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Molecular Identification of Salt Tolerant Endophytic Bacteria from Kutch, India by Sequencing of the 16S rRNA coding gene

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The importance of plant growth promoting rhizobacteria in growth promotion and their ability to elicit 'induced systemic tolerance' against abiotic stresses has been documented. However, the performance of these microbes under various abiotic stresses especially saline-sodic conditions will be of great importance in the current agricultural scenario. In present study salt tolerant endophytic bacteria isolated from four wild *Malvaceae* plant native to the different area of Kutch (*Abuliton indicum*, *Senra incana, Sida* sp., wild cotton) 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 *Staphylococcus*; 17.64% to *Bacillus*; 11.76% both to *Providencia* and *Microvirga*, whereas 5.88% were *Alcaligens.* Results obtained through sequence analysis revealed high genetic diversity across the isolates.

Key words: *Malvaceae* plant, salt tolerant endophytic bacteria, 16S rRNA Sequencing, NCBI, BLASTn

Environmental stresses such as drought, temperature, salinity, air pollution, heavy metals, pesticides, and soil pH are major limiting factors in crop production because they affect almost all plant functions. Farmers around the globe have to deal with challenges of various biotic and abiotic stress factors that reduce plant growth and productivity[1]. Salt stress is one of the major dilemmas that also cause a decline in fertile land productivity. Problems associated with salinity not only effects agriculture but also the biodiversity of that environment. About 20% of cultivated and a least half of irrigated lands around the world are severely affected by salinity[2].

Endophytic microorganisms have been defined as those that reside at some phase of their life cycle within living plant tissues[3,4]. Endophytic bacteria colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host[5]. Endophytic bacteria exert beneficial effects on host plants, such as stimulation of plant growth nitrogen fixation[6]. The endophytic bacteria also remove soil contaminants by enhancing phytoremediation and may play a role in soil fertility through phosphate solubilization and nitrogen fixation.

Halophytes are generally defined as rooted seed-bearing plants that grow in a wide variety of saline habitats from saline prone area. These highly adaptable plants, which can accrue relatively large amounts of salts. Endophytes are largely unexplored component of biodiversity, especially in the tropics. Endophytes are constantly exposed to intergeneric-genetic exchange with the host plant. Although the presence of endophytic bacteria of some of the halophytes from Kutch, Gujarat region is somewhat known but endophytic bacteria and their bioprospecting potential from live as halophytes like *Malvaceae* plant is largely unknown.

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In some halophytes, a mechanism could be considered the endophytic association between plant and rhizobacteria able to improve the plant growth in abiotic stress conditions (PGPR)[7]. Characterization of endophytic bacteria by sequencing a 300 bp portion of the 16S rRNA gene[8]. In present study attempted to elucidate the salt tolerant bacterial diversity associated with saline prone area of Kutch by sequencing of the 16S rRNA coding gene. Thus, analysis of the genetic structure of microbial populations of endophytes has practical importance; the results can be used to assess the fate of released strains and their impact on resident microbial communities.

MATERIALS AND METHODS

Plant materials

The four halophytes or salt tolerant plants collected from Kutch, Gujarat were the *Abuliton indicum* from different region Kalodungar, Bhachav and Nakhtrana, *Senra incana, Sida* sp. and wild cotton.

Isolation of endophytic bacteria

The sterile root, stem and leaves (0.1% $HgCl_2$ for 3 min and wash with distil water for 3 min) of all above plant 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 salt tolerant endophytic bacteria

The ability of crop plant endophytic bacteria isolates to tolerate a high concentration of salts NaCl was tested on N agar solid medium. N agar medium was prepared supplemented with 2.5, 5, 7.5, 10 and 12.5 % of NaCl for sodium chloride tolerance. The N agar alone was also used as control. The endophytic bacteria isolates showing growth similar to the growth as in control plates were considered as tolerant, while the isolates having little or no growth as compared to control were considered as sensitive[10].

Molecular Identification of salt tolerant endophytic bacteria

Extraction of Gemonic DNA of salt 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. Salt tolerant endophytes identified based on 16S rDNA sequencing using MicroSeq®500 16S rDNA bacterial identification kits (PN 4346298) as per manufacture protocol by using 3130XL gene sequencer.

A total of 17 endophytic strains were used for genomic DNA extraction (Figure 1) followed by the partial DNA sequencing of 16S rRNA gene amplification using 907R (5'-CCGTCAATTCCT TTRAGTTT-3') and 27F (5'AGAGTTTGATC CTGGCTCA G-3') universal primers. 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-sap with 5 µl PCR product and then preparing cycle sequencing reaction containing 7µl of purified PCR product and 13µl of sequencing module performed with one primer 27F for each samples. Sequencing analysis was achieved using 3130XL Genetic Analyser (Applied Biosystems)[11]. The quality of sequences was checked using Sequencing analysis 5.3.1. Version. Then the available DNA sequences were analysed in GenBank database using the algorithm nucleotide-BLAST (BLASTn).

RESULTS AND DISCUSSION

Isolation of Endophytic bacteria

Endophytic bacteria that reside in plant tissues without causing visible harm to the plant were successfully isolated from surface-sterilized six different *Malvaceae* family plant tissues. After performing isolation, N-agar plates incubated for 7 days under daily observation and different type of small, big and medium colonies were observed. A total 72 unique endophytic bacterial strains were isolated from *Malvaceae* family plant tissues roots, leaves and stems, respectively. The efficiency of disinfection method was checked by rolling the sterile samples on other nutrient agar plates. On these plates growth was not observed.

Screening of salt tolerant endophytic bacteria

The wild six *Malvaceae* plant samples growing in different locations were collected from Kutch, Gujarat, among 72 isolates, isolates AIN6, AIN12, AIN14, AIN15, AIN16 from *Abuliton indicum*, Nakhtrana, AIK24, AIK25, AIK28, AIK34, AIK35 from *Abuliton indicum*, Kalo dungar, AIB43, AIB44, AIB49, AIB52, AIB53 from *Abuliton indicum*, Bhachav, SI57 from *Senra incana* and WC68 from wild cotton were found salt tolerant endophytes. These *Malvaceae* family plants collected from saline area of Kutch, Gujarat, here plant play as a halophyte which having capacity to stand with saline condition. The dead point was appearance due to application with 13% of NaCl; therefore, the isolates in this work were halotolerant cleared in their maintenance and alleviate the salt suppression of plants and improving the plant

Table 1. GenBank accession numbers along with the alignments of sequences obtained with reported 16S rRNA gene sequences in GenBank and highest similarity with different bacterial genera

S. No.	Isolates	%age similarity with	Plant Species	Plant Tissue	Max Identity (%) number	Gene bank accession
1	AIN 6	*Staphylococcus saprophyticus* strain ATCC 15305	*Abuliton indicum*, Nakhtrana	Stem	96%	NR074 999.1
2	AIN 12	*Staphylococcus saprophyticus* strain ATCC 15305	*Abuliton indicum*, Nakhtrana	Root	89%	NR074 999.1
3	AIN 14	*Staphylococcus gallinarum* strain VIII1	*Abuliton indicum*, Nakhtrana	Root	95%	NR036 903.1
4	AIN 15	*Staphylococcus pasteuri* Nakhtrana	*Abuliton indicum*,	Root	88% 749.1	NR121
5	AIN 16	*Staphylococcus saprophyticus* strain ATCC 15305	*Abuliton indicum*, Nakhtrana	Root	93%	NR074 999.1
6	AIK 24	*Staphylococcus xylosus* strain JCM 2418	*Abuliton indicum*, Kalo dungar	Leaf	94%	NR113 350.1
7	AIK 25	*Staphylococcus haemolyticus* JCSC1435	*Abuliton indicum*, Kalo dungar	Leaf	93%	NR007 168.1
8	AIK 28	*Staphylococcus arlettae* strain ATCC 43957	*Abuliton indicum*, Kalo dungar	Leaf	90%	NR024 664.1
9	AIK 34	*Staphylococcus succinu s* strain AMG-D1	*Abuliton indicum*, Kalo dungar	Stem	95%	NR028 667.1
10	AIK 35	*Providencia rettgeri* strain NCTC 11801	*Abuliton indicum*, Kalo dungar	Stem	99%	NR115 880.1
11	AIB 43	*Staphylococcus arlettae* strain ATCC 43957	*Abuliton indicum*,Bhachav	Leaf	97%	NR024 664.1
12	AIB 44	*Bacillus cereus* ATCC 14579	*Abuliton indicum*,Bhachav	Leaf	97%	NR074 540.1
13	AIB 49	*Microvirga zambiensis* strain WSM 3693	*Abuliton indicum*,Bhachav	Stem	95%	NR117 847.1
14	AIB 52	*Providencia vermicola* strain OP1	*Abuliton indicum*,Bhachav	Root	99%	NR042 415.1
15	AIB 53	*Staphylococcus pasteuri indicum*,Bhachav	*Abuliton*	Root	92% 749.1	NR121
16	SI 57	*Staphylococcus xylosus* strain JCM 2418	*Senra incana*	Leaf	96%	NR113 350.1
17	WC 68	*Alcaligenes faecalis* strain NBRC 13111	Wild cotton	Leaf	99%	NR113 606.1

growth under saline stress condition. The all endophytic isolates including NaCl tolerant were used for molecular identification

Molecular Identification of salt tolerant endophytic bacteria

PCR amplification of the 16S rRNA gene using the 907R and 27F primer pair yielded amplification products of approximately 900 bp in length and differences among them were not visible in 2% agarose gel. Almost full length 16S rDNA gene was amplified using 27F and 1492R primers[12]. On the bases of 2% agarose gel 16S rRNA gene sequence analysis from 17 isolated salt tolerant endophytic bacteria band pattern showed in Figure 2. Sequencing of 16S rRNA was performed in all 17 isolates in which approximately 700-800 base pair (query length).

Overall 17 isolates, 4 Gram-negative and 13 Gram-positive isolates were obtained from different tissue of plant samples. Phylogenetic analysis based on 16S rRNA gene sequence revealed affiliation of these 17 isolates to phyla *Firmicutes* and *Proteobacteria* having five genera *Staphylococcus* (70.58% of the total number of isolates), *Microvirga, Providencia, Alcaligenes* and *Bacillus* (Table 1). Various bacterial species obtained in the present study are listed in Table 1. Each 16S rRNA sequence was aligned against GenBank database top hits using the BLASTn program by Wahyudi *et al.*[13]. Of the total number of 17 isolates analysed, 29.41% belonged to the genus *Staphylococcus*; 17.64% to *Bacillus*; 11.76% both to *Providencia* and *Microvirga*, whereas 5.88% were *Alcaligens.*

On identifying isolates at the species level, diversified bacterial communities were

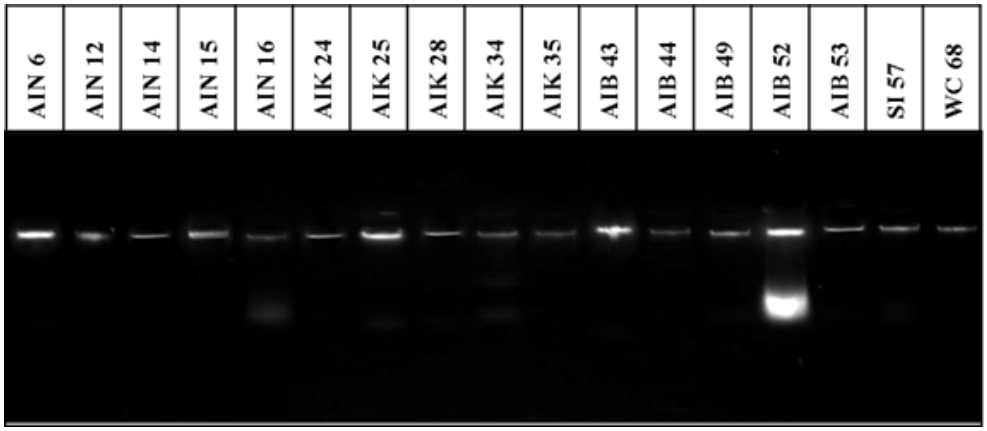

Fig. 1. Electrophoretic banding pattern of genomic DNA from 17 isolated salt tolerant endophytic bacteria on 0.8 % agarose gel

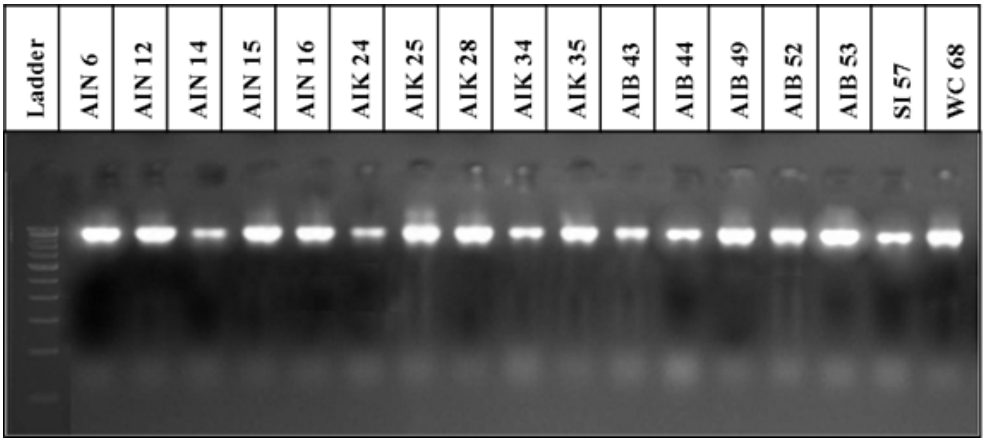

Fig. 2. 16S rRNA sequencing from 17 isolated salt tolerant endophytic bacteria on 2% agarose gel

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 *Staphylococcus* spp. Among 17 isolates, twelve i.e. AIN6, AIN12, AIN14, AIN15, AIN16, AIK24, AIK25, AIK28, AIK34, AIB43, AIB53 and SI57 isolates, *Staphylococcus* sp.

Isolates AIN6, AIN12, AIN16 gave similarity with *Staphylococcus saprophyticus* strain ATCC 15305 (NR074999.1) respectively gave 96, 86 and 93 per cent similarity, AIN14 isolates gave 95 per cent similarity with *Staphylococcus gallinarum* strain VIII1 (NR036903.1), AIN15 and AIB53 isolates respectively gave 88 and 92 per cent similarity with *Staphylococcus pasteuri* (NR121749.1), AIK24 and SI57 isolates with *Staphylococcus xylosus* strain JCM 2418 (NR113350.1) respectively 94 and 96 per cent, AIK25 isolate with *Staphylococcus haemolyticus* JCSC1435 (NR007168.1) having 93%, with *Staphylococcus arlettae* strain ATCC 43957 (NR024664.1), AIK28 gave 90% and AIB43 gave 97%, whereas isolate AIK34 gave 95% with *Staphylococcus succinus* strain AMG-D1 (NR028667.1).

Yildirim *et al.*[14] reported the mitigation of salt stress in *Raphanus sativus* by plant growth promoting rhizobacteria like *Staphylococcus kloosii* and *Kocuria erythromyxa*. Based on 16S rRNA sequences, Costa *et al.*[15] isolated 23 different genera from leaves of the common bean (*Phaseolus vulgaris*) and in that most of were *Methylobacterium* sp., *Staphylococcus* sp. and *Bacillus* sp. Based on 16S rRNA 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.

Only few isolate were different, which was the data obtained from 16S rRNA gene sequence of isolate showed that isolate AIB35 was closely related to *Providencia rettgeri* strain NCTC 11801, AIB44 was related to *Bacillus cereus* ATCC 14579 (NR074540.1) with 97% similarity., AIB 49 gave 95% similarity with *Microvirga zambiensis* strain WSM 3693 (NR117847.1), AIB52 was 99% with *Providencia vermicola* strain OP1 (NR042415.1) and WC68 was 99% with *Alcaligenes faecalis* strain NBRC 13111 (NR113606.1).

Most of the sequences of endophytes obtained were homologous with *Enterobacter* sp. and *Pseudomonas* sp. Other endophytes encountered were *Pantoea* sp., *Staphylococcus* sp., *Bravibacillus* sp., *Klebsiella* sp., *Erwinia* sp., and *Curtobacterium* sp. Endophytes isolated only in cotton were *Acinetobacter baumanii*, *Alcaligenes* sp., *Cellulomonas* sp., *Comamonas testosteroni* and *Erwinia carotovora*[16].

Egamberdieva *et al.*[17] studied salt tolerant bacteria from the rhizosphere of Uzbekistan wheat with potentially beneficial traits were isolated and characterized. Using sequencing of part of the 16S rDNA, the eight new isolates were identified as *Acinetobacter* (two strains), *Pseudomonas aeruginosa*, *Staphylococcus saprophyticus*, *Bacillus cereus*, *Enterobacter hormaechei*, *Pantoae agglomerans* and *Alcaligenes faecalis*. All these strains are potential human pathogens. Possible reasons for why these bacteria present in the rhizosphere and establish.

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. Best identified bacteria will be under such conditions, agriculture will gradually shift towards hitherto uncultivable areas such as coastal areas and waste lands and these microorganisms can contribute to sustainable agriculture under adverse conditions.

CONCLUSION

The results obtained on analysing partial sequences of the 16S rRNA region encoding gene, demonstrated that there is diversified salt tolerant endophytic bacteria inhabited in different species of the region of Kutch. This culturable halotolerant bacterium inhabiting salty and arid ecosystems has a great potential to contribute to promoting plant growth under the harsh salinity conditions. This halotolerant strains could be exploited in biofertilizer formulates to sustain crop production under saline lands. This work will be useful for the development of salt stress tolerant varieties of our important crops using genetic engineering.

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