

The Tomato Rhizosphere, an Environment Rich in Nitrogen-Fixing *Burkholderia* Species with Capabilities of Interest for Agriculture and Bioremediation[∇]

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Burkholderia strains are promising candidates for biotechnological applications. Unfortunately, most of these strains belong to species of the *Burkholderia cepacia* complex (Bcc) involved in human infections, hampering potential applications. Novel diazotrophic *Burkholderia* species, phylogenetically distant from the Bcc species, have been discovered recently, but their environmental distribution and relevant features for agro-biotechnological applications are little known. In this work, the occurrence of N₂-fixing *Burkholderia* species in the rhizospheres and rhizoplanes of tomato plants field grown in Mexico was assessed. The results revealed a high level of diversity of diazotrophic *Burkholderia* species, including *B. unamae*, *B. xenovorans*, *B. tropica*, and two other unknown species, one of them phylogenetically closely related to *B. kururiensis*. These N₂-fixing *Burkholderia* species exhibited activities involved in bioremediation, plant growth promotion, or biological control in vitro. Remarkably, *B. unamae* and *B. kururiensis* grew with aromatic compounds (phenol and benzene) as carbon sources, and the presence of aromatic oxygenase genes was confirmed in both species. The rhizospheric and endophyte nature of *B. unamae* and its ability to degrade aromatic compounds suggest that it could be used in rhizoremediation and for improvement of phytoremediation. *B. kururiensis* and other *Burkholderia* sp. strains grew with toluene. *B. unamae* and *B. xenovorans* exhibited ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity, and the occurrence of *acdS* genes encoding ACC deaminase was confirmed. Mineral phosphate solubilization through organic acid production appears to be the mechanism used by most diazotrophic *Burkholderia* species, but in *B. tropica*, there presumably exists an additional unknown mechanism. Most of the diazotrophic *Burkholderia* species produced hydroxamate-type siderophores. Certainly, the N₂-fixing *Burkholderia* species associated with plants have great potential for agro-biotechnological applications.

It is well known that hundreds of thousands of bacterial species remain to be discovered and cultured, representing a substantial reservoir of genetic diversity and great potential for biotechnological applications. Although most of the bacteria inhabiting common environments (e.g., agricultural soils and plants) have not yet been grown in culture, many of them could be cultivated using standard methods. However, for many environments, research on microbial taxonomy and ecology is lacking. Unfortunately, novel bacterial species are often described based on the analysis of a very limited set of isolates (59), commonly one to three. This is true for many bacterial species, including several belonging to the genus *Burkholderia*. For example, the species *B. kururiensis* (80), *B. sacchari* (9), *B. phenoliruptrix* (16), *B. terrae* (79), *B. tuberum*, and *B. phymatum* (73) were recently described on the basis of a single isolate analyzed, and consequently, their environmental distribution and ecological role are unknown. *B. kururiensis* and *B. sacchari* were described as species with abilities to degrade trichloroethylene and to biotechnologically produce polyhydroxyalkanoic acids, respectively, but new studies related to their ecologies or applications are largely lacking. The nitrogen-

fixing species *B. xenovorans* was described on the basis of three isolates (32); strain LB400^T was isolated from polychlorinated biphenyl (PCB)-contaminated soil in Moreau, NY, strain CAC-124 was isolated from the rhizosphere of a coffee plant cultivated in Veracruz, Mexico, and strain CCUG 28445 was recovered from a blood culture in Sweden. Although strain LB400^T is the best-studied PCB degrader, and its pathways for degradation of these compounds have been extensively characterized at the genetic and molecular levels (25, 35), strains CAC-124 and CCUG 28445 have been only partially analyzed and do not share the biphenyl-biodegrading capacities of strain LB400^T (32). Recently, one *B. xenovorans* isolate was recovered from the rhizosphere of maize cultivated in The Netherlands (62). Although the complete genome of *B. xenovorans* LB400^T was recently sequenced (12), it is noteworthy that the four extant *B. xenovorans* strains described in diverse studies have been randomly recovered from different environments and widely distant geographical regions, and there are no studies on the distribution of this PCB-degrading, nitrogen-fixing species or its association with plants. Emphasis has been given to studies of the isolation, taxonomy, and distribution of *Burkholderia* species related to human opportunistic pathogens, especially the *B. cepacia* complex species found in cystic fibrosis patients (33, 45, 52; for reviews, see references 15 and 42). In contrast, few studies have been performed on the overall diversity of the genus *Burkholderia* (61, 63), even though non-pathogenic *Burkholderia* species are frequently recovered from

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TABLE 1. Diazotrophic *Burkholderia* species isolated from the rhizospheres of Saladet variety tomato plants cultivated in different geographical regions of Mexico

| Locality | Species | Strain | Source |
|-----------------------------|-------------------------------|-----------|-------------|
| Coatlán del Río, Morelos | <i>B. xenovorans</i> | TCo-26 | Rhizoplane |
| Coatlán del Río, Morelos | <i>B. xenovorans</i> | TCo-213 | Rhizosphere |
| Coatlán del Río, Morelos | <i>B. xenovorans</i> | TCo-39 | Rhizosphere |
| Coatlán del Río, Morelos | <i>B. xenovorans</i> | TCo-382 | Rhizosphere |
| Atlatlahuacan, Morelos | <i>B. unamae</i> | TAtl-371 | Rhizosphere |
| Atlatlahuacan, Morelos | <i>B. unamae</i> | TAtl-3711 | Rhizoplane |
| Atlatlahuacan, Morelos | <i>B. unamae</i> | TAtl-3742 | Rhizosphere |
| Nepantla, State of Mexico | <i>B. unamae</i> | TNe-832 | Rhizosphere |
| Nepantla, State of Mexico | <i>B. unamae</i> | TNe-873 | Rhizosphere |
| Santa Inés, State of Mexico | <i>B. unamae</i> | TSi-883 | Rhizosphere |
| Tetecala, Morelos | <i>B. unamae</i> | TTe-829 | Rhizosphere |
| Nepantla, State of Mexico | <i>B. tropica</i> | TNe-865 | Rhizoplane |
| Nepantla, State of Mexico | <i>B. tropica</i> | TNe-831 | Rhizosphere |
| Santa Inés, State of Mexico | <i>B. tropica</i> | TSi-882 | Rhizoplane |
| Santa Inés, State of Mexico | <i>B. tropica</i> | TSi-887 | Rhizoplane |
| Santa Inés, State of Mexico | <i>B. tropica</i> | TSi-888 | Rhizoplane |
| Tetecala, Morelos | <i>B. tropica</i> | TTe-791 | Rhizosphere |
| Tetecala, Morelos | <i>B. tropica</i> | TTe-797 | Rhizosphere |
| Nepantla, State of Mexico | <i>Burkholderia</i> sp. (Bkr) | TNe-834 | Rhizosphere |
| Nepantla, State of Mexico | <i>Burkholderia</i> sp. (Bkr) | TNe-841 | Rhizosphere |
| Nepantla, State of Mexico | <i>Burkholderia</i> sp. (Bkr) | TNe-8641 | Rhizoplane |
| Nepantla, State of Mexico | <i>Burkholderia</i> sp. (Bkr) | TNe-8682 | Rhizosphere |
| Nepantla, State of Mexico | <i>Burkholderia</i> sp. (Bkr) | TNe-878 | Rhizoplane |
| Nepantla, State of Mexico | <i>Burkholderia</i> sp. | TNe-861 | Rhizoplane |
| Nepantla, State of Mexico | <i>Burkholderia</i> sp. | TNe-862 | Rhizoplane |

different environments (6, 40, 70), and despite their biotechnological potential in bioremediation and other applications (34, 70; for a review, see reference 48). Knowledge of novel diazotrophic *Burkholderia* species (11, 32, 50, 54), including legume nodule symbionts (14, 73), phylogenetically greatly distant from the *B. cepacia* complex species, has come very recently, but their environmental distribution and relevant features for agronomic and environmental applications are little known (13, 27, 32, 49).

Bacteria are involved in degradation processes of many aromatic compounds released into the environment by the decay of plant material or by anthropogenic activity. Phenolic compounds and polymers containing benzene rings (e.g., lignins) are natural aromatic compounds (21, 29). However, phenol is a man-made aromatic compound and along with its derivatives is considered a major hazardous compound in industrial wastewater. Similarly, aromatic hydrocarbons like benzene and toluene are common pollutants of soil and groundwater (78). Soil microorganisms are capable of using aromatic compounds as sole carbon sources, owing to aerobic biodegradation catalyzed by mono- or dioxygenases (3, 78). In the last few years, rhizoremediation (microbial degradation of hazardous compounds in the rhizosphere) and phytoremediation (the use of plants to extract and degrade harmful substances) have been considered alternatives for decontamination of soils. In addition, bacteria are able to exert positive effects on plants through various mechanisms. For instance, nitrogen fixation (the natural transformation of atmospheric N₂ to ammonia) contributes organic nitrogen for plant growth (28), while the bacterial enzyme 1-amino-cyclopropane-1-carboxylate (ACC) deaminase hydrolyzes ACC (the immediate precursor of ethylene) and lowers the levels of ethylene produced in developing or stressed plants, promoting root elongation (30). Some

bacteria solubilize insoluble minerals through the production of acids, increasing the availability of phosphorus and other nutrients to plants in deficient soils (55). Several bacteria improve plant growth through suppression of pathogens by competing for nutrients, by antibiosis, or by synthesizing siderophores, which can solubilize and chelate iron from the soil and inhibit the growth of phytopathogenic microorganisms (23).

This work was aimed at revealing the occurrence of nitrogen-fixing *Burkholderia* species associated with tomato (*Lycopersicon esculentum*) plants cultivated in different locations in Mexico. We found that the rhizosphere of tomato is a reservoir of different known and unknown diazotrophic *Burkholderia* species that are able to exhibit in vitro some activities involved in bioremediation, plant growth promotion, and biological control.

MATERIALS AND METHODS

Crop and locations. Saladet variety tomato (*Lycopersicon esculentum*) plants were collected in Atlatlahuacan and Tetecala (two collections from different farms), Morelos, and Nepantla and Santa Inés, State of Mexico, Mexico (Table 1).

Tomato plant samples. Eight to 10 complete flowering plants, field grown 20 m apart in each region, were randomly collected. Care was taken to keep the rhizosphere intact around the root. Samples of the rhizospheres and rhizoplanes (root surfaces) of the tomato plants were analyzed for recovery of the N₂-fixing isolates 4 to 5 hours after collection.

Media, culture conditions, and diazotrophic *Burkholderia* isolation. Rhizosphere and plant samples were treated as described previously (27). Purified colonies were assayed for nitrogenase activity by the acetylene reduction activity (ARA) method (10) with vials containing 5 ml of N-free semisolid *Burkholderia* malate-glucose-mannitol (BMGM) medium (27). ARA-positive colonies were maintained in 20% glycerol at -80°C prior to characterization.

Total DNA isolation and 16S rRNA-specific primers. Genomic DNA was isolated from bacterial cells by using published protocols (2). ARA-positive isolates were presumptively assigned to the genera *Burkholderia* and *Ralstonia* by

amplifying the 16S rRNA gene with the specific primers BuRa-16-1 and BuRa-16-2, using PCR conditions described previously (5). In addition, a new specific primer pair was designed in order to get a PCR-amplified product larger than the 409-bp amplicon obtained with primers BuRa-16-1/BuRa-16-2. *Burkholderia-Ralstonia* 16S rRNA genes were amplified using the PCR primer GB-F (5'-AG TAATACATCGGAACRTGT-3'), described previously (49), and a primer named GB-R (5'-GGSTTGGCRACCTCTGTT-3'), designed in the present study. The specificity of the GB-F/GB-R primer pair was tested with most (31 out of 40) of the well-known *Burkholderia* species as well as with *Ralstonia pickettii* and *Ralstonia solanacearum* strains. The PCR mixtures contained 20 ng of genomic DNA, 1.5 mM MgCl₂, a 250 μM concentration of the 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 denaturation for 30 s at 94°C, annealing for 45 s at 58°C, and elongation for 1 min at 72°C, followed by a final 5-min elongation at 72°C. The reaction amplified a 1,100-bp fragment.

ARA-positive isolates were confirmed as belonging to the genus *Burkholderia* by amplifying the 16S rRNA genes with the GB-F/GBN2-R-specific primer pair, using PCR conditions described previously (49).

ARDRA. Primers fD1 and rD1 were used for amplifying the 16S rRNA gene (77), using PCR conditions described previously (27). The PCR-amplified 16S rRNA genes (ca. 1.5 kb) were restricted with seven enzymes (AluI, DdeI, HaeIII, HhaI, HinfI, MspI, and RsaI), and the restriction fragments were separated as described previously (27). The restriction patterns were compared, and 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 (27). Similarities among the 16S rRNA gene sequences were estimated from the proportions of shared restriction fragments by using the method of Nei and Li (47). A dendrogram was constructed from the resulting distance matrix by using the unweighted pair group method with averages (67).

16S rRNA gene sequencing and phylogenetic analysis. Representative acetylene-reducing strains corresponding to each ARDRA genotype identified among isolates recovered from tomato plants were chosen for 16S rRNA gene sequencing. To obtain 16S rRNA sequences, PCR products were cloned as described previously (49), and the 16S rRNA gene sequences were determined at the Biotechnology Institute, UNAM (Mexico). The 16S rRNA gene sequences were deposited in the EMBL/GenBank database. These sequences were compared with previously published 16S rRNA gene sequences from *Burkholderia* species and related bacteria, such as *Ralstonia* and *Pandorea*. The multiple alignments of the sequences were performed with CLUSTAL W software (69). The tree topology was inferred by the neighbor-joining method (60), based on 1,310 DNA sites, and distance matrix analyses were performed according to Jukes and Cantor (38), using the program MEGA version 2.1 (41).

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 previously (27).

PCR amplification of *nifH* genes. Primers IGK (51) and NDR-1 (71) were used for the amplification of the *nifH* genes, using PCR conditions described previously (49). 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 (71).

Growth on aromatic compounds. Nitrogen-free semisolid BAZ mineral medium (composition in grams/liter: azelaic acid, 2.0; K₂HPO₄, 0.4; KH₂PO₄, 0.4; MgSO₄ · 7H₂O, 0.2; CaCl₂, 0.02; Na₂MoO₄ · H₂O, 0.002; FeCl₃, 0.01; bromothymol blue, 0.075), usually used for enrichment of nitrogen-fixing *Burkholderia* species (11, 27, 49, 54), was modified for testing bacterial growth on aromatic compounds. The mineral medium was supplemented with ammonium sulfate (0.05%), the azelaic acid was omitted, and aromatic compounds were included as sole carbon sources; this medium was named SAAC (salts-ammonium-aromatic compound). For growth in agar (1.8% [wt/vol]) plates, 150-μl volumes of the volatile aromatic compounds benzene, isopropylbenzene (cumene), and toluene were independently added on filter paper circles (6-cm diameters) placed in the lids of petri dishes, which were sealed with polyethylene tape and incubated upside down within closed polypropylene containers at 29°C. Naphthalene and biphenyl were tested similarly, but using 50 mg of crystals. Phenol (0.05%) was directly added to the SAAC mineral medium. SAAC medium without a carbon source was used as a negative control for bacterial growth; succinic acid (0.2%) as a carbon source was a positive control. The isolates were grown in BSE liquid medium (27) for 18 h at 29°C with reciprocal shaking (250 rpm). The cultures were harvested and the pellets washed with 10 mM MgSO₄ · 7H₂O and adjusted to an optical density (OD) of 0.25 at 600 nm (approximately 2 × 10⁸ cells per ml). Aliquots of the cultures were inoculated with a multipoint replicator on SAAC

medium plates containing the aromatic compounds described above and incubated for 4 to 10 days.

PCR amplification and sequencing of aromatic oxygenase genes. All of the isolates that were capable of growing with aromatic compounds as sole carbon sources, as well as type and reference strains of well-known diazotrophic *Burkholderia* species, were analyzed for the presence of di- and monooxygenase genes. Toluene/biphenyl dioxygenase genes were PCR amplified with the bphA668-3/bphAr1153-2 primers, using conditions described previously (78). Primers RDEG-F/RDEG-R and RMO-F/RMO-R were used for amplification of the sequences of the large subunits of toluene monooxygenases and primers PHE-F/PHE-R for amplification of phenol monooxygenases, using the PCR conditions described by Baldwin et al. (3). The reactions amplify a 466-bp fragment with both the RDEG and the RMO primer pairs and a 206-bp product with the PHE primer pair. PCR products from one or two strains from each species were cloned into the pCR2.1 vector according to the manufacturer's instructions (Invitrogen), and the aromatic oxygenase gene sequences were determined at the Biotechnology Institute, UNAM (Mexico). These gene sequences were deposited in the EMBL/GenBank database and compared with previously published sequences.

PCR amplification of *acdS* (encoding ACC deaminase) genes. Putative ACC deaminase gene sequences annotated (NCBI GenBank database) in the complete genomes of *B. xenovorans* LB400^T (accession number NC_007952), *B. vietnamiensis* G4 (NZ_AAEH000000000), *Burkholderia* sp. strain 383 (NC_007511), *B. mallei* ATCC 23344 (NC_006349), *B. pseudomallei* K96243 (NC_006351), and *Pseudomonas* sp. strain ACP (M73488) were aligned, and primers with minimal degeneracies (5' ACC [5'ACGCCGATCCARCCGCTM 3'] and 3' ACC [5'TCCAGCGTGCSTCGTTC3']) were designed for PCR amplification of *acdS* genes. The PCR mixture was composed of 20 ng of genomic DNA, 1.5 mM MgCl₂, 25 μM of each deoxynucleoside triphosphate, 5 pmol of each primer, and 1.0 U of *Taq* polymerase. PCR conditions were as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation for 45 s at 95°C, annealing for 45 s at 67°C, and elongation for 1 min at 72°C, followed by a final 5-min elongation at 72°C. The reaction amplified a 785-bp fragment.

ACC deaminase activity assay. The isolates were grown in BSE liquid medium (27) for 18 h at 29°C with reciprocal shaking (250 rpm). The cultures were harvested and the pellets washed twice with 0.1 M phosphate buffer (pH 7.5). The pellets were resuspended in 20 ml salts medium (composition in grams/liter: succinic acid, 2.0; K₂HPO₄, 1.0; KH₂PO₄, 1.0; MgSO₄ · 7H₂O, 0.5; CaCl₂, 0.13; FeSO₄, 0.013) supplemented with either 3.0 mM ACC or 3.0 mM NH₄Cl, pH, 5.7, and then the cultures were grown as described above. The cells were collected and the pellets washed twice with 0.1 M phosphate buffer (pH 7.5) before being resuspended in 1 ml 0.1 M Tris-HCl (pH 8.5) (65) and ruptured using a French press at 900 lb/in². Crude extracts were harvested by centrifugation, and the ACC deaminase activity was measured by following the production of α-ketobutyrate as described by Honma and Shimomura (37). Total protein content in extracts was determined using the method described by Bradford (8).

Phosphate solubilization. Isolates were tested using NBRIP medium agar plates containing insoluble tricalcium phosphate [Ca₃(PO₄)₂] as the sole phosphorus source (46). Phosphate solubilization assays were carried out with NBRIP medium strongly buffered with MES (morpholineethanesulfonic acid) buffer (4.4 g/liter) and on unbuffered medium. The isolates were grown in iron-restricted Casamino Acids (CAA) liquid medium (44) supplemented with succinic acid (3 g/liter) for 18 h at 29°C. The cultures were harvested and the pellets washed with 10 mM MgSO₄ · 7H₂O and adjusted to an OD of 0.25 at 600 nm. Aliquots of the cultures were inoculated on NBRIP agar plates with a multipoint replicator. The solubilization haloes around colonies and colony diameters were measured after 3, 5, and 7 days of incubation at 29°C. Halo size was determined by subtracting the colony diameter from the total diameter.

Siderophore production. The method used to detect siderophores was adapted from the universal chemical assay on chrome azurol S (CAS) agar plates (64). The time-consuming and laborious deferrization process of solutions was omitted, and piperazine was not added; MM9 growth medium was replaced by CAA medium supplemented with succinic acid (3 g/liter). The isolates were grown in CAA liquid medium for 18 h at 29°C with reciprocal shaking (250 rpm). The cultures were harvested and the pellets washed and adjusted to an OD of 0.25 as described for the phosphate solubilization assays. Aliquots of the cultures were inoculated with a multipoint replicator on modified CAS medium (CAS-CAA) and then incubated for 72 h at 29°C. Orange haloes that formed around the colonies on blue agar were considered indicative of siderophore production. Halo size was determined by subtracting the colony diameter from the total diameter. Similarly, the isolates were grown in CAA liquid medium and hydroxamate-type siderophores were identified using the Czáký test (19), after hydro-

lysis with 3 N sulfuric acid at 120°C for 30 min (53), and buffered with 3 ml of 35% sodium acetate (19). Catechol-type siderophores were identified using the Arnow test (1).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences were deposited in the EMBL/GenBank database under accession numbers EF139178, EF139179, EF139180, and EF139181 for *B. unamae* strains TNe-873, TATl-3742, TSi-883, and TATl-371, respectively; the accession numbers for *B. tropica* strains TNe-865 and TSi-887 were EF139182 and EF139183, respectively; *Burkholderia* sp. strains TNe-862, TNe-878, and TNe-841 were deposited under accession numbers EF139184, EF139185, and EF139186, respectively; and the accession numbers for *B. xenovorans* strains TCo-382 and TCo-26 were EF139187 and EF139188, respectively. The phenol hydroxylase (aromatic oxygenase) gene sequences were deposited in the EMBL/GenBank database under accession numbers EF151008, EF151009, EF151010, and EF158449 for *Burkholderia* sp. strain TNe-862, *B. unamae* strains TSi-883 and MTI-641^T, and "*B. brasiliensis*" M130, respectively. Toluene-3-monooxygenase gene sequences were deposited under accession numbers EF151011, EF151012, and EF151013 for "*B. brasiliensis*" M130, *B. kururiensis* KP23^T, and *Burkholderia* sp. strain TNe-862, respectively. The *acdS* gene sequences were deposited in the GenBank database under accession numbers EF408192 and EF408193 for *B. xenovorans* strains TCo-26 and TCo-382, respectively, and EF408194 for *B. unamae* TATl-3742.

RESULTS

Isolation. Enrichment cultures for N₂-fixing *Burkholderia* were made in N-free semisolid BAz medium, followed by further isolation and colony purification on BAc agar plates (27). Screening of 420 colonies allowed the recovery of a total of 54 isolates that showed consistent nitrogenase activity as measured by the ARA method. Although it is possible that a few isolates did not show ARA, due to suboptimal growth conditions or loss of plasmids, ARA-negative colonies were discarded, and no attempt was made to determine their taxonomic positions.

16S rRNA-specific primers. ARA-positive isolates gave PCR-amplified products of the correct size (409 bp) with primers BuRa-16-1/BuRa-16-2, confirming their taxonomic statuses as members of the genera *Burkholderia*-*Ralstonia*. In addition, use of the specific primer pair GB-F/GB-R (designed in the present study to get a PCR-amplified product of around 1,100 bp) confirmed that the ARA-positive isolates belong to the genera *Burkholderia*-*Ralstonia*. The specificity of the GB-F/GB-R primer pair was confirmed with all of the 31 type strains of the known *Burkholderia* species tested and of *Ralstonia pickettii* and *Ralstonia solanacearum* (data not shown). All of the 54 ARA-positive isolates were confirmed as belonging to the genus *Burkholderia* by amplifying the 16S rRNA genes with the GB-F/GBN2-R specific primer pair, described previously (49).

ARDRA. A total of eight ARDRA profiles were identified from among the 54 acetylene-reducing *Burkholderia* isolates recovered from the rhizosphere and rhizoplane of tomato (data not shown). One ARDRA profile identified among 11 acetylene-reducing isolates (e.g., TCo-26 and TCo-382) differed slightly from the profile of *B. xenovorans* LMG 16224. Three ARDRA genotypes identified among 11 isolates were identical to genotypes 16 (1 isolate), 17 (3 isolates), and 19 (7 isolates), described previously as belonging to the species *B. tropica* (54). Two ARDRA genotypes (14 isolates) were slightly different from that of strain MTI-641^T and from other genotypes of *B. unamae* described previously (11). One ARDRA profile similar to that of *B. kururiensis* KP23^T was identified among 12 ARA-positive isolates (e.g., TNe-841 and TNe-878), hereafter referred to in the text as *B. kururiensis*-resembling

(Bkr) group or Bkr-type isolates. One ARDRA profile (six isolates) was different from those ARDRA profiles observed in other known diazotrophic *Burkholderia* species, hereafter referred to in the text as *Burkholderia* sp.

Phylogenetic analysis of 16S rRNA gene sequences. The 16S rRNA genes from one to four acetylene-reducing strains of each ARDRA genotype identified among the isolates recovered from tomato plants cultivated on different farms were sequenced and then compared with available 16S rRNA sequences from all of the *Burkholderia* species. Analysis of the 16S rRNA genes from strains TCo-26 and TCo-382 showed 98 to 99% identity with strain LB400^T of *B. xenovorans* (NCBI sequence database accession number U86373; P. C. K. Lau and H. Bergeron, unpublished; CP000271 chromosome 2 and CP000270 chromosome 1, complete sequence), which strongly suggested that they belong to this species (Fig. 1). Similarly, 16S rRNA gene sequences from strains TSi-873, TNe-883, TATl-371, and TATl-3742, all having ARDRA profiles slightly different from that of *B. unamae* MTI-641^T, showed 98 to 99% identity with this and other strains of *B. unamae* (e.g., NCBI accession numbers AY221955, AY221956, and AY221957) (11), and isolates TNe-865 and TSi-887 showed 99% identity with *B. tropica* strains (e.g., accession numbers AJ420332, AY128103, AY321306, AY128105, and AY128104) (54). Likewise, 16S rRNA gene sequences from strains TNe-841 and TNe-878 (Bkr group) showed 97.9% identity with *B. kururiensis* KP23^T (accession number AB024310) (80) and 97.8% identity with "*B. brasiliensis*" M130 (accession number AJ238360) (unpublished), which is a species not officially validated but referred to in the relevant literature (17, 39, 57, 58, 61). According to 16S rRNA gene sequencing, the strain TNe-862 showed 98% identity with *Burkholderia* sp. legume-nodulating strains mpa3.2 and mpa2.1a (accession numbers DQ156081 and DQ156080, respectively) and 97% identity with type strain SRMrh-20^T of *B. silvatlantica* (accession number AY965240) (50).

SDS-PAGE of whole-cell proteins. Whole-cell protein extracts were prepared from 25 representative acetylene-reducing *Burkholderia* isolates (recovered from diverse tomato plants and farms) showing different ARDRA profiles and their protein profiles compared with those from type and reference strains of *Burkholderia* species that showed identical or very similar ARDRA profiles. The protein patterns of some representative strains are shown in Fig. 2. In general, the similarity in whole-cell protein profiles between isolates recovered in this study and those profiles from type or reference strains of a particular diazotrophic *Burkholderia* species is striking (for example, *B. xenovorans* LMG 16224 and strains TCo-382 and TCo-213 [Fig. 2A], isolated in this work). Similar results were observed among *B. unamae* MTI-641^T as well as *B. tropica* Ppe8^T and strains recovered from the environment of tomato plants (Fig. 2B and C). It is remarkable that *B. kururiensis* KP23^T and "*B. brasiliensis*" M130 show almost identical protein profiles (Fig. 2D). In contrast, Bkr isolates (those with ARDRA profiles similar to that of *B. kururiensis* KP23^T) showed major differences between their protein patterns and KP23^T profiles. Similarly, strains TNe-862 and TNe-861 (Table 1) showed significant differences in their SDS-PAGE profiles from those of the phylogenetically closest species, *Burkholderia* sp. strain mpa2.1a and *B. silvatlantica* strains (data not shown).

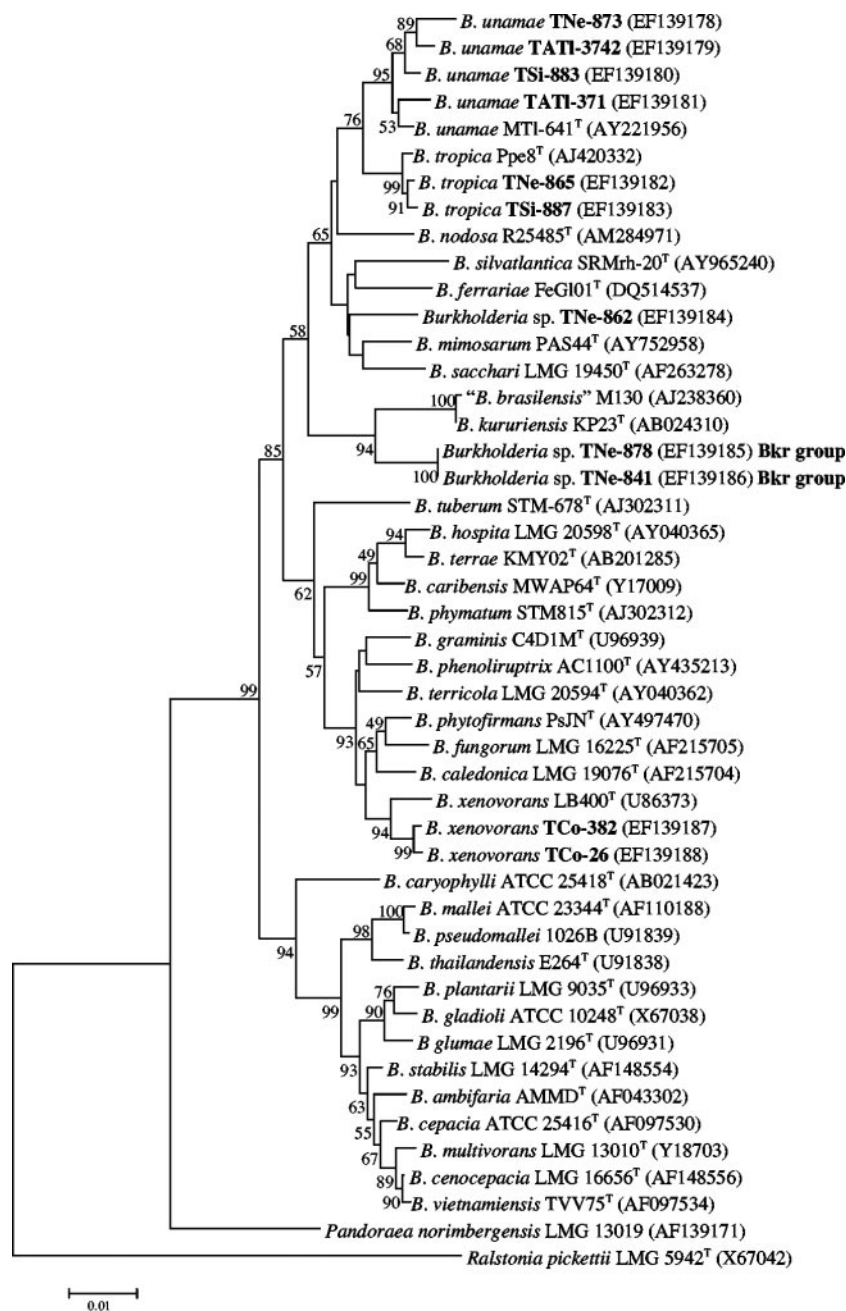


FIG. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relatedness among the nitrogen-fixing *Burkholderia* species associated with tomato plants (strain designations in bold) and related *Burkholderia* species. The bar represents one nucleotide substitution per 100 nucleotides. The nodal robustness of the tree was assessed using 1,000 bootstrap replicates. The NCBI GenBank accession number for each strain is shown in parentheses.

Bkr isolates as well as strains TNe-862 and TNe-861 appear to represent two novel diazotrophic *Burkholderia* species.

PCR amplification of *nifH* genes. Twenty-five representative acetylene-reducing *Burkholderia* isolates were analyzed (Table 1), yielding a PCR product of the expected size of 1.2 kb (data not shown) with the *nifH* primers used. These results confirmed the nitrogen-fixing abilities of the *Burkholderia* isolates.

Growth with aromatic compounds. The abilities of the diazotrophic *Burkholderia* species to grow on aromatic com-

pounds as carbon sources were variable and dependent on each species (Table 2). *B. xenovorans* strains recovered from tomato plants (Table 2) and reference strains CAC-124 and CCUG 28445 (data not shown) were unable to grow with aromatic compounds; type strain LB400^T grew with biphenyl as expected but not with any other aromatic compound tested. Interestingly, all of the *B. unamae* isolates recovered from tomato plants, except strains TAtI-3711 and TAtI-3742, were able to grow with benzene and phenol but not with biphenyl or

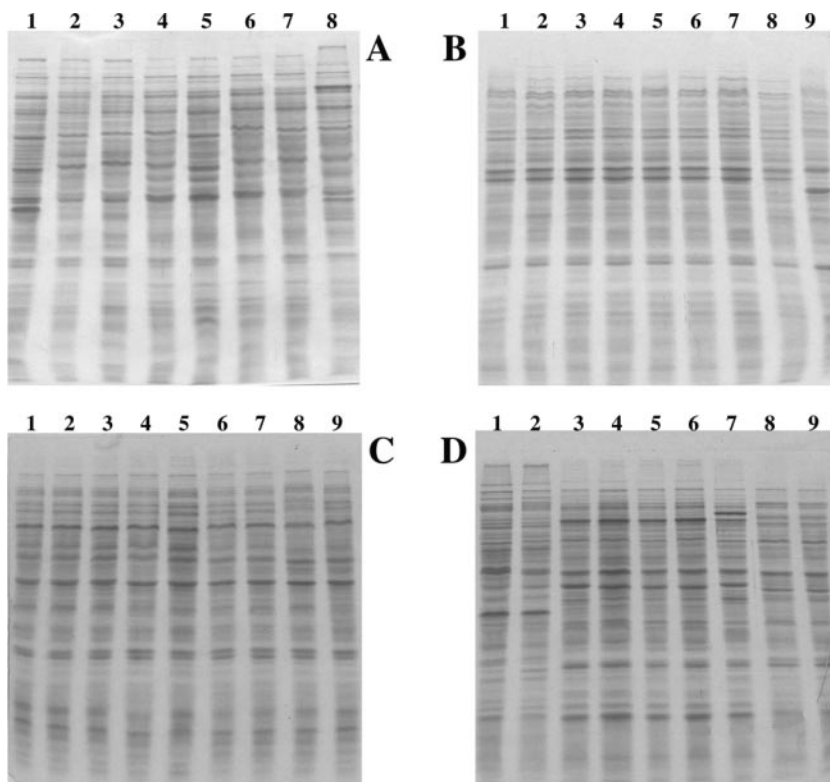


FIG. 2. Whole-cell protein profiles of representative acetylene-reducing isolates recovered in the present study, type and reference strains of known diazotrophic *Burkholderia* species, and other closely related species. (A) Lanes 1 to 7, *B. xenovorans* LB400^T, CAC-124, LMG 16224 (type and reference strains) (32), TCo-26, TCo-39, TCo-382, and TCo-213; lane 8, *B. caledonica* LMG19076^T. (B) Lanes 1 to 8, *B. unamae* TSi-883, MTL-641^T, CGC-321 (reference strain) (11), TAtl-3742, TTe-829, TNe-873, TAtl-3711, and TNe-832; lane 9, *B. sacchari* IPT101^T. (C) Lanes 1 to 9, *B. tropica* TSi-888, TSj-882, Ppe8^T, TSi-887, TTe-797, TTe-791, MTo-672 (reference strain) (54), TNe-865, and TNe-831. (D) Lane 1, “*B. brasilensis*” M130; lane 2, *B. kururiensis* KP23^T; lanes 3 to 7, *Burkholderia* sp. (Bkr group) strains TNe-8641, TNe-8682, TNe-878, TNe-841, and TNe-834; lanes 8 and 9, *Burkholderia* sp. strains TNe-862 and TNe-861.

other aromatic compounds tested (Table 2), and this feature was confirmed for seven other *B. unamae* strains, including the type strain, isolated from different plants and locations described previously (11). All of the *B. tropica* isolates recovered from tomato plants and seven other strains of this species

isolated from different plants cultivated in widely distant regions (54) were unable to grow with any aromatic compound. While Bkr isolates were able to grow only with benzene as a carbon source, *B. kururiensis* KP23^T and “*B. brasilensis*” M130 grew on toluene and phenol (Table 2). *Burkholderia* sp. strains

TABLE 2. Growth with aromatic compounds as carbon sources and PCR amplification of genes encoding aromatic oxygenases in N₂-fixing *Burkholderia* species associated with tomato plants^a

| Species or strain (n) | Presence (+) or absence (-) ^b of: | | | | | | PCR amplification of genes encoding: | | |
|---|--|--------|--------|---------|----------|-------------|--------------------------------------|---|----------------------------------|
| | Growth with aromatic compound: | | | | | | Aromatic | | |
| | Toluene | Phenol | Cumene | Benzene | Biphenyl | Naphthalene | monooxygenase | | Aromatic dioxxygenase (biphenyl) |
| | | | | | | Toluene | Phenol | | |
| <i>B. unamae</i> (5) | - | + | - | + | - | - | - | + | - |
| <i>B. unamae</i> (7) ^c | - | + | - | + | - | - | - | + | - |
| <i>B. xenovorans</i> (4) | - | - | - | - | - | - | - | - | - |
| <i>B. xenovorans</i> LB400 ^T | - | - | - | - | + | - | - | - | + |
| Bkr group (4) | - | - | - | + | - | - | - | - | - |
| <i>B. kururiensis</i> KP23 ^T | + | + | - | + | - | - | + | + | - |
| “ <i>B. brasilensis</i> ” M130 | + | + | - | - | - | - | + | + | - |
| <i>B. tropica</i> (7) | - | - | - | - | - | - | - | - | - |
| <i>B. tropica</i> (7) ^c | - | - | - | - | - | - | - | - | - |
| <i>Burkholderia</i> sp. (2) | + | + | - | + | - | - | + | + | - |

^a PCR amplification of the toluene monooxygenase gene was obtained with primers RMO-F/RMO-R but not with primers RDEG-F/RDEG-R.

^b +, good growth or gene presence; -, no growth or gene absence.

^c Includes type and reference strains.

TNe-862 and TNe-861 grew on toluene, phenol, and benzene (Table 2).

PCR amplification and sequencing of aromatic oxygenase genes. None of the nitrogen-fixing *Burkholderia* isolates associated with tomato plants (Table 1), including the *B. xenovorans* strains, yielded a PCR-amplified product with the bphAf668-3/bphAr1153-2 primers, used to amplify toluene/biphenyl dioxygenase genes (Table 2), while these primers gave PCR products of the correct sizes (525 to 531 bp) with *B. xenovorans* LB400^T as described previously (78). Diazotrophic *Burkholderia* strains that were able to grow using phenol or toluene as a carbon source yielded PCR products of the expected sizes with the specific primer pair used to amplify the corresponding aromatic oxygenase gene (Table 2). Subsequent gene sequencing and BLASTN analysis with available sequences deposited at the NCBI database confirmed the presence of phenol hydroxylases in *B. unamae* strains MTI-641^T and TSi-883, *B. kururiensis* KP23^T, “*B. brasiliensis*” M130, and *Burkholderia* sp. strain TNe-862 as well as the presence of toluene-3-monooxygenases in *B. kururiensis* KP23^T, “*B. brasiliensis*” M130, and *Burkholderia* sp. strain TNe-862. Phylogenetic trees derived from aromatic oxygenase-related sequences are illustrated in Fig. 3A and B. Although several replicates were done with different clones, the phenol hydroxylase gene fragments (203 bp) from *B. unamae* strains MTI-641^T and TSi-883 (associated with tomato) were, surprisingly but very interestingly, widely dissimilar. In contrast, the phenol hydroxylase gene fragments of *B. kururiensis* KP23^T and “*B. brasiliensis*” M130 analyzed in this work as well as the phenol hydroxylase gene sequence from *B. kururiensis* KP23^T reported previously (81) were almost identical. *B. kururiensis* KP23^T and “*B. brasiliensis*” M130 differed very slightly (99.34% identity) between their toluene-3-monooxygenase gene sequences, and these were closely related to the sequence from *Burkholderia* sp. strain TNe-862 (associated with tomato), and all of these toluene-3-monooxygenase sequences were more closely related to *B. cepacia* as well as *Ralstonia eutropha* and *R. pickettii* than to other toluene-degrading bacterial species.

PCR amplification and sequencing of *acdS* genes and ACC deaminase activity. Each of the *B. unamae* and *B. xenovorans* isolates analyzed (Table 1) yielded a 785-bp DNA fragment (data not shown) by use of the specific primer pair 5'ACC/3'ACC (designed in the present study). Gene sequencing and BLASTN analysis confirmed the presence of *acdS* genes in representative *B. unamae* and *B. xenovorans* strains associated with tomato plants. ACC deaminase activity levels were 22.93 (standard deviation [SD] = 01.68) and 25.42 (SD = 0.30) μ M α -ketobutyrate/h/mg protein in *B. unamae* strains TAtI-3742 and TSi-883, respectively, and 24.91 (SD = 0.73) μ M α -ketobutyrate in *B. xenovorans* TCo-26. All of these data clearly support the presence of the ACC deaminase enzyme and its expression in the *B. unamae* and *B. xenovorans* species.

Phosphate solubilization. Phosphate solubilization ability was variable among the diazotrophic *Burkholderia* species associated with tomato plants (Table 3). *B. tropica* exhibited the most notable phosphate-dissolving capability, showing the largest halo diameter in medium with Ca₃(PO₄)₂ as the sole P source in either the absence or the presence of MES buffer after incubation for 72 h. Although *B. unamae* TATI-371 exhibited a small solubilization halo (2 mm) in the presence of

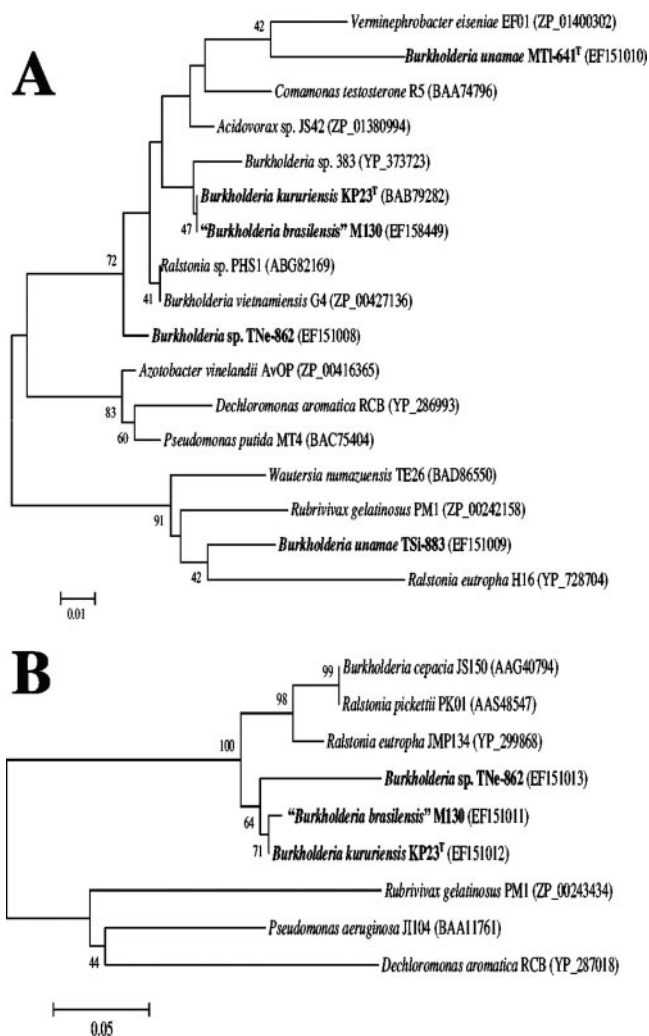


FIG. 3. Phylogenetic tree based on aromatic oxygenases showing the relatedness among the diazotrophic *Burkholderia* species associated with tomato plants (strain designations in bold) and degrader species of volatile aromatic compounds. (A) Phylogenetic analysis of phenol hydroxylase amino acid sequences; the analysis included 67 sites. (B) Phylogenetic analysis of toluene-3-monooxygenase amino acid sequences; the analysis included 153 sites. Bootstrapping analysis was used to test the statistical reliability of tree branch points. One thousand bootstrap samplings were performed. The NCBI GenBank accession number for each strain is indicated in parentheses.

MES buffer, all of the other isolates of this species as well as Bkr and *Burkholderia* sp. isolates were able to dissolve insoluble phosphates only in the absence of MES buffer after incubation for 3 to 5 days. *B. xenovorans* isolates were unable to dissolve tricalcium phosphate, except for strain CAC-124, which showed a small solubilization halo of 2 mm, but only in the absence of MES buffer.

Siderophore production. All of the diazotrophic *Burkholderia* isolates associated with tomato plants were able to produce siderophores on CAS medium agar plates (Table 3). In this medium, *B. unamae* isolates formed orange haloes (indicative of siderophore production) with diameters ranging from 5 to 8 mm, except for strain TATI-371, which produced the largest diameter halo (22 mm) among all of the diazotrophic

TABLE 3. Phosphate solubilization and biosynthesis of siderophores by N₂-fixing *Burkholderia* species associated with tomato plants

| Species or strain (<i>n</i>) | Halo size (mm) ^a on NBRI medium for Ca ₃ (PO ₄) ₂ solubilization | | | | Value for siderophore production | |
|---|---|------------------|-----------------|--------|---|-------------------------------------|
| | Without MES buffer | | With MES buffer | | Halo size (mm) ^a on CAS medium | Hydroxamate concn (μM) ^b |
| | 3 days | 7 days | 3 days | 7 days | | |
| <i>B. tropica</i> (6) | 7.1 | 9.5 | 3.0 | 4.3 | 14.2 | 0.23–1.10 |
| <i>B. tropica</i> (3) ^d | 7.3 | 9.7 | 3.0 | 4.0 | 14.0 | 0.38–0.42 |
| <i>B. unamae</i> (6) | 0.0 | 2.3 ^c | 0.0 | 0.0 | 6.5 ^c | 3.04–8.31 |
| <i>B. unamae</i> (3) ^d | 1.3 | 5.0 | 0.0 | 0.0 | 5.0 | 3.11–6.29 |
| <i>B. xenovorans</i> (4) | 0.0 | 0.0 | 0.0 | 0.0 | 16.8 | 4.70–5.91 |
| <i>B. xenovorans</i> LB400 ^T | 0.0 | 0.0 | 0.0 | 0.0 | 20.0 | 19.5 |
| Bkr group (5) | 0.0 | 1.5 | 0.0 | 0.0 | 4.6 | 0.33–1.63 |
| <i>B. kururiensis</i> KP23 ^T | 0.0 | 5.0 | 0.0 | 0.0 | 6.0 | 0.0 |
| <i>Burkholderia</i> sp. (2) | 0.0 | 0.0 | 0.0 | 0.0 | 3.0 | 0.33–0.70 |

^a Values represent the average halo sizes formed by the strains tested and are means for two replicate cultures.

^b Siderophore production range by the strains tested.

^c Halo sizes produced by *B. unamae* TAtI-371 were not included in the average, as this strain was exceptional in siderophore production and phosphate solubilization with respect to all of the strains of this species.

^d Includes type and reference strains tested.

Burkholderia strains tested. Generally, *B. xenovorans* strains produced the largest halo sizes (13 to 19 mm), followed by *B. tropica* strains (12 to 17 mm), the Bkr group (2 to 14 mm), and *Burkholderia* sp. isolates (3 mm). Except for the *Burkholderia* sp. isolates, all of these diazotrophic *Burkholderia* species produced hydroxamate-type siderophores (Table 3), but none produced catechol-type siderophores under the assay conditions used (data not shown). Generally, halo size was not correlated with amount of hydroxamate determined in liquid culture (Table 3). However, *B. unamae* TAtI-371 produced the largest amount of hydroxamate (8.3 μM) among all of the isolates recovered from the tomato environment, which otherwise produced hydroxamates in concentrations less than 5 μM (Table 3). Interestingly, *B. xenovorans* strains LB400^T, CCUG 28445, and CAC-124, included as controls in different assays, produced hydroxamates in concentrations as high as 19.5, 16, and 7.8 μM, respectively.

DISCUSSION

A total of 54 isolates recovered from the rhizospheres and rhizoplanes of tomato plants showed consistent nitrogenase activity as measured by the ARA method, and the presence of *nifH* genes was detected in all of the isolates (data not shown), confirming their diazotrophic abilities. The taxonomic statuses of all these isolates as members of the genera *Burkholderia* and *Ralstonia* were confirmed with two specific primer sets, BuRa-16-1/BuRa-16-2 (described previously [5]) and GB-F/GB-R (designed in the present study). All 54 of the diazotrophic isolates were confirmed as belonging to the genus *Burkholderia* by amplifying the 16S rRNA genes with the GB-F/GBN2-R-specific primer pair, described previously (49). A total of eight ARDRA profiles were identified from among the 54 diazotrophic *Burkholderia* isolates, and on this basis, only 25 representative N₂-fixing isolates of each ARDRA genotype identified among isolates recovered from tomato plants cultivated on different farms were further analyzed. Based on 16S rRNA sequences and whole-cell protein profiles, which provide strong evidence for the delineation of bacterial species (74), *B. xenovorans*, *B. unamae*, and *B. tropica* species were identified. In addition, two other unknown nitrogen-fixing *Burkholderia*

species were cultured in the present study, one of them closely phylogenetically related to *B. kururiensis* and the other one to legume-nodulating strains and to *B. silvatlantica*.

It is worth noting the substantial ability of the bacterial species to colonize different environments, including taxonomically distinct plants cultivated in distantly separated geographical regions. For example, *B. xenovorans* strains TCo-213 and TCo-382 were recovered from the rhizosphere of tomato in the present work, and strains CAC-124 and LMG 16224 were isolated from the rhizosphere of a coffee plant in Mexico and from a blood culture in Sweden, respectively (32). Similarly, in the present study *B. tropica* was found associated with tomato plants, but it has been isolated from sugarcane and maize varieties in Brazil, South Africa, and Mexico (49, 54). Recently, based on the 16S rRNA sequence, one isolate recovered from within dune grass (*Ammophila arenaria*) showed 99% identity with *B. tropica* strains (20). Although *B. unamae* has been found predominantly associated with sugarcane in Mexico (49), its isolation from sugarcane varieties cultivated in Brazil and South Africa (NCBI GenBank database accession number AY391282) (49) as well as from other crop plants, including maize and coffee (11), has been documented. Although 16S rRNA gene sequences from Bkr isolates associated with tomato plants showed high identity levels (97%) with *B. kururiensis* KP23^T and “*B. brasilensis*” M130, the protein patterns visualized in the Bkr isolates were clearly different from those observed in strains KP23^T and M130, which strengthens the notion that Bkr isolates do not belong to the species *B. kururiensis* or “*B. brasilensis*.” However, it is remarkable that *B. kururiensis* KP23^T and “*B. brasilensis*” M130 show almost identical protein profiles. Previously, it was suggested that strains KP23^T of *B. kururiensis* and “*B. brasilensis*” M130 belong to the same species, since both strains were able to fix N₂ in similar manners, showed the same ARDRA profile, and had identity levels of 99.9% between their 16S rRNA sequences (27). In addition, strains KP23^T and M130 differ at a single gene locus of 12 enzyme gene loci (multilocus genotypes) tested in multilocus enzyme electrophoresis assays (data not shown) and display other very similar features, such as the ability to grow using phenol and toluene as single carbon sources, with the

corresponding gene sequences (phenol hydroxylase and toluene-3-monooxygenase) being almost identical. High identity levels between *glnB* or *nifH* genes in both strains were observed previously (43). On the basis of these data, strain M130 should be considered a member of the species *B. kururiensis*, and we recommend that reference to the species "*Burkholderia brasiliensis*" be omitted in future literature to avoid further confusion. Nonetheless, the wide geographic distribution and substantial ability of *B. kururiensis* to colonize such dissimilar environments is striking; while the trichloroethylene-degrading strain KP23^T was isolated from an aquifer polluted with trichloroethylene in Japan (80), strain M130 was recovered from surface-sterilized roots of rice grown in Brazil (76). Based on these findings, it appears conceivable to find new host plants or other unexpected habitats for *B. unamae* or *B. kururiensis* and other well-known or unknown species, as observed with *B. xenovorans* and *Burkholderia* spp. recovered in the present study.

In this work, N₂-fixing *Burkholderia* isolates associated with tomato plants were assessed for their abilities to grow with some common volatile pollutant compounds as sole carbon sources. Very interestingly, all of the *B. unamae* isolates associated with tomato plants were able to grow with benzene and phenol as sole carbon sources, and the ability to grow on phenol was confirmed with the detection and sequencing of phenol monooxygenase genes by PCR. This feature was confirmed to be characteristic of this species as well as for seven other *B. unamae* strains, including type strain MTI-641, associated with different plants cultivated in widely distant regions (11). However, complete sequences of phenol monooxygenase genes from *B. unamae* strains MTI-641^T and TSi-883 should be obtained to determine the divergence between them, as these sequences differed greatly in the small (203-bp) DNA fragments analyzed. In addition, the complete phenol monooxygenase genes of more *B. unamae* strains should be analyzed to determine their evolutionary origins in populations of this species. These dissimilarities strongly contrast with the 100% identity found between phenol monooxygenase genes from *B. kururiensis* KP23^T and M130 isolated from two widely separated regions of the world. No less remarkable is the finding that *B. tropica* (54), *B. sacchari* (9), and *B. ferrariae* (72), all closely phylogenetically related to *B. unamae*, were unable to grow with the aromatic compounds benzene and phenol (data not shown). Nevertheless, very recently one *B. tropica* strain able to degrade benzene, toluene, and xylene was described (22), but this strain was identified only on the basis of PCR amplification and sequencing of 16S rRNA genes, which may be insufficient evidence to define its exact taxonomic position, considering that *B. tropica* and *B. unamae* show identity levels higher than 98% between their 16S rRNA gene sequences (11). Moreover, this *B. tropica* strain was identified (31) before *B. unamae* was described (11) and their 16S rRNA gene sequences could be compared. If the taxonomic status of the *B. tropica* strain is confirmed by means of polyphasic taxonomy criteria, then considering the inability of several *B. tropica* strains tested in this study to grow on aromatic compounds, this would indicate a case of lateral gene transfer, as has been suggested for the biphenyl pathway for PCB degradation expressed by *B. xenovorans* LB400^T but absent in two other strains of this species (12, 32). In the present study, such a

lateral gene transfer in LB400^T is supported by the finding that the *B. xenovorans* isolates (e.g., TCo-26 and TCo-382) associated with tomato plants were incapable of growing with biphenyl as the sole carbon source, and biphenyl dioxygenase genes were not PCR amplified from these strains, though this occurred, as expected, in strain LB400^T. Undoubtedly, more *B. xenovorans* isolates should be analyzed to confirm that the ability to degrade biphenyl and other aromatic compounds is a typical characteristic in this species and not a unique feature of strain LB400^T. Considering the habitat of *B. xenovorans* (soil and rhizosphere), the ability to degrade aromatic compounds originating from root exudates and root turnover could be important (12). Similarly, since about 25% of the Earth's biomass consists of compounds having a benzene ring as the main structural component (29), and phenolic compounds are widely distributed in the plant kingdom (21), the ability of *B. unamae* to use benzene and phenol as carbon sources could be advantageous for its survival in the rhizospheric and endophyte environments, both common habitats of this species (11). Unfortunately, in this study we did not analyze the endophyte environment of tomato for recovering diazotrophic *Burkholderia* species. However, the ability of *B. unamae* to grow using aromatic hydrocarbons, as well as its widespread association with different plant species (11, 49), suggests that it could be suitable for applications in rhizosphere remediation of common soil pollutants. In addition, taking into consideration the host plant range of *B. unamae* and its endophytic character, it would be of great interest to know the natural ability of this species to improve phytoremediation of volatile organic pollutants, as has been suggested with the application of engineered endophytic bacteria (4, 68).

In the present work, several bacterial plant growth-promoting mechanisms were analyzed and detected in diazotrophic *Burkholderia* isolates associated with tomato plants. In previous studies, ACC deaminase activity was proposed as a bacterial mechanism that may enhance plant growth by lowering plant ethylene levels (30), which is especially true in stressed dicots such as tomato, since monocots are less sensitive to ethylene (36). Based on these data, and supported by the occurrence of *acdS* genes (PCR amplified using a primer pair designed in this study and verified by sequencing) in *B. unamae* and *B. xenovorans* as well as by the corresponding ACC deaminase activities expressed by strains of these species associated with tomato plants, it could be suggested that both *B. unamae* and *B. xenovorans* possess the potential for improving plant growth. In fact, maize inoculation with wild-type strains of *B. unamae* promotes maize plant growth (unpublished results), and experiments using different mutants to determine the mechanism of plant growth promotion are in progress. Conversely, although ACC activity has been detected in *B. tropica* strain BM-273, and the corresponding *acdS* gene was detected in this strain and in strain BM-16 (7), we were unable either to detect ACC activity or to amplify the *acdS* gene in any of the *B. tropica* strains analyzed. This discrepancy could be attributed to the different sets of primers used for amplification in both studies. However, based on *acdS* phylogenetic analysis, an extensive horizontal transfer of *acdS* genes has been suggested, which could explain the occurrence of this gene in strains BM-273 and BM-16 (both isolated from maize plants inoculated with soil from the same field [26]) but not in *B. tropica*

strains associated with tomato plants or in seven other strains of this species recovered from plants cultivated in different locations (54). These discrepancies deserve further analysis, which was beyond the scope of this study.

In the present work, the highest mineral phosphate-dissolving capability was detected in *B. tropica* isolates; this capability was lower in *B. unamae* and *Burkholderia* sp. isolates and null in most *B. xenovorans* strains. Even though mineral phosphate-dissolving capability in most bacteria has been related to organic acid production (55), recent results indicate that this is not the only mechanism of mineral phosphate solubilization by bacteria (56). Mineral phosphate solubilization through organic acid production appears to occur in most diazotrophic *Burkholderia* species associated with tomato plants, but in *B. tropica*, an additional, unknown mechanism presumably exists, since this species solubilized tricalcium phosphate in less than 72 h even in a strongly buffered culture medium. Regardless of the mechanism used by *B. tropica*, its remarkable ability to convert insoluble mineral phosphorus to an available form is an important trait for allowing this diazotrophic species to be defined as a plant growth-promoting rhizobacterium. Seed inoculation with efficient phosphate-solubilizing bacteria is known to increase solubilization of fixed soil phosphorus and immobilized phosphates in the soil after application of mineral fertilizers, resulting in higher crop yields (23, 56).

Production of siderophores by plant growth-promoting rhizobacteria is considered to be important in the suppression of deleterious microorganisms and soilborne plant pathogens (66) and in some cases appears to trigger induced systemic resistance (18, 75). The production of hydroxamate-type siderophores by *B. unamae* and *B. tropica* strains has been described previously (11, 54) and confirmed in the present work as a characteristic feature in these species. *B. xenovorans* strain LB400^T is widely known as an effective PCB degrader, but the synthesis of hydroxamate-type siderophores in large amounts by this species is described for the first time in this work. In accordance with this finding, homologs of biosynthesis genes encoding two hydroxamate-type siderophores, pyoverdine and ornibactin, were found on chromosome 2 of *B. xenovorans* LB400^T (12). While this chromosome also carries a homolog encoding pyochelin (catechol siderophore) synthesis (12), we were unable to detect catechol-type siderophores in LB400^T liquid cultures. The lack of catechol-type siderophores detected in strain LB400^T, as well as in all of the other diazotrophic *Burkholderia* isolates, could be related to the CAA culture medium used in the assay, since pyochelin production and that of its precursor salicylic acid vary according to the minerals and carbon sources available (24). In addition, the lack of correlation between the largest orange haloes exhibited on CAS-CAA medium and the very small amounts of hydroxamate siderophores found in liquid cultures from *B. xenovorans* isolates associated with tomato plants (even smaller amounts are found in cultures from all of the *B. tropica* strains) suggests that other types of siderophores, different from hydroxamates, are produced by strains of these species. Accordingly, all of the diazotrophic *Burkholderia* species siderophore producers associated with tomato could play a major role in the biocontrol of phytopathogens, either in tomato or in other host plants of these diazotrophs.

Certainly, the nitrogen-fixing *Burkholderia* species associ-

ated with tomato plants represent a great potential for agrobiotechnological applications, which could lead toward using consortia of these species as plant growth-promoting rhizobacteria and, concomitantly, in rhizoremediation and/or for improvement of phytoremediation and for biological control of plant pathogens. However, the potential beneficial role of the *Burkholderia* species revealed in this study should be established under natural conditions, since a particular bacterial activity exhibited in the laboratory is not guaranteed to function in association with a host plant.

Undoubtedly, the isolation of *B. unamae*, *B. xenovorans*, *B. tropica*, and two other unknown N₂-fixing *Burkholderia* species, as well as their very attractive features for agronomic and environmental applications, some detected for the first time in these species, emphasizes the significance of performing studies on taxonomy and the suitability of exploring common environments, such as the rhizosphere, for isolation of bacterial species with biotechnological potential.

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