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Physiological and symbiotic diversity of *Cupriavidus necator* strains isolated from nodules of Leguminosae species

Ligiane Aparecida Florentino, Paula Marcela Duque Jaramillo, Karina Barroso Silva, Jacqueline Savana da Silva, Silvia Maria de Oliveira, Fatima Maria de Souza Moreira*

UFLA – Depto. de Ciência do Solo, C.P. 3037 – 37200-000 – Lavras, MG – Brasil.
*Corresponding author <fmoreira@dcs.ufla.br>

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ABSTRACT: Biological nitrogen fixation, performed by diazotrophic bacteria, plays an important role in the maintenance of agricultural systems, as it contributes with significant amounts of the nitrogen (N) needed for plant growth, totally or partially exempting the use of industrial N fertilisers. Twenty-five bacterial strains isolated from nodules of *Leucaena leucocephala* and *Phaseolus vulgaris* trap plants were studied. These nodules were formed after inoculation with suspensions of soil samples collected close to the root system of *Sesbania virgata*. In previous studies, these bacteria were identified as *Cupriavidus necator*. This study aimed to evaluate the ability of these strains to fix N_2 in the free-living state and to use carbon (C) sources; their resistance to antibiotics; growth in media with different pH values and salt concentrations and symbiotic efficiency with *L. leucocephala* and *P. vulgaris*. In each test, these strains were compared to *C. taiwanensis* LMG 19424^T. Although a high variability regarding antibiotic resistance, salt tolerance and use of C sources were observed among the 25 *C. necator* strains, a large group behaved similar regarding salt tolerance (20 strains) and antibiotic resistance (22 strains). *C. necator* strains behaved in a different way of LMG 19424^T. Only one of the 25 strains studied, UFLA02-69, was not able to establish symbiosis with its trap species, *P. vulgaris*. Only the strains LMG19424^T and UFLA01-672 were efficient in symbiosis with *L. leucocephala*. The ability to use C sources, grow in different pHs and salt concentrations and resistance to several antibiotics, may grant high saprophytic competence and greater competitivity to these strains in relation to the native Leguminosae-nodulating bacterial populations, suggesting potential use in inoculant strain selection studies for legumes cultivated in soils with a wide range of pH and salt concentrations.
Keywords: symbiosis, β-proteobacteria, antibiotic resistance

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Introduction

Biological nitrogen fixation (BNF), performed by diazotrophic bacteria, plays an important role in the maintenance of agricultural systems, as it contributes with significant amounts of the nitrogen (N) needed for plant growth, totally or partially exempting the use of industrial N fertilisers. This advantage becomes more evident when these bacteria establish symbiosis with legume plants, leading to the formation of root and/or stem nodules in these plants. Therefore, they may also be called legume-nodulating bacteria (LNB).

Studies on BNF experienced a major breakthrough after 2001 when it became apparent that, in addition to the α-proteobacteria subclass, LNB were also distributed in the β-proteobacteria subclass in the genera *Burkholderia* (Moulin et al., 2001) and *Cupriavidus* (Syn. *Ralstonia; Wautersia*) (Chen et al., 2001; Vandamme and Coenye, 2004; Vaneechoutte et al., 2004). Some of the 11 species of *Cupriavidus*, such as *C. metallidurans*, *C. necator* and *C. taiwanensis*, have great biotechnological potential and can be used in soil bioremediation processes. *C. necator* degrades herbicide dichlorophenoxyacetic acid (2, 4-D) (Don and Pemberton, 1981), and other aromatic compounds and chemical pollutants, such as 4-chloro-2-methylphenoxyacetic (MCPA), 3-chlorobenzoic acid (3-CB), 2,4,6-trichlorophenol and 4-fluorobenzoate (Schlomann et al., 1990; Clement et al., 1995). *C. metallidurans* and *C. taiwanensis* are resistant to several heavy metals, such as Zn, Cd, Co, Ni, Cu, Cr, Hg and Pb (Mergeay et al., 1985; 2003; Goris et al., 2001).

Besides *C. taiwanensis* (Chen et al., 2001) only *C. necator* (Silva et al., 2012) was identified until now, with the ability to establish symbiosis with legumes. Considering that these findings were relatively recent and the economic and ecological importance of these species, it is quite relevant to know the intraespecific variability of *C. necator*. Thus, the objectives of this study were (i) to evaluate the ability of these strains to fix N_2 in the free-living state and use C sources by using BIOLOG GN2; (ii) to evaluate the strains 'resistance to antibiotics and the strains' growth at different pHs and salt concentrations; and (iii) to evaluate the symbiotic efficiency with their trap species, *L. leucocephala* and *P. vulgaris*.

Materials and Methods

Origin of strains and culture characteristics

This investigation studied 25 strains of *C. necator* isolated from four soil samples, three from the municipality of Nepomuceno (21°14′ S and 45°13′ W) and one from Ribeirão Vermelho (21°13′ S and 45°02′ W) municipality, both located in southern Minas Gerais state, Brazil. These samples were collected next to the root system

of the legume *S. virgata. L. leucocephala* and *P. vulgaris*, which are promiscuous species in relation to their symbiosis with rhizobial strains, were used as LNB trap species from the soil samples (Florentino et al., 2009). These strains form isolated white colonies after three days of growth and stimulate an alkaline reaction in the medium and low production of exopolysaccharide in 79 medium (Fred and Waksman, 1928). These morphological characteristics are very similar to those exhibited by LNB of the genus *Azorhizobium* (Moreira et al., 2006). Nevertheless, *C. necator* may be differentiated by the production of exopolysaccharides, which is a little higher.

In addition to these strains, the study also used type or reference strains representing the main genera of LNB. Table 1 shows the identification of the strains, place of origin, pH values of soils, trap species or hosts of origin of LNB strains, GenBank accession numbers and 16S rDNA, GyrB, NodC and NifH gene sequences of some strains used in this study. These strains were representative of the group formed by profiles obtained by total protein electrophoretic analysis (SDS-PAGE) from a database from the University of Ghent (Silva et al., 2012).

All the strains of *C. necator* were analysed in terms of their ability to fix N_2 in the free- living state and the resistance to several antibiotics, growth in media with different pH values and salt concentrations, and ability to establish symbiosis with *L. leucocephala* and/or *P. vulgaris*. Six of these strains were evaluated for the ability to use difference carbon sources (BIOLOG GN2 microplates).

Table 1 – Identification of Legume Nodulating Bacteria strains, soil pH values, trap species or host of origin and GenBank accession numbers.

Strains identified as belonging to *Cupriavidus necator*				
Origin/Municipality/ Strains	pH of soils of origin	Trap species or Host of origin	Genbank Accession Number[1]	Reference
Nepomuceno				
UFLA01-671	7.7	*L. leucocephala*	-	Florentino et al. (2009), Silva et al.(2012)
UFLA01-657	7.3	*L. leucocephala*	GQ268825[2]	
UFLA01-668,UFLA01-672,UFLA01-673, UFLA01-674		*L. leucocephala*	-	
UFLA02-52, UFLA02-62, UFLA02-51	7.3	*P. vulgaris*	-	
UFLA02-72, UFLA02-69, UFLA02-57, UFLA02-48	5.6	*P. vulgaris*	-	
Ribeirão Vermelho				
UFLA01-669	6.8	*L. leucocephala*	HQ68403[2], HQ684042[3] HQ684059[4], Q684065[5]	Florentino et al. (2009), Silva et al. (2012)
UFLA01-659			GQ268819[2]	
UFLA01-658			GQ268824[2]	
UFLA01-661			-	
UFLA01, 662			HQ684038[3], Q684055[4] HQ684061[5]	
UFLA01-660,			-	
UFLA01-663			-	
UFLA02-55			HQ684039[3], HQ684056[4] HQ684062[5]	
UFLA02-129			GQ268820[2], HQ684037[3]	
UFLA02-71		*P. vulgaris*	HQ684034[2], HQ684043[3] HQ684060[4], HQ684066[5]	
UFLA02-58			HQ684035[2], HQ684041[3] HQ684058[4], HQ684064[5]	
UFLA02-74			-	

Type or reference strains of species from the main genera of LNB used in the study:		
Strains	Trap species or Host of origin	Reference
LMG19424[T] *C. taiwanensis*	*Mimosa pudica*	Chen et al. (2001), Vandamme and Coenye (2004)
CIAT 899[T] *Rhizobium tropici*	*P. vulgaris*	Martínez-Romero et al. (1991)
BR 827 *Sinorhizobium* sp.	*L. leucocephala*	Moreira et al. (1998)
BR 6806 *Sinorhizobium* sp. (*Ensifer adhaerens*)	*Pithecellobium dulce*	Moreira et al. (1998); Willems et al. (2003)
UFLA 03-84 *Bradyrhizobium* sp.	*Vigna unguiculata*	Lacerda et al. (2004)
BR 3804 *Mesorhizobium plurifarium*	*Chamaecrista ensiformis*	Moreira et al. (1993; 1998); De Lajudie et al. (1998)
BR 5401[T] *Azorhizobium doebereinerae*	*Sesbania virgata*	Moreira et al. (2006)
ORS 571[T] *A. caulinodans*	*S. rostrata*	Dreyfus et al. (1988)
BR 3405 *Burkholderia sabiae*	*Mimosa caesalpiniifolia*	Chen et al. (2008a)

[1]These strains were representative of groups formed by profiles obtained by total protein electrophoretic analysis (SDS-PAGE) from databases from the University of Ghent (Silva et al., 2012). GenBank accession numbers obtained by [2]16SrDNA, [3]GyrB, [4]NodC, [5]NifH gene sequencing.

Free-living N_2 fixation

The strains were grown in medium 79 and isolated colonies were inoculated in flasks containing 5 mL of LO N-free semisolid medium (Dreyfus et al., 1983) to determine their ability to fix N_2 in the free-living state. In addition to sodium lactate, which is the original carbon source of this medium, mannitol and fructose were also tested as sole carbon source. The LNB type strains from the two species of the genus *Azorhizobium*, BR5401^T (*A. doebereinerae*) (Moreira et al., 2006) and ORS571^T (*A. caulinodans*) (Dreyfus et al., 1988), were used as positive controls.

The tests were done in triplicate, and the flasks were incubated for 7 days at 28 °C to analyse the ability of the diazotrophic bacteria to fix N_2 in the free-living state, evidenced by the formation of pellicle near the surface of the culture medium. The positive controls were compared with the samples. Those that formed pellicles during this period were considered to have positive growth, whereas those that did not form a pellicle had negative growth. In addition to pellicle formation, the presence of turbidity in the culture medium was also considered to indicate bacterial growth.

Use of difference carbon sources as the only substrate

In order to test the assimilation of carbon sources, strains UFLA02-72, UFLA02-62, UFLA01-657, UFLA02-59 and UFLA01-658 were randomly selected. The type strain of *C. taiwanensis*, LMG19424^T, was also included in this test. The BIOLOG GN2 kit (Biolog, Hayward, California) was used; it has microplates containing 96 wells, identified as A1 to H12 (Table 2), with one being the control well without a carbon source (A1), and 95 wells filled with C substrates as follows: polymers (A2–A6); carbohydrates (A7–C10); carboxylic acids (D1–E12); amines and amides (F2–F4; H5–H7); amino acids (F5–F12; G1–G12); esters (C11, C12); brominated chemical (F1); aromatic compounds (H1–H4); alcohols (H8, H9); and phosphorylated compounds (H10–H12).

The strains were initially grown in 79 medium at 28 °C for three days (time needed for these strains to develop isolated colonies). Then, they were suspended in saline solution (0.85 % NaCl) until an absorbance of approximately 0.595 nm was reached. Subsequently, 150 µL of the suspension from each strain was inoculated in each of the 96 wells of the BIOLOG GN2 microplates. These plates were covered and incubated at 28 °C for 24 h. After this period, the presence of purple colour was observed, indicating the use of the carbon source by the bacteria. In this study, the results were evaluated visually, adopting a system of (+) for colour change and (-) for no colour change in the wells containing different carbon sources.

Antibiotic resistance

The antibiotic resistance test was performed aseptically in triplicate and consisted of 15 antibiotic discs containing set concentrations. First, 100 µL aliquots of each bacterial culture containing approximately 1×10^9 cells were spread in Petri dishes (diameter 88 mm) containing 79 culture medium. Then, three discs with three different antibiotics, selected randomly, were inserted into each plate. These discs were placed separated and equidistantly spaced from one another, on the plate to avoid the overlap of inhibition halos in the event of sensitivity of the strains to the compounds tested. For each type of antibiotic, there were three repetitions. The following antibiotics were used, containing the following concentrations (µg): amoxicillin–AMO (10), ampicillin–AMP (10), streptomycin–STR (10), gentamicin–GEN (10), azithromycin–AZI (15), clarithromycin–CLA (15), erythromycin–ERY (15), nalidixic acid–NAL (30), chloramphenicol–CHL (30), kanamycin–KAN (30), rifamycin–RFM (30), tetracycline–TET (30), vancomycin–VAN (30), sulphonamides–SUL (300) and bacitracin–BC (10 IU). The plates were incubated for seven days at 28 °C. After this period, the presence or absence of an inhibition halo was observed, indicating sensitivity or resistance, respectively, of LNB to the antibiotics tested.

The results of the antibiotic tests were converted into a binary matrix, and a dendrogram was generated by the UPGMA Algorithm by using the Jaccard (J) coefficient of the NTSYS-pc program, version 2.10.

Growth in different pH values and salt concentrations

One-millilitre aliquots from each strain, containing approximately 1×10^9 cells mL^{-1} obtained from log phase cultures, were transferred to sterilised 1.5-mL microtubes and centrifuged at 12,300 g at 4 °C for 10 min. The supernatant was discarded, and the cells were suspended in 1 mL of sterile saline solution (0.85 % NaCl) and centrifuged. This cell washing process was repeated three times to remove residue from the culture medium of the inoculum, which could result in false positive growth (Trannin et al., 2001; Matsuda et al., 2002; Nóbrega et al., 2004). Subsequently, 100-µL aliquots of cell suspensions washed in saline solution were inoculated in plates containing 79 medium modified to have different pH values or salt concentrations.

In order to examine the growth at different pH values, 79 medium was adjusted to a pH of 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0 by the addition of HCl or NaOH (Zerhari et al., 2000; Moschetti et al., 2005; Wei et al., 2008). The control treatment was the 79 culture medium at its original pH value of 6.8.

To determine the growth in different salt concentrations, the 79 medium was modified by adding NaCl solutions to final concentrations of 86, 171, 256, 342, 427 and 513 mM. The control treatment was the 79 culture medium in its original composition, which has 1.71 mM NaCl.

The treatments were completely randomised, with three repetitions. The plates were incubated at 28 °C for 7 days and then were evaluated for the presence (+) or absence (-) of bacterial growth.

Table 2 – Carbon sources in BIOLOG GN2 microplates by *Cupriavidus* spp. strains.

Carbon source		Strains*						Carbon source		Strains*					
A1	Control (free source C)							E1	ρ-HydroxyPhenylacetic Acid	1		3	4	5	6
A2	α-cyclodextrin							E2	Itaconic Acid	1	2				
A3	Dextrin				4			E3	α-Keto Butyric Acid	1			4	5	
A4	Glycogen							E4	α-Keto Glutaric Acid				4		
A5	Tween 40	1			4			E5	α-Keto Valeric Acid	1			4	5	6
A6	Tween 80	1	2	3	4	5	6	E6	D,L-Lactic Acid	1	2	3	4	5	6
A7	N-Acetyl-D-Galactosamine				4			E7	Malonic Acid		2	3	4	5	6
A8	N-Acetyl-D-Glucosamine				4			E8	Propionic Acid	1		3	4	5	6
A9	Adonitol							E9	Quinic Acid			3	4	5	6
A10	L-Arabinose							E10	D-Saccharic Acid						
A11	D-arabitol							E11	Sebacic Acid	1		3	4	5	6
A12	D-Cellobiose				4			E12	Succinic Acid	1	2	3	4	5	6
B1	i-Erythritol			3				F1	Bromosuccinic Acid	1	2	3	4	5	6
B2	D-Fructose				4	5	6	F2	Succinamic Acid	1	2	3	4	5	6
B3	L-Fucose							F3	Glucuronamide		2				
B4	D-Galactose							F4	L-Alaninamide	1			4		
B5	Gentiobiose				4			F5	D-Alanine	1		3	4	5	6
B6	α-D-Glucose				4			F6	L-Alanine	1		3	4	5	6
B7	m-Inositol							F7	L-Alanyl-glycine				4		
B8	α-D-Lactose							F8	L-Asparagine	1		3	4	5	6
B9	Lactulose				4			F9	L-Aspartic Acid	1		3	4	5	6
B10	Maltose				4			F10	L-Glutamic Acid	1		3	4	5	6
B11	D-Mannitol		2					F11	Glycyl-L-Aspartic Acid				4		
B12	D-Mannose		2		4			F12	Glycyl-L-Glutamic Acid				4		
C1	D-Melibiose				4			G1	L-Histidine	1		3	4	5	6
C2	β-Methyl-D-Glucoside				4			G2	Hydroxyl-L-Proline						
C3	D-Psicose							G3	L-Leucine	1		3	4	5	6
C4	D-Raffinose							G4	L-Ornithine						
C5	L-Rhamnose							G5	L-Phenylalanine	1		3	4	5	6
C6	D-Sorbitol							G6	L-Proline	1		3	4	5	6
C7	Sucrose							G7	L-Pyroglutamic Acid	1	2	3	4	5	6
C8	D-Trehalose							G8	D-Serine			3	4	5	6
C9	Turanose							G9	L-Serine	1		3	4	5	
C10	Xylitol							G10	L-Threonine	1		3	4	5	6
C11	Pyruvic Acid Methyl Ester	1		3	4	5	6	G11	D,L-Carnitine						
C12	SuccinicAcid Mono-Methyl-Ester	1		3	4	5	6	G12	γ-Amino Butyric Acid	1		3	4	5	6
D1	Acetil Acid	1	2	3	4	5	6	H1	Urocanic Acid	1		3	4	5	6
D2	Cis-Asconitic Acid	1		3	4	5	6	H2	Inosine				4		
D3	Citric Acid	1		3	4	5	6	H3	Uridine				4		
D4	Formic Acid	1	2	3	4	5	6	H4	Thymidine						
D5	D-Galactonic Acid Lactone							H5	Phenyethyl-amine						
D6	D-Galacturonic Acid		2					H6	Putrescine						
D7	D-Gluconic Acid	1		3	4	5	6	H7	2-Aminoethanol						
D8	D-Glucosaminic Acid				4			H8	2,3 Butanediol				4		
D9	D-Glucuronic Acid		2					H9	Glycerol	1	2		4	5	
D10	α-Hydroxybutyric Acid	1		3	4	5	6	H10	D,L-α-Glycerol Phosphate						
D11	β-Hydroxybutyric Acid	1	2	3	4	5	6	H11	α-D-Glucose-1-Phosphate						
D12	γ-Hydroxybutyric Acid	1	2		4	5		H12	D-Glucose-6-Phosphate						

*1 = *Cupriavidus taiwanensis* LMG19424^T; *C. necator*: 2 = UFLA02-72; 3 = UFLA02-59; 4 = UFLA02-62; 5 = UFLA01-657; 6 = UFLA01-658.

Symbiotic efficiency of *Cupriavidus necator* strains in *Leucaena leucocephala* and *Phaseolus vulgaris*

Two experiments were performed concurrently under axenic conditions in a greenhouse from Aug. to

Sep. 2009. In experiment 1, *L. leucocephala* was grown for 100 days in 250 cm^3 plastic tubes containing a sterile mixture of sand and vermiculite in a 1:2 ratio. The seeds of *L. leucocephala* were scarified by immersion in 98.08 % sulphuric acid for 20 min, followed by successive washes in sterile water. Then, these were placed in sterile plates containing moistened cotton and filter paper until the start of germination for subsequent removal of teguments and planting in tubes. The treatments consisted of the inoculation of 13 homologous strains of *L. leucocephala*, that is, strains isolated from nodules of this trap species. Treatments with the inoculating strain recommended for *L. leucocephala*, BR 827 (Faria, 1997), and with the type strain for the species *C. taiwanensis*, LMG 19424^T (Chen et al., 2001), were also included. At the time of planting, each tube received one germinated seed, and then each seed was inoculated with 1 mL of the strains cultivated in 79 liquid medium containing approximately 1 × 10^9 cells. These plants were irrigated daily with a sterilized nutrient solution from Hoagland and Arnon (1950) at one-fourth strength, containing a small quantity of mineral N (21 mg 4 L^{-1}) to act as a starter dose and prevent the inhibition of nodulation.

In experiment 2, *P. vulgaris* was grown for 40 days in 600 mL of recycled dark glass flasks containing 500 mL of the nutrient solution described by Hoagland and Arnon (1950) at one-fourth strength, containing a small quantity of mineral N (21 mg 4 L^{-1}). The glass flasks were prepared according to Florentino and Moreira (2009), and over the course of the experiment, the solution was not replaced. The *P. vulgaris* seeds were superficially disinfected by immersion in 100 % ethyl alcohol for 30 s and then in 2 % hypochlorite for 2 min, followed by consecutive washes in sterilized water. The seeds were placed in sterile plates containing moistened cotton and filter paper until the onset of germination for subsequent removal of teguments and planting in glass flasks. The treatments consisted of the inoculation of 12 homologous strains and nine of the strains tested in *L. leucocephala*. They also included a treatment with the inoculant strain for *P. vulgaris*, CIAT 899^T, and one with the type strain of *C. taiwanensis*, LMG 19424^T. At the time of planting, each glass flask received one germinated seed, and then each seed was inoculated with 1 mL of the strains cultivated in 79 liquid medium containing approximately 1 × 10^9 cells.

In both experiments (*L. leucocephala* and *P. vulgaris*), there were also two controls without inoculation: one in which the complete Hoagland and Arnon (1950) solution was used, containing 210 mg 4 L^{-1} of mineral N, and the other grown in the solution containing a low quantity of mineral N (21 mg 4 L^{-1}), as mentioned before. At the end of the growth period of each experiment, the following variables were analysed: shoot dry matter (SDM), number of nodules (NN) and relative efficiency (RE). The shoots from every treatment were subjected to a forced-air oven (65 °C to 70 °C) until reaching a constant mass to determine the weight of SDM.

At the time of experimental evaluation, the bacteria from four nodules of each plant were isolated. This isolation had the objective of confirming whether the bacteria present in the nodules exhibited the same culture characteristics typical of the genus of the strains inoculated in the seeds at the time of planting. For the isolation, the nodules were superficially disinfected according to Vincent (1970) and macerated in plates containing 79 culture medium (pH 6.8) with bromothymol blue. The bacteria were grown at 28 °C. All nodules, including those used for isolation, were counted to determine NN.

RE of each treatment was calculated by the formula RE = (inoculated SDM/SDM with N) × 100, where RE is the relative efficiency, inoculated SDM is the shoot dry matter of inoculated plant and SDM with N is the shoot dry matter of plant with high amount of mineral N.

For both experiments, a completely randomised experimental design was used with three repetitions. The values of NN, SDM and RE were grouped by the Scott-Knott test ($p < 0.05$).

Results and Discussion

Free-living N_2 fixation

There was no turbidity and/or formation of pellicles near the surfaces of the media without combined N inoculated with *C. taiwanensis* LMG 19424^T and with the strains identified as *C. necator*, indicating that these strains are not able to grow by fixing N_2 in the free-living state. These results agree with those obtained for other strains of *Cupriavidus* sp. isolated from nodules of *Mimosa pudica*, in India (Verma et al., 2004).

Of the strains use in this study, three of these were able to use fructose (UFLA 02-62, UFLA 01-657 and UFLA 01-658) and one strain were able to use mannitol (UFLA 02-72) as a carbon source in the BIOLOG GN2 kit (Table 2). Thus, the absence of growth, at least for these strains, corroborates their inability to fix N_2 in the free-living state.

The two positive control strains, ORS 571^T (*A. caulinodans*) and BR 5401^T (*A. doebereinerae*), only exhibited typical growth, in the form of one pellicle, close to the surface of the culture medium that contained sodium lactate as a carbon source.

Although this study used culture media with compositions that were slightly different from those used by Elliott et al. (2007) (JMV medium) to evaluate the growth of the LMG 19424^T strain, similar results were found, that is, the absence of growth in the media, corroborating that this strain is not able to fix N_2 in the free-living state in that conditions, i.e., semi-solid N-free media. In addition, in this study, three different sources of carbon were tested: sodium lactate, fructose and mannitol. The latter is the carbon source used in the JMV medium (Reis et al., 2004).

Free-living N_2 fixation ability enables bacteria to survive saprophytically in environments that are deficient in this nutrient; therefore, it is an adaptive advantage. Between the two genera of the β-proteobacteria class, *Cupriavidus* and *Burkholderia*, described until now as capable of nodulating legumes, species in the latter genus such as *B. tropica*, *B. vietnamiensis*, *B. kururiensis*, *B. phymatum*, *B. tuberum* and *B. unamae* have the ability to fix N_2 in the free-living state when grown in JMV semisolid medium (Reis et al., 2004; Elliott et al., 2007). In the case of *B. phymatum*, it has also been shown that it is also capable of effectively establishing symbiosis with several *Mimosa* species (Elliott et al., 2007).

Use of C sources

Table 2 shows the use of different carbon sources by the LMG 19424^T strain and the LNB strains identified as *C. necator*. The *C. taiwanensis* LMG19424^T strain used 38 sources of C, which is similar to the results found in the previous study that described the species (Chen et al., 2001). The nine *C. taiwanensis* strains (including LMG 19424^T) studied by these authors used 32 sources of carbon. For the strains identified as *C. necator*, there was variability in the use of different carbon sources, however 10 carbon sources were used by all of them.

The UFLA02-62 strain used the greatest number of these compounds (60). Then, the UFLA01-657, UFLA02-59 and UFLA01-658 strains metabolised 39, 35 and 35 carbon sources, respectively. The UFLA02-72 strain had the lowest capacity to metabolise different substrates (18), indicating the feasibility of this method to differentiate the strains and the diversity observed among the strains isolated from *L. leucocephala* and *P. vulgaris* in soils from Southern Minas Gerais state that were identified as *C. necator*.

Regarding the ability to use different carbon sources, strains UFLA01-657, UFLA02-59 and UFLA01-658 exhibited greater similarity with the *C. taiwanensis* LMG19424^T strain regarding the range of carbon sources present in the BIOLOG GN2 kit. The UFLA02-72 strain differed in most of these aspects, whereas UFLA02-62 showed intermediate results.

The assessment of the ability to use C sources, using the BIOLOG method, has been used in the phenotypic characterisation of LNB strains (Beauregard et al., 2003; 2004) and can also be used to evaluate the intraspecific diversity of diazotrophic bacteria (Reis Júnior et al., 2003).

Antibiotic resistance

The use of three antibiotic discs per plate was adequate, given that there was no overlapping of halos, even for strains that showed sensitivity. The strains identified as *Cupriavidus necator* and the type or reference strains of the species belonging to the main LNB genera exhibited variable behaviour regarding resistance to the antibiotics tested (Figure 1). The UFLA03-84 strain, from the genus *Bradyrhizobium*, was the only strain that was resistant to all of the antibiotics. This result corroborates those obtained by Florentino et al. (2010), in which this strain was also resistant to all antibiotics tested. The greater antibiotic resistance exhibited by *Bradyrhizobium* strains compared to LNB of other genera was also observed by other authors (Dowdle and Bohlool, 1985).

Regarding *C. necator* strains from southern Minas Gerais state soil samples, all strains were sensitive to RFM, NAL and TET, and all were resistant to AMP, SUL, BC, AMO and VAN. Analysing the dendrogram and the table shown in Figure 1, 20 of these strains, representing 80 % of the strains studied, formed a large group that was resistant to STR, AMP, SUL, KAN, BC, AMO, GEN and VAN and sensitive to AZI, ERY, CHL, CLA, TET, RFM and NAL. The antibiotics to which these strains were sensitive are mainly produced by several species of Actinobacteria, and their main mechanism of action is the inhibition of protein synthesis (ERY, CHL, CLA and TET) or nucleic acid synthesis (RFM and NAL) (Tortora, 2000).

By comparing the resistance exhibited by the strains that comprise the largest group of *C. necator* with that exhibited by the *C. taiwanensis* LMG 19424^T strain, it seems that they have in common resistance to five antibiotics (STR, AMP, SUL, BC and AMO). Furthermore, *C. taiwanensis* LMG 19424^T was resistant to CHL and CLA.

Out of the 15 antibiotics tested, ten are natural; that is, directly produced by microorganisms (STR, GEN, CLA, ERY, CHL, KAN, RFM, TET, VAN, BC), three are semisynthetic (SUL, AZI and NAL) and two are synthetic (AMO and AMP). Moreover, some of these antibiotics, such as AMO, AZI, CLA, ERY and SUL, can be found in soil due to veterinary use, in particular due to their use in cattle, swine and birds (Regitano and Leal, 2010). Although these strains were isolated from pasture soils, they are still sensitive to AZI, CLA and ERY. This highly similar behaviour of the majority of strains isolated from southern Minas Gerais soils, regarding antibiotic resistance can be explained. It is due by the fact that the antibiotic resistance genes, located in plasmids, can be exchanged between individuals of the same bacterial population (Walsh, 2000), suggesting that LNB strains found in the same geographical region may possess the same antibiotic resistance genes. However, this is not always observed (Xavier et al., 1998; Zerhari et al., 2000). In the case of genus *Cupriavidus* bacteria, they frequently have two chromosomes and one or two megaplasmids (Amadou et al., 2008; Janssen et al., 2010). The genes that attribute resistance to several metals are in these megaplasmids, as observed for *C. metallidurans* (*Alcaligenes eutrophus*), which can also be exchanged between bacterial species (Diels et al., 1985).

Growth in different pH values and salt concentrations

The strains of *Cupriavidus necator* were able to grow in all pH values analysed, suggesting their adaptability to environments with different values of pH,

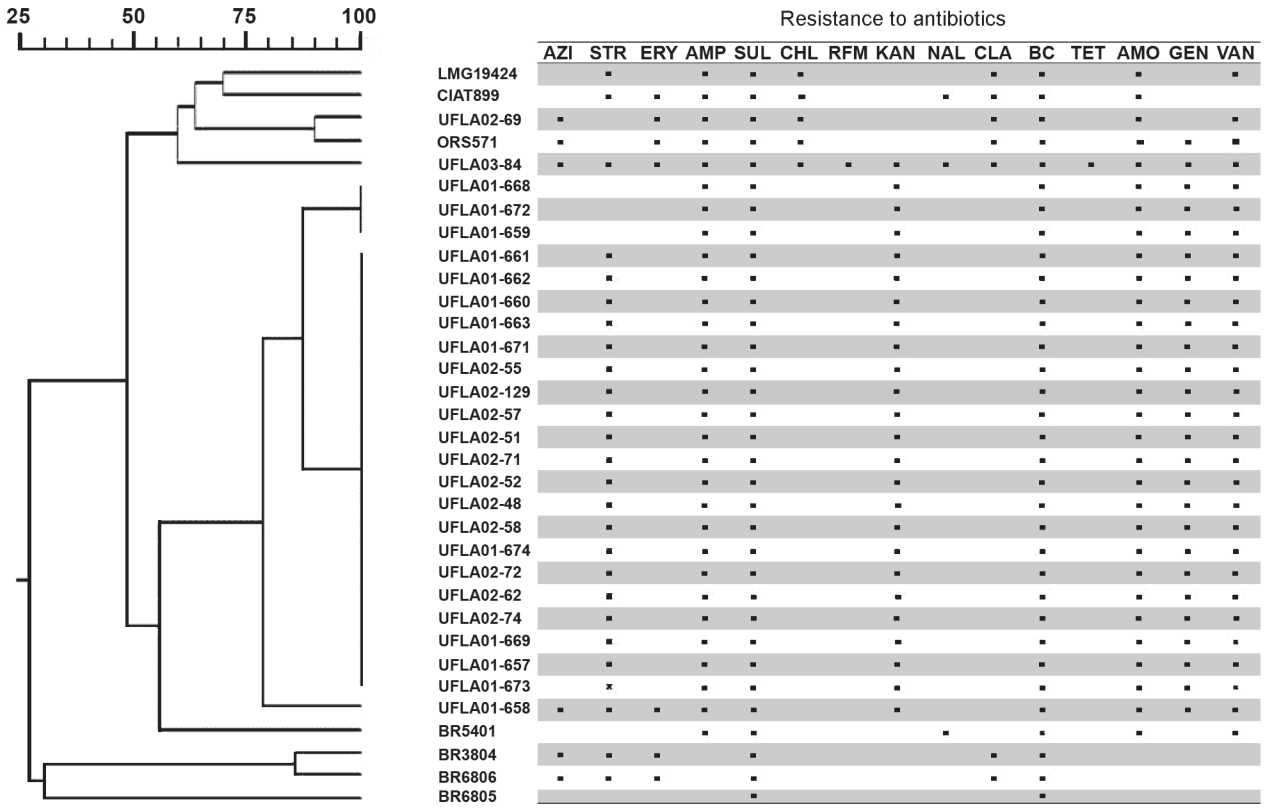

Figure 1 – Dendrogram and table representing the resistance of Legume Nodulating Bacterial strains to the 15 types of antibiotics (AZI, STR, ERY, AMP, SUL, CHL, RFM, KAN, NAL, CLA, BC, TET, AMO, GEN, VAN). The dendrogram was constructed using the UPGMA algorithm and the Jaccard coefficient (J).

including sites with high acidity, although these strains have been isolated in soils with a higher pH than those generally found in tropical soils (Table 1). For the type or reference LNB studied, only the two species from the genus *Azorhizobium*, *A. doebereinerae* (BR5401^T) and *A. caulinodans* (ORS571^T), did not grow in 79 culture medium with pH values of 4.0 or 5.0. In general, although the pH of the external environment is acidic or alkaline, the intracellular pH tends to remain close to neutrality, thus avoiding the destruction of macromolecules that are sensitive to acids or bases. In the case of the genus *Azorhizobium*, acidity, the increased concentration of H^+ ions, can interfere with the activity of extracellular enzymes responsible for the degradation of macromolecules to monomers that are essential to metabolism and in the transport of substances into the cell, leading to death of the bacterium.

The strains from the genus *Cupriavidus* grew in the highest salt concentrations tested in relation to type or reference strains of different LNB genera (Figure 2). Of these, 23 strains, with 13 being isolated from nodules of *L. leucocephala* and 10 from *P. vulgaris* and representing 92 % of the total strains studied, are able to grow in media containing up to 342 mM NaCl. The other two strains isolated from *P. vulgaris*, UFLA02-62 (non-nodulating in *P. vulgaris*) and UFLA02-72, showed growth in media containing 513 and 256 mM NaCl, respectively. The *C. taiwanensis* LMG19424^T strain grew in NaCl concentrations up to 427 mM, corroborating the results found in a previous study that described this species (Chen et al., 2001).

The LNB strains exhibited great variability regarding their ability to grow in media containing different salt concentrations (Zahran et al., 1994; Frioni et al., 2001; Shamseldin and Werner, 2005). The ability of bacteria to grow in high salt concentrations is mainly related with their ability to synthesise inorganic (K^+) or organic compounds (glycines, betaines, trehalose, glutamate and prolinetrehalose) to avoid water loss to the external environment. These compounds do not interfere in the metabolism of the cell and therefore are called compatible solutes. The cellular changes caused by salt excess in the medium can be detected by changes in protein profiles (Unni and Rao, 2001) and in surface polysaccharides (Lloret et al., 1995).

The salinity levels that inhibit the symbiosis between LNB and legumes are different from those that inhibit the individual growth of microsymbionts (Zahran, 1991). Therefore, in addition to the ability of LNB to grow in high salt concentrations, the host legume plant must also possess this characteristic for the symbiotic

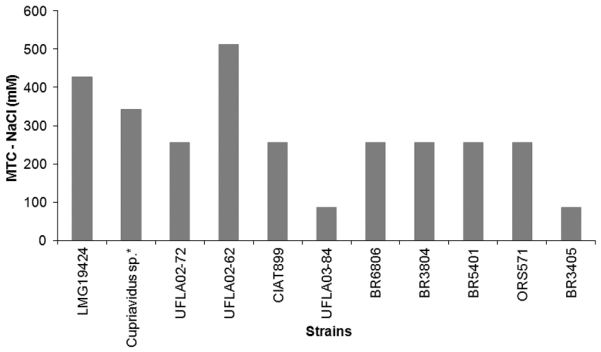

Figure 2 – Maximum tolerable concentrations (MTC) of NaCl (mM) in 79 medium of strains of *Cupriavidus* spp. (*C. taiwanensis* LMG19424^T, *C. necator**, UFLA02-72, UFLA02-62) and reference strains representative of each genus (CIAT899^T, *Rhizobium tropici*; UFLA03-84, *Bradyrhizobium* sp.; BR6806, *Sinorhizobium* sp.; BR3804, *Mesorhizobium* sp.; BR5401^T, *Azorhizobium doebereinerae*; ORS571^T, *A. caulinodans*; BR3405, *Burkholderia sabiae*). *Group consisting of 22 strains of *C. necator* that tolerate the same NaCl concentrations in 79 medium, 342 mM.

process to be successful. In some legumes, such as *L. leucocephala*, the salt concentrations that can inhibit nodulation and N_2 fixation vary from 25 to 120 mM, and the variability in these results may be due to the characteristics of the microsymbiont (Hashem et al., 1998; Anthraper and Dubois, 2003). Based on these results, *L. leucocephala* has the potential to be used in saline areas when inoculated with strains that are tolerant to salinity.

There was correlation between the growth capacity of bacteria in high salt concentrations and their ability to use different carbon sources (Table 2), given that the strains that had the highest and lowest capacities to use C sources, UFLA02-62 and UFLA02-72, respectively, were also the ones that tolerated the highest and lowest salt concentration, respectively. Strains UFLA01-657, UFLA02-59 and UFLA01-65, which metabolised an intermediate number of carbon sources, also tolerated intermediate concentrations of NaCl. The results obtained in this study are similar to those obtained for strains of *Bradyrhizobium* sp. that exhibited a direct relationship between the ability to grow in high salt concentrations and the ability to use different carbon sources (Barboza et al., 2000). These results suggests that strains that quickly adapt to use C sources can also exhibit other adaptive advantages, such as the ability to grow in high salt concentrations, in relation to other strains in the soil.

Symbiosis between *Cupriavidus necator* strains and their trap species, *L. leucocephala* and *P. vulgaris*

There was no nodulation in the treatments without inoculation, indicating the absence of contamination in the experiments. This was also corroborated by the fact that bacteria isolated from nodules exhibited identical morphological characteristics as the strains inoculated in host legume plants.

For both legume species, the treatment in which a higher mineral N concentration was added had higher SDM values to those inoculated with bacterial strains, including those treatments inoculated with the strains BR 827 and CIAT 899^T (Table 2). In the case of BR 827, studies show contradictory results, with one showing that this strain establishes efficient symbiosis with *L. leucocephala* (Gonçalves and Moreira, 2004; Moreira et al., 2010) and another showing that nodulation did not occur (Florentino et al., 2009). In the latter, the authors suggest that there may have been a problem with this strain. Nodulation did occur in our study; however, symbiosis was not as efficient as that observed by other authors, which may be due to some problem with this strain or environmental conditions, for example, high temperature, which was recorded during the experimental period. The latter justification can also be used for the inoculating strain of *P. vulgaris*, CIAT 899^T, given that plants in symbiosis are more sensitive to stresses than those that were given mineral N (Arayangkoon et al., 1990).

All strains isolated from nodules of *L. leucocephala* established symbiosis with this host legume, showing variable efficiency. The *C. necator* strain UFLA01-672 and *C. taiwanensis* strain LMG 19424^T promoted greater nodulation, development of the shoot and relative efficiency in *L. leucocephala* in relation to the inoculating strain BR 827 and the remaining strains of *C. necator*. These results are relevant given that *L. leucocephala* is a fast growing legume tree. It is used in the recovery of degraded areas, and the bacteria from the genus *Cupriavidus* have attracted interest due to their increased resistance to heavy metals (Mergeay et al., 1985; 2003; Goris et al., 2001) and ability to biodegrade recalcitrant and xenobiotic compounds (Louie et al., 2002; Trefault et al., 2004).

The selection of nodulating bacteria that exhibit these characteristics becomes important from a biotechnological point of view, given that they can establish symbiosis with legumes that are suitable for planting in areas that have suffered some type of anthropic intervention (such as mining areas). *Mimosa pudica*, when in symbiosis with the TJ208 strain of *C. taiwanensis* that is tolerant to heavy metals, absorbed a higher amount of Pb, Cu and Cd than plants that were not inoculated with this bacterial strain (Chen et al., 2008b). These results indicate that other legumes that establish symbiosis with *Cupriavidus* spp. strains, such as *L. leucocephala*, can behave similarly.

In *P. vulgaris*, out of the 21 strains tested, only UFLA02-69 was incapable of producing nodules in this host. For the remaining treatments, one observed variable behaviour in terms of nodulation, and in some cases, the number of nodules were higher than the number after inoculation with the strain recommended as an inoculant, CIAT 899^T. However, the nodules induced by these strains in *P. vulgaris* did not affect the growth of the shoots of these plants, and given that, all of the treatments were similar to the treatment without inoculation and without mineral N (Table 3), this demonstrates their

Table 3 – Nodule number (NN), shoot dry matter (SDM) and relative efficiency (RE) obtained from the inoculation of strains of *Cupriavidus necator* and *C. taiwanensis* in *Leucaena leucocephala* and *Phaseolus vulgaris*.

Treatments	*L. leucocephala*			*P. vulgaris*		
	NN	SDM	RE	NN	SDM	RE
		g per plant	%		g per plant	%
Without inoculation:						
Small dose of mineral N (21 mg 4 L^{-1})	0 e	0.17 d	23 d	0 e	0.10 c	12 c
With mineral-N[1]	0 e	0.79 a	100 a	0 e	0.84 a	100 a
Inoculated with strains recommended as inoculants:						
CIAT 899^T (*Rhizobium tropici*)	nd	nd	Nd	28 c	0.24 b	29 b
BR 827 (*Sinorhizobium* sp.)	12 c	0.22 c	28 c	nd	nd	nd
Inoculated with *Cupriavidus* sp. strains:						
LMG19424^T (*C. taiwanensis*)	19 b	0.35 b	45 b	19 c	0.12 c	15 c
C. necator strains						
UFLA01-668	13 c	0.24 c	31 c	40 a	0.14 c	17 c
UFLA01-672	33 a	0.33 b	41 b	32 b	0.10 c	12 c
UFLA01-661	14 c	0.24 c	31 c	24 c	0.13 c	15 c
UFLA01-662	6 d	0.18 d	22 d	33 b	0.10 c	12 c
UFLA01-660	14 c	0.23 c	29 c	25 c	0.10 c	12 c
UFLA01-663	14 c	0.19 d	24 d	22 c	0.11 c	14 c
UFLA01-671	16 c	0.18 d	22 d	22 c	0.10 c	12 c
UFLA01-673	12 c	0.17 d	22 d	21 c	0.10 c	12 c
UFLA01-657	13 c	0.17 d	22 d	30 b	0.12 c	14 c
UFLA01-669	10 c	0.21 c	27 c	nd	nd	nd
UFLA01-659	15 c	0.19 d	24 d	nd	nd	nd
UFLA01-680	16 c	0.21 c	27 c	nd	nd	nd
UFLA01-658	13 c	0.17 d	22 d	nd	nd	nd
UFLA02-55	nd	nd	nd	9 d	0.12 c	14 c
UFLA02-75	nd	nd	nd	18 c	0.12 c	14 c
UFLA02-57	nd	nd	nd	49 a	0.11 c	14 c
UFLA02-59	nd	nd	nd	34 b	0.11 c	13 c
UFLA02-71	nd	nd	nd	32 b	0.10 c	11 c
UFLA02-52	nd	nd	nd	25 c	0.12 c	14 c
UFLA02-48	nd	nd	nd	30 b	0.12 c	14 c
UFLA02-58	nd	nd	nd	26 c	0.12 c	14 c
UFLA02-69	nd	nd	nd	0 e	0.12 c	14 c
UFLA02-72	nd	nd	nd	12 d	0.14 c	17 c
UFLA02-62	nd	nd	nd	24 c	0.12 c	15 c
UFLA02-74	nd	nd	nd	19 c	0.12 c	14 c
CV(%)	17.11	13.54	13.69	28.49	18.31	18.01

Values followed by the same letter in the column did not differ (Scott-Knott test, $p < 0.05$); NN data were transformed into square root (x + 1); [1]Solution of Hoagland and Arnon (1950) containing 210 mg of N, with 14 mg as $NH_4H_2PO_4$, 84 mg as KNO_3 and 112 as $Ca(NO_3)_2 \cdot 4H_2O$. This solution was used at one-fourth strength.

inefficiency in fixing N_2 when in symbiosis with this host legume plant.

We highlight the fact that this the first report of the symbiosis of *C.taiwanensis* with both *Leucaena leucocephala* and *Phaseolus vulgaris*, corroborating the promiscuity of these plant species.

Conclusions

The majority of strains isolated from southern Minas Gerais state soils, identified previously as *Cupriavidus necator*, behaved differently from the *C. taiwanensis* LMG19424^T strain, which was isolated from soils in Taiwan, in terms of their use of a different range of carbon sources, growth in media with different salt concentrations and patterns of antibiotic resistance. However, all were highly versatile in terms of growing in a wide range of pH values. There was variability in the symbiotic efficiency of *C. necator* strains with their trap species *L. leucocephala*. The symbiosis of *C. necator* strains with *P. vulgaris* was always inefficient. *C. taiwanensis* is efficient in *L. leucocephala* and inefficient in *P. vulgaris*.

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