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ORIGINAL PAPER

Isolation of hydrocarbon-degrading extremely halophilic archaea from an uncontaminated hypersaline pond (Camargue, France)

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Abstract Little information exists about the ability of halophilic archaea present in hypersaline environments to degrade hydrocarbons. In order to identify the potential actors of hydrocarbon degradation in these environments, enrichment cultures were prepared using samples collected from a shallow crystallizer pond with no known contamination history in Camargue, France, with *n*-alkanes provided as source of carbon and energy. Five alkane-degrading halophilic archaeal strains were isolated: one (strain MSNC 2) was closely related to *Haloarcula* and three (strains MSNC 4, MSNC 14, and MSNC 16) to *Haloferax*. Biodegradation assays showed that depending on the strain, 32 to 95% (0.5 g/l) of heptadecane was degraded after 30 days of incubation at 40°C in 225 g/l NaCl artificial medium. One of the strains (MSNC 14) was also able to degrade phenanthrene. This work clearly shows for the first time the potential role of halophilic archaea belonging to the genera *Haloarcula* and *Haloferax* in the degradation of hydrocarbons in both pristine and hydrocarbon-contaminated hypersaline environments.

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Introduction

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. About 5% of total world waste water effluents are highly saline (Lefebvre 2005) and are generated by pesticide, chemical, pharmaceutical, and oil industries (Lefebvre and Moletta 2006). In the petroleum industry, the most abundant extraneous material in the crude oil extraction process is water (Speight 1998). Approximately ten barrels of brackish or saline water are generated for every barrel of oil produced, with salt concentrations ranging between 1 and 250 g/l (Cuadros-Orellana et al. 2006). Many wells, especially during their declining years, produce vast quantities of highly saline water, and disposal of this is both a serious and an expensive problem (Speight 1998). In addition, the Gulf war oil spill in 1991 reminded the public and the scientific community of the still largely unsolved problem of hydrocarbon degradation under saline conditions (Patzelt 2005).

The study of halophilic hydrocarbon-degrading procaryotes is based primarily on the need to find remediation measures to solve the aforementioned problems (Nicholson and Fathepure 2004; Le Borgne et al. 2008). Additional objectives are to improve the use of hypersaline-adapted enzymes in biotechnological processes (Marhuenda-Egea and Bonete 2002; Oren 2002) and to deepen our knowledge of hydrocarbonoclastic microorganisms.

Using enrichment cultures from the Great Salt Lake, Ward and Brock (1978) observed a decreasing biodegradation of

hexadecane with increasing salinity; these cultures were not able to grow on mineral oil or to mineralize hexadecane in the presence of salt concentrations above 200 g/l NaCl. However, studies carried out in later years demonstrated that hydrocarbon biodegradation by halophilic procaryotes is actually possible. The first known halophilic hydrocarbon-degrading archaea was isolated by Bertrand et al. (1990) from a salt marsh in Aigues-Mortes (Southern France) and was identified as a *Halobacterium* based on its phenotypic characteristics. This strain degrades hexadecane, eicosane, and pristane at relatively high rates (50–70% of 0.5 g/l after 30 days of incubation; Bertrand et al. 1990). Subsequently, Kulichevskaya et al. (1991) isolated a *Halobacterium* strain from hypersaline (200–350 g/l NaCl) oil-contaminated waste water in Russia. This strain degrades *n*-C_{10} to *n*-C_{30} alkanes in 300 g/l NaCl. Another extremely halophilic archaeon identified as *Haloferax mediterranei* strain M-11, growing at 100–250 g/l NaCl, has been shown to use oil as its sole source of carbon (Zvyagintseva et al. 1995). The description of the aforementioned strains, however, is not complete since their identification is based solely on their phenotypic characteristics. The capacity of certain halophilic and halotolerant bacteria to degrade benzene and toluene has also been demonstrated (Nicholson and Fathepure 2005). Recently, Al-Mueini et al. (2007) showed the degradation of pentadecane and eicosane by an actinomycete strain belonging to the genus *Actinopolyspora*, isolated from a wastewater pool on oil production site in Oman.

Although the number of known halophilic microorganisms belonging to various groups and able to grow on hydrocarbons as their sole source of carbon and energy is constantly increasing, the information available on the role played by archaea in the biodegradation of organic pollutants in hypersaline environments is still limited (Le Borgne et al. 2008). The aim of the present study was to identify the archaeal strains that can degrade hydrocarbons in such environments.

Materials and methods

Source of organisms

Water samples were collected in a shallow crystallizer pond with no known contamination history, located in the salt production area of Salins de Giraud, Camargue (France). The sampling was done using sterile 1 l plastic bottles and 25 l polypropylene containers previously washed with ethanol (97%) and water from the pond. The water had a salinity of 268 g/l NaCl, a pH value of 7.46 and an osmolarity of 9.81 Osm.

Growth conditions

The medium used for liquid and solid cultures was modified synthetic hypersaline water SHW (Bertrand et al. 1990). It was made of (g/l demineralized water): Tris–(hydroxy-aminomethane), 2; NaCl, 225; KCl, 2; KNO_3, 1; NH_4Cl, 5; $MgSO_4.7H_2O$, 24; $MnCl_2.4H_2O$ 0.04; and $CaCl_2$, 0.12. The pH value was adjusted to 7.5 and the medium was autoclaved at 110°C for 20 min. Sterile solutions of iron sulphate and potassium phosphate were added at a final concentration of 0.2 and 2 mmol/l, respectively, before incubation. Solid complex medium (SCM) was made from (g/l SHW): pepsic peptone (Difco), 5; yeast extract (Difco), 5; casamino acid (Difco), 7.5; trisodium citrate dehydrate $Na_3C_6H_5O_7.2H_2O$ (Prolabo), 3; and microbiology agar-agar (Merck), 18.

The alkanes used were sterilised as follows: *n*-hexadecane and *n*-heptadecane were autoclaved separately in the same condition as for the culture medium and added into the flask before incubation using sterile syringes, whereas *n*-eicosane was sterilised together with the culture medium.

Archaeal strains capable of using *n*-alkanes as source of carbon and energy were isolated from water samples by using an enrichment medium containing SHW and sterile *n*-hexadecane, *n*-heptadecane, and *n*-eicosane (purchased from Sigma France, 0.5 g/l each). The initial enrichments were incubated for 2 weeks at 40 ± 1°C in a reciprocal water-bath shaker (120 rpm); 1 ml of this enrichment was transferred aseptically to a second flask containing the same concentration of the same *n*-alkanes. Yeast extract, casamino acid and pepsic peptone were used at gradually decreased concentrations (2 g/l of each, 1.5 g/l, 1 g/l) for the first three replicate cultures and were not added in the subsequent four replicates. These replicates were done once every 30 days. The OD of a 1 ml aliquot of cultures incubated using only *n*-alkanes as source of carbon and energy was determined using a Milton-Roy Spectronic 401. The measure was done at 450 nm to reduce the bias caused by the colour of intermediate and final metabolites possibly produced, which absorbs at higher wave length.

At the end of enrichment procedure, the portions were streaked onto agar plates prepared with SCM. The plates were incubated at 37°C in the dark for 30 days, and single colonies were picked out and purified by multiple replicates from plate to plate. Sixteen clones were isolated (hereafter referred as strains MSNC 1 to MSNC 16) and used for biodegradation assays. One of these strains namely isolate MSNC 16 has been deposited at the Culture Collection, University of Göteborg (CCUG, Sweden, http://www.ccug.se/) under the accession number CCUG 58481.

Biodegradation assays

Strains MSNC 1 to MSNC 16 were inoculated individually in sterile 250 ml Erlenmeyer flasks containing SHW supplemented with (0.5 g/l each) sterile heptadecane and eicosane. Even if the enrichment procedure was done with a mixture of *n*-alkanes, we also tested their ability to degrade polycyclic aromatic hydrocarbons (PAHs) using a mixture of phenanthrene, dibenzothiophene, and anthracene. The PAHs were sterilised in the same way as *n*-eicosane. These flasks were then incubated in a reciprocal water-bath shaker (120 rpm) at 40 ± 1°C. Abiotic controls (without cells) were incubated under similar conditions. After 30 days, residual hydrocarbons were extracted (16 h) by liquid–liquid extraction using dichloromethane (Rathburn Chemicals Co., glass distilled grade). Squalane (>99%, GC grade, Fluka, USA) was used as an internal standard to quantify any losses during the extraction procedure. The residual hydrocarbons were then quantified by external calibration with authentic standards using a Perkin Elmer AutoSystem XL Gas Chromatograph equipped with a flame ionisation detector and a DB-1 capillary column (25 m × 0.32 mm i.d. × 0.52 µm film thickness; J & W Scientific, Inc). The oven temperature was programmed from 70 to 150°C at 15°C/min and then at 6°C/min to 320°C, at which stage it was held for 11 min.

Analysis of phenanthrene metabolites

Extracts of isolate MSNC 14 grown in SHW supplemented with the mixture of PAHs (0.5 g/l) were silylated by reaction with *N*,*O*-bis(trimethylsilyl)trifluoroacetanamide in pyridine (1:1 v/v) and were analysed by GC–MS using a Finnigan Voyager MD800 mass spectrometer (electron impact ionization) coupled to a 6890HP gas chromatograph (cool on column injection, 30 m × 0.25 mm DB5MS capillary column, oven temperature programmed from 60 to 130°C at 20°C/min and then raised to 310°C at 4°C/min, helium as the carrier gas).

DNA extraction, 16S rRNA encoding gene fragments amplification, cloning, and phylogenetic analysis

Isolated strains genomic DNA were extracted with the Microbial DNA Isolation kit according to the manufacturer's instructions (MoBio Laboratories). The gene encoding archaeal 16S rRNA fragments was obtained by PCR with the forward primer 5′-TTCCGGTTGATCCT GCC-3′ (positions 7–23, according to the *Escherichia coli* numbering scheme) and the reverse primer 5′-AAGGA GGTGATCCAGCC-3′ (positions 1541–1525) as described previously (Yang et al. 2007). The PCR conditions were modified from Yang et al. (2007): initial denaturation at 95°C for 3 min, and 30 cycles of 1 min at 95°C, 1 min at 49°C, 90 s at 72°C and 6 min at 72°C. The purified (Wizard SV Gel and PCR Clean-Up System, Promega) PCR products were cloned into pGEM-T Easy Vector System (Promega) and sequenced (GATC Biotech, http://www.gatc-biotech.com/en/index.html). The sequences were compared with GenBank database using the BLASTN software and submitted to GenBank under accession numbers FJ868731, FJ868732, FJ868734, and FJ868735 for isolated strains, and FJ868736 for strain EH4. Phylogenetic analyses were carried out using the neighbour-joining method (MEGA version 4) as described previously (Tamura et al. 2007).

Results and discussion

Sixteen archaeal strains were isolated using *n*-alkanes as substrates, but only four of them (strains MSNC 2, MSNC 4, MSNC 14, and MSNC 16) were able to grow on hydrocarbons as their source of carbon and energy. The partial 16S rRNA gene of strain MSNC 2 pointed to *Haloarcula argentinensis* (D50849) as its closest relative (99%) and the three other strains (MSNC 4, MSNC 14, and MSNC 16) were closely related to *Haloferax volcanii* (Fig. 1). To our knowledge, only one extremely halophilic archaeon (strain EH4) was known to potentially degrade *n*-alkanes and aromatic hydrocarbons to date (Bertrand et al. 1990). This strain was originally assigned to *Halobacterium* based on its phenotypic and biochemical characteristics. In order to confirm this assignment and to compare it with our new strains, we analysed the 16S rRNA of strain EH4. The results indicated that it was closely related (99%) to *Haloarcula vallismortis* (Fig. 1).

Growth rate on *n*-heptadecane ranged from 0.024 to 0.063/h (Table 1). When grown on phenanthrene, strain MSNC 14 showed a lower growth rate (0.019/h) which probably related to the low solubility of this aromatic compound in hypersaline water (Hashimoto et al. 1984; Whitehouse 1984). Cells of the strains studied were pleomorphic in shapes. They formed orange- or bright-red pigmented colonies on SCM agar plates. These characteristics were similar to those described by Torreblanca et al. (1986) concerning the member of the genera *Haloarcula* and *Haloferax*. Moreover, the colonies of these strains were circular with diameters ranging from one to five mm and they had smooth, opaque, and glistening aspects. The cross-sectional shapes of these colonies were raised, and the magnified shapes of the colonies' edge were entire.

Optimal growth temperature for *Haloarcula* is between 40 and 45°C (Torreblanca et al. 1986), whereas *Haloferax volcanii*-affiliated strains grow optimally at 45°C (Robinson et al. 2005). Both genera are able to use different

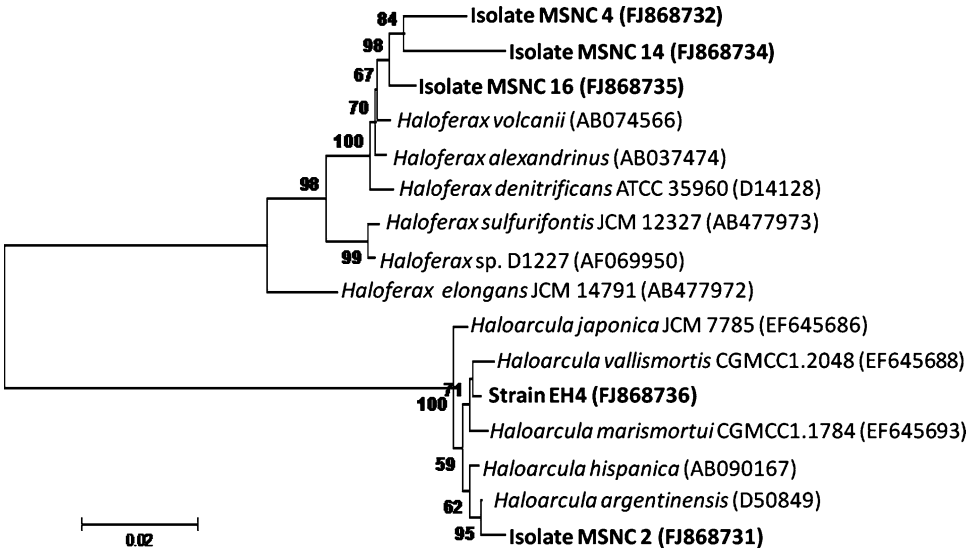

Fig. 1 Unrooted phylogenetic tree showing affiliations based on 16S rRNA sequences (1374 nucleotide positions) from the hydrocarbon-degrading halophilic archaeal strains isolated in this study (isolates MSNC, accession numbers FJ868731, FJ868732, FJ868734 and FJ868735) and strain EH4 (FJ868736) isolated by Bertrand et al. (1990). The tree was constructed using the neighbour-joining method and nucleotide substitution rates were computed using Kimura's two-parameter model. Bootstrap values (>50%) based on 1,000 resamplings are shown at branch nodes. *Scale bar* equals approximately 2% nucleotide divergence

Table 1 Growth rates (on *n*-heptadecane and phenanthrene) and percentages of model hydrocarbons degraded after 30 days of incubation by the different halophilic archaeal strains isolated

Isolates (accession no)	Closest relative in GenBank database (% identity)	Growth rate constant μ (/h)		% degradation (0.5 g/l)		
		n-C_{17}	phe	*n*-C_{17}	*n*-C_{20}	phe
MSNC 2 (FJ868731)	*Haloarcula argentinensis* D50849 (99)	0.063	n.d.	32	6	0
MSNC 4 (FJ868732)	*Haloferax volcanii* AY425724 (98)	0.030	n.d.	39	2	0
MSNC 14 (FJ868734)	*Haloferax alexandrinus* AB037474 (97)	0.033	0.019	67	30	43
MSNC 16 (FJ868735)	*Haloferax volcanii* AB074566 (99)	0.024	n.d.	95	67	0

n-C17 heptadecane, *n-C20* eicosane, *phe* phenanthrene, *n.d.* non determined

substrates as carbon sources. Few halophilic archaeal strains such as *Haloferax* sp. D1227 (Emerson et al. 1994) and *Haloarcula* sp. D1 (Fairley et al. 2002) can degrade aromatic acids. Our work clearly indicates that hydrocarbons can also constitute growth substrates for members of these two genera.

Biodegradation assays carried out with our five isolates showed 32–95% degradation of heptadecane after 30 days of incubation at 40°C. They seemed to degrade heptadecane more easily than eicosane, so heptadecane was preferred to eicosane as the growth substrate (Table 1). A similar decreasing range of biodegradation with increasing alkane chain length has been observed previously for non-halophilic as well as for halophilic hydrocarbon-degrading microorganisms. Bacteria such as *Planomicrobium alkanoclasticum* strain MAE2 selectively degrade linear and branched alkanes, but cannot degrade aromatic hydrocarbons (Engelhardt et al. 2001). The moderately halophilic bacterium *Actinopolyspora* strain DPD1 degrades pentadecane completely after 96 h, but takes 10 days to degrade about 80% of the same concentration (100 μM) of eicosane. This preference for pentadecane over eicosane could be due to enzymatic selectivity, or to decreasing hydrocarbon solubility in water (Al-Mueini et al. 2007). However, at the temperature at which our strains were incubated (40°C), eicosane is in the liquid state, which theoretically enhances its bioavailability.

Interestingly, strain MSNC 14 could degrade phenanthrene; however, degradation of anthracene and dibenzothiophene was not detected (Fig. 2). It degraded 67% of *n*-heptadecane and 43% of phenanthrene after 30 days of incubation. This degradation capacity was confirmed by the formation of 2,2′-diphenic acid in the culture medium which was associated with the decrease of the initial content of phenanthrene (Fig. 2). It has been shown that phenanthrene degradation by some bacteria (Moody et al.

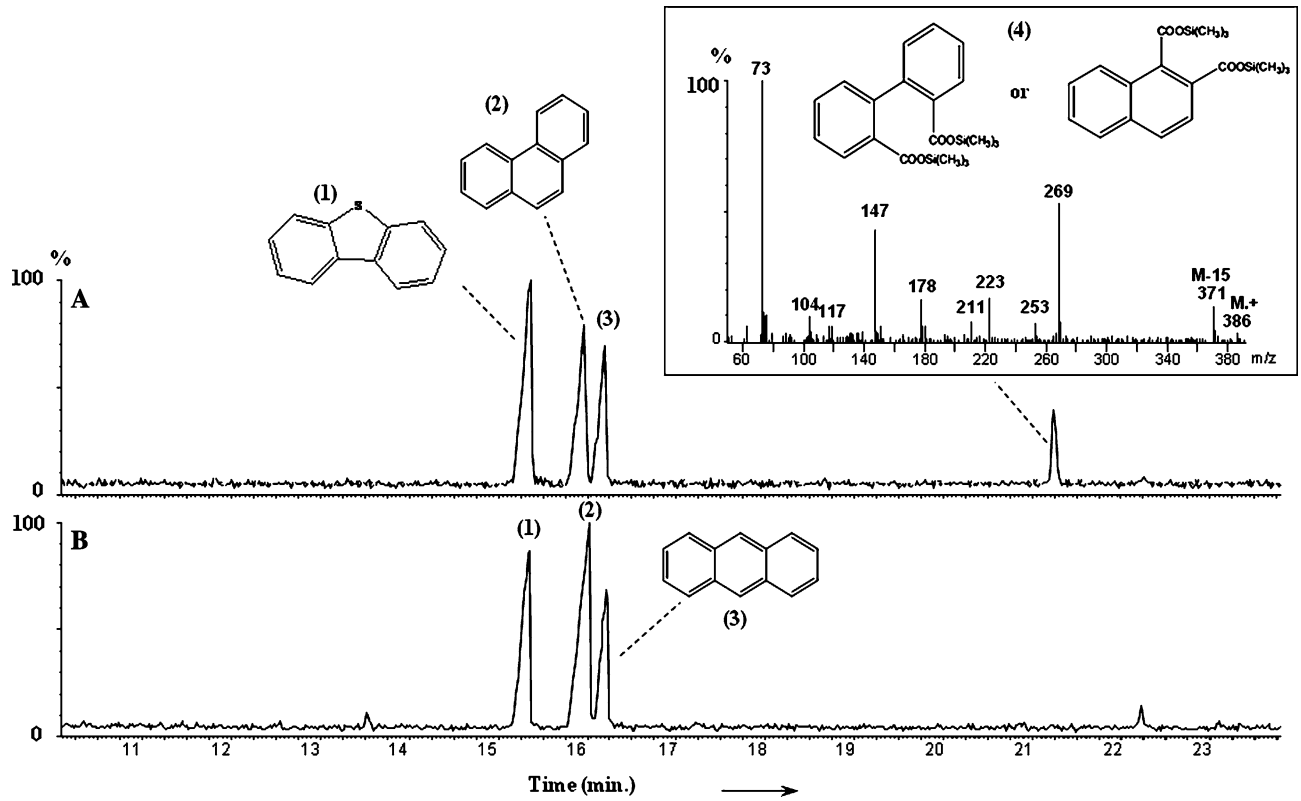

Fig. 2 Partial mass fragmentograms (m/z 147 + 269 + 327 + 371) of the total lipid extracts (silylated). **a** Culture of strain MSNC 14 grown on a mixture of *(1)* dibenzothiophene, *(2)* phenanthrene, and *(3)* anthracene (0.5 g/l each). The metabolite formed *(4)* was identified as TMS-derivatized 2,2′-diphenic acid by comparison with mass spectra library; **b** abiotic control

2001; Kim and Freeman 2005; Seo et al. 2006; López et al. 2008; Zeinali et al. 2008) or fungi (Hammel et al. 1992; Bezalel et al. 1996; Hadibarata and Tachibana 2009) starts from 9,10-dioxygenation and ortho cleavage to yield phenanthrene *cis*-9,10-dihydrodiol that is further catabolised to 2,2′-diphenic acid via 9,10-dihydroxyphenanthrene. A similar degradation pathway of phenanthrene may be involved in *Haloferax* strain MSNC 14 although this remains to be fully determined.

A comparable capacity of degrading aliphatic and aromatic hydrocarbons has been observed previously for non-halophilic and halophilic hydrocarbon-degrading bacteria. For example, a culture of the non-halophilic bacterium *Arthrobacter* sp. was shown to mineralize more than 30% of *n*-hexadecane (10 µg/ml aqueous phase) after 40 h of incubation and 69 to 71% of naphthalene (10 µg/ml water) after 71 h of incubation (Efroymson and Alexander 1991). Cell suspensions of *Rhodococcus* sp. strain Sm-1 degraded more than 92% of 5 mg/l of butane, pentane, or hexane and more than 99.8% of benzene or toluene (5 mg/l) after the same period of incubation (7 days) (Malachowsky et al. 1994). Other non-halophilic bacterial strains, namely *Pseudomonas* sp. strain BI7 and BI8, mineralized after 2 weeks of incubation 17.4 and 15.6% dodecane as well as 70 and 75% of naphthalene, respectively (Whyte et al. 1997). The recent isolation by Al-Mueini et al. (2007) of a halophilic actinomycete that degrades 80% *n*-alkanes after 10 days of incubation and fluorene (percentage not mentioned) extended the range of aromatic substrates that can be degraded by halophilic microorganisms.

Members of *Haloarcula* and *Haloferax* have been isolated from different types of hypersaline environments including salt lakes, crystallizer ponds of solar salterns (Oren 2002; Yang et al. 2007), salt flats (Ihara et al. 1997), salt marshes (Bertrand et al. 1990), and oil production areas (Zvyagintseva et al. 1995). Nicholson and Fathepure (2005) assumed that microorganisms from extreme environments with no known history or source of contamination have the potential to degrade toxic pollutants rapidly. This was observed previously for a pristine hypersaline microbial mat, in which the enzymatic requirements for the degradation of petroleum model compounds exist (Grötzschel et al. 2002). It has also been demonstrated that mats from Saudi Arabia are rich in novel halotolerant and thermotolerant microorganisms with the potential to degrade petroleum compounds at elevated salinities and temperatures (Abed et al. 2006). However, these two latter studies did not identify the potential actors of hydrocarbon

biodegradation in hypersaline environments. The present works clearly demonstrated for the first time that aliphatic hydrocarbons, and to a lesser extent aromatic hydrocarbons, can be used as source of carbon and energy by halophilic archaea isolated from hypersaline environments with no known contamination history. Our results particularly highlight the potential role of halophilic archaea belonging to the genera *Haloarcula* and *Haloferax* for aerobic bioremediation of oil-contaminated hypersaline environments. 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.

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