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Isolation and characteriation of extreme halophiles *Halomonas aquamarina* and *Halomonas marina* from trigger fish, *Abalistes stellaris* (Bloch & Schneider, 1801)

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ABSTRACT

Screening of bacteria from the skin, gills and gut of the marine triggerfish, *Abalistes stellaris* (Bloch & Schneider, 1801), collected from Vizhinjam, Kerala, India, led to the isolation of 8 halophilic bacterial strains. The isolates were able to grow optimally in culture media with 5-15% salt content. Of these, 3 extremely halophilic bacterial isolates that grew in 20-25% of salt were selected for genotypic characterisation. Bacterial strain, IJ1, isolated from skin, and strains, IJ5 and IJ6, isolated from gut of *A. stellaris* grew optimally at pH 7.0 and 5-15% NaCl at 35 °C. The cells were Gram negative short rods. According to the phenotypic characteristics and comparative partial 16SrRNA sequence analysis, the strain IJ1 was identified as *Halomonas marina* strain DSM 4741 (GenBank Accession Number: KC599209) and strains IJ5 and IJ6 as *Halomonas aquamarina* (GenBank Accession Numbers: KC620376 and KC599210 respectively). *H. aquamarina* has been reported for the first time from any environment in India and both the strains have been deposited in the microbial repository of National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, India.

Keywords: *Abalistes stellaris*, Halophiles, 16SrRNA, *Halomonas aquamarina, Halomonas marina*

Introduction

Halophilic microorganisms are one of the most important groups of microorganisms adapted to hypersaline habitats. Distinction between different kinds of halophilic microorganisms is made on the basis of their level of salt requirement and salt tolerance (Kushner, 1993).Most halophilic and all halotolerant organisms expend energy to exclude salt from their cytoplasm to avoid protein aggregation. Bacteria and eukaryotes usually accumulate neutral compatible solutes, whereas archaea prefer negatively charged solutes (Martin *et al*., 1999; Roberts, 2005). These microorganisms are the object of basic studies in relation to the origin of life in our planet and the molecular mechanisms of adaptation to saline and hypersaline conditions (DasSarma and Arora, 2002). The oldest prokaryote fossil which are 3,500 million year old stromatolites resemble the contemporary microbial mats found in hypersaline environments. Halophiles are reported to have ecological significance and promising biotechnological applications in food industry.They act as pigments, organic osmotic stabilisers, surfactants, enzymes which are able to function at low water activities, bacteriorhodopsin applications including holography, optical computers and optical memory, production of renewable energy and biodegradation of organic pollutants (Margesin and Schinner, 2001; Oren, 2002).

Moderately halophilic bacteria, like other extremophiles, have exciting and promising biotechnological applications. Not only do many of them produce compounds of industrial interest (enzymes, polymers, and osmoprotectants), but also they possess useful physiological properties which can facilitate their exploitation for commercial purposes. Firstly, most of them can grow at high salt concentrations, minimising the risk of contamination. Secondly, they grow easily and their nutritional requirements are simple. Majority of them can use a large range of compounds as their carbon and energy source. Moreover, as discussed above, many of the genetic tools developed for the non-halophilic bacteria can be applied to the moderate halophiles and hence, their genetic manipulation seems relatively simple (Ventosa *et al.*, 1998).

During the last decade, extensive studies on hypersaline environments carried out in many geographical areas have lead tothe isolation and taxonomic characterisation of a large number of moderately halophilic species. Most species are Gram-negative or Gram-positive aerobic or facultatively anaerobic moderately halophilic bacteria. Recently 16S rRNA sequence analysis have

permitted determination of the phylogenetic position of most of the moderately halophilic bacteria. The objective of the present study was to screen halophilic bacteria from selected marine fishes and to characterise those using phenotypic characteristics as well as 16S rRNA sequence analysis.

Materials and methods

Sample collection and processing

A total of five specimens of trigger fish *A. stellaris* were collected live from Vizhinjam Bay, Kerala India, during 2008. They were taken to the laboratory for screening of halophilic bacteria under sterile conditions. The fishes were anaesthetised using clove oil (0.4ml l^{-1}) for bacterial isolation. For gut isolates, the anaesthetised fish were dissected within laminar air flow cabinet on ice and the alimentary canals were removed as quickly as possible. Portions of the alimentary canal were first cleaned, then cut into pieces and slit open by a longitudinal incision. The contents were transferred to sterile petridishes and thoroughly flushed with sterilised and chilled saline (pH 7.4; 0.89% (w/v). Subsequently, the pieces of the alimentary canal were homogenised with sterile saline (10:1; v/wt). The homogenate was used as inoculum for microbial culture. For skin isolates, mucus extracts were isolated by swabbing aseptically within laminar air flow cabinet. The extracts were homogenised in a solution of 50 ml normal saline and the supernatant was used for culture. Similarly, for gill isolates, gills were macerated in 50 ml normal saline and homogenised in a vortex mixer aseptically, and the supernatant was used as inoculum for microbial culture as in the above cases.

Isolation of halophilic bacterial strains

The samples were subjected to 10 fold serial dilution and aliquots (0.1 ml) were plated onto nutrient agar, supplemented with 2% (w/v) NaCl, in duplicates. The plates were incubated at 35 °C for 48 h. Discrete and well-isolated bacterial colonies with varying morphological characteristics were selected and streaked on to nutrient agar plates to obtain pure culture. The pure cultures of bacterial strains were restreaked onto nutrient agar slants supplemented with 2-25% (2, 10, 15, 20 & 25%) NaCl. The pure cultures thus obtained were then used for further characterisation. Nutrient broth with 2% (w/v) NaCl and nutrient agar medium with 2% (w/v) NaCl were used to maintain the halophilic strains, to prepare the inocula and as the basal medium for phenotypic tests. Nutrient agar medium with 20-25% (w/v) NaCl was used for the maintenance and growth of extremely halophilic strains. The pH of the medium was adjusted to 7.2±0.2 with 1 *N* NaOH or 1 *N* HCl.

Characterization of bacterial strains

Phenotypic characterisation

Morphological tests like Gram staining and motility were determined in 24 h cultures in nutrient broth. The morphology and size of the colonies and the pigments were examined in nutrient agar after 3 days of incubation. The growth at different pH levels was determined in nutrient broth with the pH adjusted to 5, 8 and 10 using 1 *N* HCland/ or I *N* NaOH. Biochemical tests conducted for bacterial characterisation include catalase, cytochrome oxidase, penicillin sensitivity, H & L glucose O/F, sugar fermentation, cellulose hydrolysis, gelatin liquefaction, starch utilisation and phosphate solubilisation

Genotypic characterization

DNA extraction

Those strains which are able to grow in more than 15% NaCl were subjected to genotypic characterisation. Pure bacterial cultures were grown in tryptone soya broth (TSB, Oxoid, U.K.) for 2 days. The bacterial cultures were centrifuged for 10 min at 5031 *g*. A total of 10-20 mg of each bacterial culture was placed in a 1.5 ml microcentrifuge tube and resuspended in 200 µl of Tris-EDTA (TE) buffer. Bacterial DNA was extracted using the Genomic DNA Purification Kit (Genie, Bangalore, India).

Amplification of partial 16SrRNA gene sequences and phylogenetic analysis

The identification of bacteria was performed by sequence analysis of DNA coding for the 16SrRNA. Universal bacterial 16S rDNAs primers were used to amplify a fragment of 16S rDNA, 760 bp in length. The PCR reaction mixtures contained 10× PCR buffer with $(NH4)_2SO_4$, 2 m*M* dNTP Mix, 1.5 m*M* $MgCl_2$, 20 pmol of each primer, 1 ng of DNA in 10 µl, and 2 units of *Taq*DNA polymerase in a total volume of 50 µl. The PCR reactions were performed using initial denaturation during 3 min at 95 °C followed by 30 cycles of denaturation for 1 min at 95 °C, primer annealing for 1 min at 60 °C, and primer extension for 1 min at 72 °C. This procedure was followed by a final extension reaction at 72 °C for 10 min. For negative controls for PCR reactions, sterile distilled water instead of DNA was used. The PCR product was bi-directionally sequenced using the forward, reverse and internal primers. The multiple sequence alignment program Clustal W (Chenna*et al*., 2003) was used to align the 16S rRNA sequence of the strains. Sequences of rRNA genes, for comparison, were obtained from the NCBI GenBank and RPD database. Evolutionary distance matrices were calculated using the algorithm of the Kimura two-parameter model (Kimura, 1980). A phylogenetic tree was constructed using the neighbour-joining method

(Saitou and Nei, 1987) with bootstrap re-sampling (data re-sampled 100 times) to assess the degree of support for the phylogenetic branching indicated by the optimal tree.

Results and discussion

Phenotypic characterisation

The three extreme halophiles as well as the most related strains were all alkali tolerant, halophilic, Gram-negative and non-sporulating rods. The differential characteristics among the three strains under investigation and their closest phylogenetic relatives, *H. aquamarina, H. marina, H. halodurans, H. venusta* and *H. elongata,* are given in Table 1. The morphological and biochemical characteristics of the three extreme halophiles are almost similar. However, a few among these characteristics such as motility, penicillin sensitivity, acid production in the presence of sugars and some enzymatic tests differentiated

Table 1. Differential phenotypic characteristic of halophilic bacterial strains, IJ1, IJ5, IJ6 and related species of *Halomonas*

Characteristics	IJ1	IJ5	IJ6	*H. aquamarina*	*H. marina*	*H. halodurans*	*H. venusta*	*H. axialensis*	*H. elongata*
Gram stain	Gram (-)	Gram (-)	Gram (-)	Gram (-)	Gram (-)	Gram (-)	Gram (-)	Gram (-)	Gram (-)
Cell morphology	Short rod	Short rod	Short rod	Rod	Rod	Rod	Rod	Rod	Long rod
Density and Elevation	Opaque, Convex	Opaque, Convex	Opaque, Convex	-	-	-	-	-	-
Margin and configuration	Entire, Round	Entire, Round	Entire, Round	-	-	-	-	-	-
Pigmentation	Off white	Off white	Off white	Off white	Off white	Off white	Yellow	Off white	White
Motility	-	+	+	+	-	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	-	+	+	+	-	+	+	+	-
H_2S production	-	-	-	-	+	-	+	-	+
Nitrate reduction	-	-	-	+	-	-	+	+	+
Penicillin sensitivity	Sensitive	Resistant	Resistant	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gelatin liquefaction	+	-	+	-	-	-	-	-	-
Starch utilisation	+	-	-	+	+	-	-	-	-
Cellulose hydrolysis	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heat tolerance	-	-	-	-	-	-	-	-	-
Sea-salt range (% w/v)	2-20	0-25	0-25	0-20	0-20	3-20	0-20	0.5-25	0-20
Sea-salt optimum (% w/v)	5-10	5-15	5-15	7.5-10	3-15	8	0.5-7	4	11
pH range	5-10	5-10	7-10	5-10	5-10	5-10	5-10	5-12	5-10
Acid production from									
Adonitol	-	-	-	-	-	-	-	-	+
Arabinose	-	-	-	+	-	+	-	-	+
Cellobiose	-	-	+	-	-	+	+	-	+
Dulcitol	-	-	-	+	-	-	-	-	-
Fructose	+	-	+	-	+	+	+	-	+
Glucose	+	-	+	+	+	+	+	+	+
Inositol	-	-	-	+	-	+	+	-	+
Lactose	-	-	+	+	-	+	-	-	+
Mannitol	+	-	+	-	+	+	+	-	-
Mellibiose	+	-	+	+	-	-	-	-	-
Raffinose	-	-	+	-	-	-	-	-	+
Rhamnose	-	-	-	-	-	-	-	-	+
Salicin	-	-	+	-	+	+	-	-	-
Sorbitol	-	-	-	-	-	+	+	-	+
Sucrose	-	-	+	+	-	-	+	-	-
Trehalose	+	-	+	+	+	+	+	+	+
Xylose	+	-	+	-	+	+	-	-	+

Data from the present study, and for comparison from Mata *et al.,* (2002), Kaye *et al.,* (2004) and Martýnez-Checa *et al.,* (2005); n.d.: not determined

the strains from each other,. IJ1 is non-motile, oxidase-negative and sensitive to penicillin. IJ1 was able to utilise both starch and gelatin; IJ6 was able to utilise gelatin but not starch and IJ5 gave negative tests for both starch and gelatin. Among the physiological characteristics, it was observed that IJ1 grew aerobically in enrichment medium containing 2-20 % NaCl, whereas IJ5 and IJ6 were able to grow in medium containing 0-25 % NaCl. IJ5 and IJ6 had no specific requirement for NaCl. IJ1 was found to be both acid- and alkali- tolerant with an optimum at pH 7.0.

Genotypic characterisation

The 16SrRNA gene sequences of strains IJ1, IJ5 and IJ6 were determined and compared with all sequences currently available for members of the genus *Halomonas* and related taxa with the closest matches (Arahal*et al.,* 2002). Based on the nucleotide homology and phylogenetic analysis, IJ1 was detected to be *H. marina* strain DSM 4741 (GenBank Accession Number KC599209). The nearest homolog was found to be *H. halodurans* strain DSM 5160. The strain IJ5 was detected to be *H. aquamarina* (GenBank Accession Number: KC620376) and the nearest homolog was found to be *H. venusta* (GenBank Accession Number: AB362301). Strain IJ6 was detected to be *H. aquamarina* (GenBank Accession Number: KC599210) and the nearest homolog was found to be *H. axialensis* (GenBank Accession Number: AB305219). The results are presented as a phylogenetic dendrogram (Fig. 1, Fig. 2 and Fig. 3) showing that the strains IJ1, IJ5 and IJ6 as members of the genus *Halomonas*. The heterogeneity of the genus *Halomonas* was reported by Mata *et al.* (2002).

Description of Halomonas marina (strain IJ1; GenBank Accession No. KC599209)

Strain IJ1 was an alkali-tolerant, halophilic, non-sporulating, Gram-negative rod. Colonies on nutrient agar were off-white, opaque, circular and convex with entire margins. It tolerated up to 20% NaCl with optimum growth at 5-10% NaCl. Growth did not occur in the absence of NaCl. The isolate grew well in minimal media containing glucose, fructose, mannitol, mellibiose, trehalose and xylose. Catalase was present. The isolate gave negative tests for oxidase, H_2S production, nitrate reduction and cellulose hydrolysis. The strain was able to liquefy gelatin, utilise starch and was penicillin-sensitive. This species was seen to be phylogenetically related to *H. halodurans* strain DSM 5160 (Fig. 1).

Description of Halomonas aquamarina (strain IJ5;GenBank Accession No. KC620376)

Strain IJ5 wasan alkali-tolerant, halophilic, non-sporulating, Gram-negative rod. Colonies on nutrient agar were off-white, opaque, circular and convex with entire

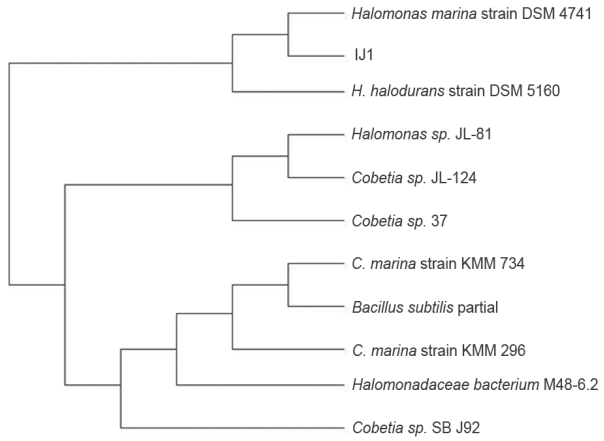

Fig. 1. Neighbour-joining tree based on 16SrRNA gene sequences showing the phylogenetic relationships of strain IJ1 and other related *Halomonas* species

margins. It tolerated up to 25% NaCl with optimum growth at 5-15 % NaCl. Growth also occurred in the absence of NaCl. Catalase was present. The isolate gave negative tests for H_2S production, nitrate reduction, gelatin liquefaction, and starch and cellulose hydrolysis. The isolate was penicillin-resistant. This species was seen to be phylogenetically related to *H. venusta* (Fig. 2).

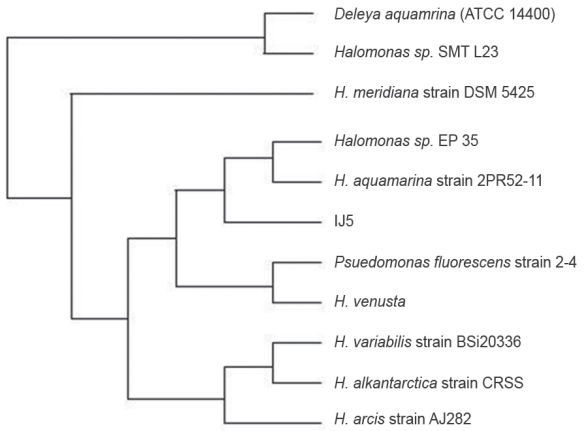

Fig. 2. Neighbour-joining tree based on 16SrRNA gene sequences showing the phylogenetic relationships of strain IJ5 and other related *Halomonas* species

Description of Halomonas aquamarina (*strain* IJ6; GenBank Accession No. KC599210)

Strain IJ6 was analkali-tolerant, halophilic, non-sporulating, Gram-negative rod. Colonies on nutrient agar were off-white, opaque, circular and convex with entire margins . It tolerated up to 25% NaCl with optimum growth at 5-15% NaCl. Growth also occured in the absence of NaCl. The isolate grew well in minimal media containing cellobiose, fructose, glucose, lactose, mannitol, mellibiose, raffinose, salicin, sucrose, trehalose and xylose. Catalase

was present. The isolate gave negative tests for H_2S production, nitrate reduction and starch and cellulose hydrolysis. The strain was able to liquefy gelatin and was penicillin-resistant. This species was seen to be phylogenetically related to *H. axialensis* (Fig. 3).

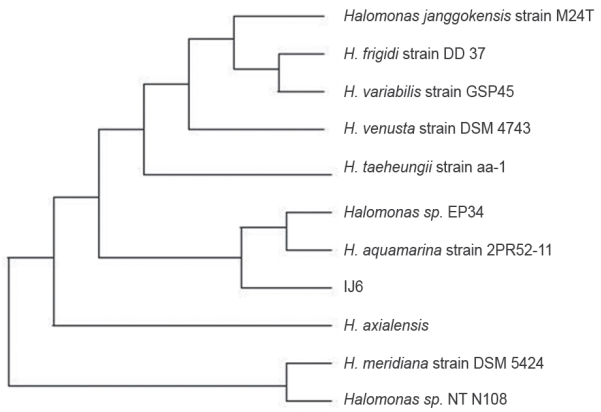

Fig. 3. Neighbour-joining tree based on 16SrRNA gene sequences showing the phylogenetic relationships of strain IJ6 and other related *Halomonas* species

Members of the genus *Halomonas* are reported from a wide variety of habitats that encompass a broad range in salinity, temperature, hydrostatic pressure, organic carbon concentration and pH (Ventosa *et al.*, 1998; Bouchotroch *et al.*, 2001; Yoon *et al.*, 2002). The success of the genus in diverse marine microbial ecosystems may be attributed to their metabolic and physiological versatility. They are capable of oxidising an extensive variety of organic compounds (Ventosa*et al.*, 1998; Mata *et al.*, 2002), which may contribute directly to their survival in the marine environment by enabling them to take advantage of many forms of transiently available nutrients.

It is the first time in India from any environment *H. aquamarina* has been reported. Due to the uniqueness and possible future application in agriculture and allied sectors, both the halophilic bacterial strains *H. aquamarina* and *H. marina* have been deposited in the culture collection at the National Bureau of Agriculturally Important Microorganisms, Mau Nath Bhanjan, Uttar Pradesh, India.

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