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Cupriavidus necator isolates are able to fix nitrogen in symbiosis with different legume species

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ABSTRACT

The aim of the present study was to identify a collection of 35 *Cupriavidus* isolates at the species level and to examine their capacity to nodulate and fix N_2. These isolates were previously obtained from the root nodules of two promiscuous trap species, *Phaseolus vulgaris* and *Leucaena leucocephala*, inoculated with soil samples collected near *Sesbania virgata* plants growing in Minas Gerais (Brazil) pastures. Phenotypic and genotypic methods applied 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, the presence of the *nodC* and *nifH* genes was also determined, and an experiment was carried out with two representative isolates in order to authenticate them as legume nodule symbionts. All 35 isolates belonged to the betaproteobacterium *Cupriavidus necator*, they possessed the *nodC* and *nifH* genes, and two representative isolates were able to nodulate five different promiscuous legume species: *Mimosa caesalpiniaefolia*, *L. leucocephala*, *Macroptilium atropurpureum*, *P. vulgaris* and *Vigna unguiculata*. This is the first study to demonstrate that *C. necator* can nodulate legume species.

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Introduction

Currently, twelve bacterial genera are able to nodulate and fix N_2 in symbiosis with Leguminosae species, including several alphaproteobacteria and two genera of betaproteobacteria, *Burkholderia* and *Cupriavidus* [8,30]. Chen et al. [8] isolated betaproteobacteria strains from root nodules of *Mimosa pudica* and *Mimosa diplotricha* introduced into Taiwan and described the novel species *Ralstonia taiwanensis*. This species was subsequently transferred to the genus *Cupriavidus* [40]. Since then, *C. taiwanensis* has been isolated from root nodules of *M. pudica*, *M. diplotricha* and *Mimosa pigra* introduced into Taiwan [6,7] and from *M. pudica* in India [43]. *C. taiwanensis* and an undetermined *Cupriavidus* sp. were also isolated from nodules of native *M. pigra* and *M. pudica* in Costa Rica [4] and native *Mimosa asperata* in the USA [1].

Except for the *Cupriavidus* sp. nodule bacteria found in Costa Rica [4] and in the USA [1], there are no reports of the occurrence of *Cupriavidus* species in root nodules of *Mimosa* in other regions, such as in Brazil which is the major centre of diversification for this legume genus [3,34]. Recently, Florentino et al. [14] obtained isolates with fast-growth alkali-reactions in culture medium from *Phaseolus vulgaris* and *Leucaena leucocephala* root nodules. The partial 16S rRNA sequences of five of these isolates were similar to members of the *Cupriavidus* genus. In the present study, it is shown that four isolates, previously identified at the genus level [14], and an additional 31 isolates from the same study [14], belonged to *Cupriavidus necator*. To confirm their ability to nodulate and fix N_2, the presence of the *nodC* and *nifH* genes was determined, and an experiment was carried out to authenticate them as legume nodule symbionts, as well as to verify their symbiotic efficiency with different legume species.

Materials and methods

Soil sampling and bacteria trapping

The 35 isolates studied in this work (Table 1) were obtained by Florentino et al. [14] from soil samples collected near *Sesbania*

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Table 1
Origin (plant species) and analysis carried out on the isolates of *Cupriavidus* obtained from pasture soils collected near *Sesbania virgata* plants in Minas Gerais, Brazil.

Isolates and their origin	Gene sequence analysis (GenBank accession number)				Inoculation experiment
	16S rRNA	*gyrB*	*nifH*	*nodC*	
Leucaena leucocephala					
UFLA01-657, UFLA01-658, UFLA01-659, UFLA01-660, UFLA01-661, UFLA01-662, UFLA01-663, UFLA01-664, UFLA01-665, UFLA01-666, UFLA01-667, UFLA01-668, UFLA01-669, UFLA01-670, UFLA01-671, UFLA01-672, UFLA01-673, UFLA01-674, UFLA01-675, UFLA01-676, UFLA01-677, UFLA01-678	UFLA01-669/(HQ684033)	UFLA01-662/(HQ684038) UFLA01-669/(HQ684042)	UFLA01-662/(HQ684055) UFLA01-669/(HQ684059)	UFLA01-662/(HQ684061) UFLA01-669/(HQ684065)	UFLA01-657
Phaseolus vulgaris:					
UFLA02-48, UFLA02-52, UFLA02-55, UFLA02-57, UFLA02-59, UFLA02-62, UFLA02-65, UFLA02-67, UFLA02-71, UFLA02-72, UFLA02-73, UFLA02-74, UFLA02-129	UFLA02-71/(HQ684034)	UFLA02-55/(HQ684039) UFLA02-71/(HQ684043) UFLA02-73/(HQ684040) UFLA02-129/(HQ684037)	UFLA02-55/(HQ684056) UFLA02-71/(HQ684060) UFLA02-73/(HQ684057)	UFLA02-55/(HQ684062) UFLA02-71/(HQ684066) UFLA02-73/(HQ684063)	UFLA02-129

virgata plants growing in distinct pasture areas in the state of Minas Gerais, Brazil. These areas were located in Nepomuceno (21°14′S and 45°13′W) and in Ribeirão Vermelho (21°13′S and 45°02′W). The isolates were captured from the soil samples by using the trap legume species *L. leucocephala* and *P. vulgaris*. These species are promiscuous plant species capable of symbiosis with more than one rhizobia species. The methodologies of trapping and isolation are described in Florentino et al. [14]. These isolates showed similar cultural characteristics in culture medium 79 [17] when compared to the *Azorhizobium* species (fast growth with alkali reaction), but they have slightly higher gum production than this species.

SDS-PAGE of whole-cell proteins

All 35 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 [28,31]. 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, Belgium). A database consisting of reference strains of established *Cupriavidus* species was available from previous studies [7,10,11,19,40,41,P. Vandamme, unpublished data, 2009]. A dendrogram based on numerical analysis of the protein profiles was constructed. The reference taxa included those with the most similar 16S rRNA gene sequences, as determined using the EzTaxon database [9].

16S rRNA and gyrB gene amplification and sequencing

The near entire 16S rRNA gene sequences were determined for two isolates (Table 1). The DNA was prepared using the alkaline lysis procedure [2]. The nearly complete sequences of the 16S rRNA gene (corresponding to positions 8–1541 in the *Escherichia coli* numbering system) were amplified by PCR using conserved primers (5′-AGAGTTTGATCCTGGCTGAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′) [10]. For the *gyrB* PCR amplification, six isolates and thirteen reference strains from established *Cupriavidus* species [37,40] were studied (Table 1). 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′) [37]. Sequencing was performed using an ABI Prism 3130xl capillary sequencer according to the manufacturer's instructions (Applied Biosystems). The sequencing primers for the 16S rRNA gene were those given by Coenye et al. [10], whereas the primers for *gyrB* were the same primers used for amplification.

Novel sequences and selected sequences of reference strains were aligned using ClustalX. Subsequently, the aligned sequences were imported into the BioNumerics version 5.1 (Applied Maths, Belgium) software for phylogenetic analyses and bootstrap analysis (1000 replicates). Phylogenetic trees were constructed using the neighbour-joining, maximum likelihood and maximum parsimony methods.

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nodC and nifH gene amplification and sequencing

Five isolates were examined for the presence of the *nodC* and *nifH* genes (Table 1). The *nodC* DNA sequence was amplified using the primers nodCF (5′-AYGTHGTYGAYGAACGGTTC-3′) and nodCI (5′-CGYGACAGCCANTTCKKCTTATTG-3′) [22]. For *nifH*, the DNA was amplified using the primers 19F (5′-GCIWTYTAYGGIAARGGIGG-3′) and 407R (5′-AAICCRCCRCAIACIACRTC-3′) [39]. The PCR products were directly sequenced on both strands using the same primers utilised in the PCR amplification. Sequencing was performed in a 3730xl sequencer.

The sequences obtained were translated to amino acids to check if the residues were conserved, and they were aligned with reference strains using ClustalW [38]. Phylogenetic trees were inferred using the neighbour-joining method, as implemented in the MEGA 4.1 package [36]. A bootstrap confidence analysis was performed with 1000 replicates.

Glasshouse experiment

The present study used two isolates previously tested regarding their ability to nodulate legume species (following Koch's postulates). Isolate UFLA01-657 was tested in *L. leucocephala*, and isolate UFLA02-52 was tested in *P. vulgaris* [unpublished data]. In this work, a glasshouse experiment examined the symbiotic abilities of two bacterial isolates from *C. necator* (UFLA02-129 and UFLA01-657) and one from *C. taiwanensis* (LMG 19424^T), with six legume species: *Mimosa caesalpiniaefolia* and *L. leucocephala* (subfamily Mimosoideae); and *S. virgata*, *Macroptilium atropurpureum*, *P. vulgaris* and *Vigna unguiculata* (subfamily Papilionoideae). Seeds of *M. caesalpiniaefolia*, *L. leucocephala*, and *M. atropurpureum* were scarified in sulphuric acid (98.8%) for 5, 35, 40 and 50 min, respectively. For *P. vulgaris* and *V. unguiculata*, the seeds were sterilised with sodium hypochlorite (2%). Seeds of the six species were germinated in Petri dishes with wet cotton and filter paper and then transplanted into sterilised Leonard pots [44], with sand and vermiculite 1:1 (v:v) in the top part and Jensen solution without nitrogen, diluted four times and sterilised [20], in the bottom part. Seeds (four seeds per pot) were inoculated with bacterial isolates and strains were cultivated in liquid medium 79 [17] containing 10^9 cells mL^{-1} (1 mL seed^{-1}). For each species, three control treatments were applied. The first control was an efficient and/or inoculant strain as a positive control: *Sinorhizobium fredii* BR 827 (for *L. leucocephala*) [16,29], *Azorhizobium doebereinerae* BR 5401^T (for *S. virgata*) [16,27], *Bradyrhizobium* sp. isolate UFLA04-0212 (for *M. atropurpureum*), *Rhizobium tropici* CIAT 899^T (for *P. vulgaris*) [26], and *Bradyrhizobium* sp. UFLA03-84 (for *V. unguiculata*) [21,35]. The reference strain *Burkholderia sabiae* BR 3405, which is known to nodulate the species *M. caesalpiniaefolia* [5], was used as a positive control. The other controls, without inoculation, received or not, mineral nitrogen (210 mg N-NH_4NO_3 kg^{-1} substrate). The experiment was carried out in a completely randomised 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 analysed statistically using the SISVAR programme, version 4.3 [13], with the effects from the treatments evaluated by the Scott-Knott test [33] with a 5% significance.

Nucleotide sequence accession numbers

The 16S rRNA, *gyrB*, *nodC* and *nifH* gene sequences were deposited in the EMBL/GenBank database. The accession numbers are shown in Table 1.

Results

Species level identification of isolates

All isolates had virtually identical whole-cell protein profiles (Fig. 1). In general, strains with highly similar whole-cell protein profiles have been shown to represent the same species [42], as a high level of whole-cell protein pattern similarity correlates with a high DNA-DNA hybridisation level. In the genus *Cupriavidus*, in particular, this was confirmed for all species examined by comparative protein profiling and DNA-DNA hybridisation studies [7,10,11,19,40,41]. For this reason, the random selection of a limited number of isolates for further species level confirmation purposes is taxonomically valid. When compared with protein profiles of type strains of all established *Cupriavidus* species [7,10,11,19,40,41, P. Vandamme, unpublished data] the highest similarity levels were obtained with the *C. necator* type strain [40]. In order to confirm this tentative identification, firstly, almost complete 16S rRNA gene sequences were determined for two randomly selected isolates (UFLA01-669 and UFLA02-71). The two sequences were 99.9–100% similar and shared 99.5–99.7% of their 16S rRNA sequence with the *C. necator* type and reference strains LMG 8453^T and LMG 1199, respectively. In the neighbour-joining phylogenetic tree, the two isolates were grouped with the *C. necator* strains with a high bootstrap support (Fig. 2), and this clustering was supported by the maximum likelihood and maximum parsimony treeing algorithms (data not shown). Secondly, the *gyrB* sequences of the same two and four additional isolates were compared with the *gyrB* sequences of *Cupriavidus* reference strains characterised in previous taxonomic studies [40] (Fig. 3). The six isolates had *gyrB* sequences that were 99.9–100% similar, and the highest similarity levels were again determined against *C. necator* reference strains (range 95.6–96.8% similarity). The diversity of *gyrB* sequences observed among the type and seven additional reference strains of *C. necator* was in the range of 97.6–98.1%.

nodC and nifH sequencing

nodC genes were detected in the isolates studied in this work. The evolutionary tree for the deduced amino acid sequences of isolates with published sequences showed that the isolates grouped with *Cupriavidus taiwanensis* LMG 19424^T (Fig. 4). The *nifH* gene was also detected, and in the amino acid phylogenetic tree the isolates were close to *C. taiwanensis* and *Burkholderia* strains (Fig. 5). It was verified that the amino acid sequences of *nodC* and *nifH* genes of *Cupriavidus* isolates had conserved regions found in other alpha- and beta-rhizobia. These results indicated the genetic ability of these isolates to nodulate legumes and fix nitrogen.

Glasshouse experiment

The symbiotic relationship of the six legume species with the two representative strains 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 efficient nodules were found in many treatments. The two strains and the type strain of *C. 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 plant species with *A. doebereinerae*. 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 nodules), *P. vulgaris* (203 nodules) and *V. unguiculata* (165 nodules). The other isolate and the type strain (UFLA02-129 and LMG 19424^T) also induced a

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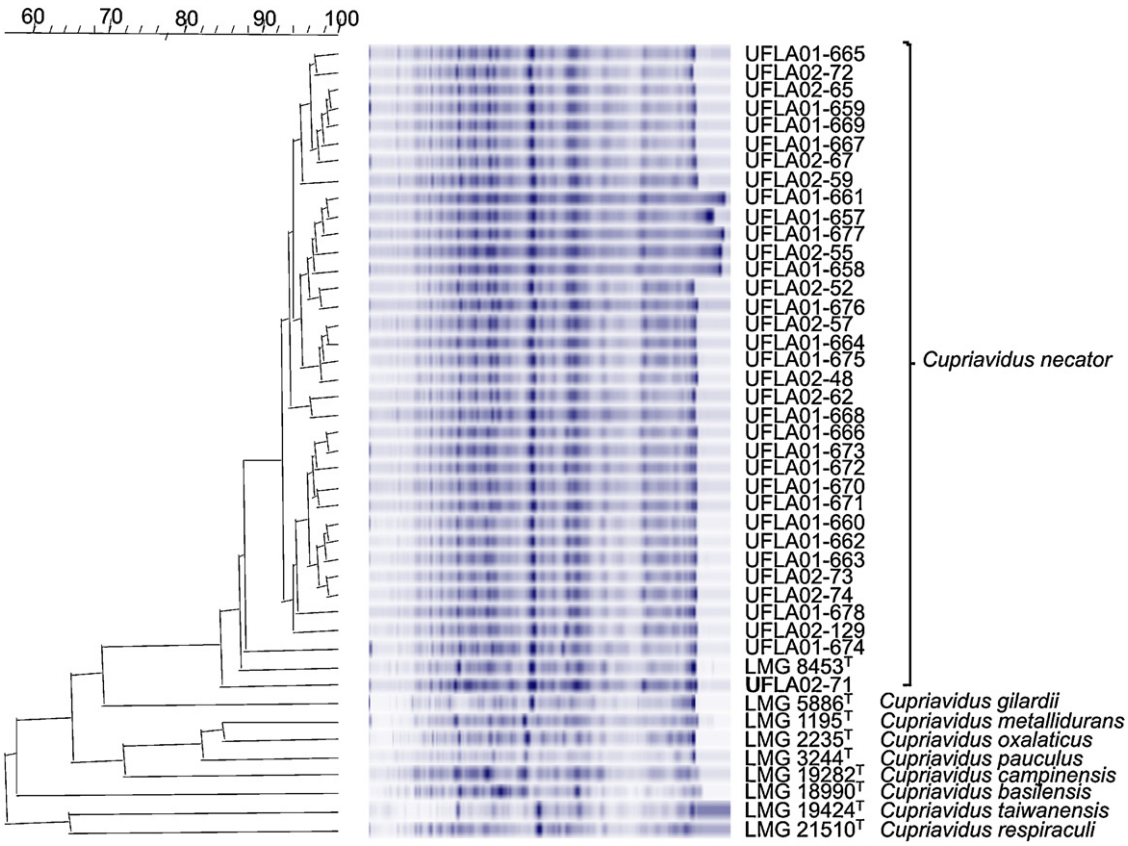

Fig. 1. Computer reconstructed whole-cell protein profiles of *Cupriavidus* isolates and reference strains, and a dendrogram based on numerical analysis of the protein profiles. The correlation level between protein profiles is expressed as a percentage similarity for convenience.

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 inoculation with *Cupriavidus* provided a better nodule number than those inoculated with strains used as positive controls. However, a high number of nodules was not always related to the efficiency of fixing N_2.

The results from shoot dry matter weight (Table 3) verified that the addition of mineral nitrogen afforded better development to all of the host species, except for *P. vulgaris*. In *P. vulgaris*, inoculation with CIAT 899^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.*

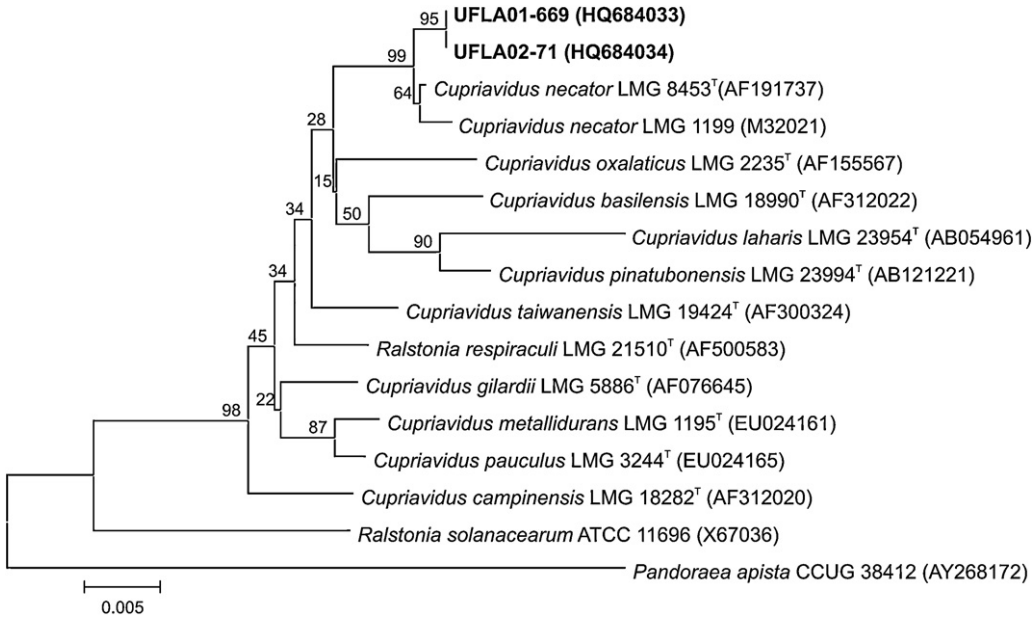

Fig. 2. Phylogenetic tree based on 16S rDNA gene sequence similarity of *Cupriavidus* isolates and strains inferred using the neighbour-joining method. Bootstrap values were based on 1000 replicates.

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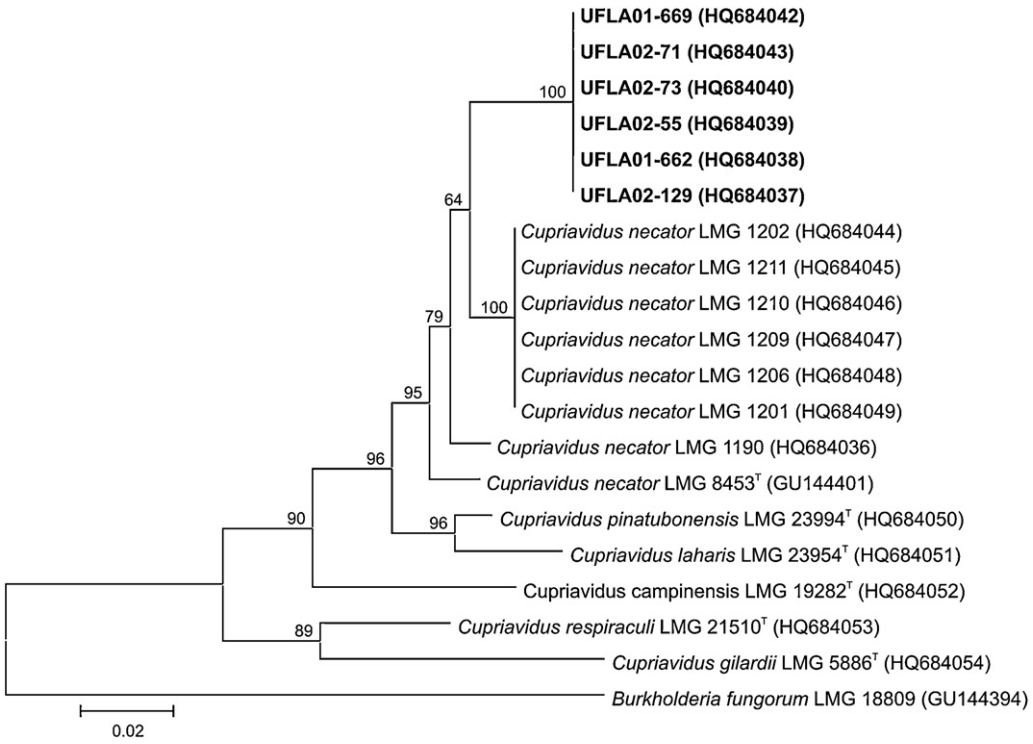

Fig. 3. Phylogenetic tree of the *gyrB* gene of *Cupriavidus* isolates and type strains inferred using the neighbour-joining method. Bootstrap values were based on 1000 replicates.

necator isolates provided moderate levels of efficiency, while inoculation in the other host plants, in general, was inefficient. For the species *L. leucocephala* and *M. caesalpiniaefolia*, the inoculant strains (positive controls) did not contribute significantly to plant growth, possibly because more time would have been required for development of symbiosis or the temperature of the season was not suitable. Nodulation by isolates of *C. necator* was confirmed by re-isolation from the nodules and it was identified by cultural characteristics.

Discussion

The occurrence of nitrogen-fixing Leguminosae-nodulating bacteria belonging to the genus *Cupriavidus* and nodulating

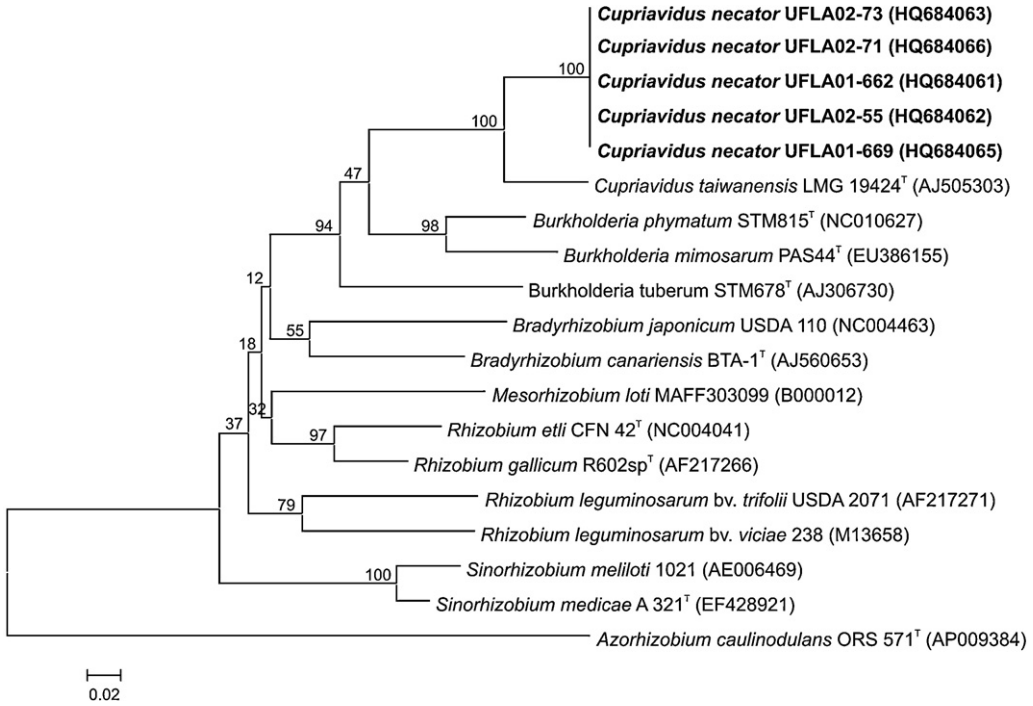

Fig. 4. Phylogenetic tree of the *nodC* protein of *Cupriavidus* isolates and alpha- and beta-rhizobia strains inferred using the neighbour-joining method. Bootstrap values were based on 1000 replicates.

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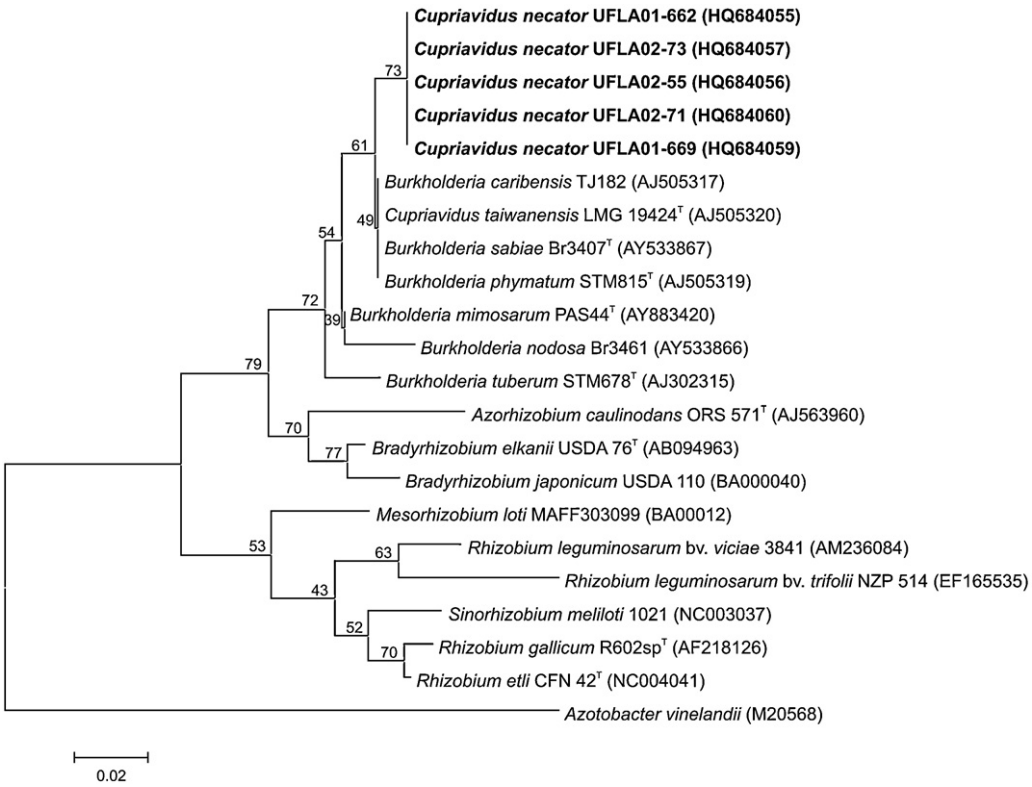

Fig. 5. Phylogenetic tree of the *nifH* protein of *Cupriavidus* isolates and alpha- and beta-rhizobia strains inferred using the neighbour-joining method. Bootstrap values were based on 1000 replicates.

Table 2
Number of nodules (n. plant^{-1}) after inoculation with *Cupriavidus necator* isolates, *Cupriavidus taiwanensis* LMG19424^T and strains of compatible rhizobia in five different legume species.

Plant species	Treatments						
	LMG 19424^T	UFLA01-657	UFLA02-129	Control 1[a]	Control 2[b]	N addition	C.V. (%)[c]
	(n. plant^{-1})[d]						
M. caesalpiniaefolia	10 c	16 c	106 a	63 b	0 d	0 d	17.76
L. leucocephala	146 a	47 b	15 b	59 b	0 c	0 c	10.43
S. virgata	0 b	0 b	0 b	253 a	0 b	0 b	11.16
M. atropurpureum	16 d	73 b	22 c	102 a	0 e	0 e	5.68
P. vulgaris	21 d	203 b	158 c	299 a	0 e	0 e	10.15
V. unguiculata	7 c	165 a	7 c	66 b	0 d	0 d	12.31

[a] Inoculation with compatible rhizobia: *M. caesalpiniaefolia* with BR 3405 reference strain [5]; *L. leucocephala* with BR 827 [16,29]; *S. virgata* with BR 5401 [16,27]; *M. atropurpureum* with UFLA04-212 efficient strain; *P. vulgaris* with CIAT 899^T [26]; *V. unguiculata* with UFLA03-84 [21,35].
[b] Uninoculated control.
[c] Coefficient of variation.
[d] Values followed by different letters in the same line are significant at a 5% probability by the Scott-Knott test [33].

Table 3
Shoot dry matter (g plant^{-1}) after inoculation with *Cupriavidus necator* isolates, *Cupriavidus taiwanensis* LMG19424^T and strains of compatible rhizobia in five different legume species.

Plant	Treatments						
	LMG 19424^T	UFLA01-657	UFLA02-129	Control 1[a]	Control 2[b]	N addition	C.V. (%)[c]
	(g plant^{-1})[d]						
M. caesalpiniaefolia	0.11 c	0.20 b	0.20 b	0.13 c	0.14 c	1.20 a	11.84
L. leucocephala	0.13 b	0.15 b	0.07 b	0.17 b	0.12 b	2.20 a	24.09
S. virgata	1.07 c	0.38 c	1.06 c	6.53 b	0.57 c	10.17 a	28.64
M. atropurpureum	0.22 c	0.12 c	0.05 c	1.25 b	0.07 c	3.25 a	21.67
P. vulgaris	0.40 b	0.59 b	1.24 a	1.27 a	0.27 b	1.23 a	23.92
V. unguiculata	0.20 c	0.29 c	0.72 c	2.75 b	0.25 c	5.98 a	15.10

[a] Inoculation with compatible rhizobia: *M. caesalpiniaefolia* with BR3405 reference strain [5]; *L. leucocephala* with BR 827 [16,29]; *S. virgata* with BR 5401 [16,27]; *M. atropurpureum* with UFLA04-212 efficient strain; *P. vulgaris* with CIAT 899^T [26]; and *V. unguiculata* with UFLA03-84 [21,35].
[b] Uninoculated control.
[c] Coefficient of variation.
[d] Values followed by different letters in the same line are significant at a 5% probability by the Scott-Knott test [33].

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Papilionoideae and Mimosoideae species in Brazilian soils was reported recently [14]. Our results demonstrated that all isolates had virtually identical whole-cell protein profiles and that randomly selected isolates shared virtually identical 16S rRNA and *gyrB* gene sequences. The whole-cell protein profiles (Fig. 1) and 16S rRNA gene sequences (Fig. 2) of isolates were indistinguishable from those of *C. necator* reference strains. As sequence analysis of protein encoding genes provides a higher taxonomic resolution compared to 16S rRNA gene sequence analysis, partial *gyrB* sequences of six isolates along with those of the type and seven established reference strains of this species [37,40] were subsequently examined. The partial *gyrB* gene sequences revealed that the *Cupriavidus* isolates represented a distinct line of descent that clustered among the other *C. necator* strains with a high bootstrap support (Fig. 3). These results demonstrated that legume nodule symbionts belonged to *C. necator* but also suggested that they represented a unique lineage within this species. However, multilocus sequence typing of a larger number of *C. necator* strains would be required to substantiate this conclusion.

This is the first time that isolates of the *C. necator* species have been reported to induce effective root nodules. To date, only *C. taiwanensis* was known as a legume nodule symbiont. *C. necator* was first described as a non-obligate predator of soil bacteria isolated from soil in the vicinity of University Park, PA, USA [24]. Reference strains of this species have so far all been isolated from soil samples in Europe, Japan and the USA [40; http://bccm.belspo.be]. Some strains are able to degrade chloroaromatic compounds [12] and are resistant to heavy metals [25]. Other strains have been studied for their ability to produce poly-β-hydroxyalkanoates, which have industrial applications [23,32].

Some lineages of betaproteobacteria probably obtained their symbiotic genes through lateral transfer from alphaproteobacteria [6,30,43]. However, in some *Cupriavidus* strains, obtained from root nodules of *Mimosa* spp. in the USA, it appears that they acquired these genes from *Burkholderia* rather than alphaproteobacteria [1]. In our study, the phylogenetic tree of deduced amino acids of *nodC* and *nifH* genes of *C. necator* strains (Figs. 4 and 5) had very similar sequences to those found in *C. taiwanensis*, and it appears therefore that they acquired these genes from betaproteobacteria.

C. necator isolates had the ability to nodulate five of the six different hosts tested. These five species were all promiscuous hosts of legume nodule symbionts. Nodulation was only absent in *S. virgata*, which shows a high degree of symbiotic specificity with *A. doebereinerae* [15,18,27]. These results corroborated a previous report [14] demonstrating that *P. vulgaris* and *L. leucocephala* are nodulated by *Cupriavidus* sp. Furthermore, the results showed that *C. necator* was also able to nodulate *M. caesalpiniaefolia*, *M. atropurpureum* and *V. unguiculata*. The results also showed an inefficient (UFLA 01-657) to high (UFLA02-129) symbiotic efficiency for *C. necator* with *P. vulgaris* and a moderate efficiency for both strains with *M. caesalpiniaefolia*, in addition to inefficient symbiotic relationships with *L. leucocephala*, *M. atropurpureum* and *V. unguiculata*.

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