Characterization of the *Shewanella oneidensis* MR-1 Decaheme Cytochrome MtrA

**EXPRESSION IN ESCHERICHIA COLI CONFERS THE ABILITY TO REDUCE SOLUBLE Fe(III) CHELATES**

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*Shewanella oneidensis* MR-1 has the metabolic capacity to grow anaerobically using Fe(III) as a terminal electron acceptor. Growth under these conditions results in the *de novo* synthesis of a number of periplasmic c-type cytochromes, many of which are multiheme in nature and are thought to be involved in the Fe(III) respiratory process. To begin a biochemical study of these complex cytochromes, the mtrA gene that encodes an approximate 32-kDa periplasmic decaheme cytochrome has been heterologously expressed in *Escherichia coli*. Co-expression of mtrA with a plasmid that contains cytochrome c maturation genes leads to the assembly of a correctly targeted holoprotein, which covalently binds ten hemes. The recombinant MtrA protein has been characterized by magnetic circular dichroism, which shows that all ten hemes have bis-histidine axial ligation. EPR spectroscopy detected only eight of these hemes, all of which are low spin and provides evidence for a spin-coupled pair of hemes in the oxidized state. Redox titrations of MtrA have been carried out with optical- and EPR-monitored methods, and the hemes are shown to reduce over the potential range −100 to −400 mV. In intact cells of *E. coli*, MtrA is shown to obtain electrons from the host electron transport chain and pass these onto host oxidoreductases or a range of soluble Fe(III) species. This demonstrates the promiscuous nature of this decaheme cytochrome and its potential to serve as a soluble Fe(III) reductase in intact cells.

Bacterial iron respiration is the process in which the Fe(III) acts as the terminal electron acceptor in an energy-conserving bacterial respiratory electron transport chain. It is an anaerobic process that provides a bacterium with a means of respiration when oxygen is absent and is distinct from the process of iron assimilation in which iron is taken up into the cell by energy-consuming systems and incorporated into cell biomass. The capacity for Fe(III) respiration is phylogenetically wide-spread, and the ecological impact in microoxic and anoxic soils and sediments is considerable (1–6). A particular problem for Gram-negative bacteria is that at circum-neutral pH, the speciation of Fe(III) is complex because the cation is present as either insoluble polynuclear oxo/hydroxo-bridged complexes or as soluble organic chelates. Thus, depending on the environment, Fe(III) is presented to bacteria in a range of forms that can vary in solubility, steric properties, and overall charge. The best characterized group of Fe(III)-respiring bacteria are species of the genus *Shewanella*, which are widespread facultative anaerobes of the γ-proteobacteria group that can express a number of terminal respiratory reductases including nitrate, nitrite, fumarate, and trimethylamine N-oxide reductases (7). During growth under iron-respiring conditions, *de novo* synthesis of a number of multiheme c-type cytochromes that may play a role in the Fe(III) respiration process occurs. Cytochrome oxidation has been observed during Fe(III) reduction, and a number of genetic loci that encode multiheme cytochromes have been implicated as being important for Fe(III) respiration (8–11). These include *cymA*, which encodes an inner membrane periplasmic tetraheme quinol dehydrogenase (CymA), and the *mtrDEF-OmcA-MtrCAB* gene cluster, which encodes three outer membrane decaheme cytochromes (MtrF, OmcA, and MtrC), two periplasmic decaheme cytochromes (MtrD and MtrA), and two putative outer membrane β-barrel proteins (MtrE and MtrB). In addition some small periplasmic tetra- and triheme cytochromes such as the small tetraheme cytochrome c and iron-induced flavocytochrome c may also play a role in Fe(III) respiration (12, 13). Consideration of the different cellular locations of these cytochromes has led to the proposal of a mechanism of electron transfer for iron respiration in which electrons are transferred from quinol via CymA to periplasmic multiheme cytochromes (10, 11, 14). These periplasmic cytochromes may then be able to nonspecifically reduce soluble Fe(III) chelates, but in order to reduce insoluble Fe(III) complexes, the electrons must then be passed to outer membrane cytochromes, e.g. OmcA.

Much of the work that has led to the development of this model for Fe(III) respiration has been done on two species, *Shewanella frigidimarina* NCIMB400 and *Shewanella oneidensis* MR-1. The genome sequence of the latter has recently been completed and reveals that there are around forty genes encoding c-type cytochromes in this organism, many of which are predicted to be multiheme in character (15). The large number of c-type cytochromes synthesized during growth in the presence of Fe(III) makes it difficult to assess the properties and function of individual cytochromes *in vivo*. In this work, we have thus begun to resolve the properties and functions of
individual cytochromes in Fe(III) respiration through heterologous expression of mtra in Escherichia coli with the aim of providing both large quantities of protein for biophysical analysis and insight into any new metabolic capacity that the expression of the gene confers.

EXPERIMENTAL PROCEDURES

Expression of mtra in E. coli—The mtra gene (GenBank™ accession number AF083240) was amplified by PCR using genomic DNA prepared from S. oneidensis MR-1 as the template, suitable homologous primers, and TaqDNA polymerase. An AccelTaq Vector™ kit (Novagen and CN Biosciences) was used to ligate the 1-kb PCR product into the β-galactosidase gene of pETBlue-1, now called pKPl. Plasmid DNA was prepared from the resulting white colonies and checked by single and double digests with FnuDI and NcoI, both cut one restriction site each on the mtra gene. Expression host cells, E. coli JM109(DE3), were transformed first with the vector pC86 containing the ccmABC-DEFGH (cytochrome c maturation) genes (16). These cells were grown and subsequently transformed with pKPl.

Growth Conditions—Cells of the E. coli JM109(DE3) (pKPl + pEC86) strain were grown aerobically at 37 °C in Luria-Bertani medium (10 g/liter tryptone, 10 g/liter NaCl, 5 g/liter of yeast extract, pH adjusted to 7.5) until they reached an optical density of approximately 1.0. They were then induced with 0.1 mM isopropyl-1-thio-galactopyranoside and aeration immediately (17, 18). For quantification of periplasmic cytochromes by heme-linked peroxidase staining as described previously (13), the cells were treated with polymixin B (1 mg/ml) to break the outer membrane and were incubated at 37 °C for 1 h. The lysed cells were spun at 15,000 × g and 4 °C for 45 min. The resulting periplasmic fraction was found to contain the cytochrome and was loaded onto a DEAE-Sepharose (fast flow) anion exchange column. Spin quantification of the EPR species was performed with an Oxford Instrument ESR-9 liquid helium flow cryostat and a dual mode EPR cavity. Spin quantification of the EPR species was performed with an Oxford Instrument ESR-9 liquid helium flow cryostat and a dual mode EPR cavity.

General Analytical Procedures and Cytochrome Oxidation Kinetics—Analyses of the cell extracts and purified proteins were routinely performed with SDS-PAGE. Gels were examined for the presence of c-type cytochromes, the gene for which was cloned into pETBlue-1 to yield pKPl. This and pEC86, which contains genes that encode cytochrome c maturation (Ccm™) and that are required for the coherent attachment of heme via thioether linkages to polypeptide chains in the periplasmic compartment, was transformed into E. coli JM109(DE3). Colonies of these cells were notably pink in color. Visible spectra of aerobically grown cultures of E. coli JM109(DE3) (pKPl + pEC86) collected between 500 and 600 nm revealed intense absorption bands at ~550 nm (Fig. 1C). This is characteristic of the α-absorbance band of reduced c-type cytochrome, which was absent in E. coli JM109(DE3) (pEC86), and E. coli JM109(DE3) (pKPl), all of which exhibited weak absorption bands at 560 nm that are characteristic of reduced b-type cytochromes and are likely to arise from endogenous cytochromes such as the cytochrome bo oxidase. Periplasmic and membrane fractions were prepared from aerobic cultures of E. coli JM109(DE3) (pKPl + pEC86) that had been induced with isopropyl-1-thio-β-galactopyranoside. Heme-stained SDS-PAGE gels of these fractions revealed the presence in both fractions of a 35-kDa heme-staining band that is the expected size of Mtra (Fig. 1B). This band was absent in E. coli JM109(DE3) (pEC86) and JM109(DE3) (pKPl + pEC86) but absent from JM109(DE3). This corresponds to the size of the heme-binding cytochrome c maturation protein CcmE, which is

The abbreviations used are: Ccm, cytochrome c maturation; Em, midpoint redox potential; NTA, nitrilotriacetic acid; HQNO, 2-n-heptyl-4-hydroxyquinoline-N-oxide; NIR, near infrared; Malto, 3-hydroxy-2-methyl-4-pyrene.
membrane-associated and encoded by pEC86. Additional faint bands at 24 kDa could be seen in membranes of JM109(DE3) (pEC86) and JM109(DE3) (pKP1 + pEC86), which correspond to the size expected of the membrane-anchored tetraheme quinol dehydrogenase, NapC, of the NaAB nitrate reductase system. A heme-staining band at around 55 kDa was present in all of the periplasm and membrane samples. This corresponds to the periplasmic pentaheme NrfA nitrite reductase protein. The observation that some of the periplasmic NrfA “sticks” to membrane preparations has been reported previously (17). The nap and nrf genes are characteristically expressed during anaerobic growth of E. coli. The synthesis of the Nap and Nrf cytochromes revealed by heme staining was confirmed by direct enzymological assays for nitrate and nitrite reductase and suggests that a low level of nap and nrf expression is occurring as a result of the cultures becoming oxygen-limited at high cell densities.

Taken together, the data from the heme-stained SDS-PAGE analysis suggest that E. coli can express mtrA and correctly locate and assemble holo-MtrA in the periplasm when mtrA is co-expressed with the ccm genes in E. coli JM109(DE3) (pKP1 + pEC86). Similar to NrfA, it seems that some MtrA will also stick to membranes and this finding suggests that it can associate with the periplasmic face of either or both inner and outer membranes. Quantification of the reduced absorption band at 552 nm in the intact cells that have synthesized MtrA with an extinction coefficient (derived from purified protein) of 280 mM$^{-1}$ cm$^{-1}$/heme reveals that around 6 nmol protein (mg cells)$^{-1}$ is produced.

**Purification and Characterization of MtrA**—The recombinant 35-kDa MtrA was purified using a combination of anion exchange and gel-filtration chromatographies (see “Experimental Procedures”). The protein stained poorly with Coomassie Blue but was judged to be pure by the absence of additional bands on Coomassie Blue-stained gels (Fig. 2A, inset). Purified protein has a ratio of $A_{408}/A_{280}$ nm of $-4.5$. An analysis of the native protein in solution by dynamic light scattering revealed
the MtrA samples to be monodisperse with an approximate molecular mass of 30 kDa, suggesting that it is monomeric in solution. An analysis using the program SignalP (21) suggested a cleavage site for the signal peptide sequence after position 34 in the MtrA pre-protein amino acid sequence (Fig. 9). This would suggest a processed molecular mass of 32,422 Da for the apoprotein and a total mass of 38,584 Da with the 10 predicted c-hemes. An analysis of MtrA by MALDI-TOF mass spectrometry gave a molecular mass value of 38,581 kDa, and this suggests that the heterologously synthesized protein does indeed bind ten hemes.

Optical Redox Titration—The optical spectrum of oxidized MtrA has a Soret (γ) band centered at 408 nm, a visible region peak at 531 nm and a shoulder at 560 nm. The spectrum is typical of low spin ferric heme. Upon reduction, the Soret band λmax shifts to 420 nm and β- and α-peaks at 525 and 552 nm that are characteristic of low spin ferrous c-type cytochromes appear (Fig. 2A). Pyridine hemochromagen assays give a value of $-132 \text{ mm}^{-1} \text{ cm}^{-1}$ heme for the MtrA Soret band, which is a value typical of low spin hemoproteins (18,22). This would correspond to an extinction coefficient of $1,320 \text{ mm}^{-1} \text{ cm}^{-1}$ for the entire protein. Absorption spectra of MtrA were collected at a range of potentials between +400 and −450 mV. An increase in absorbance at 552 nm was observed over the range of approximately −100 to −450 mV. The change in absorption at 552 nm is best fitted to two groups of hemes that titrate as $n = 1$ components and that account for 30 and 70% of the total absorption change with midpoint potentials, $E_{\text{m}}$, of −375 and −200 mV (Fig. 2B). This simple fit suggests that three hemes have near iso-midpoint potentials at −375 mV and seven hemes have near iso-midpoint potentials at −200 mV. These values are in the range that is commonly found for bis-His ligated hemes as a result of the electron-donating properties of the imidazole ring nitrogens, which serve to stabilize the oxidized state (22).

Magnetic Circular Dichroism of MtrA—The size and position of signals in UV-visible region of the spectrum, which is shown in Fig. 3A, is consistent with ferric low spin heme, with a Soret derivative cross-over at 407 nm and a peak-to-trough intensity of 97 mm$^{-1}$ cm$^{-1}$ heme. In fact, the Soret and visible region is of 97 mm$^{-1}$ cm$^{-1}$ heme. Upon reduction, the Soret band λmax shifts to 420 nm and 1 component and that account for 30 and 70% of the total absorption change with midpoint potentials, $E_{\text{m}}$, of −375 and −200 mV (Fig. 2B). This simple fit suggests that three hemes have near iso-midpoint potentials at −375 mV and seven hemes have near iso-midpoint potentials at −200 mV. These values are in the range that is commonly found for bis-His ligated hemes as a result of the electron-donating properties of the imidazole ring nitrogens, which serve to stabilize the oxidized state (22).

Electron Paramagnetic Resonance of Oxidized MtrA—The 10 K perpendicular mode X-Band EPR spectrum of air-oxidized MtrA (Fig. 4A) contains a number of low spin ($S = 1/2$) rhombic species. Normally three features are observed in rhombic spectra, but in these spectra, only the $g_{\text{max}}$ ($g_y$) features are clearly observed. The second feature is an overlay of at least two $g_y$.
incubated anaerobically, and NaNO₃, NaNO₂, or Fe(III) Maltol was added (100 nm) at the time indicated by the gmax values of 2.95 and 2.88 and a second feature in the derivative at the heme in which the histidine imidazole ring planes are approximately parallel to each other. Spin integration of the signals reveals that these two major low spin species each account for ~40% of the heme population. There is also a positive feature at g = 2.43 that is likely to arise from a small population of low spin histidine/hydroxide heme, probably from a heme or hemes in which one of the histidine ligands has become detached, and a derivative feature at g = 2.06, which may be assigned to adventitious copper.

Approximately 20% of the heme population is unaccounted for by the integrations of the low spin features. This population of heme may account for a pair of spin-coupled hemes, which could be EPR silent. However and notably, there is a broad trough at g = 1.80 that could in principle arise from a small population of low spin histidine/hydroxide heme, probably from a heme or hemes in which one of the histidine ligands has become detached, and a derivative feature at g = 2.06, which may be assigned to adventitious copper.

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EPR-monitored Potentiometric Titration of MtrA—A potentiometric titration of MtrA monitored by EPR was carried out to probe for additional heme species or extra intensity that could occur as magnetically interacting species are reduced (Fig. 4B). The addition of mediators (detailed under “Experimental Procedures”) did not change the EPR spectrum of the oxidized protein. However, at ~53 mV, a potential at which the optical redox titer does not show any reduction of the heme, the gmax at 2.88 has diminished, whereas the gmax at 2.95 has increased and now accounts for 80% of the heme concentration. This may indicate a redox-dependent conformational change in the protein that has a knock-on effect on the heme ligand geometry. At the same potential, the broad trough at g = 2.06 has increased in intensity and the adventitious copper signal at g = 2.06 in the oxidized spectrum has been reduced out of the spectrum. With further reduction, the low spin rhombic trio at g = 2.95 and g = 2.28 continue to diminish (the third feature expected at approximately g = 1.5 is not observed). Thus at ~118 mV, the 2.95 peak integrates to 50% of the heme population but accounts for only 5% of the heme content at ~298 mV. No new species are observed in the poised samples. At ~453 mV, there is a radical species at g = 2.0 in the spectrum that is likely to arise from reduced methyl or benzyl viologen.

The titer of the g = 2.95 signal and the broad trough at g = 1.80 are shown in Fig. 4B. Note that as the EPR titer measures the decrease in peak height, the y axis points have been multiplied by 1. The EPR titers follow a similar trend to the optical redox titers but accounts for only 5% of the heme content at ~298 mV. No new species are observed in the poised samples. At ~453 mV, there is a radical species at g = 2.0 in the spectrum that is likely to arise from reduced methyl or benzyl viologen.

The titer of the g = 2.95 signal and the broad trough at g = 1.80 are shown in Fig. 4B. Note that as the EPR titer measures the decrease in peak height, the y axis points have been multiplied by 1. The EPR titers follow a similar trend to the optical redox titer, but both sets of data have more positive midpoint potentials, particularly the g = 1.80 trough in which 80% of its intensity is lost with a Eₘ of ~82 mV. The peak at g = 2.96 fits to two sets of heme with midpoint potentials of ~136 and ~288.
mV (Fig. 4C). These $E_m$ values, which are offset relative to the optical titer, could be the result of collecting the spectra at 10 K. However, they are in the same potential domain as the data collected for the visible spectropotentiometric titrations and are still entirely consistent with the presence of low spin bis-His ligated hemes.

Catalytic Properties of MtrA—The catalytic properties of reduced MtrA were assessed by monitoring the oxidation kinetics of the hemes in the presence of different electron acceptors. As expected from the low redox potentials of the hemes, reduced MtrA was rapidly oxidized by air. Consequently, all of the experiments were carried out under strict anaerobic conditions in a glove box. Oxidation of the reduced MtrA was monitored at 552 nm, and the experiments revealed that all of the ferrous hemes were rapidly oxidized upon addition of Fe(III) NTA, Fe(III) EDTA, or Fe(III) Maltol (Fig. 5A, shown for Fe(III) EDTA only). The addition of fumarate resulted in no re-oxidation, whereas the addition of nitrite and nitrate resulted in a slow re-oxidation that was 1–2 orders of magnitude slower than that of Fe(III) EDTA for which a rate of approximately 5 nmol of electrons/s was obtained (Fig. 5A).

Electron Transport to Fe(III) in Intact Cells of E. coli—MtrA could play two roles in Fe(III) respiration by S. oneidensis MR-1: (i) participation in intermembrane electron transfer between the inner membrane quinol dehydrogenase (CymA) and the outer membrane cytochromes for reduction of insoluble Fe(III) species and (ii) direct reduction of soluble Fe(III) chelates using electrons derived from inner membrane quinols via the tetraheme quinol dehydrogenase CymA (Fig. 6A, scheme). E. coli has a homologue of CymA, NapC, which is involved in menaquinol oxidation as part of the NapAB nitrate reductase system (27, 28). Thus the possibility that the “foreign” periplasmic MtrA could mediate electron transport from the respiratory components of the E. coli inner membrane to soluble Fe(III) species in intact cells was investigated. Cells of E. coli JM109(DE3) ($p$KP1/H11001/EC86) were incubated under anaerobic conditions with lactate as electron donor. Spectral analysis confirmed that MtrA was reduced under these conditions as judged by the reduced absorbance maximum at 552 nm (Fig. 7A). This confirmed that E. coli could transport electrons from physiological electron donors to the S. oneidensis MtrA. Following the addition of Fe(III) NTA, rapid oxidation of the MtrA hemes was observed. Inspection of the spectrum after oxidation revealed that only the absorption peak at 552 nm disappeared (Fig. 7). The reduced cytochrome peaks at 560 nm that arise from the endogenous $b$-type cytochromes remained, and control experiments in which Fe(III) NTA was added to E. coli JM109(DE3) confirmed that the chelate did not oxidize the endogenous cytochromes (Fig. 7B). Following the consumption of the Fe(III) NTA, full re-reduction of the MtrA cytochromes in E. coli JM109(DE3) ($p$KP1 + $p$EC86) occurred. This took place without the need for addition of exogenous artificial electron donor and suggests that re-reduction was occurring via electron transport through the host respiratory system. The kinetics of the cytochrome oxidation and re-reduction was monitored.
oxidation by Fe(III) species (as already shown in Fig. 5) with purified MtrA, which showed that the rate of MtrA oxidation of Fe(III) reduction. This contrasted to the observations made (data not shown). The rates were comparable to those for addition of either nitrate or nitrite to anaerobic suspensions of cells used in these experiments; therefore, given the sensitivity of cytochrome oxidation for equimolar pulses of Fe(III) to cells of S. frigidimarina NCIMB400 that are respiring Fe(III) benzoxydamic acid. However, Fe(III) citrate was not served with R. capsulatus (29). This may be because at the high concentrations added, (pKP1 + pEC86) is also present in these experiments; therefore, given the sensitivity of cytochrome oxidation to Fe(III) NTA, rapid oxidation of the MtrA hemes still occurred but re-reduction was inhibited (Fig. 8). Thus the duration of cytochrome oxidation for equimolar pulses of Fe(III) NTA was approximately six times longer in the presence of HqNO. The same result was observed when HqNO was added to cells of S. frigidimarina NCIMB400 that are respiring Fe(III) (13). These results suggest that E. coli can feed electrons to the anaerobic quinol pool to MtrA, which can then mediate electron transfer to soluble Fe(III) species. An analysis of E. coli membranes established that the 25-kDa CymA homologue, NapC, was present in the JM109(DE3) (pKP1 + pEC86) cells used in these experiments; therefore, given the sensitivity to HqNO, this is the most probable route of electron transfer to MtrA. Other Fe(III) species that could oxidize the MtrA hemes when added to intact cell suspensions of E. coli JM109(DE3) (pKP1 + pEC86) included Fe(III) EDTA, Fe(III) Maltol, and Fe(III) benzoxydamic acid. However, Fe(III) citrate was not a good electron acceptor. This has also been previously observed with R. capsulatus, which like E. coli JM109(DE3) (pKP1 + pEC86) can reduce a range of soluble Fe(III) species (29). This may be because at the high concentrations added, Fe(III) citrate forms complex polynuclear species that cannot access the insoluble iron oxides were also unable to oxidize the MtrA in intact cells. MtrA oxidation in intact cells was also observed after the addition of either nitrate or nitrite to anaerobic suspensions (data not shown). The rates were comparable to those for Fe(III) reduction. This contrasted to the observations made with purified MtrA, which showed that the rate of MtrA oxidation by nitrate and nitrite was much slower than for the oxidation by Fe(III) species (as already shown in Fig. 5A). To study this further, reconstitution experiments of purified MtrA with purified NapAB nitrate reductase or NrfA nitrite reductase showed that it could serve as a poor electron donor to periplasmic nitrate reductase but a good electron donor to NrfA (Fig. 5B). As detailed earlier, enzymatic and heme-stained SDS-PAGE analysis (Fig. 1) revealed that the periplasmic nitrate and nitrite reductase systems were both present in the cells used in these experiments, and thus the rapid rate of oxidation of MtrA by nitrate and nitrite in intact cells can be explained as a result of these systems drawing electrons from MtrA during the reduction of nitrate to ammonium (Fig. 8).

This demonstrates that in the intact E. coli cells the foreign MtrA can integrate into the endogenous electron transport system of the host.

**CONCLUSIONS**

This work has reported the first detailed biochemical characterization of a member of the MtrA family of periplasmic decaheme cytochromes. The characterization has been facilitated through the successful heterologous expression of mtrA in E. coli, which when co-expressed with ccm genes results in the assembly of correctly targeted periplasmic decaheme cytochrome. Spectroscopic characterization has suggested that all of the heme iron ions in ferric MtrA are low spin hexacoordinate species in which histidine ligands provide both the distal and proximal (Fe(III)) ligands. The proximal ligands will be provided by each of the 10 CXXCH heme binding motifs, but an additional ten His ligands are then required for each distal site. Analysis of the S. oneidensis genome sequence reveals four paralogs of MtrA (15), the gene products of S. oneidensis. The CXXCH motifs serve as a distal ligand for another heme bound by the first motif and the conserved His after the second heme binding motif serves as a distal ligand for the heme iron. The conservation of the 10 hemes could be organized into these parallel heme binding motifs align and that an additional 11 conserved His residues can be identified (Fig. 9). If heme binding sites 3–4, 5–6, and 7–8 are considered separately, two conserved His are found to be in an arrangement of CXXCHXX/10–12HX7–9CXXCHXX. This arrangement can also be identified between hemes 2 and 3 in the small tetraheme cytochromes and the tetraheme domains of the flavocytochrome c fumarate reductases of S. frigidimarina NCIMB400 and S. oneidensis MR-1. Structures determined for these proteins (12, 30–32, 34) reveal that the two hemes bound by this motif form a near parallel heme pair in which the heme ions are around 4 Å apart, and the conserved His between the CXXCH motifs serves as a distal ligand for the heme iron bound by the first motif and the conserved His after the second heme binding motif serves as a distal ligand for another heme iron in the multiheme protein (32, 34). Thus, the present sequence analysis of the MtrA paralogues strongly suggests that a number of the 10 hemes could be organized into these parallel heme pair arrangements. The first ~190 amino acids of the decaheme MtrA paralogues contains five CXXCH heme binding motifs. If these are aligned with the ~190 amino acids of the pentaheme NrbB protein, with which MtrA shares a low sequence identity (35) and which is thought to serve as an electron donor to the NrfA nitrite reductase of E. coli (36), it can be seen that the spatial distribution of the CXXCH motifs in the primary structure are very similar, including the CXXCHXX/10–12HX7–9CXXCHXX arrangement between hemes 3 and 4. This suggests that these polypeptides will fold in a similar manner. The crystal structure of the NrfA nitrite reductase (33) shows that the protein is an ~110-kDa decaheme...
homodimer and has raised the possibility that the electron donating NrfB also binds to this complex as a decaheme homodimer. This dimer would have a molecular mass of around 35 kDa, similar in size to the decaheme MtrA, and given the similarities in primary structure this could explain why MtrA can act effectively as an electron donor for NrfA.

A key observation made in this work has been that the synthesis of MtrA confers on E. coli the capacity to couple reduction of a range of soluble Fe(III) species to its own respiratory electron transport chain. This has allowed the demonstration of the capacity in vivo for reduced MtrA as a reductase of soluble Fe(III) species. The ratio of heme to polypeptide in MtrA is ~1:28 amino acids. This compares to ~1:100 in larger multiheme enzymes such as the periplasmic nitrite reductase of S. oneidensis NCIMB400 for which structural data have recently emerged (31). This arrangement of closely packed solvent-exposed hemes has been described as one that allows for “electron harvesting.” Essentially, it might enable the protein to be highly promiscuous in its electron donating or accepting activity with the main driving force for electron transfer being thermodynamic. In the case of MtrA, the observations made in this paper support this view. The decaheme cytochrome can participate in a foreign electron transport system in which it can obtain electrons from the host cell inner membrane quinol dehydrogenase and donate electrons to host oxidoreductases. The presence of MtrA also allows the host cell to couple its own carbon metabolism to respiratory Fe(III) reduction. Al-though MtrA has a ferric reductase activity, this is not specific for any particular Fe(III) chelate, and the activity must arise from electron transfer from surface-exposed hemes that come within in long range electron transfer distance (<14 Å) of the electron accepting Fe(III) in the chelate. This is also likely to be true for the small tetraheme cytochromes that appear to be important for Fe(III) respiration in S. frigidimarina (12). This then underlies the response of Shewanella species to aerobic growth in the presence of soluble iron chelates, which as illustrated in Fig. 1, is to synthesize a large pool of multiheme periplasmic c-type cytochromes for which there are numerous genes on the chromosome and many of which may prove to have similar catalytic properties to MtrA.

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