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Two Putative *c*-Type Multiheme Cytochromes Required for the Expression of OmcB, an Outer Membrane Protein Essential for Optimal Fe(III) Reduction in *Geobacter sulfurreducens*

Byoung-Chan Kim,* Xinlei Qian, Ching Leang, Maddalena V. Coppi, and Derek R. Lovley

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

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Deletion of two homologous *Geobacter sulfurreducens* *c*-type cytochrome genes, *omcG* and *omcH*, decreased the rate of Fe(III) reduction and decreased the level of an outer membrane cytochrome critical for Fe(III) reduction, OmcB, without affecting its transcription. Expression of either gene restored Fe(III) reduction and OmcB expression, suggesting functional similarity.

The *Geobacteraceae* of the delta subdivision of the class *Proteobacteria* are a family of Fe(III)-reducing bacteria that have been intensively studied because of their important role in biological electricity production and in the bioremediation of organic contaminants, radionuclides, and toxic metals (1–3, 18, 24, 26). *Geobacter sulfurreducens*, the first *Geobacter* species for which a sequenced genome and a genetic system became available (6, 21), is one of the most intensively studied members of this environmentally important family.

One defining characteristic of *Geobacter* species is the expression of abundant *c*-type cytochromes. The genome of *G. sulfurreducens* contains over 100 putative *c*-type cytochrome-encoding genes (21). Although *c*-type cytochromes are generally involved in electron transport reactions, some heme-containing proteins have alternative physiological functions. Examples include catalase, peroxidases, bacterioferritin, and redox-sensing transcriptional regulators (5, 8, 10, 11, 29).

Genetic studies have implicated several *G. sulfurreducens* *c*-type cytochromes in Fe(III) reduction and electricity production (4, 16, 17, 20). Furthermore, *G. sulfurreducens* cytochromes can influence each other's expression. Deletion of an outer membrane-associated monoheme *c*-type cytochrome, OmcF, negatively affected Fe(III) reduction and decreased the levels of mRNAs for two previously characterized outer membrane cytochromes, OmcB and OmcC (14), one of which, OmcB, had been shown to play a critical role in Fe(III) reduction (16). Here we present another potential example of regulatory interactions between cytochromes. Two homologous multiheme cytochromes, OmcG and OmcH, were found to be involved in Fe(III) reduction and required for OmcB expression at the posttranscriptional level.

Construction and characterization of *omcG*, *omcH*, and *omcA* deletion mutants. A cluster of three putative extracellular, multiheme *c*-type cytochrome-encoding genes, designated *omcA*, *omcH*, and *omcG* (Fig. 1A), was targeted for deletion as part of a genetic screen intended to identify *c*-type cytochromes involved in Fe(III) reduction. Signal sequences were identified with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) at the N termini of both OmcA and OmcH, and analysis of the sequence upstream of the published *omcG* start codon (21) revealed the presence of an in-frame start codon followed by a signal sequence, indicating that the actual *omcG* start codon was likely to be 66 bp upstream of the published start codon. Following reannotation of the *omcG* start codon, all three cytochromes were predicted to be extracellular by two subcellular localization software packages, Psortb v2.0 and Pence proteome analyst (9, 19). All three of these cytochromes contain multiple copies of two types of heme-binding motifs, CXXCH and CXXXXCH (31), i.e., 14 and 4 copies, 19 and 5 copies, and 24 and 3 copies for *omcG*, *omcH*, and *omcA*, respectively. The predicted molecular masses of OmcG, OmcH, and OmcA following signal peptide cleavage and heme incorporation are ca. 78.7, 103.0, and 118.04 kDa, respectively. The N-terminal halves of OmcG (32A to 391G) and OmcH (30A to 384G) are 84.4% identical. There is no homology between their C termini outside of the heme binding motifs. However, the C terminus of OmcH (P480 to R901) is 70% identical to that of OmcA (P622 to K1038).

An OmcA-, OmcH-, and OmcG-deficient triple mutant (strain DLBK03) (Fig. 1A) was constructed by replacing the *omcA-omcH-omcG* cluster with a kanamycin resistance cassette via homologous recombination as previously described (14, 17, 22), using the primers indicated in Table 1. The genotype of the triple mutant was confirmed by Southern blotting genomic DNA digested with KpnI (locations of KpnI sites are indicated in Fig. 1A) as previously described (6, 14). Growth of the triple mutant and that of the wild-type strain in medium in which acetate was the electron donor and fumarate was the electron acceptor (6) were indistinguishable (data not shown). However, when log-phase acetate-fumarate-grown cultures (A_{600} = ~0.5) were inoculated (3%) into acetate-Fe(III)-citrate medium (6), the triple mutant was impaired in Fe(III) reduction (Fig. 1B). The wild-type strain completely reduced the Fe(III) in the medium within 2 days and had a doubling time of approximately 7 h, whereas the triple mutant had a doubling time of 18 h and required 6 days to complete Fe(III) reduction (Fig. 1B). Fe(II) concentrations were determined by the ferrozine assay, and cell densities were determined by

* Corresponding author. Mailing address: Department of Microbiology, 203 Morrill Science Center IVN, University of Massachusetts at Amherst, 639 North Pleasant St., Amherst, MA 01003. Phone: (413) 545-6796. Fax: (413) 545-1578. E-mail: bckim@microbio.umass.edu.

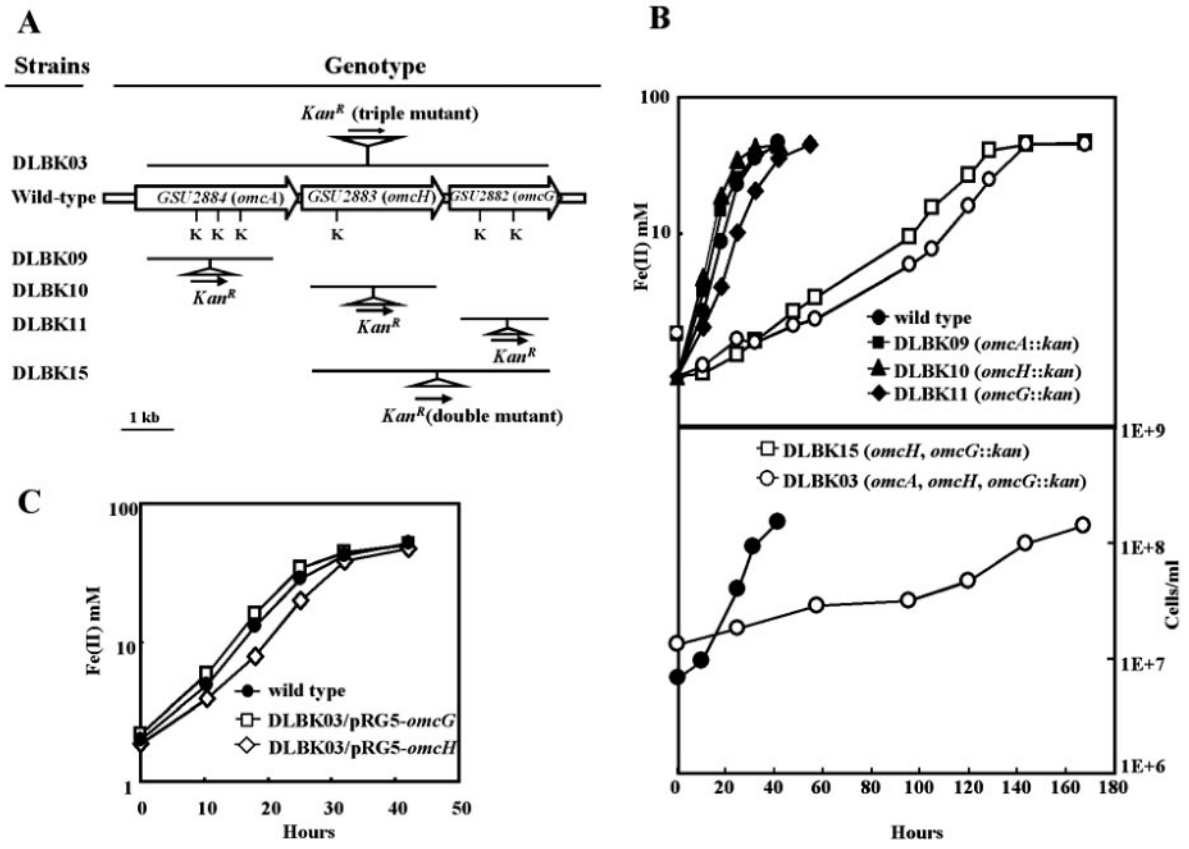

FIG. 1. Construction and characterization of mutants. (A) Wild-type and mutant genotypes. The positions of gene replacements are indicated by horizontal bars. The transcriptional orientations of the kanamycin resistance cassettes (Kan^r) are indicated by horizontal arrows. "K" indicates KpnI sites used to confirm genotypes by Southern blot analysis. GenBank accession numbers for the products of the *omcA*, *omcH*, and *omcG* genes are 39997975, 39997974, and 39997973, respectively. (B) Fe(III) reduction and growth of wild-type and mutant strains in acetate-Fe(III)-citrate medium. (C) Complementation of the triple mutant (DLBK03) by expression of either *omcG* or *omcH* in *trans*. Data are means for triplicate cultures or incubations.

epifluorescence microscopy using acridine orange staining as previously described (12, 14, 25).

To determine which gene was responsible for this phenotype, three single mutants (strains DLBK09, DLBK10 and DLBK11) (Fig. 1A) were constructed using the methods described above and the primers indicated in Table 1. The rates of Fe(III) reduction by the three single mutants were comparable to that of the wild type (Fig. 1B), indicating that deletion of more than one cytochrome was required for impairment of Fe(III) reduction. The high degree of identity between the N termini of OmcG and OmcH suggested that they might have similar physiological functions and thus be able to compensate for each other's absence. An *omcH omcG*::*kan* double mutant (strain DLBK15) (Fig. 1A; Table 1) was therefore constructed and screened as described above. The phenotype of this double mutant was comparable to that of the triple mutant (Fig. 1B).

Complementation studies were performed to investigate the functional redundancy of *omcG* and *omcH*. An *omcG* expression vector, pRG5-*omcG*, was constructed by amplifying the *omcG* coding sequence with primers Ex4776F and Ex4776R (Table 1) using previously described amplification conditions (14), inserting the coding sequence into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA), excising it with EcoRI and HindIII, and ligating it into the EcoRI and HindIII sites of the expression vector pRG5 (14). The *omcH* expression vector, pRG5-*omcH*, was constructed via a similar strategy. The *omcH* coding sequence was amplified with primers Ex4779F and Ex4779R (Table 1), and flanking EcoRI and BamHI sites were utilized for insertion of the *omcH* coding sequence into pRG5. The *omcG* and *omcH* coding sequences were subsequently sequenced to screen for PCR artifacts. Following transformation of the triple mutant with the two expression vectors, spectinomycin-resistant colonies were screened for the simultaneous presence of both the plasmid and the *omcA omcH omcG*::*kan* mutation by using the primers indicated in Table 1. A representative transformant of each type, DLBK03/pRG5-*omcG* and DLBK03/pRG5-*omcH*, was selected for phenotypic analysis. Expression of either *omcG* or *omcH* in *trans* in the triple mutant restored the wild-type phenotype (Fig. 1C), confirming that *omcG* and *omcH* have overlapping physiological functions and play a critical role in Fe(III) reduction.

Although analysis of the *G. sulfurreducens* proteome by accurate mass and time tag validation methods indicated that both OmcG and OmcH are expressed during growth on both fumarate and Fe(III) citrate (Y. R. Ding, unpublished data), it was not possible to confirm their predicted localization by

TABLE 1. Primers used in this study

Primer name	Usage(s)	Primer sequence (5′ to 3′)	Position[a]	Reference
4783-1	Recombinant PCR and Southern analysis for *omcA* deletion and *omcA-omcH-omcG* deletion	GATGGCACGGTAACCATCGC	−205 to −186 of *omcA*	This study
4783-2	Recombinant PCR for *omcA* deletion and *omcA-omcH-omcG* deletion	CCAGGCATGGGAATTCTGGT	+326 to +345 of *omcA*	This study
4783-3	Recombinant PCR for *omcA* deletion and *omcA-omcH-omcG* deletion	ACCAGAATTCCCATGCCTGG**AGTGCC ACCTGGGATGAATG**[b]	+326 to +345 of *omcA*, −274 to −255 of Kan^r in pBBR1MCS-2	This study
4783-4	Recombinant PCR for *omcA* deletion	GATGGGAGTGGCAGGAGAGAC**ATGG CAGGTTGGGCGTCGC**[b]	+2657 to +2677 of *omcA*, −51 to −33 from Kan^r stop codon in pBBR1MCS-2	This study
4783-5	Recombinant PCR for *omcA* deletion	GTCTCTCCTGCCACTCCCATC	+2657 to +2677 of *omcA*	This study
4783-6	Recombinant PCR and Southern analysis for *omcA* deletion	TGCGTCATCTCCGTGGCTC	+60 to +78 from *omcA* stop codon	This study
4779-1	Recombinant PCR and Southern analysis for *omcH* deletion and *omcH-omcG* deletion	TTCCCTTTCTCCGGCTACCC	−292 to −273 of *omcH*	This study
4779-2	Recombinant PCR for *omcH* deletion and *omcH-omcG* deletion	CGTTCATGTGGTCCGTGGCG	+246 to +265 of *omcH*	This study
4779-3	Recombinant PCR for *omcH* deletion and *omcH-omcG* deletion	CGCCACGGACCACATGAACG**AGTGCC ACCTGGGATGAATG**[b]	+246 to +265 of *omcH*, −274 to −255 of Kan^r in pBBR1MCS-2	This study
4779-4	Recombinant PCR for *omcH* deletion	GGGAACGTGTTTGGCCACT**ATGGCAG GTTGGGCGTCGC**[b]	+2418 to +2436 of *omcH*, −51 to −33 from Kan^r stop codon in pBBR1MCS-2	This study
4779-5	Recombinant PCR for *omcH* deletion	AGTGGCCAAACACGTTCCC	+2418 to +2436 of *omcH*	This study
4779-6	Recombinant PCR and Southern analysis for *omcH* deletion	TCTATGGGAGGCATGCCGTG	+242 to +261 from *omcH* stop codon	This study
4776-1	Recombinant PCR and Southern analysis for *omcG* deletion	GCCACTTCAAGCCGTCCAGG	−156 to −137 of *omcG*	This study
4776-2	Recombinant PCR for *omcG* deletion	CGAAATGGCAGTTGACGTTGG	+383 to +403 of *omcG*	This study
4776-3	Recombinant PCR for *omcG* deletion	CCAACGTCAACTGCCATTTCG**AGTGCC ACCTGGGATGAATG**[b]	+383 to +403 of *omcG*, −274 to −255 of Kan^r in pBBR1MCS-2	This study
4776-4	Recombinant PCR for *omcG* deletion, *omcH-omcG* deletion, and *omcA-omcH-omcG* deletion	GGCGCCGTATAGTTGGGAG**ATGGCAG GTTGGGCGTCGC**[b]	+1763 to +1781 of *omcG*, −51 to −33 from Kan^r stop codon in pBBR1MCS-2	This study
4776-5	Recombinant PCR for *omcG* deletion, *omcH-omcG* deletion, and *omcA-omcH-omcG* deletion	CTCCCAACTATACGGCGCC	+1763 to +1781 of *omcG*	This study
4776-6	Recombinant PCR and Southern analysis for *omcA* deletion, *omcH-omcG* deletion, and *omcA-omcH-omcG* deletion	CGCAATCAGGGTGATGACTG	+175 to +194 from *omcG* stop codon	This study
8916	Northern blot analysis of *omcB*	GGACTGCGCACCATCAAGG	+580 to +598 of *omcB*	15
8908-2	Northern blot analysis of *omcB*	GGTCAGCAGGCCACCGG	+998 to +1004 of *omcB*	16
Ex4776F	Expression of *omcG* in the triple mutant	CTTGAGGGAATGTCCGTTTC	−49 to −30 of *omcG*	This study
Ex4776R	Expression of *omcG* in the triple mutant	AAGCTTAACTACGCTCCAACGCATTG[c]	+4 to +23 from *omcG* stop codon	This study
Ex4779F	Expression of *omcH* in the triple mutant	AAAGCGAGCCGACCGAGCC	−33 to −15 of *omcH*	This study
Ex4779R	Expression of *omcH* in the triple mutant	GGATCCCCGGTGGCGAAGTTTTCTAC[d]	−1 to +19 from omcH stop codon	This study
Omcbpepnoti	Expression of OmcB peptide fragment for making OmcB antisera	GGGGCGGCCGCCGTGGCAACCAACT CGGAC[e]	+348 to +366 of *omcB*	This study
Omcbpephindiii	Expression of OmcB fragment for making OmcB antisera	GGGAAGCTTGTTGCTCATGAAATTA GCG[c]	+1173 to +1191 of *omcB*	This study

[a] Unless indicated otherwise, the A of the ATG start codon is considered position 1.
[b] The *omcA*, *omcH*, or *omcG* bases are underlined, and pBBR1MCS-2 bases are indicated by boldface.
[c] The HindIII site is underlined.
[d] The BamHI site is underlined.
[e] The NotI site is underlined.

Tris-Tricine denaturing polyacrylamide gel electrophoresis and heme staining as previously described (14), possibly due to low expression levels.

OmcB expression in the *omcA omcH omcG*::*kan* triple mutant and the complemented strains. The cytochrome contents of outer membrane-enriched fractions prepared from the wild-type and triple mutant strains by Sarkosyl extraction (23) were compared by electrophoresis and heme staining as previously described (14). A 78-kDa heme-containing protein which comigrated with OmcB, an outer membrane cytochrome previously demonstrated to play a critical role in Fe(III) reduction (16), was absent from the outer membrane fractions of the triple mutant during growth on both acetate-fumarate medium (Fig. 2A) and acetate-Fe(III)-citrate medium (data not shown). This band reappeared at wild-type levels when either *omcG* or *omcH* was expressed in the triple mutant (Fig. 2A).

Western blot analysis was performed to determine whether the 78-kDa band was in fact OmcB. In order to generate an OmcB-specific antiserum, an 846-bp fragment of the *omcB* gene (A116 to N397) was amplified using primers Omcbpepnoti and Omcbpephindiii (Table 1), digested with NotI and HindIII, and inserted into the NotI and HindIII sites of the expression vector pET15b (Novagen, WI). Competent *Escherichia coli* strain Rosetta2 (DE3) (Novagen, WI) was transformed with the resulting plasmid, pET15b-omcBpep. The His-tagged OmcB peptide was successfully overexpressed and purified by Ni-nitrilotriacetic

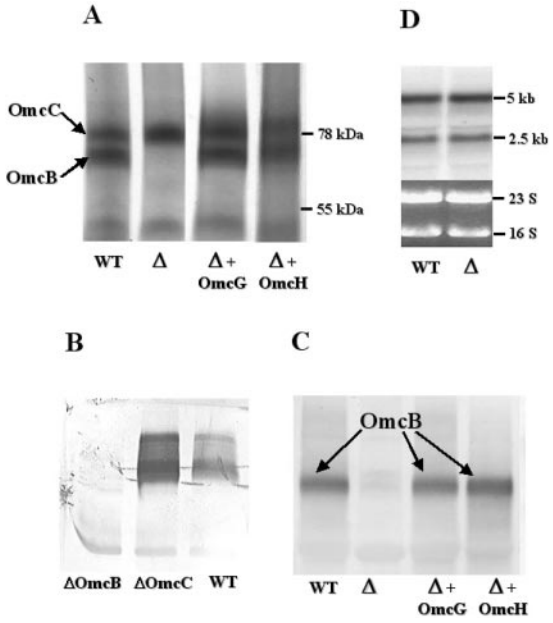

FIG. 2. OmcB expression in the triple mutant (*omcA omcH omcG*::*kan*) and complemented strains during growth on acetate-fumarate medium. (A) Cytochrome contents of outer membrane-enriched fractions of the wild-type (WT), triple mutant (Δ, DLBK03) and complemented (Δ+OmcG and Δ+OmcH, DLBK03/pRG5-*omcG* and DLBK03/pRG5-*omcH* strains, respectively) strains. Proteins (30 μg/lane) were separated by 7.5% Tris-Tricine electrophoresis and stained for heme (7, 32). (B) Specificity of anti-OmcB antiserum. A Western blot of outer membrane fractions (2.5 μg/lane) prepared from wild-type, OmcB-deficient (ΔOmcB), and OmcC-deficient (ΔOmcC) strains (16) was probed with the OmcB-specific antiserum. (C) Expression of OmcB in the triple mutant and complemented strains. Outer membrane-enriched fractions (30 μg/lane) were separated by 7.5% Tris-Tricine electrophoresis, immunoblotted, and probed with the OmcB-specific antiserum. (D) Northern analysis of *omcB* expression in the wild-type and triple mutant strains. Equal amounts of total RNA (5 μg) were loaded for each strain. Ethidium bromide staining of 16S and 23S rRNAs is shown at the bottom as confirmation of RNA quantification.

acid affinity chromatography as recommended by Novagen, and its identity was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Cross-reacting antibodies were removed by immunoabsorption using an acetone extract prepared from an OmcB-deficient mutant (16) as described by Sambrook et al. (27), except that cells were disrupted by sonication and cell debris and unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min. The specificity of the antiserum was confirmed by probing immunoblots of wild-type, OmcB-deficient, and OmcC-deficient outer membrane-enriched fractions (Fig. 2B). Outer membrane-enriched fractions were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Immunoblots were probed with the OmcB-specific antiserum according to established protocols (details are at http://aroianlab.ucsd.edu/protocols/western.htm), and immunoreactive bands were visualized using an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody and SigmaFast 5-bromo-4-chloro-3-indolylphosphatase/nitroblue tetrazolium tablets (Sigma, St. Louis, MO) according to the manufacturer's instructions.

Western blot analysis confirmed that the 78-kDa band missing from the outer membrane-enriched fraction of the triple mutant was OmcB (Fig. 2C). The OmcB protein was also undetectable in the inner membrane-enriched and soluble fractions of the triple mutant (data not shown). In addition, OmcB was detected at wild-type levels in the two complemented strains, DLBK03/pRG5-*omcG* and DLBK03/pRG5-*omcH* (Fig. 2C). Thus, expression of either *omcG* or *omcH* in *trans* fully restored both the ability to reduce Fe(III) (Fig. 1C) and wild-type levels of OmcB expression (Fig. 2A and C) in the triple mutant.

In a previous study, deletion of OmcF, a low-molecular-weight outer membrane cytochrome, eliminated expression of OmcB by dramatically decreasing the levels of *omcB* transcripts (14). The levels of *omcB* mRNA in the triple mutant were therefore assessed by Northern analysis using an *omcB*-specific probe (amplified with primers 8916 and 8908-2) (Table 1) as previously described (14, 15). The *omcB* gene is transcribed from two independent promoters within a three-gene cluster (*orf1-orf2-omcB*), resulting in the production of two transcripts, a 5-kb transcript that includes all three genes and a 2.5-kb transcript consisting of *omcB* alone (15). Both *omcB* transcripts were detected at the wild-type level in the triple mutant (Fig. 2D), indicating that deletion of OmcG and OmcH, unlike elimination of OmcF, affected OmcB expression posttranscriptionally.

Implications. This study provides additional evidence that Fe(III) reduction involves multiple interacting cytochromes. Both OmcG and OmcH play a role in Fe(III) reduction and influence the level of OmcB, a third cytochrome critical for Fe(III) reduction (16). It is likely that OmcG or OmcH may be involved in either the translation or stabilization of the OmcB protein in *G. sulfurreducens*. Unlike the case for the low-molecular-weight cytochrome OmcF, which was found to be required either for transcriptional activation of the *omcB* gene or for the stability its transcripts (14), deletion of the *omcG* and *omcH* genes did not affect levels of *omcB* mRNA. In the case of the *c*-type cytochrome nitrate reductases of *Wolinella succinogenes* and *Thermus thermophilus*, deletion of a second cytochrome which served as a membrane anchor led to accumulation of the nitrate reductase in the soluble rather than the membrane fraction (28, 33). However, failure to detect OmcB in the outer membrane of the triple mutant did not appear to be due to mistargeting or failure to incorporate heme, since OmcB could not be detected by Western blotting in either the inner membrane-enriched or soluble fractions of the triple mutant (data not shown).

One possible explanation for the effect of *omcG* and *omcH* deletion on the level of OmcB is that OmcB, OmcG, and OmcH are part of a complex required for Fe(III) reduction and the absence of OmcG and OmcH results in the accumulation and degradation of OmcB in the periplasmic space. The *G. sulfurreducens* genome contains two homologs (56 to 57% similar) of the periplasmic protease of *E. coli*, DegP, which degrades misfolded proteins, including outer membrane proteins, that accumulate in the periplasmic space (13, 30). Alternative explanations are also plausible, including direct or indi-

rect interactions between OmcG or OmcH and the intracellular signaling network that affect expression of OmcB.

Further study will be required to determine how OmcG and OmcH influence the abundance of OmcB. However, the finding that three cytochromes OmcF, OmcG, and OmcH, which influence the rate at which cells develop the capacity for Fe(III) reduction, also influence OmcB expression highlights the central role of OmcB in Fe(III) reduction by wild-type *G. sulfurreducens* and points to interactions, either direct or indirect, between the four cytochromes. In addition, this study and the previous study on OmcF (14) clearly demonstrate that *c*-type cytochromes can be involved not only in electron transfer but also in transcriptional and posttranscriptional regulation or processing in *G. sulfurreducens*.

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