



Culture-independent analysis of endophytic bacterial communities associated with Brazilian sugarcane

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ABSTRACT. Sugarcane is an economically important culture in Brazil. Endophytic bacteria live inside plants, and can provide many benefits to the plant host. We analyzed the bacterial diversity of sugarcane cultivar RB-72454 by cultivation-independent techniques. Total DNA from sugarcane stems from a commercial plantation located in Paraná State was extracted. Partial 16S rRNA genes were amplified and sequenced for library construction. Of 152 sequences obtained, 52% were similar to 16S rRNA from *Pseudomonas* sp, and 35.5% to *Enterobacter* sp. The genera *Pantoea*, *Serratia*, *Citrobacter*, and *Klebsiella* were also represented. The endophytic communities in these sugarcane samples were dominated by

the families Enterobacteriaceae and Pseudomonadaceae (class Gammaproteobacteria).

Key words: Endophytic bacteria; Microbial diversity; Sugarcane; 16S rRNA

INTRODUCTION

Endophytic bacteria live inside plants without causing damage to their hosts (Hallmann et al., 1997), and actually, these bacteria can contribute to plant growth (Rosenblueth and Martinez-Romero, 2006). This type of bacteria has been found in a broad range of agricultural plants, including rice, sugarcane, sorghum, soybean, rape seed, and sweet potatoes (Cavalcante and Döbereiner, 1988; Germida et al., 1998; Kuklinsky-Sobral et al., 2004; Sun et al., 2008; Terakado-Tonooka et al., 2008; Rout and Chrzanowski, 2009). These microorganisms are being increasingly used in agricultural practices for enhancing crop productivity and reducing the costs of pesticides and fertilizers (Perrig et al., 2007). Among the benefits conferred to the plants by endophyte inoculation are an increase in plant resistance to diseases, an increase in phosphate availability, and supply of fixed nitrogen (Araujo et al., 2002; Oliveira et al., 2002; Kuklinsky-Sobral et al., 2004). Therefore, there is great interest in the identification and characterization of endophytes, by studying the dynamics of plant bacterial populations and ecological interactions.

Before the last decade, the majority of studies on endophytic bacteria used cultivation-dependent methods. The growth conditions select fast-growing organisms, which are well adapted to the culture conditions. In contrast, cultivation-independent methods allow assessment of a larger portion of the plant microbiota (Tian et al., 2007).

Sugarcane (*Saccharum* spp) is one of the most important crops in Brazil, which amounts to a quarter of the world's production. Recently, interest in sugarcane has increased since it is the most promising sustainable source of biofuel. Alternatives for improving crop production for this Poaceae and reducing carbon dioxide emissions are under intense investigation. Most of the studies of endophytic bacteria have focused on the isolation of diazotrophic endophytes, and the organisms frequently found are *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp and *Azospirillum amazonense* (Baldani et al., 1986; Reis Junior et al., 2000).

Cultivation-dependent methods in N-free media are mainly used in these studies, suggesting that many sugarcane endophytes have not been identified, including bacteria of potential biotechnological use. In agreement with this suggestion, Fischer et al. (2011) recently showed the presence of the genera *Ideonella* and *Herbaspirillum*, as well as rhizobial *nifH* transcripts, in sugarcane.

The organisms cited above (Baldani et al., 1986; Reis Junior et al., 2000; Fischer et al., 2011) were found in stems. Although this high prevalence of classic diazotrophic bacteria has been described in sugarcane, Magnani et al. (2010) observed a dominance of Gammaproteobacteria, mainly from the families Pseudomonadaceae and Enterobacteriaceae in the stems of this plant.

The aim of the present study was to determine the dominant groups endophytically associated with sugarcane by analyzing the bacterial community in adult sugarcane stems using a culture-independent approach.

MATERIAL AND METHODS

Two healthy adult sugarcane plants (RB-72454) were collected from an experimental area of Universidade Federal do Paraná, located in Paranavaí in the northwest region of Paraná State. The plants were kept at 4°-10°C, pooled and processed within 24 h of collection.

The stems were washed in running water, followed by distilled water. The stems (cut at 15 cm from the soil) were then immersed in 70% alcohol, followed by flame sterilization of the surface. The surface of sterile stems was completely removed to avoid carryover of epiphytic bacteria, and the internal tissues of the stem (22 g) were crushed with a mortar and pestle in sterile Tris-HCl, pH 8. The extracts were used for DNA purification with the Ultra Clean Plant DNA kit (MO BIO Laboratories Inc., USA). The purified DNA was then used to amplify the 16S rRNA gene of endophytes by PCR with the primers 27f (AGAGTTTGATCC TGGCTCAG) and 16S805r (GACTACCAGGGTATCTAATCCTG) (Lane, 1991; Soares-Ramos et al., 2003), which yielded a fragment of 800 bp. The polymerase chain reaction (PCR) contained 1X PCR buffer (Invitrogen, USA), 1.5 mM MgCl₂, 200 μM of each dNTP, 5 pmol of each primer and 1 U *Taq*DNA polymerase Platinum (Invitrogen). The PCR conditions were: an initial denaturation step at 94°C for 3 min, 28 cycles at 94°C for 1 min, 62°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were cloned in the vector pCR2.1 (Invitrogen) and electrotransformed in the *Escherichia coli* DH10B. For the sequencing reaction the plasmids containing the 16S rRNA gene were isolated by alkaline lysis and further purified on Sephadex G-50 using the Millipore MultiScreen Assay System. Sequences of partial 16S rRNA genes were obtained by the dideoxy-chain termination method using the DYEnamic ET terminator cycle sequencing kit (GE Healthcare, USA) in a MegaBACE 1000 sequencer (GE Healthcare). The Phred program was used for base calling (Ewing et al., 1998) and the sequence edition was performed by BioEdit (Hall, 1999). BLASTn was used for sequence comparison (Altschul et al., 1997) and the taxonomic assignment was made using the best hit. Rarefaction curves were constructed using DOTUR 1.53 (Schloss and Handelsman, 2005). The operational taxonomic units (OTUs) were defined with a threshold of 3% dissimilarity (OTU_{0.03}). Representative sequences of each OTU and related sequences from the GenBank database were aligned with ClustalW (Thompson et al., 1994) and edited with BioEdit (Hall, 1999) to construct a phylogenetic tree from the Jukes-Cantor calculated distance matrices, using the neighbor-joining method with 1000 bootstrap replicates in the MEGA4 program (Kumar et al., 2008). Clone coverage (*C*) was calculated by the equation:

$$C = 1 - \left(\frac{n_j}{N}\right) \times 100$$

where n_j is the number of singletons and N the total number of clones (Pachiadaki et al., 2010). The sequences from the 16S rRNA clone libraries were deposited in GenBank and have the following accession numbers: HM122276 to HM122422 and HM594272 to HM594276.

For enumeration of cultivable endophytic bacteria, extracts of surface-sterilized samples of sugarcane stems were obtained, serially diluted and plated on NFbHPN (Pedrosa and Yates, 1984) and LA media. Colonies were counted after 1-5 days at 30°C.

RESULTS

Colony counting revealed 7×10^6 and 2×10^7 colony-forming units per gram of fresh sugarcane stem on NFbHPN and LA media, respectively, suggesting a high level of bacterial endophytic population.

PCR amplification using the primers 27f and 16S805r and pooled stems of sugarcane extract as template produced a single band of approximately 800 bp. Amplification of the same DNA with primers 27f and 1492r (Lane, 1991) produced a band of approximately 1.5 kb but also often produced a 2-kb band, which was presumably the result of amplification of the mitochondrial rRNA gene. When the sequences of primers 27f and 16S805r were used to search the RDPII database (Cole et al., 2009), a perfect match of both sequences with 152,954 of 1,002,421 sequences of the available 16S rRNA sequences of the domain Bacteria was observed, suggesting that this pair of primers can be used to evaluate a broad range of bacterial diversity. We, therefore, decided to use primers 27f and 16S805r to amplify the 16S rRNA gene of the microbial population of sugarcane, where the product was cloned into pCR2.1 (Invitrogen) and the inserts sequenced.

A total of 288 clones were sequenced using primer 27f, yielding 152 high-quality partial sequences of the 16S rRNA gene. The sequence length varied from 259 to 805 bp. The rarefaction curve (Figure 1) constructed using the partial sequences (138 sequences with an alignment of 320 bp) was partially saturated at 3 and 5% divergence.

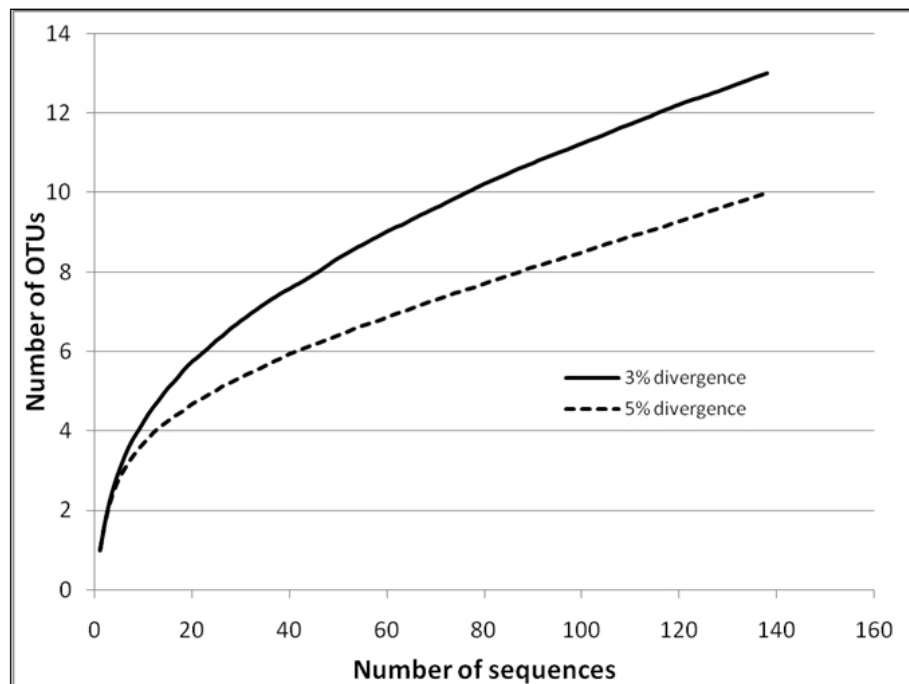


Figure 1. Rarefaction curves from 16S rRNA gene sequences of sugarcane-associated endophytes. Rarefaction curves are shown at taxa levels of species (3% divergence) and genus (5% divergence). OTUs = operational taxonomic units.

To estimate richness, the Chao index was calculated at 3% divergence and showed 18 OTUs in our sample, a number similar to that shown in the rarefaction curve. The coverage calculation based on 3% divergence showed that the sequences sampled represented 90% of the theoretical number of bacterial taxa. These analyses together indicated that the sample was representative of the bacterial endophytic diversity in sugarcane stems.

All sequences obtained had close similarity to 16S rRNA of the Gammaproteobacteria. The two main families found were Pseudomonadaceae (79 sequences) and Enterobacteriaceae (73 sequences) (Table 1). Among the latter family, 50 clones were related to the genus *Enterobacter*, 17 to *Pantoea* sp, 4 to *Klebsiella*, 1 to *Citrobacter* sp, and 1 to *Serratia* sp.

Table 1. Distribution of sugarcane endophytes within bacterial taxa according the 16S rRNA gene sequences.

No. of clones	% Total clones	Best BLAST hit		
		Classification	GenBank accession	% Identity
1	0.7	<i>Enterobacter</i> sp	FJ784695	97
3	2.0	<i>Enterobacter</i> sp	EU653006	98
1	0.7	<i>Enterobacter</i> sp	FJ870557	97
5	3.3	<i>Enterobacter ludwigii</i>	GQ380575	98
10	6.6	<i>Enterobacter ludwigii</i>	FJ859683	99
3	2.0	<i>Enterobacter cloacae</i>	EF219421	99
2	1.3	<i>Pantoea</i> sp	EU887713	99
5	3.3	<i>Pantoea agglomerans</i>	FJ603033	100
2	1.3	<i>Pantoea agglomerans</i>	EU931561	98
6	3.9	<i>Pantoea dispersa</i>	GQ246183	96
1	0.7	Uncultured <i>Serratia</i> sp	DQ279305	100
57	37.5	<i>Pseudomonas plecoglossicida</i>	GQ301534	98
5	3.3	<i>Pseudomonas putida</i>	DQ482656	99
2	1.3	<i>Pseudomonas</i> sp	EF581816	90
1	0.7	<i>Pseudomonas</i> sp	AB508856	94
6	3.9	<i>Pseudomonas</i> sp	FJ577973	94
1	0.7	<i>Pseudomonas</i> sp	FJ719329	98
1	0.7	<i>Pseudomonas</i> sp	FN421341	99
1	0.7	<i>Pseudomonas</i> sp	EU580707	99
1	0.7	Uncultured Pseudomonadaceae	EU721823	96
1	0.7	<i>Citrobacter freundii</i> ¹	GQ983053	98
16	10.5	Enterobacteriaceae ¹	EU272859	99
1	0.7	<i>Enterobacter</i> sp ¹	FN433019	88
2	1.3	<i>Pantoea ananatis</i> ¹	AY579212	98
3	2.0	<i>Enterobacter cloacae</i> ¹	AB244469	99
2	1.3	<i>Klebsiella oxytoca</i> ¹	GU003834	99
5	3.3	<i>Enterobacter</i> sp ¹	GQ360072	92
2	1.3	Enterobacteriaceae	DQ068793	99
2	1.3	<i>Enterobacter</i> sp ¹	GQ062425	99
1	0.7	<i>Pseudomonas</i> sp ¹	EF581815	90
3	2.0	<i>Pseudomonas</i> sp ¹	EF179816	99

¹Sequences annotated as “uncultured bacteria” were tentatively assigned to a taxon by comparison with the GenBank.

The phylogenetic relationships between the main bacterial taxa identified from 16S rRNA gene sequences from sugarcane endophytes and sequences retrieved from GenBank are shown in Figure 2. In agreement with previous analyses, most of the 16S rRNA gene sequences of sugarcane endophytes (identified in the figure with a black triangle and with the initial letters CA) were clustered in the families Enterobacteriaceae and Pseudomonadaceae. Among the family Enterobacteriaceae, the majority of the sequences grouped with *Enterobacter* or *Pantoea* sp.

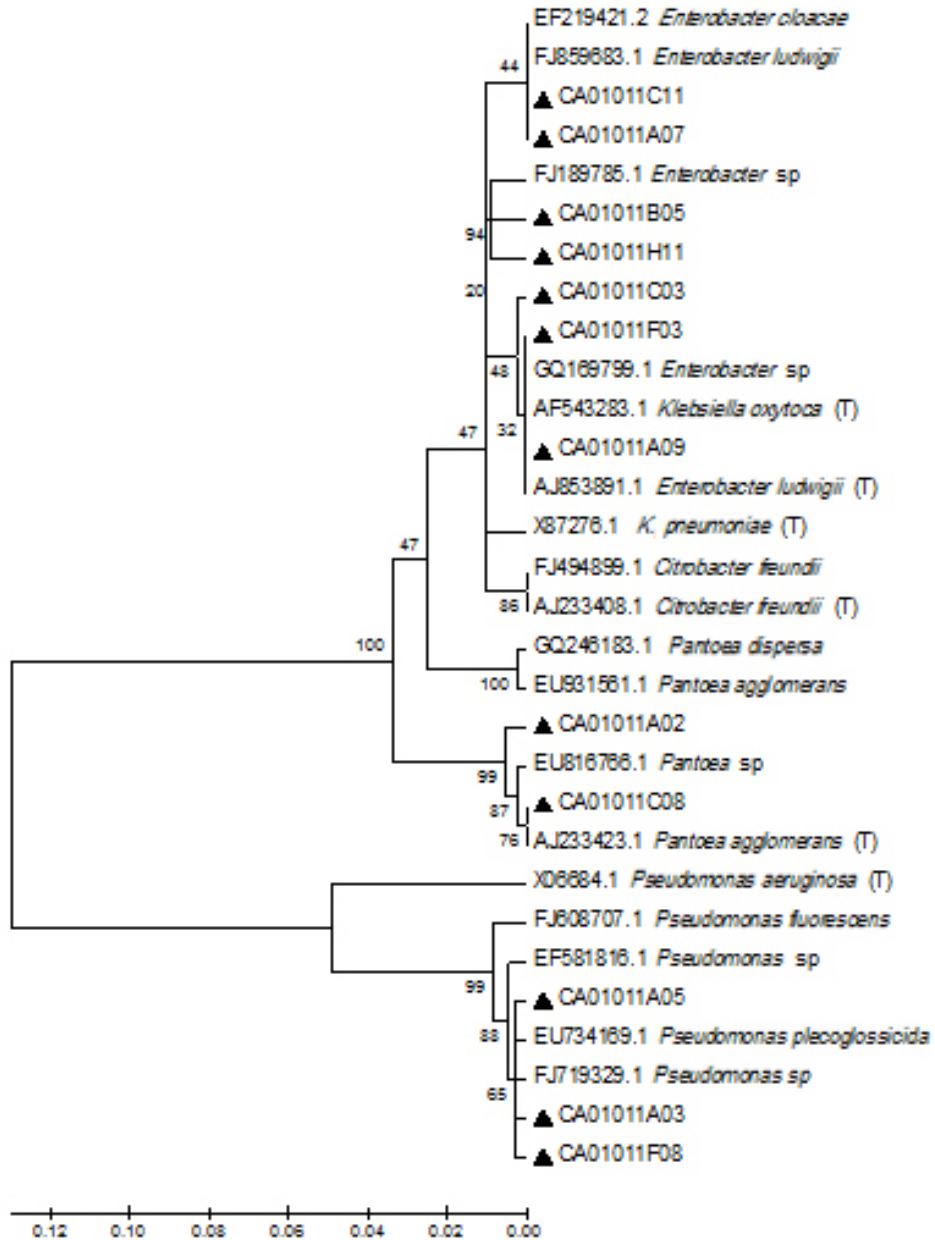


Figure 2. Phylogenetic affiliation of the endophytic bacteria from sugarcane. Unrooted phylogenetic tree positioning the sugarcane endophytes in the families Enterobacteriaceae and Pseudomonadaceae. Sequences of the 16S rRNA gene from sugarcane endophytes are indicated by a black triangle. The sequences with a (T) in the end are from type strains. The tree was constructed using the neighbor-joining method with Jukes and Cantor distance parameters from aligned sequences. The values in the nodes are percentage for 1000 bootstrap replicates. The scale bar means number of nucleotide substitution per site.

DISCUSSION

Previously, we isolated endophytes of sugarcane stems grown in Paraná State on potato medium and identified the bacteria by 16S rRNA sequencing and biochemical tests. The results revealed a prevalence of Enterobacteriaceae, with *Enterobacter* sp being the most frequently isolated bacterium (Magnani et al., 2010). In this study, we used direct amplification of the 16S rRNA gene from total DNA extracted from sugarcane stems, cloning and sequencing to identify the bacterial endophytes. Analyses of the sequences showed a dominance of Proteobacteria associated with healthy sugarcane stems. Although the number of phyla and families of Bacteria identified was relatively low, the results are consistent with other studies of endophytic diversity (Roesch et al., 2008; Prakamhang, 2009; Magnani et al., 2010; Taulé et al., 2012), which found that Proteobacteria is the group of bacteria most frequently associated with plants. Fischer et al. (2011) also found a high number of Proteobacteria with 80-100% of the sequences from 16S rRNA libraries belonging to Alphaproteobacteria of the families Acetobacteraceae, Bradyrhizobiaceae, and Rhodobacteraceae.

Apart from Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes have also often been found as endophytes of graminaceous plants in cultivation-dependent studies but with a low prevalence (Rosenblueth and Martinez-Romero, 2006). In our study, the sugarcane endophytes were distributed in the families Pseudomonadaceae and Enterobacteriaceae. The sequences related to the Pseudomonadaceae made up the largest fraction of the clone library (50% of the clones). When compared with GenBank, several 16S rRNA gene sequences were closely related to sequences obtained from bacteria that live in plants or in soil. Moreover, several clones had sequences of high similarity to sequences from Enterobacteria and Pseudomonadaceae with potential use in agriculture, such as a chlorobenzene-degrading bacterium, a copper-resistant strain from soil, and phosphate-solubilizing bacteria from tea plant rhizosphere (data not shown).

In contrast to our results, several studies found endophytic diazotrophic organisms such as *Herbaspirillum* sp, *Azospirillum* sp, and *G. diazotrophicus* in sugarcane (Reis Junior et al., 2000). These bacteria, frequently isolated in high numbers associated with sugarcane roots, stems, and leaves, and considered candidates for nitrogen fixation (James, 2000), were not found in our sample. The lack of such sequences was not due to primer bias, since analysis of the primers used in the PCR amplification revealed a 100% match with target 16S rRNA gene sequences from these diazotrophic bacteria (data not shown). Moreover, other studies also failed to identify them. For example, the *nifH*-transcripts of *H. seropedicae*, *H. rubri-subalbicans*, and *A. amazonense* were also not detected in long-term field experiments of inoculated sugarcane (Fischer et al., 2011). In addition, a low diversity was observed among the active diazotrophic bacterial community in sugarcane roots from Africa and America, with dominant phylotypes related to *Rhizobium rosettiformans*, *Sulfurospirillum multivorans*, or *Bradyrhizobium elkanii* (Burbano et al., 2011). Suman et al. (2001) showed that the number of diazotrophic endophytes isolated in LGI semi-solid medium, per gram of fresh roots of several sugarcane cultivars, ranged from 0.02 to 3.86% of the total endophytic population. Also using cultivation-dependent techniques, Rennie et al. (1982) found mainly isolates from the family Enterobacteriaceae in sugarcane, and Taulé et al. (2012) found a high prevalence of Gammaproteobacteria, including *Pseudomonas* sp, *Pantoea* sp, and *Enterobacter* sp, in the inner tissues of sugarcane stems from Uruguay. It is interesting to note that members of

the genera found in the stems of sugarcane RB-72454, such as *Enterobacter*, *Klebsiella*, and *Pseudomonas*, have been shown to fix nitrogen and promote plant growth (Rosenblueth and Martinez-Romero, 2006; Taulé et al., 2012).

Cultivation-independent methods are advantageous because they do not need the isolation of the organisms, allowing the assessment of a larger portion of microorganisms. On the other hand, in culture-dependent methods the growth conditions select mainly fast-growing organisms, well adapted to the medium conditions. Although bias in PCR amplification and the limitation in the number of sequences do not allow for a complete view of bacterial diversity, it is largely accepted that culture-independent approach can reveal the dominant organisms in an environmental sample (Ward et al., 1998).

Using a culture-independent method, Sun et al. (2008) found a larger diversity of endophytes in rice roots. A total of 52 OTUs were defined on the basis of ARDRA and 16S rRNA gene sequencing of selected clones from each OTU, which revealed bacteria belonging to Proteobacteria, Cytophaga/Flexibacter/Bacteroides (CFB) group, low G+C Gram-positive bacteria, *Deinococcus-Thermus*, and Acidobacteria. The dominant class was Betaproteobacteria (27% of the total clones), but the most dominant genus was *Stenotrophomonas* of Gammaproteobacteria (Sun et al., 2008). A taxonomic analysis of the *nifH* libraries of maize stems showed the presence of the Alpha-, Beta-, and Gammaproteobacteria classes, with no subdivision being dominant (Roesch et al., 2008). Our results suggest a lower diversity at higher taxonomic ranks of endophytes in mature sugarcane stems. This relatively lower diversity may be due in part to the tissue used (stem), the age of the plant (10 months), and geographic localization of the plantation (southern Brazil). In addition, a cultivar-dependent effect on the selection of endophytic bacteria cannot be excluded.

The results suggest that the endophytic communities in sugarcane stems, and perhaps in other Poaceae, are large and dominated by Proteobacteria. Also, it is likely that such populations can be manipulated by means of selective inoculation to favor plant productivity.

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