Diversity of Endophytic and Epiphytic Bacteria From Sugarcane in Khuzestan, Iran

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HIGHLIGHTS

- The Endophytic and Epiphytic Bacterial Flora of Sugarcane Was Studied in Khuzestan.
- Both Leaves and Sheaths Showed a High Diversity of Endophytic and Epiphytic Bacteria
- The Diversity of Bacteria in the Leaves Were Significantly More Than That of Sheaths.

Abstract: Diverse microorganisms are living as endophytes in plant tissues and as epiphytes on plant surfaces in nature. Commercial formulations of bacteria antagonist to plant pathogenic microbes and ice nucleation active bacteria have been utilized as an environmentally safe method to manage plant disease and to prevent frost damage respectively. Bacteria were isolated from the leaf and sheath of sugarcane (CP69-1026, CP57-614, CP48-103, CP73-21, and CP70-1143 cultivars) varieties grown in the field in Khuzestan province, Iran. Bacteria were found in both sheaths and leaves of sugarcane plants which they were significantly higher in density in leaves and which most were endophytic. The bacterial strains were 10 groups on the basis of the biochemical characteristic, which their 16S rRNA encoding gene from representatives were amplified and subjected to sequencing. Results of sequences analyze using blast software from the NCBI website and phylogenetic analysis showed that the representative strains belonged to a wide variety of phylogenetic groups. These results indicated that they were closely related to Burkholderia andRalstonia from β-Proteobacteria, Mesorhizobium, Ochrobactrum, Sphingomonas from α-Proteobacteria, Microbacterium,
Curtobacterium and Leifsonia from Actinobacteria and Xanthomonas from γ-Proteobacteria. This is the first report of the presence of endophytic and epiphytic bacteria from sugarcane in Khuzestan, Iran.

**Keywords:** 16S rRNA; Diversity; Endophytic bacteria; Sugarcane; Khuzestan.

**INTRODUCTION**

Sugarcane (Saccharum officinarum) is a member of the family Poaceae which is best characterized by its high sucrose amount. It is a major world industrial crop, which is used in the manufacturing of important chemicals and industrial products, including MDF, pulp and medical alcohol from molasses, brown sugar, flavored sugar, and powdery sugar. Currently, it represents one of the most important sugar sources, being cultivated in more than 50 countries. The economic significance of the sugar cane, known as the magic plant, is not overlooked by anyone, and as a strategic product, it has a special place among the agricultural production in Iran. Since a long time ago, Khuzestan Province has been one of the important centers of production of sugarcane. In some cases, production of 220 tons cane per hectare has been seen in this province. So, the cultivation of sugarcane in this province is considered a relative preference.

The aerial parts of plants including leaves, stems, buds, flowers, and fruits provide habitat for microorganisms termed the phyllosphere. Microbes can be found both as epiphytes on the plant surface and as endophytes within plant tissues [1-6]. One might expect the importance of particular abiotic factors to vary, depending upon the location of the microbial community within the plant, and this may have repercussions for the structure of microbial communities. For example, the microbial community residing in the phyllosphere (the aerial parts of plants) is faced with a nutrient poor and variable environment that is characterized by fluctuating temperature, humidity and UV radiation (Bacteria are considered to be the dominant microbial inhabitants of the phyllosphere [3, 7]. Phyllosphere bacteria can promote plant growth and both suppress and stimulate the colonization and infection of tissues by plant pathogens [3, 8]. A phyllospheric study can also be helpful to find out some relation between parasite plants and their hosts [9]. As well as, prokaryotes are directly linked to the action of plants, and various endophytes were found in sugarcane. Therefore, clarification of the diversity and function of the endophytic and epiphytic may more effectively help to clarify their roles in their hosts. However, the microbial diversity in aerial parts of sugarcane in Khuzestan is yet poorly characterized. Our main objective was to describe and compare epiphytic and endophytic bacterial communities associated with the sheath and leaves of sugarcane in Khuzestan.

**MATERIALS AND METHODS**

**Plant samples, isolation of bacteria and culture conditions**

During the winter and summer of 2017, plant samples were collected at several stages from sugar cane fields located in the north and south of Khuzestan. The cultivated of sugarcane verities which were subjected to sampling were CP69-1026 CP57-614, CP48-103, CP73-21, and CP70-1143 cultivars. All Samples were transferred to the laboratory of pathology located at the Sugar Research and Training Institute of Khuzestan. For isolation of epiphytic bacteria, sugarcane leaves from the stems and sheath were detached and placed in 100 ml sterilized distilled water containing 1 g gelatin and placed on a 120rpm shaker for 45 minutes. A loopful of the resulting suspension was streaked onto YPGA (Yeast extract: 7 g/l⁻¹, Peptone: 7 g/l⁻¹, Glucose: 7 g/l⁻¹, Agar: 15 g/l⁻¹) medium [10]. The plates were incubated at 25– 27°C for 48–72h. To isolate the endophytic bacteria, the sugar cane leaves are separated from the stems,
then the leaves were washed with sterile distilled water and their surface disinfected by washing with 70% ethanol. The stems were treated in the same way. Afterward, the leaves and stems disinfected, macerated in 100 ml sterilized distilled water containing 1 g gelatin and 1 g NaCl. The suspensions were placed on a 120 rpm shake for 30 min and appropriate cultures were made. All bacterial isolates were purified on YDC (Yeast extract: 10 g/l, Dextrose (glucose): 20 g/l, Calcium carbonate: 20 g/l, Agar: 15 g/l) medium. The appropriate amount of the bacterial strains re-suspended in sterilized distilled water and stored at 4 °C for further investigation. For the long-term storage, the bacterial strains were suspended in 15 % glycerol and kept at -70 °C.

**Biochemical characterization of the bacterial strains**

For the determination of the phenotypic features of the bacterial strains standard bacteriological methods were employed [10]. Phenotypic features include Gram reaction, oxidase and catalase activity, aerobic/anaerobic growth (O/F), levan formation, fluorescent pigment on King's B medium, growth at 40 °C, proteolytic and pectolytic activity, and colony characteristics on yeast extract-dextrose-calcium carbonate (YDC) agar medium were determined [11]. The biochemical tests were repeated twice.

**Amplification of 16S rRNA encoding genes and its sequencing**

DNA was extracted from the representative bacterial strains using the boiling cells method [10]. Genomic DNA was used as a template for the amplification of 16S rRNA encoding genes using polymerase chain reaction (PCR) with the universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3'). The reaction was performed to amplification of a fragment of approximately 1500 bp of the 16S rRNA encoding gene. The Universal PCR Kit, Gene PAK® PCR MasterMix Core (ISOGENE Laboratory, Moscow, Russia), was used and the experiments were carried out based on the manufacturer's recommendations. Each reaction test tube contains a 25µl PCR mixture which a 50-ng total DNA and 1 µl of each primer (10 pmol×µl⁻¹) were added. The PCR program was as an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 45 s, extension at 72°C for 30 s and a final extension at 72°C for 10 min. The amplified products were separated through electrophoresis using agarose gel (1.2%) under electric conditions of 100 mA in TBE buffer, stained with CinnaGen DNA safe Stain (Cat. No. F.P5082, CinnaGen Co., Tehran, Iran) and visualized under UV light.

**PCR product sequencing and phylogenetic analysis**

PCR products were subjected to sequencing (Bioneer Corporation: http://www.Bioneer.com) and the resulting sequences were aligned by using the multiple sequence alignment program, CLUSTAL W [12]. Phylogenetic analysis was performed using MEGA 6.06 software [13]. All sequences were deposited in GenBank and assigned an accession number. Phylogenetic analysis was performed using the Maximum Likelihood method [13]. The phylogenetic trees were constructed with bootstrapping (1000 replications) using MEGA 6.06 software [14].
RESULTS

Isolation and identification of the endophytic and epiphytic bacteria

A total of 390 strains of bacterial strains were isolated from sugarcane and stem leaves, all stored at 15% glycerol at -70°C. Of these 390 strains, 67 strains were selected as the representative based on the results of the biochemical tests mentioned above. Except for the species related to genus *Xanthomonas*, the rest of the Gram-negative genera have a positive oxidase and all the Gram-positive showed a negative reaction for oxidase. Results of phenotypic determination indicated that four Gram-positive bacterial species in three genera and fourteen Gram-negative bacterial species in six genera present in sugarcane leaves and stems suggesting adaptation and co-evolution of diverse bacteria with their host sugarcane plants. We found that epiphytic and endophytic bacterial populations in the leave were more diverse than the stem. The density of the endophytic bacterial population was higher than the epiphytic samples. The sugarcane leaves contain a more diverse bacterial genus than stems.

Sequence analysis

The nucleotide sequences of 16S rRNA encoding genes of 67 representatives were determined and aligned with those of reference strains in GenBank (Figure 1). Except for isolate SC112, other isolates showed high similarity (≥97%) with their closest related species. Phylogenetic analysis of 16S rRNA sequences showed that all isolates obtained in this study clustered with the type strain of each species. Most of the strains belonged to the β-proteobacteria. Results of this study allowed us to cluster the sugarcane bacteria into 4 distinct groups: Group I was composed of members of the *Burkholderiaceae* family. This group contains 45 strains, which was the most populated group. The strains of this group were clustered with *Burkholderia* and *Ralstonia*. Group II consists of bacteria from the α-Proteobacteria group include the genus, *Ochrobactrum* and *Sphingomonas*. Group III had three representatives of the Actinobacteria (a group of Gram-positive bacteria with high G+C content) which include *Leifsonia*, *Curtobacterium*, and *Microbacterium*. Group IV had a single representative of the γ-Proteobacteria phylum (SC166) related to *Xanthomonas*. This is the only genus of Gram negative bacteria that have negative oxidase (Table 1).
Table 1. The 16SrRNA sequence analyses of the endophytic and epiphytic bacteria isolated from sugarcane plants in Khuzestan province, Iran

| Groups | Characteristics of isolates | Max identity (%) | Accession no. | Separated source | Type of bacteria |
|--------|----------------------------|------------------|---------------|-----------------|-----------------|
| Group I | *Burkholderia gladioli* | 99 | MH254946 | Leaf | Endo¹ |
| β-proteobacteria | *Burkholderia gladioli* | 99 | MH256558 | Leaf | Endo |
| *Burkholderiaceae family* | *Burkholderia gladioli* | 99 | MH256493 | Leaf | Endo |
| | *Burkholderia gladioli* | 99 | MH256559 | sheath | Endo |
| | *Burkholderia gladioli* | 99 | MH256554 | Leaf | Epi² |
| | *Burkholderia gladioli* | 99 | MH256560 | Leaf | Epi |
| | *Burkholderia gladioli* | 99 | MH256555 | Leaf | Epi |
| | *Burkholderia gladioli* | 99 | MH256494 | Leaf | Epi |
| | *Burkholderia gladioli* | 99 | MH256495 | Leaf | Epi |
| | *Burkholderia gladioli* | 99 | MH256496 | sheath | Epi |
| | *Burkholderia fungorum* | 99 | MH256497 | Leaf | Endo |
| | *Burkholderia fungorum* | 85 | MH256498 | Leaf | Endo |
| | *Burkholderia fungorum* | 100 | MH256499 | Leaf | Endo |
| | *Burkholderia fungorum* | 100 | MH256500 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256501 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256502 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256503 | Leaf | Endo |
| | *Burkholderia fungorum* | 100 | MH256504 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256505 | Leaf | Epi |
| | *Burkholderia fungorum* | 99 | MH256506 | Leaf | Epi |
| | *Burkholderia fungorum* | 99 | MH256507 | Leaf | Epi |
| | *Burkholderia fungorum* | 99 | MH256508 | Leaf | Epi |
| | *Burkholderia fungorum* | 99 | MH256509 | sheath | Endo |
| | *Burkholderia fungorum* | 99 | MH256510 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256511 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256512 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256513 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256514 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256515 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256516 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256517 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256518 | Leaf | Endo |
| | *Burkholderia fungorum* | 99 | MH256519 | Leaf | Epi |
| Species                        | SC  | Year | Genbank Accession | Site       | Location   |
|-------------------------------|-----|------|-------------------|------------|------------|
| *Burkholderia fungorum*       | SC134| 99   | MH256520          | Leaf       | Epi        |
| *Burkholderia fungorum*       | SC135| 99   | MH256521          | Leaf       | Epi        |
| *Burkholderia fungorum*       | SC136| 99   | MH256522          | Leaf       | Epi        |
| *Burkholderia contaminans*    | SC137| 99   | MH256523          | sheath     | Endo       |
| *Burkholderia contaminans*    | SC138| 99   | MH256524          | sheath     | Endo       |
| *Ralstonia pseudosolanacearum*| SC156| 99   | MH256541          | Leaf       | Epi        |
| *Ralstonia pseudosolanacearum*| SC157| 99   | MH256542          | Leaf       | Epi        |
| *Ralstonia syzygi*            | SC158| 99   | MH256543          | Leaf       | Epi        |
| *Ralstonia pickettii*         | SC159| 99   | MH256544          | sheath     | Endo       |
| *Rals* tronia pickettii       | SC160| 99   | MH256545          | sheath     | Endo       |
| *Rals* tronia solanacearum    | SC161| 99   | MH256546          | Leaf       | Epi        |
| *Rals* tronia solanacearum    | SC162| 99   | MH256547          | Leaf       | Epi        |
| *Mesorhizobium huakuii*       | SC139| 99   | MH256525          | Leaf       | Epi        |
| *Mesorhizobium huakuii*       | SC140| 99   | MH256526          | Leaf       | Epi        |
| *Mesorhizobium huakuii*       | SC141| 98   | MH256527          | Leaf       | Epi        |
| *Mesorhizobium huakuii*       | SC142| 98   | MH256528          | Leaf       | Epi        |
| *Mesorhizobium huakuii*       | SC143| 99   | MH256529          | Leaf       | Epi        |
| *Ochrobactrum ciceri*         | SC144| 99   | MH256530          | sheath     | Endo       |
| *Ochrobactrum ciceri*         | SC145| 99   | MH256531          | Leaf       | Epi        |
| *Ochrobactrum ciceri*         | SC146| 99   | MH256532          | Leaf       | Epi        |
| *Ochrobactrum ciceri*         | SC147| 99   | MH256533          | Leaf       | Epi        |
| *Ochrobactrum ciceri*         | SC148| 99   | MH256534          | Leaf       | Epi        |
| *Ochrobactrum ciceri*         | SC149| 99   | MH256535          | Leaf       | Epi        |
| *Ochrobactrum ciceri*         | SC150| 99   | MH256536          | Leaf       | Epi        |
| *Ochrobactrum ciceri*         | SC151| 99   | MH256537          | Leaf       | Endo       |
| *Ochrobactrum ciceri*         | SC152| 99   | MH256538          | Leaf       | Endo       |
| *Microbacterium resistens*    | SC153| 99   | MH256539          | Leaf       | Endo       |
| *Microbacterium arborescens*  | SC154| 99   | MH256553          | sheath     | Endo       |
| *Microbacterium proteolyticum*| SC155| 97   | MH256540          | Leaf       | Epi        |
| *Leifsonia psychrotolerans*   | SC163| 99   | MH256548          | Leaf       | Endo       |
| *Curtobacterium flaccumfaciens*| SC164| 99   | MH256549          | Leaf       | Endo       |
| *Curtobacterium flaccumfaciens*| SC165| 97   | MH256550          | Leaf       | Epi        |
| *Xanthomonas campestris*      | SC166| 98   | MH256551          | Leaf       | Epi        |

1Endophytic bacteria

2Epiphytic bacteria

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Figure 1. Neighbor joining tree of 16S rRNA gene sequences from endophytic bacterial isolates from sugarcane plants.
DISCUSSION

The bacterial strains isolated from sugarcane in Khuzestan in this study were genetically diverse and belonged to divergent phylogenetic groups of bacteria from the phyla Proteobacteria and Actinobacteria. The 16S rRNA gene sequences of the strains isolated allow their classification within the genera Burkholderia,Ralstonia,Mesorhizobium, Ochrobactrum, Sphingomonas, Xanthomonas, Curtobacterium, Leifsonia, and Microbacterium. The results of this study indicate that Burkholderia has dominated the rest of the genera and found this genus more in leaves. Previously, Mendes et al. [15] separated this genus from the roots and sugarcane stems and stated that the largest population was in the root. Burkholderia species have been isolated from various sources [16,17], including sugarcane [18], rice [19], wine plants [20], onion [21], maize, and coffee [22-23]. The high frequency of Burkholderia species among the bacteria from sugarcane plants and their strong growth-inhibitory activity against F. moniliforme make these isolates potential candidates for the control of Pokkah boeng disease [15]. Previous studies have shown that strains belonging to the genus Burkholderia are effective biocontrol agents [24].

Thirteen strains belonged to the α-Proteobacteria phylum, which are in the genera Ochrobactrum, Mesorhizobium, and Sphingomonas. The Ochrobactrum strains have been isolated from various sources, mainly plant rhizospheres, clinical material and aquatic habitats [25-28]. This genus inhibited the growth of the pathogen Colletotrichum falcum on PDA plates in vitro and reduced red rot infection in vivo [28-29]. Eight strains belonged to the Microbacteiraceae, a family that includes Gram positive bacteria with high G+C within the phylum Actinobacteria. The strains SC151 to SC155 were related to Microbacterium sp.. This genus has been previously reported as endophytic bacteria in wheat [30], clover [31], and sorghum and soybeans [32]. The phylum also includes Leifsonia psychrotolerans, endophytic bacteria Which was formerly detached from the mossy soil by Ganzert et al. [33]. In the present study, Curtobacterium strains were obtained from all sugarcane leaves and comprised 2.9% of total bacteria (the strains SC163 and SC164). The strains were more than 99% similar to the species C. flaccumfaciens. The Curtobacterium strains have been isolated as typical endophytes from several woody plants like sweet-orange, coffee, and grapevine [34-36]. Previously Araujo et al. [36] found that the endophytic bacteria C. flaccumfaciens has a higher density only in asymptomatic citrus plants, and hence suggested that C. flaccumfaciens may play a key role in citrus resistance to Citrus variegated chlorosis. Several subspecies or strains of C. flaccumfaciens are known as causal agents of wilt and necrotic symptoms on horticultural and ornamental plants [37]. However, C. flaccumfaciens had also been reported as a biocontrol agent for cucumber [38] and to play a role in triggering induced systemic resistance [39]. One strain was classified in the family Xanthomonadaceae within the γ-Proteobacteria. The strain SC166 was belonged to the genus Xanthomonas, with the representation strain SC166 being 98% similar to Xanthomonas campestris. The X. campestris strains have been found in the endophytic populations from citrus and clover plants [31,35]. The difference in the bacterial association was attached to plant age, plant source, tissue type, time of sampling, and environment condition [40]. However, our present study clearly showed that the sampling time and plant tissue type could largely impression the difference in the endophytic and epiphytic association of sugarcane plants. The bacterial society associated with sugarcane shelters numerous genera with potential for plant growth propagation and disease control. These results show that analysis of the biodiversity is necessary to better our science on plant bacterial society as a former step to study the activities and utilization of endophytes and epiphytes in agriculture, environment protection, and biotechnology.

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