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Short Communication

ISOLATION AND PHYLOGENETIC ANALYSIS OF MARINE GAMMAPROTEOBACTERIA *HALOMONAS ALKALIPHILA* FROM RATNAGIRI COAST, INDIA

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ABSTRACT

Halomonas are extremophile organisms which possess the ability to thrive in high alkaline environments. They are one of the most biotechnologically active bacteria due to their molecular adaptability. There are many species of *Halomonas* identified from India but their source is restricted to only alkaline saltwater lakes or lake sediments. Current study aims at identifying *Halomonas* from marine water. The marine water sample was collected post-monsoon and subjected to screening and species isolation on Mannitol salt agar. The isolated species was analyzed for gram-staining and various biochemical testings; which confirmed the species belonged to genus *Halomonas*. Further 16s rRNA molecular identification confirmed the species to be *Halomonas alkaliphila*. Genetic distance values (Kimura-2-Parameter (K2P)) model was estimated and the phylogenetic tree was constructed using Neighbour-Joining (NJ) algorithm to aid identification. This study forms the basis for future analysis to investigate the phylogenetic diversity of bacteria between inland isolates of *H. alkaliphila* and marine isolates identified in India and elucidate its biotechnological potential.

Keywords: 16s rRNA,Extremophile,Mannitol salt agar, Phylogenetic diversity.

1. INTRODUCTION

Halophiles are extremophile organisms that can grow in very high salt concentration environments. They are generally categorized as extreme but might be moderate based on the extent of their halo-tolerance. *Halomonas* is a microbial genus that is designated as halophilic bacteria.They were first isolated from the island of Bonaire, Netherlands and classified into *halomonadaceae* family [1].Owing to their ability to adapt and survive in salty conditions, there is an increased interest among researchers to study molecular changes upon different salt exposures [2]. *Halomonas* have proven to possess great potential and can be exploited biotechnologically. Their unique physiologies have been harnessed by humans in bioremediation and the production of valuable materials.

Enzymatic activities of *Halomonas* [3, 4] have been studied for various useful applications such as degradation of phenol and catechol [5], production of polysaccharides [6], decontamination of polluted saline habitats [7], denitrification of sewage water [8], production of poly-hydroxyalkanoate [9], and degradation of petrochemicals [10]. Studies on the industrial application of *Halomonas* have also been conducted in India like bioremediation of phenol [11], production of hydrolase [12], amylase and lipase [3]. Many different species of *Halomonas* have been identified in India such as *H. campisalis* [13, 14], *H. maridiana, H. salina, H. shengliensis, H. salifodinae, H. pacifica, H. aquamarina, H. halophile* [4], *H. venusta, H. pantellerinsis, H. alkaliphila* [15],and *H. mongoliensis* [12].

Isolation of *Halomonas* sp. in India is majorly restricted to alkaline saltwater lakes (Lonarlake, Sambhar lake, Pulicat lake) or lake sediments; thus limiting its source, whereas, there many salt-tolerant species isolated from alkaline marine environment from different coastal regions of India like *Vibrio, Aeromonas, Stenotrophomonas, Pseudomonas, Bacillus* and *Fusarium*.Current study attempts to isolate and characterize *Halomonas* from Arabian Sea, west coast of Indian.

2. MATERIAL AND METHODS

The water sample was collected in a sterilized container from the sea surface, post- monsoon in the month of October, 2017 from Wayangani beach area located at 16°55'40''N and 73°16'56''E Ratnagiri coast, India. The pH, TDS, conductivity, temperature and salinity of the water was checked on the spot with the help of a water analysis kit (Electronic India). Purified strain was isolated after 24 hours of incubation on Mannitol salt agar (MSA) (Himedia).

The strain RKBR1 was analyzed for gram-staining, biochemical testing (mannose, glucose, nitrate reductase and urease)and 16s rRNA molecular identification.

The total genomic DNA was isolated by standard phenol/chloroform method [16] with slight modifications. Fragment of 16s rRNA gene was amplified by universal primers 27F: 5'AGAGTTTGATCMTGGCTCAG3'and 1492R: 5'CGGTTACCTTGTTACGACTT3' [17]. PCR was performed in 20 µl reaction volume containing 50 ng template DNA, 0.5 µM of each specific primer. The thermocycler was programmed for initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 90 sec for denaturation, annealing, and extension, with a final extension at 72°C for 5 min. The PCR amplicon product was purified using a gel extraction kit (Qiagen, Germany) following the manufacturer's protocols. Forward and reverse DNA sequencing reaction of PCR amplicon by Sanger's method of sequencing using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

The generated 16s rRNA sequence of our strain RKBR1 was verified by similarity search with the NCBI GenBank database using the BLAST tool [18] and aligned using ClustalW program [19]. The verified 16s rRNA sequence was submitted to the NCBI GenBank. Sequences of 16S rRNA genes of related species were obtained from the NCBI GenBank. Genetic distance values (Kimura-2-Parameter (K2P)) model was estimated using MEGA V. 10.0.5 software [20]. The 16s rRNA sequences were clustered using the Neighbour-Joining (NJ) algorithm to provide a graphical representation of divergence pattern and the resultant tree was constructed using 1,000 bootstrap value.

3. RESULTS & DISCUSSION

Due to seasonal variations, the temperature is lowered in post-monsoon and was recorded to be 21.42°C, the pH of the water was 6.86, the alkalinity of water remains constant throughout the year [21]. The TDS of water post-monsoon was 38.95 (g/l) and conductivity was 15.87 (mS/cm). The salinity was recorded to be 2.26(mg/l). Isolation from the environment is accomplished with the initial use of high salt selection media; therefore Mannitol salt agar (MSA) was used for isolation. The optimum pH and temperature for growth was 9.3 and 37°C respectively. White colonies were seen on MSA and further purified (labeled as RKBR1). Its growth on MSA proves these are salt-tolerant bacteria. The strain was gram-negative and rod-shaped. It gave positive results for mannose, glucose and nitrate reductase but was negative for urease test. All these features aided in the identification of the strain and found to be of the genus *Halomonas* which belongs to class *γ-Proteobacteria* [15]. BLAST analysis of 16s rRNA gene amplification identified the strain RKBR1 to be *Halomonas alkaliphila* with 99% similarity.PCR amplification of 16s rRNA resulted in 781bp nucleotides sequence which is submitted to NCBI GenBank (accession no.MK543498). The frequency of nucleotides were A: 25.48%, T: 19.85%, C:31.37% and G: 23.30%. The GC% is comparatively higher than AT% which is similar to other *H. alkaliphila* strains [22].

Table 1 shows sequence divergence value (measured as K2P distance) between intraspecific species viz., *H. alkaliphila* which ranged from 0.0063 to 0.0235 (mean 0.0177±SD 0.0044). K2P genetic distances were significantly lower between individuals of the same species than between individuals of different species within the *Halomonas* genus. Inter-specific sequence divergence between *H. alkaliphila* and *H. venusta* of the same genus ranged from 0.0000 to 0.0631 (mean 0.0213±SD 0.0035).The Neighbour-Joining tree grouped conspecific individuals in one cluster (fig 1). The phylogenetic tree revealed that the clade belonging to the species *H. alkaliphila* was grouped together separating its closely related species *H. venusta* which is in agreement with the previous report of *H. alkaliphila* [22].

Table 1: Genetic divergence values (Kimura 2 Parameter) of 16s rRNA gene sequence of selected *Halomonas* species

Halomonas alkaliphila (MK543498)		0.0055	0.0055	0.0057	0.0057	0.0057
Halomonas alkaliphila (JX122570)	0.0235		0.0021	0.0064	0.0060	0.0059
Halomonas alkaliphila (MF928149)	0.0235	0.0063		0.0019	0.0019	0.0019
Halomonas venusta (MK371741)	0.0235	0.0631	0.0055		0.0006	0.0000
Halomonas venusta (AB681589)	0.0235	0.0549	0.0047	0.0007		0.0007
Halomonas venusta (AB681651)	0.0235	0.0502	0.0055	0.0000	0.0007	

** Lower diagonal values indicate divergence values and upper diagonal values indicate standard error*

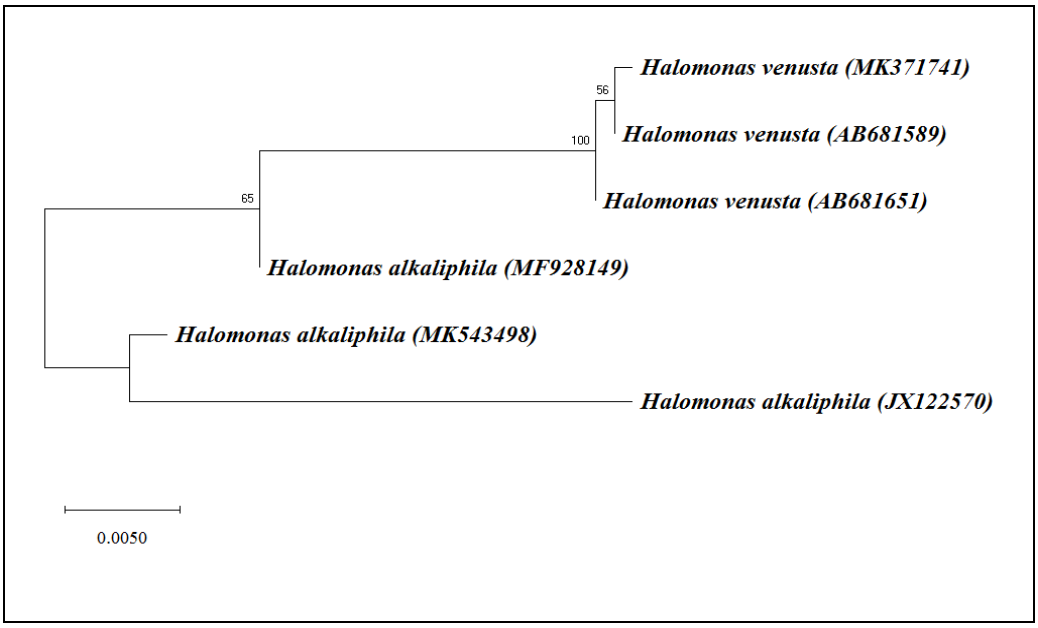

Fig 1: Phylogenetic tree based on 16s rRNA gene sequences of selected *Halomonas*species.
The tree was obtained using the neighbour-joining algorithm. GenBank accession numbers are given in parentheses. The numbers at branching points refer to bootstrap values, based on 1,000 re-samplings

4. CONCLUSION

The current study is the first report of the presence of *H.alkaliphila* in the Arabian Sea of Indian coast. This study forms the basis of future analysis to investigate Phylogenetic diversity of bacteria between inland isolates of *H. alkaliphila* and marine isolates of *H. alkaliphila* identified in India. Further, studies on different enzymatic activity and molecular evolution would be carried out to understand its phylogeny and exploit complete potential of this species.

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