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Diversity of endophytic bacteria in Brazilian sugarcane

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ABSTRACT. Endophytic bacteria live inside plant tissues without causing disease. Studies of endophytes in sugarcane have focused on the isolation of diazotrophic bacteria. We examined the diversity of endophytic bacteria in the internal tissues of sugarcane stems and leaves, using molecular and biochemical methods. Potato-agar medium was used to cultivate the endophytes; 32 isolates were selected for analysis. DNA was extracted and the 16S rRNA gene was partially sequenced and used for molecular identification. Gram staining, catalase and oxidase tests, and the API-20E system were used to characterize the isolates. The strains were divided into five groups, based on the 16S rRNA sequences. Group I comprised 14 representatives of the Enterobacteriaceae; group II was composed of Bacilli; group III contained one representative, *Curtobacterium* sp; group IV contained representatives of the Pseudomonadaceae family, and group V had one isolate with an uncultured bacterium. Four isolates were able to reduce acetylene to ethylene. Most of the bacteria isolated from the sugarcane stem and leaf tissues belonged to Enterobacteriaceae and Pseudomonaceae, respectively, demonstrating niche specificity. Overall, we found the endophytic bacteria in sugarcane to be

more diverse than previously reported.

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

INTRODUCTION

Sugarcane (*Saccharum* spp) is one of the most important crops in Brazil, with a yield of 495 M tons in 2007/2008 (UNICA, 2009), a quarter of the world's production. Its two main products are sugar and alcohol, a clean renewable alternative fuel. This crop is perhaps the most economically competitive source of ethanol and can effectively contribute to a cleaner environment. Ways of improving its productivity are subject to intense investigation in Brazil, chiefly because worldwide climate change due to the intense use of greenhouse gas-producing energy sources has resulted in substantial focus on the development of sustainable energy.

Endophytic bacteria occupy internal tissues of plants without causing damage to their hosts (Hallmann et al., 1997). This microbial community could play an important role in agriculture by conferring advantages to the plant (Mengoni et al., 2003), since endophytic bacteria can contribute to plant development by producing phytohormones (Feng et al., 2006) and siderophores (Burd et al., 1998), increasing resistance to pathogens (Reiter et al., 2002) and parasites (Hallmann et al., 1997), and promoting biological nitrogen fixation (Baldani et al., 1986) and antibiotic production (Strobel and Daisy, 2003). Understanding the diversity of plant-bacterial associations and their role in plant development is necessary if these associations are to be manipulated to increase crop production, conserve biodiversity and sustain agro-ecosystems (Germida et al., 1998; Sturz et al., 1999).

Bacterial endophytes are found in a variety of plants, such as sugar beet (Dent et al., 2004), prairie plants, agronomic crops (Zinniel et al., 2002), potato varieties (Sessitsch et al., 2002), wheat (Germida and Siciliano, 2001), and rice (Sun et al., 2008). The microbial community of endophytes colonizes plant tissues and is capable of establishing interactions not only among themselves but also with invaders such as pathogens, and in this way may influence plant development. For example, Araújo et al. (2002) described interactions between the phytopathogenic bacterium *Xylella fastidiosa* and endophytes in *Citrus sinensis*, which apparently restricted the development of the disease by suppressing the symptoms in inoculated plants.

In sugarcane, most of the research on endophytic bacteria has focused on diazotrophs, of which the main representatives are *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp (Baldani et al., 1986; Cavalcante and Döbereiner, 1988) and *Azospirillum amazonense* (Reis Júnior et al., 2000). However, the presence of diazotrophs among the total population of bacteria in sugarcane tissues seems to be low in Indian sugarcane (Suman et al., 2001). The aim of this study was to investigate the diversity of the putative endophytic population in stems and leaves of Brazilian sugarcane.

MATERIAL AND METHODS

Bacterial isolation and identification

Sugarcane plants were from a 4-year-old plantation in the northeast of Paraná State (Brazil). The plants were grown for livestock feeding. Sugarcane leaves and stems

were collected and then separated and maintained in ice until analysis. 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, and after disinfection, were flame sterilized. Stems (10 g) and leaves (10 g) were macerated separately in sterile 10 mM Tris-HCl, pH 8.0, and serially diluted to 10^{-6}. One hundred microliters of these dilutions was plated on potato-agar (Döbereiner et al., 1995) and incubated at 30°C for up to 5 days. Thirty-two isolates from stems and leaves of sugarcane, representing different types of colonies developed on agar, were randomly chosen, analyzed by gram stain, cytochrome oxidase activity (Gram-negative rods) or catalase production (Gram-positive cocci) (Koneman et al., 2001). The Gram-negative rods were plated on MacConkey medium (Miller, 1992) to identify candidate Enterobacteriaceae, and then submitted to biochemical analysis using the API-20E test (bioMérieux).

Amplification and sequencing of the 16S rRNA gene

Three milliliters of fresh cultures of the isolates in LB medium was used for DNA extraction according to Sambrook et al. (1989). Genomic DNA was used as a template in a polymerase chain reaction (PCR) with the primers Y1 (5'-TGGCTCAGAACGAACGCTG GCGGC-3') and Y2 (5'-TACCTTGTTACGACTTCACCCCAGTC-3'), allowing amplification of a fragment of approximately 300 bp of the 5' end of the 16S rRNA gene. The PCR mixtures contained 50-100 ng template DNA, 2.5 µL 10X Taq buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 4 pM Y1 and Y2 primers, and 1.0 U *Taq* DNA polymerase in 25 µL. The temperature cycles were: 94°C/30 s (once); 94°C/20 s, 58°C/20 s, 72°C/1 min (30 times), and 72°C/5 min (once).

The Y1-Y2 PCR products were purified using the AutoSeq 96 System (GE Health-Care) and sequenced using dye terminator chemistry and an ABI PRISM 377 sequencer (Applied Biosystems).

Sequence assembly and analysis

The PHRED program was used for base calling (Ewing et al., 1998). The forward and reverse sequences were assembled with the CAP3 program (Huang and Madan, 1999). Nucleotide sequence identities were determined by the BLAST and Seqmatch programs. The 16S rRNA genes have been deposited in GenBank (EF054896-EF054920/FJ966050-FJ966056).

Determination of nitrogenase activity

To test for nitrogenase activity in the Enterobacteriaceae, 100 µL of the cultures grown in LB was added to 4 mL NFDM (Dixon et al., 1977) medium containing 0.5 mM sodium glutamate in 10-mL bottles, which were sealed with suba-seals and incubated at 30°C in a rotary shaker (120 rpm) for 16 h. Acetylene (10%) was then injected into the culture vials, incubated for 1 h and analyzed for ethylene by gas chromatography. To test for nitrogenase activity in the Pseudomonads, 100 µL of the cultures grown in NfbHPN was added to inoculate 4 mL semi-solid N-free NfbHN and acetylene reduction activity was determined as described (Pedrosa and Yates, 1984).

RESULTS

Phenotypic characterization of the sugarcane isolates

Thirty-two isolates representing all colony types developed on potato-agar cultures from macerates of leaves and stems were randomly selected and stored in 50% glycerol at -20°C. Of these, 18 strains were isolated from stems (designated as CC), and 14 strains from leaves (FC). Frozen cultures were re-streaked on potato-agar, and isolated colonies of each culture were selected.

Most of the isolates were Gram-negative bacilli. The exceptions were strains CC18, a Gram-positive coccus, and strains CC38 and CC27, which were Gram-positive bacilli. All the Gram-negative bacilli were oxidase negative, but only 14 of 29 of these grew on MacConkey agar; these were tested using the API-20E kit for enteric bacteria. Based on the API and oxidase results, these 14 strains were classified as Enterobacteriaceae (Table 1), among which were *Enterobacter* (CC14, CC26, CC29, CC33, CC34, CC37, CC43, CC46, FC2P), *Pantoea* (CC16, CC21, CC47), *Kluyvera* (CC20), and *Klebsiella* (CC22). An interesting observation is that all but one strain of this group (FC2P) were isolated from sugarcane stems. The Gram-positive isolates were catalase positive.

Sequence analysis

The 5' end of the 16S rRNA gene of the 32 isolates was amplified by PCR and sequenced. At least two sequencing reactions were performed for each direction and the assembled sequences, with primer sequences removed, had lengths ranging from 275 to 301 bp.

The 16S rRNA sequences were used to search the GenBank and RDP databases using Blast and SequeMatch programs, respectively. Most of the strains (28) belonged to the gamma Proteobacteria. The results allowed clustering the sugarcane endophytic bacteria into 5 distinct groups. Group I was composed of members of the Enterobacteriaceae family. The isolates of this group were related to *Pantoea* (4), *Enterobacter* (8), *Klebsiella* (1), and *Citrobacter* (1). Again, all but one strain of the enteric bacteria group (FC2P) were isolated from sugarcane stems.

Group II comprised two isolates of the Bacilli class, which probably belong to the *Brevibacillus* (CC38) and *Staphylococcus* (CC18) genera. Group III had a single representative of the Actinobacteria phylum (CC27) related to *Curtobacterium*. Group IV contained 14 strains related to Pseudomonadaceae, which were all isolated from leaves with 2 exceptions (CC24 and CC35). Although most Pseudomonads are oxidase positive, all the isolates in this study were oxidase negative. Group V had only one isolate, related to an uncultured bacterium.

The endophytic population in the stem is more diverse than that of the leaves: the stem harbored a larger number (8) of different genera distributed in all four groups with prevalence of Enterobacteriaceae, whereas bacterial strains isolated from leaves belonged to only 2 genera with a large predominance of *Pseudomonas*.

Screening for endophytic nitrogen-fixing bacteria

Approximately 90% of these isolates showed no nitrogenase activity: only iso-

Table 1. Molecular and physiological characterization of the endophytic bacteria isolated from Brazilian sugarcane.

Isolate	Group	Max identity (%)	Closest relative based on 16S rRNA gene sequence (BLAST)	Source of the best hit on BLAST	API-20E*
CC14	Group I	98%-264 nt identical	*Citrobacter* sp	Lignin-degrading bacteria	*Enterobacter* sp
CC16	Enterobacteriaceae	100%-267 nt identical	*Enterobacter* sp	Bacteria from soy paste and soy sauce	*Pantoea* sp
CC20	family	99%-266 nt identical	*Pantoea* sp	Plant- and clinic-associated *P. agglomerans*	*Kluyvera* sp
CC21		99%-292 nt identical	*Pantoea*	Diazotrophic bacteria associated with sugarcane	*Pantoea* sp
CC22		100%-263 nt identical	*Klebsiella* sp	Wastewater treatment system reliant on N_2 fixation	*Klebsiella* sp
CC26		100%- 263 nt identical	*Enterobacter* sp	PGPR in rice	*Enterobacter* sp
CC29		100%-267 nt identical	*Erwinia* sp/ *Pantoea* sp	Diazotrophic bacteria associated with sugarcane	*Enterobacter* sp
CC33		100%-262 nt identical	*Enterobacter* sp	Urban wastewater treatment plant	*Enterobacter* sp
CC37		98%-263 nt identical	*Enterobacter* sp	Stems of field-grown soybeans	*Enterobacter* sp
CC43		100%-264 nt identical	*Enterobacter* sp	Bacteria from soy paste and soy sauce	*Enterobacter* sp
CC46		97%-261 nt identical	*Enterobacter* sp	Stems of field-grown soybeans	*Enterobacter* sp
CC47		100%-265 nt identical	*Pantoea* sp	Copper-resistant endophytic bacteria	*Pantoea* sp
FC2P		99%-263 nt identical	*Enterobacter* sp	Stem-associated bacteria in soybeans	*Enterobacter* sp
CC34		100%-263 nt identical	*Enterobacter* sp	Bacteria of soy paste and soy sauce	*Enterobacter* sp
CC38	Group II	98%-286 nt identical	*Brevibacillus* sp	Identification of a soil-born PGPR	NT
CC18	Bacilli	99%-300 nt identical	*Staphylococcus* sp	Terrestrial deep subsurface	NT
CC27	Group III Actinobacteria	100%-289 nt identical	*Curtobacterium* sp	Sepsis caused by *Curtobacterium* sp	NT
CC24	Group IV	98%-244 nt identical	*Pseudomonas* sp	Oil-degrading bacterium	NT
CC35	Pseodomonadaceae	98%-244 nt identical	*Pseudomonas* sp	Oil-degrading bacterium	NT
FC5	family	96%-239 nt identical	*Pseudomonas* sp	Bacteria isolated from Arctic seawater	NT
FC6		99%-331 nt identical	*Pseudomonas* sp	PGPR in the maize root	NT
FC10		100%-247 nt identical	*Pseudomonas* sp	A potential biocontrol agent	NT
FC12		100%-247 nt identical	*Pseudomonas* sp	A potential biocontrol agent	NT
FC16		100%-247 nt identical	*Pseudomonas* sp	A potential biocontrol agent	NT
FC18		100%-242 nt identical	*Pseudomonas* sp	Plant interactions of *Pseudomonas fluorescens*	NT
FC19		98%-243 nt identical	*Pseudomonas* sp	Bacteria from the region of a karst water rivulet	NT
FC22		98%-330 nt identical	*Pseudomonas* sp	PGPR in the maize root	NT
FC23		98%-243 nt identical	*Pseudomonas* sp	Bacteria from the upstream region of a karst water rivulet	NT
FC35		98%-242 nt identical	*Pseudomonas* sp	A potential biocontrol agent	NT
FC38		97%-242 nt identical	*Pseudomonas* sp	Marine sediment from Arctic	NT
FC40		97%-283 nt identical	*Pseudomonas* sp	Enhanced *in situ* biodegradation of toluene using a surfactant-modified zeolite support	NT
FC26	Group V	96%-239 nt identical	Uncultured bacterium	Bacteria from the region of karst water rivulet	NT

NT = not tested; nt = nucleotides; PGPR = plant growth-promoting Rhizobacteria. *Only strains that grew on MacConkey agar were tested with API-20E.

lates CC22, CC26, CC29, and CC35 reduced acetylene to ethylene. Based on 16S rRNA sequence identity, these isolated were tentatively classified as *Klebsiella*, *Enterobacter*, *Pantoea*, and *Pseudomonas*.

DISCUSSION

Plants maintain a complex ecosystem where bacterial communities interact continuously, competing for nutrients and water in the tissues of the host. Knowledge of the diversity of endophytic bacteria is important for both ecological and biotechnological studies.

Most studies of sugarcane endophytes have focused on the diazotrophic bacteria (Cavalcante and Döbereiner, 1988; Caballero-Mellado and Martinez-Romero, 1994; Olivares et al., 1996). Since factors other than biological nitrogen fixation can contribute significantly to the host, we investigated the diversity of bacteria from leaves and stems of sugarcane. In our study, the prevalence of diazotrophs was very low: only 10% of the isolates had nitrogenase activity. The absence of endophytic diazotrophs such as *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp, *Burkholderia* spp, and *Azospirillum* spp, which have been found in great numbers in sugarcane, is most notable. This discrepancy may be due to the use of nitrogen-free medium for bacterial isolation, geographic and environmental variations or a combination of these. In a study similar to ours, Suman et al. (2001) isolated endophytic bacteria from several cultivars of Indian sugarcane on LGI medium and found that the prevalence of diazotrophs varied from 3.86 to 0.02%. It is possible that by using a rich medium such as potato-agar medium, which supports the growth of many different bacteria, a distinct portion of the bacterial community was assessed, suggesting a more complex ecology of sugarcane endophytes than previously reported.

In this study, the fermenting Gram-negative bacilli were characterized both by phenotypic identification and 16S rRNA gene sequencing. The identifications obtained by these methods agreed at the genus level in 11 of 14 isolates. In the remaining cases, the identifications were of closely related genera. These discrepancies may be due to limited databases available for the phenotypic test systems and to the fact that the system used was designed for clinical diagnostics: the closest sequence matches to our isolates were always either plant endophytes or from other environmental sources, which are unlikely to be represented in the API-20E system database.

The *Enterobacter* genus was the most frequently found in the stems. *Enterobacter* has been identified as endophytes of several plants such as *Citrus sinensis*, soybean and crop plants (Araújo et al., 2002; Zinniel et al., 2002; Kuklinsky-Sobral et al., 2004). Other Enterobacteriaceae identified in sugarcane have also been previously described as endophytes. The plant growth-promoting bacterium *Kluyvera ascorbata* SUD165, resistant to heavy metals, was capable of conferring resistance to high concentrations of nickel to canola and tomato plants (Burd et al., 1998). Endophytic *Pantoea* was found in sugarcane (Loiret et al., 2004) and in soybean (Kuklinsky-Sobral et al., 2004). Representatives of these three genera have also been associated with pathogenicity (Gurtler et al., 2005; Medrano and Bell, 2007). However, the results suggest no dominance of a particular strain, indicating that a pathogenic association was unlikely. Furthermore, a completely different bacterial community, dominated by *Pseudomonas*, was identified in the leaves.

The two *Bacilli* genera identified in sugarcane have been identified before as endophytes: *Staphylococcus* was found to be associated with sweet pepper (Rasche et al., 2006) and *Brevibacillus* was found in cadmium-contaminated soils and associated with soybean (Sarkar et al., 2002; Vivas et al., 2003). *Curtobacterium*, the only representative of the Actinobacteria, was identified in orange, grape, and *Pinus* (Bell et al., 1995; Araújo et al., 2002; Idris et al., 2004), and interacting with the phytopathogenic bacterium *Xylella fastidiosa* (Lacava et al., 2004). To our

knowledge, this is the first characterization of *Brevibacillus* and *Curtobacterium* in sugarcane.

The microbial population of sugarcane leaves, colonized predominantly by *Pseudomonaceae*, and sugarcane stems, with prevalence of Enterobacteriaceae, were substantially different. Stems presumably offer a more stable niche for the bacteria, since they contain a greater diversity of genera: they are less exposed to drastic changes of their physicochemical conditions such as temperature, humidity, UV irradiation, and nutrients in the apoplast. In contrast, the more frequent variation of the environmental conditions of the leaves can restrict the growth of bacterial populations (Hirano and Upper, 2000). For example, the varying exudates of leaves impose a metabolic versatility on bacteria, a characteristic of the *Pseudomonas* genus (Mercier and Lindow, 2000; Misko and Germida, 2002). This preference for different habitats has already been shown in other plants (Sessitsch et al., 2002; Mocali et al., 2003).

The results reported here suggest that the population of sugarcane endophytes can vary depending on the plant organ analyzed. It is noteworthy that the relative number of diazotrophic endophytes recovered was low, perhaps reflecting the lack of selective pressure in the isolation procedure. Further studies will be necessary to thoroughly analyze the endophytic population of sugarcane, including collection of plants from different geographic origins and the use of culture-independent molecular analyses.

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