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MOLECULAR CHARACTERIZATION AND MODELING OF SECONDARY STRUCTURE OF 16S rRNA FROM *AEROMONAS VERONII*

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ABSTRACT: *Aeromonas veronii* is a human pathogen that causes diarrhea, wound infections and hemorrhagic septicemia. *A. veronii* isolated from Gomti river water and identified on basis of 16S rRNA sequences, which showed the high degree of homology with existing sequences. Moreover, the 16S rRNA sequences were also being used for modeling of RNA secondary structures and resembles with Gibbs free energy of other available strains. The result indicates the accurate molecular identification of *A. veronii* and its phylogeny shows the high degree of homology with diverse source of other strains.

Keywords: *A. veronii;* RNA secondary structure; Diseases; phylogeny.

INTRODUCTION

Aeromonas veronii, is a food borne bacterial pathogen causing infections in fish, animals and humans. It is Gram-negative, rod-shaped bacteria found in fresh water and have been associated with animal diseases. *A. veronii* is an opportunistic human pathogen that causes diarrhoea and extraintestinal infections, i.e., wound infection and septicaemia. *A. veronii* causes various diseases ranging from wound infections and diarrhea to septicemia in immunocompromised patients (Austin and Austin, 1999; Janda and Abbott, 1996; Isonhood and Drake, 2002). The spread of *Aeromonas* species is remarkably high it switches from one environment to other. Therefore, an immediate attention to prevent the spreading of *Aeromonas* species in new area and the development of the accurate method for identification is needed. The conventional biochemical tests used for identification of *A. veronii* are time consuming, laborious and are not always conclusive.

The ribosomal operons mainly 16S rRNA has proven to be a stable and specific molecular marker for the identification of bacteria. The copy number of 16S rDNA genes may fluctuate from 1 to 15 among different bacterial genomes. The 16S rDNA is present in scattered form in the entire genome of any bacteria. These ribosomal sequences are useful for the phylogenetic analysis and molecular taxonomy of bacteria. The 16S rDNA is a common target for the taxonomical purpose, mainly due to the mosaic composition of phylogenetically conserved and variable region within the gene.

The identification of bacteria by PCR amplification with 16S rRNA gene has been evaluated for *Aeromonas sobria, A. schubertii* and *A. jandaei* (Ash, et al., 1993) and for *A. hydrophila* and *A. veronii* (Dorsch, 1994). *A. veronii* has been isolated from the septic arthritis patient and identified by 16S rDNA PCR (Roberts, et al., 2006). Therefore, 16S rRNA is a significant target to the molecular level identification. The upstream region of 16S rRNA is known to be highly conserved in species to species so this region could also be used for the verification of the thermodynamic stability on the basis of conserved secondary structures of RNA.

There are many bioinformatics tools available to generate the RNA secondary structures like RNAdraw, RNAfold, Mfold etc. As the MFold uses the nearest neighbor energy rules to calculate the energy of the RNA secondary structures. RNA structure plays an important role in the life cycle of bacteria and provides the ability to understand evolution and stability (Zuker, 1989). Several reports have been available on RNA secondary structure of genes. The 5S rRNA sequences from different bacteria have been used previously for the generation of secondary structure (Singh and Somvanshi, 2009). The aim of present study is to identify the *Aeromonas veronii* at molecular level and predict the secondary structure for evolutionary stability.

MATERIALS AND METHODS

Isolation, media and chemicals

The water sample was collected in sterile 15 ml tube for isolation of *Aeromonas* species from the Gomti River which passes near the district Lucknow, Uttar Pradesh, India. The neat water was spread on the starch ampicillin plate (Palumbo, et al., 1985) using spread plate technique in duplicate and incubated overnight at 30^0C. Single colony was selected for primary biochemical test. All these isolates of *Aeromonas sp* were preserved in 15% glycerol at -80^0C in the laboratory. Taq DNA polymerase, dNTPs, Proteinase K, DNA ladders from Fermentas and other media, chemicals were obtained Sigma, SRL and Himedia. The primers were synthesized from Integrated DNA Technology (IDT, USA)

Biochemical test and isolation of genomic DNA

Yellow and amylolytic colonies (Kidd and Pemberton, 2002) 2-5mm in diameter were subjected to biochemical tests (Barrow and Feltham, 1992). Aero-key was used to confirm identification of *A. veronii* (Joseph and Carnahan, 1994). The cultures were stored at -80^0C in 15% (v/v) glycerol for further characterization. Isolation of genomic DNA from presumptive isolate of *A. veronii* was done by QIAGEN FlexiGene DNA Kit and it was stored at -20^OC for further molecular study.

PCR amplification and sequencing of 16S rRNA gene

PCR reaction was performed in a Thermal cycler (MJ Research PTC200). The reaction mixture 50 µl consisted of 20 ng of genomic DNA, 2.5 U of Taq DNA polymerase, 5 µl of 10 X Taq buffer (100 mM Tris-HCl, , 500 mM KCl pH-8.3) , 200µM dNTP, 10 pmoles each universal primers (forward primer 27F 5'AGAGTTTGATCCTGGCTCAG3' and reverse primer 1492R 5'TACGGTTACCTTGTTACGACTT3') and 2.0 mM $MgCl_2$ was used . Amplification includes initial denaturation at 94^0C for 5 minutes, followed by 25 cycles of denaturation 94^0C for 30 seconds, annealing temperature of primers at 50^0C for 30 seconds and extension at 72^0C for 1 minute. A final extension at 72^0C for 15 minutes was used. Five µl of the amplified product was then analyzed by submarine agarose gel electrophoresis in 1.2 % agarose gel with ethidium bromide at 8V/cm and the PCR product were visualized under Gel doc/UV transilluminator. The amplified PCR product was gel purified using the QIAGEN gel extraction kit. Total 100 ng/µl concentration of 16S rRNA amplified product was used for the sequencing with the 27F primer by the contract research service.

Construction and analysis of Phylogeny

The 16S rRNA sequences were compared and aligned with sequences deposited in the NCBI-GenBank database using BLAST (Altschul, et al., 1997) and Ribosomal Database Project II (RDP II) for identification of bacteria. The sequences were aligned in CLUSTAL X (Thompson, et al., 1997). The computed alignment was then manually checked and corrected. Pairwise evolutionary distances were computed using the Jukes and Cantor equation implemented in the MEGA4 program and a phylogenetic tree was constructed by the neighbour-joining method program available online (Tamura, *et al.,* 2007). The 16S rRNA sequences of AV25 were used for constructing phylogenetic tree. A total of 100 bootstrapped values were sampled to determine a measure of support for each node on the consensus tree.

Modeling of RNA secondary structure

The Mfold software was used for modeling of RNA secondary structure of 16S rRNA gene. This algorithm for RNA secondary structure prediction which is based on a search for minimal free energy state was used widely (Zuker, 1989). The genetic algorithm (GA) simulates the natural folding pathway which takes place during RNA synthesis. This feature not only enables new stems to be added in the growing RNA chain, but also allows structures to be removed at later stages of the simulation if other pairings are found more favorable. The NUPACK tool was also used for designing of secondary structure.

NCBI accession number

The 16S rRNA gene sequences of *A. veronii* AV25 has been submitted to NCBI- GenBank under accession number GQ891129.

RESULTS AND DISCUSSION

Aeromonas veronii is present widely in aquatic environment and known to pose an aquatic animal health risk. Gomti is one of the most important river in India, and represents a unique niche for many animals and plants. We have used the water sample for isolation of bacteria with special emphasizes on Ampicillin antibiotic resistance *A. veronii*. The presumptive isolate AV25 gave result i.e., Gram negative, motile, rod shaped and thus, produced oxidase, catalase, acid and gas from glucose. It has also produced the indole and hydrolyzed arginine and lysine. The conventional identification of bacteria on the basis of biochemical characteristics is generally not accurate as identification. These tests are although useful but they are laborious, time consuming and could give enormous identification. These biochemical tests were not always conclusive. Some of these conventional methods require the use of as many as 18 biochemical tests for species identification and six additional tests are also necessary to differentiate the species included within the *A. hydrophila* complex i.e., *A. hydrophila, A. bestiarum* and *A. salmonicida* (Janda, 1996). *A. veronii* may spread in one environment to other by infections and causes serious problems as it is also one of the most important pathogen of humans as well. Therefore, it may pose as a high risk for humans to use the *A. veronii* contaminated water and fish from Gomti or other such rivers comprising same types of bacteria. The water body may pose as carrier for several pathogens. So it is an urgent need to make surveillance program necessary to verify the quality of water timely.

In the present study, the universal primers were used for the amplification of 16S rRNA ribosomal smaller subunit as this plays an important role in the protein synthesis. The small subunit of ribosomal DNA is highly conserved in nature and can be used for molecular identification of bacteria and remain same in the process of evolution. Here, we used the 16S rRNA fragment for molecular level characterization of isolate AV25. The comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique (Clarridge, 2004).

The 16S rRNA ribosomal PCR amplified product size was 1465 bp. The 16S rRNA gene sequences showed 100% similarity with *A. veronii* in the existing NCBI database. The sources of different strains of *A. veronii* were given (Table 1). The upstream 5'- region of 16S, rRNA sequences were about 500 bp highly conserved in the bacteria. Oligonucleotide probe was designed from highly variable region may help in the rapid screening of isolates but numbers of different probes are required. Moreover, identical target region make it difficult to differentiate the pairs *A. cavaei- A. trota, A. eucrenophila- A. media and A. hydrophila- A. encheleia* (Ash, et al., 1993; Dorsch, 1994).

The upstream 5'- region of 780 bases of 16S rRNA sequences of *A. veronii* was used with seventeen other sequences which were retrieved from GenBank having different accession numbers were used for the construction of phylogenetic tree. The origin of distinct types of rRNA gene in a single genome could be explained either by the divergence evolution of each copy or lateral transfer between different species. Three major clades were obtained, while the strain *A. veronii* AV25 was present in the first clade which includes the strains from all possible environments (Figure 1). These results indicate that the *A. veronii* AV25 may pose as a pathogenic strain and causes a serious threat to animals health.

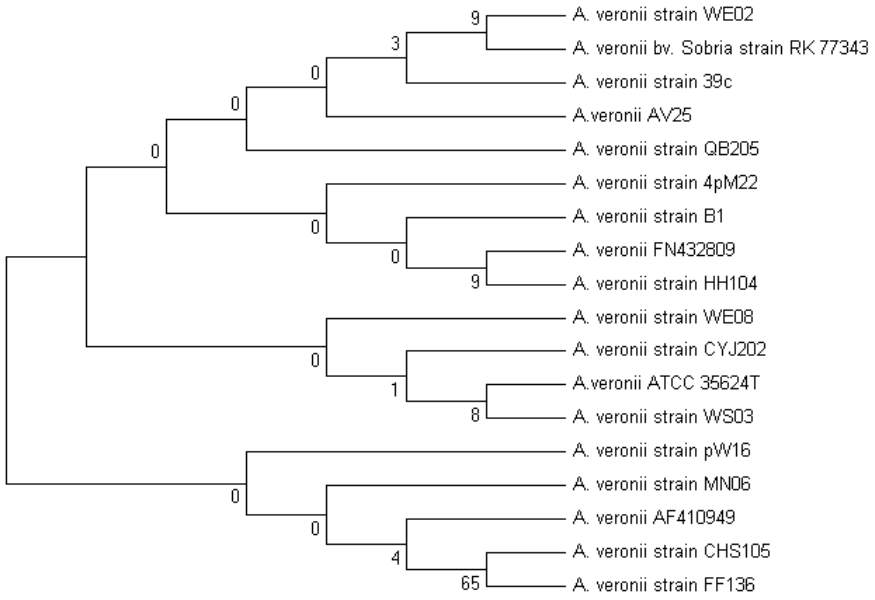

Figure 1. Unrooted phylogenetic tree based on 16S rRNA sequences of strains of *A. veronii* AV25 showing homology with different sources of *A. veronii*.

RNA secondary structure was generated using the 780 bases of 16S rRNA of *A. veronii* from all strains. The free energies for all these strains were considered and it was given (Table 1).

The secondary structure was performed for three different free energies showing *A. veronii* strains such as strain AV25 (Figure 2), strain FF136 (Figure 3) and strain WS03 (Figure 4). The free energy based approach for identification of *A. veronii* was also made and it may be better for thermodynamic use of identification methods. In our study, strain *A. veronii* AV25 showed the –322.40 kcal/mol of free energy of RNA secondary structure was also resembled with other strains from the GenBank. However, three strains FF136 and CHS105 showed the –321.60 kcal/mol and WS03 showed -329.60 kcal/mol. Total four mutations were obtained in all alignment of studied sequences. The energy variation occurs due to the mutation in the 16S rRNA sequences and also changes the stability through the evolution.

Table 1: Summary of diverse source of *A. veronii* strains with Gibbs free energy of 16S rRNA secondary structure.

Strains	Accession number	Source of strain	Free energy Kcal/mol
Aeromonas Veronii AV25	GQ891129	River water	-322.40
A. veronii strain QB205	FJ966348	Blood of black carp	-322.40
A. veronii strain CYJ202	FJ940848	Intestine of crucian carp	-322.40
A. veronii strain pW1	FJ940827	Water of freshwater	-322.40
A. veronii strain 4pM22	FJ940802	Sediment of freshwater	-322.40
A. veronii strain RK 77343	AY987763	-	-322.40
A. veronii strain HH104	EU770270	Carp intestine	-322.40
A. veronii strain MN06	EU770297	Mud from fish pond	-322.40
A. veronii strain WE08	EU770306	Water from fish pond	-322.40
A. veronii strain 39c	EU488692	Feces	-322.40
A. veronii	AF410949	-	-322.40
A. veronii strain B1	AF099024	*Cirrhinus mrigala*	-322.40
A. veronii ATCC 35624T	X74684	-	-322.40
A. veronii	FN432809	Milk sample	-322.40
A. veronii strain FF136	GQ280902	Catfish	-321.60
A. veronii strain CHS105	FJ940835	Intestine of yellow catfish	-321.60
A. veronii strain WS03	EU770309	Water from fish pond	-319.60
A. veronii strain WE02	EU770305	Water from fish pond	-322.40

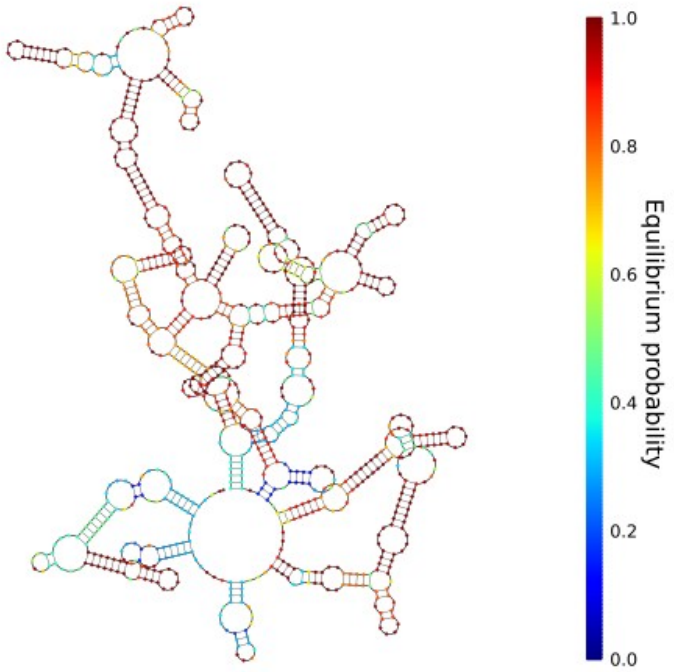

Figure 2. Modeling of RNA secondary structure of 16S rRNA of *A. veronii* AV25 (-322.40 kcal/mol).

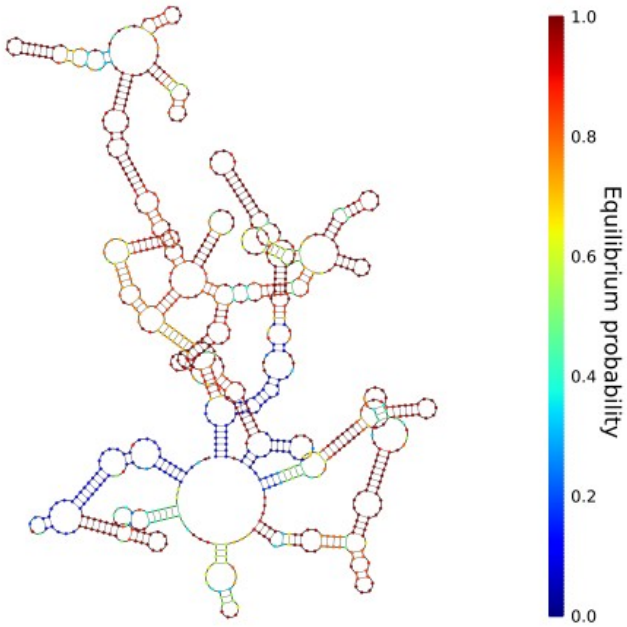

Figure 3. Modeling of RNA secondary structure of 16S rRNA of *A. veronii* strain FF136 (-321.60 kcal/mol).

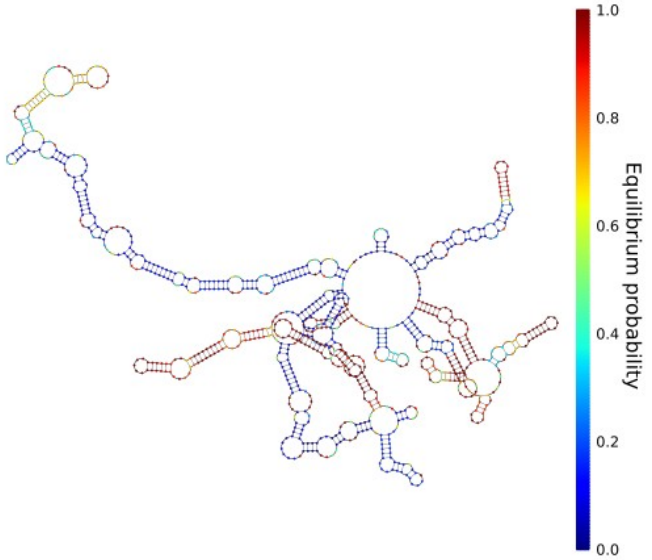

Figure 4. Modeling of RNA secondary structure of 16S rRNA of *A. veronii* strain WS03 (-319.00 kcal/mol).

We can assume that lower Gibbs free energy of RNA secondary structure provides evolutionary stability in comparison to higher temperature. It can be potential risk aquatic organisms because they are surviving at lower temperature and also reservoir for *A. veronii.* The 5S rRNA gene is a highly conserved and determined in 37 bacterial strains and the size ranged between 110-148 bp. The seven clades have been generated and RNA secondary structures from each clade have been made for evolutionary stability earlier (Singh and Somvanshi, 2009).

The 5S rRNA genes from *Sphingobium chungbukense* DJ77 have been identified and the secondary structure of 199-base-long RNA was done and it was found that two-base-longD loop was the shortest among all known 5S rRNAs. The U19-U64 non-canonical pair in the helix II region was unique feature observed in strain DJ77 among sphingomonads (Mashkova, *et al.,* 1990).

The nucleotide sequence of *Pinus silvestyris* 5S rRNA was also determined using two independent methods and then compared with other plant 5S rRNAs. It showed more than 90% sequence homology with gymnosperm 5S RNAs. The free energy analysis of 5S rRNAs from gymnosperms, angiosperms and the other higher plants revealed that the free energy of this ribosomal RNA decreases in evolution (Kwon and Kim, 2007). The secondary structure models exhibited three pairs of 16S rDNA from *Escherichia coli* and *Zea mays* chloroplast ribosome, the 18S rRNA from *Sachharomyces cerevisiae* and *Xenopus laevis* cytoplasmic ribosome, and the 12S rRNA from human and mouse mitochondrial ribosomes. The secondary structure of ribosomal rDNA has been conserved in the evolution (Zwieb, *et al.,* 1981). The Influenza virus encodes conserved non-structural gene which is thermodynamically stable during the evolution. The NS1 of Influenza A virus H5N1 strain varied and the phylogeny has been reported and the thermodynamic free energies were calculated (Somvanshi, *et al.,* 2008).

CONCLUSION

The present study was done to find rapid and accurate identification of *Aeromonas veronii* which will help in prevention and cure of diseases in various aquatic animals. These days modern epidemiologists have been using a variety of tools which provide good molecular differentiation and which can be tailored to fit the needs of the both laboratory and clinical study. Several of these molecular methods could enable the creation of large reference libraries of typed organisms to which new outbreak strains can be compared across the world in order to monitor change in bacterial populations. These techniques will be useful for species identification of bacteria and should be applicable in the studies of epidemiology, diagnosis, virulence and molecular taxonomy.

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