APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2008, p. 4277–4284 Vol. 74, No. 14
0099-2240/08/$08.00+0 doi:10.1128/AEM.02901-07

Genome-Wide Gene Expression Patterns and Growth Requirements Suggest that *Pelobacter carbinolicus* Reduces Fe(III) Indirectly via Sulfide Production▽†

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Received 21 December 2007/Accepted 21 May 2008

Although *Pelobacter* species are closely related to *Geobacter* species, recent studies suggested that *Pelobacter carbinolicus* may reduce Fe(III) via a different mechanism because it lacks the outer-surface *c*-type cytochromes that are required for Fe(III) reduction by *Geobacter sulfurreducens*. Investigation into the mechanisms for Fe(III) reduction demonstrated that *P. carbinolicus* had growth yields on both soluble and insoluble Fe(III) consistent with those of other Fe(III)-reducing bacteria. Comparison of whole-genome transcript levels during growth on Fe(III) versus fermentative growth demonstrated that the greatest apparent change in gene expression was an increase in transcript levels for four contiguous genes. These genes encode two putative periplasmic thioredoxins; a putative outer-membrane transport protein; and a putative NAD(FAD)-dependent dehydrogenase with homology to disulfide oxidoreductases in the N terminus, rhodanese (sulfurtransferase) in the center, and uncharacterized conserved proteins in the C terminus. Unlike *G. sulfurreducens*, transcript levels for cytochrome genes did not increase in *P. carbinolicus* during growth on Fe(III). *P. carbinolicus* could use sulfate as the sole source of sulfur during fermentative growth, but required elemental sulfur or sulfide for growth on Fe(III). The increased expression of genes potentially involved in sulfur reduction, coupled with the requirement for sulfur or sulfide during growth on Fe(III), suggests that *P. carbinolicus* reduces Fe(III) via an indirect mechanism in which (i) elemental sulfur is reduced to sulfide and (ii) the sulfide reduces Fe(III) with the regeneration of elemental sulfur. This contrasts with the direct reduction of Fe(III) that has been proposed for *Geobacter* species.

Extracellular electron transfer to Fe(III) (47, 69), humic substances (44, 78), electrodes (43), and other electron acceptors that are reduced at or near the outer cell surface has important environmental implications but is poorly understood. For example, dissimilatory Fe(III)-reducing microorganisms have been shown to play an important role in the biogeochemical cycles of anoxic soils and sediments and can be important agents in the bioremediation of organic and metal contaminants in the subsurface (1, 31, 64, 75, 86). Reduction of soluble humic substances provides a mechanism for long-range electron transfer in a variety of anoxic environments (44, 60, 78). Extracellular electron transfer to electrodes can convert a diversity of organic wastes and renewable biomass to electricity (6, 29, 43). The high degree of conservation of the capacity for Fe(III) reduction in hyperthermophilic microorganisms, coupled with geological evidence, suggests that Fe(III) reduction was one of the first forms of respiration (13, 33–35, 84).

Studies of *Shewanella oneidensis* and *Geobacter sulfurreducens*, the two most intensively studied microorganisms capable of extracellular electron transfer, suggest that the mechanisms for Fe(III) reduction and electricity production are markedly different in these two microorganisms. Both organisms have an abundance of *c*-type cytochrome genes, and at least some of these are required for optimal electron transfer to Fe(III) or electrodes (3, 10, 26, 30, 40, 41, 53, 57–59, 80). However, other than the heme-binding motifs, there is little homology between the cytochromes in the two organisms. In both organisms, electrically conductive appendages have been proposed to be involved in extracellular electron transfer, but these appendages are markedly different in size and apparent function (26, 69–71). Furthermore, a number of studies have demonstrated that *Shewanella* species release an electron shuttle that facilitates electron transfer between the outer surface of the cell and Fe(III) oxides (62, 63, 83, 85). An electron shuttle also appears to be involved in electron transfer to electrodes by *S. oneidensis* (39, 52). *Geothrix fermentans*, which is phylogenetically distinct from *Shewanella* and *Geobacter* species, also employs an electron shuttle in Fe(III) (61) and electrode (7) reduction.

In some instances, microorganisms indirectly transfer electrons to Fe(III) oxides or electrodes via electron shuttles that are natural environmental constituents (28, 43, 44, 60, 72, 78, 82). Potential environmental electron shuttles include humic acids (44), which contain quinone moieties (78) that can act as

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† Supplemental material for this article may be found at http://aem.asm.org/.
▽ Published ahead of print on 30 May 2008.

electron acceptors and then, once reduced to the hydroquinone state, donate electrons to Fe(III) or electrodes (6). In addition to the quinone/hydroquinone redox couple found in humic substances, potential environmental redox shuttles include the $S^0/S^=$, cysteine/cystine, and U(VI)/U(IV) redox couples (60). A pure culture example of this is *Sulfurospirillum deleyianum*, which cannot directly reduce Fe(III) but can use the cysteine/cystine or $S^0/S^=$ redox couples as shuttles for Fe(III) reduction (81).

Thus, in order to better understand the diversity of strategies for Fe(III) reduction, evaluation of the mechanisms for extracellular electron transfer in more organisms is warranted. The mechanism of Fe(III) reduction in *Pelobacter* species is of special interest because of the unique phylogenetic placement and physiology of these microorganisms (42, 51). *Pelobacter* species are phylogenetically interspersed with *Geobacter* and *Desulfuromonas* species, which completely oxidize acetate and other organic compounds with Fe(III), and often S^0, as the electron acceptor. Although *Pelobacter* species are capable of Fe(III) and S^0 reduction (42, 51), they only incompletely oxidize organic substrates with these electron acceptors and reduce smaller amounts of Fe(III) than *Geobacter* and *Desulfuromonas* species. Studies with *Pelobacter carbinolicus*, the most effective Fe(III) reducer of the *Pelobacter* species that have been evaluated, demonstrated that it lacks most of the *c*-type cytochromes that are essential for optimal Fe(III) reduction in *Geobacter sulfurreducens* (27). Furthermore, whereas *Geobacter* and *Desulfuromonas* species are capable of transferring electrons to the graphite anodes of microbial fuel cells for electricity production, *P. carbinolicus* is not (73). These results suggest that despite their close phylogenetic association with *Geobacter* and *Desulfuromonas* species, *Pelobacter* species may have a different mechanism for Fe(III) reduction.

Here we report on the results of microarray analysis of gene expression during growth on Fe(III) as well as physiological studies, both of which suggest that *P. carbinolicus* reduces Fe(III) indirectly via sulfur cycling rather than by the direct reduction of Fe(III) found in *Geobacter* species.

MATERIALS AND METHODS

Media and culture conditions. *P. carbinolicus* DSM 2380 was cultured as previously described (27). Sterile Na_2S (0.02 mM) was added to all cultures as a reductant unless otherwise noted. Fe(III) was provided as 5 mM Fe(III) nitrilotriacetic acid (NTA) (74) or 100 mM poorly crystalline Fe(III) oxide (50). Due to the salinity of the medium, much of the Fe(III) NTA was insoluble. Electron donors for Fe(III) reduction were ethanol (2 mM) or hydrogen. When hydrogen served as electron donor, media were bubbled with H_2-CO_2 (80:20) rather than N_2-CO_2, and 5 mM acetate was provided as a carbon source. Fermentative cultures contained 10 mM acetoin. Cultures were incubated at 30°C without shaking unless they contained hydrogen, in which case they were shaken at 140 rpm. Growth of Fe(III)-reducing cultures containing 0.02 mM sulfide was monitored by the ferrozine assay (48) and by counting acridine orange-stained cells by epifluorescence microscopy (49). Optical density at 600 nm of acetoin-fermenting cultures was measured with a Genesys 2 spectrophotometer (Spectronic Instruments, Rochester, NY).

Acetoin-fermenting *P. carbinolicus* cultures were cultured for more than five 1% transfers without sulfide in order to determine whether sulfate could serve as the sole sulfur source. To test the effect of sulfide and other compounds on Fe(III) reduction, cultures were amended with the following from sterile, anoxic stocks (final concentrations): sodium sulfide (0.02 and 1 mM), elemental sulfur (0.02 and 0.2 g/liter), cysteine (1 mM), L-cystine (0.1 mM), sodium thiosulfate (0.1 mM), sodium sulfite (0.1 mM), $FeCl_2$ (1.4 mM), and anthraquinone-2,6-disulfonate (AQDS; 50 μM). Control cultures lacking amendments were also initiated. Fe(III) reduction was monitored by measuring Fe^{2+} production with the ferrozine assay (48) weekly for 1 month.

Fe(III) reduction rates and cell yields. Fe(III) reduction rate was calculated from duplicate or triplicate cultures using two exponential-phase time points (t_1 and t_2): Fe(III) reduction rate = $(Fe_2 - Fe_1)/[(t_2 - t_1)(C_2 - C_1/10^7)]$, where Fe is μM of Fe(II), t is time in hours, and C is cell counts in cells ml^{-1}. Cell yield was calculated from duplicate or triplicate exponential and stationary-phase cultures as follows: cell yield = $[C/(\text{Fe}/1{,}000\ \text{ml/liter})]/10^{10}$ cells.

RNA extraction. Total RNA was extracted from triplicate mid-log-phase cultures grown with acetoin or Fe(III) NTA-ethanol using previously described methods for *P. carbinolicus* Fe(III) NTA-ethanol-grown cells (27, 32). RNA concentration and quality were determined by visualization on agarose gels and with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA was checked for DNA contamination by PCR with RNA as the template using previously described primers and PCR conditions (27).

Microarray analysis. Electrochemically synthesized oligonucleotide microarrays (Customarray 12K; CombiMatrix, Mukilteo, WA) were designed based on the *P. carbinolicus* DSM 2380 genome sequence (GenBank accession no. CP000142). Arrays consist of 11,844 different oligonucleotides, with an average of 3.5 probes per gene.

RNA amplification, labeling, hybridization, scanning, and statistical analyses were done as described elsewhere (68). Genes were considered to be significantly differentially expressed if M ($\log_2$ ratio) was ≥1 and P was ≤0.001 for at least 50% of the oligonucleotides for that gene.

Real-time RT-PCR quantification of gene expression. Primers were designed for five genes that were significantly differentially expressed in microarrays: Pcar_2144, *pilA*-99F (5′-CCAGTTTGCCGCCTATCGT-3′) and *pilA*-202R (5′-ACACCTGCCAATCGGTAAAGA-3′); Pcar_0251, *adh*-12F (5′-CAACCTCG CAGAAACCACGTA-3′) and *adh*-112R (5′-CGCCGAGTTCATTGGCTTTA-3′); Pcar_0343, *acoA*-142F (5′-GCTGTGGCTGTTGGTGTTTG-3′) and *acoA*-308R (5′-TGCATGGAACCGCCCTTA-3′); Pcar_0893, *afuA*-114F (5′-GACCG GCACTGAAATTCGTT-3′) and *afuA*-222R (5′-GGCGTCAACAGTGATGA GAAGA-3′); and Pcar_1718, *cysK*-118F (5′-CCCGGAGGCAGTGTCAAG-3′) and *cysK*-220R (5′-CACTGGTCGGTTCGATGATG-3′). Primers were tested in PCRs with *P. carbinolicus* genomic DNA extracted from acetoin-fermenting cultures with the MasterPure DNA purification kit (Epicenter Biotechnologies, Madison, WI) using previously described PCR conditions (27). PCR products were visualized by agarose gel electrophoresis, purified with the QIAquick gel extraction kit (Qiagen), and cloned with the TOPO TA cloning kit (Invitrogen), and a minimum of eight clones were sequenced to confirm that the correct product was amplified. Serial dilutions of purified PCR products made using the same primers and covering a range of 7 orders of magnitude (10^5 to 10^{11} molecules) were used as standards for real-time reverse transcription (RT)-PCR quantification as previously described (32). cDNA was made by RT as previously described (27, 32) and quantified in a GeneAmp 5700 sequence detection system, using GeneAmp 5700 SDS software (Applied Biosystems, Foster City, CA). The precision and reproducibility of the quantification were optimized, and lengths of PCR products were checked as described previously (14). Forward and reverse primers were added to the reaction mixture at a final concentration of 150 nM each along with 9.5 μl of cDNA (20 to 250 ng μl^{-1}) and 12.5 μl of Power Sybr green PCR master mix (Applied Biosystems, Foster City, CA) to a final volume of 25 μl. The temperature profile was composed of an initial incubation step at 50°C for 2 min, followed by a 10-min denaturation step at 95°C, 50 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min. Amplification and correct amplicon size were verified by agarose gel electrophoresis.

Microarray data accession number. Probe sequences and complete microarray data files are available in the NCBI Gene Expression Omnibus (GEO) under accession no. GSE8338.

RESULTS AND DISCUSSION

Fe(III) reduction rates and cell yields. In order to evaluate the mechanism for Fe(III) reduction in *P. carbinolicus*, growth rates and yields during growth on Fe(III) were quantified. Previous studies have noted that the total Fe(III) reduced in *Pelobacter* cultures is typically lower than that observed with other microorganisms (51), but rates of Fe(III) reduction in exponential-phase *P. carbinolicus* cultures and cell yield per mole of Fe(III) reduced were comparable to those reported for other Fe(III) reducers (Table 1 and Fig. 1). Growth was slower

TABLE 1. Cell-specific Fe(III) reduction rates and cell yields for *P. carbinolicus* grown in batch culture with ethanol or hydrogen as the electron donor and Fe(III) NTA or Fe(III) oxide as the electron acceptor

Acceptor	Donor	Fe(III) reduction rate [μM Fe(II)/h/10^7 cells]	Cell yield [10^{10} cells/mmol Fe(II)]
Fe(III) NTA	Ethanol	61.1 ± 36.6	1.40 ± 0.25
	Hydrogen	158.7 ± 40.4	0.94 ± 0.12
Fe(III) oxide	Ethanol	34.1 ± 4.7	1.19 ± 0.17
	Hydrogen	54.9 ± 5.4	0.65 ± 0.08

with Fe(III) oxide as the electron acceptor, than with soluble Fe(III) (Table 1), as is typical for Fe(III)-reducing bacteria (11, 49). Yields were higher with ethanol as the electron donor than with hydrogen. This may reflect differences in energy conservation with the different electron donors: via substrate-level phosphorylation during ethanol oxidation versus likely ATP generation via generation of a proton motive force across the inner membrane during hydrogen oxidation.

Although the growth yield of *P. carbinolicus* is similar to that of other Fe(III) reducers, this does not necessarily indicate a similar mechanism of extracellular electron transfer to Fe(III). The energy yield per Fe(III) reduced via chemiosmotic mechanisms is only dependent upon the number of protons pumped across the inner membrane per electron transferred. This is dependent upon the nature of the inner membrane electron carriers. Once electrons are transferred to periplasmic electron carriers, the mechanisms for electron transfer from the periplasm to the outside of the cell are not expected to contribute to energy conservation (24). Thus, organisms with substantially different routes for electron transfer through the periplasm and outer membrane might still have similar yields for growth on Fe(III). Thus, further studies were required to determine potential differences in mechanisms for Fe(III) reduction in different microorganisms.

Gene expression during Fe(III) reduction versus fermentation. Microarray analysis of gene expression during growth on extracellular electron acceptors versus other modes of growth has proven to be a useful strategy for identifying genes whose products are important in extracellular electron transfer (2, 4, 30, 54). In addition to growing via Fe(III) reduction, *P. carbinolicus* can also grow via fermentation of various substrates, including acetoin (77). Therefore, transcript levels in mid-log-phase cultures of *P. carbinolicus* growing with ethanol as the electron donor and Fe(III)-NTA as the electron acceptor were compared with transcript levels during acetoin fermentation.

At a *P* value cutoff of 0.001, 51 and 65 genes had at least twofold-higher transcript levels during Fe(III) reduction and acetoin fermentation, respectively (see Table S1 in the supplemental material). Real-time quantitative RT-PCR (qRT-PCR) was used to independently confirm microarray results for five differentially expressed genes (Table 2). qRT-PCR results indicated larger changes in expression between the two conditions than did microarrays, which has been observed repeatedly with different microarray platforms (54, 67, 68).

As expected, some of the differential gene expression was consistent with the different growth substrates. For example, a number of the genes with higher transcript levels during Fe(III) reduction with ethanol encode enzymes that catalyze a complete pathway for ethanol oxidation to acetate (Fig. 2). Seven genes in the *P. carbinolicus* genome are annotated as class IV alcohol dehydrogenases, and two of these, Pcar_0251 and Pcar_0255, were upregulated fourfold during Fe(III) reduction (Fig. 2). These two proteins have 94% amino acid identity with each other and are homologous with Fe-containing alcohol dehydrogenases that catalyze oxidation of alcohols with electron transfer to NADH (19, 21, 22). Two putative acetaldehyde dehydrogenases were also upregulated, one annotated as a tungsten-containing aldehyde:ferredoxin oxidoreductase (*aorA*; Pcar_0456) and the other as an NAD-dependent aldehyde dehydrogenase (*aldH*; Pcar_2758). Based on homology with characterized proteins, AorA is expected to produce acetate directly from acetaldehyde with electron transfer to ferredoxin (38, 55). AldH is homologous with the acetaldehyde dehydrogenase portion of multifunctional AdhE proteins in *Escherichia coli* and *Entamoeba histolytica*, which oxidize acetaldehyde to acetyl coenzyme A (acetyl-CoA) with electron transfer to NADH (9, 36).

During ethanol oxidation, one ATP is produced from acetyl-CoA by substrate-level phosphorylation by the activity of phosphotransacetylase and acetate kinase, but the genes encoding these enzymes were not differentially expressed during Fe(III) reduction or acetoin fermentation, probably because the pathway is functional during both growth conditions. Production of acetate from acetaldehyde by aldehyde:ferredoxin oxidoreductase would not produce ATP by substrate-level phosphorylation, but the reduced ferredoxin produced in this reaction can be used to produce pyruvate (for biosynthesis) from acetyl-

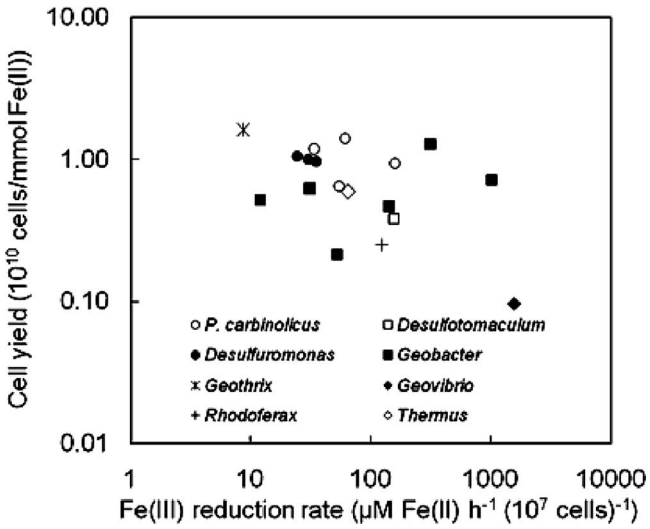

FIG. 1. Fe(III) reduction rates and cell yields for *P. carbinolicus* (Table 1) and other Fe(III)-reducing bacteria. Electron acceptors/donors and references for other bacteria are as follows: *Geobacter sulfurreducens*, Fe(III) citrate/acetate (23), Fe(III) pyrophosphate/acetate (11), Fe(III) oxide/acetate (11), and Fe(III) citrate/hydrogen (20); *Geobacter metallireducens*, Fe(III) oxide/acetate (45); *Geobacter chapellei*, Fe(III) NTA/acetate (18); *Geobacter hydrogenophilus*, Fe(III) citrate/hydrogen (18); *Desulfuromonas acetoxidans*, Fe(III) oxide/acetate (74); *Desulfuromonas palmitatis*, Fe(III) citrate/acetate (17); Fe(III) oxide/acetate (17); *Geothrix fermentans*, Fe(III) pyrophosphate/palmitate (16); *Rhodoferax ferrireducens*, Fe(III) NTA/acetate (25); and *Thermus* strain SA-01, Fe(III) NTA/lactate (37).

TABLE 2. Gene expression ratios of selected differentially expressed genes in *P. carbinolicus* Fe(III) NTA-ethanol versus acetoin-fermenting cultures determined with microarrays and real-time qRT-PCR

Gene	Name	Annotation	M value[a]	
			Microarray	RT-PCR
Pcar_2144	*pilA*	Type IV pilin structural subunit	1.56	1.72
Pcar_0251	*adh*	Alcohol dehydrogenase, class IV	2.13	3.68
Pcar_0343	*acoA*	Acetoin:DCPIP oxidoreductase, α subunit	−3.73	−7.04
Pcar_0893	*afuA*	ABC-type iron transport system, periplasmic component	−2.84	−7.69
Pcar_1718	*cysK*	Cysteine synthase A	−3.10	−13.47

[a] Positive M ($\log_2$ intensity of experimental/control) values are more highly expressed during Fe(III) reduction, and negative M values are more highly expressed during acetoin fermentation.

CoA, catalyzed by pyruvate:ferredoxin oxidoreductase (*por*; Pcar_0377), which is upregulated 2.5-fold during Fe(III) reduction (Fig. 2). The *P. carbinolicus* Por protein has 73% amino acid identity with GSU0097, the only functional pyruvate:ferredoxin oxidoreductase in *G. sulfurreducens* (79). Overall, the reactions catalyzed by the enzymes that are transcriptionally upregulated during growth on ethanol and Fe(III) are expected to produce two NADH per ethanol oxidized. The majority of the electrons are thought to contribute to energy production, but some are expected to be transferred via ferredoxin for biomass synthesis.

During fermentative growth with acetoin, a cluster of 22 genes (Pcar_0329 to Pcar_0351) encoding proteins involved in acetoin and 2,3-butanediol oxidation, was upregulated 2.1-to 4.5 fold (see Table S1 in the supplemental material). This cluster includes the genes encoding the acetoin dehydrogenase system, which was previously characterized in *P. carbinolicus* (65, 66). The only gene in this cluster that was not differentially expressed was Pcar_0336 (*acoR*), a transcriptional activator of acetoin metabolism. Other genes of interest with higher transcript levels during acetoin fermentation were genes involved in sulfate assimilation, cysteine biosynthesis, and iron uptake (see Table S1 in the supplemental material), suggesting that cysteine and iron might have been limiting during fermentative growth.

Identification of genes potentially associated with Fe(III) reduction. The genes with the greatest increase in transcript levels during growth on Fe(III) were in the cluster Pcar_0426 to Pcar_0429 (Table 3). The same orientation and similar increase in expression of these adjacent genes suggest that they may form an operon. This putative operon does not appear to be conserved in any other sequenced organism, and each gene is most similar to genes in diverse phylogenetic groups.

Pcar_0426 and Pcar_0427 are predicted to encode periplasmic thioredoxins that are 36% identical to one another. Thioredoxins maintain cellular redox potential, are involved in the oxidative stress response, and also regulate the activity of other enzymes (87). Pcar_0428 has homology with outer membrane transport proteins involved in aromatic degradation and long-chain fatty acid transport (5, 56). Pcar_0429 is annotated as an uncharacterized NAD(FAD)-dependent dehydrogenase. The N-terminal domain of Pcar_0429 has homology with pyridine nucleotide disulfide oxidoreductases, the central domain with rhodanese (sulfurtransferase), and the C-terminal domain with uncharacterized conserved proteins. Rhodanese domains are found in a wide variety of proteins, where they act as sul-

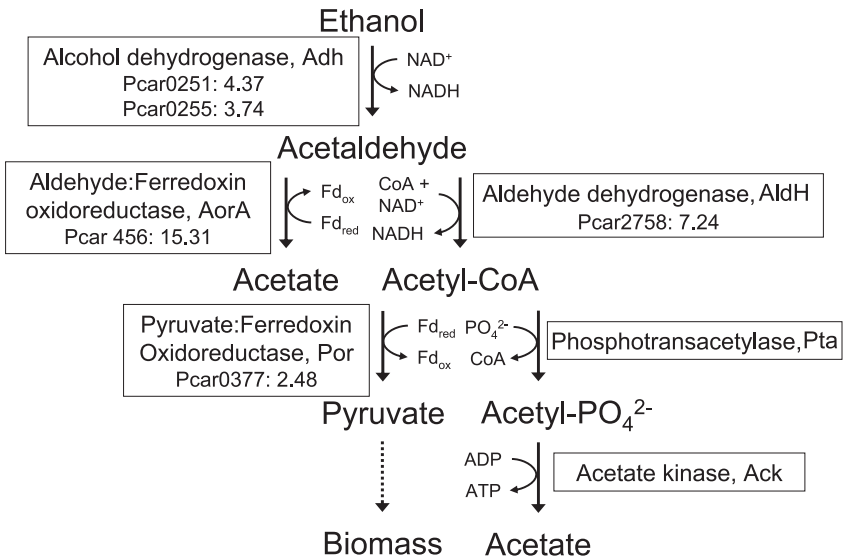

FIG. 2. Proposed pathway for ethanol oxidation in *P. carbinolicus*, based on microarrays comparing Fe(III) NTA reduction with ethanol versus acetoin fermentation. In boxes are shown enzyme names, gene numbers, and change (fold) increase for genes with higher transcript levels during ethanol oxidation.

TABLE 3. Gene cluster with the largest increase in transcript levels in *P. carbinolicus* during Fe(III) reduction with ethanol in comparison with acetoin fermentation

Gene	Annotation	M value[a]	Fold change
Pcar_0426	Conserved hypothetical protein, thioredoxin related	3.47	11.08
Pcar_0427	Conserved hypothetical protein, thioredoxin related	4.38	20.88
Pcar_0428	OMPP1/FadL/TodX family toluene transport protein	4.66	25.23
Pcar_0429	Uncharacterized NAD(FAD)-dependent dehydrogenase	4.36	20.55

[a] The M value is the $\log_2$ intensity of experimental/control.

furtransferases in a variety of metabolic and regulatory pathways (8, 15).

Unfortunately, attempts to develop a genetic system for *P. carbinolicus* have not yet been successful, making it difficult to further investigate the potential role of these proteins in Fe(III) reduction. However, the coregulation of proteins potentially involved with the reduction of sulfur-containing compounds and a putative outer membrane transport protein suggest that sulfur-containing compounds could serve as intermediates in electron transfer across the periplasm and/or outer membrane.

Although *c*-type cytochromes are generally considered to be the primary agents for extracytoplasmic electron transfer in *Geobacter* (10, 40, 41, 46, 53, 74) and *Shewanella* (3, 26, 57–59, 80) species, previous studies have demonstrated that *P. carbinolicus* has a much lower number of *c*-type cytochrome genes than closely related *Geobacter* species and that the abundance of the existing cytochromes is much lower than in *Geobacter* species (27). *P. carbinolicus* has four genes for type II cytochrome *c* biogenesis organized in two operons (27), and one operon, Pcar_1953 to Pcar_1954, was upregulated 8- to 10-fold during Fe(III) reduction (see Table S1 in the supplemental material). The second cytochrome *c* biogenesis operon (Pcar_2228 to Pcar_2229) did not show significant differences in transcript levels during Fe(III) reduction or acetoin fermentation. Furthermore, none of the 14 *P. carbinolicus c*-type cytochromes (27) had statistically significant changes in transcript levels during Fe(III) reduction or acetoin fermentation. This contrasts with the increased expression of *c*-type cytochrome genes known to be essential for optimal extracellular electron transfer in *G. sulfurreducens* (30, 54) or *Shewanella oneidensis* (2, 4) during growth with Fe(III) or an electrode as the electron acceptor. Therefore, an alternative to *c*-type cytochromes, such as the sulfur compounds suggested above, was postulated to be necessary for electron transfer to Fe(III) in *P. carbinolicus*.

The requirement in *G. sulfurreducens* for PilA, the structural type IV pilin protein, for Fe(III) oxide reduction; the apparent specific association of Fe(III) oxides with these pili; and the finding that the pili are electrically conductive has led to the suggestion that the type IV pili might function as "microbial nanowires" and serve as the final conduit for electron transfer to Fe(III) oxides in *G. sulfurreducens* (69). Pcar_2144, which encodes PilA in *P. carbinolicus*, had threefold-higher transcript levels during Fe(III) reduction compared with acetoin fermentation (see Table S1 in the supplemental material). In *G. sulfurreducens*, PilA is also likely to be involved in electron transfer through biofilms on the anodes of microbial fuel cells and/or play a structural role in biofilm formation (70, 71). *P. carbinolicus* is unable to transfer electrons to graphite electrode surfaces even though it can syntrophically grow in fuel cell systems, oxidizing ethanol to acetate and hydrogen, which *G. sulfurreducens* oxidizes with electron transfer to the anode (73). Thus, although increased expression of *pilA* during growth of *P. carbinolicus* on Fe(III) is consistent with the concept (26, 69) that pili may function as "microbial nanowires," it would be premature to conclude that the pili of *P. carbinolicus* are definitely involved electron transfer to Fe(III) without appropriate genetic studies, which at this time are not feasible.

For growth with Fe(III) as the electron acceptor, electrons must be transferred from cytoplasmic NADH to the inner membrane. *P. carbinolicus* has all of the genes for NADH dehydrogenase (complex I, Pcar_0205 to Pcar_0216), but only one of these genes had higher transcript levels during Fe(III) reduction (Pcar_0213, *nuoK*, twofold higher; see Table S1 in the supplemental material). However, there are alternatives. Other genes with higher transcript levels encoding proteins with putative NADH dehydrogenase activity include Pcar_0429, discussed above, and Pcar_1579, both of which are annotated as uncharacterized NAD(FAD)-dependent dehydrogenases. Pcar_1579 is adjacent to Pcar_1578, which has similarity to cytoplasmic membrane-bound arsenite efflux pumps (76), and related permeases.

Previous studies have suggested that flagellum-driven motility may be an important feature in Fe(III) reduction as flagella are required for cells to locate and access insoluble Fe(III) oxides (12). Seven flagellar genes were more highly expressed in *P. carbinolicus* during Fe(III) reduction (see Table S1 in the supplemental material). Gene expression, including flagellar motility, may be affected by signaling proteins encoded by five genes that have higher transcript levels during Fe(III) reduction: Pcar_2075, signal transduction protein; Pcar_0569, FOG: GGDEF domain-containing protein; Pcar_1985 and Pcar_1199, methyl-accepting chemotaxis proteins; and Pcar_2710, a two-component sensor histidine kinase (see Table S1 in the supplemental material).

Requirement for sulfide or S^0 for Fe(III) reduction. As a result of the microarray study, which suggested a potential role for sulfur compounds in Fe(III) reduction, the effect of various sulfur compounds on Fe(III) reduction was investigated. In the medium used for growth of *P. carbinolicus*, sulfate is added as sulfur source and sulfide is added as a reductant. However, *P. carbinolicus* fermented acetoin and 2,3-butanediol in the absence of sulfide for more than five 1% transfers (data not shown). Therefore, the sulfate in the medium is a sufficient source of sulfur. However, with ethanol as the electron donor and Fe(III) as the electron acceptor, growth and Fe(III) re-

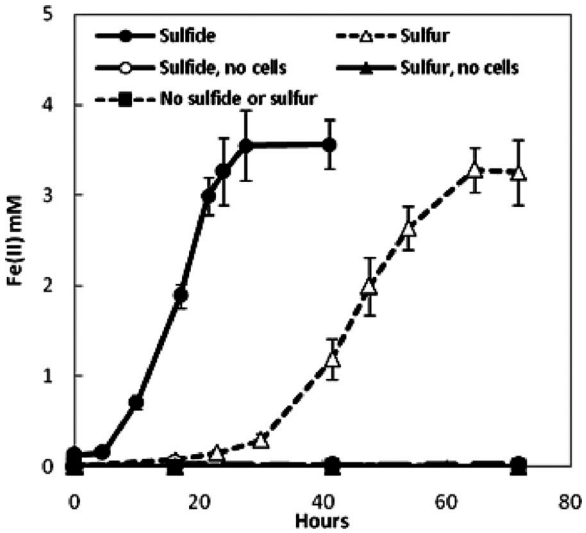

FIG. 3. Growth of *P. carbinolicus* with 2 mM ethanol and 5 mM Fe(III) NTA, in the presence and absence of 0.02 mM sulfide and 0.2 g/liter elemental sulfur. Points are averages of triplicate cultures, and error bars are standard deviations.

duction were only observed in the presence of sulfide or elemental sulfur (Fig. 3). Sulfide concentrations of 0.2 to 1.0 mM supported Fe(III) reduction (Fig. 3; data not shown), while 0.2 g/liter elemental sulfur (about 6 mM; Fig. 3) was required. Addition of 10-fold less elemental sulfur did not support Fe(III) reduction (data not shown). Elemental sulfur is insoluble, and a higher concentration may be required because insoluble sulfur is less accessible to the cells. Other compounds tested in cultures in place of sulfide or sulfur that did not support Fe(III) reduction by *P. carbinolicus* included cysteine, cystine, thiosulfate, sulfite, $FeCl_2$, and AQDS. In previous studies washed-cell suspensions of *P. carbinolicus* reduced Fe(III) in the absence of added sulfide or S^0 (51), but the amounts of Fe(III) reduced were minor.

The finding that either sulfide or S^0 is required for growth with Fe(III) as an electron acceptor, but that sulfate serves as sulfur source for fermentative growth, suggests that *P. carbinolicus* does not directly reduce Fe(III), but rather reduces Fe(III) via an indirect mechanism in which S^0 is reduced to sulfide and the sulfide reacts with Fe(III), reducing it to Fe(II) and reforming S^0, which can then initiate another reduction/oxidation cycle.

Implications. These results suggest that despite the similarity in growth yields with other dissimilatory Fe(III)-reducing microorganisms and its close relatedness to *Geobacter* and *Desulfuromonas* species, *P. carbinolicus* has a significantly different mechanism for Fe(III) reduction. The lack of increased expression of any *c*-type cytochrome genes during growth on Fe(III) versus fermentative growth suggests that none of the cytochromes that are present in *P. carbinolicus* are key in Fe(III) reduction. Three *c*-type cytochromes were previously shown to be expressed during Fe(III) reduction but not acetoin fermentation by *P. carbinolicus* (27). This study involved a nonquantitative method for detection of transcripts, and cultures grown at different times, which could account for the different results of the two studies. The substantial upregulation of genes that could be associated with reduction and transport of sulfur-containing redox-active proteins during growth on Fe(III) suggests that their role in Fe(III) reduction should be further evaluated once a genetic system for *P. carbinolicus* is developed.

The proposed mechanism of electron shuttling between *P. carbinolicus* and Fe(III) via the $S^0/S^=$ redox couple has previously been observed with other organisms (60, 81). In the case of *Sulfurospirillum deleyianum*, this form of sulfur cycling was the only mechanism by which Fe(III) was reduced (81), and this appears to also be the case for *P. carbinolicus*. In contrast, although the addition of S^0 stimulated Fe(III) oxide reduction in cell suspensions of *G. sulfurreducens* (60), *G. sulfurreducens* and other *Geobacter* species are routinely cultured on Fe(III) without sulfide or S^0, with sulfate as the sole sulfur source (49). Furthermore, *G. metallireducens*, one of the most effective Fe(III) oxide-reducing *Geobacter* species, cannot grow with S^0 as an electron acceptor (46). *Desulfuromonas acetoxidans* also does not require sulfide or S^0 for effective Fe(III) reduction (74).

The finding that *P. carbinolicus* may indirectly reduce Fe(III), whereas *Geobacter* and *Desulfuromonas* species reduce it directly, leads to the question of which mechanism of Fe(III) reduction the common ancestor of the *Geobacteraceae* possessed. Analysis of the diversity of *Geobacteraceae* genome sequences that are becoming available may aid in answering this question. The apparent diversity of mechanisms for extracellular electron transfer in the few dissimilatory Fe(III)-reducing microorganisms that have been studied to date suggests that study of electron transfer mechanism in other Fe(III) reducers is warranted in order to search for commonalities and understand differences in strategies for extracellular electron transfer.

ACKNOWLEDGMENTS

We thank Nicholas Pacelli and Jennifer Hart for technical assistance.

This research was supported by the Office of Science (BER), U.S. Department of Energy, grant no. DE-FC02-02ER63446.

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