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Adaptation to Disruption of the Electron Transfer Pathway for Fe(III) Reduction in *Geobacter sulfurreducens*

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Previous studies demonstrated that an outer membrane *c*-type cytochrome, OmcB, was involved in Fe(III) reduction in *Geobacter sulfurreducens*. An OmcB-deficient mutant was greatly impaired in its ability to reduce both soluble and insoluble Fe(III). Reintroducing *omcB* restored the capacity for Fe(III) reduction at a level proportional to the level of OmcB production. Here, we report that the OmcB-deficient mutant gradually adapted to grow on soluble Fe(III) but not insoluble Fe(III). The adapted OmcB-deficient mutant reduced soluble Fe(III) at a rate comparable to that of the wild type, but the cell yield of the mutant was only ca. 60% of that of the wild type under steady-state culturing conditions. Analysis of proteins and transcript levels demonstrated that expression of several membrane-associated cytochromes was higher in the adapted mutant than in the wild type. Further comparison of transcript levels during steady-state growth on Fe(III) citrate with a whole-genome DNA microarray revealed a significant shift in gene expression in an apparent attempt to adapt metabolism to the impaired electron transport to Fe(III). These results demonstrate that, although there are many other membrane-bound *c*-type cytochromes in *G. sulfurreducens*, increased expression of these cytochromes cannot completely compensate for the loss of OmcB. The concept that outer membrane cytochromes are promiscuous reductases that are interchangeable in function appears to be incorrect. Furthermore, the results indicate that there may be different mechanisms for electron transfer to soluble Fe(III) and insoluble Fe(III) oxides in *G. sulfurreducens*, which emphasizes the importance of studying electron transport to the environmentally relevant Fe(III) oxides.

Microorganisms in the family of *Geobacteraceae* are the predominant Fe(III) reducers in many subsurface environments where dissimilatory Fe(III) reduction plays an important role in the degradation of organic matter or the bioremediation of organic or metal contaminants (12, 13). The mechanisms by which Fe(III)-reducing microorganisms reduce Fe(III) are poorly understood. It is known that phylogenetically distinct Fe(III) reducers reduce Fe(III) oxide with significantly different mechanisms. For example, *Geothrix* and *Shewanella* species produce soluble electron shuttles and/or Fe(III)-chelating compounds to alleviate the need for direct electron transfer from the cell surface to the surface of Fe(III) oxide (20–22, 26). However, current evidence suggests that *Geobacter* species require direct physical contact between cells and extracellular electron acceptors (19), which may be achieved with appendages such as flagella and pili (3).

Earlier biochemical studies suggested that *c*-type cytochromes are likely to be involved in Fe(III) reduction in *Geobacter* species (8, 9, 16). Further genetic studies, concentrating on *Geobacter sulfurreducens*, due to the availability of a genetic system (5) and the genome sequence (17), indicated that a periplasmic cytochrome, PpcA (11); an inner membrane-associated cytochrome, MacA (2); and an outer membrane cytochrome, OmcB (10), are important for Fe(III) reduction. Based on location, PpcA and MacA were proposed to be intermediary electron transfer components whereas the outer membrane localization of OmcB and the severe impact on Fe(III) reduction when *omcB* was deleted suggested that OmcB might be the terminal Fe(III) reductase (10).

However, sequencing of the *G. sulfurreducens* genome has demonstrated that this organism contains genes for over 100 putative *c*-type cytochromes (17). A potential reason for this unprecedented number of cytochromes is that this might provide the opportunity for *G. sulfurreducens* to form multiple routes for electron transfer to Fe(III) and thus better maximize rates of Fe(III) reduction and/or provide a high degree of flexibility to adapt to disruptions in electron transfer pathways. In order to investigate this potential for adaptation further, the long-term adaptation of the OmcB-deficient mutant was studied.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *Geobacter sulfurreducens* strain DL1 (5) and DL6 (*omcB*::*cam*) (10) were routinely cultured anaerobically in NBAF (acetate/fumarate) or FWFC [acetate/Fe(III) citrate] medium at 30°C as previously described (5). Steady-state growth was investigated in chemostats under strict anaerobic conditions (N_2-CO_2 [80:20, vol/vol]) in a freshwater medium containing Fe(III) citrate (56 mM) as the electron acceptor and limited acetate (5.5 mM) as the electron donor as previously described (4, 6). The dilution rate was 0.05 h^{-1}.

Detection of cytochromes in the membrane fraction. The membrane fractions of *G. sulfurreducens* were isolated as described earlier (10). Proteins (50 μg) of membrane fractions were analyzed with Tris-Tricine denaturing polyacrylamide gel electrophoresis (1). *c*-type cytochromes were detected by staining with *N*,*N*,*N*′,*N*′-tetramethylbenzidine as previously described (7, 25). Molecular standard markers were purchased from Bio-Rad Laboratories (Hercules, CA) and were stained separately with Coomassie blue R-250.

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TABLE 1. Primers used for Northern blot analyses for *c*-type cytochrome genes whose expression levels were significantly altered in the Fe(III)-adapted OmcB-deficient mutant

Target gene	Primer	Sequences (5′-3′)	Length (bp)	Reference(s)
GSU0594	994F	CCACAGCAGTAAGGATATCG	375	This work
	994R	GCATCGATATGCAGATCCG		
GSU2494	4120F	GCACTCAAGTTCAAGCTCAAG	469	This work
	4120R	GGATGATATCGCTCTTCAGC		
GSU2495	4123F	GCTCATTCTTCACCTGTCGG	574	This work
	4123R	GATAGTGCAGCGCTTGCTTTC		
GSU2503 (*omcT*)	4140-1F	CCAACCAGTTCAGCTGCATC	382	This work
	4140-1R	GAAGGGGCCAAGGTTCTGATC		
GSU2504 (*omcS*)	4142F	ACGTTCGTGGTCTCAACAC	775	This work
	4142R	GATGGTCGTGAACTCGTATG		
GSU2731 (*omcC*)	8914	GCCAGAGTGAGGCCCAGA	543	This work; 4
	8915	GGGTGTTGTGGTAGAAGGG		
GSU2737 (*omcB*)	8916	GGACTGCGCACCATCAAGG	435	This work; 10
	8908-2	GGTCAGCAGGCCACCGG		
GSU2808	4655F	GGTGACAGTAGGAGTACCTG	354	This work
	4655R	GGTGTGACAGAGGTAGCAGAC		
GSU2811	4659F	GTGTGACCAGCTATGCTCC	395	This work
	4659R	GTCAAAGTCCAGGTCGAACC		
GSU2813	4662F	CCATCCTCTGTGCAGTCGC	599	This work
	4662R	GGTCTGTTTTCCGTCAAGG		
GSU2887	4789-1F	CCATACGAGCACCAATGAGC	445	This work
	4789-1R	GCTGTTGCTGTCAGAGGAGG		
GSU3259	5426F	CGTCTATCCTTGGCTTCGAG	426	This work
	5426R	CGATATGCCAGTGGATACC		

Analytical techniques. Protein concentration was determined with the bicinchoninic acid method with bovine serum albumin as a standard (24). Cells were counted using epifluorescence microscopy with acridine orange staining (15). Fe(II) concentrations were determined with the ferrozine assay as previously described (14). Acetate concentrations were measured with high-pressure liquid chromatography on a Hewlett-Packard series 1100 (Agilent Technologies, Inc., Albany, NY) with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) and a mobile phase of 8 mM H_2SO_4.

DNA microarray hybridization experiments and data analysis. Total RNA was isolated from two sets of identically treated chemostat cultures of both the wild type and the adapted OmcB-deficient mutant. A total of 11 replicate hybridizations were carried out: five replicate hybridizations were from one set of the biological samples, and six were from the other set. DNA microarray hybridization and data analyses were described previously (18). Briefly, total RNA (5 μg) that was isolated from steady-state continuous cultures of the wild type and the adapted OmcB-deficient mutant was used to synthesize cDNA labeled with Cy3-Cy5 fluorescent dyes, which was hybridized to gene arrays. The signal from each spot in the arrays served as a measure of the expression level of each gene and was used to calculate the expression ratio between the wild type and the mutant.

Quantitative reverse transcription-PCR (RT-PCR). For cDNA synthesis, SuperScript III RNase H^- reverse transcriptase (Invitrogen Co., Carlsbad, CA) was used according to the manufacturer's instructions with gene-specific antisense primers 8908-2 (10) and 8915 (4) for *omcB* and *omcC*, respectively.

The quantitative real-time PCR was carried out as previously described (4). Two specific primer sets, 8912/8908-2 and 8917/8915 (4), were used to determine levels of mRNA for *omcB* and *omcC*, respectively. The temperature profile was composed of an initial incubation step for 2 min at 50°C (activation of the polymerase) followed by a 10-min denaturation step at 95°C, 40 cycles of denaturation for 45 s at 95°C, annealing for 1 min at 58°C, elongation for 1 min at 72°C, and a final elongation step for 6 min at 72°C.

DNA and RNA manipulations. PCR product purification was carried out using QIAGEN PCR purification kits (QIAGEN Inc., Valencia, CA). Probes for Northern blot analysis were labeled with [α-^{32}P]dATP using a Strip-EZ DNA probe synthesis and removal kit (Ambion Inc., Austin, Texas). [α-^{32}P]dATP was purchased from PerkinElmer Life and Analytical Sciences, Inc., Boston, MA. All primers used to amplify *G. sulfurreducens* sequences were designed using the *G. sulfurreducens* genome sequence (17). QIAGEN *Taq* DNA polymerase (QIAGEN Inc., Valencia, CA) was used for all PCR amplifications.

Total RNA was isolated from mid-log cultures using RNeasy Midi kits (QIAGEN Inc., Valencia, CA) followed by treatment with RNase-free DNase (Ambion Inc., Austin, Texas). Northern blot analyses were carried out with the NorthernMax-Gly system (Ambion Inc., Austin, Texas) according to the manufacturer's instructions. All probes for Northern blot analyses were purified PCR product. Primers for amplifying probes are listed in Table 1. The PCR amplification program was as follows: 96°C for 40 s followed by 25 cycles of 96°C for 40 s, 58°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min.

RESULTS

Adaptation of OmcB-deficient mutant (DL6) to grow on soluble Fe(III). As previously reported (10), the OmcB-deficient mutant did not reduce Fe(III) citrate for periods of time well beyond those in which the wild-type cells had reduced all the Fe(III) (Fig. 1). However, after incubation for more than a

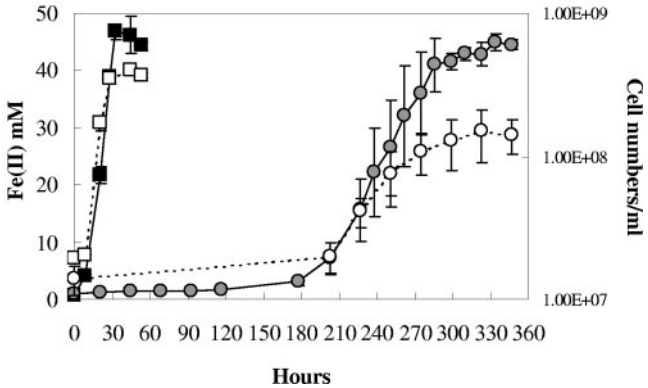

FIG. 1. Adaptation of OmcB-deficient mutant (DL6) to grow with Fe(III) citrate as the sole electron acceptor. Mid-log (optical density at 600 nm, ~0.3) fumarate-grown cells served as the inoculum. Symbols: filled or empty squares, wild-type Fe(II) or cell numbers, respectively; shaded or empty circles, OmcB-deficient mutant Fe(II) or cell numbers, respectively. Data are means ± standard deviations of triplicates.

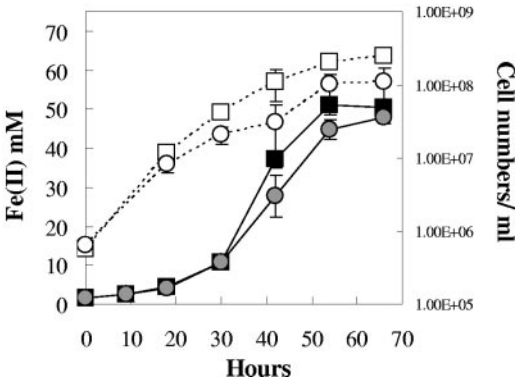

FIG. 2. Growth of the OmcB-deficient mutant after adaptation and two additional transfers on Fe(III) citrate medium. Symbols: filled or empty squares, wild-type Fe(II) or cell numbers, respectively; shaded or empty circles, OmcB-deficient mutant Fe(II) or cell numbers, respectively. Data are means ± standard deviations of triplicates.

week the OmcB-deficient mutant began to reduce Fe(III) (Fig. 1). The growth rate (doubling time, 35 h) was lower than that of the wild type (6 h), and the number of cells produced was only 38% of that of the wild type. The OmcB-deficient mutant never adapted to grow with Fe(III) oxide as the terminal electron acceptor (data not shown).

With two successive transfers of the adapted OmcB-deficient cells in Fe(III) citrate medium, the rate of Fe(III) reduction approached that of the wild type (Fig. 2). However, the final cell yield of OmcB-deficient mutant remained less than half of that of the wild type. The same pattern of adaptation was observed repeatedly when fumarate-grown cultures of the OmcB-deficient mutant were inoculated into Fe(III) citrate medium, suggesting that the adaptation was not due to spontaneous mutations; instead the growth may have resulted from a subpopulation of phase variants or cells with epigenetic modifications. Furthermore, when membrane fractions were isolated from fumarate or Fe(III) citrate-adapted cultures OmcB was not detected (Fig. 3, lanes 3 and 4). However, at least six other cytochromes, including OmcC, which is a close relative

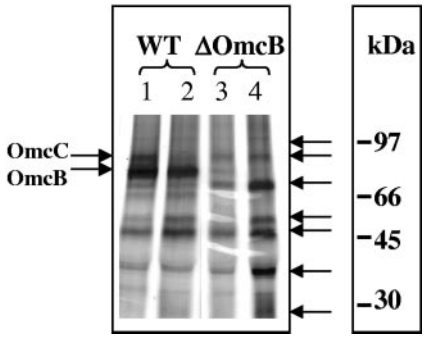

FIG. 3. Tricine-polyacrylamide gel electrophoresis and heme staining of membrane fractions prepared from wild type (WT) and the OmcB-deficient mutant. Membrane fractions were prepared from cultures grown with fumarate (lanes 1 and 3) or Fe(III) citrate (lanes 2 and 4). OmcB and OmcC are indicated with arrows on the left side of the gel. Other cytochromes which were highly expressed in the adapted Fe(III)-grown OmcB-deficient mutant are indicated with arrows on the right side of the gel.

of OmcB but is not required for Fe(III) reduction (10), were present in higher concentrations in the OmcB-deficient mutant than in wild-type cells when membrane fractions were isolated from Fe(III) citrate cultures (Fig. 3, lane 4). The higher concentration of OmcC in the adapted OmcB-deficient mutant was consistent with the finding that levels of *omcC* transcripts as determined with quantitative RT-PCR were ca. 10^3 times higher in adapted, Fe(III)-grown cells than in the wild type.

Transcriptional profile comparison of the OmcB-deficient mutant and the wild type. To further investigate potential differences in gene expression in the adapted OmcB-deficient mutant, transcript levels in continuous cultures of the adapted OmcB-deficient mutant grown with Fe(III) citrate as the electron acceptor were compared to those in wild-type cells with a whole-genome microarray (18). Although the OmcB-deficient mutant reduced Fe(III) at a rate comparable to that of the wild type in the chemostat [steady-state Fe(II) concentration, wild type versus mutant, 36.5 ± 1.2 mM versus 35.6 ± 2.9 mM, respectively; mean ± standard deviation; $n = 3$], the steady-state cell yield of the mutant was only 61% of that of the wild type (total protein of wild type versus mutant, 0.036 ± 0.003 versus 0.022 ± 0.007 mg/ml, respectively).

The adaptation of the OmcB-deficient mutant to grow on Fe(III) citrate was associated with changes in expression of a variety of genes involved in electron transfer and metabolism (Tables 2 and 3). A total of 83 genes had higher transcript levels in the OmcB-deficient mutant than in the wild type (Table 2), and 88 genes appeared to have lower transcript levels in the mutant (Table 3). Eighty-six genes had transcript levels that changed more than twofold in the OmcB-deficient mutant, with 47 genes up-regulated and 39 genes down-regulated.

The greatest increase in transcript levels was for GSU2504 and GSU2503 (Tables 2 and 4), which are cotranscribed and are predicted to encode two outer membrane *c*-type cytochromes, OmcS and OmcT, with high similarity to each other (D. Holmes et al., submitted for publication; T. Mehta et al., submitted for publication). OmcS, the cytochrome encoded by GSU2504, is readily sheared off from the outer surface of *G. sulfurreducens* and is involved in insoluble Fe(III) reduction and electricity production (T. Mehta, submitted for publication). The protein encoded by GSU2503, OmcT, has yet to be detected in any proteomic analysis of *G. sulfurreducens* (R. Ding, unpublished data). Transcripts of *omcS* and *omcT* were detected in wild-type cells during growth on insoluble Fe(III) oxide but not on soluble Fe(III) citrate (T. Mehta et al., submitted for publication). However, transcripts of both *omcS* and *omcT* were detected in the adapted OmcB-deficient mutant during growth on Fe(III) citrate (Table 2; see also Fig. 5). Thus, it is likely that the heme-staining protein recovered in higher levels from the adapted OmcB-deficient mutant (Fig. 3) with a molecular mass of ca. 45 kDa was OmcS.

Genes with significant changes in their transcript levels could be categorized into 16 different groups according to their functions (Fig. 4). The largest functional groups that had higher levels of transcripts in the OmcB-deficient mutant than in the wild type were those related to energy metabolism and genes for hypothetical proteins. Other than *omcS* and *omcT*, the other genes related to energy metabolism which had transcript levels over twofold higher in the adapted mutant were

TABLE 2. Genes that were significantly up-regulated in the adapted OmcB-deficient mutant compared to the wild type grown in acetate-limiting Fe(III) citrate continuous culture

Locus ID[a]	Common name	Role category	Log_2 ratio ± SD
GSU0944	Cystathionine beta-lyase	Amino acid biosynthesis	0.884 ± 0.059
GSU0945	Cystathionine beta-lyase		0.577 ± 0.095
GSU1906	2-Isopropylmalate synthase		0.504 ± 0.125
GSU0265	Putative membrane protein	Cell envelope	0.855 ± 0.114
GSU2078	Rod shape-determining protein		1.185 ± 0.264
GSU2497	Putative lipoprotein		1.618 ± 0.170
GSU2498	Putative lipoprotein		0.964 ± 0.095
GSU2526	Putative membrane protein		0.622 ± 0.121
GSU1140	Methyl-accepting chemotaxis protein	Cellular processes	0.873 ± 0.193
GSU1899	Virulence factor Mce family protein		0.625 ± 0.145
GSU2236	GTP pyrophosphokinase		0.947 ± 0.195
GSU2502	Spermine/spermidine synthase family protein	Central intermediary metabolism	0.723 ± 0.121
GSU2537	Biosynthetic arginine decarboxylase		0.638 ± 0.079
GSU3245	Putative DNA polymerase II	DNA metabolism	2.133 ± 0.199
GSU0193	L-Sorbosone dehydrogenase	Energy metabolism	0.716 ± 0.111
GSU0594	Cytochrome *c*		1.133 ± 0.271
GSU0782	Nickel-dependent hydrogenase, small subunit		2.165 ± 0.250
GSU0783	Nickel-dependent hydrogenase, iron-sulfur cluster-binding protein		2.547 ± 0.370
GSU0784	Nickel-dependent hydrogenase, membrane protein		2.605 ± 0.193
GSU0785	Nickel-dependent hydrogenase, large subunit		2.416 ± 0.306
GSU1640	Cytochrome *d* ubiquinol oxidase, subunit I		1.779 ± 0.776
GSU1641	Cytochrome *d* ubiquinol oxidase, subunit II		2.508 ± 0.427
GSU1707	Group II decarboxylase		0.664 ± 0.052
GSU1722	Creatinine amidohydrolase		0.570 ± 0.103
GSU1761	Cytochrome *c*		0.652 ± 0.164
GSU2018	Glycine cleavage system H protein		0.575 ± 0.112
GSU2098	Carbon monoxide dehydrogenase subunit		1.782 ± 0.286
GSU2494	Cytochrome *c*		1.863 ± 0.388
GSU2495	Cytochrome *c*		1.810 ± 0.294
GSU2501	Cytochrome *c*		0.870 ± 0.204
GSU2503	Cytochrome *c*		3.340 ± 0.154
GSU2504	Cytochrome *c*		3.676 ± 0.626
GSU2811	Cytochrome *c*		0.745 ± 0.196
GSU2812	Glutaredoxin family protein		0.815 ± 0.124
GSU2813	Cytochrome *c*551 peroxidase		0.839 ± 0.186
GSU3246	Thioredoxin peroxidase		1.049 ± 0.229
GSU1916	Phosphatidate cytidylyltransferase	Fatty acid and phospholipid metabolism	0.556 ± 0.146
GSU0192	Hypothetical protein	Hypothetical proteins	1.433 ± 0.203
GSU0208	Hypothetical protein		1.029 ± 0.354
GSU0593	Hypothetical protein		1.083 ± 0.217
GSU0647	Hypothetical protein		1.002 ± 0.139
GSU0788	Hypothetical protein		1.453 ± 0.329
GSU0919	Hypothetical protein		1.587 ± 0.326
GSU1160	Hypothetical protein		1.220 ± 0.134
GSU1309	Hypothetical protein		0.701 ± 0.216
GSU1770	Hypothetical protein		0.762 ± 0.117
GSU1947	Hypothetical protein		1.464 ± 0.098
GSU1948	Hypothetical protein		1.168 ± 0.162
GSU2496	Hypothetical protein		1.418 ± 0.374
GSU2499	Hypothetical protein		1.998 ± 0.238
GSU2500	Hypothetical protein		1.792 ± 0.532
GSU3309	Hypothetical protein		0.664 ± 0.091
GSU3310	Hypothetical protein		0.670 ± 0.160
GSU3403	Hypothetical protein		2.586 ± 0.168
GSU3409	Hypothetical protein		1.248 ± 0.215
GSU3410	Hypothetical protein		1.588 ± 0.170

Continued on following page

TABLE 2—*Continued*

Locus ID[a]	Common name	Role category	Log_2 ratio ± SD
GSU0786	Hydrogenase maturation protease	Protein fate	1.894 ± 0.178
GSU0787	TatA/E family twin-arginine translocation protein		0.820 ± 0.155
GSU1159	PfpI family intracellular protease		1.185 ± 0.245
GSU0646	tRNA (guanine-N_1)-methyltransferase	Protein synthesis	0.607 ± 0.129
GSU0648	Ribosomal protein L19		0.788 ± 0.185
GSU0537	Sensory box/GGDEF family protein	Regulatory functions	1.138 ± 0.081
GSU2506	Sigma-54-dependent DNA-binding response regulator		2.264 ± 0.345
GSU2507	Sensor histidine kinase		2.797 ± 0.432
GSU3387	AraC/XylS family transcriptional regulator		0.561 ± 0.146
GSU0189	ATP-dependent RNA helicase	Transcription	1.097 ± 0.139
GSU1161	RND family efflux transporter	Transport and binding proteins	0.949 ± 0.198
GSU1346	Sulfate ABC transporter, periplasmic sulfate-binding protein		1.047 ± 0.224
GSU1723	Mechanosensitive ion channel family protein		0.812 ± 0.177
GSU3401	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein		2.590 ± 0.255
GSU3404	Amino acid ABC transporter, ATP-binding protein		2.452 ± 0.341
GSU3405	Amino acid ABC transporter, permease protein		0.973 ± 0.187
GSU0500	GTP-binding protein TypA	Unknown functions	0.610 ± 0.086
GSU0534	RrF2 family protein		0.814 ± 0.248
GSU0664	GTP binding protein YchF		0.546 ± 0.091
GSU0769	RarD protein		1.262 ± 0.177
GSU1643	GGDEF/response regulator receiver domain protein		2.053 ± 0.162
GSU1708	Atz/Trz family chlorohydrolase		0.599 ± 0.098
GSU1877	2-Nitropropane dioxygenase family oxidoreductase		1.059 ± 0.287
GSU1945	Fibronectin type III domain protein		1.553 ± 0.177
GSU2493	NHL repeat domain protein		1.656 ± 0.166
GSU2505	NHL repeat domain protein		2.102 ± 0.573
GSU2527	Nitrate/sulfite reductase domain protein		1.200 ± 0.296

[a] ID, identification.

those associated with a nickel-dependent hydrogenase, a cytochrome *d* ubiquinol oxidase, and a carbon monoxide dehydrogenase (Table 2). The transcript of a *relA* homolog (GSU2236), whose protein product is predicted to be involved in a stringent response during stress or nutrient depletion, was ca. twofold up-regulated in the mutant. The largest functional groups that had lower levels of transcripts in the OmcB-deficient mutant than in the wild type were those coding for hypothetical proteins, mobile and extrachromosomal element functions, and transport and binding proteins (Fig. 4). Four *c*-type cytochrome genes including the *omcB* gene were among the genes with lower transcript levels than those of the wild type (Tables 3 and 4). Genes coding for acetate metabolism (acetate kinase, GSU2707, and phosphate acetyltransferase, GSU2706) were among the groups of genes with over twofold-lower transcript levels.

Validation by Northern blotting of expression ratios from transcriptional profiling. Transcript levels for cytochrome genes which were significantly different (ca. twofold) between the adapted mutant and the wild type from the whole-genome microarray analysis were evaluated with Northern blot analyses (Fig. 5). The results from the Northern analysis compared well with those from the microarray analysis. The only exception was that *omcC* was not detected as up-regulated in the microarray, but transcript levels of *omcC* were significantly higher in the Northern analysis, consistent with quantitative real-time RT-PCR results mentioned above. The predicted molecular weights of those cytochromes whose transcript levels were significantly higher (ca. twofold) than those of the wild type from both microarray and Northern analyses (Table 4) are in agreement with those protein bands that were identified from the membrane fractions of the OmcB-deficient mutant (Fig. 3, lane 4).

DISCUSSION

The results demonstrate that even though *G. sulfurreducens* has genes for ca. 30 outer membrane *c*-type cytochromes, including the closely related OmcC, it cannot fully adapt to the loss of OmcB, which is essential for the most effective growth on soluble Fe(III) as well as for any growth on Fe(III) oxide, the most abundant form of Fe(III) in most environments. As detailed below, these results suggest that outer membrane cytochromes, which are frequently viewed as rather promiscuous agents for electron transfer to a variety of extracellular electron acceptors, may in fact have quite specific functions that cannot be replicated by other cytochromes.

Adaptation of OmcB-deficient mutant for growth on soluble Fe(III). Previous short-term studies identified OmcB as an important component in electron transfer based on the finding that deleting *omcB* inhibited Fe(III) reduction and reintroducing *omcB* restored the capacity for Fe(III) reduction at a level

TABLE 3. Genes that were significantly down-regulated in the adapted OmcB-deficient mutant compared to the wild type in acetate-limiting Fe(III) citrate-grown continuous culture

Locus ID[a]	Common name	Role category	Log_2 ratio ± SD
GSU0656	Branched-chain amino acid aminotransferase	Amino acid biosynthesis	−0.765 ± 0.179
GSU0862	FolD bifunctional protein	Biosynthesis of cofactors, prosthetic groups and carriers	−0.881 ± 0.176
GSU1577	Cob(I) alamin adenosyltransferase		−0.635 ± 0.087
GSU0967	Putative membrane protein	Cell envelope	−1.843 ± 0.242
GSU1010	Slt family transglycosylase		−0.809 ± 0.186
GSU1493	Type IV pilus biogenesis protein PilC		−0.692 ± 0.128
GSU1013	Putative chemotaxis MotB protein	Cellular processes	−0.673 ± 0.191
GSU2707	Acetate kinase	Central intermediary metabolism	−1.150 ± 0.162
GSU0096	Recombination protein RecR	DNA metabolism	−0.896 ± 0.098
GSU0338	NADH dehydrogenase I; A subunit	Energy metabolism	−0.980 ± 0.156
GSU1538	Putative methylamine utilization protein MauG		−1.708 ± 0.244
GSU2612	Putative rubrerythrin/rubredoxin protein		−0.765 ± 0.098
GSU2706	Phosphate acetyltransferase		−1.189 ± 0.271
GSU2737	Cytochrome *c* (OmcB)		−0.940 ± 0.152
GSU2808	Cytochrome *c*		−0.730 ± 0.145
GSU2887	Cytochrome *c*		−1.515 ± 0.208
GSU3259	Cytochrome *c*		−1.069 ± 0.150
GSU3444	NADH dehydrogenase I B/C/D subunits		−0.669 ± 0.136
GSU3061	Squalene-hopene cyclase	Fatty acids and phospholipid metabolism	−1.180 ± 0.349
GSU0384	Hypothetical protein	Hypothetical proteins	−0.785 ± 0.190
GSU0712	Hypothetical protein		−0.867 ± 0.115
GSU0875	Hypothetical protein		−1.037 ± 0.259
GSU0964	Hypothetical protein		−1.460 ± 0.196
GSU0965	Hypothetical protein		−1.557 ± 0.543
GSU0966	Hypothetical protein		−1.731 ± 0.304
GSU0976	Hypothetical protein		−0.858 ± 0.254
GSU0977	Hypothetical protein		−0.912 ± 0.254
GSU0980	Hypothetical protein		−0.592 ± 0.176
GSU0981	Hypothetical protein		−0.847 ± 0.231
GSU0982	Hypothetical protein		−0.824 ± 0.217
GSU0988	Hypothetical protein		−0.652 ± 0.291
GSU0990	Hypothetical protein		−1.066 ± 0.296
GSU0996	Hypothetical protein		−0.597 ± 0.116
GSU1339	Hypothetical protein		−1.374 ± 0.412
GSU1943	Hypothetical protein		−1.212 ± 0.184
GSU1994	Hypothetical protein		−1.675 ± 0.211
GSU2353	Hypothetical protein		−1.264 ± 0.296
GSU2780	Hypothetical protein		−0.902 ± 0.223
GSU3079	Hypothetical protein		−0.709 ± 0.171
GSU3080	Hypothetical protein		−1.280 ± 0.150
GSU3081	Hypothetical protein		−1.051 ± 0.198
GSU3084	Hypothetical protein		−1.344 ± 0.144
GSU3085	Hypothetical protein		−1.227 ± 0.246
GSU0555	ISGsu7, transposase OrfA	Mobile and extrachromosomal element functions	−0.785 ± 0.188
GSU0556	ISGsu7, transposase OrfB		−1.069 ± 0.343
GSU0761	ISGsu7, transposase OrfB		−0.844 ± 0.289
GSU0762	ISGsu7, transposase OrfA		−0.742 ± 0.173
GSU0975	Phage tail sheath protein		−0.866 ± 0.283
GSU0986	Tail lysozyme		−0.767 ± 0.097
GSU1355	ISGsu7, transposase OrfA		−0.882 ± 0.222
GSU1356	ISGsu7, transposase OrfB		−1.029 ± 0.366
GSU1847	ISGsu7, transposase OrfA		−0.856 ± 0.212
GSU1848	ISGsu7, transposase OrfB		−1.057 ± 0.327
GSU2127	ISGsu7, transposase OrfA		−0.784 ± 0.204
GSU2128	ISGsu7, transposase OrfB		−0.969 ± 0.214
GSU2139	ISGsu7, transposase OrfA		−0.734 ± 0.198

Continued on following page

TABLE 3—*Continued*

Locus ID[a]	Common name	Role category	Log_2 ratio ± SD
GSU2140	ISGsu7,transposase OrfB		−1.009 ± 0.300
GSU2171	ISGsu7,transposase OrfB		−0.993 ± 0.263
GSU2279	ISGsu7,transposase OrfB		−0.827 ± 0.309
GSU2391	ISGsu7,transposase OrfB		−1.136 ± 0.397
GSU2392	ISGsu7,transposaseOrfA		−0.833 ± 0.253
GSU3082	ISGsu7,transposaseOrfA		−0.946 ± 0.237
GSU3083	ISGsu7, transposase OrfB		−1.249 ± 0.374
GSU0079	Cro/CI family transcriptional regulator	Regulatory functions	−0.542 ± 0.075
GSU1072	IclR family transcriptional regulator		−1.200 ± 0.414
GSU1626	GntR family transcriptional regulator		−0.626 ± 0.109
GSU2046	DNA-binding response regulator		−0.728 ± 0.091
GSU2779	MerR family transcriptional regulator		−0.966 ± 0.172
GSU1068	Sodium/solute symporter family protein	Transport and binding proteins	−1.771 ± 0.167
GSU1070	Sodium/solute symporter family protein		−1.670 ± 0.231
GSU1261	ABC transporter, ATP-binding protein		−0.534 ± 0.053
GSU1330	Metal ion efflux outer membrane protein family protein		−1.142 ± 0.400
GSU1331	RND family efflux transporter		−0.774 ± 0.144
GSU2005	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein		−1.938 ± 0.395
GSU2006	Branched-chain amino acid ABC transporter, permease protein		−1.776 ± 0.312
GSU2007	Branched-chain amino acid ABC transporter, permease protein		−1.540 ± 0.346
GSU2008	Branched-chain amino acid ABC transporter, ATP-binding protein		−1.259 ± 0.133
GSU2352	Sodium/solute symporter family protein		−1.508 ± 0.193
GSU2490	Oxalate/formate antiporter		−1.957 ± 0.510
GSU2886	TonB-dependent receptor		−1.501 ± 0.239
GSU0711	Endonuclease/exonuclease/phosphatase family protein	Unknown functions	−0.745 ± 0.136
GSU0930	Sulfur transferase		−0.758 ± 0.159
GSU1338	Heavy-metal-associated domain protein		−1.225 ± 0.341
GSU1398	SCO1/SenC family protein		−0.774 ± 0.144
GSU2010	CBS domain protein		−1.142 ± 0.215
GSU3059	Radical SAM domain protein		−0.623 ± 0.114
GSU3157	Alpha/beta fold family hydrolase		−0.725 ± 0.186
GSU3435	Ankyrin repeat protein		−0.665 ± 0.105

[a] ID, identification.

proportional to the level of OmcB production (10). Furthermore, there is a direct correlation between transcript levels of *omcB* and rates of Fe(III) reduction in continuous cultures (4). However, as shown here, after extended incubation the OmcB-deficient mutant is able to adapt and grow with soluble Fe(III) as the sole electron acceptor.

One of the more surprising aspects of this adaptation is that, during growth, adapted OmcB-deficient cultures reduced soluble Fe(III) at rates comparable to the wild type, but cell yields were substantially lower, indicating that there is less energy conservation from the pathway for Fe(III) reduction in the adapted mutant. It is unlikely that OmcB, an outer membrane protein, plays a direct role in coupling the flow of electron transport to the generation of a proton motive force, because it is expected that this is primarily established from proton pumping across the inner membrane. However, it is possible that the electron transport pathway from inner membrane electron transport components to OmcB is tightly coupled and that alternative electron transfer pathways to Fe(III) reduction, in the absence of OmcB, require electron transfer from alternative inner membrane electron transfer components that yield less proton pumping. Alternatively, the apparent need for increased biosynthesis of alternative *c*-type cytochromes, and possibly other proteins, may increase energy consumption and decrease growth yields.

The increased production of other outer membrane *c*-type cytochromes associated with adaptation in the OmcB-deficient mutant may account for the ability of the adapted mutant to reduce Fe(III) by providing an alternative route(s) for electron transfer to soluble Fe(III).

Five of the nine up-regulated *c*-type cytochrome genes with higher levels of transcripts in the OmcB-deficient mutant (GSU594, 2495, 2503 [OmcT], 2811, and 2813) were also found to have higher transcript levels when *G. sulfurreducens* was grown under electron-acceptor limiting conditions, rather than with the electron donor limiting growth (A. Esteve-Nunez, unpublished data). Thus, the physiological state associated with the disruption of the electron transport pathway in the OmcB-deficient mutant may mimic the physiological state under electron acceptor-limiting conditions, and this, as well as

TABLE 4. Genes coding for *c*-type cytochromes whose expression levels were altered significantly (ca. twofold) in the OmcB-deficient mutant versus the wild type via microarray assay[a]

Locus ID	Predicted location	Microarray $\log_2$ ratio	Predicted mol mass (kDa)	Northern blot result
GSU0594	IM	1.133	38.2	Up
GSU2494	IM	1.863	47.7	Up
GSU2495	P	1.810	69.7	Up
GSU2503	OM	3.340	45.5	Up
GSU2504	OM	3.676	45.4	Up
GSU2811	OM	0.745	52.7	Up
GSU2813	P	0.839	36.9	Up
GSU2731 (OmcC)	OM	NA	77.2	Up
GSU2737 (OmcB)	OM	−1.189	77.1	Down
GSU2808	OM	−0.730	20.4	Down
GSU2887	OM	−1.515	89.7	Down
GSU3259	IM	−1.069	57.4	Down

[a] Abbreviations: ID, identification; IM, inner membrane; P, periplasm; OM, outer membrane; NA, not applicable; mol, molecular.

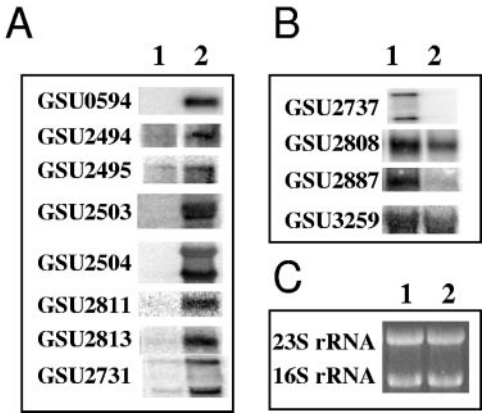

FIG. 5. Northern blot analyses of up- or down-regulated *c*-type cytochrome genes. Total RNA was isolated from mid-log Fe(III) citrate-grown cultures of wild type (lane 1) or OmcB-deficient mutant (lane 2). (A) *c*-type cytochrome genes that were identified to be up-regulated by a ≥2-fold change in the adapted OmcB-deficient mutant. (B) *c*-type cytochrome genes that were identified to be down-regulated in the adapted OmcB-deficient mutant. (C) A replicate gel was run and stained with ethidium bromide, revealing the 16S and 23S rRNA, as a loading control.

possible other physiological signals, may account for the increased production of outer membrane cytochromes.

In addition to affecting cytochrome production, the disruption of electron flow in the OmcB-deficient mutant appeared to have an impact on other aspects of metabolism. This was evident from the microarray studies which suggested that there was a decrease in transcription of several genes involved in acetate metabolism and an increase in transcript levels for genes such as a nickel-dependent hydrogenase (GSU0782 to -0787), a cytochrome *d* ubiquinol oxidase (GSU1640-1641), and a carbon monoxide dehydrogenase (GSU2098), which presumably played a role in maintaining the appropriate balance of reducing equivalents in the OmcB-deficient mutant (23, 27). Evidence that the mutant was under stress includes the apparent up-regulation of a *relA* homolog (GSU2236) which has been shown to be important in regulating levels of ppGpp and stress responses in *G. sulfurreducens* (L. DiDonato et al., submitted for publication), as well as higher transcript levels for other stress response proteins, such as thioredoxin peroxidase (GSU3246), glutaredoxin family protein (GSU2812), and proteins involved in cell envelope biosynthesis (GSU2078, 2497), etc.

Significance of failure to adapt for Fe(III) oxide reduction. The fact that the OmcB-deficient mutant was never able to adapt to grow on Fe(III) oxide emphasizes the central role that OmcB plays in the reduction of this most environmentally relevant form of Fe(III). Although it was initially considered that OmcB might function as the terminal Fe(III) reductase because of its location in the outer membrane (10), subsequent

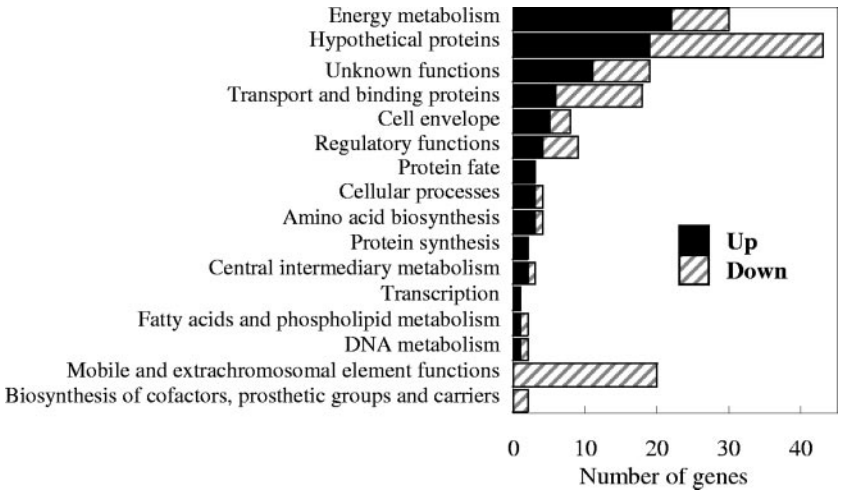

FIG. 4. Functional groups of up- or down-regulated genes from the adapted OmcB-deficient mutant. The up- or down-regulated genes of the OmcB-deficient mutant were identified by comparing expression patterns with that of the wild type as described in Materials and Methods. Functional classes are determined using the *Geobacter sulfurreducens* genome page from TIGR (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=ggs). Functional groups of up-regulated genes from OmcB-deficient mutant are represented by black bars, and functional groups of down-regulated genes are shown by gray-striped bars. The number of genes represented in each bar is indicated in the *x* axis.

studies have identified other proteins, such as the outer membrane *c*-type cytochrome OmcS (T. Mehta et al., submitted for publication) and pili (23a), that are more exposed on the outside of the cell and are specifically required for the reduction of Fe(III) oxide, but not soluble Fe(III). Thus, the critical role of OmcB in Fe(III) oxide reduction may be as an intermediary electron transfer component that establishes electron transfer to the terminal Fe(III) oxide reductase (13). The finding that the mutant deficient in OmcB can reduce soluble Fe(III), but not Fe(III) oxides, demonstrates that *G. sulfurreducens* can reduce soluble Fe(III) via mechanisms different than those required for Fe(III) oxide reduction. This is an important consideration when extrapolating results from pure cultures to Fe(III) reduction in sedimentary environments where Fe(III) oxides are the predominant Fe(III) form readily available for microbial reduction and concentrations of soluble Fe(III) are likely to be low (13, 21).

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