Rapid identification of nitrogen-fixing and legume-nodulating *Burkholderia* species based on PCR 16S rRNA species-specific oligonucleotides

Arnoldo Wong-Villarreal\textsuperscript{a,b}, Jesús Caballero-Mellado \textsuperscript{b,*}

\textsuperscript{a} Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, México
\textsuperscript{b} Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Ap. Postal 565-A, Cuernavaca, Morelos, México

**ABSTRACT**

Several novel N\(_2\)-fixing *Burkholderia* species associated with plants, including legume-nodulating species, have recently been discovered. Presently, considerable interest exists in studying the diazotrophic *Burkholderia* species, both for their ecology and their great potential for agro-biotechnological applications. However, the available methods used in the identification of these *Burkholderia* species are time-consuming and expensive. In this study, PCR species-specific primers based on the 16S rRNA gene were designed, which allowed rapid, easy, and correct identification of most known N\(_2\)-fixing *Burkholderia*. With this approach, type and reference strains of *Burkholderia kururiensis*, *B. unamae*, *B. xenovorans*, *B. tropica*, and *B. silvatlantica*, as well as the legume-nodulating *B. phymatum*, *B. tuberum*, *B. minosarum*, and *B. nodosa*, were unambiguously identified. In addition, the PCR species-specific primers allowed the diversity of the diazotrophic *Burkholderia* associated with field-grown tomato and sorghum plants to be determined. *B. tropica* and *B. xenovorans* were the predominant species found in association with tomato, but the occurrence of *B. tropica* with sorghum plants was practically exclusive. The efficiency of the species-specific primers was validated with the detection of *B. tropica* and *B. xenovorans* from DNA directly recovered from tomato rhizosphere soil samples. Additionally, using PCR species-specific primers, all of the legume-nodulating *Burkholderia* species were correctly identified, even from single nodules collected from inoculated common bean plants. These primers could contribute to rapid identification of the diazotrophic and nodulating *Burkholderia* species associated with important crop plants and legumes, as well as revealing their environmental distribution.

© 2009 Elsevier GmbH. All rights reserved.

**Introduction**

Until recently, the genus *Burkholderia* included 40 validly described species [15], but this number has increased to 58 at present. *Burkholderia* species are widely distributed in the natural environment [15], but they are also found in the hospital environment, especially the *B. cepacia* complex (Bcc) species isolated from patients with cystic fibrosis [27]. Nevertheless, most of the *Burkholderia* species are known as soil-borne bacteria, and many exhibit non-pathogenic interactions with plants [8,15,37].

Historically, the ability to fix N\(_2\), the reduction of atmospheric N\(_2\) to ammonia, in bacteria of the genus *Burkholderia* was identified only in the species *B. vietnamiensis* [21], which is a member of the Bcc [27]. Currently, 9 diazotrophic plant-associated *Burkholderia* species have been validly described, for example, *B. unamae* [8], *B. xenovorans* [22], *B. tropica* [37], and *B. silvatlantica* [34]. All of these species are able to colonize the rhizosphere and/or the endophytic environment of a wide range of taxonomically unrelated host plants, such as maize, sorghum, sugarcane, pineapple, and coffee [8,9,21,33,34,37]. *B. kururiensis*, a trichloroethylene-degrading bacterium isolated from an aquifer [51], was identified as a diazotrophic species [20]. "*B. brasilensis*" strain M130 [2], a plant-associated species never validly described, has been re-classified as *B. kururiensis* [9]. Recently, analysis of field-grown tomato plants revealed a high diversity of well-known and unknown diazotrophic *Burkholderia* species [9]. In addition, legume-nodulating N\(_2\)-fixing strains have been formally described as *B. phymatum* and *B. tuberum* [46], *B. minosarum* and *B. nodosa* [10,12]. Currently, unnamed strains with the ability to form nodules on *Macroptilium atropurpureum*, *Mimosa* species and other mimosoid legumes probably represent novel *Burkholderia* species [4,5].

*Burkholderia* strains are promising candidates for biotechnological applications [30]. Unfortunately, most of these strains belong to the Bcc, which is comprised presently of 17 species that are involved in human infections, thereby hindering potential applications.
However, it is worth noting that all of the diazotrophic *Burkholderia*, excluding *B. vietnamiensis*, comprise a group of closely related species that are phylogenetically very distant from the Bcc species [9]. In addition, transmissibility factors such as the cba gene, encoding giant cable pil [26,41], and the epidemic strain marker regulator or esnR gene [26], necessary for virulence and part of a genomic island [3], have been identified among clinical and environmental isolates of opportunistic pathogens of *B. cenocepacia* and other Bcc species, but have not been detected in the several plant-associated diazotrophic *Burkholderia* species analyzed [33].

Recently, it was shown that the novel N₂-fixing *Burkholderia* species associated with plants have great potential for agro-biotechnological applications, as they exhibit activities involved in either plant growth promotion, biocontrol or bioremediation [9], in addition to their role as nitrogen fixers in ecosystems. On this basis, an increasing interest in the isolation of N₂-fixing and legume-nodulating *Burkholderia* species would be expected. However, because the taxonomic situation within the genus *Burkholderia* is difficult, and since the number of species has continuously increased, reliable and rapid identification methods are needed. In addition, the knowledge of the novel diazotrophic *Burkholderia* species has only been gained in the last few years, and emphasis has been given to studies on taxonomy and identification methods for the *Burkholderia* genus as a whole [32,42], or the Bcc species specifically [16,29,36].

In this study, we designed species-specific primers, based on the 16S rRNA gene, which allowed easy and rapid identification of isolates belonging to most well-known nitrogen fixing *Burkholderia* species, including legume nodule symbionts, recovered in pure culture from the rhizosphere of field-grown tomato and sorghum plants, or directly from nodules collected from inoculated common bean plants. Detection of the occurrence of diazotrophic *Burkholderia* species directly from DNA recovered from tomato rhizosphere soil samples was also analyzed.

**Materials and methods**

*Design and assess of PCR 16S rRNA species-specific primers*

The 16S rRNA sequences from strains of each nitrogen-fixing *Burkholderia* species, and from all the available *Burkholderia* species in the NCBI database, were aligned in order to identify the conserved regions used to design and test PCR species-specific primers, which would provide an amplicon only with a particular species of *Burkholderia*. The multiple alignments of the sequences were performed with CLUSTAL W software [43], and the species-specific primers were designed with the OLIGO 6.71 program [39]. In *silico* analysis was performed to evaluate the specificity of the primers designed using the same software program [39]. In addition, several *Ralstonia* species (e.g., *R. pickettii*, *R. taiwanensis*, *R. garliardi*, and *R.eutropha*), a genus closely related to *Burkholderia*, were included in the *in silico* analysis. PCR species-specific primer sets and conditions are described in Table 1. The specificity of the primers was first validated with most (36 out of 58) of the well-known *Burkholderia* species, including the diazotrophs (Fig. 1). Subsequently, the species-specific primer pairs designed for each *Burkholderia* species were tested in the identification of 100 N₂-fixing isolates recovered from tomato and sorghum plants, as well as using template DNA recovered and purified directly from rhizosphere soil samples, or from nodules formed on roots of the common bean inoculated with nodulating *Burkholderia* species (see below).

**Burkholderia strains analyzed**

Type strains of the N₂-fixing and non-diazotrophic *Burkholderia* species analyzed are indicated in Fig. 1, as well as *B. kururiensis* M130 [9] that was also included in this study. Other reference strains (see legend of Fig. 1) of each N₂-fixing *Burkholderia* species tested have been described in other studies [8–10,12,22,34,37]. In addition, 100 diazotrophic *Burkholderia* recovered from tomato (40 isolates) and sorghum (60 isolates) plants were analyzed.

**Samples of plants and soil**

To obtain representative N₂-fixing *Burkholderia* species and to increase the opportunity for evaluating the species-specific primer pairs designed, the field-grown plants analyzed were chosen by considering the known high diversity of diazotrophic *Burkholderia* associated with tomato [9] and the lack of knowledge about the community of N₂-fixing *Burkholderia* species associated with sorghum, which is an important grass for animal feed as well as a source of ethanol fuel. Twenty samples of the rhizosphere (pH range 5.3–6.4) and rhizoplane (root surface) of tomato plants (*Lycopersicon esculentum* var. Saladet) field grown in Atlatlahuacan, Morelos State, Mexico, were analyzed for

---

**Table 1**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer pair (forward and reverse 5’-3’)</th>
<th>Size of PCR product (bp)</th>
<th>Annealing temp. (°C)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Btrop-F</td>
<td>TAATACATCGGAAGAAGTCGCTT</td>
<td>356</td>
<td>63.5</td>
<td>B. tropica</td>
</tr>
<tr>
<td>Btrop-R</td>
<td>GGCTCATATAGGACAGGAAGGT</td>
<td>490</td>
<td>62.0</td>
<td>B. unamae</td>
</tr>
<tr>
<td>Bunam-R</td>
<td>ATCCCGCAAGCGTGATCCG</td>
<td>567</td>
<td>52.0</td>
<td>B. kururiensis</td>
</tr>
<tr>
<td>Bunam-F</td>
<td>TTCGAAAGAATCTTCTGGG</td>
<td>580</td>
<td>65.0</td>
<td>B. xenovorans</td>
</tr>
<tr>
<td>Bkuru-R</td>
<td>TCCGAGAACGAACGACTC</td>
<td>830</td>
<td>69.0</td>
<td>B. silvatlantica</td>
</tr>
<tr>
<td>Bkuru-F</td>
<td>GCAAAGGGGGAGATCAAGAAGA</td>
<td>40</td>
<td>64.0</td>
<td>B. tuberum</td>
</tr>
<tr>
<td>Baeno-R</td>
<td>ATGCGAGTTCGCTCTGTGTT</td>
<td>561</td>
<td>62.0</td>
<td>B. phymatum</td>
</tr>
<tr>
<td>Baeno-F</td>
<td>GACGGAGTCGATTCGTGTT</td>
<td>576</td>
<td>62.0</td>
<td>B. mimosarum</td>
</tr>
<tr>
<td>Bsilva-R</td>
<td>GCGAACGTCTCCAGCTC</td>
<td>369</td>
<td>69.0</td>
<td>B. nodosa</td>
</tr>
</tbody>
</table>

PCR conditions were as follows: initial denaturation for 5 min at 94 °C, followed by 25 cycles of denaturation for 30 s at 94 °C, annealing temperature for 45 s as indicated for each species, and elongation for 1 min at 72 °C, followed by a final 5 min elongation at 72 °C.
recovery of N₂-fixing *Burkholderia* 4 h after collection. The tomato roots were shaken gently to remove loosely attached soil, and adhering soil (rhizosphere soil) samples were maintained at 4°C for 45 days prior to DNA recovery (see below). In addition, rhizosphere/rhizoplane samples of 21 sorghum (*Sorghum bicolor* L. Moench) plants (var. D65, D72 and Ambar) field grown in Yecapixtla (pH range 4.5–6.7) and Cuautla (pH range 4.8–5.5), Morelos State, were analyzed 4 h after collection, and 15 plants (var. D65, D66 and Pioneer 8641) cultivated in Irapuato (pH range 5.3–6.5), Guanajuato State, Mexico, were analyzed 48 h after collection. Plants of each variety were randomly collected with a distance of 10–15 m between plants.

Culture media and diazotrophic *burkholderia* isolation

Diazotrophic *Burkholderia* associated with tomato and sorghum plants were isolated as described previously [20]. Briefly, the roots were shaken gently to remove any loosely attached soil. The roots with adhering soil were then immersed in 10 mM MgSO₄·7 H₂O (Mgsol) containing 0.01% (v/v) Tween 20 for 5 min, and thereafter vortexed at 3000 rpm for 3 min. The resulting suspension was considered to contain bacteria from the rhizosphere/rhizoplane. Aliquots of 100 μL from the suspension were inoculated into vials containing N-free semisolid BAz medium and incubated for 5–7 days at 29°C. Thereafter, cultures were replicated 2–3 times under the same conditions. Vials with a white or yellowish pellicle were streaked onto BAc medium plates and incubated at 29°C. After 4–5 days, predominant colonies with different morphologies were individually inoculated in vials containing N-free BAz medium, incubated at 29°C for 4 days and assayed for nitrogenase activity by the acetylene reduction activity (ARA) method [7] in vials containing N-free semisolid BMGM or BAz medium [20]. ARA positive colonies were maintained in 20% glycerol at -80°C prior to characterization.

**PCR-amplification of nifH genes**

Primers IGK [35] and NDR-1 [44] were used for the amplification of the *nifH* genes using the PCR conditions described previously [33]. The reaction amplified a 1.2 kb fragment comprising the complete

---

**Fig. 1.** Specificity of the PCR primer sets for the identification of N₂-fixing and legume-nodulating *Burkholderia* species: +, PCR-amplified product; –, no amplified product. Primer names are those indicated in Table 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relatedness between the N₂-fixing plant-associated *Burkholderia* species. The bar represents 1 nucleotide substitution per 100 nucleotides. Nodal robustness of the tree was assessed using 1000 bootstrap replicates. The NCBI GenBank accession number for each type strain tested is shown in parentheses. Additional reference strains tested were: 1 *B. unamae* SCCu-23, MCo-762, CGC-72, Ts-883; TAT-371 and TNe-873; 2 *B. tropica* MTo-293, MCo-725, MTo-672 and Ts-887; 3 *B. nodosa* Br3470; 4 *B. mimosarum* Br3454 and Br3467; 5 *B. silvaticulturn* SRL-318 and PCCR-2; 6 *B. kururiensis* M130; 7 *B. xenovorans* CAC-124; TCo-382, LMG 16224; 8 *B. vietnamiensis* SxO-702 and MMi-302.
**PCR identification of isolates with 16S rRNA species-specific primers**

ARA positive isolates were subjected to identification using species-specific primers and the conditions are described in Table 1. PCR-amplified products were sequenced at the Biotechnology Institute (IBT)-UNAM (Mexico). When PCR products were not amplified, isolates were identified to the genus level as described below.

**Total DNA isolation and 16S rRNA specific primers for the genus Burkholderia**

Genomic DNA was recovered from ARA positive isolates using published protocols [1]. ARA positive isolates recovered from tomato and sorghum plants, those directly identified with the species-specific primers and conditions described in Table 1, were additionally tested and then assigned to the genera *Burkholderia* and *Ralstonia* by amplifying the 16S rRNA gene with the GB-F/GB-R primer pair (specific for genera *Burkholderia* and *Ralstonia*) under the conditions described by Caballero-Mellado et al. [9]. These isolates were confirmed as belonging to the genus *Burkholderia* by amplifying the 16S rRNA genes with the GB-F/GBN2-R primer pair specific for N2-fixing *Burkholderia*, except *B. vietnamiensis*, and very closely related species using the PCR conditions described previously [33].

**16S rRNA gene sequencing and phylogenetic analysis**

Representative ARA positive isolates, those previously identified with the species-specific primers and conditions described in Table 1 and confirmed as belonging to the genus *Burkholderia* by amplifying the 16S rRNA genes with primers GB-F/GB-R, were randomly chosen for 16S rRNA gene sequencing. Primers rD1 and rD1 were used for amplifying the 16S rRNA gene (ca. 1.5 kb) [48] using the PCR conditions described previously [20]. To obtain 16S rRNA sequences, PCR products were cloned as described previously [33], and the gene sequences were determined at the IBT-UNAM, and at Macrogen Inc. (Seoul, Korea). The 16S rRNA gene sequences were deposited in the EMBL/GenBank database. These sequences were compared with previously published 16S rRNA sequences available in the NCBI database allowed the identification of conserved regions and the design of PCR species-specific primers for *B. nodosa* (unpublished results), this plant was used for nodulation with these species and in PCR identification assays. *P. vulgaris* var. Negro Jamapa seeds were immersed in 70% ethanol for 5 min and surface sterilized in a 25% commercial bleach solution (Clorox) for 15 min, and then washed three times with distilled sterile water. Seeds were germinated on 0.75% water agar plates for 2 days at 29°C. Thereafter, the seedlings were sown in 250 ml flasks containing N-free mineral salts agar, and then inoculated with the different legume-nodulating *Burkholderia* strains and with *R. etli*, a natural symbiont of *P. vulgaris*. Plants were grown for 18 days, and then root nodules (approximately 2 mm diameter) from different plants were immersed in ethanol and surface sterilized as described above for the seeds. Single nodules were carefully squeezed, and the bacterial content collected with a micropipette tip, resuspended in 40 ml of 10 mM Mgso4 plus 0.01% Tween 20, and then the bacterial suspension was heated at 95°C for 30 min. Aliquots of 2 µl from the supernatant were subjected to PCR using the *Burkholderia* species-specific primers and conditions described in Table 1. PCR-amplified products were electrophoresed in 1% agarose gels.

**Recovery and purification of DNA from rhizosphere soil**

Total DNA was recovered from 10 g of rhizosphere soil from two different tomato plants with the PowerMax soil DNA isolation kit, according to the manufacturer’s instructions (MO BIO laboratories, Inc. CA). Purified total DNA was stored at −20°C prior to analysis.

**Detecting diazotrophic Burkholderia occurrence in soil with 16S rRNA species-specific primers**

DNA from rhizosphere soil was subjected to PCR using the species-specific primers and conditions described in Table 1. In addition, DNA from rhizosphere soil was subjected to PCR using the GB-F/GB-R primer pair, and the amplified product, a 1100 bp fragment, was used as a template in PCR assays using the species-specific primers and conditions described in Table 1. PCR-amplified products were electrophoresed in 1% agarose gels. DNA bands of the expected size for the different species-specific primers used were extracted from the gel, cloned into the pCR2.1 vector according to the manufacturer’s instructions (Invitrogen), and the gene sequences were determined at the IBT-UNAM, and at Macrogen Inc. (Seoul, Korea).

**PCR identification of Burkholderia harbored in nodules using species-specific primers**

Since the common bean (*Phaseolus vulgaris*) is nodulated by *B. tuberum* [18], as well as by *B. phosphatatum*, *B. mimosarum*, and *B. nodosa* (unpublished results), this plant was used for nodulation with these species and in PCR identification assays. *P. vulgaris* var. Negro Jamapa seeds were immersed in 70% ethanol for 5 min and surface sterilized in a 25% commercial bleach solution (Clorox) for 15 min, and then washed three times with distilled sterile water. Seeds were germinated on 0.75% water agar plates for 2 days at 29°C. Thereafter, the seedlings were sown in 250 ml flasks containing N-free mineral salts agar, and then inoculated with the different legume-nodulating *Burkholderia* strains and with *R. etli*, a natural symbiont of *P. vulgaris*. Plants were grown for 18 days, and then root nodules (approximately 2 mm diameter) from different plants were immersed in ethanol and surface sterilized as described above for the seeds. Single nodules were carefully squeezed, and the bacterial content collected with a micropipette tip, resuspended in 40 ml of 10 mM Mgso4 plus 0.01% Tween 20, and then the bacterial suspension was heated at 95°C for 30 min. Aliquots of 2 µl from the supernatant were subjected to PCR using the *Burkholderia* species-specific primers and conditions described in Table 1. PCR-amplified products were electrophoresed in 1% agarose gels.

**Results**

**Design and validation of PCR 16S rRNA species-specific primers**

Analysis of *Burkholderia* 16S rRNA sequences available in the NCBI database allowed the identification of conserved regions and the design of PCR species-specific primers for *B. unanae*, *B. silvatlantica*, *B. kururiensis*, and *B. nodosa*. In *sito* analysis supported the absolute specificity of the primers designed for these species. In *sito* analysis of the 16S rRNA sequences showed that the primers designed for the identification of *B. tropica* would potentially also amplify *B. stabilis* and *B. ubonae*, whereas the primers designed for *B. xenovorans* could amplify *B. ginsengisoli*, and those designed for the identification of *B. tuberum* would also

**SDS-PAGE of whole-cell proteins**

Preparation of whole-cell proteins from representative diazotrophic isolates, and SDS-PAGE assays were performed as described by Estrada-de los Santos et al. [20]. However, protein profiles were compared only with those of available type and reference strains of *B. tropica*, *B. xenovorans*, and *B. vietnamiensis*.

**Detection of transmissibility marker genes**

*cblA* and *esmR* genes were investigated in acetylene-reducing isolates using specific primers CBL1/CBL2 and BCESM1/BCESM2, respectively, using the PCR conditions described previously [26,41].
amplify B. ginsengisoli and B. phenoliruptrix. Similarly, the primers designed for B. phymatum would potentially also amplify B. hospita, while those primers designed for B. mimosarum could amplify B. phenazinium. Nevertheless, complete specificity of the primers was reached using the PCR conditions described in Table 1. Thus, specific PCR-amplified products were obtained for the type and reference strains of all the N₂-fixing and Burkholderia legume symbionts analyzed. The specificity of the PCR primer sets for the identification of N₂-fixing and legume-nodulating legume symbionts is illustrated in Fig. 1. Examples of PCR-amplified specific products in phylogenetically closely related diazotrophic Burkholderia species are shown in Fig. 2.

Diazotrophic Burkholderia isolation

Enrichment cultures for N₂-fixing Burkholderia were made in N-free semi-solid B₅ medium followed by further isolation and colony purification on B₄C agar plates [20]. Screening of 98 colonies isolated from different tomato plants, and 100 colonies from sorghum allowed the recovery of 40 and 60 isolates, respectively, which showed consistent nitrogenase activity as measured by the ARA method, and yielded a PCR product of the expected size (1.2 kb) with the nifH primers used (data not shown). These results confirmed the N₂-fixing ability of the Burkholderia isolates. From among the 100 diazotrophic isolates associated with tomato and sorghum plants and subjected to PCR amplification using the Burkholderia species-specific primers, 88 isolates yielded the fragment of the expected size (356 bp) for B. tropica, 7 isolates yielded the ampiclon of correct size (580 bp) for B. xenovorans, and 5 isolates did not yield a PCR-amplified product with any of the species-specific primers tested (Table 2).

16S rRNA-specific primers and phylogenetic analysis of 16S rRNA gene sequences

All of the 100 diazotrophic isolates associated with tomato and sorghum plants gave a PCR-amplified product of the correct size (1100 bp) with primers GB-F/GB-R, confirming their taxonomic position as members of the genera Burkholderia-Ralstonia, and 98 of these isolates were confirmed as belonging to the genus Burkholderia by amplifying the 16S rRNA genes with the GB-F/GBN2-R primers. Two isolates (Sfr-665 and SYE-6586) that did not yield the PCR-amplified product with the GB-F/GBN2-R primers were further identified by analysis of 16S rRNA gene sequences (Table 2).

The 16S rRNA genes from approximately 10–25% of the diazotrophic isolates from each Burkholderia species (identified with species-specific primers) associated with tomato and sorghum plants were sequenced and then compared with available 16S rRNA sequences from all of the Burkholderia species. Analysis of the 16S rRNA gene from randomly chosen N₂-fixing strains showed levels of identity higher than 99% with type or reference strains of B. tropica or B. xenovorans (Table 2). Similarly, 16S rRNA gene sequences obtained from N₂-fixing isolates that did not yield a PCR-amplified product with any of the species-specific primers tested, showed levels of identity higher than 99% with type or reference strains of B. terrae, B. phymatum or B. vietnamiensis (Table 2).

Protein electrophoregrams

Diazotrophic isolates that showed 99% identity in 16S rRNA sequences with B. tropica, B. xenovorans or B. vietnamiensis strains, also showed identical or almost identical SDS-PAGE protein profiles (evaluated by visual comparison) to those from type strains of these species (Fig. 3; data not shown for B. vietnamiensis).

Transmissibility marker genes

Two transmissibility factors associated with the highly transmissible epidemic strains of B. cenocepacia were analyzed both by PCR-amplification and Southern blot assays. Neither cblA nor esmR marker genes were detected among diazotrophic isolates by PCR amplification (data not shown).

Detecting diazotrophic Burkholderia occurrence in soil with 16S rRNA species-specific primers

PCR-amplified products, using total DNA from rhizosphere soil, were not obtained with any of the species-specific primers tested in three different assays. On this basis, total DNA recovered was subjected to PCR using the GB-F/GB-R primer pair (specific for genera Burkholderia and Ralstonia), and the amplified products were used as a template in PCR assays using the specific primers and conditions described in Table 1. With this procedure, DNA bands of the expected size were amplified exclusively with species-specific primers designed for B. tropica and B. xenovorans (data not shown). Nevertheless, additional DNA bands were
amplified with DNA extracted from control strains in nested PCRs (data not shown). Gene sequences of these PCR-amplified products (bands of 356 bp for \textit{B. tropica}, and 580 bp for \textit{B. xenovorans}) matched at 99% identity levels with \textit{B. tropica} strains (e.g., NCBI sequence database, acc. numbers AB252074, AF139183 and AY128110) described in other studies [9,37], and with \textit{B. xenovorans} strains B2-5 (acc. no. EF467847) and LB400\textsuperscript{T} (CP000271 chromosome 2 and CP000270 chromosome 1), ranging from an identity of 98–99%.

Table 2

<table>
<thead>
<tr>
<th>No. of (N_2)-fixing isolates recovered from plants\textsuperscript{a}</th>
<th>Species and no. of isolates identified\textsuperscript{b} by PCR-SSP\textsuperscript{b}</th>
<th>(N_2)-fixing strain 16S rRNA sequenced</th>
<th>EMBL acc. no. of 16S rRNA sequence from (N_2)-fixing strain</th>
<th>% identity 16S rRNA gene with nearest phylogenetic strain and EMBL acc. no.\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tomato (40)</strong></td>
<td>\textit{B. tropica} (30)</td>
<td>TAt-076, TAt-0720, TAt-0750</td>
<td>EU723239; \textit{B. tropica}</td>
<td>&gt; 99%; EF139182</td>
</tr>
<tr>
<td><strong>B. xenovorans (7)</strong></td>
<td>TAt-0771, TAt-07106</td>
<td>EU723243; \textit{B. xenovorans}</td>
<td>&gt; 99%; CP000271</td>
<td></td>
</tr>
<tr>
<td><strong>Burkholderia sp. (3)</strong></td>
<td>TAt-0728, TAt-0769, TAt-0745</td>
<td>FJ478404; \textit{B. terrae}</td>
<td>&gt; 99%; AB201284</td>
<td></td>
</tr>
<tr>
<td><strong>Sorghum (60)</strong></td>
<td>\textit{B. tropica} (58)</td>
<td>SCu-662, SCu-6583, SCu-7265, Str-6529, Str-6563, Str-6688</td>
<td>FJ436048; \textit{B. tropica}</td>
<td>&gt; 99%; EF139182</td>
</tr>
<tr>
<td><strong>Burkholderia sp. (2)</strong></td>
<td>Str-685, SYe-6586</td>
<td>FJ436049; \textit{B. vietnamiensis}</td>
<td>&gt; 99%; CP000270</td>
<td></td>
</tr>
</tbody>
</table>

Letters in strain designations refer to: T, tomato; S, sorghum; At, Atlatlahuacan; Cu, Cuautla; Ir, Irapuato; Ye, Yecapixtla.

\textsuperscript{a} Number of isolates is indicated in parentheses.

\textsuperscript{b} PCR-SSP, PCR species-specific primers.

\textsuperscript{c} \textit{Burkholderia} species and other accession numbers: \textit{B. tropica} (GenBank acc. no. EF139182, EF139183 and AY128105); \textit{B. xenovorans} \textit{LB400}\textsuperscript{T} (acc. no. CP000271 chromosome 2, and CP000270 chromosome 1, complete sequence) and TCo-26 (acc. no. EF139188); \textit{B. terrae} KMY01 (acc. no. AB201284); \textit{B. phymatum} STM815 (acc. no. CP0001043 and CP0001044 chromosome 1); \textit{B. vietnamiensis} G4 (acc. no. CP000614, CP000615 and CP000616, chromosomes 1, 2, and 3, respectively, complete sequence).

---

Fig. 3. Example of whole-cell protein profiles for representative diazotrophic isolates recovered in the present study, type and reference strains of known \(N_2\)-fixing \textit{Burkholderia}. \textbf{A}. \textit{B. tropica}, lanes 1–7: SCu-6583, TAt-0720, Str-6529, SCu-7265, TAt-0750, Ppe8\textsuperscript{T}, and MOc-725 (reference strain [37]). \textbf{B}. \textit{B. xenovorans}, lanes 1–5: TCo-382, CAC-124 (reference strain [22]), TAt-07106, TAt-0771, TCo-26 (reference strain [9]).

Fig. 4. Example of PCR-amplified products with 16S rRNA species-specific primers. PCR products correspond to a single squeezed nodule developed on common bean plants separately inoculated with: \textit{B. tuberum} STM678\textsuperscript{T}, lanes 2, 3, and 4; \textit{B. phymatum} STM815\textsuperscript{T}, lanes 6, 7, and 8; \textit{B. mimosarum} PAS44\textsuperscript{T}, lanes 10, 11, and 12; \textit{R. etli} CFN42\textsuperscript{T}, lanes 5, 9, and 13; 1 kb DNA ladder, lanes 1 and 14.

**PCR Identification of Burkholderia harbored in nodules**

Although the bacterial content from a single nodule was used for PCR assays, DNA bands with the expected size for each nodulating \textit{Burkholderia} species (see Table 1) were directly and consistently observed in three nodules from the same plant analyzed (Fig. 4).

**Discussion**

Although \textit{in silico} analysis of the 16S rRNA sequences revealed that some of the primers designed for the identification of \(N_2\)-fixing and legume-nodulating \textit{Burkholderia} could potentially
also amplify products from 1 or 2 (at the most) other species of this genus, in practice the PCR conditions overcome this limitation, since annealing temperature and oligonucleotide primer length are known to control the specificity of oligonucleotide hybridization [49]. In silico analysis of the 16S rRNA sequences from *Ralstonia* species revealed no possibility of amplification with the species-specific primers designed (data not shown). Accordingly, the different sets of primer pairs designed allowed rapid and highly specific PCR-based identification of the N2-fixing species *B. kururiensis*, *B. unanae*, *B. xenovorans*, *B. tropica*, and *B. silvatlantica*, as well as the legume-nodulating species *B. phymatutum*, *B. tuberum*, *B. mimosarum*, and *B. nodosa*. PCR tests with 57 strains from culture collections belonging to 36 *Burkholderia* species showed a high specificity of the primer sets in the identification of diazotrophic and nodulating strains of *Burkholderia* species, giving no PCR-amplified products even from closely related species. For example, *B. kururiensis* and *B. ferrariae* FeGI01T [45], identified as a N2-fixing species [28], *B. xenovorans* and *B. ginsengisoli* KMY03T, or *B. tuberum* and *B. ginsengisoli* and *B. phenoliruptrix* AC1100T (Fig. 1).

The suitability of the species-specific primers was confirmed with the identification of 100 diazotrophic isolates recovered in this study from the rhizosphere/rhizoplane of tomato and sorghum plants, 88 of them identified as *B. tropica* and 7 as *B. xenovorans* isolates, and reconfirmed with the 16S rRNA gene sequencing and whole-cell protein profiles of several representative strains of these *Burkholderia* species. Both tests are considered strong evidence for delineation of bacterial species [47]. Prevalence of both *B. tropica* and *B. xenovorans* isolates associated with tomato plants has been established previously, but using large and expensive approaches [9]. Although a detailed study on the community of N2-fixing *Burkholderia* species associated with sorghum has not been previously carried out, this work showed, surprisingly, an almost absolute occurrence (97%) of *B. tropica* in association with five sorghum varieties collected in regions up to 400 km apart. This finding strongly contrasts with previous studies in which, using virtually the same isolation strategy, *B. tropica* represented only a fraction of the total diazotrophic *Burkholderia* community associated with sugarcane, maize, coffee, tomato, *cblA* and *esmR* genes, related to transmissibility, have been found mainly in clinical isolates of *B. cenocepacia*, as well as in other *B. cepacia* complex species, both genes have also been identified from among a few environmental isolates of *B. cenocepacia* and *B. cepacia* [6,14,26,38]. In a previous study, *cblA* or *esmR* genes were not detected in isolates of *B. unanae*, *Burkholderia* NAR isolates (currently *B. silvatlantica*) or *B. tropica* [33], which was confirmed in the present study, as these genes were not detected in any of the diazotrophic *Burkholderia* isolates recovered from sorghum or tomato plants, confirming their restricted presence in clinical and environmental isolates of opportunistic pathogens of the *B. cepacia* complex. The almost absolute prevalence of *B. tropica*, particularly in sorghum plants, and the non-detection of *cblA* or *esmR* genes, would support the potential for using *B. tropica* as a plant growth promoting bacterium [9], since this species has the ability for improving maize plant growth (unpublished results). However, considering the very recent isolation of *B. tropica* from a neonatal patient [17], any agricultural development using such a species should evaluate potential risks to human health, although *B. tropica* has already been encountered in the rhizosphere or in endophytic association with several crop plants [9,20,33,37]. Accordingly, the *B. tropica* specific-primers could be an efficient tool for rapid identification of this species and for investigating its association with other host plants and their ecological distribution.

The specificity of the primers was reconfirmed, since several diazotrophic strains identified by their 16S rRNA gene sequences as belonging to other *Burkholderia* species (*B. vietnamiensis* and *B. terrae*), isolated from tomato and sorghum plants, did not yield PCR-amplified products. A notable exception was strain TAt-0745 isolated from tomato plants, which yielded a PCR-amplified product with primers Bphym-F/Bphym-R, and whose 16S rRNA gene sequence showed 99% identity with legume-nodulating *B. phymatutum* STM815T. On this basis, strain TAt-0745 could be assigned to the species *B. phymatutum*, although it was not recovered from a nodule of a legume plant. Interestingly, two strains (TAt-0728 and TAt-0769) associated with tomato plants were presumptively identified as *B. terrae*, a N2-fixing species, which has been taxonomically described with only one strain isolated from a forest soil in Korea [50]. This finding contributes towards extending our knowledge on the ecological distribution of *B. terrae*, and confirms the high diversity of diazotrophic *Burkholderia* associated with tomato described previously [9]. Certainly, the isolation of strains TAt-0728 and TAt-0769 will allow the design of 16S rRNA-specific primers for rapid and efficient identification of *B. terrae*.

PCR-products from diazotrophic *Burkholderia* species were not directly obtained from DNA extracted from the soil, apparently due to a low number of cells, since PCR-amplified products were obtained using the GB-F/GB-R primer pair, and further nested with species-specific *Burkholderia* primers. PCR products could be directly amplified by only using DNA extracted from soil inoculated with 106 cells from each of the nitrogen-fixing *Burkholderia* species (data not shown). Although nodules formed by *Burkholderia* on the roots of common bean plants are not very efficient at nitrogen fixation (unpublished results), the bacterial content from a single nodule was sufficient for directly amplifying products with the specific primers designed for the identification of the species *B. phymatutum*, *B. tuberum*, *B. mimosarum*, and *B. nodosa*. The efficiency of the specific primers for the identification of these legume symbionts directly from a single nodule could extend our knowledge on the natural host range of the nodulating *Burkholderia* species. In addition, this could be particularly significant when the sampling of nodules from legumes growing in natural conditions is restricted in order to avoid disturbance of the ecosystem, or when the number of legume plants for sampling is limited.

Apparently, there is a huge interest in N2-fixing and legume-nodulating *Burkholderia* species. Such interest is revealed by the increasing number of recent studies on the geographic and environmental distribution of diazotrophic and legume-nodulating *Burkholderia* species, including diversity and host-plant range [4,5,9,11,13,18,19,31,33], as well as on their plant growth promotion abilities [9,18,19,23,31], and other biotechnological applications, such as xenobiotic degradation processes or biological control [9,51]. In fact, complete genomes of diazotrophic (e.g., *B. unanae* MTI-641T and *B. silvatlantica* SRMh-207) and nodulating *Burkholderia* strains (e.g., *B. tuberum*) are being sequenced (http://genomesonline.org/index2.htm). Accordingly, it could be expected that more diazotrophic and nodulating *Burkholderia* isolates could be recovered in the future. On this basis, the species-specific primers designed in this study could contribute to an easy and correct identification – neither laborious nor time consuming – of many diazotrophic and legume-nodulating *Burkholderia* isolates, as well as determining their environmental distribution.

**Acknowledgments**

We thank L. Martínez-Aguilar for technical assistance, and A. Ramírez-Trujillo for his valuable advice on nodulation tests.
We are grateful to Michael Dunn (CCG-UNAM) for reading the manuscript. We acknowledge Euan K. James (University of Dundee, U.K.) and W.-M Chen (National Kaohsiung University, Taiwan) for supplying B. mimosarum and B. noda strains. We also thank J.J. Peña-Cabriales and J.A. Vera (CINVESTAV-Irapuato, Mexico) for sorghum plant collection, and José Leyva for tomato collection. A. Wong-Villarreal is a Ph.D. student in the Programa de Doctorado en Biotecnología-CEIB/UAEM, who acknowledges fellowships (Register No. 181684) from the Consejo Nacional de Ciencia y Tecnología (CONACYT) Mexico.

References


