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c-Type Cytochromes in *Pelobacter carbinolicus*▽

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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 genetic studies 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 H_2-oxidizing methanogens or acetogens (43) or (ii) with Fe(III) or S^o as an electron acceptor (34). However, these organic electron donors are only incompletely oxidized to acetate, in contrast to the ability of *Geobacter* and *Desulfuromonas* species to completely oxidize acetate and other organic electron donors to carbon dioxide (32). Yet *P. carbinolicus* appears to be capable of conserving energy from electron transfer to Fe(III) because it can grow via Fe(III) reduction with H_2 as the electron donor (34), and the cell yields per unit of Fe(III) reduced with both organic electron donors and H_2 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 lipoprotein binding motifs in signal sequences were used to predict outer membrane cytochromes (14). Proteins that were predicted to be cytoplasmic membrane associated, periplasmic, or outer membrane associated were further analyzed by BLASTP (2) to predict the function and location based on similarity to other proteins, with a cutoff *E* value of $<10^{-5}$. The predictions were based solely on sequence analysis and were not confirmed experimentally. Proteins predicted to catalyze heme biosynthesis and cytochrome *c* biogenesis were identified on the basis of similarity to such proteins in other bacteria (4, 23, 26).

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 $MgCl_2 \cdot 6H_2O$, 2.5 g of $NaHCO_3$, 0.25 g of NH_4Cl, 0.6 g of $NaH_2PO_4 \cdot H_2O$, 0.1g of KCl, and 0.14 g of $CaCl_2 \cdot 2H_2O$. 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% N_2–20% CO_2, sealed with butyl rubber stoppers, and autoclaved. Media were reduced with sterile Na_2S at a final concentration of 0.02 mM. Electron

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TABLE 1. Primers used for RT-PCR analysis of *P. carbinolicus* cytochromes and cytochrome *c* biosynthesis genes

Gene	Description	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temp for PCR (°C)
Cytochromes				
PCAR2944	Glutamate synthase, large subunit	CAGCATGCCATCAAGTTCGT	ATAATGCGTACATCGGCCG	60
PCAR2570	Cytochrome *c* family protein	TTCGCTATCTCCCTCGTTCAA	TGGCCAAAGTACGGACAATG	60
PCAR2549	Hypothetical protein	CGGCTTGCTGTTTGGTACTGT	TGAACGGCATCGAATGGTTAC	60
PCAR2529	caa_3-type cytochrome *c* oxidase, subunit II	GAGGCGGTGGACAAGGTTTT	GGAAGGCCGGGATATAAAGG	60
PCAR0558	Cytochrome *c* family protein	CTTGACGGTGATGGCATGC	CGCAAGGATATGTTCAGCCAC	60
PCAR0192	Molybdopterin oxidoreductase/ precorrin-4 methylase	CTGGCGAAAATCCTTGAACAA	AGGTAGGTGGCAAAGCGCT	60
PCAR2867	Cytochrome *c* nitrite reductase, *nrfH*	ACTTCAGTACGGACCCGACG	CCCGTTGTCATGAACTCTTCC	60
PCAR2866	Cytochrome *c* nitrite reductase *nrfA*	TCGAGAAGACCTGGGATGAGA	ACTTCAGTGCGGCAATTTCC	60
PCAR2550	Cytochrome *c*	CACGGTTCCCAAAAATTCCA	CCATCCCATTTTCAACGAGC	60
PCAR1628	Cytochrome c_7, *ppcA*	CTGTTCCGACGCCATATCAA	GCAACCACCGCAGTCTGTC	60
PCAR0152	Hypothetical protein	GGTACCGGCATCGCTTTTC	CATCAAACAACCGGGCATC	60
PCAR2984	Cytochrome *c* family protein	ATGAAAAAATGCCTTTGGATGCT	GCAACGGAAGCAGGGTTCT	60
PCAR2745	Hypothetical protein	TGGTGGCGGATTTTCTTCA	TGCAGAACATCTTCCCGGATA	60
PCAR2069	Protein-disulfide isomerase	AACAGGCCGACAAGGTTTTG	GCGGTTACAGACGATGCTCTT	60
Heme biosynthesis				
PCAR3065	*ccsA* homolog	GCGCTGGTGCTGATGATTTT	TCCCGAAAGCAGCAGATTG	55
PCAR3064	Glutamyl tRNA synthetase, *hemA*	GTCCTATGCGGCGGTGG	AAACGCACGATTTCCTGCTC	55
PCAR0266	Glutamate 1-semialdehyde aminotransferase, *hemL*	CAGCCCTGTTCGTGCATTTA	ACCCTGCCCAGATCGTTGT	55
PCAR0770	Ferrochetalase, *hemH*	GGCCGTGATTATGCGCTACT	ACGCCCTCATCCACCAAG	55
Cytochrome c biogenesis				
PCAR2229	Heme transport and ligation, *ccsB*	GCCTGCGTTCCCTGAAACT	CGCCGAGCAATATGATCAAA	55
PCAR2228	Heme transport and ligation, *ccsA*	GATTGCGTCGCGAAGGG	AATTGACATCATCCAGCGTGG	55
PCAR1954	Thioredoxin, *resA*	TTTCTGGTGCTGTCTGTCGG	GCGCAATGCTCCGGC	55
PCAR1953	Thioredoxin, *ccdA*	GCCCTGTGTATTGCCGCT	GAGAGCAAAAACGGCAATCC	55

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 cultures was monitored with ferrozine (33).

Nucleic acid extraction. Cultures used for DNA or RNA extraction were grown to mid-log phase, transferred to 50-ml conical tubes, and centrifuged at 3,150 × *g* for 20 min at 4°C. Cell pellets used for RNA extraction were frozen in liquid nitrogen and stored at −80°C. Chromosomal DNA was extracted from acetoin-grown *P. carbinolicus* cells using a FastDNA Spin kit for soil (QBiogene, Irvine, CA) or a MasterPure DNA purification kit (EPICENTRE Biotechnologies, Madison, WI). Total RNA was isolated using protocols described previously (17, 18). For acetoin-grown cells, cell pellets from 50 ml of culture were resuspended in 4 ml of Tris-EDTA-sucrose buffer (18), and RNA was extracted by the protocol used for RNA extraction from the surfaces of current-harvesting electrodes (18). For Fe(III) NTA-ethanol-grown cells, cell pellets from 50 ml of culture were resuspended in 8 ml TPE buffer (17), and RNA was extracted by using the protocol for RNA extraction from sediments (17), except that yeast tRNA was omitted. To confirm that RNA was not contaminated with DNA, PCRs were performed using RNA as the template.

PCR. Specific primers were designed for the genes for the 14 putative *c*-type cytochromes, as well as for cytochrome *c* biogenesis genes and selected heme biosynthesis genes (Table 1). Each 50-μl PCR mixture contained 10 μl of 5× Q solution (QIAGEN), 1.5 mM $MgCl_2$, 5 μl of 10× PCR buffer (QIAGEN), each deoxynucleoside triphosphate (Sigma) at a concentration of 0.02 mM, 50 pmol of each primer, 0.2 mg/ml bovine serum albumin (New England BioLabs), 1 U of *Taq* DNA polymerase (QIAGEN), and 50 ng of chromosomal DNA. PCR amplification was carried out with a PTC-200 thermal cycler (MJ Research, Waltham, MA) as follows: denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 55 or 60°C for 1 min, and 72°C for 1 min and then a final extension step at 72°C for 10 min. The annealing temperature used for each primer set is shown in Table 1. PCR products were purified from agarose gels with a QIAquick gel extraction kit (QIAGEN) and were cloned with a TOPO TA cloning kit, version R (Invitrogen). Plasmids were extracted from 8 to 12 colonies per PCR and sequenced with the M13F primer to confirm that the correct gene was amplified.

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 *Geobacter sulfurreducens* 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 $MgCl_2$ 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 (Vacufuge, Germany). The eluted tryptic peptides were desalted and concentrated with a commercial ZipTip C_{18} 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

TABLE 2. Predicted *c*-type cytochromes in *P. carbinolicus* and mRNA expression determined by RT-PCR

Gene	Annotation	No. of CXXCH motifs	Associated gene(s)	Function	mRNA expression	
					Acetoin	Fe(III) NTA-ethanol
Cytoplasmic membrane associated						
PCAR2944	Glutamate synthase, large subunit	1	None	Unknown	−	+
PCAR2570	Cytochrome *c* family protein	1	PCAR2571	In operon with another membrane-bound protein	−	−
PCAR2549	Hypothetical protein	1	PCAR2550 to PCAR2553	In operon with quinol:cytochrome *c* oxidoreductase	+	+
PCAR2529	caa_3-type cytochrome *c* oxidase, subunit II	1	PCAR2526 to PCAR2528	Terminal oxidase	+	+
Soluble periplasmic						
PCAR0558	Cytochrome *c* family protein	1	None	Unknown	+	+
PCAR0192	Molybdopterin oxidoreductase/ precorrin-4 methylase	1	PCAR0190 to PCAR0195	Unknown	+	+
PCAR2867	Cytochrome *c* nitrite reductase, *nrfH*	4	PCAR2866	Nitrite reduction, electron transfer	+	+
PCAR2866	Cytochrome *c* nitrite reductase, *nrfA*	5	PCAR2867	Nitrite reduction, catalytic subunit	+	+
PCAR2550	Cytochrome *c*	5	PCAR2549 to PCAR2553	Electron transfer from quinol:cytochrome *c* oxidoreductase	−	+
PCAR1628	Cytochrome c_7, *ppcA*	3	None	Periplasmic electron carrier	−	+
Outer membrane associated						
PCAR0152	Hypothetical protein	1	PCAR0153 to PCAR0159	Associated with Fe^{3+}-siderophore ABC transporter	+	−
PCAR2984	Cytochrome *c* family protein	2	PCAR2983	Associated with pyruvate kinase	+	+
PCAR2745	Hypothetical protein	3	None	Unknown	+	+
PCAR2069	Protein disulfide isomerase	1	None	Unknown	+	+

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_7 that belongs to a family of well-conserved cytochromes in the *Geobacteraceae*, which includes cytochrome c_7 in *Desulfuromonas 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).

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 *Desulfuromonas 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.

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

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

[a] ND, not determined.

PCAR2529 encodes subunit II of a caa_3-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 *Escherichia coli* 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 BLASTP 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 precorrin-4 methylase in the C terminus; and two that are predicted to be outer membrane bound, the triheme 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) rereduces 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 Fe^{3+}-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 c_7 and PCAR2550 encoding the periplasmic pentaheme 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

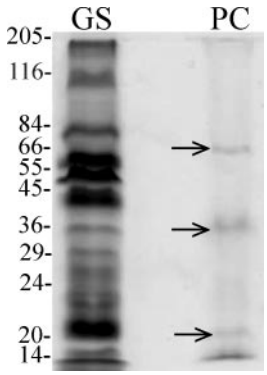

FIG. 1. Heme-stained SDS-PAGE of whole-cell protein extracted from *P. carbinolicus* (lane PC) and *G. sulfurreducens* (lane GS). Each lane contained 10 μg of protein. The bands indicated by arrows were excised for identification by mass spectrometry.

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.

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