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Degradation of 3-phenylpropionic acid by *Haloferax* sp. D1227

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Abstract *Haloferax* sp. D1227, isolated from soil contaminated with highly saline oil brine, is the first halophilic archaeon to demonstrate the utilization of aromatic compounds (i.e., benzoic acid, cinnamic acid, and 3-phenylpropionic acid) as sole carbon and energy sources for growth. The degradation of 3-phenylpropionic acid in this strain was studied to examine the strategies utilized by Archaea to metabolize aromatic compounds. Based on our findings of (1) the extracellular accumulation of cinnamic acid, benzoic acid, 3-hydroxybenzoic acid, and gentisic acid in cultures of *Haloferax* D1227 grown on 3-phenylpropionic acid, (2) the presence of an 3-phenylpropionylCoA dehydrogenase, (3) the ATP, CoA, and NAD-dependent conversion of cinnamic acid to benzoylCoA, and (4) the presence of gentisate 1,2-dioxygenase, we propose that *Haloferax* D1227 metabolizes 3-phenylpropionic acid by initial 2-carbon shortening of the side chain to benzoylCoA via a mechanism similar to fatty acid β-oxidation, followed by aromatic degradation using a gentisate pathway. The upper aliphatic pathway from 3-phenylpropionic acid to benzoic acid is regulated separately from the lower gentisate pathway.

Key words Archaea · Extreme halophiles · 3-Phenylpropionic acid · β-oxidation · Gentisate pathway · Aromatic degradation

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Introduction

Haloferax sp. D1227, isolated from soil contaminated with highly saline oil brine near Grand Rapids, MI (USA), is a halophilic archaeon requiring 2 M NaCl for optimal growth. To date, *Haloferax* D1227 is the only reported archaeon capable of aerobic metabolism of aromatic compounds (i.e., benzoic acid, cinnamic acid, and 3-phenylpropionic acid) as sole carbon and energy sources for growth (Emerson et al. 1994). Although the pathways for degradation of aromatic compounds in bacteria and fungi have been elucidated in detail (Cain 1980; Gibson and Subramanian 1984; Rochkind et al. 1987), little is known about aromatic catabolism by Archaea. Since the recognition that Archaea, consisting of methanogens, extreme thermophiles, and extreme halophiles, represents a third domain of life phylogenetically distinct from Bacteria and Eukarya (Woese et al. 1990), biochemical and genetic research has elucidated some unusual features of these organisms and provided new insights into their evolutionary relationships with eubacterial and eukaryotic organisms (Darnell and Doolittle 1986; Juez 1988; Olsen and Woese 1997). The isolation of *Haloferax* D1227 provides an opportunity to investigate the strategies utilized by Archaea for catabolism of aromatic compounds and to compare an aromatic degradation pathway in Archaea with those in Bacteria and Eukarya.

3-Phenylpropionic acid is a member of the phenylpropanoid family, comprising a wide variety of C_6-C_3 compounds synthesized by plants from phenylalanine. Phenylpropanoids are important in plant physiology for synthesis of lignin, flavonoids, insect repellents, UV protectants, and signal molecules (Hahlbrock and Scheel 1989). It has been shown that the microbial degradation of 3-phenylpropionic acid can occur via two different routes. In one route, demonstrated in a species of *Achromobacter* and two strains of *Pseudomonas* (Blakley and Simpson 1964; Coulson and Evans 1959; Dagley et al. 1965), the aromatic ring is first oxidized and opened, followed by the degradation of the resulting aliphatic segment. An alternative route for the degradation of 3-phenylpropionic acid is

catabolism of the side chain followed by aromatic ring fission. This pathway was suggested by Webley et al. (1955) on the basis of their observation of the transient accumulation of cinnamic acid and benzoic acid by *Nocardia opoca* grown with 3-phenylpropionic acid. They proposed that 3-phenylpropionic acid was metabolized by two-carbon shortening of the side chain first, via a mechanism similar to fatty acid β-oxidation, resulting in benzoylCoA. The enzymes involved in this oxidation were not investigated, nor was the degradation of benzoylCoA. Under aerobic conditions, benzoate is usually transformed into a few key intermediates including catechol, protocatechuate, and gentisate, followed by aromatic ring cleavage by ring-fission dioxygenases (Gibson and Subramanian 1984). Altenschmidt et al. (1993) have demonstrated a new aerobic benzoate degradation pathway involving CoA derivatives. Their research showed that *Pseudomonas* KB740, a facultative denitrifying *Pseudomonas* species, metabolized benzoate to 3-hydroxybenzoylCoA via benzoylCoA with further 5-hydroxylation to gentisate.

As mentioned, *Haloferax* sp. D1227 can utilize benzoic acid, cinnamic acid, and 3-phenylpropionic acid for growth. The degradation of 3-phenylpropionic acid by *Haloferax* D1227 was studied because this compound has both an aromatic ring and an aliphatic side chain in its structure. It is of interest to understand how Archaea attack such chemical structures. In this paper, we present results which indicate that the degradation of 3-phenylpropionic acid by *Haloferax* D1227 is initiated by β-oxidation of the side chain to produce benzoylCoA, which is subsequently metabolized via a gentisate pathway.

Materials and methods

Materials

All chemicals used in this study were reagent grade. 3-Phenylpropionic acid, cinnamic acid, and benzoic acid were purchased from Aldrich Chemical (Milwaukee, WI, USA); catechol, protocatechuic acid, gentisic acid, salicylic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, oleic acid, lauric acid, palmitic acid, Triton X-100, ATP, coenzymeA, and 2,2′-dipyridyl were obtained from Sigma Chemical (St. Louis, MO, USA); and HPLC grade acetonitrile was purchased from EM Science (Gibbstown, NJ, USA).

Microorganism, media, and growth conditions

Haloferax sp. D1227 has been described (Emerson et al. 1994). For growth of *Haloferax* D1227 on various carbon sources, mineral salts medium (BS3) (Emerson et al. 1994) of the following composition (in g/l) was used: $(NH_4)_2SO_4$, 0.33; KCl, 6.0; $MgCl_2{\cdot}6H_2O$, 12.1; $MgSO_4{\cdot}7H_2O$, 14.8; KH_2PO_4, 0.34; $CaCl_2{\cdot}H_2O$, 0.36; and NaCl, 100; 1 ml/l of a trace element solution (Widdel and Bak 1992) was also added before sterilization, and the pH was adjusted to 6.9 with KOH. Filter-sterilized growth substrates were added after sterilization. For rich medium (BSYT), BS3 mineral salts medium was supplemented with 3 g/l yeast extract and 3 g/l tryptone. For solid media, 15 g/l Bacto-agar was added before autoclaving. Cells were cultured aerobically in Erlenmeyer flasks containing 1/4 vol of BS3 mineral salt medium supplemented with various growth substrates. For growth on fatty acids, the sole carbon source was 1 mM oleic acid, lauric acid, or palmitic acid with or without 0.4% (v/v) Triton X-100 to disperse fatty acids (Yang and Schulz 1983). Each flask was inoculated with a mid-log-phase culture grown in BSYT-rich medium to give an initial OD_{600nm} approximately 0.02. Flasks were incubated at 37°C on a rotary shaker at 200 rpm. Growth was monitored by measuring OD_{600nm} on a Gilford DU spectrophotometer except that growth on fatty acids was determined by viable count plating.

HPLC analysis

Aromatic compounds were analyzed by reverse-phase HPLC on a Waters model chromatography (Waters, Millipore Milford, MA, USA) equipped with a Waters 486 tunable absorbance detector set at 254 nm, a Waters 746 integrator, and Waters model 501 solvent delivery system. Separation was achieved on a Nova-Pak™ C18 column (150 × 3.9 mm) at a flow rate of 0.8 ml/min. The injection volume was 20 µl. Free aromatic acids were eluted using 2% acetonitrile in 200 mM ammonium acetate buffer (pH 6.5). CoA thioesters were eluted using 15% acetonitrile in 200 mM ammonium acetate buffer (pH 5.5). For CoA derivatives for which standards were unavailable, 3 µl 10 N NaOH was added to 300-µl samples to hydrolyze CoA thioesters to their corresponding free acids (Webster et al. 1974). Aromatic acids, benzoylCoA, and 3-hydroxybenzoylCoA were identified by comparison of retention times with those of authentic standards.

Resting cell experiments with added 2,2′-dipyridyl

Cells grown on 3 mM 3-phenylpropionic acid, cinnamic acid, benzoic acid, or pyruvate were harvested when the culture (250 ml) reached mid-log phase at an OD_{600nm} of approximately 0.7. Following centrifugation at 10600 × *g* for 30 min, cells were washed twice with 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl and resuspended in 25 ml of the same buffer. Each 125-ml Erlenmeyer flask contained, in a total volume of 10 ml: 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl, 4 ml of the cell suspension, 5 mM 2,2′-dipyridyl, and 1 mM 3-phenylpropionic acid, cinnamic acid, benzoic acid, or 3-hydroxybenzoic acid. Cells were incubated at 37°C on a rotary shaker at 200 rpm. Samples were filtrated through 0.2-µm nylon filters (Scientific Resources, Eatontown, NJ, USA), and analyzed immediately by HPLC.

Preparation of cell-free extracts

Haloferax D1227 was grown in 250 ml BS3 medium containing 3 mM 3-phenylpropionic acid, cinnamic acid, and ben-

zoic acid or pyruvate. Cells in mid-log phase were harvested by centrifugation at 10600 × *g* for 30 min, washed twice with 30 ml 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl, and resuspended in 10 ml of the same buffer. Using a 4710 Series Ultrasonic Homogenizer (Cole-Parmer, Chicago, IL, USA), 2.5-ml aliquots of the suspension were sonicated four times (30 s at 50 W followed by 2 min of cooling) in an ice bath. Unbroken cells and cell debris were removed by ultracentrifugation (245300 × *g* for 1 hour at 4°C). The supernatants were then diafiltrated using Centricon-10 (MW cut-off, 10000; Amicon, Beverly, MA, USA) at 4°C with three volumes of 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl to remove small molecules in the extracts. The concentrated extracts were diluted with the same buffer to 10 ml for enzyme analysis.

Enzyme assays

All enzyme assays were performed at 37°C, unless stated otherwise.

3-Phenylpropionic acid-CoA ligase, cinnamic acid-CoA ligase, benzoic acid-CoA ligase, and 3-hydroxybenzoic acid-CoA ligase

The assay mixture for ligase activity contained, in a total volume of 0.5 ml, 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl, 0.5 mM 3-phenylpropionic acid, cinnamic acid, benzoic acid, or 3-hydroxybenzoic acid, 2 mM ATP, 2 mM coenzymeA, 10 mM $MgCl_2$ and 50 µl of cell-free extract. The ligase activity was determined by measuring the rate of the appearance of CoA thioester product using HPLC.

3-PhenylpropionylCoA dehydrogenase

To assay 3-phenylpropionylCoA dehydrogenase activity, 3-phenylpropionylCoA, which is not commercially available, was synthesized from 3-phenylpropionic acid with *Haloferax* D1227 cell-free extracts utilizing the 3-phenylpropionic acid-CoA ligase reaction described earlier. Because the reaction catalyzed by 3-phenylpropionylCoA dehydrogenase required no added cofactor, cinnamylCoA started to appear in the ligase reaction mixture at the formation of 3-phenylpropionylCoA. The 3-phenylpropionylCoA dehydrogenase activity was determined by measuring the rate of cinnamylCoA production using HPLC.

Conversion of cinnamylCoA to benzoylCoA

Because cinnamylCoA is not commercially available, it was synthesized from cinnamic acid with *Haloferax* D1227 cell-free extracts utilizing the cinnamic acid-CoA ligase reaction described here. Once the formation of cinnamylCoA ceased, NAD was added to the reaction mixture (0.5 ml) to a final concentration of 1 mM to initiate the conversion of cinnamylCoA to benzoylCoA. The simultaneous disappearance of cinnamylCoA and appearance of benzoylCoA were measured using HPLC.

3-HydroxybenzoylCoA-6-hydroxylase, benzoylCoA-3-hydroxylase, 3-hydroxybenzoate-6-hydroxylase, and benzoate-3-hydroxylase

3-HydroxybenzoylCoA-6-hydroxylase, benzoylCoA-3-hydroxylase, 3-hydroxybenzoate-6-hydroxylase, and benzoate-3-hydroxylase activities were assayed using the methods modified from those described by Niemetz et al. (1995), Kiemer et al. (1996), Wang et al. (1987), Suarez et al. (1995), van Berkel and van den Tweel (1991), and Groseclose and Ribbons (1973). The reaction mixture in a total volume of 1 ml contained 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl, or 100 mM Tris-HCl buffer (pH 7.0) containing 2 M KCl; 0.25 mM 3-hydroxybenzoylCoA, benzoylCoA, 3-hydroxybenzoate, or benzoate; 0.1 mM NADH or NADPH or in combination with 0.1 mM FAD; and 200 µl supernatant or resuspended pellet of sonicated or 0.1% Triton X-100 treated cells. The stabilizers, 0.1 mM DTT, 0.1% glycerol, and 0.1 mM EDTA, were also added to the reaction mixture, either individually or in combination. The enzyme activity was determined by measuring the appearance of hydroxylation product on HPLC.

Gentisate 1,2-dioxygenase

Gentisate 1,2-dioxygenase was assayed at room temperature by measuring the formation of maleylpyruvate at 334 nm (Lack 1959) with a Perkin Elmer double-beam 124 spectrophotometer. The reaction mixture (2 ml) contained 0.25 mM gentisic acid, 50 µl cell-free extract, and 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl. The reference cuvette contained the same reaction mixture except gentisic acid was omitted. The molar absorption coefficient for maleylpyruvate (ε = 10800) (Crawford et al. 1975) was used to calculate enzyme activity. This assay was confirmed by measurement of the disappearance of gentisate using HPLC.

Maleylpyruvate cis-trans isomerase

Because maleylpyruvate is not commercially available, it was synthesized from gentisic acid by gentisate 1,2-dioxygenase as described. When the formation of maleylpyruvate ceased, glutathione was added to the reaction mixture to a final concentration of 1 mM. The activity of maleylpyruvate *cis-trans* isomerase was determined by measuring the disappearance of maleylpyruvate at 334 nm (Lack 1959).

Protein determination

Protein concentrations of crude enzyme extracts were determined by the method of Bradford (1976) with bovine

serum albumin dissolved in 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl as the standard.

Nucleotide sequence analysis of small-subunit (SSU) rDNA

To prepare genomic DNA of *Haloferax* D1227, cells were grown in 100 ml BSYT medium and harvested at mid-log phase by centrifugation at 10600 × *g* for 30 min. After washing twice with 100 mM potassium phosphate buffer (pH 7.0) containing 2 M KCl, the pellet was resuspended in 50 ml distilled water for cell lysis. After removal of cell debris by centrifugation at 47800 × *g* for 30 min, the supernatant was extracted once with an equal volume of phenol and twice with an equal volume of phenol/chloroform. DNA in the aqueous phase was precipitated with 2 vol of ethanol and 0.1 vol of 3 M sodium acetate (pH 5.2) at −16°C overnight. After centrifugation, the pellet was rinsed with 1 ml of 70% ethanol, air dried, and resuspended in 1 ml of H_2O (Maniatis et al. 1982). This preparation was then treated with RNase at a final concentration of 25 μg/ml and used as template DNA for PCR. The PCR reaction mixture in a total volume of 100 μl contained 2 μl of template DNA (~ 100 ng), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 2.5 U of Taq DNA polymerase, and 30 pmol of each primer. Primers ARCH21BF [5′-TTCCGCTTGATCC(C/T)G CC(A/G)G-3′] and 1492R [5′-GGTTACCTTGTTACGACTT-3′] have been described (Leadbetter and Breznak 1996). The PCR reaction was carried out on Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) and thermal cycling conditions were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 2.1 min. A final extension was performed at 72°C for 6.1 min. The PCR product was purified with Geneclean II Kit (Bio 101, La Jolla, CA, USA) following the protocol recommended by the manufacturer.

Nucleotide sequencing was carried out at the Nucleic Acid Sequencing Facility of Michigan State University with an ABI Prism sequencer (Applied Biosystems). The following sequencing primers were used: ARCH 21BF, 269R [5′-TACCGA TTATCGGCACGGTG-3′], 356F [5′-AGGCGCGAAACCTTTACACT-3′], 592F [5′-CGAAGGTTCATCGGGAAATC-3′], 960R [5′-ATGTC-CGGCGTTGAGTCCAA-3′], 1196R [5′-ATTCGGGG-CATACTGACCTA-3′], 1352F [5′-CGCATTTCAATAG-AGTGCGG-3′], and 1492R. An edited, contiguous sequence was constructed from the data manually. A list of the known sequences that were most similar to the SSU rRNA sequence of *Haloferax* D1227 was obtained using the "Similarity Rank" routine at the Ribosomal Database Project (University of Illinois, Urbana-Champaign). Sequences were then aligned manually with Genome database Environment version 2.2 operating on a Sun SPARC station. Similarity matrix was constructed with the Jukes and Cantor (1969) correction for base changes. Phylogenetic trees were constructed from the same alignment by distance (DeSoete algorithm [1983]) and maximum-parsimony methods (the latter was bootstrapped with SEQBOOT). These analyses were run with PHYLIP version 3.55c.

Results

Small-subunit rRNA sequence analysis

To confirm the earlier identification of *Haloferax* D1227 as a *Haloferax* species (Emerson et al. 1994), the small-subunit (SSU) rRNA encoding gene from *Haloferax* D1227 was amplified by PCR using Archaea-specific forward primer ARCH21BF and "universal" reverse primer 1492R. Sequence corresponding to *E. coli* SSU rRNA nucleotide position 26 through 1491 was obtained (GenBank accession number AF069950). *Haloferax* D1227 showed 98.4% and 98.2% sequence similarities to *Haloferax volcanii* and *Haloferax mediterranei*, respectively. The unrooted phylogenetic tree constructed by maximum-parsimony analysis grouped *Haloferax* D1227 within the genus *Haloferax*, and this grouping was supported by a bootstrap value of 100% for the node from which this strain and the other members of the genus radiate (Fig. 1). The distance method also grouped *Haloferax* D1227 within the genus *Haloferax* (tree not shown). Of the 26 nucleotide differences between *Haloferax* D1227 and its closest relative *Hf. volcanii*, there were four dual-compensatory differences corresponding to *E. coli* positions 128 and 233, 141 and 222, 184 and 191, and 835 and 851.

Aerobic growth of *Haloferax* D1227 on aromatic compounds and fatty acids

In addition to growth on 3-phenylpropionic acid, cinnamic acid, and benzoic acid as previously described (Emerson et al. 1994), *Haloferax* D1227 also demonstrated the ability to utilize 3-hydroxybenzoic acid (1 mM) as a sole carbon source. No growth was observed with 4-hydroxybenzoic acid (1 mM), salicylic acid (1 mM), catechol (0.1 mM), protocatechuic acid (0.1 mM), gentisic acid (0.1 mM), oleic acid (1 mM), lauric acid (1 mM), or palmitic acid (1 mM). Lack of growth on gentisic acid was attributed to the inhibitory effect of either gentisic acid or its autoxidation products because growth of *Haloferax* D1227 on benzoate was inhibited when gentisic acid was added to the medium.

The metabolites detected by HPLC analysis in the cultures of *Haloferax* D1227 grown on 3 mM 3-phenylpropionic acid, cinnamic acid, or benzoic acid (Table 1) suggested that cinnamic acid, benzoic acid, 3-hydroxybenzoic acid, and gentisic acid were intermediates in 3-phenylpropionic acid degradation.

Resting cell experiments with added 2,2′-dipyridyl

To observe metabolites detected during growth in more detail, resting cell experiments were carried out with added 2,2′-dipyridyl, which is an inhibitor of gentisate 1,2-

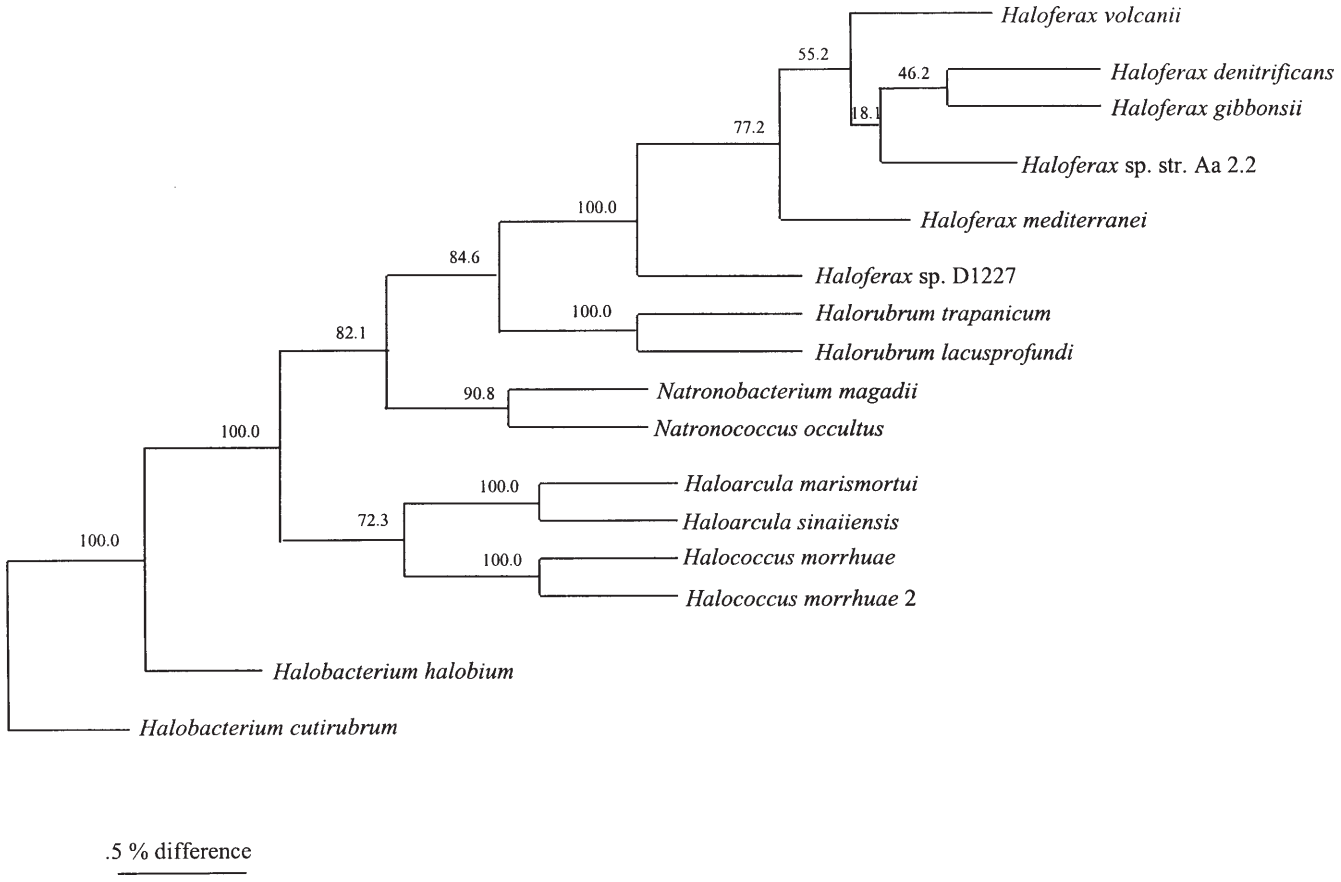

Fig. 1. Phylogenetic position of strain D1227 within the genus *Haloferax* based on a maximum-parsimony analysis. The *bar* represents a 0.5% difference in evolutionary distance. Bootstrap scores of 100 determinations are indicated at the nodes

Table 1. Aromatic metabolites observed in *Haloferax* D1227 cultures grown on various substrates

Growth substrate (3 mM)	Aromatic metabolites observed in 48-h culture	Concentration (μM)
3-Phenylpropionic acid	Cinnamic acid	0.061
	Benzoic acid	0.048
	3-Hydroxybenzoic acid	0.079
	Gentisic acid	0.035
Cinnamic acid	Benzoic acid	0.086
	3-Hydroxybenzoic acid	0.095
	Gentisic acid	0.074
Benzoic acid	3-Hydroxybenzoic acid	0.104
	Gentisic acid	0.073
Pyruvate	None	–

dioxygenase (see Enzyme activities), thus blocking further degradation of gentisic acid. When 2,2′-dipyridyl and 3-phenylpropionic acid were incubated with resting cell suspensions of *Haloferax* D1227 pregrown on 3-phenylpropionic acid, accumulations of the following metabolites were observed (in order of decreasing concentration): gentisic acid, 3-hydroxybenzoic acid, benzoic acid, and cinnamic acid (Fig. 2). The detection of these metabolites in high concentrations provided additional evidence that *Haloferax* D1227 metabolized 3-phenylpropionic acid

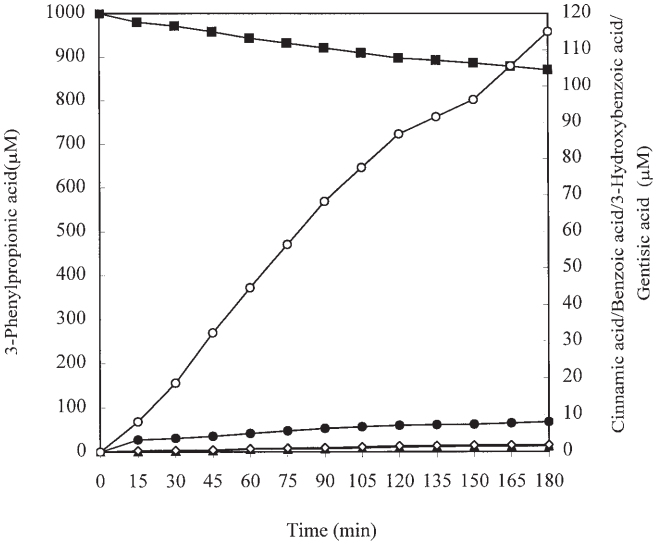

Fig. 2. Accumulation of intermediates when 3-phenylpropionic acid and 2,2′-dipyridyl were added to 3-phenylpropionic acid-grown cells. ■, 3-phenylpropionic acid; ◇, benzoic acid; ○, gentisic acid; ▲, cinnamic acid; ●, 3-hydroxybenzoic acid

via cinnamic acid, benzoic acid, 3-hydroxybenzoic acid, and gentisic acid. When the intermediates involved in the 3-phenylpropionic acid degradation pathway and 2,2′-dipyridyl were added to 3-phenylpropionic acid-grown cell suspension, the anticipated metabolites from the proposed pathway were seen, i.e., benzoic acid, 3-hydroxybenzoic acid, and gentisic acid accumulated when cinnamic acid was added as the substrate; 3-hydroxybenzoic acid and gentisic acid accumulated with benzoic acid as the substrate; and gentisic acid accumulated with 3-hydroxybenzoic acid as the substrate (data not shown).

Enzyme activities

The following enzyme activities were found in the soluble fraction of *Haloferax* D1227 cells.

3-Phenylpropionic acid-CoA ligase, cinnamic acid-CoA ligase, benzoic acid-CoA ligase, and 3-hydroxybenzoic acid-CoA ligase

Crude extracts prepared from cells grown on 3-phenylpropionic acid demonstrated ATP and coenzymeA-dependent ligase activities, which converted 3-phenylpropionic acid, cinnamic acid, benzoic acid, and 3-hydroxybenzoic acid to their corresponding CoA derivatives. Stimulation of ligase activities was observed with the addition of 10 mM $MgCl_2$ to the reaction mixtures (data not shown). These ligase activities were also detected in cells grown on cinnamic acid, benzoic acid, or pyruvate. It is not yet known whether these reactions are catalyzed by the same ligase or different ones, and also whether they are constitutive or inducible.

3-PhenylpropionylCoA dehydrogenase

The activity of 3-phenylpropionylCoA dehydrogenase, catalyzing the conversion of 3-phenylpropionylCoA to cinnamylCoA, was present in extracts of 3-phenylpropionic acid-grown cells, with a specific activity of 526 ± 19 nmol cinnamylCoA produced per min·mg protein, and also occurred in extracts of cinnamic acid-grown cells, with a specific enzyme activity of 711 ± 24 nmol cinnamylCoA produced per min·mg protein. This enzyme activity was not detectable in cells grown on benzoic acid or pyruvate. The reaction catalyzed by the 3-phenylpropionylCoA dehydrogenase proceeded without any added cofactor. When 1 mM FAD, NAD, or NADP was added to the reaction mixture, only FAD stimulated enzyme activity (50%). No conversion of 3-phenylpropionic acid to cinnamic acid was observed without both ATP and coenzyme A added to the reaction mixture. Because 3-phenylpropionylCoA dehydrogenase activity was demonstrated only in the soluble fraction of *Haloferax* D1227, membrane-associated electron-transfer proteins appear not to be involved in this dehydrogenation reaction.

Conversion of cinnamylCoA to benzoylCoA

Conversion of cinnamylCoA to benzoylCoA was observed with cell-free extracts prepared from cells grown on either 3-phenylpropionic acid or cinnamic acid. This conversion was NAD dependent over a broad concentration range with maximal conversion at 1 mM NAD. NADP and FAD were ineffective. Extracts of benzoic acid- or pyruvate-grown cells did not show the ability to transform cinnamylCoA to benzoylCoA, and no conversion of cinnamic acid to benzoic acid was detected without both ATP and coenzyme A added to the reaction mixture.

3-HydroxybenzoylCoA-6-hydroxylase, benzoylCoA-3-hydroxylase, 3-hydroxybenzoate-6-hydroxylase, and benzoate-3-hydroxylase

Benzoate and 3-hydroxybenzoate hydroxylation enzymes were not detected using the assay methods described in Materials and methods in cells grown on 3-phenylpropionic acid, cinnamic acid, or benzoic acid.

Gentisate 1,2-dioxygenase

Gentisate 1,2-dioxygenase activity was demonstrated in extracts from cells grown with 3-phenylpropionic acid, cinnamic acid, or benzoic acid with a specific activity of 1.4 ± 0.2, 1.5 ± 0.3, or 1.2 ± 0.3 μmol maleylpyruvate produced per min·mg protein, respectively. This enzyme activity was not detectable in pyruvate-grown cells. Unlike reported eubacterial gentisate 1,2-dioxygenases (Crawford et al. 1975; Harpel and Lipscomb 1990; Kiemer et al. 1996), no stimulating effect of Fe^{2+} on enzyme activity was observed, although 2,2′-dipyridyl proved to be an inhibitor for the enzyme activity (Fig. 3). The optimal salt concentration for the activity was 2 M KCl or NaCl. Detailed studies of the purified gentisate 1,2-dioxygenase and its encoding gene will be presented in a subsequent paper.

Maleylpyruvate cis-trans *isomerase*

A glutathione-dependent maleylpyruvate *cis-trans* isomerase with a specific activity of 47.6 ± 3.9 μmol/min·mg protein was present in cells grown on benzoic acid. When the ring-fission reaction of gentisate catalyzed by gentisate 1,2-dioxygenase ceased, the reaction mixture showed a product peak at 334 nm, corresponding to the absorption maximum of maleylpyruvate. A rapid decrease of the 334-nm absorbance was observed when 1 mM glutathione was added to the mixture. This change was attributed to the presence of a maleylpyruvate *cis-trans* isomerase that interconverts maleylpyruvate and fumarylpyruvate on the addition of glutathione (Lack 1959).

Pathway induction

To study the induction of the 3-phenylpropionic acid degradation pathway, 2,2′-dipyridyl and suspensions of *Haloferax*

D1227 resting cells pregrown on pyruvate, benzoic acid, cinnamic acid, or 3-phenylpropionic acid were incubated with 3-phenylpropionic acid or benzoic acid, and the appearance of metabolites in the culture was examined. When 3-phenylpropionic acid and 2,2′-dipyridyl were incubated with suspension of cells pregrown with either 3-phenylpropionic acid or cinnamic acid, metabolites of 3-phenylpropionic acid degradation were detected in the culture. However, when either a pyruvate or benzoic acid-grown cell suspension was used, no metabolite of 3-phenylpropionic acid degradation was detected (Table 2). When benzoic acid and 2,2′-dipyridyl were added to suspensions of cells grown on pyruvate or benzoic acid, 3-hydroxybenzoic acid and gentisic acid accumulated in benzoic acid-grown cells, but not in pyruvate-grown cells (data not shown). These data indicate that the upper aliphatic pathway from 3-phenylpropionic acid to benzoic acid can be induced by either 3-phenylpropionic acid or cinnamic acid, but not benzoate. The lower gentisate pathway from benzoic acid to gentisic acid is induced by benzoate.

Discussion

Although the extracellular metabolites observed during growth and in resting cell experiments suggest aerobic metabolism of 3-phenylpropionic acid via cinnamic acid, benzoic acid, 3-hydroxybenzoic acid, and gentisic acid, the enzyme data indicate its catabolism via CoA thioesters, which are not released extracellularly because of membrane impermeability. The presence of thioesterase activities converting 3-phenylpropionylCoA, cinnamylCoA, benzoylCoA, and 3-hydroxybenzoylCoA to their corresponding free acids was demonstrated in *Haloferax* D1227 cells grown on 3-phenylpropionic acid, cinnamic acid, benzoic acid, or pyruvate (data not shown). The accumulation of benzoic acid in the cultures of *Haloferax* D1227 grown on 3-phenylpropionic acid suggests that 2-carbon scission of the 3-phenylpropionic acid side chain resulting in benzoic acid precedes attack on the aromatic nucleus. The enzyme results indicate that the transformation of 3-phenylpropionic acid to benzoic acid occurs via a mechanism similar to fatty acid β-oxidation. Although the anticipated but unstable intermediates 3-hydroxy-3-phenylpropionylCoA and 3-keto-3-phenylpropionylCoA (Hilton and Cain 1990) were not detected in the incubation of cinnamylCoA and NAD with cell-free extracts, NAD-dependent conversion of cinnamylCoA to benzoylCoA was observed. The accumulation of 3-hydroxybenzoic acid and gentisic acid in benzoic acid-grown cells suggests that benzoic acid is converted to gentisic acid via 3-hydroxybenzoic acid. Although it is clear that gentisic acid serves as the substrate for gentisate 1,2-dioxygenase, we have not yet detected either benzoate or 3-hydroxybenzoate hydroxylase activity. Thus, whether benzoic acid is transformed into gentisic acid via benzoylCoA and 3-hydroxybenzoylCoA as demonstrated by Altenschmidt et al. (1993) or via free acids remains to be clarified. The proposed pathway for 3-phenylpropionic acid degradation in *Haloferax* D1227 is shown in Fig. 4.

The enzyme steps in the aliphatic upper pathway are regulated separately from the aromatic gentisate pathway. Although the upper pathway steps strongly resemble those of fatty acid β-oxidation, their induction by phenylpropanoids (i.e., 3-phenylpropionic acid and cinnamic acid) and the lack of fatty acid utilization by *Haloferax* D1227 suggest that they are distinct.

While the importance of CoA thioesters in the anaerobic degradation of aromatic compounds has long been recognized (Dangel et al. 1991), the involvement of CoA derivatives in aerobic aromatic pathways has only recently been described (Altenschmidt et al. 1993; Buder and Fuchs 1989; Crooks and Copley 1993; Vitovski 1993). Our results indi-

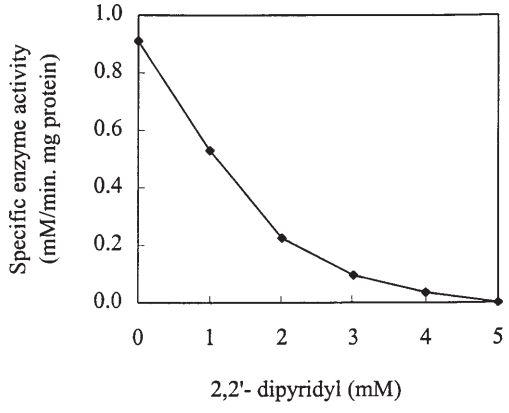

Fig. 3. Inhibition of gentisate 1,2-dioxygenase activity by 2,2′-dipyridyl

Table 2. Accumulation of intermediates when 3-phenylpropionic acid and 2,2′-dipyridyl were added to cells pregrown on various substrates

Growth substrate	Accumulation of intermediates in 3 h (μM)			
	Cinnamic acid	Benzoic acid	3-Hydroxy benzoic acid	Gentisic acid
3-Phenylpropionic acid	1.6 ± 0.3	1.9 ± 0.2	8.1 ± 1.2	115 ± 14
Cinnamic acid	0.5 ± 0.07	2.9 ± 0.4	52.5 ± 3.3	317 ± 26
Benzoic acid	0	0	0	0
Pyruvate	0	0	0	0

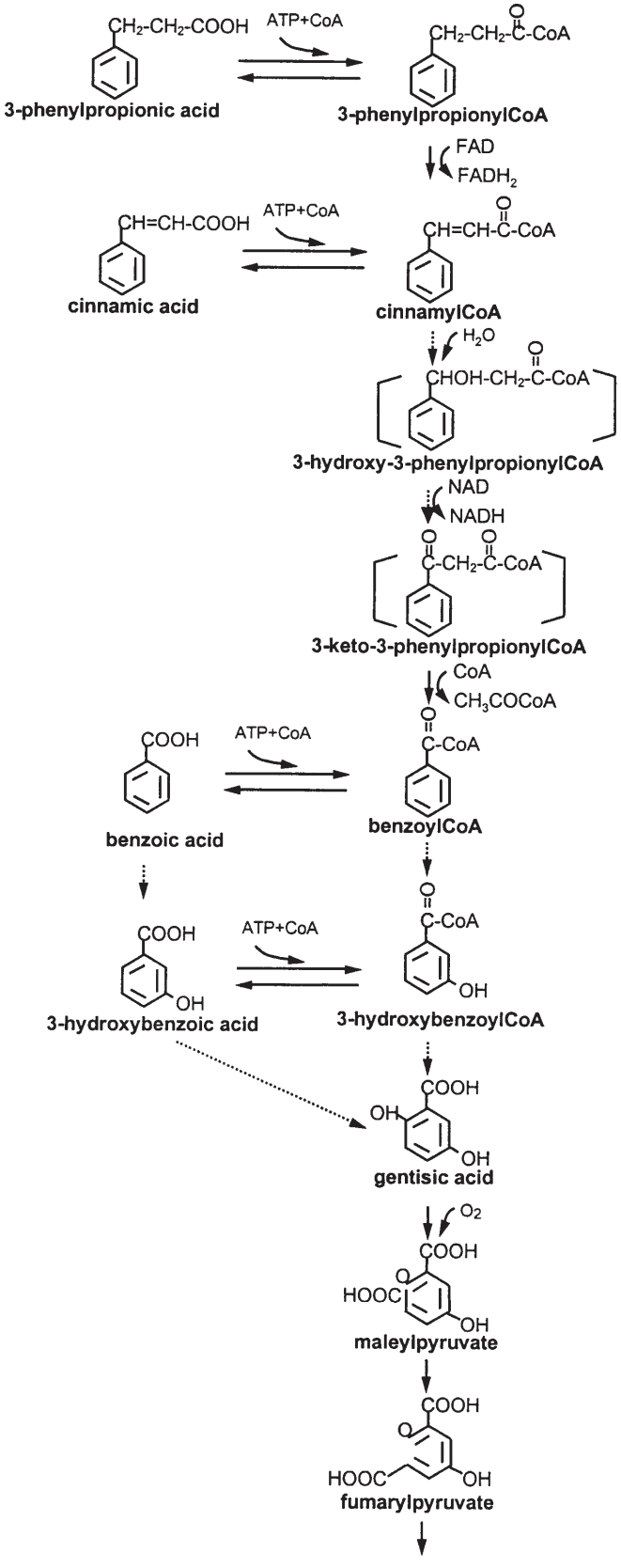

Fig. 4. Proposed pathway for the degradation of 3-phenylpropionic acid by *Haloferax* sp. D1227. Compounds in *square brackets* are intermediates not detected in this study. *Broken lines* represent postulated steps

cate the participation of CoA thioesters in the 2-carbon shortening of the side chain of 3-phenylpropionic acid, which had been postulated in Eubacteria but for which no enzyme activities have been described (Webley et al. 1955).

In summary, *Haloferax* sp. D1227 metabolizes 3-phenylpropionic acid by 2-carbon scission of the aliphatic side chain via a β-oxidation mechanism to produce benzoylCoA, which is further degraded via a gentisate pathway. Although lack of pathway details precludes in-depth comparisons, overall similarities between the *Haloferax* D1227 pathway and those of Eubacteria suggest the possibility of general pathway existence before the separation of Archaea and Bacteria.

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