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Isolation and characterization of copper resistant Exiguobacterium strains isolated from rhizosphere soil of Avicennia marina

Baskar B*, Prabakaran P.

Department of Microbiology, PRIST University (EAST CAMPUS), Thanjavur – 614904, Tamil Nadu, India

ABSTRACT

Mangrove plants are highly efficient to accumulate heavy metals through rhizosphere processes. Bacteria present on the rhizosphere sediments are obviously have the resistance against heavy metals. Hence this ideology paves the way to present study to identify the halophilic, resistant bacteria against the Copper (Cu) since it is one of the major contaminant of Pichavaram mangrove forest. A total of 10 moderately halophilic isolates were isolated from the rhizosphere soil of Avicennia marina, of which two (BPRIST019 and BPRIST020) of them showed good copper resistant character. Both the isolates were exhibited same phenotypic characters. While the 16Sr DNA sequence of BPRIST019 was showed 92% similarity with E. profundum and E. aestuarii, whereas BPRIST020 showed 93% similarity with E. profundum. The phenotypes as well 16s rDNA indicated that these strains belongs to the genus Exiguobacterium. This study for the first time demonstrated the occurrence of copper resistant bacterial strains of genus Exiguobacterium from the mangrove rhizosphere soil which suggests a new ecological role.

Keywords: isolation, rhizosphere, Avicennia marina, exibuobacterium

Corresponding author

Email: baskarmicrobiologist@gmail.com

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INTRODUCTION

Because of increasing anthropogenic activities mangroves are became a major sink for most contaminants especially for heavy metals and also act as a reducing barrier for adjacent marine environment. Since, mangrove sediments tent to accumulate more heavy metals by its reduced and anaerobic nature as well as its unique nutrient contents [18]. Mangrove plants can tolerate high concentrations of heavy metals and act as a sink for most heavy metals [16]. Normally the mangrove rhizosphere sediments will nutritiously rich with higher salinity level and also accumulate high concentration of heavy metals than the bulk sediments [22]. It will also influence on the microbial diversity of the environment particularly on bacterial diversity since it is more sensitive group than other microorganisms. In such conditions microorganisms will react to the heavy metals by several processes like transport in to membrane system, biosorption, precipitation, complexation and oxidation or reduction reactions [15]. Hence the bioremediation of heavy metals using microorganism got more attention for its potential applications in the polluted environment [7].

Copper is a necessary trace element in its ionic form for most prokaryotic and eukaryotic organisms, it can be toxic when it exceeds the desirable limits due to inhibition of metabolic reactions. Copper is one of the most important contaminant in mangrove environment and presents more in the rhizosphere soil of mangrove trees [22]. Pichavaram mangrove is one of the important mangrove forests in India, affected by more anthropogenic activities thus heavy metal contamination will be more; and also copper contamination reported many times from this environment [1, 9, 13].

Bacteria of the genus Exiguobacterium are Gram-positive aerobic (sometimes facultative anaerobe) non-spore forming bacteria, that have been frequently reported from Siberian permafrost and have been isolated from diverse environments even in extreme circumstances, including Greenland glacial ice, hot springs at Yellowstone National park, the rhizosphere of plants and the environment of food processing plants. This makes a concentration as they are likely to be specifically adapted to such environments and to carry variations in the genome correspond to such adaptations [20]. Till now only 13 species were reported from various environments and it is phylogenetically related to the genus Bacillus and related taxa [6, 20, 21]. Till now there is no report on genus Exiguobacterium form the mangrove environment.

However, much studies and reports available on the heavy metal accumulation in the rhizosphere soil of mangrove plants. There are limited studies available on heavy metal resistant halophilic rhizobacteria. This study evidently revealed the first time occurrence of copper resistant Exiguobacterium was characterized by both phenotypicallly and genotypically isolated from the rhizosphere of Avicennia marina.

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MATERIALS AND METHOD

Selection of copper resistant Bacteria and Minimum Inhibition Concentration (MIC) assay

Ten moderately halophilic bacterial isolates were isolated from rhizosphere soil of Pichvaram mangrove plant A. marina. Ten inoculums were streaked on the agar plates which is prepared with 50 µg/mL concentration of Cu^{2+} ion (using $CuCl_2$) in the basal medium (Marine agar) and plates were incubated at 37°C. After 48 hrs of incubation the results were observed. The positive isolates were subjected for the MIC assay by preparing the basal medium with increasing concentration of Cu^{2+} ion as 10 µg/mL each time and the initial concentration started with 50 µg/mL. After incubation the growth pattern was observed and MIC was identified with the concentration in which isolates failed to grow.

Phenotypic characterization of the copper resistant Isolates

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 giving various concentration of oxygen (from 10-0% with 1% declining). Then a 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, such as catalase, oxidase and nitrate reductase [11]. Carbohydrate utilization test were performed using Hi-Media carbohydrate utilization kit with 15 types of carbohydrate (Table 1), tests were carried out according to the manufacturer's instructions.

16s rDNA sequencing and Phylogenetic analysis

The genomic DNA of BPRIST019 and BPRIST020 isolates were extracted using the method described by Sohail [17]. 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 16S rRNA gene sequences available with the BLASTN search of the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed with some related Exiguobacterium and Firmicutes members, using Clustal X [19]. The method of Jukes and Cantor [8] 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). The sequence obtained in

this study was deposited in the GenBank, NCBI and their accession numbers are JF414772 and JF431411.

RESULTS AND DISCUSSION

Of the ten isolates tested, four were showed growth on the 50 µg/mL concentration of Cu^{2+}, in which two (BPRIST019 and BPRIST020) were showed normal growth where as other two were with retarded growth. Two good copper resistant isolates were subjected in to MIC assay; in which both the isolates were showed resistant up to the concentration (Cu^{2+}) of 310 µg/mL. The copper resistant capacity of the two isolates was highly comparable with the bacteria like Pseudomonas. sp [15], Escherichia coli, Pseudomonas stewartii and Staphylococcus haemolyticus [14]. Even if many reports available on the copper resistant bacteria were all isolated from the terrestrial environment and there were no much results available on mangrove associated bacteria. Hence the results of this work will lead an initiative to investigate more in this regard.

Both the isolates were showed same morphological, physiological and basic biochemical results. Both were produced orange colour rounded colonies on the marine agar media, motile Gram positive short rods, growth occurred between 10°C and 47°C; pH 5-8 and NaCl concentration at 0-18 %. The optimum temperature, pH and NaCl are 37°C, 7.0 and 3%. Respectively (Table 1). These characters of the isolates were in accordance with Group II Exiguobacterium of Vishnivetskaya et al's [20] classification, since the Group II Exiguobacterium was isolated from moderately hot, alkaline or marine environments. Both the isolates produces nitrate reductase and facultatively grows in anaerobic condition (up to 2% of oxygen), and recorded negative for oxidase (Table 1) thus these character confirms that the isolates comes under the group II Exiguobacterium, since they are able to obtain energy by reducing nitrate using anaerobic respiration [20] . Both the isolates showed positive results for citrate utilization test like some reported member of Exiguobacterium for example E. indicum, E. acetylicum and E. oxidotolerance [2]. In carbohydrate utilization tests isolate BPRIST019 utilized and produced acids from cellobiose, galactose, glucose, lactose, maltose, mannitol, melibiose and sucrose, where as isolate BPRIST020 utilized and produced acids from arabinose, cellobiose, galactose, glucose, maltose, mannitol, melibiose and sucrose (Table 1). Among the carbohydrates tested BPRIST019 have utilized the lactose without arabinose while the BPRIST020 is vice versa whereas the other carbohydrates utilization results were same. These results indicate that the isolates are differed from the reported Exiguobacterium members [2, 3, 4, 10, 12, and 21] and these two isolates may have strain level variation.

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Table 1: Phenotypic characters of the isolates showed copper resistance.

Test	BPRIST 019	BPRIST 020	Test	BPRIST 019	BPRIST 020
Colony shape	Round	Round	Oxidase	-	-
Colony color	Orange	Orange	Nitrate reductase	+	+
Gram Stain	G^{+ve}	G^{+ve}	Acid formation:		
Motility	+	+	Arabinose	-	+
pH range	5 - 8	5 - 8	Cellobiose	+	+
Optimum pH	7	7	Dextrose	-	-
Temperature range (°C)	10 - 47	10 - 47	Fructose	-	-
Optimum Tem (°C)	37	37	Galactose	+	+
NaCl (%) range	0 - 18	0 - 18	Glucose	+	+
Optimum NaCl (%)	3	3	Inositol	-	-
Anaerobic growth	+	+	Lactose	+	-
Arginine dihydrolase	-	-	Maltose	+	+
Lysine decorboxylase	-	-	Mannitol	+	+
Ornithine decorboxylase	-	-	Melibiose	+	+
Methyl Red	-	-	Rhamnose	-	-
Citrate Utilization	+	+	Salicin	-	-
Enzyme production:			Sorbitol	-	-
Catalase	-	-	Sucrose	+	+

+ Positive, - Negative

On the basis of phylogenetic analysis of 16S rDNA partial sequences, the two bacterial isolates come under the genus Exiguobacterium. Neighbour joining method were showed that, both the isolates was not formed cluster with any individual Exiguobacterium member used for the study and showed more evolutionary distance with all (Fig. 1). BLAST analysis also showed that the partial 16S rDNA sequence of BPRIST019 was showed only 92% similarity with E. profundum and E. aestuarii, in spite, BPRIST020 was showed 93 % similarity with E. profundum. The similarity at the 16S rRNA gene level showed that the two strains could be a novel species of the genus Exiguobacterium since the isolated environment is new and the general character of Exiguobacterium is that adapting diverse environment with bearing the variations in the genome level [2].

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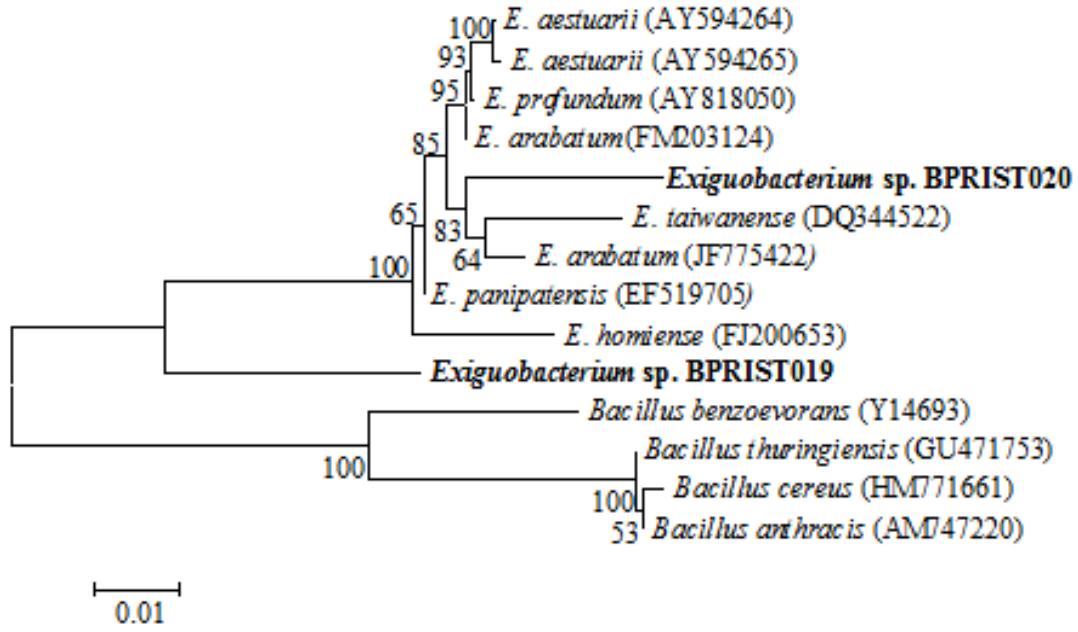

Fig. 1 Neighbour-joining phylogenetic tree based 16S rRNA gene sequences of the isolates and their closest phylogenetically neighbours. Bootstrap values are indicated at nodes. Scale bar represents observed number of changes per nucleotide position.

On the basis of above results and its treatise the two good copper resistant isolates are considered to represent novel species of the genus Exiguobacterium. Hence the isolates named as Exiguobacterium sp. BPRIST019 and Exiguobacterium sp. BPRIST020. To characterize the isolates up to species level, more phenotypic, chemotaxonomic characterization and DNA-DNA hybridization studies should be done.

Since the isolates character of withstanding wide range of pH, temperature and salt concentration and showing comparable resistant against copper, it could be used for the bioremediation of copper polluted environments. In this regard further work is in progress to identify the copper resistant mechanism and multiple tolerant studies with other important heavy metals, biocides and antibiotic due to these bacteria normally have common mechanism for all these tolerance [5, 15].

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