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Aromatic Hydrocarbon Utilization Ability of *Chromohalobacter* sp.

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Abstract
The aim of this study is to reveal the ability of utilizing different aromatic hydrocarbons (*p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene) by a halotolerant bacterial strain, *Chromohalobacter* sp., under saline conditions. The aromatic hydrocarbon degradation pathways were identified. PCR amplification was carried to define the gene zones which codify the dioxygenases of the isolates. The possible gene zones of catechol 1, 2 dioxygenase and protocatechuate 3, and 4 dioxygenase were determined. According to the PCR amplification and enzyme test results *Chromohalobacter* sp. utilizes aromatic hydrocarbons by the *ortho* cleavage of the *ß*-ketoadipate pathway. In this study, it was concluded that this isolate can be used in bioremediation studies of saline environments contaminated with aromatic hydrocarbons.
Keywords: *Chromohalobacter* sp., catechol 1, 2 dioxygenase, protocatechuate 3,4 dioxygenase.

***Chromohalobacter* sp.'nin Aromatik Hidrokarbon Kullanabilme Yeteneği**
Özet
Bu çalışmada, tuzlu koşullar altında yetiştirilen *Chromohalobacter* sp. izolatının, farklı aromatik hidrokarbonlardan (*p*-hidroksibenzoik asit, naftalen, fenatren, piren) faydalanabilme yeteneğinin ortaya konulması hedeflenmiştir. Aromatik hidrokarbonları parçalayabilme yolları belirlenmiştir. İzolatın kodladığı dioksijenaz gen bölgelerinin belirlenmesi için PCR amplifikasyonu yapılmıştır. Katekol 1,2 dioksijenaz ve protokatekol 3,4 dioksijenaz enzimlerini kodlayan muhtemel gen bölgeleri belirlenmiştir. PCR amplifikasyonu ve enzim testleri sonuçlarına göre *Chromohalobacter* sp. izolatı aromatik hidrokarbonlardan ß-ketoadipate yolunun *orto*- parçalamasını kullanarak yararlanmıştır. Bu çalışmada izolatın aromatik hidrokarbonlar ile kontamine olan tuzlu ortamların biyoremediasyon çalışmalarında kullanılabilir olduğu sonucuna varılmıştır.
Anahtar Kelimeler: *Chromohalobacter* sp., katekol 1,2 dioksijenaz, protokatekol 3,4 dioksijenaz

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INTRODUCTION

Saline environments have expanded as a result of irrigation and other uses of water. Industrial processes use salts and often release brine effluent into the environment as well as many natural geological formations that are related with hypersaline brines (Sarma and Arora 2001). Various industries like petroleum refineries generate a huge amount of oily and saline residual waters with salinities after the separation of crude oil from reservoir water (Parıltı 2010). The basic pollutants in these production waters are aromatic hydrocarbons (Salmanov et al. 2008, Moreno et al. 2011). Low molecular weight polyaromatic hydrocarbons (PAHs) such as naphthalene and phenanthrene are toxic, and high molecular weight PAHs such as pyrene and benzo[a] pyrene are carcinogenic and mutagenic (Boonchan et al. 2000). In addition, the structure of the hydrophobic polycyclic aromatic hydrocarbons, which makes them extremely difficult to clean up, allows them to remain intact for longer periods. For the restoration of PAHs contaminated soil and groundwater, bioremediation has become an accepted technology (Vogel 1996). Halophilic microorganisms may be useful for the bioremediation of contaminated

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hypersaline brine (Sarma and Arora 2001). Extremophiles are potential tools in the restoration of these contaminated environments because they have adapted to grow in such hostile environments (Moreno et al. 2011).

Moderately halophilic bacteria are widely distributed and are suspected to play an important role in the ecology of hypersaline habitats (Oren 2002, Vargas and Nieto 2004). Although mineralization of aromatic compounds and the catabolic pathways have been described in various bacteria, (Rodrigues et al. 2008, Erdoğmuş et al. 2013) this information is lacking for halophilic bacteria.

The cleavage of the aromatic ring is catalyzed by dioxygenases. This occurs through the *ortho* or *meta* cleavage pathway converting the produced metabolites into intermediates of the citrate cycle.

There is a characterization of genes encoding benzoate and *p*-hydroxybenzoate degradation in *Chromohalobacter* sp. (Kim et al. 2008) but no reports at the molecular level are available on the characterization of aromatic hydrocarbon degrading genes.

Members of *Chromohalobacter* are moderately halophilic bacteria, a group of extremophilic prokaryotes that optimally grow in 8-10% NaCl, and may grow in salt concentrations up to 30%. The broader range of temperature and pH observed for growth are 5-45°C and a pH of 5.0-10.0, respectively. In the present work, we focused on the characterization of the genes involved in the utilization of different aromatic hydrocarbons (naphthalene, phenanthrene, *p*-hydroxybenzoic acid, and pyrene) in *Chromohalobacter* sp, a halophilic bacterium, because, of the importance of the halophiles in the development and improvements in new biological strategies for the reconstruction of saline environments and waste waters.

MATERIALS AND METHODS

Chemicals

Aromatic hydrocarbons (naphthalene, phenanthrene, *p*-hydroxybenzoic acid, and pyrene) were purchased from Sigma-Aldrich and the other chemicals were purchased from Merck.

Test Microorganism

Chromohalobacter sp. was a kind gift from Dr. Josefa Anton University of Alicante, Spain. The bacterium is routinely grown in Sea Water (SW) with 20% salts (Rodriguez et al. 1985).

Determining the Aromatic Hydrocarbons Degradation Ability of *Chromohalobacter* sp.

A rapid procedure of Zhao et al. (2009) was used for determining the number of aromatic hydrocarbons degrading isolates (Zhao et al. 2009). This method is based on the adsorption of aromatic hydrocarbons using filters lying on the surface of the Mineral Salt Media (MSM) (without a carbon source). Briefly, the aromatic hydrocarbons are dissolved in ethyl ether (pyrene 2mg/mL^{-1} and *p*-hydroxybenzoic acid, naphthalene, and phenanthrene 4mg/mL^{-1}) and then 1 mL of these solutions are spread on the surface of the filter. After the *Chromohalobacter* sp. was incubated at 37°C with shaking at 150 rpm for 7 days in a 20% Sea Water Media (SW), 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, control plates without aromatic hydrocarbon were prepared and examined for growth. All tests were carried out in triplicate.

Growth status of isolate in different aromatic hydrocarbons concentrations in MSM broth

Growth of *Chromohalobacter* sp. on aromatic substrates was examined with aromatic compound concentrations ranging from 20 to 200 ppm. Each flask was inoculated with a 2 mL culture grown in an MSM medium to give an initial OD_{600} nm of approximately 0.7. The culture was incubated at 37°C with shaking at 150 rpm for 10-15 days, and growth was monitored by measuring the OD_{600} using a spectrophotometer (Fu and Oriel 1999, Arulazhagan and Vasudevan 2009). The growth rates were determined as: +; small growth (OD_{600} 0.1-0.3), ++; medium growth (OD_{600} 0.3-0.5), and +++; large growth (OD_{600} 0.5-0.8).

Preparation of Crude Cell Free Extract

The 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 and washed twice with deionized water to remove any salts that might interfere with the enzymatic activity. The cells were then resuspended in a breaking buffer (50 mM Tris HCl (pH 7.5), 1 M glycerol, 5 mM ammonium sulphate, 2.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT) and sonicated. During sonication, the cell

suspension was maintained in ice. The suspension was centrifuged at 11,000g for 3 min at 4°C. The clear supernatants obtained were used for the enzyme assays (Garcia et al. 2005). A total protein kit was used to determine the protein concentration of the biological extracts (Sigma, TP0100, USA).

Rothera Test

In determining the mode of the ring cleavage of the protocatechuate or catechol, a Rothera test was performed. For this reaction, the method of Ottow and Zolg (1974) was followed, where 2 mL of crude cell free extracts were incubated with 2 mM catechol or protocatechuic acid as the substrate. In the absence of the development of a yellow color, indicative of muconic acid semialdehyde (the extradiol cleavage product), the samples were incubated for 18 h at 28°C; solid ammonium sulphate (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 (Stanier and Ingraham 1954, Ottow and Zolg 1974).

Determination of the Activities of Dioxygenases

Protein concentrations were measured using the method of Bradford (Bradford 1976). Specific enzyme activities are reported as μmol product min-1mg protein. A 4,5 PCD (protocatechuate 4,5-dioxygenase) activity was measured by monitoring the increase in the A_{410} (ε_{410}=9,700 $M^{-1}cm^{-1}$) formation of 2-hydroxy-γ-carboxy muconic semi aldehyde (Stanier and Ingraham 1954). The 3,4 PCD (protocatechuate 3,4-dioxygenase) activity was measured by monitoring the decrease in the A_{290} (ε_{290}= 3,890 $M^{-1}cm^{-1}$) due to the oxidation of the protocatechuate (Stanier and Ingraham 1954). The 2,3 CTD (catechol 2,3-dioxygenase) activity was assayed spectrophotometrically by measuring the increase of the A_{375} (ε_{375}= 36,000 $M^{-1}cm^{-1}$) due to the formation of 2-hydroxy-muconic semi aldehyde (Fetzner et al. 1989, Hegeman 1966). The 1,2 CTD (catechol 1,2-dioxygenase) was determined by following the formation of cis-cis muconic acid at 260 nm (ε260= 16,800 $M^{-1}cm^{-1}$) (Ngai et al. 1990). The activity assays were all triplicated.

PCR Detection of Dioxygenase Enzyme Genes

The DNA extraction was carried out using a GF-1 Bacterial DNA Extraction Kit (Vivantis). The PCR detection of the dioxygenase enzyme genes was to detect the presence of two catabolic genes encoding key enzymes of the metabolic pathways, and the PCR amplification was performed using degenerate PCR primers (Table 1) (Garcia et al. 2005).

The reaction mixture (50 μL) consisted of 5 μl of 10x reaction buffer, 2.5 μl of 25 mM $MgCl_2$, 8 μl of dNTPs (200 μM each), 0.25 μl of Taq DNA polymerase, 50 pmol of the appropriate primers, and sterile distilled water.

The following program was conducted in a thermo cycler for the amplification of 1,2-CTD and 3,4 PCD encoding genes, at an initial cycle of 5 min at 95°C, denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and final extension at 72°C for 1 min. The PCR conditions for the amplification of the 1,2-CTD and 3,4 PCD encoding genes consisted of an initial cycle of 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. Similar conditions were applied for the amplification of the 2,3-CTD gene fragments, except that lower annealing temperatures were assayed (40-45°C). The amplified products were detected by electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide (Garcia et al. 2005). Then amplicons were purified with a Vivantis GF-GP-050 DNA purification kit and both strands were sequenced by using a Beckman Coulter Model: CEQ 8000 XL DNA Analyse System. The DNA sequences results of the isolate were analyzed using the BLAST program of the National Centre for Biotechnology Information (NCBI).

RESULTS

Chromohalobacter sp. was screened for the degradation of different aromatic hydrocarbons and it was determined that *Chromohalobacter* sp. used *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene as a sole carbon and energy source (Table 2). The growth of *Chromohalobacter* sp was observed in MSM broth containing different concentrations of aromatic hydrocarbons (20, 40, 80, 120, 160, and 200 ppm). The *Chromohalobacter* sp. grew well at aromatic hydrocarbons concentration of 80 and 120 ppm (Table 3). The Rothera test results showed that, *Chromohalobacter* sp. degraded aromatic hydrocarbons with 1,2 CTD by the ortho cleavage of the β-ketoadipate pathway.

Specific enzyme activities of the isolate are

Table 1. PCR primers for amplification of gene zones which codify dioxygenase enzymes of *Chromohalobacter* sp.

Gene regions	Primers	
1,2 CTD (414 bp)	cat1(5'–ACCATCGARGGYCCSCTSTAY-3')	cat3(5'-GTTRATCTGGGTGGTSAG-3')
2,3 CTD (400 bp)	cat 2.3.1 (5'-GARCTSTAYGCSGAYAAGGAR-3')	cat2.3.2 (5'-RCCGCTSGGRTCGAAGAARTA-3')
3,4 PCD (330 bp)	pro 3.4.2 (5'-GCSCCSCTSGAGCCSAACTTC-3')	pro 3.4.4 (5'-GCCGCGSAGSACGATRTCGAA-3')

Table 2. Growth status of *Chromohalobacter* sp. in different concentrations of aromatic hydrocarbons in mineral salt agarose solidified media.

Isolate	Aromatic hydrocarbons			
	p-hydroxybenzoic acid	naphthalene	phenanthren	pyrene
Chromohalobacter sp.	+	+	+	+

+(positive reaction), -(negative reaction)

Table 3. Growth status of *Chromohalobacter* sp. in different aromatic hydrocarbons concentrations in an MSM broth

PAHs	Aromatic hydrocarbons concentration (ppm)					
	20	40	80	120	160	200
p-hydroxybenzoic acid	+	++	+++	+++	+++	+
naphthalene	+	+++	+++	+++	+++	++
phenanthrene	+	+++	+++	+++	++	+
pyrene	+	+++	+++	+++	+	-

+(positive reaction), -(negative reaction) + small growth (OD_{600} 0.1-0.3), ++medium growth (OD_{600} 0.3-0.5), and +++ large growth (OD_{600} 0.5-0.8)

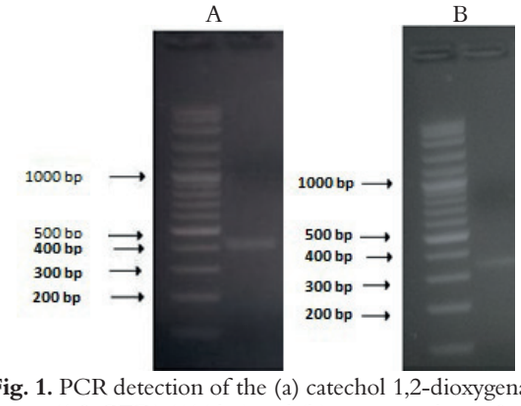

Fig. 1. PCR detection of the (a) catechol 1,2-dioxygenase (1,2-CTD) (414 bp) and (b) protocatechuate 3,4-dioxygenase (3,4-PCD) (330 bp) encoding genes. DNA standard ladder (1 kb) was loaded in the first lane and the second line was loaded PCR amplicons of *Chromohalobacter* sp.

shown in Table 4. When *Chromohalobacter* sp. was grown with p-hiydroxybenzoic acid, naphthalene, phenanthrene, and pyrene the crude extracts showed 1,2 CTD activities. In contrast, no activity towards 2,3 CTD, 3,4 PCD, and 4,5 PCD were obtained.

Garcia et al. (2005) designed some conserved sequence regions, which were used to design the degenerate PCR primers. These primers allowed us to successfully amplify specific regions of the expected sizes from the DNA of *Chromohalobacter* sp. (Figure 1). The 1,2 CTD encoding genes yielded a PCR amplification product of the expected size, approx 414 bp. This fragment of the genes coding for 1,2 CTD were positive. Primers designed for amplification of 3,4 PCD encoding genes yielded a PCR amplification product of the expected size, approx. 330 bp. This would suggest *Chromohalobacter* sp. degraded aromatic hydrocarbons with protocatechuate or catechol by the ortho-cleavage of the β-ketoadipate pathway. The determination of dioxygenase enzyme genes confirmed the results of the spectrophotometric enzyme assays and Rothera test results.

The results of the nucleotide sequence analysis are shown in Table 5. The evaluation of the sequence analysis of encoding the 1,2 CTD gene for an isolate had high similarities with the partial *cat*A gene of Halomonas organivorans (80%). The sequence analysis of encoding the 3,4 PCD gene of the isolate had a similarity to *Chromohalobacter* sp. HS2 (89%). These results showed that the isolate have gene regions of encoding 1,2 CTD and 3,4 PCD.

DISCUSSION

During the past decades, much research has been carried out on the degradation metabolism of aromatic hydrocarbons in non-halophilic bacteria. However, little information is available about the catabolism of aromatic compounds in halophilic bacteria (Garcia et al. 2005, Arulazhagan and Vasudevan 2011). Among the halophilic microorganisms, moderate halophiles constitute the most resourceful group to be used in the microbiological treatment processes (Ventosa et al. 1998). Lazaroaie reported that (2008) *C. salexigens* was able to tolerate aromatic hydrocarbons and this isolate was able to use as single source of carbon saturated or

polyaromatic hydrocarbons (naphthalene, anthracene, and phenanthrene) and monoaromatic (toluene, xylene isomers) hydrocarbons. From the present study, it may be concluded that the moderately halophilic bacterium *Chromohalobacter* sp. potentially degraded different concentrations of low and high molecular weight aromatic hydrocarbons under saline conditions with catechol by the ortho cleavage of the β-ketoadipate pathway. To our knowledge, this is the first report of moderately halophilic bacterium, *Chromohalobacter* sp., utilizing naphthalene, phenanthrene, and pyrene as the sole carbon source.

Nicholson and Fathepure (2004) have reported that benzene, toluene, ethyl benzene, and xylene compounds can be degraded by halophilic bacteria in hypersaline environments. Similar to our results, Borgne et al. (2008) detected 1,2 CTD and 3,4 PCD activities in *Halomonas organivorans* but not 2,3 CTD activity. The result of the measured enzymatic activities, *Halomonas organivorans* used the catechol *ortho* pathway for degrading the benzoic, cinnamic, salicylic, phenylpropionic and *p*-amino salicylic acid and, the protocatechuate pathway for degrading *p*-hydroxybenzoic, *p*-coumaric and ferulic acid. In addition Arulazhagan and Vasudevan (2011) reported that the halophilic isolate *Ochrobactrum* sp. strain was able to degrade different aromatic hydrocarbons such as naphthalene, fluorene, anthracene, pyrene, fluoranthene, phenanthrene, and pyrene. Similarly, Dastgheib et al. (2012) reported that a halophilic consortium, obtained from a saline soil sample, utilize phenanthrene in a wide range of NaCl concentrations. This consortium contains one culturable Halomonas strain and one unculturable strain belonging to the genus Marinobacter.

Garcia et al. (2005) also reported in their other study that the presence of the catechol branch (cat) and the protocatechuate branch (pca) of the β-ketoadipate pathway has been described in *Halomonas organivorans*. Nicholson and Fathepure (2004) demonstrated the ability of halophilic and halotolerant bacteria rapidly degrade benzene, toluene, ethyl benzene, and xylene compounds under aerobic conditions.

Kim et al. (2008) studied the *Chromohalobacter* sp. strain for the ability to grow on benzoate and *p*-hydroxybenzoate as the sole carbon and energy source and reverse transcriptase PCR experiments show that benzoate induces the expression of benzoate 1,2-dioxygenase, 1,2 CTD, and 3,4 PCD while *p*-hydroxybenzoate only induced the expression of *p*-hydroxybenzoate hydroxylase.

Table 4. Specific enzyme activities in the cell extracts of *Chromohalobacter* sp.

Aromatic hydrocarbons	Specific enzyme activities (µmol product min^{-1}mg protein)			
	1,2CTD	2,3CTD	3,4PCD	4,5CD
p-hydroxybenzoic acid	7,59±1,02	ND	ND	ND
naphthalene	12,15±2,03	ND	ND	ND
phenanthrene	6,97±1,12	ND	ND	ND
pyrene	0,47±0,10	ND	ND	ND

(ND) Not determined. Specific enzyme activities (µmol product min^{-1}mg protein)

Table 5. After sequencing the closest genes (1,2 CTD-3,4 PCD) representatives of the *Chromohalobacter* sp.

Isolate	Description	Sequence length	The closest genes response in the Gene bank (%) (Accession)
Chromohalobacter sp.	*Halomonas organivorans* partial catA gene for catechol 1,2-dioxygenase	322	80 % (FN997643.1)
Chromohalobacter sp.	Chromohalobacter sp. HS2 protocatechuate 3,4-dioxygenase beta subunit (pcaH),	378	89% (EU155151.1)

Our study showed that the enzymes of the *meta* cleavage pathway were never present, while the enzymes of the *ortho*-cleavage pathway were detected. This indicated that are *p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene converted via the *ortho*-cleavage pathway. The evaluation of the sequence analysis of encoding the 1,2 CTD gene for the isolate had high similarities with the partial *cat*A gene of *Halomonas organivorans* (80%). The sequence analysis of encoding the 3,4 PCD gene of the isolate had a similarity with *Chromohalobacter* sp. HS2.

As a brief result, we can suggest that the aromatic hydrocarbons degrading enzymes from *Chromohalobacter* sp. may be exploited to remove aromatic hydrocarbons from polluted environments safely. This strain could also be used for the development of new biological strategies and the restoration of hypersaline environments.

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