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The BH1999 Protein of *Bacillus halodurans* C-125 Is Gentisyl-Coenzyme A Thioesterase

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In this study, we have shown that recombinant BH1999 from *Bacillus halodurans* catalyzes the hydrolysis of gentisyl coenzyme A (CoA) (2,5-dihydroxybenzoyl-coenzyme A) at physiological pH with a k_{cat}/K_m of 1.6×10^6 M^{-1} s^{-1} and the hydrolysis of 3-hydroxybenzoyl-CoA with a k_{cat}/K_m of 3.0×10^5 M^{-1} s^{-1}. All other acyl-CoA thioesters tested had low or no substrate activity. The BH1999 gene is juxtaposed with a gene cluster that contains genes believed to function in gentisate oxidative degradation. It is hypothesized that BH1999 functions as a gentisyl-CoA thioesterase. Gentisyl-CoA thioesterase shares the backbone fold and the use of an active site aspartate residue to mediate catalysis with the 4-hydroxybenzoyl-CoA thioesterase of the hotdog fold enzyme superfamily. A comparative study of these two enzymes showed that they differ greatly in the rate contribution made by the catalytic aspartate, in the pH dependence of catalysis, and in substrate specificity.

The pathways leading to the degradation of environmental aromatic compounds in bacteria and fungi form the foundation for the bioremediation of industrial waste products. Studies have shown that a wide variety of aromatic compounds are degraded through pathways that converge at catechol or protocatechuate (12). These intermediates, in turn, undergo oxidative ring opening, followed by mineralization via the β-ketoadipate pathway (12). Gentisate (2,5-dihydroxybenzoate) is another common intermediate that has been implicated in the degradation pathways of 3-hydroxybenzoate (9), xylenol (20), salicylate (21), 3,6-dichloro-2-methoxybenzoate (28), and naphthalene (8). Gentisate undergoes oxidative ring cleavage (catalyzed by gentisate 1,2-dioxygenase) to maleylpyruvate. Maleylpyruvate is either cleaved to pyruvate and maleate by the enzyme maleylpyruvate hydrolase or converted to fumarylpyruvate with maleylpyruvate isomerase. The fumarylpyruvate is then cleaved to fumarate and pyruvate by fumarylpyruvate hydrolase (2, 14, 20, 22).

In *Bacillus halodurans* C-125 (26), gentisate degradation via oxidation to maleylpyruvate and cleavage to pyruvate is evidenced by the gene cluster represented in Fig. 1. The gene cluster contains two genes encoding homologs to gentisate 1,2-dioxygenase (BH2002 and BH2004, which have 32 to 37% sequence identity to the previously characterized gentisate 1,2-dioxygenase from *Sphingomonas* sp. strain RW5 [28]) and *Ralstonia* sp. strain U2 (30). In addition, the neighboring gene BH2005 encodes a homolog of fumarylacetoacetate hydrolase, an enzyme that catalyzes hydrolytic cleavage in the close analog fumarylacetoacetate (17). The presence of an isomerase homologue (BH2000) may indicate the formation of fumarylpyruvate. The upstream gene BH1999 encodes a small protein which is homologous to the 4-hydroxybenzoyl-coenzyme A (CoA) thioesterases from *Pseudomonas* sp. strain DJ12 (35% identity in amino acid sequence) (5) and strain CBS3 (31% identity in amino acid sequence) (3). It seemed plausible that BH1999 is a gentisyl-CoA thioesterase.

To test this hypothesis, the BH1999 gene was cloned for overexpression in *Escherichia coli*. Here we report the characterization of the purified protein product and present results which show that the catalytic function of this newest addition to the hotdog fold thioesterase superfamily (3) is indeed directed to gentisyl-CoA thioester hydrolysis.

MATERIALS AND METHODS

Chemicals. All restriction enzymes and T4 DNA ligase were purchased from Gibco-BRL. Oligonucleotide primers were synthesized by Gibco-BRL. DNA sequencing was performed by the DNA sequencing facility of the University of New Mexico. Benzoyl-CoA, crotonyl-CoA, *n*-propionyl-CoA, and *n*-hexanoyl-CoA were purchased from Sigma. 4-Hydroxybenzoyl-CoA, 3-hydroxybenzoyl-CoA,4-chlorobenzoyl-CoA, 4-methoxybenzoyl-CoA, phenylacetyl-CoA, 4-hydroxyphenylacetyl-CoA, 3-hydroxyphenylacetyl-CoA, and 3,4-dihydroxyphenylacetyl-CoA were synthesized as reported (16).

To a stirred solution of the benzoic acid or phenylacetic acid derivative (470 μmol) in dry tetrahydrofuran (THF) (4 ml), ethyl chloroformate (40 μl, 460 μmol), and triethylamine (50 μl, 360 μmol) were added at 25°C under N_2. A white precipitate, which formed over a period of 1 h, was removed by filtration. The resulting clear solution was added dropwise to an N_2-purged solution of 50 mg of coenzyme A lithium salt (65 μmol) in 5 ml of 50% THF–water. The pH of the solution was maintained between 7.5 and 8.0 by the addition of 0.1 N LiOH. After stirring for 1 h under N_2, the solution was acidified to pH 4 with 1 N HCl. The precipitate was removed by centrifugation (15 min), and the resulting supernatant was adjusted to pH 10. After 1.5 h (for hydrolysis of the side product), the pH was adjusted to 7 with 1 N HCl, and the mixture was then lyophilized. The residue was dissolved in deionized water and then chromatographed on a Sephadex-G25 (Amersham Pharmacia) column (120 cm by 2.5 cm) at 4°C with deionized water as the eluant.

Gentisyl-CoA was prepared by reacting 2,5-dihydroxybenzoic chloride with CoA. 2,5-Dihydroxybenzoic chloride was prepared as previously described (18). To a suspension containing 7.52 g (49 mmol) of 2,5-dihydroxybenzoic acid in 50 ml of anhydrous toluene, 0.1 mol of anhydrous pyridine was added. Thionyl chloride (7.18 g, 60 mmol) was added dropwise to the stirred solution at 10°C. The reaction mixture was then stirred at 60°C for 6 h. The solution was decanted from the residue and concentrated in vacuo at room temperature. The crude acid chloride (300 mg) was dissolved in 5 ml of anhydrous ethanol and then added dropwise to a stirred solution of 30 mg of coenzyme A lithium salt (40 μmol) in

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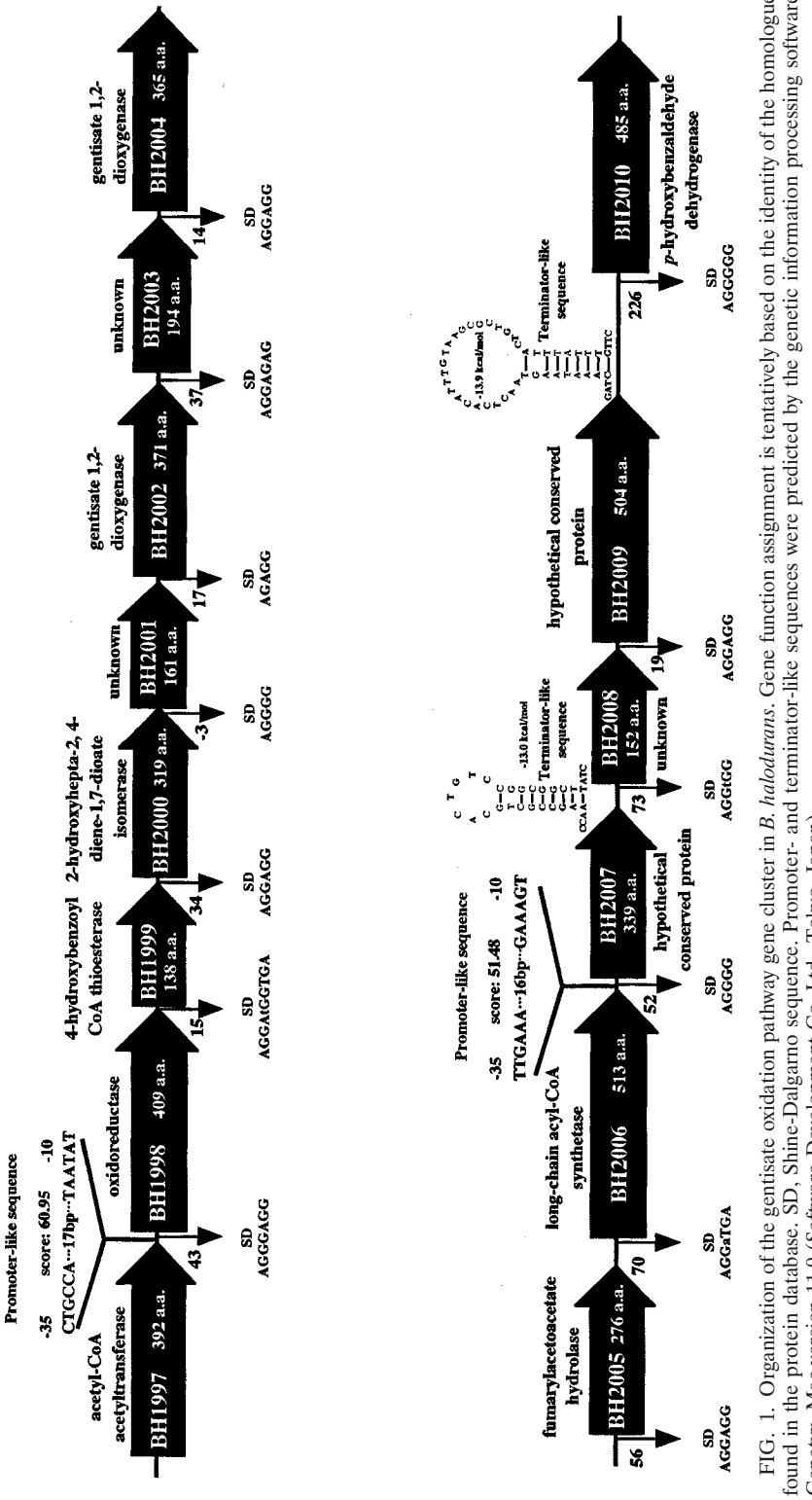

FIG. 1. Organization of the gentisate oxidation pathway gene cluster in *B. halodurans*. Gene function assignment is tentatively based on the identity of the homologue found in the protein database. SD, Shine-Dalgarno sequence. Promoter- and terminator-like sequences were predicted by the genetic information processing software Genetyx, Mac version 11.0 (Software Development Co. Ltd., Tokyo, Japan).

2 ml of 0.5 M $NaHCO_3$ (pH 8.0) at 10°C. The pH was maintained between 7 and 8 by the addition of 1.0 M LiOH in deionized water. The mixture was stirred for an additional 16 h, with the pH maintained at 7 to 8. The mixture was extracted three times with 4 ml of ethyl acetate. The water fraction was concentrated by lyophilization and then chromatographed on a Sephadex-G25 (Amersham Pharmacia) column (120 cm by 2.5 cm) at 4°C with deionized water as the eluant.

The column fractions were analyzed with a Rainin Dynamax high-pressure liquid chromatography (HPLC) system equipped with a Beckman Ultrasphere C-18 reverse-phase column (0.46 by 25 cm). The HPLC column was preequilibrated in 20% solution B (20 mM ammonium phosphate, dibasic, in 64% acetonitrile water solution, pH 6.7) and 80% solution A (20 mM ammonium phosphate, dibasic, in deionized water, pH 6.7). A sequential, linear gradient (20% B, 2 min; 20 to 65% solution B, 14 min; 65% to 80% solution B, 2 min; and 80% to 20% solution B, 2 min) was employed to elute the column at a flow rate of 1.0 ml/min. The retention time of gentisyl-CoA was 4.5 min.

The Sephadex column fractions containing pure gentisyl-CoA were pooled and concentrated by lyophilization. The yield of gentisyl-CoA was ca. 3 mg (10%). The UV spectrum of gentisyl-CoA (in water) was λ_{max} = 260 nm, ε = 17.8 mM^{-1} cm^{-1}, $\lambda_{shoulder}$ = 350 nm, ε = 4.43 mM^{-1} cm^{-1}. ^1H nuclear magnetic resonance of gentisyl-CoA (in D_2O pH 6.0): δ0.59 (s, 3H), 0.73 (s, 3H), 2.28 (t, 2H, J = 6Hz), 3.02 (t, 2H), 3.33 (m, 4H), 3.45 (d, 1H, J = 6Hz), 3.69 (d, 1H, J = 7Hz), 3.88 (s, 1H), 4.13 (s, 2H), 4.44 (s, 1H), 5.95(d, 1H, J = 5Hz), 6.65 (d, 1H, J = 9Hz, aromatic H), 6.86 (d, 1H, J = 6Hz, aromatic H), 7.00 (d, 1H, J = 3Hz, aromatic H), 7.97 (s, 1H), 8.33 (s, 1H).

Cloning and expression of the BH1999 gene in *E. coli* and protein purification. The gene encoding BH1999 from *Bacillus halodurans* C-125 (24) was amplified by PCR with the clone BH1999/pCR 2.1 and *Pfu* Turbo DNA polymerase (Stratagene). Primers containing restriction endonuclease cleavage sites for *Nde*I and *Xho*I were used. The amplification protocol employed 30 cycles of denaturation at 95°C, annealing at 45°C, and elongation at 72°C. The pET-23b vector (Novagen), which was linearized with the restriction enzymes *Nde*I and *Xho*I, was ligated to the isolated gene. The ligation product was used to transform *E. coli* JM109 competent cells (Stratagene). Plasmid was prepared using a QIAprep Spin Miniprep Kit (Qiagen). The gene sequence was confirmed by DNA sequencing. The recombinant plasmid wild-type-BH1999/pET-23b was used to transform BL21(DE3) competent cells (Novagen). The transformed cells were grown at 32°C in 1.5 liters of Luria broth (LB) containing 50 μg of carbenicillin/ml to an OD_{600} of ~0.8 and then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to an OD_{600} of ~2.0. The cells were harvested by centrifugation, resuspended in ice-cold 50 mM K^+HEPES buffer (pH 7.5) containing 2 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and lysed in a French press. Following centrifugation, the supernatant was loaded onto a Butyl Sepharose 4 fast flow column (Amersham Pharmacia Biotech, 2.5 by 25 cm) equilibrated with 1,000 ml of 10% ammonium sulfate in 50 mM potassium salt of *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (K^+HEPES buffer). A 1,200-ml 10% to 0% linear gradient of ammonium sulfate in 50 mM K^+HEPES (pH 7.5, 4°C) with a flow rate of 3 ml/min was used to elute the protein.

The desired fractions (eluted at 6% ammonium sulfate) were pooled, concentrated, and dialyzed against 50 mM K^+HEPES (pH 7.5, 4°C) containing 0.1 M KCl and 1 mM dithiothreitol. The sample was loaded onto a Bio-Gel HTP gel hydroxyapatite (Bio-Rad, 2.5 by 20 cm) column and eluted with 400 ml of a linear gradient of 0.01 M to 0.4 M K_2HPO_4 in 10 mM K^+HEPES (pH 7.5, 4°C) with a flow rate of 0.8 ml/min. The desired fractions (eluted at 0.3 M K_2HPO_4) were pooled, concentrated, and dialyzed against 10 mM 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonate (CAPSO, pH 9.5, 4°C) containing 0.5 M KCl and 1 mM dithiothreitol. The enzyme was stable under this condition for prolonged storage at −80°C. The yield of homogeneous BH1999 protein (as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] analysis) was 20 mg of protein/g of wet cells.

Site-directed mutations. Mutagenesis was carried out with a PCR strategy based on WT-BH1999/pET-23b plasmid as the template, commercial primers, the PCR kit supplied by Stratagene, and the Techgene thermal cycler manufactured by Techne (Princeton, N.J.). The PCR products were used to transform *E. coli* JM109 competent cells (Stratagene). Plasmids were prepared with a QIAprep spin miniprep kit (Qiagen). The sequence of the mutated gene was confirmed by DNA sequencing. The recombinant mutant plasmid was used to transform *E. coli* BL21(DE3) competent cells (Novagen). The D16A, D16N, and D31N mutant proteins were purified as described above for the wild-type BH1999 protein and shown to be homogeneous on the basis of SDS-PAGE analysis. The yields of homogeneous mutant BH1999 proteins were (per gram of wet cells): D16A, 15 mg/g; D16N, 15 mg/g; and D31N, 14 mg/g.

Steady-state kinetic analysis of catalyzed hydrolysis of hydroxybenzoyl-CoAs. The recombinant *Pseudomonas* sp. strain CBS3 4-hydroxybenzoyl-CoA thioesterase (31) catalyzed hydrolysis reaction of 4-hydroxybenzoyl-CoA was monitored as described (31). All other thioesterase reactions were monitored at 25°C with a 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB)-based assay in which the absorbance of 5-thio-2-nitrobenzoate at 412 nm was measured. The 5-thio-2-nitrobenzoate was produced by the reaction of DTNB with the CoA liberated from the acyl-CoA substrate upon hydrolysis. The 0.2-ml reaction solutions contained enzyme (BH1999: 0.25 μM for 4-hydroxybenzoyl-CoA, 0.015 μM for 3-hydroxybenzoyl-CoA, 0.005 μM for gentisyl-CoA, 0.42 μM for benzoyl-CoA, 0.05 μM for 4-chlorobenzoyl-CoA, 0.25 μM for 4-methoxybenzoyl-CoA, 4.0 μM for phenylacetyl-CoA, 7.5 μM for 3-hydroxyphenylacetyl-CoA, 4-hydroxyphenylacetyl-CoA, 3,4-dihydroxyphenylacetyl-CoA, crotonyl-CoA, n-propionyl-CoA and *n*-hexanoyl-CoA; *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase: 0.01 μM for 3-hydroxybenzoyl-CoA and gentisyl-CoA), various concentrations of substrate (0.5 to 5 times the K_m), DTNB (1 mM), KCl (0.2 M), and 50 mM K^+HEPES (pH 7.5, 25°C) in a quartz cuvette of 1-cm light path length.

The initial velocity data, measured as a function of substrate concentration, were analyzed with the equation below and the computer program KinetAsyst (IntelliKinetics): $V = V_{max}[S]/([S] + K_m)$, where V is the initial velocity, V_{max} is the maximum velocity, $[S]$ is the substrate concentration, and K_m is the Michaelis constant. k_{cat} was calculated from $V_{max}/[E]$ where $[E]$ is the total enzyme concentration determined by the Bradford method (4).

Gentisyl-CoA hydrolysis catalyzed by the BH1999 mutants (1.6 μM for D16A, 0.5 μM for D16N, and 0.09 μM for D31N mutants) was monitored with the DTNB assay described above. For the BH1999 D16A mutant, low activity and a small K_m prohibited accurate determination of the K_m value, and therefore only the k_{cat} value was determined (with saturating substrate concentrations).

Determination of the pH optimum for BH1999-catalyzed reactions. The k_{cat} values were determined from initial velocity data measured, at saturating levels of substrate, as a function of the reaction pH. BH1999 (0.09 μM)-catalyzed hydrolysis of gentisyl-CoA (250 μM) was monitored at 365 nm (Δε of 4.0 mM^{-1} cm^{-1}) and 25°C over the pH range of 6.0 to 11.0. The pH was maintained with a dual buffer system consisting of 50 mM 2-(*N*-morpholino)ethanesulfonate (MES) and 50 mM HEPES (pH 6.0 to 8.0); 50 mM HEPES and 50 mM *N*-tris(hydroxymethyl)methyl-3-aminopropane sulfonate (TAPS, pH 8.0 to 9.0); 50 mM TAPS and 50 mM CAPSO (pH 9.0 to 10.0); and 50 mM CAPSO and 50 mM 3-(cyclohexylamino)-1-propanesulfonate (CAPS, pH 10.0 to 11.0) containing 0.2 M KCl.

Stability of BH1999 in buffer solution at different pH values. BH1999 (7.5 μM) was incubated (at 25°C) in solutions (containing 0.2 M KCl) having different pH values, buffered with 50 mM acetate and MES (pH 4.0 to 5.0), 50 mM HEPES and MES (pH 6.0 to 8.0), 50 mM TAPS and CAPSO (pH 9.0), and 50 mM CAPSO and CAPS (pH 10.0 to 11.0). After 2 min of incubation, 0.09 μM BH1999 was assayed in 50 mM CAPSO and CAPS buffer (pH 10.0) containing 0.2 M KCl and 250 μM gentisyl-CoA by monitoring the absorbance changes at 365 nm (Δε of 4.0 mM^{-1} cm^{-1}) at 25°C.

RESULTS AND DISCUSSION

Recombinant protein purification and characterization. The *B. halodurans* BH1999 gene was cloned into the pET-23b vector for isopropylthiogalactopyranoside (IPTG)-induced overexpression in *E. coli* BL21(DE3) cells. The recombinant protein was purified by a two-step column chromatographic procedure in a yield of 20 mg/ g of wet cells. The molecular size of the denatured protein was determined by SDS-PAGE analysis to be ≈15 kDa (Fig. 2). The molecular weight of purified BH1999 protein determined by electrospray mass spectrometry is 15,739, in agreement with the theoretical value of 15,739.

Catalytic function. A search of the protein database with the NCBI BlastP search tool identified a large group of protein homologs with 20 to 35% sequence identity with the BH1999 protein. The closest homologs (31 to 35% sequence identity), and the only ones having a known enzyme function, were the 4-hydroxybenzoyl-CoA thioesterases of the 4-chlorobenzoate degradation pathways in *Pseudomonas* sp. strains CBS3 (23) and DJ-12 (5). Accordingly, it was deduced that BH1999 pro-

TABLE 1. Steady-state kinetic constants for *B. halodurans* gentisyl-CoA thioesterase- and *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase-catalyzed hydrolysis of acyl-CoAs

Enzyme and type	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
Gentisyl-CoA thioesterase				
Wild type	4-Hydroxybenzoyl-CoA	$(4.2 \pm 0.2) \times 10^{-2}$	$(1.2 \pm 0.2) \times 10^{2}$	3.6×10^{2}
Wild type	3-Hydroxybenzoyl-CoA	1.5 ± 0.1	5.0 ± 0.4	3.0×10^{5}
Wild type	Gentisyl-CoA	6.5 ± 0.2	4.0 ± 0.6	1.6×10^{6}
Wild type	Benzoyl-CoA	$(6.8 \pm 0.3) \times 10^{-2}$	$(3.3 \pm 0.4) \times 10^{2}$	2.1×10^{2}
Wild type	4-Chlorobenzoyl-CoA	7.3 ± 0.4	$(2.4 \pm 0.3) \times 10^{2}$	3.0×10^{4}
Wild type	4-Methoxybenzoyl-CoA	$(3.4 \pm 0.3) \times 10^{-1}$	$(1.0 \pm 0.2) \times 10^{2}$	3.4×10^{3}
Wild type	Phenylacetyl-CoA	$(3.4 \pm 0.2) \times 10^{-2}$	$(3.5 \pm 0.5) \times 10^{2}$	9.7×10^{1}
Wild type	3-Hydroxyphenylacetyl-CoA	$(8.1 \pm 0.3) \times 10^{-3}$	$(1.9 \pm 0.2) \times 10^{2}$	4.3×10^{1}
Wild type	4-Hydroxyphenylacetyl-CoA	No activity	—	—
Wild type	3,4-Dihydroxy-phenylacetyl-CoA	No activity	—	—
Wild type	Crotonyl-CoA	No activity	—	—
Wild type	*n*-Propionyl-CoA	$(5.8 \pm 0.5) \times 10^{-2}$	$(1.2 \pm 0.2) \times 10^{3}$	4.8×10^{1}
Wild type	*n*-Hexanoyl-CoA	$(1.3 \pm 0.1) \times 10^{-1}$	$(4.6 \pm 0.5) \times 10^{2}$	2.8×10^{2}
D16A	Gentisyl-CoA	3×10^{-3}	ND[a]	ND
D16N	Gentisyl-CoA	$(2.8 \pm 0.3) \times 10^{-2}$	9 ± 2	3.1×10^{3}
D31N	Gentisyl-CoA	$(6.9 \pm 0.2) \times 10^{-1}$	$(1.6 \pm 0.1) \times 10^{1}$	4.3×10^{4}
4-Hydroxybenzoyl-CoA thioesterase				
Wild type	4-Hydroxybenzoyl-CoA	$(1.8 \pm 0.1) \times 10^{1}$	6.0 ± 0.4	3.0×10^{6}
Wild type	3-Hydroxybenzoyl-CoA	$(1.5 \pm 0.1) \times 10^{1}$	$(2.7 \pm 0.3) \times 10^{2}$	5.5×10^{4}
Wild type	Gentisyl-CoA	8.9 ± 0.6	$(6 \pm 1) \times 10^{1}$	1.5×10^{5}

[a] ND, not determined.

tein function was related to hydroxybenzoyl-CoA thioester hydrolysis.

This idea was pursued by first testing thioesterase activity towards 4-hydroxybenzoyl-CoA. The steady-state kinetic constants obtained (Table 1) were consistent with BH1999 functioning as a thioesterase directed at a hydroxylated benzoyl-CoA substrate. This conclusion was supported by the reduced k_{cat}/K_m value for catalysis of benzoyl-CoA hydrolysis. The modest activity of the 4-hydroxybenzoyl-CoA substrate compared to what has been observed previously with the *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase (Table 1) (31) suggested that the physiological substrate of the BH1999 protein is hydroxylated at the *meta* and/or *ortho* benzoyl ring position rather than at the *para* position.

Therefore, the substrate activities of 3-hydroxybenzoyl-CoA and 2,5-dihydroxybenzoyl-CoA (gentisyl-CoA) were tested. The k_{cat} values of 3-hydroxybenzoyl-CoA and gentisyl-CoA were 36- and 155-fold higher than that measured with the 4-hydroxybenzoyl-CoA, respectively (Table 1). Furthermore, both K_m values were ≈25-fold smaller compared to the K_m measured with the 4-hydroxybenzoyl-CoA. The BH1999 protein thus prefers two-ring hydroxyl groups in its substrate, one at the C-2 position and the other at the C-5 position.

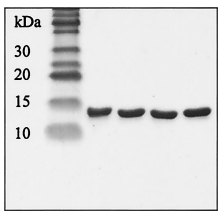

FIG. 2. SDS-PAGE gel (18% cross-link; Coomassie blue stained) of (from left to right) the Invitrogen BenchMark protein ladder and wild-type, D16N, D16A, and D31N gentisyl-CoA thioesterases.

The CoA thioesters of phenylacetate and its ring hydroxylated derivatives were tested as substrates for BH1999 to determine if this enzyme might function in the catabolism of these common aromatics. The results reported in Table 1 indicate no such role. The CoA thioesters of aliphatic acyl-CoA thioesters were also tested as substrates. These too were not active substrates.

The fact that the BH1999 protein catalyzes the hydrolysis of gentisyl-CoA efficiently (k_{cat}/K_m of 1.6×10^{6} M^{-1} s^{-1}) and the hydrolysis of 4-hydroxybenzoyl-CoA inefficiently ($k_{cat}/K_m = 3.6 \times 10^{2}$ M^{-1} s^{-1}) is a strong indicator that the BH1999 protein evolved within the hotdog fold thioesterase family specifically to catalyze gentisyl-CoA hydrolysis. In contrast, the *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase catalyzes the hydrolysis of 4-hydroxylbenzoyl-CoA efficiently ($k_{cat}/K_m = 3 \times 10^{6}$ M^{-1} s^{-1}) (31). It was not known if the high degree of substrate specificity observed with the BH1999 protein was mirrored in the *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase.

To address this issue, the substrate activities of 3-hydroxybenzoyl-CoA and gentisyl-CoA were measured with the *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase. The kinetic constants obtained are listed in Table 1. Although the k_{cat} measured for 3-hydroxybenzoyl-CoA is close to the k_{cat} observed for 4-hydroxybenzoyl-CoA as the substrate, the k_{cat}/K_m is 55-fold smaller. Similarly, the k_{cat}/K_m for *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase-catalyzed hydrolysis of gentisyl-CoA was 20-fold lower than that determined with 4-hydroxybenzoyl-CoA as the substrate. The *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase thus prefers 4-hydroxybenzoyl-CoA to the 3-hydroxybenzoyl-CoA and gentisyl-CoA. However, the level of substrate discrimination observed in the BH1999 protein is not matched in the 4-hydroxybenzoyl-CoA thioesterase. It is interesting that the *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase could function as a gentisyl-CoA

thioesterase in the cell, whereas the BH1999 protein (hereafter referred to as gentisyl-CoA thioesterase) could not fill the role of the 4-hydroxybenzoyl-CoA thioesterase.

The basis for BH1999's discrimination between the C-3- and C-4-hydroxylated benzoyl-CoAs was tested by measuring the activities of 4-chlorobenzoyl-CoA and 4-methoxybenzoyl-CoA. The substrate activity of thioesters was low but considerably higher than that of 4-hydroxbenzoyl-CoA despite the fact that both were substituted at C-4. Thus, steric hindrance is not the sole reason why the 4-hydroxybenzoyl-CoA is a poor substrate. We hypothesize that the C-4 OH may facilitate nonproductive binding through H-bonding interaction.

pH optimum for catalysis. The kinetic constants reported in Table 1 were determined at pH 7.5. Is this the pH optimum for gentisyl-CoA thioesterase? To answer this question, the k_{cat} pH profile of catalyzed gentisyl-CoA hydrolysis was measured with initial-velocity techniques. As illustrated in Fig. 3A, the pH profile is relatively flat over the pH range 6 to 8.5 and bell-shaped in the range of pH 9 to 11. The maximum turnover rate, reached at pH 10, is ≈10-fold greater than that reached at pH 7.5. Preincubation (2 min) of the enzyme (7.5 μM) in the different buffers followed by k_{cat} determination at pH 10 (assay time, 1 to 2 min) showed that the enzyme activity was not affected by preincubation at pH 4 to 10 (Fig. 3B). Above pH 10, the activity of the enzyme was rapidly lost, indicating that the drop in the pH profile (Fig. 3A) observed at pH > 10 is the result of enzyme denaturation. At pH 10, the gentisyl-CoA is ionized at one of the two ring hydroxyl groups (pK_a = 9.5 as determined by pH titration monitored at 400 nm; data not shown). Although, at this time, we have not measured the k_{cat}/K_m pH profile, we might anticipate the contribution of reduced substrate binding above pH 9 owing to the ring ionization.

Asp16 and Asp31 function in substrate binding and catalysis. The gentisyl-CoA thioesterase sequence was used, in conjunction with the 3D-PSSM search tool (15), to identify structural homologs in the SCOP protein structural database. The *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase hotdog fold (3) was identified with high confidence (E-value, 1.21e-07). The pairwise alignment of gentisyl-CoA thioesterase versus *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase sequences generated by the 3D-PSSM program is shown in Fig. 4. Residues Asp16 and Asp31 of the gentisyl-CoA thioesterase sequence aligned with Asp17 and Asp32 of *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase. Both Asp17 and Asp32 are located in the *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase active site. Asp17 is positioned to function in nucleophilic catalysis, while Asp32 is positioned to interact with the substrate benzoyl OH via bound water molecules (27, 31). The mutation of Asp17 to Asn resulted in a ≈10,000-fold decrease in k_{cat} value (31), while the mutation of Asp32 to Ser resulted in a 30-fold decrease in k_{cat} and a 7-fold increase in K_m (unpublished result).

To determine the extent to which Asp16 and Asp31 contribute to gentisyl-CoA thioesterase catalysis, site-directed mutagenesis of these residues was carried out. The two pairs of Asp to Ala and Asp to Asn mutant proteins were purified in the same manner as the wild-type enzyme. The D31A mutant proved to be unstable and was not characterized further. The steady-state kinetic constants measured for D16A, D16N, and D31N thioesterase catalyzed gentisyl-CoA hydrolysis at pH 7.5 and 25°C are listed in Table 1.

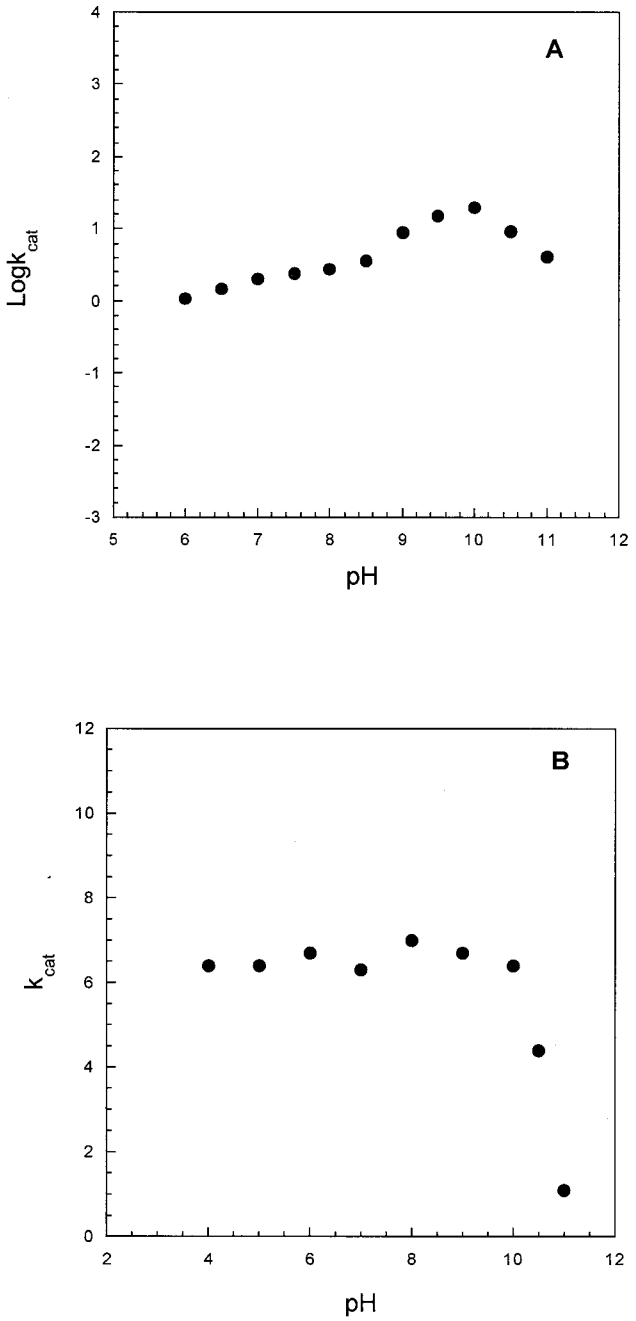

FIG. 3. (A) Kinetic pH rate profile: log k_{cat} (per second) versus pH rate for wild-type *B. halodurans* gentisyl-CoA thioesterase measured at 25°C. See the Materials and Methods for details. (B) The stability pH profile: k_{cat} (per second) of wild-type *B. halodurans* gentisyl-CoA thioesterase incubated for 2 min in the buffers used in A and then assayed at 25°C in 50 mM CAPSO and CAPS (pH 10)–0.2 M KCl buffer.

Both the D16A and D16N gentisyl-CoA thioesterases showed a large decrease (2,000- and 230-fold, respectively) in their k_{cat} values compared to the wild-type enzyme, while the D31N k_{cat} value was reduced by only a factor of 10. Because of low activity and coupled with a small K_m value, the K_m value of

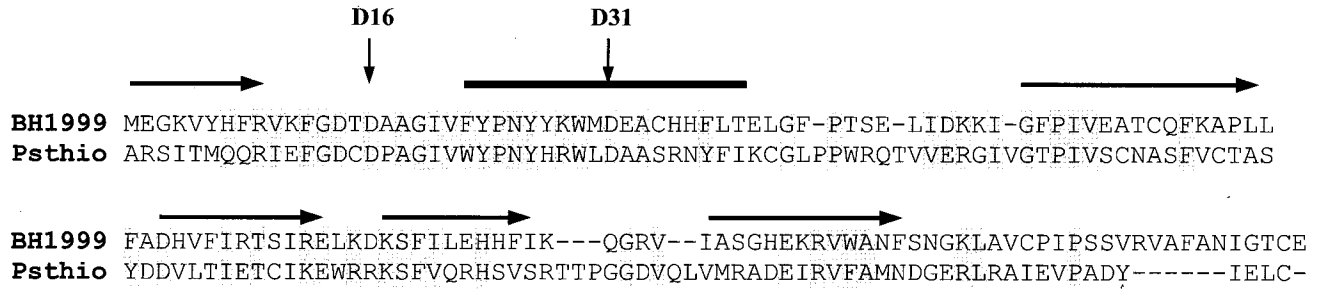

FIG. 4. Pairwise alignment of *B. halodurans* gentisyl-CoA thioesterase (BH1999) and the *Pseudomonas* 4-hydroxybenzoyl-CoA (Psthio) thioesterase generated by the 3D-PSSM program. The secondary structure is designated with a block (α helix) or an arrow (β strand). Conserved residues are shaded, and the gentisyl-CoA thioesterase residues Asp16 and Asp31 are labeled.

the D16A thioesterase could not be measured accurately. The K_m value measured for the D31N mutant, on the other hand, was found to be fourfold larger than that obtained with the wild-type enzyme (Table 1). These results suggest that Asp16 plays an important role in gentisyl-CoA catalysis while Asp31 does not.

Qualitatively, the activity losses observed with the gentisyl-CoA thioesterase Asp16 and Asp31 mutants paralleled those obtained for the *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase D17N and D32S mutants. It is, however, noteworthy that (based on the activity difference between the wild-type and Asp to Asn mutant enzymes) Asp17 contributes a rate factor of 10,000 to *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase catalysis, while Asp16 contributes a rate factor of only 230 to the gentisyl-CoA thioesterase catalysis. While it might be expected that the catalytic role assumed by Asp16 in the gentisyl-CoA thioesterase is the same as that (i.e., nucleophilic catalysis) played by Asp17 in *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase catalysis, the large difference in the observed rate contribution would suggest otherwise. Alternatively, this result may indicate reversion of the gentisyl-CoA thioesterase D16N mutant back to wild-type thioesterase (a phenomenon observed with certain dehalogenases) (29). Indeed, the D16A gentisyl-CoA thioesterase is 2,000-fold less active than the wild-type enzyme. Determination of gentisyl-CoA thioesterase structure and mechanism will be required to better understand this anomaly.

Summary and conclusions. In this study, we have shown that the *Bacillus halodurans* BH1999 protein is gentisyl-CoA thioesterase, that it possesses the novel hotdog fold previously described for the *Pseudomonas* sp. strain CBS3 4-hydroxybenzoyl-CoA thioesterase, and, like the 4-hydroxybenzoyl-CoA thioesterase, it possesses two active-site Asp residues, one of which plays an important role in catalysis. The two thioesterases are, however, distinct in several ways.

First, the k_{cat}/K_m for gentisyl-CoA thioesterase catalyzed hydrolysis of gentisyl-CoA versus 4-hydroxybenzoyl-CoA is 1.6×10^6 versus 3.6×10^2 M^{-1} s^{-1} (4400: 1) while the k_{cat}/K_m for 4-hydroxybenzoyl-CoA thioesterase catalyzed hydrolysis of gentisyl-CoA versus 4-hydroxybenzoyl-CoA is 1.5×10^5 versus 3×10^6 M^{-1} s^{-1} (1:20). The difference in the level of substrate specificity is striking. Whereas the gentisyl-CoA thioesterase is highly discriminating towards the two substrates, the 4-hydroxybenzoyl-CoA thioesterase is not.

Second, the pH optimum of the gentisyl-CoA thioesterase, as measured by the pH dependence of k_{cat}, is ≈10, whereas for the *Pseudomonas* 4-hydroxybenzoyl-CoA thioesterase, the pH optimum is between 7 and 9 (unpublished results). *B. halodurans* inhabits an alkaline environment, and consequently, the pH optima observed for its extracellular enzymes are 10 to 11.5 (25), The gentisyl-CoA thioesterase, however, is a cytoplasmic enzyme. Despite the high external pH values, the intracellular pH is maintained between 7.4 and 8.4 (13). Thus, the 10-fold increase in k_{cat} observed at pH values above the physiological pH has no apparent functional basis.

Third, the Asp catalytic groups of the two thioesterases contribute dramatically different rate enhancements. The loss of Asp17 function in the 4-hydroxybenzoyl-CoA thioesterase essentially shuts down catalysis, while loss of Asp16 function in the gentisyl-CoA thioesterase reduces it by 230-fold. This result is consistent with different catalytic functions for Asp17 and Asp16 of the two thioesterases. However, the possibility that the higher than expected activity of the D16N mutant is the result of wild-type thioesterase contamination (via Asn16 hydrolysis to Asp) has not been ruled out.

Lastly, the two thioesterases perform different physiological roles. The 4-hydroxybenzoyl-CoA thioesterase gene is located within the 4-chlorobenzoate degradation pathway operon found in 4-chlorobenzoate-degrading bacteria, including *Pseudomonas* sp. strain CBS3. Thus, despite the fact that this thioesterase can hydrolyze gentisyl-CoA at a physiologically significant rate, the actual function of the thioesterase is catalysis of 4-hydroxybenzoyl-CoA hydrolysis, the third and final step of the 4-chlorobenzoate degradation pathway.

The genome context of BH1999 (Fig. 1) suggests that the physiological function of the gentisyl-CoA thioesterase may be linked to the enzymes of the putative gentisate oxidation pathway. The origin of the gentisyl-CoA is, however, presently not known. The amino acid sequences of the proteins encoded by the neighboring genes give no indication of enzymes of an "upper" aromatic degradation pathway in which gentisyl-CoA might be formed.

The formation of gentisyl-CoA has been evidenced in other bacterial strains. For example, it has been shown that gentisyl-CoA is formed in the hydroquinone degradation pathway of anaerobic fermenting bacteria (10). Here, gentisate is generated by carboxylation of hydroquinone and then converted to gentisyl-CoA by an acyl-CoA synthetase. Reductive elimination of the hydroxyl group of gentisyl-CoA catalyzed by genti-

syl-CoA reductase produces benzoyl-CoA, which is degraded via the well-characterized benzoate pathway (7).

An early study of naphthalene degradation by *Rhodococcus* sp. strain B4 suggested gentisyl-CoA as an intermediate and thus a possible role for gentisyl-CoA thioesterase (11). Also, the first steps of a novel benzoate aerobic degradation pathway observed in a denitrifying bacterium may involve transformation of benzoate to benzoyl-CoA, benzoyl-CoA to 3-hydroxybenzoyl-CoA, and 3-hydroxybenzoyl-CoA to gentisyl-CoA (1, 19). Finally, it has been postulated that gentisyl-CoA is formed from 4-hydroxbenzoyl-CoA in halophilic *Archaea* via the action of a novel monooxygenase that catalyzes intramolecular thioester group migration (i.e., the NIH shift) (6).

The involvement of gentisyl-CoA in aromatic degradation pathways in a variety of bacteria and archaea suggest a possible role for gentisyl-CoA thioesterase as a link between certain upper aromatic degradation pathways and the (lower) gentisate oxidation pathway. In this study we have, for the first time, demonstrated the existence of a gentisyl-CoA thioesterase.

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