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Biodegradation of Aromatic Compounds by a Halophilic Archaeon Isolated from the Dead Sea.

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It is well established that aromatic compounds can be readily degraded in aerobic environments within soils, sediments and waters with salinities up to and including that of seawater. However, little is known about their metabolism in hypersaline environments. There is a growing interest in the development and optimization of bioremediation processes to deal with environments with high salinity that are contaminated with aromatic compounds. Among prokaryotes, haloarchaea are a group of microorganisms living in hypersaline environments that may have a greater potential in degrading pollutants than previously assumed and can be considered as a good environmental tool for bioremediation. We enriched and isolated 10 halophilic archaea from Dead Sea water samples on the basis of their ability to grow on p-hydroxybenzoic acid (pHBA) as the sole carbon and energy source. All isolates showed identical total lipid profiles, but are metabolically very diverse. Strain L1, which is also capable of growth on benzoic acid (BA), was chosen for biodegradation kinetics determination. When grown in BA, strain L1 produced small amounts of a compound that co-chromatographed with gentisic acid, which accumulated in the medium. The same did not occur when pHBA was the growth substrate. A discussion on the possible metabolic pathways involved is included, and a preliminary characterization of strain L1 is presented.

1. Introduction

Thalassic (sea water-derived) hypersaline environments are usually characterized by low water activity and high temperatures. Biological activity dramatically decreases as sea water concentrates, as a consequence of a_w reduction, and so does biological diversity. The Dead Sea contains 340 g/L total dissolved salts and is probably one of the most extreme examples of saline aquatic environments. Its waters are devoid of life except for local more diluted sections originated from rare abundant rainfall in the catchment area, mainly during winter, which may lead to discrete microbial blooms around the edges (Bodaker, et al, 2010). Since the pioneer studies by Benjamin Elazari Volcani on the microbiota of the Dead Sea in the early 1930s, a number of aerobic and anaerobic halophilic bacteria and archaea have been isolated from Dead Sea water and sediments, or detected through molecular or chemotaxonomic methods (Oren and Gurevich, 1995, Arahal *et al.* 1996, Rainey *et al.,* 1995, Bodaker

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et al, 2010). The unicellular green alga *Dunaliella sp*. has been recognized as the only primary producer in the Dead Sea, and glycerol, produced by *Dunaliella* cells as compatible solute, is probably one of the main sources of organic nutrients in the lake. The massive proliferation of halophilic archaea is clearly dependent on extremely rare algal blooms, observed only twice in the Dead Sea since 1980 (Oren, 1999, Bodaker et al, 2010).

Benzene ring is among the most abundant chemical substructure in nature and many compounds (from both natural and anthropogenic sources) containing benzene in their structure are major environmental pollutants (Díaz, 2004). The biodegradation of aromatic compounds by halophilic bacteria has been well studied (García et al., 2005, Mnif et al. 2009, Zhuang et al., 2010). However, we still know very little about biodegradation of aromatic substrates by microorganisms within the domain *Archaea*. A limited number of haloarchaea have been shown to metabolize aromatic substrates through a gentisate pathway (Emerson *et al.,* 1994; Fu and Oriel, 1999; Fairley *et al.,* 2002), and the mechanism of initial ring hydroxylation that results in gentisate has been described (Fairley *et al.* 2002). The only archaeal ring cleavage enzyme reported to date was cloned and purified from *Haloferax* sp. D1227, and additional enzymes catalyzing steps of a putative CoA-mediated benzoate pathway have been identified in two haloarchaeal genera (Fairley *et al.* 2006).

In a previous paper (Cuadros-Orellana et al., 2006) we reported on the isolation and properties of forty four extremely haloarchaea able to grow aerobically on aromatic acids as sole carbon and energy sources. Here, we describe the kinetics of these substrates degradation by a strain isolated from the Dead Sea, and a preliminary characterization of this isolate.

2. Materials and Methods

2.1 Environmental samples

Brine and sediment samples were collected from the Dead Sea (Jordan) in December 2002 and used for the enrichment of aromatic acid-metabolizing strains.

2.2 Culture media and growth conditions

Halophilic archaea were cultured on minimal medium (Mevarech & Werczberger, 1985), pH 7.2, amended with 0.1% (v/v) of a trace-elements solution (Dyall-Smith, 2000) and 0.1% (v/v) of a vitamin solution (Wolin, 1963). For solid medium, 1.5% (w/v) agar was used. For rich medium, minimal medium was amended with yeast extract (0.3%, w/v) and tryptone (0.3%, w/v). The growth temperature was 40º C for all strains. Batch cultures were incubated on a rotary shaker at 180 rpm.

2.4 Enrichment cultures

Enrichment cultures were prepared in Erlenmeyer flasks containing 50 mL minimal medium amended with p-hydroxybenzoic acid to a final concentration of 0.4 mM. Environmental samples were added to enrichment cultures (1:50, v/v) and incubated at 40ºC for 7 days. Three sequential transfers were performed, repeating the 1-week incubation procedure, before spreading culture aliquots onto solid medium of the same composition for colony isolation and purification.

2.5 Phenotypic characterization

Phase-contrast microscopy was used to analyze cell morphology in exponentially growing cultures. Gram staining was performed to acetic-acid fixed samples. Pigments were extracted from cells collected from 2mL aliquots of exponential phase cultures (centrifugation at 14,000 *g* for 10 min), using a mixture of acetone : methanol (1:1, v/v), for 4h, at room temperature, and the absorption spectrum was recorded using a UV-Visible Shimadzu spectrophotometer, model UV-1201, in quartz cuvettes. Cell motility was observed in wet mounts on slides previously covered with a layer of agarose to facilitate observation. Enzyme activities (catalase, oxidase, amylase, urease, lipase, caseinase and DNase), indol production and sugar acidification were determined through standard procedures. Catalase and oxidase were analyzed on colonies cultivated on rich medium and results were recorded immediately or after 5 minutes. For the other tests, results were recorded after 1 week, but negative results were considered so only after 30 days of incubation. All analyses were performed in triplicate.

2.6 Biodegradation of aromatic acids

Biodegradation experiments using p-hydroxybenzoic acid and benzoic acid as substrates were performed in triplicate 250 mL Erlenmeyer flasks containing 100 mL minimal medium amended with each substrate to a final concentration of 10 mM. An inoculum standardization procedure was undertaken, as follows: a pre-culture (50 mL) was prepared using the same medium and the same aromatic acid as substrate, and log-phase cells were collected (10,000 *g*, 5 min), washed carefully in mineral medium to prevent lysis, and resuspended in 5 mL mineral medium. Two hundred microliter-aliquots were used to inoculate the media for biodegradation experiments, resulting in an initial optical density of 0.02 at 600 nm. Cultures were incubated at 37ºC in a rotary shaker at 180 rpm. Samples were collected at 12 hour-intervals for cell density determinations (600 nm) and high performance liquid chromatographic (HPLC) analyses.

2.7 HPLC analysis

Aromatic acids on culture supernatants were analyzed using reversed-phase HPLC on a Shimadzu LC-6A model chromatograph equipped with a UV-visible detector (Shimadzu) at 254 nm. A 20 µL sample was injected in a Varian C18 column (150 x 4.6 mm) and aromatic acids were eluted using 50% methanol and 1% acetic acid in water as the solvent, at a constant flow of 0.5 mL/min and column temperature of 35ºC.

2.8 16S rRNA gene sequencing

Genomic DNA was isolated from 200 µL mid-log phase cultures in complex medium, as described by Lam (2000). PCR amplification sequencing of the 16S gene was performed using the archaea-specific primers D30 (forward, 5´-ATTCCGGTTGATCCTGC-3´) and D56 (reverse, 5´-TCGCGCCTGCGCCCCGT-3´), essentially as described by Bonfa et al., 2011.

2.9 Similarity analysis and phylogenetic tree construction

Nucleotide BLAST against the non-redundant nucleotide collection of NCBI database was performed.
A phylogenetic tree was constructed using 16S rRNA gene sequences of halophilic archaea produced in this study and obtained from the NCBI database, using *Aquifex aeolicus* as the outgroup. We used the neighbor-joining method and the Kimura-two parameter calculation model.

3. Results and Discussions

3.1 Isolation of haloarchaea strains

We isolated 10 halophilic archaea from Dead Sea water samples enrichments with 0.4 mM 4-hydroxybenzoic acid as the sole carbon source. All isolates were motile rods, and showed a similar pigment profile with absorption peaks at 526-528 nm and at 494-495 nm, except for isolate L8, which showed an additional peak at 468 nm. These absorbance values are characteristic of carotenoid pigments (bacterioruberins) of halophilic archaea (Emerson et al., 1994).

3.2 Phenotypic characterization

The 10 isolates were metabolically highly diverse, having in common the inability to hydrolyze starch, and the ability to produce oxidoreductase activity (oxidase and catalase) and indol. None, but strain L5, were able to utilize urea. All isolates showed DNAse activity, which is remarkable, considering that Arahal et al. (1996) characterized 22 strains isolated from the Dead Sea, which were tentatively assigned to 3 different genera (*Haloferax*, *Haloarcula* and *Halobacterium*), and all were DNAse-negative. Strain L1 is a motile Gram-negative and pleomorphic archaeon. It does not exhibit caseinolytic or lipolytic activity, does not have the ability to metabolize citrate and does not hydrolyze urea, showing to be the less metabolically versatile strain among all isolates. Considering that strain L1 was able to grow on mineral medium containing aromatic substrates as sole carbon and energy sources, it was chosen for further investigation of aromatic metabolism.

3.3 Aromatic acid metabolism by strain L1

Under the experimental conditions used, the lag phase of strain L1 grown in 10 mM p-hydroxybenzoic acid was very short (< 24 h) (Figure 1), a growth pattern very similar to that observed by Fairley *et al*. (2002) for *Haloarcula* sp. D1 (~20 h). When cultivated in 10 mM benzoic acid, the lag-phase of strain

L1 was reached at 48 h (Figure 2). These results suggest that, under the experimental conditions used, strain L1 was able to degrade benzoic acid more efficiently than *Haloferax* sp. D1227 (Emerson *et al.*, 1994), for which a lag-phase of approximately 72 h was observed in medium containing 5 mM benzoic acid. Discrete background growth (optical density no higher than 0.02 in the first 70 h) was observed in control cultures with no added aromatic acid, and this residual growth was probably supported by nutrients inevitably introduced together with the inoculum.

The monitoring of p-hydroxybenzoic acid and benzoic acid concentrations in the growth medium reveals a typical catabolic profile, in which the substrate removal takes place as the cell density increases. The complete disappearance of both substrates coincides with the beginning of the stationary phase of the culture, namely at 170h for p-hydroxybenzoic and at 200h for benzoic acid. The complete degradation of 4 mM p-hydroxybenzoic acid was reported for *Haloarcula* sp. D1 after 85h cultivation (Fairley *et al*., 2002) and the complete degradation of 5 mM benzoic acid by *Haloferax* sp. D1227 was observed after 215h (Emerson *et al.*, 1994). Considering that in our study we used a higher concentration of these substrates (10 mM), our results are consistent with the literature.

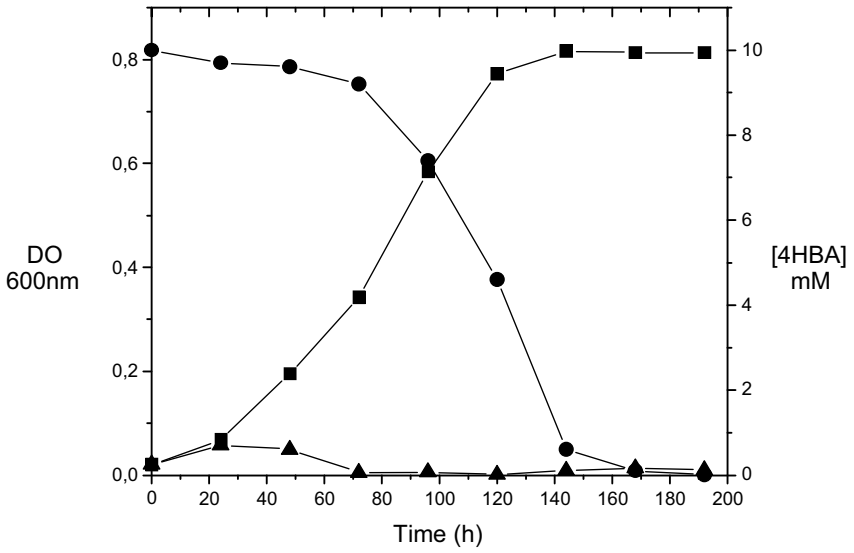

Figure 1 – Growth of strain L1 in 10 mM p-hydroxybenzoic acid. Cell density in medium supplemented with 10 mM p-hydroxybenzoic acid (■), and in medium with no added carbon source (▲). Concentration of p-hidroxybenzoic acid (●), as determined by HPLC.

Aromatic acids are often found in the environment as products of the degradation of complex organic compounds (biomass, hydrocarbons). It is likely that the metabolism of one-ring aromatic acids is a widespread feature among free-living haloarchaea. Gentisic acid has been pointed out as a metabolic intermediate in aromatic acid degradation by halophilic archaea, and gentisate 1,2-dioxygenase activity has been detected in at least two haloarchaea genera in a substrate-dependent manner (Fairley et al., 2006). We observed that benzoic acid-grown L1 cells produced micromolar amounts of a metabolite that co-chromatographed with a gentisic acid standard (Figure 2). The appearance of this metabolite coincided with the mid-log growth phase of the culture (around 100-h) and its concentration increased continuously while the original substrate (BA) was completely consumed. The same did not happen to p-hydroxybenzoic acid-grown cultures of this strain, probably because in the latter condition the metabolic intermediate(s) had too high turnover rate to allow detection.

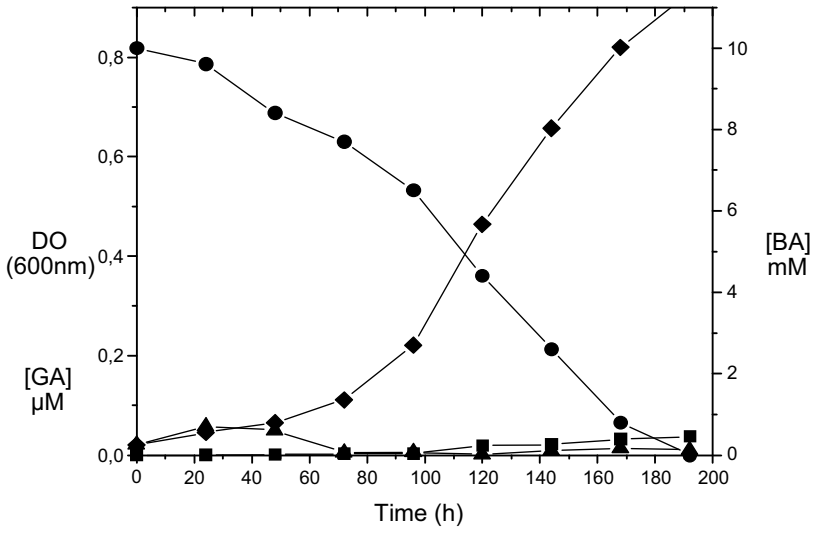

Figure 2 – Growth of strain L1 in benzoic acid. Cell density in medium with 10 mM benzoic acid (◆), and in medium with no added carbon source (▲). Concentration of benzoic acid (●) and its degradation product (■), as determined by HPLC.

3.4 Molecular characterization of strain L1

Comparative sequence analysis of the cloned 1434 bp of the 16S rRNA gene of the Dead Sea isolate L1 (Genbank accession number AY647219.1) using the Classifier algorithm from the Ribosome Database Project (http://rdp.cme.msu.edu/index.jsp) places the isolate within the unclassified Halobacteriaceae family of the Euryarchaeota with 95% confidence. Halobacteriaceae are aerobic members of the domain Archaea found in hypersaline environments and currently consist of 39 recognized genera and one unclassified grouping (Wright, 2006). Sequence similarity analysis of isolate L1 using the nucleotide Basic Local Alignment Search Tool (Blastn) against the nucleotide sequence databases available through the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov) also identified the unclassified Halobacteriaceae as having the highest similarity scores, with identities ranging from 99-96%, all of which were sequences with similar length, i.e., 100 – 92%, of that of the L1 sequence. The top scoring members of the unclassified Halobacteriaceae (99% sequence identity) consisted of a group (Genbank accession numbers JF261335.1, HQ425113.1, AJ270242.1, HQ425131.1, HQ425070.1, JN196515.1) isolated from a hypersaline salt lake in the Aran va Bidgol region of Iran. *Halobellus clavatus* strains TBN12 and TNN18, Genbank accession numbers GU951433.1 and GQ282620.1, respectively, had the next highest similarity score (95% identity over 100% of the L1 16S sequence) following those of the unclassified Halobacteriaceae matches. These strains were recently isolated from the Taibei artificial marine solar saltern in eastern China and identified as a novel genus species within the Halobacteriaceae (Cui et al., 2011).

4. Conclusions

Ten halophilic archaea from Dead Sea were isolated on the basis of their ability to grow on p-hydroxybenzoic acid (pHBA) as the sole carbon and energy source. One isolate designated L1, and identified as a member of the unclassified Halobacteriaceae family of the Euryarchaeota, when grown

on benzoic acid was shown to produce reciprocal quantities of a product identified by HPLC as gentisic acid 2,5-dihydroxybenzoic acid). The production of gentisic acid as a degradation product of benzoic acid is unusual and has recently been suggested as an intermediate in the degradation of benzoate, 3-hydroxybenzoate, cinnamate and phenylpropionate by the archaeon *Haloferax* sp. D1227.

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