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Isolation and sequence of *omcA*, a gene encoding a decaheme outer membrane cytochrome *c* of *Shewanella putrefaciens* MR-1, and detection of *omcA* homologs in other strains of *S. putrefaciens*

Judith M. Myers, Charles R. Myers *

Department of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA

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Abstract

The sequence of the *omcA* gene, which encodes a decaheme cytochrome *c* that is localized to the outer membrane (OM) of *Shewanella putrefaciens* MR-1, was determined. The 2202 bp nucleotide sequence of *omcA* encodes for 734 amino acids with a predicted molecular protein mass of 78.6 kDa. Comparison with the amino-terminal sequence of the mature protein suggests the presence of a hydrophobic leader sequence which is cleaved during translocation of the protein to the OM. This leader sequence has a lipoprotein consensus sequence for signal peptidase II at the cleavage site. The predicted mature protein is comprised of 708 amino acids with a predicted molecular mass of 75.8 kDa, but the addition of ten covalently attached heme *c* groups and covalent lipid modification to the amino-terminal cysteine increases the predicted mass to 82.7 kDa. This is consistent with its apparent mass of 83 kDa in SDS-PAGE gels. The predicted amino acid sequence for the OmcA protein shows no significant homology to known proteins. A RNA of approx. 2300 bases that hybridizes to the *omcA* gene was detected in anaerobically grown MR-1 cells. The size of this transcript is similar to the coding region of the *omcA* gene, suggesting that it is not part of a multicistronic operon. Similar to MR-1, four other strains of *S. putrefaciens* were all found to localize a majority of their membrane-bound cytochromes to the OM when grown under anaerobic conditions, and all contained an OM cytochrome of similar size to OmcA. In two of these strains, MR-4 and MR-8, a homolog of *omcA* was identified by RT-PCR and Southern blotting using primers and probes specific for *omcA* of MR-1. Western blot analysis using a polyclonal antibody to OmcA was similarly positive in strains MR-4 and MR-8. Partial nucleotide sequence analysis of these homologs demonstrated 74–77% predicted amino acid homology with OmcA of MR-1. In contrast, strains MR-30 and MR-42 tested negative for *omcA* homologs by Southern and Northern blots, RT-PCR, and Western blots.

Keywords: Outer membrane; Cytochrome; Sequence; Anaerobic respiration; (*Shewanella putrefaciens*)

1. Introduction

Shewanella putrefaciens MR-1 [1], a Gram-negative facultatively anaerobic bacterium, displays remarkable respiratory plasticity. It can couple its anaerobic growth, and link respiratory proton translocation, to the reduction of a variety of compounds including manganese(III/IV) oxides, iron(III) oxides, fumarate, nitrate, trimethylamine *N*-oxide (TMAO), and at least five other electron acceptors [1–4]. Previous studies with MR-1 implicate respiratory electron transport components, including cytochromes

* Corresponding author. Fax: +1 (414) 4566545; E-mail: cmyers@mcw.edu

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and menaquinone, in the reduction of manganese (Mn) and iron (Fe) oxides [1,3,5–8].

Given the marked insolubility of Fe and Mn oxides in water at pH 7 [9–11], MR-1 cells must have a way to link electron transport components with these extracellular insoluble metal oxides. In support of this, contact of *S. putrefaciens* cells with these oxides is necessary to mediate Mn or Fe reduction [1,12], and MR-1 localizes a majority of its formate-linked Fe(III) reductase activity in its outer membrane (OM). In addition, MR-1 localizes approx. 80% of its membrane-bound cytochromes to its OM when grown under anaerobic conditions with fumarate as the electron acceptor [13]; it has previously been demonstrated that the abilities to reduce Mn and Fe oxides are expressed in fumarate-grown cells [3]. These OM cytochromes are therefore localized where they could potentially make direct contact with the solid metal oxides at the cell surface, and could therefore play a key role in the anaerobic respiratory reduction of Mn(III/IV) and Fe(III). 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 [14–17].

A thorough understanding of these OM cytochromes is needed to better understand their potential role in anaerobic respiration. Previous studies have shown that MR-1 possesses four distinct *c*-type cytochromes in its OM, with apparent molecular masses of 150, 83, 65, and 53 kDa in SDS-PAGE gels [18]. Furthermore, the reduced forms of these OM cytochromes were rapidly re-oxidized by Fe(III) and Mn(III) [18], consistent with their putative role in metal reduction. The 83 kDa OM cytochrome was previously purified, and Western blot analysis using a specific polyclonal antibody for this protein confirmed its localization to the OM and an intermediate density membrane fraction of similar composition [18]. Its specific content in aerobically grown cells was very low relative to that of anaerobically grown cells, suggesting that a switch to anaerobic conditions significantly increases the de novo synthesis of this OM cytochrome [18].

As part of the continued studies on these OM cytochromes, this paper describes the cloning and sequence of the gene for the 83 kDa OM cytochrome (*omcA*) from MR-1. Significant levels of OM cytochromes are similarly present in other strains of *S. putrefaciens*, and two of these strains possess OmcA homologs. This represents the first report of the cloning and sequence of a cytochrome from the OM of any bacterium.

2. Materials and methods

2.1. Materials

The sources of materials were as previously described [18], with the following changes or additions: restriction enzymes were from New England BioLabs (Beverly, MA) or Pharmacia Biotech (Piscataway, NJ); the Expand High Fidelity and Expand Long Template PCR Systems were from Boehringer Mannheim (Indianapolis, IN); the TA Cloning and TOPO TA Cloning kits were obtained from Invitrogen (Carlsbad, CA), and custom oligonucleotide primers were synthesized by Operon Technologies (Alameda, CA) or Genemed Biotechnologies (South San Francisco, CA).

2.2. Bacterial strains, plasmids, media, and growth conditions

A list of the bacteria and plasmids used in this study is presented in Table 1. *Escherichia coli* strains were grown aerobically at 37°C on Luria-Bertani (LB) medium [19] supplemented, when required, with 50 μg ml^{-1} ampicillin (Ap) or kanamycin (Km). *S. putrefaciens* strains were grown at room temperature (23–25°C), under either aerobic or anaerobic conditions as previously described [13] in defined medium [3] supplemented with 15 mM lactate and vitamin-free casamino acids (0.1 g l^{-1}). For anaerobic growth, the medium was supplemented with 24 or 30 mM fumarate as the electron acceptor.

2.3. DNA manipulations, sequencing and sequence analysis

Restriction digests, ligation, cloning, subcloning, DNA electrophoresis, random-prime labeling of probes, and the isolation of genomic DNA from *S. putrefaciens* were done according to standard techniques [19]. Inverse PCR was done as described by

Ochman et al. [20]. Isolation of plasmid DNA was accomplished using the QIAprep Spin Plasmid kit (Qiagen, Chatsworth, CA). The size of DNA fragments, RNA, and proteins was estimated based on their relative electrophoretic mobilities to known standards using a computer program kindly provided by G. Raghava [21]. DNA fragments were recovered from agarose gels using GenElute Agarose Spin columns (Supelco, Bellefonte, PA), and subsequently concentrated by ethanol precipitation. Plasmids were introduced into *E. coli* by electroporation as previously described [8].

Thermal cycle DNA sequencing was accomplished using various biotinylated primers (including custom, M13/pUC, and T7) as previously described [8] except that the reactions were performed using the SequiTherm EXCEL DNA sequencing system (Epicentre Technologies, Madison, WI). Nucleotide sequence was verified by sequencing opposite strands and/or duplicate templates as appropriate.

Computer-assisted sequence analysis was done using MacVector software (IBI, New Haven, CT). Comparison to sequences on deposit in GenBank, EMBL, SWISS-PROT, and PIR-Protein was done using the Wisconsin Sequence Analysis Package of the Genetics Computer Group software, and the BLAST program [22].

2.4. Subcellular fractionation and analysis

Cytoplasmic membrane (CM), intermediate membrane (IM), OM, and soluble fractions (periplasm plus cytoplasm) were purified from cells of the various strains using an EDTA-lysozyme-Brij protocol as previously described [13]. The IM is a hybrid of CM and OM [13] and closely resembles OM on the basis of SDS-PAGE analysis [6,8,13,18,23]; such intermediate hybrid membrane fractions have been observed in other bacteria [15,16,24,25]. As in previous studies [6–8,13,18], these subcellular fractions were examined for membrane buoyant density [13], and by SDS-PAGE as adapted from Laemmli [26]. For

Table 1
Bacteria and plasmids used in this study

Bacterial strain or plasmid	Description	Reference	Source
S. putrefaciens[a]			
MR-1	Manganese-reducing strain from Lake Oneida, NY sediments	[1]	Previous study
MR-4	Manganese-reducing strain from the Black Sea water column	[57]	Previous study
MR-8	Manganese-reducing strain from the Black Sea water column	[57]	Previous study
MR-30	Manganese-reducing strain from Lake Michigan sediments	[58]	Previous study
MR-42	Manganese-reducing strain from Lake Michigan sediments	[58]	Previous study
MR-67	Manganese-reducing strain from Green Bay sediments		Unpublished
ATCC 8071	Type strain isolated from butter	[59]	American Type Culture Collection
E. coli			
JM109	*recA1* F′ *traD36 lacI*^{q}Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/e14$^-$(McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* ($r_K^-m_K^+$) *relA1 supE44*; used as host for pGEM-7Zf(+)	[60]	Promega
INVαF′	F′ *endA1 recA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 gyrA96 relA1* ϕ80*lacZ*Δ*M15*Δ(*lacZYA-argF*)*U169*; used as host for pCR2.1		Invitrogen
TOP10	F$^-$ *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15*Δ*lacX74 deoR recA1 ara-D139*Δ(*ara-leu*)*7696 galU galK rpsL* (Str^R) *endA1 nupG*; used as host for pCR2.1-TOPO		Invitrogen
Plasmids			
pGEM-7Zf(+)	3.00 kb cloning/sequencing vector; Ap^r		Promega
pCR2.1	3.9 kb TA cloning/sequencing vector; Ap^r Km^r		Invitrogen
pCR2.1-TOPO	3.9 kb TA cloning/sequencing vector; Ap^r Km^r		Invitrogen

[a]All strains were identified as *S. putrefaciens* by conventional taxonomic tests [61], and met all key taxonomic criteria of the type strain ATCC 8071.

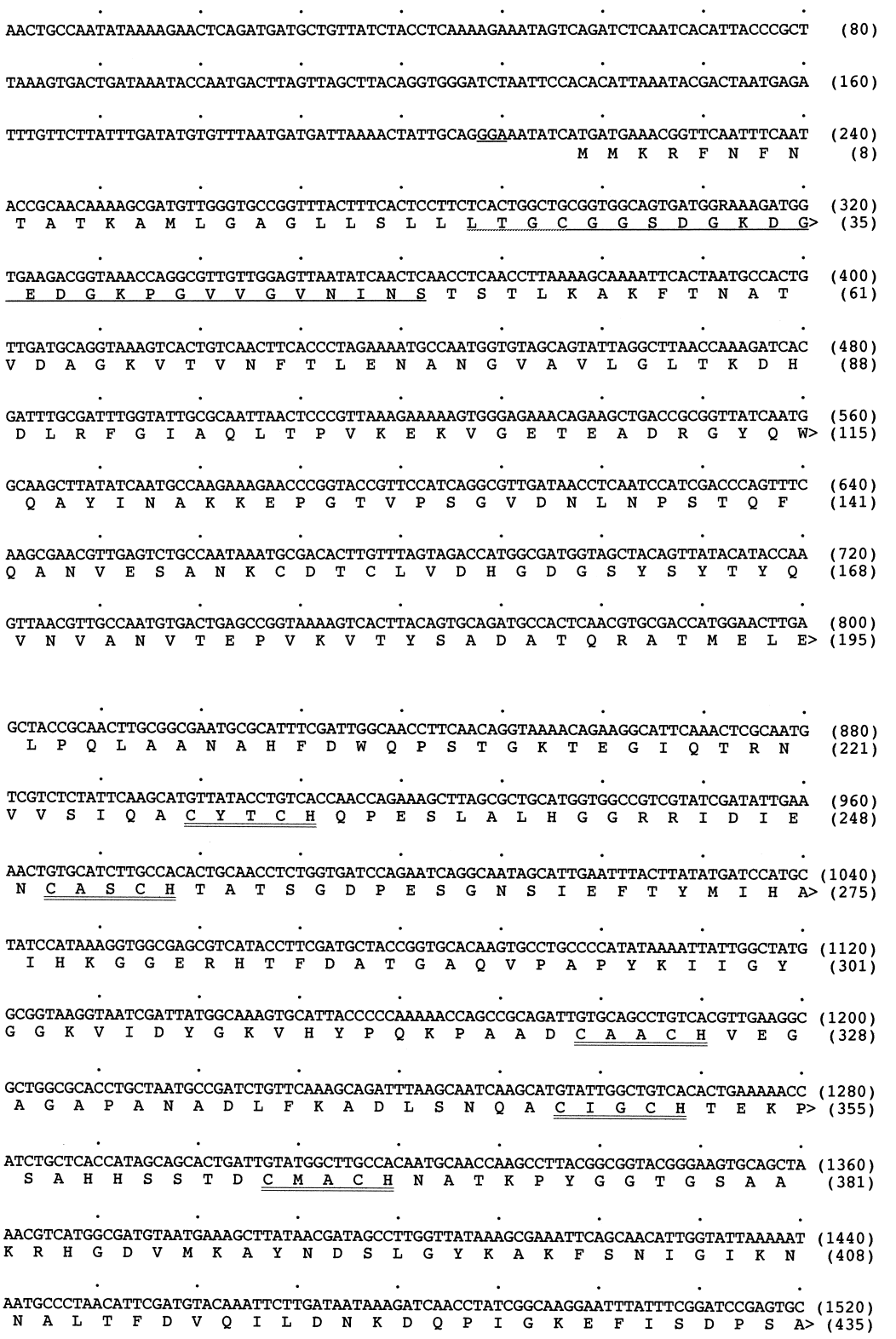
AACTGCCAATATAAAAGAACTCAGATGATGCTGTTATCTACCTCAAAAGAAATAGTCAGATCTCAATCACATTACCCGCT (80)
TAAAGTGACTGATAAATACCAATGACTTAGTTAGCTTACAGGTGGGATCTAATTCCACACATTAAATACGACTAATGAGA (160)
TTTGTTCTTATTTGATATGTGTTTAATGATGATTAAAACTATTGCAGGGAAATATCATGATGAAACGGTTCAATTTCAAT (240)
M M K R F N F N (8)
ACCGCAACAAAAGCGATGTTGGGTGCCGGTTTACTTTCACTCCTTCTCACTGGCTGCGGTGGCAGTGATGGRAAAGATGG (320)
T A T K A M L G A G L L S L L L T G C G G S D G K D G> (35)
TGAAGACGGTAAACCAGGCGTTGTTGGAGTTAATATCAACTCAACCTCAACCTTAAAAGCAAAATTCACTAATGCCACTG (400)
E D G K P G V V G V N I N S T S T L K A K F T N A T (61)
TTGATGCAGGTAAAGTCACTGTCAACTTCACCCTAGAAAATGCCAATGGTGTAGCAGTATTAGGCTTAACCAAAGATCAC (480)
V D A G K V T V N F T L E N A N G V A V L G L T K D H (88)
GATTTGCGATTTGGTATTGCGCAATTAACTCCCGTTAAAGAAAAAGTGGGAGAAACAGAAGCTGACCGCGGTTATCAATG (560)
D L R F G I A Q L T P V K E K V G E T E A D R G Y Q W> (115)
GCAAGCTTATATCAATGCCAAGAAAGAACCCGGTACCGTTCCATCAGGCGTTGATAACCTCAATCCATCGACCCAGTTTC (640)
Q A Y I N A K K E P G T V P S G V D N L N P S T Q F (141)
AAGCGAACGTTGAGTCTGCCAATAAATGCGACACTTGTTTAGTAGACCATGGCGATGGTAGCTACAGTTATACATACCAA (720)
Q A N V E S A N K C D T C L V D H G D G S Y S Y T Y Q (168)
GTTAACGTTGCCAATGTGACTGAGCCGGTAAAAGTCACTTACAGTGCAGATGCCACTCAACGTGCGACCATGGAACTTGA (800)
V N V A N V T E P V K V T Y S A D A T Q R A T M E L E> (195)
GCTACCGCAACTTGCGGCGAATGCGCATTTCGATTGGCAACCTTCAACAGGTAAAACAGAAGGCATTCAAACTCGCAATG (880)
L P Q L A A N A H F D W Q P S T G K T E G I Q T R N (221)
TCGTCTCTATTCAAGCATGTTATACCTGTCACCAACCAGAAAGCTTAGCGCTGCATGGTGGCCGTCGTATCGATATTGAA (960)
V V S I Q A C Y T C H Q P E S L A L H G G R R I D I E (248)
AACTGTGCATCTTGCCACACTGCAACCTCTGGTGATCCAGAATCAGGCAATAGCATTGAATTTACTTATATGATCCATGC (1040)
N C A S C H T A T S G D P E S G N S I E F T Y M I H A> (275)
TATCCATAAAGGTGGCGAGCGTCATACCTTCGATGCTACCGGTGCACAAGTGCCTGCCCCATATAAAATTATTGGCTATG (1120)
I H K G G E R H T F D A T G A Q V P A P Y K I I G Y (301)
GCGGTAAGGTAATCGATTATGGCAAAGTGCATTACCCCCAAAAACCAGCCGCAGATTGTGCAGCCTGTCACGTTGAAGGC (1200)
G G K V I D Y G K V H Y P Q K P A A D C A A C H V E G (328)
GCTGGCGCACCTGCTAATGCCGATCTGTTCAAAGCAGATTTAAGCAATCAAGCATGTATTGGCTGTCACACTGAAAAACC (1280)
A G A P A N A D L F K A D L S N Q A C I G C H T E K P> (355)
ATCTGCTCACCATAGCAGCACTGATTGTATGGCTTGCCACAATGCAACCAAGCCTTACGGCGGTACGGGAAGTGCAGCTA (1360)
S A H H S S T D C M A C H N A T K P Y G G T G S A A (381)
AACGTCATGGCGATGTAATGAAAGCTTATAACGATAGCCTTGGTTATAAAGCGAAATTCAGCAACATTGGTATTAAAAAT (1440)
K R H G D V M K A Y N D S L G Y K A K F S N I G I K N (408)
AATGCCCTAACATTCGATGTACAAATTCTTGATAATAAAGATCAACCTATCGGCAAGGAATTTATTTCGGATCCGAGTGC (1520)
N A L T F D V Q I L D N K D Q P I G K E F I S D P S A> (435)

Fig. 1.

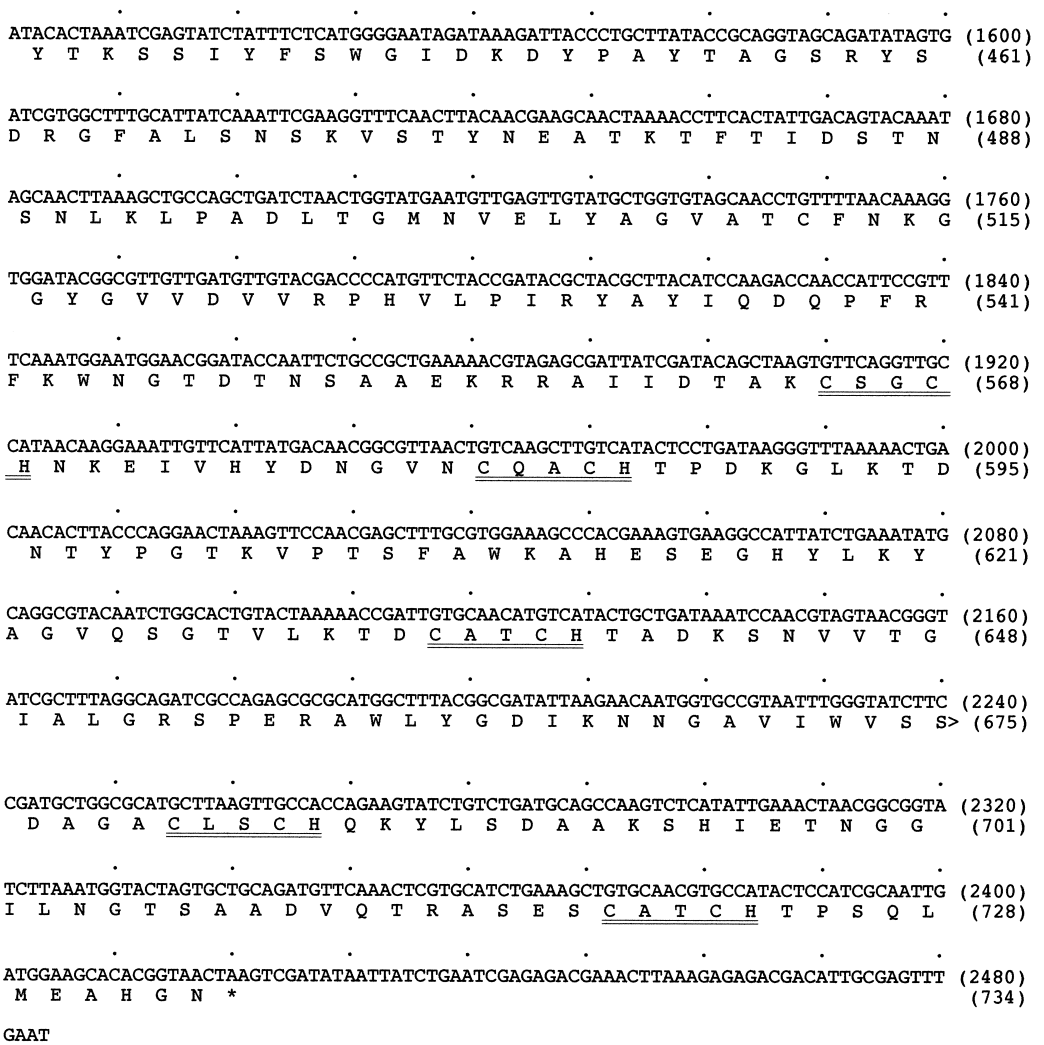

Fig. 1 (*Continued*). Nucleotide sequence of *omcA*, including flanking 5′ and 3′ regions. The inferred amino acid sequence is shown underneath with the putative ATG start codon at position 217. Other features indicated are a stop codon at positions 2419–2421 (*), a putative ribosome-binding site [30,31] at positions 208–210 (underlined). The amino-terminal sequence of the purified protein [18] is underlined (amino acids 28–49); the cysteine at position 27 is likely the first residue of the mature protein, but could not be identified, consistent with the putative lipoprotein modification of this residue. The lipoprotein consensus sequence (residues 24–27) is indicated by dotted underlining. The putative binding domains for heme *c* (CxxCH) are double-underlined and correspond to amino acids 228–232, 250–254, 321–325, 347–351, 364–368, 565–569, 582–586, 634–638, 680–684, and 719–723.

SDS-PAGE, the running gel (10% acrylamide, 0.267% bisacrylamide, 0.377 M Tris-HCl (pH 8.8), and 0.02% SDS) was polymerized in the presence of 0.024% ammonium persulfate and 6.65 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). The stacking gel (6% acrylamide, 0.16% bisacrylamide, 0.125 M Tris-HCl (pH 6.8), and 0.2% SDS) was polymerized in the presence of 0.06% ammonium persulfate and 6.65 mM TEMED. The samples were diluted as needed in 0.5 mM HEPES (pH 7.5) to equalize protein concentration, and then mixed with an equal volume of solubilization mix (0.125 M Tris-HCl (pH 6.8), 4% SDS, 21% glycerol, 0.1 M dithiothreitol, and 0.0005% bromophenol blue) prior to loading the gels. Electrophoresis was conducted with a Hoefer SE250 dual vertical slab unit (Amersham Pharmacia Biotech, Piscataway, NJ) using an electrode buffer of 49.5 mM Tris, 0.384 M glycine, and 0.1% SDS. Gels for Coomassie and heme stains were 1.5 mm thick and were run at a

constant current of 20 mA per gel, whereas gels for subsequent Western blotting were 0.75 mm thick and were run at a constant current of 10 mA per gel.

SDS-PAGE gels were stained for protein with ISS Pro-Blue (Owl Scientific, Cambridge, MA) or for heme [13] using the tetramethylbenzidine-hydrogen peroxide method of Thomas et al. [27]. Reduced-minus-oxidized cytochrome spectra were obtained as previously described [13], except that the buffer contained 0.5% Triton X-100 to aid dispersion of OM fragments. Protein was determined by the Lowry method [28] modified as described [29], with bovine serum albumin as the standard.

2.5. Northern and Southern blotting and RT-PCR

The isolation of total RNA using a hot phenol method and the removal of contaminating DNA using RNase-free DNase were done as previously described [8]. All standard precautions to prevent RNase contamination were followed [19].

Northern and Southern blot analyses were done as previously described [8] using 20–40 ng ml^{-1} biotin-labeled probe. Conditions for normal stringency consisted of overnight hybridization at 60°C, two post-hybridization washes for 5 min at 23°C in 2×SSC/0.1% SDS, and two washes for 15 min at 55°C in 0.1×SSC/0.1% SDS prior to development of the blots using the Phototope Detection system (New England BioLabs). Conditions of lower stringency consisted of overnight hybridization at 50°C, two post-hybridization washes for 5 min at 23°C in 2×SSC/0.1% SDS, and two washes for 15 min at 45°C in 1×SSC/0.1% SDS prior to development of the blots.

RT-PCR was done using the Titan One Tube RT-PCR System (Boehringer Mannheim) according to the manufacturer's instructions. Total RNA (2 μg) from each strain served as the template, and sense and antisense primers were based on the sequence of *omcA* from MR-1.

2.6. N-Terminal sequence of the 83 kDa OM cytochrome

The amino-terminal sequence of the 83 kDa OM cytochrome, which was previously purified from the OM of anaerobically grown MR-1 [18], was determined by L. Mende-Mueller of the Protein/Nucleic Acid Shared Facility of the Medical College of Wisconsin, using an Applied Biosystems model 477A pulsed liquid phase protein sequencer.

2.7. Western blotting

A previously generated polyclonal immunoglobulin (IgG) that is specific for the 83 kDa OM cytochrome of MR-1 [18] was used to screen the OM fractions isolated from various *Shewanella* strains grown under anaerobic conditions. These OM fractions were resolved by SDS-PAGE, the resolved proteins were electrophoretically transferred to a nitrocellulose membrane (BioRad TransBlot, 0.45 μm) for 1 h at 0.4 A (TE22 Tank Transfer unit, Hoefer Pharmacia Biotech, San Francisco, CA), and the membrane was developed using primary and secondary antibodies as previously described [18]. The molecular mass of positive bands was estimated based on their relative electrophoretic mobilities to known standards using a computer program kindly provided by G. Raghava [21].

2.8. Nucleotide sequence accession numbers

The nucleotide sequence and deduced amino acid sequence of this outer membrane cytochrome of MR-1 (Fig. 1) have been submitted to GenBank and have been assigned accession No. AF044272. Partial sequence data for the homologs of this gene found in strains MR-4 and MR-8 have been assigned GenBank accession Nos. AF060988, AF060989, AF060990, and AF060991.

3. Results

3.1. Sequencing of the gene for the 83 kDa OM cytochrome

The amino terminus of the previously purified 83 kDa OM cytochrome [18] was sequenced, yielding the following data: xGGSDGKDGEDGKPGVVGVNINS (x = unidentified residue). This partial protein sequence was used to design a degenerate oligonucleotide (5′-GGIAARGAYGGIGARGAYGGNAARCC-3′) for use as a primer in vector-linked

PCR. To accomplish this, MR-1 genomic DNA that was cut to completion with *Hae*III was ligated to pGEM-7Zf(+) that had previously been digested with *Sma*I and dephosphorylated using calf intestinal alkaline phosphatase. After purification by phenol/chloroform extraction and ethanol precipitation, this ligated DNA was used as a template for PCR using the degenerate primer (above) and a primer specific for the SP6 site in pGEM-7Zf(+) (5′-CTATTTAGGTGACACTATAGAATAC-3′). The products were analyzed by gel electrophoresis and compared to controls: (a) pGEM-7Zf(+) without insert used as the template; (b) the ligated DNA as template but SP6 as the sole primer. Four different annealing temperatures (52–61°C) were used; in the 52 and 55°C samples, a band (approx. 600 bp) unique to the experimental PCR was observed. This band was recovered from the gel, and ligated into pCR2.1 for nucleotide sequence analysis; the predicted amino acid sequence included an exact match to the remainder of the amino-terminal sequence (above), and included a single putative heme *c* binding site (CxxCH). These sequence data were used to design another PCR primer (5′-GTGACTGAGCCGGTAAAG-3′) which was used in an analogous strategy using as a template *Hin*dIII-digested MR-1 genomic DNA ligated in pGEM-7Zf(+). This yielded a product (approx. 200 bp) whose sequence was entirely contained within the original 600 bp product. Another round of this strategy using *Xho*I-digested MR-1 genomic DNA and another primer (5′-ATTCAAACTCGCAATGTCGTCTCT-3′) yielded a product (approx. 1570 bp) whose sequence partially overlapped the original 600 bp fragment, and encoded a total of ten heme *c* binding sites. Curiously, sequence analysis revealed that this fragment did not contain SP6 primer sequence or an *Xho*I site at either end, but was due to the MR-1-specific primer also annealing to the opposite strand approx. 1570 bp downstream. An in-frame stop codon was found 63 bp in from this downstream end, indicating that this fragment went beyond the carboxy terminus of the protein.

To obtain the nucleotide sequence upstream from that corresponding to the amino terminus of the protein, inverse PCR was done using primers oriented in opposite directions [20]. Genomic DNA from MR-1 was digested to completion with various restriction enzymes, and the resulting DNA fragments were diluted so that subsequent ligation favored the formation of monomeric circles. The resulting ligation products were used as templates for PCR using oppositely oriented primers based on the sequence known at this point (5′-ACTCCCGTTAAAGAAAAAGTGG-3′ and 5′-CATCAACAGTGGCATTAGTGAAT-3′). During sequence analysis, junctions between the ends of the original genomic fragments were identified based on the restriction site of the enzyme used to digest the genomic DNA [20]. While several enzymes were utilized to digest genomic DNA, templates which arose from digestion with *Kpn*I and *Eae*I each yielded a single product of 1.6 and 1.1 kb, respectively. Both products yielded a nucleotide sequence that went well upstream from what was known; sequence analysis of these products was discontinued once it was apparent that more than 200 bp upstream from the putative start codon had been obtained.

The nucleotide sequence data (Fig. 1) revealed a single open reading frame (ORF). The 2202 bp ORF encodes for 734 amino acids with a predicted molecular protein mass of 78.6 kDa. There are three methionine residues (positions 1, 2, and 14 in the amino acid sequence; Fig. 1) that could serve as putative start codons for this gene. The one at position 14 is probably not the start codon, as the closest ribosome-binding site [30,31] is more than 30 bases upstream from this residue. In contrast, a putative Shine-Dalgarno (S-D) sequence is observed at seven or ten residues upstream from the first and second methionine residues, respectively (Fig. 1). S-D sites in other genes are typically found at 3–15 bases upstream from the start codon [30]. Either of these methionines also has an A at the −3 position, which is the most prevalent residue at this position for other genes [30]. Either of these first two methionines could therefore potentially serve as the start codon. Comparison with the amino-terminal sequence of the mature protein (above) suggests the presence of a hydrophobic leader sequence of 25 or 26 residues which is cleaved during translocation of the protein to the OM. The deduced sequence of the mature protein consists of 708 amino acids with a predicted molecular mass of 75.8 kDa, and an estimated isoelectric point of 6.4. This isoelectric point is consistent with the protein's behavior during the ion ex-

change chromatography steps of the purification protocol [18].

This ORF, which we named *omcA* (*o*uter *m*embrane *c*ytochrome A), encodes for a protein containing ten putative binding domains for heme *c* (CXXCH) [32]. These heme *c* sites are not evenly distributed, and appear clustered in two groups of five, which span residues 228–368 and 565–723 (Fig. 1). The addition of these ten heme *c* groups, which are covalently bound to the cytochrome protein moiety [33], and the putative covalent lipid modification to the amino-terminal cysteine (see below) increase the predicted mass of the mature protein to 82.7 kDa, which is consistent with its apparent molecular mass of 83 kDa in SDS-PAGE gels [18]. Comparison to sequences on deposit in GenBank, EMBL, SWISS-PROT, and PIR-Protein failed to identify significant homology between the deduced amino acid sequence of OmcA and known sequences. Only a few short regions of OmcA demonstrated 30–40% identity with regions of cytochrome *c* precursor proteins from *Desulfovibrio vulgaris* (accession No. M63807) and *E. coli* (accession No. X72298).

A Kyte-Doolittle hydrophilicity plot of the predicted amino acid sequence of OmcA (Fig. 2) indicates a strongly hydrophobic sequence at the N terminus which contains features typical for a prokaryotic signal peptide [34]. The remainder of the protein contains a mix of hydrophilic and hydrophobic residues, although on balance it is mostly hydrophilic (Fig. 2). Its amino acid composition is 35.2% non-polar, 41.5% polar, and 23.2% acidic or basic residues. Other OM proteins tend to be similarly hydrophilic, as they are often β-type structures with multiple amphipathic antiparallel β-strands which span the OM [35–38]. The predicted secondary structure of OmcA, as calculated by the Robson-Garnier method of MacVector software, indicates the possible presence of significant β-sheet structure. However, such models are best suited for predicting the structure of soluble proteins, and experimental evidence is required to make more definitive conclusions regarding the secondary structure of OM proteins [35].

The leader sequence contains a lipoprotein consensus sequence (LxxC) [39] at the cleavage site (Fig. 1), suggesting that OmcA may be a lipoprotein. Lipoproteins, many of which are localized to the OM, contain glycerylcysteine containing two ester-linked fatty acids plus one amide-linked fatty acid at their amino terminus [39] which serves as a hydrophobic anchor for membrane attachment; this could explain why the N-terminal residue of mature OmcA, which should be a cysteine (Fig. 1), was not identifiable. While the fatty acid component of the lipoprotein modification can vary, palmitic acid is a common component of many lipoproteins [39]; if this assumption is extrapolated to OmcA, this lipid modification would add approx. 0.8 kDa to the predicted mass (see above).

3.2. Analysis for omcA expression in various strains

To assess whether the *omcA* ORF is transcribed, Northern blot analysis of total RNA from MR-1 cells grown anaerobically with fumarate as the elec-

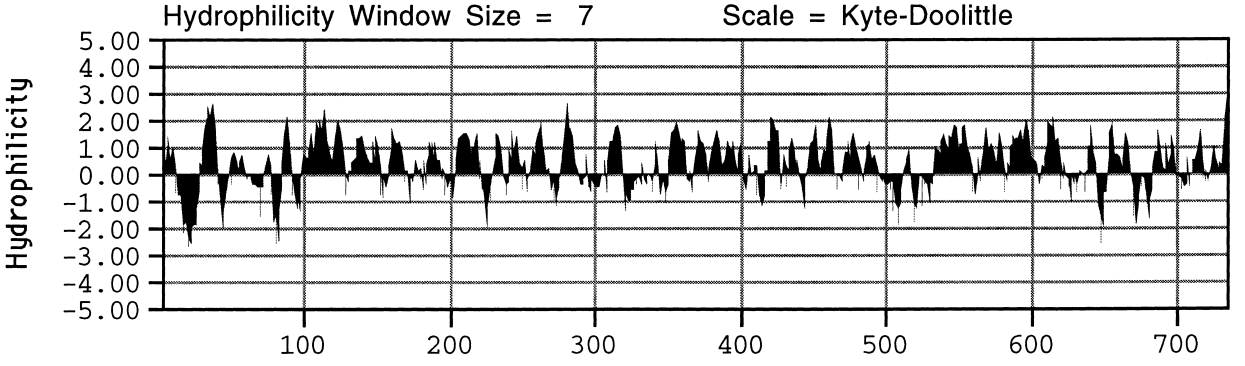

Fig. 2. Kyte-Doolittle hydrophilicity plot of the predicted unprocessed OmcA protein as generated by MacVector software. Hydrophilic regions extend upward from the 0.00 centerline, and hydrophobic regions extend below the 0.00 centerline.

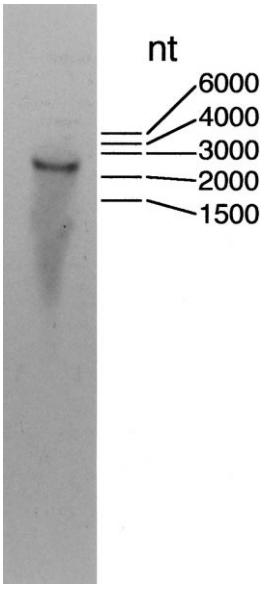

Fig. 3. Northern blot of 120 μg total RNA isolated from MR-1 grown anaerobically with fumarate. The membrane was probed with a biotin-labeled 371 bp fragment of *omcA* (spanning bases 475–845, Fig. 1). Based on relative migration to RNA markers (positions marked at right), the positive band was approx. 2200–2400 bases. X-Ray film exposure time was 6 min.

tron acceptor was conducted. Using a 371 bp fragment of *omcA* as a probe, a single RNA band migrating at approx. 2300 bases was detected in MR-1 (Fig. 3). This size is consistent with an expected transcript from the 2202 bp *omcA* ORF, and suggests that it is not transcribed as part of a multicistronic operon. No bands were seen in Northern blots of total RNA isolated from other strains of *S. putrefaciens* (MR-4, MR-8, MR-30, MR-42) grown under these same conditions. Repeat Northerns using a 'full-length' probe (fragment spanning positions 377–2383, Fig. 1) yielded the same size band in MR-1, but no signal with the other strains (not shown). This remained the case even when using less stringent conditions and lengthy film exposure times. Because the type strain of *S. putrefaciens*, ATCC 8071, grew very poorly in the defined medium under these conditions, it could not be utilized in these comparative studies.

In contrast, using the 'full-length' probe and the less stringent conditions during Southern blots of genomic DNA digested to completion with *Xho*I or *Eco*RI, positive results were obtained for MR-1, MR-4, and MR-8, whereas MR-30 and MR-42 were negative. Because of its enhanced sensitivity relative to Northern blots, RT-PCR was performed using sense and antisense primers derived from *omcA* of MR-1. Strains MR-1, MR-4, and MR-8 were all positive for a band of the expected size of 1908 bp (corresponding to nucleotides 475–2383 in Fig. 1), whereas strains MR-30 and MR-42 were negative (Fig. 4). Using these same primers for PCR of genomic DNA from these strains similarly yielded the same size band in MR-1, MR-4, and MR-8, while strains MR-30 and MR-42 remained negative. Therefore, RT-PCR, PCR of genomic DNA, and Southern blots (data not shown) all indicated the presence of *omcA* homologs in MR-4 and MR-8, and the absence of readily detectable homologs in MR-30 and MR-42.

Partial sequence analysis of the ends of the PCR products from MR-4 and MR-8 demonstrated 93.5% amino acid identity between the OmcA homologs of MR-4 and MR-8, whereas they have 73.9% and 76.6% identity, respectively, with the corresponding regions of OmcA of MR-1 (Fig. 5). Most non-identical residues are clustered at a few sites. The three heme *c* binding sites contained in these regions of

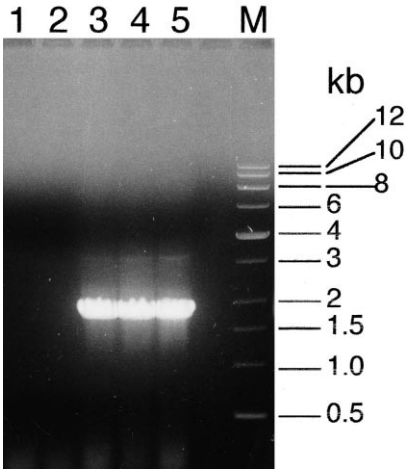

Fig. 4. RT-PCR for *omcA* expression in various strains of *S. putrefaciens*. Total RNA (2 μg) from each strain served as template and sense (5′-GATCACGATTTGCGATTTGGTATT-3′) and antisense (5′-GGCACGTTGCACAGCTTTCAGATG-3′) primers were derived from the sequence of *omcA* in MR-1. Lanes 1–5 were loaded with 15 μl of the following completed RT-PCR reactions: MR-42 (1), MR-30 (2), MR-8 (3), MR-4 (4), MR-1 (5). The sizes of the DNA markers (lane M) in kb are indicated to the right of the figure.

A

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MR-1  89 DLRFGIAQLTPVKEKVGETE--ADRGYQWQAYINAKKEPGTVPSGVDNLNPS 138
MR-4     .........h.t.atddpakp..............t...ai.......k..
MR-8     .........h.t.atddpakp..............................

MR-1 139 TQFQANVESANKCDTCLVDHGD 160
MR-4     ..yk....a.s...........
MR-8     a.y.....a.............
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B

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MR-1 614 SEGHYLKYAGVQSGTVLKTDCATCHTADKSNVVTGIALGRSPERAWLYGD-I 664
MR-4     .rrslpeicc........s......-ve..g.....e...a...t.i.pnst
MR-8     .rrslseicc........s......-ve..g.....e...a...t.i.pntt

MR-1 665 KNNGAVIWVSSDAGACLSCHQKYLSDAAKSHIETNGGILNGTSAADVQTRAS 716
MR-4     tydk.gv.............................a.....d...s........
MR-8     tydktg.............................a.....d.......k....

MR-1 717 ESCATC 722
MR-4     ......
MR-8     ......
```

Fig. 5. Predicted amino acid sequence similarities between the OmcA protein of MR-1 and its homologs in MR-4 and MR-8. Nucleotide sequences of the ends of the PCR products from MR-4 and MR-8 were obtained, and the predicted amino acid sequences aligned to the corresponding regions in OmcA of MR-1. Panels A and B represent alignments that correspond to the amino-terminal and carboxyl-terminal ends of the PCR products, respectively. Identical residues are indicated by dots, whereas lowercase letters are used to indicate residues that are different from those in MR-1. As needed, alignments were facilitated by introducing occasional gaps (–). The numbers at the beginning and end of each line of MR-1 sequence indicate the relative numbering of amino acid residues in OmcA of MR-1 (from Fig. 1). The three most C-terminal heme *c* binding sites of OmcA of MR-1 are underlined in panel B.

comparison are conserved in MR-4 and MR-8 (Fig. 5), and correspond to the three most C-terminal *c*-type hemes in OmcA.

To further investigate whether strains MR-30 and MR-42 contained a non-expressed homolog of *omcA*, PCR of genomic DNA from these strains was done using different combinations of sense (5′-GATCACGATTTGCGATTTGGTATT-3′, 5′-GTGACTGAGCCGGTAAAAC-3′, 5′-AAGCAAAATTCACTAATGCCACTG-3′, 5′-ACTCCCGTTAAAGAAAAAGTGG-3′, 5′-GCTTAGCGCTGCATGGTGG-3′) and antisense (5′-GGCACGTTGCACAGCTTTCAGATG-3′, 5′-GCTTTCAGATGCACGAGTTTGA-3′, 5′-GGGGTAATGCACTTTGCCATAATC-3′) primers derived from *omcA*. Results were negative in all cases except one in which the PCR product was larger than expected (not shown); partial sequence analysis beyond the primers themselves demonstrated no discernible nucleotide or predicted amino acid sequence homology to *omcA* (not shown). Therefore, MR-30 and MR-42 do not have readily detectable homologs to *omcA*.

3.3. Analysis of outer membrane cytochromes in various strains

Subcellular fractionation of various strains of *S. putrefaciens* that had been grown under anaerobic conditions was accomplished using a protocol previously developed for MR-1 [13]. Analysis of these fractions for membrane buoyant density and specific cytochrome content (Table 2), as well as SDS-PAGE patterns (not shown), confirmed a prominent separation of the various subcellular fractions, similar to that reported for other bacteria [14–16,25,40–44], and comparable to those in previous experiments with MR-1 [6,13,23].

Similar to results previously reported for MR-1 [13], the specific cytochrome content of the OM was markedly higher than that of the CM for each strain (Table 2). While 46–60% of total cellular cytochrome was found in the soluble fraction (Table 2), the OM contained 49–74% of total membrane cytochromes (Table 2), similar to values previously reported for MR-1 [6,7,13]. Given that the intermedi-

ate density membrane fraction (IM) closely resembles the OM, except for its lower buoyant density [6,7,13,18], 83–95% of membrane-bound cytochromes were localized to the OM plus IM in these various strains (Table 2). This is in marked contrast to other bacterial species, in which the vast majority of membrane-bound cytochromes are localized in the CM [14–16,42]. These results suggest that the localization of significant levels of cytochromes to the OM is not unique to MR-1, but is common to several strains of *S. putrefaciens*. Similar to a previous report on MR-1 [13], the cytochrome spectra (not shown) for all strains were consistent with the presence of *c*-type cytochromes in the IM, OM, and soluble fractions, and with both *c*-type and *b*-type cytochromes in the CM. Because the type strain, ATCC 8071, grew very poorly in the defined medium under these conditions, it could not be included in these comparisons.

Heme staining of SDS-PAGE gels for *c*-type cytochromes is feasible since heme *c* is covalently bound to the cytochrome protein moiety [33], and is therefore not dissociated from the protein by the strongly denaturing conditions of SDS-PAGE. Previous studies demonstrated the presence of four distinct cytochrome bands in the OM of MR-1, which migrated with apparent molecular masses of 150, 83, 65 and 53 kDa [18]. These four cytochromes were again detected in the OM of MR-1 (Fig. 6). All other strains of *S. putrefaciens* contained at least two high molecular weight cytochromes in the OM (Fig. 6). Each strain contained a prominent OM cytochrome that was approximately the size of OmcA in MR-1; the second most prominent OM cytochrome in each of

Table 2
Characteristics of subcellular fractions isolated from cells of various *S. putrefaciens* strains grown anaerobically with fumarate

Subcellular fraction[a]	% of total protein recovered	Specific cytochrome content[b]	% of total cellular cytochrome	Buoyant density (g cm^{-3})[c]
MR-1 cells				
CM	5.2	0.704	2.6	1.094
IM	5.7	1.96	8.1	1.190
OM	15.5	2.70	29.8	1.228
Soluble	73.6	1.14	59.5	–
MR-4 cells				
CM	9.2	0.696	3.6	1.087
IM	20.0	2.08	23.5	1.191
OM	20.9	2.24	26.4	1.225
Soluble	49.9	1.77	46.5	–
MR-8 cells				
CM	6.2	1.38	7.4	1.105
IM	6.5	1.98	11.2	1.190
OM	16.4	1.77	25.2	1.221
Soluble	70.9	0.930	56.3	–
MR-30 cells				
CM	4.1	1.11	2.7	1.092
IM	9.1	2.21	11.9	1.189
OM	22.4	2.81	36.8	1.241
Soluble	64.4	1.37	48.7	–
MR-42 cells				
CM	3.5	1.04	2.1	1.092
IM	12.6	2.23	16.2	1.192
OM	17.3	2.98	29.8	1.219
Soluble	66.6	1.29	52.0	–

[a]CM, cytoplasmic membrane; IM, intermediate membrane; OM, outer membrane; Soluble, periplasm plus cytoplasm.
[b]Expressed as the difference between the absorbances at the peak and trough of the Soret region from reduced-minus-oxidized difference spectra per milligram of protein.
[c]Determined by isopycnic ultracentrifugation in sucrose gradients.

these other strains appeared slightly larger than the 65 kDa OM cytochrome in MR-1 (Fig. 6). A Western blot using a previously generated antibody that is specific for OmcA [18] was positive for strains MR-4 and MR-8 (Fig. 7). These immunopositive bands comigrated with the heme-positive bands in MR-1 and MR-8 (approx. 83 kDa), and MR-4 (approx. 79 kDa). Even though the band in MR-4 is slightly smaller than that in MR-1 and MR-8, RT-PCR analysis yielded a band of very similar size in all three strains (Fig. 4), and partial nucleotide sequence analysis showed it to have significant identity to *omcA* (Fig. 5). Hence, the small size difference in the mature MR-4 protein is likely due to small sequence deletions or post-translational modifications at its amino- and/or carboxy-terminal regions. Although strains MR-30 and MR-42 each contain an OM cytochrome of approximately the same size, the anti-OmcA antibody did not react with these cytochromes (Fig. 7), even when the experiments were repeated using higher amounts of OM (not shown). The Western blot results are therefore consistent with PCR, RT-PCR, and Southern blot results for these strains (see above).

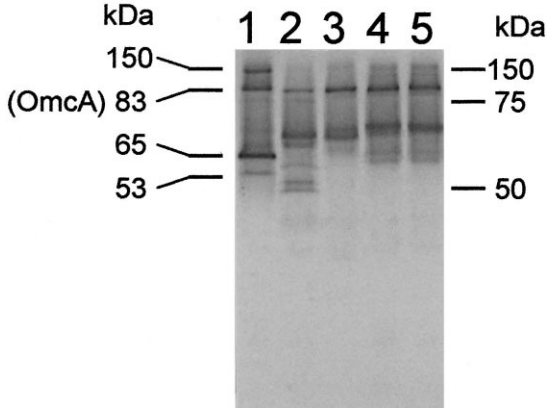

Fig. 6. Heme-stained SDS-PAGE minigel profiles of OM prepared from various strains of *S. putrefaciens* grown anaerobically with fumarate. The lanes were loaded with 5 μg OM protein from the following strains: 1, MR-1; 2, MR-4; 3, MR-8; 4, MR-30; 5, MR-42. The four OM cytochromes of MR-1 (marked by bars at left) were previously shown to have apparent molecular masses of 150, 83, 65, and 53 kDa [18]. The migration of protein standards is noted at the right of the gel. The diffusible nature of the colored product from the heme stain leads to the diffuse appearance of the heme-positive bands.

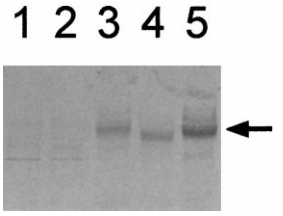

Fig. 7. Western blot of OM prepared from various strains of *S. putrefaciens* grown anaerobically with fumarate, using IgG specific for the 83 kDa OM cytochrome [18]. The lanes were loaded with 5 μg OM protein from the following strains: 1, MR-42; 2, MR-30; 3, MR-8; 4, MR-4; 5, MR-1. The band corresponding to OmcA in MR-1 is indicated by the arrow at the right of the blot.

4. Discussion

The *c*-type cytochromes are a large and diverse group of proteins that serve as components of the electron transport chains of mitochondria and many prokaryotes [32]. The amino acid or nucleotide sequences for more than 200 *c*-type cytochromes from various sources have been reported [32,45]. As a group, the functions of *c*-type cytochromes are very diverse, although many play roles in anaerobic respiratory electron transport chains in various bacteria [33,46–50]. This is also the case for two *c*-type cytochromes from *S. putrefaciens* that have been previously characterized: (a) CymA in strain MR-1 is required for the reduction of nitrate, fumarate, and Fe(III) [8], and (b) the fumarate reductase from strain NCMB400 is a flavocytochrome *c* [49].

OmcA is a decaheme cytochrome *c* with an estimated molecular mass of 82.7 kDa. Cytochromes *c* vary significantly in mass, but large multiheme cytochromes are present in several species of bacteria [51–53]. Multiheme cytochromes *c* have been previously characterized in *S. putrefaciens*, including the tetraheme cytochrome CymA from MR-1 [8], and a tetraheme flavocytochrome *c* which serves as the fumarate reductase of NCMB400 [49]. Cytochromes *c* characterized to date have between one and 16 heme groups per molecule [32], with midpoint redox potentials of the heme groups ranging from 400 mV to −400 mV in various cytochromes [32]. Most commonly, either a nitrogen atom from histidine or a sulfur atom from methionine serves to fill the sixth coordination position of the heme *c* iron, but in some cases this position is also unoccupied [32]. Exclusive

of the heme *c* binding domains, OmcA has six methionine residues and 17 histidine residues, but it is not yet known which, if any, of these residues occupy the sixth coordination positions of the ten heme irons. Additional studies will be needed to characterize the redox potentials and other physicochemical properties of OmcA.

In most bacteria, mature *c*-type cytochromes are localized at the periplasmic side of the cytoplasmic membrane, and are either free periplasmic proteins or are bound to the cytoplasmic membrane [54]. MR-1 was the first strain shown to localize the majority of its membrane-bound *c*-type cytochromes in its OM [13]. It does this only under anaerobic conditions [13,18], corresponding to required conditions for metal reduction [1]. The results of this study now make it clear that localization of the majority of membrane-bound cytochromes to the OM of anaerobically grown cells is not unique to MR-1, but is true for all strains of *S. putrefaciens* that were examined (Table 2). While only two of these strains contained an OM cytochrome with strong immunologic identity to OmcA in MR-1 (Fig. 7), all strains contained two or more higher molecular weight *c*-type cytochromes in their OM (Fig. 6). The localization of cytochromes to the OM is particularly significant, as it places them where they could potentially make direct physical contact with the extracellular Mn and Fe oxides. The ability of Fe(III) and Mn(III) to oxidize the OM cytochromes of MR-1 in vitro [18] suggests a potential role for these cytochromes in the respiratory reduction of the these metal oxides, and supports previous studies which implicated a role for cytochromes in metal reduction by MR-1 cells [1,3,5,6]. The ability of certain electron transport inhibitors to block the formate-dependent reduction of these OM cytochromes [18] is consistent with their ability to markedly inhibit the Fe(III) reductase activity of the OM [6].

Various features of OM proteins in other bacteria are important for their localization to the OM [38]. For example, many OM proteins contain either phenylalanine or tryptophan at their C terminus, and for others the sorting signature is in the last β-strand [38]. However, in other OM proteins these features cannot be found [38]. For example, even though most lipoproteins in Gram-negative bacteria are localized in the OM, they tend to lack these other OM-targeting features [38]. This is the case for OmcA, which has a very strong lipoprotein consensus sequence, but yet lacks either phenylalanine or tryptophan at its C terminus (Fig. 1). While additional work will be required to confirm the lipoprotein nature of OmcA, it is likely that this would play a prominent role in its localization to the OM of MR-1.

At present, 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. 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 [24,55,56]; however, a thorough understanding of these potential components in MR-1 is currently not available. A previous report described the requirement for a 21 kDa tetraheme cytochrome *c* (CymA) for the reduction of Fe and Mn oxides, fumarate, and nitrate by MR-1 cells [8]. Since CymA, which shares partial homology to multiheme *c*-type cytochromes in other bacteria, is ostensibly associated with the outer face of the CM, it could serve as one of the components required to shuttle electrons from the CM to electron carriers in the periplasm and/or OM of MR-1 [8]. Further studies will be necessary to elucidate the role of OmcA in MR-1, as well as the other components with which it interacts. On the basis of their prominent sequence similarities to OmcA in MR-1 (Fig. 5), the homologs in MR-4 and MR-8 will likely share a function similar to that of OmcA in MR-1. In contrast, it is difficult to predict whether the 83 kDa OM cytochromes in MR-30 and MR-42 (Fig. 6) will share an analogous function to OmcA since they were not detected as OmcA homologs on the basis of RT-PCR (Fig. 4), Western blots (Fig. 7), and Southern blots.

In summary, the 2202 bp nucleotide sequence of *omcA*, which encodes a decaheme cytochrome *c* that is localized to the OM of *S. putrefaciens* MR-1, was determined. The deduced amino acid sequence for OmcA shows no significant homology to known proteins. The leader sequence has a lipoprotein consensus sequence for signal peptidase II at the cleavage site. The predicted mass of mature OmcA, including

the ten heme *c* groups and the putative covalent lipid modification to the amino-terminal cysteine, is 82.7 kDa. A RNA of approx. 2300 bases that hybridizes to the *omcA* gene was detected in anaerobically grown MR-1 cells, suggesting that *omcA* is not expressed as part of a multicistronic operon. Similar to MR-1, all other strains of *S. putrefaciens* that were examined localized a majority of their membrane-bound cytochromes to the OM when grown under anaerobic conditions. The OM cytochromes in these other strains were generally of high molecular weight, and all strains contained an OM cytochrome similar in size to OmcA. Two of these strains express *omcA* homologs. This represents the first report of the sequence of a gene encoding an OM cytochrome.

Acknowledgements

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