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Diazotrophic *Burkholderia* Species Associated with Field-Grown Maize and Sugarcane

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Until recently, diazotrophy was known in only one of the 30 formally described species of *Burkholderia*. Novel N_2-fixing plant-associated *Burkholderia* species such as *B. unamae*, *B. tropica*, and *B. xenovorans* have been described, but their environmental distribution is scarcely known. In the present study, the occurrence of N_2-fixing *Burkholderia* species associated with different varieties of sugarcane and maize growing in regions of Mexico and Brazil was analyzed. Only 111 out of more than 900 isolates recovered had N_2-fixing ability as demonstrated by the acetylene reduction assay. All 111 isolates also yielded a PCR product with primers targeting the *nifH* gene, which encodes a key enzyme in the process of nitrogen fixation. These 111 isolates were confirmed as belonging to the genus *Burkholderia* by using a new 16S rRNA-specific primer pair for diazotrophic species (except *B. vietnamiensis*) and closely related nondiazotrophic *Burkholderia*. In Mexico, many isolates of *B. unamae* (predominantly associated with sugarcane) and *B. tropica* (more often associated with maize) were recovered. However, in Brazil *B. tropica* was not identified among the isolates analyzed, and only a few *B. unamae* isolates were recovered from one sugarcane variety. Most Brazilian diazotrophic *Burkholderia* isolates (associated with both sugarcane and maize plants) belonged to a novel species, as revealed by amplified 16S rRNA gene restriction profiles, 16S rRNA gene sequencing, and protein electrophoresis. In addition, transmissibility factors such as the *cblA* and *esmR* genes, identified among clinical and environmental isolates of opportunistic pathogens of *B. cenocepacia* and other species of the *B. cepacia* complex, were not detected in any of the plant-associated diazotrophic *Burkholderia* isolates analyzed.

Until very recently, the genus *Burkholderia* included 30 properly described species (13), but the number of novel *Burkholderia* species has continuously increased. Unfortunately, species such as *B. kururiensis* (50), *B. sacchari* (7), *B. phenoliruptrix* (12), *B. xenovorans* (21), and *B. tuberum* and *B. phymatum* (45) have been proposed on the basis of a very limited set of isolates (one to three) analyzed, and consequently, their environmental distribution and ecology are poorly understood. It is well known that bacteria of the *B. cepacia* complex (Bcc), which includes nine species or genomovars (26), may be found in soils (including polluted soils), the rhizospheres of crop plants, and water, as well as in various animal species, in humans, and in the hospital environment (13). Studies published in Europe have shown that Bcc bacteria are good colonizers of the maize rhizosphere, and they may represent one of the predominant bacterial groups in maize grown in Italy (10, 17, 19). In addition, the species *B. graminis* (48) and *B. cepacia* genomovar III (2), presently formally classified as *B. cenocepacia* (46), were shown to be abundant in maize fields analyzed in France. Although the nine species of the Bcc have been isolated from cystic fibrosis patients, *B. cenocepacia* has been the predominant species recovered (13, 32). *B. cenocepacia* cystic fibrosis strains have been shown to possess transmissibility factors such as the *cblA* gene, encoding giant cable pili (25, 37), and the epidemic strain marker regulator or *esmR* gene, designated *B. cepacia* epidemic strain marker (BCESM) (25), which is part of a genomic island (3). *cblA* and *esmR* genes have been found in a few environmental isolates (25, 34), but only a limited number of these have been analyzed.

For a long time, N_2-fixing ability in bacteria of the genus *Burkholderia* was recognized only in the species *B. vietnamiensis* (20), a member of the Bcc (13). This species has been found in the rhizospheres and in the rhizoplanes of maize, coffee, and sorghum plants cultivated in different regions of Mexico, as well as inside maize roots in the state of Morelos, Mexico (18). Recently, two nodulating N_2-fixing strains recovered from legume plants were assigned to the genus *Burkholderia* according to their 16S rRNA sequences (27), and they were formally classified as *B. phymatum* and *B. tuberum* (45). *B. kururiensis*, a trichloroethylene-degrading bacterium (50), has been identified as a diazotrophic species (18). Very recently, many diazotrophic isolates found in rhizospheric and/or endophytic association with maize and coffee plants (18) were identified as novel *Burkholderia* species, i.e., *B. unamae* (9), *B. tropica* (33), and *B. xenovorans* (21). In addition, *B. unamae* isolates have been recovered from a few field-grown sugarcane plants in Mexico (9), and a few *B. tropica* isolates have been recovered

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from within sugarcane stems cultivated in Brazil and from the rhizosphere of sugarcane in South Africa (33).

Although *B. cepacia* and *B. vietnamiensis* are species found in the rhizospheres of crop plants and both are able to promote plant growth (6, 43), their taxonomical position as members of the Bcc has limited their potential use in agriculture. Understanding the taxonomy and ecological distribution of *Burkholderia* species associated with plants is an important step towards the possibility of using some of them for improving plant growth. Hitherto a detailed study on the occurrence of N_2-fixing species of the genus *Burkholderia* associated with plants has not been described. The aim of this work was to isolate, identify, and compare the diazotrophic species of the genus *Burkholderia* associated with sugarcane and maize varieties planted in Brazil and Mexico.

MATERIALS AND METHODS

Plant samples. In Brazil, the plants of sugarcane varieties SP 70-1143 and RB 72-454 and maize variety Aventis A2345 were collected at the Experimental Campus of Embrapa Agrobiology in Seropédica, Rio de Janeiro. In Mexico, the plants of sugarcane varieties MEX 69-290 and CP 72-2086 were collected in Tuxtepec, Oaxaca state, and domestic varieties of maize plants were collected in Tlayacapan, Morelos, and in Coatepec, Veracruz state (Table 1).

Isolation, culture conditions, and biochemical tests. In Mexico, five field-grown maize plants (in flowering) from each region and 16 sugarcane plants of each variety field grown for 10 months were randomly collected, with a distance of 10 m between plants. Samples of rhizosphere and rhizoplane, as well as surface-sterilized roots and stems of maize, were analyzed for recovery of diazotrophic isolates. Rhizosphere soil and plant samples were treated as described previously (18). Briefly, the root was shaken gently to remove the loosely attached soil, and the adhering soil was rinsed in 10 mM $MgSO_4 \cdot 7H_2O$ (Mgsol). The resulting rinse solution was considered to contain the rhizosphere bacteria; this solution was 10-fold serially diluted and used for isolation of the rhizosphere bacteria. The root was subsequently washed with Mgsol containing 0.01% (vol/vol) Tween 20, followed by two rinses with Mgsol, and then immersed in Mgsol and vortexed for 3 min. The resulting suspension was considered to contain bacteria from the rhizoplane, and the solution was serially diluted and used for the bacterial isolation. N-free semisolid BAz medium was used as an enrichment culture for N_2-fixing *Burkholderia*, and BAc agar plates were used for isolation and pure cultures (18). Sugarcane roots were excised from 16 plants, and loosely adhering soil was removed; roots were then grouped into four samples, each comprising four root systems. Afterwards, a portion from the roots was cut into small pieces (1 cm), and 5 grams of each root sample was vortexed at 3,000 rpm for 3 min in Mgsol. This suspension was considered to contain bacteria from the rhizosphere-rhizoplane. Other root portions were washed with tap water and subsequently immersed for 5 min with agitation in full-strength bleach solution containing Tween 20 and rinsed three times in sterile water. Stems were cut in 10- to 15-cm pieces, immersed in 96% alcohol, and flamed for 30 to 45 seconds; thereafter, stems were surface sterilized as mentioned above for the roots. The rind was discarded under sterile conditions, and 5 grams of each stem (group of four stems) was macerated in a blender to give a 10^{-1} dilution. All macerated sugarcane samples were serially diluted, and 100-μl aliquots were placed in vials containing 5 ml of N-free semisolid BAz medium and incubated at 29°C for 4 to 5 days. Vials showing a fine subsurface pellicle were transferred to fresh semisolid BAz medium and new growth streaked out on BAc agar plates. Colonies were purified and assayed for nitrogenase activity by the acetylene reduction activity (ARA) method (8). ARA-positive colonies were maintained in 20% glycerol at −80°C prior to characterization.

In Brazil, roots were excised from four maize plants (two plants that were 2 months old [vegetative state] and two plants that were 3 months old [reproductive state]) as well as from five sugarcane plants of each variety growing for 10 months (second harvest), and loose soil was separated by agitation. From each plant, a portion of root with adhering soil was rinsed in sterile distilled water; the resulting rinse solution was 10-fold serially diluted and used for isolation of the rhizosphere bacteria. Other root portions were washed with tap water and subsequently surface sterilized with 1% chloramine T for 15 min and rinsed three times in sterile distilled water. Stems of maize and sugarcane stems or leaves were similarly disinfected with chloramine T. Surface-sterilized roots as well as stems and leaves were macerated in a blender at high speed for 3 min to give a 10^{-1} dilution. All macerated maize or sugarcane samples were serially diluted, and 100-μl aliquots were inoculated into 5 ml of N-free semisolid BAz and JMV media (33). Vials containing BAz medium were autoclaved at 121°C for 20 min, and filter-sterilized cycloheximide (200 μg/tube) was then added. After incubation for 4 to 5 days at 30°C, a fine subsurface pellicle was formed. Vials showing pellicles were transferred to fresh semisolid BAz or JMV medium and new growth streaked out on BAc agar plates. Colonies formed were inoculated into new semisolid JMV medium. All colonies obtained were transferred to LB medium and conserved for further observations. Colonies were assayed for ARA, and the isolates that exhibited activity after 13 h of incubation were used for further characterization. These isolates were maintained in 20% glycerol at −80°C prior to analysis.

TABLE 1. Nitrogen-fixing *Burkholderia* species isolated from maize and sugarcane

Crop and locality	Variety	Species	No. of isolates	Source[a]
Maize				
Mexico				
Tlayacapan, Morelos	Criollo ancho	*B. tropica*	7	Rhizosphere
				Rhizoplane
			2	Roots
Coatepec, Veracruz	Criollo	*B. tropica*	12	Rhizosphere
			1	Rhizoplane
			23	Roots
		B. unamae	2	Roots
Brazil				
Seropédica, Rio Janeiro	Aventis A2345	*Burkholderia* sp.[b]	11	Rhizosphere
			2	Roots
Sugarcane				
Mexico				
Tuxtepec, Oaxaca	CP 72-2086	*B. unamae*	28	Rh-Rp
		B. tropica	2	Rh-Rp
	MEX 69-290	*B. unamae*	3	Rh-Rp
			2	Roots
		B. tropica	1	Roots
Brazil				
Seropédica, Rio Janeiro	SP 70-1143	*B. unamae*	3	Rhizosphere
	RB 72-454	*Burkholderia* sp.[b]	5	Leaves

[a] Roots and leaves were surface sterilized. Rh-Rp, rhizosphere and rhizoplane.
[b] Corresponds to *Burkholderia* NAR group.

Acetylene-reducing isolates were analyzed with the API 20NE system. The inoculum and the assay conditions were according to recommendations of the manufacturer (bioMérieux). The results were interpreted using the API analytical profile index, which provided a percent probability of identification.

Total DNA isolation and 16S rRNA-specific primers. Genomic DNA was isolated from bacterial cells by using published protocols (1). ARA-positive isolates were presumptively assigned to the genera *Burkholderia-Ralstonia* by amplifying the 16S rRNA gene with the specific primers BuRa-16-1 and BuRa-16-2, using the PCR conditions described previously (4). ARA-positive isolates were confirmed to belong to the genus *Burkholderia* by amplifying the 16S rRNA gene with new specific primers (GB-F and GBN2-R). *Burkholderia* and *Ralstonia* 16S rRNA sequences available in the NCBI database were aligned to identify conserved regions in order to design and test specific PCR primers which would give an amplicon only with N_2-fixing *Burkholderia* species (but excluding *B. vietnamiensis*) and closely related nondiazotrophic *Burkholderia* species with greater than 97% identity between their 16S rRNA gene sequences. In the 16S rRNA gene sequences a region corresponding to positions 85 to 104 of *B. unamae* (GenBank accession no. AY221956) was identified as specific for the genus *Burkholderia*. This region was chosen to define the 20-mer forward primer GB-F (5′-AGTAATACATCGGAACRTGT-3′). Another 16S rRNA gene region, at positions 1091 to 1110 of *B. unamae*, was identified as being specific for N_2-fixing *Burkholderia* (except *B. vietnamiensis*) and closely related (97% identity or higher) nondiazotrophic *Burkholderia* species. The sequence allowed the design of the 19-mer reverse primer GBN2-R (5′-GCTCTTGCGTAGCAACTA G-3′). The specificity of the primer pair was tested with most of the well-known

Burkholderia species. The PCR mix was comprised of 20 ng of genomic DNA, 1.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, 5 pmol of each primer, and 1.0 U of *Taq* polymerase. PCR conditions were as follows: initial denaturation for 5 min at 94°C; followed by 30 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 60°C, and 1 min of elongation at 72°C; followed by a final 5-min elongation at 72°C. The reaction amplified a 1,025-bp fragment.

ARDRA. Primers fD1 and rD1 were used for the amplification of the 16S rRNA gene (49), using the PCR conditions described previously (18). The PCR-amplified 16S rRNA genes (ca. 1.5 kb) were restricted with AluI, DdeI, HaeIII, HhaI, HinfI, MspI, and RsaI. The restriction fragments were separated by electrophoresis in 3% agarose gels, and the restriction patterns were compared. Each isolate was assigned to an amplified 16S rRNA gene restriction analysis (ARDRA) genotype, defined by the combination of the restriction patterns obtained with the seven restriction endonucleases (18). Similarities among the 16S rRNA gene sequences were estimated from the proportion of shared restriction fragments by using the method of Nei and Li (29). A dendrogram was constructed from the resulting distance matrix by using the unweighted pair group method with averages (40).

16S rRNA gene sequencing and phylogenetic analysis. Strains SRMrh-20, SMrh-85, and SRCL-318, which corresponded to a predominant unknown ARDRA genotype recovered from plants that were field grown in Brazil, were chosen for 16S rRNA gene sequencing. In order to obtain the 16S rRNA sequence, PCR products were cloned into the pCR2.1 vector according to the manufacturer's instructions (Invitrogen). 16S rRNA genes were restricted in fragments from 850 and 650 bp using the enzyme EcoRI and subcloned into vector pUC18. 16S rRNA gene sequencing of strains SRMrh-20 and SRCL-318 was performed by the Biotechnology Institute, UNAM (Mexico), and strain SRMrh-85 was sequenced by Embrapa Agrobiology (Brazil). These sequences were compared with previously published 16S rRNA gene sequences from *Burkholderia* species and related bacteria such as *Pandorea* and *Ralstonia*. The multiple alignments of the sequences were performed with CLUSTAL W software (42). The tree topology was inferred by the neighbor-joining method (36), based on 1,299 DNA sites, and distance matrixes were determined according to the method of Jukes and Cantor (23) using the program MEGA version 2.1 (24).

SDS-PAGE of whole-cell proteins. Preparation of whole-cell proteins from diazotrophic isolates and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) assays were performed as described by Estrada-de los Santos et al. (18).

PCR amplification of *nifH* genes. Primers IGK (30) and NDR-1 (44) were used for the amplification of the *nifH* genes. The PCR mix was comprised of 20 ng of genomic DNA, 1.5 mM $MgCl_2$, 100 μM deoxynucleoside triphosphates, 0.005 μmol of each primer, and 1.0 U of *Taq* polymerase. The amplification was done with an initial denaturation step at 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min; with a final renaturation step for 10 min. The reaction amplified a 1.2-kb fragment comprising the entire *nifH* gene, the intergenic spacer region, and the 5′ end of the *nifD* gene (44).

Detection of transmissibility marker genes. *cblA* and *esmR* genes in acetylene-reducing isolates were investigated with the specific primers CBL1/CBL2 and BCESM1/BCESM2, respectively, using PCR conditions described previously (25, 37). In addition, total DNA from acetylene-reducing *Burkholderia* isolates was digested with EcoRI, and restriction fragments were electrophoresed and transferred from gels to nylon filters by the Southern procedure and hybridized as described previously (18). PCR-amplified *cblA* and *esmR* genes from *B. cenocepacia* strain J2315^T were used as ^{32}P-labeled probes.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of strains SRMrh-20, SMrh-85, and SRCL-318 have been deposited in the EMBL/GenBank database under accession numbers AY96520, AY965242, and AY965241, respectively.

RESULTS

Isolation and biochemical tests. The bacterial growth in N-free semisolid BAz and JMV media formed very thin and fine pellicles at a depth of 4 mm below the surface at 24 to 48 h; after 72 h the pellicles became yellowish, diffuse, and thick and moved up to the surface without pH changes in the medium. More than 900 pure isolates were recovered in BAc agar plates from the rhizosphere as well as from washed and surface-sterilized roots of maize and sugarcane plants (20 to 25 isolates from each rhizosphere and plant analyzed). However, only 111 isolates (Table 1), commonly recovered from vials inoculated with samples (100-μl aliquots) from 10^{-1} or 10^{-2} dilutions (data not shown), showed nitrogenase activity as measured by the acetylene reduction method. Although isolates were recovered from aerial parts of maize and sugarcane, very few of these, recovered from leaves but not from stems, showed acetylene reduction activity. All of the acetylene-reducing isolates recovered were gram negative and motile in N-free semisolid media at 29°C. API 20NE biochemical tests identified the isolates as *B. cepacia* (98.9 to 99.6% confidence limits based on the API analytical profile index).

16S rRNA-specific primers. ARA-positive isolates gave a PCR-amplified product of the correct size (409 bp) with primers BuRa-16-1 and BuRa-16-2, confirming their taxonomic status as members of the genera *Burkholderia-Ralstonia*. Although primers (Burk3 and BurkR) specific for the genus *Burkholderia* have been reported (38), they fail to amplify 16S rRNA genes in recently described novel N_2-fixing *Burkholderia* species such as *B. unamae* and *B. tropica* as well as in other well-known species such as the diazotrophic *B. kururiensis* and the nondiazotrophic *B. sacchari*. All of these N_2-fixing *Burkholderia* species, the diazotroph *B. xenovorans*, and the legume-nodulating, N_2-fixing *B. tuberum* and *B. phymatum*, as well as those nondiazotrophic closely related *Burkholderia* species with higher than 97% identity between their 16S rRNA gene sequences (e.g., *B. sacchari*, *B. caribensis*, *B. graminis*, *B. phenoliruptrix*, and *B. phytofirmans*), gave a PCR-amplified product of the expected size (1 kb) with the specific primer pair GB-F and GBN2-R developed in the present study (Fig. 1). However, the PCR product from *B. kururiensis* was a less intense band than those amplified from other diazotrophs (data not shown). All of the nine species of the Bcc, including the diazotroph *B. vietnamiensis* and the plant-pathogenic species (e.g., *B. caryophylli*, *B. plantarii*, and *B. gladioli*) closely related to the Bcc, as well as *Ralstonia pickettii* and *Ralstonia solanacearum*, did not give a PCR-amplified product with this primer pair (Fig. 1). The primers GB-F and GBN2-R allowed the confirmation of all the acetylene-reducing isolates recovered from maize and sugarcane as members of the genus *Burkholderia* (Fig. 1). In addition, a variable number of ARA-negative isolates (total of around 100) recovered from the rhizospheres, rhizoplanes, and plant tissues of maize and sugarcane were analyzed by PCR with primers BuRa-16-1 and BuRa-16-2. The assays revealed that over 95% of the isolates belong to the genera *Burkholderia-Ralstonia* (data not shown).

ARDRA analysis. A total of eight ARDRA profiles, clearly different from those for *Ralstonia* species, were identified from among the ARA-positive isolates recovered from field-grown maize and sugarcane in Mexico and Brazil (data not shown). Five ARDRA profiles were identified among 54 acetylene-reducing isolates recovered from the rhizosphere-rhizoplane and from surface-sterilized roots of maize growing in Mexico (Table 1; Fig. 2). Three ARDRA genotypes corresponding to the species *B. tropica* (52 isolates) and one genotype belonging to *B. unamae* (2 isolates) were identified (Table 1). In contrast, only one ARDRA profile was identified among 13 acetylene-reducing isolates associated with maize grown in Brazil, which was different from those ARDRA profiles observed in other well-known N_2-fixing *Burkholderia* species. The 13 *Burkholderia* isolates identified with such an ARDRA profile are here-

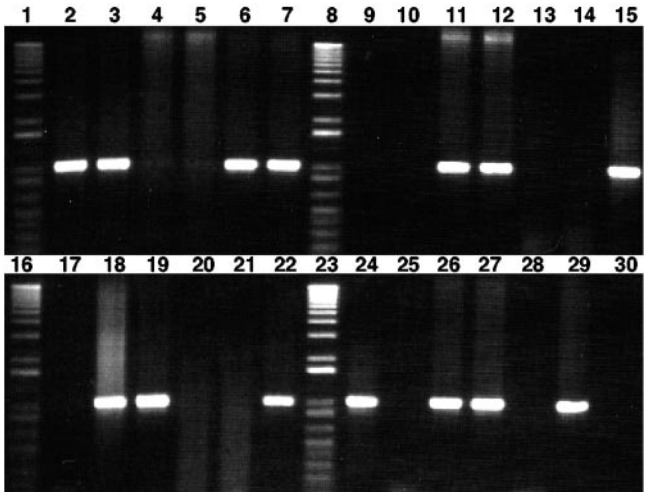

FIG. 1. PCR-amplified product of 1,025 bp obtained with the 16S rRNA-specific primer pair GB-F and GBN2-R. One-kilobase plus DNA ladder: lanes 1, 8, 16, and 23. No-DNA control, lane 30. PCR amplification in diazotrophic *Burkholderia* species (except *B. vietnamiensis*) and closely related organisms; *B. unamae*: lane 2, MTl-641^T, and lane 3, SCTx-181 isolated from rhizosphere of sugarcane (Mexico); *B. tropica*: lane 6, Ppe8^T, and lane 7, SCTx-269 isolated from surface-sterilized roots of sugarcane; type NAR isolates: lanes 11 and 12, SRMrh-20 and SRCL-318 isolated from rhizosphere of maize and surface-sterilized leaves of sugarcane, respectively; lane 15, *B. graminis* CRD1M^T; lane 18, *B. caribensis* MWAP^T; lane 19, *B. caledonica* LMG 19076^T; line 22, *B. tuberum* STM-678^T; line 24, *B. phenazinium* LMG 2247^T; lane 26, *B. sacchari* IPT101^T; lane 27, *B. phymatum* STM815^T; lane 29, *B. xenovorans* LB400^T. PCR-negative amplification in *B. cepacia* complex species and closely related species: lane 4, *B. vietnamiensis* TVV75^T; lane 5, *B. multivorans* LMG 13010^T; lane 9, *B. gladioli* ATCC10248^T; lane 10, *B. cepacia* ATCC25416^T; lane 13, *B. cenocepacia* J2315^T; lane 14, *B. glumae* LMG 2196^T; lane 17, *B. pyrrocinia* ATCC15958^T; lane 20, *B. ambifaria* LMG 11351; lane 21, *B. caryophylli* LMG 2155^T; lane 25, *Ralstonia pickettii* LMG 5942^T; lane 28, *Ralstonia solanacearum* LMG 2299^T. Other *Burkholderia* species tested (data not shown): PCR-positive amplification, *B. hospita* LMG20598^T, *B. terricola* LMG20594^T, *B. phytofirmans* PsJN^T, and *B. fungorum* LMG 16225^T; PCR-negative amplification, *B. stabilis* LMG 6997.

after referred to as the new acetylene-reducing (NAR) group or type NAR isolates. From among 36 isolates associated with sugarcane varieties grown in Mexico, three ARDRA genotypes were identified, corresponding to two genotypes of the species *B. unamae* (33 isolates) and one of *B. tropica* (3 isolates) (Table 1; Fig. 2). Associated with sugarcane varieties grown in Brazil were two identified ARDRA genotypes; one of them (three isolates recovered from variety SP 70-1143) showed a profile identical to that of *B. unamae*, and the other one (five isolates from variety RB 72-454) showed a profile identical to that of the *Burkholderia* NAR group (Table 1; Fig. 2). From among 90 ARA-negative isolates recovered from sugarcane and maize varieties cultivated in Mexico, one ARDRA genotype was predominant (data not shown). Associated with maize and sugarcane, this ARDRA genotype could correspond to the species *B. cepacia*, *B. cenocepacia*, and/or *B. ambifaria*, all of which are nondiazotrophic members of the *B. cepacia* complex. However, additional molecular tests are required for their definitive identification at the species level.

Phylogenetic analysis of 16S rRNA gene sequences. The sequences of *Burkholderia* strains SRMrh-20 and SRMrh-85, both isolated from the rhizosphere of maize, and of SRCL-318, isolated from surface-sterilized leaves of sugarcane, were compared with available 16S rRNA gene sequences from all of the *Burkholderia* species (Fig. 3). The strains SRMrh-20, SRMrh-85, and SRCL-318 were closely related, forming a cluster with a strain identified as *Burkholderia* sp. strain AB48 that was isolated from pineapple (*Ananas comosus*) in Brazil (14). Notably, strains SRMrh-20, SRMrh-85, and AB48 showed 100% identity between their 16S rRNA gene sequences and closely matched (99.77% identity) strain SRCL-318. Such an identity level strongly suggested that they belong to the same species. Comparison of the 16S rRNA gene sequences from all the well-known *Burkholderia* species showed that *B. sacchari*, a nondiazotrophic bacterium, was the closest species (97.1% identity) to the *Burkholderia* NAR group. This group, which apparently represents a novel *Burkholderia* species, clearly constituted a cluster largely distant (identity of $<96\%$) from the cluster named the *B. cepacia* complex (Fig. 3), which includes the N_2-fixing species *B. vietnamiensis*.

Protein electrophoregrams. Acetylene-reducing isolates showing ARDRA patterns identical to ARDRA genotypes of *B. unamae* or *B. tropica* also showed SDS-PAGE protein profiles (evaluated by visual comparison) that were identical or almost identical to those from type strains of these species. Type NAR isolates showed protein patterns completely different from those of known N_2-fixing *Burkholderia* species (Fig. 4).

PCR amplification of *nifH* genes. All 111 of the acetylene-reducing isolates yielded a PCR product of the expected size of 1.2 kb (data not shown) with the *nifH* primers used. These results confirmed the diazotrophy of the *Burkholderia* isolates. In addition, PCR amplification of *nifH* genes was negative in 75 representative isolates that had no acetylene reduction activity (data not shown), which confirmed their lack of N_2-fixing ability.

Transmissibility marker genes. Two transmissibility factors associated with the highly transmissible epidemic strains of *B. cenocepacia* were analyzed by both PCR amplification and Southern blot assays. No *cblA* and *esmR* marker genes were detected among diazotrophic isolates by either PCR amplification or ^{32}P hybridization assays (data not shown).

DISCUSSION

More than 900 pure isolates were recovered in BAc agar plates from the environment of maize and sugarcane plants cultivated in Mexico and Brazil. However, N_2-fixing ability was demonstrated in only 111 isolates by means of ARA assays and confirmed with the detection of *nifH* genes amplified by PCR assays. Biochemical tests based on the API 20NE system identified the N_2-fixing isolates as *Burkholderia cepacia*. However, it is known that this system is appropriate for the identification of the genus *Burkholderia*, but not at the species level (18, 39). In addition, it has been suggested that N_2-fixing ability is used as a distinctive feature for the delineation of *Burkholderia* species, which is particularly useful for the differentiation of the type species *B. cepacia* and of the nondiazotrophic Bcc species (9). In fact, both the lack of acetylene reduction activity and the impossibility of detecting the *nifH* genes along with ARDRA profiles allowed the presumptive identification of most isolates associated with maize and sugarcane as belonging to the spe-

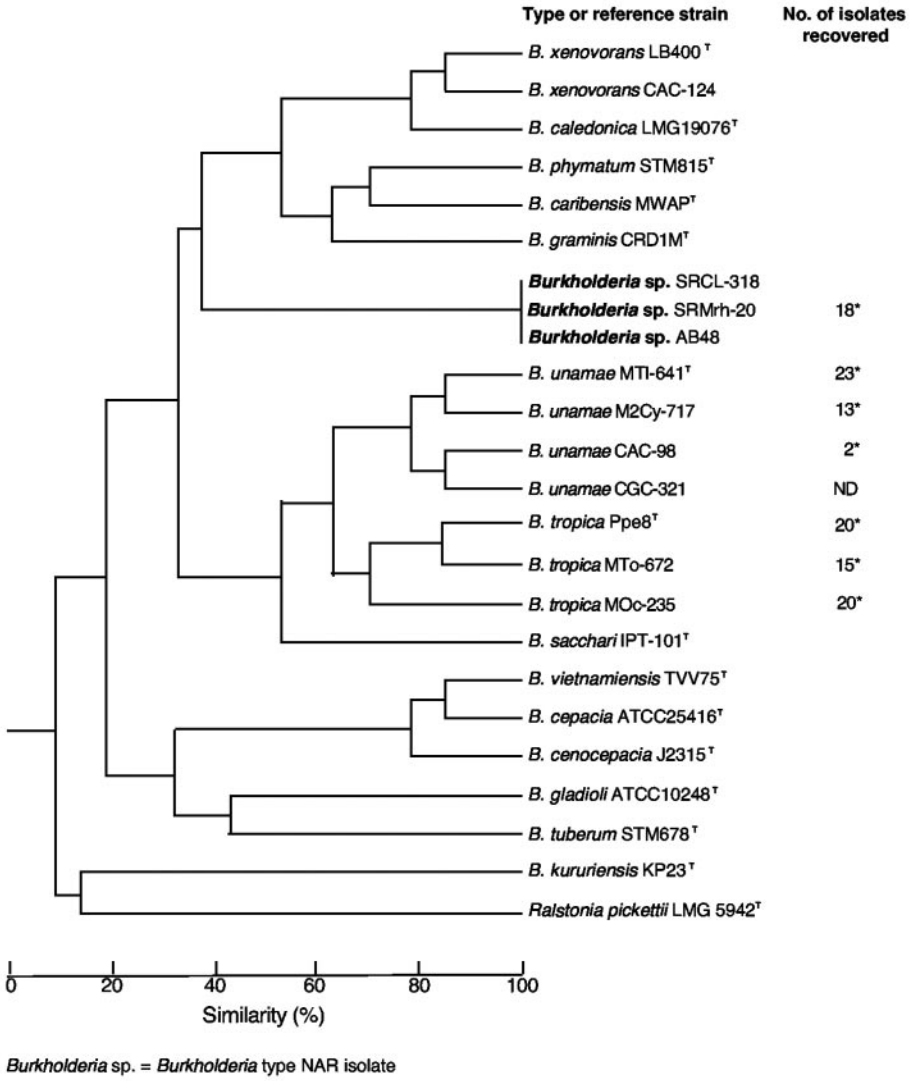

FIG. 2. Dendrogram of genetic relatedness among diazotrophic *Burkholderia* isolates recovered from maize and sugarcane plants cultivated in Mexico and Brazil and related *Burkholderia* species, based on ARDRA. The total number of isolates recovered from each diazotrophic *Burkholderia* species is indicated. ∗, ARDRA genotypes corresponding to *B. unamae* and *B. tropica* strains used as reference have been described previously (9, 33). ND, ARDRA genotype not detected.

cies *B. cepacia*, *B. cenocepacia*, and/or *B. ambifaria*. On this basis and considering the protein electrophoregrams as well as the molecular tests, the diazotrophic isolates associated with maize and sugarcane plants recovered in the present study were identified as belonging to the species *B. unamae* and *B. tropica* and one unknown *Burkholderia* species (NAR group).

In previous studies the diazotrophic species *B. unamae* and *B. tropica* were randomly recovered from the environments of both maize and sugarcane varieties cultivated in Mexico (9, 33). In the present study, predominant associations of *B. tropica* with maize and of *B. unamae* with sugarcane plants were clearly observed. This finding could be partially supported by the isolation, for the first time, of *B. unamae* from a sugarcane variety (SP 7011-43) grown in Brazil. In the present study *B. tropica* was not recovered from field-grown plants in Brazil; however, its association in low numbers with sugarcane cultivated in Brazil and South Africa has been documented (33). Though a low number of *B. unamae* isolates and no *B. tropica* isolates were recovered from field-grown plants in Brazil, these species appear to be widely distributed among geographical regions and plants. *B. unamae* has been isolated from the rhizosphere of sugarcane variety N16 cultivated in South Africa (NCBI GenBank database accession number AY391282) and from the rhizosphere of tomato plants in Mexico (unpublished results). Recently, based on the 16S rRNA sequence, one isolate recovered from within the dune grass *Ammophila arenaria* showed 99% identity with *B. tropica* strains (16). A possible explanation for the low number of *B. unamae* isolates or lack of isolation of *B. tropica* from plants in Brazil could be the dramatically uneven distribution of bacteria among individual plants, as has been observed in communities of *B. cepacia* complex species detected in the maize rhizosphere (31). A study by Tabacchioni et al. (41) showed that the genetic diversity levels of *B. cepacia* populations differ with the

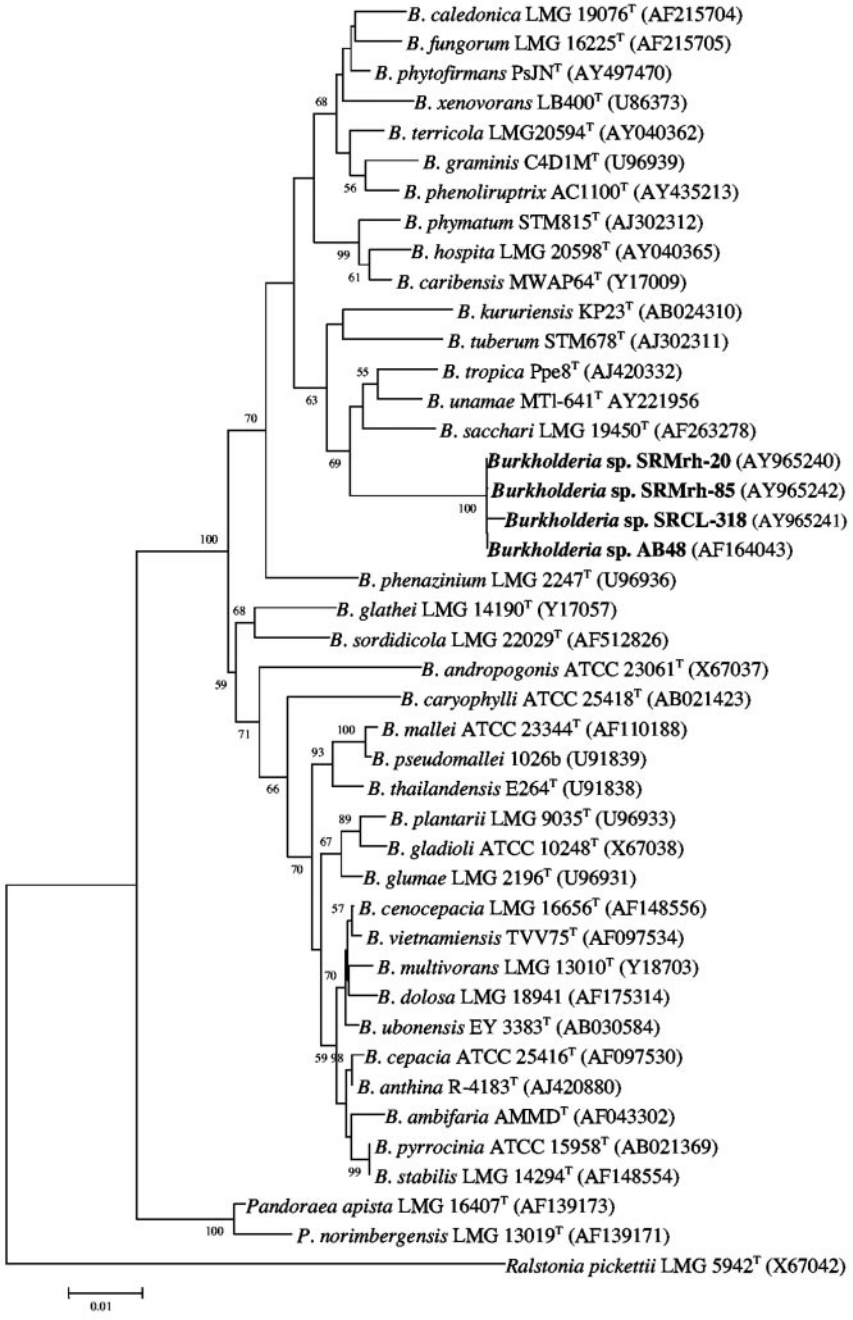

FIG. 3. Phylogenetic tree based on 16S rRNA gene sequences, showing the relatedness among the *Burkholderia* NAR group, *Burkholderia* species, and related *Betaproteobacteria*. The bar represents 1 nucleotide substitution per 100 nucleotides. The GenBank accession number for each strain is shown in parentheses.

use of different isolation media. The lack of recovery of *B. tropica* and *Burkholderia* type NAR isolates from plants grown in Brazil and Mexico, respectively, could be attributed to the biases caused by using slightly different methodologies, but such a possibility should be discounted. The main difference in methodology was the use of N-free semisolid JMV medium, which was not used in Mexico, but several *Burkholderia* type NAR isolates were recovered from N-free semisolid BAz medium and BAc agar plates, both of which were used in Mexico. Other factors should account for the lack of recovery of these bacteria. Antagonism in agar plates is a well-known event (15), and recently, antagonistic activity between *Gluconacetobacter diazotrophicus* strains was demonstrated to occur in N-free semisolid LGI medium and was confirmed on agar plates as well as in endophytic association (28). On this basis, we speculate that antagonism could occur in N-free semisolid BAz or JMV medium during the enrichment subcultures of diazotrophic *Burkholderia* species as well as under natural conditions.

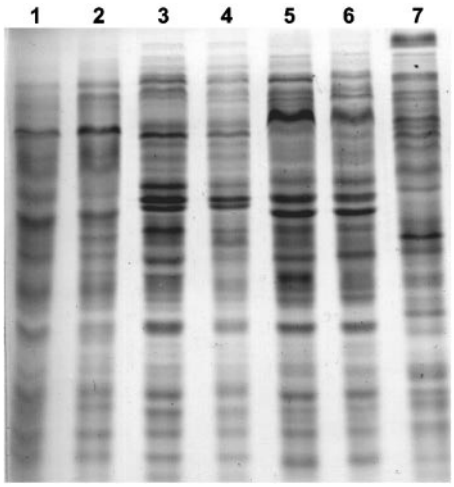

FIG. 4. Protein electrophoregrams of representative acetylene-reducing isolates recovered in the present study and type strains of known diazotrophic *Burkholderia* species. *B. tropica*: lane 1, MTl-632 isolated from rhizosphere of maize, and lane 2, Ppe8^T; *B. unamae*: lane 3, MTl-641^T; lane 4, SRCrh-274 isolated from rhizosphere of sugarcane (Brazil); type NAR isolates: lane 5, SRMrh-20, and lane 6, SRCL-318; lane 7, *B. cepacia* ATCC25416^T.

However, such antagonistic activities between *Burkholderia* species should eventually be demonstrated to occur in further studies.

16S rRNA sequences and whole-cell protein profiles are strong evidence in the delineation of new bacterial species (47). In fact, over the last few years the descriptions of many novel bacterial species have been based on data generated from 16S rRNA sequencing studies, but unfortunately they have been based on the analysis of one or two isolates (35), consequently leaving the distributions and natural habitats of the species unknown. On the basis of 16S rRNA gene sequences and protein electrophoregrams, the predominant group of N_2-fixing *Burkholderia* isolates associated with maize and sugarcane plants in Brazil could be defined as a novel species. However, we considered that more of these diazotrophic isolates from other varieties of maize and sugarcane plants or from other plants, habitats, or geographical regions would have to be recovered and analyzed with polyphasic taxonomy criteria (47) for validation of the *Burkholderia* NAR group as a new species, as well as for knowledge about their environmental distribution and natural habitats. Regardless of the taxonomic status of these N_2-fixing *Burkholderia* isolates, they are apparently common plant colonizers in Brazil, since strains SRMrh-20, SRCL-318, and AB48 were found associated with maize, sugarcane, and pineapple, respectively.

Although the *cblA* and *esmR* genes, which are related to transmissibility, have been found mainly in clinical isolates of *B. cenocepacia* as well as in other species of the *B. cepacia* complex, both genes have also been identified from among environmental isolates of *B. cenocepacia* and *B. cepacia* (5, 11, 25, 34). In the present study, *cblA* and *esmR* were not detected in any of the plant-associated diazotrophic *Burkholderia* isolates analyzed, which confirms their restricted presence in clinical and environmental isolates of opportunistic pathogens of the *B. cepacia* complex. Moreover, the lack of detection of BCESM in these N_2-fixing *Burkholderia* populations also suggests the absence of the genomic island detected in *B. cenocepacia* (3), which fulfills all the characteristic features of a bacterial pathogenicity island (22). On this basis, the results add new evidence to support the potential for using *B. unamae* and *B. tropica* as plant growth-promoting bacteria, since they have the ability to improve maize plant growth (unpublished results). Nevertheless, experiments to directly measure the probability of lateral gene transfer between opportunistic pathogen strains of the *B. cepacia* complex and other environmental *Burkholderia* taxa, such as *B. unamae* and *B. tropica*, would be necessary to have higher confidence about their safety in agricultural applications. These experiments are in progress.

In conclusion, while *B. unamae* is predominantly associated with sugarcane, *B. tropica* more often associates with maize. The isolation of *B. unamae* from field-grown sugarcane in Brazil and Mexico, as well as the isolation of *B. tropica* from maize cultivated in Mexico, and the finding of a probably-novel diazotrophic species (the *Burkholderia* NAR group) in rhizospheric and endophytic association with both maize and sugarcane in Brazil confirm the riches of the genus *Burkholderia* in nitrogen-fixing plant-associated species as well as its broad environmental and geographic distribution.

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