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Diversity of *16S rRNA* genes from bacteria of sugarcane rhizosphere soil

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Abstract

Sugarcane is an important agricultural product of Brazil, with a total production of more than 500 million tons. Knowledge of the bacterial community associated with agricultural crops and the soil status is a decisive step towards understanding how microorganisms influence crop productivity. However, most studies aim to isolate endophytic or rhizosphere bacteria associated with the plant by culture-dependent approaches. Culture-independent approaches allow a more comprehensive view of entire bacterial communities in the environment. In the present study, we have used this approach to assess the bacterial community in the rhizosphere soil of sugarcane at different times and under different nitrogen fertilization conditions. At the high taxonomic level, few differences between samples were observed, with the phylum Proteobacteria (29.6%) predominating, followed by Acidobacteria (23.4%), Bacteroidetes (12.1%), Firmicutes (10.2%), and Actinobacteria (5.6%). The exception was the Verrucomicrobia phylum whose prevalence in N-fertilized soils was approximately 0.7% and increased to 5.2% in the non-fertilized soil, suggesting that this group may be an indicator of nitrogen availability in soils. However, at low taxonomic levels a higher diversity was found associated with plants receiving nitrogen fertilizer. *Bacillus* was the most predominant genus, accounting for 19.7% of all genera observed. Classically reported nitrogen-fixing and/or plant growth-promoting bacterial genera, such as *Azospirillum*, *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Burkholderia*, were also found although at a lower prevalence.

Key words: *16S rRNA*; Biodiversity; Nitrogen fixation; Bacteria; Sugarcane; Soil

Introduction

Soil is considered to be the richest environment, with a high diversity of microorganisms belonging to the three domains of life, Bacteria, Archaea and Eukarya (1). This diversity is extreme at the species level, with approximately 50,000 bacterial species found in one soil sample (2). Investigation of bacterial diversity is an important step to assess soil conditions due to its importance in nutrient cycling, and consequently in crop productivity (3,4). Soil bacteria and, in particular, rhizosphere bacteria play an important role in many processes, such as decomposition, mineralization, biological nitrogen fixation, and denitrification (5). In addition, some bacteria associate with plants and promote growth, the so-called plant growth-promoting bacteria (6).

Sugarcane (*Saccharum* spp) was the first economically important crop in Brazil, grown since the XVI century and still has a central role in Brazil's economy, not only with respect to the export of sugar and alcohol, that yielded US$8.3 billion in 2009 (http://www.agricultura.gov.br/), but also because its production chain in Brazil is a major source of employment and an inducer of technological development. The culture of sugarcane has recently received much attention, mainly because of the interest in biofuels, particularly in bioethanol production in countries such as Brazil, USA, and China (7,8). Brazil is the leading sugarcane producer in the world with an annual production of more than 500 million tons in 2009 (http://www.unica.com.br/), with ethanol and sugar as the main products. Ways of improving productivity are subject to intense investigation in Brazil, one of them being the use of bacterial strains that are known to promote plant growth. For this purpose, a more complete knowledge about the diversity of the bacterial community associated with this crop is needed.

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The construction and sequencing of *16S rRNA* gene clone libraries is a culture-independent approach to study microbial diversity and has been a standard method for assessing bacterial communities in a variety of habitats (9,10). A disadvantage of this approach is that it is difficult to process large numbers of samples, consequently limiting statistical comparisons due to the lack of replicates. However, it is still a powerful technique that can help identify uncultured bacteria and/or dominant groups in an ecosystem. In the present investigation, we have studied the bacterial diversity of the soil adhered to the roots of sugarcane plants grown in N-fertilized or untreated soils in the northwest of Paraná State, Brazil, using a culture-independent approach.

Material and Methods

Sampling and sample preparation

Samples were collected from soil adhered to the roots of sugarcane (*Saccharum* spp; Poales order; Poaceae family), variety RB72454, submitted to two different fertilization regimes: no N fertilizer or 120 kg/ha N fertilizer. A control soil sample was collected from sugarcane RB72454 grown at the same site in the previous year, which also received N fertilization. For the comparison of N-fertilization regimes the plants were cropped for 13 months in two blocks of 10 x 10 m and about 5 m apart at the Experimental Station of the Federal University of Paraná (UFPR), located in the city of Paranavaí (23°05'30"S/052°29'04"W), State of Paraná (Brazil). Soil adhered to the roots of plant specimens was used for the *16S rRNA* gene amplification and library construction.

Harvested plants were immediately sent to the laboratory, where the roots and aerial parts were separated. The soil adhered to the roots was collected by manual shaking into tubes and immediately stored at -70°C for analysis. All materials used in plant manipulation were sterilized by autoclaving at 120°C for 30 min and, when applicable, by using 70% ethanol solution. A total of 1 g soil from each sample was used for nucleic acid extraction.

Nucleic acid extraction

The UltraClean Soil DNA Isolation kit (MoBio Laboratories, Inc., USA) was used to isolate DNA from soil microorganisms following manufacturer recommendations. DNA was recovered in Tris-HCl buffer, pH 8.0, and stored at -20°C.

16S rRNA gene amplification

The *16S rRNA* gene from rhizosphere soil microorganisms was amplified by PCR. A primer pair consisting of 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (GGYTACCTT GTTACGACTT) (11) was used to amplify nearly 1500-bp fragments of the *16S rRNA* genes. The samples were amplified in the following PCR mixture: 4 mM $MgCl_2$, 200 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, and 1 U Taq DNA polymerase in 1X buffer containing 200 mM $(NH_4)_2SO_4$ (Fermentas, Canada) in a final volume of 25 µL. The following temperature cycles were used: 94°C for 4 min, 30 cycles of 94°C for 1 min, 62°C for 45 s, and 72°C for 1 min and 30 s, and a final extension at 72°C for 10 min (12). All reactions were conducted in an Eppendorf Mastercycler thermocycler (Eppendorf, Germany).

Construction and sequencing of the *16S rRNA* gene library

PCR products were cloned using the TOPO TA cloning kit according to manufacturer recommendations. Clone libraries were constructed in the vector pCR2.1-TOPO (Invitrogen, USA). For sequencing, the recombinant plasmids were purified by the alkaline lysis method (13). Twelve random clones from each 96-well plate were checked for correct insert size by *Eco*RI digestion, and the reaction products were analyzed on 1% agarose gel at 5 V/cm for 2 h and visualized by UV (312 nm) excitation after staining with ethidium bromide (0.5 µg/L). Sequencing was performed with the 27f primer using the DYEnamic™ ET Terminator Cycle Sequencing Kit (GE Healthcare, USA). The sequencing reactions were purified by precipitation with 96% ethanol and 0.7 M ammonium acetate, and analyzed with an ABI 377 (Applied Biosystems, USA) or MegaBACE 1000 DNA sequencer (GE Healthcare).

Sequence and diversity analysis

Sequences were trimmed to remove low-quality 5' and 3' ends using the Phred base caller (14) and manually verified. Taxonomic affiliation was performed using the Classifier tool available at the Ribosomal Database Project (RDP) II site (15,16). A bootstrap cutoff value of 50% for taxonomic assignment was used for the sequences ranging from 50 to 250 bp in length, and a cutoff of 80% was applied for sequences greater than 250 bp in length. Further analyses were performed on sequences greater than 250 bp in length (394 sequences). Chimera check, sequence alignment, richness inference, and library comparisons were performed using the Mothur v.1.9.0 software (17). Alignments were performed by Mothur using the SILVA bacteria database (18). DNADIST, from the PHYLIP Package (19), was used for genetic distance calculation by the Jukes and Cantor method (20). The MEGA-BLAST algorithm (21) was used to search the NCBI GenBank database (22) to confirm taxonomical assignment of some sets of sequences.

Sequences longer than 50 bp were deposited in the GenBank database under accession Nos. HQD18044 to HQD18581.

Results

Samples of soil from the rhizosphere of sugarcane plants under two different nitrogen fertilization treatments were analyzed by a culture-independent approach using the *16S rRNA* gene library sequencing. Three samples

were selected for the *16S rRNA* gene library construction and sequencing: i) a soil sample from 12-month-old plant roots collected in September 2006, and subjected to a normal N-fertilization regime (S0N); ii) soil from 13-month-old plant roots collected in October 2007, receiving a normal N-fertilization regime (S13N); iii) soil from 13-month-old plant roots collected in October 2007, not fertilized with nitrogen (S13n). Thus, samples I and II differed in the year of cultivation, whereas sample III differed from samples I and II in terms of N fertilization.

16S rRNA gene sequencing and taxonomic identification

The *16S rRNA* gene libraries were constructed and the clones were sequenced using the primer 27f, which anneals to the 5' end of the gene. A total of 541 sequences were obtained (>50 bp); 390 (72.1%) of these were longer than 250 bp. The numbers of *16S rRNA* gene clones and sequence lengths for each library are shown in Table 1.

One of the clone sequences was marked as suspect during the analysis using RDP II tools. A detailed analysis of this sequence (R7SP1T1H06, 315 bp) showed that it was, in fact, an artifact, composed of a tandem repeat of the sequence GGATCAACTCTAGAGTTTG ATCCTGGCTCA, which contains the sequence of the 27f primer. Analysis of the primer sequence revealed the possibility that it can form a hairpin in the 5' end of the 27f primer, which could allow its extension in consecutive rounds of PCR. This anomaly was observed only once. Such artifacts have been reported and credited to problems in the amplification step (23). This sequence was removed from the dataset and not used for analysis. Different primers have been designed for diversity analysis (24), but bias for these primers were also reported (25).

Table 2 shows the distribution of the phyla obtained for the whole dataset from libraries S0N, S13N, and S13n. Over 86% of the sequences could be assigned to one of the 15 phyla detected, but only 127 (23.6%) of the sequences were assigned to the genus level, with an average confidence of 95.5% in all three libraries. The four most abundant phyla of the whole dataset were Proteobacteria (29.6%), followed by Acidobacteria (23.4%), Bacteroidetes (12.1%), and Firmicutes (10.2%; Table 2 and Figure 1). Unclassified bacteria with no phylum affiliation based on *16S rRNA* sequences accounted for 13.6% of all *16S rRNA* sequences. In the two N-fertilized samples (S0N and S13N) Proteobacteria was the most prevalent phylum, whereas Acidobacteria predominated in non-fertilized sample (S13n). Verrucomicrobia accounted for only 1.5% of all samples, but its prevalence varied from approximately 0.7% in the fertilized soils to 5.2% in the non-fertilized soil. Some phyla/classes were observed only

Table 1. Soil samples of sugarcane rhizosphere and the number of *16S rRNA* gene sequences.

Date	Library	Soil pH	Number of sequences		
			50 to 250 bp	>250 bp	Total
9/26/2006	S0N	5.2	72	84	156
10/18/2007	S13n	5.3	19	78	97
10/18/2007	S13N	5.2	60	228	288
Total			151	390	541

Samples were collected from soils under sugarcane crop, observing the following conditions: S0N = soil from 12-month-old plant roots, N-fertilized; S13n = soil from 13-month-old plant roots, no N fertilization; S13N = soil from 13-month-old plant roots, N-fertilized.

Table 2. Phylum distribution in soil of sugarcane rhizosphere.

Phylum	Library (No. of clones)			
	S0N	S13N	S13n	Total
Proteobacteria	40 (26.00%)	98 (34.03%)	21 (21.87%)	159 (29.55%)
Acidobacteria	34 (22.08%)	61 (21.18%)	31 (32.29%)	126 (23.41%)
Unclassified	30 (19.48%)	35 (12.15%)	8 (8.33%)	73 (13.57%)
Bacteroidetes	26 (16.88%)	25 (8.68%)	14 (14.58%)	65 (12.08%)
Firmicutes	8 (5.19%)	40 (13.89%)	7 (7.29%)	55 (10.22%)
Actinobacteria	14 (9.09%)	10 (3.47%)	6 (6.25%)	30 (5.58%)
Verrucomicrobia	1 (0.65%)	2 (0.69%)	5 (5.21%)	8 (1.49%)
Gemmatimonadetes	1 (0.65%)	3 (1.04%)	1 (1.04%)	5 (0.93%)
Nitrospira	0 (0.00%)	2 (0.69%)	2 (2.08%)	4 (0.74%)
Bacteria incertae sedis	0 (0.00%)	4 (1.39%)	0 (0.00%)	4 (0.74%)
OP10	0 (0.00%)	3 (1.04%)	0 (0.00%)	3 (0.56%)
Cyanobacteria	0 (0.00%)	2 (0.69%)	0 (0.00%)	2 (0.37%)
Planctomycetes	0 (0.00%)	1 (0.35%)	0 (0.00%)	1 (0.19%)
Chlorofexi	0 (0.00%)	1 (0.35%)	0 (0.00%)	1 (0.19%)
BRC1	0 (0.00%)	0 (0.00%)	1 (1.04%)	1 (0.19%)
TM7	0 (0.00%)	1 (0.35%)	0 (0.00%)	1 (0.19%)
Total	154	288	96	538

The numbers in parentheses are percent of each library. Taxonomic assignment of clones from *16S rRNA* gene libraries was obtained by using the Ribosomal Database Project Classifier tool site (http://rdp.cme.msu.edu/); cutoff values of 50 and 80% for bootstrap were used for identification of sequences measuring 50 to 250 bp and more than 250 bp in length, respectively. Samples were collected from soils under sugarcane crop, observing the following conditions: S0N = soil from 12-month-old plant roots, N-fertilized; S13n = soil from 13-month-old plant roots, no N fertilization; S13N = soil from 13-month-old plant roots, N-fertilized.

in one library. The S13n library contained 1 sequence of the BCR1 phylum and the library S13N contained the phyla Chlorofexi (1 sequence), Cyanobacteria (2 sequences), OP10 (3 sequences), Planctomycetes (1 sequence), and TM7 (1 sequence), and the Deltaproteobacteria class (2 sequences). The largest fraction of unidentified phyla was found in the S0N library (19.5%). Among Proteobacteria, the Alphaproteobacteria was the most abundant class (42.2%), followed by Betaproteobacteria (33.5%), Gammaproteobacteria (1.2%), and Deltaproteobacteria (1.2%). Sequences defined as unclassified Proteobacteria also accounted for a high proportion (18.6%) and no member of the Epsilon- or Zetaproteobacteria was found.

Sequences reliably classified at the genus level using the RDP Classifier tool were more than 250 bp in length (Table 3). *Bacillus* was the predominant genus, representing 19.7% of genus-classified sequences. To further investigate the classification of these clones, the sequences were retrieved and submitted to a MEGA-BLAST search against the GenBank database. High identities (98 to 100%) with uncultured bacteria and/or *Bacillus* isolated from agricultural and contaminated soil were obtained, and one sequence (R7SP1R1B08) showed 97% identity with an uncultured bacterium from marine sediment (AY911106).

The Alpha-, Beta-, and Gammaproteobacteria contain members of well-known diazotrophic organisms, able to establish symbiosis or association with root and/or endophytic colonization with several groups of plants. The three libraries had few representatives of the genera ofnitrogen-fixing

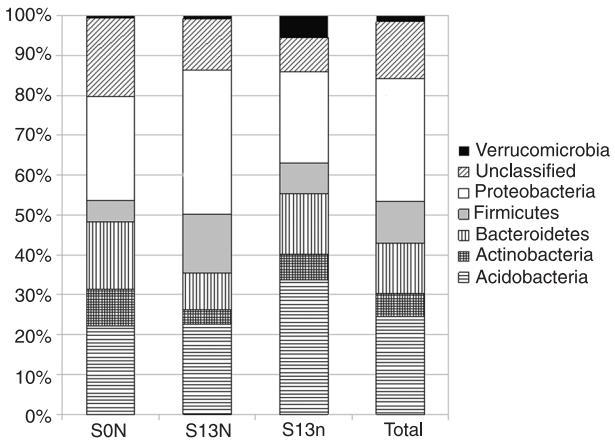

Figure 1. Distribution of the predominant phyla identified in the soil samples on the basis of *16S rRNA* gene libraries from sugarcane rhizosphere soil. Classification was achieved using the Ribosomal Database Project Classifier (http://rdp.cme.msu.edu/) with a threshold level of 50%. The bar labeled "total" shows the phylum distribution for the three samples combined. Distribution of minor phyla is not shown. Samples were collected from soils under sugarcane crop, observing the following conditions: S0N = soil from 12-month-old plant roots, N-fertilized; S13n = soil from 13-month-old plant roots, no N fertilization; S13N = soil from 13-month-old plant roots, N-fertilized.

Table 3. Genus assignment for clones from sugarcane rhizosphere on the basis of *16S rRNA* gene sequences.

Phylum/class	Genus	No. of clones
Actinobacteria	*Dactylosporangium*	1
	Lechevalieria	2
	Mycobacterium	1
	Nocardioides	1
	Rubrobacter	1
	Sinomonas	1
	Streptomyces	1
	Subtotal	8
Bacteria incertae sedis	*Ktedonobacter*	4
Bacteroidetes	*Chitinophaga*	1
	Chryseobacterium	1
	Ferruginibacter	1
	Flavisolibacter	4
	Flavobacterium	1
	Mucilaginibacter	6
	Niastella	8
	Terrimonas	9
	Subtotal	35
Firmicutes	*Bacillus*	25
	Cohnella	2
	Paenibacillus	4
	Tumebacillus	2
	Subtotal	33
Gemmatimonadetes	*Gematimonas*	5
	Subtotal	5
Nitrospira	*Nitrospira*	4
	Subtotal	4
Alphaproteobacteria	*Azospirillum*	1
	Belnapia	2
	Bradyrhizobium	3
	Hyphomicrobium	1
	Labrys	1
	Mesorhizobium	2
	Methylobacterium	1
	Novosphingobium	1
	Pseudolabrys	1
	Rhizobium	2
	Rhodoplanes	3
Betaproteobacteria	*Burkholderia*	6
	Cupriavidus	1
	Massilia	5
	Methylibium	2
	Ralstonia	1
	Ramlibacter	1
	Variovorax	2
Gammaproteobacteria	*Steroidobacter*	2
Deltaproteobacteria	*Anaeromyxobacter*	2
	Bdellovibrio	2
	Subtotal	42
Total		127

Classes are shown only within the Proteobacteria phylum.

Alphaproteobacteria able to nodulate legumes: *Bradyrhizobium* (3 sequences), *Mesorhizobium* (2 sequences), and *Rhizobium* (2 sequences). One sequence representative of *Azospirillum*, an associative nitrogen-fixing bacterium, was also found. Furthermore, 6 sequences of the *Burkholderia* genus, that contains several nitrogen-fixing species frequently isolated in association with grasses, including sugarcane, were found. A MEGA-BLAST search against the GenBank database showed high identities (97 to 100%) with sequences from strains isolated from agriculture soils and in association with plants (Table 4). Four clones were assigned to the *Nitrospira* genus with a high confidence level by the Classifier tool. However, a MEGA-BLAST search only yielded a close match to the uncultured bacteria *N. muscoviensis* and candidate *N. bockiana* (identities ranging from 90 to 94%). This genus contain species, which play an important role as nitrite-oxidizing bacteria in different ecosystems (2).

Operational taxonomic unit (OTU)-based comparison of *16S rRNA* gene libraries

The three *16S rRNA* gene libraries had a disproportionate number of sequences, with the S13N library containing 53.5% of the sequences longer than 250 bp. For statistical comparison

Table 4. MEGA-BLAST results for clones identified as symbiotic and associative bacteria.

Clone	Species	Source	Geographic source	Identity (%)
Clones matching the *Burkholderia* genus				
R7CP7R2A11	Uncultivated *Burkholderia*	Agricultural soil	USA	97
	Burkholderia strain	Forest soil		97
R4CP1R1F05		Agricultural soil	Netherlands	97
	B. hospita strain	Agricultural soil		97
	Burkholderia strain	*Mimosa pudica*	Philipines	97
R4CP1R1G07	*Burkholderia* sp		China	99
	B. cepacia strain	Maize stem (endophytic)	China	99
R4CP3R1F01	*Burkholderia* sp strain	*Mimosa pudica*	Philipines	97
	Burkholderia sp strain	*Mimosa pigra*	Costa Rica	97
R4CP3R1G05	*B. caribensis*	Soil	Mexico	99
	B. caribensis	*Indigofera suffruticosa*	Brazil (Amazon)	99
R7SP1R2G03	*Burkholderia* sp	Tomato rhizosphere	Mexico	98
	B. caribensis	Soil		98
Clones matching *Azospirillum* genus				
R7CP8R1H01	*A. brasilense* strain	*Triticum aestivum*	Greece	96
Clones matching *Bradyrhizobium* genus				
R7CP7R2F05	Uncultured Alpha-proteobacterium clone	Human gastrointestinal resection specimen		99
	Bradyrhizobium genosp Y	*Indigofera linifolia* (root nodule)	Australia	99
R7CP8R1G06	*B. japonicum*	*Arachis hypogaea*	China	98
R7CP8R2C05	Uncultured bacterium clone	Human skin, volar forearm		100
	B. elkanii	*Glycine soja*		99
Clones matching *Mesorhizobium* genus				
R7CP7R2D05	*Mesorhizobium* sp	*Pterocarpus erinaceus*	Senegal	99
	M. amorphae	*Robinia pseudoacacia*	China	98
R7CP7R2F06	*Mesorhizobium* sp	*Pterocarpus erinaceus*	Senegal	98
	M. amorphae	*Acacia angustissima*	Brazil	98
Clones matching *Rhizobium* genus				
R0SP1T1A05	*Rhizobium* sp strain	Sugarcane (endophytic)	Brazil	99
	R. tropici	Platycodon grandiflorus (root; endophytic)		99
R4CP1R1G08	*R. multihospitium*	*Cytisus striatus* (root endophyte)	Spain	99

Bacterial strains identified by best hits from a similarity search using the MEGA-BLAST algorithm against a nucleotide non-redundant NCBI database. e-values for all searches returned 0.0.

of the libraries, sequences from S13N were removed at random to obtain a comparable number of sequences in all libraries.

The Chao index indicated that the S13N library has the richest community (Chao index of 298) compared with the S0N (220) and S13n (217) libraries. The Shannon index also indicated that the S13N library was the most diverse, with a Shannon index of 4.25, followed by the S0N (4.19) and S13n (4.09) libraries. However, at the 95% confidence interval the three samples had similar richness and diversity (Table 5) with no significant difference.

Table 5. Chao and Shannon indexes for *16S rRNA* gene sequence libraries.

Sample	N	$OTU_{0.03}$	Estimate of Chao richness	Shannon diversity index
S0N	84	70	220.27 (140.70-389.39)	4.19 (4.03-4.35)
S13n	78	64	217.11 (132.66-405.41)	4.09 (3.92-4.25)
S13N	86	74	298.00 (177.75-557.62)	4.25 (4.09-4.41)

OTU definition at 3% dissimilarity. Samples were collected from soils under sugarcane crop, observing the following conditions: S0N = soil from 12-month-old plant roots, N-fertilized; S13n = soil from 13-month-old plant roots, no N fertilization; S13N = soil from 13-month-old plant roots, N-fertilized.

In the shared OTU analysis it was observed that, at 3% dissimilarity ($OTU_{0.03}$), the S13N library showed the highest number of OTUs. This library had a total of 74 OTUs, while S0N and S13n had 69 and 64 OTUs, respectively. The S13N and S13n libraries had 6 OTUs in common, which makes these two libraries the most similar. Only one sequence was shared by all three libraries. The S13N library also had the highest number of unique OTUs (86). S0N and S13N had only one in common.

Discussion

The *16S rRNA* gene bacterial diversity found among three libraries constructed from DNA extracted from soil of a sugarcane rhizosphere differed in composition. The main phylum groups found in the three libraries were Proteobacteria, Acidobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. Representatives of the Verrucomicrobia, Gemmatimonadetes, Chlorobi, Chloroflexi, Nitrospira, Cyanobacteria, Planctomycetes, TM7, Lentisoherae, and BRC1 were also found. Interestingly, the prevalence of Verrucomicrobia was approximately 10-fold higher in the sample of sugarcane that did not receive nitrogen fertilizer (Table 2).

The main classes of Proteobacteria present in the three libraries were: Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria, consistent with previous studies (26). These results agree with the phyla and proteobacterial classes that are regularly found in Brazilian soils (2,27). However, some phyla found in other agricultural Brazilian soils were not found in the present study, such as Deferribacteria and Fusobacteria. Brazilian soils are considered to have a lower diversity compared with other regions (2), a characteristic attributed to lower pH values. The soil samples studied in the present investigation had a pH of 5.2-5.3 (Table 1), and this factor alone may explain the low phylum diversity that we found, since pH has a strong influence on bacterial community composition (28). In addition, agricultural practices also influence bacterial communities. Forest soils have a higher diversity at the phylum level, whereas agricultural soils have a higher diversity at the species level (2,29). Our results agree with the observations, showing a low variation at the phylum level among the samples, with 4 phyla predominating among the 15 identified. For comparative analysis using 3% dissimilarity as OTU definition, we found an indication that the community structures of the samples from the 3 treatments might be different, suggesting that time of sampling and N fertilizer affected the bacterial communities (30).

The three libraries contained few numbers of genera with species able to endophytically colonize sugarcane plants, suggesting that the soil may not be a repository for these bacteria or that they are not part of the predominant groups. Magnani et al. using a culture-dependent (31) and a culture-independent approach (Magnani GS, Cruz LM, Weber H, Bespalhok JC, Baura V, Yates MG, et al., unpublished data) showed that the most predominant bacteria endophytically colonizing sugarcane from Southwest Brazil were *Enterobacter* and *Pseudomonas*, with few putative known nitrogen-fixing or plant growth-promoting bacteria. Suman et al. (32), using a culture-dependent approach, showed that the nitrogen-fixing bacteria constitute a small fraction of endophytic bacteria, ranging from 0.02 to 3.86% of the total community, depending on the sugarcane variety. However, among endophytic diazotrophic bacteria, *Herbaspirillum*, *Azospirillum*, and *Ideonella* were observed in high numbers in the stems of field-grown maize (30) and seem to play and important role in plant growth. It has been suggested that agricultural management of soil may significantly influence the diversity of Bacteria and Archaea (30).

Since samples S0N and S13N received N fertilization we expected that these bacterial populations would share common groups. For most groups this was not true, suggesting that N fertilization may not be the major factor influencing soil biodiversity. However, we noted that the numbers of Verrucomicrobia sequences recovered from both N-fertilized samples were significantly lower than that from the non-fertilized sample (S13n), suggesting that this group may be an indicator of N-rich soils.

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