6.0. IN SILICO ANALYSIS OF FISH PATHOGENIC BACTERIA (FPAU01-05) AND SPONGE ASSOCIATED MICROBES (THB-131 AND ACT-21) THROUGH 16S rDNA SEQUENCING

6.1. Introduction

The rRNA is the most conserved (least variable) gene in all cells. Portions of the rDNA sequence from distantly-related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. Thus the comparison of 16s rDNA sequence can show evolutionary relatedness among microorganisms.

The rRNA has been possible to estimate the historical branching order of the species, and also the total amount of sequence change. Molecular tools have a great potential to assist in isolating yet-uncultured bacteria with known rRNA sequences to further investigate or exploit these microorganisms (Kane et al., 1993). One of these molecular tools is the PCR amplification of variable regions of the genes encoding 16S rRNA (16S rDNA) by use of primers homologous to conserved regions of the gene.
Head et al. (1998) reported that, rRNA sequences play a central role in the study of microbial evolution and ecology. Particularly, the 16S rRNA genes have become the standard for the determination of phylogenetic relationships, the assessment of diversity in the environment, and the detection and quantification of specific populations. Indeed, the rRNAs combine several properties which make them uniquely suited for such diverse applications. Hence, present study was made an attempt to find out the name of the bacterial fish pathogens and sponge associated microorganisms by molecular methods.

6.2. Materials and Methods

6.2.1. Extraction of genomic DNA from fish bacterial pathogens and sponge associated bacteria.

A protocol was devised for the extraction genomic DNA from different bacterial fish pathogens. Fish pathogens (FPAU-01 to FPAU-05) and sponge associated total heterotrophic bacteria (THB-131) were grown in 20ml of nutrient broth in 100 ml conical flask at 37°C incubation in a rotary shaker (200 rpm) for overnight.
**Composition of Nutrient broth**

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<tr>
<td>Yeast extract</td>
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The volume was made to 1 litre by adding 50% seawater. pH of the medium was adjusted 7.4 ± 0.2.

Transferred 1.5 ml of bacterial culture to the eppendorf tube and centrifuged at 8,000 rpm for 2 min. The supernatant was discarded and drained well on to a tissue paper. The bacterial pellet was resuspended in 400 µl of sucrose TE buffer. To this, 32 µl of lysozyme (10 mg.ml\(^{-1}\)) was added and incubated the tubes at 37°C for 30 min. Further 100 µl of 0.5M EDTA at pH 8 and 60 µl of 10% SDS were added to it. To this, added 1.5 µl of proteinase K (20 mg.ml\(^{-1}\)) and incubated the tubes at 50°C (water bath) for 12 hrs. Brought the tube to room temperature and added 250 µl of equilibrated phenol (equilibrated with Tris HCl) mixed well and added 250 µl of chloroform. The tube was centrifuged at 10,000 rpm for 10 min and extracted twice with phenol chloroform (1:1 ratio). Again extracted the aqueous phase once with chloroform and isoamylalcohol (24:1 ratio) and collected the supernatant and precipitated with 2 volume of absolute ethanol. Centrifuged it at 10,000 rpm for 10 min and discarded the supernatant. Air dried it
completely and washed the pellet with 70% ethanol and allowed it to dry at room temperature. After complete drying, dissolved the pellet in 20 to 50 µl of sterile distilled water (or) TE and stored at -20°C.

6.2.2. Extraction of marine sponge derived actinomycete genomic DNA.

A protocol was devised for the extraction of genomic DNA extraction from different actinomycetes. ACT-21 culture was grown in 20 ml of tripticase Soya broth in 100 ml conical flask for 7 days and then centrifuged at 4,000 rpm for 5 min. The mycelial pellet was resuspended in 500 µl of 5M NaCl and transferred to a 2 ml eppendorf tube.

### Composition of Tripticase Soya broth

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The volume was made to 1 litre by adding 50% seawater.

The cells were centrifuged at 10,000 rpm for 30 sec and the pellet was resuspended in 1 ml of TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 7.5) containing 20 mg.ml⁻¹ of lysozyme and 20 mg.ml⁻¹ of RNAase A and
incubated at 37°C for 1 h. After incubation, 250 µl of TE containing 5 mg.ml⁻¹ of proteinase K and 100 µl of 10% sodium dodecyl sulphate (SDS) were added to each tube and incubated at 37°C for 1 h. The tubes were mixed by inversion after the addition of 250 µl of 5 M NaCl. Immediately thereafter, 200 µl of Cetyl trimethyl ammonium bromide (CTAB) solution (10% CTAB plus 0.7 M NaCl) was added and the tubes were heated in 65°C water bath for 10 min.

Cellular debris was removed by centrifugation (8,000 rpm for 5 min) and the supernatant solution was transferred to a 2 ml of sterile micro centrifuge tube. Proteins and lipids were removed by the addition of 0.3 volume of saturated solution and the phases were mixed by inversion and centrifuged at 12,500 rpm for 5 min. The aqueous phase was transferred to a sterile tube and the DNA was precipitated with an equal volume of isopropanol. After the genomic DNA was centrifuged (7,000 rpm for 15 min), the pellet was rinsed with 70% ethanol to remove traces of salt, dried and re-dissolved in 200 µl of TE buffer for immediate use or stored at -20°C.

6.2.3. PCR amplification of 16S rRNA

The 16S rRNA gene was amplified from genomic DNA obtained from fish pathogens and sponge associated microbes by PCR with standard universal primers.
### Organisms and Primers

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<td>Fish pathogens</td>
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<td>R-5’-ACGGCTACCTTGTTACGACTT-3’</td>
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The reaction mixture contained 25 to 50 ng of DNA, Ex Taq PCR buffer, 1.5 mM MgCl₂, 10 mM deoxynucleoside triphosphate mixture, 50 pmol of each primer and 0.5 U of Ex Taq polymerase. PCR conditions consisted of an initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 1 min, annealing 58°C for 1 min and 72°C for 1 min and final 5 min extension at 72°C. The amplification products were examined by agarose gel electrophoresis and purified by using a QIA quick PCR clean up kit with the protocol suggested by Qiagen Inc.

### 6.2.4. Sequencing of 16S rRNA gene

The 16S rRNA partial gene was sequenced by using the PCR products directly as sequencing template with above mentioned primers. All sequencing reactions were carried out with an ABI 3730 automated DNA sequencer (Applied Biosystems, Monza, Italy).
6.2.5. Sequence and phylogenetic analysis

Nucleotide sequences were compared to those in the Gene Bank Database with the Basic Local Alignment Search Tool (BLAST) algorithm to identify known closely related sequences. The DNA sequences were aligned and phylogenetic tree was constructed by neighbour joining method by using http://www.ncbi.nlm.nih.gov/blast/tree_view/blast_tree_view.cgi.

6.2.6. Secondary structure of rRNA

The 16S rRNA gene sequences of fish pathogens and sponge associated bacteria and actinomycetes were used for the construction of secondary structure models and were folded using Genbee (Brodsky et al., 1995). These secondary structures were used to assess the significance of observed differences in 16S rRNA gene sequence data.

6.3. Results

The molecular characterization of isolated fish pathogens and sponge associated microbes were characterized by using the partial 16S rDNA sequencing and the results of the BLAST analysis showed that, the sequence of the FPAU01 (680 nucleotides) showed maximum similarity (99%) with Bacillus sp. RS-1 (GenBank Acc. No. HM179550) (Plate. 1), followed by FPAU02 (720 nucleotides) showed maximum similarity (99%) with Bacillus
sp.JSG1 (GenBank Acc. No. JF937058) (Plate. 2), the FPAU03
(640 nucleotides) showed maximum similarity (100%) with Bacillus cereus
(GenBank Acc. No. FR878075) (Plate. 3), the FPAU04 (730 nucleotides)
showed maximum similarity (99%) with Bacillus sp. (GenBank Acc. No.
JF937058) (Plate.4) and FPAU05 (560 nucleotides) showed maximum
similarity (94%) with Bacillus sp. 7-8 (GenBank Acc. No. EU571145) (Plate. 5).
Based on the molecular taxonomy and phylogeny the FPAU01 was identified
as Bacillus sp. RPOCAS1 , FPAU02 was identified as Bacillus sp. RPOCAS2,
FPAU03 was identified as Bacillus cereus RPOCAS3 , FPAU04 was identified
as Bacillus sp. RPOCAS4 and FPAU05 was identified as Bacillus sp.
RPOCAS5 (RPOCAS -R- Ravikumar, P-Palaniselvan, OCAS- Oceanography
and coastal area studies) and the BLAST search results of the 16S rDNA
sequences (770 base pairs) of the sponge associated total heterotrophic
bacteria (THB-131) showed maximum similarity (96%) with Bacillus sp.,
strain H10-7 (GenBank Acc. No. FJ392727) (Plate. 6) and the
sponge associated actinomycete (ACT-21) (240 base pairs) showed
maximum similarity (99%) with Streptomyces sp.’ST-1 Sivasamy BU’
(GenBank Acc. No. GQ423725) (Plate. 7). Based on the molecular taxonomy
and phylogeny the THB-131 was identified as Bacillus sp. RPAUTHB-131
(R-Ravikumar, P- Palaniselvan, AU-Alagappa University, THB131 – total
heterotrophic bacteria 131) and the ACT-21 was identified as Streptomyces sp.
RPAUACT-21 (R-Ravikumar, P- Palani selvan, AU-Alagappa University and ACT21- actinomycetes 21). All the nucleotide sequences of 16S rDNA partial sequences were deposited in the GenBank under the accession numbers of JF899538, JF899539, JF899540, JF899541, JF899542, JN083779 and JF899543 respectively.

The present study was also made an attempt to identify the prediction of RNA secondary structure for the isolated fish pathogens and sponge associated microbes, the results suggested that, the isolate Bacillus sp. RPAUOCAS1 was showed 25 stems and 17 loops. The Bacillus sp. RPAUOCAS2 showed 25 stems and 19 loops, the Bacillus cereus RPAUOCAS3 showed 24 stems and 15 loops, Bacillus sp. RPAUOCAS4 showed 28 stems and 25 loops, Bacillus sp. RPAUOCAS5 showed 20 stems and 12 loops and sponge associates Bacillus sp., showed 32 stem and 21 loop, whereas Streptomyces showed 10 stems and 6 loops. The free energy value of Bacillus sp. RPAUOCAS1, Bacillus sp. RPAUOCAS2, Bacillus cereus RPAUOCAS3, Bacillus sp. RPAUOCAS4, Bacillus sp. RPAUOCAS5 and sponge associated microbes Bacillus sp., and Streptomyces sp., were identified as -121.8 kkal/mol, -136.9 kkal/mol, -121.3 kkal/mol, -151.2 kkal/mol, -95.0 kkal/mol, -166.7 kkal/mol, and -49.7 kkal/mol respectively (Plate. 8).
Plate 1.

(a) FPAU01 16S rDNA sequences

GAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGT
AGTAACACCGTGGGTAACCCTGCCCAATAAGACCTGGGATAACTCCCGG
AAACCCGGGCTAATACCGGATAAACATTGGAAACCACATGGGTTCGAAA
TTGAAAGCCGGCTTCCGCTGTCATTTATGGATGAGACCCCGGCTGCAATT
AGCTAGTTGTTGAGGTAACCCGCTCAACCAAGGCAAACGATGCTAGCC
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ACTCTACGGGAGGCAAGCAGTGGGAATCTTCCCAACATGGGAAAGAA
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AAACTCTGTTGTTAGGGAAGAAACAATGCTAGTTTGAATAAAGCTGCA
CCTTGACCGTACCTAACAGAGAAAGCCACCGCCTAACACGTGCAAGCA
GCCGCGTAATACGTAGGGCAAGCGTATCCGGAAATTATTGGGCG
TAAAGCCCGCGCCAGGTGTGTTCCTAAGCTGATGTGAAAGCCCAACGG
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GAGGAAGTGGAAATCACTGTTGATCAGGTGAAATGCGTACGAGAAT
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(b) Picture showing the Phylogenetic analysis (Blast) of FPAU01

Contd...
(c) Phylogenetic tree of FPAU01

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(d) Picture showing 16S rRNA gene amplification and forward DNA sequencing of FPAU01
Plate 2.

(a) FPAU02 16S rDNA sequences

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(b) Picture showing the phylogenetic analysis (Blast) of FPAU02

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</tr>
<tr>
<td>EF653781.1</td>
<td>Bacillus sp. MG-BC-6 16S ribosomal RNA gene, partial sequence</td>
<td>100%</td>
<td>0.0</td>
<td>100%</td>
</tr>
</tbody>
</table>

(c) Phylogenetic tree of FPAU02
(d) Picture showing 16S rRNA gene amplification and forward DNA sequencing of FPAU02
Plate 3.

(a) FPAU03 16S rDNA sequences

GAATGGATTAAGAGCTTGCTCTTTATGAAGTTAGCGGCGGACGGGTGA
GTAACACGTGGTAAACCTGGCCATAAAGACTGAGGATAACTCCGGA
ACCAGGGCTAATACCGGATAACATTTTTGAACCCGATGCTTACGAA
GAAAGGCGGCTCTTCGTCGATCAGTTATGGATGGAGACCCGCTCAGTTA
GCTAGTTGTTGAGTTAACGCTCAACAAAGCAAGGATGCTGTAAGAAT
ACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACCCGCCG
CTCTACCGGAGGCAGCATGAGAATCTCCGGAAATCGAGAAGCGA
TCTGACGGAACCGCAGCTGAGTGATGAAAGGCTTTCGGGTGTAAC
AATCTGTTGTTAGGAGAAACAAAGTGTAGATTGAAATAAGCTGGAC
CTTACGCGTAACCTAACCAGAAGCCAGCCGCTAACATACCTGCACGAC
GCCGCGTAATACGTAGGTGCAAGCAGTTACCCGAAATTATGGGCG
TAAAGCAGCGCCAGTGTTCATTTAAGCTCTGATGTAAGCCGACCC
CTCAACCGTGGAGGGTGCTATTGAATCTGGGAGACTGAGTGCAGGA
GAGGAAAGTGAATTCATGTGTAGCGGTGAATG

(b) Picture showing the phylogenetic analysis (Blast) of FPAU03

Contd....
(c) Phylogenetic tree of FPAU03

Contd....
(d) Picture showing 16S rRNA gene amplification and forward DNA sequencing of FPAU03
Plate 4.

(a) FPAU04 16S rDNA sequences

TCGAGCGAATGGATTAAGAGCTTGCCTCTATATGAAGTTAGCGGCGGACGGGTGAGAAACACGTGGGTAACCTGCCATAAAGACTGGGATATAACTCCGGGAAACCGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGAGCCCGCTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAATGGGAACAAATCTGTTGTTAGGGAAAGAAACAAGCTAGTTGAAATAGCTGGCACCCTTGACCGTACTTACCAATCCGAAAGCCACGGCTAATACGTAGGCCCAGCGCAGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTTGGAAGCGTAAAGCTACGCTGGTATACGGAGTCGATTGGAAACTGGGAGACTTGAGTCAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAA

(b) Picture showing the phylogenetic analysis (Blast) of FPAU04

Contd....
(c) Phylogenetic tree of FPAU04

Contd....
(d) Picture showing 16S rRNA gene amplification and forward DNA sequencing of FPAU04
Plate 5.

(a) FPAU05 16S rDNA sequences

CAACTGCATTAGGAAGACTTGCTTTATATGGACGAGTAGCGGCGGACGGGTGAGTAACTCGTGGGCAACCTGCCTGTAACATTITTTCTTGCATGCAACA AAATTGAAGATGTTTTCGCTGTAACATTACCTATGGGCGGAGGGAGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCTA GCCGACCTGAGGGTGTACGGCAACAAGGACTGAGACACGGCCGAGACCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTGTAAGTCTCTTGTTAGGAAGAGAACAAGTACAAAGTACTGCTTG TACCITGACGGTAACCTAACCAGAAAGCAGCGCTAATACCTAGTGGCACAAGCAGCGGCTAATACCTAGTGGCGAAATCCGGAATTATGGGCGTAAAGCGCGTCGCACGTCGGGCTTCTTTAAGTCCTGGATGTGTT

(b) Picture showing the phylogenetic analysis (Blast) of FPAU05

Contd....
(c) Phylogenetic tree of FPAU05

Contd....
(d) Picture showing 16S rRNA gene amplification and forward DNA sequencing of FPAU05
Plate 6.

(a) THB-131 16S rDNA sequences

TCTAAAGGTATACCTACACCAGCATTCCGGTGTTACAATAACTCTCGTGTTG
TGACTGCGGCTGTGTAGACGGCGCCCGGAAACGTATTCTACCGCCGGCATGC
TGATCCCGCGATTTAGCGGATTCGCGATTACTAGCGATTCCAGCTTCAGCATTCAATTTGCAAA
CTGCGATCCGAACTGAACTGAATGCTTGGAATTAACCTCAAATC
AGTAAAGGGGTGCTTGTTGCGGGACTTAACCCAACATCTCACAAAC
ACGAGCTGCAGCAACACATGCACCACCTGTCACCCAGCCCCCGGAAG
GGGACACGCTATCTCTAGGATTTTCACAGGATGCTAAGACCTGTTAA
GGATCCTCGGTGTTGAATTAACCCACATGCTCACCACCTGTGGTC
GGGCCCCCGTCAATCTCTTTAGTTTCTGCTGTGACCCGTAATCCTCCCC
AGGCGGATGTTCAATGCTGTCGTTGAACGACCTGCTAGCACACATTAA
ACCCTAACACTTAGACACTACGCTTTACGGCGTGGACTACCGAGGTA
TCTAAATCCGTGCTCCCACGCTTTTGGTGCTCAGGCTAGTATAG
ACCAGAGTCCCCCCACGCTGGTGTTCTCCCCACCTCTACCGCAT
TTCCAAGGTTTA

(b) Picture showing the Phylogenetic analysis (Blast) of THB-131

Contd....
(c) Phylogenetic tree of THB-131

Contd....
(d) Picture showing 16S rRNA gene amplification and forward DNA sequencing of THB-131
Plate 7.

(a) ACT-21 16S rDNA sequences

CAAGGCGACGACGGGTAGCCGGCCCTGAGAGGGCGACCGGCCACACT
GGGaCTGAGACACCGGCACAGCTTACGGGAGGCACAGTCGGGGAA
ATATTGCACTCAATGGCCAGAACCTGATGCAGCGACGCCGCGTGAGG
GATGACGGCCITTCGTTGGTAAACCTCTTTTACGCAGGGAAAGAAGCGA
AAGTGAACGTTACCTGCAGAAAGCAGCCGGCTAACCAGTGCACCA
CGCCGGCGGC

(b) Picture showing the phylogenetic analysis (Blast) of ACT-2

Contd....
(c) Phylogenetic tree of ACT-21

Contd....
(d) Picture showing 16S rRNA gene amplification and forward DNA sequencing of ACT-21
Plate 8. The RNA secondary prediction structure of fish pathogens

(a) FPAU01 (*Bacillus* sp.)     (b) FPAU02 (*Bacillus* sp.)     (c) PAU03(*Bacillus cereus*)     (d) FPAU04 (*Bacillus* sp.)

(e) FPAU05 (*Bacillus* sp.)     (f) THB-131 (*Bacillus* sp.)     (g) ACT-21 (*Streptomyces* sp.)