IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF DIAZOTROPHIC β-PROTEOBACTERIA FROM BRAZILIAN SOILS

KRISLE DA SILVA

2009

KRISLE DA SILVA

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF DIAZOTROPHIC β-PROTEOBACTERIA FROM BRAZILIAN SOILS

Thesis presented to the Federal University of Lavras as part of the requirementes of Agricultural Microbiology Program to the obtainment of the title of "Doctor".

Major Professor

Prof. Fátima Maria de Souza Moreira

LAVRAS
MINAS GERAIS - BRASIL
2009

Ficha Catalográfica Preparada pela Divisão de Processos Técnicos da Biblioteca Central da UFLA

Silva, Krisle da.
Identification and functional characterization of diazotrophic β-proteobacteria from Brazilian soils / Krisle da Silva. – Lavras : UFLA, 2009.
124 p. : il.

Tese (doutorado) – Universidade Federal de Lavras, 2009.
Orientador: Fátima Maria de Souza Moreira.
Bibliografia.

1. Fixação biológica de nitrogênio. 2. Betaproteobacteria. 3. Leguminosas. 4. Floresta Amazônica. 5. Microbiologia do solo. I. Universidade Federal de Lavras. II. Título.

CDD – 631.46

KRISLE DA SILVA

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF DIAZOTROPHIC β-PROTEOBACTERIA FROM BRAZILIAN SOILS

Thesis presented to the Federal University of Lavras as part of the requirementes of Agricultural Microbiology Program to the obtainment of the title of "Doctor".

APROVED on 26th August 2009

Prof. Peter Vandamme	UGhent
Prof. Emanuel de Souza Maltempi	UFPR
Prof. Fábio Lopes Olivares	UENF
Prof. Ricardo Magela de Souza	UFLA
Profa. Patrícia Gomes Cardoso	UFLA

Prof. Fátima Maria de Souza Moreira
UFLA
(Major Professor)

LAVRAS
MINAS GERAIS – BRASIL

A Deus, pela presença, amor incondicional, pela força para enfrentar as dificuldades e por preencher minha vida com pessoas maravilhosas.

OFEREÇO

A Ires Aurora da Silva, pelo amor, dedicação e companherismo, presentes durante toda minha vida.

DEDICO

AGRADECIMENTOS

Primeiramente, a Deus, pois cada conquista, seja ela grande ou pequena, não seria possível sem sua presença em minha vida. Essa é, sem dúvida, mais uma conquista com as bênçãos de Deus.

À Universidade Federal de Lavras, em especial ao Programa de Pós-Graduação em Microbiologia Agrícola, pela oportunidade de realização do curso.

À Fundação de Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), pela concessão da bolsa de estudos.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão da bolsa de estudos para a realização do doutorado sanduíche.

Ao projeto TSBF-CIAT/GEF-UNEP, "Conservation and Sustainable Management of Below Ground Biodiversity", pelo financiamento para execução deste trabalho.

À professora Fátima Maria de Souza Moreira, pela orientação durante o mestrado e doutorado, pela confiança, pelo exemplo e amizade.

Ao Prof. Peter Vandamme, pela excelente acolhida e pela coorientação, que foi muito positiva para a realização deste trabalho.

A todos os professores do Programa de Pós-Graduação em Microbiologia Agrícola.

Um agradecimento especial à pesquisadora Diva de Souza Andrade, pela amizade e iniciação em Microbiologia do Solo.

Aos funcionários Marlene Aparecida de Souza e Manuel Aparecido da Silva, pela contribuição na realização deste trabalho e pela amizade.

À funcionária do Laboratório de Microbiologia da Universidade de Ghent Evie de Brandt, pela paciência e contribuição a este trabalho.

A Magda Aparecida Melo, por toda a dedicação durante o doutorado e pela grande disposição.

A todos os amigos do Laboratório de Microbiologia do Solo, em especial Ligiane, Adrianinha, Rogério, Pedro e Michele Rocha.

Aos membros da banca examinadora, professores Peter Vandamme, Emanuel Maltempi de Souza, Fábio Lopes Olivares, Ricardo Magela e Patrícia, pelas sugestões e críticas construtivas dadas a este trabalho.

Às amigas de república durante mestrado e doutorado: Patrícia, Taís, Gláucia, Sílvia, Fabiana e Ludmila. Vocês foram minha nova família durante o perído em que vivemos juntas.

Às amigas que fiz na Bélgica, que fizeram com que os dias nublados e frios se tornassem mais alegres: Inês, Tatiana, Luana, Jéssica, Tânia, Margo e Luciane.

Aos meus grandes amigos e/ou irmãos: Meire, Amanda, Gláucia, Cândido e Sandro. Agradeço por todos os momentos que dividimos e que, sem dúvida, foram inesquecíveis. Tenho certeza de a nossa amizade não há distância ou tempo que desgate. Amo vocês.

As minhas queridas amigas e/ou irmãs de Londrina, Daiane e Cintia. Apesar da distância, vocês estão sempre perto, seja no meu coração ou na minha mente. Amos vocês!

A minha querida mãe, Ires Aurora da Silva, obrigada, pelo amor, exemplo de ser humano, educação e confiança. Amo você demais!

Aos meus queridos familiares, Cleonice, Kátia, Diego, Ivani, Ladislau, Pedro e Murilo, pelo grande carinho.

APRESENTAÇÃO

Nesta tese apresenta-se parte dos resultados do projeto internacional "Conservação e Manejo Sustentado da Biodiversidade do Solo", implementado em sete países tropicais - Brasil, Costa do Marfim, India, Indonesia, Quênia, Mexico e Uganda. Este projeto é coordenado pelo Tropical Soil Biology and Fertility Institute do CIAT (TSBF-CIAT) com cofinanciamento do "Global Environmental Facility" (GEF) e suporte para implementação do "United Nations Environment Program" (UNEP). O componente brasileiro do projeto é denominado BiosBrasil e é coordenado pela Universidade Federal de Lavras (UFLA) (www.biosbrasil.ufla.br). As opiniões expressas nesta publicação são de responsabilidade de seus autores e não necessariamente refletem aquelas das instituições dos autores, do "United Nations Environment Programme" ou do "Global Environmental Facility".

SUMMARY

TABLE LISTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

FIGURE LISTS

CHAPTER 2

CHAPTER 3

GENERAL ABSTRACT

SILVA, Krisle da**. Identification and functional characterization of diazotrophic β-Proteobacteria from brazilian soils.** 2009. 124 p. Thesis (Doctorate in Agricultural Microbiology) – Federal University of Lavras, Lavras, MG.*

Different isolates belonging to class β-Proteobacteria were obtained from Amazonian soils, in the Amazonas, Rondônia and Acre States and also from soils of Minas Gerais State. The isolates obtained from Amazonian soils are belonging to *Burkholderia* genus and from Minas Gerais State to *Cupriavidus* genus. All isolates were captured by using different trap plants. The *Burkholderia* isolates were captured by using siratro (*Macroptilium atropurpureum*), common bean (*Phaseolus vulgaris*), *Indigofera suffruticosa* and *Pithecellobium* sp. *Cupriavidus* isolates were obtained using common bean (*P. vulgaris*), cowpea (*Vigna unguiculata*) and leucaena (*Leucaena leucocephala*). The aims of this work were: to evalute plant growth promotion traits of *Burkholderia* isolates and to identify by genotypic and phenotypic characteristics the isolates of *Burkholderia* and *Cupriavidus.* The isolates of *Burkholderia* were evaluate by cultural characterization on 79 medium, biochemical characteristics, total protein profiles by SDS-PAGE and by plant growth promotion traits (nitrogenase activity on free nitrogen medium; solubilisation of calcium, iron and aluminium phosphates and antifungal activity against *Fusarium oxysporium* f. sp. *phaseoli*), 16S rDNA, *gyrB*, and *recA* gene sequencing and multilocus sequence typing specific to *Burkholderia cepacia* complex. The isolates of *Cupriavidus* were characterized by total protein profiles by SDS-PAGE, 16S rDNA and *gyrB* gene sequecing and *nodC* and *nifH* sequencing, beyond it, they were re-inoculed on the legumes which were isolated. The isolates of *Burkholderia* were phenotyoically diverse depending of the plant which they were captured. The most of isolates fixed nitrogen on culture medium, solubilized calcium phosphate, but only one isolate presented antifungal activity. The isolates of *Burkholderia* were identified as *B. fungorum* (isolated from siratro), *B. caribensis* (*Indigofera suffruticosa*); *B. contaminans* (common bean) and *B. lata* (*Pithecellobium* sp.). The isolates of *Cupriavidus* were identified as *C. necator*, and one of them efficiently nodulated common beans plants. This is the first report of the occurrence of biological nitrogen fixation by *B. fungorum*, *B. contaminans*, *B. lata* and *C. necator* species.

Guindance Committee: Fátima Maria de Souza Moreira – UFLA (Major Professor), Peter Vandamme – Ghent University.

RESUMO GERAL

SILVA, Krisle da. **Identificação de diazotróficos pertencentes à subclasse β-proteobactéria isolados de solos brasileiros.** 2009. 124 p. Tese (Doutorado em Microbiologia Agrícola) – Universidade Federal de Lavras, Lavras, MG.*

Diferentes isolados pertencentes à classe β-proteobactéria foram obtidos de solos da região Amazônica, compreendendo os estados do Amazonas, Rondônia e Acre, e também em áreas de pastagem de solos do estado de Minas Gerais. Os isolados obtidos da região Amazônica pertencem ao gênero *Burkholderia* e os de Minas Gerais ao gênero *Cupriavidus.* Todos os isolados foram capturados utilizando-se diferentes plantas iscas. As bactérias do gênero *Burkholderia* foram capturadas utilizando-se siratro (*Macroptilium atropurpureum*), feijão-comum (*Phaseolus vulgaris*), *Indigofera suffruticosa* e *Pithecellobium* sp. Já os isolados de *Cupriavidus* foram obtidos utilizando-se feijão (*P. vulgaris*), feijão-caupi (*Vigna unguiculata*) e leucena (*Leucaena leucocephala*). Os objetivos foram: avaliar as características de promoção do crescimento vegetal dos isolados de *Burkholderia* e identificar, por meio de características fenotípicas e genotípicas, os isolados de *Burkholderia* e *Cupriavidus*. Para os isolados de *Burkholderia* foram utilizados caracterização cultural em meio 79, caracterização bioquímica, perfil total de proteínas por meio de SDS-PAGE, características de promoção do crescimento vegetal (fixação biológica de nitrogênio atmosférico em meio de cultura livre de N; solubilização de fosfatos de cálcio, ferro e alumínio e atividade antifúngica contra *Fusarium oxysporum* f. sp. *phaseoli*), sequenciamento total do gene 16S rDNA, sequenciamento do gene *gyrB*; amplificação e sequenciamento do gene *recA* e *multilocus sequence typing* (MLST) específicos para complexo *Burkholderia cepacia*. Os isolados de *Cupriavidus* foram caracterizados quanto ao perfil total de proteínas por meio de SDS-PAGE, sequenciamento total do gene 16S rDNA, sequenciamento do gene *gyrB* e sequenciamento dos genes *nodC* e *nifH*. Além disso, eles foram autenticados na espécie em que foram isolados. Os isolados de *Burkholderia* foram diversos fenotipicamente, dependendo da planta da qual foram capturados. A grande maioria fixou nitrogênio em meio de cultura, solubilizou fosfato de cálcio, mas apenas um isolado apresentou atividade antifúngica. Os isolados de *Burkholderia* foram identificados como *B. fungorum* (isolado a partir de siratro), *B. caribensis* (*Indigofera suffruticosa*), *B. contaminans* (feijão) e *B. lata* (*Pithecellobium*). Os isolados de *Cupriavidus* foram identificados como *C. necator* e um deles

---

Comitê Orientador: Fátima Maria de Souza Moreira – UFLA (Orientador), Peter Vandamme – Universidade de Ghent.

apresentou eficiente nodulação em plantas de feijão. Este é o primeiro relato da ocorrência de fixação biológica de nitrogênio por espécies de *B. fungorum*, *B. contaminans*, *B. lata* e *C. necator*.

CHAPTER 1

1 INTRODUÇÃO GERAL

Diversos processos são mediados por microrganismos do solo e desempenham papel importante na ciclagem de nutrientes. Um desses processos é a fixação biológica de nitrogênio atmosférico, que é realizada por microrganismos procarióticos conhecidos como diazotróficos. Os diazotróficos podem ser de vida livre, estar associados a espécies vegetais ou, ainda, desenvolver simbiose com leguminosas. Os estudos com bactérias diazotróficas são de grande importância, devido à contribuição destas para o fornecimento de nitrogênio a diversos ecossistemas, naturais ou manejados.

Além da capacidade de fixar nitrogênio, é de interesse que essas bactérias possuam outras características importantes para o desenvolvimento vegetal, como solubilizar fosfatos inorgânicos, sintetizar compostos que promovam o crescimento radicular ou que apresentem efeito antagônico contra fitopatógenos. Isso porque, além da fixação biológica de nitrogênio, elas podem contribuir para o crescimento vegetal de outras culturas não leguminosas e até para leguminosas por meio de outros processos.

O gênero *Burkholderia* possui espécies diazotróficas associadas a plantas ou crescendo livremente no solo que, portanto, apresentam grande potencial como rizobactérias promotoras do crescimento vegetal (Gillis et al., 1995; Trân Van et al., 2000; Estrada-de-los-Santos et al., 2001; Reis et al., 2004; Caballero-Mellado et al., 2004; Perin et al., 2006; Caballero-Mellado et al., 2007). Também possui espécies capazes de nodular leguminosas (Moulin et al., 2001; Vandamme et al., 2002a; Chen et al., 2006, 2007, 2008a)

Dentro do gênero *Cupriavidus*, da classe β-proteobactéria, também foi descoberta, recentemente, uma espécie fixadora de nitrogênio, *C. taiwanenis*, capaz de formar simbiose com leguminosas do gênero *Mimosa* (Chen et al., 2001). Estudos recentes demonstraram que *C. taiwanenis* também tem potencial

para utilização em fitorremediação de áreas contaminadas com metais pesados (Chen et al., 2008b).

No Brasil, foi relatada a ocorrência de diversas espécies de *Burkholderia* fixadoras de nitrogênio (Reis et al., 2004; Perin et al., 2006; Chen et al., 2006, 2008a). No entanto, há somente um relato para *Cupriavidus* (Florentino et al., 2009).

A floresta Amazônica é considerada um reservatório mundial de biodiversidade. Mas, os estudos com diazotróficos nesta região ainda são poucos, considerando-se a sua vasta extensão. Alguns isolados obtidos na região Amazônica, nos estados de Rondônia e Acre, utilizando siratro (*Macroptilium atropurpureum*) (Pereira, 2000), foram identificadas por meio do sequenciamento parcial do gene 16S rRNA como *Burkholderia* sp. (Moreira, dados não publicados). Em outros estudos na região amazônica (estado do Amazonas), utilizando como plantas-iscas siratro e feijão-comum (*Phaseolos vulgaris*) em diferentes sistemas de uso da terra, foram encontrados isolados que, por meio do sequenciamento parcial do gene 16S rRNA, também foram identificados como pertencentes ao gênero *Burkholderia* (Barberi, 2007; Lima et al., 2009). Foi relatada também a ocorrência de *Cupriavidus* sp. isolado de solos próximos a plantas de *Sesbania virgata*, capturadas utilizando diferentes plantas iscas consideradas promíscuas em dois municípios do sul do estado de Minas Gerais (Florentino et al., 2009). Contudo, é necessária a identificação destes isolados.

Assim, este trabalho foi realizado com os seguintes objetivos:

- avaliar algumas características funcionais de estirpes de *Burkholderia* isoladas de solos da região Amazônica;
- identificar os isolados de *Burkholderia* e *Cupriavidus*, obtidos de solos da região Amazônica e do sul do estado de Minas Gerais, respectivamente.

2 REFERENCIAL TEÓRICO

2.1 O gênero *Burkholderia*

Bactérias do gênero *Burkholderia* foram descritas, pela primeira vez, por Burkholder (1950) como um patógeno vegetal associado ao apodrecimento dos bulbos de cebola. No entanto, o gênero só foi criado em 1992, por Yabuuchi et al., com base em características fenotípicas, composição de ácidos graxos, homologia DNA-DNA e sequenciamento do gene 16S rDNA, de sete espécies até então consideradas como pertencentes ao gênero *Pseudomonas*, que foram, então, transferidas para o novo gênero.

O gênero *Burkholderia* pertence à classe β-proteobactéria. São microrganismos gram-negativos em forma de bastonetes móveis, com três a vários flagelos. Atualmente, o gênero *Burkholderia* tem 56 espécies descritas (Euzéby, 2009)[1] com grande diversidade: patógenos humanos, animais e vegetais (Burkholder, 1950; Isles et al., 1984; Whitlock et al., 2007), promotores do crescimento vegetal (Trân Van et al., 2000; Peix t al., 2001), fixadores de nitrogênio (Estrada-de-los-Santos et al., 2001; Moulin et al., 2001) e degradadores de poluentes (Nelson et al., 1987; Fries et al., 1997; Radway et al., 1998; Zhang et al., 2000), entre outros.

A grande diversidade presente neste gênero pode ser resultante da organização e do tamanho do seu genoma. O genoma de difrentes estirpes de *B. cepacia* possui de dois a quatro cromossomos de diferentes tamanhos e contém um grande número de sequência de inserções (Lessie et al., 1996). Este número elevado de sequência de inserções pode ter um papel importante na habilidade desta bactéria de se adaptar a diferentes ambientes por meio de transferência genética e mutação (Tabacchioni et al., 2002).

[1] Available in: <http**://** www.biosbrasil.ufla.br >. Access: 18 Apr. 2009.

2.2 *Burkholderia* spp. como bactérias promotoras do crescimento vegetal (PGPB)

Diversas espécies de bactérias são consideradas benéficas em seu ambiente natural. Algumas delas podem contribuir para o crescimento vegetal e são conhecidas como rizobactérias promotoras do crescimento vegetal - "plant growth-promoting rhizobacteria" (PGPB) (Kloepper & Schroth, 1978). Muitas espécies de *Burkholderia* são conhecidas como PGPB e são de grande interesse para o uso em ambientes agrícolas. A fixação biológica de nitrogênio é um processo que também ocrrem em espécies deste gênero, tanto em simbiose com leguminosas (Moulin et al., 2001) quanto em associações com vegetais ou vida livre (Estrada-de-los-Santos et al., 2001) e esta característica contribui para a aquisição deste nutriente em diversos ambientes.

O fósforo é um elemento essencial para plantas, no entanto, apenas 5% do fosfato total do solo estão disponíveis (Epstein, 1972). O fósforo se encontra no solo na forma orgânica (derivada de plantas e microrganismos) e inorgânica (adicionado via fertilizantes). A adubação com fósforo é uma prática comum em solos agrícolas, mas, a grande maioria do fósforo aplicado acaba se tornando indisponível para as plantas, pois é rapidamente imobilizado por íons de ferro e alumínio em solos ácidos e por íons de cálcio em solos alcalinos. Muitos microrganismos têm a capacidade de solubilizar fosfatos precipitados com ferro, alumínio e cálcio. Essa solubilização tem sido atribuída à liberação de diversos ácidos orgânicos. O ácido glucônico foi o principal responsável pela solubilização de trifosfato de cálcio em uma estirpe de *B. cepacia* (Lin et al., 2006), mas outros ácidos também foram detectados.

Um efeito indireto na promoção do crescimento vegetal por estirpes de *Burkholderia* é por meio de efeitos benéficos no controle de fitopatógenos. Diferentes mecanismos estão envolvidos no controle de fitopatógenos por espécies de *Burkholderia,* incluindo secreção de aleloquímicos (antibióticos e

sideróforos), competição por nutrientes e indução de resistência sistêmica. A capacidade em atuar contra diversos fitopatógenos presentes no solo, causadores de "tombamento" em plantas, foi relatada para *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, *B. pyrrocinia*, *B. vietnamiensis* e *B. phytofirmans*, principalmente no controle de *Fusarium* spp., *Phythium* spp., *Phytophthora capsici*, *Rhizoctonia solani* e *Botrytis cinerea* (Bowers & Parker, 1993; Burkhead et al., 1994; Moon et al., 1996; Bevivino et al., 1998; Lee et al., 2000; Peix et al., 2001). Além de fungos fitopatogênicos, há relatos de que *Burkholderia* spp. também produz antibióticos contras leveduras e bactérias (Cain et al., 2000).

Diversas substâncias com atividade antimicrobiana já foram detectadas em *Burkholderia* spp., como pirrolnitrina, xilocandinas, derivados de quinolinas, altericidinas, produtos voláteis, entre outros (Arina et al., 1964; Lee et al., 1994; Moon et al., 1996; Vial et al., 2007). O antibiótico pirrolnitrina (fenilpirrole clorinado) é o antifúngico mais estudado em *Burkholderia.* Este metabólito secundário foi isolado, primeiramente, de *B. pyrrocinia* (Arina et al., 1964). A pirrolnitrina impede o desenvolvimento de vários fungos por meio da inibição da cadeia de transporte de elétrons do sistema respiratório (El-Banna & Winkelmann, 1998). Estirpes de *Burkholderia* também podem induzir respostas de defesa da planta, como relatado para *B. phytofirmans* (Compant et al., 2005), a qual pode, ainda, atuar diretamente sobre o fitopatógeno *Botritys cinerea* (Ait Barka et al., 2002).

A produção de compostos de baixo massa molecular denominados de sideróforos, que atuam na quelatização de íons de ferro, também é conhecido por inibir o crescimento de alguns fungos fitopatogênicos (Compant et al., 2008), competindo por ferro onde este elemento é limitante. Em estirpes do complexo *B. cepacia* há pelos menos quatro tipos de sideróforos que são produzidos (Bukovits et al., 1982; Sokol, 1984; Meyer et al., 1989; Stephan et al., 1993; Meyer et al., 1995; Barelmann et al., 1996 apud Thomas, 2007).

A produção de fito-hormônios por estirpes de *Burkholderia* também pode atuar no crescimento vegetal. *B. vietnamiensis* promoveu o crescimento de plantas de arroz e esse acréscimo além da fixação biológica de nitrogênio também foi atribuído à produção de fito-hormônios (Trân Van et al., 2000). Estirpes de *Burkholderia* também podem auxiliar no crescimento vegetal mantendo baixos os níveis de etileno na planta por meio da ação da enzima ACC deaminase (Glick et al., 1998). Esta enzima foi detectada, primeiramente, em um isolado de *Burkholderia* obtido de nódulos de *Mimosa pudica* (Pandey et al., 2005) e, posteriormente, foi detectada em *B. xenovorans*, *B. unamae* e *B. phytofirmans* (Sessitsch et al., 2005; Caballero-Mellado et al., 2007). *B. cepacia* e *B. vietnamiensis* isolados da rizosfera de milho e arroz, respectivamente, também produziram ácido indol acético e promoveram o crescimento de plantas de pepino (Bevivino et al., 1994). No entanto, são poucos os estudos quanto à produção de fito-hormônios por estipes de *Burkholderia.*

Estirpes de diazotróficos de *B. tropica, B. unamae*, *B. xenovorans* e *B. kururienses* isoladas da rizosfera de plantas de tomate, no México, apresentaram atividades envolvidas na biorremediação (cresceram utilizando fenol, benzeno ou tolueno), produção de sideróforos e solubilização de fosfatos, o que pode ter grande potencial em aplicações agrobiotecnológicas (Caballero-Mellado et al., 2007).

A estirpe SAOCV2 de *B. cepacia* promoveu o crescimento de plantas de feijão (*P. vulgaris*), o que foi atribuído à mobilização de fósforo, à atividade antifúngica contra *Fusarium* e à promoção da nodulação por rizóbio (Peix et al., 2001).

A inoculação da estirpe de *B. vietnamiensis* TVV75 promoveu um acréscimo na produção de grãos de arroz de 13% a 22%, em experimento realizado em condições de campo, no Vietnã (Trân Van et al., 2000).

O gênero *Burkholderia* também tem sido estudado quanto à sua capacidade de degradação de poluentes, devido à sua grande versatilidade na utilização de fontes de carbono. A espécie ***B. kururiensis*** foi isolada de amostras de um aquífero poluído com tricloroetileno (TCE), no Japão, utilizando uma única fonte de carbono fenol (Zhang et al., 2000).

A espécie *B. cepacia* G4 tem recebido especial atenção pela sua capacidade de degradar compostos como hidrocarbonetos aromáticos e solventes clorados (Radway et al., 1998). O TCE é degradado por *B. cepacia* G4 cometabolicamente utilizando tolueno ou outro composto aromático como única fonte de carbono (Nelson et al., 1987). A degradação de TCE por uma população nativa de um aquífero contaminado foi de moderadamente eficiente a não eficiente e o sucesso dessa degradação foi limitado pela presença de tolueno e fenol como fontes de carbono, mas a população nativa não foi tão eficiente como *B. cepacia* G4 em degradar o TCE (Fries et al., 1997). A oxidação do tolueno é realizada pela enzima tolueno mono-oxigenase (TomA), que também é a enzima responsável pela degradação do TCE (Mars et al., 1996). O gene que codifica para a formação do tolueno monooxigenase está localizado no plasmídeo e é conhecido como pTOM. *B. xenrovorans* estirpe LB400^{T} é conhecida pela habilidade de degradar bifenil policlorinados (Goris et al., 2004). Assim, essas bactérias possuem potencial para futuros trabalhos com biorremediação de áreas contaminadas, o que tem despertado o interesse dos pesquisadores.

2.3 Estirpes de *Burkholderia* e fixação biológica de nitrogênio

Determinados grupos de microrganismos são de grande interesse nos estudos de diversidade, devido aos benefícios decorrentes de suas atividades, como, por exemplo, as bactérias diazotróficas. As bactérias diazotróficas, por meio do processo conhecido como fixação biológica do nitrogênio (FBN), são responsáveis pelo fornecimento deste elemento em diversos ecossistemas. As

bactérias que nodulam leguminosas (BNL) certamente se destacam por apresentar elevada importância econômica, que está relacionada à sua ampla distribuição geográfica e também à maior eficiência no processo de FNB, devido à parceria mais evoluída entre o vegetal e o microrganismo (Moreira & Siqueira, 2006).

Existem cerca de 20.000 espécies de leguminosas descritas no mundo, mas somente uma pequena proporção tem sido estudada quanto à sua capacidade para nodulação. Além disso, a maioria das espécies estudadas quanto à nodulação é de herbáceas, principalmente as produtoras de grãos, como a soja (*Glycine max* L.) e o feijão (*Phaseolus vulgaris* L.) (Moreira & Siqueira, 2006). Embora a maioria das espécies de leguminosas se concentre nas regiões tropicais, muitos estudos já realizados foram conduzidos em regiões temperadas. A capacidade nodulífera da maioria das leguminosas e as bactérias que formam simbioses com plantas em regiões tropicais ainda são desconhecidas.

Atualmente, existem somente 11 gêneros indicados como simbionte que nodulam leguminosas, como *Rhizobium* (Frank, 1889), *Bradyrhizobium* (Jordan, 1984), *Azorhizobium* (Dreyfus et al., 1988), *Sinorhizobium* (Chen et al., 1988), *Mesorhizobium* (Jarvis et al., 1997), *Allorhizobium* (Lajudie et al., 1998), *Burkholderia* (Moulin et al., 2001), *Methylobacterium* (Sy et al., 2001), *Devosia* (Rivas et al., 2002), *Ralstonia*/*Cupriavidus* (Chen et al., 2001) e *Ochrobactrum* (Trujillo et al., 2005). Dentre estes gêneros, *Burkholderia* e *Cupriavidus* são os únicos simbiontes pertencentes à classe β-proteobactéria, sendo todos os demais α-proteobactéria.

Até 2001, todos os gêneros de bactérias capazes de nodular leguminosas descritos faziam parte da classe α-proteobactéria e eram denominados vulgarmente como rizóbios. No entanto, Moulin et al. (2001) encontraram dois isolados obtidos a partir de nódulos de *Asphalatus carnosa*, oriundo da África do Sul e *Machaerium lunatum*, da Guiana Francesa, que eram distantes

filogeneticamente dos então conhecidos rizóbios. Por meio do sequenciamento do gene 16S rDNA, verificou-se que estes isolados pertenciam à subclasse β-proteobactéria. Estes foram capazes de nodular siratro (*Macroptilium atropurpureum*) e apresentaram os genes associados à nodulação e à fixação de nitrogênio (*nod* e *nif*). Estes isolados foram então descritos como *Burkholderia tuberum* (estirpe STM678^T obtida de nódulos *A. carnosa*) e *B. phymatum* (STM815^T obtida de *M. lunatum*) (Vandamme et al., 2002a). Atualmente, seis espécies são descritas como nodulíferas.

Dois isolados obtidos a partir de nódulos de *Mimosa diplotricha* e *Mimosa pudica*, em Twain, foram identificados como *B. caribenis* (Vandamme et al., 2002a). *B. caribensis* foi primeiramente identificado como uma bactéria produtora de exopolissacarídeos e responsável pela formação de microagregados em um vertisolo, na Martinica (Achouak et al., 1999). O isolado obtido na Martinica, LMG18531^T, não foi capaz de nodular e fixar nitrogênio atmosférico (Vandamme et al., 2002a). Assim, na espécie *B. caribensis*, a característica para nodulação em leguminosas pode ou não estar presente.

Posteriormente, quatorze isolados obtidos a partir de nódulos de *Mimosa pigra* e *Mimosa scabrella*, em Taiwan, Venezuela e Brasil, foram descritos como *B. mimosarum* (Chen et al., 2006). Três isolados obtidos de nódulos de *M. scabrella* e *M. bimucronata* nativas do Brasil foram descritos como uma nova espécie de *Burkholderia* capaz de nodular e fixar nitrogênio, *B. nodosa* (Chen et al., 2007). Outra espécie descrita como nodulífera, a última descrita até o momento, *B. sabiae*, foi baseada em dois isolados oriundos de nódulos de *M. caesalpiniifolia*, no Brasil (Chen et al., 2008a). Exceto por *B. tuberum* e *B. phymatum*, todas as outras espécies de *Burkholderia* foram isoladas a partir de nódulos de espécies de *Mimosa*. Apesar de *B. phymatum* e *B. tuberum* terem sido isoladas de espécies da subfamilia Fabaceae, quando realizada a reinoculação no hospedeiro original estas não formaram nódulos (Elliot et al.,

2007a; Elliot et al., 2007b). Assim, estas estirpes podem ter sido isoladas de nódulos de plantas que não são os hospedeiros originais, mas se encontravam em uma fase transitória nestas leguminosas.

Diversos trabalhos têm ressaltado a afinidade de leguminosas da subfamília Mimosoideae por espécies nodulíferas do gênero *Burkholderia.* A maioria de 190 isolados de nódulos de *M. pudica* e *M. diplotricha* em Taiwan foram identificados como pertencente à β-proteobactéria (Chen et al., 2003). Outros vinte isolados de plantas de *Mimosa* oriundas do Brasil e Venezuela eram pertencentes ao gênero *Burkholderia* (Chen et al., 2005). Quarenta e quatro de 51 isolados de nódulos de quatro plantas da subfamília Mimosoideae (*M. casta*, *M. pigra*, *M. pudica*, *Abarema macradenia* e *Pithecellobium hymenaeafolium*), no Panamá, também eram pertencentes ao gênero *Burkholderia* (Barret & Parker, 2005).

Em trabalhos recentes também foi constatado que espécies de *Burkholderia* são capazes de formar nódulos efetivos em leguminosas da subfamília Papilionoideae (Elliot et al., 2007b; Garau et al., 2009). Foi verificado que a estirpe de *B. tuberum* foi capaz de formar nódulos efetivos em siratro (*M. atropurpureum*) e cinco espécies de *Cyclopia* (Elliot et al., 2007b). Duas estirpes de *Burkholderia* sp. isoladas de nódulos de *Rhynchosia ferulifolia* na África do Sul foram autenticadas e formaram nódulos efetivos, sendo os tratamentos com a inoculação dessas estirpes similares ao tratamento com fornecimento de nitrogênio mineral. Devido ao grande número de espécies de leguminosas existentes e ao pouco conhecimento da capacidade nodulífera da grande maioria, há necessidade de mais estudos para compreender as relações simbióticas entre bactérias do gênero *Burkholderia* que é tão diverso, e espécies da família Leguminosae.

Também foram isolados de nódulos de leguminosas estirpes de *Burkholderia* sp., similar ao grupo conhecido como complexo *Burkholderia*

cepacia. A estirpe STM1441 foi isolada de *Alysicarpus glumaceus* (Fabaceae), no Senegal e foi identificada como *Burkholderia cepacia* genomovar VI (Vandamme et al., 2002a). Em Madagascar, também foi encontrado um isolado obtido de nódulos de *Dalbergia* sp. que, por meio do sequenciamento do gene 16S rDNA, foi identificado como uma estirpe do complexo *B. cepacia* (Rasolomampianina et al., 2005).

Também há relatos de estirpes de *B. fungorum* isoladas a partir de nódulos de leguminosas (Barret & Parker, 2006; Vandamme et al., 2007b), no entanto, sua habilidade em nodular não foi confirmada.

Diversas espécies do gênero *Burkholderia* também são capazes de fixar nitrogênio atmosférico em vida livre ou em associações com vegetais.

A primeira espécie diazotrófica descrita foi *B. vietnamiensis* (Gillis et al., 1995). Esta bactéria foi isolada da rizosfera de plantas de arroz cultivado no Vietnã. Análises características fenotípicas e genotípicas mostraram que os isolados fixadores de nitrogênio oriundos de plantas de arroz formaram um grupo distinto, juntamente com dois isolados de origem clínica. Estes foram então descritos como *B. vietnamiensis* e todos foram capazes de fixar nitrogênio atmosférico.

A espécie *B. kururiensis* foi isolada de amostras de um aquífero poluído com tricloroetileno (TCE), no Japão (Zhang et al., 2000) e a capcidade capacidade de fixar nitrogênio foi descrita posteriormente (Estrada-de-los-Santos et al., 2001).

B. tropica foi isolada da rizosfera e também de tecidos de plantas de cana-de-açúcar, milho e teosinte, no Brasil, no México e na África do Sul (Reis et al., 2004). *B. unamae* foi isolado da rizosfera, rizoplano e tecidos de plantas de milho, cana-de-açúcar e café cultivadas no México (Caballero-Mellado et al., 2004). *B. xenovorans* possui alta capacidade de degradar compostos de bifenilpoliclorados (PCB) (Goris et al., 2004), mas também é conhecida como

diazotrófica (Estrada-de-los-Santos et al., 2001; Caballero-Mellado et al., 2007). Novas estirpes isoladas da rizosfera, raízes e folhas de milho de cana-de-açúcar cultivado no Brasil, capazes de fixar nitrogênio atmosférico, foram identificadas como *B. silvatlantica* (Perin et al., 2006). *B. terrae* e *B. ginsengisoli* também são espécies fixadoras de nitrogênio, tendo sido isoladas de solo de floresta e campo de ginseng, respectivamente, na Coreia do Sul (Yang et al., 2006; Kim et al., 2006).

2.4 Complexo *Burkholderia cepacia* (Bcc)

O complexo *Burkholderia cepacia* foi proposto para compreender um grupo de espécies similares, originalmente referidas como *B. cepacia* genomovar I, II, III, IV e V (Vandamme et al., 1997). Estudos taxonômicos revelaram que esses microrganismos compreendem um grupo heterogêneo de estirpes, com elevada similaridade na sequência do gene 16S rDNA (98-100%) e moderado nível de hibridação DNA-DNA, coletivamente referido como o complexo *B. cepacia* (Bcc). Atualmente, este grupo consiste de 17 espécies: *B. cepacia* (Yabuuchi et al., 1992), *B. multivorans* (Vandamme et al., 1997), *B. cenocepacia* (Vandamme et al., 2003), *B. stabilis* (Vandamme et al., 2000), *B. vietnamiensis* (Gillis et al., 1995; Vandamme et al., 1997), *B. dolosa* (Vermis et al., 2004), *B. ambifaria* (Coenye et al., 2001b), *B. anthina* (Vandamme et al., 2002b), *B. pyrrocinia* (Vandamme et al., 2002b), *B. latens*, *B. diffusa*, *B. arboris*, *B. seminalis*, *B. metallica* e *B. ubonensis* (Vanlaere et. al., 2008a), *B. contaminans* e *B. lata* (Vanlaere et. al., 2009). A composição da sequência do 16S rDNA indica um grupo distinto das demais espécies do gênero *Burkholderia* (Coenye et al., 2001c).

Bactérias do Bcc podem ser encontradas em solos, águas, rizosfera de plantas, em humanos, em animais e em hospitais (Coenye & Vandamme, 2003). Espécies deste complexo incluem fixadores de nitrogênio e promotores de

crescimento vegetal (Trân Van et al., 2000), fitopatógenos (Burkholder, 1950), patógenos de animais (Neubauer et al., 2005) e de humanos (Isles et al., 1984). Em humanos estirpes do Bcc, são considerados patógenos oportunistas, principalmente para pessoas com fibrose cística. Fibrose cística é a doença hereditária mais frequente em populações brancas, ocorrendo em aproximadamente um para cada 3.000 nascimentos (O'Sullivan & Freedman, 2009). Devido à grande resistência a antibióticos, a infecção por espécies de *Burkholderia* pode causar um rápido declínio da função pulmonar e, apesar da taxa infecção por esta bactéria ser relativamente baixa em pessoas com fibrose cística, o risco de morbidade e mortalidade é elevado (LiPuma, 1998).

2.5 Complexo *B. cepacia* X ambiente

O estudo da dinâmica populacional do complexo *B. cepacia* em ambientes naturais tem sido objetivo de vários trabalhos, devido ao grande interesse no uso dessas bactérias como promotores do crescimento vegetal, na biorremediação de xenobióticos. Além disso, a atenção em relação a esses microrganismos tem crescido, devido ao fato de serem patógenos oportunistas para pessoas imunodeficientes, principalmente aquelas com fibrose cística (Tabacchione et al., 2002; Coenye & Vandamme, 2003; Baldwin et al., 2007; Mahenthiralingam et al.; 2008).

Estes estudos são importantes para esclarecer se ambientes naturais constituem um reservatório de bactérias do complexo *B. cepacia* para pacientes com fibrose cística ou imunodeficientes. Bactérias do complexo *B. cepacia* são comuns na rizosfera de plantas milho (Ramette et al., 2005), arroz (Trân Van et al., 2000), algodão (Parke & Gurian-Sherman, 2001), café (Estrada-de-los-Santos et al., 2001), etc. Também são endofíticos de diversas plantas, como arroz (Singh et al., 2006), *Citrus sinensis* (Araujo et al., 2002) e milho (Estrada-de-los-Santos et al., 2001).

Especulações sobre ambientes naturais como reservatório para a aquisição de infecções causadas por *B. cepacia* possuem evidências diretas e indiretas (Coenye & Vandamme, 2003). A evidência indireta surge de observações que medidas de controle de infecções têm reduzido, mas não elimina, novas infecções (frequentemente com isolados apresentando novas carcaterísticas genotípicas). Já evidências diretas foram obtidas de estudos de classificação genotípica.

Alguns marcadores de patogenicidade têm sido associados ao complexo *B. cepacia*. Entre eles, dois genes: *esmrR* e *cblA* (Mahenthiralingam et al., 1997; Sajjan et al., 1995). Durante estudo de *Random amplified polymorphic DNA*, ou RAPD, em espécies do complexo *B. cepacia*, foi verificado um fragmento de DNA conservado entre estirpes epidêmicas, mas que era ausente em estirpes não epidêmicas e raras em isolados ambientais (Mahenthiralingam et al., 1997). Este marcador de DNA foi chamado de BCESM (sigla para *Burkholderia cepacia epidemic strain marker*) e a análise da sequência desse fragmento revelou uma ORF de 834 pares de base, denominado gene *esmR*. O produto deste gene é homólogo a reguladores transcricionais negativos. Posteriormente, foi descoberto que este marcador BCESM é exclusivo da espécie *B. cenocepacia* (LiPuma et al., 2001). Outro marcador, o gene *cblA*, relacionado à expressão de um tipo de pilus (*Cable pilus*) está associado à aderência à mucina e a células epiteliais do hospedeiro (Sajjan et al., 1995).

Outro estudo foi realizado buscando avaliar fatores de virulência em isolados de amostras clínicas de pacientes com fibrose cística e isolados coletados da rizosfera de milho, ambos na Itália (Bevivino et al., 2002). Neste estudo, a maioria do isolados clínicos correspondeu a *B. cenocepacia* e os isolados ambientais a *B. cenocepacia* e *B. pyrrocinia.* Foi verificada a presença do marcador BCESM em 61% dos isolados clínicos e em 15% dos isolados ambientais. Já para o gene *cblA*, foi encontrado em apenas um isolado clínico.

Outras características de virulência foram avaliadas, como resistência a antibióticos, atividade proteolítica e hemolítica e também a capacidade de causar danos a tecidos vegetais. Tanto os isolados clínicos quanto ambientais foram funcionalmente equivalentes quanto a essas características. Esses resultados, mais a presença do BCESM em 15% dos isolados ambientais, reforça a hipótese de o ambiente servir como um reservatório para estirpes patogênicas do Bcc (Bevivino et al., 2002).

No entanto, esses marcadores não são absolutos para a habilidade de *B. cenocepacia* em causar infecção e não estão associados a todas as estirpes que causam infecções em pacientes com fibrose cística (LiPuma et al., 2001). Por meio de *recA*-RFLP, são conhecidas duas linhagens de *B. cenocepacia*, III-A e III-B. O marcador BCESM está presente em mais de 77% da linhagem III-A, mas é ausente na maioria III-B (Baldwin et al., 2004). Mas, quando estes dois marcadores estão presentes em estirpes coletadas em pacientes com fibrose cística, têm sido documentadas alta transmissibilidade, virulência e mortalidade (Mahenthiralingam et al., 2001). A linhagem ET12, mais comum no Canadá e no Reino Unido, tem esses dois marcadores. No entanto, nos EUA, eles estão presentes em apenas 23% das estirpes de *B. cenocepacia* encontradas em pacientes com fibrose cística. Isso sugere que não existe apenas um fator que leva à virulência, mas sim vários outros fatores que ainda são desconhecidos.

Por meio do estudo do genoma de *B. cenocepacia*, foi verificado que, além de possuir três cromossomos e um plasmídeo, 10% do genoma correspondem a ilhas genômicas, presentes nos três cromossomos (Mahenthiralingam et al., 2005). O marcador BCESM está presente em uma dessas ilhas, que apresentam genes característicos de patogenicidade e também do metabolismo bacteriano. Existem também várias regiões de sequências de inserção, que estão associados à plasticidade do genoma desta bactéria.

B. cenocepacia, estirpe PHDC, espécie mais frequentemente isolada de pacientes com fibrose cística nos EUA, também foi isolada de solos agrícolas (LiPuma et al., 2002). Por meio da análise de sequências por multilocus (MLST), foi comparada uma coleção de 381 isolados clínicos com 233 isolados de amostras ambientais contendo nove espécies do complexo *B. cepacia*. Destes, 81 isolados clínicos foram idênticos, por meio de MSLT, a uma grande faixa dos isolados de amostras ambientais (Baldwin et al., 2007).

O solo constitui um ambiente natural para muitos bactérias patogênicas (Berg et al., 2005), incluindo *Burkholderia.* Assim, existe a preocupação com relação ao fato de a inoculação com essas estirpes que possuem características que promovam o crescimento vegetal, mas também estão associados a doenças, poderem aumentar a disseminação desta bactéria em humanos. Em estudo utilizando *B. ambifaria* inoculadas em sementes de ervilha, verificou-se que, após seis semanas do plantio, a densidade populacional de bactérias do tratamento inoculado a do tratamento sem inoculação foram similares (King & Parke, 1996). Esses dados sugerem que, após um período, a população natural reestabelece o equilíbrio e que a inoculação não aumenta o reservatório natural da rizosfera com a introdução de patógeno oportunista.

Além das bactérias do complexo *B. cepacia*, outras espécies do gênero *Burkholderia* também são patógenos de humanos e animais. A doença melioidose é causada por estirpes de *B. pseudomallei*, ocorrendo, principalmente, no sudeste da Ásia e norte da Austrália, causando pneumonia e septicemia (Dance, 1990; White, 2003). *B. pseudomallei* é uma saprófito encontrado em solos úmidos que, em contato com pessoas predispostas, podem causar infecção (White, 2003). A doença conhecida como mormo, que ataca principalmente cavalos, é causada pela *B. mallei*, mas também pode infectar mulas e burros (Neubauer et al., 2005). *B. mallei* é um patógeno obrigatório de

mamíferos e pode ser transmitido a humanos via contato com animais infectados (Whitlock et al., 2007).

Estes resultados mostram que mais estudos devem ser realizados para permitir a utilização desses microrganismos na agricultura, sem causar danos à saúde humana ou animal.

2.6 Estudos de identificação de *Burkholderia* spp.

Estudos de identificação de bactérias envolvem vários testes morfológicos, bioquímicos e moleculares. Para o gênero *Burkholderia*, uma abordagem taxonômica polifásica utilizando testes diagnósticos múltiplos é necessária para uma identificação segura.

Testes fenotípicos, como caracterização cultural, utilização de fontes carbono, caracterização morfológica celular, análise de proteínas totais por SDS-PAGE (dodecil sulfato de sódio - gel de poliacrilamida), composição celular de ácidos graxos e testes moleculares, como sequenciamento do gene 16S rDNA, sequenciamento do gene *recA*, RFLP (polimorfismo de tamanho dos fragmento de restrição), ARDRA (análise de restrição do DNA ribossomal amplificado), hibridização DNA-DNA e conteúdo de G+C, entre outros, têm sido amplamente empregados para a carcaterização do gênero *Burkholderia* (Tabela 1).

A caracterização genotípica de bactérias diazotróficas é ferramenta útil para o conhecimento da diversidade destes organismos e também para compreender como os sistemas de uso do solo podem afetar a população desses microrganismos. O sequenciamento de genes que codificam para o RNA ribossomal (16S, 23S e 5S) tem sido amplamente empregado para estudos de diversidade. Os RNAs ribossomais são considerados cronômetros moleculares, pois são moléculas universais com funções altamente específicas estabilizadas ao longo da evolução e não sofrem influência por mudanças no meio ambiente.

Atualmente, o gene 16S rDNA é o mais utilizado para inferir a respeito das relações filogenéticas entre bactérias (Woese, 1991).

A análise do gene 16S rDNA produz valores numéricos de similaridade que podem ser utilizados como limites para definição de espécies e gêneros. Esta molécula é especialmente útil para nível taxonômico acima de espécies, em que, por exemplo, um gênero pode ser definido por bactérias que apresentem 95% de similaridade em suas sequências. Foi observado que organismos com similaridade genômica acima de 70%, geralmente, são compatíveis com mais de 97% de similaridade nas sequências do gene 16S rDNA (Rosselló-Mora & Amann, 2001). No entanto, o 16S rDNA não tem o poder de resolução em espécies, ou seja, ele não discrimina estirpes dentro da mesma espécie, podendo existir diferentes espécies com sequências 16S rDNA quase idênticas ou idênticas, micro-heterogeneidade dentro de uma mesma espécies e, ainda, organismos únicos com duas ou mais sequências divergentes. Assim, outras regiões do DNA de procariotos devem ser utilizadas.

Especialmente para o gênero *Burkholderia*, os estudos de taxonomia e identificação são complexos e o sequenciamento do gene 16S rDNA não pode ser utilizado como a principal análise para distinguir todas as espécies (Payne et al., 2005).

Para a identificação deste e de outros grupos diversos de bactérias, outros genes estão sendo utilizados. Esses genes são denominados de "housekeeping genes", ou seja, são genes que codificam funções metabólicas fundamentais e sempre são expressos e fornecem uma discriminação suficiente para a classificação de bactérias (Maiden, 2006). Os "housekeeping gene" evoluem mais rápido do que o 16S rDNA e podem representar uma alternativa útil ou um complemento aos genes ribossomais (Tayeb et al., 2008). No entanto, apenas os genes *recA*, *gyrB*, *rpoD* e *rpoB* se revelaram muito úteis na

discriminação de espécies do gênero *Burkholderia* (Mahenthiralingam et al., 2000; Payne et al., 2005; Maeda et al., 2006; Tayeb et al., 2008).

O gene *gyrB* codifica para a subunidade β da DNA girase, uma enzima responsável por introduzir superenovelamentos negativos no DNA bacteriano, atividade fundamental na replicação dos cromossomos (Watt & Hickson, 1994). O gene *rpoD* codifica um dos fatores sigma e *rpoB* subunidade beta da RNA polimerase (Lonetto et al., 1992; Tayeb et al., 2008), ou seja, participa da transcrição do DNA. Esses genes foram utilizados como um método de detecção e identificação de espécies fitopatogênicas de *Burkholderia* em arroz (Maeda et al., 2006).

O gene *recA* codifica para a proteína RecA, que está entre as proteínas mais conservadas em bactérias e, por isso, tem sido empregado em estudos de identificação e classificação bacteriana (Karlin et al., 1995; Mahenthiralingam et al., 2000; Gaunt et al., 2001; Vermis et al., 2002; Payne et al., 2005). O gene *recA* (recombinase A) codifica para a proteína multifuncional envolvida no sistema de reparo S.O.S do DNA bacteriano. Estudos de caracterização de espécies do gênero *Burkholderia* com o gene *recA* têm facilitado e esclarecido a posição taxonômica de diversas estirpes (Payne et al., 2005).

A determinação da similaridade DNA-DNA é uma técnica padrão para o delineamento de espécie em procariotos (Rosselló-Mora & Amam, 2001). No entanto, esta é uma técnica que não pode ser realizada em muitos laboratórios, o que restringe o seu uso. Em 1998, a técnica denominada "Multilocus Sequence Typing", ou MLST, foi proposta como um método de caracterização bacteriana portátil e universal, utilizando o patógeno humano *Neisseria meningitidis* como exemplo (Maiden et al., 1998). Um esquema de MLST foi desenvolvido para a identificação do complexo *B. cepacia* e mostrou-se altamente válido para a identificação de espécies deste complexo (Baldwin et al., 2005). Com o auxílio

da técnica de MLST, sete novas espécies do complexo *B. cepacia* foram descritas (Vanlaere et al., 2008a; Vanlaere et al., 2009).

Dados acumulados de cerca de 1.000 isolados do complexo *B. cepacia* analisados demonstraram que um nível de 3% de divergência das sequências concatenadas corresponde a 70% de hibridização DNA-DNA, que é o limite para a delimitação de espécies (Vanlaere et al., 2009).

2.7 O gênero *Cupriavidus*

O gênero *Cupriavidus*, classe β-proteobactéria, foi descrito baseado em um único isolado N-1^T obtido de um solo aos arredores do University Park, Ilinóis, EUA (Makkar & Casida, 1987). Esta estirpe é um predador não obrigatório de fungos e outras bactérias e foi descrita como *C. necator.*

O gênero *Ralstonia* foi criado em 1995 para acomodar espécies que eram antes descritas como *Alcaligenes eutrophus*, *Pseudomonas solanacearum* e *Pseudomonas picketti* (Yabuuchi et al., 1995). Posteriormente, diversas espécies foram sendo descritas dentro do gênero *Ralstonia*, como *Ralstonia gilardii* (Coenye et al., 1999), *Ralstonia paucula* (Vandamme et al., 1999), *Ralstonia basilensis* (Steinle et al., 1998), *Ralstonia oxalaticus* (Sahin et al., 2000), *Ralstonia taiwanensis* (Chen et al., 2001), *Ralstonia campinensis* e *Ralstonia metallidurans* (Goris et al., 2001), *Ralstonia mannitolytica* (De Baere et al., 2001), *Ralstonia insidiosa* (Coenye et al., 2003a) e *Ralstonia respiraculi* (Coenye et al., 2003b).

Análise de sequências do gene 16S rDNA revelou que as espécies do gênero *Ralstonia* revelou que elas compreendiam duas linhagens, com uma dissimilaridade de sequências maior que 4%, levando à proposição um novo gênero designado de *Wautersia* (Vaneechoutte et al., 2004). A linhagem 1 compreendia *R. pickettii*, *R. insidiosa*, *R. mannitolilytica*, *R. solanacearum* e *R. syzygii* comb. nov. (anteriormente *Pseudomonas syzygii*). A linhagem 2

compreendia *Ralstonia eutropha*, *R. basilensis*, *R. campinensis*, *R. gilardii*, *R. metallidurans*, *R. oxalatica*, *R. paucula*, *R. respiraculi* e *R. taiwanensis* passou para o novo gênero *Wautersia*.

No entanto, a estirpe tipo do recém-criado gênero *Wautersia*, *W. eutropha* LMG1199^T e *Cupriavidus necator* LMG 8453^T apresentavam perfis proteicos e teor de G+C altamente similares, similaridade do gene 16S rDNA de 99,7% e valores de hibridização DNA-DNA acima de 70% (Vandamme & Coenye, 2004). Então, conclui-se que *W. eutropha* e *C. necator* pertenciam a mesma espécie.

A regra 42 do Código Internacional de Nomenclatura de Bactéria especifica que se dois ou mais taxa do mesmo nível são unidos, o nome mais velho legítimo permanece. Assim, o nome do gênero *Cupriavidus* tem prioridade sobre o nome *Wautersia* e os outros membros do gênero *Wautersia* foram reclassificados para *Cupriavidus.* Atualmente, são descritas 11 espécies dentro do gênero *Cupriavidus*: *Cupriavidus necator* (Makkar & Casida, 1987); *Cupriavidus basilensis*, *Cupriavidus pauculus*, *Cupriavidus gilardii*, *Cupriavidus oxalaticus*; *Cupriavidus taiwanensis*, *Cupriavidus metallidurans* e *Cupriavidus campinensis*, *Cupriavidus respiraculi* (Vandamme & Coenye 2004, comb. nov.); *Cupriavidus laharis* e *Cupriavidus pinatubonensis* (Sato et al., 2006). As principais técnicas utlizadas para a identificação dessas espécies são apresentadas na Tabela 2.

O gênero compreende espécies isoladas de diversos ambientes, como solo, áreas vulcânicas, água, lodo, humanos e plantas e apresentam características diversas, como predadores de fungos e bactérias, resistência a metais, biodegradadação de xenobióticos, produtores de exopolissacarídeos de interesse industrial, causadores de infecções humanas e bactérias fixadoras de nitrogênio que nodulam leguminosas (Don & Pemberton, 1981; Makkar &

Casida, 1987; Ramsay et al., 1990; Coenye et al., 1999; Vandamme et al., 1999; Goris et al., 2001; Chen et al., 2001; Sato et al. 2006).

2.8 O gênero *Cupriavidus* e a fixação biológica de nitrogênio

Dentro do gênero *Cupriavidus*, atualmente, uma única espécie está descrita como bactéria fixadora de nitrogênio atmosférico. Oito isolados coletados de nódulos de *Mimosa pudica* e *Mimosa diplotricha*, em Taiwan e um isolado clínico de um paciente com fibrose cística foram descritos como *Ralstonia*/*Cupriavidus twainensis* (Chen et al., 2001).

Em estudo sobre ocorrência de simbiontes em *M. pudica* e *M. diplotricha* em 14 áreas, em Taiwan, *C. taiwanensis*, entre 190 isolados, foi a bactéria dominante associada a essas leguminosas (Chen et al., 2003). Isolados de *C. taiwanensis* também foram encontrados em nódulos de *M. pudica* do norte e do sul da Índia (Verma et al., 2004). Devido ao fato de sua ocorrência ter sido relatada somente na Ásia, acreditava-se que esta bactéria fosse oriunda dessa região. No entanto, espécies de *Mimosa* são oriundas de regiões tropicais e neotropicais da África do Sul, se estendendo do sul dos EUA até a Argentina (Allen & Allen, 1981). Mas, estudos posteriores verificaram que a simbiose *C. taiwanensis* não está limitada a Taiwan e à Índia. Isolados de *C. taiwanensis* em nódulos de *M. pigra* e *M. pudica*, na Costa Rica (Barret & Parker, 2006) e também em nódulos de *M. asperata*, no Texas, EUA (Andam et al., 2007), já foram obtidos.

Estudos sobre a ocorrência deste gênero ainda são poucos. No Brasil, ainda não foi verificada a presença de *C. taiwanensis* em nódulos de *Mimosa* spp. No entanto, foi relatada a ocorrência de *Cupriavidus* sp. isolados de solos da rizosfera de *Sesbania virgata*, capturado utilizando-se três espécies de plantas promíscuas diferentes, feijão-comum (*Phaseolus vulgaris*), feijão-caupi (*Vigna unguiculata*) e leucena (*L. leucocephala*) (Florentino et al., 2009).

TABELA 1 Número de isolados e principais testes utilizados para a identificação e a classificação de espécies do gênero *Burkholderia*.

Espécies Descritas	Isolados*	Morfologia de colônias/celular	Fontes de C	Análise de proteínas totais	Composição de ácidos graxos	RFLP ou ARDRA	16S rDNA	*recA*	G+C %	Hibridização DNA-DNA
(1) *B. ambifaria* LMG 19182^T	19	-/+	+	-	+	+	+	+	+	+
(2) *B. andropogonis* LMG2129^T	22	-/+	+	-	+	-	+	-	+	+
(3) *B. anthina* LMG20980^T	19	+/-	+	+	+	+	+	+	+	+
(4) *B. arboris* ES0263AT	17	+/+	+	-	-	-	+	+	+	+
(5) *B. bryophila* 1S18T	14	-/+	+	+	-	-	+	-	+	+
(6) *B. caledonica* LMG19076^T	7	-/+	+	+	+	-	+	-	+	+
(7) *B. caribensis* MWAP64^T	21	+/+	+	-	-	+	+	-	+	+
(8) *B. caryophyli* ATCC25418^T	1	-/+	+	-	+	-	+	-	+	+
(9) *B. cenocepacia* LMG16656^T	6	+/+	+	+	-	-	+	+	+	+
(8) *B. cepacia* ATTCC25416^T	1	-/+	+	-	+	-	+	-	+	+
(10) *B. contaminans* J2956^T	9	+/+	+	-	-	+	+	+	+	+

Continua...

Continua...										
[4] *B. diffusa* AU1075^T	14	+/+	+	-	-	-	+	+	+	+
[11] *B. dolosa* LMG18943^T	18	-/+	+	-	+	+	+	+	+	+
[12] *B. endofungorum* HKI456^T	1	+/+	+	-	+	-	+	-	-	+
[13] *B. ferrariae* FeGl01^T	1	+/+	+	-	+	-	+	+	+	+
[6] *B. fungorum* LMG16225^T	9	-/+	+	+	+	-	+	-	+	+
[14] *B. ginsengisoli* KMY03^T	1	+/+	+	-	+	-	+	-	+	+
[8] *B. gladioli* NCPPB1891^T	1	-/-	+	-	+	-	+	-	+	+
[15] *B. glathei* LMG14190^T	2	-/+	+	+	+	-	+	-	+	+
[16] *B. glumae* NIAS 1169^T	1	+/+	+	-	+	-	+	-	+	+
[17] *B. graminis* ATCC 700544^T	19	+/+	+	-	+	-	+	-	-	+
[22] *B. hospita* LMG20598^T	29	-/+	+	+	+	-	+	-	+	+
[19] *B. kururiensis* KP23^T	1	+/+	+	-	+	-	+	-	+	-
[10] *B. lata* 383^T	11	+/+	+	-	-	+	+	+	+	+
[4] *B. latens* FIRENZE3^T	6	+/+	+	-	-	-	+	+	+	+
Continua...										

Continua...										
[8] *B. mallei* ATCC23344^T	1	-/+	+	-	+	-	+	-	+	+
[5] *B. megapolitana* A3^T	3	-/+	+	+	-	-	+	-	+	+
[4] *B. metallica* AU0553^T	5	+/+	+	-	-	-	+	+	+	+
[20] *B. mimosarum* PAS44^T	14	+/+	+	+	+	-	+	-	+	+
[15] *B. multivorans* LMG13010^T	15	-/+	+	+	+	-	+	-	+	+
[21] *B. nodosa* Br3437^T	3	+/+	+	+	+	-	+	-	-	+
[22] *B. oklahomensis* C6786^T	4	+/+	+	-	-	-	+	-	+	+
[17] *B. phenazinium* LMG2247^T	1	+/+	+	-	+	-	+	-	+	+
[23] *B. phenoliruptrix* AC1100^T	1	+/+	+	+	+	-	+	-	+	+
[24] *B. phymatum* LMG 21445^T	1	+/+	+	+	+	+	+	+	+	+
[25] *B. phytofirmans* PsJN^T	8	-/+	+	-	+	-	+	-	+	+
[26] *B. plantarii* JCM5492^T	1	+/+	+	-	+	-	+	-	+	+
Continua...										

Continua...										
[8] *B. pseudomallei* ATCC23343^T	1	+/+	+	-	+	-	+	-	+	+
[15] *B. pyrrocinia* LMG14191^T	1	-/+	+	+	+	-	+	-	+	+
[12] *B. rhizoxinica* HKI454^T	1	+/+	+	-	+	-	+	-	-	+
[27] *B. sabiae* Br3407^T	2	-/+	+	+	+	-	+	-	+	+
[28] *B. sacchari* IPT101^T	1	+/+	+	+	+	-	+	-	+	+
[29] *B. sartisoli* RP007^T	3	+/+	+	+	+	-	+	-	+	+
[30] *B. sediminicola* HU2-65W^T	1	+/+	+	-	+	-	+	-	+	+
[4] *B. seminalis* AU0553^T	21	+/+	+	-	-	-	+	+	+	+
[31] *B. silvatlantica* LGM 23149^T	21	+/+	+	+	+	-	+		-	+
[32] *B. soli* GP25-8^T	1	+/+	+	-	+	-	+	-	+	+
[33] *B. sordidicola* KCTC 12081^T	2	+/+	+	-	+	-	+	-	+	+
[34] *B. stabilis* LMG14294^T	21	-/+	+	+	+	-	+	-	+	+
[35] *B. terrae* KMY02^T	1	+/+	+	-	+	-	+	-	+	+
[18] *B. terricola* LMG20594^T	17	-/+	+	+	+	-	+	-	+	+
[36] *B. thailandensis* E264^T	1	+/+	+	-	-	-	+	-	-	-
Continua...										

Continua...										
[37] *B. tropica* LGM 22274^T	41	+/+	+	+	-	+	+	-	+	+
[23] *B. tuberum* LMG 21444^T	1	+/+	+	+	+	+	+	+	+	+
[38] *B. ubonensis* NCTC 13147^T	1	+/+	+	-	+	-	+	-	+	+
[39] *B. unamae* MTI-641^T	20	+/+	+	+	+	+	+	-	-	+
[2] *B. vietnamiensis* TVV75^T	12	+/+	+	-	-	-	+	-	+	+
[40] *B. xenovorans* LB400^T	3	-/+	+	+	+	+	+	-	+	+

[1] Coenye et al. (2001b); [2] Gillis et al. (1995); [3] Vandamme et al. (2002a); [4] Vanlaere et al. (2008a); [5] Vandamme et al. (2007a); [6] Coenye et al. (2001a); [7] Achouak et al. (1999); [8] Yabuuchi et al. (1993); [9] Vandamme et al. (2003); [10] Vanlaere et al. (2009); [11] Vermis et al. (2004); [12] Partida-Martinez et al. (2007); [13] Valverde et al. (2006); [14] Kim et al. (2006); [15] Vandamme et al. (1997); [16] Urakami et al. (1994); [17] Viallard et al. (1998); [18] Goris et al. (2003); [19] Zhang et al. (2000); [20] Chen et al. (2006); [21] Chen et al. (2007); [22] Glass et al. (2006); [23] Coenye et al. (2005); [24] Vandamme et al. (2002b); [25] Sessitsch et al. (2005); [26] Urakami et al. (1994); [27] Chen et al. (2008a); [28] Brämer et al. (2001); [29] Vanlaere et al. (2008b); [30] Lim et al. (2008); [31] Perin et al. (2006); [32] Yoo et al. (2007); [33] Lim et al. (2003); [34] Vandamme et al. (2000); [35] Yang et al. (2006); [36] Brett et al. (1998); [37] Reis et al. (2004); [38] Yabuuchi et al. (2000); [39] Caballero-Mellado et al. (2004); [40] Goris et al. (2004).

***** Número de isolados utilizados no estudo de descrição.

TABELA 2 Número de isolados e principais testes utilizados para a identificação e a classificação de espécies do gênero *Cupriavidus.*

Espécies Descritas	Isolados*	Morfologia de colônias/celular	Fontes de C	Análise de proteínas totais	Composição de ácidos graxos	RFLP ou ARDRA	16S rDNA	G+C	Hibridização DNA-DNA
(1) *C. basilensis* RK1^T	1	+/+	+	-	-	-	+	-	-
(2) *C.campinensis* WS2^T	8	+/+	+	+	+	-	+	+	+
(3) *C. Gilardii* LMG5886^T	10	-/+	+	+	+	-	+	+	+
(4) *C. Laharis* 1263a^T	1	+/+	+	+	-	-	+	+	+
(2) *C. matallidurans* CH34^T	17	+/+	+	+	+	-	+	+	+
(5) *C.necator* N-1^T	1	+/+	+	-	-	-	-	+	-
(6) *C. oxalaticus* ^{T}	1	+/+	+	-	-	-	+	+	+
(7) *C. pauculus* LMG3244^T	12	+/+	+	+	-	+	+	+	+
(4) *C. pinatubonensis* 1245^T	9	+/+	+	+	-	-	+	+	+
(8) *C.respiraculis* AU3313^T	4	-/+	+	+	+	-	+	-	-
(9) *C. taiwanensis* LMG19424^T	9	-/+	+	-	-	+	+	+	+

(1) (Steinle et al., 1999) Vandamme & Coenye (2004), comb. nov.; (2) (Goris et al., 2001) Vandamme & Coenye (2004), comb. nov.; (3) (Coenye et al., 1999) Vandamme & Coenye (2004), comb. nov. ; (4) Sato et al. (2006); (5) Makkar & Casida (1987); (6) (Sahin et al.,

2000) Vandamme & Coenye (2004), comb. nov.; [7] (Vandamme et al., 1999) Vandamme & Coenye (2004), comb. nov.; [8] (Coenye et al., 2003) Vandamme & Coenye (2004), comb. nov.; [9] (Chen et al., 2001) Vandamme & Coenye (2004), comb. nov.

* Número de isolados utilizados no estudo de descrição.

3 REFERÊNCIAS BIBLIOGRÁFICAS

ACHOUAK, W.; CHRISTEN, R.; BARAKAT, M.; MARTEL, M. H.; HEULIN, T. *Burkholderia caribensis* sp. nov., an exopolysaccharide-producing bacterium isolated from vertisol microaggregates in Martinique. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 49, n. 2, p. 787-794, Apr. 1999.

AIT BARKA, E.; GOGNIES, S.; NOWAK, J.; AUDRAN, J-C.; BELARBI, A. Inhibitory effect of endophyte bacteria on Botrytis cinerea and its influence to promote the grapevine growth. **Biological Control**, Orlando, v. 24, n. 2, p. 135-142, June 2002.

ALLEN, O. N.; ALLEN, E. K. **The leguminosae:** a source book of characteristics, uses and nodulation. Wisconsin: University of Wisconsin, 1981. 812p.

ANDAM, C. P.; MONDO, S. J.; PARKER, M. A. Monophyly of *nodA* and *nifH* Genes across Texan and Costa Rican Populations of *Cupriavidus* Nodule Symbionts. **Applied and Environmental Microbiology**, Washington, v. 73, n. 14, p. 4686-4690, July 2007.

ARAUJO, W. L.; MARCON, J.; MACHERONI, W. J. R.; VAN ELSAS, J. D.; VAN VUURDE, J. W. L.; AZEVEDO, J. L. Diversity of endophytic bacterial populations and their interaction with Xylella fastidiosa and citrus plants. **Applied and Environmental Microbiology,** Washington, v. 68, n. 10, p. 4906-4914, Oct. 2002.

ARIMA, K.; IMANAKA, H.; KOUSAKA, M.; FUKUDA, A.; TAMURA, G. Pyrrolnitrin, a new antibiotic substance, produced by *Pseudomonas*. **Agricultural and Biological Chemistry,** Tokyo, v. 28, n. 8, p. 575-576, Aug. 1964.

BALDWIN, A.; MAHENTHIRALINGAM, E.; DREVINEK, P.; VANDAMME, P.; GOVAN, J. R.; WAINE, D. J.; LIPUMA, J. J.; CHIARINI, L.; DALMASTRI, C.; HENRY, D. A.; SPEERT, D. P.; HONEYBOURNE, D.; MAIDEN, M. C. J.; DOWSON, C. G. Environmental *Burkholderia cepacia* Complex Isolates in human infections. **Emerging Infectious Diseases**, Atlanta, v. 13, n. 3, p. 458-461, Mar. 2007.

BALDWIN, A.; MAHENTHIRALINGAM, E.; THICKETT, K. M.; HONEYBOURNE, D.; MAIDEN, M. C.; GOVAN, J. R.; SPEERT, D. P.; LIPUMA, J. J.; VANDAMME, P.; DOWSON, C. G. Multilocus sequence typing scheme that provides both species and strain differentiation for the *Burkholderia cepacia* complex. **Journal of Clinical Microbiology**, Washington, v. 43, n. 9, p. 4665-4673, Sept. 2005.

BALDWIN, A.; SOKOL, P. A.; PARKHILL, J.; MAHENTHIRALINGAM, E. The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic Island Encoding Both virulence and metabolism-associated genes in *Burkholderia cenocepacia.* **Infection and Immunity**, Washington, v. 72, n. 3, p. 1537-1547, Mar. 2004.

BARBERI, A. **Diversidade e eficiência de bactérias que nodulam feijoeiro de diferentes sistemas de uso da terra na Amazônia Ocidental.** 2007. 121p. Tese (Doutorado) – Universidade Federal de Lavras, Lavras.

BARRET, C. F.; PARKER, M. A. Prevalence of *Burkholderia* sp. nodule symbionts on four mimosoid legumes from Barro Colorado Island, Panama. **Systematic and Applied Microbiology**, Amsterdam, v. 28, n. 1, p. 57-65, Jan. 2005.

BARRET, C. F.; PARKER, M. A. Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. In Costa Rica. **Applied and Environmental Microbiology**, Washington, v. 72, n. 2, p. 1198-1206, Feb. 2006.

BERG, G.; EBERL, L.; HARTMANN, A. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. **Environmental Microbiology**, Washington, v. 7, n. 11, p. 1673-1685, Nov. 2005.

BEVIVINO, A.; DALMASTRI, C.; TABACCHIONI, S.; CHIARINI, L.; BELLI, M. L.; PIANA, S.; MATERAZZO, A.; VANDAMME, P.; MANNO, G. *Burkholderia cepacia* Complex Bacteria from Clinical and Environmental Sources in Italy: Genomovar Status and Distribution of Traits Related to Virulence and Transmissibility. **Journal of Clinical Microbiology**, Washington, v.40, n.3, p.846-851, Mar. 2002.

BEVIVINO, A.; TABACCHIONI, S.; CHIARINI, L.; CARUSI, M. V.; GALLO, M. D.; VISCA, P. Phenotypic comparison between rhizosphere and clinical isolates of *Burkholderia cepacia*. **Microbiology**, Spencers Wood, v. 140, n. 5, p. 1069-1077, May 1994.

BEVIVINO, A.; SARROCCO, S.; DALMASTRI, C.; TABACCHIONI, S.; CANTALE, C.; CHIARINI, L. Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia*: effect of seed treatment on disease suppression and growth promotion of maize. **FEMS Microbiology Ecology,** Amsterdam, v. 27, n. 3, p. 225-237, Dec. 1998.

BOWERS, J.; PARKE, J. Epidemiology of *Pythium* damping-off and *Aphanomyces* root rot of peas after seed treatment with bacterial agents for biological control. **Phytopathology,** Saint Paul, v. 83, n. 12, p. 1466-1473, Dec. 1993.

BRÄMER, C. O.; VANDAMME, P.; da SILVA, L. F.; GOMEZ, J. G. C.; STEINBÜCHEL, A. *Burkholderia sacchari* sp. nov., a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 51, n. 5, p. 1709-1713, Sept. 2001.

BRETT, P. J.; DESHAZER, D.; WOODS, D. E. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 48, n. 1, p. 317-320, Jan. 1998.

BURKHEAD, K. D.; SCHISLER, D. A.; SLININGER, P. J. Pyrrolnitrin Production by Biological Control Agent *Pseudomonas cepacia* B37w in Culture and in Colonized Wounds of Potatoes. **Applied and Environmental Microbiology**, Washington, v. 60, n. 6, p. 2031-2039, June 1994.

CABALLERO-MELLADO, J.; MARTÍNEZ-AGUILAR, L.; PAREDES-VALDEZ, G.; ESTRADA-DE LOS SANTOS, P. *Burkholderia unamae* sp. nov., an N_2-fixing rhizospheric and endophytic species. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 54, n. 4, p. 1165-1172, July 2004.

CABALLERO-MELLADO,J.; ONOFRE-LEMUS, J.; ESTRADA-DE LOS SANTOS, P.; MARTINEZ-AGUILAR, L. The Tomato Rhizosphere, an Environment Rich in Nitrogen-Fixing *Burkholderia* Species with Capabilities of Interest for Agriculture and Bioremediation. **Applied and Environmental Microbiology**, Washington, v. 73, n. 16, p. 5308-5319, Aug. 2007.

CAIN, C. C.; HENRY, A. T.; WALDO III, R.; CASIDA JR.; L. J.; FALKINHAM III, J. O. Identification and Characteristics of a Novel *Burkholderia* Strain with Broad-Spectrum Antimicrobial Activity. **Applied and Environmental Microbiology**, Washington, v. 66, n. 9, p. 4139-4141, Sept. 2000.

CHEN, W. X.; YAN, G. H.; LI, J. L. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 38, n. 4, p.392-397, Apr. 1988.

CHEN, W. M.; DE FARIA, S. M.; CHOU, J. H.; JAMES, E. K.; ELLIOTT, G. N.; SPRENT, J. I.; BONTEMPS, C.; YOUNG, J. P. W.; VANDAMME, P. *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia*. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 58, n. 9, p. 2174-2179, Sept. 2008a.

CHEN, W. M.; DE FARIA, S. M.; JAMES, E. K.; ELLIOTT, G. N.; LIN, K. Y.; CHOU, J. H.; SHEU, S. Y.; CNOCKAERT, M.; SPRENT, J. I.; VANDAMME, P. *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 57, n. 5, p. 1055-1059, May 2007.

CHEN, W. M.; JAMES, E. K.; COENYE, T.; CHOU, J. H.; BARRIOS, E.; DE FARIA, S. M.; ELLIOTT, G. N.; SHEU, S. Y.; SPRENT, J. I.; VANDAMME, P. *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 56, n. 8, p. 1847-1851, Aug. 2006.

CHEN, W. M.; JAMES, E. K.; PRESCOTT, A. R.; KIERANS, M.; SPRENT, J. I. Nodulation of *Mimosa* spp. by the β-proteobacterium *Ralstonia taiwanensis*. **Molecul**ar **Plant-Microbe Interactions**, Saint Paul, v. 16, n. 12, p. 1051-1061, 2003.

CHEN, W. X.; LAEVENS, S.; LEE, T. M.; COENYE, T.; DE VOS, P.; MERGEAY, M.; VANDAMME, P. *Rasltonia taiwanensis* sp. nov. isolated from nodules of *Mimosa* species and sputum of a cystic fibrosis patient. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 51, n. 5, p. 1729-1735, Sept. 2001.

CHEN, W-M.; WU, C.H.; JAMES, E. K.; CHANG, J-S. Metal biosorption capability of *Cupriavidus taiwanensis* and its effects on heavy metal removal by nodulated *Mimosa pudica*. **Journal of Hazardous Materials**, Amsterdam, v. 151, n. 2-3, p. 364-371, Mar. 2008b.

COENYE, T.; FALSENT, E.; VANCANNEYT, M.; HOSTE, B.; GOVANT, J. R. W.; KERSTERS, K. and VANDAMME, P. Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. **International Journal of Systematic Bacteriology,** Spencers Wood, v. 49, n. 2, p. 405-413, Apr. 1999.

COENYE, T.; GORIS, J.; VOS, P.; VANDAMME, P.; LIPUMA, J. J. Classification of *Ralstonia pickettii*-like isolates from the environment and various clinical samples as *Ralstonia insidiosa* sp. nov. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 53, n. 4, p. 1075-1080, July 2003a.

COENYE, T.; HENRY, D.; SPEERT, D. P.; VANDAMME, P. *Burkholderia phenoliruptrix* sp. nov., to accommodate the 2,4,5-trichlorophenoxyacetic acid and halophenol-degrading strain AC1100. **Systematic and Applied Microbiology**, Amsterdam, v. 27, n. 6, p. 623-627, Nov. 2004

COENYE, T.; LAEVENS, S.; WILLEMS, A.; OHLEN, M.; HANNANT, W.; GOVAN, J. R. W.; GILLIS, M.; FALSEN, E.; VANDAMME, P. *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 51, n. 3, p. 1099-1107, May 2001a.

COENYE, T.; MAHENTHIRALINGAM, E.; HENRY, D.; LIPUMA, J. J.; LAEVENS, S.; GILLIS, M.; SPEERT, D. P.; VANDAMME, P. *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 51, n. 4, p. 1481-1490, July 2001b.

COENYE, T.; VANDAMME, P. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. **Environmental Microbiology**, Washington, v.5, n.9, p.719-729, Sept. 2003.

COENYE, T.; VANDAMME, P.; GOVAN, J. R.; LIPUMA, J. J. Taxonomy and identification of the *Burkholderia cepacia* complex. **Journal of Clinical Microbiology**, Washington, v. 39, n. 10, p. 3427-3436, Oct. 2001c.

COENYE, T.; VANDAMME, P.; LIPUMA, J. J. *Ralstonia respiraculi* sp. nov., isolated from the respiratory tract of cystic fibrosis patients. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 53, n. 5, p. 1339-1342, Sept. 2003b.

COMPANT, S.; DUFFY, B.; NOWAK, J.; CLEMENT, C.; BARKA, E. A. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. **Applied and Environmental Microbiology**, Washington, v. 71, n. 9, p. 4951-4959, Sept. 2005.

COMPANT, S.; NOWAK, J.; COENYE, T.; CLEMENT, C.; BARKA, E. A. Diversity and occurrence of *Burkholderia* spp. in the natural Environment. **FEMS Microbiology Reviews**, v.32, n.4, p.607-626, Apr. 2008.

DANCE, D. A. B. Melioidosis. **Reviews in Medical Microbiology**, London, v. 1, n. 1, p. 143-150, Aug. 1990.

DE BAERE, T.; STEYAERT, S.; WAUTERS, G.; DE VOS, P.; GORIS, J.; COENYE, T.; SUYAMA, T.; VERSCHRAEGEN, G.; VANEECHOUTTE, M. Classification of *Ralstonia pickettii* biovar 3/'thomasii' strains (Pickett 1994) and of new isolates related to nosocomial recurrent meningitis as *Ralstonia mannitolytica* sp. nov. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 51, n. 2, p. 547-558, Mar. 2001.

DE LAJUDIE, P.; WILLEMS, A.; NICK, G.; MOREIRA, F.; MOLOUBA, F.; HOSTE, B.; TORCK, U.; NEYRA, M.; COLLINS, M. D.; LINDSTRÖM, K.; DREYFUS, B.; GILLIS, M. Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 48, n. 2, p. 369-382, Apr. 1998.

DON, R. H.; PEMBERTON, J. M. Properties of Six Pesticide Degradation Plasmids Isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. **Jounal of Bacteriology**, Washington, v. 145, n. 2, p. 681-686, Feb. 1981.

DREYFUS, B.; GARCIA, J. L.; GILLIS, M. Characterization of *Azorhizobium caulinodulans* gen. nov. sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 38, n. 1, p. 89-98, Jan. 1983.

EL-BANNA, N.; WINKELMANN, G. Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against streptomycetes. **Journal of Applied Microbiology**, Oxford, v. 85, n. 1, p. 69-78, July 1998.

ELLIOT, G. N.; CHEN, W. M.; CHOU, J. H.; WANG, H. C.; SHEU, S. Y.; PERIN, L.; REIS, V. M.; MOULIN, L.; SIMON, M. F.; BONTEMPS, C.; SUTHERLAND, J. M.; BESSI, R.; DE FARIA, S. M.; TRINICK, M. J.; PRESCOTT; A. R.; SPRENT, J. I.; JAMES, E. K. *Burkholderia phymatum* is a highly effective nitrogenfixing symbiont of *Mimosa* spp. and fixes nitrogen *ex planta*. **New Phytologist,** Cambridge, v. 173, n. 1, p. 168-180, Oct. 2007a.

ELLIOTT, G. N.; CHEN, W.G.; BONTEMPS, C.; CHOU, J-H. ; YOUNG, J. P. W.; SPRENT, J. I. JAMES, E. K. Nodulation of Cyclopia spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum*. **Annals of Botany,** Exeter, v. 100, n. 7, p. 1403-1411, Sept. 2007b.

EPSTEIN, E. **Nutrição mineral das plantas**: princípios e perspectivas. Rio de Janeiro: Livros Técnicos e Científicos, 1975. 341p.

ESTRADA-DE LOS SANTOS, P.; BUSTILIOS-CRISTALES, R.; CABALLERO-MELLADO, J. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. **Applied and Environmental Microbiology**, Washington, v. 67, n. 6, p. 2790-2798, June 2001.

EUZÉBY, J. S. **List of prokaryotic names with standing in nomenclature - Genus *Burkholderia*.** Toulouse: École Nationale Vétérinaire de Toulouse, [2009]. Available in: <http://www.bacterio.cict.fr/b/burkholderia. html>. Access: 18 Apr. 2009.

FLORENTINO, L. A.; GUIMARAES, A. P.; RUFINI, M.; SILVA, K.; MOREIRA, F. M. S. *Sesbania virgata* stimulates the occurrence of its microsymbiont in soils but does not inhibit microsymbionts of other species. **Scientia Agricola**, Piracicaba, v. 66, n. 5, p. 667-676, Sept. 2009.

FRIES, M. R.; FORNEY, L. J.; TIEDJE, J. M. Phenol- and toluene-degrading microbial populations from an aquifer in which successful trichloroethene cometabolism occurred. **Applied and Environmental Microbiology**, Washington, v. 63, n. 4, p. 1523-1530, Apr. 1997.

GARAU, G.; YATES, R. J.; DEIANA, P.; HOWIESON, J. G. Novel strains of nodulating *Burkholderia* have a role in nitrogen fixation with papilionoid herbaceous legumes adapted to acid, infertile soils. **Soil Biology and Biochemistry,** Elmsford, v. 41, n. 1, p. 125-134, Jan. 2009.

GAUNT, M. W.; TURNER, S. L.; RIGOTTIER-GOIS, L.; LLOYD-MACGILP, S. A.; YOUNG, J. P. W**.** Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 51, n. 6, p. 2037-2048, June 2001.

GILLIS, M.; VAN, T. V.; BARDIN, R.; GOOR, M.; HEBBAR, P.; WILLEMS, A.; SEGERS, P.; KERSTERS, K.; HEULIN, T.; FERNANDEZ, M. P. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N2-fixing isolates from rice in Vietnam. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 45, n. 2, p. 274-289, Apr. 1995.

GLASS, M. B.; STEIGERWALT, A. G.; JORDAN, J. G.; WILKINS, P. P.; GEE, J. E. *Burkholderia oklahomensis* sp. nov., a *Burkholderia pseudomallei*-like species formerly known as the Oklahoma strain of *Pseudomonas pseudomallei*. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 56, n. 9, p. 2171-2176, Sept. 2006.

GLICK, B. R.; PENROSE, D. M.; LI, J. A model for the lowering of plant ethylene concentrations by plant growth promoting rhizobacteria. **Journal of Theoretical Biology**, London, v. 190, n. 1, p. 63-68, Jan. 1998.

GORIS, J.; DE VOS, P.; CABALLERO-MELLADO, J.; PARK, J.; FALSEN, E.; QUENSEN III, J. F.; TIEDJE, J. M.; VANDAMME, P. Classification of the biphenyl- and polychlorinated biphenyl-degrading strain LB400$^{(T)}$ and relatives as *Burkholderia xenovorans* sp. nov. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 54, n. 5, p. 1677-1681, Sept. 2004.

GORIS, J.; DE VOS, P.; COENYE, T.; HOSTE, B.; JANSSENS, D.; BRIM, H.; DIELS, L.; MERGEAY, M.; KERSTERS, K.; VANDAMME, P. Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov., and *Ralstonia basilensis* Steinle et al. 1998 emend. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 51, n. 5, p. 1773-1782, Sept. 2001.

GORIS, J.; DEJONGHE, W.; FALSEN, E.; DE CLERCK, E.; GEERAERTS, B.; WILLEMS, A.; TOP, E. M.; VANDAMME, P.; DE VOS, P. Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- and B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. **Systematic and Applied Microbiology**, Amsterdam, v. 25, n. 3, p. 340-352, Oct. 2002.

ISLES, A.; MACLUSKY, I.; COREY, M.; GOLD, R.; PROBER, C.; FLEMING, P.; LEVISON, H. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. **Journal of Pediatrics**, New York, v. 104, n. 2, p. 206-210, Feb. 1984.

JARVIS, B. D. W. ; VAN BERKUM, P.; CHEN, W. X.; NOUR, S. M. ; FERNANDEZ, M. P.; CLEYET-MAREL, J. C.; GILLIS, M. Transfer of *Rhizobium loti, Rhizobium huakuii, Rhizobium ciceri, Rhizobium mediterraneum* and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 47, n. 3, p. 895-898, July 1997.

JORDAN, D. C. Rhizobiaceae Conn 1938. In: KRIEG, N. R.; HOLT, J. D. BERGEYS'S. **Manual of systematic bacteriology**. London: Willians and Wilkins, 1984. p. 234-244.

KARLIN, S.; WEINSTOCK, G. M.; BRENDEL, V. Bacterial classifications derived from RecA protein sequence Comparisons. **Journal of Bacteriology**, Washington, v. 177, n. 23, p. 6881-6893, Dec. 1995.

KIM, H. B.; PARK, M. J.; YANG, H. C.; AN, D. S.; JIN, H. Z.; YANG, D. C. *Burkholderia ginsengisoli* sp. nov., a beta -glucosidase-producing bacterium isolated from soil of a ginseng field. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 56, n. 11, p. 2529-2533, Sept. 2006.

KING, E. B.; PARKET J. L. Population density of the biocontrol agent *Burkholderia cepacia* AMMDRl on four pea cultivars. **Soil Biology and Biochemistry**, Elmsford, v. 28, n. 3, p. 307-312, Mar. 1996.

KLOEPPER, J. W.; SCHROTH, M. N. Plant growth-promoting rhizobacteria on radishes. In: INTERNATIONAL CONFERENCE ON PLANT PATHOGENIC BACTERIA, 4., 1978, Angers. **Proceedings…** Angers: Station de Pathologie Vegetale et Phytobacteriologie, 1978. v. 2, p. 879-882.

LEE, C. H.; KEMPF, H. J.; LIM, Y.; CHO, Y. L. Biocontrol Activity of *Pseudomonas cepacia* AF2001 and Anthelmintic Activity of Its Novel Metabolite, Cepacidine A. **Wourld Journal of Microbiology and Biotechnology**, Seoul, v. 10, n. 4, p.568-571, July 2000.

LEE, C. H.; KIM, S.; HYUN, B.; SUH, J. W.; YON, C.; KIM, C.; LIM, Y.; KIM, C. Cepacidine A: a novel antifungal antibiotic produced by *Pseudomonas cepacia*. I. Taxonomy, isolation and biological activity. **Journal of Antibiotics**, Tokyo, v. 47, n. 12, p. 1402-1405, Dec. 1994.

LESSIE, T. G.; HENDRICKSON, W.; MANNING, B. D.; DEVEREUX, R. Genomic complexity and plasticity of Burkholderia cepacia. **FEMS Microbiology Letters,** Amsterdam, v. 144, n. 2/3, p. 117-128, Nov. 1996.

LIM, J. H.; BAEK, S. H.; LEE, S. T. *Burkholderia sediminicola* sp. nov., isolated from freshwater sediment. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 8, n. 3, p. 565-569, Mar. 2008.

LIM, Y. W.; BAIK, K. S.; HAN, S. K.; KIM, S. B.; BAE, K. S. *Burkholderia sordidicola* sp. nov., isolated from the white-rot fungus *Phanerochaete sordida*. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 53, n. 5, p. 1631-1636, Sept.2003.

LIMA, A. S.; NOBREGA, R. S. A.; BARBERI, A.; SILVA, K.; FERREIRA, D. F.; MOREIRA, F. M. S. Nitrogen-fixing bacteria communities occuring in soils under different uses in the Western Amazon Region as indicated by nodulation of siratro (*Macroptilium atropurpureum*). **Plant and Soil**, The Hague, v. 319, n. 1/2, p. 127-145, Jan. 2009.

LIN, T. F.; HUANG, H. I.; SHEN, F. T.; YOUNG, C. C. The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by *Burkholderia cepacia* CC-Al74. **Bioresource Technology,** Essex, v. 97, n. 7, p. 957-960, May 2006.

LIPUMA, J. J. *Burkholderia cepacia*. Management issues and new insights. **Clinics in Chest Medicine**, Philadelphia, v. 19, n. 3, p. 473-486, Sept. 1998.

LIPUMA, J. J.; SPILKER, T.; COENYE, T.; GONZALEZ, C. F. An epidemic *Burkholderia cepacia* complex strain identified in soil. **The Lancet**, London, v. 359, n. 9322, p. 2002-2003, June 2002.

LIPUMA, J. J.; SPILKER, T.; GILL, L. H.; CAMPBELL, P. W.; LIU, L., MAHENTHIRALINGAM, E. Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. **American Journal of Respiratory and Critical Care Medicine,** New York, v. 164, n. 1, p. 92-96, July 2001.

LONETTO, M.; GRIBSKOV, M.; GROSS, C. A. The sigma 70 family: sequence conservation and evolutionary relationships. **Journal of Bacteriology**, Washington, v. 174, n. 12, p. 3843-3849, June 1992.

MAEDA, Y.; SHINOHARA, H.; KIBA, A.; OHNISHI, K.; FURUYA, N.; KAWAMURA, Y.; EZAKI, T.; VANDAMME, P.; TSUSHIMA, S.; HIKICHI, Y. Phylogenetic study and multiplex PCR-based detection of *Burkholderia plantarii*, *Burkholderia glumae* and *Burkholderia gladioli* using *gyrB* and *rpoD* sequences. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 56, n. 5, p. 1031-1038, May 2006.

MAHENTHIRALINGAM, E.; BALDWIN, A; DOWSON, C. G. *Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology. **Journal of Applied Microbiology**, Oxford, v. 104, n. 6, p. 1539-1551, June 2008.

MAHENTHIRALINGAM, E.; BISCHOF, J.; BYRNE, S. K.; RADOMSKI, C.; DAVIES, J. E.; AV-GAY, Y.; VANDAMME, P. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia cepacea* genovars I and III. **Journal of Clinical Microbiology,** Washington, v. 38, n. 9, p. 3165-3173, Sept. 2000.

MAHENTHIRALINGAM, E.; SIMPSON, D. A.; SPEERT, D. P. Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. **Journal of Clinical Microbiology**, Washington, v. 35, n. 4, p. 808-816, Apr. 1997.

MAHENTHIRALINGAM, E.; URBAN, T. A.; GOLDBERG, J. B. The Multifarious, Multireplicon *Burkholderia Cepacia* complex. **Nature Reviews**, London, v. 3, n. 2, p.144-156, Feb. 2005.

MAHENTHIRALINGAM, E.; VANDAMME, P.; CAMPBELL, M. E.; HENRY, D. A.; GRAVELLE, A. M.; WONG, L. T.; DAVIDSON, A. G.; WILCOX, P. G.; NAKIELNA, B.; SPEERT, D. P. Infection with *Burkholderia cepacia* complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace *Burkholderia multivorans*. **Clinical Infectious Diseases**, Boston, v. 33, n. 9, p. 1469-75, Nov. 2001.

MAIDEN, M. C. J. Multilocus sequence typing of Bacteria. **Annual Review of Microbiology**, Palo Alto, v. 60, p. 561-588, Oct. 2006.

MAIDEN, M. C.; BYGRAVES, J. A.; FEIL, E.; MORELLI, G.; RUSSELL, J. E.; URWIN, R.; ZHANG, Q.; ZHOU, J.; ZURTH, K.; CAUGANT, D. A.; FEAVERS, I. M.; ACHTMAN, M.; SPRATT, B. G. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. **Proceedings of National Academy of Sciences**, Washington, v. 95, n. 6, p. 3140-3145, Mar. 1998.

MAKKAR, N. S.; CASIDA, L. E. JR. *Cupriavidus necator* gen. nov., sp. nov.: a nonobligate bacterial predator of bacteria in soil. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 37, n. 4, p. 323-326, Oct. 1987.

MARS, A. E.; HOUWING, J.; DOLFING, J.; JANSSEN, D. B. Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. **Applied and Environmental Microbiology**, Washington, v. 62, n. 3, p. 886-891, Mar. 1996.

MOON, S.S.; KANG, P. M.; PARK, K. S.; KIM, C. H. Plant growth promoting and fungicidal 4-quinolinones from Pseudomonas cepacia. **Phytochemistry**, New York, v. 42, n.2, p. 365 368, May 1996.

MOREIRA, F. M. S.; SIQUEIRA, J.O. **Microbiologia e bioquímica do solo.** 2. ed. Lavras: UFLA, 2006. 729p.

MOULIN, L.; MUNIVE, A.; DREYFUS, B.; MASSON, C. B. Nodulation of legumes by menbers of the β-subclass of Proteobacteria. **Nature**, London, v. 411, p. 948-950, June 2001.

NELSON, M. J. K.; MONTGOMERY, S. O.; MAHAFFEY, W. R.; PRITCHARD, P. H. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. **Applied and Environmental Microbiology**. Washington, v. 53, n. 5, p. 949-954, May 1987.

NEUBAUER, H.; SPRAGUE, L. D.; ZACHARIA, R.; TOMASO, H.; AL DAHOUK, S.; WERNERY, R.; WERNERY, U.; SCHOLZ, H. C. Serodiagnosis of *Burkholderia mallei* infections in horses: state-of-the-art and perspectives. **Journal of veterinary medicine:** serie B**,** Infectious diseases and veterinary public health, Berlin, v. 52, n. 5, p. 201-205, June 2005.

O'SULLIVAN, B. P.; FREEDMAN, S. D. Cystic fi brosis. **The Lancet**, London, v. 373, n. 9358, p. 1891-1901, Feb. 2009.

PANDEY, P.; KANG, S. C.; MAHESHWARI , D. K. Isolation of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica***. Current Science**, Bangalore, v. 89, n. 1, p. 177-180, July 2005.

PARKE, J. L.; GURIAN-SHERMAN, D. Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. **Annual Review of Phytopathology**, Palo Alto, v. 39, n. 1, p. 225-258, Sept. 2001.

PARTIDA-MARTINEZ, L. P.; GROTH, I.; SCHMITT, I.; RICHTER, W.; ROTH, M.; HERTWECK, C. *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 57, n. 11, p. 2583-2590, Nov. 2007

PAYNE, G. W.; VANDAMME, P.; MORGAN, S. H.; LIPUMA, J. J.; COENYE, T.; WEIGHTMAN, A. J.; JONES, T. H.; MAHENTHIRALINGAM, E. Development of a *recA* gene-based identification approach for the entire *Burkholderia* genus. **Applied and Environmental Microbiology,** Washington, v. 71, n. 7, p. 3917-3927, July 2005.

PEIX, A.; MATEOS, P. F.; RODRIGUEZ-BARRUECO, C.; MARTINEZ-MOLINA, E.; VELAZQUEZ, E. Growth promotion of common bean (*Pasheolus vulgaris* L.) by a strain of *Burkholderia cepacia* under growth chamber conditions. **Soil Biology and Biochemistry**, Elmsford, v. 33, n. 14, p. 1927-1935, Nov. 2001.

PEREIRA, E. G. **Diversidade de rizóbios isolados de diferentes sistemas de uso da terra na Amazônia**. 2000. 93p. Tese (Doutorado) – Universidade Federal de Lavras, Lavras.

PERIN, L.; MARTÍNEZ-AGUILAR, L.; PAREDES-VALDEZ, G.; BALDANI, J. I.; ESTRADA-DE LOS SANTOS, P.; REIS, V. M.; CABALLERO-MELLADO, J. *Burkholderia silvatlantica* sp. nov., a diazotrophic bacterium associated with sugar cane and maize. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 56, n. 8, p. 1931-1937, Aug. 2006.

RADWAY, J. C.; SANTO-DOMINGO, J.W.; HAZEN, T.C.; WILDE, E.W. Evaluation of biodegradation potential of foam embedded *Burkholderia cepacia* G4. **Biotechnology Letters**, v. 20, n.7, p. 663-666, July 1998.

RAMETTE, A.; LIPUMA, J. J.; TIEDJE, J. M. Species abundance and diversity of *Burkholderia cepacia* complex in the environment. **Applied and Environmental Microbiology**, Washington, v. 71, n. 3, p. 1193-1201, Mar. 2005.

RAMSAY, B. A.; LOMALIZA, K.; CHAVARIE, C.; DUBE, B.; BATAILLE, P. Production of poly-(ρ-hydroxybutyric-co-3-hydroxyvaleric) acids. **Applied and Environmental Microbiology**, Washington, v. 56, n. 7, p. 2093-2098, July 1990.

RASOLOMAMPIANINA, R.; BAILLY, X.; FETIARISON, R.; RABEVOHITRA, R.; BÉNA, G.; RAMAROSON, L.; RAHERIMANDIMBY, M.; MOULIN, L.; DE LAJUDIE, P.; DREYFUS, B.; AVARRE, J.-C. Nitrogen-fixing nodules from rose wood legumes trees (*Dalbergia* spp.) endemic to Madagascar host seven different genera belonging to α- and ß-Proteobacteria. **Molecular Ecology,** Oxford, v. 14, n. 14, p. 4135-4146, Nov. 2005.

REIS, V. M.; ESTRADA-DE LOS SANTOS, P.; TENORIO-SALGADO, S.; VOGEL, J.; STOFFELS, M.; GUYON, S.; MAVINGUI; P.; BALDANI, V. L. D.; SCHMID, M.; BALDANI, J. I.; BALANDREAU, J.; HARTMANN, A.; CABALLERO-MELLADO, J. *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 54, n. 6, p. 2155-2162, Nov. 2004.

RIVAS, R.; VELÁZQUEZ, E.; WILLEMS, A.; VIZCAÍNO, N.; SUBBA-RAO, N. S.; MATEOS, P. F.; GILLIS, M.; DAZZO, F. B.; MARTÍNEZ-MOLINA, E. A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f) Druce. **Applied and Environmental Microbiology**, Washington, v. 68, n. 11, p. 5217-5222, Nov. 2002.

ROSSELLÓ-MORA, R.; AMANN, R. The species concept for prokaryotes. **FEMS Microbiology Reviews**, Amsterdam, v. 25, n. 1, p. 39-67, Jan. 2001.

SAHIN, N.; ISIK, K.; TAMER, A. U.; GOODFELLOW, M. Taxonomic position of ‘‘*Pseudomonas oxalaticus*’’ strain Ox1T (DSM 1105T) (Khambata and Bhat, 1953) and its description in the genus *Ralstonia* as *Ralstonia oxalatica* comb. nov**. Systematic and Applied Microbiology**, Amsterdam, v. 23, n. 2, p. 206-209, June 2000.

SAJJAN, U. S.; SUN, L.; GOLDSTEIN, R.; FORSTNER, J. F. Cable (Cbl) type II pili of cystic fibrosis-associated *Burkholderia (Pseudomonas) cepacia:* nucleotide sequence of the *cblA* major subunit pilin gene and novel morphology of the assembled appendage fibers. **Journal of Bacteriology**, Washington, v. 177, n. 4, p. 1030-1038, Feb. 1995.

SATO, Y.; NISHIHARA, H.; YOSHIDA, M.; WATANABE, M.; RONDAL, J. D.; CONCEPCION, R. N.; OHTA, H. *Cupriavidus pinatubonensis* sp. nov. and *Cupriavidus laharis* sp. nov., novel hydrogen-oxidizing, facultatively chemolithotrophic bacteria isolated from volcanic mudflow deposits from Mt. Pinatubo in the Philippines. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 56, n. 5, p. 973-978, May 2006.

SESSITSCH, A.; COENYE, T.; STURZ, A. V.; VANDAMME, P.; AIT BARKA, E.; SALLES, J. F.; VAN ELSAS, J. D.; FAURE, D.; REITER, B.; GLICK, B. R.; WANG-PRUSKI, G.; NOWAK, J. *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 55, n. 3, p. 1187-1192, May 2005.

SINGH, R. K.; MISHRA, R. P. M.; JAISWAL, H. K.; KUMAR, V.; PANDEY, S. P.; RAO, S. B.; ANNAPURNA, K. Isolation and identification of natural endophytic rhizobia from rice (*Oryza sativa* L.) through rDNA PCR-RFLP and sequence analysis. **Current Microbiology**, New York, v. 52, n. 5, p. 117-122, May 2006.

STEINLE, P.; STUCKI, G.; STETTLER, R.; HANSELMANN, K. W. Aerobic mineralization of 2,6-dichlorophenol by *Ralstonia* sp. strain RK1. **Applied and Environmental Microbiology**, Washington, v. 64, n. 7, p. 2566-2571, July 1998.

SY, A.; GIRAUD, E.; JOURAND, P.; GARCIA, N.; WILLEMS, A.; DE LAJUDIE, P.; PRIN, Y.; NEYRA, M.; GILLIS, M.; BOIVIN-MASSON, C.; DREYFUS, B. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. **Journal of Bacteriology**, Washington, v. 183, n. 1, p. 214-220. Jan. 2001.

TABACCHIONI, S.; BEVIVINO, A.; DALMASTRI, C.; CHIARINI, L. *Burkholderia cepacia* complex in the rhizosphere: a minireview. **Annals of Microbiology**, Milan, v. 52, n. 1/3, p.103-117, 2002.

TAYEB, L. A.; LEFEVRE, M.; PASSET, V., DIANCOURT, L.; BRISSE, S.; GRIMONT; P. A. D. Comparative phylogenies of *Burkholderia*, *Ralstonia*, *Comamonas*, *Brevundimonas* and related organisms derived from *rpoB*, *gyrB* and rrs gene sequences. **Research in Microbiology**, Paris, v. 159, n. 3, p. 169-177, Apr. 2008.

THOMAS, M. S. Iron acquisition mechanisms of the *Burkholderia cepacia* complex. **Biometals**, London, v. 20, n. 3/4, p. 431-452. June 2007.

TRÂN VAN, V.; BERGE, O.; NGO, K. E. S.; BALANDREAU, J.; HEULIN, T. Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. **Plant and Soil**, The Hague v. 218, n. 1/2, p. 273-284, Jan. 2000.

TRUJILLO, M. E.; WILLEMS, A.; ABRIL, A.; PLANCHUELO, A. M.; RIVAS, R.; LUDEÑA, D.; MATEOS, P. F.; MARTÍNEZ-MOLINA, E.; VELÁZQUEZ, E. Nodulation of *Lupinus albus* by strains of *Ochrobacterium lupine*. **Applied and Environmental Microbiology**, Washington, v. 71, n. 3, p. 1318-1327, Mar. 2005.

URAKAMI, T.; ITO-YOSHIDA, C.; ARAKI, H.; KIJIMA, T.; SUZUKI, K. I.; KOMAGATA, K. Transfer of *Pseudomonas plantarii* and *Pseudomonas glumae* to *Burkholderia* as *Burkholderia* spp. and description of *Burkholderia vandii* sp. nov. **International Journal of Systematic and Bacteriology**, Spencers Wood, v. 44, n. 2, p. 235-245, Apr. 1994.

VALVERDE, A.; DELVASTO, P.; PEIX, A.; VELÁZQUEZ, E.; SANTA-REGINA, I.; BALLESTER, A.; RODRÍGUEZ-BARRUECO, C.; GARCÍA-BALBOA, C.; IGUAL, J. M. *Burkholderia ferrariae* sp. nov., isolated from an iron ore in Brazil. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 56, n. 10, p. 2421-2425, Oct. 2006.

VANDAMME, P.; COENYE, T. Taxonomy of the genus *Cupriavidus*: a tale of lost and found. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 54, n. 6, p. 2285-2289, Nov. 2004.

VANDAMME, P.; GORIS, J.; CHEN, W. M.; DE VOS, P.; WILLEMS, A. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. **Systematic and Applied Microbiology**, Amsterdam, v. 25, n. 4, p. 507-512, Dec. 2002a.

VANDAMME, P.; GORIS, J.; COENYE, T.; HOSTE, B.; JANSSENS, D.; KERSTERS, K.; DE VOS, P.; FALSEN, E. Assignment of Centers for Disease Control group IVc-2 to the genus *Ralstonia* as *Ralstonia paucula* sp. nov. **International Journal of Systematic Bacteriology,** Spencers Wood, v. 49, n. 2, p.663-669, Apr. 1999.

VANDAMME, P.; GOVAN, J.; LIPUMA, J. Diversity and role of *Burkholderia* spp. In: COENYE, T.; VANDAMME, P (Eds.). ***Burkholderia*: molecular microbiology and genomics. Wymondham: Horizon Scientific, 2007b. p. 1-28.

VANDAMME, P.; HENRY, D.; COENYE, T.; NZULA, S.; VANCANNEYT, M.; LIPUMA, J. J.; SPEERT, D. P.; GOVAN, J. R.W.; MAHENTHIRALINGAM, E. *Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools. **FEMS Immunology and Medical Microbiology,** Amsterdam, v. 33, n. 2, p. 143-149, June 2002b

VANDAMME, P.; HOLMES, B.; COENYE, T.; GORIS, J.; MAHENTHIRALINGAM, E.; LIPUMA, J. J.; GOVAN, J. R. W. *Burkholderia cenocepacia* sp. nov. a new twist to an old story. **Research in Microbiology**, Paris, v. 154, n. 2, p. 91-96, Mar. 2003.

VANDAMME, P.; HOLMES, B.; VANCANNEYT, M.; COENYE, T.; HOSTE, B.; COOPMAN, R.; REVETS, H.; LAUWERS, S.; GILLIS, M.; KERSTERS, K.; GOVAN, J. R. W. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 47, n. 4, p. 1188-1200, Oct. 1997.

VANDAMME, P.; MAHENTHIRALINGAM, E.; HOLMES, B.; COENYE, T.; HOSTE, B.; DE VOS, P.; HENRY, D.; SPEERT, D. P. Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV). **Journal of Clinical Microbiology**, Washington, v. 38, n. 3, p. 1042-1047, Mar. 2000.

VANDAMME, P.; OPELT, K.; KNÖCHEL, N.; BERG, C.; SCHÖNMANN, S.; DE BRANDT, E.; EBERL, L.; FALSEN, E.; BERG, G. *Burkholderia bryophila* sp. nov. and *Burkholderia megapolitana* sp. nov., moss-associated species with antifungal and plant-growth-promoting properties. **International Journal of Systematic and Evolutionary Microbiology**, Reading, v. 57, n. 10, p. 2228-2235, Oct. 2007a.

VANEECHOUTTE, M.; KAMPFER, P.; DE BAERE, T.; FALSEN, E.; VERSCHRAEGEN, G. *Wautersia* gen. nov., a novel genus accommodating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of *Ralstonia* [*Pseudomonas*] syzygii (Roberts et al., 1990) comb. nov. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 54, n. 2, p. 317-327, Mar. 2004.

VANLAERE , E.; LIPUMA, J .J.; BALDWIN, A.; HENRY, D.; DE BRANDT , E.; MAHENTHIRALINGAM, E.; SPEERT, D.; DOWSON, C.; VANDAMME, P. *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. nov., novel species within the *Burkholderia cepacia* complex. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v.58, n.7, p.1580-1590, July 2008a.

VANLAERE, E.; BALDWIN, A.; GEVERS, D.; HENRY, D.; DE BRANDT, E.; LIPUMA, J. J.; MAHENTHIRALINGAM, E.; SPEERT, D. P.; DOWSON, C.; VANDAMME, P. Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 59, n. 1, p. 102-111, Jan. 2009.

VANLAERE, E.; VAN DER MEER, J. R.; FALSEN, E.; SALLES, J. F.; DE BRANDT, E.; VANDAMME, P. *Burkholderia sartisoli* sp. nov., isolated from a polycyclic aromatic hydrocarbon-contaminated soil. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 58, n. 2, p.420-423, Feb. 2008b.

VERMA, S. C.; CHOWDHURY, S. P.; TRIPATHI, A. K. Phylogeny based on 16S rDNA and *nif*H sequences of *Rasltonia taiwanensis* strains isolated from nitrogen-fixing nodules of *Mimosa pudica*, in India. **Canadian Journal of Microbiology**, Ottawa, v. 50, n. 5, p. 313-322, May 2004.

VERMIS, K.; COENYE, T. ; MAHENTHILARINGAM, E.; NELIS, E. J.; VANDAMME, P. Evaluation of species-specific recA-based PCR tests for genomovar level identification within the *Burkholderia cepacia* complex. **Journal of Medical Microbiology**, Washington, v. 51, n. 11, p. 937-940, Nov. 2002.

VERMIS, K.; COENYE, T.; LIPUMA, J. J.; MAHENTHIRALINGAM, E.; NELIS, H. J.; VANDAMME, P. Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 54, n. 2, p. 689-691, Mar. 2004.

VIAL, L.; GROLEAU, M.C.; DEKIMPE, V.; DÉZIEL, E. *Burkholderia* diversity and versatility: an inventory of the extracellular products. **Journal of Microbiology and Biotechnology,** Seoul, v. 17, n. 9, p. 1407-1429, Sept. 2007.

VIALLARD, V.; POIRIER, I.; COURNOYER, B.; HAURAT, J.; WIEBKIN, S.; OPHEL-KELLER, K.; BALANDREAU, J. *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas*] *phenazinium*, [*Pseudomonas*] *pyrrocinia* and [*Pseudomonas*] *glathei* as *Burkholderia*. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 48, n. 2, p. 549-563, Apr.1998.

WATT, P. M.; HICKSON, I. D. Structure and function of type II DNA topoisomerases. **Biochemistry Journal**, Oxford, v. 303, n. 3, p. 681-695, Nov. 1994.

WHITE, N. J. Melioidosis. **The Lancet,** London, v. 361, n. 9370, p. 1715-1722, 2003.

WHITLOCK, G. C.; ESTES, D. M.; TORRES, A. G. Glanders: off to the races with *Burkholderia mallei*. **FEMS Microbiology Letters**, Amsterdam, n. 2, v. 277, p. 115-122, Dec. 2007.

WOESE, C. Prokaryote Systematics: the evolution of a science. In: The prokaryotes. In: BALOWS, A. (Ed.). **The prokaryotes**: a handbook on the biology of bacteria, ecophysiology, isolation, identification, applications. 2. ed. New York: Springer-Verlag, 1991. Chapter 1, v. 1, p. 3-18.

YABUUCHI, E.; KAWAMURA, Y.; EZAKI, T.; IKEDO, M.; DEJSIRILERT, S.; FUJIWARA, N.; NAKA, T.; KOBAYASHI, K. *Burkholderia uboniae* sp. nov., L-arabinose-assimilating but different from *Burkholderia thailandensis* and *Burkholderia vietnamiensis*. **Microbiology and Immunology**, Tokyo, v. 44, n. 4, p. 307-317, Apr. 2000.

YABUUCHI, E.; KOSAKO, Y.; OYAIZU, H.; YANO, I.; HOTTA, H.; HASHIMOTO, Y.; EZAKI, T.; ARAKAWA, M. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. **Microbiology and Immunology**, Tokyo, v. 36, n. 12, p. 1251-1275, Dec. 1992.

YABUUCHI, E.; KOSAKO, Y.; YANO, I.; HOTTA, H.; NISHIUCHI, Y. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. **Microbiology and Immunology**, Tokyo, v. 39, n. 11, p. 897-904, Nov. 1995.

YANG, H. C.; IM, W. T.; KIM, K. K.; AN, D. S.; LEE, S. T. *Burkholderia terrae* sp. nov., isolated from a forest soil. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 56, n. 2, p. 453-457, Feb. 2006.

YOO, S. H.; KIM, B. Y.; WEON, H. Y.; KWON, S. W.; GO, S. J.; STACKEBRANDT, E. *Burkholderia soli* sp. nov., isolated from soil cultivated with Korean ginseng. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 57, n. 1, p. 122-125, Jan. 2007.

ZHANG, H.; HANADA, S.; SHIGEMATSU, T.; SHIBUYA, K.; KAMAGATA, Y.; KANAGAWA, T.; KURANE, R. *Burkholderia kururiensis* sp. nov., a trichloroethylene (TCE)-degrading bacterium isolated from an aquifer polluted with TCE. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 50, n. 2, p. 743-749, Mar. 2000.

CHAPTER 2

DIAZOTROPHIC *Burkholderia* spp. (*B. fungorum*, *B. contaminans* and *B. lata*) ISOLATED FROM THE AMAZON REGION EXHIBIT PHENOTYPICAL, FUNCTIONAL AND GENETIC DIVERSITY

1 RESUMO

O gênero *Burkholderia* é conhecido por sua grande diversidade, inluindo espécies de vários habitats. Neste trabalho, isolados do gênero *Burkholderia* foram obtidos a partir de nódulos de espécies de leguminosas de solos da Amazônia. Assim, o objetivo foi avaliar os isolados de *Burkholderia* obtidos de solos da Amazônia, quanto às características fenotípicas e funcionais que podem ser utilizadas para promover o crescimento vegetal e determinar a posição filogenética desses isolados. Quarenta e quatro isolados foram obtidos utilizando-se siratro (*Macroptilium atropurpureum*) como planta isca e dois isolados (UFLA02-27 e UFLA02-28) foram obtidos utilizando feijão (*Phaseolus vulgaris*). Outros dois isolados foram obtidos a partir de dois nódulos de outras espécies de leguminosas coletados no campo, INPA89A a partir de *Indigofera suffruticosa* e INPA42B a partir de *Pithecellobium* sp. As carcterísticas avaliadas foram: carcterização cultural em meio 79, assimilação de diferentes fontes de carbono, atividade enzimática, solubilização de fosfatos, atividade da nitrogenase e atividade antifúngica contra *Fusarium oxysporium* f. sp. *phaseoli*. Perfil de proteínas totais, sequenciamento dos genes 16S rDNA, *gyrB*, *recA* e análise de sequência por multilocus (MLST) foram utilizados para identificar esses isolados. Os isolados apresentaram diferentes características culturais e bioquímicas, dependendo da espécie de leguminosas a partir da qual eles foram obtidos. Somente o isolado INPA89A não solubilizou fosfato de cálcio e não apresentou atividade da nitrogenase em meio de cultura livre de nitrogênio. O isolado UFLA02-27 e as estirpes de *B. cepacia* LMG1222^T e *B. vietnamiensis* LMG10929^T apresentaram atividade antifúngica contra *Fusarium oxyporium* f. sp. *phaseoli*. Os isolados obtidos a partir de nódulos de siratro foram identificados como pertencentes a *B. fungorum*; UFLA02-27 e UFLA02-28, isolados a partir de nódulos de feijão, foram identificados como *B. contaminans*; INPA89A, isolado de *Indigofera suffruticosa*, foi identificado como *B. caribensis* e INPA42B, isolado de *Pithecellobium* sp., foi identificado como *B. lata*.

Keywords: *Burkholderia fungorum*, complexo *Burkholderia cepacia*, β-proteobactéria, 16S rDNA, *gyr*B; MLST.

2 ABSTRACT

The genus *Burkholderia* is known for its large diversity, including species from numerous habitats. In this work, isolates were obtained from root nodules of legume species from Amazonian soils. The aims of this work were to evaluate *Burkholderia* isolates obtained from Amazonian soils for phenotypic and functional characteristics that can be used to promote plant growth and to determine the phylogenetic position of these isolates. Forty-four isolates were obtained using siratro (*Macroptilium atropurpureum*) as the trap species, and two isolates (UFLA02-27 and UFLA02-28) were obtained using common beans (*Phaseolus vulgaris*). Two other isolates were obtained from two field nodules of other plant species, INPA89A from *Indigofera suffruticosa* and INPA42B from *Pithecellobium* sp. The evaluated characteristics were the following: colony characterisation on "79" medium, assimilation of different carbon sources, enzymatic activities, solubilisation of phosphates, nitrogenase activity and antifungal activity against *Fusarium oxysporium* f. sp. *phaseoli*. Total protein profiles, 16S rDNA, *gyrB*, and *recA* gene sequencing and multilocus sequence typing were used to identify these isolates. The isolates presented different cultural and biochemical characteristics depending on the legume species from which they were isolated. Only the isolate INPA89A was not able to solubilise calcium phosphate and did not present nitrogenase activity under free-living conditions. The isolate UFLA02-27 and the strains of *B. cepacia* LMG1222^T and *B. vietnamiensis* LMG10929^T had antifungal activity against *Fusarium oxyporium* f. sp. *phaseoli*. The isolates obtained from siratro nodules were identified as belonging to *B. fungorum*; UFLA02-27 and UFLA02-28, isolated from common bean plants, were identified as *B. contaminans*; INPA89A, isolated from *Indigofera suffruticosa,* was identified as *B. caribensis*; and INPA42B, isolated from *Pithecellobium* sp., was identified as *B. lata.*

Keywords: *Burkholderia fungorum*; *Burkholderia cepacia* complex; β-Proteobacteria; 16S rDNA; *gyrB*; MLST.

3 INTRODUCTION

Nitrogen fixation is carried out by many species of prokaryotes that are referred to as diazotrophs. Certain diazotrophs can develop symbiotic relationships with legume species by forming structures known as nodules. Thus, these microorganisms can be called nitrogen-fixing Leguminosae-nodulating bacteria (NFLNB). Currently, there are 11 genera able to nodulate and fix N_2 in symbiosis with Leguminosae species, and these genera include both α- and β-Proteobacteria.

The genus *Burkholderia* of the class β-Proteobacteria was created to accommodate seven species from the *Pseudomonas* genus (Yabuuchi et al., 1992). Currently, 56 species of *Burkholderia* have been described (Euzéby, 2009), and new species are frequently discovered. This genus has a great diversity of species, and isolates have come from humans, animals and environmental sources (Coenye &Vandamme, 2003).

It was only recently reported that some strains of *Burkholderia* are able to nodulate species of Leguminosae (Moulin et al., 2001). Currently, six species are described as NFLNBs: *B. tuberum, B. phymatum* and *B. caribensis* (Vandamme et al., 2002); *B. mimosarum* (Chen et al., 2006); *B. nodosa* (Chen et al., 2007); and *B. sabiae* (Chent et al., 2008). There are also reports of strains of the *B. cepacia* complex (Bcc) isolated from root nodules of legumes (Rasolomampianina et al., 2005). The Bcc is a group of phenotypically similar species or genomovars that have been isolated from clinical specimens of human origin, particularly from cystic fibrosis patients (Coenye & Vandamme, 2003).

In recent years, there has been considerable interest in using *Burkholderia* as plant-growth-promoting rhizobacteria (PGPR). In addition to symbiotic N_2-fixation, other characteristics of PGPR are attributed to this genus. Previous reports described *Burkholderia* strains as phosphate solubilising

bacteria (Peix et al., 2001; Caballero-Mellado et al., 2007). They are also described as having the ability to fix N_2 as free-living or endophytic bacteria (Estrada-de-los-Santos et al., 2001), and several strains have been used as biocontrol agents because of their ability to inhibit many fungi that cause plant diseases (Peix et al., 2001; Li et al., 2009). This genus is also able to produce phytohormones and secrete siderophores, among other abilities (Compant et al., 2008).

The identification of species in the genus *Burkholderia* is complex, as the 16S rRNA gene sequence used in the taxonomy of prokaryotes cannot be used for reliable identification. Housekeeping genes showed better separation of related species, including those of *Burkholderia* genus, and they must be used in identification and species definition (Tayeb et al., 2008). In particular Bcc species can be reliably separated by *recA* gene and multilocus sequence analyses (Mahenthiralingam et al., 2000; Baldwin et al., 2005).

The Amazonian forest is known for its large diversity of plant and animal species. By partial 16S rDNA sequencing of 88 isolates that were representative of 1890 isolates from Amazonian soil collected using siratro (*Macroptilium atropurpureum*) as the trap species, six different genera were found that were related to the well-known Leguminosae nodulating bacteria *Azorhizobium*, *Rhizobium, Bradyrhizobium, Mezorhizobium, Sinorhizobium* and *Burkholderia* (Lima et al., 2009), revealing the high microbial diversity in this region. When common bean plants (*Phaseolus vulgaris*) were used as the trap species, isolates belonging to *Burkholderia* spp. were also captured (Barberi, 2007).

The aims of the present study were to evaluate the *Burkholderia* isolates obtained from Amazonian soils for the phenotypical and functional characteristics that can be used to promote plant growth and to determine the phylogenetic position of these isolates.

4 MATERIAL AND METHODS

4.1 Origin of isolates

This study is part of the "Conservation and Sustainable Management of Below-Ground Biodiversity" (CSM-BGBD) project, the Brazilian component of which is called BiosBrasil[2]. The project is co-funded by Global Environment Facility (GEF) with implementation support from the United Nations Environment Programme (UNEP). CSM-BGBD is being carried out in seven countries (Brazil, Ivory Coast, India, Indonesia, Kenya, Mexico and Uganda). The studied area was located in the Benjamin Constant Municipality, Northwest Amazonas state, on the triple border of Brazil, Colombia and Peru, within the geographic coordinates 4°20′ and 4°26′ South and 69°36′ and 70°2′ West. This area includes the communities of Guanabara II and Nova Aliança and the town of Benjamim Constant, situated approximately 1,100 km west of Manaus at the upper Solimões River. Fidalgo et al. (2005) characterised the sampling grids, respective sampling points and land use systems (LUS) in the area using six categories: Pristine Forest (PF); Pasture (PA); Agriculture (AG); Agroforestry (AF); Young Second Forest (YSF) and Old Second Forest (OSF). Ninety-eight sampling points were distributed in six grids, approximately 9 ha each, in order to include all of the representative LUSs of the region. Each sampling point was geo-referenced and was part of a pre-established grid. The distance between points was generally 100 m but was reduced to 50 m in some cases where more replicates per LUS were necessary. Soil sampling was performed during the first two weeks of March 2004. At each sampling point, sub-samples were collected in two concentric circles with a 3-6 m radius. The samples were taken from 0-20 cm layers using an auger. The soil samples used for microbiological analysis

[2] Available in: <http:// www.biosbrasil.ufla.br >. Access: 12 Jan. 2009.

(about 300 g) were placed in sterile Nasco® plastic bags and stored at 4°C until further use.

Six strains used in this study are from the project "Alternatives to Slash-and-Burn/International Centre for Research in Agroforestry" (ASB/ICRAF). These isolates were collected in the Amazonian region in the Rondônia and Acre States. Soil samples were collected in 1997 and were taken from 0-20 cm depths in four land use systems: Pasture, Agriculture, Agroforestry and Young Second Forest (Lima et al., 2005) (Table 1).

TABLE 1 Main characteristics of the land use systems (LUSs) and *Burkholderia* isolates captured in each area using Siratro (*Macroptilium atropurpureum*) or common beans (*Phaseolus vulgaris*) as the trap plant, or from the root nodules of *Indigofera suffruticosa* and *Pithecellobium* sp. collected in the field.

LUS	State*/region	Characteristics	Isolates
Pristine Forest (PF)	AM - Western	Sub region of the low Amazon plateaux	**UFLA04-164(R-39720), UFLA04-165 (R-39722)**
	AM - East		**INPA42B (R-39750)****
Pasture (PA)	AM - Western	Implanted in 1970, with imperial grass (*Axonopus scoparius*). After decline, approximately 11 years after implanting, it was substituted with *Brachiaria brizantha*, *B. humidicola* and (*Paspalum notatum flugge*), currently presenting weed species. Pasture reform is carried out by planting seedlings in hill plots. The grasses are cut three times a year with a sickle, hoe and axe.	**UFLA04-222 (R-39736), UFLA04-223 (R-39734), UFLA04-224 (R-39735)**
	RO - Western	Ten years old. Some trees and stumps. Many termite mounds. Utisols.	**ST5-1 (R-39743)**
Agriculture (AG)	AM - Western	Areas of annual crops (cassava, corn, sugar cane and pineapple) and semi-perennial crops (banana).	**UFLA04-131 (R-39700), UFLA04-132 (R-39701), UFLA04-133 (R-39702), UFLA04-134 (R-39703), UFLA04-135 (R-39706), UFLA04-136 (R-39704), UFLA04-148 (R-39713), UFLA04-162 (R-39721)**
	RO- Western	Crop of manihot	**ST1-2 (R-39741)**
Agroforestry (AF)	AM - Western	Characterized by being mostly formed by spontaneous secondary forest species generation enriched by planting species of economic interest such as “cupuaçu” (*Theobroma*	**UFLA04-23 (R-39707), UFLA04-53 (R-39698), UFLA04-59 (R-39712), UFLA04-130 (R-39699),**

Continues...

Continues...

		grandflorum), pupunha-type heart of palm (*Bactris gassipaes*), banana, pineapple, coffee and others.	**UFLA04-138 (R-39705)**
	RO - Western	Coffee with rubber tree (12 years). Vegetal cover of 70% with some species of pioneer trees. Few dead branches or stumps	**ST7-12 (R-39742), ST9-7* (R-39740)**
Young Second Forest (YSF)	AM - Western	Less than 5 years after cultivation of the area.	**UFLA04-142 (R-39708), UFLA04-143 (R-39711), UFLA04-144 (R-39709), UFLA04-146 (R-39710), UFLA04-149 (R-39716), UFLA04-154 (R-39714), UFLA04-155 (R-39715), UFLA04-159 (R-39718), UFLA04-160 (R-39717), UFLA04-161 (R-39719), UFLA04-166 (R-39723)**
	AC Western	Stony soils with low fertility, poorly developed forest	**ST11-6 (R-39744)**
	RO - Western	Natural cover of 2 years after common beans and maize cultivation	**ST4-8 (R-39745) INPA89A (R-39749)****
Old Second Forest (OSF)	AM - Western	More than 5 years after cultivation of the area.	**UFLA04-168 (R-39728), UFLA04-210 (R-39727), UFLA04-213 (R-39730), UFLA04-215 (R-39729), UFLA04-216 (R-39726), UFLA04-217 (R-39733), UFLA04-218 (R-39731), UFLA04-219 (R-39732), UFLA04-235 (R-39725), UFLA02-28 (R-39747)***, UFLA02-27 (R-39748)***

*AM – Amazonas State; AC – Acre; RO – Rondônia State; ** INPA89A – *Indigofera suffruticosa*; INPA42B – *Pithecellobium* sp.;***Isolates collected using common beans (*Phaseolus vulgaris*).

4.2 Preliminary identification

A total of 190 isolates obtained in different works from Amazonian soils using siratro (*Macroptilium atropurpureum*) (Pereira, 2000; Lima et al., 2009) and common bean plants (*Phaseolus vulgaris*) (Barberi, 2007) as the trap plant were investigated by partial 16S rDNA sequencing. Forty-six isolates were identified as belonging to the genus *Burkholderia* (Table 1). We also included two isolates collected from root nodules of *Indigora suffruticosa* (INPA89A) and *Pithecellobium* sp. (INPA42B) in the field of Young Second Forest and Pristine Forest, respectively (Magalhães & Silva, 1987; Moreira et al., 1993). The isolates obtained in each LUS are shown in Table 1.

4.3 Phenotypic Characterisation

4.3.1 Cultural characterisation

At first, the cultural characterisation of 48 isolates and type strains of *Burkholderia* was performed on 79 culture medium (Fred & Waksman, 1928) according to pH after growth (acid, alkaline, neutral); time needed for the appearance of isolated colonies (fast — 2-3 days; intermediate — 4-5 days; slow — 6-10 days); colony diameter, border, elevation, colour and surface; exopolysaccharide production (few, moderate, abundant) and consistency; indicator absorption; and light transmission (opaque, brilliant, translucent) according to Jesus et al. (2005). We also included *Burkholderia* type strains (*Burkholderia phymatum* LMG21445^T, *Burkholderia tuberum* LMG21444^T, *Burkholderia nodosa* LMG23741^T, *Burkholderia mimosarum* LMG23256^T, *Burkholderia cepacia* LMG1222^T, *Burkholderia caribensis* LMG18531^T, *Burkholderia silvatlantica* LMG23149^T, *Burkholderia tropica* LMG22274^T, *Burkholderia unamae* LMG22722^T, *Burkholderia vietnamiensis* LMG10929^T and *Burkholderia xenoverans* LMG21463^T).

4.3.2 Biochemical characterisation

Some isolates (UFLA04-155, ST1-2, ST6-3, ST4-8, INPA42B, INPA89A and UFLA02-27) that were representative of the groups obtained by the morphological and physiological characterization on "79"medium were chosen for APIZYM and API20 NE tests. These tests were performed according to the manufacturer's recommendations (bioMerieux).

4.3.3 SDS-PAGE of whole-cell proteins

The 44 isolates obtained using siratro as the trap species were grown on nutrient agar (CM3; Oxoid) supplemented with 0.04% (w/v) KH_2PO_4 and 0.24%(w/v) $Na_2HPO_4.12H_2O$ (pH 6.8) and incubated at 28°C for 48 h. Whole-cell protein preparations and SDS-PAGE were performed as previously described (Pot et al., 1994). Densitometric analysis, normalisation and interpolation of the protein profiles and numerical analysis using Pearson's product-moment correlation coefficient were performed using the gelcompar 4.2 software package (Applied Maths). The protein profiles were compared with the data bank of the Laboratory of Microbiology de Ghent (LMG), Ghent University, which has approximately 72,000 protein profiles.

4.4 Plant Growth Promotion Traits

4.4.1 Phosphate solubilisation assay

For the phosphate solubilisation assay used on all of the isolates, we used a medium containing 5 g of yeast extract, 10 g of glucose and 20 g of agar made up to 1 L with water. The phosphates used were $CaHPO_4$ (2.6 g L^{-1}) and $Fe(PO_4)_3$ (4.33 g L^{-1}) (Silva-Filho & Vidor, 2001), both at pH 6.8. Aluminium phosphate was made by mixing K_2HPO_4 (6 g) and $AlCl_3$ (5.34 g), then adding medium at pH 4.5 (Hara & Oliveira, 2004).The isolates were maintained for 15 days under incubation at 28^{o}C. Bacterial colonies forming clear zones were considered to be phosphate solubilisers. We also included *Burkholderia* type

strains (*Burkholderia phymatum* LMG21445^T, *Burkholderia tuberum* LMG21444^T, *Burkholderia nodosa* LMG23741^T, *Burkholderia mimosarum* LMG23256^T, *Burkholderia cepacia* LMG1222^T, *Burkholderia caribensis* LMG18531^T, *Burkholderia silvatlantica* LMG23149^T, *Burkholderia tropica* LMG22274^T, *Burkholderia unamae* LMG22722^T, *Burkholderia vietnamiensis* LMG10929^T and *Burkholderia xenoverans* LMG21463^T).

4.4.2 Nitrogenase activity

The 48 isolates were evaluated for their ability to grow by fixing N_2 in free-nitrogen LO semi-solid medium (Deyfrus et al., 1983) with three different carbon sources (10 g of lactate, 10 g of mannitol and 10 g fructose). After the formation of typical thick pellicles near the medium surface, nitrogenase activity was confirmed using the acetylene reduction assay (ARA) following the methodology described by Dilworth (1966). The production of ethylene was verified by gas chromatography (Varian Star 3400 c*x*). We included the *Burkholderia* and *Azorhizobium* type strains (BR5401^T - *Azorhizobium doebereinerae* and ORS571^T - *Azorhizobium caulinodans*).

4.4.3 Antifungal-activity of *Burkholderia* isolates

Some isolates (UFLA04-155, ST1-2, ST6-3, ST4-8, INPA42B, INPA89A and UFLA02-27) that were representative of the groups obtained by the morphological and physiological characterization on "79"medium were chosen for the antifungal activity test. The antifungal activities of *Burkholderia* isolates were tested against *Fusarium oxysporum* f. sp. *phaseoli* using methods modified from Peix et al. (2001). Discs (6 mm) of *Fusarium* mycelium growing in PDA (Potato Dextrose Agar) were transferred to plates of the same medium, to which three different bacteria had been streaked. The plates were incubated

for three days at 28°C. After the incubation, the isolates that inhibited fungal growth were verified. Each isolate was tested in triplicate.

4.5 Genetic Characterisation

4.5.1 16S rDNA sequencing

Six isolates obtained from root nodules of siratro (UFLA04-55, UFLA04-130, UFLA04-136, UFLA04-155, UFLA04-219 e UFLA01-223) and the isolate obtained from *Indigofera suffruticosa,* INPA89A, were chosen for 16S rRNA sequencing. Nearly full-length 16S rRNA genes from the isolates were amplified with the primer pair 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) from log phase cultures in 79 medium. An aliquot of 10 μL of lysed cells was used for the 50 uL PCR reaction (0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.2 μM each primer, 1 U *Taq* DNA polymerase, 1 X PCR Buffer and Milli-Q water). The Eppendorf Mastercycler® (Germany) was used for the PCR. An initial denaturation at 94°C for 5 min was followed by 40 PCR cycles consisting of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, elongation at 72°C for 1.5 min, and a final elongation for 7 min. Amplified products were separated on 1% agarose gels and visualised under UV light. The PCR products were purified with Microcon™ filters (Millipore). Single pass sequencing of PCR amplified rDNAs was performed with the 27F and 1492R primers in a 3730xl sequencer.

The sequences were assembled using DNASTAR SeqMan Version 5.0. Sequences were selected based on the Phred value being equal to or greater than 20 and were compared with GenBank sequences using the Basic Local Alignment Search Tool (NCBI). Only sequences with more than 1300 bp were used in the phylogenic analyses. Phylogenetic trees were inferred by the neighbour-joining method using Kimura's 2-parameter model (Kimura, 1980) as

implemented in the MEGA 4.1 package (Tamura et al., 2007). A bootstrap confidence analysis was performed with 1,000 replicates.

4.5.2 *gyr*B gene sequencing

Thirteen isolates (UFLA04-53, UFLA04-130, UFLA04-136, UFLA04-148, UFLA04-155, UFLA04-218, UFLA04-219, UFLA04-223, ST7-12, ST9-7 and INPA89A) and an additional 69 type strains of *Burkholderia fungorum*, *B. graminis* and *B. phytofirmans* and *Burkholderia caribensis* were utilised for *gyrB* sequencing. To prepare genomic DNA, the strains were grown in trypticase soy agar for two days at 28°C. Then, the cells were lysed using the alkaline lysis procedure (Baele et al., 2000). The PCR was performed using the primers gyrB-20-F 5'- GAC AAY GGB CGY GGV RTB CC-3' and gyrB-21-R 5'- ACR CCR TTR TTC AGG AAY GA-3', which were redesigned from primers designed by Tayeb et al. (2008) and originally designed from the *Burkholderia*, *Rasltonia*, *Cupriavidus* and *Pandorea*. A 1 µL aliquot of lysed cells was used for the 25 uL PCR reaction (0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.1 µM each primer gyrB-20-F and gyrB-21-R, 0.5 U *Taq* DNA polymerase, 1 X PCR Buffer and Milli-Q water). The reaction used an initial denaturation at 94°C for 5 min followed by 35 PCR cycles consisting of denaturation at 94°C for 1 min, annealing at 65°C for 1 min 15 sec, elongation at 72°C for 2 min, and a final elongation for 5 min. Amplified products were separated on 1.0% agarose gels and visualised under UV light. The obtained PCR products were purified in the Robot Sample Preparation System (Tecan, Switzerland). Sequencing was performed using an ABI Prism 3130xl capillary sequencer according to the manufacturer's instructions (Applied Biosystems).The sequencing primers for *gyr*B were the same as those used for the amplification.

The sequence results were analysed in Bionumerics version 5.1 (Applied Maths) and compared with GenBank sequences using the Basic Local

Alignment Search Tool (NCBI). The obtained sequences were aligned with clustalW, and phylogenetic trees were inferred by the neighbour-joining method and constructed using Bionumerics version 5.1.

4.5.3 Bcc-specifc *rec*A gene and MLST

Three isolates obtained from root nodules of common bean plants (UFLA02-27 and UFLA02-28) and one from *Pithecellobium* sp. (INPA42B) showed similarity with strains of the Bcc based on 16S rRNA sequencing (data not shown). The DNA from the isolates was also amplified with primers for the *recA* gene specific to the Bccs BCR1 and BCR2, as described by Mahenthiralingam et al. (2000). Then, we used Multilocus sequence typing (MLST) in an attempt to identify these isolates.

MLST analysis was performed as previously described (Baldwin et al., 2005). A phylogenetic tree of concatenated sequences (2773 bp), including fragments of seven genes (atpD [443 bp], gltB [400 bp], gyrB [454 bp], recA [393 bp], lepA [397 bp], phaC [385 bp] and trpB [301 bp]), from each isolate was constructed based on the neighbour-joining method using MEGA software package version 3 (Baldwin et al., 2005; Kumar et al., 2004). The significance of branching within the trees was evaluated by bootstrap analysis of 1000 computer-generated trees. The program DnaSP version 4.1[3] (Rozas et al., 2009) was used to calculate the mean number of nucleotide substitutions per site (i.e., the percentage divergence of concatenated allele sequences) between populations based on a Jukes-Cantor method (Naser et al., 2007; Rozas et al., 2003). The standard deviation was calculated to show how widely the values were spread in the dataset. Evidence for clonal or recombining populations was estimated by measuring the extent of linkage (using the standardised index of association [sIA]) between alleles at different loci around the chromosome as

[3] Available in: < http://www.ub.es/dnasp/DnaSP32Inf.html >. Access: 18 Apr. 2009.

described previously (Baldwin et al., 2005; Haubold & Hudson, 2000; Maynard Smith et al., 1993). An sIA not significantly greater than 0 after 1000 computer randomisations suggests that a single species population (monophyletic) is in linkage equilibrium (freely recombining), while a population with an sIA significantly greater than 0 ($P<0.001$) is considered to be in linkage disequilibrium (clonal) (Baldwin et al., 2005; Haubold & Hudson, 2000). Nucleotide sequences of each allele and allelic profile and sequence types for all strains analysed in this study are available from the Bcc MLST website (http://pubmlst.org/bcc/) developed by Keith Jolley and sited at the University of Oxford (Jolley et al., 2004).

5 RESULTS

5.1 Phenotypic Characterisation

5.1.1 Cultural characterisation

The 48 isolates obtained from Amazon soils presented different morphological characteristics depending on the legume species from which they were isolated. The isolates obtained using siratro as the trap plant presented similar characteristics on 79 media, and they were different from those obtained from *Indigofera suffruticosa* and *Pitechellobium* sp. (INPA 89A and INPA42B) and common bean plants (UFLA02-27 and UFLA02-28) (Table 2). All isolates presented fast growth on 79 media at 28^{o}C, taking only two to three days for the appearance of isolated colonies. However, other characteristics, such as pH reaction on culture medium, exopolysaccharide production, colour, elevation, consistency indicator absorption and light transmission were variable.

5.1.2 Biochemical characterisation

Only the isolate INPA42B was able to assimilate all twelve of the carbon sources tested (Table 2). For the enzymatic activity, all isolates presented a positive reaction for catalase, C_4-Esterase, C_8-Esterase lipase, leucine aminopeptidase, alkaline phosphatase and acid phosphatase activities. The following features were absent in all of the investigated strains: nitrite reduction; indole production; arginine dihydrolase; valine aminopepetidase; trypsin; chymotrypsin; α-galactosidase; β-galactosidase; β-glucuronidase; α-glucosidase; β-glucosaminidase and α-fucosidase.

5.1.3 SDS-PAGE of whole-cell proteins

The results of SDS-PAGE of whole-cell proteins from the 46 tested isolates are shown in Figure 1. When the results were compared with the LMG

collection data bank, which contains 72,000 protein profiles, we verified that the isolates presented the same protein profile as *Burkholderia fungorum* type strains (LMG15693^T, LMG15688^T, LMG15692^T, LMG16226^T and LMG16225^T).

TABLE 2 Cultural and biochemical characteristics of *Burkholderia* isolates obtained from Amazonian soils.

CHARACTERISTICS	Siratro isolates (*M. atropurpureum*)	INPA89A (*I. suffruticosa*)	INPA42B (*Pithecellobium* sp.)	UFLA02-27 (*P. vulgaris*)
Cultural Characteristics on 79 medium:				
Medium pH	Neutral	Acid	Alkaline	Acid
Growth*	Fast	Fast	Fast	Fast
Exopolysaccharide production**	M/A	A	M	A
Color	Cream	Cream	White	Yellow
Elevation	Convex	Convex	Flat	Convex
Consistency	Gum	Gum	Butter	Gum
Indicator absorption	+/-	+	-	+
Light transmission	Translucent	Brilliant	Opaque	Brilliant
Assimilation of:				
Glucose	+	+	+	+
Fructose	+	-	+	+
Arabinose	+	+	+	+
Mannose	+	+	+	+
Mannitol	+	+	+	+
N-acetyl-glucosamine	+	+	+	+
Maltose	-	-	+	-
Gluconate	+	+	+	+
Caprate	-	-	+	+
Adipate	+	-	+	+
Malate	+	-	+	+
Citrate	+	-	+	+
Phenyl-acetate	+	-	+	+

Continues...

Continues...				
Activity of:				
Nitrate reduction	+	-	+	-
Catalase	+	+	+	+
Oxidase	+	-	-	+
β-glucosidase	-	-	+	+
Gelatinase	-	-	+	+
Urease	+	+	-	-
C_4-Esterase	+	+	+	+
C_8-Esterase lípase	+	+	+	+
C_{14}-Lipase	-	-	+	+
Leucine aminopeptidase	+	+	+	+
Phosphatase alkaline	+	+	+	+
Phosphatase acid	+	+	+	+
Phosphoamidase	-	+	+	+
Cystine aminopeptidase	+	-	-	-
α-Mannosidase	-	-	-	+
ARA***:				
Fructose	+	-	+	+
Lactate	+/-	-	+	+
Mannitol	+/-	-	+	+

* Fast: 2-3 days for the appearance of isolated colonies;
**A: abundant; M: moderate;
*** Acetilene reduction assay in tree different carbon sources (lactate, mannitol and fructose);
Characteristics are scored as: + positive, - negative, +/- variable, depending on the isolate utilised.

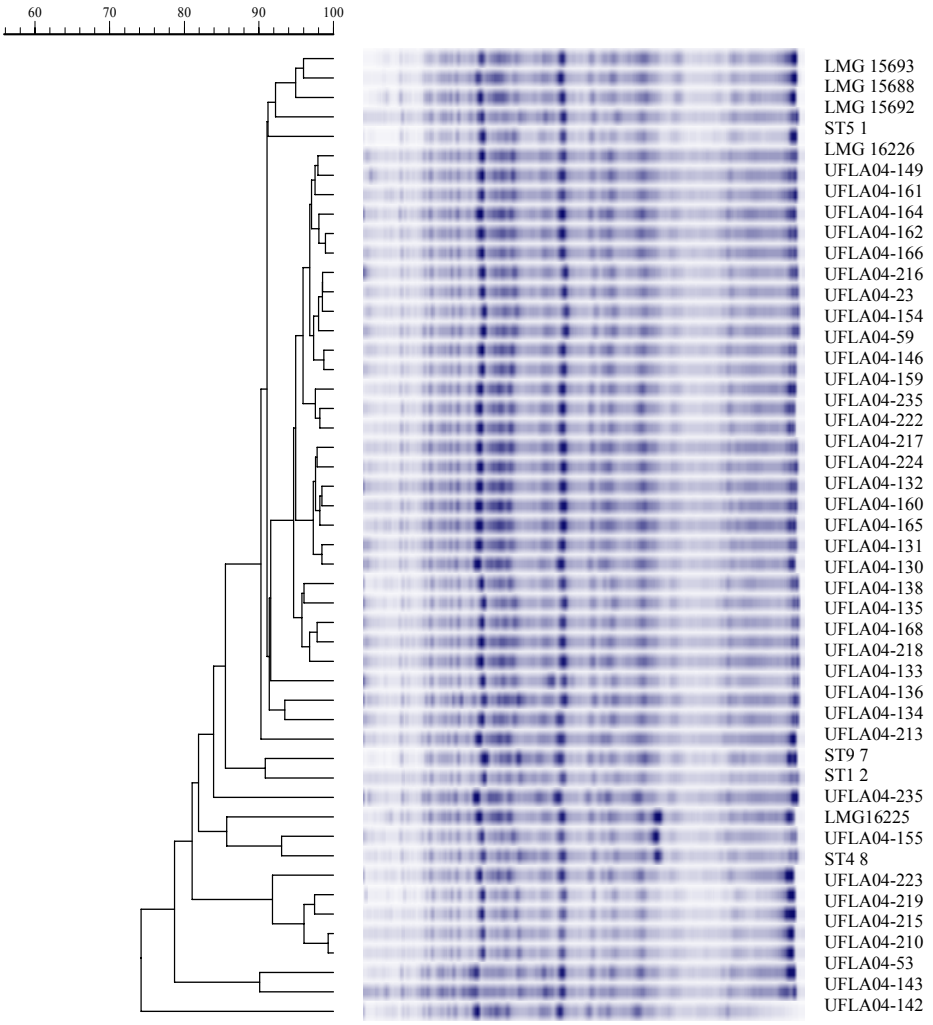

FIGURE 1 Dendrogram derived from UPGMA of correlation coefficients between the whole-cell protein patterns of *Burkholderia* isolates obtained from Amazonian soils captured using siratro (*Macroptilium atropurpureum*) as the trap plant and some *B. fungorum* type strains.

5.2 Plant Growth Promotion Traits

5.2.1 Phosphate solubilisation assay

All of the isolates except UFLA04-158 and INPA89A were able to solubilise calcium phosphate (Figure 2). The type strains of *B. cepacia* LMG1222^T, *B. tropica* LMG22274^T, *B. silvatlantica* LMG23149^T and *B. vietnamiensis* LMG10929^T were also able to solubilise calcium phosphate. No isolates or type strains were able to solubilise iron and aluminium phosphate.

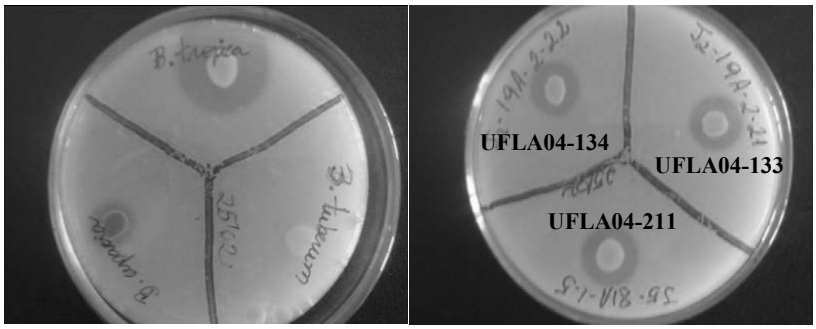

FIGURE 2 Solubilisation of calcium phosphate by certain type strains and isolates of *Burkholderia.* Obs.: *B. tuberum* did not solubilise calcium phosphate.

5.2.2 Nitrogenase activity

All isolates trapped using siratro, the isolates UFLA02-27 (common beans) and INPA42B (*Pithecellobium* sp.) (Table 2) and some *Burkholderia* type strains (*B. unamae* LMG22722^T, *B. tropica* LMG22274^T, *B. silvatlantica* LMG23149^T, *B. vietnamiensis* LMG10929^T, *B. xenoverans* LMG21463^T, *B. nodosa* LMG23741^T, *B. tuberum* LMG21444^T, *B. caribensis* LMG18531^T) presented positive ARA results on media with fructose as a carbon source. Only

one isolate (INPA89A) and two type strains (*B. cepacia* LMG1222^T and *B. mimosarum* LMG23256^T) did not reduce acetylene on the media tested. The positive controls BR5401^T (*Azorhizobium doebereinerae*) and ORS571^T (*Azorhizobium caulinodans*) presented positive ARA results for almost every tested carbon source, except on media containing mannitol. Most of the strains were ARA negative on mannitol.

5.2.3 Antifungal-activity of *Burkholderia* isolates

The antifungal-activity of the *Burkholderia* isolates and type strains were tested against *Fusarium oxysporum* f. sp. *phaseoli*, but only one isolate (UFLA02-27) trapped by common bean plants (Figure 3A), and two type strains *B. cepacia* LMG1222^T (Figure 3B) and *B. vietnamiensis* LMG10929^T presented this characteristic.

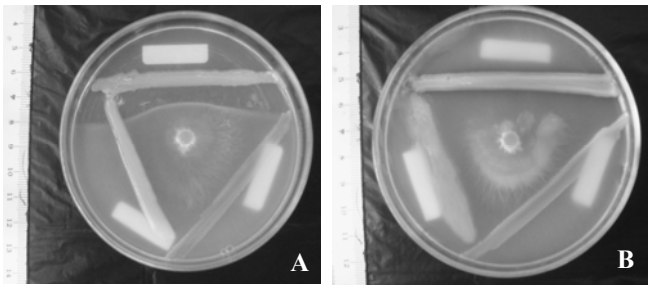

FIGURE 3 Antifungal-activity of *Burkholderia* isolates and strains tested against *Fusarium oxysporium* f. sp. *phaseoli* in potato dextrose agar. A- Isolate UFLA02-27; B- Strain *B. cepacia* LMG1222^T.

5.3 Genetic Characterisation

5.3.1 16S rRNA sequencing

By 16S rDNA sequencing, some isolates (UFLA04-130, UFLA04-155, UFLA04-53, UFLA04-223, UFLA04-219 and UFLA04-136) presented 100% identity with type strains of the *Burkholderia fungorum* species. The phylogenetic tree (Figure 4) showed that the isolates were grouped with the *B. fungorum* type strains with a strong bootstrap value. The isolate INPA89A presented 99% identity with *B. caribensis.*

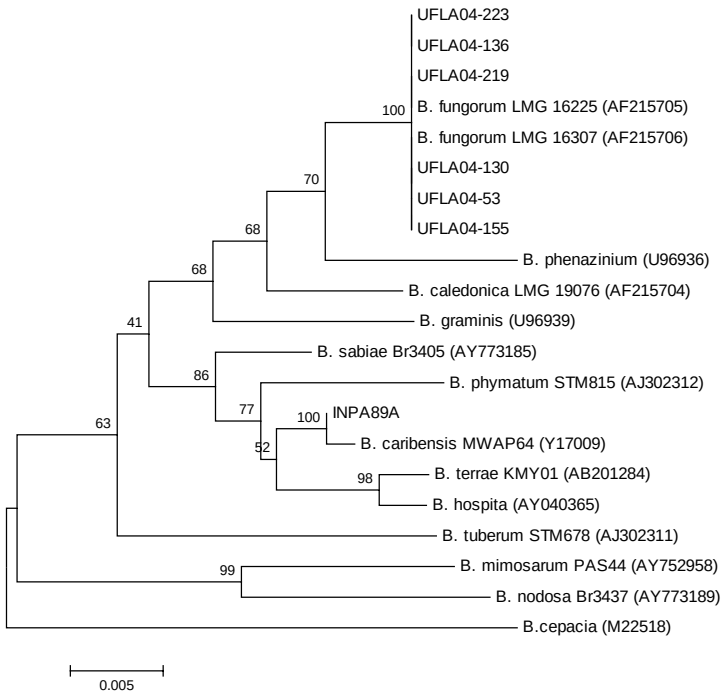

FIGURE 4 Phylogenetic tree of isolates and *Burkholderia* type strains based on 16S rDNA sequences. Phylogenies were estimated by the neighbour-joining method. Bootstrap values were based on 1,000 trials.

5.3.2 *gyr*B genes amplification and sequencing

The results of *gyrB* sequencing are shown in Figure 5. The results were similar to those found for the 16S rDNA sequencing. The isolates obtained from siratro nodules formed a group with the isolates and *B. fungorum* type strains. The isolate INPA89A, which was obtained from an *Indigofera suffruticosa* nodule, was grouped with strains of *B. caribensis.*

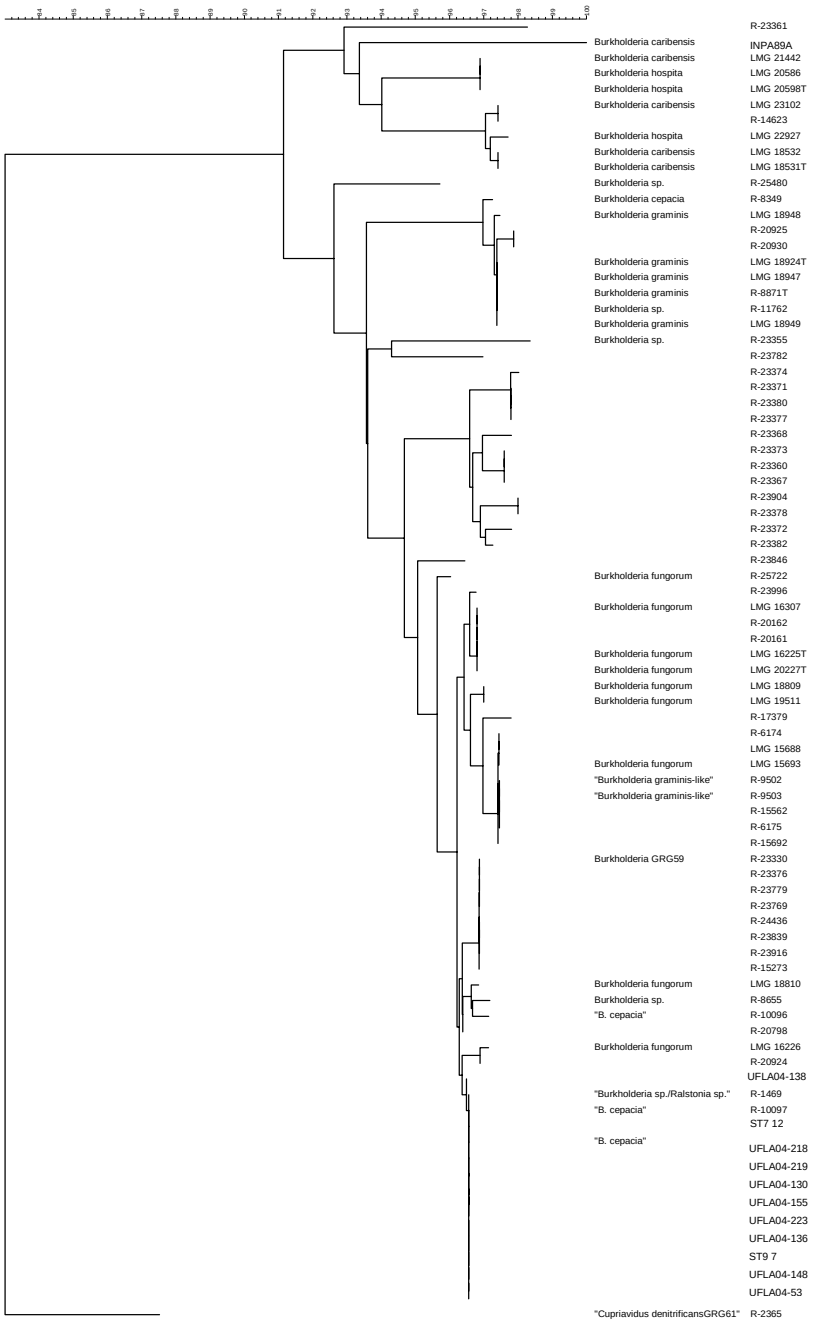

FIGURE 5 *gyrB* tree. The tree was constructed using the neighbour-joining method.

5.3.3 Bcc-specifc *rec*A gene and MLST

Some isolates (UFLA02-27, UFLA02-28 and INPA42B) showed similarity with *Burkholderia cepacia* strains based on partial 16S rRNA sequencing (data not shown). About 1000 bp of the *recA* gene from isolates UFLA02-27, UFLA02-28 and INPA42B were successfully amplified using the Bcc specific primer pair BCR1/BCR2, indicating that these strains are members of the Bcc. Then, the *recA* sequencing and MLST were used to determine the phylogenetic position of these isolates. The isolates UFLA02-27 and UFLA02-28 presented 99% similarity with *B. contaminans* and other *B. cepacia* strains*;* the isolate INPA42B presented 98% similarity with *B. lata* and *B. contaminans.*

The results of the MLST are presented in Figure 6. By MLST, two isolates, UFLA02-27 (R-39747) and UFLA02-28 (R-39748), were closely related to *B. contaminans*, and the isolate INPA42B (R-39750) was closely related to *B. lata*.

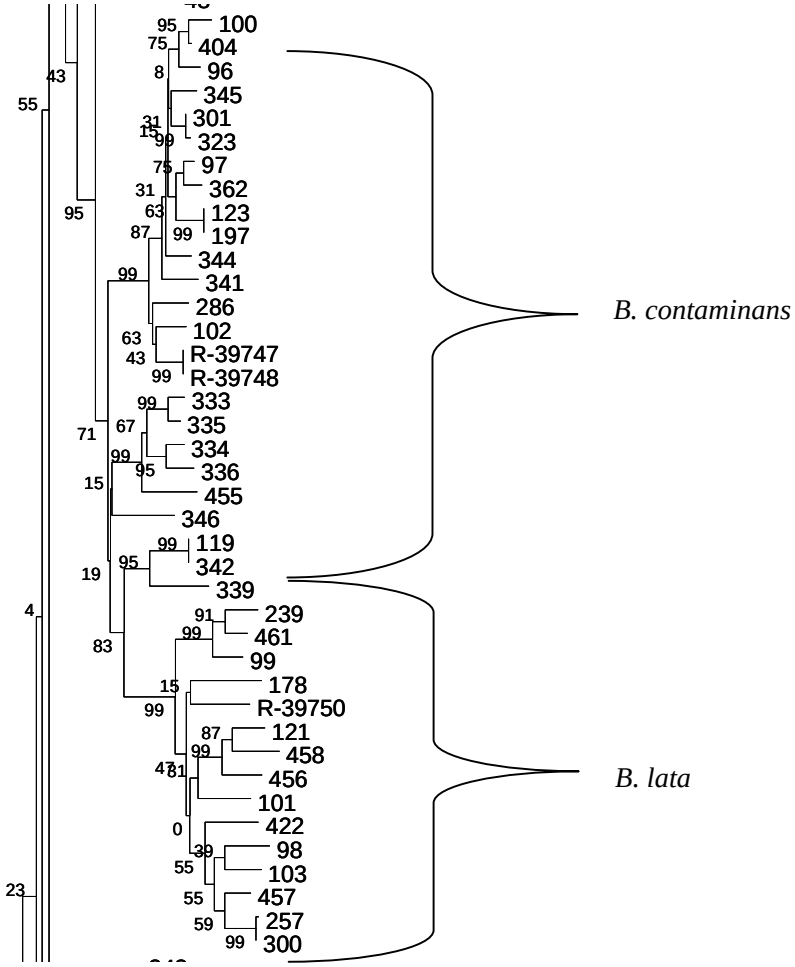

FIGURE 6 Phylogenetic analysis of concatenated nucleotide sequences from 6 loci (*phaC* was omitted), using the neighbour-joining method.

6 DISCUSSION

In the present study, we investigated the phenotypic and functional characteristics of bacterial isolates obtained from Amazonian soils trapped with different legume hosts (*M. atropurpureum*, *P. vulgaris*, *Indigofera suffruticosa* and *Pithecellobium* sp.). We used genotypic characteristics such as 16S rDNA, *gyr*B and *recA* gene sequencing and multilocus sequencing typing (MLST) to determine the phylogenetic position of these isolates.

6.1 Phenotypic Characteristics and Plant Growth Promotion Traits

The isolates presented a diversity of cultural and biochemical characteristics, showing the diversity of *Burkholderia* genus. Most of them were able to solubilise calcium phosphate (96%) and fix N_2 on nitrogen free-medium (97%), and one isolate presented antifungal activity against *Fusarium oxysporum*.

Several works have reported the ability of *Burkholderia* strains to solubilise inorganic phosphates (Babu-Khan et al., 1995; Nahas, 1996; Peix et al., 2001; Caballero-Mellado et al., 2007), and this can be used to increase the available phosphate.

In our study, all isolates except for one (INPA89A) were ARA positive on nitrogen free medium. Numerous strains of *Burkholderia* are known for their ability to fix N_2 as free-living bacteria (Estrada-de-los-Santos et al., 2001). Some strains are known to be able to nodulate and fix N_2 on media, such as *B. phymatum* and *B. nodosa* (Elliot et al., 2007). In this work, *B. tuberum* also presented these features. We did not detect *nodC* genes in these isolates, but *nifH* was present (data not shown). Our results showed for the first time that *B. fungorum* is able to solubilise calcium phosphate and fix N_2.

The ability of Bcc strains to inhibit fungal growth has been shown (Bowers & Parker, 1993; Burkhead et al., 1994; Moon et al., 1996; Bevivino et al., 1998; Lee et al., 2000; Peix et al., 2001). In our study, we verified that the two strains and one isolate belonging to the Bcc (*B. cepacia* LMG1222^T, *B. vietnamiensis* LMG10929^T and UFLA02-27) presented this characteristic. The isolate UFLA02-27 was identified as *B. contaminans,* a member of the Bcc. Recently, it was shown that this species is also able to inhibit the growth of fungi (Gu et al., 2009).

All of these isolates were obtained from root nodules of different hosts, but when they were tested for their ability to nodulate siratro, they failed in several experiments. Thus, these isolates can be considered endophytic.

6.2 Genotipic Characterisation

The strains obtained from Amazon soils using siratro as the trap species were isolated by Lima et al. (2009). These authors used the morphological and physiological characterization on "79" medium to distribute the 1890 isolates into 112 groups. Representative isolates from these 112 groups had their 16S rRNA genes partially sequenced. Twelve of them were identified as belonging to the *Burkholderia* genus. We investigated the strains present in these 12 groups to better chracterise them. Pereira (2000) also obtained isolates from Amazon soils using siratro as the trap species and these isolates had similar cultural characteristics as those found by Lima et al. (2009). After protein profiling and 16S rRNA and *gyrB* gene sequencing, we could conclude that the isolates obtained from siratro nodules and the isolate INPA89A from *Indigofera suffruticosa* nodules belong to *B. fungorum* and *B. caribensis*, respectively. *B. fungorum* was described by Coenye et al. (2001) and has been isolated from human, animal, fungal and environmental samples. There are also some reports of isolating strains closely related to *B. fungorum* from the root nodules of

legume species (Barret & Parker, 2005; Vandamme et al., 2007). In this study, bacteria were recovered from Amazonian soils at different places and times, but their ability to nodulate is not clear. These isolates were obtained from the root nodules of siratro plants inoculated with soil solution diluted from 10^{-1} to 10^{-4}, indicating that *B. fungorum* is abundant in these soils and that it could be found in the Amazonian soils of all land use systems.

B. caribensis was isolated from vertisol on the island of Martinique and was found to be responsible for the formation of microaggregates due the production of exopolysaccharides (Achouak et al., 1999). Later, two isolates recovered from root nodules of *Mimosa* spp. were identified as belonging to *B. caribensis* (Vandamme et al., 2002).

Three isolates, UFLA02-27, UFLA02-28 and INPA42B, belong to *B. cepacia*, and, based on the results of *recA* gene sequencing and MLST, we could clarify the phylogenetic position of these isolates within the Bcc. The isolates obtained from root nodules of the common bean plant (UFLA02-27 and UFLA02-28) belong to *B. contaminans*, and the isolate from the root nodules of *Pithecellobium* sp**.** (INPA42B) belongs to *B. lata*. These two species were recently described by Vanlaere et al. (2009), who established that these species are members of a diverse group known as Takon K. These species are found in human, animal, and environmental sources. In Madagascar, an isolate of Bcc was also found in a nodule of *Dalbergia* sp. (Rasolomampianina et al., 2005).

The isolates of *B. fungorum* obtained from siratro nodules did not form nodules when re-inoculated in this legume. The isolates of *B. caribensis* (INPA89A), *B. contaminans* (UFLA02-27, UFLA02-28) and *B. lata* (INPA42B) also did not form nodules in siratro and common bean plants. We did not detect *nodC* genes in these isolates, but we only used one primer that was designed for α-Proteobacteria (Laguerre et al., 2001). Therefore, these isolates can act as endophytic bacteria.

7 CONCLUSIONS

Forty-eight isolates obtained were identified as *B. fungorum* (44 isolates), *B. caribensis* (one isolate), *B. contaminans* (two isolates) and *B. lata* (one isolate).

The genus *Burkholderia* is abundant in Amazonian soils.

Almost all the isolates studied presented plant growth promotion traits.

These isolates can be considered as edophytic bacterial.

8 REFERENCES

ACHOUAK, W.; CHRISTEN, R.; BARAKAT, M.; MARTEL, M. H.; HEULIN, T. *Burkholderia caribensis* sp. now, an exopolysaccharide-producing bacterium isolated from vertisol microaggregates in Martinique. **International Journal of Systematic and Evolutionary Mcrobiology**, Spencers Wood, v. 49, n. 2, p. 787-794, Apr. 1999.

BABU-KHAN, S.; YEO, T. C.; MARTIN, W. L.; ROGERS, R. D.; GOLDSTEIN, A. H. Cloning of a mineral phosphate-solubilizing gene from *Pseudomonas cepacia.* **Applied and Environmental Microbiology**, Washington, v. 61, n. 3, p. 972-976, Mar. 1995.

BAELE, M.; BAELE, P.; VANEECHOUTTE, M.; STORMS, V.; BUTAYE, P.; DEVRIESE, L. A.; .VERSCHRAEGEN, G.; GILLIS, M.; HAESEBROUCK, F. Application of tRNA intergenic spacer PCR foridentificationof Enterococcus species. **Journal of Clinical Microbiology**, Washington, v. 38, n. 11, p. 4201-4207, Nov. 2000.

BALDWIN, A.; MAHENTHIRALINGAM, E.; THICKETT, K. M.; HONEYBOURNE, D.; MAIDEN, M. C.; GOVAN, J. R.; SPEERT, D. P.; LIPUMA, J. J.; VANDAMME, P.; DOWSON, C. G. Multilocus sequence typing scheme that provides both species and strain differentiation for the *Burkholderia cepacia* complex. **Journal of Clinical Microbiology**, Washington, v. 43, n. 9, p. 4665-4673, Sept. 2005.

BARBERI, A. **Diversidade e eficiência de bactérias que nodulam feijoeiro de diferentes sistemas de uso da terra na Amazônia Ocidental.** 2007. 121p. Tese (Doutorado em Ciência do Solo) – Universidade Federal de Lavras, Lavras.

BARRET, C. F.; PARKER, M. A. Prevalence of *Burkholderia* sp. nodule symbionts on four mimosoid legumes from Barro Colorado Island, Panama. **Systematic and Applied Microbiology**, Amsterdam, v. 28, n. 1, p. 57-65, Jan. 2005.

BEVIVINO, A.; SARROCCO, S.; DALMASTRI, C.; TABACCHIONI, S.; CANTALE, C.; CHIARINI, L. Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia*: effect of seed treatment on disease suppression and growth promotion of maize. **FEMS Microbiology Ecology,** Amsterdam, v. 27, n. 3, p. 225-237, Nov. 1998.

BOWERS, J.; PARKE, J. Epidemiology of *Pythium* damping-off and *Aphanomyces* root rot of peas after seed treatment with bacterial agents for biological control. **Phytopathology,** Saint Paul, v. 83, n. 12, p. 1466-1473, Dec. 1993.

BURKHEAD, K. D.; SCHISLER, D. A.; SLININGER, P. J. Pyrrolnitrin Production by Biological Control Agent *Pseudomonas cepacia* B37w in Culture and in Colonized Wounds of Potatoes. **Applied and Environmental Microbiology**, Amsterdam, v. 60, n. 6, p. 2031-2039, June 1994.

CABALLERO-MELLADO, J.; ONOFRE-LEMUS, J.; ESTRADA-DE LOS SANTOS, P.; MARTINEZ-AGUILAR, L. The Tomato Rhizosphere, an Environment Rich in Nitrogen-Fixing *Burkholderia* Species with Capabilities of Interest for Agriculture and Bioremediation. **Applied and Environmental Microbiology**, Amsterdam, v. 73, n. 16, p. 5308-5319, Aug. 2007.

CHEN, W. M.; FARIA, S. M. ; JAMES, E. K.; ELLIOT, G. ; LIN, K. Y. ; CHOU, J. H. ; SHEU, S. Y. ; CNOKAERT, M. ; SPRENT, J. ; VANDAMME, P. *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 57, n. 5, p. 1055-1059, May 2007.

CHEN, W. M.; FARIA, S. M.; CHOU, J. H.; JAMES, E. K.; ELLIOTT, G. N.; SPRENT, J. I.; BONTEMPS, C.; YOUNG, J. P. W.; VANDAMME, P. *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia.* **International Journal of Systematic and Evolutionary Mcrobiology**, Spencers Wood, v. 58, n. 9, p. 2174-2179, Sept. 2008.

CHEN, W. M.; JAMES, E. K.; COENYE, T.; CHOU, J. H. ; BARRIOS, E.; FARIA, S. M.; ELLIOT, G.; SHEU, S. Y.; SPRENT, J. ; VANDAMME, P. *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 56, n. 8, p. 1847-1851, Aug. 2006.

COENYE, T.; LAEVENS, S.; WILLEMS, A.; OHLEN, M.; HANNANT, H.; GOVAN, J. R. W.; GILLIS, M.; FALSEN, E.; VANDAMME, P. *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. **International Journal of Systematic and Evolutionary Mcrobiology**, Spencers Wood, v. 51, n .3, p. 1099-1107, May 2001.

COENYE, T.; VANDAMME, P. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. **Environmental Microbiology**, Oxford, v. 5, n. 3, p. 719-729, May 2003.

COMPANT, S.; NOWAK, J.; COENYE, T.; CLEMENT, C.; BARKA, E. A. Diversity and occurrence of *Burkholderia* spp. in the natural Environment. **FEMS Microbiology Reviews**, Amsterdam, v. 32, n. 4, p. 607-626, July 2008.

DILWORTH, M. J. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. **Biochemica et Biophysica Acta**, Amsterdam, v. 127, n. 2, p. 285-294, Oct. 1966.

DREYFUS, B.; GARCIA, J. L.; GILLIS, M. Characterization of *Azorhizobium caulinodulans* gen. nov. sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 38, n. 1, p. 89-98, Jan. 1988.

ELLIOT, G. N.; CHEN, W. M.; CHOU, J. H.; WANG, H. C.; SHEU, S. Y.; PERIN, L.; REIS, V. M.; MOULIN, L.; SIMON, M. F.; BONTEMPS, C.; SUTHERLAND, J. M.; BESSI, R.; DE FARIA, S. M.; TRINICK, M. J.; PRESCOTT; A. R.; SPRENT, J. I.; JAMES, E. K. *Burkholderia phymatum* is a highly effective nitrogenfixing symbiont of *Mimosa* spp. and fixes nitrogen *ex planta*. **New Phytologist,** Cambridge, v. 173, n. 1, p. 168-180, Jan. 2007.

ESTRADA-DE LOS SANTOS, P.; BUSTILIOS-CRISTALES, R.; CABALLERO-MELLADO, J. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. **Applied and Environmental Microbiology**, Whashington, v. 67, n. 6, p. 2790-2798, June 2001.

EUZÉBY, J. S. **List of prokaryotic names with standing in nomenclature - Genus *Burkholderia*.** Toulouse: École Nationale Vétérinaire de Toulouse, [2009]. Available in: <http://www.bacterio.cict.fr/b/burkholderia. html>. Access: 18 Apr. 2009.

FIDALGO, E. C. C.; COELHO, M. R.; ARAÚJO, F. O.; MOREIRA, F. M. S.; SANTOS, H. G.; BREFIN, M. L. M. S.; HUISING, J. **Levantamento do uso da terra de seis áreas amostrais relacionadas ao projeto "Conservation and sustainable management of below-ground biodiversity: Phase 1", Município de Benjamin Constant (AM)**. Seropédica: Embrapa, 2005. Apostila.

FRED, E. B.; WAKSMAN, S. A. **Laboratory manual of general microbiology**. New York: McGraw-Hill Book, 1928. 143 p.

GU, G.; WANG, N.; CHANEY, N.; SMITH, L.; LU, S-E. AmbR1 is a key transcriptional regulator for production of antifungal activity of *Burkholderia contaminans* strainMS14. **FEMS Microbiology Letter**, Amsterdam, v. 297, n. 1, p. 54-60, Aug. 2009.

HARA, F. A. S.; OLIVEIRA, L. A. Características fisiológicas e ecológicas de isolados de rizóbios oriundos de solos ácidos e álicos de Presidente Figueiredo, Amazonas. **Acta Amazônica,** Manaus, v. 34, n. 3, p. 343-357, jul./set. 2004.

HAUBOLD, B.; HUDSON, R. R. LIAN 3.0: detecting linkage disequilibrium in multilocus data, linkage analysis. **Bioinformatics,** Oxford, v. 16, n. 9, p. 847-848, Sept. 2000.

JESUS, E. C.; FLORENTINO, L. A.; RODRIGUES, M. I. D.; OLIVEIRA, M. S.; MOREIRA, F. M. S. Leguminosae nodulating bacteria diversity from three land use systems in Brazilian Western Amazon. **Pesquisa Agropecuária Brasileira**, Brasília, v. 40, n. 8, p. 769-776, ago. 2005.

JOLLEY, K. A., CHAN, M.S.; MAIDEN, M. C. mlstdbNet -distributed multi-locus sequence typing (MLST) databases. **BMC Bioinformatics**, Oxford, v. 5, n. 86, p. 1-8, July 2004.

KIMURA, M. A. simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. **Journal of Molecular Evolution,** New York, v. 16, n. 2, p. 111-120, June 1980.

KUMAR, S.; TAMURA, K.; NEI, M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. **Briefings in Bioinformatics**, London, v. 5, n. 2, p. 150-163, June 2004.

LAGUERRE, G.; BARDIN, M.; AMARGER, N. Isolation from soil of symbiotic and nonsymbiotic *Rhizobium leguminosarum* by DNA hybridization. **Canadian Journal of Microbiology**, Ottawa, v. 39, n. 12, p. 1142-1149, Dec. 2001.

LEE, C.H.; KEMPF, H. J.; LIM, Y.; CHO, Y. L. Biocontrol activity of *Pseudomonas cepacia* AF2001 and anthelmintic activity of its novel metabolite, Cepacidine A. **Wourld Journal of Microbiology and Biotechnology**, Seoul, v. 10, n. 4, p. 568-571, July 2000.

LI, X.; QUAN, C.S.; YU, H.Y.; WANG, J.H.; FAN, S.D. Assessment of antifungal effects of a novel compound from *Burkholderia cepacia* against *Fusarium solani* by fluorescent staining. **Wourld Journal of Microbiology and Biotechenology**, Seoul, v. 25, n. 1, p. 151-154, Jan. 2009.

LIMA, A. S.; NÓBREGA, R. S. A.; BARBERI, A.; SILVA, K.; FERREIRA, D. F.; MOREIRA, F. M. S. Nitrogen-fixing bacteria communities occurring in soils under different uses in the Western Amazon Region as indicated by nodulation of siratro (*Macroptilium atropurpureum*). **Pland and Soil**, The Hague, v. 319, n. 1/2, p. 127-145, June 2009.

MAHENTHIRALINGAM, E.; BISCHOF, J.; BYRNE, S. K.; RADOMSKI, C.; DAVIES, J. E.; AV-GAY, Y.; VANDAMME, P. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia cepacea* genovars I and III. **Journal of Clinical Microbiology,** Washington, v. 38, n. 9, p. 3165-3173, Sept. 2000.

MOON, S.-S.; KANG, P. M.; PARK, K. S.; KIM, C. H. Plant growth promoting and fungicidal 4-quinolinones from *Pseudomonas cepacia.* **Phytochemistry,** New York, v. 42, n. 2, p. 365-368, May 1996.

MOULIN, L.; MUNIVE, A.; DREYFUS, B.; MASSON, C. B. Nodulation of legumes by members of the ß-subclass of proteobacteria. **Nature**, London, v. 411, n. 6840, p. 948-950, June 2001.

NAHAS, E. Factors determining rock phosphate solubilization by microorganisms isolated from soil. **Wourld Journal of Microbiology and Biotechnology**, Seoul, v. 12, n. 6, p. 567-572, Nov. 1996.

NASER, S. M.; DAWYNDT, P.; HOSTE, B.; GEVERS, D.; VANDEMEULEBROECKE, K.; CLEENWERCK, I.; VANCANNEYT, M.; SWINGS, J. Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 57, n. 12, p. 2777-2789, Dec. 2007.

PEIX, A.; MATEOS, P. F.; RODRIGUEZ-BARRUECO, C.; MARTINEZ-MOLINA, E.; VELAZQUEZ, E. Growth promotion of common bean (*Pasheolus vulgaris* L.) by a strain of *Burkholderia cepacia* under growth chamber conditions. **Soil Biology and Biochemistry**, Elmsford, v. 33, n. 14, p. 1927-1935, Nov. 2001.

PEREIRA, E. G. **Diversidade de rizóbios isolados de diferentes sistemas de uso da terra na Amazônia.** 2000. 93p. Tese (Doutorado em Ciência do Solo) – Universidade Federal de Lavras, Lavras.

POT, B.; VANDAMME, P.; KERSTERS, K. Analysis of electrophoretic whole-organism protein fingerprints. In: GOODFELLOW, M.; O'DONNELL, A. G. (Ed.). **Chemical Methods in Prokaryotic Systematics**, Chichester: J. Wiley, 1994. p. 493-521.

RASOLOMAMPIANINA, R.; BAILLY, X.; FETIARISON, R.; RABEVOHITRA, R.; BÉNA, G.; RAMAROSON, L.; RAHERIMANDIMBY, M.; MOULIN, L.; DE LAJUDIE, P.; DREYFUS, B.; AVARRE, J.C. Nitrogen-fixing nodules from rose wood legumes trees (*Dalbergia* spp.) endemic to Madagascar host seven different genera belonging to α- and ß-Proteobacteria. **Molecular Ecology,** Oxford, v. 14, n. 13, p. 4135-4146, Nov. 2005.

ROZAS, J.; LIBRADO, P.; SÁNCHEZ-DELBARRIO, J. C.; MESSEGUER, X.; ROZAS, R. **DNA Sequence Polumorphism version 4.1.** Barcelona: Universitat de Barcelona, 2009. Available in: <http://www.ub.es/dnasp/DnaSP32Inf.html >. Access: 18 Apr. 2009.

ROZAS, J.; SANCHEZ-DELBARRIO, J. C.; MESSEGUER, X.; ROZAS, R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. **Bioinformatics**, Oxford, v. 19, n. 18, p. 2496-2497, Dec. 2003.

SMITH, M. J.; SMITH, N. H.; O'ROURKE, M.; SPRATT, B. G. How clonal are bacteria? **Proceedings of the National Academy of Sciences,** Washington, v. 90, n. 10, p. 4384-4388, May 1993.

TAMURA, K.; DUDLEY, J.; NEI, M.; KUMAR, S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. **Molecular Biology and Evolution**, Chicago, v. 24, n. 8, p. 1596-1599, Aug. 2007.

TAYEB, L. A.; LEFEVRE, M.; PASSET, V.; DIANCOURT, L.; BRISSE, S.; GRIMONT; P. A. D. Comparative phylogenies of Burkholderia, Ralstonia, Comamonas, Brevundimonas and related organisms derived from rpoB, gyrB and rrs gene sequences. **Research in Microbiology**, Paris, v. 159, n. 3, p. 169-177, Apr. 2008.

TROPICAL SOIL BIOLOGY AND FERTILITY INSTITUTE, CIAT. Conservation and Sustainable Management of Below Ground Bio Diversity. **Projeto BiosBrasil**. Available in: < http://www.unb.br/ib/zoo/bios/>. access: 15 july 2009.

VANDAMME, P.; GORIS, J.; CHEN, W. M.; DE VOS, P.; WILLENS, A. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. **Systematic Applied Microbiology**, Amsterdam, v. 25, n. 4, p. 507-512, Dec. 2002.

VANDAMME, P.; GOVAN, J.; LIPUMA, J. Diversity and role of *Burkholderia* spp. In: COENYE, T.; VANDAMME, P (Ed.). ***Burkholderia*:** molecular microbiology and genomics. Wymondham: Horizon Scientific, 2007. p.1-28.

VANLAERE, E.; BALDWIN, A.; GEVERS, D.; HENRY, D.; DE BRANDT, E.; LIPUMA, J. J.; MAHENTHIRALINGAM, E.; SPEERT, D. P.; DOWSON, C.; VANDAMME, P. Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. **International Journal of Systematic and Evolutionary Mcrobiology**, Spencers Wood, v. 59, n. 1, p. 102-111, Jan. 2009.

YABUUCHI, E.; KOSAKO, Y.; OYAIZU, H.; YANO, I.; HOTTA, H.; HASHIMOTO,Y.; EZAKI, T.; ARAKAWA, M. Proposal of *Burkholderia* gen. nov. and transfer of seven species of genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes, 1981) comb. nov. **Microbiology and Immunology**, Tokyo, v. 36, n. 12, p. 1251-1275, Dec. 1992.

CHAPTER 3

Cupriavidus necator ISOLATES ARE ABLE TO NODULATE AND FIX NITROGEN IN DIFFERENT LEGUME SPECIES

1 RESUMO

Espécies de bactérias que nodulam e fixam nitrogênio em Leguminosae representa um grupo importante de organismos cuja diversidade tem sido revelada constantemente. Isto é relevante para β-proteobactéria, que foi recentemente identificada como simbionte da família Leguminosae. Este trabalho foi realizado com o objetivo de identificar, por meio de testes fenotípicos e genotípicos, 45 isolados com características culturais similares (crescimento rápido e reação alcalina em meio de cultura e baixa produção de exopolissacarídeos), dos quais cinco foram identificados como pertencentes ao gênero *Cupriavidus* por meio do sequenciamento parcial do gene 16S rDNA em um trabalho anterior. Estes isolados foram previamente obtidos a partir de nódulos radiculares de três espécies promíscuas de leguminosas inoculadas com amostras de solos coletadas próximo a plantas de *Sesbania virgata*, em Minas Gerais, Brasil. Métodos fenotípicos e genotípicos para este estudo foram análise de proteínas totais por SDS-PAGE e sequenciamento dos genes 16S rDNA e *gyrB*. Para confirmar a capacidade para nodular e fixar nitrogênio, também foi verificado a presença dos genes *nodC* e *nifH*, e um experimento com dois isolados representativos para autenticar e verificar a eficiência simbiótica com seis espécies de leguminosas: *Mimosa caesalpiniaefolia*, *Leucaena leucocephala*, *Sesbania virgata*, *Macroptilium atropurpureum*, *Phaseolus vulgaris* e *Vigna unguiculata*. Os perfis proteicos dos 45 isolados apresentaram seis grupos diferentes. O sequenciamento dos genes 16S rDNA e *gyrB* mostrou que, dos 45 isolados, 37 pertencem ao gênero *Cupriavidos* e foram identificados como *Cupriavidus necator*. Estes isolados também apresentaram os genes simbióticos *nodC* e *nifH* e dois isolados testados foram capazes de nodular cinco espécies de leguminosas diferentes. Este é o primeiro relato que demosntra que *Cupriavidus necator* pode nodular eficientemente espécies de leguminosas.

Palavras-chave: *Cupriavidus necator*, β-proteobactéria, 16S rDNA, *gyr*B, *nod*C, *nif*H, fixação simbiótica de nitrogênio.

2 ABSTRACT

Nitrogen fixing Leguminosae nodulating species represent an agriculturally important group of organisms from which diversity has been revealed day by day. This is particularly relevant for β-Proteobacteria, which were recently identified as Leguminosae symbionts. The aim of this work was to identify, by genotypic and phenotypic tests, forty-five isolates with similar cultural characteristics (fast-growth, alkali reaction in culture media and low exopolysaccharide production), from which five have been identified as belonging to the *Cupriavidus* genus by partial 16S rDNA sequencing in previous work. These isolates were previously obtained from the root nodules of three promiscuous species inoculated with pasture soil samples collected near *Sesbania virgata* plants in Minas Gerais, Brazil. Genotypic and phenotypic methods for this study were SDS-PAGE of whole-cell proteins, and 16S rRNA and *gyrB* gene sequencing. To confirm the ability to nodulate and fix N_2, we also searched for the *nodC* and *nifH* genes, and we carried out an experiment with two representative isolates to authenticate and verify their symbiotic efficiency with six species: *Mimosa caesalpiniaefolia*, *Leucaena leucocephala*, *Sesbania virgata*, *Macroptilium atropurpureum*, *Phaseolus vulgaris* and *Vigna unguiculata.* The protein profiles of the forty-five isolates were distributed among six different groups. 16S rRNA and *gyrB* sequencing showed that thirty-seven isolates belong to the species *Cupriavidus necator*. These isolates also presented the *nodC* and *nifH* genes, and the two representative isolates were able to nodulate five different promiscuous legume species. This is the first report to demonstrate that *Cupriavidus necator* can efficiently nodulate legume species.

Keywords: *Cupriavidus necator*; β-Proteobacteria; 16S rDNA; *gyr*B; *nod*C; *nif*H; Symbiotic nitrogen fixation.

3 INTRODUCTION

Nitrogen fixation is a process carried out by prokaryotes called diazotrophs. Some diazotrophic species establish a symbiotic relationship with legume species by forming root or stem nodules and thus can be named nitrogen-fixing Leguminosae-nodulating bacteria (NFLNB). Currently, there are 11 genera that are able to nodulate and fix N_2 in symbiose with Leguminosae species. NFLNB includes both α- and β- Proteobacteria. The first NFLNB members belonging to β-Proteobacteria were reported by Moulin et al. (2001). Since then, six NFLNB species of the genus *Burkholderia* (Vandamme et al., 2002; Chen et al., 2006, 2007, 2008) and one species of the genus *Cupriavidus* (Chen et al., 2001) have been described.

Sesbania virgata is a Leguminosae, of the sub-family Papilionoideae, which is native to South America. These plants can develop specific symbiosis with bacteria of the species *Azorhizobium doebereinerae* (Moreira et al., 2006). Florentino et al. (2009) evaluated the occurrence of *A. doebereinerae* and of other NFLNB near the rhizosphere of *Sesbania virgata* using five promiscuous legume species as traps. They found a group of forty-five isolates with fast-growth alkali-reactions in culture media (like *Azorhizobium*). Five of these isolates were identified as members of the *Cupriavidus* genus by 16S rDNA partial sequencing.

The genus β-Proteobacterium *Cupriavidus* was described in 1987 by Makkar & Casida, based on a single isolate obtained from soil samples. The isolate was described as *Cupriavidus necator*, and it is a non-obligate bacterial predator of fungi and bacteria. Later, Vaneechoutte et al. (2004) split the genus *Ralstonia* and described a new genus *Wautersia*, with *W. eutropha* as the type species. Then, it was verified by Vandamme & Coenye (2004) that the type

species of the genus *Wautersia* is actually *Cupriavidus necator* (Makkar & Casida, 1987), the type species of the genus *Cupriavidus*. Rule 42 of the International Code of Nomenclature of Bacteria specifies that if two or more taxa of the same rank are united, the oldest legitimate name is retained. As a consequence, the genus name *Cupriavidus* has priority over *Wautersia*. Therefore, all other members of the genus *Wautersia* were reclassified into *Cupriavidus*. Currently, the genus *Cupriavidus* comprises 11 species: *Cupriavidus necator* (Makkar & Casida, 1987); *Cupriavidus basilensis*; *Cupriavidus pauculus*; *Cupriavidus gilardii*; *Cupriavidus oxalaticus*; *Cupriavidus taiwanensis*; *Cupriavidus metallidurans* and *Cupriavidus campinensis*; *Cupriavidus respiraculi* (Vandamme and Coenye 2004, comb. nov.); *Cupriavidus laharis* and *Cupriavidus pinatubonensis* (Sato et al., 2006). Only *C. taiwanensis* is known as a NFLNB.

The product of the highly conserved 16S rDNA gene has a fundamental role in protein synthesis. Because of the highly conserved nature of the 16S rDNAs, they can not be solely used for bacterial species definition. However, they can be used for a first phylogenetic affiliation (Roselló-Mora & Amann, 2001). Thus, for closely related organisms, other genes must be used to resolve phylogenetic relationships. For example, nucleotide sequences for housekeeping protein-coding genes evolve more rapidly than 16S rDNA and may provide useful alternatives to identifications based on 16S rDNA (Tayeb et al., 2008). Thus a housekeeping gene like *gyrB* can be used because it is distributed universally among bacterial species and it has already been used to resolve phylogenetic relationships (Yamamoto & Harayama, 1998). The *gyrB* gene is responsible for the DNA gyrase B subunit, an enzyme that plays a crucial role in the replication of chromosomes by introducing negative supercoils into DNA (Watt & Hickson, 1994).

Many genes are involved in symbiotic nitrogen fixation. Some strains of β-Proteobacteria can acquire these genes from α-Proteobacteria (Chen et al., 2003; Moulin et al., 2001) or from close relatives of the same class (Andam et al., 2007). Nodulation occurs when the plant stimulates, by specific flavonoids, the expression of NFLNB genes. *nod* genes such as *nodC* are common in NFLNBs. The NodC protein acts as an *N*-acetylglucosaminyltransferase that is involved in the formation of the Nod factor backbone (Geramia et al., 1994). The *nif* genes are important because they are responsible for N_2 fixation, being the nitrogenase iron protein gene *nifH* a good fixation marker (Ueda et al., 1995).

The aim of this work is to identify, by genotypic and phenotypic tests, forty-five isolates with similar cultural characteristics (fast-growth alkali reaction in culture media and low exopolysaccharide production), from which five have been identified as belonging to the *Cupriavidus* genus by partial 16S rDNA sequencing in previous work.

4 MATERIAL AND METHODS

4.1 Soil sampling and bacteria trapping

Isolates studied in this work were obtained by Florentino et al. (2009). In their work, soil samples near five *Sesbania virgata* plants were collected in February 2006 from distinct pasture areas in the state of Minas Gerais, Brazil. Three were located in Nepomuceno (21º14' S and 45º13' W) while the other two were in Ribeirão Vermelho (21º13' S and 45º02' W). Two composite soil samples were taken from each plant. One was taken close to the stem of each plant and another 10 m away from it, totalling ten composite samples. Samples were taken at a depth of 0-20 cm. Each composite sample was made up of four simple samples. During sampling, litter was removed and tools were flame-sterilized before each compound sampling to prevent contamination. Ten samples were kept in plastic bags and preserved in a cold chamber (4ºC) until inoculation of the soil suspensions into the following legume species: *S. virgata; Leucaena leucocephala*; *Macroptilium atropurpureum*; *Phaseolus vulgaris*; and *Vigna unguiculata*. Except for *S. virgata*, these species are promiscuous plant species capable of symbiosis with more than one NFLNB species. *S. virgata*, *L. leucocephala* and *M. atropurpureum* were grown for 40 days while *P. vulgaris* and *V. unguiculata* were grown for 30 days under greenhouse conditions. To isolate NFLNB, four nodules were randomly sampled per plant, for each of the five nodulating legume species. Nodules were disinfected with alcohol and hydrogen peroxide (H_2O_2) for 30 and 60s, respectively, and washed with sterilized water six times successively before maceration in culture medium 79. Isolates were characterized in culture medium 79 (Fred & Waksman, 1928), pH 6.8 at 28ºC. Among other isolates, forty-five were captured from promiscuous *L. leucocephala*, *P. vulgaris* and *V. unguiculata* species. These isolates showed

similar cultural characteristics when compared to the *Azorhizobium* species (fast growth with alkali reaction) (Table 1), however they have slightly higher gum production than this species. Five of them were identified as members of the *Cupriavidus* genus by 16S rDNA partial sequencing.

TABLE 1 Origin and identification of *Cupriavidus* isolates obtained from pasture soils collected near of *Sesbania virgata* plants.

Trap-plants	**Isolates**
Leucena (*Leucaena leucocephala*)	UFLA01-658 (R-39765), UFLA01-657 (R-39766), UFLA01-660 (R-39767), UFLA01-661 (R-39770), UFLA01-662 (R-39772), UFLA01-663 (R-39774), UFLA01-664 (R-39778), UFLA01-665 (R-39783), UFLA01-666 (R-39784), UFLA01-667 (R-39785), UFLA01-668 (R-39787), UFLA01-669 (R-39788), UFLA01-670 (R-39789), UFLA01-659 (R-39791), UFLA01-671 (R-39792), UFLA01-672 (R-39793), UFLA01-673 (R-39794), UFLA01-674 (R-39799), UFLA01-675 (R-39801), UFLA01-676 (R-39802), UFLA01-677 (R-39804), UFLA01-678 (R-39805)
Common bean (*Phaseolus vulgaris*)	UFLA02-129 (R-39761), UFLA02-48 (R-30762), UFLA02-52 (R-39769), UFLA02-53 (R-39771), UFLA02-55 (R-39773), UFLA02-56 (R-39775), UFLA02-57 (R-39776), UFLA02-58 (R-39777), UFLA02-59 (R-39780), UFLA02-62 (R-39782), UFLA02-65 (R-39790), UFLA02-67 (R-39795), UFLA02-69 (R-39796), UFLA02-70 (R-39797), UFLA02-71 (R-39798), UFLA02-72 (R-39800), UFLA02-73 (R-39803), UFLA02-74 (R-39806)
Cowpea (*Vigna unguiculata*)	UFLA03-40 (R-39779)

4.2 SDS-PAGE of whole-cell proteins

The forty-five isolates were grown on nutrient agar (CM3; Oxoid) supplemented with 0.04% (w/v) KH_2PO_4 and 0.24% (w/v) $Na_2HPO_4 \cdot 12H_2O$ (pH 6.8) and incubated for 48 h at 28°C. Preparation of whole-cell proteins for SDS-PAGE was performed as described previously (Pot et al., 1994). Densitometric analysis, normalization and interpolation of the protein profiles and numerical analysis using Pearson's product-moment correlation coefficient were performed using the GelCompar 4.2 software package (Applied Maths, Belgium).

4.3 16S rDNA and *gyr*B genes amplification and sequencing

16S rDNA gene sequencing was performed in representative isolates of the protein profile groups obtained previously (UFLA02-50, UFLA02-58, UFLA02-56, UFLA01-669 and UFLA02-71). The strains were grown in trypticase soy agar for two days at 28°C. Then, the DNA was prepared using the alkaline lysis procedure (Baele et al., 2000) from cells grown for two days. An aliquot of 5 µL of lysed cells was used in the 50 µL PCR reaction (0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.1 µM of each primer, 1 U *Taq* DNA polymerase, 1 X PCR Buffer and Milli-Q water). The nearly complete sequences of the 16S rRNA gene were amplified by PCR using the primers pA and (5'-AGAGTTTGATCCTGGCTGAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') (Coenye et al., 1999). For the *gyrB* PCR amplification, only isolates identified by 16S rDNA sequencing as belonging to the *Cupriavidus* genus were studied (UFLA02-58, UFLA01-669, UFLA02-71). Also, four other isolates from the largest group, obtained by protein profiling, were included (UFLA02-129, UFLA01-662, UFLA02-55 and UFLA02-73) and twenty-seven strains of the *Cupriavidus* species. The PCR reaction was performed using the primers gyrB1F (5'- GAC AAC GGC CGC GGS ATT CC-3') and gyrB2R (5'- CAC GCC GTT GTT CAG GAA SG-3') (Tayeb et al., 2008). A 5 µL of lysed cells was used for the 50 µL PCR reaction (0.2 mM

dNTP, 1.5 mM $MgCl_2$, 0.1 µM of each primer gyrB1F and gyrB2R, 1 U *Taq* DNA polymerase, 1 X PCR Buffer and Milli-Q water). The obtained PCR products were purified directly with QIAquick Gel Extraction-PCR purification columns (QIAGEN, Germany). Sequencing was performed using an ABI Prism 3130xl capillary sequencer according to the manufacturer's instructions (Applied Biosystems). The sequencing primers for 16S rDNA were those given by Coenye et al. (1999); the primers for *gyrB* were the same primers used for amplification.

The sequence results were analyzed by Bionumerics version 5.1 (Applied Maths, Belgium) and compared with EMBL-EBI sequences using the Basic Local Alignment Search Tool. The obtained sequences were aligned with clustalW and phylogenetic trees were inferred by the neighbour-joining method, constructed using the Bionumerics version 5.1 software.

4.4 *nod*C and *nif*H genes amplification and sequencing

All forty-five isolates were examined for the *nod*C gene. However, for *nodC* sequencing we chose the same isolates from which the 16S rDNA and *gyr*B genes were sequenced (UFLA02-58, UFLA01-669, UFLA02-71, UFLA01-662, UFLA02-55 and UFLA02-73), with the exception of isolate UFLA02-129. *nifH* was also sequenced for the same isolates.

The *nod*C DNA sequence was amplified using the primers nodCF (5'-AYGTHGTYGAYGAACGGTTC-3') and nodCI (5'-CGYGACAGCCANTTCKKCTTATTG-3') (Laguerre et al., 2001). A 5 µL aliquot of genomic DNA was used for the 50 µL PCR reaction (0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.8 µM of each primer nodCF and nodCI, 1 U *Taq* DNA polymerase, 1 X PCR Buffer and Milli-Q water). For *nif*H, the DNA was amplified using the primer described by Ueda et al. (1995), 19F (5'-GCIWTYTAYGGIAARGGIGG-3') and 407R (5'-

AAICCRCCRCAIACIACRTC - 3'). A 50 µl PCR reaction (0.2 mM dNTP, 2.5 mM $MgCl_2$, 2 µM of each primer 19F and 407R, 1 U *Taq* DNA polymerase, 1 X PCR Buffer and Milli-Q water). The PCR products were directly sequenced on both strands using the same primers utilized in the PCR amplification. Sequencing was performed in a 3730xl sequencer.

The obtained sequences were aligned with clustalW (Thompson et al., 1994). Phylogenetic trees were inferred using the Maximum Parsimony method, as implemented in the MEGA 4.1 package (Tamura et al., 2007). A bootstrap confidence analysis was performed with 1,000 replicates.

4.5 Glasshouse experiment

In previous work, Florentino et al. (2009) tested four isolates from this current study to verify their ability to nodulate legume species (following Koch's Postulates) Isolate UFLA01-657 was tested in *L. leucocephala*, and isolates UFLA02-58, UFLA02-56 and UFLA02-52 were tested in *Phaseolus vulgaris.* These isolates represent two groups obtained by protein profiling. In this work, a glasshouse experiment examined the symbiotic abilities of two bacterial isolates from the *Cupriavidus* genus (UFLA02-129 and UFLA01-657 are representatives of the largest group obtained by protein profiling), including LMG19424^T (*C. taiwanensis*), with six legume species: *Mimosa caesalpiniaefolia* and *L. leucocephala* (sub-family Mimosoideae); and *Sesbania virgata*, *M. atropurpureum, Phaseolus vulgaris* and *Vigna unguiculata* (sub-family Papilionoideae). Seeds of *M. caesalpiniaefolia*, *L. leucocephala*, and *M. atropurpureum* were scarified in sulphuric acid (98.8%) for 5, 35, 40 and 50 minutes, respectively. For *P. vulgaris* and *V. unguiculata*, the seeds were sterilized with sodium hypochlorite (2%). Seeds of the six plants were germinated in Petri dishes with wet cotton and filter paper and then transplanted into sterilized Leonard pots (Vincent, 1970), with sand and vermiculite 1:1 (v:v)

on the top part and Jensen solution without nitrogen, diluted four times and sterilized (Jensen, 1942), in the bottom part. Seeds (four seed per pot) were inoculated with bacterial isolates and strains and cultivated on liquid medium 79 (Fred & Waksman, 1928) containing 10^9 cells ml^{-1} (1 ml $seed^{-1}$). For each species three control treatments were applied, using an efficient and/or inoculant strain as a positive control: *Burkholderia sabiae* BR3405 (for *M. caesalpiniaefolia)*, *Sinorhizobium fredii* BR827 (for *L. leucocephala), Azorhizobium doebereinerae* $BR5401^T$ (for *S. virgata),* the unidentified isolate UFLA04-0212 (for *M. atropurpureum), Rhizobium tropici* $CIAT899^T$ (for *P. vulgaris)*, and *Bradyrhizoium* sp. UFLA 03-84 (for *V. unguiculata).* The other controls without inoculation, received or not, mineral nitrogen (210 mg kg^{-1} of N). The experiment was carried out in a completely randomized design with three replicates. After 60 days, the plants were harvested, and the number of nodules and shoots were evaluated, with respect to dry matter weight. The data were analyzed statistically using the SISVAR program, version 4.3 (Ferreira, 2000), with the effects from the treatments evaluated by Scott-Knott test, with a 5% significance.

5 RESULTS

5.1 SDS-PAGE of whole-cell proteins

Whole-cell proteins were analyzed to verify the diversity among the forty-five isolates (Figure 1). After numerical analysis of the profiles, six different groups of profiles were obtained at 80% similarity levels: the first group has thirty-five isolates; the second group has two isolates (UFLA02-56 and UFLA02-69); the third group has four isolates (UFLA02-49, UFLA02-50, UFLA02-51 and UFLA02-64); the fourth group has two isolates (UFLA02-58 and UFLA03-40); the fifth (UFLA02-53) and sixth (UFLA02-61) have one isolate each. Then, based on these results, we chose representative isolates from these groups for 16S rDNA sequencing.

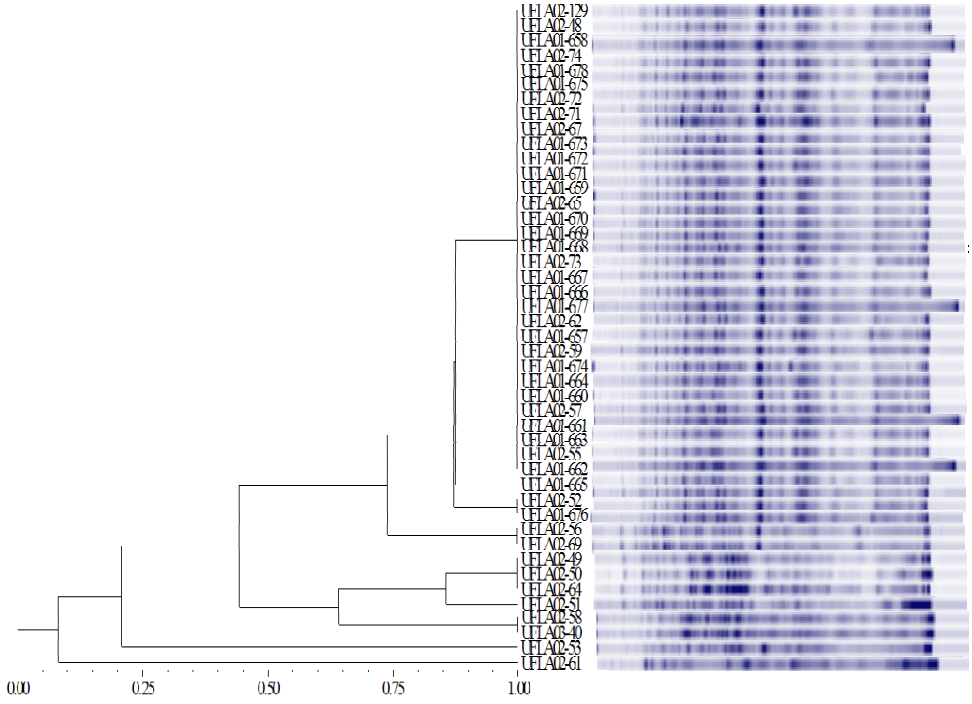

FIGURE 1 Dendrogram derived from UPGMA of correlation coefficients between the whole-cell protein patterns of *Cupriavidus* isolates obtained from the rhizosphere of *S. virgata*, captured by the root nodules of promiscuous species.

5.2 16S rDNA and *gyr*B sequencing

The 16S rDNA sequences of the different protein profile groups showed that some of them do not belong to the *Cupriavidus* genus and instead belong to *Pseudomonas* (the third group), *Mitsuaria* (the fifth group, UFLA02-53) and *Stenotrophomonas* (the sixth group, UFLA02-61) (data not shown). Isolates

UFLA01-669 and UFLA02-71 from the first group (comprising thirty-five isolates) and UFLA02-58 from the fourth group belong to the *Cupriavidus* genus. These isolates showed a high similarity with type strains of *Ralstonia eutropha*, (synonym of *Cupriavidus necator*): 99.1% (UFLA02-58); 99.8% (UFLA01-669); and 99.3% (UFLA02-71). Isolates UFLA01-669, UFLA02-58 and UFLA02-71 were grouped together into one cluster with two type strains of *C. necator* (LMG1199^T and ATCC43291^T) using a bootstrap of 95% (Figure 2).

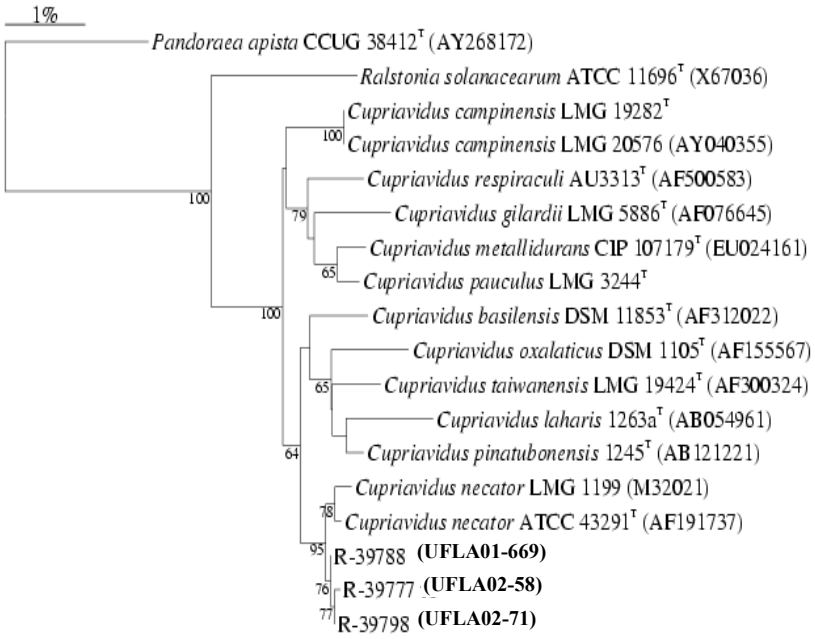

FIGURE 2 Phylogenetic tree based on 16S rDNA gene sequence similarity. Cluster analysis was based on the neighbour-joining method. Bootstrap values were based on 1000 trials.

For *gyrB* gene sequencing, apart from the three isolates identified as *C. necator* by 16S rDNA gene sequencing, we included four more isolates from the first group of protein profiles (UFLA02-129, UFLA01-662, UFLA02-55 and UFLA02-73). The *gyrB* gene fragment that was sequenced was about 400 bp, and the phylogenetic tree is shown in Figure 3. The seven isolates were also grouped in a cluster with type strains of *Cupriavidus necator*. Therefore, the topology of the *gyrB* and 16S rDNA trees showed similar results. After these results, thirty-seven out of the forty-five isolates were identified as *Cupriavidus necator.*

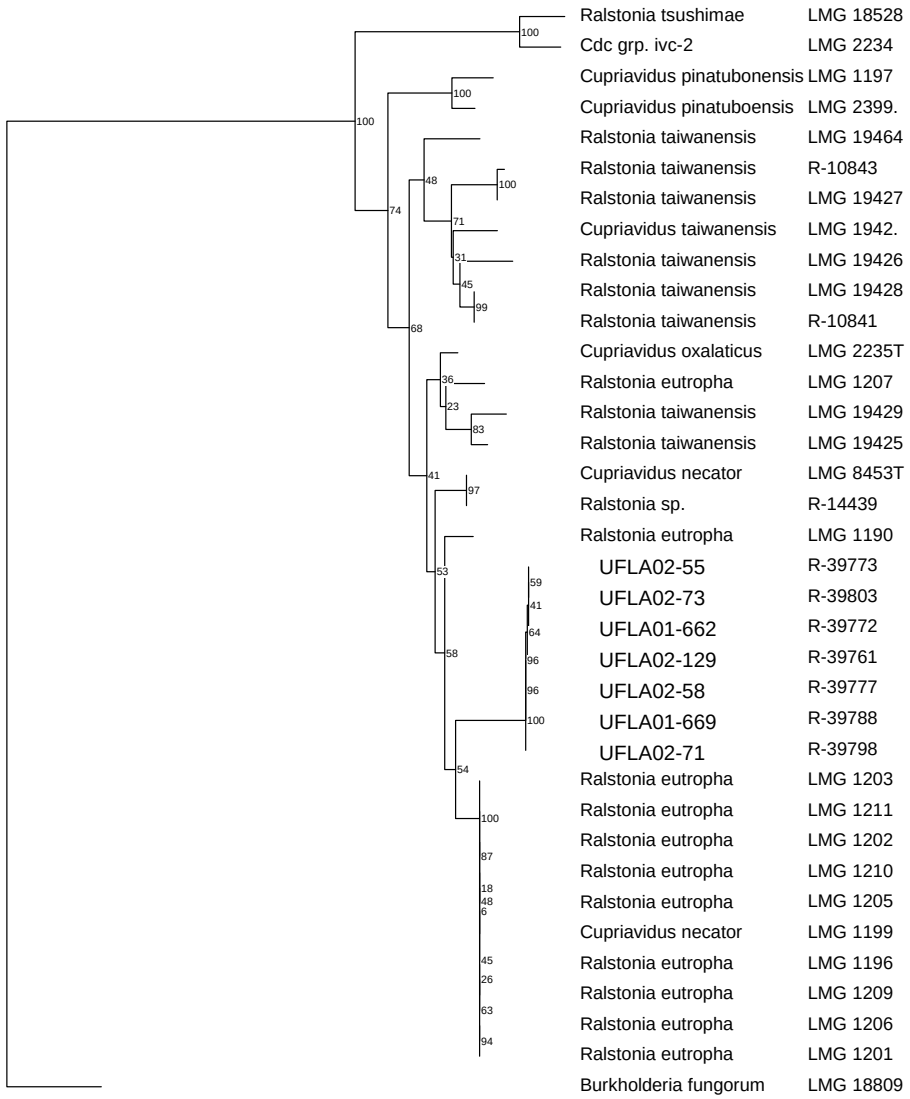

FIGURE 3 *gyrB* tree of isolates and type strains of *Curpiavidus*. The tree was constructed using the neighbour-joining method. Bootstrap values of 1000 replications.

5.3 *nodC* and *nifH* sequencing

The *nodC* gene was not detected in five isolates (UFLA02-49, UFLA03-40, UFLA01-668, UFLA02-72 and UFLA01-678) out of the thirty-seven identified as *C. necator*, probably because of problems with the reaction. Other thirty-two, the PCR product of the *nodC* gene presented an amplicon of roughly 900 bp (Figure 4A). The sequencing results from the six representative isolates (UFLA02-58, UFLA01-669, UFLA02-71, UFLA01-662, UFLA02-55 and UFLA02-73) showed two distinct sequences. The phylogenetic tree of *nodC* (Figure 4) shows five similar sequences in a separated group close to the type strain of *C. taiwanenis* LMG19424^T. Isolate UFLA02-58 presented a distinct sequence that was closest to the α-proteobacteria.

For *nifH* sequences (Figure 6), the six isolates presented an amplicon of approximately 400 bp (Figure 4B). The phylogenetic tree (Figure 6) yielded different results from those obtained from the *nodC* genes. Only one isolate was close to *C. taiwanensis* LMG19424^T the UFLA02-55. The others were grouped with the α-proteobacteria.

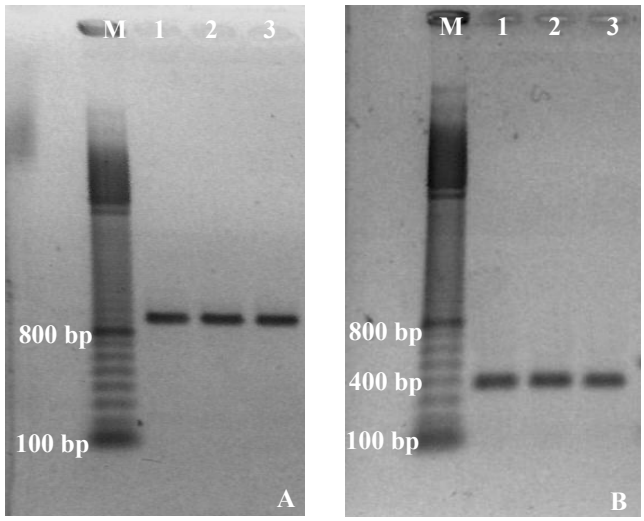

FIGURE 4 PCR product of *nod*C (A) and *nif*H (B) genes of *C. necator* isolated near the rhizosphere of *S. virgata*. M- DNA Marker: 100 Base-Pair Ladder (Amersham); 1- UFLA02-52; 2- UFLA02-129; and 3- UFLA02-48.

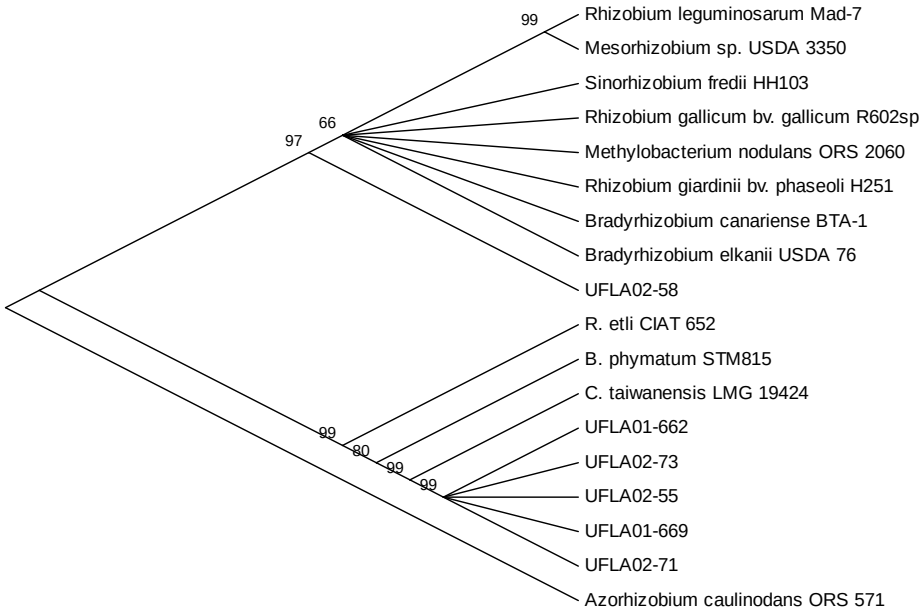

FIGURE 5 Phylogenetic tree of *nod*C genes inferred using the Maximum Parsimony method. Only bootstrap probability values greater than 50% (over 1000 replicates) are indicated at the branching points.

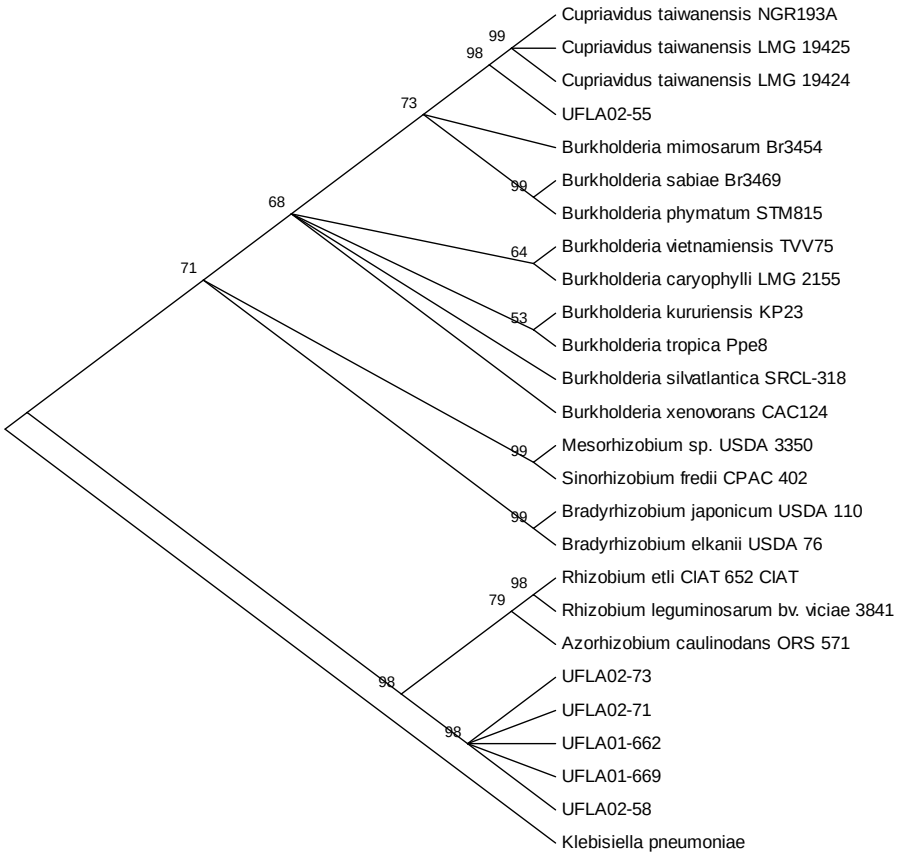

FIGURE 6 Phylogenetic tree of *nif*H genes inferred using the Maximum Parsimony method. Only bootstrap probability values greater than 50% (over 1000 replicates) are indicated at the branching points.

5.4 Glasshouse experiment

The symbiotic relationship of the six legume species to the two representative isolates is presented in Table 2. Treatment without inoculation did not present nodules, indicating the absence of contamination. It was also possible to verify that the experimental conditions were adequate for the expression of symbiosis as nodules were found in many treatments. The two isolates and the type strain of *Cupriavidus taiwanensis* were able to nodulate five of the six legume species tested. They were not able to nodulate *S. virgata,* corroborating the specificity of this species. There was high variability among the number of nodules induced by the *Cupriavidus* isolates in the different hosts. Isolate UFLA01-657 induced a higher number of nodules in a higher number of species: *M. atropurpureum* (73), *P. vulgaris* (203) and *V. unguiculata* (165). The other isolate and the type strain (UFLA02-129 and LMG19424^T) also induced a high number of nodules in *L. leucocephala* and *M. caesalpiniaefolia*, respectively. In plants from *M. caesalpiniaefolia, L. leucocephala* and *V. unguiculata*, it was possible to verify that the inoculation with *Cupriavidus* provided better nodule number than those inoculated with recommended strains.

According to the results from shoot dry matter weight (Table 3), we verified that mineral nitrogen addition afforded better development to all of the host species, except for *P. vulgaris*. In *P. vulgaris*, inoculation with CIAT899^T (positive control) and UFLA02-129 provided results that were similar to those found in the treatment with mineral nitrogen. In *M. caesalpiniaefolia*, the symbiotic relationship with *C. necator* isolates provided moderate levels of efficiency, while the inoculation in the other host plants, in general, was inefficient. Some inoculated strains were not able to fix N_2 because more time was required to establish the process or the season was not suitable.

TABLE 2 Nodule number for host legumes after inoculation with *Cupriavidus necator* isolates, the *Cupriavidus taiwanensis* LMG19424^{T} type strain and strains recommended as inoculants for each legume species [(1)].

Treatments	*Mc*[2]	*Ll*[3]	*Sv*[4]	*Ma*[5]	*Pv*[6]	*Vu*[7]
LMG 19424^{T}	10 c	146 a	0 b	16 d	21 d	7 c
UFLA01-657	16 c	47 b	0 b	73 b	203 b	165 a
UFLA02-129	106 a	15 b	0 b	22 c	158 c	7 c
Inoculant strain	63 b BR3405^{T}	59 b BR827^{T}	253 a BR5401^{T}	102 a UFLA04-212	299 a CIAT899^{T}	66 b U03-84
Negative control	0 d	0 c	0 b	0 e	0 e	0 d
Mineral N (210 mg.kg^{-1})	0 d	0 c	0 b	0 e	0 e	0 d
CV (%)	17.76	10.43	11.16	5.68	10.15	12.31

[2]*M. caesalpiniaefolia*, [3]*L. leucocephala*, [4]*S. virgata*, [5]*M. atropurpureum*, [6]*P. vulgaris*, [7]*V. unguiculata*

[(1)]Values followed by different letters in the same column are significant at 5% probability by Scott-Knott test.

TABLE 3 Shoot dry matter (g plant^{-1}) of legume species after inoculation with isolates of *Cupriavidus necator*, type strains of *Cupriavidus twainensis* and strains that were recommended as inoculants for each legume species [(1)].

Treatments	*Mc*[2]	*Ll*[3]	*Sv*[4]	*Ma*[5]	*Pv*[6]	*Vu*[7]
LMG 19424^{T}	0,11 c	0,13 b	1,07 c	0,22 c	0,40 b	0,20 c
UFLA01-657	0,20 b	0,15 b	0,38 c	0,12 c	0,59 b	0,29 c
UFLA02-129	0,20 b	0,07 b	1,06 c	0,05 c	1,24 a	0,72 c
Inoculant strain	0,13 c BR3405^{T}	0,17 b BR827^{T}	6,53 b BR5401^{T}	1,25 b UFLA04-212	1,27 a CIAT899^{T}	2,75 b U03-84
Negative control	0,14 c	0,12 b	0,57 c	0,07 c	0,27 b	0,25 c
Mineral N (210 mg.kg^{-1})	1,20 a	2,20 a	10,17 a	3,25 a	1,23 a	5,98 a
CV (%)	11.84	24.09	28.64	21.67	23.92	15.10

[2]*M. caesalpiniaefolia;* [3]*L. leucocephala,* [4]*S. virgata,* [5]*M. atropurpureum,* [6]*P. vulgaris,* [7]*V. unguiculata*

[(1)]Values followed by different letters in the same column are significant at 5% probability by Scott-Knott test.

Nodulation by isolates of *C. necator* were confirmed by re-isolation from the nodules and identified by cultural characteristics.

6 DISCUSSION

The occurrence of nitrogen-fixing Leguminosae-nodulating bacteria (NFLNB) belonging to genus *Cupriavidus* and nodulating Papilionoideae in Brazilian soils was reported recently (Florentino et al., 2009). The sequences of 16S rRNA from these isolates showed a high similarity and a close phylogenetic relationship with *Cupriavidus necator.* This result was confirmed by sequencing a housekeeping gene, *gyrB*, which was used for *Cupriavidus* spp. identification, as demonstrated by Tayeb et al. (2008).

This is the first time that isolates of the *Cupriavidus necator* species have been reported to induce effective root nodules. Until now, only *Cupriavidus twainensis* was known as a NFLNB. This species was isolated from *Mimosa* plants in Taiwan, India, Costa Rica and EUA (Chen et al, 2001; Chen et al., 2003; Verna et al., 2004; Chen et al., 2006; Barret & Parker, 2006; Adam et al., 2007).

C. necator was described by Makkar & Casida (1987) as a non-obligate predator of soil bacteria isolated from soil in the vicinity of University Park, PA, USA. This species occurs in soil and human clinical specimens (Vandamme & Coenye, 2004). Some strains are able to degrade chloroaromatic compounds (Don & Pemberton, 1981) and are resistant to heavy metals (Mergeay et al., 1985). Other strains have been studied for their ability to produce poly-β-hydroxyalkanoates, which have industrial applications (Ramsay et al., 1990; Madison & Huisman, 1999).

The genome of *C. taiwanenis* (Amadou et al., 2008) has a high similarity with the genome of C. *necator* (syn. *C. eutrophus* H16), yet they have divergent plasmids. Genome analysis revealed that *C. taiwanensis* has *nod* and *nif* genes located in the plasmid. Thus, it uses the same strategy for nodulation as

classical rhizobia. However, no *nod* and *nif* genes were reported in the genome of *C. necator* until now. *C. necator* and *C. taiwanensis* contain genes that are associated with virulence, being phylogenetically close to pathogens. This may suggest that the bacteria genus is genetically adapted to ecological transitions between mutualism and parasitism in either sense.

In our study, we verified that the *C. necator* isolates presented the ability to nodulate five of the six different hosts tested. These five were all promiscuous host species. Nodulation was only absent in *S. virgata,* which shows a high degree of symbiotic specificity with *Azorhizobium doebereinerae* (Gonçalves & Moreira, 2004; Moreira et al., 2006; Florentino & Moreira, 2007). These results corroborated a previous report by Florentino et al. (2009) concerning *P. vulgaris* and *L. leucocephala* and showed that *C. necator* were was also able to nodulate *M. caesalpiniaefolia*, *M. atropurpureum* and *V. unguiculata*. Our results showed a high symbiotic efficiency for *C. necator* with *P. vulgaris* and a moderate efficiency with *M. caesalpiniaefolia*, in addition to inefficient symbiotic relationships with *L. leucocephala* and *V. unguiculata*.

The *nodC* genes were detected in the isolates of this work, even when primers designed for α-Proteobacteria from the sequences of *Rhizobium*, *Sinorhizobium*, *Mezorhizobium* and *Bradyrhizobium* (Laguerre et al., 2001) were used. The *nodC* sequences were close to those from *C. tawianensis,* but they have a common origin with α-Proteobacteria. The *nodABC* genes have been characterized as common *nod* genes in α-Proteobacteria. The phylogeny of the *nifH* genes was not congruent with the phylogeny of the *nod*C gene, and only one isolate was close to *C. twainensis*, even though the *nodC* and *nifH* sequences have a common origin in α-Proteobacteria. These isolates could have acquired these genes from α-Proteobacteria, corroborating the results from other works in which strains of β-Proteobacteria probably obtained their symbiotic genes

through lateral transfer from α-Proteobacteria (Moulin et al., 2001; Chen et al., 2003; Verma et al., 2004).

7 CONCLUSIONS

Thirty-seven isolates were identified as belonging to *Cupriavidus necator*.

These isolates were able to nodulate and fix N_2 in different legume species.

This is the first work to demonstrate that *C. necator* can nodulate legume species.

8 REFERENCES

AMADOU, C.; PASCAL, G.; MANGENOT, S.; GLEW, M.; BONTEMPS, C.; CAPELA, D.; CARRERE, S.; CRUVEILLER, R.; DOSSAT, C.; LAJUS, A.; MARCHETTI, M.; POINSOT, V.; ROUY, Z.; SERVIN, B.; SAAD, M.; SCHENOWITZ, C.; BARBE, V.; BATUT, J.; MEDIGUE, C.; MASSON-BOIVIN, C. Genome sequence of the β-rhizobium *Cupriavidus taiwanenis* and comparative genomics of rhizobia. **Genome Research**, Cold Spring Harbor, v. 18, n. 9, p. 1472-1483, Sept. 2008.

ANDAM, C. P.; MONDO, S. J.; PARKER, M.A. Monophyly of *nodA* and *nifH* Genes across Texan and Costa Rican Populations of *Cupriavidus* Nodule Symbionts. **Applied and Environmental Microbiology**, Washington, v. 73, n. 14, p. 4686-4690, July 2007.

BAELE, M.; BAELE, P.; VANEECHOUTTE, M.; STORMS, V.; BUTAYE, P.; DEVRIESE, L. A.; .VERSCHRAEGEN, G.; GILLIS, M.; HAESEBROUCK, F. Application of tRNA intergenic spacer PCR foridentificationof Enterococcus species. **Journal of Clinical Microbiology**, Washington, v. 38, n. 11, p. 4201-4207, Nov. 2000.

BARRET, C. F.; PARKER, M. A. Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. In Costa Rica. **Applied and Environmental Microbiology**, Washington, v. 72, n. 2, p. 1198-1206, Feb. 2006.

CHEN, W. M.; FARIA, S. M.; CHOU, J. H.; JAMES, E. K.; ELLIOTT, G. N.; SPRENT, J. I.; BONTEMPS, C.; YOUNG, J. P. W.; VANDAMME, P. *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia*. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 58, n. 9, p. 2174-2179, Sept. 2008.

CHEN, W. M.; FARIA, S. M.; JAMES, E. K.; ELLIOTT, G. N.; LIN, K. Y.; CHOU, J. H.; SHEU, S. Y.; CNOCKAERT, M.; SPRENT, J. I.; VANDAMME, P. *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 57, n. 5, p. 1055-1059, May 2007.

CHEN, W. M.; JAMES, E. K.; COENYE, T.; CHOU, J. H.; BARRIOS, E.; DE FARIA, S. M.; ELLIOTT, G. N.; SHEU, S. Y.; SPRENT, J. I.; VANDAMME, P. *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 56, n. 8, p. 1847-1851, Aug. 2006.

CHEN, W. M.; JAMES, E. K.; PRESCOTT, A. R.; KIERANS, M., SPRENT, J. I. Nodulation of *Mimosa* spp. by the β-proteobacterium *Ralstonia taiwanensis*. **Molecular Plant-Microbe Interactions**, Saint Paul, v. 16, n. 12, p. 1051-1061, 2003.

CHEN, W. X.; LAEVENS, S.; LEE, T. M.; COENYE, T.; DE VOS, P.; MERGEAY, M.; VANDAMME, P. *Rasltonia taiwanensis* sp. nov. isolated from nodules of *Mimosa* species and sputum of a cystic fibrosis patient. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 51, n. 5, p. 1729-1735, Sept. 2001.

COENYE, T.; FALSENT, E.; VANCANNEYT, M.; HOSTE, B.; GOVANT, J. R. W.; KERSTERS, K.; VANDAMME, P. Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. **International Journal of Systematic Bacteriology,** Spencers Wood, v. 49, n. 2, p. 405-413, Apr.1999.

DON, R. H.; PEMBERTON, J. M. Properties of Six Pesticide Degradation Plasmids Isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. **Jounal of Bacteriology**, Washington, v. 145, n. 2, p. 681-686, Feb. 1981.

FERREIRA, D. F. Análises estatísticas por meio do SISVAR para Windows versão 4. 0. In: REUNIÃO ANUAL DA REGIÃO BRASILEIRA DA SOCIEDADE INTERNACIONAL DE BIOMETRIA, 45., 2000, São Carlos. **Anais...** São Carlos: UFSCar, 2000. p. 235.

FLORENTINO, L. A.; GUIMARAES, A. P.; RUFINI, M.; SILVA, K.; MOREIRA, F. M. S. *Sesbania virgata* stimulates the occurrence of its microsymbiont in soils but does not inhibit microsymbionts of other species. **Scientia Agricola**, Piracicaba, v. 66, n. 5, p. 667-676, set. 2009.

FRED, E. B.; WAKSMAN, S. A. **Laboratory manual of general microbiology**. New York: McGraw-Hill Book, 1928. 143 p.

GONÇALVES, M.; MOREIRA, F. M. S. Specificity of the Legume *Sesbania virgata* (Caz.) Pers. and its Nodule Isolates *Azorhizobium johannae* with other Legume Hosts and Rhizobia. I. **Symbiosis**, Phyladelphia, v. 36, n. 1, p. 57-68, Jan. 2004.

JORDAN, D. C. Rhizobiaceae Conn 1938. In: KRIEG, N. R.; HOLT, J. D. BERGEYS'S. **Manual of systematic bacteriology.** London: Willians and Wilkins, 1984. p. 234-244.

LAGUERRE, G.; NOUR, S. M.; MACHERET, V.; SANJUAN, J.; DROUIN, P.; AMARGER, N. Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. **Microbiology**, v. 147, n. 4, p. 981-993, Apr. 2001.

MADISON, L. L.; HUISMAN, G. W. Metabolic Engineering of poly(3-Hydroxyalkanoates): from DNA to plastic. **Microbiology and Molecular Biology Reviews**, New York, v. 63, n. 1, p. 21-53, Mar. 1999.

MAKKAR, N. S.; CASIDA, L. E. J.R. *Cupriavidus necator* gen. nov., sp. nov.: a nonobligate bacterial predator of bacteria in soil. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 37, n. 4, p. 323-326, Oct. 1987.

MARGEAY, M.; NIES, D.; SCHLEGEL, H. G.; GERITS, J. CHARLES, P. VAN GIJSEGEM, F. *Alcaligenes eutrophus* CH34 Is a Facultative Chemolithotroph with Plasmid-Bound Resistance to Heavy Metals. **Journal of Bacteriology**, Washington, v. 162, n. 1, p. 328-334, Apr. 1985.

MOULIN, L.; MUNIVE, A.; DREYFUS, B.; MASSON, C. B. Nodulation of legumes by menbers of the β-subclass of Proteobacteria. **Nature**, London, v. 411, p. 948-950, June 2001.

POT, B.; VANDAMME, P.; KERSTERS, K. Analysis of electrophoretic whole-organism protein fingerprints. In: GOODFELLOW, M.; O'DONNELL, A. G. (Ed.). **Chemical Methods In Prokaryotic Systematics** Chichester: J. Wiley, 1994. p. 493-521.

RAMSAY, B. A.; LOMALIZA, K.; CHAVARIE, C.; DUBE, B.; BATAILLE, P. Production of poly-(ρ-hydroxybutyric-co-3-hydroxyvaleric) acids. **Applied and Environmental Microbiology**, Washington, v. 56, n. 7, p. 2093-2098, July 1990.

ROSSELLÓ-MORA, R.; AMANN, R. The species concept for prokaryotes. **FEMS Microbiology Reviews**, Amsterdam, v. 25, n. 1, p. 39-67, Jan. 2001.

SATO, Y.; NISHIHARA, H.; YOSHIDA, M.; WATANABE, M.; RONDAL, J. D.; CONCEPCION, R. N.; OHTA, H. *Cupriavidus pinatubonensis* sp. nov. and *Cupriavidus laharis* sp. nov., novel hydrogen-oxidizing, facultatively chemolithotrophic bacteria isolated from volcanic mudflow deposits from Mt. Pinatubo in the Philippines. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 56, n. 5, p. 973-978, May 2006.

TAMURA, K.; DUDLEY, J.; NEI, M.; KUMAR, S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. **Molecular Biology and Evolution**, Chicago, v. 24, n. 8, p. 1596-1599, Aug. 2007.

TAYEB, L. A.; LEFEVRE, M.; PASSET, V.; DIANCOURT, L.; BRISSE, S.; GRIMONT; P. A. D. Comparative phylogenies of *Burkholderia*, *Ralstonia*, *Comamonas*, *Brevundimonas* and related organisms derived from *rpoB*, *gyrB* and rrs gene sequences. **Research in Microbiology**, Paris, v. 159, n. 3, p. 169-177, Apr. 2008.

THOMPSON, J. D.; HIGGINS, D.G.; GIBSON, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. **Nucleic Acids Research**, Oxford, v. 22, n. 22, p. 4673-4380, Nov. 1994.

UEDA, T.; SUGA, Y.; YAHIRO, N.; MATSUGUCHI, T. Remarkable N2-fixing Bacterial Diversity Detected in Rice Roots by Molecular Evolutionary Analysis of *nif*H gene sequences. **Journal of Bacteriology**, Washington, v. 177, n. 5, p. 1414-1417, Mar. 1995.

VANDAMME, P.; COENYE, T. Taxonomy of the genus *Cupriavidus*: a tale of lost and found. **International Journal of Systematic and Evolutionary Microbiology**, Spencers Wood, v. 54, n. 6, p. 2285-2289, Nov. 2004.

VANDAMME, P.; GORIS, J.; CHEN, W. M.; DE VOS, P.; WILLEMS, A. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. **Systematic and Applied Microbiology**, Amsterdam, v. 25, n. 4, p. 507-512, Dec. 2002.

VANEECHOUTTE, M.; KAMPFER, P.; DE BAERE, T.; FALSEN, E.; VERSCHRAEGEN, G. *Wautersia* gen. nov., a novel genus accommodating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of *Ralstonia* [*Pseudomonas*] syzygii (Roberts et al.1990) comb. nov. **International Journal of Systematic and Evolutionary Microbiology,** Spencers Wood, v. 54, n. 2, p. 317-327, Mar. 2004.

VERMA, S. C.; CHOWDHURY, S. P.; TRIPATHI, A. K. Phylogeny based on 16S rDNA and *nif*H sequences of *Rasltonia taiwanensis* strains isolated from nitrogen-fixing nodules of *Mimosa pudica*, in India. **Canadian Journal of Microbiology**, Ottawa, v. 50, n. 5, p. 313-322, May 2004.

WATT, P. M.; HICKSON, I. D. Structure and function of type II DNA topoisomerases. **Biochemistry Journal**, Oxford, v. 303, n. 3, p. 681-695, Nov. 1994.

YAMAMOTO, S.; HARAYAMA, S. Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of *gyr*B, *rpo*D and 16S rRNA genes. **International Journal of Systematic Bacteriology**, Spencers Wood, v. 48, n. 3, p. 813-819, July 1998.