16S rRNA based identification of Aeromonas sp. kumar by constructing phylogenetic tree and identification of Regulatory elements from the Harmful Red tide bloom, Gulf of Mannar.

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Abstract

A bacterial strain, designated Aeromonas sp. kumar, was isolated from a water sample collected from Red tide Bloom occurred in the region of Gulf of Mannar region, Puthumadam Coast, India and the strain was identified using 16S rRNA based identification. During the sample collection, microbiology analysis was done to study the morphology of the bacteria. Pure culture of strain was maintained through out the study. DNA was isolated and sequenced using 16S rRNA primers. A length of 1452 nucleotide was sequenced and was put in public data base for obtaining accession number. The sequence was studied using MEGA 4, to estimate the evolutionary distances and to construct the Phylogenetic tree. Along with that Regulatory elements and Transcription factors were studied using BPROM tool. In genetics, a promoter is a region of DNA that facilitates the transcription of a particular gene. Promoters are typically located near the genes they regulate, on the same strand and upstream (towards the 5' region of the sense strand). The objective of the study is to predict the regulatory elements which are -10 box, -35box and three Transcription Factors (rpoD19, rpoD17 and araC) with their binding sites in the 16S rRNA gene of Aeromonas sp. Kumar. The gene bank accession number for 16S rRNA gene of Aeromonas sp. Kumar is FJ896014.

Key words: Red tide Bloom; PCR; Neighbor-joining method; BPROM; Promoter region;

An algal bloom occurred between Mandapam and Keezhakarai region in the Gulf of Mannar coast during 8th to 12th October. The water was like thick soup green in color. Extensive patches of green coloration were observed in the coast of Puthumadam region. This was due to the outbreak of a massive algal bloom along the coast known as "Red tide" which refers to the discoloration of ocean surface caused by the blooming of planktonic organisms. In laboratory studies, of the water sample collected during the bloom reveals the presence of *Noctiluca Scintillans*[1,2] which shows the evidence of bioluminescence. The *Noctiluca's* are marine dinoflagellates which are of peculiar order. It is usually bioluminescent when distributed, as are various other dinoflagellates and large blooms can sometimes be seen as flickering lights on the ocean. *Aeromonadaceae* falls between the family *Vibrionaceae* and *Enterobacteriaceae*[3] on basis of a collection of molecular genetic data and its phylogenetic relationship. *Aeromonas* are autochthonous to aquatic environment and are usual microbiota of fish amphibians and other animals[4]. The family *Aeromonadaceae* is represented by four genera, *Aeromonas, Tolumonas*, *Oceanimonas*, *Oceanisphaera* and *Zobellella* at the time of writing[5]. Of the above genera, *Aeromonas* is the largest containing about 20 species plus 12 sub-species[6]. In the present research communication, we report the genotypic characterization of *Aeromonas sp. kumar* based on phylogenetic analysis and to identify their regulatory elements. It is quite challenging to collect samples in a form suitable for subsequent analysis together with environmental data about the sampling site. Because ocean is highly heterogeneous in nature and proper attention must be paid to the replication, frequency and location of sampling. The water samples were collected in the coastal waters of Puthumadam, Gulf of Mannar, where the bloom was heavy. The sample was collected using a sterile bottle and immediately stored in the ice. Then sample is brought to the laboratory tests for further analysis. The microbial population in the bloom environment is large and complex. These microbes may be

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present in extremely large in numbers. A variety of techniques have been developed whereby isolation into pure culture can be accomplished. The water samples bought to the laboratory were serially diluted and poured into Zobell Marine Agar. The plates were incubated at 28° C for a period of 48 hrs. After the incubation the morphology of predominant colonies were studied. The bacteria were identified as Gram negative bacteria. It is thought that the Gram negative envelope provides a structure better suited to support the life in nutritionally dilute aquatic environment than Gram positive bacteria cell wall[7]. A gram negative bacterium acts in protection from certain toxic substances. Eg. Fatty acids and Antibiotics. Overnight cultures were grown aseptically for the extraction of DNA. All extractions were done using the hexadecyltrimethylammonium bromide (CTAB) method[8]. In this procedure, all steps were carried out in 2 ml eppendorf tubes and centrifugations were at 14,000 rpm in a micro centrifuge. The samples of about 1.5 ml of culture is taken along with 175 l CTAB buffer (2% CTAB; 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 0.2% 2-mercaptoethanol), 2 l proteinase K (20 mg/ml) was added, and samples were incubated at 55°C for 2 hr. Further extractions were done with 150 l of chloroform, isoamyl alcohol (24:1) and 150 l of phenol: chloroform: isoamyl alcohol (25:24:1with 5 min centrifugations between steps. The DNA was precipitated by addition of 150 l isopropanol (–20°C) and pelleted by a 20 min centrifugation. The DNA was washed with 400 l of 70% ethanol, centrifuged for 5 min, air dried, and resuspended in 30 l of distilled water. This procedure was altered to some extent which suits our laboratory conditions. Amplification of the 16S rRNA gene from the strain *Aeromonas sp. kumar* was done with primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'- TACGGYTACCTTGTTACGACTT-3'). Standard PCRs were performed on 2 l of template in a 25 l volume containing 0.4 M of each primer. 0.125 mM dNTPs, 2.0 mM Mgcl2, ChromTaq DNA Polymerase assay buffer (10X), and ChromTaq DNA Polymerase enzyme. Thermal cycling conditions were as follows: 94°C for 2 min, then 38 cycles (94°C for 30 sec, 58°C for 50 sec, 72°C 1'30'' sec)

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followed by 72°C for 10 min. The amplified products was separated by the dideoxy chain terminator method, using the BigDye Terminator kit (Perkin-Elmer), followed by capillary electrophoresis on ABI 310 Genetic analyzer (Applied Biosystems). An almost-complete (1453 nt) 16S rRNA gee sequence of strain *Aeromonas sp. kumar* was used as the query to search for homologous sequences in the GenBank database. Sequence analysis revealed that its closest relative (97 % similarity) was *Aeromonas Hydrophilla HC960715 – 1* followed by *Aeromonas sp. WW7*, *Aeromonas Veronii strain 457c, Aeromonas veronii strain WE08, Aeromonas Veronii strain MN06, Aeromonas veronii strain IH317, Aeromonas veronii strain IH103, Aeromonas veronii br. Sobria strain AE33, Aeromonas veronii br. sobria strain RK77343, Aeromonas sp. NLEPA-1607*. With the same confidence level (97 % similarity) there were some uncultured bacterium clone strains namely *aaa29f05, YCC116, aab17a12, 11b49b03, aaa35g11, aaa30e04*. This strain shows less similarity with *Oceanimonas, Oceanisphaera, Tolumonas* and *Zobellella*. Sequence from its closest relative of about 22 type strains representing different species of the genera *Aeromonas, Tolumonas, Oceanimonas, Oceanisphaera* and *Zobellella* of the family *Aeromonadaceae* and of the genus *Vibrio* within the family *Vibrionaceae* were used for phylogenetic analysis. The above sequences were multi-aligned using Clustal W[9], a general purpose software program for multiple sequence alignment and edited manually. Aligned Sequences were studied using MEGA 4[10] software for phylogenetic inference. To obtain a confidence value for the aligned sequence dataset, bootstrap analysis of 100 replications was done using MEGA 4. Phylogenetic tree was constructed using the reference strains by neighbour joining method based on Kimura two parameter distance[11]. Irrespective of the tree generation topologies software packages used, the overall tree topologies were similar in all cases. Phylogenetic analysis revealed the strain *Aeromonas sp. kumar* fall with the radiation of the family *Aeromonadaceae* (neighbour-joining analysis shown in Figure 1). The phylogenetic analysis also shows that the closest relative was Aeromonas veronii strain CYJ108[12] from the *Aeromonas*

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cluster and the *Oceanimonas, Oceanisphaera, Tolumonas* and *Zobellella* cluster with a very high bootstrap value. *Escherichia coli ATCC 11775* was used as an outgroup. All along with the DNA sequence of *Aeromonas sp. kumar* 16S rRNA (Accession No. FJ896014) gene from the Genbank as an input sequence for the identification of regulatory elements analysis because this strain has maximum similarity (97% identity) during BLAST[13]. Bacterial promoter prediction program, BPROM[14] were used to identify the position of the promoter i.e. Transcription Start Site (TSS), -10 box and -35 box in input sequence. It also predicts the Transcription Factor (TF) with their binding sites. Genomic regulatory elements were frequently represented by DNA motifs. These promoters contain conserved sequences which are required for specific binding of RNA polymerase and transcription initiation. The main objective is to discuss the transcriptional regulatory elements, more specifically, promoter elements and DNA binding sites that are bound by the TFs. In the 16S rRNA *Aeromonas sp. kumar* FJ896014, there were two predicted promoter gene contains three signals name Transcription Start Site (TSS), -10 box and -35 box from the stop and start position of the input sequence, respectively in Figure 2. The details about the three TF rpoD19, rpoD17 and araC are listed below in Table. 1. There is no TF for the second promoter region in Figure. 2. In conclusion, the r-RNA based analysis is a central method in microbiology used not only to explore the microbial diversity but also used as a method for bacterial identification.

In the present study genomic DNA was extracted from the strain and 16S rRNA sequence was done and a total of 1453bp of 16S rRNA was obtained. The PCR targeting conserved nucleic acid sequence of 16S rRNA gene of bacterial isolate as a molecular tool is used to identify the presence of *Aeromonas sp. kumar* in the water sample during Red tide bloom, Gulf of Mannar. A phylogenetic tree was constructed and the closest relative was *Aeromonas veronii strain CYJ108.* Along with that the promoter region containing signals were identified using BPROM for the identification

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of their binding sites. The data provide a better idea to understand the mechanisms of transcription regulation of 16S rRNA gene in *Aeromonas sp. kumar*.

Figure 1. The evolutionary history was inferred using the Neighbor-Joining method[15]. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates)[16] is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method[17] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 664 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

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>gi|226973714|gb|FJ896014.1| Aeromonas sp. kumar 16S ribosomal RNA pseudoge
 Length of sequence-      1453
 Threshold for promoters -  0.20
 Number of predicted promoters -      2
 Promoter Pos:   1218 LDF-  4.80
 -10 box at pos.   1203 TGCTACAAT Score    72
 -35 box at pos.   1182 TTACGG    Score     9
 Promoter Pos:    440 LDF-  3.13
 -10 box at pos.    425 TGGTAAGCT Score    61
 -35 box at pos.    405 TTTCAG    Score    30

 Oligonucleotides from known TF binding sites:

 For promoter at   1218:
     rpoD19:  ACGTGCTA at position    1200 Score -  12
     rpoD17:  GCTACAAT at position    1204 Score -   8
       araC:  ACAGAGGG at position    1219 Score -  12
 No such sites for promoter at    440
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Figure 2. Output of BPROM tool using SoftBerry.

TF	TF binding site	Position of TF binding site	Score	Reference	Function
rpoD19	ACGTGCTA	1200	12	-	Putative protein
rpoD17	GCTACAAT	1204	8	-	Putative protein
araC	ACAGAGGG	1219	12	Swiss-Prot Entry - P0A9E0	This protein controls the expression of at least six genes that are involved in the transport and catabolism of L-arabinose. It regulates initiation of transcription of the araBAD operon and it also controls its own synthesis. The L-arabinose operon displays both positive and negative

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		regulation through araC

Table 2. Details of Putative TF binding sites with sequence and function found in the Aeromonas sp. kumar 16S rRNA gene in the first promoter region.

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