Funct Integr Genomics (2009) 9:15–25
DOI 10.1007/s10142-008-0094-7

REVIEW

Diversity of promoter elements in a *Geobacter sulfurreducens* mutant adapted to disruption in electron transfer

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Received: 27 June 2008 / Revised: 3 July 2008 / Accepted: 7 July 2008 / Published online: 2 August 2008

Abstract The delta-proteobacterium, *Geobacter sulfurreducens*, can obtain energy by coupling the oxidation of organic matter to the reduction of insoluble Fe(III) or the anode of a microbial fuel cell. Because Fe(III) oxide or the anode surface, in contrast to oxygen, nitrate, or sulfate, is not soluble nor can it be reduced readily, *Geobacter* species have developed mechanisms which allow electrons to be delivered across outer membrane to the cell surface. OmcB is an outer-membrane *c*-type cytochrome important for *G. sulfurreducens* Fe(III) respiration. In the absence of OmcB, cells lost the ability to reduce soluble or insoluble Fe(III). However, the *omcB* deletion mutant can slowly adapt to growth on soluble Fe(III) over prolonged incubation in the medium with acetate as the electron donor. We discuss available information about predicted or experimentally validated promoters and transcription regulatory sites identified upstream of operons with transcriptional expression significantly changed in the adapted *omcB* mutant. DNA sequences of upstream regions of coregulated operons in the adapted mutant are divergent, suggesting the presence of recognition sites for different transcriptional regulators and indicating that adaptation of the *omcB* mutant to growth on soluble Fe(III) has shifted the relevant expression networks involved to a more diverse molecular basis.

Keywords *Geobacter sulfurreducens* · Promoter · *c*-type cytochrome · Transcription

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Bacteria in the family *Geobacteraceae*, which belongs to the δ subdivision of Proteobacteria, participate in the bioremediation of toxic metals, radionuclides, and the coupling of the oxidation of organic contaminants to the reduction of Fe (III), Mn(VI), and a graphite electrode of a microbial fuel cell (Anderson and Lovley 1999; Rooney-Varga et al. 1999; Roling et al. 2001; Andrews et al. 2003; Ortiz-Bernad et al. 2004; Lovley 2006a, b; Sung et al. 2006). The ability of *Geobacteraceae* to utilize insoluble electron acceptors is unusual when compared to a number of other taxonomic groups that can utilize only soluble electron acceptors, such as fumarate, nitrate, sulfate, or oxygen, which can cross cell membranes easily (Lovley et al. 2004; Mehta et al. 2005; Shi et al. 2007). Due to these properties and to the ability of *Geobacteraceae* to bioremediate metal and organic contaminants and to generate bioenergy, molecular mechanisms and biological components involved in their electron transfer have been actively investigated (Holmes et al. 2002, 2004, 2006; Bond and Lovley 2003; Kim et al. 2005, 2006; Mehta et al. 2005, 2006; Shelobolina et al. 2007).

Geobacter sulfurreducens, a pure culture model representative from the *Geobacteraceae* family, has been extensively studied to determine its molecular, genetic, and physiological features; its genome sequence[1] and a genetic system for mutation analysis are available; multiple gene expression experiments and comparative proteomics analysis have been carried out, and an online database of its operon organization and regulatory sequence elements has been developed for this model organism (Caccavo et al. 1994; Coppi et al. 2001; Methé et al. 2003, 2005; Giometti 2006; Núñez et al. 2006; Krushkal et al. 2008).

The genome of *G. sulfurreducens* contains more than 100 genes encoding *c*-type cytochromes (Methé et al. 2003). *c*-type cytochromes, which contain heme groups acting as electron carriers, are important components of the electron transfer system in *G. sulfurreducens* and are necessary for optimal Fe(III) reduction or electricity production (Lovley et al. 2008). In earlier studies, the *ppcA* (GSU0612) and the *macA* (GSU0466) deletion mutants demonstrated defective Fe(III) reduction (Lloyd et al. 2003; Butler et al. 2004); the *omcS* (GSU2504) and the *omcE* (GSU0618) mutants entirely lost their ability to reduce insoluble Fe(III) or the electrodes but not to reduce soluble Fe(III) (Mehta et al. 2005; Holmes et al. 2006). Most recently, OmcZ (GSU2076), another outer-membrane cytochrome, was demonstrated to be essential for electricity production, but not for soluble or insoluble Fe(III) reduction (K. P. Nevin and B.-C. Kim, personal communication).

The *omcB* (GSU2737) mutant lost its ability to reduce both soluble and insoluble Fe(III), demonstrating its importance in Fe(III) reduction (Leang et al. 2003). The loss of ability for soluble or insoluble Fe(III) reduction by the *omcB* deletion mutant was contrasted with its continued ability to reduce uranium U(VI) (Shelobolina et al. 2007) or to produce electricity (Holmes et al. 2006), which may indicate the differences between molecular pathways of Fe (III) and U(VI) or electrode reduction by *G. sulfurreducens*.

Not only are some *c*-type cytochromes directly involved in Fe(III) reduction, but some also influence the expression of other *c*-type cytochromes (Kim et al. 2005, 2006). In the absence of OmcF (GSU2432), transcription of the *omcB* gene was low or not detectable, resulting in cells' inability to reduce Fe(III) (Kim et al. 2005). In contrast, in the absence of OmcG (GSU2882) and OmcH (GSU2883), the transcriptional level of the *omcB* gene was similar to that of the wild type, but the OmcB protein was not detected in the outer-membrane fraction of the mutant (Kim et al. 2006). These studies underscore the importance of the presence of OmcB for normal Fe(III) reduction levels and the complexity and possible redundancy among roles of different *c*-type cytochromes in *G. sulfurreducens*.

[1] GenBank accession number AE017180

Importance of OmcB in electron transfer

OmcB, a *c*-type cytochrome with 12 heme-binding domains, has been shown to be tightly associated with the outer membrane and is partially exposed to the outer surface of the cell (Leang et al. 2003; Qian et al. 2007). In the absence of OmcB, *G. sulfurreducens* was unable to reduce either soluble or insoluble Fe(III) (Fe(III) citrate and crystalline Fe(III) oxide, respectively; Leang et al. 2005). Cells were able to reduce Fe(III) when the *omcB* gene was expressed in trans in the *omcB* deletion mutant, suggesting that OmcB is involved in electron transfer to Fe(III) (Leang et al. 2003). Interestingly, during a prolonged incubation in medium with acetate as the electron donor and Fe(III) citrate as the electron acceptor, the *omcB* deletion mutant was able to adapt to growth on soluble Fe(III), although the cell yield was only a third of that of the wild type (Leang and Lovley 2005). Below, we refer to the *omcB* deletion mutant that had grown on soluble Fe(III) after a long period of incubation (over 7 days versus 2–3 days for wild type) as an adapted *omcB* deletion mutant.

Leang et al. (2005) performed genome-wide transcription profiling of the adapted *omcB* deletion mutant in order to investigate regulatory mechanisms of its adaptation. The changes in gene expression in the adapted *omcB* deletion mutant (*G. sulfurreducens* strain DL6, *omcB::cam*) were compared to those of the wild type (strain DL1; Leang et al. 2005)[2]. Both strains were grown in steady-state chemostats under strictly anaerobic conditions in a freshwater medium containing Fe(III) citrate as the electron acceptor and limited acetate as the electron donor. Gene expression microarray analysis revealed that genes having most significantly upregulated transcript levels in the adapted mutant strain encoded *c*-type cytochromes, including outer-membrane cytochromes OmcS (GSU2504) and OmcT (GSU2503; Leang et al. 2005). Other genes whose transcript levels were differentially expressed in the adapted *omcB* deletion mutant included those classified into functional categories of energy metabolism, amino acid biosynthesis, cell envelope, cellular processes, and transport and binding (Leang et al. 2005).

Transcriptional regulation in *G. sulfurreducens* is controlled by multiple sigma factors

While a number of individual components playing roles in electron transfer and metal reduction by *G. sulfurreducens*

[2] Descriptions of the microarray experiments, quantitation data, and array design are available from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/); accession number GSE8983

have been identified (Leang et al. 2003; Lloyd et al. 2003; Afkar et al. 2005; Kim et al. 2005, 2006; Reguera et al. 2005; Holmes et al. 2006; Shelobolina et al. 2007), detailed insights into the mechanisms of their regulation remain to be elucidated. Available information suggests that diverse regulatory networks are involved in electron transfer to Fe(III).

G. sulfurreducens has homologs of six RNA polymerase sigma factors. These include RpoD, RpoS, RpoH, FliA, a single FecI–RpoE homolog with similarity to both *Escherichia coli* FecI and RpoE sigma factors, and RpoN (Methé et al. 2003; Yan et al. 2006, 2007; Ueki and Lovley 2007). Experimental and in silico approaches have identified *G. sulfurreducens* promoters for RpoD, RpoS, RpoH, and RpoN, including RpoD-, RpoS-, and RpoN-regulated promoters in the upstream regions of differentially expressed operons in the adapted *G. sulfurreducens omcB* deletion mutant (Leang et al. 2005; Yan et al. 2006, 2007; Ueki and Lovley 2007).

RpoD (σ^{70}) is a major sigma factor, which is involved in the expression of housekeeping genes, including those encoding components of ribosomal subunits, polymerases, and other proteins (Hengge-Aronis 2002a, b; Gruber and Gross 2003). RpoS (σ^{38}), belonging to the σ^{70} family, is a global regulator of *G. sulfurreducens* gene expression, which is involved in stationary-phase survival, oxygen stress response, and the effective reduction of Fe(III) oxides (Núñez et al. 2004, 2006; Yan et al. 2006). Its role in the stringent response of *G. sulfurreducens* has also been strongly suggested (Krushkal et al. 2007). In *G. sulfurreducens*, both RpoS and RpoD were found to recognize similar −35/−10 elements promoter sequences, which are significantly similar to RpoD-regulated promoters of *E. coli* (Yan et al. 2006).

An alternative sigma factor, RpoN (σ^{54}), is essential for *G. sulfurreducens*, and an *rpoN* deletion mutant could not be isolated (C. Leang et al., manuscript in preparation). Overexpression of RpoN affects a significant number of genes whose products are involved in a variety of cellular functions, including C_4-dicarboxylate transport, central metabolism, and cytochromes essential for Fe(III) reduction (C. Leang et al., manuscript in preparation). Experimental evidence (C. Leang et al., manuscript in preparation) suggests that, similar to other bacteria, RpoN in *G. sulfurreducens* regulates promoters containing conserved −24 and −12 sequence elements (Wang and Gralla 1998).

Genome-wide studies of *G. sulfurreducens* have identified multiple putative RpoS-, RpoD-, and RpoN-regulated promoters (Yan et al. 2006; C. Leang et al., manuscript in preparation). Among them were multiple promoter elements identified in the upstream regions of operons with significantly altered transcript levels in the adapted *omcB* deletion mutant. The regulation of operons under the control of predicted RpoD- or RpoN-dependent promoters is most likely to involve additional transcriptional regulators. For example, the operon containing the *omcB* gene itself is regulated by multiple RpoS-dependent promoters located within open reading frames, which is an unusual feature for bacterial promoters (Leang and Lovley 2005).

Attempts to search for conserved overrepresented motifs in the upstream regions of either up- or down-coregulated operons in the *omcB* deletion mutant failed to identify conserved transcriptional regulatory sites similar to those for well-studied *E. coli* transcriptional factors (J. Krushkal et al., unpublished data). This sequence divergence of upstream regulatory regions suggests that the regulatory mechanisms for the Fe(III) reduction pathway and the adaptation to growth on soluble Fe(III) are likely to involve many regulatory factors, including possible participation of different hierarchical cascades of transcriptional regulation.

Available evidence from *E. coli* suggests that many of its outer-membrane proteins are regulated via a variety of mechanisms that involve sensor kinase-dependent and response regulator-dependent regulation, small RNA (sRNA) molecules, an alternative sigma factor RpoE (σ^{24}), and an anti-sigma E factor RseA (Guillier et al. 2006), suggesting that the control of adaption in the *omcB* deletion mutant may also involve a diversity of regulatory mechanisms. It is plausible that regulation of outer-membrane components in *G. sulfurreducens* may differ substantially from that of *E. coli*. For example, the RseA anti-sigma factor is absent from the genome of *G. sulfurreducens* (J. Krushkal, unpublished data).

Expression of several genes with regulatory roles was altered in the adapted *omcB* mutant (Leang et al. 2005) suggesting their involvement in the regulation of gene expression in response to the loss of OmcB. Genes GSU0537 (encoding sensory box–GGDEF family protein), GSU2506 (encoding RpoN-dependent DNA-binding response regulator), GSU2507 (encoding sensor histidine kinase), and GSU3387 (encoding a transcriptional regulator from the AraC–XylS family) were significantly upregulated, while GSU0079 (encoding a transcriptional regulator from the Cro–CI family), GSU1072 (encoding a transcriptional regulator from the IclR family), GSU1626 (encoding a transcriptional regulator from the GntR family), GSU2046 (encoding a DNA-binding response regulator), and GSU2779 (encoding a transcriptional regulator from the MerR family) were significantly downregulated.

RpoN-dependent promoter elements upstream of operons with significant expression changes in the adapted *omcB* deletion mutant

RpoN-regulated promoters were predicted in the genome of *G. sulfurreducens* (C. Leang et al., manuscript in prepara-

tion) using the PromScan software (Studholme et al. 2000), based on their similarity to 186 RpoN promoter elements from 47 bacterial species (Barrios et al. 1999). Five RpoN-dependent promoters were identified in noncoding upstream of target genes that belong to downregulated operons in the adapted *omcB* deletion mutant, and six promoters were located in the noncoding regions upstream of genes from upregulated operons (Table 1). Most RpoN-dependent downregulated target operons are involved in transport and binding, which may suggest a common mechanism for the adapted omcB deletion mutant in coping with proton–anion imbalance caused by the loss of OmcB. Upregulated genes in the adapted *omcB* deletion mutant, which were predicted to be regulated by RpoN-dependent promoters, include genes involved in signal transduction, which may suggest the activation of a different pathway for dissimilatory Fe(III) reduction (Table 1). RpoN-regulated promoters were also identified upstream of upregulated genes involved in energy metabolism, cell envelope, and those encoding hypothetical proteins with yet unknown functions. At least two target genes, GSU0596 and GSU2526, have been annotated as coding for RpoN-dependent response regulators (Table 1), in agreement with predicted regulation of their promoters by RpoN.

−35/−10 RpoS-dependent promoter elements upstream of operons with significant expression changes in the adapted *omcB* mutant

Yan et al. (2006) predicted RpoS- and RpoD-regulated promoters in the genome of *G. sulfurreducens* using sequence similarity searches and microarray transcriptional profiling of the *rpoS* deletion mutant. Most likely, RpoS-regulated promoters were identified in the upstream regions of significantly downregulated operons of the *rpoS* deletion mutant. Promoter prediction included a search for conserved single-block sequence motifs by the AlignACE software (Roth et al. 1998) as well as a search which aimed to identify two conserved sequence blocks, separated by a spacer of 15–21 bp long (Yan et al. 2006), using BioProspector and BioOptimizer software (Liu et al. 2001; Jensen and Liu 2004). Additional −35/−10 promoter elements, including likely RpoS-, RpoD-, and RpoH-regulated promoters, were found using sequence similarity searches to promoter matrices of *G. sulfurreducens* and *E. coli*, using the ScanACE software (Robison et al. 1998).

A number of putative RpoS-dependent promoter elements were identified in the upstream regions of operons with significant changes in expression levels in the adapted *omcB* deletion mutant as compared to the wild type (Tables 2 and 3; Yan et al. 2006). Because sequences of RpoS- and RpoD-regulated promoters were shown to be very similar in *G. sulfurreducens* (Yan et al. 2006), prediction about whether or not a particular σ^{70} family promoter may be RpoS-dependent was made based on whether it was part of a conserved group of promoter elements identified upstream of significantly downregulated target operons in the *rpoS* deletion mutant (Yan et al. 2006). Promoters were suggested to be RpoS-regulated based on the following criteria: (1) they were identified as RpoS-regulated promoter elements using a search for conserved overrepresented motifs in the analysis of RpoS transcriptome (Núñez et al. 2006; Yan et al. 2006) and (2) their downstream target operons displayed significant downregulation in the *rpoS* deletion mutant (Núñez et al. 2004). A number of the operons in the adapted *omcB* mutant that were significantly downregulated in the *rpoS* deletion mutant fall in the functional categories of "energy metabolism" and "transport and binding" (Leang et al. 2005; Núñez et al. 2006). One of the significantly downregulated genes in the adapted *omcB* deletion mutant, GSU1538 (annotated as MauG, a putative methylamine utilization cytochrome *c* peroxidase), was experimentally demonstrated to be under the control of RpoS, with its promoter elements experimentally validated (Table 3; Yan et al. 2006). Presence of multiple RpoS-dependent promoters upstream of significantly upregulated or downregulated genes in the adapted *omcB* deletion mutant (Tables 2 and 3) may suggest that the RpoS response mechanism may have been activated in order to compensate for the loss of OmcB and/or the physiological impact resulting from the loss of OmcB.

Putative −35/−10 RpoD-like promoter elements

The absence of common sequence motifs similar to known transcription factor binding site matrices in the upstream regions of significantly upregulated and downregulated operons in the adapted *omcB* deletion mutant indicates that diverse regulatory mechanisms are likely to be involved in their transcription. For example, GSU0982 (encoding phage protein D), which belongs to the operon containing genes GSU0979–GSU0983, was downregulated in both *omcB* and *rpoS* deletion mutants, but no RpoS-dependent −35/−10 promoters were identified in the upstream region of this operon (Table 3). This suggests that the involvement of RpoS in the regulation of this particular operon may be indirect. In addition, several operons, identified as downregulated in the *rpoS* deletion mutant, were predicted to have RpoD-regulated promoters using similarity searches to −35/−10 promoter elements of *G. sulfurreducens* and *E. coli*, suggesting their indirect regulation by RpoS (Yan et al. 2006; Tables 2 and 3). For example, an operon containing GSU2005–GSU2010 (Table 3) encodes branched-chain amino acid ABC transporters. Experimental evidence

Table 1 RpoN promoters identified in noncoding regions upstream of significantly upregulated or downregulated genes in the adapted *omcB* deletion mutant

Promoter sequence	Genome positions	Strand	Target gene cluster		
			Gene ID	Gene function	Gene subrole
Upregulated operons					
CTGGCATACGGGGTGCA	628383–628399	–	GSU0597	Hypothetical protein	Unknown
			GSU0596	σ^{54}-dependent response regulator	Signal transduction
			GSU0595.1[b]	Conserved hypothetical protein	Unknown
			GSU0594[a]	Cytochrome c family protein	Energy metabolism
			GSU0593[a]	Hypothetical protein	Unknown
ATGGTACGGCTACTGCA	985792–985808	–	GSU0920.1[b]	Conserved hypothetical protein	Unknown
			GSU0919[a]	Hypothetical protein	Unknown
ATGGCAAATATTTAGCT	2135268–2135284	–	GSU1948[a]	Hypothetical protein	Unknown
			GSU1947[a]	Hypothetical protein	Unknown
CTGGCATGGATATTGTT	2765711–2765727	–	GSU2505.1[b]	Hypothetical protein	Unknown
			GSU2506[a]	Response regulator (Rec-σ^{54} interaction-HTH8)	Signal transduction
TTGGCATGTAAATTGTA	2784628–2784644	–	GSU2526[a]	Membrane protein, putative	Cell envelope
			GSU2525	Nitroreductase family protein	Unknown function
GTGGCACGGTCACTGCT	3751277–3751293	+	GSU3409[a]	Hypothetical protein	Unknown
			GSU3410[a]	Hypothetical protein	Unknown
Downregulated operons					
TTGGTATACAGGTTGCT	1039939–1039955	+	GSU0964[a]	Hypothetical protein	Unknown
			GSU0966[a]	Hypothetical protein	Unknown
			GSU0967	Membrane protein, putative	Cell envelope
ATGGCACGGCCTGTGTA	1455058–1455074	+	GSU1331[a]	Efflux transporter, RND family, MFP subunit	Transport and binding
			GSU1332	Heavy metal efflux pump, CzcA family	Transport and binding
			GSU1333	Hypothetical protein	Unknown
CTGGCACGCCAATTGGA	1465280–1465296	+	GSU1338[a]	Heavy metal transport–detoxification domain protein	Unknown function
ATGGCACTGTAGTTGCT	2198355–2198371	+	GSU2006[a]	Branched-chain amino acid ABC transporter, permease protein	Transport and binding
			GSU2007[a]	Branched-chain amino acid ABC transporter, permease protein	Transport and binding
			GSU2008[a]	Branched-chain amino acid ABC transporter, ATP-binding protein	Transport and binding
			GSU2009	Branched-chain amino acid ABC transporter, ATP-binding protein	Transport and binding
			GSU2010[a]	CBS domain protein	Unknown function
ATGGTATGCAAGTTGCT	2735628–2735644	+	GSU2490[a]	Oxalate–formate antiporter, putative	Transport and binding

Listed are predicted RpoN promoter elements (C. Leang et al., manuscript in preparation) and their target gene clusters. Target gene clusters were defined as all downstream genes predicted to belong to an operon (Krushkal et al. 2007) adjacent to an RpoN promoter and to have the same strand orientation with that promoter. Genes are listed in the order of their transcription.

[a] Significantly downregulated genes in the *omcB* deletion mutant (Leang et al. 2005)

[b] Genes that were added to the curated genome reannotation of the *G. sulfurreducens* after the initial sequencing effort of the *G. sulfurreducens* genome (Methé et al. 2003), and the expression change of such genes in the *omcB* deletion mutant was not measured (Leang and Lovley 2005).

Table 2 −35/−10 promoter elements predicted in the upstream regions of likely RpoS-regulated operons containing upregulated genes in the adapted *omcB* deletion mutant

Prediction software	Gene ID	Function	Gene subrole
ScanACE	GSU1159	Intracellular protease, PfpI family	Protein fate
	GSU1160[a]	Hypothetical protein	Unknown
	GSU1161	Efflux transporter, RND family, MFP subunit	Transport and binding
	GSU1162	ABC transporter, ATP-binding protein	Transport and binding
	GSU1163	ABC transporter, permease protein	Transport and binding
	GSU1164	ABC transporter, permease protein	Transport and binding
ScanACE	GSU1640	Cytochrome d ubiquinol oxidase, subunit I	Energy metabolism
	GSU1641[a]	Cytochrome d ubiquinol oxidase, subunit II	Energy metabolism
BioProspector/BioOptimizer, ScanACE	GSU1945[a]	Fibronectin type III domain protein	Unknown function
BioProspector/BioOptimizer, ScanACE	GSU2811[a]	Cytochrome c Hsc, 2 heme-binding sites	Energy metabolism
	GSU2812	Glutaredoxin family protein	Energy metabolism
	GSU2813[a]	Cytochrome c551 peroxidase	Energy metabolism
	GSU2814	Rubrerythrin	Energy metabolism

Sequences of predicted RpoS- and RpoD-regulated promoters were reported by Yan et al. (2006) and are available from the GSEL database (Krushkal et al. 2008; http://www.geobacter.org/research/gsel/). Promoter sequences for each operon are also available upon request from J. Krushkal. Each gene cluster is represented by the name of the software which predicted the promoter sequences, followed by the complete list of genes in the target operon. Genes are listed in the order of their transcription. Most likely RpoS-regulated promoters (underlined) were identified using the search for conserved overrepresented sequence motifs in the upstream regions of significantly downregulated operons in the *rpoS* deletion mutant using AlignACE and BioProspector, followed by optimization by the BioOptimizer software. Additionally, −35/−10 promoters (which may be either RpoS- or RpoD-regulated) were found using similarity searches to RpoS and RpoD promoter matrices of *G. sulfurreducens* and *E. coli* using the ScanACE software (Yan et al. 2006). A number of predicted promoters were identified using multiple methods.
[a] Significantly downregulated genes in both the *omcB* and the *rpoS* deletion mutants (Leang et al. 2005; Núñez et al. 2006)

suggests that it is regulated by a sigma factor from the σ^{70} family (C. Leang et al, manuscript in preparation), while no RpoS-regulated promoters have been identified in its upstream region (Yan et al. 2006). While operons containing genes GSU2005–GSU2010 and GSU0979–GSU0983 were downregulated in the *rpoS* mutant, none of them belong to the most significantly downregulated expression cluster suggested to be under direct RpoS control (Yan et al. 2006). These and some additional operons are likely to be indirectly regulated by RpoS with the help of additional components of the regulatory cascade.

Many additional −35/−10 promoters are not RpoS-regulated (Tables S3 and S4). Transcription of many of them is likely to be directed by RpoD, the housekeeping sigma factor (Gruber and Gross 2003) or by other σ^{70} family members, with involvement of additional regulatory mechanisms. For example, conserved −35/−10 promoters were found upstream of an operon containing GSU3404–GSU3406 (Yan et al. 2006), which was significantly upregulated in the adapted *omcB* deletion mutant. This operon encodes amino acid ABC transporter proteins. Some of the suggested RpoD-dependent promoters upstream of significantly downregulated genes have been verified experimentally using primer extension analysis, particularly, those upstream of GSU1538 (encoding a putative methylamine utilization protein MauG; Yan et al. 2007) and GSU2046 encoding a DNA-binding response regulator (T. Ueki and D. R. Lovley, personal communication).

An extensive overlap between RpoD and RpoH sigma factors in binding to −35/−10 promoters has been demonstrated in *E. coli* (Wade et al. 2006), and *G. sulfurreducens* has a functional RpoH ortholog (Ueki and Lovley 2007). However, none of the differentially expressed genes in the *omcB* deletion mutant (Leang and Lovley 2005) were located downstream of known RpoH-regulated promoters or CIRCE binding sites for the HrcA repressor protein (Rodionov et al. 2004; Ueki and Lovley 2007), suggesting that the adaptation of the *omcB* deletion mutant is unlikely to involve the heat shock regulon. To date, no binding sites have been identified for the *G. sulfurreducens* sigma factor with sequence similarity to both FecI and RpoE (Yan et al. 2007), and therefore it remains unknown whether any of the predicted −35/−10 promoters could be regulated by that sigma factor.

Relevance of reported promoters to gene regulation in the adapted *omcB* deletion mutant

The adapted *omcB* deletion mutant, grown on soluble Fe (III), had a similar Fe(III) reduction rate, but only one third of growth yields compared to the wild type (Leang and Lovley 2005). Gene expression microarray profiling of this

Table 3 −35/−10 promoter elements identified in the upstream regions of likely RpoS-controlled operons containing downregulated genes in the *omcB* deletion mutant

Prediction software	Gene ID	Function	Gene subrole
AlignACE, ScanACE	GSU0079[b]	Transcriptional regulator, Cro–CI family	Regulatory functions
AlignACE, BioProspector/BioOptimizer, ScanACE	GSU0716	Hypothetical protein	N/A
	GSU0715	Hypothetical protein	N/A
	GSU0714	Hypothetical protein	N/A
	GSU0713	Hypothetical protein	N/A
	GSU0712[b]	Hypothetical protein	N/A
	GSU0711[b]	Endonuclease–exonuclease–phosphatase family protein	Unknown function
	GSU0710	Hypothetical protein	N/A
	GSU0709	Predicted P-loop ATPase	N/A
No -35/-10 promoters identified	GSU0979	Hypothetical protein	Hypothetical proteins
	GSU0980	Hypothetical protein	N/A
	GSU0981	Uncharacterized protein with LysM domain	Hypothetical proteins
	GSU0982[b]	Similar to COG3500: phage protein D	Hypothetical proteins
	GSU0983	Conserved hypothetical protein	Hypothetical proteins
ScanACE	GSU0990[b]	Hypothetical protein	N/A
	GSU0991	Glycosyl transferase, group 1 family protein	Cell envelope
	GSU0992	Hypothetical protein	N/A
ScanACE	GSU0996[b]	Hypothetical protein	N/A
ScanACE	GSU1012	Membrane protein, putative	Cell envelope
	GSU1013[b]	Chemotaxis MotB protein, putative	Cellular processes
	GSU1014	Smr domain protein	Unknown function
	GSU1015	Hypothetical protein	N/A
	GSU1016	Potassium uptake protein, Trk family	Transport and binding
ScanACE	GSU1069	Hypothetical protein	Hypothetical proteins
	GSU1068[b]	Sodium–solute symporter family protein	Transport and binding
ScanACE	GSU1071[b]	Hypothetical protein	Hypothetical proteins
	GSU1070[b]	Sodium–solute symporter family protein	Transport and binding
AlignACE, BioProspector/BioOptimizer, ScanACE	GSU1399.1[c]	FOG: GGDEF domain	Unknown function
	GSU1399	Magnesium and cobalt transport protein CorA	Transport and binding
	GSU1398[b]	SCO1–SenC family protein	Unknown function
	GSU1397	Cytochrome c family protein, putative	Energy metabolism
	GSU1396	Hypothetical protein	N/A
AlignACE, BioProspector/BioOptimizer, ScanACE (*Experimentally validated promoters*)[a]	GSU1538[b]	Methylamine utilization protein MauG, putative	Energy metabolism
BioProspector/BioOptimizer, ScanACE	GSU1994[b]	Hypothetical protein	N/A
ScanACE	GSU2005[b]	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein, putative	Transport and binding
	GSU2006[b]	Branched-chain amino acid ABC transporter, permease protein	Transport and binding
	GSU2007[b]	Branched-chain amino acid ABC transporter, permease protein	Transport and binding
	GSU2008[b]	Branched-chain amino acid ABC transporter, ATP-binding protein	Transport and binding
	GSU2009	Branched-chain amino acid ABC transporter, ATP-binding protein	Transport and binding
	GSU2010[b]	CBS domain protein	Unknown function
ScanACE	GSU2353[b]	Hypothetical protein	Hypothetical proteins
	GSU2352[b]	Sodium–solute symporter family protein	Transport and binding

Explanation of abbreviations and promoter prediction approaches are provided in the legend to Table 2

[a] RpoS-regulated promoters upstream of GSU1538 were experimentally validated using primer extension analysis (Yan et al. 2006).

[b] Significantly downregulated genes in both the *omcB* and the *rpoS* deletion mutants (Leang et al. 2005; Núñez et al. 2006)

[c] Genes that was added to the curated genome reannotation of the *G. sulfurreducens* after the initial sequencing effort of the *G. sulfurreducens* genome (Methé et al. 2003), and the expression change of this gene in the *omcB* deletion mutant was not measured (Leang and Lovley 2005).

Table 4 Promoters and additional transcriptional regulatory sites in the upstream regions of operons containing *c*-type cytochrome genes that demonstrated significant expression changes in the adapted OmcB deletion mutant

GSU	Gene symbol and annotation	Predicted promoters	References for biological role	Additional sites
Upregulated genes				
GSU0594	Cytochrome *c*, 7 heme-binding sites	RpoN, RpoD		ModE
GSU1761	*omcR*; cytochrome *c*, 3 heme-binding sites	RpoD		
GSU2495[a]	Cytochrome *c*, 26 heme-binding sites	RpoD		Attenuator
GSU2501[a]	Cytochrome *c*, 6 heme-binding sites	RpoD		Attenuator
GSU2503[b]	*omcT*; cytochrome *c*, 6 heme-binding sites	RpoD	(Mehta et al. 2005; Shi et al. 2007)	Fur
GSU2504[b]	*omcS*; cytochrome *c*, 6 heme-binding sites	RpoD	(Mehta et al. 2005; Shi et al. 2007)	Fur
GSU2731[c]	*omcC;* cytochrome *c,* 12 heme-binding sites	RpoS	(Leang et al. 2003; Leang and Lovley 2005)	Fur
GSU2811[d]	Cytochrome *c* Hsc, 2 heme-binding sites	RpoS		Attenuator
GSU2813[4]	cytochrome *c* peroxidase, 2 heme-binding sites, ccpA-2 RpoS	(Butler et al. 2004)		Attenuator
Downregulated genes				
GSU2494	Cytochrome *c*, 10 heme-binding sites	RpoD		
GSU2808	Cytochrome *c*, 6 heme-binding sites	RpoD	(Methé et al. 2005)	
GSU2887	Cytochrome *c*, 27 heme-binding sites	RpoD	(Kim et al. 2005)	Attenuator
GSU3259	Cytochrome *c*, 7 heme-binding sites	RpoD		

The list of upregulated and downregulated *c*-type cytochrome genes is from Leang et al. (2005). Not listed is GSU2737 (the *omcB* gene deleted in the mutant strain), which has been shown to be controlled by multiple RpoS-regulated promoter elements (Leang and Lovley 2005).
Attenuator An uncharacterized transcriptional attenuator element predicted by Merino and Yanofsky (2005).
[a] Genes GSU2495 and GSU2501 belong to the same operon, according to the predicted operon organization of the *G. sulfurreducens* genome (Krushkal et al. 2007).
[b] Genes GSU2503 (*omcT*) and GSU2504 (*omcS*) belong to the same operon (Krushkal et al. 2007).
[c] Gene GSU2731 (*omcC*) was demonstrated to be upregulated using real-time quantitative RT-PCR and Northern blot analysis, but no evidence for its upregulation could be obtained from gene expression microarray analysis, most likely due to a high degree of DNA sequence similarity shared by the *omcC* and the *omcB* genes (Leang et al. 2005).
[d] Genes GSU2811 and GSU2813 belong to the same operon.

mutant demonstrated that transcription of genes involved in acetate metabolism were decreased, while increased expression levels were observed for genes encoding a nickel-dependent hydrogenase, cytochrome *d* ubiquinol oxidase, and carbon monoxide dehydrogenase and for outer-membrane *c*-type cytochromes other than OmcB, presumably for maintaining the balance of reducing equivalents (Leang and Lovley 2005).

Identification of RpoN- and RpoS-regulated promoter elements upstream of operons with significantly altered expression levels in the adapted *omcB* deletion mutant suggests that, in *G. sulfurreducens*, RpoN and RpoS regulate a group of genes related to central metabolism and Fe(III) reduction. In agreement with this observation, in two separate microarray studies investigating the RpoS and RpoN regulons of *G. sulfurreducens*, expression of genes whose products were involved in central metabolism and Fe(III) reduction was significantly altered in the *rpoS* deletion mutant and/or the RpoN overexpressing strain (Núñez et al. 2006; C. Leang et al., manuscript in preparation).

Regulation of *c*-type cytochromes

A number of RpoN-, RpoS-, and RpoD-dependent promoter elements were identified upstream of operons containing the most upregulated cytochrome genes and some downregulated cytochrome genes (Table 4). Expression of several of these genes was significantly altered in the *rpoS* deletion mutant and/or in the rel_{Gsu} deletion mutant (DiDonato et al. 2006; Núñez et al. 2006; Krushkal et al. 2007). The latter mutant lacks Rel_{Gsu}, a RelA/SpoT homolog which controls levels of 3′, 5′ bispyrophosphate, ppGpp, a participant in stringent response (DiDonato et al. 2006). Changes in expression of the *c*-type cytochromes listed in Table 4 for the adapted *omcB* deletion mutant may be compensating for the loss of OmcB.

Genes encoding OmcS and OmcT, both of which contain six heme-binding motifs, were significantly upregulated in the adapted *omcB* deletion mutant, exhibiting the highest differential expression levels (Leang et al. 2005; Mehta et al. 2005). These genes were predicted and experimentally validated to belong to the same operon (Mehta et al. 2005;

Krushkal et al. 2007). Mehta et al. (2005) found OmcS to be essential for Fe(III) oxide reduction and to be involved in electricity production. Interestingly, neither the *omcS* nor the *omcT* genes were expressed during *G. sulfurreducens* growth on soluble Fe(III), and deletion of both the *omcS* and the *omcT* genes did not affect the ability of *G. sulfurreducens* to reduce soluble Fe(III) (Mehta et al. 2005). This suggests that the marked increase in their expression in the adapted *omcB* deletion mutant was directly affected by the absence of OmcB. The expression of the *omcS* gene was significantly increased in the rel_{Gsu} deletion mutant grown in steady-state chemostat cultures (Krushkal et al. 2007). Analysis of this operon with other coregulated operons in this mutant suggested the presence of a binding site for ferric uptake repressor, Fur, in its upstream region (Table 4), as their common mode of coregulation (Krushkal et al. 2007); the presence of this Fur site was further confirmed by transcriptional profiling of the *fur* deletion mutant (R. O'Neil et al., manuscript in preparation). In many bacterial species, Fur controls iron homeostasis (Griggs and Konisky 1989; Harvie et al. 2005). A number of −35/−10 promoters were identified in the upstream region of the *omcS–omcT* operon (Yan et al. 2006). They do not appear to be RpoS-regulated based on transcriptional profiling of the *rpoS* deletion mutant (Núñez et al. 2006) and, therefore, their transcription is likely to be directed by RpoD or by other sigma factors from the σ^{70} family.

In addition to the *omcS–omcT* operon, Fur binding sites were identified upstream of the *omcC* gene (Table 4). The latter gene has been suggested to be unimportant for Fe(III) reduction and is regulated through RpoS-dependent promoters located within upstream open reading frames (Leang et al. 2003; Leang and Lovley 2005; Yan et al. 2006). Fur binding sites were also found upstream of a number of both upregulated and downregulated genes that did not encode *c*-type cytochromes. Many of these genes showed differential patterns of expression in the rel_{Gsu} deletion mutant and across multiple perturbation conditions, suggesting their Fur-dependent regulation (DiDonato et al. 2006; Krushkal et al. 2007; Mahadevan et al. 2008). However, because Fur regulatory sites were suggested to be present in the upstream regions of both upregulated and downregulated operons in the adapted *omcB* deletion mutant, their differential patterns of expression are likely to be affected by additional mechanisms. Similarly, GSU0594, which encodes a *c*-type cytochrome (Table 4), and several other significantly upregulated and downregulated genes were found to have binding sites for ModE, a regulator of molybdate homeostasis, in their upstream regions (Yan et al. 2007), but their differential patterns of expression suggest that ModE may not be a common mechanism for their expression changes.

The regulation of the adapted *omcB* deletion mutant likely extends beyond direct binding of transcription factors to DNA in the upstream regions of regulated operons. Merino and Yanofsky (2005) predicted *G. sulfurreducens* sequence elements regulated by transcriptional attenuation, which involves changes in secondary mRNA structure between terminator and antiterminator structures in response to the presence of metabolites (Jackson and Yanofsky 1973; Yanofsky 1981, 1988, 2000; Vitreschak et al. 2004). Merino and Yanofsky (2005) reported gene clusters that are likely regulated by transcriptional attenuation and have homologs in the Clusters of Orthologous Groups database (http://www.ncbi.nlm.nih.gov/COG/; Tatusov et al. 1997, 2001). Their predictions (Merino and Yanofsky 2005)[3] included transcription attenuator sequences upstream of multiple genes encoded for *c*-type cytochromes of *G. sulfurreducens*, including uncharacterized transcriptional attenuator elements located upstream of operons containing several *c*-type cytochrome genes, GSU2495, GSU2501, GSU2811, GSU2813, and GSU2887 (Table 4). The presence of these attenuator sequences further suggests the diversity of regulatory mechanisms involved in the adaptation of the *omcB* deletion mutant to growth on soluble Fe(III).

Multiple promoters

Sequence similarity searches of individual upstream regions intimated the presence of multiple RpoD-dependent promoter elements (Yan et al. 2006). While some of them may represent different biologically important promoter regions that may be activated under different conditions, other sequence elements, particularly those with scores exceeding the lower score threshold, may be false-positive predictions which are known to be more frequent in the noncoding regions (Huerta et al. 2006; Yan et al. 2007). However, a number of reported multiple promoters in the adapted *omcB* deletion mutant have been confirmed experimentally, e.g., promoters upstream of GSU1538 (encoding a putative methylamine utilization protein MauG; Yan et al. 2007). Therefore, it is possible that certain genes with altered expression levels in the *omcB* deletion mutant adapted to growth on soluble Fe(III) may be controlled by specific promoters appropriate for this adaptation.

Acknowledgements We are grateful to E. Anderson (University of Virginia) and J. Peeples (University of Tennessee, Memphis) for editorial assistance. We are also grateful to R. O'Neil and M. Coppi (University of Massachusetts, Amherst) for providing gene expression data on Fur-dependent regulation of *G. sulfurreducens*, to T. Ueki for sharing his results of primer extension analysis of RpoD-dependent promoters, and to K. Nevin and B.-C. Kim for information about the

[3] http://cmgm.stanford.edu/~merino/

OmcZ cytochrome. We also thank B. Methé (JCVI) for microarray data submission to the GEO database and B. Palsson (UCSD) for helpful discussions about sigma-factor-dependent regulation.

This research was supported by the Office of Science (BER), US Department of Energy, Cooperative Agreement No. DE-FC02-02ER63446.

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