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OmcF, a Putative c-Type Monoheme Outer Membrane Cytochrome Required for the Expression of Other Outer Membrane Cytochromes in Geobacter sulfurreducens  

Byoung-Chan Kim,* Ching Leang, Yan-Huai R. Ding, Richard H. Glaven, Maddalena V. Coppi, and Derek R. Lovley

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

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Dissimilatory Fe(III)-reducing bacteria can obtain energy by coupling the oxidation of organic compounds to the reduction of Fe(III) (31). Molecular analysis of the composition of microbial communities has shown that the Geobacteraceae, a family of dissimilatory Fe(III)-reducing bacteria in the delta subdivision of the class Proteobacteria, are the predominant Fe(III)-reducing microorganisms in a wide variety of sedimentary environments in which Fe(III) reduction is the principal terminal electron-accepting process (1, 42, 48, 49, 54, 55). Some of these bacteria can use alternate electron acceptors, such as the radionuclide U(VI) (32), vanadium (43), humic substances (30), and insoluble graphite electrodes (5). The genome of Geobacter sulfurreducens has been reported to be associated with the outer membrane. Thus, elimination of OmcF may impair Fe(III) reduction by influencing expression of OmcB, which has previously been demonstrated to play a critical role in Fe(III) reduction.

**MATERIALS AND METHODS**

Bacterial strains and culture conditions. E. coli strain DH5α [F supE44 ΔlacU169 (Δ80 lacZΔM15) hsdR17 (rK- mK-) recA1 endA1 gyrA96 thi-1 relA1] (15, 38) and E. coli strain TOP10 [F- mcrA Δ(mcrA-mrr) ΔlacZΔM15 ΔlacX74 ΔrecA deoR araD139 (ΔaraD139)7697 galU galK rpsL (Str)] (Invitrogen Co., Carlsbad, CA) were used for DNA manipulations and PCR product subcloning, respectively. G. sulfurreducens strains DLBK01 (omcF::kan) and DLBK01/pRGS-omcF (omcF::kan/pRGS-omcF) were produced from G. sulfurreducens strain DL1 (7) as described below. G. sulfurreducens strains were obtained from the American Type Culture Collection (Rockville, MD).
Deletion of the *omcF* gene via single-step gene replacement. Single-step gene replacement was performed essentially as previously described (28). To disrupt the *omcF* gene, a linear 2.2-kb DNA fragment, containing the kanamycin resistance marker (*Kan*) flanked by ca. 0.5 kb of sequence upstream and downstream of *omcF* (Table 1 and Fig. 1), was generated by recombinant PCR (28, 37). The sequence upstream of *omcF* was amplified with primers 4017-1 and 4017-2 (Table 1). The sequence downstream of *omcF* was amplified with primers 4017-5 and 4017-6 (Table 1). The kanamycin resistance cassette was amplified from plasmid pBBR1MCS-2 (24) with primers 4017-3 and 4017-4 (Table 1). Following recombinant PCR with the three primary PCR products, the final 2.2-kb fragment was amplified with distal primers 4017-1 and 4017-6. The PCR conditions were similar to those described previously (11, 28), except that the annealing temperature was 60°C. Electroporation, mutant isolation, and genotyping confirmation were performed as previously described (11, 28). One mutant strain, designated DLBK01 (*omcF::kan*), was chosen as the representative strain (Fig. 1). Deletion of the *omcF* gene was confirmed by Southern blot analysis and sequencing. This process was performed as previously described (11, 27). The 2.2-kb probe used for Southern blot analysis was amplified from the *omcF*-deficient mutant (strain DLBK01) with primers 4017-1 and 4017-6 (Table 1) and labeled with digoxigenin (Roche Diagnostics Corp., Indianapolis, IN). Hybridization was performed with digoxigenin nucleic acid detection kit (Roche Diagnostics Co., Indianapolis, IN). To ensure that the *omcB* and *omcC* genes had not been accidentally disrupted during deletion of the *omcF* gene, PCR and Southern blot analyses were performed as previously described (11, 27). The 2.2-kb probe for Southern blot analysis was amplified from the *omcF*-deficient mutant with primers 8916 and 8916-2 and primers 8914 and 8915, respectively (Table 1). The resulting plasmid was designated pRG5. The complete DNA & RNA purification kit (Epicentre Technologies, Madison, WI). was used for all PCR amplifications. 

**RESULTS**

**Analysis of the *omcF* gene.** The genome of *G. sulfurreducens* contains more than 100 genes predicted to encode c-type cytochromes based on the presence of the CXXCH heme c binding motif (36). Thirty-one of these cytochromes are predicted to be localized in the outer membrane (http://psort.nibb.ac.jp/form.html). The smallest of these putative outer membrane c-type cytochromes is the product of the *omcF* gene, which encodes a 104-amino-acid protein with a single heme-binding motif and a prokaryotic membrane lipoprotein lipid attachment site and signal sequence (Fig. 2). Following heme incorporation and cleavage of the signal sequence, the molecular mass of OmcF is predicted to be approximately 9.4 kDa. A BLAST search of the National Center for Biotechnology Information database revealed that the closest relatives of OmcF are found in the *Geobacteraceae*. The genomes of *G. sulfurreducens* and *Geobacter metallireducens* encode related monoheme c-type cytochromes which are 57% and 50% similar to OmcF, respectively (Fig. 2). Outside the *Geobacteraceae*, OmcF is most similar to the soluble monoheme cytochromes of cyanobacteria and planktonic algae (Fig. 2). The cytochromes serve as electron donors to cytochrome c oxidase in cyanobacteria (2, 13) and transfer electrons from cytochrome *b*₅ to photosystem I in both cyanobacteria and unicellular algae (2, 13, 23). OmcF and the *c*₅ family of c-type cytochromes share a 74-amino-acid domain (Fig. 2, G27 to 5401).
V100 of OmcF) which includes 15 strictly conserved residues, including the heme-binding motif and a methionine residue (M78) that serves as an axial ligand for heme iron (60). The 14-amino-acid domain of OmcF is 64 to 65% similar to those found in the related soluble cytochromes of G. metallireducens and G. sulfurreducens and 53 to 59% similar to those of various algal and cyanobacterial ε6 cytochromes (Fig. 2).

**Expression of omcF.** Expression of omcF was detected in mRNA prepared from Fe(III) citrate-grown cells by RT-PCR when cDNA was synthesized with either omcF-specific or random primers. In contrast, omcF expression could be detected in mRNA prepared from fumarate-grown cells only when cDNA was synthesized with omcF-specific primers (Fig. 3A). Because the levels of omcF cDNA synthesized with specific

### Table 1. Primers used in this study

<table>
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<tr>
<th>Primer</th>
<th>Use</th>
<th>Sequence (5’ to 3’)</th>
<th>Position*</th>
<th>Reference</th>
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<tr>
<td>4017-1</td>
<td>Recombinant PCR and Southern blot analysis</td>
<td>ACAGAGGGGTCAGAGGGGAGC</td>
<td>−514 to −497 of omcF</td>
<td>This study</td>
</tr>
<tr>
<td>4017-2</td>
<td>Recombinant PCR</td>
<td>GACGGATCCGAGAGGGGGAAG</td>
<td>−18 to −1 of omcF</td>
<td>This study</td>
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<tr>
<td>4017-3</td>
<td>Recombinant PCR</td>
<td>CGAGGGGGGTCAGAGGGGGGAAG</td>
<td>−18 to −1 of omcF, −274 to −255 of Kan’ in pBBR1MCS-2</td>
<td>This study</td>
</tr>
<tr>
<td>4017-4</td>
<td>Recombinant PCR</td>
<td>CTCATTGGGAAAGCTCAACGATGGTTGGCGTGC</td>
<td>−1 to 18 from omcF stop codon, −51 to −33 from Kan’ stop codon in pBBR1MCS-2</td>
<td>This study</td>
</tr>
<tr>
<td>4017-5</td>
<td>Recombinant PCR</td>
<td>CGTGGGAGCACTCTCATGAG</td>
<td>−1 to 18 from omcF stop codon</td>
<td>This study</td>
</tr>
<tr>
<td>4017-6</td>
<td>Recombinant PCR and Southern blot analysis</td>
<td>CCTTTCAGCGGGGAGCCACG</td>
<td>−567 to −548 from omcF stop codon</td>
<td>This study</td>
</tr>
<tr>
<td>ComF2</td>
<td>Expression of the omcF gene in trans</td>
<td>CGAGAAAAGCGAGAGAAGG</td>
<td>−63 to −44 of omcF</td>
<td>This study</td>
</tr>
<tr>
<td>ComR3</td>
<td>Expression of the omcF gene in trans</td>
<td>AAGCTTCGGAACGAGGGCTCTCATGGA</td>
<td>−14 to 8 from omcF stop codon</td>
<td>This study</td>
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<tr>
<td>8916</td>
<td>RT-PCR and Northern blot analysis of omcB</td>
<td>GGACTGCGCACCATCAAGG</td>
<td>580 to 598 of omcB</td>
<td>26</td>
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<tr>
<td>8908-2</td>
<td>RT-PCR and Northern blot analysis of omcB</td>
<td>GGTCTGACGGCCACCG</td>
<td>998 to 1004 of omcB</td>
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<tr>
<td>8914</td>
<td>RT-PCR and Northern blot analysis of omcC</td>
<td>GGGTGGGCGAGAAGGAC</td>
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<tr>
<td>8915</td>
<td>RT-PCR and Northern blot analysis of omcC</td>
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<tr>
<td>RT4017F</td>
<td>RT-PCR and Northern blot analysis of omcF</td>
<td>ATGAGAGGGCTTGCCCCTG</td>
<td>1 to 21 of omcF</td>
<td>This study</td>
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<tr>
<td>RT4017R</td>
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<td>This study</td>
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<tr>
<td>RT4018F</td>
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<td>GCATGGCCACCATCAAGG</td>
<td>1037 to 1056 of orfA</td>
<td>This study</td>
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<td>RT4018R</td>
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<td>This study</td>
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<tr>
<td>RT4016F</td>
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<td>CTTGCGGGAAGCCAGGAG</td>
<td>421 to 420 of orfC</td>
<td>This study</td>
</tr>
<tr>
<td>RT4016R</td>
<td>RT-PCR analysis of orfC</td>
<td>TGTGCAGTAAAGGATGAG</td>
<td>901 to 920 of orfC</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Unless otherwise indicated, the A of the ATG start codon is considered position 1.

1. The omcF bases are underlined, and pBBR1MCS-2 bases are indicated by boldface type.

2. The underlining indicates positions −1 to 18 from the omcF stop codon, and the boldface type indicates positions −51 to −33 from the Kan’ stop codon in pBBR1MCS-2.

3. HindIII site is underlined.

**FIG. 1.** (A) Structure of gene cluster containing omcF (DL1) and mutation scheme for OmcF-deficient strain DLBK01. The omcF gene was replaced with a kanamycin cassette in mutant DLBK01 (omicF::kan). The gene replacement is indicated by a horizontal bar. The transcriptional orientation of the kanamycin resistance cassette (Kan’) is the same as that of omcF and is indicated by a horizontal arrow. “S” indicates Smal sites. The current annotation (36) of the genes surrounding omcF is as follows: orfA, ATP-dependent protease; orfC, putative membrane protein; and orfd, SFPH/band 7 domain protein. (B) Confirmation of omcF::kan genotype by Southern blot analysis. Wild-type and OmcF-deficient mutant genomic DNA were cleaved with Smal, blotted, and probed with a 2.2-kb DNA fragment amplified from the omcF::kan strain with primers 4017-1 and 4017-6 (Table 1). The expected labeled fragments are 0.9 kb and 0.42 kb long for the wild type (WT) and 2.1 kb and 0.39 kb long for the mutant.
primers are likely to be much higher than those generated with random primers, this result suggested that _omicF_ expression is significantly higher during growth on Fe(III) citrate than during growth on fumarate. The results of the Northern blot analysis (Fig. 3B) were consistent with this conclusion, and a smeared _omicF_ transcript at roughly 3 kb was significantly more abundant during growth on Fe(III) citrate than during growth fumarate. This result is consistent with proteomic studies which detected OmcF only in Fe(III) citrate-grown cells, not in fumarate-grown cells (Ding, unpublished data).

**Characterization of **_OmcF-deficient (omicF::kan)_** mutant. To investigate the physiological function of OmcF, a deletion mutant ( _omicF::kan_ , strain DLBK01) was constructed by homologous recombination (Fig. 1). The OmcF-deficient mutant grew as well as the wild type when fumarate was provided as the electron acceptor (Fig. 4A). However, when washed suspensions of the OmcF-deficient mutant were suspended in bicarbonate buffer and supplied with Fe(III) citrate as the electron acceptor, the rates of Fe(III) reduction with acetate and hydrogen as the electron donors were only 4.4% and 2.7% of the rates observed for the wild type, respectively (Fig. 4B). The ability of the OmcF-deficient mutant to reduce Fe(III) was also impaired under growth conditions (Fig. 4C). When acetate-fumarate-grown wild-type cells were inoculated into acetate-Fe(III) citrate medium, they completely reduced the Fe(III) in the medium within 3 days. In contrast, Fe(III) reduction by the OmcF-deficient mutant was not detectable until 6 days (150 h) after inoculation (Fig. 4C). Expression of the _omicF_ gene in _trans_ fully restored the ability of acetate-fumarate-grown cultures of the mutant to reduce Fe(III) in cell suspension assays (Fig. 4B) and also enabled the mutant to grow in Fe(III) citrate medium with a lag phase, doubling time, and maximum cell density that were comparable to those of the wild-type strain (Fig. 4C).

After approximately 150 h of incubation in Fe(III) citrate medium, the OmcF-deficient mutant developed the ability to grow using Fe(III) citrate as an electron acceptor. However, the doubling time and the final cell yield for the OmcF-deficient mutant were ca. 145% and 70%, respectively, of those for the wild type (15.3 h and 1.11 × 10⁶ cells/ml for the mutant and 10.3 h and 1.55 × 10⁶ cells/ml for the wild-type strain). During four subsequent transfers in Fe(III) citrate medium, the mutant grew with a much shorter lag phase but consistently grew more slowly than the wild type (data not shown). In order to determine if acquisition of the ability to reduce Fe(III) citrate was reversible, the adapted mutant was transferred four times (3% inoculum) in acetate-fumarate medium. Despite extensive culturing in acetate-fumarate medium, the adapted mutant retained the ability to grow on acetate-Fe(III) citrate medium with approximately the same lag phase (20 h), doubling time (15.2 h), and cell yield (1.06 × 10⁸ cells/ml) observed for the adapted mutant prior to culturing on fumarate. However, it was clear that the adaptation had not restored the original pathway for Fe(III) reduction found in the wild-type strain, as washed cell suspensions of the fumarate-grown adapted mutant reduced Fe(III) citrate only at rates comparable to those of the unadapted mutant (Fig. 4B).

The long lag phase required for the initial growth of the OmcF-deficient mutant in Fe(III) citrate medium and the stability of the adapted phenotype suggested that growth of the OmcF-deficient mutant on Fe(III) citrate might have resulted from selective growth of a subpopulation of phase variants or cells with a secondary mutation. Due to the possibility of genetic differences between the OmcF-deficient strain cultured exclusively on acetate-fumarate medium and the strain that was subjected to selective pressure in acetate-Fe(III) citrate medium, the OmcF-deficient strain that had been adapted for growth on Fe(III) citrate was designated the Fe(III) citrate-adapted (FC-adapted) strain.

**Cytochrome content of the OmcF-deficient mutants. In order to gain insight into the subcellular localization of OmcF, the cytochrome protein compositions of outer membrane-enriched fractions prepared from the wild-type and the OmcF-deficient cultures were compared. No difference in the abundance of low-molecular-mass (<10-kDa) cytochromes was vis-
TOF MS, three peptides corresponding to OmcF (DVAAYIR, TLARARR, and TLARARREANGIRTVR) were identified. Thus, consistent with predictions based on sequence analysis, OmcF appears to be an outer membrane-associated c-type cytochrome. As expected from the expression studies discussed above and other proteomic studies (Ding, unpublished data), OmcF was substantially more abundant during growth on Fe(III) than during growth on fumarate.

Differences in the higher-molecular-weight cytochromes present in the wild-type and OmcF-deficient outer membrane-enriched fractions were apparent (Fig. 5). Of particular interest was a heme-staining band migrating at ca. 78 kDa that was missing from the outer membrane-enriched fraction of the OmcF-deficient mutant (Fig. 5A) during growth on both fumarate and Fe(III) citrate. This band was resolved into two discrete bands following electrophoresis on a lower-percentage Tricine gel (Fig. 5B). The electrophoretic mobilities and expression patterns of these two bands resembled those of two previously described outer membrane cytochromes, OmcB and OmcC (27). The band with the lower electrophoretic mobility yielded tryptic peptides corresponding to OmcB (AYLGTTRP, GILFFNAHPYFYR, CADCHDGTTAVATSDFATAESR, and DVMGAANLHLHDPGAYANR), and the other band yielded peptide sequences corresponding to OmcC (ATIDADGILGFVNSHTAGQOGLTGYYEYATR, LA GADRPNITFILDWQOSAHGGK, TTASGTIEGYVIR, and GILFFNAHPYFYR). Another high-molecular-weight heme-staining band with an electrophoretic mobility similar to that of OmcC was present in outer membrane-enriched fractions prepared from the F(III) citrate-grown OmcF-deficient strain (Fig. 5B). However, when this band was analyzed by MALDI-TOF MS, no peptide corresponding to OmcC was detected. Instead, three peptides corresponding to the putative outer membrane c-type cytochrome GSU2887 (GenBank accession number GI-39997978) were identified (LYVSSISSSR, LYVSISSSRTLR, and VVLRLYSISISSSSRTLR). Restoration of omcF expression in trans restored expression of OmcB and OmcC to nearly wild-type levels. Thus, OmcF expression appeared to be required for expression of OmcB and OmcC during growth on both fumarate and Fe(III) citrate.

In addition to GSU2887, deletion of OmcF resulted in overexpression of a 45-kDa cytochrome during growth on Fe(III) citrate (Fig. 5A and B). MALDI-TOF MS analysis of a tryptic digest of this protein yielded the amino acid sequences HPGANGKFGATIALGLNSYK, SLGSGYAFANQVAAPVSYN, and FNLAYEFTITADGNSYGTDPNSSLOGLR. These sequences identified the cytochrome as OmcS, a previously studied outer membrane c-type cytochrome (35).

Changes in the expression patterns of two additional cytochromes, which had molecular masses of ca. 25 kDa and 21 kDa, were visible during growth of the OmcF-deficient mutant on both fumarate and Fe(III) citrate (Fig. 5A). The 25-kDa cytochrome was overexpressed in the OmcF-deficient mutant, whereas expression of the 21-kDa cytochrome was lower in the OmcF-deficient mutant than in the wild type. In both cases, alterations in expression were more pronounced during growth on Fe(III) citrate. Restoration of omcF expression in trans restored wild-type expression patterns for all four cytochromes (Fig. 5A and B).
Expression of omcB and omcC. In order to gain further insight into the mechanism by which deletion of omcF reduced the abundance of OmcB and OmcC, Northern analysis of omcB and omcC expression was performed. The omcB and omcC genes are located in a tandem chromosomal duplication consisting of two three-gene clusters: orf1-orf2-omcB and orf1-orf2-omcC (27). Both omcB and omcC are transcribed from two independent promoters in their individual clusters, which results in the production of two transcripts for each gene, a 5-kb transcript that includes all three genes and a 2.5-kb transcript consisting of omcB or omcC alone (26). The two omcB and omcC transcripts were visible in mRNA prepared from wild-type cells growing on either fumarate or Fe(III) citrate but were absent in the OmcF-deficient strains growing in either medium (Fig. 6A and B). Restoration of omcF expression in trans restored the levels of the omcB and omcC transcripts to the levels present in wild-type cells (Fig. 6A and B). Similar results were obtained when omcB expression and omcC ex-
pression were evaluated by RT-PCR (data not shown). The failure to detect omcB and omcC transcripts in the OmcF-deficient mutant did not appear to be due to the quality of the mRNA prepared from the OmcF-deficient mutant, as the levels of 16S and 23S rRNA present in the various preparations were comparable (Fig. 6C) and expression of the open reading frames immediately upstream and downstream of omcF was detected by RT-PCR (data not shown).

FIG. 5. Heme staining and Tricine-polyacrylamide gel electrophoresis of outer membrane-enriched fractions prepared from wild-type, OmcF-deficient (omcF::kan), FC-adapted OmcF-deficient (FC-adapted omcF::kan), and complemented (omcF::kan/pRG5-omcF) strains. Outer membrane-enriched fractions were prepared from cultures grown in acetate-Fe(III) citrate or acetate-fumarate medium by Sarkosyl extraction (41). Outer membrane proteins (10 μg for panel A, 30 μg for panel B) were resolved on a 15% (A) or 7.5% (B) Tris-Tricine polyacrylamide gel and stained for heme. In order to resolve OmcB and OmcC (27), the gel shown in panel B was run until all proteins having molecular masses less than 30 kDa were off the gel.

FIG. 6. Northern blot analysis of omcB and omcC expression. Total RNA was extracted from acetate-fumarate- or acetate-Fe(III) citrate-grown cultures of wild-type, OmcF-deficient (omcF::kan), and complemented (omcF::kan/pRG5-omcF) strains and hybridized with 32P-labeled probes for omcB (A) and omcC (B). Equal amounts of mRNA (5 μg/lane) were loaded for each strain, and RNA quantification was confirmed by ethidium bromide staining (C). The probes were specific for bp 508 to 1004 of omcB and bp 285 to 897 of omcC.

DISCUSSION

Our results indicate that OmcF, a small, outer membrane, c-type cytochrome, plays an important role in Fe(III) reduction in G. sulfurreducens. Elimination of OmcF alters the abundance of as many as six outer membrane c-type cytochromes. This includes the loss of OmcB, an outer membrane cytochrome that is required for optimal Fe(III) reduction (27). This is the first example of a requirement for one outer membrane cytochrome for the production of another outer membrane cytochrome that is necessary for Fe(III) reduction in G. sulfurreducens.

Potential roles of OmcF. The most readily apparent phenotype of the OmcF-deficient mutant was its inability to reduce Fe(III) citrate. Deletion of another outer membrane c-type cytochrome, OmcB, also results in impairment of Fe(III) citrate reduction (27). Like the OmcF-deficient mutant, the OmcB-deficient mutant adapted to grow with Fe(III) citrate as the electron acceptor and eventually reduced Fe(III) nearly as fast as the wild type, but the cell yields were about one-half those of the wild type (25). Adaptation of the OmcB-deficient mutant to reduce Fe(III) was associated with increased production of other outer membrane c-type cytochromes, most notably OmcS (25). It is interesting that the reduced capacity of the OmcF-deficient mutant to grow with Fe(III) citrate as an
electron acceptor was also associated with the loss of OmcB and that adaptation of the OmcF-deficient mutants was associated with increased production of OmcS. The lack of OmcS during growth of the FC-adapted OmcF-deficient mutant on fumarate and the inability of the fumarate-grown FC-adapted OmcF-deficient mutant to reduce Fe(III) in cell suspension further suggest that increased production of OmcS was an important part of the adaptation of the OmcF-deficient mutant to grow on Fe(III).

These results suggest that the inability of the OmcF-deficient mutant to reduce Fe(III) may be due to the lack of OmcB. However, this cannot be concluded with certainty because the presence of another outer membrane c-type cytochrome (molecular mass, ca. 21 kDa) which has an unknown function was also diminished in the mutant. Production of OmcC, a homolog of OmcB, was also lower in the OmcF-deficient mutant, but genetic studies indicate that OmcC is unlikely to play a critical role in Fe(III) reduction (27).

The mechanism by which the loss of OmcF results in loss of OmcB and other cytochromes is not known. The similarity of OmcF to cytochrome $c_6$ of cyanobacteria and planktonic algae suggests that it may serve as an important electron carrier in $G. sulfurreducens$ (2, 13, 23). If OmcF is part of a complex with one or more of the cytochromes that were not present in the OmcF-deficient mutant, then the absence of OmcF might result in accumulation of partially folded components of the complex, which, as seen in E. coli (44–46, 59), could activate an envelope stress response influencing gene expression.

Alternatively, OmcF may be part of a signal transduction pathway that ultimately influences either the transcriptional activation of the operon or the presence of cytochromes in the mature cell. OmcF is feasible to cytochrome $c_6$, which can be influenced by the relative abundance of Fe(III) and Fe(II). Another possibility is that OmcF is part of a signaling pathway involving proteolysis. Northern analysis suggested that the $omcF$ transcript is poly-cistronic (Fig. 6B). The gene immediately upstream of $omcF$ (orfA) is homologous (57% similar) to the gene encoding the regulatory Lon protease of E. coli.

Considerable additional investigation is required to determine the physiological role of OmcF. However, this study clearly demonstrated that OmcF is one of several cytochromes that play a critical role in Fe(III) reduction in G. sulfurreducens. Thus, Fe(III) reduction is likely to be a complex process involving multiple steps and complexes with many potential levels of regulation.

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