

## NOTES

### Microarray Transcription Profiling of a *Shewanella oneidensis* *etrA* Mutant

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**DNA microarrays were used to examine the effect of an insertional mutation in the *Shewanella oneidensis* *etrA* (electron transport regulator) locus on gene expression under anaerobic conditions. The mRNA levels of 69 genes with documented functions in energy and carbon metabolism, regulation, transport, and other cellular processes displayed significant alterations in transcript abundance in an *etrA*-mutant genetic background. This is the first microarray study indicating a possible involvement of EtrA in the regulation of gene expression in *S. oneidensis* MR-1.**

In recent years, it has become apparent that microorganisms have developed complex regulatory mechanisms for controlling gene expression in response to alterations in growth conditions (3, 23, 26). For example, *Escherichia coli* Fnr (fumarate-nitrate reduction regulator) mediates global changes in gene expression during transitions between aerobic and anaerobic growth (12). Fnr homologs have been described for a number of bacterial species (7, 16, 22, 29), including *Shewanella oneidensis* MR-1 (20) (formerly *Shewanella putrefaciens* MR-1 [28]), a facultatively anaerobic metal-reducing bacterium (19). The predicted *S. oneidensis* EtrA (electron transport regulator) protein (20) shares a high degree of amino acid sequence identity with *E. coli* Fnr and with the analogous Anr (anaerobic regulator of arginine deiminase and nitrate reductase) protein from *Pseudomonas aeruginosa* (50.8 and 73.6% identity, respectively), thus suggesting the possibility that *etrA* is involved in regulating anaerobic energy metabolism in MR-1 (20). Subsequent experiments, however, demonstrated that insertional inactivation of the *etrA* gene had no significant physiological effect on the respiratory growth of *S. oneidensis* under anaerobic conditions (17). In this study, we used partial DNA microarrays to examine the transcriptional effects of an insertional disruption in the chromosomal *etrA* locus under fumarate- and nitrate-reducing conditions. Our results indicated that *etrA* mutation affects the mRNA levels of various functionally grouped genes involved in energy metabolism, transcriptional regulation, biosynthesis, and other cellular functions, although, as shown previously (17), the presence of *etrA* is not essential for anaerobic growth and reduction of electron acceptors by *S. oneidensis*.

#### Generation and phenotype analysis of the *etrA* mutant strain.

*S. oneidensis* and *E. coli* strains were grown as described previously (18, 21). *S. oneidensis* strain DSP-10, a spontaneous rifampin-resistant derivative of the wild type, was used as a parental strain to generate an *etrA* null allele by integrative disruption with the suicide plasmid pKNOCK-Km<sup>r</sup> (1). Briefly, an internal fragment (247 bp) of the *etrA* gene was amplified by PCR with primers 5398IM-F (5'-AGGTGATGAACAGATCACAGG-3') and 5398IM-R (5'-TGCGTTTTTCTTACTCAGTAGC-3') and cloned into the *EcoRV* site of plasmid pKNOCK-Km<sup>r</sup>. The resulting construct was propagated in *E. coli* S17-1/ $\lambda_{pir}$  and subsequently transferred into *S. oneidensis* DSP-10 by conjugation, essentially as described elsewhere (27). Integration of the plasmid into the *etrA* locus was verified by PCR amplification with external primers 5398F (5'-GCCGCTAGTGGG TGTGCAAT-3') and 5398R (5'-TCCTAGCA TTACCCGCCAAGAGA-3'), which are complementary to sequences flanking the *S. oneidensis* *etrA* gene. As expected, a product of approximately 709 bp in length was amplified from the parental DSP-10 DNA, whereas a 3-kb product was amplified from *etrA*::pKNOCK-Km<sup>r</sup> DNA (data not shown), verifying disruption of the *etrA* gene.

The resulting *etrA* mutant strain, designated ETRA1, was compared to DSP-10 for anaerobic utilization of different electron acceptors as described elsewhere (4, 18). While no significant differences were found between the mutant and DSP-10 strains in their abilities to reduce and/or grow on MnO<sub>2</sub>, Fe(OH)<sub>3</sub>, Fe(III) citrate, thiosulfate, sulfite, dimethyl sulfoxide (DMSO), and trimethylamine-*N*-oxide (TMAO), the ETRA1 cells displayed a decrease of up to 30% in initial growth rates when fumarate or nitrate was used as an electron acceptor (data not shown). The DSP-10 strain displayed a higher initial growth rate when fumarate rather than nitrate was used as an electron acceptor ( $0.76 \pm 0.03$  versus  $0.53 \pm 0.01$  h<sup>-1</sup>), while the ETRA1 strain grew at similar rates in the presence of

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either fumarate or nitrate ( $0.51 \pm 0.04$  and  $0.47 \pm 0.04 \text{ h}^{-1}$ , respectively). The results of physiological studies agreed with previous findings (17), further supporting the observation that EtrA is not essential for anaerobic growth and respiration of *S. oneidensis* MR-1.

**Gene expression profiling.** To examine the functional role of EtrA in the regulation of anaerobic metabolism in *S. oneidensis* MR-1, transcription profiles of DSP-10 and ETRA1 strains under fumarate- and nitrate-reducing conditions were compared by using partial DNA microarrays. A complete list of the MR-1 genes represented on the microarray is available online ([http://www.esd.ornl.gov/facilities/genomics/partial\\_microarrays.html](http://www.esd.ornl.gov/facilities/genomics/partial_microarrays.html)). Sample preparation, probe synthesis, and microarray procedures were performed as described previously (27). For each condition, a total of four independent hybridization experiments were performed, including two biological replicate experiments with fluorescent dye reversal.

Following signal intensity quantification and normalization, 69 genes showed significant alterations in mRNA abundance, as defined previously (27), in the ETRA1 mutant under fumarate- and/or nitrate-reducing conditions (Fig. 1). Based on the genome sequencing results (The Institute for Genomic Research, unpublished data), responsive genes fell into five putative functional categories: (i) electron transport, (ii) intermediary carbon metabolism, (iii) transcription regulation, (iv) substrate transport and binding, and (v) biosynthesis, assembly, and other cellular processes.

The upstream regulatory regions of all genes displaying altered mRNA levels in ETRA1 were analyzed for the presence of Fnr consensus sequences (TTGATN<sub>4</sub>ATCAA). Previous studies have shown that the *etrA* gene of *S. oneidensis* MR-1 is able to complement an *fnr* mutant of *E. coli* (20), suggesting that the cellular functions and the specific DNA-binding recognition sites for both proteins are highly similar, possibly identical. Based on the information about the Fnr motif conservation in *E. coli*, which was obtained from the RegulonDB database ([http://www.cifn.unam.mx/Computational\\_Genomics/regulondb](http://www.cifn.unam.mx/Computational_Genomics/regulondb)), a percent identity cutoff equal to or greater than 70% was selected. The search results identified 36 potential Fnr-binding sites located in the putative regulatory regions of 26 of 52 responsive operons or genes that were affected by the disruption of *etrA* (Table 1).

Of these 52 genes, 31 encoded putative components of the electron transport chain, including a predicted formate dehydrogenase (open reading frames [ORFs] 1752, 1754, and 3005), DMSO reductase (ORFs 3454, 3455, 3457, and 3458), and three fumarate reductases (ORFs 1863, 2389, 2390, and 2987). While the transcription levels of *frdC* and *fccA* were consistently repressed in ETRA1 under both fumarate- and nitrate-reducing conditions, the mRNA levels of the *frdAB* operon decreased slightly only in the presence of fumarate (Fig. 1A). Analysis of hybridization signal intensities suggested that the transcription levels of the *frdAB* operon were strongly repressed in both the DSP-10 and ETRA1 strains under nitrate-reducing conditions. A similar expression pattern was observed for ORF 2952, which encodes a homolog of the *Desulfovibrio desulfuricans* diheme split-Soret cytochrome *c* that was previously implicated in sulfate and thiosulfate reduction (8) (Fig. 1A). Genes encoding a periplasmic nitrate reductase (*napAH*; ORFs 2849 and 2851) and prismane (ORF

3388) showed an approximately two- to threefold increase in mRNA levels in an *etrA*<sup>+</sup> background under fumarate-reducing conditions, while the expression of these same genes under nitrate-reducing conditions was either unaffected or exhibited marginal decreases. Previous microarray analysis of the wild-type *S. oneidensis* MR-1 indicated that mRNA levels of the nitrate reductase-encoding operon *napBHGA* and a prismane-encoding gene increased substantially (8- to 56-fold) in response to nitrate relative to levels found with growth on fumarate (5). Together, these data suggest that expression of the *S. oneidensis nap* gene cluster and the prismane-encoding gene may be subject to two levels of global control.

Inactivation of the *etrA* locus did not alter the transcription levels of the *tor* operon, which encodes the putative TMAO reductase, or of a second DMSO reductase gene cluster present in the genome of *S. oneidensis*. These findings are in accordance with the physiological evidence indicating that DMSO and TMAO respiration in *S. oneidensis* is not affected by *etrA* inactivation. It also suggests that gene duplication in MR-1 may contribute to the wild-type levels of DMSO reduction in the ETRA1 strain.

Genes exhibiting increased mRNA levels in the ETRA1 mutant were largely those associated with aerobic respiration, including those encoding NADH dehydrogenase, cytochrome *cbh*<sub>3</sub> and *d* oxidases, and tricarboxylic acid cycle components (Fig. 1A and B). In addition, genes for a conserved hypothetical protein (ORF 3290) and a putative oxidoreductase (ORF 3280) of unknown function exhibited a twofold decrease in mRNA levels under fumarate- and nitrate-reducing conditions. Sequence analysis of the conserved hypothetical protein revealed 76% identity to another hypothetical protein (GenBank accession no. PA2776) from *P. aeruginosa* PAO1 and 67% identity to a putative OrdL oxidoreductase (GenBank accession no. U38543) from *E. coli*. The deduced amino acid sequence of the putative oxidoreductase gene was 59% identical to that of the *ordL* gene product (GenBank accession no. PA5309) from *P. aeruginosa* PAO1.

Putative Fnr motifs were located upstream of all 11 operons which displayed decreased mRNA levels in ETRA1 and which are putatively involved in anaerobic respiration and the reduction of DMSO, fumarate, and nitrate. Highly conserved Fnr motifs were found upstream of the *hydABC*, *frdA*, *napDAHGB*, *cydAB*, *bfr*, and *ccoNOQP* operons (Table 1). These findings are consistent with previous promoter studies indicating that the same orthologous genes in *E. coli* are directly regulated by Fnr (10, 14, 24).

The *etrA* mutation affected the transcription levels of 9 putative regulatory genes and 12 genes involved in substrate transport and binding (Fig. 1C and D). Of these effects, the most notable was the approximately twofold increase in mRNA abundance that was observed for the ferric uptake regulator (*fur*) gene in the ETRA1 strain. Fur, a classic iron-responsive repressor, has been shown to negatively control the expression of siderophore biosynthesis and other iron acquisition genes in a number of organisms (2), including *S. oneidensis* (27). Putative iron acquisition and siderophore-binding genes (ORFs 870, 1755, 1761, 1988, 2304, 3607, and 3813) exhibited a 2- to 11-fold decrease in mRNA levels under fumarate- and nitrate-reducing conditions. In addition, a putative Fnr-binding motif was found upstream of the *fur* regulatory gene (Table 1).

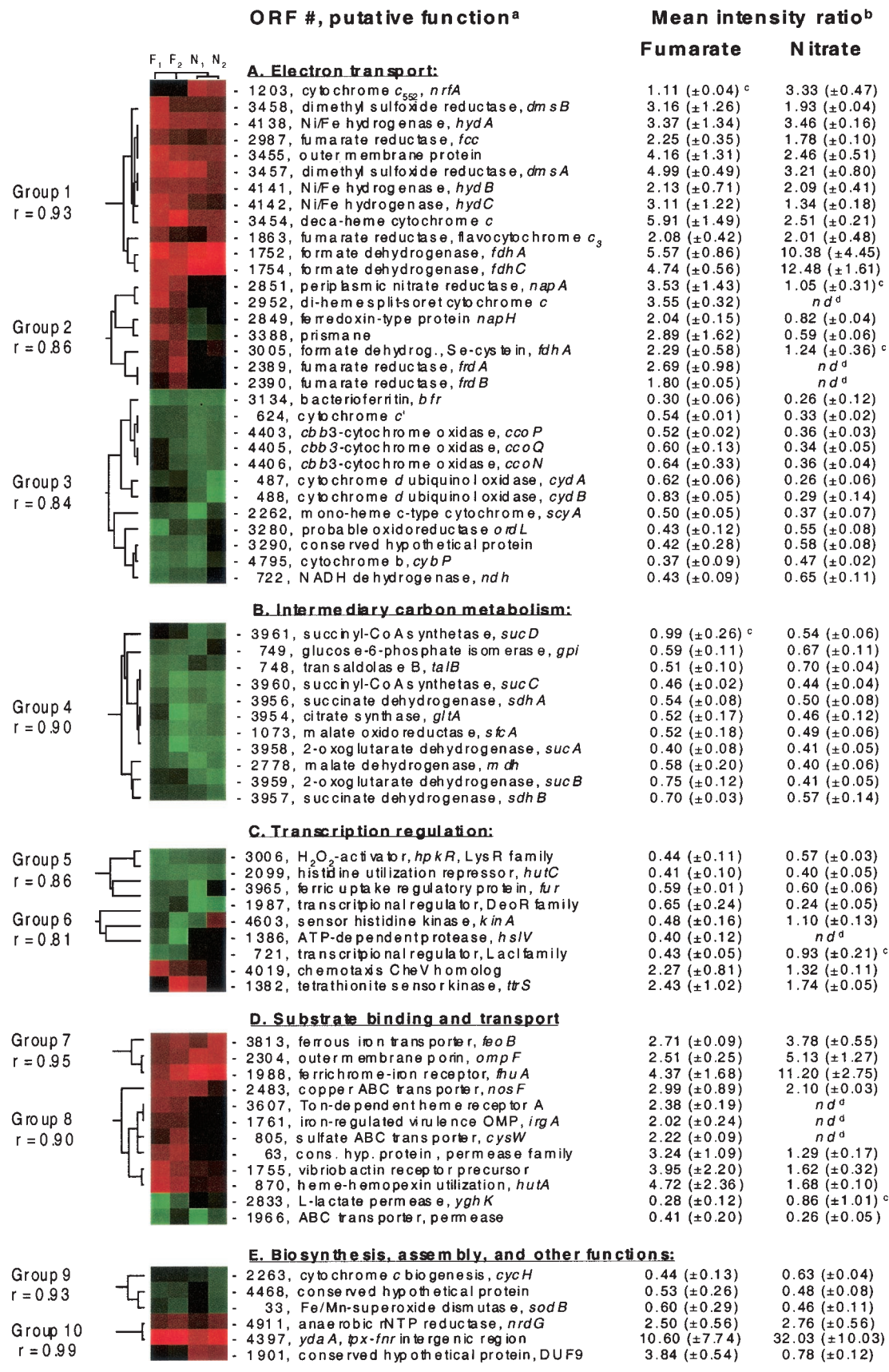


TABLE 1. Putative Fnr motifs located upstream of the genes affected by the *etrA* mutation

Gene or operon and product (ORF)	Putative Fnr box <sup>a</sup>	Position (bp) <sup>b</sup>
<i>nrfA</i> , cytochrome <i>c</i> <sub>552</sub> , nitrite reductase (1203)	TTGAT . . . . cgCAA	83
<i>frdA</i> , fumarate reductase, flavocytochrome <i>c</i> <sub>3</sub> (1863)	aTGAA . . . . ATCAA	272
Diheme split-Soret cytochrome <i>c</i> (2952)	TcGAT . . . . AcCAA	192
<i>fccA</i> , fumarate reductase (2987)	gTGAT . . . . tTCAA	210
<i>fdhA</i> , selenocysteine formate dehydrogenase (3005)	cTGAT . . . . ATaAA	222
Prismane, 6Fe-6S protein (3388)	gTGAT . . . . cTCTA	119
<i>fdhABC</i> , formate dehydrogenase (1752)	cTGtT . . . . ATCAA	55
<i>frdABC</i> , fumarate reductase (2389)	TTGAT . . . . cTCAG	105
<i>napDAHGB</i> , nitrate reductase (2848–2852)	TTGAT . . . . ATCgA	119
<i>dmsAB</i> , DMSO reductase (3454–3458)	gTGAT . . . . tTCAA	169
<i>hydABC</i> , Ni-Fe hydrogenase (4138–4142)	TTGAT . . . . ATCAA	100
<i>cydAB</i> , cytochrome <i>d</i> ubiquinol-oxidase (487 and 488)	TTGAT . . . . ATCAA	338
<i>scyA</i> , monoheme <i>c</i> cytochrome (2262)	TTGAT . . . . tTCcA	170
<i>bfr</i> , bacterioferritin (3134)	TTGAT . . . . ATCAA	43
<i>ordL</i> , probable oxidoreductase (3280)	TaGAT . . . . AaCAG	200
<i>ccoPOQN</i> , cytochrome <i>cb</i> <sub>3</sub> oxidase (4403)	TTGAT . . . . ATCAA	212
<i>ccoPOQN</i> , cytochrome <i>cb</i> <sub>3</sub> oxidase (4404)	TTGAc . . . . cTCAA	169
<i>ccoPOQN</i> , cytochrome <i>cb</i> <sub>3</sub> oxidase (4405)	TTGAc . . . . ATCAA	106
<i>ccoPOQN</i> , cytochrome <i>cb</i> <sub>3</sub> oxidase (4406)	TTGAa . . . . cgCAA	163
<i>fur</i> , ferric uptake regulatory protein (3965)	TTGAa . . . . cgCAA	163
<i>kinA</i> , sensor histidine kinase (4603)	TTGAc . . . . AgCAA	176
Ferrichrome-iron receptor (1988)	TTGAT . . . . ATCAc	103
<i>ompF</i> , outer membrane porin (2304)	gTGAT . . . . AatAA	166
<i>feoB</i> , ferrous iron transporter (3813)	TTGAT . . . . ATtAg	369
	TTGAT . . . . gTaAA	87
<i>nrdG</i> , anaerobic ribonucleotide reductase (4911)	tTGAT . . . . ATCAc	121
	TTGtT . . . . ATCAA	63
<i>sodB</i> , Fe-Mn superoxide dismutase (33)	aTGAT . . . . ATCAc	87
<i>cycH</i> , cytochrome <i>c</i> biogenesis (2263)	TTGAT . . . . AaagA	344
<i>ydaA</i> , <i>tpx-fnr</i> intergenic region (4397)	TTGAT . . . . AaCAA	36
Conserved hypothetical protein (4468)	gTGAT . . . . ATCAc	314

<sup>a</sup> Nucleotides matching those of the *E. coli fur* consensus sequence (TTGATNNNNATCAA) are shown in uppercase letters. The selected identity cutoff is 70%.

<sup>b</sup> The position of the putative *fnr* box *x* is given as the distance from the 5' end of the motif to the presumed translation start codon.

These observations suggest that EtrA influences the transcription of other regulatory genes; however, it is unclear whether a mechanism of coordinate regulation is operating in *S. oneidensis* to control anaerobic respiration.

A significant decrease in mRNA levels (10- to 30-fold) was displayed by the putative *ydaA* gene, whose predicted product is distantly related to a family of universal stress proteins. The *ydaA* gene is located 90 bp downstream of the *etrA* stop codon and is transcribed in the same orientation as *etrA*. Within the intragenic *etrA-ydaA* region, we identified a conserved Fnr-binding site (TTGAT N<sub>4</sub> AACAA) that was located 36 bp upstream of the predicted translational start for *ydaA* and which displayed a 90% identity to the consensus Fnr-binding site sequence (TTGAT N<sub>4</sub> ATCAA). Although a potential *rho*-independent site was identified downstream of *etrA*, we

cannot exclude the possibility that the *etrA* mutation has a polar effect on the transcription of *ydaA*.

**Concluding remarks.** The *S. oneidensis* MR-1 *etrA* gene encodes a putative DNA-binding protein that shares a high degree of sequence identity with *E. coli* Fnr. Despite the absence of a discernible physiological effect for ETRA1, the microarray data indicated that disruption of the chromosomal *etrA* locus affects the mRNA levels of 69 *S. oneidensis* genes (Fig. 1). The results also suggest that EtrA is functional and that it participates, either directly or indirectly, in gene regulation under anaerobic conditions. One possible explanation for the observed ETRA1 phenotype could be the presence of other regulatory genes that encode proteins with similar functions. It was previously suggested that another gene similar to *etrA* is present in MR-1 (20), but no genes exhibiting high sequence

FIG. 1. Pairwise average-linkage clustering analysis of the 69 *S. oneidensis* MR-1 genes exhibiting altered mRNA levels in the ETRA1 mutant. Hierarchical clustering, based on pairwise correlations across all experimental points, groups together genes of known similar function (A to E) and also provides a means of obtaining leads as to the function of unknown or poorly characterized genes (9). Each column indicates a separate biological replicate (F<sub>1</sub> and F<sub>2</sub> refer to fumarate-reducing conditions; N<sub>1</sub> and N<sub>2</sub> refer to nitrate-reducing conditions). Red and green colors indicate genes that are induced and repressed, respectively, in the presence of *etrA*. The Pearson correlation coefficients (*r*) are displayed to the left of the nodes. a, sequence information provided as a courtesy by The Institute for Genomic Research (unpublished results). The ORF numbers provided here are for the purpose of tracking gene locations and sequence information; they may change once the complete version of *S. oneidensis* MR-1 genome sequence is released. b, relative mRNA levels presented as the ratio of the dye intensity of the parental strain to that of the *etrA* mutant averaged across two biological replicate experiments. Values in parentheses indicate the standard deviation for each mean expression ratio. c, value not significantly different from 1 at a *P* of 0.05 based on a one-tailed *t* test. d, unable to determine expression ratio for this ORF due to the very low levels of hybridization intensities in both DSP-10 and ETRA1 strains (*nd*).

identity to those for either EtrA or Fnr were identified in the *S. oneidensis* genome. Sequence analysis, however, revealed a putative cyclic nucleotide-binding protein that shows 88 and 44% identity at the amino acid level to the *E. coli* Crp and *Bacillus subtilis* Fnr regulators, respectively. Although comparison of the deduced amino acid sequences of the *S. oneidensis* *crp* and *etrA* genes did not reveal any substantial degree of homology (21% identity and 45% similarity), the products of both of these genes belong to the same family of transcriptional regulators (15). Previous studies of Fnr- and Crp-activated promoters have shown that Fnr and Crp are structurally and mechanistically similar (11). Also, evidence exists demonstrating a reciprocal recognition of binding sites where the Crp regulator can bind and activate transcription from natural Fnr-binding sites (25). Based on this information, we cannot rule out the possibility that the presence of a Crp homolog or other regulator(s) of energy metabolism in *S. oneidensis* alleviates the effect of *etrA* inactivation. Previous studies of genes involved in anaerobic metabolism in *E. coli* have demonstrated that gene expression is often controlled by several regulatory elements such as ArcA, Fnr, Crp, and NarL (6, 13, 25). Given the metabolic diversity and genome plasticity of *S. oneidensis*, it is possible that coordinate regulation by multiple transcription factors may alleviate the effects of the *etrA* inactivation. Further studies are necessary to elucidate the complex regulatory networks and mechanisms controlling energy metabolism in *S. oneidensis* MR-1.

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