JOURNAL OF BACTERIOLOGY, Feb. 1997, p. 634–642 Vol. 179, No. 3
0021-9193/97/$04.00+0

4-Hydroxybenzoyl Coenzyme A Reductase (Dehydroxylating) Is Required for Anaerobic Degradation of 4-Hydroxybenzoate by *Rhodopseudomonas palustris* and Shares Features with Molybdenum-Containing Hydroxylases

JANE GIBSON,[1] MARILYN DISPENSA,[2] AND CAROLINE S. HARWOOD[2]*

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853,[1] and Department of Microbiology, University of Iowa, Iowa City, Iowa 52242[2]

Received 30 August 1996/Accepted 13 November 1996

The anaerobic degradation of 4-hydroxybenzoate is initiated by the formation of 4-hydroxybenzoyl coenzyme A, with the next step proposed to be a dehydroxylation to benzoyl coenzyme A, the starting compound for a central pathway of aromatic compound ring reduction and cleavage. Three open reading frames, divergently transcribed from the 4-hydroxybenzoate coenzyme A ligase gene, *hbaA*, were identified and sequenced from the phototrophic bacterium *Rhodopseudomonas palustris*. These genes, named *hbaBCD*, specify polypeptides of 17.5, 82.6, and 34.5 kDa, respectively. The deduced amino acid sequences show considerable similarities to a group of hydroxylating enzymes involved in CO, xanthine, and nicotine metabolism that have conserved binding sites for [2Fe-2S] clusters and a molybdenum cofactor. Cassette disruption of the *hbaB* gene yielded a mutant that was unable to grow anaerobically on 4-hydroxybenzoate but grew normally on benzoate. The *hbaB* mutant cells did not accumulate [^{14}C]benzoyl coenzyme A during short-term uptake of [^{14}C]4-hydroxybenzoate, but benzoyl coenzyme A was the major radioactive metabolite formed by the wild type. In addition, crude extracts of the mutant failed to convert 4-hydroxybenzoyl coenzyme A to benzoyl coenzyme A. This evidence indicates that the *hbaBCD* genes encode the subunits of a 4-hydroxybenzoyl coenzyme A reductase (dehydroxylating). The sizes of the specified polypeptides are similar to those reported for 4-hydroxybenzoyl coenzyme A reductase isolated from the denitrifying bacterium *Thauera aromatica*. The amino acid consensus sequence for a molybdenum cofactor binding site is in HbaC. This cofactor appears to be an essential component because anaerobic growth of *R. palustris* on 4-hydroxybenzoate, but not on benzoate, was retarded unless 0.1 μM molybdate was added to the medium. Neither tungstate nor vanadate replaced molybdate, and tungstate competitively inhibited growth stimulation by molybdate.

Benzoate, in the form of its coenzyme A (CoA) thioester, is widely regarded as a key intermediate in the anaerobic degradation of a large variety of naturally occurring and man-made aromatic compounds by photosynthetic and nitrate- or sulfate-reducing bacteria (14, 15, 17, 18). Many of the bacterial strains isolated by anaerobic growth on benzoate are also able to utilize the closely related compound 4-hydroxybenzoate (4-OHBen) as a carbon source in the absence of oxygen. Studies with the purple nonsulfur phototrophic bacterium *Rhodopseudomonas palustris* (11, 16) and with the denitrifier *Thauera aromatica* (formerly *Pseudomonas* sp. strain K172) (3) have shown that the first step in the utilization of 4-OHBen is the formation of 4-hydroxybenzoyl (4-OHbenzoyl)-CoA and that specific CoA ligases are involved in this reaction. Subsequent reductive dehydroxylation of 4-OHbenzoyl-CoA to form benzoyl-CoA has been demonstrated in *T. aromatica* and *R. palustris*. The enzyme responsible for this conversion, 4-OHbenzoyl-CoA reductase, has been purified from *T. aromatica*, and its reaction mechanism has been examined (7, 8, 26) (Fig. 1).

Several genes encoding enzymes involved in the degradation of aromatic compounds in *R. palustris* have been identified (12, 16) and appear to be clustered in an approximately 25-kb region of the chromosome. Insertion of an antibiotic resistance cassette into an open reading frame divergently transcribed from the gene encoding 4-OHBen-CoA ligase, the first enzyme of 4-OHBen degradation, resulted in the generation of a mutant that was unable to grow on 4-OHBen. Sequencing of this region revealed three open reading frames with sequence similarities to bacterial hydroxylating enzymes (21, 37, 43) as well as to eukaryotic xanthine dehydrogenases (1, 28). The work described here suggests that these genes encode the *R. palustris* 4-OHbenzoyl-CoA reductase (dehydroxylating) and that 4-OHBen is obligatorily metabolized via benzoyl-CoA when it is used as a carbon source for phototrophic growth.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this work are described in Table 1. *R. palustris* cultures were grown anaerobically in light in defined mineral medium (PM) at 30°C as described previously (29). Benzoate and 4-OHBen were each added to a final concentration of 3 mM, and succinate was added to a final concentration of 10 mM. The effects of transition elements (Mo, W, V, and Cr) on growth were investigated by growing cells in screw-cap culture tubes that had been rinsed in concentrated nitric acid and then boiled in three changes of water from a quartz still. The mineral salts stock (35) used to prepare media for these experiments contained no molybdate. Growth was followed spectrophotometrically at 660 nm. *Escherichia coli* strains were grown aerobically in LB medium (42) and anaerobically in completely filled tubes or dilution bottles of LB supplemented with 10 mM glucose and 20 mM KNO_3. Antibiotics were used at the concentrations (in micrograms per milliliter) indicated in parentheses: for *R. palustris*, kanamycin (100), gentamicin (100), and tetracycline (100); for *E. coli*, kanamycin (100), gentamicin (10), tetracycline (25), and ampicillin (100).

Cloning and DNA manipulations. Standard protocols were used for DNA cloning and transformation and for plasmid DNA purification (2, 42). Chromosomal DNA was purified as described previously (12). DNA fragments were purified from agarose gels by using a GeneClean spin kit from Bio101 (La Jolla,

* Corresponding author. Mailing address: Department of Microbiology, University of Iowa, Iowa City, IA 52242. Phone: (319) 335-7783. Fax: (319) 335-7679. E-mail: caroline-harwood@uiowa.edu.

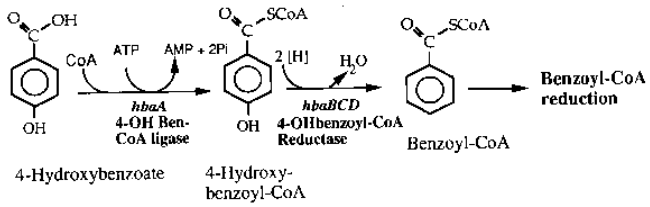

FIG. 1. Pathway of anaerobic 4-OHBen degradation by *R. palustris.*

Calif.). Southern hybridizations were carried out with a Genius kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). A TA cloning kit (Invitrogen Corp., San Diego, Calif.) was used for cloning some PCR products. Most recombinant plasmids were generated in pUC vectors and were maintained in *E. coli* DH5α. Broad-host-range plasmids were mobilized into *R. palustris* from *E. coli* S17-1 by conjugation (11).

Cloning of the DNA containing the *hbaBCD* genes. In previous work, we had cloned and sequenced *hbaA*, which encodes 4-OHBen-CoA ligase (16). We sequenced DNA upstream of *hbaA* from plasmid pMD402 (Fig. 2) and found one complete (*hbaB*) and one partial (*hbaC*) open reading frame. DNA adjacent to this region was present in a kanamycin-resistant (Km^r) cosmid clone obtained from an *R. palustris* strain (CGA604) that has a Km^r cassette inserted in *hbaA* (12, 13). An 11.2-kb *Sac*I fragment from the cosmid clone was subcloned into pUC19, yielding plasmid pMD416 (Fig. 2), which includes DNA containing the entire *hbaBCD* sequence.

DNA sequencing and computer analysis. The sequences of both strands of DNA were determined by the dideoxy-chain termination procedure using both universal and custom-synthesized primers (University of Iowa DNA Core Facility) and plasmids pMD402 and pMD416 as templates (Table 1 and Fig. 2). Nucleotide and amino acid sequence data were analyzed with Gene Inspector version 1.01 (Textco Inc., West Lebanon, N.H.) and the Wisconsin sequence analysis package version 8.0 (Genetics Computer Group, Madison, Wis.). Open reading frames were identified by an ATG start site and standard stop codons. Rare codon analysis, using the codon usage table of *Rhizobium meliloti*, an organism with a G+C content similar to that of *R. palustris*, was used to choose the correct open reading frames and to eliminate frameshifts based on sequencing errors. Similar sequences from SWISS-PROT and GenBank databases were identified by using the BLAST network service at the National Center for Biotechnology Information (Bethesda, Md.). Pairwise amino acid sequence comparisons were made by using the GAP program from the Wisconsin package.

Construction of *hbaB* mutants. Two *R. palustris* mutants, CGA502 and CGA506, were constructed by insertional inactivation of the *hbaB* gene. The 1.3-kb Km^r GenBlock cassette (Pharmacia Biotech, Piscataway, N.J.) and the 5.2-kb promotorless *lacZ*-Km^r cassette derived from pUTminiTn*5lacZ1* (10) were each separately inserted into the *Pst*I site of the *hbaB* gene in pMD409 (Fig. 2) and cloned into plasmids pRK415 and pJQ200mp18, respectively, to generate pMD502 and pMD422 (Fig. 2). Each of these plasmids was introduced into CGA009 by conjugal transfer (11). A recombinant (CGA502) from the mating with S17-1(pMD502) was identified by screening for kanamycin resistance and tetracycline sensitivity. A recombinant (CGA506) from the mating with S17-1(pMD422) was identified by plating on PM medium containing succinate, kanamycin, and 5% sucrose. The chromosomal insertions in both strains were verified by Southern analysis (data not shown).

Expression of *hbaBCD* in *E. coli.* A 4-kb fragment containing *hbaBCD* was amplified from pMD416 by PCR, using an Expand HiFidelity kit from Boehringer Mannheim. Dimethyl sulfoxide (5%) was included in the PCR mixture. The primers P66-*Nde*I (AGCGCCATATGGATGAAACGATGATGCGA) and P65-*Bam*HI (GCTGAGGATCCGGCGAATTGGGCTGA) introduced restriction sites (underlined) to facilitate directional cloning into pT7-7 to give plasmid pMD419 (Table 1). A fragment containing *hbaD* alone was amplified from pMD416 by using primers P78-*Nde*I (AGGGCAGCCATATGACCGCCTTGA ATGCGT) and P65-*Bam*HI and cloned into pT7-7, resulting in plasmid pMD420.

For expression of *hbaBCD*, the recombinant plasmids were transformed into *E. coli* BL21(DE3). Cells were then grown in 5 ml of LB plus ampicillin to an optical density at 660 nm of 0.3 to 0.5, washed twice in M9 medium (2), resuspended in 5 ml of M9 plus 0.02% 18 amino acids without methionine or cysteine, and incubated with shaking at 37°C for 30 min (2). Aliquots of 0.5 ml were incubated with 800 μM isopropylthiogalactopyranoside (IPTG) at 37°C with shaking for 45 min to induce expression of the T7 polymerase. *E. coli* protein synthesis was stopped by incubation with 1.6 mg of rifampin per ml for 5 min at 42°C and then 20 min at 37°C. Cells were pulse-labeled with 10 μCi of L-[^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) for 5 to 10 min, pelleted, and resuspended in 50 μl of sample buffer. Labeled proteins were separated by sodium dodecyl sulfate-gel electrophoresis and localized by autoradiography (32).

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
E. coli strains		
DH5α	F$^-$ λ$^-$ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* ϕ80d*lacZ*Δ*M15*	GIBCO-BRL
S17-1	*thi pro hdsR hdsM*$^+$ *recA*; chromosomal insertion of RP4-2 (Tc::Mu, Km::Tn*7*)	44
BL21(DE3)	Contains chromosomal copy of T7 RNA polymerase gene, inducible by IPTG, *hsdS gal* (λ *cIts*857 *ind*1 *S*am7 *nin*5 *lacUV5*-T7 gene 1)	46
R. palustris strains		
CGA009	Wild type; spontaneous Cm^r derivative of CGA001	29
CGA502	*hbaB*::Km^r	This work
CGA506	*hbaB*::*'lacZ*-Km^r	This work
Plasmids		
pT7-7	2.4-kb expression vector containing the T7 RNA polymerase promoter followed by a strong ribosome binding site upstream of an *Nde*I restriction site; Ap^r	47
pJQ200mp18	6.1-kb broad-host-range suicide vector containing *sacB* gene; Gm^r	39
pRK415	10.5-kb broad-host-range vector; IncP1, Tc^r	27
pHRP314	pUC1813 with promotorless *lacZ* gene and Km^r gene from pUTminiTn*5lacZ1*; Ap^r Km^r	36
pUC18, pUC19	ColE1, *lacZ* Ap^r	51
pACYC184::Km	1.3 kb-Km^r GenBlock from Pharmacia cloned into the *Eco*RI site of pACYC184	Pharmacia
pMD402	pUC18 containing 7.8-kb *Sph*I fragment from CGA401 clone bank; Km^r cassette is in *Sal*I site of the *hbaA* gene; Ap^r Km^r	16
pMD409	pUC18 containing 1.3-kb *Xho*I fragment from pMD402 containing *hbaB* gene; Ap^r	This work
pMD416	pUC19 containing 11.2-kb *Sac*I fragment from pPE310; contains *hbaABCD* and adjacent DNA; a Km^r cassette is in the *Sal*I site of *hbaA*; Ap^r	This work
pMD419	Ap^r; PCR product containing *hbaBCD* cloned into *Nde*I-*Bam*HI sites of pT7-7	This work
pMD420	Ap^r; PCR product containing *hbaD* cloned into *Nde*I-*Bam*HI sites of pT7-7	This work
pMD422	pJQ200mp18 containing 1.3-kb *Xho*I fragment from pMD416, containing the *hbaB* gene, with 5.2-kb promotorless *lacZ*-Km^r cassette from pHRP314 cloned into *Pst*I site; Gm^r Km^r	This work
pMD502	pRK415 containing 1.3-kb *Xho*I fragment from pMD402 with Km^r cassette in *Pst*I site of *hbaB* gene	This work

[a] Ap = ampicillin, Km = kanamycin, Gm = gentamicin, Tc = tetracycline, *'lacZ* = promotorless *lacZ* gene.

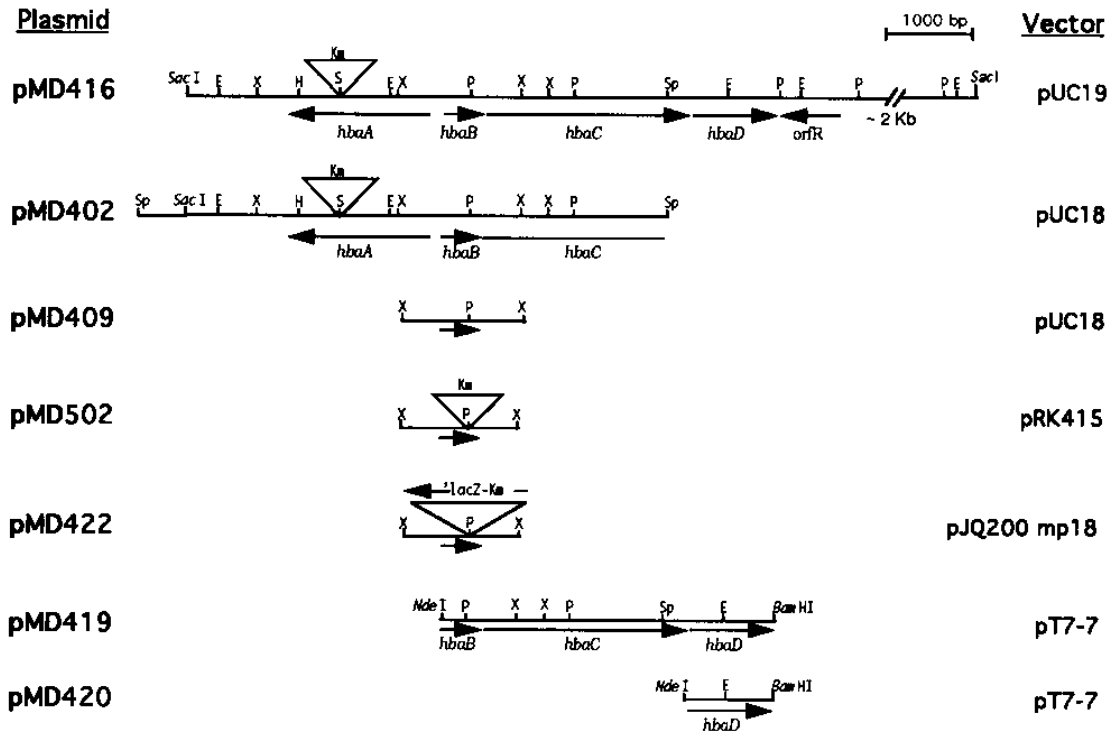

FIG. 2. Restriction maps of clones carrying *hbaBCD* and other constructions used in this study. The restriction sites as well as the locations and orientations of the coding regions were determined by nucleotide sequencing. The orientations of coding regions are indicated by arrows. Restriction enzyme sites: E, *Eco*RI; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; X, *Xho*I.

Preparation of 4-OHbenzoyl-CoA. 4-OHbenzoyl-CoA was prepared enzymatically by using partially purified 4-OHBen-CoA ligase (16) and purified by using a Waters ODS SepPak (Millipore Corp., Milford, Mass.) as previously described (38).

Anaerobic preparation of cell extracts. Cultures of *R. palustris* grown in 900-ml Roux bottles were harvested and washed once under anoxic conditions with about 100 ml of buffer containing 50 mM potassium phosphate (pH 7.0), 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 mM $Na_2S_2O_4$. Unless used immediately, the cell pellets were frozen in liquid N_2. To prepare extracts, cells were suspended in 1 to 2 volumes of the same buffer (freshly prepared from anaerobic stocks) and broken by sonication under argon gas or by passage through a French press (Aminco, Silver Spring, Md.). The broken cell suspensions were transferred to an anaerobic chamber (Coy Laboratories, Grass Lake, Mich.) and then centrifuged for 10 min at approximately 5,000 × *g*. Some samples were centrifuged outside the chamber at high speed (200,000 × *g*, 45 min) to sediment the membranes.

Assay of 4-OHbenzoyl-CoA reductase. Reductive dehydroxylation of 4-OHbenzoyl-CoA was assayed by measuring the formation of benzoyl-CoA in a reaction mixture containing 4-OHbenzoyl-CoA, reduced methyl or benzyl viologen, and cell extract. The reactions were carried out in the anaerobic chamber at 22 to 24°C in a 100-μl volume containing 50 mM potassium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM benzyl or methyl viologen, 1 to 2 μl of 50 mM $Na_2S_2O_4$ to reduce the dye, and 0.5 mM 4-OHbenzoyl-CoA. The reactions were started by adding cell extract (0.1 to 0.2 mg of protein) and terminated by adding 10 μl of 2 M $HClO_4$. Precipitated protein was removed by centrifugation, and the supernatant fluids were neutralized by adding 50 μl of 1 M $KHCO_3$. The rate of benzoyl-CoA formation was determined by reversed-phase high-pressure liquid chromatography, using a Waters C_8 Nova Pak column (3.9 by 150 mm; Millipore) together with a Waters 996 photodiode array detector and a linear gradient starting with 20% methanol (B) in 20 mM potassium phosphate (pH 6.0) (A) and rising to 80% B:20% A over 30 min, at a flow rate of 1 ml/min. 4-OHbenzoyl-CoA was stable after acidification or in the presence of boiled enzyme.

Other analytical procedures. Protein was estimated by dye binding using the Bio-Rad (Richmond, Calif.) protein assay reagent. Bovine serum albumin was used as the protein standard. Rapidly appearing intracellular products formed by whole cells from [U-^{14}C]4-OHBen were analyzed as previously described (33) except that the solvent for thin-layer chromatography (TLC) was 5 parts *n*-butanol:3 parts H_2O:1 part acetic acid.

Nucleotide sequence accession number. The GenBank accession number for the sequence shown in Fig. 6 is U65440.

RESULTS

Involvement of *hbaBCD* in 4-OHBen degradation. Nucleotide sequence analysis of approximately 4 kb of DNA upstream of *hbaA*, the structural gene for 4-OHBen-CoA ligase, revealed three divergently transcribed open reading frames, designated *hbaB*, *hbaC*, and *hbaD*, which were so closely spaced as to suggest they might form an operon (Fig. 2). Insertional inactivation of the *hbaB* gene with a Km^r cassette generated a mutant (CGA502) that was unable to utilize 4-OHBen as a carbon source for anaerobic growth. However, slow growth (doubling times of 40 h, as opposed to about 14 h for the wild type) sometimes occurred after lags of at least 4 days in cultures transferred to 4-OHBen from succinate or benzoate. The DNA from one of these strains had a hybridization pattern different from that of the original mutant in Southern blots probed with the 1.3-kb *Xho*I fragment from pMD409. Experiments with CGA502 were therefore carried out with cultures maintained on succinate or benzoate and incubated with 4-OHBen for periods of generally less than 24 h. A second mutant, CGA506, with a larger 5.2-kb insert placed into the same site, consistently failed to grow on 4-OHBen under anaerobic conditions but grew with wild-type doubling times on benzoate and succinate. The mutants were unimpaired in aerobic growth on 4-OHBen. The behavior of these mutants suggests that the *hbaB* gene is required for anaerobic 4-OHBen degradation.

Short-term uptake of [^{14}C]4-OHBen. Illuminated whole cells of the *hbaB* mutant, CGA502, that had been grown with benzoate but exposed to 4-OHBen for 18 h accumulated both [^{14}C]4-OHBen and [^{14}C]benzoate at wild-type rates (33) under anaerobic conditions. Autoradiograms of TLC-separated com-

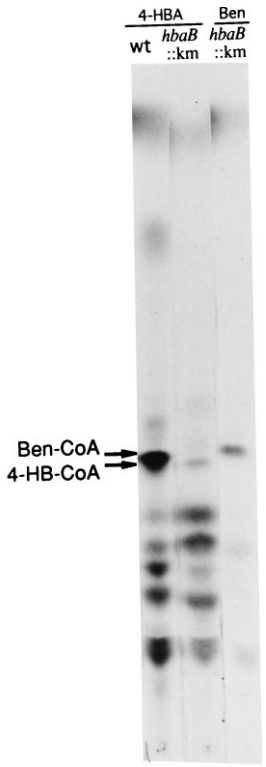

FIG. 3. Autoradiogram of intermediates formed intracellularly by whole cells incubated with either [^{14}C]4-OHBen or [^{14}C]benzoate (Ben) (5 μM) for 1 min. Intracellular contents were extracted in boiling water and separated by TLC before exposure to X-ray film. Wild-type (wt) *R. palustris* was grown on 4-OHBen. The *hbaB* mutant, CGA502, was grown with benzoate, and 4-OHBen was added during the exponential phase of growth, 18 h before harvest. Spots labeled as Ben-CoA (benzoyl-CoA) and 4-HB-CoA (4-OHbenzoyl-CoA) comigrated with authentic standards. Benzoyl-CoA standard was obtained from Sigma Chemical Co. (St. Louis, Mo.).

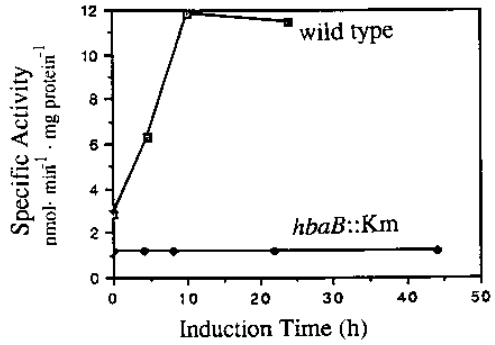

FIG. 4. Induction of 4-OHbenzoyl-CoA reductase activity in wild-type (strain CGA009) and *hbaB* mutant (strain CGA502) cells. Cultures were grown in replicate bottles anaerobically with 2 mM benzoate. When the benzoate concentration fell to between 0.4 and 0.5 mM, 4-OHBen (2 mM) was added to each bottle (time zero). The entire contents of a bottle were harvested at the times indicated; then cell extracts were prepared and reductase activities were assayed as described in Materials and Methods.

pounds formed by whole cells revealed, however, that whereas wild-type *R. palustris* converted 4-OHBen to both 4-OHbenzoyl-CoA and benzoyl-CoA, the *hbaB* mutant failed to produce any benzoyl-CoA and formed only 4-OHbenzoyl-CoA (Fig. 3). As in earlier experiments (33), a series of more polar, and as yet unidentified, compounds contained the greater part of the radioactivity in these chromatograms, even in extracts prepared at the shortest uptake periods (10 s) examined.

Activity of 4-OHbenzoyl-CoA reductase. The activity of 4-OHbenzoyl-CoA reductase was determined by measuring the formation of benzoyl-CoA in crude extracts of wild-type *R. palustris*. Specific activities ranging from 12 to 20 nmol · min^{-1} · mg of protein^{-1} were obtained after low-speed centrifugation. Following ultracentrifugation (200,000 × *g*, 45 min) to sediment membranes, the specific activity increased to 30 to 50 nmol · min^{-1} · mg of protein^{-1} without a change in activity per milliliter, indicating that the enzyme was soluble. The inclusion of 10 μM flavin mononucleotide or flavin adenine dinucleotide (FAD), or inclusion of 1 to 100 μM molybdate or tungstate, did not stimulate reductase activity. In extracts of uninduced wild-type cells (grown on succinate or benzoate), activity was at or below the detection limit (about 1.5 nmol · min^{-1} · mg of protein^{-1}).

In benzoate-grown wild-type cells, 4-OHbenzoyl-CoA reductase activity increased with time after induction with 4-OHBen (Fig. 4). CGA502 (*hbaB*::Km^r) cells, by contrast, had a very low level of reductase activity that did not increase when benzoate-grown cultures were supplemented with 4-OHBen at a comparable stage of growth. In another experiment in which incubation of CGA502 cultures with 4-OHBen was extended to 96 h, the specific activity of the final sample was only 1.8 nmol · min^{-1} · mg of protein^{-1}. No conversion of 4-OHbenzoyl-CoA to benzoyl-CoA was detected after 30 min in extracts of strain CGA506, the second *hbaB* mutant (with 0.54 mg of protein, more than twice the quantity used with the wild type), after incubation of the benzoate-grown culture with 4-OHBen for 20 h as described above. These data indicate that the *hbaB* mutants are specifically defective in the synthesis of 4-OHbenzoyl-CoA reductase.

Expression of *hbaBCD* in *E. coli*. The predicted sizes of the HbaB, HbaC, and HbaD proteins (17.6, 82.6, and 34.6 kDa, respectively) are very similar to the sizes of the three subunits of the 4-OHbenzoyl-CoA reductase purified from *T. aromatica* (7). Expression and selective labeling with [^{35}S]methionine of the proteins encoded by *hbaBCD* from *E. coli* BL21(DE3)(pMD419) are shown in Fig. 5. The three subunit sizes of 17, 34, and 82 kDa correspond well to the predicted molecular weights. Expression of *hbaB* greatly exceeded that of the other two genes, presumably because the start codon of *hbaB* is adjacent to the T7 polymerase promoter and strong ribosome binding site of pT7-7 (47). Improved expression of HbaD was seen in similar experiments with the smaller clone, *E. coli* BL21(DE3)(pMD420), in which *hbaD* is adjacent to the ribosome binding site (Fig. 5).

Enzymatic conversion of 4-OHbenzoyl-CoA to benzoyl-CoA was not detected in anaerobically prepared extracts of BL21(DE3)(pMD419) cells that had been grown in filled bottles as described in Materials and Methods. Since anaerobic growth of this strain in LB with nitrate plus glucose was poor, pMD419 was also transformed into JM109(DE3), an *E. coli* strain that grew more vigorously and denitrified actively. However, no formation of benzoyl-CoA from 4-OHbenzoyl-CoA was detected in anaerobically prepared extracts of this strain following 2 to 5 h of induction with 0.2 to 0.5 mM IPTG.

Nucleotide sequence of *hbaBCD* and amino acid sequence comparisons. The predicted translated products of *hbaBCD* contain 163, 774, and 327 amino acids. The complete DNA and deduced amino acid sequences are shown in Fig. 6.

The predicted polypeptides are similar in size and amino acid sequence to molybdenum-containing hydroxylases (30, 50). Several prokaryotic enzymes belonging to this family are

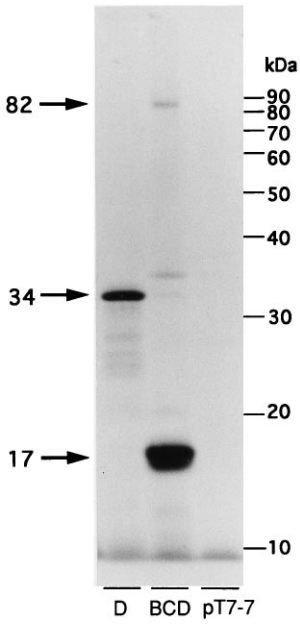

FIG. 5. Expression of the HbaB, -C, and -D proteins in *E. coli*. A T7 RNA polymerase expression system was used as described in the text. An autoradiogram of a sodium dodecyl sulfate–12% polyacrylamide gel is shown. The HbaD protein (34 kDa) was expressed from plasmid pMD420. HbaB (17 kDa), HbaC (82 kDa), and HbaD were expressed from plasmid pMD419. The lane marked pT7-7 is the vector control. The 10-kDa protein ladder from GIBCO-BRL (Gaithersburg, Md.) was used to calibrate the autoradiogram.

nicotine dehydrogenase from *Arthrobacter nicotinovorans* (*ndhABC*), carbon dioxide dehydrogenase from *Oligotropha carboxidovorans* (*coxSLM*), and carbon monoxide dehydrogenase from *Pseudomonas thermocarboxydovorans* (*cutABC*) (21, 37, 43). These enzymes consist of three subunits similar in size to HbaB, HbaC, and HbaD. Xanthine dehydrogenase, well studied in several eukaryotic organisms, is a single polypeptide with clearly defined domains that can be cleaved by trypsin digestion into three fragments which are close in size to the subunits of the other enzymes (1). This enzyme catalyzes the hydroxylation of xanthine to uric acid through the operation of a self-contained electron transport chain that is initiated by the interaction of the substrate with a molybdopterin cofactor followed by electron transfer through a flavin and two [2Fe-2S] clusters to a final electron acceptor; NAD or oxygen (24).

Amino acid comparisons between HbaB, -C, and -D and the corresponding subunits of similar proteins are summarized as follows. The smallest subunit, encoded by *hbaB*, has approximately 50% identity with the subunits encoded by *cutC*, *coxS*, and *ndhB* and 41% identity with the 20-kDa fragment of xanthine dehydrogenase from *Drosophila melanogaster*. A sequence alignment is shown in Fig. 7. Both the large (HbaC) and the intermediate-size (HbaD) subunits have between 22 and 29% amino acid identities with the corresponding subunits from the other enzymes.

HbaB contains motifs indicative of two [2Fe-2S] clusters. Putative ligands for a ferredoxin-like [2Fe-2S] center found in HbaB are cysteines 49, 54, 57, and 68, since they are arranged in the motif -C-X_4-C-X_2-C-X_N-C- (Fig. 7), which corresponds with the general sequence for ligand binding regions of a number of enzymes known to contain this type of iron-sulfur cluster (22, 48). This arrangement of cysteines is conserved in HbaB, CoxS, CutC, NdhB, and the 20-kDa fragment of xanthine dehydrogenase with respect to motif, amino acid environment, and position in the protein. Possible ligands for a second [2Fe-2S] center (cysteines 108, 111, 143, and 145) are conserved among the small subunits of molybdenum-containing hydroxylases as well as in HbaC (Fig. 7). The exact spacings and environments for this type of cluster have not been as well defined, however (9, 48).

HbaC contains a molybdenum cofactor binding motif. A -GGGFGA- sequence found in residues 242 to 247 in HbaC (Fig. 8) corresponds closely to the conserved sequence -GGG FGG- from xanthine dehydrogenases (19), which has been shown to be involved in binding the molybdenum cofactor by mutational analysis in *D. melanogaster* (25). This motif is highly conserved in molybdenum-containing hydroxylases in terms of sequence, relative location within the protein, and amino acid environment (Fig. 8) and is probably involved in binding a molybdenum cofactor in HbaC. Several studies have implicated the general sequence -GXGXXG/A- in dinucleotide cofactor binding (34, 49), and it is possible that residues 568 to 574 (-GSGAQA-) of HbaC are involved in this capacity.

Effect of molybdate on growth of *R. palustris*. The finding that HbaC contains a molybdenum cofactor binding sequence (25) prompted us to investigate the effects of molybdate and related transition elements on the anaerobic growth of *R. palustris* on various carbon sources. Omission of molybdate from media did not affect growth on benzoate or succinate, even after five successive passages (Table 2). However, growth on 4-OHBen was reproducibly slower in molybdate-depleted medium, and addition of tungstate at micromolar concentrations further retarded growth on 4-OHBen but not on other carbon sources. The inhibitory effect of tungstate was abolished by addition of 0.1 to 1 μM molybdate, and neither vanadate nor chromate could replace molybdate in restoring growth rate in the absence of added molybdate or in the presence of tungstate. A growth requirement for molybdate is thus restricted to cultures utilizing 4-OHBen, suggesting that an enzyme specifically needed for its utilization may contain a molybdenum cofactor. The residual growth of cells on 4-OHBen was probably due to the presence of traces of molybdate in the other medium components, since special steps, other than cleaning the glassware, were not taken to eliminate this possibility.

DISCUSSION

Reductive dehydroxylations are likely to be critical to the biodegradation of plant-derived biomass in anaerobic environments. Aromatic compounds derived from lignin, the second most abundant polymer on earth, are often methoxylated or hydroxylated. Methyl groups are removed to leave hydroxyl substituents, which in turn are removed by reductive dehydroxylases to generate benzoyl-CoA (15, 23). The anaerobic degradation of many industrially produced phenolics, including phenol, *p*-cresol (4-methylphenol), and *m*-cresol (3-methylphenol), also depends on reductive dehydroxylation. Phenol is carboxylated to form 4-OHBen (31), whereas *p*-cresol and *m*-cresol are converted to 4-OHBen and 3-OHBen, respectively, by methyl group oxidation (5, 6).

Work reported here confirms the critical role of 4-OHbenzoyl-CoA reductase by showing that this enzyme is essential for anaerobic growth of *R. palustris* on 4-OHBen. Although the behavior of some poorly characterized mutants of *R. palustris* had suggested that an alternative breakdown route for 4-OHBen, not involving reduction to benzoate or its CoA derivative, might exist (23, 33), the behavior of the insertion mutants described here strongly suggests that such a route, if it exists, could be of only minor quantitative significance.

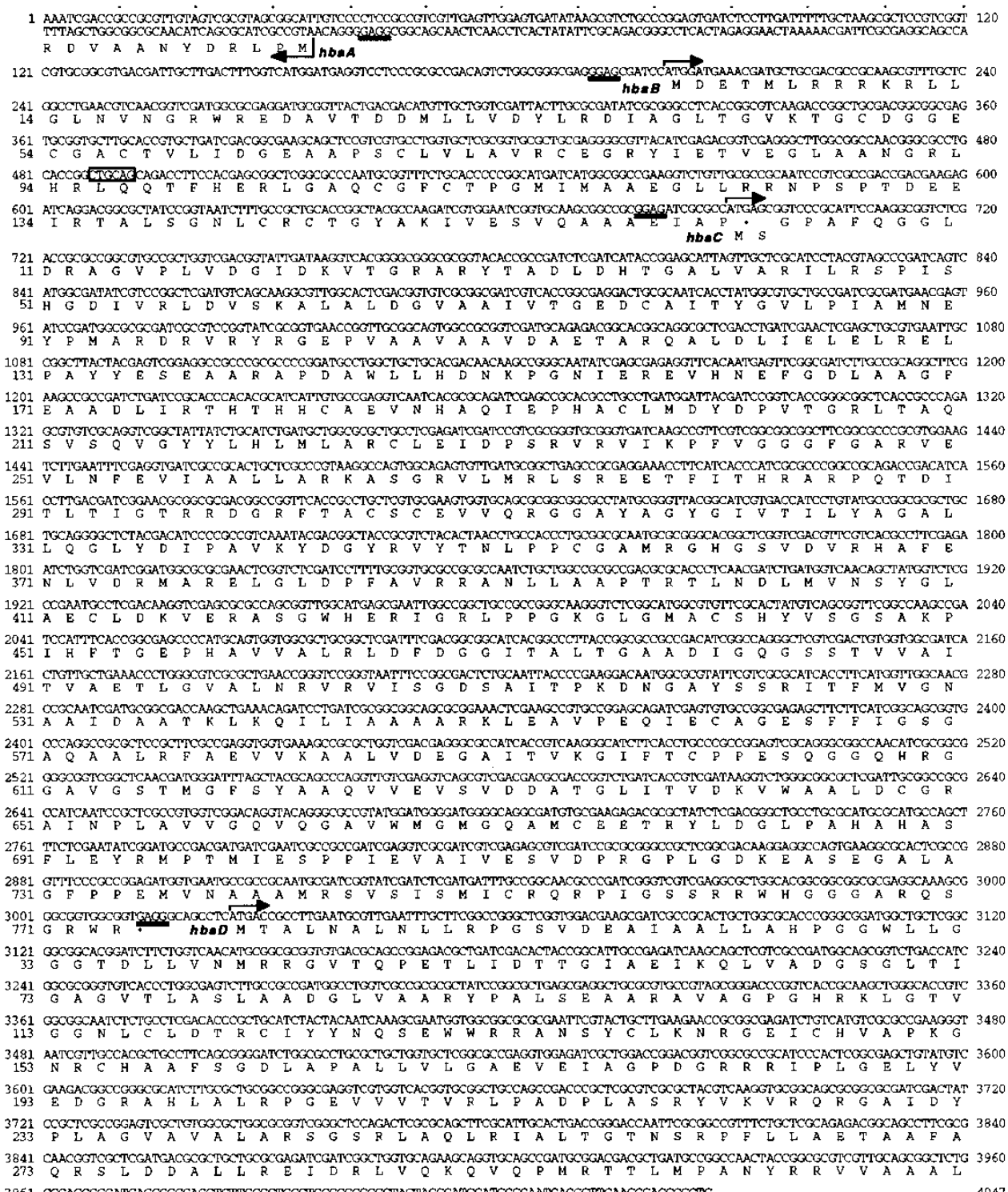

FIG. 6. Nucleotide and deduced amino acid sequences of the *hbaBCD* genes. The sequence between *hbaB* and *hbaA* and the beginning amino acid sequence of *hbaA* are also shown. The start codon of each gene is indicated by an arrow. Stop codons are indicated by dots. Potential ribosome binding sites are underlined. The boxed sequence from 487 to 492 indicates the *Pst*I site into which cassettes were cloned for insertional inactivation of *hbaB*.

The gene sequences presented predict that the *R. palustris* 4-OHbenzoyl-CoA reductase is a molybdenum-containing hydroxylase, a proposal that is supported by the finding that cells require molybdate when growing on 4-OHBen but not on other carbon sources. The *hbaBCD* genes show significant similarities to genes that encode enzymes involved in oxidoreductions of purine derivatives and of CO or CO_2, although the order of the genes in the corresponding operons is variable.

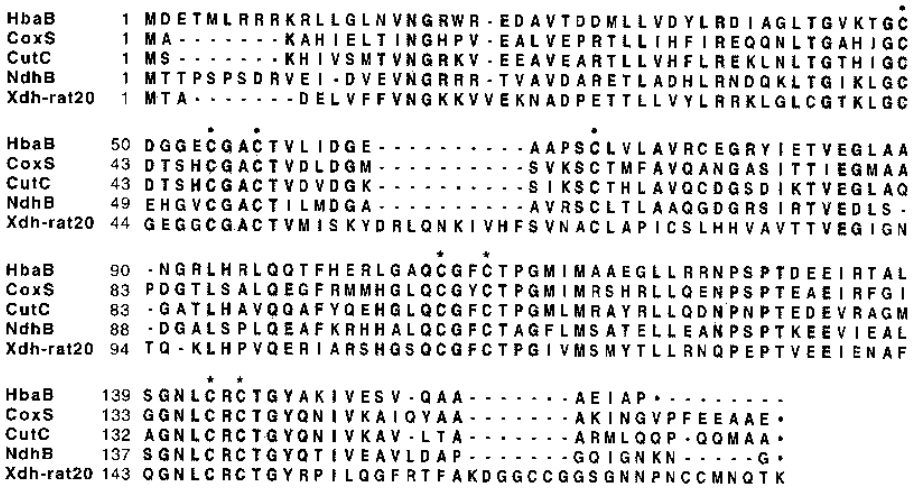

FIG. 7. Multiple sequence alignments of HbaB and the [2Fe-2S]-containing subunits from similar proteins. Amino acid sequences: HbaB, 4-OHbenzoyl-CoA reductase from *R. palustris*; CoxS, carbon dioxide dehydrogenase from *O. carboxidovorans* (43); CutC, carbon monoxide dehydrogenase from *P. thermocarboxydovorans* (37); NdhB, nicotine dehydrogenase from *A. nicotinovorans* (21); Xdh-rat20, 20-kDa fragment from xanthine dehydrogenase from rat (1). Gaps were introduced to enhance alignment score. Identical amino acids are shaded. Dots indicate conserved cysteines of a ferredoxin-like [2Fe-2S] cluster; asterisks indicate conserved cysteines of a second [2Fe-2S] cluster.

The similarities are strongest in the small (17 to 29-kDa) subunits, where up to 50% of the amino acids are identical, and [2Fe-2S] cluster binding motifs are clearly conserved. Although the overall amino acid identities in the large and intermediate-size components (HbaC and -D; 85 and 35 kDa, respectively) with the other enzymes are only between 20 and 30%, the sequences associated with binding a molybdenum cofactor in the large subunit appear to be conserved. Most molybdenum-containing hydroxylases are flavoproteins (30, 50), and although definitive evidence is lacking, it is generally agreed that FAD is associated with the intermediate-size fragment of xanthine dehydrogenase (24). However, an FAD binding motif is not readily identifiable from sequence comparisons of xanthine dehydrogenases with each other (19) or in comparisons of the intermediate-size subunits of molybdenum-containing hydroxylases from prokaryotes. Likewise, no FAD binding motif is discernible in the predicted HbaD subunit of the *R. palustris* 4-OHbenzoyl-CoA reductase. Determination of the significance of the possible dinucleotide cofactor binding site in HbaC will require characterization of the purified enzyme.

The molybdenum-containing hydroxylases that are homologous to the 4-OHbenzoyl-CoA reductase described here are all derived from aerobic sources and do not show the sensitivity to molecular oxygen characteristic of 4-OHbenzoyl-CoA reductase. Although reductive dehydroxylation of 4-OHbenzoyl-CoA to benzoyl-CoA is an energetically difficult reaction (8, 26), the similarities described here suggest that it is carried out by an enzyme that has a basic structure quite widely adapted for use among aerobic reactions.

The size similarity between HbaBCD and the subunits of the *T. aromatica* 4-OHbenzoyl-CoA reductase (17, 35, and 75 kDa) suggests that the two enzymes are similar. The purified *T. aromatica* enzyme was found to be an iron-sulfur protein, but its spectrum showed no evidence for a flavin component. Molybdenum was present at no more than 0.1 mol/mol of protein. However, the enzyme was inactivated by cyanide and azide, a characteristic feature of molybdoenzymes (7). A definitive comparative analysis of the enzymes from the denitrifying bacterium and the phototroph will require a determination of the sequences of the corresponding genes from *T. aromatica*, as well as purification and characterization of the 4-OHbenzoyl-CoA reductase from *R. palustris*. Expression of *R. palustris* 4-OHbenzoyl-CoA reductase to high levels in *E. coli* would be helpful in this regard. However, initial attempts to express active enzyme from cloned *hbaBCD* genes were not successful. A number of factors may have contributed to this. First, only the first subunit, HbaB, was strongly expressed when all three genes were cloned into the expression vector pT7-7 (Fig. 5),

FIG. 8. Amino acid sequence alignment in the region of the presumed molybdenum cofactor binding site. The boxed glycine, when changed to a glutamate in xanthine dehydrogenase of *D. melanogaster*, results in a molybdenum deficiency of the mutant enzyme (25). Amino acid sequences: HbaC, 4-OHbenzoyl-CoA reductase from *R. palustris*; CoxL, carbon dioxide dehydrogenase from *O. carboxidovorans* (43); CutA, carbon monoxide dehydrogenase from *P. thermocarboxydovorans* (37); NdhC, nicotine dehydrogenase from *A. nicotinovorans* (21); Xdh-rat85, 85-kDa fragment from xanthine dehydrogenase from rat (1); Xdh-dm85, 85-kDa fragment from xanthine dehydrogenase from *D. melanogaster* (25).

TABLE 2. Effects of molybdate, tungstate, and vanadate on growth rates of *R. palustris* wild-type cells in media lacking molybdate

Addition	Generation time (h)[a]	
	Benzoate[b]	4-OHBen
None	11	22
0.1 μM molybdate	10.5	11
1.0 μM molybdate	10.5	11.5
0.1 μM tungstate	10.5	28
1.0 μM tungstate	ND	68
1.0 μM vanadate	10.5	24

[a] Average of two to three separate experiments. The generation time for growth on 4-OHBen in standard mineral medium is between 11 and 14 h. ND, not determined.

[b] Carbon sources were added to a final concentration of 3 mM.

while the other two did not become nearly as heavily labeled as would be expected from their size and methionine contents had they been produced in stoichiometric proportions. Thus, quantities of the second and third subunits sufficient to give measurable enzyme activity may not have been produced. Mixed extracts prepared from induced BL21(DE3) transformants carrying genes separately subcloned as *hbaBCD* and *hbaCD* were also inactive (data not presented). This finding could indicate that the active enzyme is not easily reconstituted in vitro from separately synthesized subunits. Another problem may have lain in the availability of the correct molybdenum cofactor. Although the JM109 host was clearly capable of producing the factor needed for active denitrification in *E. coli* (45), this may not be the same as the putative molybdenum cofactor for the *R. palustris* enzyme.

Reductive dehydroxylation of 2-OHbenzoyl-CoA and 3-OHbenzoyl-CoA has been demonstrated in vitro (4, 7). Dihydroxylated compounds can also serve as substrates; reductive dehydroxylation of protocatechuyl-CoA (3,4-dihydroxybenzoyl-CoA) to 3-OHbenzoyl-CoA has been shown to occur (20). It will be interesting to see if the enzymes that catalyze these reactions are also molybdenum-containing dehydroxylases. Trihydroxylated aromatic compounds, such as gallate, are not reductively dehydroxylated to form a benzoyl-CoA derivative but instead are converted under anaerobic conditions to phloroglucinol, a trihydroxybenzene with symmetrically placed hydroxyl groups that is easily reduced to a nonaromatic derivative. A molybdenum-containing transhydroxylase that converts pyrogallol to phloroglucinol, with the involvement of 1,2,3,5-tetrahydroxybenzene as a cosubstrate and coproduct, has been described. This enzyme, which is a heterodimer with two dissimilar subunits, is not sensitive to oxygen and is likely related to molybdenum-containing hydroxylases (40, 41).

ACKNOWLEDGMENTS

This work was supported in part by grants from the Department of Energy, Division of Energy Biosciences (DE-FG02-86ER13495 to J.G. and DE-FG02-95ER20184 to C.S.H.) and by the U.S. Army Research Office (DAAH04-95-0124 and -0315 to C.S.H.).

We thank Joe Perrotta for analysis of the radioactive intracellular products.

REFERENCES

1. **Amaya, Y., K. Yamazaki, M. Sato, K. Noda, T. Nishino, and T. Nishino.** 1990. Proteolytic conversion of xanthine dehydrogenase from the NAD-dependent type to the O_2-dependent type. J. Biol. Chem. **265:**14170–14175.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Greene Publishing Associates, New York, N.Y.
3. **Biegert, T., U. Altenschmidt, C. Eckerskorn, and G. Fuchs.** 1993. Enzymes of anaerobic metabolism of phenolic compounds: 4-hydroxybenzoate-CoA ligase from a denitrifying *Pseudomonas* species. Eur. J. Biochem. **213:**555–561.
4. **Bonting, C. F. C., and G. Fuchs.** 1996. Anaerobic metabolism of 2-hydroxybenzoic acid (salicylic acid) by a denitrifying bacterium. Arch. Microbiol. **165:**402–408.
5. **Bonting, C. F. C., S. Schneider, G. Schmidtberg, and G. Fuchs.** 1995. Anaerobic degradation of *m*-cresol via methyl oxidation to 3-hydroxybenzoate by a denitrifying bacterium. Arch. Microbiol. **164:**63–69.
6. **Bossert, I. D., G. Whited, D. T. Gibson, and L. Y. Young.** 1989. Anaerobic oxidation of *p*-cresol mediated by a partially purified methyl-hydroxylase from a denitrifying bacterium. J. Bacteriol. **171:**1117–1122.
7. **Brackmann, R., and G. Fuchs.** 1993. Enzymes of anaerobic metabolism of phenolic compounds. 4-Hydroxybenzoyl-CoA reductase (dehydroxylating) from a denitrifying *Pseudomonas* species. Eur. J. Biochem. **213:**563–571.
8. **Buckel, W., and R. Keese.** 1995. One electron redox reactions of CoASH esters in anaerobic bacteria—a mechanistic proposal. Angew. Chem. Int. Ed. **34:**1502–1506.
9. **Cammack, R.** 1992. Iron-sulfur clusters in enzymes: themes and variations. Adv. Inorg. Chem. **38:**281–322.
10. **De Lorenzo, V., I. Cases, M. Herrero, and K. N. Timmis.** 1993. Early and late responses of TOL promoters to pathway inducers: identification of postexponential promoters in *Pseudomonas putida* with *lacZ-tet* bicistronic reporters. J. Bacteriol. **175:**6902–6907.
11. **Dispensa, M., C. T. Thomas, M.-K. Kim, J. A. Perrotta, J. Gibson, and C. S. Harwood.** 1992. Anaerobic growth of *Rhodopseudomonas palustris* on 4-hydroxybenzoate is dependent on AadR, a member of the cyclic AMP receptor protein family of transcriptional regulators. J. Bacteriol. **174:**5803–5813.
12. **Egland, P. G., J. Gibson, and C. S. Harwood.** 1995. Benzoate-coenzyme A ligase, encoded by *badA*, is one of three ligases able to catalyze benzoyl-coenzyme A formation during anaerobic growth of *Rhodopseudomonas palustris* on benzoate. J. Bacteriol. **177:**6545–6551.
13. **Egland, P. G., and C. S. Harwood.** Unpublished results.
14. **Elder, D. J. E., and D. J. Kelly.** 1994. The bacterial degradation of benzoic acid and benzenoid compounds under anaerobic conditions: unifying trends and new perspectives. FEMS Microbiol. Rev. **13:**441–468.
15. **Fuchs, G., M. E.-S. Mohamed, U. Altenschmidt, J. Koch, A. Lack, R. Brackmann, C. Lochmeyer, and B. Oswald.** 1994. Biochemistry of anaerobic biodegradation of aromatic compounds, p. 513–553. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
16. **Gibson, J., M. Dispensa, G. C. Fogg, D. T. Evans, and C. S. Harwood.** 1994. 4-Hydroxybenzoate-coenzyme A ligase from *Rhodopseudomonas palustris*: purification, gene sequence, and role in anaerobic degradation. J. Bacteriol. **176:**634–641.
17. **Gibson, J., and C. S. Harwood.** 1994. Anaerobic utilization of aromatic carboxylates by bacteria, p. 298–313. *In* R. G. Chaudhry (ed.), Biological degradation and bioremediation of toxic chemicals. Timber Press, Portland, Oreg.
18. **Gibson, J., and C. S. Harwood.** 1995. Degradation of aromatic compounds by non-sulfur purple bacteria, p. 991–1003. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
19. **Glatigny, A., and C. Scazzocchio.** 1995. Cloning and molecular characterization of *hxA*, the gene coding for the xanthine dehydrogenase (purine hydroxylase I) of *Aspergillus nidulans*. J. Biol. Chem. **270:**3543–3550.
20. **Gorny, N., and B. Schink.** 1994. Anaerobic degradation of catechol by *Desulfobacterium* sp. strain Cat2 proceeds via carboxylation to protocatechuate. Appl. Environ. Microbiol. **60:**3396–3400.
21. **Grether-Beck, S., G. L. Igloi, S. Pust, E. Schilz, K. Decker, and R. Brandsch.** 1994. Structural analysis and molybdenum-dependent expression of the pAO1-encoded nicotine dehydrogenase of *Arthrobacter nicotinovorans*. Mol. Microbiol. **13:**929–936.
22. **Harayama, S., A. Polissi, and M. Rekik.** 1991. Divergent evolution of chloroplast-type ferredoxins. FEBS Lett. **285:**85–88.
23. **Harwood, C. S., and J. Gibson.** 1988. Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. **54:**712–717.
24. **Hille, R., and T. Nishino.** 1995. Xanthine oxidase and xanthine dehydrogenase. FASEB J. **9:**995–1003.
25. **Hughes, R. K., W. A. Doyle, A. Chovnick, J. R. S. Whittle, J. F. Burke, and R. C. Bray.** 1992. Use of *rosy* mutant strains of *Drosophila melanogaster* to probe the structure and function of xanthine dehydrogenase. Biochem. J. **285:**507–513.
26. **Kasmi, E. A., R. Brackmann, G. Fuchs, and S. W. Ragsdale.** 1995. Hydroxybenzoyl-CoA reductase: coupling kinetics and electrochemistry to derive enzyme mechanisms. Biochemistry **34:**11668–11677.
27. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
28. **Keith, T. P., M. A. Riley, M. Kreitman, R. C. Lewontin, D. Curtis, and G. Chambers.** 1987. Sequence of the structural gene for xanthine dehydrogenase (rosy locus) in *Drosophila melanogaster*. Genetics **116:**67–73.
29. **Kim, M.-K., and C. S. Harwood.** 1991. Regulation of benzoate-CoA ligase in *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. **83:**199–204.
30. **Kretzer, A., K. Frunkze, and J. R. Andreesen.** 1993. Catabolism of isonicotinate by *Mycobacterium* sp. INA1: extended description of the pathway and purification of the molybdoenzyme isonicotinate dehydrogenase. J. Gen. Microbiol. **139:**2763–2772.
31. **Lack, A., and G. Fuchs.** 1992. Carboxylation of phenylphosphate by phenol carboxylase, an enzyme system of anaerobic phenol metabolism. J. Bacteriol. **174:**3629–3636.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
33. **Merkel, S. M., A. E. Eberhard, J. Gibson, and C. S. Harwood.** 1989. Involvement of coenzyme A thioesters in anaerobic metabolism of 4-hydroxybenzoate by *Rhodopseudomonas palustris*. J. Bacteriol. **171:**1–7.
34. **Möller, W., and R. Amons.** 1985. Phosphate-binding sequences in nucleotide-binding proteins. FEBS Letters **86:**1–7.
35. **Ornston, L. N., and R. Y. Stanier.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. **241:**3776–3786.
36. **Parales, R. E., and C. S. Harwood.** 1993. Regulation of the *pcaIJ* genes for aromatic acid degradation in *Pseudomonas putida*. J. Bacteriol. **175:**5829–5838.
37. **Pearson, D. M., C. O'Reilly, J. Colby, and G. W. Black.** 1994. DNA sequence

of the *cut A*, *B* and *C* genes, encoding the molybdenum containing hydroxylase carbon monoxide dehydrogenase, from *Pseudomonas thermocarboxydovorans* strain C2. Biochim. Biophys. Acta **1118:**423–428.
38. **Perrotta, J. A., and C. S. Harwood.** 1994. Anaerobic metabolism of cyclohex-1-ene-1-carboxylate, a proposed intermediate of benzoate degradation, by *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. **60:**1775–1782.
39. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene **127:** 15–21.
40. **Reichenbecher, W., A. Brune, and B. Schink.** 1994. Transhydroxylase of *Pelobacter acidigallici*: a molybdoenzyme catalyzing the conversion of pyrogallol to phloroglucinol. Biochim. Biophys. Acta **1204:**217–224.
41. **Reichenbecher, W., A. Rudiger, P. M. Kroneck, and B. Schink.** 1996. One molecule of molybdopterin guanine dinucleotide is associated with each subunit of the heterodimeric Mo-Fe-S protein transhydroxylase of *Pelobacter acidigallici* as determined by SDS/PAGE and mass spectrometry. Eur. J. Biochem. **237:**406–413.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Schübel, U., M. Kraut, G. Mörsdorf, and O. Meyer.** 1995. Molecular cloning of the gene cluster *coxMSL* encoding molybdenum-containing carbon monoxide dehydrogenase of *Oligotropha carboxidovorans*. J. Bacteriol. **177:**2197–2203.
44. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
45. **Stewart, V.** 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. **52:**190–232.
46. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
47. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
48. **Werth, M. T., G. Cecchini, A. Manodori, B. A. C. Ackrell, I. Schröder, R. P. Gunsalus, and M. K. Johnson.** 1990. Site-directed mutagenesis of conserved cysteine residues in *Escherichia coli* fumarate reductase: modification of the spectroscopic and electrochemical properties of the [2Fe-2S] cluster. Proc. Natl. Acad. Sci. USA **87:**8965–8969.
49. **Wierenga, R. K., M. C. H. De Maeyer, and W. G. L. Hol.** 1985. Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. Biochemistry **24:**1346–1357.
50. **Wootton, J. C., R. E. Nicolson, J. M. Cock, D. E. Walters, J. F. Burke, W. A. Doyle, and R. C. Bray.** 1991. Enzymes depending on the pterin molybdenum cofactor; sequence families, spectroscopic properties of molybdenum and possible cofactor-binding domains. Biochim. Biophys. Acta **1057:**157–185.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains; nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.