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Direct Correlation between Rates of Anaerobic Respiration and Levels of mRNA for Key Respiratory Genes in *Geobacter sulfurreducens*

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The predominance of *Geobacter* species in environments in which Fe(III) reduction is important has suggested that Fe(III) reduction rates might be estimated in *Geobacter*-dominated environments by assessing in situ activity with molecular techniques. To determine whether mRNA levels of key respiratory genes might be correlated with respiration rates in *Geobacter sulfurreducens*, studies were conducted with fumarate as the electron acceptor and acetate as the limiting electron donor in anaerobic continuous cultures. Levels of mRNA for a fumarate reductase gene, *frdA*, quantified by real-time reverse transcription-PCR were directly correlated with fumarate reduction rates. In similar studies with Fe(III) as the electron acceptor, mRNA levels for *omcB*, a gene for an outer membrane *c*-type cytochrome involved in Fe(III) reduction, were positively correlated with Fe(III) reduction rates. Levels of mRNA for *frdA* and *omcB* were also positively correlated with fumarate and Fe(III) reduction rates, respectively, when growth was limited by the availability of fumarate or Fe(III), but mRNA levels were higher than in acetate-limited cultures. Levels of mRNA for *omcC*, which encodes a *c*-type cytochrome highly similar to OmcB but not necessary for Fe(III) reduction, followed patterns different than those of *omcB*. This agrees with the previous finding that OmcC is not involved in Fe(III) reduction and suggests that changes in mRNA levels of *omcB* are related to its role in Fe(III) reduction. These results demonstrate that mRNA levels for respiratory genes might be used to estimate in situ Fe(III) reduction rates in *Geobacter*-dominated environments but suggest that information on environmental conditions and/or the metabolic state of *Geobacter* species is also required for accurate rate estimates.

Dissimilatory Fe(III) reduction is one of the most geochemically significant processes in anoxic sedimentary environments, but estimating in situ rates of Fe(III) reduction is difficult. Dissimilatory Fe(III) reduction plays an important role in the anaerobic degradation of organic matter and influences sediment chemistry through the dissolution of Fe(III) oxides and the resultant release of soluble Fe(II), phosphate, and trace metals, as well as the production of Fe(II) minerals (20, 21). The ability of some dissimilatory Fe(III) reducers to degrade organic contaminants (6, 22, 29) and the abundance of Fe(III) oxides in many subsurface environments (17) can lead to significant removal of organic contaminants from groundwater via Fe(III) reduction. Fe(III)-reducing microorganisms can reduce toxic metals such as uranium (28), chromium (18), cobalt (3), and technetium (13). Stimulating the growth and activity of Fe(III)-reducing microorganisms in uranium-contaminated subsurface sediments can be an effective strategy for removing uranium from contaminated groundwater (1, 5).

Estimates of the in situ rates of Fe(III) reduction in subsurface environments could provide helpful insights into sediment biogeochemistry and would aid in modeling bioremediation by Fe(III)-reducing microorganisms. However, suitable methods for accurately measuring in situ rates of Fe(III) reduction are not yet available. This contrasts with most other anaerobic respiratory processes, for which good assessment techniques are available. For example, rates of sulfate reduction are typically estimated by monitoring the reduction of tracer quantities of $^{35}SO_4^{2-}$ (8), but reduction of Fe(III) cannot be measured by tracer techniques because there is rapid isotope exchange between Fe(III) and Fe(II) forms (39). Rates of Fe(III) reduction can be estimated from the accumulation of Fe(II) in sediments over time (24, 46). However, most of the Fe(II) produced is in solid phases and most sediments are sufficiently heterogeneous that sediments must be mixed prior to sampling in order to obtain reliable estimates of Fe(II) accumulation. This mixing can affect rates of microbial metabolism, especially in subsurface environments (4). Furthermore, long incubation times in sealed containers are typically required to detect significant increases in Fe(II) (24), which raises serious questions about the relationship of the measured rates to in situ activity.

An alternative strategy for measuring Fe(III) reduction might be to monitor mRNA levels of key genes involved in Fe(III) reduction. In some instances, environmental mRNA levels have been correlated with rates of microbial activity. For example, it has been shown that *nahA* transcript levels correlated positively with [^{14}C]naphthalene mineralization rates, soil naphthalene concentration, and *nahA* gene frequency in soils (7). In some, but not all, instances direct correlations between levels of mercuric reductase (*merA*)-specific transcripts and Hg(II) volatilization rates have been observed (11, 33). However, it is less clear whether a direct correlation between rates of anaerobic respiration and levels of mRNA for key respiratory genes can be expected. For example, studies with *Escherichia coli* found that expression of genes for the reduction of fumarate, dimethyl sulfoxide, and nitrate changed little with increased growth rates or actually decreased (9, 47). Furthermore, unlike some respiratory process that are highly conserved, the mechanisms for Fe(III) reduction may be sig-

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TABLE 1. Primers used for real-time PCR assays for quantitative detection of respiratory genes

Target gene	Primer[a]	Sequence	Length of amplicon (bp)	Reference
omcB	8912	5′-CCCACTTCGACAACTATTCG-3′	212	This study
	8908-2	5′-GGTCAGCAGGCCACCGG-3′		12
omcC	8917	5′-GGTCTTCACCCAGATCTCG-3′	232	This study
	8915	5′-GGGTGTTGTGGTAGAAGGG-3′		This study
frdA	frdA-2	5′-GAACTCGGTTACAACGTTG-3′	144	This study
	frdA-Q5	5′-GATGGTGTCATAGAAGAGACG-3′		This study

[a] Sense and antisense primers for each gene are listed in sequence.

nificantly different in phylogenetically distinct organisms (19, 35, 36).

Molecular analyses of the structure of the microbial community in a variety of subsurface environments in which Fe(III) reduction is important have demonstrated that *Geobacter* species are typically the predominant Fe(III)-reducing microorganisms (1, 10, 40, 41, 43, 44). This enrichment of a single group of microorganisms contrasts with the much greater diversity of microorganisms found in many other environments. Thus, if it is feasible to determine the rates of Fe(III) reduction in *Geobacter* species in situ, it might be possible to estimate rates of Fe(III) reduction in these *Geobacter*-dominated subsurface environments.

Geobacter sulfurreducens serves as a pure-culture model for the *Geobacter* species that predominate in subsurface environments (15, 16, 32). Since current methods in common use for mRNA quantification are relatively insensitive (2, 31), we developed real-time reverse transcription (RT)-PCR assays to quantify the expression levels of respiratory genes in *G. sulfurreducens*. Here we report that there is a direct correlation between rates of Fe(III) and fumarate reduction in *G. sulfurreducens* and levels of mRNA for key genes involved in Fe(III) and fumarate respiration.

MATERIALS AND METHODS

Culturing conditions and growth media. *G. sulfurreducens* strain PCA (3) was grown in continuous culture at various growth rates under strict anaerobic conditions (N_2/CO_2 [80:20, vol/vol]) in a cysteine-free freshwater medium as previously described (A. Esteve-Núñez et al., submitted for publication). The acetate-limited cultures contained 5.5 mM acetate and either 30 mM fumarate or 56 mM Fe(III) citrate. For growth under electron acceptor-limiting conditions the acetate concentration was 10 mM while fumarate or Fe(III) citrate was supplied at 10 or 20 mM, respectively. Cells were grown in 200-ml water-jacketed glass vessels, maintained at 30°C, and stirred at a constant speed of 600 rpm with a magnetic bar (Esteve-Núñez et al., submitted). The flow rate was maintained constant with a peristaltic pump. Steady-state cell growth was expected after the medium input reached 5 volumes of the culture vessel, and this was confirmed by measuring cell density and fumarate, succinate, and Fe(II) concentrations.

Total RNA extraction and RT-PCR conditions. Cells were harvested from each chemostat vessel by centrifugation at 5,000 × *g* for 15 min at 4°C. Total RNA was isolated immediately with a QIAGEN RNA Midi kit (QIAGEN Inc., Valencia, Calif.), and the extracted RNA was treated with RNase-free DNase (Ambion Inc., Austin, Tex.). DNA contamination was checked by agarose gel electrophoresis following RT-PCR by performing control experiments in which no reverse transcriptase was added to extracted RNA prior to the PCR step. RNA concentration was determined by measuring A_{260} with a Biophotometer (Eppendorf, Hamburg, Germany) and additionally verified by fluorometry with the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, Oreg.). Purified RNA was stored at −80°C.

One microgram of total RNA served as the template for cDNA synthesis. For reverse transcription reactions SuperScript III RNase H^- reverse transcriptase (200 U; Invitrogen Life Technologies, Rockville, Md.) was used in accordance with the manufacturer's instructions. Either random hexamers (2.5 μM final concentration; Invitrogen Life Technologies) or gene-specific antisense primers (1 μM; Sigma Genosys, The Woodlands, Tex.) (Table 1) were used for cDNA synthesis. The cDNA synthesis conditions were as follows: incubation at 55°C for 60 min (25°C for 5 min prior to incubation at 55°C for random primers) and enzyme inactivation at 70°C for 15 min. To remove RNA complementary to the cDNA, *E. coli* RNase H (2 U) was added and the reaction mixture was incubated at 37°C for 20 min, followed by rapid cooling to 4°C. The cDNA samples were stored at −20°C before PCR analysis. Two cDNA syntheses per RNA sample were performed.

Primer design and optimization. Primers used to amplify *G. sulfurreducens* sequences were designed in accordance with the desired criteria by the TaqMan system (GeneAmp 5700 SDS user manual; Applied Biosystems, Foster City, Calif.) and the *G. sulfurreducens* genome sequence (32). Primers were further evaluated and optimized for the application with the TaqMan system with the Primer Express software (version 1.5; Applied Biosystems). For each assay, primer concentrations and annealing temperatures were experimentally optimized to obtain specific amplification. The resulting conditions were experimentally checked with genomic DNA isolated from *G. sulfurreducens* cultures. All primers were synthesized by Sigma Genosys Oligofactory.

In order to determine whether the designed primers were suitable, gene-specific qualitative PCR (without addition of SYBR green) was performed prior to quantitative PCR. PCR products were amplified from cDNA generated by reverse transcription with the appropriate primers (Table 1) under the following conditions: 96°C for 40s; 27 cycles of 96°C for 40s, 55°C (*frdA*) or 58°C (*omcB* and *omcC*) for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were checked with ethidium bromide-stained agarose gel, and the specificity of PCR products was verified by sequence analysis.

Real-time PCR quantification. Real-time RT-PCR is the most sensitive and flexible method for the detection of low-abundance mRNA (49). The assays were performed with a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Quantification of samples was carried out by determining the threshold cycle value and by comparing results to a standard curve to determine the starting copy number. The threshold cycle was proportional to the logarithm of the target molecule number. The number of target molecules was automatically calculated by the GeneAmp 5700 SDS software (version 1.3). The principles of real-time PCR and more details have been described by Bustin, Raeymaekers, and Suzuki et al. (2, 38, 45). The precision and reproducibility of quantification were carefully optimized. In order to eliminate intra-assay and interassay variations, the same PCR run was performed in four replicates and the separate PCR runs were performed in triplicate, respectively. In addition, correct lengths of PCR products were checked by agarose gel electrophoresis to ensure that the fluorescent signal obtained in the real-time PCR originated from specific PCR products and not from artifacts.

Dilution series of purified RT-PCR products were used as calibration standards for the real-time PCR quantification. The cDNAs, which were generated as described above, were amplified with the assay primers (Table 1), and the resulting amplicons were purified with a QIAquick PCR purification kit in accordance with the manufacturer's (QIAGEN) instructions. The purified RT-PCR products were then quantified by measurement of A_{260} with a Biophotometer (Eppendorf), and the concentration was verified by fluorometry with the PicoGreen double-stranded DNA quantitation kit (Molecular Probes). The concentration of RT-PCR products was converted to numbers of target molecule per microliter, and the *omcB* or *frdA* standards were prepared for serial dilution and stored at −20°C. The detection limits of all PCR assays were determined from four independent measurements with dilution series of purified RT-PCR products (standards) in a real-time PCR (10^9 to 10^1 target molecules per reaction). All assays had a minimum sensitivity of 10^1 to 10^2 target molecules per reaction (Fig. 1).

PCR was performed with 96-well optical reaction plates (Applied Biosystems)

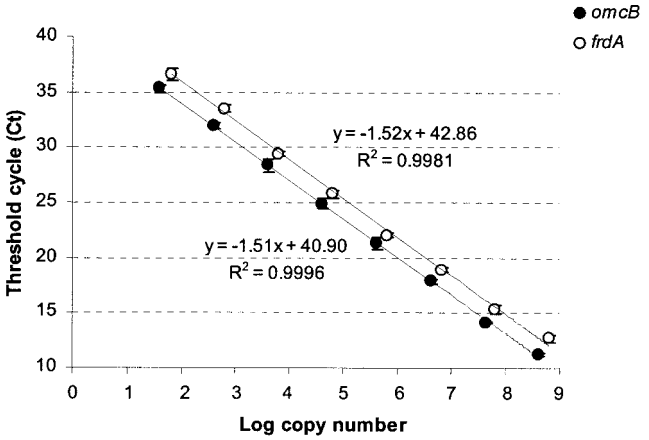

FIG. 1. Standard curves for target amplicons *omcB* and *frdA*. The symbols represent means ± standard deviations of four replicates of PCR amplifications of the standard samples. The lines are trend lines from the linear regression. Amplicon lengths are 212 (*omcB*) and 144 (*frdA*) bp. The lower detection limit was about 40 to 70 copies of target mRNA. The copy numbers of unknown samples were calculated after real-time amplification from the linear regression of the standard curve.

sealed with optical caps on the GeneAmp 5700 Sequence Detection System. A 5-μl sample of known (standards) or unknown (cDNA) amounts of template DNA was added to 25 μl of 2 × TaqMan PCR Master mix (Applied Biosystems) and a 400 nM final concentration of each amplification primer in a final volume of 50 μl.

The temperature profile was composed of an initial incubation step of 2 min at 50°C (activation of the polymerase), followed by a denaturation step of 10-min at 95°C; 40 cycles of denaturation for 45 s at 95°C, annealing for 1 min at 55 or 58°C, and elongation for 1 min at 72°C; and a final elongation step of 6 min at 72°C. Four negative controls (reaction mixtures with no template) were included in each real-time PCR run. The size of the PCR products was verified by agarose gel electrophoresis.

Analytical techniques. Cell density of *G. sulfurreducens* during growth on fumarate was monitored at 600 nm with a Spectronic Genesys 2 spectrophotometer (Spectronic Instruments, VWR, Boston, Mass.). Reduction of Fe(III) citrate was assessed by monitoring Fe(II) concentrations with ferrozine assays as previously described (26). Total iron concentrations were determined with hydroxylamine as previously described (27). Fumarate, succinate, and acetate concentrations were measured by high-pressure liquid chromatography on a Hewlett Packard series 1100 (Agilent Technologies, Inc., Albany, N.Y.) with a Bio-Rad Aminex HPX-87H column (300 by 7.8 mm) and a mobile phase of 8 mM H_2SO_4. Protein concentrations were determined by the bicinchoninic acid method with bovine serum albumin as the standard (42).

The specific fumarate reduction rate or Fe(III) reduction rate at each growth rate was calculated from the protein concentration and the amount of succinate production or Fe(II) production, respectively.

RESULTS

Expression of *frdA* and *omcB* under acetate-limited conditions. When *G. sulfurreducens* was grown with acetate as the electron donor and fumarate as the electron acceptor in continuous cultures in which acetate availability limited growth, levels of mRNA for the fumarate reductase gene, *frdA*, were directly correlated ($R^2 = 0.99$) with rates of fumarate reduction and growth rates (Fig. 2A and B). The number of copies

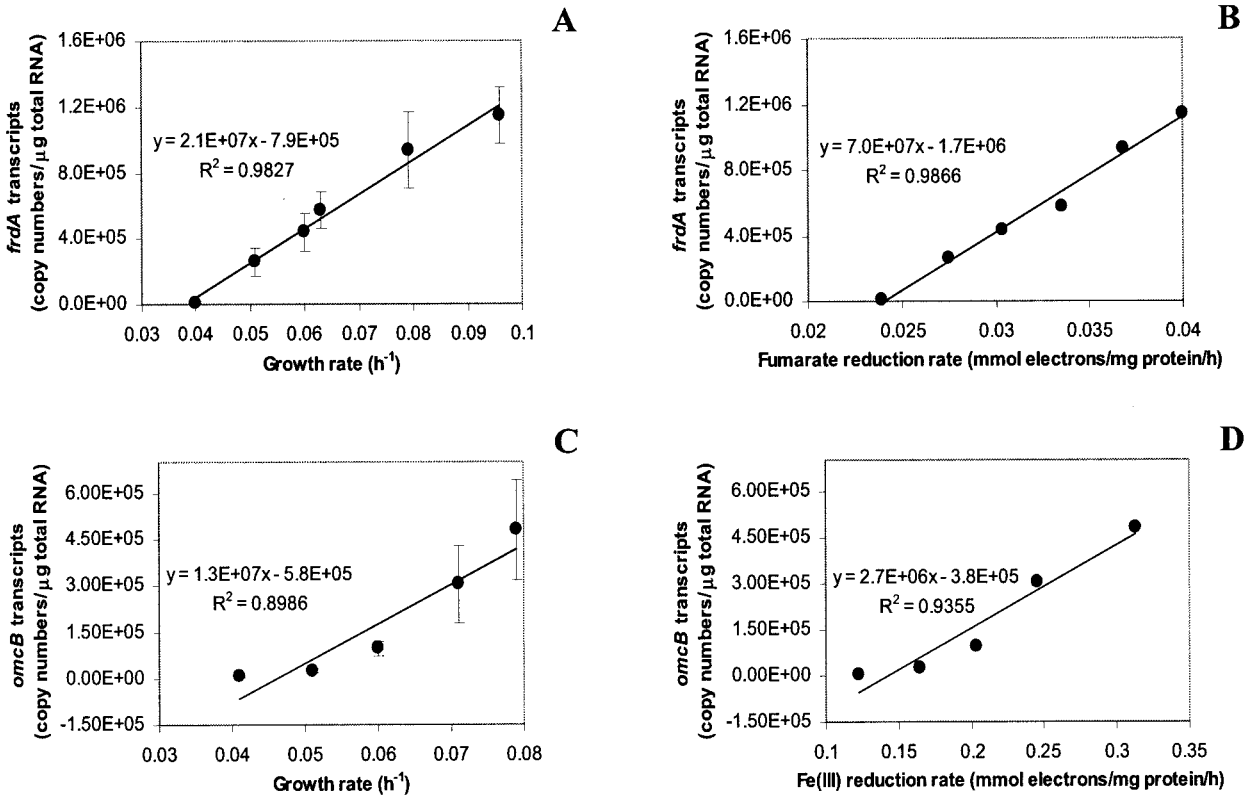

FIG. 2. Expression of respiratory genes in *G. sulfurreducens* grown at various growth rates with fumarate (30 mM) (A, B) or ferric citrate (56 mM) (C, D) as the terminal electron acceptor and acetate (5.5 mM) as the limiting electron donor. Data are means ± standard deviations of triplicates.

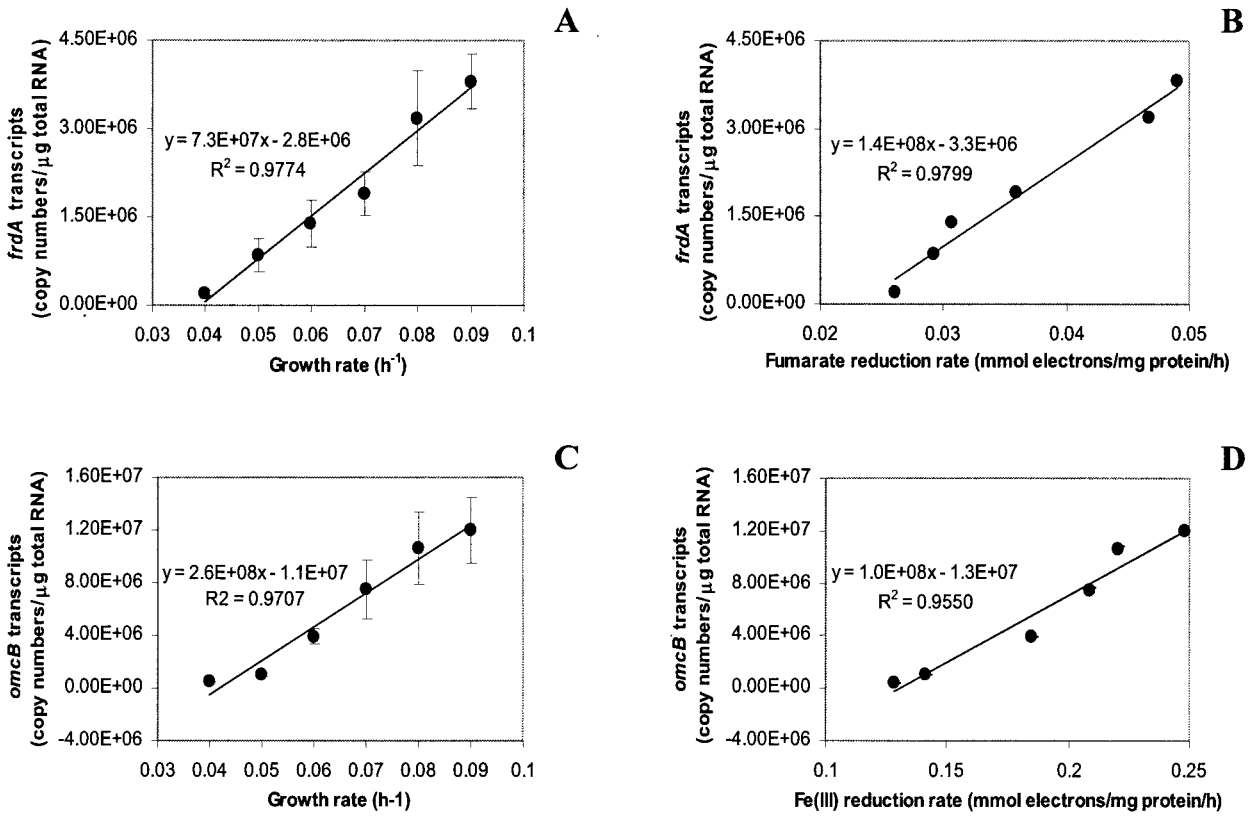

FIG. 3. Expression of respiratory genes in *G. sulfurreducens* grown at various growth rates with fumarate (10 mM) (A, B) or ferric citrate (20 mM) (C, D) as the limiting terminal electron acceptor and acetate (10 mM) as the electron donor. Data are means ± standard deviations of triplicates.

of *frdA* transcripts increased about 100-fold relative to the total RNA over the range of growth rates evaluated (Fig. 2A).

In a similar manner, there was a strong positive correlation ($R^2 = 0.94$) between levels of mRNA for *omcB*, a gene for an outer membrane *c*-type cytochrome required for Fe(III) reduction (12, 30) and the rate of Fe(III) reduction (Fig. 2D). Levels of mRNA for *omcB* increased more than 60-fold over the range of growth rates evaluated (Fig. 2C).

Expression of *frdA* and *omcB* under electron acceptor-limited conditions. In order to determine whether levels of mRNA during growth when the electron acceptor was limiting were similar to those when the electron donor was limiting, *G. sulfurreducens* cells were grown with excess electron donor (acetate) and either limited fumarate or Fe(III) citrate. The levels of mRNA for *frdA* and *omcB* were positively correlated with the rates of fumarate and Fe(III) reduction, respectively (Fig. 3). The number of copies of *frdA* mRNA was ca. threefold higher relative to the total RNA under the fumarate-limited conditions (Fig. 3A) versus the acetate-limited conditions (Fig. 2A). The relative levels of *omcB* mRNA in Fe(III)-limited cultures were ca. 20-fold higher (Fig. 3C) than those in acetate-limited cultures (Fig. 2C).

Differential expression of *omcB* and *omcC*. In order to further evaluate whether expression of *omcB* could be specifically related to Fe(III) reduction, the expression of *omcC* was also evaluated. Although *omcC* and *omcB* are 79% identical in nucleotide sequences (73% identity in peptide sequences) and may have arisen from a gene duplication event, OmcB is required for Fe(III) reduction but OmcC is not (12). At the same dilution rate, *omcB* transcript levels were lower than *omcC* transcript levels during growth on fumarate but were higher during growth on Fe(III) (Fig. 4). The difference in *omcB* and *omcC* transcript levels during growth on Fe(III) was particularly pronounced under Fe(III)-limited conditions, in which levels of *omcB* mRNA increased in comparison to those in acetate-limited cultures whereas *omcC* transcript levels in Fe(III)-limited cultures were substantially lower than those under acetate-limited conditions (Fig. 4).

DISCUSSION

The results demonstrate that there is a positive correlation between levels of mRNA for key respiratory genes and rates of reduction of the relevant electron acceptor in *G. sulfurreducens*. As detailed below, this provides the possibility of estimating rates of Fe(III) reduction, and possibly other types of respiration, in *Geobacter*-dominated subsurface environments. However, the finding that the levels of mRNA associated with a given rate of Fe(III) is substantially influenced by whether metabolism is limited by electron donor or electron acceptor availability suggests that it may be necessary to monitor the pattern of expression of several genes in order to evaluate whether metabolism is electron donor or electron acceptor

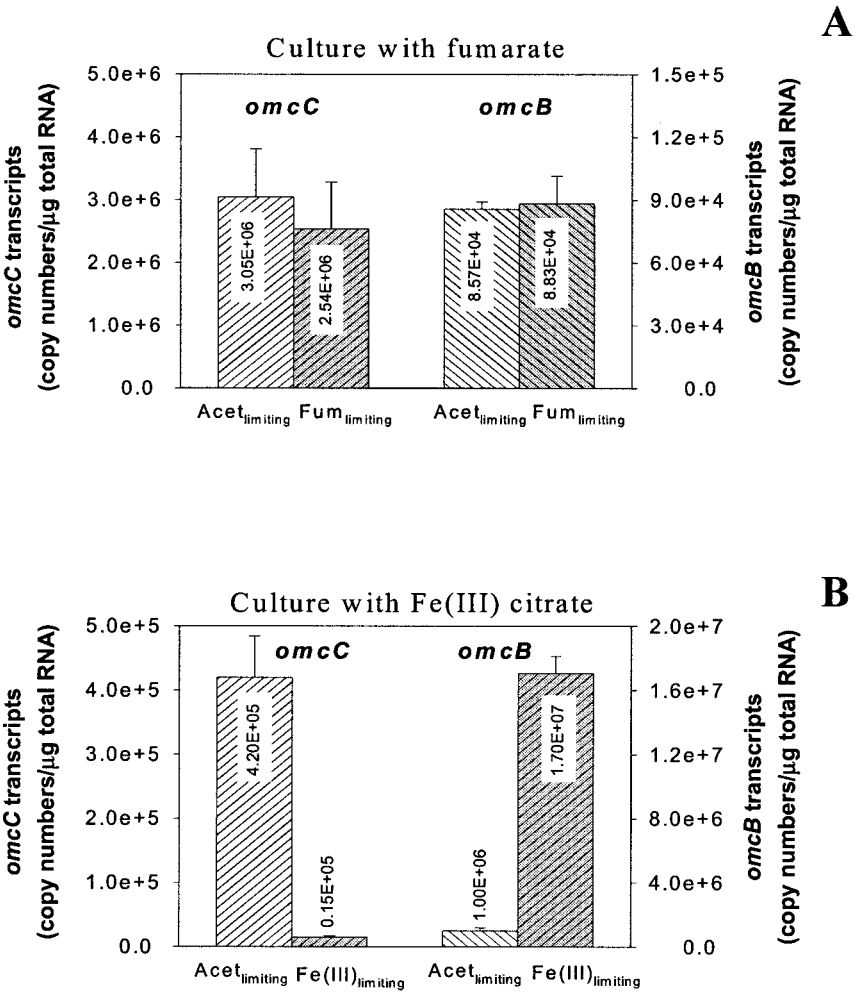

FIG. 4. Differential expression of respiratory genes in *G. sulfurreducens* grown at the same growth rate ($\mu = 0.08\ h^{-1}$) under acetate (Acet)-limiting versus terminal electron acceptor-limiting conditions: A, *omcC* and *omcB* gene expression in culture with fumarate (Fum); B, *omcC* and *omcB* gene expression in culture with Fe(III). Data are means ± standard deviations of triplicates.

limited before it will be possible to accurately estimate in situ respiration rates.

Fumarate respiration. Initial studies of fumarate respiration were conducted because this is the best-understood form of respiration in *G. sulfurreducens*. A cluster of three genes, *frdCAB*, with high homology to the three-gene operon for the fumarate reductase in *Wolinella succinogenes* is present in the *G. sulfurreducens* genome, and disruption of *frdA* eliminates the capacity for fumarate reduction in *G. sulfurreducens* (J. E. Butler and D. R. Lovley, unpublished data).

The strong correlation between mRNA levels for *frdA* and rates of fumarate reduction further verify that *frdA* is involved in fumarate respiration. The regulatory mechanisms that account for the apparent increase in the transcription of *frdA*, and presumably other genes involved in fumarate reduction, are unknown. Whether there was an increase in the levels of the fumarate reductase associated with higher levels of *frdA* mRNA was not documented. However, producing more of a terminal reductase when conditions favor higher rates of reduction of the electron acceptor could be an adaptive response. In a similar manner, it has been noted that levels of mRNA for the *dsrAB* genes, the key, highly conserved genes coding for the dissimilatory sulfite reductase in sulfate reduction (37, 48), increase in dissimilatory sulfate-reducing microorganisms as rates of sulfate reduction increase (34; L. Hayes and D. R. Lovley, Abstr. 102nd ASM Gen. Meet. Am. Soc. Microbiol., abstr. N-95, p. 321-322, 2002).

In contrast, in *E. coli*, *frdA* expression changed little as the cell growth rate increased from 0.12 to 0.60 h^{-1} under anaerobic conditions and there was also no increase, or a slight decrease, in the expression of genes associated with the reduction of dimethyl sulfoxide or nitrate as growth rates were increased (47). The growth rates evaluated in *E. coli* were substantially higher than those at which *G. sulfurreducens* can be grown. This suggests that phylogenetically distinct microorganisms may use different mechanisms for controlling the production of respiratory proteins or that at high growth rates different forms of regulation are more effective.

Fe(III) reduction. Mechanisms for electron transport to Fe(III) in *G. sulfurreducens* are not as well understood as those for fumarate reduction; however, it has been demonstrated that the gene encoding OmcB, an outer membrane *c*-type

cytochrome, is required for Fe(III) reduction (12). Deletion of *ppcA*, a gene for a smaller, periplasmic *c*-type cytochrome, also had an effect on Fe(III) reduction (14), but Fe(III) reduction was only inhibited approximately 40% compared to nearly complete inhibition of Fe(III) reduction in the OmcB-deficient mutant (12). Furthermore, the OmcB protein is predicted to be localized in the outer membrane, where it might directly interact with Fe(III) (12), whereas PpcA is periplasmic and thus likely to be an intermediary in electron flow to Fe(III) and other extracellular electron acceptors (14). Thus, of the genes that encode proteins currently known to be important in Fe(III) reduction in *G. sulfurreducens*, the *omcB* gene is the one most specifically associated with Fe(III) reduction.

The direct correlation between levels of *omcB* mRNA and rates of Fe(III) reduction further suggests that OmcB is a key component in electron transfer to Fe(III), as does the finding that levels of *omcB* were substantially higher during growth on Fe(III) than on fumarate. It is notable that levels of mRNA for the closely related gene *omcC* were higher during growth on fumarate than during growth on Fe(III) and were lower than levels of *omcB* RNA during growth on Fe(III), especially when Fe(III) availability was the factor limiting growth. These results suggest that, in contrast to *omcB*, transcription of *omcC* is regulated by factors other than those involved in a physiological response to the use of Fe(III) as an electron acceptor. This is consistent with the finding that knocking out *omcC* does not affect Fe(III) reduction, indicating that, despite their high degree of similarity, OmcB plays a key role in electron transfer to Fe(III) whereas OmcC does not (12).

Implications for estimating in situ respiration rates. The direct correlation between levels of mRNA for two key respiratory genes and rates of respiration suggests that it may eventually be possible to monitor in situ respiration in environments in which *Geobacter* species predominate. It is encouraging that a similar response was observed from two fundamentally different types of respiration. Whereas fumarate reduction takes place at the inner membrane, current evidence suggests that Fe(III) reduction takes place at or near the outer membrane.

The higher levels of *omcB* and *frdA* mRNA in cultures limited by electron acceptor [Fe(III) or fumarate] availability in comparison with cultures growing at the same rate but limited by electron donor (acetate) availability suggest that expression of the relevant terminal reductases is regulated in order to produce more of the appropriate terminal respiration genes as the availability of the electron acceptor decreases. This result has important implications for evaluating rates of respiration in *Geobacter*-dominated communities because the relative levels of mRNA for respiratory genes are affected not only by the rate of electron acceptor reduction but also by the availability of the electron acceptor. Thus, it may not be possible to specify Fe(III) reduction rates based on levels of mRNA for a single respiratory gene. Initial studies were conducted with acetate availability as the limiting factor because in many subsurface environments respiration is likely to be limited by the availability of the primary electron donor for anaerobic respiration, acetate. However, in some instances, Fe(III) could be the limiting factor. For example, as Fe(III) is depleted from sediments as a result of Fe(III) reduction, Fe(III) eventually becomes limiting and the predominant terminal electron-accepting process switches from Fe(III) reduction to sulfate reduction (23, 25). Furthermore, when acetate is added to the subsurface in millimolar quantities in order to stimulate dissimilatory metal reduction in uranium-contaminated aquifers, the availability of Fe(III) rather than acetate might limit the activity of Fe(III)-reducing microorganisms (1). Determining unequivocally whether a *Geobacter* population is either electron donor or Fe(III) limited from standard geochemical measurements would be difficult. Therefore, analysis of mRNA levels for a suite of genes, some of which could provide information on the factors limiting growth and activity, might be necessary in order to estimate in situ rates of respiration from levels of mRNAs for key respiratory genes.

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