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Haloferax sp. D1227, a halophilic Archaeon capable of growth on aromatic compounds

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Abstract. A pink-pigmented halophilic Archaeon, Strain D1227, was isolated from soil contaminated with oil brine and shown to be a member of the genus *Haloferax*, based on: (1) its hybridization with a 16S rRNA probe universal for the Archaea; (2) its resistance to a broad spectrum of antibiotics that affect Bacteria; (3) its requirement for at least 0.86 *M* NaCl and 25 m*M* Mg^{2+} for growth; (4) its possession of C_{50}-carotenoids characteristic of the halophilic Arachaea; (5) the thin layer chromatographic pattern of its polar lipids, which was identical to that of other species of *Haloferax*; and (6) its pleomorphic cell morphology. However, in contrast to the known species of Archaea, *Haloferax* strain D1227 was able to use aromatic substrates (e.g., benzoate, cinnamate, and phenylpropanoate) as sole carbon and energy sources for growth. Physiologically similar organisms, such as *Haloferax volcanii, Haloferax mediterrani, Haloarcula vallismortis,* and *Haloarcula hispanica*, could not grow on these aromatic substrates. When grown on ^{14}C-benzoate, strain D1227 mineralized 70% of the substrate and assimilated 19% of the ^{14}C-label into cell biomass. In addition to growth on aromatic substrates, D1227 was also capable of growth on a variety of carbohydrates and organic acids. Optimum growth of strain D1227 occurred at 45°C in media containing 1.7–2.6 *M* NaCl and 100 m*M* Mg^{2+}. Under optimum growth conditions, the cell shape varied from that of an oblate spheroid on mineral salts medium alone, to disc-shaped, irregular or triangular cells on the same medium amended with yeast extract and tryptone. To our knowledge, this is the first unequivocal demonstration of the ability of an Archaeon to grow by mineralization of aromatic substrates, and it adds a new dimension to our appreciation of the physiological diversity of this group of prokaryotes.

Key words: Halophilic Archaea – *Haloferax* – Aromatic-ring mineralization – Benzoate – Cinnamate – Phenylpropanoate – Oil brine

Abbreviations: *Ha., Haloarcula*; *Hf., Haloferax*

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It is well known that many Bacteria, both aerobes and anaerobes, are able to catabolize a wide variety of aromatic compounds as sole carbon and energy sources (Gibson and Subramanian 1984; Schink et al. 1992). Among aerobic Bacteria in particular, the mechanisms of ring cleavage and pathways used to degrade aromatic compounds, such as toluene, benzoate, or halogenated benzoates, have been elucidated in detail, both enzymatically and genetically (for reviews see Gibson and Subramanian 1984; Burlage et al. 1989). The pathways for degradation and utilization of phenolic compounds in fungi have also been described in detail (Jones et al. 1993). In contrast, the ability of Archaea to grow by mineralization of aromatic compounds has never been demonstrated.

Among the three major groups of Archaea, i.e., the methanogens, the thermophilic sulfur utilizers, and the extreme halophiles, the latter group has the most metabolic diversity. Methanogens, although important members of anaerobic mixed-cultures capable of degrading aromatic substrates to CO_2 and CH_4, are not known to degrade aromatic rings themselves and are largely limited to C_1 compounds and acetate for growth (Schink et al. 1992). Likewise, many of the thermophilic sulfur oxidizers are strict anaerobes and have limited substrate ranges (Huber and Stetter 1992; Segerer and Stetter 1992; Stetter 1992; Zillig 1992), although members of the genus *Sulfolobus* show significant metabolic versatility (Grogan 1989) and might be candidates for aromatic ring catabolism. Among the halophilic Archaea, members of *Haloarcula* and *Haloferax* use a variety of carbohydrates and organic acids as sole carbon and energy sources (Tindall 1992). However, to our knowledge, it has not yet been demonstrated that any members of these or other genera of halophilic Archaea are capable of growth solely on aromatic compounds (Oren et al. 1992).

During an investigation of a microbial community in soil contaminated with oil brine at an oil well site in Michigan, we isolated a halophilic Archaeon capable of growth on benzoate, cinnamate, or phenylpropanoate as sole carbon and energy source. We report here the initial characterization of this organism.

Materials and methods

Source and isolation of strain D1227

The source material for enrichment of organisms was the top 5 cm of coarse, sandy soil surrounding an oil well near Grand Rapids, Michigan (43°N; 84°W). The soil was blackened with oil and smelled strongly of hydrocarbons. Salt crystals were often visible in the soil matrix. This particular oil well has been in production for over 50 years, and numerous small spills have occurred at the site during this time.

The primary enrichment was done by using a diffusion gradient chamber that allowed the establishment of multiple, continuous diffusion gradients in the X-Y planes of a $5 \times 5 \times 1.5$ cm arena. In this chamber, gradients of 1.7–5.2 *M* NaCl in an east/west orientation, and 0–10 m*M* sodium *p*-toluate in a north-south direction, were employed to select for halophilic microorganisms capable of degrading toluate and related aromatic compounds. Details about the gradient system and its use in studying halophilic, aromatic-degrading bacteria will appear elsewhere (D. Emerson et al. 1994; D. Emerson et al., manuscript in preparation).

The basal medium used for the primary enrichment contained (g/l): $MgCl_2 \cdot 6H_2O$, 19.5; $MgSO_4 \cdot 7H_2O$, 29.0; $CaCl_2 \cdot 2H_2O$, 1.1; KCl, 6.0; $NaHCO_3$, 0.2; yeast extract, 0.1; tryptone, 0.1. The pH was adjusted to 7.0 with KOH prior to heat sterilization. In addition, the medium within the gradient chamber was thickened with 0.15% agarose in which organisms could grow and move in response to the imposed gradients of salt and toluate. After gradients of salt and toluate had been partially established for 3 days, the surface of the gel was inoculated with 2 ml of supernatant fluid of a 1:20 dilution (in growth medium) of soil from the contaminated site. Bacteria were allowed to grow for 6 days at 37°C.

Prolific growth occurred in portions of the gel; from a region containing approximately 3.8 *M* NaCl and 2 m*M* toluate, a sample was taken and diluted into pour plates containing the same basal medium amended with 3.8 *M* NaCl and 1.5 m*M* toluate. These plates were incubated at 37°C and examined periodically with a stereomicroscope for colony development. After several days, pink colonies were observed in a 10^{-5} dilution pour plate; these represented 5–10% of the total CFU. Cells from these pink colonies were picked and streaked for isolation. One of the isolates, strain D1227 (deposited with the American Type Culture Collection (ATCC) no. 51408, Rockville, Md.) was then used for further characterization.

Growth and maintenance of D1227 and other halophiles

For tests of carbon source utilization, a defined mineral salts medium (BS 3) of the following composition (in g/l) was used: $NH_4(SO)_4$, 0.33; KCl, 6.0; $MgCl_2 \cdot 6H_2O$, 12.1; $MgSO_4 \cdot H_2O$, 14.8; KH_2PO_4, 0.34; and $CaCl_2 \cdot 2H_2O$, 0.36. NaCl was also incorporated, usually at a final concentration of 1.7 *M*, unless stated otherwise, and the pH was adjusted to 6.8–6.9 with KOH prior to sterilization. A trace element solution (1 ml/l, Widdel and Bak 1992) was also added prior to sterilization. After sterilization, a vitamin solution (1 ml/l) (Wolin et al. 1963) was added that contained all the vitamins listed by the authors except thioctic acid. When a richer medium (BSYT) was desired, BS 3 medium was supplemented with 0.3% (w/v) yeast extract and 0.3% (w/v) tryptone, and trace elements and vitamins were omitted. The pH was adjusted to 6.8. For solidified medium, 1.5% (w/v) agar was added prior to autoclaving.

Other halophilic strains

For comparative studies, the following halophilic Archaea were obtained from the ATCC: *Haloarcula hispanica,* ATCC 33960; *Ha. vallismortis,* ATCC 29715; *Haloferax volcanii,* ATCC 29605; *Hf. mediterranei,* ATCC 33500. These strains were maintained on BSYT medium containing 3.4 *M* NaCl.

Growth on aromatic compounds

Cells were cultured in 125 ml Erlenmeyer flasks obtaining 50 ml sterile BS 3 medium and amended with the filter-sterilized test compound. Each flask was inoculated with 1 ml of a late log phase culture grown either in BS 3 medium with glucose, or in BSYT medium. Flasks were incubated at 37°C on a rotary shaker set at 200 rpm. If growth was evident, the culture was transferred to fresh medium containing the same test compound. After two transfers resulting in growth on a particular compound, a time course of growth and substrate disappearance was determined. The OD (600 nm) of a 1-ml aliquot of the culture was first determined using a Gilford DU spectrophotometer. The same sample was then centrifuged at 8000 *g* for 4 min. The supernatant was diluted at least 1:2 with 100 m*M* HCl, and stored at −20°C until analysis by high-pressure liquid chromatography (HPLC), as described below.

HPLC analysis

Aromatic compounds were analyzed by reversed phase HPLC with an instrument (Waters, Millipore Corp. Milford, Mass., USA) equipped with a Brownlee Spheri-5, RP-18 5 µm column (100 × 4.6 mm; Applied Biosystems, Foster City, Calif., USA). Samples were separated by using a gradient of 100 m*M* phosphate buffer-methanol solvent (pH 2.6, increasing methanol) at a flow rate of 1.3 ml/min. Eluted compounds were detected by using a UV detector set at 212 nm.

Catabolism of ^{14}C-benzoate

Strain D1227 was grown to mid-log phase in 5 ml BSYT medium. Cells were collected by centrifugation, washed once in BS 3 medium alone, and then resuspended in 5 ml BS 3 medium containing 1.2 m*M* uniformly ring-labeled ^{14}C-benzoate (Sigma Chemical, St. Louis, Mo., USA) with a specific activity of 0.166 mCi/mmol. The culture was held in 30-ml serum vials and incubated at 37°C in a shaking water bath. A control vial contained only non-radioactive benzoate and cells. After cells in the control vial had utilized all the benzoate (5 days), as determined by HPLC, the contents of the experimental vial were acidified with 10 µl 0.1 *N* H_2SO_4 and the $^{14}CO_2$ present in a 1 ml sample of headspace gas was trapped in phenethylamine for liquid scintillation spectroscopy (LSS). Cells in a 1 ml subsample of the culture were concentrated by centrifugation, lysed by dilution with deionized H_2O, and re-acidified; the ^{14}C-content of the cellular lysate was also determined. For LSS, samples were mixed with 4 ml Bio-Safe II liquid scintillation cocktail, and the radioactivity was measured using an LKB 1211 Rackbeta liquid scintillation counter with appropriate correction for self-absorption of radiation.

Oligonucleotide probes

The following oligomers from universally conserved 16S rRNA sequences for the domain Bacteria (probe EUB 338), or the domain Archaea (probe ARCH 915) were used: EUB 338, 5′-GCTGCCTCCCGTAGGAGT-3′ (Amann et al. 1990a); ARCH 915, 5′-GTGCTCCCCCGCCAATTCCT-3′ (Stahl and Amann 1991). The probes were designed, synthesized, and purified by the method of Amann et al. (1990b), and they were labeled with tetramethylrhodamine-5-isothiocyanate (TRITC). For hybridization studies mid-late log phase cultures of strain D1227, as well as *Ha. hispanica* and *Hf. volcanii* (positive controls), were harvested by centrifugation, and washed with a buffer consisting of 30 m*M* phosphate buffer, pH 7.2, and 1.5 *M* NaCl. The washed cells were concentrated by centrifugation and stored at −20°C in the buffer containing 50% ethanol. A 10-µl drop of this suspension was spotted into the well of a precoated (with 0.25% gelatin) toxoplasmosis slide (Bellco Glass, Vineland, N.J., USA), allowed to air dry, and then dehydrated in 50%, 80%, and 95% (v/v) ethanol, 3 min each. *Escherichia coli* K-12, which served as a negative control, was

treated identically except that 30 m*M* phosphate buffer (pH 7.2) containing 0.15 *M* NaCl was used. Specimens were hybridized at 46°C according to the procedure of Manz et al. (1992). Following hybridization, they were viewed under a Leitz Orthoplan epifluorescence microscope, employing a rhodamine fluorescence filter set.

Other taxonomic analyses

The Mg^{2+} requirement of strain D1227 was determined by growing the cells at 37°C in 125-ml flasks containing 50 ml BS 3 medium with 2.2 *M* NaCl and varying the total concentration of Mg^{2+} from 1 to 120 m*M*. The shaker speed was 200 rpm. Mg^{2+} was added as a 1 : 1 ratio of $MgSO_4 \cdot H_2O$ and $MgCl \cdot 6H_2O$. For determination of the temperature optimum, cells were grown in 125-ml flasks containing 50 ml BSYT medium in thermostated shakers (200 rpm) set at 30°, 37°, 45°, or 55°C. For determination of the pH optimum, cells were grown in screwcap tubes with 10 ml BS 3 medium containing 5 m*M* glucose and the following buffers (Sigma): 10 m*M* Mes, pH 5.5 or 6.5; 10 m*M* Mops, pH 7.5; or 10 m*M* Taps, pH 8.5. The tubes were incubated at 30°C on a rotary shaker. For the determination of growth on nonaromatic substrates, cells were cultured in screw cap tubes containing BS 3 medium and 5 m*M* of the test carbon source, which was added to heat-sterilized BS 3 medium from a filter-sterilized stock solution. An exception was starch, which was added to BS 3 prior to heat sterilization. For all growth experiments, growth was assessed by measuring the maximum OD (600 nm) attained by the cells.

The capacity of strain D1227 to denitrify was determined by growing the cells anaerobically in 15 ml BS 3 medium with 10 m*M* glucose in 150 mm screwcap tubes sealed with butyl rubber septa. One set of tubes contained 10 m*M* KNO_3 and one set did not; the tubes were incubated on a rotary shaker at 30°C. Glass Durham tubes were used to check for gas production. Catalase activity was determined by adding a drop of 0.3% H_2O_2 directly to a colony and observing bubble formation. Oxidase activity was determined by spotting a colony onto filter paper saturated with oxidase reagent (Difco, Detroit, Mich., USA). Antibiotic susceptibility was determined by spread-plating 100 µl of a log phase culture of strain D1227 onto BSYT plates containing 3.4 *M* NaCl and adding diffusion disks containing various antibiotics. The plates were incubated at 37°C for 1 week and then the zone of inhibition, or lack thereof, was recorded. In addition, bacitracin sensitivity was determined by growing strain D1227 at 30°C in liquid BS 3 medium with 5 m*M* glucose in the presence of 5, 50, and 100 µg/ml bacitracin.

Electron microscopy

Cells of strain D1227 were grown either in BS 3 broth with 10 m*M* glucose or in BSYT broth, until late log phase (OD_{600} = 1.6). Cells were harvested by centrifugation, and pellets were resuspended in 2.5 ml 0.1 *M* phosphate buffer, pH 7.2, containing 1.6 *M* NaCl, 50 m*M* $MgSO_4 \cdot 7H_2O$ and 50 m*M* $MgCl_2 \cdot 6H_2O$. The cells were then fixed overnight at 5°C with 2.5% (v/v, final concentration) glutaraldehyde. The fixed samples were washed three times in the phosphate buffer containing NaCl and Mg^{2+} salts, embedded in agar (made up in the same buffer), and then treated with OsO_4 for 1 h. The specimens were dehydrated in a graded ethanol-propylene oxide series and embedded in PolyBed 812 (Electron Microscopy Sciences, Fort Washington, Pa., USA) resin for thin sectioning.

Lipid analysis

For analysis of polar lipids, *Ha. hispanica, Ha. vallismortis, Hf. volcanii, Hf. mediterranei,* and strain D1227 were grown at 37°C for 3 days in 150 ml BSYT medium containing 3.4 *M* NaCl. Each culture was harvested by centrifugation and cells were washed once in 0.1 *M* phosphate buffer (pH 7.0) containing 3.4 *M* NaCl. Following procedures described by Kates (1986), total lipids were extracted from cell pellets by a modified Bligh-Dyer technique, and the polar lipids were separated from the neutral lipids by precipitation with cold acetone. Chloroform extracts of the polar lipid fraction were spotted onto silica gel thin-layer chromatography (TLC) plates and developed once in chloroform : methanol : acetic acid : water (85 : 22.5 : 10 : 4). Glycolipids were detected by spraying the plates with α-naphthol followed by $H_2SO_4 : H_2O$ (95 : 5) (Kates 1986). The plates were heated for a few minutes in an oven at approximately 100°C until purple spots, indicative of glycolipids, developed. The plates were then charred at 110°–120°C to develop all lipid spots.

To determine the pigments associated with the neutral lipid fraction, strain D1227 was grown to late log phase at 37°C in BSYT liquid medium. Cells were harvested by centrifugation and the pellet was extracted three times with acetone. The resulting orange extract was concentrated under a stream of N_2, and an absorption spectrum of the resulting sample was recorded from 300 to 600 nm by using a dual-beam spectrophotometer (Perkin-Elmer, Norwalk, Conn., USA).

Results

General characteristics of Haloferax strain D1227

The colony and cell morphologies of all pink-pigmented isolates originally obtained from the diffusion gradient chamber were similar; therefore, one clone, designated strain D1227, was chosen for further study. When grown on BSYT agar plates for 1 week at 37°C, colonies were smooth, round, and red to pink in color with a diameter of 2–3 mm. In BSYT liquid medium at 37°C, cells had a doubling time of approximately 4 h; in BS 3 medium with 10 m*M* glucose, cells had a doubling time of approximately 6 h. The optimum of NaCl concentration for growth was 1.7–2.6 *M*, but growth occurred over the range of 0.86–5.2 *M* NaCl. At concentrations of ≤ 1.3 *M* NaCl, the cells appeared as swollen spheres. They lysed instantly when diluted with deionized H_2O. The optimum temperature for growth was 45°C; very little growth occurred at 55°C. The optimum pH for growth was between 6.5 and 7.5; there was no growth at pH 5.5, and limited growth at pH 8.5. Strain D1227 did not grow anaerobically, nor did inclusion of nitrate in the medium permit growth under anaerobic conditions. The cells were catalase- and oxidase-positive.

Cell morphology and effects of Mg^{2+}

Cells of strain D1227 were pleomorphic and their morphology varied with the nature of the medium (Fig. 1). In BS 3 medium with glucose as energy source, cells tended to be oblate spheroids (Fig. 1c). In BSYT medium, the pleomorphy was more pronounced, with cells ranging from rod-shaped to triangular to curled disks of indeterminate shape (Fig. 1d). Shortly after isolation, rotating cells were occasionally seen in log phase cultures; however, contemporary cultures no longer show any evidence of motility. Gas vacuoles were never observed by phase-contrast microscopy or electron microscopy.

The concentration of Mg^{2+} in the growth medium had a profound effect on cell growth and shape. Strain D1227 did not growth at Mg^{2+} concentrations below 5 m*M*. At 25 m*M* Mg^{2+}, the growth rate was about 30% of that at 100 m*M* Mg^{2+}, and the cells were large spheres (Fig. 1a) with an average cell diameter of 2.5 µm (SD = 0.18; n = 15). At 50 m*M* Mg^{2+}, cell growth was similar to that at 100 m*M*

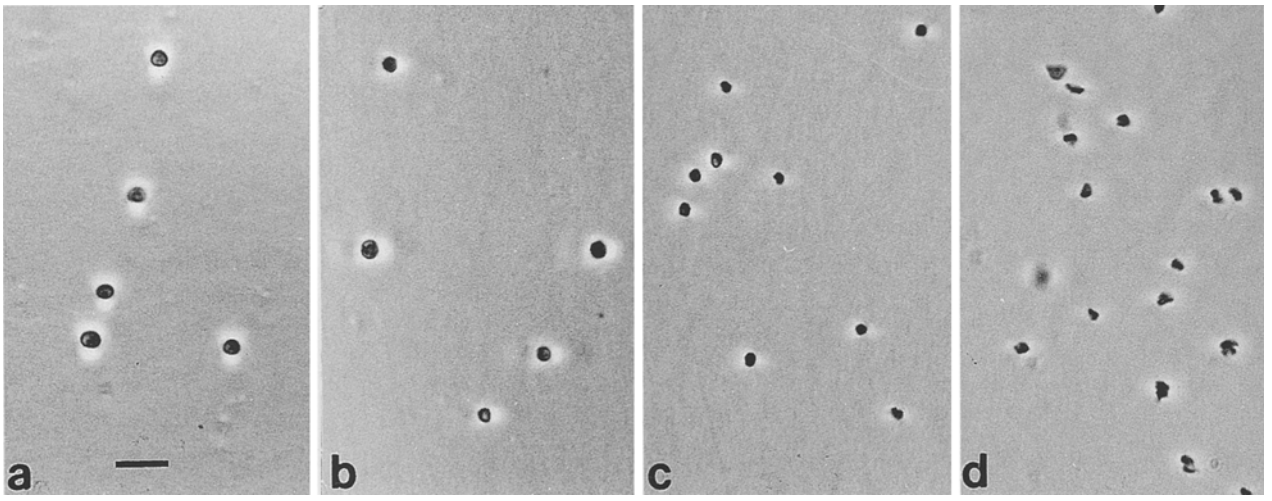

Fig. 1a–d. Light micrographs of D1227 demonstrating the effect of growth conditions on cell morphology. Panels **a** to **c** show growth in BS 3 medium with 10 m*M* glucose and **a** 25 m*M* Mg^{2+}; **b** 50 m*M* Mg^{2+}; **c** 120 m*M* Mg^{2+} (full strength BS 3). Panel **d** illustrates the cell morphology of cells grown in BSYT medium. *Bar* = 5 μm

Mg^{2+}, but cell shape was still spherical (Fig. 1b) with an average cell diameter of 2.2 μm (SD = 0.23, n = 15). At 100 m*M* Mg^{2+} or above, cells were oblate spheroids (Fig. 1c) as described above and had a diameter of 1.9 μm (SD = 0.23; n = 15).

Electron microscopy revealed even more precisely the differences in morphology of D1227 when grown in BS 3 vs BSYT liquid medium. Cells grown in BS 3 medium were nearly coccoid with some flattening of the edges (Fig. 2a), whereas cells grown in BSYT medium were more disk-shaped and pleomorphic (Fig. 2b). Under both growth conditions the cell walls were morphologically similar.

16S rRNA oligonucleotide probes

Based on the salt requirement, colony pigmentation and cell morphology, it appeared likely that strain D1227 was a member of the Archaea. This was confirmed by the observation that the cells hybridized with an archaeal probe (ARCH 915) but not a bacterial probe (EUB 338, results not shown). The same was true for *Haloarcula hispanica,* but the opposite was true for cells of *Escherichia coli.* The hybridization procedure appeared to release a good deal of cytoplasmic material from the D1227 cells, as they went from being phase dark after fixation to nearly phase transparent after hybridization. As a result, the fluorescent signal was weak in cells hybridized with the archaeal probe, but was nonexistent in the same cells hybridized with the bacterial probe. The same problem was encountered with *Haloferax volcanii*; however, *Ha. hispanica* maintained more phase density and yielded a stronger fluorescent signal.

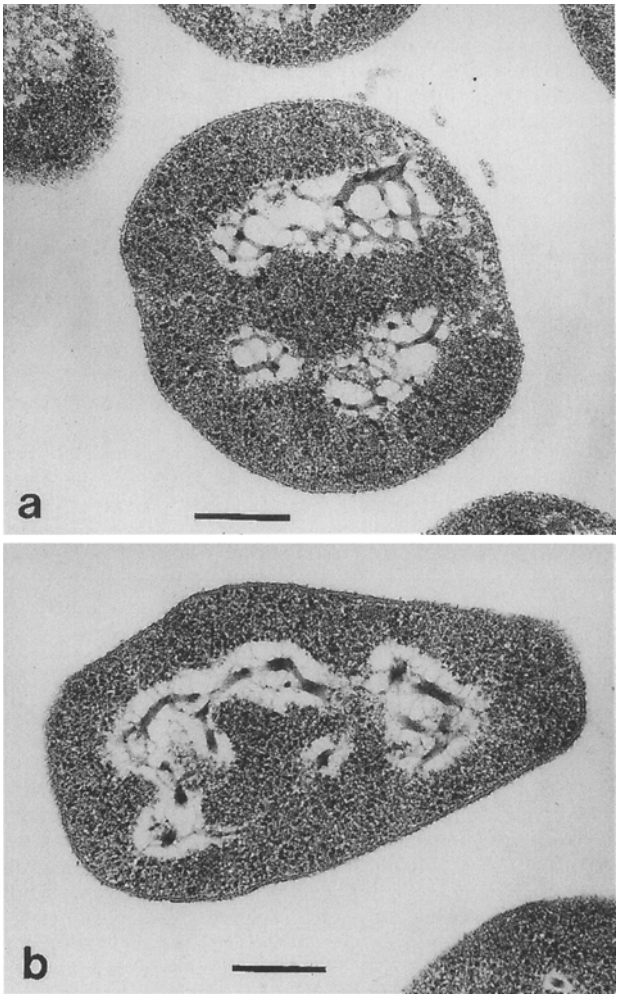

Fig. 2a, b. Transmission electron micrographs of D1227 grown on various media. **a** BS 3 medium with 10 m*M* glucose. **b** BSYT medium. *Bars* = 0.5 μm

Lipid analysis

The polar lipids of strain D1227 were compared to those of other authentic halophilic Archaea by TLC. The profile exhibited by D1227 was identical to that of the two *Haloferax* species, which were, in turn, distinct from those of the *Haloarcula* species (Fig. 3). Based on comparison of this profile with that observed by Torreblanca et al. (1986, Fig. 2) a sulfated diglycosyl diether (S-DGD) and a di-

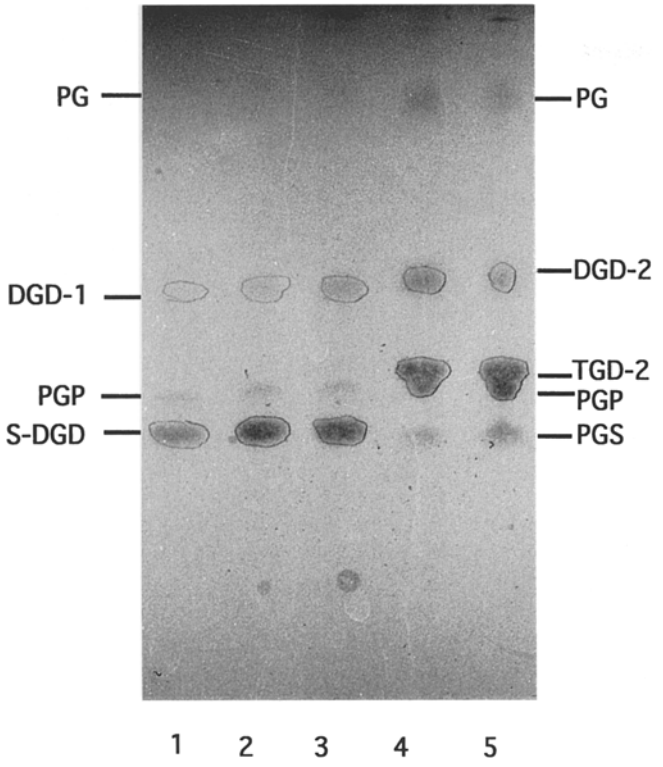

Fig. 3. Comparison of polar lipid profiles of known halophiles and D1227 by TLC. *Lane 1, Haloferax mediterranei; lane 2, Hf. volcanii; lane 3, D1227; lane 4, Haloarcula vallismortis; lane 5, Ha. hispanica.* One the left side of the panel are listed the lipids that putatively correspond to the genus *Haloferax* by comparison with the chromatograms of Torreblanca et al. (1986). On the right side are the polar lipids that putatively correspond to the genus *Haloarcula.* The *circled spots* tested sugar-positive. PG, phosphatidylglycerol; DGD-1, diglycosyl diether-1; PGP, phosphatidylglycerophosphate; S-DGD, sulfated-diglycosyl diether; DGD-2, diglycosyl diether-2; TGD-2, triglycosyl diether-2; PGS, phosphatidylglycerosulfate

glycosyl ether (DGD-1) could be tentatively identified. These are signature lipids for the genus *Haloferax.* By comparison, the *Haloarcula* species both appeared to contain a triglycosyl diether (TGD-2) and an unknown diglycosyl ether (DGD-2), which are signature lipids of this genus. The phosphatidylglycerol (PG) spot for *Ha. mediterranei* was not visible in this chromatogram (Fig. 3), and the PG spots for *Ha. volcanii* and D1227 were only faintly visible. In other chromatograms that were developed twice in the solvent system, the PG bands were much more evident for these organisms, but the other bands were less well resolved.

Treatment of strain D1227 with acetone extracted all of the pigment from the cells. The absorption spectrum of the acetone extract showed maxima at 468, 493, and 527 nm. These values correspond closely to the absorption maxima in acetone near 465, 490, and 525 nm for the C_{50}-carotenoids neo-bacterioruberin (R_1), bacterioruberin (R_2), monhydrobacterioruberin (R_3), and bisanhydrobacterioruberin (R_5) (Kushwaha et al. 1975). Minor absorption peaks were observed at 385 and 368 nm, which are also diagnostic for these chromophores. These carotenoids are all characteristic of the halophilic Archaea (Kushwaha et al. 1975).

Table 1. Nonaromatic substrates tested for growth of D1227 in BS 3 medium[a]. Strain D1227 was inoculated into duplicate screw-cap tubes containing 10 ml of medium with 5 m*M* of the test substrate and incubated at 30°C on a rotary shaker

Compound	Growth[b]	Acid produced[c]
Starch	0.14	–
Maltose	0.36	+
Sucrose	0.42	+
Trehalose	0.40	+
Arabinose	0.34	+
Xylose	0.34	+
Glucose	0.32	+
Glycerol	0.23	–
Acetate	0.11	–
Pyruvate	0.32	–
Lactate	0.58	–
Proline	0.11	–

[a] No growth occurred on the following substrates: mannose, galactose, lactose, ribose, xylitol, sorbitol, mannitol, succinate, and betaine

[b] Growth is expressed as the maximum OD (600 nm) obtained

[c] Acid production was determined by measuring the pH of the medium following growth; + indicates the final pH was at least 1.5 units lower than the initial pH of 6.8

Antibiotic susceptibility

Strain D1227 was resistant to ampicillin (10 μg), bacitracin (50 μg), chloramphenicol (30 μg), erythromycin (15 μg), penicillin (10 U), polymyxin B (300 U), streptomycin (10 μg), tetracycline (30 μg), and vancomycin (30 μg). It was sensitive to novobiocin (5 μg) and bacitracin (100 μg).

Growth on aromatic compounds

Although strain D1227 was originally isolated on a medium containing sodium *p*-toluate, ironically the cells did not grow on toluate alone (Table 1). Presumably, cells may have grown in the primary isolation medium at the expense of small amounts of yeast extract and tryptone that were also present, or on degradation products of toluate that were formed by other microbes in the enrichment. Nevertheless, subsequent screening established that strain D1227 could grow on a limited number of aromatic compounds as sole carbon and energy source, inluding benzoate, cinnamate, and phenylpropanoate. Growth on benzoate and cinnamate (Fig. 4a, b), as well as phenylpropanoate (results not shown), resulted in complete disappearance of the aromatic compounds; however, such growth on these substrates was always characterized by a longer lag time than for growth on glucose. For example, when D1227 was grown on 5 m*M* glucose the lag time was <24 h, while the lag time for growth on 5 m*M* benzoate was approximately 3 days and was independent of previous growth history. Increasing the concentration of benzoate increased the duration of the lag phase; cells grown on 20 m*M* benzoate had a lag time of 5 days and grew with doubling times of >10 h, suggesting that high benzoate concentrations were toxic.

When D1227 was grown on ring-labeled ^{14}C-benzoate, we could account for 89% of the radiolabel: 70% was re-

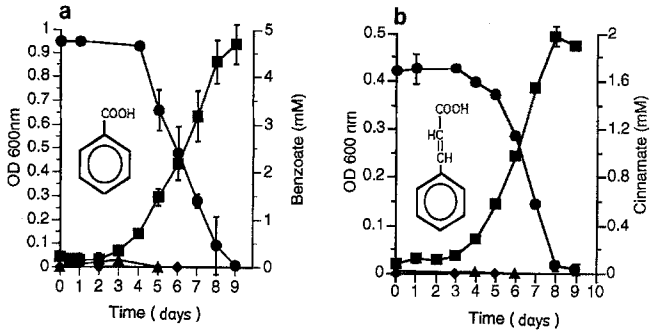

Fig. 4a, b. Growth of strain D1227, *Haloferax volcanii,* and *Haloarcula hispanica* on 5 m*M* benzoate and 2 m*M* cinnamate. **a** Growth of D1227 on benzoate (■) and disappearance of substrate (●); growth of *Hf. volcanii* (▲), and *Ha. hispanica* (◆) on benzoate. **b** Growth of D1227 on cinnamate (■) and disappearance of substrate (●); growth of *Hf. volcanii* (▲), and *Ha. hispanica* (◆) on cinnamate. Note that the *Y*-axis for **a** and **b** differ. The error bars represent one standard deviation from the mean. For both substrates, unioculated controls incubated identically to the inoculated flasks showed no loss of substrate, nor was there any disappearance of substrate in the *Hf. volcanii* or *Ha. hispanica* cultures (data not shown)

covered directly as $^{14}CO_2$ and 19% was recovered in the particulate cell material after cell lysis and acidification. Presumably much of this latter portion had been incorporated into cell biomass. Thus, it appeared that D1227 was capable of mineralizing most of the benzoate carbon to CO_2.

Strain D1227 was also tested for growth on a number of other aromatic compounds; however, none of the following were utilized (m*M*): ferulate (2), syringate (2), *o-*, *m,- p*-toluates (1 each), *o-*, *m-*, *p*-cresols (1 each), phenol (1), 1-hydroxybenzoate (2), benzene (1), toluene (1), *o-*, *m-*, *p*-xylenes (1 each), 2,4-dichloropenoxyacetic acid (1), 2-chlorobenzoate (0.5), 3-chlorobenzoate (1), 4-chlorobenzoate (1), biphenyl (0.5), *p*-hydroxybenzoate (1), α-naphthol (1) *p*-nitrophenol (1), 2-aminophenol (1), phenylalanine (2), and tryptophan (2).

Comparison with other halophilic Archaea

No growth or degradation of benzoate or cinnamate was found for *Ha. hispanica* or *Hf. volcanii,* nor did they grow on phenylpropanoate. *Ha. vallismortis* and *Hf. mediterranei* also did not grow on either benzoate or cinnamate; they were not tested on phenylpropanoate.

Growth on other substrates

Various nonaromatic substrates were used as sole carbon and energy sources for growth of strain D1227, including carbohydrates, and organic and amino acids (Table 1). Enough acid was produced from each of the carbohydrates to lower the pH of the medium from 6.7 to <5.0.

Discussion

The halophilic Archaea were originally thought to be fastidious microbes that required complex media and utilized primarily amino acids for growth (reviewed in Tindall 1992). However, this view changed with the report of strains capable of growth in defined, mineral salts medium (Rodriguez-Valera et al. 1980). This capacity to grow under more stringent nutritional conditions has become more widely recognized, and members of *Haloarcula* and *Haloferax* are now known to use a variety of carbohydrates and organic acids as sole carbon and energy sources (Tindall 1992). Nutritionally, strain D1227, isolated in the present study, resembles these two genera with respect to the substrates it can use as sole carbon and energy sources. However, strain D1227 also reveals an important new dimension in the catabolic repertoire of the Archaea, by virtue of its ability to conserve energy for growth by mineralization of the aromatic compounds benzoate, cinnamate, and phenylpropanoate.

We are aware of only one other report suggesting that halophilic Archaea may degrade aromatic compounds (Bertrand et al. 1990). This unidentified archaeon, designated EH4, was isolated from a salt marsh in France. It was reported to degrade 24% of 9-methyl anthracene (initial concentration, 2.6 m*M*), as well as lesser amounts of phenanthrene, anthracene, and acenaphthene, to undetermined products in 30 days. However, there was no evidence that such "biodegradation" included mineralization or even limited degradation of the aromatic nuclei, as opposed to modification of the parent substrate. Nor was it reported whether such putative biodegradation increased the growth rate or yield of EH4. The organism was not tested with any of the aromatic compounds on which strain D1227 grew. Another recent paper reported growth by several strains of halophilic Archaea, isolated from oil fields in Russia, on crude petroleum (Kulichevskaya et al. 1992); however, no mention was made of whether these isolates could utilize aromatic compounds present in crude petroleum.

Taxonomy

A summary of the important taxonomic characteristics of strain D1227 and its comparison with *Hf. volcanii* and *Ha. hispanica* is presented in Table 2. Compared to the halophilic Archaea as a whole, which generally grow best at approximately 3.4 *M* NaCl, the optimum NaCl concentration for growth of strain D1227 is 1.7–2.6 *M*. In this respect, it resembles *Hf. volcanii,* which was isolated from the Dead Sea and also has a salt optimum of 1.7–2.6 *M* (Mullakhanbhai and Larsen 1975). The pleomorphic morphology of D1227 is one of its most striking characteristics and is common to members of the genera *Haloferax* and *Haloarcula*. The requirement for a high Mg^{2+} concentration and the dependence of cell shape on the Mg^{2+} concentration is a trait of *Haloferax* species (Tindall 1992) and *Haloarcula japonica* (Takashina et al. 1990). What differentiates strain D1227 from the genus *Haloarcula* are its polar lipids, which are diagnostic of the genus *Haloferax.*

Within the genus *Haloferax*, strain D1227 appears most closely related to *Hf. volcanii* both in salt requirement and morphology (compare our Fig. 2 with Figs. 7 and 8 of Mullakhanbhai and Larsen 1975). The most important differences between these two organisms are nutritional: strain D1227 utilizes aromatic compounds, whereas *Hf. volcanii* does not. More minor nutritional differences are that *Hf. volcanii* utilizes lactose (Torreblanca et al. 1986)

Table 2. Comparison of discriminatory phenotypic features of D1227, and of *Haloferax volcanii* and *Haloarcula hispanica*[a]

Organism	Strain D1227[b]	*Hf. volcanii*[c]	*Ha. hispanica*[d]
Colony type	Pink, 2–3 mm diameter	Red-orange, 1–2 mm diameter	Red-orange, 2 mm diameter
Cell morphology	Pleomorphic, disk-shaped	Pleomorphic, disk-shaped	Pleomorphic rods
Carbon sources utilized[e]			
Lactose	–	+	+
Succinate	–	+	+
Benzoate	+	–	–
Cinnamate	+	–	–
Phenylpropanoate	+	–	–
NaCl requirement (40°C)			
Range	0.86–5.2 *M*	1.0–5.0 *M*	2.5–5.2 *M*
Optimum	1.7 –2.6 *M*	2.5 *M*	4.3 *M*
Mg^{2+} requirement	50–100 m*M*	100–200 m*M*	5 m*M*
pH optimum	6.5–7.6	ND	7
Temperature optimum	45°C	45°C	35–40°C
Polar lipids[f]	S-DGD, PGP, DGD-1, PG	S-DGD, PGP, DGD-1, PG	PGS, PGP, TGD-2, DGD-2, PG

[a] The table lists primary phenotypic differences between the organisms. All three are chemoheterotrophs, contain bacterioruberins, are oxidase- and catalase-positive, and are susceptible to the antibiotics novibiocin and bacitracin
[b] Ref.: this work
[c] Refs.: Mullakhanbhai and Larsen 1975; Torreblanca et al. 1986; Kauri et al. 1990; this work
[d] Refs.: Juez et al. 1986; Torreblanca et al. 1986; this work
[e] Lists only difference in carbon source utilization by the three organisms. All the other carbon sources utilized by D1277 and listed in Table 1 are also utilized by *Hf. volcanii* and *Ha. hispanica*
[f] See legend to Fig. 3 for abbreviations
ND, not determined

and succinate (Kauri et al. 1990), whereas strain D1227 does not. However, because nutritional differences may not accurately reflect phylogenetic differences, we have deferred attaching a species epithet to this organism until a more detailed phylogenetic and biochemical analysis can be carried out.

Another interesting aspect of the physiology of strain D1227 is the profound morphological difference between cells grown in BS 3 medium with glucose (uniform oblate spheres) and those grown with yeast extract and tryptone (pleomorphic disks). At present, we do not know the physiological basis for these differences. The NaCl and Mg^{2+} salt concentrations of these two media should be essentially the same and thin sections of cells grown in each medium did not reveal any striking differences in cell wall structure that might account for the difference in morphology. However, we cannot rule out the possibility that the richer medium may allow for production of a more robust proteinaceous S-layer that is not immediately obvious by the TEM analysis we employed. The S-layer of other halophilic Archaea is known to influence cell morphology (Cohen et al. 1991; Nakamura et al. 1992; Nishiyama et al. 1992).

Ecological and evolutionary considerations

Oil brine is not generally thought of as a common habitat for halophilic Archaea, which are normally isolated from salterns, salt lakes, and similar environments (Oren 1993). Thus, the isolation of strain D1227 from topsoil contaminated with oil brine is somewhat curious and raises the question of its origin. It seems unlikely that D1227 is a native inhabitant of the topsoil from the site where it was isolated, since the salinity of these soils is normally well below that allowing survival of this organism. However, the airborne dissemination of strain D1227 to this site, possibly in conjunction with salt crystals, cannot be ruled out. Still, it seems the most likely explanation for its presence is that it was associated with the subterranean brine and pumped up with the oil and its associated production water. Michigan oil fields are somewhat unusual in the high concentrations of brine they contain. Corroborating evidence for this conjecture comes from the recent discovery of halophilic Archaea in Russian petroleum deposits (Kulichevskaya 1992). Extreme halophiles also have been reported in association with non-oil-bearing deep brine deposits in the United States (Vreeland and Huval 1991). Benzoate would probably be a common constituent of such oil brines, since it is a common intermediate in the breakdown of many aromatic hydrocarbons. Indeed, this prompted us to test strain D1227 for utilization of benzoate, even though it did not grow on toluate. It is also interesting that when the medium contained yeast extract and tryptone, strain D1227 tolerated and even grew well in the presence of saturating concentrations of potent solvents such as toluene and benzene, although there was no evidence for its ability to metabolize these aromatic hydrocarbons (S. Chauhan, unpublished results). These results indicate that strain D1227 has the ability to survive in oil brine and suggest that its possession of aromatic ring cleaving pathways may present it with a clear advantage in such habitats.

The nature of the aromatic ring cleaving pathway and its evolution is perhaps the most interesting question associated with this organism. Since the Archaea represent one of the three domains of life, their evolutionary distance from Bacteria, known to catabolize aromatic compounds aerobically, is great (Woese 1987). There is much interest in the evolution of these pathways in the Bacteria, especially regarding the genes encoding oxygenases that mediate the initial ring destabilizing reactions that lead to aromatic ring catabolism (Horn et al. 1991; Harayama et al. 1992).

Biochemical and genetic analysis of strain D1227 should reveal whether it degrades aromatic compounds by a pathway similar to one found in Bacteria and, if so, whether this may represent a case of convergent evolution or gene exchange between phylogenetically disparate organisms.

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