Isolation and Cultivation of Halophilic Archaea from Solar Salterns Located in Peninsular Coast of India

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Citation

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Abstract

Two brightly red-pigmented, motile, rod and triangular-shaped, extremely halophilic archaea were isolated from saltern crystallizer ponds located in peninsular coast of India. They grew optimally at salt concentrations between 25 and 35% and did not grow below 20% salts. Thus, these isolates are among the most halophilic organisms known within the domain Bacteria. The isolate HA3 showed optimal growth at 42°C whereas HA9 showed optimal growth at 52°C. These haloversatile microorganisms were presumed as new strains of Haloarcula. H. quadrata (HA3) showed unusual broad spectrum antibiotic resistance pattern. The isolate HA9 was named as H. vallismortis var. cellulolytica due to its peculiar cellulolytic activity, though full taxonomic description is pending.

INTRODUCTION

Though the oceans are invariably considered as largest saline body, hypersaline environments are, particularly, those containing salt concentrations in excess of seawater (3.5% total dissolved salts). Many hypersaline bodies derive from the evaporation of seawater and are called thalassic (DasSharma and Arora, 2001). The thalassic environment is obvious in the crystallizer ponds of solar salterns. Despite the prevailing extreme environment, a great diversity of microbial life has been observed in hypersaline bodies of greater than 3.5 mol/L NaCl, a point at which only a few extreme halophiles can grow (Anton et al., 1999). These extreme halophiles grow best at the highest salinities (3.4–5 mol/L NaCl), forming dense blooms, and resulting in the red colour of many salterns (Guixa-Boixereu et al., 1996). Common species of halobacteria are rod-, cocci- or disc-shaped, although triangular and even square-shaped species exist. Many are pleomorphic, especially when the ionic conditions of the media are altered, and most lyse below 1–1.5 mol/L NaCl. Halobacteria are classified as archaea (and are also called halophilic archaea or haloarchaea) and belong to the family Halobacteriacea. More than eleven genera have been reported, Halobacterium, Haloarcula, Halococcus, Haloferax, Halorubrum, Halobaculum, Natronobacterium, Natronococcus, Natrialba and Natromonas, and an eleventh genus, Haloterrigena, has also been proposed (Rodriguez-Valera et al., 1981; Grant and Larsen, 1989; Kamekura and Dyall-Smith, 1995; Oren et al., 1999; Ventosa et al., 1998). A unique feature of halobacteria is the purple membrane, specialized regions of the cell membrane that contain a two-dimensional crystalline lattice of a chromoprotein, bacteriorhodopsin. Bacteriorhodopsin contains a protein moiety (bacteriorhodopsin) and a covalently bound chromophore (retinal) and acts as a light-dependent transmembrane proton pump (Krebs and Khorana, 1993).

Solar salterns consist of a series of shallow ponds connected in a sequence of increasingly saline brines located along the peninsular coast of India was chosen for the isolation of haloarchaea. Obviously cystallizers are almost completely dried ponds and have salinity of above 30% (Benlloch et al., 1995). Therefore, microorganisms present in the cystallizers are invariably haloarchaea. Since the haloarchaea obviously grow optimum between 25% to saturated concentration of NaCl, the possible contamination during the isolation procedure is scanty. The analysis of microbial diversity has shifted in the last two decades from cultivation-dependent approaches to 16S rRNA-based cultivation-independent approaches, which led to the discovery of many novel microbial taxa. Nevertheless, this approach also has important limitations and is often confined to naming 16S rDNA clones through sequence similarity and speculation on their ecophysiology on the grounds of this similarity. Therefore, cultivation is still the method of choice to understand fully the physiology and complex ecological interactions in which microorganisms engage. The present

study was initiated to achieve isolation, characterization and cultivation of halophilic archaea from solar salterns located in the southeast coast of India.

MATERIAL AND METHODS

HALOARCHAEAL ISOLATION

The saltern soil sample was collected from Kanyakumari coast (peninsular coast) of India. The sampling site was flourished with more than 5000 salterns, which makes a congenial environment for the growth of haloarchaea. Isolations were performed on modified DSC-97 medium (Casamino acids – 7.5 g, Yeast extract – 10.0 g, Trisodium citrate – 3.0 g, KCl – 2.0g, MgSo4.7H2O – 20.0 g, FeCl2 – 0.023 g, NaCl – 250 g, Agar-agar – 15 g, distilled water – 1000 ml, pH 7.4). After incubating at 30 °C for 4 days, 9 visible colonies, designated as HA1-9, were transferred and subcultured until pure culture was obtained. The isolates were maintained as a glycerol suspension (20%, w/v) at - 20 °C.

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

The cultures for morphology were prepared by incubating the test strain on DSC-97 medium 37 °C for 3 d. The morphological properties of colonies were observed as colour, margin and elevation. Cultural characteristics were observed using starch agar media, Czapek medium, Nutrient agar, Gelatin agar, Casein agar, Simmon's citrate medium and SIM medium. All media were supplemented with 25% NaCl. Biochemical tests such as ONPG, nitrate reduction, phenylalanine deaminase, and amino acid decarboxylase were performed after MacFaddin (2000). Liquefaction of gelatin was tested using DSC-97 supplemented with gelatin (12%, w/v). Hydrolysis of urease was detected using basal medium (1 g glucose, 1 g casein-peptone, 1.98 g disodium hydroxyphosphate, 1.51 g monobasic potassium phosphate, 0.5 g magnesium sulfate, 250 g NaCl, 0.012 g phenol red, pH 9.0, 1 L distilled water). Urea was filter-sterilized and added to a final concentration of 2% (w/v). Catalase production was assayed by using 0±3% hydrogen peroxide with colonies taken from CM plates. Production of H2S was determined using Triple-sugar iron agar (Difco). Fermentation reaction was tested using phenol red basal medium supplemented with appropriate carbohydrates and saturated concentration of NaCl.

PHYSIOLOGICAL AND ANTIBIOTIC SENSITIVITY TESTS

All tests were done at 30 °C, unless otherwise specified. In order to find out the halophilic range of isolates, they were inoculated in DSC-97 medium having varied concentration of NaCl as 0 to 35% (saturated concentration). The temperature ranges for growth were determined at varied incubation in 25,35,45,55,65,75,80 and 100°C. Rhodopsin pigments were extracted from cell pellets with methanol/acetone (1:1, v/v) and absorption spectra were recorded against the solvent in a spectrophotometer (Geneys10) (Anton et al., 2002). Both bacterial strains were subjected to in vitro antibiotic sensitivity test (Kitajima et al., 1996) using standard antibiotic discs (Himedia). The radial width of the zones outside the antibiotic discs was measured in mm. The results were interpreted based on measurement of zone of inhibition (mm) (a) sensitive: equal to, greater than or not less than 20-mm (b) intermediate: ≥ 15-mm (c) resistant: ≤ 8-mm.

RESULTS AND DISCUSSION

The supplementation NaCl had direct effect on the growth of halophiles. The number of colonies grown on the nutrient media was consecutively decreased with the increase of NaCl concentration. Among the 9 isolates (HA1 – HA9), HA 3 and HA9 were showed optimal growth at 35% NaCl and were grown well at saturated salt. Based on the NaCl requirement, the chosen isolates were grouped under ‘haloversatile' which require 25-35% NaCl for optimum growth. It was noteworthy, the colonies of HA3 further grow like a crystalline tree on the media whereas HA9 grows as square type crystals. The characteristic crystalline structure (accumulation of NaCl) was increased with the growth of colonies. It may be a peculiar characteristic of haloarchaea. It was presumed that the haloarchaea might increase the rate of crystallization process in the solar salterns. Halophilic microorganisms can be conveniently grouped according to NaCl requirements for growth (Ventosa et al., 1998). Larsen (1986) defined moderate halophiles as organisms growing optimally between 5 and 20% NaCl. The Salinibacter strains isolated from saltern crystallizer ponds were extremely halophilic and grow optimum at 20-30% NaCl (Anton et al., 2002). All these strains could grow in solutions saturated with NaCl. High salt concentrations were not required for the maintenance of cell shape and cells did not lyse when suspended in distilled water. The isolate HA3 showed optimal growth at 42°C whereas HA9 showed optimal growth at 52°C.

The morphological, biochemical and physiological characteristics of the isolates are presented in Table 1.

Figure 1

Table 1: Characteristics of HA3 and HA9

Characteristics	HA3	HA9	Reference strains		
			H. vallismortis (ATCC 29715)	*H. quadrata* (ATCC 700850)	*H. argentinensis* (ATCC 700875)
Morphology	Small rods	Square	Pleomorphic Rods	Square and flat	Small rods
Gram stain	-	-	-	-	-
Motility	Motile	Motile	Motile	Motile	Motile
NaCl for optimal growth	35%	35%	25 %	15-25%	26%
Temp range					
Optimum temperature	40-45ºC	45-53ºC 52ºC	30-45ºC 40ºC	45-53ºC 53ºC	40ºC 40ºC
pH range	42ºC				
Optimum pH	5-8	7-7.2 7.4	5.5-8.5 7.4-7.5	6.5-7 7-7.2	7 7.4
Acid form:	7.2				
Fructose					
Galactose		-	+	-	+
Mannose	+	+	-	+	+
	-	-	-	-	+
Hydrolysis of Starch	-				
Gelatin		+	+	+	+
Catalase	+	-	-	-	-
Cellulase	-	+	+	+	+
Urease	+	-	-	-	-
Caesinase	+	-	-	-	-
	-	-	-	-	-
Indole production	-	+		-	-
Nitrate reduction	+	-	+	+	+
	+		+		
H2S Production		+		+	-
	+		+		
ONPG					
Rhodopsin		+	+	+	+
	+	+	+	+	+
	+				

The isolate HA9 had a peculiar characteristic feature as it grow as square type colonies whereas HA3 grow as discrete yellowish colonies. The colony morphology of HA9 was flat with undulated margin. HA3 was entire and raised colonies. Both the isolates were Gram negative motile rods (HA3) and square shaped (HA9). Invariably the isolates contained intra-cellular catalase and extra-cellular amylase enzymes. The isolate HA3 showed cellulose degradation, which was not reported for any of the reference strains. It was presumed that the isolate HA3 might have an active role in the biotransformation of humus in the soil of solar salterns. Both isolates produced hydrogen sulfide and indole, but nitrate reduction was restricted to HA3. The production of beta-galactosidase was observed for both isolates. Based on the morphological, biochemical physiological characteristics and comparison with characteristics of reference strains and standard reaction given in Bergy's manual of Systematic Bacteriology (Grant and Larsen, 1989), the present isolates were tentatively identified as Haloarcula vallismortis (HA3) and Haloarcula qudrata (HA9). Since the isolate HA3 showed cellulolytic activity, the isolate was presumed as new strain and denoted as Haloarcula vallismortis var. cellulolytica. Both isolates were deposited as freeze dried cultures in the depository (ICBM) of Department of Biotechnology (ICBM6 & 7 respectively). H. vallismortis was known for its potential of bacterial rhodopsins (Kitajima et al., 1996). Literature evidenced that mostly the haloarchaea species appeared as unculturable (Bolhuis et al., 2004).

One of these uncultivated microorganisms, the enigmatic square archaeon first described more than 20 years ago by A. E. Walsby (1980). This intriguing microorganism was detected by conventional microscopy in brine samples collected from a salt crust forming the surface of a hypersaline pool on the Sinai Peninsula. Based on the presence of a regular hexagonal surface layer, similar to that of other halobacteria, Walsby suspected that the square prokaryotes belong to the group of the ‘Archaebacteria'. Later, this morphotype was shown to belong to the group of halophilic archaea and named as Haloarcula quadrata (Oren et al., 1999, Anton et al., 1999; Benlloch et al., 1995; Benlloch et al., 2001). They are found in large amounts in solar salterns especially at the end of the concentration process leading to NaCl precipitation. Cultivation is essential in order to unravel the molecular basis of the unique physiological and morphological characteristics of this remarkable organism. The present findings helpful to isolate, maintain and explore the biopotentials of H. quadrata.

The antibiogram indicated that H. quadrata was resistance against all the tested antibiotics whereas H. vallismortis was sensitive to all the antibiotics tested except ampicillin (Table 2). The exact mechanism behind in this board spectrum of resistant pattern was unknown. In general, a bacterium resistant to antibiotics indicates its history exposed with antibiotics earlier. However this phenomenon may not applicable for H. quardata as there was no possible means of antibiotic contamination in salterns. Since the isolate showed a broad-spectrum resistance pattern, it could be used as test organism to screen broad-spectrum antibacterial agents instead of screening for a battery of test organisms.

Figure 2

Table 2: Antibiogram of HA3 and HA9

Antibiotics	Concentration (µg / disc)	BTL9	BTL3
Ampicillin	10	R	R
Chloramphenicol	20	R	S
Erythromycin	15	R	S
Tetacyclin	30	R	S
Glyoxacillin	20	R	I
Nalidic acid	10	R	S
Amoxicillin	15	R	S
Oxytetracyclin	20	R	I
Gentamycin	30	R	S
Novobiocin	10	R	S
Bacitracin	30	R	S
Pencillin –g	10	R	S
Ciprofloxacin	5	R	I
Neomycin	20	R	I
Irimethoprine	10	R	S
Streptomycin	15	R	S
Ciprofloxacin	5	R	S
Cephalexin	30	R	S
Norfloxacin	10	R	S

R-Resistant, I-Intermediate, S-Sensitive

It is noteworthy that bright-red pigmentation is common in microorganisms inhabiting salt lakes and saltern ponds (Anton et al., 2002). Pigmentation was noticed in both species and showed maximum rhodopsin pigmentation in incubation under mercury lamb (1000 lux). Pigments showed absorption maxima at 482 nm and a shoulder at 506–510 nm. Members of the Halobacteriaceae possess C-50 carotenoids of the bacterioruberin group. The role of this pigmentation in protecting against the harmful intensities of sunlight to which the cells are exposed in their natural environment was shown many years ago (Dundas and Larsen, 1962). The red colour of most members of the Halobacteriaceae has been used in the past as an easily recognizable character to discriminate between archaeal and bacterial members of the prokaryote community (Rodriguez-Valera et al., 1981).

In summary, the isolates HA3 and HA9 showed optimum growth at 25% to saturated concentration of NaCl. These haloversatile microorganisms were presumed as new strains. H. quadrata showed unusual broad spectrum antibiotic resistance pattern. The isolate HA9 was named as H. vallismortis var. cellulolyticus due to its peculiar cellulolytic activity, though full taxonomic description is pending. Both isolates showed no synergistic growth with other contaminants of solar saltern. The present study concluded that the haloarchaea could be isolated and maintained as pure culture for the exploration of biopotentials.

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References

r-0. Anton J, Oren A, Benlloch S, Rodriguez-Valera F, Amann R and Rossello-Mora R (2002) Salinibacter ruber gen. nov., sp. nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. Int. J. Sys. Evol. Microbiol. 52: 485-491
r-1. Anton J, Llobet-Brossa E, Rodriguez-Valera F, Amann R (1999) Fluorescence in situ hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. Environ. Microbiol. 1: 517 523
r-2. Bauer AW, Kirby WM, Sherris KC, Turck M (1996) Antibiotic susceptibility testing by a standardized single disc method. Am. J Clin. Pathol. 45: 493-496
r-3. Benlloch S, Martinez-Murcia AJ, Rodriguez-Valera F (1995) Sequencing and bacterial and archaeal 16S rRNA genes directly amplified from a hypersaline environment. Syst. Appl. Microbiol. 18: 574–581
r-4. Benlloch S, Lopez-Lopez A, Casamayor EO, Ovreas L, Goddard V, Daae FL Smerdon G, Massana R, Joint I, Thingstad F, Pedrós-Alió C, Rodríguez-Valera F (2002) Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern, Environ. Microbiol. 4: 349 360
r-5. Benlloch S, Acinas SG, Anton J, Lopez-Lopez A, Luz SP, Rodriguez-Valera F (2001) Archaeal biodiversity in crystallizer ponds from a solar saltern: culture versus PCR. Microbiol. Ecol. 41: 12 19
r-6. Bolhuis H, te Poele EM, Rodriguez-Valera F (2004) Isolation and cultivation of Walsby's square archaeon. Environ. Microbiol. 6: 1287
r-7. DasSharma S, Arora P (2001) Halophiles In: Encyclopedia of Life Sciences, Nature Publishing Group.
r-8. Dundas ID, Larsen H (1962) The physiological role of the carotenoid pigments of Halobacterium salinarium. Arch. Mikrobiol.44: 233-239
r-9. Grant WD, Larsen H (1989) Extremely halophilic archaebacteria order Halobacteriales ord. nov. In: Staley JT, Bryant MP, Pfennig N, Holt J G (Eds), Bergey's manual of systematic bacteriology, Vol.3. Williams & Wilkins, Baltimore, Md. pp. 2216–2233
r-10. Guixa-Boixereu N, Caldero´n-Paz JI, Heldal M, Bratbak G, Pedros-Alio C (1996) Viral lysis and bacterivory as prokaryotic loss factors along a salinity gradient. Aquat. Microb. Ecol. 11: 215–227
r-11. Kamekura M, Dyall-Smith ML (1995) Taxonomy of the family Halobacteriaceae and the description of two new genera Halorubrobacterium and Natrialba. J. Gen. Appl. Microbiol. 41: 333-350
r-12. Kitajima T, Hirayama J, Ihara K, Sugiyama Y, Kamo N, Mukohata Y (1996) Novel bacterial rhodopsins from Haloarcula vallismortis. Biochem. Biophys. Res. Commun. 220: 341-345
r-13. Krebs MP, Khorana HG (1993) Mechanism of light-

dependent proton translocation by bacteriorhodopsin. J. Bacteriol. 175: 1555–1560
r-14. Larsen H (1986) Halophilic and halotolerant microorganisms: an overview and historical perspective. FEMS Microbiol. Rev. 39: 3-7
r-15. MacFaddin JF (2000) Biochemical Tests for Identification of Medical bacteria. 3rd edition. Williams & Wilkins
r-16. Oren A, Ventosa A, Gutierrez MC, Kamekura M (1999) Haloarcula
quadrata sp. nov., a square, motile archaeon isolated from a brine pool in Sinai (Egypt). Int. J. Sys. Bacteriol. 49: 1149-1155
r-17. Rodriguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A (1981) Characteristics of the heterotrophic bacterial populations in hypersaline environments of different salt concentrations. Microb. Ecol. 7: 235-243
r-18. Ventosa A, Nieto JJ, Oren A (1998) Biology of moderately halophilic aerobic bacteria. Microbiol. Mol. Biol. Rev. 62: 504-544
r-19. Walsby AE (1980) A square bacterium. Nature 283: 69 71.

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