

Aromatic Hydrocarbon Degradation by Halophilic Archaea Isolated from Çamaltı Saltern, Turkey

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Abstract The aims of the present study were to identify the halophilic Archaea that can degrade aromatic hydrocarbons (namely, *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene) and to determine their catabolic pathways in the process of degrading the hydrocarbons. It was determined nine archaeal isolates used *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene as sole carbon and energy sources. The isolates were identified as *Halobacterium piscisalsi*, *Halorubrum ezzemoulense*, *Halobacterium salinarium*, *Haloarcula hispanica*, *Haloferax* sp., *Halorubrum* sp., and *Haloarcula* sp. by 16S rRNA gene sequences. Activity of catechol 1,2 dioxygenase and protocatechuate 3,4 dioxygenase enzyme of the *ortho* cleavage pathway were detected. Determination of the genes of these dioxygenases was also shown. This

study clearly demonstrated for the first time that *Halorubrum* sp. and *H. ezzemoulense* among the isolates were able to grow at 20 % (w/v) NaCl, utilizing *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene as the sole carbon sources.

Keywords Aromatic hydrocarbons · Archaea · Catechol 1, 2 dioxygenase · Protocatechuate 3, 4 dioxygenase

1 Introduction

Aromatic compounds can be defined as organic molecules containing one or more aromatic rings. There are three major categories: heterocyclics, substituted aromatics, and polycyclic aromatic hydrocarbons (PAHs). PAHs are a group of chemicals that contain two or more fused aromatic rings (Seo et al. 2010). Their wide spread in the environment is of great concern since many of them have been shown to be toxic, mutagenic, and carcinogenic to all living organisms (Samanta et al. 2002; Xue and Warshawsky 2005). The US Environmental Protection Agency has classified 16 PAHs as “priority pollutants,” and increasing attention has been dedicated to control the release of such compounds (US EPA et al. 1998). One of the most effective and economical ways to remove these aromatic pollutants is microbial degradation (Alexander 1981; Parales et al. 2002; Parales and Haddock 2004). In recent years, the biodegradation of PAHs has received great attention,

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and a variety of microorganisms have been reported to play important roles in remediation process (Saxena and Thakur 2005; Tapilatu et al. 2010; Arulazhagan and Vasudevan 2011; Lease et al. 2011). PAH degradation appears to be associated with certain phylogenetic groups of bacteria, particularly *Sphingomonas*, *Burkholderia*, and *Pseudomonas* amongst Gram-negative species and *Mycobacterium* and *Rhodococcus* amongst Gram-positive species. Because high and fluctuating salinity promotes the loss of cell wall integrity, protein denaturalization, and changes in osmotic pressure, the biological treatment of industrial hypersaline wastewaters and the bioremediation of polluted hypersaline environments are not possible with conventional microorganism (Oren et al. 1992; Pieper and Reineke 2000; Perneti and Di Palma 2005). Studies demonstrated that hydrocarbon biodegradation by halophilic prokaryotes is actually possible (Buchan et al. 2000; Nicholson and Fathepure 2004; Garcia et al. 2005; Arulazhagan and Vasudevan 2009). Especially, halophilic Archaea capable of degrading hydrocarbon have been the subject of growing attention in recent years due to problems encountered by the oil industry in hypersaline waste water removal and decontamination of oil polluted salt marshes (Tapilatu et al. 2010). The first known halophilic hydrocarbon-degrading Archaea was isolated by Bertrand et al. (1990) from a salt marsh in Aigues-Mortes and was identified as a *Halobacterium*. Subsequently, Kulichevskaya et al. (1991) isolated a *Halobacterium* strain from hypersaline oil-contaminated waste water. This strain degrades *n*-C10 to *n*-C30 alkanes. Another extremely halophilic Archaea identified as *Haloferax mediterranei* strain M-11 has been shown to use oil as its sole source of carbon (Zvyagintseva et al. 1995). Garcia et al. (2005) described the characterization of a novel moderate halophile, *Halomonas organivorans*, able to degrade different aromatic compounds. The capacity of certain halophilic and halotolerant bacteria to degrade benzene and toluene has also been demonstrated (Nicholson and Fathepure 2005). Tapilatu et al.'s (2010) work clearly showed that Archaea belonging to the genera *Haloarcula* and *Haloferax* could degrade the hydrocarbons.

Biodegradation of aromatic compounds has been one of the most intensively studied aspects of microbial catabolism in both bacteria and eukaryotic microorganisms. In contrast, little is known about the metabolism of aromatic compounds by microorganisms within the domain Archaea (Borgne et al. 2008). Further studies of

the pathways and genes involved in hydrocarbon degradation and of hydrocarbon degrading extremely halophilic Archaea in various types of hypersaline environments are clearly needed (Tapilatu et al. 2010).

The aims of the present study were to identify the halophilic Archaea that can degrade aromatic hydrocarbons (namely, *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene) and to determine their catabolic pathways in the process of degrading the hydrocarbons. This work has given us the analysis of an Archaea community able to degrade aromatic compounds, and the isolation and characterization of new isolates constitutes a preliminary step toward understanding the ecology and fate of aromatic compounds in saline habitats.

2 Materials and Methods

2.1 Chemicals

p-Hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene were purchased from Sigma-Aldrich, USA (purity 99 %), and all other chemicals were purchased from Merck.

2.2 Source of the Micro-Organisms and Identification

The isolates used in the study were collected from brine samples of Çamaltı Saltern in Turkey in 2007. Isolates were maintained on sea water agar (Mutlu et al. 2008), and a total of 15 archaeal isolates were used for screening of the aromatic hydrocarbons degradation.

Only the isolates degrading PAHs were subjected to the identification tests. Extraction of genomic DNA, polymerase chain reaction (PCR)-mediated amplification of 16S rDNA and direct sequencing of the purified PCR product were performed as described in a previous study (Mutlu et al. 2008). 16S rDNA sequences (>1,350 bases) of closely related taxa of the bacterial isolates were retrieved from the NCBI database by using basic local alignment search tool. These sequences were aligned using the CLUSTAL X program (Thomson et al. 1997).

2.3 Determination of Aromatic Hydrocarbons Degradation Ability of the Isolates

A rapid procedure was used to determine the number of aromatic hydrocarbon-degrading isolates (Zhao et

al. 2009). This method is based on adsorption of aromatic hydrocarbons by cellulose acetate/nitrate filters lying on the surface of a mineral salt media (MSM) (without carbon source). Briefly, the aromatic hydrocarbons were dissolved in ethyl ether (*p*-hydroxybenzoic acid, naphthalene, phenanthrene 4 mg/ml⁻¹ and pyrene 2 mg/ml⁻¹), and then 1 ml of these solutions was added to the center of the filter disk and spread on the surface of the filter. Ethyl ether evaporated in the laminar flow. After the cultures were incubated at 37 °C with shaking at 150 rpm for 7 days at 20 % SW (sea water media), 1 ml of culture solutions was spread on the surface of the filter. These plates were examined regularly for growth and colony formation. To determine whether these colonies can utilize aromatic hydrocarbons as the sole carbon and energy source, some control plates without aromatic hydrocarbon were prepared and examined for growth. All tests were carried out in triplicate.

2.4 Growth Status of Isolates in Different Aromatic Compounds Concentrations

Growth of the strains on aromatic substrates including *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene was investigated at aromatic compound concentrations ranging from 20 to 200 ppm. Cells were grown in MSM until late exponential phase (A_{600} , 0.7) and inoculated to sterile MSM supplemented with aromatic compounds as a sole carbon and energy. The cultures were incubated at 37 °C with shaking at 150 rpm for 10–15 days, and their growths were monitored by measuring OD₆₀₀ using spectrophotometer (Fu and Oriol 1999; Arulazhagan and Vasudevan 2009). Solution without inoculated media was used as references. The growth rates were determined as: +, little growth (OD₆₀₀ 0.1–0.3); ++, medium growth (OD₆₀₀ 0.3–0.5); +++, well growth (OD₆₀₀ 0.5–0.8).

2.5 Preparation of Crude Cell-Free Extract

Cells were grown in MSM in the presence of aromatic compounds. They were harvested at the middle exponential phase by centrifugation at 6,000×*g* for 5 min. The cells were washed twice using deionized water and centrifuged after each washing under the same conditions. The cells were re-suspended in breaking buffer (50 mM Tris-HCl [pH 7.5], 1 M glycerol, 5 mM ammonium sulfate, 2.5 mM MgCl₂, 1 mM

EDTA, 1 mM DTT) and sonicated. During sonication, the cell suspension was maintained in ice. The suspensions were centrifuged at 11,000×*g* for 3 min at 4 °C. The clear supernatants obtained were used for enzyme assays (Garcia et al. 2005). Total protein kit was used to determine the protein concentration of biological extracts (Sigma, TP0100, USA).

2.6 Rothera Test

The presence of ring cleavage dioxygenase activity was identified colorimetrically in crude cell-free extracts by Rothera reactions (Stanier and Ingraham 1954; Ottow and Zolg 1974). For this reaction, 2 ml crude cell-free extracts were incubated with 2 mM catechol or protocatechuic acid as substrate. The absence of development of a yellow color is indicative of muconic acid semi-aldehyde (the extradiol cleavage product). The samples were incubated for 18 h at 28 °C. Solid ammonium sulfate (1 g), concentrated ammonium hydroxide (0.5 ml), and %1 sodium nitroprusside (5 drops) were then added. Development of a deep purple color determined visually is indicative of an *ortho* pathway intermediate β-ketoadipate.

2.7 Determination of Specific Activities of Dioxygenase Enzymes by Spectrophotometer

Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activity assays were performed spectrophotometrically using UV/Vis spectrophotometer with quartz cuvettes by measuring the rate of increase in absorbance at 260 nm ($\epsilon_{260}=16,800 \text{ M}^{-1} \text{ cm}^{-1}$) or 375 nm ($\epsilon_{375}=36,000 \text{ M}^{-1} \text{ cm}^{-1}$) due to the formation of *cis*, *cis*-muconate or 2-hydroxymuconic semialdehyde, respectively (Ngai et al. 1990; Hegeman 1966; Fetzner et al. 1989; Garcia et al. 2005). Protocatechuate 3,4-dioxygenase was measured by determining the decrease at 290 nm ($\epsilon_{290}=3,890 \text{ M}^{-1} \text{ cm}^{-1}$) due to the oxidation of protocatechuate (Stanier and Ingraham 1954). Protocatechuate 4,5-dioxygenase activity was assayed by following the formation of 2-hydroxy-γ-carboxy muconic semi-aldehyde (Stanier and Ingraham 1954; Garcia et al. 2005). 2-Hydroxy-γ-carboxy muconic semi-aldehyde was measured by monitoring the increase at 410 nm ($\epsilon_{410}=9,700 \text{ M}^{-1} \text{ cm}^{-1}$). All experiments were performed with three replications, and specific enzyme activities were reported as

micromoles product per minute per milligram protein (Garcia et al. 2005).

2.8 PCR Detection of Dioxygenase Enzyme Genes

DNA extraction of the aromatic hydrocarbon-degrading isolates was carried out by using GF-1 Bacterial DNA Extraction Kit (Vivantis). PCR amplification was performed using degenerate PCR primers. The primers for the amplification were the following: *cat1* (5'-ACCATCGARGGYCCSCTSTAY-3' and *cat3* (5'-GTTRATCTGGGTGGTSAG-3') for catechol 1,2-dioxygenase (1,2-CTD) genes, *pro3.4.2* (5'-GSCCSCTSGAGCCSAACTTC-3') and *pro3.4.4* (5'-GCCGCGSAGSACGATRTCGAA-3') for amplification of protocatechuate 3,4 dioxygenase (3,4-PCD) genes (Garcia et al. 2005).

Briefly, the composition of the PCR mixture (50 μ l) was 5 μ l of 10 \times reaction buffer (Promega), 2.5 μ l of 25 mM MgCl₂, 8 μ l of dNTPs (200 μ M each), 50 pmol of the appropriate primers, 0.25 μ l of Taq polymerase (Promega), and sterile distilled water to adjust the total volume to 50 μ l. The reaction was subjected to 35 cycles of amplification of 1,2-CTD and 3,4 PCD encoding genes (initial cycle of 5 min at 95 $^{\circ}$ C, denaturation at 94 $^{\circ}$ C for 1 min, annealing at 50 $^{\circ}$ C for 1 min); this was followed by 1 min of extension at 72 $^{\circ}$ C. Similar conditions were applied for amplification of the 2,3-dioxygenases (2,3-CTD) gene fragments, except that lower annealing temperatures were assayed (40–45 $^{\circ}$ C). The amplified products were analyzed on 1 % (w/v) agarose gels stained with ethidium bromide and photographed with UV illumination (Garcia et al. 2005).

3 Results and Discussion

Fifteen isolates were screened for aromatic hydrocarbons degradation, but only nine of them were able to grow on hydrocarbons as their source of carbon and energy. These Archaea isolates were identified as *Haloferax* sp. (isolates C-24 and C-27), *Halobacterium piscisalsi* (C-37), *Halobacterium salinarum* (C-51), *Halorubrum ezzemoulense* (C-41 and C-46), and *Halorubrum* sp. (C-43). Their similarity percentage was between 97 % and 99 %. Isolate C-50 was identified as *Haloarcula hispanica* whereas isolate C-52 was identified as *Haloarcula* sp. with a similarity of 90 % and

93 %, respectively. The phylogenetic tree (Fig. 1) was constructed by using the neighbor-joining method. This indicated that the isolates were part of the cluster within genus *Halobacterium*, *Haloferax*, *Halorubrum*, and *Haloarcula* (Tables 1 and 2). As shown in Table 2, nine archaeal isolates used *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene as sole carbon and energy sources, but isolates *H. piscisalsi* C-37 and *H. salinarum* C-51 did not use only *p*-hydroxybenzoic acid. Aerobic degradation of aromatic compounds occurs in some members of the extremely halophilic Archaea (Fairley et al. 2002). Nicholson and Fathepure (2004) demonstrated the ability of halophilic and halotolerant bacteria to rapidly degrade benzene, toluene, ethyl benzene, and xylene compounds under aerobic conditions. Bertrand et al. (1990) indicated that *Halobacterium* was known to potentially degrade *n*-alkanes and aromatic hydrocarbons. In addition, Emerson et al. (1994) demonstrated that *Haloferax* strain D1227 was able to use aromatic substrates as sole carbon and energy sources for its growth. Tapilatu et al. (2010) results particularly highlight the potential role of halophilic Archaea belonging to the genera *Haloarcula* and *Haloferax* for aerobic bioremediation of oil contaminated hyper-saline environments. Halophilic Archaea belonging to the genera *Haloarcula*, *Halobacterium*, and *Haloferax* are adapted to high concentrations of halogenated hydrocarbons, such as trichlorophenols, or the insecticides lindane and DDT (Oesterhelt et al. 1998).

Our isolates (except *H. piscisalsi* C-37 and *H. salinarum* C-51) were able to grow in *p*-hydroxybenzoic

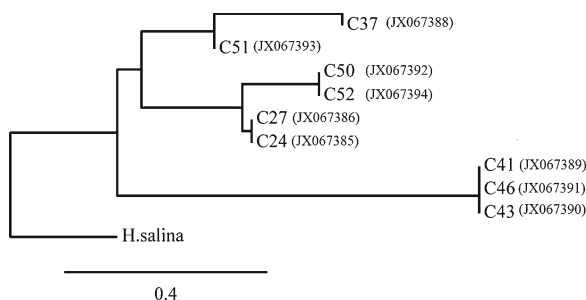


Fig. 1 16 S rRNA gene sequence-based dendrogram showing the phylogenetic position of the aromatic hydrocarbons degrading archaeal isolates. The tree was constructed using the neighbor-joining method (Saitou and Nei 1987) and rooted using *Halomonas salina* (a member of Bacteria) as out-group. GenBank accession numbers are given beside the strains. The scale bar represents one nucleotide substitution per 4,000 sequence positions

Table 1 Identification of 16S rRNA genes of aromatic hydrocarbon-degrading Archaea isolated from Çamaltı Saltern

Isolate no.	Closest gene bank match
C-24	97 % <i>Haloferax</i> sp. BankIt1537931 C24 JX067385
C-27	99 % <i>Haloferax</i> sp. BankIt1537931 C27 JX067386
C-37	98 % <i>Halobacterium piscicarsi</i> BankIt1537931 C37 JX067388
C-41	99 % <i>H. ezzemoulense</i> BankIt1537931 C41 JX067389
C-43	98 % <i>Halorubrum</i> sp. BankIt1537931 C43 JX067390
C-46	99 % <i>H. ezzemoulense</i> BankIt1537931 C46 JX067391
C-50	90 % <i>H. hispanica</i> BankIt1537931 C50 JX067392
C-51	98% <i>H. salinarium</i> BankIt1537931 C51 JX067393
C-52	93 % <i>Haloarcula</i> sp. BankIt1537931 C52 JX067394

acid. It was noted that some isolates did not grow at 200 ppm concentration of *p*-hydroxybenzoic acid. All isolates were able to grow at every concentration of two-ringed naphthalene. Similarly, isolates were able to grow in phenanthrene-containing (tree-ringed) media, but some of them did not grow at the concentration of 200 ppm like what occurred in *p*-hydroxybenzoic acid (mono-aromatic hydrocarbon). None of the isolates were able to grow at 200 ppm pyrene (four-ringed) concentration but were able to grow at lower

concentrations. The optimal aromatic hydrocarbons concentrations for isolate growth were observed to be as 80 and 120 ppm.

Under aerobic conditions, degradation pathways of a variety of aromatic hydrocarbons converge at either protocatechuate or catechol. In the *meta* cleavage pathway, the ring fission occurs adjacent to one of the hydroxyls (extradiol cleavage), and the main enzymes involved in this fission are the catechol 2,3-CTD and protocatechuate 4,5-dioxygenases (van der Meer et al. 1992). Ring-cleavage dioxygenases constitute central enzymes in the bacterial recycling of aromatic compounds. In the β -ketoadipate pathway, catechol and protocatechuate are cleaved between their two hydroxyl groups by 1,2-CTD or protocatechuate 3,4-PCD. Catechol 2,3-dioxygenases constitute a group of enzymes that are considered crucial for degradation of a wide range of aromatic compounds in contaminated habitats (Moharikar et al. 2003). These pathways have been identified in the main aromatic compound degrader bacteria, including species of the genera *Acinetobacter*, *Alcaligenes*, *Bacillus*, and *Pseudomonas* (van der Meer et al. 1992; Harwood and Parales 1996).

Aerobic halophilic bacteria degrade phenol mainly by the *meta* and *ortho* cleavage pathways. Some bacteria degrade phenol using one of these two pathways, while others may degrade phenol using both pathways (Jiang et al. 2006; Borgne et al. 2008). The

Table 2 Growth status of isolates in different aromatic hydrocarbon concentrations in MSM broth

Isolates	PAH concentration (ppm)																							
	<i>p</i> -Hydroxy-benzoic acid						Naphthalene						Phenanthrene						Pyrene					
	20	40	80	120	160	200	20	40	80	120	160	200	20	40	80	120	160	200	20	40	80	120	160	200
C-24	+	++	+++	+++	++	-	+	++	+++	+++	++	+	+	++	+++	+++	++	-	+	++	+++	+++	+	-
C-27	+	++	+++	+++	+	-	+	++	+++	+++	++	+	+	++	+++	+++	++	-	+	++	+++	+++	+	-
C-37	-	-	-	-	-	-	+	++	+++	+++	++	+	+	++	+++	+++	+	-	+	++	+++	+++	+	-
C-41	+	++	+++	+++	++	+	+	++	+++	+++	+++	++	+	++	+++	+++	++	+	+	+++	+++	+++	++	-
C-43	+	++	+++	+++	++	+	+	++	+++	+++	+++	++	+	++	+++	+++	++	+	+	+++	+++	+++	++	-
C-46	+	++	+++	+++	++	+	+	++	+++	+++	+++	++	+	++	+++	+++	++	+	+	+++	+++	+++	++	-
C-50	+	+	+++	+++	++	+	+	++	+++	+++	+++	+	+	++	+++	+++	++	+	+	++	+++	+++	+	-
C-51	-	-	-	-	-	-	+	++	+++	+++	+++	+	+	++	+++	+++	++	-	+	++	+++	+++	+	-
C-52	+	+	+++	+++	+++	+	+	++	+++	+++	+++	+	+	+++	+++	+++	++	+	+	+++	+++	+++	++	-

Plus signs positive reaction, minus signs negative reaction: + little growth (OD₆₀₀ 0.1–0.3), ++ medium growth (OD₆₀₀ 0.3–0.5), +++ well growth (OD₆₀₀ 0.5–0.8)

accumulation of catechol and *cis, cis*-muconic acid has been observed during the growth of several *Halomonas* strains on phenol, indicating that these bacteria used the *ortho* pathway (Maskow and Kleinstauber 2004; Borgne et al. 2008). Another study indicated that catechol 1,2 and protocatechuate 3,4-dioxygenase activities were detected in *H. organivorans* (Garcia et al. 2005). According to the measured enzymatic activities, *H. organivorans* preferentially used the catechol *ortho* pathway for degrading the benzoic, cinnamic, salicylic, phenylpropionic, and *p*-aminosalicylic acids and the protocatechuate pathway for degrading the *p*-hydroxybenzoic, *p*-coumaric, and ferulic acids (Garcia et al. 2005).

The most straightforward approach to characterize degradation pathways is to analyze the catabolic enzymes (Cao et al. 2008). Therefore, after determining the presence of the enzymes by using Rothera test, their activities were measured by spectrophotometer. Rothera test results showed that our isolates could degrade the aromatic hydrocarbons with either protocatechuate or catechol by the *ortho* cleavage of the β -keto adipate pathway because there was no development of a yellow *meta* cleavage product.

Activity of catechol 1,2 dioxygenase enzyme of the *ortho* cleavage pathway could be detected in isolates [except C-52 (*Haloarcula sp.*)] grown on *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene. Activity of protocatechuate 3,4 dioxygenase enzyme of the *ortho* cleavage pathway could be detected in cells of *Haloarcula sp.* C-52 grown on *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene. The enzymes of the modified *meta* cleavage pathway were never present, while the enzymes of the *ortho* cleavage pathway were detected in all cultures. This indicated that are *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene converted via the *ortho* cleavage pathway (Table 3). Spectrophotometric enzyme assays confirmed the results of the Rothera tests. Similarly to our findings, Song (2009) reported that all isolates from pine litter were able to grow in the presence of aromatic hydrocarbons, degraded protocatechuate, or catechol by the *ortho* cleavage of the β keto adipate pathway.

Ortho cleavage pathway is frequently chromosomally encoded and is widely distributed in soil bacteria and fungi, constituting the major pathway for aromatic compounds catabolism in these organisms. The *cat* genes are usually organized in a single cluster (Harwood and Parales 1996). From genome analysis

Table 3 Specific enzyme activity of crude cell-free extracts of different isolates

Isolates	Specific enzyme activities ($\mu\text{mol product min}^{-1}\text{mg protein}^{-1}$)															
	<i>p</i> -Hydroxy-benzoic acid			Naphthalene			Phenanthrene			Pyrene						
	1,2 CTD	2,3CTD	3,4PCD	4,5CD	1,2CTD	2,3CTD	3,4PCD	4,5PCD	1,2CTD	2,3CTD	3,4PCD	4,5PCD	1,2CTD	2,3CTD	3,4PCD	4,5PCD
C-24	7.64±1.72	ND	21.85±3.04	ND	6.53±0.51	ND	ND	ND	8.43±1.22	ND	ND	ND	9.28±1.32	ND	ND	ND
C-27	10.84±2.04	ND	34.40±2.95	ND	10.10±1.65	ND	ND	ND	3.52±0.31	ND	ND	ND	8.61±0.97	ND	ND	ND
C-37	–	–	–	–	4.55±0.88	ND	ND	ND	6.36±0.87	ND	ND	ND	4.46±0.12	ND	ND	ND
C-41	9.34±3.00	ND	ND	ND	10.03±2.75	ND	ND	ND	9.77±2.12	ND	ND	ND	11.28±1.74	ND	ND	ND
C-43	2.54±0.98	ND	ND	ND	10.47±3.15	ND	ND	ND	5.53±0.36	ND	ND	ND	12.21±2.01	ND	ND	ND
C-46	2.38±0.48	ND	ND	ND	9.72±1.84	ND	ND	ND	4.91±0.99	ND	ND	ND	7.19±1.45	ND	ND	ND
C-50	9.32±1.75	ND	ND	ND	11.42±2.42	ND	ND	ND	4.24±1.02	ND	ND	ND	7.39±1.32	ND	ND	ND
C-51	–	–	–	–	8.20±1.24	ND	ND	ND	6.36±1.24	ND	ND	ND	5.83±0.99	ND	ND	ND
C-52	ND	ND	13.21±2.51	ND	ND	ND	14.67±1.18	ND	ND	ND	20.00±2.18	ND	ND	ND	15.21±2.58	ND

ND not determined, 1,2 CTD catechol 1,2 dioxygenase, 2,3 CTD catechol 2,3 dioxygenase, 3,4 PCD protocatechuate 3,4 dioxygenase, 4,5 CD protocatechuate 4,5 dioxygenase

for *Pseudomonas putida* KT2440, it has been predicted that at least four main pathways encoded by the chromosomal DNA in *P. putida* for the catabolism of aromatic compounds could exist (Jimenez et al. 2002). For some strains, the presence of plasmids makes this species more catabolically versatile in the biodegradation of aromatic compounds. The use of one pathway or the other is dependent on the microbial species and the nature of the growth substrate. When benzoate is used as the sole growth substrate, different biodegradation pathways have been found for different species or different strains (Cao et al. 2008).

Garcia et al. (2005) designed some conserved sequence regions, which were used to design the degenerate PCR primers cat1 and cat3, and pro3.4.2 and pro3.4.4. The primers for the amplification were the following: cat1 and cat3 for 1,2-CTD genes, designed from two conserved regions of different catA proteins, spanning residues 100–106 and 232–237 in IsoB from *Acinetobacter radioresistens* (accession no. AAG16896); pro3.4. and pro3.4.4 are designed from conserved regions of PcaH, coding for the b-subunit of the enzyme (residues 118–124 and 224–230 of PcaH from *Pseudomonas aeruginosa* PA01, accession no. NP_248843). These primers allowed us to successfully amplify specific regions of the expected sizes from the DNA of different archaeal isolates (Figs. 2 and 3). 1,2-CTD-encoding genes yielded a PCR amplification product of the expected size approximately 414 bp. This fragment of the genes coding for 1,2-CTD were positive for eight isolates (except C-52) (Table 4). Primers designed for amplification of 3,4-PCD-encoding genes yielded a PCR amplification product of the expected size approximately 330 bp. Four isolates (C-24, C-27,

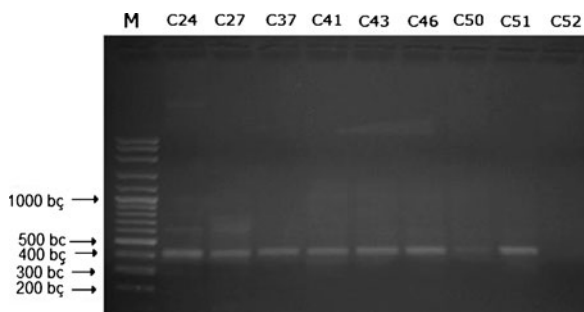


Fig. 2 PCR detection of the catechol 1,2-dioxygenase (1,2-CTD)-encoding genes. Numbers on the different lanes indicate the pollutant-degrading strain. DNA standard ladder (1 kb) was loaded in the first lane

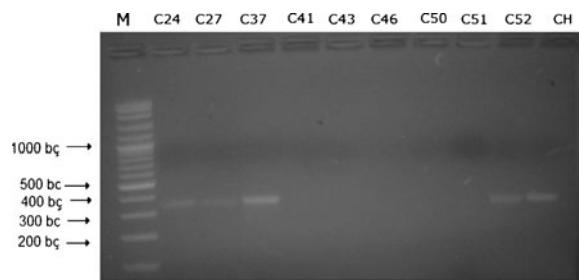


Fig. 3 PCR detection of the protocatechuate 3,4-dioxygenase (3,4-PCD)-encoding genes. Numbers on the different lanes indicate the pollutant-degrading strain. DNA standard ladder (1 kb) was loaded in the first lane

C-37, C-52) were positive for amplification of a 330-bp internal fragment of the genes coding for 3,4-PCD (Table 4). This would suggest that isolates degraded aromatic hydrocarbons with protocatechuate or catechol by the *ortho* cleavage of the β -keto adipate pathway. Determination of dioxygenase enzyme genes confirmed the results of the spectrophotometric enzyme assays and Rothera test results.

The present work clearly demonstrated for the first time that Archaea *Halorubrum sp.*, *H. ezzemoulense* are able to grow at 20 % (w/v) NaCl, utilizing *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene as the sole carbon sources. The results showed that isolates have genes regions of encoding catechol 1,2-dioxygenase and/or protocatechuate 3,4-dioxygenase. The comparison of sequences of archaeal

Table 4 The results of PCR amplification of catabolic genes of isolates

Isolates	1,2-CTD primers ^a Gene region (414 bp)	3,4-PCD primers ^b Gene region (330 bp)
C-24	+	+
C-27	+	+
C-37	+	+
C-41	+	–
C-43	+	–
C-46	+	–
C-50	+	–
C-51	+	–
C-52	–	+

^a 1,2-CTD primers (cat1 and cat3 primers)

^b 3,4-PCD primers (pro 3.4.2 and pro 3.4.4 primers)

halophilic dioxygenases with non-halophilic counterpart will help us in determining the necessary conditions for the halophilic enzymes to be stable at high salt concentrations.

4 Conclusions

This study showed for the first time that *Halorubrum* sp., *H. ezzemoulense*, are able to grow at salty conditions containing aromatic hydrocarbons. None of our isolates had specific enzyme activities of *meta* pathways. The results indicated that the isolates studied have gene regions of encoding catechol 1,2 dioxygenase and/or protocatechuate 3,4 dioxygenase. It has been suggested that PAH-degrading enzymes from *Archaea* (*Haloferax* sp., *Halorubrum* sp., *H. piscisalsi*) may be exploited to remove aromatic hydrocarbons from the polluted environments safely.

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