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TAXONOMÍA DE BURKHOLDERIAS FIJADORAS DE NITRÓGENO ASOCIADAS AL MAÍZ

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The adequacy of characterization of a bacterium is a reflextion of time; it should be as full as modern techniques make possible. Unfortunately, one now regarded as adequate is likely, in 10 years time, to be hopelessly inadequate!

Cowan, 1965.

INDICE

RESUMEN

En general se acepta que la fijación biológica de nitrógeno es un proceso llevado a cabo por un reducido número de especies bacterianas. Desde la creación del género *Burkholderia* en 1992 y durante varios años, este género ha estado representado por una sola especie diazótrofa, *B. vietnamiensis*, descrita en 1995. La investigación desarrollada en esta tesis doctoral revela la existencia de múltiples especies fijadoras de nitrógeno del género *Burkholderia* así como su asociación rizosférica y endófita con plantas de interés agrícola como maíz, sorgo, caña de azúcar y cafeto cultivadas en diferentes regiones geográficas de México y también en asociación con plantas de teocintle creciendo en forma silvestre. El agrupamiento de los aislados diazótrofos del género *Burkholderia* mediante ARDRA (Amplified rDNA Restriction Analysis) mostró la existencia de 7 especies desconocidas. El análisis taxonómico con un enfoque polifásico, que incluye metodologías que revelan las características fenotípicas y genómicas, de dos de los grupos diazótrofos aislados con mayor frecuencia reveló que ambos corresponden a nuevas especies. Una de éstas será descrita con el nombre de *Burkholderia tropicalis* y la otra con el de *Burkholderia unamae*. La caracterización de las especies diazótrofas del género *Burkholderia* y su comparación con otras conocidas reveló la capacidad diazótrofa de las especies *B. caryophylli* y *B. kururiensis*. Los resultados en conjunto contribuyen a mostrar que la fijación de nitrógeno no es un mecanismo restringido a un número reducido de especies bacterianas. Además, en este trabajó se demostró por primera vez la asociación endófita de *B. vietnamiensis* con plantas cultivadas en el campo. Es conocido que el género *Burkholderia* es bioquímicamente versátil, característica que ha sido aprovechada en procesos biotecnológicos. Considerando estos antecedentes, resultaría interesante y de gran valor determinar la capacidad de *B. "tropicalis"* y *B. "unamae"* para expresar algunas de las características que han dado importancia biotecnológica a este género, tal es el caso de las actividades que involucran el control biológico de fitopatógenos, la degradación de compuestos xenobióticos o la producción de compuestos de interés en la industria. También de gran importancia será el evaluar la capacidad de *B. "tropicalis"* y *B. "unamae"* para promover el crecimiento de las plantas con las que se asocia así como para disminuir el uso de los fertilizantes y el impacto de estos en el ambiente.

SUMMARY

The ability to fix nitrogen among the microorganisms seems to be rare according to the small number of diazotrophic species described. For a long time N_2-fixing ability was only recognized in *B. vietnamiensis*. In the present study it was showed the existence of many N_2-fixing *Burkholderia* species, as well as, their rhizospheric and endophytic association with maize, sorghum, sugarcane, teosinte and coffee cultivated on distant geographical regions in Mexico. The clustering of the N_2-fixing strains carried out by ARDRA (Amplified rDNA Restriction Analysis) reveled 7 unknown species. The taxonomic analysis using a polyphasic approach included strategies that showed the phenotypic and genomic features from two selected groups of diazotrophs. These bacteria belong to two new species, *B. tropicalis* and *B. unamae* will be the names proposed. The analysis of the capacity to fix nitrogen by *Burkholderia* isolates included also described *Burkholderia* species. Interestly, *B. caryophylli* and *B. kururiensis* were able to express this activity. This results show that the capacity to fix nitrogen in the genus *Burkholderia* it is not restricted to one species. More over, this is the first time that was showed the endophytic association of *B. vietnamiensis* to field plants. It is well known that the genus *Burkholderia* is biochemicaly versatile, feature that has been used in biotechnology. Taking this into account it would be worth to explore weather *B. "tropicalis"* and *B. "unamae"* share the same features described on *Burkholderia* species, such as biological control, pesticides degradation or production of biotechnological important compounds. It would be also important to evaluate the capacity of *B. "tropicalis"* and *B. "unamae"* to promote plant growth.

INTRODUCCIÓN

Se considera que las primeras formas de vida que habitaron nuestro planeta, hace alrededor de 3,700 millones de años, fueron los microorganismos. Estos seres diminutos que escapan a la visión humana y que normalmente pueden ser vistos a través de un microscopio, han servido como base para crear la atmósfera de nuestro planeta y contribuido a generar la vida que conocemos actualmente. El mundo microbiano es basto en número y diversidad, siendo cerca de 6,000 especies bacterianas las que se conocen en la actualidad (Staley y Gosink 1999). Es conocido que la distribución de las bacterias es cosmopolita. Los estudios de biogeografía y biodiversidad han revelado la existencia de bacterias en ambientes muy diversos, ampliándose con esto las fronteras del conocimiento sobre las estrategias y límites de la vida (Staley y Gosink 1999; Tiedje y Stein 1999). El cultivo de los microorganismos ha sido tradicionalmente una herramienta útil para aislar y determinar la diversidad bacteriana existente en distintos ambientes. Sin embargo, se sabe que este tipo de procedimientos pueden conducir a la obtención de resultados y conocimientos parciales (Wagner y col. 1993). Un gramo de suelo puede contener hasta 10 mil millones de microorganismos pertenecientes, posiblemente, a miles de especies diferentes (Roselló-Mora y Amann 2001). La última década ha traído consigo el desarrollo de diferentes métodos moleculares que han revolucionado el concepto de la diversidad bacteriana. Estos métodos se basan típicamente en el aislamiento de ADN microbiano directamente de un ambiente como el suelo o una planta y se complementan con el uso de la reacción en cadena de la polimerasa (PCR) para amplificar la secuencia de ADN de interés. En el caso de los estudios de taxonomía y diversidad bacteriana el análisis del gen ribosomal 16S ha sido de gran utilidad, no obstante, cualquier secuencia conservada de ADN puede servir para el mismo propósito. En la actualidad, el número de especies bacterianas en el planeta se estima en el rango 10,000 a más de 10^9 (Staley y Gosink 1999), lo cual sobrepasa con mucho las 6000 especies descritas.

El estudio sobre la diversidad de bacterias descritas ha permitido el aprovechamiento de infinidad de microorganismos con fines biotecnológicos. Sin embargo, el aislamiento bacteriano ha limitado el conocimiento sobre la diversidad bacteriana real. Los estudios filogenéticos sobre bacterias que no han sido cultivadas podrían contribuir al aislamiento de estos microorganismos basándose en las propiedades de las especies cultivables más

cercanas. El aislamiento de microorganismos no cultivados puede conducir a conocer nuevas o mejores propiedades de las conocidas en la actualidad y que pudieran ser de gran utilidad biotecnológica. La conservación y explotación de la biodiversidad es una prioridad a nivel mundial.

El conocimiento de la diversidad microbiana de un hábitat como el suelo, una planta o el tracto digestivo de una termita es considerado de gran importancia debido a que puede conducir a un mejor y mayor entendimiento de la ecología de ese ambiente. Por esta razón se requiere de un censo en nuestro planeta para conocer los microorganismos que lo habitan y determinar cual es el papel que desempeñan en los distintos ambientes.

El aislamiento bacteriano es definitivamente un punto esencial para describir taxonómicamente una nueva especie, así como para estudiar sus características fisiológicas y genéticas y de esta manera conocer, por ejemplo, la diversidad metabólica bacteriana. Desde el reconocimiento de las bacterias, hace apenas unos pocos cientos de años, miles de ellas han sido identificadas surgiendo la necesidad de organizarlas en grupos con características similares. La taxonomía bacteriana se inició con el estudio de las características fenotípicas de los microorganismos, no obstante, con el transcurso del tiempo fueron apareciendo nuevas herramientas que a su vez mejoraron el sistema de clasificación. El conocimiento basado en los estudios de la fisiología bacteriana y de las propiedades del ADN trajo consigo la quimiotaxonomía y la biología molecular, respectivamente. Particularmente, los estudios de ADN condujeron al análisis del gen ribosomal 16S. Esta molécula y su homóloga en los organismos eucariotes (gen 18S ribosomal) permitieron definir el "árbol universal de la vida", constituido por los dominios Archaea, Bacteria y Eucarya (Woese y col. 1990). Sin embargo, cada uno de los métodos utilizados en la identificación, tanto de quimiotaxonomía como de ADN, producen resultados parciales en la caracterización de una especie bacteriana. Esto se debe a que las técnicas por si solas no poseen el suficiente poder de resolución para identificar a un microorganismo particular, ya que solamente se analiza una pequeña parte de la información total de la bacteria. En general, los taxónomos concuerdan en la actualidad en que para obtener una clasificación confiable se deben utilizar herramientas que exploren las características fenotípicas y genómicas de una especie bacteriana. La aplicación de estas estrategias se ha denominado taxonomía polifásica (Vandamme y

col. 1996), la cual es esencial para definir una nueva especie de acuerdo al Código Internacional en Nomenclatura Bacteriana (CINB). Una especie bacteriana es referida como un grupo de cepas que comparten un nivel de reasociación de 70 % o mayor y con una ΔTM de 5 ºC o menor (Vandamme y col. 1995). Como la anterior existen muchas mas definiciones de especie (Roselló-Mora y Amann 2001), esto se debe a que actualmente existe un debate sobre el significado de este término (Cohan 2002). No obstante, esta definición es funcional y aun cuando los métodos utilizados en la identificación bacteriana que existen en la actualidad no son estrictamente perfectos para definir una especie bacteriana, cumplen con los objetivos principales de la taxonomía, son rápidos y confiables. Además, para cumplir con estos requisitos es de gran importancia que la clasificación de una especie esté basada en el análisis del mayor número de cepas posibles. Se ha calculado que 25 cepas son necesarias para la adecuada descripción de una especie bacteriana, en tanto que el límite menor aceptable es de 10 cepas (Roselló-Mora y Amann 2001). Este número, desafortunadamente, no ha sido tomado en cuenta en la descripción de numerosas nuevas especies, existiendo publicaciones que basaron la descripción en una sola cepa, por ejemplo, *Burkholderia kururiensis* (Zhang y col. 2000), *B. sacchari* (Brämer y col. 2001), *B. thailandensis* (Brett y col. 1998) y *B. uboniensis* (Yabuuchi y col. 2000).

El Comité Internacional en Bacteriología Sistemática ha propuesto la categoría provisional de *Candidatus* para facilitar el manejo de la información obtenida de bacterias no cultivables. La definición de *Candidatus* se refiere a una bacteria de la cual solamente se conoce una secuencia de ADN pero las características para definirla como nueva especie, según el CINB, son desconocidas (Murray y Stackebrandt 1995).

ANTECEDENTES GENERALES

Los microorganismos son esenciales para el funcionamiento de los ciclos biogeoquímicos en nuestro planeta lo cual es fundamental para el mantenimiento de la vida (Madigan y col. 1997). El nitrógeno forma parte de uno de los ciclos más importantes debido a que es una molécula primordial para el crecimiento de los seres vivos. Este elemento es requerido para la síntesis de proteínas y ácidos nucleicos, entre otras moléculas. El mayor reservorio de nitrógeno en nuestro planeta lo constituyen las rocas primarias (98 %) (Foth y Ellis 1988), en donde el nitrógeno se encuentra atrapado como molécula de N_2 (Burns y Hardy 1974). Sin embargo, es el N_2 de la atmósfera (2 %) la fuente principal para el suelo debido a que el atrapado en las rocas primarias no puede ser aprovechado directamente por las plantas. En la atmósfera, el N_2 constituye el 79 % de todos los elementos que la integran y son los organismos ubicados en los dominios Archaea y Bacteria los únicos con la capacidad enzimática de romper y utilizar esta molécula. Este proceso es denominado fijación biológica de nitrógeno (FBN). El conocimiento derivado sobre los estudios de la FBN ha mostrado que este proceso en la naturaleza es realizado por un pequeño número de especies bacterianas. Algunas familias y géneros fijadores de nitrógeno se muestran en la Tabla 1. Debido a que el N_2 constituye hasta ahora el reservorio más importante de nitrógeno disponible para los organismos vivientes, la capacidad para utilizar este elemento es de gran importancia ecológica.

La FBN es un proceso en el cual se requiere una gran cantidad de energía, bajo condiciones óptimas la nitrogenasa cataliza la siguiente reacción (Eady 1996):

$$\mathbf{N_2 + 8e^- + 8H^+ + 16ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16Pi}$$

La enzima responsable de llevar a cabo la reacción anterior es la nitrogenasa. Esta enzima es extremadamente sensible al oxígeno y se encuentra formada por dos subunidades. La subunidad I, Fe+Mo (dinitrogenasa reductasa) codificada por los genes *nifDK* y la subunidad II, Fe (dinitrogenasa) codificada por el gen *nifH* (Burgess y Lowe 1996). Esta nitrogenasa posee molibdeno (Mo) en su estructura, no obstante, existen otros tipos de nitrogenasas que contienen otros metales, por ejemplo, una de ellas posee vanadio (V) en la subunidad I (genes *vnfDGK*), en tanto que existe una tercera

que solamente posee fierro (Fe) en su estructura (genes *anfDGK*) (Eady 1996). El cuarto tipo de nitrogenasa es completamente distinto a los tres anteriores. Esta nitrogenasa fue detectada en la especie *Streptomyces thermoautotrophicus* e involucra tres componentes, St1 (dinitrogenasa reductasa, MoFeS), St2 (manganeso superóxido reductasa) y St3 (CO-deshidrogenasa) (Gadkari y col. 1992; Ribbe y col. 1997). La diferencia de esta nitrogenasa con las tres anteriores radica en su insensibilidad al oxigeno y a su incapacidad para reducir el acetileno a etileno o etano; ésta, es una actividad que rutinariamente se utiliza para detectar y/o medir la actividad nitrogenasa. De los 4 sistemas descritos, el más estudiado corresponde al de la nitrogenasa que contiene FeMo.

Existen bacterias diazótrofas que poseen más de un tipo de nitrogenasa como es el caso de *Klebsiella pneumoniae*, distintas especies de *Azotobacter* y *Clostridium*, entre otras (Newton 1993) y existen también, bacterias fijadoras de nitrógeno que poseen más de una copia de los genes que codifican para esta enzima, por ejemplo, *Rhizobium etli* alberga tres copias del gen *nifH* y dos de los genes *nifDK* (Quinto y col. 1985).

La amplificación y secuencia del gen *nifH* han servido para conocer la diversidad de bacterias diazótrofas en distintos ambientes sin necesidad de utilizar medios de cultivo, por ejemplo, se han analizado ambientes como el suelo (Rösch y col. 2002), la rizosfera (Lovell y col. 2000; Ueda y col. 1995), los océanos (Zehr y col. 1998) o el intestino de diversas termitas (Ohkuma y col. 1999). Estos trabajos han descrito una gran cantidad de secuencias *nifH* desconocidas, las cuales parecen corresponder a especies bacterianas no identificadas.

El interés que se ha tomado por las bacterias fijadoras de nitrógeno radica en la posibilidad de que donen el nitrógeno fijado a plantas de interés agrícola, contribuyendo a resolver de esta manera algunos de los problemas causados por el uso de los fertilizantes provenientes de la agricultura intensiva además de disminuir el uso de los recursos naturales no renovables utilizados para la producción de fertilizantes a través de síntesis química (Foth y Ellis 1988). Un ejemplo de lo anterior es el mayor florecimiento de las algas en las aguas costeras, por lo cual se ha formado una gran zona "sin oxígeno" ("sin vida") en el Golfo de México (Tilman y col. 2001). Es importante tomar en consideración que el incremento en el uso de los fertilizantes puede conducir en un futuro a pérdidas significativas en la biodiversidad así como a cambios en la

composición y funcionamiento de los ecosistemas terrestres y acuáticos (Tilman y col. 2001). Con una población mundial de 6,000 millones de personas en la actualidad, que se estima aumentará a 7,500 millones para el año 2020 y a cerca de 9,000 millones en el año 2050 (Tilman y col. 2001), tendrán que realizarse importantes avances en la agricultura para tratar, por un lado, de sostener la alimentación mundial y por otro minimizar el daño que produciría el uso de los fertilizantes requerido para mantener el nivel de tal producción alimenticia.

Una de las alternativas propuestas para combatir los problemas generados por el uso de los fertilizantes es el estudio de la diversidad de las bacterias diazótrofas. En el dominio Bacteria existen microorganismos que fijan nitrógeno en vida libre o en asociación con organismos superiores. Entre estas últimas, destacan las asociaciones de *Rhizobium*-plantas leguminosas, *Frankia*-plantas actinorrícicas y cianobacterias-plantas primitivas. El resultado de las dos primeras simbiosis es la formación de un nódulo en el cual la bacteria fija nitrógeno proporcionándoselo a la planta y ésta a su vez le retribuye a la bacteria con fuentes de carbono. Recientemente, fue mostrada la capacidad de *Burkholderia* y *Ralstonia* para formar nódulos en asociación con plantas leguminosas, (Chen y col. 2001; Moulin y col. 2001; Moulin y col. 2002). Estos dos géneros bacterianos se encuentran clasificados dentro de la subclase β-Proteobacteria, en tanto que los distintos géneros del grupo de los rhizobia se ubican en la subclase α-Proteobacteria (Tabla 1), éstos habían sido considerados tradicionalmente y hasta ese momento como las únicas bacterias capaces de nodular plantas leguminosas. Para estos grupos de microorganismos se han propuesto los términos de α-rhizobia y β-rhizobia (Moulin y col. 2002). A diferencia de los diazótrofos simbióticos las bacterias de vida libre, como su nombre lo indica, pueden fijar nitrógeno sin formar una simbiosis con organismos superiores. Las bacterias diazótrofas que colonizan las raíces de las plantas han sido denominadas como asociativas (Phillips y Martínez-Romero 2000). Algunos de los géneros diazótrofos más documentados en la literatura son *Azoarcus*, *Azospirillum*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum* y *Klebsiella*, entre otros. Estos géneros bacterianos se han encontrado asociados a plantas como maíz, trigo, arroz y café, entre otras. Una lista detallada sobre los sistemas de fijación de nitrógeno asociativos mas estudiados se muestra en la Tabla 2. En los años recientes, especial interés ha recaído en aquellos géneros bacterianos endófitos, entre ellos *Gluconacetobacter*, *Azoarcus* y

Herbaspirillum dada la posibilidad de que aporten el nitrógeno fijado directamente en el interior de la planta.

Es importante, como se mencionó anteriormente, conocer la diversidad bacteriana en el ambiente e investigar la existencia de otras asociaciones entre plantas y bacterias. El propósito final es tratar de llegar, por ejemplo, a una agricultura sostenible y para ello es de fundamental importancia la búsqueda, el aislamiento y la caracterización de bacterias que puedan cumplir con este objetivo. Por esta razón, en los años recientes el género *Burkholderia* ha cobrado gran importancia debido a que algunas de sus especies expresan distintas capacidades que pudieran ser aprovechadas en la biotecnología agrícola, entre ellas la fijación de nitrógeno.

El género *Burkholderia*, transferido del género *Pseudomonas*, fue descrito en 1992 por Yabuuchi y col. con base en una caracterización fenotípica que incluyó la composición de ácidos grasos y lípidos celulares, y una caracterización genómica en la cual se determinaron la secuencia del gen ribosomal 16S y valores de reasociación ADN-ADN. El género *Burkholderia* se encuentra ubicado en la familia Burkholderiaceae en la subclase de las Beta proteobacterias (Tabla 1). En su origen el grupo *Burkholderia* se encontraba constituido por 7 especies, sin embargo, dos de ellas fueron reclasificadas posteriormente en el género *Ralstonia* (Yabuuchi y col. 1995). Desde la descripción del género *Burkholderia* ha surgido un número importante de especies, ascendiendo en la actualidad a 28 (Tabla 3). El género *Burkholderia* se encuentra constituido también por un grupo de especies y genomovares que han sido denominadas como el "complejo *B. cepacia*". Un genomovar es definido como una nueva especie, la cual, puede ser distinguida de otras especies del género por sus características genómicas pero no por características fenotípicas. El "complejo *B. cepacia*" ha estado constituido hasta por 9 genomovares; los genomovares II, IV, V, VII, VIII y IX han sido nombrados como *B. multivorans*, *B. stabilis*, *B. vietnamiensis*, *B. ambifaria*, *B. anthina* y *B. pyrrocinia*, respectivamente. Los genomovares I, III y VI aún no han sido descritos formalmente. La cepa tipo de *B. cepacia* se encuentra representada por el genomovar I. Existe también la propuesta de 5 nuevas especies, *B. "brasilensis"*, *B. "sordicola"* , *B. "tropicalis"*, y *B. "kirkii"* como especie "Candidatus" (Van Oevelen y col. 2002).

El género *Burkholderia* es bastante versátil en cuanto a su capacidad para habitar distintos ambientes (Tabla 3). Por ejemplo, algunas de sus especies se han encontrado

en el suelo, en ambientes húmedos, asociadas o como patógenos de plantas, residentes de hongos e incluso existen varias especies patógenas para el ser humano. *B. cepacia*, *B. mallei* y *B. pseudomallei*, son tres de las especies del género más importantes en el área de la salud debido a que causan enfermedades en el humano. *B. cepacia* surgió en los '80s como un patógeno oportunista en pacientes con la enfermedad genética fibrosis quística, FQ (Jones y col. 2001), *B. mallei* causa la enfermedad llamada "glanders" (Woods y Sokol 2000) y *B. pseudomallei* la enfermedad denominada melioidosis (Dance 2000).

La FBN en el género *Burkholderia* se resumía en 1995 a una sola especie, *B. vietnamiensis* (Gillis y col. 1995). Esta especie fue aislada de la rizosfera de plantas de arroz inoculadas con suelo proveniente de Vietnam y cultivadas en el laboratorio. Posteriormente, en el año 2001 se encontró que una "cepa" de *Burkholderia* sp., endosimbionte obligado del hongo micorrícico *Gigaspora margarita*, poseía los genes para llevar a cabo la FBN (Minerdi y col. 2001). En este mismo año se encontró que *B. kururiensis*, aislada de un manto acuífero contaminado con tricloroetileno (Zhang y col. 2000), poseía los genes *nifHDK* así como ser capaz de reducir acetileno en diferentes medios de cultivo (Estrada-de los Santos y col. 2001). Recientemente, tres cepas del género *Burkholderia* fueron aisladas de los nódulos de plantas leguminosas, todas ellas capaces de formar nódulos y aun cuando se detectó la presencia del gen *nifH* solamente una de ellas fijó nitrógeno en simbiosis con muchas plantas leguminosas tropicales (Moulin et al., 2001; 2002). Dos de estas cepas han sido propuestas como *B. tuberum* y *B. phymatum* (Vandamme y col. 2002). Además de las tres cepas nodulantes de *Burkholderia* también fueron detectadas dos cepas en los nódulos de plantas leguminosas que taxonómicamente corresponden a *B. caribensis*, ambas cepas fueron capaces de nodular y fijar nitrógeno en el nódulo (Moulin com. pers.; Vandamme y col. 2002). *B. "brasilensis"* y *B. "tropicalis"*, dos especies diazótrofas que han sido propuestas recientemente, fueron aisladas del interior de plantas de arroz y caña de azúcar, respectivamente (Hartman y Reis, com. pers.). Resultaría interesante determinar si las especies diazótrofas *B. vietnamiensis*, *B. "tropicalis"* y *B. "brasilensis"*, son capaces de nodular plantas leguminosas.

Existen otras capacidades que poseen algunas de las especies del género *Burkholderia*, tal es el caso del biocontrol de patógenos de plantas que ejerce *B.*

cepacia, entre ellos *Rhizoctonia solani* (Kang y col. 1998), *Phytium* spp. y *Fusarium* spp. (Hebar y col. 1998), los cuales deben su importancia en la agricultura por ocasionar la enfermedad llamada "damping-off" (ahogamiento). *B. cepacia* ha ofrecido una alternativa al uso de fungicidas los cuales ocasionan efectos dañinos en la salud humana. El mecanismo de biocontrol por *B. cepacia* es desconocido, sin embargo, frecuentemente se le atribuye a la producción de metabolitos antifúngicos que incluyen antibióticos y sideróforos. Otra capacidad expresada por cepas de *B. cepacia* es la de degradar compuestos complejos por lo que se ha propuesto su uso en la biorremediación de suelos y mantos acuáticos contaminados con herbicidas (Haugland y col. 1990) e hidrocarburos clorados (Folsom y col. 1990). Las lipasas producidas por *B. cepacia* y *B. glumae* son comercializadas para la producción de detergentes e ingredientes de alimentos, entre otros usos (Jaeger y Reetz 1998). La producción de polímeros relacionados al compuesto PHB (polibetahidroxibutirato) con propiedades termoplásticas y de biodegradabilidad es de gran importancia comercial (Steinbüchel 1991). Algunas cepas mutantes de *B. cepacia* son capaces de sintetizar este compuesto con gran eficiencia, reduciendo los costos de producción (Silva y col. 2000). En experimentos de inoculación se observó que *B. vietnamiensis* es capaz de promover el crecimiento de plantas de arroz, así como de aumentar la producción del grano (Trân Van y col. 2000). La fijación de nitrógeno es el mecanismo al que se atribuye el efecto observado. A pesar de todas las ventajas descritas, *B. vietnamiensis*, *B. cepacia* y el resto de las especies que constituyen el "complejo *B. cepacia*" han sido aisladas, desafortunadamente, de las expectoraciones de pacientes que padecen FQ. La FQ es una enfermedad genética y fatal que afecta aproximadamente a 30,000 niños y adultos en los Estados Unidos (Parke y Gurian-Sherman 2001). Considerando la tasa de mortalidad de pacientes con FQ que adquieren *B. cepacia* (aproximadamente el 20 % de ellos experimentan una afección pulmonar fatal), se ha propuesto una moratoria sobre el uso de las especies del "complejo *B. cepacia*" en experimentos dirigidos a la agricultura (Holmes y col. 1998). En enero de 2002, la Agencia de Protección al Medio Ambiente (EPA por sus siglas en inglés) propuso que el uso de las especies del "complejo *B. cepacia*" se restringiera a la investigación.

Tabla 1. Microorganismos fijadores de nitrógeno.

Dominio Bacteria	Familia	Género
1 Bacterias púrpuras (Proteobacterias)		
1.1 Alfa	Acetobacteraceae	*Gluconacetobacter*
		Rhodopila
	Gpo. Rhizobiaceae	
	"Beijerinckiaceae"	*Beijerinckia*
		*Derxia**
	"Bradyrhizobiaceae"	*Bradyrhizobium*
		*Photorhizobium^*
		Rhodoblastus
		Rhodopseudomonas
	Brucellaceae	*Mycoplana*
	Hiphomicrobiaceae	*Ancylobacter*
		Azorhizobium
		Blastochloris
		Rhodomicrobium
		Xanthobacter
	"Methylocystaceae"	*Methylocystis*
		Methylosinus
	"Methylobacteriaceae"	*Methylobacterium*
	"Phyllobacteriaceae"	*Mesorhizobium*
	Rhizobiaceae	*Agrobacterium*
		Rhizobium
		Sinorhizobium
	"Rhodobiaceae"	*Rhodobium*
	"Rhodobacteraceae"	*Rhodobacter*
		Rhodovolum
	Rhodospirillaceae	*Azospirillum*
		Magnetospirillum
		Phaeospirillum
		Rhodospirillum
	No clasificada	*Aquaspirillum*
		Renobacter
1.2 Beta	Alcaligenaceae	*Alcaligenes*
	"Burkholderiaceae"	*Burkholderia*
	Comamonadaceae	[*Pseudomonas*]
		Rubrivivax
	"Oxalobacteraceae"	*Herbaspirillum*
	"Ralstoniaceae"	*Ralstonia*
	"Rhodocyclaceae"	*Azoarcus*
		Rhodocyclus
1.3 Gamma	Chromatiaceae	*Allochromatium*
		Marichromatium
		Thiocapsa
		Thiococcus
		Thiocystis

Continúa en la siguiente página

Continuación Tabla 1.

Dominio Bacteria	Familia	Género
1 Bacterias púrpuras (Proteobacterias)		
1.3 Gamma	Ectothiorhodospiraceae	*Ectothiorhodospira*
	Enterobacteriaceae	*Citrobacter*
		Enterobacter
		Klebsiella
		Pantoea
		Raoultella
	Methylococcaceae	*Methylobacter*
		Methylococcus
		Methylomonas
	Pseudomonadaceae	*Azomonas*
		*Azorhizophilus^*
		Azotobacter
		Pseudomonas
	Beggiatoaceae	*Beggiatoa*
	Vibrionaceae	*Vibrio*
		Listonella
	No clasificada	*Acidithiobacillus*
1.4 Delta	"Desulfovibrionaceae"	*Desulfovibrio*
	Thermodesulfobacteriaceae	*Thermodesulfobacterium*
	No clasificada	*Desulfobacter*
		Desulfomicrobium
1.5 Epsilon	Campylobacteraceae	*Arcobacter*
2 Bacterias Gram +		
2.1 Firmibacteria (bajo contenido de GC)		
	Clostridiaceae	*Clostridium*
	"Heliobacteriaceae"	*Heliobacillus*
		Heliobacterium
	"Paenibacillaceae"	*Paenibacillus*
	Peptococcaceae	*Desulfosporosinus*
		Desulfotomaculum
		Propionispira
2.1 Firmibacteria (alto contenido de GC)		
	Frankiaceae	*Frankia*
	Propionibacteriaceae	*Propionibacterium*
	Streptomycetaceae	*Streptomyces**
3 Bacterias verde-sulfurosas		
	Chlorobiaceae	*Chlorobium*
		Chloroherpeton
		Pelodyction
		Prosthecochloris

Continúa en la siguiente página

Continuación Tabla 1.

Dominio Bacteria	Familia	Género
4 Cianobacterias (Algas azul-verde)		
	Chroococcales (orden)	*Cyanothece*
		Gloeocapsa
		Gloeothece
		Synechococcus
		Synechocystis
	Nostocales (orden)	*Anabaena*
		Anabaenopsis
		Aphanizomenon
		Calothrix
		Cylindrospermum
		Nodularia
		Nostoc
		Scytonema
		Tolypothrix
	Oscillatoriales (orden)	*Lyngbya*
		Microcoleus
		Oscillatoria
		Phormidium
		Plectonema
		Pseudanabaena
		Spirulina
		Trichodesmium
	Pleurocapsales (orden)	*Chroococcidiopsis*
		Dermocarpa
		Myxosarcina
		Pleurocapsa
		Stanieria
		Xenococcus
	Prochloraceae	*Prochloron*
		Richelia
	Stigonematales (orden)	*Chlorogloeopsis*
		Fischerella
		Mastigocladus

Dominio Archaea	Familia	Género
Reino Euryachaeota		
Halófilos extremos	Halobacteriaceae	*Halobacterium*
Methanobacteriales	Methanobacteriaceae	*Methanobacterium*
		Methanothermobacter
	Methanothermaceae	*Methanothermus*
Methanococcales	Methanococcaceae	*Methanococcus*
		Methanothermococcus
Methanomicrobiales	Methanomicrobiaceae	*Methanoplanus*
Methanosarcinales	Methanosarcinaceae	*Methanosarcina*
		Methanolobus

"", nombre propuesto; *, no existe en Taxonomy Browser: ^, no existe en LBSN; [], no clasificada.
Tomado de Young 1992, Phillips y Martínez-Romero 2000. Nombres científicos actualizados en el Taxonomy Browser de la base de datos NCBI (National Center for Biotechnology Information) y LBSN (Listed of bacterial names with standing in nomenclature).

Tabla 2. Bacterias diazótrofas asociativas.

Géneros bacterianos	Planta
Achromobacter	*Oryza sativa*
Arcobacter	*Spartina alterniflora*
Azoarcus	*Leptochloa fusca, Oriza* spp.
Azospirillum	*Cynodon dactylon, Digitaria decumbes, Leptochloa fusca Oriza sativa, Panicum maximum, Pennisetum purpureum Saccharum* spp., *Zea mays*
Azotobacter	*Paspalum notatum var. batatais, Pennisetum purpureum Saccharum* spp.
Beijerinckia	*Leptochloa fusca, Saccharum* spp.
Burkholderia	*Coffe arabica, Oriza sativa, Saccharum* spp., *Zea mays*
Enterobacter	*Leptochloa fusca*
Gluconacetobacter	*Coffea arabica, Saccharum* spp.
Herbaspirillum	*Pennisetum purpureum, Saccharum* spp.
Klebsiella	*Leptochloa fusca, Pennisetum purpureum, Saccharum* spp.
Pantoea	*Pennisetum purpureum*
Paenibacillus	*Saccharum spp., Triticum*
Rhizobium	*Triticum, Oriza sativa, Zea mays*

Tabla 3. Especies del género *Burkholderia.*

	Especie	Fuente de aislamiento
1.	*B. ambifaria*	Rizosfera de chícharo
2.	*B. andropogonis*	*Sorghum bicolor*
3.	*B. anthina*	Rizosfera de plantas ornamentales
4.	*B. "brasilensis"* *	Endófito de *Oryza sativa*
5.	*B. caledonica*	Rizosfera
6.	*B. caribensis*	Suelo
7.	*B. caryophylli* ¥	*Dyanthus caryophillus*
8.	*B. cepacia*	*Allium cepa*
9.	*B. fungorum*	*Phanerochaete chrysosporium*
10.	*B. gladioli*	*Gladiolus* sp.
11.	*B. glathei*	Suelo
12.	*B. glumae*	*Oryza sativa*
13.	*B. graminis*	Rizosfera de maíz
14.	*B. hospita*	Suelo agrícola
15.	*B. kirkii* "Candidatus"	*Psychotria kirkii*
16.	*B. kururiensis* ¥	Agua contaminada con TCE
17.	*B. mallei*	Glándula humana
18.	*B. multivorans*	Paciente con FQ
19.	*B. phenazinium*	Suelo
20.	*B. phymatum***	Nódulos de *Machaerium lunatum*
21.	*B. plantarii*	*Oryza sativa*
22.	*B. pseudomallei*	Infección humano
23.	*B. pyrrocinia*	Desconocida
24.	*B. sacchari*	Suelo de plantación de caña de azúcar
25.	*B. "sordicola"*	*Phanerochaete sordida*
26.	*B. stabilis*	Paciente con FQ
27.	*B. terricola*	Suelo agrícola
28.	*B. thailandensis*	Suelo de plantación de arroz
29.	*B. "tropicalis"* ¥	Endófito de caña de azúcar
30.	*B. tuberum* **	Nódulos de *Aspalathus carnosa*
31.	*B. ubonensis*	Suelo
32.	*B. vietnamiensis* *	Rizosfera de arroz

* Especies fijadoras de nitrógeno. ** Especies que albergan los genes de nodulación. ¥ Especies descritas como diazótrofas en esta tesis.
TCE, Tricloroetileno. FQ, Fibrosis quística.

ANTECEDENTES PARTICULARES

En diferentes ocasiones se ha observado la presencia de bacterias fijadoras de nitrógeno en el interior de distintas plantas sin que estas causen un daño aparente a sus hospederos. Algunas de las especies diazótrofas descritas como endófitas son *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp. y *Azoarcus* spp., entre otras. Dentro del género *Burkholderia* se reportan dos especies diazótrofas endófitas, *B.* "*brasilensis*" y *B.* "*tropicalis*".

El trabajo que antecedió esta tesis doctoral fue dirigido al aislamiento de bacterias diazótrofas endófitas de plantas de maíz con el objetivo de conocer la diversidad bacteriana asociada a esta planta (Estrada-de los Santos 2000). En este trabajo el enriquecimiento y enumeración de las especies diazótrofas endófitas se llevó a cabo utilizando tres medios de cultivo semigelificados distintos, LGI (Cavalcante y Döbereiner 1988) con modificaciones en la concentración de sacarosa, NFb (Döbereiner 1980) con ácido málico como fuente de carbono y un medio en el que se uso la glucosa como fuente carbonada. El resultado de esta estrategia condujo al aislamiento de distintas cepas fijadoras de nitrógeno, destacando las enterobacterias, *Azospirillum* y *Burkholderia*, siendo aisladas las cepas de este último género en el medio LGI modificado. El análisis de la secuencia del gen ribosomal 16S de una de las cepas de *Burkholderia* reveló niveles de identidad en el rango de 93 a 96 % con la secuencia de las especies descritas del género, sugiriendo la existencia de una nueva especie fijadora de nitrógeno. Este resultado motivó un análisis posterior más amplio de las cepas de *Burkholderia* fijadoras de nitrógeno. Los resultados previos y los obtenidos durante el inicio de los estudios de doctorado se muestran a continuación en el trabajo "A N_2-fixing endophytic *Burkholderia* sp. associated with maize plants cultivated in Mexico. Can. J. Microbiol. 48, 285-294. 2002.

Considerando el hallazgo descrito se plantearon nuevos objetivos dirigidos a un estudio más profundo sobre la diversidad de especies fijadoras de nitrógeno del género *Burkholderia* asociadas con la planta de maíz.

A N_2-fixing endophytic *Burkholderia* sp. associated with maize plants cultivated in Mexico

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Abstract: In the frame of a survey of potentially endophytic N_2-fixing *Burkholderia* associated with maize in Mexico, its country of origin, the soil of an indigenous maize field near Oaxaca was studied. Under laboratory conditions, plant seedlings of two ancient maize varieties were used as a trap to select endophyte candidates from the soil sample. Among the N_2 fixers isolated from inside plant tissues and able to grow on PCAT medium, the most abundant isolates belonged to genus *Burkholderia* (API 20NE, *rrs* sequences). Representative isolates obtained from roots and shoots of different plants appeared identical (*rrs* and *nif*H RFLP), showing that they were closely related. In addition, their 16S rDNA sequences differed from described *Burkholderia* species and, phylogenetically, they constituted a separate deep-branching new lineage in genus *Burkholderia*. This indicated that these isolates probably constituted a new species. An inoculation experiment confirmed that these N_2-fixing *Burkholderia* isolates could densely colonize the plant tissues of maize. More isolates of this group were subsequently obtained from field-grown maize and teosinte plants. It was hypothesized that strains of this species had developed a sort of primitive symbiosis with one of their host plants, teosinte, which persisted during the domestication of teosinte into maize.

Key words: endophytic bacteria, nitrogen-fixation, *Zea mays*, *Burkholderia*.

Résumé : Lors d'une étude des *Burkholderia* endophytes fixatrices d'azote associées au maïs dans son pays d'origine, le Mexique, le sol d'un champ de maïs traditionnel de la région d'Oaxaca a été étudié. Des jeunes plantes de maïs de deux variétés traditionnelles furent utilisées comme pièges, en conditions de laboratoire, pour sélectionner les souches candidates endophytes à partir du sol. Les tissus de ces plantes montrèrent la présence de bactéries fixatrices d'azote poussant sur le milieu PCAT, identifiées comme *Burkholderia* (API 20NE). Ces isolats présentaient des profils de restriction identiques pour les gènes *nif*H et *rrs*. Leurs séquences 16S rDNA différaient de celles des espèces connues de *Burkholderia* et l'étude de leur phylogénie a montré qu'ils constituaient un phylum séparé. Ces résultats suggèrent qu'ils constituent une nouvelle espèce de *Burkholderia*. Un essai d'inoculation en serre a montré que cette bactérie était bien capable de coloniser densément les tissus du maïs. De nouveaux isolats comparables furent obtenus ultérieurement de maïs au champ et même de téosinte. Ces résultats permettent d'émettre l'hypothèse que des souches de ce type de *Burkholderia* ont développé une sorte de symbiose primitive avec la téosinte, qui a été préservée durant sa domestication en maïs.

Mots clés : bactéries endophytes, fixation d'azote, *Zea mays*, *Burkholderia*.

Introduction

Hebbar et al. (1992*a*) first reported the occurrence and abundance of *Pseudomonas cepacia* in the rhizosphere of maize in some soils in the United States and Australia. At that time our knowledge of this bacterial group was limited, and novel data have changed our perception of it significantly. First, this taxon was transferred to a new genus, *Burkholderia* (Yabuuchi et al. 1992), as *Burkholderia cepacia*, along with six other species of *Pseudomonas*. This *B. cepacia* species was then found to include five (Vandamme et al. 1997), and later, seven (Vandamme et al. 2000; Coenye at al. 2001*b*) closely related genomic species, forming what is now called the "*cepacia* complex"

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(Vandamme et al. 1997). Other species of environmental origin were then added to this genus, including *Burkholderia graminis* (Viallard et al. 1998), *B. caribensis* (Achouak et al. 1999), *B. kururiensis* (Zhang et al. 2000), *B. ubonensis* (Yabuuchi et al. 2000), *B. caledonica* and *B. fungorum* (Coenye et al. 2001*a*), and *B. sacchari* (Brämer et al. 2001). The current number of described *Burkholderia* species is 25, of which many could correspond to what Hebbar et al. (1992*a*) described as *P. cepacia*.

In a more recent survey conducted in France, Hebbar et al. (1994) confirmed that "*B. cepacia*" could be abundant in some maize fields. Identification was based upon API strips that, again, precluded precise identification. Recently, more surveys of maize-associated *Burkholderia* have been published in Europe that take into account recent changes in the taxonomy of *Burkholderia*. Two species have been shown to be abundant in a maize field studied in France: a new species, *B. graminis* (Viallard et al. 1998), and *B. cepacia* genomovar III (Balandreau et al. 2001). The latter is a *B. cepacia* complex genomic species involved in the "*cepacia* syndrome" (Isles et al. 1984). In three regions of Italy, Fiore et al. (2001) collected 120 *cepacia* complex isolates from maize fields. These isolates belonged to several species, viz *B. cepacia* sensu stricto (genomovar I), *Burkholderia vietnamiensis* (genomovar V; Gillis et al. 1995), *B. ambifaria* (genomovar VII; Coenye et al. 2001*c*), and *B. cepacia* genomovar III. At that time, *B. cepacia* complex strains had been isolated only from non-native plants: maize in Europe and wheat and lupin in Australia (Balandreau et al. 2001). As maize is native to Mexico, it was felt that a comparison of maize-associated *Burkholderia* species from Mexico and Europe would be of interest.

In the course of a survey of the predominant N_2-fixing bacteria in Mexico, Estrada-de-los-Santos et al. (2001) found that *B. vietnamiensis* was the predominant N_2-fixing bacterial partner in the state of Morelos. In other regions of Mexico, other *Burkholderia* isolates were obtained that did not fit the present state of taxonomy of this genus. Among these isolates, some were obtained from stem and surface-sterilized roots, suggesting an endophytic life style. The present article presents recent data on the biology, ecology, and taxonomy of some of these *Burkholderia* isolates obtained from an area where maize was grown following ancestral practices. These isolates were characterized in the frame of a France–Mexico collaborative program on the diversity changes in maize-associated *Burkholderia* during the domestication of the host plant and its exportation to Europe.

Materials and methods

Maize plants and soil sampling

The rhizosphere soil of the indigenous maize Tsaa poo'p mook (cv. Piedra blanca) was sampled from a field in Totontepec, in the mountains near the city of Oaxaca, Mexico. The field had always been cultivated in the Indian way, without fertilizers or pesticides. Three soil samples were collected 3 m apart around the field, in February, during the dry season 3 months after harvest.

Media and growth conditions of bacteria

All bacterial strains used in this study are listed in Table 1. N-free semisolid LGI medium (Döbereiner et al. 1995) was modified by increasing the cane sugar from 5 to 10 g/L, omitting the vitamins, and adjusting the pH to 6.5. This modified medium, called LGIM, was used for the enrichment and enumeration of N_2-fixing bacteria. Vials containing 5 mL of N-free LGIM medium were sterilized at 121°C for 20 min, and filter-sterilized cycloheximide (20 μg/mL) was added. To isolate and cultivate N_2-fixing bacteria, LGIM agar plates supplemented with 50 mg of yeast extract/L were used. When dealing with *Burkholderia*, a modification of the selective PCAT medium (Burbage and Sasser 1982) was used: tryptamine and chlorothalanil were omitted and bromothymol blue (75 mg/L) was added. The pH was adjusted to 5.7 and the medium was sterilized at 121°C for 20 min. The incubation temperature was 29°C.

Isolation and enumeration

The rationale for isolating endophytic N_2-fixing bacteria was based on using plant seedlings as a trap to select endophyte candidates from the soil sample, followed by maceration of plant tissues, dilution, and inoculation of appropriate selective media.

This strategy was applied here to isolate the most abundant N_2-fixing endophytic *Burkholderia* associated with maize. For this purpose, seeds of two indigenous maize varieties (Piedra blanca and Rojo) were surface-sterilized with a bleach solution (1.2% hypochloride) for 6 min. Seeds were then washed three times in sterile distilled water and transferred to LB (Luria–Bertani) agar plates to germinate (at 29°C). After germination, individual seedlings were transferred to 250-mL flasks containing sterile vermiculite (90 g). Flasks with plants were inoculated with 5 g of rhizosphere soil resuspended in 100 mL of water and incubated at 29°C in a growth cabinet. Control seedlings received only 100 mL of water. Plant tops were left growing outside the flasks through plastic foam plugs. After 15 and 30 days of growth, the whole plants were washed in sterile distilled water and immediatly surface-sterilized by immersion in 1% chloramine T for 10 min. After washing three times in sterile distilled water, roots were separated from shoots and some samples were rolled on LB agar plates to check for surface sterilization. Roots or shoots were then macerated in a blender in 10 mM $MgSO_4 \cdot 7H_20$ to give a 10^{-1} dilution. Tenfold serial dilutions were used to inoculate (in triplicate) N-free semisolid LGIM. After 96–120 h of incubation, vials were assayed for acetylene reduction activity (ARA), as described previously (Mascarúa-Esparza et al. 1988). Nitrogenase-positive vials with a surface pellicle were replicated into N-free semisolid LGIM, incubated for 96 h, and once more assayed for ARA. Bacterial growth from nitrogenase-positive vials was then streaked onto LGIM agar plates and incubated for 72–96 h. In general, two to three morphological types of colonies were observed. All the colony types were numbered and only one representative of each type was transferred to N-free semisolid LGIM and assayed for ARA as described above. Thereafter, all the acetylene-reducing colonies were checked for purity and their ability to grow on modified PCAT agar plates. At this stage,

Table 1. Strains used in this study.

	Taxonomy	Source	Ecology
Endophytic strains			
CFN-BM04	*Burkholderia* sp.	This work	Roots of 15-day-old maize cv. Rojo
CFN-BM16	*Burkholderia* sp.	This work	Shoots of 15-day-old maize cv. Rojo
CFN-BM229	*Burkholderia* sp.	This work	Shoots of 15-day-old maize cv. Piedra blanca
CFN-BM242	*Burkholderia* sp.	This work	Roots of 15-day-old maize cv. Piedra blanca
CFN-BM265	*Burkholderia* sp.	This work	Shoots of 30-day-old maize cv. Piedra blanca
CFN-BM273	*Burkholderia* sp.	This work	Roots of 30-day-old maize cv. Piedra blanca
LMG 19232 (WS 11.7)	*Burkholderia cepacia* gnv. III	Balandreau et al. 2001	Wheat shoot endophytes (South Australia)
Maize rhizosphere strains			
CFN15	*Azospirillum lipoferum*	This work	Maize rhizosphere (Mexico)
ATCC 700544^T (C4D1M)	*Burkholderia graminis*	Viallard et al. 1998	Maize rhizosphere (France)
C3B1M (R-13371)	*Burkholderia cepacia* gnv. III	Balandreau et al. 2001	Maize rhizosphere (France)
m35b (R-13370)	Undetermined	Balandreau et al. 2001	Maize rhizosphere (France)
Reference strains (used as 16S rDNA sequences)			
Ral-3	*Burkholderia ambifaria*	Coenye et al. 2001*c*	Soybean rhizosphere
ATCC 23061^T	*Burkholderia andropogonis*	Gillis et al. 1995	Plant pathogen
LMG 19076^T	*Burkholderia caledonica*	Coenye et al. 2001*a*	Soil
LMG 18531^T	*Burkholderia caribensis*	Achouak et al. 1999	Vertisol microaggregates in Martinique
ATCC 25418^T	*Burkholderia caryophylli*	Yabuuchi et al. 1992	Carnation pathogen
ATCC 25416^T	*Burkholderia cepacia* (gnv. I)	Yabuuchi et al. 1992	Onion sour skin
LMG 12614	*Burkholderia cepacia* (gnv. III)	Vandamme et al. 1997	Cystic fibrosis sputum (U.K.)
LMG 18941	*Burkholderia cepacia* (gnv. VI)	Coenye et al. 2001*b*	Cystic fibrosis sputum (U.S.A.)
ATCC 33664^T	*Burkholderia cocovenenans*	Gillis et al. 1995	Fermented coconut (Bongkrek)
LMG 16225^T	*Burkholderia fungorum*	Coenye et al. 2001*a*	Fungal associate
LMG14190^T	*Burkholderia glathei*	Vandamme et al. 1997	Fossil soil
ATCC 33617^T	*Burkholderia glumae*	Urakami et al. 1994	Rice pathogen
ATCC 10248^T	*Burkholderia gladioli*	Yabuuchi et al. 1992	Gladiolus bulb pathogen
KP23^T	*Burkholderia kururiensis*	Zhang et al. 2000	Soil
LMG 13010^T	*Burkholderia multivorans* (gnv. II)	Vandamme et al. 1997	Cystic fibrosis sputum (Belgium)
LMG 2247^T	*Burkholderia phenazinium*	Viallard et al. 1998	Soil
LMG 9035^T	*Burkholderia plantarii*	Urakami et al. 1994	Rice pathogen
Strain 1026b	*Burkholderia pseudomallei*	Yabuuchi et al. 1992	Melioidosis
ATCC 15958^T	*Burkholderia pyrrocinia*	Vandamme et al. 1997	Soil
LMG 14294^T	*Burkholderia stabilis* (gnv. IV)	Vandamme et al. 2000	Cystic fibrosis sputum (Belgium)
ATCC 700348^T	*Burkholderia thailandensis*	Brett et al. 1998	
LMG 16020^T	*Burkholderia vandii*	Urakami et al. 1994	Orchid rhizosphere
LMG 10929^T	*Burkholderia vietnamiensis* (gnv. V)	Gillis et al. 1995	Rice rhizosphere
LMG 16407^T	*Pandorea apista*	Coenye et al. 2000*a*	Cystic fibrosis patient (Denmark)
LMG 18087^T	*Pandorea pnomenusa*	Coenye et al. 2000*a*	Cystic fibrosis patient (U.K.)
LMG 18819^T	*Pandorea sputorum*	Coenye et al. 2000*a*	Cystic fibrosis patient (U.S.A.)
LMG 18106^T	*Pandorea pulmonicola*	Coenye et al. 2000*a*	Cystic fibrosis patient (Canada)

Table 1 (*concluded*).

	Taxonomy	Source	Ecology
Biotechnological strains (used as 16S rDNA sequences)			
DhA-54	*Burkholderia* sp.	Moore depositor, 1998	Grows on resin acids
LB400	*Burkholderia* sp.	Erickson and Mondello 1992	Grows on chloro-biphenyl
N2P5	*Burkholderia* sp.	Mueller et al. 1997	PAH (polycyclic aromatic hydrocarbon) degrader
N2P6	*Burkholderia* sp.	Mueller et al. 1997	PAH (polycyclic aromatic hydrocarbon) degrader
N3P2	*Burkholderia* sp.	Mueller et al. 1997	PAH (polycyclic aromatic hydrocarbon) degrader
RP007	*Burkholderia* sp.	Laurie and Lloyd-Jones 1999	PAH (polycyclic aromatic hydrocarbon) degrader
VUN10013	*Burkholderia* sp.	Boochan et al. depositors, 1998	PAH (polycyclic aromatic hydrocarbon)

Note: Type strains are indicated by a superscript T; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, México; gnv., genomovar; ATCC, American Type Culture Collection, Rockville, Md., U.S.A.; LMG, Culture Collection, Laboratorium voor Microbiologïe, State University of Ghent, Ghent, Belgium.

the corresponding most probable numbers (MPN) of PCAT-growing and ARA-positive isolates were calculated using the McCrady tables. All the isolates were maintained in 20% glycerol at –80°C until analyzed. One colony of each morphological type per plate from the highest dilution was selected for further analysis. These precautions allowed us to focus on the most abundant N_2-fixing population and to avoid analyzing several colonies belonging to the same clone.

Phenotypic identification

The isolates were presumptively identified using the API 20NE system (bioMérieux, La Balme les Grottes, France). The results were interpreted using the API analytical profile index, which provided percentage of identification. *Azospirillum* identification was based on biochemical tests, as described previously (Mascarúa-Esparza et al. 1988).

DNA preparation

DNA was extracted by thermal shock. From one to three colonies were dispersed in 50 µL of sterile water; tubes were heated to 100°C in boiling water and then put in liquid N_2 for a few seconds. Cell debris was centrifugated down and the supernatant used for PCR.

16S rDNA and *nifH* amplification

The oligonucleotides used to amplify the 16S rDNA gene of strain BM273 (see Table 1) were described previously (Normand et al. 1992; Viallard et al. 1998). The forward primer was FGPS-1509′-153(5′ATGGA(AG)AG(TC)TTACATCCTGGCTCA 3′), with a T_m of 66–70°C, calculated after Suggs et al. (1981); the reverse primer was FGPS4-281bis (5′AAGGAGGGG ATCCAGCCGCA 3′), with a T_m of 66°C. The 16S rDNA gene of strain BM16 (see Table 1) was PCR-amplified with the primers fD1 (5′ AGAGTTT GATCCTGGCTCAG 3′) and rD1 (5′ AAGGAGGTGATCCAGCC 3′) (Weisburg et al. 1991). PCR was performed directly in a final volume of 50 µL under a thin layer of paraffin oil; the reaction mixture contained 1 µL of extracted DNA, 5 µL of buffer (10 µM Tris-HCl (pH 8.2); 1.5 mM $MgCl_2$, 50 mM KCl; 0.01% w/v gelatin), 20 µM of each dNTP (Pharmacia Biotech, U.S.A.), 0.5 µM of each primer, and 2.5 U of Taq DNA polymerase (Gibco-BRL, Cergy-Pontoise, France). Amplifications were carried out on a Perkin Elmer dry-block thermocycler, using the following program: 3 min at 95°C; followed by 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C); with a final extension of 10 min at 72°C. To check for amplification efficiency, 5 µL of the amplification product was run on a 2% horizontal agarose gel in TBE buffer (0.1 M Tris, 90 mM boric acid, 1 mM EDTA, pH 8.4) at 4 V/cm. The PCR amplification products were visualized by ethidium bromide staining. For amplified ribosomal DNA restriction analysis (ARDRA), approximately 200 ng of the amplified 16S rDNA gene fragment (ca. 1.5 kb) was restricted with 5 U each of endonucleases *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, and *Rsa*I. The restriction-fragment patterns were determined by electrophoresis in 3% agarose gels and compared.

PCR primers used for *nifH* amplification (Poly et al. 2001) were forward primer IGK (5′ TGCGA(TC)CC (CG)AA(AG)GC(CG)GACTC 3′), with a T_m of 62–68°C, and reverse primer GEM2 (5′AT(CG)GCCATCAT(TC)TC(AG) CCGGA 3′), with a T_m of 60–64°C. PCR amplifications were performed in a total volume of 50 µL. The final concentration of each primer was 0.5 µM. Other reagents were 200 mM of each dNTP, 2 U of Expand High Fidelity DNA polymerase, and 1× PCR buffer (as specified by the manufacturer; Boehringer Mannheim). PCR was run for 30 cycles of 1 min at 94°C (denaturation), 1 min at 55°C (annealing), 2 min at 72°C (extension), with a final extension of 7 min at 72°C for the last cycle. Amplification products were submitted to electrophoresis in a 2% agarose gel and stained with ethidium bromide. For restriction fragment length polymorphism (RFLP) analysis, approximately 200 ng of the amplified *nifH* gene fragment (ca. 0.324 kb) was restricted with 5 U each of the endonucleases *Hae*III, *Nde*II, *Mnl*I, and *FNU*4H1, following the manufacturer's recommendations. The restriction-fragment patterns were determined by electrophoresis in 8% acrylamide gels, stained with Zyber

Green, photographed under UV (302 nm) light, and compared.

DNA sequencing and phylogenetic analysis

For strain BM273, the 16S rDNA gene and *nifH* were sequenced by Genome Express S.A. (Grenoble, France). For strain BM16, the 16S rDNA sequence was obtained with an ALF sequencer (Pharmacia Biotech).

DNA sequences were aligned using the multiple alignment CLUSTAL W software (Thompson et al. 1994). Sites involving insertions–deletions (indels) were excluded from all analyses. Evolutionary distances between sequence pairs corrected for multiple substitutions were computed using the Jukes and Cantor (1969) model. The phylogenetic tree was inferred using the neighbor-joining (NJ) method (Saitou and Nei 1987). Bootstraps were performed as previously described (Felsenstein 1985). The PHYLO_WIN and SEAVIEW graphic tools for phylogenetic analyses were used (Galtier et al. 1996).

Endophytic colonization of maize

To test the endophytic behaviour of bacterial isolates, the maize indigenous cultivar Rojo and the hybrid cultivar B-555 were used. Seeds were surface-sterilized with hypochloride and germinated as described in Isolation and enumeration, above. Germinated seeds were inoculated by immersing roots in a bacterial suspension containing washed cells (1×10^7 CFU/mL) of a mid-logarithmic-phase culture. Seedlings were then transferred to a 250-mL flask containing sterile vermiculite (90 g) and 90 mL of a N-free nutrient solution composed of (mg/L): K_2HPO_4 54, KH_2PO_4 109, NaCl 12, $CaCl_2$ 344, $MgSO_4 \cdot 7H_2O$ 185, $FeSO_4 \cdot 6H_2O$ 1, $ZnSO_4 \cdot 7H_2O$ 1, H_3BO_3 1, $CuSO_4 \cdot 5H_2O$ 0.03, $MnSO_4 \cdot 4H_2O$ 0.1, and $Na_2MoO_4 \cdot 2H_2O$ 0.03. Two plants of each cultivar were inoculated with isolate BM16 and, as a control, two other plants were inoculated with *Azospirillum lipoferum* CFN15, a strain isolated from the rhizosphere of maize cv. Rojo during another survey in the same location. As an absolute control, two plants were left uninoculated. After 15 days, the plants were harvested and surface-sterilized with chloramine T. Roots and shoots were macerated separately and diluted; the bacterial cells were quantified by the MPN method. For each sample, aliquots of the highest-dilution tubes were streaked onto modified PCAT agar plates. For each plate, three colonies were characterized by multilocus enzyme electrophoresis (MLEE) profiles, following the procedures described by Caballero-Mellado et al. (1995), to verify that these colonies corresponded to the inoculated isolates.

Nucleotide sequence accession numbers

Sequences were deposited in GenBank under the accession numbers AF312214 (isolate BM16) and AF312213 (isolate BM273).

Results

Estimation and identification of the *Burkholderia* population

Following inoculation of vials containing N-free semisolid LGIM with plant macerates, large pale-yellow surface pellicles were observed. After plating dilutions on LGIM agar, three morphological colony types were obtained. All were gram-negative bacteria. The most abundant colonies were yellow, 2–3 mm in diameter, round, mucous, smooth, and convex with translucent margins. When transferred to modified PCAT medium, these colonies were 1–2 mm in diameter, round, white, smooth, and convex with entire margins, and the surrounding medium turned blue. Colonies with this morphology ranged from 5×10^2 to 9×10^3 CFU/g root (fresh weight) of and from 5×10^2 to 2.2×10^5 CFU/g shoot (fresh weight). Colonies of the other two morphology types were smaller and represented less than 10^2 CFU/g root or shoot (fresh weight). It is important to point out that the analysis of uninoculated plants showed the presence of very few bacterial colonies and that these were clearly different morphologically from those found in inoculated plants.

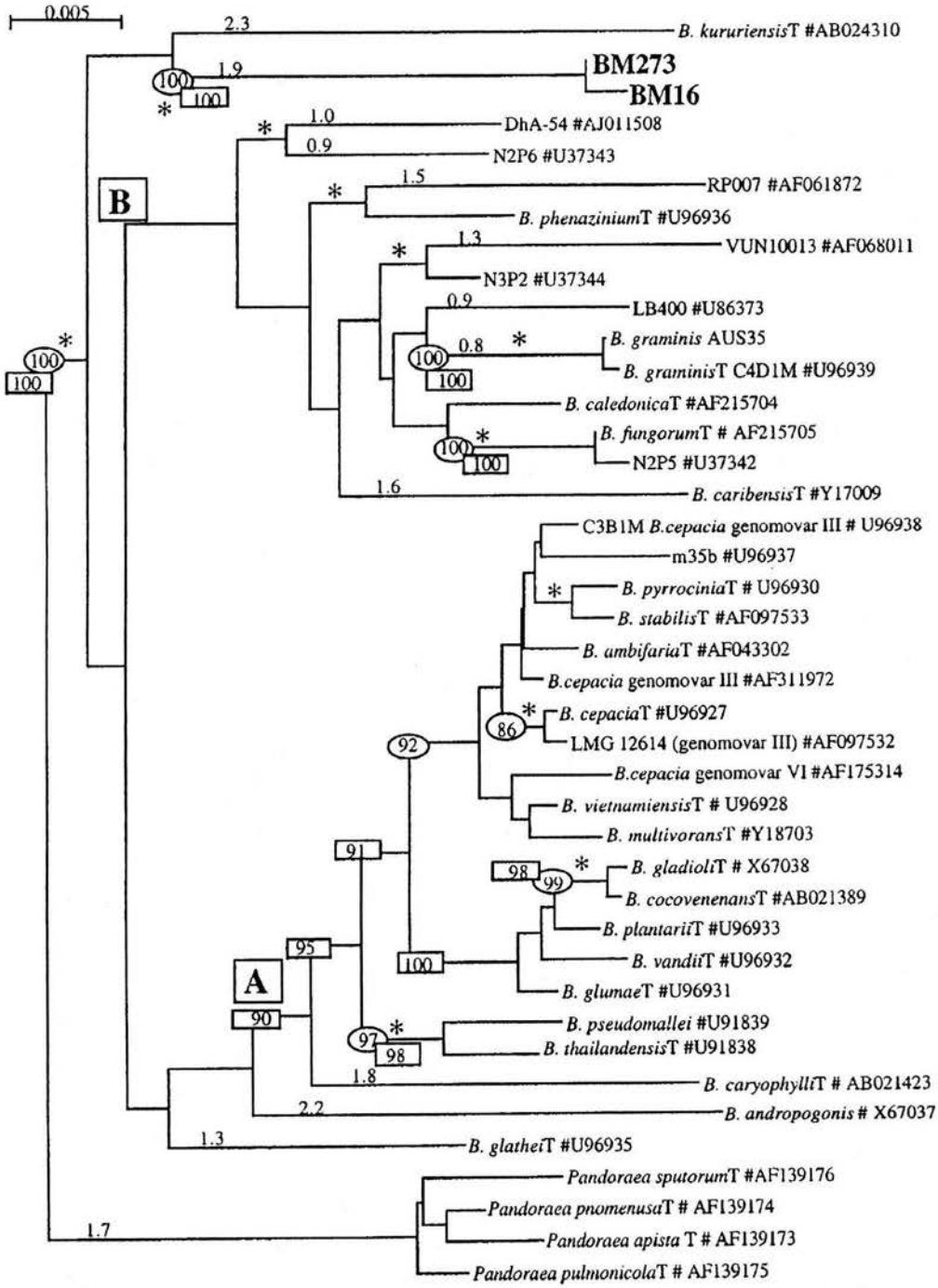
For a preliminary identification, PCAT-growing and ARA-positive isolates were used to inoculate API 20NE strips. Among the 38 selected isolates, only 14, originating from different inoculated plants, matched known taxa in the API data base. Four isolates corresponded to *Enterobacter cloacae*, three to *Azospirillum* spp., and one to *Klebsiella pneumoniae*. Six isolates belonging to the most abundant N_2-fixing colonies were identified as *B. cepacia*. The percentage of identification ranged from 86.2 to 99.7%, which is considered acceptable for a genus level determination. Further characterization focused on the group of isolates belonging to *Burkholderia*.

16S rDNA data

ARDRA of the six isolates that were presumptively identified as *B. cepacia* showed that they all gave identical restriction patterns with seven different endonucleases. Two isolates, BM273 and BM16, obtained from the highest dilutions, were selected for 16S rDNA sequencing. The former was isolated from roots of maize cv. Piedra blanca and the latter from the shoot of maize cv. Rojo (Table 1). Their sequences were 99.9% similar.

The 16S rDNA sequences of isolates BM273 and BM16 were aligned with those of all 16S rDNA sequences presently available for the genus *Burkholderia* and with sequences of the closely related genus *Pandoraea*, using 1435 positions. Phylogenetic relationships between the 41 selected sequences of this data set were inferred using the Jukes and Cantor (1969) distance-matrix method and a parsimony analysis. The two approaches gave similar results (Fig. 1), with the following main significant observations: (*i*) all 16S rDNA sequences from the genus *Burkholderia* formed a distinct cluster that was clearly separate from sequences of the closely related genus *Pandoraea* (for 100% of the bootstrap replicates using the distance-matrix method and among 100% of the 44 most parsimonious trees requiring 558 steps); (*ii*) sequences of isolates BM273 and BM16 clearly belonged to the cluster of *Burkholderia* sequences, but represented a distinct novel lineage within this group; (*iii*) the closest 16S rDNA neighbor of the BM273 and BM16 sequences could not be identified with a high confidence level using this data set. Multiple analyses were performed to identify the sequences affecting the resolution of the phylogenetic relationships. Two sequences were found to affect the robustness of the analyses, viz. the 16S rDNA sequences

Fig. 1. Phylogenetic analysis of 16S rDNA sequences from a representative set of *Burkholderia* strains, including isolates BM273 and BM16 from maize. Phylogenetic relationships were estimated using 16S rDNA sequences from most if not all species of *Burkholderia* and representative sequences from species of the closely related genus *Pandoraea*. Distances were computed according to Jukes and Cantor (1969), and the tree was built using the neighbor-joining (NJ) method (Saitou and Nei 1987); 1328 DNA sites (of which 106 are informative) were used. Horizontal distances on the NJ tree are proportional to evolutionary divergences expressed in substitutions per 100 sites. Scale is given on the NJ tree and some distances are given on the tree branches. Bootstrap values above 85% are given in ovals for the original data set and in squares for values derived from the same data set but not considering the Ban-23061 and Bgl-14190 16S rDNA sequences. Vertical separations are for clarity only. Asterisks indicate lineages observed among 100% of the most parsimonious trees (44 trees, requiring 558 steps) that were derived from the same data set. The parsimony analysis was performed using the DNAPARS program of the PHYLIP package (Felsenstein 1985). GenBank accession numbers for the 16S rDNA sequences used are indicated following "#". Species type strains are followed by "T".



of *Burkholderia andropogonis* (Ban-23061) and *B. glathei* (Bga-14190). The NJ phylogenetic tree (distance-matrix method) obtained with the modified data set (39 sequences instead of 41, excluding the above two sequences) clearly showed a cluster of sequences, designated "A", comprising the *cepacia* complex, *Burkholderia gladioli*, *B. cocovenenans*, *B. plantarii*, *B. vandii*, *B. glumae*, *B. pseudomallei*, *B. thailandensis*, and *B. caryophilli*. The cepacia complex was well individualized in 92% of bootstraps. French maize isolates m35b and C3B1M were members of this complex; strain C3B1M belongs to *B. cepacia* genomovar III, whereas m35b belongs to another undescribed species of this *cepacia* complex (Balandreau et al. 2001). In the NJ tree, another cluster appeared, designated "B," that included the other sequences from *Burkholderia* and the sequences from isolates BM273 and BM16 (Fig. 1). The closest named species of the latter appeared to be *B. kururiensis*; however, the number of substitutions differentiating them from *B. kururiensis* is much larger than the number differentiating any other two species inside the genus *Burkholderia*, showing that they are separate species. It is noteworthy that this data set did not position the *B. andropogonis* and *B. glathei* sequences and that these could still be part of either of these two main clusters. Within cluster A, several subgroups could be resolved with a high confidence level (see Fig. 1), but within cluster B, no significant subgroups could be detected.

Endophytic status of *Burkholderia* strains

We selected isolate BM16, which had been recovered from the highest dilution of shoot tissue, to test endophytic behaviour. Following inoculation with isolate BM16, large numbers of bacterial colonies were recovered from surface-sterilized plant tissues: up to 10^3 CFU/g root (fresh weight) and up to 10^5 CFU/g shoot (fresh weight). The identity of these colonies was confirmed by MLEE profiles. In contrast, no bacteria were recovered from the inside tissues of control plants, either uninoculated or inoculated with *A. lipoferum* CFN15.

Nitrogen fixation

As mentioned above, all *Burkholderia* isolates could reduce acetylene. We tested the presence of *nifH* genes in isolates BM273 and BM16 by PCR using *nifH*-specific primers. The PCR products obtained gave identical RFLP patterns (data not shown), confirming the close relationship between these two strains. The BM273 *nifH* gene has been partially sequenced (324 bp). The closest sequence found in GenBank (using BLAST) corresponded to the *nifH*1 gene of *Rhizobium* sp. strain ORS571 (Norel and Elmerich 1987).

Discussion

Several published reports show that nonpathogenic bacteria can be present in tissues of many species of healthy plants (Döbereiner et al. 1993; Reis et al. 2000), including sugar cane (Cavalcante and Döbereiner 1988), coffee (Jimenez-Salgado et al. 1997), pineapple (Tapia-Hernández et al. 2000), maize (McInroy and Kloepper 1995*a*, 1995*b*), rice (Engelhard et al. 2000), wheat, and lupin (Balandreau et al. 2001). The nature, numbers, and localization of these endophytic bacteria are diverse. Bacteria involved include *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., *Azoarcus* spp., and *Burkholderia* spp.

Data presented in this study show that, in Mexico, soils under conditions of traditional sustainable agriculture contain bacterial populations of a new group of N_2-fixing *Burkholderia*. These isolates are able to colonize maize tissues in large numbers—up to 9×10^3 CFU/g root (fresh weight) and up to 2.5×10^5 CFU/g shoot (fresh weight)—following seed inoculation, without any disease symptoms. This level of internal colonization in maize is comparable with levels found by Hebbar et al. (1992*a*, 1992*b*) in Kairi (Australia). The population level of *Burkholderia* sp. found internally associated with rice roots in Italy by Engelhard et al. (2000) is also comparable. Direct localization studies have not yet been performed to confirm the endophytic habit of these *Burkholderia* isolates, but strong evidence comes from the inoculation experiment comparing *Burkholderia* isolate BM16 and a rhizosphere isolate of *A. lipoferum*, CFN15. As expected, only *Burkholderia* isolate BM16 could colonize the plant tissues. In addition, in the uninoculated control treatment in this experiment, no *Burkholderia* colonies were found in plant tissues, showing that the seeds did not carry any endophytic *Burkholderia*.

During preparation of this report, more endophytic isolates of this new *Burkholderia* group (identified by ARDRA) were obtained directly from field-grown maize plants in Mexico. Population levels were comparable with those reported here (up to 10^3 CFU/g root (fresh weight) and up to 10^5 CFU/g shoot (fresh weight)). In addition, similar endophytic isolates were obtained from teosinte plants growing in Tepoztlan, 35 km from Cuernavaca in the state of Morelos, Mexico.

No maize endophytic *Burkholderia* were described in Italy by Fiore et al. (2001). In two preliminary experiments conducted near Lyon (France), K. Ophel-Keller (personal communication) studied surface-sterilized maize stems and found no endophytes (on PCAT); in contrast, using the same methodology, endophytic *Burkholderia* were obtained from surface-sterilized stems and roots of wheat and lupin in Australia. Their densities were very low in planta, ranging from 10^1 to 10^2/g tissue (fresh weight). These isolates belong to *B. cepacia* genomovar III (Balandreau et al. 2001); they do not fix nitrogen and are similar to other rhizosphere bacteria, suggesting that their presence results from an opportunistic inwards colonization from the rhizosphere. In the present study, the large internal populations seen without coincident disease symptoms, and the ability to fix nitrogen, suggest a possible contribution to the nitrogen nutrition of the host plants, which is consistent with the traditional indian way of growing maize without nitrogen fertilizers.

Some reports of *Burkholderia* closely associated with plants have included 16S rDNA sequencing. Three almost complete 16S rDNA sequences are found in GenBank that are very similar (99%) to the 16S rDNA sequences of isolates BM273 and BM16. These are for strains PPe8 (accession No. AJ420332), AB98 (AF164045), and TFD3 (AF184929). Strain PPe8 represents a group of "isolate E"-type bacteria isolated by Döbereiner et al. (1993) from sugarcane, as reported by Hartmann et al. (1995), who classified

them as *Burkholderia* sp. Strain AB98 was isolated from a pineapple fruit (Magalhaes et al. 2001). PPe8 and AB98 16S rDNA have been provisionally assigned the name *Burkholderia tropicalis* (Magalhaes et al. 2001). Strain TFD3 is a 2,4-D-degrading *Burkholderia* isolated in Michigan (Velicer et al. 1999). These three strains and our isolates display a high level of sequence similarity and therefore probably represent a single species. 16S rDNA sequences and phylogeny support the idea that this group is probably a new species of the genus *Burkholderia*. A taxonomically valid description of this species is in preparation.

Strains of the new *Burkholderia* group found in this study are remarkable for being both endophytic and able to fix nitrogen. These features support the hypothesis that members of this species developed a sort of primitive symbiosis with teosinte, one of their host plants, that persisted during the domestication of teosinte into maize. Most likely, this association was lost when maize was exported as seeds from America to Europe during the sixteenth century. Research is in progress to confirm the absence of this N_2-fixing *Burkholderia* group in maize cultivated in Europe and to evaluate the possibility that inoculation with it could improve maize growth. Results obtained with *B. vietnamiensis* inoculation (Tran Van et al. 2000) have already shown the very high potential of *Burkholderia* as plant growth promoting rhizobacteria. Moreover this practice might decrease maize colonization by potentially hazardous *B. cepacia* genomovar III (Balandreau et al. 2000).

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OBJETIVO DEL TRABAJO DE TESIS

El objetivo inicial de este trabajo fue contribuir con el conocimiento sobre la existencia de especies fijadoras de nitrógeno del género *Burkholderia* asociadas con plantas de maíz, así como conocer sobre su distribución en el medio ambiente. No obstante, este objetivo fue ampliado para conocer la asociación de estas bacterias con otras plantas de interés agrícola.

OBJETIVOS PARTICULARES

1. Aislamiento de especies del género *Burkholderia* fijadoras de nitrógeno habitantes de la rizosfera, del rizoplano y de los tejidos internos de plantas de maíces criollos e híbridos, así como, de plantas cafeto, teocintle, caña de azúcar y sorgo.

2. Identificación de especies del género *Burkholderia* asociadas con el maíz, cafeto, caña de azúcar, sorgo y teocintle, así como análisis de su distribución en el medio ambiente.

RESULTADOS

Aislamiento de diazótrofos del género *Burkholderia* spp.

Considerando la ubiquidad de las bacterias, para llevar a cabo el primer objetivo de este trabajo de tesis doctoral, referido al aislamiento de especies diazótrofas del género *Burkholderia*, se trató de analizar el mayor número posible de muestras vegetales. Se analizaron distintas variedades de plantas de maíz y cafeto, así como algunas plantas de sorgo cultivadas en diferentes regiones de México. Además, se investigó la presencia bacteriana en la rizosfera (suelo influenciado por los exudados de las raíces), rizoplano (superficie de la raíz) y los tejidos internos de raíz, tallo y hojas de las plantas de maíz y sorgo. En el caso de las plantas de cafeto solamente se analizó la rizosfera y el rizoplano. Por otro lado, la estrategia para el aislamiento de las especies diazótrofas utilizada anteriormente fue modificada (Estrada-de los Santos 2000; Estrada y col. 2002). Algunas de las especies del género *Burkholderia* han sido aisladas utilizando el medio de cultivo PCAT (Burbage y Passer 1982), el cual esta constituido por el ácido azelaico como fuente de carbono. Este compuesto es un ácido dicarboxílico (ácido 1,7-heptanodicarboxilico, $C_9H_{16}O_4$) que se encuentra naturalmente en plantas gramíneas. El ácido azelaico proporciona cierta selectividad al medio PCAT debido a que es una fuente de carbono compleja que no puede ser utilizada por muchos otros microorganismos.

El aislamiento y enumeración de los aislados fue llevado a cabo utilizando el medio PCAT, el cual fue modificado disminuyendo la concentración de agar y omitiendo la triptamina como fuente de nitrógeno (medio BAz). La selección de las colonias bacterianas se realizó en placas con este medio de cultivo pero adicionado con extracto de levadura. Las modificaciones realizadas en la estrategia de aislamiento incrementaron considerablemente el número de cepas de *Burkholderia,* comparado con el trabajo que antecedió a esta investigación, sin embargo, entre los diazótrofos aislados se encontró que algunos de ellos pertenecían a la familia *Enterobacteriaceae*. Fue así, que se diseñó un nuevo medio de cultivo (BAc) para dar una mayor selectividad a la estrategia de aislamiento de las especies diazótrofas del género *Burkholderia*. El medio BAc contiene ácido azelaico como fuente de carbono y como fuente de nitrógeno la citrulina, la cual fue seleccionada previa evaluación de diferentes aminoácidos. La selectividad de este medio fue dada por la capacidad de algunas cepas de *Burkholderia* spp. para utilizar el

ácido azelaico y la citrulina y a la incapacidad de las enterobacterias, y de otros grupos bacterianos encontrados en asociación con plantas, como *Gluconacetobacter diazotrophicus*, *G. johannae*, *G. azotocaptans*, *Azospirillum* spp. y *Rhizobium* spp. para crecer en este medio de cultivo. La fijación de nitrógeno de las cepas de *Burkholderia* fue determinada mediante ensayos de ARA y detección de los genes estructurales de la nitrogenasa, *nifHDK*.

El agrupamiento de los aislados diazótrofos de *Burkholderia* se realizó a través de la metodología ARDRA (Amplified rDNA Restriction Analysis). Esta estrategia permitió identificar 16 genotipos de *Burkholderia* fijadores de nitrógeno. Entre estos diazótrofos, se identificó a *B. vietnamiensis* (genotipos 1 y 2), aislada originalmente de la rizosfera de arroz (Gillis y col. 1995). En la presente investigación, *B. vietnamiensis* fue encontrada tanto en la rizosfera como en el rizoplano y como endófito de plantas de maíz y asociada también a plantas de café y sorgo cultivadas en diferentes regiones geográficas de México. Otras de las cepas diazótrofas quedaron agrupadas dentro del "complejo *B. cepacia*" (genotipos 5, 6, 7 y 8), por lo que resultaría interesante conocer si éstas corresponden a nuevos genomovares o pueden ser identificadas como nuevas especies pertenecientes a este complejo. Además, varios de los genotipos (13, 14, 15, 16, 17, 18, 19, 20 y 21) de diazótrofos, con un número importante de aislados, formaron un gran grupo en el cual ninguna de las especies conocidas de *Burkholderia* quedó contenida, así como tampoco el resto de las especies conocidas de este género que fueron analizadas posteriormente. Considerando el valor del método empleado (ARDRA) estos genotipos podrían corresponder a dos o quizá tres especies nuevas del género *Burkholderia*. Los genotipos 13, 14 y 15 corresponderían a una nueva especie, en tanto que los genotipos 16, 17, 18 y 19 a una segunda y el 20 y 21 a una tercera nueva especie. Además, en este trabajo se observó que *B. kururiensis*, especie descrita en 2000, y *B. "brasilensis"*, recientemente propuesta como una nueva especie, poseen los mismos perfiles con la metodología ARDRA. El análisis de la secuencia del gen ribosomal 16S mostró un 99.9 % de identidad entre ambas especies, resultado que sugiere que corresponden a una misma especie. Sin embargo, se requieren de más estudios para determinar si esta hipótesis es correcta. Otro hallazgo importante fue que la cepa tipo KP23^{T} de *B. kururiensis* tiene la capacidad para reducir acetileno cuando es cultivada en presencia de diferentes fuentes de carbono. Esta capacidad no fue

analizada cuando fue descrita como nueva especie. Además, la presencia de los genes *nifHDK* en esta especie fue revelada con ensayos de hibridación. Ambos ensayos nos permitieron proponer a *B. kururiensis* como especie fijadora de nitrógeno.

En conclusión, el trabajo desarrollado para cubrir el primer objetivo de esta tesis doctoral permitió describir la riqueza del género *Burkholderia* en especies diazótrofas y su amplia distribución en el ambiente, considerando que fueron aisladas de distintas regiones de la planta (rizosfera, rizoplano, endófitos de raíz y tallo), así como de plantas (maíz, café y sorgo) pertenecientes a diferentes familias, las cuales fueron cultivadas en distintas regiones geográficas de México (Estados de Chiapas, Morelos, Oaxaca, Querétaro y Veracruz) con características climáticas diferentes.

La totalidad de los resultados sobre el aislamiento e identificación parcial de las cepas de *Burkholderia* spp. se muestra a continuación en el trabajo "*Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide enviornmental and geographic distribution. Appl. Environ. Microbiol. 67, 2790-2798. 2001.

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

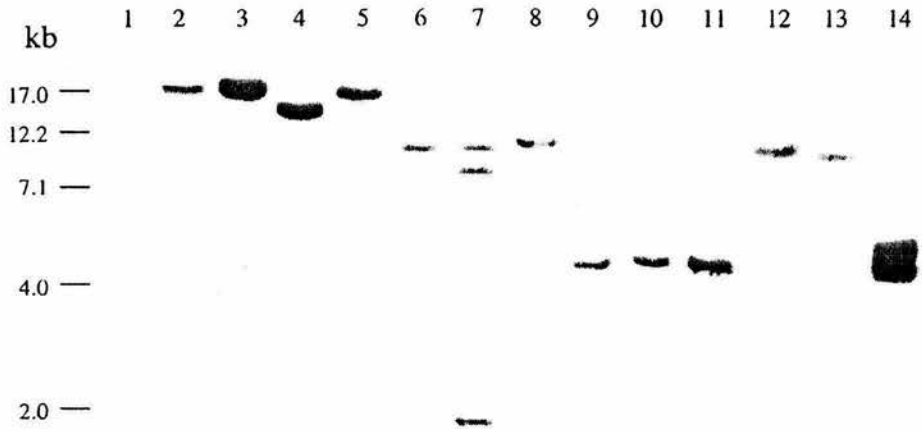

FIG. 1. Autoradiogram of a Southern blot of total *Eco*RI-digested DNA hybridized with the *nifHDK* probe of *R. etli* CFN 42. Lane 1, *B. cepacia* ATCC 29352 used as a negative control; lane 2, *B. vietnamiensis* TVV75^T. Lanes 3 through 12 are examples of representative N_2-fixing *Burkholderia* isolates;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

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

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

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

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

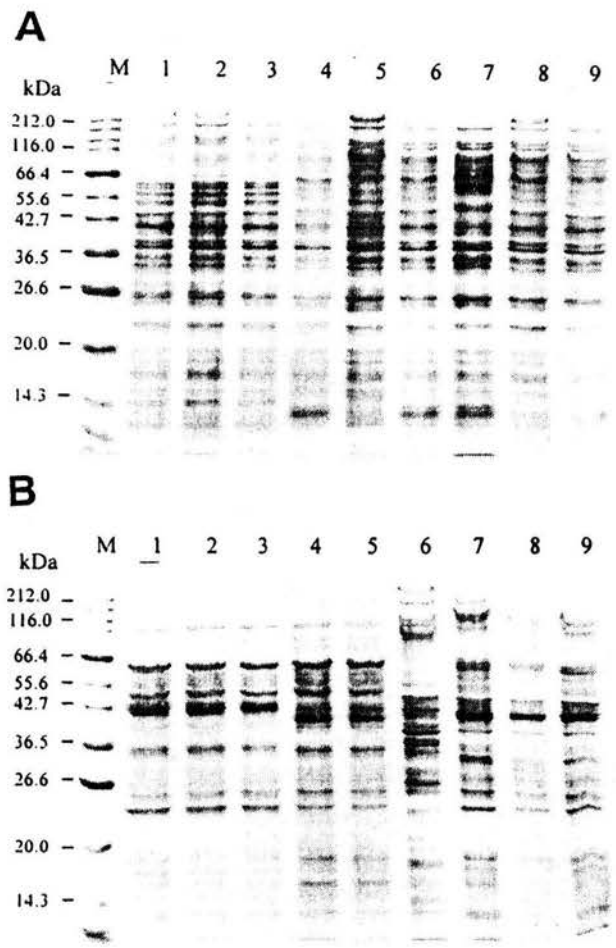

FIG. 2. Protein electrophoregrams (SDS-PAGE) of representatives N_2-fixing *Burkholderia* strains. (A) *B. vietnamiensis* isolates recovered: from maize, lane 1, MMi-324; lane 2, MMi-1547; lane 3, MMi-1556; lane 9, MMi-302; from coffee, lane 6, CCE-312; lane 7, CCE-101; lane 8, CCE-201; from sorghum,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

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

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

DISCUSSION

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

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

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

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

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

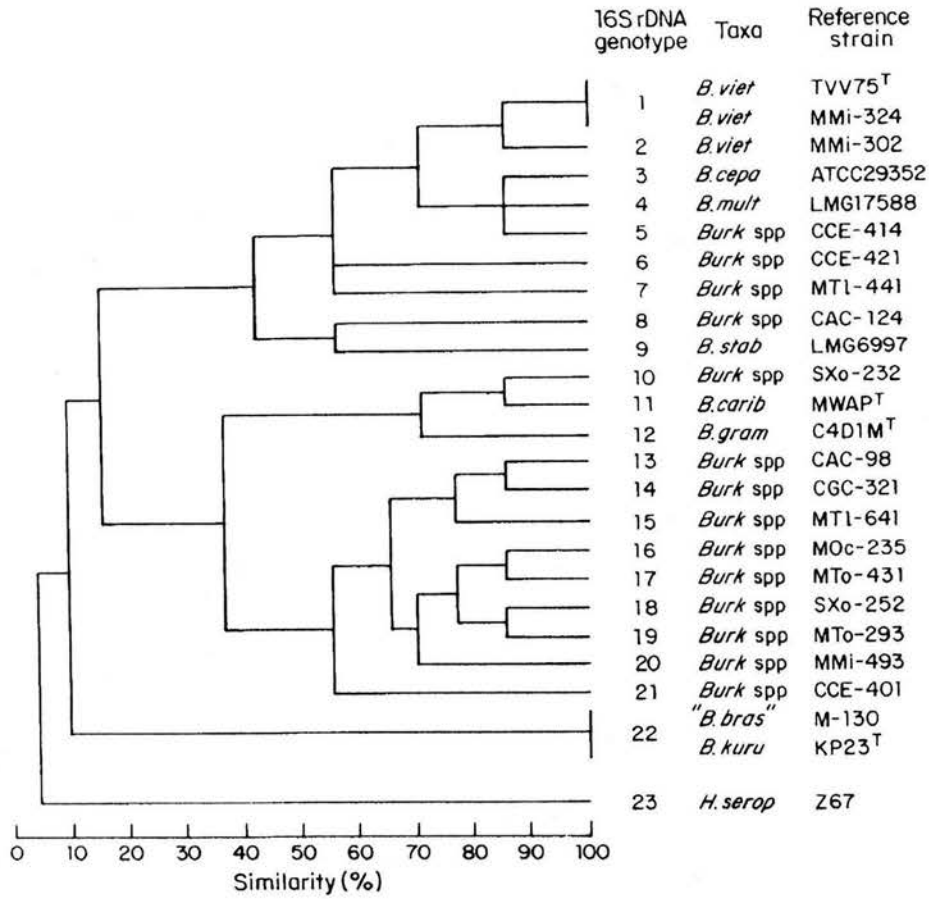

FIG. 3. Dendrogram of genetic relationships among N_2-fixing *Burkholderia* isolates recovered from maize, coffee, and sorghum plants and related species of *Burkholderia* based on ARDRA analysis. *Burk, Burkholderia; B. viet, B. vietnamiensis; B. cepa, B. cepacia; B. mult, B. multivorans; B. stab, B.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

regions with a sustainable agriculture where fertilizer has not been used for generations. It is conceivable that the occurrence of *B. vietnamiensis* (4×10^6 CFU/g of rhizosphere soil and 4×10^4 CFU/g of fresh tissue of roots) might contribute to the growth of the maize plant and hence to crop production, as has been observed with field inoculation of rice with *B. vietnamiensis* TVV75T (37, 38).

Although the ability to fix N_2 is not reported to be a common feature among the known species of the genus *Burkholderia*, we have found that this bacterial genus is very rich in diazotrophic species, as was demonstrated with ARA assays and confirmed with the presence of *nifHDK* genes. Our results confirm that diazotrophy is a common property among bacteria. However, the ability of some bacterial species to fix N_2 is unknown because this feature is not routinely evaluated when a new species is described (46). This is especially true for the *B. kururiensis* species. In this study, both the presence of *nifHDK* genes in strain KP23T of *B. kururiensis* and the ARA assays revealed that this species is capable of fixing N_2 with selected carbon sources under microaerophilic conditions. It is known that many N_2-fixing bacterial species do not show such ability except under special growth conditions (46). Nevertheless, this possibility does not explain the erratic acetylene reduction activity observed with some isolates corresponding to the 16S rDNA genotypes 16, 17, 18, and 19. Further studies are required to resolve the observed discrepancies.

Interestingly, strains KP23T of *B. kururiensis* and M-130 of "*B. brasilensis*" were capable of fixing N_2 in a similar manner, and both strains showed the same ARDRA profile. In addition, an analysis of the 16S rRNA sequences revealed 99.9% similarity between *B. kururiensis* KP23T and "*B. brasilensis*" M-130 (data not shown), suggesting that both strains belong to the same species. This finding emphasizes the wide geographic and environmental distribution of the bacterial species. While *B. kururiensis* KP23T was recovered from an aquifer polluted with trichlorethylene in Japan (47), the "*B. brasilensis*" M-130 strain was found to be plant associated in Brazil (V. L. D. Baldani, G. Kirchhof, V. M. Reis, E. de Oliveira, I. J. Baldani,

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

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

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

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Identificación y distribución en el ambiente de diazótrofos del género *Burkholderia*

El estudio de las especies fijadoras de nitrógeno del género *Burkholderia* asociadas a plantas continuó con la identificación de los genotipos descritos anteriormente (Estrada de los Santos y col. 2001). Las cepas seleccionadas para su identificación fueron el grupo correspondiente a los genotipos 16, 17 y 19. Para cumplir este objetivo se llevó a cabo el aislamiento de nuevas cepas de los tres genotipos con el propósito de contar con una colección de cepas más amplia y conocer su distribución en el medio ambiente. La estrategia utilizada para el aislamiento de las cepas fue descrita anteriormente (Estrada de los Santos y col. 2001).

El aislamiento de cepas correspondientes a los genotipos 16, 17 y 19 fue realizado a partir de plantas de maíz cultivadas en distintas regiones de la República Mexicana así como de plantas de teocintle creciendo en forma silvestre en México. En colaboración con el grupo del Dr. Jacques Balandreau (Université Claude Bernard Lyon1, Francia) se analizaron plantas de caña de azúcar provenientes de Sud África.

La caracterización de las cepas diazótrofas incluyó métodos fenotípicos como la capacidad de crecimiento en medio de cultivo semigelificado, morfología colonial en placa, crecimiento en distintos medios de cultivo en placa, así como crecimiento a diferentes temperaturas y pH, análisis de los perfiles bioquímicos (API 20NE), uso de fuentes de carbono mediante las galerías API 20NE y API 50CH. La caracterización fenotípica fue complementada con la determinación de los perfiles de proteínas totales (SDS-PAGE). La caracterización genómica de los aislados diazótrofos incluyó la agrupación mediante la metodología ARDRA, secuencia y análisis filogenético del gen ribosomal 16S y ensayos de reasociación ADN-ADN.

La estrategia de taxonomía polifásica utilizada para la caracterización de los aislados diazótrofos mostró que las cepas de los genotipos 16, 17 y 19 pertenecen a la especie *B. "tropicalis"*. A continuación se describen los resultados que permitieron la identificación de esta especie.

Taxonomía de *Burkholderia tropicalis*

La falta de información sobre métodos para el aislamiento e identificación de esta especie bacteriana condujo a la extraordinaria coincidencia de encontrar que las cepas correspondientes a los genotipos 16, 17 y 19 pertenecen a la especie *B. "tropicalis"*, dado que al inicio de nuestra investigación esta especie no había sido descrita oficialmente, existiendo en ese tiempo en la base de datos GenBank solamente la secuencia parcial del gen ribosomal 16S. La propuesta oficial de *B. "tropicalis"* como nueva especie fue enviada recientemente a la revista Internacional Journal of Systematic and Evolutionary Microbiology (IJSEM), quedando condicionada su aceptación y publicación al desarrollo de experimentos adicionales que complementen su caracterización (Hartmann y Reis com. pers.). El trabajo desarrollado en la presente investigación fue enviado para su publicación al IJSEM casi en forma paralela al trabajo de Hartmann, respetando el nombre de *B. "tropicalis"* a pesar de no existir su descripción formal pero con el propósito de complementar la información que sería reportada sobre esta especie. Sin embargo, debido a la política de la revista de publicar solo nuevas especies o metodologías, el manuscrito fue rechazado a pesar de su "valioso contenido". La decisión de rechazo de nuestro estudio fue protestada al Editor en jefe del IJSEM, Dr. Aidan Parte, quien sugirió la fusión de ambos manuscritos. En el momento de escribir esta Tesis hemos recibido la propuesta de Hartmann (autor responsable del manuscrito) para fusionar ambos trabajos y hacer la descripción conjunta de *B. "tropicalis"* como nueva especie, toda vez que esta fusión enriquecerá la información taxonómica y sobretodo los conocimientos sobre la ecología de esta especie bacteriana, producto de nuestro estudio.

Los resultados sobre la descripción de *B. "tropicalis"*, derivados de este trabajo doctoral, son mostrados en el manuscrito "Isolation, taxonomy and distribution of *Burkholderia tropicalis*" que fue enviado inicialmente a la revista IJSEM y se muestran al final de esta sección. Algunos puntos sobresalientes se comentan a continuación.

La estrategia para el aislamiento de los aislados diazótrofos, basada en el uso combinado del ácido azelaico y la citrulina, desempeño un papel determinante para la recuperación de los genotipos 16, 17 y 19. La metodología ARDRA utilizada para la identificación de los genotipos diazótrofos reveló a un nuevo genotipo, designado 19a, correspondiente a dos cepas aisladas de caña de azúcar en Sud África. En este estudio

se observó que la suma de los fragmentos del gen 16S digerido con la enzima *HinfI* de las cepas del genotipo 17 de *B. "tropicalis"* fue mayor a 1.5 Kb, el cual es el tamaño del gen ribosomal 16S sin digerir. Se conoce que algunas bacterias pueden albergar hasta 15 copias de operones ribosomales y que entre ellas pueden existir diferencias internas. Una situación similar podría explicar el resultado obtenido con el genotipo 17.

La caracterización fenotipica, mediante el uso de las galerías API 20NE, mostró que los genotipos 16, 17, 19 y 19a poseen un 98.9 a 99.6 % de identidad con *B. cepacia*, sin embargo, el análisis del conjunto de pruebas basadas en la capacidad de crecimiento en medio de cultivo semigelificado, actividad reductora de acetileno, morfología colonial en placa, crecimiento en distintos medios de cultivo en placa, así como crecimiento a diferentes temperaturas y pH, y el uso de distintas fuentes de carbono, mostraron que los aislados son diferentes a *B. cepacia* y a otras especies diazótrofas de *Burkholderia* como *B. vietnmiensis* y *B. kururiensis*. Sin embargo, el uso de las mismas estrategias reveló una gran similitud entre los genotipos 16, 17, 19 y 19a y la cepa Ppe8 de *B. "tropicalis"*. Así mismo, los perfiles proteicos de los genotipos diazótrofos fueron casi idénticos a *B. "tropicalis"*.

El análisis de la secuencia del gen ribosomal 16S así como los ensayos de reasociación ADN-ADN mostraron niveles de similitud superiores a los indicados para definir una especie, en este caso, valores que ubicaron a los genotipos diazótrofos en la especie *B. "tropicalis"*.

Es importante destacar que de acuerdo al análisis de la secuencia del gen ribosomal 16S, los distintos genotipos de *B. "tropicalis"* se encuentran filogenéticamente alejados de los patógenos oportunistas agrupados en el "complejo *B. cepacia*". Esta situación nos ha hecho reflexionar en la posibilidad de usar a la especie *B. "tropicalis"* en agrobiotecnología, considerando su capacidad para fijar nitrógeno, para producir sideróforos (Tenorio-Salgado y col. 2002) y para colonizar el ambiente rizosférico y endófito de plantas pertenecientes a familias diferentes cultivadas en regiones geográficamente alejadas y con climas que abarcan desde el templado sub-húmedo a cálido húmedo.

Title

Isolation, taxonomy, and distribution of *Burkholderia tropicalis*

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SUMMARY

For a long time, N_2-fixing ability in bacteria of the genus *Burkholderia* was recognized only in the species *B. vietnamiensis*. Recently, the richness of the genus *Burkholderia* in unknown diazotrophs associated with plants was shown. A group of these N_2-fixing isolates with identical or very similar 16S rDNA restriction patterns (designated as ARDRA genotypes 16, 17, and 19) was selected, and a polyphasic taxonomy study was performed including new isolates recovered from diverse regions of Mexico and South Africa. This study included microscopic and colony morphology, API 20NE tests, growth on different culture media at different pH and temperatures, as well as assimilation of different carbon sources, and whole-cell protein patterns of 37 strains. In addition, 16S rDNA sequence analysis and DNA-DNA reassociation were performed with representative strains. The genotypes 16, 17, 19, and a new genotype designated as 19a showed very similar phenotypic features with strain Ppe8 of *B. tropicalis*, just recently proposed as a novel species. Analysis of 16S rDNA sequences showed 99.2 to 99.9 % similarity to strains Ppe8 and AB98 of *B. tropicalis,* and 96 % of similarity to "*B. cepacia* complex" strains. The DNA-DNA reassociation value among strain Ppe8 and strains with genotype 16, 17, 19, and 19a was higher than 70 %. All of these results revealed that the strains analyzed belong to *B. tropicalis*. This species was isolated from the rhizosphere and as an endophyte of maize and teosinte plants as well as from roots of sugarcane plants grown in geographic regions with climates ranging from temperate subhumid to hot humid, and it appears to flourish better in slightly acidic soils. Synthesis of hydroxamate-type siderophores is a characteristic feature of *B. tropicalis*.

INTRODUCTION

The genus *Burkholderia,* including seven species, was proposed in 1992 (Yabuuchi *et al.*, 1992). Since then there has been a steady increase in the description of new *Burkholderia* species (Urakami *et al.*, 1994; Gillis *et al.*, 1995; Vandamme *et al.*, 1997; Brett *et al.*, 1998; Achouak *et al.*, 1999; Zhang *et al.*, 2000; Coenye *et al.*, 2001; Vandamme *et al.*, 2002). Nevertheless, some of these species have been reclassified to other genera, for instance *B. solanacearum*

(presently *Ralstonia solanacearum*) (Yabuuchi *et al.*, 1995), and *B. norimbergensis* (presently *Pandoraea norimbergensis*) (Coenye *et al.*, 2000). Currently, there exist 24 different *Burkholderia* species, *B. cepacia* being the type species of the genus (Yabuuchi *et al.*, 1992). Moreover, two nodulating diazotrophic strains recovered from legume plants were assigned to the genus *Burkholderia* according to their 16S rRNA sequences (Moulin *et al.*, 2001). In addition, the EMBL/GenBank data base contains the 16S rRNA sequences of two N_2-fixing bacteria, *Burkholderia brasilensis* and *Burkholderia tropicalis* (accession numbers AF164045 and AJ238360, respectively) which have been proposed very recently as novel species (V.M. Reis & A. Hartmann pers. comm.).

For a long time, N_2-fixing ability in bacteria of the genus *Burkholderia* was recognized only in the species *B. vietnamiensis* (Gillis *et al.*, 1995). This species was isolated from the rhizosphere of young rice plants grown on a Vietnamese soil under laboratory conditions (Tran Van *et al.*, 1994). Recently, the analysis of N_2-fixing bacteria associated with maize and coffee plants grown under natural field conditions revealed both the presence of *B. vietnamiensis* as well as the richness of the genus *Burkholderia* in unknown diazotrophs (Estrada-de los Santos *et al.*, 2001). These unknown N_2-fixing isolates showed different amplified 16S rDNA restriction (ARDRA) profiles corresponding to fourteen the ARDRA genotypes identified. However, some isolates with slightly different ARDRA profiles showed almost identical whole-cell protein patterns and other common phenotypic and genotypic features such as nitrogenase activity with different carbon sources and identical *nifHDK* hybridization patterns. On these bases, and taking into account the abundance as well as the wide geographic and environmental distribution of isolates corresponding to 16S rDNA genotypes 16, 17, and 19, as they were designated previously (Estrada-de los Santos *et al.*, 2001), an extended phenotypic and genotypic analysis was performed including new isolates recovered from Mexico and South Africa.

In the present study, we show evidence which indicate that the isolates with ARDRA profiles 16, 17, and 19 belong to the novel species *B. tropicalis* proposed

recently. We also describe the climatic distribution of *B. tropicalis* and show that this species has different host plants such as maize, teosinte, and sugarcane.

METHODS

Isolation. N_2-fixing *Burkholderia* isolates were recovered using two different strategies. In Mexico, the diazotrophic isolates were recovered as described previously (Estrada-de los Santos *et al.*, 2001) from the rhizosphere, rhizoplane and inner tissues of maize and teosinte plants cultivated in different climatic regions. *Burkholderia* strains recovered in South Africa were isolated from unsterilized roots of sugarcane cultivated in the South Africa Sugar Experiment Station of Mount Edgecombe (Kwazulu Natal). Roots were washed gently with tap water and blended in a Waring blender in 0.8% (w/v) NaCl to give a 10^{-1} dilution. Serial 10-fold dilutions were prepared and the 10^{-3} and 10^{-4} dilutions were plated onto PCAT medium (Burbage & Sasser, 1982). After incubation for 48 h bacterial colonies were transferred to PCAT agar plates once more and purified on triptic soy agar (TSA) plates. Isolates were maintained in 20 % (v/v) glycerol at -80 ^{0}C prior to analysis.

Bacterial strains. The source of thirty-seven *Burkholderia* isolates analyzed is shown in Table 1. Six of these strains were from a collection described previously (Estrada-de los Santos *et al.*, 2001) which correspond to ARDRA genotypes 16, 17 and 19. Among many new N_2-fixing *Burkholderia* isolates obtained from each sample (rhizosphere, rhizoplane or plant tissue), we included only one representative strain from each sample. Type strains of *Burkholderia* species included in the analysis were *B. cepacia* LMG1222^T, *B. vietnamiensis* TVV75^T, *B. caribensis* MWAP64^T, *B. graminis* C4D1M^T, *B. kururiensis* KP23T^T and *B. tropicalis* Ppe8 (LMG21393).

Phenotypic characterization. Strains were grown at 29 ^{0}C unless otherwise indicated. An inoculum was prepared by growing isolates for 12 h in BSE medium (Estrada-de los Santos *et al.*, 2001). The cultures were washed twice in 10 mM $MgSO_4$ and then adjusted to a 0.2 optical density (3 x 10^6 ufc ml^{-1}). Each culture was streaked on solid media to determine the phenotypic features.

Colony morphology was examined on BAc agar plates (Estrada-de los Santos *et al.*, 2001) and growth was recorded after four days. Growth at 37 and 42 ^{0}C, as well as at pH 4.5, 5.5, and 6.5 were determined in BSE agar medium. Growth on MacConkey agar (DIFCO) plates as well as on BCSA medium and BCSA-vancomycin (2.5 mg l^{-1}) medium (Henry *et al.*, 1997) was determined after 72 h. Growth on MacConkey medium was determined at 29 and 37 ^{0}C. Two replicates were used for each characteristic examined. In addition, strains were analyzed with API 20NE, API 50CH and Biolog systems. In the case of API 20NE and API 50CH tests the strains were streaked on BSE medium and incubated for 48 h. Several colonies were harvested with a swab to prepare a bacterial suspension with turbidity equivalent to 4 McFarland and also several colonies grown on BAc agar plates (Estrada-de los Santos *et al.*, 2001) were harvested to determine oxidase reaction which is a complementary test in the API 20NE system. The bacterial suspension for both tests was performed according to recommendations of the manufacture (bioMérieux). The results for API 50CH galleries were obtained after 6 days of incubation. When the Biolog system was used, the strains were incubated on Biological Universal Growth Medium at 30 ^{0}C for 24 h. Colonies were removed from the agar plate with a sterile swab and transferred into a tube containing inoculating fluid using a turbidemeter to measure the cell density. Then, 150 μl of this suspension was added to each of the 96 wells in Biolog GN2 Microplates and incubated at 30 ^{0}C. Colour formation in the wells was measured after 24 h using an optical density (OD590 nm) microplate reader. The quantitative data of carbon source utilization by each strain was transformed to categories using the CategVar module in ADE-4 software.

Siderophore production. Siderophores were detected using the universal chemical assays on chromeazurol-S (CAS) agar plates, and in CAS solution as described previously (Schwyn & Neilands, 1987). Hydroxamate-type siderophores were identified using the Czàky test (1948), and catechol-type siderophores using the Arnow test (1937).

SDS-PAGE of whole-cell proteins. Strains were grown in BSE medium and the preparation of whole-cell proteins as well as SDS-PAGE assays were performed as described previously (Estrada-de los Santos *et al.*, 2001).

Total DNA isolation. Strains were grown in BSE medium for 12 h and centrifuged at 12,300Xg for total DNA preparation according to Ausubel *et al.*, (1987).

PCR and amplified DNA restriction analysis (ARDRA). Primers fD1 and rD1 were used for the amplification of the 16S rRNA gene (Weisburg *et al.*, 1991) using the PCR conditions described previously (Estrada-de los Santos *et al.*, 2001). The amplified 16S rDNAs (ca. 1.5 kb) were restricted with 5 U each of *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hinf*I, *Msp*I, and *Rsa*I. The restriction fragments were revealed by electrophoresis in 3% agarose gels and the patterns were compared. Each isolate was assigned to one of ARDRA genotype 16, 17 or 19 as described previously (Estrada-de los Santos *et al.*, 2001).

16S rRNA gene sequencing. Strains MOc-725, MTo-672 and MTo-293, which correspond to ARDRA genotypes 16, 17 and 19, respectively (Estrada-de los Santos *et al.*, 2001) were chosen for 16S rRNA gene sequencing. In order to get 16S rRNA sequence, PCR products were cloned into the pCRII vector according to the manufacturer instructions (Invitrogen). 16S genes were restricted in small fragments (from 0.8- to 0.3-kb) using *Eco*RI and subcloned into vector pUC18. 16S rRNA gene sequences were performed by Medigenomix (Germany). The sequence of both strands was determined using universal primers for pUC18 vector.

Phylogenetic analysis of 16S rDNA gene sequences. All of the 16S rDNA gene sequences were deposited in the EMBL/GenBank data base (accession numbers in Fig. 2). These sequences were compared with previously published 16S rDNA from *Burkholderia* species and related bacteria such as *Herbaspirillum seropedicae* and *Ralstonia pickettii*. Phylogenetic relationships were estimated according to Jukes and Cantor (1969) and the tree was constructed by the Neighbor-Joining (NJ) method (Saitou and Nei 1987). The alignment included 1284 DNA sites.

Species specific PCR primers. Available *Burkholderia* 16S rRNA sequences were aligned to identify regions with some degree of specificity in order to design and test PCR primers which would give an amplicon only with *B. tropicalis*. In the 16S sequence a region corresponding to positions 456-475 of *E. coli* (GenBank accession V00348) was identified which is specific for *B. tropicalis*. A BLAST in GenBank retrieved only *B. tropicalis* strains and two unidentified bacteria found in a sponge (Althoff *et al.*, 1998); all other hits were eucaryotic sequences. This region was chosen to define the following (17-mer) forward primer: 5'TCCCTGGTCCTAATATG3'. Moreover, in a previous work (Pallud *et al.*, 2001) another 16S region was identified as nearly *Burkholderia* specific and tested as a probe. The same sequence allowed the design of a reverse primer (positions 1240-1256): 16-mer: 5'CAACCCTCTGTTCCGA3'. PCR conditions were as follows: initial denaturation for 7 min at 95 ^{0}C, followed by 35 cycles of 1 min denaturation at 94 ^{0}C, 1 min annealing at 48 ^{0}C, and 1 min elongation at 72 ^{0}C, followed by a final 15 minutes elongation at 72 ^{0}C.

DNA-DNA relatedness analysis. DNA-DNA homology was based on relative levels of hybridization to ^{32}P-labeled DNA as described previously (Estrada-de los Santos *et al.*, 2001).

RESULTS

Phenotypic characterization. All of the strains corresponding to either ARDRA genotype 16, 17, and 19 were Gram negative, motile in N-free semisolid media at 29 ^{0}C. The bacterial growth in N-free semisolid BAz medium formed very thin and fine pellicles at a depth of 4 mm below the surface at 24 h. After 72 h the pellicles become yellowish, diffuse, thick and moved up to the surface without pH changes in the medium. Transmission electron microscopy revealed that the strain BM273 (genotype 19) was a slightly curved rod and possessed three polar flagella (Fig. 1) as well as peritrichous fimbriae (data not shown). The colonies of isolates with genotype 16, 17, or 19 growing on BAc medium plates were yellowish, round, smooth, and convex, from 1-2 mm in diameter with entire margins after incubation for 4 days at 29 ^{0}C as described previously (Estrada-de

los Santos *et al.*, 2001). API 20NE biochemical tests identified the isolates as *B. cepacia* (98.9 to 99.6 % confidence limits based on the API analytical profile index). However, *B. cepacia* is not able to fix nitrogen whereas all of these isolates and the strain Ppe8 of *B. tropicalis* grew and showed the ability to reduce acetylene in N-free semisolid BAz medium (Table 2), except for strain RASC. All of the isolates with ARDRA genotype 16, 17, or 19 and strain Ppe8 grew on MacConkey agar medium at 29 ^{0}C but not at 37 ^{0}C, nor did they grow on BCSA medium with or without vancomycin. All of these isolates, including strain Ppe8, grew on BSE agar medium at 37 ^{0}C but not at 42 ^{0}C; they exhibited good growth on BSE medium at pH from 4.5-6.5 but poor growth was observed from pH 7.0-7.5. All of the strains with genotype 16, 17 or 19 reduced nitrate, showed urease activity but not esculin hydrolysis, liquefaction of gelatin, nor indole production. Phenotypic characteristics for the differentiation of N_2-fixing *Burkholderia* species and the type species of the genus *Burkholderia* are showed in Table 2.

The assimilation profile of 49 carbon sources was very similar among isolates with ARDRA genotypes 16, 17, 19, 19a and strain Ppe8 (Table 3) suggesting that all of them belong to a same species. Differences in the usage of carbon sources by these isolates and other related *Burkholderia* species are shown in Table 3. The N_2-fixing *Burkholderia* isolates with genotype 16, 17, 19 or 19a were analyzed for the ability to oxidize 95 compounds (BIOLOG). All of the strains were able to oxidize Tween 40, Tween 80, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, α-D-glucose, D-mannitol, mannose, L-rhamnose, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, citric acid, D-gluconic acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, D,L-lactic acid, propionic acid, quinic acid, sebacic acid, succinic acid, bromosuccinic acid, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, hydroxy-L-proline, L-phenylalanine, L-proline, γ-aminobutyric acid, and 2-aminoethanol. The strains were unable to oxidize α-cyclodextrin, dextrin, glycogen, N-acetyl-D-galactosamine, erythritol, α-D-lactose, maltose, melibiose, methyl β-D-glucoside, D-raffinose, sucrose, turanose, xylitol, D-galactonic acid lactone, D-glucoronic acid, γ-hydroxybutyric acid, α-ketovaleric

acid, malonic acid, glucoronamide, glycyl-L-aspartic acid, D-serine, urocanic acid, uridine, phenylethylamine, and glucose-1-phosphate. Variable results were obtained for the oxidation of D-sorbitol, formic acid, D-galacturonic acid, D-saccharic acid, L-serine, alaninamide, L-pyroglutamic acid, inosine, *myo*-inositol, acetic acid, D-glucosaminic acid, α-hydroxybutyric acid, DL-alanine, L-histidine, gentiobiose, lactulose, D-psicose, itaconic acid, L-ornithine, D,L-carnitine, putrescine, α-ketobutyric acid, α-ketoglutaric acid, succinamic acid, L-threonine, glucose-6-phosphate, D-trehalose, glycyl-L-glutamic acid, 2,3-butanediol, glycerol, glycerolphosphate, and L-leucine.

Siderophore production. All of the isolates with genotype 16, 17, and 19 showed the ability to produce siderophores. Ninety-two percent of the strains produced hydroxamates as the main type of siderophore.

SDS-PAGE of whole-cell proteins. Whole-cell protein extracts were prepared from 37 N_2-fixing *Burkholderia* isolates and from several related species. The protein patterns of some representative strains corresponding to ARDRA genotypes 16, 17, 19, and 19a are shown in Fig. 2. N_2-fixing *Burkholderia* strains isolated from maize, teosinte, and sugarcane plants growing in different regions of Mexico and South Africa showed almost identical protein patterns among them and with the strain Ppe8 of *B. tropicalis*. These proteins patterns were clearly different from other N_2-fixing and from non N_2-fixing *Burkholderia* species (Fig. 2). Interestingly, strain KP23^T of *B. kururiensis* and *B. brasilensis* M130 showed very similar protein electrophoregrams (Fig. 2).

ARDRA. Previously, ARDRA genotypes 16, 17, and 19 were identified with seven restriction enzymes (Estrada-de los Santos *et al.*, 2001). These genotypes showed identical ARDRA profiles with enzymes *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Msp*I, and *Rsa*I but they were distinguished with the enzyme *Hin*fI (Fig. 3). *B. tropicalis* strain Ppe8 showed an ARDRA profile identical to genotype 19. Strains LM1-376.8, LM2-376.3 and RASC showed the same profiles as genotype 19 except with *Hae*III, and therefore they were designated as ARDRA genotype 19a (Table 1). The size of the 16S rDNA gene amplified with fD1 and rD1 primers was approximately 1.5 kb. When the 16S rDNA gene was restricted with each of the

seven different enzymes the sum of the fragments was less than or equal to 1.5 kb, except with the *Hinf*I enzyme. In the case of strains corresponding to ARDRA genotype 17 the sum of the 16S rDNA fragments restricted with the *Hinf*I enzyme was greater than 2.0 kb (Fig. 3, lane 8). This result was always obtained despite to use different pools of purified DNA in PCR assays. Bacterial contamination of the culture was eliminated as a possible cause.

Phylogenetic analysis of 16S rDNA gene sequences. The sequence of strains MOc-725, MTo-672 and MTo-293 from genotypes 16, 17 and 19, respectively, were compared with available 16S rDNA sequences from all of the *Burkholderia* species (Fig. 4). The strains MOc-725, MTo-672 and MTo-293 were closely related, forming a cluster with the strains BM16 and BM273 described in a previous study (Estrada *et al.*, 2002) as well as with the strains Ppe8 and AB98 of *B. tropicalis*. The percentage of similarity among the 16S rDNA sequences of these strains ranged from 99.2 to 99.9. The N_2-fixing species *B. tropicalis* clearly constituted a cluster separated from the cluster formed by the other diazotrophic species *B. kururiensis*/*B. brasilensis*, but was largely distant (similarity < 96 %) from the cluster named "*B. cepacia* complex", a group of opportunistic pathogen species in patients with cystic fibrosis (Vandamme *et al.*, 1997), which include the N_2-fixing species *B. vietnamiensis*. *B. sacchari*, a non-diazotrophic bacterium, was the closest specie to the *B. tropicalis* cluster (97.2 % of similarity). 16S similarity levels with *Herbaspirillum seropedicae* and *Ralstonia pickettii*, taxa belonging to the β-subclass of *Proteobacteria*, were 89.9 and 85.8 %, respectively.

Species specific PCR. Table 4 shows that amplification was strictly species specific.

DNA-DNA relatedness analysis. Strains corresponding to genotypes 16, 17, and 19, were selected to estimate the DNA-DNA reassociation levels with strain Ppe8 of *B. tropicalis*. The DNA-DNA reassociation values between Ppe8 and strain MOc-725 (genotype 16), MTo-672 (genotype 17) and MTo-293 (genotype 19) were 87.3, 97.3 and 94.0 %, respectively. Strains LM1-376.8, LM2-376.3 and RASC (genotype 19a) showed DNA-DNA reassociation values of 92.3, 90.2 and

74.0 %, respectively, with strain Ppe8 of *B. tropicalis*. The DNA-DNA reassociation values between strain Ppe8 and strains *B. cepacia* LMG1222^T, *B. caribensis* MWAP64^T, *B. graminis* C4D1M^T, *B. kururiensis* KP23T^T and *Herbaspirillum seropedicae* Z67, were 15.1, 9.8, 10.1, 16.7 and 8.1 %, respectively.

DISCUSSION

Recently, we showed that the genus *Burkholderia* is rich in N_2-fixing bacteria which are widely distributed in the environment (Estrada-de los Santos *et al.*, 2001). In the present study, N_2-fixing *Burkholderia* were repeatedly isolated from the rhizosphere and rhizoplane as well as from the endophyte environment of maize and teosinte using the culture media BAz and BAc described previously (Estrada-de los Santos *et al.*, 2001). The successful recovery of these diazotrophic bacteria is based in the semiselective enrichment using the N-free semisolid azelate medium (BAz), and to the subsequent isolation and culturing on BAc medium plates, which contain citruline as a nitrogen source. Colonies of *Burkholderia* (N_2-fixing or not) grow yellowish, round, smooth and convex on BAc medium plates, and they turn the culture medium color from green to a deep blue color. Colonies with this morphology and with the ability to reduce acetylene to ethylene into N-free media can be presumptively considered diazotrophic *Burkholderia*.

The isolation of diazotrophic *Burkholderia* sp. from rice roots and sugarcane stems has been claimed several times (Baldani *et al.*, 1997; Kirchhof *et al.*, 1997, Reis *et al.*, 2000). These N_2-fixing bacteria have been provisorily named *Burkholderia brasilensis* (Weber *et al.*, 1999) and *Burkholderia tropicalis* (Magalhaes *et al.*, 2001). In addition, 23S rRNA oligonucleotide probes have been designed for identification of *B. brasilensis* and *B. tropicalis* (Hartman *et al.*, 1995; Weber *et al.*, 1999), and their complete 16S rDNA sequences have been deposited recently in the GenBank database (accession numbers AJ238360 and AJ420332, respectively). Nevertheless, hitherto there is no report of a specific approach for the isolation from *B. brasilensis* and *B. tropicalis*, nor description of

their colony morphology or phenotypic features, criteria which contribute to recognizing a bacterial species. This unusual situation led to the exceptional coincidence of finding that the *Burkholderia* isolates with ARDRA genotype 16, 17, 19, and 19a, recovered with different strategies as well as from different host plants and environments (Estrada-de los Santos *et al.*, 2001; Estrada *et al.*, 2002), belong to the species *B. tropicalis*, just recently submitted as a proposed new diazotrophic bacterium (V.M. Reis & A. Hartmann, pers. comm.). Our claim is based on the high similarity of phenotypic and genotypic traits between the *Burkholderia* isolates with genotype 16, 17, 19, or 19a and the strain Ppe8 of *B. tropicalis*, as well as by the low resemblance of these isolates with other *Burkholderia* species. Similar phenotypic characteristics include the ability or inability to grow on different culture media, temperatures and range of pH, as well as the assimilation patterns of carbon sources, and the almost identical whole-cell protein profiles. In addition, isolates with genotypes 16, 17, and 19 showed a similarity of 16S rDNA sequences higher than 99.2 % with strains Ppe8 and AB98 of *B. tropicalis* and levels of DNA-DNA reassociation higher than 70 % (range from 74 to 97 %) with total DNA from strain Ppe8. All of these features agree with similarity criteria recommended for the delineation of bacterial species (Vandamme *et al.*, 1996).

In this study we found that the sum of the restriction fragments of 16S rDNA with the enzyme *Hinf*I from strains of *B. tropicalis* genotype 17 was higher than the size of the unrestricted 16S rDNA. A possible explanation could be due to the amplification of distinct copies of these genes, some of which lack one of the restriction sites. It is known that bacteria have up to 15 copies of ribosomal operons and that they have internal differences (Mylvaganam & Denis, 1992, Klappenbach *et al.*, 2000). *B. cepacia* contain up to six operons of ribosomal genes (Lessie *et al.*, 1996) and recently, Salles *et al.* (2002) showed that *Burkholderia* sp. from environmental samples had multiple 16S rDNA operons.

Previously, we reported the ability from diazotrophic *Burkholderia* isolates, corresponding to ARDRA genotypes 16, 17, and 19, to reduce acetylene with different carbon sources, and we showed the presence of *nifHDK* genes in some

of these isolates (Estrada-de los Santos *et al.*, 2001). These characteristics have been confirmed with many isolates tested in the present study (data not shown), which confirm that the ability to fix nitrogen is a typical feature of *B. tropicalis*.

Hitherto, N_2-fixing ability had been only described in the species *B. vietnamiensis* (Gillis *et al.*, 1995) and *B. kururiensis* (Estrada-de los Santos *et al.*, 2001) among all of the well-known species of the genus *Burkholderia*. In this framework, the diazotrophic ability can be used as a distinctive feature for the delineation of *Burkholderia* species. *B. tropicalis* can be phenotypically differentiated from *B. kururiensis* by its ability to grow on MacConkey and BAc medium plates at 29 ^{0}C. Moreover, *B. tropicalis* can be differentiated from *B. vietnamiensis* and *B. kururiensis* by its inability to grow on BSE medium at 42 ^{0}C. Both *B. tropicalis* and *B. kururiensis* can be differentiated from *B. vietnamiensis* by their inability to grow on MacConkey medium at 37 ^{0}C, and they are incapable of growth on BCSA and BCSA-vancomycin medium plates. In addition, *B. tropicalis* can be differentiated from *B. vietnamiensis* by its ability to assimilate adonitol, rhamnose, and L-fucose as well as its inability to assimilate dulcitol, amygdalin, sucrose, D-raffinose, and D-tagatose. *B. tropicalis* can be differentiated from *B. kururiensis* by its ability to assimilate cellobiose and β-gentiobiose and by its inability to assimilate xylitol, L-arabitol and 5-ketogluconate. Taking into account the almost identical 16S rDNA sequences (99.9 %) between *B. kururiensis* and *B. brasilensis* described previously (Estrada-de los Santos *et al.*, 2001), and because many phenotypic features were identical (data not shown) or very similar (e.g., protein patterns; Fig. 2) in both species, *B. brasilensis* was not included in comparative analyses with other diazotrophic *Burkholderia*.

Burkholderia species are soil bacteria whose natural habitats are moist environments, the rhizosphere, and the surface of plant roots (Woods & Sokol, 2000). In this study, *B. tropicalis* was isolated from the rhizosphere and as endophyte of maize and teosinte plants as well as from roots of maize and sugarcane plants grown in geographic regions with climates ranging from temperate subhumid to hot humid. In contrast, *B. tropicalis* was isolated from

sugarcane plants growing in a tropical environment in Brazil. In Mexico, the distinct *B. tropicalis* genotypes were not recovered from the rhizosphere nor from maize plants grown in four different regions with semihot or hot dry climates (data not shown). Moreover, *B. tropicalis* was found in the rhizosphere and associated with plants growing in soils with pH in the range from 4.5 to 7.1. However, when a few maize plants were collected from soils with pH higher than 7.5, *B. tropicalis* isolates were not recovered. This result agrees with the good growth of *B. tropicalis* on culture media with pH 4.5-6.5. Bacteria of the genus *Burkholderia* are considered to be characteristic of neutral pH environments (Liesack *et al.*, 1997) but recent evidence suggests a high abundance of *Burkholderia* in acidic soils (Nogales *et al.*, 2001). Although more studies should be carried out, *B. tropicalis* appears to flourish better in slightly acidic soils and its distribution limited to geographic regions with humid or subhumid climates.

Because *B. tropicalis* is largely distant from the diazotrophic species *B. vietnamiensis* as well as from the other species included in the "*B. cepacia complex*" (Vandamme *et al.*, 1997), and taking into account the abilities of *B. tropicalis* to fix nitrogen, to produce siderophores as well as its substantial capability for colonizing different host plants from widely distant geographical regions, we considered it important to assess the agricultural potential of this species, both for promoting plant growth and as a biocontrol agent.

Because *B. tropicalis* has been referred to in diverse studies but considering that information on its isolation and identification was lacking, we summarized the characteristics of this novel N_2-fixing bacterium. Cells are slightly curved rods (approximately 1.5 μm long and 0.7 μm wide) with three polar flagella. Isolates are Gram negative, oxidase and catalase positive. Growth and acetylene reduction to ethylene is observed in N-free semisolid media. *B. tropicalis* strains grow on BAc plates showing colonies yellowish, round, smooth, convex with entire margins and 1 mm diameter after incubation for 4 days at 29 ^{0}C. Isolates grew on agar media at 29 ^{0}C but not at 42 ^{0}C. At 37 ^{0}C, they grew on BSE medium but not on MacConkey medium. Strains did not grow on BCSA medium with or without vancomycin. Phenotypic characteristics for the differentiation of

N_2-fixing *Burkholderia* species and the type species of the genus *Burkholderia* are shown in Table 2. Nitrate is reduced; there is urease activity but not esculin hydrolysis, nor liquefaction of gelatin, or indole production. Additional phenotypic characteristics are listed in Table 3. The species *B. tropicalis* is constituted by strains, including the strain Ppe8, with 4 different ARDRA genotypes, and their 16S rDNA sequence show more than 99.2 % similarity. The accession numbers of the 16S rDNA sequences in the EMBL/GenBank data base of strains MTo-293, MTo-672, and MOc-725 are AY128103, AY128104, and AY128105, respectively. *B. tropicalis* strains were deposited in the American Type Culture Collection (ATCC) with the following numbers: strain MOc-725 (ATCC BAA-567), strain MTo-672 (ATCC BAA-568) and strain MTo-293 (ATCC BAA-569).

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Table 1. Source and locality from *Burkholderia tropicalis* strains analyzed.

ARDRA Profile	Strain designation	Source	pH of soil	Locality	Climate[a] class	Reference
16	MCh-1054	Maize roots*	6.76	Chilpancingo, Guerrero[1]	1	This study
16	MCh-1057	Maize rhizoplane	6.76	Chilpancingo, Guerrero	1	This study
16	MCo-7712	Maize roots*	6.69	Coatepec, Veracruz[1]	2	This study
16	MOc-255	Maize rhizosphere	6.40	Ocotepec, Morelos[1]	3	Estrada *et al.*, 2001
16	MOc-332	Maize rhizosphere	6.22	Ocotepec, Morelos	3	This study
16	MOc-725	Maize rhizoplane	6.35	Ocotepec, Morelos	3	Estrada *et al.*, 2001
16	MOc-3412	Maize roots*	6.55	Ocotepec, Morelos	3	This study
16	MTe-73523	Maize roots*	5.04	Tepoztlan, Morelos[1]	1	This study
16	MTe-7363	Maize rhizoplane	5.04	Tepoztlan, Morelos	1	This study
16	MTl-5681	Maize roots*	4.56	Tlayacapan, Morelos[1]	1	This study
16	MTl-582	Maize rhizosphere	4.84	Tlayacapan, Morelos	1	This study
16	TTe-225	Teosinte rhizosphere	7.10	Tepoztlan, Morelos	1	This study
17	MCa-9022	Maize rhizosphere	5.15	Cacaohatan, Chiapas[1]	4	This study
17	MCo-7931	Maize rhizosphere	6.89	Coatepec, Veracruz	2	This study
17	MCu-831	Maize rhizoplane	7.07	Cuernavaca, Morelos[1]	1	This study
17	MMi-786	Maize rhizoplane	nd	Miacatlán, Morelos[1]	3	Estrada *et al.*, 2001
17	MSj-805	Maize rhizosphere	6.20	San J. Atenco, Puebla[1]	5	This study
17	MTl-6311	Maize rhizoplane	4.87	Tlayacapan, Morelos	1	This study
17	MTo-432	Maize rhizoplane	nd	Totontepec, Oaxaca[1]	2	Estrada *et al.*, 2001
17	MTo-672	Maize stem*	nd	Totontepec, Oaxaca	2	This study
17	MXo-435	Maize rhizoplane	7.15	Xoxocotla, Morelos[1]	3	This study
17	MXo-437	Maize rhizosphere	7.15	Xoxocotla, Morelos	3	This study
17	TSj-832	Teosinte rhizosphere	6.29	San J. Atenco, Puebla	5	This study
17	TTe-1910	Teosinte stem*	7.03	Tepoztlan, Morelos	1	This study
19	BM-16	Maize stem*	nd	Totontepec, Oaxaca	2	Estrada *et al.*, 2002
19	BM-273	Maize roots*	nd	Totontepec, Oaxaca	2	Estrada *et al.*, 2002
19	MCo-761	Maize roots*	6.79	Coatepec, Veracruz	2	This study
19	MCo-8562	Maize rhizoplane	6.62	Coatepec, Veracruz	2	This study
19	MCu-82	Maize rhizosphere	7.07	Cuernavaca, Morelos	1	This study
19	MCu-833	Maize roots*	7.07	Cuernavaca, Morelos	1	This study
19	MCu-842	Maize rhizoplane	7.07	Cuernavaca, Morelos	1	This study
19	MSj-8432	Maize rhizosphere	6.29	San J. Atenco, Puebla	5	This study
19	MTo-16	Maize rhizosphere	nd	Totontepec, Oaxaca	2	Estrada *et al.*, 2001
19	MTo-293	Maize stem*	nd	Totontepec, Oaxaca	2	Estrada *et al.*, 2001
19a	LM1-376.8	Sugarcane roots	nd	Tongaat, KwaZulu-N[2].	1	This study
19a	LM2-376.3	Sugarcane roots	nd	Tongaat, KwaZulu-N.	1	This study
19a	RASC	Activated sludge		Oregon[3]		Suwa *et al.*, 1996

[a] Climate class codes: 1, semihot subhumid; 2, semihot humid; 3, hot subhumid; 4, hot humid; 5, temperate subhumid.
* Surface-sterilized.
[1] México; [2] South Africa; [3] United States of America.
nd, not determinated.

Table 2. Phenotypic characteristics of N_2-fixing *Burkholderia* species and *B. cepacia*^{T}

Characteristics	**Genotypes 16, 17, and 19 (n=34)**	***B. tropicalis* Ppe8**	***B. vietnamiensis* (n=30)**	***B. kururiensis* KP23^T**	***B. cepacia* LMG1222^T**
Growth in N-free semisolid BAz medium	+	+	+	+	–
C_2H_2 reduction activity (N_2-fixation)	+	+	+	+	–
Growth on BAc medium	+	+	+	–	+
Growth on MacConkey medium at 29 ^{0}C	+	+	+	–	+
Growth on MacConkey medium at 37 ^{0}C	–	–	+	–	+
Growth on BCSA medium	–	–	+	–	+
Growth on BCSA-vancomycin medium	–	–	+	–	+
Growth on BSE agar medium at 37 ^{0}C	+	+	+	+	+
Growth on BSE agar medium at 42 ^{0}C	–	–	+	+	+

+, good growth; –, no growth

Table 3. Phenotypic characteristics of *Burkholderia tropicalis* and related species.

Substrate	1[1]	2	3	4	5	6	7	8	9
Glycerol	+	+	+	+	+	+	+	+	+
D-Arabinose	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	-	+
L-Xylose	d-	-	d-	-	-	-	+	+	-
Adonitol	+	+	+	+	+	-	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-
Rhamnose	+	+	+	+	-	-	+	+	+
Dulcitol	-	-	-	-	+	+	-	-	-
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	-	-	+	+	-	-	-
Arbutin	-	-	-	-	+	-	-	-	-
Salicin	d+	-	+	-	+	+	-	-	-
Cellobiose	+	+	+	+	+	+	-	+	+
Maltose	-	-	-	-	-	-	-	-	+
Lactose	d+	-	d+	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	+	+	-	+	-
Trehalose	+	d-	d+	-	+	+	-	+	-
D-Raffinose	-	-	-	-	+	+	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	+	+	+
β-Gentiobiose	+	+	+	+	+	+	-	-	-
D-Lyxose	+	d-	+	-	+	+	+	+	-
D-Tagatose	-	-	-	-	+	+	-	-	-
D-Fucose	+	+	+	+	-	+	+	-	-
L-Fucose	+	+	+	+	+	-	+	+	+
L-Arabitol	-	-	-	-	+	-	+	+	+
5-Ketogluconate	-	-	-	-	+	-	+	-	-
Esculin hydrolysis	-	-	-	-	+	-	-	-	-

All of the strains tested assimilated the following substrates: L-arabinose, D-arabitol, D-fructose, galactose, gluconate, D-glucose, inositol, 2-ketogluconate, malate, mannitol, D-mannose, phenylacetate, sorbitol, and D-xylose.

None of the strains assimilated erythritol, inulin, melezitose, methyl α-glucoside, methyl α-D-mannoside, methyl β-xyloside, starch, and D-turanose.

[1] *B. tropicalis* strains tested: 1, genotype 16 (n=9); 2, genotype 17 (n=10); 3, genotype 19 and 19a (n=12); 4, Ppe8. Other *Burkholderia* species tested: 5, *B. cepacia* LMG1222^T; 6, *B. vietnamiensis* TVV75^T; 7, *B. kururiensis* KP23^T; 8, *B. graminis* C4D1M^T; 9, *B. caribensis* MWAP64^T.

B. tropicalis strains: +, more than 95 % of the strains gave a positive reaction; -, more than 95 % of the strains gave a negative reaction; d+, between 80 and 95 % of the strains gave a positive reaction; d-, between 80 and 95 % of strains gave a negative reaction.

Table 4. Species specific PCR amplification of *B. tropicalis*. A 784 nucleotides amplicon was obtained (+) only with *B. tropicalis* strains, not (-) with representatives of other *Burkholderia* species.

Strain designation	ARDRA Profile	PCR result	References
B. tropicalis SMi-583	16	+	Estrada *et al.*, 2001
B. tropicalis MMi-786	17	+	Estrada *et al.*, 2001
B. tropicalis MTo-431	17	+	Estrada *et al.*, 2001
B. tropicalis BM-16	19	+	Estrada *et al.*, 2002
B. tropicalis BM-273	19	+	Estrada *et al.*, 2002
B. tropicalis MTo-293	19	+	Estrada *et al.*, 2001
B. tropicalis LM1-376.8	19a	+	This work
B. tropicalis LM2-376.3	19a	+	This work
B. caledonica LMG 19076 ^{T}	nd	-	Coenye *et al.*, 2001
B. caribensis MWAP64 ^{T}	nd	-	Achouack *et al.*, 1999
B. cepacia LMG 1222 ^{T}	nd	-	Yabuuchi *et al.*, 1992
B. cepacia genomovar III (6 strains tested)	nd	-	Balandreau *et al.*, 2001
B. fungorum LMG 16225 ^{T}	nd	-	Coenye *et al.*, 2001
B. graminis C4D1M ^{T}	nd	-	Viallard *et al.*, 1998
B. phenazinium LMG 2247 ^{T}	nd	-	Viallard *et al.*, 1998
B. thailandensis ATCC 700388 ^{T}	nd	-	Brett *et al.*, 1998
B. vietnamiensis TVV75 ^{T}	nd	-	Gillis *et al.*, 1995

nd, not determinated

Figure legends

Fig. 1. Transmission electron micrograph of strain BM-273 of *B. tropicalis* cells showing three polar flagella. Negative staining (aqueous sodium silicotungstate 1 % for 30 sec.) Photo courtesy of René Rohr (Lyon1 University).

Fig. 2. Protein electrophoregrams (SDS-PAGE) of selected *B. tropicalis* strains and *Burkholderia* reference strains. Lanes 1 through 13, *B. tropicalis* strains MOc-725, TTe-225, MTe-73523, MTo-293, LM1-376.8, LM2-376.3, MTo-672, TTe-1910, MXo-435, MCh-1057, TSj-832, MSj-8432, Ppe8, lane 14, *B. vietnamiensis* TVV75^{T}; lane 15, *B. kururiensis* KP23 ^{T}; lane 16, *B. brasilensis* M130; lane 17, *B. caribensis* MWAP64 ^{T}; lane 18, *B. cepacia* LMG1222 ^{T}.

Fig. 3. ARDRA profiles of *B. tropicalis* strains. Lanes: 1 and 6, 100 base pairs molecular marker (GIBCO BRL). Lanes 2 through 5, 16S rDNA amplified gene digested with *Dde*I; lane 2, strain MOc-725; lane 3, strain MTo-672; lane 4, strain MTo-293; lane 5, strain Ppe8. Lanes 7 through 10, 16S rDNA amplified gene digested with *Hinf*I; lane 7, strain MOc-725; lane 8, strain MTo-672; lane 9, strain MTo-293; lane 10, strain Ppe8.

Fig. 4. Phylogenetic tree based on 16S rRNA sequences showing the relatedness among *B. tropicalis*, *Burkholderia* species and related β-*Proteobacteria*. The bar represents 1 nucleotide substitution per 100 nucleotides. Bootstrap probabilities (Kumar et al., 1993) are indicated at the branch points. The GenBank accession number for each strain is shown in parentheses.

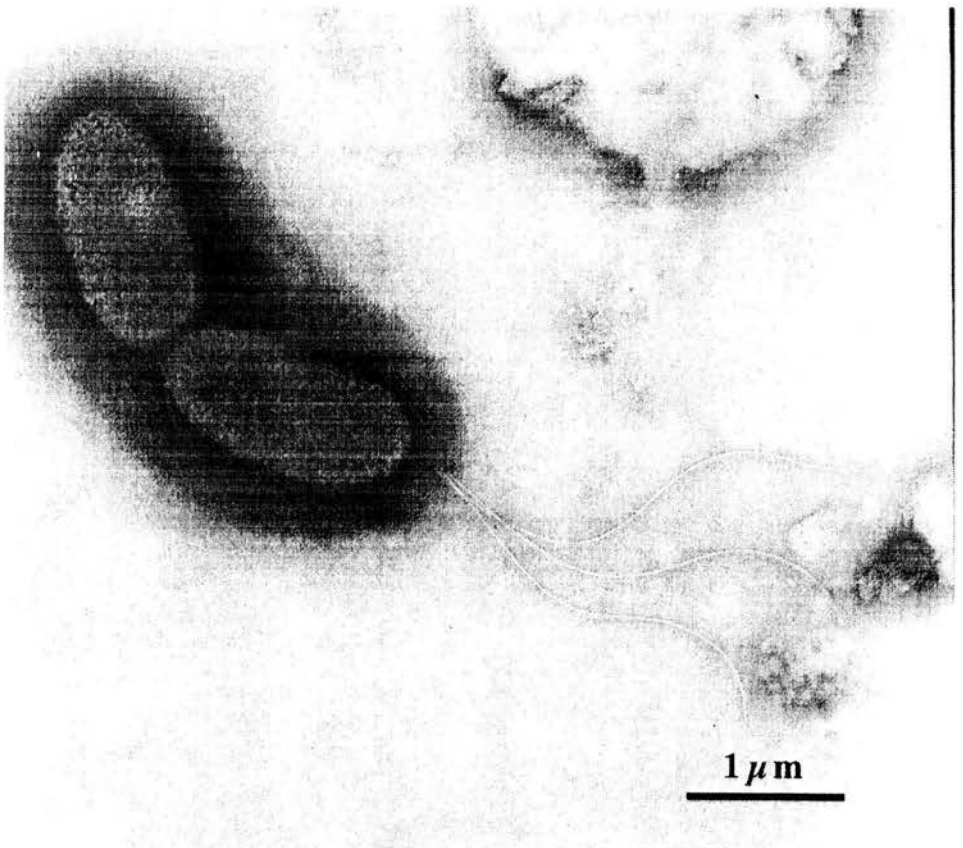

Fig. 1

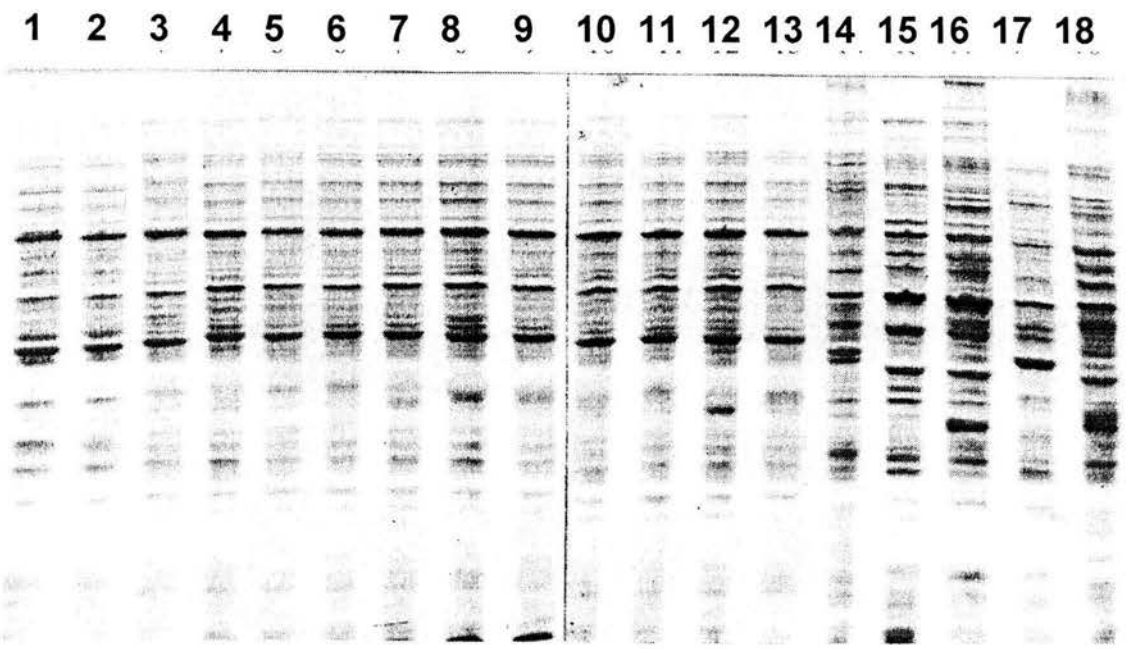

Fig. 2

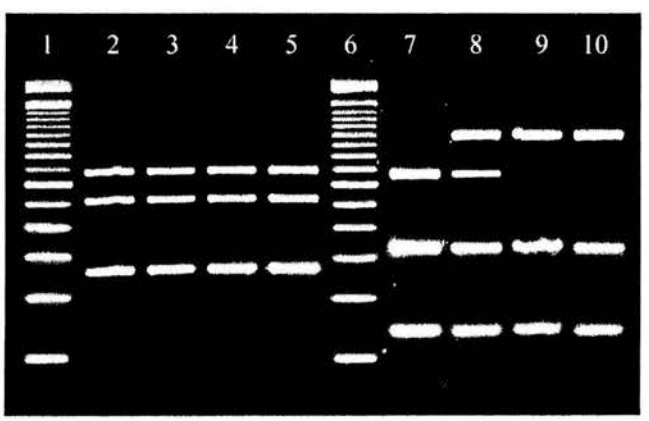

Fig. 3

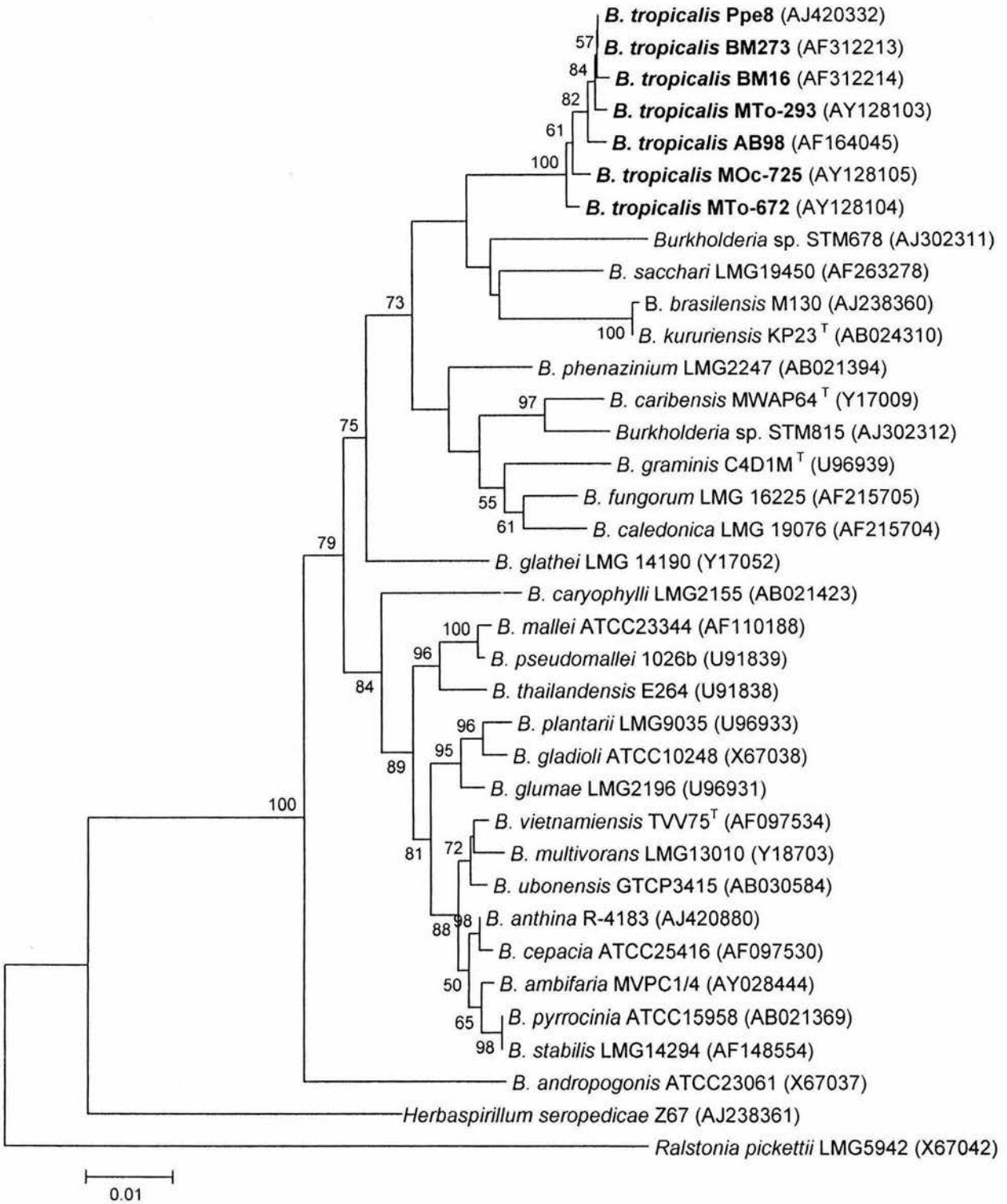
B. tropicalis Ppe8 (AJ420332)
B. tropicalis BM273 (AF312213)
B. tropicalis BM16 (AF312214)
B. tropicalis MTo-293 (AY128103)
B. tropicalis AB98 (AF164045)
B. tropicalis MOc-725 (AY128105)
B. tropicalis MTo-672 (AY128104)
Burkholderia sp. STM678 (AJ302311)
B. sacchari LMG19450 (AF263278)
B. brasilensis M130 (AJ238360)
B. kururiensis KP23 T (AB024310)
B. phenazinium LMG2247 (AB021394)
B. caribensis MWAP64 T (Y17009)
Burkholderia sp. STM815 (AJ302312)
B. graminis C4D1M T (U96939)
B. fungorum LMG 16225 (AF215705)
B. caledonica LMG 19076 (AF215704)
B. glathei LMG 14190 (Y17052)
B. caryophylli LMG2155 (AB021423)
B. mallei ATCC23344 (AF110188)
B. pseudomallei 1026b (U91839)
B. thailandensis E264 (U91838)
B. plantarii LMG9035 (U96933)
B. gladioli ATCC10248 (X67038)
B. glumae LMG2196 (U96931)
B. vietnamiensis TVV75T (AF097534)
B. multivorans LMG13010 (Y18703)
B. ubonensis GTCP3415 (AB030584)
B. anthina R-4183 (AJ420880)
B. cepacia ATCC25416 (AF097530)
B. ambifaria MVPC1/4 (AY028444)
B. pyrrocinia ATCC15958 (AB021369)
B. stabilis LMG14294 (AF148554)
B. andropogonis ATCC23061 (X67037)
Herbaspirillum seropedicae Z67 (AJ238361)
Ralstonia pickettii LMG5942 (X67042)
0.01

Fig. 4

RESULTADOS ADICIONALES

Taxonomía de *B. unamae*

El trabajo en conjunto en nuestro laboratorio permitió continuar con el aislamiento y caracterización de nuevas cepas correspondientes a los genotipos 13, 14 y 15 descritos previamente (Estrada-de los Santos y col. 2001). La estrategia para el aislamiento de los aislados diazótrofos fue similar a la utilizada con *B. "tropicalis"*. La caracterización inicial de los aislados incluyó el análisis de la secuencia del gen ribosomal 16S de las cepas de *Burkholderia* MCo-762 (genotipo 13), MTl-641 y SCCu-23 (genotipo 15) que fue amplificado con los oligonucleótidos universales fD1 y rD1 (Weisburg y col. 1991) y clonado en el vector pCR2.1 (TA Cloning Kit, Invitrogen). El gen 16S fue subclonado en fragmentos de menor tamaño utilizando el vector pUC18 (Messing 1983) para facilitar el proceso de secuencia. Los distintos fragmentos del gen ribosomal 16S fueron enviados a la empresa Medigenomix (Alemania) para su secuenciación. Las secuencias obtenidas fueron analizadas comparando con aquellas existentes en la base de datos EMBL/GenBank del género *Burkholderia*. La relaciones filogenéticas fueron estimadas de acuerdo a Jukes y Cantor (1969) y el árbol filogenético fue construido utilizando el método de Neighbor-Joining (Saitou y Nei 1987). El análisis de las secuencias mostró que las cepas diazótrofas poseen en promedio un porcentaje de similitud de 99.3 entre las cepas diazótrofas correspondientes a los genotipos 13, 14 y 15 siendo *B. sacchari* la especie mas cercana (~ 98.5 % de similitud; Figura 1). La segunda especie más cercana a los genotipos 13, 14 y 15 son el grupo de cepas correspondientes a *B. "tropicalis"* (~ 98.1 % de similitud). Un nivel de similitud de 97.0 % es considerado como el limite para la separación de especies bacterianas. No obstante, este límite no es absoluto y deben considerarse características fenotípicas y genómicas en conjunto. Es importante resaltar que el grupo de cepas correspondientes a los genotipos 13, 14 y 15 se encuentra alejado del conjunto de especies patógenas oportunistas denominado "complejo *B. cepacia*" (~ 95.8 % de similitud). Los resultados genómicos adicionales al análisis de la secuencia del gen 16S, así como características fenotípicas del grupo de diazótrofos estudiados en nuestro laboratorio, revelan que los genotipos 13, 14 y 15 representan una nueva especie dentro del género *Burkholderia*, para la cual se propone el nombre de *B. "unamae"*. Un manuscrito sobre la taxonomía polifásica de esta nueva especie esta siendo elaborado en nuestro grupo de trabajo.

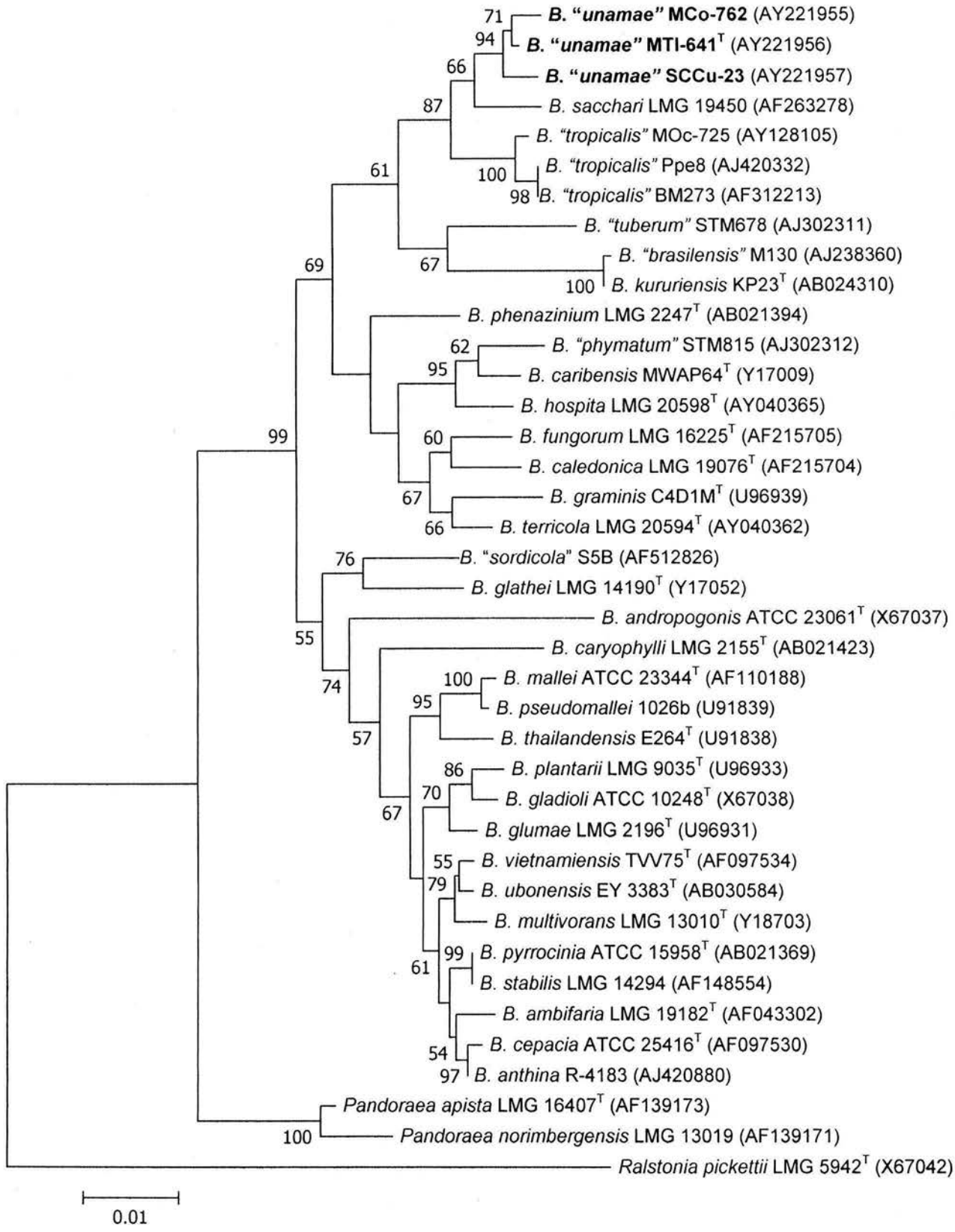

Figura 1. Relaciones filogenéticas basadas en el análisis del gen ribosomal 16S de especies conocidas de *Burkholderia* y *B. "unamae"*. El análisis incluyó 1324 nucleótidos.

Detección y secuencia parcial del gen *nifH* de diazótrofos de *Burkholderia*

Introducción

La descripción de nuevas especies bacterianas, incluyendo a las fijadoras de nitrógeno, se ha dado en años recientes y ha repercutido en un aumento en el conocimiento sobre la diversidad bacteriana. Al inicio de esta tesis, dentro de las especies del género *Burkholderia* solamente se conocía a *B. vietnamiensis* como la única fijadora de nitrógeno (Gillis et al. 1995). Además, en algunas revisiones se ha hecho alusión a la existencia de cepas diazótrofas de *Burkholderia* spp. (Kirchhof y col. 1997; Reis y col. 2000). Como parte de los estudios desarrollados en este trabajo, mostramos la existencia de numerosos aislados diazótrofos pertenecientes al género *Burkholderia* (Estrada-de los Santos et al., 2001). No obstante, en la actualidad solo existe en la base de datos del EMBL GenBank la secuencia de los genes estructurales de la nitrogenasa de una "cepa" de *Burkholderia* sp. (Número de acceso AF194084), endosimbionte del hongo micorrícico *Gigaspora margarita* (Minerdi y col. 2001) y la secuencia del gen *nifH* de la cepa STM678 de *B. tuberum*, nodulante de plantas leguminosas (Número de acceso AJ302314) (Moulin y col. 2001).

Dada la existencia de un número importante de aislados fijadores de nitrógeno identificados como *Burkholderia* spp. y de especies en proceso de ser validadas, así como al escaso conocimiento sobre la secuencia del gen *nifH* de estos diazótrofos, el objetivo de este trabajo fue determinar la presencia y secuencia de este gen en los diazótrofos de este género bacteriano. El material y métodos utilizados se muestran en el Anexo 1.

Actividad Reductora de acetileno (ARA)

La ARA fue una de las pruebas utilizadas para determinar la actividad nitrogenasa de 14 cepas de *Burkholderia,* las cuales fueron descritas anteriormente (Estrada-de los Santos y col. 2001, Estrada y col. 2002). Todas las cepas de *Burkholderia* spp. analizadas mostraron ARA, así como las cepas tipo de las especies *B. "brasilensis"*, *B. "tropicalis"*, *B. vietnamiensis* y *B. kururiensis*, confirmándose su actividad nitrogenasa nuevamente. Además de las 4 especies diazótrofas referidas, se encontró que la cepa tipo de *B. caryophylli* fue capaz de reducir el acetileno a etileno.

Amplificación del gen *nifH*

La presencia del gen *nifH* en los aislados reductores de acetileno de *Burkholderia* fue determinada utilizando los oligonucleótidos IGK y NDR. La amplificación dio como resultado un fragmento de aproximadamente 1.2 kilobases (kb) (Figura 2). Este fragmento corresponde al gen *nifH* y a la parte inicial del gen *nifD*, incluyendo el espacio entre estos dos genes.

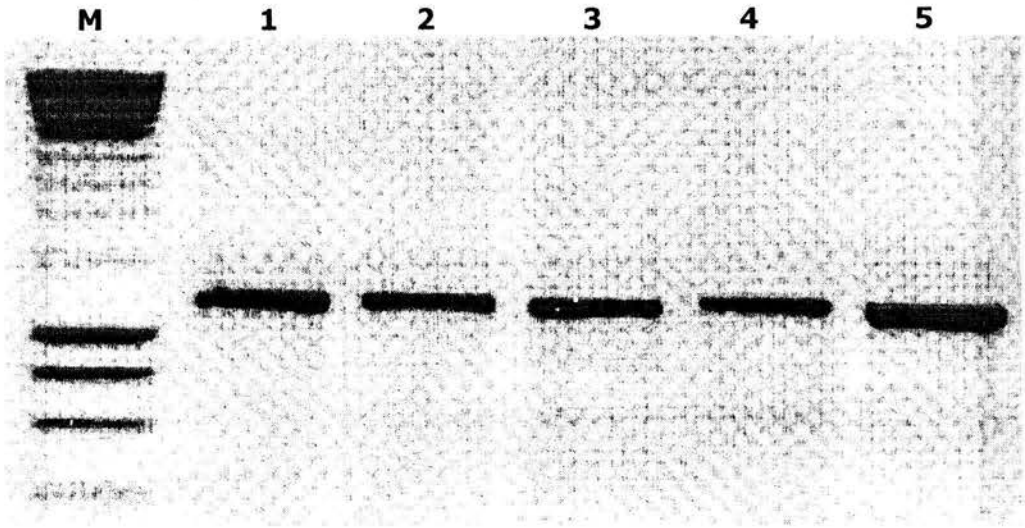

Figura 2. Amplificación del gen *nifH*.
Cepas utilizadas: carril 1, *Burkholderia* sp. CAC-98; carril 2, *B. "unamae"* MTl-641; carril 3, *B. vietnamiensis*^{T}; carril 4, *Burkholderia* sp. CBN-516; carril 5; *B. caryophylli*^{T}. M, marcador de peso molecular 200 pb (Eurogentec).

Con la mayoría de las cepas diazótrofas se observó una banda de amplificación de 1.2 kb, sin embargo, en algunas de ellas se observaron fragmentos de mayor o menor tamaño al esperado. Algunas de estas bandas, en adición a aquellas de 1.2 kb, fueron tomadas directamente del gel de agarosa y purificadas (QIAquick Gel Extraction kit, Qiagen). Los fragmentos de ADN fueron utilizados como guía para la amplificación de un fragmento interno del gen *nifH* con los oligonucleótidos PolR y PolF. Estos oligonucleótidos amplificaron un fragmento de ADN de 0.4 kb a partir de cada una de las bandas de 1.2 kb (Figura 3). Con algunas bandas de 1 kb amplificadas con los oligonucleóticos KAD y NDR, por ejemplo de la cepa *Burkholderia* sp. MTl-441, se

produjo una banda de 0.4 kb (Figura 3, Tabla 4), mientras que con algunas bandas mayores a 1.2 kb analizadas no se obtuvo el producto esperado, tal es el caso de *Burkholderia* sp. SXo-232 y *B. caribensis*^{T} (Figura 3, Tabla 4).

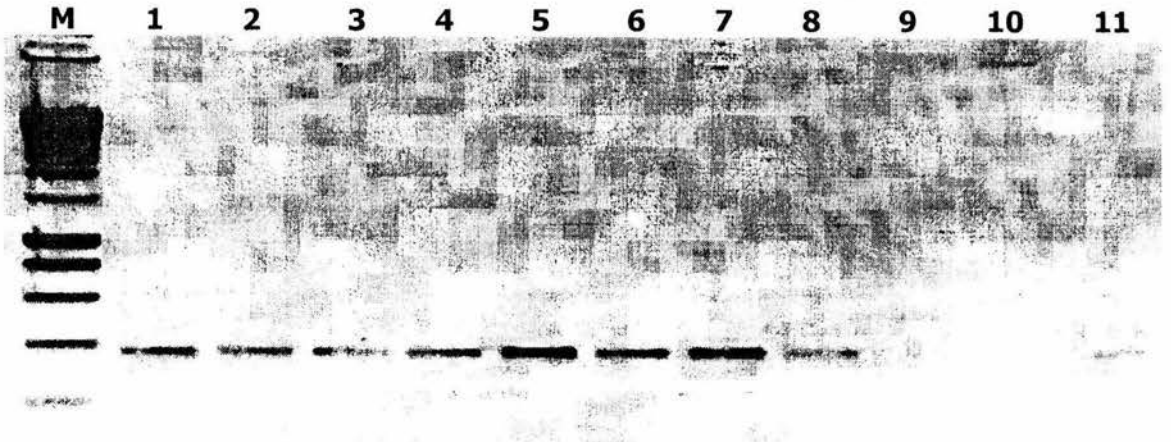

Figura 3. Amplificación de un fragmento interno del gen *nifH*. Carril 1, *Burkholderia* sp. CAC-98; carril 2, *B. "unamae"* MTI-641; carril 3, *B. vietnamiensis*^{T}; carril 4, *Burkholderia* sp. CBN-516; carril 5, *B. caryophylli*^{T}; carril 6, *B. vietnamiensis* SXo-702; carril 7, *Burkholderia* sp. CCE-312; carril 8, *Burkholderia* sp. MTI-441; carril 9, *Burkholderia* sp. SXo-232; carril 10, *B. caribensis*^{T}; carril 11, *B. "tropicalis"* BM16. M, Marcador de peso molecular 200 pb (Eurogenetec)

Tabla 4. Amplificación del gen *nifH* y de un fragmento interno del mismo.

Cepa	Amplificación IGK-NDR	Amplificación PolF-PolR
Burkholderia sp. CAC-98	1.2 Kb	0.4 Kb
B. "unamae" MTI-641	1.2 Kb	0.4 Kb
B. vietnamiensis TVV75^{T}	1.2 Kb	0.4 Kb
Burkholderia sp. CBN-516	1.2 Kb	0.4 Kb
B. caryophylli^{T}	1.2 Kb	0.4 Kb
B. vietnamiensis SXo-702	1.2 Kb	0.4 Kb
Burkholderia sp. CCE-312	1.2 Kb	0.4 Kb
Burkholderia sp. MTI-441	1.0 Kb	0.4 Kb
Burkholderia sp. SXo-232	2.0 Kb	No amplificación
B. caribensis^{T}	2.0 Kb	No amplificación
B. "tropicalis" BM16	1.2 kb	0.4 Kb

Secuencia parcial del gen *nifH*

Las cepas *B. vietnamiensis*^{T}, *B. caryophylli*^{T}, *Burkholderia* sp. CAC-98, *B. "unamae"* MTI-641 y *Burkholderia* sp. CBN-516 fueron seleccionadas para llevar a cabo la secuenciación y análisis del gen *nifH*. Un promedio de 600 pb de secuencia del gen *nifH* fue obtenido con cada uno de los oligonucleótidos (IGK y NDR). Las secuencias parciales fueron analizadas utilizando el sistema BlastN de la base de datos NCBI (Tabla 5). Las secuencias parciales del gen *nifH* pertenecientes a las distintas cepas analizadas fueron similares a un fragmento del genoma de la cepa LB400 de *B. fungorum* (Número de acceso NZ_AAAC01000309) la cual se encuentra en proceso de secuenciación. Las secuencias fueron también similares al gen *nifH* de la cepa STM678 de *B. tuberum*, la cual es una de las cepas nodulantes de plantas leguminosas.

Tabla 5. Análisis de la secuencia parcial del gen *nifH* de *Burkholderia* spp.

Cepas analizadas	Similitud con:	%
B. "unamae" CAC-98	*B. fungorum* LB400	90
	B. tuberum STM678	87
B. "unamae" MTI-641	*B. fungorum* LB400	89
	B. tuberum STM678	88
B. caryophylli^T	*B. fungorum* LB400	89
	B. tuberum STM678	88
B. vietnamiensis TVV75^T	*B. tuberum* STM678	89
	B. fungorum LB400	89

El análisis fue realizado utilizando el sistema BlastN de la base de datos NCBI. Se analizó la secuencia obtenida con el oligonuclótido IGK.

El gen *nifH* de la cepa *B. "tropicalis"* MTo-293 fue amplificado y utilizado como sonda para llevar a cabo ensayos de hibridación con el ADN total de los distintos genotipos fijadores de nitrógeno de *Burkholderia*. Las cepas de los genotipos 16, 17, 19 (correspondientes a *B. "tropicalis"*) analizadas produjeron una señal de hibridación de aproximadamente 3.5 Kb. Las cepas de *B. vietnamiensis* así como la cepa tipo, TVV75^T, mostraron una señal mayor a 10 kb. Los genotipos 13, 14, 15 (correspondientes a *B. "unamae"*) y el genotipo 21 produjeron una banda de hibridación de 3.5 Kb o una de aproximadamente 9.0 Kb. Las especies de *Burkholderia* que dieron una señal de hibridación fueron *B. "brasilensis"* M130, *B. "tropicalis"* Ppe8 (~ 3.5 Kb) y *B. kururiensis* KP23^T (~ 9.0 Kb).

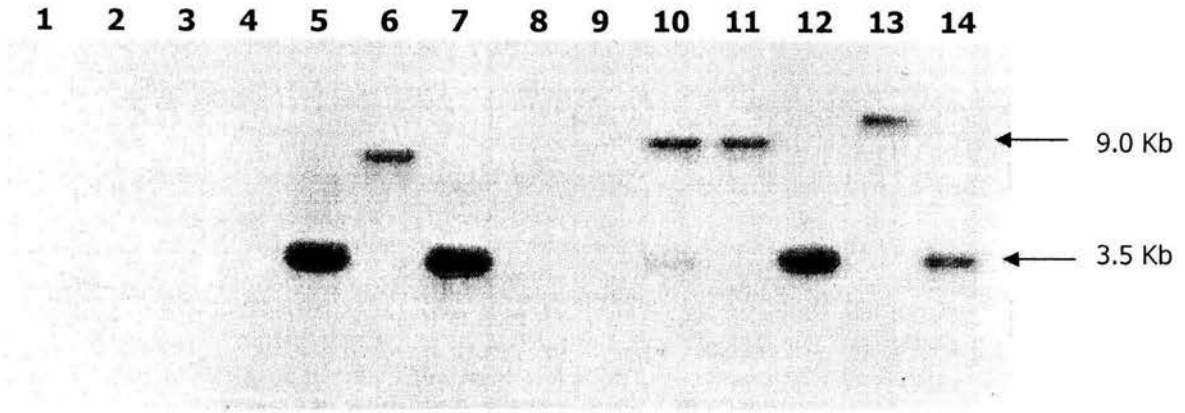

Figura 4. Hibridación tipo Southern blot utilizando como sonda el gen *nifH* amplificado de la cepa MTo-293 de *B. "tropicalis"*. Carril 1, *B. caribensis*^{T}; carril 2, *B. plantarii*^{T}; carril 3, *B. pyrrocinia*^{T}; carril 4; *B. caledonica*^{T}; carril 5, *B. "brasilensis"* M130; carril 6, *B. kururiensis*^{T}; carril 7, *B. "tropicalis"* Ppe8; carril 8, *B. sacchari*^{T}; carril 9, *B. cepacia*^{T}; carril 10, *B. "unamae"* M2Cy-711; carril 11, *B. "unamae"* M2Cy-717; carril 12, *B. "unamae"* M2Cy-626; carril 13, *B. "tropicalis"* LM1-376.8; carril 14, *B. "tropicalis"* LM2-376.3.

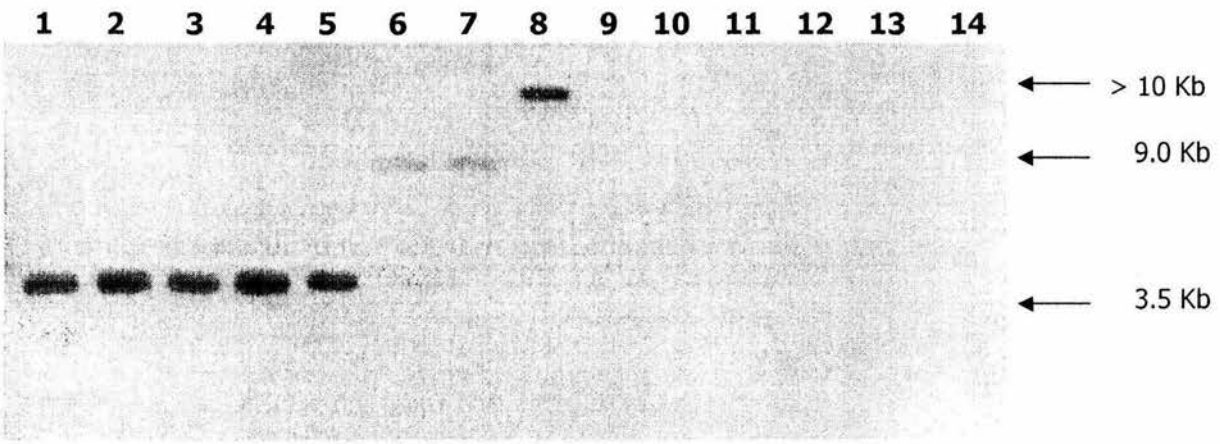

Figura 5. Hibridación tipo Southern blot utilizando como sonda el gen *nifH* amplificado de la cepa MTo-293 de *B. "tropicalis"*. Carril 1, *B. "tropicalis"* MTo-293; carril 2, *B. "tropicalis"* BM16; carril 3, *B. "tropicalis"* BM273; carril 4; *B. "tropicalis"* MSj-8432; carril 5, *Burkholderia* sp. CCE-401; carril 6, *Burkholderia* sp. CBN-25; carril 7, *Burkholderia* sp. CAC-92; carril 8, *B. vietnamiensis*^{T}; carril 9, *B. graminis*^{T}; carril 10, *B. glathei*^{T}; carril 11, *B. gladioli*^{T}; carril 12, *B. fungorum*^{T}; carril 13, *caryophylli*^{T}; carril 14, *B. phenazinium*^{T}.

Discusión

La importancia de la fijación de nitrógeno en la agricultura ha conducido a realizar distintos estudios dirigidos a resolver los problemas causados por el uso de los fertilizantes nitrogenados. Entre estos estudios el aislamiento de fijadores de nitrógeno y con ello su caracterización han sido temas de exploración de gran interés. Sobre esta misma línea, el trabajo adicional desarrollado en esta tesis doctoral fue dirigido hacia la investigación sobre la información contenida en bacterias diazótrofas del género *Burkholderia* para fijar nitrógeno. Las características analizadas fueron la capacidad de las cepas para reducir acetileno, amplificación del gen *nifH* y ensayos de hibridación con este gen. En general, la amplificación del gen *nifH* coincidió con la capacidad de los aislados diazótrofos para reducir acetileno, excepto en el caso de *Burkholderia* sp. SXo-232. Esta cepa redujo el acetileno, sin embargo, los oligonucleótidos IGK y NDR condujeron a la amplificación de un fragmento de 2.0 Kb, a partir del cual no se logró la amplificación del segmento interno. Es posible que la secuencia del gen *nifH* sea un tanto diferente al grado que fue imposible lograr la amplificación del segmento interno del gen con los oligonucleótidos Pol1 y Pol2. Desafortunadamente, esta especie bacteriana no fue analizada en ensayos de hibridación de ADN con la sonda del gen *nifH*, lo cual, habría sido útil para conocer de manera aproximada la similitud de este gen con el gen *nifH* de la cepa MTo-293 de *B. "tropicalis"*.

Un hallazgo que contribuyó a aumentar nuestro conocimiento sobre la diversidad de fijadores de nitrógeno del género *Burkholderia* fue la capacidad diazotrófica de la cepa tipo de *B. caryophylli*.

Por otro lado, resultó muy interesante encontrar que las secuencias parciales del gen *nifH* de los aislados diazótrofos son similares a un fragmento de ADN de la cepa LB400 de *B. fungorum*. Esta cepa y en general la especie *B. fungorum* no ha sido reportada como una fijadora de nitrógeno. En este estudio la cepa tipo de *B. fungorum* fue analizada en su capacidad para reducir acetileno así como la amplificación e hibridación del gen *nifH*, resultando negativa ambas pruebas. Sería interesante analizar distintas cepas de *B. fungorum* en especial la cepa LB400 que se encuentra en proceso de secuenciación para conocer si tiene la capacidad para fijar nitrógeno y reconocerla como una especie diazótrofa más en el género *Burkholderia*.

Aún cuando los estudios desarrollados en esta fase de investigación se encuentran incompletos, ya que las secuencias del gen *nifH* son parciales y solamente un número pequeño de cepas fueron analizadas, resultaría interesante continuar con este trabajo y obtener la secuencia completa del gen *nifH* de los distintos diazótrofos. Esto podría dar una idea sobre la historia de este gen en la evolución de los fijadores de nitrógeno. Otro aspecto de interés sería analizar aquellas cepas bacterianas reductoras de acetileno cuyo *nifH* no pudo ser amplificado o revelado en los ensayos de hibridación. Existe la posibilidad de que pueda tratarse de una nitrogenasa distinta de las conocidas.

ASPECTOS RELEVANTES DEL TRABAJO DE TESIS

La taxonomía es un área del conocimiento que cuenta con una gran variedad de herramientas que en los últimos años han servido para la descripción de numerosas especies bacterianas, dando con ello sentido a la diversidad bacteriana. En este proyecto doctoral se ha puesto de manifiesto que el género *Burkholderia* esta constituido por una amplia diversidad de especies fijadoras de nitrógeno, las cuales son habitantes comunes del ambiente rizosférico y endófito de plantas de distintas familias entre las que destaca el maíz por su relevancia en la sociedad mexicana y en el mundo.

La diversidad de especies diazótrofas del género *Burkholderia* se expresa en el número de genotipos que muy probablemente representan a 7 nuevas especies.

El análisis taxonómico de uno de los grupos mayoritarios de aislados diazótrofos, correspondientes a los genotipos, 16, 17 y 19, mostró que pertenecen a la especie *B. "tropicalis"*. La identificación de esta especie diazótrofa fue una extraordinaria coincidencia considerando la escasa información existente en la literatura al inicio de este trabajo, aun en la actualidad esta especie no ha sido descrita oficialmente. De acuerdo a los resultados en este proyecto, *B. "tropicalis"* parece encontrarse distribuida ampliamente en el ambiente. Además, investigaciones posteriores en nuestro laboratorio sobre especies diazótrofas del género *Burkholderia* han mostrado que *B. "tropicalis"* se encuentra frecuentemente asociada con plantas en particular con el maíz.

La diversidad de diazótrofos en el género *Burkholderia* también se manifiesta por la existencia de cepas predominantes en el ambiente, las cuales corresponden a los genotipos 13, 14 y 15. Estos genotipos constituyen la propuesta de *Burkholderia unamae* como nueva especie fijadora de nitrógeno. *B. "unamae"* al igual que *B. "tropicalis"* se encuentra distribuida ampliamente en el ambiente en asociación con plantas de distintas familias.

Este trabajo reveló también la existencia de otros genotipos fijadores de nitrógeno del género *Burkholderia* que muy probablemente representan nuevas especies. Estos aislados fueron descritos como los genotipos 6, 7, 8, 20 y 21 (Estrada de los Santos y col. 2001). En particular, la cepa CAC-124 (genotipo 8) ha sido identificada como una nueva especie (P. Vandamme com. pers.). Sin embargo, para la descripción formal de nuevas especies se requiere obtener nuevos aislamientos de los distintos genotipos, incluyendo el genotipo 8, considerando el escaso número de cepas con el que se cuenta

actualmente, lo que limita cualquier análisis taxonómico serio. Además, no cumpliría las reglas sobre el número de cepas necesarias para definir una nueva especie bacteriana ni sería útil para conocer la ecología de la especie particular.

Otro resultado de interés, producto de este trabajo de investigación, fue la identificación de *B. vietnamiensis* en asociación natural con plantas de maíz, cafeto y sorgo. Esta especie no había sido detectada en asociación con plantas cultivadas en el campo ni tampoco en asociación endófita. *B. vietnamiensis* fue originalmente aislada de la rizosfera de plantas arroz cultivadas en el laboratorio (Gillis y col. 1996).

El conocimiento sobre bacterias diazótrofas del género *Burkholderia* incluyó también a las especies *B. kururiensis* y *B. caryophylli.* Es muy común que durante la descripción de una nueva especie no se determine la capacidad para fijar nitrógeno por las cepas analizadas, lo cual se debe a que esta prueba no forma parte de las características más importantes que sirven para definir a una especie bacteriana. Es muy probable que la capacidad diazotrófica sea expresada también por cepas de las especies descritas formalmente. Resultaría importante y de gran valor incluir la capacidad para fijar nitrógeno como una prueba fenotípica rutinaria en los estudios de taxonomía bacteriana, considerando que esta característica fue de fundamental importancia en este trabajo de investigación para seleccionar a los grupos diazótrofos y distinguirlos de los no fijadores de nitrógeno.

Burkholderia es un género versátil tomando en cuenta las capacidades expresadas por las especies que lo constituyen. Es probable que al estudiar este género se encuentren nuevos e interesantes procesos, como fue el caso de la nodulación de plantas leguminosas (Moulin y col. 2001). Dadas las características polifásicas encontradas en especies del género *Burkholderia* resulta de gran interés continuar su estudio, especialmente dirigido a su uso en agrobiotecnología.

PERSPECTIVAS DEL TRABAJO DE INVESTIGACIÓN

El trabajo descrito en este proyecto de tesis doctoral ha conducido al planteamiento de preguntas que resultaría interesante contestar.

Es fundamental llevar a cabo la identificación de los genotipos descritos anteriormente (Estrada-de los Santos 2001). Un enfoque polifásico nos permitirá incrementar el conocimiento taxonómico y de la diversidad de diazótrofos del género *Burkholderia* asociados a plantas.

Es conocido que algunas de las especies de *Burkholderia* albergan múltiples cromosomas así como plásmidos (Lessie y col. 1996; Wigley y Burton 2000). ¿Las cepas diazótrofas aisladas en este trabajo de investigación poseen tal estructura genómica? ¿La información para fijar nitrógeno u otra actividad de interés biotecnológico se encuentra en alguno o en ambos componentes celulares? Algunos avances realizados en nuestro grupo de investigación muestran la presencia de mega plásmidos en las especies diazótrofas del género *Burkholderia* (Cabellos-Avelar com. pers.), y actualmente se están llevando a cabo estudios para determinar si en ellos se encuentra contenida la información para fijar nitrógeno, como se ha demostrado la capacidad para nodular en la cepa STM815 de *B. phymatum.* Explorar la estabilidad de los plásmidos en las especies de *Burkholderia* es otro aspecto de valor, considerando que algunos de los aislados diazótrofos han perdido la capacidad para fijar nitrógeno. Sobre esta misma línea, conocer la organización y tamaño del genoma, tanto el porcentaje correspondiente a los cromosomas como de plásmidos, en una bacteria ayudaría a explicar las diferencias que pueden presentarse en ensayos de reasociación ADN-ADN.

Algunas de las especies del género *Burkholderia* expresan actividades de gran importancia para el hombre, entre las que destacan el control biológico, la biorremediación y la producción de compuestos de importancia industrial. Sería de gran valor conocer si las nuevas especies expresan alguna de estas actividades, además de la fijación de nitrógeno, y si estas pueden favorecer el crecimiento de las plantas, contribuir en la recuperación de suelos contaminados con xenobióticos o ayudar a disminuir el costo de la producción de algún compuesto de interés industrial. Estudios iniciados en nuestro grupo de investigación han mostrado que algunos de los aislados diazótrofos producen sideróforos (Tenorio-Salgado y col. 2002), los cuales podrían ser utilizados en el control biológico de fitopatógenos.

Se han mencionado anteriormente los problemas causados por el "complejo *B. cepacia*" en pacientes con FQ así como el riesgo de utilizar a estas especies con fines agrobiotecnológicos. Aun cuando los factores de virulencia y mecanismos de patogenicidad en *B. cepacia* no han sido elucidados, se han identificado algunos marcadores de patogenicidad que albergan las cepas pertenecientes a uno de los genomovares (III) de mayor peligro para los pacientes con FQ (Clode y col. 2000). Uno de los marcadores descritos es el gen *cblA* que codifica para el llamado pili cable (Richardson y col. 2001). Determinar la presencia de estos marcadores de patogenicidad en los aislados diazótrofos del género *Burkholderia* con la idea de conocer si podrían representar un peligro para la población de pacientes con FQ es otra área de estudio de interés en nuestro grupo de investigación.

Resultaría interesante analizar si *B. "tropicalis"* o *B. "unamae"* pueden llegar a establecerse en suelos con características diferentes a las locales considerando su ausencia en suelos europeos (J. Balandreau com. pers.; Balandreau y col. 2001; Fiore y col. 2001) y determinar a través de experimentos de inoculación si son capaces de promover el crecimiento de plantas. La inoculación con *B. vietnamiensis* a plantas de arroz ha mostrado el potencial de *Burkholderia* como una bacteria promotora del crecimiento (Tran Van y col. 2000). Sobre esta misma línea, si *B. "tropicalis"* o *B. "unamae"* son capaces de colonizar suelos europeos convendría analizar si estas bacterias pueden competir y desplazar a otras especies de *Burkholderia*, en especial a las pertenecientes al "complejo *B. cepacia*". No obstante, más importante resultaría determinar si pudiera darse transferencia de material genético, en particular los determinantes de patogenicidad, entre estos dos grupos considerando que las especies del "complejo *B. cepacia*" representan un riesgo para los pacientes con FQ.

Es muy probable que existan otros mecanismos en el género *Burkholderia* que pudieran ser explotados en biotecnología. Hasta ahora *Burkholderia* ha mostrado ser un género de gran versatilidad bioquímica. Con toda seguridad, el análisis de la secuencia del genoma de algunas especies de *Burkholderia* contribuirá al conocimiento de actividades de gran interés para el hombre.

ANEXO 1

Material y métodos para la detección y secuencia parcial del gen *nifH* de diazótrofos de *Burkholderia*

Cepas bacterianas. Las cepas diazótrofas analizadas fueron aisladas de plantas de maíz, cafeto, sorgo y caña de azúcar (Estrada-de los Santos y col. 2001; Estrada-de los Santos y col. 2003, Caballero-Mellado y col. 2003, manuscritos en preparación). Las especies bacterianas del género *Burkholderia* utilizadas en el ensayo se muestran en la Tabla 6.

Tabla 6. Especies de *Burkholderia* utilizadas para la amplificación del gen *nifH*.

Cepas	Referencia
B. andropogonis LMG2129^T	Gillis y col. 1995
B. "brasilensis" M130	No publicado
B. caledonica LMG19076^T	Coenye y col. 2001
B. caribensis MWAP64^T	Achouak y col. 1999a
B. cepacia LMG1222^T	Yabuuchi y col. 1992
B. gladioli LMG2216^T	Yabuuchi y col. 1992
B. glathei LMG 14190^T	Viallard y col. 1998
B. glumae ATCC33617^T	Urakami y col. 1994
B. graminis C4D1M^T	Viallard y col. 1998
B. graminis C5A1M	Viallard y col. 1998
B. phenazinium LMG2247^T	Viallard y col. 1998
B. plantarii LMG9035^T	Urakami y col. 1994
B. pyrrocinia ATCC15958^T	Viallard y col. 1998
B. thailandensis ATCC700388	Brett y col. 1998
B. vietnamiensis TVV75^T	Gillis y col. 1995

Actividad reductora de acetileno. La ARA se determinó utilizando el medio de cultivo semigelificado BMGM (Estrada-de los Santos y col. 2001), cuya característica importante es que posee tres fuentes de carbono. Cada una de las cepas fue inoculada por duplicado en el medio de cultivo BMGM e incubadas a 29 ^{0}C. Al término de 4 días, las cepas fueron analizadas en su capacidad para reducir acetileno como fue descrito anteriormente (Mascarúa-Esparza y col. 1988).

Aislamiento de ADN total. Las cepas bacterianas fueron cultivadas en el medio de cultivo BSE (Estrada-de los Santos y col. 2001). Las cepas fueron incubadas en agitación a 29 ^{0}C durante 24 horas y centrifugadas a 12,300 x g. El aislamiento de ADN fue realizado de acuerdo a Ausubel y col. (1987).

Amplificación del gen *nifH*. La amplificación del gen *nifH* se llevó a cabo a partir del ADN total de los distintos diazótrofos utilizando los oligonucleótidos IGK delantero (Poly y col. 2001) y NDR reverso (Normand com. pers.). También, se usaron los oligonucleótidos internos del gen *nifH* PolF delantero y PolR reverso (Poly y col. 2001). Las condiciones para la amplificación del gen *nifH,* utilizando ambos pares de oligonucleótidos, fue: 1 ciclo a 94 ^{0}C, 5 minutos. 35 ciclos a 94 ^{0}C, 1 minuto; 57 ^{0}C, 1 minuto y 72 ^{0}C, 2 minutos. Finalmente un ciclo a 72 ^{0}C por 7 minutos. La mezcla para la PCR fue (concentración final en 20 μL de reacción): Buffer, 0.1 %; DMSO, 0.1 %; $MgCl_2$, 1.5 mM; cada dNTP, 20 μM; cada oligonucleótido, 0.1 μM; Taq polimerasa, 1 U (Gibco BRL). Los productos de la PCR fueron analizados en geles de agarosa al 2 % y revelados con bromuro de etidio.

Análisis de la secuencia parcial del gen *nifH*. Los segmentos de ADN amplificados con los oligonucleótidos IGK y NDR correspondientes al gen *nifH* de algunas de las cepas analizadas fueron enviados a la empresa OLIGO EXPRESS (Francia) para su secuenciación. Las secuencias obtenidas fueron comparadas con las existentes en la base de datos NCBI (National Center for Biotechnology Information).

Hibridación con el gen *nifH*. Cepas representantes de los genotipos 1, 8, 13, 14, 15, 16, 17, 18, 19, 19a y 21 (Estrada-de los Santos 2001; Estrada-de los Santos 2003, Caballero-Mellado y col. 2003, manuscritos en preparación) así como distintas especies del género *Burkholderia* fueron seleccionadas para llevar a cabo ensayos de hibridación con el gen *nifH*. El ADN de los distintos aislados fue digerido con la enzima *Eco*RI y los fragmentos obtenidos fueron analizados en geles de agarosa al 1 % para posteriormente ser transferidos a membranas de nylon e hibridados como se describió previamente (Caballero-Mellado y col. 1994).

Preparación de la sonda para hibridación. El gen *nifH* de la cepa MTo-293 fue amplificado con los oligonucleotidos IGK y NDR utilizando el sistema Expand High Fidelity PCR (Roche). El fragmento amplificado fue clonado en el vector pCR2.1 del sistema TA Cloning Kit (Invitrogen) y transformado en la cepa competente DH5α de *Escherichia coli*. El gen *nifH* fue obtenido utilizando enzimas de restricción presentes en el vector de clonación para posteriormente llevar a cabo el ensayo de hibridación.

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