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Biochimica et Biophysica Acta 1326 (1997) 307–318

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Outer membrane cytochromes of *Shewanella putrefaciens* MR-1: spectral analysis, and purification of the 83-kDa *c*-type cytochrome

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Received 12 December 1996; accepted 30 January 1997

Abstract

The metal-reducing bacterium *Shewanella putrefaciens* MR-1 is known to localize a majority of its membrane-bound cytochromes to its outer membrane when grown under anaerobic conditions. In this study, pyridine hemochrome spectra confirmed that these outer membrane cytochromes are *c*-type, and electrophoretic data demonstrated the presence of four distinct outer membrane cytochromes, with apparent molecular masses of 150, 83, 65, and 53 kDa. Fourth-order derivative analysis of 77 K spectra of the outer membrane revealed four spectrally distinct *c*-type hemes, with peaks at 545.4, 548.0, 550.6, and 552.6 nm. Outer membrane cytochromes in the reduced state were rapidly re-oxidized by oxidized iron and manganese, which have previously been shown to serve as electron acceptors for anaerobic respiration in this bacterium. The 83-kDa outer membrane cytochrome was purified and a specific polyclonal antibody was generated against this protein. Western blot analysis demonstrated that the vast majority of this protein was localized to the outer membrane and an intermediate density membrane fraction of similar composition. Its levels, but not its subcellular distribution, were somewhat influenced by the electron acceptor used to support anaerobic growth, with levels higher in fumarate-grown cells relative to iron(III)- or trimethylamine *N*-oxide-grown cells. Its specific content in cells grown under aerobic conditions was only 14% of that of fumarate-grown cells, suggesting that a switch to anaerobic conditions significantly increases the de novo synthesis of this outer membrane cytochrome.

Keywords: Outer membrane; Cytochrome; Anaerobic respiration; Respiration; Manganese; Iron; (*Shewanella putrefaciens*)

1. Introduction

Shewanella putrefaciens MR-1 [1–6] is a Gram-negative facultatively anaerobic bacterium that can couple its anaerobic growth, and link respiratory proton translocation, to the reduction of a variety compounds including fumarate, nitrate, TMAO, manganese(III/IV) oxides, iron(III) oxides, and others [2,3,7,8]. Previous studies implicate respiratory electron transport components, including cytochromes, in the reduction of manganese (Mn) and iron (Fe) oxides by MR-1. Antimycin A and HQNO (2-heptyl-4-hydroxyquinolone *N*-oxide) inhibit cellular Mn(IV) and Fe(III) reduction [2,9], proton translocation in response to Mn(IV) and Fe(III) [3], and the Fe(III) reductase activity of its outer membrane [6]. Formate, an effective electron donor in anaerobic respiration

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PII S0005-2736(97)00034-5

but not in substrate level phosphorylation [10,11], can serve as an electron donor for the reduction of Fe(III) and Mn(IV) by MR-1 [6,8,9]. The requirement of menaquinone for Fe(III) reduction, and a marked preference for menaquinone for Mn(IV) reduction, provide further support that the metal reducing systems in MR-1 are linked to anaerobic respiration [12].

Since Fe and Mn oxides are essentially insoluble in water at pH 7 [13–16], any proposed mechanisms of respiratory reduction of Fe(III) and Mn(III/IV) must include contact of the cells and at least some of their electron transport components with the insoluble metal oxides. Indeed, contact of *S. putrefaciens* cells with these oxides is necessary to mediate Mn or Fe reduction [2,17]. Possible ways to accomplish this would be to either (i) excrete solubilization factors that would extract Mn or Fe oxide molecules from extracellular oxides and transport them to the periplasm or cytoplasmic membrane or (ii) localize at least some electron transport components in the outer membrane (OM), where they could potentially make direct contact with the solid substrates at the cell surface. Consistent with this latter scenario, we have previously reported that MR-1 localizes approximately 80% of its membrane-bound cytochromes to its OM when grown under anaerobic conditions [4]. Cells grown under these same conditions have active Fe(III) and Mn(IV) reductase activities [3,6], with the majority of Fe(III) reductase activity in the OM [6].

This novel cytochrome distribution is in contrast to that of other bacteria in which the cytochromes are typically confined to the cytoplasmic membrane and periplasm [18–22]. These OM cytochromes are in a subcellular location where they could potentially make direct contact with the extracellular insoluble metal oxides, and could therefore play a key role in the anaerobic respiratory reduction of Mn(III/IV) and Fe(III). A thorough understanding of these OM cytochromes is needed to better understand their potential role in anaerobic respiration.

This paper provides spectral and electrophoretic evidence that there are at least four distinct cytochromes in the OM of anaerobically grown MR-1. All of these OM cytochromes are *c*-type. The purification and differential expression of one of these OM cytochromes is also described. This represents the first report of the purification of a cytochrome from the outer membrane of any bacterium.

2. Materials and methods

2.1. Materials

Tris, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (Hepes), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), fumaric acid disodium, and disodium EDTA were purchased from Research Organics (Cleveland, OH). Acrylamide and glycine were from EM Science (Gibbstown, NJ), sodium cholate was from Calbiochem (San Diego, CA), and Z3-12 (*N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) was from Sigma (St. Louis, MO). Chromatography media were purchased from Pharmacia Biotech (Piscataway, NJ). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA), U.S. Biochemical (Cleveland, OH), Sigma (St. Louis, MO), or Aldrich (Milwaukee, WI). CentriCell® ultrafiltration units (30 000 mol. wt. cut-off) were from Polysciences (Warrington, PA).

Adult New Zealand female white rabbits (specific pathogen-free) were purchased from Hazelton Labs (Denver, PA). The animals were maintained under conditions approved by the Institutional Animal Care and Use Committee (IACUC) and the Animal Resource Center (AAALAC accredited) of the Medical College of Wisconsin.

2.2. Organism and growth conditions

Shewanella putrefaciens MR-1 [2] was grown at room temperature (23–25°C) to mid-logarithmic phase as previously described [4]. Anaerobic growth was in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) with an atmosphere of 5 to 6% hydrogen (balance nitrogen). For aerobic growth, cultures of 500 ml in 2800-ml Fernbach flasks were shaken continuously at 200 rpm on an orbital shaker. Growth was in defined medium [3] supplemented with 15 mM lactate and vitamin-free Casamino acids (0.1 g liter^{-1}). For anaerobic growth, the medium was supplemented with either 20 mM trimethylamine *N*-oxide (TMAO) or 24 mM fumarate as the electron acceptor. For growth on TMAO, the medium was also supplemented with 30 mM Hepes to buffer against alkalinization by the product trimethylamine.

2.3. Subcellular fractionation and analysis

Cytoplasmic membrane (CM), intermediate membrane (IM), outer membrane (OM), and soluble fractions (periplasm plus cytoplasm) were purified from MR-1 cells using an EDTA-lysozyme-Brij protocol as previously described [4]. The IM is a hybrid of CM and OM [4]; such intermediate hybrid membrane fractions have been observed in other bacteria [20,21,23–25]. The separation and purity of these subcellular fractions were assessed by spectral cytochrome content [4], NADH-oxidase activity [21], membrane buoyant density [4], and SDS-PAGE gels [26,27]. SDS-PAGE gels were stained for protein with Coomassie blue or for heme as previously described [4]. Protein was determined by the method of Lowry et al. [28] modified as described [29], with bovine serum albumin as the standard.

2.4. Low-temperature (77 K) difference spectroscopy

Dithionite-reduced minus air-oxidized difference spectra were obtained at 77 K according to Scott and Poole [30] using the low-temperature accessory of an Aminco DW-2000 spectrophotometer (SLM Instruments, Urbana, IL) operated in the split beam mode. Spectra were recorded using sample holders with a 2-mm pathlength, a scan rate of 0.5 nm s^{-1} and a spectral bandwidth of 0.5 nm. Fourth-order derivative analysis of the spectra [30] was accomplished using the Savitsky-Golay algorithm supplied with the DW-2000 software.

2.5. Pyridine hemochrome spectra

Pyridine hemochrome spectra were determined essentially as described by Rieske [31]. A membrane sample containing 20 mg protein was treated at 0–4°C successively with 10 ml acetone, 10 ml chloroform/methanol (2:1), and 10 ml ethanol to remove the lipids and precipitate the proteins. The resulting pellets were extracted three times with 5 ml acetone containing 0.05 ml 2.4 M HCl. Both the resulting pellet and pooled supernatant fraction (the latter was first evaporated to dryness under a stream of N_2), were mixed with 1 ml pyridine and 1 ml 0.2 M KOH. Each sample was then divided equally between two quartz cuvettes. The contents of one cuvette were oxidized with 50 μl 50 mM potassium ferricyanide, and the other reduced with a few grains of sodium dithionite. The reduced-minus-oxidized spectra were recorded on the DW-2000 using a slit width of 2.0 nm and a scan rate of 2 nm s^{-1}. To prevent potential heme degradation, the pyridine spectra were done as quickly as possible after adding the pyridine and KOH [32].

2.6. Purification of the 83-kDa OM cytochrome

The 83-kDa OM cytochrome was purified from the OM of cells grown under anaerobic conditions with fumarate as the electron acceptor. To remove loosely associated proteins, the OM was treated at 23°C with 20.9 mM cholate in buffer A (20 mM K_2HPO_4 (pH 7.4), 1 mM EDTA, 0.02% azide, 5% glycerol) containing 9.4 mM DTT and 0.185 M NaCl (final concentrations which accounted for the volume of OM); the cholate/protein ratio was 9:1 (w/w) with protein at 1 mg ml^{-1}. After thorough stirring, the suspension was sonicated four times (30 s each) with interspersed 1–2 min periods of cooling at 23°C. The suspension was stirred an additional 10 min, and then centrifuged for 97 min at 50 000 rpm ($302\,000 \times g$) at 4°C in a Beckman 50.2Ti rotor. The cytochromes remained with the pellets which were resuspended in a total volume of 3.0 ml buffer A. To digest any remaining cell wall material, the suspended pellet was treated with 12 mg ($5.6 \cdot 10^5$ U) lysozyme for 1 h at 23°C; 500 U mutanolysin was added and incubation was continued for another hour at 23°C. The sample was diluted with buffer A and centrifuged in the 50.2Ti rotor as above. The cytochromes remained with the pellet which was subsequently solubilized at 23°C in buffer A containing 0.19 mM NaCl, 9.5 mM DTT, and 49.6 mM Z3-12 (final concentrations which accounted for the volume of OM); the Z3-12/protein ratio was 16.5:1 (w/w) with a final protein concentration of 1 mg ml^{-1}. After stirring for 10 min at 23°C, the solubilized OM was sonicated twice (30 s each) with interspersed 1 min periods of cooling at 23°C. This was centrifuged for 101 min at 52 000 rpm ($303\,800 \times g$) at 4°C in a Beckman 55.2Ti rotor. The supernatant fractions which contained the cytochromes were pooled and concentrated by ultrafiltration to a final volume of approximately 1.9 ml. This OM concentrate was applied to a Sephacryl

S-200 HR gel filtration column at 4°C (1.6 × 84 cm), and eluted with buffer A containing 0.5 M NaCl, 0.1 mM DTT, and 14.9 mM Z3-12 at a flowrate of 0.33 ml min^{-1}. The fractions were screened by heme- and silver-stained SDS-PAGE. The fractions containing the 83-kDa OM cytochrome were pooled and concentrated by ultrafiltration, and then dialyzed (14 000 mol. wt. cut-off) against buffer E (5 mM K_2HPO_4 (pH 7.4), 1 mM EDTA, 0.02% azide, 5% glycerol, 14.9 mM Z3-12, 0.1 mM DTT). The sample was applied to a DEAE-Sephacel ion exchange column at 4°C (1.6 × 20 cm). Buffer E was pumped through the column at a flowrate of 0.25 ml min^{-1} for 1.6 h to remove nonadherent proteins. The column was then developed using a 300 ml 0 to 0.5 M linear NaCl gradient in buffer E, followed by 100 ml 1 M NaCl in buffer E. The fractions were screened by heme- and silver-stained SDS-PAGE, and it was determined that the 83-kDa OM cytochrome had eluted at approximately 0.20 to 0.25 M NaCl. The fractions containing the 83-kDa OM cytochrome were pooled and concentrated by ultrafiltration, and then dialyzed (50 000 mol. wt. cut-off) at 4°C against 50 mM Tris-HCl (pH 6.9), 1 mM EDTA, 0.02% azide, 4 mM Z3-12. At this point, the 83-kDa OM cytochrome was purified to near homogeneity, and contained only a few minor contaminants. On the basis of protein content, the yield at this point was 1.4% of the original OM protein. In selecting fractions at the various steps, the goal was to optimize purity at the expense of recovery.

Final purification was achieved by preparative gel electrophoresis on a 10% SDS-PAGE gel. The 83-kDa OM cytochrome was clearly visible in the gel as an orange band; this band was excised and stored at −20°C. The band was thawed at 23°C in 0.9% saline, homogenized four times with a Potter–Elvehjem tissue homogenizer, and then extruded several times through a 27-gauge needle.

2.7. Generation of antibodies

Prior to immunization, preimmune serum was obtained from the rabbit. The initial injection consisted of 220 μg purified 83-kDa cytochrome. The immunogen was injected intradermally dispersed among 30–40 sites on the back of each rabbit. Intradermal booster injections of 110 μg of purified 83-kDa cytochrome were administered at 14 and 28 days after the initial injection. The rabbit was bled at day 35 to test the antibody response. A terminal bleed was done soon thereafter when it was determined that the desired antibody had been obtained.

2.8. Purification and characterization of antibodies

A purified immunoglobulin G (IgG) fraction was obtained from the immune and preimmune sera using ammonium sulfate fractionation and ion exchange chromatography as previously described [33,34]. Nonspecific antibodies were removed from preimmune and postimmune purified IgGs by absorption with autoclaved *Escherichia coli* JM109 [35] cells as described [36].

The IgG preparation was characterized by indirect Western blotting. Subcellular fractions of MR-1 were resolved by SDS-PAGE [26,27] using an acrylamide concentration of 10% in the running gel. Resolved proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad TransBlot, 0.45 μm) for 1.5 h at 100 volts (Electroblot system, Bio-Rad Laboratories, Richmond, CA) as described [37] except that the transfer buffer was 25 mM Tris/192 mM glycine. The membranes were briefly rinsed in deionized water, wrapped in plastic wrap, and stored at −20°C until development.

Thawed membranes were developed at room temperature as follows [38]: after blocking with 1% bovine serum albumin in TTBS (20 mM Tris-HCl, 0.05% Tween 20, 500 mM NaCl (pH 7.5)) for 3 h to prevent nonspecific binding, the membranes were incubated for 2 h with primary antibody (IgG) diluted to a final concentration of 2 μg ml^{-1} in blocking solution. The membrane was washed five times (5 min each) with TTBS, and then incubated for 30 min with secondary antibody (goat anti-rabbit IgG-alkaline phosphatase, Pierce) diluted 1:15000 in blocking solution. The membrane was washed three times in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl (pH 7.5)) and developed using the ImmunoPure NBT/BCIP Substrate Kit (Pierce). Densitometry was performed on the immunoblots using an AMBIS Optical Imaging System (AMBIS, San Diego, CA) with AMBIS Quantprobe Software (version 3.0). The molecular mass of protein bands was estimated based on their relative electrophoretic mobilities to known

standards using a computer program kindly provided by G. Raghava [39].

3. Results

Subcellular fractions of MR-1 were prepared as in previous studies [4–6]; a thorough characterization of fractions prepared by this method has been described [4]. Analysis of these fractions for NADH-oxidase activity, considered a marker for the CM [21,40], demonstrated that its specific activity was 7.5-fold greater in the CM than in the OM (Table 1). Analysis for membrane buoyant density and specific cytochrome content (Table 1), as well as SDS-PAGE patterns (Fig. 1), confirmed a prominent separation of the various subcellular fractions, which resembled those in previous experiments [4–6]. An absolute separation of these subcellular compartments has not been achieved in any bacterium, but these MR-1 fractions are separated to an extent similar to that reported for other bacteria [18–21,25,40–43], and are comparable to the analogous fractions from previous experiments [4–6].

3.1. Spectral and electrophoretic characterization of OM cytochromes

In our original report on the cytochrome content and distribution of anaerobically grown MR-1 cells, room temperature cytochrome spectra demonstrated that the OM, IM, and soluble fractions contain *c*-type cytochromes, whereas the CM contains both *b*- and *c*-type cytochromes [4]. In this study, pyridine

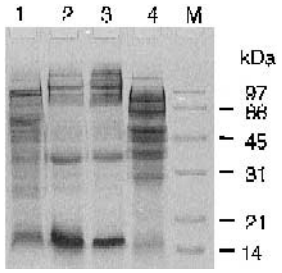

Fig. 1. SDS-PAGE profiles of subcellular fractions prepared from MR-1 cells grown anaerobically with fumarate. The gel was stained for protein with Coomassie blue. Lanes 1–4 were loaded with 50 μg protein of each of the following fractions: CM (1), IM (2), OM (3), and soluble (4). The molecular mass markers (M) were 97, 66, 45, 31, 21, and 14 kDa.

hemochrome spectra confirm the presence of heme *c* in the OM (Fig. 2). Since *c*-type heme is covalently attached to the protein moiety via thioether linkage, heme *c* ($\lambda_{max} \sim 550$ nm) remains with the acetone-HCl-insoluble material. Heme *b* ($\lambda_{max} \sim 560$ nm), which should be extracted into the acetone-HCl-soluble fraction, was not detected in the OM (Fig. 2). Pyridine hemochrome spectra also showed that the IM contains only heme *c*, while the CM contains both *b*- and *c*-type heme (not shown).

In previously published room-temperature cytochrome spectra of the OM, the α peak appeared as a single peak with a maximum at 552 nm [4]. Cytochrome spectra were performed at 77 K to improve the resolution, increase the signal-to-noise ratio, and resolve the individual components of the α peak. At 77 K, the α peak shifted to 550 nm (Fig. 3); this 2-nm shift to the blue end of the spectrum is expected

Table 1
Characteristics of membrane fractions isolated from cells grown anaerobically with fumarate [a]

Subcellular fraction [b]	Buoyant density (g cm^{-3}) [c]	Specific NADH-oxidase activity (nmol min^{-1} mg of protein^{-1})	Specific cytochrome content [d]
CM	1.085 ($\pm$0.009)	35.8 ($\pm$3.68)	0.503 ($\pm$0.093)
IM	1.194 ($\pm$0.001)	5.81 ($\pm$2.53)	1.40 ($\pm$0.126)
OM	1.237 ($\pm$0.003)	4.78 ($\pm$1.02)	1.86 ($\pm$0.141)
Soluble	–	1.32 ($\pm$0.41)	0.971 ($\pm$0.016)

[a] All values represent mean ($\pm$S.D.) for two different preparations.
[b] CM, cytoplasmic membrane; IM, intermediate membrane; OM, outer membrane; soluble, periplasm plus cytoplasm.
[c] Determined by isopycnic ultracentrifugation in sucrose gradients.
[d] Expressed as the difference between the absorbances at the peak and trough of the Soret region from reduced-minus-oxidized difference spectra per mg of protein.

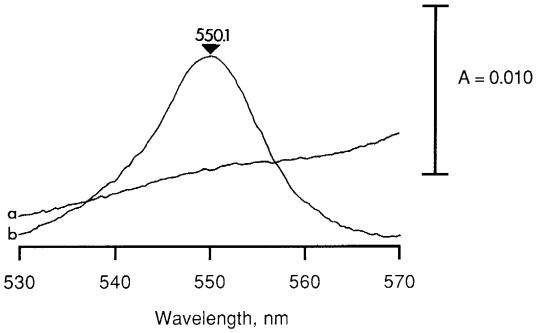

Fig. 2. Redox difference spectra of the pyridine hemochromes of the OM purified from fumarate-grown anaerobic MR-1 cells. No peaks were seen in the acetone-HCl-soluble material (a), which should contain heme *b* if present. A peak at 550.1 nm (heme *c*) is clearly evident in the acetone-HCl-insoluble material (b). The scan covered 520–600 nm; no peaks were seen in the portions of the scan not shown.

for 77 K spectra [30,44–46]. Fourth-order derivative analysis [46,47] of this 77 K spectrum of the OM revealed four spectrally distinct *c*-type hemes, with peaks at 545.4, 548.0, 550.6, and 552.6 nm (Fig. 3). In repeat spectra, four peaks were always distinguishable at wavelengths within 0.4 nm of these values. The positions of the maxima of the fourth-order derivative approximate the absorption maxima of the individual components which comprise the 77 K spectrum [45,46]. However, the heights of the peaks in the fourth-order derivative spectra do not necessarily reflect the relative concentrations of cytochromes [48].

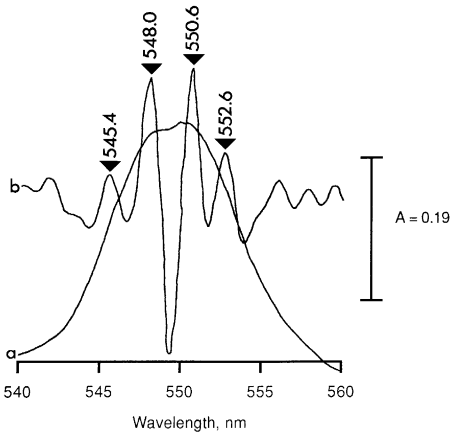

Fig. 3. Representative 77 K reduced-minus-oxidized difference spectrum of OM purified from anaerobically grown MR-1 (a), and 4th-order derivative of this spectrum (b). While not shown, scans covered the range of 400–700 nm; other peaks were the Soret at 422.2 nm and the β peak at 521.2 nm.

Heme staining of SDS-PAGE gels for *c*-type cytochromes is feasible since heme *c* is covalently bound to the cytochrome protein moiety [44], and is therefore not dissociated from the protein by the strongly denaturing conditions of SDS-PAGE. Previous heme-stained SDS-PAGE minigels with high protein loading (50 μg) revealed three OM cytochrome bands [4]. However, since the colored product from the heme stain is diffusible, these prominent bands were diffuse in appearance [4]. By using different acrylamide percentages, reducing the protein load to 2.5 μg, and also shortening the development time of the heme stain, sharper bands were obtained (Fig. 4). Under these modified conditions, four distinct OM cytochrome bands were seen (Fig. 4), which migrated with apparent molecular masses of 150, 83, 65 and 53 kDa. While each of the four peaks identified by the fourth-order derivative analysis of the 77 K spectrum of the OM (Fig. 3) may represent the four heme *c* proteins as identified by heme-stained SDS-PAGE (Fig. 4), it is possible for a single protein to display a split α absorption peak [45,49].

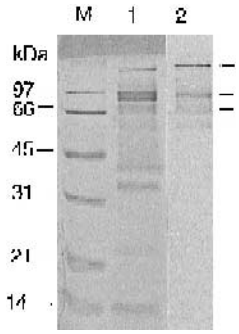

Fig. 4. SDS-PAGE profiles of OM prepared from anaerobically grown cells. The gel was cut in half and stained for protein (1), or for heme (2). Lane M contains molecular mass markers of the following sizes: 97, 66, 45, 31, 21, and 14 kDa. Based on average relative migration in several gels, the four OM cytochromes (marked by bars at right) exhibit apparent molecular masses of 150, 83, 65, and 53 kDa. The size of the 150-kDa cytochrome is estimated from other experiments in which a 200-kDa standard was included. Since the colored product from the heme stain is diffusible, the protein load was low (2.5 μg) and the heme development was stopped early to prevent the heme-positive bands from becoming diffuse in appearance.

3.2. Oxidation of OM cytochromes by Fe(III) and Mn(III)

We conducted in vitro spectral studies to examine the potential role of OM cytochromes in Fe(III) and Mn(III) reduction. All experiments were established in the anaerobic chamber using solutions that had been pre-equilibrated to anoxic conditions. They were sealed in anaerobic cuvettes with plunger devices (SLM Instruments) that allowed for later addition of the oxidant without compromising the anaerobic conditions; they were then scanned using an Aminco DW-2000 spectrophotometer. The OM cytochromes were first reduced by the physiologic electron donor formate (Fig. 5). Subsequent addition of Fe(III) citrate resulted in the rapid reoxidation of the OM cytochromes (Fig. 5). Neither sodium citrate nor water caused this reoxidation suggesting that it was related to the presence of Fe(III) (Fig. 5). The electron transport inhibitors pCMPS (*p*-chloromercuriphenylsulfonate), HQNO (2-heptyl-4-hydroxyquinolone *N*-oxide), and KCN blocked the reduction of the OM cytochromes by formate (Fig. 5); this is consistent with their ability to markedly inhibit Fe(III) reductase activity in purified OM [6] and in MR-1 cells [9]. The effects of KCN could be due to its marked inhibition of formate dehydrogenase, while the effects of the other inhibitors are not related to formate dehydrogenase [6].

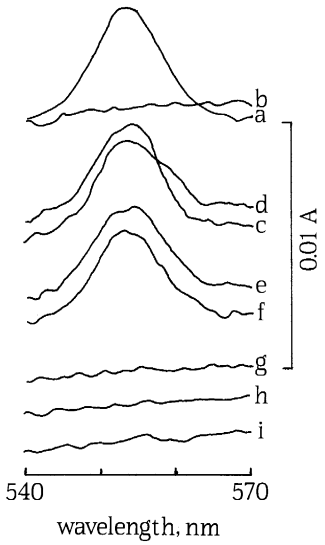

Fig. 5. Reduced OM cytochromes can transfer electrons to Fe(III). The experiments were performed in stirred anaerobic cuvettes (3.0 ml total volume) in: 28 mM phosphate (pH 7.5), 0.567 mM FMN, and purified OM (96 μg protein); the sample cuvette also contained 10 mM formate (pH 7.5). The cuvettes were set up in the anaerobic chamber and sealed to maintain anaerobic conditions. Periodic wavelength scans monitored the reduction of cytochromes in the sample cuvette. When cytochrome reduction had reached a maximum (approximately 15–30 min), a potential electron acceptor (e.g. ferric citrate) or control compound (e.g. sodium citrate) was added, using an anaerobic sealed plunger device, to both cuvettes to a final concentration of 10 mM. Wavelength scans were repeated at 1-min intervals. Formate-reduced OM cytochromes (a, c, e) were rapidly reoxidized by the addition of Fe(III) citrate (b), but not by water (d) or sodium citrate (f). The addition of 10 μM HQNO (g), 0.5 mM pCMPS (h), and 0.2 mM KCN (i) prevented formate-mediated cytochrome reduction. Paired scans were slightly offset from one another in the vertical axis so that individual scans could be discerned.

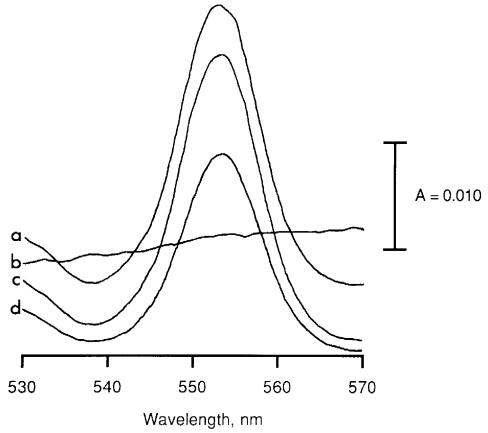

Fig. 6. Reduced OM cytochromes can transfer electrons to Mn(III). The experiments were performed as per Fig. 4. Formate-reduced OM cytochromes (a, c) were rapidly reoxidized by the addition of Mn(III) acetylacetonate (b), but not by Zn(II) acetylacetonate (d). Paired scans were slightly offset from one another in the vertical axis so that they could be discerned.

Analogous experiments were conducted to examine the potential role for OM cytochromes in Mn(III) reduction. OM cytochromes that had been reduced by formate were reoxidized by the addition of Mn(III) acetylacetonate, but not by Zn(II) acetylacetonate (Fig. 6), suggesting that the cytochrome oxidation was due to the addition of the Mn(III).

3.3. Purification of the 83-kDa OM cytochrome and generation of a specific antibody

OM proteins are generally more difficult to solubilize than cytoplasmic membrane proteins, and, once

solubilized, may adhere tenaciously to other proteins. Overcoming these two hurdles was therefore the initial focus of our purification strategies. The process was one of extensive trial and error and several different approaches were tried. Several nondenaturing nonpolar and zwitterionic detergents that have been used for the purification of other bacterial outer membrane proteins were tried, alone and in combination; these included Triton X-100, Lubrol PX, CHAPS (3-{(3-cholamidopropyl)dimethylammonio}-1-propanesulfonate), cholate, Z3-12, Z3-14 (*N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate), and octyl β-D-thioglucopyranoside. While all but cholate could solubilize the majority of cytochromes from EDTA-treated OM, subsequent purification of the OM cytochromes was most successful when Z3-12 was used. Several different chromatographic columns were tried: DEAE-Sephacel and DEAE-Sepharose CL-6B for anion exchange; CM-Sepharose CL-6B for cation exchange; Bio-gel HTP for hydroxylapatite; and Sephadex G-100, Sephadex G-150, Sephacryl S-200, Sepharose CL-6B, and Sepharose CL-4B for gel filtration. Trial and error was used to ascertain which ones provided the most optimal separation, and to ascertain the best order of use.

The procedures described in the methods section above resulted in the best separation of the 83-kDa OM cytochrome from the other OM proteins. It was purified to near homogeneity following gel filtration and anion exchange chromatography on DEAE-Sephacel (Fig. 7). Final purification was achieved by preparative gel electrophoresis. The gel-purified protein was used as an antigen to inject rabbits.

3.4. Characterization of antibody generated against the purified 83-kDa cytochrome

Initial characterization of the purified IgGs by Western blotting showed that in addition to reactivity with the expected protein, there were also several minor bands seen (not shown). These minor bands were seen in both pre-immune and post-immune IgG, and likely were the result of antibodies generated against common bacterial cell wall or outer membrane components [36]. Pre-adsorption of these purified IgG preparations with autoclaved *E. coli* JM109 cells according to Gruber and Zingales [36] resulted in the removal of these non-specific antibodies; the resulting preimmune IgG showed no reactivity with OM from MR-1, while the post-immune IgG showed high specificity for the 83-kDa OM cytochrome (Fig. 8).

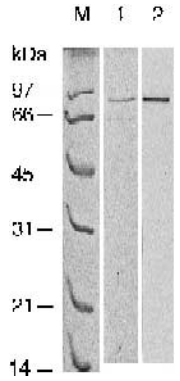

Fig. 7. SDS-PAGE of the 83-kDa OM cytochrome after DEAE-Sephacel chromatography (1,2). Lanes M and 1 were stained for protein using a silver stain, and lane 2 was stained for heme. The molecular mass markers (M) were 97, 66, 45, 31, 21, and 14 kDa.

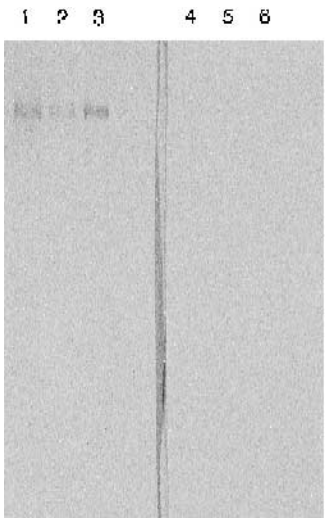

Fig. 8. Western blot using IgG specific for the 83-kDa OM cytochrome. The lanes were loaded as follows: 1 and 4, 83-kDa OM cytochrome after DEAE-Sephacel chromatography (approximately 0.1 μg protein); 2 and 5, 0.5 μg OM from fumarate-grown MR-1; 3 and 6, 1.0 μg OM from fumarate-grown MR-1. Lanes 1–3 were probed with IgG specific for the 83-kDa OM cytochrome, and lanes 4–6 with IgG from pre-immune serum. The band recognized by the IgG in lanes 1–3 migrated at a molecular mass of 83 kDa.

Table 2
Subcellular distribution of the 83-kDa cytochrome in cells grown anaerobically with fumarate [a]

Subcellular fraction [b]	Net specific content [c]	% of total protein recovered	% 83-kDa protein in each fraction [d]
CM	8.48 (±0.48)	9.89 (±1.44)	1.38 (±0.18)
IM	107. (±1.40)	15.2 (±0.14)	26.7 (±0.71)
OM	100. (±1.31)	31.3 (±1.98)	51.4 (±2.40)
Soluble	28.7 (±0.75)	43.6 (±3.32)	20.6 (±1.91)

[a] All values represent mean (±S.D.), $n = 2$.
[b] CM, cytoplasmic membrane; IM, intermediate membrane; OM, outer membrane; soluble, periplasm plus cytoplasm.
[c] Net specific content per 5 μg protein based on the net visual intensity assigned by the AMBIS imaging system relative to the background intensity which was subtracted from all values. The values are relative to those of the OM whose mean was arbitrarily set at 100.
[d] Calculated from average net specific content and individual total protein recoveries.

3.5. Subcellular distribution and expression of the 83-kDa OM cytochrome

The subcellular distribution of the 83-kDa OM cytochrome in MR-1 cells grown under anaerobic conditions with fumarate as the electron acceptor was examined by Western blotting. The specific content of this cytochrome was very high in the OM and IM relative to the CM and soluble fractions (Table 2); this agrees with previous heme-stained SDS-PAGE gels [4]. When accounting for the total protein distribution among these fractions, approximately 78% of the 83-kDa OM cytochrome protein was localized to the OM and IM fractions. While the exact nature of the IM is unknown, it closely resembles the OM except for its lower buoyant density (Fig. 1, Table 1). It is possible that it is a subpopulation of the OM; previous studies also suggest the possibility that the IM may at least in part represent Bayer's junction-like adhesion sites between the CM and OM [23,50,51]. Given its strong resemblance to the OM, however, it is not surprising that approximately one-fourth of the 83-kDa cytochrome is found in the IM.

Western blots indicated that approximately 20% of the 83-kDa cytochrome was in the soluble fraction (Table 2). This is greater than what has been seen in heme-stained SDS-PAGE gels [4]. This may be the result of the ability of the Western blot to detect additional populations of this protein. While heme-stained SDS-PAGE gels only detect mature cytochrome which is predominantly in the OM and IM [4], Western blots would also detect the apocytochrome in the cytoplasm, and periplasmic cytochrome en route to the outer membrane. We cannot exclude the possibility that the nonionic detergent Brij 58 used during the subcellular fractionation caused the release of a small amount of this cytochrome into the soluble fraction. However, we have previously shown that these OM cytochromes are not significantly removed from the OM by treatment with chaotropic agents, increased concentrations of Brij 58, or with 2% Triton X-100/10 mM $MgCl_2$ [4], indicating that these are integral membrane proteins. Nonetheless, the data clearly indicate that the vast majority of this 83-kDa cytochrome is associated with the OM and IM.

Western blots of OM from cells grown under various conditions indicated that the 83-kDa OM cytochrome was present in cells grown under anaerobic conditions with fumarate, TMAO, and Fe(III) as electron acceptors (Table 3). For anaerobically grown cells, the content was greatest in cells grown on fumarate, and least on cells grown on TMAO (Table

Table 3
Specific content of the 83-kDa cytochrome in the outer membrane of cells grown with different electron acceptors

Growth conditions	Electron acceptor	Net specific content [a]
Aerobic	O_2	14.4 (±0.64)
Anaerobic	fumarate	100. (±5.66)
Anaerobic	Fe(III)	71.1 (±2.83)
Anaerobic	TMAO	53.7 (±18.1)

[a] Net specific content per 5 μg protein based on the net visual intensity assigned by the AMBIS imaging system relative to the background intensity which was subtracted from all values. The values are relative to those of fumarate-grown cells whose mean was arbitrarily set at 100. All values represent mean (±S.D.), $n = 2$.

3). However, its specific content in the OM of cells grown under aerobic conditions was only 14% of that of fumarate-grown cells. This is consistent with heme-stained SDS-PAGE gels and cytochrome spectral data from previous reports [4,5]. While the specific content of this cytochrome was influenced by the electron acceptor, its relative subcellular distribution was not affected by the various growth conditions (data not shown).

4. Discussion

The *c*-type cytochromes are a large and diverse group of proteins that are widespread in both mitochondrial and prokaryotic electron transport chains [52]. The amino acid sequences for more than 200 *c*-type cytochromes from various sources have been obtained [52,53]. As a group, the functions of *c*-type cytochromes are very diverse, although many play roles in anaerobic electron transport chains including those responsible for the use of fumarate, nitrate, nitrite, and TMAO in various bacteria [44,54–58]. The ability of the OM cytochromes of MR-1 to be oxidized by Fe(III) and Mn(III) in vitro (Figs. 5 and 6) suggests a potential role for these cytochromes in vivo in the respiratory reduction of the these metal oxides, and is consistent with previous studies which implicated a role for cytochromes in metal reduction by MR-1 [2,3,6,9]. The ability of HQNO, pCMPS, and KCN to block the formate-dependent reduction of these OM cytochromes is in agreement with their ability to markedly inhibit the formate-dependent Fe(III) reductase activity of the OM [6].

In most other bacteria, mature *c*-type cytochromes are localized at the periplasmic side of the bacterial cytoplasmic membrane, and are either bound to the cytoplasmic membrane or are free periplasmic proteins [59]. MR-1 was the first strain for which a significant content of cytochromes was noted in its outer membrane [4]. Since in many bacterial species only crude membrane preparations have been analyzed, it is possible that the localization of cytochromes to the OM of MR-1 is not unique. The localization of cytochromes to the outer membrane of MR-1 is particularly significant, as it places them where they could potentially make direct physical contact with the extracellular Mn and Fe oxides. A complete oxidation of all of the formate-reduced cytochromes was seen with Fe(III) and Mn(III) (Figs. 5 and 6) which suggests a potential role for all of the OM cytochromes. By itself, however, these spectral data are not sufficient to prove an in vivo role. It is possible that the oxidation of the OM cytochromes by Fe(III) and/or Mn(III) (Figs. 5 and 6) represents a chemical and not a true enzymatic process. However, even if it is 'chemical', it is possible that this is the nature of cellular electron transport in MR-1 to insoluble Fe(III) or Mn(III/IV) oxides. One possible scenario is that these OM cytochromes can be 'chemically' oxidized via contact with insoluble metal oxides at the surface of the cell. Alternatively, it is possible that this cytochrome oxidation represents a true enzymatic process. At this point, it is not known whether these OM cytochromes themselves can directly transfer electrons to the metal oxides, or whether they serve as intermediate electron carriers to as yet other unidentified electron transport components. It is also unknown whether some of these cytochromes interact with each other as components of an electron transport chain or if each represents a different branch. Regardless of the mechanism of cytochrome oxidation, the data in Figs. 5 and 6 supply evidence for a transfer of electrons from formate, via OM cytochromes, to Fe(III) and Mn(III). In MR-1 cells, the electron transport components of the CM and OM could be linked by electron transport components present in the periplasm or in putative adhesion sites between the CM and OM [23,50,51]; however, a thorough understanding of these potential components in MR-1 is currently not available.

A role for cytochromes in metal reduction is not unprecedented; in vitro studies suggest that cytochrome c_3 of *Desulfovibrio vulgaris* may mediate uranium(VI) reduction [60]; however, the subcellular distribution of this cytochrome c_3 is not so relevant because, unlike Mn(III/IV) and Fe(III), U(VI) is water-soluble.

In other species, many *c*-type cytochromes are expressed only under anaerobic conditions, although some are also present under aerobic conditions [4,59]. The synthesis of these OM-localized cytochromes in MR-1 under anaerobic conditions coincides with the anaerobic reduction of Mn and Fe oxides by MR-1 cells [3,6]. In contrast to anaerobically grown cells, cytochrome spectra and heme-stained SDS-PAGE

gels had indicated that aerobically grown MR-1 cells had very low levels of cytochromes in the OM and other subcellular fractions [4–6]. Similarly, the total cytochrome content of *S. putrefaciens* NCMB 400 cells is significantly increased by a switch from aerobic to anaerobic conditions [61]. However, it was not clear from these previous results whether the switch to anaerobic conditions induced the de novo synthesis of these OM cytochromes or just the maturation or OM localization of previously synthesized precursors. Since the Western blots indicated that the level of the 83-kDa cytochrome in aerobic cells is approximately 14% of that in anaerobic fumarate-grown cells (Table 3), it would seem that the synthesis of these OM cytochromes is significantly increased when cells are placed under anaerobic conditions. However, the specific details of this regulatory mechanism are not yet known. However, a consistent increase in the anaerobic synthesis of all cytochromes in MR-1 is not the explanation, as the proportion and relative distribution of many other cytochromes in MR-1 is markedly different in aerobic versus anaerobic cells [4].

The data in Table 3 further indicate that, even under anaerobic conditions, the specific content of the 83-kDa OM cytochrome can be influenced by the electron acceptor used for growth of the cells. For example, the content of this cytochrome in TMAO-grown cells is approximately 50% of that in fumarate. This is less of a difference than predicted by previous spectral data in which the total specific cytochrome content in TMAO-grown cells was only 25 to 35% of that in fumarate-grown cells [4,6,12]. This could be the result of the ability of the antibody to also detect apocytochrome, or it could be that the specific content of the 83-kDa cytochrome is higher than that of some of the other OM cytochromes under these conditions.

Anaerobic growth with the different electron acceptors does not, however, apparently influence the subcellular distribution of the 83-kDa cytochrome. This agrees with previous cytochrome spectra and heme-stained SDS-PAGE gels which demonstrated that the relative subcellular distribution of cytochromes is similar in TMAO- vs. fumarate-grown cells (i.e. 75–87% of the membrane-bound cytochromes localized to the OM) [4,6,12]. Similarly, it has been reported that the cytochrome complement of anaerobic *S. putrefaciens* NCMB 400 cells is independent of the electron acceptor used but the total cytochrome content in fumarate-grown cells is consistently higher [61]. However, we have not examined the potential effect of all possible electron acceptors on cytochrome content and distribution in MR-1 cells, so we cannot exclude the possibility that growth with other electron acceptors may result in marked differences from our current findings.

In summary, four distinct *c*-type cytochromes are evident in the OM of anaerobically grown MR-1. These cytochromes are oxidized by oxidized forms of iron and manganese, which have previously been shown to serve as electron acceptors for anaerobic respiration in MR-1. The 83-kDa OM cytochrome was purified, and Western blots using specific antisera for this protein demonstrated that the vast majority is localized to the OM and an intermediate density membrane fraction of similar composition. Its specific content is markedly increased by a switch from aerobic to anaerobic conditions, suggesting that its de novo synthesis is significantly increased under anaerobic conditions. Other studies are currently underway in our lab to discern the in vivo role of these OM cytochromes.

Acknowledgements

This work was supported by National Institute of Health grant 5R21GM50786-02 to C.R.M.

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