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Study of Stress Proteins Induced by Temperature Stress in Extremely Halophilic Archaea, *Haloferax mediterranei* RT18

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A B S T R A C T

The survival of any cell mainly depends upon the ability of it to rapidly adapt to changes in the natural environment in which they live. Halophilies that live in extreme salt conditions have to face different types of stress like temperature change, high salt concentrations, chemical changes and radiation changes. When such stress conditions occur, these organisms induces the production of certain stress proteins, which depends upon the type of stress these organisms have faced. In the present study, halophilic archaea was isolated from sea water sample from Marine drive area of Mumbai, India. It was exposed to temperature stress and the soluble stress proteins were isolated. The differential proteins expressed in response to stress were studied using SDS-PAGE and identified using MALDI-TOF MS-MS analysis. The identified stress protein, beta proteasomal subunit has been found to play a main role in maintaining protein homeostasis. This study shows that the haloarchaea when exposed to stress, upregulated the production of stress proteins and proteasomal assembly that gives an insight to mechanism of stress physiology in archaeabacteria.

Introduction

The living organisms in oceans and seas have developed extraordinary ability to deal with extreme environments like changes in temperature, chemicals and radiations. When certain limiting factors of the environment like sunlight and nutrients availability, pressure and temperature conditions, salinity when goes beyond the normal tolerable levels, there is a loss of protein's native conformation followed by their proteolysis (Nath and Bharathi, 2011).

The study of how these organisms cope up with such stress conditions has been a topic of research in recent years.

Molecular chaperons or stress proteins are the proteins that maintain protein homeostasis by regulating protein folding. They recognize proteins of non-native structure and help them in proper folding to give them a native structure (Imai *et al.*, 2003). These proteins are present in cell in

normal conditions in very low concentrations. When there is stress to the organism, the newly formed proteins are not able to fold correctly in its tertiary structure and forms Defective Ribosomal Products (DRiP's). Since the main function of chaperones is to correctly fold the non-native proteins and to ensure its smooth functioning, they are found to be expressed in higher concentrations in stress conditions.

Chaperones usually work in conjugation with proteasomes, which are another class of proteins responsible for protein degradation. The misfolded proteins are directed to proteasomes by chaperones where are degraded by the Ubiquitin-Proteasome system (UPS). These pair of proteins prevents accumulation of abnormally formed proteins by their due destruction (Imai *et al.,* 2003).

Halophilic archaea are a group of extremophiles that live in high salinity regions like sea water and coastal regions. They face different types of stress like high salinity, temperature, pH conditions, radiation and chemical changes. Like other organism, archaea too possess chaperones and proteasomal proteins in normal conditions and whose concentration gets elevated in stress conditions. Hsp40 (DnaJ), Hsp60 (chaperonins), Hsp70 and small heat-shock proteins (sHsp) have also been studied in archaeal system (Macario *et al.,* 1999).

The present study attempts to study the expression of proteins in an extremely halophilic archaeon, *Haloferax mediterranei* in response to temperature stress.

Materials and Methods

Collection of sample and isolation of haloarchaea:

Surface water sample of the Arabian Sea was collected from the Marine drive area of Mumbai, India in sterile plastic container in the month of January 2014. The water sample was enriched in Sehgal and Gibbon's medium (SG) containing (g l^{-1}) Casamino acids- 7.5; Yeast Extract- 10, potassium chloride- 2, trisodium citrate- 3, $MgSO_4$- 20; NaCl- 150; pH- 7.2 (Sehgal and Gibbons, 1960) at 40°C for 7 days at 100 rpm in a rotary shaking incubator until intense pink coloration was observed. Thereafter, the sample was isolated on Sehgal and Gibbon's agar medium with 15% NaCl as described by Digaskar *et al.* (2015). The isolated cultures were stored as glycerol stocks at -20°C till further use.

Morphological and Biochemical characterization of the haloarchaea

Isolated archaeal strains were studied for their phenotypic and biochemical characterization according to Bergey's Manual of Systematic Bacteriology, Second Edition, Volume I (1984). Standard biochemical tests were used for identification of the isolate as described by Oren *et al.* (1997). Gram staining was performed as described by Dussault (1955). The optimum salt required for growth was tested by culturing the isolate in SG medium containing different concentrations of NaCl from 0-35% NaCl. The confirmatory test for archaea was performed by testing the growth of the isolate in SG medium containing Chloramphenicol (20mg/ L) and sodium taurocholate (0.25 g/L) (Enachea *et al.,* 2006). The production of catalase, oxidase, urease and formation of indole was performed as prescribed by Oren *et al.* (1997). Anaerobic growth of the isolate in presence of DMSO, Arginine and KNO_3 as electron acceptors was tested as described by Oren and Truper (1990). The culture was inoculated in standard growth medium (g/L) (Yeast extract- 10, Casamino acids-7.5g, NaCl- 250, $MgSO_4 . 7H_2O$- 40, KCl- 2,

Trisodium Citrate- 3, Trace solution- 10ml/L). Trace solution contains (in 100 ml, $FeCl_2.4H_2O$- 2.3mg, $CaCl_2$. $7H_2O$- 7mg; $MnSO_4.H_2O$-0.3mg; $ZnSO_4$- 0.44mg; $CuSO_4.5H_2O$- 0.050mg, pH- 7.2) (Asker and Ohta, 1999) with DMSO (5g/L), Arginine (5g/L) and KNO_3 (30 mM) respectively in separate tubes and kept in dark conditions for 7-10 days. The growth was measured in terms of the absorbance of the broth at 600 nm (Hartmann *et al.,* 1980; Oren and Trüper, 1990). The effect of magnesium and manganese on growth of the isolate was studied. The isolate was cultured in SG medium containing 15% NaCl supplemented with various concentrations of $MgSO_4$ and $MnCl_2$ (0 mM - 500 mM). Screening of isolate for presence of four enzymes (Amylase, Protease, Gelatinase and Lipase) was performed as described by Digaskar *et al.* (2015). The utilisation of sugar was studied by inoculating the isolate in medium containing 0.05% of sugar (Lactose, Maltose, Sucrose and Glucose) (Oren *et al.,* 1997).

Identification of the isolate using 16S rRNA sequencing

The isolate was identified using 16S rRNA sequencing using Sangers method and the sequence was assembled using Chromas Pro v1.34 assembly tool. Using BLASTn search, the sequences were compared with 16S rRNA sequences available in the Gene Bank databases (Altschul *et al.,* 1997). Multiple sequence alignments were performed using CLUSTAL X version 2 (Thompson *et al.,* 1994). Phylogenetic tree was constructed from evolutionary distances with the help of neighbor-joining method of MEGA 6 program (Kumar *et al.,* 2004; Bhorgave *et al.,* 2012). The 16S rRNA sequences of the isolated haloarchaea were submitted to NCBI Gene Bank Database and all the culture was deposited in Microbial Culture Collection, NCCS, Pune, India.

Effect of Temperature stress on haloarchaea

The isolate was cultured in 250 ml shake flask in SG medium containing 15 % NaCl at 40°C for 7 days and then the culture was exposed to temperature stress by incubating the culture in incubator at 70°C, 55 °C and 4 °C for 4 h. The growth in terms of absorbance at 600 nm was monitored.

Preparation of cell lysate and 1D- gel electrophoresis

The cultures exposed to stress were subjected to centrifugation at 10, 000 rpm for 10 min. The cell pellet was resuspended and treated in buffer (5 mM Tris-HCl, pH 8.0, 10 mM SDS, 2-mercaptoethanol/1 mM PMSF (freshly prepared). Unbroken cells and debris were removed by centrifugation at 8000 rpm for 10 min at 4°C, and the supernatant containing the soluble fraction of proteins was transferred into a separate tube. Subsequently, the lysate was digested with 100 μg/ml DNase, and 40 μg/ml RNase, for 60 min at 37°C to remove nucleic acids (Shukla, 2006). The proteins of the samples treated with temperature stress were subjected to SDS-PAGE as described by Laemmli (1970).

Trypsin digestion and extraction of peptides from gel

Samples were resolved through 1-D gel electrophoresis. Proteins band that were present in the stressed samples and absent in the samples without stress were identified and were excised from Commassie stained gels and washed with water and twice with 50 % acetonitrile for 15 min. Gel pieces were then washed with a 1:1 solution of 0.1 M NH_4HCO_3 and acetonitrile for 15 min. For destaining, gel pieces were incubated in 10 mM DTT/0.1 M NH_4HCO_3 for 45 min at

56°C to reduce the protein, followed by incubation in 55 mM iodoacetamide/0.1 M NH_4HCO_3 for 30 min at room temperature in the dark for alkylation.

Supernatants were discarded and gel pieces were washed with 100 µl NH_4HCO_3, followed by two washes (5 min each with vortexing and brief centrifugation) with 100 µl (or enough to cover) of 25 mM NH_4HCO_3in 50% acetonitrile.

The gel particles were dehydrated to complete dryness and rehydrated with trypsin digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$, 12.5 ng/µl trypsin) was added in a final volume of 25 µl.

Tubes were incubated on ice for 45 min, after which 25 mM NH_4HCO_3 was added and tubes were further incubated overnight at 37°C. The supernatant was removed into a clean siliconized tube and extracted twice in 50 % acetonitrile and 5 % formic acid and acetonitrile.

The mixture was vortexed 20–30 min and centrifuged (Shukla, 2006). Supernatant was pooled into a separate tube and analysed by MALDI.

Identification of protein by MALDI-TOF MS

One µL of the peptide digest extracted from a gel piece was premixed with equal volume of matrix and spotted on a matrix-assisted laser desorption ionization (MALDI) plate.

Peptide mass fingerprint (PMF) data was acquired on the MALDI TOF- mass spectrometer (Ultraflex II, Bruker Daltonics, Germany) in the reflector mode. The data was searched against Swiss-Prot database using MASCOT search engine (Govekar *et al.,* 2012).

Results and Discussion

Collection of the sample and isolation of the haloarchaea:

Marine Drive is a 4.3-kilometre-long area in South Mumbai in Mumbai city. It is a natural bay which is a 'C'-shaped six-lane concrete road along the coast (Fig. 1). The sea water sample in this study was collected from this region of Mumbai. The temperature of the sea water sample was between 35-37°C and pH was in the range of 8-9.

Previously, haloarchaea have been isolated from salterns of Mumbai (Digaskar *et al.,* 2015; Pathak and Sardar, 2014). However, this is probably the first report of isolation of halophilic archaea from low salinity area (around 3–4% NaCl) of Arabian Sea adjoining Marine drive area of Mumbai, India.

Morphological and Biochemical identification of the isolate

The culture after enrichment showed faint pink colour colonies on SG medium with 15% NaCl and it was designated as SII. Phase contrast image of the isolate confirmed the coccoid morphology (Fig. 2).

The SII isolate was gram negative in nature with coccoid morphology. The colonies were less than 1 mm in size with entire margin, translucent opacity and concave elevation

Biochemical tests like salt tolerance, catalase, oxidase, anaerobic growth, ability to utilize $MgSO_4$ and $MnCl_2$, sugar utilization were performed for the isolate and the results are represented in table 1.

The organism could tolerate a salt concentration of upto 25% with anaerobic growth in presence of DMSO, Arginine and KNO_3 as electron acceptors. It was oxidase positive and catalase negative. It could utilize $MgSO_4$ in the range of 5–500 mM which proved that the presence of magnesium ion is must for the growth of the organism. Similarly, it grew in the presence of manganese ion in a concentration ranging from 10–200 mM. Higher concentration of $MnCl_2$ also inhibited the growth of the organism. It could utilize Glucose and Sucrose however it failed to utilize Lactose and Maltose sugars. Hydrolysis of Starch and Tween 80 was done but hydrolysis of Gelatin and Casein was not exhibited by the organism.

Identification of the isolate using 16S rRNA sequencing:

On the basis of 16S rRNA sequencing, biochemical and morphological characterization, the SII isolate was identified as *Haloferax mediterranei* RT18. It was deposited in NCBI Genbank with Accession number KP712891. It belongs to family *Halobacteriacece,* which consists of extremely halophilic organisms. This organism was isolated from evaporation ponds of sea water near Alicante, Spain, for the first time (Rodriguez-Valera *et al.,* 1980).

It has been found that this organism exhibits rapid growth than other members of *Halobacteriaceae* and demonstrates excellent genome stability and metabolic efficiency even at high salt concentrations (Rodriguez-Valera *et al.,* 1983). Because of this outstanding ability of this organism, it has proved as a good model to study haloarchaeal metabolism and physiology studies for several years (Jäger *et al.,* 2002). The evolutionary relationship between the organism in the current study and other organisms is given in figure 3.

Effect of temperature stress on *Hfx. mediterranei*

The growth of the isolate at different temperatures in the range of 10°C, 25°C, 37°C, 40°C, 55°C, 70°C for 7–10 days was studied as in Fig.4. Growth in terms of absorbance was measured at 600 nm. When the organism is exposed to stress, the growth is retarded for few hours. This is evident due to the period of adaptation of the organism to stress. The organism demonstrated fast growth at 40°C. It was observed that when the organism was exposed to 70°C, there was no growth for the first four days, after which the growth increased. The initial growth response of the organism to the temperatures 24°C, 37°C and 55°C was good with eventual decrease, whereas the growth at 10°C was retarded. Fig 4 exhibits the optimum temperature required for growth of the isolate to be 40°C.

Identification of stress proteins expressed due to temperature stress in *Hfx mediterranei*

The proteins differentially expressed in the isolate in response to stress were studied using SDS-PAGE. These over expressed proteins may be attributed to the production of chaperonins or proteasomes. The protein bands expressed differentially in response to stress were excised from the gel and use for trypsin digestion.The extracted gel pieces were treated with reducing, alkylating agents, trypsin enzyme and prepared for MALDI-TOF MS analysis. The MALDI analysis revealed the protein that was expressed in the stress samples was beta subunit of Proteasome protein which is a component of Ubiquitin Proteasomal System (UPS).

Table.1 Morphological and biochemical characters of the isolate SII

Sr. No.	Morphological and Biochemical characteristics	Result
	Colony characteristic	
1.	Size (mm)	> 1
2.	Shape	Circular
3.	elevation	Concave
4.	margin	Entire
5.	consistency	Translucent
6.	Colony Pigmentation	Faint Pink
7.	Gram Nature	Gram Negative cocci
8.	Motiliy	Non motile
9.	**NaCl requirement for growth (%)**	10-25
10.	Optimum NaCl requirement	25
11.	**pH requirement for growth**	7-9
12.	Optimum pH	7.2
13.	**Temperature requirement for growth (˚C)**	37-42
14.	Optimum Temperature	40
15.	Lysis in Distilled Water	+
16.	Oxidase test	+
17.	Catalase test	-
	Hydrolysis of:	
18.	Starch	+
19.	Casein	-
20.	Gelatin	-
21.	Tween 80	+
	Anaerobic growth in presence of:	
22.	Arginine	+
23.	KNO_3	+
24.	DMSO	+
	IMViC test:	
25.	Indole Production	-
26.	MR	-
27.	VP	-
28.	Citrate Utilization	-
29.	Urease Test	-
30.	H_2S Production	-
31.	Reduction of Nitrate to Nitrite	-
	Utilization of Sugars:	
32.	Maltose	-
33.	Lactose	-
34.	Glucose	+
35.	Sucrose	+
36.	Requirement of $MgSO_4$ for growth (mM)	5-500
37.	Requirement range of $MnCl_2$ for growth (mM)	10-200
	Growth in Presence of:	
38.	Chlormaphenicol	+
39.	Sodium Taurocholate	-

Table.2 Description of the identified protein based on Peptide mass finger print (PMF) data acquired on the MALDI TOF-TOF Protein analyzer (Ultraflex II, Bruker Daltonics) in the reflector mode. The data was searched against SwissProt database using MASCOT search engine with a peptide mass tolerance of 100 ppm

Band Description	Intensity coverage	Digest Match Score/ Mascot	Putative molecular weight	Putative pI Isoelectric point	Matched Sequence	Comments	Gene
Band 1 expressed in response to temperature stress of 70°C *Haloferax mediterranei*	82 %	44	22.91kDA	8.7	Proteasome subunit beta 2 OS=Sulfolobus solfataricus (strain ATCC 35092 / DSM 1617 / JCM 11322 / P2) GN=psm	Three Threonine residues at site 15-17	Psm B
Band 2 expressed in response to temperature stress of 70°C *Haloferax mediterranei*	83.0 %	45	22.9 kDA	8.1	Proteasome subunit beta	Three Threonine residues at site 15-17	Psm B

with a peptide mass tolerance of 100 ppm

Fig.1 Marine drive sea water: Site of sample collection

Fig.2a) Haloarchaea SII isolated from the water sample on SG medium, b) Gram staining of the isolate SII (1000X) c) Phase contrast micrograph of the organism (400X)

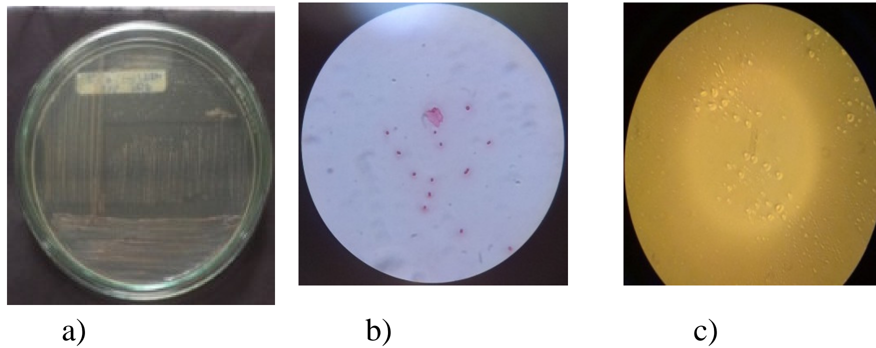


Fig.3 Effect of temperature stress on *Haloferax mediterranei* RT 18

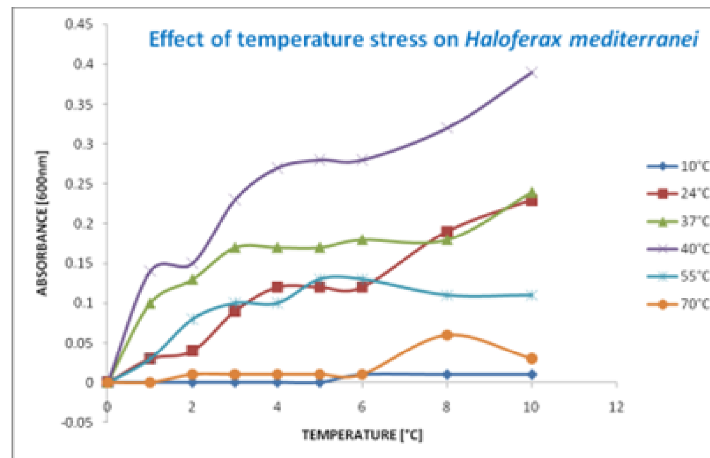
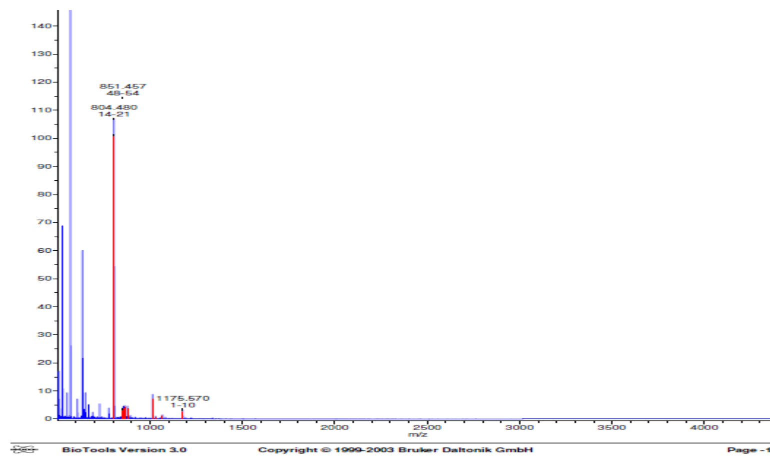


Fig.4 Graph of Atomic unit versus m/z generated by MALDI-TOF MS analysis of *Haloferax mediterranei* RT18 stress proteins expressed in response to temperature stress



The proteasomal protein is usually found in cytosol but may also be present in micrsomal and nuclear fractions (Brooks *et al.,* 2000; Wojcik and DeMartino, 1999). The main component of this 20S proteasome is beta subunit along with alpha subunit. It forms the core of 20S proteasome assembly which functions in the degradation of the misfolded proteins (Table 2). [Fig. 5] shows a graph of atomic unit against the mass/charge ratio of the protein. The identified protein is a low molecular weight protein (22.91kDa).

Two bands which were differentially expressed in stress sample were chosen for MALDI-TOF analysis. The molecular weight of the identified protein was estimated to be 22.91 kDa and 22.9 kDa respectively and isoelectric point (pI) was found out to be 8.1 and 8.2. The gene identified was Psm B (Proteasome subunit beta) in both the bands. It is a part of the Universal Proteasome System that is involved in proteolysis of damaged or unneeded proteins in response to stress and interplays with chaperonin to ensure protein quality and readjustment of composition of proteome following stress. Shukla (2006) studied expression of such chaperones (Dna J, GrpE, sGsp-1 etc) in *Halobacterium* sp. NRC-1 and found that this organism has ability to adapt to extreme conditions due to expression of the chaperones in stress conditions. Chaperonin proteins have also been expressed and isolated from *Haloferax volcanii* by Kapatai *et al.* (2006). Similarly the proteins of the proteasomal system have been demonstrated to be expressed in response to perchlorate stress in *Haloarcula argentiensis* (Chitnis and Thombre, 2014). Protein degradation using proteasome is the main component of cell's machinery that maintains protein homeostasis (Tsvetkov *et al.,* 2013). This protein often works in association with heat shock proteins.

The effect of temperature stress on archaea was studied. In the current investigation, production of beta proteasomal protein a part of the UPS that works in conjunction with chaperones was demonstrated. These organisms on subjection to multiple stresses and upregulate the production of 20S proteasome along with chaperonin proteins, heat shock proteins and many other stress proteins for protection against these factors. This archaeal model and the stress induced chaperonins and proteasomes in these organisms still remain largely unexplored. Studying the stress proteins can provide useful insights of the cellular mechanisms of repair and survival of these halophiles under extreme stress. All the work done in this field of stress physiology shows that these halophilic organisms, like others also express stress proteins and have evolved various mechanisms to protect themselves from the stress conditions.

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