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Isolation, characterization and growth response of biofilm forming bacteria *Bacillus pumilus* from the sea grass, *Halodule pinifolia* off Kanyakumari coast

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Bacteria from the biofilms of the blade of sea grass, *Halodule pinifolia* occurring along the sea grass beds off Kanyakumari coast (Tamil Nadu) were isolated. Bacteria identified included *Pseudomonas, Staphylococcus, E.coli* and *Bacillus* species. Predominant biofilm bacterium was characterized as *Bacillus pumilus* (Accession No. HM006706) by standard biochemical and molecular methods. Sea grass biofilm associated *B. pumilus* strain was aerobic, Gram positive, rod-shaped, spore-forming motile bacteria. The 16s rDNA gene sequence of the new isolate *B. pumilus* had shown maximum similarity (96%) with other *B. pumilus* strains PRE14, NHIC3, CT3. Growth response due to interaction of *B. pumilus* in the presence of sea grass extract was evaluated by inoculating in different concentration of sea grass extract. Results indicated that more colony forming units (4.6×10^{11} CFUs) in Zobell marine broth supplemented with 20.0% of the water extract of *Halodule pinifolia*.

[Keywords: Bacteria, biofilm, colony forming units, sea grass extract, growth response]

Introduction

Sea grasses host specialized communities of bacteria on their surfaces[10,25]. They form biofilm and these bacteria play a significant role in the nutrition of marine angiosperms[7]. Surface properties of the plant tissue, nutrient, water availability and productivities of the colonized bacteria strongly influence the biofilm structure[24]. Biofilms in marine habitat play the key ecological role of sustaining of populations of invertebrate grazers[26].

The inclination for bacteria to colonize surfaces is a double-edged sword, they can prove either beneficial or potentially destructive[13]. These organisms can promote plant growth by deterring insect and animal herbivory[3] by releasing chemicals that prevent grazing or biofouling by other organism[2] or they may be phytopathogens. Sometime excessive growth of biofilm reduces photosynthetic productivity of sea grass due to shading of light. Within the bacterial community on plants, this complex interaction depends on external environmental factors and the interaction between plant and microorganisms and among the bacteria[4,8,16].

Sea grass *H. pinifolia* is one of the most common and ubiquitous seagrasses in the shores of Kanyakumari. Ability of bacteria to develop biofilms on seagrass surface is a useful feature that is often taken advantage for the design of bioremediation. The 16s rRNA based studies has become the most common method for assessing microbial communities and such approach has revolutionized the field of microbial ecology. Considering the importance of the biofilm forming bacteria, the present study was undertaken to detect the diversity of biofilm forming bacteria which adhere on the sea grass blade and to identify and characterize the predominant bacteria by 16s rDNA method and to infer about the interaction and growth characteristics by analyzing the difference in the their growth behaviour in the presence of sea grass extract.

Materials and Methods

Isolation and characterization of marine biofilm bacteria from sea grass blades

Sea grass *Halodule pinifolia* from Kanyakumari coast were collected in plastic bags containing sterile seawater from submerged marine rocks during the low tide (Plate 1). They were washed with sterile seawater to remove loosely attached epiphytes. Sea grass blades were cut as 1sq cm pieces and aseptically transferred to Zobell marine Agar (Hi Media 2216) plates. After incubating for 18 h at 37^{o}C, those

Plate 1—Station location

colonies growing around the sea grass blades were segregated and purified. Purified typical bacterial colonies were characterized by standard biochemical tests, antibiotic sensitivity pattern and 16s RNA sequence data method.

Morphological characterization of the colonies were documented using standard microbiological approaches[9] and based on morphological appearance such as colony colour, motility, elevation, colony shape, cell shape etc. Standard biochemical tests were carried out to identify the bacterial strain upto genus level[14] and the bacterial isolates were identified. Predominant strain present in the seagrass blades were further characterized using molecular tools because it is difficult to identify the bacteria in lab by biochemical characterization alone. The 16s rDNA gene of the isolates were sequenced (ABI 3100 sequencer and genotyper; Genie) after the DNA isolation and amplification. The sea grass *Halodule pinifolia* blades were thoroughly rinsed with distilled water. The blades were then ground in a mixer grinder in 5 mL of water. This aqueous extract was made bacteria free by passing though 0.2 µm syringe bacterial filters. The clean filtrate obtained was then kept in sterile containers in refrigerator for further use.

The isolated and characterized bacterium was allowed to grow in 25 mL of Zobell marine broth for 18 h at 37°C. After 18 h of incubation, 0.1 mL of bacterial culture was added to four different conical flasks containing 25 mL of the above broth. To three conical flasks containing Zobell marine broth were supplemented with different volumes of filtered sea grass extract to get 4, 20 and 40% of the extract in the medium. One conical flask without extract was kept as control and all these were incubated for 18 h at 37°C. After incubation for 18 h, the serially diluted culture are plated on Zobell marine agar and incubated for 18 h at 37°C. After 18 h of incubation, the colony forming units (CFU) were enumerated.

Results

Morphological and biochemical characteristics

The colonies isolated from *H. pinifolia* blades exhibited different colony morphotypes. Microscopically, rod shaped cells were more abundant in the blades. All are motile forms. Gram staining revealed that rod shaped cells were Gram negative *E. coli* and *Pseudomonas* and Gram positive *Bacillus*. Cocci were represented by the Gram positive *Staphylococcus.* Morphological characteristics are given in Table 1. In the biochemical tests, positive response of *Bacillus* was noted in the amylase test. However the rest of the isolates were negative for Gelatinase. Except *Pseudomonas,* negative results were noticed in caseainase. Acid/Gas test showed positive for all the isolates (Table 2).

Molecular characterization was made only for the predominant strain. Results of 16s rDNA data indicated that the biofilm forming bacteria based on nucleotides homology and phylogenetic analysis was characterized as *Bacillus pumilus*. Bacteria was Gram

Table 1—Morphological characteristics of biofilm forming bacteria on *Halodule pinifolia* (seagrass) blades

Bacterial strains	Morphological tests							
	Gram stain	Morphology	Motility	Elevation	Colour	Colony shape	Cell shape	Margin
Staphylococcus	+	Cocci	-	Flat	Cream	Irregular	Round in tetrads	Undulate
Pseudomonas	-	Rod	+	Convex	Cream	Circular	Uniform bacillary	Entire
E. coli	-	Rod	+	Convex	Cream	Circular	Short rod	Regular
Bacillus	+	Rod	+	Slightly convex	Cream	Circular	Long rods	Undulate

Table 2—Biochemical characterization of biofilm forming bacteria on *Halodule pinifolia* blades

Bacterial strains	Biochemical tests										
	Catalase	MR	VP	Amylase	Gelatinase	Oxidase	Casaenase	Acid/Gas	Indole	Urease	NaCl
Staphylococcus	+	-	-	-	-	-	-	+	-	-	6%
Pseudomonas	+	-	-	-	-	+	+	-	-	-	5%
E. coli	+	+	-	-	-	-	-	+	+	-	2%
Bacillus	+	+	+	+	-	+	-	+	-	-	2%

Table 3—Alignment View

Alignment View	ID	Alignment results	Sequence description
	H4	0.91	Studied Sample
	AM1237349	0.96	*Bacillus pumilus*
	EU880532	0.96	*Bacillus pumilus* St. PRE14
	EU221329	0.96	*Bacillus pumilus* St. NM1C3
	EU660356	0.96	*Bacillus pumilus* St. CT3
	AJ831844	0.96	*Bacillus aerophilus* St. 28K
	AJ831841	0.96	*Bacillus stratosphericus* St. 41KF2a
	EU652063	0.95	*Bacillus anthracis* St. me-12
	EU384279	0.95	*Streptomyces* sp.
	EU931563	0.94	*Bacillus subtilis* St. ZFJ-8
	AJ831842	0.96	*Bacillus altitudinis* St. 41KF2b

positive, rod shaped that displayed mucoid colonial phenotypes. This strain displayed a cream pigmentation in Zobell marine agar plates. Phylogenetic analysis based on 16S rDNA sequence placed this strain as a member of the genus, *Bacillus.* The nearest homolog genus was found to be *Streptomyces* species and this strain is closely related to species *Bacillus stratosphericus*. The identified *B. pumilus* strain showed (96%) homology with other *B. pumilus* strains such as St.PRE14, St.NMIC3, St. CT3. The phylogenetic tree made in MEGA3.1. software using Neighbor Joining Method is given in Fig. 1.

Results on the culturing *B. pumilus* in the sea grass extract showed a profound growth response. *Bacillus pumilus* exhibited growth in the medium containing 4% of sea grass extract. Still higher growth was recorded in medium containing 20% of the extract. However, the low colony forming units was recorded in medium supplemented with 40% of the extract. Nevertheless, the control medium without the sea grass extract showed very low bacterial growth.

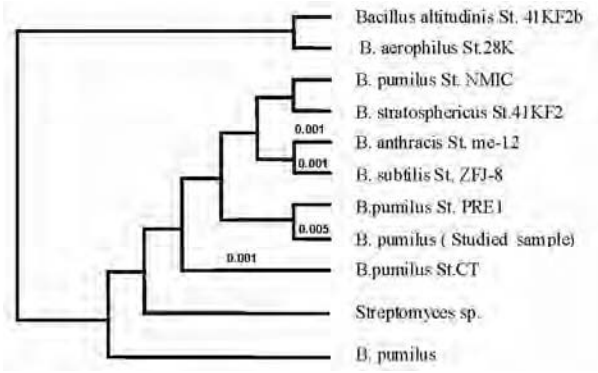

Fig. 1

The Alignment view using combination of NCBI Genbank and Table and RDP Database is given in Table 3.

Discussion

Plant leaves host complex assemblages of bacteria[29]. Leaves of aquatic plants support very active bacterial communities that are thought to be influenced by plant primary production[15]. Various researches describe the diversity of organisms

Table 4—Growth of *Bacillus pumilus* in media supplemented with different percentage of sea grass *Halodule pinifolia* extract

Percentage of *Halodule pinifolia* extract	Colony Forming Units*
Control	4×10^8
4.0	1.5×10^{11}
20	4.6×10^{11}
40	2.8×10^{10}

*Mean value

attached to marine abiotic surfaces[12] but the associated bacterial community present in the seagrass blades and their interaction with the host was negligible. Research results from the Northern Gulf of Elat indicated broad microbial diversity associated with the seagrass *Halophila stipulaceae*[28]. Epiphyte bacterial communities such as Bacteroidetes, Alphaproteobacteria, Betaproteobacteria were identified from three sea grass species from the East African coast[10]. In India, there is no published information about characterization of biofilm forming bacteria on seagrass, and their interaction with host surface.

Biofilm forming bacteria associated with sea grass *Halodule pinfolia* blade was characterized as *Staphylococcus, Pseudomonas, E. coli* and *Bacillus.* The predominant bacteria was characterized as *Bacillus pumilus* by 16s rDNA sequence method. (Accession number HM006706). The presence of *Pseudomonas* species were previously reported in seagrass *Halophila stipulaceae*[28]. The presence of *E. coli* in terrestrial plants was previously reported[6] and these bacteria have the ability to colonize corn, bean under humid condition. There are also few reports that indicate *E. coli* are capable of surviving on the phyllosphere of fresh water[30,23,19] while the presence of *Staphylococcus* in seagrass *Halophila ovalis* was also reported[27]. The predominant *Bacillus* species are ubiquitous and diverse both in the terrestrial and marine ecosystem[22]. This species are able to produce highly hydrophobic spores that are able to adhere firmly to various inert substrata. *Bacillus pumilus* was reported to be the second most dominant species among aerobic spore forming bacteria[18]. *Bacillus* from seawater and marine bottom deposits are halotolerant and they are able to propogate and metabolize under marine condition. The bacteria *Bacillus pumilus* are aerobic, Gram positive, catalase positive, rod shaped and motile bacteria.

The occurrence of bacteria *Bacillus* in seagrass blades were already reported[1] and the *Bacillus* species are among the most common bacteria found to colonize seagrasses and it is likely that they could play a role in the biocontrol of the vascular plant pathogens[1]. *Bacillus* spores are also resistant to unfavorable conditions such as low or no nutrient availability, extreme desiccation, hydrogen peroxide, ultraviolet light, gamma radiation or chemical disinfection[20].

The relation between plants and biofilm can be quite varied. Bacteria physically interact with plants in diverse ways. A common feature of this interaction is surface colonization in which the microbe adhere to external and internal plant tissues as individual cells and in clusters[24]. Bacteria that are successful in establishing pathogenic or symbiotic interactions have developed multiple ways to protect themselves[11].

Plants serve as a mechanical support or plants may provide some nutrients for the microbes. A comparative study made on the adhesion of epiphytic bacteria and marine free living saprophytic and pathogenic bacteria on seagrass leaves and abiotic surfaces to prove the bacteria plant symbiotrophic relationship. *Cytophaga* sp KMM 3552 and *Pseudomonas citrea* KMM 461, on *Zostera marina* seagrass blades showed increased number of viable cells (i.e.) 3-7-fold after 60h of incubation when compared to abiotic surface[18]. In the present study also the sea grass extract supplemented media had supported comparatively faster growth rate of the biofilm forming bacteria. Higher bacterial numbers such as 4×10^{11} CFUs were recorded which corroborated earlier reports of sea grass blades supporting the diverse array of bacteria[18].

The growth response of biofilm forming *B. pumilus* in the presence of sea grass *Halodule pinifolia* extract showed that the growth of the bacterial cells are more in the medium containing 4% of the sea grass extract, than the control (medium containing no sea grass extract). Maximum growth of bacterial cells occurred in the medium containing 20% of the sea grass extract. But very high concentration such as the medium containing 40% of extract showed lower bacterial growth than the medium supplemented with 20% of the sea grass extract indicating that the higher concentration of the extract could reduce the growth of the bacterium.

This result indicated that the transportation of nutrient in and around sea grass beds could enhance

the adhesion of the bacteria *Bacillus pumilus* in the natural conditions. The associated bacteria acquire these necessary nutrition such as vitamins, polysaccharides and fatty acids from the plant host and thus the growth of associated microorganism gets promoted and benefited[2]. Earlier reports proved that *B. pumilus* acts as a biocontrol agent in agriculturally important crops such as tomato, reducing significantly whitefly crawlers, Nymphs and pupae which threatened the plants[17]. Apart from that fungicidal activity against Mucoraceae and *Aspergillus* species was also reported[5]. Results of earlier research suggest that the bioactivity of *B. pumilus* might be vital for the health of seagrasses assuming the roles similar to those reported earlier in the terrestrial plants[14].

The predominant nature of *Bacillus* and the growth response showed that a beneficial interaction that exist between surface bacteria *B. pumilus* and the sea grass *H. pinifolia*. The present study provides a platform for further studies of interaction between marine bacteria in surface associated communities and between biofilm and eukaryotic host surface and the exploration of such beneficial biofilm bacteria will be of interest in marine biotechnology.

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