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Diversity of Cultivated Endophytic Bacteria from Sugarcane: Genetic and Biochemical Characterization of *Burkholderia cepacia* Complex Isolates[▽]

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Bacteria were isolated from the rhizosphere and from inside the roots and stems of sugarcane plants grown in the field in Brazil. Endophytic bacteria were found in both the roots and the stems of sugarcane plants, with a significantly higher density in the roots. Many of the cultivated endophytic bacteria were shown to produce the plant growth hormone indoleacetic acid, and this trait was more frequently found among bacteria from the stem. 16S rRNA gene sequence analysis revealed that the selected isolates of the endophytic bacterial community of sugarcane belong to the genera of *Burkholderia*, *Pantoea*, *Pseudomonas*, and *Microbacterium*. Bacterial isolates belonging to the genus *Burkholderia* were the most predominant among the endophytic bacteria. Many of the *Burkholderia* isolates produced the antifungal metabolite pyrrolnitrin, and all were able to grow at 37°C. Phylogenetic analyses of the 16S rRNA gene and *recA* gene sequences indicated that the endophytic *Burkholderia* isolates from sugarcane are closely related to clinical isolates of the *Burkholderia cepacia* complex and clustered with *B. cenocepacia* (gv. III) isolates from cystic fibrosis patients. These results suggest that isolates of the *B. cepacia* complex are an integral part of the endophytic bacterial community of sugarcane in Brazil and reinforce the hypothesis that plant-associated environments may act as a niche for putative opportunistic human pathogenic bacteria.

Brazil is one of the world's largest sugarcane producers and has considerable influence over the international sugar market. Brazilian sugarcane is primarily used to produce sugar and alcohol. Production has increased over time to approximately 26 million tons of sugar and 16 million m^3 of alcohol in 2006. Although ethanol has been used as an alternative source of fuel in Brazil since 1980, it is currently receiving worldwide interest as a biofuel to replace, at least in part, gasoline, thereby contributing to a reduction in carbon emissions (33). Consequently, sustaining and enhancing the growth and yield of sugarcane have become a major focus of research. The growth and performance of sugarcane in the field are adversely affected by a number of abiotic and biotic factors, including a wide range of fungal and bacterial diseases. Pokkah boeng, caused by the fungus *Fusarium moniliforme*, is one of the most widespread diseases and may cause serious yield losses in commercial sugarcane plantings (68). *F. moniliforme* can be disseminated horizontally by airborne spores or crop debris and vertically through seed pieces. Current control strategies involve the use of resistant varieties and fungicide applications. The efficacy of both control measures, however, is limited, and there is an increasing need for novel and environmentally sound strategies to control this and other diseases of sugarcane.

The overall goal of this study was to isolate and characterize beneficial bacteria that are intimately associated with sugarcane and have the potential to control pathogens and to promote the growth and yield of sugarcane. Among the plant-associated microorganisms, endophytic bacteria are regarded as a largely untapped resource for the discovery of isolates with novel antifungal and plant growth-promoting traits (52, 71, 77, 78). For several crops, endophytic bacteria have shown beneficial effects on plant growth and health, and the main modes of action described are nitrogen fixation, production of phytohormones and antifungal compounds, and induced systemic resistance (17, 38, 44, 52, 60, 71, 76). For sugarcane, studies on endophytic bacteria have focused on *Gluconacetobacter diazotrophicus* and its abilities to fix nitrogen (9, 24) and to inhibit *Xanthomonas albilineans*, the causal agent of leaf scald disease (8). However, information on the antifungal traits and different mechanisms involved in plant growth promotion, beyond nitrogen fixation, is limited. Moreover, no comprehensive analysis of the frequency, diversity, and activities of endophytic bacterial communities of sugarcane has, to our knowledge, been performed to date.

In this study, bacteria were isolated from the rhizosphere and from inside the roots and stems of sugarcane plants grown in the field in Brazil. We specifically focused on delineation of the cultivated endophytic bacterial isolates and characterization of their salient metabolic features. The diversity and putative identities of the cultivated endophytic bacteria were determined by genomic DNA fingerprinting by 16S rRNA gene and *recA* (13) sequence analyses. Antifungal activities toward *F. moniliforme* and specific metabolites produced by the endophytic bacteria were determined in bioassays, PCR-based analysis, and chromatography (thin-layer chromatography [TLC] and reverse-phase high-performance liquid chromatography [RP-HPLC]).

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MATERIALS AND METHODS

Bacterial isolation, media, and growth conditions. The bacterial strains used in this study were isolated from sugarcane plants (cv. SP80-1842) grown for 3 months in an experimental field located in Piracicaba, Brazil (22°41′S 47°33′W). Rhizosphere and root endophytic bacteria were isolated from root segments collected at a depth of 5 to 15 cm from the stem base. The root segments were washed in tap water to remove adhering soil particles. Rhizosphere bacteria were isolated by vigorously shaking 2 g of root segments in 200 ml of PBS buffer (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]) for 1 h, followed by dilution plating on tryptic soy agar (TSA; Difco, Le Pont de Claix, France) supplemented with 50 mg ml^{-1} of the fungicide imazalil (Agricur, Brazil) and incubation at 28°C. For the isolation of root endophytes, washed root segments were surface sterilized by sequential washing in 70% ethanol for 1 min, sodium hypochlorite (2%, vol/vol) for 3 min, and 70% ethanol for 30 s and two rinses with ample sterilized distilled water. Surface sterilization was verified by plating aliquots (100 μl) of the sterile distilled water used in the final rinse onto TSA plates. The surface-sterilized root segments were ground in a mortar with a pestle by using 2 ml of PBS buffer, and 100-μl aliquots were plated on TSA supplemented with 50 mg ml^{-1} imazalil. Plates were incubated at 28°C, and the colonies were counted after 10 days. Stem endophytic bacteria were isolated from stem sections sampled at 15 and 30 cm from the stem base. Segments of 5 cm were washed and surface sterilized as described for the roots. Afterwards, the epidermis was aseptically removed and plant extract was collected from the stem section by centrifugation at 3,000 × *g* for 10 min (46). Aliquots of 100 μl were plated onto TSA supplemented with imazalil and incubated at 28°C, and colonies were counted after 10 days. All bacterial isolates were stored at −80°C in 40% (vol/vol) glycerol.

Genotypic characterization of endophytic bacteria. To determine the genotypic diversity of the isolated bacteria, BOX-PCR analysis with the BOX-1AR primer (42) was performed according to the protocol described in detail by Rademaker et al. (67). Amplifications were carried out with an MJ Research PTC-200 thermocycler. Six-microliter aliquots of the PCR products were loaded onto a 25-cm 1.5% agarose gel in 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]) and run at 40 V for 16 h. The gel was stained with ethidium bromide and visualized under UV light. Image analysis was performed with GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium).

Bacterial isolates were sent to Macrogen Inc. (Seoul, Korea) to have the nearly full-length 16S rRNA gene (~1,500 bp) sequenced according to company specifications. Sequencing of the gene for RecA recombinase (*recA*, 1,000 bp) was performed by BaseClear (Leiden, The Netherlands) with primers BCR1 and BCR2 (54). Sequences were examined and edited with the BioEdit Sequence Alignment Editor (35). The Basic Local Alignment Tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and sequence match of the Ribosomal Database Project (http://rdp.cme.msu.edu) were used to search for similar known sequences. These sequences were aligned with CLUSTAL_X version 1.8 (80). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 3.1 (45). Neighbor-joining consensus trees were obtained with the Kimura two-parameter substitution model (41) and bootstrap test (28). In the 16S rRNA gene and *recA* gene analyses conducted, we focused primarily on comparisons and best hits with sequences of well-characterized (type) strains from the *Burkholderia cepacia* complex experimental strain panel (16, 56).

Phenotypic characterization of bacterial strains. The activity of the bacterial strains toward hyphal growth of *F. moniliforme* strain CBS 218.76 was performed on one-fifth-strength potato dextrose agar medium (pH 7.0) (Oxoid, Hampshire, England) as described previously (22). Indoleacetic acid (IAA) production was determined by using a modification of the qualitative method developed by Bric et al. (11). Six strains were spot inoculated onto 1/10-strength TSA plates amended with 5 mM L-tryptophan (Sigma, Steinheim, Germany) and overlaid with a nitrocellulose membrane (Amersham, Buckinghamshire, England). After incubation for 24 h at 25°C, the membrane was removed from the plate and treated with Salkowski reagent (2% [wt/vol] 0.5 M $FeCl_3$ in 35% perchloric acid) for 15 min at room temperature. IAA-producing bacteria were identified by a red halo on the membrane surrounding the bacterial colony.

Protease activity was evaluated on agar medium containing (per liter) 15 g of skim milk powder, 4 g of blood agar base, and 0.5 g of yeast extract (Oxoid, Hampshire, England). Inoculated plates were incubated at 27°C for 24 h, and extracellular protease activity was detected by the presence of a halo surrounding the bacterial colony.

Detection and analysis of antibiotic genes and metabolites. PCR-based screening for genes involved in the biosynthesis of pyrrolnitrin, 2,4-diacetylphloroglucinol, phenazines, and pyoluteorin was performed as described by De Souza and Raaijmakers (21). Special attention was given to the *prnD* gene, which is involved in catalyzing the oxidation of the amino group of aminopyrrolnitrin to a nitro group to form pyrrolnitrin (reviewed in reference 21). The 1,092-bp *prnD* gene was partially (666 bp) sequenced by BaseClear (Leiden, The Netherlands) with primers PRND1 and PRND2 (21). Pyrrolnitrin detection by TLC was performed as described by De Souza and Raaijmakers (19). Pure pyrrolnitrin was used as a reference and was kindly provided by M. A. de Waard of the Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands. For detection of the antibiotics 2,4-diacetylphloroglucinol, phenazines, and pyoluteorin, RP-HPLC linked to a photodiode array spectrophotometer was used by following protocols described previously (10).

To determine the activity of metabolites in cell extracts of cultures of the bacterial isolates, TLC plates were sprayed with a spore suspension (10^6 spores ml^{-1}) of *Cladosporium cucumerinum* in potato dextrose broth (Difco, Le Pont de Claix, France) and incubated at 25°C for 5 days.

Site-directed mutagenesis. To assess the role of pyrrolnitrin in fungal growth inhibition, a pyrrolnitrin-deficient mutant of endophytic strain ESS4 was obtained by site-directed mutagenesis of the *prnD* gene. A 396-bp fragment of the *prnD* gene of strain ESS4 was amplified with primers PRNhF (5′-TTTTTAAGCTTTGCACTTCGCGTTCGAGAC-3′) and PRNxR (5′-TTTTTTCTAGACGAGATGAGCATGTGCATG-3′), which contain restriction sites for HindIII and XbaI (underlined), respectively. These primers were designed on the basis of an alignment of the GenBank sequences AF161183, AF161186, CP000150, CP000125, CP000441, PFU74493, and CP000076. After digestion with HindIII and XbaI, the 396-bp fragment was cloned into plasmid pMP5285 (43) and transferred into a spontaneous rifampin-resistant derivative of strain ESS4 by triparental mating. Putative *prnD* mutants were selected on Pseudomonas Agar F medium (Difco, Le Pont de Claix, France) containing 200 μg ml^{-1} rifampin and 1,200 μg ml^{-1} kanamycin. The disruption of the *prnD* gene was confirmed by PCR amplification with primers PRND2 (21) and P5285R (5′-CCAGATAGCCCAGTAGCTG-3′). Primer PRND2 targets a specific sequence in the *prnD* gene, whereas primer P5285R anneals to the plasmid flanking region cointegrated into the *prnD* gene. The amplified 600-bp PCR product was checked for the presence of the HindIII restriction site. The deficiency in pyrrolnitrin production was determined by TLC analysis of cell extracts of the putative *prnD* mutant culture.

Nucleotide sequence accession numbers. The sequences obtained in this study were deposited in GenBank (for the accession numbers, see Table 2).

RESULTS AND DISCUSSION

Frequency, diversity, and identity of endophytic bacteria from sugarcane. The population density of culturable bacteria in the rhizosphere of sugarcane was approximately 20-fold higher than the density of bacterial endophytes in the roots (Table 1). In the stem, bacterial density was several orders of magnitude lower than in the roots and rhizosphere of sugarcane plants (Table 1). This distribution pattern, in which lower plant parts harbor higher frequencies of endophytes, confirms and extends results reported previously for corn (31), pea (25), and soybean (44) plants. A total of 154 bacterial isolates was randomly selected and subjected to BOX-PCR analysis to assess the genotypic diversity. On the basis of a cutoff similarity value of 70% (58, 59), 24 distinct genotypic groups were defined among the stem endophytes, 23 groups were defined among the root endophytes, and 25 groups were defined among the rhizosphere isolates (Table 1). Dual-culture plate assays revealed that 20, 39, and 25% of the bacterial isolates selected from the stem, root, and rhizosphere, respectively, significantly inhibited the hyphal growth of *F. moniliforme*, the causal agent of Pokkah boeng disease of sugarcane (Table 1).

On the basis of BOX-PCR and antagonistic activity against *F. moniliforme*, 18 endophytic bacterial isolates, consisting of 11 isolates from the root and 7 from the stem, were selected for 16S rRNA gene sequencing. Analysis of the 16S rRNA gene sequences revealed that most (13 out of 18) of the endophytic isolates belong to the genus *Burkholderia*, whereas the other 5

TABLE 1. Frequency, diversity, and activity of bacteria isolated from the rhizosphere, roots, and stems of sugarcane plants grown in the field in Brazil

Isolate source	Mean density (CFU g^{-1} [fresh wt]) ± SEM[a]	No. of randomly selected isolates	No. of BOX-PCR groups	No. (%) of isolates showing:	
				Inhibition of *F. moniliforme* growth	IAA production
Stem endophytes	$(2.87 \pm 1.33) \times 10^2$	49	24	10 (20)	36 (74)
Root endophytes	$(3.27 \pm 2.01) \times 10^6$	44	23	17 (39)	20 (46)
Rhizosphere	$(7.90 \pm 2.43) \times 10^7$	61	25	15 (25)	8 (13)

[a] Mean values of 18 replicates are shown.

isolates were classified as *Pantoea*, *Pseudomonas*, and *Microbacterium* isolates (Table 2). Previous studies have shown that isolates belonging to these bacterial genera promote the growth of different crops and are able to control specific plant diseases. For example, the rice endophyte *Pantoea agglomerans* YS19 showed nitrogen-fixing activity in vitro, produced four different phytohormones, including IAA, and promoted plant growth (29). Recently, a new endophytic nitrogen-fixing *Pantoea* sp. was isolated from sugarcane plants in Cuba (53), although its role in plant growth promotion has not been established. Endophytic *Pseudomonas fluorescens* strains Endo2 and Endo35 induced systemic disease protection against *Macrophomina phaseolina*, the causal agent of dry root rot of black gram (40). Also, endophytic *Burkholderia* species have received increased interest in the last years because of their capacity to fix nitrogen and their potential to promote plant growth. *Burkholderia* species have been isolated from several crops (5, 79), including sugarcane (61), rice (23), wine plants (18), onion (75), maize, and coffee (26, 27).

Genotypic characterization of endophytic *Burkholderia* isolates. The high frequency of *Burkholderia* species among the endophytic bacteria from sugarcane plants and their strong growth-inhibitory activity against *F. moniliforme* (Table 3) make these isolates potential candidates for the control of Pokkah boeng disease. Previous studies have shown that strains belonging to the genus *Burkholderia* are effective biocontrol agents (6) and represent the active ingredient in several commercially available biocontrol products, including Deny (Helena Chemicals, Memphis, TN) and Intercept (Soil Technologies Corp., Fairfield, IA). However, many strains of different species belonging to the so-called *B. cepacia* complex may act as opportunistic pathogens of humans, especially of cystic fibrosis (CF) patients and immunocompromised individuals (34). Strains belonging to the *B. cepacia* complex are resistant to a wide range of therapeutic antibiotics, and in spite of stringent control policies, the number of infections in hospitals caused by *B. cepacia* complex strains has not decreased (15, 72). The *B. cepacia* complex consists of at least nine discrete genomic species (described previously as genomovars I through IX) (16, 56), and all of these genomovars contain strains that are able to infect humans or to colonize plants (3). Therefore, accurate identification of the *Burkholderia* isolates from sugarcane plants is a crucial step toward further development of these isolates for biological control of Pokkah boeng and other sugarcane diseases.

Over the past years, a range of molecular markers have been tested to characterize *Burkholderia* species from environmental and clinical origins (7, 12, 30, 50, 73). In most of these studies, including a recent multilocus sequence typing (MLST) study (4, 19), the 16S rRNA gene and the *recA* gene were shown to be good markers by which to characterize and discriminate *Burkholderia* isolates (2, 13, 64, 69, 81). In the present study, sequencing and phylogenetic analyses were performed on both the 16S rRNA gene (~1,500 bp) and *recA* (~1,000 bp) of all 13

TABLE 2. Genetic characteristics of endophytic bacteria isolated from sugarcane plants[a]

Strain	Plant tissue	16S rRNA gene					*recA* gene				
		Accession no.	Hit in NCBI database	Genomovar	% Identity	Reference strain	Accession no.	Hit in NCBI database	Genomovar	% Identity	Reference strain
ESS21	Stem	EF602568	*Microbacterium testaceum*		99	SE017					
ESR94	Root	EF602564	*P. fluorescens*		99	ATCC 13525					
ESS10	Stem	EF602554	*Pantoea stewartii*		99	LMG2715					
ESS12	Stem	EF602555	*Pantoea ananatis*		99	LMG20106					
ESS29	Stem	EF602556	*Pantoea ananatis*		99	LMG20106					
ESS2	Stem	EF602551	*B. cenocepacia*	III	100	AU1054	EF602569	*B. cenocepacia*	III	100	CEP511
ESS9	Stem	EF602553	*B. cenocepacia*	III	100	AU1054	EF602573	*B. cenocepacia*	III	100	CEP511
ESR100	Root	EF602566	*B. cenocepacia*	III	100	HI2424	EF602581	*B. cenocepacia*	III	99	HI2424
ESR99	Root	EF602565	*B. cenocepacia*	III	100	HI2424	EF602571	*B. cenocepacia*	III	99	HI2424
ESR60	Root	EF602557	*B. cenocepacia*	III	99	HI2424	EF602574	*B. cenocepacia*	III	99	CEP511
ESR108	Root	EF602567	*B. cenocepacia*	III	99	LGM12615	EF602572	*B. cenocepacia*	III	99	MRL10
ESS4	Stem	EF602552	*B. cenocepacia*	III	99	LGM12615	EF602576	*B. cenocepacia*	III	98	MRL10
ESR90	Root	EF602562	*B. cepacia*	I	99	ATCC 25416	EF602580	*B. cenocepacia*	III	98	MRL10
ESR85	Root	EF602560	*B. cepacia*	I	99	ATCC 25416	EF602579	*B. cenocepacia*	III	98	MRL10
ESR92	Root	EF602563	*B. cepacia*	I	99	ATCC 25416	EF602570	*B. cenocepacia*	III	98	MRL10
ESR63	Root	EF602558	*B. cepacia*	I	99	ATCC 25416	EF602578	*B. cepacia*	I	98	ATCC 17759
ESR73	Root	EF602559	*B. cenocepacia*	III	99	LGM12615	EF602577	*B. cepacia*	K	99	R9929
ESR87	Root	EF602561	*B. cepacia*	I	99	ATCC 25416	EF602575	*B. cepacia*	-	97	J503

[a] The isolates represent 18 genotypic groups based on BOX-PCR analysis.

TABLE 3. Phenotypic characteristics of endophytic bacteria isolated from sugarcane plants

Strain	Antagonism toward *F. moniliforme*[a]	IAA	Protease	Pyrrolnitrin		Growth at 37°C
				PCR	TLC	
ESS21	−	+[b]	+	−	−	+
ESR94	++	+	+	+	+	−
ESS10	+	+	−	−	−	+
ESS12	+	+	−	−	−	+
ESS29	+	+	−	−	−	+
ESS2	+++	+	+	−	−	+
ESS9	+++	+	+	−	−	+
ESR100	++	−[b]	+	−	−	+
ESR99	+++	+	+	−	−	+
ESR60	+++	+	+	−	−	+
ESR108	+++	−	+	+	+	+
ESS4	+++	+	+	+	+	+
ESR90	+++	−	+	+	+	+
ESR85	+++	−	+	+	+	+
ESR92	+++	−	+	+	+	+
ESR63	+++	−	+	+	+	+
ESR73	++	−	+	+	+	+
ESR87	+++	+	+	+	+	+

[a] −, no inhibition; +, zone of inhibition of 1 to 3 mm; ++, zone of inhibition of 4 to 8 mm; +++, zone of inhibition of 9 to 13 mm.
[b] +, positive; −, negative.

endophytic *Burkholderia* isolates (Table 2; Fig. 1 and 2). The results showed that the endophytic *Burkholderia* isolates from sugarcane plants cluster closely with the *B. cepacia* complex type strains and distantly from other *Burkholderia* species that do not belong to the *B. cepacia* complex (Table 2; Fig. 1 and 2). On the basis of a BLAST analysis of both 16S rRNA gene and *recA* gene sequences, sugarcane isolates ESS2, ESS9, ESR100, ESR99, and ESR60 are closely related (identities ranging from 99 to 100%) to well-characterized *B. cenocepacia* (gv. III) strains, including strains isolated from CF patients and fully sequenced strains (Table 2). *B. cenocepacia* (gv. III), as well as *B. multivorans* (gv. II) and *B. dolosa* (gv. VI), mostly harbors strains from clinical sources (3). *B. cenocepacia* strain AU1054 was isolated from the blood of a CF patient and was, on the basis of various genotyping methods, characterized as a representative of the *B. cenocepacia* PHDC clonal lineage (14). On the basis of 16S rRNA gene analysis, the sugarcane endophytic isolates ESS2 and ESS9 were 100% identical to strain AU1054 (Table 2; Fig. 1). Consistent with the 16S rRNA gene analysis, also the analysis of the *recA* gene sequences showed that isolates ESS2 and ESS9 presented 100% identity to *B. cenocepacia* CEP511 (Table 2; Fig. 2), a type strain recovered from a CF patient (Sydney, Australia) and representative of an epidemic strain spread among several patients (55). Similarly, endophytic isolates ESR100, ESR99, and ESR60 were closely (identities, 99 to 100%) related to *B. cenocepacia* strain HI2424 on the basis of 16S rRNA gene and, in part, *recA* sequence analyses (Table 2; Fig. 1 and 2). *B. cenocepacia* strain HI2424 was initially obtained from an onion field but was later also characterized as being another representative of the *B. cenocepacia* PHDC clonal lineage, which causes extensive infection in CF patients (51). On the basis of analyses of the *recA* gene sequences, isolates ESR108, ESS4, ESR92, ESR85, and ESR90 clustered and are most closely related to strain MRL-10, an environmental strain classified by MLST (4) as *B. cenocepacia* (gv. IIIE) (Table 2; Fig. 2). Considering the *recA* gene phylogeny, isolate ESR87 was less related to a strain of the *B. cepacia* complex, revealing 97% identity with strain J503 (Table 2) and only 96% identity with *B. cenocepacia* strain CEP511 (Fig. 2). On the basis of 16S rRNA gene phylogenetic analysis, the endophytic *Burkholderia* isolates obtained in this study clustered distantly from *B. sacchari* (AF263278) and *B. tropicalis* (AJ420332), two isolates obtained previously in Brazil from sugarcane plantation soil and from a sugarcane stem, respectively (Fig. 1). Also on the basis of phylogenetic analysis of the *recA* sequences, the endophytic *Burkholderia* isolates obtained in this study clustered distantly from *B. sacchari* (AJ550303) isolated from sugarcane plantation soil in Brazil (Fig. 2). The presence of *B. cenocepacia* III-like isolates in endophytic bacterial populations of sugarcane plants supports and further extends the conclusions of previous studies (2, 20, 65) that *B. cenocepacia* gv. III and lineage III-B occur in natural habitats, which may be a niche for opportunistic human pathogenic bacteria. In vitro assays further showed that all 13 endophytic *Burkholderia* isolates from sugarcane plants were able to grow at 37°C (Table 3). It should be emphasized, however, that a complete MLST analysis should be undertaken, as well as infection studies to categorically match the sugarcane isolates with well-characterized clinical isolates of the *B. cepacia* complex. Nevertheless, 16S rRNA gene and *recA* gene phylogenetic analyses suggest that the endophytic *Burkholderia* strains isolated from sugarcane are closely related to well-characterized *B. cepacia* complex type strains. To date, most studies on the occurrence of *B. cepacia* complex strains in natural habitats have focused on the maize rhizosphere (19, 20, 63, 65). The results of our study indicate, for the first time, that *B. cepacia* complex-related isolates are an integral part of the endophytic bacterial community of sugarcane.

Biochemical characterization of endophytic *Burkholderia* isolates. Many of the 154 randomly selected bacterial isolates from the rhizosphere, roots, and stems of sugarcane plants produced the plant growth hormone IAA (Table 1). Most of the IAA-producing isolates were found among the stem endophytes, followed by root endophytes and rhizosphere isolates (Table 1). IAA production was detected in 6 of the 13 endophytic *Burkholderia* isolates, 5 of which were closely related to *B. cenocepacia* gv. III (Table 2; Fig. 1 and 2). The observation that IAA production is more prevalent among the bacterial endophytes than among rhizosphere bacteria of sugarcane is consistent with the results of a previous study performed with soybean (44). Although the effect and role of IAA production by endophytic bacteria in growth promotion of sugarcane need to be investigated, this trait was considered one of the major mechanisms involved in maize growth promotion by the rhizosphere bacteria *Gluconacetobacter azotocaptans* DS1, *Pseudomonas putida* CQ179, and *Azospirillum lipoferum* N7 under greenhouse conditions (60). Several studies have reported that endophytic microbial communities originate from the soil and rhizosphere (25, 47). The observation that the frequency of IAA-producing bacteria is higher in the stems than in the rhizosphere of sugarcane plants suggests that the plant selects for endophytic bacteria with this trait or that IAA-producing bacteria harbor other traits that allow them to more effectively reach and establish themselves in the inner plant tissue.

To identify metabolites involved in the growth inhibition of

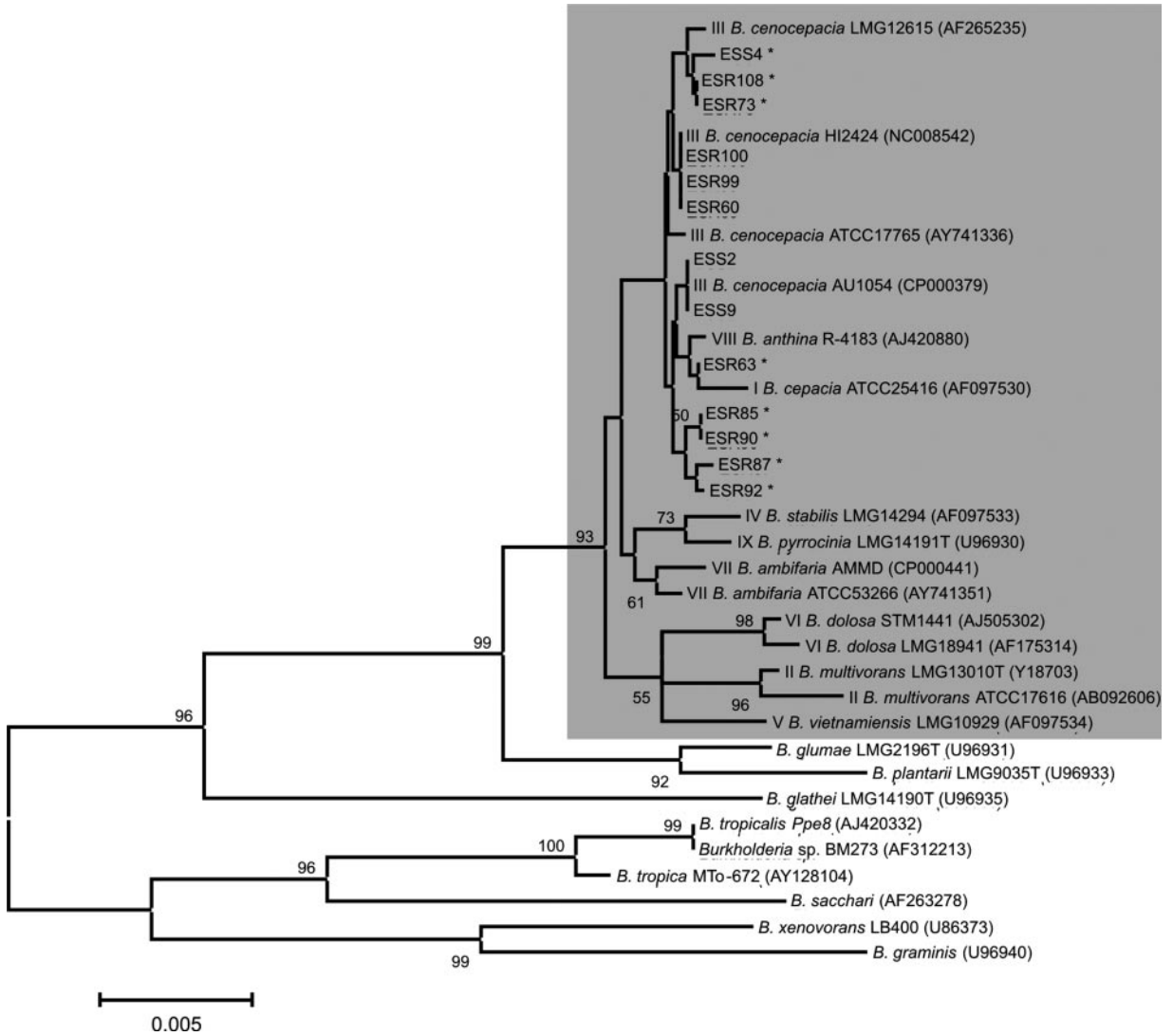

FIG. 1. Neighbor-joining tree of 16S rRNA gene sequences from endophytic bacterial isolates from sugarcane plants. ESS stands for endophyte sugarcane stem, and ESR stands for endophyte sugarcane root. Sequences were obtained from databases, and the accession numbers are in parentheses. The shaded box contains (type) strains belonging to the *B. cepacia* complex and includes the endophytic isolates from sugarcane plants. The roman numerals in front of the species and strain names are *B. cepacia* complex genomovar numbers. The isolates from sugarcane plants that produce pyrrolnitrin are indicated by asterisks. The Kimura two-parameter substitution model was used, and the nodes are supported by 1,000 bootstrap replications. Bootstrap values above 50% and the genetic distance scale are shown.

F. moniliforme, the bacterial endophytes were subjected to a range of tests, including enzymatic assays, PCR-based detection of antibiotic genes, and chromatography (TLC and RP-HPLC). All of the bacterial endophytes, except the three *Pantoea* species, produced extracellular proteases (Table 3). None of the bacterial endophytes produced hydrogen cyanide, a volatile compound found in several antagonistic *Pseudomonas* species (70, 82), and all of the isolates were negative for chitinase and biosurfactant production (data not shown). PCR-based detection revealed that none of the 18 endophytic isolates harbored specific genes involved in the biosynthesis of the antibiotics 2,4-diacetylphloroglucinol, phenazines, and pyoluteorin; the lack of production of these three antibiotics was confirmed by RP-HPLC analysis of culture extracts of the 18 endophytic isolates (data not shown). PCR-based detection of *prnD*, one of the four genes involved in pyrrolnitrin biosynthesis (48), and TLC analyses revealed that the endophyte *Pseudomonas* sp. strain ESR94 and eight of the endophytic *Burkholderia* isolates produced the antibiotic pyrrolnitrin (Table 3; Fig. 3A). On the basis of 16S rRNA gene and *recA* gene sequences, the pyrrolnitrin-producing endophytic *Burkholderia* isolates clustered (Fig. 1 and 2), suggesting that *prn* genes may be used as an additional molecular marker to further distinguish between strains and species within the *B. cepacia* complex. Results of the study by Seo and Tsuchiya (74) showed that among a collection of *B. cepacia* complex strains, 74% were positive in PCR-based detection of the *prnC* gene; most of the *prnC*-positive isolates belonged to *B. cepacia* gv. I, comprising isolates from both clinical and environmental sources. On the basis of their results, Seo and Tsuchiya (74) suggested that

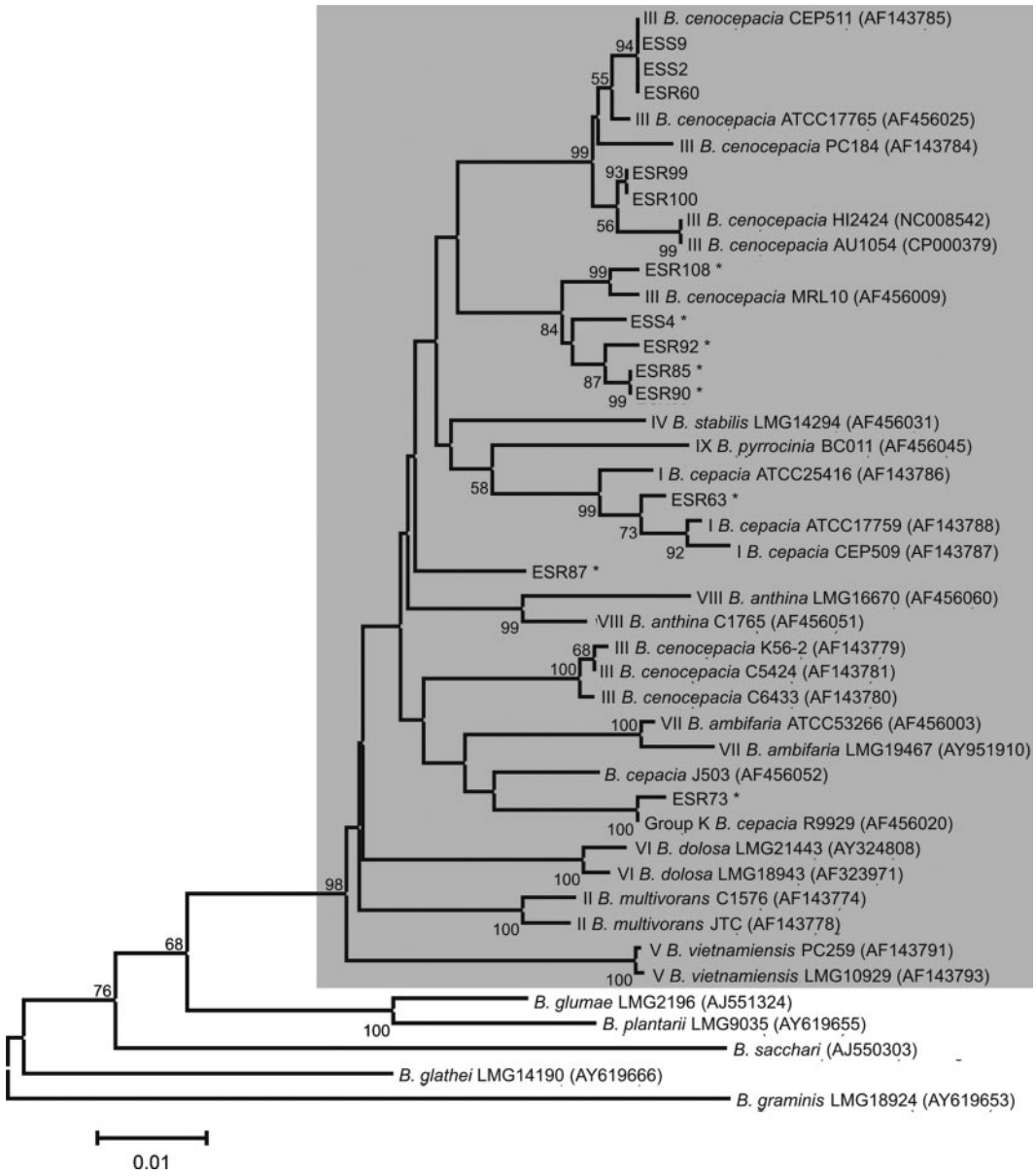

FIG. 2. Neighbor-joining tree of *recA* gene sequences from endophytic bacterial isolates from sugarcane plants. ESS stands for endophyte sugarcane stem, and ESR stands for endophyte sugarcane root. Sequences were obtained from databases, and the accession numbers are in parentheses. The shaded box contains (type) strains belonging to the *B. cepacia* complex and includes the endophytic isolates from sugarcane plants. The roman numerals in front of the species and strain names are *B. cepacia* complex genomovar numbers. The isolates from sugarcane plants that produce pyrrolnitrin are indicated by asterisks. The Kimura two-parameter substitution model was used, and the nodes are supported by 1,000 bootstrap replications. Bootstrap values above 50% and the genetic distance scale are shown.

restriction fragment length polymorphism analysis of the *prnC* gene could be used as a marker to distinguish *Burkholderia* species. However, on the basis of in silico analysis of fully sequenced *B. cepacia* complex genomes included in the 16S rRNA gene and *recA* phylogeny in this study (Fig. 1 and 2), the type strains *B. cenocepacia* HI2424 and AU1054, isolated from onion and from the blood of a CF patient, respectively, do not contain the pyrrolnitrin gene cluster. Furthermore, strains *B. ambifaria* AMMD, isolated from healthy pea plants, and *B. cenocepacia* PC184, isolated from CF patients, do contain the genes for pyrrolnitrin biosynthesis. This apparent lack of correlation between the origin of the *Burkholderia* isolates and the presence of *prn* genes, suggests that this trait is probably of minor value as an additional marker to distinguish between clinical and environmental *Burkholderia* isolates.

Role of pyrrolnitrin in growth inhibition of *F. moniliforme* by endophytic *Burkholderia*. Pyrrolnitrin [3-chloro-4-(2′-nitro-3′-chlorophenyl)pyrrole] is an antifungal metabolite with activities against a range of plant and human pathogenic fungi (37, 49). Pyrrolnitrin was originally described for *Burkholderia pyrrocinia* (1) and later for *Pseudomonas*, *Enterobacter*, *Myxococcus*, and *Serratia* (36). Pyrrolnitrin plays a key role in the antifungal

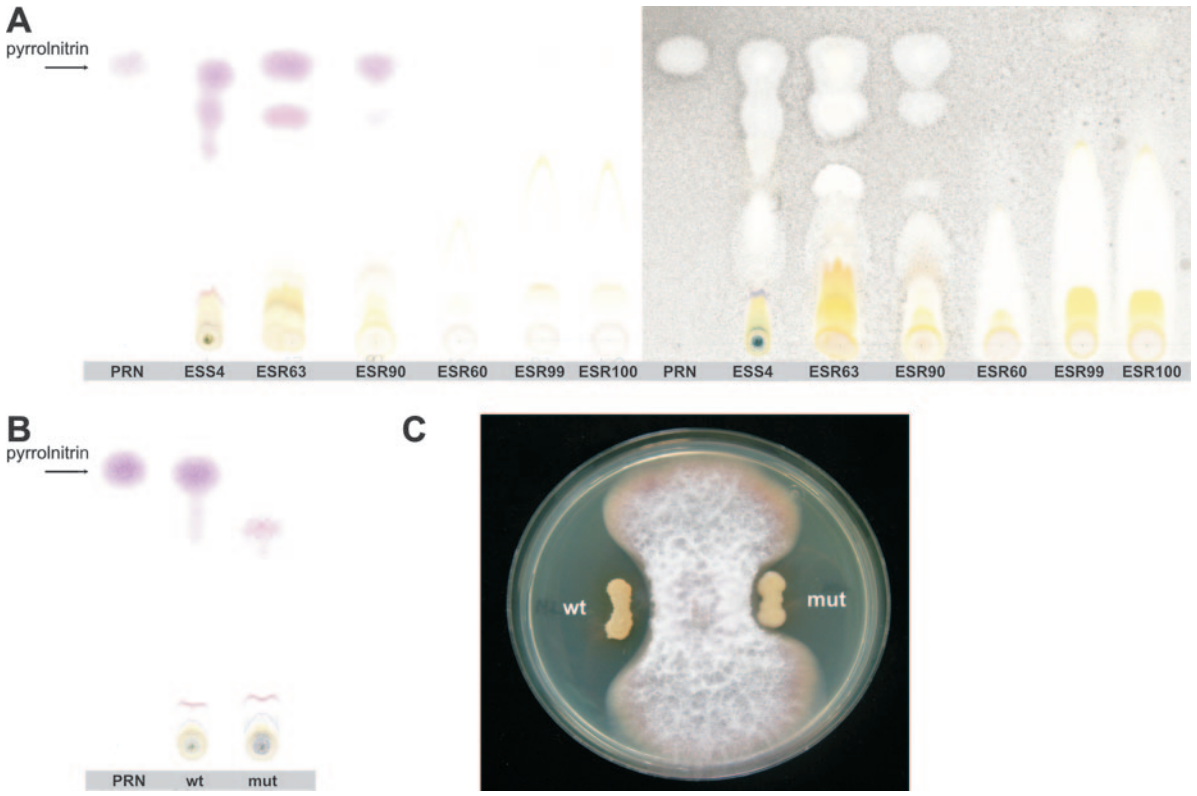

FIG. 3. TLC analysis of pyrrolnitrin production by endophytic bacteria from sugarcane plants and growth inhibition of *F. moniliforme* by *Burkholderia* sp. strain ESS4 and its *prnD* mutant. (A) Pyrrolnitrin is detected in cell extracts of cultures of the endophytic bacterial strains ESS4, ESR63, and ESR90 from sugarcane plants by spraying TLC plates with Ehrlich's reagent. Pure pyrrolnitrin (PRN; R_f is 0.48) was included as a control and is indicated by an arrow. TLC plates were subsequently sprayed with a spore suspension of the fungus *Cladosporium cucumerinum*, and the white zones indicate areas where fungal growth was inhibited. (B) TLC analysis of cell extracts from cultures of wild-type (wt) strain ESS4 and its *prnD* mutant (mut); pyrrolnitrin was detected in the wild-type strain but not in the mutant. (C) Dual-culture assay with *Burkholderia* strain ESS4 (wt), its *prnD* mutant (mut), and the fungus *F. moniliforme* (inoculated in the center).

activities of specific *Pseudomonas* strains (49, 66) and has been postulated to play a role in the natural suppressiveness of some soils against root rot caused by *Rhizoctonia solani* (32). To further clarify the relative importance of pyrrolnitrin production by the endophytic *Burkholderia* isolates in growth inhibition of *F. moniliforme*, site-directed mutagenesis of the *prnD* gene was performed with *Burkholderia* isolate ESS4. The partial *prnD* gene sequence of strain ESS4 obtained in this study (accession number EF602550) was 94% identical to that of type strain *B. cepacia* AMMD (CP000441). Site-directed mutagenesis resulted in 6 (out of 15) putative *prnD* mutants. Disruption of the *prnD* gene in these mutants was confirmed by PCR analysis, and loss of pyrrolnitrin production was confirmed in TLC analysis (Fig. 3B). Assays with *F. moniliforme* showed that the *prnD* mutant was slightly less effective at inhibition of hyphal growth than its parental strain (Fig. 3C), suggesting that most of the activity against *F. moniliforme* is related to other, as yet unknown, metabolites. *Burkholderia* species are well known for their production of a range of antifungal metabolites, including phenylacetic acid, hydrocinnamic acid, 4-hydroxyphenylacetic acid, and 4-hydroxyphenylacetate methyl ester (39, 57). The role of these compounds, if present in the isolated strains, in the control of fungal diseases and endophytic colonization of sugarcane remains to be investigated.

Conclusion. The endophytic bacterial community associated with sugarcane harbors multiple genera with potential for plant growth promotion and disease control. On the basis of 16S rRNA gene and *recA* sequence and phylogenetic analyses, most of the selected endophytic isolates obtained from sugarcane plants belonged to the genus *Burkholderia* and are closely related to clinical isolates of the *B. cepacia* complex. These results exemplify and support the conclusions of Parke and Gurian-Sherman (62) that accurate identification and classification of antagonistic and environmental *Burkholderia* isolates are essential before application to seeds and planting material for disease control and plant growth promotion. The relatively high frequency of different *B. cepacia* complex species associated with sugarcane plants reinforces the hypothesis of Berg et al. (5) that plant-associated environments may act as a reservoir for opportunistic human pathogenic bacteria. To categorically match the endophytic *Burkholderia* isolates from sugarcane with well-characterized clinical isolates of the *B. cepacia* complex, a complete MLST analysis should be conducted, followed by infection and epidemiological studies.

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