Molecular phylogeny and plant growth promoting traits of endophytic bacteria isolated from roots of seagrass *Cymodocea serrulata*

Polpass Arul Jose, Ilangoval Shanmuga sundari, Kunjukrishnan Kamalakshi Sivakala & Solomon Robinson David Jebakumar*

Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai - 625 021, India

[E.mail: jsolomon_mrna@yahoo.com ]

Received 5 April 2013; revised 19 September 2013

In this study, phylogeny and growth promoting attributes of bacterial endophytes isolated from root tissue of seagrass were investigated. Sequence analysis of 16S rDNA among the isolates displayed the presence of bacterial members affiliated to six different genera: *Vibrio, Photobacterium, Bacillus, Aerococcus, Saccharomonospora* and *Kocuria*. Most of the isolates shared high level 16S rDNA sequence similarity with bacteria previously reported from marine water column, crustaceans, corals and terrestrial rhizosphere. Plant growth promoting (PGP) attributes including ability to fix nitrogen, solubilize phosphate and produce ammonia, acetoin and indolic compound were assessed in all the isolates. Genetically diverse, endophytic isolates with different PGP abilities showed close relatedness with bacterial inhabitants of marine ecosystem and likely to have ecological relevance. It implies the need of further studies to validate the relationship between the isolated endophytes and seagrass for sustenance of marine ecology.

[Keywords: Endophytes, *Cymodocea serrulata*, Seagrass, Phylogeney, PGP traits ]

Introduction

Diverse communities of endophytic bacteria are in association with roots of terrestrial and aquatic plants, without exerting any adverse effects to the host\(^1\). The endophytic bacterial population comprises both Gram-positive and Gram-negative bacteria\(^2\); often favour plant growth either by ensuring the availability of limited nutrients or responding to pathogens, stress and pollutants through mutualistic bacteria-plant interactions\(^3-7\). There have been several reports on composition and ecological roles of symbiotic bacterial communities associated with terrestrial and fresh water plants; however, the presence and relevance of endophytic bacterial communities in marine plants remain almost unexplored\(^8\).

Seagrasses are primary producers, developing lush and highly productive meadows in shallow coastal environments with several ecological and biogeochemical significances\(^9-11\). Seagrasses stabilize water quality by filtering suspended matter in the water column\(^12\), prevent surface fouling by inhibiting marine pathogens\(^13,14\) and provide a link between sediment and water column nutrient cycles\(^15-16\). These ecological roles of seagrasses are significantly contributed by the rich associated bacterial communities\(^17-19,14\). Microbial activities in seagrass bed sediments showed strong seasonality and were highest when the plants were actively growing\(^20,21\) and suggest mutual interactions between the seagrasses and associated microbial communities.

Earlier studies on bacterial communities associated with seagrasses have been focused on
bacterial communities in seagrass-bed sediments or associated with seagrass surfaces either roots or shoots. Whereas, studies centred on bacterial communities inhabiting endophytically within the seagrass tissues remain scarce with limited reports. Prevalent endophytic bacterial communities comprising Proteobacteria, Actinobacteria and Firmicutes have been isolated from leaf tissues of Thalassia testudinum. Desulfovibrio zosterae has been isolated from the surface-sterilized roots of Z. marina. Subsequently, Clostridium glycolicum has been isolated from the rhizoplane and deep cortex cells of Halodule wrightii. More recently, endophytic bacterial community of tissues of seagrass Posidonia oceanica has been studied through culture-independent approach using denaturing gradient gel electrophoresis technique, revealing presence of bacterial endophytes that differed among locations and tissue types. While it is obvious that certain endophytic bacteria occur in seagrass tissues, the scope and scale of those bacteria are largely unknown.

There are 13 species of seagrass found in Gulf of Mannar and Palk Bay, among this Cymodocea serrulata is the dominant species. Previous studies on Cymodocea serrulata have been concerned with their extracts and bacteria associated with stem and leaf for antibacterial activity against clinical pathogens. Whereas, studies on diversity of bacteria endophytically associated with Cymodocea serrulata remains scarce. This study was undertaken to isolate and define phylogenetic diversity of bacterial population associated with roots of seagrass Cymodocea serrulata. To obtain information about the biological roles of root-associated bacteria, a series of Plant Growth Promoting (PGP) traits were assessed among the isolates.

**Materials and Methods**

Whole plant samples of the seagrass Cymodocea serrulata were collected in February 2012 from seagrass beds in the vicinity of Kasuwari Island, Gulf of Mannar (fig. 1, Latitude 8° 52’ 18.0” N and Longitude 78° 13’ 20.4” E). The collected samples were placed in sterile plastic bag with a minimal volume of seawater from the collection site and transported with ice to the laboratory. Fresh healthy plants with no sign of obvious injury were washed with sterile seawater and processed within 24 h for isolation of associated bacteria.

Seagrass sample was washed with sterile seawater and roots were separated. Roots of the seagrass were carefully cut with sterile knife and surface sterilized with disinfectant solution containing 2% sodium hypochlorite and 0.1% Tween 20. Samples were rinsed with sterile milliQ water to remove disinfectant and ground in sterile mortar and pestle. Ground suspension was serially diluted and 0.1 mL of each dilution was spread over Zobell Marine Agar medium (Himedia, India) and natural sea water based isolation medium IM131 contained 10 g of starch, 4 g of yeast extract, 2 g of peptone and 18 g of agar in 1 L of sterile seawater. Plates were incubated at 29°C for 1 to 6 weeks. Morphologically distinct colonies obtained were further streaked onto same medium to obtain pure cultures. Stock cultures were made in Zobell Marine Broth with 20% sterile glycerol and stored at -20°C.

Effectiveness of the surface sterilization was validated according to a previously described method. Surface sterilized roots were soaked in 5 mL sterile milli Q and stirred for 1 min. The milli Q (500 µL) was then spread onto Zobell Marine Agar and incubated for 7 d at 29°C. Absence of bacterial growth on the medium was an indication of good surface-sterilization and successful elimination of epiphytic bacteria.

The bacterial isolates were grown in Zobell Marine Broth (Himedia, India) at 37°C for 12 to 72 h and genomic DNA was extracted following standard
phenol–chloroform extraction procedure. To amplify bacterial 16S rDNA, a pair of eubacterial universal primer was used: 27 F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R 5'- GGT TAC CTT GTT ACG ACT T-3'. PCR conditions for amplifying bacterial 16S rDNA were as described earlier. Amplification reaction was performed in thermal cycler (MyCycler, Bio-Rad, USA) and the PCR products were examined by electrophoresis through 1% agarose, stained with ethidium bromide, and visualized on a UV transilluminator.

16S rDNA sequences of bacterial isolates have been deposited in the Gen Bank database under the accession numbers from KC012643 to KC012651.

The partial 16S rDNA sequences of the isolated bacterial strains were initially analyzed and edited by using BioEdit software and the resulted sequences were compared with those available in the GenBank with BlastN algorithm. Sequences were aligned using the multiple sequence alignment program CLUSTAL X with default parameters and the data were converted to PHYLIP format using Bioedit. Phylogenetic tree was inferred by using suitable programs of the PHYLIP (phylogeny inference package) version 3.68. Nucleotide distances were calculated according to the algorithm of Jukes and Cantor by using DNADIST and a neighbour-joining tree was constructed using NEIGHBOUR from the PHYLIP program package. Bootstrap analysis was performed on 1000 random samples taken from the multiple alignments.

In vitro screening of isolates for PGP traits

All the bacterial isolates were qualitatively screened for various growth-promoting traits according to Yadav et al. with minor modifications. Bacterial strains were evaluated for their ability to salubilize inorganic phosphate on Pikovskaya’s agar plates. In vitro indole acetic acid (IAA) production was assayed based on the method described by Hamdali et al. Fresh culture of isolates was inoculated into 5ml of nutrient broth supplemented with 3 mg/mL of L-tryptophan and incubated at 28°C for 5 days. After incubation, the bacterial cells were removed by centrifugation at 10,000 rpm for 10 min. A drop of orthophosphoric acid and 2mL of Salkowaski’s reagent was added to 1mL of the supernatant and incubated at room temperature for 20 min. The development of pink colour was taken as indication for the production of IAA. Production of ammonia was screened in tubes containing 10 mL of peptone water inoculated and incubated with fresh cultures of isolates for 48 h at 30°C. After incubation, about 0.5 mL of Nessler’s reagent was added to each tube and observed for development of brown to yellow colour, positive indication for ammonia production. Production of acetoin was tested among the isolates using standard Voges-Proskauer test in 2 mL of MR-VP broth. The ability of bacteria to fix the atmospheric nitrogen and to grow in nitrogen free medium was tested on Jenkinson’s medium. Isolates were spot inoculated and incubated at 28°C for 3 days. Presence of growth on the medium was a positive indication of nitrogen fixing activity.

All the isolates were subsequently screened for three extracellular enzymatic activities which possibly support plant growth by degrading organic materials associated with the sediments. Isolates were screened qualitatively for amylase activity on starch agar medium containing starch as carbon source. The isolates were spot inoculated and incubated for 3 d at 28°C. After the incubation colonies were flooded with iodine solution and left for 10 mins. Formation of clear zone around the colonies was considered as positive result for amylase activity. Production of protease was screened by spot inoculating the isolates on nutrient agar supplemented with 10% skimmed milk (w/v) and incubating for 2-4 d at 37°C. After the incubation, the formation of the zone of clearance around the colony was taken as an indication of protease activity. Isolates were screened for cellulase activity by spot inoculating them on cellulase activity indicator agar medium prepared with 0.5% carboxymethylcellulose (w/v) and incubating for 48-72 h at 37°C. After incubation, the plates were flooded with 0.5% Congo red solution for 30 min, rinsed with water, and then washed twice with 1 M NaCl. Development of yellow halo around the
colonies against red background was taken as positive indication of cellulose activity1.

Results

A total of 9 morphologically unique bacterial isolates were obtained from roots of seagrass Cymodocea serrulata. Among the two isolation media employed for culturing seagrass associated endophytic bacterial population, natural sea water based isolation agar favoured isolation of 78% of isolates while the Zobell marine agar favoured only 22%.

PCR amplification of 16S rDNA using a set of universal eubacterial specific primers: 27 F and 1492R yielded a single amplicon of ~1500 bp for all the isolates. 16S rDNA of all the isolates were sequenced and acquired sequences were in size range between 1379 bp and 1441 bp. In 16S rDNA based phylogenetic tree (fig. 2), the isolates located in three major clusters affiliated to Gammaproteobacteria, Firmicutes and Actinobacteria.

The most abundant group of isolates was affiliated with the phylum Proteobacteria (Gammaproteobacteria), comprising two different genera Vibrio and Photobacterium accounting for 45% of all bacterial isolates. Isolates JSA02, JSA05 and JAJ06 were found to be Vibrio species while JAJ04 was identified as Photobacterium species. Isolate JAS02 had 99.4% sequence similarity with type strain Vibrio sagamiensis (GenBank AB428909), luminous marine bacteria isolated from sea water46. Other strains JSA05 and JSA06 fell in the same clad in the phylogenetic tree with 99.9% and 99.8% sequence similarity respectively with Vibrio owensii (GenBank GU018180), isolated from cultured crustaceans in Australia47. Photobacterium species JAJ04 shared maximum sequence similarity (99.9%) with type strain Photobacterium rosenbergii (GenBank AJ842344), associated with coral bleaching48.

The second most dominant bacterial group in the seagrass root associated endophytic bacterial community was the phylum Firmicutes, comprising two different genera Bacillus and Aerococcus accounting for 33% of total population. Among the 9 bacterial isolates, 2 species belongs to the genus Bacillus. Of these, strain JSA08 was clustered with nearest type strain Bacillus gibsonii (GenBank X76446) showing 99.5% similarity. Isolate JSA03 shared 99.8% sequence similarity with plant growth promoting bacterium Bacillus methylotrophicus (GenBank: EU194897), isolated from rice rhizosphere49. Aerococcus strain JSA07 was 99.6% identical to the type strain Aerococcus urinaeequi (GenBank D87677).

The actinobacterial isolates belonged to the genera Saccharomonospora and Kocuria representing 22% of entire isolates. Isolate JSA09 was identified as Saccharomonospora species, shares 98.5% sequence similarity with the type strain
Saccharomonospora glauca (GenBank Z38003) isolated from moldy hay. Another actinomycete strain JSA01 was clustered with type strain Kocuria rhizophila (GenBank Y16264) isolated from rhizoplane of the narrow-leaved cattail.

**In vitro screening of isolates for PGP traits**

All the 9 isolates were screened for their multiple PGP traits and summarized in Table 1. Phosphate solubilising ability was observed in 3 (39%) isolates. Actinomycete isolate Kocuria sp. JSA01 showed highest phosphate solubilisation activity with highest zone diameter. Other isolates Saccharomonospora sp. JSA09 and Vibrio sp. JSA05 showed moderate and mild phosphate solubilising activities respectively. Production of growth promoting substance indole acetic acid was observed in 4 (44%) isolates, which includes Kocuria sp. JSA01, Vibrio species JSA02, JSA05 and JSA06. Ammonia production was detected in 6 (62%) isolates. All the bacterial isolates were found to be able to fix nitrogen and grew well in nitrogen deficient medium. Acetoin production was observed only with Vibrio sp. JSA06 while others failed to produce it.

Proteolytic activity was detected in 3 (38%) endophytic bacterial isolates associated with the seagrass roots. Actinomycete strain, Saccharomonospora sp. JSA09 showed maximum proteolytic activity whereas the bacilli strain, Bacillus sp. JSA08 showed minimum activity. Production of extracellular cellulase and amylase were detected in 8 (89%) and 6 (66%) isolates respectively.

**Discussion**

The present study provides the first report of culture dependent endophytic bacterial communities associated with roots of a seagrass, Cymodocea serrulata from the Gulf of Mannar. In this study, 9 different endophytic isolates belonging to six different bacterial genera have been isolated. Sequencing and BLAST analysis of 16S rDNA of isolates revealed the presence of high G+C Gram-positive, low G+C Gram-positive and Proteobacteria groups among the endophytic bacterial population. Presence of specialized bacterial phylotypes in roots of seagrass has previously been reported only through culture-independent approach. Phylogenetic analysis based on 16S rDNA sequences assigned the isolates into five genera; Vibrio, Photobacterium, Bacillus, Aerococcus, Saccharomonospora and Kocuria, categorised in three phyla: Gammaproteobacteria, Firmicutes and Actinobacteria. Prevalence of endophytic bacteria belong to Gammaproteobacteria, Actinobacteria and Firmicutes has been observed in

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Phosphate Solubilization</th>
<th>IAA Production</th>
<th>NH3 Production</th>
<th>Nitrogen Fixation</th>
<th>Acetoin Production</th>
<th>Protease Activity</th>
<th>Cellulase Activity</th>
<th>Amylase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kocuria sp. JSA01</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vibrio sp. JSA02</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus sp. JSA03</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Photobacterium sp. JSA04</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio sp. JSA05</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vibrio sp. JSA06</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aerococcus sp. JSA07</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus sp. JSA08</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saccharomonospora sp. JSA09</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Positive for PGP trait; - Negative for PGP trait; *ND – Not detected
leaf tissue of *Thalassia testudinum*\(^{26}\). Most dominant group of endophytes found in the roots of *Cymodocea serrulata* affiliated with Gammaproteobacteria comprised two genera *Vibrio* and *Photobacterium*. This dominance of Gammaproteobacteria is consistent with previous reports on bacterial communities associated with root surfaces of several seagrasses\(^{24,25}\). Furthermore, a recent report on endophytic bacterial population associated with seagrass *Posidonia oceanica* also suggested abundance of gammaproteobacteria in root tissues\(^{8}\). Most of the endophytes isolated from root tissues of *Cymodocea serrulata* shared high level 16S rDNA sequence similarity with bacteria of rhizosphere or marine ecosystem.

The endophytes tested in this study exhibited multiple PGP traits that may promote plant growth. Multiple PGP attributes of endophytic bacterial population associated with seagrass roots are consistent with views that bacterial-seagrass root interactions are important to plant productivity\(^{52-54}\). Phosphate solubilising bacteria ensure availability of soluble phosphate to the plants by solubilising the insoluble phosphate which is normally unavailable for plants\(^{39}\). In this study, *Saccharomonospora* sp, *Kocuria* sp and a *Vibrio* sp. were found to be positive for phosphate solubilisation. Very recently, phosphate solubilising bacteria have also been isolated from seagrass rhizosphere soil. IAA is an important plant growth hormone render positive effect on root growth and morphology. In this study, four endophytes showed positive result for IAA production and it is consistent with earlier reports of IAA production in seagrass-associated bacteria from other seagrass *Vallisneria americana*\(^{55}\). Moreover, established seagrass tissue cultures have also been shown to benefit from auxins\(^{56,57}\). Capability to fix nitrogen is widespread among many different bacteria isolated from seagrass rhizosphere\(^{55}\). All the isolates tested in this study exhibited nitrogen fixing ability. Presence of nitrogen fixing bacteria has also been identified among the endophytic population associated with *Posidonia oceanica* through culture-independent study\(^{8}\). Ammonia, acetoin, protease, amylase and cellulase production also constitute a very important part of plant growth promotion. The observed PGP attributes are similar to that of bacteria associated with terrestrial rhizosphere\(^{57,59}\) and suggest that interactions between bacteria and seagrass roots might be similar to those described in better studied terrestrial plants\(^{59-61,39}\).

**Conclusion**

This is the first report of isolation and identification of endophytic bacterial communities associated with surface sterilized root tissues of seagrass *Cymodocea serrulata*, suggesting the presence of three phylotypes comprising diverse bacterial genera in seagrass roots. PGP attributes of the endophytic community reveals their possible biological role within the root tissues of the seagrass.

**Acknowledgements**

Authors are grateful for the funding from ‘Research Fellowship in Science for Meritorious Students Scheme’ of University Grant Commission, India and also thankful to the UGC-MRP Scheme for accessing the funded resource facilities in our laboratory.

**References**


56. Koch, E.W., Durako, M.J., In vitro studies of the submerged angiosperm *Ruppia maritima*: auxin and cytokinin


