c-Type Cytochromes in *Pelobacter carbinolicus*

Shelley A. Haveman,* Dawn E. Holmes, Yan-Huai R. Ding, Joy E. Ward, Raymond J. DiDonato, Jr., and Derek R. Lovley

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

Received 15 May 2006/Accepted 14 August 2006

Previous studies failed to detect c-type cytochromes in *Pelobacter* species despite the fact that other close relatives in the Geobacteraceae, such as *Geobacter* and *Desulfuromonas* species, have abundant c-type cytochromes. Analysis of the recently completed genome sequence of *Pelobacter carbinolicus* revealed 14 open reading frames that could encode c-type cytochromes. Transcripts for all but one of these open reading frames were detected in acetoin-fermenting and/or Fe(III)-reducing cells. Three putative c-type cytochrome genes were expressed specifically during Fe(III) reduction, suggesting that the encoded proteins may participate in electron transfer to Fe(III). One of these proteins was a periplasmic triheme cytochrome with a high level of similarity to PpcA, which has a role in Fe(III) reduction in *Geobacter sulfurreducens*. Genes for heme biosynthesis and system II cytochrome c biogenesis were identified in the genome and shown to be expressed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of protein extracted from acetoin-fermenting *P. carbinolicus* cells contained three heme-staining bands which were confirmed by mass spectrometry to be among the 14 predicted c-type cytochromes. The number of cytochrome genes, the predicted amount of heme c per protein, and the ratio of heme-stained protein to total protein were much smaller in *P. carbinolicus* than in *G. sulfurreducens*. Furthermore, many of the c-type cytochromes that have indicated are required for optimal Fe(III) reduction in *G. sulfurreducens* were not present in the *P. carbinolicus* genome. These results suggest that further evaluation of the functions of c-type cytochromes in the Geobacteraceae is warranted.

*Pelobacter* species seem to be an anomaly within the family Geobacteraceae. They are phylogenetically intertwined with *Geobacter* and *Desulfuromonas* species and have the capacity to use Fe(III) as an electron acceptor (29, 34), yet they were previously found to lack c-type cytochromes (34, 43–47), which are abundant in *Geobacter* and *Desulfuromonas* species and are thought to be important in electron transfer to Fe(III) in these organisms (8, 25, 28, 30, 35, 41). This has led to questions about the evolution of the different genera within the Geobacteraceae and about the true role of c-type cytochromes in Fe(III) reduction in this family. In fact, the apparent lack of c-type cytochromes in *Pelobacter* but conservation of the structural gene for electrically conductive pilin is one line of evidence suggesting that pili serve as the electrical conduit between the outer surface of Geobacteraceae cells and Fe(III) oxides (40).

*Pelobacter* species are common in anaerobic subsurface environments (16, 22, 37, 50, 52). *Pelobacter carbinolicus*, which grows by fermentation of butanediol, acetoin, and ethylene glycol to ethanol and acetate, was isolated from marine mud (43). *P. carbinolicus* can also grow by oxidizing ethanol and other alcohols (i) in coculture with *H₂*-oxidizing methanogens or acetogens (43) or (ii) with Fe(III) or S⁰ as an electron donor and H₂ are equivalent to those for *Geobacter* species (E. S. Shelobolina, unpublished data).

Analysis of the recently completed genome sequence of *P. carbinolicus* DSM2380 (www.jgi.doe.gov) led to the surprising finding that this organism contains genes predicted to encode c-type cytochromes, as well as genes for heme biosynthesis and cytochrome c biogenesis. Here we report that most of these c-type cytochrome genes are expressed under one or more growth conditions and that low levels of c-type cytochromes can be detected biochemically.

**MATERIALS AND METHODS**

Bioinformatics. The predicted protein-encoding sequences in the *P. carbinolicus* genome sequence (www.jgi.doe.gov) were searched for CXXCH heme c binding motifs using the FindPatterns algorithm of the Genetics Computer Group Wisconsin Package, version 10.3 (Accelrys Inc., San Diego, CA). The subcellular location of the CXXCH-containing putative proteins was predicted using several programs, including PSORTb (12), SubLoc (19), TMPred (15), and SignalP (5). Conserved LXXC CXXCH-containing putative proteins was predicted using several programs, including PSORTb (12), SubLoc (19), TMPred (15), and SignalP (5). Conserved LXXC CXXCH-containing putative proteins was predicted using several programs, including PSORTb (12), SubLoc (19), TMPred (15), and SignalP (5).

Media and culture conditions. *P. carbinolicus* DSM2380 was cultured at 30°C under strictly anaerobic conditions in media containing (per liter) 9.0 g of NaCl, 2.7 g of MgCl₂ · 6H₂O, 2.5 g of NaHCO₃, 0.25 g of NH₄Cl, 0.6 g of NaH₂PO₄ · H₂O, 0.1 g of KCl, and 0.14 g of CaCl₂ · 2H₂O. Vitamins and minerals (10 ml liter⁻¹ each) were added from stock solutions (31). Media were dispensed into anaerobic pressure tubes or bottles, and the tubes or bottles were gassed with 80% N₂–20% CO₂, scaled with butyl rubber stoppers, and autoclaved. Media were reduced with sterile Na₂S at a final concentration of 0.02 mM. Electron Fe(III) reduction with H₂ as the electron donor (34), and the cell yields per unit of Fe(III) reduced with both organic electron donors and H₂ are equivalent to those for *Geobacter* species (E. S. Shelobolina, unpublished data).

*Corresponding author. Mailing address: Department of Microbiology, University of Massachusetts, Amherst, MA 01003. Phone: (413) 577-0217. Fax: (413) 545-1578. E-mail: haveman@microbio.umass.edu.

Published ahead of print on 25 August 2006.
donors were added from sterile, anaerobic stock solutions at a final concentration of 10 mM (acetoin [3-hydroxy-2-butanone]) or 2 mM (ethanol). Fe(III) was provided in ethanol-containing cultures in the form of Fe(III) nitrilotriacetic acid donors were added from sterile, anaerobic stock solutions at a final concentration of 10 mM (acetoin [3-hydroxy-2-butanone]) or 2 mM (ethanol). Fe(III) was provided in ethanol-containing cultures in the form of Fe(III) nitrilotriacetic acid (NTA) at a final concentration of 5 mM (41). Due to the salinity of the medium, much of the Fe(III) was insoluble. Cell growth on acetoin was monitored by measuring the optical density at 600 nm with a Genesys 2 spectrophotometer (Spectronic Instruments, Rochester, NY). Fe(II) production in Fe(III) NTA was monitored with ferrozine (33).

Cytochromes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Forward primer (5'–3')</th>
<th>Reverse primer (5'–3')</th>
<th>Annealing temp for PCR (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCAR2944</td>
<td>Glutamate synthase, large subunit</td>
<td>CAGCATCGCTACGGTACG</td>
<td>ATATGGCGTACATGCGCG</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2570</td>
<td>Cytochrome c family protein</td>
<td>TCGTATCCTCCGTTGC</td>
<td>TGGCCTTTAGCAGCAAT</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2549</td>
<td>Hypothetical protein</td>
<td>CGGCGCTGTGGTGGTACTG</td>
<td>TGAACGGCATGAATGTTAC</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2529</td>
<td><em>ca</em> -type cytochrome c oxidase, subunit H</td>
<td>CTTGCGGTTGAGACGAAG</td>
<td>GGAGGCGGGATATGAAGG</td>
<td>60</td>
</tr>
<tr>
<td>PCAR0558</td>
<td>Cytochrome c family protein</td>
<td>CTGGGAAATTCCTGAACCA</td>
<td>AGTATTGCTGGCAAAAGCG</td>
<td>60</td>
</tr>
<tr>
<td>PCAR0192</td>
<td>Molybdopterin oxidoreductase/ci-precissin-4-methylase</td>
<td>CTGCCTGACTATCTG</td>
<td>CCGGCTTGTAGAACCTTCC</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2867</td>
<td>Cytochrome c nitrite reductase, mhf</td>
<td>ACCTGCTACTGGGCGCAAG</td>
<td>CATCAAGAAACCGGCAATC</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2866</td>
<td>Cytochrome c nitrite reductase mnf</td>
<td>TGGAAGACACTGGGATGAGA</td>
<td>ACTCGATGGCCGAAATTCCT</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2550</td>
<td>Cytochrome c</td>
<td>CACGGTTCACAAATTCACCA</td>
<td>CATCCCATTCTCAACACG</td>
<td>60</td>
</tr>
<tr>
<td>PCAR1628</td>
<td>Cytochrome c, ppcA</td>
<td>CTGTTGCGGACCATATCAA</td>
<td>GCAACACCACCAATGCCG</td>
<td>60</td>
</tr>
<tr>
<td>PCAR0152</td>
<td>Hypothetical protein</td>
<td>GATCTCCATGGTTTCTCTA</td>
<td>CATCAAAACGGGCAATC</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2864</td>
<td>Cytochrome c family protein</td>
<td>ATGAAATAATGCGGTTGATGCT</td>
<td>GCAACAGAAGCGGTTCTCT</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2745</td>
<td>Hypothetical protein</td>
<td>TGGTGGCGGATTTTCTTCA</td>
<td>TGCAGAATCATCCCGGATA</td>
<td>60</td>
</tr>
<tr>
<td>PCAR2069</td>
<td>Protein-disulfide isomerase</td>
<td>AACAGCCGCAAGAGTGGTTTG</td>
<td>GCGTGTACAGACGATGCTT</td>
<td>60</td>
</tr>
</tbody>
</table>

**Heme biosynthesis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'–3')</th>
<th>Reverse primer (5'–3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCAR3065</td>
<td>ccsA homolog</td>
<td>GCCGTTGATGCTGATGTTTT</td>
</tr>
<tr>
<td>PCAR3064</td>
<td>Glutamyl tRNA synthetase, hemA</td>
<td>GTCTATCGGCGGATGGTGG</td>
</tr>
<tr>
<td>PCAR2026</td>
<td>Glutamate 1-semialdehyde</td>
<td>CAGCCGGTTGTCGCTATTAA</td>
</tr>
<tr>
<td>PCAR0770</td>
<td>Ferrochelatase, hemH</td>
<td>GCGCGGTTGATGCGCTACT</td>
</tr>
</tbody>
</table>

**Cytochrome c biogenesis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'–3')</th>
<th>Reverse primer (5'–3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCAR2229</td>
<td>Heme transport and ligation, ccsB</td>
<td>GCTCGGTTGTCCTGAAACT</td>
</tr>
<tr>
<td>PCAR2228</td>
<td>Heme transport and ligation, ccsA</td>
<td>GAGGTGTCTGCGGAGG</td>
</tr>
<tr>
<td>PCAR1954</td>
<td>Thioricin, resA</td>
<td>TTTCTGGTGTCGTCTGC</td>
</tr>
<tr>
<td>PCAR1953</td>
<td>Thioricin, ccsA</td>
<td>GCCCTGTTGTATGCCGCT</td>
</tr>
</tbody>
</table>

**RT-PCR.** Reverse transcription (RT) was performed with an Enhanced Avian First Strand synthesis kit (Sigma) as described previously (17). Two negative controls lacking reverse transcriptase or RNA were included for each gene. PCRs were performed as described above, using 5 µl of the RT reaction mixture as the template for PCR. As described above for PCR products, RT-PCR products were gel purified, TOPO cloned, and sequenced.

**Proteomics.** Late-log-phase acetoin-grown *P. carbinolicus* cells or fumarate-acetate-grown *Gebacter sulfureducens* cells were harvested by centrifugation at 3,150 × g for 20 min at 4°C, and the cell pellets were stored at −20°C. Cells were washed in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl₂ and Complete protease inhibitor cocktail (Roche). Washed cells were suspended in the same buffer at a concentration of 0.2 g (wet weight) of cells per ml of buffer. The cells were lysed by sonication at 4°C. The cell debris was removed by centrifugation at 9,000 × g for 15 min, and the supernatant contained whole-cell protein. Ten micrograms of protein from each organism was separated on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, and c-type cytochromes were heme stained as described previously (11, 51).

Heme-stained protein bands were excised from the gel and washed with 500 µl of MilliQ water for 30 min at 20°C to remove the chemical residue. The gel pieces were dehydrated in 200 µl of 50 mM ammonium bicarbonate in 50% acetonitrile for 1 h, followed by acetonitrile for 30 min. Each gel piece was digested in 20 to 40 µl digestion buffer (20 mM ammonium bicarbonate containing 75 ng trypsin) at 37°C overnight. The supernatant was recovered, and the remaining peptides were extracted from the gel piece by washing it with 80% acetonitrile–1% formic acid in water. The extracts were pooled, and the volume was reduced to 5 to 10 µl with a Speedvac (Vaculfe, Germany). The eluted tryptic peptides were desalted and concentrated with a commercial ZipTip C₁₈ pipette tip (Millipore). Peptides were detected by matrix-assisted laser desorption ionization–time of flight mass spectrometry as previously described (20). Peptide mass fingerprints were analyzed by using the MS-FIT Protein Prospector program (UCSF Mass Spectrometry Facility).

**RESULTS AND DISCUSSION**

**c-Type cytochrome genes.** The *P. carbinolicus* genome sequence contains 58 CXXCH motifs in 42 predicted proteins. Individual predicted proteins contain between one and five
CXXCH motifs. Twenty-eight of the putative c-type cytochromes are predicted to be cytoplasmic and therefore not expected to bind heme c. Of the remaining 14 protein sequences, 4 are predicted to be associated with the cytoplasmic membrane, 6 are predicted to be periplasmic, and the other 4 may be outer membrane associated (Table 2).

One gene (PCAR1628) is predicted to encode a cytochrome c, which belongs to a family of well-conserved cytochromes in the Geobacteraceae, which includes cytochrome c7 in Desulfomonas acetoxidans (3, 6) and Geobacter metallireducens (1) and PpcA in G. sulfurreducens (28). In G. sulfurreducens this cytochrome appears to function as a periplasmic intermediary electron carrier between cytoplasmic electron donors and outer membrane-associated Fe(III) reductase (28).

PCAR2984 encodes a putative outer membrane diheme cytochrome c and is conserved in Geobacteraceae genomes upstream of a gene for pyruvate kinase. A mutant lacking the homolog in G. sulfurreducens (GSU3332) was incapable of reducing poorly crystalline Fe(III) oxides and was deficient in reduction of U(VI), suggesting that this cytochrome may play a role in electron transfer to extracellular electron acceptors (E. S. Shelobolina, unpublished data).

PCAR2069 encodes a putative outer membrane-associated Fe(III) reductase (28).

PCAR2867 and PCAR2866 encode proteins that are homologous to the two subunits of cytochrome c nitrite reductase, which catalyze nitrite reduction to ammonia in many bacteria (48) and are conserved in the Geobacteraceae. In P. carbinolicus PCAR2866 the unique CXXCK heme binding motif (48) is replaced by a CXXCH motif. The role of cytochrome c nitrite reductase in P. carbinolicus, which is not known to use nitrite as an electron acceptor, could be similar to that in Desulfovibrio vulgaris, in which nitrite reduction is not coupled to growth and cytochrome c nitrite reductase is used for nitrite detoxification (13, 39).

The periplasmic pentaheme cytochrome c encoded by PCAR2550 is part of a conserved operon (PCAR2550 to PCAR2553) that is predicted to encode a cytoplasmic membrane-bound quinol:cytochrome c oxidoreductase. A homologous complex was purified from Chloroflexus aurantiacus, and homologous operons are found in individual members of seven bacterial phyla, suggesting that the complex has been laterally transferred (53). A similar but distinct complex has been found in the genome sequences of G. metallireducens, D. vulgaris, and Desulfovibrio desulfuricans but not in the G. sulfurreducens sequence (53) or in the draft sequence of Desulfomonas acetoxidans (www.jgi.doe.gov). The gene encoding cytoplasmic membrane-bound monoheme cytochrome c, PCAR2549, is immediately upstream of PCAR2550, but this cytochrome has no significant similarity to other proteins.
PCAR2529 encodes subunit II of a caa₃-type cytochrome c oxidase (encoded by PCAR2526 to PCAR2529), which is homologous to the oxidases in other members of the Geobacteraceae, as well as the characterized oxidase in Rhodothermus marinus (42). G. sulfurreducens has a cytochrome c oxidase (36) and has been shown to grow with low levels of oxygen as a terminal electron acceptor (27).

PCAR0152 encodes a putative outer membrane cytochrome with no significant similarity to other proteins. This gene is located between the genes for an ABC-type transporter with homology to the Fep ferric enterobactin transporter (7) and therefore may play a role in the uptake of chelated Fe(III).

The functions of the remaining six putative cytochromes cannot be predicted by sequence homology, either because they have no significant BLAST hits or because they are similar to proteins that are not known to bind heme c. These cytochromes include two that are predicted to be cytoplasmic membrane bound, the cytochrome encoded by PCAR2570 and the glutamate synthase homolog encoded by PCAR2944; two periplasmic cytochromes, one encoded by PCAR0558 and one encoded by PCAR0192, the latter of which is homologous to molybdopterin oxidoreductase in the N terminus and pre-corrin-4 methylase in the C terminus; and two that are predicted to be outer membrane bound, the trimeric cytochrome encoded by PCAR2745 and the protein disulfide isomerase encoded by PCAR2069.

**Heme biosynthesis and cytochrome c biogenesis genes.** In order for the putative cytochromes to be functional, heme c must be covalently bound to the proteins in the periplasm. Formation of c-type cytochromes requires heme biosynthesis, transport of heme and apoprotein to the periplasm, and covalent attachment of heme to CXXCH motifs. *P. carbinolicus* possesses all of the genes required for heme biosynthesis in four regions of the genome (Table 3). Consistent with its anaerobic physiology, *P. carbinolicus* contains a homolog of the oxygen-independent protoporphyrinogen oxidase HemG (encoded by PCAR0772), which catalyzes the penultimate step in heme biosynthesis, but not HemY, which catalyzes the same reaction in an oxygen-dependent manner (10). Likewise, it contains a homolog of the oxygen-independent coproporphyrinogen III oxidase HemN (encoded by PCAR0110), but it lacks the oxygen-dependent form, HemF (10). hemACD are located in an operon with genes encoding phosphoheptose isomerase, siroheme synthase, and a cytochrome biogenesis protein homolog (PCAR3062 to PCAR3067). hemB is located downstream of this operon in a dicistronic operon containing a gene encoding a conserved hypothetical protein (PCAR3060 and PCAR3061).

The steps after heme biosynthesis are collectively referred to as the cytochrome c biogenesis pathway. There are three different systems for cytochrome c biogenesis, called systems I, II, and III (24, 38). System II is found in gram-positive bacteria, β- and ε-Proteobacteria, and chloroplasts (23, 24). *P. carbinolicus*, along with other members of the Geobacteraceae, has genes for the system II cytochrome c biogenesis pathway (49). The four genes fall into two operons (Table 3) encoding four integral membrane proteins. PCAR2228 and PCAR2229 encode CcsA and CcsB homologs, which transport heme to the periplasm and may catalyze the covalent linkage of heme to apocytochrome c (24). ResA (encoded by PCAR1954) is a thioredoxin that reduces the cysteines of the apocytochrome c so that heme can be attached, and CcdA (encoded by PCAR1953) reductases ResA (24).

**mRNA expression.** Most of the putative c-type cytochrome genes were expressed during fermentation and/or during Fe(III) reduction; the only exception was PCAR2570 (Table 2). PCAR0152, encoding the cytochrome associated with genes for the Fe³⁺-siderophore ABC transporter, was expressed during fermentation but not during Fe(III) reduction, supporting the hypothesis that this cytochrome has a role in iron uptake during iron limitation. Three cytochrome genes were expressed during Fe(III) reduction but not during fermentation, including PCAR1628 encoding the periplasmic cytochrome c₁ and PCAR2550 encoding the periplasmic penta-heme cytochrome in the quinol:cytochrome c oxidoreductase. Together, these two cytochromes could transport electrons from the quinone pool to the outer membrane. The gene encoding a cytoplasmic

---

**TABLE 3. Predicted *P. carbinolicus* heme biosynthesis and cytochrome c biogenesis genes and mRNA expression determined by RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Designation</th>
<th>Description</th>
<th>mRNA expression (acetoin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>heme biosynthesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCAR3065</td>
<td>hemA</td>
<td>CcsA homolog</td>
<td>+</td>
</tr>
<tr>
<td>PCAR3064</td>
<td></td>
<td>Glutamyl tRNA synthase</td>
<td>+</td>
</tr>
<tr>
<td>PCAR3063</td>
<td>hemC</td>
<td>Porphobilinogen deaminase</td>
<td>ND</td>
</tr>
<tr>
<td>PCAR3062</td>
<td>hemD</td>
<td>Uroporphyrinogen-III synthase/methyltransferase</td>
<td>ND</td>
</tr>
<tr>
<td>PCAR3061</td>
<td>hemB</td>
<td>ALA dehydratase</td>
<td>ND</td>
</tr>
<tr>
<td>PCAR0266</td>
<td>hemL</td>
<td>Glutamate 1-semialdehyde aminotransferase</td>
<td>+</td>
</tr>
<tr>
<td>PCAR0769</td>
<td>hemE</td>
<td>Uroporphyrinogen-III decarboxylase</td>
<td>ND</td>
</tr>
<tr>
<td>PCAR0770</td>
<td>hemH</td>
<td>Ferrocyclotetase</td>
<td>+</td>
</tr>
<tr>
<td>PCAR0110</td>
<td>hemN</td>
<td>Coproporphyrinogen-III oxidase</td>
<td>ND</td>
</tr>
<tr>
<td>PCAR0772</td>
<td>hemG</td>
<td>Protoporphyrinogen-IX oxidase</td>
<td>ND</td>
</tr>
<tr>
<td>Cytochrome c biogenesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCAR2229</td>
<td>ccsB</td>
<td>Heme transport and ligation</td>
<td>+</td>
</tr>
<tr>
<td>PCAR2228</td>
<td>ccsA</td>
<td>Heme transport and ligation</td>
<td>+</td>
</tr>
<tr>
<td>PCAR1954</td>
<td>resA</td>
<td>Thioredoxin for reduction of CXXCH cysteines</td>
<td>+</td>
</tr>
<tr>
<td>PCAR1953</td>
<td>ccdA</td>
<td>Thioredoxin for reduction of ResA</td>
<td>+</td>
</tr>
</tbody>
</table>

* ND, not determined.

---
membrane-bound cytochrome related to glutamate synthase was also expressed only during Fe(III) reduction, but the role of this cytochrome is not clear.

Expression of selected genes in the heme biosynthesis pathway was determined by RT-PCR. These genes included the genes encoding the enzymes for first two steps in the pathway (hemA and hemL) and the final step (hemH), all of which were expressed during fermentation (Table 3). A ccsA homolog in the same operon as hemA was also expressed. The four cytochrome c biogenesis genes were expressed as well (Table 3). Therefore, all of the genes necessary for heme biosynthesis and cytochrome c biogenesis are present and expressed in P. carbinolicus, which allows functional c-type cytochromes to be produced.

**Protein expression.** Previous attempts to detect cytochromes in P. carbinolicus by difference spectroscopy of crude cell extracts and membrane fractions of fermentatively grown cells (43) or of intact washed cells (34) were unsuccessful. Likewise, no cytochromes were detected by difference spectroscopy of fermentatively grown cells, but when whole-cell protein from these cells was electrophoresed on SDS-PAGE gels and heme stained, three bands were detected (Fig. 1). The same amount of protein from G. sulfurreducens produced many more bands and contained much more heme-containing protein (Fig. 1). Proteins in the three heme-stained P. carbinolicus bands were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry, and the results were in agreement with the RT-PCR results. The following three identified cytochromes were all predicted to be soluble: cytochrome c nitrite reductase encoded by PCAR2866, molybdopterin oxidoreductase/precorrin-4 methylase encoded by PCAR0192, and the cytochrome c family protein encoded by PCAR0558.

**Implications for Pelobacter physiology.** Our results demonstrate for the first time that P. carbinolicus contains c-type cytochromes. As noted above, the functions of some of these cytochromes can be inferred from homology with cytochrome genes encoding known functions in other organisms. However, definitive elucidation of the functions of the genes in P. carbinolicus with genetic approaches has not been possible yet because techniques for generating specific mutations via homologous recombination that have been successful in G. sulfurreducens (8, 9, 20, 25, 28, 35) have not worked well in P. carbinolicus.

The detection of transcripts for three c-type cytochrome genes during growth on Fe(III) but not under fermentative conditions suggests that the cytochromes may be specifically involved in Fe(III) reduction. It is notable that one of these differentially expressed cytochrome genes encodes a triheme, periplasmic cytochrome that is highly conserved in the Geobacteraceae and is essential for optimal Fe(III) reduction in G. sulfurreducens (28). However, the triheme cytochrome is much less abundant in P. carbinolicus than in G. sulfurreducens, and G. sulfurreducens contains five homologs of this protein (36) compared to just one homolog in P. carbinolicus. Furthermore, P. carbinolicus lacks genes for many c-type cytochromes that have been found to be required for optimal Fe(III) reduction in G. sulfurreducens. These cytochromes include the inner membrane cytochrome MacA (8) and the outer membrane cytochromes OmcB (25), OmcS (35), and OmcE (35) that are thought to be involved in electron transfer to Fe(III). Also missing are OmcF, OmcG, and OmcH, which are outer membrane cytochromes that may play a regulatory role during Fe(III) reduction (20, 21). Not only is the overall number of c-type cytochrome genes in P. carbinolicus much lower than that in G. sulfurreducens, 14 versus 111 (36), but also the number of hemes in P. carbinolicus cytochromes, 5 or less, is generally lower than the number found in many G. sulfurreducens cytochromes, which can have as many as 27 hemes.

The differences between the c-type cytochrome contents of P. carbinolicus and G. sulfurreducens could conceivably be linked to factors related to metabolism of acetate, which G. sulfurreducens can use as a sole electron donor for Fe(III) reduction but P. carbinolicus cannot use (32). Alternatively, they could be related to differences in the environmental conditions in the preferred habitats of the organisms. However, further studies to definitively determine the functions of the c-type cytochromes in both organisms are necessary before substantive conclusions can be made.

**ACKNOWLEDGMENT**

This research was supported by grant DE-FC02-02ER63446 from the Genomics: GTL Program of the Office of Science (BER), U.S. Department of Energy.

**REFERENCES**

20. Kim, B. C., C. Leang, Y. H. Ding, R. H. Glaven, M. V. Coppi, and D. R.  
18. Lonergan, D. J., H. L. Jenter, J. D. Coates, E. J. Phillips, T. M. Schmidt, and  
17. Hayashi, S., and H. C. Wu.  
14. Dailey, H. A.  