JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2015. Vol. **9**(3), p. 2429-2433

Molecular Identification of Drought Tolerant Endophytic Bacteria from Grasses of Kutch by Sequencing of the 16s rRNA Coding Gene

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(Received: 01 April 2015; accepted: 09 July 2015)

Endophytic bacteria from seven species of grasses native to the banni region of Kutch (*Dactylactenium sindicum*, *Cenchrus biflorus, Sporobolus fertilis, Alelurops lagopoides, Eleusine indica, Chlorius barbata* and *Cenchrus ciliaris*), were isolated and identified, through partial sequencing of the 16s rRNA encoding gene. The sequence of 17 isolates deposited in NCBI and compared to sequences- 29.41% belonged to the genus *Bacillus*; 17.64% to *Staphylococcus;* 11.76% both to *Methylobacterium* and *Pseudomonas*, Where as 5.88% were *Brevibacillus, Enterobacter, Pantoea, Ochrobacterium* and *Micrococcus.* Results obtained through sequence analysis revealed high genetic diversity across the isolates. This is the first report concerning the isolation and identification of drought tolerant endophytic bacteria in these grass species of banni region from Kutch.

Key words: Grasses, Drought tolerant endophytic bacteria, 16s rRNA Sequencing, NCBI, BLASTn.

Plant microbe interactions that promote development of plant and plant health have been the subject of considerable interest. Plants constitute vast and diverse niches for endophytic organisms. Among the microorganisms, endophytic bacteria occupy internal tissues of plants without causing damage to their hosts. An understanding of the mechanisms enabling these microorganisms to interact with plant will be essential to fully achieve the biotechnological potential of efficient plant–bacterial partnerships for a range of applications[1, 2].

Endophytic communities are formed mainly by fungi and bacteria. It is estimated that every plant-species constitutes a possible host for endophytic microorganisms, which in the vast majority and despite their biotechnological potential remain unidentified. Although the interaction between these microorganisms and their respective host plants is not, as yet, fully understood, over recent years they have been progressively more extensively employed, either in agriculture[3] or in the production of compounds with therapeutic application, such as taxol[4].

The banni region (Kutch, Gujarat) is an internationally recognized unique grassland stretch of western India. The climate of the banni is arid therefore, the temperature remain high during most of the time and it reaches a maximum of 48-49°C during May-June (the hottest months) and in banni region the dominant family is *Poaceae* (grasses) about 32%. The wild type of flora in every genes are vigorous and withstand the external stresses. It is not surprising that biomes characterized as extremely biodiverse are also believed to harbor significant richness and variety of microorganism populations. There is a report on endophytic microorganisms isolated endophytic bacteria from arboreal species of the

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Amazon and identification by sequencing of the 16s rRNA encoding gene and *Bacillus, Pantoea* and two non culturable samples were identified[5]. A 31 bacterial endophytes from switchgrass and bacterial endophytes were identified as *Microbacterium testaceum, Curtobacterium flaccumfaciens, Bacillus subtilis, Bacillus pumilus, Pseudomonas fluorescens, Sphingomonas parapaucimobilis, Serratia* sp. and *Pantoea ananatis*[6]. By 16s rRNA encoding gene[7], a great proportion of the isolates belong to genus *Bacillus* and *Pantoea* which were isolated from drought tolerant brazilian catci. Thus, more in-depth knowledge of this microbiota, as well as the interactions it maintains with host-plants and the environment, is an essential variable in the development of conservation strategies directed to sustaining environmental balance, thereby preserving biodiversity as a whole, in efforts that may pave the way for its biotechnological application[8]. In present study attempted to elucidate the drought tolerant bacterial diversity associated with grasses of Kutch by sequencing of the 16s rRNA coding gene.

MATERIALS AND METHODS

Plant materials

Plants collected from banni region (between North latitudes of 23°19' and 23°52' N and East longitudes of 68°56' to 70°32' E Kutch, Gujarat) were the *Dactylactenium sindicum, Cenchrus biflorus, Sporobolus fertilis, Alelurops lagopoides, Eleusine indica, Chlorius barbata* and *Cenchrus ciliaris.*

Isolation of endophytic bacteria

The sterile root, stem and leaves(0.1% $HgCl_2$ for 3 min and wash with distill water for 3 min) of all above grasses were used to isolate the endophytic bacteria under aseptic conditions. Plant material was then suspended in 0.05 M phosphate saline buffer and ground with a sterilized mortar and pestle.The crushed samples were inoculated on nutrient agar media at 28°C for 5 to 7 days under observation[9].

Screening of drought tolerant endophytic bacteria

Bacteria were selected based on their ability to grow in nutrient broth with different water potentials -0.15 Mpa, -0.49Mpa, -1.03 Mpa was prepared by adding the appropriate concentrations like 10% , 20% and 30% of Polyethylene glycol (PEG 6000), respectively[10].

Molecular Identification of drought tolerant endophytic bacteria

Extraction of Gemonic DNA of drought tolerant endophytic bacteria were carried out using invitrogen charge Switch® gDNA Mini Bacteria Kit. DNA was isolated from 1 day old cultures in a nutrient broth. Drought tolerant endophytes identified based on 16srDNA sequencing using MicroSeq®500 16s rDNA bacterial identification kits(PN 4346298) as per manufacture protocol by using 3130XL gene sequencer.

The primers 907R (5'-CCGTCAATTCCTTTRAGTTT-3') and 27F (5'-AGAGTTTGATCCTGGCTCAG-3') were used for amplification of 16s rRNA gene. The total PCR reaction was 25.0 µl comprising 2µl dNTPs, 1µl Forward primer, 1µl Reverse primer, 2.5 µl Taq buffer,0.5µl Taq enzyme,1µl demonic DNA with 17µl nuclease free water. The reactions conditions were 96°C for 10 min followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, primer extension at 60°C for 45 min, followed by the final extension at 60°C for 10 min. Then 10µl of PCR products per lane were running in 2% agarose gel electrophoresis using a 1.5 kb DNA mass ladder. Samples were purified using 2 µl Exo-ap with 5 µl PCR product and then preparing cycle sequencing reaction containing 7µl of purified PCR product and 13µl of seguencing module performed with one primer 27F for each samples. Sequencing analysis was achieved using 3130XL Genetic Analyzer (Applied Biosystems)[11].The quality of sequences was checked using Sequencing analysis 5.3.1. Version. Then the available DNA sequences were analyzed in GenBank database using the algorithm nucleotide-BLAST (BLASTn).

RESULTS AND DISCUSSION

Isolation of Endophytic bacteria:

All the grasses yielded the endophytic bacteria from leaves, stems and roots. A total 78 unique bacterial endophytic strains were isolated from grasses.

Screening of drought tolerant endophytic bacteria

Among the 78 endophytic bacteria tested, the best 19 completely drought tolerant bacterial isolates were CEB9, CEB 12, CEB 14, CEB

15, SM 19, EI 36, EI 41, EI 42, EI 45, EI 47,EI 49, CHB 54,CHB 58, CHB 59, CHB 60, CHB 61, CC 70,CEB 75,CEB 76 which grow at 30% (-10.3 bar/-1.03Mpa) PEG 6000 concentration. These 19 completely drought tolerant bacterial isolates were used for molecular identification.

Molecular Identification of drought tolerant endophytic bacteria

All amplified products produced a single band with approximately 900 base pair in length and differences among them were not visible in 2% agarose gel (fig.1). Sequencing of 16s rRNA was performed in all 19 isolates but results were obtained for only 17 isolates in which approximately 700-800 base pair (query length) were observed whereas rest of two were not gave enough sequences to analyzed it's data properly. Based on the nucleotide sequences each of the isolates was assigned to 9 different genera (table 1). In terms of class, most isolates belonged to Firmicutes (52.94% of the total number of isolates), followed by α-Proteobacteria (23.52%) and after γ-proteobacteria (17.64%). Whereas, lastly Actinobacteria (5.88%). Of the total number of 17 isolates analyzed, 29.41% belonged to the genus *Bacillus*; 17.64% to *Staphylococcus*; 11.76% both to *Methylobacterium* and *Pseudomonas*, Where as 5.88% were *Brevibacillus, Enterobacter, Pantoea, Ochrobacterium* and *Micrococcus.*

On identifying isolates at the species level, diversified bacterial communities were observed. When sequences obtained in the present analysis were compared to those deposited in GenBank using BLASTn, it was observed that one same given sequence was actually similar to those sequences of more than one species (table 1), specially with *Bacillus* spp. Among 17 isolates, five were *Bacillus* spp., from which CEB12, EI47 and CEB75 isolates gave 93, 92 and 92 per cent similarity, respectively with *Bacillus cereus* strain JCM 2152(NR 102506.1) and CEB 15 and EI 41 isolates gave 90 and 93 per cent similarity with *B. amyloliquefaciens* strain NBRC15535(NR 041455.1) and *B. amyloliquefaciens* strain MPA1034(NR 117946.1). Three were *Staphylococcus* spp. in which CEB 14 and CHB 58 isolates gave 95 and 94 per cent similarity with *Staphylococcus saprophyticus* strain ATCC15305(NR074999.1), respectively while CHB 60 isolate gave 92 per cent similarity with *Staphylococcus saprophyticus* strain NBRC102446(NR114090.1). The CHB 54 and CHB 59 isolates gave 93 and 91 per cent similarity, respectively with *Methylobacterium populi* BJ001(CP001029.1). Based on 16s rRNA

Table 1. DNA sequence identity of bacterial 16s rRNA partial sequencing of drought tolerant endophytic isolates

Isolates	Identity	Plant Species	Plant Tissue	Max Identity (%)	Accession Number (Genbank)
CEB 9	*Enterobacter cloacae* strain DSM	*Cenchrus biflorus*	Root	97	NR117679.1
CEB 12	*Bacillus cereus* strain JCM 2152	*Cenchrus biflorus*	Stem	98	NR102506.1
CEB 14	*Staphylococcus saprophyticus* strain ATCC15305	*Cenchrus biflorus*	Stem	98	NR074999.1
CEB 15	*Bacillus amyloliquefaciens* strain NBRC 15535	*Cenchrus biflorus*	Leaf	93	NR041455.1
EI 36	*Micrococcus luteus* strain NCTC2665	*Eleusine indica*	Root	93	NR075062.2
EI 41	*Bacillus amyloliquefaciens* strain MPA1034	*Eleusine indica*	Stem	93	NR117946.1
EI 45	*Pseudomonas synxantha* strain NBRC103159	*Eleusine indica*	Stem	92	NR113583.1
EI 47	*Bacillus cereus* strain JCM 2152	*Eleusine indica*	Stem	92	NR102506.1
EI 49	*Pseudomonas cedrina* strain CFML96-198	*Eleusine indica*	Stem	96	NR042147.1
CHB 54	*Methylobacterium populi* BJ001	*Chlorius barbata*	Root	94	CP001029.1
CHB 58	*Staphylococcus saprophyticus* strain ATCC15305	*Chlorius barbata*	Root	97	NR074999.1
CHB 59	*Methylobacterium populi* BJ001	*Chlorius barbata*	Root	94	CP001029.1
CHB 60	*Staphylococcus saprophyticus* strain NBRC102446	*Chlorius barbata*	Stem	92	NR114090.1
CHB 61	*Brevibacillus brevis* NBRC 100599	*Chlorius barbata*	Leaf	90	NR041524.1
CC 70	*Ochrobacterium intermedium* strain NBRC15820	*Cenchrus ciliaris*	Root	93	NR113812.1
CEB 75	*Bacillus cereus* strain JCM 2152	*Cenchrus biflorus*	Leaf	92	NR102506.1
CEB 76	*Pantoea ananatis* AJ13355	*Cenchrus biflorus*	Root	97	NR074740.1

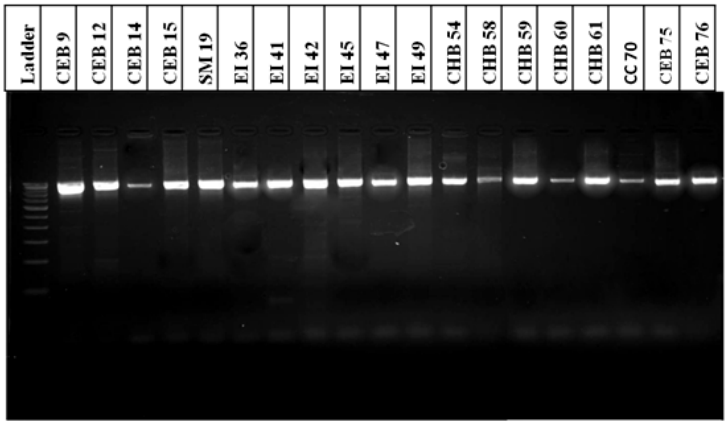

Fig. 1. 16s rRNA banding pattern of drought tolerent endophytic bacteria

sequences,[12] isolated 23 different genera from leaves of the common bean (*Phaseolus Vulgaris*) and in that most of were *Methylobacterium* spp., *Staphylococcus* spp. and *Bacillus* spp.

The EI 45 and EI 49 gave 92 and 96 per cent similarity with *Pseudomonas synxantha* strain NBRC103159(NR113583.1) and *Pseudomonas cedrina* strain CFML96-198(NR042147.1), respectively. Whereas CHB 61 was 90% similar with *Brevibacillus brevis* NBRC 100599(NR041524.1)., CEB 9 was 97% similar with *Enterobacter cloacae* strain DSM(NR117679.1), CEB 76 was 97% similar with *Pantoea ananatis*AJ13355(NR 074740.1), CC 70 was 93% similar with *Ochrobacterium intermedium* strain NBRC15820(NR113812.1) and EI 36 was 93% similar with *Micrococcus luteus* strain NCTC2665(NR075062).

The results indicates that *Bacillus* spp. were been most prevalent. There were six different *Bacillus* spp. identified from Barzilian catci[7], the genus was deeply studied and high frequency of *Bacillus* spp. detected can be attributed to the ability of endospore formation, that enable bacteria to thrive unfavourable environmental conditions such as heat, radiation, drought, starvation and salt, also other members of the same family *Brevibacillus* sp. and *pantoea* spp. from *Enterobacteriaceae* family.

The diversity of plant growth promoting bacteria associated with wheat (*Triticum aestivum*) under drought condition using 16s rRNA gene sequences. The results revealed that 134 strains belonged to 3 phyla namely actinobacteria (18 %), firmicutes (38 %) and proteobacteria (43%) with 38 distinct species of 17 genera. Three major classes were formed in which proteobacteria were most predominant phylum followed by firmicutes. The 17 strains belonged to phylum firmicutes were grouped into three families of bacilli namely *Bacillaceae* (11 strains *Bacillus* spp); Second cluster of phylum actinobacteria consist of five strains, *Arthrobacter humicola, Corynebacterium callunae, Kocuria* sp., *Micrococcus luteus* and *Micrococcus* sp. Phylum proteobacteria consist three grouped of γ-proteobacteria (3 strain *Methylobacterium* spp.), and γ- proteobacteria (11 strains *Acinetobacter* sp., *Pantoea ananatis, Pseudomonas* spp.). Overall, *Micrococcus* from actinobacteria, *Bacillus* from firmicutes, and *Pseudomonas* from proteobacteria were the most frequently recovered genera[13].

Gram positive and Gram negative endophytic bacteria have already been isolated from many tissue types in numerous plant-species. Different media used for bacterial isolation could be a factor affecting bacterial community diversity recovered from grasses. For accurate identification of the bacteria in comparison of the 16s rRNA sequence, other analytical approaches like the analysis of fatty acids and phospholipids can be used. Therefore, the need for a better distinction between the two has become the central topic of several taxonomy studies.

CONCLUSION

The results obtained on analyzing partial sequences of the 16s rRNA region encoding gene,

demonstrated that there are diversified drought tolerant endophytic bacteria inhabited in different species of the grasses from banni region of Kutch. The isolation and identification of bacterial genera may have potential biotechnological applications. This is the first report to identify the drought tolerant bacteria from the grasses of Kutch.

ACKNOWLEDGEMENTS

This Research work was funded by Junagadh Agricultural University, Junagadh, Gujarat. The sequencing was performed at Department of Biotechnology, JAU, Junagadh.

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