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-phenylpropionyl-CoA dehydrogenase, (3) the ATP, CoA, and NAD-dependent conversion of cinnamic acid to benzoyl-CoA, 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 benzoyl-CoA 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

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 C₆-C₃ 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 *Nocardiopsis opaca* 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, *Haloflex* sp. D1227 can utilize benzoic acid, cinnamic acid, and 3-phenylpropionic acid for growth. The degradation of 3-phenylpropionic acid by *Haloflex* sp. 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 *Haloflex* sp. 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, coenzyme A, 3-hydroxybenzoylCoA, and benzoylCoA were purchased from Aldrich Chemical (Milwaukee, WI, USA); and HPLC grade acetonitrile was purchased from EM Science (Gibbstown, NJ, USA).

**Microorganism, media, and growth conditions**

*Haloflex* sp. D1227 has been described (Emerson et al. 1994). For growth of *Haloflex* sp. D1227 on various carbon sources, mineral salts medium (BS3) (Emerson et al. 1994) of the following composition (in g/l) was used: (NH₄)₂SO₄, 0.33; KCl, 6.0; MgCl₂·6H₂O, 12.1; MgSO₄·7H₂O, 14.8; KH₂PO₄, 0.34; CaCl₂·H₂O, 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, and the pH was adjusted to 6.9 with KOH. Growth was monitored by measuring OD₆₅₀nm 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, 3μl 10N 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 OD₆₅₀nm 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 filtered through 0.2-μm nylon filters (Scientific Resources, Eatontown, NJ, USA), and analyzed immediately by HPLC.

**Preparation of cell-free extracts**

*Haloflex* sp. D1227 was grown in 250 ml BS3 medium containing 3 mM 3-phenylpropionic acid, cinnamic acid, and ben-