7. Molecular characterization of the active fish venom gland associated bacterial strain
INTRODUCTION

Regeneration of nutrients in most marine ecosystems is facilitated by marine microbes which are responsible for the mineral cycling and sustenance of living things (Hunter-Cevera et al., 2005). Microbes also play vital roles for the consortia of marine organisms in various forms. Corals cannot survive without the bacterial associations on their surfaces. Similarly, bacteria associated with squid eggs have been demonstrated to protect the eggs from fungal infection. Biofilm bacteria are known to broadcast attraction cues that affect the colonization of invertebrate larvae on it. Thus marine microbial diversity is most essential part of the marine microflora which gives life to the ecosystem (Hunter-Cevera et al., 2005).

Marine microbes have various means to tolerate and grow in extreme conditions like psychrophilic, thermophilic and halophilic regions. This is possible by either changing their biochemistry or creating barriers to evade the harsh conditions out of their cells (Hunter-Cevera et al., 2005). On these conditions they produce diversified metabolites in self or into external environments. Complex metabolic diversity of marine microbes makes the oceans a rich source of biological material for bioprospecting. Unique range of secondary metabolites, enzymes, polymers and metabolic processes can be found in marine habiting microbes. It is likely that many more useful products could be found as research progresses. The biotechnological potential of these organisms is a major driving
force behind the efforts to characterize marine microbial diversity (Hunter-Cevera et al., 2005).

Numerous natural products from marine invertebrates show striking structural similarities to metabolites of microbial origin (Radjasa et al., 2007b), suggesting that they are the true source of these metabolites or are intricately involved in their biosynthesis (Proksch et al., 2002; Thiel and Imhoff, 2003). More than hundreds of new compounds are being discovered from marine flora and fauna including microbes every year (Donia and Hamann, 2003). Such novel compounds are produced by (Lie and Zhou, 2002) high percentage of marine bacteria (Kasanah and Hamann, 2004). More than 99% of microorganisms are not culturable by conventional culture technique (Amann et al., 1995). Such non cultivable bacteria can be identified by their 16s rRNA gene sequence which has led to many revolutionary findings in studies of microbial ecology (Bull et al., 1992).

Advanced techniques of molecular biology such as Polymerase Chain Reaction (PCR), in particular the application of degenerated primers of Non-Ribosomal Peptide Syntetases (NRPS) to amplify gene fragments from peptide producers has allowed screening on the presence of non ribosomal peptides among secondary metabolite-producing microorganisms (Marahiel et al., 1997; Radjasa and Sabdono, 2003; Radjasa et al., 2007a). Cytotoxic potential of
marine gliding bacteria isolated from marine specimens such as biofilms, invertebrates and plant materials of Thai sea water and their 16S rRNA genes were amplified by the polymerase chain reaction (PCR) technique (Sangnoi et al., 2009). This study has been taken with the objective of identifying the strain F3 which showed wide antibacterial activity upto species level with following techniques.

**MATERIALS AND METHODS**

Bacterial strains were isolated from *P. lineatus* venom gland and screened by antibacterial activity. Morphology and biochemical tests were used to identify the strains upto genus level (Simbert and Krieg, 1997; Sneath et al., 1986).

Strain (F3) with wide spectral antibacterial activity was identified upto species level by PCR amplification of the 16s rRNA gene, BLAST analysis and comparison with sequences in the GenBank nucleotide database. Species level identification was done based on nearest phylogenetic neighbor with > 99% sequence similarity (Hentschel et al., 2001).

**Morphological and biochemical characterization**

Purified strains isolated from venom gland were characterized using morphological and biochemical tests according to standard methods such as indole, methyl red, Voges Proskeur, citrate utilization, catalase test (3% H₂O₂),
oxidase test (Kovacs, 1956), glucose, sucrose and lactose assimilation (1% w/v) (Hansen and Sorheim, 1991). Cell morphology by Gram staining and motility by hanging drop method were performed using light microscopy. Isolates were tentatively identified to genus level by comparing their phenotypic characteristics and biochemical test results to typed strains (Chelossi et al., 2004).

**Molecular identification and characterization of F3 strain**

**Isolation of genomic DNA**

Exponentially growing F3 bacterial cells were harvested by centrifugation at 1150 rpm for 15 minutes and suspended in 50ml of colony lysis solution (10 mM Tris-Hcl, pH 7.5; 10 mM EDTA and 50 mg/ml of proteinase K) for molecular identification and phylogenetic analysis. The reaction mixture was incubated at 55 °C for 15 min followed by proteinase K inactivation at 80 °C for 10 min. The reaction mixture was centrifuged at 15,000 rpm at 4 °C for 15 min. The supernatant containing genomic DNA was directly used as template in PCR reaction.

**Presequencing treatment**

Gel elution purification is a technique used to remove the non specific amplification. The DNA was purified from the agarose gel using the following protocol (Chromous Genomic DNA isolation kit). The DNA fragment was excised from the agarose gel with a clean sharp scalpel. The gel slice was weighed in a
colourless tube and 3 volumes of Buffer QG (from Chromous Genomic DNA isolation kit) were added to 1 volume of gel (100 mg ~ 100 µl) and incubated at 50 °C for 10 min until it was completely dissolved. The tube was gently mixed every minute, to ensure complete dissolving of the gel. One gel volume of isopropanol was transferred to the spin column (Chromous quick spin column) placed in a collection tube, centrifuged for 1 min at 1000 rpm; flow-through was discarded and 500 µl of buffer QG was added and centrifuged as above. The column was washed with 600 µl of buffer QE, centrifuged, flow-through was discarded and the column was centrifuged for additional 1 min to complete elimination of buffer, 15 µl water (Milli Q) was added to the centre of the membrane; the column was let to stand for 1 min and then centrifuged for 1 minute. The eluted DNA was used for further amplification or sequencing. The above step was repeated and the final product obtained was checked in the gel and then further used for DNA sequencing.

After PCR amplification the excess of deoxyribonucleotides and primers in samples, which are not utilized during amplification process, should be removed before sequencing reaction or they will interfere with the quality of the results. Hence PCR product purification was done enzymatically using EXO/SAP (Exonuclease-Shrimp Alkaline phosphatase) purification. EXO/SAP was prepared using 0.5 µl of Exonuclease 1 mixed with 0.5 µl of Shrimp Alkaline Phosphatase. 5 µl of PCR product was taken into a PCR eppendorf tube to which
1 µl of EXO/SAP enzyme was added. Then the PCR was carried out with the conditions as mentioned above. This in vitro technique allows a small amount of DNA to be amplified exponentially.

**Polymerase Chain Reaction (PCR)**

PCR amplification of almost full length 16s rRNA gene was carried out with bacteria specific primers, 16s rRNA specific forward (16s forward primer-5' AGAGTRTGATCMTYGCTWAC-3') and reverse primer (16s Reverse Primer-5' CGYTAMCTTWTTACGRCT-3') (Pidiyar et al., 2002). A 25 µl reaction mixture contained 10 ng of genomic DNA, 1x reaction buffer (10mM Tris Hcl, pH 8.8 at 25 °C, 1.5 mM MgCl₂, 50 mM KCl and 0.1% Triton x 100), (2.5 mM each) deoxynucleoside triphosphate (dNTPs) and 3 U of Taq DNA polymerase. PCR was performed in an automated Gene Amp PCR system Thermalcycler (ABI12720) under the following conditions: The initial denaturation at 94 °C for 5 min was followed by denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 2 min (the above steps were operated for 35 cycles) and final extension at 72 °C for 15 min expected PCR product of around 1.5 kb was checked by electrophoresis of 5 µL of the PCR product on 1% agarose gel in 1XTBE buffer and stained with standard concentration of Ethidium Bromide.
Sequencing

PCR product was directly sequenced using the ABI 3130 Genetic Analyzer. The amount of dNTPs and the concentration of the primer used during the PCR were optimized so that there would be no unincorporated dNTPs or excess of primers present in the products. A master mix and SEQ PCR Reaction Mixture (Big Dye Terminator Ready Reaction Mix: 4µl; Template (100 ng/µl) - 1µl; Primer (10 pmol/µl)- 2 µl; Milli Q Water- 3 µl) was prepared in a 1.5 ml tube and dispersed equally into a Micro amp (Applied Biosystem Micro Amp) 96 well plate. The PCR of the Micro amp plate containing the DNA and the mixture was carried out in the Thermal cycler ABI12720 in the conditions mentioned, i.e. in the 1st (Initial denaturation) step 96 °C for 10 sec, 2nd (Denaturation) step 96 °C for 10 sec, 3rd (Hybridization) 50 °C for 5 sec, 4th (Elongation) 60 °C for 4 mins and finally last 4 °C. The PCR was carried out for three hours after optimizing the conditions after which the plate was processed.

Construction of phylogenetic tree

The 16s rRNA gene sequence obtained from the isolated F3 strain was compared with other bacterial sequences by using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search for their pair wise identities. Multiple alignments of this sequence with the sequence available in the data bank were carried out by CLUSTALX 2 version of DDBJ and the phylogenetic tree was constructed in PHYLIP 3.6 using the Neighbor-Joining (NJ) method with
bootstrap value and NJ belongs to the distance-matrix method. The analyzed sequence deposited in GenBank.

**Accession numbers of sequenced isolate**

The nucleotide sequences of the isolate sequenced in this study have been submitted to the GenBank database: [http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) to get accession number.

**RESULTS**

**Morphological and biochemical characterization**

About five bacterial strains were isolated from the fish *P. lineatus* venom gland. The obtained results of staining and biochemical test suggested that F1, F2, F3, F4 and F5 strains were *Micrococcus* sp., *Bacillus* sp., *Staphylococcus* sp., *Pseudomonas* sp. and *Vibrio* sp. respectively (Table 7.1).

Strains F1, F2 and F3 were identified as Gram positive while the rest viz., F4 and F5 were identified as Gram negative. Regarding motility, F2, F4 and F5 were observed to be motile and F1 and F3 non motile. Biochemical tests performed with F1 strain was positive for catalase and oxidase and negative to indole test (Table 7.1). F2 strain showed positive results for Methyl Red, Glucose, Sucrose, Oxidase and Catalase while Indole, Voges-Proskauer, Citrate
and Lactose utilization were negative. F3 strain showed positive results to glucose while negative to catalase and oxidase.

F4 strain showed positive results to Citrate utilization, Oxidase including Catalase and negative response to Indole, Methyl Red, Lactose utilization, Voges-Proskaur, Glucose utilization and Sucrose utilization. In the case of F5 strain, Indole, Lactose utilization, Citrate utilization, Glucose utilization, Oxidase and Catalase were positive and Sucrose utilization and Voges-Proskaur were negative.

**Molecular characterization of F3 bacterial strain**

The molecular characterization of active strain F3 was identified as *Staphylococcus equorum* (JN792134). PCR amplification performed with universal primers produced extracted ~1.5kb product (Fig 7.1) which was sequenced eventually. For phylogenetic characterization, closely related species of F3 were downloaded for the rRNA gene database. For multiple alignments, CLUSTALX 2 version was used. Phylogeny was constructed using neighbor joining method employing bootstrap analysis (Fig 7.2). The results revealed that strains belonged to *Staphylococcus equorum* clustered with strong boot strap value (99%). The accession number was given for strain *Staphylococcus equorum* as JN792134.
Phylogenetic analysis of 16S rRNA gene (rDNA) sequences served as a useful tool for characterization of microorganisms. Such molecular approaches exploring microbial diversity in many different environments have yielded several novel microorganisms that may be physiologically significant (Barns et al., 1993; Fuhrman et al., 1993; Giavannoni et al., 1990; Gray and Herwig, 1996; Haddad et al., 1995; Kuske et al., 1997).

In the present study five strains viz., F1, F2, F3, F4 and F5 were isolated from the venom gland of fish P. lineatus. Based on morphological and biochemical tests and were identified as Micrococcus sp., Bacillus sp., Staphylococcus sp., Pseudomonas sp. and Vibrio sp. respectively. In a similar study, Jebasingh (2008) isolated Gram positive bacteria Bacillus megaterium from marine venomous mollusc Conus virgo.

Anand et al. (2006) observed the dominance of Vibrio, Pseudomonas and Bacillus among the sponge associated bacteria. Pseudomonas, being commonly present in seawater, has also been reported to be associated with marine organism like sponge (Thakur et al., 2003; Romananko et al., 2005). Jebasingh (2008) found Pseudomonas strain from the tubeworm (Hydroides sp.), biofilm and barnacle (Balanus amphitrite) which is corroborated with the present study.
Phylogenetic analysis on 16S rRNA gene sequence showed that the strain F3 (GenBank accession number JN792134) is closest to the species *Staphylococcus equorum* JN230520 with 99% bootstrap value upon phylogenetic tree analysis. The genus *Staphylococcus* is found to be more common in marine environment. Lu et al. (2011) reported 395 pure bacterial strains from East China Sea including 10 species of *Staphylococcus*. Anand et al. (2006) isolated *Staphylococcus equorum* with antimicrobial activity from the sponge *Spongia* sp. and also observed the dominance of *Vibrio, Psuedomonas* and *Bacillus*.

Many associated bacterial strains were identified by molecular techniques. Wang et al. (2008a) reported that TTX (Tetrodotoxin) producing strains with the dominance of *Vibrio* sp. isolated from toxic marine gastropod *Nassarius semiplicatus* by partial 16S ribosomal DNA (rDNA) sequencing and *Bacillus* sp. were also reported. Chelossi et al. (2004) studied viable epibiotic microbial community of the marine sponge *Petrosia ficiformis* and characterized using classical and molecular techniques. He found antimicrobial activity in several isolates, two of which were identified as *Rhodococcus* sp. and *Pseudomonas* sp. by partial 16S rRNA gene sequencing.

Reports regarding molecular characterization of active strain from venom gland of fish are scarce. Best of our knowledge this is the first study from the Gulf
of Mannar region in India. Isolated active strain was identified by phylogenetic gene sequence.

**Table 7.1: Microscopical and biochemical test of isolated strain from the venom gland of fish *P. lineatus***

<table>
<thead>
<tr>
<th>S.no</th>
<th>Test</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Grams staining</td>
<td>+/coccı</td>
<td>+/rod</td>
<td>+/coccı</td>
<td>-/rod</td>
<td>-/rod</td>
</tr>
<tr>
<td>2.</td>
<td>Motility</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Biochemical test - Indole</td>
<td>-</td>
<td>-</td>
<td>NP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Methyl Red</td>
<td>NP</td>
<td>+</td>
<td>NP</td>
<td>-</td>
<td>NP</td>
</tr>
<tr>
<td>5.</td>
<td>Voges-Proskuer</td>
<td>NP</td>
<td>-</td>
<td>NP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>Citrate utilization</td>
<td>NP</td>
<td>-</td>
<td>NP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7.</td>
<td>Glucose utilization</td>
<td>NP</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8.</td>
<td>Sucrose utilization</td>
<td>NP</td>
<td>+</td>
<td>NP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td>Lactose utilization</td>
<td>NP</td>
<td>-</td>
<td>NP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10.</td>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11.</td>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+ Positive; - Negative; NP not performed)
Fig 7.1: 16s rRNA gene product of the strain F3
(Lane description: L: 500bp DNA ladder; 1. ~1.5kb 16s rRNA fragment amplified)

Fig 7.2: 16s rRNA gene phylogenetic analysis of strain F3 with related 16s rRNA sequences found in GenBank database

- **Salinicoccus kunningensis**  DQ837380<sup>T</sup>
- **Staphylococcus arlettæ**  AB009933<sup>T</sup>
- **Staphylococcus cohnii**  D83361<sup>T</sup>
- **Staphylococcus equorum**  AB009939<sup>T</sup>
- **Staphylococcus succinus**  AJ320272<sup>T</sup>
- **Staphylococcus xylosus**  D83374<sup>T</sup>
- **Staphylococcus saprophyticus**  AP008934<sup>T</sup>