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Multichromosomal Genome Structure and Confirmation of Diazotrophy in Novel Plant-Associated *Burkholderia* Species[▽]

Lourdes Martínez-Aguilar,[1] Rafael Díaz,[1] Juan José Peña-Cabriales,[2] Paulina Estrada-de los Santos,[1] Michael F. Dunn,[1] and Jesús Caballero-Mellado[1]*

Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Ap. Postal No. 565-A, Cuernavaca, Morelos, México,[1] *and Centro de Investigación y de Estudios Avanzados, Km 9.6 Libramiento Norte Carr. Irapuato-León, Irapuato, Guanajuato, México*[2]

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Pulsed-field gel electrophoresis and 16S rRNA hybridization experiments showed that multichromosome genome structures and very large genome sizes (6.46 to 8.73 Mb) are prevalent in novel plant-associated *Burkholderia* species. $^{15}N_2$ isotope dilution assays revealed unambiguous diazotrophy in these novel species. *nifH* gene sequence analysis, often used to determine phylogenetic relatedness among diazotrophs, showed tight clusters of *Burkholderia* species, which were clearly distinct from those of other diazotrophs.

Multireplicon genomes occur in members of the alpha, beta, and gamma subdivisions of *Proteobacteria*, but most species in each subdivision contain only a single chromosome (4). Genome structure studies of *Betaproteobacteria* of the *Burkholderia* genus have been directed mainly to species included in the *Burkholderia cepacia* complex, opportunistic human pathogens having large genomes (6 to 9 Mb) consisting usually of two or three chromosomes (17). The more than 40 bacterial species presently classified as *Burkholderia* are ubiquitous in nature (10). Until recently, among *Burkholderia* species, only *B. vietnamiensis*, a member of the *B. cepacia* complex (10) that exists in the rhizospheres of several plants and inside of maize (11) and sugarcane (15), was recognized to participate in N_2 fixation (13). Recently, *B. kururiensis* (33) was identified as a diazotroph (11), and two N_2-fixing legume-nodulating strains (18) were formally classified as *B. phymatum* and *B. tuberum* (31). Novel diazotrophic *Burkholderia* species found among rhizospheric and endophytic isolates associated with important crop plants, including *B. unamae* (5), *B. xenovorans* (14), *B. tropica* (26), and *B. silvatlantica* (23), were recently described (5, 6, 14, 23, 26, 27). More recently, *B. mimosarum* and *B. nodosa* were formally described as legume-nodulating species (8, 9). All of the presumptively diazotrophic *Burkholderia* species, excluding *B. vietnamiensis*, comprise a group of closely related species that are phylogenetically distant from the *B. cepacia* complex (5, 6, 14, 23, 26), but their genomic organizations, genome sizes, and *nifH* gene sequences are unknown.

In this study, the aim was to determine if the novel plant-associated, presumptively N_2-fixing *Burkholderia* species contain large multireplicon genomes like those in *B. cepacia* complex species and to evaluate unambiguously their abilities to fix nitrogen using $^{15}N_2$ incorporation assays. In addition, the genomic localization patterns of *recA* and *nif* genes were resolved; *nifH* gene sequences were determined for many of the strains examined and used for phylogenetic analyses.

The strains of *Burkholderia* species analyzed are shown in Table 1. Growth conditions were as described by Perin et al. (23). For pulsed-field gel electrophoresis (PFGE), genomic DNA was prepared (19) and electrophoresis was performed with a CHEF DRIII system (Bio-Rad) using 1× Tris-acetate-EDTA buffer at 13.5°C. Replicons were separated using two sets of conditions: an 800-s pulse, an angle of 106°, and a voltage of 110 V for 72 h in 0.8% agarose for the separation of replicons of 0.9 to 3.5 Mb and an 1,800-s pulse, an angle of 106°, and a voltage of 110 V for 76 h in 0.7% agarose for replicons larger than 3.5 Mb. Replicons separated by PFGE were transferred (28) onto Hybond-N membranes (Amersham), ^{32}P-labeled probes were prepared using the Rediprime II random prime labeling system (Amersham), and the membranes were hybridized with a PCR product amplified with primers specific for each gene as described below. The 16S rRNA genes of most *Burkholderia* strains were localized by hybridizing membranes with a PCR product amplified with primers fD1 and rD1 (32) as described previously (11). 16S rRNA gene probes (ca. 1.5 kb) were PCR products from *B. unamae* MTl-641^T. PFGE profiles of representative *Burkholderia* species are shown in Fig. 1, and replicon numbers and sizes for all species analyzed are summarized in Table 1. The genomes of three strains each of *B. unamae* and *B. silvatlantica* contained four replicons, with average sizes ± standard deviations of 3.31 ± 0.06 Mb for the largest replicon and 1.11 ± 0.14 Mb for the smallest. The three strains of *B. tropica* each contained five replicons, and the replicons ranged in size from 3.24 ± 0.06 Mb for the largest to 0.53 ± 0.08 for the smallest. According to sequence data, *B. xenovorans* strains LB400^T and CAC-124 (7) have genomes containing three and two replicons, respectively, with total genome sizes larger than (LB400^T) or similar to (CAC-124) those of the *B. unamae*, *B. tropica*, and *B. silvatlantica* strains described above. Single strains of *B. vietnamiensis* and *B. sacchari* each displayed three

* Corresponding author. Mailing address: Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Ap. Postal 565-A, Cuernavaca, Morelos, México. Phone: (52 777) 329-17-03. Fax: (52 777) 317-55-81. E-mail: jesuscab@ccg.unam.mx.

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TABLE 1. Results from analyses of strains of *Burkholderia* species to determine genome structures; locations of 16S rRNA, *recA*, and *nifH* genes; and nitrogen-fixing abilities[a]

Species	Strain	Estimated size (Mbp) of replicon:					Genome size (Mbp)	Replicon location(s) of:			% Excess of ^{15}N	Amt (ng) of N fixed
		1	2	3	4	5		16S rRNA	*recA*	*nifH*		
B. unamae	MTl-641^T	3.34	1.81	1.39	1.15		7.69	1, 2, 3, 4	1	4	0.079	461 ± 4[b]
	SCCu-23	3.36	1.91	1.44	1.23		7.94	1, 2, 3, 4	1	4	0.307	1,187 ± 50[b]
	MCo-762	3.39	1.82	1.36	0.81		7.38	1, 2, 3, 4	1	4	0.289	52 ± 0[c]
B. tropica	Ppe8^T	3.21	1.86	1.64	1.37	0.650	8.73	1, 2, 4	1	5	0.319	742 ± 16[b]
	MTo-672	3.33	1.77	1.52	1.34	0.450	8.41	1, 2, 4	1	5	0.289	11 ± 0[c]
	MOc-725	3.19	1.75	1.50	1.33	0.500	8.27	1, 2, 4	1	5	0.032	299 ± 21[b]
B. silvatlantica	SRMrh-20^T	3.27	1.87	1.55	1.22		7.91	1, 2, 3, 4	1	1	0.301	9 ± 0[c]
	SRCL-318	3.27	1.84	1.61	1.10		7.82	1, 2, 3, 4	1	1	0.288	30 ± 0[c]
	PPCR-2	3.22	2.06	1.58	1.16		8.02	1, 2, 3, 4	1	1	0.286	24 ± 0[c]
B. xenovorans	LB400^T	4.90	3.36	1.47			9.73*	1, 2*	1*	2*	0.284	39 ± 0[c]
	CAC-124	4.47	3.36*				7.83*	1, 2*	1			
	CAC-124	4.39	3.32				7.71	1, 2	1	2	0.396	1,362 ± 123[b]
B. vietnamiensis	TVV75^T	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.267	77 ± 0[c]
	MMi-302	3.33	2.35	1.22			6.90	1, 2, 3	1	3	0.471	3,186 ± 342[b]
B. kururiensis	KP23^T	3.67	2.79				6.46	1, 2	1	2	0.291	24 ± 0[c]
	M130	3.67	2.97				6.64	1, 2	1	2	0.127	1,232 ± 43[b]
B. sacchari	LMG 19450^T	3.09	2.30	1.14			6.53	1, 2, 3	1	Absent	ND	ND

[a] ND, not detected or not determined; *, genome size data and data on the presence of genes are from reference 7.
[b] Result (mean ± standard deviation) obtained after incubation for 21 days.
[c] Result (mean ± standard deviation) obtained after incubation for 13 days.

replicons, while two strains of *B. kururiensis* each contained only two. The similarity in replicon size and number between *B. kururiensis* strains KP23^T and M130 strengthens our contention that the not yet officially described species "*B. brasilensis*" strain M130 mentioned in the literature (3) is instead a strain of the species *B. kururiensis* (6). Total genome sizes (6.46 to 6.90 Mb) for *B. vietnamiensis*, *B. kururiensis*, and *B. sacchari* were somewhat smaller than those for the *Burkholderia* species discussed above, and the percentages of the total genome size corresponding to the different replicons were essentially identical for the strains having the same numbers of replicons. For sequenced *Burkholderia* genomes that contain megaplasmids, these replicons accounted for less than 5% of the total genome size. In all of the sequenced *Burkholderia* genomes, the smallest chromosome accounts for a minimum of 10% of the genome. For the genomes analyzed in this study (Table 1), only

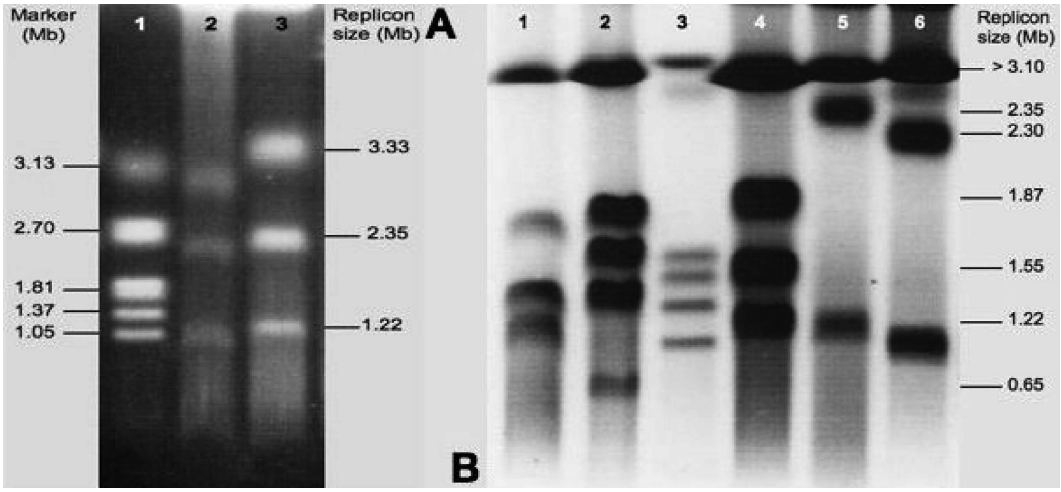

FIG. 1. PFGE analysis of undigested whole-genome DNA. (A) Results obtained using an 1,800-s pulse for 76 h in 0.7% agarose. Lanes: 1, *Hansenula wingei* (DNA marker); 2, *B. sacchari* LMG 19450^T; and 3, *B. vietnamiensis* MMi-302. (B) Results obtained using an 800-s for 72 h in 0.8% agarose. Lanes: 1, *B. unamae* MTl-641^T; 2, *B. tropica* Ppe8^T; 3, *H. wingei* (DNA marker); 4, *B. silvatlantica* SRMrh-20^T; 5, *B. vietnamiensis* MMi-302; and 6, *B. sacchari* LMG 19450^T.

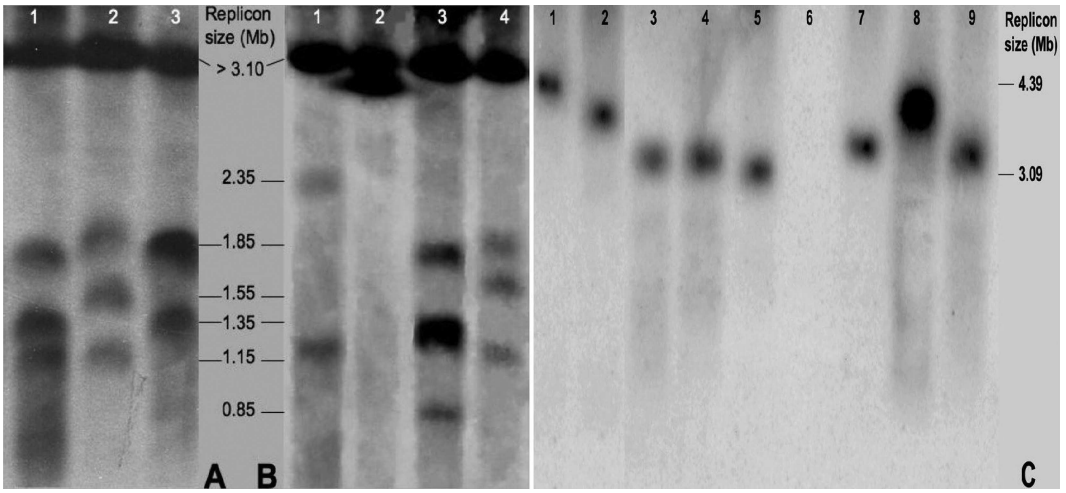

FIG. 2. Autoradiograms of a Southern blot of undigested whole-genome DNA hybridized with a 16S rRNA gene probe (replicons resolved by PFGE with an 800-s pulse for 72 h in 0.8% agarose) (A and B) and a *recA* gene probe (replicons resolved by PFGE with an 1,800-s pulse for 76 h in 0.7% agarose) (C). (A) Lanes: 1, *B. unamae* MTl-641^T; 2, *B. silvatlantica* SRMrh-20^T; and 3, *B. tropica* Ppe8^T. (B) Lanes: 1, *B. vietnamiensis* MMi-302; 2, *B. xenovorans* CAC-124; 3, *B. unamae* MCo-762; and 4, *B. silvatlantica* SRCL-318. (C) Lanes: 1, *B. xenovorans* CAC-124; 2, *B. kururiensis* KP23^T; 3, *B. vietnamiensis* MMi-302; 4, *B. tropica* Ppe8^T; 5, *B. sacchari* LMG 19450^T; 6, *Schizosaccharomyces pombe* (DNA marker); 7, *B. unamae* MTl-641^T; 8, *B. kururiensis* M130; and 9, *B. silvatlantica* SRMrh-20^T.

the smallest replicon encountered in the *B. tropica* strains accounted for less than 10% of the total genome size. Based on size, all of the replicons resolved in this study, except for the smallest from *B. tropica*, were probably chromosomes. The presence of 16S RNA genes is indicative of a replicon's being a chromosome rather than a megaplasmid (4). Southern hybridizations with 16S rRNA as a probe showed that the gene was present on all of the replicons from each species except for replicons 3 and 5 from *B. tropica* (Fig. 2A and B) and replicon 3 from *B. xenovorans* LB400^T (7).

Sequence analysis of the *recA* housekeeping gene, present in only one copy in the sequenced genomes, provides an important method for distinguishing between *B. cepacia* complex species (17). The *recA* gene (ca. 869 bp) of *B. tropica* Ppe8^T was amplified using the BUR1/BUR2 primers (21), and the PCR product was used as a probe to localize the *recA* genes of the *Burkholderia* strains. Consistent with genome sequence data for different *Burkholderia* species (www.ncbi.nlm.nih.gov/genomes/lproks.cgi), in which *recA* is always carried on a chromosome (usually the largest one), we found *recA* present only on the largest replicon of each species analyzed in this study (Table 1; Fig. 2C). On this basis, *recA* genes may also be useful for differentiating between the novel *Burkholderia* species analyzed in this work, in addition to 16S rRNA sequences and other features used in their taxonomic identification.

The *nifH* genes of *Burkholderia* strains were PCR amplified with primers IGK (24) and NDR-1 (30) by using mixtures and conditions similar to those described previously (30). *nif* genes

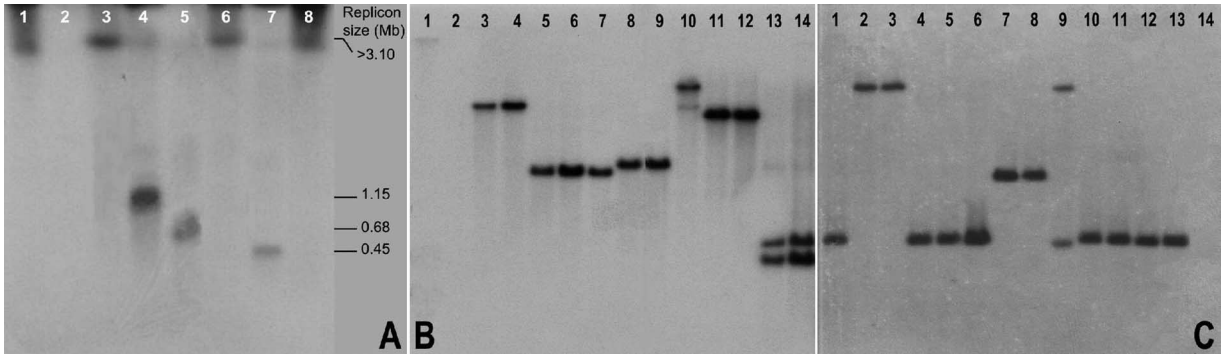

FIG. 3. (A) Autoradiogram of a Southern blot of undigested whole-genome DNA (replicons resolved by PFGE with an 800-s pulse for 72 h in 0.8% agarose) hybridized with a *nifH* gene probe. Lanes: 1, *B. kururiensis* KP23^T; 2, *B. sacchari* LMG 19450^T; 3, *B. silvatlantica* SRMrh-20^T; 4, *B. unamae* MTl-641^T; 5, *B. tropica* Ppe8^T; 6, *B. silvatlantica* SRCL-318; 7, *B. tropica* MTo-672; and 8, *B. xenovorans* CAC-124. (B and C) Autoradiograms of a Southern blot of the total EcoRI DNA fingerprints hybridized with a *nifH* gene probe (B) and with a *recA* gene probe (C). (B) Lanes: 1, DNA marker 1 kb Plus; 2, *B. sacchari* IPT-101^T; 3 and 4, *B. unamae* SCCu-23 and MTl-641^T; 5, 6, and 7, *B. tropica* Ppe8^T, MOc-725, and MTo-672; 8 and 9, *B. silvatlantica* SRMrh-20^T and SRCL-318; 10, *B. vietnamiensis* MMi-302; 11 and 12, *B. kururiensis* KP23^T and M130; and 13 and 14, *B. xenovorans* CAC-124 and LB400^T. (C) Lanes: 1, *B. sacchari* LMG 19450^T; 2 and 3, *B. unamae* SCCu-23 and MTl-641^T; 4, 5, and 6, *B. tropica* Ppe8^T, MOc-725, and MTo-672; 7 and 8, *B. silvatlantica* SRMrh-20^T and SRCL-318; 9, *B. vietnamiensis* MMi-302; 10 and 11, *B. kururiensis* KP23^T and M130; 12 and 13, *B. xenovorans* CAC-124 and LB400^T; and 14, DNA marker 1 kb Plus.

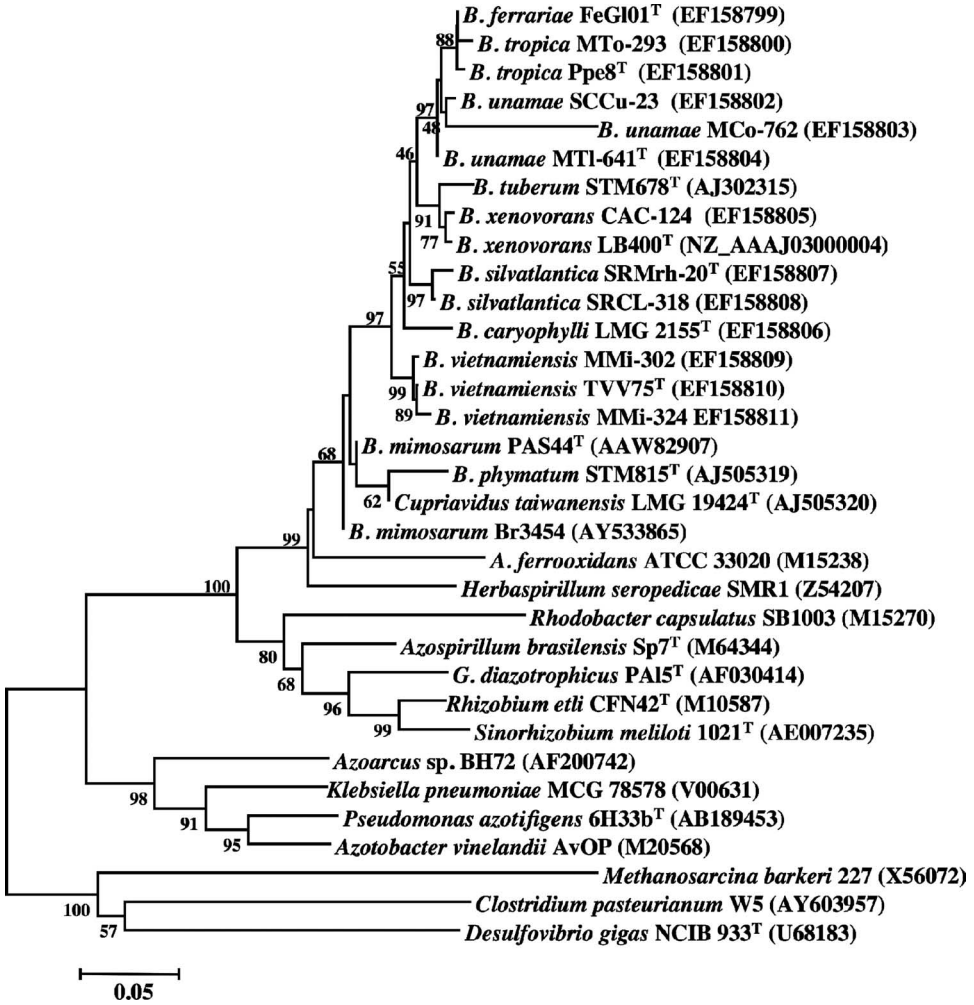

FIG. 4. Phylogenetic inference for nitrogen-fixing *Burkholderia* species and other diazotrophic bacteria, based on NifH amino acid sequences. The analysis included 205 sites. A bootstrapping analysis was used to test the statistical reliability of tree branch points; 1,000 bootstrap samplings were performed. The NCBI GenBank accession numbers for the *nifH* gene sequences are indicated in parentheses.

were localized by hybridizing membranes with *nifH* gene probes (1:1 mixtures of PCR products) from *B. unamae* MTl-641^T and *B. xenovorans* LB400^T. For analysis, total DNA was digested with EcoRI, electrophoresed, blotted, and hybridized under the conditions described previously (11). The conditions for the cloning and sequence analysis of *nifH* genes were the same as those described previously (30). A new primer pair to amplify the second complementary strand of the *nif* genes was designed; the forward primer nifH-IF (5′-GGCSATGTACGC GGCSAAC-3′) and the reverse primer nifH-IR (5′-GTTSGC CGCGTACATSGCC-3′) corresponded to *B. unamae* MTl-641^T at positions 442 and 460, respectively. PCR products (1.2 kb) amplified with primers IGK and NDR-1 were used as templates in PCR assays using the primer sets NDR-1/nifH-IF and IGK/nifH-IR under the PCR conditions described above. The products amplified with primers IGK/nifH-IR (450 bp) and NDR-1/nifH-IF (750 bp) were cloned into pCR2.1 (Invitrogen) and sequenced. For the phylogenetic analysis, NifH protein sequences were retrieved from the NCBI database. Deduced amino acid sequences were aligned using Clustal W (29), and neighbor-joining phylogenies were constructed with MEGA 3.1 (16) by using pairwise deletion of gaps and missing data. For all of the *B. unamae* and *B. tropica* strains, *nifH* hybridized only with the smallest replicon, while for all of the *B. silvatlantica* strains, it hybridized only with the largest (Table 1; Fig. 3A). For *B. xenovorans* strains, *nifH* hybridized only with the second largest replicon. Southern blots of total EcoRI-digested DNA hybridized with either a *nifH* or *recA* probe showed a single hybridization band for all of the *Burkholderia* species analyzed, with the exception of the *B. xenovorans* strains, which contain an EcoRI site in their *nifH* gene sequences (Fig. 3B), and *B. vietnamiensis* MMi-302, which apparently contains an EcoRI site in its *recA* sequence (Fig. 3C), as occurs in the *recA* gene on chromosome 1 of *B. vietnamiensis* G4 (GenBank accession no. NC_009256). These results confirm that *nifH* and *recA* genes are present in only one copy in these genomes.

The majority of the available *nifH* gene sequences of diazotrophic *Burkholderia* species were obtained in this study. *nifH* gene phylogenies are often used to determine relatedness among diazotrophic bacteria (25). All of the *Burkholderia* species analyzed (Table 1) formed a tight cluster with other plant-

associated *Burkholderia* species and *Cupriavidus taiwanensis* LMG 19424^T (Fig. 4). This cluster was well separated from others containing symbiotic or free-living diazotrophs from the alpha, gamma, and delta subdivisions of the *Proteobacteria*, as well as members of the *Archaea* and *Firmicutes*. However, it is noteworthy that the legume-nodulating *Burkholderia* species, except *B. tuberum*, formed a single cluster that included *Cupriavidus taiwanensis*, a diazotroph nodulating *Mimosa* species that is closely related to the genus *Burkholderia* and whose *nifH* gene may be derived from a *Burkholderia* ancestor (1).

Previous analyses of the nitrogen-fixing abilities of *Burkholderia* species have been based on acetylene reduction activity and/or the presence of *nifH* genes (5, 11, 13, 14, 23, 26). Since relating acetylene reduction activity to the actual quantity of nitrogen fixed is prone to error (12) and the presence of *nifH*, encoding nitrogenase reductase (a key enzyme in the process of nitrogen fixation), only implies the ability of an organism to fix nitrogen, in this study $^{15}N_2$ isotopic dilution experiments were developed to unequivocally confirm diazotrophy. For these experiments, cells grown in BAz liquid medium (11), lacking azelaic acid and containing succinic acid (5 g/liter) and yeast extract (0.5 g/liter), were harvested by centrifugation and washed three times with sterile deionized water. Inocula were standardized using the Bradford method. Each suspension was combined with $^{15}N_2$ (Isotec; 99% $^{15}N_2$ atom excess) and sterile air in a Vacutainer tube to give an isotopic dilution equivalent to a 74.25% $^{15}N_2$ atom excess. The cultures were incubated for 13 or 21 days at 28°C. The percentage of ^{15}N atom excess was determined by emission spectrometry (2) using an atomic absorption spectrophotometer (model NOI-6E; Fischer Analysen Instrumente, Leipzig, Germany). This is the first time that diazotrophy in *Burkholderia* has been demonstrated using $^{15}N_2$, quantitatively confirming the N_2-fixing abilities of all of the *Burkholderia* strains analyzed (Table 1), as well as that of the legume-nodulating *B. tuberum* STM678^T (data not shown). No assay detected nitrogen fixation in *B. sacchari*, which lacks *nifH*.

We have shown here that novel N_2-fixing, plant-associated *Burkholderia* species, as well as the closely related nondiazotrophic species *B. sacchari*, possess two to five large replicons, usually chromosomes. This finding extends the observation that large (usually >7.0-Mb) multichromosomal genomes are a characteristic feature of the genus *Burkholderia*. Although the selective advantage conferred by multireplicons is not clear, it has been proposed previously that in *B. cepacia* complex and *Vibrio* species the presence of multichromosomal genomes is essential and advantageous for the fitness of the species in particular environments (17, 20). The large genomes and chromosomal multiplicity in the novel diazotrophic *Burkholderia* species analyzed in this study may explain their great abilities to colonize the rhizospheres and endophytic environments of a wide range of host plants (5, 6, 13, 22, 23, 26), which along with their metabolic versatility in using volatile compounds (6, 33), as well as their ability to fix nitrogen, produce siderophores, and solubilize insoluble phosphates (6), are probably significant for the survival and success of *Burkholderia* species in different environments.

Nucleotide sequence accession numbers. The *nifH* gene sequences determined in this study were deposited in GenBank under accession numbers EF158799 through EF158811.

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