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Characterization of amplified polymerase chain reaction *glnB* and *nifH* gene fragments of nitrogen-fixing *Burkholderia* species

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ABSTRACT

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Aims: To clone and sequence polymerase chain reaction (PCR)-amplified *glnB* and *nifH* genes of the nitrogen-fixing bacteria *Burkholderia brasilensis* strain M130, *B. tropicalis* strain PPe8 and *B. kururiensis* strain KP23.

Methods and Results: The *glnB* and *nifH* gene fragments were amplified by PCR using universal degenerated primers. A very high percentage of similarity for the *nifH* (100%) and *glnB* (96%) genes was observed between strains M130 and KP23. A similarity of 100% for the *nifH* gene was also observed between strains M130 and PPe8. However, the identity for the *glnB* gene was 98% and the similarity 88%. The phylogenetic tree of the *nifH* gene showed a very high degree of similarity to the 16S rDNA gene.

Conclusions: The nitrogen-fixing bacteria of the *Burkholderia* genus formed a cluster separated from the other species of the genus mainly when the *nifH* rather than the *glnB* gene was used to construct the phylogenetic tree.

Significance and Impact of the Study: Knowledge of the *nifH* and *glnB* gene sequences of *B. brasilensis*, *B. tropicalis* and *B. kururiensis* will support new studies on the diversity of these diazotrophs in natural environments.

Keywords: *glnB*, *nifH*, PCR, nitrogen fixation, *Burkholderia*, phylogeny.

INTRODUCTION

The genus *Burkholderia* comprises over 25 species; however, the species *Burkholderia vietnamiensis* is the only nitrogen-fixing bacterium officially described (Gillis *et al*. 1995). Two other new nitrogen-fixing bacteria belonging to the genus *Burkholderia* have been described and provisionally named *B. brasilensis* (strain M130) and *B. tropicalis* (strain PPe8) (Baldani 1996). Recently, a new species, named *B. kururiensis* (strain KP23), was identified as a trichloroethylene-degrading bacterium (Zhang *et al*. 2000) and its ability to fix nitrogen in a similar manner to strain M130 was demonstrated by De Los Santos *et al*. (2001). Two other strains (STM678 and STM815) of the genus *Burkholderia* have also been very recently described as possessing nitrogen fixation ability; furthermore, they could establish a symbiosis with legume plants (Moulin *et al*. 2001). The genus *Burkholderia* is very attractive because of its widespread ecology and physiological characteristics. It has been found in environments such as soil, water and the plant rhizosphere (Parke and Gurian-Sherman 2001).

Minerdi *et al*. (2001) were the first to identify and report the characterization of *nifHDK* genes isolated from the arbuscular fungus spores of *Gigaspora margarita* but they strongly suggested that these genes belonged to the genome of the endosymbiont *Burkholderia*.

The functional *nifH* gene has highly conserved regions as well as a great divergence in other regions; it can, therefore, be used to evaluate the phylogenetic relationships among nitrogen-fixing micro-organisms (diazotrophs) of several groups (Ueda *et al*. 1995). Since the *nifH* gene only occurs in nitrogen-fixing micro-organisms, it has been used to monitor the presence of these diazotrophs, e.g. in pure cultures (Franke *et al*. 1998), in soil (Widmer *et al*. 1999) as

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well as in plants (Ueda *et al*. 1995; Lovell *et al*. 2000). It has also been detected in arbuscular mycorrhizal fungus spores (Minerdi *et al*. 2001), marine environments (Zehr *et al*. 1998) and termite guts (Ohkuma *et al*. 1996; Kudo *et al*. 1998).

The PII protein, encoded by the *glnB* gene, is involved in the regulation of glutamine synthetase activity and the global nitrogen regulation system. It also regulates adenylyltransferase (ATase) and the NifA protein activity in several nitrogen-fixing bacteria. In addition, its role has been demonstrated in nitrate utilization (*Rhizobium leguminosarum* and *Azospirillum brasilense*) and in ammonia and methylammonia transport (*A. brasilense*) (Arcondéguy *et al*. 2001). Recently, a fragment of the *glnB* gene was cloned and sequenced from *A. amazonense* (Potrich *et al*. 2001).

The objective of this work was to partially sequence the *nifH* and *glnB* genes in different diazotrophic *Burkholderia* species and compare them with those sequences of other nitrogen-fixing organisms present in the GenBank database, with a view to understanding the diversity of these genes in nitrogen-fixing bacteria of the genus *Burkholderia*.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study were M130 (*B. brasilensis*), PPe8 (*B. tropicalis*) and KP23 (*B. kururiensis*). The first two strains were obtained from the Embrapa Agrobiologia Culture Collection (Rio de Janeiro, Brazil). Dr Yoichi Kamagata (National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan) donated the third strain.

Polymerase chain reaction amplification of the *nifH* and *glnB* genes

The total DNA was extracted according to the hexadecyltrimethylammonium bromide (CTAB) method (Sambrook *et al*. 1989). For the *nifH* gene, the degenerated primers described by Ueda *et al*. (1995) were used. The reaction mixture contained: 1 × buffer, 2·5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} deoxynucleotide triphosphate (dNTPs), 100 pmol of each primer and 2 U of Taq DNA polymerase (Promega, Madison, WI, USA) in a final volume of 50 μl. The reaction conditions with 10 ng of DNA template were: 4 min at 94 °C, 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C (for 35 cycles) and 4 min at 72 °C. The *glnB* gene primers were kindly provided by Dr Irene Schrank Universidade Federal do Rio Grande do Sul (UFRGS, Brazil): up 5′-GCCATCATTAAGCCGTTCAA-3′ and down 5′-AAGATCTTGCCGTCGCCGAT-3′. The reaction mixture contained: 1 × buffer, 2·5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTPs, 100 pmol of each primer and 1 U of Taq DNA polymerase (Promega) in a final volume of 25 μl. The reaction conditions with 200 ng of DNA template were: 5 min at 95 °C, 30 s at 52 °C, 30 s at 72 °C and 30 s at 94 °C (for 36 cycles), 2 min at 52 °C, 5 min at 72 °C. The polymerase chain reaction amplification was performed in a thermal cycler (PTC-100; MJ Research, Waltham, MA, USA). Fragments of approx. 400 bp (*nifH*) and 200 bp (*glnB*) were obtained and cloned using the pGEM-T Easy Vector (Promega). The clones were sequenced using the PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

Sequence alignment and phylogenetic analysis

Sequence data were analysed using Genedoc (Nicholas and Nicholas 1997). Sequence multiple alignments were carried out with ClustalW (Thompson *et al*. 1994) and the phylogenetic trees constructed by the neighbour-joining method (Saito and Nei 1987). The MEGA package (Kumar *et al*. 1993), with the distance of Jukes and Cantor, was used (Jukes and Cantor 1969). The other program parameters were maintained unchanged. Bootstrap analyses of 1000 replicates were established (Felsenstein 1985).

Nucleotide sequence accession numbers

The nucleotide sequences of the *nifH*, *glnB* and 16S rDNA genes of several nitrogen-fixing bacteria were obtained from GenBank and used to construct the phylogenetic trees.

RESULTS

In general, the amino acid sequences of the *glnB* gene of the *Burkholderia* species showed a similarity level higher than 82% when compared with the GlnB proteins of other nitrogen-fixing bacteria, such as *Herbaspirillum seropedicae*, *R. leguminosarum*, *Bradyrhizobium japonicum*, *A. brasilense*, *Klebsiella pneumoniae* and *Azotobacter vinelandii* (Table 1). A very high similarity level (96%) was verified between the amino acid sequences of the *glnB* gene for *B. brasilensis* strain M130 and *B. kururiensis* strain KP23. A much lower similarity level (88%) was observed between strains M130 and PPe8. On the other hand, a similarity level higher than 90% was observed between the species of the genus *Burkholderia* and *H. seropedicae*. In the phylogenetic tree of the *nifH* gene, the strains belonging to the genus *Burkholderia* formed a cluster with high bootstrap values (Fig. 1). The only exception was the *Burkholderia* strain isolated from *Gigaspora* (Minerdi *et al*. 2001), which showed a greater similarity to the species belonging to the α-Proteobacteria

Table 1 Similarity (%) of the *glnB* gene amino acid sequences

Organism	*B. kururiensis*	*B. tropicalis*	*H. seropedicae*	*R. leguminosarum*	*Br. japonicum*	*A. brasilense*	*Kl. pneumoniae*	*Az. vinelandii*
Burkholderia brasilensis AY098587 96	88	94	86	88	86	90	90	
Burkholderia kururiensis AY098589	85	91	83	85	84	88	86	
Burkholderia tropicalis AY098591		90	82	88	86	88	86	
Herbaspirillum seropedicae U86073			85	91	92	92	91	
Rhizobium leguminosarum X04880				90	86	83	84	
Bradyrhizobium japonicum M26753					94	88	86	
Azospirillum brasilense X51499						88	91	
Klebsiella pneumoniae AJ006531							86	
Azotobacter vinelandii U91902								

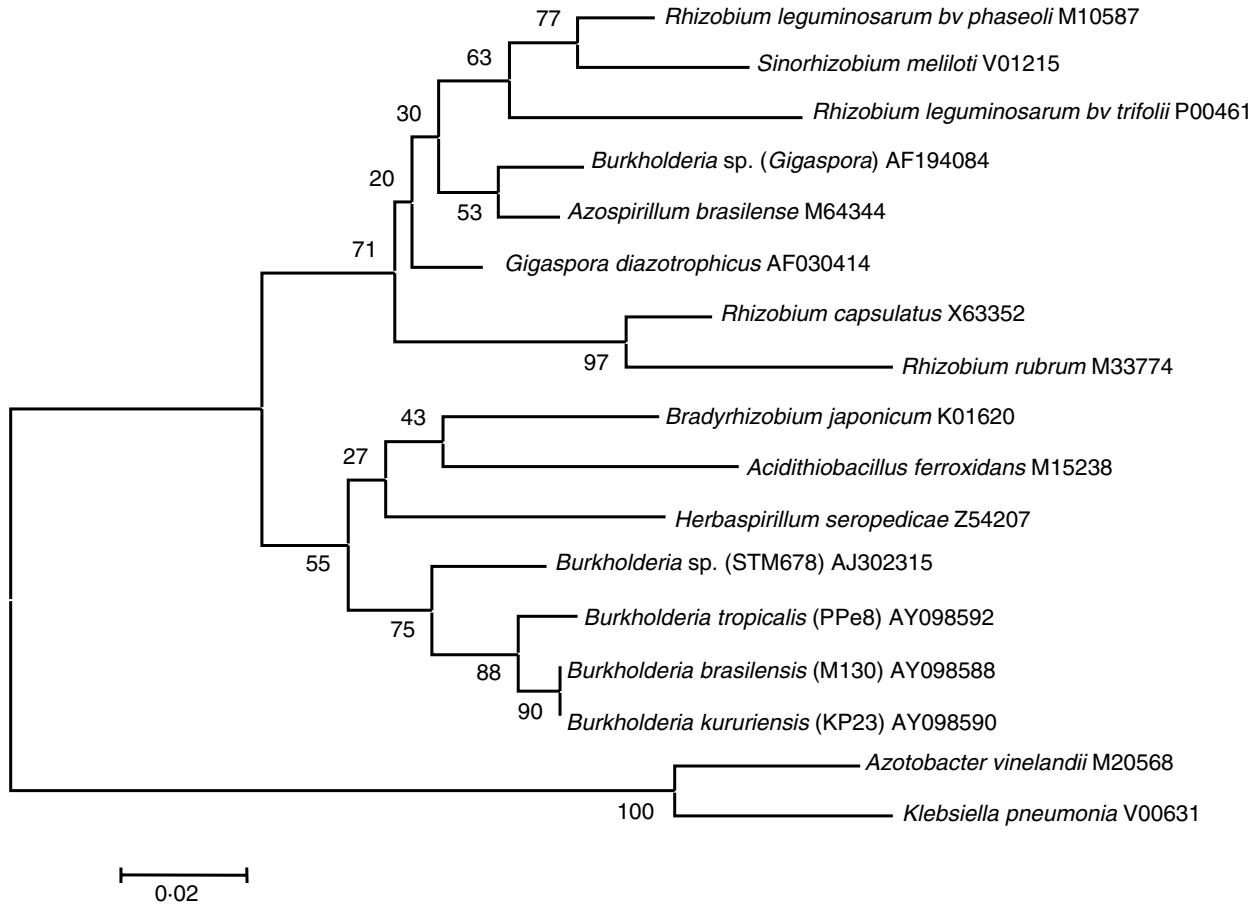

Fig. 1 Phylogenetic analysis of *nifH* gene amino acid sequences of several nitrogen-fixing bacteria constructed by the NJ method. The bootstrap values (1000 replicates) are indicated in the grouping base. The scale bar indicates the distance in substitutions by nucleotide

group. The phylogeny of the *nifH* genes was consistent with that obtained with the 16S rDNA gene (Figs 1 and 2). Although the bootstrap values were low (Fig. 1), the phylogenetic trees obtained with the partial sequences were always consistent with those obtained for the complete *nifH* gene sequences (Young 1992; Ueda *et al.* 1995; Achouak *et al.* 1999).

Very high similarity (above 99%) was observed among the amino acid sequences of the *nifH* gene for the species of the genus *Burkholderia* tested (*B. brasilensis*, *B. kururiensis*, *B. tropicalis* and *Burkholderia* sp. strain STM678). *Burkholderia tropicalis* (strain PPe8), isolated from sugar cane (Baldani 1996; Baldani *et al.* 2000), showed high identity with *B. brasilensis* (98%), *B. kururiensis* (98%) and

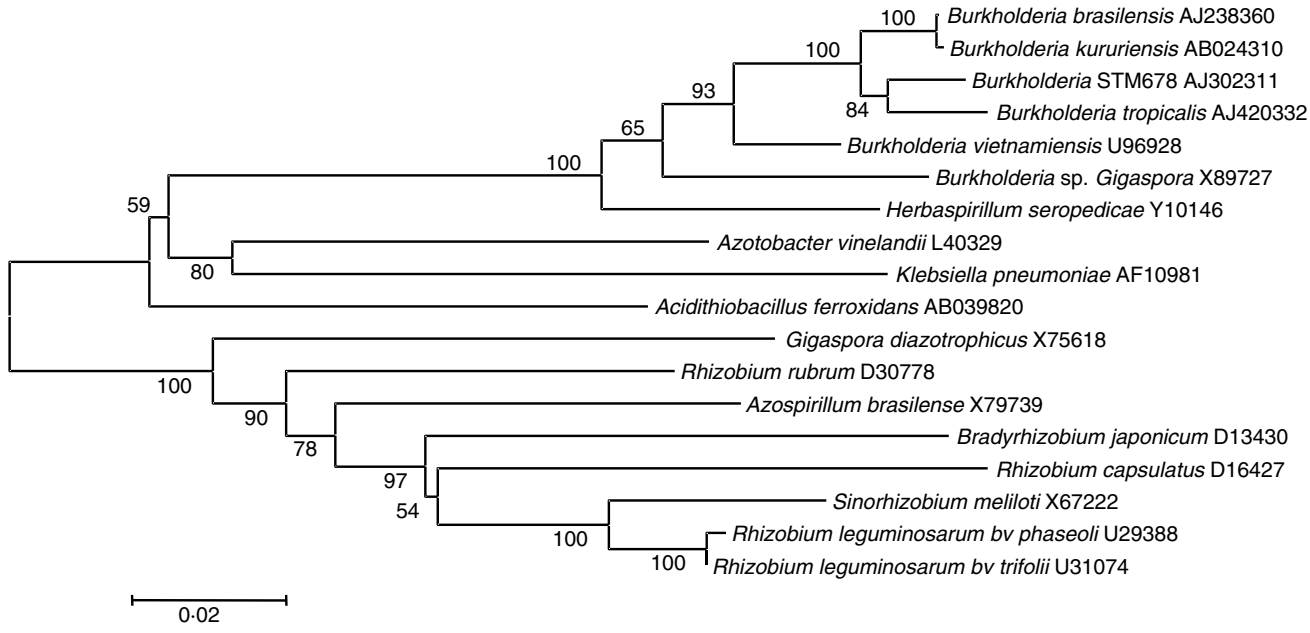

Fig. 2 Phylogenetic analysis of 16S rDNA gene sequences of nucleotides constructed by the NJ method. The bootstrap values (1000 replicates) are indicated in the grouping base. The scale bar indicates the distance in substitutions by nucleotide

Burkholderia sp. strain STM678 (95%). An identity and similarity of 100% was observed between *B. brasilensis* and *B. kururiensis*. Due to the similarity between the *nifH* gene sequences of *B. brasilensis* and *B. kururiensis* strains, the nucleotide sequences of the 16S rDNA gene of both species were compared. An identity of 99·93% was observed between the species, differing in only one nucleotide.

DISCUSSION

In this study, the partial *nifH* and *glnB* gene sequences of the nitrogen-fixing species *B. brasilensis*, *B. tropicalis* and *B. kururiensis* are reported. It is shown that the bacteria of the *Burkholderia* genus formed a cluster separated from the other species. Interestingly, the amino acid sequences of the *glnB* gene of *B. tropicalis* presented much higher similarity to the *glnB* sequences of *H. seropedicae* than to the *glnB* sequences of *B. brasilensis* and *B. kururiensis*.

The amino acid sequence alignment demonstrated the presence of the residue Tyr-51 for all species from the *glnB* gene. This residue is a site for uridylylation, usually present in the PII protein when the bacteria are grown in low nitrogen concentrations. It is known to play a role in the deadenylylation of glutamine synthetase by ATase (Arcondéguy *et al*. 2001). Genes homologous to *glnB* have been found in *A. amazonense*, *A. brasilense*, *H. seropedicae* and other bacteria, suggesting the presence of two copies of *glnB*-like genes in these organisms (Arcondéguy *et al*. 2001; Potrich *et al*. 2001). The phylogenetic tree obtained with the amino acid sequences determined from the *nifH* gene of the *Burkholderia* species showed a similar topology to that described by other authors (Ueda *et al*. 1995; Ohkuma *et al*. 1996; Franke *et al*. 1998; Vermeiren *et al*. 1999).

The observation that the *Burkholderia* sp. isolated from the fungus *G. margarita* is close to *A. brasilense* was also verified by Minerdi *et al*. (2001) who attributed this effect to the occurrence of lateral transfer of the gene. The similarity of the phylogenetic trees inferred from 16S rDNA and *nifH* genes supports the hypothesis that the *nifH* gene has descended vertically (Ueda *et al*. 1995; Ohkuma *et al*. 1996). These authors also suggested that the preservation of this gene in several organisms is due to evolution from a common ancestor, instead of a lateral transfer in recent times. However, other studies indicate a possible lateral transfer of *nif* genes, explaining why the operon *nifHDK* of *H. seropedicae* (β-proteobacteria) has a high similarity to *Br. japonicum* (α-proteobacteria) (Hurek *et al*. 1997; Vermeiren *et al*. 1999). Similarly, it has also been detected in *Acidithiobacillus ferroxidans*, a γ-proteobacteria that is closely related to *Br. japonicum* (Kelly and Wood 2000).

The similarity values of the sequences from *nifH* (100%) and 16S rDNA (99%) genes suggest that the bacterium *B. brasilensis* strain M130 (Baldani *et al*. 1997; Cruz *et al*. 2001) is closely related to the species *B. kururiensis* described by Zhang *et al*. (2000). De Los Santos *et al*. (2001) also suggested this hypothesis, based on the amplified ribosomal DNA (rDNA) restriction analysis (ARDRA) profile and nitrogen-fixing ability of strains KP23 and M130. However,

additional DNA : DNA studies showed that this is not the case (V. L. Baldani, personal communication). Similar high values of 16S rDNA (99·38%) were also observed for the species *H. seropedicae* (strain Z67) and *H. rubrisubalbicans* (strain M4) (Baldani *et al*. 1996), although they showed very low DNA : DNA hybridization values. The higher similarity for the *nifH* gene is probably due to a more conserved region that needs to be preserved during the evolution of the bacteria. It should be pointed out that only part of the gene was sequenced and other regions may provide new information that could modify the degree of similarity.

The genus *Burkholderia* has been intensively studied in the last few years because of its widespread ecological and physiological characteristics. Knowledge of the *nifH* and *glnB* genes will support new studies on the diversity and role that these diazotrophic *Burkholderia* species play in the environment.

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