *S. oneidensis* genome. TorC belongs to the same family as CymA although it possesses a fifth heme group in an additional C-terminal domain. It has also been shown that menaquinol is required for respiration with DMSO in *S. oneidensis* but not with TMAO (*19*). It thus appears that DMSO respiration requires menaquinol (or a modified low-potential derivative) and CymA, whereas TMAO respiration requires ubiquinol and TorC.

The *E. coli* DMSO reductase system consists of a heterotrimeric, membrane-bound enzyme comprising a MGD-containing catalytic subunit (DmsA), an iron−sulfur protein (DmsB) responsible for electron transfer, and an integral membrane subunit with no apparent cofactors (DmsC) (*31*, *32*). The corresponding enzyme from *Rhodobacter capsulatus* is a soluble, periplasmic molybdoenzyme, encoded by the DorA gene that is closely related to DmsA from *E. coli*. Electrons are delivered to the enzyme by a pentaheme *c*-type cytochrome associated with the membrane (*33*). DorC is a member of the NapC/NirT family and shows high similarity to TorC from *E. coli*. Analysis of the *S. oneidensis* genome sequence revealed the presence of two DmsA and DmsB homologues, each encoded in a small operon, but intriguingly no homologues of DmsC were found (www.tigr.org). DmsA1 (SO1429) and DmsA2 (SO4358) show 46.7% and 39.2% identity to DmsA from *E. coli* K12-MG1655. DmsB1 (SO1429) and DmsB2 (SO4357) are about 60% identical to DmsB from *Yersinia pestis*. We suggest that CymA delivers electrons directly to the DmsB subunit of DMSO reductase. If this is the case, we can view the DMSO reductase system in *S. oneidensis* as a hybrid consisting of features of the two well-characterized systems described above. Two more genes are found in these *dms* operons. Open reading frames SO1427 and SO4360 encode a homologue of MtrA, a decaheme *c*-type cytochrome associated with the outer membrane and implicated in metal reduction (*34*), whereas SO1428 and SO4359 appear to encode outer membrane proteins with no known cofactors. The possible role of the products of these reading frames in DMSO respiration is unclear.

The *S. oneidensis* genome also encodes a homologue of NrfA, a pentaheme *c*-type cytochrome that catalyzes the six-electron reduction of nitrite to ammonia. The crystal structures of this enzyme isolated from *E. coli*, *W. succinogenes*, and *Sulfurospirillum deleyianum* have been determined (*35*−*37*). Electrons are delivered to NrfA in *W. succinogenes* and *E. coli* by the membrane-bound tetraheme *c*-type cytochrome NrfH (*18*, *38*), a homologue of CymA. *Shewanella* possesses an open reading frame (SO3980) with 79.3% similarity to NrfA from *E. coli*, but no NrfH homologue is found nearby that might be cotranscribed. Since our results implicate CymA in nitrite reduction in *Shewanella*, we propose that the role of NrfH is taken by CymA in transferring electrons from quinols to the periplasmic NrfA nitrite reductase.

In this work we have demonstrated how a soluble form of CymA transfers electrons efficiently to the soluble fumarate reductase of *S. oneidensis*. Furthermore, we have shown in phenotypic studies that $\text{CymA}_{\text{sol}}$ restores fumarate respiration in the *cym*A knockout strain and that CymA appears crucial in the pathways leading to the reduction of DMSO and nitrite. CymA is therefore a key player in the branched respiratory pathways of *Shewanella*, supplying electrons to at least five different terminal reductases (Figure 5).

## ACKNOWLEDGMENT

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