Density and diversity of diazotrophic bacteria isolated from Amazonian soils using N-free semi-solid media

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ABSTRACT: Non-symbiotic diazotrophic bacteria are amongst the most important functional groups of soil-dwelling microorganisms. These bacteria contribute to plant growth predominantly through biological N_2 fixation. Here, we evaluated the density and diversity of non-symbiotic diazotrophic bacteria in soils taken from diverse land use systems (LUS) in Amazonia using nitrogen-free media. A total of 30 soil samples were collected from the following LUS: pristine forest, young secondary forest, old secondary forest, agroforestry, agriculture and pasture. Bacterial density was evaluated by the most probable number (MPN) method utilizing N-free semi-solid media with varied compositions (JNFb, NFb, LGI and Fam). Individual isolates were characterized by colony and cellular morphology as well as total protein profiles and nitrogenase activity. Isolate genotypes were determined by partial 16S rDNA sequences. No typical diazotrophic growth in the JNFb medium was observed. Bacterial densities in the NFb medium were higher in the agriculture and agroforestry soil samples. In LGI and Fam media, bacterial densities were highest in the pasture soil samples. Overall, 22 isolates with high phenotypic diversity were obtained. Eleven isolates exhibited nitrogenase activity. Sequences of 16S rDNA genes of 14 out of 19 isolates had similarities below 100 % with known nitrogen-fixing species. Isolates were identified as belonging to the *Burkholderia, Enterobacter, Serratia, Klebsiella*, and *Bacillus* genera. A higher number of isolates from pasture soil samples were isolated, with the majority of these belonging to the *Burkholderia* and *Bacillus* genera. Among the isolates, unknown sequences were obtained, possibly indicating new species. Taken together, these data demonstrate that Fam, NFb, and LGI semi-solid media allowed the growth of diazotrophic bacteria belonging to different phylogenetic lines.
Keywords: *Burkholderia* sp., *Enterobacter* sp., *Serratia* sp., *Bacillus* sp., *Klebsiella* sp.

Introduction

Biological nitrogen fixation (BNF), the enzymatic reduction of N_2 to ammonia, is important because it converts nitrogen in a form that living organisms can utilize for biosynthesis. BNF by diazotrophic bacteria is an important process when N is a limiting factor for the growth of organisms. Diazotrophic bacteria are found in symbiosis with plants forming root nodule structures, or as free-living bacteria in the rhizosphere or as associative bacteria in a large variety of plant species, mainly monocotyledons such as rice (*Oryza sativa* L.) (Döbereiner and Pedrosa, 1987; Khammas et al., 1989; Gillis et al., 1995; Xie and Yokota, 2005), sugar cane (*Saccharum officinarum* L.) (Döbereiner and Ruschel, 1958; Reis et al., 1994; Baldani et al., 1997), corn (*Zea mays* L.) (Döbereiner and Ruschel, 1958; Magalhães et al., 1979; Reis et al., 2004; Caballero-Mellado et al., 2004), and wheat (*Triticum aestivum*, L.) (Neal and Larson, 1976; Baldani et al., 1983). Non-symbiotic (associative or free-living) diazotrophic bacteria also promote plant growth by BNF and through the production of metabolites that stimulate root growth.

The Amazonian forest ecosystem is considered a great reservoir of biodiversity. Plant species diversity promotes a greater diversity of microorganisms associated with the plants and in the adjacent soils. Studies involving culture-independent approaches verified the high microbial diversity present in Amazonian soils (Borneman and Triplett, 1997; Jesus et al., 2009). Other authors have shown that a variety of diazotrophic bacteria are able to nodulate legumes (Moreira et al., 1998; Lima et al., 2005; Lima et al., 2009). However, only a few studies have been conducted regarding the occurrence of non-symbiotic diazotrophic bacteria in Amazonian soils (Sylvester-Bradley et al., 1980; Magalhães et al., 1983; Magalhães and Döbereiner, 1984). Thus the objective of this work was to evaluate the density and diversity of non-symbiotic diazotrophic bacteria from soils under different land use systems in the Amazon, using N-free media currently known to favor the growth of diazotrophics.

Materials and Methods

This study is part of the "*Conservation and Sustainable Management of Below-Ground Biodiversity*" project, supported by the Global Environmental Facility (GEF) and implemented by the "*United Nations Programme*" (UNEP); it was conducted in Brazil, Ivory Coast, India, Indonesia, Kenya, Mexico and Uganda. In Brazil, the

project is named BiosBrasil (www.biosbrasil.ufla.br). The area of study is located in Benjamin Constant, Northwest Amazonas state, on the triple border of Brazil, Colombia and Peru (4°20' - 4°26' S and 69°36' - 70°2' W). Fidalgo et al. (2005) characterized the sampling grids and points. Additionally, land use systems (LUS) in the area were assessed pristine forest, old second forest, young second forest, agroforestry, agriculture and pasture. Thirty soil samples were collected as follows: six from the pristine forest; three from the old second forest; four from the young second forest; five from agroforestry areas, six from areas of agriculture and six from pastures.

Soil samples (10 g) were submitted to successive serial dilutions (10^{-1} to 10^{-8}) in a salt solution (0.85 % NaCl), and 0.1 mL aliquots of the diluted suspensions were inoculated onto semi-solid culture media known to favor the growth of certain diazotrophic species, but also permit the growth of other diazotrophic species (Nóbrega et al., 2004). The media used were as follows: JNFb (*Herbaspirillum* spp.), NFb (*Azospirillum* spp.), LGI (*Azospirillum amazonense*) (Döbereiner et al., 1995), and Fam (*Azospirillum amazonense*) (Magalhães and Döbereiner, 1984), with three replications. The inoculated media were kept for 14 days in growth chambers at 27 ºC. The population of diazotrophics was estimated using the Most Probable Number (MPN) technique, calculated through the "Most Probable Number Estimate software" (MPNES) (Woomer et al., 1988). The data were analyzed statistically by Scott-Knott test ($p \leq 0.05$) using the SISVAR program, version 4.3 (Ferreira, 2008).

Diazotrophic bacteria isolations were performed from all the cultures that presented typical growth, i.e., the formation of a pellicle near the medium surface. Pellicles located 4 mm or less below surface were also considered. Colony characterization of the isolates was conducted on potato culture medium after a growth period of five days at 27 ºC. The following colony characteristics were evaluated: days for the appearance of isolated colonies, average diameter, shape, gum (polysaccharide) production and color. The following type and reference strains were included for comparison: BR11001^T (*Azospirillum brasilense*), BR11140^T (*Azospirillum amazonense*), BR11340^T (*Burkholderia* sp.), BR11080^T (*Azospirillum lipoferum*), BR11175^T (*Herbaspirillum seropedicae*), KBC1^T (*Azospirillum irakense*), and BR9004 (*Burkholderia* sp. isolated from woody legumes, Moreira, unpublished data). Values of 0 and 1 were attributed to the absence or presence of each characteristic, respectively, and their similarity was calculated using the Jaccard (S_j) coefficient ($S_j = a/a + b + c$). In this equation, a is the presence of a characteristic in both individuals, b is the presence of a characteristic in one individual and its absence in the other, and c is the absence of a characteristic in one individual and its presence in the other. All evaluated characteristics have the same value. The isolates and the strains were grouped by the UPGMA (average linkage clustering) method and graphically depicted as a dendrogram (NTSYS-pc, version 2.1t).

The isolates and the type and reference strains were evaluated for cell morphology using optical phase contrast microscopy. The observed characteristics were as follows: width, movement, shape and the presence of poly-β-hydroxybutyrate (PHB). The isolates were also Gram stained.

To evaluate the nitrogenase activity, both the isolates and reference strains were grown in 10 mL vials containing 5 mL of the same semi-solid isolation medium from where they were isolated, until pellicle formation. Nitrogenase activity was evaluated using the acetylene reduction assay (ARA). Assays were performed following a methodology described by Dilworth (1966). Ethylene production was verified by means of gas chromatography (Varian Star 3400 c*x*).

The isolates and reference strains were streaked out twice onto solid tryptone yeast (TY) medium. Next, the isolates were grown for 4 days at 28 ºC in liquid TY medium to reach the end of the exponential growth period; the cultures were then centrifuged and the cells extracts were washed twice with NaPBS buffer. Afterwards, 70 mg of cells were weighed and placed into tubes containing 0.9 mL sample buffer and 0.1 mL of 20 % SDS. These mixtures were then heated for 10 min at 95 ºC and latter centrifuged at 80 g × 100 for 10 min at 4 ºC. Next, samples were analyzed by polyacrylamide gel electrophoresis (PAGE); the Laemmli (1970) method, modified by Jackman (1985) was used. All steps were highly standardized. Values of 0 and 1 were attributed to the absence or presence of bands, respectively, and their similarity was calculated using the Jaccard coefficient (S_j). A dendrogram was inferred as described in the colony characterization section.

Near full-length 16S rDNA genes were amplified utilizing 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) primers (Lane, 1991). Briefly, 5 μL aliquots of cells lysed by boiling at 95 ºC were used for PCR. In a total volume of 50 μL, reactions were set up as follows: 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.2 μM of each primer, 1 U *Taq* DNA polymerase, 1 X PCR Buffer and Milli-Q water. Reactions were run on an Eppendorf Mastercycler® (Germany). An initial denaturation at 94 ºC for 5 min was followed by 40 cycles consisting of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, elongation at 72 °C for 1.5 min; further, the protocol included a final elongation period of 7 min. Amplified products were separated on 1 % agarose gels and visualized under UV light. The bands of interest were cut from the gels and purified using the QiAquick Gel Extraction Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. The PCR products were used directly for partial sequencing using the primers 27F and 1492R. Sequencing was performed using a MegaBACE™ 500.

Sequences were compared with the GenBank, using the basic local alignment search tool (NCBI) (http://www.ncbi.nlm.nih.gov, access in Nov. 2009). Novel sequences and selected reference strain sequences were aligned

using the ClustalW (Thompson et al., 1994). Phylogenetic trees were inferred by the neighbor-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. The 16S rDNA gene partial sequences were deposited in the EMBL/GenBank database under the accession numbers HM598436 to HM598450 and DQ78790.

Results and Discussion

No growth was observed on the JNFb medium. Diazotroph density ranges on NFb, LGI and Fam media were, respectively (cell numbers g^{-1} soil), 0.035×10^3 to 9.18×10^3, 0 to 2.30×10^3, and 0 to 4.24×10^3. The mean density (cell numbers g^{-1} soil) in each medium was 1.05×10^3 for NFb, 0.28×10^3 for LGI and 0.39×10^3 for Fam. The mean densities per LUS are presented in the Figure 1. In the NFb medium, while agroforestry and agriculture samples exhibited the highest density values, pristine forest samples had the lowest values. In the LGI and Fam media, the density was highest in pasture soils, probably because in this LUS, there is a predominance of grasses that are host plants for diazotrophic bacteria (Magalhães and Döbereiner, 1984). This happens even in mined soils when revegetated with grasses, where the highest diazotrophic bacteria densities were found, when compared to re-vegetation without grasses (Melloni et al., 2004). NFb medium cultures exhibited higher diazotroph densities compared to LGI and Fam media cultures. Fam media cultures, except for old second forest samples, had higher density values compared to samples cultured in LGI medium.

After successive streak outs for purification, 22 isolates were obtained. The soil origin, isolation medium and isolate identification are presented in Table 1. Isolates were obtained from 14 out of the tested 30 soil samples. Bacteria were isolated from all LUS, except from the old secondary forest; almost half of the isolates (9) were obtained from pasture soils. The highest number of isolates (14) was obtained from Fam medium cultures. This could be due to the higher bacterial densities in Fam media pasture soil cultures. This medium was also more efficient in culturing diazotrophic bacteria from the roots and the rhizospheres of Orchidaceae and other plants compared to LGI medium (Lange and Moreira, 2002), most likely because of the presence of micronutrients that

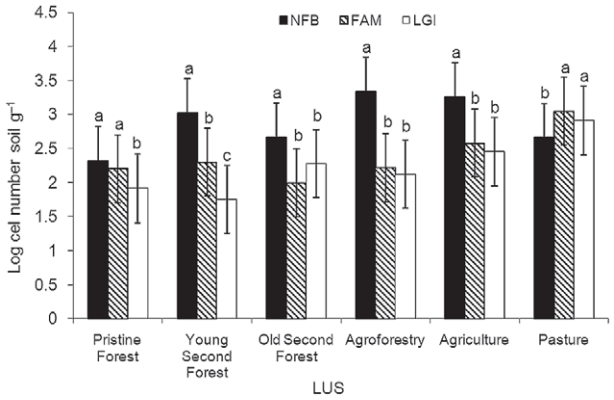

Figure 1 – Mean density of associative diazotrophic bacteria per different land use systems (LUS) in NFb, LGI and Fam media. Means followed by the same letter in the same colour bars are not different (Scott-knott test range, $p \leq 0.05$).

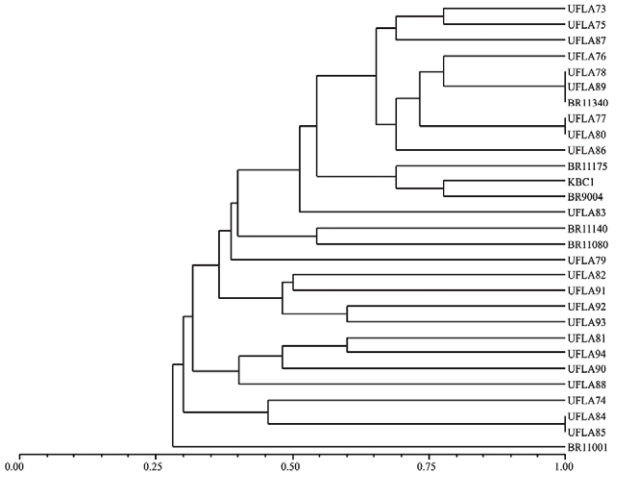

Figure 2 – Dendrogram based on ten cultural characteristics of isolates obtained from the soil of different land use systems (LUS) in Amazonian. Type and reference strains were also included. All isolates and type and reference strains were cultured on potato medium. Reference and type strains were as follows: BR11001^T (*Azospirillum brasilense*), BR11140^T (*Azospirillum amazonense*), BR11340^T (*Burkholderia* sp.), BR11080^T (*Azospirillum lipoferum*), BR11175^T (*Herbaspirillum seropedicae*), KBC1^T (*Azospirillum irakense*) and BR9004 (*Burkholderia* sp.).

Table 1 – Land use systems (LUS) and media from which isolates were obtained from Amazonian soils.

LUS	Isolates obtained in different media		
	NFb	LGI	Fam
Pristine forest	UFLA74	-	UFLA76
Young second forest	-	-	UFLA78, UFLA82
Agroforestry	-	UFLA90, UFLA91	UFLA77, UFLA80, UFLA81
Agriculture	UFLA73	UFLA92, UFLA93	UFLA79
Pasture	UFLA75	UFLA94	UFLA83, UFLA84, UFLA85, UFLA86, UFLA87, UFLA88, UFLA89

stimulate microorganism growth. Fam medium also could have provided better survival conditions for diazotrophic bacteria during the isolation process.

Colony characterization of isolates and type and reference strains revealed high phenotypic diversity, with 25 phenotypically groups being identified (Figure 2). The only similar characteristic among all isolates was days of growth for the appearance of isolated colonies (1 to 2 days). On a cellular level, isolates also exhibited diverse morphology (Table 2). Cell width varied between 0.55 and 1.10 μm and three isolates were Gram positive (UFLA75, UFLA85 and UFLA94) (Table 2). The cell shapes found were vibrioid, cocci and rods; the presence of bacterial chains was not observed. Three types of cell movements were observed and are described as follows: quick with trajectory, slow and trembling and quick twist or wave-like. Few isolates presented the same cell movement of the strains BR11001^T and BR11140^T (quick twist and wave-like); predominately, isolates were observed to be slow and trembling. Bacteria with cell characteristics distinct from *Azospirillum* spp. (typical vibrioid) were isolated, indicating that N-free semi-solid media favored the growth of other diazotrophic species. Similar findings have been published for Fam and NFB media (Magalhães and Döbereiner, 1984; Fernandes et al., 2001; Tripathi et al., 2002; Nóbrega et al., 2004). Eleven of the 22 isolates exhibited nitrogenase activity (Table 2). In five of these, the activity was low. It was not possible to determine the nitrogenase activity of isolate UFLA78, because this isolate lost its ability to develop the characteristic pellicle growth after successive cultivation.

The protein profiles of isolates UFLA78, UFLA84, UFLA85, UFLA88 and UFLA90 were not revealed following by SDS-PAGE analysis. After numerical analysis (data not shown) and visual comparison, only three isolates presented identical protein profiles (UFLA80, UFLA86 and UFLA89), indicating that the protein profiles, similar to the morphological characterization, showed a high diversity among the isolates and type of strains. Sequencing results were used to group the protein profiles of isolates by phylum and class (Figure 3).

Table 2 – Cell morphology and mobility, Gram test and nitrogenase activity of 22 isolates from land use systems (LUS) of Amazonian isolates and type and reference strains.

Isolates	Cell Shape	Width	Cell Mobility*	Gram	C_2H_4
		m			nmol h^{-1} $culture^{-1}$
UFLA73	vibrioid	1.10	Q/T	-	161.51
UFLA74	cocci	0.55	S/Tre	-	0
UFLA75	vibrioid	0.94	Q/T	+	360.10
UFLA76	vibrioid	0.72	QT/W	-	0
UFLA77	cocci	0.72	S/Tre	-	0
UFLA78	vibrioid	0.61	S/Tre	ne	ne
UFLA79	rod	0.83	S/Tre	-	0
UFLA80	rod	0.55	QT/W	-	89.45
UFLA81	vibrioid	ne	Q/T	-	6.01
UFLA82	rod	0.66	Q/T	-	22.76
UFLA83	vibrioid	0.77	S/Tre	-	0
UFLA84	ne	ne	ne	+	0
UFLA85	ne	ne	ne	+	36.63
UFLA86	ne	ne	ne	-	0
UFLA87	vibrioid	0.55	S/Tre	-	0
UFLA88	rod	0.55	Q/T	-	0
UFLA89	rod	0.83	Q/T	-	ne
UFLA90	rod	0.77	S/Tre	-	0.90
UFLA91	rod	0.72	S/Tre	-	1.54
UFLA92	rod	0.61	S/Tre	-	0.30
UFLA93	rod	0.77	S/Tre	-	1.62
UFLA94	vibrioid	0.77	S/Tre	+	2.23
BR11140^T	vibrioid	ne	QT/W	-	ne
BR11001^T	vibrioid	0.88	QT/W	-	773.50

*Mobility: slow and trembling (S/Tre), quick and trajectory (Q/T), quick twist and wavelike (QT/W). ne, not evaluated.

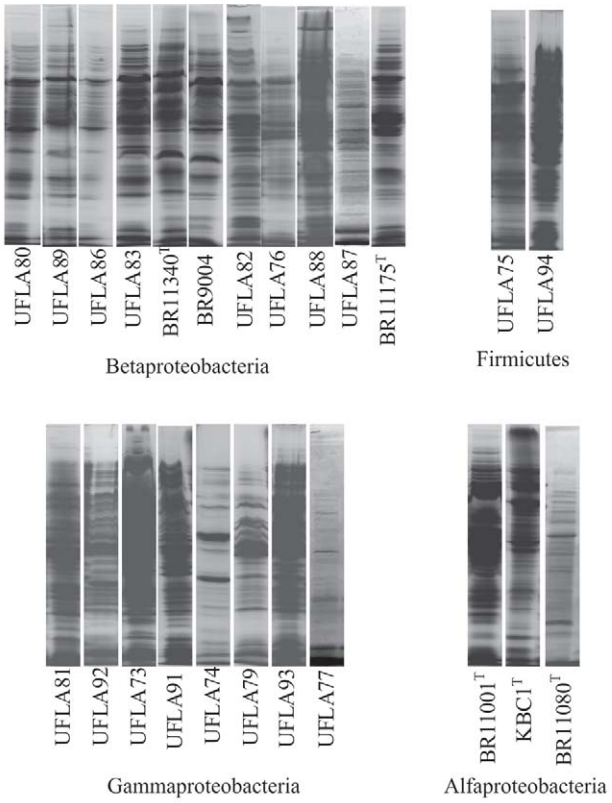

Figure 3 – Protein profiles obtained by polyacrylamide gel electrophoresis of isolates obtained from the soil of land use systems (LUS) in Amazonian. Reference and type strains were as follows: BR11001^T (*Azospirillum brasilense*), BR11340^T (*Burkholderia* sp.), BR11080^T (*Azospirillum lipoferum*), BR11175^T (*Herbaspirillum seropedicae*), KBC1^T (*Azospirillum irakense*) and BR9004 (*Burkholderia* sp.).

Partial sequences of the 16S rDNA gene of 19 isolates were obtained. A phylogenetic tree of 13 of the isolates was constructed with partial sequences of forward primers (Figure 4). Four isolates, UFLA77, UFLA80, UFLA87 and UFLA90, were sequenced only with the reverse primer and are not presented in the tree. The 19 isolates belonged to the Proteobacteria and Firmicutes phyla.

Proteobacteria isolates were distributed in two classes, Betaproteobacteria and Gammaproteobacteria. Eight isolates of the Betaproteobacteria class belonged to the *Burkholderia* genus (UFLA76, UFLA80, UFLA82, UFLA83, UFLA86, UFLA87, UFLA88 and UFLA89). Two isolates exhibited 100 % 16S rDNA gene sequence similarity with *Burkholderia cepacia* complex strains. While UFLA82 was grouped strongly with *Burkholderia pyrrocinia* and *Burkholderia multivorans* strains, UFLA88 was grouped with *Burkholderia vietnamiensis* (Figure 4). The *Burkholderia cepacia* complex is a group of phenotypically similar species or genomovars with high (98 to 99 %) 16S rDNA sequence similarity to strains that have been isolated from environmental and human clinical specimens, particularly those from cystic fibrosis patients (Coenye and Vandamme, 2003). *B. vietnamienses* is a diazotrophic member of *B. cepacia* complex isolated from rhizosphere macerates of rice in Vietnam (Gillis et al., 1995). Four strains were grouped with *Burkholderia tropica* strains, three (UFLA76, UFLA80 and UFLA89) with high (99 to 100 %) and one (UFLA83) with low (93 %) 16S rDNA sequence similarity. Although the 16S rDNA gene of isolate UFLA86 was not sequenced, it had identical protein profiles of the isolates UFLA80 and UFLA89 that were closely related to *B. tropica.* The sequence of UFLA87 using the reverse primer, exhibited 96 % similarity to the 16S rDNA sequences of *B. tropica* and *B. unamae* strains. However, when a phylogenetic tree was constructed with data obtained from the reverse primer (data not shown), this isolate was closer to *B. unamae* strains. *B. tropica* and *B. unamae* strains are associative diazotrophic bacteria that have been isolated from the rhizospheres or as endophytics of maize, sugarcane, teosinte and coffee (Reis et al., 2004; Caballero-Mellado et al., 2004).

For the Gammaproteobacteria isolates, most isolates belonged to the Enterobacteriaceae family with *Klebsiella*, *Enterobacter*, and *Serratia* genera represented. Isolates UFLA79 and UFLA93 exhibited similarities of 96 % and 99 %, respectively, to the 16S rDNA sequence of *Enterobacter oryzae,* a diazotroph recently described that was isolated from the surface-sterilized roots of the wild rice species *Oryza latifolia* (Peng et al., 2009). Isolate UFLA93 grouped

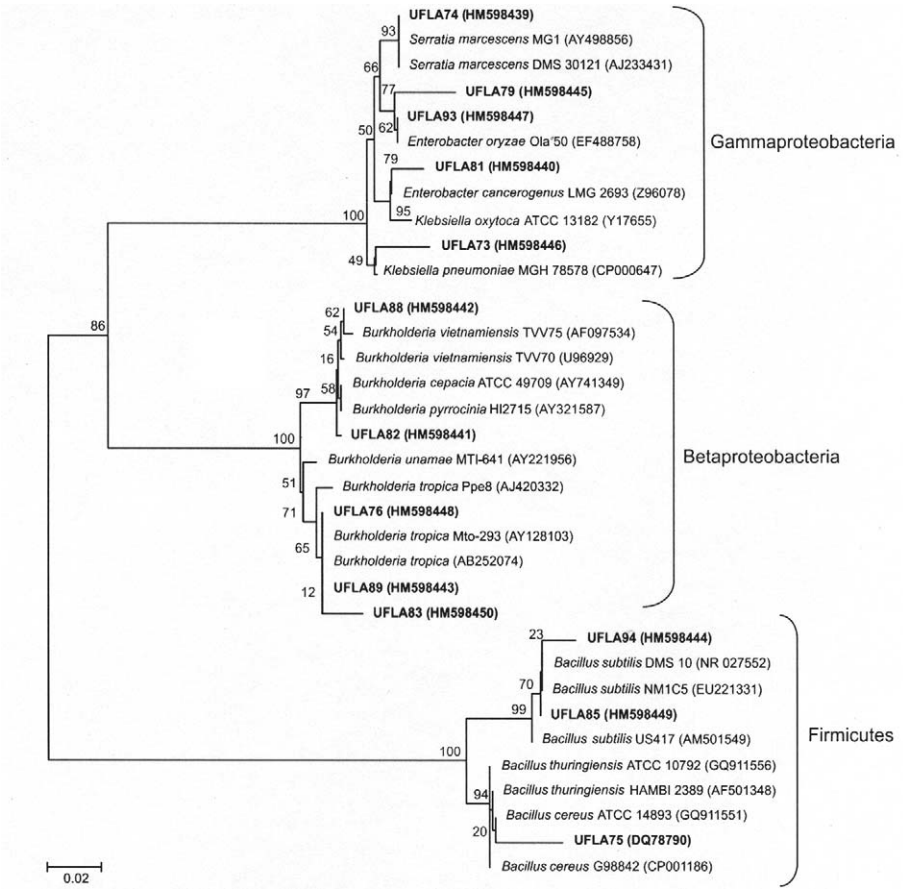

Figure 4 – Un-rooted tree estimated by the isolates obtained from the soil of land use systems (LUS) in Amazonian based on 16S DNA partial sequences. Alignment size was 350 bp, and gaps were omitted. The tree was estimated by the neighbor-joining method. Bootstrap values are based on 1,000 trials.

strongly with *E. oryzae* (Figure 4). With the reverse primer, isolate UFLA77 exhibited 97 % similarity to the 16S rDNA gene of *E. oryzae*. One isolate, UFLA81, had 98 % 16S rDNA sequence similarity with *E. cancerogenus,* bacteria known as plant pathogen (Dickey and Zumoff, 1988). *Klebsiella* isolate UFLA73 was more similar to the nitrogen-fixing *Klebsiella pneumoniae*, with 96 % 16S rDNA gene sequence similarity. Another isolate, UFLA90, was more similar o *Enterobacter* sp., but only exhibited 94 % similarity. Isolate UFLA74 had 100 % sequence similarity to *Serratia marscens*, which is both a human pathogen and a diazotroph found in cotton, maize (McInroy and Kloepper, 1995), rice (Gyaneshwar et al., 2001) and rice rhizospheres (Tripathi et al., 2002).

In the phylum Firmicutes, isolates UFLA85 and UFLA94 were more similar to *Bacillus subtilis,* with 99 % and 97 % 16S rDNA sequence similarity, respectively; additionally, UFLA75 was more similar to *B. cereus* and *B. thuringiensis*, with 94 % sequence similarity. The genus *Bacillus* is also known to have nitrogen-fixing species, including *B. cereus* and *B. subtilis* (Xie et al., 1998; Ding et al., 2005; Xie et al., 2006). In our study, all *Bacillus* isolates exhibited acetylene reduction indicating the presence of nitrogenase.

Two isolates, UFLA78 and UFLA84, could not be sequenced and were therefore not identified or classified into any class, phyla or genus. Although the sequences of UFLA90 and UFLA91 isolates were of low quality, they were close to the Gammaproteobacteria class.

A high phenotypic (colony appearance, cell morphology and protein profiles) and genotypic diversity was verified among isolates. There was no relationship among groups based on the analyzed characteristics. Groups formed in the colony characterization dendrogram (Figure 2) and in the protein profile analysis (Figure 3) are not the same as those formed by the 16S rDNA neighbour-joining tree analysis (Figure 4). Further, isolates closely related in the 16S rDNA tree (Figure 4) exhibited different protein profiles (Figure 3). Because protein profile analysis yields discriminative information at or below species level (Vandamme et al., 1996), and most of the isolates demonstrated 16S rDNA gene sequence similarity below 100 % for known nitrogen-fixing species, we conclude that these isolates could represent new species of diazotrophic bacteria. Thus, Amazonian soils have diverse populations of diazotrophic bacteria; further, NFb, LGI and Fam semi-solid media were able to support the growth of different diazotrophic lineages.

Gammaproteobacteria class isolates were observed in samples from all LUS, except pastures. While Betaproteobacteria isolates were found in almost all LUS except agriculture, and Firmicutes isolates were found only in pasture soils. A study conducted in the same area found that the bacterial community structure and composition (evaluated by T-RFLP, cloning and sequencing) were related to land use, likely through the effects of soil attributes; further, while Firmicutes were found to be present predominately in primary forest and old second forest samples, Betaproteobacteria (including *Burkholderia*) and Gammaproteobacteria were found mainly in primary forest samples (Jesus et al., 2009). Another study, evaluating the density, diversity and efficiency of diazotrophic bacteria able to nodulate siratro plants (*Macroptilium atropurpureum*) in Amazonian identified Betaproteobacteria (*Burkholderia* sp.) in cultivated soils (agriculture and agroforestry) and other LUS (primary forest) through 16S rDNA partial sequencing (Lima et al., 2009). In our study, we verified that while Gammaproteobacteria and Firmicutes could be detected with NFb, LGI and Fam media, Betaproteobacteria (e.g., *Burkholderia*) were not detected using NFb medium. *Burkholderia* was the main genus found in our study. In a previous study, most isolates obtained from *Araucaria angustifolia* roots and soil using NFb, JNFb and LGI media also belonged to the *Burkholderia* genus (Neroni and Cardoso, 2007). These results show that different methodologies are necessary to detect the overall bacterial diversity.

Conclusions

Density and diversity of diazotrophic bacteria was influenced by LUS. NFb, LGI and Fam media allowed the isolation of different lineages of non-symbiotic diazotrophic bacteria. The obtained isolates exhibited high phenotypic and genotypic diversity; however, no relationships were observed among the groups based on the different characteristics. Furthermore, the isolates obtained may represent new species of non-symbiotic diazotrophic bacteria.

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