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2-Hydroxycyclohexanecarboxyl Coenzyme A Dehydrogenase, an Enzyme Characteristic of the Anaerobic Benzoate Degradation Pathway Used by *Rhodopseudomonas palustris*

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A gene, *badH*, whose predicted product is a member of the short-chain dehydrogenase/reductase family of enzymes, was recently discovered during studies of anaerobic benzoate degradation by the photoheterotrophic bacterium *Rhodopseudomonas palustris*. Purified histidine-tagged BadH protein catalyzed the oxidation of 2-hydroxycyclohexanecarboxyl coenzyme A (2-hydroxychc-CoA) to 2-ketocyclohexanecarboxyl-CoA. These compounds are proposed intermediates of a series of three reactions that are shared by the pathways of cyclohexanecarboxylate and benzoate degradation used by *R. palustris*. The 2-hydroxychc-CoA dehydrogenase activity encoded by *badH* was dependent on the presence of NAD^+; no activity was detected with $NADP^+$ as a cofactor. The dehydrogenase activity was not sensitive to oxygen. The enzyme has apparent K_m values of 10 and 200 μM for 2-hydroxychc-CoA and NAD^+, respectively. Western blot analysis with antisera raised against purified His-BadH identified a 27-kDa protein that was present in benzoate- and cyclohexanecarboxylate-grown but not in succinate-grown *R. palustris* cell extracts. The active form of the enzyme is a homotetramer. *badH* was determined to be the first gene in an operon, termed the cyclohexanecarboxylate degradation operon, containing genes required for both benzoate and cyclohexanecarboxylate degradation. A nonpolar *R. palustris badH* mutant was unable to grow on benzoate or cyclohexanecarboxylate but had wild-type growth rates on succinate. Cells blocked in expression of the entire cyclohexanecarboxylate degradation operon excreted cyclohex-1-ene-1-carboxylate into the growth medium when given benzoate. This confirms that cyclohex-1-ene-1-carboxyl-CoA is an intermediate of anaerobic benzoate degradation by *R. palustris*. This compound had previously been shown not to be formed by *Thauera aromatica*, a denitrifying bacterium that degrades benzoate by a pathway that is slightly different from the *R. palustris* pathway. 2-Hydroxychc-CoA dehydrogenase does not participate in anaerobic benzoate degradation by *T. aromatica* and thus may serve as a useful indicator of an *R. palustris*-type benzoate degradation pathway.

Aromatic compounds in the form of plant phenolics, plant-derived lignin monomers, and aromatic hydrocarbons are among the most abundant compounds on earth (22, 39). Despite the intrinsic resonance stability of the aromatic ring, many microbes can degrade aromatic compounds for use as carbon and energy. Two different biochemical strategies are used by microbes to accomplish the degradation of aromatic molecules, depending on the availability of oxygen. Under aerobic conditions, oxygenases hydroxylate aromatic rings to initiate the degradation process (13, 15). Under anaerobic conditions, reductive reactions relieve the resonance of the aromatic ring. In recent years, research has established that structurally diverse aromatic compounds are converted to benzoate or benzoyl-coenzyme A (CoA) as a starting intermediate for a central anaerobic pathway of aromatic ring reduction culminating in ring cleavage (16, 17).

The purple nonsulfur phototrophic bacterium *Rhodopseudomonas palustris* and the denitrifying bacterium *Thauera aromatica* have served as model systems for the study of anaerobic benzoate degradation. Initial physiological studies with these two organisms indicated that the pathway of anaerobic benzoate degradation can be divided into several segments that include (i) CoA thioester formation, (ii) ring reduction, (iii) introduction of a hydroxyl group, and (iv) ring fission. Based on this, it was assumed that *R. palustris* and *T. aromatica* used identical pathways to degrade benzoate. However, subsequent biochemical and molecular studies have made it clear that the benzoate degradation pathways used by these two bacteria are not exactly the same (16). The two organisms have similar benzoyl-CoA reductase enzymes, but once benzoyl-CoA is reduced to a cyclohexadienecarboxyl-CoA intermediate, the next step in *T. aromatica* is a hydration of the ring, whereas the next probable step in *R. palustris* is a two-electron reduction to give cyclohex-1-ene-1-carboxyl-CoA as an intermediate. Two slightly different sets of reactions follow which lead to the formation of two different ring fission products: pimelyl-CoA in the case of *R. palustris* and 3-hydroxypimelyl-CoA in the case of *T. aromatica* (Fig. 1).

Despite differences in the structures of the intermediates, a dehydrogenation reaction is required in each pathway to accomplish the conversion of a ring hydroxyl to a carbonyl. This reaction has not yet been studied for *R. palustris*, but we have proposed that it is catalyzed by the BadH protein (10). The *badH* gene is present in a cluster of benzoate degradation genes, and the predicted BadH protein has similarity to short-chain dehydrogenases (10). BadH does not have any significant amino acid sequence identity to the hydroxyacyl-CoA dehydrogenase (Had) used by *T. aromatica* to degrade benzoate (7, 16). Here we report the purification and characterization of

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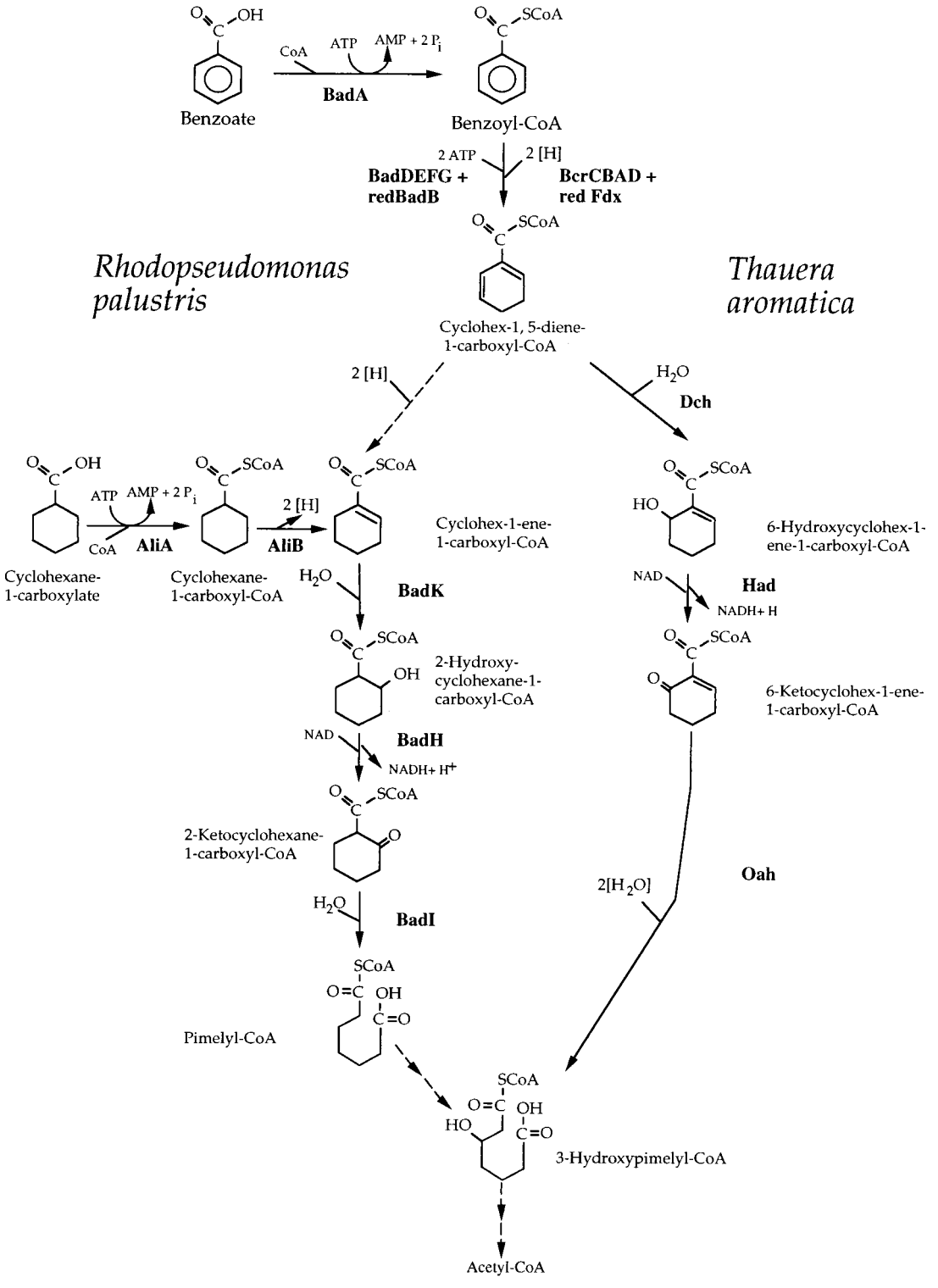

FIG. 1. Comparison of anaerobic benzoate degradation by *R. palustris* and *T. aromatica*. Reactions involved in funneling cyclohexanecarboxylate into the anaerobic benzoate degradation pathway in *R. palustris* are shown. Solid arrows indicate enzymatic activities that have been purified from either *R. palustris* or *T. aromatica* (1, 6, 11, 23, 25, 26, 33). Dashed arrows indicate hypothetical enzymatic reactions. Assignment of gene products from *R. palustris* (Fig. 2) and *T. aromatica* (7) to specific steps is indicated. redBadB, reduced BadB; redFdx, reduced ferredoxin.

BadH from *R. palustris*. We also demonstrate that *badH* is the first gene in an operon involved in cyclohexanecarboxylate utilization by this organism and is an essential gene for anaerobic benzoate degradation. A possible advantage of the *R. palustris*-type benzoate pathway as opposed to the *T. aromatica*-type pathway is that it provides a route for the utilization of the alicyclic acids cyclohexanecarboxylate and cyclohex-1-ene-1-carboxylate as growth substrates.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
E. coli strains		
DH5α	F⁻ λ⁻ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* ϕ80d*lacZ*ΔM15	Gibco-BRL
S17-1	*thi pro hdsR hdsM*⁺ *recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn*7*)	38
R. palustris strains		
CGA009	Wild-type strain; spontaneous Cm^r derivative	21
CGA702	*badH*::Km^r (polar)	This study
CGA720	*badH*::Km^r (nonpolar)	This study
Plasmids		
pUC18	High-copy-number cloning vector; ColE1 *lacZ* Ap^r	45
pJQ200KS	Mobilizable suicide vector; *sacB* Gm^r	35
pUC18K2	pUC18 containing nonpolar Km^r cassette	29
pTrcHisA	Six-histidine fusion protein expression vector; ColE1 *lacI*^{q} Ap^r	Invitrogen
pPE304	pHC79; cosmid cloning vector containing 35-kb insert including *badH*; Ap^r	10
pDP101	pUC18 with 2.6-kb fragment from pPE304 containing *badH*; Ap^r	This study
pDP203	pDP101 with Km^r gene at the *Stu*I site in *badH*	This study
pDP204	pJQ200KS; *sacB* suicide vector containing Km^r cassette at the *Stu*I site of *badH*	This study
pDP212	pDP101 with nonpolar Km^r gene at the *Stu*I site in *badH*	This study
pDP213	pJQ200KS; *sacB* suicide vector with nonpolar Km^r cassette at the *Stu*I site of *badH*	This study
pDPHisH1	pTrcHisA vector containing *badH*; Ap^r	This study

[a] Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Cm^r, chloramphenicol resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *R. palustris* was grown anaerobically in defined mineral medium at 30°C as described previously (11). Carbon sources were added to a final concentration of 3 mM, except succinate, which was used at a final concentration of 10 mM. Growth was monitored spectrophotometrically at 660 nm. Cultures were illuminated with 40- or 100-W incandescent light bulbs. Cells were harvested in the mid- to late logarithmic phase of growth by centrifugation, washed once in 20 mM Tris hydrochloride (pH 7.2) (Tris buffer), and stored at −70°C until used. *Escherichia coli* strains used for protein expression were grown with shaking at 30°C in Luria-Bertani medium supplemented with ampicillin (100 μg/ml).

Synthesis of acyl-CoA thioesters. Cyclohex-1-ene-1-carboxyl-CoA and pimelyl-CoA were synthesized from mixed anhydrides as described by Merkel et al. (30), except that the final alkali treatment step was omitted. The procedure for pimelyl-CoA synthesis yielded mainly pimelyl-CoA and only small amounts of pimelyldi-CoA. 2-Hydroxycyclohexanecarboxyl-CoA (2-hydroxychc-CoA) was synthesized enzymatically from cyclohex-1-ene-1-carboxyl-CoA using crotonase (EC 4.2.1.17) purchased from Sigma (St. Louis, Mo.). 3-Hydroxypimelyl-CoA was synthesized enzymatically using pimelyl-CoA as the starting substrate and partially purified pimelyl-CoA dehydrogenase and dehydropimelyl-CoA hydratase activities from benzoate-grown cells of *R. palustris* (M. Emig, unpublished data). Acetoacetyl-CoA and 3-hydroxybutyryl-CoA were purchased from Sigma. Acyl-CoA thioesters were purified using C_{18} reverse-phase Sep-Pack cartridges (Millipore Corp., Milford, Mass.) as described previously (33). High-pressure liquid chromatography (HPLC) analysis using an Ultrasphere octyldecyl silane (C_{18}) reverse-phase column (4.6 mm by 25 cm) (Beckman Instruments, Fullerton, Calif.) was used to confirm that the purified CoA thioester substrates were free of contaminating CoASH. The solvent system used was a mixture of 20 mM ammonium acetate (pH 6.0) and methanol. The column was equilibrated with 20% methanol, and elution was done by a linear gradient of 20 to 80% methanol in 30 min. The absorbance of the effluent was monitored by scanning the region from 210 to 260 nm using a model 996 photodiode array detector (Waters Associates, Milford, Mass.). The products of the synthesis reactions were analyzed by electrospray ionization mass spectrometry at the University of Iowa High-Resolution Mass Spectrometry Facility to confirm that they had the expected molecular mass. Since CoASH and acyl-CoA thioesters have essentially the same extinction coefficients at 254 nm, the acyl-CoA thioester substrates were quantitated spectrophotometrically at this wavelength according to a standard curve of known quantities of CoASH.

Cloning and DNA and RNA manipulations. Standard protocols were used for DNA cloning and transformation (4). Plasmids were purified using QIAprep spin columns (Qiagen Inc., Chatsworth, Calif.), and restriction fragments used for cloning were extracted from agarose gels with GeneClean spin (Bio 101, La Jolla, Calif.). *R. palustris* chromosomal DNA was isolated as described previously (9). The access reverse transcription-PCR system (Promega Corp., Madison, Wis.) was used to determine the transcriptional organization of the *badHIaliBA* and *badK* genes. Total RNA was isolated from benzoate-grown *R. palustris* cells using the SV total RNA isolation system (Promega Corp.).

Construction of *R. palustris* chromosomal mutants. A *badH*::Km^r (kanamycin resistant) nonpolar mutant (strain CGA720) was constructed by inserting the 0.85-kb Km^r cassette from pUC18K2 (29) into a unique *Stu*I site on the plasmid pDP101 to yield the plasmid pDP212. The plasmid pDP101 contains the entire *badH* gene cloned into pUC18. The *badH*::Km^r nonpolar construct was subcloned into the suicide vector pJQ200KS (35) to generate the plasmid pDP213. The *badH*::Km^r nonpolar construct (pDP213) was then introduced into *R. palustris* by conjugation from *E. coli* S17-1 (38). A *badH*::Km^r polar mutant (strain CGA702) was constructed by inserting the 1.3-kb Km^r GeneBlock cassette (Pharmacia) into a unique *Stu*I site on the plasmid pDP101 to yield the plasmid pDP203. The construct was cloned into the suicide vector pJQ200KS (35) to generate the plasmid pDP204. The *badH*::Km^r construct (pDP204) was then introduced into *R. palustris* by conjugation from *E. coli* S17-1 (38). Recombinants were selected as described previously (9). The insertion of the nonpolar Km^r cassette into the *badH* open reading frame was confirmed by PCR amplification of the *badH* region of the *R. palustris* chromosome from wild-type (0.8-kb product) and mutant (1.6-kb product) chromosomal DNA. The insertion of the polar Km^r GeneBlock cassette into the *badH* open reading frame was confirmed by Southern blot hybridization.

Enzyme activities. Standard assay conditions for hydroxyacyl-CoA dehydrogenase activity were 20 mM Tris-HCl (pH 9.0), 1.5 mM NAD, 0.5 mM hydroxyacyl-CoA substrate, 60 mM hydrazine, and appropriate amounts of protein. Activity was also monitored in the reductase direction using a solution containing 20 mM Tris-HCl (pH 7.0), 100 μM NADH, and 50 μM acetoacetyl-CoA, because 2-hydroxychc-CoA dehydrogenase is active with this substrate. Activity was measured spectrophotometrically by monitoring absorbance at 340 nm, and a molar extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH was used to calculate activities. The standard assay was used to estimate K_m values for hydroxyacyl-CoA substrates from double-reciprocal plots with substrates from 1.25 μM to 2 mM. Inhibition experiments were performed using the standard assay conditions except that enzyme was incubated on ice with the test compound for 15 min prior to addition to the assay mixture. 2-Ketocyclohexanecarboxyl-CoA hydrolase activity was measured as described previously (33).

Analysis of *R. palustris badH*::Km (CGA702) culture supernatants. CGA702 was grown in the light in defined mineral medium containing 10 mM succinate and 1.5 mM benzoate at 30°C. Aliquots (1.0 ml) were removed at various time points, cells were removed by centrifugation, and 0.5 ml of the resulting supernatant was analyzed by HPLC using an Ultrasphere octyldecyl silane (C_{18}) reverse-phase column (4.6 mm by 25 cm) (Beckman Instruments). The solvent system used was a mixture of 20 mM ammonium acetate (pH 6.0) and methanol. The column was equilibrated with 1% methanol, and elution was done by a linear gradient of 1 to 50% methanol in 30 min. The absorbance of the effluent was monitored by scanning the region from 210 to 260 nm using a model 996 photodiode array detector (Waters Associates). Peaks were identified by spectral properties and comigration by HPLC with authentic standards.

Preparation of *R. palustris* cell extracts. Cells were grown to late logarithmic phase, harvested by centrifugation, washed, and suspended in approximately 2 volumes of 20 mM Tris-HCl (pH 7.4) buffer containing 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5 μg of DNase/ml, 5 μg of RNase/ml, and 0.5 mM

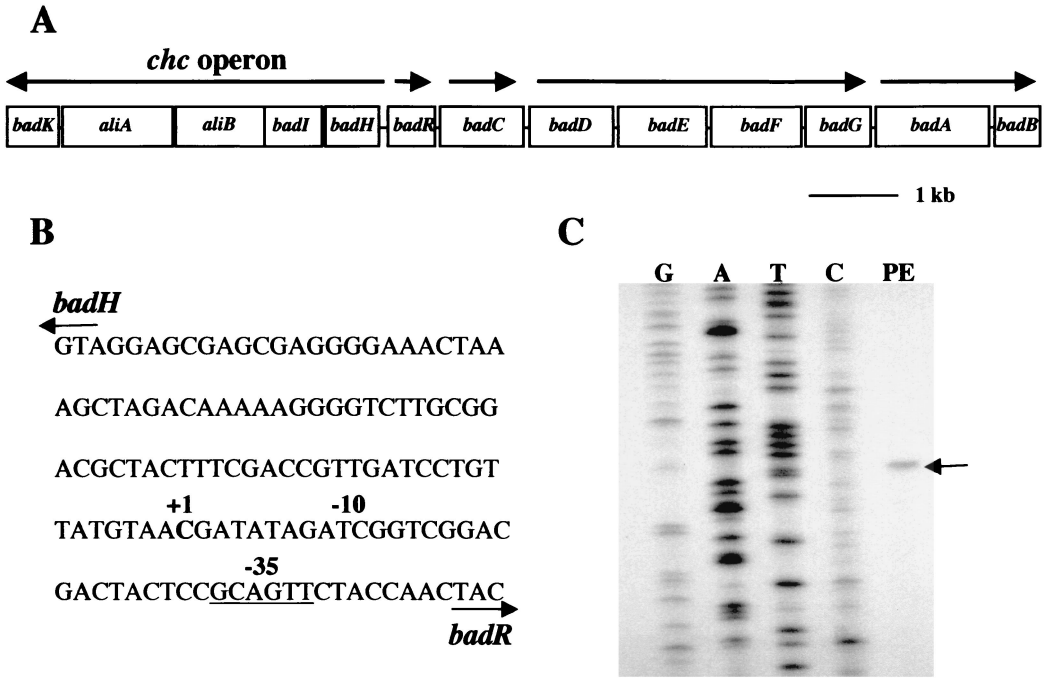

FIG. 2. (A) Map of *bad* (benzoic acid degradation) gene cluster from *R. palustris*. Arrows indicate transcriptional units. The genes encode enzymes of anaerobic benzoate or cyclohexanecarboxylate degradation, as indicated in Fig. 1. (B) Nucleotide sequence of the *badH* promoter region, with numbering indicating the start site of *badH* transcription (+1) and the −10 and −35 (underlined) nucleotides. (C) Mapping of the *badH* transcriptional start site by primer extension. The position of the primer extension product is indicated by the arrow (lane PE). RNA for the primer extension reaction was isolated from benzoate-grown cells. A sequence ladder generated with the same primer is shown.

phenylmethylsulfonyl fluoride. Cells were lysed by sonication, and cell debris was removed by centrifugation at 10,000 × *g* for 30 min at 4°C. The resulting supernatant was then centrifuged at high speed (100,000 × *g*) for 1 h at 4°C. The supernatant from the second centrifugation was termed crude cell extract.

Construction of a histidine-BadH fusion expression strain. A His-BadH fusion was constructed by PCR amplification of *badH* from pDP101 using primers containing *Eco*RI and *Bam*HI restriction sites. The *Eco*RI- and *Bam*HI-digested PCR product was ligated into the *Eco*RI and *Bam*HI restriction sites of the pTrcHisA vector (Invitrogen, Carlsbad, Calif.) to generate the plasmid pDPHisH1, resulting in an N-terminal six-histidine tag sequence and a factor Xa cleavage site in the recombinant BadH fusion protein. The plasmid pDPHisH1 was introduced into *E. coli* DH5α by transformation according to standard protocols (4).

Purification of histidine-tagged BadH by affinity chromatography. The His-BadH fusion protein was expressed in *E. coli* DH5α Cells were grown to an optical density at 660 nm of 0.6 and were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 to 4 h. Cells from 1 liter of culture were harvested by centrifugation, washed, and resuspended in binding buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl). Cells were lysed by passage through a French pressure cell at 85 MPa, and cell debris was removed by centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant from the centrifugation was termed crude cell extract. All subsequent steps were carried out at 4°C. Crude cell extract (typically 300 to 500 mg of protein) was loaded onto a 5-ml Hitrap chelating column (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) that had been charged with $NiSO_4$ and equilibrated with binding buffer. The column was washed extensively with binding buffer and then was washed with binding buffer containing 5% elution buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, 1 M imidazole) for 30 min at 1 ml/min. A linear gradient of 5 to 50% elution buffer in binding buffer was then passed through the column at a flow rate of 1 ml/min over a period of 25 min. Fractions (2 ml) were collected and assayed spectrophotometrically for 2-hydroxychc-CoA dehydrogenase activity. The enzyme activity was eluted at approximately 0.5 M imidazole. The activity-containing fractions from the affinity column were pooled and exchanged into 20 mM Tris-HCl (pH 7.4) using a 5-ml Hitrap desalting column at a flow rate of 1 ml/min. The activity-containing fractions were adjusted to 50% glycerol and stored at −20°C. Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Other analytical procedures. The native apparent molecular mass of His-BadH was determined by gel filtration chromatography using a Progel TSK G3000SW HPLC column (0.75 by 7.5 mm) (Supelco, Bellefonte, Pa.) equilibrated with 20 mM Tris-HCl (pH 7.5)–30 mM NaCl. The following protein molecular weight standards were used to calibrate the column: chymotrypsinogen A, 25,000; bovine serum albumin, 66,000; aldolase, 158,000; and ferritin, 440,000. SDS-PAGE was carried out with 12% acrylamide gels by standard procedures (24). Separated proteins were visualized by staining with Coomassie blue R-250. Molecular weight standards were from Bio-Rad Laboratories (Richmond, Calif.). The protein concentration was estimated by the dye-binding assay (Bio-Rad) using bovine serum albumin as a standard.

Immunoblotting. Polyclonal antiserum was prepared from a rabbit inoculated with purified His-BadH at Covance Research Products Inc. (Denver, Pa.). Cell extracts of *R. palustris* were typically separated on SDS–12% polyacrylamide gels and electroblotted onto an Immobilon polyvinylidene difluoride membrane (Millipore), and antigens were visualized using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) together with nitroblue tetrazolium and bromochloroindolyl phosphate as chromogenic substrates (4).

Computer analysis of DNA sequences. The BLAST network services at the National Center for Biotechnology Information (Bethesda, Md.) were used to search protein databases for similar sequences (3). Amino acid sequence similarities were calculated using the GAP program from the University of Wisconsin Genetics Computer Group software package (version 9.0) (8). The multiple sequence alignment was constructed using the CLUSTALW multiple sequence alignment program at the Baylor College of Medicine Human Genome Center (42). The program BOXSHADE (version 3.21) was used to shade aligned sequences.

RESULTS

***badH* is the first gene in an operon that contains genes for cyclohexanecarboxylic acid degradation.** The *badH* gene is the first gene in a cluster of five genes (*badH*, *badI*, *aliB* [formerly *badJ*], *aliA*, and *badK*) that are predicted to be transcribed in the same direction (Fig. 2). These genes are adjacent to genes known to be required for anaerobic benzoyl-CoA reduction (10). The genes *badI*, *aliB*, *aliA*, and *badK* have been assigned functions in either benzoate or cyclohexanecarboxylate degradation (Fig. 1). Reverse transcription-PCR amplification of total RNA isolated from benzoate-grown *R. palustris* cells showed that the five genes are organized as an operon. The transcriptional start site of the *badHIaliBAbadK* operon (henceforth called the chc degradation operon) was located 81

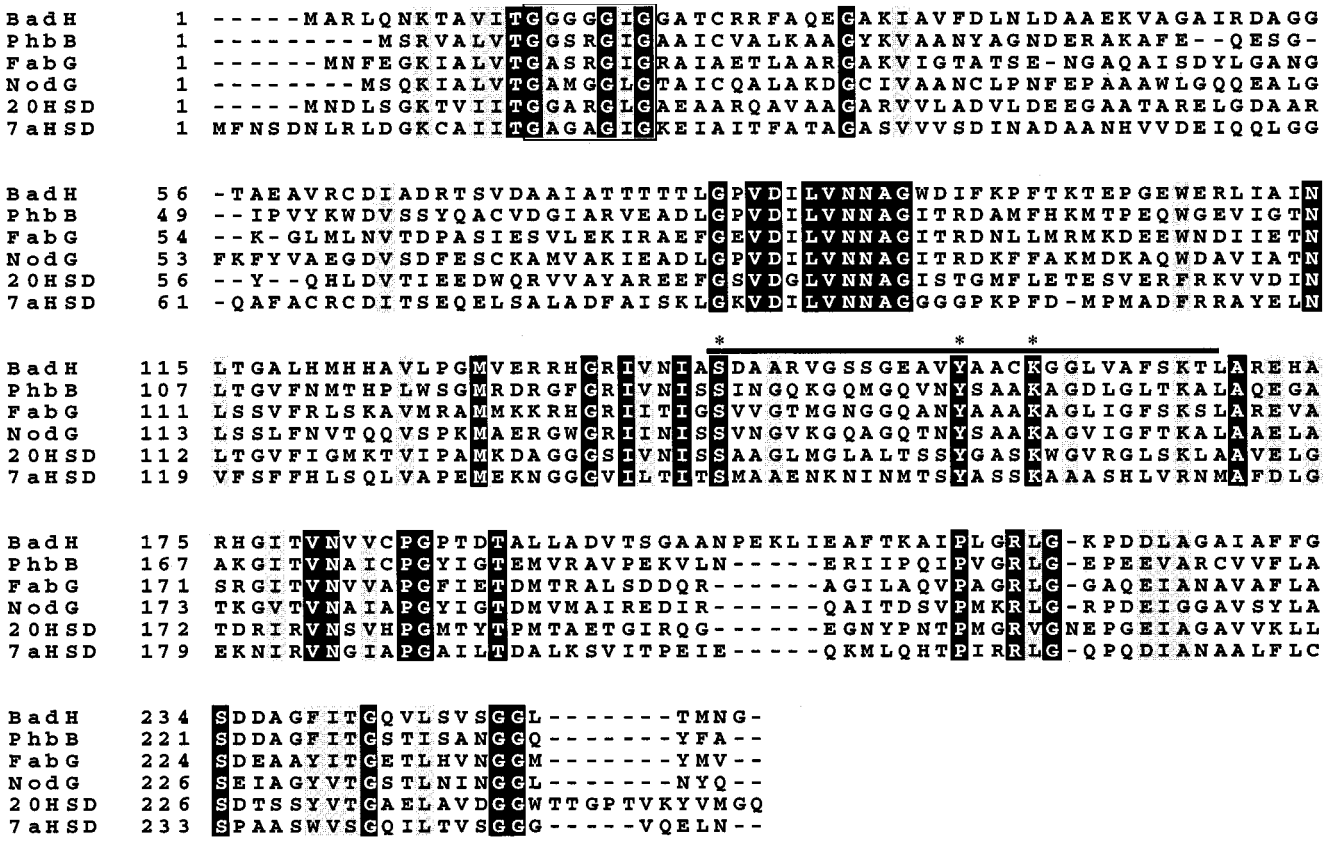

FIG. 3. Amino acid sequence alignment of BadH with selected members of the SDR family. The abbreviation, enzyme, number of amino acids, organism, and accession number, respectively, for each family member follow: BadH, 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase, 255, *R. palustris*, and U75363 (10); PhbB, acetoacetyl-CoA reductase, 241, *Rhizobium meliloti*, and P50205 (43); FabG, 3-ketoacyl-acyl carrier protein reductase, 244, *E. coli*, and P25716 (36); NodG, nodulation protein G, 246, *Azospirillum brasilense*, and P17611 (44); 20HSD, 20β-hydroxysteroid dehydrogenase, 255, *S. exfoliatus*, and P19992 (28); and 7αHSD, 7α-hydroxysteroid dehydrogenase, 255, *E. coli*, and P25529 (46). Alignment was created using CLUSTALW (version 1.74), and shading was done with the program BOXSHADE. Black shading indicates 100% amino acid identity, and gray shading indicates 100% similarity. *, active-site amino acids. The boxed sequence is the proposed pyridine nucleotide-binding site, and the solid black line denotes the SDR family signature (20).

bp upstream of the *badH* initiation codon. The *badH* promoter region has a −35 sequence similar to the consensus RNA polymerase sigma 70 subunit recognition element but no obvious −10 consensus sequence.

Molecular characteristics of *badH*. The *badH* gene is similar in deduced amino acid sequence to members of the short-chain dehydrogenase/reductase (SDR) family of enzymes (19, 20). Most members of the SDR family of enzymes are NAD(P)-dependent oxidoreductases of 250 to 300 amino acids that contain no metal cofactors and have a wide range of substrate specificities that include alcohols, sugars, prostaglandins, and hydroxysteroids (19, 20, 34). BadH is most similar to eubacterial acetoacetyl-CoA reductases involved in polyhydroxyalkanoate and fatty acid biosynthesis (about 40% amino acid identity) (36, 43). It is 37% identical to 7α-hydroxysteroid dehydrogenase from *E. coli* and 36% identical to 20β-hydroxysteroid dehydrogenase from *Streptomyces exfoliatus*. The *badH* open reading frame is predicted to encode a protein of 255 amino acids with a molecular mass of 26 kDa and a pI of 6.3. Signature features of members of the SDR family include a GxxxGxG motif near the N terminus, proposed to be a nucleotide-binding domain, and three highly conserved amino acids (a serine, a tyrosine, and a lysine) near the middle of the protein that have been implicated in catalysis (12, 28, 40, 41). BadH has these features (Fig. 3).

BadH is a 2-hydroxychc-CoA dehydrogenase. The sequence of reactions that we had proposed for the benzoate degradation pathway included the oxidation of 2-hydroxychc-CoA to 2-ketocyclohexanecarboxyl-CoA. We were able to measure 2-hydroxychc-CoA dehydrogenase activities of 45 and 35 nmol of 2-hydroxychc-CoA oxidized min^{-1} mg of $protein^{-1}$ in cell extracts of *R. palustris* that had been grown on benzoate and cyclohexanecarboxylate, respectively. The level of activity detected in succinate-grown cells was fivefold lower (7 nmol min^{-1} mg of $protein^{-1}$). 2-Hydroxychc-CoA dehydrogenase activity was not sensitive to oxygen. Based on its amino acid sequence, we hypothesized that BadH catalyzed this activity. The *badH* gene was therefore cloned into a histidine fusion expression vector to create the plasmid pDPHisH1. When the histidine-tagged BadH protein was expressed in *E. coli*, 2-hydroxychc-CoA dehydrogenase activity was detected, with a specific activity of 350 nmol min^{-1} mg of $protein^{-1}$. This activity was purified 18-fold to homogeneity by affinity chromatography (Fig. 4). Typical yields of purified enzyme per liter of culture were 5 to 10 mg, with a final specific activity of 6,500 nmol min^{-1} mg of $protein^{-1}$. The enzyme could be stored at −20°C in 50% glycerol for several months without significant loss of activity, but enzyme stored without glycerol became inactive within several days. Purified His-BadH had NAD^+-dependent dehydrogenase activity with 3-hydroxybutyryl-CoA

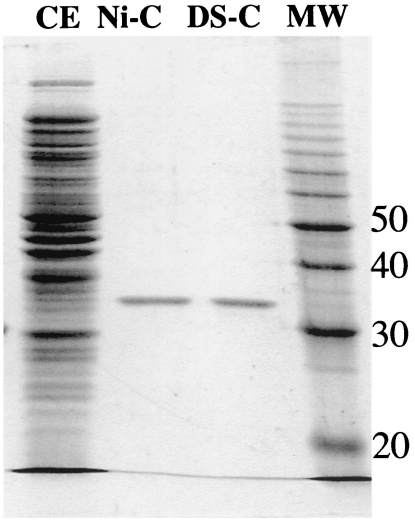

FIG. 4. SDS-PAGE analysis of active protein fractions obtained during purification of His-BadH. Lanes: CE, crude cell extract (20 μg); Ni-C, nickel column pooled fractions (1 μg); DS-C, Hitrap desalting column pooled fractions (1 μg); MW, protein ladder (Bio-Rad). Numbers on the right are molecular weights, in thousands.

as well as with 2-hydroxychc-CoA, but no activity was detected with 3-hydroxypimelyl-CoA as a substrate. Activity could be detected in the reverse direction with acetoacetyl-CoA when NADH was supplied as a cofactor. The rate of activity of the purified His-BadH protein with 2-hydroxychc-CoA as a substrate fell off rapidly (Fig. 5). This inhibition could be relieved by addition of hydrazine, which can react with the keto group of the reaction product to form a hydrazone, irreversibly removing the product (32). Similar results were obtained with an addition of purified 2-ketocyclohexanecarboxyl-CoA hydrolase. These data indicate that the equilibrium of the dehydrogenase reaction lies on the side of the hydroxyacyl-CoA substrate. In view of these findings, 60 mM hydrazine was routinely added to 2-hydroxychc-CoA assay mixtures.

The apparent K_ms for 2-hydroxychc-CoA and for NAD^+ were 10 μM (± 5 μM) and 200 μM, respectively. The K_m for 2-hydroxybutyryl-CoA was 70 μM. No activity was detected with $NADP^+$ as a cofactor. The pH optimum for the 2-hydroxychc-CoA dehydrogenase reaction with purified His-BadH was about pH 9.5 in 20 mM Tris-HCl buffer. Activity at pH 10.0 was the same as that at pH 9.5, while activities at pHs 9.0, 8.0, and 7.0 were 86, 46, and 19%, respectively, of that at pH 9.5.

Active His-BadH had a native molecular mass of 115 kDa as determined by gel filtration chromatography and a subunit molecular mass of 32 kDa as determined by SDS-PAGE analysis. The predicted molecular mass of the histidine-tagged form of BadH is 30 kDa. This indicates that the active enzyme is a homotetramer.

Construction and analysis of a *badH* mutant. To determine if BadH is required for growth of *R. palustris* on aromatic or alicyclic acids, a nonpolar mutation was made in the *badH* gene by insertion of a kanamycin resistance gene cassette. This cassette contained translational stop codons in all three reading frames at the 5′ end of the kanamycin resistance gene and a ribosome binding site and translational start sites at the 3′ end to ensure translation of downstream genes. The *badH* mutant (strain CGA720) that was generated in this way was unaffected in its expression of *badI*, the gene immediately downstream of *badH*, as determined by Western blot analysis with BadI antiserum. Also, extracts of *badH* mutant cells grown on succinate plus benzoate had BadI (2-ketocyclohexanecarboxyl-CoA hydrolase) enzymatic activity.

The *badH* mutant was unable to grow anaerobically on benzoate, cyclohexanecarboxylate, or cyclohex-1-ene-1-carboxylate, but it had wild-type doubling times on succinate. It also failed to grow aerobically on cyclohexanecarboxylate or cyclohex-1-ene-1-carboxylate. Wild-type *R. palustris* cells grow well on these two alicyclic acids under aerobic conditions, whereas benzoate degradation is sensitive to oxygen. No 2-hydroxychc-CoA dehydrogenase activity was detected in extracts of the *badH* mutant grown on any of the carbon sources tested. Polyclonal antiserum that was prepared against purified His-BadH reacted with a protein of 27 kDa (the predicted size of BadH) that was present in extracts of benzoate-grown wild-type cells of *R. palustris*. No BadH protein was detected in extracts of succinate-grown cells or in extracts of *badH* mutant cells grown on benzoate plus succinate (data not shown).

A mutant blocked in expression of the chc degradation operon excretes cyclohex-1-ene-1-carboxylate when given benzoate. In addition to creating the *badH* nonpolar mutation described above, we also inserted an unaltered kanamycin resistance gene into *badH* to generate a mutant that, due to polar effects of the insertion, was defective in expression of all genes of the chc degradation operon. This polar *badH* mutant was, as expected, unable to grow on benzoate, cyclohexanecarboxylate, or cyclohex-1-ene-1-carboxylate. We also found that mutant cells growing on succinate in the presence of 1.5 mM benzoate excreted substantial amounts of the alicyclic acid cyclohex-1-ene-1-carboxylate (Fig. 6). This was probably derived from cyclohex-1-ene-1-carboxyl-CoA that was formed from benzoate and that accumulated intracellularly in the blocked mutant.

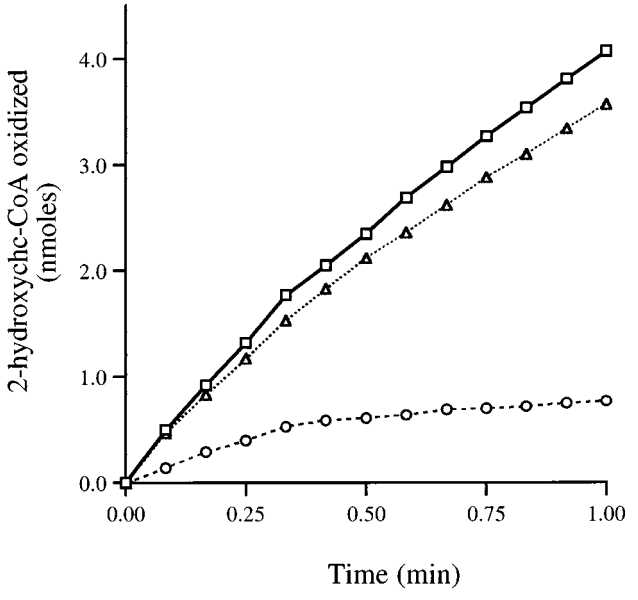

FIG. 5. Time course of 2-hydroxychc-CoA dehydrogenase activity. Activity was measured spectrophotometrically by monitoring the reduction of NAD^+ at 340 nm using standard assay conditions as described in Materials and Methods. Reaction mixtures contained purified His-BadH (0.8 μg) plus purified 2-ketocyclohexanecarboxyl-CoA hydrolase (10 μg) (triangles), hydrazine (60 mM) (squares), or neither hydrazine nor 2-ketocyclohexanecarboxyl-CoA hydrolase (circles).

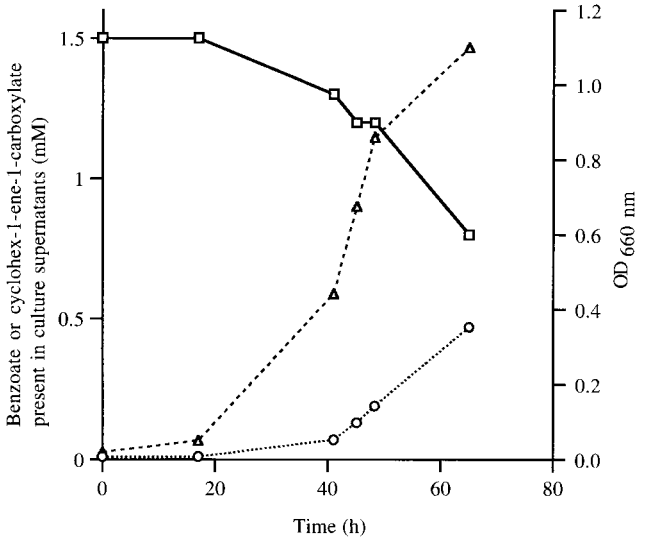

FIG. 6. Time course of anaerobic benzoate transformation by cultures of CGA702 (*badH*::Km^r). Cells were grown anaerobically in mineral medium containing 10 mM succinate and 1.5 mM benzoate. Aliquots were removed at various time points, cells were removed by centrifugation, and supernatants were analyzed by C_{18} reverse-phase HPLC for the presence of benzoate (squares) and cyclohex-1-ene-carboxylate (circles). Growth (represented by triangles) was monitored spectrophotometrically by monitoring optical density at 660 nm ($OD_{660\ nm}$). Data points are averages of duplicates.

DISCUSSION

The ability to degrade benzoate and various aromatic compounds that are processed through benzoyl-CoA is widespread among taxonomically diverse bacteria, including phototrophs, denitrifiers, fermentative bacteria, sulfate reducers, and iron-reducing bacteria (3, 5, 14, 18, 27). To date, however, detailed studies of anaerobic benzoyl-CoA degradation have been carried out with just two species, the denitrifier *T. aromatica* and the phototroph *R. palustris*. From this work, it is evident that at least two variants of the benzoyl-CoA degradation pathway exist (Fig. 1) (16). Work presented here provides further evidence in favor of the pathway that we have proposed for *R. palustris*. Previously we reported the cloning and sequencing of a cluster of five genes, *badK*, *aliA*, *aliB*, *badI*, and *badH*, that we hypothesized to have a role in the degradation of both benzoate and the alicyclic acid cyclohexanecarboxylate (10). Here we demonstrate that these genes are organized as an operon. We also provide evidence that cyclohex-1-ene-1-carboxyl-CoA itself, and not a hydroxylated derivative, is an intermediate in anaerobic benzoate degradation by showing that a mutant blocked in the transcription of the chc degradation operon accumulates cyclohex-1-ene-1-carboxylate in the growth medium when it metabolizes benzoate.

Each of the two known variants of the anaerobic benzoate degradation pathways may have advantages. The *T. aromatica* pathway accomplishes the conversion of benzoyl-CoA to 3-hydroxypimelyl-CoA in just four enzymatic steps, compared to seven steps for *R. palustris* (16). On this basis, the *T. aromatica* pathway is more efficient. The *R. palustris* pathway, on the other hand, provides a point of entry for alicyclic acids, like cyclohexanecarboxylate, to be degraded. At this point, it is not known whether additional variants of the benzoyl-CoA degradation pathway will be found in other bacteria. The fermentative bacteria deserve special consideration, however, because of the severe energetic constraints under which they operate. Bacteria of the genus *Syntrophus* can grow with benzoate as a sole carbon and energy source when they are in coculture with hydrogen-consuming bacteria, such as methanogens or sulfate reducers, but energy calculations show that they cannot possibly generate sufficient ATP to support growth by using either of the benzoyl-CoA degradation pathways shown in Fig. 1 (31, 37). Thermodynamic calculations suggest that a four-electron rather than a two-electron reduction of benzoyl-CoA would not necessarily require the hydrolysis of ATP. A modification of the pathway, such that benzoyl-CoA is reduced directly to cyclohex-1-ene-1-carboxyl-CoA, would allow sufficient ATP to be generated for growth. This postulated pathway for fermenters would be very similar to the proposed *R. palustris* pathway, but with the omission of a cyclohexadienecarboxyl-CoA as an intermediate.

With this report, the functions of each of the five gene products of the chc degradation operon have now been confirmed either by N-terminal amino acid sequencing of purified enzymes or by heterologous expression of recombinant enzyme activities in *E. coli*. The *badI* gene product has been purified from *R. palustris* and shown to catalyze the hydrolytic cleavage of 2-ketocyclohexanecarboxyl-CoA (33). The gene *aliB* encodes an acyl-CoA dehydrogenase that has been heterologously expressed, purified, and shown to catalyze the oxidation of cyclohexanecarboxyl-CoA to cyclohex-1-ene-1-carboxyl-CoA (Emig, unpublished data). The gene *aliB* was formerly named *badJ* (10). We propose that this gene be renamed *aliB* because we have now determined that it functions to funnel the alicyclic compound cyclohexanecarboxylate into the benzoate pathway and does not appear to have a direct role in anaerobic benzoate degradation (Emig, unpublished data). The gene *aliA* encodes cyclohexanecarboxylate-CoA ligase. This enzyme has been purified from *R. palustris*, and the N-terminal amino acid sequence was determined (23). The gene *badK* encodes an enoyl-CoA hydratase that has previously been demonstrated to have activity with cyclohex-1-ene-1-carboxyl-CoA (10). Finally, the *badH* gene described here was expressed and shown to encode a 2-hydroxychc-CoA dehydrogenase that is required for anaerobic growth on benzoate as well as for growth on cyclohexanecarboxylate under either aerobic or anaerobic conditions.

2-Hydroxychc-CoA dehydrogenase activity may prove to be a useful indicator of an *R. palustris*-type, as opposed to a *T. aromatica*-type, benzoyl-CoA degradation pathway. The substrate for this enzyme can be prepared comparatively easily. Cyclohex-1-ene-1-carboxyl-CoA is easily prepared from its commercially available free acid, and it can then be hydrated to form 2-hydroxychc-CoA using commercially available crotonase. The benzoate degradation pathway used by *T. aromatica* includes a 6-hydroxycyclohex-1-ene-carboxyl-CoA dehydrogenase that is an NAD^+-dependent alcohol dehydrogenase rather than an SDR, as is BadH (7, 26). This enzyme has been purified and found not to be active with 2-hydroxychc-CoA as a substrate (26). *T. aromatica* cell extracts do have a 2-hydroxychc-CoA dehydrogenase activity, but in contrast to *R. palustris*, the activity is not inducible by anaerobic growth on benzoate (26).

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