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Burkholderia, a Genus Rich in Plant-Associated Nitrogen Fixers with Wide Environmental and Geographic Distribution

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The genus *Burkholderia* comprises 19 species, including *Burkholderia vietnamiensis* which is the only known N_2-fixing species of this bacterial genus. The first isolates of *B. vietnamiensis* were recovered from the rhizosphere of rice plants grown in a phytotron, but its existence in natural environments and its geographic distribution were not reported. In the present study, most N_2-fixing isolates recovered from the environment of field-grown maize and coffee plants cultivated in widely separated regions of Mexico were phenotypically identified as *B. cepacia* using the API 20NE system. Nevertheless, a number of these isolates recovered from inside of maize roots, as well as from the rhizosphere and rhizoplane of maize and coffee plants, showed similar or identical features to those of *B. vietnamiensis* TVV75^T. These features include nitrogenase activity with 10 different carbon sources, identical or very similar *nifHDK* hybridization patterns, very similar protein electrophoregrams, identical amplified 16S rDNA restriction (ARDRA) profiles, and levels of DNA-DNA reassociation higher than 70% with total DNA from strain TVV75^T. Although the ability to fix N_2 is not reported to be a common feature among the known species of the genus *Burkholderia*, the results obtained show that many diazotrophic *Burkholderia* isolates analyzed showed phenotypic and genotypic features different from those of the known N_2-fixing species *B. vietnamiensis* as well as from those of *B. kururiensis*, a bacterium identified in the present study as a diazotrophic species. DNA-DNA reassociation assays confirmed the existence of N_2-fixing *Burkholderia* species different from *B. vietnamiensis*. In addition, this study shows the wide geographic distribution and substantial capability of N_2-fixing *Burkholderia* spp. for colonizing diverse host plants in distantly separated environments.

There exist many examples of the wide geographic distribution of *Rhizobium* species in symbiotic association with legumes (see reference 23). Commonly, this association is referred to as being restricted to legume plants with the exception of the genus *Parasponia* (41). Recently, *Rhizobium leguminosarum* bv. trifolii and *Azorhizobium caulinodans* have also been found in natural endophytic association with field-grown rice (12, 45). Similarly, *Gluconacetobacter* (formerly *Acetobacter*) *diazotrophicus* was considered in early studies as an endophyte associated only with sugarcane and with two other sucrose-accumulating plants (11). However, in the last few years *G. diazotrophicus* has been found in endophytic association with multiple host plants such as *Coffea arabica* (19), *Eleusine coracana* (22), and *Ananas comosus* (36), all of which were cultivated in very distant geographical regions. A similar picture has been described for *Azoarcus* species. The first *Azoarcus* species, *Azoarcus indigens* and *Azoarcus communis*, were described in association with Kallar grass cultivated in Pakistan (30). Interestingly, one of the strains used in this study was isolated in France in 1982 from a refinery oil sludge and identified as *A. communis*. Recently, *Azoarcus indigens* has also been isolated from field-grown rice cultivated in Nepal (12). *Azoarcus tolulyticus* has been recovered from a variety of environments and regions (13, 48). These data suggest that *Azoarcus* spp. are widely distributed. Apparently, many bacterial species are able to flourish in very different and distant habitats. Staley (34) pointed out that the bacterial biogeography data tend to support the hypothesis that many bacteria are cosmopolitan in their distribution. However, the geographic and environmental distribution of many bacterial species remain unknown. For instance, *Burkholderia vietnamiensis* was discovered in association with roots of rice plants grown in a Vietnamese soil (15). To date, this species has not been isolated from any other plant.

The genus *Burkholderia* comprises 19 species, which includes soil and rhizosphere bacteria as well as plant and human pathogens (1, 35, 39, 42, 47). In addition, the GenBank database contains the 16S rRNA sequence (accession number AJ238360) of a N_2-fixing bacterium, *"B. brasilensis"*, which has not been officially described. *B. vietnamiensis* is the only N_2-fixing species of this bacterial genus validly described (15).

Over the last few years there has been an increasing interest in *B. cepacia*, the type species of the genus, because of its wide distribution in natural and clinical environments (18, 31, 39). *B. cepacia* is recognized for its abilities to promote maize growth (5), to enhance crop yields (8, 35), and to suppress many soilborne plant pathogens (5, 17, 25), as well as to degrade diverse pesticides (9, 26). Similarly, *B. vietnamiensis* has attracted interest because of its abilities to promote rice plant growth and to enhance grain yield (37, 38). However, both *B. cepacia*, particularly the named genomovar III and *B. vietnamiensis* have been cultured from patients with cystic fibrosis

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(39). Recently, it has been described that *B. cepacia* genomovar III is a common plant-associated bacterium (3).

The determination of the extent and distribution of microbial diversity on the planet will help in understanding the role of specific microbial taxa in their natural habitats (34). In addition, studies on the diversity of plant-associated bacteria may contribute to the discovery of new beneficial plant-microbe interactions.

In this study, we report on the association of N_2-fixing *Burkholderia* species with field-grown maize and coffee plants cultivated in widely separated geographical regions of Mexico. Our results show that *B. vietnamiensis* is widely distributed and reveal the existence of new N_2-fixing *Burkholderia* spp. We also report on the ability of *B. kururiensis* to fix N_2, a feature hitherto unknown in this species.

MATERIALS AND METHODS

Plant samples. From four to eight complete maize and coffee plants grown under field conditions were collected in different geographical regions of Mexico. The origins of maize and coffee plants analyzed are summarized in Table 1.

Media and cultural conditions. A nitrogen-free semisolid medium (BAz) was used as an enrichment culture and for the enumeration of N_2-fixing *Burkholderia* species. BAz medium had the following composition (in grams/liter): azelaic acid, 2.0; K_2HPO_4, 0.4; KH_2PO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $Na_2MoO_4 \cdot H_2O$, 0.002; $FeCl_3$, 0.01; bromothymol blue, 0.075; and agar, 2.3. The medium was adjusted with KOH to pH 5.7. Vials containing 5 ml of BAz medium were autoclaved at 121°C for 20 min, and filter-sterilized cycloheximide (200 μg/tube) was then added. PCAT medium is considered selective for *B. cepacia* (6). Because azelaic acid is used as a carbon source for most of the known *Burkholderia* species and tryptamine is used by *B. cepacia* but not by many other *Burkholderia* species (15), the PCAT medium was modified. Tryptamine was omitted as a nitrogen source to avoid the overgrowth of *B. cepacia* and to allow the growth of N_2-fixing *Burkholderia* species. In this modified medium (PCATm) chlorothalanil was omitted as well, and bromothymol blue (75 mg/liter) was added. A BAc medium (0.2% azelaic acid, 0.02% L-citrulline, 0.04% K_2HPO_4, 0.04% KH_2PO_4, and 0.02%, $MgSO_4 \cdot 7H_2O$) was also used for isolation and culturing of *Burkholderia* species. The pH was adjusted to 5.7, and the medium was sterilized at 121°C for 20 min prior to the addition of filter-sterilized (pore size, 0.22 μm) citrulline as the sole nitrogen source. In addition to N-free BAz medium used as an enrichment culture and for acetylene reduction activity (ARA) assays, we also tested a modified BAz medium, one lacking azelaic acid but supplemented with a single carbon source (0.5% fructose, glucose, sucrose, mannitol, glycerol, malate, succinate or 0.2% azelate, benzoate, or propionate) or with three carbon sources (0.2% malic acid, glucose, and 0.1% mannitol). This medium was named BMGM. *Burkholderia* spp. isolates were grown in BSE medium (0.5% succinate, 0.04% K_2HPO_4, 0.04% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract; pH 6.5) for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) assays, and BDN medium (0.02% peptone, 0.01% yeast extract, 0.04% K_2HPO_4, 0.04% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$; pH 6.5) for total DNA isolation.

Isolation and enumeration. The root was shaken gently to remove the loosely attached soil, and the adhering soil was rinsed in 9.0 ml of 10 mM $MgSO_4 \cdot 7H_2O$ (Mgsol). The resulting rinse solution containing the rhizosphere bacteria was serially diluted with Mgsol. The root was subsequently washed with Mgsol containing 0.01% (vol/vol) Tween 20, followed by two rinses with Mgsol, and then immersed in 9.9 ml of Mgsol and vortexed for 3 min. The resulting suspension, which was considered to contain bacteria from the rhizoplane, was serially diluted with Mgsol. The vortexed root was immersed for 5 min under agitation in full-strength bleach solution containing Tween 20 and rinsed three times in sterile H_2O. The roots were rolled on to Luria-Bertani agar plates to verify root surface sterilization and then were macerated in a blender in a known volume of Mgsol, and the suspension was serially diluted. The aerial parts of maize plants were surface sterilized for 10 min and treated as described above for the root samples. The macerates were serially diluted with Mgsol and used to calculate the most probable number (MPN) and for recovering the *Burkholderia* endophytic community. Vials containing N-free semisolid BAz medium were inoculated with 100-μl aliquots from diluted samples and incubated for 5 to 7 days at 29°C. Thereafter, cultures were replicated once more under the same conditions. Vials with a white or yellowish pellicle at a depth of 1 to 4 mm below of surface were streaked onto PCATm and BAc medium plates and incubated at 29°C. After 4 to 5 days, predominant colonies with different morphology were individually inoculated in vials containing N-free BAz medium, incubated at 29°C for 4 days and assayed for ARA as described previously (24). When ARA was not detected in N-free BAz medium, the isolates were inoculated in N-free semisolid BMGM medium, incubated for 3 days before the ARA assays were carried out. All the acetylene-reducing colonies were further verified for culture purity, and then the colony morphology was recorded and their distribution was used to calculate the MPN of N_2-fixing bacteria, using the McCrady tables. Three replicates per 10-fold dilution were made from each sample. Three N_2-fixing isolates from each colony morphology type were chosen from the highest dilutions of each sample. These isolates were maintained in 20% glycerol at −80°C prior to analysis.

Phenotypic characterization. The isolates were presumptively identified with the API 20NE system (bioMérieux). The results were interpreted by using the API analytical profile index, which provided the percentage of identification. In addition, representative isolates were evaluated for their ability to reduce acetylene using glucose, fructose, sucrose, mannitol, glycerol, malate, succinate, azelate, benzoate, or propionate, as the single carbon sources. Acetylene was injected after the cultures were incubated at 29°C for 72 h, but when azelate, benzoate, and propionate were tested the incubation was for 4 days.

SDS-PAGE. Cultures were grown in BSE medium with reciprocal shaking (200 rpm) for 15 h at 29°C, and 1.0-ml samples were harvested by centrifugation at 12,300 × *g* for 10 min. The pellet were resuspended in 70 μl of 0.125 M Tris-HCl, 4% SDS, 20% glycerol, and 10% mercaptoethanol at pH 6.8. Aliquots of 10 μl were used for SDS-PAGE performed as described by Laemmli (20).

Total DNA isolation and amplified DNA restriction analysis (ARDRA). Cultures were grown in BDN medium for 24 h and centrifuged at 12,300 × *g* for total DNA preparation as described previously (2). The 16S ribosomal DNA (rDNA) genes from N_2-fixing *Burkholderia* isolates were PCR amplified with the primers fD1 and rD1 (44), using *Taq* polymerase (Boehringer-Roche). The PCR conditions consisted of an initial denaturing cycle (94°C, 3 min), 35 amplification cycles (94°C, 1 min; 55°C, 1 min; 72°C, 2 min), and then a final elongation cycle (72°C, 5 min). The PCR amplified 16S rRNA gene fragments (ca. 1.5 kb) were restricted with 5 U each of *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, and *Rsa*I. The lengths of the restriction fragments of the different 16S rRNA genes were determined by electrophoresis in 3% agarose gels, and the restriction patterns from each isolate were compared. Each isolate was assigned to a 16S rDNA genotype, defined by the combination of the restriction patterns obtained with the seven restriction endonucleases. Similarities among the 16S rRNA gene sequences were estimated from the proportion of shared restriction fragments by the method of Nei and Li (28). A dendrogram was constructed from the resulting distance matrix using the unweighted pair group method with averages (UPGMA) (32).

***nifHDK* hybridizing patterns and DNA-DNA relatedness analysis.** Total DNA was digested with *Eco*RI, and restriction fragments were electrophoresed, blotted, and hybridized as described previously (7). Total *Eco*RI DNA digests from *Burkholderia* isolates were hybridized with pCQ12, which contains a 4.1-kb segment of the *nifHDK* region of *R. etli* CFN 42 (29). DNA-DNA homology was based on relative levels of hybridization to ^{32}P-labeled DNA from *B. vietnamiensis* TVV75^T. DNA-DNA hybridization was for 12 h at 65°C, and the nylon filters were washed once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 10 min, once in 1× SSC at 55°C for 5 min, and once in 0.1× SSC at 65°C for 5 min. The percentage of total homologous reassociation was calculated for each strain tested as described previously (19).

RESULTS

Isolation and enumeration. The inoculation of N-free semisolid BAz medium with samples from rhizosphere soil of maize and coffee plants as well as with maize plant tissues, followed by subsequent streaking on the PCATm and BAc culture media, allowed the recovery of more than 200 N_2-fixing isolates. Although many N_2-fixing isolates obtained from each sample of rhizosphere, rhizoplane, or plant tissues were recovered and analyzed, we included only one representative isolate recovered from each sample in Table 1.

Bacterial growth in N-free semisolid BAz medium resulted in the formation of surface pellicles with different characteristics and acetylene reduction activities. In this medium some cultures formed pellicles at a depth of 1 to 2 mm below the

TABLE 1. Representative nitrogen-fixing *Burkholderia* strains associated with maize and coffee plants

16S rDNA genotype	Taxon	Strain	Source[a]	Cultivar	Plant	Location code[b]
1	*B. vietnamiensis*	MMi-324	Rhizosphere	VS-535	Maize	1
1	*B. vietnamiensis*	MMi-334	Rhizoplane	VS-535	Maize	1
1	*B. vietnamiensis*	MMi-353	Rhizoplane	VS-535	Maize	1
1	*B. vietnamiensis*	MMi-1486	Rhizosphere	VS-535	Maize	1
1	*B. vietnamiensis*	MMi-1537	Roots*	VS-535	Maize	1
1	*B. vietnamiensis*	MMi-1547	Rhizoplane	VS-535	Maize	1
1	*B. vietnamiensis*	MMi-1556	Roots*	VS-535	Maize	1
1	*B. vietnamiensis*	MMi-1577	Roots*	VS-535	Maize	1
1	*B. vietnamiensis*	MMi-1776	Rhizosphere	VS-535	Maize	1
1	*B. vietnamiensis*	CCE-101	Rhizoplane	Caturra	Coffee	5
1	*B. vietnamiensis*	CCE-115	Roots†	Caturra	Coffee	5
1	*B. vietnamiensis*	CCE-201	Rhizoplane	Caturra	Coffee	5
1	*B. vietnamiensis*	CCE-211	Roots†	Caturra	Coffee	5
1	*B. vietnamiensis*	CCE-303	Rhizoplane	Caturra	Coffee	5
1	*B. vietnamiensis*	CCE-312	Roots†	Caturra	Coffee	5
2	*B. vietnamiensis*	MMi-302	Rhizosphere	VS-535	Maize	1
2	*B. vietnamiensis*	MMi-313	Rhizosphere	VS-535	Maize	1
2	*B. vietnamiensis*	MMi-344	Roots*	VS-535	Maize	1
5	*Burkholderia* sp.	CCE-414	Roots†	Caturra	Coffee	5
6	*Burkholderia* sp.	CCE-421	Rhizosphere	Caturra	Coffee	5
7	*Burkholderia* sp.	MTl-441	Rhizosphere	Landrace	Maize	3
8	*Burkholderia* sp.	CAC-124	Rhizosphere	Arabiga	Coffee	7
13	*Burkholderia* sp.	CAC-98	Rhizosphere	Arabiga	Coffee	7
13	*Burkholderia* sp.	CAC-142	Rhizosphere	Arabiga	Coffee	7
13	*Burkholderia* sp.	CAC-369	Rhizosphere	Arabiga	Coffee	7
13	*Burkholderia* sp.	CAC-382	Rhizoplane	Arabiga	Coffee	7
14	*Burkholderia* sp.	CGC-321	Rhizosphere	Garnica	Coffee	7
15	*Burkholderia* sp.	MTl-641	Rhizosphere	Landrace	Maize	3
15	*Burkholderia* sp.	CGC-72	Rhizoplane	Garnica	Coffee	7
15	*Burkholderia* sp.	CAC-112	Rhizoplane	Arabiga	Coffee	7
15	*Burkholderia* sp.	CGC-316	Rhizosphere	Garnica	Coffee	7
16	*Burkholderia* sp.	MOc-235	Rhizosphere	Landrace	Maize	2
16	*Burkholderia* sp.	MOc-255	Rhizosphere	Landrace	Maize	2
16	*Burkholderia* sp.	MOc-725	Rhizoplane	Landrace	Maize	2
17	*Burkholderia* sp.	MMi-786	Rhizoplane	VS-535	Maize	1
17	*Burkholderia* sp.	MTo-41	Rhizosphere	Landrace	Maize	4
17	*Burkholderia* sp.	MTo-431	Rhizosphere	Landrace	Maize	4
17	*Burkholderia* sp.	MTo-432	Rhizoplane	Landrace	Maize	4
17	*Burkholderia* sp.	MTo-452	Rhizoplane	Landrace	Maize	4
18	*Burkholderia* sp.	CBN-516	Rhizosphere	Bourbon	Coffee	6
18	*Burkholderia* sp.	CBN-523	Rhizoplane	Bourbon	Coffee	6
18	*Burkholderia* sp.	CBN-721	Rhizoplane	Bourbon	Coffee	6
18	*Burkholderia* sp.	CBN-724	Rhizoplane	Bourbon	Coffee	6
19	*Burkholderia* sp.	MTo-16	Rhizosphere	Landrace	Maize	4
19	*Burkholderia* sp.	MTo-293	Stem	Landrace	Maize	4
20	*Burkholderia* sp.	MMi-493	Rhizosphere	VS-535	Maize	1
21	*Burkholderia* sp.	CCE-401	Rhizoplane	Caturra	Coffee	5
21	*Burkholderia* sp.	CBN-15	Rhizoplane	Bourbon	Coffee	6
21	*Burkholderia* sp.	CBN-23	Rhizoplane	Bourbon	Coffee	6
21	*Burkholderia* sp.	CBN-25	Rhizosphere	Bourbon	Coffee	6
21	*Burkholderia* sp.	CAC-92	Rhizosphere	Arabiga	Coffee	7

[a] *, Surface-sterilized roots; †, unwashed roots.
[b] Location codes: 1, Miacatlán, Morelos State; 2, Ocotepec, Morelos State; 3, Tlayacapan, Morelos State; 4, Totontepec, Oaxaca State; 5, El Eden, Chiapas State; 6, La Neblina, Querétaro State; 7, Coatepec, Veracruz State (all in Mexico).

surface, while others formed pellicles 4 mm below the surface. The pellicles were white, whitish, or yellowish and dense and fine or thick and diffuse. In general, pH changes were not observed in N-free BAz medium, but the growth of some isolates resulted in a slightly raised pH as indicated by a green-blue color in the medium.

Predominant colonies on PCATm medium were yellowish, round, smooth, flat or convex with entire margins and a diameter of from ≤1.0 to 1.5 mm. Many but not all of these isolates reduced acetylene in N-free semisolid BAz medium. However, more N_2-fixing isolates were detected when tested in the N-free semisolid BMGM medium. Many N_2-fixing isolates formed white colonies, while others formed whitish or yellowish colonies on BAc agar. However, all of the colonies were round and smooth, with entire margins varying in diameter from 1 to 2 mm. White colonies were flat or slightly convex, and these isolates turned the medium from green to deep blue, while isolates with whitish or yellowish colonies were convex

TABLE 2. ARA by representative N_2-fixing *Burkholderia* isolates and strains of related species

16S rDNA genotype	Taxon	Reference strain	ARA with[a]:									
			Fru	Glu	Scr	Man	Gly	Suc	Mal	Aze	Ben	Pro
1	*B. vietnamiensis*	TVV75^T	88	−	+	38	+	88	+	78	+	90
1	*B. vietnamiensis*	MMi-324	34	−	−	24	+	84	+	22	+	24
1	*B. vietnamiensis*	MMi-1537	30	−	+	26	+	118	+	28	+	28
1	*B. vietnamiensis*	MMi-1547	24	−	+	30	+	100	+	22	+	22
1	*B. vietnamiensis*	CCE-201	26	−	+	22	+	172	+	29	+	26
1	*B. vietnamiensis*	CCE-312	40	50	+	36	+	123	+	48	+	130
1	*B. vietnamiensis*	CCE-101	50	34	+	32	+	156	+	10	+	24
1	*B. vietnamiensis*	SXo-702	42	40	+	32	+	128	+	24	+	22
2	*B. vietnamiensis*	MMi-302	60	−	−	42	+	82	+	33	+	52
2	*B. vietnamiensis*	MMi-344	78	36	+	66	+	94	+	70	+	72
3	*B. cepacia*	ATCC 29352	−	−	−	−	−	−	−	−	−	−
5	*Burkholderia* sp.	CCE-414	86	−	+	22	+	104	+	74	+	44
6	*Burkholderia* sp.	CCE-421	28	−	−	<10	+	114	+	46	−	<10
8	*Burkholderia* sp.	CAC-124	79	48	+	41	+	48	+	34	+	36
11	*B. caribensis*	MWAP64^T	−	−	−	−	−	−	−	−	−	−
12	*B. graminis*	C4D1M^T	−	−	−	−	−	−	−	−	−	−
13	*Burkholderia* sp.	CAC-382	28	62	+	34	+	218	+	58	−	26
13	*Burkholderia* sp.	CAC-98	173	254	−	94	+	236	+	220	+	130
14	*Burkholderia* sp.	CGC-321	112*	48	+	36	+	436	+	56	−	26
15	*Burkholderia* sp.	MTl-641	72*	345	+	86	+	152	+	206	+	206
15	*Burkholderia* sp.	CGC-72	165	256	±	142	+	346	+	180	+	142
16	*Burkholderia* sp.	MOc-235	48*	−	+	62*	+	68*	±	32	−	28
16	*Burkholderia* sp.	MOc-725	82	−	+	48	+	198	+	54	+	104
16	*Burkholderia* sp.	SMi-583	112	−	+	49	+	194	+	64	−	42
17	*Burkholderia* sp.	MMi-786	40	−	+	<10*	−	216	±	−	−	−
17	*Burkholderia* sp.	MTo-431	64	−	+	42*	−	156*	+	−	−	−
18	*Burkholderia* sp.	SXo-252	76	−	+	<10*	−	164	+	<10*	±	−
18	*Burkholderia* sp.	CBN-516	60*	72*	−	16*	−	312	+	16*	±	−
18	*Burkholderia* sp.	CBN-721	86*	66*	−	<10	−	184	+	<10*	±	−
19	*Burkholderia* sp.	MTo-293	80	96	±	38	−	166	+	40	−	−
19	*Burkholderia* sp.	MTo-16	152	40	±	58	+	196	+	66	−	208
20	*Burkholderia* sp.	MMi-493	52	28*	−	46	+	220	+	44	−	62
21	*Burkholderia* sp.	CCE-401	34	34	+	20	−	158	+	20	−	−
21	*Burkholderia* sp.	CBN-23	34	15	+	24	−	270	+	44	−	−
21	*Burkholderia* sp.	CAC-92	24	60	+	22	−	80	+	38	−	−
22	*B. kururiensis*	KP23^T	69	160	+	46	+	132	+	68	−	−
22	"*B. brasilensis*"	M-130	30	78	±	26	+	74	±	<10*	−	−

[a] Values represent nanomoles of C_2H_4/h/culture and the means of two replicate cultures. When inconsistent ARA was observed, up to six replicates were done in independent experiments. +, Positive activity; −, negative activity; ± or *, inconsistent activity. Fru, fructose; Glu, glucose; Scr, sucrose; Man, mannitol; Gly, glycerol; Mal, malate; Suc, succinate; Aze, azelate; Ben, benzoate; Pro, propionate.

and turned the medium a light blue color. However, several N_2-fixing isolates were not able to grow on BAc medium plates. MPN values for colonies with the features described varied from 4×10^6 CFU/g of rhizosphere soil to 4×10^5 CFU/g of fresh tissue of maize.

Phenotypic characterization. Biochemical tests based on the use of API 20NE showed that a majority of the N_2-fixing isolates recovered on PCATm agar plates belonged to the species *B. cepacia* (81.1 to 99.6% confidence limits based on the API analytical profile index), but some diazotrophic isolates were identified as *Pseudomonas aureofaciens* with confidence limits from 63.5 to 89.7%. Similarly, N_2-fixing isolates growing on BAc medium plates were identified as *B. cepacia* and *P. aureofaciens*. However, several isolates recovered from PCATm agar plates but incapable of growing on BAc medium were identified as belonging mainly to *Enterobacter cloacae* and *Klebsiella pneumoniae* subsp. *pneumoniae* (data not shown). Frequently, strains of these species were isolated from within the roots and stems of maize plants.

The ability to fix N_2 varied among the different diazotrophs isolated from the maize and coffee plants (Table 2). All of these isolates were capable of N_2 fixation with fructose, mannitol, malate, and succinate as single carbon sources, but this ability was variable with other carbon substrates. *B. vietnamiensis* TVV75^T and several isolates recovered from maize and coffee plants showed ARA with all of the carbon sources tested except glucose. However, the isolates CCE-312 and CCE-101 were capable of reducing acetylene when glucose was used as a single carbon source. This inability to reduce acetylene when glucose was the carbon source was also observed with the collection of *B. vietnamiensis* strains TVV69, TVV72, and TVV115 (data not shown) recovered from the rhizosphere of rice (15). Inexplicably, isolates corresponding to the 16S rDNA genotypes 16, 17, 18, and 19 showed an inconsistent ARA even among replicates from the same assay. Frequently, one or two of three replicates did not exhibit ARA. The carbon source was eliminated as a possible cause of this inconsistency because these isolates were able to grow with glucose, fructose, sucrose,

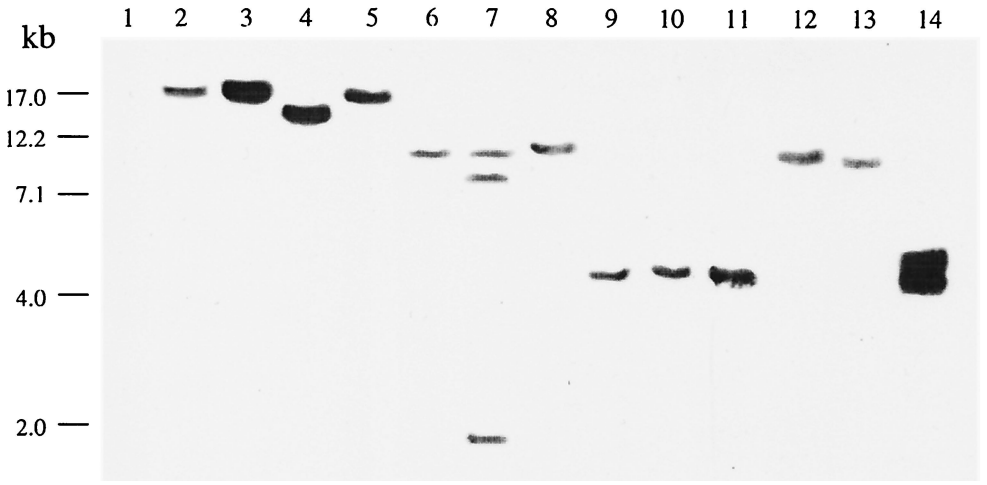

FIG. 1. Autoradiogram of a Southern blot of total *Eco*RI-digested DNA hybridized with the *nifHDK* probe of *R. etli* CFN 42. Lane 1, *B. cepacia* ATCC 29352 used as a negative control; lane 2, *B. vietnamiensis* TVV75^T. Lanes 3 through 12 are examples of representative N_2-fixing *Burkholderia* isolates; lane 3, MMi-1486; lane 4, CCE-101; lane 5, MMi-302; lane 6, CCE-414; lane 7, CCE-421; lane 8, CGC-321, lane 9, MMi-786; lane 10, SXo-252; lane 11, MTo-293; lane 12, CAC-92; lane 13, *B. kururiensis* KP23^T; lane 14, *R. etli* CFN42.

mannitol, succinate, and other carbon sources when NH_4NO_3 is supplied as nitrogen source (data not shown). Also, contamination was discarded as a possible cause of inconsistency. Interestingly, *B. kururiensis* KP23^T was capable of fixing N_2 when grown on several carbon sources (Table 2).

***nifHDK* hybridizing patterns.** The *nifHDK* patterns of representative *Burkholderia* isolates are shown in Fig. 1. Several isolates showed an *nifHDK* hybridization pattern identical (e.g., MMi-1486) or very similar (e.g., CCE-101) to that of the *B. vietnamiensis* type strain. In addition, other groups of N_2-fixing *Burkholderia* isolates showed a hybridization pattern of the *nifHDK* genes different from that of the *B. vietnamiensis* type strain. A band hybridizing to the *nifHDK* genes of *R. etli* was observed in total *Eco*RI DNA fingerprints from *B. kururiensis* KP23^T. The *nifHDK* hybridization pattern of *B. kururiensis* was different from that of *B. vietnamiensis* but was very similar to that of strain CAC-92 recovered from a coffee plant. These results confirmed the ability of the *Burkholderia* isolates to fix N_2, even by isolates (e.g., MMi-786 and SXo-252) which showed an inconsistent ARA.

Protein electrophoregrams. The whole-cell protein patterns of representative N_2-fixing *Burkholderia* strains are shown in Fig. 2. Several isolates (e.g., MMi-1547 and MMi-1556) recovered from the rhizosphere, rhizoplane, and inside of maize roots, as well as isolates (e.g., CCE-101 and CCE-312) from the roots and rhizosphere of coffee plants showed protein patterns very similar to those of the *B. vietnamiensis* type strain (Fig. 2A). The differences were mostly observed in the 55.6- to 116-kDa region. In addition, other groups of N_2-fixing isolates recovered from the maize and coffee environment showed almost identical protein electrophoregrams (Fig. 2B), but those (e.g., MOc-235 or MTl-641) were clearly different from the protein patterns of *B. vietnamiensis* (Fig. 2B) and from other known *Burkholderia* species (data not shown). Interestingly, a few N_2-fixing isolates (e.g., SXo-702 and SXo-252) recovered from the sorghum plant (cv. D-65) environment in Xoxocotla, Morelos State (data not shown), exhibited very similar protein patterns to those of the isolates recovered from maize plants in Morelos State but were almost indistinguishable from the protein patterns of isolates (e.g., CCE-312 and CBN-516) from coffee plants cultivated in Chiapas State at a distance of about 1,200 km (Fig. 2A and B).

ARDRA profiles. Although ARDRA has been used successfully to differentiate *Rhizobium* (21) and *Burkholderia* (31) species, coefficients of similarity to define species limits do not exist. However, in the present study, *B. vietnamiensis* and *B. cepacia–B. multivorans*, as well as *B. graminis* and *B. caribensis*, were differentiated by coefficients of 70% similarity (Fig. 3). On this basis, we used the level of 70% similarity as indicative of a separate species for the diazotrophic *Burkholderia* recovered from the maize and coffee plant environment. Twenty-two different 16S rDNA genotypes were identified among the strains of known *Burkholderia* species analyzed and the N_2-fixing isolates recovered from maize and coffee plants (Fig. 3). Only the 16S rDNA genotypes 1 and 15 were recovered from both maize and coffee plants, while genotypes 2, 16, 17, and 19 were identified only among isolates recovered from maize plants, and the genotypes 5, 6, 8, 13, 14, 18, and 21 were identified only among isolates recovered from coffee plants (Table 1). One isolate (SMi-583) recovered from the surface-sterilized roots of a sorghum plant showed an ARDRA profile identical (16S rDNA genotype 16) to that of isolates (e.g., MOc-235 and MOc-725) recovered from maize plants. Interestingly, several N_2-fixing isolates recovered from maize and coffee plants showed ARDRA profiles identical (16S rDNA genotype 1) or almost identical (16S rDNA genotype 2) to that of the *B. vietnamiensis* strains TVV75^T, TVV69, and TVV72 (Fig. 3). In addition, other N_2-fixing isolates corresponding to the 16S rDNA genotypes 13 to 21 were phylogenetically related to *B. caribensis* and *B. graminis* but were clearly different and largely distant (15% similarity) from the diazotrophic species *B. vietnamiensis* and from *B. cepacia, B. multivorans*, and *B. stabilis* (Fig. 3), all of which are included in the named "*B. cepacia* complex" (39). Strain KP23^T of *B. kururiensis* and "*B.*

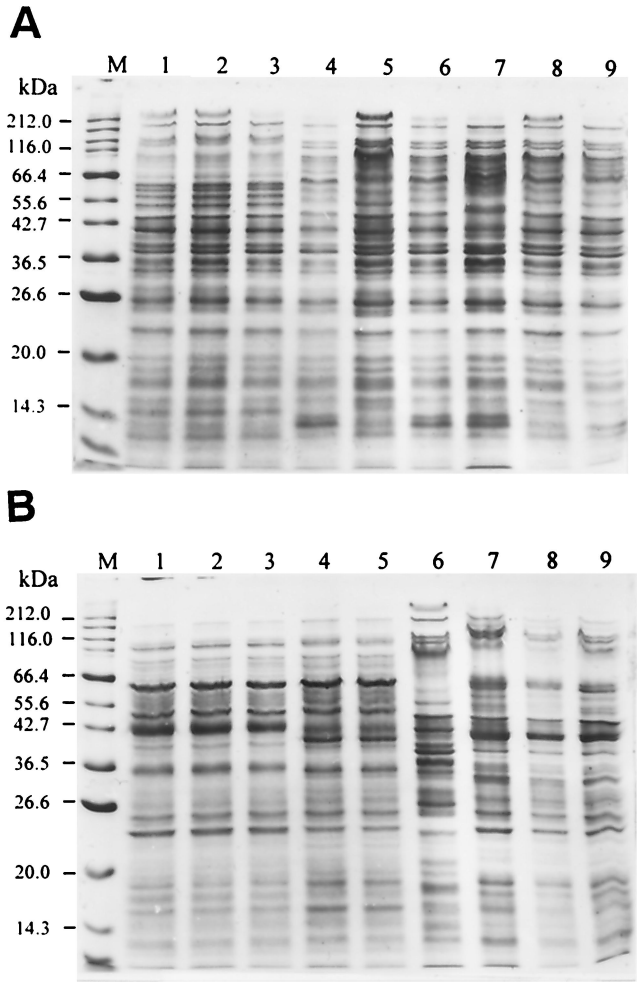

FIG. 2. Protein electrophoregrams (SDS-PAGE) of representatives N_2-fixing *Burkholderia* strains. (A) *B. vietnamiensis* isolates recovered: from maize, lane 1, MMi-324; lane 2, MMi-1547; lane 3, MMi-1556; lane 9, MMi-302; from coffee, lane 6, CCE-312; lane 7, CCE-101; lane 8, CCE-201; from sorghum, lane 4, SXo-702; from rice, lane 5, type strain TVV75^T. M, protein marker. (B) N_2-fixing *Burkholderia* spp. Lane 1, sorghum isolate SXo-252; coffee isolates, lane 2, CBN-516; lane 3, CBN-721; lane 7, CAG-98; lane 8, CGC-321; maize isolates, lane 4, MOc-235; lane 5, MOc-255; lane 9, MTl-641; rice isolate, lane 6, *B. vietnamiensis* TVV75^T. M, protein marker.

brasilensis" M-130 showed the same ARDRA profiles obtained with the seven restriction enzymes used.

DNA-DNA relatedness. Twenty-five *Burkholderia* strains were analyzed in the DNA-DNA reassociation assays. Nine N_2-fixing *Burkholderia* strains analyzed corresponding to the 16S rDNA genotypes 1 and 2 constituted a homogeneous group related to *B. vietnamiensis* TVV75^T, with DNA homology values ranging from 65 to 102%. One or two N_2-fixing isolates corresponding to each of the 16S rDNA genotypes 13 to 18 and genotype 21 exhibited very low DNA homology levels, ranging from 6 to 13% with the reference strain TVV75^T. The type strains *B. caribensis, B. graminis, B. kuruiensis*, and *"B. brasilensis"* exhibited low DNA homology (14 to 38%) with the same reference strain TVV75^T, as is expected for different species.

DISCUSSION

In this study, we report the isolation of many diazotrophs from inside maize roots as well as from the rhizosphere and rhizoplane of maize and coffee plants cultivated in distant geographical regions of Mexico. The successful recovery of N_2-fixing *Burkholderia* spp. associated with maize and coffee plants is partially attributed to the semiselective enrichment using N-free semisolid BAz medium, as well as to the subsequent isolation on PCATm agar plates and growth on BAc medium. Although some strains of the family *Enterobacteriaceae* were isolated from maize plants on PCATm agar plates, they cannot grow with citrulline on the BAc medium. The selectivity of the BAc medium was based on the ability of *Burkholderia* spp. to grow with azelaic acid and citrulline (15), substrates which are not used by bacteria associated with coffee plants such as *G. diazotrophicus* (19), *G. johannae*, and *G. azotocaptans* (14) nor by other common plant-associated bacteria such as *Azospirillum* and *Rhizobium* spp. (data not shown).

Phenotypic identification of the N_2-fixing isolates with the API 20NE system showed that most of the isolates recovered on PCATm medium and all of the isolates which grew on BAc medium plates belonged to *B. cepacia* and a few belonged to *P. aureofaciens*. Recently, the inability to differentiate *B. cepacia* from *P. aureofaciens* or from other *Burkholderia* species using the API 20NE identification system has been reported (31). However, among the 51 *B. cepacia* isolates analyzed in that study only one was misidentified at the genus level. On this basis, the N_2-fixing isolates recovered from the environment of maize and coffee plants were considered to belong to the genus *Burkholderia*. PCR amplification of 16S rDNA genes with selected primers described previously (4) confirmed that these N_2-fixing isolates are members of the genus *Burkholderia* (data not shown).

A number of *Burkholderia* isolates recovered from the environment of maize and coffee plants showed very similar or identical features to those of type strain TVV75^T of *B. vietnamiensis*. These features include N_2-fixing ability, almost identical protein patterns, and identical ARDRA profiles. In addition, these isolates showed high levels of DNA-DNA reassociation (mean homology, 81%) with total DNA from strain TVV75^T. Taking into account that bacteria with very similar protein patterns possess high genome similarity (40) and that a bacterial genomic species includes strains with 70% or greater DNA-DNA relatedness (33), these isolates were assigned to the species *B. vietnamiensis*.

It is worth noting that the first isolates of *B. vietnamiensis*, including the type strain TVV75^T, were recovered from the rhizosphere of young rice plants grown on a Vietnamese soil in a phytotron (38). In the present study, the isolates of this species were recovered from the rhizosphere and rhizoplane of maize and coffee plants grown under natural field conditions, as well as endophytically from the maize plants. Moreover, this study shows the substantial capability of the bacterial species for colonizing different host plants and environments. This is further supported by the recovery of *B. vietnamiensis* isolates (e.g., SXo-702) from the rhizoplane of sorghum plants.

In Mexico, maize has been traditionally cultivated for thousands of years, and even today this crop is grown in many rural

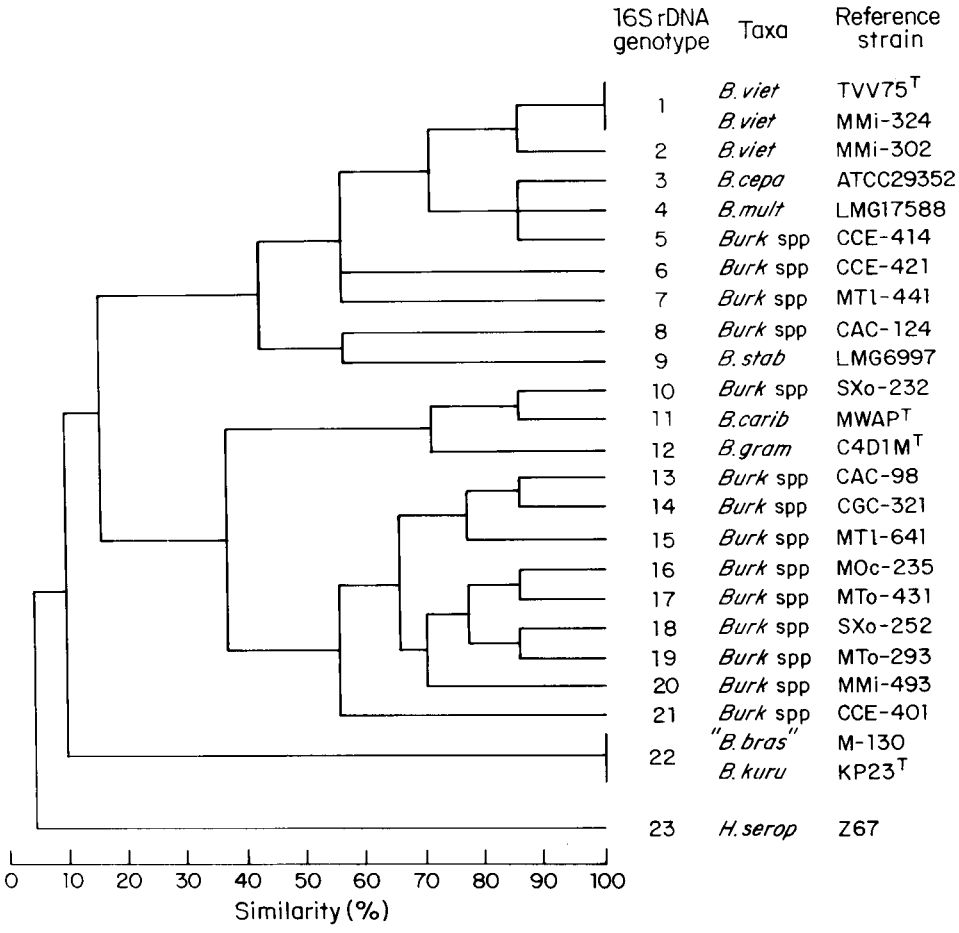

FIG. 3. Dendrogram of genetic relationships among N_2-fixing *Burkholderia* isolates recovered from maize, coffee, and sorghum plants and related species of *Burkholderia* based on ARDRA analysis. *Burk, Burkholderia; B. viet, B. vietnamiensis; B. cepa, B. cepacia; B. mult, B. multivorans; B. stab, B. stabilis; B. carib, B. caribensis; B. gram, B. graminis; B. kuru, B. kururiensis; "B. bras," "B. brasilensis"*; and *H. serop, Herbaspirillum seropedicae*. Isolates identified with a same 16S rDNA genotype, in addition to reference strain: genotype 1, MMi-334, MMi-353, MMi-1486, MMi-1537, MMi-1547, MMi-1556, MMi-1577, MMi-1776, SXo-702, CCE-101, CCE-115, CCE-201, CCE-211, CCE-303, and CCE-312 and reference strains TVV69 and TVV72; genotype 2, MMi-313 and MMi-344; genotype 13, CAC-142, CAC-369, and CAC-382; genotype 15, CAC-112, CGC-72, and CGC-316; genotype 16, MOc-255, MOc-725, and SMi-583; genotype 17, MMi-786, MTo-41, MTo-432, and MTo-452; genotype 18, CBN-516; CBN-523, CBN-721, and CBN-724; genotype 19, MTo-16; genotype 21, CBN-15, CBN-23, CBN-25, and CAC-92.

regions with a sustainable agriculture where fertilizer has not been used for generations. It is conceivable that the occurrence of *B. vietnamiensis* (4×10^6 CFU/g of rhizosphere soil and 4×10^4 CFU/g of fresh tissue of roots) might contribute to the growth of the maize plant and hence to crop production, as has been observed with field inoculation of rice with *B. vietnamiensis* TVV75^T (37, 38).

Although the ability to fix N_2 is not reported to be a common feature among the known species of the genus *Burkholderia*, we have found that this bacterial genus is very rich in diazotrophic species, as was demonstrated with ARA assays and confirmed with the presence of *nifHDK* genes. Our results confirm that diazotrophy is a common property among bacteria. However, the ability of some bacterial species to fix N_2 is unknown because this feature is not routinely evaluated when a new species is described (46). This is especially true for the *B. kururiensis* species. In this study, both the presence of *nifHDK* genes in strain KP23^T of *B. kururiensis* and the ARA assays revealed that this species is capable of fixing N_2 with selected carbon sources under microaerophilic conditions. It is known that many N_2-fixing bacterial species do not show such ability except under special growth conditions (46). Nevertheless, this possibility does not explain the erratic acetylene reduction activity observed with some isolates corresponding to the 16S rDNA genotypes 16, 17, 18, and 19. Further studies are required to resolve the observed discrepancies.

Interestingly, strains KP23^T of *B. kururiensis* and M-130 of "*B. brasilensis*" were capable of fixing N_2 in a similar manner, and both strains showed the same ARDRA profile. In addition, an analysis of the 16S rRNA sequences revealed 99.9% similarity between *B. kururiensis* KP23^T and *"B. brasilensis"* M-130 (data not shown), suggesting that both strains belong to the same species. This finding emphasizes the wide geographic and environmental distribution of the bacterial species. While *B. kururiensis* KP23^T was recovered from an aquifer polluted with trichlorethylene in Japan (47), the "*B. brasilensis*" M-130 strain was found to be plant associated in Brazil (V. L. D. Baldani, G. Kirchhof, V. M. Reis, E. de Oliveira, I. J. Baldani,

N. Springer, W. Ludwig, A. Hartmann, and J. Döbereiner, NCBI GenBank database, accession number AJ238360, 1999).

In this study, most of the N_2-fixing isolates analyzed showed phenotypic (colony morphology, N_2-fixing ability, and protein patterns) and genotypic (ARDRA and *nifHDK* profiles) features different from those of the known N_2-fixing species *B. vietnamiensis*, as well as from those of *B. kururiensis*, previously unknown to be a diazotrophic species. In addition, DNA-DNA reassociation assays confirmed the existence of N_2-fixing *Burkholderia* species different from *B. vietnamiensis*. Nevertheless, a polyphasic taxonomy analysis, as recommended by Vandamme et al. (40), is required for the validation of novel N_2-fixing *Burkholderia* species.

Among the bacteria associated with the rhizosphere and roots of maize plants, *B. cepacia* seems to be one of the predominant species (10, 27). Because of its abilities, *B. cepacia* is considered as a potential agricultural agent (5, 8, 9, 17, 25, 26, 35). However, despite the undoubted economic and ecological benefits of utilizing *B. cepacia* in agriculture, there exist diverse opinions on the use of this bacterial species (16, 18, 43) because of its importance as an opportunistic pathogen in nosocomial infections and in patients with cystic fibrosis. Taking into account this fact as well as the wide geographic distribution and the riches of N_2-fixing *Burkholderia* isolates associated with maize, coffee, and sorghum plants, we consider it important to assess the N_2-fixing *Burkholderia* genotypes distantly related to *B. cepacia*, both for their ability to promote plant growth and for their potential as biocontrol and bioremediation agents.

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