Purification and Magneto-optical Spectroscopic Characterization of Cytoplasmic Membrane and Outer Membrane Multiheme c-Type Cytochromes from *Shewanella frigidimarina* NCIMB400*

(Received for publication, June 1, 1999, and in revised form, November 3, 1999)

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Two membranous c-type cytochromes from the Fe(III)-respiring bacterium *Shewanella frigidimarina* NCIMB400, CymA and OmcA, have been purified and characterized by UV-visible, magnetic circular dichroism, and electron paramagnetic resonance spectroscopies. The 20-kDa CymA is a member of the NapC/NirT family of multiheme cytochromes, which are invariably anchored to the cytoplasmic membrane of Gram-negative bacteria, and are postulated to mediate electron flow between quinols and periplasmic redox proteins. CymA was found to contain four low-spin c-hemes, each with bis-His axial ligation, and mid-point reduction potentials of +10, −108, −136, and −229 mV. The 85-kDa OmcA is located at the outer membrane of *S. frigidimarina* NCIMB400, and as such might function as a terminal reductase via interaction with insoluble Fe(III) substrates. This putative role is supported by the finding that the protein was released into solution upon incubation of harvested intact cells at 25 °C, suggesting an attachment to the exterior face of the outer membrane. OmcA was revealed by magneto-optical spectroscopies to contain 10 low-spin bis-His ligated c-hemes, with the redox titer indicating two sets of near iso-potential components centered at −243 and −324 mV.

A number of Gram-negative bacteria can utilize Fe(III) as a terminal electron acceptor to support anaerobic growth (1). Since access of this respiratory substrate to the periplasmic compartment of the cell is denied by the formation of highly insoluble polymeric Fe(III) oxyhydroxides at circumneutral pH, outer membrane (OM)1 proteins may be required for reduction to occur. An unprecedented pathway would thus have to exist in which electrons were transferred from primary dehydrogenases to the cell exterior, with the concomitant generation of a proton-electrochemical gradient across the cytoplasmic membrane (CM). It is now becoming apparent that such a model might be fitted to the reduction of insoluble forms of Fe(III) by the versatile facultative anaerobe *Shewanella putrefaciens*. This bacterium has been demonstrated to grow on Fe(III) oxyhydroxides with H₂, formate, lactate, or pyruvate serving as the electron source (2). Other insoluble terminal electron acceptors used by *S. putrefaciens* include Mn(IV) oxides (3) and elemental sulfur (4). A characteristic of *S. putrefaciens* cultured either anaerobically or under microaerobic conditions is the high content of c-type cytochromes in the cell, located both in the periplasm (5) and membrane (6) fractions.

An essential component of the electron transport pathway to Fe(III) in *S. putrefaciens* is a 21-kDa tetraheme c-type cytochrome, CymA, as revealed by transposon mutagenesis studies in strain MR-1 (7). Primary structure analysis has indicated CymA to be a member of the widespread NapC/NirT redox family (7). These multiheme proteins are invariably anchored to the CM, and are postulated to oxidize insoluble quinols and reduce soluble periplasmic enzymes such as nitrate or nitrite reductases (8). The soluble domain of NapC from *Paracoccus denitrificans* has recently been expressed as a periplasmic protein and spectroscopically characterized (8). The four c-hemes present were noted to be low-spin, with bis-His axial ligation, and mid-point reduction potentials in the range −235 to −56 mV (8).

In a novel finding for Gram-negative bacteria, several c-type cytochromes have been found to reside at the OM of anaerobically grown *S. putrefaciens* MR-1 (6). When reduced, the cytochromes in OM fractions are readily reoxidized upon addition of Fe(III), suggesting that they may be involved in reduction of the metal cation by intact cells (9). An 83-kDa c-type cytochrome, OmcA, has been purified from OM fractions of *S. putrefaciens* MR-1 (9). The sequence of the *omcA* gene indicates that the translated protein possesses an N-terminal phospholipid attachment site and 10 CXXCH c-heme binding motifs (10). Further genetic analysis has revealed *omcA* to be part of a 13-kilobase gene cluster, *mtrDEF-omcA-mtrCAB* which is predicted to encode four further c-type cytochromes. MtrC and MtrF are putative decaheme proteins located at the OM, while MtrA and MtrD are putatively decaheme but located in the periplasmic compartment of the bacterium. Two non-heme OM proteins, MtrE and MtrR, are also predicted to be encoded by this DNA sequence, and transposon mutagenesis has demonstrated the expression of MtrB to be essential for the respiration of Fe(III) by *S. putrefaciens* MR-1 (11).

Among several other species of *Shewanella* capable of Fe(III) respiration is *Shewanella frigidimarina* (formerly *S. putrefaciens*) NCIMB400 (12). Studies on the anaerobic metabolism of this bacterium have mainly focused on a 64-kDa periplasmic tetraheme flavocytochrome c₃, Fcc₃, which functions as a physiological fumarate reductase (13). A related protein has been reported to be present in *S. putrefaciens* MR-1 (14). Small tetraheme c-type cytochromes of approximately 12 kDa have

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*This work was supported by Wellcome Trust Project Grant 048547/Z/96/Z (to D. J. R. and P. S. D.) and a Biotechnology and Biological Sciences Research Council (BBSRC) Quota Studentship (to S. J. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: OM, outer membrane; CM, cytoplasmic membrane; PAGE, polyacrylamide gel electrophoresis.

2 GenBank accession number AF083240.
also been purified from both \textit{S. frigidimarina} NCIMB400 and \textit{S. putrefaciens} MR-1.

In this paper we present the purifications of membranous \textit{c}-type cytochromes from \textit{S. frigidimarina} NCIMB400 that correspond to the CymA and OmcA proteins from \textit{S. putrefaciens} MR-1. The subsequent detailed magneto-optical spectroscopic study of CymA is the first to be reported for any protein in a native form from the Nap/Cir/T family, while similar characterization of OmcA is the first for any hemoprotein located at a bacterial OM.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Bacterial Strains and Growth Conditions—} \textit{S. frigidimarina} NCIMB400 and the type strain of \textit{S. putrefaciens} (NCIMB10471; ATCC8071) were purchased from the National Collections of Industrial and Marine Bacteria (Aberdeen, United Kingdom). \textit{S. putrefaciens} MR-1 was supplied by Dr. G. A. Reid, University of Edinburgh, UK. The temperatures employed for cell growth were 25 °C (\textit{S. frigidimarina} NCIMB400 and \textit{S. putrefaciens} MR-1) or 30 °C (\textit{S. putrefaciens} type strain). Microaerobic growth was achieved in the basal medium of Luria-Bertani (g/liter tryptone 10; yeast extract 5; NaCl 10) adjusted to pH 7.5 with NaOH, and supplemented with 50 mM dl-lactate. Cultures of \textit{S. frigidimarina} NCIMB400 were grown at 250 rpm for 15 min in 2-liter foam-plugged flasks until the late logarithmic phase of growth was attained. Anaerobic growth of the \textit{Shewanella} species with 50 mM Fe(III) citrate were achieved by the protocol of Dobbin et al. (16).

\textbf{Cell Fractionation and Membrane Preparation—} Microaerobically or anaerobically grown cells of \textit{Shewanella} species were harvested by centrifugation at 15,900 \( \times \) g and 4 °C for 10 min, and resuspended in 20 mM NaHEPES, pH 7.5 (20 ml per 1-liter culture). Cell breakage was achieved by two passages through a chilled French pressure cell operated at 3,000 p.s.i. Membranes were pelleted by ultracentrifugation at 235,000 \( \times \) g and 4 °C for 1 h, then washed with 20 mM NaHEPES, pH 7.5. Ultracentrifugation was repeated prior to final resuspension in the same buffer (10 ml per 1-liter culture).

\textbf{Detergent Solubilization—} Membrane fractions derived from cells of \textit{S. frigidimarina} NCIMB400 grown either microaerobically or anaerobically with Fe(III) citrate were employed in solubilizations with Triton X-100. Membranes (45 mg/ml protein) were stirred with the detergent (20 mg/ml) in 20 mM NaHEPES, pH 7.5, at 4 °C for 1 h. Unsolubilized material was then removed by ultracentrifugation at 235,000 \( \times \) g and 4 °C for 1 h.

\textbf{Protein Purifications—} Both the 20-kDa CymA and 85-kDa OmcA hemoproteins were purified from detergent-solubilized membrane fractions of \textit{S. frigidimarina} NCIMB400 cells that had been grown in microaerobic cultures totalling 20 liters. All chromatographic, dialysis, and concentration steps were performed at 4 °C. Solubilized material was dialyzed against 20 mM Tris-HCl, pH 8.0, then loaded onto a DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech) anion exchange column (250–500 mM NaCl were applied over 10 column volumes to elute CymA). Membranes (45 mg/ml protein) were stirred with the detergent (20 mg/ml) in 20 mM NaHEPES, pH 7.5, at 4 °C for 1 h. Unsolubilized material was then removed by ultracentrifugation at 235,000 \( \times \) g and 4 °C for 1 h.

\textbf{Redox Titrations—} Mediated spectrophotometric redox potentialmetry was undertaken using methodology described by Dobbin et al. (16). Titrations with dithionite of 3.2 \( \mu \text{M} \) oxidized CymA and 4.4 \( \mu \text{M} \) oxidized OmcA were performed under argon atmosphere at 15 °C in 100 mM Tris-HCl, pH 8.0, and 100 mM NaHEPES, pH 7.5, respectively, with these buffers each containing 0.2 mg/ml dodecyl maltoside. After addition of dithionite, a quenching time of 10 min was allowed before acquisition of a spectrum in the range 500–700 nm. The reduction potentials reported for the hemes of CymA and OmcA are referenced to the SHE.

\textbf{Enzymatic Activity Assays—} Duroquinol and menaquinol were prepared by the method of Reiske (20). Donation of electrons from these reduced species (50 \( \mu \text{M} \)) to the oxidized 20-kDa CymA cytochrome (2 \( \mu \text{M} \)) in 20 mM NaHEPES, pH 7.5, containing 0.2 mg/ml dodecyl maltoside, was monitored by scanning in the region 500–600 nm using an Hitachi U3000 spectrophotometer. The reaction between the dithionite-reduced 85-kDa OmcA cytochrome (1 \( \mu \text{M} \)) and Fe(III)-EDTA (100 \( \mu \text{M} \)) in identical buffer was analyzed by stopped-flow spectrophotometry using protocols detailed by Dobbin et al. (16).

\textbf{RESULTS}

\textbf{Membranous Cytochromes Present in \textit{Shewanella} Species—} The presence of potentially homologous \textit{c}-type cytochromes in membrane fractions of \textit{Shewanella} species was investigated by SDS-PAGE. Both the type and MR-1 strains of \textit{S. putrefaciens}, and also \textit{S. frigidimarina} NCIMB400, were found to produce four major c-heme containing membranous proteins during the anaerobic respiration of Fe(III) (Fig. 1, lanes 1–3). Moreover, the apparent molecular masses for these cytochromes, namely 85, 70, 55, and 20 kDa, were equivalent for the three \textit{Shewanella} species. In \textit{S. putrefaciens} MR-1, the cytochrome of apparent molecular mass 85 kDa has previously been purified from OM fractions (9) and designated OmcA. The gene encoding this protein has been characterized, and suggests that mature OmcA is an OM decamethylene \textit{c}-type cytochrome featuring an \textit{N}-terminal lipid modification (10). Previous SDS-PAGE analysis after fractionation of \textit{S. putrefaciens} MR-1 membranes has demonstrated the 70-kDa hemoprotein of this bacterium to also be located at the OM (6). In view of nucleotide sequencing data for the \textit{mtrC} gene of \textit{S. putrefaciens} MR-1, which is predicted to encode an OM decaheme protein of a near equivalent molecular mass, the 70-kDa cytochrome apparent on heme-stained gels is most probably the putative MtrC pro-
tein. Also in *S. putrefaciens* MR-1, the cytochrome of apparent molecular mass of 20 kDa has been identified as a CM protein, and designated CymA (7). The nucleotide sequence data for cymA suggests the mature protein to be tetraheme, and a transposon mutation in the gene causes an absence of the 20-kDa cytochrome in SDS-PAGE gels of the CM fraction (7). Based on these previous findings, the 85-, 70-, and 20-kDa membranous cytochromes from *S. putrefaciens* MR-1, the cytochrome of apparent mass 85 kDa, purified samples from *S. frigidimarina* NCIMB400 apparent in our SDS-PAGE analysis were considered to most probably be respective homologs of the OmcA, MtrC, and CymA proteins from *S. putrefaciens* MR-1.

**Localization of the 85- and 70-kDa Cytochromes to the OM of *S. frigidimarina* NCIMB400—**SDS-PAGE analysis of the membrane fraction of *S. frigidimarina* NCIMB400 grown microaerobically also revealed the 85- and 70-kDa hemoproteins to be present (Fig. 2, lane 1). However, the relative abundance of the 70-kDa cytochrome compared with the 85-kDa protein was markedly higher in membranes from cells cultured by Fe(III) respiration (Fig. 1, lane 3) as opposed to microaerobically (Fig. 2, lane 1). Using methods previously applied to *S. putrefaciens* MR-1 (6), which involved preparation of CM and OM fractions using sucrose density gradients, the 70- and 85-kDa cytochromes of *S. frigidimarina* NCIMB400 were isolated for use in the bacterial supernatant (data not shown). Furthermore, compelling evidence for these hemoproteins having such subcellular location was obtained from an experiment simply involving resuspension in buffer of harvested intact cells of *S. frigidimarina* NCIMB400 from a microaerobic culture. Following incubation at 25 °C and subsequent centrifugation, SDS-PAGE analysis of the supernatant demonstrated both the 85- and 70-kDa membranous *c*-type cytochromes to have been extracted into the buffer solution (Fig. 2, lane 2). By appraisal of band intensities, removal of the 70-kDa hemoprotein from intact cells was more easily facilitated during the incubation. As no periplasmic cytochromes were found in the supernatant by SDS-PAGE, cell lysis was deemed not to have occurred. Comparable results were obtained using intact cells from cultures grown by Fe(III) respiration. In addition to localizing the 85- and 70-kDa membranous cytochromes to the OM of *S. frigidimarina* NCIMB400, and thus providing further evidence for these proteins being homologs of the *S. putrefaciens* MR-1 OmcA and putative MtrC respectively, the intact cell incubation experiment suggests both hemoproteins to be anchored at the external face of the OM. Furthermore, all

**Membranous c-Type Cytochromes of *S. frigidimarina* NCIMB400**

![Fig. 1. Heme-stained SDS-PAGE (15%) profiles of membrane fractions from *Shevanella* species, and purified proteins from *S. frigidimarina* NCIMB400.](image-url)

![Fig. 2. Heme-stained SDS-PAGE (10%) profiles of membrane fraction from *S. frigidimarina* NCIMB400, protein removed during incubation of intact cells, and purified protein.](image-url)
sequence data for cytochromes of similar structure and function in these Shewanella species (12), this degree of identity is strongly supportive of the 85-kDa cytochrome from S. frigidimarina NCIMB400 being a homolog of the OmcA protein of S. putrefaciens MR-1. Characterization of the omcA gene has predicted the first amino acid in the mature OmcA protein from S. putrefaciens MR-1 to be cysteine, and this residue has been proposed to form a lipid attachment site to the OM of the bacterium (10).

UV-Vis and MCD Spectroscopies—The visible absorption spectra obtained for the fully reduced 20- and 85-kDa membranous cytochromes from S. frigidimarina NCIMB400 are presented in Fig. 3, A and B, respectively. Both give absorbance maxima that are characteristic of c-heme containing proteins, namely α-, β-, and γ-bands centered at 552, 523, and 420 nm, respectively. The visible absorption spectra obtained for the fully-reduced 20- and 85-kDa cytochromes in solution saturated with CO are also shown in Fig. 3, A and B, respectively, and the changes in features caused by the presence of this potential heme ligand indicate interactions to occur with both proteins. The binding of CO to ferrous heme can occur either at a vacant sixth co-ordination site or by displacement of an axial ligand from the polypeptide chain. Expected spectral shifts upon CO ligation to reduced c-type cytochromes are increases in the α- and β-band wavelength maxima accompanied by a decrease in the γ-band wavelength maximum (21). The traces obtained for the reduced 20- and 85-kDa cytochromes in the presence of CO show a combination of features in the αβ-region that represent both ferrous heme and CO-ligated ferrous heme. Not all of the ferrous hemes in these multiheme species thus interact with CO, and a bound:unbound ratio of 1:1 can be estimated for both proteins.

Quantifications of hemes in the 20- and 85-kDa c-type cytochromes were based on assumptions that the M₄ values of the corresponding apoproteins were 20,000 and 80,000. The pyridine hemochrome spectra then indicated nearest integer values of 4 and 10 mol of c-heme to be present per mole of polypeptide in the holoproteins. These data provide further support for the cytochromes purified from S. frigidimarina NCIMB400 being respective homologs of the putative tetraheme CymA (7) and decaheme OmcA (10) from S. putrefaciens MR-1.

The UV-vis absorption spectra of both the air-oxidized 20- and 85-kDa membranous c-type cytochromes from S. frigidimarina NCIMB400 (Figs. 4A and 5A, respectively) were found not to be altered by addition of ferricyanide. The full oxidation of these proteins in as-prepared samples was confirmed by room temperature MCD spectra collected in the UV-vis region (Figs. 4B and 5B), which featured none of the characteristic signatures of ferrous heme. Over the wavelength range 300–600 nm, intense low-spin ferric heme MCD bands have been shown to invariably dominate those derived from high-spin ferric heme (22). In the Soret region of MCD spectra (∼400 nm), a single low-spin heme is known to give rise to a derivative shaped band with a peak to trough intensity of approximately 150 M⁻¹ cm⁻¹ 1 T⁻¹, and the MCD spectra in Figs. 4B and 5B are plotted against concentrations calculated from this value. The UV-vis absorption spectra in Figs. 4A and 5A are plotted against concentrations similarly derived, and indicate the molar extinction coefficients per low-spin heme at 410 nm implied by MCD spectroscopy for the fully-oxidized 20- and 85-kDa cytochromes as 138,000 and 121,000, respectively. The presence of low-spin ferric heme in both oxidized proteins is sup-

![Fig. 3. Visible absorption spectra of the fully reduced 20- and 85-kDa membranous cytochromes from S. frigidimarina NCIMB400. A, 1.4 μM 20-kDa cytochrome. B, 0.7 μM 85-kDa cytochrome. The solid and broken lines, respectively, represent spectra obtained before and after saturation with CO gas. Both proteins were buffered in 20 mM NaHEPES, pH 7.5, containing 0.2 mg/ml dodecyl maltoside. Complete reduction of the hemes present was achieved by adding excess dithionite.](http://www.jbc.org/Downloadedfrom/)

![Fig. 4. UV-visible absorption and MCD spectra of air-oxidized 20-kDa cytochrome from S. frigidimarina NCIMB400, and MCD spectrum of the dithionite-reduced protein. A, UV-vis absorption spectrum of 1.5 μM air-oxidized protein. B and D, room temperature MCD spectra of air-oxidized protein in the UV-vis and near-IR regions, respectively (concentrations 15 μM at <460 nm, and 100 μM at >460 nm). C, MCD spectrum of 85 μM dithionite-reduced protein in the UV-vis region. The cytochrome was buffered in 50 mM NaHEPES-D₂O, pH 7.5, containing 0.2 mg/ml dodecyl maltoside.](http://www.jbc.org/Downloadedfrom/)
ported by the forms and intensities of $\alpha,\beta$-MCD bands observed. UV-visible absorption and MCD spectroscopies of the 20- and 85-kDa cytochromes also indicate no high-spin ferric hemes to be featured. Characteristic charge transfer bands (largely porphyrin $\rightarrow$ ferric heme) for this state, which appear at $\sim 630$ nm as a distinct shoulder in a UV-vis spectrum and as a derivative shaped feature in a MCD spectrum, were not observed for either protein.

The charge-transfer band for low-spin ferric heme occurs in the near-IR region (800–2500 nm). Although rarely detected by absorption spectroscopy, this band may be readily located by MCD, with the peak wavelength being an indicator of the axial ligands to the heme iron (22, 23). The near-IR MCD spectra of the oxidized 20- and 85-kDa membranous c-type cytochromes from *S. frigidimarina* NCIMB400 show positive bands, at 1500 and 1510 nm, respectively, and each exhibit side structures to higher energy (Figs. 4D and 5D). Both proteins thus give characteristic forms of low-spin charge transfer bands, and these occur at wavelengths that are typical for hemes with bis-His ligation (22, 23). However, examples are known of both Hisamine (24) and Met-His$^-$ (25) coordination yielding low-spin charge transfer bands at similar wavelengths. Met-His$^-$ can be ruled out here since the coordination of sulfur to low-spin ferric heme iron, either as Met or Cys, gives rise to additional ligand $\rightarrow$ Fe(III) charge transfer MCD bands in the 650–750 nm region (26), and these were not observed for either the 20- or 85-kDa oxidized cytochromes (Figs. 4B and 5B).

The UV-vis room temperature MCD spectra of the dithionite-reduced forms of the 20- and 85-kDa cytochromes are presented in Figs. 4C and 5C, respectively. Both are dominated by an extremely sharp 550-nm derivative-shaped heme $\alpha$-band which is typical of the low-spin ferrous state. High-spin ferrous heme can in principle be detected by MCD transitions between 700 and 1000 nm, although the signals are some 3 orders of magnitude weaker than the $\alpha$-band described above for low-spin ferrous heme and their observation requires high sample concentration. Fig. 5C shows that at the maximal concentration of 3 mM total heme achieved for the 85-kDa cytochrome, weak transitions were noted in the 700–1000 nm region, especially at around 755 nm. However, the intensities of these signals indicate that they represent substantially less than 1 in 10 of the hemes present in the protein. The reduced 20-kDa cytochrome could only be concentrated to 340 $\mu$M heme, and so while Fig. 4C appears to show a proportionally larger band near 755 nm to that noted for the 85-kDa cytochrome, the signal to noise ratio is insufficient for confident quantification. More significantly, however, a splitting is observed in the Soret band of the reduced 20-kDa cytochrome MCD spectrum (Fig. 4C), where high- and low-spin ferrous states may contribute with comparable intensities. The additional detail would be consistent the presence of high-spin ferrous heme, and a level of up to one in four of those present in the 20-kDa protein may be suggested by this observation.

**EPR Spectroscopy**—The MCD sample of the membranous 20-kDa cytochrome from *S. frigidimarina* NCIMB400 was also examined using X-band EPR spectroscopy at 10 K (Fig. 6A). The features observed at $g = 2.93, 2.24,$ and $\sim 1.5$ are typical of $S = 1/2$ low-spin ferric hemes with two His ligands of parallel orientation (27). Only several minority species were otherwise detected, namely low levels of high-spin ferric heme at $g = 5.8$ (estimated as 2% of total heme), adventitious ferric iron at $g = 4.3,$ and a radical at $g = 1.99.$ The nature of the hemes observed in the 20-kDa cytochrome by EPR is thus in some agreement with MCD data on the protein. Concentrations of the species giving rise to the low-spin ferric iron triplet were estimated by integration of the $g = 2.93$ feature in duplicate samples of the protein and comparison with a 1 mM Cu(II)EDTA standard using the method of Aasa and Vanngard (28). This revealed the EPR signal to only represent approximately 10–20% of the low-spin ferric heme detected by electronic absorption and MCD spectroscopies. It is therefore apparent that three out of the four hemes contained in the 20-kDa cytochrome are rendered EPR silent, and this presumably arises from spin coupling between redox centers. The coupling is most likely to be predominantly exchange in character, as the maximum dipolar coupling expected between bis-His ligated heme would not be sufficient to abolish an EPR spectrum at the X-band. Spin coupling between EPR silent hemes has previously been revealed by Mössbauer spectroscopy of the pentaheme nitrite reductase from *Desulfuviobrio desulfuricans* (29).

The 10 K X-band EPR spectrum of the membranous 85-kDa cytochrome from *S. frigidimarina* NCIMB400 (Fig. 6D) also contains $S = 1/2$ low-spin rhombic features at $g = 2.99, 2.27,$ and 1.54, that are again characteristic of heme ligated with two parallel His residues. A small shoulder observed to the low-field side of the $g = 2.99$ peak was resolved at lower temperature and increased microwave power (Fig. 6C) as an asymmetric signal at $g = 3.61.$ This is typical for the $g_\perp$ feature of a “high-$g_{\perp}$” spectrum, which is commonly encountered for low-spin ferric hemes with two perpendicular His ligands (27). Integration of the $g = 2.99$ peak demonstrated this species to account for $\sim 21\%$ of the total low-spin ferric heme detected by pyridine hemochrome assays. Similar integration of the $g =$
The amplitude of 1 millitesla for 100 m perpendicular mode X-band EPR spectra were collected at a modulation amplitude of 1 millitesla for each protein in 50 mM NaHEPES-D₂O, pH 7.5, containing 0.2 mg/ml dodecyl maltoside. The g-values for each peak are indicated. A, the 20-kDa protein, at 10 K using 2.1 mW microwave power, and at 5 K using 32 mW microwave power, respectively.

3.61 feature was not as straightforward to perform due to the poorly defined baseline. However, based on estimations of the other g values that will form a triplet with this feature (30), the species was calculated to account for approximately 20% of the total low-spin heme content. In the region of 60% of the low-spin ferric hemes in the 85-kDa cytochrome were therefore not detected by X-band EPR, and again this may arise from spin coupling between centers. The high-spin ferric heme (g = 5.97) present in the protein was estimated as 0.2% of total heme.

**Redox Titrations**—Values obtained for ΔA₅₅₂=ΔA₅₆₃ (i.e. the absorbance at the α-max wavelength for reduced c-heme less the absorbance at an isosbestic point) in the spectrophotometric mediated reductive titration of the S. frigidimarina NCIMB400 20-kDa membranous cytochrome were plotted as a function of E (Fig. 7A). The protein can be seen to have progressed from fully oxidized to fully reduced over the approximate potential range +50 to −300 mV. The best fit to the points was obtained with a theoretical curve comprising four Nernstian components of equal amplitude centered at +10, −108, −136, and −229 mV (Fig. 7A). Data from the redox titration of the 20-kDa cytochrome is thus supportive of the findings from the pyridine hemochrome assay and MCD spectroscopy, in that the protein is indicated to feature four c-hemes, and these have low midpoint reduction potentials which suggest the binding of two axial His ligands.

An equivalent plot was made for values obtained in the titration of the S. frigidimarina NCIMB400 85-kDa membranous cytochrome (Fig. 7B), demonstrating this protein to be reduced over the approximate potential range −180 to −400 mV. The best fit to the points was obtained with two Nernstian components, centered at −243 and −324 mV, and given respective weightings of 30 and 70% (Fig. 7B). Data from the redox titration of the 85-kDa cytochrome is thus also supportive of the findings from pyridine hemochrome and MCD analysis, in that the protein is suggested to feature 10 c-hemes, and these may be grouped, at near isopotential, around two low midpoints characteristic of bis-His axial ligation.

**Enzymatic Activities**—Electron donation to the 20-kDa cytochrome by both duroquinol and menaquinol was observed using visible spectroscopy (results not shown). Reactions were noted to cease within 2 min, with the absorbance increase at the α-band wavelength maximum of 552 nm indicating that approximately one of the four c-hemes present in the protein was reduced using either quinol. These data provide further support for the 20-kDa membranous cytochrome purified from S. frigidimarina NCIMB400 being a homolog of the putative tetraheme CymA protein from S. putrefaciens MR-1 (7), which has been postulated to function as a quinol oxidase on the basis of primary structure analysis.

The reaction between 1 μM of the reduced 85-kDa cytochrome (i.e. 10 μM electrons) and 100 μM Fe(III)EDTA was monitored in a stopped-flow spectrophotometer (results not shown). The kinetics of heme oxidation observed at the α-band wavelength maximum of 552 nm were biphasic, consistent with kₐ₉ values of 206 s⁻¹ and 35 s⁻¹ contributing 60 and 40%, respectively, to the amplitude. This rapid donation of electrons to an Fe(III) chelate by the reduced 85-kDa cytochrome provides evidence that the protein, which we have demonstrated to be anchored to the exterior face of the OM, may function as a terminal Fe(III) reductase during anaerobic growth of S. frigidimarina NCIMB400.

**DISCUSSION**

This work has demonstrated that the membranous cytochromes CymA and OmcA are common to both the Fe(III)-respiring microorganisms S. frigidimarina NCIMB400 and S. putrefaciens MR-1. Subsequent magneto-optical analyses of the proteins from S. frigidimarina NCIMB400 have revealed each to exclusively feature bis-His ligated low-potential c-hemes. Such a characterization for the 20-kDa tetraheme CymA is the first to be presented for any member of the NapC/NirT redox protein class in a native form. CymA has previously been included in this family of CM-anchored cytochromes based upon primary structure analysis of the putative protein from S. putrefaciens MR-1 (7). A recent report from our laboratory has
detailed spectroscopic studies on a water-soluble form of NapC from *P. denitrificans* (8). NapC is believed to pass electrons from insoluble quinols to the periplasmic nitrate reductase NapAB in this bacterium. The engineered NapC protein was also found to contain four bis-His ligated c-hemes, with midpoint reduction potentials of −56, −181, −207, and −235 mV. In our present work, the corresponding values for the hemes of detergent-solubilized CymA were found to be +10, −108, −136, and −229 mV. The similar nature of the hemes present in NapC and the CymA from *S. frigidimarina* NCIMB400 supports the notion that CymA is a member of the Nap/C/NirT family, and moreover suggests that the two proteins may perform comparable functions in phylogenetically distinct bacteria. Previous primary structure analysis has identified four conserved His residues which may provide heme ligands in several members of the Nap/C/NirT family, including the putative CymA from *S. putrefaciens* MR-1 (8). Biophysical data reported here for CymA from *S. frigidimarina* NCIMB400 thus also supports the hypothesis that bis-His axial ligation is a defining feature of the NapC/NirT hemoproteins (8). Our MCD spectroscopy of CymA indicates that one of the four hemes present may lose a His ligand provided by the polypeptide chain when in the fully reduced state and become a high-spin complex. Further work is required to establish whether this is common among NapC/NirT family members, and moreover of any functional significance.

A potential role for CymA in electron transport from CM-entrapped quinols to periplasmic oxidoreductases has been indicated by studies in *S. putrefaciens* MR-1, with a transposon mutant in cymA losing the ability to respire nitrate, fumarate, and Fe(III) (7). In *P. denitrificans*, the non-energy conserving and cytochrome bc1 complex-independent electron transfer from quinol to nitrate via the periplasmic NapAB is believed to be a means of dissipating excess reductant, which is accumulated during aerobic growth on highly reduced carbon substrates such as butyrate (31). The low potentials noted for the c-hemes of the engineered NapC protein are thus in some agreement with this proposed role, as the ubiquinol pool will be most reduced when electrons are supplied from longer chain organic acids. In *Shewanella* species, the reducing conditions experienced during the anaerobic respiration of nitrate, fumarate, and Fe(III) would seem likely to be sufficient to drive electron flow from the quinol pool to CymA. Furthermore, several strains of *S. putrefaciens* have been demonstrated to contain menaquinones and methylmenaquinones (32), which would be expected to feature lower midpoint reduction potentials than ubiquinones. Regarding putative electron acceptors for CymA in *S. frigidimarina* NCIMB400, the heme midpoint potentials of the periplasmic tetraheme flavocytochrome c_{554} fumarate reductase Fcc_{2} have been reported as −102, −146, −196, and −238 mV (33). Our characterization has thus revealed CymA not to feature markedly lower potential hemes than the redox centers found in a protein it is proposed to reduce during a mode of anaerobic respiration. Moreover, CymA contains three c-hemes of near iso-potential (±10 mV) to those noted in Fcc_{2}. This data is in some contrast to findings for the NapC and NapAB proteins of *P. denitrificans*. NapC, which in an engineered soluble form features similar low-potential c-hemes to those of CymA, is believed to pass electrons to hemes of midpoint potentials −15 and +80 mV contained in the NapB c-type cytochrome (34).

Our magneto-optical spectroscopic characterization of the 85-kDa decaheme OmcA from *S. frigidimarina* NCIMB400 is the first to be presented for any cytochrome residing at a bacterial OM. The homolog of this protein in *S. putrefaciens* MR-1 has previously been purified, but only subjected to analysis by visible spectroscopy (9). The OM c-type cytochromes of *S. putrefaciens* MR-1 have been postulated to be involved in electron transport to extracellular Fe(III), based primarily upon the oxidation of the reduced hemes in OM fractions of strain MR-1 noted when Fe(III) is added (9). Data from the present study demonstrates the purified OmcA from *S. frigidimarina* NCIMB400 to be capable of rapidly passing electrons to an Fe(III) chelate. That OmcA may function as a terminal Fe(III) reductase in the intact cell is also suggested by our localization of the protein to the exterior face of the OM. OmcA could therefore interact with insoluble Fe(III) that is unable to access the periplasm, and reduce this respiratory substrate by long-range electron transfer. Such a mechanism might be expected since the midpoint reduction potentials of the bis-His ligated c-hemes present in OmcA will be considerably lower than the corresponding values for any Fe(III) in an oxyhydroxide polymer.

The release of a 70-kDa c-type cytochrome from *S. frigidimarina* NCIMB400 intact cells upon incubation at 25 °C implies that the protein is also most likely anchored to the outside face of the OM, and thus might similarly act as a terminal Fe(III) reductase. Based upon our membrane analyses of *Shewanella* species by SDS-PAGE, this 70-kDa hemoprotein is suggested to be a homolog of the putative MtrC decaheme cytochrome of *S. putrefaciens* MR-1. The subcellular arrangements in *S. frigidimarina* NCIMB400 of OmcA and the 70-kDa MtrC homolog indicate that these proteins will be unable to accept electrons directly from CymA, as might have been expected if one of the two 5-heme clusters probably present in each were situated at the periplasmic face of the OM. Thus a periplasmic redox protein, and/or an OM redox protein facing the periplasm, is likely to also be involved in mediating electron flow from CymA to Fe(III) species at the cell exterior. Nucleotide sequencing data for *S. putrefaciens* MR-1 indicates mtrC to be in the same operon as mtrB and mtrA, which are predicted to encode a non-heme β-barrel OM protein and a decaheme periplasmic c-type cytochrome, respectively (11). Furthermore, mtrCAB is located immediately downstream of *omcA* on the *S. putrefaciens* MR-1 chromosome. Genetic evidence thus strongly suggests MtrA to be involved in electron transport to the OM cytochromes MtrC and OmcA in *S. putrefaciens* MR-1. We have recently purified from *S. frigidimarina* NCIMB400 a homolog of the putative *S. putrefaciens* MR-1 MtrA protein, and demonstrated the c-hemes present to be titrated from fully oxidized to fully reduced in the potential range −80 to −400 mV.3 Taken with the data for CymA and OmcA from *S. frigidimarina* NCIMB400, which yield reductive titers in the potential ranges +50 to −300 mV and −180 to −400 mV, respectively, it thus might be proposed that electron transport from CM-entrapped quinols to Fe(III) at the cell exterior occurs in this bacterium via a network of low-potential bis-His ligated c-hemes contained in cytochromes of varying subcellular locations. However, there appears to be no general increase in heme potentials on progression of this putative network, and certain individual midpoint values are near replicated among the different redox proteins.

Other pathways involving multiheme proteins in bacterial electron transport include passage from the octaheme hydroxylamine oxidoreductase, HAO, to the tetraheme cytochrome c_{554}, Cyt c_{554}, in *Nitrosomonas europaea*. As HAO has been shown by x-ray crystallographic analysis to exist as a trimer (35), and this multimerization is reasoned to be essential for stabilization and catalytic function, the HAO-Cyt c_{554} electron

3 M. Ellington, S. J. Field, C. S. Butler, P. S. Dobbin, and D. J. Richardson, unpublished data.
Membranous c-Type Cytochromes of *S. frigidimarina* NCIMB400

transfer complex is thought to be consistent of 36 hemes. Comparable data regarding reduction potential ranges to that presented here for the membranous c-hemes of *S. frigidimarina* NCIMB400 have also been obtained for the HAO (36) and Cyt c554 (37) of *N. europae*, with the lowest midpoint values noted for these proteins being −390 and −226 mV, respectively. Furthermore, x-ray crystallography has revealed a number of structural similarities between the tetraheme core of Cyt c554 (38) and four of the hemes of HAO (35). Based upon studies of the cytochromes in *S. frigidimarina* NCIMB400 and *N. europae*, it would therefore seem that a simple consideration of only the thermodynamics of the individual heme equilibrium midpoints is irrelevant to electron transport through large low-potential networks of such redox centers. With specific respect to Fe(III) respiration by *S. frigidimarina* NCIMB400, both x-ray crystallographic studies and reconstitution experiments with purified cytochromes from different subcellular fractions may in the future provide further information as to how electrons are passed from CM-embedded (naptho)quinol to insoluble Fe(III) situated at the cell exterior.

Acknowledgments—We are grateful to Ann Reilly and Jeremy Thornton for technical assistance, and thank Dr Graeme Reid (University of Edinburgh) for useful discussions.

REFERENCES

1. Lovley, D. R. (1997) *FEMS Microbiol. Rev.* **20**, 365–313
2. Lovley, D. R., Phillips, E. J. P., and Lonergan, D. J. (1989) *Appl. Environ. Microbiol.* **55**, 700–706
3. Myers, C. R., and Nealson, K. H. (1988) *Science* **240**, 1319–1321
4. Moser, D. P., and Nealson, K. H. (1996) *Appl. Environ. Microbiol.* **62**, 2100–2105
5. Morris, C. J., Gibson, D. M., and Ward, F. B. (1990) *FEMS Microbiol. Lett.* **69**, 259–262
6. Myers, C. R., and Myers, J. M. (1992) *J. Bacteriol.* **174**, 3429–3438
7. Myers, C. R., and Myers, J. M. (1997) *J. Bacteriol.* **179**, 1143–1152
8. Boldan, M. D., Sears, H. J., Cheesman, M. R., Ferguson, S. J., Thomson, A. J., Berks, B. C., and Richardson, D. J. (1998) *J. Biol. Chem.* **273**, 28785–28790
9. Myers, C. R., and Myers, J. M. (1997) *Biochim. Biophys. Acta* **1326**, 307–318
10. Myers, J. M., and Myers, C. R. (1998) *Biochim. Biophys. Acta* **1373**, 237–251
Purification and Magneto-optical Spectroscopic Characterization of Cytoplasmic Membrane and Outer Membrane Multiheme c-Type Cytochromes from *Shewanella frigidimarina* NCIMB400

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*J. Biol. Chem.* 2000, 275:8515-8522.
doi: 10.1074/jbc.275.12.8515

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