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Outer Membrane *c*-Type Cytochromes Required for Fe(III) and Mn(IV) Oxide Reduction in *Geobacter sulfurreducens*

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The potential role of outer membrane proteins in electron transfer to insoluble Fe(III) oxides by *Geobacter sulfurreducens* was investigated because this organism is closely related to the Fe(III) oxide-reducing organisms that are predominant in many Fe(III)-reducing environments. Two of the most abundant proteins that were easily sheared from the outer surfaces of intact cells were *c*-type cytochromes. One, designated OmcS, has a molecular mass of ca. 50 kDa and is predicted to be an outer membrane hexaheme *c*-type cytochrome. Transcripts for *omcS* could be detected during growth on Fe(III) oxide, but not on soluble Fe(III) citrate. The *omcS* mRNA consisted primarily of a monocistronic transcript, and to a lesser extent, a longer transcript that also contained the downstream gene *omcT*, which is predicted to encode a second hexaheme outer membrane cytochrome with 62.6% amino acid sequence identity to OmcS. The other abundant *c*-type cytochrome sheared from the outer surface of *G. sulfurreducens*, designated OmcE, has a molecular mass of ca. 30 kDa and is predicted to be an outer membrane tetraheme *c*-type cytochrome. When either *omcS* or *omcE* was deleted, *G. sulfurreducens* could no longer reduce Fe(III) oxide but could still reduce soluble electron acceptors, including Fe(III) citrate. The mutants could reduce Fe(III) in Fe(III) oxide medium only if the Fe(III) chelator, nitrilotriacetic acid, or the electron shuttle, anthraquinone 2,6-disulfonate, was added. Expressing *omcS* or *omcE* in *trans* restored the capacity for Fe(III) oxide reduction. OmcT was not detected among the sheared proteins, and genetic studies indicated that *G. sulfurreducens* could not reduce Fe(III) oxide when *omcT* was expressed but OmcS was absent. In contrast, Fe(III) oxide was reduced when *omcS* was expressed in the absence of OmcT. These results suggest that OmcS and OmcE are involved in electron transfer to Fe(III) oxides in *G. sulfurreducens*. They also emphasize the importance of evaluating mechanisms for Fe(III) reduction with environmentally relevant Fe(III) oxide, rather than the more commonly utilized Fe(III) citrate, because additional electron transfer components are required for Fe(III) oxide reduction that are not required for Fe(III) citrate reduction.

The mechanisms for electron transfer to insoluble Fe(III) oxides in *Geobacter* species are of interest because insoluble Fe(III) oxides are the primary source of Fe(III) for dissimilatory Fe(III) reduction (27, 42) and because *Geobacteraceae* are the predominant Fe(III)-reducing microorganisms in diverse sedimentary environments in which Fe(III) oxide reduction is an important process (17, 47, 48, 52, 53). Previous studies have demonstrated that the mechanism by which *Geobacter* species reduce Fe(III) oxides is distinct from that of other well-studied organisms, including *Shewanella* and *Geothrix* species. *Shewanella* and *Geothrix* species do not have to directly contact Fe(III) oxides in order to reduce them (42, 43). These species produce both electron shuttles, which promote the indirect transfer of electrons from the cell surface to Fe(III) oxides, and compounds that solubilize Fe(III) oxides. In contrast, *Geobacter* species need to directly contact Fe(III) oxides in order to reduce them (44). Current evidence suggests that *Geobacter* species may locate Fe(III) oxides via chemotaxis (8), establish contact with Fe(III) oxides via pili, and transfer electrons to the exterior of the cell and then onto Fe(III) oxides (26, 31, 46).

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Since the earliest studies of electron transfer in *Geobacter* species (15, 34), it has been speculated that cytochromes are involved in electron transfer to Fe(III). Recent studies have focused on the role of *c*-type cytochromes in Fe(III) reduction in *Geobacter sulfurreducens*. This organism, which was isolated from hydrocarbon-contaminated soil (7), was chosen for detailed study because it is closely related to the *Geobacter* species which predominate in many subsurface environments (17) and because both its complete genome sequence (39) and a genetic system (10) are available. Several *c*-type cytochromes that are involved in Fe(III) reduction in *G. sulfurreducens* have been identified (6, 11, 23, 25). These include MacA, which is thought to be associated with the periplasmic surface of the inner membrane (6), PpcA (25) and related periplasmic low-molecular-weight cytochromes (11), and OmcB, an outer membrane *c*-type cytochrome (23). However, the genome of *G. sulfurreducens* contains over 100 genes for putative *c*-type cytochromes, substantially more than are found in other organisms, including the intensively studied Fe(III) reducer *Shewanella oneidensis* (39). Some of these cytochromes may also play a role in Fe(III) oxide reduction.

The insoluble nature of Fe(III) oxides and the requirement for direct contact for reduction by *Geobacter* species indicate that electrons are transferred to the outside of the cell and onto the Fe(III) oxide surface. If this is the case, then it seems likely that some of the most important proteins in electron

TABLE 1. Peptides from trypsin digestion of band A and band B shown in Fig. 1

Band	Peptide[a]
A	(R)ILGGTGYQPK
	(R)AHASGFDSMTR(F)
	(R)SVNEMTAAYYGR(T)
	(K)FGATIAGLYNSVK(K)
	(R)FVDGSIATTGLPIK(N)
	(R)RFVDGSIATTGLPIK(N)
	(K)NSGSYQNSNDPTAWGAVGAYR(I)
	(K)SLSGSYAPANOVPAAVAPSTYNR(T)
	(R)FNLAYEFTTIADASGNSIYGTDPNTSSLQGR(S)
B	(K)VHDNTDAPFLR(T)

[a] The letters in parentheses indicate cleavage sites for the enzyme trypsin, which cleaves after K and R residues.

transfer to Fe(III) oxides are located on the outer surface of the cell. In this study, we identified two *c*-type cytochromes that are loosely associated with the cell surface of *G. sulfurreducens* growing on the insoluble electron acceptor Mn(IV) oxide. Genetic studies indicate that these cytochromes are involved in the reduction of Fe(III) and Mn(IV) oxides but are not required for the reduction of soluble Fe(III).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Geobacter sulfurreducens* strain DL1 (7) was used to construct the mutants Δ*omcT*::*spec*, Δ*omcS*::*spec*, and Δ*omcE*::*kan*. The strains were cultured anaerobically at 30°C in medium with acetate (20 mM) as the electron donor and either Fe(III) citrate (56 mM), fumarate (40 mM) (10), Fe(III) oxides (100 mM), or Mn(IV) oxides (10 mM) as the electron acceptor, as previously described (7, 30).

Isolation and identification of OmcS and OmcE. *G. sulfurreducens* was grown on Mn(IV) oxide medium as described above until mid-log phase. The cell culture was subjected to shearing forces in a Waring blender at room temperature at low speed for 2 min. This method was a modified version of protocols described for the isolation of extracellular proteins (50, 57). To ensure that shearing did not compromise cell integrity, sheared preparations were stained with acridine orange and observed by epifluorescence microscopy with a Nikon Eclipse E600 microscope (Nikon, Inc., NY). Following shearing and microscopic examination, medium components and cells were removed by centrifugation at 8,000 × *g* for 20 min. Proteins in the supernatant were precipitated with 45% ammonium sulfate overnight at 4°C followed by centrifugation at 30,000 × *g* for 2 h at 4°C. The pellet was resuspended in 10 mM Tris-HCl (pH 7.8). The proteins were analyzed by 12% nonreducing Tris-Tricine denaturing polyacrylamide gel electrophoresis (2). To identify *c*-type cytochromes, the gel was heme stained as previously described (14).

The protein bands corresponding to the heme-containing bands were excised from the Coomassie brilliant blue-stained gel, trypsin digested (in the presence of 0.01% *n*-octylglucopyranoside), and subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry (Kratos Axima CFR; Kratos Analytical, Manchester, England). The sequences of the peptides (Table 1) were used to identify the corresponding genes in the *G. sulfurreducens* genome (39).

Deletion mutant construction and complementation. Single-step gene replacement was performed to construct deletion mutants with deletions in *omcS*, *omcT*, and *omcE* (see Fig. 2). To construct the *omcS* deletion mutant, a 1.6-kbp fragment including the upstream sequence of *omcS*, a spectinomycin resistance (Spec^r) marker, and the sequence downstream of *omcS* was generated by recombinant PCR (40). The upstream region (396 bp, positions −460 to −63) and downstream sequences (410 bp, positions +1215 to +1625) were amplified from *G. sulfurreducens* genomic DNA with the following primer combinations: OmcS_1f (5′-CGGCCTGGGCAACTACTACATC-3′) with OmcS_1r (5′-CGGCTTTGTGTCCCCACCTTGCG-3′) and OmcS_2f (5′-GACCGCTGCCTACTATGGCCGT-3′) with OmcS_2r (5′-GCACGACGAGCTCTGGTCC-3′), respectively. Total genomic DNA was isolated with a genome DNA kit (Bio101, Inc., Carlsbad, Calif.). The spectinomycin cassette was amplified from pSJS985Q (49), using primers Spec_OmcSf (5′-CGCAAGGTGGGGACACAAAGCCGAGCACAGGATGACGCCTAACAA-3′) and Spec_OmcSr (5′-ACGGCCATAGTAGGCAGCGGTCATAGTCTCCCCAGCTCTCTA-3′). The PCR conditions were as follows: 94°C for 1 min; 94°C for 30 seconds; 25 cycles of 94°C for 30 seconds, 53°C for 1 min, and 72°C for 2.5 min; and a final extension of 72°C for 10 min. PCR fragments were gel purified using a QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA). Recombinant PCR was performed using the three PCR products as a template, and the final product was amplified using the distal primers OmcS_1f and OmcS_2r. The PCR conditions were as follows: 95°C for 2 min; 30 cycles of 95°C for 40 seconds, 68.5°C for 2 min, and 74°C for 2 min; and a final extension of 75°C for 7 min.

The linear fragment for construction of the *omcT* deletion mutant was generated using a similar strategy and PCR conditions. The upstream (381 bp, positions −106 to +275) and downstream (348 bp, positions +1262 to +1610) regions of *omcT* were amplified with the following primer combinations: OmcT_1f (5′-GCCACAATCTGATGTTGTGTG-3′) with OmcT_1r (5′-TCTGCGGTACTGATGTGGT-3′ and OmcT_2f (5′-CCTGTGCAACAAGTGTC-3′) with OmcT_2r (5′-CGAAGAGCCAGCTACCCAG-3′), respectively. The spectinomycin resistance cassette was amplified with Spec_OmcTf (5′-ACCACATCAGTACCGCAGAAGCACAGGATGACGCCTAACAA-3′) and Spec_OmcTr (5′-GACACTTGTTGCACAGGATAGTCTCCCCAGCTCTCTA-3′).

The linear fragment for construction of the *omcE* deletion mutant was also constructed by recombinant PCR. The primers OmcE_1f (5′-GCTGCTTGTGTCGACTTGTGC-3′) and OmcE_1r (5′-GGTCGGTGTTCGTCGCCTTG-3′) were used to amplify a 647-bp fragment containing the upstream, flanking region and the 5′ end of *omcE* (−396 to +251). Primers OmcE_2f (5′-CACCCTCTACTCGTCCAGC-3′) and OmcE_2r (5′-TCATGGAACCCACGAATCAC-3′) were used to amplify a 639-bp downstream region (+335 to +974). The kanamycin cassette was amplified from pBR1MCS-2 (22), using primers Kan_OmcEf (5′-CAAGGCGACGAACACCGACCATGTCAGCTACTGGGCTATC-3′) and Kan_OmcEr (5′-GCAGAAGAGCGAGATGGAGTCGAAACTCTGTGAGGCAG-3′). Following recombinant PCR, the final linear fragment was amplified with the distal primers OmcE_1f and OmcE_2r. The PCR conditions were as follows: 94°C for 1 minute; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 3 minutes; and 72°C for 5 minutes.

Electroporation, mutant isolation, and confirmation were performed as previously described (10). Gene disruption was confirmed by PCR and Southern hybridization as previously described (10), and one isolate of each of the three deletion mutants was chosen as a representative for further study.

In order to perform complementation studies, the genes *omcS*, *omcT*, and *omcE* were inserted into the EcoRI and BamHI restriction sites of the expression vector pMJG (6), derived from the plasmid pCM66 (38), to generate Δ*omcS*::*spec*/pMJG-*omcS*, Δ*omcT*::*spec*/pMJG-*omcT*, and Δ*omcE*::*spec*/pMJG-*omcE*, respectively. The *omcS*, *omcT*, and *omcE* coding regions were amplified from *G. sulfurreducens* genomic DNA using the following primer combinations: omcTfor (5′-GGATCCGATGTTGTGTGATTCCGC-3′) with omcTrev (5′-GCGAATTCGATAGTGGATCGATTGCTGGA-3′), omcSfor (5′-GGGCCGGATCCGGAGGAAATGATGAAAAAGGGG-3′) with omcSrev (5′-GGGCGGAATTCGTCCTTGGCGTGGCACTTGTTGC-3′), and omcEfor (5′-CCGCGGATCCAGGAGGTTCTTTTATGAGAAGCGAAG-3′) with omcErev (5′-CCCCGGAATTCCTTCTTGTGGCAACCCAGACAGAG-3′), respectively (restriction sites are underlined). Plasmid DNA purification was carried out using Mini plasmid purification kits and PCR purification kits (QIAGEN, Inc., Valencia, Calif.). The deletion mutants Δ*omcT*::*spec*, Δ*omcS*::*spec*, and Δ*omcE*::*kan* were transformed with pMJG-*omcS*, pMJG-*omcT*, and pMJG-*omcE*, respectively, as previously described (6). Gentamicin-resistant transformants were isolated and screened for the presence of the appropriate plasmids and mutations. One of each of the three types of transformants was chosen as a representative for further study.

Gene expression studies. The expression of the *omcS*, *omcT*, and *omcE* genes during the growth of *G. sulfurreducens* with different electron acceptors was analyzed by reverse transcription-PCR (RT-PCR) and Northern blotting. RNA was isolated from mid-log-phase cultures using RNeasy Mini kits (QIAGEN Inc.), followed by DNase treatment with RNase-free DNase (Ambion, Inc., Austin, TX). RT-PCR was performed using the Superscript II RT-PCR system (Invitrogen, Inc., Carlsbad, CA). The primers used were OmcSf (5′-TCGTTTTGTTGACGGCAGCATC-3′) with OmcSr (5′-AGCGCGGTGTCGTCGATCT-3′), OmcEf (5′-CGGGAGCCGCCAGCATCAA-3′) with OmcEr (5′-TGCACTCCCCGGTCTTCATGG-3′), and OmcTf (5′-GCTTCAAGACTCTTCCCGTC-3′) with OmcTr (5′-GGTGTTCATGCTCCCCGG-3′). Isolated RNA was tested for DNA contamination by using it as a template in PCR.

For Northern analysis, RNAs (1.5 μg/lane) were loaded into a 0.8% denaturing formaldehyde-agarose gel and transferred to a charged nylon membrane with the TurboBlotter Rapid Downward transfer system (Schleicher and Schuell Bioscience, GmbH, Germany). Probes were amplified using the RT-PCR primers described above. Probes were labeled with [α-^{32}P]dCTP (Perkin-Elmer, MA)

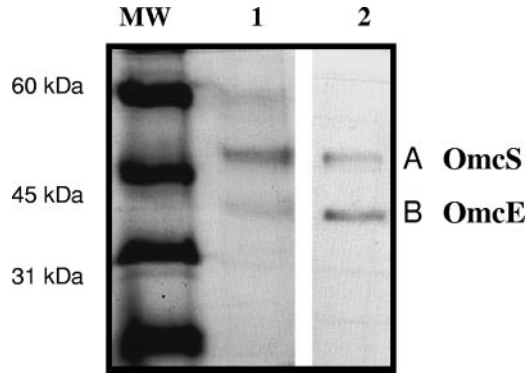

FIG. 1. Isolation of outer membrane cytochromes OmcS and OmcE. Loosely associated outer membrane proteins were isolated from *G. sulfurreducens* cells grown on Mn(IV) oxides by shearing. Proteins were resolved by 12% nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained for total proteins with Coomassie blue (lane 1) or for heme (lane 2). No heme-staining bands other than those shown were detected on the entire gel.

with a commercial labeling kit (NEBlot kit; New England Biolabs Inc., Beverly, MA).

Analytical techniques. The concentration of Fe(II) in Fe(III)-reducing cultures was measured with the ferrozine assay as previously described (28). Protein concentrations were measured with a bicinchoninic acid kit (Sigma-Aldrich, Inc.). Mn(IV) oxide reduction was detected visually by a change in the medium color from black (oxidized) to gray (reduced).

RESULTS

Identification of outer membrane cytochromes in *G. sulfurreducens*. In order to gain insight into the proteins associated with the outer surface of *G. sulfurreducens*, Mn(IV) oxide-grown cultures were subjected to shearing to recover proteins that were loosely associated with the cell surface. Cells were grown with Mn(IV) oxide rather than Fe(III) oxide because Mn(IV) oxide provided larger cell numbers and cells could be more readily disassociated from the Mn(IV) oxide particles. Shearing did not break open the cells, as evidenced by a visual lack of cell lysis and the fact that soluble proteins, such as the abundant periplasmic cytochrome PpcA (24, 25), were not detected. Two of the most abundant proteins released by shearing stained positively for heme (Fig. 1). These two bands were excised, digested with trypsin, and analyzed by mass spectrometry. Mass spectrometry indicated that the molecular mass of the protein in band A was 51.7 kDa and that the protein in band B had a molecular mass of 27.9 kDa. The peptide sequences detected (Table 1) indicated that band A was encoded by the open reading frame GSU2504 (gi-39997599) in the *G. sulfurreducens* genome and that band B was encoded by open reading frame GSU0618 (gi-39997598).

The presence of six CXXCH heme-binding motifs suggests that GSU2504 encodes a hexaheme *c*-type cytochrome. Analysis with SubLoc V1.0 (18) (http://www.bioinfo.tsinghua.edu.cn/SubLoc/) suggested that the protein is targeted to the outer membrane, which is consistent with its loose association with intact cells. The protein was therefore designated *o*uter *m*embrane *c*ytochrome *S*, or OmcS.

The program SignalP (http://www.cbs.dtu.dk/services/SignalP/) indicated that OmcS has a signal sequence with a predicted cleavage site between positions 25 and 26. However, some proteins have N-terminal signal sequences that assist in secretion and are not cleaved by the signal peptidase, instead forming a transmembrane anchor (19, 55), and the topology prediction programs HMMTOP (http://www.enzim.hu/hmmtop/html/submit.html), SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) suggested that the N terminus of OmcS is not cleaved, but rather forms a transmembrane helix anchoring the otherwise hydrophilic protein in the outer membrane. The predicted molecular masses for the protein, including the incorporated hemes, with or without cleavage, are 46.5 and 49 kDa, respectively, which are lower than that measured with mass spectrometry. This suggests that there may be post-transcriptional modifications to the protein.

Immediately downstream of the *omcS* gene is another open reading frame with 62.6% amino acid sequence identity to OmcS (GSU2503, gi-39997598), whose product was designated OmcT (Fig. 2). Application of the algorithms listed above indicated that OmcT, like OmcS, is likely a hexaheme outer membrane *c*-type cytochrome. However, even though there is high sequence homology between OmcS and OmcT, only peptides which can be uniquely attributed to OmcS were recovered (Table 1) from band A, suggesting that OmcT was not a predominant protein sheared from the outer cell surface.

The protein encoded by GSU0618, the other heme-staining protein that was sheared from the cells, contains four heme-binding motifs and was predicted with SubLoc V1.0 to be targeted to the outer membrane. Thus, this protein was designated outer membrane cytochrome E, or OmcE. As with OmcS, SignalP predicted that there is a signal sequence that is cleaved, but HMMTOP, SOSUI, and TMHMM suggested that this sequence forms a transmembrane helix. If this is so, then the rest of OmcE is predicted to be hydrophilic and thus is likely to be displayed on the outside of the cell. Without cleavage, OmcE has a predicted molecular mass of 27.6 kDa after the addition of the four heme groups, which compares well with the results from mass spectrometry. This is considerably less than the molecular weight that would be predicted from the position of the protein in the gel (Fig. 1). The anomalous electrophoretic mobility of OmcE may have been due to the fact that electrophoresis was performed under nonreducing conditions in order to permit heme staining (37).

Expression of *omcS*, *omcT*, and *omcE*. RT-PCR expression analysis revealed that *omcS* and *omcT* were expressed when insoluble Fe(III) oxide was the terminal electron acceptor, but not during growth on Fe(III) citrate (Fig. 3A). The *omcS* and *omcT* genes were also expressed during growth with fumarate as the sole electron acceptor. In contrast, *omcE* was expressed during growth on all three electron acceptors (Fig. 3A). Northern analysis of gene transcripts of *omcS* and *omcT* demonstrated that *omcS* was present in two transcripts, of ca. 1.5 and 2.5 kb, whereas *omcT* was only present in the larger, 2.5-kb transcript (Fig. 3B). These results suggest that *omcS* is transcribed both individually and in an operon with *omcT*, whereas *omcT* is only transcribed with *omcS*.

Impact of deleting *omcS*, *omcT*, or *omcE*. Deleting *omcE* had no impact on growth with fumarate or Fe(III) citrate as the sole electron acceptor (data not shown). In contrast, the *omcE* deletion inhibited Fe(III) oxide reduction for ca. 30 days (Fig.

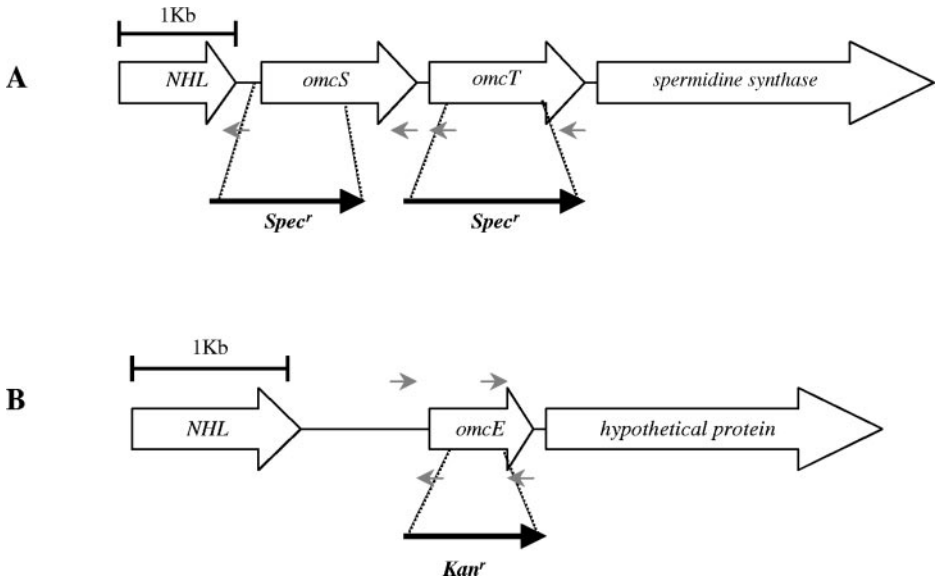

FIG. 2. Construction of *omcS*, *omcT*, and *omcE* deletion mutants. (A) *omcS* and *omcT*; (B) *omcE*. Gray arrows indicate primer positions. Dashed lines indicate the stop and start positions of deletions. Black arrows indicate the direction of antibiotic resistance cassettes for spectinomycin (Spec^r) or kanamycin (Kan^r). NHL indicates that the genes encode a protein with an N (asparagine)-H (histidine)-L (leucine) repeat domain.

4A). After that time, the *omcE* deletion mutant reduced Fe(III) oxide, but at a lower rate than in the wild type. The reduction of Mn(IV) oxide was also affected, and the mutant had a 2-week lag phase, in contrast to the wild type. If the Fe(III) chelator nitrilotriacetic acid (NTA) was added to the Fe(III) oxide medium in order to chelate Fe(III), the *omcE* deletion mutant reduced Fe(III) oxide at a rate comparable to that of the wild type (Fig. 5). Likewise, when the electron shuttle anthraquinone-2,6-disulfonate (AQDS), which also alleviates the need for direct electron transfer from the cell to Fe(III) oxide (32), was added, the deletion mutant reduced Fe(III) oxide (Fig. 5). Expressing *omcE* in *trans* restored the capacity for Fe(III) oxide reduction, but at a rate somewhat lower than that of the wild type (Fig. 4A). This is consistent with the general finding that the present method for expressing genes in *trans* in *G. sulfurreducens* often does not result in sufficient expression to replicate the protein levels found in the wild type (6, 23).

Deleting *omcS* or *omcT* inhibited Fe(III) oxide reduction even in long-term incubations (Fig. 4) but had no impact on the reduction of fumarate or Fe(III) citrate (data not shown). The reduction of Mn(IV) oxide was also inhibited in the *omcS* mutant. Both mutants reduced Fe(III) oxide in the presence of NTA or AQDS (Fig. 5). The expression of *omcS* in *trans* in the *omcS* mutant restored the capacity for Fe(III) oxide reduction (Fig. 4B). However, expressing *omcT* in *trans* in the *omcT* mutant did not permit growth on Fe(III) oxide (Fig. 4B).

In order to evaluate this further, the expression of *omcS* and *omcT* in the two mutants was evaluated. Northern blot analysis of the expression of *omcS* and *omcT* in fumarate-grown cells did not detect *omcS* in the *omcT* deletion mutant, and no *omcT* transcripts were detected in the *omcS* deletion mutant (Fig. 3B). When RT-PCR analysis was used to gain more sensitivity, a low level of *omcS* expression could be detected in the *omcT* deletion mutant, but no *omcT* was detected in the *omcS* deletion mutant (Fig. 3C). These results demonstrated that deleting either *omcS* or *omcT* negatively impacted the expression of the other cytochrome gene.

DISCUSSION

Although many studies have suggested that outer membrane *c*-type cytochromes are likely to play an important role in Fe(III) reduction, this is the first report of outer membrane cytochromes that are specifically required for the reduction of insoluble Fe(III) oxides, but not soluble, chelated Fe(III). As discussed below, these results suggest that there may be distinct differences in the mechanisms for the reduction of soluble Fe(III), which is rare in most environments, and insoluble Fe(III) oxides, which are the most important form of microbially reducible Fe(III) in many soils and sediments.

Properties and role of OmcS and OmcE. The results strongly suggest that OmcS and OmcE are located on the outer membrane of *G. sulfurreducens*. The fact that OmcS and OmcE are predicted to have heme-binding sites and stain positively for heme suggests that these putative *c*-type cytochromes are likely involved in electron transfer. A potential role for outer membrane cytochromes in electron transfer to Fe(III) has been proposed earlier (3, 4, 23, 35, 36, 41). Therefore, it may not be surprising that deleting *omcS* or *omcE* inhibited the reduction of Fe(III) oxide and that the capacity for Fe(III) oxide reduction was restored when *omcS* or *omcE* was expressed in *trans* in the mutants.

The surprise was that the deletion of these genes had no impact on the reduction of soluble Fe(III). Such specificity for Fe(III) forms has not previously been observed in cytochrome mutants. Notably, the mutants reduced Fe(III) in Fe(III) oxide medium if the medium was supplemented with the Fe(III)

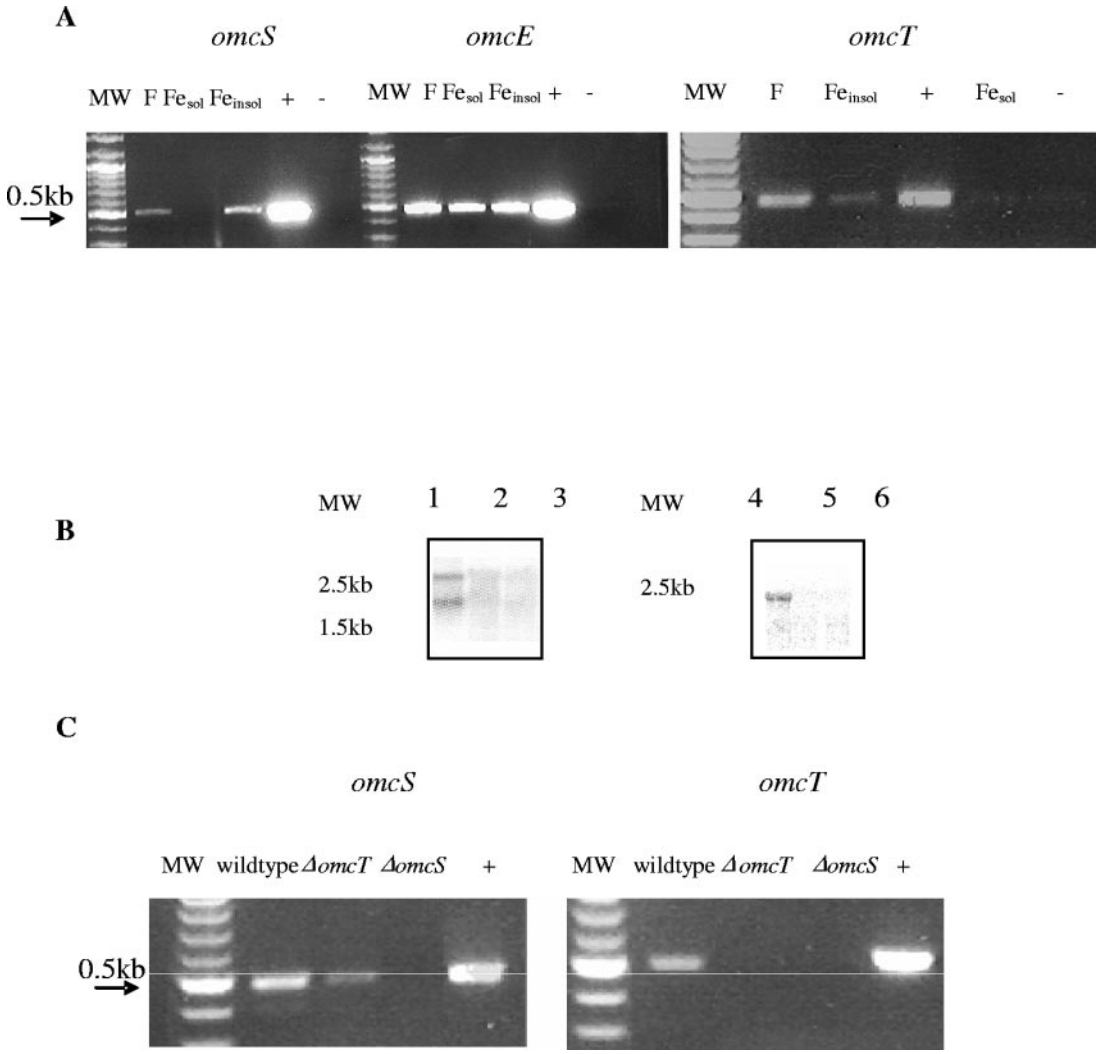

FIG. 3. Expression of *omcS*, *omcT*, and *omcE*. (A) The expression of *omcS*, *omcT*, and *omcE* was measured by RT-PCR. RNAs were extracted from wild-type cells grown in the presence of fumarate (F), ferric citrate (Fe_{sol}), and insoluble Fe(III) oxide (Fe_{insol}). Negative (−) controls were performed without cDNA synthesis, and positive controls (+) were performed by using genomic DNA as the template. (B) Northern analysis of *omcS* and *omcT* expression in cells grown on fumarate. The expression of *omcS* in the wild type (lane 1) and the Δ*omcS*::*spec* (lane 2) and Δ*omcT*::*spec* (lane 3) mutants is shown. The expression of *omcT* in the wild type (lane 4) and the Δ*omcS*::*spec* (lane 5) and Δ*omcT*::*spec* (lane 6) mutants is also indicated. (C) Expression of *omcS* and *omcT* in deletion mutants. The strains were cultured with fumarate as the electron acceptor, and genomic DNA was used as a template for positive controls. MW, molecular size marker.

chelator NTA or the electron shuttling compound AQDS. NTA chelates and solubilizes Fe(III) oxide (29). AQDS serves as a soluble electron shuttle (32, 33) and promotes the reduction of Fe(III) oxide in wild-type *Geobacter* species and other Fe(III) reducers by alleviating the need for direct contact between Fe(III) reducers and Fe(III) oxides (43). These results indicate that although the reduction of soluble Fe(III), AQDS, and Fe(III) oxides is likely to take place at or near the cell surface, additional components are required for electron transfer to Fe(III) oxides. This is more likely to reflect the soluble nature of chelated Fe(III) or AQDS than other differences between the properties of these soluble electron acceptors and Fe(III) oxide. For example, although the midpoint redox potential of the Fe(III) citrate/Fe^{2+} redox couple (+372 mV) (54) is more positive than that for poorly crystalline Fe(III) oxide/Fe^{2+} (+150 mV) (56), the midpoint redox potential for oxidized AQDS/reduced AQDS (−184 mV) (5) is lower. Furthermore, given the fact that Fe(III) oxide is reduced chemically nearly as readily as Fe(III) citrate, and more readily than AQDS, it seems unlikely that a redox carrier that would reduce Fe(III) citrate or AQDS would not also transfer electrons to Fe(III) oxide. However, in order for such electron transfer to take place, the electron transfer protein must contact the Fe(III) oxide. It is likely that Fe(III) citrate and AQDS can interact with electron transport constituents in the outer membrane, or possibly even the periplasm, which will not effectively contact insoluble Fe(III) oxides.

In order to best understand the mechanisms of Fe(III) reduction in soils and sediments, it is important to know the mechanisms of electron transfer to Fe(III) oxides. Soluble

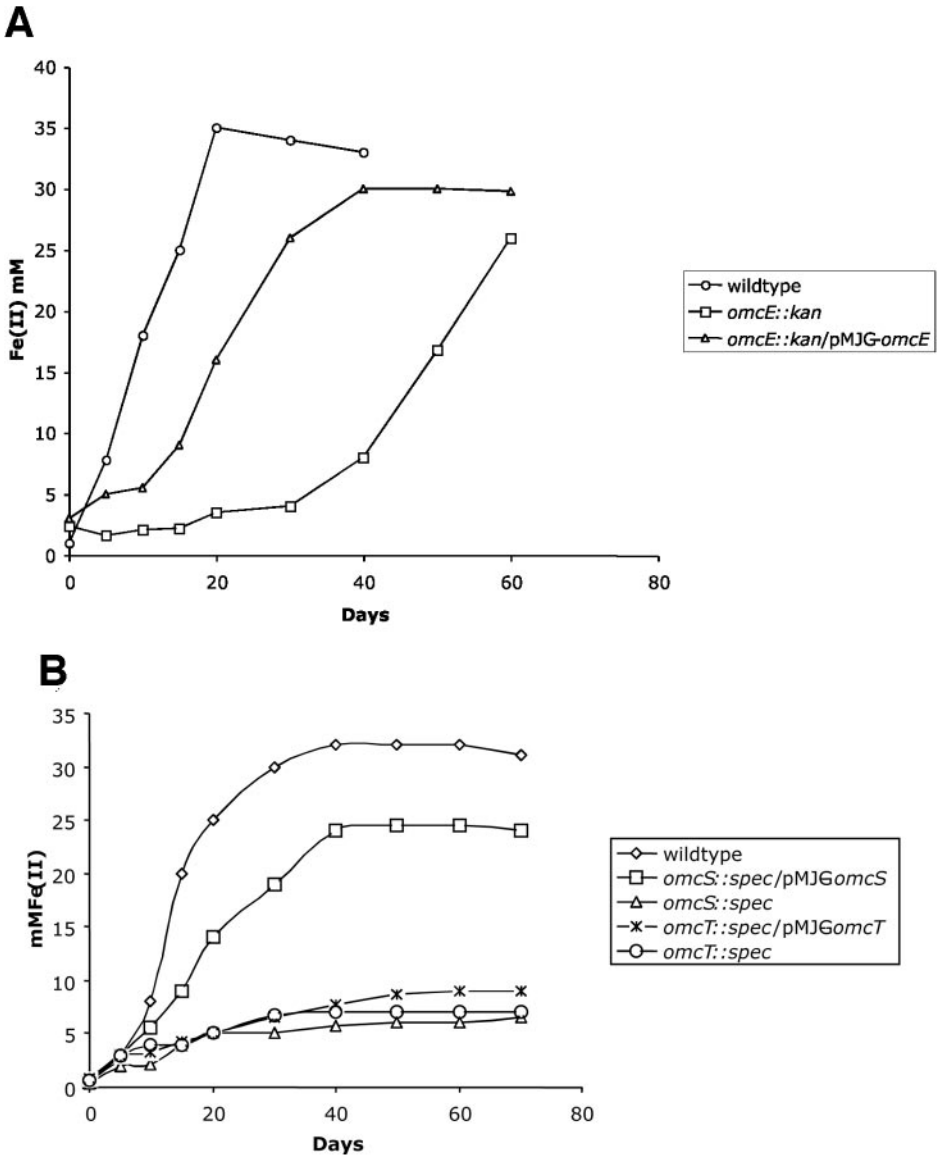

FIG. 4. Reduction of Fe(III) oxide. (A) Production of Fe(II) by reduction of insoluble Fe(III) by wild-type *G. sulfurreducens*, *G. sulfurreducens* Δ*omcE*::*kan*, and Δ*omcE*::*kan* complemented with Δ*omcE*::*kan*/pMJG-*omcE*. (B) Production of Fe(II) from insoluble Fe(III) reduction by wild-type *G. sulfurreducens*, *G. sulfurreducens* Δ*omcS*::*spec* and Δ*omcT*::*spec* deletion mutants, and the mutants complemented in *trans*. The cultures were grown with acetate as the electron donor and insoluble Fe(III) oxide as the electron acceptor. The results shown are the means of triplicate cultures for each strain.

Fe(III) is not expected to be abundant in most environments (43). Insoluble Fe(III) oxides (27, 42), and possibly structural Fe(III) in clays (20, 21, 51), are the most abundant source of Fe(III) for microbial reduction.

Thus, in the absence of other data, mutants that cannot reduce soluble Fe(III) may not reveal the most environmentally relevant Fe(III) reductases. This is an important consideration because most studies on the mechanisms of electron transfer to Fe(III) have used soluble Fe(III) as the Fe(III) source for detailed studies. In nearly all instances, the only forms of Fe(III) that the organisms were grown on were soluble forms. Yet the finding that *omcS* was expressed during growth on Fe(III) oxide but not on Fe(III) citrate demonstrates that components important in Fe(III) oxide reduction may even be missing in cells grown on soluble Fe(III).

The actual role of OmcS and OmcE in Fe(III) oxide reduction cannot be definitively stated from the data presently available. The *omcE* mutant did slowly adapt to reduce Fe(III) oxide in the absence of OmcE, suggesting that one or more as yet undetermined components can partially compensate for the loss of OmcE. A similar adaptation was not observed in the *omcS* mutant, suggesting that OmcS is essential for Fe(III) oxide reduction. The localization of OmcS and OmcE on the outside of the cell suggests that they have a higher likelihood of serving as the terminal step in electron transfer to Fe(III) oxides than other *c*-type cytochromes that are known to be

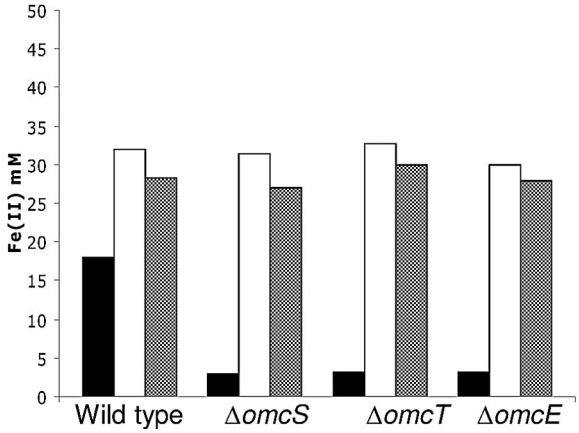

FIG. 5. Fe(III) reduction by the wild type and various mutants. The graph shows the reduction of Fe(III) oxide (black bars), Fe(III) oxide supplemented with AQDS (gray bars), and Fe(III) oxide supplemented with NTA (white bars) by the wild type and the Δ*omcS*::*spec*, Δ*omcT*::*spec*, and Δ*omcE*::*kan* mutants. Fe(II) was measured after 10 days. The results shown are the means of triplicate incubations.

required for Fe(III) reduction but are localized on the inner membrane or in the periplasm (6, 11, 25). The outer membrane *c*-type cytochrome OmcB (23) is required for Fe(III) oxide reduction. However, the fact that OmcB is also required for the reduction of Fe(III) citrate and does not appear to be exposed on the outside of the cell suggests that it may be an intermediary electron carrier in Fe(III) oxide reduction.

Recent results have demonstrated that *G. sulfurreducens* requires pili to reduce Fe(III) oxides and that these pili are conductive (46). Furthermore, Fe(III) oxides appear to be more associated with pili than with the outer surface of the cell (46). Therefore, it has been proposed that the pili of *G. sulfurreducens* function as "microbial nanowires," transferring electrons to Fe(III) oxides. If so, then OmcS and OmcE are not the terminal Fe(III) oxide reductases but may be involved in electron transfer to the pili.

Role of OmcT. Sequence analysis suggests that OmcT is likely to have biochemical properties similar to those of OmcS, but OmcT was not detected in the proteins sheared from intact cells. Proteomic studies have also demonstrated that OmcS is about 40-fold more abundant in Fe(III) oxide-grown cells than OmcT (12).

Transcripts of *omcT* could not be detected in the *omcS* deletion mutant, and deleting *omcT* greatly diminished the expression of *omcS*. According to bioinformatic analysis, the *omcS* and *omcT* genes are predicted to be in the same operon (J. Krushkal, personal communication), consistent with the recovery of *omcS* and *omcT* in the same transcript. It is not uncommon to find that the deletion of a gene impacts the expression of downstream genes when the two genes are in the same operon because the secondary structures located in the 5′ region often confer stability to the downstream mRNA (16) and the unstable free 5′ end becomes susceptible to cleavage by RNase E (1, 13, 16). For example, in the Fe(III) reducer *Shewanella oneidensis* (formerly *Shewanella putrefaciens* strain MR-1), *mtrC*, *mtrA*, and *mtrB* are transcribed in an operon, and the deletion of *mtrC* leads to the loss of the *mtrAB* transcript (4). The stability of the *omcS* transcript could be affected by the deletion of *omcT*, as seen for the *malEFG* operon of *Escherichia coli*, where deletion of the downstream region between *malE* and *malF* causes a decrease in the *malE* transcript (45).

From the studies in which *omcT* was expressed in *trans*, it is apparent that the presence of OmcT in the absence of wild-type levels of OmcS is not sufficient for Fe(III) oxide reduction. In contrast, the ability to restore Fe(III) oxide reduction when *omcS* was expressed in *trans* in the *omcS* deletion mutant demonstrates that the presence of OmcS in the absence of OmcT is sufficient for Fe(III) oxide reduction, since the *omcS* deletion mutant did not express *omcT*. This has been confirmed in subsequent studies. A double mutant in which *omcS* and *omcT* were both deleted was constructed, and the expression of *omcS* in *trans* again restored the capacity for Fe(III) oxide reduction. However, the expression of *omcT* in *trans* did not restore the Fe(III) reduction phenotype.

Although it might be expected that OmcT and OmcS would have similar functions based on their sequence similarity, previous studies have suggested that in *G. sulfurreducens*, *c*-type cytochromes with similar sequences can have different functions (11, 23). For example, OmcB is required for Fe(III) reduction, but OmcC, which has a predicted amino acid sequence which is 79% identical to that of OmcB, is not required for Fe(III) reduction (23). Furthermore, *omcC* has patterns of expression under various environmental conditions that are markedly different from those of *omcB*, further suggesting a different function for OmcC (9). In a similar manner, multiple small periplasmic *c*-type cytochromes in *G. sulfurreducens* with similar sequences appear to have different functions (11).

In summary, this study is the first description of *c*-type cytochromes specifically required for the reduction of Fe(III) oxide, but not soluble Fe(III). This emphasizes the importance of actually studying growth and reduction on environmentally relevant Fe(III) oxides in order to understand the most environmentally relevant mechanisms of Fe(III) reduction. Further studies on the role of OmcS and OmcE and their potential interactions with each other and other electron transfer proteins involved in extracellular electron transfer are warranted.

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