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RESEARCH ARTICLE

CHARACTERIZATION OF MANGROVE ASSOCIATED NITROGEN FIXING HALOPHILIC BACTERIUM *Paenibacillus* sp.

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ABSTRACT

Conservation of mangrove through improved mangrove nursery technologies using bacterial bio-fertilizers will be appropriate solution for deforestation. Members of the genus *Paenibacillus* widely reported for its nitrogen fixing character from various environments. In this study we identified a halophilic nitrogen fixing bacterium from Pichavaram mangrove rhizosphere soil of *Avicennia marina*, which is dominant flora in the forest. The bacterium showed 2017±32.60 nmol ethylene mg protein^{-1} of nitrogenase activity hence it is highly comparable with all other nitrogen fixers reported in the genus. Further the bacterium characterized by 16S rRNA gene sequence and phenotypic characters. 16S rRNA gene sequence showed 98% similarity with *P. xylanexeden* and *P. amylolyticus* whereas 99% similarity with *P. pabuli*, however phenotypic characters are totally differed from these three species. On the other hand phenotypic characters fairly comparable with nitrogen fixing *P. brasilensis* but showed only 94% similarity in 16S rRNA gene sequence. Hence the bacterium was named as *Paenibacillus* sp. BPRIST073. This study indicated the first time in reporting the occurrence of nitrogen fixing *Paenibacillus* from mangrove rhizosphere.

INTRODUCTION

As a result of increasing anthropogenic activities and climatic changes deforestation of the mangrove ecosystem reached an alarming state, to control this intensive care should be taken by using various conservation mode (FAO, 2007; Kathiresan and Bingham, 2001). Mangroves are unique inter-tidal wetland ecosystem comprises specialised woody plants as a major flora. This provides good nursery ground for many commercially important aquatic organisms (Kathiresan, 2000). Nitrogen is one of the important limiting factors present low concentrations in mangrove environments, which affects highly on the vegetation of the mangrove plants. Hence microbial transformations are the important factor to overcome deficit of nitrogen for the mangrove plants. Because of high concentration of the salt and anaerobic soil condition of the mangrove environment, it is important to find halophilic diazotrophic bacteria present in that environment for the conservation through improve the growth of mangrove seedlings (Ravikumar *et al* ., 2004). Pichavaram mangrove is one of the important mangrove forests in India, largely influenced by human activities, and has *Avicennia marina* as the dominant flora (Alongi *et al.*, 2005). *Paenibacillus* are the gram positive rods, aerobic or facultative anaerobic bacteria having the ability of producing endospores. Several species namely *P. polymyxa*, *P. macerans*, *P. azotofixans*, *P. peoriae*, *P. graminis*, *P. odorifer*, *P. bracilensis, P. zanthoxyli* and *P. forsythiae* reported widely for its diazotrophic nature from rhizospheric systems of different environments (Ma and Chen, 2008; Ma *et al*., 2007). Members of this genus also reported in the mangrove rhizosphere environments as a phosphate solubilizer and hydrocarbon degrader (Daane *et al.*, 2001; Vazquez *et al.*, 2000). Though the genus *Paenibacillus* reported widely for its nitrogen fixation there are no much reports available from the mangrove rhizosphere. In this study we identified a halophilic diazotrophic bacterium with excellent nitrogen fixing capacity and characterized the potent bacterium, using 16S rRNA gene sequence analysis and phenotypic characters.

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MATERIALS AND METHODS

Selection of potential isolate

Five moderately halophilic bacteria used in this study were isolated from the rhizosphere soil of Pichavaram mangrove plant *A. marina* and showed growth on the nitrogen free medium. To evaluate the nitrogenase activity of the isolates, modified AOB medium was used for the Acetylene Reduction Assay (ARA) as described by Holguin *et al*., 1992 using Gas chromatography. The nitrogenase activity expressed as nmol ethylene produced mg protein^{-1} of the cells as described by Berge *et al*., 2002.

16S rRNA gene sequencing and phylogenetic analysis

The genomic DNA of the isolate was extracted as described by Sohail, 1998. PCR amplification was performed using

50 µL 10 x Taq buffer A (GeNei, India) containing 20 ng of the template DNA, 2.5 mM of each deoxynucleotide triphosphate (dATP, dGTP, dTTP and dCTP), 1µm concentration of each primer pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3'), and 3 U of Taq DNA polymerase. These reactions were subjected to initial denaturation of 92ºC for 2 min. and 10 s followed by 35 cycles at 92ºC for 1 min., 48ºC for 30 s and 72ºC for 2 min. and 10 s and a final extension step of 72ºC for 6 min. and 10s using GeneAmp® PCR system 9700 (Applied Biosystems, USA). The partial sequencing of purified PCR product was undertaken at Oscimium Biosciences, Bangalore India, using the above mentioned forward and reverse primer. The 16S rRNA gene sequence of the isolate was compared with the sequences available with the BLASTN search of the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed with some closely related members, using Clustal X (Thompson *et al.*, 1994). The method of Jukes and Cantor, 1969 was used to calculate evolutionary distances. Phylogenetic dendrogram was constructed by the neighbour-joining method and tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 4.1 (Molecular Evolutionary Genetic Analysis).

Phenotypic characterization

From the observation of Gram staining and motility tests, the strain was cultivated in Zobell marine medium (Hi-Media) and the anaerobic growth were tested by incubating the culture without oxygen. Production of endospore was determined by Malachite green staining. Series of physical and biochemical tests were performed such as growth on various pH, temperature and NaCl concentration, aminoacid decorboxylase test, methyl red and Simmons citrate. Enzyme production tests i.e. catalase, oxidase and nitrate reductase (Lanyi, 1987). Carbohydrate utilization test were executed by means of Hi-Media carbohydrate utilization kit with 15 types of carbohydrate (Table 1). Enzymatic hydrolytic activity of the test strain was carried out using spot inoculation method with 2% w/v of the following supplements in the basal medium; Skimmed milk (for caesin), Tween – 80 and Starch.

RESULTS AND DISCUSSION

Among the five isolates used in the present study one of them showed excellent nitrogen fixing activity and the strain was designated as BPRIST073. The isolate showed maximum nitrogenase activity of 2017±32.60nmol ethylene (mg protein^{-1}) than the other nitrogen fixers reported in the genus *Paenibacillus viz*., *P. polymyxa* (3.2±0.55), *P. macerans* (2.48±0.2), *P. odorifer* (37.62±18.9), *P. graminis* (272.9±7.9) and *P. forsythia* (1537.0±10.9) whereas *P. azotofixans* (2256.8±46.9) and *P. durus* (2256.8±46.9) were equally comparable with our isolate (Ma and Chen, 2008; Ma *et al*., 2007). These results pokes hope on the bacterium for the conservation of the mangrove vegetation through the nursery development in the nitrogen limiting environment. 16S rRNA gene sequence showed 98% similarity with *P. xylanexeden* and *P. amylolyticus* and 99% similarity with *P. pabuli*. Phylogenetic analysis was also revealed the same by forming the cluster with *P. pabuli* (Fig. 1). Thus the bacterium may come under the genus of *Paenibacillus*. The sequence of the

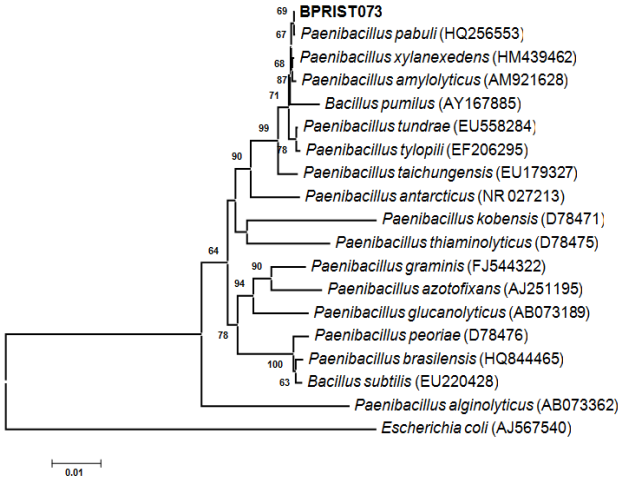

Fig. 1 Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequence of the isolate and its phylogenetically closest neighbours. Bootstrap values are indicated at nodes. Scale bar represents observed number of changes per nucleotide position.

Table 1. Phenotypic characters of the isolate BPRIST073

Test	Results	Test	Results
Colony color	White	Gelatinase	+
Gram Stain	G^{+ve}, Rod	Nitrate reductase	+
Spore formation	+	Acid formation:	
Motility	+	Arabinose	+
pH range:	2-10	Cellobiose	+
Optimum pH	7	Dextrose	+
Temperature range :	15-45°C	Fructose	+
Optimum Temperature	37°C	Galactose	+
NaCl range in %:	1-12	Glucose	+
Optimum NaCl %	2	Inositol	-
Anaerobic growth	+	Lactose	+
Arginine dihydrolase	+	Maltose	+
Lysine decorboxylase	-	Mannitol	+
Ornithine decorboxylase	+	Melibiose	-
ONPG test	-	Rhamnose	-
Indole	-	Salicin	+
Methyl Red	+	Sorbitol	-
Voges Proskauer	+	Sucrose	-
Simmons Citrate	+	Hydrolysis of:	
Enzyme production:		Casein	+
Catalase	+	Starch	+
Oxidase	+	Tween 80	+
Urease	-	Egg yolk (lipase)	+

$^{+}$ Positive, $^{-}$ negative

isolate was submitted in the GenBank, NCBI and the accession number was JF700522. The phenotypic characters (Table 1) of the isolate showed that the bacterium was Gram positive rods, endospore forming motile bacteria, produces white colour irregular colonies in an anaerobic conditions, resists wide range of pH 2-10 with the optimum of pH 7, observed no growth in the 0% NaCl concentration and tolerated up to 12% NaCl with optimum of 2% and resisted the temperature up to 45°C and grows in 15°C. These characters evidenced that the bacterium has wide range of adaptability in various environmental stresses. As mangrove has more frequency in the changing environmental parameters, the bacterium could be an indigenous microflora of the ecological niche. From the biochemical tests isolate showed positive results for Arginine dihydrolase, Ornithine decorboxylase, Voges Proskauer, Simmons Citrate, and

negative for Lysine decorboxylase, ONPG and indole. In case of enzymes catalase, oxidase, gelatinase and Nitrate reductase were produced and not showed urease production. Where as in the 15 carbohydrates tested i.e. Arabinose, Cellobiose, Dextrose, Fructose, Galactose, Glucose, Lactose, Maltose, Mannitol and Salicin were utilized by the isolate. Enzymes such as amylases, proteases and lipases were produced for hydrolysing the substrates used (Casein, Starch, Tween 80 and Egg yolk). On the whole, the results of the biochemical tests not comparable with any of three species though of good similarity in BLAST analysis. Since *P. pabuli* is unable to produce nitrate reductase, Arginine dihydrolase and oxidase, but shows positive for ONPG test. In case of *P. xylanexeden* and *P. amylolyticus* were differed (Heyndrickx *et al.*, 1996; Nelson *et al.*, 2009). However biochemical results were maximum comparative with *P. brasilensis*, but showed 94% sequence similarity in 16S rRNA gene sequence analysis with poor query coverage and score value. Interestingly among the four comparable species only *P. brasilensis* reported for its nitrogen fixing capacity (Weid *et al*., 2002). On the basis of above results and its treatise the potent nitrogen fixing isolate was considered to represent novel species of the genus *Paenibacillus*. Hence the isolates designated as *Paenibacillus* sp. BPRIST073. To characterize the isolate up to species level, more phenotypic, chemotaxonomic characterization and DNA-DNA hybridization studies should be done. Since the nature of adaptability to various environmental stresses, survive in the rhizosphere soil of the mangrove and notable nitrogen fixing capacity, the bacterium *Paenibacillus* sp. BPRIST073 can be used as a biofertilizer for the various mangrove nurseries.

REFERENCES

Alongi, D.M., A.L Ramanathan, L. Kannan, F. Tirendi, L.A. Trott and Prasad, M.B. 2005. Influence of human induced disturbance on benthic microbial metabolism in the Pichavaram mangroves, Vellar–Coleroon estuarine. *Marine Biol.,* 147: 1033–1044.

Berge, O., Guinebretiere, M.H., Achouak, W., Normand, P. and Heulin, T. 2002. *Paenibacillus graminis* sp.nov. and *Paenibacillus odorifer* sp. nov., isolated from plant roots, soil and food. *Int. J. Syst. Evol. Microbiol.,* 52: 607–616.

Daane, L.L., Harjono, I., Zylstra, G.J. and Haggblom, M.M., 2001. Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of saltmarsh plants. *Appl. Environ. Microbiol.,* 67: 2683–2691.

FAO. 2007. The world's mangroves 1980 – 2005. Food and agriculture organization of the United Nations. Rome.

Heyndrickx, M., Vandemeulebroecke, K., Scheldeman, P., Kersters, K., Devos, P., Logan, N.A., Aziz, A.M., Ali, N. and Berkeley, R.C.W. 1996. A Polyphasic Reassessment of the Genus *Paenibacillus*, Reclassification of *Bacillus lautus* (Nakamura 1984) as *Paenibacillus lautus* comb. nov. and of *Bacillus peoriae* (Montefusco *et al*., 1993) as *Paenibacillus peoriae* comb. nov., and Emended Descriptions of *P. lautus* and of *P. peoriae. Int. J. Syst. Evol. Microbiol.,* 46: 988-1003.

Holguin, G., Guzman, M.A. and Bashan, Y. 1992. Two new nitrogen-fixing bacteria from the rhizosphere of mangrove trees: Their isolation, identification and in vitro interaction with rhizosphere Staphylococcus sp. *FEMS Microbiology Ecology.,* 101: 207-216.

Jukes, T.H. and Cantor, C.R., 1969. Evolution of protein molecules. In Mammalian protein 242 metabolism (Ed.) Munro, H.N. 21.132. New York: Academic Press.

Kathiresan, K. 2000. A review of Studies on Pichavaram man grove, southeast India. *Hydrobiologia*., 430: 185-205.

Kathiresan, K. and Bingham B. L. 2001. Biology of mangrove and mangrove ecosystems. *Advances in Marine Biology*, 40: 81-251.

Lanyi, B. 1987. Classical and rapid identification methods for medically important bacteria. *Methods Microbiol.,* 19: 1–67.

Ma, Y., Zhang, J. and Chen, S., 2007. *Paenibacillus zanthoxyli* sp. nov., a novel nitrogen-fixing species isolated from the rhizosphere of *Zanthoxylum simulans*. *Int. J. Syst. Evol. Microbiol.,* 57: 873–877.

Ma, Y.C. and Chen, S.F., 2008. *Paenibacillus forsythiae* sp. nov., a nitrogen-fixing species isolated from rhizosphere soil of Forsythiamira. *Int. J. Syst. Evol. Microbiol.,* 58: 319–323.

Nelson, D.M., Glawe, A.J., Labeda, D.P., Cann, I.K.O. and Mackie, R.I., 2009. *Paenibacillus tundrae* sp.nov. and *Paenibacillus xylanexedens* sp.nov., psychrotolerant, xylan-degrading bacteria from Alaskantundra. *Int. J. Syst. Evol. Microbiol.,* 59: 1708–1714.

Ravikumar, S., Kathiresan, K., Ignatiammal, S.T.M., Selvam, M.B. and Shanthy, S., 2004. Nitrogen-fixing azotobacters from mangrove habitat and their utility as marine biofertilizers. *Journal of Experimental Marine Biology and Ecology,* 312: 5–17.

Sohail, M., 1998. A simple and rapid method for preparing genomic DNA from gram-positive bacteria. *Mol. Biotechnol.,* 10: 191-193.

Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.,* 22: 4673–4680.

Vazquez, P., Holguin, G., Puente, M.E., Cortes, A.L. and Bashan, Y., 2000. Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biol. Fertil. Soils.,* 30: 460–468.

Weid, I., Duarte, G.F., Elsas, J.D. and Seldin, L., (2002). *Paenibacillus brasilensis* sp.nov., a novel nitrogen-fixing species isolated from the maize rhizosphere in Brazil. *Int. J. Syst. Evol. Microbiol.,* 52: 2147–2153.

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